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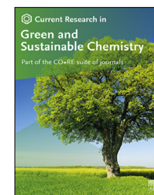

Optimization of ultrasound-assisted extraction of total phenolics and flavonoids from the leaves of *Lobelia nicotianifolia* and their radical scavenging potential

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ABSTRACT

Optimization of the ultrasound-assisted extraction (UAE) for total phenolics content (TPC) and total flavonoid content (TFC) from leaves of *Lobelia nicotianifolia* Roth. was worked out. Full factorial design (FFD) was used to study the effects of three independent variables (methanol ratio, temperature, and extraction time), each with three levels. The optimal combination for maximum efficiency of extraction and antioxidant activity was 75.25% methanol, 62.72 °C temperature, and 9.44 min duration. The extract made under these conditions had higher TPC and TFC of 23.78 mg TAE/g dry weight and 20.21 mg QE/g dry weight, respectively. Furthermore, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays of the extract showed higher antioxidant potential with 95.78% and 97.26% radical scavenging activity (%RSA), respectively. Scanning electron micrograph (SEM) of leaves showed ultrasonication-induced morphological changes and cellular damage. Fourier-transform infrared (FTIR) analysis of conventional extraction and extracts obtained by UAE showed remarkable differences in peaks. Therefore, the UAE method seems to be superior to the conventional method for extracting total phenolics and flavonoids.

1. Introduction

Lobelia, a tropical and subtropical genus of the family Campanulaceae, is known for its bioactivities and bioactive secondary metabolites [1]. *Lobelia* spp. are known to have CNS, drug abuse, and multidrug resistance activities [2–5]. They also improved memory and used to treat Alzheimer's disease [6,7]. The principal bioactive compounds of *Lobelia* are the piperidine class of alkaloids such as lobeline, norlobeline, lobelanidine, and norlobelanine [7,8]. *Lobelia* is also known to have terpenoids, phenolics, flavonoids, and coumarin [1]. Recent studies have shown anticancer phenolics and coumarins in *Lobelia chinensis* [9]. Wild and cultivated species of *Lobelia,* including *Lobelia nicotianifolia* Roth. (Wild tobacco) have been reported from India. It is commonly used in folk medicine to treat pain, asthma, bronchitis, fever, eye diseases, snake and dog bites, and healing wounds. It is antibacterial, and its antioxidants potential was also studied [10–12]. The annual demand for *L. nicotianifolia* in India is about ten metric tons/year [13], indicating its medicinal and commercial value. Preliminary screening with LC-HRMS of *L. nicotianifolia* has revealed 18 phenolic compounds (data not shown), which showed similarity with different *Lobelia* species [1]. This putative identification of *L. nicotianifolia* leaf extracts has revealed the presence of effective antioxidant and anticancer phenolics and flavonoids [14].

Plant phenolics are attracting researchers due to their cardioprotective role and pharmaceutical applications such as antioxidants, anticancer, antibacterial, and anti-inflammatory agents [15]. Extraction is a critical step in the recovery and isolation of phenolic compounds. However, it needs to be optimized by considering their chemical nature and interfering substances [16,17]. Extraction of phenolic compounds and their bioactivities are governed by solvent, temperature, and extraction duration [18]. Biologically active compounds such as phenolics are usually present in small quantities. Moreover, extraction methods

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; FDD, Full factorial design; FTIR, Fourier-transform infrared; RSA, Radical scavenging activity; SEM, Scanning electron micrograph; TFC, Total flavonoids content; TPC, Total phenolics content; UAE, Ultrasound-assisted extraction.

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Table 1
Independent variables used in a full factorial design.

Symbols	Independent variables	Levels		
		−1	0	1
X_1	Methanol concentration (%)	50	75	100
X_2	Extraction temperature (°C)	50	60	70
X_3	Extraction time (min)	5	10	15

that give higher yields may disturb their functional properties [19]. Several methods for extracting plant phenolics have been documented, reviewed, and categorized into traditional/conventional and advanced/green methods [20]. Extraction of phenolics with stirring, maceration, cold pressing, squeezing, and hydrodistillation are standard conventional methods. However, these methods are inconvenient for industrial and commercial applications due to the loss of phenolic compounds caused by their oxidation, ionization, and hydrolysis [21–23]. Therefore, advanced extraction methods like microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), high-pressure assisted extraction (HPAE), high voltage electric discharges assisted extraction (HVED), pulsed electric fields assisted extraction (PEF), and supercritical fluid extraction (SFE) methods, collectively called ‘green extraction methods,’ were developed. These methods reduce energy and chemical consumption and can be used at an industrial scale [24]. UAE is based on the cavitation effect, which causes physical and mechanical damage in plant materials and enhances extraction recovery [25]. UAE is an eco-friendly extraction method that consumes less solvent and energy, has less extraction time and expenses with a higher product recovery than conventional methods [26–31]. Though UAE is better than conventional methods, it needs to be optimized as per the target plant system using advanced statistical tools [27,29,32–35].

In light of this, the present investigation was undertaken to optimize UAE parameters for the extraction of total phenolics and flavonoids from leaves of *L. nicotianifolia* and testing effects of extraction parameters on the antioxidant potential of extracts thus prepared. For optimization, three factors were considered, viz., methanol (%), extraction temperature (°C), and time (min). Further, extracts were tested for their antioxidant potential by using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinbis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Scanning electron microscopy (SEM) was used to assess the UAE's effect on leaf cell structure. Fourier transformed infrared spectroscopy (ATR-FTIR) was used to assess leaf samples' chemical alterations before and after ultrasonication treatment.

2. Materials and methods

2.1. Plant material and chemicals

Plants of *L. nicotianifolia* were collected from the Kas lake region (17°43′24″N, 73°48′47″E) of Satara district, Maharashtra, India, and identified using the Flora of Maharashtra [36]. A herbarium specimen was submitted to Naoroji Godrej Centre for Plant Research (Voucher number NGCPR-1901). Organic solvents (HPLC grade), tannic acid (GRM7541:100G), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (MB255-1G), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (RM2798-1G), and Folin–Ciocalteu's phenol reagent (RM10822-100 ML) were procured from Himedia, India. Quercetin (Q) (RM6191-100G) was obtained from Sigma, USA. The ultrasonication unit was purchased from Lifecare Equipment, India. The rotary evaporator (PBU-6D) was obtained from Superfit, India. Spectrophotometric analysis (quantification of TPC, TFC, and antioxidant potential) was performed using Shimadzu UV-1900 UV–Vis Spectrophotometer (Shimadzu, Japan).

2.2. Experimental design

2.2.1. Effect of ultrasonic frequencies on the extraction of phenolic compounds

A pilot experiment was performed using ultrasound frequencies of 25, 35, 45, 55, and 65 kHz. The efficiency of these frequencies was

Table 2
Input factors (natural and coded values) and levels selected for a full factorial design where responses are total phenolics content (TPC mg TAE/g dw), total flavonoids content (TFC mg QE/g dw), and DPPH and ABTS (% RSA) for *L. nicotianifolia* leaves.

Run	Independent Variables			Responses			
	Natural Values (Coded Values)						
	X_1	X_2	X_3	TPC	TFC	DPPH	ABTS
	Methanol (%)	Temperature (°C)	Time (min)				
1	50 (−1)	50 (−1)	5 (−1)	15.92	12.76	47.16	50.35
2	50 (−1)	50 (−1)	10 (0)	17.28	14.02	50.75	53.23
3	50 (−1)	50 (−1)	15 (1)	16.04	12.29	42.10	42.52
4	50 (−1)	60 (0)	5 (−1)	18.14	14.53	53.69	58.17
5	50 (−1)	60 (0)	10 (0)	19.94	15.29	55.89	60.97
6	50 (−1)	60 (0)	15 (1)	18.84	14.08	52.62	56.08
7	50 (−1)	70 (−1)	5 (−1)	16.16	15.47	62.94	61.64
8	50 (−1)	70 (−1)	10 (0)	17.91	16.64	67.97	65.35
9	50 (−1)	70 (−1)	15 (1)	16.37	15.62	61.08	60.68
10	75 (0)	50 (−1)	5 (−1)	19.34	16.60	73.36	74.11
11	75 (0)	50 (−1)	10 (0)	19.81	17.88	78.58	77.56
12	75 (0)	50 (−1)	15 (1)	19.20	17.03	77.04	72.54
13	75 (0)	60 (0)	5 (−1)	20.93	18.09	91.41	93.61
14	75 (0)	60 (0)	10 (0)	22.65	20.02	96.47	98.21
15	75 (0)	60 (0)	15 (1)	19.80	16.04	85.01	84.98
16	75 (0)	70 (−1)	5 (−1)	21.39	17.84	84.31	87.74
17	75 (0)	70 (−1)	10 (0)	22.42	18.81	92.76	95.38
18	75 (0)	70 (−1)	15 (1)	21.89	16.80	88.40	89.15
19	100 (1)	50 (−1)	5 (−1)	18.18	15.74	54.99	74.18
20	100 (1)	50 (−1)	10 (0)	19.40	16.42	58.67	77.04
21	100 (1)	50 (−1)	15 (1)	18.06	15.37	56.02	72.35
22	100 (1)	60 (0)	5 (−1)	18.33	14.90	66.86	69.72
23	100 (1)	60 (0)	10 (0)	19.93	13.86	68.35	71.97
24	100 (1)	60 (0)	15 (1)	17.01	13.05	58.93	64.55
25	100 (1)	70 (−1)	5 (−1)	18.01	12.35	56.79	61.84
26	100 (1)	70 (−1)	10 (0)	18.29	12.29	60.45	62.29
27	100 (1)	70 (−1)	15 (1)	17.03	10.65	55.22	54.63

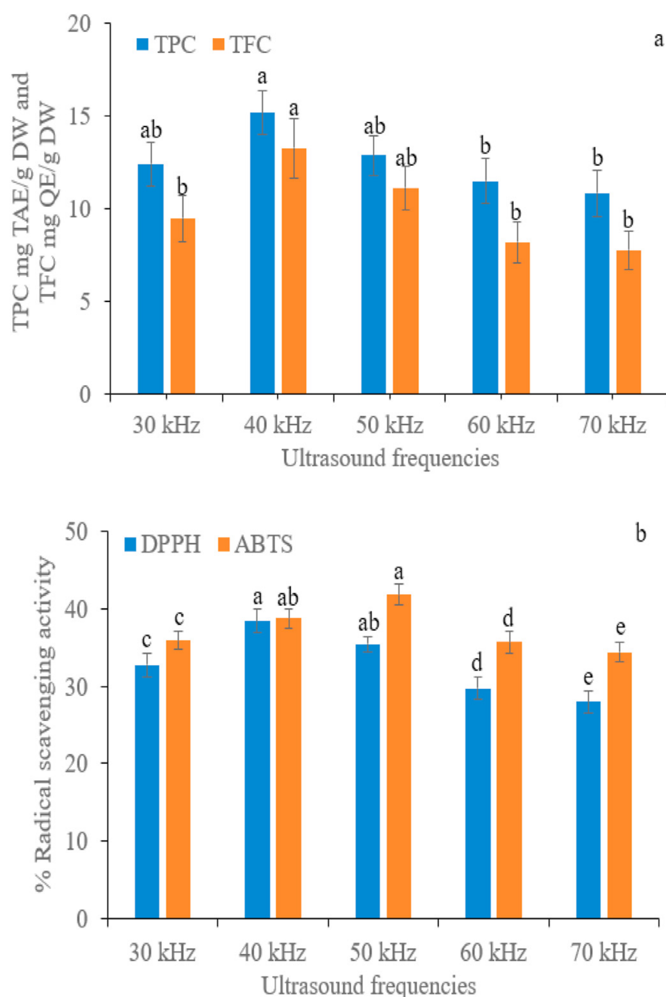

Fig. 1. The effect of different ultrasound frequencies on (a) total phenolics and total flavonoid content and (b) % RSA in DPPH and ABTS assay.

investigated in terms of yield (TPC and TFC) and its antioxidant assays. UAE was performed in 75% methanol for 10 min at 60 °C and.

2.2.2. Full factorial design

The solvent percentage, extraction time, and extraction temperature determine the UAE's yields and subsequent antioxidant activities [37]. A full factorial design (FFD) was used to test different UAE conditions (Table 1) to identify the most efficient combination to optimize the UAE process.

2.3. Determination of total phenolic and flavonoid contents

TPC and TFC in leaf extracts of *L. nicotianifolia* were determined using Folin–Ciocalteu's phenol and $AlCl_3$ methods with slight modifications [38]. For TPC analysis, 50 μL extract was mixed with 200 μL of Folin–Ciocalteu's phenol reagent (2 N) and 1000 μL of 7.5% sodium carbonate. The reaction mixture was incubated for 30 min at 25 °C. For TFC analysis, leaf extract was added to 2% $AlCl_3$ in equal proportion. The reaction mixture was incubated at room temperature for 1 h. The TPC and TFC were quantified at 765 and 420 nm, respectively, using a UV–Vis spectrophotometer (Shimadzu UV-1900). Tannic acid and quercetin were used as standard phenolic and flavonoid substances, respectively, to prepare calibration curves of absorbance against concentration (0–250 μg mL^{-1}). Concentrations of TPC and TFC were expressed as mg of tannic acid equivalent (TAE)/g dw and quercetin equivalent (QE)/g dw, respectively.

2.4. Determination of antioxidant activity

2.4.1. DPPH radical-scavenging activity

The percentage radical scavenging activity (%RSA) of *L. nicotianifolia* leaf extract against DPPH radicals was evaluated as per Zheleva-Dimitrova et al. [39] with some modifications. In short, 1000 μL of extract (100–500 μg mL^{-1}) was added to 4000 μL of 0.2 mM methanolic DPPH. The resulting reaction mixture was incubated at room temperature for 30 min in the dark. The activity was measured at 517 nm. Ascorbic acid was used as a reference standard. The following equation was used to calculate the ability of extracts to quench DPPH radicals:

$$\text{DPPH RSA } (\%) = (\text{Abs S})/\text{Abs C} \times 100 \tag{1}$$

2.4.2. ABTS radical scavenging assay

Two stock solutions were prepared for ABTS assay as per Zheleva-Dimitrova et al. [39], viz., 7 mM ABTS and 2.4 mM potassium persulfate designated as A and B, respectively. A working solution (ABTS solution) was prepared by mixing equal volumes of A and B. The mixture was allowed to mature for 14 h at room temperature. One thousand μL of ABTS solution was diluted with methanol to get an absorbance of 0.706±0.01 at 734 nm. To 1000 μL leaf extract, 1000 μL of ABTS solution was added, and absorbance was measured at 734 nm. The ABTS radical quenching capacity was compared with ascorbic acid, and the following

Table 3
ANOVA and regression coefficients of the quadratic polynomial models and regression coefficients in coded units for TPC, TFC, DPPH, and ABTS.

Term	TPC		TFC		DPPH		ABTS	
	Coef	P-Value	Coef	P-Value	Coef	P-Value	Coef	P-Value
$\beta 0$ (Intercept)	22.423	0	18.655	0	91.99	0	92.56	0
Linear								
$\beta 1$ (Methanol)	0.353	0.069 NS	−0.280	0.045 *	1.946	0.008 **	4.603	0.00 **
$\beta 2$ (Temperature)	0.289	0.132 NS	−0.075	0.573 NS	4.218	0.00 **	2.072	0.035 *
$\beta 3$(Time)	−0.099	0.596 NS	−0.340	0.017 *	−0.699	0.304NS	−1.565	0.103
Quadratic								
$\beta 11$	−2.078	0.00 **	−2.418	0.00 **	−19.392	0.000 **	−16.50	0.00 **
$\beta 22$	−0.711	0.014 *	−0.817	0.00 **	−3.457	0.002 **	−3.23	0.024 *
$\beta 33$	−0.947	0.002 **		NS	−3.457	0.002 **	−3.66	0.012 *
Cross Product								
$\beta 12$		NS		NS	−2.838	0.000 **	−4.989	0.00 **
$\beta 13$		NS	−1.206	0.00 **		NS		NS
$\beta 23$		NS		NS		NS		NS
P Value of Model	NA	0.00 **	NA	0.00 **	NA	0.00 **	NA	0.00 **
S	0.9378	NA	0.6693	NA	3.3741	NA	4.66	NA
R Sq %	0.8145	NA	0.9322	NA	0.9640	NA	.9243	NA
R Sq (Adj) %	0.7589	NA	0.9118	NA	0.9507	NA	.8965	NA

NA- Not Applicable, NS - Not significant, * Significant at $P \leq 0.05$, and **Significant at $P \leq 0.01$.

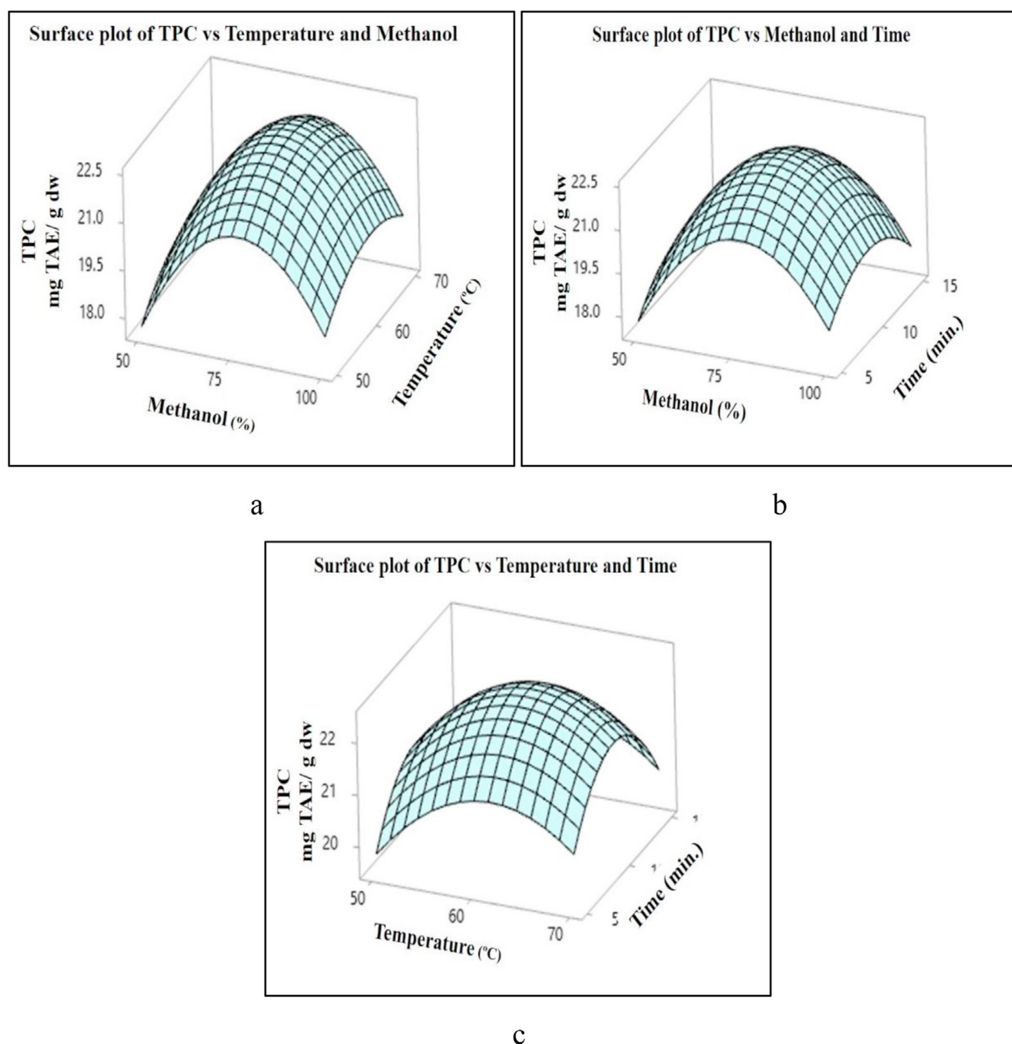

Fig. 2. Surface plots showing the effect of methanol percentage (X1), temperature (°C) (X2), and time (min) (X3) on the extraction of TPC from leaves of *L. nicotianifolia*.

formula was used to calculate percentage inhibition:

$$\text{ABTS RSA } (\%) = \text{Abs C} - \text{Abs S}/\text{Abs C} \times 100 \quad (2)$$

In Equations (1) and (2), the Abs C is the absorbance of methanolic DPPH/ABTS radical, and Abs S is the absorbance of DPPH/ABTS radical solution mixed with *L. nicotianifolia* leaf extract or reference standard.

2.5. Optimization of UAE conditions using a full factorial design

The extraction method was optimized by multiple regression on data collected using FFD. It is simple to design, execute, and estimate quadratic relations for three variables of three levels [40], hence adopted for this study. However, the frequency used for ultrasonication was based on the single factor experimental design. An FFD consisted of collecting data on all combinations of all factors' levels to achieve process optimization. Three independent variables (inputs) tested were the percentage of methanol, temperature, and time. Each of these factors was used at three levels coded as −1, 0, and 1 for low, medium, and high levels, respectively. Table 2 shows a design matrix consisting of 27 experimental runs.

The data obtained from these 27 experimental runs were fitted to the following second-degree polynomial regression equation by multiple regression:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j$$

In this equation, y is response (outputs); β_o is a constant (or model intercept); βi is the linear coefficient of variables; βii is coefficient of a square of the parameter; βij is cross-product coefficient, and ***xi*** and ***xj*** are independent variables.

2.6. Scanning electron microscopy

Morphological changes induced by ultrasonication of the leaves of *L. nicotianifolia* were studied using an electron microscope (Sigma 300, Zeiss). Processed samples were mounted on aluminum stubs with colloidal graphite and sputter-coated with gold using a JFC-1200 fine coater (JEOL). The observations were made at an accelerating voltage of 5 kV under a high vacuum.

2.7. FTIR analysis

FTIR analysis in this study was carried as per Altemimi et al. [29] with some modifications. The viscous leaf extract was loaded in an ATR-FTIR spectroscope (Shimadzu IRAffinity-1S 00466). Thirty scans were made between the range of 4000–500 cm^{-1}. After every scan, a new reference of background air spectra was recorded. The ATR plate was cleaned by

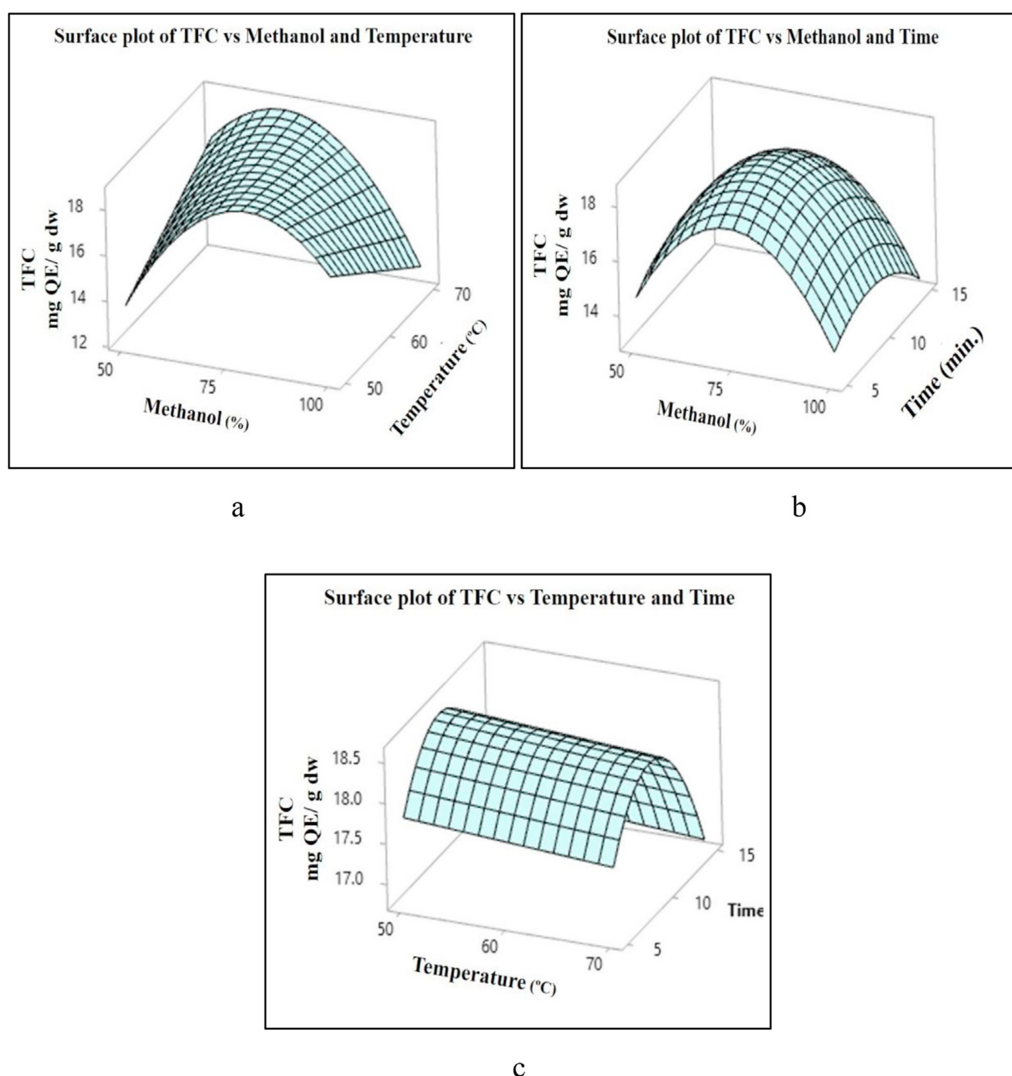

Fig. 3. Surface plots representing the effect of methanol percentage (X1), temperature (°C) (X2), and time (min) (X3) on the extraction of TFC from the leaves of *L. nicotianifolia*.

wiping it carefully with 70% acetone twice, followed by drying with soft tissue before the next run. IR solution software was used to process data.

2.8. Statistical analysis

All experiments were replicated three times, and the values presented in the tables are the mean of three readings. Minitab 17 was used for constructing a design matrix, graph, and data analysis. The F-test (ANOVA) were performed at $P < 0.05$ (significant), and $P < 0.01$ (very significant). The mathematical model's predicted optimized condition was verified using the percentage of relative change (PRC) between experimental and predicted values [41].

3. Results and discussion

3.1. Optimization of UAE conditions by full factorial design

When other influential factors such as temperature (60 °C), methanol (75%), and time (10 min) were kept constant, the amount and bioactivity of TPC and TFC extracted by ultrasonication were influenced by ultrasound frequency used during extraction (Fig. 1a and b). Maximum TPC, TFC, and % RSA in DPPH assay were observed at 40 kHz, whereas the highest % RSA in ABTS was observed at 50 kHz. Thus, the extractability of TPC and TFC was poor at very low or very high frequencies. The lower frequency could be insufficient to extract these compounds. In contrast, higher frequencies might have caused bubbles' collapse, thereby reducing the production and intensity of cavitation in liquid [31]. A similar loss of yield at higher frequencies was also observed by Altemimi et al. [29] and Bendicho and Lavilla [42]. FFD was optimized based on these single-factor experiments. This design had three input factors, each with three levels, and thus 27 runs. The influence of these 27 combinations on response variables (TPC, TFC, and % RSA) are shown in Table 2.

3.2. Fitting the model

The main effect plots showed a non-linear relation between the predictor and response variable. To estimate regression coefficients with multiple regression analyses, the second-order polynomial models for TPC and TFC and two antioxidant assays viz., DPPH and ABTS were fitted. ANOVA was used to assess the significant differences and good fit of the model. The results are shown in Table 3. All models were highly significant ($P < 0.01$), suggesting their reliability and adequacy. The model's R^2 values were 0.8145, 0.9322, 0.9646, and 0.9243 for TPC, TFC, DPPH, and ABTS, respectively, indicating a higher correlation between the predicted values and the experimental data. The adjusted R^2 values were also very high to advocate for the model's high significance [43].

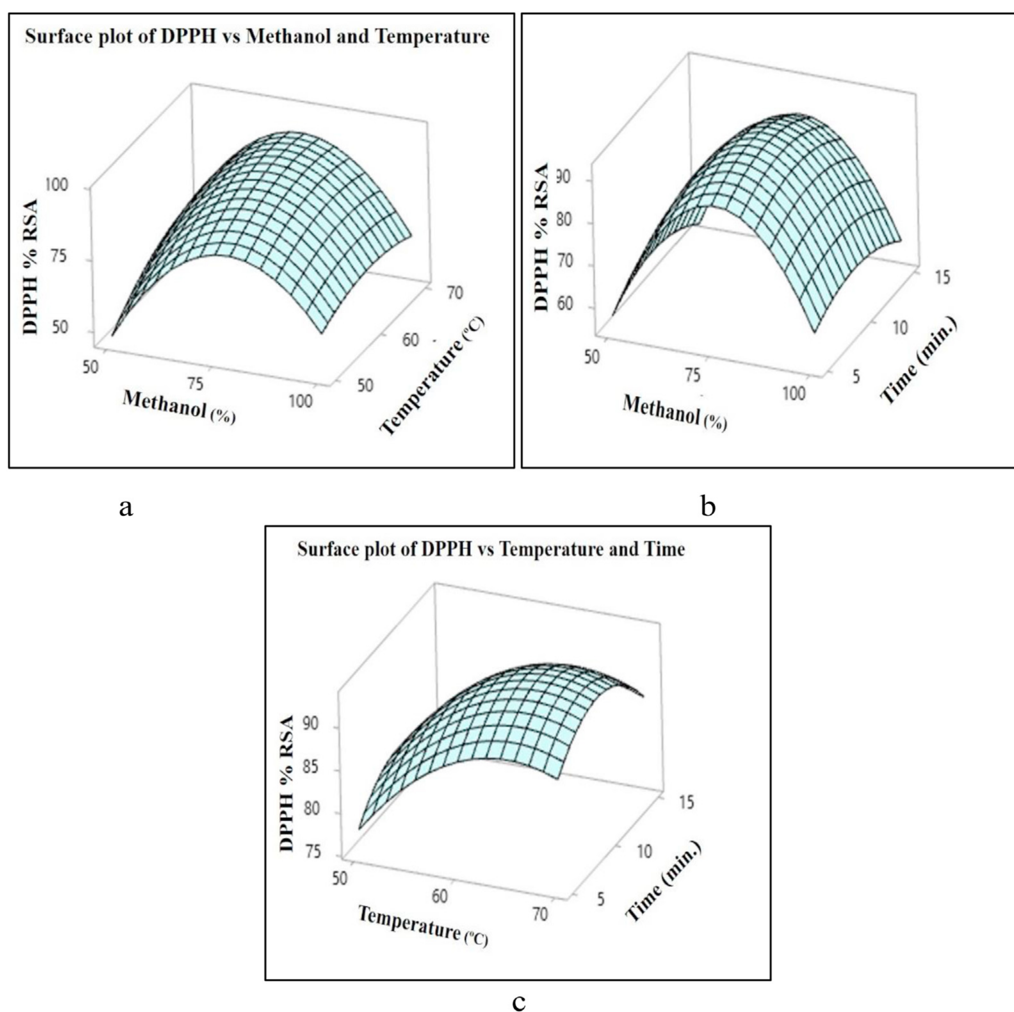

Fig. 4. Surface plots representing the effect of methanol percentage (X1), temperature (°C) (X2), and time (min) (X3) on %RSA in DPPH assay.

3.3. Analysis of TPC and TFC

A 3-dimensional surface plot was drawn with one variable kept at a medium level and the other two variables at their experimental range. The significance of each coefficient for the models shown in Table 3 confirms fitting a quadratic model. Fig. 2 shows 3-dimensional surface plots that describe the effects of methanol, temperature, and time on TPC recovery. The quadratic effect of methanol was the most important ($P < 0.01$), followed by that of time ($P < 0.01$) and temperature ($P < 0.05$). A linear effect of methanol, temperature, and time was non-significant ($P > 0.05$). In this study, TPC's concentration ranged from 15.92 to 22.65 mg TAE/g dry weight (Table 2). With an increase in the methanol ratio (50–75%) and temperature (50–60 °C), TPC increased to 22.5 mg TAE/g dw (Fig. 2a). In contrast, 100% methanol and the temperature of 70 °C reduced the TPC. TPC's maximum yield was observed at a methanol concentration of 76.25%. For methanol and temperature, 76.80% of methanol and (63.20 °C) temperature maximized the TPC (Fig. 2a). The maximum yield of TPC was obtained in 9.81 min at a methanol concentration of 76.94% (Fig. 2b). The relationship between temperature and time was non-liner, and the combination of 61.69 °C temperature and 9.52 min extraction gave a maximum yield of TPC (Fig. 2c).

The optimized extraction method for TFC from the leaves of *L. nicotianifolia* yielded TFC in the range of 10.65–20.02 QE/g dw (Table 2). Fig. 3 illustrates the surface plots describing the effects of methanol concertation, temperature, and time on TFC. The methanol concertation and time had significant linear effects ($P < 0.05$). The quadratic effect of methanol concertation and temperature were the most important influential factors ($P < 0.01$), followed by an interactive effect between methanol and time ($P < 0.01$). More or less same TFC was observed at all levels of temperature and 70–90% of methanol (Fig. 3a). The maximum TFC yield was observed at 75.24% methanol and 59.33 °C. Interestingly, mixed linear and quadratic relations were seen for methanol percentage and time (Fig. 3b). The maximum TPC yield was obtained at 73.38% methanol and a 9.05 min duration of extraction. The quadratic effect of time was statistically insignificant and showed a quadratic relation graphically. The TFC yield increased with an increase in the extraction duration. However, it decreased beyond 10 min of extraction duration. As seen from Fig. 3c, almost the same TFC was observed between 55 and 65 °C at around 10 min. An increased TFC was observed at the initial stages of temperature and time combinations, which was decreased beyond the combination of 60 °C temperature and 10 min of extraction. Like TPC, the combination of 61.69 °C and 9.52 min of extraction resulted in the maximum TFC yield.

The extraction of phenolics was influenced by the optimized binary solvent system, temperature, and time. Methanol was the solvent of choice for extracting phenolic compounds from the leaves of *L. nicotianifolia*, and its superiority has been well documented for species such as *Limnophila aromatica* (Lamk.) Merr. [44] and *Ocimum basilicum* L. [45]. The optimized alcohol and water ratios are the key factors in the extraction of phenolic compounds. Water helps in the swelling of plant tissue, while alcohol helps dissolve and recover phenolic compounds [46, 47]. The influence of solvent concentration, time, and temperature set

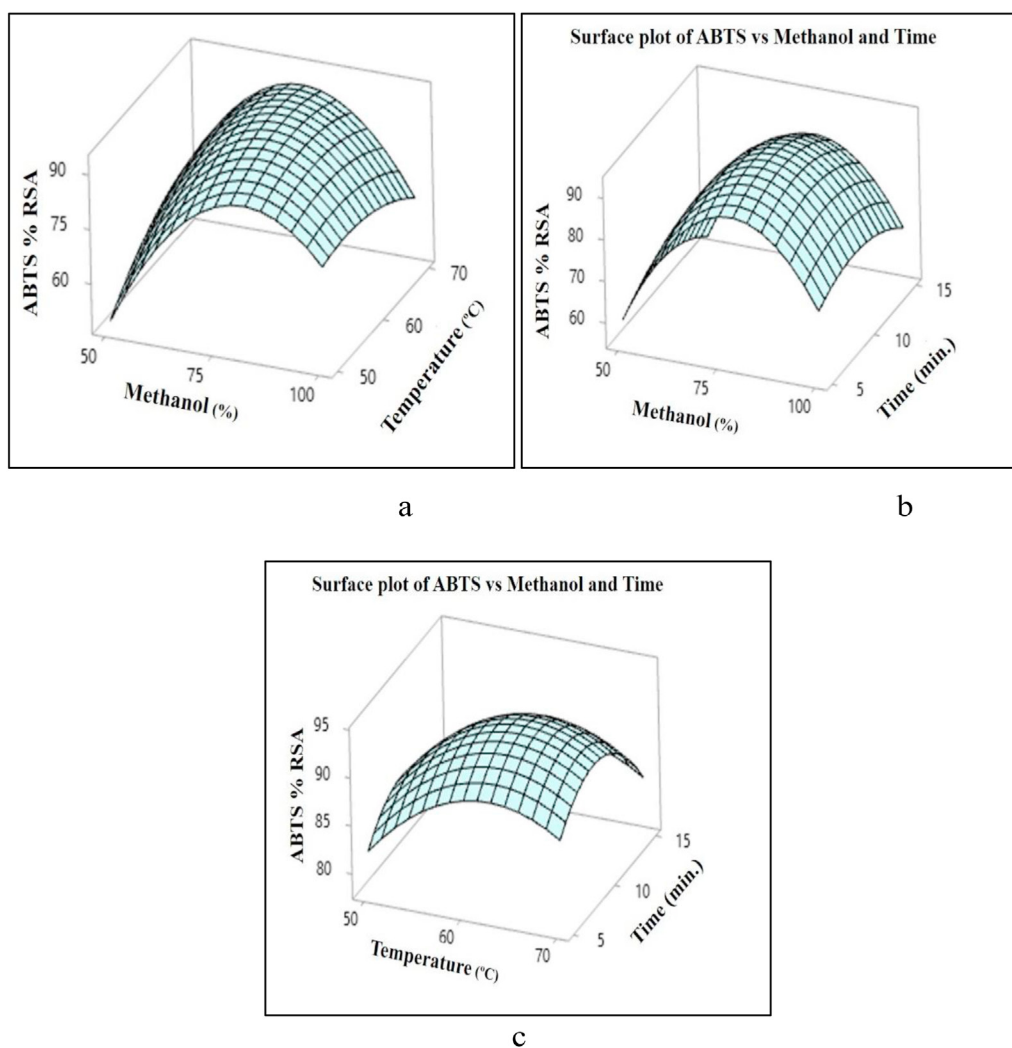

Fig. 5. Surface plots representing the effect of methanol percentage (X1), temperature (°C) (X2), and time (min) (X3) on %RSA in ABTS assay.

Table 4
The predicted values using the optimal conditions for UAE for TPC and TFC from the leaves of *L. nicotianifolia* and their % RSA in DPPH and ABTS assays.

Response	Fit	Predicted values at 95%	Experimental values
TPC	22.44	(20.258, 24.625)	23.78 ± 1.02
TFC	18.64	(16.942, 19.984)	20.21 ± 0.78
DPPH	93.04	(85.16, 100.92)	95.78 ± 0.45
ABTS	93.06	(82.16, 103.97)	97.26 ± 0.69

during ultrasonication was reported for fenugreek seeds [37] and orange leaves [48]. The recovery of phenolic compounds is higher in ultrasonication-based extraction due to cavitation and rapid formation and collapse of air bubbles that break the cell wall releasing the phenolic compounds [30,37,49]. In the present investigation, though the UAE seemed to be helpful for the extraction of phenolics from the leaves of *L. nicotianifolia,* a decrease in the TPC and TFC was observed at higher temperatures and extended extraction. These results are in line with those of Balasubramaniam et al. [47], Sharma et al. [50], and Bamba et al. [51] for different plant species. Different phenolic compounds are degraded or denatured due to prolonged extraction and higher temperature during extraction [28,29,37]. Ameer et al. [52] have reviewed the UAE's efficiency in different plants and concluded that the degradation due to oxidative pyrolysis might cause a reduced yield of bioactive principles.

3.4. Analysis of antioxidant assay

Fig. 4 shows the surface plots describing the relationship between extraction parameters (methanol percentage, temperature, and time) and % RSA in the DPPH assay. It shows a liner and significant effect ($P < 0.05$). A quadratic and interaction effect of methanol percentage, temperature, and time was significant. A maximum % RSA in DPPH assay was seen when methanol concentration was 75% at temperatures between 55 and 70 °C (Fig. 4a). Maximum % RSA in the DPPH assay was reached with 77.93% methanol and 65.49 °C. With 75% methanol, the same % RSA was observed for 2.5–12.5 min. It indicates that with an increase in the percentage of methanol at a constant temperature, the % RSA in DPPH assay increases initially but decreases after 75% methanol (Fig. 4b). The maximum % RSA was observed with a methanol concentration of 77.68% and a time of 9.86 min. The maximum % RSA was observed at 9.39 min time and 65.23 °C (Fig. 4c), beyond which it decreased in all directions.

The surface plots (Fig. 5) describe the effects of methanol percentage, temperature, and time on % RSA in ABTS assay. A linear and significant effect is seen for methanol percentage and temperature. A quadratic effect of methanol percentage, temperature, and time was substantial. However, the interactive effect of methanol percentage and temperature was statistically significant. The plot reveals that the % RSA in the ABTS assay increases for the same percentage of methanol for a given temperature. The % RSA was maximum between 70 and 85% and the

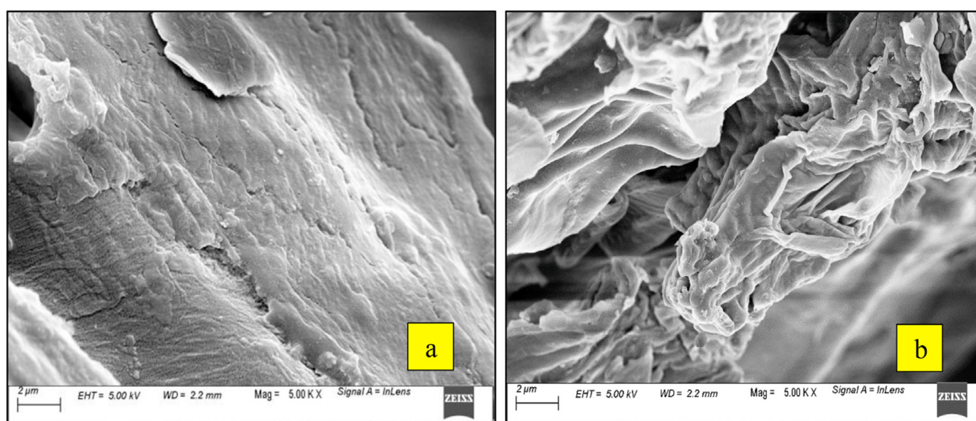

Fig. 6. Scanning electron micrographs of (a) leaf fragments in the extract of *L. nicotianifolia* extracted without ultrasonication, (b) leaf fragments in the ultrasound-assisted extraction (75% methanol, 60 °C temperature, and 10 min).

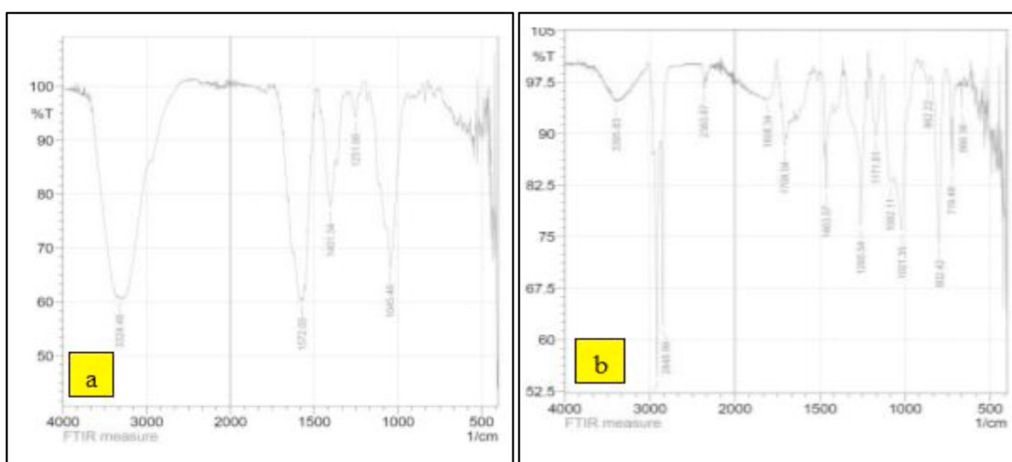

Fig. 7. Attenuated total reflection Fourier transformed infrared (FTIR) spectroscopic spectra of (a) untreated leaves of *L. nicotianifolia* and (b) leaves extracted with UAE (75% methanol, 60 °C, and 10 min].

temperature range of 53–70 °C (Fig. 5a).

The maximum % RSA in ABTS assay was observed when methanol concentration was 77.93% at 62.01 °C (Fig. 5b). It also describes a marked plateau of maximum % RSA between 5.1 and 12.5 min, where methanol concentration was between 70 and 85%. The shape of the surface plot in Fig. 5c is a perfect dome with a peak near 62.58 °C at 9.20 min.

The extraction parameters also influenced the antioxidant potentials of the resultant extracts. The ABTS and DPPH are stable free radicals. Their scavenging is indicated by a change in color in the presence of antioxidants. In the DPPH assay, the antioxidants reduce the purple methanolic DPPH solution to yellow. In the ABTS assay, the blue-green ABTS solution becomes colorless due to the presence of antioxidants. The antioxidant potential of plants is strongly correlated with phenolic compounds because of their hydroxyl groups at *ortho* and *para* positions contributing to the antioxidant property [53].

In the present investigation, the antioxidant activity was influenced by methanol concentration, which might be correlated with its polarity and viscosity. Similar results were noted in *Medicago sativa* L. [28] and *Averrhoa carambola* L. [54]. The % RSA in leaf extracts of *L. nicotianifolia* was increased with the increasing magnitude of extraction parameters. These results corroborate previous studies showing that the higher levels of ultrasonic extraction parameters reduce the antioxidant potential. Such reduced antioxidant activity might be attributed to the degradation of antioxidants in the extracts [29,30,54]. The reduced % RSA in the ultrasonication-assisted leaf extracts of *L. nicotianifolia* may be due to the degradation and oxidization of phenolic compounds with increasing temperature and extraction duration [55]. In this study, antioxidant assays with DPPH and ABTS have shown different % RSA for the same extracts. The differences in the phenolic compounds extracted and their association with antioxidant assays may be responsible for such differences. These results support the observations reported by Giorgis et al. [56].

3.5. Verification of the model-predicted optimal conditions

The objective of this study was to achieve higher extraction of TPC and TFC and achieve higher % RSA in DPPH and ABTS assays. A combination of 75.25% methanol, 62.72 °C temperature, and 9.44 min extraction duration was the optimum combination to achieve maximum extraction of phenolics and flavonoids from the leaves of *L. nicotianifolia.*

The optimum condition was obtained by the simultaneous optimization of several response variables described by Derringer and Suich [57]. By following these optimum conditions, the predicted response values ($P = 0.05$) for TPC and TFC were 22.44 mg TAE/g dw and 18.64 mg QE/g dw, respectively. For % RSA in DPPH and ABTS antioxidant assay, the predicted values were 93.04% and 93.06%, respectively. Considering the values presented in Table 4, experiments were performed in triplicates to test the validity of the experimental input and output values.

From this experiment, higher TPC (23.78 348 mg TAE/g dw), TFC (20.21 mg QE/g dw), and % RSA in DPPH assay (95.78%) and ABTS assay (97.26%) was achieved (Table 4). Hence, the optimized UAE model can be reasoned to have the potential to extract TPC and TFC to a greater extent and subsequently a higher antioxidant activity.

3.6. Structural changes in plant tissues after extraction

UAE caused morphological changes in the leaves of *L. nicotianifolia* (Fig. 6a and b). The UAE-extracted leaves showed beaked, wrinkled, ruptured, and fragmented cell walls. UAE creates micro-pores and structural changes through cavitation in the plant samples, which leads to enhanced diffusion and washing out the cell contents [29,30,41,54].

3.7. FTIR analysis

ATR–FTIR spectroscopy is a rapid, non-invasive, and cost-effective method to analyze functional groups, structural information, and molecular features in crude extracts [47,58,59]. The FTIR spectra of the control leaf samples and those subjected to UAE are shown in Fig. 7. The control sample showed five peaks (3324.46, 1572.05, 1401.34, 1251.86, and 1045.46) whereas, leaves extracted with UAE showed fourteen peaks (3395.83, 2848.98, 2363.87, 1808.34, 1708.04, 1463.07, 1260.54, 1171.81, 1082.11, 1021.35, 862.22, 802.42, 719.48, and 668.36). The spectra for control (Fig. 7a) and UAE extracted leaves (Fig. 7b) show differences in the peak numbers, which might indicate a remarkable role the UAE has in extracting phytoconstituents.

4. Conclusion

In the present study, UAE of *Lobelia nicotianifolia* leaves, with three independent variables having three levels each, was used to optimize the yield of total phenolics (TPC) and flavonoids (TFC) and their subsequent antioxidant activity. Methanol percentage, temperature, and time seemed to be critical factors to obtain higher TPC and TFC yields and their bioactivity (% RSA in ABTS and DPPH assays). The ultra-sonication causes an extensive rupturing of the plant tissue. UAE might have reduced the chemical changes in the phenolics and flavonoids, which in turn might have contributed to the higher antioxidant potential of the extracts. Thus, the proposed optimized UAE seems to have better efficiency in extracting plant phenolics and flavonoids with improved antioxidant potential.

CRediT authorship contribution statement

Saurabha B. Zimare: Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization, Investigation, Supervision, Writing – review & editing. **Ganesh D. Mankar:** Conceptualization, Methodology, Software, Data curation, Writing – original draft, Software, Validation, Writing – review & editing. **Rajkumar B. Barmukh:** Software, Validation, Methodology, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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