Food Research 7 (5) : 361 - 367 (October 2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Black rice (*Oryza sativa* L. *indica*) bran ethanolic extract improved insulin levels and total antioxidant capacity in type 2 diabetic rats

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Article history:
Received: 22 December 2021
Received in revised form: 12 February 2022
Accepted: 18 July 2022
Available Online: 31 October 2023

Keywords:
Black rice bran,
Insulin levels,
Total antioxidant capacity,
Type 2 diabetes mellitus

DOI:
https://doi.org/10.26656/fr.2017.7(5).1024

Abstract

Type 2 Diabetes Mellitus (T2DM) showed a decrease in insulin levels and Total Antioxidant Capacity (TAC). Black rice (*Oryza sativa* L. *indica*) bran contained a phytochemical constituent that could ameliorate insulin levels and TAC in T2DM. This study aimed to determine the effect of black rice bran ethanolic extract on insulin levels and TAC in T2DM rats. The black rice bran ethanolic extract was prepared using the maceration method. It was a true experimental study with pre-post-test randomized control group design using forty-two male Wistar rats which were divided into six groups: healthy control, T2DM control (C-), T2DM + Metformin (C+) and T2DM + black rice bran ethanolic extract at the doses of 15 mg/200 g BW (T1), 30 mg/200 g BW (T2) and 60 mg/200 g BW (T3). The treatment was given oral gavage for 21 days. The results showed that there was a significant increase in insulin levels and TAC in treatment groups compared with the T2DM control ($p<0.05$). The improvement in TAC from the treatment of black rice bran ethanolic extract was the same as metformin. It could be concluded that black rice bran ethanolic extract increased insulin levels and TAC in T2DM rats with the most effective dose of 60 mg/200 g BW. The result indicated that black rice bran ethanolic extract at a dose of 60 mg/200 g BW was as effective as metformin in increasing TAC.

1. Introduction

Diabetes Mellitus is one of the greatest public health challenges worldwide. The World Health Organization (2019) reported that the number of people with diabetes is expected to increase from 171 million in 2000 to 366 million in 2030. Based on the Diabetes Atlas 10th Edition from the International Diabetes Federation (2021), the number of people with Type 2 Diabetes Mellitus (T2DM) continues to rise in most nations, with over 90% of undiagnosed diabetics residing in middle and developing countries, with Africa, Southeast Asia, and the Western Pacific accounting for more than half of all undiagnosed cases. Diabetes Mellitus, if not properly diagnosed and treated, causes complications that lead to comorbidities and mortality (Lotfy *et al*., 2017). The inability of pancreatic cells to secrete insulin to maintain normal blood glucose levels is a common pathophysiology of T2DM (Saisho, 2015). This impaired insulin secretion was caused by a decrease in insulin secretory capacity, a decrease in pancreatic cell mass, or both (Cantley and Ashcroft, 2015).

Oxidative stress plays a role in the pathogenesis of insulin resistance, impaired insulin secretion and impaired hepatic glucose in T2DM (Maslov *et al*., 2019). In the presence of an imbalance in the antioxidant defence system, either due to weakened endogenous antioxidants or deficiency of exogenous antioxidants, oxidative stress subsequently worsens in T2DM. A previous study found that patients with T2DM, with or without complications, had lower TAC than healthy people (Ramazani *et al*., 2019; Lotfi *et al*., 2020). Oxidative stress in pancreatic cells in T2DM occurs due to hyperglycemia, hyperlipidemia, and inflammation. Exposure of pancreatic cells to excessive levels of free fatty acids and hyperglycemia can lead to β-cell apoptosis (Oguntibeju, 2019), impaired insulin gene expression and decreased insulin secretion (Oh *et al*., 2018). Currently, the involvement of lifestyle changes and functional food has been conducted as a strategy in T2DM prevention (Alkhatib *et al*., 2017). Plants with high antioxidant activity can be utilized to treat T2DM (Rajendiran *et al*., 2018).

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

Black rice (*Oryza sativa* L. *indica*) bran has become increasingly popular for daily consumption because of its high phytochemical contents which are beneficial for health and disease prevention. As a by-product of black rice milling, black rice bran is quite accessible and affordable. Black rice bran has a high antioxidant content such as flavonoids, especially anthocyanins, and phenols. Despite its benefits, the use of black rice bran is relatively limited (Issara and Rawdkuen, 2016). Cyanidin 3-glucoside, the major anthocyanin in black rice bran, is beneficial for improving insulin resistance (Belwal *et al*., 2017). Black rice cereal enriched with the brans extract has been shown to be able to improve blood glucose levels, insulin resistance, MDA and inflammation in hyperglycemic rats (Krisbianto *et al*., 2016). In alloxan-induced diabetic rats, black rice bran ethanolic extract was demonstrated to have an anti-diabetic effect, and C3G ethanolic extract also improved hyperinsulinemia in diabetic nephropathy rats (Wahyuni *et al*., 2016; Zheng *et al.*, 2020). Anthocyanins also showed antioxidant properties as a free radical scavenger, thus reducing oxidative stress (Martín *et al*., 2017). Despite the benefits of black rice bran as an anti-diabetic agent, prior research on the effect of administering black rice bran ethanolic extract on insulin levels and antioxidant status was limited, and no specific testing on the T2DM rats model was performed. Therefore, this study aimed to determine the effect of black rice (*Oryza sativa* L. *indica*) bran ethanolic extract on insulin levels and TAC in T2DM rats.

2. Materials and methods

2.1 Ethical statement

The experiments were approved by the Health Research Ethics Commission of the Faculty of Medicine, Universitas Diponegoro, with the number 18/EC/H/FK-UNDIP/II/2021.

2.2 Preparation of black rice bran ethanolic extract

Black rice bran was obtained from organic and good quality black rice from the Jeliteng cultivar, produced by the Al-Barokah Rice Farmers Group in Semarang Regency, Indonesia. Black rice bran was macerated using an acidified solvent (Ethanol: HCl = 85:15) with a ratio of 10:1 (v:w) for 48 hrs and was occasionally stirred. The extract was filtered using Whatman No. 1, followed by evaporation using a vacuum rotary evaporator at a temperature of 70^{o}C into a thick extract and stored in a freezer at -20^{o}C until use (Rukmana *et al.*, 2017).

2.3 Animal and treatments

A total of forty-two male Wistar rats (*Rattus norvegicus*, obtained from Center for Food and Nutrition Studies, Universitas Gadjah Mada, Special Region of Yogyakarta, Indonesia) aged 8 weeks, weighing 150-200 g were selected for this study. The rats were kept in individual stainless-steel cages with a setting of a 12-12-hour dark-light cycle at a temperature of 23±2^{o}C and humidity of 50±5^{o}C. Rats were divided into six groups, healthy control (N), T2DM control (C-), T2DM+metformin treatment at a dose of 9 mg/200 g BW (C+) and T2DM+black rice bran ethanolic extract treatment at doses of 15, 30 and 60 mg/200 g BW rats (T1, T2 and T3, respectively). Each rat was given a standard feed of 20 g per day and ad libitum water during acclimatization and treatment. The treatment of metformin and black rice bran ethanolic extract was carried out for 21 days.

2.4 Type 2 Diabetes Induction

T2DM induction was performed in C-, C+, T1, T2 and T3 groups after acclimatization for 7 days. The rats were fed a high-fat diet of 20 mg/day for 14 days, followed by intraperitoneal injections of streptozotocin (Cayman Chemical, Michigan, USA) and nicotinamide (Sigma-Aldrich, Missouri, USA). A single dose of nicotinamide (110 mg/kg BW, diluted in saline buffer) was injected 15 mins before the injection of streptozotocin (45 mg/kg BW, diluted in citrate buffer). Three days after STZ-NA administration, blood glucose levels were measured. Rats were considered to develop T2DM if blood glucose levels were > 250 mg/dL (Ghasemi *et al.*, 2014).

2.5 Measurement and laboratory analysis

Blood samples were taken after the T2DM induction and after the intervention ended. The rats fasted 8-12 hours before blood sampling. Approximately 3 mL of blood drawn from the retro-orbital plexus was centrifuged for 15 mins at 4,000 rpm to yield serum. Blood glucose levels were measured by the GOD-PAP method (DiaSys, Holzheim, Germany). TAC was measured using the Total Antioxidant Capacity Assay Kit (FRAP) (Fine Test, Wuhan, China). Insulin levels were measured using the enzyme-linked immunosorbent assay (ELISA) method (Fine Test, Wuhan, China).

2.6 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics software. The normality of the data was tested using the Shapiro–Wilk test. The significance between pre- and post-intervention of all parameters was analyzed by the paired t-test. Differences between the groups of rats were analyzed by one-way ANOVA followed by a post hoc test for the normally distributed data and by the Kruskal–Wallis test followed by the Mann-Whitney test

https://doi.org/10.26656/fr.2017.7(5).1024

when the data were not normally distributed. Differences were considered to be significant if $p < 0.05$.

3. Results

3.1 Body weight changes

There was a significant increase in body weight after being fed a high-fat diet for 2 weeks in the T2DM induction group (C-, C+, T1, T2 and T3) compared to the N group ($p < 0.05$). Then, following STZ-NA injection, body weight in the T2DM induction group was significantly lowered. The C-group continued to experience weight loss over the 21-day treatment period. In contrast, C+, T1, T2 and T3 groups presented an improvement in body weight (Figure 1).

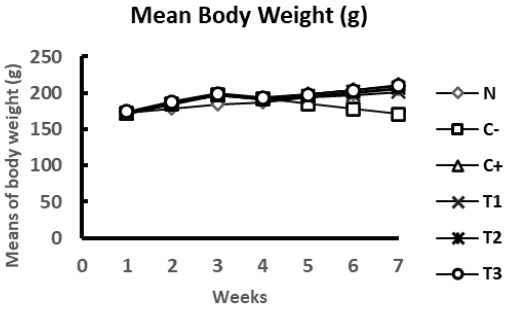

Figure 1. Changes in rats' body weight between week 1 until 7 of the intervention. Six groups of rats (n= 7 each group) consist of N: healthy control, C-: T2DM control and C+: metformin 9 mg/200 g BW; T1: black rice bran extract 15 mg/200 g BW; T2: black rice bran extract 30 mg/200 g BW; T3: black rice bran extract 60 mg/200 g BW.

3.2 Blood glucose level

Figure 2 shows that the blood glucose levels of the T2DM-induced group before the treatment exceeded 250 mg/dL. The rat's blood glucose level in the normal control group (N) remained relatively stable at a normal level. On the contrary, the diabetic control group (C-) continuously suffered from persistent hyperglycemia (266.956±3.140 mg/dL). Both treatments with black rice bran ethanolic extract (P1, P2 and P3) and metformin (C+) significantly decreased blood glucose level and also manifested a significant difference with C-group ($p<0.05$). Among the groups treated with black rice bran ethanolic extract, T3 group presented the best improvement of blood glucose levels (95.885±1.334 mg/dL) compared with T1 and T2 group (139.629±4.730 mg/dL and 110.400±2.640 mg/dL, respectively). No significant difference was observed in blood glucose levels after the intervention in the T3 group compared with the C+ group.

3.3 Insulin levels

Before the treatment period, T2DM-induced groups had a significant decrease in insulin levels compared to the N group (Table 1). After 21 days, intervention with black rice bran extracts and metformin presented an increase in insulin levels ($p < 0.05$). Significant differences in insulin levels after the intervention were observed in the C+, T1, T2, and T3 groups compared to

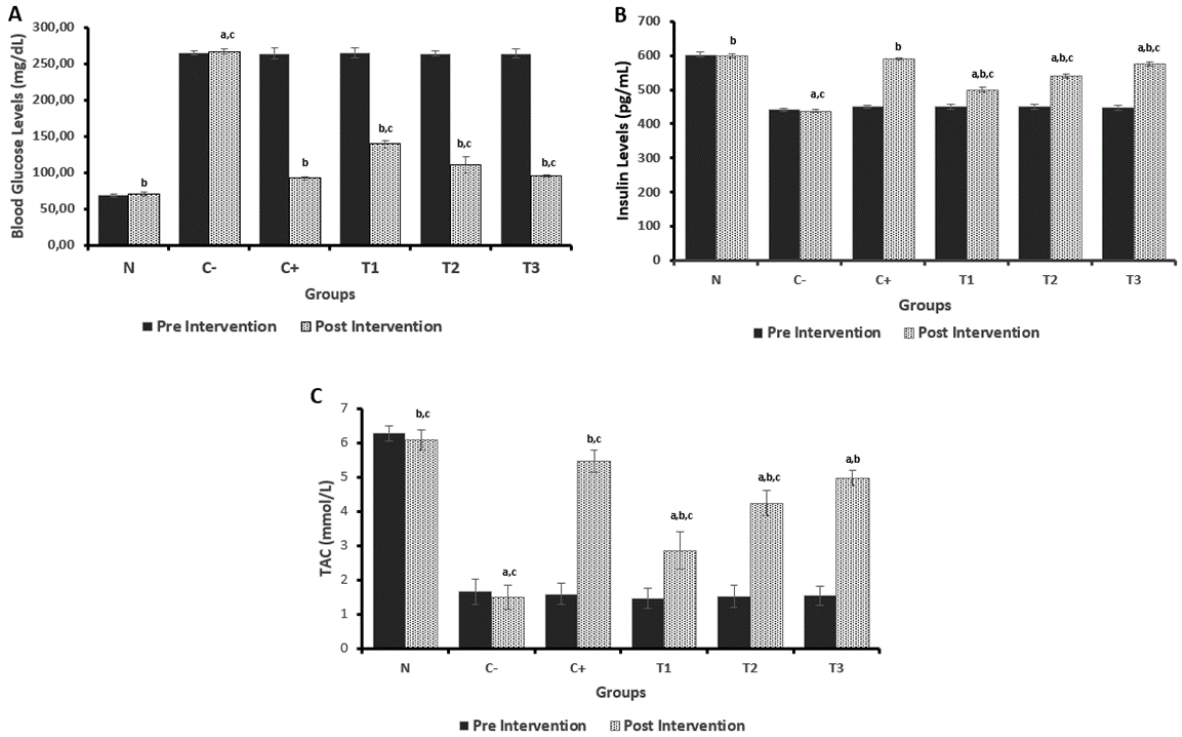

Figure 2. Comparison of blood glucose levels (A), insulin levels (B) and total antioxidant capacity (C) before and after intervention in the N, C-, C+, T1, T2 and T3 groups. Six groups of rats (n = 7 each group) consist of N: healthy control, C-: T2DM control and C+: metformin 9 mg/200 g BW; T1: black rice bran extract 15 mg/200 g BW; T2: black rice bran extract 30 mg/200 g BW; T3: black rice bran extract 60 mg/200 g BW. The error bars represent the standard deviation of the mean. $^{a}p < 0.05$ compared to N, $^{b}p < 0.05$ compared to C-, $^{c}p < 0.05$ compared to C+.

https://doi.org/10.26656/fr.2017.7(5).1024

Table 1. The effect of black rice bran extract on insulin levels, TAC and blood glucose levels.

Outcome Measure	Groups						
	N	C-	C+	T1	T2	T3	P
Insulin (pg/mL)							
Pre	603.34±7.54	441.47±4.31	451.06±4.52	450.07±7.21	450.40±8.12	447.13±6.22	0.000*
Post	599.37±7.01^{b}	436.70±4.50^{a,c}	590.79±3.46^{b}	499.95±6.24^{a,b,c}	539.59±5.15^{a,b,c}	575.84±4.95^{a,b,c}	0.000*
p	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	
Δ	-3.96±1.13^{c}	-4.79±1.41^{c}	139.72±5.95^{a,b}	49.88±6.28^{a,b,c}	89.18±11.38^{a,b,c}	128.71±8.98^{a,b,c}	0.000^{y}*
TAC (mmol/L)							
Pre	6.28±0.22	1.66±0.37	1.59±0.31	1.47±0.29	1.51±0.32	1.54±0.27	0.000*
Post	6.09±0.29^{b,c}	1.49±0.34^{a,c}	5.46±0.33^{a,b}	2.86±0.53^{a,b,c}	4.24±0.35^{a,b,c}	4.98±0.22^{a,b}	0.000*
p	0.004*	0.000*	0.000*	0.001*	0.000*	0.000*	
Δ	-0.19±0.11^{c}	-0.17±0.06^{c}	3.87±0.51^{a,b}	1.39±0.56^{a,b,c}	2.73±0.51^{a,b,c}	3.43±0.37^{a,b}	0.000^{y}*

Values are presented as mean±SD. Six groups of rats (n = 7 each group) consists of N: healthy control, C-: T2DM control and C+: metformin 9 mg/200 g BW; T1: black rice bran extract 15 mg/200 g BW; T2: black rice bran extract 30 mg/200 g BW; T3: black rice bran extract 60 mg/200 g BW. p: Paired T-Test, P: One-Way Anova, ^{y}Kruskall-wallis, *significant $p < 0.05$, $^{a}p < 0.05$ compared to N, $^{b}p < 0.05$ compared to C-, $^{c}p < 0.05$ compared to C+.

the C-group ($p < 0.05$). At the end of the intervention, insulin levels in the C+ group were similar to those in the N group, as evidenced by no significant difference between the two groups. Insulin level increase in T3 was significantly better than T1 and T2 groups, although it was still lower than the value presented in the C+ group (Figure 2).

3.4 Total antioxidant capacity

Changes in TAC of rats in the six groups were presented in Table 1. There was a significant decrease in TAC before treatment in T2DM-induced groups. At the end of the treatment, there was a significant increase in TAC-treated C+, T1, T2 and T3 groups compared to the C-group ($p < 0.05$). A significant difference was still observed in the treatment group compared to the N group ($p < 0.05$). Among the groups treated with black rice bran ethanolic extract, the highest dose (T3) delivered the best improvement of TAC. Furthermore, there was no significant difference observed in TAC in the T3 group compared to that in the C+ group ($p < 0.05$), indicating a similar improvement of TAC between those treatments (Figure 2).

4. Discussion

According to the findings of this study, both the black rice bran ethanolic extracts and metformin raised body weight in rats in a manner comparable to that of healthy rats. The loss in body weight seen in the C-group was due to the fact that the C-group did not receive any treatment. STZ injection causes physiological abnormalities such as weight loss, increased appetite, and thirst in order to compensate for the decrease in ATP and glycogen. Signals suggesting a shortage of energy sources such as ATP and glycogen cause an increase in ATP formation via gluconeogenesis from fat and muscle mass. Insulin resistance leads to a failure to store glucose as glycogen, resulting in continuous energy synthesis via gluconeogenesis and, as a result, weight loss (Al-Attar and Alsalmi, 2019). On the other hand, weight gains in intervention groups (C+, T1, T2 and T3) were observed. Anthocyanin consumption has been linked with body weight changes in hyperglycemic rats (Skovsø, 2014). A previous study demonstrated a weight gain in STZ-NA-induced mice after the intervention of brown rice and black soybean extracts for 35 days (Rahayu *et al*., 2019). This weight gain might be caused by improved insulin production and secretion by pancreatic beta cells, thus decreasing lipolysis and fatty acid oxidation (Arner *et al.,* 2018).

The results of this study showed that black rice bran ethanolic extract reduced blood glucose levels in T2DM rats. Blood glucose levels after treatment with black bran ethanolic extract at a dose of 60 mg/200 g BW were equivalent to those in the metformin group at a dose of 9 mg/200 g BW. This finding was consistent with prior research (Nakamura *et al*., 2017; Sari and Wahyuni, 2017; Zhang *et al*., 2013). The role of anthocyanins in black rice bran extracts against hyperglycemia occurs through several mechanisms such as increased glucose uptake and metabolism, increased glucose transport via the GLUT4 transporter and PI3K-dependent pathway mechanisms, upregulation of the GLUT1, GLUT4 and PI3K p85a, and increased Akt phosphorylation (Houghton *et al*., 2019). Previous *in vivo* studies showed that anthocyanin improves blood glucose levels and glucose-insulin tolerance by suppressing ROS production and increasing GSH levels and antioxidant enzyme activity, as well as preventing oxidative stress induced by endoplasmic reticulum stress (Zhang *et al*., 2013).

This study confirmed that black rice bran ethanolic

https://doi.org/10.26656/fr.2017.7(5).1024

extract increased insulin levels in T2DM rats. The increase in insulin levels from the administration of black rice bran ethanolic extract compared with T2DM control groups in this study was in accordance with previous studies (Sarikaphuti *et al*., 2013; Chen *et al*., 2018; Krisbianto *et al*., 2016; Chayati *et al*., 2019). The administration of anthocyanin improves insulin levels through various mechanisms, for example, anthocyanin-rich chokeberry methanolic extract improved insulin secretion and reduced ROS in TC3 cell cultures through the protection of the enzymatic antioxidant system against H_2O_2 and glucotoxicity in pancreatic cells (Rugina *et al*., 2015). C3G isolated from mulberry fruit ethanolic extract was found to exhibit cytoprotective effects such as reducing ROS production and lipid peroxidation and preventing apoptosis in hyperglycemic MIN6N pancreatic cells. This indicated that the antidiabetic role of anthocyanins was associated with an amelioration in oxidative stress and an increase in the antioxidant defense system (Lee *et al*., 2015). Likewise, another research utilizing bayberry ethanolic extract resulted in a hypoglycemic effect and improved insulin secretion due to the antioxidant and cytoprotective effects of anthocyanins, thereby reducing oxidative stress. In that study, the expression of PDX-1, Ins-2, and insulin levels also showed an improvement compared to the control group (Sun *et al*., 2012). Furthermore, anthocyanins promote AMPK activation, which improves glucose absorption and insulin secretion by pancreatic cells. AMPK activation is accompanied by an increase in GLUT4 in white adipose tissue and skeletal muscle. C3G also increases adiponectin mRNA levels in white adipose tissue, thus inhibiting INS-1 cell death and consequently increasing insulin secretion (Rózańska and Regulska-Ilow, 2018).

Aside from anthocyanin, it is known that black rice bran contains other phytochemical constituent such as flavonoids and phenols. Preliminary investigation in our study revealed that the total phenolic, total flavonoid and total anthocyanin content of the black rice bran ethanolic extract were 492.60±1.70 mgGAE/100 g, 170.95±0.92 mgQE/100 g, and 5.77±0.15 mgC3GE/100 g, respectively, as well as 55.7% of antioxidant activity (Eviana, 2021; Monikasari, 2021). Several flavonoids found in black rice bran are quercetin, apigenin and catechin (Ghasemzadeh *et al*., 2018). Flavonoids modulate insulin secretion through regulation in Ca^{2+} flux by L-type Ca^{2+} channels (L-VDCC) mechanism, accumulation of intracellular (PKA-mediated) cAMP and activation of CaMK II or gene transcription factors PDX-1, GLP-1, IRS-2, or Insig-1. Flavonoids in black rice bran improve insulin levels through modulation of Ca^{2+}, either through the mobilization influx increase of Ca^{2+} from the endoplasmic reticulum through L-VDCC, inhibition of NFκB pathway, repairment of oxidative damage in pancreatic cells by decreasing DNA damage, ROS production, lipid peroxidation protein carboxylation and restoration of β-cell apoptosis (Soares *et al*., 2017; Al-Ishaq *et al*., 2019). Most phenolic compounds found in black rice bran are protocatechuic acid, ferulic acid, syringic acid, caffeic acid and p-coumaric acid (Ghasemzadeh *et al*., 2018). These phenolic compounds played an important role in modulating the expression of genes involved in pancreatic cell dysfunction and insulin secretion through several mechanisms: 1) synergistic action between polyphenols and phenolic acids in targeting signalling molecules such as transcription factors, 2) reduction of radical damage in β-cell dysfunction through its antioxidant activity and 3) trigger factors responsible for insulin secretion (Saji *et al*., 2020).

Hyperglycemia reduced TAC in humans and experimental animals due to the increased AGE-induced free radical formation and increased oxidative stress triggered by various biological changes such as glucotoxicity induced by the polyol pathway (Chen *et al*., 2018). This study showed that after 21 days, TAC in the groups treated with black rice bran ethanolic extract and metformin was higher than that in the T2DM control group. TAC in the group treated with black rice bran ethanolic extract at a dose of 60 mg/200 g BW was similar to TAC in the group treated with metformin at a dose of 9 mg/200 g BW. Previous studies also presented similar results (Li *et al.*, 2015; Krisbianto *et al.*, 2016; Chusak *et al.*, 2020). C3G has been shown to protect against ROS-induced DNA or cellular damage. Anthocyanins' antioxidant action is mediated through an increase in the body's antioxidant defenses as well as a direct effect of anthocyanins on scavenging ROS (Belwal *et al*., 2017). Exogenous antioxidant enhances the endogenous antioxidant defence system by providing hydrogen atoms from hydroxyl groups to radical compounds, resulting in a more stable phenolic hydroxyl (Niki, 2010). A large amount of hydroxyl groups from the flavonoid framework and the double bonds in the C ring were often linked to anthocyanins' capability to scavenge free radicals. Furthermore, anthocyanin in black rice bran inhibits enzymes that generate superoxide anions, chelates transition metals involved in the radical production process, and prevents peroxidation by reducing alkoxy and peroxy-radicals (Gowd *et al.*, 2017).

4. Conclusion

Black rice bran ethanolic extract improved insulin levels and TAC in T2DM rats. The dose of 60 mg/200 g BW of black rice bran ethanolic extract showed a better effect in improving these parameters. Black rice bran

https://doi.org/10.26656/fr.2017.7(5).1024

ethanolic extract showed comparable effectiveness to metformin in improving the TAC of T2DM rats.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to express our gratitude to the Animal Laboratory of the Center for Food and Nutrition Studies at Universitas Gadjah Mada for their assistance during the animal experimentation.

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