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Research Article
Total Phenolic Content and Antioxidant Activity of Anthocyanin Extract from Purple Yam (*Dioscorea alata* L.) Flour Using Different Solvents

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Abstract

Background and Objective: Purple yam (*Dioscorea alata* L.) is a potential source of natural antioxidants due to its relatively high anthocyanin content. Anthocyanin is a natural source of antioxidants, acting as a free radical scavenger with a role in aging, cancer and degenerative illness prevention. Using a variety of solvents and acids, this study aimed to extract purple yam flour anthocyanins with high antioxidant activities. **Methodology:** Anthocyanins were extracted using methanol and ethanol-based solvents that were acidified with hydrochloric acid (HCl), citric acid or tartaric acid. The resulting extracts were assayed for anthocyanin and total phenolic contents, antioxidant activity (expressed as % radical-scavenger activity [%RSA]) and ferric reducing antioxidant power (FRAP). All data were analyzed using one-way analysis of variance. The differences were analyzed using Duncan's multiple range test. The $p<0.05$ was considered significant. Pearson's correlation coefficient analysis among anthocyanins, total phenolics contents and antioxidant activities were calculated using Microsoft Excel, 2007. **Results:** Results showed that the methanol/HCl (MeH) solvent could be used to extract anthocyanin from purple yam flour more thoroughly than other solvents. Total phenolic contents were not significantly different between MeH and methanol/tartaric acid (MeT) extracts (5.18 mg GA/100 g extract). Antioxidant activities of MeH and MeT anthocyanin extracts were not significantly different (69.87% RSA and FRAP of 50.27 μmol ε ferro L^{-1}). Anthocyanin and total phenol contents correlated significantly with RSA and FRAP. **Conclusion:** This study suggested that anthocyanins and phenols purple yam flour are an abundant natural antioxidant sources, while the best solvent for the extraction was an acidified polar solvent.

Key words: Purple yam flour, anthocyanin, extraction, solvent, antioxidant activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Purple yam (*Dioscorea alata* L.) is a species of yam, a tuberous root vegetable that contains anthocyanins similar to potatoes[1]. The anthocyanin content of purple yam is 31 mg/100 g dry weight[2]. The anthocyanin content of purple yam is comparable with that of purple potatoes (16-57 mg/100 g DW)[3] and purple sweet potatoes (35.98-41.18 mg/100 g DW)[4]. Anthocyanin is a natural source of antioxidants, acts as a free radical scavenger and plays a role in aging, cancer and degenerative illness prevention[5]. Anthocyanin also protects the liver, reduces coronary artery disease symptoms and enhances retinal function[6]. Anthocyanin is a natural pigment found in vegetables, fruits, leaves and tubers. Anthocyanins may appear red, blue or purple (depending on pH) and are water-soluble[6]. Anthocyanin is one of the subclasses of phenolic phytochemicals. It is derived from flavonol and is the glycosylated form of anthocyanidin[7]. The basic structure of anthocyanin consists of an anthocyanidin moiety. When the anthocyanidins are found in their glycoside form (bonded to a sugar moiety) they are known as anthocyanins[8]. The types of anthocyanin in yam are cyanidin and peonidin which are acylated with sinapic acid[9]. Purple yams contain a variety of acylated anthocyanins that exhibit higher levels of antioxidant activity than nonacylated compounds[10].

Anthocyanins can be extracted from tubers and used as natural colorants that play a role as a natural antioxidant. Anthocyanin is a polar compound, so it requires polar solvent for its extraction process[6]. The polar solvents commonly used for the anthocyanin extraction include methanol, ethanol and water[11,12]. The extraction of flavonoid compound groups can be performed in an acidic environment since acid works well in denaturing plant cell membranes and dissolving anthocyanin pigments, so that acid can move out of the cell and prevent flavonoid oxidation[13]. Anthocyanin is stable and in acidic pH, it has a light color, which gradually changes with increasing pH, becoming colorless around a pH of 4.0-5.7[14]. When anthocyanin is at pH 1, the flavylium cation (red colour) is the predominant species and contributes to the purple and red colours, whereas at pH values ranging from 2-4, the blue quinoidal species are predominant[14].

Isolation of anthocyanin pigments from plants is typically done using a solvent extraction process[15]. Anthocyanins are polar molecules and consequently are more soluble in polar solvents, however, extraction conditions are also key factors in their overall solubility. Extracting anthocyanins from fruits and vegetables, including purple-fleshed sweet potato powder, purple corn, red and blackcurrants and grapes has shown that the extraction using methanol was suitable[6]. This study had been conducted in order to extract anthocyanin using water acidified with citric, acetic or tartaric acids as a solvent for red raspberry (*Rubus idaeus* L.) fruits. The experimental results demonstrated that most of the total anthocyanin extract was obtained from the tartaric acid-based solvent. Tartaric acid has the highest dissociation constant compared to other acids[16]. A more acidic solution (pH closer to 1) caused the anthocyanin pigment to exist in the colored flavylium or oxonium cation form. Measurement of its absorbance resulted in the highest anthocyanins content[17]. Research has been conducted on anthocyanin extraction from senduduk (*Melastoma malabathricum* L.) fruit using a methanol-HCl solvent, which was more effective than using methanol-citric acid solvent[7]. A study about anthocyanin extraction from mangosteen (*Garcinia mangostana*) skin fruit demonstrated that the acidic solvents concentration used for the extraction greatly affected the extract yield. A higher acid concentration (pH close to 1) produced more anthocyanin extracts, because anthocyanins flavylium cations are shaped and colored, so that a large absorbance measurement is obtained[18]. Research was conducted in order to extract the highest anthocyanin concentration using acidified methanol from purple-fleshed sweet potatoes[6]. Several extraction methods have been proposed in order to obtain extracts rich in anthocyanins, these methods are usually based on solvents such as methanol, ethanol, acetone, water or mixtures. The addition of a small amount of HCl or formic acid is recommended to prevent the degradation of the acylated compounds[19].

In a study of sweet potato storage roots, it was recently reported that the relationship between anthocyanin content and radical-scavenging activity correlated positively. The correlation coefficients between radical-scavenging activity (RSA) and anthocyanin content ranged from 0.606-0.687[20]. Another study concerning correlations between antioxidant activity and anthocyanin in flesh-coloured (R^2 = 0.659) has been found[21]. Total phenol contents in sweet potatoes were highly correlated with the free radical 1,1-Diphenyl-1-picryhydrazyl (DPPH) values (R^2 = 0.820). Therefore, total phenol content can serve as a useful indicator for sweet potato antioxidant activities[22].

This study aimed to determine the effects of solvent type (methanol or ethanol) and acid type (HCl, citric or tartaric acid) on anthocyanin extraction with high antioxidant activity from purple yam flour and to assess the relationship between

antioxidant activities and anthocyanin concentrations in addition to phenolic compounds.

MATERIALS AND METHODS

Materials: The primary material, purple yam, was obtained from the local market in Godean, Sleman, Yogyakarta, Indonesia. They weighed approximately 2 kg per tuber. The chemicals used were free radical DPPH, Folin-Ciocalteu reagent, gallic acid (GA) obtained from Sigma Chemical Co., St Louis, United States and ethanol, methanol, HCl, sodium carbonate, sodium nitrite, aluminium trichloride, sodium hydroxide (Merck KGaA, 64271 Darmstadt, Germany), citric and tartaric acids, acetate buffer, ferric tripyridyltriazine (Fe^{3+}-TPTZ) and ferric chloride.

Apparatus included a UV-VIS 1240 spectrophotometer, Ohaus analytical balance, pH meter (HI 2210), vortex, laboratory glassware, cabinet drier and rotary evaporator (Laborata 4000 tipe Heizbad HB digit, Heidolph, Germany).

Methods

Production of purple yam flour: The yam tubers were peeled, washed and cut into slices (3 cm thickness). The yam slices were steam-blanched (8 min), cooled to room temperature and dried in a cabinet drier (50°C, 10 h). The dried yam slices were ground with a blender and sieved through a 80 mesh sieve in order to obtain the yam flour.

Extraction of anthocyanin: The experiment was carried out by extracting anthocyanin from purple yam flour. Each 150 g portion of purple yam flour was extracted with 600 mL of methanol and ethanol that was acidified with 1% (v/v) HCl, 3% (v/v) citric acid[23] or 1% (v/v) tartaric acid[18].

Afterwards, the extraction was performed with the following combination of treatments: (1) Methanol+1% (v/v) HCl (MeH), (2) Methanol+3% (v/v) citric acid (MeS), (3) Methanol+1% (v/v) tartaric acid (MeT), (4) Ethanol+1% (v/v) HCl (EH), (5) Ethanol+3% (v/v) citric acid (ES) and (6) Ethanol+1% (v/v) tartaric acid (ET). The solution was further homogenized for 15 min at room temperature (30°C). After being homogenized, it was then stored at 4°C for 12 h. The resulting extract was filtered through Whatman filter paper No. 1 and evaporated at 50°C until no residual solvent remained. The resulting anthocyanin extract was kept at 4°C for further analysis.

Determination of total anthocyanin: Total anthocyanin concentration was determined and each of aliquot of 0.4 mL of extract was transferred into two different reaction tubes[24]. The extracts were mixed with 2.6 mL of 0.225 M potassium chloride buffer (pH 1) in the first tube and 2.6 mL of 0.4 M sodium acetic buffer (pH 4.5) in the second tube. The absorbance values from the two samples were measured in a spectrophotometer at 520 and 700 nm after 15 min. The absorbance value (A) was calculated using the following Eq:

$$A = (A_{520}-A_{700})\ pH1-(A_{520}-A_{700})\ pH4.5 \quad (1)$$

Total anthocyanin was calculated as cyanidin-3-glycoside (the most common anthocyanin in nature)[14], using a molar extinction coefficient (ε) of 26.900 L cm^{-1} and molecular weight (MW) of 449.2[25] according to the following Eq:

$$\text{Total anthocyanin } (\text{mg L}^{-1}) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L} \quad (2)$$

DF is the dilution factor (3 mL/0.4 mL) and L is the cell path length (1 cm).

Determination of antioxidant activity (DPPH method): Antioxidant activity was measured in order to determine the DPPH radical scavenging capacity of the extract[26]. Anthocyanin extract (0.2 mL) at a concentration of 1.000 ppm was added with 3.8 mL of 0.1 mM DPPH and then vortexed for 3 min. After 30 min of incubation, the absorbance was measured using a spectrophotometer at a wavelength of 517 nm[27]. The free radical scavenging activity was expressed as percentage radical scavenging activity (RSA), which indicated percentage DPPH inhibition:

$$RSA\,(\%) = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}} \quad (3)$$

Determination of antioxidant activity (FRAP method): The ferric reducing antioxidant power (FRAP) method was used to determine the antioxidant ability for reducing Fe^{3+} [28]. Ferric reducing antioxidant power (FRAP), depends on the reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe (II)-TPTZ) by a reluctant[29]. The FRAP reagent was prepared according to following steps: (1) 300 mM acetate buffer (pH 3.6) was added to 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ (ratio 10:1:1), (2) 3 mL of FRAP reagent was mixed with 100 mL of sample (at a concentration of 1.000 ppm) and 300 mL aquadest, (3) after mixing, the samples was vortexed for 1 min and left for 4 min and (4) The absorbance was then read at 593 nm. A FRAP

value was calculated using an Fe^{2+} calibration curve (4.3-137.5 mg L^{-1}) with $R^2 = 0.99$ and expressed as mg of ferro equivalent per gram of extract (mg ε ferro g^{-1} extract)[30].

Determination of total phenolic content: Total phenolic content was determined with the Folin-Ciocalteu method using gallic acid as a standard[31]. Anthocyanin extracts (50 µL each) were mixed thoroughly with 250 µL of Folin-Ciocalteu reagent. After 1 min, 750 mL of 20% $NaCO_3$ was added to the mixture and vortexed. Aquadest was added to the resulting mixture up to 5 mL and allowed to stand at room temperature for 5 min. After that period, the absorbance was then measured at 760 nm against a blank. The calibration curve was prepared using 31.88-510.00 mg gallic acid L^{-1} with $R^2 = 0.99$[32]. Total phenolic content was expressed as mg of gallic acid equivalents (GA) per g of weight extract[33].

Statistical analysis: The results were presented as mean and standard deviation and all of the analyses were performed in triplicate. All resulting data were tabulated and analyzed using one way analysis of variance (ANOVA). The mean differences were tested using Duncan's Multiple Range Test (DMRT) and $p<0.05$ was considered significant. Pearson's correlation coefficient analysis among anthocyanin content, total phenolics and antioxidant activities (DPPH and FRAP methods) were calculated using Microsoft Excel, 2007.

RESULTS AND DISCUSSION

Total anthocyanins: The total anthocyanins in the MeH (247.35 mg/100 g extract) and MeT (151.29 mg/100 g extract) extracts were higher than the other treatments (MeS, EH, ES and ET) (Fig. 1). Bridger *et al.*[6] reported that anthocyanins are polar molecules and consequently are more soluble in polar solvents. Hosseini *et al.*[34] reported that methanol is commonly used for phenol and anthocyanins extraction from fruits and vegetables. The acid type has an effect on the concentration of extracted anthocyanins. The use of HCl and tartaric acid yielded more anthocyanins than that of citric acid. Tartaric acid and HCl have larger dissociation constants than citric acid. Tartaric acid's dissociation constant is 9.04×10^{-4} and HCl is 1.00[35]. The higher the dissociation constant, the stronger the acid. More acidic conditions (pH closed to 1) would result in more antocyanin pigments in the form of flavylium cation or colored oxonium, so that the absorbance measurement showed a larger amount of antocyanin. However, more acidic conditions would cause more vacuolar cell wall lysis, so that it would yield more antocyanin pigments[18].

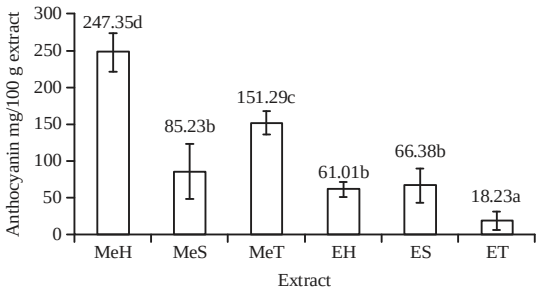

Fig. 1: Total anthocyanin extract from purple yam flour using different solvents

This result was consistent with a previous study reported by Naczk and Shahidi[36], in which anthocyanins were extracted from plant materials with an acidified organic solvent, most commonly methanol. Anthocyanin extraction from black sorghum with 1% HCl in methanol and 70% aqueous acetone, showed that the acidified methanol solvent was more effective for extraction than the acetone solvent[37]. Bridgers *et al.*[6] researched the effects of different solvents (70% ethanol, 70% acidified ethanol, 70% methanol and 70% acidified methanol) used to extract anthocyanins from purple-fleshed sweet potatoes. Extraction of anthocyanins from purple-fleshed sweet potatoes with acidified solvents resulted in 16-46% more anthocyanins than obtained by using non-acidified ethanol and methanol solvents.

The type of acid used for anthocyanin extraction affected the extract from purple yam flour. The different amounts of total anthocyanin extracts were expected as a result of difference in acid dissociation constants. The acid dissociation constant for HCl, tartaric and citric acids were 1.00, 9.20×10^{-4} and 7.45×10^{-4}, respectively[35]. The higher the dissociation constant, the stronger the acid. This occurred due to the higher quantity of hydrogen ions released into the solution. Tensika *et al.*[16] reported that a more acidic solution (pH is closer to 1) will cause anthocyanin pigments to exist in the colored flavylium or oxonium cation forms and higher absorbance measurements demonstrated higher quantities of anthocyanins.

Total phenolic content: As shown in Fig. 2, the phenolic components found in extracts after using MeH solvent (5.53 mg ε GA/100 g extract) and MeT solvent (4.83 GA/100 g extract) were higher than that found in extracts with other solvents (MeS, EH, ES and ET). The polar phenolic compound was easily dissolved in polar solvents[35], noting that methanol (relative polarity constant 0.76) was more efficient as an extraction solvent for antioxidant components than ethanol (relative polarity constant 0.65) and water (relative

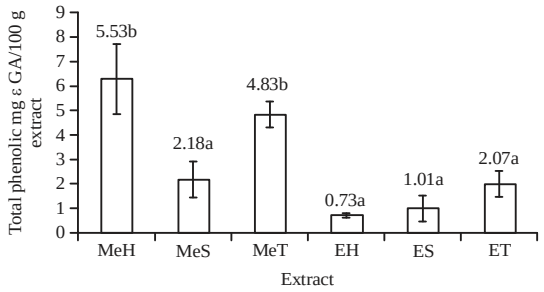

Fig. 2: Total phenolic content of anthocyanin extract from purple yam flour using different solvents

polarity constant 1.00). According to Boeing *et al.*[38], ethanol was less efficient for antioxidant compound extraction than methanol, even if their polarities were similar. This may be due to the low solvation provided by ethanol, probably because of the presence of the ethyl radical that is longer than the methyl radical in methanol, resulting in a lower solvation of antioxidant molecules. Antioxidants, including phenolics are found in various plant parts (such as fruits, leaves, tubers) and have been reported to have multiple biological effects including antioxidant activity[39]. According to a study on mulberry (*Morus atropurpurea* Roxb.), it was demonstrated that acidified methanol solvent was more capable of extracting phenolic compounds when compared with an acidified acetone solvent[40]. Another study on sweet potato varieties cultivated in Korea Park *et al.*[41], indicated that pure methanol was much better than pure ethanol for phenolic compound extraction. Phenolic compound extraction with 100% methanol was about 2.4 times higher than that with 100% ethanol[42]. A study with different solvents (hexane, chloroform, ethyl acetate and methanol) for phenolic compound extraction from vegetable residues[39], showed the methanolic extracts contained the highest total phenolic content.

Antioxidant activity of anthocyanin extract from purple yam flour: Several methods have been employed to evaluate *in vitro* antioxidant activities of different plant materials, of which FRAP, DPPH and oxygen radical antioxidant capacity (ORAC) activities are the most common. Those methods have different reaction mechanisms, so the results obtained depend on the method used. For this reason, it is recommended to use at least two methods to provide a reliable antioxidant capacity of the sample[38]. Purple yam's anthocyanin extract's antioxidant activity was measured by two different methods (1) DPPH and (2) FRAP. The DPPH method is based on free radical DPPH reduction in methanol in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form of DPPH-H, thus, DPPH turns pale yellow. On the other hand, the FRAP method is based on the ability of phenolic compounds to reduce a ferroin analog, the Fe^{3+} complex of tripyridyltriazine ($Fe[TPTZ]^{3+}$), to an intensive blue colored Fe^{2+} complex ($Fe[TPTZ]^{2+}$) under acidic conditions.

Table 1: Correlation among antioxidant activity (RSA%, FRAP), anthocyanin content and phenolic content

Antioxidant activity	Anthocyanin content	Phenolic content
RSA (%)	0.843**	0.779**
FRAP (μmol ε ferro L^{-1})	0.867**	0.900**

**Correlation is significant at 0.05 level

As seen in Fig. 3, antioxidant activities (such as RSA% and FRAP) of anthocyanin extracts treated with MeH and MeT were stronger compared to other treatments (MeS, EH, ES and ET). According to this study, the highest extract anthocyanins and phenolic content in purple yam flour was obtained from the MeH and MeT solvents. Anthocyanins and phenolic compounds are bioactive that determine antioxidant activity. Purple yams contain a variety of acylated anthocyanins that exhibit higher levels of antioxidant activity[10]. Phenolic compounds are usually responsible for the antioxidant properties of fruits and vegetables[38]. The findings from the present study are in agreement with the experimental results from extraction of phenolic compounds in berries[38]. That study explained that methanol was more efficient as a solvent for antioxidant extraction compared to ethanol, water and acetone. Studies in yams indicate high content of phenolic compounds associated with antioxidant activities[2,42]. Phenolic compounds are groups of chain-breaking antioxidants, scavenge free radicals and stop the propagation of free radical chain reactions. The antioxidant activity of phenolic phytochemicals occurs mainly a result of their redox potential, which results from mechanisms such as free radical scavenging activity, metal chelating activity and singlet oxygen quenching ability, thus, these compounds can protect cells against oxidative damage caused by reactive oxygen species[43]. A study concerning antioxidant activity in garlic found that the material with high phenolic content was in agreement with high antioxidant activity[44]. Tang *et al.*[45], reported that the FRAP values followed a similar trend as the DPPH values for sweet potato samples.

Correlation among anthocyanin content, RSA% and FRAP: As shown in Table 1. the antioxidant activities (RSA% and FRAP) from anthocyanin extracted from purple yam flour significantly and positively correlated with anthocyanin content and total phenolic compounds. It is indicated that antioxidant activity of anthocyanin extract from purple yam flour was determined among others by anthocyanin and total

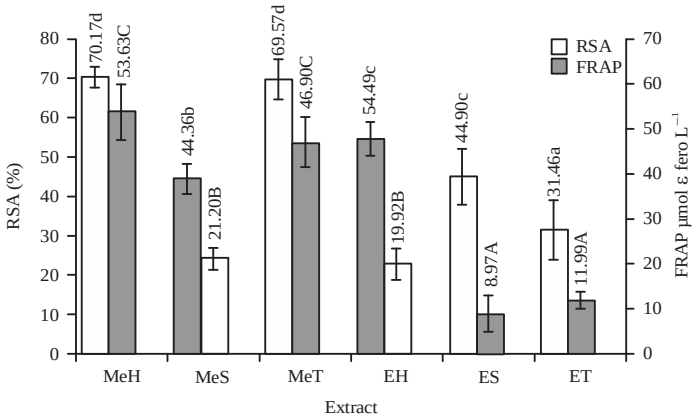

Fig. 3: Radical scavenger activity (RSA%) and ferric reducing antioxidant power (FRAP) of anthocyanin extract from purple yam flour using different solvents
Values are the mean of triplicates

phenolic contents. Several previous studies support our study. In a study by Awika *et al.*[37] concerning antioxidant properties in black sorghum, it was demonstrated that antioxidant activity (µmol TE g^{-1}) was positively correlated with anthocyanin content ($R^2 = 0.94$). According to a study by Siti Azima *et al.*[46], it was shown that *Garcinia mangostana* peel, *Syzigium cumini* and *Clitoria ternate* extracts showed positive correlations ($R^2 = 0.992$) between anthocyanin content and antioxidant activity (FRAP) in anthocyanin-containing extracts in addition to positive correlations with antioxidant activity (RSA%) of extracts with anthocyanin content ($R^2 = 0.859$). The antioxidant activity and total phenolic content in *Lantana camara* leaves presented a total phenolic content that had a positive correlation with antioxidant activity using DPPH method ($R^2 = 0.998$) and FRAP method ($R^2 = 0.949$)[28]. In a study of sweet potato storage roots by Hamouz *et al.*[20], it was recently reported that the relationship between anthocyanin content and radical-scavenging activity was positively correlated. The correlation coefficients between radical-scavenging activity (RSA) and anthocyanin content ranged from 0.606-0.687. Another study concerning correlations between antioxidant activity and anthocyanin in flesh-coloured potatoes ($R^2 = 0.659$) has been found[21]. Total phenolic contents in sweet potatoes were highly correlated with DPPH ($R^2 = 0.820$) values. Therefore, total phenolic content can serve as a useful indicator for sweet potato antioxidant activities[22]. According to the study about antioxidant activity of the sweet potato, *Ipomoea batatas* (L.) Lam., cultivars grown in Egypt[47], a high positive correlation ($R^2 = 0.988$) was found between antioxidant activity assayed by DPPH and total phenolic content of sweet potato.

CONCLUSION

The highest yield of anthocyanin extract from purple yam flour was obtained in cases in which MeH solvent (247 mg/100 g extract) was used for extraction. Total phenolic content was not significantly different between MeH and MeT extracts (5.18 mg GA/100 g extract). Antioxidant activities of anthocyanin MeH and MeT extracts were not significantly different (69.87% RSA and FRAP 50.27 µmol ε ferro L^{-1}). Anthocyanin and total phenolic contents were highly correlated with antioxidant activity (RSA% and FRAP).

SIGNIFICANCE STATEMENT

The study results show that purple yam flour contains anthocyanin and phenolic compounds were potent as natural antioxidants. This study discovered the type of solvent and acid for anthocyanin extraction from purple yam flour that could be beneficial for obtaining anthocyanin extracts with high antioxidant activity. This study provides a scientific information on the type of solvent(s) and acid(s) that are useful for extracting the anthocyanin purple yam flour with high antioxidant activity.

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