Pak. J. Biochem. Mol. Biol., 2020, 53(2-3):33-58

Research paper

Biochemical characterization of red rice cultivar from Thatta, Pakistan

Asma Javed[2] and M. Kamran Azim[1]*

[1]Department of Biosciences, Mohammad Ali Jinnah University, Karachi, Pakistan.

[2]HEJ Research Institute of Chemistry, University of Karachi, Karachi, Pakistan.

*corresponding author: kamran.azim@jinnah.edu

Abstract:

Rice is one of the basic diet constituents of many people around the globe. There are different colored varieties of rice like black, red, brown (due to anthocyanin pigment). Colored rice pigments possess antioxidant activities. Red rice is a specific variety of rice grown in different parts of world including Thatta district of Sindh, Pakistan. This study was conducted to analyze the biochemical properties of red rice cultivar of Thatta. Red rice grain samples collected from Thatta were subjected to phenol extraction to estimate the free and bound fraction of phenols. Results revealed higher fraction of bound phenol in all the cultivars (78% of bound phenol content). Phenolic compounds not only protect plants from toxic effects, pests and pathogens but also have different applications in medicines and cosmetics. Gas chromatography coupled mass spectrometry (GC-MS) identified several volatile compounds in red rice grains. Total 18 compounds were identified out of which 5 belongs to ester group and rest of the compounds belongs to amide group with the higher proportion of amides in all the samples as compared to esters. N-(Trifluoroacetyl)pyrrolidine was detected as the major compound among all samples.SDS-PAGE was used to determine protein profile of red rice grains. Results obtained from SDS-PAGE of the rice grains showed similar pattern of proteins in rice cultivars. Few polymorphic bands that could be used to distinguish the cultivars were also detected. Major bands were commonly present in all the cultivars indicating that genes coding these proteins are conserved.

Keywords:protein profile, GCMS, Rice chemistry, Rice metabolites.

Introduction:

In Asia, rice (*Oryza sativa* L.) is most important staple food.Rice is classified as a typical monocotyledon plant, which is considered as semi-aquatic plant because of its semi-waterlogged condition [1].The origin of the domestic rice is controversial. Genetic evidence showed that both the *indica* and *japonica* Asian rice forms originated in China, 8200-13,500 years ago from *Oryza rufipogon***,** wild rice from a single domestication [2]. Rice genome variation map indicated that rice domestication took place in China in Pearl River valley [3].On the basis of the molecular clock, it is estimated that rice first domesticated during 8,200 -23,500 years ago, which is consistent to the known archaeological data on the subject, although exact date is unknown [2].

The annual production of rice worldwide is more than 700 million tons and known to cover total area of approximately 158 million hectares. It has been estimated that globally 90% of the rice is produced in Asia (i.e. 640 million tons of rice). Latin America produces around 25 million tons of rice while Sub-Saharan Africa produces approximately 19 million tons. Small farms of 0.5-0.3 hectares are mostly used to grow rice in sub Saharan Africa and Asia. In a very poor rain fed conditions, rice yields range from less than 1 ton per hectares and in intensive temperate irrigated system it is more than 10 tons per hectares. Rice is productive in many conditions and can tolerate wide range of environments where

other crops would fail (Ricepedia: http://ricepedia.org/).

China and India are the world's largest rice producers. Even though India's rice harvested area is higher than China but its yield is lower because most of the rice growing areas of India are not well irrigated whereas rice growing belt in China are cultivated on irrigated land.Indonesia, Bangladesh, Vietnam, Mya nmr, and Thailandare major rice producers after China and India. The average rice production of these seven countries in 2006-08 was30 million tons which is equivalent to more than 80% of the world rice production.

Although Asia is dominant in rice production and consumption, rice is also very important in other parts of the world. In Africa, for example, rice has been the main staple food – defined as the food, among the three main crops, that supplies the largest amount of calories in parts of western Africa and for some countries in the Indian Ocean. In Africa, rice consumption has been increased which is more than the production of the rice, and the needs have been fulfilled by increasing the imports. In 2006-08 more than 40% of the African rice production was from Western Africa which is now known as the main rice producing sub-region. Major rice producers of paddy in 2006-08 were Egypt, Nigeria and Madagascar with a sharecontribution as respective; 7.0 million tons, 3.8 million tons and 3.2 million tons of rice production. (http://ricepedia.org/).

Rice crop is economically important for Pakistan being second foreign exchange earner after cotton. In Pakistan, rice holds 11% of total cultivated area. After India, Vietnam and Thailand; Pakistan is the 4th largest rice exporting country in the world. About 0.573 million metric tons Basmati rice is exported to the Middle East, Europe and America; whereas, 3.759 million metric tons' coarse rice is exported to different countries of Asia and Africa. Pakistan earned US$ 1.86 billion foreign exchange from the export of rice during 2015-16. The biggest producer of rice in the country is the Punjab province. During 2015-16, rice was cultivated on 4.399 million acres and production was 3.502 million-tones. The Punjab contributes 51% to Pakistan's rice production, while other provinces i.e.; Sindh, Baluchistan and Khyber Pakhtunkwa contribute 39%, 9%, and 1% respectively (https://aari.punjab.gov.pk/).

ROLE OF RICE IN DIET

Rice being a primary source of nutrients holds important role in diet [4], especially in the developing countries, where animal proteins are high priced. Rice contributes nearly 28-54% proteins in the Asian diet. Rice supplies 27% of the world's nutritional energy and 20% of overall nutritional protein [5]. Rice is an excellent crop because it is one of those plants that synthesize and store both major classes of proteins, i.e., prolamins and glutelins in sub-cellular compartments [6].

COLOURED (PIGMENTED) RICE

Rice is known with the white color but in the traditional rice growing areas of Asia purple, red, brown, black, yellow colored rice has been grown.In ancient time, coloured rice has been given importance because of their special characteristics such as exclusive taste and medicinal value. For the royals of China, black rice was the most-liked food. In various parts of Sri Lanka, India, and Bhutan red rice has been considered important [7].

Coloured rice has distinctive flavour and colour but they are not normally consumed as a part of diet because of the hard texture of the cooked coloured rice. Nonetheless, pigments in coloured rice and their beneficial effects have been known long ago. Colour compounds anthocyanin and flavonoids occur naturally in coloured rice. Pigmented rice has gained popularity due to its bioactive compounds such as α-tocopherols, γ-oryzanol, phenolic compounds and for its antioxidants. Acetylated procyanidins is a prevalent anthocyanin known to possess

antioxidantactivity [8]. Anthocyanins,proanthocyanidins have been reported as a major phenolic compound in red rice [9]and cyanidin-3-glucoside as a major phenolic compound in black rice [10].The antioxidant activities of coloured pigments of rice have been reported [8,11,12]. Vitamin E isomers, C-oryzanols, carotenoids and bioactive fat-soluble components are rich in pigmented rice. Brown rice is highly nutritious because its outer layer remains intact during processing which is known to contain beneficial vitamins and fibres. Saturated fatty acids including palmitic acid and stearic acidare rich in coloured rice [13]. Coloured rice varieties can be used as a source of natural antioxidants [14]. Pigmented rice has been reported to heal iron deficiency and known to have anticarcinogenic, antioxidant, antiallergic, antiatherosclerosis activities [15].

Bran layer of brown rice contains many minerals and vitamins. Red rice is rich in zinc and iron while purple and black rice are known to have fat, crude fiber and also rich in protein. Anthocyanins are the key colour pigments of the red, black, purple rice. Anthocyanins are well known for their antioxidant and free radical scavenging activities and various beneficial health effects (Ricepedia: http://ricepedia.org/). In Southeast Asia, pigmented rice has a long history for human consumption [11]. Antioxidant activities ofblack rice, red Thai, dark purple and red brown rice have been extensively studied. It has reported that pigmented rice has high phenolic contents and antioxidant activities in comparison to non-pigmented rice (white rice) [16,17]. Anthocyanin and polyphenolic compounds are reported to be abundant in black rice [18,19]. Coloured rice bran contains anthocyaninwhich is known to have an antidiabetic and inhibitory activity of reductase enzyme [20,21]. Shao and co-workers [22] studied anthocyanins and phenolic acids in endosperm, bran and embryo of white, black and red rice andreported that the bran layer contained highest amount of total phenolic content (TPC) i.e. 7.35 mg GAE/g and contributed 60% of phenolics in white, 86% in red, and 84 % in black rice. In bran, Cis-p-coumaric was found to be present in bound form whereas cis-sinapic acidfound in free form in both bran and embryo. In black rice the anthocyanins were identified to be identified to be peonidin-3-O-glucoside and Cyanidin-3-O-glucoside. In bran of black rice Cyanidin-3-O-rutinoside was detected [22].

RED RICE

Rice having a red bran layer is known as red rice. The bran layer colour varies from light red to dark red. After milling it gives slight red shade. The coloured pigments are present only in a bran layer which contain anthocyanins, polyphenols and have antioxidant activities. After removal of bran layer, red rice appears to be white alike white rice. Red rice has 2-3 times higher zinc and iron content in comparison to white rice [7]. Chinese varieties of red rice '*Bloody Sticky*' and '*Dragon Eyeball*' reported to have high iron content [23].

Red rice grains are covered with light to dark colour of husk, occur in three types weedy, wild and cultivated.

CULTIVATED RED RICE

Before white rice, red rice had importance in nearly all the rice growing countries of Asia such as Japan, Korea, Sri Lanka, India, Philippines, Japan and China. In China, around 20% of the germplasm collected were of red rice and 1.26% of the total ricegrowing area is taken by the different coloured rice [24]. In Bhutan, red rice is grown on around 30% of the area. In an evaluation program conducted under the Directorate of Rice Research (Hyderabad, India), for biotic stress 28.31% of the entries out of 12,750 entries were coloured rice. Out of which, 10.48% were red, 9.41% brown and 8.40% purple. The Central Rice Research Institute (CRRI), (Cuttack, Orissa, India) collection had higher number of red rice. The National Bureau of Plant Genetic Resources (NBPGR) conducted a

survey from 1991 to 1998 and documented/registered 21% and 35% varieties of red rice in Manipur and Orissa [7,25].

Red rice was common in East, West, and hilly tracts of the West and Northeast in India. Few varieties of red rice were also reported from Rajasthan, plains of Punjab, Gujarat and western Uttar Pradesh. Red rice was known to be highly tolerable to harsh environmental conditions such as deep water, infertile soils and mountain lands [7]. Majority of the red rice are coarse grained but diversity does exist as in white rice. Red rice is scented and non-scented, short and long grained, glutinous and non-glutinous, early maturing and late maturing. Himalayan red rice (long grained red rice) has got attention of exporters.*Patni* of Maharashtra *Matta* of Kerala, *Matali* and *Jatu* of the Kulu valley in Himachal Pradeshare few famous red rice varieties. During the British period the Commissioner of Kulu was so fond of *Jatu* rice that he used to send it to his family in England [7].

USES OF RED RICE

In India, red rice is contemplated as medicinal and rich in nutrition. Red *gunja* rice used for making bread and chapatti as well as eaten as whole grain [26]. In south India, glutinous rice is consumed. *Jatu* red rice is consumed for its taste and aroma. In Himachel Pradesh, *Matali* and *Lal dhan* used for the cure of fever and blood pressure. In Karnataka, *Atikaya* and *Kari kagga* are used as tonic and for coolness [7]. In China, red rice is used for preparing tart, cosmetics, vinegar, and red rice yeast for medicinal purpose. Fermentation of yeast *Monascus purpurea* over red rice is the way to prepare red rice yeast which is used for treatment of stomach, indigestion, and promotes blood circulation, bruised muscles. Red rice yeast is marketed all over the world as a cholesterol lowering product [24]. In Japan, red rice is used for making coloured noodles, cakes and red sake. Red rice is a favourite food of people of Sri Lanka and is also used as nutraceutical [7].

SPECIAL FEATURES

Red rice holds many notable features, besides being nutritive and medicinal importance. It has been experienced that black and red rice are more impervious to storage pests and insect as compare to brown rice. Red rice has stored and maintained their original state since 1905 as reported in Japan, while white rice was badly damaged. Resistance and hardiness of *Jatu* rice of Himachal Pradesh and *Patni* rice of Maharashtra are generally known. [7].

Besides storage capability, red rice varieties have also shown resistance to various adverse agro climatic conditions such as alkalinity, resistance to diseases and pests, salinity, flood, drought [24].

The objective of this study was to carry out biochemical analysis of red rice of Thatta, Sindh, Pakistan. Red rice is an unexplored commodity of Sindh province though it has been grown and consumed in district Thatta of Sindh, Pakistan for decades. Farmers usually grow it on a small scale just for their consumption. In Thatta, red rice is known as Dhamaka or Dhamako.

MATERIALS AND METHODS

SAMPLE COLLECTION

Red rice samples were purchased from the local market in Thatta, Sindh, as well as from Research Institute of Thatta, Sindh. Basmati rice samples were purchased from local market in Karachi, Pakistan.

TOTAL PHENOLIC CONTENT OF RED RICE GRAINS

EXTRACTION OF FREE PHENOLICS

To extract free phenolics, 0.5 g of ground rice were treated with 80% of 8 mL aqueous methanol twice in a sonic bath at 35°C for 1 hour. Both the supernatants were combined after centrifugation at 12,300 rpm and their pH was adjusted to 4.5−5.5 using 6 M HCl [27].

EXTRACTION OF BOUND PHENOLICS

To extract bound phenolics, the residues obtained from free phenolics were washed

using 20 mL of distilled water. After removal of water, samples were blended twice with 20 mL of 0.4 M NaOH for 2 hours in a sonic bath then centrifuged at 12,300 rpm for 25 minutes. Both the supernatants were combined and pH was adjusted to 4.5−5.5 using 6 M HCl [27].

DETERMINATION OF TOTAL PHENOLIC CONTENT

Total phenolic content (TPC) was determined by Folin–Ciocalteu method. To 0.2 mL sample extract, 5 mL of distilled water and 0.5 mL of Folin–Ciocalteu reagent were added. After incubation for 5 minutes at room temperature, the mixture was neutralized with 1.5 mL of 20% Na_2CO_3 and mixed well by vortexing. After incubation for 30 minutes, absorbance of the mixture was measured at 765 nm with a UV–VIS spectrophotometer. Gallic acid (0–800 mg/L) was used as a reference standard, and the results were expressed as milligrams of Gallic acid equivalents per kilograms of the sample (mg GAE/kg sample) [28]. Experiment was repeated three times and results were presented as means. Results of this experiment were statistically analyzed using t-test (unpaired) at significance level $p< 0.05$.

GAS CHROMATOGRAPHY MASS SPECTROMETRYOF RED RICE GRAINS

SAMPLE PREPARATION

Samples were prepared by using optimized method as described by [29]. Rice grains were de-hulled and dipped in liquid nitrogen prior to grinding. 0.3 g of ground rice samples were taken, 3 mL methanol/water (4:1 v/v) mixture was followed by addition of 100 µL of caprylic acid solution (0.3 mg/mL) as an internal standard. Samples were vortexed for 1 minute and incubated for 30 minutes, followed by 60 minutes of sonication and centrifugation at 20,000 rpm for 10 minutes. 2 mL of supernatant was collected and freeze dried.

DERIVATIZATION OF SAMPLE

Samples were derivatized using 90 µL of N, O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) and 80 µL of pyridine. Water bath was set at 75°C and mixtures were placed and heated in water bath for 45 minutes before being transferred to GC vials. Derivatized samples were analysed within 24 hours after derivatization [29].

GC-MS ANALYSIS

GC-MS analysis was performed using 7890A gas chromatography (Agilent technologies, USA), equipped with an Agilent Technology GC autosampler 120 (PAL LHX-AG12) and coupled to an Agilent 7000 Triple Quad system (Agilent Technologies, USA). TM TRACE TM ZB5 column (Thermo Scientific Inc., USA) was used having 30 mlength, ID 0.25 mm, 0.25 µm film thickness (P/N 260F142P). Injector was Instant Connect SSL at 285°C temperature. Split flow was maintained to 12 mL/minute. Injection mode was split at volume of 1:10. Carrier gas was Helium gas at constant flow rate of 1.2 mL per minute. Oven temperature was initially set to 60°C for 4 minutes, which was increased to 8°C / minute to 170°C, and retained to 4°C / minute to 300°C. The temperature was increased in post run to 300 °C for 15 minutes. For GC-MS, the Electron Ionization at 70 eV was used. Data acquired in full scan mode within range of 40-600 Da at an acquisition rate of 250 ms with ion source temperature of 300°C.

PROTEIN ELECTROPHORESIS OF RED RICE GRAINS

SAMPLE PREPARATION

The samples of rice grains were washed with autoclaved distilled water to remove any impurity and then dried. Two grams of rice seeds were ground to fine paste in sterile mortar and pestle with liquid nitrogen and incubated with 800 µL of extraction buffer (0.25 M Tris HCl, 20% Glycerol, 5% SDS, 0.002% Bromophenol Blue, 0.05% DTT) in Eppendorf tubes. Extracts were incubated at 4°C for 30 minutes then centrifuged at 10,000 rpm for

10 minutes. Supernatant was collected and diluted employing sample diluting buffer (1:2) (Tris-HCl (0.5 M), pH 6.8, 4% SDS, 20% glycerol, 0.5 mL distilled water, 0.02% bromophenol dye, 5% beta mercaptoethanol) used for SDS-PAGE. Samples were kept at -20°C until use.

ELECTROPHORESIS GEL PREPARATION

RESOLVING GEL: The resolving gel was prepared by mixing 2.625 mL of distilled water, solution A (Acrylamide 15 g, Bisacryl 0.4 g, water 50 mL) 3.13 mL, solution B (Tris 18.15, water 50 mL, pH 8.8) 1 mL, solution D (1% SDS, water 50 mL) 0.75 mL, solution E (10% APS [Ammonium per sulphate (freshly prepared)]) 0.3785 mL, TEMED 0.008 mL. After mixing the gel was poured in vertical SDS-PAGE assembly. Resolving gel was poured first to the gel plates and allowed to stand for 15-20 minutes for solidification. It was overlaid with 100-200 µL of distilled water for uniform gel surface.

STACKING GEL: Stacking gel was prepared by mixing distilled water 2 mL, 1 mL of solution A, 1.25 mL of solution C (Tris 3 g, water 50 mL, pH 6.8), 0.5 mL of solution D, 0.25 mL of solution E, TEMED 0.008 mL. After mixing it was poured over resolving gel between SDS-PAGE plates and a stacking gel comb was immediately inserted into to the gel solution to make wells to load samples. It was kept on room temperature for at least 2 hours for polymerization.

Sample application: The samples were mixed with sample diluting buffer (mentioned above) in 1:2 ratios and heated at 90°C for 5 minutes on heating block prior to loading. Glass plates were fixed with vertical electrophoresis apparatus, filled with running buffer (0.325% Tris, 1.44% glycine, 0.1% SDS). The samples were loaded (20 µL) in each well. Molecular weight marker PageRuler^{TM} Prestained Protein Ladder of 11-170 kDa (Fermentas Life Sciences) was also loaded. The electrophoresis apparatus was connected to an electric power supply. A current of 50 volts was applied to the gel until the dye front had moved away from the wells then the current was increased to 100 volts and the gel was run further until dye reached to the bottom of gel. The glass plates were detached from the apparatus and separated. The orientations of the gels were marked before the process of staining.

After electrophoresis, the gels were transferred to container filled with commasie blue staining solution (1% Commasie brilliant blue, 37.2% Acetic acid, 25% Methanol). Container was covered and subjected to gradual agitation for an hour at room temperature. For destaining, gels were placed in destaining solution containing 10% acetic acid with gentle shaking at 37°C. This process was repeated two to three times to de-stain the gels completely. The gels were photographed and/or stored in 10% glacial acetic acid.

SCORING AND SDS-PAGE DATA ANALYSIS

The bands of proteins were scored as 1 (present) and 0 (absent) and each band was considered and scored. The Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc, version 2) using Dice's coefficient was used to determine similarity index and construct dendrogram by Unweighted Pair Group Method with Arithmetic means (UPGMA).

RESULTS AND DISCUSSION

Beside white rice, rice of different colors such as purple, red and black rice are also grown in different countries [7]. Red rice grains have red color due to anthocyanin compounds and considered beneficial for health [18]. This study was conducted on red rice grown in Thatta district of Sindh province. Red rice (and other pigmented rice) have been cultivated and consumed in different countries for a long time. Several studies on pigmented rice (red, purple, black, brown), their metabolites and other volatile aroma compounds, including antioxidant compounds and anthocyanin pigments have been conducted [30-32].

Nutritional quality of red rice varieties [33], antidiabetic potential of red and purple rice bran extracts [34], oligomers and polymers of proanthocyanidins, total phenolics, flavonoids [35,36], changes in physical, cooking, textural [37], and other phytochemical profiles [38,39] have been reported from around the world.

Diseases like cancer, cardiovascular disorders, diabetes are on the rise. Keeping these issues in mind scientists are paying more attention to the quality traits of food such as mineral content, antioxidant properties, and glycemic index. Several experiments were conducted to analyze the antioxidant and nutrient content of coloured rice which showed that red rice has higher antioxidant property than white and black rice. Red rice contains polyphenols which is correlated with the antioxidant properties. In health-conscious consumers, red rice could gain importance due to its beneficial properties. In Japan, new cultivars of red rice have been introduced and being used as functional food [7, 40].

TOTAL PHENOLIC CONTENT (TPC)

The phenolic content of red rice samples from Thatta (both free and bound) was quantified by Folin-Ciocalteu reagent (Table 1). Figure 1 showed that concentration of bound phenolic was higher than free phenolic fractions in all the samples of red rice. The total phenolic content (TPC) in free form ranged between 352.5 mg GAE/kg to 1725 mg GAE/kg while bound TPC ranged from 2685 mg GAE/kg to 4630 mg GAE/kg. The free TPC in highest amount was found in sample “S” i.e. 1725 mg GAE/kg followed by sample “SCI” 1090 mg GAE/kg, “J” 540 mg GAE/kg and “M” 352.5 mg GAE/kg. The highest bound TPC fraction was found in sample “J” i.e. 4630 mg GAE/kg followed by sample “SCI” 3085 mg GAE/kg, “M” 2722.5 mg GAE/kg, “S” 2685 mg GAE/kg. Total TPC ranged from 3074.7 mg GAE/kg to 5170 mg GAE/kg, the highest TPC amount was found in sample encoded “J” i.e. 5170 mg GAE/kg. The percentage of free phenolic content was 22% while the percentage of bound phenolics was 78%.

Our results showed that red rice contained bound TPC in higher amount than in free form. On the contrary, higher amount of free phenolics compared to bound phenolics was reported in previous studies [41,22]. In black rice, higher amount of bound phenolics compared to free phenolics was observed [42,22]. It is also reported that content of bound phenolic was found to be higher compared to free phenolic in germinated brown and white rice [36]. It has been reported that 62% of total phenolics were bound phenolics in rice [43]. In brans of light brown rice of Bengal and Cocodrie, bound phenolic contributed40-50% to the total phenolics [41].

However, in the rice of same colour amount of TPC varied [32]. Black and red rice contain on average 24-41% of bound phenolic compounds and 59-76% of free phenolic compounds. Distribution of phenols in grains is not uniform at the cellular and subcellular levels. Free phenolics are found in the cell vacuoles while bound phenolic are present in cell walls of plants. Some of the phenolics might increase on exposure to UV radiation, wounding, air pollution, pathogen or parasite infection and extreme temperatures. Rice breeding, cultivation techniques, process of ripening, growing conditions (e.g. fertilization and altitude), effect the phenolics level in grains. Different climates are also known to influence phenolic composition and content [41,44].

Rice bran (especially rice brans of light colour) contains significant amounts of bound phenolics. According to few studies, bound phenolics fraction should not be ignored while studying antioxidants of cereal grains [43,45]. Gorinstein and colleagues [46] reported that bound phenolics were not included in total phenolic content and their antioxidant activities in vegetables and fruits were underestimated.

CHARACTERIZATION OF VOLATILE COMPOUNDS IN RED RICE GRAINS

In this study, grains of red rice were treated with methanol followed by GC-MS (non-targeted) analysis in order to identify major volatile metabolites in red rice grown in Thatta. An array of metabolites including sugars, sugar alcohols, fatty acid methyl esters and organic acids have been reported in coloured rice grains based on GC-MS [30,47].

The volatile components of red rice (saturated, unsaturated and non-fatty acid compounds) was separated by GC and identification of compounds was carried out by Mass Spectroscopy (MS). These red rice compounds were identified by comparing the mass spectra of peaks with those available in the NIST mass spectral library, (Wiley registry NIST 11) above 70% similarity index.

Table 1: Total phenolic content (TPC) of red rice and contributions of free and bound fractions to the total phenolic content. Results are presented as means having highly significant differences from each other (p=0.005).

SAMPLES	FREE TPC (mg GAE/kg)	BOUND TPC (mg GAE/kg)	TOTAL TPC (mg GAE/kg)
M	352.5±12.676	2722.5±4.193	3074.7±71.657
J	540±73.557	4630±11.060	5170±41.177
S	1725±23.075	2685±22.368	4410±70.750
SCI	1090±93.412	3085±24.419	4175±14.998

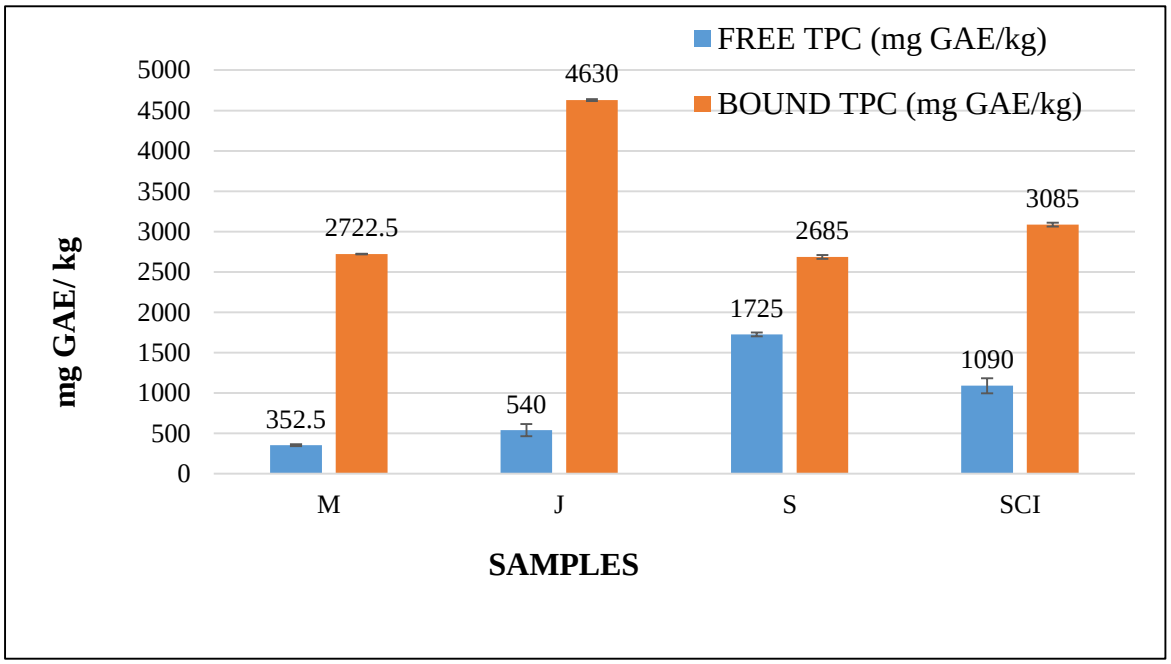

Figure 1: Comparison of free and bound phenolics in red rice varieties.

Metabolites identified in red rice are presented in Table 2. The metabolites identified belonged to amide and ester group of compounds. Collectively 18 compounds were identified out of which 5 were esters, and rest of the compounds belonged to amide group. The analysis showed that amides present in red rice were comparatively in higher amount than ester.

Nine compounds identified from sample encoded "J" (Figure 2) i.e. N-(Trifluoroacetyl)pyrrolidine (89.02%), Pentadecanoic acid, pyrrolidide (0.32%), Pyrrolidine, 1-(1-oxo-9-octadecenyl)- (0.31%), 9-Hexadecenoic acid, pyrrolidide (3.5%), 1-Pyrrolidinecarboxaldehyde (0.32%), 9,12-octadecanoic acid, pyrrolidide (2.17%), Hexadecanoic acid, pyrrolidide (2.13%), Hexadecanoic acid, methyl ester (0.45%), 2-Propanoic acid, 3-(4-methoxyphenyl)-, ethyl ester (0.04%).

Twelve compounds identified from sample encoded "M" (Figure 3) i.e. N-(Trifluoroacetyl)pyrrolidine (97.54%), 2-Piperidinone (0.11%), Pentadecanoic acid, pyrrolidide (0.07%), Pyrrolidine, 1-(1-oxo-9-octadecenyl)- (0.15%), 8,11-Octadecadienooic acid, pyrrolidide (0.51%), 9-Hexadecenoic acid, pyrrolidide (0.27%), 1-Pyrrolidinecarboxaldehyde (0.25%), Hexadecanoic acid, pyrrolidide (0.46%), Methyl-tetradecanoate (0.06%), Hexadecanoic acid, methyl ester (0.26%), 9,12-Octadecadienoic acid, methyl ester

(0.27%), 9- Octadecenoic acid (Z)-, methyl ester (0.08%).

Twelve compounds identified from sample encoded "SCI" (Figure 4) i.e. N-(Trifluoroacetyl)pyrrolidine (89.02%), Decanoic acid, pyrrolidide (2.13%), Pyrrolidine, 1-(1-oxo-9-octadecenyl)-(0.5%), 9-Hexadecenoic acid, pyrrolidide (0.35%), 1-Pyrrolidinecarboxaldehyde (0.32%), Dodecanoic acid, pyrrolidide (2.13%), 9,12-octadecanoic acid, pyrrolidide (2.17%), Hexadecanoic acid, pyrrolidide (2.13%), Hexadecanoic acid, methyl ester (0.45%), 9,12-Octadecadienoic acid, methyl ester (0.88%), 9- Octadecenoic acid (Z)-, methyl ester (0.88%), 2-Propanoic acid, 3-(4-methoxyphenyl)-, ethyl ester (0.28%).

Nine compounds were detected from sample encoded "S" (Figure 5) i.e. N-(Trifluoroacetyl)pyrrolidine (95.87%), Decanoic acid, pyrrolidide (0.68%), Pyrrolidine, 1-(1-oxo-9-octadecenyl)-(0.28%), Pyrrolidine, 1-(12-methyl-1-oxo-tetradecyl) (0.78%), Pyrrolidine, 1-(1-oxo-10-octadecynyl)(0.86%), 1-Pyrrolidinecarboxaldehyde (0.25%), Hexadecanoic acid, methyl ester (0.49%), 9, 12-Octadecadienoic acid, methyl ester (0.65%), 9- Octadecenoic acid (Z)-, methyl ester (0.15%).

The present GC-MS data showed that N-(Trifluoroacetyl)pyrrolidine acid has the highest peak area (≥ 90%) and has highest peak height in all the samples analysed (red rice) as compared to the other compounds (Figure 6).

Fatty acids arealiphatic carboxylic acid with different lengths of hydrocarbon chains at one end and terminal carboxyl (-COOH) group at the other end. Fatty acids mostly found to have carbon atoms between 12 and 22, form lipids in microorganisms, plants and animals in a reaction with glycerol and are unbranched [48].

Palmitic acid (Hexadecanoic acid), 9 octadecanoic acid (Oleic acid), 9,12 octadecanoic acid (Linoleic acid) have been reported in rice [49]; methyl tetradecanoate, 9 octadecanoic methyl ester, 9,12 octadecanoic acid methyl ester, hexadecanoic acid methyl ester, dodecanoic acid, pentadecanoic acid, 9 hexadecanoic acid, octadecanoic acid were reported in various cultivars of oats [50]. Long-chain fatty acids including dodecanoic, decanoic, octanoic and tetradecanoic acids slightly affect distillate taste. Fatty acids that contribute to aroma, primarily esters, dodecanoic, decanoic, octanoic are most important esters found in pomace brandies [51].

Decanoic, hexanoic and octanoic fatty acids usually produce unpleasant odors of greasy oil, lard or spoiled cheese, rancid fat [52,53]. Hexadecanoic acid abundantly found in the essential oils of *Cynomorium songaricum.* (Z)-9-octadecenoic acid was accumulated in the oils of *C.songaricum* [29,54]. Trabelsi and his colleague [55] studied phytochemical profile of *Pistacia lentiscus* and found that the major fatty acids were 9- Octadecenoic acid (oleic acid) palmitic acid (hexadecanoic acid) and linoleic acid (9,12 Octadecadienoic Acid). Hexadecanoic acid has reported as the major compound in essential oils of harmal and other saturated fatty acids such as tetradecanoic acid, pentadecanoic acid, hexadecanoic acid and octadecanoic acid was also found in harmal essential oils. Unsaturated fatty acids called (Z)-9-hexadecenoic acid, (Z, Z) - 9, 12-octadecadienoic acid were also reported in harmal oils [56].

Linoleic acid trimethylsilyl ester and Oleic acid, trimethylsilyl ester were observed in red rice. It is reported that Linoleic acid trimethylsilyl ester and Oleic acid trimethylsilyl ester are present in Acacia Sieberiana stem bark extract and possesss antiinflammatory, antimenorrhagic, antiprostatitic, cancer-preventive, hepatoprotective properties [27].

Amines are poly-functional and can give a pleasant aroma like floral, sweet or fruity to rice. Amines are very reactive substances and widely used as additives or monomers in the manufacture of food material or materials in contact with food. It is well

known that amines are not stable in something of fatty nature. They usually react with the components to form amides [58].

Pyrrolidine is a flavoring agent present in food stuff in trace amount. It's found to be present in milk, cheese, bread, stalks, coffee, beer and fatty fish as well as carrot and tobacco. It is found in many pharmaceutical drugs such as bepridil and procyclidine . It also forms the basis for the aniracetam, piracetam (racetam compounds).Pyrrolidine possess radical scavenging, anti-tumor, anti-inflammatory, hepatoprotective and metal chelator activities [59,60,61,62,63,64].

Table 2: List of metabolites detected in red rice by GC-MS (ND= not detected**).**

COMPOUNDS	PERCENTAGE (%)			
AMIDES	**J**	**M**	**SCI**	**S**
N-(Trifluoroacetyl)pyrrolidine	89.02	97.54	89.02	95.87
2-Piperidinone	ND	0.11	ND	ND
Pentadecanoic acid, pyrrolidide	0.32	0.07	ND	ND
Decanoic acid, pyrrolidide	ND	ND	2.13	0.68
Pyrrolidine, 1-(1-oxo-9-octadecenyl)-	0.31	0.15	0.5	0.28
Pyrrolidine, 1-(12-methyl-1-oxo-tetradecyl)-	ND	ND	ND	0.78
Pyrrolidine, 1-(1-oxo-10-octadecynyl)	ND	ND	ND	0.86
8,11-Octadecadienooic acid, pyrrolidide	ND	0.51	ND	ND
9-Hexadecenoic acid, pyrrolidide	3.5	0.27	3.5	ND
1-Pyrrolidinecarboxaldehyde	0.32	0.25	0.32	0.25
Dodecanoic acid, pyrrolidide	ND	ND	2.13	ND
9,12-octadecanoic acid, pyrrolidide	2.17	ND	2.17	ND
Hexadecanoic acid, pyrrolidide	2.13	0.46	2.13	ND
ESTERS				
Methyl tetradecanoate	ND	0.06	ND	ND
Hexadecanoic acid, methyl ester	0.45	0.26	0.45	0.49
9,12-Octadecadienoic acid, methyl ester	ND	0.27	0.88	0.65
9- Octadecenoic acid (Z)-, methyl ester	ND	0.08	0.88	0.15
2-Propanoic acid, 3-(4-methoxyphenyl)-, ethyl ester	0.04	ND	0.28	ND

Figure 2: GC-MS Chromatogram of methanol extract of seeds of red rice ("J").

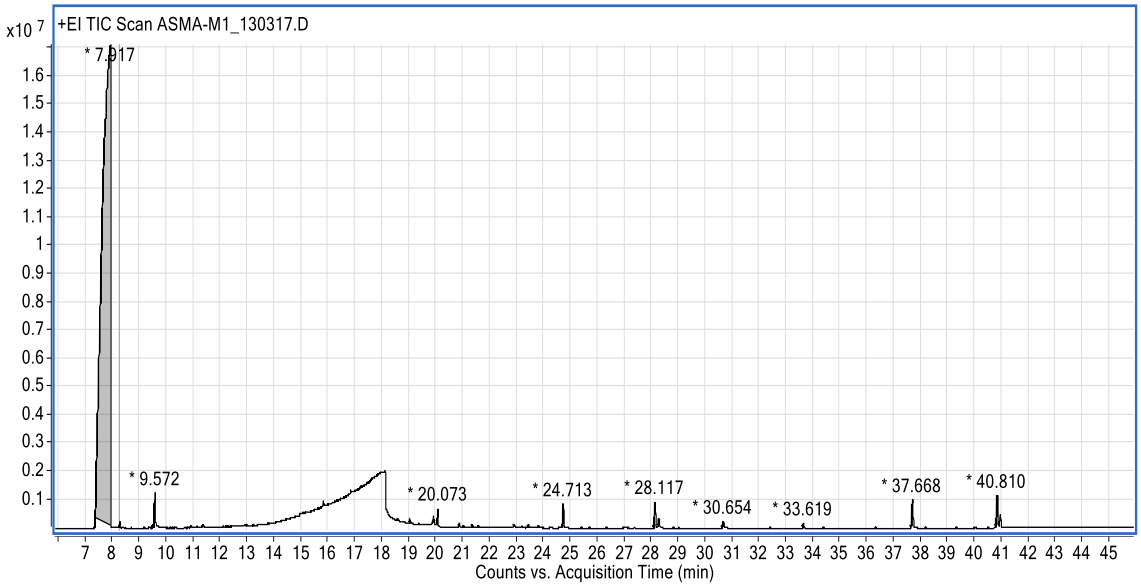

Figure 3: GC-MS Chromatogram of methanol extract of seeds of red rice ("M").

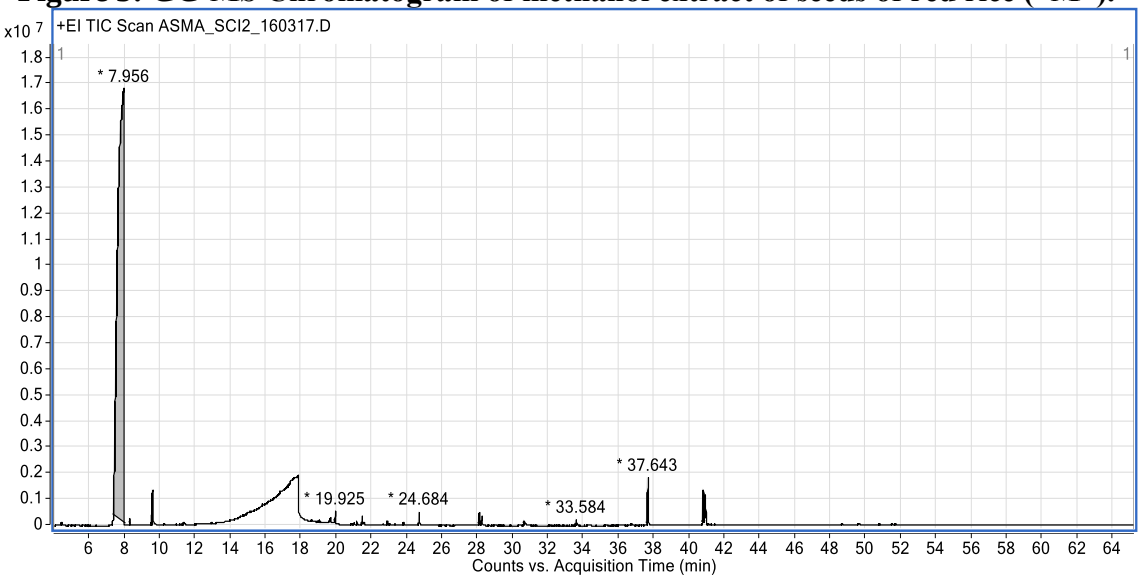

Figure 4: GC-MS Chromatogram of methanol extract of seeds of red rice ("SCI").

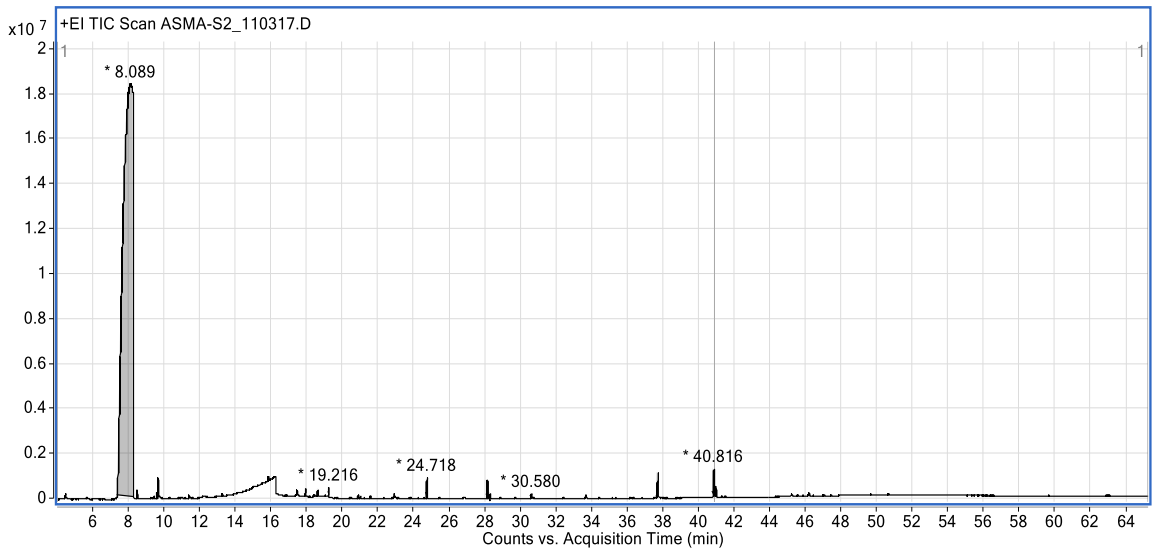

Figure 5: GC-MS Chromatogram of methanol extract of seeds of red rice ("S").

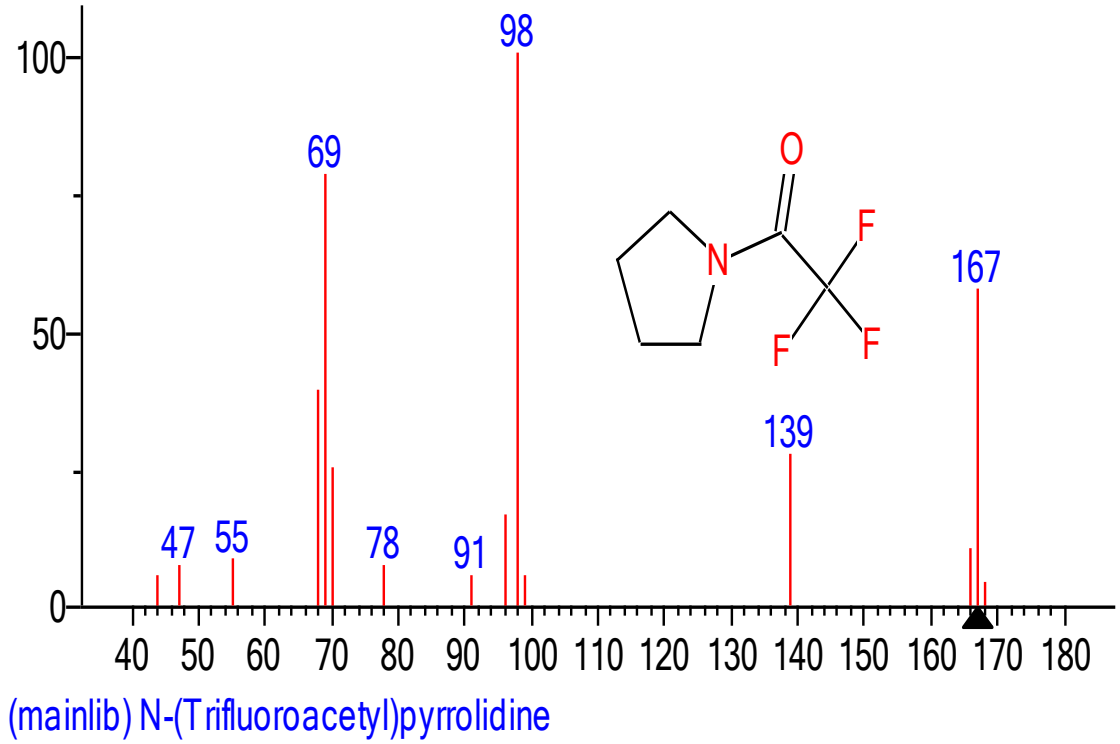

Figure 6: Mass spectrum of N-(Triflouroacetyl)pyrrolidine.

PROTEIN PROFILE OF RED RICE GRAINS

Protein profile of 23 red rice varieties was carried out using SDS-PAGE. Rice grains were processed for protein electrophoresis using denaturing conditions (SDS-PAGE).

Banding pattern obtained from SDS-PAGE of red rice showed that all the cultivars contained similar pattern of protein bands. However, few polymorphic bands could be used to distinguish the cultivars as shown in Figures 7-10. According to mobility of proteins on gel, banding pattern divided into four regions "A", "B", "C", "D". Region "A" contained 6 bands more or less deeply stained with one major band present at 7th position with molecular weight ranging from 130-55 kDa. This region was common in all the cultivars analyzed. Region "B" contained proteins of molecular weight 43-34 kDa. Proteins of Molecular weight (MW) of the bands ranged from 148 kDa to 12 kDa with three major bands of MW 59 kDa, 33 kDa, 17 kDa was determined using a standard curve of mobility of marker proteins. region "C" were between 33-12 kDa and region "D" had proteins of molecular weight less than 12 kDa. Regions "B", "C" and "D" were contained polymorphic regions among all the cultivars. These regions consisted of several thin bands with one major band at the bottom "B" and "C" region. One prominent band can be seen in MC25 and MC2 in region "B". One prominent band is present in "C" region of MC3, MC21, MC22 and MC27. Region "D" contained 3 low molecular weight protein bands. This region contained some very prominent proteins with clearly stained bands as seen in MC61, MC3, and MC27. Three prominent bands present in MC61, MC3, 2 bands in MC27, 2 thin bands in MC165 while 1 prominent band at the bottom of MC29 and MC4, MC87, AJ and RR.

Table 3: Rice samples analyzed using SDS-PAGE.

No.	CODES	No.	CODES
01	MC2	13	MC61
02	MC3	14	MC65
03	MC4	15	MC86
04	MC22	16	MC87
05	MC25	17	MC106
06	MC27	18	MC110
07	MC29	19	MC116
08	MC30	20	MC117
09	MC31	21	MC165
10	MC49	22	RED RICE (RR)
11	MC55	23	AJ
12	MC56	18	MC110

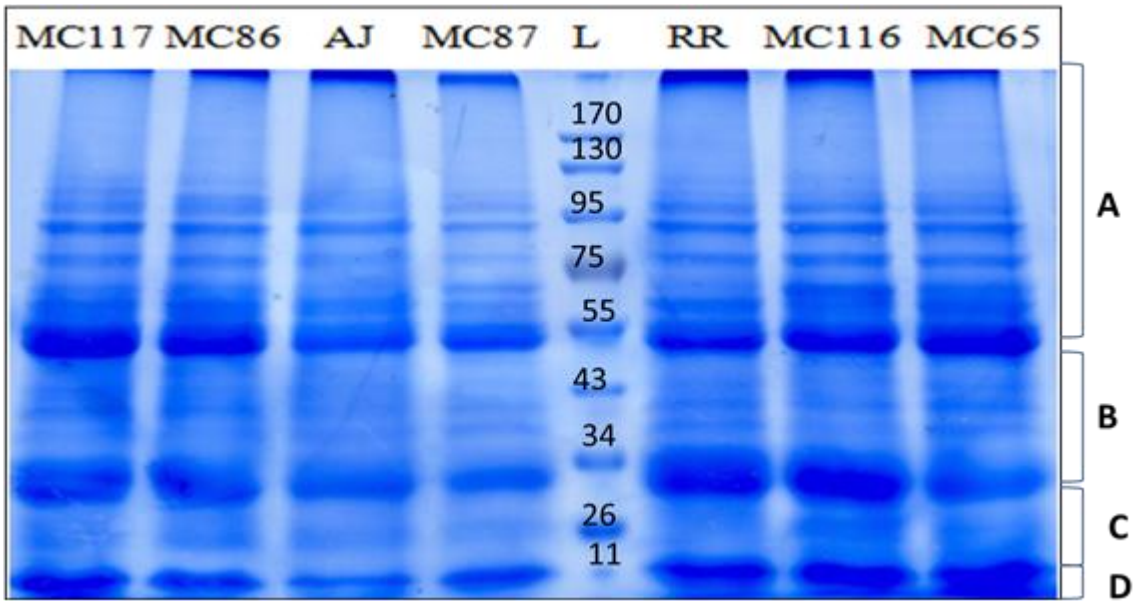

Figure 7: Electrophoretic bands produced by SDS-PAGE of seed storage proteins of red rice cultivars where L = Protein ladder.

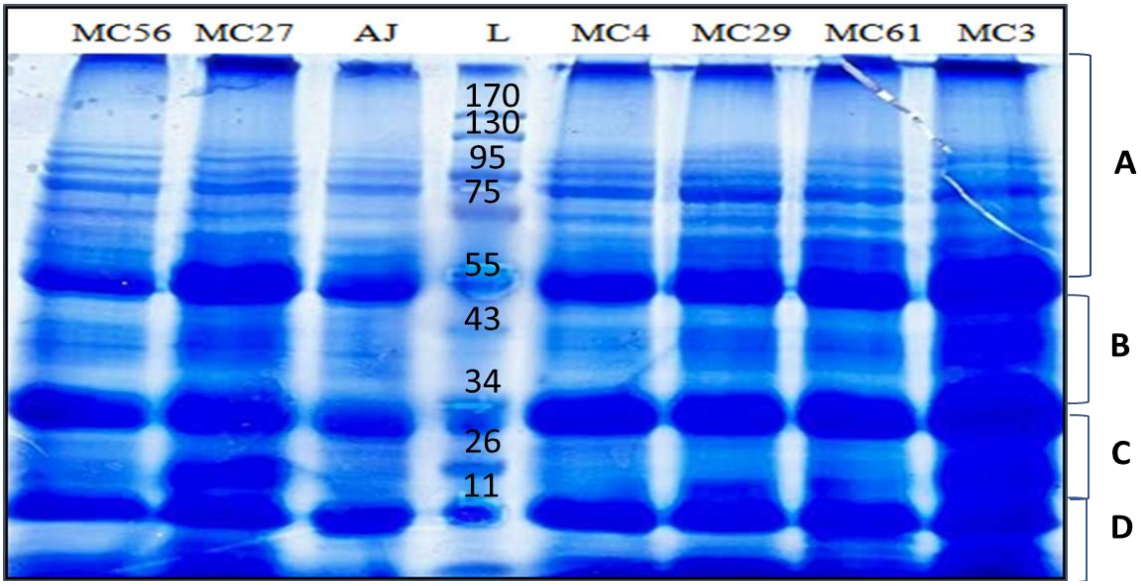

Figure 8: Electrophoretic bands produced by SDS-PAGE of seed storage proteins of red rice cultivars where L = Protein ladder.

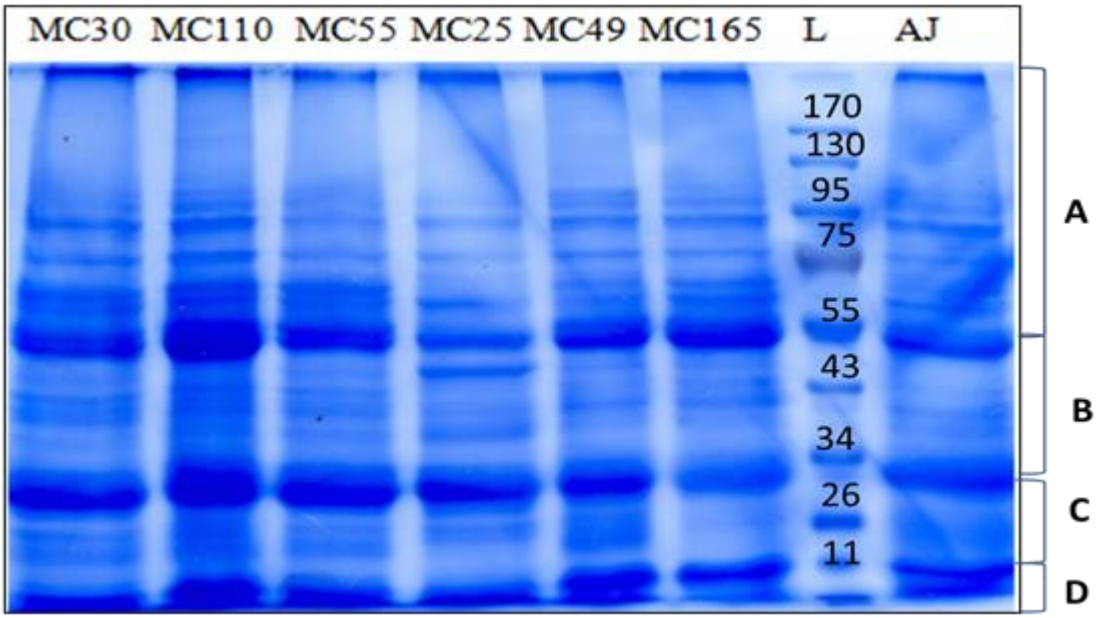

Figure 9: Electrophoretic bands produced by SDS-PAGE of seed storage proteins of red rice cultivars where L = Protein ladder.

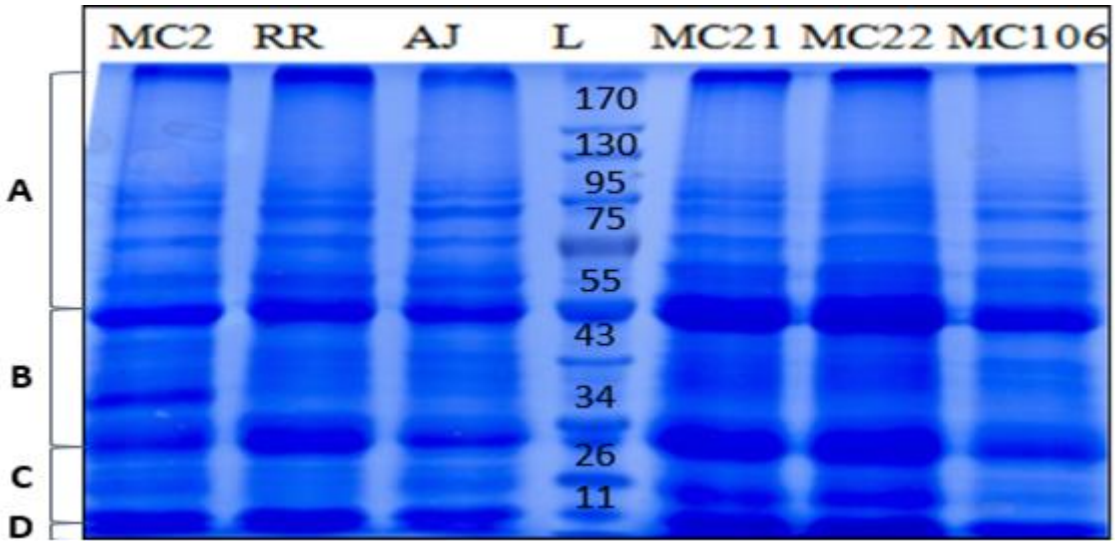

Figure 10: Electrophoretic bands produced by SDS-PAGE of seed storage proteins of red rice cultivars where L = Protein ladder.

SIMILARITY INDEX

Similarity matrices of red rice cultivars was generated using Dice's coefficient is presented in Table 4. The similarity values of protein ranged from 0.774 to 1.000. The highest similarity i.e. 1.000 was observed between MC56 and MC27, MC29 and MC3, MC21 and MC165, MC30 and MC110, AJ and MC22, MC25 and MC49. Lowest similarity was observed between RR and MC106 and MC4. The rest of cultivars showed similarity in the range of 0.812 to 0.972.

CLUSTER ANALYSIS

The dendrogram constructed using similarity values to calculate the correlation among the red rice cultivars is presented in Figure 11. The cluster analysis divided red rice cultivars in two main clusters at 90% similarity. The cluster 1 showed similarity of 85% consisted of only one cultivar i.e. MC106. The cluster 2 showed similarity of 90% and contained rest of the cultivars. Cluster 2 is further divided into two sub-clusters (I, II). The sub-cluster I is smallest with only two cultivars i.e. MC87 and MC65. Sub-cluster II was largest and contained rest of the cultivars. MC25 and MC49, AJ and MC22, MC30 and MC110, MC21 and MC165, MC56 and MC27, MC29 and MC3 showed 100% similarity.

Protein markers of seeds are highly polymorphic and can be effectively used as tool for the identification of different varieties in plants and to study genetic variability among them. Seed proteins are largely independent of environmental influence [65]. Genotypes of specific plant species can easily be distinguished by protein profile of seeds because seed proteins are highly stable [66]. SDS-PAGE is easiest way for the assessment of genetic diversity. Protein profiling of seeds can be used for different purposes. For example, for cultivars and species identification, germplasm characterization, biosystematics analysis and to determine phylogenetic relationship among different species [67]. Evolutionary and taxonomic problems of various crop plants can be successfully resolved by electrophoretic patterns of seed proteins [68,69]. Cluster analysis based on SDS-PAGE have been used as a powerful tool for differentiating genotypes of *Vigna mungo* and *Vigna radiata* [70]. On the basis of genetic dissimilarities in seed proteins, SDS-PAGE analysis provides strong means for discrimination of genotypes [71]. Nonetheless, few of the studies [72,73] pointed out that SDS-PAGE was not effective in identification of cultivars.

Extensive work has been done on rice seed protein profiling based on SDS-PAGE [74,75]. Our results of 23 rice cultivars (including red rice from Thatta) showed very narrow level of inter-cultivar polymorphism as shown by similar band pattern. We could not find variations in major bands among the cultivars tested, however variation in minor bands was present in most of the cultivars. Uniformity in the major protein bands clearly states that gene coding these proteins are highly conserved [76]. Few studies are in support of our finding as they reported a medium to low level of inter-cultivar variation among seed protein of rice genotypes [73,77]. Among eleven genotypes of rice 0-80% of polymorphism was reported [78]. In wheat, 84.0% polymorphism among 10 genotypes was observed [76]. Significant polymorphism in Kabuli Chickpea genotypes has also been reported [68], while 0-100% polymorphism was observed in different genotypes of Capsicum [80].

In the present analysis, samples showed low level of diversity, hence two-dimensional (2D) electrophoresis can be used to further characterize the proteins in red rice grains.

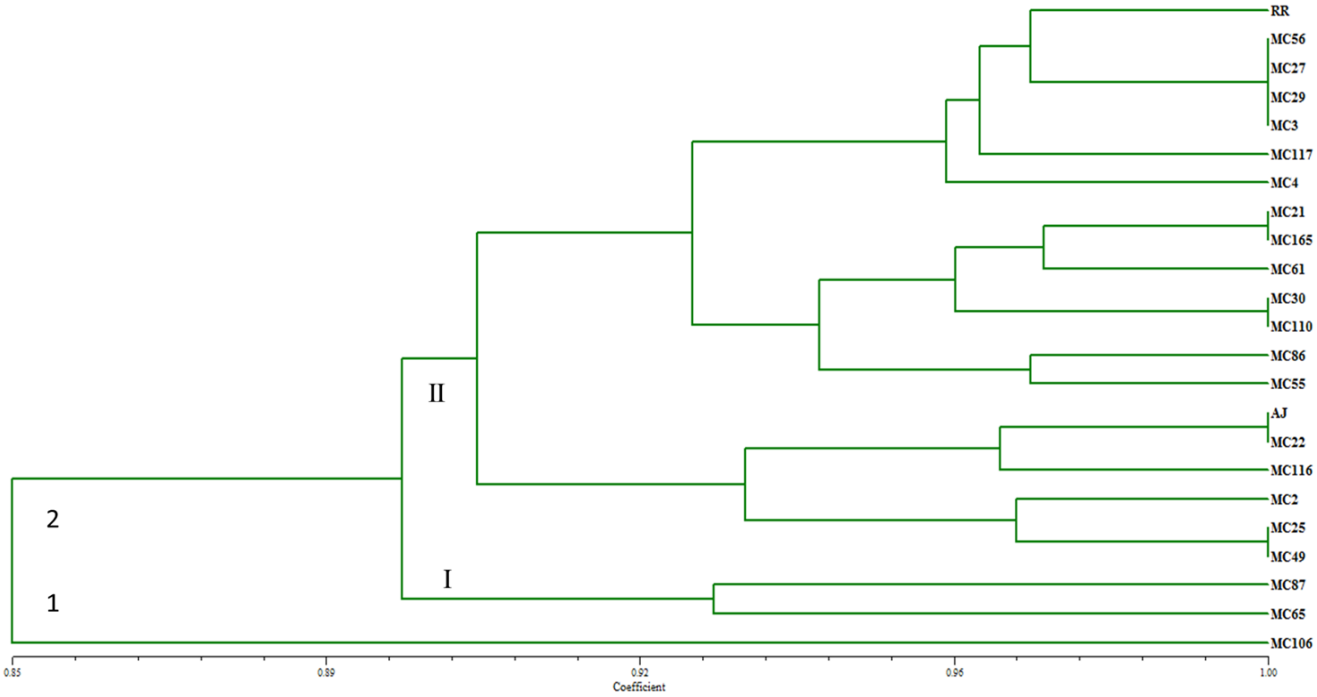

Figure11: Dendrogram of red rice cultivars based on data generated by SDS-PAGE.

REFERENCES

1- Kende, H., Van Der Knaap, E., & Cho, H. T. (1998). Deepwater rice: a model plant to study stem elongation. Plant Physiology, 118(4), 1105-1110.

2- Molina, J., Sikora, M., Garud, N., Flowers, J. M., Rubinstein, S., Reynolds, A., Huang, P., Jackson, S., Schaal, B. A., Bustamante, C. D., Boyko, A. R., & Purugganan, M. D. (2011). Molecular evidence for a single evolutionary origin of domesticated rice. Proceedings of the National Academy of Sciences, USA, 108(20), 8351-8356.

3- Huang, X., Kurata, N., Wei, X., Wang, Z. X., Wang, A., Zhao, Q., Zhao, Y., Liu, K., Lu, H., Li, W., Guo, Y., Lu, Y., Zhou, C., Fan, D., Weng, Q., Zhu, C., Huang, T., Zhang, L., Wang, Y., Feng, L., Furuumi, H., Kubo, T., Miyabayashi, T., Yuan, X., Xu, Q., Dong, G., Zhan, Q., Li, C., Fujiyama, A., Toyoda, A., Lu, T., Feng, Q., Qian, Q., Li, J., & Han, B. (2012). A map of rice genome variation reveals the origin of cultivated rice. Nature, 490(7421), 497-501.

4- Khush, G. S. (1997). Origin, dispersal, cultivation and variation of rice. Plant Molecular Biology, 35(1-2), 25-34.

5- Bashir, K., Khan, N. M., Rasheed, S., & Salim, M. (2007). Indica rice varietal development in Pakistan: an overview. Paddy and Water Environment, 5(2), 73-81.

6- Muench, D. G., Wu, Y., Coughlan, S. J., & Okita, T. W. (1998). Evidence for a cytoskeleton-associated binding site involved in prolamine mRNA localization to the protein bodies in rice endosperm tissue. Plant Physiology, 116(2), 559-569.
7- Ahuja, U., Ahuja, S. C., Chaudhary, N., & Thakrar, R. (2007). Red rices–past, present and future. Asian Agri-History, 11(4), 291-304.
8- Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., Furuta, S., Suda, I., & Sato, T. (2002). Polymeric procyanidins as radical-scavenging components in red-hulled rice. Journal of Agricultural and Food Chemistry, 50(26), 7524-7529.
9- Nawa, Y., & Ohtani, T. (1992). Property of pigments in rice hulls of various colors. Journal of Food Industry, 11, 28-33.
10- Osawa, T. (1999). Protective role of rice polyphenols in oxidative stress. Anticancer Research, 19(5A), 3645-3650.
11- Hu, C., Zawistowski, J., Ling, W., & Kitts, D. D. (2003). Black rice (Oryza sativa L. indica) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. Journal of Agricultural and Food Chemistry, 51(18), 5271-5277.
12- Ichikawa, H., Ichiyanagi, T., Xu, B., Yoshii, Y., Nakajima, M., & Konishi, T. (2001). Antioxidant activity of anthocyanin extract from purple black rice. Journal of Medicinal Food, 4(4), 211-218.
13- Minatel, I. O., Han, S. I., Aldini, G., Colzani, M., Matthan, N. R., Correa, C. R., Fecchio, D., & Yeum, K. J. (2014).
14- Moko, E. M., Purnomo, H., Kusnadi, J., & Ijong, F. G. (2014). Phytochemical content and antioxidant properties of coloured and non-coloured varieties of rice bran from Minahasa, North Sulawesi, Indonesia. International Food Research Journal, 21(3), 1017.
15- Deng, G. F., Xu, X. R., Zhang, Y., Li, D., Gan, R. Y., & Li, H. B. (2013). Phenolic compounds and bioactivities of pigmented rice. Critical Reviews in Food Science and Nutrition, 53(3), 296-306.
16- Muntana, N., & Prasong, S. (2010). Study on total phenolic contents and their antioxidant activities of Thai white, red and black rice bran extracts. Pakistan Journal of Biological Sciences, 13(4), 170.
17- Yodmanee, S., Karrila, T. T., & Pakdeechanuan, P. (2011). Physical, chemical and antioxidant properties of pigmented rice grown in Southern Thailand. International Food Research Journal, 18(3).
18- Ryu, S. N., Park, S. Z., & Ho, C. T. (1998). High performance liquid chromatographic determination of anthocyanin pigments in some varieties of black rice. Journal of Food and Drug Analysis, 6(4), 729-736.
19- Zhang, M. W., Guo, B. J., Zhang, R. F., Chi, J. W., Wei, Z. C., Xu, Z. H., Zhang, Y., & Tang, X. J. (2006). Separation, purification and identification of antioxidant

compositions in black rice. Agricultural Sciences in China, 5(6), 431-440.
20- Kim, M. K., Kim, H. A., Koh, K., Kim, H. S., Lee, Y. S., & Kim, Y. H. (2008). Identification and quantification of anthocyanin pigments in coloured rice. Nutrition Research and Practice, 2(1), 46-49.
21- Yawadio, R., Tanimori, S., & Morita, N. (2007). Identification of phenolic compounds isolated from pigmented rices and their aldose reductase inhibitory activities. Food Chemistry, 101(4), 1616-1625.
22- Shao, Y., Xu, F., Sun, X., Bao, J., & Beta, T. (2014). Identification and quantification of phenolic acids and anthocyanins as antioxidants in bran, embryo and endosperm of white, red and black rice kernels (Oryza sativa L.). Journal of Cereal Science, 59(2), 211-218.
23- Rood, M. A. (2000). Red menace. Rice Journal, 103, 18-20.
24- Chaudhary, R. C. (2003). Speciality rices of the world: effect of WTO and IPR on its production trend and marketing. Journal of Food, Agriculture and Environment, 1(2), 34-41.
25- Krishna, M., & Sharma, A. C. (1970). Manipur-rich in rice germplasm. Oryza, 7(1), 45-50.
26- Rani, S., & Krishnaiah, K. (2001). Current status and future prospects of improving traditional aromatic rices. Specialty Rices of the World: Breeding, Production, and Marketing. New Delhi: Oxford IBH Publishers.
27- Kotásková, E., Sumczynski, D., Mlček, J., & Valášek, P. (2016). Determination of free and bound phenolics using HPLC-DAD, antioxidant activity and in vitro digestibility of Eragrostis tef. Journal of Food Composition and Analysis, 46, 15-21.
28- Sumczynski, D., Bubelova, Z., Sneyd, J., Erb-Weber, S., & Mlcek, J. (2015). Total phenolics, flavonoids, antioxidant activity, crude fibre and digestibility in non-traditional wheat flakes and muesli. Food Chemistry, 174, 319-325.
29- Zhou, J., Ma, C., Xu, H., Yuan, K., Lu, X., Zhu, Z., Wu, Y., & Xu, G. (2009). Metabolic profiling of transgenic rice with cryIAc and sck genes: An evaluation of unintended effects at metabolic level by using GC-FID and GC–MS. Journal of Chromatography B, 877(8-9), 725-732.
30- Frank, T., Reichardt, B., Shu, Q., & Engel, K. H. (2012). Metabolite profiling of coloured rice (Oryza sativa L.) grains. Journal of Cereal Science, 55(2), 112-119.
31- Sukhonthara, S., Theerakulkait, C., & Miyazawa, M. (2009). Characterization of volatile aroma compounds from red and black rice bran. Journal of Oleo Science, 58(3), 155-161.
32- Chen, X. Q., Nagao, N., Itani, T., & Irifune, K. (2012). Anti-oxidative analysis, and identification and quantification of anthocyanin pigments in different coloured rice. Food Chemistry, 135(4), 2783-2788.

33- Gunaratne, A., Wu, K., Li, D., Bentota, A., Corke, H., & Cai, Y. Z. (2013). Antioxidant activity and nutritional quality of traditional red-grained rice varieties containing proanthocyanidins. Food Chemistry, 138(2), 1153-1161.
34- Boue, S. M., Daigle, K. W., Chen, M. H., Cao, H., & Heiman, M. L. (2016). Antidiabetic potential of purple and red rice (Oryza sativa L.) bran extracts. Journal of Agricultural and Food Chemistry, 64(26), 5345-5353.
35- Chen, M. H., McClung, A. M., & Bergman, C. J. (2016). Concentrations of oligomers and polymers of proanthocyanidins in red and purple rice bran and their relationships to total phenolics, flavonoids, antioxidant capacity and whole grain colour. Food Chemistry, 208, 279-287.
36- Tian, S., Nakamura, K., & Kayahara, H. (2004). Analysis of phenolic compounds in white rice, brown rice, and germinated brown rice. Journal of Agricultural and Food Chemistry, 52(15), 4808-4813.
37- Pandey, S., Asha, M. R., & Jayadep, A. (2016). Changes in physical, cooking, textural properties and crystallinity upon iron fortification of red rice (Jyothi). Journal of Food Science and Technology, 53(2), 1014-1024.
38- Kim, G. R., Jung, E. S., Lee, S., Lim, S. H., Ha, S. H., & Lee, C. H. (2014). Combined mass spectrometry-based metabolite profiling of different pigmented rice (Oryza sativa L.) seeds and correlation with antioxidant activities. Molecules, 19(10), 15673-15686.
39- Pereira-Caro, G., Cros, G., Yokota, T., & Crozier, A. (2013). Phytochemical profiles of black, red, brown, and white rice from the Camargue region of France. Journal of Agricultural and Food Chemistry, 61(33), 7976-7986.
40- Itani, T., & Ogawa, M. (2004). History and recent trends of red rice in Japan. Japanese Journal of Crop Science, 73(2), 137-147.
41- Min, B., Gu, L., McClung, A. M., Bergman, C. J., & Chen, M. H. (2012). Free and bound total phenolic concentrations, antioxidant capacities, and profiles of proanthocyanidins and anthocyanins in whole grain rice (Oryza sativa L.) of different bran colours. Food Chemistry, 133(3), 715-722.
42- Kong, S., & Lee, J. (2010). Antioxidants in milling fractions of black rice cultivars. Food Chemistry, 120(1), 278-281.
43- Adom, K. K., & Liu, R. H. (2002). Antioxidant activity of grains. *Journal of Agricultural and Food Chemistry*, *50*(21), 6182-6187.
44- Naczk, M., & Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. Journal of Pharmaceutical and Biomedical Analysis, 41(5), 1523-1542.
45- Pérez-Jiménez, J., & Saura-Calixto, F. (2005). Literature data may underestimate the actual antioxidant capacity of cereals. Journal of Agricultural and Food Chemistry, 53(12), 5036-5040.

46- Gorinstein, S., Cvikrova, M., Machackova, I., Haruenkit, R., Park, Y. S., Jung, S. T., Yamamoto, K., Ayala, A. L. M., Katrich, E., & Trakhtenberg, S. (2004).
47- Shu, X. L., Frank, T., Shu, Q. Y., & Engel, K. H. (2008). Metabolite profiling of germinating rice seeds. Journal of Agricultural and Food Chemistry, 56(24), 11612- 11620.
48- Chow, C. K. (2007). Fatty acids in foods and their health implications. United States: CRC press
49- Yasumatsu, K., & Moritaka, S. (1964). Fatty acid compositions of rice lipid and their changes during storage. Agricultural and Biological Chemistry, 28(5), 257-264.
50- Ačanski, M. M., Pastor, K. A., & Vujić, D. N. (2016). Authenticity of Lipid and Soluble Sugar Profiles of Various Oat Cultivars (Avena sativa). *International Journal of Nutrition and Food Engineering*, *10*(3), 151-156.
51- Silva, M. L., Malcata, F. X., & Revel, G. D. (1996). Volatile contents of grape marcs in Portugal. Journal of Food Composition and Analysis, 9(1), 72-80.
52- Ferreira, V., Ortín, N., Escudero, A., López, R., & Cacho, J. (2002). Chemical characterization of the aroma of Grenache rose wines: Aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. Journal of Agricultural and Food Chemistry, 50(14), 4048-4054.
53- Rogerson, F. S. S., & De Freitas, V. A. P. (2002). Fortification spirit, a contributor to the aroma complexity of Port. Journal of Food Science, 67(4), 1564-1569.
54- Zhou, Y. B., Ye, R. R., Lu, X. F., Lin, P. C., Yang, S. B., Yue, P. P., Zhang, C. X., & Peng, M. (2009). GC–MS analysis of liposoluble constituents from the stems of Cynomorium songaricum. Journal of Pharmaceutical and Biomedical Analysis, 49(4), 1097-110.
55- Trabelsi, H., Cherif, O. A., Sakouhi, F., Villeneuve, P., Renaud, J., Barouh, N., Boukhchina, S., & Mayer, P. (2012). Total lipid content, fatty acids and 4-desmethylsterols accumulation in developing fruit of Pistacia lentiscus L. growing wild in Tunisia. Food Chemistry, 131(2), 434-440.
56- Moussa, T. A., & Almaghrabi, O. A. (2016). Fatty acid constituents of Peganum harmala plant using Gas Chromatography–Mass Spectroscopy. Saudi Journal of Biological Sciences, 23(3), 397-403.
57- Elmezian, H. (2016). Determination of Amoxicillin in Co-Amoxiclav and Urine Samples by Differential Pulse Adsorptive Stripping Voltammetry. Analytical Chemistry, 16(13).
58- Paseiro-Cerrato, R., Rodríguez-Bernaldo de Quirós, A., Sendón, R., Bustos, J., Sánchez,J. J., López-Hernández, J., & Paseiro-Losada, P. (2015). Instability of

an aromatic amine in fatty food and fatty food simulant: characterisation of reaction products and prediction of their toxicity. Food Additives & Contaminants: Part A, 32(1), 100-109.
59- Fleming, J., Ghose, A., & Harrison, P. R. (2001). Molecular mechanisms of cancer prevention by selenium compounds. Nutrition and Cancer, 40(1), 42-49.
60- Hong, T. M., Teng, L. J., Shun, C. T., Peng, M. C., & Tsai, J. C. (2009). Induced interleukin-8 expression in gliomas by tumor-associated macrophages. Journal of Neuro-Oncology, 93(3), 289-301.
61- Lee, D. Y., & Fiehn, O. (2008a). High quality metabolomic data for Chlamydomonas reinhardtii. Plant Methods, 4(1), 7.
62- Lee, C. H., Kim, S. H., & Lee, S. M. (2008b). Effect of pyrrolidine dithiocarbamate on hepatic vascular stress gene expression during ischemia and reperfusion. European Journal of Pharmacology, 595(1), 100-107.
63- Li, X., Li, Y., & Xu, W. (2006). Design, synthesis, and evaluation of novel galloyl pyrrolidine derivatives as potential anti-tumor agents. Bioorganic & Medicinal Chemistry, 14(5), 1287-1293.
64- Natarajan, M., Gibbons, C. F., Mohan, S., Moore, S., & Kadhim, M. A. (2007). Oxidative stress signalling: a potential mediator of tumour necrosis factor α-induced genomic instability in primary vascular endothelial cells. The British Journal of Radiology, 80(special_issue_1), 13-22.
65- Gepts, P., & Bliss, F. A. (1986). Phaseolin variability among wild and cultivated common beans (Phaseolus vulgaris) from Colombia. Economic Botany, 40(4), 469-478.
66- Chittora, M., & Purohit, S. D. (2012). Optimization of SDS-PAGE conditions and analysis of seed protein diversity in Abrus precatorius genotypes with different seed coat colour. International Journal of Life Science Biotechnology and Pharma Research, 1(2), 268-277.
67- Sammour, R. H. (1991). Using electrophoretic techniques in varietal identification, biosystematic analysis, phylogenetic relations and genetic resources management. Journal of Islamic Academy of Sciences, 4(3), 221-226.
68- Hameed, A., Shah, T. M., Atta, B. M., Iqbal, N., Haq, M. A., & Ali, H. (2009). Comparative seed storage protein profiling of Kabuli chickpea genotypes. Pakistan Journal of Botany, 41(2), 703-710.
69- Ladizinsky, G., & Hymowitz, T. (1979). Seed protein electrophoresis in taxonomic and evolutionary studies. Theoretical and Applied Genetics, 54(4), 145-151.
70- Ghafoor, A., Ahmad, Z., Qureshi, A. S., & Bashir, M. (2002). Genetic relationship in Vigna mungo (L.) Hepper and V. radiata (L.) R. Wilczek based on morphological traits and SDS-

PAGE. Euphytica, 123(3), 367-378.

71- Khan, S. A., Shinwari, Z. K., & Rabbani, M. A. (2013). Study of total seed protein pattern of rice (Oryza sativa L.) breeding lines of Pakistan through SDS- PAGE. Pakistan Journal of Botany, 45(3), 871-876.

72- Ahmad, F., & Slinkard, A. E. (1992). Genetic relationships in the genus *Cicer* L. as revealed by polyacrylamide gel electrophoresis of seed storage proteins. *Theoretical and Applied Genetics*, *84*(5-6), 688-692.

73- De Vries, I. M. (1996). Characterization and identification of Lactuca sativa cultivars and wild relatives with SDS-electrophoresis (Lactuca sect. Lactuca, Compositae). Genetic Resources and Crop Evolution, 43(3), 193-202

74- Thanh, C. V., & Hirata, Y. (2002). Seed storage protein diversity of three rice species in the Mekong Delta. Biosphere Conservation, 4(2), 59-67.

75- Yousaf, Z., Masood, S., Shinwari, Z. K., Khan, M. A., & Rabani, A. (2006). Evaluation of taxonomic status of medicinal species of the genus Solanum and Capsicum based on poly acrylamide gel electrophoresis. Pakistan Journal of Botany, 38(1), 99.

76- Ali, Z., Qureshi, A. S., Ali, W., Gulzar, H., Nisar, M., & Ghafoor, A. (2007). Evaluation of genetic diversity present in pea (*Pisum sativum* L.) germplasm based on morphological traits, resistance to powdery mildew and molecular characteristics. *Pakistan Journal of Botany*, *39*(7), 2739-2747.

77- Sultana, T., Ghafoor, A., & Ashraf, M. (2005). Genetic divergence in lentil germplasm for botanical descriptors in relation with geographic origin. Pakistan Journal of Botany, 37(1), 61-69.

78- Khan, M. W., Khan, I. A., Ahmad, H., Ali, H., Ghafoor, S., Afzal, M., Khan, F. A., Shah, M., & Afridi, S. G. (2010). Estimation of genetic diversity in walnut. Pakistan Journal of Botany, 42(3), 1791.

79- Siddiqui, M. F., & Naz, N. (2009). Protein landmarks for diversity assessment in wheat genotypes. African Journal of Biotechnology, 8(9).

80- Akbar, A., Ahmed, H., Ghafoor, S., & Khan, A. I. (2010). Phylogeny and genetic diversity studies in Capsicum using seed storage proteins. *Current Research Journal of Biological Sciences*, *2*(4), 250.

Red Rice grown in Thatta, Pakistan.