Comparative analyses of the nutraceutical potentialities of selected Indian traditional black rice (*Oryza sativa* L.) landraces

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ABSTRACT

The present investigation aims to explore the importance of some traditional black rice varieties with their nutraceutical properties. The beneficial properties of these varieties remain unknown to the majority of the population due to the inadequate data. Decorticated rice grains of four black rice varieties were evaluated and compared with two commonly consumed white rice varieties - Gobindabhog (traditional aromatic) and BPT 5204 (modern high yielding variety). The sugars and total lipid levels were similar in Gobindabhog and BPT 5204. The black rice varieties, however, had considerably greater protein, thiamin, riboflavin, pantothenic acid, pyridoxine, and antimicrobial activities compared to the two selected white rice varieties. The ICPMS analysis of black rice grains showed that the former also had higher levels of Mn, Fe, and Zn than the white rice varieties. However, BPT 5204 contained a greater amount of Cu than the black rice varieties. Total flavonoid and phenolic content, as well as antioxidant potentiality of black rice varieties were also considerably higher than those of the white rice varieties.

Key words: *Black rice, nutraceutical property, B complex vitamins, micronutrients*

1. INTRODUCTION

Rice (*Oryza sativa*) is one of the three most important cereals, being the staple food for more than half of the world's population (Khush, 1997). India is the center of origin of indica rice, characterized by numerous agronomic and nutraceutical properties (Deb, 2017). Black (purple) rice is becoming popular for their putative nutritional value, and some phytochemical properties, many of which are not yet established. Although many phenotypic characters of different rice varieties seem to be largely genetically determined (Kovach et al., 2009), micronutrient contents of rice may vary with farm soil characteristics (Baxter et al., 2012). Popularly grown modern white rice varieties are known to contain

very low levels of essential micronutrients (Deepa et al., 2008), which upon continuous consumption, lead to micronutrient deficiency and would require fortification with metallic micro-nutrients and vitamins (Ye et al., 2000). In contrast, many folk rice landraces are inherently rich in nutraceutical properties (Deb, 2017; Shylaraj et al., 2018). In the States of Tamil Nadu and Kerala, for instance, folk medicine prescribes consumption of Nyavara rice for treating patients suffering from a variety of neurotic disorders. In West Bengal, Kabiraj-sal rice is recommended for convalescing patients. Garib-sal was traditionally used to cure gastro-enteric infection (Deb et al., 2015). Recent studies indicate that iron and zinc contents in many traditional rice landraces are significantly higher

than that of modern cultivars (Anandan et al., 2011; Deb et al., 2015). Folk medicine in West Bengal prescribes Parmaisal for improving growth in children (Deb, 2017). Micronutrients like vitamin E (α-tocopherol), B complex vitamins (riboflavin, thiamin and niacin), minerals such as iron, zinc, and certain alkaloids have already been identified in a large number of rice landraces, developed and grown by indigenous farmers over centuries (Deb, 2017). A considerable amount of β-carotene (provitamin-A) is also found in the bran of some red and black rice varieties from the Philippines, Malaysia, Vietnam and Thailand (Frei, Siddhuraju and Becker, 2003). There are dozens of such rice varieties with various nutraceutical properties, mentioned in folk medicine, awaiting detailed scientific investigation and validation.

We report here the results of our estimation of the concentrations of a few nutritionally important heavy metals, B complex vitamins, total antioxidant activity, total protein, soluble sugars, starch, total carotenoids, total phenolics, and flavonoids in selected indigenous black rice varieties, and compared to two popularly consumed rice varieties with white kernels.

2. MATERIALS AND METHODS

2.1. Plant materials

Samples of Kalo Bhat, Chak Hao Poireithon, Huggi Bhatta, Tiki and Gobindabhog rice were collected from Centre of Interdisciplinary Studie's conservation farm Basudha (http://www.cintdis.org/basudha), located in Rayagada district of Odisha (19° 42' 32.0" N, 83° 28' 8.4" E). BPT-5204, a popular modern rice cultivar, was procured from Rajendranagar market, Hyderabad, India.

In the laboratory, all rice samples were decorticated manually by rubbing against a pumice stone, keeping the bran layer intact, and ground to fine powder using mortar and pestle, and stored in - 20°C for biochemical analysis. Analytical grade chemicals from Sigma-Aldrich (India) were used for the study. De-ionized double-distilled water was used during sample preparation for all experiments.

2.2. Morphological characterization of traditional rice varieties

Different morphological characteristics of the selected rice varieties were recorded, following Biodiversity International (2007) manual. Details of the morphological features are provided in Table 1.

2.3 Scanning Electron Microscopy (SEM)

Scanning electron micrograph of the native back rice landraces and two white rice varieties were obtained using a scanning electron microscope (Zeiss EVO LS10). Flour of raw and cooked rice seeds were mounted on platinum coated aluminum stubs and the surface morphology was scanned. Selected regions were photographed (Fig. 2a & Fig. 2b).

2.4 Estimation of total soluble sugar and total starch

Total soluble sugar and total soluble starch were analyzed spectrophotometrically following the method described by Chow and Landhausser (2004), with slight modifications. Briefly, 100 mg of the powered rice sample was taken in a 2 ml micro-centrifuge tube. 2 ml

Table 1. Grain morphological characters of the selected rice varieties.

Characters	Kalo Bhat	Chak Hao Poireithon	Huggi Bhatta	Tiki	Gobindabhog	BPT5204 (Samba Mahsuri)
Grain: Length (mm)	9.40	9.00	9.40	8.55	6.10	7.35
Decorticated grain: Length (mm)	6.93	6.67	7.60	5.75	4.40	5.32
Bran Colour	Longitudinal half black	Black	Dark purple	Longitudinal half black	White	White
Decorticated grain: Aroma	Moderate	Nil	Strong	Nil	Strong	Nil
Lemma and palea: colour	Purple spot	Black	Black furrows	Purple furrows	Gold	Straw
Grain: Width (mm)	3.30	3.42	3.47	3.55	2.20	2.06
Decorticated grain: width (mm)	2.53	2.58	2.57	2.70	1.80	1.84
Awn length (mm)	0	14.0	23	0	0	0
1000 grains weight (g)	25.3	25.8	25.0	32.6	10.5	15.5

of 80% ethanol was added. The tube was placed in a water bath for 15 minutes at 90°-95° C, centrifuged at 3000 rpm for 5 minutes, and the supernatant collected in a separate test tube. The extraction procedure was repeated two more times and the combined supernatant from the three extractions was used for total soluble sugar estimation. After oven drying at 80°C for 2 h, the residue was used for total soluble starch estimation.

For total soluble sugar estimation, 10 µl from the combined supernatant was taken in a 15 ml test tube, to which 990 µl of 80% ethanol and 5 ml Anthrone reagent were added and placed in a boiling water bath for 8 minutes. After rapid cooling on ice, the absorbance was taken using a spectrophotometer (Jasco V730 Bio, Japan) at 630 nm. For total soluble starch estimation, the oven-dried sample (after soluble sugar extraction) was taken in a 15 ml test tube. After adding 5 ml of 1.1% HCl, the tube was placed in a boiling water bath for 30 minutes. After cooling at room temperature, the solution was filtered. 10 µl of the filtrate was taken in a 15 ml test tube, to which 990 µl of 1.1% HCl and 4 ml Anthrone reagent was added. The tube was placed in a boiling water bath for 11 minutes. After rapid cooling on ice, the absorbance was taken using a Jasco V730 Bio, Japan spectrophotometer at 630 nm. For soluble sugar and soluble starch, standard glucose (SRL, India) and potato starch (SRL, India) were used respectively, for standard curve preparation. The procedure was repeated thrice for each rice variety. This total starch includes amylose, amylopectin and resistant starch contained in the rice kernel. Separate investigation was carried out of the amylose content of different rice varieties following the method described by Juliano (1971). The results were expressed in percentage of dry weight (g%). The results were repeated thrice.

2.5 Estimation of soluble protein

Total Soluble protein was determined spectrophotometrically using Bradford method (Bradford, 1976), the protein was extracted following the method described in Croy et al. (1980) with slight modifications. 250 mg powdered rice sample was taken in a 2 ml micro-centrifuge tube, into which 1 ml of 0.25 (M) sodium phosphate buffer (pH 8) and 2% NaCl were added. After freezing and thawing for 3 times, the tubes were centrifuged at 13000 rpm for 20 minutes. The supernatant was further diluted to 10 times with the same extraction buffer in a separate tube and Bradford reagent added. After 15 minutes of incubation, absorbance was measured at 595 nm in a spectrophotometer (Jasco V730 Bio, Japan). The standard curve was prepared using standard BSA (Chromous, India) using Bradford reagent. The procedure was repeated for three replications.

2.6 Estimation of total lipid

Total lipid was extracted following the method described by Bligh and Dyer (1959) with slight modifications, as follows. 500 mg of rice powder was soaked in Methanol, water, and chloroform at 2:1:1 ratio in an airtight glass vial and kept at 4°C for overnight. After separation of three layers, 1 ml of the lower chloroform phase was taken out in a small vial and evaporated to dryness using N_2 gas. Total lipid was quantified gravimetrically as mg/g of dry weight of the sample. The estimations were made in triplicate for all rice samples.

2.7 Metal detection and analysis

Heavy metal detection and analysis were performed using NexION 300X - Perkin Elmer's inductively coupled plasma mass spectrometry (ICPMS) at the Indian Institute of Technology- Madras, as described by Sen Gupta et al. (2017). The procedure was repeated five times for all samples.

2.8 Estimation of phenolics and flavonoids

Rice powder was soaked overnight with methanolic water (7:3 v/v) at 4°C. Total phenolic content (TPC) of the methanolic extract of the samples was estimated by Folin-Ciocalteu method (Harborne 1973), measuring spectrophotometrically at 765 nm (Jasco V-730 Bio, Japan) and expressed as mg of gallic acid (SRL, India) equivalent (GAE) per 100 g dry weight. Total flavonoid content (TFC) was estimated from methanolic extract of the samples by adding 10% (w/v) aluminum chloride, 5% (w/v) sodium nitrite and water in a ratio of 1:1:1:7 (Goufo and Trindade, 2014). Optical Density was measured spectrophotometrically at 510 nm (Jasco V-730 Bio, Japan) after 25 minutes of incubation and the result was expressed as mg of quercetin equivalent (QE) per 100 gm dry weight of rice grain. The results were obtained from three replications.

2.9 Estimation of antioxidant activity

2.9.1 Scavenging ability by DPPH radical assay

Antioxidant potentialities (AP) of the samples were measured by the radical scavenging property of 2-diphenyl-1-picrylhydrazyl (DPPH, SRL, India) (Brand-Williams et al., 1995). 0.16 mM aqueous methanolic (3:7 v/v) DPPH was added to the sample aliquot in 3:1 ratio and the degree of decolorization was measured at 517 nm in a UV-spectrophotometer (Jasco V-730, Bio, Japan) after 30 minutes. incubation in dark. The IC50 value (the concentration at which 50 percent of the DPPH free radicals are inhibited) of the sample was calculated against the standard ascorbic acid, and the AP was expressed as the inverse of the IC50 value. The results were repeated thrice in all cases.

2.9.2 Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay is a method to estimate the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. The development of blue colored Fe^{2+}-TPTZ complex (Fe^{2+} tripyridyltriazine) increases the absorbance at 593 nm. The method described in Kaushik et al. (2012) was used for this assay using a spectrophotometer (JASCO V-730, Bio, Japan). The results were calculated by standard curves prepared with known concentrations of $FeSO_4$, and were expressed as mM $FeSO_4$/g dry weight. The results were averaged from three replications in all cases.

2.10 Estimation of total carotenoids

Carotenoids were extracted as per the method described by Howe and Tanuminardio (2006) from dried ground rice seeds. The carotenoid content in the petroleum-ether extract was determined spectrophotometrically, the absorbance was measured spectrophotometrically (Jasco V730 Bio, Japan) at the wavelength of 450 nm. The concentration of total carotenoids was calculated using the response factors as follows:

$$\text{Total carotenoid} = (A \times d \times V) / E^1\% \times w$$

where: A is absorbance, d is dilution factor, $E^1\%$ is coefficient of absorbency (2592 for petroleum-ether) at 1cm path length, w is weight of the sample (g), and V is volume (ml). The results are expressed in μg/g.

2.11 Analysis of B complex vitamins

Extraction of B complex vitamins were carried out following the method described in Puwastien et al. (2011). Extracted samples were subjected to High-Performance Liquid Chromatography (HPLC, Waters, UK). Gradient of two mobile phases were: methanol (A) and water with 0.02% aqueous H3PO4 (B) were set at: 0% A + 100% B for 3 minutes; 10% A + 90% B for 10 minutes; 30% A + 70% B for 15 minutes and 30% A + 70% B for 35 minutes. The injection volume was 20μl. The flow rate was kept at 1 ml/min and analytes were scanned at 210 nm wavelength. The peaks were identified by comparing the relative retention time with proper peak integration, co-chromatography with standard and calibration against absorption spectra obtained from the analytical grade chemicals (Sigma, India). All vitamins were quantified by using standard validation method and finally, the amounts of the analytes were expressed as mg/100g of the dry weight of the sample. The electrospray ionization (ESI) source of the Thermo LTQ mass spectrometer was used for the identification of B complex vitamins in the extracts of rice samples. The data were acquired using Xcalibur Quant software. The diluted samples were sprayed by applying a voltage of 5 kV in both the ionization modes with a mass range of m/z 50-1000. The capillary temperature was set to 250°C, N_2 sheath gas flow rate was 10 arbitrary units, the solvent flow rate was set at 5 μL/min, and tube lens voltage was ±100 V. Collision induced dissociation (CID) fragmentation was carried out for the specific ion peaks from the MS analysis with an isolation width of 1.0 m/z. Utilizing the spectral details, the B complex vitamins were annotated using databases line PubChem, MassBank, etc. and also published literature (Xu et al., 2020).

2.12 Antimicrobial activity testing

Two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and one Gram-negative bacterial species (*Escherichia coli*) were selected in this study to evaluate the antibacterial properties of the rice landraces. The bacterial species were chosen based on their pathogenic characteristics. Working concentrations of approximately 105-10 cfu/ml through dilution was used for antibacterial activity assays. The methanolic extracts of the black and white rice varieties

were dissolved in DMSO (2%) as a non-toxic solvent to prepare the stock solution and the concentration was maintained at 80 - 100 mg/ml. The solution was sterilized through 0.22 μm membrane filter. The agar diffusion test was done according to Bauer (1966) with some modification. Bacterial cultures were added (approximately 105-10 cfu/ml) (100 μl) to separate petri dishes containing NA (15 ml). The inoculation of bacterial cells on nutrient agar petri dishes and rice extracts were laid over these dishes. The plates were incubated for 18-24 h at 37°C and thereafter, the growth of bacteria was determined below the nanofibrous scaffolds (zone of inhibition). The presence of antimicrobial activity is indicated by the absence of bacterial growth directly below the rice extracts. The inhibition zone was measured (mm). The Broth method was carried out to determine the minimum inhibitory concentration (MIC) following the method described in EUCAST (2003).

2.13 Statistical analysis

Pearson's correlation between pairs of biochemical characters of each rice variety was estimated. The number of replications was small (df = 2), the resolution of confidence limit was narrowed to p <0.01, at which the strongest correlations between the pairs of biochemical properties were identified.

3 RESULTS & DISCUSSIONS

3.1 Rice grain morphology

The morphological characters of the four black rice varieties and two popularly consumed white rice varieties are presented in Table 1. Grain colour, size, shape, and weight are considered as important criteria for understanding the quality and various physical properties of rice. Decorticated rice kernels of Kalo Bhat, Chak Hao Poireithon, Huggi Bhatta, and Tiki are black pigmented, whereas, Gobindabhog and BPT 5204 are white. Kalo Bhat, Huggi Bhatta, and Gobindabhog are aromatic. The photograph of the spikelets (top row) and decorticated (bottom row) grains of rice varieties used in this study, is provided in Fig. 1. Rice is a single amyloplast consists of several polyhedral starch granules (Watson & Dikeman, 1977). The surfaces of starch granules were observed to be more or less smooth in native rice samples. Images of dehusked flour from black and white rice varieties are presented by

Fig. 1. Photograph of the spikelets (top row) and decorticated (bottom row) grains of rice varieties used in this study.

scanning electron microscopy (SEM). During cooking, because of water absorption, the starch granule size increases / swells. During swelling, the shapes of the granules are preserved, but irregular granule folding occurs as temperature increases, leading to disintegration and loss of shape. The SEM images of these varieties of cooked rice are also presented below the SEM images of corresponding raw rice Fig. 2a & Fig. 2b. The cooking process breaks down the swollen semi-crystalline starch molecules, leading to irreversible changes in the properties of the starch, such as swelling of granules, loss of birefringence and solubility of starch. In the SE micrographs, the changes occurring after cooking are clearly visible, indicates leaching out due to the combination of linear molecules, protein and fiber dispersion.

It was observed that decorticated grains of the black rice varieties are superior in terms of length, width and 1000 grain weight than white rice varieties. Due to the presence of aroma and other superior qualities, Kalo Bhat and Gobindabhog rice are popular in market and are used for making various food dishes (Paul, 2013).

3.2 Macronutrients composition of rice grain samples

Rice is a major source of energy to majority of the population worldwide (Inabangan-Asilo et al., 2019). Humans need to consume a certain amount of

Table 2. Macronutrient composition of rice samples.

Variety of rice	Soluble starch (g%)	Total soluble sugar (g%)	Digestible Amylose (g%)	Soluble protein (g%)	Total lipid (mg/gm)
Kalo Bhat	34.55±0.34*	4.04±0.06*	18.63±1.04	3.54±0.16	16±0.62
Huggi Bhatta	34.12±0.45*	7.8±0.03	19.3±0.48	5.56±0.13**	26±0.38
Tiki	53.78±0.36*	4.94±0.09*	22.59±0.56	4.83±0.04**	14±0.23
Chak Hao Poireithon	46.16±0.27*	5.52±0.07	19.97±0.34	5.53±0.11**	17±0.14
Gobindabhog	65.74±0.36	13.78±0.11	24.5±0.43	2.86±0.06	16±0.13
BPT 5204	65.23±0.43	8.33±0.1	19.81±0.28	2.31±0.02	14±0.25

Values are mean ± standard deviation of three replications. * Significant difference was observed at $p<0.01$ and ** Significant difference was observed at $p < 0.05$.

macronutrients, micronutrients to grow normally and remain healthy during their life (Welch and Graham, 2004). Rice grain contains most of these beneficial components for humans, some are present in large amounts, while others only in small quantities. For instance, according to Indian Food Composition Table 2017, 100g of brown rice grains contained 9.16±.75 g protein, 1.24±.08 g lipid and 74.80±.85 g carbohydrate (Longvah et al., 2017). In our samples, all these components were found in good amount and there was no unimodal difference in the macronutrient compositions among the black and white rice varieties (Table 2). However, the soluble starch content in black rice varieties (~ 35 - 54 g%) is significantly lower (p <0.01) than the white rice varieties (~ 65 - 66 g%), while black rice varieties (except Kalo Bhat) contained significantly higher soluble protein (~ 4.83 to 5.5 g%), in contrast to (< 3.0 g%) in white rice varieties (pairwise t = 19.571, p <0.05). Carbohydrates are the major component of the rice grain. There are two structural components of starch, namely, amylose, and amylopectin. Amylose content serves as one of the most important factors for evaluating the quality of rice products. Because all the rice samples studied contain amylose below 40 percent, they are non-waxy in nature, following Bao et al. (2006)'s criteria. Shewry (2007) reported that rice protein contains all the 10 essential amino acids. Presence of a high amount of soluble protein in black rice varieties indicated that rice proteins had similar hypocholesterolemic effects as reported by Yang and Kadowaki (2009). Major portions of the lipid in the rice grains are in the embryo, aleurone layer and also in the starchy endosperm. The lipid content in the samples of black rice varieties range from 16 - 26 mg/g. A major proportion of this lipid is fatty acid. A higher percentage of lipid in the grains may protect human liver from oxidative damage caused by lipid peroxidation. Nam et al. (2008) showed that lipids in rice bran are more effective in the improvement of overall cholesterol metabolism and antioxidant status as it contains several important fatty acids like, linoleic (18:2) acid, oleic (18:1) acid and palmitic (16:0) acid. Among the black rice varieties, Huggi Bhatta and Kalo Bhat contain a significant amount of omega-3 and omega-6 fatty acid (Ray et al., 2021. Unpublished data, MS in preparation) which may contribute to the therapeutic potentiality of these varieties.

Table 3. Concentration (ppm) of nutritionally important minerals in different rice samples.

Rice landrace	Cr	Mn	Fe	Cu	Zn	Co
Kalo Bhat	1.61±0.04	53.13±0.37*	6.09±0.26	3.12±0.03	40.03±0.01	0.07±0.001
Huggi Bhatta	7.2±0.11*	50.04±0.46*	25.73±0.69*	2.79±0.06	56.26±0.05*	0.46±0.003
Tiki	8.51±0.02*	62.54±0.54*	25.46±0.42*	0.56±0.04	36.91±0.03	0.24±0.002
Chak Hao Poireithon	9.38±0.21*	34.61±0.72	21.71±0.35*	8.23±0.13	25.25±0.02	0.37±0.002
Gobindabhog	8.94±0.03	39.47±0.63	17.83±0.22	7.39±0.18	22.89±0.03	0.37±0.001
BPT 5204	1.9±0.02	34.12±0.36	8.91±0.17	12.91±0.16	37.91±0.02	0

Values are mean ± standard deviation of five determinations. Values are in ppm. * Significant difference was observed at p<0.01.

Protein influences the nutritional quality of rice. In this study the protein content was high (>3.5%) for the black rice varieties. Especially Huggi Bhatta and Chak Hao Poireithon showed the highest protein values (5.56 g% and 5.53 g%), and therefore they are good for consumption. The digestible amylose content of all varieties was between 18.6 g% to 24.5 g% and no significant differences were found between rice varieties.

3.3 Selected mineral composition

Mineral contents in the black rice samples are given in Table 3. It was found that the black rice varieties contained 2-to-4-fold higher Cr, 2-to-3-fold higher Fe and a higher amount of other mineral micronutrients than the white rice varieties, specifically BPT 5204. Among the black varieties, Chak Hao Poireithon contained the highest amount of Cr (9.38 mg/kg), Tiki contained the highest amount of Mn (62.54 mg/kg), and Huggi Bhatta contained the highest amount of Fe (25.73 mg/kg), Zn (56.26 mg/kg) and Co (0.46 mg/kg). Copper is found to be the highest (12.91 mg/kg) in white rice BPT 5204. These minerals are essential for normal metabolic function, production of essential hormones and enzymes and are required components for a balanced diet. A positive correlation was observed between Fe and Cr ($p < 0.01$) in black rice varieties. As shown in Table 3, black rice Huggi Bhatta and Tiki are an excellent source of essential minerals among the studied rice varieties and could be helpful to prevent various nutritional deficiencies in the human body. Presence of high level of Zn and Fe in the black rice varieties might be beneficial for human body, as the recommended limit of daily intake of Fe (11.8 to 58.6 mg/day) and Zn (3 to 14 mg/day) could be achieved by consuming these rice varieties (WHO, 2004). Huggi Bhatta with the highest amount of Fe, Zn, and Co, may be prescribed to patients suffering from anemia. Traditionally, people of West Bengal used to feed iron-rich rice for lactating mothers, to cure their peri-partum anemia (Deb, 2017). Heavy metal micronutrient-rich rice varieties have a long history of treating various diseases and malnutrition as they have selective therapeutic properties (Deb, 2017).

## 3.4 Phenolics and antioxidant potentialities

Phenolics are a group of natural antioxidants that have an important role in pharmacological functions. As

Table 4. B complex vitamins and selected phytochemicals in different rice samples.

Variety name	Thiamin (mg/100g)	Riboflavin (mg/100g)	Niacin (mg/100g)	Pantothenic acid (mg/100g)	Pyridoxine (mg/100g)	Biotin (mg/100g)	Cyanoco-balamin (mg/100g)	DPPH (IC_{50}) in mg/ml	FRAP (mM $FeSO_4$/g dw)	TPC (mg gae/100 gmdw)	TFC (mg qe/100 gmdw)	Total Carotenoid (µg/g)
Kalo Bhat	0.11±0.01	0.56±0.02*	0.19±0.01*	2.9±0.22*	0.2±0.001	0.01±0.001	ND	3.01±0.02*	3.58±0.02*	384.69±7.59*	198.51±5.29*	29.73±0.28*
Huggi Bhatta	0.67±0.01*	0.04±0.002	0.19±0.02*	ND	ND	0.24±0.01*	ND	1.44±0.01*	2.95±0.03*	495.11±15.88*	215.73±11.82*	32.86±0.53*
Tiki	1.38±0.08*	0.3±0.01	0.13±0.03	0.81±0.04	0.11±0.002	ND	ND	2.34±0.04*	3.45±0.01*	324.72±10.64*	150.87±9.36	21.19±0.44*
Chak Hao Poireithon	0.64±0.01*	0.31±0.01	0.8±0.02	2.69±0.02*	0.1±0.001	0.01±0.001	ND	3.97±0.03	1.71±0.02	212.62±11.22*	165.42±7.59	15.39±0.63
Gobindabhog	ND	0.01±0.001	0.34±0.03	ND	ND	ND	ND	25.63±0.13	1.12±0.02	15.88±0.68	76.43±1.26	1.19±0.03
BPT 5204	0.37±0.02	0.02±0.001	0.05±0.001	ND	ND	ND	ND	ND	0.49±0.01	29.5±0.83	34.33±0.96	2.26±0.12

Represented values are mean ± standard deviation of three determinations. *Significant difference was observed at p<0.01. ND: Not detected.

Kala bhat   Hugi bhatta   Tiki

(2a)

Chakhao Poireiton   Gobinda bhog   BPT

(2b)
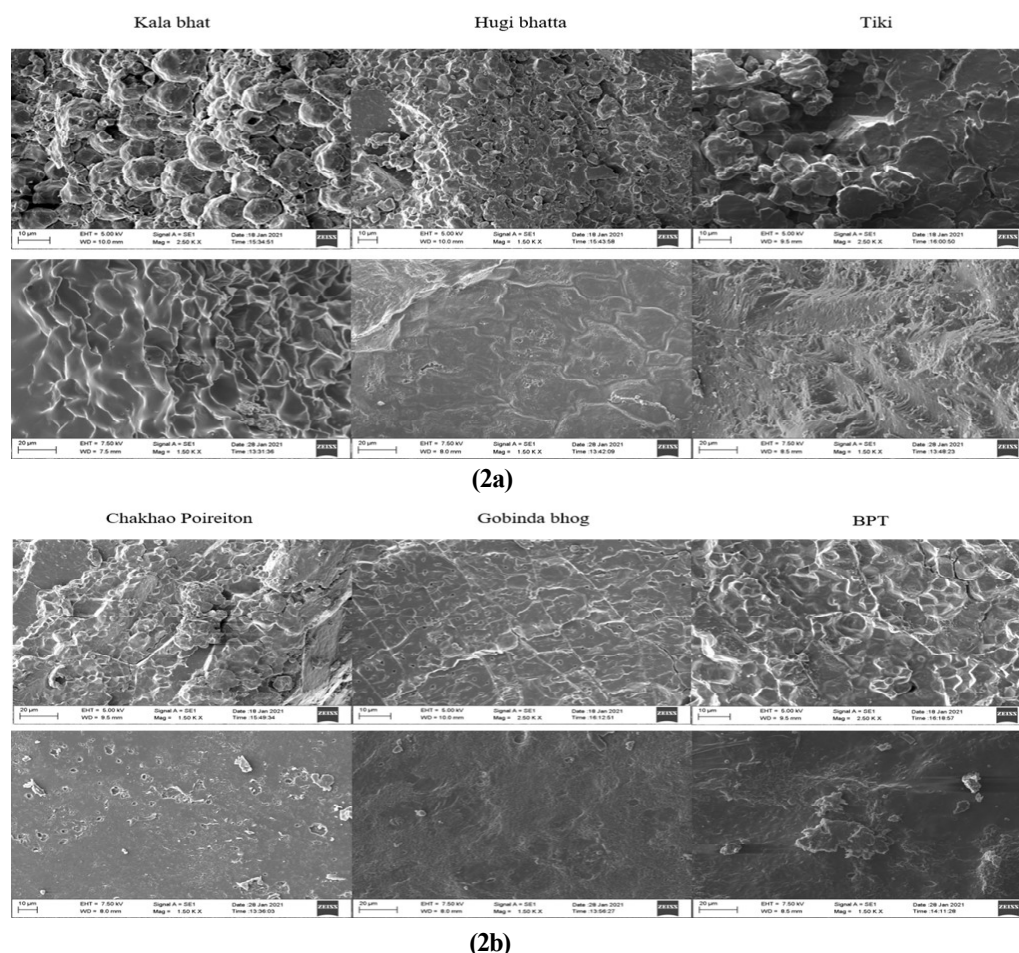
Fig. 2a. & 2b. Scanning electron microscopic (SEM) images of the samples. Images of raw rice grains (top row) and corresponding cooked rice grains (bottom row).

shown in Table 4, higher TPC (Total Phenolic Compound) was observed in Huggi Bhatta (495 mg GAE/100g) followed by Kalo Bhat (384.69 mg GAE/100g), Tiki (324.72 mg GAE/100g) and Chak Hao Poireithon (212.62 mg GAE/100 g). TPC was also found in lower amounts in white rice varieties. BPT 5240 contained higher TPC (29.50 mg GAE/100g) than Gobindabhog (15.88 mg GAE/100g). The higher content of polyphenols in the darkly pigmented rice grains (black) can be related to the difference in pericarp colour. The pericarp colour pigments are derived from various polyphenols, whose concentrations in the grain vary among genotypes. Plant phenolics like isoflavonoids and stilbenes have been found to be beneficial to human health (Cornwell, Cohick and Raskin, 2004). Because flavonoids promote antioxidant and anti-inflammatory activities and prevent cancer cell

growth, estimation of the flavonoid contents alongside the total antioxidant activity were carried out. The highest flavonoid content was found in Huggi Bhatta rice grain (215.73 mg QE/100g) followed by Kalo Bhat (198.51 mg QE/100g), Chak Hao Poireithon (165.42 mg QE/100g) and Tiki (150.87 mg QE/100g). The flavonoid contents of the two white rice varieties, Gobindabhog (76.43 mg QE/100g) and BPT 5204 (34.33 mg QE/100g) are presented in Table 4. The potentiality to inhibit the 50 percent concentration of the DPPH free radicals (IC_{50}) is a measure of antioxidant activity. Lower IC_{50} values indicate greater antioxidant potentiality. The free radical scavenging activity was found higher in black rice varieties. Among all the rice varieties (Table 4), Gobindabhog showed lowest antioxidant potentiality (25.63 mg/ml) where as in BPT 5204, no antioxidant potentiality was found (Table 4).

IC_{50} value was found highest in Huggi Bhatta (1.44 mg/ml) followed by Tiki (2.34 mg/ml), Kalo Bhat (3.01 mg/ml) and Chak Hao Poireithon (3.97 mg/ml) which could be due to high pigments and high content of phytoconstituents. The phytochemicals were significantly higher in black rice varieties than the two white rice varieties. A strong positive correlation was found between antioxidant activity and TPC in all the black rice varieties (at p <0.01), clearly indicating that higher TPC value is largely responsible for their antioxidant activities. This suggested that black rice varieties are a richest source of phenolic antioxidants and could be beneficial of health due to the presence of high level of nutritionally important phytochemicals.

3.5 Total Carotenoid contents

Total Carotenoid contents of four black rice and two white rice varieties were represented in Table 4. The results have shown that black rice Huggi Bhatta contained the highest concentration of total carotenoid (32.86 µg/g) followed by Kalo Bhat (29.73 µg/g), Tiki (21.19 µg/g) and Chak Hao Poireithon (15.39 µg/g) which were significantly (p<0.01) higher than the white rice varieties. Among the white rice varieties, Gobindabhog contained the lowest amount of carotenoid (1.19 µg/g). Due to the presence of pigments, carotenoid contents were significantly higher in the black rice grains than in the white rice varieties. A positive correlation was also observed between TPC and TFC, suggesting their functionality and biological properties as nutraceuticals. Carotenoids are naturally occurring pigments that are present in hundreds of forms in the food chains and human diet. The presence of high level of carotenoid in food could prevent different types of cancer, heart diseases and oxidation of lipoprotein. Dietary carotenoids provide benefit for ocular function

and health (Eggersdorfer and Wyss, 2018). The presence of a high level of total carotenoids in black rice grains implies a therapeutic importance of these landraces in human health and nutrition.
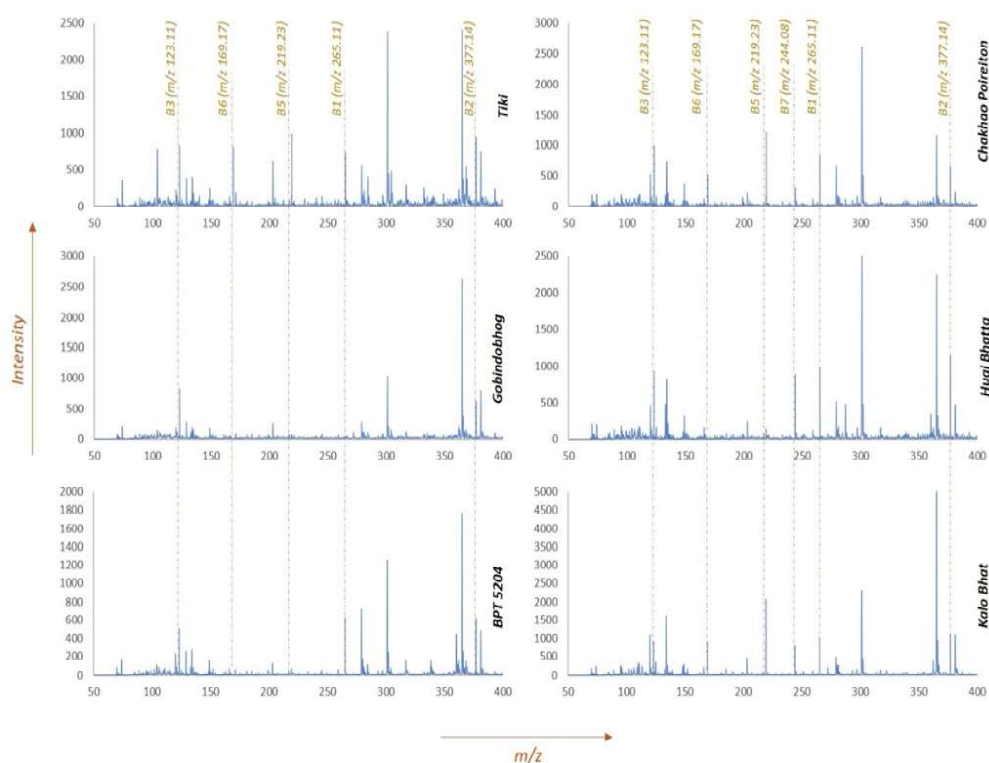
3.6 Vitamin B complex

The concentrations of different B complex vitamins in the rice varieties examined here are given in Table 4. Vitamin B_1 (Thiamin) was present in the range of 0.11 to 1.38 mg/100g, vitamin B_2 (riboflavin) 0.01 to 0.56 mg/100g, vitamin B_3 (niacin) 0.05 to 0.80 mg/100g, vitamin B_5 (pantothenic acid) 0.81 to 2.69 mg/100g, vitamin B_6 (pyridoxine) 0.10 to 0.20 mg/100g and vitamin B_7 (biotin) 0.01 to 0.24 mg/100g in different rice varieties. Vitamin B_{12} (cyanocobalamin) was not detected in any rice varieties. In Kalo Bhat and Chak Hao Poireithon, all the B complex vitamins except B12 were detected. Among the white rice varieties, BPT 5204 contained more B complex vitamins than Gobindabhog. Further, mass spectrometric identification of B complex vitamins in the extracts of rice samples confirmed the presence of the vitamin molecules in rice grains (Fig. 3). Thiamin (m/z 265.11), riboflavin (m/z 377.14), and niacin (m/z 123.11) were detected in all the rice samples. However, pantothenic acid (m/z 219.23), pyridoxine (169.17), and biotin (m/z 244.08) were detected only in Tiki, Kalo Bhat, and Chak Hao Poireithon rice grains. So, the rice varieties used in this study, are a good source of thiamin (except Gobindabhog), riboflavin and niacin. Moderate contents of such B complex vitamins in black rice varieties could be useful in treating various malnutritional problems such as, weakness, muscle wasting, etc. The functional relationship (Fig. 4a) as well as the variability of B complex vitamins, namely Thiamin, Riboflavin and Niacin with the antioxidant potentiality, have been

Table 5. Measurement of the zone of inhibition for antibacterial activity testing by agar diffusion method.

Rice variety	Concentration of extract	Diameter of the inhibition zones around the well (mm)			
		E. coli	*S. warneri*	*S. aureus*	*B. subtilis*
Kalo Bhat	20 mg/ml	3.1 ± 0.2	2.9 ± 0.2	4.6 ± 0.3	3.5 ± 0.3
Huggi Bhatta	20 mg/ml	3.2 ± 0.2	3.6 ± 0.3	4.1 ± 0.2	3.2 ± 0.2
Tiki	20 mg/ml	4.6 ± 0.3	4.8 ± 0.3	6.9 ± 0.4	8.3 ± 0.5
Chak Hao Poireithon	20 mg/ml	3.7 ± 0.2	4.5 ± 0.2	4.2 ± 0.2	6.9 ± 0.3
Gobindabhog	20 mg/ml	n.a	n.a	n.a	n.a
BPT	20 mg/ml	n.a	n.a	n.a	n.a

n.a: not applicable (zone of inhibition not found)

**Fig. 3.** ESI-MS Identification of different B complex vitamins in extracts of rice samples.

represented in (Fig. 4b). We considered the three B complex vitamins as they are found in all rice varieties used in this study. The darker region indicates the higher antioxidant activity which corresponds to the concentration of specific B complex vitamins in the grains. It appears that the presence of B complex vitamins in rice increases the antioxidant activity of the grains. In black rice varieties, due to the presence of all three B complex vitamins, the antioxidant potentiality was higher than the white rice varieties. According to Sinbad et al. (2019) certain vitamins such as vitamin C, E and B complex could act as an antioxidant by reducing the oxidative stress in human body. Therefore, it can be concluded that the presence of B complex vitamins in rice grains might have a direct impact on the antioxidant potentiality of rice varieties.

3.7 Antimicrobial activities

Antimicrobial properties of the rice extract were examined against selected Gram-positive and Gram-negative foodborne pathogenic bacteria. Agar diffusion method was used and the ability of the bacteria to

produce visible growth in presence of rice extract was investigated and presented in Table 5. All the black rice extracts were found to have some antibacterial properties against all tested bacteria (Fig. 5). Specifically, Kalo Bhat and Tiki demonstrated more sensitivity than other black rice varieties. Extract of Gobindabhog and BPT did not show any antibacterial activity against any of the bacteria. In case of Black rice varieties, the diameter of the inhibition zone ranged between 2.9 ± 0.2 mm to 8.3 ± 0.2 mm. MIC (minimum inhibitory concentration) determination reveled that black rice extracts were found to inhibit the growth of all the Gram-positive and Gram-negative bacteria. The best antibacterial potentiality of the back rice (Kalo Bhat) extract was found against *S. aureus*. Both in agar diffusion assay and MIC assay, Gram-positive bacteria were more susceptible than Gram-negative bacterial.

Bacterial species taken for this study are common gut commensals, and also cause human infections, like wound infection, urinary tract infection, endocarditis, etc. Therefore, this study indicates an
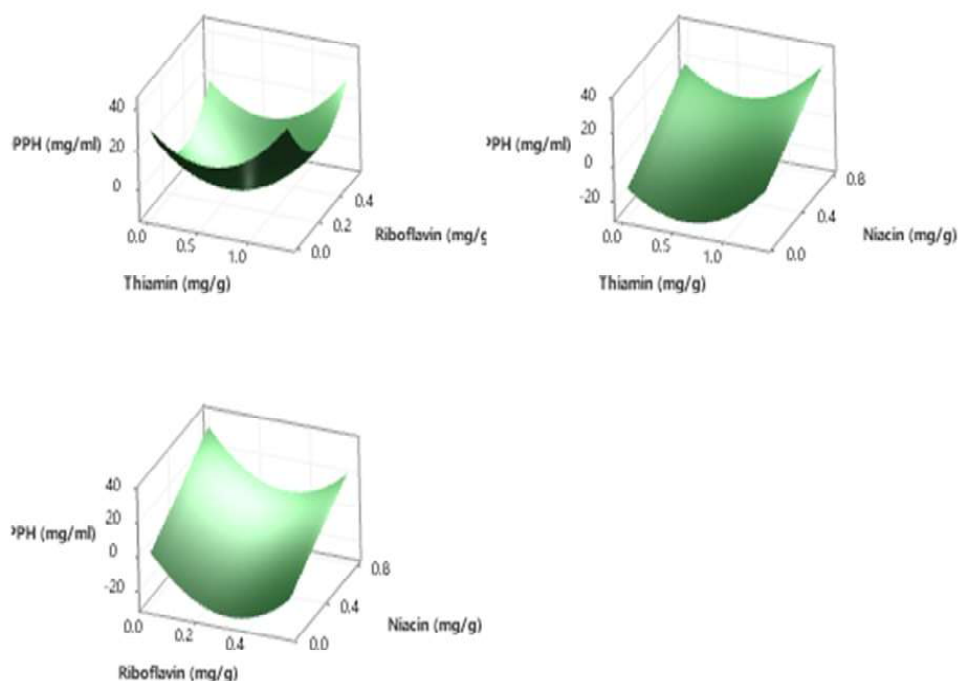
Fig. 4a. Surface plot of the functional relationship of B complex vitamins with antioxidant potentiality among rice varieties.
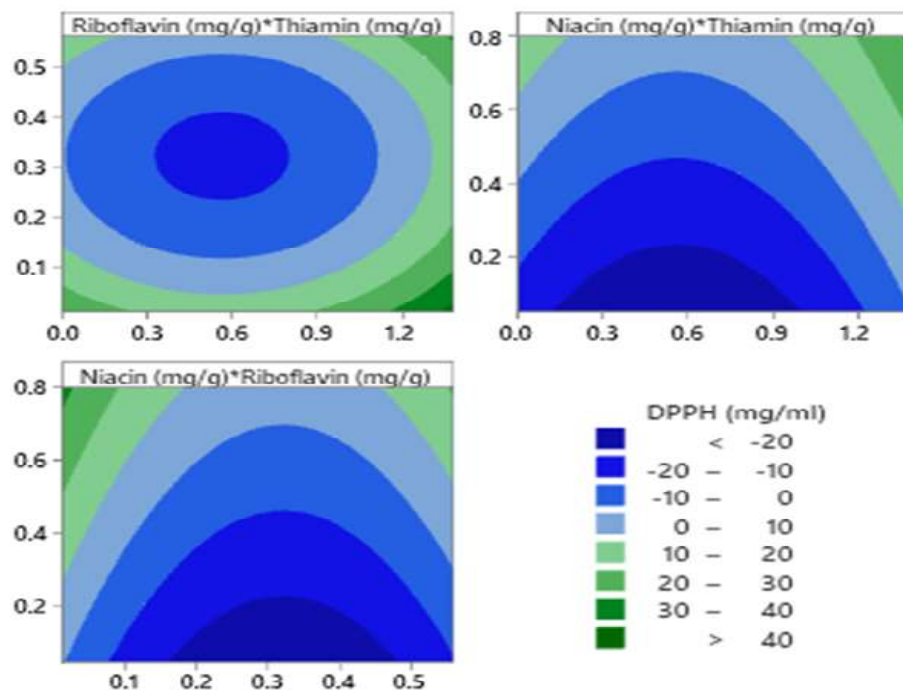

Fig. 4b. Contour plot of antioxidant potentiality with the variability of B complex vitamins among rice varieties.

important role of the extract of the back rice varieties as a potential bactericidal agent.

3.8 Relationships among different nutrients

From a pair wise correlation matrix of selected biochemical characters, we identified the rice varieties with strong relationships (p<0.01) between pairs of biochemical characters, and are presented in Table 6A
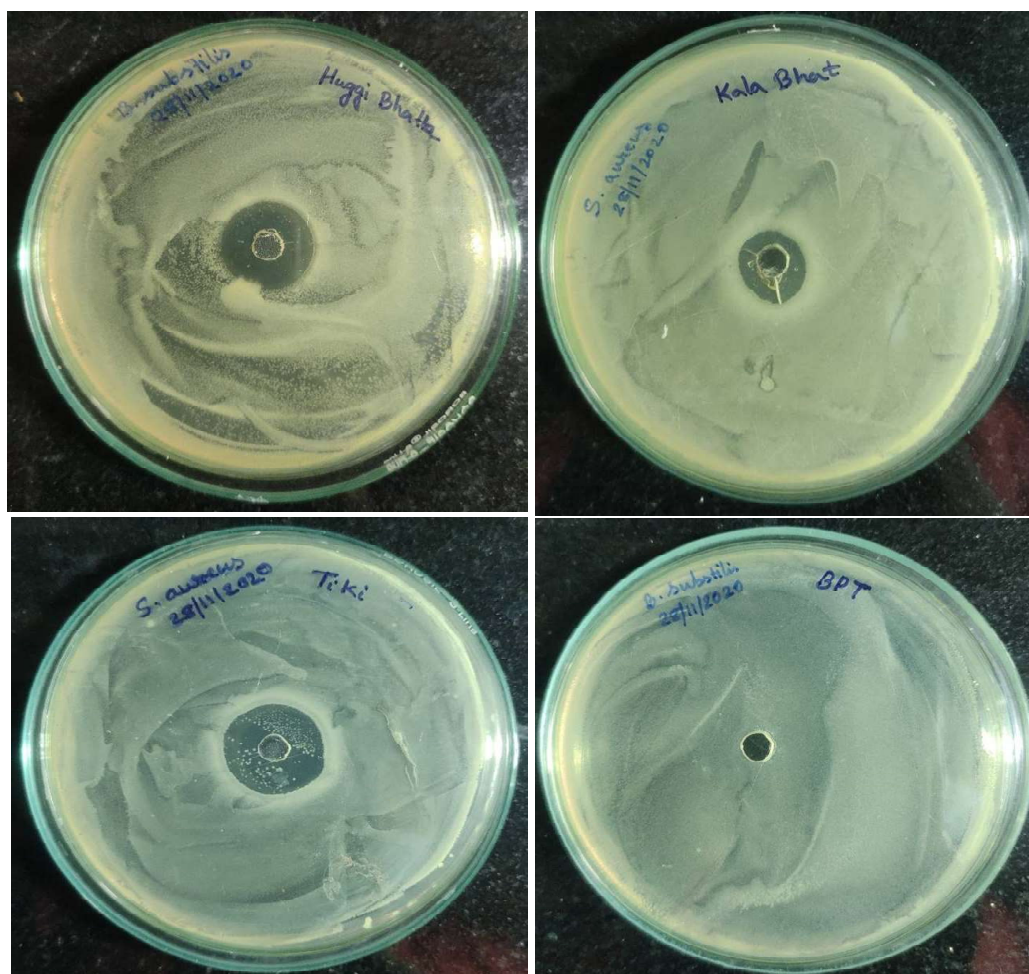
Fig. 5. Selected photographs of the nutrient agar plates showing zone of inhibition of the black rice extracts implying their antibacterial activities and one white rice plate showing no antibacterial activity.

to 6F. From these Tables, any inherent propensity for different biochemical properties to express together is likely to be detected. However, an examination of the relationships does not reveal any explicit unimodal relationship for all rice varieties. It appears that Fe and Zn are directly ($p<0.01$) correlated in Kalo Bhat and Chak Hao Poireithon and both the white rice varieties, while the relationship is strongly inverse in the case of Huggi Bhatta. Starch and protein contents are strongly related in both the white rice varieties, whereas, this relation does not hold for the black rice, except in Huggi Bhatta rice. Zn and Carotenoid contents are directly correlated ($p<0.01$) in Kalo Bhat and Chak Hao Poireithon, however the relationship does not exist in case of other rice varieties. In Kalo Bhat, Chak Hao

Poireithon and BPT rice varieties, vitamin B1 is directly correlated ($p<0.01$) with Fe and Zn, whereas, the relationship does not exist for other rice varieties. Starch and Zn contents are inversely correlated ($p<0.01$) in all the rice varieties except Kalo Bhat. Tekin et al. (2018) reported correlation between vitamins and minerals in wheat. In this study, Vitamin B2 showed a strong positive correlation with Zn ($p<0.01$) only in Tiki. An analysis with large number of rice samples could be helpful to understand the correlation between B complex vitamins and minerals. Antioxidant potentiality shows a strong positive correlation ($p<0.01$) with vitamin B_1, B_2, and carotenoids (Table 6A to 6F) in all black rice varieties. However, there are either negative correlation or no correlation found between the

biochemical characters in other rice varieties. In view of the absence of a unimodal relationship between the biochemical properties, it is plausible that molecular marker-based Quantitative Trait Locus (QTL) analyses may be required to understand the relationships of the biomolecular pathways of synthesis of these nutrient molecules. However, QTL and molecular biological assessment are beyond the scope of this study.

4. CONCLUSION

This study shows that decorticated black rice varieties have significantly greater protein, heavy metal micronutrients, B complex vitamins, phenols, flavonoids, and antioxidant activities than the popularly consumed white rice varieties. It is also reported that B complex vitamins significantly impact the contents of minerals and antioxidant potentiality in black rice varieties. High levels of thiamin, riboflavin, antioxidant activities, flavonoid and carotenoids are likely to underlie the therapeutic properties of the black rice varieties as reported in folk medicine. This study is the first scientific investigation on the nutraceutical attributes of selected farm-saved Indian traditional black rice landraces. Further studies are in progress to characterize the bioactive phytochemicals in these and other rice landraces in order to understand their nutraceutical importance.

Authors Contribution

D.D. & P.R. conceived the research program and designed the study. P.R, A.K.S, T.S, & S.T.M. verified the analytical methods. D.D, T.S, & T.P. encouraged P.R, A.K.S, & S.T.M. to perform all the biochemical analyses, provided the access and instrumental facilities for various analyses and supervised the findings of this work. D.D & P.R performed the computations. All authors discussed the results and contributed to the final manuscript.

Declaration of Interest

The authors declare no competing financial interest.

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