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FLAVONOID AND ANTIOXIDANT ACTIVITY ANALYSIS OF ANTHOCYANIN BLACK RICE BRAN EXTRACT (ABRIBE) CV CEMPO IRENG ORIGIN FROM INDONESIA

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ABSTRACT

Black rice is an indigenous food in Indonesia that is rich in anthocyanins, a group of plant pigments that are also found in berries. Despite the similarity in anthocyanin content, the high cost and limited availability of berries have restricted their use for extraction. Therefore, this study aimed to determine the total anthocyanin, flavonoid content, and antioxidant activity of black rice bran. To achieve this, the extraction was conducted with maceration using ethanol-citric acid and freeze-drying techniques. The process involved several stages, such as making black rice bran powder, maceration, homogenization, filtration, evaporation, and freeze-drying. The UV-Vis spectrophotometer was used to determine the total anthocyanin and flavonoid content, as well as antioxidant activity. The result showed a total anthocyanin content and the total flavonoid content of 2.48 ± 0.17 mg/gram and 3.76 ± 0.000 mg QE/g freeze-dried extract, respectively. Furthermore, the antioxidant activity of black rice bran CV Cempo Ireng extract and ascorbic acid as a standard was measured in terms of half maximal inhibitory concentration (IC_{50}) in the form of percentage inhibition (%I), with values of 112.42 ± 2.368 and 7.18 ± 0.042 µg/mL, respectively.

Keywords: Anthocyanin, Flavonoid, Antioxidant Activity, Black Rice Bran, Cempo Ireng

INTRODUCTION

Anthocyanins are natural pigments that belong to the flavonoids group **(Martin *et al.*, 2017)** located in water-soluble cells. These pigments occur in the form of glycosylation **(Khoo *et al.*, 2017)** and are responsible for producing red, purple, and blue pigmentation in flowers **(Martin *et al.*, 2017; Khoo *et al.*, 2017).** Anthocyanins are acyl glycosides of anthocyanidins in fruits, vegetables, and cereal grains. They occur as polyhydroxylated or methoxylated derivatives of flavylium or 2- phenylbenzopyrilium **(Dwiatmini & Afza, 2018; Castañeda-Ovando *et al.*, 2009)**. The contents of anthocyanins in berries, currants, grapes, and some tropical fruits, such as red-fleshed dragon fruit are very high **(Khoo *et al.*, 2017)**. In addition to red and purple fruits, these substances are abundant in colored grains like black rice **(Murdifin *et al.*, 2015).** The consumption of berries is limited in Indonesia due to their high cost and restricted availability. As an alternative, black rice, which is rich in anthocyanins, has emerged as a comparable food source. Black rice is characterized by its high levels of anthocyanins, a group of plant pigments also found in berries.
Indonesia is a major producer of pigmented rice, specifically black rice, following China and India **(Prasad *et al.*, 2019)**. Among the various types of Indonesian black rice, Cempo Ireng has the highest total anthocyanin content compared to others, as reported by **Pratiwi *et al.* (2019) and Kristamtini & Wiranti (2017)**. Approximately 43% of anthocyanins are contained in the aleurone layer, namely black rice bran **(Pratiwi *et al.*, 2015)**. Cyanidin-3-O-glucoside and peonidin 3-O-glucoside are anthocyanins found in black rice bran **(Apridamayanti *et al.*, 2017)**. Cempo Ireng has a total anthocyanin content of 428.38 mg/100g, which is slightly lower than *Vaccinium corymbosum* blueberry (cultivar CVAC5.001) of 430 mg/100g **(Kristamtini & Wiranti, 2017; Peña-Sanhueza *et al.*, 2017)**.
The optimal anthocyanin content in black rice is influenced by several factors, such as solvent, temperature, time, solid/liquid ratio, and particle size **(Maulida & Guntarti, 2015; Le *et al.*, 2019)**. Previous studies have shown that the use of acidified solvent produces more optimal anthocyanin contents. However, its stability is influenced by various factors, such as light, storage temperature, pH, concentration, solvent, oxygen, chemical structure, pressure, enzymes, and metal ions **(Yousuf *et al.*, 2016)**. Anthocyanins are very unstable and susceptible to degradation **(Giusti & Wrolstad, 2003)**. To maintain their stability, techniques such as freeze-drying and frozen storage are often used, allowing for the preservation of the extract for more than a year **(Syamaladevi *et al.*, 2011)**.
Anthocyanins and flavonoids are well-known sources of antioxidants **(Hanifa *et al.*, 2020)**. Flavonoids are members of the phenol family and have a high antioxidant capacity due to their ability to eliminate free radicals and prevent excessive accumulation **(Chen *et al.*, 2022; Takagaki *et al.*, 2019; Ghorbani, 2017; Yan *et al.*, 2016; Sasaki *et al.*, 2007)**. Anthocyanin is a type of flavonoid found in black rice and plays an essential role in antioxidant activity **(Suryanti *et al.*, 2020; Seo *et al.*, 2011)**. Flavonoids have the ability to scavenge nitric oxide and oxygen **(Choi *et al.*, 2018).** Their compounds act as reductants, free radical scavengers, and singlet oxygen quenchers, thereby exhibiting reducing, scavenging, and singlet oxygen formation-quenching properties **(Pattananandecha *et al.*, 2021)**.
Anthocyanins act as antioxidants primarily through two mechanisms, namely the transfer of a single electron and donor of hydrogen atom **(Putri *et al.*, 2022)**. During single electron transfer, antioxidants (AH+) donate electrons to free radicals, reducing the oxidized intermediate to a stable state. In contrast, in the mechanism of the hydrogen atom donor, free radicals (R•) are converted into a more stable form by removing hydrogen atoms from antioxidants (AH+). According to **Tena *et al.* (2020)**, both mechanisms occur concurrently in most cases.
This study aims to determine the total anthocyanin content, total flavonoid content, and antioxidant activity of Indonesian black rice bran extract using citric acid−ethanol solvent.

MATERIAL AND METHODS

Material

Unhulled dry rice was obtained from Cigudeg, Bogor processed with HW60AN Huller (Yanmar, Indonesia). The rice was winnowed to separate the skin-cracked rice from the empty unhulled types. Afterward, a total of 200 grams of black rice was polished for one and a half minutes with a Japanese Satake polisher. Sieving was then carried out with a mesh size of 60 to obtain fine particles **(Maulida & Guntarti, 2015; Pramitasari & Angelica, 2020)**. The resulting black rice bran powder was stored at 20°C in a dark dry plastic wrap, vacuumed, and kept away

from light until ready for extraction **(Maulida & Guntarti, 2015; Pramitasari & Angelica, 2020; Thao *et al.*, 2015).**
Material prepared for extraction include black rice bran powder, 96% ethanol, 20% (w/v) citric acid, 0.025 M KCl at pH 1, and 0.4 M sodium acetate at pH 4.5 **(Maulida & Guntarti, 2015; Pramitasari & Angelica, 2020; Giusti & Wolrstad, 2001)**.

Anthocyanin Extraction Procedure

Maceration of Black Rice Bran Powder

A total of 30 grams of black rice bran powder was macerated with an ethanol-citric acid solution at room temperature for 24 hours. The ratio of black rice bran powder and the solvent is 1:10 (w/v) **(Maulida & Guntarti, 2015; Pramitasari & Angelica, 2020)**. The maceration process, which involves soaking black rice bran powder in a solvent to soften the material, was performed in triplicate. The mixture was stored in a dark room to avoid reaction with light and changes **(Maulida & Guntarti, 2015; Thao *et al.*, 2015; Widarta *et al.*, 2013)**. During these 24 hours maceration processes, homogenization was conducted in the first and last two hours using a magnetic stirrer.

Filtration, Evaporation, and Freeze-drying

The macerate was filtered using Toyo filter paper no. 5B in a butchner funnel pores placed in a flask connected to a vacuum pump **(Maulida & Guntarti, 2015)**. Furthermore, It was evaporated at a temperature of 50°C using an IKA® HB10 vacuum rotary evaporator (VirtualExpo Group, UK) and a speed of 30 rpm with a digital IKA® RV10 vacuum rotary evaporator (VirtualExpo Group, UK). This process was continued until the ethanol was completely evaporated. The filtrate was placed in a brown bottle (Amber) and frozen at -20°C for 12 hours. It was then freeze-dried for 72 hours and the extract of black rice bran was stored at -20°C **(Syamaladevi *et al.*, 2011)**.

Determination of Total Anthocyanin Content

Total anthocyanin content was determined from the freeze-dried extract of black rice bran. The absorbance value of the freeze-dried extract was analyzed by the pH difference method (pH 1 and 4.5) using a Genesys™ 150 UV-Vis Spectrophotometer (ThermoFisher Scientific, USA). The absorbance and correction factor for this process is 512 nm and 700, respectively, using KCl and Na acetate according to Giusti and Wrolstad method **(Pramitasari & Angelica, 2020; Giusti & Wrolstad, 2001; Amelia *et al.*, 2013)**. One ml of extract was placed in each of the two 5 ml test tubes. KCl at a pH of 1 was added to the first tube, and the second was added with Na acetate at a pH of 4.5 to the mark limit and shaken until dissolved. The solution was left for fifteen minutes, and their absorbance values were measured at two wavelengths (λvis-max and 700 nm). The determination of the total anthocyanin content was conducted in triplicate. The anthocyanin concentration and absorbance values were calculated using the following equation **(Giusti & Wrolstad, 2001)**:

$$A = [(A_{\lambda vis\text{-}max} - A_{700})\,pH\,1 - (A_{\lambda vis\text{-}max} - A_{700})\,pH\,4.5]$$

$$MA\ (mg/L) = \frac{A \times MW \times DF \times 1000}{\epsilon \times L}$$

Where:
A = Absorbance
MA = Monomeric anthocyanin
MW = Molecular weight (449.2 g/mol)
DF = Dilution factor
Ɛ = Molar extinction coefficient (26900 L/cm/mg)
L = Cuvette width (1 cm)

The total anthocyanin content in the freeze-dried extract was determined based on the following calculation:

$$TAC\ (mg/gram) = \frac{MA\ (mg/L) \times V\ (L)}{W\ (gram)}$$

TAC = Total anthocyanin content in the freeze-dried extract
V = Volume in liter
W = Weight of freeze-dried extract

Determination of the Total Flavonoid Content

The total flavonoid content was determined according to the method of **Aiyegoro and Okoh (2010)**. One milliliter of the sample (1 mg/ml) was combined with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate, and 5.6 ml of distilled water. This mixture was allowed to stand at room temperature for 30 minutes. The absorbance of the reaction mixture was determined using a UV-visible spectrophotometer at 420 nm. Afterward, the total flavonoid content in extracts was quantified as mg quercetin equivalent/gram freeze-dried extract. The determination of the total flavonoid content was conducted in triplicate.

Antioxidant Activity Analysis

The antioxidant activity of the extract was assessed using a 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay. This assessment was based on the procedure described by **Jaradat *et al.* (2015)**. A concentration of 0.002% w/v DPPH solution was mixed with methanol and incubated in the dark for 30 minutes at room temperature before taking absorbance measurements at 517 nm. The assay was conducted in triplicate and the percentage of antioxidant activity was determined to measure the scavenging capacity of the extract, using ascorbic acid as a standard. The following formula was used for calculation **(Jaradat *et al.*, 2015)**:

$$\text{DPPH free radical scavenging activity (\%)} = (A_{control} - A_{extract/standard})/A_{control} \times 100\%$$

where,
$A_{control}$ = The absorbance of DPPH and methanol
$A_{extract/standard}$ = The absorbance of DPPH and extract or standard
BioDataFit edition 1.02 was used to compute the antioxidant half maximal inhibitory concentration (IC_{50}) for the extract and standard.

RESULTS AND DISCUSSION

Total Anthocyanin Content

The total anthocyanin content of freeze-dried black rice bran extract using ethanol solvent acidified with citric acid was 2.48 ± 0.17 mg/gram higher than 0.34 ± 0.000 mg/gram from the results of Pramitasari and Angelica **(Pramitasari & Angelica, 2020)**. It was also higher in sweet purple potatoes extracted with ethanol and citric acid than in aquadest and citric acid (0.83 ± NA mg/gram and 0.17 ± NA mg/gram, respectively) **(Chen *et al.*, 2019)**.
The high total anthocyanin content produced by black rice bran extract is attributed to the increased polarity similar to ethanol solvent **(Widarta, Nocianitri & Sari, 2013).** Ethanol, being a polar solvent, dissolved anthocyanin effectively, following the principle of "like dissolves like" **(Amelia *et al.*, 2013)**. According to Widarta *et al.*, black rice bran extracted with ethanol solvent produces a total anthocyanin content of 0.33 ± NA mg/gram **(Widarta, Nocianitri & Sari, 2013)**. This is higher than the result of 0.30 ± NA and 0.25 ± NA mg/gram of bran produced from extraction with methanol and aquadest solvents in black rice bran, respectively **(Widarta, Nocianitri & Sari, 2013)**. The ethanol solvent is lower in toxicity compared to the methanol **(Abdel-Aal & Hucl, 1999)**. Therefore, the extraction was not tested with methanol solvent, and no toxic effects were observed in the study conducted by Guo *et al.*, with experimental animals using black rice extraction with ethanol **(Guo *et al.*, 2007).**

Table 1 TAC of Indonesian black rice bran extract CV Cempo Ireng and the comparative study

Source of ANC	Type of Solvent	TAC (mg/g FDE)	Reference
Black Rice Bran	Ethanol-Citric Acid	2.48 ± 0.170	This study
Black Rice	Ethanol-Citric Acid	0.34 ± 0.000	Pramitasari & Angelica, 2020
Sweet Purple Potato	Ethanol-Citric Acid	0.83 ± NA	Chen *et al.*, 2019
Sweet Purple Potato	Aquadest-Citric Acid	0.17 ± NA	Chen *et al.*, 2019
Black Rice Bran	Ethanol	0.33 ± NA	Widarta, Nocianitri & Sari, 2013
Black Rice Bran	Methanol	0.30 ± NA	Widarta, Nocianitri & Sari, 2013
Black Rice Bran	Aquadest	0.25 ± NA	Widarta, Nocianitri & Sari, 2013

TAC: Total anthocyanin content (Data are presented as mean + standard deviation (SD)); ANC: Anthocyanin; FDE: Freeze-dried extract; NA: Not available

Anthocyanin stability is pH-dependent **(Hosseini *et al.*, 2016)**, and the addition of acid can help denature the cell wall membrane and stabilize the anthocyanins in solution **(Martin, 2017; Rodriguez-Saona & Wrolstad, 2001; Nisha & Narayan, 2020)**. Extracts in an acidic environment attract more anthocyanin pigments into the solvent **(Ermiziar, Raskita & Latifa, 2017)**. This is because the acid added to the extract helps digest the black rice cell wall. Therefore, the extracts can be released very effectively in large quantities **(Halee *et al.*, 2018)**. In addition to stabilizing anthocyanins, acids can also change the original form of pigments in the tissues by breaking the bond to metals, co-pigments, or other factors **(Rodriguez-Saona & Wrolstad, 2001)**. Citric acid was chosen to acidify the solvent based on the pKa value. The smaller the value, the more stable the anthocyanins and the more acidic. The pKa of acetic, citric, and tartaric acid was 4.76, 3.09, and 2.98, respectively **(Hubbermann *et al.*, 2006)**. A stronger acid released more hydrogen ions into the solution, which is reflected by a higher dissociation constant **(Tensiska, Sukarminah & Natalia, 2006)**. Citric acid was chosen over tartaric acid despite the similar pKa values, and it was more commonly used in the extraction of foodstuffs that are rich in anthocyanins. According to Chang *et al*., the acidification of ethanol solutions with citric acid is preferable to HCl to avoid harmful residues, specifically when used in food products **(Chang *et al*., 2012)**. Citric acid and ethanol can be used safely because they are non-toxic for the extraction of pigments and bioactive compounds in black rice compared to HCl and methanol which are toxic to humans **(Castañeda-Ovando *et al*., 2009; Abou-Arab *et al*., 2011; Nisha and Narayanan, 2020; Pedro, Granato & Rosso, 2016; Delgado-Vargas *et al*., 2000; Amanda, Santoni & Darwis, 2015).**

Total Flavonoid Content

The total flavonoid content of freeze-dried black rice bran extract using ethanol solvent acidified with citric acid was 3.76 ± 0.000 mg Quercetin equivalent/gram. This value was higher than black rice bran extract from other studies, as shown in Table 2.

Table 2 TFC of Indonesian black rice bran extract CV Cempo Ireng and the comparative study

Type of black rice	TFC (mg QE/g FDE)	Reference
Cempo Ireng	3.76 ± 0.000	This study
D Youzinuo 161	1.98 ± 0.037	Shao *et al*., 2018
Heimi No. 1	3.14 ± 0.271	Shao *et al*., 2018
Heixiannuo No. 3	3.62 ± 0.158	Shao *et al*., 2018
Black Kavuni	1.09 ± 0.103	Thanuja & Parimalavalli, 2020
Sintoheugmi	0.83 ± 0.140	Choi *et al*., 2018
Chiang Mai	1.93 ± 0.030	Pengkumsri *et al*., 2015
Le'leng	0.62 ± NA	Hanifa *et al*., 2020

TFC: Total flavonoid content (Data are presented as mean ± standard deviation (SD)); QE: Quercetin equivalent; FDE: Freeze-dried extract; NA: Not available

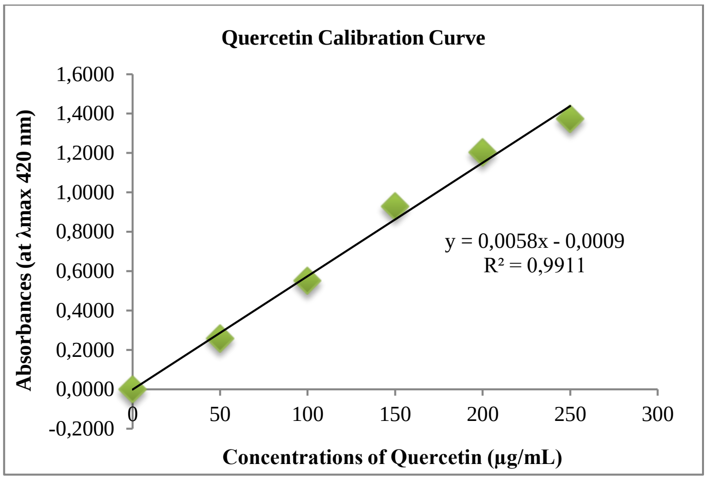


Figure 1 Calibration Curve of Quercetin

The calibration curve of quercetin in Figure 1 exhibited linearity with a correlation coefficient of r = 0.99, showing a strong positive correlation between the concentrations of quercetin and its corresponding absorbances.

Antioxidant Activity of ABRiBE

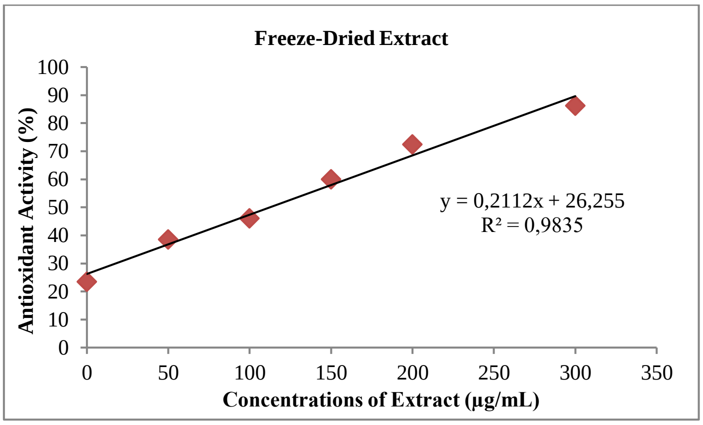
Ichikawa *et al.* (2001) showed that purple-black rice and blueberry extract have 10 to 25 times higher antioxidant activity compared to other sources. The IC_{50} value, which represents the concentration of antioxidants required to inhibit 50% of free radicals, is used to determine the antioxidant activity values **(Suryanti *et al*., 2020; Budaraga & Salihat, 2020).** The total antioxidant activity of black rice anthocyanin acts against DPPH free radical scavenging **(Fatchiyah *et al*., 2020)**, and the IC_{50} of ABRiBE in this study was 112.42 ± 2.368 μg/mL. An IC_{50} value < 50 μg/mL indicates that an antioxidant exhibits very strong activity. Furthermore, an IC_{50} value in the range of 50 - 100 μg/mL, 101 - 150 μg/mL, and 151 - 200 μg/mL indicates strong, moderate, and weak antioxidant activity, respectively **(Budaraga & Salihat, 2020; Molyneux, 2004).** The antioxidant of the ABRiBE in this study showed moderate activity. **Tyagi *et al.* (2022)** also exhibit moderate activity antioxidant (IC_{50} in the black rice extract of 109.617 ± 0.74 μg/mL). According to **Seo *et al.* (2011)**, colored rice in Korea exhibits weak antioxidant activity with IC_{50} of 246.9 ± 11.95, 287.39 ± 13.26, and 381.3 ± 20.57 μg/mL for Heungjinju, Shinti heugmi, and Heungseol, respectively. **Apridamayanti *et al.* (2017)** reported that the black rice Cempo Ireng has a weak antioxidant activity with an IC_{50} of 200.960 ± NA μg/mL.

Table 3 Antioxidant activity of the extract in ethanol − citric acid solvents determined by DPPH assay

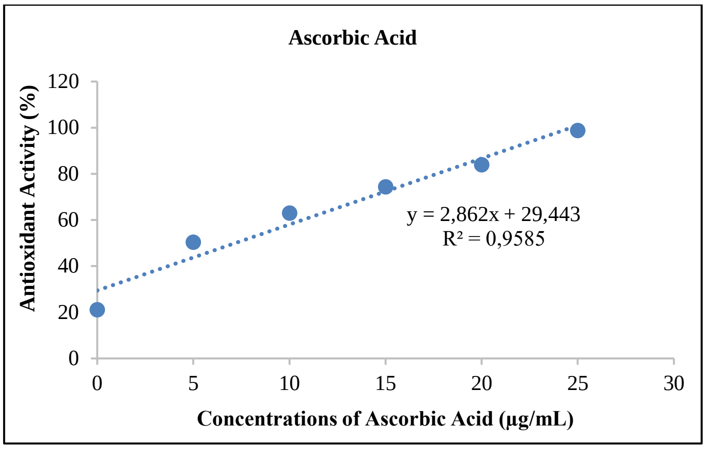
Absorbance Groups	A	Antioxidant Activity (%)	IC_{50}
$A_{control}$	0.81	3.68	
$A_{extract}$			112.42 ± 2.368
0	0.62	23.46 ± 0.004	
50 μg/mL	0.50	38.46 ± 0.007	
100 μg/mL	0.44	46.01 ± 0.017	
150 μg/mL	0.32	60.00 ± 0,127	
200 μg/mL	0.22	72.39 ± 3.156	
300 μg/mL	0.11	86.17 ± 0.316	
$A_{ascorbic\ acid}$			7.18 ± 0.042
0	0.64	21.02 ± 0.325	
5 μg/mL	0.40	50.31 ± 0.116	
10 μg/mL	0.30	62.98 ± 0.057	
15 μg/mL	0.21	74.36 ± 0.089	
20 μg/mL	0.13	83.85 ± 0.620	
25 μg/mL	0.01	98.79 ± 0.035	

An antioxidant that contributed to the inhibition of free radical DPPH molecules was shown by the percentage of inhibition (%I) **(Budaraga & Salihat, 2020).** The antioxidant of ABRiBE exhibited 86.17 ± 0.316% as a percentage of inhibition (%I), which is higher than 33.51 ± 2.77% of Cempo Ireng black rice in the report of **Putri *et al.* (2022).** According to **Apridamayanti *et al.* (2017)**, the concentration of 500 μg/mL Cempo Ireng extract inhibits the free radicals by 81.46 ± NA%. Furthermore, the ABRiBE showed a percentage of inhibition (%I) higher on the extract concentration of 300 μg/mL than the results of **Apridamayanti *et al.* Hetharia *et al.* (2020)** reported that the antioxidant activity of black rice extract is 76.81 ± NA% with 100 μg/mL. The antioxidant activity of black rice by **Ponnappan *et al*. (2017)** shows 86.12 ± 0.05%, while Chakhao Amubi and Chakhao Poireiton black rice show 70.28 ± NA% and 60.84 ± NA%, respectively, according to the results of **Asem *et al.* (2015)**. The antioxidant activity of Korean Heugjunjubyeo black rice is 40.39 ± NA% as obtained by **Park *et al.* (2008)**, and Chinese Brown Himi black rice has 70.82 ± NA% **(Saenkod *et al.*, 2013)**. **Murdifin *et al.* (2015)** showed that the examination of black rice DPPH activity has a very low inhibition value of 22.22 ± 1.05%. According to **Noorlaila *et al.* (2018)**, the antioxidant activity of black rice is 88.72 ± NA% and 79.29 ± 0.64% in the results of **Xie *et al.* (2020).** Furthermore, the antioxidant activity of Indonesian black rice from several studies is 66.27 ± NA%, 6.51 ± NA%, 48.77 ± NA%, and 46.20 ± NA% **(Azis *et al.*, 2015; Suhartatik *et al.*, 2015; Dwiyanti *et al.*, 2013; Wanti *et al.*, 2015, respectively)**, while Black Kavuni rice from India has 25.13 ± 1.92% **(Thanuja & Parimalavalli, 2020).**

In this study, the correlation between the antioxidant activity and freeze-dried black rice bran extract was 0.98 ($p < 0.00001$), indicating a very strong positive relationship with antiproliferative activity compared to 0.65 of **Chen *et al.* (2022)**. **Mapoung *et al.* (2023)** showed that the total anthocyanin and flavonoid content in the black rice extract have a strong positive correlation with antioxidant activity, as indicated by r values of 0.88 ($p < 0.01$) and 0.91 ($p < 0.01$), respectively. **Thanuja & Parimalavalli (2020)** show a very strong negative and moderate correlation (r = -0.98 and r = +0.82) between total anthocyanin and flavonoid content in black rice and antioxidant activity, respectively. According to **Nindita *et al.*** (2018), there is a moderate positive correlation between total anthocyanin content in black rice and antioxidant activity, as indicated by r = 0.81 ($p < 0.001$).



(a)



(b)

Figure 2 The antioxidant activity of extract (a) and standard (b)

Lao Kuldilok *et al.* (2011) showed that anthocyanins contribute significantly to total flavonoid content and antioxidant activity. The antioxidant actions of anthocyanin on the body prevent free radicals, reactive oxygen species, and reactive nitrogen species **(Ilmi *et al.*, 2018).**

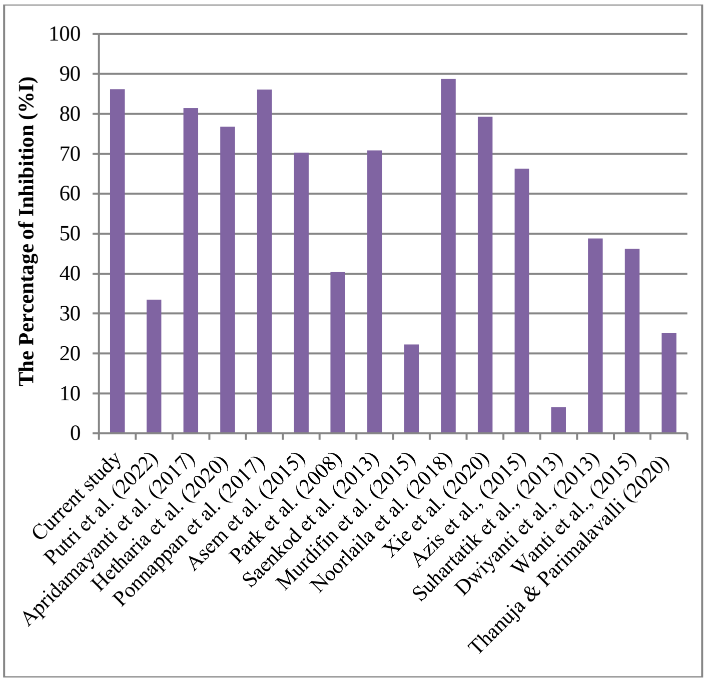


Figure 3 The antioxidant activity of black rice from several studies

CONCLUSION

The total anthocyanin and flavonoid content in ABRiBE Cempo Ireng from Indonesia was found to be higher than black rice varieties, and it also exhibited high antioxidant activity.

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