 https://doi.org/10.3311/PPch.42486

Periodica Polytechnica Chemical Engineering, 70(2), pp. 246–259, 2026

Uncovering the Potential of Indonesian *Rubus fraxinifolius* Fruit: Optimization of Green Extraction with Simplex-Lattice-Design and Phytochemical Analysis

Fahmi Achmad Saputra[1*], Endah Dwi Hartuti[2], Lira Windriawati Listriyani[3], Siti Zulaeha[2], Cokorda Istri Meyga Semarayani[2], Reza Pahlevi Rudianto[4], Tubagus Bahtiar Rusbana[5], Salmah Mutia Rahman[5], Nezly Nurlia Putri[5], Indria Puti Mustika[3], Lily Ismaini[2]

[1] Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research and Innovation Agency (BRIN), Jl. Raya Bogor Km. 46 Cibinong 16911, West Java, Indonesia
[2] Research Center for Applied Botany, National Research and Innovation Agency (BRIN), Jl. Raya Bogor Km. 46 Cibinong 16911, West Java, Indonesia
[3] Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Jl. Raya Bogor Km. 46 Cibinong 16911, West Java, Indonesia
[4] Research Center for Polymer Technology, National Research and Innovation Agency (BRIN), B.J. Habibie Science and Technology Area, South Tangerang 15314, Banten, Indonesia
[5] Food Technology Department, Faculty of Agriculture, Universitas Sultan Ageng Tirtayasa, Jl. Raya Palka Km.03 Sindangsari, Pabuaran, Serang 42163, Banten, Indonesia
[*] Corresponding author, e-mail: fahm008@brin.go.id

Received: 17 October 2025, Accepted: 18 May 2026, Published online: 24 June 2026

Abstract

Unlocking the full potential of *Rubus fraxinifolius* (*R. fraxinifolius*) fruit as a source of high-value ingredients necessitates efficient and green extraction protocols. This study employed ultrasound-assisted extraction (UAE) coupled with a simplex lattice design (SLD) to systematically optimize the ethanol-water solvent for maximizing the phenolic (TPC), flavonoid (TFC), and antioxidant capacities. The optimum *R. fraxinifolius* extract (ORFE) was subsequently profiled using gas chromatography-mass spectrometry (GC-MS). Our models revealed key complexity: with TPC maximizing in absolute ethanol, while TFC and antioxidant capacity peaked in distinct hydroethanolic mixtures. Numerical optimization identified an overall optimal composition of 92.2% ethanol and 7.8% water, which was experimentally validated to yield high TPC (106.2 mg gallic acid equivalent /g), TFC (30.114 mg quercetin equivalent/g), and potent 2,2-diphenyl-1-picrylhydrazyl scavenging capacity (91.2% inhibition). GC-MS analysis of ORFE revealed a unique chemical signature, identifying key semi-volatile contributors including anethole, γ-tocopherol, and a substantial alkaloid fraction. We conclude that the extract's potent bioactivity reflected the concerted action of the substantial non-volatile polyphenolic load, evidenced by the high TPC and TFC results, and the specific semi-volatile bioactive compounds identified by GC-MS. This work establishes a scientifically validated, green protocol for producing a chemically characterized, high-potency antioxidant ingredient from *R. fraxinifolius* for nutraceutical applications.

Keywords

rubus fraxinifolius, ultrasound-assisted extraction, simplex-lattice design, phytochemical profiling

1 Introduction

The fruit of *Rubus fraxinifolius* (*R. fraxinifolius*), a plant with a rich history of ethnobotanical use in the Rosaceae family from Indonesia, represents a potent reservoir of bioactive phytochemicals. Its chemical profile is abundant in flavonoids, such as quercetin and myricetin, and phenolic acids that have been strongly linked to a spectrum of health benefits, including powerful antioxidant, anti-inflammatory, and anticancer activities [1–3]. However, efficiently extracting these valuable metabolites from the complex fruit matrix presents a significant extraction challenge, demanding a strategic selection of both technique and solvent [4]. Prior investigations into *R. fraxinifolius* fruit have primarily utilized conventional maceration techniques, which are characterized by low efficiency, high solvent consumption, and a lack of systematic optimization [5, 6]. These studies lacked the multi-objective

Cite this article as: Saputra, F. A., Hartuti, E. D., Listriyani, L. W., Zulaeha, S., Semarayani, C. I. M., Rudianto, R. P., Rusbana, T. B., Rahman, S. M., Putri, N. N., Mustika, I. P., Lily Ismaini, L. "Uncovering the Potential of Indonesian *Rubus fraxinifolius* Fruit: Optimization of Green Extraction with Simplex-Lattice-Design and Phytochemical Analysis", Periodica Polytechnica Chemical Engineering, 70(2), pp. 246–259, 2026. https://doi.org/10.3311/PPch.42486

approach necessary to understand how solvent polarity affects the co-extraction of diverse metabolite classes.

To address this, modern green extraction technologies offer a promising alternative to conventional methods like maceration. Among these, ultrasound-assisted extraction (UAE) is particularly effective. By employing acoustic cavitation to disrupt plant cell walls, UAE accelerates mass transfer, leading to higher yields in shorter times while preserving heat-sensitive compounds [7–9]. Yet, the efficiency of the UAE is highly dependent on being paired with an optimal solvent system. For this purpose, aqueous ethanol mixtures are exemplary; their tunable polarity is ideal for co-extracting a broad spectrum of bioactive compounds, from polar phenolics to less-polar flavonoids, all while maintaining a safe and environmentally friendly profile [10, 11].

Optimizing the ethanol-water ratio is therefore crucial for maximizing the yield and subsequent biological activity of the extract. Methodologies such as response surface methodology (RSM) employing a simplex lattice design (SLD) offer a powerful statistical approach to systematically investigate solvent interactions and identify the ideal composition with minimal experimental effort [12, 13]. While such optimization based on bulk phytochemical content (total phenolic content (TPC), total flavonoid content (TFC)) and overall antioxidant activity is a vital first step, it does not reveal the specific chemical constituents responsible for the observed effects. A deeper phytochemical characterization is necessary to elucidate the molecular basis of the extract's bioactivity and identify key marker compounds. Hyphenated techniques like gas chromatography-mass spectrometry (GC-MS) [14] are indispensable for this purpose, enabling the profiling of semi-volatile compounds such as fatty acids [15], terpenes [16], and potent lipophilic antioxidants like tocopherols [17], which may significantly contribute to the overall functional properties of the extract.

Unlike previous studies that relied on stochastic maceration or single-factor optimization, this work addresses the complex solubility behavior of *R. fraxinifolius* metabolites through a multidimensional SLD approach, specifically targeting the potential interplay between its unique alkaloid fraction and phenolic antioxidants. We aimed to first employ a SLD to optimize the UAE solvent system for maximal total phenolic content, total flavonoid content and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity from *R. fraxinifolius* fruit extract (RFE). Subsequently, we performed a detailed GC-MS phytochemical profiling on the resulting optimum *R. fraxinifolius* extract (ORFE) to identify the potential chemical contributors to its potent bioactivity. This work seeks to provide a scientifically validated, eco-friendly extraction protocol for producing a high-value, functionally potent ingredient from *R. fraxinifolius*, which could serve as a valuable foundation for future applications in the nutraceutical and functional food industries.

2 Experimental

2.1 Plant material and preparation

Fresh fruits of *R. fraxinifolius* were procured from local farmers in traditional markets in the Cibodas Botanical Garden, West Java, Indonesia. The samples were sorted based on a specific maturity, where only fruits at the fully ripe stage were selected. This stage was defined by a uniform red coloration and a characteristic softening of the fruit texture. After being procured, the samples were placed immediately in sterile bags and transported to the laboratory in a cool box. Upon arrival, the fruits were cleaned, drained, and separated from the leaves and stems for further analysis. Subsequently, the fruits were dried in a hot air oven at a constant temperature of 60 °C until a constant weight was achieved, indicating a final moisture content of <10%. The dried fruits were then pulverized into a fine powder using a high-speed grinder (Jing Gong HC-800Y, Jinhua, China) and sieved to obtain a uniform particle size of 50–80 mesh (0.297–0.177 mm). The powdered sample was kept in sealed containers in a cool, dark, and dry location until extraction.

2.2 Chemicals and reagents

Ethanol (96%, food grade) was used for the extraction process. Deionized water was obtained from a reverse osmosis system. The following analytical grade chemicals and reagents were used for phytochemical and antioxidant assays: Folin-Ciocalteu reagent, gallic acid (≥98%), quercetin (≥95%), anhydrous sodium carbonate, aluminum chloride, sodium acetate and 2,2-diphenyl-1-picrylhydrazyl. All chemicals and reagents were purchased from Merck (Darmstadt, Germany).

2.3 Optimization with simplex lattice design

A simplex lattice design was strategically employed in this study, effectively allowing for a comprehensive investigation into the impact of solvent concentration on total phenolic content, total flavonoid content, and antioxidant activities. This design models the responses based on all possible combinations of ethanol and water as solvents. In total, we conducted eight distinct extractions,

as detailed in Table 1. The selected response parameters (Y_i dependent variables) were total phenolic content , total flavonoid content and antioxidant capacities (C).

2.4 Preparation of *R. fraxinifolius* extract

The dried fruits of *R. fraxinifolius* were extracted using an ultrasonic homogenizer (Digital Sonifier 450, Branson, Danbury, USA) equipped with a probe. The sample was suspended in water-ethanol-solvent according to the experimental design (Table 1). The extraction was conducted for a total duration of 10 min at 40% amplitude, using pulsed cycles of 20 s on and 5 s off to minimize solvent evaporation, and a 20 kHz frequency [18]. Following extraction, the crude mixture was centrifuged at 17,000 g for 10 min at 4 °C (MX-301, Tomy Kogyo Co., Ltd., Tokyo, Japan) to separate the supernatant fromthe solid residue. The solvent was subsequently removed from the collected supernatant under reduced pressure at 40 °C using a rotary evaporator (RE100-Pro, Biobase, Jinan, China). The RFE was precisely weighed to determine the extraction yield and subsequently stored at −20 °C for further analysis as needed.

2.5 Total phenolic content analysis

Initially, 10 mg of dried RFE were dissolved in ethanol to get a 10 mg/mL stock solution. Subsequently, 30 microliters of solution were transferred into 96-well plates, followed by the addition of Folin-Ciocalteu reagent with the same volume. The mixtures were mixed gently on the Eppendorf Mixmate® for 1 min and incubated at room temperature for 5 min. A 160 µL sodium carbonate (5.25%) was added to the well and then incubated at room temperature for 30 min with protection from light. Absorbance was recorded at 750 nm using Varioskan™ LUX Multimode Microplate Read (Thermo Scientific™). A serial dilution of gallic acid (0-100 µg/mL) was applied as reference for phenolic compounds. Total phenolic content was expressed as mg gallic acid equivalents per gram of extract (mg/g) [19]. The TPC quantification was conducted in triplicate experiments.

Table 1 Experimental design of simplex lattice design

Run	Independent variables	
	Ethanol (v/v%)	Water (v/v%)
1	0	100
2	0	100
3	25	75
4	50	50
5	50	50
6	75	25
7	100	0
8	100	0

2.6 Total flavonoid content analysis

The TFC was evaluated by a colorimetric assay [19]. Fifty microliters of extract solutionwas transferred to the 96-well plate, followed by the addition of 100 µl ethanol into all wells. Immediately, 20 µL of aluminium chloride (10%) was added to each well and the plate was incubated for 3 min at room temperature. By the end of incubation, 20 µL of sodium acetate 1 M were transferred, followed by 60 µl of ethanol. The absorbance was recorded using Varioskan™ LUX Multimode Microplate Read (Thermo Scientific™) at 430 nm after 40 min of incubation at room temperature and protected from light. As compound references, quercetin was prepared in ethanol and analyzed with the same protocol. Total flavonoid content was expressed as mg quercetin equivalents per gram of extracts (mg/g). The TFC quantification was performed in triplicate experiments.

2.7 2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity

The antioxidant capacity of RFE was evaluated against the DPPH as a free radical indicator in a colorimetric assay [20]. Analysis was conducted in a single point extract final concentration of 500 µg/mL in triplicate experiments. One hundred microliters of RFE were transferred into 96-well assay plates. Immediately, 100 µl of 0.2 mM DPPH solution was added to all wells. The reaction mixture was then mixed on a microplate shaker (Eppendorf Mixmate®) for 1 min, followed by incubation at room temperature in the protection from light for 30 min. In this experiment, ethanol was assigned as the negative control (0% DPPH scavenging capacity) and 0.20 mg/mL vitamin C as the positive control (100% DPPH scavenging capacity). The absorbance was recorded at 517 nm using the Varioskan LUX Multimode Microplate Reader from Thermo Fisher Scientific.

2.8 Gas chromatography-mass spectrophotometry analysis

The analysis was performed on an Agilent Gas Chromatograph coupled to an Agilent 19091S-433:93.92873 DB-5MS UI 5% Phenyl Methul Silox (Agilent Technologies, Santa Clara, CA, USA). The GC was equipped with an HP-5ms Ultra Inert capillary column (30 m × 250 µm × 0.25 µm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min.

The injection volume was 1 μL, performed in splitless mode. The injector temperature was maintained at 280 °C. The oven temperature was programmed as follows: initial temperature of 40 °C held for 1 min, then ramped at a rate of 10 °C/min to 300 °C and held for 4 min. The total run time was 65 min. The raw GC-MS data files were processed using Agilent MassHunter Qualitative Analysis software. Automated peak deconvolution, integration, and identification were performed by comparing the acquired mass spectra against the National Institute of Standards and Technology (NIST) 2020 Mass Spectral Library. A compound was considered tentatively identified if its mass spectrum exhibited a match factor of >80%. Following identification, all compounds were manually curated and classified into distinct chemical groups (alkaloids, terpenoids, carbohydrates, fatty acids, phenolics, alkanes, and others) based on their foundational chemical structures. The relative abundance of each compound was determined via peak area normalization, and the cumulative percentage for each chemical class was calculated by summing the relative abundances of its constituent compounds.

3 Results and discussion

3.1 Model fitting

To model the effects of solvent composition on TPC, TFC, and antioxidant capacity (Table 2), various polynomial equations (linear, two-factor interaction, quadratic, and cubic) were fitted to the experimental data. The optimal predictive model for each response was selected based on the criteria of a non-significant lack-of-fit and the highest values for both the coefficient of determination (R^2) and adjusted R^2, as depicted in Table 3. The selected models demonstrated a robust fit, with R^2 and adjusted R^2 values exceeding 0.80. Furthermore, an adequate precision value greater than 4 for all responses confirmed a high signal-to-noise ratio, indicating model reliability [21].

Table 2 Simplex lattice experimental design and results of total phenolic content, total flavonoid content and antioxidant capacity from RFE

Run	Independent variables		Responses		
	Ethanol (v/v%)	Water (v/v%)	TPC (mg GAE/g)	TFC (mg QE/g)	Antioxidant capacity (%)
1	0	100	37.3 ± 1.68	23.2 ± 4.72	40.95 ± 1.13
2	0	100	36.2 ± 1.67	25.7 ± 4.22	42.23 ± 1.15
3	25	75	38.0 ± 1.73	23.0 ± 1.05	50.81 ± 3.85
4	50	50	63.8 ± 3.36	25.2 ± 0.41	77.50 ± 2.53
5	50	50	64.3 ± 3.36	25.6 ± 0.41	74.59 ± 2.55
6	75	25	102.1 ± 5.88	32.3 ± 5.87	91.23 ± 1.46
7	100	0	114.6 ± 6.71	31.1 ± 0.15	90.20 ± 0.74
8	100	0	119.4 ± 6.71	31.2 ± 0.14	89.53 ± 0.73

Table 3 ANOVA for model optimization

Responses and fitted model	TPC cubic	TFC cubic	Antioxidant capacity (%) cubic
Lack of fit	$p = 0.2125$	$p = 0.1719$	$p = 0.1308$
R^2	0.9975	0.9335	0.9961
Adjusted R^2	0.9957	0.8836	0.9932
Adequate precision	49.2029	10.2347	40.6350

Model accuracy was validated by comparing experimental results with predicted values (Figs. 1 and 2). A strong positive correlation was observed, with data points clustering closely around the regression line. Concurrently, residual plots displayed a random distribution, confirming the absence of systematic error. These statistical evaluations collectively affirm the suitability and predictive power of the developed RSM models.

3.2 Effect of solvent composition on total phenolic content

The composition of the extraction solvent exerted a significant influence on the TPC of RFE, as depicted in Fig. 3(a). The TPC values ranged substantially from 36.2 to 119 mg gallic acid equivalent (GAE)/g of dry extract, underscoring the critical role of solvent selection (Table 1). Notably, 100% ethanol (run 8) yielded the highest TPC (119 mg GAE/g), whereas the pure aqueous solvent (100% water) resulted in the lowest yield (36.2 mg GAE/g). This observation indicates that the phenolic constituents within *R. fraxinifolius* fruits exhibit preferential solubility in less polar solvents like ethanol.

To quantitatively model the relationship between the solvent mixture and TPC, a regression analysis employing a special cubic model was applied. The resulting predictive equation is given as

$$Y_{TPC} = 117.169A + 36.9894B - 46.8518AB + 128.053AB(A - B) \quad (1)$$

where Y_{TPC} represents the TPC response, while A and B denote the proportions of ethanol and water, respectively. The regression coefficients as depicted in Eq. (1) reveal that both linear components (A, B) and the interaction terms (AB, $AB(A-B)$) exerted a significant influence on TPC ($p < 0.05$). The large, positive coefficient for ethanol (A) confirms its dominant role in the extraction of phenolic compounds. Furthermore, the significance of the interaction terms suggests a complex non-linear interaction

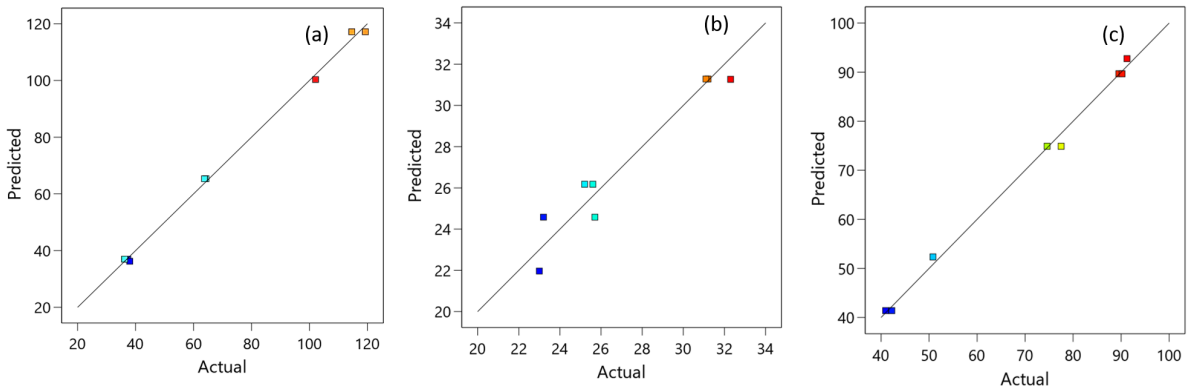

Fig. 1 Predicted versus experimental values for the response model (a) TPC, (b) TFC, and (c) antioxidant capacity

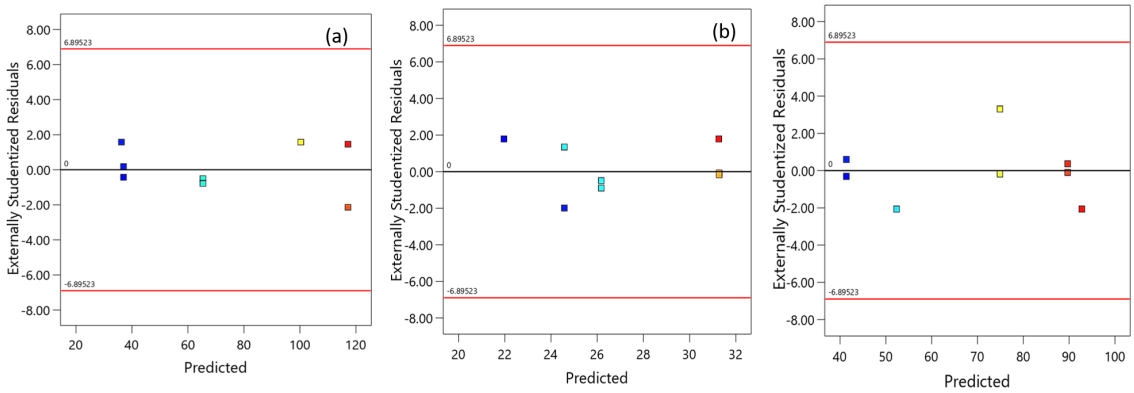

Fig. 2 Residuals versus predicted values illustrating the adequacy of the response models for (a) TPC, (b) TFC, (c) antioxidant capacity

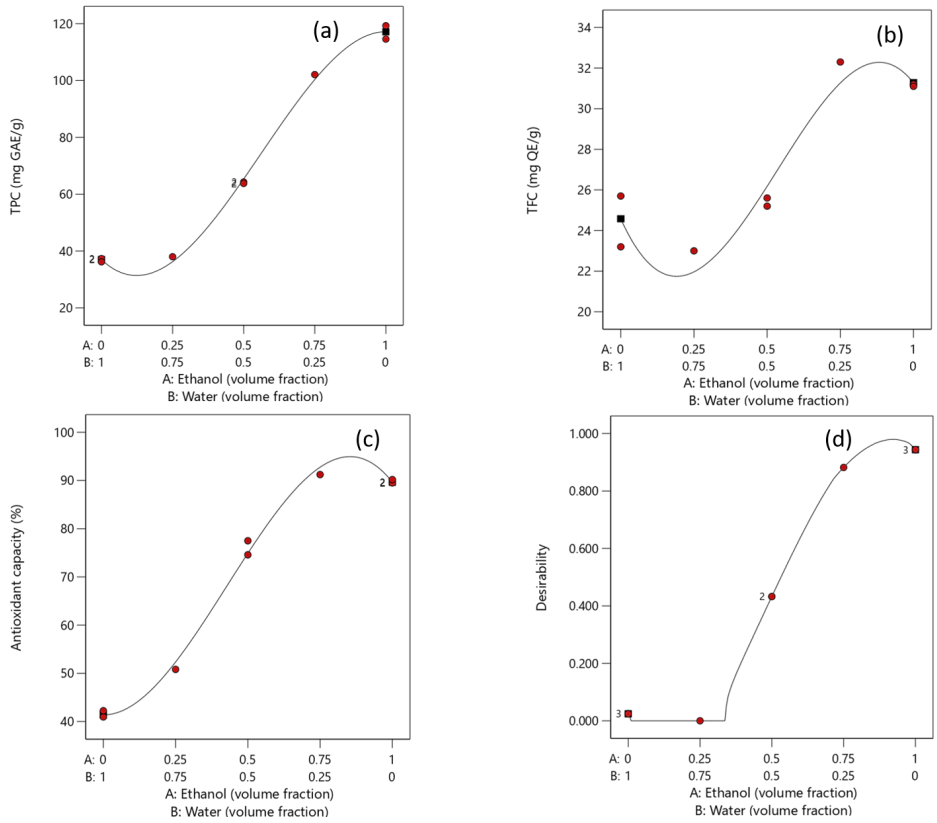

Fig. 3 Effect of solvent composition on the response model (a) TPC; (b) TFC; (c) antioxidant capacity; and (d) optimization plot. The x-axis represents the ethanol–water solvent composition expressed as volume fractions (0–1), corresponding to 0–100% ethanol and 100–0% water, respectively.

between ethanol and water at specific ratios, reinforcing the necessity of optimizing this binary solvent system.

These findings are in agreement with previous studies reporting that hydroethanolic mixtures enhance the extraction of phenolics from various plant matrices [22, 23]. Ethanol is reported to facilitate the disruption of the structural integrity of the plant cell wall, thereby facilitating the release of intracellular bioactive components [24]. Crucially, the polarity of the solvent system plays a pivotal role; hydroethanolic mixtures create a medium of intermediate polarity that is often optimal for extracting a broader spectrum of phenolic compounds, which may be sparingly soluble in either pure water or absolute ethanol alone [25].

The maximum TPC obtained in this study (119 mg GAE/g) is comparable to or higher than values reported in the literature for RFE using conventional extraction methods. For instance, methanolic maceration of RFE has been reported to have a TPC of 11.09 mg GAE/g [5] and 8.7 mg GAE/g [6]. While other research reported varied results using Soxhlet extraction, with the ethyl acetate extract yielding 8.25 mg GAE/g, the high value from their methanol extract (202.21 mg GAE/g) [26] could potentially be attributable to prolonged extraction times at high temperatures, which can co-extract non-phenolic interfering substances.

The higher extraction yield observed in our work may be related to the use of UAE, which can enhance mass transfer during the extraction process. The phenomenon of acoustic cavitation generated by ultrasonic waves accelerates mass transfer by inducing cell wall disruption and fragmentation, thereby enhancing solvent penetration and solute diffusion [27]. This mechanism not only leads to a higher TPC yield but also operates at lower temperatures and in significantly shorter times. This minimizes the risk of thermal degradation of labile phenolic compounds, affirming UAE as a more potent and efficient technique than conventional maceration or Soxhlet extraction for bioprospecting *R. fraxinifolius*.

3.3 Effect of solvent composition on total flavonoid content

The TFC from RFE was profoundly dependent on the solvent composition, revealing a complex relationship graphically elucidated in the model plot, as depicted in Fig. 3(b). The plot demonstrates a distinct, non-linear parabolic trend, underscoring the complex interplay between ethanol and water [28]. The TFC ranged from 23.0 to a peak of 32.3 mg quercetin equivalent (QE)/g of dry extract. Notably, the model predicts, and the observed results indicate that the optimal yield was achieved not with a pure solvent, but with a hydroethanolic mixture of approximately 75% ethanol. The yield curve exhibits a notable non-linear interaction effect at low ethanol concentrations (25%) before a steep, synergistic increase towards the peak, followed by a slight decline as the solvent approached absolute ethanol.

$$Y_{TFC} = 31.2794A + 24.5794B - 7.0117AB + 31.73AB\left(A - B\right), \quad (2)$$

where Y_{TFC} represents the *TFC* response. This trend is a direct visual representation of the special cubic model as depicted in Eq. (2), where significant negative (*AB*) and positive higher-order (*AB*(*A*−*B*)) interaction terms characterize the curvature. From a phytochemical standpoint, this optimum suggests that the 75% ethanol mixture provides the ideal polarity to solubilize the predominant flavonoids in the fruit, which likely exist as moderately polar glycosides. The presence of 25% water is therefore essential, increasing the solvent's affinity for these compounds compared to less-polar absolute ethanol.

The maximum TFC obtained in this study (32.3 mg QE/g) is higher than that obtained using conventional agitated maceration, even under comparable solvent conditions. For instance, our work significantly exceeds the 5.82 ± 0.02 mg catechin equivalent (CE)/g reported in previous work [5]. More pointedly, previous work [6] reported just 7.91 ± 0.19 mg CE/g despite using a conceptually similar solvent system, 80% a methanol and continuous agitation on an orbital shaker for 2 h. This stark difference highlights the limitations of conventional methods, which rely on passive diffusion and low kinetic energy. In contrast, the UAE employed here utilizes high-energy acoustic cavitation to generate microjets and shockwaves that enhance solvent penetration and accelerate mass transfer. While our result is lower than the 58.71 ± 2.65 mg QE/g reported by [26] from an unripe fruit extract, their use of the high-temperature, long-duration Soxhlet method introduces potential confounding factors such as thermal degradation or co-extraction of interfering substances. Therefore, the UAE-based approach provides an optimized solvent system and shows potential for rapid and selective extraction of flavonoids from ripe *R. fraxinifolius*.

3.4 Effect of solvent composition on antioxidant capacity

The DPPH radical scavenging assay effectively evaluates the antioxidant capacity of the RFE, systematically considering the influence of solvent composition. The scavenging capacity varied substantially across the solvent

gradient, ranging from approximately 40% to a maximum of 91.2% at a fixed concentration of 500 µg/ml, as visualized in Fig. 3(c). The scavenging capacity ranged from approximately 40% in pure water to a maximum of 91.2%. Notably, the model predicts that this optimal capacity is achieved not with a pure solvent, but within a specific hydroethanolic mixture of approximately 80% ethanol. This parabolic trend is accurately described by the special cubic regression model (Eq. (3)), where the statistical significance ($p < 0.05$) of the interaction terms suggests a complex non-linear relationship between ethanol and water in producing an extract with optimum antioxidant function.

$$Y_{DPPH} = 89.6732A + 41.3982B + 37.4337AB + 86.84AB(A - B), \quad (3)$$

where Y_{DPPH} represents the *DPPH* response To understand the phytochemical drivers behind this distinct antioxidant profile as depicted in Eq. (3), a correlation with the corresponding phytochemical yields is essential. A comparative analysis reveals that the optimal solvent for antioxidant capacity 80% ethanol occupies an intermediate position between the optima for TPC (Fig. 3(a)) and TPC (Fig. 3(b)). This intriguing result indicates that absolute ethanol yielded the highest TPC, 80-85% produced the highest TFC and antioxidant capacity (Fig. 3), suggesting that antioxidant activity depended more on the composition and reactivity of the extracted phenolic constituents than on TPC alone. The hydroethanolic solvent likely enhanced the recovery of moderately polar antioxidant compounds through increased polarity and improved matrix penetration [13, 29]. Additionally, the parabolic nature of the antioxidant curve closely correlated with the TFC trend, indicating that polar flavonoids are major contributors. However, the shift of the antioxidant peak towards a higher ethanol concentration implies that less-polar phenolics, more efficiently extracted by nearly absolute ethanol, also play a crucial role as potent radical scavengers.

We verified the model predictions relating solvent composition to antioxidant capacity through eight runs of laboratory analysis. The maximum DPPH scavenging capacity of RFE was observed in the ethanol/water (75:25, v/v) mixture with the capacity value of 91.23% ± 1.46 (Table 1). Slightly lower results were shown by the extraction process using absolute ethanol, which produced DPPH capacities with values of 90.20% ± 0.74 and 89.53% ± 0.73. On the other hand, the antioxidant capacity declines as the decrease of ethanol and water ratio decreases which results in aqueous environment. An equal part of ethanol/water (50:50 v/v) could extract a group of compounds that reduce scavenging capacity; 77.50% ± 2.53 and 74.59% ± 2.55. Fruit samples extracted with aqueous solvent tend to exhibit weak antioxidant capacity as seen in this study. A mixture of ethanol/water (25:75, v/v) generated a scavenging capacity of 50.81% ± 3.85, followed by water solvent which resulted in the smallest capacity; 42.23% ± 1.15 and 40.95% ± 1.13. The choice of solvent and extraction techniques is a critical protocol in the extraction to obtain targeted phytochemical constituents. Isolation of antioxidant-related compounds from the plant matrix, including phenol group, was carried out efficiently by polar solvents such as methanol and ethanol [30]. The results in Table 2 clearly illustrate the effectiveness of polar solvents for recovering plant-based antioxidant compounds.

Plant-derived antioxidants, in this case fruit fraction, have shown some benefits in health-promoting and disease-preventing effects. The potency of *R. fraxinifolius* fruit was characterized in this study by high antioxidant capacity (91.2%) at 500 µg/ml, suggesting its potential as a natural antioxidant agent. UAE offers alternative extraction techniques to improve the recovery of antioxidant-related compounds from plant parts. Previous study that conducted RFE in conventional maceration technique has been reported by [6] (IC_{50} of 765 µg/mL for ethanol, where IC_{50} means half-maximal inhibitory concentration) and [5] (IC_{50} of 86.00 µg/mL). Although an exceptionally low IC_{50} (19.74 µg/ml) was reported by [26] for an extract from unripe fruit, this result is likely influenced by the fruit's unique maturational stage. Ultimately, the ability of our optimized UAE process to efficiently liberate the specific blend of moderately polar flavonoids and less-polar phenolics is proposed to contribute to the high functional activity observed. These results suggest that the technique is suitable for producing a chemically complex and biologically active extract from ripe *R. fraxinifolius*.

3.5 Model recommendation and validation

Fig. 3(d) depicts the optimum point that has been utilized with SLD-RSM. The factor and response were valued with a desirability value. The high desirability value indicates that the chosen conditions converge towards optimal extraction [31], reflecting a high level of efficiency and utility in addressing the extraction objectives [32]. The ideal conditions determined through numerical optimization indicated that a combination of 92% ethanol and 8% water yielded the highest TPC at 115.32 mg GAE/g, TFC at 32.178 mg QE/g, and an antioxidant capacity of 93.86% scavenging, along with a high desirability score of 0.979.

The optimized parameter was then used to test the validity of the model. The validation was based on the use of the 95% Prediction Interval (PI), which provides a more stringent assessment compared to a simpler error metric or confidence interval [33]. The 95% PI is defined by its lower and upper boundaries, denoted as PI low and PI high, respectively. This range constitutes the operational window within which a new experimental outcome is expected to fall with 95% confidence [34]. Following this framework, triplicate experiments were executed, and their mean values were benchmarked against the model's 95% PI ranges, as delineated in Table 4.

The empirical results demonstrated a clear and decisive validation of the model. The error rates were found to be consistently low across all responses (2.51-3.86%). Such minimal deviations underscore the model's precision in addition to its predictive accuracy [35]. The values that were observed fall within the confidence interval surrounding the mean of the predicted values, confirming that the numerical optimization was successful. Beyond mathematical rigor, this successful validation using the 95% PI highlights its practical relevance [34] for both academia and industry. For academic purposes, it confirms the high reproducibility of the empirical equations. From an industrial perspective, this narrow prediction interval functions as a defined tolerance margin for quality control. It provides manufacturers with a high degree of certainty that scaling up [36] this optimized extraction will yield consistent bioactive recoveries, thereby minimizing batch-to-batch variability and ensuring a predictable, commercially viable process.

Furthermore, a comparative evaluation of the optimized UAE and conventional maceration under equivalent solvent conditions was conducted to assess extraction performance. The results showed that UAE significantly enhanced flavonoid recovery, yielding a TFC of 31.5 mg QE/g compared to 11.9 mg QE/g obtained by maceration. In contrast, maceration resulted in a higher TPC (231.1 mg GAE/g) than UAE (110.87 mg GAE/g), indicating that extraction efficiency may vary depending on compound class. In terms of antioxidant activity, the UAE extract exhibited stronger radical scavenging capacity, with a lower IC_{50} value (145.7 µg/mL) compared to maceration (186.1 µg/mL). These findings suggest that UAE improves the recovery of flavonoids and enhances antioxidant activity, likely due to cavitation effects that promote cell wall disruption and mass transfer [37, 38] whereas maceration may favor the extraction of certain phenolic compounds [39]. Overall, the inclusion of this comparative analysis confirms the effectiveness of the optimized green extraction approach and provides a more comprehensive evaluation of its performance relative to conventional methods.

3.6 Metabolite profilling optimum *R. fraxinifolius* extract with gas chromatography – mass spectrometry

The comprehensive metabolite profile of the optimum *R. fraxinifolius* extract was elucidated by GC-MS to provide a molecular-level explanation for its previously determined high bioactivity, as depicted in Table 5 and Fig. 4. The curated data revealed a unique phytochemical signature, compositionally dominated by three major classes: carbohydrate, phenolic compounds (24.20%), and

Table 4 Predicted values from the optimization point and model validation for the UAE, comparing with maceration

Responses	Predicted	95% prediction interval (PI)	Experimental mean*	Error (%)	Maceration
TPC (mg GAE/g)	115.32	109.14; 132.73	110.87 ± 4.41	3.86	231.1 ± 4.31
TFC (mg QE/g)	32.18	28.76; 35.60	31.51 ± 1.28	2.07	11.9 ± 1.27
Antioxidant capacity (%)	93.87	89.81; 98.62	91.52 ± 0.61	2.51	n.d**
DPPH scavenging IC_{50} (µg/mL)	n.d	n.d	145.7 ±2.18	n.d	186.1 ± 2.03

* Values represent the mean of triplicate experiments (n = 3).
** n.d: not determined.

Table 5 Bioactive compounds present in ORFE provided by GC-MS analysis

No	Retention time (min)	Compound	Formula	Area (%)
Alkaloid				
1	4.8054	1H-Imidazole, 1,5-dimethyl-	$C_5H_8N_2$	8.098
2	8.9266	1,3-Dimethylimidazole-2(3H)-thione	$C_5H_8N_2S$	1.745
3	10.8675	N-Amino-1,2,3,4-tetrahydroquinolin	$C_9H_{12}N_2$	14.270
Alkane				
4	10.3760	Undecane, 4-methyl-	$C_{12}H_{26}$	0.769
5	14.5854	Pentadecane	$C_{15}H_{32}$	0.861

Table 5 Bioactive compounds present in ORFE provided by GC-MS analysis (continued)

No	Retention time (min)	Compound	Formula	Area (%)
6	15.0013	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	1.355
7	15.5054	3-Ethyl-2,6,10-trimethylundecane	$C_{31}H_{64}$	0.298
8	15.5936	Nonadecane	$C_{19}H_{40}$	0.257
9	16.3624	Tetracosane, 1-iodo-	$C_{24}H_{49}I$	0.286
10	16.5767	Eicosane	$C_{20}H_{42}$	0.460
11	16.7909	10-Methylnonadecane	$C_{20}H_{42}$	1.212
12	16.8917	Hentriacontane	$C_{31}H_{64}$	0.374
13	16.9548	Octacosane, 2-methyl-	$C_{29}H_{60}$	0.972
14	17.2824	2-Methylhexacosane	$C_{27}H_{56}$	0.729
15	18.0638	Octadecane	$C_{18}H_{38}$	0.252
16	19.0343	Octacosane	$C_{28}H_{58}$	0.803
17	20.2064	Tetradecane, 2-methyl-	$C_{15}H_{32}$	0.182
18	23.6596	Heneicosane	$C_{21}H_{44}$	0.164
19	24.4662	Pentacosane	$C_{25}H_{52}$	0.045
20	25.2350	Hexacosane	$C_{26}H_{54}$	0.046
Carbohydrate-derived				
21	6.6329	2,5-Furandione, dihydro-3-methylene-	$C_5H_4O_3$	2.495
22	9.0526	Methyl 2-furoate	$C_6H_6O_3$	0.554
23	9.6198	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	5.536
24	11.4598	5-Hydroxymethylfurfural	$C_6H_6O_3$	17.619
Fatty acid				
25	5.3221	Hexadecanoic acid, 3-hydroxy-, methyl ester	$C_{19}H_{36}O_2$	0.262
26	6.2926	5-Hexenoic acid	$C_6H_{10}O_2$	0.457
27	6.4312	Hexanoic acid	$C_6H_{12}O_2$	0.229
28	7.5403	Heptanoic acid	$C_7H_{14}O_2$	2.624
29	19.7526	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	2.689
30	20.0299	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	0.782
31	20.4962	Nonahexacontanoic acid	$C_{69}H_{138}O_2$	0.235
32	21.0508	11-octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	0.424
33	21.4793	9-Octadecenoic acid, (E)-	$C_{18}H_{34}O_2$	4.263
34	21.6431	Octadecanoic acid	$C_{18}H_{36}O_2$	0.979
35	21.9582	Carbonic acid, eicosyl vinyl ester	$C_{23}H_{44}O_3$	0.158
36	24.7434	Octadecane, 1-(ethenyloxy)-	$C_{23}H_{44}O_3$	0.038
37	25.9911	sulfurous acid, 2-propyl tetradecyl ester	$C_{17}H_{36}O_3S$	0.030
Others				
38	9.1157	2-Octen-4-ol	$C_8H_{16}O$	0.441
39	20.8869	d-Manno-l-gluco-octonic acid	$C_8H_{16}O_8$	0.165
40	22.0590	1 (2H) -Naphtalenone, octahydro-, trans -	$C_{10}H_{16}O$	0.317
41	23.0168	Hexasiloxane, tetradecamethyl-	$C_{14}H_{42}O_5Si_6$	0.920
42	25.1215	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	$C_{13}H_{39}O_5Si_6$	0.136
Phenolic				
43	7.1244	Anethole	$C_{10}H_{12}O$	23.788
44	29.1167	ɣ-Tocopherol	$C_{28}H_{48}O_2$	0.411
Terpenoid				
45	8.6746	7-Oxabicyclo[4.1.0]heptan-2-one, 6-methyl-3-(1-methylethyl)-	$C_{10}H_{14}O_2$	0.824
46	16.1986	Sesquicineole	$C_{15}H_{26}O$	0.446

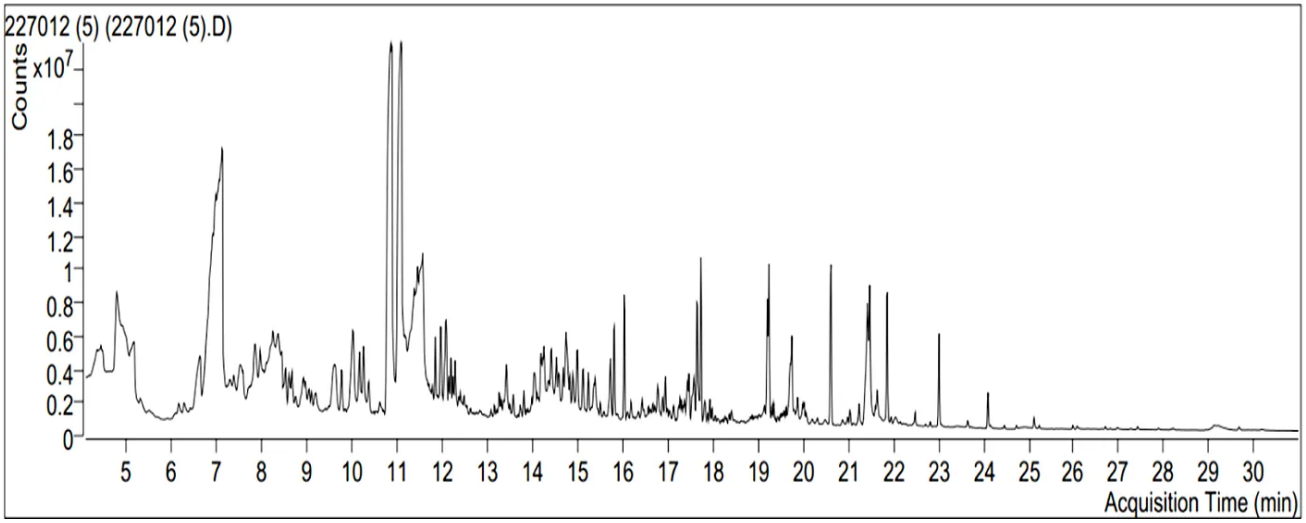

Fig. 4 GC-MS chromatogram showing the chemical profile of the ORFE

alkaloids (24.11%), as visualized in Fig. 5. The detailed analysis of these groups, particularly the identification of specific bioactive compounds, allows for a robust interpretation of the high TPC, TFC, and antioxidant capacity (93.87% inhibition) of the ORFE.

A revealing feature of the ORFE is the profound abundance of 5-hydroxymethylfurfural (5-HMF) at 17.62%. The formation of 5-HMF is primarily driven by the acid-catalyzed dehydration of endogenous hexose sugars – specifically fructose and glucose – during the thermal drying process of the Rubus fruit [40, 41]. This provides critical insight into the sample's history, as thermal load required for effective moisture removal facilitates the Maillard reaction and sugar degradation. While often considered a processing marker, it is crucial to note that 5-HMF itself is a well-documented antioxidant [42]. Therefore, due to its sheer concentration, it must be considered a significant contributor to the overall bioactivity, representing a key component of the extract's final chemical identity.

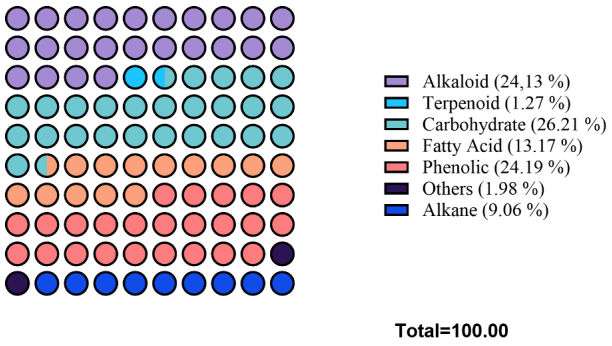

Fig. 5 Relative peak area (%) of phytochemical classes identified in ORFE represented as a 10 × 10 dot plot

The potent antioxidant capacity is likely associated with the presence of key phenolic compounds. Among these, anethole (23.79%) was putatively identified, a phenylpropanoid known for its antioxidant properties [43–45], suggesting it as a significant contributor to the observed activity. Mechanistically, anethole may contribute to this observed activity through its conjugated double-bond system, which allows for resonance stabilization of free radicals [46]. Furthermore, the analysis identified γ-tocopherol (vitamin E). Although modest in abundance (0.41%), γ-tocopherol is an exceptionally potent, chain-breaking lipophilic antioxidant [47, 48] that operates via a hydrogen atom transfer (HAT) mechanism, where the phenolic hydroxyl group donates a hydrogen atom to neutralize peroxyl radicals [49, 50]. Its successful extraction is a testament to the efficacy of the high-ethanol (92%) solvent system, potentially providing a molecular basis for the extract's radical scavenging ability [51]. The possible collective interaction of these compounds, likely supported by the minor terpenoid fraction, is proposed to contribute to the extract's antioxidant power as observed by GC-MS.

In addition to phytochemical composition, safety considerations are essential when evaluating the nutraceutical potential of *R. fraxinifolius* fruit extracts. 5-HMF, a compound formed during the Maillard reaction, is commonly present in a wide range of food products and has been reported to exhibit low toxicity, with no clear evidence of carcinogenicity in short- and long-term studies, aside from findings in female mice that are considered to have limited relevance to humans [52]. Anethole, a naturally occurring terpenoid found in various aromatic plants, has also been extensively studied and is reported

to be non-toxic at low concentrations [53, 54] as well as non-genotoxic and non-carcinogenic under typical conditions of use [43, 55]. Collectively, these findings suggest that the major compounds identified in the present study are unlikely to pose significant health risks at normal exposure levels; however, further toxicological evaluation of the extract as a whole is recommended to confirm its safety for nutraceutical applications.

Finally, this detailed semi-volatile profile must be reconciled with the exceptionally high TPC (115.32 mg GAE/g) and TFC (32.18 mg QE/g) values. While the GC-MS profiling provides a detailed identification of the volatile and semi-volatile constituents in *R. fraxinifolius*, it is primarily suited for thermally stable and relatively non-polar molecules. Furthermore, although the assignment of metabolites such as anethole was based on high library match factors from the NIST database, these identifications should be considered tentative and require future confirmation using authentic standards or complementary spectroscopic approaches. Consequently, highly polar and high-molecular-weight compounds, such as complex tannins and glycosylated flavonoids – which likely contribute to the elevated phenolic and flavonoid totals – may remain undetected in the current GC-MS profile.

Furthermore, the optimization was strategically focused on solvent composition, while other physical UAE parameters such as sonication time, amplitude, and temperature, were kept constant based on established protocols. While this isolation of variables was necessary for the SLD model's clarity, it suggests a window for future multivariate optimization of the physical extraction environment. Consequently, the current chemical profile should be viewed as a complementary rather than an exhaustive characterization. Future work employing liquid chromatography – mass spectrometry/mss spectrometry [56] and in vitro toxicity assays is therefore necessary to fully elucidate the non-volatile constituents and confirm the safety profile of this highly active extract.

4 Conclusion

This study successfully employed and validated SLD-RSM to optimize the UAE from *R. fraxinifolius* fruit, concurrently maximizing three key responses: TPC, TFC and DPPH radical scavenging capacity. An optimal solvent system consisting of 92.2% ethanol and 7.8% water was experimentally validated, showing improved extraction efficiency and functional characteristics of ORFE under the studied conditions. The investigation revealed that its bioactivity is underpinned by a complex interplay between different phytochemical classes. This synergy arises from the combined action of the substantial non-volatile polyphenolic load, evidenced by the high TPC and TFC results, and a unique profile of potent semi-volatile compounds, including anethole, γ-tocopherol, and a significant alkaloid fraction, as identified by GC-MS.

Ultimately, this work provides not only a scientifically validated, green extraction protocol for *R. fraxinifolius* but also a more nuanced understanding of the chemical basis for its potent bioactivity. The successful application of UAE was critical in achieving the effective co-extraction of these chemically diverse, synergistic components. These findings establish a robust scientific foundation for developing *R. fraxinifolius* as a source of high-value, chemically characterized ingredients for the nutraceutical and functional food industries. Future work should integrate complementary liquid chromatography-mass spectrometry analysis to characterize the non-volatile phenolic fraction, which would further validate the synergistic effects proposed in this work.

Acknowledgement

The authors express their gratitude for the facilities, scientific, and technical support from the Advanced Characterization Laboratory, National Research and Innovation Agency, through E-Layanan-Sains (ELSA) – BRIN.

References

[1] Tajner-Czopek, A., Gertchen, M., Rytel, E., Kita, A., Kucharska, A. Z., Sokół-Łętowska, A. "Study of Antioxidant Activity of Some Medicinal Plants Having High Content of Caffeic Acid Derivatives", Antioxidants, 9(5), 412, 2020. https://doi.org/10.3390/antiox9050412

[2] Neves, K. O. G., Silva, S. O., Cruz, M. S., Mar, J. M., Bezerra, J. A., ..., Santos, A. D. C. "Investigation of the Influence of the Extraction System and Seasonality on the Pharmacological Potential of Eugenia Punicifolia Leaves", Molecules, 30(3), 713, 2025. https://doi.org/10.3390/molecules30030713

[3] Farahmandfar, R., Asnaashari, M., Sayyad, R. "Comparison Antioxidant Activity of Tarom Mahali Rice Bran Extracted from Different Extraction Methods and Its Effect on Canola Oil Stabilization", Journal of Food Science and Technology, 52(10), pp. 6385–6394, 2015. https://doi.org/10.1007/s13197-014-1702-2

[4] Hamid, H., Thakur, N. S., Rana, N., Thakur, A. "Effect of Different Solvents on Extraction of Phenolic Antioxidants by Reflux Method from Wild Pomegranate Flavedo, Their Antioxidant and Antibacterial Properties", Indian Journal of Pharmaceutical Sciences, 84(4), pp. 838–847, 2022. https://doi.org/10.36468/pharmaceutical-sciences.979

[5] Abu Bakar, M. F., Ismail, N. A., Isha, A., Mei Ling, A. L. "Phytochemical Composition and Biological Activities of Selected Wild Berries (*Rubus Moluccanus* L., *R. Fraxinifolius* Poir., and *R. Alpestris* Blume)", Evidence-Based Complementary and Alternative Medicine, 2016(1), 2482930, 2016. https://doi.org/10.1155/2016/2482930

[6] Shamsudin, N. A., Matawali, A., Gansau, J. A. "Comparison of Antioxidant Activity and Phytochemical Content of Borneo Wild Berry, *Rubus Fraxinifolius* (Rogimot)", Transactions on Science and Technology, 6(1), pp. 36–41, 2019

[7] Muck, R., Weinberg, Z. G., Contreras-Govea, F. E. "Silage Extracts Used to Study the Mode of Action of Silage Inoculants in Ruminants", Agricultural and Food Science, 22(1), pp. 108–114, 2013. https://doi.org/10.23986/afsci.6717

[8] Putranto, S., Rahayu, S., Ciptadi, G., Nanda Widhaningrum, S. "Effect of a Combination of Water Clover (*Marsilea Crenata*) Ethanol Extract and Moringa Leaf (*Moringa Oleifera*) Ethanol Extract on Follicle and Oocyte Diameter of Goat: *In Vitro* Study", Biotropika: Journal of Tropical Biology, 11(2), pp. 101–105, 2023. https://doi.org/10.21776/ub.biotropika.2023.011.02.05

[9] Kim, Y. S., Yuk, H. J., Kim, D. S. "Effect of Jakyakgamcho-Tang Extracts on H_2O_2-Induced C2C12 Myoblasts", Molecules, 26(1), 215, 2021. https://doi.org/10.3390/molecules26010215

[10] Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., Ju, Y.-H. "Effect of Extraction Solvent on Total Phenol Content, Total Flavonoid Content, and Antioxidant Activity of *Limnophila Aromatica*", Journal of Food and Drug Analysis, 22(3), pp. 296–302, 2014. https://doi.org/10.1016/j.jfda.2013.11.001

[11] Chinnarasu, C., Prabha, K. "Potential for Fourier Transform Infrared Spectroscopy toward Predicting Phytochemicals with Potent Antioxidant Activity in Leaves of *Annona Squamosa* Linn", Indian Journal of Pharmaceutical Sciences, 85(1), pp. 267–272, 2023. https://doi.org/10.36468/pharmaceutical-sciences.1091

[12] Mosca, F., Hidalgo, G. I., Villasante, J., Almajano, M. P. "Continuous or Batch Solid-Liquid Extraction of Antioxidant Compounds from Seeds of *Sterculia apetala* Plant and Kinetic Release Study", Molecules, 23(7), 1759, 2018. https://doi.org/10.3390/molecules23071759

[13] Psarrou, I., Oreopoulou, A., Tsimogiannis, D., Oreopoulou, V. "Extraction Kinetics of Phenolic Antioxidants from the Hydro Distillation Residues of Rosemary and Effect of Pretreatment and Extraction Parameters", Molecules, 25(19), 4520, 2020. https://doi.org/10.3390/molecules25194520

[14] Rizvi, S. N. R., Afzal, S., Khan, K.-R., Aati, H. Y., Rao, H., Ghalloo, B. A., Shahzad, M. N., Khan, D. A., Esatbeyoglu, T., Korma, S. A. "Chemical Characterisation, Antidiabetic, Antibacterial, and In Silico Studies for Different Extracts of *Haloxylon stocksii* (Boiss.) Benth: A Promising Halophyte", Molecules, 28(9), 3847, 2023. https://doi.org/10.3390/molecules28093847

[15] Fărcaș, A. C., Socaci, S. A., Chiș, M. S., Dulf, F. V., Podea, P., Tofană, M. "Analysis of Fatty Acids, Amino Acids and Volatile Profile of Apple By-Products by Gas Chromatography-Mass Spectrometry", Molecules, 27(6), 1987, 2022. https://doi.org/10.3390/molecules27061987

[16] Oulad El Majdoub, Y., Alibrando, F., Cacciola, F., Arena, K., Pagnotta, E., Matteo, R., Micalizzi, G., Dugo, L., Dugo, P., Mondello, L. "Chemical Characterization of Three Accessions of *Brassica juncea* L. Extracts from Different Plant Tissues", Molecules, 25(22), 5421, 2020. https://doi.org/10.3390/molecules25225421

[17] Mathe, E., Sethoga, L., Mapfumari, S., Adeniran, O., Mokgotho, P., Shai, J., Gololo, S. "Phytochemical Screening and Characterization of Volatile Compounds from Three Medicinal Plants with Reported Anticancer Properties Using GC-MS", Life, 14(11), 1375, 2024. https://doi.org/10.3390/life14111375

[18] Leksawasdi, N., Taesuwan, S., Prommajak, T., Techapun, C., Khonchaisri, R., ..., Khemacheewakul, J. "Ultrasonic Extraction of Bioactive Compounds from Green Soybean Pods and Application in Green Soybean Milk Antioxidants Fortification", Foods, 11(4), 588, 2022. https://doi.org/10.3390/foods11040588

[19] Sari, K. R. P., Ikawati, Z., Danarti, R., Hertiani, T. "Micro-Titer Plate Assay for Measurement of Total Phenolic and Total Flavonoid Contents in Medicinal Plant Extracts", Arabian Journal of Chemistry, 16(9), 105003, 2023. https://doi.org/10.1016/j.arabjc.2023.105003

[20] Scherer, R., Godoy, H. T. "Antioxidant Activity Index (AAI) by the 2,2-Diphenyl-1-Picrylhydrazyl Method", Food Chemistry, 112(3), pp. 654–658, 2009. https://doi.org/10.1016/j.foodchem.2008.06.026

[21] Sharayei, P., Azarpazhooh, E., Einafshar, S., Zomorodi, S., Zare, F., Ramaswamy, H. S. "Optimization of Wall Materials for Astaxanthin Powder Production from Shrimp Shell Extract Using Simplex Lattice Mixture Design", Journal of Food Processing and Preservation, 2024, 9794290, 2024. https://doi.org/10.1155/2024/9794290

[22] Radzali, S. A., Markom, M., Saleh, N. M. "Co-Solvent Selection for Supercritical Fluid Extraction (SFE) of Phenolic Compounds from *Labisia pumila*", Molecules, 25(24), 5859, 2020. https://doi.org/10.3390/molecules25245859

[23] Bisognin, D. A., Luz, L. V. da, Lencina, K. H., Santos, C. O. dos, Sautter, C. K. "Contents of Total Phenolics and Flavonoids in and Antioxidant Activity of *Ilex paraguariensis* Leaves", Pesquisa Agropecuária Brasileira, 54, e00856, 2019. https://doi.org/10.1590/s1678-3921.pab2019.v54.00856

[24] Aminina, N. M., Karaulova, E. P., Vishnevskaya, T. I., Yakush, E. V., Kim, Y.-K., Nam, K.-H., Son, K.-T. "Characteristics of Polyphenolic Content in Brown Algae of the Pacific Coast of Russia", Molecules, 25(17), 3909, 2020. https://doi.org/10.3390/molecules25173909

[25] Aguilar-Piloto, G., Negrón-Diaz, A. C., Moo-Huchin, V. M., Ramírez-Sucre, M. O., Delgadillo-Díaz, M., Cuevas-Glory, L. F., Sauri-Duch, E. "Ultrasound-Assisted Extraction (UAE) of Phenolic Compounds from *Brosimum Alicastrum* Fruit and Their Antioxidant Capacity", Ecosistemas y Recursos Agropecuarios, 10(NEIII), e3655, 2023. https://doi.org/10.19136/era.a10nNEIII.3655

[26] Desmiaty, Y., Elya, B. "Unripe Fruit of *Rubus Fraxinofolius* as a Potential Source of Antioxidant and Antielastase Agent", International Journal of Applied Pharmaceutics, 13(SI2), pp. 78–81, 2021. https://doi.org/10.22159/ijap.2021.v13s2.15

[27] Hoo, D. Y., Low, Z. L., Low, D. Y. S., Tang, S. Y., Manickam, S., Tan, K. W., Ban, Z. H. "Ultrasonic Cavitation: An Effective Cleaner and Greener Intensification Technology in the Extraction and Surface Modification of Nanocellulose", Ultrasonics Sonochemistry, 90, 106176, 2022. https://doi.org/10.1016/j.ultsonch.2022.106176

[28] Md Yusof, A. H., Abd Gani, S. S., Zaidan, U. H., Halmi, M. I. E., Zainudin, B. H. "Optimization of an Ultrasound-Assisted Extraction Condition for Flavonoid Compounds from Cocoa Shells (*Theobroma cacao*) Using Response Surface Methodology", Molecules, 24(4), 711, 2019. https://doi.org/10.3390/molecules24040711

[29] Lee, J.-E., Jayakody, J., Kim, J.-I., Jeong, J.-W., Choi, K.-M., Kim, T.-S., Seo, C., Azimi, I., Hyun, J., Ryu, B. "The Influence of Solvent Choice on the Extraction of Bioactive Compounds from Asteraceae: A Comparative Review", Foods, 13(19), 3151, 2024. https://doi.org/10.3390/foods13193151

[30] Sultana, B., Anwar, F., Ashraf, M. "Effect of Extraction Solvent/ Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts", Molecules, 14(6), pp. 2167–2180, 2009. https://doi.org/10.3390/molecules14062167

[31] Suhendar, D., Rahmadini, D. P., Pikoli, M. R., Sriherwanto, C., Wahjono, E., ..., Nurhayati, N. "Immobilization of Penicillin G Acylase (PGA) on Cellulose Nanocrystalline (CNC) Produced by *Escherichia coli* BL21 PET28-PrPGA", Biocatalysis and Biotransformation, 43(4), pp. 424–436, 2025. https://doi.org/10.1080/10242422.2025.2508467

[32] Giang, D. H., Le, B. T. N., Minh, N. T. T., Thuy, N. T. T., Dang, N. H., Anh, H. L. T., Tung, N. N., Dat, N. T. "Optimization of the Extraction Conditions and Evaluation of Bioactivities of the Phenolic Enrichment From *Pandanus amaryllifolius* Leaves", Journal of Analytical Methods in Chemistry, 2025(1), 5256388, 2025. https://doi.org/10.1155/jamc/5256388

[33] Nourani, V., Paknezhad, N. J., Tanaka, H. "Prediction Interval Estimation Methods for Artificial Neural Network (ANN)-Based Modeling of the Hydro-Climatic Processes, a Review", Sustainability, 13(4), 1633, 2021. https://doi.org/10.3390/su13041633

[34] Coskun, A. "Prediction Interval: A powerful statistical tool for monitoring patients and analytical systems", Biochemia Medica, 34(2), pp. 175–185, 2024. https://doi.org/10.11613/BM.2024.020101

[35] Anis, N., Ahmed, D. "Modelling and Optimization of Polyphenol and Antioxidant Extraction from *Rumex Hastatus* by Green Glycerol-Water Solvent According to Response Surface Methodology", Heliyon, 8(12), e11992, 2022. https://doi.org/10.1016/j.heliyon.2022.e11992

[36] Saputra, F. A., Khayrani, A. C., Fauzantoro, R. A., Marwanta, E., Mahsunah, A. H., ..., Herianto, G. "Optimizing S-Allyl-Cysteine (SAC) Content in Extract Black Garlic (*Stamilic*) for Health Supplement", AIP Conference Proceedings, 3080, 070001, 2024. https://doi.org/10.1063/5.0199929

[37] Liu, F.-F., Li, Y.-Q., Sun, G.-J., Wang, C.-Y., Liang, Y., Zhao, X.-Z., He, J.-X., Mo, H.-Z. "Influence of Ultrasound Treatment on the Physicochemical and Antioxidant Properties of Mung Bean Protein Hydrolysate", Ultrasonics Sonochemistry, 84, 105964, 2022. https://doi.org/10.1016/j.ultsonch.2022.105964

[38] Osorio-Tobón, J. F. "Recent Advances and Comparisons of Conventional and Alternative Extraction Techniques of Phenolic Compounds", Journal of Food Science and Technology, 57(12), pp. 4299–4315, 2020. https://doi.org/10.1007/s13197-020-04433-2

[39] Singla, M., Sit, N. "Application of Ultrasound in Combination with Other Technologies in Food Processing: A Review", Ultrasonics Sonochemistry, 73, 105506, 2021. https://doi.org/10.1016/j.ultsonch.2021.105506

[40] Martins, F. C. O. L., Alcantara, G. M. R. N., Silva, A. F. S., Melchert, W. R., Rocha, F. R. P. "The Role of 5-Hydroxymethylfurfural in Food and Recent Advances in Analytical Methods", Food Chemistry, 395, 133539, 2022. https://doi.org/10.1016/j.foodchem.2022.133539

[41] Herrero, M., Castro-Puyana, M., Rocamora-Reverte, L., Ferragut, J. A., Cifuentes, A., Ibáñez, E. "Formation and Relevance of 5-Hydroxymethylfurfural in Bioactive Subcritical Water Extracts from Olive Leaves", Food Research International, 47(1), pp. 31–37, 2012. https://doi.org/10.1016/j.foodres.2012.01.008

[42] Li, W., Qu, X.-N., Han, Y., Zheng, S.-W., Wang, J., Wang, Y.-P. "Ameliorative Effects of 5-Hydroxymethyl-2-Furfural (5-HMF) from *Schisandra chinensis* on Alcoholic Liver Oxidative Injury in Mice", International Journal of Molecular Sciences, 16(2), pp. 2446–2457, 2015. https://doi.org/10.3390/ijms16022446

[43] Freire, R. S., Morais, S. M., Catunda-Junior, F. E. A., Pinheiro, D. C. S. N. "Synthesis and Antioxidant, Anti-Inflammatory and Gastroprotector Activities of Anethole and Related Compounds", Bioorganic & Medicinal Chemistry, 13(13), pp. 4353–4358, 2005. https://doi.org/10.1016/j.bmc.2005.03.058

[44] Rostami-Faradonbeh, N., Amini-Khoei, H., Zarean, E., Bijad, E., Lorigooini, Z. "Anethole as a Promising Antidepressant for Maternal Separation Stress in Mice by Modulating Oxidative Stress and Nitrite Imbalance", Scientific Reports, 14(1), 7766, 2024. https://doi.org/10.1038/s41598-024-57959-2

[45] Marinov, V., Valcheva-Kuzmanova, S. "Review on the Pharmacological Activities of Anethole", Scripta Scientifica Pharmaceutica, 2(2), 14, 2015. https://doi.org/10.14748/ssp.v2i2.1141

[46] Raposo, A., Raheem, D., Zandonadi, R. P., Suri, N., Olukosi, ..., Calina, D. "Anethole in Cancer Therapy: Mechanisms, Synergistic Potential, and Clinical Challenges", Biomedicine & Pharmacotherapy, 180, 117449, 2024. https://doi.org/10.1016/j.biopha.2024.117449

[47] Sipoloni, V. M., Bassicheto, M. C., de Oliveira, M. V., de Souza, A. B. dos S., Alves, G. M., Veiga, T. A. M. "Nonconventional Techniques in Plant Alkaloid Extraction: A Decade of Progress (2014–2023)", Chemistry & Biodiversity, 22(8), e202403225, 2025. https://doi.org/10.1002/cbdv.202403225

[48] Vega, E. N., González-Zamorano, L., Cebadera, E., Barros, L., da Silveira, T. F. F., ..., Morales, P. "Wild *Myrtus communis* L. Fruit By-Product as a Promising Source of a New Natural Food Colourant: Optimization of the Extraction Process and Chemical Characterization", Foods, 14(3), 520, 2025. https://doi.org/10.3390/foods14030520

[49] Caruso, F., Pedersen, J. Z., Incerpi, S., Belli, S., Sakib, R., Rossi, M. "Interaction between Vitamins C and E When Scavenging the Superoxide Radical Shown by Hydrodynamic Voltammetry and DFT", Biophysica, 4(2), pp. 310–326, 2024. https://doi.org/10.3390/biophysica4020022

[50] Olbińska, E., Trela-Makowej, A., Larysz, W., Orzechowska, A., Szymańska, R. "The Effect of α-Tocopherol Incorporated into Different Carriers on the Oxidative Stability of Oil in Water (O/W) Emulsions", Colloids and Surfaces B: Biointerfaces, 230, 113536, 2023. https://doi.org/10.1016/j.colsurfb.2023.113536

[51] Ponphaiboon, J., Krongrawa, W., Aung, W. W., Chinatangkul, N., Limmatvapirat, S., Limmatvapirat, C. "Advances in Natural Product Extraction Techniques, Electrospun Fiber Fabrication, and the Integration of Experimental Design: A Comprehensive Review", Molecules, 28(13), 5163, 2023. https://doi.org/10.3390/molecules28135163

[52] Abraham, K., Gürtler, R., Berg, K., Heinemeyer, G., Lampen, A., Appel, K. E. "Toxicology and Risk Assessment of 5-Hydroxymethylfurfural in Food", Molecular Nutrition & Food Research, 55(5), pp. 667–678, 2011. https://doi.org/10.1002/mnfr.201000564

[53] Ponte, E. L., Sousa, P. L., Rocha, M. V. A. P., Soares, P. M. G., Coelho-de-Souza, A. N., Leal-Cardoso, J. H., Assreuy, A. M. S. "Comparative Study of the Anti-Edematogenic Effects of Anethole and Estragole", Pharmacological Reports, 64(4), pp. 984–990, 2012. https://doi.org/10.1016/S1734-1140(12)70895-2

[54] Smith, R., Adams, T., Doull, J., Feron, V., Goodman, J., ..., Sipes, I. "Safety Assessment of Allylalkoxybenzene Derivatives Used as Flavouring Substances — Methyl Eugenol and Estragole", Food and Chemical Toxicology, 40(7), pp. 851–870, 2002. https://doi.org/10.1016/S0278-6915(02)00012-1

[55] Yea, S. S., Jeong, H.-S., Choi, C. Y., Park, K.-R., Oh, S., Shin, J.-G., Yun, C.-H. "Inhibitory Effect of Anethole on T-Lymphocyte Proliferation and Interleukin-2 Production through down-Regulation of the NF-AT and AP-1", Toxicology in Vitro, 20(7), pp. 1098–1105, 2006. https://doi.org/10.1016/j.tiv.2006.01.020

[56] Zhou, J., Zheng, X., Yang, Q., Liang, Z., Li, D., Yang, X., Xu, J. "Optimization of Ultrasonic-Assisted Extraction and Radical-Scavenging Capacity of Phenols and Flavonoids from *Clerodendrum cyrtophyllum* Turcz Leaves", PLoS ONE, 8(7), e68392, 2013. https://doi.org/10.1371/journal.pone.0068392