

Optimization of microwave-assisted extraction of polyphenols and antioxidant activity in *Reichardia tingitana* and *Atriplex halimus* via response surface methodology

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Abstract

The demand for natural, plant-derived polyphenols is growing in both academic and industrial sectors, owing to their strong therapeutic potential, including antioxidant, antiviral, anti-inflammatory, and anticancer properties. A comprehensive study investigating the polyphenolic profile and antioxidant potential of *Reichardia tingitana* and *Atriplex halimus* utilizing microwave extraction technique, supported by response surface methodology-based optimization, remains unexplored. Comparison of the polyphenol contents in the mentioned plants extracted with microwave-assisted methods demonstrated that *R. tingitana* possess high phenolic contents (93.48-123.35 mg GAE/g DW) than *A. halimus* (54.64–84.28 mg GAE/g DW). The single factor optimization experiments demonstrated that 50% ethanol-water mixture outperform in extracting polyphenols (102.45–123.35 for *R. tingitana* and 63.39–84.28 for *A. halimus*) followed by 50% acetone-water and methanol/water respectively, whereas ethyl acetate-water resulted in the lowest TPC (93.48–109.73 mg GAE/g DW for *R. tingitana* and 54.64–70.57 mg GAE/g for *A. halimus*) respectively. The MAE extraction parameters (MW power, temperature, time and ethanol%) were further optimized by response surface methodology with Box-Behnken design. The outcomes of the Box-Behnken design demonstrated that optimum TPC contents could be extracted with 500 W MW power at 75 °C temperature, 7 min of contact time and 70% ethanol concentration, providing the polyphenol yield (*R. tingitana* = 129.43 mg GAE/g DW and *A. halimus* = 91.87 mg/g DW plant extract). Finally, the optimized plant extracts were evaluated for antioxidant activities using ABTS assay. Prominent correlation was found to exist between the antioxidant activities and polyphenol contents of the extracts.

1. Introduction

Plant-based antioxidants, including vitamins E and C, as well as polyphenols including phenolic acids, flavonoids, catechins, and anthocyanins, are receiving growing attention for their significance health benefits and in contexts of dietary components (Ferguson, 2001). There is a rising trend in studying and utilizing plant-based antioxidants for their potential benefits in scientific research and in industries such as dietary supplements, pharmaceuticals, and cosmetics. This is primarily attributed to their potent biological activities, unlike many synthetic antioxidants that may act as carcinogenesis promoters (Čanadanović-Brunet et al., 2006). Medicinal plants and fruits have currently gained considerable demand for their polyphenol content, particularly due to their antioxidant potentials (Singh et al., 2002).

Reichardia tingitana L. Roth (poppy-leafed Reichardia) is a bitter annual species of the *Asteraceae* family, typically found in rocky and mountainous terrains. *R. tingitana* has been the subject of extensive investigations focusing on its phytochemical profile and a wide spectrum of biological effects. Extracts from this species demonstrate marked antimicrobial potential (Abd-ElGawad et al., 2020). Phytochemical analyses of its aerial parts have led to the identification of numerous bioactive constituents, such as phenolics, tannins, flavonoids, coumarins, phytosterols, lactones, and essential oils (Salama et al., 2002; Abdel-Mogib et al., 1993).

Atriplex halimus L. (Mediterranean saltbush), a salt-loving shrub from the *Amaranthaceae* family, thrives in arid and semi-arid climates of the Mediterranean zones, mostly below 900 m in altitude (Walker et al., 2014). Ethnopharmacological evidence points to its application in treating cardiac and gastrointestinal disorders, poisoning, tumors, elevated blood pressure, and rheumatism (Taïbi et al., 2021; Karous et al., 2021; Qasem, 2015). *A. halimus* extracts have been reported to contain phenolic acids, flavonoids, alkaloids, fatty acids, carbohydrates, organic acids, amino acids, and vitamins (Morad et al., 2023; Zeghib et al., 2021; Bouaziz et al., 2021; Benhammou

et al., 2009). The phenolic compounds, in particular, possess significant antioxidant, antidiabetic, and antibacterial properties (Cunha et al., 2004; Hafizur et al., 2015; Badhani et al., 2015).

In recent decades, researchers have devoted significant attention to identifying and isolating bioactive substances from plants, mostly utilizing conventional extraction techniques (maceration, refluxing, and soxhlet extraction, among others) (Row and Jin, 2006; Wang et al., 2009; Vovk et al., 2005). However, there are a number of drawbacks to conventional extraction methods, including their high solvent, energy, and time consumption as well as the loss of phytochemicals during extended extraction at high temperatures (Routray and Osarat 2013). The past few years have seen the development of innovative extraction technologies aimed at extracting phenolic compounds from plant materials (Nkhili et al., 2009; Xia et al., 2006; Jun et al., 2010; Chang et al., 2000).

Microwave-assisted extraction typically exhibits clear benefits over the conventional extraction approach, including shorter extraction times, fewer energy and solvent usage, higher extraction yields with more selectivity, and improved target extract quality (Chen et al., 2005; Kaufmann and Christen 2002). Through the generation of high-temperature zones, microwaves enhance the extraction process by promoting cell wall breakdown, thereby enabling efficient transfer of active ingredients into the solvent.

Choosing the appropriate solvent is crucial during extraction. Being polar in nature, polyphenols dissolve rapidly in polar liquids (water, ethanol, methanol, and acetonitrile and their mixes) (Sultana et al., 2009). Water, organic solvents, and their combinations are commonly employed in the extraction of plant-derived polyphenols (Musa et al., 2011).

Although some but few investigations have examined the polyphenolic composition of *R. tingitana* and *A. halimus*, employing only traditional extraction. However, a significant research gap remains concerning the application of modern extraction techniques, particularly microwave-assisted extraction (MAE) for extracting polyphenols from the selected plants, and the process optimization by response surface methodology. The current research involved an investigation on modern extraction strategy (MAE) for extracting polyphenols evaluating their antioxidant activity from the selected plants. Furthermore, the influence of various key MAE parameters on polyphenol yield was assessed and optimized through single-factor experiments and response surface methodology.

2. Experimental

Materials

Gallic acid and the Folin–Ciocalteu reagent were procured from Alfa Aesar Chemical Co., Ltd. (Shanghai, China). Milli-Q ultra-pure water (Merck, Germany) was used to prepare aqueous solutions. Analytical-grade solvents including acetonitrile and ethanol were sourced from Merck (Germany).

Fresh aerial parts of the plants *Reichardia tingitana* and *Atriplex halimus* were collected from the local flora of Dera Ismail Khan, Pakistan. The plants were identified by Dr. Muhammad Faizan, Associate professor, Department of Botany, University of Science and Technology Bannu, where the voucher samples (Accession No. C-043 and No. 251) has been preserved there. The aerial part (stems and leaves) were shade dried and crushed into a fine powder and subsequently filtered with 30-mesh screen to improve surface area for efficient extraction.

Polyphenol extraction via microwave-assisted technique

Plant polyphenols were isolated using a digitally controlled microwave oven (Samsung MDS-6G) with power settings that could be adjusted from 200 to 1000 W. The powdered plant sample (2.5 g) was dispersed in 50 mL of the 50% aqueous-organic solvents (ethyl-acetate water, acetone-water, acetonitrile-water, methanol-water and ethanol-water). For single-factor optimization experiments, the samples were subjected to microwave extraction at power level of 400 W, at varying temperature of 60, 70, 80 and 90°C for extraction duration of 2, 4, 6, 8 and 10 minutes. The extraction was performed at varying temperatures (60–90°C) and contact time (2–10 minutes). After extraction via MAE, each sample was filtered using Whatman No. 1 filter paper and adjusted to a total volume of 100 mL. From the resulting supernatant, 1 mL was withdrawn for determination of total polyphenol content.

Determination of Total Polyphenols (TPs)

Quantification of total phenolics in the plant extract was carried out via the Folin-Ciocalteu assay, as per earlier published protocols (Lee et al., 2013). Specifically, 2.5 mL of a 10% Folin-Ciocalteu reagent was added to 0.5 mL of extract diluted at a 1:10 ratio. After 4 minutes of reaction, 2 mL of 7.5% (w/v) Na₂CO₃ was added, and the mixture was then incubated at 25°C for 2 hours in the dark. The SHIMADZU UV-1700 UV-Vis spectrophotometer was used to measure absorbance at 760 nm. A standard curve of gallic acid (10–100 µg/mL) served to determine total phenolic content, and the results were expressed as mg GAE/g dry extract.

Response surface methodology-based optimization of polyphenols

Following the results of single-factor analyses, the optimization of extraction parameters for TPC yield was carried out using a 4-factor, 3-level Box–Behnken design within RSM. The variables examined were microwave intensity (A), temperature (B), contact time (C), and ethanol concentration (D). Table 1 depicts the coded levels and their corresponding actual values for each factor.

Table 1
Levels of variables (coded and actual) used in the MAE experimental design

Codes	Independent variable	Low	Centre	High
		-1	0	+1
A	Microwave power	400	500	600
B	Temperature (°C)	70	75	80
C	Contact iime (min)	6	7	8
D	Ethanol concentration (%)	60	70	80

29 experimental runs were designed by BBD experimental design. Following the BBD experimental data's regression analysis, a second-order polynomial model as represented by **Eq. (1)** was followed to interpret the data.

$$Y = \beta + \sum \beta X + \sum \beta X + \sum \beta XX (1)$$

Where:

- Y = response variable

- X_i, X_j = coded input variables
- β_0 = Constant (intercept of the model)
- $\beta_i, \beta_{ij}, \beta_{ij}$ = regression coefficients (linear, quadratic, and interaction coefficients, respectively)

ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Assay

The antioxidant capacity of the plant infusions, obtained under different extraction conditions, was determined using the ABTS radical scavenging assay following the procedure of Re et al., 1999. ABTS^{•+} formation was carried out by mixing equal volumes of 7.2 mM ABTS with 2.5 mM potassium persulfate. Following a 12-hour incubation in the dark at ambient temperature, the stock solution was diluted 80-fold in water until the absorbance at 734 nm reached 0.70 ± 0.05 . A 150 μ l aliquot of either the 80-fold diluted plant infusion, Trolox standard solution (0–0.8 mM in ethanol), or water (used as the control) was added to 2.4 ml of the ABTS^{•+} reagent and mixed thoroughly. The mixture was kept in the dark at room temperature for two hours, after which its absorbance at 734 nm was recorded, with water serving as the blank. The antioxidant capacity was evaluated using a standard calibration curve prepared from Trolox concentrations of 0–80 μ M and expressed as mmol L⁻¹ TE per g sample.

Statistical analysis

Design-Expert version 13.0 (Stat-Ease Inc.) was employed for the statistical analyses, including Response Surface Methodology and Box-Behnken Design. Additionally, the same program was used to assess the model fittings Analysis of Variance, and response surface plots for each analyte. ANOVA was applied to test the statistical significance of extraction parameters affecting the polyphenol yield, based on a 95% confidence level ($p < 0.05$).

To optimize the extraction parameters for polyphenol yield, a statistical and mathematical approach using experimental design was applied. For each single-variable study, three replicates were performed, and the data are given as mean $\pm$ standard deviation. A multiple regression model was fitted to the results to quantify the relationships between independent variables and the response.

3. Results and discussion

Effect of various extraction parameters and solvents on the total polyphenol contents of plant extracts

To evaluate the favorable extraction conditions for optimizing total polyphenol yield, microwave-assisted methods was applied to powdered materials of *Reichardia tingitana* and *Atriplex halimus* using 50% combinations of various aqueous-organic solvents (aqueous/ethyl acetate, acetone, acetonitrile, methanol and ethanol) at various extraction temperature and time. The corresponding TPC outcomes are depicted in Table 2 and Fig. 1.

Table 2
Variation of polyphenol contents in selected plants with solvent types, temperature and contact time

<i>Reichardia tingitana</i>						<i>Atriplex halimus</i>				
Extraction time						Extraction time				
Solvents/ Temperature	2	4	6	8	10	2	4	6	8	10
EtOAc-H ₂ O										
60 °C	96.15	99.47	103.32	106.56	109.73	56.54	60.26	64.18	67.38	70.57
70 °C	±1.24	±1.52	±2.01	±1.92	±1.63	±	±	±	±	±
80 °C						1.30	0.96	1.54	1.56	1.79
90 °C	99.28	102.64	105.36	109.61	105.86	59.72	63.67	65.79	69.16	65.45
	±1.56	±1.73	±1.97	±2.49	±2.31	±	±	±	±	±
						0.67	1.27	1.27	2.12	2.01
	102.52	105.39	103.81	100.54	98.38	62.83	65.35	63.21	60.46	57.82
	±2.23	±2.41	±1.51	±1.54	±1.20	±	±	±	±	±
						1.23	0.67	1.75	0.68	1.05
	105.73	102.55	99.37	96.83	93.48	65.78	62.75	60.3	57.53	54.64
	±1.87	±1.64	±1.06	±1.32	±1.03	±	±	±	±	±
						1.64	1.08	0.81	1.26	0.78
Acetone-Me ₂ CO-H ₂ O										
60 °C	106.84	109.59	113.74	117.65	120.26	66.79	70.23	73.85	77.58	81.17
70 °C	±2.36	±1.59	±2.11	±3.31	±2.56	±	±	±	±	±
80 °C						1.18	1.18	1.68	2.51	1.58
90 °C	110.35	114.66	117.53	115.34	112.68	70.62	74.31	78.12	76.29	72.46
	±3.12	±3.36	±3.21	±2.72	±1.83	±	±	±	±	±
						2.21	1.56	2.09	1.84	2.13
	113.25	116.36	112.57	109.17	106.15	73.55	75.91	71.67	68.83	65.26
	±2.45	±2.86	±1.57	±1.85	±0.79	±	±	±	±	±
						1.53	2.15	0.98	0.96	0.85
	110.75	107.16	104.58	101.36	97.62	69.85	66.35	62.74	59.49	56.65
	±1.64	±1.95	±1.92	±1.23	±1.19	±	±	±	±	±
						1.37	1.13	1.47	1.27	1.24
CAN-H ₂ O										
The findings were presented as mean values (n = 3) ± standard deviation										

	<i>Reichardia tingitana</i>					<i>Atriplex halimus</i>				
60 °C	98.29	101.08	106.46	109.75	112.56	57.27	60.54	64.38	68.47	72.28
70 °C	±0.93	±1.27	±1.25	±2.49	±1.87	±	±	±	±	±
80 °C						0.59	0.85	1.28	1.38	1.35
90 °C	101.61	105.85	109.63	112.45	115.39	60.23	63.84	67.65	71.53	75.25
	±1.14	±1.69	±2.23	±1.66	±2.58	±	±	±	±	±
						1.36	1.23	0.88	2.16	2.21
	105.81	109.26	113.43	117.36	114.52	64.53	68.69	72.34	76.56	72.37
	±1.47	±2.38	±1.88	±3.26	±3.15	±	±	±	±	±
						1.72	1.57	1.20	1.67	1.16
	109.47	112.86	116.58	112.45	111.66	67.35	71.73	68.59	65.87	62.16
	±2.05	±2.89	±3.34	±1.35	±2.31	±	±	±	±	±
						2.01	2.19	0.79	1.25	0.75
MeOH-H2O										
60 °C	100.72	103.54	107.24	111.43	115.61	60.55	64.28	67.73	71.52	75.43
70 °C	±1.29	±1.75	±2.17	±2.15	±1.25	±	±	±	±	±
80 °C						0.91	1.25	1.39	1.47	1.67
90 °C	104.32	108.44	112.17	116.51	119.42	64.67	68.39	72.48	76.35	79.64
	±2.51	±2.48	±3.05	±3.07	±2.67	±	±	±	±	±
						1.24	1.71	1.87	2.13	2.14
	108.21	111.68	109.37	106.28	102.68	69.12	73.36	70.15	67.53	63.71
	±1.58	±2.75	±1.39	±1.24	±0.82	±	±	±	±	±
						1.29	2.23	1.53	1.26	1.23
	112.48	110.36	107.72	103.25	99.89	72.63	69.42	65.85	61.49	57.29
	±3.27	±1.86	±1.76	±0.87	±1.06	±	±	±	±	±
						1.85	1.37	1.16	0.74	1.12
EtOH-H ₂ O										
60 °C	102.45	105.68	109.57	113.49	117.64	63.39	67.36	71.42	75.52	78.92
70 °C	±2.35	±1.65	±2.57	±1.72	±3.12	±	±	±	±	±1
80 °C						1.07	0.76	1.30	1.92	.06
90 °C	106.53	111.12	115.61	119.82	123.35	68.46	72.84	76.53	80.65	84.28
	±1.52	±2.28	±3.21	±3.21	±2.85	±	±	±	±	±
						1.52	1.29	1.65	1.63	2.13
	110.48	115.67	119.26	122.45	118.69	73.34	77.58	81.37	83.87	79.16
	±1.86	±2.69	±3.69	±2.44	±2.68	±	±	±	±	±
						1.63	1.68	2.11	2.24	1.85
The findings were presented as mean values (n = 3) ± standard deviation										

<i>Reichardia tingitana</i>					<i>Atriplex halimus</i>					
114.83	118.46	115.25	112.65	108.86	77.15	80.73	77.58	74.49	70.66	
±3.21	±3.57	±1.82	±1.68	±1.26	±	±	±	±	±	
					2.14	2.16	1.65	1.32	1.57	
The findings were presented as mean values (n = 3) ± standard deviation										

Among the various plants used for extraction of polyphenols, it was observed that *R. tingitana* afforded more TPC contents ranging from 93.48–123.35, mg GAE/g dry weight as compared to *A. halimus* ranging from 54.64–84.28 across various solvents, temperature and extraction time. These findings were in line with the previous reports.

Salama et al. [6] documented that the shoot extract of *R. tingitana* contains phenolics (143.68 mg GAE/g dry extract), flavonoids (32.41 mg CE/g dry extract), and tannins (96.57 mg GAE/g dry extract). According to Guedri et al., 2024, the total polyphenols in dry powdered leaves of *Atriplex halimus*, sampled from various populations across different regions, ranged between 51.3 and 101.0 mg GAE/g DW.

Effect of solvent

Extraction yields of polyphenols varied significantly with solvent composition. Maximum TPC was achieved using a 50% ethanol-water solvent across various temperature and contact time (102.45–123.35 mg GAE/g DW for *R. tingitana* and 63.39–84.28 mg GAE/g for *A. halimus*) followed by 50% acetone-water mixtures (97.62–120.26 mg GAE/g for *R. tingitana* and 56.65–81.17 mg GAE/g for *A. halimus*) and methanol/water (99.89–119.42 mg GAE/g for *R. tingitana* and 57.29–79.64 mg GAE/g for *A. halimus*) respectively, whereas ethyl acetate-water resulted in the lowest TPC (93.48–109.73 mg GAE/g for *R. tingitana* and 54.64–70.57 mg GAE/g dry weight for *A. halimus*) respectively.

Prior investigations have demonstrated notable differences in TPC from diverse plant sources when extracted with different solvents. This is attributed to the diverse polarity and chemical nature of phenolic compounds, which significantly influence their solubility and, consequently, their extractability in various solvents.

Several studies have reported higher TPC levels with 50% ethanol compared to other solvents (Bhebhe et al., 2016). Vural et al., 2020, examined the effectiveness of different solvent systems, comprising 50% and 75% aqueous ethanol and methanol, pure solvents, and water for extracting total phenolics from black tea, green tea, blackberry, and black mulberry. Results revealed that black tea, when extracted using 50% ethanol, produced the maximum TPC, a trend also noted in mate tea. Conversely, green tea and blackberry had higher TPC when extracted with 50% methanol, while 70% ethanol was more efficient for black mulberry.

50% aqueous ethanol is commonly used solvent for extracting polyphenols from various plant materials due to its affordability, safety, reusability, and suitability for food and cosmetic applications (Durling et al., 2007; Alothman et al., 2009). Sultana et al., 2009, also reported that aqueous-ethanol and methanol extracts gave the highest TPC from medicinal plants.

Turkmen et al., 2006, found that mate tea extracted with 50% acetone yielded the highest polyphenol concentration (132.5–120.4 mg GAE/g) across the various extraction techniques. Do et al., 2014, also explored the extraction of phenolic compounds from *L. aromatica* by applying water and graded concentrations (50%, 75%, and 100%) of methanol, ethanol, and acetone. Their findings showed that 50% aqueous acetone yielded the highest TPC (33.67

mg GAE/g), surpassing both 50% ethanol (32.94 mg GAE/g) and 70% methanol (32.92 mg GAE/g). According to Turkmen et al., 2007, 50% acetone extracted the highest amount of polyphenols from black tea (TPC: 98.19–114.01), with DMF being the next most effective (95.60–109.36). In contrast, 50% ethanol and methanol resulted in lower TPCs of 74.35–86.31 and 58.38–68.69, respectively.

According to Sepahpour et al., 2018, 80% acetone yielded the highest TPC from turmeric, torch ginger, and lemongrass, with 80% ethanol and 80% methanol showing comparatively lower efficiency. In contrast, curry leaf extracts showed optimal TPC yield with 80% ethanol, followed by methanol and acetone. The higher extraction efficiency of acetone could be linked to its suitability for dissolving lipophilic phenolics and its ability to disrupt protein–polyphenol associations (Ferreira and Pinho, 2012; Jakobek, 2015). For curry leaf, the enhanced extraction efficiency observed with 50% ethanol and 50% methanol could be attributed to the fact that many of its phenolic compounds are glycosylated, including quercetin-3-glucoside, kaempferol-O-glucoside, and similar derivatives (Singh et al., 2011). These glycosylated structures exhibit higher solubility in alcoholic solvents than in 80% acetone. This behavior can be attributed to differences in solvent polarity, with the dielectric constant serving as a useful indicator. The higher polarity of water–alcohol mixtures relative to water–acetone mixtures makes them more effective for dissolving polar substances.

Similar to our findings, Cha et al., 2013, demonstrated that 80% ethanol was the most efficient solvent for extracting TPC and TFC from *Opuntia humifusa* fruit, followed by 80% methanol and acetone ($p > 0.05$). Conversely, our findings contrast with those of Do et al., 2014 and Xiong et al., 2012, who observed higher TPC and TFC in 70–80% acetone extracts of *Osmanthus fragrans* seeds and *Limnophila aromatica* compared to methanol or ethanol extracts at equivalent concentrations.

Phenolic compounds, being polar, are best extracted using hydro-alcoholic mixtures, especially those based on ethanol (Muñiz-márquez et al., 2013). This is due to the combined effect of the solvents: ethanol helps dissolve the phenolics, while water swells the plant matrix, improving solvent penetration.

In polyphenol extraction, the solvent's vapor pressure, alongside its polarity, solubility, and safety, plays a crucial role. Low vapor pressure solvents, due to their higher boiling points, enable extraction at elevated temperatures with minimal solvent loss, thereby enhancing polyphenol dissolution and diffusion. On the other hand, high vapor pressure solvents such as acetone (184 mmHg), methanol (88 mmHg), and acetonitrile (73 mmHg) at 25°C are more volatile and pose challenges in high-temperature extractions. Ethanol, with a lower vapor pressure of 59 mmHg at 25°C, remains stable and safe even above 70°C, making it preferable for techniques like microwave-assisted extraction. Ethanol-water mixtures thus strike a balance between efficiency, safety, and sustainability in both conventional and green extraction methods.

Effect of Temperature

Among the critical parameters affecting TPC, temperature significantly influences extraction efficiency. As evident in Fig. 1b, enhancing the temperature from 60°C to 70 and 80°C (depends upon the solvents) enhanced TPC extraction. However, a decline in TPC was noted at temperatures above 70 and 80°C. The rise in TPC yield at higher temperatures can be attributed to better mass transfer, increased solubility, and lower solvent viscosity (Celli et al., 2015; Hossain et al., 2012). Conversely, overly high temperatures can degrade phenolic compounds due to reduced surface tension and increased vapor pressure, thereby lowering the extraction rate (Carrera et al., 2012; Tao et al., 2014). According to previous reports, suitable extraction temperatures enhance the release of bioactive constituents, however, elevated temperatures may cause degradation of phenolic and flavonoid

compounds (Ma et al., 2008; Vega-Gálvez et al., 2009; Wang et al., 2019). Therefore, the temperature range of 70–80°C, yielding the maximum TPC, was chosen as the optimal condition for further optimization using RSM.

Extraction Time

The effect of varying contact times on plant extract TPC was investigated by performing extractions over different durations (2 to 10 minutes). The maximum TPC value was recorded at the extraction time of 6–8 minutes, as Fig. 1c illustrates. Increasing the ultrasonic time can help bioactive chemicals dissolve completely. However, elevated temperature above 8 minutes resulted in decline in the polyphenol contents, which may be due to degradation of the polyphenols at prolonged extraction under high microwave intensity.

According to Sahin, 2013, a process consisting of two primary phases is responsible for the rise in phenolic contents as a function of time. The first phase, referred to as "washing," lasts for the first ten to twenty minutes of extraction. During this stage, dissolution of soluble surface components occurs, significantly boosting the extraction rate and enabling recovery of nearly 90% of the total phenolic content (Ma et al., 2008). In the second stage, known as the "slow extraction" phase, diffusion facilitates the transfer of solute mass from the matrix into the solvent. Longer ultrasonic periods, however, may result in increased energy waste and bioactive component breakdown. Consequently, the extraction duration of 30 minutes was chosen for the further research, taking into account the enhancement of extraction effectiveness and energy conservation.

Response surface methodology-based optimization of polyphenol extraction

Response surface methodology, a potent optimization approach for assessing several factors and the ways in which they interact with each other and with response which otherwise could not be studied with single-factor experiments. Additionally, it takes less time and effort and involves fewer experiments. RSM has been used extensively for extraction parameter optimization (Salar Bashi et al., 2012; Dahmounea et al., 2013; Liu et al., 2013). The parameters that effecting the extraction yield were statistically analysed and optimized using RSM based Box-Behnken experimental design.

To further enhance polyphenol yield, an RSM using a Box-Behnken Design (BBD) with 4-factors at 3-levels was adopted. BBD generated 29 experimental trials and the total phenolic content (TPC) obtained were: 116.17 to 129.43 mg GAE/g DW for *Reichardia tingitana* and 78.31 to 91.87 mg GAE/g for *Atriplex halimus* (Table 3).

Table 3
BBD experimental design and the response (TPC) values

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2
Std	Run	A:MW power	B:Temp	C:Time	D:Ethanol conc.	TPC-1	TPC-2
		watt	0C	min	%	mg/g	mg/g
13	1	500	70	8	70	128.42	90.81
11	2	600	75	6	70	127.18	89.56
14	3	400	80	7	70	122.83	84.89
12	4	600	75	8	70	124.95	87.21
28	5	400	75	7	60	117.51	79.67
27	6	500	75	7	70	129.29	91.73
26	7	600	75	7	80	121.49	83.76
18	8	400	75	7	80	116.98	79.12
3	9	500	75	7	70	129.43	91.87
1	10	500	70	7	80	119.03	81.22
19	11	400	70	7	70	116.17	78.31
10	12	500	80	7	80	123.85	85.95
22	13	500	75	8	80	123.62	85.73
4	14	500	75	7	70	129.16	91.59
17	15	500	75	7	70	129.02	91.46
16	16	600	75	7	60	121.78	83.97
2	17	500	80	7	60	124.26	86.39
7	18	500	80	6	70	127.88	90.27
5	19	500	75	7	70	128.87	91.32
8	20	500	70	7	60	119.88	82.16
9	21	400	75	8	70	122.58	84.67
25	22	500	70	6	70	120.81	83.08
21	23	500	75	8	60	125.78	88.11
6	24	600	70	7	70	126.97	89.34
24	25	500	75	6	60	120.13	82.39
15	26	500	80	8	70	126.46	88.75
29	27	500	75	6	80	119.27	81.41

Factor 1			Factor 2	Factor 3	Factor 4	Response 1	Response 2
20	28	400	75	6	70	118.29	80.46
23	29	600	80	7	70	125.19	87.45
Predicted mean						129.12	91.59
Standard deviation ($\pm$)						1.0096	1.0433

Fitting the model and ANOVA assessment

According to the model fit analysis, the quadratic model is the most appropriate for describing total polyphenol content extracted from both the selected plants (TPC-1 and TPC-2), as evidenced by the strong alignment between the adjusted and predicted R² value for the TPCs from respective plants as shown in Table 4.

Table 4
Statistical summary of model fitting for polyphenol contents in various plants

	Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	
TPC-1	Linear	0.0514	< 0.0001	0.2003	0.0782	
	2FI	0.7836	< 0.0001	0.0920	-0.2454	
	Quadratic	< 0.0001	0.0027	0.9411	0.8321	Suggested
TPC-2	Linear	0.0561	< 0.0001	0.1934	0.0719	
	2FI	0.7953	< 0.0001	0.0800	-0.2578	
	Quadratic	< 0.0001	0.0022	0.9399	0.8285	Suggested
TPC-1 = <i>Reichardia tingitana</i> , TPC-2 = <i>Atriplex halimus</i>						

The analysis of variance (ANOVA) results for the BBD model are presented in Table 5.

Table 5
ANOVA of the quadratic models for total polyphenol contents

TPC-1					TPC-2				
Source	Sum of Squares	Mean Squares	F-value	p-value	Sum of Squares	Mean Squares	F-value	p-value	Significance
Model	470.42	33.60	32.96	< 0.0001	491.95	35.14	32.28	< 0.0001	***
A-MW power	91.85	91.85	90.11	< 0.0001	97.30	97.30	89.38	< 0.0001	***
B-Temp	30.69	30.69	30.10	< 0.0001	29.39	29.39	27.00	0.0001	***
C-Time	27.76	27.76	27.23	0.0001	27.33	27.33	25.11	0.0002	**
D-Ethanol conc.	2.17	2.17	2.13	0.1669	2.52	2.52	2.32	0.1503	
AB	17.81	17.81	17.47	0.0009	17.94	17.94	16.48	0.0012	*
AC	10.63	10.63	10.43	0.0061	10.76	10.76	9.88	0.0072	*
AD	0.0144	0.0144	0.0141	0.9071	0.0289	0.0289	0.0265	0.8729	
BC	20.39	20.39	20.00	0.0005	21.39	21.39	19.65	0.0006	**
BD	0.0484	0.0484	0.0475	0.8307	0.0625	0.0625	0.0574	0.8141	
CD	0.4225	0.4225	0.4145	0.5301	0.4900	0.4900	0.4501	0.5132	
A ²	125.10	125.10	122.72	< 0.0001	132.76	132.76	121.95	< 0.0001	***
B ²	23.73	23.73	23.28	0.0003	25.89	25.89	23.78	0.0002	**
C ²	13.83	13.83	13.57	0.0025	14.97	14.97	13.75	0.0023	*
D ²	191.54	191.54	187.90	< 0.0001	202.71	202.71	186.21	< 0.0001	***
Lack of Fit	14.08	1.41	29.13	0.0027	15.05	1.51	32.08	0.0022	*
TPC-1 = <i>Reichardia tingitana</i> , TPC-2 = <i>Atriplex halimus</i>									

The regression models were observed to be statistically significant, as indicated by the very low p-value ($p < 0.0001$) and high F-value (32.96 for TPC-1 and 32.28 for TPC-2). Therefore, the model is well-suited for predicting and interpreting the extracted TPC values.

Figure 2 (a and b) demonstrates the individual effects of single variable on the total polyphenol content in *R. tingitana* and *A. halimus*, respectively. MW power (A), temperature (B), and contact time (C) significantly affected TPC yields ($p < 0.05$). In contrast, ethanol concentration (D) did not have a statistically significant impact ($p > 0.05$). According to the F-values presented in the ANOVA Table 5, the influence of the parameters on TPC yield followed the order: MW power (A) > temperature (A) > contact time (C) > ethanol concentration (D) with corresponding F-

values of 90.11, 30.10, 27.23 and 2.13 for TPC-1 and 89.38, 27.00, 25.11 and 2.32 for TPC-2. A higher F-value and lower p-value denote a pronounced impact of a variable on the outcome.

Response surface analysis of the interactive effects of extraction parameters on total polyphenol content

The impact of interactions among independent variables on total polyphenol content was assessed via 2D interaction plots, contour diagrams, and 3D response surface views generated from the model outlined in Eq. (1).

Figures 3 and 4 (a–f) present the interaction plots illustrating the paired effects of independent variables on the TPC yield of *R. tingitana* and *A. halimus*, respectively. According to the interaction plots, temperature and time (BC) exhibit the strongest combined influence on TPC content, as shown in Figs. 3 and 4(c), followed by MW power with temperature (AB) and time (AC) in Figs. 3 and 4(a, b). In contrast, interactions involving the ethanol concentration with other parameters (AD, BD, CD) are non-significant, with minimal differences in TPC values at both high and low levels of parameters, as seen in Figs. 3 and 4(d–f). These outcomes align with the ANOVA results in Table 5, where significant BC, AB and AC interactions are indicated by low p-values and high F-values. The descending order of interaction effects on TPC is: BC > AB > AC > CD > BD > AD.

Figure 3–4(c) illustrates that TPC content exhibited a significant rise with increasing temperature at both low and high time levels, indicating a pronounced temperature–time (BC) interaction. The increase was more pronounced at high level of contact time, underscoring the synergistic effect of these two parameters. Similarly, significant interactions were also observed for the paired combination of MW power-temperature (Fig. 3-4a), and MW power-time (Fig. 3-4b).

The results of the interaction plots were also supported by the response surface plots demonstrated in Figs. 5 and 6 (a–f). The steepness of the response surface plots serves as a measure of the interaction strength between paired factors influencing TPC content (Miao et al., 2020). Notably, the response surfaces corresponding to the interactions of temperature-time (BC), MW power-temperature (AB), and MW power-time (AC) exhibit more pronounced slopes steepness as depicted in Figs. 5, 6 (a–c), indicating a stronger effect on TPC yield. In contrast, the response surface plots for the interactions of ethanol concentration with MW power (AD), temperature (BD) and time (CD) are relatively flat with low slope steepness, suggesting minimal paired effects on TPC yield, as illustrated in Figs. 5, 6 (d–f).

The findings of the response surface plots were, in some cases, not fully aligned with the statistical outcomes outlined in the ANOVA table. Specifically, while the ANOVA indicated a more pronounced effect for the BC interaction as reflected by a lower p-value and a higher F-value in comparison to AB and AC interactions, however, the response surface plots demonstrated a comparatively lower slope steepness for the BC interaction as compared to AB and AC interactions.

RSM optimization revealed that MW power of 500 W, 60°C temperature, 25 min contact time, and a 70% ethanol concentration yielded maximum TPC: 129.43 mg GAE/g for *R. tingitana* and 91.87 mg GAE/g for *A. halimus*.

Percentage contribution of each term in total polyphenol contents

As presented in Figures S1 and S2, the Pareto and normal plots, along with the percentage contributions listed in Table 6, highlight the relative importance and statistical relevance of the parameters and their interactions in

influencing polyphenol levels.

The significance of each factor and their interaction effect can be determined from the height of each bar in the Pareto chart (**Figure S1** and **S2**). Moreover, the statistical significance of each factor is shown against the reference distribution (reference line), which visually determine which effect deviate more or less from the reference line. The factor which lies far from the reference line/ or cross a reference line (usually based on the t-distribution) is considered statistically more significant.

Table 6 shows that among the single factors the MW power-A is the major contributor for TPC yield in each plant with percentage contribution of (23.18% for TPC-1 and 23.46% for TPC-2) followed by the temperature-B with %age contribution of (7.74% for TPC-1 and 7.09% for TPC-2) and contact time-C (7.00% for TPC-1 and 6.59% for TPC-2), while the ethanol concentration-D has minimum contribution (0.547% for TPC-1 and 0.607% for TPC-2). Among the interaction terms only BC (temperature-time), AB (MW power-temperature) and AC (MW power-time) terms contribute significantly with contribution of (5.14%, 4.49% and 2.68% for TPC-1 and 5.16%, 4.33% and 2.59% for TPC-2 respectively), while the ethanol concentration term, when combined with other factors, did not provide significant contribution to the outcomes (**Table 6**).

Regression analysis

Using multiple regression analysis, the experimental results were analyzed, and a polynomial regression equation was constructed through response surface methodology with the Box–Behnken Design to express the empirical relationship between the response (Y) and the independent variables (Xi) as shown below.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \dots \text{(Eq. 1)}$$

Where:

- Y = response variable,
- X_i = coded independent variables, representing ethanol concentration (A), temperature (B), contact time (C), and solvent-to-sample ratio (D), respectively
- β_0 = intercept (constant term),
- $\beta_i, \beta_{ii}, \beta_{ij}$ = regression coefficients.

Based on these models and the respective regression coefficients, the predicted response values for total polyphenol content were calculated and are presented in **Table S1**.

$$Y_1(\text{Response/TPC-1}) = 129.15 + 2.77X_1 + 1.60X_2 + 1.52X_3 + -0.425X_4 - 2.11X_{12} + 1.63X_{13} + 0.06X_{14} - 2.26X_{23} + 0.11X_{24} - 0.325X_{34} - 4.39X_1^2 - 1.91X_2^2 - 1.46X_3^2 - 5.43X_4^2$$

$$R^2 = 0.9706; \text{Adjusted } R^2 = 0.9411; \text{Predicted } R^2 = 0.8321$$

$$Y_2(\text{Response/TPC-2}) = 91.59 + 2.85X_1 + 1.56X_2 + 1.51X_3 - 0.458X_4 - 2.12X_{12} - 1.64X_{13} + 0.085X_{14} - 2.31X_{23} + 0.125X_{24} - 0.35X_{34} - 4.52X_1^2 - 2.00X_2^2 - 1.52X_3^2 - 5.59X_4^2$$

$$R^2 = 0.9700; \text{Adjusted } R^2 = 0.9399; \text{Predicted } R^2 = 0.8285$$

Antioxidant activity of various tea infusions and its correlation with polyphenol contents

Different extraction settings produced plant extracts with distinct antioxidant activities, largely due to variations in their phenolic profiles. As indicated in Table 7, variations in MW power (A), extraction temperature (B), contact time (C), and ethanol concentration (D) were applied to evaluate their role in determining antioxidant activity and their connection with phenolic content.

Table 7
Influence of changing extraction settings on the ABTS radical scavenging efficiency in plant infusions.

Std	Run	A:MW power	B:Temp	C:Time	D:Ethanol conc.	TPC-1	TPC-2	ABTS-1	ABTS-2
		watt	0C	min	%	mg/g	mg/g	mM TE/g sample	mM TE/g sample
13	1	500	70	8	70	128.42	90.81	1625.36	1518.56
11	2	600	75	6	70	127.18	89.56	1575.64	1469.83
14	3	400	80	7	70	122.83	84.89	1352.27	1251.28
12	4	600	75	8	70	124.95	87.21	1461.35	1355.65
28	5	400	75	7	60	117.51	79.67	1082.15	997.23
27	6	500	75	7	70	129.29	91.73	1696.65	1587.36
26	7	600	75	7	80	121.49	83.76	1267.29	1179.38
18	8	400	75	7	80	116.98	79.12	1054.23	975.52
3	9	500	75	7	70	129.43	91.87	1713.29	1604.27
1	10	500	70	7	80	119.03	81.22	1146.67	1047.36
19	11	400	70	7	70	116.17	78.31	1025.36	956.31
10	12	500	80	7	80	123.85	85.95	1402.38	1298.92
22	13	500	75	8	80	123.62	85.73	1381.78	1277.81
4	14	500	75	7	70	129.16	91.59	1682.39	1572.53
17	15	500	75	7	70	129.02	91.46	1665.31	1558.62
16	16	600	75	7	60	121.78	83.97	1288.74	1203.18
2	17	500	80	7	60	124.26	86.39	1434.54	1327.42
7	18	500	80	6	70	127.88	90.27	1597.28	1489.76
5	19	500	75	7	70	128.87	91.32	1654.49	1546.54
8	20	500	70	7	60	119.88	82.16	1189.19	1104.17
9	21	400	75	8	70	122.58	84.67	1321.58	1224.73
25	22	500	70	6	70	120.81	83.08	1239.35	1157.21
21	23	500	75	8	60	125.78	88.11	1512.34	1407.64
6	24	600	70	7	70	126.97	89.34	1558.33	1449.72
24	25	500	75	6	60	120.13	82.39	1208.62	1131.42
15	26	500	80	8	70	126.46	88.75	1539.21	1431.26
TPC-1 = <i>Reichardia tingitana</i> ; TPC-2 = <i>Atriplex halimus</i> ; ABTS-1 = <i>R. tingitana</i> ; ABTS-2 = <i>A. halimus</i>									

Std	Run	A:MW power	B:Temp	C:Time	D:Ethanol conc.	TPC-1	TPC-2	ABTS-1	ABTS-2
29	27	500	75	6	80	119.27	81.41	1162.26	1073.54
20	28	400	75	6	70	118.29	80.46	1115.46	1016.12
23	29	600	80	7	70	125.19	87.45	1479.87	1382.23
TPC-1 = <i>Reichardia tingitana</i> ; TPC-2 = <i>Atriplex halimus</i> ; ABTS-1 = <i>R. tingitana</i> ; ABTS-2 = <i>A. halimus</i>									

The findings indicated a substantial relationship between phenolic composition and antioxidant capacity, supported by high correlation coefficients ($r = 0.999$ and 0.998 for TPC-1/ABTS-1, and TPC-2/ABTS-2 respectively), as depicted in correlation plots and the scatterplots (**Figure S3**). These findings align with previous studies indicating a strong association between phenolic content and antioxidant activity, suggesting that phenolic compounds are the primary contributors to the extract's antioxidant potential (Zaroug et al., 2014; Sir Elkhathim et al., 2018). Siddhuraju and Becker, 2003, examined the DPPH scavenging potential of different solvent extracts derived from various tea varieties. It was observed that the 50% aqueous ethanol extracts possessed the most pronounced antioxidant activity (26.62–61.39% inhibition), outperforming both the 50% aqueous methanol (30.35–44.71%) and water extractions (18.82–23.65%). This suggests that antioxidant activity is closely tied to phenolic content, with the 50% ethanol–water mixture being the most efficient solvent system for phenolic extraction.

4. Conclusion

In the current study, polyphenols from *Reichardia tingitana* and *Atriplex halimus* were extracted using microwave-assisted extraction techniques, using various extracting conditions such as solvent types, temperature and contact time. Comparison of the various parameters revealed that 50% ethanol-water mixture yielded the highest phenolic content, followed by acetone-water and aqueous methanol, while 70–80 °C temperature and 6–8 minutes of contact time were found optimum parameters for maximum TPC yields. Following the single-factor experiments, the optimal ranges of parameters were subjected to further optimization via a sequential Box–Behnken experimental design employing response surface methodology. Through RSM analysis, the most favorable extraction settings were found to be MW power (500 W), temperature (75 °C), contact time (7 min) and ethanol% (70%). A regression model was employed to establish the relationship between the tested factors and polyphenol yield. A Pareto chart was constructed to demonstrate the extent to which extraction parameters and their interactions affected the polyphenol content.

Of the four variables considered, TPC yield was most strongly influenced by MW power, followed by temperature, and time. No significant effect of ethanol concentration variation on the extraction process was observed. In terms of paired effects, temperature combined with time had the most notable influence on TPC yield, followed by MW power combined with temperature and and time. No significant paired effects were observed between ethanol concentration and the other factors. The antioxidant activity of the extracts, obtained under varying parameters, showed a strong correlation with their polyphenol content.

This research highlights that the use of optimized extraction conditions can lead to greater recovery of health-promoting compounds, especially those with antioxidant properties. Further investigations are recommended to explore the availability and behaviour of polyphenols in the studied plants under a broader range of extraction parameters.

Abbreviations

MAE: Microwave assisted extraction; TPC: Total polyphenol contents; RSM: Response surface methodology; BBD: Box behnken design; DW: dry weight, EtOAc: Ethyl acetate; AcMe₂: Acetone; EtOH: Ethanol; MeOH: Methanol; ACN: Acetonitrile

Declarations

Conflict of interest

The authors declare that there are no financial or personal relationships that could have influenced the outcome of this research.

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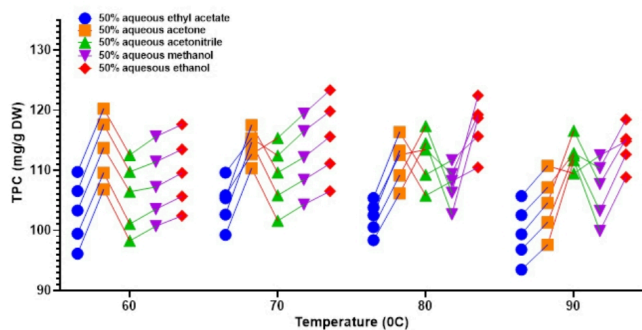
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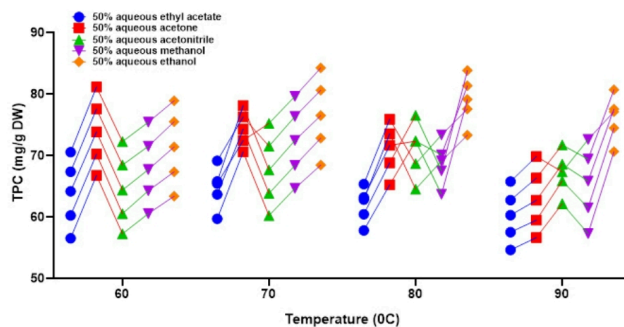
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Figures



(a)

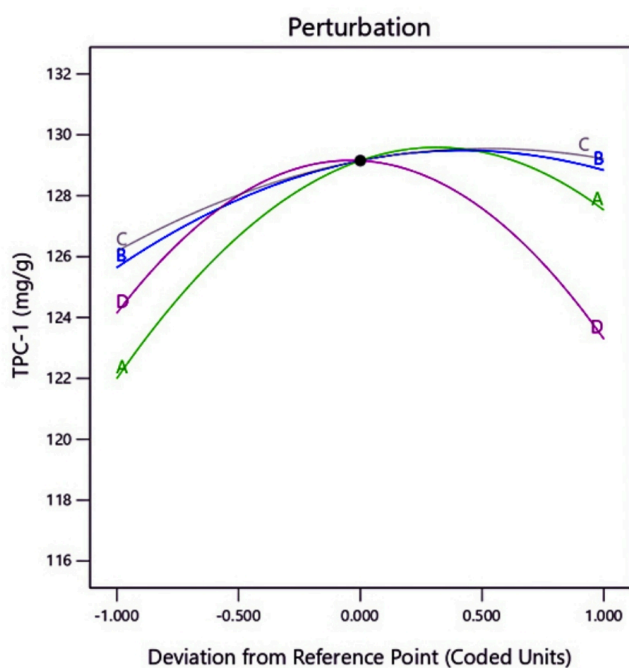


(b)

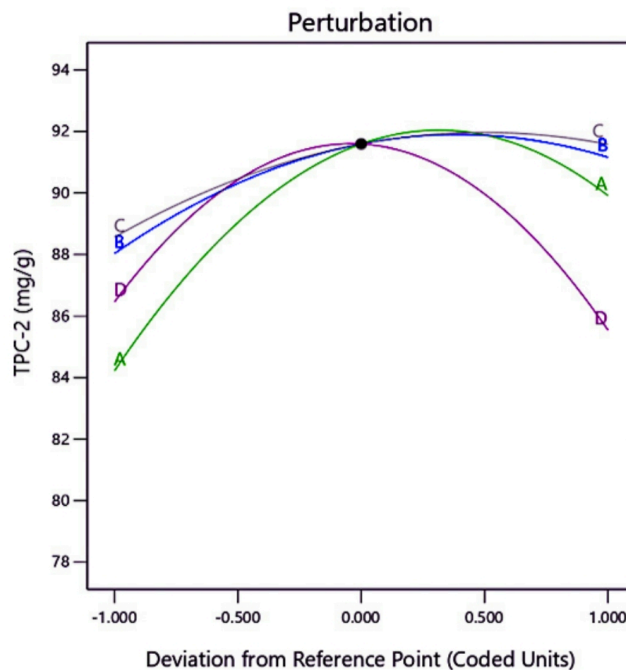
Figure 1

Variation of total phenolic content in (a) *Reichardia tingitana* (b) *Atriplex halimus* with various solvent types and temperature.

Note: the various value symbols at different temperatures represent the TPC contents at different extraction times (2-10 minutes)



(a)



(b)

Figure 2

Graphs showing the impact of single factors on the TPC outcome of (a) *R. tingitana* and (b) *A. halimus*: MW power-A; temperature-B; contact time-C; ethanol concentration-D

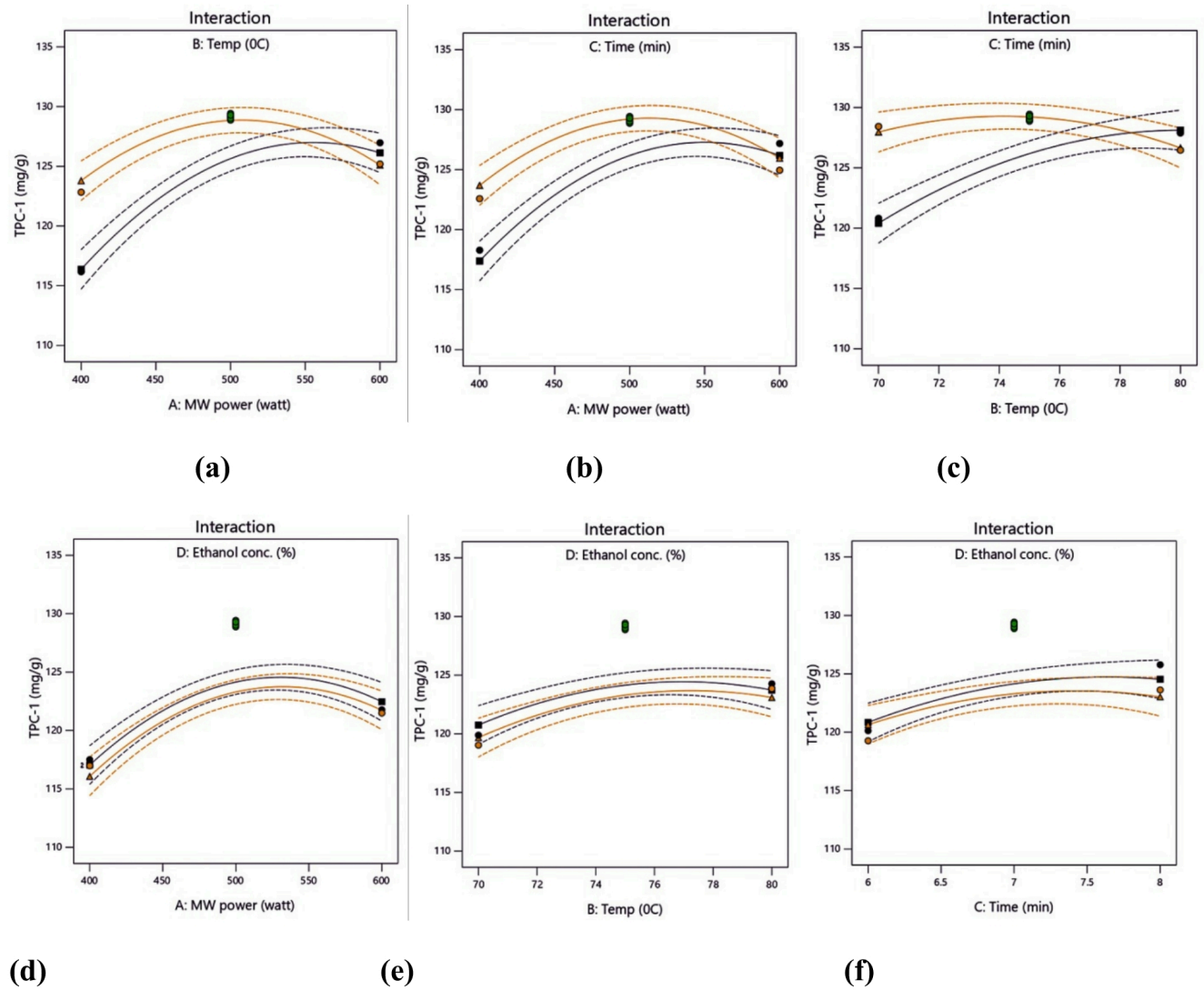


Figure 3

Interaction plots of paired-factors effect on the TPC yield in *R. tingitana*: (a) MW power-temperature; (b) MW power-time; (c) temperature-time; (d) MW power-ethanol%; (e) temperature-ethanol%; (f) time-ethanol%.

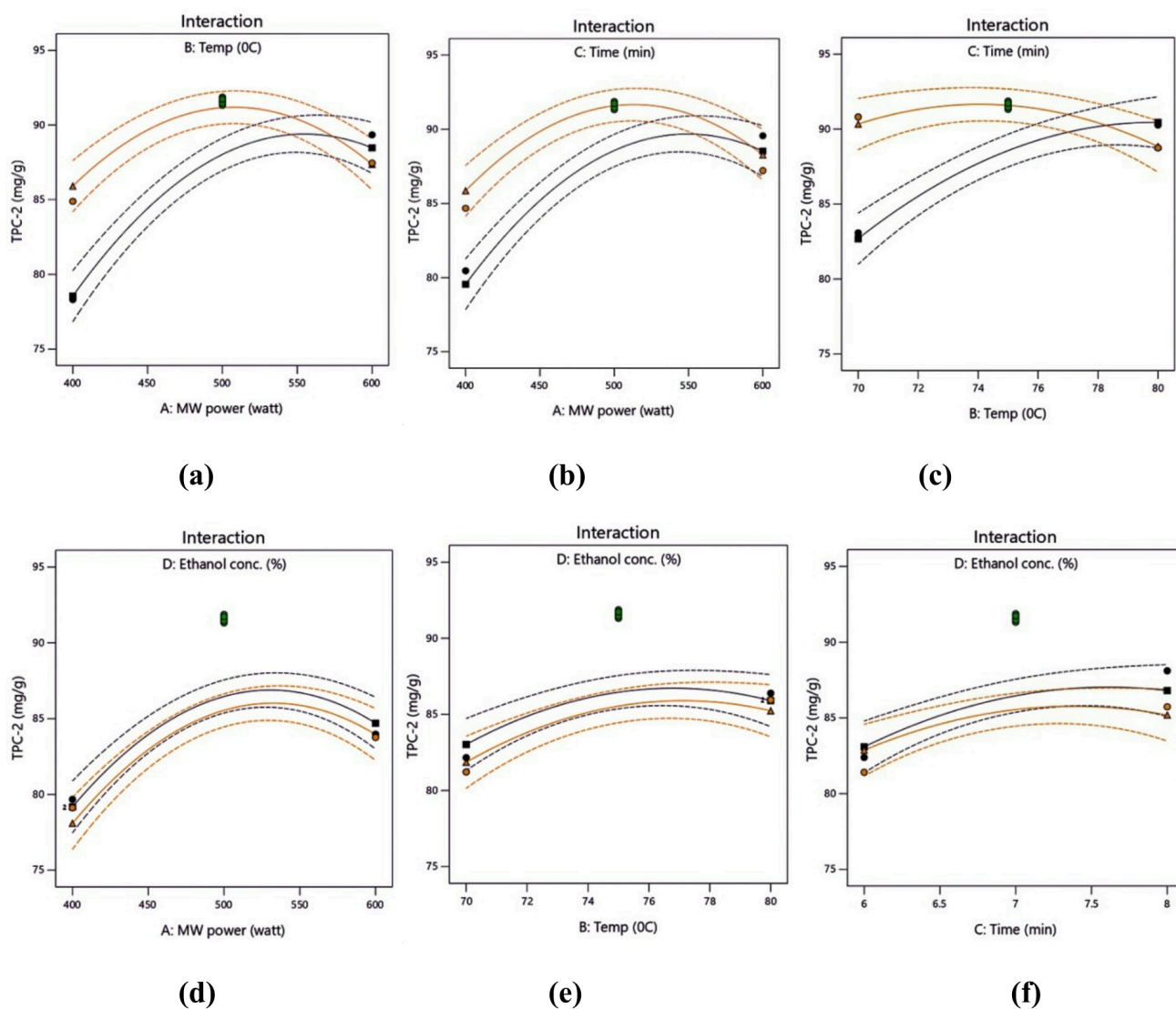


Figure 4

Interaction plots evaluating the paired-effect of variable on the TPC yield of *A. halimus*: (a) MW power vs. temperature; (b) MW power vs. time; (c) temperature vs. time; (d) MW power vs. ethanol concentration; (e) temperature vs. ethanol concentration; (f) time vs. ethanol concentration.

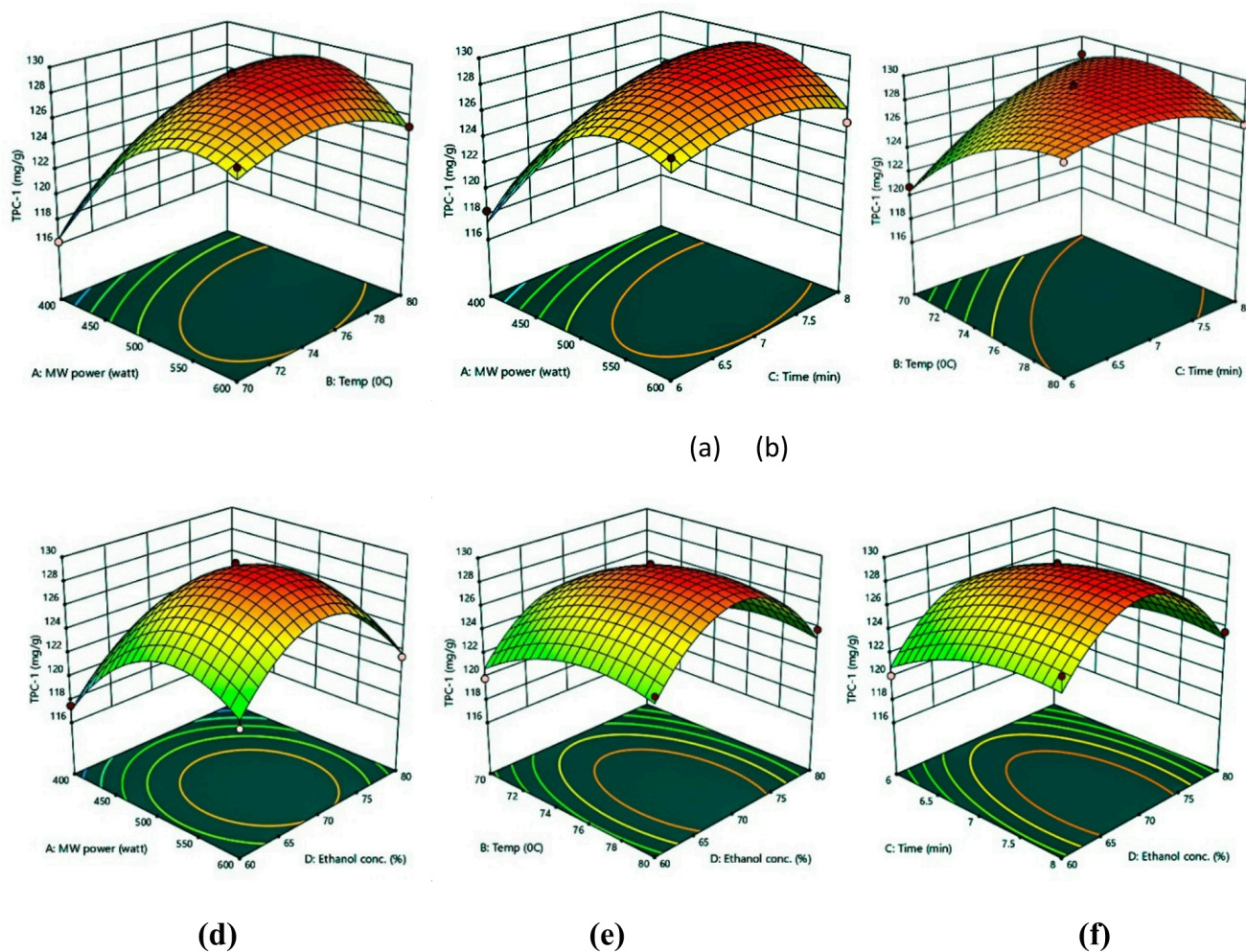


Figure 5

Response surface 3D diagrams depicting the interaction-effect between the variables influencing the TPC yield in *R. tingitana*: (a) MW power vs temperature, (b) MW power vs time, (c) temperature vs time, (d) MW power vs ethanol%, (e) temperature vs ethanol%, and (f) time vs ethanol%.

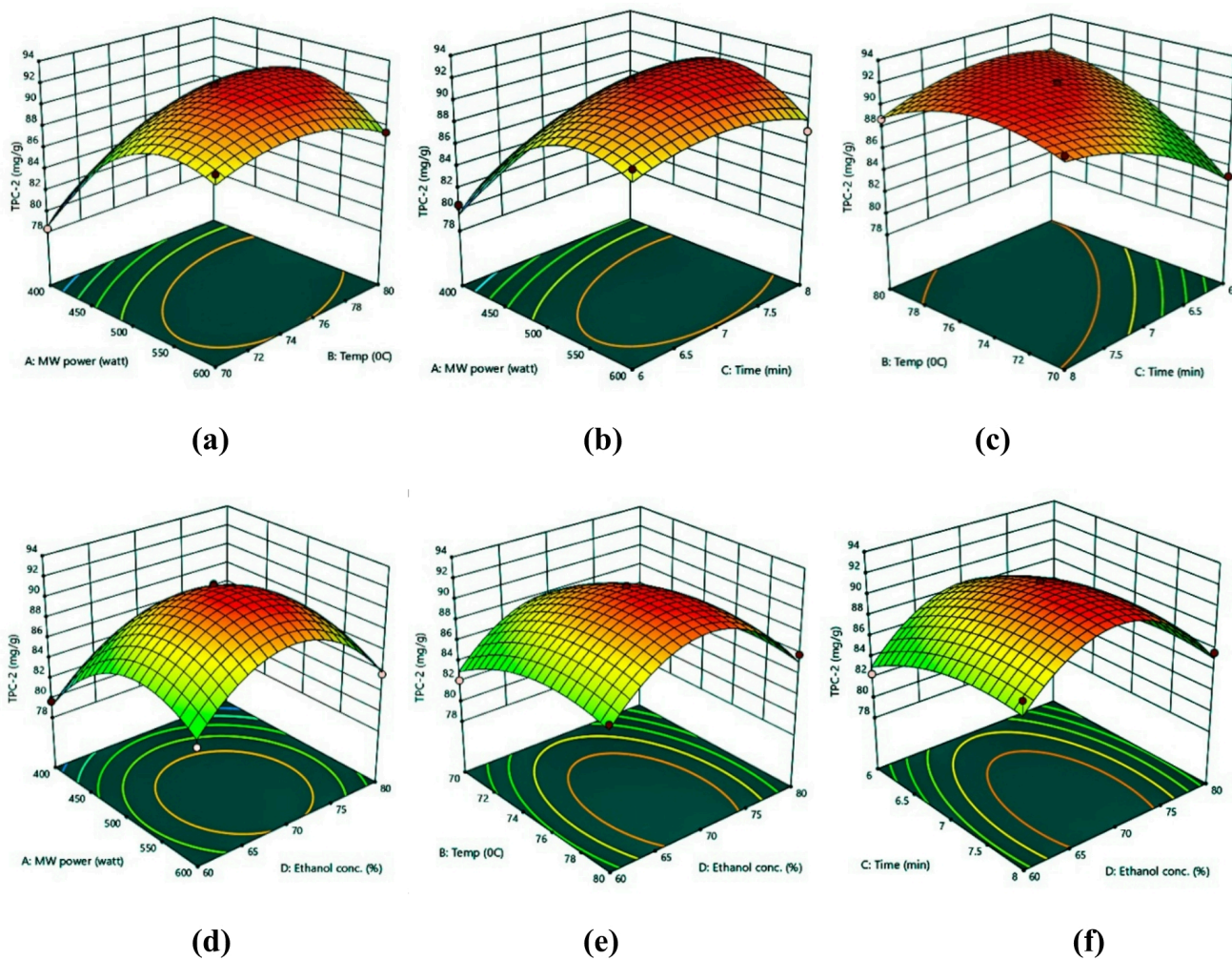


Figure 6

The response surface graphs for the combined effects of parameters on the TPC yield of *A. halimus*: (a) MW power-temperature, (b) MW power-time, (c) temperature-time, (d) MW power-ethanol concentration, (e) temperature-ethanol concentration and (f) time-ethanol concentration.

Supplementary Files

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