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Anthocyanin Content and Antioxidant Activity of Red Chrysanthemum (*Chrysanthemum morifolium* Ramat.) at Different Flower Ages

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ABSTRACT

Chrysanthemum sp. is a floricultural plant of the Asteraceae family with high economic value. The anthocyanin pigment in red chrysanthemum acts as an antioxidant, the content of which can be influenced by genetic factors such as the physiological age of the flower. This study aimed to determine the effect of flower age on anthocyanin content and antioxidant activity in red chrysanthemum plants. The age of red chrysanthemum used was 115 Days After Planting (DAP) (early bloom stage), 120 DAP (half-bloom stage), 125 DAP (blooming stage), and 134 DAP (wilted flower). The anthocyanin content was analysed using spectrophotometric methods. Antioxidant activity was determined by the DPPH method and then the absorbance was measured using a UV-Vis spectrophotometer. The results showed that the highest anthocyanin content was obtained at the age of 134 DAP at 3.56 mg/g, followed by the age of 115 DAP at 2.40 mg/g, then at 125 DAP at 1.95 mg/g and the lowest at 120 DAP at 1.69 mg/g. The highest antioxidant activity was shown in chrysanthemum flowers aged 115 DAP which had an IC_{50} value of 288.85 g/ml. The research shows that wilted chrysanthemum flowers still contain anthocyanins so it can be used in various industrial fields such as chrysanthemum tea and additives for soap.

Keywords: Anthocyanin, antioxidant, flower age, red chrysanthemum

INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is in the Asteraceae family which has market prospects and high economic value. Chrysanthemum plants are widely used as cut flowers and pot flowers. Potted chrysanthemums usually use plants that have an ideal height for planting in pots, which is around 24 – 35 cm (Kurnia, 2017). The use of chrysanthemum flowers still needs to be explored by considering the content of secondary metabolites.

The chrysanthemum flower is also used because of the presence of secondary metabolites in it, including pigment content. Secondary metabolites in chrysanthemum flower parts have properties such as antipyretic, antibiotic, anti-inflammatory and natural pesticides (Kim *et al*., 2018). Plant secondary metabolites are influenced by endogenous factors, including physiological age. Red chrysanthemum (*C. morifolium*) has a high anthocyanin pigment and acts as an antioxidant. Anthocyanins are organic chemical compounds that give the effect of orange, red, purple, blue to black, which are found in parts of flowers, leaves and stems (Du *et al*., 2015; Batubara *et al*., 2016). Anthocyanins are anthocyanidins which bind to 3-glucosides, whereas in the aglycone state anthocyanins are anthocyanidins. Anthocyanidins bind to monosaccharides (glucose, galactose, pentose and ramnose) to form ester bonds, forming anthocyanins (Lee *et al*., 2017).

Anthocyanin biosynthesis is regulated by three proteins including R2R3MYB transcription factors (TFs), basic helix loop helix (bHLH) TFs and WD40 Repeat Proteins (WDR) that form ternary MBW complexes and regulate anthocyanin biosynthetic gene expression (Yue *et al*., 2018). Anthocyanin biosynthesis is strongly

influenced by various physical and chemical factors. Various physical factors, such as temperature, light intensity and quality, moisture content, nutrients, and minerals have been identified as having a major influence on the accumulation of anthocyanin content in flowers. Chemical factors in the form of environmental pH affect the intensity of colour and pigmentation of flowers (Mekapogu *et al*., 2020).

Antioxidants in chrysanthemum plants can be found in the leaves and flowers. The basic property of an antioxidant is that it helps to limit oxidative damage in the human body by preventing or detecting a chain of oxidative propagation by stabilising the produced radical. Substances with antioxidant capacity can act through multiple mechanisms such as hydrogen atom transfer (HAT), single electron transfer (SET) or the ability to chelate transition metals (Santos-Sánchez *et al*., 2019).

Internal factors affecting the content of anthocyanin pigments in chrysanthemum flowers can be analysed based on the stage of flower development. According to Gantait and Pal (2010), the highest anthocyanin content in several chrysanthemum cultivars was obtained at the age of 15 days after opening the flowers. Most of the anthocyanins have a relatively high content in the early flowering and bud stages. At the bud stage, anthocyanins accumulate and are gradually degraded, accompanied by fading flower colour. The aim of the research is to determine the effect of flower age on anthocyanin content and antioxidant activity in red chrysanthemum plants. The research is expected to be useful in providing information regarding the harvesting age of chrysanthemums that produce the highest anthocyanin content. This also supports the development of potential utilisation of chrysanthemum flower waste as a source of antioxidants, based on their antioxidant activity.

MATERIALS AND METHODS

The research was done from April 2022 to July 2022 in the Laboratory of Plant Structure and Function Biology, Department of Biology, Faculty of Science and Mathematics, Diponegoro University. Samples of flowers were obtained from Chrysanthemum Village, Bandungan, Semarang. The criteria for the chrysanthemum sample as shown in Figure 1 were: early blooming with a diameter of ± 2.5 cm (115 days after planting/ 115 DAP), half blooming flowers with a diameter of ± 4.5 cm (120 days after planting/ 120 DAP), blooming flowers with diameter ± 6.5 cm (125 days after planting/ 125 DAP), wilted flowers (134 days after planting/ 134 DAP). Flower diameter and fresh weight were measured. The petal of the chrysanthemum flower was separated from the other parts and then cleaned of adhering dirt.

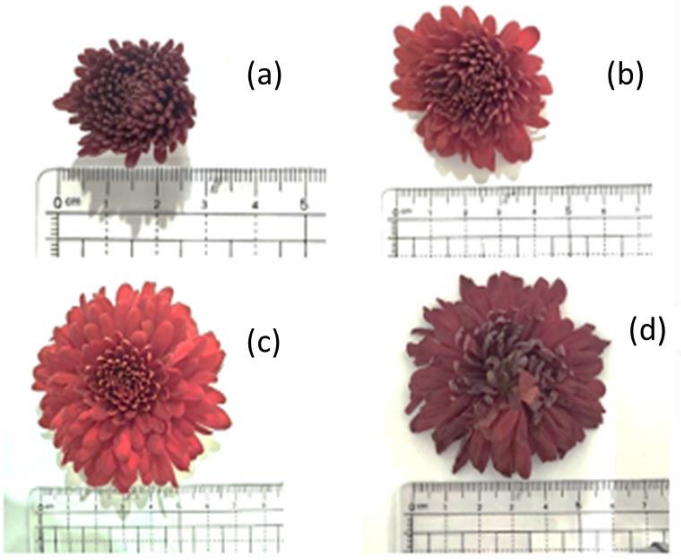

Figure 1. Stages of chrysanthemum flowering. (a) Early blooming stage (115 DAP), (b) Half blooming stage (120 DAP), (c) Blooming flowers (125 DAP) and (d) Wilted flower (134 DAP); DAP – Days after planting

Total Anthocyanin Content

Samples were extracted by grinding fresh chrysanthemum flowers using a mortar and pestle. A total of 1.5 g of the sample was weighed and then immersed in 5% HCl in distilled water with a ratio of 1:10 in a dark glass bottle. The mixture was stored in the refrigerator at 4 °C for 24 hours. After 24 hours the mixture was filtered using filter paper.

Total anthocyanin was determined by spectrophotometric method with a modified method from Hasidah *et al*. (2017). Identification of total anthocyanin (mg/g) was carried out by taking 10 ml of the extracted filtrate and then diluting it to 20 ml with 96% ethanol: 1.5 N HCl with a ratio of 85:15. Then the mixture was homogenised and its absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 535 nm. The total anthocyanin content was calculated using the Lees and Francis (1972) formula:

$$\text{Anthocyanin (mg/g)} = \frac{(absorbance\ x\ DF)}{98.2\ x\ w\ (g)} \text{ x } 100$$

where DF = Dilution factor; 98.2 = ε value value (molar absorption of anthocyanin pigment in 96% ethanol:HCl 1.5 N (85:15), which refers to the absorbance of anthocyanin in a wavelength of 535 nm; W = weight of sample (1.5 g)

Antioxidant Activity Test

Examination of antioxidant activity was carried out using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method with modifications (Molyneux, 2004). For extraction, 20 g of fresh flower samples were crushed using a mortar and pestle then soaked in 100 ml of 96% ethanol for 24 h. 0.4 mM DPPH reagent was prepared by dissolving 15.7 mg of DPPH powder in 100 ml of 96% ethanol. Chrysanthemum flower extract stock solution of 1000 ppm was prepared by 10 ml of chrysanthemum extract added with 10 ml of 96% ethanol. Absorption measurement of DPPH blank by using 0.4 mM DPPH solution was taken with a pipette of 2 ml, then add 2 ml of 96% ethanol. This solution was homogenised and left for 30 min in the dark, then the absorption was measured with a UV-Vis spectrophotometer at a wavelength of 515 nm.

Measurement of antioxidant activity of chrysanthemum flower extract by using 2 ml of ethanol extract of chrysanthemum with several concentration variations, 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml were added to 2 ml of 0.4 mM DPPH. The mixture was homogenised and left for 30 min in the dark, then the absorption was measured with a UV-Vis spectrophotometer at a wavelength of 515 nm. Antioxidant activity was calculated using the Molyneux formula (2004) as follows:

$$\text{\% Inhibition} = \frac{Abs\ blanko - Abs\ sampel}{Abs\ blank}\ x\ 100\%$$

Antioxidant activity was expressed by the IC_{50} value. To determine the IC_{50} value, a relationship curve was made between % inhibition and extract concentration to obtain a linear regression equation: y = ax+b.

Data Analysis

The data obtained were then analysed using Completely Randomized Design (CRD) and Analysis of Variance (ANOVA) at the 95% confidence level to determine the effect of the treatment. If there was a significant difference, a further test was carried out using Duncan's Multiple Range Test (DMRT) to determine the most optimum effect.

RESULTS AND DISCUSSION

Anthocyanin Content

The anthocyanin content in red chrysanthemum flowers is shown in Table 1. The anthocyanin content of 1.5 g of chrysanthemum extract at flower age 115 DAP was 2.40 mg/g, flower age 120 DAP was 1.69 mg/g, flower age 125 DAP of 1.95 mg/g and the age of interest 134 DAP of 3.56 mg/g.

Table 1. Anthocyanin content of red chrysanthemum flowers at different flower ages

Flower ages (DAP)	Anthocyanin (mg/g)
115	2.40[b]
120	1.69[d]
125	1.95[c]
134	3.56[a]

Note: Numbers followed by different letters show significantly different results based on Duncan's test with a 95% confidence level.

Flower ages of 115 DAP (early bloom), 120 DAP (half bloom), and 125 DAP (perfect bloom) are chrysanthemum flowers that are still in the growth phase. As the age of the flower increases, the anthocyanin content decreases. These results are in accordance with several previous studies that the highest anthocyanin content was in the early stages of interest and then further degraded as the flowering stage occurred in *Brunfelsia calycina* (Luo *et al*., 2017) and three *Rosa rugosa* hybrid cultivars (*R. rugosa* "Zizhi", *R. rugosa* "Fenzizhi" and *R. rugosa* "Baizizhi") (Zhao *et al*., 2019). Anthocyanins are already produced at the bud stage, then decrease and are not detected at the half-opening stage (Zhao *et al*., 2019).

Table 1 shows that the anthocyanin content decreased at the age of 120 DAP by 1.69 mg/g. The decrease in anthocyanin content is thought to be due to a degradation process that exceeds its biosynthesis. This is in accordance with the opinion of Povero *et al*. (2011) and Liu *et al*. (2018), which stated that the anthocyanin content depends on the balance between biosynthesis and degradation. The decrease in anthocyanin is caused by a downregulation of anthocyanin biosynthesis which is triggered by a decrease in the expression of anthocyanin activator, namely R2R3 MYB TFs. This decrease in expression can be triggered by enzyme activity and light. According to Riaz *et al*. (2016), the enzymes that most degrade anthocyanins are called anthocyanases, some of the enzymes that play a role are glycosidases, peroxidases, and phenolases. Marszalek *et al*. (2017) added that these degradation enzymes can be produced by plants or in plant tissues as a response to environmental stress. Research by Vaknin *et al*. (2005), one of the things that is suspected as a trigger for the degradation of anthocyanins is due to the presence of peroxidase enzymes present in the vacuoles, which allows oxidation and reduces the anthocyanin content. According to Movahed *et al*. (2016) overexpressed VviPrx31, encoding a grapevine class III peroxidase, in petunia and caused anthocyanin reduction in petunia petals under heat stress, indicating active anthocyanin degradation. It indicated that VviPrx31 is responsible for anthocyanin degradation at high temperature.

At 125 DAP, the anthocyanin content increased compared to 120 DAP but was still lower than 115 DAP. Light is another important parameter in anthocyanin stability. Light can stimulate the synthesis and accumulation of anthocyanins. In this case, light can increase the rate of anthocyanin biosynthesis in perfect blooming flowers. The regulation of structural genes of anthocyanins is induced by the same MBW complex that commonly includes two imperfect myeloblastosis protein repeats (R2R3MYB) transcription factors, basic helix-loop-helix (bHLH), and WD40 proteins (Petroni & Tonelli, 2011; Zhang *et al*., 2019). According to Albert *et al*. (2009), high light can induce the MYB transcription factor which determines the biosynthesis of anthocyanins, especially the cyanidin type. MYB transcription factors (MYB TFs) could positively or negatively regulate anthocyanin biosynthesis. MYB TFs regulate the signalling pathways by MYB TFs during anthocyanin biosynthesis including jasmonic acid (JA) signalling pathway, cytokinins (CKs) signaling pathway, temperature-induced, light signal, 26S proteasome pathway, NAC TFs, and bHLH TFs (Li *et al*., 2022). This is supported by the opinion of Aramwit *et al.* (2010) that, the longer the exposure of the extract to light, the more anthocyanin content and its antioxidant activity will decrease. Therefore, the anthocyanin content in the blooming flower (125 DAP) was not as high as that of the initial flower blooming (115 DAP).

Flowers aged 134 DAP (withered flowers) had the highest amount of anthocyanin and accumulated in wilted flowers (Table 1). This is because the 134 DAP flowers have dark red flowers (Figure 1). According to Chen *et al*. (2012), anthocyanin pigments are water soluble and localised in various organs, including leaves,

flowers and fruit. The pink to red purple colour of the chrysanthemum petals indicates the presence of anthocyanin, while the lighter colour of the petals which is not as intense indicates a small accumulation of anthocyanin. Anthocyanin total increased as the colour of the flowers changed from white to blue to pink to purple to red-purple. The cyanidin derivatives accounted for the highest proportion, and the pelargonidin derivatives accounts for the lowest proportion of total anthocyanins (Chen *et al*., 2023).

Antioxidant Activity

Based on the results of the IC_{50} values (Table 2), it can be seen that at all ages the chrysanthemum flowers have IC_{50} values ranging from 288-1817. The flower age of 115 DAP has an IC_{50} value of 288.85 µg/ml; the IC_{50} value at 120 DAP was 1817.46 µg/ml; the IC_{50} value at 125 DAP was 551.00 µg/ml; the IC_{50} value at 134 DAP was 1174.97 µg/ml. According to Molyneux (2004), an IC_{50} value between 200 – 1000 µg/ml means that the substance is less active but can still be used as an antioxidant. This low antioxidant activity is suspected because the anthocyanin content is less than optimal compared to other flowers such as roses. Research by Zhao *et al.* (2019) found that the anthocyanin content of roses (*Rosa rugosa*) can reach 4.30 mg/g. Research reported that the anthocyanin content of the red chrysanthemum variety 'Tata red' was 0.19 mg/g (Gantait & Pal, 2010), three chrysanthemum cultivars with different flower colours indicated that anthocyanin contents varied among them. 'Z1', with deep red rayflorets, accumulated 2.83 mg/gFW compared with 0.08 mg/gFW in 'Z2' which had pink flower colours, and 'Z3' which had yellow flowers that no anthocyanin was by detected (Xiang *et al*., 2015). This suggested that the difference in anthocyanin contents was the main reason for the different colours of ray florets of chrysanthemum flower cultivars. Based on these results, it can be seen that the anthocyanin content of each species is different.

The IC_{50} value of red chrysanthemum flowers showed results consistent with the anthocyanin content, except for the age of the flowers at 134 DAP (Table 2). The antioxidant activity of anthocyanins is due to changes in the expression of several genes related to anthocyanin biosynthesis during the flower growth phase. According to a study by Liu *et al*. (2022), the transcription of each gene encoding anthocyanin biosynthesis varies at the age of the flower, such as the CmCHS and CmCHI genes showing the highest transcription levels during early and full bloom, while the least is transcribed in half-opened flowers.

Table 2. Antioxidant activity at various ages of red chrysanthemum flowers

Flower age (DAP)	Regression equation	IC_{50} (µg/ml)
115	y = -0.2027x + 108.55 R^2 = 0.9979	288.85
120	y = -0.0272x + 99.435 R^2 = 0.9722	1817.46
125	y = -0.1039x + 107.25 R^2 = 0.9849	551.00
134	y = -0.0277x + 99.139 R^2 = 0.9844	1174.97

Flower age of 115 DAP (early blooming flower) has the highest antioxidant activity with an IC50 value of 288.85. The antioxidant activity of chrysanthemum flowers is due to the high anthocyanin content of 2.40 mg/g (Table 1) and is supported by the content of other chemical compounds besides anthocyanins. Compounds such as saponins, steroids, flavonoids, tannins, terpenoids, alkaloids (Yulianti *et al*., 2019), polyphenols and lignin (Han *et al*., 2017), quercetin3-Oglucoside, diosmetin7-O-glucuronide Delphinidin 3-O-β-Dglucoside- 5-O-(6-Omalonylβ-Dglucoside) (Lin & Harnly, 2010) were present and synthesised in early blooming chrysanthemum flowers. Wilted flowers (134 DAP) were perfectly blooming flowers which were dried in the air for nine days until wilted, had the highest anthocyanin content but low antioxidant activity (Table 2). This might happen because the wilted flowers have entered the postharvest stage and are not supported by the presence of other secondary metabolites to increase their antioxidant activity. This is in line with the opinion of Yun *et al*. (2012), which mentioned that postharvest storage has an unavoidable and mostly negative effect on biological processes. The resulting effects include degradation of proteins, lipids and nucleic acids and cell dysfunction, disintegration and death.

Previous research has shown that besides anthocyanins, the essential oils present in chrysanthemum flowers also have antioxidant activity. This is in accordance with Kim *et al*. (2018), the DPPH radical scavenging activity of chrysanthemum essential oil at a concentration of 10 mg/ml was higher in the full flowering phase group (59.7 – 0.9%) compared to the vegetative phase group (44.1 – 2.0%) and the preflowering phase group (44.8 – 1.1%). According to Youssef *et al*. (2020), the essential oil obtainned from *C. morifolium* flowers reveals considerable antioxidant potential with an IC_{50} value in the DPPH test, 1.90 and 2.89 mg/ml. Antioxidant activity can be interpreted by the synergistic interactions between all essential oil components in addition to their action as pro-oxidants.

Fresh Weight and Flower Diameter

The fresh weight of the flower is the weight when the flower is still alive and is weighed immediately after being harvested. Based on the results of further tests on the fresh weight and diameter of the red chrysanthemum flowers at different flower ages (Table 3), the fresh weight of the flowers at 115 DAP was 1.12 g; age 120 DAP of 1.46 g; age 125 DAP of 2.84 g; and the age of 134 DAP of 1.82 g. Age of 125 DAP showed the highest flower fresh weight of 2.84 g, presumably because of the high water content. According to Ramdani (2018), wet weight shows the amount of water content contained in plant tissues or organs whose levels are influenced by environmental factors such as temperature and humidity. In addition to high water content, photosynthate content also affects fresh weight.

Table 3. Fresh weight and flower diameter at different flower ages

Flower age (HST)	Parameter	
	Fresh weight (g)	Flower diameter (cm)
115	1.12^{d}	2.52^{s}
120	1.46^{c}	4.86^{r}
125	2.84^{a}	6.62^{p}
134	1.82^{b}	6.1^{q}

Note: Numbers followed by different letters show significantly different results based on Duncan's test with a 95% confidence level.

Flower diameter is a generative plant growth which is characterised by an increase in flower size (Table 3). Diameter at all ages were significantly different flowers. The largest flower diameter is found in blooming flowers, which is 6.62 cm. The increase in the physiological age of the plant is followed by the increase in the growth rate of the plant which has an impact on the increase in the fresh weight of the plant and the diameter of the chrysanthemum flower. According to Fu *et al*. (2012), sunlight absorbed by chlorophyll is processed by plants for photosynthesis. This is also in accordance with Amthor (2010), to carry out photosynthesis plants need nutrients, water and sunlight. The process of photosynthesis produces photosynthates which will be passed on by the transport network to all parts of the plant including flowers thereby increasing the diameter of the flowers. The amount of photosynthate produced from the photosynthesis process will be converted into biomass. A large amount of biomass will produce a high fresh weight of chrysanthemum flowers (Zhu *et al*., 2010).

Chrysanthemum flowers aged 134 HST (wilted flowers) experienced a decrease in fresh weight and flower diameter due to water content in the flowers. According to Maguire *et al*. (2000), transpiration and respiration are the physiological processes that most influence postharvest weight loss in plants. This is supported by the opinion of Xanthopoulos *et al*. (2017) that, there are three main ways in which the process of aerobic respiration can contribute to the loss of overall plant weight: carbon dioxide gas, water vapour during transpiration and respiratory energy for more evaporation of the water.

This study was intended to determine the anthocyanin content and antioxidant activity at each age of the chrysanthemum flower so that the right harvest time could be determined by looking at the highest content. The research shows that wilted chrysanthemum flowers still contain anthocyanins so that they can be used as a source of nutrition or as a natural stain. Research by Santosa *et al*. (2019) resulted in wilted chrysanthemum flowers or chrysanthemum waste that can be used to make nutritious food preparations such as chrysanthemum tea, chrysanthemum chips, and chrysanthemum

candies. According to Nurcahya *et al*. (2021), essential oil from wilted and unfit for sale chrysanthemum flowers can be used as an additive in making bath soap and also as a natural colouring agent. Knowledge of the metabolite content of chrysanthemum can expand its use in various industrial fields.

CONCLUSION

Based on the research that has been done, it can be concluded that the age of the flowers reduces the anthocyanin content and antioxidant activity of red chrysanthemum. Red chrysanthemum flowers aged 134 DAP or wilted flowers had the highest anthocyanin content of 3.56 mg/g. Red chrysanthemum flowers aged 115 DAP or early blooming flowers had the highest antioxidant activity of 288.85 μg/ml. The anthocyanin content of chrysanthemums is still quite low, so it is necessary to optimise cultivation to increase anthocyanin content.

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