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ORIGINAL ARTICLE

Valorization of unsalable *Amaranthus tricolour* leaves by microwave-assisted extraction of betacyanin and betaxanthin

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Abstract

Betacyanin and betaxanthin are natural colouring compounds found in *Amaranthus tricolour* leaves. Microwave-assisted extraction was performed to extract betacyanin and betaxanthin from the unsalable leaves. A set of different process parameters like microwave power, temperature and time was applied in this study. The combination of 450-W microwave power, 90 °C temperature and 15 min of extraction time resulted in the highest amount of betacyanin (71.95 mg/g of dw) recovery. The highest amount of betaxanthin (42.30 mg/g of dw) was extracted at 200-W microwave power, 60 °C temperature and 15 min of extraction time. Green extraction was done by using water as the solvent based on the solubility of compounds being highest in it. The combined effect of time and temperature was also found to be significant for the extraction. The betacyanin and betaxanthin also exhibited good scavenging ability against superoxide and hydrogen peroxide. The recovery was maximum at higher temperatures with a shorter time of extraction. The SEM images showed that the plant matrix ruptured due to microwaves. The FTIR spectroscopy showed the existence of betacyanin and betaxanthin in the leaf extract. The microwave technique was also proved to be better than ultrasound extraction and Soxhlet extraction. The colour change in the powdered sample was observed after extraction which shows the efficient recovery of pigments. The *A. tricolour* leaves are the cheaper source of betacyanin and betaxanthin. These compounds not only possess colouring properties but also exhibit antioxidant and medicinal properties. Hence, betacyanin and betaxanthin extracted from these leaves can be used as additives and colourants in food products.

Keywords *Amaranthus tricolour* leaves · Betacyanin · Betaxanthin · Extraction · Microwave

1 Introduction

The green leafy vegetables have higher moisture content due to which they possess high water activity. The dead and damaged part of these vegetables are the by-product of their processing. The reutilization of such waste has been done by composting and landfill. But, they often lead to the release of unpleasant smell and undesirable microbial growth [1, 2]. The leafy vegetables are susceptible to deterioration because of their perishable nature and higher surface area to volume ratio. It also suffers a loss in quality due to several rapid biochemical and physiological changes [3]. The leafy vegetables suffer postharvest losses during peak season which remains a constraint in its handling, distribution and marketing. A rare and unique postharvest difficulty during minimal processing of the leafy vegetables is encountered when the leaves are chopped off from the whole plant. This hinders their capability of nutrient intake from the main plant [4]. The postharvest loss of more than 50% in leafy vegetables depends on various environmental and biological factors [5]. The goal of green extraction is to acquire energy-efficient extraction with faster rate, minimal processing, miniaturized equipment and improved heat and mass transfer [6].

One such perishable leafy vegetable is *Amaranthus tricolour*. It is an annual plant having purple-red colour leaves belonging to the Amaranthaceae family. It is found in the Southern and South-eastern parts of Asia. Its leaves are consumed as cooked vegetable and salad as well. It is widely consumed in India, Bangladesh and some African countries. The leaves of *A. tricolour* are a rich source of minerals,

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vitamins and phytochemicals and contain unique phytochemical called betacyanin and betaxanthin which gives the reddish dark colour to the leaves. Betacyanin and betaxanthin are nitrogen-containing compounds known to possess several health benefits, such as to counter cardiovascular diseases, arthritis, cancer, retinopathy and cancer cataracts [7]. Reactive oxygen species (ROS) like superoxides radical (O_2^-) and hydrogen peroxide (OH·) radicals are known to cause degradation of biomolecules which can lead to several chronic and life-threatening diseases. Since betacyanin and betaxanthin possess a high antioxidant potential, they can also quench these ROS [8]. Commercially, betacyanin and betaxanthin are extracted from the roots and stems of beet. Many pieces of literature have also reported about the extraction of betalains from *Beta vulgaris*, *Opuntia ficus-indica* and *Hylocereus undatus* [9–11]. In general, solvent extraction and maceration are widely used for betacyanin and betaxanthin extraction. The conventional techniques are expensive because of the need for a higher amount of solvent, energy, plant sample and extraction time. These processes also lead to loss of phytochemicals being extracted and in some cases instability of the compound [12]. The intention is to achieve a higher extraction efficiency along with a reduction in the simultaneous extraction of undesired compounds. Hence, to accomplish these requirements, green solvents can be used with innovative and non-conventional eco-friendly extraction technology. The betacyanin and betaxanthin extraction from prickly pear fruits was done by maceration. The microwave extraction was compared to maceration for the extraction of betacyanin and betaxanthin from beetroot was done. It was observed that a higher amount of betacyanin and betaxanthin were extracted which was found to less in maceration [13].

Microwave-assisted extraction (MAE) involves the use of microwaves for the extraction of compounds from their respective matrix. Microwaves are electromagnetic radiation of wavelength in the range of 1 to 1 mm and the frequency ranges from 300 MHz to 300 GHz [14]. Nature of plant matrix, solvent type, temperature, time, pressure, solvent concentration, solvent volume and particle size are important factors impacting microwave process [15]. Reduction in usage of solvent volume and sample amount is the major motive of using microwave technology leading to recovery at low cost [16]. Water is considered a green solvent and has several advantages over other organic solvents. It is economical, eco-friendly, nontoxic, non-polluting and favours clean processing. The efficient extraction of phytochemicals from a selected source is very important for the economy of the process. Researchers have used response surface methodology (RSM) and Box-Behnken design (BBD) for optimization of parameters for phytochemical extraction using conventional and non-conventional technologies [17].

A three-level BBD was employed for the optimization of microwave power, extraction temperature and extraction time. Four responses recorded for the optimization were Ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), betacyanin content and betaxanthin content. The optimum parameters for efficient extraction of betacyanin and betaxanthin contents from the unsalable *A. tricolour* leaves are yet to be researched and reported. Moreover, water as a green solvent has not been used till now for the extraction of betacyanin and betaxanthin pigments. Hence, we have proposed this method for the optimization of extraction parameters from these leaves. The range of process variables was 200–700 W for microwave power, temperature range of 30–90 °C and time range was 5–15 min. FTIR spectroscopy was used to determine the existence of betacyanin and betaxanthin on the basis of functional groups. The MAE technique was also compared to ultrasound-assisted extraction (UAE) and Soxhlet extraction.

2 Materials and methods

2.1 Chemicals

DPPH and TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) were purchased from Himedia laboratories, India. Sodium acetate was purchased from Avantor performance materials, India. Trolox was purchased from Sigma Aldrich. Ferric chloride, hydrochloric acid and acetic acid were purchased from Loba Chemie Pvt. Ltd. India. Ethanol and methanol were purchased from Thermofisher Scientific, India. Merck Millipore water was used for the preparation of reagents and stock solutions.

2.2 Plant sample

The unsalable *A. tricolour* leaves were procured from the local vegetable market of Raipur, Chhattisgarh, India. The leaves were rinsed with clean potable water to remove physical impurities. The leaves were dried in a hot air oven at 40 °C for 24 h. The size reduction of dried leaves was done to obtain a uniform powder. The powdered leaves were stored in an air-tight container and stored at room temperature (28 ± 2 °C) until further use.

2.3 Microwave-assisted extraction

The extraction experiments were carried out according to the experimental design. Seventeen experimental runs were performed in a microwave-ultrasound-UV reactor provided by Nutech Analytical Technologies Pvt. Ltd., India (Model: NuWav Pro 2450 MHz frequency). The microwave was

operated in batch mode. The duty cycle of the microwave was 50%, 0.5 s on and 0.5 s with a time base of 1 s. Two grams of powdered sample was mixed in ultrapure water (ratio of powdered sample:water was 1:80) in a four-neck round-bottom flask as mentioned by Zin et al. [18]. A platinum probe for temperature detection was assembled in the flask. A condenser was also fitted to the flask to prevent solvent evaporation. The samples were filtered through Sartorius Filter paper No. 292 and the filtrate was centrifuged at 4000 rpm for 10 min by a benchtop centrifuge provided by Remi Laboratory instruments (Model: Neya 8). The supernatant was collected and stored in screw-capped tubes at temperature below 10 °C, until further analysis. All the experiments were done in triplicate.

2.4 Betacyanin and betaxanthin content

The spectrophotometric method was used for the determination of betacyanin and betaxanthin content [19]. The leaf extract was diluted appropriately prior to analysis. The absorbance was measured separately for betacyanin and betaxanthin content at the wavelength of 536 nm and 485 nm. The following equation was used to calculate the betacyanin and betaxanthin content and it was expressed as milligram per gram of dried samples, as

$$\text{Betacyanin (Betaxanthin) Content} = \frac{A \times DF \times MW \times 1000}{\varepsilon \times i} \quad (1)$$

where MW is the molecular weight betacyanin = 550 g/mol, $A = A_{536}\text{nm} - A_{650}\text{nm}$, ε (molar extinction coefficient in $L \times mol^{-1} \times cm^{-1}$) = 60,000. Similarly, for betaxanthin, MW (molecular weight) = 339 g/mol, $A = A_{485}\text{nm} - A_{650}\text{nm}$, ε (molar extinction coefficient in $L \times mol^{-1} \times cm^{-1}$) = 48,000, DF is the dilution factor and *i* is the path length in centimetre.

2.5 Antioxidant profile

The antioxidant profile was analysed by ferric reducing antioxidant power (FRAP) assay and DPPH assay [20]. FRAP reagent was prepared by mixing 10 mM TPTZ with 20 mM $FeCl_3$ and 300 mM acetate buffer in a ratio of 1:1:10. Two hundred sixty microlitres of the extract was mixed with 3.74 ml of FRAP reagent. The absorbance of the reaction mixture was recorded at 593 nm wavelength after 5 min of incubation. Trolox was taken as standard and its calibration curve was plotted for the determination of FRAP. The results were expressed in terms of Trolox equivalent. Similarly, for DPPH activity, 100 µl of the extract was mixed with 3.9 ml of 0.1 mM DPPH reagent and the reaction mixture was incubated in dark for 1 h. The absorbance was taken at 517 nm and the below equation was used to calculate the DPPH activity, expressed in percentage.

$$\text{DPPH Activity}\ (\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100 \quad (2)$$

2.6 Superoxide and hydrogen peroxide radical scavenging assay

The potential of betalains to inhibit the detrimental activity of ROS like superoxide and hydrogen peroxide was determined by the methods described by Alam et al. [20]. A stock solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50 mM) having a 7.4 pH. The absorbance of hydrogen peroxide solution was recorded at 230 nm. *A. tricolour* leaf extract was added to hydrogen peroxide in a 1:1 ratio. The absorbance of the reaction mixture was taken at 230 nm after 10 min of incubation time. Phosphate buffer solution of same concentration without hydrogen peroxide was taken as the blank sample. For the determination of superoxide radical scavenging ability, a reaction mixture was prepared to generate superoxide anion radicals. This reaction mixture contained 0.5 ml NADH (0.936 mM), 0.5 ml NBT solution (0.3 mM), 0.5 ml Tris-HCl buffer (16 mM; pH 8.0) and 1 ml *A. tricolour* extract. 0.5 ml of PMS solution (0.12 mM) was added to start the reaction. The absorbance was measured at 560 nm after incubation period of 5 min at 25 °C.

2.7 Changes in morphology and colour

The morphological changes in the plant matrix were determined by analysing the powdered samples before and after MAE. The micrographs of samples were taken by scanning electron micrograph (SEM) (model: Zeiss). The pictures of the powdered sample were taken before and after MAE. The powdered sample was separated from the extract and dried in a hot air oven at 50 °C temperature after extraction.

2.8 Experimental design

2.8.1 Selection of variables

The microwave power, extraction temperature and extraction time were the chosen variables for the experiments. The design of experiments (DOE) was calculated on the basis of BBD. Preliminary experiments were conducted to finalize the 3 levels of variables, i.e., microwave power (200, 450 and 700 W), temperature (30 °C, 60 °C and 90 °C) and time (5 min, 10 min and 15 min). The responses considered for this optimization study were DPPH antioxidant activity, FRAP activity, betacyanin content and betaxanthin content. About 17 experimental runs were conducted for the optimization of MAE of *A. tricolour* leaves.

2.8.2 Design of experiments

The mean values of dependent parameters obtained from the triplicates were fitted to a second-order polynomial model as follows:

$$Y = \beta o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 AB + \beta_5 AC + \beta_6 BC + \beta_7 A^2 + \beta_8 B^2 + \beta_9 C^2 \quad (3)$$

In Eq. (3), β_o, β_1, β_2, β_3, β_4, β_5, β_6, β_7, β_8 and β_9 are the regression coefficient for constant, linear, quadratic and interactive term respectively. A, B and C are independent variables, i.e., microwave power, temperature and time. *Y* is the response to be calculated by the model equation.

2.9 Ultrasound-assisted extraction and Soxhlet extraction

The UAE and Soxhlet extraction methods were carried out to compare the efficiency of MAE with these techniques. Both the extraction experiments were conducted at the optimum conditions obtained after preliminary research on unsalable *A. tricolour* leaves. Distilled water was also used as a solvent in these experiments. A sonication probe preinstalled in the microwave reactor was used for the UAE experiment. The optimum conditions of the UAE were 40 °C and 30 min of extraction time. Similarly, a Soxhlet apparatus was used for the Soxhlet extraction of betacyanin and betaxanthin. The optimum variables for Soxhlet extraction were 15 h of extraction time at 90 °C temperature. The extracts of *A. tricolour* leaves were evaluated for DPPH activity, FRAP value, betacyanin and betaxanthin content.

2.10 FTIR spectroscopy

The *A. tricolour* leaf extract was analysed for the functional group using FTIR spectroscopy (model: Bruker, Alpha). The extract was mixed with dry KBr to form a KBr thin disc. Furthermore, the disc was kept over the sample cup of a diffuse reflectance accessory. The IR spectrum was within 4000–400 cm^{-1} for the investigation of functional groups. The analysis was conducted at room temperature (25 °C). Peak integration was done to accurately identify the peaks obtained from the spectrum. IUPAC (International Union of Pure and Applied Chemistry) table for the IR spectrum was considered as a reference for the interpretation of the spectrum.

2.11 Statistical analysis of experimental data

The statistical analysis of the experimental data was conducted by ANOVA (analysis of variance) in Design Expert software (Version 11.0.3.0). The comparison of experimental data was done on the basis of 95% ($p < 0.05$) significance level. Regression coefficients were used to analyse the results obtained by the *F* test. R^2, Adj. R^2 and sum of square values were used to compare and validate the adequacy of models. The response surface plots were constructed by using coefficients from the regression model equations.

3 Results and discussion

3.1 Fitting the model

The optimization of MAE for *A. tricolour* leaves was conducted by BBD including three variables, 3 levels and 5 centre point. Table 1 illustrates the 17 experimental runs with process variables and their responses. The significance of each coefficient was checked on the basis of *p* values obtained. The model terms were found to be significant, highly significant and remarkably significant when the values of probability (*p*) were less than 0.05, 0.01 and 0.001 while the model terms were insignificant when these values were greater than 0.05. On the basis of ANOVA, it was found that the model was remarkably significant for all the responses ($p < 0.0001$). The lack of fit for each model was insignificant ($p > 0.05$), demonstrating that the established model satisfactorily describes the relationship between the variables and responses. The values of R^2 and Adj. R^2 was close to 1, which displays a high degree of relationship between the experimental and predicted values. When the *F* value is high and the *p value* is low, the process variables have a significant effect. The results obtained by the mathematical model has shown that the *p*-values were relatively low, indicating the significance of the model. The combined impact of variables on responses can be analysed from the 3D surface graphs.

Table 2 shows the ANOVA designed for DPPH, FRAP, BC and BX. The R^2 (coefficient of determination) value for DPPH was 0.95, which signifies a 95% similarity between experimental and predicted data. The *p* value of the model was obtained to be 0.0008 which suggests the significance of the model. For DPPH, the *p* value of models A, AC and B^2 were 0.006, 0.006 and < 0.0001, respectively. This indicates that microwave power (A), microwave power-extraction time (AC) and temperature (B) had a significant impact on DPPH activity. The model *F* value was estimated to be 15.26; it shows the ability of the model in predicting the % DPPH activity. The R^2 value of FRAP was 0.98 which was close enough to an adjusted R^2 of 0.97. The experimental value was found to be in good agreement with the predicted value for the FRAP values. The entire model was observed to be highly significant for FRAP values which can be understood by the *p* value of < 0.0001. The FRAP values of *A. tricolour* was also significantly affected by the process variables since their *p* values were remarkably significant

Table 1 BBD experimental design for microwave-assisted extraction of *Amaranthus tricolour* leaves

Experimental Run	Microwave Power (W)	Temperature (°C)	Time (minutes)	DPPH activity (%)	FRAP power mMTrolox/g	Betacyanin content (mg/g)	Betaxanthin content (mg/g)
1	450	60	10	78.4615 ± 0.12	0.5386 ± 0.22	56.28333 ± 0.41	27.1906 ± 0.18
2	450	60	10	78.4615 ± 0.05	0.5386 ± 0.47	56.28333 ± 0.38	27.1906 ± 0.41
3	450	30	5	79.7800 ± 0.91	0.3789 ± 0.29	47.32 ± 0.55	29.8037 ± 0.52
4	200	60	5	76.7846 ± 0.35	1.0977 ± 0.33	16.86667 ± 0.16	34.3700 ± 0.19
5	200	60	15	78.6153 ± 0.17	0.9547 ± 0.72	71.95 ± 0.33	42.3043 ± 0.29
6	450	60	10	78.4615 ± 0.29	0.5386 ± 0.10	56.28333 ± 0.02	27.1906 ± 0.61
7	200	90	10	79.8231 ± 0.08	0.8326 ± 0.27	40.425 ± 0.23	26.5400 ± 0.53
8	700	60	15	78.6923 ± 0.16	0.3533 ± 0.45	29.79167 ± 0.47	17.5900 ± 0.34
9	700	60	5	79.8461 ± 0.99	0.7032 ± 0.77	58.66 ± 0.11	23.3768 ± 0.86
10	450	30	15	80.1538 ± 0.87	0.3341 ± 0.15	51.51667 ± 0.28	28.0381 ± 0.67
11	450	90	5	80.6153 ± 0.20	0.8134 ± 0.80	50.23333 ± 0.93	24.7893 ± 0.77
12	450	60	10	78.4615 ± 0.017	0.5386 ± 0.19	56.28333 ± 0.21	27.1906 ± 0.58
13	450	90	15	80.0769 ± 0.020	0.3214 ± 0.37	61.59 ± 0.46	27.7556 ± 0.25
14	200	30	10	80.7692 ± 0.081	0.7359 ± 0.44	37.87 ± 0.82	37.3200 ± 0.49
15	700	30	10	80.9231 ± 0.15	0.2734 ± 0.38	32.81667 ± 0.35	13.5100 ± 0.31
16	700	90	10	80.7692 ± 0.23	0.5942 ± 0.27	39.52 ± 0.42	19.1800 ± 0.92
17	450	60	10	78.4615 ± 0.39	0.5386 ± 0.91	56.28333 ± 0.63	27.1906 ± 0.26

DPPH, 2,2-diphenyl-1-picrylhydrazyl; *FRAP*, Ferric reducing antioxidant power

($p < 0.001$). The reliability of this model in the prediction of the yield of FRAP can be understood by the model F value (61.08). The betacyanin content of *A. tricolour* leaves was also significantly affected by extraction time ($p < 0.05$) and temperature ($p < 0.05$). Among the combined effect of process variables, only AC (microwave power-extraction time) were significantly affecting betacyanin content. The p value (< 0.0001) depicted that the model was remarkably significant for betacyanin content. The R^2 (0.99) shows a high similarity of experimental and predicted values of betacyanin content. The model F value (116.54) obtained from the ANOVA table shows the model was reliable to predict the betacyanin content. For betaxanthin content, the R^2 (0.99) showed that experimental and predicted values were close enough. The microwave power ($p < 0.0001$) and extraction temperature ($p = 0.0006$) had a highly significant effect on the betaxanthin content. The model was found to be remarkably significant for the betaxanthin content with a p value less than 0.0001. The extraction time (alone) ($p = 0.097$) did not show any significant impact on the recovery of betaxanthins. However, it significantly affected the process under the influence of microwave power ($p < 0.0001$) and extraction temperature ($p = 0.006$).

3.2 Investigation of the adequacy of models

The adequacy of models and the correlation between experimental and predicted values was analysed by diagnostic plots. The diagonal line shows the predicted values while the data points represent the experimental values. The closer the straight line and data points are, the better the correlation between the experimental and predicted values are. A normal probability graph of the residual values was used for the confirmation of the normality assumption. Since the data points were found to lie close to the straight line, it was concluded that the data followed a normal distribution. Figure 1 shows the graph between the predicted and actual data for % DPPH activity, FRAP reducing power, betacyanin and betaxanthin contents. Figure 2 shows the normality graphs for % DPPH activity, FRAP reducing power, betacyanin and betaxanthin contents.

3.3 Effect of process variables on antioxidant activity

The assessment of antioxidant activity plants cannot be done by one method because of the complex nature of the phytochemical reactions. Hence, at least two methods are suggested to evaluate the antioxidant potential. DPPH and FRAP were the two methods adopted in this research to evaluate antioxidant potential [21]. The highest value of DPPH activity (80.92%) was observed for extraction with 700 W microwave power for 10 min at a temperature of 30 °C. However, the extraction with 200 W microwave power at 60 °C temperature for 5 min resulted in the lowest DPPH activity (76.78%). Betacyanin is reported to have good hydrogen donating potential and effectively scavenge the DPPH radicals. Betacyanin extracted from *Basella alba* fruit has a scavenging

Table 2 ANOVA table for DPPH, FRAP, BC and BX

Source	Sum of squares	df	Mean	*F* value	*p* value	
DPPH						
Model	20.39185	9	2.265761	15.2551	0.000819	Significant
A-Power	2.245578	1	2.245578	15.11921	0.005988	
B-Temp	0.014583	1	0.014583	0.098186	0.763148	
C-Time	0.032805	1	0.032805	0.220875	0.652663	
AB	0.15692	1	0.15692	1.056521	0.338211	
AC	2.227017	1	2.227017	14.99424	0.006114	
BC	0.208087	1	0.208087	1.401024	0.275185	
A^2	0.201668	1	0.201668	1.357807	0.282085	
B^2	15.05276	1	15.05276	101.3484	< 0.0001	
C^2	0.16139	1	0.16139	1.086619	0.331879	
Residual	1.039674	7	0.148525			
Lack of fit	1.039674	3	0.346558			
Pure error	0	4	0			
R^2	0.9515					
Adj R^2	0.8891					
FRAP						
Model	0.87391	9	0.097101	61.0845	< 0.0001	Significant
A-Power	0.35986	1	0.35986	226.3812	< 0.0001	
B-Temp	0.08802	1	0.08802	55.37176	0.0001	
C-Time	0.132522	1	0.132522	83.36734	< 0.0001	
AB	0.012546	1	0.012546	7.89243	0.0262	
AC	0.01069	1	0.01069	6.724673	0.0358	
BC	0.050016	1	0.050016	31.464	0.0008	
A^2	0.156563	1	0.156563	98.49061	< 0.0001	
B^2	0.063126	1	0.063126	39.71109	0.0004	
C^2	0.008819	1	0.008819	5.547858	0.0507	
Residual	0.011127	7	0.00159			
Lack of fit	0.011127	3	0.003709			
Pure error	0	4	0			
R^2	0.9874					
Adj R^2	0.9713					
BC						
Model	2975.293	9	330.5882	116.5384	< 0.0001	Significant
A-Power	4.998068	1	4.998068	1.761911	0.2260	
B-Temp	61.855	1	61.855	21.80502	0.0023	
C-Time	218.0742	1	218.0742	76.87516	0.0001	
AB	4.302167	1	4.302167	1.516593	0.2579	
AC	1761.971	1	1761.971	621.1269	< 0.0001	
BC	12.8164	1	12.8164	4.518016	0.0711	
A^2	765.8534	1	765.8534	269.9774	< 0.0001	
B^2	111.1863	1	111.1863	39.19522	0.0004	
C^2	9.733334	1	9.733334	3.431179	0.1064	
Residual	19.85712	7	2.836732			
Lack of fit	19.85712	3	6.619041			
Pure error	0	4	0			
R^2	0.9934					
Adj R^2	0.9848					
BX						

Table 2 (continued)

Source	Sum of squares	df	Mean	*F* value	*p* value	
Model	751.8127	9	83.53474	217.3726	< 0.0001	Significant
A-Power	559.075	1	559.075	1454.815	< 0.0001	
B-Temp	13.53788	1	13.53788	35.22803	0.0006	
C-Time	1.401243	1	1.401243	3.646288	0.0978	
AB	67.65063	1	67.65063	176.0392	< 0.0001	
AC	47.06818	1	47.06818	122.48	< 0.0001	
BC	5.59766	1	5.59766	14.56613	0.0066	
A^2	1.617303	1	1.617303	4.208516	0.0794	
B^2	24.93153	1	24.93153	64.87637	0.0001	
C^2	33.94734	1	33.94734	88.33717	< 0.0001	
Residual	2.69005	7	0.384293			
Lack of fit	2.69005	3	0.896683			
Pure error	0	4	0			
Cor total	754.5027	16				
R^2	0.9964					
Adj R^2	0.9919					

DPPH, 2,2-diphenyl-1-picrylhydrazyl; *FRAP*, Ferric reducing antioxidant power; *BC*, betacyanin content; *BX*, betaxanthin content

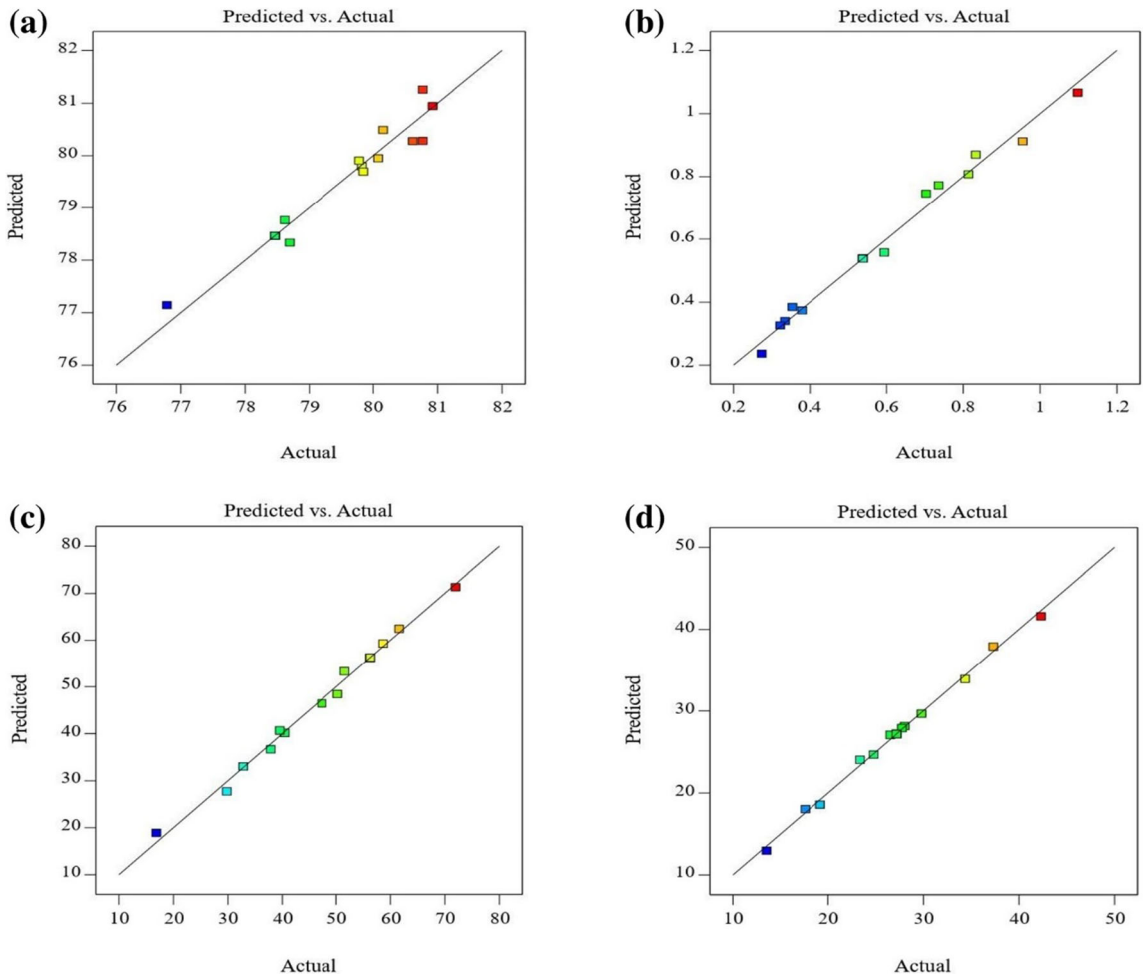

Fig. 1 Comparison between predicted values and actual values **a** % DPPH, **b** FRAP, **c** betacyanin content, **d** betaxanthin content

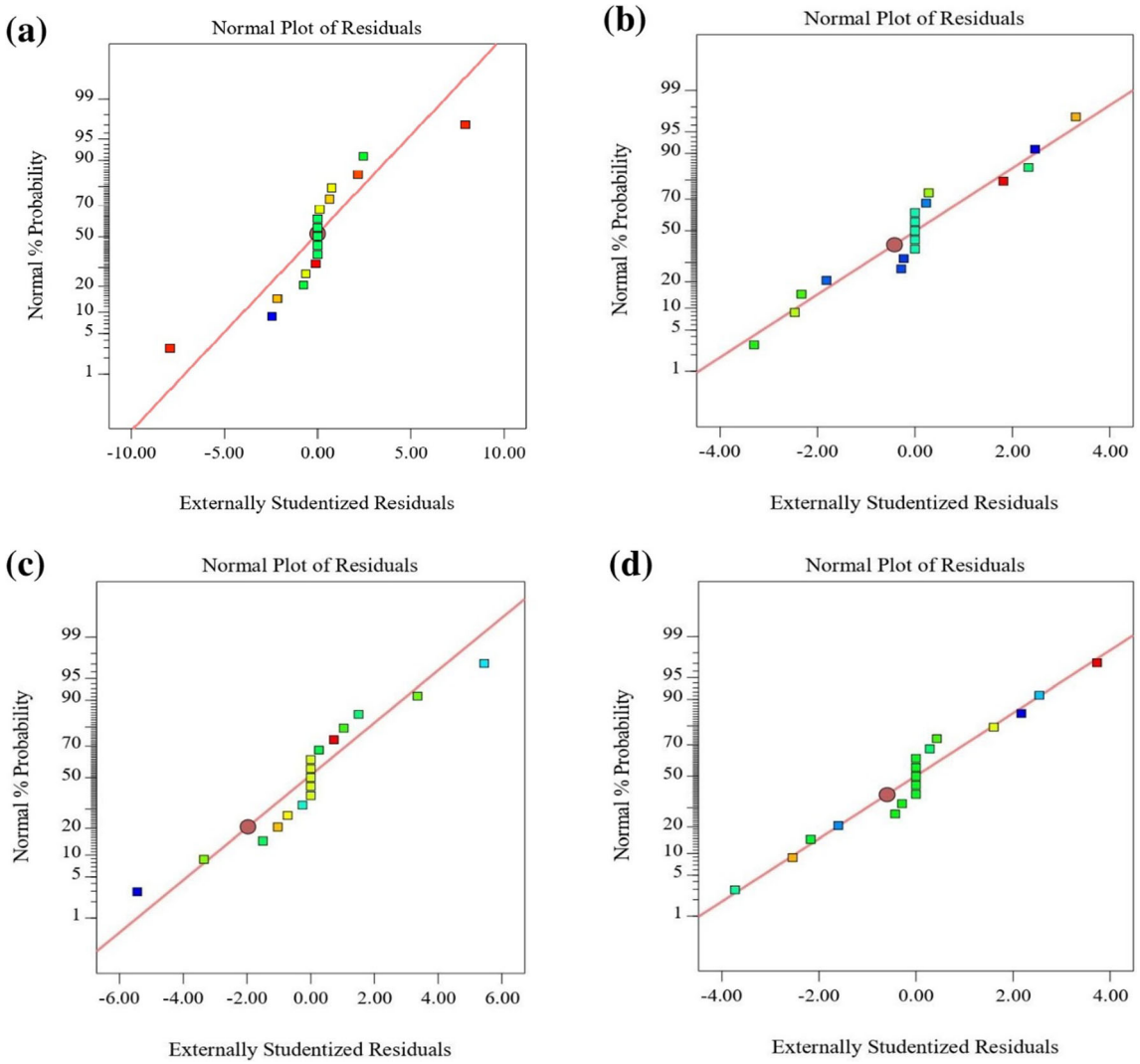

Fig. 2 Normality graph **a** % DPPH, **b** FRAP, **c** betacyanin content, **d** betaxanthin content

ability of up to 54% while those extracted from *A. tricolour* leaves are comparatively higher. This difference might be due to the higher betacyanin content extracted from the *A. tricolour* leaves by MAE [22]. The second-order polynomial equation for the DPPH activity was

$$\begin{aligned}\text{DPPH} = {} & 78.46 + 0.5298\text{A} - 0.0427\text{B} + 0.0640\text{C} \\ & + 0.1981\text{AB} - 0.7462\text{AC} - 0.2281\text{BC} \\ & + 0.2189\text{A}^2 + 1.89\text{B}^2 - 0.1958\text{C}^2 \end{aligned} \quad (4)$$

The experimental and predicted values were in good agreement with each other which is illustrated by the $R^2 = 0.996$. The ANOVA results depict that microwave power had a significant effect on the extraction. The combination of microwave power and time also exhibited a significant effect on the extraction.

Figure 3a depicts the combined effect of time and microwave power on microwave extraction. A higher DPPH activity was observed when the microwave power was at 700 W for 5 min of extraction. Similarly, when temperature and power are considered in Fig. 3b, the 3D plot reflects that the higher temperature (90 °C) and the lower temperature (30 °C) both affect the DPPH activity. A precise conclusion can be made by considering the 3D plot of time and temperature illustrated in Fig. 4. It can be observed from these plots that DPPH activity is again higher either for low and high temperature when the extraction time is less. But, it decreases with respect to increasing time. Hence, when the simultaneous effect of all three parameters is considered, it can be concluded that high microwave power is effective with low temperature and short time for extraction of betalains. Betalains are known to be thermolabile compounds which are degraded when exposed to high temperature (90 °C) for a long time period [23], thus showing low DPPH activity at a high temperature of extraction.

The reducing power is reported to be dependent on antioxidant activity. The phytochemicals possess a reducing ability

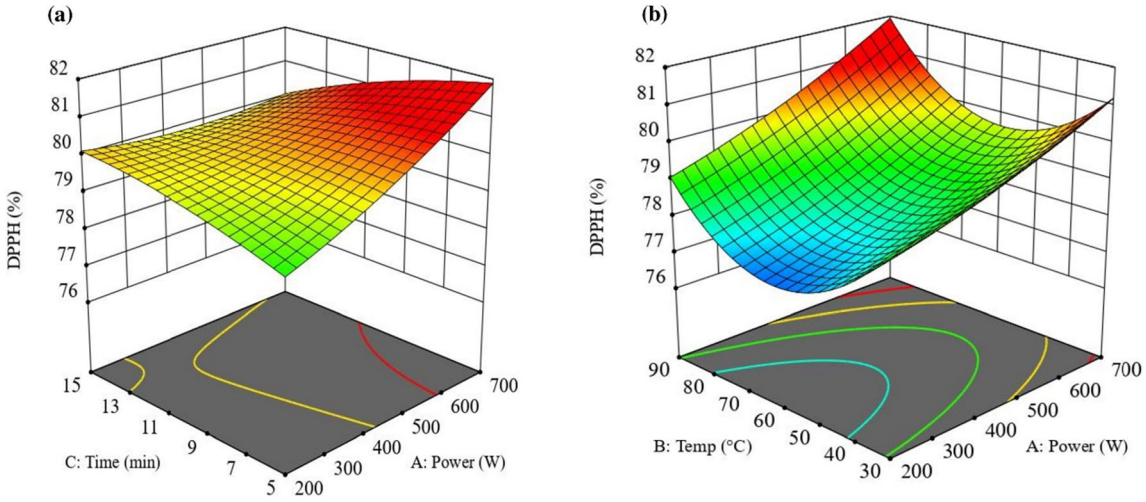

Fig. 3 **a** Effect of time and power on DPPH antioxidant activity. **b** Effect of temperature and power on DPPH antioxidant activity

which is identified when they break the free radical chain by donating the hydrogen ion [23]. The ferric-ferricyanide complex gets reduced to the ferrous-ferricyanide complex due to the reducing ability of betacyanin and betaxanthin. The model was found to be significant for reducing the power of betalains showing a 0.0008 p value ($p < 0.05$). The R^2 value of the model was 0.951 while the Adj. The R^2 value was 0.889. The second-order polynomial equation for FRAP value is given by the below equation

$$\begin{aligned} \text{FRAP} = {} & 0.5387 + 0.2121\text{A} + 0.1049\text{B} - 0.1287\text{C} \\ & + 0.0560\text{AB} - 0.0527\text{AC} - 0.1118\text{BC} \\ & + 0.1928\text{A}^2 - 0.1224\text{B}^2 + 0.0458\text{C}^2 \end{aligned} \quad (5)$$

The highest FRAP value of 1.097 mM Trolox/g was obtained at 200 W microwave power, 5 min time and 60 °C temperature. The lowest value of FRAP (0.273 mM Trolox/g) was calculated when the extraction was carried out for 10 min at 30 °C with 700 W of microwave power. Betacyanins are also reported to possess a high electron-donating ability. The reducing power of betacyanin and betaxanthin extracted from *A. tricolour* leaves was observed to a lot better than those extracted from *B. alba* fruits. The presence of reductones can be a potential reason for higher reducing power. These reductones are known to donate hydrogen atom leading to the breakdown of the radical chain [22]. The extraction of betacyanin and betaxanthin was also significantly ($p < 0.05$) affected by time and temperature.

Figure 5 illustrates that the FRAP value increased at high temperature and low microwave power. The increasing microwave power and extraction temperature result in a decreased FRAP value. This clearly indicates that the reducing power of betalains is decreased at higher temperature with higher microwave power. The antioxidant activity depends on the chemical structure and betalain content of the plant source [24].

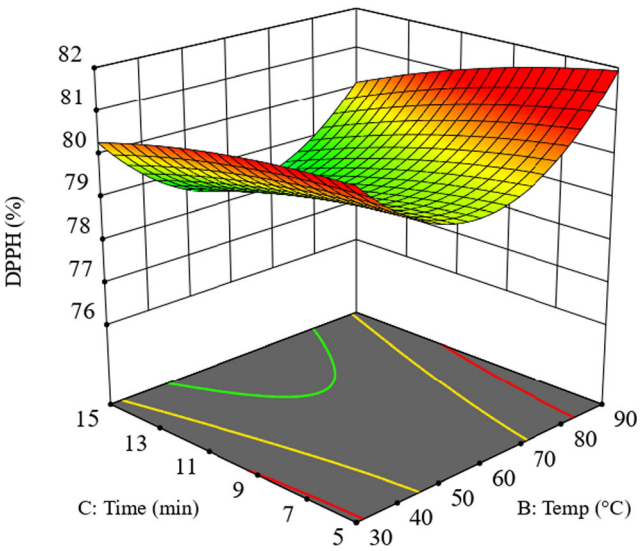

Fig. 4 Effect of time and temperature on DPPH antioxidant activity

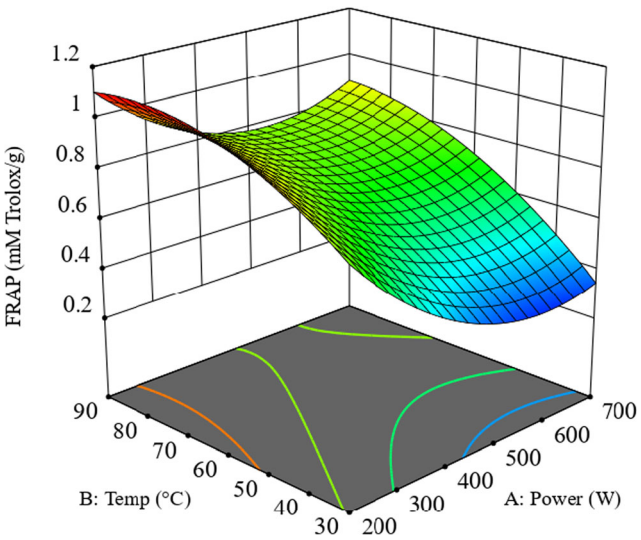

Fig. 5 Effect of temperature and power on FRAP value

3.4 Betacyanin and betaxanthin content

Betacyanin and betaxanthin are the major compounds among the betalains found in *A. tricolour* leaves. The betacyanin content was calculated by the polynomial equation mentioned below

$$\begin{aligned}\text{Betacyanin Content} = {} & 56.28 - 0.79A + 2.78B + 5.22C \\ & + 1.04AB - 20.99AC \\ & + 1.79BC - 13.49A^2 - 5.14B^2 \\ & + 1.52C^2 \end{aligned} \quad (6)$$

The highest content of betacyanin (71.95 mg/g) was extracted at 60 °C temperature for 15 min with 200 W microwave power. The lowest betacyanin content (16.87 mg/g) was calculated with parameter as 200 W microwave power, 60 °C temperature and 5 min of extraction time. Acidified methanol was used for betacyanin extraction from *Basella alba* fruits. The total betacyanin content obtained from the extract was 15 μg/g which is comparatively low as compared to the betacyanin content obtained in this research [22]. From the above observations, it can be speculated that betacyanins can be extracted efficiently at lower microwave power. However, a longer extraction time is required for better extraction with high temperature. Figure 6a illustrates the effect of time and power.

When high microwave power was applied for extraction, the betacyanin was not extracted in a high amount. Betacyanin content was observed to be high at high temperature and low microwave power, as shown in Fig. 6b. Betacyanin was degraded when the extraction was performed at higher microwave power and high temperature. It was observed that time and temperature have a linear relationship for the extraction of betacyanin (Fig. 7). The betacyanin content was found to increase with increasing time and temperature. Higher betacyanin content can be obtained by conducting the extraction at lower power for a long time and at high temperature. When low microwave power is used, high dipole rotation is created thus increasing the temperature of the extraction solvent [24]. A similar observation was reported in the literature when efficient extraction of betalains was observed for longer extraction time at higher temperatures [12].

The betaxanthin content of *A. tricolour* leaves was evaluated for each extract. The highest amount (42.30 mg/g) of betaxanthin extracted was observed with 200 W microwave power for 15 min of extraction time at 60 °C temperature. The lowest value (13.51 mg/g) of betaxanthin content was observed when extraction was performed at 30 °C temperature for 10 min with 700 W microwave power. The polynomial equation for betaxanthin content is mentioned below

$$\begin{aligned}\text{Betaxanthin Content} = {} & 27.19 - 8.36A - 1.30B - 0.41C \\ & + 4.11AB - 3.43AC \\ & + 1.18BC - 0.61A^2 - 2.43B^2 \\ & + 2.84C^2 \end{aligned} \quad (7)$$

Figure 8a depicts the combined effect of microwave power and temperature on the extraction of betaxanthin. It can be clearly observed that with low microwave power and low temperature, higher betaxanthin content was extracted. Similarly, Fig. 8b shows that a long time period is required with low microwave power to extract the maximum quantity of betaxanthin. When the extraction is carried out at a low temperature for a long time period, the amount of betaxanthin extracted is more. The recovery of betaxanthin is less when the extraction is done at a higher temperature for long time periods. The betaxanthin undergoes isomerization due to the influence of heat. The betalamic acid is condensed to form indicaxanthin due to excess and continuous exposure to heat. Hence, the thermal degradation of betaxanthin takes place

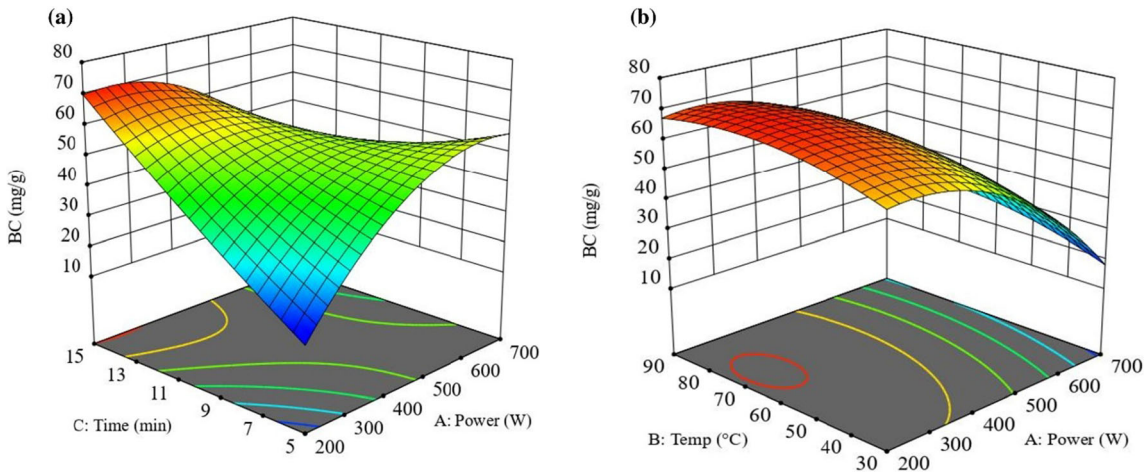

Fig. 6 **a** Effect of time and power on betacyanin content. **b** Effect of temperature and power on betacyanin content

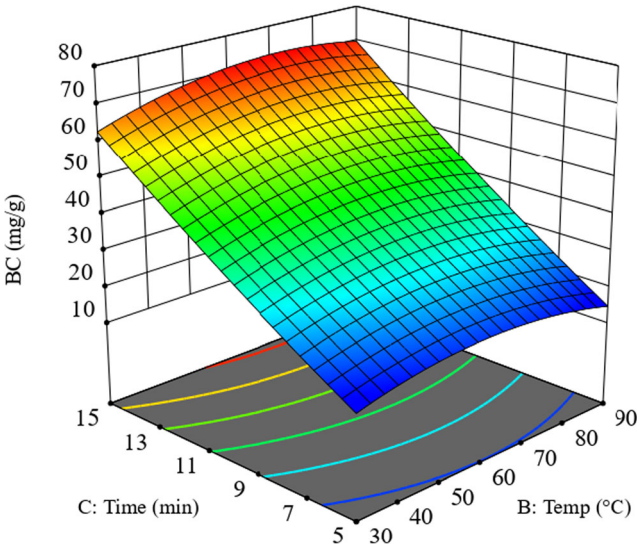

Fig. 7 Effect of temperature and time on betacyanin content

when the extraction is carried out at a high temperature for a long time duration [25].

3.5 Detection and identification of functional groups

The identification of betacyanin and betaxanthin was done by analyzing the FTIR spectrum obtained. The analysis was done individually for the extract obtained from MAE, UAE and Soxhlet extraction. Table 3 shows the wave number (cm^{-1}), stretching, functional group and the intensity of the bonds. Figure 9 depicts the FTIR spectrum obtained for the *A. tricolour* leaf extracts. The FTIR analysis showed the presence of more functional groups in the MAE extract as compared to UAE extract and Soxhlet extract. For the MAE extract, the strong C–O stretch of phenols was detected at wave numbers 1013, 1032 and 1055 cm^{-1}. The O–H bending of phenols of medium intensity was detected at 1346 and 1398 cm^{-1}. The strong stretching of CH_3, CH_2 and CH were identified at 2844, 2865, 2922, 2938 and 2967 cm^{-1} representing the alkane groups. A primary amine functional group was found at 1629 cm^{-1} and 3423 cm^{-1}, which reflects the presence of betacyanin and betaxanthin. Three other O–H bending of phenols were detected at 1346, 1398 and 3650 cm^{-1} wave numbers. The O–H stretch observed at 3678, 3693, 3751, 3807, 3822, 3841, 3855, 3872 and 3904 cm^{-1} represents the presence of water molecules. The other functional groups identified were disulphides at 514 and 537 cm^{-1} may be due to the presence of amino acids. The cis-distributed functional group of alkenes was detected at 663 cm^{-1}. At 790 cm^{-1}, a functional group of esters was observed. The extract obtained by the UAE of *A. tricolour* leaves also showed the presence of different functional groups in FTIR analysis. The presence of the primary amine group was detected at 1055 and 1509 cm^{-1} which signifies the presence of betacyanin and betaxanthin. The deformation of CH and CH_3 groups was identified at 618, 1375 and 1459 cm^{-1}. The carbonyl groups (C=O) were found at 1257, 1616 and 1734 cm^{-1} wave numbers. The strong intensity of C=O groups of carboxylic acid was also detected at 2846 and 2914 cm^{-1} wave numbers. At 3353 cm^{-1}, the OH group of carboxylic acid was detected with strong intensity. Similarly, the extract obtained from the Soxhlet extraction showed the least functional groups as seen in the FTIR graph. 772 and 3448 cm^{-1} wave numbers represented the existence of primary amines signifying the occurrence of betacyanin and betaxanthin. The other functional groups identified at 1219, 1465, 1635, 1734, 2850 and 2918 cm^{-1} were CN stretch, C=C symmetry, C=O, CH_3, CH_2 and CH groups. The existence of amine groups in the extract indicates the presence of

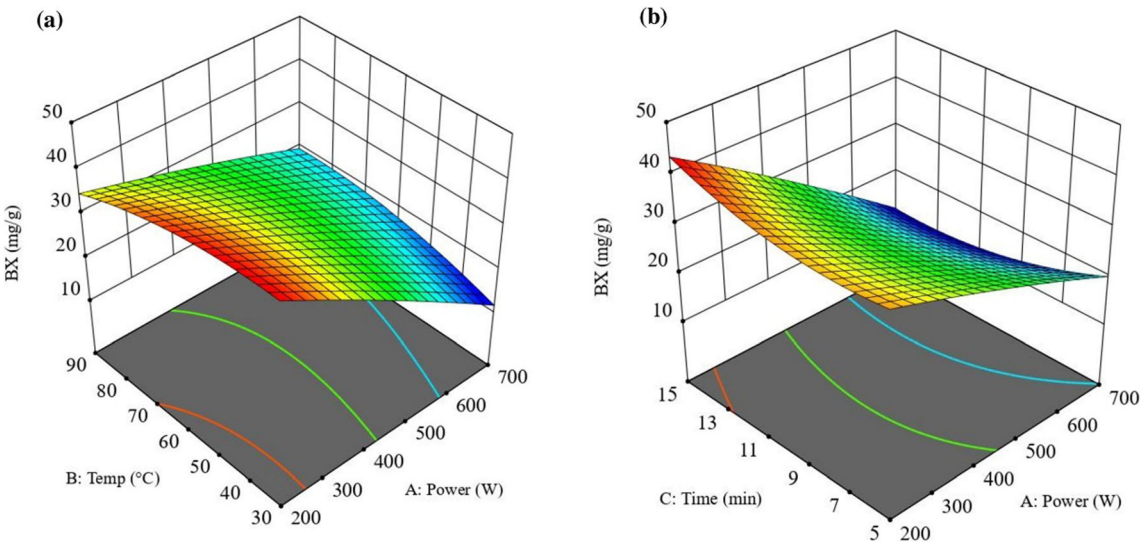

Fig. 8 **a** Effect of temperature and power on betaxanthin content. **b** Effect of time and power on betaxanthin content

Table 3 Functional groups identified by FTIR in MAE extract, UAE extract and Soxhlet extraction extract of unsalable *A. tricolour* leaves

MAE extract		UAE extract		Soxhlet extraction extract	
Wave number (cm^{-1})	Stretch and functional group	Wave number (cm^{-1})	Stretch and functional group	Wave number (cm^{-1})	Stretch and functional group
514	Disulphide	505	Disulphide	772	NH_2 & NH amine
537	Disulphide	527	Disulphide	1219	CN amine
663	Cis-disubstituted alkene (C–H vinyl)	536	Disulphide	1465	CH_2 & CH_3 deformation alkane
790	S-OR esters	558	Disulphide	1635	C=C symmetry alkene
1013	Phenols (C–O)	569	Disulphide	1734	C=O aldehyde
1032	Phenols (C–O)	618	CH deformation	2850	CH_3, CH_2 & CH alkane
1055	Phenols (C–O)	1055	CN (amine)	2918	CH_3, CH_2 & CH alkane
1346	O–H bending (phenols)	1257	O–C carboxylic acids	3448	NH 1 amine
1398	O–H bending (phenols)	1375	CH_3 deformation		
1455	CH_2 and CH_3 deformation (alkanes)	1459	CH_3 deformation		
1629	NH_2 scissoring (1 amine)	1509	NH amide		
2075	C=C bending vibration (alkyne)	1616	C=O carboxylic acids		
2844	CH_3, CH_2 & CH; 2 or 3 bands (alkane)	1734	C=O aldehyde & ketone		
2865	CH_3, CH_2 & CH; 2 or 3 bands (alkane)	2846	O–H carboxylic acids		
2922	CH_3, CH_2 & CH; 2 or 3 bands (alkane)	2914	O–H carboxylic acids		
2938	CH_3, CH_2 & CH; 2 or 3 bands (alkane)				
2967	CH_3, CH_2 & CH; 2 or 3 bands (alkane)				
3423	NH 1 amine				
3650	OH (free) alcohols or phenols				
3678	Water OH stretch				
3693	Water OH stretch				
3751	Water OH stretch				
3807	Water OH stretch				
3822	Water OH stretch				
3841	Water OH stretch				
3855	Water OH stretch				
3872	Water OH stretch				
3904	Water OH stretch				

MAE extract, FTIR data of extract obtained from MAE; UAE extract, FTIR data of extract obtained from UAE; Soxhlet extract, FTIR data of extract obtained from Soxhlet extraction

betacyanin and betaxanthin in the extracts was also reported [26–28]. The FTIR results also show that the greater number of other phytochemicals recovered by MAE was greater than UAE and Soxhlet extraction. The higher proportion of these compounds might have also contributed to enhanced antioxidant potential in these leaves.

3.6 Optimal solution of MAE of unsalable *A. tricolour* leaves

The experimental runs performed as per the BBD has led to an optimal solution of MAE. The desirability function from the numerical optimization was used to obtain the optimal solution. A maximized desirability function is produced from the data points after numerical optimization. The goals and boundaries must be defined to find the desirability function. For the optimal solution, process variables and their responses are combined to obtain the desirability function. The desirability value of 0.881 was found for 200 W microwave power, 31.45 °C temperature and 15 min of extraction time. The responses predicted by the mathematical model were 80.923% DPPH activity, 0.861 mM Trolox/g FRAP, 63.30 mg/g betacyanin content and 43.44 mg/g betaxanthin content (Table 4).

3.7 Validation of predicted model and optimal solution

To examine the fitness of the model and to verify the authenticity of optimized conditions, extraction experiments were

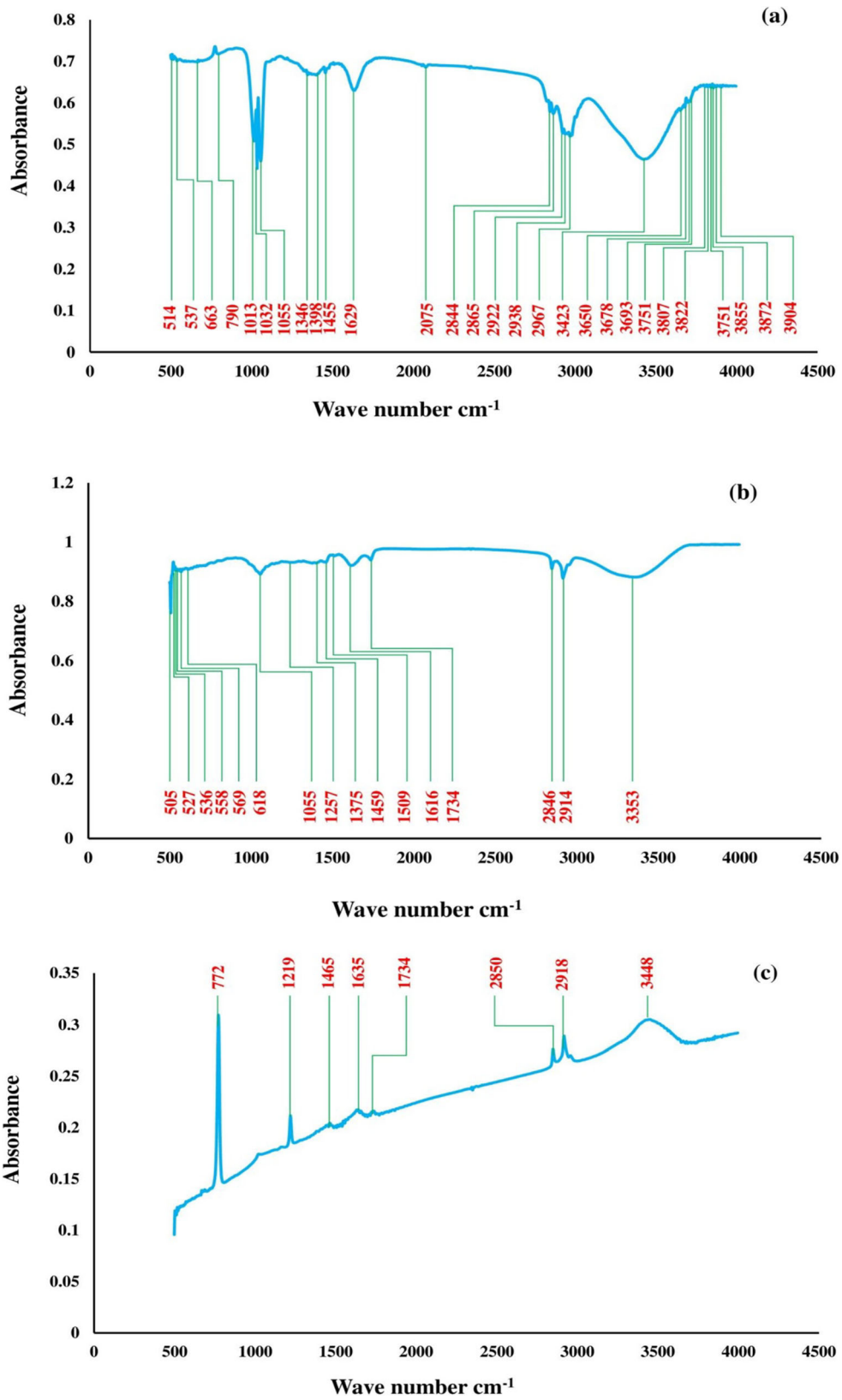

Fig. 9 **a** FTIR spectroscopy of MAE extract. **b** FTIR spectroscopy of UAE extract. **c** FTIR spectroscopy of Soxhlet extraction extracts

performed. Table 3 illustrates the results obtained from the experiment conducted at optimum conditions. 80.15%, 0.857 mM Trolox/g, 62.54 mg/g and 43.29 mg/g were the experimental values obtained for DPPH activity, FRAP value,

Table 4 % DPPH, FRAP, betacyanin and betaxanthin content of extracts obtained from MAE, UAE and Soxhlet extraction

	MAE	UAE	Soxhlet extraction
% DPPH	80.15 ± 0.15	74.31 ± 0.49	59.88 ± 0.36
FRAP (mM Trolox/g)	0.857 ± 0.19	0.633 ± 0.51	0.594 ± 0.40
BC (mg/g)	62.54 ± 0.28	55.92 ± 0.18	44.15 ± 0.52
BX (mg/g)	43.29 ± 0.33	35.27 ± 0.22	28.91 ± 0.06

% DPPH, DPPH antioxidant activity; FRAP, FRAP reducing power; BC, betacyanin content; BX, betaxanthin content

betacyanin content and betaxanthin content, respectively. A 95% level of significance was observed between the validation data and predicted data. Hence, it can be inferred that the model had good fitness for the extraction experiments.

3.8 Comparison of MAE with UAE and Soxhlet extraction

The responses recorded for the optimum extraction conditions of MAE, UAE and Soxhlet extraction are shown in Table 4. The % DPPH activity, FRAP radical scavenging potential, betacyanin content and betaxanthin content for UAE at optimum conditions were 74.31%, 0.633 mM Trolox/g, 55.92 mg/g and 35. 27 mg/g, respectively. Similarly, the DPPH activity, FRAP value, betacyanin content and betaxanthin content for Soxhlet extraction at optimum conditions were 59.88%, 0.594 mM Trolox/g, 44.15 mg/g and 28.91 mg/g, respectively. The ultrasounds produced a cavitation effect in the extraction solvent. The formation and collapse of bubbles occur by the virtue of this cavitation releasing energy. This phenomenon causes heating of solvent and disruption of plant matrix and cell wall liberating the betacyanin and betaxanthin [29]. However, prolonged extraction under the influence of ultrasound caused the degradation of betacyanin and betaxanthin [30]. Hence, the antioxidant potential also decreased for the extracts obtained by the UAE. Similarly, in the case of Soxhlet extraction, the plant matrix was exposed to a high temperature for a long time. The breakdown of the cell wall caused the release of betacyanin and betaxanthin but a longer extraction time also caused the degradation of these compounds. Moreover, high temperature also caused the loss of solvent leading to reduced efficiency of extraction [31]. From the results obtained, it is clear that the MAE was a better method for the extraction of betacyanin and betaxanthin from unsalable *A. tricolour* leaves.

3.9 Superoxide and hydrogen peroxide radical scavenging assay

Superoxides generate ROS like singlet oxygen and hydroxyl radical, after decomposition, consequently, initiating lipid peroxidation and damage to DNA nucleotides thus damaging cellular structures. Lipid peroxidation is also facilitated by the generation of hydroxyl radicals due to the biochemical activity of superoxides [32]. Hydrogen peroxide is also one of the unstable by-products of cellular metabolism. Its action can result in more severe consequences when it works with ROS like superoxide and can damage lipids, DNA and the nucleus. Hence, it becomes very necessary to quench these ROS species. The H_2O_2 scavenging activity is evaluated by the measurement of colour changes by oxidation reduction of the single-electron exchange reaction. These betalain pigments neutralize H_2O_2 to water by donating electron which can validate the mechanism of H_2O_2 scavenging activity [33]. The superoxide and hydrogen peroxide radical scavenging activity of betalains extracted at optimum extraction conditions were 78.91 ± 0.71% and 72.96 ± 0.50%, respectively. These results were found to be similar to the ability of *B. alba* fruits extract to scavenge superoxide and hydrogen peroxide radicals [22].

3.10 Effect of water as a solvent on MAE

Non-thermal extraction technique along with the water was suggested to prevent the degradation of betacyanin and betaxanthin during the extraction [34]. MAE is primarily affected by the type of solvent selected to recover the desired phytochemical. It depends on the mass transfer of solute, interaction with plant matrix and dielectric properties of the solvent. The water is known to have higher dielectric properties in comparison to alcohols regardless of the microwave power. Water may enhance the diffusion of the desired compound by absorbing more heat than alcohol [35]. The betacyanin and betaxanthin contents of prickly pear fruit were in the range 11 mg/100 g to 14 mg/100 g and 18 mg/100 g to 25 mg/100 g, respectively. The range of betacyanin and betaxanthin content in the research obtained was 16 mg/g to 61 mg/g and 13 mg/g to 42 mg/g, respectively. The recovery of betacyanin and betaxanthin was significantly higher than reported. The plausible reason for this result can be the use of water as a solvent for the MAE since betacyanin and betaxanthin are hydrophilic in nature. They are also known as scavengers of cationic radicals [36]. Water as a solvent might have also undergone behavioural change in its subcritical area to acquire the characteristics of an organic solvent [37]. It was reported that the extraction of betalains was better due to the polarity of water in comparison to alcoholic solvents. Another reason might be the interaction between phytochemicals and hydrophilic biomolecules like carbohydrates or their carboxyl end. However, degradation of the desired compound was also observed when extraction was carried out at high temperature for a longer period [29].

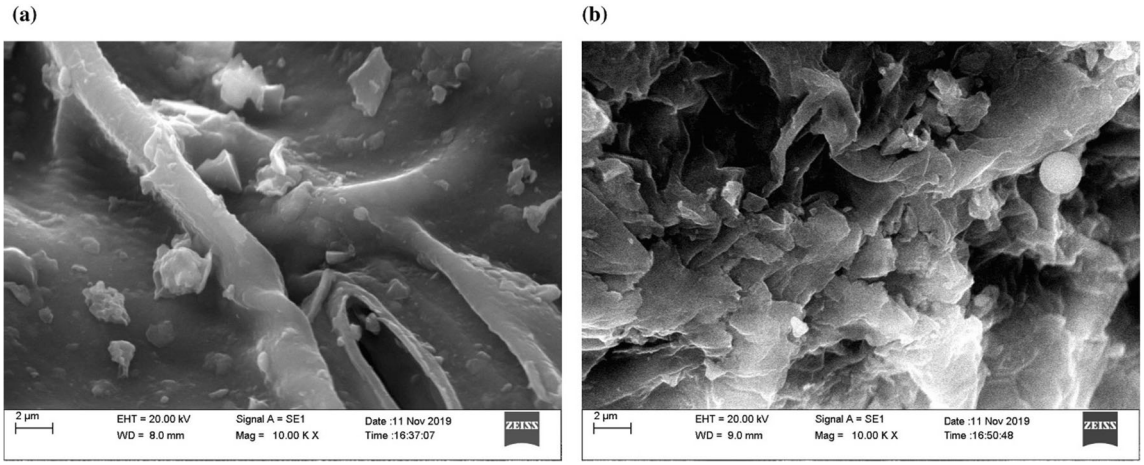

Fig. 10 SEM images of plant samples. **a** Before MAE. **b** After MAE

3.11 Change in morphology and colour

The micrographs of samples were taken at a magnification of 10,000×. Figure 10 illustrates the images of SEM of powdered samples. It is clearly visible that the external surface was uniform and smooth prior to microwave extraction (Fig. 10a). However, the microwaves have severely damaged the surface of the powdered sample (Fig. 10b). Similar results of the effect of microwaves on the morphology of plant matrix were reported for extraction of pectin from the skin [38]. It was reported that the betalains are mainly localized in the cell vacuoles of leaves, more specifically, into the subdermal and epidermal layers of the tissues [9]. These layers might have been ruptured either by the microwaves or by the heat generated by them. Hence, the solvent could have been diffused easily into the plant matrix. This phenomenon of heat and mass transfer facilitated the extraction of betacyanin and betaxanthin by MAE. The change in colour of powdered samples can be observed from Fig. 11a and b. The figure shows that the powdered sample had a purple-red colour before MAE. The betacyanin and betaxanthin present in the leaves of *A. tricolour* were responsible for the colour. During the extraction, microwaves were able to release these pigments from the plant matrix. Moreover, betacyanin and betaxanthin were highly soluble in water which was used as a green solvent. Water and microwave were found to be efficient in the extraction of betacyanin and betaxanthin pigment which led to the colour loss of powdered sample as illustrated in Fig. 11b.

4 Conclusion

The optimization of the process variables depicted that at a temperature of 31 °C for 15 min of extraction time and with 200 W microwave power, the betacyanin and betaxanthin extraction is effective and efficient. The experimental data calculated for the set of extraction experiments shows that low microwave power and less temperature can be used for a longer duration for the recovery of betalains. The mass transfer, i.e. diffusion of the betacyanin and betaxanthin pigments was influenced by microwaves thus enhancing the extraction. Whenever the extraction was done at higher temperatures for a

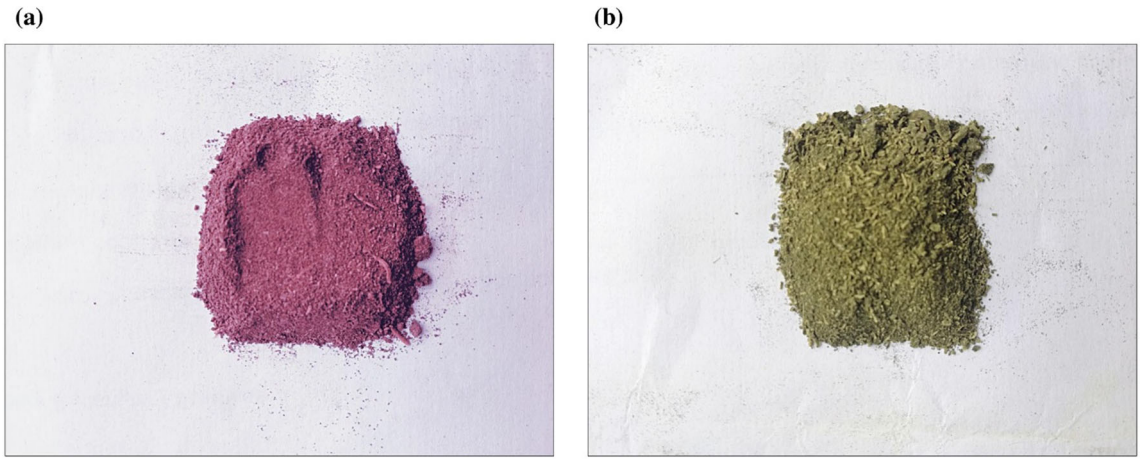

Fig. 11 Images of the powdered sample. **a** Before MAE. **b** After MAE

long duration, the betacyanin and betaxanthin pigments were degraded due to prolonged thermal exposure. Moreover, high antioxidant activity and better reducing power of *A. tricolour* leaves are directly linked to the betacyanin and betaxanthin content of leaves. FTIR spectroscopy confirmed the presence of betacyanin and betaxanthin in the extract. It was also established that MAE is better than UAE and Soxhlet extraction in terms of recovery of phytochemicals. The micrograph images showed that the microwaves were able to disintegrate the plant matrix thus facilitating the extraction. This phenomenon was further validated by the colour change in powdered samples after the MAE. Water as a green solvent was proved to be an efficient medium for extraction of the betacyanin and betaxanthin pigments. Furthermore, this method can be widely used for the recovery of plant pigments soluble in water by MAE.

Authors' contributions The experiments and analysis were performed by Alok Sharma. The research was planned and designed by Bidyut Mazumdar. The research paper was written and drafted by Amit Keshav.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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