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Application of Multi-Level Factorial Design in Optimisation of Ultrasound-assisted Extraction (UAE) Condition from *Erythrina variegata* L. Leaves on Antioxidant Activities

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ABSTRACT

Erythrina variegata L. (dedap), from the Fabaceae family, has been widely used as an anti-inflammatory, antimicrobial, antioxidant and wound healing agent. There is a lack of literature data regarding the optimisation of extraction parameters from this plant on antioxidant activities, particularly involving Ultrasound-assisted Extraction (UAE). Therefore, this study aims to determine the optimum extraction parameter for the UAE technique from *E. variegata* leaves extracts on in-vitro antioxidant activities by applying a multi-level factorial design. The solvent (methanol) concentration (X_1) and extraction time (X_2) were the independent variables, while the dependent variables are antioxidant assays: DPPH radical scavenging activity (Y_1), ferric reducing antioxidant power (FRAP) (Y_2) and total phenolic content (TPC) (Y_3). The results showed that the optimised extraction condition was at 100% solvent concentration and 60 minutes of extraction time,

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which demonstrated 0.301 ± 0.013 mg/mL, 54.624 ± 0.143 mg $FeSO_4$/g and 29.433 ± 0.062 mg GAE/g of DPPH activity, FRAP assay and TPC values, respectively. However, the highest extraction yield of 18.56% was obtained when extracted at only 25% solvent concentration and 60 minutes. This research has provided basic scientific evidence that optimum UAE conditions for *E. variegata* are crucial to maximising antioxidant activity.

Keywords: Antioxidant, *Erythrina variegata* L., extraction parameters, multi-level factorial design, ultrasound-assisted extraction

INTRODUCTION

Medicinal plants have been acknowledged as an alternative treatment for many ailments, including those in the genus *Erythrina*. This genus comprises approximately 110 species and belongs to the family of Fabaceae (Amir et al., 2011). One of the genera *Erythrina* cultivated in Malaysia is *Erythrina variegata* L. (synonym *Erythrina indica* L.), which is also recognised as the "Indian coral tree" or "Tiger's claw" and is locally referred to as "dedap" in Malaysia. It is a fast-growing tree that originates mainly in tropical and subtropical regions of the Asia-Pacific (Keerthana et al., 2024). This species is characterised by its striking flowers, trifoliate leaves, and rapid growth, reaching heights of up to 60 feet. Different parts of the plant, such as leaves, barks and flowers, have been employed by traditional practitioners in folk medicine as a nervine sedative, antiasthmatic, antiepileptic, antiseptic, and astringent (Kavitha et al., 2023; Kumar et al., 2010). Due to its diverse pharmacological potential, *E. variegata* has become a subject of increasing interest for phytochemical and pharmacological research.

Species from the *Erythrina* genus have enormous sources of secondary metabolites, particularly flavonoids, isoflavones, and pterocarpans, which possess strong antioxidant properties (Jiménez-Cabrera et al., 2020). These compounds are effective in neutralising reactive oxygen species (ROS), scavenging free radicals, and inhibiting lipid peroxidation (Kavitha et al., 2023; Sakat & Juvekar, 2010). Antioxidants derived from *E. variegata* have shown promising *in-vitro* activity, including high DPPH radical scavenging capacity, indicating their potential role in preventing oxidative stress-related ailments such as cardiovascular disorders, inflammation, and cancer (Kavitha et al., 2023; Laveena & Chandra, 2018). Plant-based compounds have drawn researchers' interest nowadays as potential alternatives for synthetic antioxidants due to their safety and low toxicity to vertebrates (Baranitharan et al., 2019). Consequently, further exploration of *E. variegata* as a natural antioxidant source may contribute to the development of safe, plant-based therapeutic agents.

The extraction process is a cornerstone in natural product research, which involves desired bioactive compounds that are separated from the raw material. Continuous initiatives are being made to obtain and discover better extraction techniques that are

more economical and efficient (Pandey et al., 2024). Extraction technique, solvent type, solvent concentration and also extraction time and extraction temperature will influence the extraction efficiency of bioactive compounds and antioxidant potential in the extract (Wong et al., 2014). In this study, ultrasound-assisted extraction is employed due to its proven efficiency in extracting phenolic and flavonoid compounds, which are key contributors to antioxidant activity (El Baakili et al., 2023). It is a modern extraction technique that applies high-frequency ultrasonic waves to create acoustic cavitation in the extraction medium, which can cause the disruption of plant cell walls and facilitate the drug surface area for solvent penetration (Demesa et al., 2024). Furthermore, the UAE method allows for fine-tuning of variables such as sonication time, amplitude, solvent concentration, and solid-to-liquid ratio. By optimising these parameters using statistical tools like response surface methodology (RSM), UAE can maximise extraction efficiency while minimising degradation of target compounds (Belwal et al., 2016; Damayanti et al., 2021). Despite the reported antioxidant potential of *E. variegata*, limited studies have focused on optimising extraction parameters using ultrasound-assisted extraction (UAE). In particular, there is a lack of systematic studies employing a multilevel factorial design to evaluate the interaction between extraction variables and their combined effect on antioxidant activity. Therefore, this study aims to address this gap by optimising UAE conditions (solvent concentration and extraction time) to maximise antioxidant recovery from *E. variegata* leaves by applying a multi-level factorial design.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents used were of analytical grade. Folin-Ciocalteu reagent, gallic acid monohydrate, 2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri-2-pyridinyl-1,3,5-triazine (TPTZ), and hydrochloric acid were procured from Sigma Aldrich, Germany. Meanwhile, sodium carbonate anhydrous, sodium acetate trihydrate, acetic acid, iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), and iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) were purchased from QRëC, Malaysia. Methanol and ascorbic acid were acquired from R&M, Malaysia, and Thermo Fisher, USA, respectively.

Sample Collection and Preparation

Fresh *Erythrina variegata* L. leaves were collected from a local cultivator at Jasin, Melaka, in December 2024. It is verified by Dr. Khairil Mahmud, a botanist from the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia (UPM), with the voucher specimen number (KM0116/24), and it was deposited at the Herbarium Unit, Universiti Putra Malaysia (UPM), for future reference. The leaves were cleaned, air-dried in the shade for around a week or until the moisture content reduced to about 10% (Rahim et al., 2022). It was then ground into powder and kept in sealed plastic bags.

Sample Extraction (Ultrasound-assisted Extraction (UAE))

The UAE procedure was conducted as reported by Zahari et al. (2025) with slight alterations. The powdered *E. variegata* leaves were mixed with methanol at various concentrations ranging from 0%, 25%, 50%, 75%, and 100% v/v, following a 1:10 ratio of solid to solvent. The mixture was then placed in an ultrasonic cleaner (Elma E-30-H, Germany) with a power of 280 W and frequency of 37 kHz setting for varying extraction times of 30, 45, and 60 minutes and at a fixed extraction temperature of 60 °C. The sample was then centrifuged for 15 minutes at 4200 rpm, followed by filtration using Whatman filter papers. The resulting filtrate was evaporated under reduced pressure at 60 °C with a rotary evaporator (Buchi Switzerland R-100, Switzerland) until a constant weight was obtained, and the extraction yield was calculated as in Equation 1. The crude extract was stored in a vial at -20 °C for further antioxidant testing.

$$\text{Extraction yield (\%)} = (W_{ext} / W_p) \times 100 \quad [1]$$

where W_p is the mass of the dry plant and W_{ext} is the mass of crude extract

The fixed conditions of ultrasonic power (280 W) and frequency (37 kHz) were selected based on the method by Zahari et al. (2025) of the ultrasonic bath, and previous studies also reported that effective cavitation within this range for phenolic extraction (Dzah et al., 2020; Gao et al., 2022). Meanwhile, the extraction temperature was fixed at 60 °C to enhance mass transfer while preventing thermal degradation of heat-sensitive bioactive compounds (Dzah et al., 2020). These parameters were kept constant to allow focused optimisation of solvent concentration and extraction time, which are known to significantly influence extraction efficiency. The solvent concentration range (0-100% methanol) was selected to represent a wide polarity spectrum, as solvent polarity significantly influences the extraction of phenolic compounds. Similarly, extraction time (30-60 minutes) was chosen based on commonly reported UAE durations that balance extraction efficiency and compound stability (Dzah et al., 2020; Gao et al., 2022; Kaushik & Barmanray, 2025).

Experimental Design

A multi-level factorial design was applied to determine the optimum UAE condition of *E. variegata* leaves extract to achieve maximum antioxidant activities. The multi-level design is a unique type of full factorial design that permits a random number of levels for the studied factors in an experiment, which encompasses every possible combination of factor level (Elkanayati et al., 2024). In this study, the solvent concentration (X_1) and extraction time (X_2) were the independent variables to optimise the response variables,

which are antioxidant assays: DPPH radical scavenging activity (Y_1), ferric reducing antioxidant power (FRAP) (Y_2) and total phenolic content (TPC) (Y_3). The independent variables (factors) and their varying levels are shown in Table 1. The experimental runs were built using a statistical software package, Design Expert® (Version 13, State Ease, Inc., Minneapolis, USA) and generated a total of 15 runs.

Table 1
Levels of independent variables used in the multilevel factorial design

Factors	Unit	Levels	Values
Solvent Concentration (X_1)	% (v/v)	5	0, 25, 50, 75, 100
Extraction Time (X_2)	min	3	30, 45, 60

Antioxidant Activity - DPPH Radical Scavenging Activity

The antioxidant activity of *E. variegata* leaves extract was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Itam et al., 2021) with minor changes. In this method, DPPH methanolic solution (0.1 mM) was prepared freshly, and 2 mL of the DPPH solution was mixed with the sample extract (1 mL). The mixture was thoroughly vortexed for about 1 minute and then kept in the dark for 30 minutes. The measurement of the absorbance reading was taken at 517 nm using a UV-Vis spectrophotometer (T60, PG Instruments, UK). The percentage of DPPH radical scavenging activity was calculated using the following Equation 2:

$$\text{DPPH radical scavenging activity (\%)} = [(A_0 - A_1) / A_0] \times 100 \quad [2]$$

where A_0 represents the absorbance of the control, and A_1 represents the absorbance of the extract or standard

Additionally, the DPPH radical scavenging activity of the positive control (ascorbic acid) and all *E. variegata* leaves extracts was evaluated and expressed as an IC_{50} value, which indicates the concentration of the extracts required to scavenge 50% of the radicals. Five different concentrations varying from 0.1, 0.25, 0.5, 1 and 2 mg/mL of each sample were prepared, and the IC_{50} value was determined through linear regression between inhibition percentage and sample concentration. The IC_{50} value classified antioxidant activity as follows: IC_{50} < 50 µg/mL (very strong), IC_{50} = 50-100 µg/mL (strong), IC_{50} = 101-250 µg/mL (moderate), IC_{50} = 250-500 µg/mL (weak), and IC_{50} > 500 µg/mL (inactive) (Itam et al., 2021).

Antioxidant Activity - Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was performed according to the procedure described by Chaves et al. (2020) with minor changes. *E. variegata* leaves extract (0.2 mL) was mixed with FRAP reagent (3.8 mL). The FRAP reagent was freshly prepared by combining 1 part of 10 mM TPTZ, 1 part of 20 mM $FeCl_3 \cdot 6H_2O$ and 10 parts of 300 mM sodium acetate buffer (pH 3.6). The mixture was then incubated in the dark at 37°C for 10 minutes. After incubation, the absorbance reading was assessed at 593 nm using a UV-Vis spectrophotometer. A blank sample was prepared by replacing the extract with an equal volume of methanol. The FRAP results were expressed as milligram equivalents of $FeSO_4$ per milligram of dry weight. A standard calibration curve was plotted using $FeSO_4$ solutions at different concentrations (20, 40, 60, 80, and 100 mg/mL).

Total Phenolic Content (TPC)

The total phenolic content in the extracts was analysed using the Folin-Ciocalteu colourimetric method with minor modifications (Proestos et al., 2013). Briefly, *E. variegata* leaves extract (500 µL) was added to a volumetric flask sized 10 mL that contained 2.5 mL of Folin-Ciocalteu reagent (diluted 10 times and freshly prepared) and left in the dark for 5 minutes at ambient temperature. Then, 2 mL of sodium carbonate (7.5% w/v) was added, and the flask was filled to volume with distilled water. The mixture was shaken vigorously and allowed to stand in the dark for 120 minutes. The absorbance was then measured at 760 nm using a UV-Vis spectrophotometer. Calibration was performed using a standard curve prepared with various concentrations (0.02, 0.04, 0.06, 0.08, and 0.10 mg/mL) of gallic acid, and the results were expressed as milligrams of gallic acid equivalent (mg GAE) per gram of extract. All measurements were conducted in triplicate for each extract.

Statistical Analysis

All analyses were carried out in triplicate, and the findings were conveyed as means ± standard deviation (SD). Differences between the variables were evaluated for significance using the one-way ANOVA with Tukey-LSD analysis procedure when $p<0.05$. All analyses were performed using OriginPro 2018 software (Aranda-Ledesma et al., 2024).

RESULTS AND DISCUSSION

Extraction Yield

Table 2 shows the results of the extraction yield obtained for each trial run generated on the ultrasound-assisted extraction condition. Extraction yield was measured by dividing the mass of extract by the mass of dry matter to determine the effect of extraction efficiency on *E. variegata* leaves extract.

Table 2
Extraction yield obtained from E. variegata *leaves extract*

Run No.	Extraction Parameters		Extraction Yield (%)
	Solvent (Methanol) Concentration (%)	Extraction Time (min)	
1	0	30	13.00
2	25	30	16.00
3	50	30	17.44
4	75	30	15.92
5	100	30	5.56
6	0	45	5.20
7	25	45	15.00
8	50	45	17.48
9	75	45	10.52
10	100	45	7.08
11	0	60	12.08
12	25	60	18.56
13	50	60	18.24
14	75	60	9.40
15	100	60	8.52

The highest yield was obtained when extracted at 25% solvent concentration and 60 minutes (Run 12) with 18.56%. In this study, the solvent used was methanol, with varying from 0 to 100% concentration. The solvent concentration of 25% had the highest yield, which may be due to the solvent polarity increasing and improving solvent penetration to the plant matrix, allowing a wider range of compounds to be extracted (Gil-Martín et al., 2022). However, the extraction process may be disrupted if the solvent is too concentrated (Zhang et al., 2018). This is agreed in a previous study that stated the extraction yield for water-methanol is higher than that of organic solvents alone (Dhanani et al., 2017). In addition, prolonged extraction time at higher temperatures can often lead to greater extraction yields, but it can also lead to degradation of the extracted compounds. For this study, the extraction yield was higher when extracted for a longer time (60 minutes), but the temperature used was fixed at 60 °C. This condition maintains the composition of bioactive compounds, particularly polyphenols, which may be damaged at higher temperatures exceeding 60 °C as established in prior studies (Dzah et al., 2020; Hlatshwayo et al., 2025).

As mentioned previously, the higher extraction yield observed at lower solvent concentrations (25%) may be attributed to the extraction of highly polar compounds such as sugars, proteins, and other non-phenolic constituents. In contrast, higher solvent concentrations (100%) assist the extraction of less polar phenolic and flavonoid compounds, which are predominantly responsible for antioxidant activity.

This explains the observed discrepancy between extraction yield and antioxidant activity. Solvent polarity plays a critical role in determining the selectivity and efficiency of compound extraction, influencing the solubility of different phytochemicals (Lučić & Onjia, 2025). Furthermore, ultrasound-assisted extraction enhances the release of intracellular compounds through cavitation and cell wall disruption, improving the recovery of bioactive compounds (Shen et al., 2023). Similar findings have been reported where increased extraction yield does not necessarily correspond to higher bioactivity, particularly antioxidant activity, due to differences in extract composition and compound selectivity (Demesa et al., 2024).

Optimisation of Extraction Parameters - Model Fitting

A multi-level factorial design was employed to optimise the ultrasound-assisted extraction conditions of *E. variegata* leaves extracts for maximising the antioxidant activities. Table 3 showed the outcomes of the independent variables (solvent concentration, X_1; extraction time, X_2) and their influence on the response variables (antioxidant activities - DPPH, Y_1; FRAP, Y_2; TPC, Y_3) across 15 experimental runs on UAE. These three assays were chosen to comprehensively assess antioxidant potential, as it is suggested to perform at least two or more complementary assays due to the complexity of antioxidant mechanisms. The DPPH assay is a widely used method that evaluates the ability of antioxidants to neutralise free radicals by donating hydrogen atoms or electrons, thus reflecting free radical scavenging capacity (Gulcin & Alwasel, 2023). Meanwhile, FRAP measures the ability of an antioxidant to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, indicating the reducing power and potential metal-chelating activity of the extract. The TPC assay basically quantifies the phenolic compounds that strongly correlated with antioxidant activity, as phenolics contribute significantly to the scavenging and reducing capacity of plant extracts (Chaves et al., 2020). The primary aim of this study was to optimise extraction parameters to maximise antioxidant activity and phenolic recovery, which is a common approach in UAE studies before detailed compound identification.

The table shows that the extraction parameters had a significant effect on antioxidant activity. Run 15 (solvent concentration of 100% and extraction 60 minutes) had significantly higher antioxidant activities (IC_{50}, 0.272 ± 0.003 mg/mL for DPPH; 54.624 ± 1.983 mg $FeSO_4$/g for FRAP) and TPC values (29.541 ± 0.306 mg GAE/g) as compared to other experimental runs. The enhanced antioxidant activity observed at higher methanol concentrations can be attributed to the selective extraction of phenolic compounds such as flavonoids, isoflavones, and phenolic acids, which are known to be soluble in organic solvents. Methanol has been widely reported as an effective solvent for extracting these compounds due to its polarity, which is able to penetrate plant matrices of *E. variegata* leaves extract better and leads to an enhancement of extraction efficiency

Table 3
Experimental design with the response variable of antioxidant activities

Run No.	Extraction Parameters		Antioxidant Activities		
	Solvent (Methanol) Concentration (%)	Extraction Time (min)	DPPH, IC_{50} (mg/mL)	FRAP (mg $FeSO_4$/g)	TPC (mg GAE/g extract)
Ascorbic Acid	-	-	0.189 ± 0.071^{a}	-	-
1	0	30	0.321 ± 0.002^{c}	29.249 ± 0.488^{d}	26.689 ± 0.501^{b}
2	25	30	0.490 ± 0.001^{d}	30.344 ± 2.100^{d}	28.276 ± 1.361^{a}
3	50	30	0.442 ± 0.004^{d}	32.628 ± 1.176^{d}	24.578 ± 1.391^{c}
4	75	30	0.421 ± 0.003^{d}	24.399 ± 0.236^{e}	27.711 ± 1.212^{b}
5	100	30	0.288 ± 0.003^{b}	35.225 ± 1.239^{c}	28.828 ± 1.142^{a}
6	0	45	0.357 ± 0.004^{c}	40.544 ± 0.482^{c}	14.544 ± 0.199^{e}
7	25	45	0.485 ± 0.002^{d}	32.253 ± 0.895^{d}	23.542 ± 0.511^{d}
8	50	45	0.461 ± 0.003^{d}	31.783 ± 2.397^{d}	24.161 ± 1.952^{c}
9	75	45	1.158 ± 0.004^{f}	33.911 ± 3.802^{d}	24.403 ± 0.458^{c}
10	100	45	1.755 ± 0.008^{f}	45.112 ± 5.541^{b}	25.734 ± 0.874^{c}
11	0	60	1.064 ± 0.001^{f}	37.447 ± 1.034^{d}	24.241 ± 0.176^{c}
12	25	60	0.377 ± 0.001^{c}	32.534 ± 5.216^{d}	28.451 ± 0.537^{a}
13	50	60	0.490 ± 0.001^{d}	44.799 ± 2.401^{b}	27.335 ± 0.269^{b}
14	75	60	0.516 ± 0.002^{e}	37.071 ± 1.067^{d}	26.366 ± 0.292^{b}
15	100	60	0.272 ± 0.003^{b}	54.624 ± 1.983^{a}	29.541 ± 0.306^{a}

Note. Different letters in each column indicate significant differences ($p<0.05$); DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = Ferric reducing antioxidant power; TPC = Total phenolic content; Ascorbic acid was used as a positive control for DPPH assay

for phenolics and antioxidants (Ahmed et al., 2024; Jiménez-Cabrera et al., 2020). The antioxidant activity of the extract was also compared with the positive control (ascorbic acid), which exhibited a moderate radical scavenging activity (IC_{50}, 0.189 ± 0.071 mg/mL), and it is well-known to act as a reducing agent that effectively reduces the DPPH radical (Jiménez-Cabrera et al., 2020).

In addition, a longer extraction time gives sufficient time for the penetration of phenolic compounds into the solvent and improves the antioxidant activities (Uma et al., 2010). However, a contradictory finding was observed for the lowest value of DPPH (run 10; solvent concentration 100% and 45 minutes), FRAP (run 4; concentration 75% and 30 minutes) and TPC (run 6; concentration 0% and 45 minutes). This may be due to the fact that the extraction time was inadequate to extract more antioxidant compounds (Mungwari et al., 2024). The UAE method generally involves the use of ultrasound that is significantly able to preserve the chemical integrity of bioactive compounds under standard conditions, which is important in maintaining the antioxidant properties of the extract (Demesa et al., 2024).

Table 4 depicts the analysis of variance (ANOVA) findings for the UAE condition on antioxidant activities. ANOVA was performed to reveal the significance of each independent variable (extraction parameters) and their interaction on the measured antioxidant responses. The reliability and adequacy of the model were evaluated using *p*-values and F-values, where low *p*-values ($p<0.05$) confirm the statistical relevance and high F-values indicate strong model significance (Pimsamarn et al., 2024; Prakoso et al., 2022). Based on the table, factors X_1 (solvent concentration), X_2 (extraction time) and X_1*X_2 interaction (extraction parameters condition) have a *p*-value of less than 0.05, denoting that both factors combined play a crucial role in determining antioxidant response. Furthermore, the model F-value obtained was high, with 6208.75 (DPPH), 175018.67 (FRAP), and 151651.35 (TPC), suggesting that the regression models were highly significant and not influenced by random variation. It should be noted that the possibility of this high F-value being caused by noise is only a 0.01% (Bayat & Shokri, 2021; Madhu et al., 2018). This indicates that the model for all antioxidant activities has a significant impact on the changes in the extraction conditions.

The model summary statistics for ultrasound-assisted extraction (UAE), including the coefficient of determination (R^2), adjusted R^2, predicted R^2, standard deviation (SD), mean, coefficient of variation (CV), and adequate precision, are shown in Table 5. The R^2 value is a main indicator of how well the experimental data fit the regression model, with values approaching 1.0 reflecting excellent model performance (Aguilar-Ascon et al., 2020). In this study, the R^2 obtained for DPPH, FRAP and TPC was 0.9898, 0.9856 and 0.9927, respectively, which reveals that up to 98% of the data fit the regression model for all assays studied.

Table 4
ANOVA analysis of extraction parameters on antioxidant activities

Source	DPPH					FRAP					TPC				
	SS	**DF**	**MS**	**F-value**	***p*-value**	**SS**	**DF**	**MS**	**F-value**	***p*-value**	**SS**	**DF**	**MS**	**F-value**	***p*-value**
Model	7.12	14	0.5085	6208.75	< 0.0001	2319.69	14	165.69	175018.67	< 0.0001	550.90	14	39.35	151651.35	< 0.0001
X_1	0.72	4	0.1797	21925.40	< 0.0001	912.20	4	228.05	$2.409e^{+05}$	< 0.0001	197.32	4	49.33	$1.901e^{+05}$	< 0.0001
X_2	1.58	2	0.7900	96357.15	< 0.0001	1002.54	2	501.27	$5.295e^{+05}$	< 0.0001	223.52	2	111.76	$4.307e^{+05}$	< 0.0001
X_1X_2	4.82	8	0.6026	73498.32	< 0.0001	404.95	8	50.62	53467.99	< 0.0001	130.06	8	16.26	62653.64	< 0.0001
Residual	0.0002	28	$8.198e^{-06}$			0.0265	28	0.0009			0.0073	28	0.0003		
Corrected Total	7.12	44				2319.73	44				550.91	44			

Note. DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = Ferric reducing antioxidant power; TPC = Total phenolic content; DF = degree of freedom; SS = Sum of squares; MS = Mean square; X_1 = solvent concentration (%), X_2 = extraction time (min)

Table 5
Model summary statistics of extraction parameters on antioxidant activities

Terms	DPPH	FRAP	TPC
R^2	0.9898	0.9856	0.9927
Adjusted R^2	0.9753	0.9714	0.9837
Predicted R^2	0.9666	0.9653	0.9638
Adequate Precision	845.28	1598.33	1517.44
Standard Deviation	0.00	0.03	0.02
Mean	0.59	36.40	25.63
CV (%)	0.48	0.09	0.06

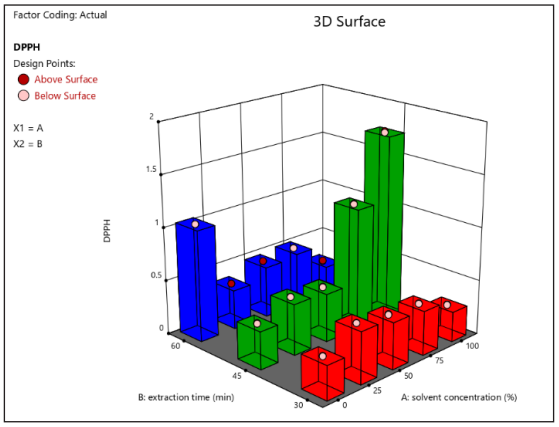
Note. DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = Ferric reducing antioxidant power; TPC = Total phenolic content; CV = Coefficient of variance

Moreover, the difference between predicted R^2 and adjusted R^2 was less than 0.2, indicating strong agreement between them. Meanwhile, adequate precision, which measures the signal-to-noise ratio of the model, further confirmed the reliability of the results. The adequate precision values obtained were 845.28 (DPPH), 1598.33 (FRAP), and 1517.44 (TPC), which are above the minimum acceptable threshold of 4.0 (Bayat & Shokri, 2021). Besides that, the obtained CVs were 0.48% (DPPH), 0.09% (FRAP), 0.06% (TPC) and these values were below 10% that implies good precision and reliability of the experiments (Rahim et al., 2022).

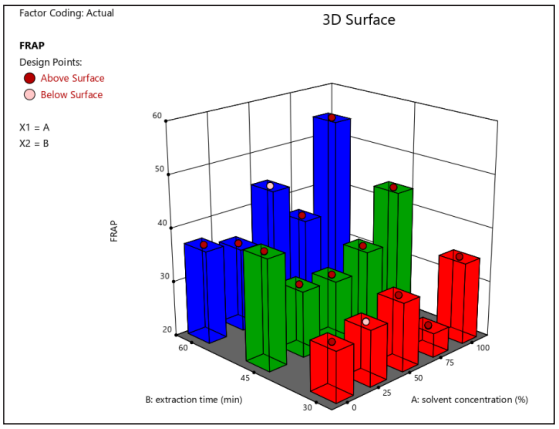
Effect of Extraction Parameters on Antioxidant Activities

Figure 1 illustrates the 3D surface plot of the independent variables (solvent concentration and extraction time) on the antioxidant activities, where Figure 1a - DPPH; Figure 1b - FRAP; Figure 1c - TPC for ultrasound-assisted extraction (UAE). For the DPPH activity, the results showed that when extracted at 100% of solvent concentration and 60 minutes of extraction time, it had a low IC_{50} value of 0.272 mg/mL, which indicates high antioxidant activity. Similar results were shown on FRAP and TPC, where, under the same conditions, they revealed a potential FRAP activity of more than 50 mg $FeSO_4$/g and almost 30 mg GAE/g of TPC values. This agrees with a previous study that stated an optimal condition for UAE is likely around higher solvent concentration (75% to 100%) and longer extraction times (Wang et al., 2013).

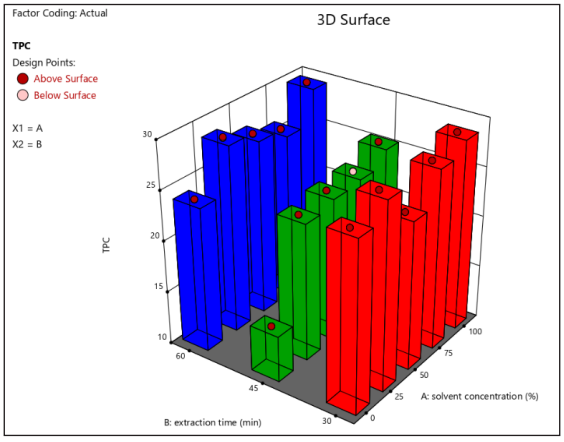
From Figure 1, the improved extraction efficiency observed in this study can be explained by the mechanism of UAE cavitation. During the UAE, ultrasonic waves generate microbubbles that undergo rapid growth and violent collapse, producing localised high pressure, temperature, and microjets. These effects disrupt plant cell walls, increase solvent penetration, and enhance the release of intracellular bioactive compounds (Demesa et al., 2024; Shen et al., 2023).



(a)



(b)



(c)

Figure 1. 3D response surface plots of *E. variegata* leaves extract showing the effect of extraction parameter conditions for ultrasound-assisted extraction (UAE) on (a) DPPH; (b) FRAP; and (c) TPC

Earlier research demonstrated that direct contact between sound waves in the UAE technique and the extraction medium allows better interaction and elevates the extraction of target compounds from cellular plant matrices (Dzah et al., 2020). This method has also demonstrated a significant influence on the bioactivity of phenolic compounds by considering the solvent used, temperature, and duration of extraction (Ramesh et al., 2024). Previous studies have demonstrated that UAE yields higher phenolic content and antioxidant activity when compared to conventional methods, such as maceration and Soxhlet extraction, primarily due to reduced thermal degradation and enhanced mass transfer (Ahmed et al., 2024; Ramesh et al., 2024).

Model Validation

The optimum UAE condition was verified by utilising the desirability of the responses using the statistical software. Table 6 displayed the summary of the validation process on the antioxidant activities of *E. variegata* leaves extract. The optimised UAE condition was at 100% solvent concentration (X_1) and 60 minutes of extraction time (X_2) and exhibited an IC_{50} value of 0.301±0.013 mg/mL for DPPH radical scavenging activity (Y_1), 54.624 ± 0.143 mg $FeSO_4$/g of FRAP values (Y_2) and 29.433 ± 0.062 mg GAE/g of TPC values (Y_3). This is similar to a previous study on *Mesembryanthemum edule* L. shoots that showed high phenolic and antioxidant properties when extracted only with 100% methanol (Dzah et al., 2020; Falleh et al., 2012). The experimental values should match the predicted values under this optimal condition, with the percentage of errors being less than 10% (Bohui et al., 2024). The results also presented that there is no significant difference (p>0.05) between the predicted and experimental findings of the antioxidant activities. This proves that the application of a multi-level factorial design, which involves a design with variable factor levels, is valid for predicting the antioxidant activities in the selected range of extraction conditions.

Table 6
Summary of the validation process on both extraction techniques on the antioxidant activities

Antioxidant Activities	Predicted Value	Experimental Value	Percentage of Error (%)
DPPH, IC_{50} (mg/mL)	0.272	0.301 ± 0.013[a]	9.74
FRAP (mg FeSO4/g)	54.666	54.624 ± 0.143[a]	0.08
TPC (mg GAE/g)	29.552	29.433 ± 0.062[a]	0.40

Note. DPPH = 2,2-diphenyl-1-picrylhydrazyl (DPPH); FRAP = Ferric reducing antioxidant power; TPC = Total phenolic content

CONCLUSION

In summary, optimisation of extraction conditions for *Erythrina variegata* leaves extracts was successfully carried out using a multi-level factorial design for ultrasound-assisted extraction (UAE). Design-Expert software enabled accurate prediction and validation of responses for all antioxidant activities (DPPH, FRAP and TPC). The optimised extraction condition was at 100% solvent concentration (X_1) and 60 minutes of extraction time (X_2), which exhibited an IC_{50} value of 0.301±0.013 mg/mL for DPPH radical scavenging activity (Y_1), 54.624 ± 0.143 mg $FeSO_4$/g of FRAP values (Y_2) and 29.433 ± 0.062 mg GAE/g of TPC values (Y_3). This indicated that the UAE method has high extraction efficiency in terms of extraction yields (18.56%) and across all measured antioxidant activities in *E. variegata* leaves extracts. This method is straightforward, less time-consuming, and relatively cheaper than other conventional extraction methods, such as maceration and Soxhlet extraction. Therefore, it can be summarised that the identified optimised UAE conditions could have potential for large-scale application in tropical agricultural processing, particularly in the development of products involving *E. variegata* leaves extracts in a food or nutraceutical industry, as this method is considered a scalable and efficient technique. Future work involving LC-MS/MS analysis is recommended to further elucidate the specific compounds responsible and establish a clearer relationship between extraction conditions and metabolite composition.

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