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Phytochemical profiling, antioxidant capacities and anthocyanin compositions of the pigmented rice (*Oryza sativa*) of north-east India

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ABSTRACT

The association between the consumption of pigmented rice (*Oryza sativa* L.) and the improvement of human health is generating a great deal of interest among the researchers. An experiment was conducted during 2020 and 2021 at Assam Agricultural University, Jorhat, Assam to study the phytochemical profiling, antioxidant capacities and anthocyanin compositions of the 14 pigmented rice genotypes. The results were compared with the well-known traditional pigmented rice variety from Manipur, Poreiton Chakhao. The total phenolic content, total flavonoid content and total anthocyanin content ranged from 37.101 to 493.611 mg gallic acid equivalent/100 g, 53.316 to 151.667 mg quercetin equivalent/100 g and 1.006 to 13.904 mg cyanidin-3-chloride equivalent/100 g in the studied rice genotypes. The amount of rice showing 50% DPPH free radical scavenging activity (IC 50 value) ranged from 6.610 to 29.376 mg. The HPLC analysis revealed presence of both cyanidin-3-glucoside and peonidin-3-glucoside in the black pigmented rice genotypes, viz. TTB Black Rice 7, Chakhao-1, Chakhao-2 and TTB Black Rice 11. However, in the red rice genotypes analysed (Balam and Nepali Chakuwa), these two anthocyanins were not detected.

Keywords: Anthocyanins, Antioxidants, DPPH, HPLC, Pigmented rice

Rice (*Oryza sativa* L.) is an important cereal cultivated worldwide and a significant staple food of a large section of the world's population (Mudoi and Das 2019). Reddy *et al.* (2018) reported that in developing countries it is the major source of carbohydrates and even proteins providing 21% of dietary energy and 15% of protein to global population. Rice is popularly consumed in the form of white rice but recently due to increased awareness about the health benefits and economic importance of pigmented rice, it is being preferred amongst the population and is gaining a good platform. Pigmented rice are black, red or purple in colour which is due to the presence of anthocyanins and proanthocyanidins in their bran layer (Mohan *et al.* 2010). Anthocyanins are a group of reddish to purple water-soluble flavonoids belonging to the class of phenols and are present in the pericarp layers of the rice grain (Chaudhary 2003). Anthocyanins possessing antioxidant activity is one among the bioactive components as nutraceutical and conventional medicine owing to the nutritional benefits of pigmented rice over white rice. Proanthocyanidins, a compound found in red rice, are condensed tannins produced as an end product of flavonoid biosynthetic pathway. They are also found to have a wide range of health beneficial properties like antioxidant, antitumor and immune stimulating properties (Rauf *et al.* 2019). The pigmented rice is a crucial future crop for the planet to counter the first health problems in growing population and supply ample of nutrients to the body that are not present in the white rice.

Assam, being rich in indigenous rice germplasm produces different cultivars of both white and pigmented rice genotypes (Mudoi and Das 2019). Special amongst these are the Bao dhan (deep water rice), generally pigmented, grown and conserved by farmers over ages and have potential to be explored owing to their neutraceutical and economic importance. Bhuvaneswari *et al.* (2020) studied the profile of anthocyanins in Chakhao genotypes and revealed cyanidin-3-O-glucoside (C3G) and peonidin-3-O-glucoside (P3G) as the major anthocyanins. Some other researchers, Abdel-Aal *et al.* (2006), Lee (2010), Achparaki *et al.* (2012), Pereira-Caro *et al.* (2013) and Pengkumsri *et al.* (2015) also studied the profile of anthocyanins in black pigmented rice genotypes of different areas globally and reported cyanidin -3- glucoside and peonidin -3- glucoside as the major anthocyanin present.

Owing to our study, this is the first and foremost report considering the anthocyanin compositions of the pigmented rice genotypes of Assam. Thus, the current research was undertaken with the aim to explore and characterise the

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rich pigmented rice germplasm of Assam which will pave the way for its improvement and popularity.

MATERIALS AND METHODS

Collection of cultivars: The present study was carried out at Department of Biochemistry and Agricultural Chemistry, Assam Agricultural University, Jorhat, Assam during 2020 and 2021. A total of 13 pigmented rice genotypes were used in the present study and Poreiton Chakhao was taken as a reference cultivar (Table 1). The genotypes were cultivated and harvested as per their package and practices based on the type of rice at Regional Agricultural Research Station (RARS), AAU, Titabar.

Processing of rice grains for storage: Rice grains were de-husked using a de-husker (Satake Corporation, Hiroshima, Japan). The dehusked rice samples were stored refrigerated (at 5°C) within food grade plastic container until used for analysis. Prior to analysis the rice grains were powdered using mortar and pestle.

Total phenolic content: The total phenol content (TPC) was estimated by the method given by Singleton *et al.* (1999) using Folin Ciocalteau reagent.

The rice flour (0.5 g) was extracted with 80% methanol (Hi-media) under agitation for 30 min using a magnetic stirrer. The mixture was then centrifuged at 10,000 rpm for 20 min and the supernatant was collected. The residues were re-extracted twice under the same conditions. The collected supernatants were then evaporated to dryness and the residue was dissolved in 7 ml distilled water.

For the estimation of TPC, 0.1–0.3 ml of the sample extract was taken and the volume was made up to 1 ml with distilled water. Adding 0.5 ml Folin Ciocalteau reagent (Hi-media), standing time of 3 min was given. After that 2 ml of 20% sodium carbonate (Hi-media) solution was added. The reaction mixture was mixed thoroughly with the help of a vortex and the test tubes were placed in boiling water bath for exactly 1 min. It was then cooled and absorbance was measured at 650 nm against a reagent blank. The replication was done in triplicate and the average value was calculated out. By using the standard curve of Gallic acid (Hi- Media), the TPC of the pigmented rice genotypes were analysed and expressed as mg Gallic acid equivalent (GAE) per 100 g dry wt.

Table 1 Genotypes used in the present study

Genotype	Place of collection	Types of rice
Amona Bao	RARS, Titabar	Deep water rice
Balam	RARS, Titabar	Winter rice
Betu	RARS, Titabar	Autumn rice
Chakhao- 1	RARS, Titabar	Locality specific
Chakhao-2	RARS, Titabar	Locality specific
Dol Bao	RARS, Titabar	Deep water rice
Kokua Bao	RARS, Titabar	Deep water rice
Lal Dhupa	RARS, Titabar	Winter rice
Nepali Chakuwa	RARS, Titabar	Winter rice
Negheri Bao	RARS, Titabar	Deep water rice
Poreiton Chakhao	Imphal, Manipur	Black rice of Manipur
Singphow Bora	RARS, Titabar	Winter rice
TTB Black Rice (AAU 1491-4, line 7)	RARS, Titabar	Winter rice
TTB Black Rice (AAU 1347-2, line 11)	RARS, Titabar	Winter rice

Total flavonoid content: The total flavonoid content (TFC) was measured by colorimetric method, given by Wu and Ng (2008). Sample was extracted in a similar manner as that of TPC. For the estimation of TFC, 0.3 ml of the extracted sample were diluted up to 1 ml by addition of 0.7 ml distilled water. Following which 0.15 ml of 5% sodium nitrite (SRL) and 0.15 ml of 10% aluminium chloride (Sigma) was added and the reaction mixture was given a standing time 6 min. Then 4% NaOH (2 ml) (Hi- media) was added to the mixture and mixed well, and kept for 15 min. The absorbance was measured at 510 nm. The replication was done in triplicate and the average value was calculated out. Quercetin (Hi-media) was used as the standard and the TFC of the analysed samples were expressed in the form of mg quercetin equivalent (QE) per 100 g dry wt.

Total anthocyanin content: For determination of total anthocyanin content (TAC), the spectrophotometric method reported by Abdel-Aal and Hucl (1999) was followed. The samples were extracted using acidified methanol i.e. 0.1 N HCl and methanol in a volume ratio of 85:15. The solvent and sample ratio was maintained at 10:1. The reaction mixture was first incubated in a shaker for 30 min (protected from light) and then centrifuged following which the supernatant was collected and kept aside. The residues were re-extracted twice under the same conditions and the supernatants were collected together and the absorbance was measured at 525 nm using a spectrophotometer against a reagent blank. The replication was done in triplicate and the average value was calculated out. Cyanidin -3-chloride was used as the standard and the TAC of the analysed samples were expressed in the form of mg cyanidin-3-chloride equivalent per 100 g dry wt.

DPPH radical scavenging activity: The methanolic extract of the samples was analysed for free radical scavenging activity by the procedure given by Brand-Williams *et al.* (1995) using stable 2,2- diphenyl-1-picryl hydrazyl radical (DPPH) (Hi- media). An aliquot (0.1 to 0.3 ml) of methanolic extract of sample (0.5 g of sample extracted with 15 ml of methanol) was mixed with 2.7 ml of freshly prepared 0.004% DPPH in methanol (after making the volume up to 0.3 ml) and kept in dark for 30 min at room temperature. The absorbance was measured at 517 nm against blank (only methanol) and 0.3 ml of methanol mixed with 2.7 ml of DPPH was used as a control. The replication was done in triplicate and the average value was calculated out.

The DPPH free radical scavenging activity was calculated as:

$$\text{Scavenging activity (\%)} = \frac{\text{(Absorbence of control – Absorbence of sample)}}{\text{(Absorbence of control)}} \times 100$$

From the above data, IC 50 values representing 50% DPPH radical scavenging activity was determined for each of the sample.

Extraction for HPLC analysis: 1 g of the samples were extracted in 20 ml of acidified methanol (1 N HCl, 85:15 v/v) and filtered through 0.2 micrometer syringe filter suitable for HPLC analysis. Cyanidin-3-glucoside and peonidin-3-glucoside (Cayman Chemical) were taken as standards and solution was prepared by dissolving 1 mg of each standard in 1 ml of acidified methanol. 100 µl of the standard was further diluted with 900 µl of acidified methanol and 20 µl was injected into the system.

Chromatographic analysis was performed on Hitachi Chromaster 3000 series HPLC system equipped with an autosampler and a diode array detector. The separation of anthocyanins was carried out through a Cosmosil MS-2 C18 column (300 mm × 4.6 mm ID, pore size 5 µm) at ambient temperature. A 20 µl of the sample was injected into the HPLC system and a gradient flow was maintained (Table 2). Mobile phases and gradients used were 0.1% phosphoric acid in water (solvent A) and 100% acetonitrile (solvent B). The flow rate was set to 1 ml/min and the elution of the compounds were monitored at 200–600 nm wavelength in a scan mode.

Table 2 Gradient profile of the mobile phases used for the separation of anthocyanins

Time (min)	Solvent A (%)	Solvent B (%)
0–5	90	10
7	75	25
10–14	60	40
16–20	40	60
22–24	20	80
26–30	90	10

Statistical analysis: Statistical analyses were performed using IBM SPSS V25.0 programme with three independent replicates for each data set. Significance among different samples was calculated using one way ANOVA with Duncun's multiple range test ($P \leq 0.05$).

RESULTS AND DISCUSSION

The TPC, TFC, TAC and antioxidant activity of pigmented rice genotypes were analysed and compared to the black rice cultivar of Manipur, Poreiton Chakhao (Table 3). The genotypes differed significantly regarding TPC, TFC, TAC and antioxidant activity.

Anthocyanin profile of genotypes corresponding to high amount of TPC, TFC and TAC, viz. TTB Black Rice (AAU 1491-4, line 7), Chakhao-1, Chakhao-2, Balam, Nepali Chakhua, Poreiton Chakhao and TTB Black Rice (AAU 1347-2, line 11) were further performed by HPLC. The retention time and absorbance peaks of the analysed samples were compared with the standards, viz. cyanidin-3-glucoside, peonidin -3- glucoside (Fig 1).

As per our study, black rice genotypes have higher amount of TPC, TFC and TAC. Genotypes TTB Black Rice 11, TTB Black Rice 7, Chakhao 1 and Chakhao 2 representing the black rice of Assam are found to be promising genotypes. Whereas Nepali Chakuwa, Singphow

Table 3 Phytochemical analysis of pigmented rice genotypes of Assam

Genotype	Total phenol content (mg GAE/100 g)	Total flavonoid content (mg QE/100 g)	Total anthocyanin content (mg cyanidin-3-chloride equivalent/100 g)	Antioxidant activity (IC 50 value in mg)
Poreiton Chakhao	453.426 ^{k}	170.926 ^{h}	21.667 ^{i}	6.310 ^{a}
TTB Black Rice 11	493.611 ^{j}	151.667 ^{g}	13.905 ^{h}	6.611 ^{a}
TTB Black Rice 7	250.926 ^{i}	121.111 ^{f}	10.280 ^{g}	8.771 $^{b\,c}$
Chakhao-2	203.426 ^{h}	118.148 ^{f}	8.152 ^{f}	7.643 $^{a\,b}$
Chakhao- 1	188.333 ^{g}	110.000 ^{e}	6.101 ^{e}	9.005 $^{b\,c}$
Nepali Chakuwa	134.074 ^{f}	78.519 ^{d}	1.896 ^{d}	10.039 ^{c}
Singphow Bora	117.593 ^{e}	77.407 ^{d}	1.905 ^{d}	14.283 ^{d}
Betu	107.280 $^{d\,e}$	62.526 ^{b}	1.277 ^{b}	9.074 $^{b\,c}$
Negheri Bao	94.259 $^{c\,d}$	66.111 $^{b\,c}$	1.637 ^{c}	14.837 ^{d}
Lal Dhupa	91.667 ^{c}	73.148 $^{c\,d}$	1.655 ^{c}	18.910 ^{e}
Kokua Bao	67.037 ^{b}	66.111 $^{b\,c}$	1.068 ^{a}	26.564 ^{f}
Balam	66.574 ^{b}	66.111 $^{b\,c}$	1.655 ^{c}	14.618 ^{d}
Dol Bao	52.593 ^{b}	74.630 ^{d}	1.006 ^{a}	27.320 ^{f}
Amona Bao	37.101 ^{a}	53.316 ^{a}	1.158 $^{a\,b}$	29.377 ^{g}

Data within the same column with the same letters are nonsignificant ($P \leq 0.05$)

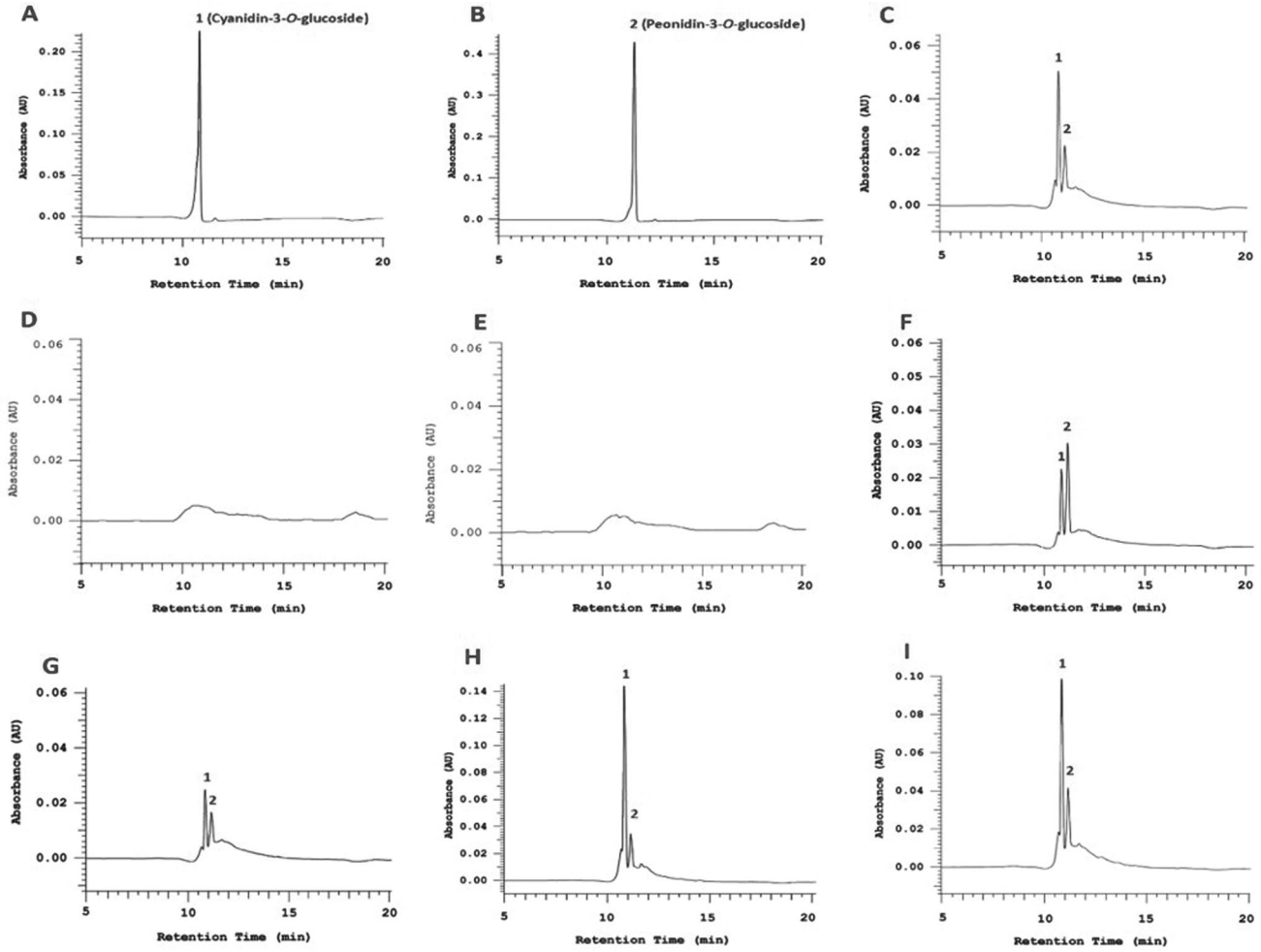

Fig 1 HPLC detection of the anthocyanin compounds in the crude extracts of the rice samples.
A, C-3-O-G; B, P-3-O-G; C, TTB Black Rice (AAU 1491-4, line 7); D, Balam; E, Nepali Chakuwa; F, Chakhao-2; G, Chakhao-1; H, Poreiton Chakhao; I, TTB Black Rice (AAU 1347-2, line 11).

bora, Betu, Balam, Lal dhupa are red rice cultivars and have comparatively lower amount of TPC, TFC and TAC.

Our results are in line with that of Saikia *et al.* (2012) who reported TPC, TFC and TAC in Poreiton Chakhao to be 245 mg GAE/100 g and 123.75 mg QE per 100g and 35.87 mg cyanidin-3-glucoside equivalent per 100 g (6% polished sample) and comparatively higher amount was found in our analysed samples. However, Asem *et al.* (2015) reported a higher quantity of TPC and TAC i.e. 577 mg GAE/100 g and 74 mg cyanidin-3-glucoside equivalent per 100 g in unpolished Poreiton Chakhao. The present findings were also comparable to the findings of Samyor *et al.* (2016) who studied two dehusked red rice genotypes of Arunachal Pradesh and reported the phenol content in the range of 262–350 mg GAE/100 g brown rice. Pathak *et al.* (2016) studied the dehusked pigmented glutinous rice variety of Assam and reported the total flavonoid content to be 60.76 mg QE per 100 g. Jayaraman *et al.* (2019) studied the anthocyanin content in dehusked red rice genotypes and reported that it ranged from 9.26 to 15.20 mg cyanidin-3-glucoside equivalent per 100 g.

Further Saikia *et al.* (2012) reported 94.19% DPPH scavenging activity for 10 mg polished sample (6%) of Poreiton Chakhao where as Pramai and Jiamyangyuen, (2016) reported the IC 50 value to be 1.56 to 10.10 mg in black rice genotypes (unpolished) and 1.87–3.94 mg in red rice genotypes (unpolished) of Thailand. Our study also showed a comparable trend with IC 50 value ranging from 6.3 to 9.0 mg in black rice genotypes.

It was seen that TPC, TFC and TAC are positively correlated contributing to the antioxidant properties of pigmented rice genotypes. Additionally, better antioxidants tend to have lower IC 50 value which is well elucidated in the Table 4 having negative correlation with the phenolics group.

Table 4 Correlation among TPC, TFC, TAC and IC 50 of the analysed rice genotypes

	TPC	TFC	TAC	IC 50
TPC	1	+ 0.955	+ 0.94	-0.687
TFC	-	1	+ 0.97	-0.672
TAC	-	-	1	-0.616
IC 50	-	-	-	1

However, the traditional red rice variety Betu showed the IC 50 value (9.074 mg) which is comparable to Chakhao-1 (9.005 mg). Lower IC 50 values observed for most of the traditional red genotypes of Assam signified probable role of proanthocyanidins in scavenging DPPH free radical. In some studies reported by Pradipta *et al.* (2020) and Mackon *et al.* (2021), proanthocyanidins were found to be responsible for red colour of the red rice genotypes contributing towards its antioxidant properties (Rauf *et al.* 2019).

Genotypes further characterised by HPLC showed the presence of cyanidin-3- glucoside and peonidin-3-glucoside in genotypes, viz. TTB Black Rice (AAU 1491-4, line 7), Chakhao-1, Chakhao-2, Poreiton Chakhao and TTB Black Rice (AAU 1347-2, line 11). Amongst these 5 genotypes except Chakhao-2, cyanidin-3-glucoside was present in higher amount than peonidin-3-glucoside which is discernibly expressed in the graph (Fig 1). Reports by Hu *et al.* (2003) and Abdel-Aal *et al.* (2006) also stated that the cyanidin-3-glucoside and peonidin-3-glucoside were the major anthocyanins for pigmented rice of China and Canada, respectively. Pengkumsri *et al.* (2015) also reported cyanidin-3- glucoside, peonidin-3-glucoside and cyanidin chloride as the major anthocyanins present in pigmented rice genotypes from Thailand. However, in the red rice genotypes analysed, viz. Balam and Nepali Chakuwa no peak was detected at the absorbance range 460–550 nm, which might be due to either their presence below the detectable level or total absence. The findings of the present study regarding the presence of anthocyanins in Chakhao genotypes are in agreement with that already reported by Bhuvaneswari *et al.* (2020). They also found cyanidin-3-glucoside and peonidin-3-glucoside in Chakhao genotypes.

To our very best, this is the first ever report regarding the specification of anthocyanins in pigmented rice germplasm of Assam and the results of the present study were in well accordance with that of the other researcher's report on pigmented rice genotypes from both India and abroad. It was also found that in the red rice cultivars anthocyanins were not present in sufficient levels but, owing to their antioxidant properties it could be suggested that proanthocyanidins might be an important to be detectable compound which could be further characterised in the future studies. This assumption can be supported by the report of Pradipta *et al.* (2020) who stated that proanthocyanidins are the major phenolic compounds in red rice genotypes. Mackon *et al.* (2021) also reported that higher anthocyanin content led to black coloured rice bran and higher proanthocyanin content led to red coloured rice bran. Hence, characterisation of red rice germplasm of this region for their proanthocyanidin compounds could be an important area of research owing to the growing health benefits and economic importance of pigmented rice genotypes.

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