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Co-pigmentation with catechin derived from Indonesian gambier increases the stability of black rice anthocyanin in isotonic sports drinks during one-month storage in 4°C

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Abstract

Anthocyanins are water-soluble pigments, which are found abundantly in plants. However, anthocyanins are unstable and break due to several factors. The stability of anthocyanins could be increased by applying co-pigmentation with the addition of flavonoids, polyphenols, alkaloids, amino acids, organic acids, or self-association. This study aimed to observe the effect of co-pigmentation with catechin from Indonesian gambier (*Uncaria gambir*) to enhance the stability and antioxidant activity of black rice anthocyanin in isotonic sports drink during storage. Anthocyanins extracted from black rice (30 mg/L) were used for isotonic sports drink formulation. The addition of 0.1 µg/L catechins to the drink with catechin: drink ratio (1:1) significantly increased antioxidant activity (89.30±1.12%). In a storage period of one month at room temperature (25°C), anthocyanin content and antioxidant activity in isotonic sports drinks showed a significant decrease than that at 4°C. Catechin co-pigmentation in the drinks increased anthocyanin stability and antioxidant activity compared to that of control (drinks without catechin). In conclusion, co-pigmentation with catechin derived from Indonesian gambier could be applied to enhance the stability and antioxidant activity of black rice anthocyanin in isotonic sports drink during storage at cold temperature (4°C) for one month.

1. Introduction

Physical exercise is an activity carried out to improve and maintain body fitness. During this activity, the body needs energy, fluids, and other nutrient intakes (Stohs and Kitchens, 2013). Isotonic sports drink has a role in restoring body fluids and electrolytes to maintain their performance. It also contains carbohydrates that could be used as a source of energy (Galaz, 2013). An isotonic sports drink contains a mineral salt concentration similar to human body fluids so that it is easily absorbed in the digestive system. It typically contains carbohydrates ranging from 6-9% and electrolytes derived from mineral salts (Galaz, 2013).

The production of free radicals such as reactive oxygen species (ROS) naturally occurs in the human body due to metabolism (Bean, 2013). Moderate to heavy intensity exercise could increase metabolism followed by increased free radical production, which causes oxidative stress (Nielsen, 2013). When doing aerobic exercise such as long-distance running, there was an increase in the amount of oxygen needed so that there could be hyperoxics in the mitochondria, which causes increasing the production of ROS (Berawi and Averianti, 2017). The increase in free radicals could exceed the capacity of the body to produce antioxidants. Hence it requires antioxidants obtained from food or beverage intake (Sharma, 2014).

Anthocyanins are water-soluble pigments found in flowers, fruits, tubers, and cereals. Anthocyanins are polyhydroxyl derivatives of 2-phenyl-benzopyrylium, which have antioxidant activity (Wong, 2018). Black rice is a food that contains anthocyanin (Zhang *et al.*, 2010). The dominant type of anthocyanin in black rice is cyanidin-3-glucoside. Cyanidin has higher antioxidant activity than other types of anthocyanins (Chaiyasut *et al.*, 2016), so black rice has the potential to be developed as a food product with high antioxidants.

The commercial isotonic sports drinks that have been sold in Indonesia does not contain antioxidants to reduce free radicals production during exercise. Extraction is a process to obtain the advantage of anthocyanins. This extract could be added to the isotonic sports drink to provide an antioxidant effect. However, anthocyanins extract is unstable and break due to temperature, pH, and

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light (Giusti and Wallace, 2009). The decrease in anthocyanin impacts the decrease in antioxidant activity (Mustofa and Suhartatik, 2018). Anthocyanin stability could be increased by applying co-pigmentation with compounds such as flavonoids, polyphenols, alkaloids, amino acids, organic acids, or anthocyanin itself (Mazza, 2018). Mustofa and Suhartatik (2018) reported that the addition of Maya flower extract as a co-pigment compound in isotonic sports drinks containing black glutinous rice anthocyanin could increase the stability of anthocyanin in storage for up to three weeks. Catechin is one of the flavonoid compounds found in tea leaves and gambier (*Uncaria gambir*) plants (Yuliarti, 2009). Gambier plant was found in Indonesia and had a high catechin content. Rahmawati *et al*. (2012) revealed that gambier plants from three different areas in Indonesia contained 40.94-80.71% of catechin.

Kanha *et al*. (2019) showed that co-pigmentation with catechin could increase the stability of anthocyanin. However, catechin derived from Indonesian gambier as a co-pigment compound has never been done in isotonic sports drinks containing black rice anthocyanin. Hence, the purpose of this study was to observe the effect of co-pigmentation with catechin from Indonesian gambier to enhance the stability and antioxidant activity of black rice anthocyanin in isotonic sports drink during one-month storage in 4 and 25°C.

2. Materials and methods

2.1 Materials

The materials used in this research were black rice from a local distributor (Yogyakarta, Indonesia), Uncaria gambir catechin powder 95% (CV. Kempan, Indonesia), distilled water (Amidis, Indonesia), citric acid (Koepoe Koepoe, Indonesia), sucrose, fructose, food-grade mineral salts (calcium lactate, potassium chloride, and magnesium chloride), strawberry flavour, 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich, USA), potassium chloride (Merk, Germany), sodium acetate (Merk, Germany), and methanol (Merk, Germany). All chemicals were analytical grade.

2.2 Sample preparation

The whole black rice was stored in cold storage at 4°C before being used. Black rice was ground into powder using a food processor. Then, it was sieved using a 40-mesh sieve. Black rice powder was stored in a dark container at 4°C before further processing.

2.3 Extraction of black rice anthocyanin

Black rice anthocyanin was extracted using the maceration method according to Aguilera *et al*. (2016) with some modifications. A total of 50 g of black rice powder was weighed and put into Erlenmeyer, then 2% citric acid solution was added. The ratio of the sample and the solvent used was 1:3 (w/v). The samples were put into a water bath shaker (GFL, Germany) at a temperature of 50°C for 4 hours. The extract obtained was centrifuged (Eppendorf, Germany) at 32,000×*g* for 5 mins, then the filtrate was filtered using the filter paper. The extract was stored at -20°C in dark conditions.

2.4 Analysis of total anthocyanins content

Total anthocyanins content was analyzed using the pH differential method (Giusti and Wrostald, 2001). A total of 0.1 mL of extract was each added with 2 mL of buffer solution pH 1 (potassium chloride) and pH 4.5 (sodium acetate). Then homogenized using a vortex (ThermoFisher, USA) and incubated for 5 minutes. Extracts at pH 1 and 4.5 were measured for their absorbance at a wavelength of 512 nm and 700 nm using a UV-Vis Spectrophotometer (ThermoFisher Scientific, USA). The absorbance results obtained were calculated using the equations:

$$A = [(A_{512\,nm} - A_{700\,nm})\ pH\ 1.0] - [(A_{512\,nm} - A_{700\,nm}) \quad (1)$$

Total anthocyanins content was calculated as mg cyanidin-3-glucoside equivalent per g dried weight:

$$\text{Total monomeric anthocyanin content} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L} \quad (2)$$

Where A = measured absorbance, MW = molecular weight for cyanidin-3-glucoside (449.2 g/mol), DF = dilution factor, ε = Molar absorbance unit of cyanidin-3-glucoside (26,900 L/(cmxmol), L = cuvette length (1 cm) and 1000 = conversion factor from milliliters to liters

2.5 Formulation of anthocyanin isotonic sports drink

The drink composition refers to Mustofa and Suhartatik (2018) with some modifications (Table 1). All ingredients were mixed well in a bottle. Then the drink was warmed at 50°C for 5 mins to inhibit the growth of microbes that caused contamination.

Table 1. Composition of anthocyanin Isotonic sports drink

Component	Concentration
Water (L)	1
Black rice anthocyanin extract (mg/L)	30
Sucrose (%)	5
Fructose (%)	2
Strawberry flavour (mL/L)	1
Cl^- (mEq/L)	21.5
Na^+ (mEq/L)	17
K^+ (mEq/L)	4
Ca^{2+} (mEq/L)	1
Lactate- (mEq/L)	1
Mg^{2+} (mEq/L)	0.5

2.6 Analysis of antioxidant activity

Analysis of antioxidant activity was performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition method (Krings and Berger, 2001). A total of 0.1 mL of anthocyanin extract and sports drink were each mixed with 2 mL of 0.1 mM DPPH reagent. The reference used was 1% ascorbic acid. The absorbance of each sample was measured using a UV-Vis spectrophotometer (ThermoFisher Scientific, USA) at a wavelength of 517 nm. The percentage of antioxidant activity was expressed in the following equation:

$$\%\ \text{antioxidant activity} = 1 - \frac{A1 - A2}{A0} \times 100\% \qquad (3)$$

Where A1 = absorbance of the sample + DPPH, A2 = absorbance of the sample + methanol, and A0 = absorbance of methanol + DPPH

2.7 Measurement of anthocyanin stability in isotonic sports drink during storage

Stability assay was carried out by measuring total anthocyanins content and antioxidant activity (Giusti and Wrostald, 2001; Krings and Berger, 2001; Pangestu *et al*., 2020) in isotonic drink formula without catechin (formula 1) as control compared to the drink with the addition of catechin derived from Indonesian gambier as co-pigment (formula 2). A total of 0.1 µg/L catechin was added to the drink with catechin: drink ratio (1:1). The drinks were stored at room (25°C) and cold temperature (4°C), respectively. Analysis was carried out every three days during the one month of storage.

2.6 Statistical analysis

The data from the stabilities assay were processed using IBM SPSS statistics 24 software. The statistical method used was Analysis of Variance (ANOVA) followed by the Duncan's test at $p<0.05$.

3. Results and discussion

3.1 Antioxidant activity of black rice anthocyanin isotonic sports drinks

The antioxidant activity of black rice anthocyanin isotonic sports drink added with catechin (formula 2) was not significantly different with black rice anthocyanin extract and ascorbic acid as a reference ($p>0.05$). Meanwhile, the black rice anthocyanin isotonic sports drink without catechin showed a significantly lower antioxidant activity than other samples (Figure 1).

Anthocyanin extract could inhibit the free radicals due to electrons donation to free radicals (Ullah *et al*., 2019). The antioxidant activity of black rice came from anthocyanin and other phenolic compounds such as ferulic acid and p-coumaric acid (Zhang *et al*., 2010; Zhang *et al*., 2015). Phenolic compounds could also be extracted from black rice as they were also soluble in polar solvents such as water (Barchan *et al*., 2014). The anthocyanin isotonic sports drink also showed high antioxidant activity with a percentage above 50% (Figure 1). However, the anthocyanin isotonic sports drink added with catechin showed a significantly higher antioxidant activity than the control. This might happen due to the catechin contribution, as it was a type of flavonoid with high antioxidant activity (Yeni *et al*., 2017). In line with our research, Tan *et al*. (2018) revealed co-pigmentation using catechin hydrate improved the stability of encapsulated blueberry and elderberry anthocyanins extracts.

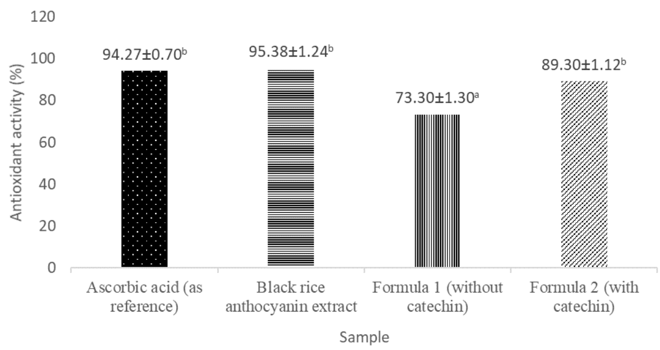

Figure 1. Antioxidant activity of black rice anthocyanin isotonic sports drinks. Different superscript notations above each bar indicate statistically significant differences ($p<0.05$).

3.2 Stability of black rice anthocyanin in isotonic sports drinks during storage

Based on Figure 2, total anthocyanins content stability during storage for 30 days in both formula 1 (control) and 2 (with catechin) were higher at 4°C than those at room temperature (25°C) ($p<0.05$). Total anthocyanins content in both formula 1 and 2 at room temperature was decreased significantly ($p<0.05$) from 30.47 to 14.77 mg/L (51.52%) and from 29.72 to 15.78 mg/L (46.9%), respectively. Meanwhile, total anthocyanin content in both formula 1 and 2 at 4°C was decreased significantly ($p<0.05$) from 30.47 to 25.3 mg/L (16.96%) and from 29.72 to 26.63 mg/L (10.4%), respectively.

Anthocyanin degradation could be inhibited by applying co-pigmentation. It might happen due to the formation of bonds through intermolecular and intramolecular interactions between anthocyanin and co-pigment molecules that would stabilize its colour (Teixeira *et al*., 2013). Anthocyanin would bind non-covalently with catechin compounds as co-pigment (Ghasemifar and Saeidian, 2014). The interaction between anthocyanin and co-pigment could inhibit the hydration reaction, which changes the anthocyanin

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structure from the red flavylium cation to the colourless form of carbinol pseudobase (Mazza, 2018). Pan *et al.* (2014) reported that anthocyanin in blueberries decreased during storage. However, the addition of the flavonoid C-glycoside compound from pigeon pea leaves as a co-pigment could inhibit anthocyanin reduction compared to the control.

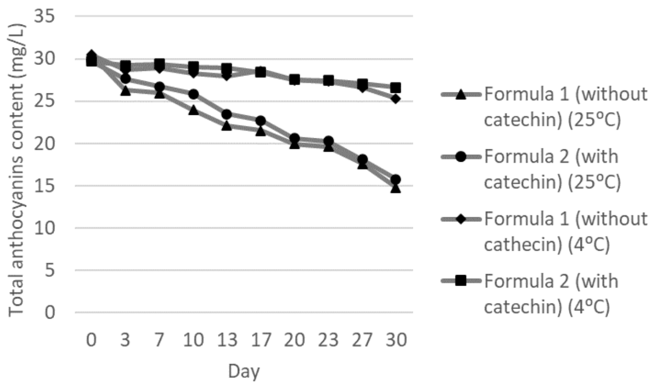

Figure 2. Stability of black rice anthocyanin in isotonic sports drinks during storage

Anthocyanin isotonic sports drinks at cold temperature (4°C) showed much less anthocyanin degradation than those at room temperature (Figure 3). Anthocyanins tend to be more stable at low temperatures and more easily degraded at high temperatures (Sui 2017). These were in line with Sinela *et al*. (2017) stated anthocyanin placed at 4°C during storage was more stable and only decreased by 17% compared to that at temperatures above 20°C which could degrade by more than 50%. Besides temperature, pH and other compounds added into the drink could be contributing to the anthocyanin stability. The anthocyanin isotonic sports drink that was made had a low acidity level with a pH of 3.8. This acidic condition could also make anthocyanin more stable. The composition of sugar and salt in isotonic sports drinks could also affect the stability of anthocyanin. Sugar and salt could reduce water activity, which impacts increasing anthocyanin stability (Ammari and Schroen, 2016; Lavelli and Kerr, 2019).

3.3 Antioxidant activity in black rice anthocyanin isotonic sports drinks during storage

The stability of antioxidant activity during storage at room temperature in formula 2 (with catechin) was significantly higher than formula 1 ($p<0.05$). The decrease of antioxidant activity at room temperature in both formula 1 and 2 were 14.72% and 12.83%, respectively. Meanwhile, there was a significant decrease ($p<0.05$) of 9.32% in formula 1 and 3.01% in formula 2 at 4°C (Figure 3).

A decrease in antioxidant activity could occur due to anthocyanin degradation that impacts decreased electrons donation to DPPH radicals (Ge and Ma, 2013). Anthocyanin could be degraded into phenolic compounds such as protocatechuic acid during storage. This compound was also known to have antioxidant activity (Sinela *et al*., 2017). The contribution of another compound to antioxidant activity caused a decrease of antioxidant activity not as much as the anthocyanin content. The addition of catechin could reduce the degradation of antioxidants in isotonic sports drinks due to co-pigmentation in anthocyanins (Roger *et al*., 2014). The decrease of antioxidant activity after the storage period for 30 days showed that the isotonic sports drinks still could scavenge the free radicals.

4. Conclusion

The stability of black rice anthocyanin and antioxidant activity in isotonic sports drinks could be influenced by co-pigmentation and storage temperature. The addition of catechin as a co-pigment in anthocyanin isotonic sports drinks could reduce the degradation of anthocyanin and decrease antioxidant activity during 30 days of storage. Storage at low temperature (4°C) showed a significantly lower decrease in anthocyanin than at room temperature. Further research is needed to optimize the catechin concentrations to obtain the optimal stability of anthocyanin isotonic sports drinks.

Conflict of interest

The authors declare no conflict of interest.

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