



*Research article*

## **Antioxidant activity and inhibition of $\alpha$ -amylase and $\alpha$ -glucosidase in fermented black rice bran-based analog rice**

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**Abstract:** Analog rice is an alternative food that can also be a functional food. Analog rice has the same shape as rice grains, can be made from non-rice flour, and can be consumed like white rice. The purpose of this study is to determine the effect as an antidiabetic of the addition of fermented black rice bran (FBB) and non-fermented black rice bran (NFBB) on analog rice based on in vitro assays. This research was conducted in three stages: analog rice was made from the raw materials of sorghum, yellow soybean, black soybean, FBB and NFBB; analysis of the phytochemical characteristics of analog rice; evaluation of DPPH-radical scavenging; and analysis of the inhibitory effects of agents  $\alpha$ -amylase and  $\alpha$ -glucosidase. Increased phenol, flavonoid, and anthocyanin content were found in analog rice with the addition of FBB. In addition, analog rice with the addition of FBB also had antioxidant activity and higher inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase activity with a range of 54.50–65.52%, 63.16–65.51% and 60.27–62.09% respectively compared to analog rice with the addition of NFBB. The results of this study indicate that analog rice with the raw materials of sorghum, beans and the addition of FBB has potential as an antidiabetic food.

**Keywords:** antihyperglycemic; digestive enzyme; functional food; radical-scavenging

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## 1. Introduction

Diabetes is a disease chronic caused by non-infectious metabolic disorder, characterized by hyperglycemic conditions or high blood glucose levels [1]. The most common diabetes is type 2 diabetes (T2D) which is caused by lifestyle factors and diet that causes obesity [2]. Postprandial hyperglycemia can be reduced by modulating the absorption of glucose in the body as it reduces the hydrolysis of carbohydrates. Pancreatic  $\alpha$ -amylase is an enzyme in the initial stages of starch hydrolysis, and  $\alpha$ -glucosidase subsequently hydrolyzes di- and oligosaccharides into glucose in the intestine [3]. Some bioactive components act as inhibitors that can inhibit the activity of the enzymes  $\alpha$ -amylase and  $\alpha$ -glucosidase, which can reduce the absorption rate of glucose and postprandial hyperglycemia. Digestive enzyme inhibitors are generally found in functional foods that contain many bioactive components such as polyphenols [4]. The condition of hyperglycemia will also encourage the formation of free radicals that trigger a state of oxidative stress in the body. Oxidative stress will reduce insulin secretion and inhibit glucose uptake in muscle cells and adipose [5], because oxidative stress can trigger different expressions of some genes involved [6]. Therefore the body needs antioxidant-rich foods to prevent oxidative stress. Testing the activity of antioxidants and inhibitors of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes can be used as an approach to evaluate the antidiabetic potential of a functional food.

Analog rice is an artificial rice which has the shape of white rice granules. It can be made from non-rice flour, cooked and consumed as white rice, and has high functional properties [7]. Therefore, analog rice can be consumed as a functional food and as an alternative staple food. Analog rice made from a mixture of corn, sago, yellow soybeans, and rice bran contains phenols, flavonoids, resistant starches, high food fiber and has a low IG value [8–11]. Analog rice from sorghum, red palm oil, yellow soybeans, and rice bran has been reported to have properties that inhibits colon cancer [12].

In this study, analog rice was made using the raw materials of sorghum, yellow soybeans, black soybeans, and rice bran which are still underutilized. The selection of raw materials was based on the results of previous studies that show that sorghum [13], yellow soybeans [14], black soybeans [15], and rice bran [16] have antioxidant activity, are able to inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes, reduce blood glucose levels, increase insulin levels and regenerate  $\beta$ -pancreatic cells. Rice bran, which is a byproduct of hulling rice, still contains many bioactive components, food fiber and phytochemicals, so it can be used as a raw material for food products such as bakery products, drinks, and meatballs [17]. Adding rice bran to food products can also prevent chronic diseases such as cancer and cardiovascular disease [18]. Fermentation by the solid state fermentation (SSF) method can increase the amount of phenolic and antioxidant activity of rice bran which has been widely reported by several researchers [17,19,20]. Bran fermented for 4 days can increase phenolic compounds, flavonoids,  $\gamma$ -orizanol,  $\alpha$ -tocopherol, and have antioxidant activity and inhibition of  $\alpha$ -amylase [21]. There have been several studies on diabetes that have focused on bioactive components in functional foods. However, there have been no studies on antidiabetic activity of analog rice. Therefore this study was conducted to determine the effect of adding fermented black rice bran (FBB) and non-fermented black rice bran (NFBB) to analog rice on its potential as an antidiabetic. The results of this study are expected to provide scientific information about the potential of analog rice derived from local Indonesian raw materials as an antidiabetic.

## 2. Materials and methods

### 2.1. Analog rice preparation

The materials used in this study are Numbu cultivar sorghum from a local farmer in Gadog, Indonesia, Grobogan cultivar yellow soybean from BB Biogen, Indonesia, Detam 1 cultivar black soybeans from Balitkabi in Malang, Indonesia, and Cempo Ireng cultivar black rice bran prepared from a paddy which was bought from local farmers in Yogyakarta, Indonesia. The skin of the black rice was polished by rice mill processing (Satake Grain Testing Mill, Japan). The FBB was fermented by the SSF method using *Rhizopus oligosporus* [21].

### 2.2. Analog rice

Analog rice production was carried out following [7] with modifications to the temperature of extrusion. The analog rice was made in three stages: (1) mixing the raw material (flour) for 10 minutes with a dry mixer, (2) extruding process using the twin screw extruder (Berto BEX-DS-2256) at 100 °C, (3) the final product of analog rice granules was dried at 60 °C for 3 hours. Four analog rice formulas were made (the formulas are subject to a patent application): AR1 analog rice from a mixture of sorghum, yellow soybean, and NFBB; AR2 analog rice from a mixture of sorghum, black soybean, and NFBB; AR3 analog rice from a mixture of sorghum, yellow soybean, and FBB; and AR4 analog rice from a mixture of sorghum, black soybean, and FBB.

### 2.3. Sample extraction

Analog rice was ground in a blender (Maspion, Indonesia), and passed through an 80 mesh sieve. A sample extraction was prepared [22] with slight modifications. Analog rice and 70% ethanol (1:10) were mixed for 24 hours, and then filtered with number 41 filter pads. The residue was re-extracted with 70% ethanol. All supernatants were collected and centrifuged at 1207 xg for 20 minutes. The ethanolic extract was evaporated using a rotary vacuum evaporator at 50 °C for 1 hour, and then exhaled with N<sub>2</sub> gas until all the solvents were removed and stored at -18 °C until use. The extract was used for all analysis. All experiments were done in triplicate.

### 2.4. Analysis of phytochemical composition

The total phenolic content was determined using the Folin-Ciocalteu reagent [19]. Absorbance was measured at 765 nm using a UV spectrophotometer. Gallic acid was used as a standard. Results were expressed as milligrams equivalent of gallic acid per 100 grams of sample (mgGAE/100g sample).

The total flavonoid content was measured using Quercetin as a standard [23]. The absorbance was measured using a UV-VIS spectrophotometer at 432 nm. The results obtained were expressed in milligrams equivalent of quercetin per 100 grams sample (mgQE/100g sample).

Total anthocyanin content was measured based on the pH difference method [24]. The absorbance was measured at 510 and 700 nm. The absorbance value was calculated as  $A = [(A_{510} - A_{700}) \text{ at pH } 1 - (A_{510} - A_{700}) \text{ at pH } 4.5]$ . The total anthocyanin was expressed as milligrams of

cyanidin-3-glucoside (C3G) per gram of sample using a molar coefficient of 26.900 and a molecular weight of (BM) 449.

Analysis of  $\gamma$ -oryzanol was performed using high performance liquid chromatography (HPLC, Agilent Technologies 1200 series) [25]. As much as 20  $\mu$ L of the standard and extract samples were injected into a C-18 column (Zorbax Eclipse XDB C-18 column 4.6 x 150 nm) with a flow rate of 1 mL/minute. A mixture of methanol and acetonitrile (35:65) was used during the mobile phase using the HPLC UV-VIS detector at a wavelength of 325 nm. Results were expressed as milligrams per grams of dried sample (mg/g db).

$\alpha$ -tocopherol was analyzed following the method 971.30 [26]. 20 $\mu$ L of extract was injected into HPLC (Agilent Technologies 1200 series) at 280 nm wavelength, then into a C-18 column (Zorbax Eclipse XDB C-18 column 4.6 x 150 nm), and a UV-VIS detector, methanol:isopropanol (98:2) as mobile phase, with a flow rate of 1 mL/minute. Results were expressed as milligrams per grams of dried sample (mg/g db).

### 2.5. DPPH radical-scavenging method

The DPPH-radical scavenging method was used to measure antioxidant activity [27]. The absorbance of the solution was measured at a wavelength of 517 nm using a UV-VIS spectrophotometer. DPPH-radical scavenging was expressed as a percentage calculated as  $[1 - (\text{abs of sample} / \text{blank abs})] \times 100$ .

### 2.6. $\alpha$ -Amylase inhibition assay

Inhibition of the  $\alpha$ -amylase was measured according to the method of Thalapaneni et al. [28]. Briefly, the extract sample was reacted with the amylase enzyme solution, then incubated at 37 °C for 10 minutes. 1% starch solution was added and re-incubated at 37 °C for 10 minutes. The 3,5-dinitrosalicylate (DNS) reagent was added, incubated for 5 minutes in boiling water, then as much as 5 mL of distilled water was added and the absorbance was measured using a UV-VIS detector spectrophotometer at 540 nm. The  $\alpha$ -amylase inhibition was calculated as  $[(\text{abs control} - \text{abs sample}) / \text{abs control}] \times 100$ .

### 2.7. $\alpha$ -Glucosidase inhibition assay

The  $\alpha$ -glucosidase assay was carried out based on the method of Sancheti et al. [29]. A phosphate buffer solution 0.1 M (pH 7), 4-nitrophenyl  $\alpha$ -Dglucopyranoside 0.5 mM, 10  $\mu$ L extract sample and 25  $\mu$ L  $\alpha$ -glucosidase enzyme solution (1 mg/mL in 0.01 M phosphate buffer, pH 7) were reacted, and then incubated at 37 °C for 30 minutes. The reaction was stopped by adding sodium carbonate 100  $\mu$ L (0.2 M). The inhibition of  $\alpha$ -glucosidase was measured using a microplate reader at a wavelength of 410 nm. The percent inhibition was calculated as  $[1 - (\text{abs sample} / \text{abs control})] \times 100$ .

### 2.8. Statistical analysis

Data were analyzed using one way analysis of variance (ANOVA) and post hoc Duncan's multiple range test ( $p < 0.05$ ). Data values were expressed as mean  $\pm$  standard deviation (Mean  $\pm$  SD).

### 3. Results and discussion

#### 3.1. Total phenols, flavonoids and anthocyanins

Phenolic content in analog rice ranged from 429.31–758.61 mg GAE/100g (Table 1) and was significantly different ( $p < 0.05$ ). Analog rice with the addition of FBB has a higher phenolic content compared to rice with the addition of NFBB in the same type of soybeans; AR4 > AR2 and AR3 > AR1. This result shows that the added FBB can increase the phenolic content of analog rice products. This is in accordance with the results of previous studies which showed that the phenolic content in FBB increased by 57.39% due to the fermentation process [21]. In this study it was found that analog rice using black soybeans with the addition of FBB and NFBB had a higher phenolic content, compared to yellow soybeans. This can be attributed to the higher phenolic content of black soybeans compared to yellow soybeans (258.02 mg GAE/100g versus 155.15 mg GAE/100g, data were not shown). This means that the phenolic content of analog rice is also strongly influenced by the raw materials used. This result was also supported by previous research [9] which stated that analog rice using raw materials of sorghum had a total phenolic content of 18 mgGAE/100g, while analog rice from composit sorghum and yellow soybeans had 24 mgGAE/100g.

The flavonoid content of analog rice ranged from 235.03–374.62 mgQE/100g and was significantly different ( $p < 0.05$ ). The highest content was found in AR4 followed by AR2, AR3 and AR1. As with phenols, the flavonoid content of analog rice in the same type of soybeans with the addition of FBB is higher than with the addition of NFBB. Black soybeans in the AR4 and AR2 have a higher flavonoid content when compared to analog rice using yellow soybeans in AR3 and AR1. This is presumably because black soybeans have a higher flavonoid content. According to [30], the flavonoid content in black soybeans is 6 times higher (2.57 mg/g) than yellow soybeans (0.41 mg/g).

Anthocyanin content of analog rice is found to be higher with the addition of FBB than NFBB. The highest amount of anthocyanin obtained in this study was 78.88 mg/g (AR4) followed by AR3 (72.26 mg/g), then AR2 (70.89 mg/g) and AR1 (42.51 mg/g). The addition of FBB which has an anthocyanin content of 96.38 mg/g [21] contributed to the increase in anthocyanin levels of analog rice products. Black soybeans are also reported to contain 1.044 mg/g of anthocyanins [31], so AR2 using black soybeans and NFBB has high levels of anthocyanins as well (Table 1).

The addition of FBB to analog rice can increase total phenolic, flavonoid and anthocyanin content. The SSF technique is an alternative to increase the free phenolic compounds and antioxidant activity in food products [32]. Koji prepared from raw beans uses the SSF technique with *Rhizopus sp.*, which has high antioxidant activity and is associated with increased total phenolic and anthocyanin content [33]. During the SSF process, hydrolysis of the glycosidic bonds into free aglycones occurred, which contributed to the high value of antioxidant activity [34].

#### 3.2. The $\gamma$ -oryzanol and $\alpha$ -tocopherol compound

The  $\gamma$ -oryzanol compound ranges from 2.84–3.36 mg/g (Table 1), not significantly different among the four analog rice formulas. The total amount of  $\alpha$ -tocopherol obtained was significantly different for all four analog rice formulas. The highest amount of  $\alpha$ -tocopherol was 126.16  $\mu$ g/g in AR4 followed by AR3 which had 86.18  $\mu$ g/g with added FBB, and the lowest content was found in AR2 (24.75  $\mu$ g/g) and AR1 (14.11  $\mu$ g/g) which contained added NFBB. The amount of  $\gamma$ -oryzanol

and  $\alpha$ -tocopherol compounds obtained in analog rice are higher when compared to the content of  $\gamma$ -oryzanol (0.041 mg/g) and  $\alpha$ -tocopherol (3  $\mu$ g/g) in white rice [35]. This is presumably due to the addition of black rice bran which contains a lot of  $\gamma$ -oryzanol 1.85–4.21 mg/g [36] and  $\alpha$ -tocopherol 43.57–150  $\mu$ g/g [37]. According to [36] the addition of FBB to analog rice contributes to the increase of  $\gamma$ -oryzanol and  $\alpha$ -tocopherol. Enzyme activity during the fermentation process is also capable of releasing bound forms of  $\gamma$ -oryzanol and  $\alpha$ -tocopherol into free forms and have high bioavailability [39].

**Table 1.** Chemical composition of analog rice\* (db).

Content	AR1	AR2	AR3	AR4
Total Phenol (mgAGE/100g)	429.31 $\pm$ 0.72 <sup>a</sup>	636.56 $\pm$ 0.82 <sup>c</sup>	513.41 $\pm$ 1.06 <sup>b</sup>	758.61 $\pm$ 0.93 <sup>d</sup>
Total Flavonoid (mgQE/100g)	235.03 $\pm$ 0.96 <sup>a</sup>	354.97 $\pm$ 0.044 <sup>c</sup>	318.72 $\pm$ 1.24 <sup>b</sup>	374.62 $\pm$ 0.87 <sup>d</sup>
Total Anthocyanin (mgC3G/g)	42.51 $\pm$ 0.44 <sup>a</sup>	70.89 $\pm$ 0.10 <sup>b</sup>	72.26 $\pm$ 0.24 <sup>c</sup>	78.88 $\pm$ 0.18 <sup>d</sup>
$\gamma$ -Oryzanol (mg/g)	2.84 $\pm$ 0.42 <sup>a</sup>	2.77 $\pm$ 0.36 <sup>a</sup>	3.37 $\pm$ 0.37 <sup>a</sup>	3.36 $\pm$ 0.39 <sup>a</sup>
$\alpha$ -Tocopherol ( $\mu$ g/g)	14.11 $\pm$ 0.29 <sup>a</sup>	24.75 $\pm$ 3.16 <sup>b</sup>	86.18 $\pm$ 0.25 <sup>c</sup>	126.16 $\pm$ 0.75 <sup>d</sup>

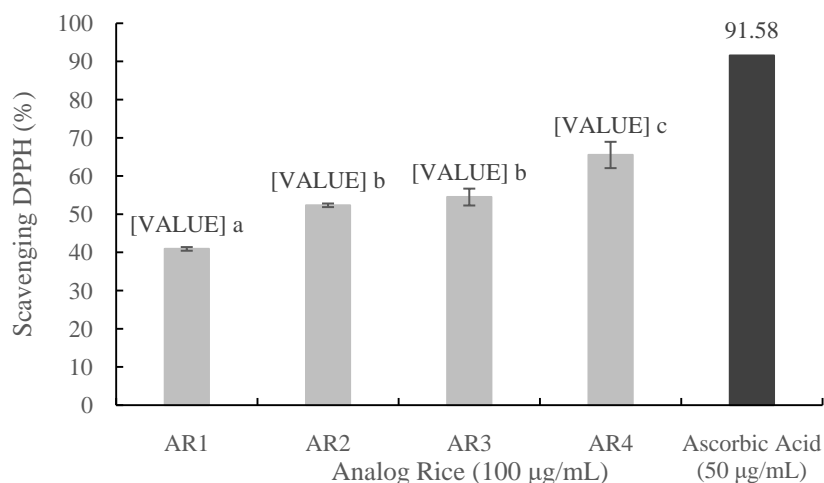
\*AR1: sorghum, yellow soybeans, and NFBB; AR2: sorghum, black soybeans and NFBB; AR3: sorghum, yellow soybeans and FBB; AR4: sorghum, black soybeans and FBB. Means  $\pm$  standard deviasi with the same letter on the same row differ no significantly at the test level of 5%.

$\gamma$ -oryzanol and  $\alpha$ -tocopherol are antioxidant components, making up 38.7–41.1% and 1.2% of the total antioxidant components (respectively) found in bran. The  $\gamma$ -oryzanol is the main antioxidant, and  $\alpha$ -tocopherol is found in smaller amounts. The  $\alpha$ -tocopherol can prevent oxidation of the body by donating hydrogen atoms from its hydroxyl groups to free radicals that often occur in diabetics [40].

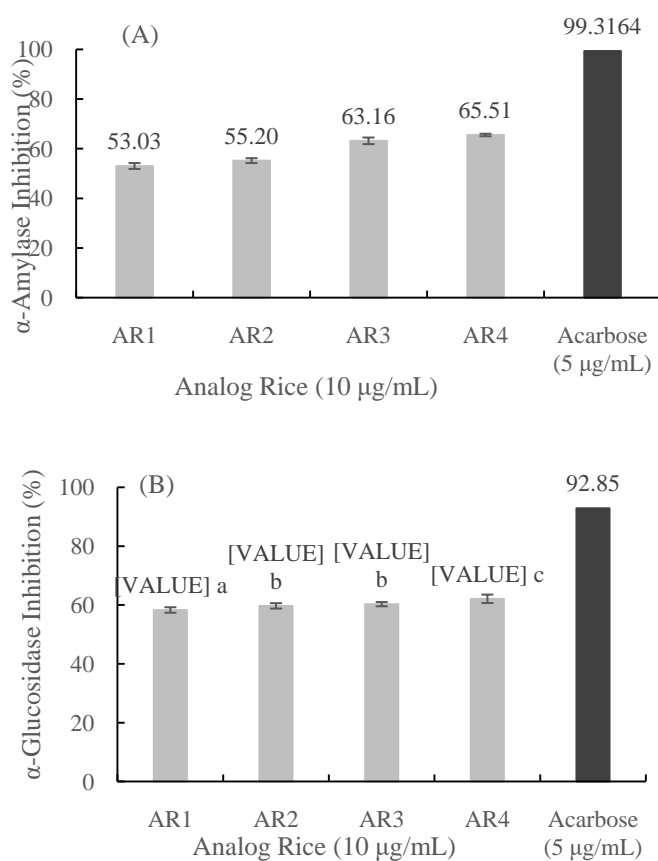
### 3.3. Scavenging ability of DPPH radicals

Antioxidant activity in the analog rice was determined using the DPPH-radical scavenging method. The results showed that the scavenging ability of DPPH radicals ranged from 40.92–65.52% (Figure 1). The lowest antioxidant activity was found in AR1 and the highest was found in AR4. Antioxidants are naturally present in food, and can be increased by fermentation techniques [41]. Soy products fermented using the SSF technique have high antioxidant activity compared to non-fermented products [42]. This study also found that analog rice with the addition of FBB has high antioxidant activity. The phenolic aglycone obtained from fermentation has the highest antioxidant activity compared to the glycoside form [43]. It is also thought to be related to the number of free hydroxyl groups phenolic compounds and flavonoids obtained during SSF. The hydroxyl group can be a hydrogen donor for DPPH free radicals so antioxidant activity increases [44].

Paiva et al. [45] stated that antioxidant activity has a positive correlation with phenolic components. This study also found a positive correlation between phenolic components and antioxidant activity, namely  $r = 0.90$ . DPPH free radical scavenging activity is also associated with high anthocyanin content in analog rice. This is in accordance with research by Laokuldilok et al. [40] which stated that pigmented rice bran showed high free radical scavenging activity–6 times that of white rice bran. In addition, it is also supported by a positive correlation value ( $r = 0.92$ ) between anthocyanins and antioxidant activity.



**Figure 1.** Antioxidant activity of analog rice (100 µg/mL). Different letters indicate a significantly difference by Duncan's Multiple Range Test ( $p < 0.05$ ). Description of analog rice refers to Table 1.



**Figure 2.** Inhibitory  $\alpha$ -amylase (A) and  $\alpha$ -glucosidase (B) in analog rice. Different letters indicate a significantly difference by Duncan's Multiple Range Test ( $p < 0.05$ ). Description of analog rice refers to Table 1.

### 3.4. Inhibitory effects of analog rice against $\alpha$ -amylase and $\alpha$ -glucosidase

$\alpha$ -Amylase and  $\alpha$ -glucosidase are enzymes that catalyze the degradation of starch into glucose for absorption by the body. Inhibition of these enzymes is useful for controlling excessive blood glucose and regulating hyperglycemia that causes diabetes [46]. Previous studies have reported that inhibition of the  $\alpha$ -glucosidase enzyme by inhibitors can be one of the most effective ways to control type 2 diabetes [47].

The anti-amylase activity of analog rice is presented in Figure 2A. The percentage of inhibition of analog rice to  $\alpha$ -amylase ranges from  $53.03 \pm 1.18\%$ – $65.51 \pm 0.51\%$ . Analog rice with the addition of FBB significantly ( $p < 0.05$ ) had higher anti-amylase activity ranging from 63.16–65.51%, compared to analog rice with NFBB only 53.03–55.20%. The inhibitory activity of analog rice to  $\alpha$ -glucosidase ranged from 58.29–62.09% (Figure 2B). The highest inhibitory activity was obtained in AR4, which was made from black soybeans and FBB (62.09%), and it was significantly different ( $p < 0.05$ ) from other analog rice.

The inhibitory activity of analog rice to  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes is thought to be related to the phenolic content in analog rice. Analog rice with the addition of FBB has higher total phenolic content due to the fermentation process being carried out. According to Sompong et al. [48] pigmented rice bran such as black rice bran is a rich source of phenolic components. The polyphenol and flavonoid content found in several foods is very effective at inhibiting  $\alpha$ -amylase and  $\alpha$ -glucosidase activity [49]. Phenolic compounds are able to inhibit  $\alpha$ -amylase enzyme activity by binding to the  $\alpha$ -amylase reactive side and changing its catalytic effect [50]. The polyhydroxyl group plays an important role in  $\alpha$ -glucosidase inhibition [51]. In addition, polyphenols and flavonoids can mimic the pyranosil portion of  $\alpha$ -glucosidase [52]. This study also found a positive correlation between total phenols and  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition ( $r = 0.60$  and  $r = 0.87$ ). Previous studies also reported that there was a positive correlation between total phenols and anti-amylase activity ( $r = 0.68$ ) [53], and with  $\alpha$ -glucosidase inhibitory activity [54]. The opposite has been reported by [11]; carrots, pumpkin, and radishes where phenolic content was not found, showed low inhibitory activity against  $\alpha$ -amylase (<8.5%) and  $\alpha$ -glucosidase (<2.5%).

## 4. Conclusions

The results of this study indicate that analog rice with the raw materials sorghum, black soybeans, and fermented black rice bran (AR4) has the highest amount of total phenols, flavonoids, anthocyanins,  $\gamma$ -oryzanol and  $\alpha$ -tocopherol compared to the others. The analog rice also had the highest inhibitory activity against DPPH free radicals,  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. The findings of this study show that analog rice made from fermented rice bran has potential as an anti-diabetes functional food.

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## Conflict of interest

All authors declare no conflicts of interest.

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