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Antihyperglycemic Effect and Antioxidants Properties of Black Rice (*Oryza sativa* L. *indica*) Cereal and Anthocyanin Extract on Health and Histopathology of Hyperglycemic Rats

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Abstract: Black rice (*Oryza sativa* L. *indica*) is rich in anthocyanin hence it could be used as functional food such as cereal for hyperglycemic patient. Black rice cereal supplemented with black soybean (*Glycine max* L. *Merr*) (RSC) was prepared as isocaloric feed for three groups of hyperglycemic rats. The first group (F0) was treated only with RSC, while the other two also receive 40 ppm (F4) and 80 ppm (F8) black rice bran anthocyanin extract (BRE). Non- hyperglycemic and hyperglycemic rats which were fed with standard feed were used as control (C) and hyperglycemic group (H) respectively. After 6 weeks experiments, blood glucose level, insulin resistance and MDA value were decreased in treatment groups, which were more significant in F4 and F8 than F0, while FRAP was increased. RSC and BRE alleviated inflammatory and steatosis in pancreas, liver and kidney as shown by the tissue preparation with Hematoxylin and Eosin (H &E) staining.

Key words: Black rice cereal, anthocyanin extract, hyperglycemia, insulin, antioxidant, histopathology

INTRODUCTION

Anthocyanin pigment is a natural antioxidant which is belong to the phenolic compounds (flavonoid) and provide protection from various types of oxidants through several mechanisms (Castaneda-Ovando *et al.*, 2009; Kong *et al.*, 2003). Its antioxidant activity has been shown inhibit neuronal and cardiovascular diseases, anticarcinogen, antineoplastic, antiviral and antiinflammatory (Konczak and Zhang, 2004; Stintzing and Carle, 2004). Anthocyanin has antidiabetic activity by stimulated the secretion of insulin from pancreatic β cells and suppress postprandial glucose levels (Ghosh and Konishi, 2007). Anthocyanin from black rice has been shown to have high antioxidant activity and able to suppress insulin resistance and plasmic oxidants level (Guo *et al.*, 2007).

Black rice (*Oryza sativa* L. *indica*) is a rice cultivar which is rich in dark anthocyanin pigment on its aleuron layer. It only gains its popularity around a decade in Java although this island historically has some varieties of black rice such as *cempo ireng*, *melik* and *jlitheng* (Kristantini, 2009). While black steamed rice was referred in the 19th century Javanese culture encyclopedia *Serat Centhini*, it is debatable whether it was intended for black rice or white rice which was colored with black glutinous rice (Haryono, 1998; Sunjata *et al.*, 2014). But it historically has been known as the food for the kings of Surakarta Sultanate (Kristantini, 2009). Unfortunately, black rice is not a

preferable staple food even for diabetic patients because of its relative hard texture and distinct flavor than common white rice. Black rice processing into cereal is expected can improve its sensory quality while still obtaining its benefits as functional food. However, cooking or processing may reduce anthocyanin level and antioxidant potential of black rice by more than 60% (Hartati, 2012; Hiemori *et al.*, 2009). Putri (2015) found that black rice anthocyanin extract which was heated up to 135°C had a very high degradation rate which lead to complete destruction of anthocyanin. However, Kurilich *et al.* (2015) found that cooking process increase the bioavailability of anthocyanin because the heat would destroy the cell walls and would be more easily to be accessed by our digestive system.

This study was evaluating whether the processing of black rice into cereal still retains its antihyperglycemic and antioxidative properties on hyperglycemic rats. The cereal was substituted with black soybean (*Glycine max* L. *Merr*) to increase the anthocyanins level and nutritional properties. Furthermore, different dose of black rice bran anthocyanin extracts (BRE) fortification was done to alleviate the deleterious effect of processing on black rice cereal supplemented with black soybean (RSC).

MATERIALS AND METHODS

Chemicals and reagents: The reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ),

1,1,3,3-Tetraethoxypropane (TEP), 2-thiobarbituric acid, streptozotocin (STZ) and nicotinamide (NA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-(+)- ascorbic acid (vitamin C) was purchased from J.T. Baker (Selangor, Malaysia). Glucose GOD FS kit was purchased from DiaSys (Holzheim, Germany). Rat Insulin (INS) ELISA kit was purchased from Qayee-Bio (Shanghai, China). Ethanol 96% and citric acid were also purchased from Sigma-Aldrich. Other chemicals were kindly provided by Food Chemical and Biochemical Laboratory, Faculty of Agriculture Technology, Gadjah Mada University (UGM), were from Merck Millipore.

Cereal (RSC), anthocyanins extract (BRE) and feed preparation: Black rice variety Toraja was purchased from organic farmland in Malang, East Java. Black soybean variety Mallika was purchased from local distributor. Black rice bran flour was obtained by grinded it with dry grinder (BL-301 GS G/Y) for 1-2 min. The anthocyanins extraction process was according to Kristiana *et al.* (2012). Black rice bran was macerated for three hours on ethanol-citric 3%w/v (1:10), filtered, then evaporated with distilling rotary evaporator (IKA RV 06-ML 1-B) for 2 h.

The cereal composition was as follow: 63.23% black rice flour, 9.26% black soybean flour, 6.51% coconut oil, 2.71% olive oil, 1.62% sesame oil and 16.67% arenga sugar, all in w/w. The dough was thinned and baked in the oven at 140°C for 15 min (Astuti and Marsono, 2015). Dose of BRE was calculated by mg anthocyanin per kg body weight (bw) of rat, i.e., 40 and 80 ppm.

AIN-93M was used as the standard feed for rat (Reeves *et al.*, 1993). AIN-93M Mineral Mix and AIN-93-VX Vitamin Mix were purchased from MP Biomedicals (Santa Ana, CA, USA). Cereal feed isocalori was prepared by substitute 20% calori of standard feed with RSC, i.e., 180 g RSC per kg cereal feed as shown in Table 1.

Total anthocyanins (TA) and phenolic content (TPC) analysis: TA in cereal and anthocyanin extract were analyzed by pH-differential method (Giusti and Wrolstad, 2001). Feed (1 g) was diluted with ethanol-citric 3% (w/v) until 10 mL. Each aliquot (0,1 mL) were mixed with 0,9 mL potassium chloride 0.025 M (pH 1) and sodium acetate 0,4 M (pH 4.5) buffer respectively. Absorbance was measured at 530 nm and 700 nm using spectrophotometer (Spectronic 200) after 15 min incubation. Molecular weight ($M_w = 449.2$ g/mol) and molar absorptivity ($\epsilon = 26900$ L/mol.cm) were regarded as cyanidin-3-glycoside.

TPC using Folin-Ciocalteu reagent was based on the method by Shui and Leong (2006). Gallic acid (0-200 mg/ml) was used as standard and the absorbance was measured at 765 nm. The data was served as mg Gallic Acid Equivalent (GAE)/g.

DPPH, FRAP and MDA analysis: Antioxidant activity by DPPH method was based on Sompong *et al.* (2011) with modification. DPPH 0.1 M was prepared by diluted 0.0039 g DPPH with ethanol 96% until 100 mL. DPPH solution (1 mL) was mixed with 200 μ L aliquot and the absorbance was measured at 515 nm after 30 min incubation. The data was served as mg vitamin C equivalent (VCE)/L with the help of vitamin C standard curve (0-40 mg/L).

Antioxidant capacity by FRAP method was described by Shui and Leong (2006) with $FeSO_4 \cdot 7H_2O$ for standard curve (0-1000 μ M). The absorbance was measured at 593 nm and the data was serves as μ mol/L.

MDA analysis by TBARS method was described by Wuryastuti *et al.* (1996). The absorbance was measured at 532 nm and the data was served as nmol/mL blood plasma.

Determination of blood glucose, insulin and HOMA2-IR: Rat's blood plasma was used to determine blood glucose and insulin level. Blood glucose determination was following the procedure from Glucose GOD FS kit (DiaSys) while insulin level from Rat Insulin (INS) ELISA kit (Qayee-Bio) procedure. HOMA2-IR was calculated using HOMA2 calculator (Lib 2.2.3) from <http://www.dtu.ox.ac.uk>.

Animal experimental: Twenty five male Sprague Dawley rats about 2 months old were acclimated for 3 days with standard feed. The rats were divided into five groups: non-hyperglycemic rats as control (C) and untreated hyperglycemic rats (H) fed by standard feed; and hyperglycemic rats fed by cereal feed (F0), cereal diet+40 ppm BRC (F4) and cereal diet+80 ppm BRC (F8). Hyperglycemic condition in rats was induced by STZ 65 mg/kg and NA 230 mg/kg (Szkudelski, 2012). The experiment last for 6 weeks. The rats were weighing and the blood samples were taken via *retro-orbital plexus* per week after 8-12 hours fasting. After 6 weeks, pancreas, liver and kidney were collected and were stained with H&E stain which was conducted by Pathology Laboratorium, Faculty of Veterinary Medicine, UGM. All procedures related to animal experiment in this study was approved by Ethical Clearance Committee of LPPT, UGM, Indonesia (Approval number: 306/KEC-LPPT/VIII/2015).

Design and statistical analysis: Completely Randomized Design was used for rats experimental. The data were subjected to statistical analysis using one-way analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) at the 95% confidence level. *In vitro* results were expressed as means \pm standard deviation (SD) while *in vivo* results as means \pm standard error by means (SEM).

RESULTS AND DISCUSSION

Cereal feed and anthocyanins extract specification:

The specification of cereal feed and BRE were shown in Table 2. The results showed that cereal feed still has antioxidant properties even after the processing. Putri (2015) reported that heating anthocyanin extract at 121°C would destroy all anthocyanin in 20 min, but the BRC was baked at 140°C for 15 min. It is likely that other components of the cereal brought protective effect to anthocyanin from oxidation during heating.

The anthocyanins level of extract was close to Putri (2015) who used the same variety of black rice, i.e., 1.35 mg/g rice bran, but higher than Widarta *et al.* (2013) and Monika *et al.* (2013) who used different variety of black rice. The phenolic content was lower than Monika *et al.* (2013) but higher than Widarta *et al.* (2013). Chakuton *et al.* (2012), Kristamtini (2014) and Sugiat *et al.* (2010) found that the rendement, total anthocyanin and phenolic content of different varieties of black rice varies greatly.

Blood glucose, insulin and insulin resistance: The average blood glucose levels of the rats were shown at Fig. 1. Five days after STZ induction, the hyperglycemic groups had significantly increased blood glucose levels, but were down gradually on the groups threatened with RSC and BRE. Takikawa *et al.* (2010) explained that anthocyanins would activated AMP-activated Protein Kinase (AMPK) on white adipose tissues, muscles and liver, thus increasing glucose transporter 4 (Glut4) and increasing the absorption and utilization of glucose on muscles and white adipose. However, Masiello *et al.* (1998) reported that the dose of 65 mg/kg STZ along with 230 mg/kg NA only affected the blood glucose level but not insulin level which was relative stable until 9 weeks of experiments. The result was similar with present study as shown in Table 3.

Anthocyanin able to stimulate insulin secretion of pancreatic β cells (Ghosh and Konishi, 2007). Corkey (2011) explained that hyperinsulinemia might cause insulin resistance and indicated prediabetes type 2. The increasing or decreasing of insulin level doesn't always predetermine insulin resistance, although Pearson correlation test (data not shown) showed that insulin resistance had much stronger correlation to insulin level than glucose level. All treatment groups except F0 had decreasing of insulin level and insulin resistance although the difference between week 0 and 6 (by t-test) were not significant. It showed that cereal feed alone fail to alleviate insulin resistance, but cereal feed with BRE fortification were able to decrease insulin resistance.

The HOMA2-IR result was likely correlated with the increasing of body weight of each groups. Abassi *et al.* (2002) found that insulin resistance had a tendency to increase along with the increase of Body Mass Index (BMI). The mean±SEM body weight of C and F0 groups on the last week were 295.2±9.9 and 274.6±11.8 g, respectively, while F4 and F8 were 214.3±12.3 and

Table 1: Composition of standard feed AIN-93M and cereal feed per 1000 g

Composition	AIN-93M (g)	Cereal feed (g)
Corn starch	620.7	505.8
RSC	-	180
Casein	140	118.9
Sucrose	100	100
Soybean oil	40	13.9
Na-CMC	50	50
Mineral mix	35	30.9
L-Cystine	1.8	1.8
Vitamin mix	10	10
Cholin bitartrate	2.5	2.5

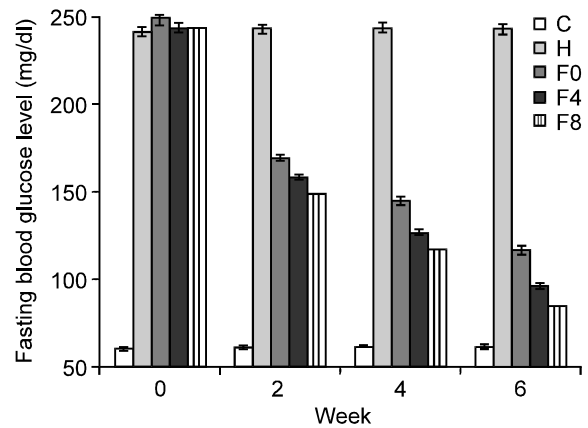


Fig. 1: Fasting blood glucose level (mg/dl), mean±SEM

208.2±14 g, respectively. Keep in mind that rat feed contains complex carbohydrate and sugar with approximately 348.50 and 346.32 kcal/100 g for each standard and cereal feed.

Antioxidant activities: Hyperglycemic condition generates oxidative stress through some mechanisms, i.e., glucose autooxidation, polyol pathway and protein glycation (Droge, 2002; Blaton, 2002). The higher the oxidative stress, the more antioxidant which is used to maintain body homeostasis which mean the plasmic antioxidant level might be low. The FRAP analysis on blood samples aimed to determine the antioxidant ability of cereal diet and anthocyanins extract to reduce Fe^{III}-TPTZ into its ferro form as shown in Fig. 2 (Benzie and Strain, 1996). The higher the value showed the higher of antioxidant level on blood samples. The data showed that cereal feed and BRE increased the plasmic antioxidant capacity on treatment groups.

Meanwhile, malondialdehyde (MDA) is the final form of lipid oxidation and it is appears on the surface of blood vessels in diabetic patients (Yan *et al.*, 1994) and so it can be used as a parameter of lipid oxidation (de Zwart *et al.*, 1999). The higher of MDA value mean the higher of lipid oxidation activity in hyperglycemic rats. Fig. 3 shows that cereal feed and BRE able to reduce plasmic MDA value and so had a positive effect on the health of hyperglycemic rats.

Table 2: Specification of cereal feed and BRE

	Cereal feed	BRE
Rendemen (%w/w)	-	47.60
Total antocyanin (mg/g), mean (SD)	0.06 (0.01)	1.74 (0.06)
Total phenolic (mgGAE/g), mean (SD)	7.05 (0.22)	10.21 (3.23)
DPPH (mgVCE), mean (SD)	14.66 (0.75)	13.89 (0.88)
FRAP (µmol/L), mean (SD)	592.67 (50.92)	527.23 (44.50)

Table 3: Insulin level and HOMA2-IR

	C	H	F0	F4	F8
Insulin level (µIU/ml)					
Week 0, Mean±SEM	23.11±1.38 ^a	24.57±3.32 ^a	31.17±1.25 ^{ab}	43.09±3.05 ^b	34.87±3.52 ^c
Week 6, Mean±SEM	23.55±1.76 ^a	26.10±3.58 ^a	44.68±2.13 ^b	40.42±5.30 ^b	36.42±1.69 ^b
HOMA2-IR					
Week 0, Mean±SEM	2.59±0.14 ^a	3.95±0.51 ^b	5.15±0.24 ^c	6.76±0.42 ^d	5.55±0.52 ^c
Week 6, Mean±SEM	2.66±0.20 ^a	4.23±0.58 ^b	5.77±0.25 ^c	5.01±0.69 ^{bc}	4.42±0.18 ^b

Description: Different superscript on the same line show a significant different (p>0.05)

Table 4: Semiquantitative histopathologic reading

	C	H	F0	F4	F8
Pancreas					
Pancreatitis (%)	0	20 (1 ¹)	0	0	0
Pancreatic steatosis (%)	0	20 (1 ¹)	0	0	0
Liver					
Necrosis (%)	60 (2 ¹ , 1 ²)	100 (1 ¹ , 2 ² , 2 ³)	60 (1 ¹ , 2 ²)	80 (3 ¹ , 1 ²)	80 (2 ¹ , 1 ² , 1 ³)
Steatohepatitis (%)	60 (2 ² , 1 ³)	0	0	60 (2 ¹ , 1 ²)	20 (1 ²)
Kidney					
Nephritis (%)	60 (1 ¹ , 2 ²)	60 (2 ¹ , 1 ²)	60 (2 ¹ , 1 ²)	0	20 (1 ¹)
Renal steatosis (%)	20 (1 ²)	20 (1 ²)	0	0	0

Description: Numbers in parentheses show the number of rats which were diagnosed per group of five rats, while the superscript numbers show the level of damage in scale of 0 to 3. Level 0 means no damage was observed, while level 1, 2 and 3 means <25%, 25-50% and >50% damage was observed respectively

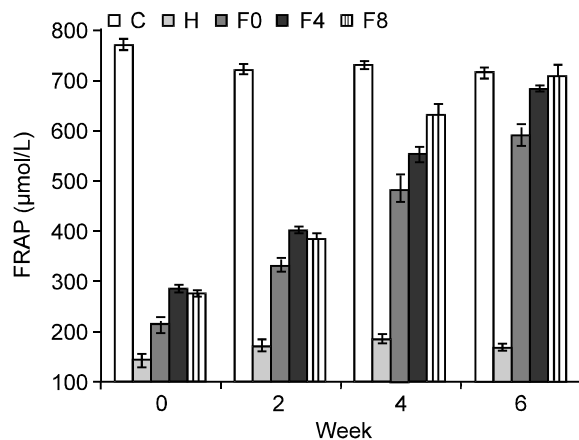


Fig. 2: Plasmic FRAP value (µmol/L), mean±SEM

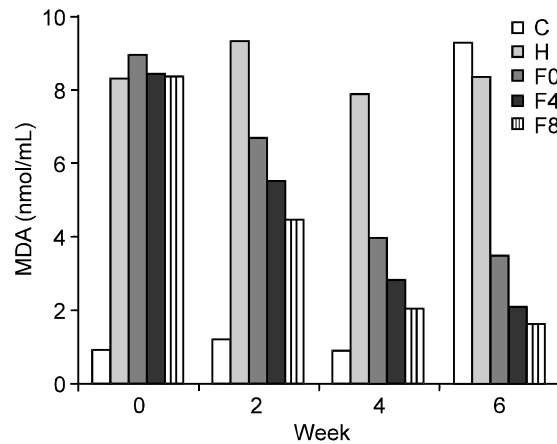


Fig. 3: Plasmic MDA value (nmol/mL), mean±SEM

Histopathology of pancreas, liver and kidney: The consumption of RSC and BRE alleviated pancreatitis, liver necrosis, nephritis, pancreatic steatosis and renal steatosis, but not steatohepatitis (Table 4). Jankowski *et al.* (2000) reported that anthocyanin could inhibit the development of acute pancreatitis by reduce the swelling and decrease lipid peroxidase and deaminase activity. The same result was shown on nephritis or renal inflammation data.

STZ induction will damage the DNA of pancreatic β cells which lead to the increase the activity of poly (ADP-

ribose) polymerase (PARP-1) to repair the damage, but this will increase the use of intracellular ATP and NAD⁺ which lead to cell necrosis (Szkudelski, 2012). Aboonabi *et al.* (2014) reported that the anthocyanin from red pomegranate could alleviate the damage on the liver of diabetic rats, while Asgary *et al.* (2014) found that cornelian cherry anthocyanin extract could alleviate inflammation on liver of diabetic rats. This present study found that on the treatment groups, the necrotic level of the liver were decreasing compared to hyperglycemic group.

The data showed that cereal feed and BRE could alleviate pancreatic and renal steatosis but steatohepatitis was not found in hyperglycemic group. Jang *et al.* (2012) reported that black rice extract could alleviate hepatic steatosis by increase the expression of fatty acid metabolism-related genes on the rats. In present study, steatohepatitis was not found on hyperglycemic group probably due to fat reserve on the rat bodies were greatly depleted. Darmono (1995) explained that steatohepatitis was the lowest level liver damage while necrosis was the highest. The rats on hyperglycemic group were very thin and weak and had cloudy red eyes.

Conclusions: Fortification RSC with BRE evidently able to improve the health profile of hyperglycemic rats by increasing FRAP value, decreasing MDA value, depressing blood glucose level and alleviate the damage caused by hyperglycemia on pancreas, liver and kidney. But RSC alone fail to alleviate insulin resistance which was showed by the value of HOMA2-IR. The higher dose of BRE had higher effect on health profile of hyperglycemic rats.

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