Chapter 4

Ultrasound-assisted extraction of anthocyanin-rich extract from black rice bran and its stability study under different temperature and pH

4.1. Introduction

Naturally occurring phytochemicals have gained lots of attention as they provide certain health beneficial properties and also act as functional food ingredient [11]. Bioactive phytochemicals such as carotenoids, phytosterols, flavonoids, tocopherols, tocotrienols, ɤ-oryzanol, phenolics and other compounds function as antioxidants. These phytochemicals are also known to present naturally in aromatic black rice providing a relatively strong free-radical scavenging and antioxidant effects [23]. The color pigments in rice (dark purple, black, and red) are on account of anthocyanin and proanthocyanin compounds present in the bran [13]. The colored rice varieties have been documented to contain high phenolic content and antioxidant activities than the white rice [37]. These rice varieties have gained great attention and curiosity from the researcher and consumer preferences due to its functional properties. These properties are most important for boosting memory and body's defences [26].

The colored rice varieties have been documented to contain high phenolic content and antioxidant activities than the white rice [37]. The main phenolic acids found in those cultivars are synaptic acid, caffeic acid, protocatechuic acid, p-coumaric acid, vanillic acid, and ferulic acid that are found accumulated in a single layer of cells in the seed coat [29, 30]. Anthocyanins, a group of polyphenolic flavonoids, are reported to provide potential health benefits in minimizing the risk of cancers, heart diseases, diabetes, obesity, etc. [29, 30, 35]. These naturally occurring antioxidants can scavenge free radicals that improve the oxidative stress, inhibitory effect on cancer cell proliferation, lowering the cancer development and heart diseases [25]. Considering their nontoxicity and high biocompatibility to human body, anthocyanins drew increasing attention from food manufacturers and researchers for their usage as natural pigments and antioxidants.

In recent years, extractions of anthocyanins and polyphenolic compound have been reported using various novel extraction techniques [10, 14, 19, 31, 32, 36, 39]. Novel extraction such as ultrasound-assisted extraction (UAE) is considered to be an effective method for the extraction of anthocyanin compounds from solid samples due to its rapidity,

simplicity, low-cost, and low solvent volumes [9]. The novel extraction is based on the formation of high frequency ultrasonic waves that are capable of causing cavitation due to the expansion and contraction cycles experienced by the material. These cycles disrupt the cell walls of the solid matrix and this favors the penetration of the solvent and mass transfer, thus increasing the extraction rate. However, conventional extraction method is the traditional solvent extraction method for extracting the valuable compounds. This method usually uses higher temperature and longer time which can also degrade bioactive compounds in the extracts inducing low extraction efficiency of stable anthocyanins. UAE and conventional extraction of anthocyanin was performed and compared to pursue high recovery of polyphenols and anthocyanins with high antioxidant activity [36]. Moreover, the extraction yields are also dependent on the method of extraction process [21]. Again, supercritical fluid extractions are expensive, equipment costs are high and blockage in the system might occur [5]. Ultrasound-assisted extraction (UAE) can offer higher solvent extraction efficiency such as mass transfer, diffusion, disruptions of cells in plants, penetration of solvent and effects of capillary [7]. However, extraction efficiency depends on certain factors that include types of solvent and its concentration, time of extraction and temperature, solid-solvent ratio, pH, etc. Among all solvents, ethanol has been found as a suitable solvent to provide good yields of bioactive compounds [4].

To achieve optimized interaction parameters of the extraction process, empirical or statistical methods can be performed [16]. Response surface methodology (RSM) allows determination of effects of variables and interactions on the response factors using quantitative data. It is an effective statistical tool to optimize the responses thereby reducing the experiment numbers required. It also evaluates the parameters effect and their interaction by establishing a mathematical model. One-factor-at-a-time approach is an empirical method in which one factor vary at a time while all other factors are kept constant [31]. However, little knowledge is *accessible* in the published article about the use of RSM in UAE of phytochemicals from rice bran [7]. Many researches have been carried out on optimization of the extraction process for several phytochemicals [10, 25]. Rice bran extract have been proven good antioxidant, tyrosinase inhibition activities and can also be applied to the health food, medical, etc. [36]. In the modern days, increasing demand of healthy foods and beverages from consumers has been brought up in many parts of the world. Hence, development of functional foods and beverages with various antioxidants as functional ingredients has been enlightened.

However, stability of such compounds depends on external environment and chemical factors such as pH, metal ions, exposure to light, temperature, oxygen and enzymatic activities [1]. Stability study of these bioactive compounds have been studied and review by B¹kowska-Barczak [3]. The stability of such compounds specially anthocyanin pigments depend on different factors such as the pigment structure. With the increased in hydroxylation of anthocyanin, the stability decreases however, with increased methylation leads to increase in stability [33]. Microencapsulation is one technique to entrap such active compounds within another substance called wall material. The main principle of this technique is to protect the core material from adverse environmental conditions. Various encapsulation techniques have been successfully applied for entrapping bioactive components. A dual emulsion prior to the process of complex coacervation has been adopted for encapsulation process to improve the stability which could be obtained by the electrostatic interaction of biopolymers (oppositely charged), creating complex coacervated membranes with decreased solubility and liquid-liquid phase separation [17].

So, in this study, optimization of extraction of phenolic compounds, flavonoids, anthocyanin and antioxidant activity was carried out using ultrasound-assisted extraction targeting the maximum anthocyanin content. The kinetic model study of anthocyanin extraction was also carried out. The stability study of the anthocyanin under different pH and temperature were evaluated. The research objective was planned so as to develop functional foods as per literature published regarding the consumer interest in expanding the health beneficial properties thus providing an impulse for divulgation of new research on the microencapsulating anthocyanin.

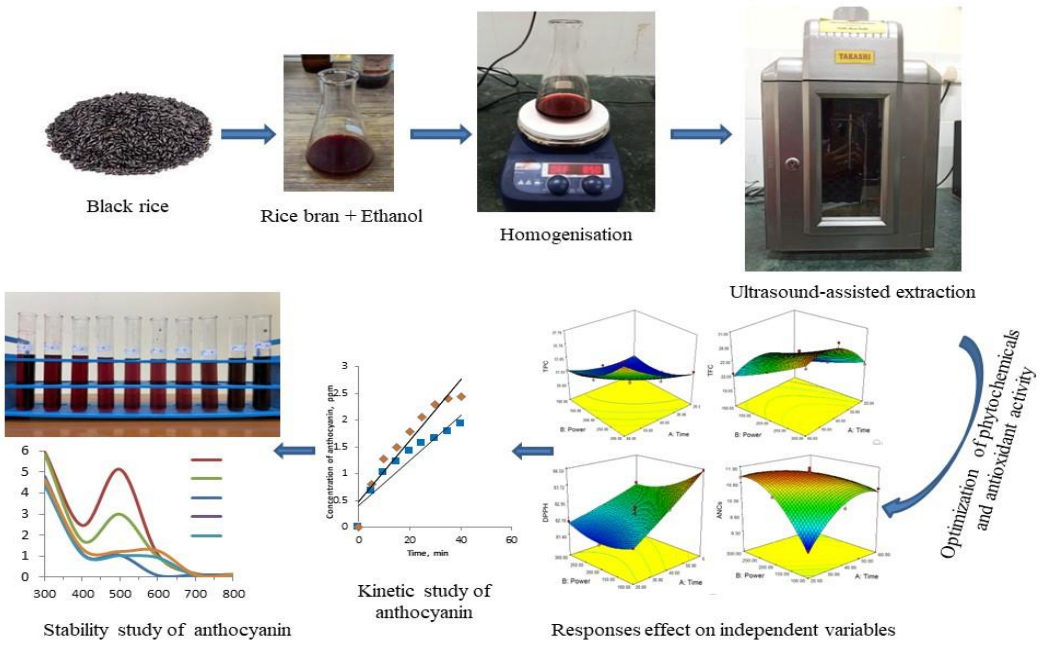

Fig. 4.1. Pictorial representation of UAE of phytochemicals

4.2. Materials and Methods

4.2.1. Rice samples, chemicals and reagents

Black rice paddy (*Oryza sativa L. indica*) was collected from the local farmer of Imphal (Manipur, India). Milling was performed using rice dehusker (APL-5KC30GF029T, Marathon, India) and foreign materials were cleaned manually. Rice bran was collected by milling black rice using rice polisher (NF366, Marathon, India). Fine particles were obtained using 500 µm mesh and packed in an air tight container and kept in the refrigeration for further use. Chemicals such as Folin-Calcalteu reagent, gallic acid, quercetin and ethanol were obtained from Sigma-Aldrich and Sisco Research Laboratories Pvt. Ltd.

4.2.2. Preliminary trial

Preliminary experiments of extraction process were performed under sonication in order to select suitable solvent, range of extraction time and power [8]. The rice bran samples were extracted using different solvents such as acidified ethanol and methanol (1 M HCl solution). The bran to solvent ratio for the extraction process was kept fixed at 1:25 w/v. Treatment time at different intervals 10, 20, 30, 40, 50, 60, 70 and 80 min and the ultrasound power of 50-350 W were carried out for the extraction process. The response

was studied using “one-factor-at-a-time” method on the extraction yield. The effect of one parameter was analyzed on the yield while the other parameter was kept fixed.

4.2.3. Extraction process

4.2.3.1. Ultrasound-assisted extraction of phenolics, flavonoids and anthocyanin

The extraction of bioactive compounds from black rice bran was performed using Ultrasonic Homogenizer (U500, Takashi, Japan) providing a power of maximum 500 W and frequency of 25 kHz. The homogenizer equipment also provides the amplitude percentage of 1 %- 99 %, which refers to the percentage of maximum power used [2]. Extract was prepared by dissolving 1 g of black rice bran in 25 mL acidified ethanol solution (70 % ethanol, 1 N HCl). According to the preliminary trials, acidified ethanol (1 N HCl) provided the best result as compared to other solvents and the same was used for the extraction process. The solution was given sonication at different combination of independent variables as per experimental design Table 4.1. After sonication, the solution was homogenized using a magnetic stirrer for 15 min. Centrifugation was performed at 4500 rpm for 15 min in order to obtain the supernatant. It was the stored under refrigeration (4 ºC) for further analysis.

4.2.3.2. Experimental design and optimization

Response surface methodology (RSM) was applied for multiple regression evaluation to determine the optimum condition of extraction process for total phenolic contents, total flavonoid contents, DPPH radical scavenging activity and anthocyanin content from black rice bran. According to the preliminary study, two independent variables extraction time (X_1) and ultrasound power (X_2) were selected. For the optimization of UAE process, amplitude percentage of 20, 40 and 60 % have been selected for analyzing the response variables which is also same as 100, 200 and 300 W. The effects for both the treatment time and ultrasound power were evaluated for determining the significant difference using the design expert software (version 7, Stat-Ease, Inc. USA). Faced centered composite design (FCCD) was performed to determine the response patterns and the optimum combination of variables. Total 13 experiments were conducted for the extraction of phenolics, flavonoids, anthocyanin and antioxidant activity. Polynomial equation was fitted to data to attain regression model equation. The significance of the terms was analyzed by Analysis of variance (ANOVA) in the regression equation. Response surface plots were generated and the numerical optimization was carried out. The effect of the

independent variables was studied using the model on the responses TPC, TFC, DPPH and anthocyanin content. The real value for the independent variables is given in Table 4.1.

4.2.4. Extract analysis

4.2.4.1. Total phenolic content

Extract was analyzed for the total phenolic content (TPC) by Folin–Ciocalteu method with some modification as described by Reddy et al., [27]. 200 µL aqueous extracts was pipetted out in a test tube and 1 mL of diluted FCR [(Folin-Ciocalteu reagent) (1 FCR:10 distilled water)] was added into it. The solution was mixed well and 1 mL of Na_2CO_3 solution was added. Stirring of the solution was done followed by dilution with deionized water to make up the final volume of 5 mL. The dispersion was allowed to stand for 1 h in the dark at room temperature. Afterwards, the absorbance was noted at 765 nm using a spectrophotometer (Spectronic 20D+, Thermo Scientific, USA). Gallic acid was used for measurement of standard. TPC were expressed as mg gallic acid equivalents per gram of black rice extract.

4.2.4.2. Total flavonoid content

The same aqueous extract was analyzed for the total flavonoid contents (TFC) as described by Mir et al., [20] with some modification. 250 µL extracts were taken in a test tube, diluted it with 1.25 mL distilled water and addition of 75 µL of $NaNO_2$ (5 %) solution into it. The mixture was kept in the dark allowed to stand at room temperature for 5-6 min and further addition of 150 µL of 10 % $AlCl_3$ solution into it. The dispersion was allowed to stand in the dark for 5 min and further addition of 0.5 mL of 1 M NaOH solution was done. The final dispersion was diluted with 3 mL with distilled water to make up the final volume to 5 mL and shaken vigorously. Absorbance was recorded immediately at 510 nm using a spectrophotometer (Spectronic 20D+, Thermo Scientific, USA). TFC were expressed as mg quercetin equivalents per g of black rice extract.

4.2.4.3. Antioxidant activity

The antioxidant activity was determined using DPPH radical scavenging activity by modifying the method described previously by Mir et al., [20]. 100 µL extract was taken in a test tube and addition of freshly prepared 1.4 mL 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanolic solution (0.2 mM) into it and shaken thoroughly. Further, 3.5 mL of methanol was added into the solution and allowed to stand in dark for 30 min. The

absorbance (Abs.) was then recorded at 517 nm. The scavenging activity was calculated by using the formula given in Eq. (4.1).

DPPH radical scavenging activity (%) = [1- (Abs. $_{517nm\ control}$ /Abs. $_{517\ sample}$)] × 100 (4.1)

4.2.4.4. Total anthocyanin content

The total monomeric anthocyanin pigment content of the rice bran samples was measured by following the pH differential methods [28]. Extract 0.5 mL was mixed with 3.5 mL potassium chloride buffer (0.025 M, pH 1). The solution was stirred and vortexed and then allowed to stand for 15 min. Again, the extract was mixed with sodium acetate buffer (0.025 M, pH 4.5), vortexed and allowed to stand for 15 min. The absorbance of the sample for both the solutions were recorded at 515 and 700 nm against the blank acidified ethanol using a spectrophotometer (Spectronic 20D+, Thermo Scientific, USA).

The TAC was analyzed based on the cyanidin-3-glucoside content using Lambert-Beer Law as follows (Eq. 4.2):

TAC (mg/L) = [(Abs. × Extract volume × MW × 10^6) / (1000 × ε × M)]/1000 (4.2)

Where: MW = molecular wt. of cyanidin-3-glucoside (449.2 g/mol);

ε = molar absorptivity of cyanidin-3-glucoside (25,965 L/cm mol); and

M= mass.

4.2.5. Kinetics study of anthocyanin extraction

The same extraction was carried out with the same extraction techniques of UAE (2.2.1). The solute to solvent ratio was kept at ratio of 1: 25 (g/mL). Different experiments were carried out at different time intervals under the optimized condition of UAE. Experimental data for ultrasound-assisted extraction of anthocyanin were analyzed and fitted to kinetic model. The data were compared with conventional extraction process of anthocyanin. For conventional extraction process, the solution was kept in the orbital shaker at 800 rpm under control environment condition for the kinetic study. After extraction, the supernatant was filtered and kept under refrigeration for further analysis.

The first order kinetic model equation is given in the Eq. (4.3).

$C_t = C_\infty (1- e^{-kt})$

$\Rightarrow \ln [(C_\infty - C_t)/C_\infty] = -kt$ (4.3)

Where, C_t is the Concentration of the reactant or product at time t, C_∞ is the corresponding concentration at the end point and k is the first order rate constant (s^{-1}) and t is time (s).

4.2.6. Stability study of phytochemicals

The phenolic, flavonoids and anthocyanin compounds are known to be sensitive and affected by external environment such as temperature, pH, light, oxygen, etc. However, these compounds are an ideal component for application in functional foods. Therefore, prior to formulation process in a food matrix, the stability of these active compounds needs to be determined in all processing steps [11]. Following phytochemicals effects were analyzed under different temperature and color effect on differrent pH.

4.2.6.1. Effect of temperature

The same extract under optimized condition were treated and maintained with different temperatures at 30, 40, 50, 60, 70, 80 and 90 ˚C for 30 min. The effect of heat treatment on the optimized extract were evaluated by modifying the method described by Jiang et al., [15] The solution was vortexed for 3 min and the extract was collected. Then, the total phenolics, flavonoids, anthocyanin content and antioxidant activity under these temperatures were determined. Finally, the effect of temperatures on phytochemical compounds were studied.

4.2.6.2. Effect of pH

The pH of the optimized extract was maintained using 1 M HCl viz. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11. The effect of pH on the extract was evaluated. The stability study of anthocyanin extract on different pH was carried out by modifying the process described in Wahyuningsih et al., [35].

4.2.6.3. Effect of color / Characterization of anthocyanin extracts in various pH

The color potency of anthocyanin extract was measured on eleven pH conditions (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11). Each solution was measured at highest wavelength using Hunter Lab colorimeter (Ultrasound VIS, Hunder Lab. Inc., USA) that absorbed the range on CIE color scale L*, a* and b*.

4.2.7. Statistical analysis

Model fitted were analyzed for each response by multiple regressions. Analysis of variance (ANOVA) was used to determine the significance of the model. Lack of fit test, regression coefficient (R), Adj. regression coefficient (Adj. R^2), predicted regression coefficient (Pred. R^2), adequate precision and residual sum of squares were used to analyze the model adequacies. Optimization of extraction parameters was carried out using mathematical tool of RSM. All the analyses apart of RSM have been performed in triplicates manner. Data have been depicted as mean ± SD. Significant differences tests between means have been analyzed using Duncan's multiple range test at the level of significance ($p \leq 0.05$) using SPSS Statistics 20.

4.3. Results and Discussion

4.3.1. Phytochemical extraction and its antioxidant activity

According to preliminary trial, acidified ethanol provided the highest yield. Different solvents have variation in the phenolics, flavonoids, anthocyanin contents and its antioxidant activity and the maximum value was observed in ethanolic solution. This might be because of higher diffusion taking place in ethanol as compared to other solvents. Similarly, the effect of extraction time was observed to be more prominent at longer duration (50 and 60 min) as compared to extraction in short duration (10, 20 min). Moreover, increasing in extraction time (upto 80 min) only utilizes energy and solvent evaporation in the extraction process. After certain interval of time, solute concentrations in the matrix becomes stable and no additional diffusion takes place in the extraction process [22]. Again, prolonged diffusion of these compounds in the extraction solvent led to oxidation of phenolic compounds [8] which leads to reduction in antioxidant activity. Therefore, for additional exploration, extraction time was selected from 20 to 60 min.

4.3.2. Model fitting

A face centered composite design (FCCD) was employed to attain the optimum extraction condition maximizing the responses, which was analyzed using Design Expert software (version 7, Stat-Ease, Inc. USA). The experimental design for each run and responses is presented in Table 4.1. Mean experimental responses for 13 different combinations of experimental runs varied from 30.16 to 37.62 mg gallic acid/g TPC, 20.16 to 30.70 mg quercetin/g TFC and 9.42 to 11.27 mg cyanidin-3-glucoside/L ANCs respectively (Table

4.1). DPPH radical scavenging assay was used to find out the antioxidant activity of the bran extracts and Table 4.1 showed to vary from 81.48 to 84.45 %.

The real values of independent variables for each run are also shown. The developed correlation between dependent and independent variables is given in terms of 2^{nd} order polynomial equation and regression analysis was followed. The regression equations so obtained from the developed model for the extraction process are shown in Eq. (4.4, 4.5, 4.6 and 4.7).

$$TPC = +35.92 - 0.16 \times X_1 - 0.05 \times X_2 + 4.4875 \times 10^{-3} \times X_1 \times X_2 + 1.90 \times 10^{-3} \times X_1^2 + 1.19 \times X_2^2 \quad (4.4)$$

$$TFC = +8.82 + 0.64 \times X_1 - 2.56 \times 10^{-4} \times X_2 + 3.76 \times 10^{-4} \times X_1 \times X_2 - 7.73 \times X_1^2 + 3.59 \times X_2^2 \quad (4.5)$$

$$ANCs = +4.12 + 0.17 \times X_1 + 0.04 \times X_2 - 3.47 \times 10^{-4} \times X_1 \times X_2 - 1.31 \times 10^{-3} \times X_1^2 - 0.51 \times 10^{-4} \times X_2^2 \quad (4.6)$$

$$DPPH = +81.61 + 0.07 \times X_1 - 0.01 \times X_2 - 1.83 \times 10^{-4} \times X_1 \times X_2 + 1.57 \times 10^{-4} \times X_1^2 + 3.78 \times 10^{-5} \times X_2^2 \quad (4.7)$$

Where, X_1 and X_2 denote extraction time and power respectively.

The obtained data from FCCD were analyzed using surface regression responses and have developed a 2^{nd} order polynomial relationship with R^2 value of 0.94 for TPC, 0.95 for TFC, 0.98 for ANCs and 0.87 for DPPH respectively shown in Table 4.2. This indicates that the sample variation of 94.10 %, 95.41 %, 98.05 and 86.98 % for extraction of TPC, TFC, anthocyanin content and antioxidant activity respectively attributed to the independent variables and only 5.9 %, 4.59 %, 1.95 % and 13.04 % could not be explained by the model. Again, the adjusted R^2 value can be compared to R^2 (Table 4.2) and the regression model is best fitted model as the difference is found negligible. The obtained regression parameters for ANOVA are shown in Table 4.2.

Table 4.1. Faced centered composite design of two variables with their observed responses

Sl. no.	Extraction time, X_1 (min)	Ultrasound power, X_2 (W)	TPC (mg gallic acid/g)	TFC (mg quercetin/g)	DPPH (%)	ANCs (mg cyanidin-3-glucoside/L)
1	20	100	30.38	20.16	81.94	9.43
2	20	200	30.16	21.25	81.48	10.63
3	20	300	32.43	23.39	82.04	10.98
4	40	100	30.84	22.67	83.09	10.31
5	40	200	31.80	26.24	82.76	11.27
6	40	200	30.38	25.38	82.59	11.14
7	40	200	31.20	26.60	82.76	11.09
8	40	200	30.75	26.70	81.61	11.05
9	40	200	31.52	26.77	82.43	11.20
10	40	300	33.07	30.70	82.27	10.78
11	60	100	31.98	22.32	84.45	10.65
12	60	200	32.89	25.22	83.25	10.43
13	60	300	37.62	28.56	83.09	9.42

From Table 4.2, it can be observed that the model and regression developed were found to be significant at $p < 0.05$ and the lack of fit was insignificant ($p > 0.05$) for all the responses. The lack of fit thus implies that the model developed were found right for the data obtained for all experiments and showed best correlation among the variables. It is not possible to obtain an ideal fit that creates the lack of fit not significant and model significant in a bio-system where there is repetition of experiment [7].

Table 4.2. ANOVA table for fitted model of phytochemical contents and antioxidant activity

Sources	Sum of squares	F	p value	C.V %	R^2	Adj R^2	Pred R^2	Adeq. precision
TPC	43.37	22.32	0.0004	1.95	0.9410	0.8988	0.6760	16.32
TFC	103.13	29.09	0.0002	3.36	0.9541	0.9213	0.6384	17.58
DPPH	6.52	9.35	0.0053	0.45	0.8698	0.7768	0.7613	11.30
ANCs	4.48	70.39	<0.0001	1.06	0.9805	0.9666	0.8791	23.13

4.3.3. Effect of process variables on phenolic and flavonoid content

The effect of the process variables and their mutual correlation on extraction of phenolic and flavonoid contents were examined by plotting 3-dimensional surface response curves of multiple quadratic regression models (Fig. 4.2). The response surfaces were generated by plotting Z-axis verses two process variables. The only important parameter for selecting the optimum extraction time is thermal degradation of phytochemical compounds taking place with variation with respect to time. With the increased in extraction time from 20 to 40 min, the total phenolic and flavonoid content increases shown in Table 4.1. While increasing in extraction time to 60 min, the highest TPC (37.62 mg gallic acid/g) and TFC (28.56 mg quercetin/g) were found. Both extraction time and power have significant effect on toral phenolic and flavonoid content. It was also reported that the TPC was reduced when the extraction time further increased to 90 min in Thai rice bran extracts [24].

The model equation (Eq. 4.4) showed that both the linear terms; extraction time and power have a negative effect on TPC while the interaction term for both has a positive effect on TPC. From Table 4.1, it is also observed that increase in extraction time, TPC increases; and the same for power too. The effect of the process variables and their mutual interaction on the TPC during extraction process was investigated from the response surface curve of non-linear regression models [Fig. 4.2 (a) and (b)]. Again, the extraction time has positive effect while power has negative effect on TFC (Eq. 4.5). This can also be observed from Table 4.1 that with increasing extraction time from 20 to 60 min, the TFC increases. However, from Fig. 4.2 (b), the response surface curve slightly increases up to increase in

extraction time up to 40 min and with further increase in extraction time up to 60 min, the TFC decreases. This decrease in TFC may be due to volatilization of the flavonoid compounds caused by heat generation due to sonication (increase in power) and some unwanted reactions like enzymatic degradation and oxidation. Increasing treatment time using ultrasound induces disintegration of phytochemical compounds [39]. It has also been reported previously that the ultrasound-assisted extraction intensifies the efficiency of extraction process with increased yields and reducing the extraction time [7].

4.3.4. Effect of process variables on anthocyanin content and antioxidant activity

Fig. 4.2 (c and d) showed the effect of extraction time and power on total anthocyanin content and antioxidant activity of bran extract. As observed in Table 4.1, the total anthocyanin content was found highest (11.27 mg cyanidin-3-glucoside/L) at the central point (40 min, 200 W). It may be due to the presence of a stable form of the red flavylium cation in the bran extract. Moreover, the result illustrated that increasing in ultrasound power from 100 to 300 W (20 min) increases the total anthocyanin content and antioxidant activity. Whilst the maximum DPPH radical scavenging activity was found to be 84.45 % at 60 min, 100 W. Ultrasonication leads to growth and bubbles cavitation in the liquid surface generated by ultrasound irradiation [6]. However, examining this study, the rise in temperature upto (60 ˚C) due to sonication was observed and hence, has enhanced the extraction process making it more efficient for extraction (upto 60 min). Therefore, the study was conducted only in the combination using extraction time (20 to 60 min) and power (100-300 W).

Prolonged treatment of extraction time and power can lead to decomposition of anthocyanins. This may be due to formation of colorless structures where the cation (red flavylium) gets saturated by hydration to colorless carbinol [12]. In the combination (60 min and 300 W), increase in temperature (up to 60-70 ˚C) was observed that influence the phytochemical compounds and DPPH radical scavenging activity. The rise in temperature due to sonication at prolonged extraction time could degrade some bioactive compounds which were produced into the extracting solvent [32]. This increase in temperature may be the reason for decomposition of bioactive compounds such as phenolics, flavonoids and anthocyanins that causes decreased in their antioxidant activity at higher extraction time (60-90 min). However, significant difference was not observed in DPPH values at 60 and 90 min of extraction time [24]. Hence, it can be also observed in Fig. 1 (d) that with

increase in ultrasound power from 100 to 300 W (60 min), the total anthocyanin content decreases showing the decomposition of some of the anthocyanin compound forming colorless structures. The degradation of anthocyanin in the extract may be due to various sonochemical reactions, including free radical generation, polymerisation/depolymerisation and some other reactions.

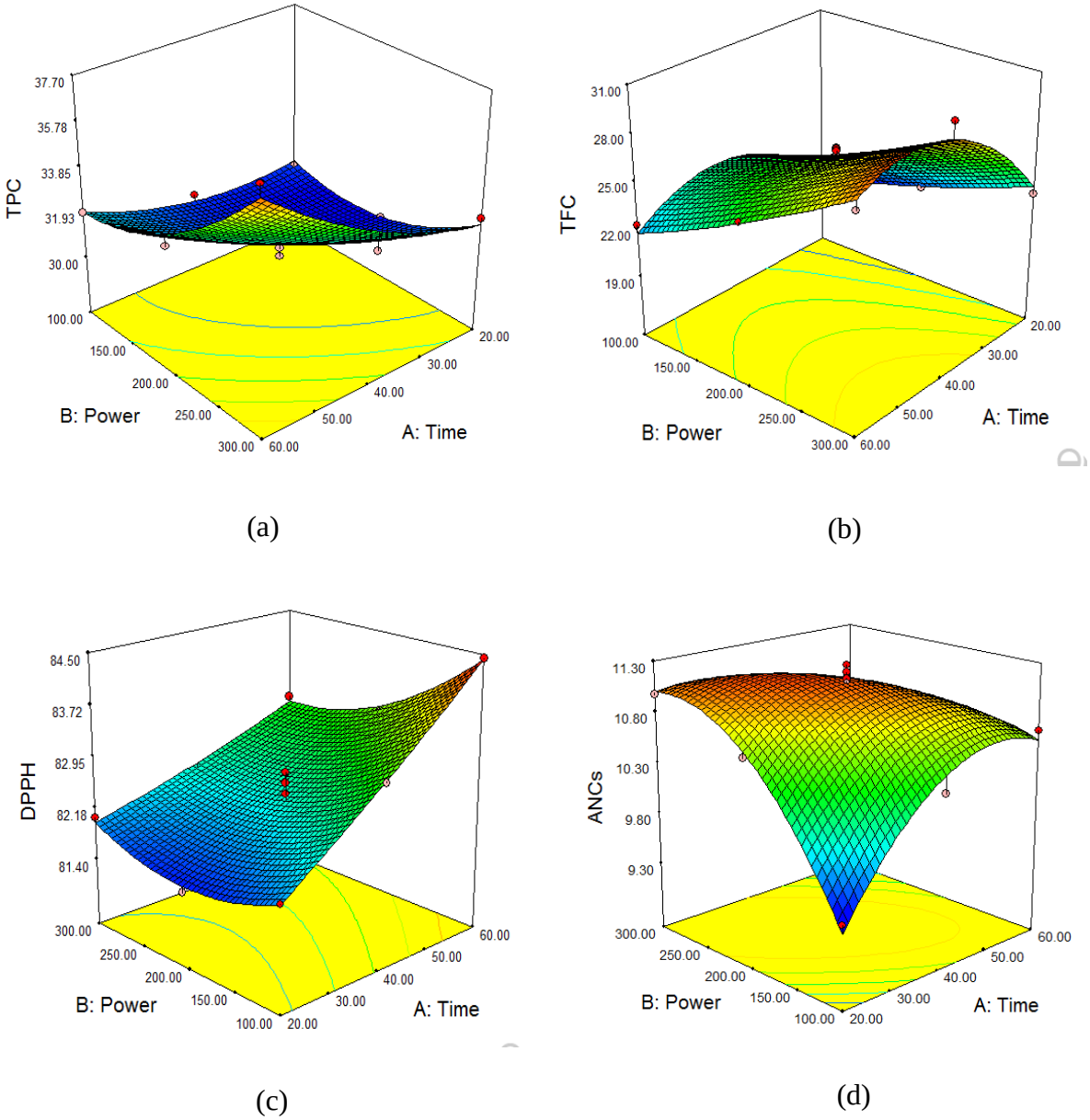

Fig. 4.2. 3-D plots for the effect of extraction time and power on (a) total phenolic content (b) total flavonoid content (c) DPPH radical scavenging activity (d) total anthocyanin content

4.3.5. Optimization and validation of extraction process

The main aim for optimization step was to attain the highest response variables. The maximized responses were total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC) and antioxidant activity (DPPH). The optimization of extraction condition of TPC, TFC, TAC and DPPH from black rice bran were performed. From Table 4.1, it could be observed that the model developed (Eq. 4.4, 4.5, 4.6 and 4.7) can efficiently predict the TPC, TFC, TAC and DPPH value in the UAE extract. Therefore, the model developed was used to optimize the parameters for UAE. Based on the desirability, the optimal condition was selected for the highest content of phytochemical contents and antioxidant activity and was obtained at 33.78 min and 234.01 W. The optimized conditions with their responses for the experimental value of TPC was found to be higher (32.69 mg gallic acid/g) than the predicted value (31.21 mg gallic acid/g), while for TFC, the predicted value (26.41 mg quercetin/g) is higher than the experimental value (26.08 mg quercetin/g). Similarly, the DPPH value was observed to be higher in predicted value (82.09 %) and ANCs value was higher in experimental value (11.63 mg cyanidin-3-glucoside/L) than the predicted value (11.16 mg cyanidin-3-glucoside/L) (Table 4.3).

Table 4.3. Predicted and experimental values of UAE of phytochemical compounds

Values	TPC (mg gallic acid/g)	TFC (mg quercetin/g)	DPPH (%)	ANCs (mg Cyanidin-3-glucoside/g)
Predicted value	31.21	26.41	82.09	11.16
Experimental value	32.69	26.08	80.24	11.63

4.3.6. Extraction kinetics

It was observed that the anthocyanin extraction behaviour was suitable for the first order kinetics as shown in Fig. 4.3. The reaction rate constant of the ultrasound assistant extraction (0.0574) was higher than conventional extraction process (0.0424). Similar results were also obtained reported by Das et al., [8]. The propagation of ultrasound waves and subsequent cavitation have been linked to yield improvement in ultrasound-assisted

extraction. High shear stress caused by the ultrasonic wave accelerates the bulk transfer of ANCs compounds from the rice bran. Additionally, the ultrasonic wave leads to particles disintegration, that creates additional surface area and boosts mass transfer. However, cavitation bubbles burst and create macro-turbulence, speeding up eddy diffusion and internal diffusion by causing collisions of high-velocity particles and agitation in the bran's microporous particles. Therefore, the ultrasound assistance extraction showed the high reaction rate constant in term of ANCs in the bran extract over conventional extraction process.

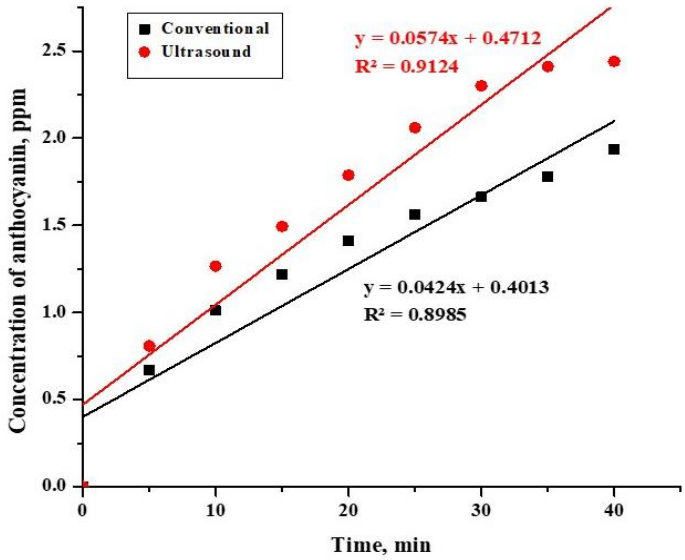

Fig. 4.3. Kinetic study of anthocyanin extraction for UAE and conventional extraction

4.3.7. Stability study

Stability study was performed by determining the effect of temperature, pH and color under optimum condition of the UAE process. The optimum condition was maximized on the response ANCs considering other responses (TPC, TFC and AA) in range and the optimum condition parameters were 33.78 min and 234.01 W. Under this condition the stability study of anthocyanin was determined.

4.3.7.1. Effect of temperature

The extraction process was conducted at 30, 40, 50, 60, 70 and 80 ˚C under the optimized condition. The temperature has an influence on all the responses (TPC, TFC, AA and ANCs). As observed in Table 4.4, there is an increase in the total phenolic content with

the increase in temperature from 30 to 50 ˚C. But with further increasing the temperature to 80 ˚C, the TPC decreases. As also discussed in section in 3.4, prolonged treatment of bioactive compounds at high temperature causes degradation [31]. Similarly, the anthocyanin content shows the same trend with increasing temperature. Decomposition of the phenolic components might be the reason causing to reduce the anthocyanin content leading to brown in color. This result could be associated to the absorption spectrum of anthocyanins and phenolic compounds and the absorbance spectrum is highest at 520 nm for anthocyanins [37] and 517 nm for phenolic compounds [33]. However, total flavonoid content remains almost the same content although there is rise in temperature. The antioxidant activity also increases with the increase in temperature. There is significant difference at $p \leq 0.05$ in total phenolic content, total flavonoid content, DPPH and anthocyanin content at different temperature as shown in Table 4.4. Moreover, ultrasound is also assisted causing implosion of bubbles in the solution matrix, may decompose these bioactives which was released in the extracting solvent [31].

Table 4.4. Effect of temperature on phytochemicals and antioxidant activity

Temperature (˚C)	**TPC (mg gallic acid/g)**	**TFC (mg quercetin/g)**	**DPPH (%)**	**ANCs (mg Cyanidin-3-glucoside/L)**
30	34.75 ± 0.53^{b}	24.40 ± 0.68^{a}	84.93 ± 1.38^{d}	10.58 ± 0.57^{c}
40	35.28 ± 0.33^{b}	25.45 ± 0.40^{a}	83.84 ± 0.77^{cd}	10.71 ± 0.56^{c}
50	37.58 ± 1.16^{c}	33.06 ± 1.63^{b}	76.91 ± 0.67^{a}	11.84 ± 0.10^{d}
60	35.13 ± 0.90^{b}	35.37 ± 0.97^{c}	82.82 ± 0.54^{bc}	8.77 ± 0.70^{b}
70	32.81 ± 0.78^{a}	33.57 ± 1.10^{bc}	82.90 ± 1.51^{bc}	8.50 ± 0.47^{b}
80	31.36 ± 0.90^{a}	34.51 ± 0.80^{bc}	81.25 ± 1.21^{b}	7.53 ± 0.31^{a}

All data are the mean ± SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$)

4.3.7.2. Effect of pH on anthocyanin

Anthocyanin is being affected by several factors such as structure of pigment, temperature, light, copigments, metal ions, pH, oxygen, enzymes and antioxidants [17]. The molecular structure of some anthocyanins found to be more stable than others [32]. It was reported that increasing hydroxylation reduces stability while increasing methylation increases it. The present study indicates that at acidic conditions (low pH), anthocyanins are red shown in Fig. 4.4, higher the pH value (alkaline conditions) anthocyanins provide color fading and darker in color. These anthocyanins (red-colored pigments) are mainly in the form of flavylium cations [3] and are more stable at low pH solution. This flavylium cation developed allows the anthocyanin to be more soluble in water. Figure 4.4 indicates the absorption spectrum of ANCs shift to a higher wavelength. In general, the spectrum exhibits a peak of ANCs at wavelength range from 400-600 nm. pH 1 provide the spectra with higher absorption and appear more bright red in color which again appear little fade in color in pH 3 and 5 and so on. When exposed to heat, it exhibits less stable causing the color to fade and browning. The reason for this color fading is that at reduced pH, the cyanidin molecule is protonated and creates a positive ion or cation, as the pH increases the molecules become deprotonated, at higher pH the molecule forms a negative ion or anion [34].

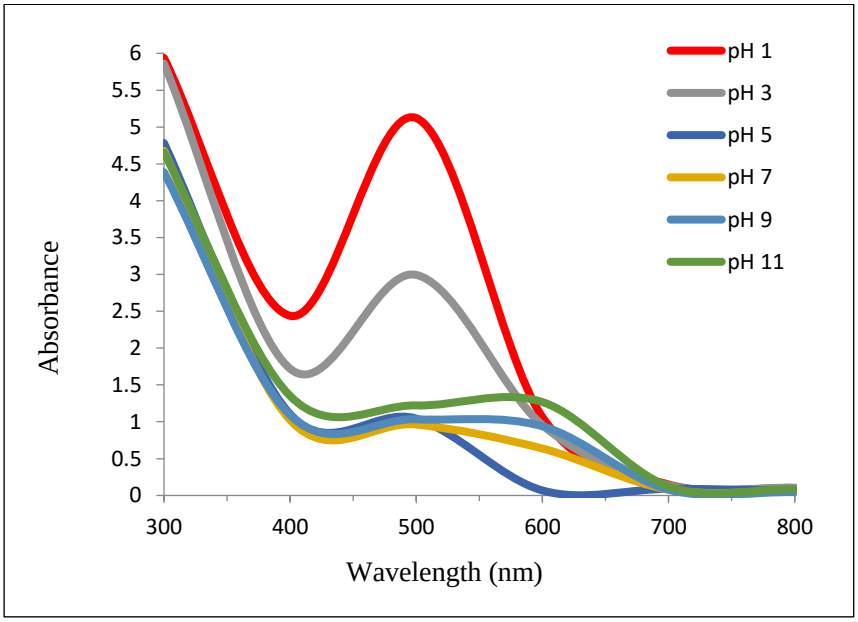

Fig. 4.4. Effect of pH

4.3.7.3. Color stability of anthocyanin at different pH

The color of anthocyanins is dependent on the pH of the solution which is due to the structure of anthocyanins having an ionic nature [32]. The molecular structure of anthocyanins tends to change at different pH which is shown in Fig. 4.5. It was observed in Table 4.5 that at acidic condition, more lightness and redness was observed which reduces with increase in pH value. The value of L* indicates lightness (100) to darkness (0), a* indicates redness (100) to greenness (0) and b* indicates blueness (100) to yellowness (0). With increasing pH value, L* value decreases which indicate that the color change to darkness from lightness. a* value also reduces which indicate that the color tends to change to yellowness. There is no significant difference in the L* value as observed in Table 4.5. However, a* and b* value differ significantly at $p \leq 0.05$.

Table 4.5. Color stability at different pH

pH	Parameters		
	*L**	*a**	*b**
1	23.69 ± 0.92^{a}	3.76 ± 0.49^{e}	0.25 ± 0.04^{abcd}
2	23.31 ± 0.89^{a}	3.61 ± 0.34^{e}	0.31 ± 0.04^{bcd}
3	23.37 ± 0.27^{a}	2.77 ± 0.20^{d}	0.38 ± 0.04^{cde}
4	23.38 ± 0.41^{a}	$2.53\pm0.^{27cd}$	0.36 ± 0.08^{cde}
5	23.46 ± 0.51^{a}	2.45 ± 0.25^{cd}	0.47 ± 0.20^{de}
6	23.38 ± 0.51^{a}	2.23 ± 0.22^{bc}	0.61 ± 0.22^{e}
7	23.53 ± 0.31^{a}	1.92 ± 0.06^{b}	0.59 ± 0.33^{e}
8	23.32 ± 0.62^{a}	1.89 ± 0.15^{b}	0.23 ± 0.07^{abcd}
9	23.25 ± 0.20^{a}	1.16 ± 0.09^{a}	0.08 ± 0.01^{ab}
10	23.36 ± 0.36^{a}	1.14 ± 0.20^{a}	0.03 ± 0.01^{a}
11	23.25 ± 0.13^{a}	1.12 ± 0.18^{a}	0.12 ± 0.02^{abc}

All data are the mean ± SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$)

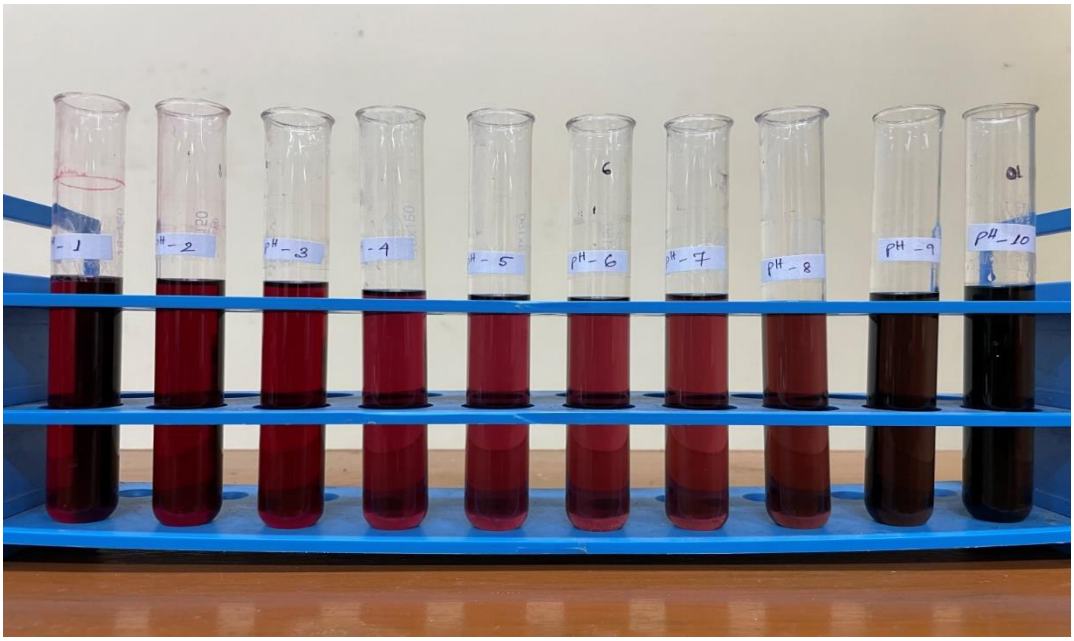

Fig. 4.5. Color of anthocyanin extract in various pH

4.4. Conclusions

The study concluded that ultrasound-assisted extraction could be an effective method for extraction of active plant material. Considering the proposed objectives and the results obtained, the optimum conditions in term of ultrasound power and extraction time were evaluated for maximum extraction of phenolics, flavonoids, anthocyanin and antioxidant activity and found to be 33.78 min and 234.01 W. The effects of ultrasound power and extraction time on the responses were evaluated and their mutual correlations were determined with empirical mathematical explanation. However, the results revealed that prolonged treatment of extraction time and power can lead to decomposition of anthocyanins. Cyanidin-3-glucoside is the predominated anthocyanin found in black rice bran as compared to peonidin-3-D-glucoside. The anthocyanin was successfully encapsulated with gelatin and acacia gum as wall materials using dual emulsion technique. The study suggested that the microcapsules thus developed could be used as a potential source of stable nutraceuticals in pharmaceutical industries working toward systems for controlled release of natural antioxidant (anthocyanins) and other water-soluble bioactive compounds which can also replace synthetic antioxidants.

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