Results and Discussion

METHODOLOGY

The methodology adopted for the present study entitled “Nutraceutical Potentials of Black Rice (Oryza Sativa L.) And Its Hypoglycaemic Activity in Streptozotocin Induced Diabetic Rats” is dealt under the following phases:

3.1 Phase I : Assess the physico-chemical characteristics of black rice and white rice

3.2 Phase II : Conduct nutrient analysis in raw and cooked samples of black and white rice

3.3 Phase III : Study the acceptability of black rice in traditional recipes

3.4 Phase IV : Evaluate the nutraceutical potentials of black rice and white rice in raw and cooked forms

3.5 Phase V : Determine *in vitro* and *in vivo* hypoglycaemic potentials of black rice in streptozotocin induced diabetic rats

3.6 Phase VI: Statistical analysis and interpretation of data

Black rice is one of the colored rice variety of *Oryza sativa L.* (Gramineae), that is cultivated in South East Asia, Korea, China and Japan. Unmilled black rice contains a dark purple to black coloured pigment known as anthocyanins in the protective outer layer, endosperm. The colour depends upon the blend of anthocyanidins. Black rice is a whole grain cereal which is rich in fiber, minerals, polyphenols and many antioxidant compounds. Numerous studies have pointed out that black rice possess immense beneficial properties like antioxidant activity, α-glucosidase inhibitory, antimutagenic, hepatoprotective, hyaluronidase inhibitory, and anti-inflammatory activity.
3.1 PHASE I

ASSESS THE PHYSICO - CHEMICAL CHARACTERISTICS OF BLACK RICE AND WHITE RICE

3.1.1 Sampling of Rice

Sampling of rice should be taken from same geographical area in order to maintain the homogeneity of properties. Thus, the aim of sampling is to secure apportion of the material that satisfactorily represents the whole. The samples should be collected from the homogeneous area to avoid the variation in results. Figure 8 shows the area in which black rice is grown in Tamilnadu and the locale from which the samples were collected for the study.

FIGURE 8
STUDY LOCALE
The following steps were adopted in sampling:

- According to the systematic sampling method, Black rice locally known as kavuni raw rice and white rice locally known as Ponni raw rice were collected from the village of Keelapoongudi, Karaikudi district, Tamilnadu, India (Lat: 10° 5' 47.256" and Long: 78° 51' 22.4172") of 25 kg individually for the homogeneous environmental and soil conditions. White rice was used as control because it is used widely in Tamilnadu for daily consumption.

- The collected rice samples were packed in air tight pouches to avoid any possible contamination before bringing to laboratory. The samples were stored in airtight plastic pouches in the laboratory at room temperature.

- The analysis of the samples was carried out immediately after the preparation of samples to avoid the storage effect and time lapse between collection of samples and analysis.

3.1.2 Preparation of Samples

i. Preparation of raw rice powder

The raw samples of black rice and white rice were powdered separately in a mixer grinder for two minutes and stored in the air tight plastic pouches.

ii. Preparation of pressure cooked rice powder

One hundred gram of white rice was weighed and washed with deionized water to remove impurities. After decanting excess water completely, the rice was mixed with deionized water in the ratio of 1:2 (W/V) and cooked for 20 min at 15 psi in a pressure cooker. The pressure cooked white rice sample was dried in a hot air oven at 30°C and powdered finely. Similarly, One hundred gram of black rice was weighed, washed with deionized water to remove impurities. After removing excess water, the rice was cooked in 1:4 ratio of water for about 45 minutes in the pressure cooker at 15 psi. The pressure cooked black rice sample was dried in a hot air oven at 30°C and powdered finely. The powders were stored in airtight plastic pouches separately at room temperature. Deionized water has no scale of mineral and ion traces. The reason for using deionized water for washing and cooking the rice samples are not to interact or affect the mineral content in the samples.
iii. Preparation of conventionally cooked rice powder

One hundred gram of white rice was weighed and washed with deionized water to remove impurities. After decanting thoroughly, water in the ratio of 1:5 (W/V) was added to the rice and cooked by conventional method for 38 mins. The excess water was drained out. The conventionally cooked white rice sample was dried in a hot air oven at 30°C and powdered finely. In a similar way, black rice was prepared in conventional cooking method. One hundred gram of black rice was weighed and washed with deionized water to remove impurities. After pouring out the excess water, the rice was cooked in 1:7 ratio of water for about 55 mins by conventional cooking method. The conventional cooked black rice sample was dried in a hot air oven at 30°C and powdered finely. The powders were stored in the airtight plastic pouches separately at room temperature.

When compared to white rice, black rice took long time to cook both in pressure cooking and conventional cooking method because it is unhusked. For both type of cooking, the black rice is ready when two thirds of the grains have burst open to reveal the cream – white interior. The prepared and powdered samples of raw and cooked varieties of black rice and white rice were subjected to the analysis which was done in triplicates. Plate 3 and 4 show the raw and cooked samples of black rice and white rice.

PLATE 3
RAW AND COOKED SAMPLES OF BLACK RICE
3.1.3 Evaluation of Physico-chemical Characteristics of Black Rice and White Rice

Rice is the staple food which is consumed as a whole grain. Rice grain quality is evaluated by four components such as cooking quality, nutritional content, grain characteristics and physico-chemical property. Out of the above, cooking quality and physico-chemical property is more important with regard to acceptance. The endosperm possess certain physical and chemical characteristics basically due to amylose content (Webb, 1985; Juliano, 1998; Unnevehr et al., 1992), protein content (Hsieh and Brunner, 1976), gel consistency (Cagampang et al., 1972), gelatinization temperature (Juliano, 1972) and physical properties of cooked rice grain are considered as an important factors (Khatoon and Prakash, 2007).

The physico-chemical characteristics of raw rice grains of black rice and white rice were studied using standard techniques. Grain length and breadth were measured by slide caliper. Classification of rice based on length indicates three major classes wiz long (>6 mm in length), medium (5-6 mm in length) and short (<5 mm in length). The sub-classification pertaining to size and shape includes
length / breadth ratio; slender (ratio more than 3); bold (ratio 2-3); round (ratio less than 2) (Dihti et al., 2002). Thousand kernel weight, bulk density, porosity, volume expansion, optimal cooking time, amylose, elongation ratio and index, equilibrium moisture content on soaking and density were measured (Bhattacharya and Sowbhagya, 1972; Swamy et al., 1971; Sidhu et al., 1975; Juliano, 1998; Oghbaei and prakash, 2010).

3.2 PHASE II

CONDUCT NUTRIENT ANALYSIS IN RAW AND COOKED SAMPLES OF BLACK AND WHITE RICE

The powders of raw and cooked samples of black and white rice were subjected to nutrient analysis following the standardized procedures outlined by Manual of laboratory techniques (Raghuramulu et al., 2003). Contents of macronutrient, micronutrients, total moisture, carbohydrates, crude fiber, protein, fat, ash, thiamine, riboflavin, niacin, iron, sodium, calcium, phosphorous, magnesium, selenium, zinc and potassium were analyzed.

3.2.1 Estimation of Amino Acid Content

The qualitative and quantitative estimation of amino acids were done using an automatic amino acid analyzer. Filter derived amino acid sample (20 µl) was injected into single column and analyzed using sodium buffer system (Liu et al., 1995). Based on the retention of the standard amino acids, the amino acids in the samples were identified.

3.2.2 Estimation of Fatty Acid Content

Miller and Berger (1985) outlined the procedure of fatty acids in the food sample. Quantitative estimation of fatty acid was done using NEON 11 gas chromatography instrument. Fatty acids in lipid sample were converted to respective methyl esters using saponification reagent, methylation reagent, extraction solvent and base wash. The fatty acids were identified and quantified by comparing the peaks with that given by standard fatty acid methyl esters.
3.3 PHASE III

STUDY THE ACCEPTABILITY OF BLACK RICE IN TRADITIONAL RECIPES

The sensory attributes such as colour, appearance, flavor, texture and taste assess the uniqueness of the food. The discipline of sensory examination requires the use of panel members, in which test outcome are recorded based on their responses to the products under test. Traditional recipes were prepared using black and white rice; the recipes were completely replaced by the black rice with white rice which is commonly in use, to know the acceptability of black rice. Seven point hedonic rating scale was used to evaluate the sensory characteristics of the developed recipes. The sensory evaluation was carried out in the laboratory as per the standard procedure administered by 50 semi-trained panel members (Bianchi et al., 2009). Water was provided to panel members before and after tasting the recipe.

The breakfast items such as idli, dosa, adai, aapam, paniyaram (karam and sweet), idiyappam, roti, sweet and karam kolukattai, puttu, pongal, sweet pongal, laddu and kali were tried out using organoleptic qualities. Lunch items included boiled rice (pressure cooked, conventionally cooked), vegetable biriyani, tamarind rice, mint rice, coconut rice, lemon rice, tomato rice and khanji, some of the snack items such as vadagam, murukku, sweet balls and kolukattai (savoury, sweet and milk). The mean scores of the organoleptic evaluation were calculated on the basis of the total scores obtained.

3.4 PHASE IV

EVALUATE THE NUTRACEUTICAL POTENTIALS OF BLACK RICE AND WHITE RICE IN RAW AND COOKED FORMS

Nutraceuticals or functional ingredients are active compounds, phytochemicals and pro-vitamins that help in maintaining good health and combating disease. Polyphenols and bioflavonoids form potent antioxidants due to their ability to scavenge free radical scavenging ability in an efficient manner (Langley, 2000). Nevertheless, antioxidant rich foods reduce oxidative damage in the human body by scavenging free radicals and prevent from degenerative diseases (Mau et al., 2001; Gulcin et al., 2002). Rice contains abundant amount of
antioxidants like phenolic compounds, tocopherols and $\gamma$-oryzanol. It is the main ingredient that is widely used to prevent oxidative deterioration in lipids thereby retaining the quality of food. The present study was carried out to evaluate the potential of rice varieties as a source of antioxidants and to compare the antioxidant level of black rice and white rice in methanolic and water extracts under different preparation methods.

3.4.1 Preparation of Extracts

The powdered rice samples of black rice and white rice were taken and subjected to successive solvent extraction. The extraction was carried out for 16 hours using the solvents namely petroleum ether, chloroform, ethyl acetate, ethanol and water in the increasing order of polarity (Figure 9).

**FIGURE 9**

FLOW CHART FOR SUCCESSIVE SOLVENT EXTRACTION

```
50g of Powdered Rice Material

<table>
<thead>
<tr>
<th>Extract</th>
<th>Solvent</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum Ether Extract</td>
<td>Petroleum Ether</td>
<td></td>
</tr>
<tr>
<td>Extract with 250ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform Extract</td>
<td>Chloroform</td>
<td></td>
</tr>
<tr>
<td>Extract with 250ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl Acetate Extract</td>
<td>Ethyl Acetate</td>
<td></td>
</tr>
<tr>
<td>Extract with 250ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol Extract</td>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td>Extract with 250ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water Extract</td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>Extract with 250ml Water</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```
The powder was extracted with solvent in the ratio of 1:5 (w/v) for 16 hours by using soxhlet apparatus. Whatman filter paper No.2 was used to filter and rotator was used to remove the solvents with reduced pressure at 40ºC. The extract then kept in pre-weighed flask to facilitate drying. Further, the residue after extraction was used for subsequent extraction by other solvents. The powdered samples of black rice and white rice were subjected to solvent extraction successively until the secondary metabolites are absorbed by solvents. Based on increasing order of polarity, the solvents were selected for extraction.

3.4.2. Phytochemical Screening of Rice Extract

The phytochemical screening of rice extract was carried out using the method of Harborne (1987) and Trease and Evans (1996).

i. Test for alkaloids

Two ml aliquot of the extract was treated with Dragenderoff’s reagent and Mayer’s reagent. The presence of alkaloids was confirmed by the formation of orange or red and white precipitate.

ii. Test for flavonoids

a) Shinoda test: One ml of the extract was treated with magnesium turnings and one to two drops of concentrated HCl. Formation of pink or red color showed the presence of flavonoids.

b) One ml of the extract was treated with one ml of ferric chloride. Flavonoids were confirmed, if brown colour was formed.

iii. Test for tannins and phenolic compounds

a) To one ml of the extract, few ml of 15 per cent neutral FeCl₂ was added. Tannins were confirmed if dark blue or bluish black colour was formed.

b) To one ml of the extract, few ml of gelatin was added. Presence of tannins and phenolic compounds were confirmed by the formation of white precipitate

c) To few ml of lead tetra acetate solution, one ml of extract was added. The presence of tannins and phenolic compounds were confirmed by the formation of precipitate.
iv. Test for carbohydrates

Fehling’s or Benedicts test: Fehling’s solution / Benedict’s solution was treated with one ml of the extract. Reducing sugar was confirmed if red or yellow colour precipitate was observed.

v. Test for cardioglycosides

Salkowski test: The extract of 0.5 ml was dissolved in 2 ml of chloroform and to form a lower layer, sulphuric acid was added along the sides carefully to facilitate the formation of lower layer. Steroidal ring is confirmed if a reddish brown colour developed at the interface.

vi. Test for saponins

a) Frothing test: Twenty ml of distilled water mixed with one ml of diluted alcoholic extract and shaken in a graduated cylinder for 15 minutes. The saponins were confirmed if a layer of foam formed measured atleast one cm.

b) One ml of the extract alcoholic was treated a few drops of vanillin solution and concentrated sulphuric acid. The development of deep violet colour showed the presence of saponins.

vii. Test for oils and fats

Spot Test: Extract was pressed in between two-filter papers confirm the presence of oil.

viii. Test for Terpenoids

a) Horizon test: Terpenoids were confirmed if red or yellow precipitate was obtained.

b) Liebermann test: Three ml of acetic acid along with few drops of concentrated sulphuric acid was treated with one lm of extract. Terpenoid were confirmed if red to blue colour appeared.

3.4.3 Extract Recovery Percentage

Extract recovery percentage is the process of separation of active components of samples of a given sample. Selective solvents are used for
Results and Discussion

The polarity of the components is the key for extraction. Based on it, the solvents diffuse the sample (Tiwari, 2001). The extract recovery percentage can be calculated using the formula:

\[
\text{Per cent} = \frac{(\text{Extract + Container (g)} - \text{Empty Container (g)}) \times 100}{\text{Sample Weight (g)}}
\]

3.4.4 Assay of Enzymatic Antioxidants

In humans, antioxidant systems such as enzymatic and nonenzymatic work together and protect the body from free radical damage thus preventing the onset of degenerative diseases at various interfaces (Cadenas and Packer, 1996; Sies, 1996; Halliwell, 2000; Halliwell and Gutteridge, 2002). constitute the first line of defense is Superoxide dismutase (SOD) which act against reactive oxygen species, Oxidative stress in cells are prevented by the decomposition of hydrogen peroxide to water and oxygen (Sies, 1996). Polyphenol oxidase, peroxidase, glutathione peroxidase, Glutathione-s-transferase, ascorbate oxidase glutathione reductase, thioredoxin, peroxidase, thiols and disulfide bonding are the various enzyme buffers systems that protect the each and every cell.

3.4.4.1 Preparation of enzyme extract

Rice samples were powdered and homogenized (1g) in an electrical mixer with M/150 phosphate buffer at a pH 7.0 and centrifuged at 4°C. Cold phosphate buffer was added to the sediment and disturbed at regular intervals. This extraction is repeated once or twice. It should be ensured that the extraction time did not exceed 24 hours. The supernatant was extracted and used for analysis.

Enzymatic antioxidants like superoxide dismutase (SOD) (Das et al., 2000), Glutathione peroxidase (GPx) activity (Rotruck et al., 1973), Glutathione-S-transferase (GST) (Habig et al., 1974), peroxidase (Addy and Goodman., 1972), catalase (Sinha, 1972), polyphenol oxidase (PPO) (Sadasivam and Manickam, 1996), ascorbate oxidase (Vines and Oberbacher., 1965) were analysed for the six rice samples.
3.4.4.2 Assay of Enzymatic Antioxidants

i. Assay of Superoxide Dismutase (SOD) activity

For the analysis of superoxide dismutase (SOD) activity, the method given by Das et al. (2000) was followed. According to this method, one hundred ml of sample extract was added to the reaction mixture and incubated. Riboflavin was added for the generation of superoxide radical. One ml of Griess reagent was added. The production of red azo compound was measured.

ii. Assay of Peroxidase activity

Pyrogallol is used as substrate for assay of peroxidase (Addy and Goodman, 1972).

\[
\text{Peroxidase} \quad \text{H}_2\text{O}_2 + \text{Pyrogallol} \rightarrow 2\text{H}_2\text{O} \rightarrow \text{Purpurogallin (Donor)} \rightarrow \text{Oxidized Donor}
\]

Three ml of buffered pyrogallol and 0.5 ml of one per cent H\textsubscript{2}O\textsubscript{2} are the reaction mixture, to which 0.1 ml enzyme extract was added. The extinction coefficient of pyrogallol that is oxidized was used for calculation of peroxidase activity.

iii. Assay of Catalase activity

Assay of catalase activity was carried out by the method of Sinha, (1972). Rapid decomposition of hydrogen peroxide is caused by catalase. The production of chromic acetate determines the activity of catalase. Catalase activity was expressed in terms of μmoles of H\textsubscript{2}O\textsubscript{2} consumed/min/mg protein.

iv. Assay of Glutathione Peroxidase (GPx) activity

Ellman’s method given by Rotruck et al. (1973), was followed for assay of glutathione peroxidase activity. The amount of unreacted GSH was measured. The development of colour at 412 nm was measured to determine the glutathione peroxidase activity.

v. Assay of Ascorbate Oxidase activity

The method postulated by Vines and Oberbacher, (1965) was followed for assay of ascorbate oxidase. The activity of enzymes present is directly proportional
to the extent of oxygen consumed during oxidation of ascorbic acid. Hence, reduced oxidation of ascorbic acid oxidase may reflect as reduction in acid peak absorption.

vi. Assay of Glutathione-S-Transferase (GST)

The method given by Habig et al. (1974) was followed for assay of Glutathione-S-transferase. 340 nm absorbance was used to measure the enzyme activity.

vii. Assay of Polyphenol Oxidase (PPO)

The method postulated by Sadasivam and Manickam, (1996) was followed for the assay of polyphenol oxidase. Polyphenol oxidase catalyses the hydroxylation of monophenols to o-diphenols and it further catalyse the oxidation of o-diphenols to produce o-quinones.

3.4.5 Quantitative Analysis of Phytochemicals in Aqueous and Ethanolic Extracts of Raw and Processed Samples of Black and White Rice

Phytochemicals are the chemicals produced by plants are not essential nutrients that may affect health. Since plant based foods are diverse mixtures of bioactive compounds, knowledge on the health benefits of individual phytochemicals is linked to the health benefits of foods which contains such phytochemicals.

According to Goffman and Bergman (2004), the higher antioxidative effect and phenolic content of coloured rice can be compared with bran colour of rice. Pentose phosphate, shikimate and phenylpropanoid pathways in plants are the main derivatives of the secondary metabolites, phenolic compounds (Randhir et al., 2004). Flavonoids are the compounds with a C3 – C6 – C3 skeleton that consists of two aromatic rings joined by the three carbon links; they include anthocyanins, flavonones, flavanols, flavanoids and flavanones. Flavonoids are located in the pericarp of all cereals.

Weighed 0.5 g of rice samples, ground in a mortar and pestle and prepared ethanolic and aqueous extracts of rice samples separately. The supernatant was collected in a volumetric flask after the extracts were centrifuged at 2000 rpm for ten
minutes. The evaporated residue was then dissolved in water for the estimation of total phenolic and flavanoid content.

3.4.5.1 Estimation of Total Flavonoid content (TFC)

Flavonoids reacts with aluminium chloride in ethanolic solution forms a yellow colour which was read colorimetrically at 420nm (Ordonezet al., 2006). To 0.5 ml of sample solution, 0.5 ml of Two per cent AlCl₃ in ethanol solution was added and incubated at room temperature for one hour. UV visible spectrophotometer was used to measure the absorbance at 420 nm. A standard graph was prepared using the quercetin and the total flavonoid content was expressed as quercetin equivalent (mg/g).

3.4.5.2 Estimation of Total Phenol content (TPC)

Singleton and Rossi(1965)method was used to estimate the total phenolic content. 0.1 ml of the sample extract was taken along with Three ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent was added. Two ml of 20 per cent sodium carbonate was added exactly after three minutes and thoroughlymixed. Boiling water bath was prepared and the tubes were kept in it exactly for one minute. The tubes were cooled and measured at 650nm in spectrophotometer. The standard graphs were plotted with various concentrations of gallic acid.

3.4.5.3 Estimation of Tannin content

Tannins are wide spread in nature and possibly in all plant materials. Tannin content was carried out by method given by Schenderl, (1970). Tannin reduces phosphomolybdic acid in alkaline condition to lower oxides of molybdenum when Na₂CO₃ and folin-Denis reagent are added.

3.4.6 Assay of Non - Enzymatic Antioxidants

Non-proteinaceous antioxidants have low molecular weight. It is present either inside or outside the cell in a lipid or aqueous environment. Glutathione can be synthesized by the body whereas vitamins C and E should be obtained from the diet. Reduced glutathione (GSH), source of thiol group, is present in the intracellular compartment of the cells and acts as anantioxidant by scavenging a free radical species. Reduced glutathione is a vital factor for glutathione peroxidase.
Ellman, (1972) method was used to estimate total reduced glutathione. The method given by Rosenberg (1992) was followed for the vitamin E estimation.

3.4.7 In vitro Radical Scavenging Assays

The relation between free radicals and disease comes under the concept of ‘oxidative stress’. The different levels of antioxidant defense are effectively kept in check the production of pro-oxidants in the form of reactive oxygen species and reactive nitrogen species in human body. Reactive Oxygen Species can be classified into oxygen centered radicals and oxygen centered non radical. Oxygen centered radicals are superoxide anion ($O_2^-$), hydroxyl radical (OH$^-$), peroxyl radical (ROO$^-$), hydrogen peroxide ($H_2O_2$), singlet oxygen (O$_2$) and alkoxyl radical (RO$^-$). Other reactive species are nitrogen species such as nitric oxide (NO$^-$), nitrogen dioxide (NO$_2$) and peroxide nitrite (OONO$^-$). Reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chloride species are mainly produced in animals and human under physiologic and pathologic condition (Gilbert, 2000; Evans and Halliwell, 2001).

3.4.7.1 Preparation of extracts

The powdered black rice and white ricein raw, pressure cooked and conventionally cooked samples were weighed and 50 gram were taken exactly from each. 250ml of ethanol and water were taken for homogenization and the homogenate was left in a shaker for 24 hrs at 27°C. Whatman No. 1 filter paper was used to filter the above. The residue was again subjected to extraction with 250 ml of respecting solvents. The supernatant of all extracts were mixed together. The extract was then dried and stored at 4°C until further used. Fifty mg of ethanol or aqueous extract of various rice samples were used for in vitro antioxidant scavenging assays.

3.4.7.2 In vitro Radical Scavenging Activity

DPPH radical scavenging was determined by the method of Shimada et al. (1992). According to the method of Oyaizu, (1986), reducing power assay was measured. The method given by Benzie and Strain, (1996) was followed for the FRAP assay to estimate the total antioxidant potential of sample. Method given
byRe et al. (1999) was used for ABTS$^+$ radical scavenging activity. By the method of Ruch et al. (1989), hydrogen peroxidescavenging activity was measured. Hydroxyl radical scavenging assay was determined by the method of Klein et al. (1991).

### 3.4.8 Quantification of Anthocyanin of Black Rice and White Rice in Raw and Cooked Forms

Anthocyanins are water-soluble glycosides and acylglycosides of anthocyanidins and they are pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Black rice contains rich anthocyanins in the aleurone layer. In particular, these components are associated with healthy effects such as dietary antioxidant, anti-inflammatory compounds and chemopreventive agents (Ling et al., 2001) and strong free radical scavenging activities (Choi et al., 2007). Total anthocyanin content of rice samples was determined by pH differential method (Giusti and Wrolstad, 2001).

### 3.4.9 Quantification of Polyphenols of Black Rice and White Rice in Raw and Cooked Forms by HPLC Method

Polyphenols are the copious antioxidants present in our diet (Scalbert and Williamson, 2000). In addition to their antioxidant properties, polyphenols may have other biological activities including anti-oestrogenic, anti-inflammatory effects, anticarcinogenic and anti-mutagenic which prevent human health from the degenerative diseases (Bravo, 1998). The modified method of Naveena and Bhaskarachary (2013) were used for RP-HPLC quantification of individual and total polyphenolic content of black rice and white rice in raw and cooked forms.

### 3.5 PHASE V

**DETERMINE IN VITRO AND IN VIVO HYPOGLYCAEMIC ACTIVITY OF BLACK RICE IN STREPTOZOTOCIN INDUCED DIABETIC RATS**

**3.5.1 In vitro Inhibitory Potential of Black Rice and White Rice in Raw and Cooked Forms Against α-Glucosidase and α- Amylase Enzymes**

Diabetes as a result of deficit insulin leads to hyperglycemia. Plant extract proves to be effective in ethanomedical treatment of diabetes and is widely accepted as alternative medicine. Current knowledge of the activity of intestinal enzymes such as α-amylase and α-glucosidase in carbohydrate digestion and
Results and Discussion

56

Nutraceutical Potentials of Black Rice (Oryza sativa L.) and Its Hypoglycaemic Activity in Streptozotocin Induced Diabetic Rats

Glucose absorption lead to the development of advancement in pharmacological agents. As a part of this research, investigation was done on the inhibitory effects of black rice and white rice in raw and cooked forms against α-amylase and α-glucosidase activity in controlling blood glucose level. The α-glucosidase inhibitory activity was measured by the method of Nishioka et al. (1998). The α-amylase inhibitory activity was determined by the method of Hansawasdi et al. (2000). For both assays, acarbose was used as a standard.

3.5.2 In vivo Hypoglycaemic Activity of Black Rice in Streptozotocin Induced Diabetic Rats

Diabetes is usually accompanied by excessive production of the molecules of reactive oxygen species (ROS) and/or poor antioxidant defense systems, which damages bio-molecules. Hyperglycaemia is main cause of oxidative stress that occupies a vital position in pathophysiology of Diabetes mellitus. Research indicates that in both experimental and clinical studies suggest that oxidative stress caused by hyperglycemia plays a major role in pathogenesis of diabetes mellitus.

Streptozotocin induced experimental diabetes mellitus is an idyllic model to study the hypoglycaemic activity of test samples and its constituents. Streptozotocin generates excessive reactive oxygen species as a result of destruction of pancreatic cell and thus induces diabetes mellitus. Lipid peroxidation formation and breakage of DNA in pancreatic cell is well documented.

The phytochemicals are indicative of its potential in the treatment of hyperglycaemic hence the present work was thus designed to investigate the hypoglycaemic effect of the black rice in healthy and streptozotocin induced diabetic rats with the objective to focus on its modifying effect on carbohydrate metabolizing enzyme activities, antioxidant activity and lipid peroxidation in streptozotocin induced diabetic rats.

3.5.2.1 Preparation of the sample

From the phase 1 to 4, raw and cooked samples of black rice and white rice were analysed. Among all, raw sample of black rice showed higher nutrient levels, antioxidant content and showed higher radical scavenging activities followed by pressure cooked sample of black rice among all the rice samples. Though the raw
sample of black rice showed higher levels in nutrient and antioxidant content, the pressure cooked sample of black rice was chosen for the further study due to the consumption form by humans. The composition of stock diet was based on the procedure mentioned in the manual of laboratory techniques (Raghuramulu et al., 2003). Instead of wheat flour (15 per cent), the pressure cooked sample of black rice were added and made into pellet with all other ingredients as given in the NIN manual of laboratory techniques (Jang et al., 2012).

3.5.2.2 Selection and maintenance of animals

Wistar strain male albino rats of 150-180g were selected for the study. The cages used were maintained in hygienic condition. Cage was spacious enough. The rats were fed with pellets supplied by M/S Hindustan Lever Limited, Bangalore, India and water ad libitum.

3.5.2.3 Experimental induction of Diabetes mellitus

Diabetes mellitus was induced in overnight-fast of 12 hours in albino wistar rats by single intraperitoneal injection of streptozotocin (STZ 45mg/kg body weight) dissolved in freshly prepared 0.1M citrate buffer (pH 4.5) (Kanchana et al., 2011). After 72 hours of STZ injection, fasting blood glucose levels were tested and rats which showed fasting blood glucose level exceeding 200 mg/kg were considered as diabetic and selected for further study.

3.5.2.4 Experimental design

The study was approved by Institutional Animal Ethical Committee (Ref. No: J.Sridevi/ 10PH081/ IAEC/ KMCP/ 40/ AIHS/ Ph.D). The experimental rats were divided into five groups each containing six animals. Table 4 shows the experimental design for animal study.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Experimental Design</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control, normal healthy rats</td>
<td>10 ml/Kg of normal saline for 90 days</td>
</tr>
<tr>
<td>Group II</td>
<td>A single intra peritoneal injection of 45 mg/kg bw of streptozotocin were given to induce diabetes</td>
<td>No treatment</td>
</tr>
<tr>
<td>Group III</td>
<td>Rats were induced with diabetes as mentioned in group 2</td>
<td>Treated with glipizide(10 mg/kg bw) orally for a period of 90 days</td>
</tr>
<tr>
<td>Group IV</td>
<td>Rats were induced with diabetes as mentioned in group 2</td>
<td>Diabetic rats received modified pellet diet of pressure cooked black rice for 90 days.</td>
</tr>
<tr>
<td>Group V</td>
<td>Perzi control, normal healthy rats(Non- diabetic rats)</td>
<td>Non- diabetic rats received modified pellet diet of pressure cooked black rice for 90 days.</td>
</tr>
</tbody>
</table>

### Collection of blood, liver, kidney and pancreas

After 90 days of experimental period, under mild chloroform anesthesia, cervical dislocation was done to sacrifice the animal. Blood was collected on decapitation (Raghuramalu et al., 2003) and centrifuged were separate the serum at 1500 rpm for 10 mins. The liver, kidney, and pancreas were excised immediately and ice-cold physiological saline was used for washing. The serum and organs collected were used for biochemical experiments (Plate 5).
PLATE 5
EXPERIMENTAL ANIMALS

Ad libitum feeding and Water

Dissection of Rat
3.5.3. Biochemical Estimations

After 90 days of hypoglycaemic study, all rats were sacrificed. Retro orbital plexus method was used to withdraw blood from the rats (Kanchana et al., 2011) and the biochemical estimations were carried out in blood, liver and pancreas samples of control and experimental rats in each group and alterations occurring in this rats were assessed by analyzing the following biochemical parameters:

- Derived parameter - body weight
- Hematological parameter in serum
- Glycemic profile in serum
- Renal markers in serum
- Lipid profile serum
- Hepatic marker enzymes in serum
- Protein levels in serum
- Lipid peroxidation in liver and kidney
- Enzymatic antioxidants in liver and kidney
- Non-enzymatic antioxidants in liver and kidney
- Membrane bound enzymes in liver and kidney
- Glycoproteins in liver and kidney
- Carbohydrate metabolizing enzymes in liver and kidney
- TCA cycle markers in liver and kidney
- Histopathology of liver, kidney and pancreas

3.5.3.1 Hematological estimations

RBC, WBC and platelet were estimated by the method of Sanderson and Philips (1981).
3.5.3.2 Renal markers in serum

Natelson et al., (1951) were used for urea estimation. Caraway (1963) was used for uric acid analysis. Owen et al., (1954) method were used for creatinine analysis.

3.5.3.3 Glycaemic profile in Serum

Drabkin and Austin (1932) method was used to estimate the Haemoglobin content. Sasaki et al., (1972) method was used for the estimation of glucose. Glycosylated haemoglobin level was analysed by using Diatek assay kit.

3.5.3.4 Lipid profile in serum

Zak (1977) method was used for cholesterol analysis. Triglyceride was estimated by the method of Rice (1970). HDL, LDL and VLDL - cholesterol was estimated by the method of Warnick et al., (1985). Horn and Mehanan (1981) method was used for free fatty acid estimation.

3.5.3.5 Protein levels in serum

Lowry et al. (1951) method was used for estimation of total protein. Albumin and globulin were determined by the method of Wolfson (1948).

3.5.3.6 Hepatic Marker enzymes in serum

Reitman and Frankel (1957) method was used for the estimation of aspartate transaminase and alanine transaminase. Lactate dehydrogenase and Acid phosphatase was estimated by the method of King (1965).

3.5.4 Biochemical Estimations in Liver and Kidney

3.5.4.1 Liver peroxidation

By the method of Uchiyama and Mihara (1978), lipid peroxidation was estimated in liver and kidney tissues

3.5.4.2 Enzymic Antioxidants

Superoxide dismutase was analyzed by the Das et al., (2000) method. Sinha (1972) method was used for the estimation of catalase. Glutathione peroxidase was

3.5.4.3 Non-Enzymic Antioxidants

Vitamin C was estimated by the method of Omaye et al., (1971). Vitamin E was analysed by the method of Rosenberg (1992).

3.5.4.4 Membrane Bound Enzymes

Bonting (1970) was used for the estimation of Na$^+/K^+$ATPase. Mg$^{2+}$ ATPase was analyzed by the method of Ohnishi et al. (1982)

3.5.4.5 Glycoproteins

The hexokinase activity was measured by the method of Brandstrup et al. (1957). Wagner, (1979) method was used for the estimation of hexosamine.

3.5.4.6 Carbohydrate metabolism enzymes

King (1965) method was used for the estimation of aldolase. The phosphoglucoisomerase was estimated by the method of Horrocks et al. (1963). The activity of the enzyme Glucose-6-Phosphatase was determined by method of King, (1965). The activity of the enzyme Fructose-1, 6-bisphosphatase was analysed by the method of Gancedo and Gancedo, (1971).

3.5.4.7 TCA cycle markers

The Malate Dehydrogenase was measured by the method of Mechler et al. (1948). The Isocitrate dehydrogenaseactivity was analyzed by the method of Bell and Barron (1960).

3.5.5 Histopathological Investigation

Histopathological examination was done by the method of Dunn, (1974). The liver, kidney and pancreas samples were conserved in 10 per cent commercial formalin. The tissues were then processed by standard histopathological technique (i.e.) dehydration through graded isopropyl alcohol, cleaning through xylene and
impregnated in paraffin wax for two hours. Then wax blocks were made, sections were used for cutting microtone and stained by haematoxylin eosin method and photographed. The liver, kidney and pancreas were preserved 10 per cent commercial formalin immediately from animal. Liver, kidney and pancreatic tissues were placed in ten per cent formalin saline (ten per cent formalin in nine per cent sodium chloride) for one hour to rectify shrinkage due to higher concentration of formalin and processed further for tissue staining by the standard procedure.

3.6 PHASE VI

STATISTICAL ANALYSIS AND INTERPRETATION OF DATA

The data was consolidated and tabulated and subjected to statistical analysis with appropriate tools using the SPSS version 16.0 version. The following were the statistical tests applied in the different phases.

Phase I

Descriptive statistics (mean and standard deviation) was used to represent the morphological, physical, chemical and cooking properties of black and white rice.

Phase II

Descriptive statistics (mean) was used to represent the basic distribution of nutrients.

Phase III

Descriptive statistics (mean and standard deviation) was used to represent the acceptability scores of black rice and white rice in breakfast, lunch and snack recipes. Two way ANOVA was used to compare the significant difference between the groups and the attributes in the groups using Agres stat (version 3.1).

Phase IV

Descriptive statistics (mean and standard deviation) was used to represent the quantification of phytonutrients and polyphenols (total and individual), enzymatic and non-enzymatic antioxidants and in vitro radical scavenging assays.
Phase V

The experimental results are expressed as the Mean ± SD. The data were subjected to Anova and the significance of the difference between groups were calculated by Duncan Multiple Range Test (DMRT) (Duncan, 1957) using Irristat version 3.1. A value of p<0.05 was considered to indicate a significant difference between groups.

Validation of Data

The procedures involved in the biochemical analysis in blood were standardized in normal rat blood samples. The analysis of biochemical parameters in the test samples of the present investigation was carried out after this. Proper quality control was maintained in the protocol throughout the study period. The investigator herself carried out the entire analytical procedures. She underwent prior training in handling of animals and the procedures involved in animal experimentation. Further, she also underwent training in phytochemical analysis and animal protocols to conduct the in vivo hypoglycaemic study. Hence, there is no probability of bias or error in obtaining the analytical data in the present study.

Limitation of Methodology

The present study on in vivo of hypoglycaemic activity was carried out only in animal models. Due to paucity of time within the research period, in vivo hypoglycaemic activity in Type – II diabetic subjects could not be conducted.

The research design of the study is depicted in figures 10, 11, 12, 13 and 14.
Results and Discussion

Nutraceutical Potentials of Black Rice (Oryza sativa L.) and Its Hypoglycaemic Activity in Streptozotocin Induced Diabetic Rats

PHASE I

FIGURE 10

PHYSICO - CHEMICAL CHARACTERISTICS OF BLACK RICE AND WHITE RICE

- Thousand kernel weight
- Hundred kernel weight
- Broken kernel weight
- Length/width ratio
- Bulk density
- Gel consistency
- Equilibrium moisture content

- Optimal cooking time
- Elongation Ratio
- Elongation Index
- Porosity
- Density
- Amylose content
- Gruel solid loss

Black rice and White rice

Raw Sample
Results and Discussion

Nutraceutical Potentials of Black Rice (Oryza sativa L.) and Its Hypoglycaemic Activity in Streptozotocin Induced Diabetic Rats

PHASE II

FIGURE 11
NUTRIENT ANALYSIS IN RAW AND COOKED SAMPLES OF BLACK AND WHITE RICE

Black rice and White rice

- Raw sample
- Pressure cooked sample
- Conventionally cooked sample

Nutrient Analysis for six Samples

Macronutrients
- Moisture
- Total carbohydrate
- Protein
- Fat
- Fibre
- Ash
- Essential and non-essential amino acids
- Free fatty acids, saturated fatty acids, monounsaturated fatty acids and poly unsaturated fatty acids

Micronutrients
- Thiamine
- Riboflavin
- Niacin
- Zinc
- Iron
- Calcium
- Phosphorus
- Potassium
- Sodium
- Magnesium
FIGURE 12

ACCEPTABILITY OF BLACK RICE IN TRADITIONAL RECIPES

- Black rice and White rice
- Pressure cooked sample

**Breakfast Recipes**
- Idli
- Dosa
- Adai
- Aapam
- Idiyappam
- Roti
- Puttu
- Pongal
- Khanji
- Kali

**Lunch Recipes**
- Boiled rice (Pressure cooked)
- Boiled rice (Conventionally cooked)
- Vegetable biriyani
- Tamarind rice
- Mint rice
- Tomato rice
- coconut rice
- Lemon rice

**Snack items**
- Vadagam
- Murukku
- Sweet balls
- Kolukattai (sweet)
- Kolukattai (savoury)
- Kolukattai (milk)
- Laddu
- Sweet Pongal
FIGURE 13
NUTRACEUTICAL POTENTIALS OF BLACK RICE AND WHITE RICE IN RAW AND COOKED FORMS

- Black rice and White rice
  - Raw sample
  - Pressure cooked sample
  - Conventionally cooked sample

  - Qualitative analysis of preliminary phytochemical screening in different solvents
  - Total anthocyanins
  - Quantification of total and individual polyphenols by HPLC

  - Non enzymatic antioxidants
    - Vitamin E
    - Total Reduced Glutathione
  - Enzymatic antioxidants
    - Superoxide dismutase
    - Catalase
    - Peroxidase
    - Glutathione peroxidase
    - Glutathione – S – Transferase (GST)
    - Polyphenol Oxidase (PPO)
    - Ascorbate Oxidase
FIGURE 14 (Contd…)

NUTRACEUTICAL POTENTIALS OF BLACK RICE AND WHITE RICE IN RAW AND COOKED FORMS

PHASE IV

Black rice and White rice

Raw sample
Pressure cooked sample
Conventional cooked sample

Ethanol and Aqueous extract for six samples

Antioxidant analysis for 12 samples

- Total Phenols
- Total flavonoids
- Tannins

In vitro radical scavenging activities
- DPHH radical scavenging activity
- ABTS radical scavenging activity
- Nitric oxide radical scavenging activity
- Hydroxyl radical scavenging activity
- Hydrogen peroxide radical scavenging activity
- Reducing power assay
- FRAP
FIGURE 15

IN VITRO AND IN VIVO HYPOGLYCAEMIC POTENTIALS OF BLACK RICE IN STREPTOZOTOXIN INDUCED DIABETIC RATS

**In vitro** hypoglycaemic potentials of black and white rice

- Black rice and White rice
  - Raw sample
  - Pressure cooked sample
  - Conventional cooked sample
  - \(\alpha\) - Amylase inhibiting activity
  - \(\alpha\) - Glucosidase inhibiting activity
  - In vitro glycaemic index

**In vivo** hypoglycaemic potentials of black rice in streptozotocin induced diabetic rats

- Selection of albino rats (Male-Wistar strain)
  - Grouping of rats (30 rats – 6 rats in each number)
    - Control Group
    - Diabetic Control
    - Diabetic + Std. Drug
    - Diabetic + Black
    - Non-Diabetic + Black rice

After 90 days of hypoglycaemic study, all rats from each group were sacrificed

Parameter evaluated

- Haematological parameters
- Lipid profiles
- Derived parameters
- Serum and renal biomarkers
- Serum protein profiles
- Lipid peroxidation in liver and kidney
- Enzymatic and Non – enzymatic antioxidants
- Glycoproteins
- Glycolytic and TCA cycle markers
- Membrane bound enzymes
- Histopathological Examination