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The Content of Chlorophyll, and Antioxidant Activity of Malabar plum (*Syzygium jambos*) Leaves at Different Developmental Stages

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Abstract

Malabar plum [*Syzygium jambos* (L.) Alston.] is a tropical plant which is used as a medicinal plant, because it contains secondary metabolites, especially in the leaves. The different leaves developmental stages can affect physiological changes, especially metabolic processes, so it is suspected to affect the antioxidant content and activity. The objective of this research was to study the difference of leaves morphology, chlorophyll contents, antioxidant contents, and activity at the different leaves developmental stages. Samples were taken from Kaliboto Village, Purworejo, Central Java. The leaf color measurement was using colorimetry; determination of chlorophyll, carotenoids, ascorbic acid, and total phenol content is was using spectrophotometry; and antioxidant activity was using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. Qualitative data were analyzed descriptively, while quantitative data were analyzed by ANOVA tests. The results showed that the higher level of leaves development, the higher pigment content, total phenol, and antioxidant activity ascorbic acid content in the mature leaves is lower when compared to the young and old leaves. The results of this research provide the information that can support the use of Malabar plum leaves in traditional medicinal activity and pharmaceutical industry, as well as basic information for plant breeding.

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INTRODUCTION

Antioxidants are compounds that have a function to neutralize free radicals (Winarsi, 2007). Natural antioxidants are obtained from green plants because it contains many secondary metabolites that potential as antioxidants (Sayuti & Yenrina, 2015). Some of them are good consumed fresh, such as tomato papaya (Li et al., 2015), and wild garlic *Allium ursinum* (Sahnoun et al., 2017) and the others have to be processed first, such as tomato (Iswari & Susanti, 2016), Pine (*Pinus merkusii*, *Pinus oocarpa*, *Pinus insularis*) and Agathis (*Agathis loranthifolia*) (Tillah et al., 2017), Obat Pahit (*Bauhinia semibifida*, *Cnestis palala* and Penawa Root) (Fitmawati et al., 2017).

Syzygium jambos belongs to the Myrtaceae family. It is a typical tropical plant which contains many secondary metabolite compounds (Mohanty & Cock, 2010), such as ascorbic acid, phenols, caffeine, kaempferol, gallic acid, rutin, chlorogenic acid, quercetin, and flavonoids (Bonfanti et al., 2013).

Plants developments are influenced by external factors such as soil, moisture, light, water, and internal factors such as genes, hormones, the anatomical structure of organs, morphology of organs and metabolite content (Sumenda et al., 2011). The leaves are plant organs that play a significant role. The leaves undergo morphological and physiological changes as development progresses. Based on the morphology of leaves, development is divided into three stages, i.e., juvenile stage (young stage), the early adult stage (mature stage), and the late adult stage (old stage) (Tsukaya, 2002; Thomas, 2017).

Metabolic processes in the different developmental stages of a leaf can be affected by the changes of morphological and environmental characteristics, so that, the content of both primary and secondary metabolic compounds will differ at the different stages of development (Fleming, 2005; Sayuti & Yenrina, 2015). The defense mechanism in plants requires a complex process consisting of non-enzymatic and enzymatic components. The antioxidant components play a role in scavenging Reactive Oxygen Species (ROS). Under normal conditions, ROS is synthesized in small quantities and acts as a secondary messenger of intracellular signals which mediates the response of plants to environmental conditions, but under unfavorable environmental conditions, the production of ROS will increase. It will lead to oxidative damage of lipids, proteins, and nucleic acids. Plants can overcome oxidative damage by increasing endogenous antioxidant defenses (Sharma et al., 2012). Soybean [*Glycine max* (L.) Merr.] cultivar Grobogan produces phenylalanine, ammonia-lyase activity, and phenolic acid composition when exposed to the multiple stress of purple nutsedge (*Cyperus rotundus* L.) interference and drought (Darmanti et al., 2018). A standard method used to determine antioxidant activity is by using DPPH (1,1 diphenyl 2 picrylhydrazyl), with the principle of determining the IC50 value that indicating the 50% free radical inhibition by the sample being tested (Dehpour et al., 2009).

Several previous kinds of research on Malabar plum by Islam et al. (2012), Bonfanti et al. (2013), and Dhanabalan et al. (2014) were about the content and antioxidant activity of *S. jambos* leaves, but it was not describing the leaves criteria used. So, it is necessary to study the differences of different development leaves stages on antioxidant content and activity. The purposes of our research were to examine morphology, chlorophyll content, antioxidant content (i.e. ascorbic acid and total phenol), and antioxidant activity of *S. jambos* leaves at different developmental stages. The results of this research were expected to add information, support the use and further research of *S. jambos* in traditional medicinal activities, the pharmaceutical industry, and for plant breeding and conservation.

METHODS

The sample used in this research was *S. jambos* leaves taken from Kaliboto Village, Bener Sub-District, Purworejo District, Central Java. Plant life was around 3-5 years. Based on the segment arrangement from the top petiole, leaves in segment 1-3 were assumed to be the young leaves, leaves in segment 4-6 were assumed to be the mature leaves (early adult stage), while leaves in segment 7 and after they were assumed to be the old leaves (the late adult phase). This research used Completely Randomized Design (CRD) one factor with the leaves developmental stages, i.e., young leaves, mature leaves, and old leaves.

Determination of S. jambos leaf sample

The selection of Malabar plum leaves was based on different developmental level, i.e. young, mature, and old leaves. The leaf color measurement was using a digital colorimeter by Xenon lights to determined coordinate values of L*, hunter-*a**, and hunter-*b** (Suyatma, 2009).

Determination of chlorophyll and carotenoids content

The method used in this study was following Hendry and Grime (1993) research: 0.1g of fresh leaves sample was crushed in a mortar and added with acetone 80% as much as 10ml. After it was filtered, then the filtrate was measured for its absorbance at λ: 645, 663, and 480nm. The calculation of chlorophyll and carotenoid levels are were as follows:

Chlorophyll a mg/g = ((12.7 x A663) – (2.69 x A645)) x 10-1

Chlorophyll b mg/g = ((22.9 x A645) – (4.68 x A663)) x 10-1

Chlorophyll total mg/g = ((8.02 x A663)+ (20.2 x A645)) x 10-1

Carotenoid µmol/g = ((A480 + (0.114 x A663) –(0.638 x A645)) x V x 10^3)/(112.5 x 0.1 x 10)

Determination of ascorbic acid content

Based on the method used by Darmanti et al. (2016): a 0.5g of fresh leaves sample was crushed and homogenized with 3ml of 7% sulfosalicylic acid and centrifuged at 10000g for 10minutes. The supernatant was used as a sample extract. The mixed solution that consisted of 2ml $Na_2MoO_4$2%; 2ml H_2SO_4 0.15M; 1ml $NaHPO_4$ 15mM and 1ml of sample extract was incubated for 40 minutes at 60°C and then cooled at the room temperature. Then, it was centrifuged at a rate of 3000g for 10 minutes. The absorbance value was measured at λ= 660nm. Ascorbic acid content was calculated by the standard ascorbic acid curve.

Determination of total phenol content

Determination of total phenol content was conducted based on the method used by Darmanti et al. (2016): a 0.1g of fresh leaves sample was crushed and homogenized using 0.5ml of methanol, then filtered with filter paper and the final volume used was 1ml. Then, mix the 20µl of extract solution with 1.58ml of sterile water and 100µl folin-ciocalteau reagent, and incubated for 8 minutes. Furthermore, 300ml of 7.5% sodium carbonate was added and incubated at 30°C for 30 minutes. The absorbance value was measured at λ=769nm. Total phenol is calculated using the calibration curve of standard gallic acid.

Determination of antioxidant activity

The measurement of antioxidant activity was by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH), the method used in this study has followed the method by Banerjee et al. (2005): a 2g of fresh leaves sample was crushed and homogenized using 10ml of methanol. Then, the solution was shaken until separated between the waste and the solution. The formed solution was used as the extract. DPPH weighing 0.002g was dissolved in 50ml of 95% ethanol. Fifty milligrams of fresh leaves were crushed and dissolved in 5ml methanol as a sample solution of 10,000ppm, then the series of 0, 200, 300, 400, and 500ppm were made. Each sample solution was taken as much as 0.5ml, respectively, added with 1.5ml of 0.1mM DPPH reagent, vortexed and incubated in the dark room with room temperature for 30 minutes. Absorbance was read at λ=517nm. Inhibition percentage was calculated by the formula: % inhibition =(Control Absorbance-Sample Absorbance)/(Control Absorbance) x 100%

Then, curves were made with the x-axis of antioxidant activity and the y-axis of the inhibition to determine the linear regression equation that was used to determine the value of the IC50. The value of IC50 vitamin C was used as a comparative antioxidant activity of the sample. IC50 of vitamin C was determined by a standard curve of vitamin C in series concentrations of 0, 2, 4, 6, 8, 10ppm (Afriani et al., 2014).

Data analysis

Qualitative data were analyzed descriptively. Quantitative data was tested for its homogeneity, followed by one-way Analysis of Variance (ANOVA) test at 95% confidence level and, followed by Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

The results showed that *S. jambos* leaves morphology at different developmental phases stages did not show any morphological differences. The morphological characteristics of leaves were as follows: the integer leaf edge, bare surface strand (glaber), penninervis repeats, acute-shaped of the base and the end of the leaf, so that the base form can be said lanceolate-shaped (Figure 1). It is consistent with Tjitrosoepomo (2009) that stated that the base of the leaf is separated by the stem and if the end of the leaf forms a tapered angle (<90^0) is called acutus, and if the leaf extends in the center is called lanceolate.

There are three hunter color values, i.e., hunter-a, b, and L. Hunter-a: value (-) shows green color, then value (+) shows red color. Hunter-b: value (-) shows blue color, and value (+) shows yellow color. Lightness (L): the higher the leaf color, the brighter the color. The working principle of the colorimeter is using light that is fired on the surface of the sample, so brightness is

preferred in this test, in addition to the yellow color also supports the lightness level of the sample (Suyatma, 2009). Based on the results of the color test with colorimeter showed that the value of hunter-a, hunter-b and the lightness (L) in leaves: the value of hunter-a shows a decrease in young and old leaves decreased significantly when compared with mature leaves. However, the hunter-b and lightness (L) values showed a decrease in young leaves, and then the value of older leaves decreased significantly when compared to the mature leaves (Table 1). The values of hunter-a and hunter-b are read on the chromaticity diagram showing the difference in leaf color of the young leaves are brownish green, mature leaves are light green and old leaves are dark green.

The result of pigment test showed that the higher leaf growth, the higher the content of chlorophyll-a, chlorophyll-b, total chlorophyll and carotenoid, but a significant increase was only shown by old leaves (Table 2). This condition is caused by the presence of the other pigment contents in the leaves, such as anthocyanin that give a red, purple, and blue color. It is in accordance with the statement of Ogunwenmo et al. (2007), that leaves color difference, other than caused by carotene, phaeophytin, and xanthophyll content. In addition to the above differences in leaf, color variation are also related, further according to Papafotiou et al. (2007), differences in the color of *S. jambos* leaf is also associated with modification of anthocyanin structure, co-pigmentation with other flavonoid compounds, and the formation of complex compounds with metal ions. Meanwhile, according to İnanç (2011), the leaves color change can also be caused by the leaves developmental stage that is in the process of chlorophyll metabolism that is on the formation of chlorophyll.

Mature leaves that have light green color indicates that chlorophyll-*a* is already formed and giving the green color on the leaves. The old leaves are dark green because the content of chlorophyll pigments (chlorophyll-a,b, and total pigments) are higher than the other leaves. Change in the color of the mature leaves to the old leaves marked by the change in light green to dark green. This is according to Maulid and Laily (2015), which states that in old leaves there is a synthesis of chlorophyll-b from chlorophyll-a which is characterized by changing the color of light green leaves (on mature leaves) to dark green (old leaves).

Carotenoids are yellow to orange pigments found in thylakoid along with chlorophyll-a and chlorophyll-b (Lakitan, 2010). Carotenoid levels are influenced by its role in protecting chlorophyll in the process of photosynthesis, the higher chlorophyll content then the higher carotenoid content. Carotenoids play a role in absorbing solar light energy that can be forwarded to chlorophyll-a (Pratama, 2009). Also, carotenoids also serve to protect chlorophyll from the high light intensity in order to avoid oxidative damage by O_2. According to Suryaningrum et al. (2006) carotenoids and chlorophyll are classified as potential antioxidant compounds and can be converted into essential vitamins. Carotenoid biosynthesis requires light of increasing enzyme activity (Bramley, 2002). The enzyme used to initiate the biosynthesis of carotenoids is the phyto-synthase enzyme derived from the psy-1 and psy-2 gene (Simkin et al., 2003).

The results of the analysis of showed that ascorbic acid content of mature leaves was lower than that of young and old leaves, while the young leaf content was similar to that of old leaves (Table 3). It is because the ascorbic acid transports from the mature leaves to the immature leaves because younger leaves require ascorbic acid for cell division and growth. It is consistent with the statement of Ayua et al., (2016), that the young leaves require more vitamin C, but ascorbic acid synthesis cannot meet its physiological processes. It is in line with the statement of Davey et al. (2006) that ascorbic acid plays a role in cell growth and the process of photosynthesis. Transport of ascorbic acid from the older leaves to the younger leaves is through the phloem network (Franceschi & Tarlyn, 2002).

Table 1. *S. jambos* Leaf color at different developmental stages

Treatment	Leaf Color Coordinate		
	Hunter-a*	Hunter-b*	Lightness (L)*
Young leaf	-7.050^{c}	39.100^{e}	33.090^{h}
Mature leaf	-24.010^{a}	46.060^{d}	36.060^{g}
Old leaf	-21.460^{b}	20.180^{f}	21.160^{i}

Numbers followed by the same letter in the same column shows not significantly different results by DMRT ($p< 0.05$).

Table 2. The contents of chlorophyll-a (mg/g), chlorophyll-b (mg/g), total chlorophyll (mg/g) and carotenoids (µmol/g) of *S. jambos* leaves at different developmental stages

Treatment	Leaf Chlorophyll Content			Carotenoids (µmol/g)
	Chlorophyll-a (mg/g)	Chlorophyll-b (mg/g)	Total chlorophyll (mg/g)	
Young leaf	0.370[b]	0.200[b]	0.560[b]	15.650[h]
Mature leaf	0.400[b]	0.170[b]	0.580[b]	18.280[h]
Old leaf	0.640[a]	0.270[a]	0.910[a]	26.000[g]

Numbers followed by the same letter in the same column shows not significantly different results by DMRT ($p < 0.05$).

Table 3. The contents of ascorbic acid (mg/g), total phenol (mgGAE/g) and IC50 (ppm) of *S. jambos* leaves at different developmental stages

Treatment	Ascorbic Acid Content (mg/g)	Total Phenol Content (mgGAE/g)	IC_{50} Value (ppm)
Young leaf	122.200[a]	82.175[c]	223.390[a]
Mature leaf	87.520[b]	152.224[b]	215.030[b]
Old leaf	124.200[a]	213.341[a]	171.640[c]
Vitamin C (Control)	-	-	12.480[d]

Numbers followed by the same letter in the same column shows not significantly different results by DMRT ($p < 0.05$).

Ascorbic acid accumulates in the chloroplast. High chlorophyll content in old leaves shows a high amount of chloroplast, so ascorbic acid of old leaves is higher than mature leaves; this is consistent with the result of this study (Table 3). According to Winarsi (2007), ascorbic acid is found in chloroplasts, cytosols, vacuoles, and extracellular compartments. It is in line with the statement of Franceschi and Tarlyn (2002), that the ability of the older leaves to produce ascorbic acid from GAL-l increased 3 to 10 times higher, while the rate of utilization of ascorbic acid is low.

The results showed that higher phase of leaf development, the higher total phenolic content and antioxidant activity (Table 3). It is in line with the statement of Achakzai et al. (2009), that older leaves require a more extensive environmental defense system and thus require more secondary metabolites as well. While young leaves require many nutrients to grow and carry out various metabolic processes. Phenol compounds are synthesized via a cyclic pathway with the main enzyme phenylalanine ammonia-lyase (PAL) forming cinnamic acid compounds and their derivatives such as flavonoids, isoflavones, pterocarpans, stilbene, coumarin, phenolamines, aurones, chalcones, lignans and lignins (Halbwirth et al., 2009 and Darmanti et al., 2018). Agati et al., (2012), states that flavonoids are one of the most abundant phenolic compounds in leaves that can increase the total phenol levels. Flavonoids are synthesized in chloroplasts and nuclei and act as antioxidants.

IC50 analysis shows that higher rate of leaf development, the lower IC50 value which means that the higher the rate of leaf development, the higher antioxidant activity. Yu and Cheng (2007), stated that IC50 values indicate the concentration of antioxidants needed to reduce 50% free radicals, so the smaller the value of IC50, then the higher its antioxidant activity. Armala (2009) stated that the ability of antioxidants in reducing free radicals divided into several groups of antioxidant activity: IC50 value below 50ppm is a powerful antioxidant, 50-100ppm including in high/strong category, the value of 101-150ppm includes in the medium category, and the value of >150ppm includes in the weak category. Based on the classification, the antioxidant activity of Malabar plum level is low. However, a study by Dhanabalan et al. (2014) revealed the results of an analysis of the activity of Malabar plum leaves antioxidant from India with methanol, ethanol and chloroform solvents, respectively are 159.57ppm, 114.56ppm, and 132.91ppm. While the research of Islam et al. (2012) showed that, the antioxidant activity of Malabar plum leaves is 14.10ppm, which classified as very strong/ high. The difference in the results of this study is possibly because of the difference in the *S. jambos* variety and the solvent used.

Ascorbic acid in the antioxidant activity test is used as a positive or comparative control

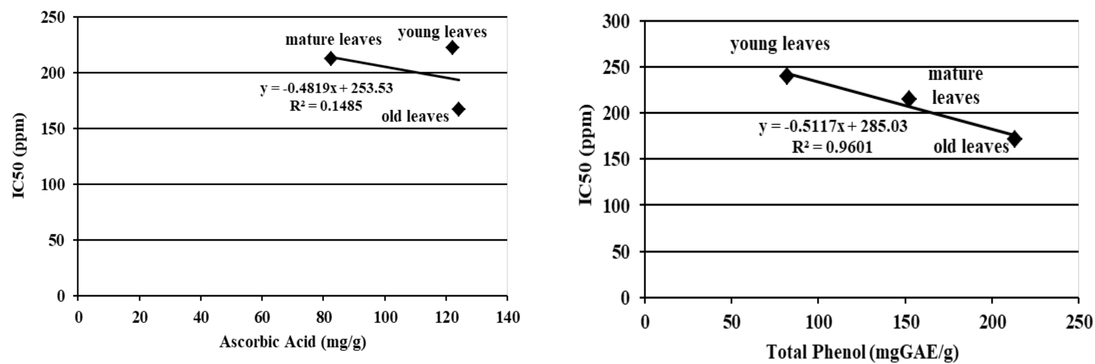

Figure 2. Correlation of ascorbic acid, total phenolic content and antioxidant activity of *S. jambos* leaves at different developmental stages

of the antioxidant activity of the sample. Ascorbic acid used as a positive control, according to Afriani et al. (2014), because of ascorbic acid, is included in the class of antioxidants capable of counteracting various free radicals. Ascorbic acid antioxidant activity in this study showed a value of 12.48ppm, so the antioxidant activity of ascorbic acid in this study showed a much higher result compared with the results of Malabar plum leaves sample. The IC50 value of vitamin C in a study by Haryoto et al. (2007) was 3.72ppm, while in a study by Firman et al. (2016) was 26.76ppm.

The correlation test between ascorbic acid (x) and IC50 (y) shows the correlation coefficient of r2 = 0.1485 with the equation of line y = -0.4819x + 253.53. These results indicate that the correlation is 14.85%. Whereas the correlation of total phenolic content (x) with IC50 (y) leaf sample has a correlation coefficient r2 = 0.9601 with the equation of line y = -0.5117x + 285.03. These results indicate that 96% of the antioxidant activity of *S. jambos* leaves is the result of contributions from phenolic compounds, while ascorbic acid contributes only 14%. These results indicate that antioxidant activity is the result of contributing ascorbic acid content, so there is no apparent correlation between ascorbic acid and antioxidant activity. According to Zheng and Wang (2001) study that stated that in some herbs, there is a correlation between antioxidant activity and phenolic content, so most of the antioxidant activity is determined by total phenol.

This research was intended to verify the content and antioxidant activity of *S. jambos* leaves in each developmental stages of leaves. This is to follow up the previous studies about Malabar plum leaves without examining the stages of leaves development (Islam et al., 2012; Bonfanti et al., 2013, and Dhanabalan et al., 2014). The findings show that the old leaf stage has the highest content and antioxidant activity (ascorbic acid and phenol) compared to the mature and young leaves stage. This information is essential as a recommendation for the community to choose the old leaves for herbal medicine. Scientifically, this research also proves that the main component of photosynthesis, namely chlorophyll, is also higher in older leaves. Furthermore, the results of research on *S. jambos* leaves can be used in the pharmaceutical industry.

CONCLUSION

The higher stages of *S. jambos* leaf development, higher chlorophyll-a, chlorophyll-b, the total chlorophyll, the carotenoid, the total phenol, and the antioxidant activity, but the ascorbic acid levels of young and old leaves are higher than the mature leaves. There is a high correlation between total phenol content and antioxidant activity, where the antioxidant activity of *S. jambos* leaves is 96%, determined by total phenol content.

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