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Preventive effect of black rice antioxidant extract on oxidative stress induced by ethyl alcohol

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The present study investigated the black rice ethanolic extract (BREE) protective effect as natural antioxidants against oxidative stress induced by ethyl alcoholic (EA) in male rats. BREE flavonoids and phenols were determined by high performance liquid chromatography (HPLC). The study was conducted on 40 male Wistar rats weighing 170±2 g, the animals divided into 4 equal groups. The first group was given distilled water (DW) and used as a negative control (NC) group. The second group was administrated EA (6 g/kg bw/day) and used as positive control (PC) group. The other groups of rats were administrated [BREE 125 or 250 mg/kg bw + EA 6 g/kg bw/day]. Blood samples were collected after 10 days. Lipid profile, thiobarbituric acid (TBA), superoxide dismutase (SOD), glutathione (GSH) and F2-isoprostanes (F2-isoPs) were determined. The results indicate that the rats treated with EA 6 g/kg bw/day showed a significant (p≤0.05) increase in the levels of total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), TBA and F2-isoprostanes and significant (p≤0.05) decrease in high-density lipoprotein-cholesterol (HDL-C), SOD and GSH levels. The rise in serum TC, LDL-C, TBA and F2-isoprostanes were significantly attenuated by BREE 125 or 250 mg/kg bw + EA 6 g/kg bw/day. Also, the levels of serum HDL-C, SOD and GSH in BREE 125 or 250 mg/kg bw + EA 6 g/kg bw/day groups showed a significant (p≤0.05) increase as compared to PC group. The current results ascertained the beneficial effects of BREE in controlling oxidative stress induced by ethyl alcohol in male rats.

Key words: Antioxidants, black rice extract, ethanol, oxidative stress, free radicals, F_2-isoprostanes, rats.

INTRODUCTION

Rice (*Oryza sativa* Linn.) is the principle and important cereal crop for over half of the world (Clampett et al., 2002; Hansakul et al., 2011) (Figure 1). It plays an important role in the relation between the diet and health, because it contains many phytochemicals as bio-active compounds such as ϒ-oryzanol, phenylpropanoids, tocopherols, tocotrienols, phenolics, and flavonoids compounds (Clampett et al., 2002). Colored rice having

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red, purple and black rice hulled are considered to be a healthy food, especially black rice which has many health benefits because it contains a lot of antioxidative phytochemicals (higher amount of phenolic compounds and flavonoids) and anthocyanins (Ryu et al., 2003; Lum and Chong, 2012) which adds as reducing agents, metalion cheaters and free radical scavengers (Lum and Chong, 2012). Additionally, anthocyanins may reduce the risks of hypercholesterolemia [decreased total cholesterol (TC), low density lipoprotein (LDL) and triglycerides (TG)], antioxidants, cancer, inflammatory process, atherosclerosis and anti-inflammatory activities (Chen et al., 2006; Walter and Marchesan, 2011).

Moreover, phenolic compounds, flavonoids and anthocyanins have many properties such as antioxidant activity, nonmutagenic and nontoxicity, therefore, considered to be significant health implications (De Pascual-Teresa et al., 2002) and widely used in food industry (Kong et al., 2003). Previous studies reported that black rice pigments improved lipid profile and reduced oxidative stress in the liver induced by alcohol, which is due to imbalance between antioxidants and pro-oxidant systems in liver (Valcheva-Kuzmanova et al., 2004; Nordmann et al., 1992), control of lipid in blood and related diseases (Ling et al., 2001) and prevention of Alzheimer's (Miyake et al., 2012).

Ethanol alcohol has an important role of lipid peroxidation and oxidative stress because it is soluble in water and lipids. Thus, it can be spread through stomach and oesophagousmucos membrane and appears in urine and expired air. Then, it is ingested and oxidized in liver (Abraham et al., 2002). Oxidative stress is induced by alcohol due to metabolism of ethanol. Many studies indicated that there are three pathways of ethanol metabolic in the body of human, which include the following enzymes: (1) alcohol dehydrogenase, which catalyzes ethanol to form acetaldehyde which results in the free radical formation. Hydride ion of ethanol transferred to NAD^+ (Cunningham et al., 2001), also causes concomitant changes in the levels of NADH/NAD+ redox ratios and NADH (Das et al., 2005; Mantle and Preedy, 1999); (2) Microsomal ethanol oxidation system (MEOS), cytochrome p450 isoenzymes ($2E_1$, $1A_2$, $3A_4$) is catalyzed by ethanol oxidation (Lieber and DeCarli, 1970). 2E1 isoenzyme may be a significant catalyst for reactive oxygen species (ROS) formation in the consumer of alcohol and generated higher amounts of H_2O_2 (Nordsblom and Coon, 1977) and increased hydroxyl radicals generation (Klein et al., 1983). The formation of ROS such as H_2O_2 and superoxide anion (O^{-2}) represents important cause of oxidative injury in many diseases associated with free radical formation. In the presence of trace amounts of transition metals (most frequently Fe) O^{-2} and H_2O_2 generate highly-reactive hydroxyl radicals, which are then responsible for the oxidation of biological constituents (Albano, 2006), and catalase (Das et al., 2005; Mantle and Preedy, 1999).

Ethanol ingestion leads to increment of TG in blood (Zima, 1993), serum cholesterol and hepatic cholesterol ester levels (Schrocder et al., 1995) and accumulation of very low density lipoprotein (vLDL) and LDL in blood (Frohlich, 1996). Catabrese et al. (1995) indicated high levels of fatty acid ethyl esters and free fatty acids (FFA) in liver, brain, heart and kidney of rats treated with ethanol. The measurement of changes in endogenous antioxidant enzyme activity is considered a fairly sensitive biomarker of the response to oxidative stress. The cells were protected from ROS damage by glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) are primary antioxidant enzymes (Das and Vasudevan, 2007).

Zhaohua et al. (2010) evaluated the protective effect of anthocyanin-rich extract from black rice (AEBR) on chronic ethanol-induced biochemical changes in male Wistar rats and reported that rats treated with AEBR showed a better profile of the antioxidant system with normal glutathione S-transferase (GST), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities. The results demonstrate that AEBR has a beneficial effect in reducing the adverse effect of alcohol. Quantification of F2-isoprostanes (F2-isoP), prostanoids produced by non-enzymatic free radical-catalyzed peroxidation of arachidonic acid, in plasma or urine is sensitive and specific index of lipid peroxidation (LP) *in vivo* (Roberts and Morrow, 1993; Delanty et al., 1996). Moreover, ethanol-stimulated lipid peroxidation is linked to the impairment of mitochondrial oxidative phosphorylations (Bailey and Cunningham, 2002). Because alcohol-induced several organs injury, which has been linked to oxidative stress, the effect of black rice extract as natural antioxidant against oxidative stress induced by ethyl alcohol in male rats was investigated.

MATERIALS AND METHODS

Preparation of black rice ethanolic extract

Black rice grains (*O. sativa* Linn. 2015) (Figure 1) were obtained from Field Crops, Research Institute, Agriculture Research Center, Giza, Egypt. Blender (Waring 2-L Laboratory Blender- The Lab Depot Inc) was used to ground black rice (Figure 2A). The black rice extract was extracted from weighed portion (100 g) of black rice with ethyl alcohol (150 ml) at room temperature for 24 h. Filter paper Whatman No.4 was used to filter the ethanolic extract of black rice. Solvent extraction was repeated twice and the extract solution (Figure 2B) was combined and kept at 4°C until used.

High-performance liquid chromatography (HPLC)

Flavonoids/compounds and phenols/compounds were determined by Agilent 1100 HPLC equipped at 330 and 280 nm, respectively, with multiwavelength detector, Auto-sampler, degasser, quaternary pump and column compartment set at 35°C. This compound was fractionated by column zorbox ODS 5 µm, 4.6 × 250 mm. The mobile phase flow rate was set at 1 ml/min (Pascale et al., 1999; Pirjo et al., 2002).

Figure 1. Black rice grains.

A **B**

Figure 2. (A) Black rice powder. **(B)** Black rice ethanolic extract.

Biological methods

The adult albino male rats [(40 animals), weighing 170±2 g] were obtained from Vaccination Center, Helwan, Giza, Egypt, then transported to Animal House of Ophthalmology Research Institute, Giza, Egypt. Rats were housed in individual cages with screen bottoms and fed for ten days on basal diet (cellulose 5%, corn starch 70%, corn seed oil 10%, casein 10%, vitamins mixture 1%, and salt mixture 4%). The rats were weighed after equilibration, then divided into four groups (ten animals per each); everyone was assigned to one of the four diet groups; first group: normal or negative Control (NC) received normal diet, Second group: Positive Control (PC) treated orally with EA (6 g/kg bw/day), third and fourth groups: treated orally with EA (6 g/kg bw/day) + BREE (125 and 250 mg/kg bw/day, respectively). Approval was obtained from Institutional Review Board (IRB): 2015-10-239. Blood samples were collected according to Schermer (1967). To obtain blood serum, each sample was centrifuged (1500×g) at 4°C for 30 min in the dry clean centrifuge tube.

Biochemical assays in serum

The methods described by Allain et al. (1974), Fossati and Prencipe (1982), and Lopez-Virella et al. (1977) were used to determine Total cholesterol (TC), Triglyceride (TG) and high density lipoprotein cholesterol (HDL-C), respectively. The formula of Friedewald et al. (1972) was used to calculate the low-density

Table 1. Phenols and flavonoids components in BREE analyzed using HPLC.

Phenols		Flavonoids	
Compound	**mg/100 g**	**Compound**	**mg/100 g**
Benzoic	2.798	Rosmarinic	0.129
Cinnamic	1.983	Hispertin	2.309
Pyrogallal	4.003	Kampferol	0.351
Protocatchuoic	4.005	Quercetin	6.819
Hydroytyrozol	2.623	Luteolin	0.224
Gallic	0.600	Apignen	0.502
Coumarin	3.982	Naringin	1.901
Salycilic acid	7.813	Narenginin	1.400
Ferulic	0.015	Quercetrin	3.602
Vanilic	1.202	Hypersoide	0.371
Caffeic	0.812	Rutin	6.586
Catechein	4.589	Hisperdin	2.345
Catechol	0.609	---	---
Ellagic	2.207	---	---
Cholorogenic	4.998	---	---
Caffein	1.209	---	---
Total	43.448 (mg/100 g)	Total	26.539 (mg/100 g)

BREE, Black rice ethanolic extract.

lipoprotein cholesterol (LDL-C) levels for serum samples. Also, the methods described by Ohkuma et al. (1982) and Pegg (2005) were used to measure the enzymatic activity of superoxide dismutase (SOD) and thiobarbituric acid (TBA) in the serum blood samples. The instructions of Morrow and Roberts (1997) were used to measure F2-isoprostanes (F2-IsoPs) by using a competitive enzyme-linked immunoassay (ELISA) kit (Cayman Chemical, Ann Arbor, MI). All other chemicals used were purchased from Algomhorya company, Giza, Egypt.

Statistical analysis

The standard error of mean (SEM) was used to express the results. One way analysis of variance (ANOVA) followed by Fischer's LSD test was used to measure the intergroup variation. Statistical significance was considered at $P \leq 0.05$. The statistical analysis was done using the Jandel Sigma Stat Statistical Software version 2.0.

RESULTS AND DISCUSSION

Table 1 shows phenolic contents (PCs) and flavonoids contents (FCs) in BREE analyzed by using HPLC. PCs and FCs were 43.448 and 26.539 mg/100 g, respectively. The highest compounds of PCs and FCs are salicylic acid (7.813 mg/100 g) and quercetin (6.819 mg/100 g), respectively. PCs was also observed by Lum and Chong (2012), they found that PCs ranged from 22.59 to 329.53 mg/kg in Malaysia rice. Also, Salgado et al. (2010) found that PCs contents in black rice amount to 23.78 mg/g. China black rice contained the highest PCs as compared to white and red rice; also, ethanol (70%) extracts contained more PCs and FCs as compared to water (25 and 50°C) extracts (Chanida et al., 2013). Also, black rice had the highest FCs when compared with white and red rice; it has content FCs range from 16.98 to 158.47 mg/kg (Chanida et al., 2013; Melissa and Enio, 2011). Wang et al. (2014) determined the phenolic constituents in black rice by HPLC-MS/MS and they found that the negatives mode detected many components including p-coumaric acid, vanillic acid, gallic acid, ferulic acid, syringic acid, caffeic acid, protocatechuic acid, p-hydroxybenzoic acid, rutin, and quercetin. Also, Loypimai et al. (2016) studied the black colorant powder (BCP) and they showed higher concentrations of gallic acid (253.29 to 257.57 mg/g), caffeic acid (129.34 to 136.12 mg/g), ferulic acid (630.74 to 663.34 mg/g), total phenolics (187.18 to 201.61 mg gallic acid equivalent (GAE/g) and lutein (70.6 to 72.4 mg/g). Total anthocyanins of black rice extract contained 416.92 ± 0.63 mg (Hao et al., 2015). Additionally, cyanidin-3-glucoside content was 11 times higher than peonidin-3-glucoside, which was consistent with previous reports (Hou et al., 2013; Lee, 2010).

The data in Table 2 shows the results of initial, final, gain and daily gain body weight in NC, PC and EA (6 g/kg bw) + BREE, (125 and 250 mg/kg bw) administration rats. The body weights in ethanol treated group rats were found to be significantly ($P \leq 0.05$) reduced as compared to the control, which is similar to the previous reports (Lieber, 1994; Hou et al., 2010); the reason may be due to malabsorption and food intake loss (Lieber, 1994). The excessive alcohol ingestion disturbs the metabolism of most nutrients in diet resulting in malnutrition (Lieber,

Table 2. Effect of BREE on body weight of control and ethanolic administrated rats

Treatment	Initial body weight	Final body weight	Gain body weight	Daily Gain body weight
NC	170.40[a] ±0.75	191.00[a] ±0.71	20.60[a] ±0.24	2.06[a] ±0.02
PC	170.60[a] ±0.81	143.20[c] ±0.80	-27.40[d] ±0.81	-2.74[d] ±0.08
G1 (125 mg/kg bw BREE)	171.40[a] ±0.60	183.60[b] ±0.60	12.20[c] ±0.37	1.22[c] ±0.04
G2 (250 mg/kg bw BREE)	170.80[a] ±0.73	189.00[a] ±0.71	18.20[b] ±0.49	1.82[b] ±0.05
LSD	2.183	2.120	1.572	0.157

BREE, Black rice ethanolic extract; TC, Total cholesterol; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; Gand G2 Groups, 1 and 2. Values are mean (dev for 10 rats in each group. NC, Compared with negative control group: P ≤ 0.05; PC, Compared with positive control group: P ≤0.05 (One-way ANOVA followed by Fischer's LSD test).

Table 3. Effect of BREE on TC, HDL-C and LDL-C activities in control and ethanolic administrated rats.

Treatment	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
NC	148.85[d] ±0.09	107.98[b] ±0.37	38.91[d] ±0.13
PC	288.39[a] ±0.41	97.97[d] ±0.25	193.78[a] ±0.27
G1 (125 mg/kg bw BREE)	198.80[b] ±0.07	101.38[c] ±0.17	97.99[b] ±0.03
G2 (250 mg/kg bw BREE)	150.16[c] ±0.13	108.81[a] ±0.17	41.64[c] ±0.18
LSD	0.670	0.760	0.524

BREE, Black rice ethanolic extract; TC, Total cholesterol; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; Gand G2 Groups, 1 and 2. Values are mean (dev for 10 rats in each group. NC, Compared with negative control group: P ≤ 0.05; PC, Compared with positive control group: P ≤0.05 (One-way ANOVA followed by Fischer's LSD test).

2000). Also, the results indicated that rats administered EA (6 g/kg bw) + BREE, (125 and 250 mg/kg bw) gave the best results compared to NC (Table 2). This protective effect may be due to the presence of polyphenols and nutrients present in BREE (Hou et al., 2010; Jang et al., 2012; Hou et al., 2013) which might have detoxified the liver and improved the body weight to near normal levels.

Effect of BREE on TC, HDL-C and LDL-C levels in serum

Levels of TC, HDL-C and LDL-C in serum of experimental and NC rats are presented in Table 3. Treatment with ethyl alcohol caused a severe significant increase in TC and LDL-C levels (P≤0.05) in blood serum, while decrease of blood serum HDL-C level (P≤0.05) than NC. The altered redox state secondary to the oxidation of ethanol promotes lipogenesis through enhanced formation of acylglycerols. The depressed oxidative capacity of the mitochondria injured by chronic alcohol feeding also contributes to the development of the fatty liver. Accumulation of fat acts as a stimulus for the secretion of lipoproteins and the development of hyperlipemia. At the early stage of alcohol abuse, when liver damage is still minimal, hyperlipemia will prevail, whereas hypolipemia occurs in the later stages with severe liver injury (Lieber and Savolainen, 1984). On the other hand, pretreatment of rats with BREE (125 and 250 mg/kg body weight) reduced the formation of HDL-C, LDL-C and TC of serum when compared with the ethanol group, the serum TC and LDL-C levels decreased significantly, while the serum HDL-C level increased significantly in BREE group. It is our investigation that these positive effects of BREE may be due to phenols, flavonoids and cyanidin-3 glucoside, because they are known to exert anti-oxidative effect (Halliwell, 2007; Cvorovic et al., 2010; Guo et al., 2008). These results agree with the results of Hou et al. (2010) who found that TG and TC levels were increased in serum of rats after administration of ethanol and were decreased in serum of rats after administration of black rice anthocyanin-rich extract. TG, TC and LDL were decreased significantly (P≤0.05) and HDL was significantly higher (P≤0.05) in plasma of mice fed with black rice extract diet (Chiang et al., 2006).

Effect of BREE on TBA, SOD, GSH and F2-isoprostanes activities in serum

Levels of TBA, SOD, GSH and F2-isoPs changes in PC and NC group rats and in rats after administration of EA (6 g/kg bw) + BREE (125 and 250 mg/kg bw) are as shown in Table 4. Data indicated significant (P≤0.05) increase in the levels of TBA and F2-isoPs (19.68nmol/L and 0.6991 pg/ml), respectively in PC as compared to NC (6.60 nmol/L and 0.2532 pg/ml), respectively. Treatment with ethanol results in the hepatic accumulation of MDA and decrease in antioxidant are increased oxidative stress and free radical mediated tissue damage in rats

Table 4. Effect of BREE on TBA, SOD, GSH and F2-isoprostanes activities in control and ethanolic administrated rats.

Treatment	TBA (nmol/L)	SOD (U/mL)	GSH (nmol/ml)	F2-isoP (pg/ml)
NC	6.60^{d} ±0.11	25.56^{b} ±0.20	28.77^{b} ±0.07	0.2532^{c} ±0.001
PC	19.68^{a} ±0.22	15.10^{d} ±0.07	12.10^{d} ±0.06	0.6991^{a} ±0.003
G1 (125 mg/kg bw BREE)	9.24^{b} ±0.11	22.54^{c} ±0.13	21.33^{c} ±0.10	0.3271^{b} ±0.002
G2 (250 mg/kg bw BREE)	7.90^{c} ±0.07	26.06^{a} ±0.10	29.04^{a} ±0.09	0.2513^{c} ±0.001
LSD	0.42449	0.40097	0.253	0.005

BREE, Black rice ethanolic extract; TC, Total cholesterol; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; Gand G2 Groups, 1 and 2. Values are mean (dev for 10 rats in each group. NC, Compared with negative control group: P ≤ 0.05; PC, Compared with positive control group: P ≤0.05 (One-way ANOVA followed by Fischer's LSD test).

and human (Minana et al., 2002). The reduced levels may be due to ROS being generated during alcohol metabolism that lead to lipid peroxidation and GSH oxidation.

The important tripeptide non-enzymatic antioxidant is GSH, which is thought to be an important endogenous defense molecule against peroxidative cellular destruction membranes. It reacts directly with electrophilic metabolites and ROS, serves as a substrate for several enzymes and prevents essential thiol groups from oxidation. In the present study, GSH concentration was reduced significantly in alcoholic-treated group rats (Table 4) which were in support with several reports (Jang et al., 2012; Chiang et al., 2006). Administration of black rice ethanolic extract with ethanol significantly altered the activities of both the non-enzymatic and enzymatic antioxidants to near the levels of normal group.

Interestingly, SOD and GSH were significantly (P≤0.05) lower (15.10 u/mL and 12.10 nmol/ml) in PC rats, respectively than NC (25.56 u/mL and 28.77 nmol/ml), respectively. On the other hand, BREE (125 and 250 mg/kg bw) treated groups significantly (P≤0.05) alleviate the TBA, SOD, GSH and F2-isoprostanes to near normal levels (Table 4). This decrease could be due to ROS inefficient scavenging which might be implicated to oxidative inactivation of enzymes (Jayaraman et al., 2009), which proves to be a potent antioxidant, a finding that correlates with recent reports (Chiang et al., 2006; Arulmozhi et al., 2012; Hsieh et al., 2008). Furthermore, the antioxidant property and the oxygen-radical scavenger of the extract may therefore be due to the presence of polyphenolic high content compounds such as anthocyanins and flavonoids (Mira et al., 2009; Yawadio et al., 2007; Zhang et al., 2010). Hou et al. (2010) found that ethanol treatment caused a severe increase in serum MDA and decrease in GSH concentration (P≤0.05) in rats, while, black rice anthocyanin-extract reduced the formation of serum MDA and restored the levels of non-enzymatic antioxidant in tissues. Leiber et al. (1995) have reported increased hepatic F2-isoPs in liver biopsy specimens from alcohol fed baboons. Other investigators have reported increased plasma F2-isoP levels in alcoholic hepatitis and cirrhosis patients (Lieber, 1997; Delanty et al., 1996; Aleynik et al., 1998).

Conclusion

The results showed that BREE has a protective action against EA induced oxidative stress; this protective effect is mainly due to its antioxidant properties and free radical scavenging activity which has been suggested as a possible mechanism of action of BREE against ethanolic toxicity. It indicates the therapeutic values of black rice and their potential role in preventing cardiovascular diseases. This observation points to a new direction when trying to understand the physiological function of black rice extract as a benefit to human health.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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