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Evaluating the Antioxidant Activity and Stability of Pigmented Rice Extract

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ABSTRACT

This study aims to evaluate the antioxidant activity and stability of the extract of pigmented rice. Pigmented rice powder from 6 different varieties including Subang, Jowo Melik, Andel Abang, Bukittinggi, Cempo Merah, and Cempo Ireng) was macerated using ethanol with concentrations of 96% and 70%. Next, the rice extract was tested for stability to pH (1, 3, 5, 7, 9) and temperature (20°, 40°, 60°, 80°C). Results revealed that extract of Subang black glutinous rice using 96% ethanol resulted the highest antioxidant activity of 11.11±2.04 mg FeSO4/g, with an overall anthocyanin, flavonoids, phenolic contents of respectively 28.22±1.14 mg CE/g, 2.91±1.13 mg QE/g, and 3.46±1.59 mg GAE/g. The physiochemical properties of the rice extract were affected by the difference of pH. The increase in pH resulted in a decrease in antioxidant activity, total anthocyanin, and the values of color attributes (L, a*, b*, C*, and ºHue) of the rice extracts. Temperature did not affect the physiochemical properties of the extract. The antioxidant activity, total anthocyanin, and color attributes of the rice extract were typically stable when the temperature rose from 20°C to 80°C. The extract at pH 1 and 80°C treatment exhibited slightly higher yield based on antioxidant activity, total anthocyanin content, a* value, and ºHue with the values of 16.92±1.73 mg FeSO4/g, 33.90±2.33 mg CE/g, 21.55±0.54, and 30.04±0.57, respectively.*

1. INTRODUCTION

Indonesia is one of the largest pigmented rice producers in the Asia region besides China, Japan, South Korea, Thailand, Vietnam, and India (Arifa *et al*., 2021; Yangmuangmorn & Prom-u-Thai, 2021). Red rice, black rice, and black glutinous rice are the types of pigmented rice that are commonly consumed. Anthocyanin is the major pigment found among various types of pigmented rice, which contributes to the dark color of rice grains (Suarti *et al*., 2021). This pigment possesses several health benefits, such as hypoglycemic effects, anti-inflammatory, and antioxidant (Pang *et al*., 2018; Danastry *et al*., 2021).

Several factors can influence the chemical characteristics of pigmented rice extracts, namely rice varieties and plant environmental conditions, such as sunlight, rainfall intensity, land altitude, and nutrients contained in the soil. (Kristamtini *et al*., 2018; Maulani *et al*., 2019; Yangmuanmorn & Prom-u-Thai, 2021).

An extraction process using the maceration method could be operated to obtain and utilize the anthocyanin compound in pigmented rice. Ethanol is one of the common solvents used for extraction of anthocyanin. Different ethanol concentrations can influence the chemical characteristics of pigmented rice extracts due to the polarity difference of the solvent. According to Agustin & Ismayati (2015), extraction of anthocyanin with 96% ethanol produces the highest anthocyanin content, but with lower yields. On the other hand, Nurhidajah *et al*. (2022) stated that anthocyanin extraction using a lower ethanol concentration could increase both yield and anthocyanin content.

Besides ethanol concentration, pH differences also influence the content of anthocyanin in the pigmented rice extract. According to Oancea (2021) and Shidiqqi *et al*. (2021), anthocyanin possesses the best stability in acidic conditions (pH < 3), while in more basic conditions, the chemical structure of anthocyanin will be degraded into a chalcone structure, which contributes to blue-green color of the solution. Extraction performed with the addition of 2% citric acid (w/v) in 80% ethanol produces the highest anthocyanin content (Jaberi *et al*., 2022).

Temperature can impact the stability of the extract. Higher temperatures can cause anthocyanin damage, resulting in a cloudier solution. Another factor that affects the extract's stability is temperature. High temperatures will damage the anthocyanin, which leads to a cloudier color of the solution. According to Mustofa & Suhartatik (2018), copigmented anthocyanin in black glutinous rice extract decreased from 25 mg/L to 2.82 mg/L during heating treatment at 50 -70℃. Based on the above description, ethanol concentration, temperature, and pH differences influence the antioxidant activity of pigmented rice extract. Hence, this study aims to determine the impact of pH and temperature on the antioxidant activity and stability of pigmented rice extract.

2. MATERIALS AND METHODS

2.1. Materials & Chemicals

Three pigmented rice including organic black rice (Cempo Ireng "Javara"; Jowo Melik "Healthy Choice"), organic red rice (Cempo Merah "Healthy Choice"; Andel Abang "Javara"), and black glutinous rice (Subang; Bukittingi "Ubek Selero Minang") were obtained from West Java, Central Java, & West Sumatera through Tokopedia. Other materials including food grade ethanol 96% & 70%, HCl 37%, KCl, sodium acetic anhydrate, sodium acetic trihydrate, acetic acid glacial, Folin-Ciocalteu Reagent, $NaCO_3$, $AlCl_3$, methanol *pro analysis*, TPTZ, $FeCl_3$, $FeSO_4.7H_2O$, *quercetin*, gallic acid, citric acid monohydrate, Na_2HPO_4, NaOH, and glycine.

2.2. Research Method

2.2.1. Sample Preparation

Six pigmented rice varieties were cleaned and dried using a cabinet dryer at 50 ºC for 4 h. The dry rice grains were ground using a grinder and sieved through 60-mesh sieves (Pramitasari & Angelica, 2020). The pigmented rice powder, which passed through 60-mesh sieves was then kept in a dark airtight container.

2.2.2. Extraction of Pigmented Rice

In the first stage of research, the extraction of 6 pigmented rice powders was conducted using the maceration method. Ethanol 96% and 70% were used as the solvent with the addition of citric acid 2% (Nurhidajah *et al.*, 2022; Sholihah *et al.*, 2021). The sample-to-solvent ratio was 1:10 (w/v). A magnetic stirrer was used to mix the solution for 15 min. Then, the maceration process was operated for 48 h in a dark environment at room temperature. After that, the solution was strained off using Whatmann filter paper No.1. The filtrate obtained was then evaporated at 50 ºC by using a rotary evaporator. Each of the pigmented rice extract was stored in a lightproof glass bottle at 0-5 ºC.

2.2.3. Antioxidant Activity and Stability of Rice Extract

The chosen rice extract from the first stage was continued to the second research stage. In this stage, the rice extract was analyzed based on the stability of anthocyanin and antioxidant activity using different temperature treatments (20ºC, 40ºC, 60ºC, 80ºC) and pH treatments (1, 3, 5, 7, 9) for 15 minutes (Fendri *et al*., 2020).

2.3. Experimental Design

The experimental was designed for the first research stage was a completely randomized with 2 factors, 2 replications, and a Duplo for each replication. The first factor was ethanol concentration (96% and 70%), while the second factor was rice varieties (Andel Abang, Jowo Melik, Cempo Ireng, Ketan Hitam Subang, Ketan Hitam Bukittinggi, Cempo Merah). For the second stage, the experimental design was completely randomized one-factorial with 3 replications and a Duplo for each replication. The treatments used in this stage are pH and temperature differences. Statistical analysis was done to analyze the obtained data using ANOVA and Duncan's post-hoc test on IBM SPSS Version 25.

2.4. Data Analysis

In the sample preparation stage, two analyses were conducted: rice powder yield (Syafutri *et al*., 2020) and moisture content analysis (AOAC, 2005). In the first research stage, several analyses were done which were extraction yield (Danastry *et al.,* 2021), moisture content (AOAC, 2005) with modification, total anthocyanin content (AOAC, 2005) with modification, antioxidant activity (Sadeer *et al*., 2020) with modification, total phenolic (Nurhidajah *et al.*, 2022) and flavonoid (Nurhidajah *et al.*, 2022) with modification. In the second research stage, several analyses were done which were total anthocyanin content (AOAC, 2005) with modification, antioxidant activity (Sadeer *et al*., 2020) with modification, maximum wavelength (Fendri *et al*., 2020), and color intensity (Maulani *et al*., 2019).

2.4.1. Total Anthocyanin Content

The content of total anthocyanin was measured using the pH differential method, which involves assessing structural changes in anthocyanin forms and absorbance at pH 1.0 and 4.5. The extracts of pigmented rice were separately diluted with 0.025 M buffer at pH 1 and 0.4 M sodium acetate buffer at pH 4.5. Each extract sample was then further diluted to obtain an absorbance reading. The absorbance of the blend was analyzed using a UV-Vis spectrophotometer at wavelength $\lambda_{vis\text{-}max}$ and 700 nm. The absorbance and total anthocyanin content of a sample can be calculated according to Equation (1) and (2), respectively.

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 4.5} \quad (1)$$

$$\text{Total anthocyanin content (mg CyE/g)} = \frac{A\ x\ MW\ x\ DF\ x\ 1000}{\varepsilon\ x\ l}\ x\ \frac{V}{W} \quad (2)$$

where A is the absorbance, MW = 449.2 g/mol is the molecular weight of cyanidin-3-glucoside, DF is the dilution factor, V is the volume of solvent (mL), ε = 26,900 $L.mol^{-1}.cm^{-1}$ is the molar absorptivity, W is weight of the sample, and l = 1 cm is the cell path length.

2.4.2. Total Flavonoid Content

A total of 0.05 g of rice extract sample was liquefied in ethanol (according to treatment) in a 10 mL volumetric flask until the volume reached 10 mL. Exactly 0.5 mL of the solution was taken and supplemented with 0.1 mL of 10% $AlCl_3$, 0.1 mL of CH_3COONa, 1.5 mL of ethanol 70% or 96%, and 2.8 mL of distilled water. The blend was incubated at room temperature (25°C) for 30 min. The absorbance was measured at a wavelength of 415 nm using a spectrophotometer. The standard Quercetin solution was prepared with a concentration range of 0 – 25 mg/L, which was then used to calculate the flavonoid content of pigmented rice extract. The content of total flavonoid was presented in milligrams of quercetin equivalents mass per gram of extract sample (mg QE/g sample).

2.4.3. Total Phenolic Content

The procedure involved dissolving 0.01 g of rice extract sample in ethanol to make a total volume of 10 mL in a 10 mL flask. Then, 0.2 mL of this solution was mixed with 1.8 mL of distilled water and 1 mL of 10% Folin-Ciocalteu reagent in a test tube. The mixture was then incubated for 5 min. After that, 5 mL of 7.5% Na_2CO_3 was added and incubated for 30 min more. Absorbance was determined at a wavelength of 765 nm using a spectrophotometer. The standard Gallic Acid solution was made with a concentration range of 20 – 100 mg/L, which was then used to calculate the phenolic content of pigmented rice extract. Total phenolic was quantified as the weight of gallic acid equivalent per gram of extract mass (mg GAE/g).

2.4.4. Determination of Antioxidant Activity

A total of 0.01 g of extract is dissolved in 10 mL of ethanol (concentration according to treatment). FRAP (Ferric Reducing Antioxidant Power) reagent preparation was carried out by mixing 0.01 M TPTZ (2,4,6-tripyridil-s-triazine) stock solution dissolved in 0.04 M HCl, 0.3 M acetate buffer solution, and 0.02 M FeCl3 with a ratio of 1:10:1 (v/v/v) respectively. 0.1 mL of sample extract solution was mixed with 2.4 mL of FRAP reagent. The absorbance was tested with a visible spectrophotometer at a wavelength of 593 nm. The standard $FeSO_4$ solution was prepared with a concentration range of 0.2 mM – 1 mM, which was then used to determine the equivalent antioxidant capacity of pigmented rice extract.

2.4.5. UV-Vis Color Spectrum Measurement

The measurements involved dissolving 0.5 g of pigmented rice extract sample in 50 mL of buffer solution. The solution was then transferred into a cuvette, and the absorbance was measured at a wavelength ranging from 400 to 700 nm using a UV-Vis spectrophotometer to determine the maximum absorbance value and wavelength.

2.4.6. Color Measurement

Color intensity analysis of pigmented rice extract was conducted using Chromameter. The color parameters were represented in L^* (brightness intensity), a^* (green-red color intensity), b^* (blue-yellow color intensity), and C^* (Chroma value, level of vibrance or dullness of the color). The °*Hue* value was calculated based a^* and b^* values according to the Equation (3). Hutchings (1999) provides classification of color groups based on the value of °*Hue* as presented in Table 1.

$$°Hue = \arctan (b^*/a^*) \quad (3)$$

Table 1. Color grouping based on °*Hue* value

°*Hue*	Color	°*Hue*	Color
342° – 18°	Purple – Red	162° – 198°	Green
18° – 54°	Red	198° – 234°	Blue – Green
54° – 90°	Red – Yellow	234° – 270°	Blue
90° – 126°	Yellow	270° – 306°	Blue – Purple
126° – 162°	Yellow – Green	306° – 342°	Purple

Source: Hutchings (1999)

3. RESULTS AND DISCUSSION

3.1. Pigmented Rice Powders Characteristics

Each rice sample that had been dried and milled into powder was analyzed based on its yield and moisture content. The result is shown in Table 2. Rice powder yield ranged from 56.68–73.54%. Syafutri *et al.* (2020) also stated the similar rice powder yield from red rice grains which was 51.58–64.17%. However, other studies show higher rice powder yields from black rice and red rice grains, which were 92.55–92.63% and 89.69–90.66%, respectively (Anugrahati & Aurielle, 2021; Karfinto & Anugrahati, 2022). The difference in yield results could occur due to several factors, such as particle size, milling method, and milling time. According to Marlisa *et al.* (2020), a greater yield of powdered food samples is produced when a longer milling or grinding time is taken.

Table 2. Physicochemical characteristics of pigmented rice powders

Rice Varieties	Extract yield (%)	Moisture content (% w.b.)
Cempo Ireng	67.89±0.64	7.96±0.12
Jowo Melik	67.79±2.55	7.21±0.06
Andel Abang	56.68±2.1	7.19±0.02
Cempo Merah	64.68±2.4	6.51±0.08
Ketan Hitam Subang	73.54±3.66	7.21±0.11
Ketan Hitam Bukittinggi	67.01±3.08	6.77±0.09

Note: % w.b. = wet basis

From Table 2, the moisture content ranged between 6.51–7.96%. These results have fulfilled the maximum moisture content requirements of rice flour (13% w.b.) and glutinous rice flour (12% w.b.) (BSN, 1998; BSN, 2009). According to Aini *et al*. (2016), a flour or powdered food sample can be stored for a long period if the moisture content is below 10%.

3.2. Physicochemical Characteristics of Pigmented Rice Extracts

The parameters used in analyzing pigmented rice extracts were extraction yield, moisture content, total anthocyanin content, total flavonoid, total phenolic content, and antioxidant activity.

3.2.1. Moisture Content

The difference in ethanol concentration has a significant effect on the extract's moisture content ($p<0.05$), while the rice varieties do not ($p>0.05$). Statistical analysis results explain that there is an interaction between ethanol concentration and rice varieties toward extract's moisture content ($p<0.05$). Based on Figure 1, the moisture content of pigmented rice extracts ranges from 15.31±4.81% to 24.30±0.45%. These pigmented rice extracts can be classified into the thick extract category, which requires moisture content between 5–30% (Utami *et al.*, 2020). Lower moisture content will reduce the risk of microbial contamination, like bacteria and mold, and prolong the shelf life of the extract (Utami *et al*., 2020).

The moisture content of pigmented rice extracts with 96% ethanol tends to be higher than 70% ethanol. According to Rohaeni *et al*. (2015), increasing ethanol concentration exhibits a negative correlation with the extracts' moisture content. This differentiation could occur due to the hygroscopic properties of ethanol, hence, the ability to bind with water molecules increases as the ethanol concentration increases (Sucipto *et al*., 2021). Please briefly clarify why the moisture content of Jowo Melik and Ketan Hitam Bukit Tinggi was significantly lower than the other varieties.

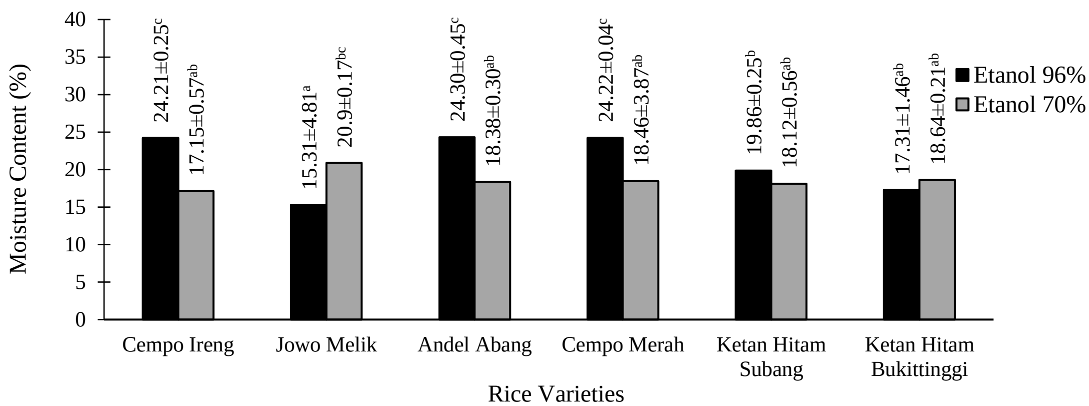

Figure 1. Impact of ethanol concentration and rice varieties on moisture content of pigmented rice extracts. (Different superscripts after mean values specify significant differences, $p<0.05$)

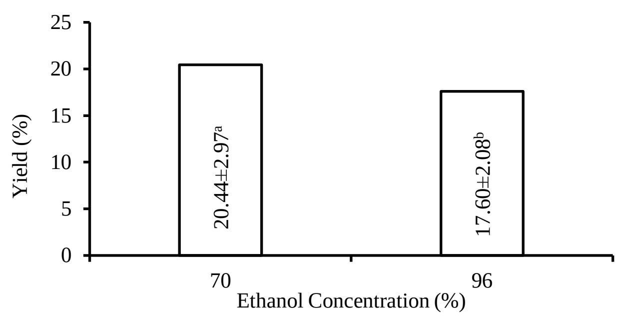

Figure 2. Impact of ethanol concentration and rice varieties on the extraction yield of pigmented rice extracts. (Different superscripts after mean values specify significant differences, $p<0.05$)

3.2.2. Extraction Yield

The ethanol concentration difference shows a significant effect on the extract's yield ($p<0.05$), while rice varieties don't ($p > 0.05$). Statistical analysis results explain that there is no interaction between ethanol concentration and rice varieties toward the extract yield ($p > 0.05$). Based on Figure 2, pigmented rice extraction yield with 70% ethanol exhibits a higher yield than 96% ethanol. This result is in accordance with the previous study by Agustin & Ismayati (2015), which found that anthocyanin extraction yield showed a lower yield as the ethanol concentration increased. A negative correlation between ethanol concentration and extraction yield could occur because of the high moisture content in the powdered pigmented rice samples. Thus, the free water within the samples blocks the surface contact between rice particles and the solvent during the maceration process (Kartika *et al*., 2022; Hikmawanti *et al*., 2020).

3.2.3. Total Anthocyanin Content

The ethanol concentration difference and rice varieties show a significant effect on the extract's total anthocyanin content ($p<0.05$). Statistical analysis results explain that there is an interaction between ethanol concentration and rice varieties toward the extract's total anthocyanin content ($p<0.05$). Based on Figure 3, most of the pigmented rice extracts with 96% ethanol exhibited a relatively higher total anthocyanin content than 70% ethanol, except the Bukittinggi rice extract. This phenomenon could happen due to the glycosidic bond difference in the anthocyanin compound within the pigmented rice, which contributes to different polarities (Tan *et al*., 2022). A chemical compound that has the most similar polarity to the solvent could dissolve more easily into the solvent, hence increasing the efficacy of the extraction process (Suhendra *et al*., 2019).

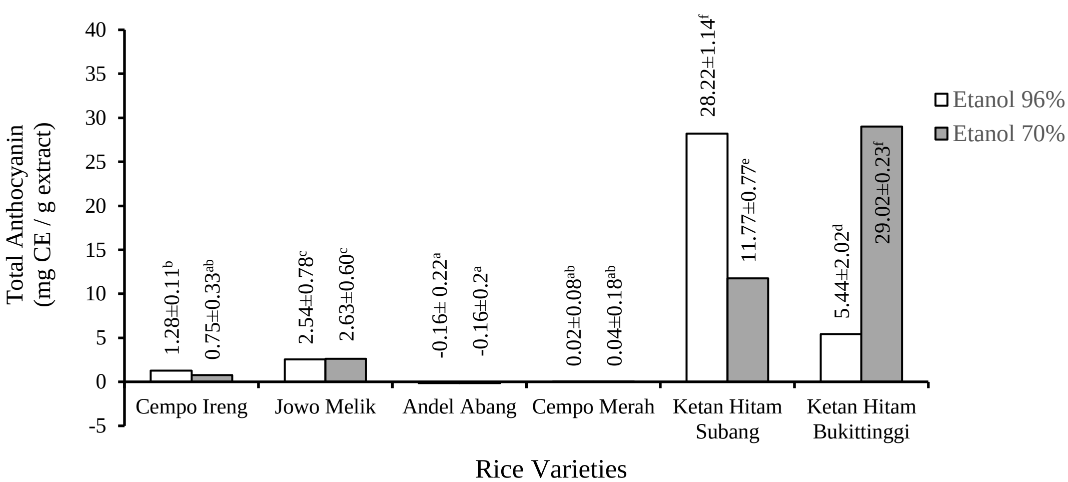

Figure 3. Impact of ethanol concentration and rice varieties on the total anthocyanin content of pigmented rice extracts. (Different superscripts after mean values specify significant differences, $p<0.05$)

From Figure 3, Andel Abang and Cempo Merah rice extracts have the lowest anthocyanin content. Research conducted by Thitipramote *et al.* (2016) also showed that the anthocyanin content of both red jasmine rice and red Japanese rice could not be detected. According to Hosada *et al.* (2018), there's a possibility of another pigment compound besides anthocyanin, which is proanthocyanidin, that dominates and contributes the most color to red rice grains. From Figure 3, the anthocyanin content in black rice extracts (Cempo Ireng and Jowo Melik) is significantly lower than in black glutinous rice extracts (Subang and Bukittinggi). A previous study also stated similar results that anthocyanin content in several black glutinous rice extracts and black rice extracts ranged from 57–442 mg/100g and 12–129 mg/100g, respectively (Yangmuanmorn & Prom-u-Thai, 2021). This can be caused by several factors, such as the physical color of the rice grains and the growth environment, like rainfall and sunlight intensity, land altitude, and soil nutrients (Kristamtini *et al*., 2018; Maulani *et al*., 2019; Yangmuanmorn & Prom-u-Thai, 2021).

3.2.4. Antioxidant Activity

The ethanol concentration difference shows no significant effect on the extract's yield ($p > 0.05$), whereas rice varieties do ($p < 0.05$). Statistical analysis results explain that there is an interaction between ethanol concentration and rice varieties toward the extract's yield ($p<0.05$). Based on Figure 4, the antioxidant activity of pigmented rice extracts ranges between 1.03±0.01 to 11.11±2.04 mg $FeSO_4$/g. The extracts with 96% ethanol showed relatively higher results than 70% ethanol, except for the Bukittingi rice extract. 96% ethanol polarity is less polar than 70% ethanol because fewer water molecules are attached to it (Yusof *et al*., 2020). Bioactive compounds that contribute to antioxidant activity in pigmented rice have a more non-polar structure, which makes it easier to bind with 96% ethanol. Besides that, different rice varieties also show a significant effect on the antioxidant activity of pigmented rice extracts (Chen *et al*., 2022). This result is mainly caused by the difference in the bioactive compound profile and its proportion in each of the pigmented rice samples, which contribute to the antioxidant activity (Rajendran *et al*., 2018).

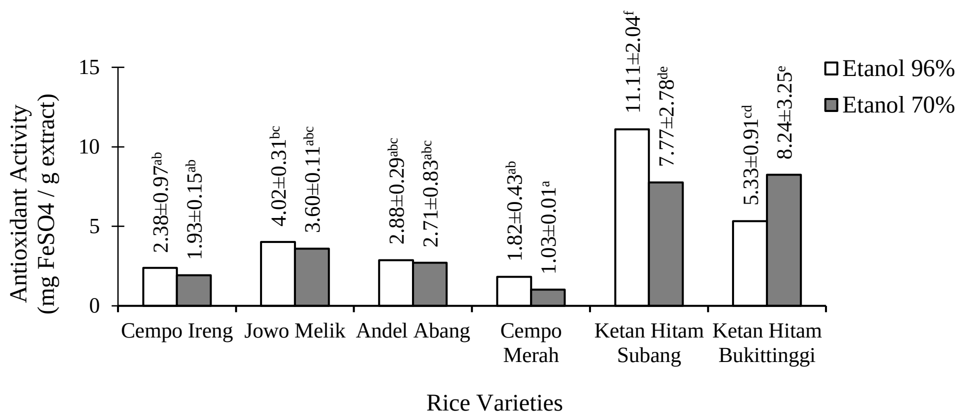

Figure 4. Impact of ethanol concentration and rice varieties on antioxidant activity of pigmented rice extracts. (Different superscripts after mean values specify significant differences, $p<0.05$)

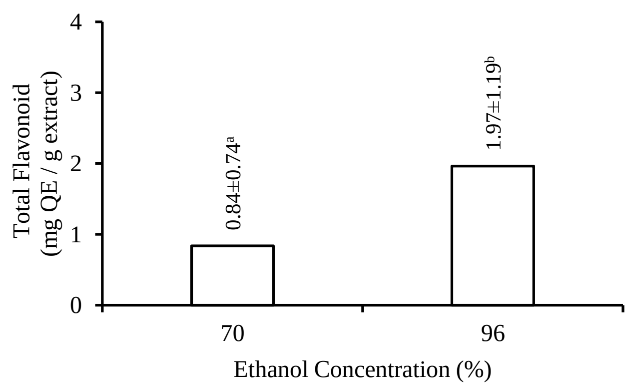

Figure 5. Impact of ethanol concentration on total flavonoid content of pigmented rice extracts (Different superscripts after mean values specify significant differences, $p<0.05$)

3.2.5. Total Flavonoid Content

The ethanol concentration difference and rice varieties show a significant effect on the extract's total flavonoid content ($p<0.05$). However, statistical analysis results explain that there is no interaction between ethanol concentration and rice varieties toward the extract's total flavonoid content ($p>0.05$). Based on Figure 5, the total flavonoid produced by pigmented rice extracts with 96% ethanol is significantly higher (1.97±1.19 mg QE/g) than 70% ethanol (0.84±0.74

mg QE/g). This result is not coherent with the study conducted by Sholihah *et al.* (2021) and Nurhidajah *et al*. (2022), which stated that the highest flavonoid content from black rice extracts was produced with 55% ethanol. However, the flavonoid content decreased as the ethanol concentration got higher (Sholihah *et al*., 2021; Nurhidajah *et al*., 2022). This deviation might be caused by the bioactive compound polarity properties of pigmented rice extracts, which tend to dissolve in a more non-polar solvent, which is 96% ethanol (Suhendra *et al*., 2019).

From Figure 6, it can be observed that the highest flavonoid was produced by Subang black glutinous rice extract, with a value of 2.91±1.13 mg QE/g. This result is in accordance with Chen *et al.* (2022), where black rice extracts range from 1.13 to 3.26 mg QE/g. Based on Figure 3 and Figure 4, the total flavonoid result has a similar pattern to the total anthocyanin and antioxidant activity. This explains that the anthocyanin within the pigmented rice greatly contributes to the flavonoid content (Pramitasri & Angelica, 2020). Flavonoid compounds are normally tested for efficacy based on their ability to act as free radical scavengers or chelating agents of Fe^{2+} ions (Maulani *et al*., 2019).

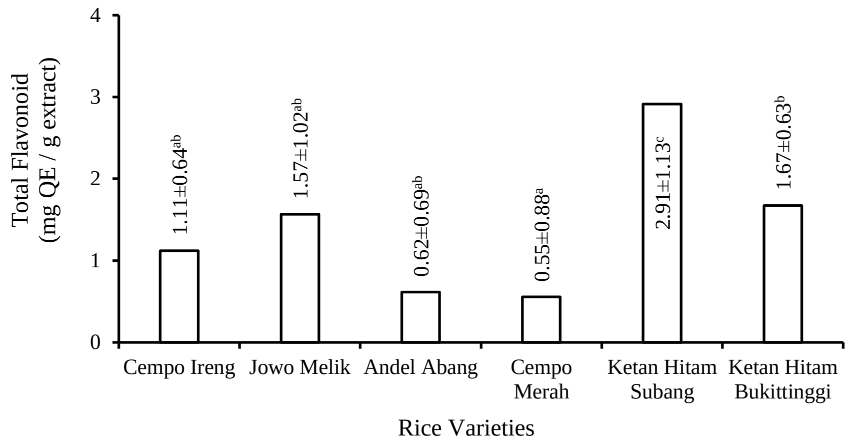

Figure 6. Impact of rice varieties on total flavonoid content of pigmented rice extracts. (Different superscripts after mean values specify significant differences, $p<0.05$)

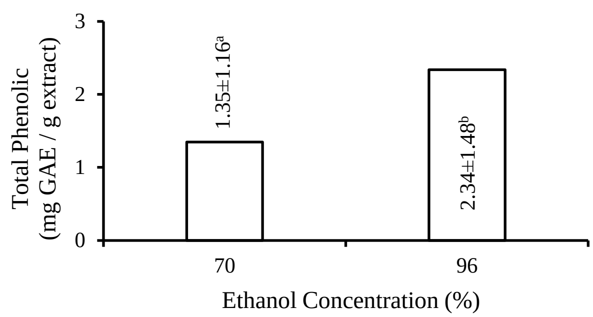

Figure 7. Impact of ethanol concentration on total phenolic content of pigmented rice extracts. (Different superscripts after mean values specify significant differences, $p<0.05$)

3.2.6. Total Phenolic Content

The ethanol concentration difference and rice varieties show a significant effect on the extract's total phenolic content ($p<0.05$). However, statistical analysis results explain that there is no interaction between ethanol concentration and rice varieties toward the extract's total phenolic content ($p>0.05$). Based on Figure 7, pigmented rice extracts with ethanol 96% exhibited a significantly higher result than 70% ethanol with a value of 2.34±1.48 mg GAE/g and 1.35±1.16 mg GAE/g, respectively. Total phenolic has a positive correlation with total flavonoid since both parameters show 96% ethanol resulting in a higher value which can be seen in Figure 5. Nurhidajah *et al*. (2022) also stated that flavonoid content and phenolic content have a positive correlation when different ethanol concentrations were used in the extraction process.

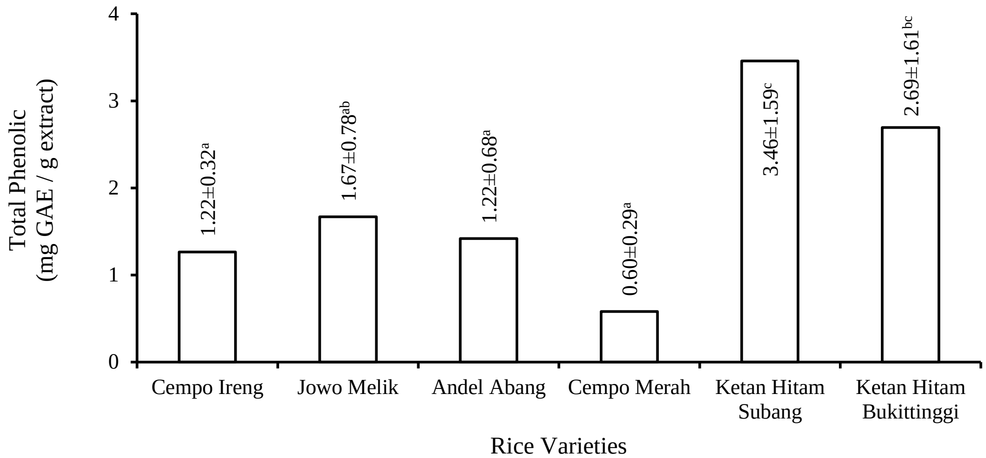

Figure 8. Impact of rice varieties on total phenolic content of pigmented rice extracts (Different superscripts after mean values specify significant differences, $p<0.05$)

From Figure 8, the highest phenolic content is produced by Subang black glutinous rice extract at a value of 3.46±1.59 mg GAE/g. Compared to Figure 6, there is a constructive correlation between phenolic content and flavonoid content since both parameters exhibit the same pattern. This result indicates the flavonoid compound in pigmented rice contributes the most to its total phenolic compound (Chen *et al*., 2022). Besides the flavonoid group, there are other compounds commonly found in pigmented rice, such as gallic acid, syringic acid, caffeic acid, and ferulic acid, which also contribute to the total phenolic compound of pigmented rice extract (Lang *et al*., 2019; Santos *et al*., 2021; Chen *et al*., 2022).

3.3. Determination of the Best Pigmented Rice Extract

Based on the total anthocyanin and antioxidant activity, Subang black glutinous rice extract with 96% ethanol showed the best result, with a value of 28.22±1.12 mg CE/g and 11.11±2.04 mg $FeSO_4$/g, respectively. Although Subang extract with 96% ethanol was slightly lower than Bukittinggi extract with 70% ethanol (29.02±0.23 mg CE/g), it showed no significant difference when analyzed statistically.

The total flavonoid and phenolic content of pigmented rice extracts exhibited that the use of 96% ethanol was more effective in extracting those compounds than 70% ethanol. Based on rice varieties, Subang black glutinous rice extract has the highest total flavonoid and phenolic content among other extracts. Thus, Subang black glutinous rice extract with 96% was used for further analysis in the second research stage.

3.4. Effect of pH Difference on Pigmented Rice Extract Stability

The stability of the pigmented rice extract test was conducted based on these parameters, such as total anthocyanin content, antioxidant activity, maximum wavelength, and color intensity.

3.4.1. Total Anthocyanin Content

Based on statistical analysis, the pH difference shows a significant effect on the total anthocyanin of the pigmented rice extract ($p<0.05$). Based on Figure 9, the increase in pH shows a negative correlation with the extract's total anthocyanin. Anthocyanin is relatively stable from pH 1 – 5 since there is no significant difference. On the other hand, the extract started to show a significant anthocyanin decrease at pH 7 – 9. This result is coherent with the literature showing that anthocyanin has the best stability in acidic conditions (Oancea, 2021). When the pH starts to shift into a neutral or basic condition (pH 7 – 9), the anthocyanin structure will be degraded into a quinoidal base, which contributes to a blue color forming (Fendri *et al*., 2018).

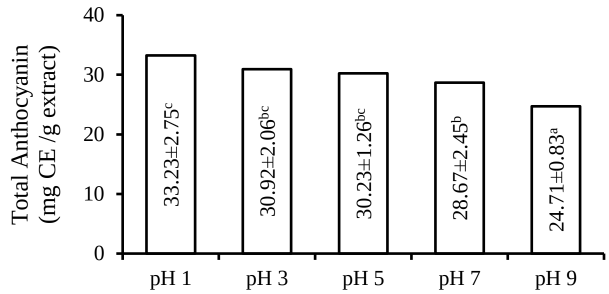

Figure 9. Impact of pH difference on total anthocyanin content of pigmented rice extract (Different superscripts after mean values specify significant differences, $p<0.05$)

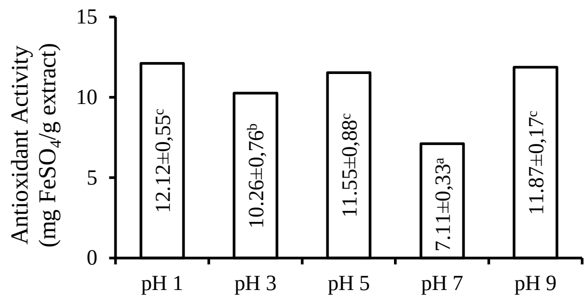

Figure 10. Impact of pH difference on antioxidant activity of pigmented rice extract (Different superscripts after mean values specify significant differences, $p<0.05$)

3.4.2. Antioxidant Activity

Based on statistical analysis, the pH difference has a significant effect on the antioxidant activity of the pigmented rice extract ($p<0.05$). Based on Figure 10, Subang black glutinous rice extract exhibits the highest antioxidant activity at pH 1, amounting to 12.12±0.55 mg $FeSO_4$/g. This result indicates that the extract has the most optimum condition for reducing or chelating Fe^{3+} ions at pH 1 (Pramitasari & Angelica, 2020). However, as pH increased, the antioxidant activity showed a lower value at pH 3 – 7. According to Wahyuningsih *et al*. (2017), the decrease in antioxidant value also correlates with the color degradation and lower anthocyanin amount of the extract. Lower anthocyanin content causes lower reducing power or radical scavenging activity, hence, the antioxidant activity detected through this test will show a decreased value.

From Figure 10, there is a significant increase in antioxidant activity at pH 9, with a value of 11.87±0.17 mg $FeSO_4$/g. The increase in antioxidant value could be caused by the anthocyanin structure degradation into a chalcone form, which contributes to blue color forming within the extract (Shidiqqi *et al*., 2021). The blue color forming within the sample causes the absorbance bias to the sample since the reduced Fe^{3+} ions into Fe^{2+} ions indicator also exhibits a dark blue color (Sadeer *et al*., 2020).

3.4.3. Maximum Wavelength

Maximum wavelength determination was performed towards Subang black glutinous rice extract in several pH levels. The test was conducted using a UV-VIS spectrophotometer at 400 – 700 nm. The results are displayed in Table 3. Based on Table 3, it can be observed that the increase of pH resulted in the change of maximum wavelength of extract Subang rice. The highest absorbance was at pH 1 (1.202), whilst the lowest was at pH 5 (0.217). According to Fendri *et al*. (2020), anthocyanin extract performs the best stability at pH 1. The decrease in absorbance value from pH 1 to pH 5 indicates that there is a color degradation process within the extract. This result is in accordance with the literature that the color changes in anthocyanin structure occur between pH 1 – pH 4.5 due to its oxonium form (red) changing to its hemiketal form (colorless) (Oancea, 2021).

Table 3. Maximum wavelength of the Subang rice extract in different pH levels

pH	λ max (nm)	Absorbance
1	510	1.202
3	515	0.650
5	525	0.217
7	545	0.362
9	575	0.647

From Table 3, the absorbance at pH 7 shows a higher value than pH 5. The rise in absorbance value is mainly because the extract at pH 7 forms a quinoidal base, which exhibits a more intense color than at pH 5, since its anthocyanin structures are mostly composed of hemiketal form (colorless) (Oancea, 2021). The absorbance keeps rising at pH 9, but the maximum wavelength change also occurs at 575 nm. According to Qi *et al.* (2022), the anthocyanin wavelength ranges between 465–560 nm. The maximum wavelength at pH 9 is beyond that range, hence, it cannot be classified as an anthocyanin molecule. In a basic solution, anthocyanin structure changes to chalcone form, which contributes to blue–green color formation (Wahyuningsih *et al*., 2017).

3.4.4. Color Intensity

Based on statistical analysis, the pH difference shows a significant effect on the lightness (*L*), a*, b*, C*,* and *ºHue* of the pigmented rice extract ($p<0.05$). Based on Table 4, *L** value of the extract is quite stable from pH 1 – 3. However, as the pH increases, the *L** value changes become more susceptible. The highest *L** value is produced at pH 5 (53.00±0.36), which is mainly caused by hemiketal structure formation and leads to a colorless anthocyanin (Oancea, 2021). The *a** value of the extract is relatively stable at pH 1 – 3, ranging between 19.10±2.09 to 18.73±1.89. A high *a** value indicates a higher intensity of the red color. The more intense red color produced by the extract is mainly caused by the formation of the oxonium structure of anthocyanin molecules, which usually happens at low pH levels (Oancea *et al.*, 2021).

Table 4. Effect of pH difference on L*, a*, b*, C*, and ºHue value of Subang rice extract

Color Parameter	pH 1	pH 3	pH 5	pH 7	pH 9
*L**	46.26±1.43^{c}	47.58±0.86^{c}	53.00±0.36^{a}	50.26±1.51^{b}	40.92±0.55^{d}
*a**	19.10±2.09^{a}	18.73±1.89^{a}	5.75±0.79^{c}	5.55±1.03^{c}	10.45±0.42^{b}
*b**	10.16±1.65^{a}	8.34±1.30^{b}	1.04±0.08^{c}	1.50±0.25^{c}	-2.11±0.08^{d}
*C**	21.64±2.62^{a}	20.51±2.25^{a}	5.85±0.79^{c}	5.75±1.06^{c}	10.66±0.43^{b}
ºHue	27.93±1.22^{b}	23.97±1.10^{c}	10.33±0.64^{e}	15.15±0.31^{d}	348.56±0.14^{a}

Note: Different superscripts indicate significant differences ($p<0.05$)

From Table 4, the *b** value shows a negative correlation as the pH increases. The highest *b** value is produced at pH 1 (10.16±1.65), while the lowest value is produced at pH 9 (-2.11±0.08). A positive *b** value indicates the yellow color, while a negative *b** value indicates the blue color of the extract (Maulani *et al*., 2019). The results show a negative *b** value at pH 9, which is primarily caused by quinoidal structure formation and contributes to a more blue-colored anthocyanin within the extract (Shidiqqi *et al*., 2021).

The *C** value of the extract is relatively stable at pH 1 – 3 (21.64±2.62 to 20.51±2.25). A higher *C** value indicates a more intense color exhibited by the extract, while a lower *C** value indicates a duller color.These results also correlate with the *L**, *a**, and *b** values of the extract, which show a high value at pH 1 – 3 due to oxonium structure formation, whereas at pH 5 – 7 show a decline due to hemiketal structure formation and contribute to a duller color (Oancea, 2021; Shidiqqi *et al*., 2021).

ºHue value of the extract at pH 1 – 3 can be classified into a red color group since the results of the values are still in the range of 18° – 54°. As the pH increases from pH 5 – 9, the °*Hue* values can be classified into red–purple color group since the results are in the range of 342° to 18° (Hutchings, 1999).

3.4.5. Pigmented Rice Extract Stability (pH)

The stability of Subang black glutinous rice extract showed good stability at pH 1 – 3. The extract exhibited a total anthocyanin content of 33.23±2.75 to 30.92±2.06 mg CE/g and antioxidant activity of 12.12±0.55 to 10.26±0.76 mg $FeSO_4$/g. The *L**, *a**, *b**, and *C** values at pH 1 – 3 also resulted in a higher value and a more intense red color compared to other pH levels. °*Hue* value determinations were also following the *L**, *a**, *b**, and *C** results, which showed the extract at pH 1 – 3 was classified into a red group, whereas pH 5 – 9 was classified into a red–purple group.

3.5. Effect of Temperature on Extract Stability

The stability of the pigmented rice extract test was conducted based on these parameters, such as total anthocyanin content, antioxidant activity, maximum wavelength, and color intensity.

3.5.1. Total Anthocyanin Content

Based on statistical analysis, the temperature difference showed no significant effect on the total anthocyanin content of the extract (p>0.05). The anthocyanin ranged between 33.90±2.33 to 31.84±0.93 mg CE/g. These results indicate that the anthocyanin in the Subang rice extract is relatively stable during heat treatment. One of the factors that could influence stability is the glycosidic bond of the anthocyanin structure. Loypimai *et al.* (2016) stated that cyanidin-3-glucoside showed great stability at 60 – 100 °C compared to other glycosidic bonds of anthocyanin molecules.

3.5.2. Antioxidant Activity

Based on statistical analysis, the temperature difference showed no significant effect on the antioxidant activity of the extract (p>0.05). The antioxidant activity of the rice extract ranged between 15.84±2.25 to 17.70±1.02 mg $FeSO_4$/g. These results indicated the bioactive compounds, such as anthocyanin, flavonoid, and phenolic compounds, still functioned well to reduce or scavenge free radicals, like Fe^{3+} ions, regardless of the heating treatment from 40 – 80 °C. Pramitasari &Angelica (2020) stated that the amount of anthocyanin left within the extract had a positive correlation and contributed to its antioxidant activity.

3.5.3. Maximum Wavelength

Maximum wavelength determination was performed towards Subang black glutinous rice extract in several temperatures. The test was conducted using a UV-VIS spectrophotometer at 400 – 700 nm. The results are displayed in Table 5. The maximum wavelength of the extract does not show any significant changes during the heat treatment. The absorbance shows a slight decrease at 40 – 60 °C. However, there was a slight increase in absorbance when the extract was heated at 80 °C. This phenomenon could happen due to the formation of other compounds besides anthocyanin during the heating process (Nasrullah *et al*., 2020).

Table 5. Maximum wavelength of the Subang rice extract in different temperatures

Temperature (°C)	λ max (nm)	Absorbance
20	510	1.216
40	510	1.201
60	515	1.126
80	515	1.173

3.5.4. Color Intensity

Based on statistical analysis, the temperature difference showed no significant effect on the *L** and *a** values of the extract (p>0.05). However, the temperature showed a significant effect on the *b**, *C**, and °*Hue* values of the extract (p<0.05). The *L** value ranged between 45.97±0.41 to 47.77±1.50. The highest *L** value was produced at 60 °C. This result is in accordance with the absorbance data in Table 5, which indicates a slight color degradation within the extract. According to Maulani *et al.* (2019), the higher the *L** values of the extract, the closer its color to white. Color degradation also indicates a smaller amount of anthocyanin left within the extract (Maulani *et al*., 2019).

The *a** value of the extract ranged between 18.46±2.28 to 21.55±0.54. The lowest *a** value was produced at 60 °C, which indicates a slightly lower red color intensity than other treatments. This result follows the *L** value at 60 °C which exhibited a slight color degradation of the extract due to the heating process (Nasrullah *et al*., 2020).

The *b**, *C**, and *ºHu*e values statistically showed a significant difference between each treatment. However, according to Hutchings (1999), all the rice extracts from 20 – 80 °C did not exhibit different colors since all the results were in the range of 18 – 54 °C, which is the red color group.

3.5.5. Pigmented Rice Extract Stability (Temperature)

Subang black glutinous rice extract was relatively stable during heat treatments until 80ºC. The extract exhibited anthocyanin content of 32.65±0.83 to 33.90±2.33 mg CE/g and antioxidant activity of 15.84±2.25 to 16.92±1.73 mg $FeSO_4$/g. The physical parameters, which are the *L**, *a**, *b**, *C**, and *ºHue* values, also showed good stability of the extract at 20 – 80 °C. The red color intensity did not result in drastic changes, which was supported primarily by the insignificant changes of *a** value and *ºHue* values.

4. CONCLUSION

Rice varieties and ethanol concentrations significantly affect the physichochemical characteristics of pigmented rice extract. Higher concentrations of ethanol resulted in increased levels of anthocyanin, antioxidant activity, total flavonoid, and phenolic content in the extract. The Subang black glutinous rice extract using 96% ethanol showed the best results, with antioxidant activity of 11.11±2.04 mg $FeSO_4$/g, anthocyanin content of 28.22±1.14 mg CE/g, overall flavonoid content of 2.91±1.13 mg QE/g, and a total phenolic content of 3.46±1.59 mg GAE/g.

The difference of pH showed a significant effect on the physicochemical properties of the pigmented rice extract. As the pH increased, the total anthocyanin, antioxidant activity, and color of the extracts (*L**, *a**, *b**, *C**, and *ºHue*) were significantly decreased. However, the heat treatments did not affect the physicochemical characteristic of the extract significantly. As the temperature increased from 20 – 80 °C, the total anthocyanin, antioxidant activity, and color parameters (*L**, *a**, *b**, *C**, and *ºHue*) remained constant. The result of this study showed that the optimum condition for the extract was in a very acidic solution (pH 1) at 80 °C with a total anthocyanin content of 33.90±2.33 mg CE/g, antioxidant activity of 16.92±1.73 mg $FeSO_4$/g, *a** value of 21.55±0.54, and *ºHue* of 30.04±0.57.

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