IJPSR (2024), Volume 15, Issue 12 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 27 June 2024; received in revised form, 16 July 2024; accepted, 29 July 2024; published 01 December 2024

EVALUATION OF CYANIDIN-3-O-GLUCOSIDE FROM DIFFERENT BLACK RICE VARIETIES OF MANIPUR UTILIZING FTIR SPECTROSCOPY AND HPTLC METHOD

Thongam Sofianandi [1], Dhaneshwaree Asem [2], Nikki Konthoujam [1], Antopia Laishram [1], Ojit Keithellakpam [1], Robindro Laishram [2], Nanaocha Sharma [*1] and Pulok Kumar Mukherjee [1]

Institute of Bioresources and Sustainable Development (IBSD) [1], Department of Biotechnology, Government of India, Imphal - 795001, Manipur, India.
Department of Nanotechnology [2], School of Technology, North-Eastern Hill University, Shillong - 793022, Meghalaya, India.

Keywords:

Black rice, Phytochemical analysis, Bioactive compound, Cyanidin-3-O-glucoside, FTIR, HPTLC

Correspondence to Author:
Dr. Nanaocha Sharma

Scientist E,
Institute of Bioresources and Sustainable Development (IBSD), Department of Biotechnology, Government of India, Imphal - 795001, Manipur, India.

E-mail: sharma.nanaocha@gmail.com

ABSTRACT: Black rice is a rice species, *Oryza sativa Linne* (Gramineae), which is sticky, high in nutrients, and primarily grown in Asia. In recent years, the health benefits of black rice varieties have been reported due to the presence of a bioactive compound, anthocyanin, which has highly potent activity with high macronutrients. The present study aimed to evaluate and quantify the metabolites, focusing on describing the structure of Cyanidin-3-O-glucoside (CY3G) as a biomarker of anthocyanin present in the extract of black rice. Four different black rice varieties black rice extracts of Poireiton (BREP), Kokngangbi (BREK), Amubi (BREA), and Sempak (BRES) were collected, and phytochemical analyses performed using different standard methods. Compared to other black rice varieties, Amubi, BREA has the highest phenolic (63.23±0.76 mg/g GAE), flavonoid (28.09±0.68 mg/g QE), and anthocyanin (17.69±0.45 mg C/g DEW) content. It also exhibited the highest potent antioxidant activity, with IC_{50} values of 29.1 ± 1.11 mg/ml in the ABTS assay and 98.83 ± 0.61 mg/ml in the DPPH assay. The FTIR spectroscopy analysis result shows a similar intensity of the peak absorbance in the 400–4000 cm^{-1} region of functional groups related to the biomarker compound CY3G in the black rice sample. The HPTLC analysis generated polynomial calibration curves for the 100–600 ng spot -1 concentration range for both the standard biomarker and sample, yielding a satisfactory correlation coefficient, R0.999. The retention factor (RF) and spectral correlation confirmed the method's specificity.

INTRODUCTION: Rice is a staple food for most people in various regions. Black rice is one kind of rice that has been consumed as a functional food due to its usefulness for health in China and other Eastern Asian nations since ancient times.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.15(12).3497-10

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.15(12).3497-10

It is a member of the *Oryza sativa Linne* (Gramineae) rice family, renowned for its high nutrient content and sticky texture. In Asian countries, it is widely consumed combined with white rice to improve the taste, colour, and nutritional content.

In Manipur, black rice is known as “Chak-Hao”, which means delicious and used to prepare a variety of dishes, including pudding, porridge, black rice cake, pasta, noodles, bread, wine, and flavour-infused yogurt. It is a good source of plant-

based protein as well as fibre. Including black rice in the diet may therefore be good for health. It is also known as purple rice, pigmented rice, heaven rice, king's rice, etc. because it gives a purple to black colour to the pericarp layer due to the presence of a major bioactive pigmented compound called anthocyanin, which gives the rice its different colours like dark purple, brown, red, etc. content located in the pericarp layer.

Of all rice varieties, it has the highest concentrations of dietary fibre, protein and antioxidants. Antioxidants serve as the first line of defence against free radical damage. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function, and may play a part in cancer, heart disease, stroke, and other diseases of aging. Antioxidants can stabilize or deactivate free radicals before they cause cell damage. Some recent studies also revealed that the existence of bioactive compounds in black rice variants has been linked to numerous human health benefits. The groups of phytochemicals found in whole grains can be classified as phenolic, carotenoids, vitamin E compounds, lignans, β-glucan and inulin [1].

One of the most abundant groups of phyto-chemicals in whole grains is phenolics, which are considered as natural antioxidants and act as radical scavengers to induce oxidative stress, causing damage to macromolecules such as proteins, lipids, and DNA [2]. Further phenolic compounds were sub-grouped into phenolic acids, flavonoids, coumarins and tannins, according to most of the literature. Flavonoids can be further divided into subclasses depending on the type of heterocycle involved, such as flavonols, flavones, isoflavones, flavanones and anthocyanidins. Kaempferol, quercetin, and myricetin are part of the flavonols, which are a subgroup of flavonoids [3], and quercetin in particular is frequently present in the tissues of plants and used as a standard for determining the total flavonoid content of black rice extract. According to another study, when the amount of purple rice flour in the biscuit mixture grew, so did the digestibility of the protein. The biscuits made entirely of purple rice flour had the lowest predicted glycemic index (PGI) compared to others [4]. Consuming black rice can also have several other positive impacts, such as anti-inflammation, preventing some cancers, detoxifying the body, improving cardiovascular health by balancing the body's levels of HDL and LDL cholesterol, etc [5, 6]. Therefore, black rice has been considered a useful and functional food due to the presence of bioactive compounds, mainly anthocyanins, in sufficient amounts [7].

Anthocyanins are water-soluble pigments primarily obtained from plants. Six main types of anthocyanin commonly occur in nature, namely Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin and Petunidin [8]. Among these, Cyanidin-3-O-glucoside (CY3G) is the major anthocyanin, which is composed of 93% of total anthocyanin in black rice and produces red, purple and brown colors in plants [9]. It is proven that switching from artificially processed to naturally occurring foods high in bioactive components, such as black rice with its aromatic and flavorful qualities, can have a positive impact on one's health. Anthocyanins are absorbed in the intestines and transported to the liver via the portal vein. They are digested, secreted, and then reabsorbed in the enterohepatic circle to resume the entire route.

After absorption, anthocyanins are processed by phase I and phase II enzymes, which form hydroxylated, glucuronidated, sulfated and methylated molecules primarily in the liver but at renal and enterocyte levels [10]. Interest in anthocyanin research is increasing due to its nutritional value and biological activity. Therefore, consumption of anthocyanin-rich diets influences the gut microbiota by maintaining energy and satiety while inhibiting the accumulation of body fat. Wistar rats were given anthocyanin cyanindin-3-O-glucoside for eight weeks, which significantly suppressed Rothia and Romboutsia while boosting Clostridium [11]. From this study, we can verify anthocyanin's potential beneficial effects on the gut microbiota. Anthocyanin extract has been shown to improve eyesight, reduce cholesterol levels, increase insulin production, protect the liver, and more [12]. All these benefits of anthocyanin compounds like cyaniding 3-O-glucoside, which have a variety of functional properties, including protection against cytotoxicity, anti-obesity, anti-neurodegenerative activity and higher antioxidant scavenging activity than white rice varieties, are the

reasons why black rice is preferable to other varieties. Considering these benefits of black rice, consumers might be persuaded to increase their consumption of black rice, which would lead to both nutritional and economic benefits in every rice-consuming country. Therefore, black rice is getting more and more popular these days due to the benefits revealed by the previously investigated studies, which include prolonging life and preventing and treating diseases and disorders like diabetes, cancer, heart disease and high blood pressure [13].

Ultimately, efficacy depends on the kind of secondary metabolite present in the sample, which determines the biological activities of the plant portion used. Therefore, understanding the benefits of black rice can provide important information to improve human health by encouraging the consumption of black rice and its use in food product development. Most of the prior studies concentrated on rice's chemoprofilling studies and little is known about the structural identification of the compounds present in different black rice varieties. Considering these gaps, the present study provides new insights into the evaluation and quantification of metabolites present in four different varieties of black rice using FTIR spectroscopy and the HPTLC method. FTIR spectroscopy is used to determine the compound through the stretching and bonding patterns of the chemical compound. It has many unique advantages over other methods, like easy and fast operation, high reliability and sensibility, high speed of measurement procedure, low amount of sample is necessary, no need to perform complex and time-consuming extractions, and non-destructive to plant samples. In crude extracts of four distinct black rice varieties (black rice extracts of Poireiton-BREP, Kokngangbi, BREK, Amubi, BREA and Sempak, BRES), FTIR analysis carried out a similar intensity peak absorbance of functional groups, which identified the chemical bond in the bioactive compound as Cyanidin-3-O-glucoside. The main anthocyanin compound, cyanidin-3-O-glucoside, was quantified as a standard biomarker in various black rice varieties using the HPTLC method. These findings have implications for improving human health through increasing black rice consumption, as well as the development of functional food products.

MATERIALS AND METHOD:

Sample Collection and Location: The current study employed four different varieties of black rice (*Oryza sativa* L.), namely Poireiton (BREP), Kokngangbi (BREK), Amubi (BREA) and Sempak (BRES), based on their availability, consumption, and cultivation in the local area.

FIG. 1: FOUR DIFFERENT BLACK RICE VARIETIES A) POIREITON, B) KOKNGANGBI, C) AMUBI, AND D) SEMPAK

All samples were collected from the local farmers in different districts of Manipur, namely Ukhrul, Tamenglong, Thoubal and Imphal-East district, Manipur **Fig. 1** and **Table 1**. The geographical position (longitude and latitude) of the four sampling sites was taken with the help of a GPS machine. All paddy rice samples were dehulled and polished using a rice dehusker and rice-milling machine. After being crushed into a coarse powder using an electric grinder, the samples were sieved *via* mesh size 80 to remove any remaining dust or debris. The samples were stored at -20 °C before the further procedure.

TABLE 1: LOCATION DETAILS OF THE SAMPLING SITE

Sample	Sampling site	Location
Poireiton, BREP	Kazora, Ukhrul Dist.	N25°03’56.4” E094°20’40.6”, Elevation of1801m

Kokngangbi, BREK	Bamgaijaeng village,Tamenglong Dist.	N24°93'663", E093°39712", Elevation of 182m
Amubi, BREA	Elangkhangpokpi, Thoubal Dist.	N24°26'23.0" E093°55"57.5", Elevation of 780m
Sempak, BRES	Haraorou, I/E Dist.	N24°91'89.6", E093°98'27.2", Elevation of 784m

Chemicals and Reagents: All chemicals utilized were of analytical grade. Quercetin dehydrates, gallic acid, anhydrous sodium carbonate (Na_2CO_3), aluminum trichloride, sodium acetate, ferric chloride hexahydrate ($FeCl_3.6H_2O$), Folin-Ciocalteu reagent, Dragendorff's reagent, mercuric chloride, potassium iodide, iodine, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt (ABTS) and ascorbic acid were acquired from Sigma-Aldrich Company. Sodium hydroxide (NaOH), ammonia, glacial acetic acid, hydrochloric acid (HCl), sulfuric acid (H_2SO_4), chloroform and citric acid are from E. Merck Himedia. There was no extra purification before employing any of the compounds or reagents.

Preparation of Extracts: According to the experimental run, the appropriate amount of methanol was put into an Erlenmeyer flask (1000 mL). The powdered rice samples were extracted using the maceration process with 70% methanol as the solvent. Ground coarse powders of black rice were treated with a 70% methanol solution for 72 hours at room temperature. The re-extraction process was carried out until the substance became colorless. The homogenates were then filtered with Whatman filter paper, the solvent evaporated using a rotary evaporator at 50 °C, and the crucible containing the sample was placed in an oven for 1 hour. The crude extracts are stored until they can be analysed. Following this, the crude extract recovered was weighed to determine yield using the following formula:

Yield % = (Weight of black rice crude extract after evaporation) / (Weight of black rice coarse powder before extraction) × 100

Quantitative Determination of Metabolites:

Total Phenolic Content: The total phenolic content of four different black rice extracts was carried out by the Folin-Ciocalteu method using gallic acid as a standard (5, 10, 25, 50, 100, and 200 µg/ml) [14]. The extracts (1 mg) were diluted in 1 mL of methanol and 100 µL from each concentration was mixed with 500 µl of water, followed by 100 µl of Folin-Ciocalteu reagent. The mixture was incubated in total darkness for 6 minutes at room temperature. After this incubation, add 100µl of 7% sodium carbonate and 50µl of distilled water to the reaction mixture. The absorbance of the test solution was observed after 90 minutes of incubation at 760 nm using a UV spectrophotometer (UV2550, Shimadzu, Japan). A calibration curve was built using various concentrations of gallic acid. The results were expressed as milligrams gallic acid equivalent (GAE)/g DEW (dry extract weight). All the experiments were performed in triplicate. **Fig. 3** shows the standard gallic acid curve and regression equation used to calculate the total phenolic content of the extracts.

Total Flavonoid Content: The total flavonoid content of different Black rice variety extracts was determined by an Aluminum chloride complex forming assay using Quercetin as a standard [14]. The calibration curve of quercetin was developed with dilutions of (5, 10, 25, 50, 100, and 200 µg/ml concentrations, which were prepared in methanol **Fig. 5**. In a 96-well plate, 100 µl of each concentration was mixed with 500 µl of distilled water and then followed by 100 µl of 5% 1M sodium acetate and allowed to stand for 6 min. Then 150 µl of 10% Aluminium chloride solution was added and allowed to stand for 5 minutes, after which 200 µl solution of 1M Sodium hydroxide was added sequentially. After 40 minutes of incubation, the absorbance of this reaction mixture was measured at 510 nm using a UV spectrophotometer (UV2550, Shimadzu, Japan). The same procedure was repeated three times and the total flavonoid content was calculated as quercetin equivalent (QE)/g DEW (dry extract weight).

Anthocyanin Content: Anthocyanin content was determined by using the pH differential method [15, 16]. 1 ml of methanolic extract of black rice solution was pipetted into two different test tubes (A1 and A2) containing 1 ml of 0.01% HCL in 95% ethanol solution, followed by the addition of 10 ml of 2% HCL (pH 0.8) in A1 and 10 ml of Citric buffer (0.2 mM Na_2HPO_4 % and 0.1M mM citric acid; pH 3.5) in A2. Both of these were vertex 2 minutes for uniform mixing and finally, absorbance was

recorded at 520nm against 70% methanol instead of extract as a blank. Anthocyanin content (AC) was determined using the following formula:

$$AC\ (Cyanidin/g) = (A1\text{-}A2 \times MW \times DF \times CF1 \times CF2) / (\varepsilon \times 1)$$

Where, A1 is absorbance at 2% HCL solution, A2 is absorbance at citric buffer solution, MW is molecular weight of Cyanidin-3-O-glucoside (449 g/mol), DF is dilution factor (10 mL/10mg), CF1 is 106 (µg/g), CF2 is 1 L/1000 mL, ε is molar extinction coefficient of cyanidin-3-O-glucoside (26,900 L/mol.cm) and l is path length (1 cm), respectively.

Free Radical Scavenging Activity:

ABTS Assay: 2, 2′-azino-bis-3-ethylbenzthiazoline - 6-sulphonic acid (ABTS) free radical scavenging assays were carried out to measure the radical scavenging activity of Black rice [17]. To make the ABTS positive radical cation, 7 mM of ABTS stock solution was combined with 2.45 mM of potassium persulfate (final concentration), and the mixture was then allowed to lie in the dark at room temperature for 12–16 hours, or until the reaction was complete and the absorbance was stable. The ABTS working solution was prepared by diluting the stock solution to get an absorbance of 0.8 units at 734 nm using a UV-visible spectrophotometer. Standards concentrations ranging from 0-100 (5, 10, 15, 20, 30, 40) µg/ml were prepared. After reaching equilibrium at room temperature, 50 µL of the test sample was mixed with 100 µL of the ABTS solution. After 6 minutes of incubation, the absorbance was measured at 734 nm. The results were expressed as ascorbic acid equivalent antioxidant capacity (AAEAC)/g of DEW and gallic acid equivalent (GAE)/g of DEW. All samples were tested in triplicate. The % scavenging activity of both the standard and samples was calculated for each concentration and the graph is plotted in **Fig. 7**. The ABTS radical scavenging activities were measured by the following formula:

$$\%\ Radical\ scavenging\ activity = 1 - (sample\ absorbance) / (control\ absorbance) \times 100$$

DPPH Assay: The free radical scavenging activity of black rice extract was measured using the 1.1-diphenyl-2-pikrihidrazil (DPPH) free radical scavenging method [1]. When DPPH is oxidized, it gives a rich violet hue to methanol. An antioxidant chemical compound reduces DPPH by giving it an electron, which causes it to shift from deep violet to yellow. A freshly prepared 0.1 M solution of DPPH was prepared in methanol. 100µl DPPH solution was mixed with 100µl of various concentrations (10, 20, 40, 80, 150, 300, and 500 µg/ml) of extracts in a 96-well plate method and incubated in a dark room for 20 min at 27 °C. The absorbance of the samples was recorded at 517 nm after incubation using a spectrophotometer (UV 2550, Shimadzu, Japan). Ascorbic acid and gallic acid were used as positive controls **Fig. 8**. The DPPH radical-scavenging activity was determined using the following formula:

$$\%\ radical\ scavenging\ activity = (control\ absorbance - (sample\ absorbance) / (control\ absorbance) \times 100$$

Evaluation of Metabolites:

FTIR Analysis: Fourier Transforms Infrared Spectroscopy (ECO-ATR, LASER CLASS 1 Bruker, Germany) was used to identify the functional groups of the control and black rice extract samples. The analysis was made in an infrared spectrometer recording the spectrum range between 4000 and 400 cm^{-1}, with a resolution of $1cm^{-1}$ and an accumulation of 100 scans (scan rate 0.5 cm^{-1}/s). As a blank, potassium bromide was used. The ATR plate was cleaned properly and a background scan was performed before taking each spectrum for analysis [18].

FIG. 2: FTIR INSTRUMENT

HPTLC Analysis: Extracts were examined using the HPTLC (High Performance Thin Layer Chromatography) method. After experimenting with different solvent systems, the best result was obtained using an 8:2 ratio of methanol and water. Samples were placed on pre-coated silica gel 60 F_{254} sheets (10 x 20 cm) as a stationary phase. The chromatograms were then scanned using a

densitometer at 540 nm. The R_f values and fingerprint details were recorded using WIN CATS software.

Statistical Analysis: The analyses were performed in triplicate, and the findings were provided as mean ± SD. One-way non-parametric ANOVA was performed to study the overall phytochemical analysis parameters of four different black rice samples at a significance threshold of $p < 0.0001$ with GraphPad.

RESULTS AND DISCUSSION:

Extraction Yield: The extraction yield of four different black rice varieties was obtained ranging from 17.5 to 22.25 %.

TABLE 2: EXTRACTION YIELD OF DIFFERENT BLACK RICE EXTRACTS

Methanolic extract	Extraction yield (% w/w)
Poireiton, BREP	20%
Kokngangbi, BREK	17.5%
Amubi, BREA	22.25%
Sempak, BRES	18.75

Quantitative Determination of Metabolites: As mentioned above, four different black rice varieties were examined for total phenols, flavonoids, and free radical scavenging activity. The phytochemical analysis of crude methanolic extracts of various black rice varieties revealed the presence of alkaloids, phenols, steroids, cardiac glycosides, flavonoids, and anthocyanin.

Total Phenolic Content: A standard graph of gallic acid shown in **Fig. 3** was used to determine the total phenolic content of four different varieties of black rice samples. Total phenolic content was calculated as mg of gallic acid equivalent (GAE) per gram DEW (dry extract weight) (Y = 0.0034x +0.0016, R^2 = 0.9987). The experiment was performed three times.

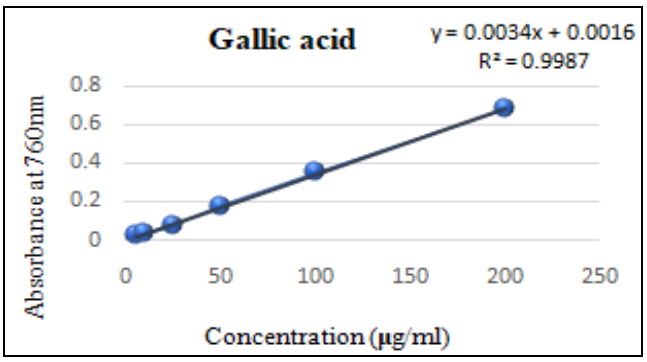


FIG 3: STANDARD CURVE OF GALLIC ACID FOR TPC

The total phenolic content of all methanolic extracts of black rice of different varieties ranged from 32.72 ± 0.50 to 63.23 ± 0.76 mg GAE/g DEW, as shown in **Table 3**. The highest amount of phenolic content is found in the BREA (63.23 ± 0.76 mg GAE/g DEW) variety of Black rice, followed by the BREP (49.98 ± 0.89 mg GAE/g DEW) variety and the BRES variety has the least content (32.72 ± 0.50 mg GAE/g DEW). When compared to other varieties of black rice, Amubi, BREA has the highest phenolic content, which allows it to absorb free radicals and chelate metal ions, catalysing the generation of ROS, which promotes lipid peroxidation.

TABLE 3: QUANTITATIVE ANALYSIS OF TOTAL PHENOLIC CONTENT

Sample	TPC mg GAE /g DEW
Poireiton, BREP	49.98 ± 0.89
Kokngangbi, BREAK	48.34± 0.45
Amubi, BREA	63.23 ± 0.76
Sempak, BRES	32.72 ± 0.50

*All data are expressed as mean ± SD of triplicate calculation.

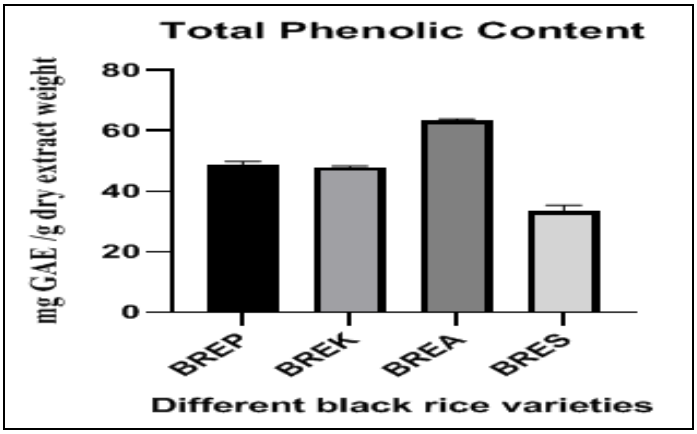


FIG. 4: TPC OF FOUR DIFFERENT BLACK RICE VARIETIES

Total Flavonoid Content: The total flavonoid content of four distinct black rice varieties was determined using a standard graph of quercetin **Fig. 5**.

Total phenolic content was calculated as mg of gallic acid equivalent (GAE)/g DEW (dry extract weight) (Y = 3.9683x-0.017, R^2 = 0.9964). The experiment was performed three times.

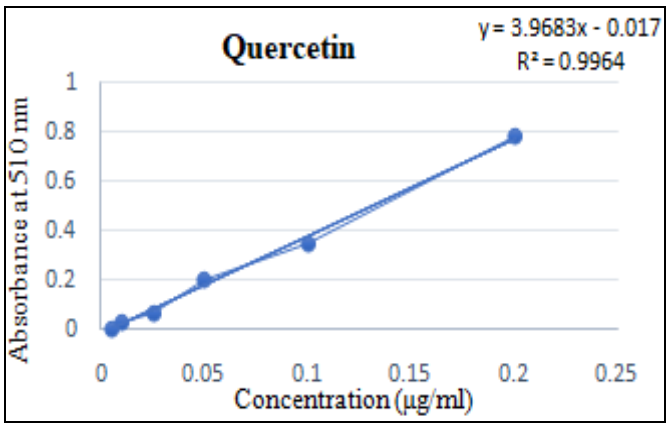


FIG. 5: STANDARD CURVE OF QUERCETIN FOR TFC

The total flavonoid content of all methanolic black rice varieties ranged from 18.37 ± 0.67 to 28.09 ± 0.68 mg QE/g DEW, as shown in **Table 4**. The highest amount of flavonoid content is also at Amubi, BREA 28.09 ± 0.68 mg QE/g DEW, followed by Poireiton, BREP 26.78 ± 0.67 mg QE/g DEW compared to other varieties.

Such flavonoid molecules are important because they help the human body fight illnesses and can act as powerful antioxidants. Therefore, such compounds may function together with other phytochemical substances present in black rice and make it medicinally and nutritionally useful.

TABLE 4: QUANTITATIVE ANALYSIS OF TOTAL FLAVONOID CONTENT

Sample	TFC mg QE/g DEW
Poireiton, BREP	26.78 ± 0.52
Kokngangbi, BREAK	23.5± 0.79
Amubi, BREA	28.09 ± 0.68
Sempak, BRES	18.37± 0.67

*All data are expressed as mean ± SD of triplicate calculation.

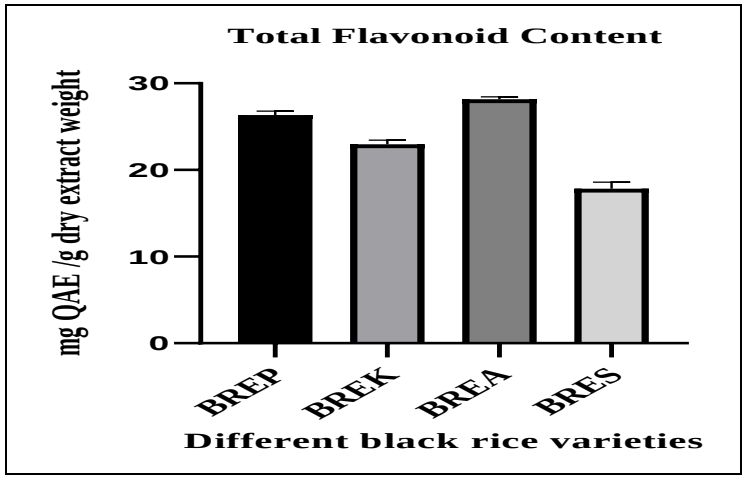


FIG. 6: TFC OF FOUR DIFFERENT BLACK RICE VARIETIES

Total Anthocyanin Content: The overall anthocyanin content obtained varied amongst these distinct types of black rice, ranging from 8.39 to 17.69 mg C (cyanidin)/g DEW (dry extract weight).

The highest concentration was found in the Amubi, BREA variety of black rice, with a value of 17.69 mg C/g DEW.

It suggests that this kind has higher antioxidant activity than other types of black rice. Moreover, the findings revealed that the anthocyanin level of rice was associated with grain colour. **Table 5** compares the anthocyanin concentrations of four distinct kinds of black rice.

TABLE 5: TOTAL ANTHOCYANIN CONTENT OF FOUR DIFFERENT BLACK RICE VARIETIES

Sample	TAC (mg C/g DEW)
BREP	13.7 ± 0.92
BREK	15.48 ± 0.61
BREA	17.69 ± 0.45
BRES	8.39 ± 0.53

* All data are expressed as mean ± SD of triplicate calculation.

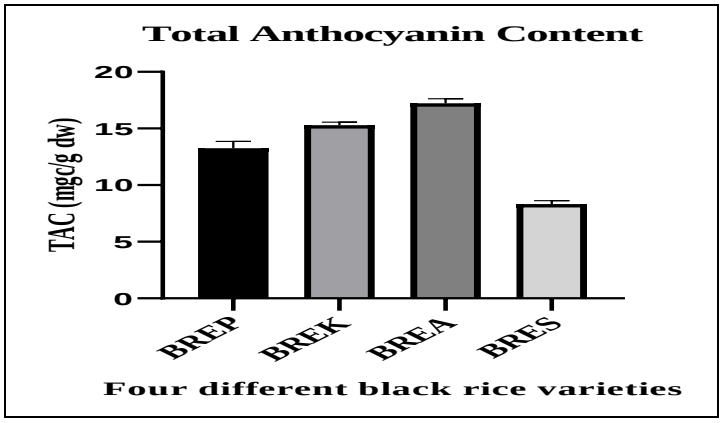


FIG. 7: TOTAL ANTHOCYANIN CONTENT OF DIFFERENT BLACK RICE VARIETIES

Free Radical Scavenging Activity:

ABTS and DPPH Assay: The radical scavenging activity of black rice is associated with the phytochemical compounds present in it. The antioxidant activity of various black rice samples was assessed using the ABTS and DPPH assays. A single assay to determine antioxidant capabilities would not provide a precise result since the antioxidant activity of plant extract is impacted by a variety of factors, including the test system, reagent preparation, and extract composition. Therefore, it is important to carry out more than one type of assay. DPPH is a nitrogen-centered free radical with an odd electron, producing a high absorption at 517 nm, whereas the ABTS assay relies on the antioxidant compound's capacity to scavenge the ABTS radical with a minimal amount of extract. These two assays are simple, inexpensive, and often used methods for determining antioxidant activity, with consistent findings.

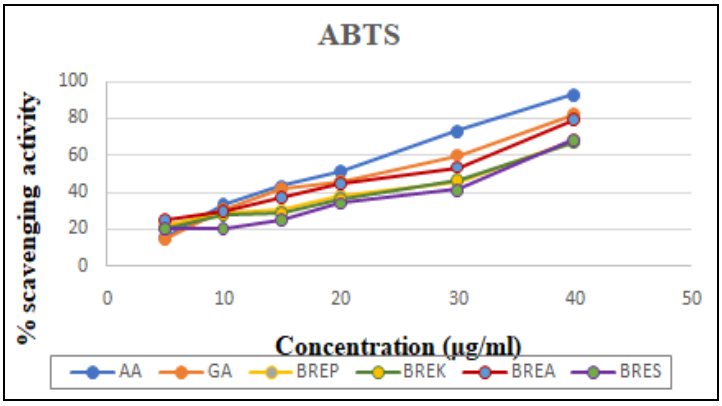


FIG. 8: ABTS RADICAL SCAVENGING ACTIVITY OF DIFFERENT BLACK RICE VARIETIES

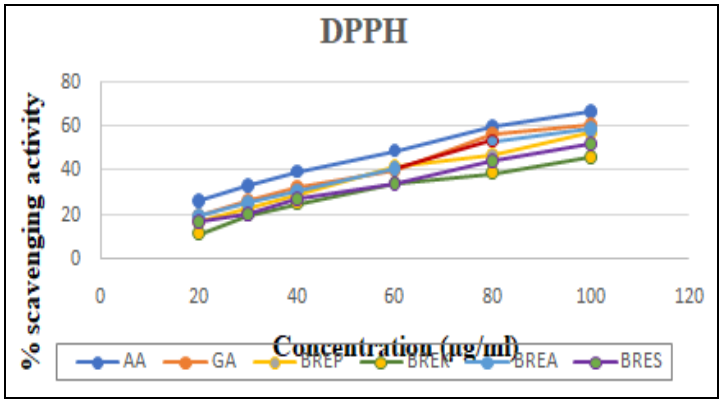


FIG. 9: DPPH RADICAL SCAVENGING ACTIVITY OF DIFFERENT BLACK RICE VARIETIES

The results of ABTS and DPPH were derived using calibration graphs of standard ascorbic acid and gallic acid, which were linear over the calibration range with R^2 values of 0.9913 and 0.9972,

respectively. For ABTS assay **Fig. 7**, compared with other black rice varieties, Amubi, BREA variety showed the highest IC50 value (29.1 ± 1.11 mg/ml), followed by Poireiton, BREP (28.3 ± 0.51 mg/ml), Kokngangbi, BREK (21.76 ± 0.79 mg/ml) in ABTS assay, and least content in Sempak, BRES (16.91 ± 0.55 mg/ml). For the DPPH assay, the black rice variety Amubi, BREA was found to be most potent (98.83 ± 0.61 mg/mL) compared to Poireiton, BREP (387.61 µg/mL), Kokngangbi, BREAK (85.76 ± 1.01 mg/ml), and Sempak, BRES (76.87 ± 0.70 mg/mL) **Fig. 8**. This could be because the Amubi, BREA variety of black rice has more antioxidant chemical compounds than the other varieties, which neutralize DPPH and ABTS radicals. A prior study also indicated that black rice was more beneficial than red rice in terms of antioxidant activity [19]. The results of the DPPH and ABTS assays for samples are presented in **Table 6**.

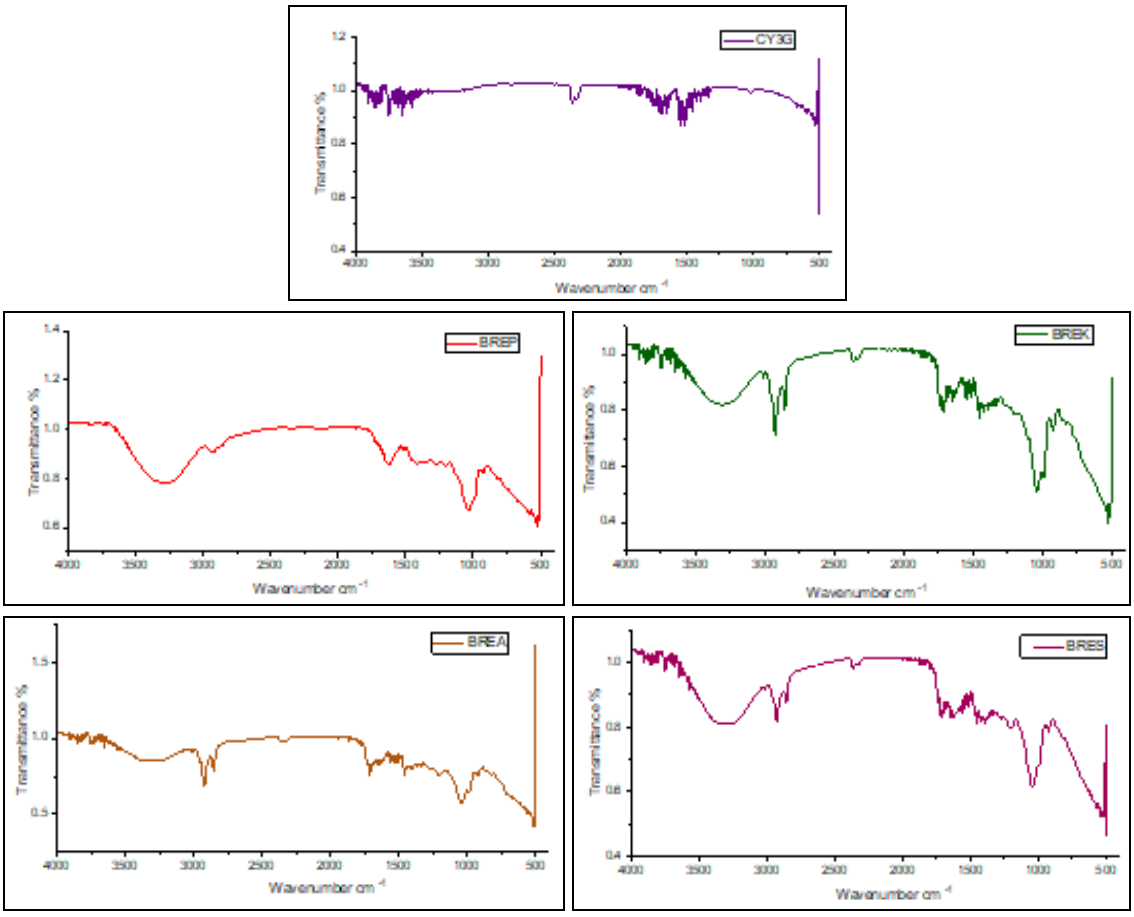
TABLE 6: RADICAL SCAVENGING ACTIVITY OF FOUR DIFFERENT BLACK RICE VARIETIES USING ABTS AND DPPH RADICALS

Sample	Radical Scavenging Activity IC50 (mg/mL)	
	ABTS	DPPH
Poireiton, BREP	28.3 ± 0.51	97.08 ± 0.53
Kokngangbi, BREAK	21.76 ± 0.79	85.76 ± 1.01
Amubi, BREA	29.1 ± 1.11	98.83 ± 0.61
Sempak, BRES	16.91 ± 0.55	76.87 ± 0.70

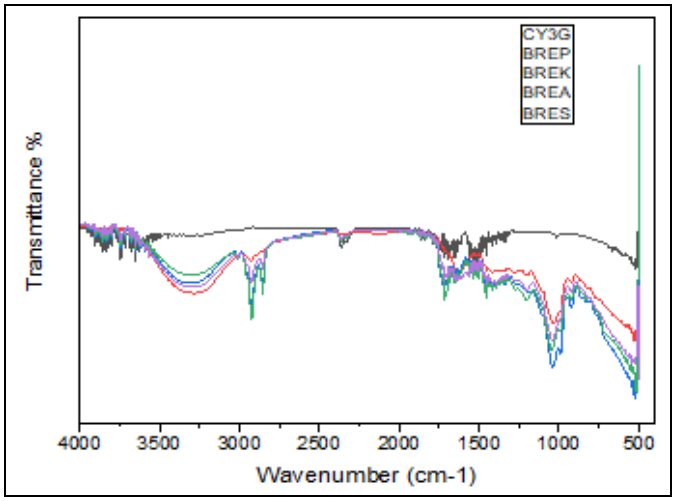
*All data are means of triplicate measurement ± standard deviation.

Evaluation of Metabolites:

FTIR Analysis: FTIR analysis successfully showed the spectrum band region of phytochemical compounds in the black rice sample compared to the standard, cyanidin-3-O-glucoside. The results of FTIR analysis of four different varieties of black rice samples [Poireiton (BREP), Kokngangbi (BREK), Amubi (BREA) and Sempak (BRES)] and the standard Cynaidin-3-O-glucoside (CY3G) are depicted in **Fig. 10** and **Table 7.**



(A)



(B)

FIG. 10: FTIR SPECTRUM OF (A) STANDARD CYANIDIN-3-GLUCOSIDE (CY3G) AND FOUR DIFFERENT VARIETIES OF BLACK RICE SAMPLE POIREITON (BREP), KOKNGANGBI (BREK), AMUBI (BREA) AND SEMPAK (BRES), AND (B) COMBINED SPECTRA OF CY3G AND FOUR DIFFERENT BLACK RICE VARIETIES, IN 400-4000CM^{-1} REGION.

The spectra can be used to determine isomer configuration from transmittance or absorbance, as well as frequency wavenumber among functional groups. Functional groups on organic macromolecules absorb IR radiation at specific wavelengths, creating a complex spectrum that serves as a fingerprint of the cellular composition [20]. However, a biological sample is a very complex matrix comprising a variety of chemicals, the interactions of which are represented in the vibrational spectrum. The use of standard references to generate spectra may impose a bias since molecular interactions are not precisely identified. Surprisingly, standardization of spectra was appropriate for comparing metabolite levels between black rice samples and the reference biomarker, cyanide-3-O-glucoside. When spectra are just baseline corrected, correlations are significantly higher, indicating that this data treatment can be used to compare different varieties. This study focused on the analysis of FTIR in the mid-IR spectrum mid-IR spectrum (400-4000 cm^{-1}) in a baseline-corrected way. Data were analysed using Origin Pro software.

TABLE 7: FTIR SPECTRA FOR CY3G AND FOUR DIFFERENT VARIETIES OF BLACK RICE SAMPLES

Wavenumber (cm^{-1})					Probable Functional GROUP
CY3G	BREP	BREK	BREA	BRES	
3748	3741	3741	3741	3741	O-H of Alcohol and phenol
3324	3293	3309	3345	3293	N-H Stretching; Aliphatic primary amines
2920	2929	2920	2920	2929	N-H stretching of amine salt, C-H of alkane
2850		2857	2850		C-H of alkane
2354	2361	2354	2354	2361	C≡C stretching of alkyne.
1710	1703	1717	1710	1703	C-H bending of aromatic compound
1625	1636	1636	1650	1636	C=C stretching of alkene, C=C stretching of Cyclic alkene, N=H bending of amine
1459	1456	1462	1455		N=O stretching of nitro compound
1413	1407	1413	1413	1407	O-H bending of alcohol
1201	1206	1201	1201	1206	C-O stretching of tertiary alcohol/C-O stretching of an ester
1038	1037	1038	1038	1037	C-O-C vibration in cellulose and hemicellulose
925	925	923	925	925	-CH=CH$_2$ bending of alkene (mono-substituted)
684	679	769	720	679	C=C bending of alkene (Ortho di-substituted benzene)
517	521	521	521	521	O-C-O plane bending of carboxylic or esters

The FTIR study successfully demonstrated that the spectrum band region of the main phytochemical components in black rice samples is identical to the standard biomarker, Cyanidin-3-O-glucoside. Similar functional groups in all samples suggest the presence of functional groups with benzene

structures. Benzene structures can be found in phenols, flavonoids, and anthocyanins. While these data showed similar actions in both black rice and regular biomarkers, they highlighted that anthocyanin molecules are found in black rice, which has powerful biological activities.

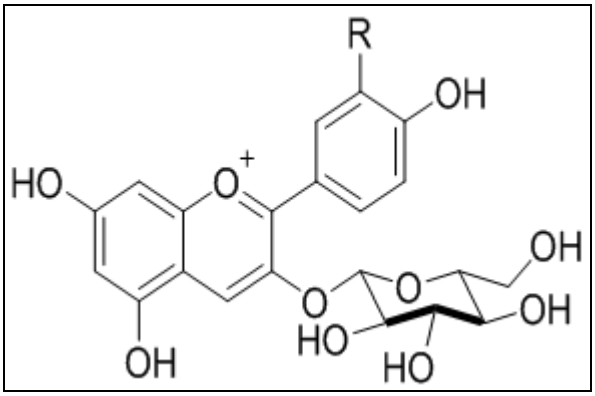


FIG. 11: STRUCTURE OF CYANIDIN-3-O-GLUCOSIDE. (IUPAC: 2-[2-(3,4-dihydroxy phenyl)-5,7-dihydroxychromenylium-3-yl]oxy-6-(hydroxyl-methyl)oxane-3,4,5-triol;chloride)

Functional groups that are commonly owned by Cyanidin-3-O-glucoside and four different varieties of black rice are 3741 and 3748 cm^{-1} (Hydroxyl, O-H bending of alcohol and phenol), 3324, 3309, 3343 and 3293 cm^{-1} (N-H stretching of aliphatic primary amine), 2920 and 2929 cm^{-1} (N-H stretching of amine salt & C-H of alkane), 2850 and 2857 cm^{-1} (C-H of alkane), 2354 and 2361 cm^{-1} (C=C stretching of alkyne), 1710, 1717 and 1703 cm^{-1}(C-H bending of the aromatic compound), 1625, 1636 and 1650 cm^{-1} (C=C stretching of alkene, bending of amine), 1459, 1462 and 1455 cm^{-1} (N-O stretching of a nitro compound), 1413 and 1407 cm^{-1} (O-H bending of alcohol), 1201 and 1206 cm^{-1} (C-O stretching of tertiary alcohol and ester), 1038 and 1037 cm^{-1} (C-O-C vibration in cellulose), 925 and 923 cm^{-1} (-CH= CH2 bending of alkene mono substituted), 684, 679, 720 and 769 cm^{-1} (C=C bending of alkene, ortho-disubstituted benzene), 521 and 517 cm^{-1} (O-C-O plane bending carboxylic ester) **Fig. 8** and **Table 5**.

However, some features in BREP, such as the peak at 2850 cm^{-1}, and in BRES, 2850 and 1459 cm^{-1}, are not detected. Different peak intensities at specific wavenumbers are detected depending on the bioactive chemical availability of the sample.

In this study, a linear correlation was obtained between the standard biomarker and sample at the majority of wavenumbers, but a strong linear correlation is obtained in some wavenumbers corresponding to the vibration of O-C-O bending of carboxylic ester, -CH= CH2 bending of alkene mono-substituted, C-O-C vibration in cellulose, C-O stretching of tertiary alcohol and ester, and O-H bending of alcohol after baseline correction. According to Fatchiya *et al.* 2020 [21] and Arora *et al.* 2021 [18], as compared to the standard, the peak at 4000–3500 cm^{-1} was attributed to medium to sharp intermolecular bonded hydroxyl groups (OH stretching) of alcohols and phenols.

The bands at 3400 and 1650 cm^{-1} represent molecular deformation in starch molecules induced by stretching and angular deformation of the OH bonds in proportion to moisture content, whereas the band at 2930 cm^{-1} represents the axial displacement of C-H bonds in lipids [22]. The absorbance around the band of 2900 cm^{-1} is attributed to the C-H stretching of alkane and the N-H stretching of secondary amine salt [18].

The intensity of such peaks implies that lipids or carbohydrates are present in black rice. Amide bands of C-H bending, which are commonly detected between 1700 and 1600 cm^{-1}, have been reported to provide information about the prevalence of secondary metabolites in proteins [23].

The fingerprint region has been identified as a conspicuous peak in the 1200-600 cm^{-1} range. Thus, the peaks in the range of 950–1150 cm^{-1} might be interpreted as evidence of the cross-linking between the starch molecules and other non-starch elements, especially the protein [24].

The absorbance band ranges from 900 to 700 cm^{-1} ascribed to the C=C, C-H meta-di-substituted benzene aromatic bond, and C-N and C=S stretching in thiocarbonyls [25, 26]. The peak region between 595 and 510 cm^{-1} was assigned to the O-C-O symmetric or planar bending of carboxylic or esters, which is the fingerprint peak of the particular sample. The FTIR spectra verified the presence of plane bending vibrations of O-C-O in both Standard, CY3G at 517 cm^{-1} and four different black rice varieties at 521 cm^{-127}.

HPTLC Fingerprint Analysis: The HPTLC profiles of CY3G and four different black rice varieties are presented in **Fig. 12**. In this study, we

standardized the extraction methods and quantified the content of a major bioactive compound in black rice, cyanide-3-O-glucoside, as a biomarker, which showed an R_f value of 0.71 using ethyl acetate: formic acid: water (8:2:2, v/v) as a mobile phase [28].

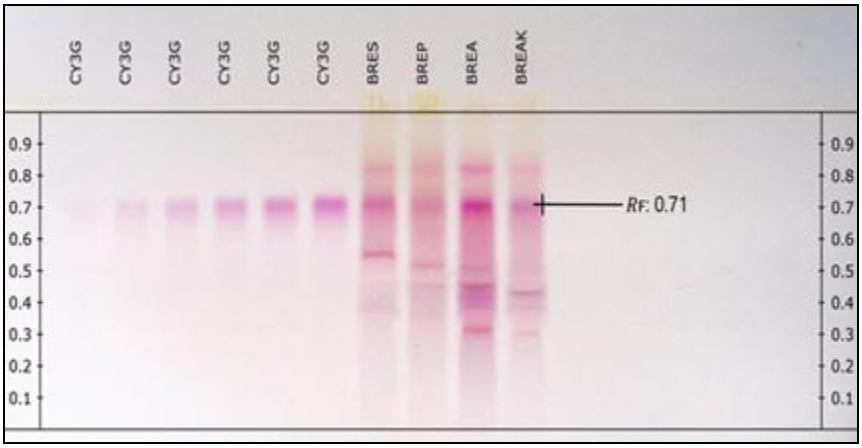


FIG. 12: HPTLC CHROMATOGRAM OF STANDARD CY3G AND FOUR DIFFERENT BLACK RICE VARIETIES UNDER WHITE LIGHT AT 540NM

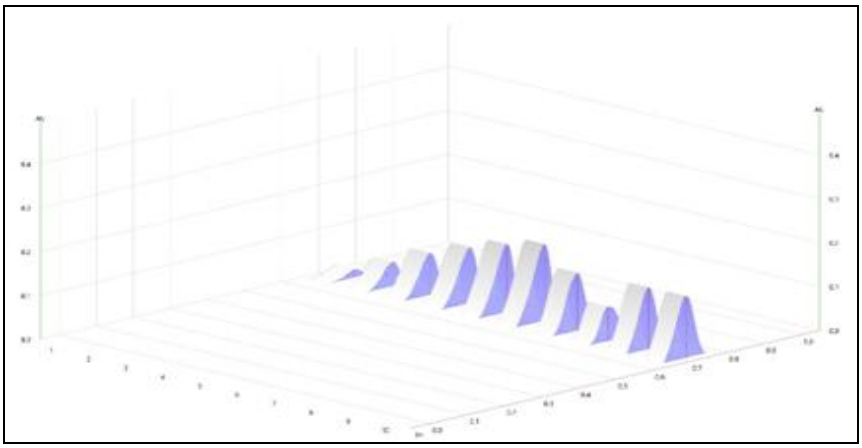


FIG. 13: 3D OVERLAY OF HPTLC CHROMATOGRAM OF ALL TRACKS I, E, TRACK 1-6: CY3G, TRACK 7: BRES, TRACK 8: BREP, TRACK 9: BREA, TRACK 10: BREK AT THE WAVELENGTH OF 540NM

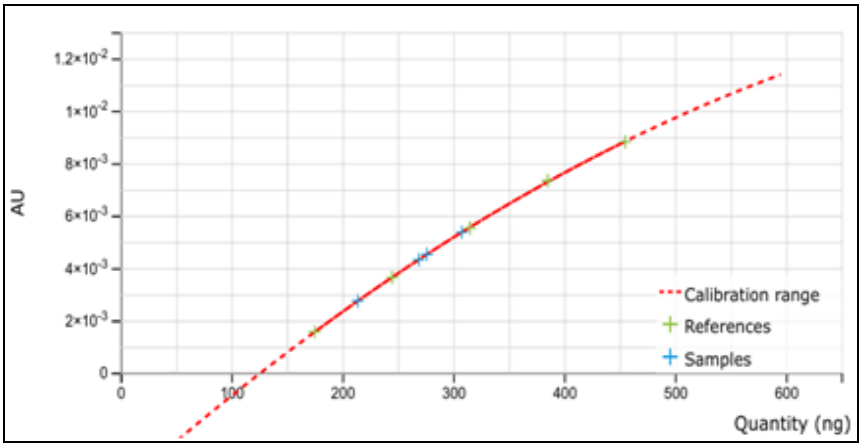


FIG. 14: AREA CALIBRATION GRAPH OF CYANIDIN-3-O-GLUCOSIDE AND DIFFERENT VARIETIES OF BLACK RICE EXTRACTS

The CY3G content was maximum in BREA (Amubi -30.76µg/ml), followed by BREP (Poireiton-27.63µg/ml), BREK (Kokngangbi-26.89µg/ml), and minimum amount in BRES (Sempak-21.40µg/ml) **Fig.15**.

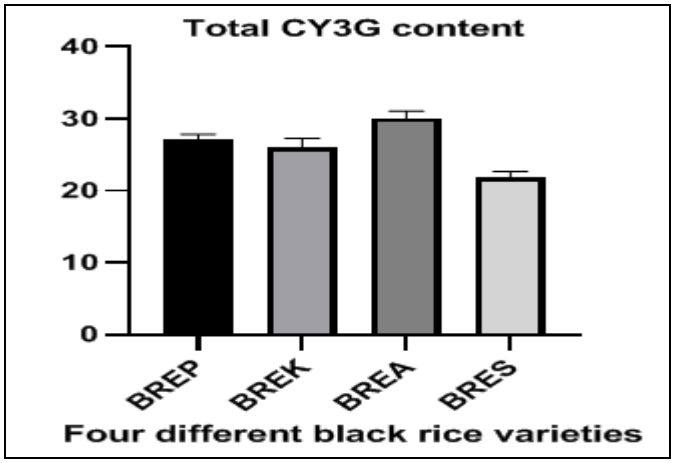


FIG. 15: CY3G CONTENT IN FOUR DIFFERENT BLACK RICE VARIETIES QUANTIFIED BY HPTLC METHOD

CONCLUSION: The findings showed that black rice varieties contain high levels of compounds, which have a high content of nutritional and medicinal values with high macronutrients. From the result, the total content of phenolic, flavonoid, anthocyanin, and antioxidant activity differed among the varieties of black rice. The maximum content was obtained in the Amubi variety of black rice, BREA, and exhibited strong antioxidant activity, followed by Poireiton, BREP compared to other varieties of black rice. It can be concluded that this variety has a high phytochemical distribution and nutritional significance. Based on the resulting data from the phytochemical analysis, the identification of major bioactive compounds is needed. So, FTIR spectroscopy methods are developed suitably for the structural identification of the cellular metabolite, performing baseline correction on spectra at 400–4000 cm^{-1} of biomolecules. The chemical structure of the major bioactive compound present in the black rice was identified as anthocyanin, mainly Cyanidin-3-O-glucoside, using FTIR spectroscopy analysis.

The FTIR spectra of standard biomarkers and research samples show mostly similar peak absorbance that represents similar functional groups, which is consistent with the basic structure of CY3G. These data suggest that the structural similarities between anthocyanin, CY3G, and all varieties of black rice result in marked similarities in biological activity effects. However, large amounts of accurate and high-throughput bioinformatics are required for now and into the future. It can be concluded that this, FTIR spectroscopy method will be able to create a non-invasive tool for the structural identification of many compounds. In addition, the HPTLC densitometric method was successfully validated for the quantification of cyanidin-3-O-glucoside from several black rice varieties. This is an ongoing study, and further advanced studies of other spectroscopic and *in-vivo* studies are required to evaluate the different activities of black rice.

ACKNOWLEDGMENTS: The authors are grateful to the Department of Biotechnology for financially supporting this work: Sanction No. BT/PR45213/NER/95/1993/2022. The authors are also thankful to the Department of Nanotechnology, School of Technology, NEHU, Shillong, for providing access to their laboratories, which was a great help in conducting the FTIR analyses.

CONFLICT OF INTEREST: NIL

REFERENCES:

1. Pinta W, Aninbon C, Kaewtaphan P and Kunyanee K: Effects of Parboiling on Chemical Properties, Phenolic Content and Antioxidant Capacity in Colored Landrace Rice. Foods 2024; 13(3): 393.
2. Khan J, Khan MZ, Ma Y, Meng Y, Mushtaq A, Shen Q and Xue Y: Overview of the Composition of Whole Grains' Phenolic Acids and Dietary Fibre and Their Effect on Chronic Non-Communicable Diseases. International Journal of Environmental Research and Public Health 2022; 19(5): 3042.
3. Laoué J, Fernandez C and Ormeño E: Plant Flavonoids in Mediterranean Species: A Focus on Flavonols as Protective Metabolites under Climate Stress. Plants 2022; 11(2): 172.
4. Klunklin W and Savage GP: Biscuits: A Substitution of Wheat Flour with Purple Rice Flour. Advances in Food Science and Engineering 2018; 2(3): 81-97.
5. Das M, Dash U, Mahanand SS, Nayak PK and Kesavan RK: Black rice: A comprehensive review on its bioactive compounds, potential health benefits and food applications. Food Chemistry Advances 2023; 3: 100462.

6. Sangma HC and Parameshwari S: Health benefits of black rice (*Zizania aqatica*) - a review. Materials Today: Proceedings 2023; 80(3): 3380-3384.
7. Rahim MA, Umar M, Habib A, Imran M, Khalid W, Lima CMG, Shoukat A, Itrat N, Nazir A, Ejaz A, Zafar A, Awuchi CG, Sharma R, Santana RF and Emran TB: Photochemistry, Functional Properties, Food Applications, and Health Prospective of Black Rice. Journal of Chemistry 2022; (2755084): 1-12.
8. Merecz-Sadowska A, Sitarek P, Kowalczyk T, Zajdel K, Jęcek M, Nowak P and Zajdel R: Food Anthocyanins: Malvidin and Its Glycosides as Promising Antioxidant and Anti-Inflammatory Agents with Potential Health Benefits. Nutrients 2023; 15(13): 3016.
9. Wahyuni AS, Munawaroh R and Dai M: Antidiabetic mechanism of ethanol extract of black rice bran on diabetic rats. National Journal of Physiology, Pharmacy, and Pharmacology 2016; 6(2): 106-110.
10. Lila MA, Burton-Freeman B, Grace M and Kalt W: Unraveling Anthocyanin Bioavailability for Human Health. Annual Review of Food Science and Technology 2016; 7: 375-393.
11. Verediano TA, Stampini Duarte Martino H, Dias Paes MC and Tako E: Effects of Anthocyanin on Intestinal Health: A Systematic Review. Nutrients 2021; 13(4): 1331.
12. Rahim Abd, Arjuna A, Pakki E, Syaiful SA, Rewa AM, Alam G and Murdifin M: Antioxidant and HPTLC study of black glutinous rice extract from south Sulawesi Indonesia. International Journal of Pharmacognosy and Phytochemical Research 2016; 8: 771-776.
13. Li Q, Zhang F, Wang Z, Feng Y and Han Y: Advances in the Preparation, Stability, Metabolism, and Physiological Roles of Anthocyanins: A Review. Foods 2023;12(21):3969.
14. Shirazi OU, Khattak MAK, Shukri NAM and MN Nasyriq A: Determination of total phenolic, flavonoid content, and free radical scavenging activities of common herbs and spices. Journal of Pharmacognosy and Phytochemistry 2014; 3(3): 104-108.
15. Silva HR, Assis DC, Prada AL, Junior JOCS, Sousa MB, Ferreira AM, Amado JRR, Carvalho HO, Santos AVTLT and Carvalho JCT: Obtaining and characterization of anthocyanins from *Euterpe oleracea* (açaí) dry extract for nutraceutical and food preparations. Brazillian Journal of Pharmacognosy 2019; 29(5): 677-685.
16. Sholikhah U, Parjanto P, Handoyo T and Yunus A: Anthocyanin Content in Some Black Rice Cultivars. IOP Conference Series: Earth and Environmental Science 2021; 709(1): 012076.
17. Khatun S and Mollah MD: Analysis of black rice and some other cereal grains for protein, sugar, polyphenols, antioxidant, and anti-inflammatory properties. Journal of Agriculture and Food Research 2024; 16: 101121.
18. Arora S, Virdi IK, Sharanagat VS, Kheto A, Dhua S, Suhag R, Kumar R, Kumar Y and Patel A: Roasting of black rice (*Oryza sativa* L.): change in physico-functional, thermo-pasting, antioxidant and anthocyanin content. Journal of Food Measurement and Characterization 2021; 15: 2240 - 2250.
19. Sompong R, Siebenhandl-Ehn S, Linsberger-Martin G and Berghofer E: Physicochemical and Antioxidative Properties of Red and Black Rice Varieties from Thailand, China, and SriLanka. Food Chemistry 2011; 1: 132-140.
20. Wagner H, Liu Z, Langner U, Stehfest K and Wilhelm C: The use of FTIR spectroscopy to assess quantitative changes in the biochemical composition of microalgae. J Biophotonics 2010; 3(8-9): 557-566.
21. Fatchiyah F, Sari DR, Safitri A and Cairns J: Phytochemical Compound and Nutritional Value in Black Rice from Java Island, Indonesia. Systematic Reviews in Pharmacy 2020; 11: 414-421.
22. Ribeiro VHdA, Cavalcanti-Mata MERM, Almeida RLJ and Silva VMdA: Characterization and Evaluation of Heat–Moisture-Modified Black and Red Rice Starch: Physicochemical, Microstructural and Functional Properties. Foods 2023; 12(23): 4222.
23. Duque SMM, Leong SY, Agyei D, Singh J, Larsen N and Oey I: Understanding the impact of Pulsed Electric Fields treatment on the thermal and pasting properties of raw and thermally processed oat flours. Food Research International 2020; 129: 108839.
24. Sun Q, Gong M, Li Y and Xiong L: Effect of dry heat treatment on the physicochemical properties and structure of proso millet flour and starch. Carbohydrate Polymers 2014; 110: 128-134.
25. Jogihalli P, Singh L, and Sharanagat VS: Effect of microwave roasting parameters on functional and antioxidant properties of chickpea (*Cicer arietinum*). LWT - Food Science and Technology 2017; 79: 223-233.
26. Sharanagat VS, Suhag R, Anand P, Deswal G, Kumar R, Chaudhary A, Singh L, Singh Kushwah O, Mani S, Kumar Y and Nema PK: Physico-functional, thermo-pasting, and antioxidant properties of microwave-roasted sorghum [Sorghum bicolor (L.) Moench]. Journal of Cereal Science 2019; 85: 111-119.
27. Gonultas O and Candan Z: Chemical Characterization and FTIR Spectroscopy of Thermally Compressed Eucalyptus Wood Panels. Maderas. Cienciay Tecnologia 2018; 20: 431-442.
28. Bationo RK, Dabiré CM, Abdoulaye Y, Kaboré B, Sawadogo O, Koala M, Palé E and Nébié RHC: Major anthocyanin quantification, free radical scavenging properties, and structural identification in *Cymbopogon giganteus* extracts. African Journal of Pure and Applied Chemistry 2023; 17(3): 32-46.

How to cite this article:
Sofianandi T, Asem D, Konthoujam N, Laishram A, Keithellakpam O, Laishram R, Sharma N and Mukherjee PK: Evaluation of cyanidin-3-o-glucoside from different black rice varieties of Manipur utilizing FTIR spectroscopy and HPTLC method. Int J Pharm Sci & Res 2024; 15(12): 3497-10. doi: 10.13040/IJPSR.0975-8232.15(12).3497-10.