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Ultrasound-assisted extraction (UAE) of antioxidant phenolics from *Corchorus olitorius* leaves: a response surface optimization

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Abstract

Background Besides fibre production, *Corchorus olitorius* is considered a nutritional and medicinal plant in traditional medicine. Its leaves possess different antioxidant compounds and display various biological properties. This study optimized the ultrasound-assisted extraction (UAE) parameters like temperature, time, solvent concentration, and liquid–solid ratio for total flavonoids (TFC) and total polyphenols content (TPC) from leaves employing response surface methodology (RSM).

Results Findings demonstrated that under the optimized conditions, the highest extraction yield of total flavonoids (7.17 mg QE/g DW) and total polyphenol content (13.92 mg GAE/g DW) were recorded with the ethanol concentration 70.92%, temperature 68.06 °C, liquid–solid ratio 48.80 mL/mg, and ultrasound irradiation time 37.20 min. The optimum value of TFC (6.96 mg QE/g DW) and TPC (13.38 mg GAE/g DW) from the experiment of verification of optimized conditions was close to the predicted value and significantly superior to the conventional heat reflux extraction (HRE). LC–MS and HPLC analysis of the optimized extract from UAE demonstrated the existence of six major phenolic compounds, including chlorogenic acid, isoquercetin, hyperoside, adhyperforin, 1,3-di-*O*-caffeoylquinic acid, and 3,4-di-*O*-caffeoylquinic acid. Furthermore, the antioxidant test of the UAE leaves extract revealed an excellent 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$•) and hydroxyl radical scavenging with IC_{50} values of 226.29, 199.53 and 402.02 µg/mL, respectively, compared to HRE with 336.31 µg/mL, 253.86 µg/mL, 520.08 µg/mL.

Conclusions The developed optimization method could contribute to the good recovery of natural antioxidants from *C. olitorius* in the pharmaceuticals and food industries.

Keywords UAE, *Corchorus olitorius*, RSM, Extraction optimization, Characterization, Antioxidants

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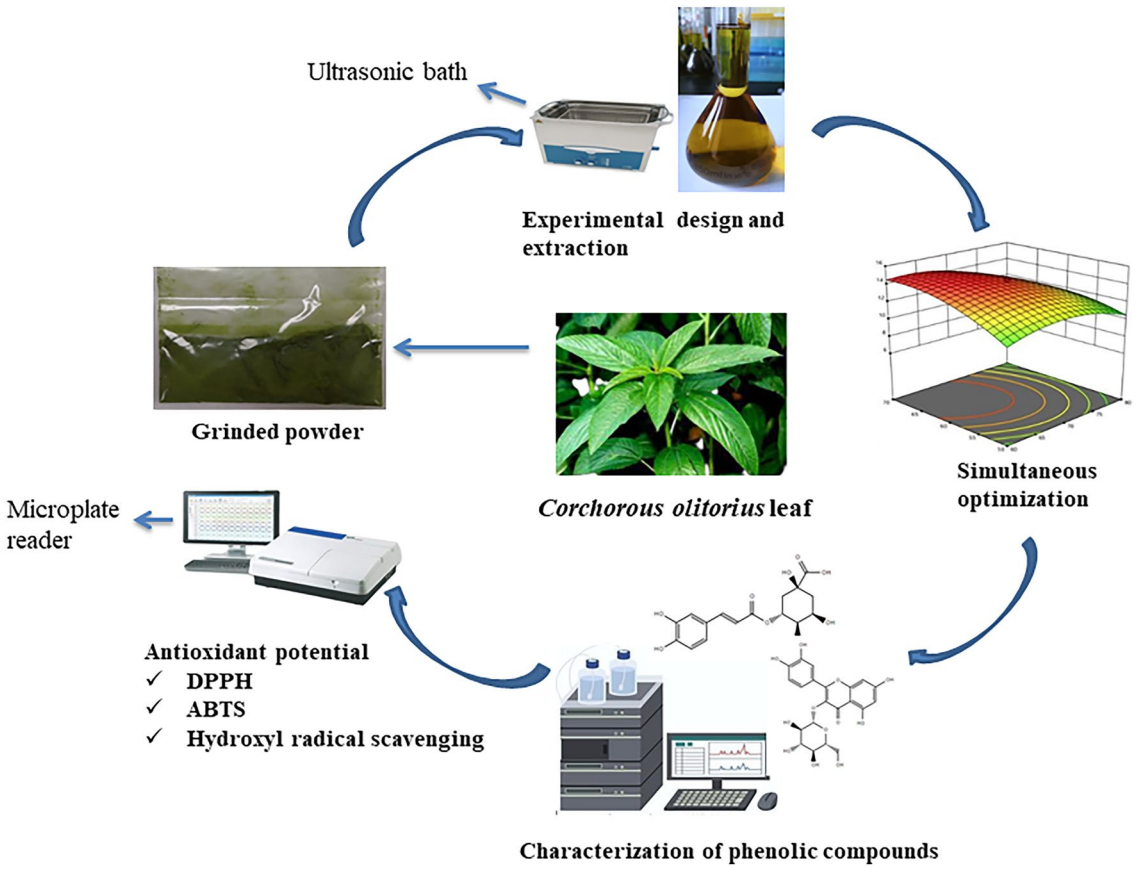
Graphical Abstract



Introduction

It has been reported that phenolic compounds from plants are an important group of natural antioxidants, and their use in the food, cosmetic, and pharmaceutical industries has gained worldwide attention due to antioxidant activities [1]. *Corchorus olitorius* (Tiliaceae) is an important green leafy vegetable which have great medicinal potential, and considerable in several tropical areas, including India, Bangladesh, tropical Asia, Sudan, and Egypt, as well as in tropical Africa, Japan, and South America [2]. The different parts of *C. olitorius* possess a range of bioactive compounds like polysaccharides, phenolics, flavonoids, cardiac glycosides, fatty acids, triterpenoids, sterols, ionones, antitumor promoters, such as phytol and monogalactosyldiacylglycerol [3–5], where its leaves display various biological properties, including diuretic, hypoglycemic, antiobesity, analgesic, gastroprotective, anti-inflammatory, and antipyretic, antibacterial, antifungal and antimicrobial, antidiabetic, antitumor function [6–9]. Besides, the leaf has been used as folk medicine to treat cystitis, dysuria, fever, gonorrhoea, laryngitis, diarrhoea, and vomiting, and these beneficial effects are linked to bioactive components. Hence, *C. olitorius* could be a significant source of high-value bioactive compounds used in confectionery, nutraceutical, and pharmaceutical purposes.

The extraction of desired antioxidants is important for determining the quantity and form of bioactive compounds from solid samples. The isolation and characterization of bioactive compounds are strongly influenced by various factors when selecting an effective extraction process. However, the optimal conditions for bioactive compounds derived from different plants are not globally consistent [10]. In addition, traditional extraction methods such as heat reflux, maceration, decoction, and soxhlet are inefficient, expensive, and time-consuming [11]. Many variables like the type of solvent, extraction temperature, solvent/dispensing ratios, and extraction time have influenced the extraction of bioactive from plant materials [12, 13]. Therefore, optimizing species-specific

optimum extraction conditions for bioactive substances and pharmacological efficiencies is crucial. Fortunately, ultrasonic-assisted extraction (UAE) has been recognized as the most efficient method for extracting antioxidant compounds while also being environmentally friendly. It is a simple, rapid, energy-saving, low-cost approach and offers an excellent selectivity of the targeted compounds compared to traditional technologies [14]; since ultrasound-generated media can decompose cell walls and effectively speed up the transfer process due to cavitation, vibration, crushing, and mixing effects [15, 16]. In addition, UAE may prevent the degradation of bioactive compounds by heat, but it can also enhance the quality of products [17].

The conventional optimization using single-factor analysis to optimize each level extraction variable is inadequate to differentiate between cross interaction of different factors. Thus statistical design experiments are important to achieve a meaningful model of different variables by conducting minimal tests. Response surface methodology (RSM) is a potent statistical tool for providing adequate data on multivariable system modelling. It reduces the number of experiments and experimental errors, and evaluates several independent parameters and their relationship in a single investigation [18, 19]. This method was first introduced for chemical processing and now effectively applying UAE procedures to examine optimal conditions for recovering bioactive constituents like phenolic compounds, polysaccharides, anthocyanins, and protein notably from diverse food matrices [20–24].

In previous research, the extraction yield between UAE and supercritical fluid extraction (SFE) for antioxidant from *C. olitorius* leaves has been compared where time, solvent volume, and temperature was considered as parameters [25]. Since, previous research was limited to comparing the extraction yield of two methods and there has no comprehensive literature available on the effects of extraction factors for the recovery of total polyphenols and total flavonoids, therefore this research was the first attempt in detail to investigate the interactions between the parameters through a polynomial mathematical model in UAE from *C. olitorius* leaves. Then, verify the developed UAE method and compare it to traditional heat reflux extraction (HRE). Additionally, performed the profiling of phenolic compounds and investigated the extracts' antioxidant capabilities obtained from optimal conditions.

Materials and methods

Plant material and preparation of extract

Plant materials

The young leaves of *C. olitorius* (T8 variety) were picked 45 days after sowing (DAS) from the experimental field of the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha, China. The collected leaves were dried until their weight remained constant using natural ventilation. Then dried leaves were crushed into the powdered form using a grinding mill. Further, the powder was also transferred through a standard sieve and stored in an airtight polyethylene bag at 4 °C until extraction and analysis.

Chemicals and equipment

Folin–Ciocalteu's reagent, sodium carbonate (Na_2CO_3), gallic acid (HPLC grade) was obtained from Tianjin Section Co. Ltd (Tianjin, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,2-azino-bis (3-ethylbenothiazoline-6-sulphonic acid) ($ABTS^{+}$•), hydrogen peroxide, and acetonitrile (HPLC grade) was collected from Shanghai Macklin, China. Aladdin Chemistry Co., Ltd. (Shanghai, China) supplied the quercetin and ascorbic acid. All solvents and reagents were of analytical grade, and all aqueous solutions were made with double distilled water.

The water-bath sonicator (KQ5200DE, 40 kHz) was collected from Kunshan Ultrasonic Instrument Co., (Jiangsu, China). The grinding machine and microplate reader were received from Kemio Chemical Reagent Co., Ltd. (Tianjin, China). The spectrophotometer (UV 2700, Shimadzu, Japan) was used in this study.

Ultrasound-assisted extraction and single-factor experiment

The ultrasound-assisted extraction of *C. olitorius* leaves was accomplished utilizing water-bath sonicator apparatus with the frequency set at 40 kHz and 200 W nominal power. First, dried powder (1 g) of *C. olitorius* leaves was extracted in 10 mL cylindrical glass tubes. To evaluate the impact of the independent factor on the TFC and TPC, the dried sample powder was soaked in ethanol solvents (ranging from 40 to 90%, w/w). At the same time, ultrasound time was set at 60 min, temperature 70 °C, and 30 mL/g liquid–solid ratio. To establish the ideal extraction temperature, temperature ranges (30–80 °C) were applied under the following criteria: ethanol concentration of 60% (based on the above step results), extraction period of 40 min, and liquid–solid ratio of 30 mL/g. Finally, the influence of liquid–solid ratio 10–60 mL/g on extraction efficiency was assessed utilizing the following criteria: ethanol concentration 60%, time 40 min, and temperature 70 °C (based on the results from the preceding phase). The supernatant was collected after centrifuging the treated samples at 5000 rpm for 10 min. At − 20 °C, the crude extract was kept for further analysis. According to the single-factor experimental data, next section describes a series of studies under the experimental design model.

CCD for extraction optimization

Each RSM design variable's maximum and minimum levels were established depending on the outcomes of single-factor experiments in TFC and TPC. Therefore, the four independent factors, including A: ethanol concentration (50–90%), B: temperature (40–80 °C), C: time (30–70 min), and D: liquid–solid ratio (20–60 mL/g), were investigated to maximize the UAE of flavonoids and polyphenols. The interaction between factors may be studied and optimized in the full factor space. The independent variables, their corresponding codes, and the RSM utilization levels for each variable are shown in Table 1. The CCD method of RSM led to 30 experimental runs, all generated with Design Expert software version 13, which included six central point replicates performed (Table 2). Using CCD as the experimental design provides a better prediction and is a more precise factor impact evaluation [26]. The extraction yield (v/v) (Y) was used as the response variable for experimental designs. Randomized second-order expanded polynomial regression models were used to minimize unexpected variability in observed responses when fitting experimental results. The mathematical quadratic response equation is as follows:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j, \quad (1)$$

where Y denotes response value (TFC, TPC); β_0 reflects the intercept coefficient of the model; β_j, β_{jj}, and β_{ij} are the linear, quadratic, and interactive coefficients, respectively. X_i and X_j are independent coded variables. K shows the number of variables.

Total flavonoids content (TFC) determination

A colorimetric approach reported by Adegbaju et al. was used to calculate the total flavonoids content [27]. In brief, 1 mL $NaNO_2$ (5%, w/v), 2 mL $AlCl_3$ (10%), and 1 mL NaOH (1 mol/L) solution were mixed into 1 mL leaves extract. By adding deionized water, the final volume was set to 5 mL and left for 10 min for incubation. Afterward, the absorbance at 510 nm was then compared to the mixture without the sample. Quercetin was used as a standard, and the findings were presented regarding quercetin equivalent (QE) mg per g dry weight (DW).

Total polyphenols content (TPC) determination

The sample's total polyphenols content was analysed using the colorimetric technique of Folin–Ciocalteu (FC) [28]. A blue colour solution was produced by combining the Folin–Ciocalteu reagent and 10% $NaCO_3$. Shortly, 0.5 mL extract was combined with 0.2 mL Folin–Ciocalteu reagent, 0.8 mL 10% (w/w) Na_2CO_3, and 1.5 mL distilled water. Mixes attained a brilliant blue colour by keeping the combinations at room temperature for 60 min. A spectrophotometer read the absorbance solutions at 765 nm compared to the reagent blank. Gallic acid equivalents (mg GAE/g DW) were used as a reference to determine the total polyphenols content of the sample.

Verification of models

The optimal conditions for extracting the total flavonoids and polyphenols content from *C. olitorius* leaves were validated by comparing actual experimental values to predicted values in the final reaction regression equations for ethanol concentration, extraction temperature, extraction time, and liquid–solid ratio. To ensure the appropriateness and accuracy of the optimized conditions of developed models, verification experiments with optimized parameters were carried out. The findings are summarized in Table 5.

Comparison of conventional heat reflux extraction (HRE) and ultrasound-assisted extraction (UAE)

Heat reflux extraction (HRE) is a typical bioactive compound extraction method, which requires a more extended extraction period and high temperatures [29]. However, newer procedures, like ultrasonic extraction, have not yet been adopted for the exceptionally strong medicinal plant. A comparison between the HRE and UAE was conducted to establish the reliability of the UAE technique developed in the present study. The HRE of TFC and TPC was performed with slight alterations to the parameters optimized in UAE. In short, 1.0 g of the

Table 1 Levels of independent central composite design (CCD) variables

Independent variable	Symbol	Level				
		$-\alpha$	−1	0	1	$+\alpha$
Ethanol concentration (%)	*A*	50	60	70	80	90
Ultrasonic temperature (°C)	*B*	40	50	60	70	80
Ultrasonic time (min)	*C*	30	40	50	60	70
Liquid–solid ratio (mL/g)	*D*	20	30	40	50	60

Table 2 Designed experiments and results for response surface analysis

Std	Ethanol (Conc., %, v/v)	Temperature (°C)	Time (min.)	Liquid–solid Ratio (mL/g)	TFC (mg QE/g)	TPC (mg GAE/g)
1	60	50	40	30	5.16	8.43
2	80	50	40	30	5.07	9.05
3	60	70	40	30	6.48	13.52
4	80	70	40	30	4.86	9.42
5	60	50	60	30	5.7	10.05
6	80	50	60	30	6.07	9.34
7	60	70	60	30	6.12	13.42
8	80	70	60	30	5.78	9.6
9	60	50	40	50	6.52	7.97
10	80	50	40	50	4.66	8.89
11	60	70	40	50	7.22	12.44
12	80	70	40	50	4.93	9.12
13	60	50	60	50	6.97	13.19
14	80	50	60	50	6.47	10.21
15	60	70	60	50	6.69	14.17
16	80	70	60	50	5.46	10.46
17	50	60	50	40	5.79	10.64
18	90	60	50	40	4.26	6.74
19	70	40	50	40	5.92	7.61
20	70	80	50	40	7.04	11.51
21	70	60	30	40	4.5	11.37
22	70	60	70	40	6.32	13.55
23	70	60	50	20	6.34	10.99
24	70	60	50	60	7.21	11.58
25	70	60	50	40	7.53	13.72
26	70	60	50	40	7.16	14.57
27	70	60	50	40	7.22	13.93
28	70	60	50	40	7.58	14.46
29	70	60	50	40	7.13	13.79
30	70	60	50	40	7.45	14.45

dried sample was extracted by maintaining 70% ethanol concentration, liquid–solid ratio 37 mL/mg at 70 °C, under reflux, which was 48 min.

Qualitative analysis of the phenolic composition

Characterization of phenolic compounds from optimized leaf extract of *C. olitorius* was characterized as previous literature [30]. Leaf extract of *C. olitorius* obtained under optimum conditions was filtered through a 0.45-µm-diameter nylon membrane into a 2-mL vial before injection. An Agilent 1260 HPLC equipped with a UV–Vis DAD detector was utilized for HPLC analysis. Chromatographic separation of analytes was achieved by utilizing a C18 reverse phase column (Waters Xbridge™, 250 × 4.6 mm^2 i.d., 5 µm, Milford, USA). The mobile phase comprised solvent A (0.1% acetic acid in H_2O) and B (acetonitrile) and was eluted using a linear gradient: 0–45 min, 10–100% A; 45–55 min, 100% B. The flow rate was set to 0.8 mL/min and the column temperature was set at 25 °C. At 254 nm, the chromatogram was taken, and the full-scan mode was selected for mass detection with a range of 100 to 1000 m/z.

LC–MS analysis was performed using a Shimadzu (Kyoto, Japan) coupled with binary pumps LC-30AD, an autosampler SIL-30AC, a CTO-20AC column, a degasser DGU-20A3R oven, and a reversed-phase column (2.1 mm × 100 mm; 4 µm). Formic acid in water (0.2%, v/v) and formic acid in acetonitrile (0.2%, v/v) were used as mobile phases A and B, respectively. The injection volume was 5 µL, the column temperature was 40 °C, and the flow rate was 0.5 mL/min. All analyses were done in triplicate. It compared the retention times and UV/VIS spectra of corresponding standards under the same

conditions, allowing for identifying the phenols present in extracts from *C. olitorius*.

Evaluation of antioxidant potential

DPPH• radical scavenging activity

The DPPH• scavenging effect of leaves obtained by UAE and HRE was assessed and compared using the technique described in the previous study with minor modifications [28]. The antioxidants reduced the stable DPPH radical to the yellow-coloured diphenyl picrylhydrazine. In short, 2 mL of anhydrous ethanol containing 0.2 mM DPPH was combined with 2 mL of sample extract with 0.1–0.6 mg/mL concentrations. The reduced DPPH• radical absorbance was read at 517 nm against a blank after 30 min in the dark, and Vit-C was used as a positive control. According to the following equation, the scavenging rate was defined:

$$\text{Scavenging ability}(\%) = \left[\left(A_{\text{control}} - A_{\text{sample}}/A_{\text{control}}\right] \times 100, \quad (2)$$

where $\text{Abs}_{\text{control}}$ was termed as control absorbance and $\text{Abs}_{\text{sample}}$ was regarded as sample absorbance. The test was performed in triplicate, and the IC_{50} is the concentration of sample required for 50% scavenging of DPPH free radical, which was determined from the plot between % inhibition and concentration of the extract.

$ABTS^{+}$• radical scavenging activity

The $ABTS^{+}$• radical inhibitor assay of extract obtained from UAE and HRE was carried out according to the methodology planned by Berg et al. with minor adjustments [31]. At first, phosphate-buffered saline (0.01 M, pH 7.4) was used to dissolve ABTS to a concentration of 7 mM. Then, an equal volume of 2.45 mM $K_2S_2O_8$ solution was added to the initial ABTS solution and allowed to stand in the dark for 16 h to produce ABTS+•. The ABTS+• solution was diluted with distilled water until it absorbed 0.70 ± 0.002 nm at 734 nm. The prepared ABTS+• solution was reacted with 0.5 mL of extract, and the mixture was then incubated in the dark for 10 min. A UV–Vis microplate reader was employed to read the absorbance at 734 nm, and the Vit-C was utilized as a comparison reference. The following equation was used to compute the inhibition percentage of radical scavenging:

$$\text{Scavenging ability}(\%) = \left(A_{\text{control}} - A_{\text{sample}}/A_{\text{control}}\right) \times 100, \quad (3)$$

where A_{control} was regarded as control absorbance and $\text{Abs}_{\text{sample}}$ was regarded as sample absorbance. The test was performed in triplicate, and the IC_{50} was determined from the plot between % inhibition and concentration of the extract.

Hydroxyl radical scavenging activity

Like DPPH• and $ABTS^{+}$• radical scavenging potential, hydroxyl radical scavenging was evaluated according to the literature with slight alterations [32]. A tube was filled with 2 mL of various concentrations (0.1–0.6 mg/mL) of extract, 1 mL of 9 mM salicylic acid ethanol, 1 mL of 9 mM $FeSO_4$, and 3 mL of distilled water. The reaction was introduced by adding 1 mL of 8.8 mM H_2O_2 and was incubated for 30 min at 37 °C. At 510 nm, the mixture's absorbance was measured, and Vit-C was utilized as a positive control of this work. The scavenging activity has been computed as follows:

$$\text{Scavenging ability}(\%) = \left(1 - \frac{A_1 - A_2}{A_j}\right) \times 100, \quad (4)$$

where A_1 is the sample absorbance, A_2 is called mixture absorbance with deionized water rather than a sample; and A_j reflects the water absorbance instead of H_2O_2. The test was performed in triplicate, and the IC_{50} was determined from the plot between % inhibition and concentration of the extract.

Statistical analyses

All extraction, TFC and TPC determination, and antioxidant activities assays were performed in triplicates. Design-Expert v13 (Stat-Ease Inc., Minneapolis, USA) was employed to determine quadratic polynomial model coefficients and design the extraction conditions throughout response surface methodology (RSM) with their respective 3D graphs. Analysis of variance (ANOVA) was analysed using the SPSS software package v25, and $p < 0.05$ was the threshold for significant differences. The findings were presented as mean ± standard error ($n = 3$).

Results

Single-factor experiment

Effect of extraction temperature on the TFC and TPC

The temperature has a tremendous impact on extraction performance; there is no doubt about that. The increased ultrasonic temperature may lead to a greater diffusion coefficient of the compounds of interest and enhance the solubility of those compounds [33]. It was demonstrated that the extraction temperature significantly contributes to UAE (Fig. 1d). The TFC and TPC yield increased dramatically with increasing extraction temperature from 30 to 60 °C. It may be caused by a decline in solvent viscosity and surface tension that causes high vapour pressure.

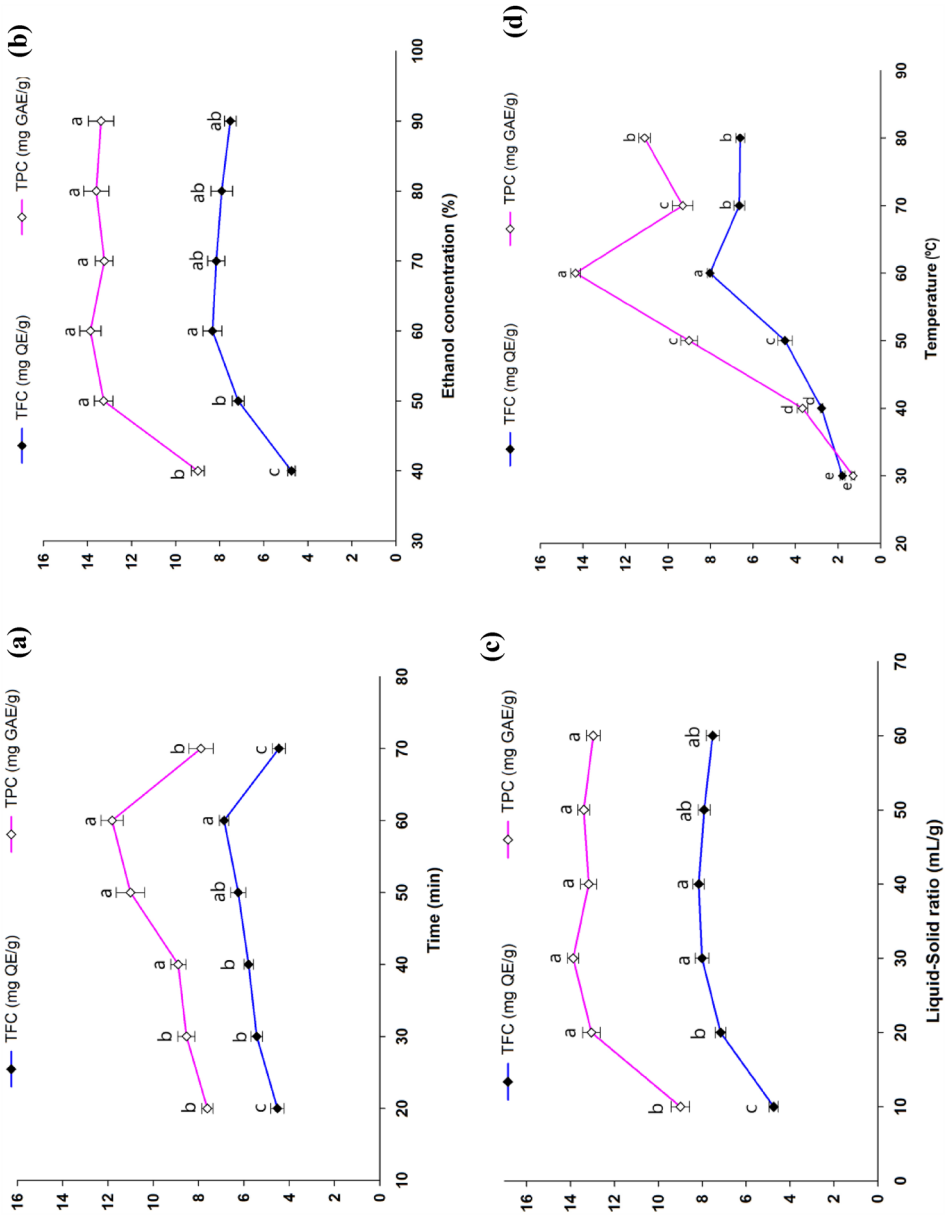


Fig. 1 Effect of four independent variables on the TFC and TPC content. **a** Ultrasonic time, **b** ethanol concentration, **c** liquid–solid ratio, and **d** temperature. The results were presented as mean with standard error ($n = 3$)

Nevertheless, production of TFC and TPC declined at a higher extraction temperature of more than 60 °C. Therefore, the optimal extraction temperature was selected as 60 °C. Similar results were obtained in *Pteris cretica,* where a temperature of 60 °C gave the highest yield of total flavonoid [34]. Our experiment finding may be due to hot spots that induced deterioration of the viscosity of solvent and an acceleration of the movement of molecules as temperatures increased; even degradation of sensitive flavonoids can occur at higher temperatures [35]. High extraction temperatures of more than 80 °C have not been evaluated as higher temperatures are unlikely to lead to significant results or adverse effects due to the deterioration of the analytes [36]. One of the possible explanations for this was the disruption of the flavonoid structure at high temperatures [37].

Effect of time of extraction on the TFC and TPC

Additionally, extraction time governs the reducibility of phenolic compounds, and the scheme should be considered during the optimization process. The influence of ultrasound time on the total extraction yield of flavonoids and polyphenols is displayed in Fig. 1a. The impact of the ultrasonic period (20 to 70 min) on TFC and TPC was investigated, with the following conditions: ethanol concentration 50% (v/v), ultrasonic temperature 60 °C, and liquid–solid ratio of 25 mL/g. At 60 min of extraction, the yield of the flavonoids (6.87 mg QE/g DW) and polyphenols (11.82 mg GAE/g DW) reached their peak, followed by a rapid decline. It can be clarified that extended extraction time leads the phenolic compounds present in the extract to decompose, which decreases its extraction yield [38]. It appears that a 60-min extraction time was considered the best extraction time.

Effect of ethanol concentration on the TFC and TPC

In the food industry, aqueous ethanol is a widely known solvent for extracting phenolic compounds from plants, where ethanol increases TPC recovery by disrupting solute–plant matrix bonds. At the same time, water facilitated the swelling of cell material [39]. So, selecting a suitable ethanol concentration is crucial in improving extraction performance. Figure 1b illustrates that the TFC and TPC content increased as the ethanol concentration increased from 40 to 60% but declined rapidly with ethanol concentration higher than 60%, indicating that different ethanol–water systems exhibited varying degrees of extraction efficiency and revealed adding a certain amount of water may boost extraction effectiveness. This is because water can enhance the polarity of aqueous ethanol. Therefore, 60% of ethanol was picked for the subsequent study. A similar finding reported that the highest total flavonoid yield was observed in *Pteris cretica* at 60% ethanol [34].

Effect of liquid–solid ratio on the TFC and TPC

A certain amount of changes in the liquid–solid ratio might boost the extraction efficiency, possibly due to a greater concentration difference [40, 41]. In this study, liquid–solid ratios ranging from 10 to 60 mL/g were introduced to evaluate the impact on TFC and TPC yield, while the other three variables remained constant with the 60 °C ultrasonic temperature, 30 min ultrasonic time, and 50% (v/v) ethanol concentration. It was observed from the results, the liquid-to-material ratio influenced the yield of extraction (Fig. 1c). The TFC and TPC yield increased by the increasing liquid–solid ratio from 10 to 30 mL/g, and decreasing and fluctuated pattern developed when the liquid–solid ratio of more than 30 mL/g was induced. The maximum value for TFC (8.16 mg QE/g DW) and TPC (13.88 mg GAE/g DW) was attained at 30:1 mL/g. It was probably due to increased contact between the solvent and sample, which enhances mass transfer and then increases the polyphenols solubility in plant cells [42], and a similar tendency in tea polyphenols extraction employing a microwave, as stated by authors [43]. Therefore, a liquid–solid ratio of 30 mL/g was selected for further CCD experiments.

Model fitting

The optimization experiment performed the CCD in triplicate, which comprised four factors, five levels, and six centre point runs. The experimental conditions and responses from 30 runs are given for the preferences of suitable extraction processes that have affected better return for the total flavonoids content (TFC) and polyphenols content (TPC). The appropriateness of the chosen model was assessed by observing R^2, adj-R^2, coefficient of variance, F-value, and p-values. The significance of each coefficient is illustrated in Tables 3 and 4, respectively. A quadratic polynomial model suggested by the software has been selected and adopted for all independent variables and responses [44]. The statistical regression analyses were subsequently performed on the experiments and the relationship between the response and the independent variables could be estimated using the following second-order polynomial equation:

$$\begin{aligned} Y_{TFC} &= 7.34 - 0.4425A + 0.1317B \\ &\quad + 0.3333C + 0.2258D - 0.2125AB \\ &\quad + 0.2600AC - 0.2525AD - 0.2025BC \\ &\quad - 0.0975BD + 0.0100CD - 0.5862A^2 \\ &\quad - 0.2225B^2 - 0.490C^2 - 0.1487D^2, \end{aligned} \tag{5}$$

Table 3 Analysis of variance (ANOVA) of regression equation for optimization of TFC

Source	Sum of squares	*df*	Mean square	*F*-value	*p*-value	Significance
Model	26.99	14	1.93	29.23	< 0.0001	***
A-Ethanol conc. (%)	4.70	1	4.70	71.26	< 0.0001	***
B-Temperature (°C)	0.4161	1	0.4161	6.31	0.0239	*
C-Time (min)	2.67	1	2.67	40.43	< 0.0001	***
D-Liquid–solid ratio (mL/g)	1.22	1	1.22	18.56	0.0006	***
AB	0.7225	1	0.7225	10.96	0.0048	**
AC	1.08	1	1.08	16.40	0.0010	***
AD	1.10	1	1.10	16.72	0.0010	***
BC	0.6561	1	0.6561	9.95	0.0066	**
BD	0.1521	1	0.1521	2.31	0.1496	Ns
CD	0.0016	1	0.0016	0.0243	0.8783	Ns
A^2	9.43	1	9.43	142.94	< 0.0001	***
B^2	1.36	1	1.36	20.59	0.0004	***
C^2	6.59	1	6.59	99.86	< 0.0001	***
D^2	0.6069	1	0.6069	9.20	0.0084	**
Residual	0.9892	15	0.0659			
Lack of fit	0.7927	10	0.0793	2.02	0.2272	Not significant
Pure error	0.1966	5	0.0393			
Cor total	27.98	29				
Std. dev	0.2568					
Mean	6.19					
C.V.%	4.15					
Press	4.85					
R^2	0.9646					
Adjusted R^2	0.9316					
Predicted R^2	0.8267					
Adeq precision	17.7873					

***Highly significant ($P < 0.001$); **quite significant ($0.001 < P < 0.01$); *significant ($0.01 < P < 0.05$)

$$\begin{aligned} Y_{TPC} &= 14.19 - 1.04A + 0.9508B \\ &\quad + 0.6650C + 0.2000D - 0.8000AB \\ &\quad - 0.3337AC - 0.0675AD - 0.3312BC \\ &\quad - 0.1975BD + 0.4763CD - 1.352A^2 \\ &\quad - 1.13B^2 - 0.4098C^2 - 0.7035D^2, \end{aligned} \tag{6}$$

where *A*, *B*, *C*, and *D* represent the ethanol concentration, extraction temperature, time, and liquid–solid ratio, respectively.

The findings were further analysed, and the model's suitability was estimated by linear regression and ANOVA, as presented in Tables 3 and 4. The results show that models for TFC and TPC are remarkably significant ($p < 0.001$), and the high coefficient (R^2) and high adjusted determination coefficient (adj-R^2) for all responses (TFC 0.96 and 0.93; TPC 0.97 and 0.95, respectively) reflect a strong association between the expected and experimental results. A significant correlation was found when the difference between the observed and predicted values was less than 0.2. Additionally, the *p*-value for lack of fit was non-significant ($p > 0.1$) in all cases, indicating that the models accurately predicted the variations. Experimental accuracy and trust worthiness have been monitored utilizing the coefficient of variance. The researchers indicated that the coefficient of variation (CV) was less than 10%, confirming the experimental run's accuracy and reliability [45]. Low variation coefficients (4.15 and 4.70 for TFC and TPC, respectively) showed good experimental precision and consistency in the current study. In conclusion, the two model equations have established good adaptability and compliance with the preceding principles.

Response surface analysis

Effect of UAE variables on total flavonoid (TFC) extraction

The structure of three-dimensional (3D) response surface plots provides precise information concerning the impacts of two independent factors on a dependent

Table 4 Analysis of variance (ANOVA) of regression equation for optimization of TPC

Source	Sum of squares	*df*	Mean square	*F*-value	*p*-value	Significance
Model	156.67	14	11.19	39.78	< 0.0001	***
A-Ethanol conc. (%)	25.83	1	25.83	91.83	< 0.0001	***
B-Temperature (°C)	21.70	1	21.70	77.13	< 0.0001	***
C-Time (min)	10.61	1	10.61	37.73	< 0.0001	***
D-Liquid–solid ratio (mL/g)	0.9600	1	0.9600	3.41	0.0845	Ns
AB	10.24	1	10.24	36.40	< 0.0001	***
AC	1.78	1	1.78	6.34	0.0237	*
AD	0.0729	1	0.0729	0.2591	0.6181	Ns
BC	1.76	1	1.76	6.24	0.0246	*
BD	0.6241	1	0.6241	2.22	0.1571	Ns
CD	3.63	1	3.63	12.90	0.0027	**
A^2	50.16	1	50.16	178.30	< 0.0001	***
B^2	35.32	1	35.32	125.56	< 0.0001	***
C^2	4.61	1	4.61	16.37	0.0011	**
D^2	13.58	1	13.58	48.26	< 0.0001	***
Residual	4.22	15	0.2813			
Lack of fit	3.49	10	0.3494	2.41	0.1720	Not significant
Pure error	0.7253	5	0.1451			
Cor total	160.89	29				
Std. dev	0.5304					
Mean	11.27					
C.V. %	4.70					
Press	21.17					
R^2	0.9738					
Adjusted R^2	0.9493					
Predicted R^2	0.8684					
Adeq precision	21.0264					

***Highly significant ($P < 0.001$); **quite significant ($0.001 < P < 0.01$); *significant ($0.01 < P < 0.05$)

variable in three dimensions, while some other variables remain at a constant value of 0. An elliptical contour or saddle structure suggests strong interactions between the corresponding parameters, in contrast to the minimal parameter interactions shown by a circular contour graph [46]. Table 3 reveals the Y_{TFC} model was remarkably significant that had a high *F*-value (74.12) and small *p*-value (< 0.0001) derived from the ANOVA. The recovery yield of TFC from *C. olitorius* leaf extract varied from 4.26 to 7.58 mg QE/g dry weight of the sample. In contrast, experimental run no. 28 reported the highest content, followed by 25, while the lowest was found in experimental run 18. Furthermore, the regression coefficient indicated that all linear terms (*A*, *B*, *C*, *D*) and quadratic terms (A^2, B^2, D^2) could significantly ($p < 0.05$) affect the TFC.

The interactions between two variables (*AB*, *AC*, *AD*, *BC*, *BD*, and *CD*) on the response variable (Y_{TFC}) are presented graphically in Fig. 2a–f. The ethanol concentration and temperature (*AB*) interactive effect are shown in Fig. 2a, demonstrating the extraction performance was improved significantly by increasing the extraction temperature with reduced ethanol concentration in ultrasound, extraction performance was improved significantly. This is because the solvent's polarity can be reduced by weakening the phenolic matrix by using high temperatures throughout the extraction, which ultimately increases the solubility of the desired compound [47]. The TFC was positively influenced by temperature, while the higher ethanol with increased temperature recovery of TFC decreased.

According to Table 3, there was a surprisingly strong ($p < 0.001$) positive effect on TFC that resulted from the interaction between ultrasonic time and ethanol concentration (*AC*). The yield of TFC steadily increased as the extraction period and ethanol concentration was raised from 40–55 min and 60–70%, respectively. However, the present investigation's showed that TFC production had increased over time with reduced ethanol concentration levels. Therefore, ultrasonic time seemed to be one major

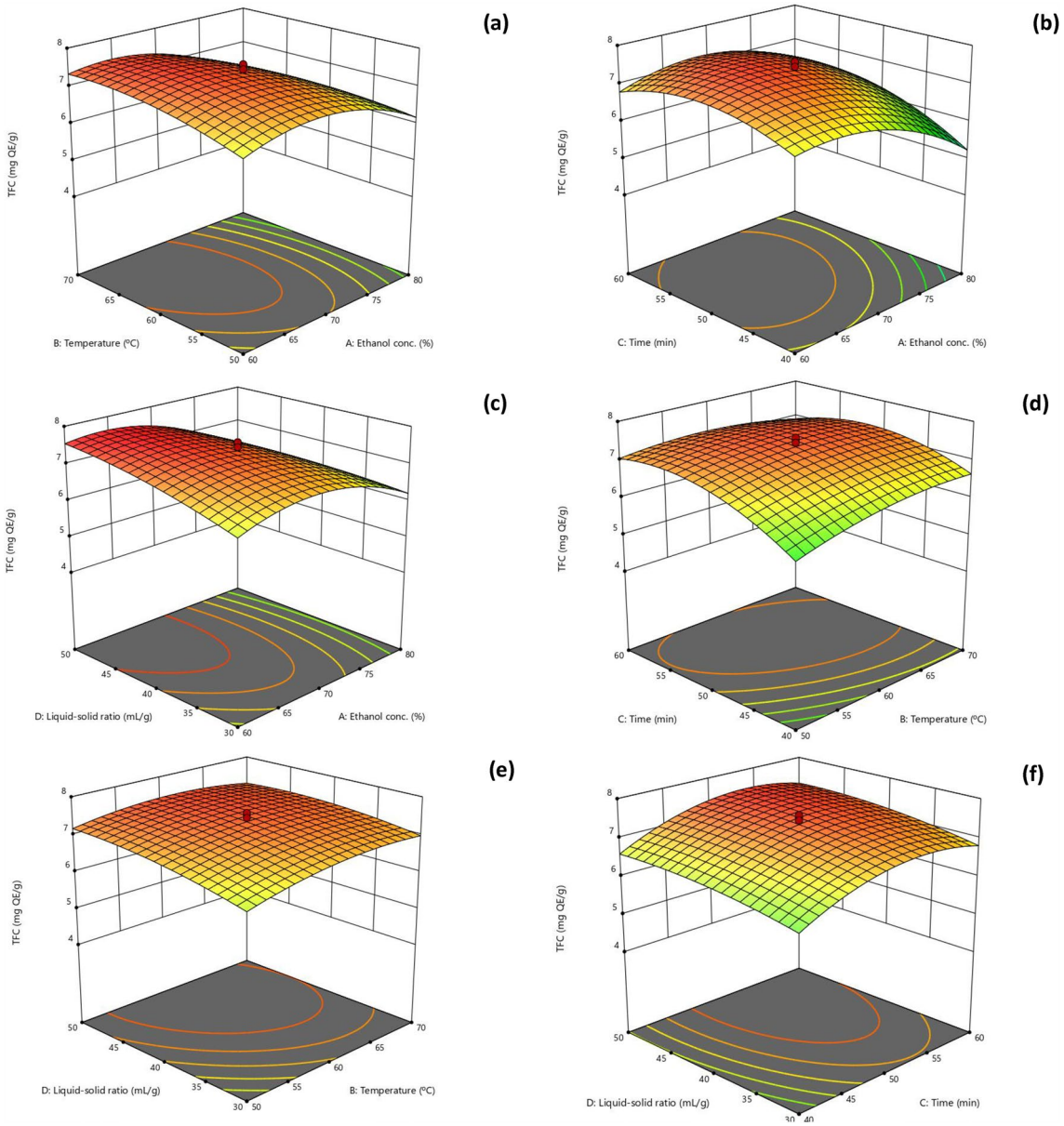


Fig. 2 Response surface graphs displayed the interaction between different extraction variables (ethanol concentration, time, temperature and liquid–solid ratio) on TFC

factor in the extraction method. Low to medium ethanol concentration was observed to increase TFC recovery, whereas higher solvent concentration significantly decreased the yield. The alteration in solvent polarity likely caused the effect due to adding a certain amount of water. The accomplishment was supported by researchers who stated that a higher ethanol concentration and longer extraction time decreased the TFC yield [24].

It can be noticed from Table 3 and Fig. 2c, the relationship between the ethanol level and the liquid–solid ratio (*AD*) demonstrated a remarkably significantly positive effect on TFC when the other two variables were fixed. The 3D graph observed that TFC extraction yield improved with increasing liquid–solid ratio at low-level ethanol concentration. In other words, the yield of TFC dramatically increased with an increasing liquid–solid ratio when ethanol concentration was beyond 70%.

The significant mutual interaction between temperature and time (*BC*) on flavonoid yield is shown in Fig. 2d whenever the liquid–solid ratio and temperature were constant. 3D graph represented that the production of TFC gradually increased with heightening the

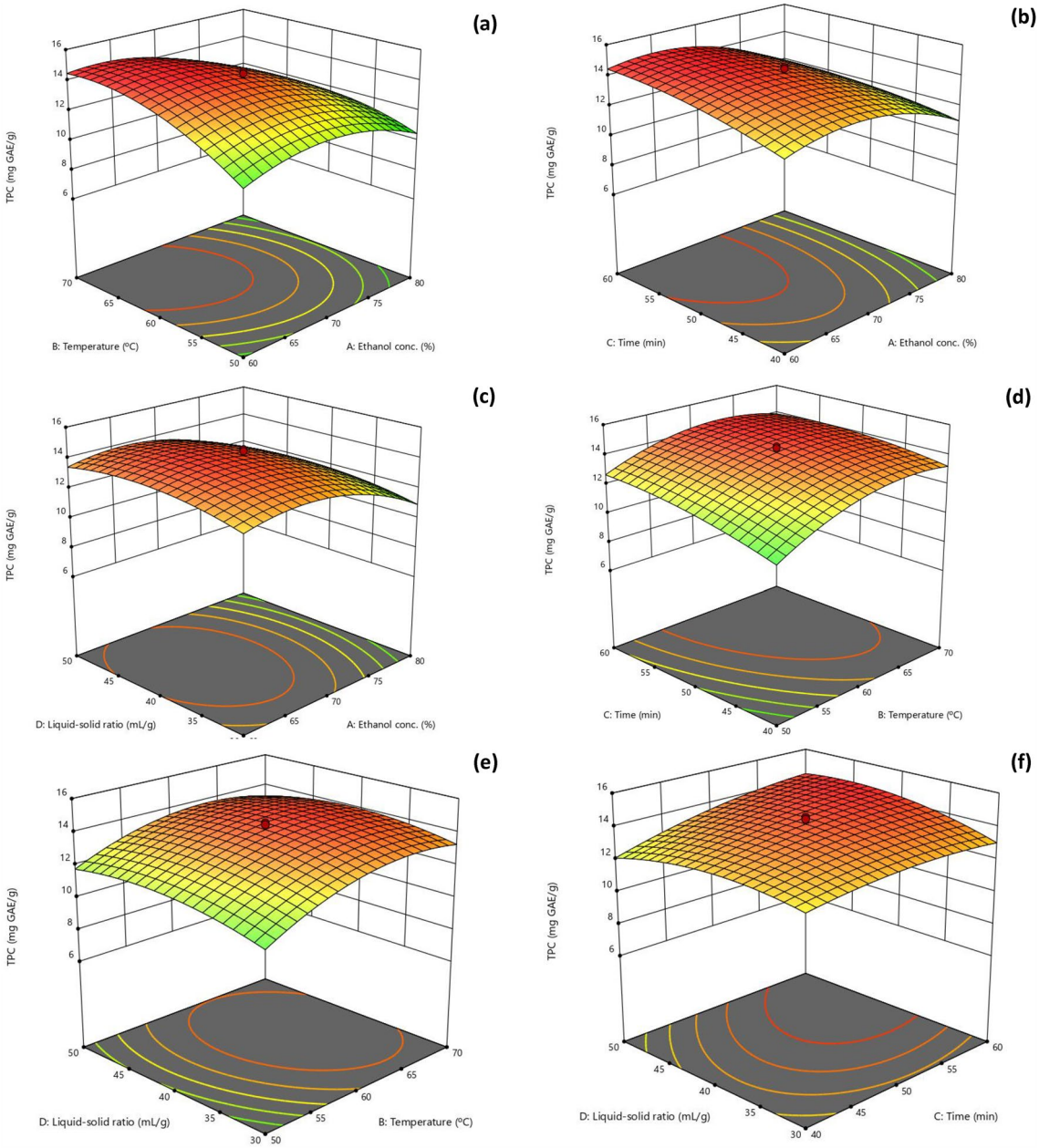


Fig. 3 Response surface graphs displayed the interaction between different extraction variables (ethanol concentration, time, temperature and liquid–solid ratio) on TPC

extraction temperature and time ratio. But TFC yield declined with increased irradiation temperature over an extended treatment time. This result may imply that the higher temperature and time of the reaction would have a negative impact on the solid–liquid phase separation. In agreement with a previous study suggested that prolonged treatment lowers the yield of flavonoids [48]. This could be due to the molecular movement speeds up and decomposition of flavonoids at high ultrasound [21].

Effect of UAE variables on total polyphenol content (TPC) extraction

According to the results of the ANOVA presented in Table 4, the model was statistically significant both in terms of its high *F*-value (61.68), as well as the low *p*-value ($p < 0.0001$). Furthermore, coefficient values (CV = 4.70) and adequate precision (adeq. precision = 21.03) indicate the ability to identify relations between the extraction variables and TPC responses in a deduced model.

Generally, the actual relation between response (TPC) and independent variables has been adequately represented for all of the statistical parameters obtained from the model. The total polyphenol content (TPC) recovered in the leaf extract ranged from 6.74 to 14.57 mg GAE/g DW sample. Figure 3a describes how ethanol concentration and temperature (AB) influence the production of total polyphenol content. It was observed that the extraction yield enhanced with increased extraction temperature and lower ethanol concentration, where temperature varied from 50 to 70 °C with a medium level of ethanol concentration, the phenols extraction yield was highest. Similar findings on the influence of higher temperature improved the TPC extraction efficiency on *Angelica keiskei* [41] and *Hylocereus polyrhizus* [24]. However, the phenomenon could be explained that increasing temperature reduces the solvent surface tension while vapour pressure increases, which facilitates the formation of cavitation bubbles at the lower acoustic intensity and positively influences phenolic extraction yield [11, 49].

The interaction between ethanol concentration and ultrasonic time (*AC*) had a statistically significant ($p < 0.0001$) positive impact on TPC, as demonstrated in Fig. 3b. It was noticed that the extraction efficacy had been improved, with a progression in ethanol concentration and a longer period of ultrasound. It was observed that increased extraction time (40–60 min) and increasing ethanol concentration increased the yield of TPC. However, beyond 70% ethanol concentration, the extraction yield decreased. It was reported that a higher ratio of water to solvent facilitated the recovery yield of TPC by disrupting the bond between the solutes and plant matrices as well as enhanced cell material swelling [39]. Table 4 reveals a remarkable ($p < 0.001$) positive relationship between the ultrasonic period and liquid–solid ratio (*CD*) on the yield of TPC. The maximum phenolic was obtained with a liquid–solid ratio of 40–45 mL/g and extraction time of 40–60 min, as shown in Fig. 3f. It has been reported that the availability of adequate water in a solvent may enhance the yield of extraction by favouring the contact surface between the plant matrix and solvent, hence enhancing the extraction rate [50]. Nevertheless, when the liquid–solid ratio was maintained at the highest level of 50 mL/g for 50 min, no noticeable improvement

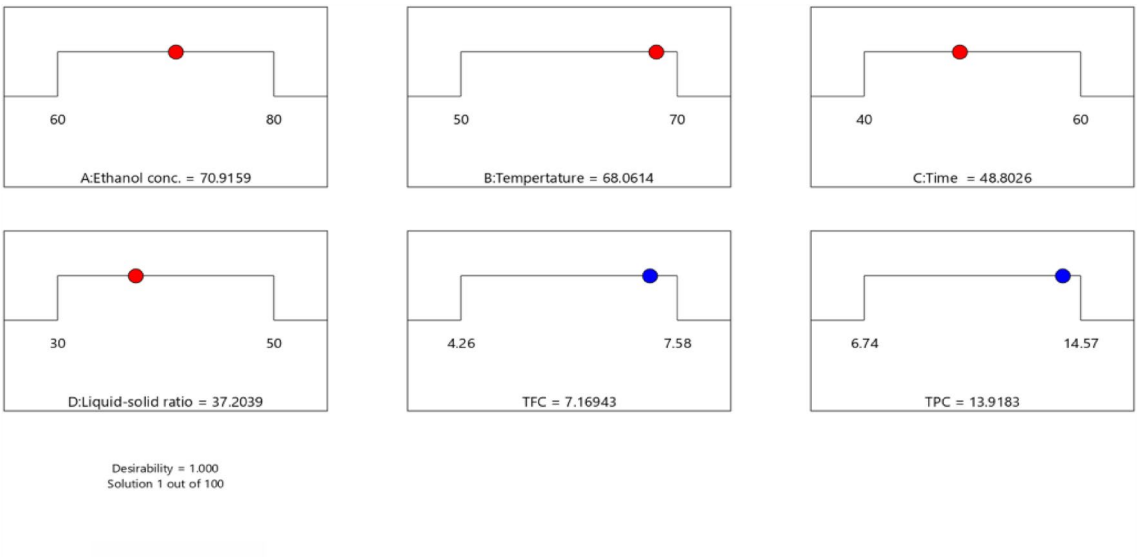


Fig. 4 Optimal parameters represented by desirability ramp

Table 5 Total flavonoids and total polyphenols content were obtained by optimization condition of ultrasonic-assisted extraction (UAE) and heat refluxed extraction (HRE)

Response variables	UAE extraction method		HRE
	Experimental value	Predicted value	
Total flavonoids content (mg QE/g DW)	6.96 ± 0.18	7.17	3.63 ± 0.25
Total polyphenols content (mg GAE/g DW)	13.38 ± 0.43	13.92	8.24 ± 0.90

Results were presented as average values ± standard error

was observed in TPC extraction, as the value declined continuously.

Optimization of UAE extraction conditions, verification, and comparison with the conventional method

The RSM was utilized to achieve the maximum-suited combination of factors and levels. UAE parameters were optimized concerning a determined extract yield based on the CCD model and process factor's significant linear, quadratic, and interactive effects. The maximum desirability of TFC (7.58 mg QE/g DW) extraction conditions was as follows: ethanol concentration 70%, ultrasonic temperature 60 °C, ultrasonic time 50 min, and 40 mL/g liquid–solid ratio obtained from the std of 28. The highest TPC (14.57 mg GAE/g DW) production was recorded in similar extraction conditions to TFC. Under the UAE optimized conditions, the predictive value of TFC and TPC extraction yields had 7.17 mg QE/g DW and 13.92 mg GAE/g DW, respectively, with extraction parameters as ethanol concentration 70.92%, temperature 68.06 °C, extraction time 48.80 min and liquid–solid ratio 37.20 mL/g (Fig. 4). In model appropriateness verify experiments, results gave a yield of TFC and TPC of 6.96 ± 0.18 mg QE/g DW and 13.38 ± 0.43 mg GAE/g DW, respectively (Table 5). Experimental results for TFC and TPC were significantly closer to the corresponding expected values, implying that CCD optimization was accurate and adequate to reflect the expected optimization.

In a comparison study of HRE and UAE, the findings indicated that the TFC and TPC obtained by UAE were higher in optimized terms compared to the value obtained by HRE (TFC: 3.63 ± 0.25 mg QE/g DW and TPC: 8.24 ± 0.90 mg GAE/g DW) (Table 5). It was believed that UAE induces cavitation that disrupts cells wall and promotes the mass transfer of solutes from plant cells to solvent for extraction, which may explain the high yield of antioxidants [15]. This finding was consistent with the literature that offered higher potency of the yield of bioactive compounds using UAE than traditional HRE [18, 51]. Therefore, the optimization condition obtained from this study could be helpful guideline information for the further industrialization of the UAE process.

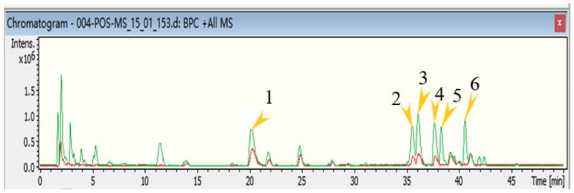


Fig. 5 LC–MS chromatograms of the compound from leaves extract of *C. olitorius*

Identification of phenolic compounds

Without a doubt, the chemical constituents of *C. olitorius* largely contribute to pharmacological efficacy. Consequently, extracts obtained under optimal conditions were analysed by HPLC to investigate the polyphenols profile and the relevant MS and UV data presented in Table 6. The chromatogram of UAE extract was recorded in both negative ion and negative ion modes, and characterized compounds were numbered according to retention time (Fig. 5). The detected compounds were identified by comparing retention times, UV and mass spectrometry (MS) data to theoretical fragmentation that had been previously published. A total of six potential phenolic compounds were identified from the leave extracts, including chlorogenic acid, isoquercetin, hyperoside, adhyperforin, 1,3-di-*O*-caffeoylquinic acid, and 3,4-di-*O*-caffeoylquinic acid. Authors identified by the corresponding conversion of the peak area and the associated elution periods were 19.30, 33.9, 34.4, 35.9, 37.3, and 39.1 min, respectively, are shown in Table 6. Compounds were identified at different wave lengths: chlorogenic acid at 326 nm; isoquercitrin, and hyperoside at 254 nm and 355 nm, adhyperforin at 276 nm; 1,3-di-*O*-caffeoylquinic acid and 3,4-di-*O*-caffeoylquinic acid at 330 nm. Most of the identified phenolic compounds have previously been reported in *C. olitorius* [52], while new ones, namely adhyperforin were reported in leaves for the first time. But, the numbers of identified compounds are less reported by authors

Table 6 Compounds identified in *C. olitorius* leaves extract using LC–MS and HPLC

Peak	Retention time	Name	Molecular weight	$[M+H]^+$(*m/z*)	$[M-H]^-$ (*m/z*)	UV
1	20 min/19.3 min	Chlorogenic acid	354	355	353, 191	326
2	35.5/33.9	Isoquercitrin	464	465, 303	463	254, 355
3	36.2/34.4	Hyperoside	464	465, 303	463	254, 355
4	37.7/35.9	Adhyperforin	550	551	549	276
5	39.3/37.3	1,3-Di-*O*-caffeoylquinic acid	516	517, 163	515, 353, 161	330
6	41.2/39.1	3,4-Di-*O*-caffeoylquinic acid	516	517, 163	515, 353, 161	330

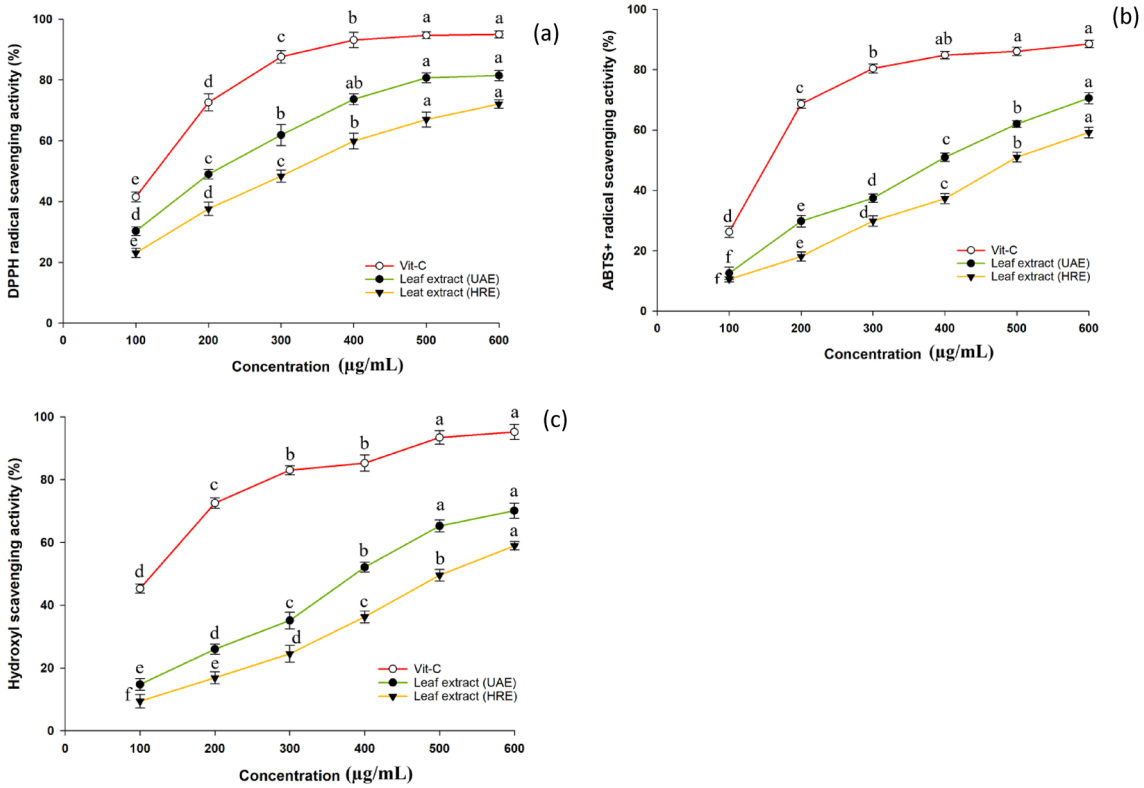


Fig. 6 Antioxidant activity of *C. olitorius* extracts. **a** Radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH•), **b** Radical scavenging activity of $ABTS^{+}$•, and **c** Hydroxyl radical scavenging activity. Results were presented as average values ± standard error

[52, 53]. This difference may affect the phytochemical composition of medicinal plants because of genetic and biological diversity, environmental factors, and seasonal variations [28, 54–56]. Pharmacological and nutritional investigations have revealed that these identified compounds possess various beneficial properties, including antioxidant, bacteriostasis, anticancer, and anti-inflammatory properties exhibited in earlier studies [52, 57]. Moreover, these identified compounds and other unidentified compounds may be responsible for the antioxidant activity observed in the UAE of *C. olitorius*. The combined effects of these phenolic acids and other components may influence the extract's antioxidant activity by scavenging free radicals and suppressing lipid peroxidation or metal chelation.

Antioxidant activities of the *C. olitorius* leaf extracts

DPPH radical scavenging capacity

Antioxidant properties of natural compounds can be evaluated by measuring the DPPH scavenging activity, and the mechanism is that the DPPH is conceived with antioxidants for its hydrogen-donating ability [12]. Outcomes revealed a significant positive association with increasing concentration (100–600 µg/mL of extracts and ascorbic acid) shown in Fig. 6a, and both UAE and HRE extraction scavenge potential was low level compared to Vit-C. Furthermore, when UAE extracts concentration increased by 600 µg/mL the antioxidant activity (81.50 ± 1.74%) was comparable to Vit-C (95.0 ± 1.17%). The IC_{50} value of UAE (226.29 µg/mL) was lower than HRE (336.31 µg/mL), and these results agreed with the findings of Biswas et al. [28], indicating that there is good potential for scavenging DPPH radicals. These results showed that leaf extracts possess DPPH scavenging because of flavonoid presence and the position of hydroxyl groups [58].

$ABTS^{+}$• radical scavenging activity

The extracts' capacity to neutralize $ABTS^{+}$• free radicals indicates their antioxidant activity [59]. Figure 6b illustrates the performance of the extract obtained from UAE and HRE in terms of the ability to scavenge $ABTS^{+}$• radicals at various concentrations. Results revealed that the $ABTS^{+}$• radical scavenging activity appeared to be predominantly dose-dependent. The value of the scavenging effect of UAE ranged from 12.36 ± 0.58% to 67.39 ± 1.24%, whereas HRE ranged from 11.72 ± 0.86% to 59.21 ± 1.75%, which was comparatively lower than Vit-C. The IC_{50}

value of the UAE, HRE, and Vit-C was (199.53 µg/mL), (253.86 µg/mL) and (67.45 µg/mL), respectively. According to these findings, it could be concluded that *C. olitorius* extract is a powerful scavenger of $ABTS^{+}\bullet$ radical.

Hydroxyl radical scavenging activity

The hydroxyl radical, one of the most reactive free radicals, interacts with DNA's purine and pyrimidine bases, triggering free radical chain reactions that affect the general aging process and tissue damage [60]. Figure 6c shows that the superoxide anion scavenging rates were correlated with the concentration and increased with increasing concentration. Concentration-dependent antioxidant activity showed the IC_{50} value of UAE was 402.02 µg/mL and 520.08 µg/mL for HRE, whereas 25.48 µg/mL for ascorbic acid. These results proved that the *C. olitorius* leaf extract possesses a noticeable hydroxyl radical scavenging ability, which increased proportionally to the extract concentration.

Conclusion

This study very early attempted to optimize the extraction factors using UAE with RSM to recover TFC and TPC from the *C. olitorius*. The mathematical models of RSM indicated that all parameters significantly influenced the extraction process and parameters were found to have linear, quadratic, and interactive effects. Results showed that predicted optimal conditions produced an experimental value of total flavonoids (6.96 ± 0.18 mg QE/g DW) and total polyphenols (13.38 ± 0.43 mg GAE/g DW) were quite close to predicted values of total flavonoids (7.17 mg QE/gDW) and total polyphenols (13.92 mg GAE/g DW), whereas the UAE were significantly higher than conventional HRE. Results from the LC–MS revealed that six phenolic compounds including chlorogenic acid, isoquercetin, hyperoside, adhyperforin, 1,3-di-*O*-caffeoylquinic acid, and 3,4-di-*O*-caffeoylquinic acid are identified in extracts as major compounds. The *C. olitorius* extract displayed significant dose-dependent DPPH•, $ABTS^{+}\bullet$, and hydroxyl radical scavenging activities. The present work results can be utilized for designing the experiment for further separate antioxidants from *C. olitorius.* Furthermore, these improved extraction processes are needed to scale up for massive quantities synthesis of antioxidants for application in drug and food industries.

Author contributions

DL, AB, and SD conceptualization and designed the experiment. AB and SD material preparation, data analysis, and original drafting of the article. AX, YD, and RR software, formal analysis, data curation, and validation. AB, DA, ZMB, and LL writing review and editing. DL supervision, review, and final approval for submission of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The data used to support the findings of this research are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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