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LC-QTOF-MS–based Metabolite Profiling and Evaluation of Anti-diabetic Activity (α-amylase and α-glucosidase) of Traditional Kavuni Rice *In vitro*

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Abstract

Rice is a staple food for more than 50% of the global population and there is close relationship between rice eaters and the prevalence of diabetes. Among the landraces, 'Kavuni' is considered as a traditional brownish black variety which is extensively cultivated in the southern part of India, Tamil Nadu. The Tamil Nadu Agricultural University is among the first State Agricultural University to release therapeutic rice in 2023 as Co.57. This study aimed to identify and quantify the major anti-diabetic compounds present in Kavuni rice grain extracted using various solvents. Metabolite profiling using LC-QTOF-MS analysis has revealed that 31 phytochemicals detected among them anthocyanins and flavonoids are predominant. Ethanolic extract of Kavuni rice grain had the highest concentration of anthocyanins, cyanidin 3-O-glucoside (61.31 ± 0.04 mg/g). The highest antioxidant activities in the ethanolic extract of Kavuni rice were detected as 89.51 ± 0.1% and 82.97 ± 0.09% measured using anti-oxidant assays. Further, Kavuni rice had the highest inhibition of carbohydrate digestive enzymes (α-amylase and α-glucosidase) 82.30% and 70.21%, respectively. The data clearly demonstrated that land race "Kavuni" carries phytochemicals that are known to regular postprandial hyperglycemia by inhibiting anti-diabetic enzyme could lower the risk of developing Type-2 diabetes.

Keywords: Kavuni rice • Anthocyanins • LC-QTOF-MS • Anti-oxidant capacity • Anti-diabetic activity

Introduction

Diabetes Mellitus (DM) is one of the most important chronic metabolic disorders that pose threat to human life across the globe. According to World Health Organization (WHO), global diabetic population is anticipated to hit 418 million by the year 2025 and 552 million by 2030 [1]. Moreover, reports showed that majority of diabetic patients (more than 90%) are affected from non-insulin dependent Type 2 Diabetes Mellitus (T2DM). Destruction of pancreatic β-cells with the involvement of Reactive Oxygen Species (ROS) which triggers the activation of several signaling pathways and activates apoptosis by inflammatory cytokines leads to nature spiking higher post glucose level, (hyperglycemia) which is closely associated with a higher risk of cardiovascular diseases, retinopathy and other dysfunction [2]. Since there is no effective cure for DM, a therapeutic approach is mainly focused on identifying low Glycemic Index (GI) diet which promotes a decline in the postprandial hyperglycemia. Several reports have shown that the natural sources such as fruits, vegetables and spices (bitter melon, raspberry, black cumin) are rich in phenolics which are known to contain abundance of ant-oxidants and reduce the impact of DM [3]. The polyherbal solutions from medicinal plants have reached to the stage of clinical trials and also the adoption of these niche foods in the regular diet is not practically possible. Consequently, there is urgent need to identify a staple food source have both nutraceutical anti-hyperglycemic potential.

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Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population. Among the countries spread across the globe, India and China are predominantly rice consuming populations [4]. Over the years, thousands of rice varieties have been domesticated by these two countries with a focus to increase the productivity and nutritional qualities [5]. Recently, landraces are given importance as these genotypes possess nutritional and medicinal properties. India is considered as one of the highly bio diversified countries carrying a wide array of rice germplasms with varying metabolites that are closely associated with therapeutic properties [6]. Recently, the Department of Biotechnology, Government of India, New Delhi, has taken painstaking efforts of assembling more than 20,000 landraces and undertaken a mega project to inventorying them for inclusion in the research program to explore their potentials and utilizing them to tolerate abiotic and biotic stresses, for biofortification and for therapeutic properties [7]. Despite the fact that the grain contains low amounts of minerals and essential nutrients, the people in Asia eat rice on a day today basis to meet the energy requirement [8]. As population continues to grow in Asia particularly in India which commensurate with the increase in number of people eat rice, the prevalence of diabetes gets increased. This led to the population suffer from DM primarily with a wide range of associated complications such as cardiovascular diseases, retinal disorders and obesity [9].

Having its huge consumption, the development and dissemination of rice with nutritional and therapeutic values is gaining importance due to their healthy prospective and also considered as functional food ingredients [10]. Pigmented rice genotypes are characterized in terms of nutraceutical starch, phytochemicals like carotenoids, flavonoids and polyphenols, dietary fibre, resistant starch and consumption of these traditional pigmented rice varieties helps in improving human health. Some of the pigmented rice genotypes, *viz*., Njavara from kerala and mapillai samba from Tamil Nadu can functions as promising therapeutic for cancer, diabetes and other medicinal values [11,12]. The wide spectrum of functional metabolites bound in cell wall of the grains of pigmented rice have gained interest owing to their ability to inhibit enzymes responsible for anti-diabetic treatment, as these molecules are not found in white rice. However, traditional cooking of those pigmented rice varieties results in decreased phytochemical content which results in less anti-oxidant activity and the phenomenon that happens during cooking

might be thermal degradation and matrix softening effect [13]. Also, the beneficial effects of those pigmented rice varieties were remain unexplored and the phytochemicals present in it might undergo numerous physical and chemical changes during cooking. Hence, the objective of our study was to extract dietary phytochemicals from traditional dark brownish black rice variety "Karuppu Kavuni" using different solvents. There are only a few or limited reports on the complete chemoprofiling of "Karuppu Kavuni" rice variety. Also, the study aimed to evaluate the anti-oxidant capacity and *in vitro* assessment of potential α-amylase and α−glucosidase inhibitory activity of traditional "karuppu Kavuni" from Tamil Nadu, South India. The results of our study can be useful in eludicating the different classes of chemical compounds present in the variety, promoting for developing novel oral nutraceutical product for regular human consumption.

Materials and Methods

Source of plant material

Sample of improved traditional brownish black therapeutic rice variety "Karuppu Kavuni" (CO 57) was obtained from the Department of Rice, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India in May, 2021. The well dried seeds with optimum moisture content (approx.10%) were de-husked manually, powdered and stored at 4°C until analysis.

Chemicals and reagents

Ethyl acetate (AR grade) was purchased from HIMEDIA, India. Ethanol (AR grade 99.9% purity) from Changshu Hongsheng Fine Chemicals and Co. Ltd., China. Acetone (LR grade) was purchased from Sigma-Aldrich, Bangalore, India. Deionized water for all aqueous solutions was purified by a Milli-Q Water purification system (Millipore, MA, USA). Standards such as gallic acid and quercetin were purchased from Sigma-Aldrich, Bangalore, India. Microfilters (0.45 µm) membranes were used to filter the extraction solvents from the mixture prepared for LC-QTOF-MS analysis. Cyanidin-3-O-glucoside standard with HPLC purity (99%) were obtained from Sigma-Aldrich, Bangalore, India. α-amylase and α-Glucosidase (from *Saccharomyces cerevisiae*) with 4-nitrophenyl-α-D-glucopyranoside were purchased from Sigma-Aldrich. Acarbose were procured from HIMEDIA chemical laboratories (Mumbai, India).

Extraction of metabolites from rice grains

Kavuni rice grains were dried under shade at room temperature of 25 ± 2 °C and pulverized using a mechanical grinder to a coarse powder. The powdered rice grains (100 g) were extracted using an orbital shaker with different solvents like water, ethanol, ethyl acetate and acetone based on polarity in the ratio of 1:2 (W/V of grain powder/solvent) for each extracting solvent. The resulting extracts were collected and filtered through Whatman No. 40 filter paper and evaporated to dryness with a rotary evaporator (Heidolph Model-G3, Germany) under the conditions of 60 rpm and 45 °C temperature. The concentrated grain extract was stored at 4 °C for further analysis.

LC-QTOF-MS conditions

LC-QTOF-MS analysis was carried out using a Shimadzu LC-9030 system with LC were carried out in Nexera X2 module with SIL30AC auto-sampler injection volume of 10 µl. The chromatographic conditions were optimized based on the mobile phase composition, gradient and flow rate and sample injection volume. The optimization was done by changing those parameters to get enhanced resolution, analysis time and peak shape. The operating parameters were optimized as follows: drying gas (N_2) flow, 8 L/min; drying gas temperature, 250 °C. The gradient mobile phase consisted of water (A) and acetonitrile (B). LC separation was achieved using Shimadzu Shim-pack GIST C_{18} reverse phase column with the dimension of 2.1 mm × 150 mm. The sample (1 mg/ml) was prepared by dissolving in each solvent and filtered through a 0.45 µm filter before injection. The identification of compounds was based on the high-resolution accurate mass analysis with score value (on a scale of 0–100) of the measured mass (m/z) with respect to their theoretical formula. Considering the MS conditions, both positive and negative ionization mode was used to obtain better tandem mass spectra and high-resolution mass spectra using LC 8040 mass spectrometer.

ATR- FTIR spectroscopy

Dried extracts of Kavuni grains were placed on the ATR crystal of FTIR and the spectral data was collected using FT-IR 6800 Type A model (JASCO International Co., Ltd., Japan). The spectra data was processed and generated at a resolution of 4 cm^{-1} in the frequency range (ν) of 4000 cm^{-1} to 400 cm^{-1}. Air was scanned as the background spectrum before each measurement.

Estimation of total phenolic and flavonoid content

The estimation of total phenolic and flavonoid content for four different solvents obtained Kavuni rice grain was performed based on the earlier method adopted from our laboratory with some modification [14]. The samples were prepared at the concentration of 1 mg/ml. The chemical mixture was prepared by adding 20 µl of sample with 90 µl of 10% Folin-ciocalteu reagent and 0.2 ml of 8% $NaHCO_3$. The samples were thereafter incubated under dark conditions for 30 min. Gallic acid was used as standard in the concentration range 0.2-1 µg/ml. The absorbance was measured at 765 nm using a UV-Vis spectrophotometer (SPECORD PLUS, Analytik Jena AG, Germany). The Total Phenol Content (TPC) was calculated as mg gallic acid equivalent/ g of sample.

The Ttotal Flavonoids Content (TFC) was also determined by UV-Vis spectrophotometric assay (SPECORD PLUS, Analytik Jena AG, Germany) with minor modifications. The samples were prepared in a different concentration range (10-100 µg/ml) using methanol. Initially, 5 µl of 3% $NaNO_3$, 50 µl of 1M NaOH and 10 µl of 15% $AlCl_3$ were added in a chemical mixture. The absorbance of total flavonoids was determined at 420 nm. The content of total flavonoids was calculated as mg of quercetin equivalent/g of sample. The results were represented as mean ± Standard Deviation (SD) (n=3).

Method validation and quantitative estimation of major anthocyanin in Kavuni rice grain

Quantification of Cyanidin 3-O-glucoside content: Major anthocyanin (Cyanidin 3-O-glucoside) (98% HPLC purity) present in the Kavuni grain extracts were quantified using UV-Vis spectrophotometer (SPECORD PLUS, Analytik Jena AG, Germany). The calibration curve was drawn using a range of concentration of 10-50 µg/ ml against the absorbance of the standard was measured at λ_{max}=290 nm and λ_{max}=536 nm. Similarly, a linear range of standard was created by the value of the least square regression co-efficient R2=0.999 and expressed in terms of milligrams of per gram of leaf extract. The Limit of Quantification (LOQ) was determined by taking LOQ as the lowest concentration (0.001 mg) at which acceptable levels of precision and accuracy were observed.

Antioxidant activity

DPPH radical scavenging assay: DPPH radical scavenging ability was detected using the method described by Kifle ZD and Enyew EF [15] with a minor modification. In brief, 200 µl of sample solution with different concentrations were mixed evenly with 200 µl of 0.1 mM of DPPH methanolic solution using a 96 well microplate reader. The reaction mixtures were incubated at 37 °C for 30 min in the dark and the absorbance was recorded at 515 nm using the Epoch-2 Microplate reader (BioTek Instruments, Inc., USA). After 30 min, unreacted DPPH radical was determined colorimetrically at 515 nm by blanking against the control. The radical scavenging activity was expressed in percentage and was calculated according to the following equation:

$$\text{Scavenging rate (\%)} = [A_0 - (A_1 - A_2)]/A_0 \times 100$$

Where A0 is the absorbance of DPPH solution (Control), A1 is the absorbance of DPPH solution with sample and A2 is the absorbance of the sample (Blank). Each sample was determined three times and averaged.

ABTS radical scavenging assay: The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity of each sample was determined using Zhang H, et al. [16] with some modification.

Briefly, 10 mg ABTS was dissolved in 2.6 mL of potassium per sulfate solution (3 mM) and was kept in the dark condition at 4°C for 16 h before use. A fresh ABTS working solution was prepared by diluting ABTS stock solution in pH 7.4 phosphate buffer solution to obtain the solution with the absorbance of 0.70 at =735 nm. In brief, a known amount of sample was added to 3 mL of fresh ABTS solution and was shaken vigorously and kept in the dark for 20 min at room temperature and the sample absorbance was read at λ= 735 nm using the Epoch-2 plate reader at an interval of 3 min (BioTek Instruments, Inc., USA). All the samples were performed in triplicates and averaged.

Scavenging rate (%) = $100 \times (A_{blank} - A_{sample})/A_{blank}$

Where A_{sample} is the absorbance of ABTS with the sample and A_{blank} refers to the absorbance of ABTS without the sample (Control)

In vitro anti-diabetic activity

α-amylase inhibitory assay: The α-amylase inhibitory activity was determined using a published protocol by Shanmugam H, et al. [17] with some modification. In brief, 20 µl (50-250 µg/ml) of each sample were added into 96 microplate well and the total volume was made up to 120 µL with Phosphate Buffer Solution (PBS pH 6.9) was added and the mixture was incubated at 25 °C in a water bath for 10 min. The positive control, acarbose (20 µL) was used. Starch and α-amylase (45 µl) were added sequentially to all the wells and incubated at 25 °C for 1 h for the conversion of starch to glucose. The reaction was terminated by adding 50 µl of Dinitro Salicylic Acid (DNSA) and the color was developed by heating the microplate at 60 °C for 1 h. The absorbance was measured at 540 nm using Epoch-2 microplate reader (Bio Tek Instruments, Inc., USA). The percentage of enzyme inhibition was calculated using the formula:

Inhibition rate (%)=$100 \times (A_{blank} - A_{sample})/A_{blank}$

Where A_{sample} is the absorbance with sample and A_{blank} refers to the absorbance without the sample (Control).

α-glucosidase inhibitory assay: The α-glucosidase (α-Glu) inhibitory assay was determined using the method as described by Zhao JQ, et al. [18] with slight modification. Twenty microliters of each sample solution (50-250 µg/mL) obtained from Kavuni grain extract were mixed with 10 µL of α-glucosidase (0.135 mg/mL) solution and 200 µL of 20 mM Phosphate Buffer Solution (PBS pH 6.8) were mixed and was maintained at 37 °C for 10 min. Then 10 µL of 20 mM 4-nitrophenyl-α-D- glucopyranoside was added to the mixture and kept at 37 °C for 30 min. The reaction was terminated by adding 100 µL of 0.2 M sodium carbonate solution and the absorbance was measured at 405 nm using Epoch-2 microplate reader (Bio Tek Instruments, Inc., USA). The inhibitory rate was calculated using the following equation:

Inhibition rate (%) = $100 \times (A_{blan} - A_{sample})/A_{blank}$

Where A_{sample} is the absorbance with extract and A_{blank} refers to the absorbance without extract (Control).

Statistical analysis

The statistical analysis of all of the experimental data was expressed as mean value ± Standard Deviation (SD) (n=3). The significance of the difference between mean was determined by one-way ANOVA test followed by Pearson correlation coefficient at p value <0.05.

Results and Discussion

Tentative identification and characterization of bioactive compounds by LC-QTOF-MS

The LC- QTOF-MS is an advanced technique used to identify the major phytochemicals and holistic nutritional benefits of Kavuni rice grains. The comprehensive characterization was performed using in-house well-established protocols and data were fed into the peak view software V.1.1. (AB SCIEX, USA). The full-scan spectra clearly indicated that the major phytochemicals were obtained under both in positive and negative ionization mode ($[M-H]^-$/$[M+H]^+$). The eluted phytochemicals were identified based on the respective retention times, measurement of accurate molecular mass and isotope peak pattern. The Table 1 showed the list of tentatively identified phytochemicals with their retention time, molecular formulae, error (in ppm) and their corresponding mass (m/z) with respect to their theoretical formula of identified compounds from Kavuni grain extract using different solvents. The total ion chromatograms of the Kavuni grain extract using different solvents were obtained using the optimized LC-QTOF-MS system and their mass spectrum is shown in Figure 1. A total 31 phytochemicals were identified in positive and negative ion mode from Kavuni rice grain extracts which includes anthocyanins derivatives, flavonoid aglycones, flavonoid glucosides and some carotenoid compounds.

In the present study, it is found that extract of Kavuni grains are rich in anthocyanin derivatives in positive ionization mode. The protonated accurate mass and mass error of the identified anthocyanin is presented in Table 1. A peak eluted at Retention Time (RT) 3.12, 3.89, 4.10, 4.12 and 5.32 min represents the characteristic flavylium cation. From water and ethanolic extract of Kavuni rice grains, the detected anthocyanins with a precursor ion $[M+H]^+$ at m/z of 432.10, 417.11, 484.85 have been assigned as apigenin 4-O-glucoside, pelargonidin 3-*O*-rhamnoside and cyanidin 3-O-glucoside respectively. The presence of cyanidin 3-O-glucoside in black rice is also confirmed by results reported by Hou Z, et al. [19] and Hao J, et al. [20]. In addition, two cyanidin derivatives with $[M-H]^-$ precursor ion was tentatively identified in acetone extract of Kavuni rice grains as isorhamnetin 3-O-glucoside and pelargonidin 3-O-glucoside. Earlier reports have shown that anthocyanins could be characterized by both negative and positive modes [21,22]. Our study is more reliable with Will and Dietrich that most abundant anthocyanins (cyanidin 3-O-glucoside) preferred to be identified under positive mode of ionization.

Kavuni rice grains are determined to be rich in flavonoid aglycones includes flavones, flavonols, isoflavones and flavanones. Peaks eluted at Retention Time (RT) of 1.30, 2.40, 2.989 and 5.65 min were identified as flavonoid aglycone derivatives. For the flavonol, reference compound Flavan-3-ol, m/z of the $[M-H]^-$ a precursor ion 224.95. The precursor ion undergoes a loss of neutral mass unit (56 Da) and displayed a product ion at m/z at 226.09 $[M-H-H_2O]^-$, $[M-H-CO]^-$ which confirmed the presence of the target compounds [23]. The data are conformity with the presence of flavonoid derivatives in Kavuni rice [24,25].

Two carotenoids (Xanthophylls) were tentatively identified in ethyl acetate and acetone extracts of Kavuni rice grains. However, no carotenoids have been detected in water and ethanolic extracts. Lutein having the precursor ion at m/z 568.9 with $[M-H]^-$ mode and zeaxanthin 568.9 with $[M+H]^+$ mode were also detected lutein (therapeutic carotenoid) a retinol equivalent compound found with meagre quantities in Kavuni rice grains [26,27]. Overall, the data clearly indicated that the Kavuni rice grain extract carries a wide array of phytochemicals that serve as the richest source anti-oxidants and their associated health benefits (Table 1) (Figure 1).

Functional group characterization using FT-IR

The IR spectra of Kavuni rice grain extract showed the functional groups that correspond to the specific spectra in the extract. The crude extract obtained from the grain extract of Kavuni rice were subjected to FT-IR analysis, thereby leading to the major functional group of the active components that were identified based on the peaks observed separately in the infrared region. The obtained results of the absorbance spectra of the extract fall between the wavenumber ranges of 4000-400 cm^{-1} are shown in Figure 2. The broad bands detected in water and ethanolic extracts between 3300-3200 cm^{-1} have been linked to –OH stretching vibrations, suggesting the existence of phenolic hydroxyl compounds, in concordance with previous reports. The characteristic peak that appeared at 2974 cm^{-1} shows the presence of alkane stretching vibrations, whereas the peak at 2922 cm^{-1} to 2853 cm^{-1} in ethyl acetate and acetone extracts confirmed the presence of carboxylic acid C=O (H bond) nitriles C=N stretching vibrations in the extracts. The peak at 1649 cm^{-1} verified the presence of alkene C=C stretching present in the extract. The peak owing to 1451 cm^{-1} showed the presence of C-O-H groups in the compound, which confirms the carboxylic acid and its derivatives in the ethanolic extract of Kavuni rice grain, representing the presence of anthocyanins that serves as

Table 1. Secondary metabolites identified in different solvents of kavuni rice extract by LC-QTOF-MS technique in positive and negative ionization mode.

Extraction Solvent	RT[a] (min)	Experimental Mass m/z[b] Value	Theoretical Mass m/z Value	Molecular Formula	Mass Error (%)[c]	Tentative Identification of Compounds
Water Extract	2.5	176.95	175.95	$C_6H_4Cl_2O_2$	0.5	3,6-dichlorocatechol
	3.10	223.00	222.01	$C_7H_8O_6S$	0.4	1-Methyl-pyrogallol-3-O-sulfate
	5.15	355.10	354.11	$C_{16}H_{20}O_9$	-0.04	Ferulic acid 4-glucoside
	5.65	397.20	396.19	$C_{24}H_{28}O_5$	1.2	8-(3,6-Dimethyl-2-heptenyl)-4',5,7-trihydroxyflavanone
	1.15	178.90	180.04	$C_9H_8O_4$	0.28	3,5-Dihydroxycinnamic acid
	1.30	224.95	226.09	$C_{15}H_{14}O_2$	-0.43	Flavan-3-ol
	2.40	341.15	342.11	$C_{19}H_{18}O_7$	0.76	3,5,6,7-Tetramethoxyflavone
	3.12	431.15	432.10	$C_{21}H_{20}O_{10}$	0.23	Apigenin 4'-O-glucoside
	5.20	683.25	684.23	$C_{30}H_{34}O_{18}$	-0.32	8-Hydroxyhesperetin 7-[6-acetylglucosyl-(1 -> 2)-glucoside]
Ethanolic Extract	2.45	365.15	364.379	$C_{17}H_{16}O_9$	0.43	Xanthotoxol glucoside
	4.10	482.10	484.85	$C_{21}H_{21}O_{11}Cl$	0.12	Cyanidin 3-O-glucoside chloride
	2.66	207.00	208.07	$C_{11}H_{12}O_4$	-0.54	Methyl ferulate
	2.83	255.30	256.07	$C_{15}H_{12}O_4$	0.67	Liquiritin rhamnoside
	3.09	305.00	306.04	$C_{11}H_{14}O_8S$	-0.47	4-Hydroxy-5-(dihydroxyphenyl)-valeric acid-O-sulphate
	4.12	415.15	417.11	$C_{21}H_{21}O_9$	0.786	Pelargonidin 3-rhamnoside
Ethyl Acetate Extract	1.98	158.15	157.08	$C_{11}H_{11}N$	0.123	2,6-Dimethylquinoline
	2.632	202.15	201.11	$C_{13}H_{15}NO$	0.964	2,3-Dihydro-6-methyl-5-(5-methyl-2-furanyl)-1H-pyrrolizine
	2.89	246.20	245.10	$C_{14}H_{15}NO_3$	-0.472	N-Phenylacetyl pyroglutamic acid
	1.58	122.15	123.06	C_7H_9NO	-0.327	4-(Methylamino)phenol
	3.09	240.15	241.11	$C_{12}H_{19}NO_2S$	-0.439	2-(4-Methyl-5-thiazolyl)ethyl hexanoate
	5.32	465.30	466.73	$C_{21}H_{22}O_{12}$	0.763	Taxifolin 7-O-glucoside
	6.89	588.50	590.9	$C_{39}H_{58}O_4$	0.871	Beta-Sitosterol ferulate
Acetone Extract	2.09	117.10	116.08	$C_6H_{10}O_3$	0.472	2-Oxovaleric acid
	2.25	138.95	138.03	$C_7H_6O_3$	0.673	Alpha-furyl methyl diketone
	3.039	194.1	193.09	$C_7H_{15}NO_5$	-0.474	N-methylglucosamine
	5.79	567.10	568.9	$C_{40}H_{56}O_2$	-0.532	Lutein
	5.79	567.10	568.9	$C_{40}H_{56}O_2$	-0.398	Zeaxanthin
	3.65	475.35	477.32	$C_{15}H_{12}O_4$	0.732	Isorhamnetin 3-O-glucoside
	3.89	431.35	433.24	$C_{21}H_{12}O_{10}$	0.739	Pelargonidin 3-O-glucoside
	2.989	387.15	388.11	$C_{20}H_{20}O_8$	0.631	3'-Hydroxy-4',5,6,7,8-pentamethoxyflavone
	4.89	521.10	522.4	$C_{23}H_{22}O_{14}$	0.834	3,4',5,6-Tetrahydroxy-3',7-dimethoxyflavone 3-glucuronide

a major phytochemical responsible for anti-oxidant and anti-diabetic activity. In addition to that, bands corresponding to skeletal stretching vibration (1087 cm^{-1}, 1044 cm^{-1}) signifies the contribution of aromatic rings and C-O-C group of flavonoids in water and ethanolic extract of Kavuni rice grains. However, those peaks are absent in ethyl acetate and acetone extracts. Our data demonstrate that the bioactive marker compounds extracted from the grains of Kavuni rice are strongly influenced by the solvent polarity resulting in varied and selective extraction of chemical moieties. The FT-IR analysis has further proved the presence of major phytochemicals such as anthocyanins, flavonoids and phenolics in the Kavuni rice grains (Figure 2).

Total Phenolic (TPC) and Flavonoid Contents (TFC) in Kavuni rice grain extracts

Plant phenolics are one of the most important classes of compounds acting as a principal anti-oxidants or free radical terminators [28]. In our experiment, the highest TPC was exhibited by ethanolic extract of Kavuni rice grain as 29.91 ± 0.12 mg/g dry weight gallic acid equivalence whereas the lowest TPC was recorded by acetone extract 23. 64 ± 0.09 mg/g gallic acid. From results, it was found that polar solvents are efficient in extracting total phenolic content in Kavuni rice grain due to solubility index of phenolic compounds. In addition, this may also be due to the pericarp colour (blackish brown grain colour) of Kavuni rice grains which substantially increased the phenolic content in extracts [29]. Our study further confirmed that adds evidence to the finding that ethanolic extract is more efficient in extracting phenolic compounds quantitatively from Kavuni rice grains.

The total phenolic content of the black pigmented traditional rice is in the range of 43.19 ± 0.54 mg GAE/100 g which was four times higher than the conventional non-pigmented varieties. Despite the fact that traditional pigmented rice genotypes are rich in phytochemicals but during the process of cooking, complex physical and chemical changes are undergone in phenolic compounds such as degradation, polymerization and release of loosely bound phenolics from aleurone layer that led to the loss of phenolics [30]. Even after loss of phytochemicals, the average TPC of cooked pigmented rice was higher by 77% than non-pigmented rice varieties [31]. The data clearly have shown that the TPC depends on the chemical composition of the grains.

Flavonoids are the most treasured phytochemicals as pro-oxidants and stimulate body's natural antioxidant system playing a major role in regulating various biological functions with potent antioxidant capacity and anti-diabetic activity [32]. The total flavonoid content was also high in ethanolic extracts of Kavuni rice grains 47.5 ± 0.23 mg/g dry weight quercetin equivalence and the least was found in acetone extract 25.2 ± 0.14 mg/g dry weight quercetin equivalence. The data are in agreement with the research findings of Meera K, et al. [33] who recorded the total flavonoid content of 44.08 mg catechin/g was extracted from black Kavuni rice. Further, scientists have reported that the black rice possesses higher flavonoid content than brown and red rice varieties which may be due to the deposition of dietary flavonoids in the aleuronic layer

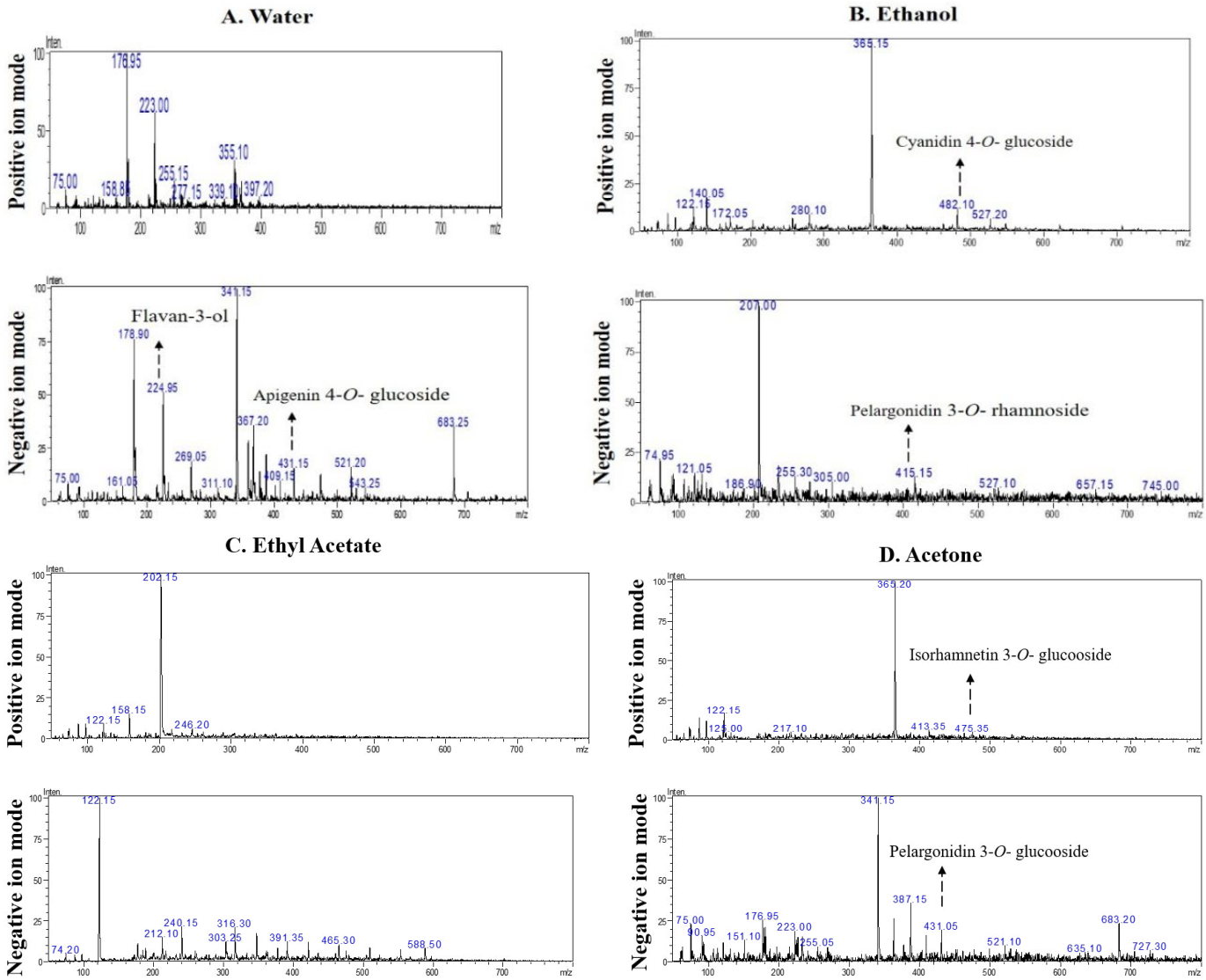

Figure 1. LC-QTOF-MS total ion chromatogram of kavuni grain extract using different solvents in positive and negative ionization mode **A)** Water extract, **B)** Ethanol extract, **C)** Ethyl acetate extract and **D)** Acetone extract.

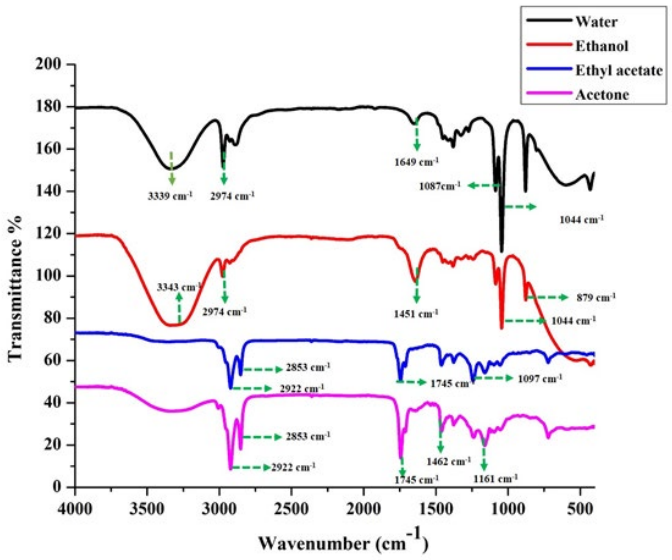

Figure 2. FT-IR analysis of traditional kavuni rice grain extract using different solvents with their characteristics peaks.

of the rice grains. The flavonoid contents of pigmented varieties are known to vary with cultivation techniques, growing conditions, genotypes of the cultivar and extraction techniques. Devraj L, et al. [34] reported that pigmented rice varieties had higher flavonoid content than the conventional non-pigmented rice varieties. Hence, it is noteworthy that Kavuni rice cultivars are a remarkable source of functional foods (Figure 3).

Quantification of major anthocyanins (Cyanidin 3-O-glucoside)

Anthocyanins represent a sub-class of water-soluble flavonoids responsible for the colors of various fruits, vegetables and cereals. Cyanidin-3-*O*-glucoside is the major anthocyanin present in the grains of black rice, which accounts for up to 89.6% of total anthocyanins and the content of anthocyanins in Kavuni rice extracted using different solvents are presented in Figure 4A [35]. In the present study, quantitative determination of anthocyanins in Kavuni rice grain is based on the maximum absorbance from UV-Vis spectroscopy at 536 nm and the absorption spectra are shown in Figure 4B. Interestingly, the highest cyanidin-3-O-glucosidecontent is observed from ethanolic extract (61.31 ± 0.04 mg/g) followed by water extract (45.20 ± 0.10 mg/g) Figure 4A.

However, the content of anthocyanins in different black rice varieties may vary. Gong ES, et al. [36] identified two anthocyanins (cyanidin 3-O-glucoside, peonidin 3-glucoside) whereas identified four anthocyanins (cyanidin 3-O-glucoside, peonidin 3-glucoside, cyanidin 3-rutinoside, cyanidin-3,5-diglucoside) in black rice. Besides, the content of anthocyanins is greatly varied with 5.2-163.3 mg/100 g of 10 different black rice cultivars [37]. The differences in anthocyanin contents in rice varieties vary with varieties, extraction and

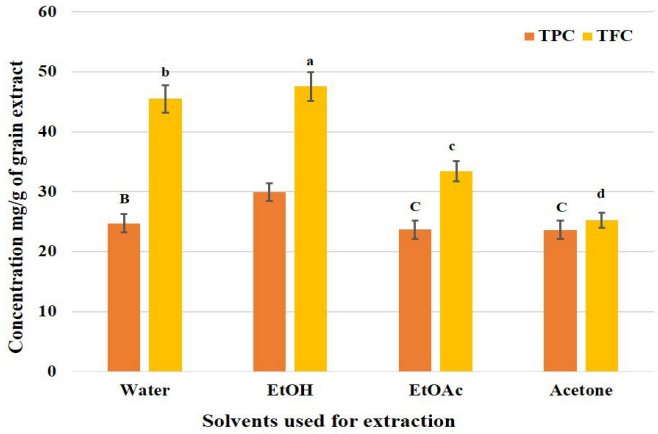

Figure 3. Total Phenolic (TPC) and Flavonoid Content (TFC) present in kavuni rice grain extract using different solvents EtOH-Ethanol; EtOAc-Ethyl acetate. The values are represented as mean ± SD with statistical significance indicated by * (one-way ANOVA, p<0.05) and "ns" as not significant.

quantification methods. The anthocyanin content in black rice and red rice cultivars were 3.5 and 4.3 mg/g, respectively, while no anthocyanins detected in brown and white rice varieties (Figure 4) [38].

Anti-oxidant activity

Intake of dietary polyphenols from pigmented rice landraces involve in free-radical redox reaction resulting in decreased risk of disease incidence associated with oxidative stress. Antioxidant activity was conducted to test the therapeutic capacity of rice grains that neutralized free radicals. In brief, total anti-oxidant activity assay is based on electron transfer or hydrogen atom transfer mechanism-based assays. Hence, the total anti-oxidant activity of Kavuni rice grain extract from different solvents was assessed using two different assays.

DPPH radical scavenging activity of Kavuni rice grain extract: Phytochemicals present in the different solvent extracts of Kavuni rice grains with anti-oxidant activities react with DPPH, a nitrogen centered radical and gets converted to 1, 1-diphenyl-2-picryl hydrazine (DPPH-H), because of its electron donating ability to reduce any compound including metals, carbonyls and radicals [39]. Figure 5A shows the DPPH radical scavenging activity of Kavuni rice extracted using different solvents in the concentration range of 50-250 µg/mL. Earlier finding indicated that different black rice varieties contained different polyphenols including anthocyanins which exerts anti-oxidant activities [40,41]. Ethanolic extract of Kavuni rice grain at a concentration of 200 µg/ml exhibited maximum DPPH radical scavenging activity (89.51 ± 0.1%). The minimum DPPH scavenging activity was recorded with acetone extract of Kavuni rice grains (28.72 ± 0.02%). Our results are in concordance with Ghasemzadeh A, et al. [42] where black rice extracts was found to have higher DPPH activity than red and brown rice cultivars. In addition to that Rajendran V, et al. [43] reported the highest free radical inhibition in pigmented rice varieties (Kala namak) recorded 89.01% scavenging ability compared to non-pigmented rice variety (Seera samba) with 59.01%. This variation in radical scavenging activity may be due to variation in phytochemicals present in pigmented and non-pigmented rice varieties.

ABTS radical scavenging activity: ABTS scavenging activity is initiated by oxidation of radical cation ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonate) with potassium persulfate and its reduction in the presence of hydrogen-donating anti-oxidants. In the study, the ABTS scavenging activity was highest in ethanolic extract (82.9 ± 0.09%) at the concentration range of 200 µg/mL and the lowest ABTS scavenging activity was recorded with ethyl acetate and acetone extract (5.722 ± 0.3%) at a concentration of 50 µg/mL and (4.86 ± 0.2%) with a concentration range of 150 µg/mL respectively. Interestingly, in ABTS radical scavenging assay the total anti-oxidant capacity is contributed by both hydrophilic and hydrophobic compounds present in the extract [44]. Previous reports revealed that the anti-oxidant activity in rice grains positively correlates with the concentration of total phenolics and flavonoids present in the extract. Hence, our result clearly signifies that the ethanolic extract of Kavuni rice grain could be having higher level of phenolics and flavonoids compared with other solvents which contributes to higher anti-oxidant activity. Also, the content of anthocyanins, more predominantly cyanidin 3-O-glucoside, extracted from aleurone layer of Kavuni rice grains may results in higher anti-oxidant activity, promoting them as strong free radical scavenger (Figure 5).

In vitro anti-diabetic activity of Kavuni rice grain extract

Inhibition of α-amylase activity: Inhibition of α-amylase enzyme activity is used as one of the major strategies in the treatment of diabetes which has been proven to be most effective to decrease postprandial hyperglycemia, at the intestinal blood interphase, by targeting the carbohydrate hydrolysis and mobilization of glucose into the blood [45]. Commercially, this can be achieved by using chemical inhibitors like acarbose, miglitol and voglibose. However, the continuous usage of chemical inhibitors can cause severe gastrointestinal side effects such as abdominal pain, hepatitis, pancreatitis and other cardiovascular diseases. Therefore, there is a need to identify and explore the enzyme inhibitors from natural sources having fewer side effects.

Our experimental results obtained from Kavuni rice grain extract extracted using different solvents against the positive control acarbose resulted in maximum α-amylase inhibition of 82.30% with ethanolic extracts at the concentration range of 200 µg/mL, while water extract recorded in minimum α-amylase inhibition of 24.102% at the concentration of 250 µg/mL. The α-amylase inhibitory effect of the ethanolic extracts is comparable to the clinically established drug at similar concentration. For example, at a concentration of 100 µg/mL, the ethanolic extract showed 72.2% inhibitory while acarbose showed 74.96% inhibitory activity. From the results, it suggests that the activity of the enzymes was significantly suppressed by ethanolic extracts of Kavuni rice grains which might be due to higher amounts of phytochemical constituents (phenols, flavonoids and anthocyanins) present in it. Hence, significant inhibition of α-amylase enzyme activity might be due to the presence of hydroxyl group present in polyphenolic compounds those complexes with proteins, which might promote inhibitory activity [46]. Ali Asgar MD [47] reported that in Type-2 diabetic patients, the enhanced activity of α-amylase enzyme may be due to non-covalent interactions occurs between polyphenolic compounds and proteins. Also, the phenolic compounds can

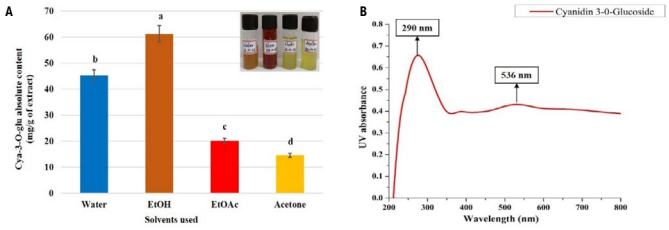

Figure 4. A) Quantification of cyanidin-3-*O*-glucoside in kavuni rice extracts using different solvents EtOH-Ethanol; EtoAc- Ethyl acetate and **B)** UV-Vis absorption spectra of standard cyanidin-3-*O*-glucoside with λ_{max} = 290 nm and λ_{max} =536 nm. The values are represented as mean±SD with statistical significance indicated by * (one-way ANOVA, p<0.05) and "ns" as not significant.

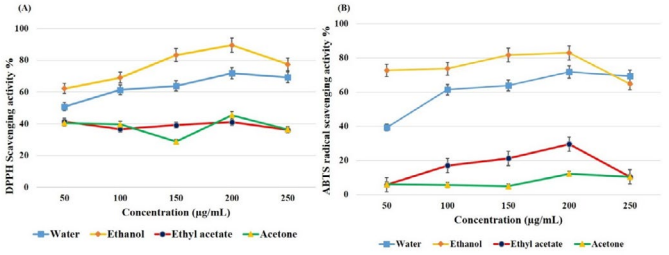

Figure 5. A) DPPH antioxidant activity recorded in kavuni rice grain extracts obtained from different solvents at various concentrations and **B)** ABTS antioxidant activity in kavuni rice grain extract using different solvents at various concentrations. The values are represented as mean±SD with statistical significance indicated by * (one-way ANOVA, p<0.05) and "ns" as not significant.

form hydrogen bonds with polar group of enzymes could result in managing postprandial hyperglycemina condition. Our results confirm that higher amount of phytochemical constituents extracted from ethanolic solvent has shown good inhibition against α-amylase enzyme and can be consumed in managing Type 2 diabetic condition (Figure 6).

Inhibition of α-glucosidase activity: Inhibition of α-glucosidase enzyme activity is one of the therapeutic approaches in preventing type 2 diabetes by delaying the glucose absorption in the blood [48]. As shown in Figure 7, the maximum α-glucosidase enzyme activity was observed from ethanolic extracts of Kavuni rice grains with 70.21% at the concentration of range of 200 µg/mL and the minimum α-glucosidase enzyme activity from acetone extracts of 6.69% at the concentration of range of 50 µg/mL. From the results, it was found that ethanolic extract of Kavuni rice grains retained higher levels of intact anthocyanins present in the aleurone layer of rice. And this was in agreement Barik SK, et al. [49] confirming that anthocyanins extracted from black currant berries recorded a maximum α-glucosidase inhibitory activity compared with that of phenolic compounds present in the samples. Moreover, it was observed that the structure of anthocyanins consists of anthocyanin aglycones and glycosides, which helps in hydrolyzing α-glucosidase enzyme in the most effective way than other phenolic compounds.

Our finding suggests that the anti-hyperglycemic effect of Kavuni rice grains were mainly driven by the synergistic effect of the phenolic compounds and anthocyanins. Also, by supporting our results indicating that from *in silico* studies it has been found that there is an intricate binding pattern between phytochemicals present in grain extract and digestive enzymes used for the study [50]. Hence, our result of carbohydrate-hydrolyzing enzymes inhibition supports the view that Kavuni rice grain consumption could regulate the postprandial glucose condition in diabetic patients with minimal side effects (Figure 7) [51].

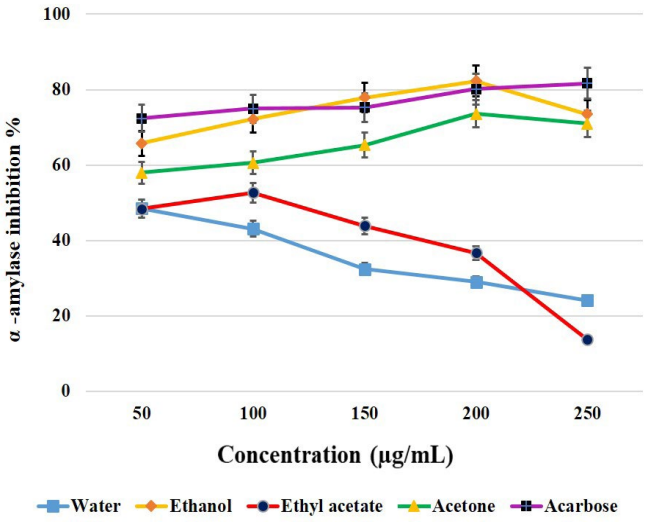

Figure 6. α-amylase inhibition activity recorded in kavuni rice grain extract with different solvents against commercial chemical inhibitor, acarbose. The values are represented as mean ± SD with statistical significance indicated by * (one-way ANOVA, p<0.05) and "ns" as not significant.

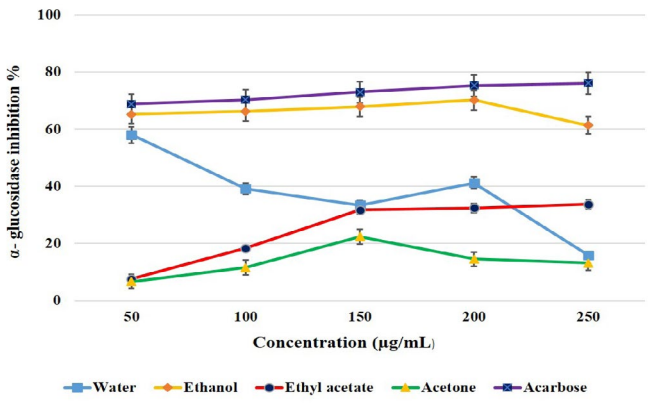

Figure 7. α-glucosidase enzyme inhibition activity of kavuni rice grain extract with different solvents against commercial chemical inhibitor, acarbose. The values are represented as mean ± SD with statistical significance indicated by * (one-way ANOVA, p<0.05) and "ns" as not significant.

Conclusion

The consumption of therapeutic Kavuni rice has been shown to reduce postprandial hyperglycemia, which is a rich source of polyphenols specially anthocyanins (cyanidin 3-O-glucoside). The *in vitro* anti-diabetic activity data presented here showed that the ethanolic extracts of Kavuni rice grain are effective at inhibiting α-amylase in the mouth and α-glucosidase in the intestine using different solvents. LC-QTOF-MS analysis confirmed that anthocyanins are the major phytochemicals extracted from Kavuni rice grains using ethanolic extracts. Also, the present study recorded differential responses to total phenolic and flavonoid content extracted from Kavuni rice grains with different solvents and also assessing the extracts for anti-oxidant capacity. Results confirmed the maximum anti-oxidant capacity by DPPH assay and ABTS assay have been correlated with higher total phenolic and flavonoid content extracted from ethanolic solvents are predominantly having higher amount of major phytochemicals (anthocyanins, flavonoids and phenols). FT-IR analysis further confirms the presence of major phytochemicals obtained from Kavuni rice grain extract through functional groups identification. Hence, the major phytochemical specifically anthocyanins extracted from Kavuni rice grain has been confirmed to possess free radical scavenging anti-oxidant activity and α-amylase and α-glucosidase inhibiting starch blocker activity and can be used in treating Type-2-diabetes. The information obtained from the present study will be directed towards developing novel nano nutraceutical product with enhanced anti-diabetic activity.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of Interest

The authors declare no conflict of interest.

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