A Study on Bioactive Content, Antioxidant Activity, and α-Amylase Inhibition of Black Rice Grown in Turkey

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ABSTRACT
The primary aim of this study was to determine the antioxidant activity of black rice (Orzya sativa L. japonica var.) which grown in our country for the first time. The antioxidant activity and individual bioactive compounds of black rice, extracted with aqueous ethanol (60% v/v) of ground to fine powder, were assessed. α-amylase inhibition assay of black rice extract was also analysed. At 500 µg/ml concentration, the black rice extract exhibited 76.35% inhibition on the peroxidation of linoleic acid, 94.08% activity on the β-carotene bleaching test, 77.30% DPPH scavenging activity, and 32.65% metal chelating capacity. It also showed ferrous ion reducing capability. The determination of bioactive contents were found as 83.01±0.51 µg GAE/mg, 21.3±0.89 µg QE/mg, and 14.11±1.25 µg/mg for total phenolic, flavonoid, and anthocyanin contents, respectively.

Key words: α-amylase inhibition, black rice; DPPH scavenging activity; phenolics.

1. INTRODUCTION

Turkey is one of the most favored countries in terms of agricultural production due to the following factors: good ecological and climatological conditions, land property and a rich crop pattern (Surek 1997). According to 2011 data, grains have planted especially wheat and other cereals such as maize, rice, barley, rye etc. in 73.6 % of cultivated areas of Turkey. White rice is one of the most important cereal products which have been increase production in Turkey. According to the Summary of Turkey Agricultural Statistics (2011), in 2009, 2010 and 2011 planted rice fields were 967.541, 990.000, 994.000 decares and the quantities of the products of these areas are also 750.000, 860.000 and 900.000 tons, respectively. In Turkey, the main rice growing regions are the northwestern (Marmara-Thrace) and the northern parts of the

country (Black Sea region). A large part of the white rice cultivation is being done in Edirne located in Thrace, and mostly cultivated varieties are Rocca, Baldo, Surek-95, Ergene, Veneria, Meric, and Ipsala (Surek 1997). In spite of too much production and consumption of white rice in our country, coloured rice varieties hadn't been known and made cultivation since 2009 that black rice was first harvested in Turkey. Black and the other coloured rice species, which has been consumed for a long time in Asia, especially Chine, Korea, Japan and many countries in Southeast Asia are reported as potent sources of antioxidants and as functional food (Sompong *et al.* 2011; Kong & Lee 2010; Chiang *et al.* 2006). Recent studies demonstrated that coloured rice species and their crude extracts had various biological activities, including antioxidant, antidiabetic, anticarcinogenic and antiatherosclerosis effects (Deng *et al.* 2013).

Coloured rice cultivation has just began in our country because of increased culinary and its bioactive contents interest so no data is available about black rice, which was firstly planted in Turkey. The objective of this study was to determine antioxidant properties, bioactive contents, and α-amylase inhibitory activity of that black rice. Therefore in this work we investigated antioxidant properties of black rice extract by total antioxidant activity assays with thiocyanate method and β-carotene bleaching method, scavenging effect on 1,1-diphenyl-2-picyrl-hydrazyl (DPPH) radicals, reduction power assay and metal chelating capacity. Total phenolic, total flavonoid, anthocyanin contents were determined, and black rice extract studied for possible α-amylase inhibitory activity on starch breakdown *in vitro.*

2. MATERIALS AND METHODS

2.1. Materials

Black rice (*Orzya sativa* L. *japonica* var.) used in this study was grown in Edirne province, Turkey, 2011. All chemicals and reagents used were of analytical grade and were purchased from various suppliers including Sigma-Aldrich (Munich, Germany), Merck and Riedel-de Haen (Seelze, Germany).

2.2. Preparation of crude extract

Black rice (*Orzya sativa* L. *japonica* var.) was ground to fine powder by using Waring blender (Waring Commercial, USA). Twenty gram grounded flour was extracted with 60% (v/v) aqueous ethanol under shaking bath at 100 rpm for 3 h at 35°C and then filtered. The sample was re-extracted with fresh solvent for a further 3 h and then filtered. The solvents were combined, and concentrated under vacuum and then freeze-dried. The extract was stored at 4°C until usage.

2.3. Determination of total phenolic content (TPC)

The total soluble phenolic content was determined by the Folin-Ciocalteu's procedure (Slinkard & Singleton 1977). Briefly 1 ml of extract solution was transferred to an erlenmeyer flask and the volume was adjusted to 46 ml by the addition of distilled water. Then, 1 ml of Folin-Ciocalteu reagent was added to the mixture, followed after 3 min by 3 ml of Na_2CO_3 solution (2% w/v). The mixture was shaken and the absorbance was measured at 760 nm with a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan) after incubation for 2 h at room temperature. Quantification was done on the basis of the standard curve of gallic acid concentration ranging between 50 to 500 µg/ml. The amount of the total phenolic content is expressed as µg of gallic acid equivalent (GAE) in gram extract. The following equation was used:

Absorbance = 0.0013 x Gallic acid (µg) – 0.0349 (r^2=0.9991)

2.4. Determination of total flavonoid content (TFC)

Total flavonoid content was determined using a method based on the formation of a complex flavonoid–aluminium (Quettier-Deleu *et al.* 2000). Quercetin was used to make the calibration curve. One milliliter of

extract solution was mixed with 1 ml of 2% (w/v) aluminium chloride methanolic solution. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer, and total flavonoid content was expressed as µg quercetin equivalent (QE) by using an equation that was obtained from standard quercetin graph. The equation is given below:

Absorbance = 0.0153 x Quercetin (µg) (r^2=0.9877)

2.5. Determination of monomeric anthocyanin content (TAC)

Total monomeric anthocyanin content of black rice extract was determined using the pH-differential method (Giusti & Wrolstad 2001). Black rice extract in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5) were measured at 510 nm and 700 nm, respectively. The content of total anthocyanin was calculated as mg of Cyd-3-glu by using a molar absorptivity (ε) of 26.900 and a molecular weight of 445.2.

2.6. The antioxidant activity determination by thiocyanate method

The antioxidant activity of black rice extract was identified according to the thiocyanate method (Pan *et al.* 2007). Linoleic acid emulsion was prepared with linoleic acid and Tween 20 in phosphate buffer (0.04 M; pH 7.0). A reaction solution, containing extract, linoleic acid emulsion and phosphate buffer (0.04 M; pH 7.0) was put in a test tube. The mixed solution was incubated in darkness at 37 °C. The amount of peroxide was determined by reading the absorbance at 500 nm, after reaction with $FeCl_2$ (0.1 ml; 20 mM in HCl 3.5% v/v) and thiocyanate (0.1 ml; 30% w/v) at intervals during incubation. The solution without added extract was used as blank sample. The inhibition of lipid peroxidation in percent was calculated by the equation:

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

where A_0 is the absorbance of the control reaction and A_1 is the absorbance of black rice extract. BHA was used as standard compound.

2.7. β-Carotene bleaching assay

The antioxidant activity of extract was evaluated by the β-carotene/linoleate model system (Miller & Luiz-Larrea 2002). 2.5 mg of crystalline β-carotene were dissolved in 100 ml round-bottom flask containing 10 ml of chloroform, and 200 µl of linoleic acid and 2 ml of Tween 40 were added into this solution. After the removal of chloroform by evaporation, 500 ml of distilled water were added to the flask under vigorous stirring. Afterwards, 3 ml of the mixture were pipetted into test tubes containing 750 µl of the sample. As soon as the tubes were shaken, their zero time absorbances were immediately measured at 490 nm using a spectrophotometer, and then the tubes incubated at 50°C for 120 min. Control contained ethanol in place of the extract. Each sample was read against blank solution. The blank solution was prepared in a similar way, except the addition of β-carotene. After 120 min, the absorbance of the mixtures was read at 490 nm. The total antioxidant activity (TAA) was expressed as percent of inhibition relative to the control after a 120 min incubation period (Al-Saikhan *et al.* 1995), as shown below:

$$TAA = 100 (DR_C - DR_S) / DR_C$$

where

DR_C = degradation rate of control = ln(a/b) / 120;

DR_S = degradation rate of sample= ln(a/b) / 120;

a and b = absorbance of samples and controls at 0 and 120 min.

2.8. DPPH radical scavenging ability

The free radical scavenging activity of extract was evaluated with DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl radical) using Blois method (1958). One milliliter of extract and BHT was added to 4 ml of 0.1 mM DPPH in ethanol, and the mixture was kept at room temperature. After 30 min the absorbance was measured at 517 nm with a spectrophotometer (Shimadzu UV-1601). The control solution was prepared by adding all reagents except the sample;

$$\text{The antiradical activity (\%)} = [(A_{Control} - A_{Sample}) / A_{Control}] \times 100$$

DPPH radical scavenging ability was calculated using overhead ratio where $A_{control}$ is the absorption of the DPPH solution, A_{sample} is the absorption of the extract/standard. The EC_{50} value of DPPH activity was calculated from the graph of antioxidant activity percentage against different extract/standard concentrations.

2.9. Reducing power assay

The reducing power of the extract was determined according to the Oyaizu method (1986). One milliliter of extract and BHT was mixed with 2.5 ml of phosphate buffer (0.2 M; pH 6.6) and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v), and then the mixture was incubated at 50°C for 20 minutes. Afterwards, 2.5 ml of trichloroacetic acid (10% w/v) were added to the mixture and centrifuged at 3,000xg (Hettich Rotina 38) for 10 min. The upper layer solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml; 0.1% w/v), and the absorbance was measured at 700 nm. The control was prepared without the extract. The increased absorbance of the reaction mixture indicated the reduced power.

2.10. Metal chelating capacity

The chelating activity of ferrous ions by black rice extract was measured according to Dinis *et al*. (1994) with a slight modification. The extract was added to 50 µl of 2 mM $FeCl_2$ and the solution was incubated at room temperature for 30 minutes. After the incubation process, 100 µl of 5 mM ferrozine were added. As the next step, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured at 562 nm against the same mixture, without the extract, as a blank. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following equation:

$$\text{Metal chelating activity (\%)} = [1 - (A_1 / A_0)] \times 100$$

where A_0 was the absorbance of the blank and A_1 was the absorbance of the extract.

2.11. α-Amylase inhibition assay

α-Amylase inhibition assay was performed according to Apostilidis *et al*. (2007). A starch solution (0,5 % w/v) was obtained by stirring 0.25 g of starch in 50 ml of 20 mM sodium phosphate buffer with sodium chloride, pH 6.9, at 50 °C for 10 min. The α-amylase solution was prepared by mixing 0.05 g of porcine pancreatic α-amylase (16 U/mg) in 100 ml of 20 mM sodium phosphate buffer with sodium chloride, pH 6.9. Black rice extract was dissolved concentration at 1 mg/ml. The colorimetric reagent was prepared mixing 3,5-dinitrosalicylic acid solution with sodium potassium tartrate and sodium hydroxide.

Equal volumes (100 µl) of extract and enzyme solution were incubated at 25 °C for 10 min. After the preincubation, 100 µl of starch solution was added to tubes and samples were incubated at 25 °C for further 10 min. One milliliter dinitrosalicylic acid was added. The tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. Then 50 µL was removed from tubes and transferred to the wells of 96-well microplate reader (Thermo Fisher Scientific, Finland). The reaction mixture was diluted by adding 200 µl distilled water, and absorbance was measured at 540 nm. The absorbance of sample blank (buffer instead of enzyme solution) and a control (buffer instead of sample extract) were also performed. The final

extract absorbance was obtained by subtracting its corresponding sample blank reading. The α-amylase inhibitory activity was calculated as percentage inhibition, as follows:

$$\text{Inhibition (\%)} = [(Abs_{Control} - Abs_{Sample}) / Abs_{Control}] \times 100$$

All analyses were carried out in triplicate and results were expressed as mean ± SD (standard deviation).

3. RESULTS AND DISCUSSION

3.1. Total phenolic, flavonoid and anthocyanins contents

The level of bioactive compounds in black rice extract is presented in Table I. The total phenol content of black rice was determined by Folin-Ciocalteu assay and found as 83.01±0.51µg GAE/mg extract. The total flavonoid and anthocyanin levels of extract were found as 21.3±0.89 µg QE/mg and 14.11±1.25 µg/mg, respectively. In another study, Kong and Lee (2010) show that total polyphenplic concentration was 18.2±0.59 and 13.3±0.31 mg GAE/g; the flavonoid content was 3.39±0.05 and 2.13±0.02 mg KE/g; the anthocyanin content was 1.89±0.05 and 1.00±0.03 mg total anthocyanins/g for whole grains from two black rice species. Sompong *et al.* (2011) were determined total anthocyanin contents as 256.61±7.66, 109.52±0.32, 244.83±2.13 mg/100 g, and total phenolic contents as 665.16±22.05, 336.69±0.72, 475.87±19.22 mg/100 g for three black rice varieties. Different results may be due to extraction conditions, kind of solvent, properties of soil, climate conditions and where grain was grown in areas.

Potential health benefits of flavonoids are associated with the powerful hydrogen-donating and also their reducing properties (Slinkard & Singleton 1977). Anthocyanins are a group of water soluble components, and are responsible for the colors of numerous fruits, vegetables, cereals and flowers. Several anthocyanins have been isolated and identified from colored rice (Chiang *et al.* 2006; Hiemori *et al.*, 2009; Frank *et al.*, 2012) and it was found that the cyanidin-3-glucoside and peonidin-3-glucoside are the predominant anthocyanins in black rice.

Table 1: The level of bioactive compounds in black rice extract[a]

	Black rice extract
Total phenolic content (µg GAE/mg)	83.01 ± 0.51
Total flavonoid content (µg QE/mg)	21.3 ± 0.89
Anthocyanin content (µg/mg)	14.11 ± 1.25

[a]Mean values ± standard deviation; n=3.

3.2. Antioxidant assays

The inhibitory effect of black rice extract on the peroxidation of linoleic acid at concentration within the range of 50-500 µg/ml was determined using the ferric thiocyanate (FTC) method. From the FTC results (Figure 1), the percentage inhibition of linoleic acid peroxidation at all concentrations was showed strong antioxidant activity, as compared to BHA, but it was not concentration-dependent manner. The inhibition effect of extract was found higher even at 50 µg/ml concentrations (73.25%±1.48). At same concentration, inhibition of lipid peroxidation found to be 82.25%±2.19 for BHA.

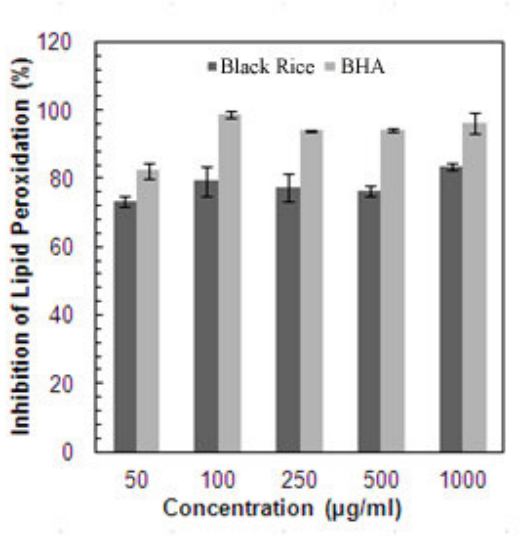

Figure 1. Antioxidant activities of black rice extract and standard compound in lipid peroxidation assay. (Data are mean ± SD, n=3)

It was also evaluated the antioxidant potential of black rice extract by the β-carotene bleaching method. In the β-carotene/linoleic acid model system, linoleic acid produces hydroperoxides during incubation at 50°C, and β-carotene undergoes rapid discoloration in the presence of hydroperoxides (Wettasinghe & Shahidi 1999). However, hydroperoxides formed in the system can be neutralized by the antioxidants from the extracts. Black rice extract exhibited effective antioxidant activity at range of concentrations (50-500 µg/ml) and the activities were determined to be comparable those of BHT standard compound (Figure 2). β-carotene bleaching activities of BHT and black rice extract were found in the range of 97.2%±0.63-98.95%±0.57 and 79.88%±3.37-94.89%±2.57, respectively. In a general evaluation, the results showed that the lipid peroxidation was effectively inhibited both methods. The other studies have also reported that black rice extract has excellent antioxidant activitiy (Park *et al.* 2008; Sompong *et al.* 2011).

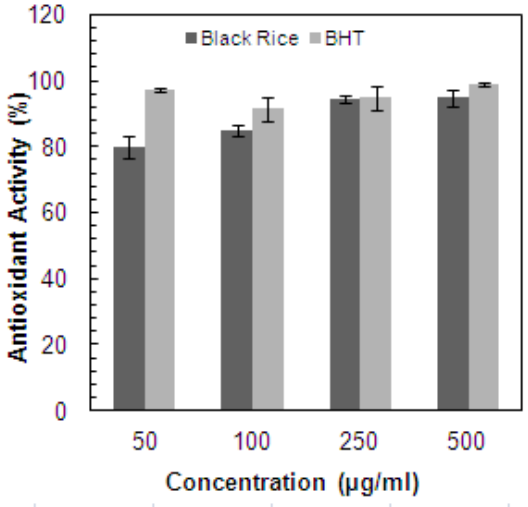

Figure 2. β-carotene bleaching activities of black rice extract and standard compound. (Data are mean ± SD, n=3)

The stable DPPH radical was used to investigate free radical scavenging capacities of extract. DPPH$^{\bullet}$ scavenging effect of black rice extract were examined at different concentration (25-500 µg/ml). Free radical scavenging activity of black rice extract increased with increasing concentration. As shown in Figure 3, black rice extract at 500 µg/ml of concentration was potent in scavenging (77.3%±0.4) the DPPH$^{\bullet}$ generated *in vitro,* when compared to BHT (84.15%±0.35). Sompong *et al*. (2011) have also reported similar findings on DPPH$^{\bullet}$ scavenging activity of three black rice varieties. EC_{50} values of black rice extract and BHT were 0.29 mg/ml and 0.11 mg/ml, respectively. Lower EC_{50} value indicates higher antioxidant activity.

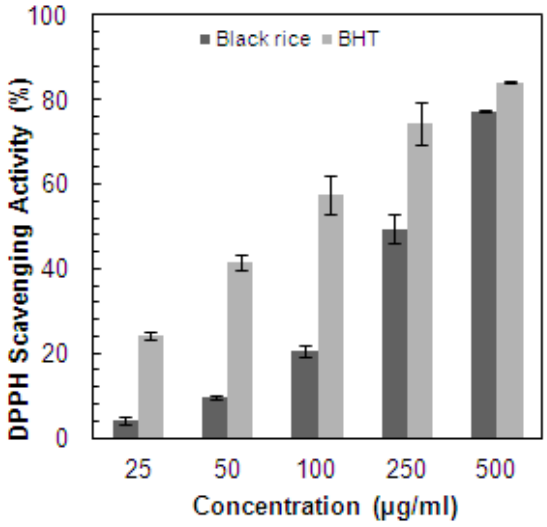

Figure 3. DPPH radical scavenging activities of black rice extract and standard compound. (Data are mean ± SD, n=3)

Fe^{3+} - Fe^{2+} transformation was investigated in the presence of extract for the measurement of reductive power. In the method, the increasing absorbance indicates increasing reductive potential. Figure 4 shows that the reductive capacities of black rice extract compared with BHT. The reducing power of sample and BHT increased with concentration-dependent manner (from 25 to 1000 µg/ml). However, at these different concentration, black rice extract did not show an effective reducing power as much as BHT.

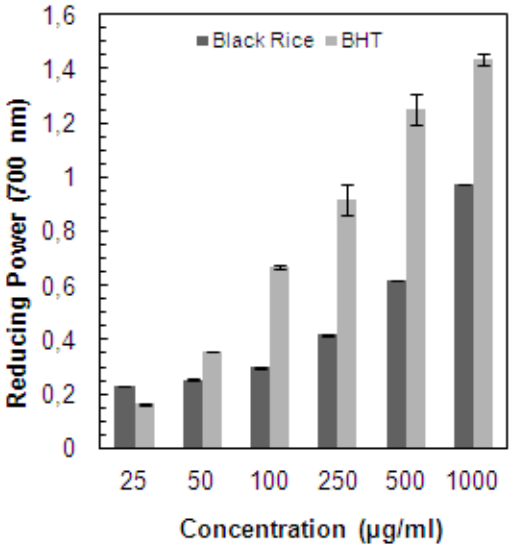

Figure 4. Reducing capacities of black rice extract and standard compound. (Data are mean ± SD, n=3)

The chelating activity of ferrous ions by black rice extract was estimated, and the percentage of inhibition of ferrozine-Fe^{2+} complex formation due to extract was calculated. Figure 5 shows the metal chelating capacity of black rice extract. The iron chelating ability of black rice extract was far lower than that of EDTA because of its strong chelator properties. The sample showed highest chelating activity at 1000 µg/ml concentration as 39.54%±3.02. We attribute in part, the chelating activity to the presence of phytic acid in black rice. In the study of Kong and Lee (2010), the phytic acid content was determined to be 47.5±0.51 and 33.4±0.56 mg/g sample in whole grain of two black rice cultivars.

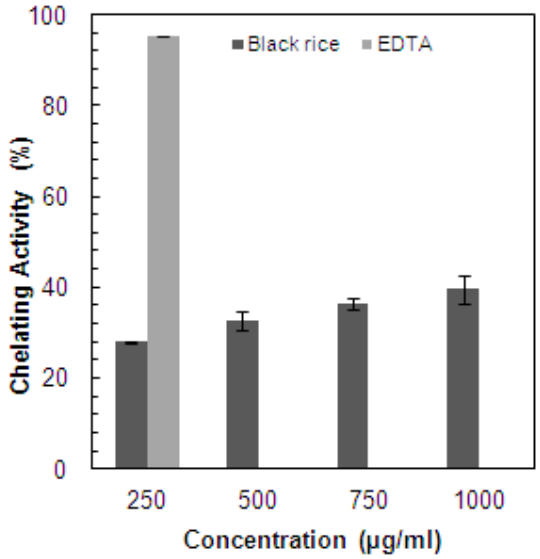

Figure 5. Metal chelating capacity of black rice extract and EDTA. (Data are mean ± SD, n=3)

3.3. In vitro inhibition of porcine pancreatic α-amylase

Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both (WHO 1999). One of an important therapeutic approach for treating diabetes is to decrease the postprandial hyperglycemia by suppressing glucose absorption through the inhibition of the carbohydrate-hydrolyzing enzymes, α-amylase and α-glucosidase, in the digestive tract. In this respect, inhibitory effect of black rice extract against α-amylase was assayed. At the concentration of 1 mg/ml, black rice extracts showed α-amylase inhibitory activity of 18.55%±4.31. The inhibitory effects of dietary polyphenols against α-amylase have attracted great interest in recently (Asgar 2013; Xiao *et al.* 2013). Tadera *et al.* (2006) reported that the flavonoids quercetin, myricetin, epigallocatechin gallate, and cyanidin were efficient inhibitors of α-amylase. Hence, the phenolic compounds contained in the extract of black rice are responsible for the α-amylase inhibitory activity observed.

CONCLUSIONS

In the present study, we aimed to determine antioxidant properties and to assess some biocomponents of black rice started to be grown in the last years in Turkey. Our results demonstrated that methanol extract from black rice showed strong DPPH scavenging effect and antioxidant ability. These results could help rice producers to promote black rice and also help to be known it in our country. Black rice generally used as an ingredient snacks and desserts in some regions. In Turkey, as in other countries, it can be aimed to use black rice in food industry due to its unusual color and sweet nutty flavor. However additional studies might be required to determine in more detail investigation on black rice and its application.

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