

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

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Abstract

This study was conducted to optimize the ultrasound-assisted extraction (UAE) parameters for total flavonoids and total polyphenols content from *Corchorus olitorius* leaves employing response surface methodology (RSM). Based on single-factor investigations, a four-factor-five-level Central Composite Design (CCD) with 30 experimental runs has been applied. Results demonstrated that all models were significant and had good accuracy of polynomial quadratic equations ($p < 0.05$) with a non-significant lack of fit at $p > 0.05$ and adjusted regression coefficient (R^2) is > 0.95 . Under the optimized conditions, the highest extraction yield of total flavonoids (TFC) (7.17 mg QE/g DW) and total polyphenol content (TPC) (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 validation of optimized conditions, that were close to the predicted value and significantly superior to the conventional heat reflux extraction (HRE). Furthermore, the antioxidant test of the leaves extracts revealed an excellent 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺•) and hydroxyl radical scavenging with IC₅₀ values of 226.29, 199.53 and 402.02 µg/mL, respectively. The results indicate that optimization of UAE extraction conditions is essential for recovery of bioactive compounds in pharmaceuticals and food industries.

Introduction

Studies have already shown that phenolic compounds from plants are an important group of natural antioxidants, and their use in food, cosmetic, and pharmaceutical industries has gained worldwide attention due to antioxidant activities [1]. *Corchorus olitorius* (Tiliaceae) is an important green leafy vegetable that also has considerable in several tropical areas, including India, Bangladesh, tropical Asia, Sudan, Egypt, as well as in tropical Africa, Japan, and South America, which have the great medicinal potential [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 [6–10]. 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 the presence of bioactive components. Hence, *C. olitorius* could serve as 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 taken into consideration when selecting an effective extraction process. However, the optimal conditions for bioactive compounds derived from different plants are not globally consistent [11]. Besides, traditional extraction methods such as heat reflux, maceration, decoction, and

soxhlets are inefficient, expensive, and time-consuming [12]. Many variables like type of solvent, extraction temperature, solvent/dispersing ratios, and extraction time have influenced the extraction of bioactive from plant materials [13, 14]. Therefore, optimizing species-specific optimum extraction conditions for bioactive substances and pharmacological efficiencies is crucial. Fortunately, ultrasonic-assisted extraction (UAE), which 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 [15]; since ultrasound-generated media can decompose cell walls and effectively speed up the transfer process due to cavitation, vibration, crushing, and mixing effects [16, 17]. In addition, UAE may prevent the degradation of bioactive compounds by heat, but it can also enhance the quality of products [18].

The conventional optimization using single-factor analysis to optimize each level extraction variable is inadequate to differentiate between cross interaction of different factors. Thus, to achieve a meaningful model of different variables by conducting minimal tests, statistical design experiments are important. Response surface methodology (RSM) is a potent statistical tool for provide adequate data on multivariable system modeling, reduce the number of experiments and experimental errors, evaluates several independent parameters and their relationships in a single investigation [19, 20]. This method was first introduced for chemical processing and now effectively applying UAE procedures to examine optimal conditions for recover bioactive constituents like phenolic compounds, polysaccharides, anthocyanins, and protein notably from diverse food matrices [21–25].

In previous research, it has been reported that supercritical fluid extraction (SFE) demonstrated more efficiencies than UAE where time, solvent volume, and temperature was utilized as parameters for optimization leaves of *C. olitorius* extract [26]. As there has no comprehensive literature available on the effects of UAE factors for the recovery of antioxidant phenolics, therefore this research was the first attempt in detail to optimize the extraction conditions of UAE in *C. olitorius* and test the interactions between the parameters through a polynomial mathematical model. Then, verify the developed UAE method and compare it to traditional heat reflux extraction (HRE).

Materials And Methods

Plant Material and Preparation of Extract

Plant materials

The young leaves of *C. olitorius* were picked 45 days after sowing (DAS) from the experimental field of research of the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha, China. Collected leaves were dried under natural ventilation until reached a constant weight. 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 equipments

Folin–Ciocalteu's reagent, Sodium carbonate (Na_2CO_3), Gallic acid (HPLC grade) was obtained from Tianjin Section Co. Ltd (Tianjin, China). 1,1-diphenyl-2 trinitrophenylhydrazine (DPPH•), 2,2-azinobis (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). Grinding machine and Microplate reader received from Kemio Chemical Reagent Co., Ltd. (Tianjin, China). The spectrophotometer (UV 2700, Shimadzu, Japan) was used in this study.

Single-factor experiments

Dried powder (1g) of *C. olitorius* leaves was taken 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 40–90%, w/w) while ultrasound time was set at 60 min, temperature 70 °C, and 30 mL/g liquid-solid ratio. To establish the ideal extraction temperature, ranges of temperature (40–90 °C) were applied under the following criteria: Ethanol concentration 60% (based on the above step results), extraction period 40 min, and liquid-solid ratio of 30 mL/g. Finally, the influence of liquid-solid ratio 10–50 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, section 2.4 describes a series of studies carried out under the experimental design model.

CCD for extraction optimization

The maximum and minimum levels of each RSM design variable 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. In the full factor space, the interaction between factors may be studied and optimized. The CCD method of RSM led to 30 experimental runs, all of which were generated with Design Expert software version 11, which included six central point replicates performed (Table 2). The use of CCD as the experimental design provides a better prediction, and it is a more precise factor impact evaluation [27]. The independent variables, their corresponding codes, and the RSM utilization levels for each of the variables are shown in Table 1. The extraction yield (mg/g) (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

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

Independent Variable	Symbol	Level				
		- α	-1	0	1	+ α
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

Std	Ethanol (Conc., %, v/v)	Temperature (°C)	Time (min.)	Liquid-Solid Ratio (mL/g)	TFC (mg QE/g)	TPC (mg GAE/g)
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

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

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 flavonoid content determination

A colorimetric approach reported by Adegbaaju et al. 2020 was used to calculate the total flavonoid content [28]. 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 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 in terms of quercetin equivalent (QE) mg per g dry weight (DW)

Total polyphenols content determination

Analysis of total polyphenols content in the sample was carried out using the colorimetric technique of Folin-Ciocalteu (FC) [29]. A blue color solution was produced by combining the Folin–Ciocalteu reagent and 10% NaCO_3 . In 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. Keeping the combinations at room temperature for 60 min mixes attained a brilliant blue color. A spectrophotometer was used to read the absorbance solutions compared to the reagent blank. Gallic acid equivalents (mg GAE/g DW) were used as a reference to determine the polyphenols content of the sample.

Verification of models

Validation of the optimal conditions for extracting the total flavonoid and polyphenols content from *C. olitorius* leaves was accomplished 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. Validation is essential to ensuring the appropriateness and accuracy of the optimized conditions. To validate the developed models, triplicate experiments with optimized parameters (slightly changed, ethanol concentration 70%, temperature 70 °C, time 37 min, and liquid-solid ratio 48 mL/mg) were carried out. The findings are summarized in Table 5.

Conventional heat reflux extraction (HRE)

Heat reflux extraction (HRE) is a typical bioactive compound extraction method, which requires a more extended extraction period and high temperatures [30]. 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 in order 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 that had been optimized in UAE. In short, 1.0 g of the dried sample was extracted by maintain 70% ethanol concentration, liquid-solid ratio 48 mL/mg at 70 °C, under reflux, which was 37 min. The HRE was conducted and then the extract was processed in accordance with procedures stated in section 2.3.

Evaluation of antioxidant potential

DPPH• radical scavenging activity

The DPPH• scavenging effect of leaves obtained by UAE was assessed using the technique described in the previous study with minor modifications [29]. The antioxidants reduced the stable DPPH radical to the yellow-coloured diphenylpicrylhydrazine. In short, 2 mL of anhydrous ethanol containing 0.2 mM DPPH was combined with 2 mL of sample extract with 0.1–1.0 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.

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

Where, Abs_{control} was termed as control absorbance and Abs_{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 was carried out according to the methodology planned by Berg et al. 1999 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 hours to produce ABTS⁺. The

ABTS+• solution was diluted with distilled water prior to use until it had an absorbance of 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:

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

Where, A_{control} was regarded as control absorbance and A_{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

The extract's potential to scavenge hydroxyl radicals was evaluated in accordance to literature with slight alterations [32]. A tube was filled with 2 mL of various concentrations (0.1–1.0 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 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

$$\text{Scavenging ability (\%)} = (1 - \frac{A_1 - A_2}{A_0}) \times 100\%$$

Where A_1 is the sample absorbance, A_2 is termed as 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 as well as 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 conducted by using SPSS software package v25, and $p < 0.05$ as threshold for significant differences. The findings were presented as mean $\pm$ 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, as well as 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 °C to 60 °C. It may be caused by a decline in solvent viscosity and surface tension that causes high vapour pressure.

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 temperature 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 temperature [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 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 were 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. After 60 min of extraction, the flavonoids (6.87 mg QE/g DW) and polyphenols (11.82 mg GAE/g DW) yield 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 decreased its extraction yield [38]. It appears that a 60-minute 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 increased TPC recovery by disrupting solute-plant matrix bonds while water facilitated swelling of cell material [39]. So, the selection of suitable ethanol concentration is crucial in improving extraction performance. Figure 1b illustrated that the TFC and TPC content increased as the ethanol concentration increased from 40–60% but declined rapidly by ethanol concentration higher than 60%, indicating that different ethanol-water systems exhibit 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. It has been reported a similar finding 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 efficiency of extraction, 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 40 mL/g, and decreasing and fluctuated pattern developed when the liquid-solid ratio of more than 40 mL/g was induced. The maximum value for TFC (8.16 mg QE/g DW) was recorded at 40:1 mL/g 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 under the higher ratio of solvent, 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, for further CCD experiments, a liquid-solid ratio of 30 mL/g was selected.

Model Fitting

The optimization experiment performed the CCD in triplicate, which comprised four factors, five levels, and six center 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 flavonoid content (TFC) and total polyphenols content (TPC). The appropriateness of the chosen model was assessed by observing R^2 , $\text{adj-}R^2$, coefficient of variance, F -value, and p -values. The significance of each coefficient has been 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 analyzes 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:

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					

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

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
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 ($0.01 < P < 0.05$)						

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					

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

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
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)						

$$Y_{TFC} = 7.34 - 0.4425A + 0.1317B + 0.3333C + 0.2258D - 0.2125AB + 0.2600AC - 0.2525AD - 0.2025BC - 0.0975BD + 0.0100CD - 0.5862A^2 - 0.2225B^2 - 0.490C^2 - 0.1487D^2$$

$$Y_{TPC} = 14.19 - 1.04A + 0.9508B + 0.6650C + 0.2000D - 0.8000AB - 0.3337AC - 0.0675AD - 0.3312BC - 0.1975BD + 0.4763CD - 1.352A^2 - 1.13B^2 - 0.4098C^2 - 0.7035D^2$$

Where A, B, C, and D represent the ethanol concentration, extraction temperature, time, and liquid-solid ratio, respectively.

The findings have been further analysed, and the model's suitability was estimated by employing linear regression and ANOVA, as presented in Table 3 and Table 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 trustworthiness 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 accurateness and reliability [45]. In the current study, low variation coefficients (4.15 and 4.70 for TFC and TPC, respectively) showed good experimental precision and consistency. 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 variable in three dimensions, while some other variables remain at 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 revealed 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, whereas 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}) have been presented graphically in Figs. 2a-2f. The ethanol concentration and temperature (AB) interactive effect is shown in Fig. 2a, demonstrated that by increasing the extraction temperature with reduced ethanol concentration in ultrasound, extraction performance was improved significantly. This reason is due to the solvent's polarity can be reduced by weakening the phenolic matrix by using high temperature throughout the extraction, which ultimately leads to an increase in 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 results of the present investigation had shown that TFC production had increased over time with reduced ethanol concentration levels. Therefore, ultrasonic time seemed to be one major influencing factor in the method of extraction. It was observed that low to medium ethanol concentration increased the recovery of TFC, whereas higher solvent concentration significantly decreased the yield. The effect was likely caused by the alteration in solvent polarity due to the addition of 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 [25].

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. From the 3D graph, it was observed that TFC extraction yield improved with increasing liquid-solid ratio at low-level ethanol concentration. In other words, the yield of TFC dramatically went up with an increasing liquid-solid ratio when ethanol concentration was beyond 70%.

The significant mutual interaction between temperature and time (BC) on flavonoid yield was 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 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 [22].

Effect Of Uae Variables On Total Phenolic Content (TPC) Extraction

According to the results of the ANOVA, which are presented in Table 4, the model was statistically significant both in terms of its high F -value (61.68), as well as low p -value ($p < 0.0001$). Furthermore, coefficient values ($CV = 4.70$) and adequate precision (adeq. precision = 21.17) indicate the ability to identify relations between the variables of extraction and responses of TPC in a deduced model. Thus, it is clear that the model is unlikely to occur because of noise. 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 phenolic content (TPC) that was recovered in the leaf extract ranged from 6.74 to 14.57 mg GAE/g DW sample. Figure 3a described how ethanol concentration and temperature (AB) influence the production of total phenolic content. It was also observed that the yield of extraction 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 were reported on the influence of higher temperature improved the TPC extraction efficiency on *Angelica keiskei* [41] and *Hylocereus polyrhizus* [25]. 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 influenced phenolic extraction yield in extract [12, 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 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 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 favoring 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, there was no noticeable improvement observed in TPC extraction, as the value declined continuously.

Optimization Of Uae Extraction Conditions, Verification, And Comparison With Conventional Methods

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 as well as significant linear, quadratic, and interactive effects of the process factor. The maximum desirability of TFC (7.58 mg QE/g DW) extraction conditions was as follows: concentration of ethanol 70%, ultrasonic temperature 60 °C, ultrasonic time 50 min, and 40 mL/g liquid-solid ratio obtained from the Std of 28. Whereas, 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). It was observed that 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 [16]. This finding was consistent with the literature that offered higher potency of the yield of bioactive compounds using UAE than traditional HRE [19, 51]. Therefore, the optimization condition obtained from this study could be helpful guideline information for the further industrialization of the UAE process.

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 [13]. The antioxidant activity of *C. olitorius* leaf extract was evaluated and compared with known antioxidant ascorbic acid (Vit-C). Outcomes revealed a significant positive association with increasing concentration (100–600 µg/mL of extracts and ascorbic acid) shown in Fig. 5a, and that the extraction scavenges was low levels results compared to Vit-C at the same concentration. Furthermore, when extracts concentration increased to 600 µg/mL, the antioxidant activity of extracts ($81.50 \pm 1.74\%$) was comparable to Vit-C ($95.0 \pm 1.17\%$). The IC_{50} value of *C. olitorius* leaves extract was 226.29 µg/mL, agreed to the findings of Biswas et al.[29], indicating that good potential for scavenging DPPH radicals. These results showed that leaves extracts possess DPPH scavenging because of flavonoids presence and the position of hydroxyl groups [52].

ABTS⁺• radical scavenging activity

The radical cation-derived ABTS is known as the most often employed organic radical for determining the antioxidant activity of natural products [53]. Additionally, the extracts capacity to neutralize ABTS⁺• free radicals is a good indicator of their antioxidant activity [54]. Figure 5b illustrated the performance of the *C. olitorius* extract in terms of its ability to scavenge ABTS⁺• radicals at various concentrations, ranging from 50 to 300 µg/mL. Results revealed that the ABTS⁺• radical scavenging activity of the *C. olitorius* extract and ascorbic acid appeared to be predominantly dose-dependent, and the scavenging rate of the leaf extracts or the ascorbic acid positively correlated. The value of the scavenging effect ranged from 12.36 ± 0.58 to 67.39 ± 1.24, which was comparatively lower than Vit-C at the same concentration. The IC₅₀ value of the *C. olitorius* extract and Vit-C were 199.53 µg/mL and 67.45µg/mL, respectively. According to these findings, it could be concluded that *C. olitorius* extract is a powerful scavenger of ABTS⁺• radical.

Hydroxyl Radical Scavenging Activity

The hydroxyl radical, which is 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 [55]. The scavenging activity was due to chelating ions such as Fe²⁺ and Cu⁺ inhibiting hydroxyl radical generation rather than direct scavenging [56]. Figure 5c showed that the superoxide anion scavenging rates were correlated with the concentration and increased with increasing concentration of *C. olitorius* extract and ascorbic acid. A concentration-dependent antioxidant activity was similar to Vit-C, with an IC₅₀ value of 402.02 µg/mL, whereas 25.48 µg/mL for ascorbic acid. These results proved that the *C. olitorius* leaf extract possesses the noticeable hydroxyl radical-scavenging ability, which increased proportionally to the extract concentration.

Table 5
Total flavonoids and phenolic 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 phenolics content (mg GAE/g DW)	13.38 ± 0.43	13.92	8.24 ± 0.90

Conclusions

This study very early attempted to optimize the extraction factors using CCD along with RSM for recovery of TFC and TPC from the *C. olitorius* in UAE. The mathematical models of RSM indicated that all parameters significantly influenced the extraction process and allowing for the incorporation of a

quadratic model. In validation experiments, results were quite consistent to predicted values by the corresponding model, and were significantly higher than conventional HRE. These results indicated that UAE is more effective and feasible approach than HRE for isolating these compounds. Additionally, the *C. olitorius* extract displayed significant dose-dependent DPPH•, ABTS⁺•, and hydroxyl radical scavenging activities. The present work results can serve as a real practical contribution to the topic for designing the experiment for the separation of antioxidants and comprehensive application of *C. olitorius* in drug and food industries.

Abbreviations

ABTS	2,2'-azino-bis(3 ethylbenzothiazoline-6-sulphonic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant potential
GAE	Gallic acid equivalents
QE/g DW	Quercetin per gram dry weight
ROS	Reactive oxygen species
TFC	Total flavonoids content
TPC	Total polyphenols content
UAE	Ultrasound-assisted extraction

Declarations

Author Contributions All authors contributed to the study conception and design. Conceptualization and material preparation was performed by Defang Li, Ashok Biswas and Susmita Dey. Experiment performed data analysis and participated in original drafting the article by Ashok Biswas and Susmita Dey. Formal analysis and validation were performed by Aiping Xiao, Yong Deng, Ziggiju Mesenbet Birhanie and Jiangjiang Zhang. Review and editing the article performed by Siqi Huang and [Liangliang Liu](#). Defang Li Supervised the project, reviewed and final approved for submission of manuscript. All authors have read and agreed to the published version of the manuscript.

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

Competing Interests The authors declare no competing interests.

Ethics Approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflicts of Interest The authors declare that they have no potential conflicting interests in relation to the study in this research.

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Figures

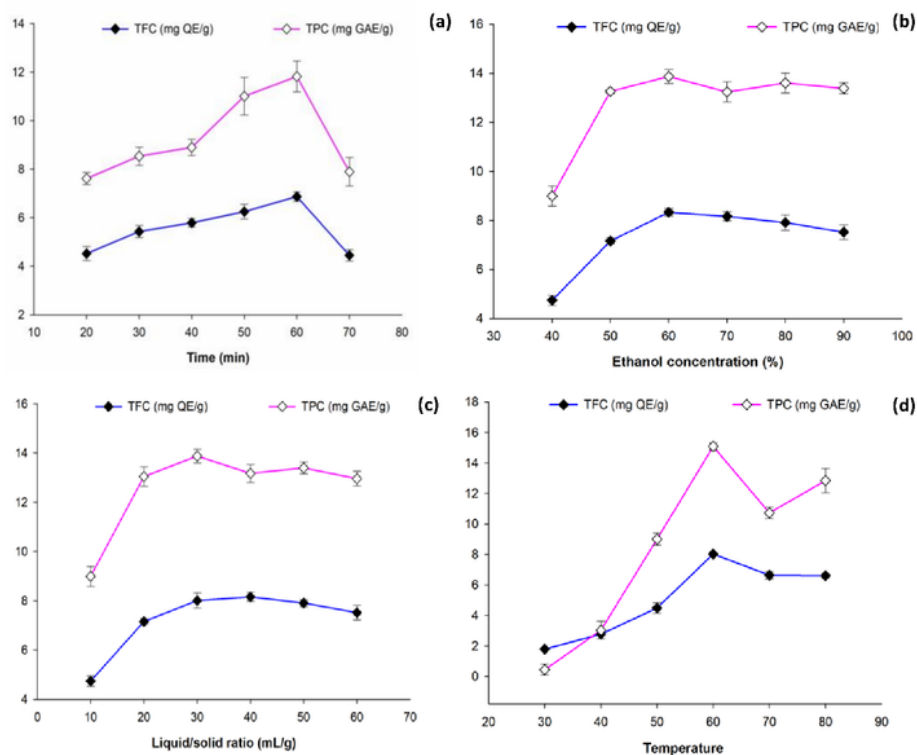


Figure 1

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 errors ($n=3$).

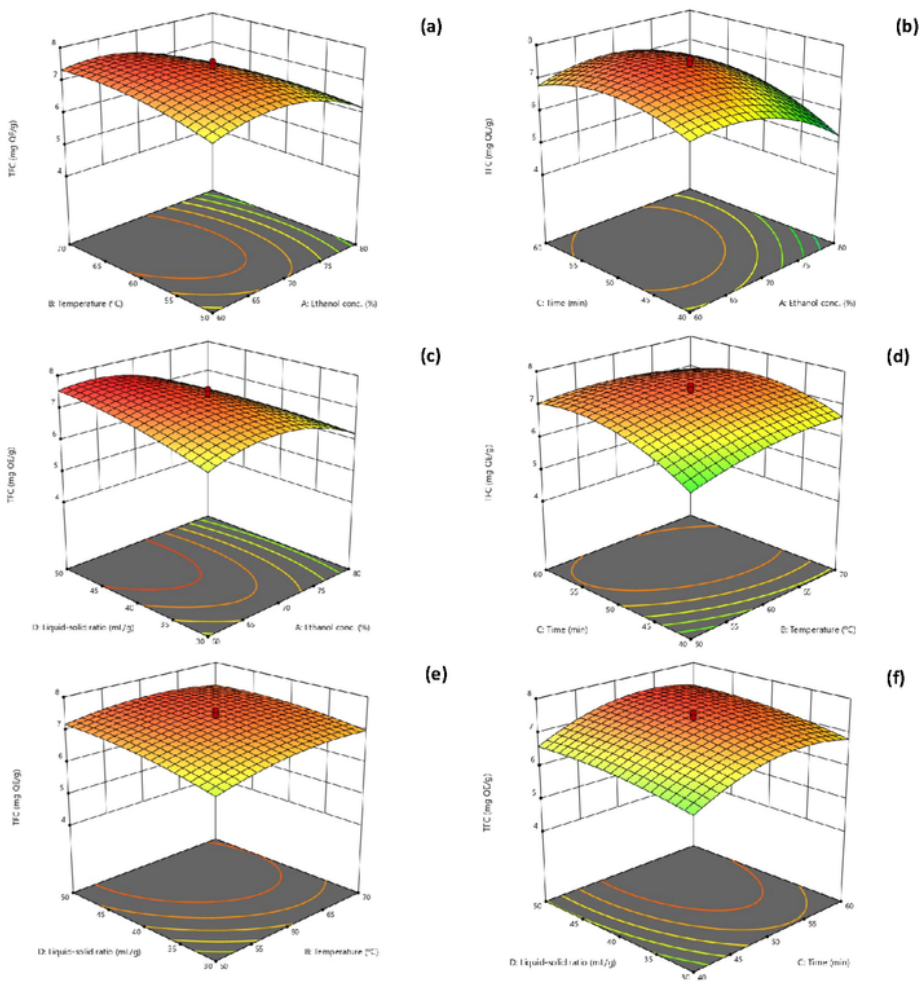


Figure 2

The interaction of extraction variables on TFC

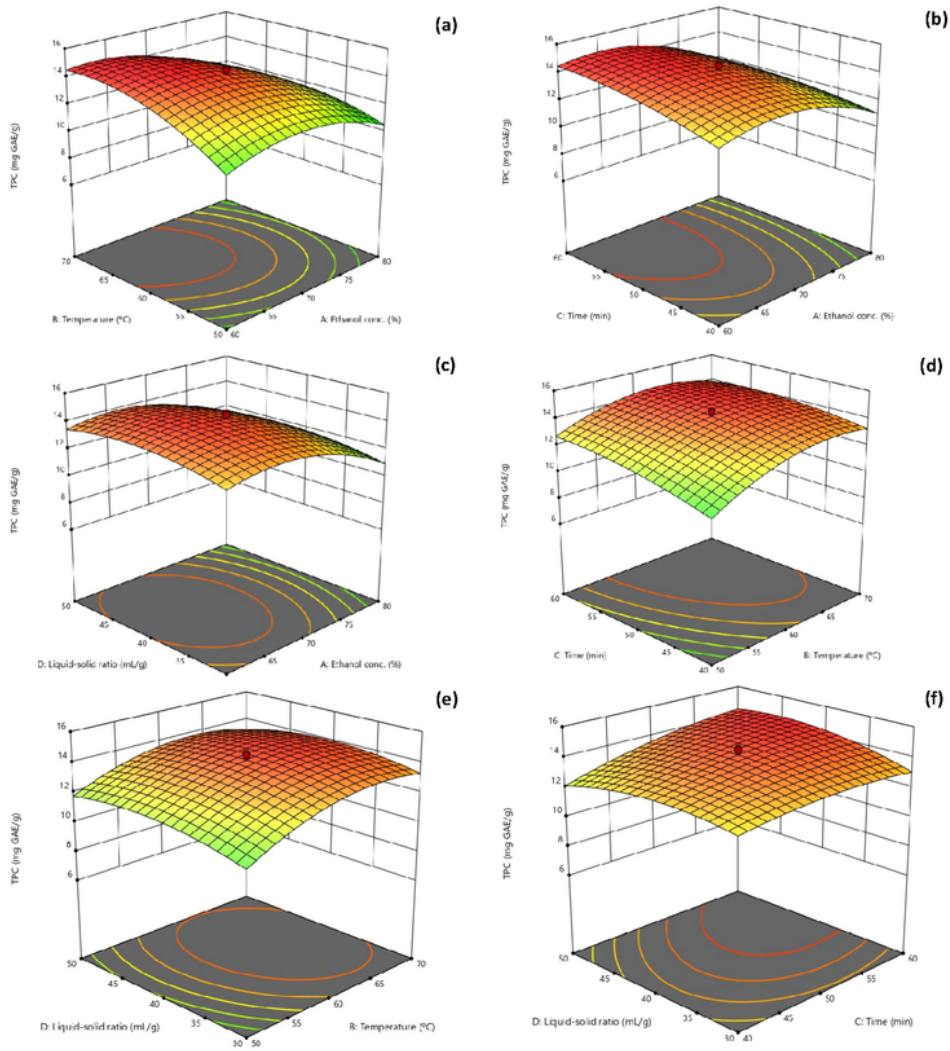


Figure 3

The interaction of extraction variables on TPC

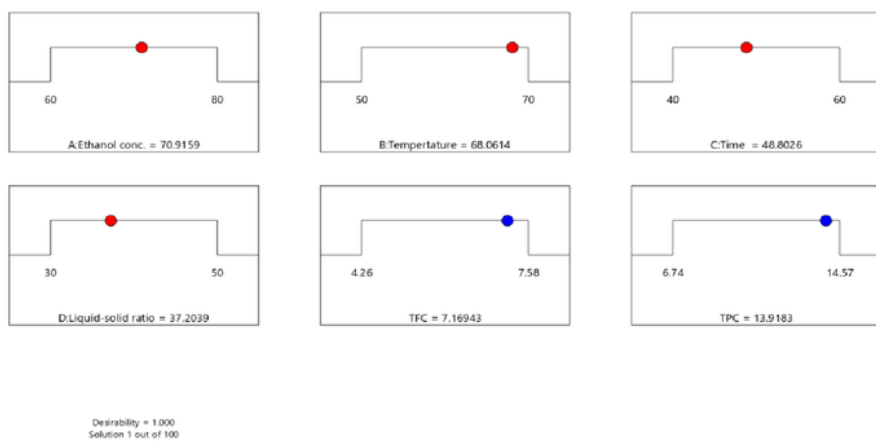


Figure 4

Parameters optimization represented by desirability ramp

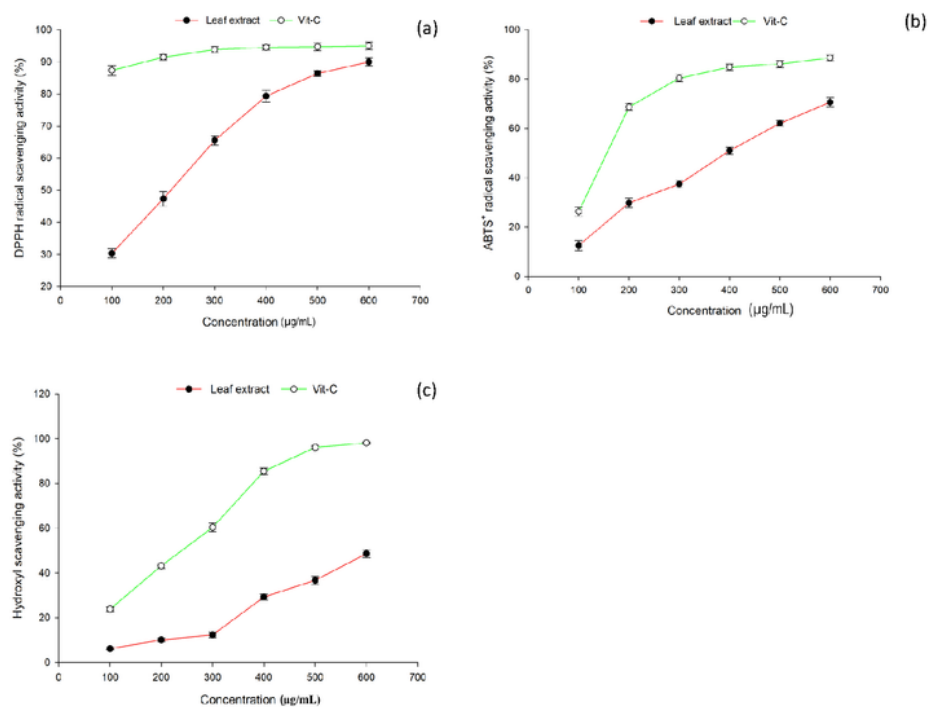


Figure 5

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 $\pm$ standard errors.

Supplementary Files

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- [Graphicalabstract.pdf](#)