Chapter 2

To identify the phytochemical contents of bhimkol (*Musa balbisiana*) banana blossom and optimization of their extraction using supercritical fluid extraction (SCFE) and ultrasound assisted extraction (UAE)

2.1 Introduction

In today's modernized world, livelihood is leading the population towards the profound change in terms of more good diet habits with the aspiration of more natural and healthy food products [16]. Research on bioactive compounds especially in phytochemicals in natural food products possessing high antioxidant activities and health beneficial properties has increased at a great pace [37]. Bhimkol blossom is mostly consumed in some Southeast Asian countries [8, 35] as one of the priority dish. According to ancient beliefs and prevailing literature, banana blossom possesses various medicinal properties for some disorders such as diabetes, cancer [26], bronchitis, constipation, ulcers, lactating disorder [19], dysentery, menorrhagia [14], diuretic problem and wound healing problem [23]. The sap is used to treat allergic reactions when there are bites and stung by insects [19]. Other blossom varieties e.g., *Musa paradisiaca*, *Musa acuminate* [28, 31], *Musa* sp. var. Nanjangud rasa bale [25], *Musa sapientum* [18] are reported to have rich bioactive compounds *viz*., polyphenols, terpenoids, alkaloids [2], steroids [19] etc. Phytochemicals in blossoms are also reported to possess some health beneficial properties such as anti-inflammation [18], anti-cancer property [32], anti-diabetic property [7] as discussed in previous Chapter 1. Nevertheless, banana blossoms are underutilized agricultural by-products of banana plants [33] and generally it is discarded after harvesting of banana fruits and utilization of other plant parts. Although banana blossom has been consumed as a food source since the ancient time, their consumption level is low because of less awareness of their edibility and their medicinal values [6]. Therefore it is very imperative to encourage highly in utilizing the unexplored food commodity in terms of environmental factors and health perspective.

The present study explores the better ways to extract phytochemical constituents by using ultrasound-assisted extraction (UAE), supercritical fluid extraction (SCFE), and conventional extraction method, in bhimkol banana blossom. This study has also focused on the impacts of extraction parameters on the antioxidant property and phytochemical compounds. As every vegetable has a different composition of phytochemicals which imparts their uniqueness [10] in terms of taste and medicinal properties, therefore, it is necessary to study the impact of extraction parameters on phytochemicals in addition to the techniques. The understanding of the effects of various techniques and optimized extraction conditions will lead to better quantification, effective application of

technologies and natural products in processing and examining of foods having maximum medicinal properties.

2.2 Material and methods

2.2.1 Chemicals and reagents

Chemicals and reagents used in the present study were of high purity analytical grade and the HPLC grade analytical standards and solvents were purchased from Sigma-Aldrich (USA).

2.2.2 Plant material

Bhimkol banana (*Musa balbisiana*) blossom (the inflorescence part) was collected from Tezpur University, Assam, India (latitude: 26.7005 and longitude: 92.8248). The sample was taxonomically identified in the Department of Botany, Gauhati University, India (Accession No.18999).

Fig. 2.1 Various parts of Bhimkol blossom (*Musa balbisiana*): (a) Bhimkol blossom (inflorescence), (b) Bract, (c) Male flowers, and (d) Spadics

Blossoms were washed with distilled water immediately after harvesting and separated into three major parts (Fig. 2.1); bract (Br), male flowers (MF), and spadics (Sp) for the evaluation of major phytochemicals. Parts were chopped into smaller pieces distinctly as Br, MF, Sp, and whole blossom (WB), and then dried in a hot air oven (Advantage lab, AL01-05-100, Belgium) at 45^{o}C for 24 h. Dried samples were ground to powder and stored in airtight containers at 4^{o}C for further analysis.

2.2.3 Methods for extraction of phytochemicals

2.2.3.1 Conventional solid-liquid extraction

Powder sample of Br, MF, Sp, and WB were mixed in an extraction solvent of 70% ethanol at 2:30 ratios in a 100 ml beaker, individually. Each sample solution was poured into 50 ml centrifuge tubes and centrifuged (Eppendorf, 5430R, Germany) at 10,000 rpm in 30^{o}C for 20 min by following method of Zhu et al. [39]. Supernatants were collected and the remaining portion was discarded. The supernatant of each sample was filtered using Whatman Number1 filter paper and the filtrate was allowed to evaporate using a rotary evaporator (IKA, RV10, India) at 40^{o}C. Dried sample materials were stored in an airtight container at 4^{o}C for further analysis.

2.2.3.2 Ultrasound-assisted extraction (UAE)

Powder of whole blossom was mixed in an extraction solvent of 70% ethanol at a 2:30 ratio in a 100 ml beaker. Following the Priyadharshini and Bakthavatsalam [24] and Manga et al. [21] methods with slight modification, the mixtures were ultrasonicated (Q Sonica, Q700, USA) with 220 Å probe (12.7 mm diameter) at frequency of 20 kHz as per the experimental design generated by RSM-CCD (Design Expert 11) as shown in Table 2.1. Extraction parameters *viz*., temperature (40 to 60^{o}C), extraction solvent (70%), extraction amplitude (25 to 45%), and extraction time (20 to 40 min) were selected. Temperature, amplitude, and extraction time were considered as independent variables, whereas the extraction solvent (70% ethanol), sample and solvent ratio (2:30) and at 75% duty cycle (15 s pulse on and 5 s pulse off) [13] were kept as an independent fixed variable. The total phenolic content (TPC) and antioxidant activity were taken as dependent variables. During the extraction process, probe was immersed 1.5 times the diameter of the tip and without touching the bottom of the solution in 100 ml beaker for better extraction.

After the complete extraction process, the supernatant of each sample was filtered using Whatman No.1 filter paper , and the filtrate was allowed to evaporate using a rotary evaporator at 40^{o}C. Dried sample materials were stored at 4^{o}C for further analysis.

Table 2.1 Coded response surface methodology-central composite design (RSM-CCD) design for ultrasonic assisted extraction (UAE) method

Run	Temperature (°C)	Amplitude (%)	Time (min)	TPC (mg GAE/100g)	Antioxidant activity (DPPH %)
1	-1.000	-1.000	-1.000	1750.26	81.98
2	1.000	-1.000	-1.000	1861.57	82.01
3	-1.000	1.000	-1.000	1852.6	75.69
4	1.000	1.000	-1.000	1956.6	77.32
5	-1.000	-1.000	1.000	1771.44	78.04
6	1.000	-1.000	1.000	1973.06	75.93
7	-1.000	1.000	1.000	1852.84	77.05
8	1.000	1.000	1.000	2050.13	76.54
9	-1.000	0.000	0.000	1853.13	76.72
10	1.000	0.000	0.000	1990.92	76.48
11	0.000	-1.000	0.000	1773.63	74.82
12	0.000	1.000	0.000	1875.45	71.98
13	0.000	0.000	-1.000	1830.55	77.79
14	0.000	0.000	1.000	1864.35	75.10
15	0.000	0.000	0.000	1832.13	74.22
16	0.000	0.000	0.000	1860.93	73.41
17	0.000	0.000	0.000	1848.65	74.52
18	0.000	0.000	0.000	1861.05	74.00
19	0.000	0.000	0.000	1842.43	75.40
20	0.000	0.000	0.000	1847.96	74.53

TPC, Total phenolic content; GAE, Gallic acid equivalent; DPPH, Diphenyl-picrylhydrazyl.

2.2.3.3 Supercritical fluid extraction (SCFE)

In phytochemical extraction by supercritical fluid, the SCFE chiller was set at 5^{o}C which took about 20 min to lower down up to the desired temperature (from room temperature, 25^{o}C). Cloth bags in the size of a tea sachet were prepared and then 2 g of whole blossom powder was put for the individual run and kept inside the extraction vessel of

the SCFE extraction system (Waters, SCF100, USA). SCFE system contains chiller (Julabo, FL601, Germany), supercritical fluid (CO_2) input device, pressure valves, back pressure regulators, heat exchanger, extraction chambers, and a computer set up where system data logger controlled the whole programs automatically. With the help of a pump, the flow of supercritical fluid and modifier fluid (ethanol) was regulated, where temperature, pressure, flow rates of CO_2 were controlled by the data logger in the system.

All the extraction parameters are categorized as independent variables, independent fixed variables, and dependent variables. The independent variables ranges were selected as 40 to 60^oC, 70 to 210 bar, 5 to 9 g/min, and 20 to 40 min for temperature, pressure, CO_2 flow rate and extraction time, respectively. Independent fixed variables selected were 70% ethanol as modifier fluid concentration and flow rate at 1.5 ml/min whereas, dependent variables for the study were TPC (mg GAE/100g) and antioxidant activity (%). The experimental design was prepared using RSM-CCD (Design Expert 11) (Table 2.2) by following the methods of Wang et al. [34] and Shi et al. [29]. After extraction of each run, the effluents were collected in 50 ml centrifuged tubes and then filtered using Whatman No.1 filter paper. After that whole filtrates of extract were evaporated separately by using a rotary evaporator at 40^oC. Each evaporated solute was stored in airtight glass vials separately at 4^oC for further dependent variable analysis.

Table 2.2 Coded response surface methodology-central composite design (RSM-CCD) design for supercritical fluid extraction (SCFE) method

Run	Temperature (°C)	Pressure (bar)	Time (min)	CO_2 flow rate (g/min)	TPC mg GAE/100g	Antioxidant activity (DPPH %)
1	-1.000	-1.000	-1.000	-1.000	1690.99	76.48
2	1.000	-1.000	-1.000	-1.000	2095.11	77.76
3	-1.000	1.000	-1.000	-1.000	1732.03	78.10
4	1.000	1.000	-1.000	-1.000	2321	78.82
5	-1.000	-1.000	1.000	-1.000	1759.57	77.22
6	1.000	-1.000	1.000	-1.000	2280.67	77.85
7	-1.000	1.000	1.000	-1.000	2028.41	79.34
8	1.000	1.000	1.000	-1.000	2750.37	79.41
9	-1.000	-1.000	-1.000	1.000	1743.09	77.55
10	1.000	-1.000	-1.000	1.000	2463.35	78.21
11	-1.000	1.000	-1.000	1.000	2010.96	78.51
12	1.000	1.000	-1.000	1.000	2937.08	78.62
13	-1.000	-1.000	1.000	1.000	1857.09	76.99
14	1.000	-1.000	1.000	1.000	2714.33	77.01
15	-1.000	1.000	1.000	1.000	2352.76	78.46
16	1.000	1.000	1.000	1.000	2949.86	77.92
17	-1.000	0.000	0.000	0.000	1678.93	77.73
18	1.000	0.000	0.000	0.000	2400.54	78.10
19	0.000	-1.000	0.000	0.000	1912.69	77.01
20	0.000	1.000	0.000	0.000	2282.47	78.28
21	0.000	0.000	-1.000	0.000	1855.86	76.69
22	0.000	0.000	1.000	0.000	2131.04	76.71
23	0.000	0.000	0.000	-1.000	1656.34	77.56
24	0.000	0.000	0.000	1.000	2008.64	77.35
25	0.000	0.000	0.000	0.000	1871.21	77.22
26	0.000	0.000	0.000	0.000	1899.9	77.54

27	0.000	0.000	0.000	0.000	1901.34	77.80
28	0.000	0.000	0.000	0.000	2403.12	76.78
29	0.000	0.000	0.000	0.000	1936.05	77.33
30	0.000	0.000	0.000	0.000	2001.78	77.10

TPC, Total phenolic content; GAE, Gallic acid equivalent; DPPH, Diphenyl-pecrylhydrazyl.

2.2.4 Extraction yield

According to Zohra et al. [40], extraction yields for both UAE and SCFE were calculated as the ratio of the weight of evaporated solute (W1) to the weight of the sample taken for extract (W2) as shown in Equation 1.

$$\% \text{ Yield} = \frac{W1}{W2} \times 100 \quad (1)$$

2.2.5 Determination of responses (TPC and antioxidant activity)

Determination of TPC

TPC was determined using Folin-Ciocalteau reagent as described by Ćetković et al. [11] with slight modification. The powder of the extracted sample was dissolved in ethanol at 1 mg/ml and the standard curve of gallic acid was prepared at 0.05 to 0.45 µg/ml concentrations individually in separate test tubes. From the sample and standard solutions along with blank, 0.5 ml was aliquoted separately in test tubes and then 2.5 ml of 1:10 Folin-Ciocalteau reagent (FCR) was mixed in each test tube. After three minutes of rest, 2 ml of 7.5% Na_2CO_3 was added to the mixture in the test tubes. The absorbance was read at 760 nm using a spectrophotometer (AG22331, Eppendorf, Germany) and TPC was expressed in gallic acid equivalent (mg GAE/100 g).

Determination of antioxidant activity

The antioxidant activity was estimated by DPPH inhibition assay as described by Al-Huqail et al. [3] with slight modification. A sample solution of sample extract and methanol at 1 mg/ml was prepared in a test tube. DPPH solution of 10^{-4} M (24 mg in 100 ml methanol) was prepared and absorbance was noted at 517 nm. Then 3 ml of DPPH solution was added in 100 µl of the sample solution and absorbance was again read at 517 nm. DPPH radical scavenging activity (antioxidant activity) was calculated as the

ratio of the difference between absorbance of DPPH solution ($A_{control}$) and absorbance of sample and DPPH mixture (A_{sample}) to the absorbance of DPPH solution (Equation 2).

$$\% \text{ Antioxidant activity} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad 2$$

2.2.6 Determination of phytochemicals

2.2.6.1 Phytochemicals estimation by the conventional method

2.2.6.1.1 Total flavonoid content (TFC)

Total flavonoid content was determined following Chang et al. [12] protocol with slight modification. The sample extract was dissolved in 95% ethanol at 20 µg/ml concentration in a test tube. Five standard solutions of catechin were prepared from 25 to 100 µg/ml concentrations in separate test tubes. From the sample and standard solutions along with blank, 0.5 ml was aliquoted and adjusted to 2 ml volume with 95% ethanol in each test tube. To each mixture, 0.1 ml of aluminum chloride solution (10%) was added and then 0.1 ml of 1 M potassium acetate was added. Each mixture was diluted with 2 ml distilled water and then incubated for 40 min at room temperature (25^{o}C) in a dark chamber. The absorbance was read at 415 nm and the TFC was expressed in catechin equivalent (mg CE/100g).

2.2.6.1.2 Tannin

Tannin content was determined by the Follin-Denis reagent method as described by Mahmood et al. [20]**.** Follin-Denis reagent was prepared by dissolving 100 g of sodium tungstate (Na_2WO_4) in 750 ml distilled water in 1000 ml beaker and then 20 g of phosphomolybdic acid and 50 ml of 85% phosphoric acid were added. The whole mixture solution was refluxed for 2 h and diluted up to 1000 ml after getting cooled. A saturated Na_2CO_3 solution was prepared by dissolving 8.75 g of anhydrous Na_2CO_3 in 25 ml of distilled water at 70^{o}C in a 250 ml conical flask. Na_2CO_3 solution was cooled overnight and used immediately on the experiment day. Five tannic acid standard solutions were prepared at 3-15 µg/ml in test tubes. The sample extract of 0.1 g was dissolved in 80 ml distilled water in a 250 ml conical flask and boiled for 30 min. After that, the mixture was diluted up to a mark and filtered using a Whatman No.1 filter paper in a conical flask. Then from the sample solution, five standard solutions (3-15 µg/ml)

along with a blank, 2.5 ml was aliquoted and adjusted to 4 ml volume with distilled water in 20 ml test tubes. Each mixture was added with 1.25 ml of Follin-Denis reagent and then 2.5 ml of Na_2CO_3 solution was added. After 30 min of incubation in a dark chamber, the absorbance was recorded at 760 nm.

2.2.6.1.3 Phytate

The determination of phytate content was done by following the Sivakumaran and Kothalawala [30] method, where 0.2 g of sample extract was dissolved in100 ml of 2% HCl for 3 h in a 250 ml conical flask and then filtered by using Whatman No.1 filter paper in a 250 ml conical flask. After that 50 ml of that filtrate was added with 10 ml distilled water and 10 ml 0.3% ammonium thiocyanate. The mixture was titrated with $FeCl_3$ solution (5.8 g $FeCl_3$ in 100 ml of 3% $C_2HCl_3O_2$) until the final solution turned slightly yellowish. The titrated volume was noted and analyzed. All the experiments were carried out in triplicate to minimize error. The phytate (%) was calculated by the formula (y x 1.19 x 100) where y is the multiplication of titer value and 0.00195 (concentration of iron (III) in g/ml).

2.2.6.1.4 Cyanogenic glycosides

At first alkaline picrate solution was prepared by mixing 25 g of sodium carbonate and 5 g picric acid in 1000 ml distilled water in a 1000 ml beaker. By following AOAC [5] protocol, Whatman No.1 filter paper of 12 cm long and 0.5 cm wide was saturated with alkali picrate solution for 2 hr in a 200 ml beaker. A standard solution of potassium cyanide was prepared by mixing 5 ml of alkaline picrate solution with 5 ml potassium cyanide (100 µg/ml) in a 100 ml conical flask and heated in boiling water for 5 min. Five concentrations with aliquot 0.1 to 1 ml standard solutions were diluted up to 10 ml with distilled water in 20 ml test tubes and closed with a stopper. After cooling down of the test tubes, the absorbance was noted at 625 nm. Then, sample extract of 0.1 g was mixed by magnetic stirrer at 1000 rpm in 25 ml distilled water with 3 to 4 drops of chloroform in a 500 ml conical flask. The saturated filter paper was hanged with the help of a cork stopper inside a mixed sample containing a conical flask. Then it was incubated for 24 h at room temperature (25^oC). If hydrocyanic acid is present in the sample then the saturated filter paper placed in it is reduced to reddish. The color formed in the filter

paper was eluted in a test tube containing 10 ml distilled water and comparison was carried out with a standard solution in 625 nm.

2.2.6.1.5 Alkaloids

Alkaloid content was determined by the α-solanine standard as a control as described by AOAC [5]. Standard solanine solution was prepared by adding 0.2 to 3 mM α-solanine in 96% ethanol-20% H_2SO_4 mixture (1:1) in test tubes. The sample extract was dissolved in 5% acetic acid at 20 ml/g in a 100 ml beaker and filtered through a muslin cloth in a 100 ml conical flask. The filtrate was heated in a shaking water bath (Equitron, 8406, India) at 70^oC for 15 min without agitation (or 0 rpm). Concentrated NH_4OH was added dropwise till the pH 10 was obtained and then centrifuged at 1000 rpm for 10 min to discard the supernatant. Residue collected after discarding supernatant was washed with 1% NH_4OH and re-centrifuged at the same condition as before. Again residue (crude solanine) was collected after discarding the supernatant. Then crude solanine obtained was oven-dried at 50^oC and then weighed. For the control, a standard α-solanine of known quantity (50 mg) and 50 mg crude solanine obtained was dissolved separately in a mixture of 96% ethanol and 20% H_2SO_4 (1:1). Both the mixtures were aliquoted at 1 ml in 5 ml of 60% H_2SO_4 separately. After 5 min, 5 ml formaldehyde reagent (0.5% in 60% H_2SO_4) was added and allowed to stand for 3 h at room temperature, and finally, the absorbance was noted at 570 nm against the blank solution.

2.2.6.2 HPLC analysis

RP-HPLC (Waters, 2690, US) gradient elution was performed to detect phytochemicals present and compared among the three samples extracts (conventional extraction method, UAE and SCFE) with an ultraviolet detector (Waters, 2489, US) where non-polar stationary phase column (Symmetry 300^{TM} C18-4.3 x 250 mm, 5 µm) was used with a binary pump (Waters, 1525, US). Powdered sample extracts from conventional extraction, UAE, and SCFE were dissolved in methanol at 1 mg/ml concentrations in separate clean glass vials and filtered through 0.2 µm Whatman syringe filter (nylon) in sterilized vials separately. Filtrate samples were collected in small glass vials and labeled. Similarly, phytochemical analytical standards (*viz*., chlorogenic acid, syringic acid, tannic acid, saponin, ferulic acid, caffeic acid, rutin, quercetin, catechin, gallic acid,

sinapic acid, coumarin) were prepared at three concentration each from 0.2 to 1 mg/ml (in methanol) separatley.

Following the method of Saikia et al. [28], acidified Milli-Q water (0.1% acetic acid, pH 3.2) was used as a mobile phase A and methanol as mobile phase B in gradient mode as 80% A (0-8 min), 65% A (9-12 min), 45% A (13-16 min), 30% A (17-20 min), 20% A (21-30 min), 10% A (31-40 min) and finally washing the column with 80% A (41-50 min). Prepared samples and standards were injected at 20 µl in the HPLC injector using a glass syringe at a flow rate of 0.8 ml/min and read at 254 nm in triplicate. Quantification of all detected phytochemicals in sample was obtained through the linear regression with their respective standard phytochemicals.

2.2.6.3 Determination antioxidant activity in major phytochemicals detected

Three major phytochemicals detected in HPLC will be evaluated for their antioxidant activities by DPPH method as described above *2.5*.

2.2.7 Statistical analysis and optimization of extraction

Optimizations of extractions using UAE and SCFE were done by the RSM-CCD face-centered and implementation of two-way ANOVA in a quadratic equation by determination of dependent variables (TPC and antioxidant activity) as shown in Table 2.1 and Table 2.2. Following the method of Shi et al. [29], the total numbers of experiments for Optimization in UAE for three independent variables were twenty experimental runs whereas for SCFE it was thirty experiments of four independent variables. To minimize the uncontrolled variance effect, the experiments were performed randomly. For estimation of the experimental error, each level of independent parameters was performed in three levels as -1, 0, and +1 for face-centered central points. The equation for the polynomial model was expressed in the quadratic process.

After analyzing the significance of the model by ANOVA, the experimental runs were repeated to obtain a fit model (significant model with p-value ≤ 0.05) and to obtain the desired correlation coefficient (R^2) in the entire process. The response surface of each parameter was studied. After analyzing both dependent parameters, optimization was done by setting the independent parameters to “in-range” option and “maximum” range for dependent parameters in RSM. After setting the both ranges, the optimized condition

for each dependent parameter were noted. All the quantification experiments were performed in triplicate and standard deviations were noted to avoid error in the result. All the quantifications were expressed in mean±standard deviation. Mean±standard deviation values with different superscript letters are significant differences ($p<0.05$) analyzed by ANOVA in SPSS (IBM SPSS Statistics 20).

2.3 Results and discussion

2.3.1 Extraction yield

In the extraction of phytochemicals in bhimkol blossom, the extraction yield percentage obtained by SCFE, UAE, and conventional methods were 10.10±0.65%, 9.8±0.13%, and 7±0.89%, respectively. Extraction yield variations might be attributed to the different extraction efficiencies and breakdown of phytochemicals and the present results are in line with the extraction yields obtained by Zohra et al. [40] for *Dysphania ambrosioides* (L.) extract.

2.3.2 Optimization and influence of extraction parameters on responses

During the extraction of phytochemicals by UAE and SCFE, the RSM-CCD face-centered was performed to study the effect of extraction parameters (*viz*., temperature, pressure, CO_2 flow rate, extraction time in SCFE and temperature, amplitude, and extraction time in UAE) on responses (TPC and Antioxidant activities) as presented in Table 2.1. The R^2 was obtained at a significant p-value <0.05 after analyzing the quadratic model fit by ANOVA for each response and regression coefficient. A significant model and non-significant lack of fit with the R^2 higher than 80% were obtained and shown in Tables 2.3, 2.4, 2.5, and 2.6.

Table 2.3 ANOVA table (Quadratic model) of response 1 (TPC) for ultrasound assisted extraction (UAE)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.032E+05	9	11467.64	107.99	< 0.0001	significant
A-Temperature	56551.90	1	56551.90	532.53	< 0.0001	
B-Frequency	20945.27	1	20945.27	197.23	< 0.0001	
C-Time	6772.49	1	6772.49	63.77	< 0.0001	
AB	16.94	1	16.94	0.1595	0.6980	
AC	4213.62	1	4213.62	39.68	< 0.0001	
BC	189.15	1	189.15	1.78	0.2116	
A^2	12917.05	1	12917.05	121.64	< 0.0001	
B^2	2304.71	1	2304.71	21.70	0.0009	
C^2	100.31	1	100.31	0.9446	0.3540	
Residual	1061.95	10	106.19			
Lack of Fit	445.57	5	89.11	0.7229	0.6348	not significant
Pure Error	616.37	5	123.27			

At R^2 = 0.9898; Predicted R^2 =9615; Adjusted R^2 = 9806; Coefficient of variation (%CV) = 0.5518; Standard deviation = 10.31.

Table 2.4 ANOVA table (Quadratic model) of response 2 (antioxidant activity) for ultrasound assisted extraction (UAE)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	117.29	9	13.03	57.29	< 0.0001	significant
A	0.144	1	0.144	0.633103	0.4447	
B	20.164	1	20.164	88.65196	< 0.0001	
C	14.71369	1	14.71369	64.68942	< 0.0001	
A2	15.43463	1	15.43463	67.85905	< 0.0001	
B2	1.898627	1	1.898627	8.347402	0.0161	
C2	13.48105	1	13.48105	59.27004	< 0.0001	
AB	1.28	1	1.28	5.627579	0.0391	
AC	2.2898	1	2.2898	10.06721	0.0099	
BC	14.045	1	14.045	61.74949	< 0.0001	
Residual	2.274513	10	0.227451			
Lack of Fit	0.0878	5	0.0176	0.0401	0.9985	not significant
Pure error	2.19	5	0.4373			

At R^2 = 0.9810; Predicted R^2 = 0.9721; Adjusted R^2 = 0.9639; Coefficient of variation (%CV) = 0.6261; Standard deviation = 0.4769.

Table 2.5 ANOVA table (Quadratic model) of response 1 (TPC) for supercritical fluid extraction (SCFE)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.672E+06	14	2.623E+05	13.29	< 0.0001	significant
A-Temperature	2.039E+06	1	2.039E+06	103.32	< 0.0001	
B-Pressure	4.506E+05	1	4.506E+05	22.83	0.0002	
C-Time	2.166E+05	1	2.166E+05	10.98	0.0047	
D-CO2	4.118E+05	1	4.118E+05	20.87	0.0004	
AB	6865.37	1	6865.37	0.3479	0.5641	
AC	209.74	1	209.74	0.0106	0.9193	
AD	46717.58	1	46717.58	2.37	0.1447	
BC	13294.67	1	13294.67	0.6736	0.4246	
BD	13649.83	1	13649.83	0.6916	0.4187	
CD	4235.73	1	4235.73	0.2146	0.6498	
A^2	33893.45	1	33893.45	1.72	0.2097	
B^2	76845.82	1	76845.82	3.89	0.0672	
C^2	12012.16	1	12012.16	0.6087	0.4474	
D^2	22346.08	1	22346.08	1.13	0.3041	
Residual	2.960E+05	15	19735.57			
Lack of Fit	93124.26	10	9312.43	0.2295	0.9771	not significant
Pure Error	2.029E+05	5	40581.85			

At R^2 = 0.9254; Predicted R^2 = 0.7732; Adjusted R^2 = 0.8558; Coefficient of variation (%CV) = 6.62; Standard deviation = 140.48.

Table 2.6 ANOVA (Quadratic model) of response 2 (Antioxidant activity) for supercritical fluid extraction (SCFE)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	16.25	14	1.16	27.42	< 0.0001	significant
A-Temperature	0.6124	1	0.6124	14.47	0.0017	
B-Pressure	7.19	1	7.19	169.99	< 0.0001	
C-Time	0.0016	1	0.0016	0.0379	0.8482	
D-CO2	0.2048	1	0.2048	4.84	0.0439	
AB	0.3108	1	0.3108	7.34	0.0161	
AC	0.4193	1	0.4193	9.91	0.0066	
AD	0.3752	1	0.3752	8.86	0.0094	
BC	0.2525	1	0.2525	5.97	0.0274	
BD	0.4258	1	0.4258	10.06	0.0063	
CD	1.67	1	1.67	39.47	< 0.0001	
A^2	1.17	1	1.17	27.65	< 0.0001	
B^2	0.4189	1	0.4189	9.90	0.0067	
C^2	0.7636	1	0.7636	18.04	0.0007	
D^2	0.1166	1	0.1166	2.75	0.1178	
Residual	0.6349	15	0.0423			
Lack of Fit	0.0097	10	0.0010	0.0078	1.0000	not significant
Pure Error	0.6251	5	0.1250			

At R^2 = 0.9624; Predicted R^2 = 0.9530; Adjusted R^2 = 0.9273; Coefficient of variation (%CV) = 0.2447; Standard deviation = 0.2057.

According to Saikia et al. [28], a significant lack of fit implies inaccuracy of experimental data leading to failure of the model used. In the present study the quadratic model in the polynomial process was obtained significant, therefore, further experiments were performed for optimization. At maximum ranges of responses (dependent variables) in the optimization step, 3D responses of various extraction parameters over the dependent variables were noted (Fig. 2.2 and 2.3).

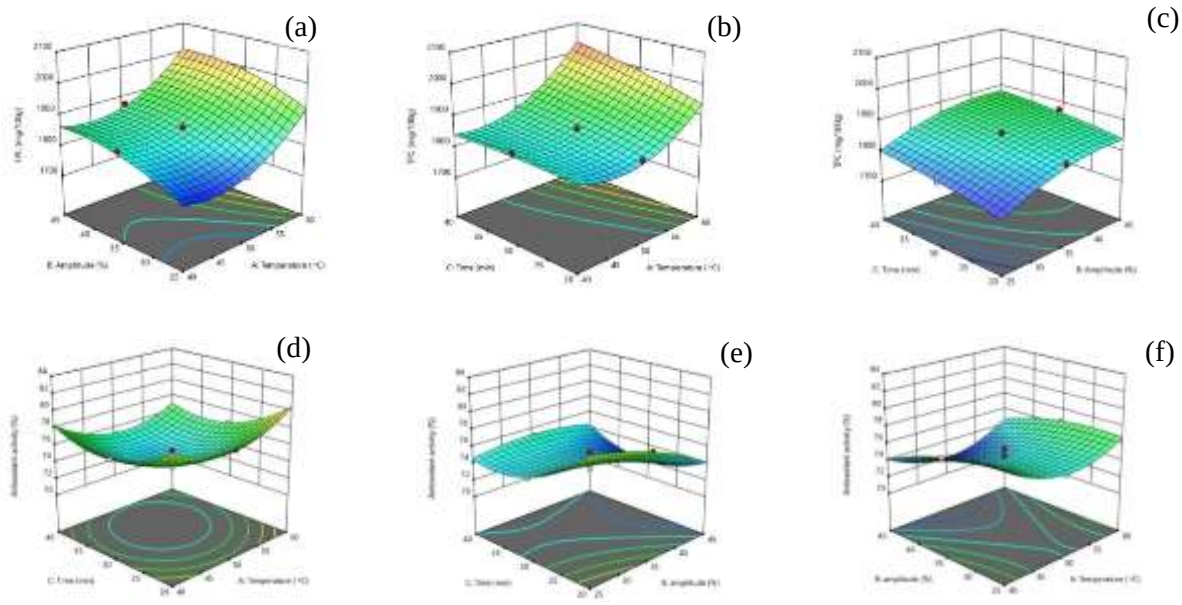

Fig. 2.2 Response surface methodology (RSM) graphs for ultrasound assisted extraction (UAE); (a) Amplitude-temperature (AB) for TPC, (b) Temperature-time (AC) for TPC, (c) Amplitude-time (BC) for TPC, (d) Amplitude-temperature (AB) for Antioxidant activity (e) Temperature-time (AC) for Antioxidant activity (f) Amplitude-temperature (AB) for Antioxidant activity

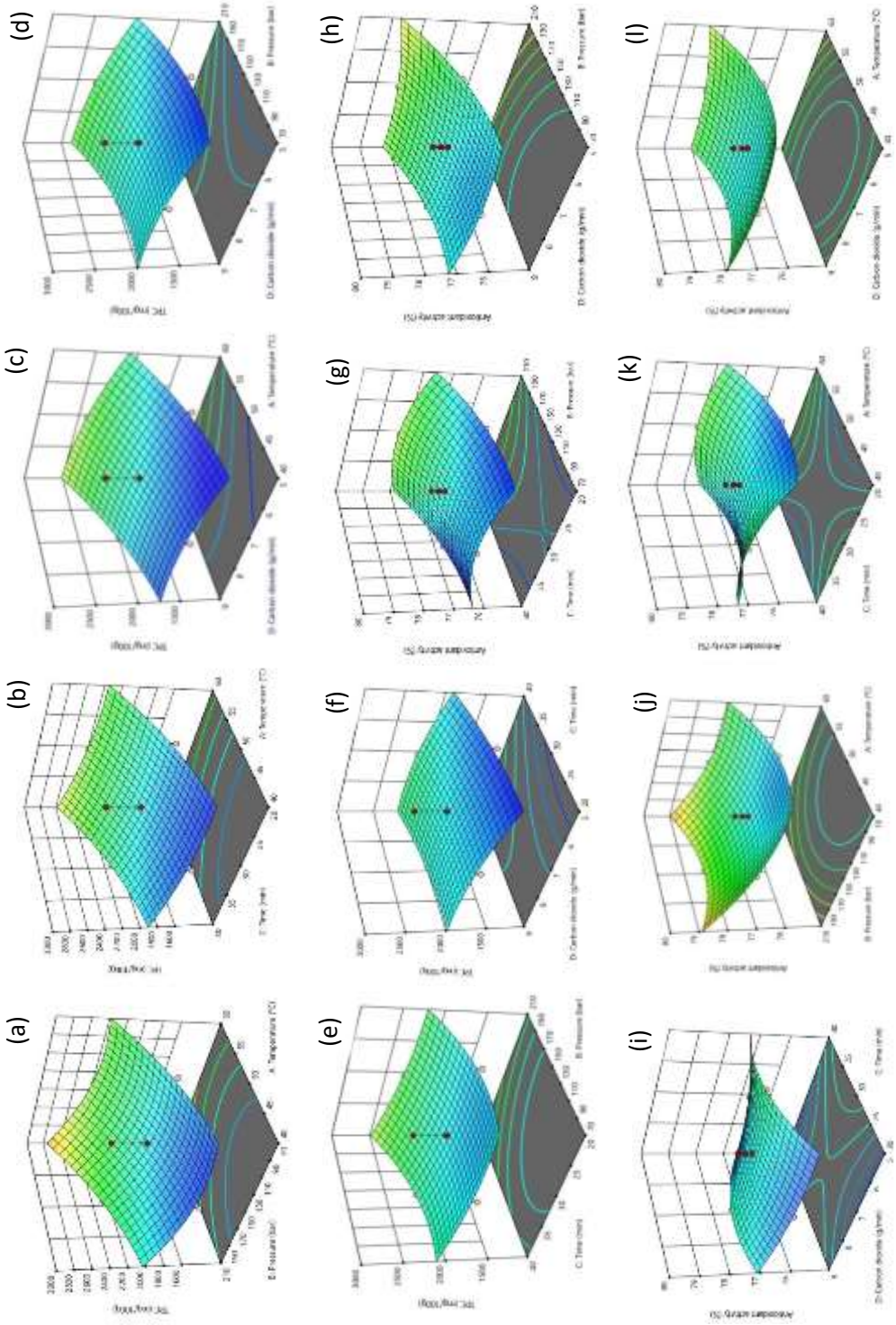

Fig. 2.3 Response surface methodology (RSM) graphs for supercritical fluid extraction(SCFE); (a) Temperature-pressure (AB) for TPC, (b) Temperature-time (AC) for TPC, (c) Temperature-carbon dioxide(AD) for TPC, (d) Pressure-time (BC) for TPC, (e) Pressure-Carbon dioxide (BD) for TPC, (f) Time-carbon dioxide (CD) for TPC, (g) Temperature-pressure (AB) for antioxidant activity, (h) Temperature-time (AC) for antioxidant activity, (i) Temperature-carbon dioxide(AD) antioxidant activity, (j) Pressure-time (BC) for antioxidant activity, (k) Pressure-Carbon dioxide (BD) for antioxidant activity, and (l) Time-carbon dioxide (CD) for antioxidant activity

2.3.2.1 Ultrasound-assisted extraction (UAE)

In phytochemical extraction by UAE, independent parameters *viz*., temperature, amplitude and time were coded as A, B and C, respectively. From the 3D responses (Fig. 2.2 and coded Equations 3 and 4), at fixed variable of 70% ethanol extraction solvent, increased temperature, time and amplitude evinced higher TPC response. The effect of each independent parameter (*viz*., temperature, time, and amplitude) was different. Temperature and time exhibited the highest positive effect while amplitude and time showed the highest negative effect on TPC. The rising of temperature enhances the solubility of phytochemicals, however, higher temperature (above 80^{o}C) might degrade certain phenolic compounds [36].

$$Y_{TPC} = +1850.71 + 75.20\,A + 45.77\,B + 26.02\,C - 1.46\,AB + 22.95\,AC - 4.86\,BC + 68.54\,A^2 - 28.95\,B^2 - 6.04\,C^2 \quad (3)$$

$$Y_{Antioxidantactivity} = +74.30 - 0.1200\,A - 1.42\,B - 1.21\,C + 0.4000\,AB - 0.5350\,AC + 1.33\,BC + 2.37\,A^2 - 0.8309\,B^2 + 2.21\,C^2 \quad (4)$$

In case of antioxidant activity, the effect of time and amplitude (BC) exhibited a higher positive effect (Equation 4 and Fig. 2.2). A lower time with increased temperature gave higher antioxidant activity. According to Golmohamadi et al. [15], the antioxidant property is much sensitive to extended extraction time with higher extraction frequency, and beyond it which might degrade the antioxidant releasing compound(s). In contrary to that, it was recorded that the maximum antioxidant compounds were released at maximum temperature with lesser extraction time (within the parameters performed in this study). Similar effects of parameters were also reported by Boungo Teboukeu et. al. [9] and Golmohamadi et al. [15] which corroborates the UAE extraction method for increase of antioxidant activity up to 60^{o}C in limited extraction time. The optimized condition for phytochemical extraction by UAE was obtained at 60^{o}C temperature, 35% amplitude, and 20 min of extraction time in both dependent responses (TPC and antioxidant activity).

2.3.2.2 Supercritical fluid extraction (SCFE)

In SCFE, the independent parameters *viz*., temperature, pressure, CO_2 flow rate and time were coded as A, B, C and D, respectively. The solvent concentration (70% ethanol) and

flow rate of solvent were taken as fixed variables. Each independent parameter (*viz*., temperature, pressure, time, and CO_2 flow rate) exhibited a different effect on TPC response. From the 3D response (Fig. 2.3), it can be seen that increased pressure and temperature showed higher TPC response, while temperature and time (AD) exhibited the highest positive effect on TPC (Equation 5). Overall, the TPC response increased with the increasing pressure, temperature, time, and CO_2 flow rate whereas the combinatorial negative effect of time and CO_2 flow rate (CD) on TPC (Equation 5) was noted. Among all independent parameters, temperature (A) showed a maximum impact on the TPC response, and the similar results were reported on *Prunus persica* extract by Mezzomo et al. [22].

$$Y_{TPC} = +1963.80 + 336.58\,A + 158.22\,B + 109.70\,C + 151.26\,D + 20.71\,AB + 3.62\,AC + 54.04\,AD + 28.83\,BC + 29.21\,BD - 16.27\,CD + 114.38A^2 + 172.22\,B^2 + 68.09\,C^2 - 92.87\,D^2 \quad (5)$$

$$Y_{Antioxidant\ activity} = +77.27 + 0.1844\,A + 0.6322\,B + 0.0094\,C - 0.1067\,D - 0.1394\,AB - 0.1619\,AC - 0.1531\,AD + 0.1256\,BC - 0.1631\,BD - 0.3231\,CD + 0.6721\,A^2 + 0.402\,B^2 - 0.5429\,C^2 + 0.2121\,D^2 \quad (6)$$

In 3D response of antioxidant activity (Fig. 2.3), the pressure and temperature combination revealed the highest antioxidant activity. The highest positive effect was exhibited by temperature and CO_2 flow rate (AD) on the other hand the most negative effect by time and CO_2 flow rate (CD) on antioxidant activity (Equation 6). Among all independent parameters, pressure showed the most desirable effect. Similar results were also reported by Yi et al. [38] that the antioxidant activity decreases with higher extraction pressure and temperature (above 80^oC) however, TPC increases.

Table 2.7 Observed and the predicted value of responses TPC and antioxidant activities for ultrasound assisted extraction (UAE) and supercritical fluid extraction (SCFE) method in response surface methodology (RSM)

Extraction methods	Response	Observed value	Predicted value	SD	SE
UAE	TPC (mg GAE/100g)	1898.20	1938.29	10.30	3.54
	Antioxidant activity (%)	80.13	80.56	0.47	0.16
SCFE	TPC (mg GAE/100g)	2750.37	2665.05	140.48	114.01
	Antioxidant activity (%)	79.41	79.40	0.20	0.16

TPC, Total phenolic content; GAE, Gallic acid equivalent. SD, Standard deviation; SE, Standard error.

The validation of optimized conditions was noted as 60^{o}C temperature, 210 bar pressure, 40 min, and 5 g/min CO_2 flow rate for both responses. Observed values at the optimized conditions for UAE and SCFE methods are given in Table 2.7 along with the predicted values.

2.3.3 Comparison of extraction methods on the recovery of TPC and antioxidant activities

The three extraction methods *viz*., conventional extraction method, UAE, and SCFE, were compared based on extraction yield, dependent responses obtained (TPC and antioxidant activity), and extraction time. The results revealed that the SCFE extraction method is more efficient based on extraction yield, TPC, and antioxidant activities (Fig. 2.4).

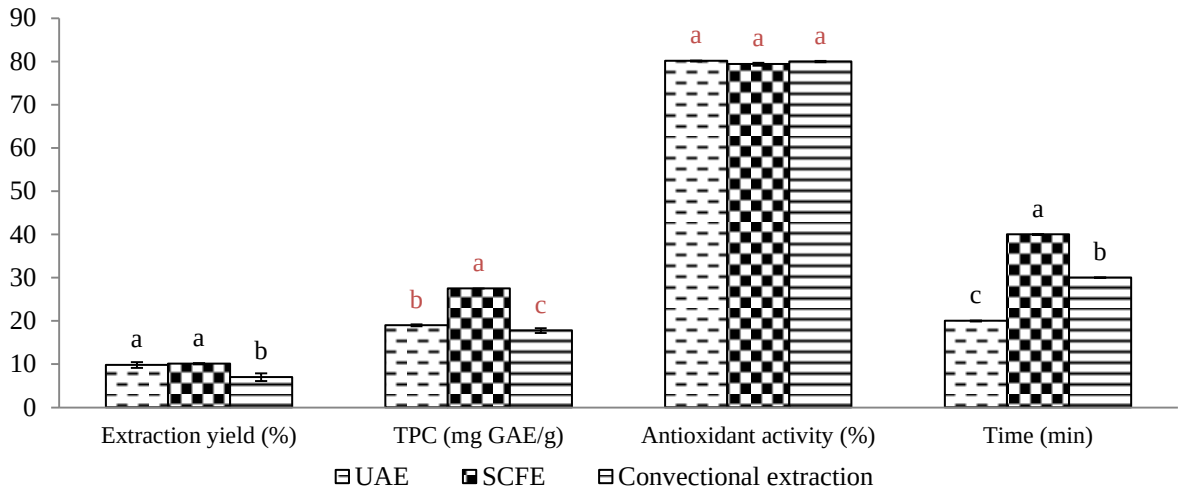

Fig. 2.4 Comparative bar diagram of conventional extraction, ultrasound assisted extraction (UAE), and supercritical fluid extraction (SCFE) with respect to extraction yield, TPC, antioxidant activity and extraction time. Error bar with different letters are significant different values (p<0.05)

As compared to SCFE the UAE method is more economic and more accessible. Nonetheless, the extraction efficiency of SCFE is much better than UAE. In the three extraction methods, solvent:sample ratio was kept as fixed parameter as it was reported for its positive effects on the dependent responses obtained (TPC and antioxidant activity) by Castro-López et al. [10]. Other than 70 % ethanol (extraction solvent) in all three extraction methods used (SCFE, UAE and conventional extraction method), SCFE uses CO_2 gas as main extraction solvent which makes more costlier than other extraction methods (UAE and conventional extraction method) performed in this study. On the basis of energy consumption also, the hierarchy of energy consumption increases as UAE<conventional extraction method<SCFE [4]. According to specifications of centrifuge (Eppendorf, 5430R, Germany), energy consumption goes upto 1650 W to raise 14000 rpm in room temperature. UAE system (Q Sonica, Q700, USA) consumes 700 W to raise 20kHz, 100% amplitude at room temperature for probe 220 Å (12.7 mm diameter). According to Waters SCFE product specification [17], energy consumption of SCFE system is moderate, 4.931 MWh/day at maximum temperature.

2.3.4 Phytochemical determination by conventional methods

Most phytochemicals present in the plant samples can be directly related to the original color of the fresh sample [27]. Plant samples with higher polyphenol contents are mostly seen as a darker color. In the phytochemical estimation of all four major parts of bhimkol banana plant; WB (whole bhimkol blossom), Br, MF, and Sp, the majority of the phytochemical contents were found in the WB. The flavonoids, TPC, tannin, and phytate obtained in the WB were 333.20±0.33 mg CE/100g, 1870.21±0.55 mg GAE/100g, 14.3±0.00 mg TAE/100g, 46.90±0.07 mg phytic acid/100g, respectively. On the other hand, the cyanogenic glucosides, and alkaloids content were 0.0001±0.00%, and 2.78±0.33 mg/100g, respectively (Table 2.8). The lowest phytochemical contents were found in the innermost part of the blossom i.e. Sp.

Table 2.8 Phytochemical evaluation of major parts of bhimkol blossom using a conventional method of determination

Bhimkol blossom parts	TFC (mg CE/100g)	TPC (mg GAE/100g)	Tannin (mg TAE/100g)	Phytate (mg phytic acid/100g)	Cyanogenic glycosides (%)	Alkaloids (mg/100g)
WB	333.20± 0.33^{a}	1870.21± 0.55^{a}	14.3± 0.00^{c}	46.90± 0.07^{b}	0.0001± 0.00^{a}	2.78± 0.33^{b}
Br	243± 0.97^{b}	1610.66± 0.10^{b}	11.5± 0.75^{d}	47.39± 1.12^{b}	ND	3.81± 0.02^{a}
Mf	214± 1.40^{c}	830.84± 0.00^{c}	15.2± 0.55^{b}	29.33± 1.20^{c}	ND	2.13± 0.15^{bc}
Sp	202± 0.45^{d}	822.48± 0.14^{d}	16.63± 0.03^{a}	30.03± 0.15^{a}	ND	1.89± 0.47^{bc}

WB, Whole blossom; Br, Bract; MF, Male flower; Sp, Spadics; TFC, Total flavonoid content; TPC, Total phenolic content; CE, Catechin equivalent; GAE, Gallic acid equivalent; TAE, Tannic acid equivalent; ND, Not detected; Data presented as mean ± standard deviation (n=3); Values in the same column with different superscript letters are significantly different (p<0.05).

The outermost part of the blossom (Br) showed the highest content of phytochemicals as compared to the innermost parts of the blossom (MF and Sp). The color of the banana blossom from the innermost part to the outermost layer was equally distributed from lightest cream color to purple color (Fig. 2.1).

2.3.5 Phytochemical determination by HPLC at optimized condition

In RP-HPLC (reverse-phase high-performance liquid chromatography), twelve phytochemical analytical standards (*viz*., chlorogenic acid, syringic acid, tannic acid, saponin, ferulic acid, caffeic acid, rutin, quercetin, catechin, gallic acid, sinapic acid, and coumarin) were run first and retention time was recorded at 254 nm (Table 2.9). According to Abdel-Hameed et al. [1], sharp phytochemicals chromatograms along with many derivatives can be observed between 230 to 360 nm. Likewise, a good peak of phytochemicals was eluted at 254 nm at different retention times.

Table 2.9 Retention time of phytochemical standards in RP-HPLC at 254 nm

Sl. No.	Phytochemical standards	Retention time (in min)
1	Chlorogenic acid	13.56
2	Syringic acid	16.09
3	Tannic acid	17.26
4	Saponin	18.71
5	Ferulic acid	18.46
6	Caffeic acid	15.42
7	Rutin	19.18
8	Quercetin	22.78
9	Catechin	9.73
10	Gallic acid	4.83
11	Sinapic acid	18.36
12	Coumarin	18.21

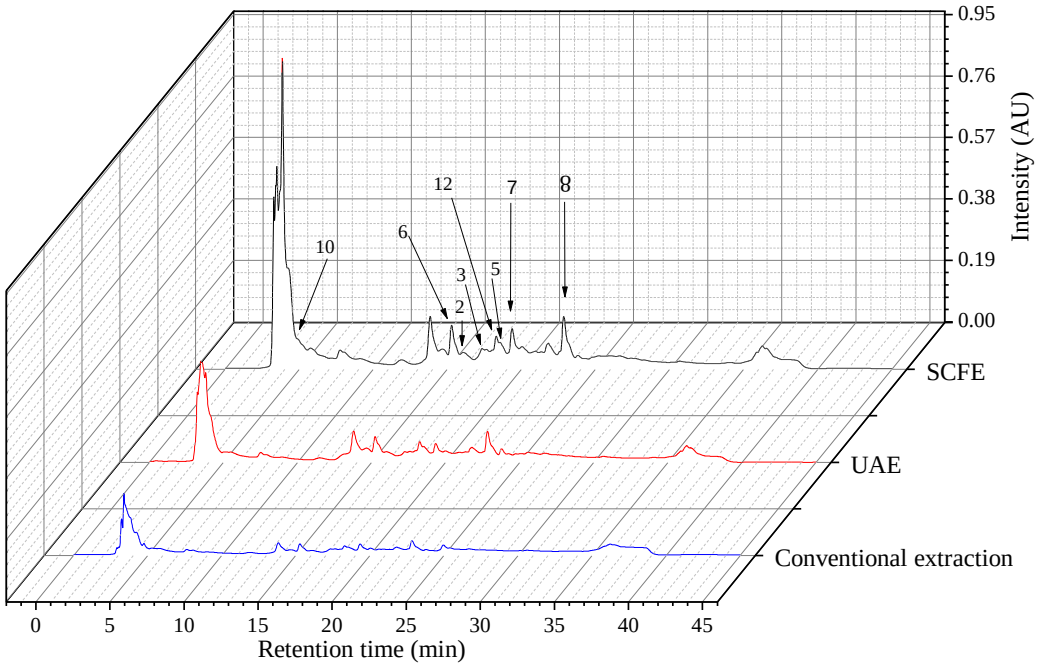

Fig. 2.5 RP-HPLC Chromatograms of phytochemical compounds detected of whole bhimkol blossom extracts from conventional extraction, ultrasound assisted extraction (UAE), and supercritical fluid extraction (SCFE) at 254 nm. (2-syringic acid, 3- tannic acid, 5-ferulic acid, 6-caffeic acid, 7-rutin, 8- quercetin, 10-gallic acid, 12-coumarin)

HPLC chromatograms of phytochemical analysis of extracts by UAE, SCFE, and conventional extraction methods are represented in Fig. 2.5. The retention time of each peak was compared with the standard phytochemicals and analyzed. Major peaks identified in the chromatograms of all extracts were *viz*., caffeic acid, rutin, quercetin, gallic acid, and coumarin at 254 nm (Fig. 2.5). Quercetin was observed at the highest peak at a retention time of 22.82 min. An unidentified peak was observed at 18.25 min retention time which might be a derivative of phytochemical compound present in the sample extracts. Phytochemicals identified from SCFE extract was seen with higher peaks (higher intensities) than other extracts (UAE and conventional extraction) at 254 nm. During quantification of phytochemicals in extracts from all three extraction methods (UAE, SCFE and conventional extraction method), quercetin was found to be in major quantity among other phytochemicals detected (Table 2.10). Quercetin was recorded as the highest (303.39±0.01 mg/100g) in SCFE extract as compared to the

remaining methods and the concentration of quercetin was found in the order of SCFE>UAE>conventional extraction method.

Table 2.10 Phytochemical contents detected by HPLC in bhimkol blossom extracts from supercritical fluid extraction (SCFE), ultrasound assisted extraction (UAE) and conventional extraction method

Phytochemicals detected by HPLC	SCFE (mg/100g)	UAE (mg/100g)	Conventional extraction method (mg/100g)
Syringic acid	21.75±0.02^{a}	19.59±0.40^{b}	19.04±0.04^{c}
Tannic acid	27.19±0.02^{a}	11.07±0.03^{b}	8.04±0.04^{c}
Ferulic acid	16.05±0.03^{a}	15.82±0.14^{b}	15.69±0.18^{b}
Caffeic acid	20.70±0.04^{a}	19.15±0.04^{b}	17.97±0.07^{c}
Rutin	23.23±0.05^{a}	20.70±0.10^{b}	20.26±0.09^{c}
Quercetin	303.39±0.01^{a}	285.58±0.10^{b}	273.22±0.03^{c}
Gallic acid	0.06±0.00^{a}	ND	ND
Coumarin	12.75±0.01^{a}	11.94±0.04^{b}	10.56±0.07^{c}

ND, Not detected; Data presented as mean ± standard deviation (n=3); Values in the same row with different superscript letters are significantly different (p<0.05).

2.3.6 Antioxidant activity of major phytochemicals detected by HPLC

Evaluation of antioxidant activities of three major phytochemicals detected *viz*., quercetin, rutin and syringic acid in bhimkol blossom extracts from SCFE, UAE and conventional extraction method were done. Antioxidant activity (%) evaluated by DPPH inhibition assay of quercetin, rutin and syringic acid are illustrated in the Fig. 2.6. Antioxidant activity shown by bhimkol blossom extract might be due to the presence of rutin, quercetin, syringic acid and along with other phytochemicals detected.

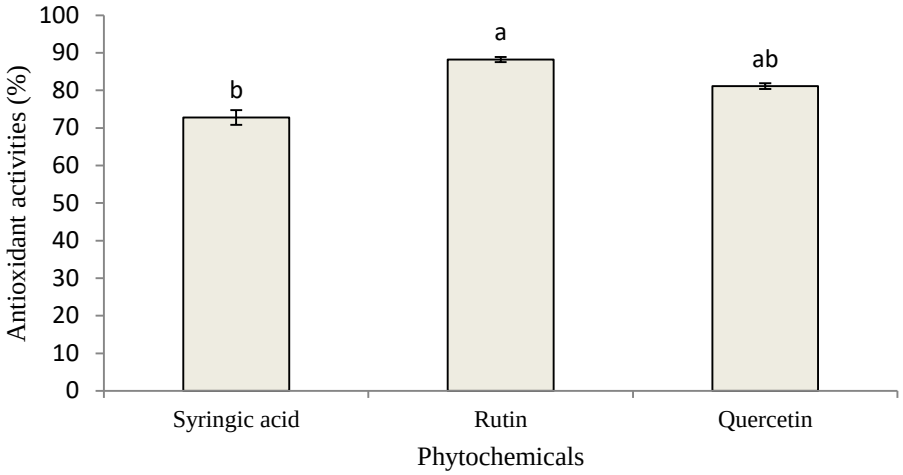

Fig. 2.6 Antioxidant activities of major phytochemicals detected in HPLC. Error bar with different letters are significant different values (p<0.05)

2.3.7 Plausible reasons for different phytochemical values

The results revealed that the identification and estimation of phytochemicals depend on the various extraction parameters. Although the whole phytochemical composition of plant species depends on the growing environment and condition, on the other hand, the estimation of phytochemicals and their decomposition depend on sample preparation and detection method. As stated in 3.2.1, 3.2.2, and 3.3, variable impacts of each extraction parameters and extraction techniques on phytochemicals were observed. According to Golmohamadi et al. [15], in UAE extraction, the ultrasound energy is the primary factor, where sound waves are highly responsible for the breakage of cell membranes of the plant sample and make it easier in breaking large phytochemical compounds into a simpler form. In the UAE system, the type of probe used for exerting sound energy, and the apparatus used might also affect the extraction process. Selection of the probe is necessary depending on the viscosity of the sample and solvent mixture. In SCFE, the pressure applied in supercritical fluid plays a critical role in extracting the phytochemical compounds from the sample with the help of extracting solvent. As phytochemical solubility varies with the polarity of the solvent used for extraction, the combination of exerted pressure at selected temperature and extraction time was the primary reason for extraction efficiency in SCFE. In the conventional extraction method, the use of centrifugal force is employed to extract the phytochemical-rich supernatant. Larger compounds like starch, cellulose, and fibers settled down as sediment and the

supernatant of phytochemicals floated after centrifugation at high gravitational force. Other parameters *viz*., temperature and time encouraged the phytochemical extraction up to a certain limit. Beyond the limit, the applied temperature might harm the chemical bonding of the specified compound and consequently, it can decrease the anti-oxidative properties of the phytochemical compounds [10]. They also stated that the ratio of the sample and solvent mixture is also important, where enough solvent is required for phytochemicals to get dissolve.

2.4 Conclusion

Bhimkol banana blossom is very rich in TPC, flavonoid, tannin, phytate, and alkaloids and this further accentuate the age old belief of medicinal compounds present in it. Phytochemical extraction by SCFE was found to be the most efficient method compared to the UAE and conventional extraction methods and evinced better recovery of TPC and antioxidant activity. Significant phytochemical compounds were detected in each optimized extracts of SCFE and UAE and better extraction of it in the former than the latter. The phytochemicals present in the bhimkol banana blossom are the main factors for antioxidant activities and they may be responsible for many other medicinal properties. Detail *in vivo* and *in vitro* studies on the health beneficial property of phytochemical-rich bhimkol blossom is further required.

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