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Anthocyanin, nutrient contents, and antioxidant activity of black rice bran of *Oryza sativa* L. 'Cempo Ireng' from Sleman, Yogyakarta, Indonesia

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ABSTRACT The chemical contents and health benefits of black rice bran of some rice cultivars have been investigated. However, there has been little research on the 'Cempo Ireng' cultivar from Sleman, Yogyakarta. The aim of this present study was to determine the anthocyanin, antioxidant activity, and macro- and micronutrients contents of black rice bran from this local cultivar. The anthocyanin in the black rice bran was extracted using the maceration method with methanol as a solvent. The extract obtained was separated through a preparative thin layer chromatography (TLC) of silica GF254 and a mobile phase composed of n-butanol, acetic acid, and water. Two fractions were collected and analyzed for the anthocyanin content. The preparative TLC spots were separated for further detection and measurement of cyanidin 3-O-glucoside using HPLC followed by LC-MS. The antioxidant activity of the fractions were measured using the DPPH free radical scavenging method. The results showed that the anthocyanin in fraction 1 was identified as cyanidin 3-O-glucoside (66.1 ± 10.6 µg/g). The IC_{50} of fractions 1 and 2 were 200.96 and 218.36 µg/mL, respectively. Analysis of the macro- and micronutrients revealed that the black rice bran of 'Cempo Ireng' had nutrient contents comparable with other rice cultivars. Therefore, this local black rice bran can be used as a source of antioxidants and macro– and micronutrients.

KEYWORDS anthocyanin; antioxidant; black rice bran; nutrient contents

1. Introduction

Black rice is becoming more popular and is now consumed as a functional food due to its health benefits. Black rice contains macro- and micronutrients such as carbohydrate and protein, and the micronutrients such as vitamins and minerals which are higher than those in white rice. Kristamtini et al. (2012) and Ichikawa et al. (2004) reported that the mineral content of black rice such as Fe, Zn, Mn and P is higher than white rice, and that the mineral content in black rice depends on the variety and type of soil where it is grown. In addition to the nutrient contents, black rice also contains bioactive compounds including anthocyanin.

The anthocyanin component in black rice is known to have health benefits such as antioxidant activity against ROS (Park et al. 2008), reducing oxidative stress on mice (Xia et al. 2006), anticancer activity (Hyun and Chung 2004) and anti-inflammatory activity (Tsuda et al. 2003). Black rice has better physical properties than white rice. It is a soft and fragrant rice due to the amylose content in black rice of about 22.97%. The other phytochemical contents are beta-caroten and anthocyanin, about 804.16 mg/100 g and 393.93 ppm respectively (Kristamtini and Purwaningsih 2009). In Indonesia, several local black rice cultivars have been studied, however, their phytochemical content and health benefits still need further investigation (Pratiwi and Purwestri 2017).

The previous studies showed that the black rice dietary of local cultivar 'Cempo Ireng' affected an increase in HDL (High-Density Lipoprotein) in hyperlipidemic rats and decreased LDL (Low-Density Lipoprotein) in hyperlipidemic rats, and the level of cholesterol concentration decreased significantly (Pratiwi et al. 2014). The methanolic extract fraction of rice bran of this cultivar is also a potential candidate for preventing the growth of cervical cancer HeLa cells (Pratiwi et al. 2015). Furthermore, the black rice bran extracts from different cultivars ('Toraja', 'Woja Laka' and 'Cempo Ireng') have a different response cytotoxic activity in different cells, i.e. HepG2 cells, Raji cells and Vero cells (Rukmana et al. 2016).

Rice bran is a by-product of rice milling and consists of the pericarp, seed coat, nucellus and aleurone (Juliano 1985). The aleurone layer in black rice has been reported to contain anthocyanin as well. The content of antho-

cyanin in black rice has been reported by Ryu et al. (1998) and Yoshimura et al. (2012) and, based on these studies, the black rice is rich in anthocyanin in the aleuron layer. Xia et al. (2006) reported that consumption of aleurone layer extracts can reduce cholesterol and triglyceride levels in the blood of mice deficient in Apo-E. The type of anthocyanins in the aleurone layer are known as cyanidin 3-O-glucoside and peonidin 3-O-glucoside.

This study was conducted with the aim of determining anthocyanin, quantification of total anthocyanin, antioxidant activity of the extract fraction of black rice bran and to measure the protein, carbohydrate, lipid, minerals and water contents in the black rice bran 'Cempo Ireng'. The result of this study is expected to provide more information on anthocyanin and nutrient contents in black rice bran. Furthermore, this black rice bran can be developed as an alternative source of potential functional foods.

2. Materials and methods

2.1. Plant material

Black rice 'Cempo Ireng' was described by Pratiwi et al. (2015). This rice was obtained from Sayegan, Sleman, Yogyakarta, Indonesia.

2.2. Chemicals

Methanol, HCl, acetic acid, phosphoric acid, formic acid, n-butanol, silica powder F 254 (Merck) and standard of Cyanidin 3-O-glucoside (Polyphenols Laboratories AS 1201-1), H_2SO_4, HNO_3, solution mixture of boric acid with 1% solution of methyl bromo cresol green and red, K_2SO_4, $K_3Fe(CN)_6$, $Na_2S_2O_3$, Na-tungstate, KCl, KI, $ZnSO_4.7H_2O$, selenium, Iod 2%, amylase standard, n-hexane, petroleum ether, petroleum benzine, Benedict solution, glucose standard, Nelson solution, alcohol 80%, $CaCO_3$, Na-dried oxalate (Merck), standard minerals Ca, P, and Fe (Sigma Aldrich), 2,2-diphenyl-1- picrylhydrazyl (DPPH) (Sigma-Aldrich).

2.3. Sample preparation

Black rice bran was powdered with a sample mill cyclotec machine and sieved through a 100 mesh sieve.

2.4. Analysis of anthocyanins

The total anthocyanin content in the rice bran was measured by the pH-differential method (Lee et al. 2005). The rice bran was dissolved in KCL buffer pH 1.0 and buffer CH_3COONa pH 4.5 and absorbance of the solution was measured using a spectrophotometer at 520 and 700 nm. The content of total anthocyanin was calculated with the formula:

Total anthocyanin mg/L =

$$\frac{\{Abs_{(510-700nm)pH1.00} - Abs_{(510-700nm)pH4.5}\} \times MW \times DF \times 1000}{\varepsilon \times 1} \quad (1)$$

where ε is the molar extinction of cyanidin 3-O-glucoside (26.900 L $\times$ mol^{-1} $\times$ cm^{-1}); MW is the molecular weight of cyanidin 3-O-glucoside (449.2); DF is the dilution factor; and 1 is cuvette thickness (1 cm).

The anthocyanin fraction was prepared using the modified methods of Abdel-Aal et al. (2006) and Kim et al. (2008). Three g of black rice bran was extracted using the combination of methanol:HCl 1N (85:15). Analysis of the chemical content of the extract was carried out using analytical TLC silica F254 and was then separated using preparative TLC silica GF254 as the stationary phase and n-butanol:acetic acid:water (4:1:5) as the mobile phase. The results of preparative TLC were detected under visible light and UV 366 nm and the spot was marked. The spot result in preparative TLC was separated and dissolved in methanol:HCl (85:15 v/v) and then characterized using HPLC (Hitachi UV-Vis detektor L-2420) and LC-MS (Accela PDA, autosampler 1250 Orbitrap Thermo exative). The mobile phase of HPLC was phosphoric acid:acetonitrile (80:20), stationary phase was C-18 column (cromasil 250x4.6 mm) and mobile phase of LC-MS corresponding to Abdel-Aal et al. (2006) research was formic acid 6% (A) dan methanol 100% (B) in gradient flow rate, stationary phase was C-18 column (hypersil bold 50 x 2.1 mm). HPLC data were compared with the retention time of each fraction with standard cyanidin 3-O-glucoside and followed by analysis using LC-MS to study the m/z of fractions and compared with the m/z theoretical compound of cyanidin 3-O-glucoside.

2.5. The activity of radical scavenging assay against 2.2-diphenyl-1-picrylhydrazyl (DPPH)

The antioxidant activities of the fractions from viscous rice bran extract were carried out using DPPH scavenging radical activity. One mL of 0.1 mM solution of DPPH in methanol was mixed with the solution of the fraction of viscous rice bran extract by dissolving four concentrations of 50, 100, 250 and 500 µg/mL, and methanol was added to the solution upto 5 mL. The solution was incubated for 30 min and then absorbance was measured with the spectrophotometer at 515 nm, as was carried out by Brand-Williams et al. (1995) and Kikuzaki et al. (2002).

2.6. Analysis of macro- and micronutrients

Carbohydrates (AOAC), proteins (Kjeldahl), lipids (Shoxtec Analyzer), amylase (Spectrophotometer) and minerals Ca, P and Fe (ICP-MS) were analyzed using the standard method AOAC 944.02. All data is presented as the mean ± standard deviation.

3. Results and discussion

3.1. Separation and identification of anthocyanin

Black rice bran of 'Cempo Ireng' Sayegan, Yogyakarta was extracted by the maceration method using methanol and 1N HCl in the ratio 85:15 v/v. The resulting filtrate

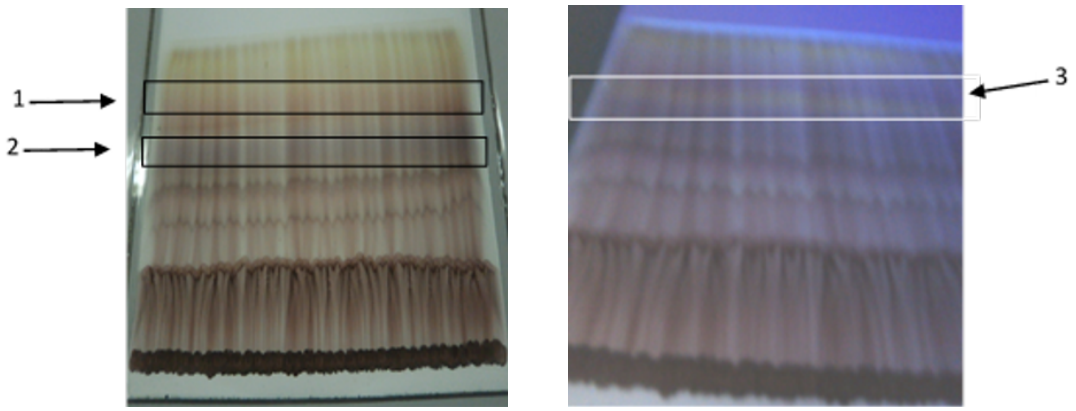


FIGURE 1 Crude extract black rice bran with thin layer chromatography preparative silica 254 with stationary phase and mobile phase n-butanol:acetic acid:water (4:1:5). (a) Spot lines detection with visible light; and (b) with UV 366 nm. Fractions 1, 2, and 3 are in the direction of the arrow.

was then separated using liquid-liquid partition and by using n-hexane as the solvent to separate nonpolar compounds such as lipids. About 120 g of the sample of black rice bran was extracted, resulting in 19.4 g of viscous extract. The amount of the yield of viscous extract is 16.2%. The determination of the total anthocyanin content using pH differentiation method in black rice bran is obtained 8.2 ± 0.8 mg/g.

Viscous extract was separated with a preparative thin layer chromatography (TLC) using the mobile phase n-butanol:acetic acid:water (4:1:5). The profile of separated fractions on thin layer chromatography can be seen in Figure 1 which shows three fractions of viscous extract. The result of fraction separation is shown in the direction of the arrow in Figure 1. The data of the separation results of the viscous extract with thin layer cellulose chromatogram can be seen in Table 1. The retention factor (hRf) of each fraction has a different spot according to UV 254 nm and visible light detections. The retention factor is a quantitative indication of how far a molecule of a compound is brought into the mobile phase. The data from Table 1 shows the comparison of the retention factor of each fraction with a marker compound. In this study we used cyanidin 3-O-glucoside as a marker. Table 1 shows that fraction 1 has a similar retention factor with cyanidin 3-O-glucoside, which means that quantitatively fraction 1 has similarities in chemical properties with cyanidin 3-O-glucoside. Fractions 2 and 3 have different hRf's.

TABLE 1 Chromatogram of viscous extract separated with cellulose thin layer chromatography as a stationary phase.

No.	Compound	hRf	Visible light	UV 254
1	Cyanidin 3-O-glucoside	0.56	Magenta	Damping
2	Fraction 1	0.56	Magenta	Damping
3	Fraction 2	0.71	Magenta	Damping
4	Fraction 3	0.81	Yellow	Yellow Florescence

The data from this study can be seen in Table 1 and is continued in the characteristics of fractions 1 and 2. The election of fraction 1 based on qualitative test (hRf) with TLC separation indicated that fraction 1 is cyanidin 3-O-glucoside.

3.2. Characterization of BRB anthocyanin with HPLC and LC-MS

The HPLC data shows a typical chromatogram for each fraction (Figure 2 and 3). The data was obtained from the retention time and area from each fraction. Chromatograms of fractions 1 and 2 are presented in Figure 2 and 3. From these results we can choose which fraction to continue characterization by using LC-MS to confirm the molecular weight and fragmentation of the molecule compound.

Fraction 1 has a retention time (rt) at 4.44 min (Figure 2) and fraction 2 has rt at 6.97 min (Figure 3). The separation results of fraction 1 and 2 with HPLC are known to have different retention times compared with cyanidin 3-O-glucoside as standard compound at 3.78 min. Based

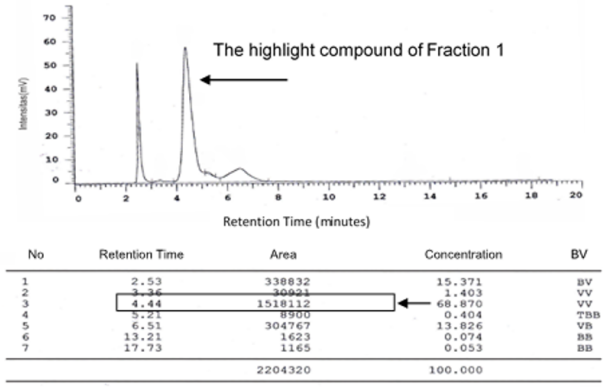
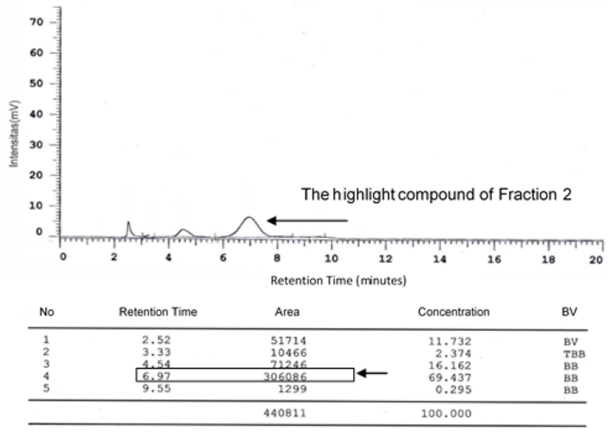


FIGURE 2 A typical chromatogram of fraction 1 separation with HPLC (retention time shown by the arrow direction).



No	Retention Time	Area	Concentration	BV
1	2.52	51714	11.732	BV
2	3.33	10466	2.374	TBB
3	4.54	71246	16.162	BB
4	6.97	306086	69.437	BB
5	9.55	1299	0.295	BB
		440811	100.000	

FIGURE 3 A typical chromatogram of fraction 2 separation with HPLC (retention time shown by the arrow direction).

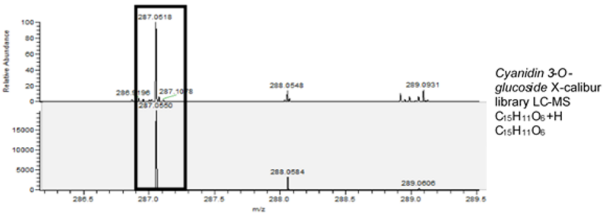


FIGURE 4 Confirmation of ion peak at molecular weight m/z 449 in LC-MS spectrum.

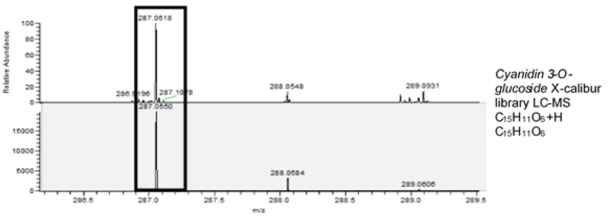


FIGURE 5 Confirmation of fragmented ion peak at m/z 287 in LC-MS spectrum.

on these results, the examination of fraction 1 is continued by using LC-MS. Confirmation of the peak in fraction 1 was carried out using LC-MS ESI positive ion mode. The LC-MS was eluted with methanol and 6% formic acid.

This study used LC-MS with ESI positif ion mode, the molecular data is $[M+H]^+$ and fragmented ion molecular is $[M+H-X]^+$. Based on the result from fragmentation of fraction 1 compared with X-calibur library data on the LC-MS instrument and data from another research of black rice can be seen in Table 2. The spectrum of fraction 1 has four main ion molecules that are m/z 287.0518 (55%); m/z 306.9616 (63%); m/z 326.0408 (100%); and m/z 449.1037 (27%). The molecular weight of fraction 1 is indicated as m/z 449.1037, while the theoretically cyanidin 3-O-glucoside compound is m/z 449.1078. This study confirmed that the specific spectrum of cyanidin 3-O-glucoside is m/z 449 and m/z 287, and showed that the fraction 1 has a specific spectrum of cyanidin 3-O-glucoside such as the theoretical specific spectrum. Based on the molecular weight and fragment ions shown, fraction 1 can be identified as cyanidin 3-O-glucoside (m/z 287 ion fragment cyanidin aglycon and m/z 449 molecular weight cyanidin 3-O-glucoside). The cyanidin 3-O-glucoside in the glucoside or sugar compound is hexose (m/z 162). The fragment of hexose in this compound was m/z 306.9616 (63%) and m/z 326.0408 (100%).

It can be confirmed that cyanidin 3-O-glucoside is the main compound in fraction 1 of methanolic extract of black rice bran 'Cempo Ireng'. This characterization process was done with a LC-MS instrument by comparing the molecular weight (m/z) of each fraction with theoretical molecular weight (m/z) from data base LC-MS instrument, and presented in Table 2. This present study suggests that the molecular weight (m/z) of fraction 1 is 449.1037 and the theoretical ion m/z of Cyanidin 3-O-glucoside compound is 449.1078 (Figure 4). Based on the LC-MS spectrum with ion confirmation through ion fragmentation, the information of fraction 1 has fragmented ion in m/z 287 with molecular weight m/z 449, as is shown in Figure 5.

Kim et al. (2008) and Lee (2010) suggested that confirmation of ion peak based on molecular weight of cyanidin 3-O-glucoside is m/z 449 and ion fragmentation of molecular weight of cyanidin is m/z 287. Meanwhile, Kim et al. (2008) and Yoshimura et al. (2012) reported that fragmentation of cyanidin 3-O-glucoside showed the loss of hexose. Hexose is a sugar compound with molecular weight m/z of 162. Based on these results of structural characterization using LC-MS, it can be concluded that the fraction 1 is thought to be cyanidin 3-O-glucoside or a compound

TABLE 2 Identification of mass spectrum on fraction 1 using LC-MS.

Name	Retention time (min)	Ion Molecule $[M]^+$ (m/z)			Ion Fragment $[M-X]^+$ (m/z)		
		Experiment	Software Xcalibur library	Other research*	Experiment	Software Xcalibur library	Other research*
Fraction 1	1.23	449.10	449.10	449.00	287.00	287.00	287.00
Standard of Cyanidin 3-O-glucoside	1.28	449.10	449.10		287.00	287.00	

*Other research (Kim et al. 2008; Lee 2010)

TABLE 3 The activity of free radical scavenging.

No.	Concentration (µg/mL)	Fraction 1 Activity (%)	Fraction 2 Activity (%)
1.	50	27.88	25.66
2.	100	39.97	34.41
3.	250	61.59	62.74
4.	500	81.46	80.79

in which the sugar bound is hexose. The concentration of cyanidin 3-O-glucoside in the black rice brand 'Cempo Ireng' is obtained as 76.78 ± 12.37 ppb.

3.3. The activity of radical scavenging assay against 2.2-diphenyl-1-picrylhydrazyl (DPPH)

In the 2.2-diphenyl-1-picrylhydrazyl (DPPH) assay (Table 3), fraction 1 had IC_{50} 200.96 µg/mL, while fraction 2 had IC_{50} 218.36 µg/mL. In this study's antioxidant activity we used IC_{50} as a parameter. The concentrations of IC_{50} explain how particular radical scavenging can inhibit the free radical by as much 50%.

A previous study reported that coloured rice in Korea has antioxidant activity (IC_{50}) for 'Heungjinju', 'Shintiheugmi' and Heungseol, 246.9, 287.39 and 381.33 µg/mL respectively (Seo et al. 2011). In accordance with this study, the black rice brand 'Cempo Ireng' has an antioxidant activity (IC_{50}) of fraction 1 and fraction 2, i.e. 200.960 µg/mL and IC_{50} 218.361 µg/mL respectively. The reducing power of inhibiting free radical activity can be compared with synthetic antioxidants, according to Park et al. (2008). The study used BHT, BHA and α-tocopherols at 100 µg/mL scavenging activity is 64.61%; 69.67% and 32.68% respectively. It could be suggested that black rice brand 'Cempo Ireng' is an important nutritional food source for human health.

3.4. Analysis of macro- and micronutrients

The analysis of macronutrients in this study was to measure reducing and non-reducing carbohydrates (total carbohydrates), and proteins, and lipids which are 9.86 ± 1.11%; 12.56 ± 1.49%; 11.65 ± 0.27%; and 10.84 ± 0.09% respectively. The micronutrients are composed of minerals Ca, P, Fe with the levels of 38.45 ± 1.86 ppm; 22,565.5 ± 2.314 ppm; 91.46 ± 4.07 ppm, respectively (Table 4).

Carbohydrate especially glucose is a main energy source for the human body. Simple carbohydrates are digested faster than complex carbohydrates. Proteins are the main structural component of the human body and also act as enzymes of metabolic reactions in the body. This study showed that protein content is 11.65±0.26% which is higher than the protein content in white rice (8%) (Haryadi 2008). Lipid content in black rice bran in this study is 10.84 ± 0.09%. Meanwhile, lipid content in black rice bran from Japan is 15%, and 3.8% of it is oryzanol. Oryzanol is a potential compound to reduce total cholesterol in blood plasma (Zawistowski et al. 2009).

TABLE 4 Macro- and micronutrient content of BRB 'Cempo Ireng'.

No.	Macro- and micronutrients	Content
1.	Reducing carbohydrates (%)	12.56 ± 1.49
2.	Non-reducing carbohydrates (%)	9.86 ± 1.11
3.	Amilosa (%)	2.27 ± 0.09
4.	Proteins (%)	11.65 ± 0.27
5.	Lipids (%)	10.85 ± 0.09
6.	Phosphor (P) (ppm)	22,565.50 ± 2.30
7.	Ferrum (Fe) (ppm)	91.46 ± 4.07
8.	Calcium (Ca) (ppm)	38.45 ± 1.86

Note: Water content was 8.65%.

Calcium and phosphor are the dominant minerals related in bone and tooth formation and it is important in muscle contraction. The iron mineral is important for heme formation in red blood cells (Soetan et al. 2010). Overall, this study of macro- and micronutrients of BRB 'Cempo Ireng' suggested that this material can be used as a staple food which is good enough for protein, lipid and mineral sources.

4. Conclusions

This study concludes that fraction 1 of the methanolic extract of black rice bran contains cyanidin 3-O-glucoside, in which the glucoside is hexose. The activity of fraction 1 and 2 of the methanolic extract of this rice bran against DPPH free radical scavengers has a similar antioxidant activity. The nutrient content of black rice bran 'Cempo Ireng' for total carbohydrate, proteins, lipids, minerals Ca, P and Fe are comparable with other rice.

Acknowledgments

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Authors' contributions

R, YAP designed the study. AP carried out the laboratory works. AP, R, RP, YAP, WAST analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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