

Antioxidant and toxicity properties of anthocyanin extracted from red flower of four tropical shrubs

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Abstract. Kuspradini H, Rosiarto AM, Putri AS, Kusuma IW. 2016. Antioxidant and toxicity properties of anthocyanin extracted from red flower of four tropical shrubs. Nusantara Bioscience 8: 135-140. Some of flowers are colored by anthocyanins and make their own shade profile. Anthocyanins are natural colorants which have gained a growing interest due to their extensive range of colors and beneficial health effects. Anthocyanin content, antioxidant and toxicity activity of four red flowers (*Bougainvillea glabra*, *Jatropha integerrima*, *Melastoma malabathricum*, and *Mussaenda philippica*) has been evaluated in this study by pH-differential, DPPH and Brine Shrimp Lethality method, respectively. In this present work, 1% HCl-ethanol and ethanol solvent were used for the extraction. The extractive yield was varied in different extraction system, 5.80 to 13.30% in 1% HCl-ethanol and 6.29 to 8.81% in ethanol solvent. The IC₅₀ of antioxidant from different extraction systems were also varied, whereas the acidified ethanol extracts showed the highest scavenging activity than the ethanol extracts, 11.67 to 46.38 ppm and 79.08 to 133.35 ppm, respectively. The free radical scavenging action of 1% HCl-ethanol extracts of flower is in the order as *J. integerrima* > *M. malabathricum* > *M. philippica* > *B. glabra* while in the ethanol extracts are in the order as *M. malabathricum* > *J. integerrima* > *B. glabra* > *M. philippica*. In the group of 1% HCl-ethanol extracts, *Jatropha integerrima* Jacq was the richest in anthocyanin content (15.83 mg CGE/100 g DW) and showed the strongest antioxidant activity (11.67 ppm) and not toxic. Therefore, these can be regarded as a potential pigment source for food and natural product applications.

Keywords: Anthocyanin, antioxidant, flower, toxicity

INTRODUCTION

Secondary metabolites have been widely used as a dye, poison, flavors, medicines and others. One of secondary metabolites group is polyphenols. Polyphenols can be categorized into two main groups: the flavonoids and phenolic acids (Scalbert et al. 2005), with non-flavonoid polyphenols being less prevalent. Anthocyanins are compounds derived from the flavonoid compound. Anthocyanin is the pigment that plays an important role in the effects of color on most of the plants in the world. Besides its attractive color, anthocyanins are also beneficial to health, with potential beneficial physiological effects and have also been observed to possess potent antioxidative properties (Jonna et al. 2006). This sub-group of flavonoids is extremely important and is associated with the pigments found in plant tissues.

In this study, we have extracted anthocyanin pigments from several locally available red and purple flowers especially from one of the endemic plants in East Kalimantan. Karamunting (*Melastoma malabathricum* L.; Melastomataceae) is a shrub, the plant is one of the most common weeds that grow wildly and abundantly throughout the tropics, especially in the moist areas, and can be found in the Indian Ocean Islands, throughout South and South-East Asia, China, Taiwan, Australia, and the South Pacific Ocean (Wong 2008). Many parts of *M.*

malabathricum have been used in herbal remedies for the treatment of various human ailments. The Malay populations in Malaysia have used the leaves and shoots of *M. malabathricum* for the treatment of wounds, post-natal care, prevention of scars from smallpox infection, stomach ulcers, dysentery and diarrhea (Sulaiman et al. 2004). Batavia (*Jatropha integerrima* Jacq; Euphorbiaceae) is an erect ornamental shrub, native to West Indies that grows commonly in south parts of India (Krishnan and Paramathma 2009). Various parts of *J. integerrima* are traditionally used as purgative, styptic, emetic, in treatment of warts, tumors, rheumatism, herpes, pruritis, toothaches, scabies, eczema and ringworm (Kirtikar and Basu 2002). Bougainvillea (*Bougainvillea glabra* Choisy; Nyctaginaceae) is an ornamental flowering plant which having distinguishable medicinal properties. It has a thorny woody, smooth leaves, and which one that is great for produce brilliant color (El-Quesni 2007; Jawla 2012; Joshny 2012). Bougainvillea originated and popular in the tropical and warm climates areas such as South America, Southern California, Florida, and the Caribbean (Schoellhorn and Alfarez 2002). The parts of this plant used as antidiabetic, anti-inflammatory, antiallergenic, antithrombotic, antiviral, analgesic, antimycotic, and virostatic (Adebayo et al. 2009; Perales and Leysa 2012). The population of Mandsaur district in Madhya used this plant as traditional practitioner in variety of disorders like

diarrhea, reduce stomach acidity, cough and sore throat, blood vessels and leucorrhea (decoction of dried flowers), and hepatitis (decoction of the stem) (Sheeja et al. 2005). Nusa indah (*Mussaenda philippica* A. Rich; Rubiaceae) distributed in throughout India, South East Asia. It has greatly enlarged calyx leaf-like and small flowers which is tubular yellowish orange corolla. The plant used in traditionally as dysentery, jaundice, emollient and snake bites. The leaves and sepals of *M. philippica* may help to control epilepsy (Kar et al. 2014; McLaughlin and Garofalo 2004).

Anthocyanins are the principal water-soluble pigments responsible for the red, blue, and purple colors of terrestrial plants. Anthocyanins are omnipresent in our plant-based diet, have little or no known toxicity (Nabae, et al. 2008). In recent years, anthocyanins are becoming increasingly popular in the food research owing to their several beneficial health effects, such as antioxidant, anticancer, antidiabetic, antiatherogenic, and antimicrobial activities (Castaneda-Ovando et al. 2009; Lee and Choung 2011; Lee et al. 2011).

The aim of this study was to show the added value of several red and purple flowers as a good source of anthocyanin and antioxidant. We decided to compare the antioxidant and toxicity potential of selected flowers by the diphenyl-1-picrylhydrazyl (DPPH) and Brine Shrimp Lethality Test (BSLT) methods, respectively. Phenols, flavonoids, and anthocyanins content were also investigated.

MATERIALS AND METHODS

Extraction

Sample was collected at Education Forest of Universitas Mulawarman, Samarinda, East Kalimantan, Indonesia. The samples used in this research are *Bougainvillea glabra*, *Jatropha integerrima*, *Melastoma malabathricum*, and *Mussaenda philippica*. The flowers petals were extracted with 1% HCl-ethanol and ethanol 96% solvents. Liquid extract was concentrated by rotary evaporator.

Total phenol content

To determine total phenol content used Folin-Ciocalteu reagent using the method of John et al. (2014) with slight modification. 1 mg of extract was diluted with 10 mL DMSO. 200 µL of diluted extract mixed by 0.5 mL aquadest, 0.25 mL Folin-Ciocalteu reagent and 1.25 mL of 7.5% sodium carbonate (Na_2CO_3), incubated for 1 hour. Absorbance measured at 700 nm using Shimadzu UV-VIS 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Gallic acid used as standard to make calibration curve.

Total flavonoid content

To determine total flavonoid content used colorimetric assay using the method of John et al. (2014) with slight modification. 1 mg of extract was diluted with 10 mL DMSO. 200 µL of diluted extract mixed by 0.3 mL aquadest; 0.1 mL of 5% NaNO_2 ; 0.1 mL of 10% AlCl_3 , and

0.5 mL of 1 M NaOH. The solution was incubated for 10 minutes and absorbance measured at 480 nm using Shimadzu UV-VIS 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Catechin used as standard to make calibration curve.

Total anthocyanin content

To determine total anthocyanin content used pH differential method using the method of Sutharut and Sudarat (2012) with slight modification. 5 mg extract was diluted with 6 mL pH 1.0 buffer of KCl and pH 4.5 buffer of sodium acetate. The solution was incubated for 15 minutes and absorbance measured at 510 and 700 nm using Shimadzu UV-VIS 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Cyanidin-3-glucoside was used as equivalent data.

Antioxidant assay

To determine antioxidant activity used DPPH free radical scavenging method, using the method of Sahu et al. (2013) with slight modification. 33 µL of diluted extract was mixed by 467 µL and 500 µL of 27% DPPH. The final concentration of sample is 100, 50, 25, 12.5 ppm. The solution was incubated for 20 minutes and absorbance measured at 519 nm using Shimadzu UV-VIS 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Ascorbic acid was used as comparative control.

Toxicity assay

To determine toxicity activity used BSLT (brine shrimp lethality test) method, using the method of Mirzaei et al. (2013). Twenty mg of extract was diluted with 2 mL ethanol. 250, 125, and 50 µL diluted extract was evaporated at vial bottle. Put 10 nauplii (larvae) of *Artemia salina* at the sample, then add sea water until volume 5 mL. The plant extracts were tested at concentration 100, 250 and 500 ppm.

Brine shrimp eggs (*Artemia salina*) were hatched in artificial seawater. After 48 hours of incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette and used for the assay. Survivors were counted after 24 hours, and the percentages of lethality at each concentration were recorded according to Abbot's formula:

$$\%M = [(m_e - m_b) / (10 - m_b)] * 100$$

Where, m_e = dead shrimp in the sample and m_b = shrimp dead in the blank

LC_{50} values were obtained from the best-fit line plotted concentration versus percentage lethality.

Data analysis

The mean results of the percentage inhibition of antioxidant and brine shrimp mortality against the concentrations were plotted using the Microsoft Excel computer program, which also gives the regression equations. The regression equations were used to calculate IC_{50} and LC_{50} value.

RESULTS AND DISCUSSION

Extraction

In the present work, 1% HCl-ethanol and ethanol were used for the extraction. The extractive yield of all the plants is shown in Fig.1. The extractive yield was considerably more in 1% HCl-ethanol than in ethanol. The extractive yield in 1% HCl-ethanol ranged from 5.80 to 13.30% while in ethanol the range was from 6.29 to 8.81%. The minimum 1% HCl-ethanol extractive yield was in *M. malabathricum* while maximum 1% HCl-ethanol extractive yield was in *B. glabra* while the minimum ethanol extractive yield was in *M. philippica* while maximum yield was in *J. integerrima*.

The acidified ethanol solvents were more effective to extract *M. philippica* and *B. glabra* than the ethanol solvents. The 1% HCl-Ethanol giving in *M. philippica* and *B. glabra* yield of 8.78% and 13.30%, which is higher than those obtained using ethanol solvent. This suggests that anthocyanins of *M. philippica* and *B. glabra* flower can dissolve more easily in acidified ethanol solvent. In

contrast ethanol solvent was more effective to extract *M. malabathricum* and *J. integerrima* flowers in high yield (8.01% and 8.81% respectively).

Following the paradigm of solute dissolution of "like-dissolves-like", the use of ethanol in anthocyanins extraction might be related to the physical solvent property of liquid intermolecular cohesive pressure (Reichardt and Welton 2011). According to the pH of the medium, anthocyanins may change from intensely red or orange under acidic conditions (pH (pH < 2) due to the presence of eight conjugated double bonds carrying a positive charge (Horbowicz et al. 2008).

Total phenolic, total flavonoid, and total anthocyanin content

The results of the total phenolic content determination of the examined plant extracts, using Folin-Ciocalteu method, are presented in Table 2. The content of total phenols in extracts, expressed as gallic acid equivalents (GA) per 100 g of fresh weight sample, ranged between 841.85 to 4200 mg GA/g.

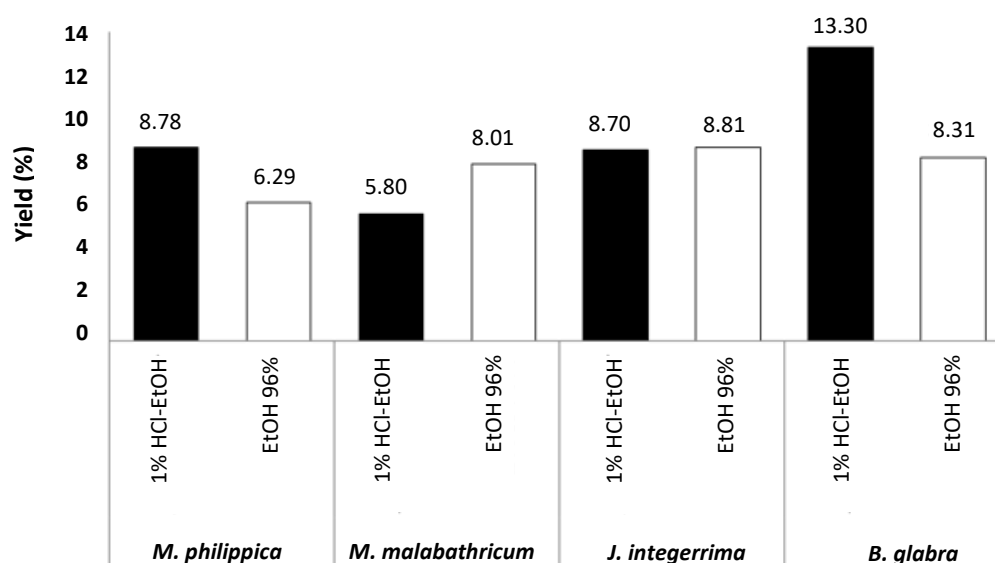


Figure 1. The yield of four different flower extract of four tropical shrubs

Table 2. Total phenolic, total flavonoid and total anthocyanin content of four flowers in different extracts of four tropical shrubs

Sample	Total phenolic mg GAE* / 100 g FW **		Total flavonoid mg CE * / 100 g FW**		Total anthocyanin mg CGE* / 100 g FW**	
	1% HCl-EtOH	EtOH	1% HCl-EtOH	EtOH	1% HCl-EtOH	EtOH
<i>M. philippica</i>	906.96	673.51	1,323.56	980.01	12.68	nd***
<i>M. malabathricum</i>	1,182.70	1,276.24	688.85	1,265.92	6.61	30.42
<i>J. integerrima</i>	2,609.70	4,200.93	2,130.63	1,040.66	15.83	102.38
<i>B. glabra</i>	1,659.58	841.85	2,410.05	1,162.69	11.70	0.15

Note: * GAE = Gallic Acid Equivalent, CE = Catechin Equivalent, CGE = Cyanidin-3-Glucosyde Equivalent, ** FW = Fresh Weight, *** nd = not detected

In relation to the solvent used, high concentrations of phenolic compounds were found in *J. integerrima* ethanol extract (4200.93 mg GA/100g) and 1% HCl-Ethanol extract of *M. philippica* (673.51 mg GA/100g) contained the lowest phenolic content. High concentrations of flavonoids compounds were found in *B. glabra* 1% HCl-Ethanol extract (2,410.05 mg CE/100g) and the lowest flavonoid content was obtained in 1% HCl-Ethanol extract of *M. malabathricum* (688.85 mg CE/100g). The higher anthocyanin content was revealed in ethanol extract of *J. integerrima* (102.38 mg CGE/100g), and the 1% HCl-Ethanol of *B. glabra* extract contained the lowest anthocyanin content (0.15 mg CGE/100g).

Antioxidant and toxicity

The results indicate the IC₅₀ value of 1% HCl-ethanol and ethanol flower extracts ranging from 11.67-161.68 ppm and 119.65-133.35 ppm, respectively. The free radical scavenging action of 1% HCl-ethanol extracts of flower are in the order as *J. integerrima* > *M. malabathricum* > *M. philippica* > *B. glabra*. The extracts, which showed the strongest DPPH radical scavenging activity, are *J. integerrima* and *M. malabathricum*, while the others show moderate antioxidant properties.

Brine Shrimp Lethality Test (BSLT) was used to prescreening bioactivity of extract which had toxicity and it was determined in LC₅₀ value. LC₅₀ value is a concentration which may kill 50% of the test subject. Following the procedure of Mirzaei et al. (2013), the lethality of the extracts to brine shrimp was determined on *Artemia salina* after 24 hours of exposure of the samples and comparing them relative to the positive control. The study reveals maximum mortality took place at a concentration of 10 µg/mL. The degree of lethality was found to be directly proportional to the concentration of the extracts. The LC₅₀ values of the plant extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentration of the extracts and the best-fit line was obtained from regression analysis by polynomial (Table 3).

Increased extract concentrations were associated with increased mortality rates. All extracts showed 0% mortality at 100 ppm, only ethanol extracts of *J. integerrima* and *B. glabra* showed mortality on 250 ppm and 500 ppm. *J. integerrima* and *B. glabra* extract demonstrated 46-66% and 36-86%. All the extracts in this study giving LC₅₀ values greater than 100 µg/mL (243.351 to more than 500 ppm).

Correlation of TPC, TFC, TAC and antioxidant in different group of extracts

Correlation between specific classes of compounds and antioxidant activity in different group of extracts were also investigated. Obtained results are presented in Table 4. Correlation coefficients were obtained from graphs against amount of appropriate class of polyphenol compounds in different extraction system.

Table 3. Inhibitory concentration of flower in different extract

Sample	Solvent	Antioxidant	Toxicity
		IC ₅₀ (ppm)	LC ₅₀ (ppm)
<i>M. philippica</i>	1% HCl-EtOH	44.74	nd (> 500)
	1% HCl-EtOH	119.65	nd (> 500)
<i>M. malabathricum</i>	EtOH	17.86	nd (> 500)
	1% HCl-EtOH	133.35	nd (> 500)
<i>J. integerrima</i>	EtOH	11.67	nd (> 500)
	1% HCl-EtOH	119.65	243.351
<i>B. glabra</i>	EtOH	46.38	nd (> 500)
	1% HCl-EtOH	121.17	325.157

Note: nd = not detected (the shrimps still alive until 500 ppm)

Table 4. Correlation of total phenolics, flavonoids, and anthocyanins with antioxidant activity of four different flowers

Polyphenolic compound	Correlation coefficients (r)	
	Group of extracts	
	1% HCl-EtOH	EtOH
Total phenolics	0.58	0.69
Total flavonoids	0.67	0.69
Total anthocyanin	0.95	0.79

Results showed that there is strong correlation between total anthocyanin ($r = 0.95$) content in 1% HCl-EtOH extract and antioxidant, while moderate correlation was observed with total phenolic ($r = 0.58$) and flavonoid ($r = 0.67$) in 1% HCl-EtOH extracts. The same trend was also observed for the group of ethanol extracts, strong correlation between total anthocyanin ($r = 0.79$) content and antioxidant, while moderate correlation was observed with total phenolic ($r = 0.69$) and flavonoid ($r = 0.69$).

Discussion

Various solvent systems have been used for the extraction of polyphenols from plant materials. Extraction yield is dependent on the solvent and the method of extraction. Furthermore, solvent polarity will play a key role in increasing phenolic solubility. Water and aqueous mixtures of ethanol, methanol, and acetone are commonly used in plant extraction (Mohday et al. 2010) Ethanol were used because of the good solubility of anthocyanins in polar solvents, according to principle of "like dissolve like". The acids were added to enhance its stability. The greatest yield of extracts from several flowers was obtained from ethanol 96% acidified with 1% HCl solvent (Figure 1). Extraction with solvent contained HCl resulted in highest yield value than that using ethanol 96%, except *M. malabathricum*. This can be explained that the use of HCl may cause pigment degradation during concentration, especially the occurrence of acid hydrolysis of labile acyl and sugar residues (Strack and Wray 1989).

Solubility of phenolics may vary from simple to complex structures, and is affected by the polarity of solvent (s) used. It is very difficult to develop a universal extraction procedure suitable for extraction of all plant phenolics. Therefore, the phenolic extracts from plant

materials are always a diversified mixture of plant phenolics soluble in the solvent system used. Solvents, such as methanol, ethanol, propanol, acetone, ethyl acetate, dimethylformamide, and their combinations have been used for the extraction of phenolics, often with different proportions of water (Luthria and Mukhopadhyay 2006; Zadernowski et al. 2005). Solvent, extraction time, solid-liquid ratio, mixing time and extraction temperature are the factors which have great impacts on the extraction yield. Extraction yield is dependent on the solvent and the method of extraction. Furthermore, solvent polarity will play a key role in increasing phenolic solubility. Water and aqueous mixtures of ethanol, methanol, and acetone are commonly used in plant extraction (Sun and Ho 2005).

Even the yield of total anthocyanin of *J. integririma* and *M. malabathricum* in ethanol extract were higher than acidified ethanol, we observed that the acidified ethanol extract of those flowers displayed potent biological properties, as antioxidant. *J. integririma* and *M. malabathricum* from acidified ethanol extracts showed the strongest activity with IC₅₀ value at 11.67 and 17.86 ppm, respectively. So, the anthocyanin obtained in acidified ethanol should be more specific than in the ethanol extract itself. Solvent extraction of anthocyanins was the first step in the determination of total and individual anthocyanins before quantification, purification, separation, and characterization of (Kong et al. 2003) and generally involves the use of acidified methanol or ethanol. The use of acid will stabilize anthocyanin in flavylium cation form, which is red at low pH (Rivas-Gonzalo 2003). Anthocyanins consist of hydrocarbon, which is water resistant, but they also have the polyphenol compounds that are highly soluble in water and polarized solvent. That's why the suitable solvent for anthocyanins extraction should be chosen from the organic solvent and then mixes with water, which is safe for human health and cheap (Thao et al. 2015).

Brine shrimp results presented in Table 3 show that all of the extracts in this study were exhibited very low toxicity and virtually non-toxic on the shrimps. The lower the LC₅₀ value would indicate high toxicity effect, whereas the higher LC₅₀ showed that the sample has low toxicity. They are giving LC₅₀ values more than 200 µg/mL. According to Mbawambo et al. (2007) and Moshi et al. (2010), the extract that has LC₅₀ value greater than 100µg/mL showed no significant toxicity against brine shrimp. This signified that all these extracts might not be toxic to human. Moshi et al. (2007) provide circumstantial evidence that plant extracts with LC₅₀ values below 20 µg/mL have a likelihood of yielding anticancer compounds.

Anthocyanins have great potential in food and pharmaceutical industries because they act as antioxidants by donating hydrogen to highly reactive radicals (Lapornik et al. 2005). They can increase the protection of the body against diseases such as cancer and they are in the class of antitumor compounds in medical science. The mechanism for enhancing body protection by anthocyanins lies in the fact that they scavenge free radicals in the body and contribute to reduce the oxidative stress (Silva et al. 2007).

Moderate to strong linear relationship between responses of TPC, TFC, and TAC versus their antioxidant activities of flower extracts in this study was assessed, which suggested that anthocyanin compounds in flower extracts contributed significantly to their antioxidant potential. There was a good relationship between total anthocyanin content and total antioxidant activity in extracts of different flowers, indicating anthocyanin may be the major contributor to the total antioxidant activities of red and purple flowers in this study. Several data in the literature on the relationship between the polyphenol content of plants and their antioxidant activity are sometimes contradictory. Some authors have observed such a high correlation between the polyphenol content of plants and their antioxidant activity (Vasco et al. 2008) others found no such correlation exists or only a very weak one (Souri et al. 2008).

It can draw a conclusion that total anthocyanin content is responsible for the antioxidant activity. A high correlation was demonstrated between total anthocyanins content and antioxidant capacities. Polyphenols are a class of antioxidant compounds that are present in many fruits, vegetables and plant-derived beverages, and products such as tea, coffee, chocolate, fruit juice and red wine. Phenolic compounds result from secondary metabolism in fruits and vegetables. They are produced as a defense barrier against seed dispersal, microorganisms, and UV radiation. These compounds are natural pigments and can be used to add natural flavors to foods and vegetables (Pandey and Rizvi 2009). The extract showed good antioxidant activity thus makes it suitable as a promising pigment source for food applications.

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