Optimization of ultrasound and microwave assisted extractions of polyphenols from black rice (*Oryza sativa* cv. Poireton) husk

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Abstract Phenolic compounds were extracted from the husk of milled black rice (cv. Poireton) by using a combination of ultrasound assisted extraction and microwave assisted extraction. Extraction parameters were optimized by response surface methodology according to a three levels, five variables Box–Behnken design. The appropriate process variables (extraction temperature and extraction time) to maximize the ethanolic extraction of total phenolic compounds, flavonoids, anthocyanins and antioxidant activity of the extracts were obtained. Extraction of functional components with varying ethanol concentration and microwave time were significantly affected by the process variables. The best possible conditions obtained by RSM for all the factors included 10.02 min sonication time, 49.46 °C sonication temperature, 1:40.79 (w/v) solute solvent ratio, 67.34% ethanol concentration, and 31.11 s microwave time. Under the given solutions, the maximum extraction of phenolics (1.65 mg/g GAE), flavonoids (3.04 mg/100 g), anthocyanins (3.39 mg/100 g) and antioxidants (100%) were predicted, while the experimental values included 1.72 mg/g GAE of total phenolics, 3.01 mg/100 g of flavonoids, 3.36 mg/100 g of anthocyanins and 100% antioxidant activity. The overall results indicated positive impact of co-application of microwave and ultrasound assisted extractions of phenolic compounds from black rice husk.

Keywords Black rice · Polyphenols · Ultrasound assisted extraction · Microwave assisted extraction · Response surface methodology

Introduction

The black rice has many nutritional characteristic and fall under the categories of underutilized crops because of its limited consumption. Black rice is rich in phytic acid, flavonoids, polyphenols, γ -oryzanol and vitamin E which are beneficial with respect to human health. These antioxidant compounds play a vital role by eliminating reactive oxygen species (ROS) such as superoxide anion radicals and lipid peroxide that can lower cholesterol content (Ichikawa et al. 2001; Nam et al. 2008). Black rice consists of some vital secondary metabolites localised in the pericarp and aleurone layers of the seeds such as peonidin-3-*O*-glucoside anthocyanins, and cyanidin-3-*O*-glucoside (Abdel-Aal et al. 2006). There are many anthocyanins which have been identified in pigmented rice by various authors such as peonidin-3-glucoside and cyanidin-3-glucoside (Hu et al. 2003); 5-diglucoside, pelargonidin-3, malvidin, cyanidin-3-glucoside and cyanidin-3, 5-diglucoside (Zhang et al. 2006); cyanidin-3-glucoside and pelargonidin-3-glucoside (Yawadio et al. 2007). Pal et al. (2016) has shown that germinated brown rice has more nutritional value as compared to brown rice and it can be utilized as functional ingredients in the preparation of rice-based products. During germination in brown rice, higher accumulation of γ -aminobutyric acid, histidine, arginine, proline, methionine and acidic amino acids have been observed. Germinated brown rice has also shown increase in content of ferulic acid, vanillic acid, caffeic acid, syringic acid and p-coumaric acid. Husk and bran are the

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products with low value fraction and are an outcome of commercial rice-milling process. Due to the inedible nature of rice husk it is applied as low-value waste materials in various non-food applications. However, rice husk has been identified to provide important nutritional benefits such as protection of rice seed from oxidative stress by using an antioxidant defence system (Ramarathnam et al. 1988).

Several types of new techniques of extraction viz., microwave assisted extraction (MAE), solvent extraction, supercritical fluid extraction, and ultrasound assisted extraction (UAE) are available for extracting value added functional food from plant based sources (Wang and Weller 2006). Among the above mentioned UAE is a fairly economical process, due to its simple and low instrument related requirements and is an exclusive substitute of conventional extraction techniques. Acoustic cavitation induced in the solvent due to ultrasound wave is being considered as the main factor for increase in extraction (Wang et al. 2008). Mechanical effects produced by ultrasound leads to higher insertion of solvent through cavity formation in sample matrix, thus enhancing area of contact across the solid–liquid phase, and therefore a quick diffusion of solute from the solid phase to the solvent takes place (Rostagno et al. 2003). Application of higher ultrasonic frequency leads to the enhancement of OH radical formation; although phenolic compounds play a vital role of avoiding the reaction with OH radicals due to their hydrophilic nature (Chowdhury and Viraraghavan 2009). UAE is the best option considering its less involvement in chemical reactions and reduced time of extraction and also avoids the loss of chemical properties of targeted compounds (Wang and Weller 2006).

Generally in soxhlet extraction, maceration and heat reflux are the main conventional methods for extracting polyphenols with low yields in spite of its easy operation (Masawaki et al. 1996; Derksen et al. 1998, 2002). Higher rate and superior recovery coupled with reducing solvent consumption and extraction time has grabbed the attention for MAE especially in cosmetic, pharmaceutical and food industries. Several authors have reported the effects of MAE factors, such as extraction time, microwave power, particle size, solvent concentration and solvent–solute ratio (Chen et al. 2007; Hemwimon et al. 2007; Rostagno et al. 2007; Spigno and DeFaveri 2009; Xiao et al. 2008).

Classical method of optimizing the medium mainly involves the changing of one independent factor and keeping rest of the factors constant. Interaction among the factors poses as the major barrier in finding the optimum with respect to multifactor experimental design, and also makes the conventional methods time-consuming (Liu and Tzeng 1998). Response surface methodology (RSM) is a vital tool for optimization which reduces the number of

experiments and provide sufficient information to make it more acceptable statistically (Giovanni 1983). This statistical tool helps to examine the effect of test factors on the response, determination of interrelationship among the test variables and description of the combined effect of them. RSM can be described as an agglomeration of statistical tools and techniques for finding the functional relationship between response variables and a set of design variables (Venter 1998). Recently, Singh et al. (2017) also found the UAE assisted acetone extraction conditions from RSM for extraction of total phenol content, total flavonoids content, total antioxidant activities, ferric reducing power and DPPH radical scavenging activity and revealed higher yield in hull, than in whole mung bean or cotyledon, and the yield was higher than conventional method. Box–Behnken designs are available for 3–7 factors and require only 3 levels, coded as -1 , 0 and $+1$. It allows prediction of the combined effects of three controlled parameters. Fewer numbers of runs are required as it minimizes the number of factor combination (Chonga et al. 2013). The husk of black rice which is usually treated as waste material has been found to contain high polyphenols during our preliminary investigation and therefore the present study has been primarily focussed to optimize the extraction of polyphenols from the husk by using UAE and MAE for potential development of functional foods.

Materials and methods

Materials

Black rice cv. Poireton was collected from five different cultivation zones of Imphal region of Manipur, India and the husk was removed using a mechanical dehusker (RTE-07, A-GRAIN, India) (Jha et al. 2015). The husks were pooled, dried and a mechanical grinder was used to powder it, and was passed through a 0.841 mm sieve and stored in dehydrated condition for further use.

Experimental design

Optimization of the experimental design and evaluation of important effects like quadratic effects and interaction effects due to formulation ingredients and their impact on extraction yields of polyphenols was carried out by using Box–Behnken design (BBD). Following the principle of BBD, sonication temperature of 40–60 °C, sonication time of 10–40 min, solute: solvent ratio of 1:20–1:50, ethanol concentration of 40–70% and microwave time of 20–60 s, were chosen as the process variables and evaluated in a 46 sets of experiments and selected variables had significant effects on the yields. Five factors chosen were designated

Table 1 Independent variables and their levels employed in the optimization process

Variables	Code	Units	Low	Centre	High
Sonication time	X ₁	Min	10	25	40
Sonication temperature	X ₂	°C	40	50	60
Microwave time	X ₃	s	20	40	60
Ethanol concentration	X ₄	%	40	55	70
Solute:solvent	X ₅	Ratio	1:20	1:35	1:50

as X₁, X₂, X₃, X₄, and X₅ and (Table 1) and factors were represented as three levels, and coded as +1, 0 and −1 for high, intermediate and low value, respectively. Test variables were coded according to the Eq. 1.

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (1)$$

where, x_i represents the coded value of an independent variable; X_0 is the actual value of an independent variable at center point; X_i denotes actual value of an independent variable and ΔX is the step change value of an independent variable. A second-order polynomial model was fitted for predicting the optimal point and to correlate relationship between independent variables and the responses of total phenolic content, flavonoid, anthocyanins and antioxidant activities. Equation 2 represents for the five factors and is given as follows.

$$Y = B_0 + \sum_{i=1}^5 B_i x_i + \sum_{i=1}^4 \sum_{j=i+1}^5 B_{ij} x_{ij} + \sum_{i=1}^5 B_{ii} x_i^2 \quad (2)$$

where, Y represents the response variable for various yields in real values; B_j , B_i and B_{ij} are the regression coefficients of variables for intercept quadratic, linear, and interaction terms, respectively; X_i and X_j are independent variables ($i \neq j$).

Ultrasound assisted extraction (UAE)

Black rice husk powder (2 g) was taken in a flask and UAE was carried out with a useful volume of 5 L in a sonication water bath (Sonorex RK510H, Bandelin, Germany). The working frequency and power were selected with a value at 35 kHz and 160/640 W, respectively.

Microwave assisted extraction (MAE)

The same flask used in the UAE was then subjected to MAE and was carried out using microwave oven (MC-8087ABR, LG Electronics, India) with constant frequency of 2450 MHz.

Centrifugation after extraction

The centrifugal temperature, duration and rotational speed were set at 4 °C, 15 min and 10,000 rpm, respectively. The supernatant was mixed with anhydrous ethanol to make the final volume of the extract to 50 mL. The extracts were preserved at 4 °C till the analysis was accomplished.

Analysis for total phenolic compounds (TPC)

This was carried following the standard method of Bray and Thorpe (1954) and TPC was expressed in terms of gallic acid equivalent (mg GAE/100 mL).

Estimation of flavonoids

Total flavonoid content was determined by aluminium chloride method using catechin as a standard (Zhishen et al. 1999).

Estimation of anthocyanins

Method described by Ozso et al. (2008) with some modifications was used for determination of total anthocyanins. Total anthocyanin (mg/mL) of extracted solution was expressed by using the absorbance (B) of a 1% w/v solution of malvidin-3-glucoside and the relationship is given in Eq. (3).

$$\begin{aligned} \text{Total anthocyanins (mg/mL)} \\ = A \times \text{dilution factor} \times \frac{1000}{B} \end{aligned} \quad (3)$$

Determination of antioxidant activity

The phosphor molybdenum complex method (Prieto et al. 1999) was used to determine the total antioxidant activity.

HPLC analysis for total phenolic compounds

The samples were filtered using Whatman No 2 filter paper. Rotary vacuum evaporator was used to evaporate the filtrate and during evaporation water and ethanol were removed followed by washing with hexane which removed non-polar compounds and 10 mL of the mixture (adjusted to 50 mL) was adjusted to a pH of 7.0. A series of cartridges (C₁₈ Sep Pak) which were pre-conditioned using methanol was used to filter the mixture and 20 µL of the filtrate was used for estimation by standard method. Phenolic compounds were detected using an UV detector at 215 nm. The filtration was carried out using acclaim 120 C₁₈ column (5 µm, 120 Å) with a size of 4.6 × 250 mm. A combination of two solvents formed the solvent system

Table 2 Experimental layout and responses for the five factors and four variables as given by the Box–Behnken design

Run	St (min)	ST (°C)	S:S (g:mL)	EC (%)	MT (s)	TPC (mg/100 mL)	Fl (mg/100 mL)	An (mg/100 mL)	Aa (%RSA)
1	10	40	1:35	55	40	1.69	2.47	4.01	100.00
2	10	50	1:20	55	40	0.98	0.81	1.18	69.71
3	10	50	1:35	40	40	1.24	1.31	1.79	57.25
4	10	50	1:35	55	20	1.46	1.05	5.48	100.00
5	10	50	1:35	55	60	1.17	1.20	1.71	92.01
6	10	50	1:35	70	40	1.80	4.31	1.87	100.00
7	10	50	1:50	55	40	1.68	1.40	3.97	100.00
8	10	60	1:35	55	40	1.91	1.33	2.38	92.76
9	25	50	1:50	70	40	0.94	2.25	1.56	95.73
10	40	50	1:35	70	40	1.30	4.06	1.19	97.96
11	25	50	1:50	55	20	1.04	1.06	2.98	100.00
12	25	40	1:35	70	40	1.23	2.89	1.85	80.49
13	25	40	1:50	55	40	1.37	1.19	2.99	99.08
14	25	60	1:50	55	40	1.51	1.27	3.25	90.71
15	40	50	1:50	55	40	1.42	1.48	2.73	100.00
16	25	50	1:50	55	60	0.66	0.59	0.47	62.27
17	25	50	1:35	70	60	1.25	2.63	1.51	86.44
18	25	50	1:35	40	20	1.08	0.94	1.61	77.51
19	25	50	1:35	55	40	0.91	1.15	3.76	100.00
20	25	50	1:20	40	40	0.73	0.63	0.53	88.48
21	25	60	1:35	70	40	1.30	1.55	1.30	94.43
22	25	50	1:35	55	40	1.50	1.46	4.16	100.00
23	25	50	1:35	55	40	1.25	1.38	3.62	100.00
24	25	50	1:35	55	40	1.25	1.29	3.60	100.00
25	25	60	1:35	55	20	1.12	1.06	2.14	100.00
26	25	50	1:35	70	20	1.43	3.59	1.33	62.27
27	25	50	1:35	55	40	0.88	1.50	3.90	86.10
28	25	40	1:35	40	40	1.51	1.78	2.42	73.24
29	25	60	1:20	55	40	0.99	0.86	1.19	76.77
30	25	60	1:35	55	60	0.88	0.47	0.54	47.96
31	25	40	1:20	55	40	1.59	1.10	2.39	84.95
32	40	50	1:35	55	60	0.59	0.38	0.59	85.32
33	25	50	1:35	55	40	1.31	1.69	5.04	80.12
34	40	60	1:35	55	40	0.97	1.00	1.11	100.00
35	25	60	1:35	40	40	0.78	1.94	1.62	71.19
36	40	50	1:20	55	40	0.93	0.73	1.17	78.63
37	40	40	1:35	55	40	1.51	1.32	2.72	100.00
38	25	50	1:20	55	60	0.66	0.58	0.69	54.84
39	40	50	1:35	40	40	1.01	1.13	1.20	60.79
40	25	50	1:20	55	20	1.01	0.67	0.96	75.84
41	25	50	1:35	40	60	0.60	2.22	1.27	67.11
42	40	50	1:35	55	20	1.09	1.17	1.58	99.45
43	25	50	1:20	70	40	1.07	1.70	0.72	83.46
44	25	50	1:50	40	40	1.09	1.61	2.59	65.80
45	25	40	1:35	55	20	1.20	1.94	2.54	79.00
46	25	40	1:35	55	60	1.02	1.42	1.66	81.42

St Sonication time, ST sonication temperature, SS solute:solvent ratio, EC ethanol concentration, MT microwave time, TPC total polyphenol content, Fl flavonoid content, An anthocyanin content, Aa antioxidant activity

Table 3 Analysis of variance showing the linear, quadratic interaction and lack of fit of the response variables

Source of variation	df	Response variables							
		TPC		Flavonoid content		Anthocyanin content		Antioxidant activity	
		Sequential sum of square	F	Sequential sum of square	F	Sequential sum of square	F	Sequential sum of square	F
Regression	20	3.51	4.28***	26.63	4.50***	59.74	7.21***	6625.68	2.37*
Quadratic	5	1.24	6.06***	13.28	8.98***	30.32	14.64***	2349.31	3.36*
Linear	5	1.70	4.81***	11.03	3.84*	24.73	4.36***	2746.71	2.98*
Square	10	0.57	0.057 ^{ns}	2.33	0.34 ^{ns}	4.70	0.35 ^{ns}	1529.66	0.78 ^{ns}
Residual error	10	1.02	–	2.36	–	3.52	–	1267.59	–
Lack of fit	20	0.73	0.63 ^{ns}	6.85	3.18 ^{ns}	8.85	1.46 ^{ns}	3101.76	1.94 ^{ns}
Pure error	5	0.29	–	0.54	–	1.51	–	399.16	–
Corr total	45	4.53	–	34.02	–	70.10	–	10,126.59	–
R ²	–	0.7740	–	0.7828	–	0.8523	–	0.6543	–
Adjusted R ²	–	0.5932	–	0.6090	–	0.7341	–	0.3777	–

^{ns} not significant

* Significant at $p \leq 0.05$; ** significant at $p \leq 0.01$; *** significant at $p \leq 0.001$

Table 4 Estimated regression coefficients of the fitted second order polynomial for response variables

Coefficients	Estimated coefficients			
	TPC	Flavonoid content	Anthocyanin content	Antioxidant activity
B ₀	1.18***	1.38***	4.00***	94.36*
B ₁	−0.19***	−0.16 ^{ns}	−0.63***	0.65 ^{ns}
B ₂	−0.10*	−0.29*	−0.44*	−1.52 ^{ns}
B ₃	0.11*	0.24 ^{ns}	0.73***	6.31*
B ₄	0.14***	0.71***	−0.11 ^{ns}	8.71**
B ₅	−0.16***	−0.12 ^{ns}	−0.64**	−7.30*
B ₁₁	−0.16*	0.14 ^{ns}	−0.67**	2.87 ^{ns}
B ₂₂	0.15*	0.003958 ^{ns}	−0.72**	−2.28 ^{ns}
B ₃₃	−0.095 ^{ns}	−0.47*	−1.11***	−6.10 ^{ns}
B ₄₄	−0.036 ^{ns}	0.92***	−1.53***	−11.44**
B ₅₅	−0.21***	−0.21 ^{ns}	−1.30***	−10.66*
B ₁₂	−0.19*	0.20 ^{ns}	0.0031 ^{ns}	1.81 ^{ns}
B ₁₃	−0.050 ^{ns}	0.040 ^{ns}	−0.31 ^{ns}	−2.23 ^{ns}
B ₁₄	−0.068 ^{ns}	−0.018 ^{ns}	−0.023 ^{ns}	−1.39 ^{ns}
B ₁₅	−0.053 ^{ns}	−0.23 ^{ns}	0.70*	−1.53 ^{ns}
B ₂₃	0.18	0.081	0.37	−0.046
B ₂₄	0.20	−0.37	0.064	4.00
B ₂₅	−0.017	−0.017	−0.18	−13.62
B ₃₄	−0.12	−0.11	−0.31	8.74
B ₃₅	−0.006494	−0.098	−0.56	−4.18
B ₄₅	0.075	−0.56	0.13	8.64
Probability of F value	0.0004	0.0003	0.0001	0.0215
Probability of lack of fit	0.7923	0.1016	0.3588	0.2380

^{ns} not significant

* Significant at $p \leq 0.05$; ** significant at $p \leq 0.01$; *** significant at $p \leq 0.001$

Fig. 1 Variation in response variable yield due to interaction of **a** effect of ethanol concentration and solvent solute ratio on yields of total polyphenol, **b** effect of solute solvent ratio and sonication time on yields of flavonoid, **c** effect of microwave time and solvent solute ratio on yields of flavonoid, **d** effect of microwave time and sonication temperature on yields of flavonoid, **e** effect of ethanol concentration and sonication temperature on yields of anthocyanin, **f** effect of solvent solute ratio and sonication temperature on yields of anthocyanin, **g** effect of ethanol concentration and sonication temperature on antioxidant activity

which consisted of Eluent A—acidified water (pH adjusted to 2.64 with dilute HCl) and Eluent B—acidified water: acetonitrile (20:80). Elution was conducted by a gradient run at a steady flow rate of 1.5 mL/min. All the samples were 10 fold diluted prior to analysis. For validation of the method used, standard curves for the phenolic compound standards were plotted in the range of 0.01–0.1, 1–10, 10–100 and 100–500 ppm based on their content in the samples. The calibration curve equations and coefficient of determination of linearity (R^2 value) were derived from the plots, and the limits of detection (LoD) and limits of detection quantification (LoQ) for the various standard phenolic compounds were obtained from the Eqs. 4 and 5, respectively (Shrivastava and Gupta 2011).

$$LOD = 3.3 \times \frac{\text{intercept of the calibration curve}}{\text{slope of the calibration curve}} \quad (4)$$

$$LOQ = 10 \times \frac{\text{intercept of the calibration curve}}{\text{slope of the calibration curve}} \quad (5)$$

Statistical analysis

Design-Expert (version 8.0.7.0) was used for establishing the experimental design and analysis of data. Statistical analysis and analysis of variance (ANOVA) were used for model prediction. Coefficient of determination (R^2 value) was used to express the fitness of the polynomial model equation.

Results and discussion

Optimization and modelling of the extraction process from rice husk

The extraction method was optimized with reference to TPC, flavonoid, anthocyanin and antioxidant compounds from rice husk using UAE as well as MAE. The different independent variables (Table 1) employed for experimental design were identified by using BBD. Based on the identified levels, experimental variables were evaluated and are presented in Table 2. The analysis of variance

(ANOVA) was carried out and showed the linear, quadratic interaction and lack of fit of the response variables (Table 3). The value exhibited a satisfactory fit (Eq. 2) which was mathematically allowable at $p \leq 0.05$ and enough with suitable R^2 values. Data of regression coefficients (Table 4) was used in equations of prediction after ignoring the non-significant terms. Three-dimensional surface plots were generated using the appropriate model fitted Eq. 2 for the assessment of the existing relationships among dependent and independent factors.

Impact of independent variables on extraction of total phenolic compounds (TPC)

Experimental values showing the extraction of TPC from rice husk were obtained by UAE and MAE (Table 2). The statistical analysis of experimental data was carried out which revealed regression coefficients as well as significance of TPC and are shown in Tables 3 and 4. On evaluation of statistical analysis, results for significant values were noted with $p \leq 0.001$. Extraction time had the highest impact on the processing of TPC. Subsequently the presence of TPC in rice husk was established and it was consistent with the findings of maximum yields of TPC from rice husk when extraction was done for a longer duration. The importance of the second-order polynomial model equation was checked by F-test (ANOVA) values for total phenolic compound (Tables 3 and 4) and was utilized in predictive equations as given in Eq. 6.

$$Y = 1.18 - 0.19X_1 - 0.10X_2 + 0.11X_3 + 0.14X_4 - 0.16X_5 - 0.16X_1^2 + 0.15X_2^2 \quad (6)$$

Three-dimensional surface plots were generated using Eq. 4 to estimate the optimal level of variables for the UAE as well as MAE of TPC from black rice husk. Process variables significantly affected the extraction of TPC from the rice husk. Increase in solute solvent ratio, microwave time, ethanol concentration, sonication time (Fig. 1a) concomitantly increased TPC. It increased initially linearly with increase in microwave time at constant ethanol concentration. However, a marginal increase was observed when ethanol concentration, microwave time and sonication time increased, keeping solute solvent ratio constant.

Impact of independent variables on extraction of flavonoids

Experimental values showing the extraction of flavonoids from rice husk were obtained by UAE and MAE (Table 2). The statistical analysis of experimental data was carried out which revealed regression coefficients as well as

Table 5 Estimated optimum conditions of independent factors and predicted and experimental values of the responses

Factor	Optimal condition	
Sonication time	10.02 min	
Sonication temperature	49.46 °C	
Solute: solvent	1/40.79 g/mL	
Ethanol concentration	67.34%	
Microwave time	31.11 s	
Responses	Predicted value	Experimental value
TPC (mg/g GAE)	1.65	1.72
Flavonoid content (mg/100 g)	3.04	3.01
Anthocyanin content (mg/100 g)	3.39	3.36
Antioxidant activity (%)	100	100

significance of flavonoids (Tables 3 and 4) and were used for a final predictive equation (Eq. 7).

$$Y = 1.38 - 0.16X_1 - 0.29X_2 + 0.24X_3 + 0.71X_4 - 0.12X_5 + 0.14X_1^2 + 0.003958X_2^2 \quad (7)$$

The result exhibited that extraction time affected the flavonoid content ($p \leq 0.001$). When the sonication time was unchanged, the flavonoid content increased in tandem with increase in microwave time, sonication temperature and solute solvent ratio and reached 1.45 mg/100 g, 1.08 mg/100 g and 1.41 mg/100 g, respectively. Figure 1b illustrated that at a fixed temperature, flavonoid content increased with increase in sonication time, solute solvent ratio and microwave time. At constant microwave time it increased with increase in sonication time, ethanol concentration and solute solvent ratio (Fig. 1c). Similarly, at a constant solute solvent ratio it increased with increase in sonication time, sonication temperature and microwave time (Fig. 1d).

Impact of independent variables on extraction of the anthocyanin contents

The statistical analysis of experimental data was carried out which revealed regression coefficients as well as significance of anthocyanin (Tables 3 and 4) and were used for a final predictive Eq. 8.

$$Y = 4.00 - 0.63X_1 - 0.44X_2 + 0.73X_3 - 0.11X_4 - 0.64X_5 - 0.67X_1^2 - 0.72X_2^2 \quad (8)$$

The results revealed extraction time as a highly significant factor at $p \leq 0.001$ having an impact on the

extraction of anthocyanin, and it was consistent with the increased yields of anthocyanin from rice husk when extraction was carried out for a longer duration. When the sonication time was unchanged, the anthocyanin content increased gradually with increase in ethanol concentration, microwave time, solute solvent ratio and sonication temperature and noted 2.59 mg/100 g, 1.32 mg/100 g, 3.35 mg/100 g and 2.87 mg/100 g, respectively. It increased with increase in microwave time, sonication time (Fig. 1e) and sonication temperature when ethanol concentration was constant. It also increased with increase in microwave time, sonication temperature, ethanol concentration and sonication time when solvent solute ratio was fixed (Fig. 1f).

Impact of independent variables on extraction of the antioxidant activity

The statistical analysis of experimental data was carried out which revealed regression coefficients as well as significance of antioxidant (Tables 3 and 4) and were used for a final predictive equation (Eq. 9).

$$Y = 94.36 + 0.65X_1 - 1.52X_2 + 6.31X_3 + 8.71X_4 - 7.30X_5 + 2.87X_1^2 - 2.28X_2^2 \quad (9)$$

The result clearly revealed extraction time as a highly significant factor at $p \leq 0.05$ on the extraction of antioxidant activity. The antioxidant activity evinced a direct proportional relationship with the increase of ethanol concentration, solute solvent ratio and microwave time and reached 92.75 and 99.57 and 88.18%, respectively at a constant sonication time. A noticeable increase was observed with an increment of sonication time and also showed incremental nature with increase of solute solvent ratio, ethanol concentration and microwave time at constant sonication temperature. When sonication temperature was constant, antioxidant activity increased (90.34%) with increase of microwave time and thereafter decreased (Fig. 1g).

Optimization of the parameters

All the generated models explained the variation of response and the R^2 values for TPC, flavonoids, anthocyanin and RSA were 0.7740, 0.7828, 0.8523 and 0.6543, respectively while adjusted R^2 values were 0.5932, 0.6090, 0.7341 and 0.3777, respectively (Table 3). The values of R^2 and adjusted R^2 indicated that the model has established the desired relationship between chosen parameters. The suitable conditions obtained by RSM for all the factors included 10.02 min sonication time, 49.46 °C sonication temperature, 1/40.79 (g/mL) solute solvent ratio, 67.34%

ethanol concentration and 31.11 s microwave time for the maximum extraction of TPC (1.65 mg/g GAE), flavonoids (3.04 mg/100 g), anthocyanin (3.39 mg/100 g) and RSA (100%). Under the given solutions, the experimental values for the extraction of phenolics from black rice husk were found to be 1.72 mg/g GAE of TPC, 3.01 mg/100 g of flavonoids, 3.36 mg/100 g of anthocyanin and 100% of RSA, which correlated with the predicted values (Table 5).

The mechanism of sonication is mainly ascribed to the cavitation, and proliferation of acoustic waves and upon cavitation, physical, chemical and mechanical effects are generated. It results in collapsing of material matrix and enhances the rate of recovery of polyphenolic compounds from material matrix to the solvent (Al-Dhabi et al. 2017). When solid–liquid extraction is assisted by ultrasounds the possible benefits of their application in extraction are an intensification of mass transfer, improved solvent penetration into the matrix of husk by increasing the contact surface area. The collapse of cavitation bubbles near the cell walls is expected to produce the cell disruption together with a good penetration of the solvent into the cells, through the ultrasonic jet (Da Porto et al. 2013).

Ghafoor et al. (2009) reported similar studies with grape seed and achieved maximum TPC (5.44 mg GAE/100 mL) through UAE and the optimal conditions were 56.03 °C temperature, 53.15% ethanol concentration and 29.03 min time as obtained by RSM. Highest antioxidant activity (12.31 mg/mL) was obtained with 60.65 °C extraction temperature, 53.06% ethanol concentration and 30.58 min extraction time. Maximum total anthocyanin (2.28 mg/mL) was predicted for 55.13 °C temperature, 52.35% ethanol concentration and 29.49 min time. Experimental values obtained by using the prescribed conditions revealed TPC 5.41 mg GAE/100 mL, antioxidant activity 12.28 mg/mL, and total anthocyanin 2.29 mg/mL of the grape seed extract. In another study Peng et al. (2013) reported the optimal condition from RSM for UAE in buckwheat and was 35% methanol concentration, 60 °C temperature, and 21 min time for the maximum yield of total flavonoid 4.06% and using these conditions the experimental total flavonoid obtained was 3.94%. Zhao et al. (2014) also reported the optimal condition from RSM for UAE from olive leaf and was 50% ethanol concentration, 1/250 g/mL solute solvent ratio, 27.5 min extraction duration for the highest total phenolic compounds 4.28% and almost similar values (4.29%) was extracted experimentally using the above mentioned conditions. Duan et al. (2015) employed RSM for MAE from Chinese bayberry and reported the optimal conditions of solid to liquid ratio, extraction temperature, and extraction time viz., 1:50, 80 °C, and 15 min, respectively. Under these conditions the experimental yield of anthocyanin content and antioxidant activity was 2.95 mg g⁻¹ and 279.96 µmol g⁻¹ trolox equivalent,

respectively on a dry weight basis. Shi et al. (2014) also observed that the optimal condition by RSM for MAE from *Eclipta alba* to determine wedelolactone yield and found 208 W microwave power, 90% ethanol concentration, 26.5 min irradiation time and 33 mL g⁻¹ solvent-to solid ratio. In another experiment, the optimal condition by RSM for MAE from mulberry fruits (*Morus alba* L.) was studied by Teng and Lee (2013) and found the optimal conditions of ethanol concentration of 40%, irradiation time of 8 min and microwave power of 210 W; and the observed results for total phenolic and flavonoid content were 11.14 mg/g dw and 36.88 mg/g dw, respectively (Fig. 2).

Conducting similar studies by Singh et al. (2017) with hull of mung bean reported that the optimal condition given by RSM for UAE was 55% acetone concentration, 53 °C extraction temperature and 114 min extraction duration and the predicted and experimental values were 52.39 µmol AAE/g and 51.93 ± 0.60 µmol AAE/g, respectively for total antioxidant activity. Carciocchi et al. (2015) also obtained the optimal condition by RSM for UAE from quinoa (*Chenopodium quinoa*) seeds as 80% ethanol concentration and 60 °C extraction temperature. The predicted response values were 103.6 mg GAE/100 g TPC, 25.0 mg QE/100 g total flavonoid content and 28.6% DPPH radical scavenging for antioxidant activity, while the corresponding experimental conditions were 102.86 mg GAE/100 g, 26.93 mg QE/100 g and 28.9% DPPH radical scavenging activity, respectively. Hiranrangsee et al. (2016) also obtained the optimal conditions by RSM for extraction of anthocyanins and total phenolic content by using UAE from pericarp of mangosteen (*Garcinia mangostana* L.) and found the best optimal condition for extraction heating time, sample to solvent and amplitude of ultrasound were 5 min, 1:8.5 and 94%, respectively. Under these conditions, the predicted value for anthocyanin and total phenolic content were 25.29 mg Cyn-3-Glu/100 g and 2493.56 ± 40.84 mg GAE/100 g, respectively, while the experimental values were 23.54 ± 0.31 mg Cyn-3-Glu/100 g and 1975.00 ± 74.34 mg GAE/100 g, respectively.

HPLC analysis for phenolic compounds of black rice husk

The results for validation of the HPLC method used for analysis of the phenolic compounds are presented in Table 6. From the results, it was evident that the methodology used was suitable for the detection and quantification of the phenolic compounds studied. The characteristic polyphenols profile of black rice husk obtained by extraction under conventional method and optimal condition are given in Table 7. Five phenolic compounds were identified in black rice husk through conventional solvent extraction viz., ferulic (18.77 ppm) quercetin (5.44 ppm) quinic acid

Fig. 2 HPLC Chromatograms of the two different extracts, viz. **a** Conventional solvent extraction and **b** extraction under optimized condition

Table 6 Calibration curve equations, coefficient of determination of linearity (R^2 value), limits of detection (LoD) and limits of detection quantification (LoQ) of the various phenolic compounds in HPLC study

Phenolic compound	Retention time (min)	Calibration curve equation	R^2	LoD (ppm)	LoQ (ppm)
Gallic acid	7.16	$y = 11.16x + 0.122$	0.995	0.036	0.109
p-Coumaric acid	32.75	$y = 0.461x + 0.108$	0.989	0.773	2.342
Ferulic acid	35.71	$y = 0.480x + 0.100$	0.999	0.688	2.083
Quercetin	39.63	$y = 0.480x - 0.011$	0.994	0.076	0.229
Salicylic acid	38.42	$y = 0.590x + 0.105$	0.994	0.587	1.780
Quinic acid	38.20	$y = 0.194x - 5.559$	0.994	94.560	286.546
Apigenin	40.07	$y = 0.353x + 0.007$	0.992	0.065	0.198
Syringic acid	26.43	$y = 0.825x - 0.171$	0.982	0.684	2.073
Chlorogenic acid	22.79	$y = 0.307x + 0.066$	0.994	0.709	2.150
Catechin	23.16	$y = 0.675x + 0.020$	0.997	0.098	0.296

Table 7 Amount of phenolic compounds detected by HPLC in extracts of normal and optimized extraction process

Phenolic compound	Concentration (ppm)	
	Conventional solvent extraction	Extraction under optimized condition
Gallic acid	–	2.98
p-Coumaric acid	–	31.19
Ferulic acid	18.77	20.45
Quercetin	5.44	46.84
Salicylic acid	–	17.95
Quinic acid	2052.25	2682.37
Apigenin	0.93	1.34
Syringic acid	38.33	65.34
Chlorogenic acid	–	123.65
Catechin	–	1.72

(2052.25 ppm), apigenin (0.934 ppm) and syringic acid (38.33 ppm). On the other hand, under the obtained optimal conditions, nine phenolic compounds were identified viz., gallic acid (2.98 ppm), p-coumaric acid (31.19 ppm), ferulic acid (20.45 ppm), quercetin (46.84 ppm), salicylic acid (17.95 ppm), quinic acid (2682.37 ppm), apigenin

(1.34 ppm), syringic acid (65.34 ppm), chlorogenic acid (123.65 ppm) and catechin hydrate (1.72 ppm). The presence of these compounds in black rice husk is desirable as these flavonoidic compounds exhibit antipyretic, analgesic, antiinflammatory, antiarthritic, antioxidant and immunomodulatory properties (Mradu et al. 2012). Free

radical scavenging and antioxidant activities of cereals, vegetables and fruits are the known resultant of phenolic compounds and causing various biological effects. Primarily it can be attributed to chelating of transition metals, their antioxidant activities in scavenging free radicals and inhibition of peroxidation (Halliwell 2001). Of late, phenolics have acquired the centre stage in the field of food research mainly due to its biology and health benefits. Researchers are intensifying the research leading to simplification of extraction, separation and quantification of these compounds from natural sources. The focus is mainly to establish the whole process in an environmental friendly and comprehensive manner (Khoddami et al. 2013). Hence, from the results it was evident that this methodology developed for the extraction of the bioactive phenolic compounds is more effective as compared to the conventional solvent extraction process.

Conclusion

Box–Behnken design employed for the extraction of phenolic compounds from husk of black rice revealed significant effect of sonication time, sonication temperature, solute solvent ratio, ethanol concentration and microwave time on the yield. Furthermore, interaction of ethanol concentration and solute solvent ratio revealed good optimization yields. The results favourably corroborate black rice husk as an important source of phenolic compounds, which can be best extracted using the combination of ultrasonication and microwave assisted method. This can provide new opportunities in various industries like food, nutritional, cosmetic and pharmaceuticals, and more specifically in the food industries to develop functional foods.

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