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Article

Determination of Anthocyanin Content and Antioxidant Activity in Sidenuk Black Rice Mutant (*Oryza sativa* var. *sidenuk*)

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Abstract. The Sidenuk black rice mutant is the latest development of Sidenuk Rice which is known to excel in high rice production and is resistant to pests. Due to the many health benefits offered by black rice and no data on anthocyanin content and antioxidant activity of the mutant black rice variety Sidenuk, further research is needed. This study aims to analyze the anthocyanin content and antioxidant activity of Sidenuk black rice mutants (*Oryza sativa* var. *sidenuk*). The yield of concentrated macerate of Sidenuk black rice mutant produced was 23.25%. Based on the results obtained in the qualitative test and information from the journal, it indicates that Sidenuk black rice mutant contains anthocyanin compounds, but not in Sidenuk white rice mutant. The cyanidin 3-glucoside compound was the most abundant anthocyanin compound in the Sidenuk black rice mutant, followed by peonidin-3-glucoside. The total anthocyanin content in black rice is 1.07 ± 0.04 mg/g. This data is higher than black glutinous rice but lower than other black rice varieties. The IC_{50} value of Sidenuk black rice mutant is 36.02±5.09 µg/mL, which is classified as strong antioxidant and is the strongest of all black rice varieties in this study.

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1. Introduction

Free radicals and oxidative stress are two major factors that play a role in the development of various chronic diseases, such as cancer, heart disease, diabetes, and Alzheimer's [1-2]. Exposure to free radicals can come from internal (body metabolism) and external sources (pollution, radiation, and unhealthy lifestyle) [3]. To combat the negative effects of free radicals, the body needs antioxidants. Antioxidants are compounds that can neutralize free radicals and prevent cellular damage [4].

One potential source of natural antioxidants is black rice [5]. Black rice is known to have a higher antioxidant content than other types of rice, mainly due to the presence of anthocyanin pigments [6]. Anthocyanins are a group of flavonoid compounds that give black rice its purple or black color, and have various health benefits. Previous research has highlighted several key characteristics and potential health benefits of black rice including as antioxidants [7-8], anti-inflammatory [9-10], anticancer [11-12], and antidiabetic [13-14]. Black rice also contains other beneficial compounds, such as fiber to promoting digestive health and aiding in weight management [15-16]; minerals, including iron, magnesium, and zinc [17-18]; vitamins, such as vitamin B1 (thiamine) and vitamin E [19-20].

There are various varieties of black rice in Indonesia. One of the latest black rice developments is the Sidenuk black rice mutant [21]. Sidenuk black rice mutant is a local black rice variety that has great potential to be developed. However, because the Sidenuk black rice mutant is a new black rice variety compared to other varieties, research regarding the anthocyanin content and antioxidant activity of the Sidenuk black rice mutant is not yet available.

Sidenuk black rice mutants have several advantages compared to other black rice, including higher yield potential and potential for tolerance to suboptimal environmental conditions [22-23]. Sidenuk black rice mutant comes from the Sidenuk rice parent which has a higher yield potential compared to other rice varieties. This makes it more attractive to farmers and has the potential to increase overall black rice production. In addition, Sidenuk rice is known to have good tolerance to less than optimal environmental conditions, such as drought and pest and disease attacks [24]. This makes it more adaptive and sustainable to be planted in various land conditions. With these advantages, Sidenuk black rice mutant has great potential to be developed as a functional food source that is rich in antioxidants and beneficial for health.

This study aims to analyze the anthocyanin content and antioxidant activity of Sidenuk black rice mutants (*Oryza sativa* var. *sidenuk*). This study use the pH differential method to determine the total anthocyanin content, LC-HRMS to determine the type of anthocyanin and the DPPH method to test the antioxidant activity of the Sidenuk black rice mutant (*Oryza sativa* var. *sidenuk*). The pH differential method is a quick quantitative analysis to determine the concentration of anthocyanins in a sample [25]. This method is not only fast but also convenient than the HPLC method, because doesn't require an anthocyanin standard, which can be expensive to purify [26]. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) can accurately determine the mass of molecular and fragment ions of unknown components and reducing false positives or negatives, making it crucial in obtaining comprehensive and reliable profiles in sample analysis [27-28]. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay has several advantages over other methods for measuring antioxidant activity, including easy to perform, doesn't require a generator to create the radical compared to ABTS [29], fast, inexpensive, accurate, reproducible results, highly sensitive, efficient for thermally unstable compounds, and versatile [30].

This study is expected to provide valid scientific information on anthocyanin content and antioxidant activity in Sidenuk black rice mutant, as well as become the basis for the development of innovative and competitive black rice-based food products. This research is also expected to contribute to the preservation and development of local black rice varieties, as well as increasing public awareness of the importance of consuming healthy and antioxidant-rich foods.

2. Experimental Section

2.1. Plant samples

The samples used in this research were Sidenuk black and white rice mutant flour grinded with pestle and mortar. Sidenuk black and white rice mutants were harvested from rice fields in the Maninjau area, West Sumatra, Indonesia. The materials used in this study were methanol, citric acid, 2M HCl, 2M NaOH, n-butanol, acetic acid, distilled water, TLC plate. Potassium chloride buffer (KCl 0.025M, pH 1), distilled water, sodium acetate buffer (0.4M, pH 4.5), concentrated HCl, quercetin, DPPH (2,2-diphenyl-1-picrylhydrazyl), methanol, and vial tubes.

2.2. Research Method Flowchart

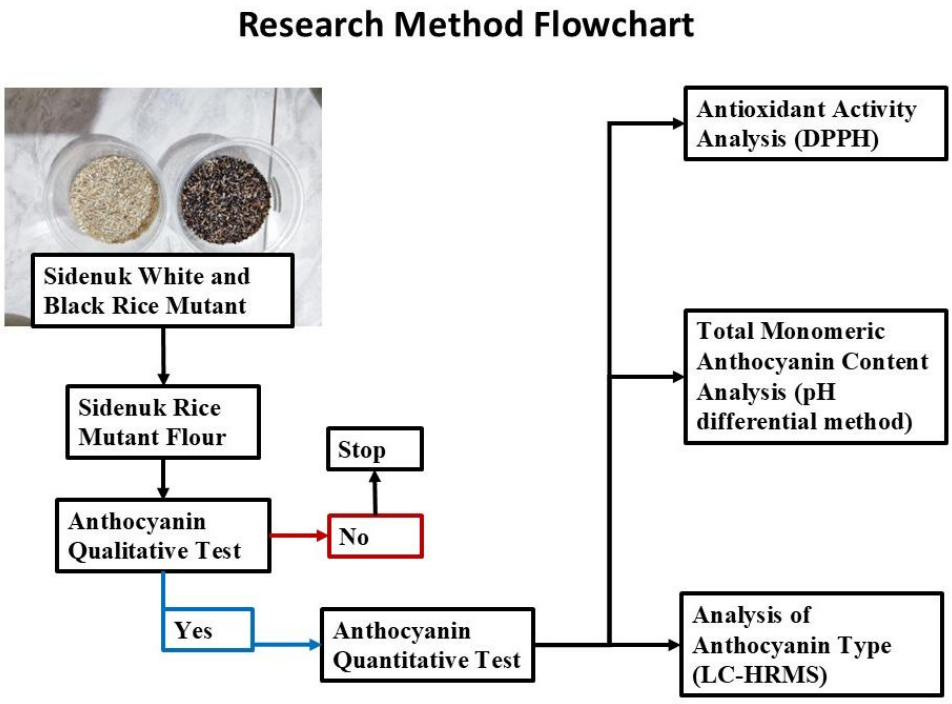


Figure 1. Research Method Flowchart

2.3. Sidenuk Black Rice Mutant Extraction

A total of 50 g of Sidenuk black rice mutant flour was put into an extraction container, then 500 ml of pH 3 methanol solvent (acidified with citric acid) was added [31]. The Sidenuk white rice mutant was extracted using the same solvent as a negative control. The extraction container was tightly closed and stored in a dark place for 24 hours. The macerate obtained was separated from the pellet using Whatman No. 1 filter paper. The black rice flour pellet was re-macerated up to three times to maximize the extraction of secondary metabolite compounds. Furthermore, the macerate was concentrated using a rotary vacuum evaporator (rotavapor) at a temperature of 40°C. The concentrated macerate was stored in a tightly closed dark bottle at refrigerator temperature (10±2°C) until used for analysis. The concentrated macerate obtained was weighed and compared with the initial weight of the black rice flour before extraction [31].

$$\text{Yield (\% w/w)} = \frac{weight\ of\ black\ rice\ extract\ (g)}{weight\ of\ black\ rice\ flour\ (50g)} x100\%$$

The reason for choosing methanol pH 3 solvent in this study is because first, methanol solvent is classified as a polar solvent, so that it can bind polar anthocyanin compounds, based on the principle of "like dissolves like" [31]. Second, methanol solvent has a boiling point of 64.96°C. This temperature is close to the stability temperature of anthocyanin compounds (60 – 70°C), so that methanol can evaporate first before damaging the anthocyanin compounds during rotary vacuum evaporator (rotavapor) process [32]. Last, acidic conditions (pH ~ 3) stabilize the anthocyanin molecule, specifically its flavylium cation form, so acids are often incorporated into the solvent. Weak acids such as formic, citric, or acetic acid are preferred for this purpose, because strong acids can actually degrade the anthocyanins [33].

2.4. Anthocyanin Qualitative Color Test

The color test on anthocyanin qualitative is carried out in two steps. The first step is to heat the sample with 2M HCl at a temperature of 100°C for 5 minutes, then observe the color of the sample. If the red color in the sample does not change (stable), this indicates the presence of anthocyanin. The second step is to add the sample with 2M NaOH drop by drop. The anthocyanin content is indicated by the color change of the sample to red if HCl is added and to greenish blue and fades slowly if NaOH is added [34-35]. This method was repeated three times to increase the credibility of the results.

2.5. Thin Layer Chromatography (TLC) Test

The mobile phase (solvent) BAA is prepared by adding n-butanol, acetic acid, and distilled water in a ratio of 4:1:5, then the top layer is taken. The mobile phase is put into a glass container as much as 1-5 ml (try not to exceed the start line on the plate). Black rice anthocyanin extract is spotted on the TLC plate using a capillary pipette. The plate is put into a container containing the mobile phase, then the container is closed with a glass lid. Wait for the movement of the mobile phase from the start line to the finish line. The movement of the spots is calculated in the form of Rf values. This method was repeated three times to increase the credibility of the results. The Rf value of anthocyanin in the BAA mobile phase is moderate, namely in the range of 0.10-0.40 [35-36].

$$Rf\ Value = \frac{The\ distance\ traveled\ by\ the\ spot\ from\ the\ starting\ line}{The\ distance\ traveled\ by\ the\ solvent\ from\ the\ start\ line\ to\ the\ finish\ line}$$

2.6. LC-HRMS

LC-HRMS analysis was performed by Corpora Science. The Liquid Chromatography (LC) instrument used was the Vanquish™ Horizon UHPLC with Binary Pump model from Thermo Scientific™ (Germering, Germany) with an analytical column of the Accucore™ Phenyl Hexyl model measuring 100 mm length x 2.1 mm ID x 2.6 µm particle size (Lithuania). There were two (2) mobile phases used, namely A: MS grade Water with 0.1% Formic Acid and B: MS grade Acetonitrile with 0.1% Formic Acid. The LC specifications used were a flow rate of 0.3 mL/min, a total run time of 25 minutes, a column temperature of 40 °C, and an injection volume of 5 µL. The LC process began with 5% mobile phase B, then gradually increased to 90% for 16 minutes, held at 90% for 4 minutes, then reduced back to 5% B for 25 minutes.

The High Resolution Mass Spectrometry (HRMS) instrument used was the Orbitrap™ Exploris 240 HRMS model (Bremen, Germany) from Thermo Scientific™. The HRMS parameters used were Acquisition mode: Full MS/dd-MS2; Polarity: Positive and negative (polarity switching); Full MS resolution: 60.000 FWHM; Scan Range: 70-800 m/z; Maximum Injection Time: 100 ms; Intensity threshold: 5000; Charge states: 1; Mass tolerance: 5 ppm; dd-MS2 resolution: 22.500 FWHM; Normalized Collision Energy: 30, 50, 70; Collision gas: Nitrogen. The ion source used was produced with an Optamax™ NG Heated Electrospray Ionization (H-ESI) machine, with the following Spray Voltage specifications: Positive (3500 V), Negative (2500 V); Sheath gas: 35 Arbitrary Units (AU); Auxiliary gas: 7 Arbitrary Units (AU); Sweep gas: 1 Arbitrary Units (AU); Ion Transfer Tube

Temperature: 300 °C; Vaporizer Temperature: 320 °C. The HRMS process began with 10 µL of sample diluted in 1 mL of HPLC grade methanol, then vortexed for 60 seconds. The supernatant was filtered through a 0.22 µm nylon filter, and the sample was ready for injection.

2.7. Total Monomeric Anthocyanin Content Analysis (pH differential method).

Determination of monomeric anthocyanin content was carried out using the pH differential method. Potassium chloride buffer (0.025M KCl, pH 1) was prepared by adding 0.186g KCl into 98 ml of distilled water. The pH was measured and then adjusted to pH 1 by adding concentrated HCl. The solution was transferred into a 100 ml volumetric flask and distilled water was added up to 100 ml. Sodium acetate buffer (0.4M, pH 4.5) was prepared by adding 5.443 sodium acetate into 96 ml of distilled water. The pH was measured and then adjusted to pH 4.5 by adding concentrated HCl. The solution was transferred into a 100 ml volumetric flask and distilled water was added up to 100 ml [12]. The concentrated macerate of Sidenuk black rice mutant was serially diluted with each buffer (pH 1 and 4.5 buffers). The absorbance of dilute macerates of pH 1 and pH 4.5 were measured using UV-Vis spectrophotometer at wavelengths of 520 and 700 nm respectively [37-38]. This method was repeated three times to increase the credibility of the results. The concentration of anthocyanin pigments was expressed as cyanidin-3-glucose equivalents, calculated as follows:

$$Anthocyanin\ content\ (mg/L) = \frac{(\Delta A \times MW \times DF \times 1000)}{\epsilon \times 1}$$

ΔA is the result of the difference in absorbance of pH1 with pH 4.5 (A = A(λ520 – λ700) pH 1 - A(λ520 – λ700) pH 4.5); MW is the molecular weight (449.2 g/mol for cyanidin-3-glucoside); DF is the dilution factor; ϵ is the molar extinction coefficient 26.900 L.mol-1.cm-1; 1 is the cuvette width in cm; 1000 is the conversion factor from g to mg. Results are reported as monomeric anthocyanins, expressed as cyanidin-3-glucose equivalents in mg/L.

Anthocyanin pigments, when in their single-molecule (monomeric) form, change color depending on the acidity (pH) of their environment. At a very acidic pH (1.0), they exhibit a colored form (oxonium). As the pH becomes less acidic (around 4.5), they shift to a colorless form (hemiketal). The intensity of the color, measured by light absorption at 520 nm, is directly related to how much monomeric anthocyanin is present. The measurements are standardized against a specific type of anthocyanin, cyanidin-3-glucose. However, anthocyanins can also exist in a larger, polymerized form due to degradation. These polymerized anthocyanins don't change color with pH and are not measured because they absorb light at both low and higher pH values, making it difficult to distinguish them from the monomeric forms at pH 4.5. Absorbance readings at 700nm are taken to account for any cloudiness in the sample. If the diluted sample is too cloudy, it should be clarified by centrifugation or filtration prior to measurement. It's important to use a filter that won't bind the anthocyanins, such as a Millipore membrane filter with a 1.2µm pore size [38-39].

2.8. Antioxidant Activity Analysis (DPPH)

Macerate of Sidenuk black rice mutant was diluted with serial dilutions of 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, and 1 mg/L. Quercetin was diluted with serial dilutions of 3 mg/L, 1.5 mg/L, 1 mg/L, 0.5 mg/L, and 0.1 mg/L. Quercetin was used as a comparison (positive control). DPPH 0.167 mM was made by dissolving 3.9 mg of DPPH in 60 ml of methanol. A total of 3 ml of sample was put into a closed vial tube, then added with 3 ml of DPPH 0.167 mM. The sample was incubated in a dark room for 30 minutes. The absorbance value of the sample was calculated using a UV-Vis spectrophotometer at a wavelength of 517 nm. This method was repeated five times to increase the credibility of the results. The sample's ability to inhibit DPPH is calculated by:

$$\%\ Inhibition = \frac{(A0 - A1)}{A0} \times 100\%$$

A0 is DPPH before the sample is given. A1 is DPPH after the sample is given. After that, a linear regression curve is made with the X axis showing the sample concentration and the Y axis showing the % inhibition, until the equation y = bx + a is obtained. The IC50 value is calculated by [40-41]:

$$X = \frac{(50 - a)}{b}$$

3. Results and Discussion

3.1. Yield of Concentrated Black Rice Extract

The yield of concentrated extract of Sidenuk black rice mutant produced was 23.25% (Table 1). The yield of methanol extract of black rice is calculated by comparing the weight of the extract after evaporation to the weight of the rice before extraction [42]. The extract is then filtered and evaporated under pressure using a rotary vacuum evaporator (rotavapor) to produce a 'concentrated' extract. The extract is said to be 'concentrated' because there is no more solvent that can be sucked by the rotary evaporator. Several factors can influence the amount of methanol extract obtained from black rice. These include the duration of the extraction process, the temperature at which the extraction is carried out, the concentration of the methanol solution used, and the pH level of that solution [43]. These results are lower to the yield of black glutinous rice extract with methanol solvent, which is 25.30% [40] but higher than other four varieties of black rice methanol extract [42], as shown in Table 1. These results show that acidifying methanol to pH 3 using a weak acid (citric acid) will increase the yield of the concentrated extract.

Methanol at a pH of 3 was selected as the solvent for this study for three key reasons. First, as a polar solvent, methanol effectively dissolves polar anthocyanin compounds, adhering to the principle of "like dissolves like" [31]. Second, its boiling point of 64.96°C is close to the optimal temperature range for anthocyanin stability (60-70°C). This proximity allows the methanol to evaporate during rotary evaporation without damaging the anthocyanins [32]. Finally, the acidic pH of approximately 3 stabilizes the anthocyanins by promoting the flavylium cation form. This is achieved using weak acids, such as formic, citric, or acetic acid, as strong acids can degrade these compounds [33].

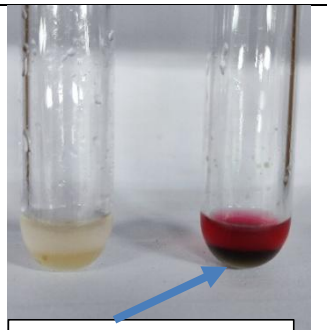
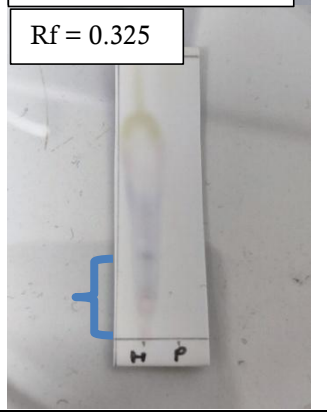
Table 1. Yield of concentrated extract of Sidenuk black rice mutant

No	Sample	Solvent	Yield (%)
1	Sidenuk black rice mutant	Methanol pH 3 (acidified with citric acid)	23.25
2	Black glutinous rice [40]	Methanol p.a	25.30
3	Black Rice Poireiton [42]	Methanol 70%	20%
4	Black Rice Kokngangbi [42]	Methanol 70%	17.5%
5	Black Rice Amubi [42]	Methanol 70%	22.25%
6	Black Rice Sempak [42]	Methanol 70%	18.75

3.2. Qualitative Results of Anthocyanin

The results of the qualitative flavonoid test (Table 2) showed that the Sidenuk black rice mutant contained anthocyanin as indicated by a color change to red after the addition of HCl, the formation of a blue-green haze after the addition of NaOH, and the formation of a purple spot with Rf 0.32 in the thin layer chromatography (TLC) test. Meanwhile, the white rice sample did not show anthocyanin content.

Table 2. Qualitative test results of Sidenuk black rice mutants

No	Test	Literature [34]	Sample	Results	Figure
1	Heated with 2M HCl at 100°C for 5 minutes	Red	Black Rice	Red (+)	
			White Rice	No red color is formed (-)	
2	Add 2M NaOH drop by drop.	Red, then turns blue-green and fades.	Black Rice	Red, then forms a blue-green mist, fades and settles at the bottom of the tube (+)	
			White Rice	No blue color is formed (-)	
3	TLC with BAA (n-butanol, acetic acid, and distilled water)	Rf (0.1 – 0.4)	Black Rice	Rf = 0.32 (+)	
			White Rice	n/a (-)	

Anthocyanins change color at different pH levels because their molecular structure changes along with changes in the pH of the solution from acidic to basic and vice versa. The anthocyanin molecule undergoes 'protonation', that is, a proton (H^+) is attached to the phenol group at a low pH of around 3. Anthocyanins in this environment, are positive ions, or cations, and absorb light in the blue-green spectrum (around 450-560nm), making them visible red to human eyes. However, when the pH of the environment increases, anthocyanin molecules undergo 'deprotonation', so that protons (H^+) are removed from the phenol group. As a result, the light absorption of the molecule shifts to absorb light in the yellow-orange spectrum (around 570-620nm), giving it a bluish-purple appearance to the human eye. If the pH is lowered again, the protons reattach to the molecule, changing the light absorption once again and returning it to its original color [44].

Based on Table 2, it can be seen that on the H track, the Sidenuk black rice mutant extract produced two purple spots in visible light with Rf values of 0.163; and 0.325, while on the P track, the white rice extract did not produce purple spots. Based on Harborne (1996) in [34]), the Rf value of anthocyanin is in the range of 0.1 - 0.4, so both spots are included in the anthocyanin compound. Based on research by [45], the most concentrated spot, namely at an Rf value of 0.325, is suspected to be a cyanidin monoglucoside compound. This compound is in accordance with the literature [46] which states that the most abundant anthocyanin compound in black rice is cyanidin 3-monoglucoside. The spot at Rf 0.163 is suspected to be a cyanidin 3-arabinoside-5-glucoside compound [45]. The green color on the "H" track is suspected to be chlorophyll because it produces a red color in UV light at a wavelength of 366nm.

3.3. LC-HRMS Chromatogram

The types of anthocyanins in the Sidenuk black rice (*Oryza sativa*) mutant were detected using LC-HRMS. A total of 99 compounds were detected by LC-HRMS. Among the 99 compounds, there were 5 anthocyanin compounds in the Sidenuk black rice mutant, namely cyanidin 3-glucoside, peonidin-3-glucoside, malvidin, petunidin 3-glucoside, and delphinidin 3-glucoside (Figure 2 and Table 3). The cyanidin 3-glucoside compound was the most abundant anthocyanin compound in the Sidenuk black rice mutant, followed by peonidin-3-glucoside (Figure 3).

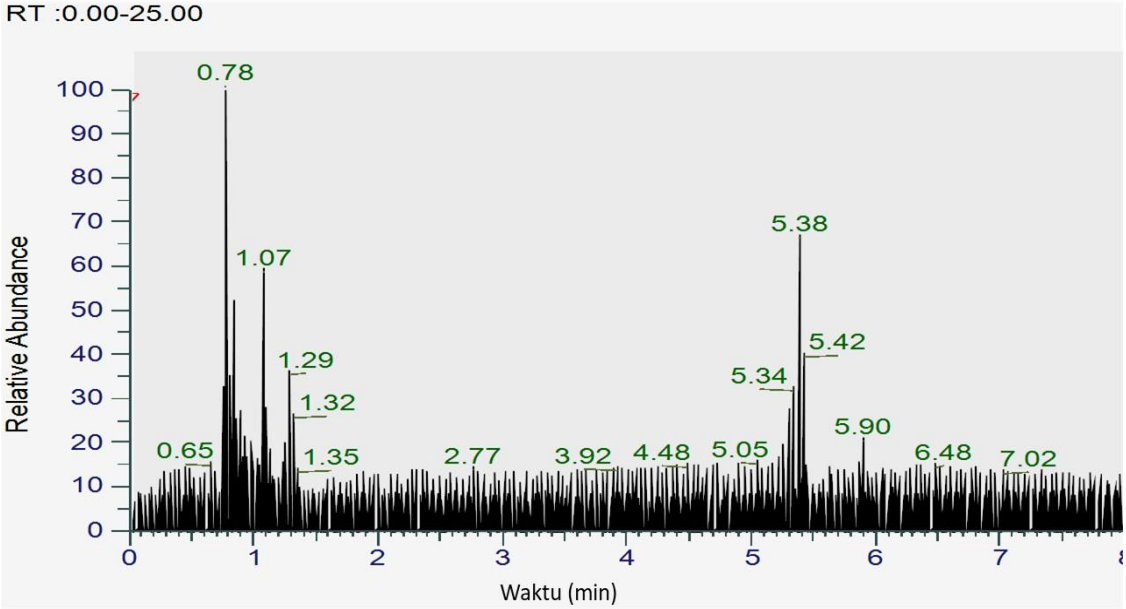


Figure 2. Total Ion Chromatogram of Sidenuk black rice mutant anthocyanins

Table 3. Retention time and area of the sidenuk black rice mutant anthocyanin compounds

Name	RT [min]	Area
Cyanidin 3-glucoside	0.807	48594422.56
Peonidin-3-glucoside	4.222	16451549.23
Malvidin	6.815	3315233.48
Petunidin 3-glucoside	4.696	1829135.63
Delphinidin 3-glucoside	4.197	807403.95

The data obtained from LC-HRMS is semi-quantitative data, meaning that the numerical data obtained can only estimate the concentration of compounds in the sample, without any measurement. This is because unknown screening does not use a standard curve for each compound obtained. Total ion chromatogram data shows the relative abundance of a compound shown at a certain retention time (minutes). The retention time on the x-axis is the time required for the compound to move through the column. While the relative abundance on the y-axis is the percentage of a compound compared to the total area of all peaks in the chromatogram. The area data in Table 3 shows the concentration of each substance, which means that the larger the area under the curve, the higher the concentration of the substance [47] The anthocyanin data obtained shows that the black color of the Sidenuk black rice mutant is influenced by the composition of the five anthocyanins obtained, namely cyanidin 3-glucoside, peonidin-3-glucoside, malvidin, petunidin 3-glucoside, and delphinidin 3-glucoside.

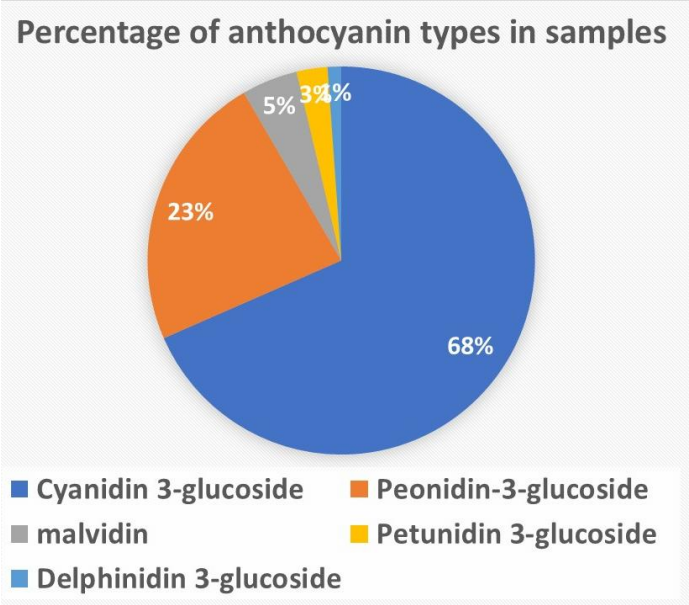


Figure 3. Percentage of anthocyanin types of Sidenuk black rice mutants

3.4. Total Monomeric Anthocyanin Content (pH differential method)

Determination of the total monomeric anthocyanin content was carried out using pH differential method, with the concentration of anthocyanin pigments expressed as cyanidin-3-glucose equivalents. The data in Table 4 shows that the anthocyanin content in black rice is 1074.74 mg/L or if converted to mg/g, the content is 1.07 mg/g. This means that each gram of Sidenuk black rice mutant contains 1.07 mg cyanidin-3-glucose equivalents. Sidenuk black rice mutants have a higher total monomeric anthocyanin content than black glutinous rice [40], as shown in Table 4. However, other black rice varieties have even higher levels of anthocyanin content.

Anthocyanins can be found as either single molecules (monomeric) or in chains of molecules (polymeric). Monomeric anthocyanins change color based on acidity (pH). In highly acidic conditions (pH 1.0), they are colored (oxonium form), while in less acidic conditions (around pH 4.5), they become colorless (hemiketal form). The color intensity, measured by light absorption at 520nm, reflects the amount of monomeric anthocyanin present and is compared to a standard anthocyanin, cyanidin-3-glucose. Polymeric anthocyanins, formed from degradation, don't change color with pH and aren't measured at 520nm because they absorb light across a range of pH values, interfering with the monomeric anthocyanin measurement. Turbidity in the sample is assessed by measuring light absorption at 700nm. Cloudy samples should be clarified by centrifugation or filtration using a filter that doesn't bind anthocyanins, like a 1.2µm pore size Millipore membrane filter, before measurements are taken [38-39].

Several factors influence the amounts of anthocyanins in black rice, such as the specific type of rice, the temperature at which it's dried, and how it's stored. Different black rice varieties, like Toraja compared to Banjaregara [48], have varying anthocyanin levels. Higher drying temperatures decrease the anthocyanin content, likely because they cause these compounds to bind together, reducing the measurable amount [49]. Finally, proper storage is crucial; storing black rice in a nitrogen-rich environment helps preserve its anthocyanin content [50]. Other factors that can destroy anthocyanins include light, oxygen, enzymes, ascorbic acid, thermal treatment, sulfur dioxide or sulfite salts, metal ions, and copigments [51].

Anthocyanin production, is also controlled by both external environmental cues and internal hormonal signals. Several environmental factors, including light quality (particularly blue and red wavelengths), salinity, drought, cold stress, nutrient deficiencies (nitrogen and phosphorus), and UV radiation, can trigger changes in anthocyanin levels [52-53]. These compounds play a crucial role in plant defense against pests and diseases. Furthermore, various phytohormones, such as abscisic acid, jasmonic acid, ethylene, and gibberellin, exert regulatory control over anthocyanin biosynthesis, with each hormone capable of both promoting and inhibiting its production depending on the specific context [54].

Sidenuk black rice mutants may have lower anthocyanin levels than other black rice varieties due to their origin from mutated white rice. Because of that, the process of forming the enzymes needed in the anthocyanin biosynthesis process may not be as effective as the wild type version of black rice. The consequence is a lower anthocyanin content than other black rice varieties. However, this hypothesis needs to be proven further. The inappropriate selection of solvents and extraction methods also hinders the efficient extraction of anthocyanins.

Table 4. Anthocyanin content of Sidenuk black rice mutants and other varieties

No.	Black Rice Cultivar	Anthocyanin Content mg/g
1	Sidenuk black rice mutant	1.07 ± 0,04
2	Black glutinous rice [40]	$0.04 \pm 1.73 \times 10^{-4}$
3	Black Rice Poireiton [42]	13.7 ± 0.92
4	Black Rice Kokngangbi [42]	15.48 ± 0.61
5	Black Rice Amubi [42]	17.69 ± 0.45
6	Black Rice Sempak [42]	8.39 ± 0.53

3.5. Antioxidant Activity (DPPH)

The IC50 value of the Sidenuk black rice mutant is 36.027 µg/mL meaning that a concentrated extract concentration of the Sidenuk black rice mutant of 36.027 µg/mL is needed to inhibit free radicals by 50% (Table 5). When compared to the IC50 value of quercetin, the antioxidant activity of black rice is 1.035% of the activity of quercetin.

Antioxidant activity was measured using the DPPH assay and UV-vis spectrophotometry. This test assesses a compound's ability to donate a hydrogen atom and neutralize the DPPH radical, indicated by a color change (from purple to yellow). A greater color change suggests stronger antioxidant capacity. This capacity is quantified by the IC50 value, representing the concentration of the compound needed to inhibit 50% of the DPPH radicals. A lower IC50 signifies higher antioxidant activity. Specifically, IC50 values are categorized as: powerful (<10µg/ml), strong (10-50µg/ml), mild (50-100µg/ml), weak (100-250µg/ml), and inactive (>250µg/ml) [55].

Based on these categories, the IC50 value of black rice is in the strong antioxidant activity range, namely in the range 10 and 50µg/ml. The results of the IC50 value of black rice obtained in this study are better than the study [40] which produced an IC50 of 94.624µg/ml in the methanol extract sample of black glutinous rice. The Sidenuk black rice mutant also has better antioxidant activity than other rice varieties. Thus, even though it produces lower anthocyanin content, the antioxidant activity of the Sidenuk black rice mutant is the strongest compared to other black rice varieties in this study.

An antioxidant's effectiveness is influenced by several key factors. These include its chemical structure, temperature, the substance being protected from oxidation, its concentration, interactions with other compounds (both synergistic and pro-oxidant), and the physical environment. An antioxidant's structure dictates how readily it reacts with free radicals and other reactive oxygen species (ROS), directly affecting its activity. Both concentration and location within the system, such as interface distribution, are also crucial. Furthermore, the reaction kinetics, including the reaction

rate with a specific oxidant, the reaction's thermodynamics, and the antioxidant's overall reactivity, play a significant role in its protective ability, both immediately and over time [56].

Besides the main antioxidant compounds, plant extracts contain other substances that influence measured antioxidant activity. These include various phenolic compounds (known for their strong antioxidant properties, working by donating electrons or hydrogen), flavonoids (considered major contributors to plant antioxidant activity), and other plant-derived antioxidants like lignin, stilbenes, and tannins [57]. Certain metabolites, such as sucrose, betaine, fructose, ascorbic acid, glycine, and arginine, as well as compounds like glucose, epicatechin, gallic acid, and citric acid, can also contribute to the measured antioxidant activity [58]. Furthermore, the solvent used for extraction is critical, as its polarity significantly impacts the recovery of phenolic compounds and thus the overall measured antioxidant activity [59].

Quercetin was used as a comparison because it has the same main chain structure as anthocyanin and is a pigment that has antioxidant activity (Figure 4). A concentration of 0.167 mM in DPPH was chosen so that the DPPH color remains purple when it meets quercetin at the highest concentration, but not too concentrated so that the absorbance value becomes more than 1.4. The highest wavelength value of DPPH in this study was 517 nm. Determination of DPPH concentration and wavelength is needed to see changes in absorbance values before and after sample administration at a wavelength of 517 nm, as well as changes in DPPH color from purple to pale yellow due to hydrogen transfer from the sample to DPPH [60]

Table 5. Antioxidant activity of quercetin, Sidenuk black rice mutant, and other varieties

No.	Sample	Antioxidant activity IC50 (µg/mL)
1	Quercetin	$3.73 \times 10^{-1} \pm 7.11 \times 10^{-2}$
2	Sidenuk black rice mutant	36.03 ± 5.09
3	Black glutinous rice [40]	94,624
4	Black Rice Poireiton [42]	97.08 ± 0.53
5	Black Rice Kokngangbi [42]	85.76 ± 1.01
6	Black Rice Amubi [42]	98.83 ± 0.61
7	Black Rice Sempak [42]	76.87 ± 0.70

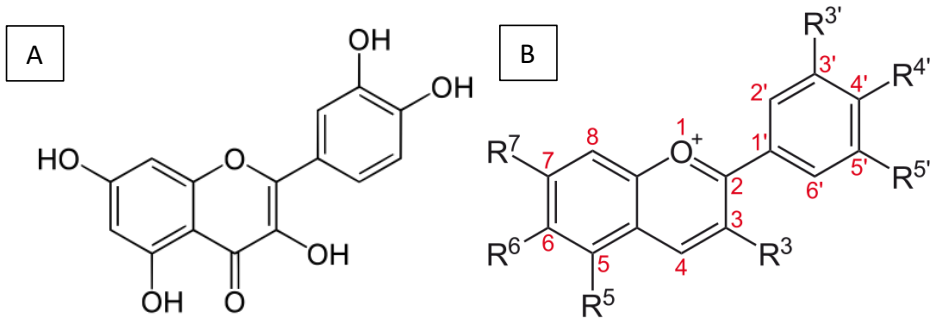


Figure 4. Comparison between the main chains of Quercetin (A) and Anthocyanin (B)

4. Conclusion

The Sidenuk black rice mutant yielded 23.25% concentrated macerate. Tests confirmed the presence of anthocyanins, particularly cyanidin 3-glucoside and peonidin-3-glucoside, which were absent in the white rice mutant. The black rice contained 1.07 ± 0.04 mg/g of total anthocyanins, a higher amount than black glutinous rice but less than other black rice varieties. With an IC50 value of 36.02±5.09 µg/mL, the Sidenuk black rice mutant exhibited strong antioxidant activity, surpassing that of other black rice varieties in this study.

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