Antioxidant activity and compound constituents of gamma-irradiated black rice (Oryza sativa L.) var. Cempo Ireng indigenous of Indonesia

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Abstract. Suryanti V, Riyatun, Suharyana, Sutarno, Saputra OA. 2020. Antioxidant activity and compound constituents of gamma-irradiated black rice (Oryza sativa L.) var. Cempo Ireng Indigenous of Indonesia. Biodiversitas 21: 4205-4212. Nowadays, black rice is gaining consumer interest because of its health benefit. Due to the high content of antioxidant compounds such as phenolics and flavonoids, the nutritional profile of black rice is much better than any other rice varieties. Anthocyanins, pigment with powerful antioxidant properties, give a vibrant color to the rice. The antioxidant activity and chemical constituents of the non-irradiated and gamma-irradiated black rice Oryza sativa L. var. Cempo Ireng were investigated. The total phenolic content was determined based on the reaction of the Folin-Ciocalteu reagent with samples. Total anthocyanin was determined by the pH differential method. Antioxidant activity was fulfilled using DPPH method. The results revealed that non-irradiated and gamma-irradiated black rice were categorized as potent antioxidants. It is noted that irradiation increased antioxidant activity and changed the chemical components of black rice. Both of non-irradiated and irradiated black rice contains simple phenolics and flavonoids, including anthocyanins. Non-irradiated and irradiated black rice possess similar types of secondary metabolites, with different chemical content. The non-irradiated black rice contains anthocyanins of cyanidin-3-O-glucoside, whereas the irradiated black rice possesses anthocyanin of peonidin-3-O-glucoside. Additionally, irradiated black rice contains terpenoids, which increased its antioxidant activity compared to the control.

Keywords: Antioxidant activity, irradiated black rice, Oryza sativa, total anthocyanins, total phenolics

INTRODUCTION

Rice (Oryza sativa L.) is consumed by nearly half of the world’s population and considered one of the most important cereal crops. Black rice, a pigmented rice, is becoming more popular as a functional food (Pratiwi and Purwesti 2017). Black rice consumption inhibits the cancer cell invasion, prevents cardiovascular disease, and reduces the risk of fatty liver diseases, diabetes, and obesity (Pratiwi et al. 2015; Rathna Priya et al. 2019; Rukmana et al. 2016). The health benefits of black rice are associated with its nutritional values and antioxidant components (Hu 2003; Zhang et al. 2010; Bolea et al. 2016; Suttiarporn et al. 2016; Batubara et al. 2017). Black rice has a high content of fat, protein, and crude fiber. It also contains phenolic compounds, such as p-coumaric acid, caffeine acid, and ferulic acid, protocatechuic acid, vanillic acid, and hydroxyl benzoic acid, which are responsible for the antioxidant activity (Walters and Marchesan 2011). Furthermore, black rice contains flavonoids, including anthocyanins, which play an important role in antioxidant activity (So et al. 2011). Cyanidin-3-O-glucoside and peonidin-3-O-glucoside are anthocyanins usually found in black rice (Figure 1). Cyanidin-3-O-glucoside is the main anthocyanins, forming to 94% of the total anthocyanins content (Chen et al. 2012; Hou et al. 2013; Hao et al. 2015; Apridamayan et al. 2017). The color of black rice is caused by anthocyanin pigments (Park et al. 2008; Lee 2010; Loypimai et al. 2016). Nowadays, phenolic- and flavonoid-rich natural food have gained interest in nutrition and food science (He and Giusti 2010; Cisowski et al. 2011). These compounds possess aromatic rings having at least one hydroxyl group that can act as electron donors. Their hydroxyl group can directly engage in antioxidant action (Walter and Marchesan 2011; Khoo et al. 2017; Suryanti et al. 2020).

![Figure 1. Structure of cyanidin-3-O-glucoside and peonidin-3-O-glucoside](image-url)
The cultivation of black rice is very rare due to its plant height, sensitivity to the natural enemy, long harvest period, and low productivity. A mutation is often used to overcome the limitation of the crops (Harding et al. 2012; El-Degwy 2013; Marcu et al. 2013; Shao et al. 2013). It has been reported that mutation induction through irradiation improves the quality of local black rice cultivar (Hartanti et al. 2017). The grains of black rice var. Cempo Ireng irradiated with gamma-ray at a dose of 200 and 300 Gy, namely BR-200 and BR-300 results in shorter harvesting time, reduced plant height, and enhanced stress tolerance (Patni et al. 2008). It also changes the anthocyanin content of the wild variety of black rice (Purwanto et al. 2019). However, the nutritient content, i.e. moisture, lipids, proteins, carbohydrates, and fibers contents in gamma-irradiated black rice var. Cempo Ireng was not significantly different from non-irradiated rice.

The nutritional value of BR-200 was slightly better than that of BR-300 (Riyatun et al. 2017). Although the antioxidant activity and chemical compounds of the black rice have been reported, studies on the antioxidant activity and chemical compounds of irradiated black rice are still limited. Therefore, this paper describes the antioxidant activity and chemical compound diversities of irradiated black rice in comparison to non-irradiated variety.

MATERIALS AND METHODS

Materials

Non-irradiated black rice (BR-NI) and irradiated black rice (Oryza sativa L. cv. Cempo Ireng) were used in this research. The irradiated black rice used in this study was the third generation of gamma-irradiated black rice with doses 200 and 300 Gy (BR-200 and BR-300). 2,2-diphenyl-(1-picrylhydrazyl) (DPPH) and gallic acid were purchased from Sigma Aldrich. Other analytical grade chemicals were obtained from E-Merck and used without further purification.

Sample preparation

Black rice grains (200 g) were grounded into a fine powder and macerated three times with ethanol at room temperature for 24 h. The filtrate was collected and evaporated using a rotary evaporator to get concentrated extracts.

Gas Chromatography-Mass Spectroscopy (GC-MS)

The ethanol extract of BR-NI and BR-200 was analyzed by Shimadzu QP2010S GC-MS. The GC-MS was run using EI 70 Ev ionizing type, Rtx 5 Ms column (30 m length x 0.25 mm ID), injector temperature of 300°C, column temperature of 70°C, splitless injection method, detector temperature of 300°C and the carrier gas was He with the operating pressure of 13.7 kPa.

Liquid Chromatography-Mass Spectrometry (LC-MS)

The ethanol extract of BR-NI and BR-200 was analyzed by LC-MS Waters 2489 with a UV-Vis detector. The column temperature was 35°C and the solvent used was a mixture of solution A (aqua dest: formic acid = 9:1) and solution B (aquabidest: acetonitrile: formic acid = 6:3:1) with a flow rate of 1 mL/min for 25 mins. The solvents gradient used for the initial 5 mins was 75% solution A and 25% solution B, the second 5 mins were 71% solution A and 29% solution B, the third 5 mins was 66% solution A and 34% solution B, the fourth 5 mins was 62% solution A and 38% solution B, 57% A and 43% solution B, the last 5 mins was 100% solution B. Their absorbance was measured at 520 nm. MS analysis was used ESI ionization.

Determination of total phenolics

The total phenolics were analyzed by a modified method of Doymaz and Karasu (2018). In 5 mL volumetric flask, gallic acid (100 ppm, 1 mL) was mixed with Folin-Ciocalteu reagent (0.5 mL) and left for 1 minute. Four ml of 7.5% Na2CO3 solution was added to the mixture and left for a further 1 minute. The samples were analyzed using UV-Vis Spectroscopy (Perkin Elmer Precisely Lambda 25 UV-Vis) at 10-minute intervals until reaching equilibrium state. The same procedure was applied for obtaining the standard curves of gallic acid with concentrations of 25, 50, 75, 100 ppm. The ethanol extract of black rice (100 ppm) was analyzed for total phenolics content. Total phenolics were quantified using formula (1), where C is gallic acid concentration determined from the calibration curve (g/L), V is the volume of sample extract (L) and m is the weight of the sample extract (K). Total phenolics are expressed as milligram of gallic acid equivalents (GAE) per gram of dry.

Phenolics Total (g GAE /Kg) = \( \frac{C \times V}{m} \)  

Determination of total anthocyanins

The determination of anthocyanin content was carried out using the pH-differential method (AOAC 2005-02). Ethanol extract (0.05 g) was put into a tube and added with 4 mL of KCl buffer solution (pH 1) and 4 mL of CH3COONa buffer solution (pH 4.5). After 2 hours, the samples were filtered and their absorbance was measured at 520 nm and 700 nm wavelengths using UV-Vis Spectroscopy (Perkin Elmer Precisely Lambda 25 UV-Vis).

The anthocyanin content was calculated using formula 2, where A is (\( A_{520-A_{700}} \))\(_{\text{H+}}\)-(\( A_{520-A_{700}} \))\(_{\text{H+}}\), \( \varepsilon \) is the molar extinction of cyanind-3-O-glucoside (26,900 L.mol⁻¹.cm⁻¹), I is cuvette thickness (1 cm), Mw is the molecular weight of cyanind-3-O-glucoside (449.2 g/mol), DF is dilution factor, V is the volume of sample extract, W is the weight of sample extract.

\[ TA = \frac{A}{\varepsilon \times Mw \times DF \times \frac{V}{W}} \times 100\% \]  

Cyanin-3-O-glucoside, the most common pigment in nature, is selected as a standard for the evaluation of total anthocyanins contents.

Determination of antioxidant activity

The antioxidant activity was determined using DPPH scavenging radical activity by following the method of
Salazar-Aranda et al. (2011). The stock solution (100 ppm) was diluted to the concentrations of 12.5, 25, 50, 75, and 100 ppm in a 5 mL volumetric flask. Vitamin E concentrations (2.5, 5, 10, 12.5, and 20 ppm) was used as a positive control. One mL of DPPH solution in methanol (100 ppm) was added to the mixture and was left for 30 minutes in a dark condition. The mixture was then analyzed at the λ<sub>max</sub> wavelength. The λ<sub>max</sub> wavelength was obtained previously by measuring the DPPH solution (100 ppm) at a wavelength of 800–400 nm. Antioxidant activity was assessed using formula (3), where A is absorbance of sample and B is the absorbance of the black ethanol extract of black rice. The color changes of DPPH solution from purple to yellow is observed when the radical is quenched by antioxidant.

### RESULTS AND DISCUSSION

The ethanol extract of black rice grain was obtained in 14–15% yield as a viscous yellow oil. These extracts were analyzed for total phenolic and total anthocyanin contents (Table 1). The total phenolic content determination was performed based on the reaction of the Folin-Ciocalteu reagent with samples. The resulting blue-colored solution intensity is proportional to the amount of phenolics present. Total anthocyanin is determined based on changes in anthocyanin structure at pH 1 and pH 4.5. The difference in absorbance between the two buffer solutions is due to the total content of monomeric anthocyanin pigments. The non-enzymatic brown pigments and polymerized anthocyanin pigments are omitted from the absorbance calculation. They do not show reversible structural transformation as a function of pH.

Antioxidant activity of the extracts was determined using DPPH method. DPPH radical is widely used as a substrate for evaluating antioxidant activity because DPPH is stable radical, and its simplicity testing and accuracy.

The color changes of DPPH solution from purple to yellow is observed when the radical is quenched by antioxidant. The DPPH radical scavenging activities of vitamin E and black rice are presented in Figure 2. The IC<sub>50</sub> values were presented in Table 2. The lower the IC<sub>50</sub> value, the better the antioxidant activity. The radical scavenging capacity of the black rice extracts exhibited concentration-dependent.

### Table 1. Total phenolics and total anthocyanins content of the black rice extracts

| Samples | Total phenolics content (mg GAE/g) | Total anthocyanins content (g Cyanidin-3-O-glucoside/kg) |
|---------|-----------------------------------|------------------------------------------------------|
| BR-NI   | 34.12 ± 0.78                     | 0.092                                                |
| BR-200  | 37.63 ± 0.89                     | 0.123                                                |
| BR-300  | 42.15 ± 0.93                     | 0.154                                                |
| Vitamin E | 43.53 ± 0.99                  | 0.184                                                |

### Table 2. IC<sub>50</sub> values of vitamin E and black rice

| Sample | IC50 (ppm) | Antioxidant activity |
|--------|------------|----------------------|
| BR-NI  | 47.99      | Very strong          |
| BR-200 | 32.67      | Very strong          |
| BR-300 | 43.53      | Very strong          |
| Vitamin E | 3.53       | Very strong          |

### Figure 2. DPPH free radical scavenging activity of vitamin E and black rice extracts in different concentrations

### Table 3. Chemical composition of non-irradiated black rice BR-NI and 3<sup>rd</sup> generation of gamma-irradiated black rice by GC-MS

| Rt     | %    | BR-NI Compound                  | Rt     | %    | BR-200 Compound                  |
|--------|------|---------------------------------|--------|------|-----------------------------------|
| -      | -    | -                               | 16.375 | 5.08 | Geyrene                           |
| 9.24   | 4.37 | -                               | 25.775 | 1.94 | Caryophyllene                     |
| 30.76  | 3.05 | -                               | 25.233 | 1.73 | Selinene                          |
| 47.27  | 3.58 | Decanoic acid                   | 27.467 | 2.04 | Cadinene                          |
| 47.99  | 1.73 | Hexadecanoic acid               | 28.925 | 1.73 | Octadecanolic acid               |
| 36.974 | 23.55| Hexadecanoic acid               | 4207   | 12.5 | 9,12-Octadecadienoid acid        |
| 10.21  | 1.73 | 9,12-Octadecadienoid acid       | 47.079 | 9.24 | Cyclohexane ethanamine            |
| 54.86  | 9.24 | 9,12-Octadecadienoid acid       | 44.014 | 10.21| 2-Methyl-, 2-(dimethylamino)ethyl ester|
| 10.50  | 12.5 | 2-Methyl-, 2-(dimethylamino)ethyl ester | 47.079 | 9.24 | Cyclohexane ethanamine            |
Figure 3. GC chromatogram of ethanolic extract of nonirradiated black rice BR-NI

Figure 4. GC chromatogram of ethanolic extract of 3rd generation of gamma-irradiated black rice BR-200

Figure 5. LC-MS chromatogram of non-irradiated black rice BR-NI
Figure 6. LC-MS chromatogram of 3rd generation of gamma-irradiated black rice BR-200

Table 4. Chemical composition non-irradiated black rice BR-NI by LC-MS

| No. | Rt   | m/z    | Compound                                                                 |
|-----|------|--------|--------------------------------------------------------------------------|
| 1   | 0.476| 185.40 | Methyl-4-hydroxy-3-methoxybenzoate                                       |
| 2   | 0.817| 148.07 | -                                                                         |
| 3   | 1.192| 778.83 | -                                                                         |
| 4   | 1.634| 268.22 | Tectochrysin                                                              |
| 5   | 2.009| 450.11 | Cyanidin-3-O-glucoside                                                   |
| 6   | 2.350| 404.21 | -                                                                         |
| 7   | 2.827| 126.43 | Thymine                                                                  |
| 8   | 3.099| 726.57 | -                                                                         |
| 9   | 3.542| 330.28 | 2-(3-hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one              |
| 10  | 3.678| 933.78 | -                                                                         |
| 11  | 4.398| 397.22 | Ergosta-4,6,22-trien-3-ol                                                 |
| 12  | 4.564| 382.79 | -                                                                         |
| 13  | 4.871| 797.57 | -                                                                         |
| 14  | 5.177| 229.00 | Myristic acid                                                             |
| 15  | 5.348| 872.67 | -                                                                         |
| 16  | 5.995| 257.26 | Methyl-9-methyltetradecanoate                                            |
| 17  | 6.779| 925.75 | -                                                                         |
| 18  | 7.119| 383.45 | -                                                                         |
| 19  | 7.460| 669.58 | -                                                                         |
| 20  | 7.869| 120.82 | 4-vinyl phenol                                                            |
| 21  | 8.005| 913.62 | -                                                                         |
| 22  | 8.277| 729.05 | -                                                                         |
| 23  | 8.618| 858.68 | -                                                                         |
| 24  | 9.061| 472.41 | -                                                                         |
| 25  | 9.436| 112.32 | Heptenal                                                                  |
| 26  | 9.776| 316.73 | -                                                                         |
Table 5. Chemical composition of the 3rd generation of gamma-irradiated black rice BR-200 eluded by LC-MS

| No. | Rt   | m/z        | Compound                                    |
|-----|------|------------|---------------------------------------------|
| 1   | 0.408| 384.11     | -                                          |
| 2   | 0.613| 296.44     | 3,7,11,15-tetramethyl-2-hexadecen-1-ol      |
| 3   | 1.429| 860.48     | -                                          |
| 4   | 1.600| 408.24     | -                                          |
| 5   | 1.906| 647.18     | -                                          |
| 6   | 2.314| 302.30     | 7-hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one |
| 7   | 3.199| 759.86     | -                                          |
| 8   | 3.335| 621.60     | -                                          |
| 9   | 3.948| 789.44     | -                                          |
| 10  | 4.356| 464.50     | peonidine-3-O-glucoside                     |
| 11  | 4.663| 971.05     | -                                          |
| 12  | 5.173| 420.75     | -                                          |
| 13  | 5.718| 167.67     | 4,7,7-trimethylbicyclo[3,3,0]octan-2-one    |
| 14  | 6.194| 457.57     | -                                          |
| 15  | 6.841| 174.88     | -                                          |
| 16  | 7.385| 118.18     | -                                          |
| 17  | 7.555| 682.72     | -                                          |
| 18  | 7.828| 904.27     | -                                          |
| 19  | 7.998| 574.63     | -                                          |
| 20  | 8.542| 719.73     | -                                          |
| 21  | 9.019| 127.41     | 5-hydroxymethyl-2-furancarboxaldehyde       |
| 22  | 9.257| 926.22     | -                                          |
| 23  | 9.802| 773.69     | -                                          |

The non-irradiated black rice (BR-NI) has the highest total phenolics and anthocyanins contents. The total phenolics content of BR-200 is higher than that of BR-300, whereas the total anthocyanins contents are lower than that of BR-300. All samples can be categorized as strong antioxidants since all samples have IC$_{50}$ values less than 50 ppm. Remarkably, both the irradiated black rice have lower IC$_{50}$ values than that of the non-irradiated black rice. In this case, BR-200 has lower IC$_{50}$ values than that of BR-300, indicating that the BR-200 has a higher antioxidant activity than the BR-300. Furthermore, the BR-NI and BR-200 were subjected to GC-MS and LC-MS analysis to identify the chemical constituents. The GC spectra of BR-NI and BR-200 revealed 13 and 18 peaks, respectively (Figures 3 and 4). The retardation time (R$_r$) and compounds of BR-NI and BR-200 detected by GC-MS are presented in Table 3. The LC spectra of BR-NI and BR-200 displayed 26 and 23 peaks, respectively (Figures 5 and 6). The retardation time (R$_r$) and mass-to-charge ratio (m/z) of compounds of BR-NI and BR-200 eluted by LC-MS are presented in Tables 3 and 4, respectively.

**Discussion**

The results of GC-MS analysis of BR-NI and BR-200 showed that the non-irradiated and gamma-irradiated black rice extracts have the same 4 major components, i.e. hexadecanoic acid; 9,12-octadecadienoic acid; 2-methyl-, 2-(dimethylamino) ethyl ester and cyclohexaneethanamine (Table 3). Several compounds in BR-200 were not detected in BR-NI, which are classified as terpenoids, such as geyrene, carophyllene, selinene, and cadinene. Decanoic acid was also only be found in BR-200.

The results of LC-MS analysis of BR-NI showed that the identified compounds can be categorized as 1 aliphatic aldehyde (heptanal), 1 aromatic (1-ethyl-3-methylbenzene), 2 simple phenolics (methyl-4-hydroxy-3-methoxybenzoate and 4-vinylphenol), 3 flavonoids (tectochrysin, cyanidine-3-O-glucoside and 2-(3-hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one) and 1 sterol (ergosta-4,6,22-trien-3-ol). There is a flavonoid, chlorophyll a, without the Mg$^{2+}$ ion. The identified compounds of BR-200 can be categorized as 1 aliphatic aldehyde (3,7,11,15-tetramethyl-2-hexadecen-1-ol), 1 furan (5-hydroxymethyl-2-furancarboxaldehyde), 1 macrocycle (bicyclo (4,7,7-trimethylbicyclo[3,3,0]octan-2-one) and 2 flavonoids (7-hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one and peonidine-3-O-glucoside).

Among the identified compounds in BR-NI, the phenolics and flavonoids are responsible for antioxidant activity. Methyl-4-hydroxy-3-methoxybenzoate or methyl vanillate present in cow’s milk and beer are known to have antioxidant activity (Khan 2019). 4-Vinylphenol or 4-hydroxy styrene is also found in beer and wine having a hydroxy group at position 4 (Fulcrand, 1996). Tectochrysin is a flavone substituted by a hydroxy group at position 4 and a methoxy group at position 7 respectively. Tectochrysin is a major compound in propolis and has been known to have antioxidant activity that can inhibit the growth of human colon cancer cells (Park et al. 2015). Cyanidin-3-O-glucoside is an anthocyanin which is a reddish-purple pigment in fruits and vegetables, and as the main pigment in red-colored vegetables and berries (Khoo et al. 2017). It possesses a good antioxidant activity for radical scavenging capacity against superoxide but not hydroxyl radicals (Olivas-Aguirre et al. 2016, Stintzing et
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