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Anthocyanin profile of *Syzygium oleana* young leaves and fruits using triple quadrupole mass spectrometer: Identification of a new peonidin

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Abstract. Anggraini T, Syukri D, Manasikan TW, Nakano K. 2020. Anthocyanin profile of *Syzygium oleana* young leaves and fruits using triple quadrupole mass spectrometer: Identification of a new peonidin. *Biodiversitas* 21: 5893-5900. Anthocyanin is pigment present in many red, blue and purple colored plants that can be used as stable food-safe colorings and also offer health benefits as antioxidants. *Syzygium oleana*, with its dark purple fruit and red leaves, is one hitherto unexplored source of anthocyanin. This study is the first to establish the anthocyanin profile of *S. oleana* leaves and fruit exploiting the speed and accuracy of Multiple Reaction Monitoring (MRM) with a triple quadrupole mass spectrometer. The anthocyanin compounds in the leaves and fruit of *S. oleana* were found to be derivatives of agliconpeonidin, cyanidin derivative, delphinidin. It was found that while both leaves and fruit of *S. oleana* contain the anthocyanin precursors cyanidin, delphinidin, petunidin, and peonidin. Fruit contains the anthocyanin malvidin and a large amount of petunidin not present in the leaves. In detail: anthocyanin found in *S. oleana* leaves were Cyanidin 3-galactoside, cyanidin with m/z 611, Delphinidin 3-O- β -D-glucopyranoside, and unknown peonidin. Anthocyanin in *S. oleana* fruits was cyanidin with m/z 449.1, delphinidin 3-O- β -D-glucopyranoside, petunidin with m/z 476, Malvidin 3-O- β -D-glucopyranoside, and an unknown peonidin. Fruit could be a better anthocyanin source and more effective as colorant than leaves, while leaves contain a stronger as yet unidentified antioxidant.

Keywords: Anthocyanins, antioxidant, *Syzygium oleana*, triple quadrupole mass spectrometer

INTRODUCTION

Antioxidants are bioactive compounds found in various plants that have been shown to effectively treat many diseases (Elfalleh et al. 2019; Akhtar et al. 2018). Some plant antioxidants are claimed to reduce some cancers and have anti-inflammatory and anti-bacterial properties (Kamble and Gacche 2019; Toubane et al. 2017; Sepahvand et al. 2014). Polyphenols are one class of antioxidants that frequently occur in leaves and fruit (Oyenihi and Smith 2019; Russo et al. 2017). The polyphenols isoquercitrin, quercetin 3-O-xyloside, quercetin 3-O-arabinoside, and quercetin 3-O-rhamnoside have been found in apple leaves, while chlorogenic acid, isoquercetin, rutin, kaempferide, and quercetin are present in mulberry leaves (Lu et al. 2019; Zhang et al. 2018).

Anthocyanins, water-soluble pigments, are one class of polyphenols that occur in most species in the plant kingdom and are responsible for the red, purple, and blue coloring in many fruits, leaves, and seeds. For instance, strawberries are rich in anthocyanin glycosides and cyanidin-3-O- β -glucoside, pelargonidin-3-O-rutinoside, cyaniding-3-O glucoside (Nowicka et al. 2019). Anthocyanin content can be influenced by plant hormones, light, temperature, soil condition, and nutrients (Das et al. 2012).

Due to the growing health awareness of consumers, the food industry is under pressure to replace synthetic colorants with naturally occurring compounds. Natural

pigments like anthocyanins are one source of these pigments commercially available to replace synthetic dyes. For instance, the edible *Oxalis triangularis* contains anthocyanins delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin which provide a purple colorant for food (Pazmino-Duran et al. 2001).

Anthocyanins as natural pigmented compounds and antioxidants have been found to provide health benefits. Cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-rutinoside, peonidin-3-glucoside, and peonidin-3-rutinoside from red-fleshed sweet cherries have been found to effectively protect Caco-2 cells (Leong et al. 2017). An anthocyanin extract is reported to inhibit Akt-mTOR signaling by inducing maturation of acute myeloid leukemia cells, and also has potential to inhibit human colon, breast, lung, and gastric tumor cells (Bontempo et al. 2015; Bowen-Forbes et al. 2010). Delphinidin-3-glucoside, petunidin-3-O-glucoside, and malvidin-3-O-glucoside inhibit the activity of α -glucosidase, α -amylase, dipeptidyl peptidase-IV, reactive oxygen species, and decrease glucose uptake into the human body (Mojica et al. 2017). Anthocyanin in purple basil (*Ocimum basilicum* L.) has been found to have potential anti-inflammatory properties (Szymanowska et al. 2015). Anthocyanins from red cabbage effectively reverse the signs of intestinal injury, including myeloperoxidase activity and quantity of ileum mucus (Tong et al. 2017).

In the last decade, HPLC coupled with tandem mass spectrometry (LC-MS/MS) has been used to rapidly and

sensitively quantify small molecules such as anthocyanin. The common aglycons of anthocyanin compounds in plants are limited in number consisting mainly of cyanidin, pelargonidin, malvidin, petunidin, delphinidin, and peonidin, and can be identified using the m/z ratio.

The many species of *Syzygium* are a rich source of a variety of functional compounds. *S. aromaticum* contains essential oil. *S. samarangense* (B1.) Merr. et Perry has been found to contain triterpenoids and sycamarins A-D. *S. grijsii* contains lignans and polyphenols. *S. cumini* contains the anthocyanin delphinidin-3,5-diglucoside (Hu et al. 2018; Diaz-Urbe et al. 2019; Radunz et al. 2019; Yao et al. 2013). The ornamental *Syzygium oleana* (*S. oleana*), with its young red leaves and deep purple fruit, is rich in anthocyanins which could also be potential sources of food-safe red and purple pigments that may also provide health benefits (Anggraini 2017). Ultrasonication is an effective method to extract compounds from plant samples, and time of extraction is a determining factor in the yield of the extract. In this study, the researcher studied the potential of leaves and fruit of *S. oleana* as a source of colorants and antioxidant food additives. A simple method for identification of the anthocyanin in the young leaves and mature fruit of *S. oleana* was developed using a combination of liquid chromatography and mass spectrometry (LC-MS), and profile of anthocyanins was obtained using MRM which is known to provide more sensitive results than other mass spectroscopy measurement methods. However, until now no information has been available about the identity and properties of these anthocyanins.

MATERIALS AND METHODS

Plant materials

Young leaves (red color) and ripe fruit (deep purple color) of *S. oleana* were collected from Andalas University Campus, Padang, West Sumatra, Indonesia.

Sample preparation

Syzygium oleana fruit: About 300 g of flesh and the seed of *S. oleana* were separated. The flesh homogenized at ambient temperature and the extract filtered through filter paper (Whatman No. 4). *S. oleana* leaves. The extraction process of leaves used about 300g sample of leaves in distilled water using a 1: 1 ratio by weight.

Anthocyanin identification

1 ml sample of extract was placed in 0.9 ml of acidic methanol (Formic acid 1%), and filtered through a 0.20 μ M Sartorius RC for analysis. A high-performance liquid chromatography-Mass spectrometry (HPLC/MS/MS) analysis of anthocyanins was performed according to Leong et al. (2017) by using UFLCXR Shimadzu (Kyoto, Japan). The two solvents were used in the mobile phase where solvent A consisted of 0.1 % formic acid in water and solvent B used 0.1 % formic acid in acetonitrile. The elution was isocratic at 1% of solvent B for the first 2 min, then a linear gradient of B from 1 % to 45 % was used for

20 min, followed by a linear gradient of B of 45 to 99% for another 5 min and finally a linear gradient of B from 99 to 1% for 3 min. The flow rate was 0.2 mL/min. Mass spectra were obtained using a triple quadrupole ion-tunnel mass spectrometer equipped with analyst software (Q-TRAP 4500, AB Sciex, Framingham, MA, USA). Approximately 5 μ L/min of the HPLC eluate was separated using a reverse-phase chromatographic column, (3 μ m, 2 \times 150 mm, Cadenza, CD-C18, Imtakt, Kyoto, Japan) then delivered to the ESI source. The precursors of all anthocyanidins, including cyanidin (MW 287), delphinidin (MW 303), malvidin (MW 331), peonidin (MW 301), pelargonidin (MW 271), petunidin (MW 317) were scanned for simultaneously during analysis of all samples. Anthocyanins were ionized using a Turbo-VTM ion source in positive mode and were detected using multiplex MRM for specific productions. The m/z value of each aglycon of anthocyanin were 287, 271, 303, 317,331, and 301 for cyanidin, pelargonidin, delphinidin, petunidin, malvidin, and peonidin respectively. Precursor ions were set in the range of m/z of 400-1000. Identified of m/z anthocyanins were confirmed based on the online lipid map database, Lipidomics Gateway

(<http://www.lipidmaps.org/data/structure/LMSDSearch.php?Mode=ProcessClassSearch&LMID=LMPK12>).

Total monomeric anthocyanin content

The total monomeric anthocyanin content was determined as described by Coklar and Akbulut (2017) using the differential method with modification. Each sample (rind, pulp, seeds, and the whole fruit) was homogenized then 1 g was added to 9 mL of methanol and 1 mL of 27% HCl. 0.5 ml sample of crude extract was poured into two reaction tubes. KCl buffer (0.025 M) pH 1 was added to the first, and 9.5 mL sodium acetate buffer (0.4 M) pH 4.5 to the second. After 15 minutes the absorbance of the sample was measured using a spectrophotometer at 515 nm and 700 nm. The anthocyanin content (cyanidin-3-glucoside equivalents, mg/100g) was calculated as follows:

$$\text{Anthocyanin content} = \frac{A \times MW \times DF \times 10^3}{\epsilon \times l}$$

Where; A = pH 1.0 (A 520 – A 700 nm) – pH 4.5 ((A 520 – A 700 nm); MW = molecular weight of cyanidin-3-glucoside (449.2 g/mol); DF = dilution factor and ϵ = molar extinction coefficient of cyanidin-3-glucoside (26.900 L/mol x cm)

Solvent extraction

For sample preparation, *S. oleana* leaves and fruit was weighted 20 g, added with 100 ml ethanol and the mixture was placed in a water bath (Elmasonic P30, Elma Hans Schmidbauer GmbH, Sinden, Germany) for 30 min, 60 min, and 120 min at room temperature to solubilize bioactive compounds from the *S. oleana* leaves and fruit.

DPPH (*Dyphenyl Pycryl Hydrazyl*) radical scavenging activity

DPPH assays were determined as described by Zhang et al. (2018). One gram samples (rind, flesh, seeds, whole fruit, leave and bark) were added to 10 mL of either water, methanol, or ethanol then homogenized. The crude extract samples were mixed with 3.9 ml of methanol and 1 ml of a DPPH solution (1mM in methanol) in a test-tube and the absorbance measured at 517 nm after 30 minutes incubation. DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity} = \left[1 - \frac{A_{517}(\text{sample})}{A_{517}(\text{blank})} \right] \times 100 \%$$

RESULTS AND DISCUSSION

Anthocyanin profile of *S. oleana* Leaves

According to Lipidomics, there are seven precursors of anthocyanin, but, just six common anthocyanins classified by Martin-Bueno et al. (2012); delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. The presence of anthocyanin is evident in the color of a plant. It can be predicted by hyperspectral imaging. For example, Nogales-Bueno et al. (2016) found hyperspectral imaging accurately predicted levels of extractable anthocyanin in grape skins. A number of deeply colored berries owe their coloring to high anthocyanin content. Raspberry, strawberry, sour cherry, black currant, and blueberry have a range of anthocyanin with associated antioxidant activity (Szymanowska and Baraniak 2019; Gornas et al. 2016; Zhao et al. 2017). The leaves of perilla and other red leaves are also a source of anthocyanin (Dar et al. 2019; Lee et al. 2018). *S. oleana* produces a bright red color in young leaves and a deep purple in the matured fruit suggesting the presence of various anthocyanin as shown in Figure 1.

MRM provided a rapid and sensitive analysis of the anthocyanin profile. The anthocyanin profile of *S. oleana* leaves can be seen in Table 1 while the anthocyanin profile of the fruit can be seen in Table 2.

Two kinds of cyanidin, one kind of delphinidin, and one kind of peonidin were found in *S. oleana* leaves. Based on lipidomics gateway data, the possible identification of these anthocyanins from the leaves was Cyanidin 3-galactoside, cyaniding with m/z 661 (possibly cyanidin 3-C2-glucosyl galactoside), cyanidin 3,5-diglucoside, Cyanidin 3,3' diglucoside, and cyanidin 3,4' diglucoside and Delphinidin 3-O-β-D-glucopyranoside. The m/z value of the peonidin did not correspond to any on this database so remains unknown

Figure 2 shows the MRM profile of the chromatogram of anthocyanin compounds in *S. oleana* leaves.

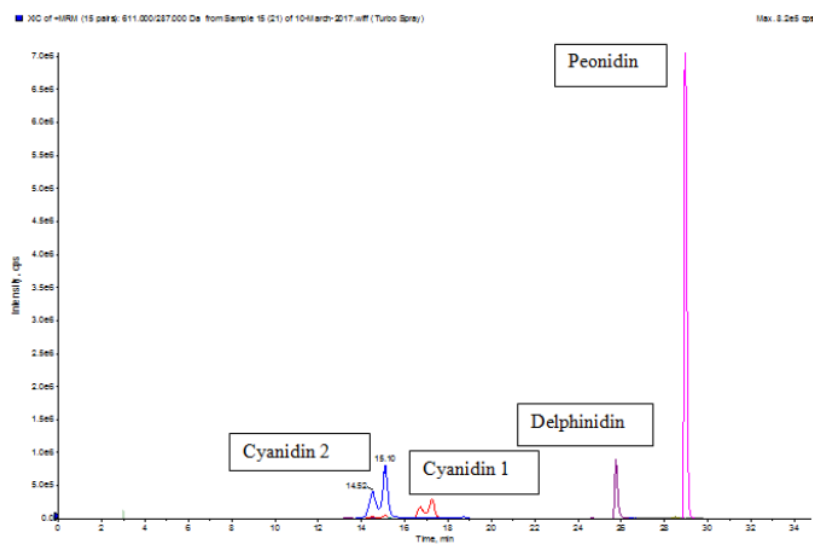
Search results of online databases indicate more than one possible identity for some of the aglycon. This is because each anthocyanin can bind to sugar or the isomer of that sugar and there is more than one possible bonding position for the glycoside bond of a particular sugar. There were three main anthocyanin components found. The derivatives of the peonidin compound are anthocyanin major having pairs Q1/Q3 = 582.7/301. No compounds from peonidin with BM 479.1 could be found on online metabolomic database Lipidmaps indicating the characterization of this peonidin compound needs further research. There were three other anthocyanins identified, derivatives of two types of cyanidin and one of delphinidin. Table 2 shows possible identification of these anthocyanins based on matches on Lipidmaps. The cyanidin with Q1 449.1 is assumed to come from Cyanidin 3- galactoside that has an isomer in their constituent sugars, glucose and galactose. Cyanidine with Q1 661.0, is assumed to be one of the 5 anthocyanin derivative compounds that have the same MR with constituent sugars containing different glycoside bonds and/or positions.



Figure 1. *Syzygium oleana* as ornamental plant (A), *S.oleana* fruits (B, C)

Table 1. Anthocyanin profile of *Syzygium oleana* leaves

Number	Product ion m/z (Q3)	Precursor ions (Q1)m/z	Predicted anthocyanin (lipid maps)	Retention time	Intensity
1	287	449.1	Cyanidin Cyanidin 3-galactoside/C ₂₁ H ₂₁ O ₁₁	17.25	3.00E+05
2		661.0	Cyanidin 3-galactoside 5-glycoside/C ₂₇ H ₃₁ O ₁₆ Cyanidin 3-C2-glycosylgalactoside/C ₂₇ H ₃₁ O ₁₆ Cyanidin 3,5-diglycoside/C ₂₇ H ₃₁ O ₁₆ Cyanidin 3,3' diglycoside/C ₂₇ H ₃₁ O ₁₆ Cyanidin 3,4' diglycoside/C ₂₇ H ₃₁ O ₁₆	15.1	8.00E+05
3	303	465.1	Delphinidin Delphinidin 3-O-β-D-glucopyranoside/C ₂₁ H ₂₁ O ₁₂	25.75	9.10E+05
4	301	582.7	Peonidin Unknown	28.94	7.00E+06

**Figure 2.** Chromatogram of *Syzygium oleana* leaves**Table 2.** Anthocyanin profile of *Syzygium oleana* fruits

Number	Product ion m/z (Q3)	Precursor ions (Q1)m/z	Predicted anthocyanin (lipid maps)	Retention time	Intensity
1	287	449.1	Cyanidin Cyanidin 3-galactoside/C ₂₁ H ₂₁ O ₁₁ Cyanidin 3-O glucoside/C ₂₁ H ₂₁ O ₁₁	17.35	4.00E+06
2	303	466.1	Delphinidin Delphinidin 3-O-β-D-glucopyranoside/C ₂₁ H ₂₁ O ₁₂	16.17	9.00E+05
3	317	479	Petunidin Petunidin 3-galactoside/C ₂₂ H ₂₃ O ₁₂ Petunidin 3-glycoside/C ₂₂ H ₂₃ O ₁₂	18.08	8.00E+05
4	331	493.2	Malvidin Malvidin 3-O-β-D-glucopyranoside/C ₂₃ H ₂₅ O ₁₂	20.92	2.00E+06
5	301	582.7	Peonidin Unknown	28.95	5.00E+06

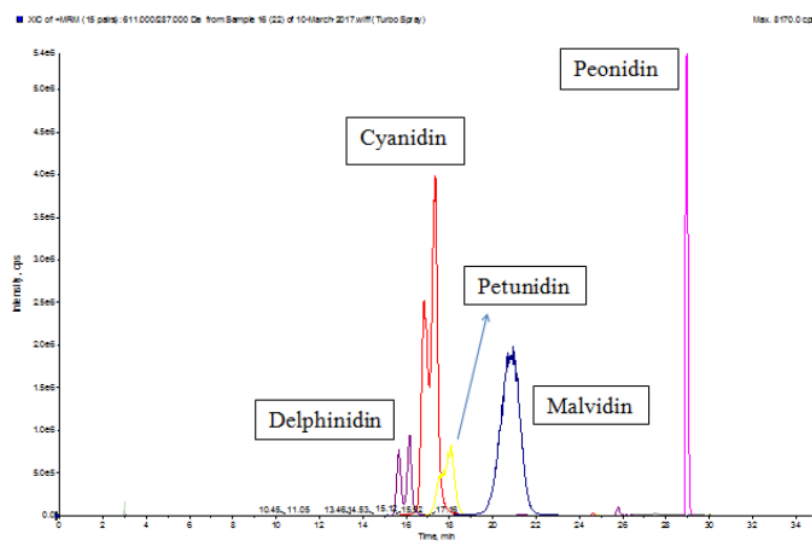


Figure 3. Chromatogram of *Syzygium oleana* fruits

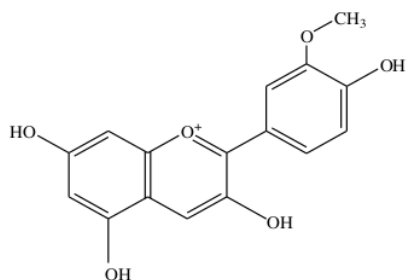


Figure 4. Peonidin structure

Anthocyanin found in *S. oleana* fruits were cyanidin, delphinidin, petunidin, malvidin, and peonidin (Table 2, Figure 3). The cyanidin had m/z of 449.1 (which could possibly be Cyanidin 3-galactoside or Cyanidin 3-O glucoside). Delphinidin was identified as 3-O- β -D-glucopyranoside. The petunidin with 476 m/z could possibly be Petunidin 3-galactoside or Petunidin 3-glucoside. The malvidin matched malvidin 3-O- β -D-glucopyranoside. The same unknown peonidin as was in the leaves was also found in the fruit. In fact, the unidentifiable derivative compound of peonidin present in the leaves is the main anthocyanin constituent of the fruit.

Syzygium oleana young leaves and mature fruit contain slightly different anthocyanin in different quantities resulting in different colors. The leaves contain one cyanidin not present in the fruit, while the fruit contains malvidin and petunidin which are not detectable in the leaves and are responsible for the deep purple coloring. The peonidin is a member of a class of non-toxic compounds, which is in the cationic form of flavylium in acid and in

alkaline takes the purple-blue quinoidal basic form. The structure of peonidin can be seen in Figure 4. Peonidin can be used as a food-safe colorant due to its stability up to pH 8 (Rajan et al. 2018). Peonidin has 4 -OH groups and has been found to be a more reactive radical scavenger than quercetin and can decrease inflammatory gene expression (Rajan et al. 2018).

By using MRM, up to 152 phenolic compounds of rose wines can be detected in 30 minutes without sample purification (Lambert et al. 2015). Meanwhile, Jaitz et al. (2010) needed 20 minutes to quantify 28 polyphenols in cocoa extracts.

The anthocyanin identified in the *S. oleana* leaves and fruits is also common in other plants. Petunidin-3-glucoside is a major anthocyanin in *Lycium ruthenicum* Murray fruit and has been shown to have the potential to reduce monosodium urate crystal-induced inflammation (Zhang et al. 2019). Cyanidin is a very common anthocyanin in fruit. Cyanidin 3-O glucoside has been found to be the major anthocyanin in grape skin and cyanidin 3-galactoside in apple skin. Malvidin 3-O glucoside is the major anthocyanin in ripe black and red grapes (Ferreira et al. 2017).

The use of *S. oleana* will develop the improvement of food colorant since anthocyanin are very also stable in high temperature (Lao and Giusti 2017; Osorio et al. 2010). This first report about anthocyanin profile in *S. oleana* will helpful for another researcher for product development.

Total monomeric anthocyanin content

Data for total monomeric anthocyanin of extract leaves and fruit of *S. oleana* extracted with methanol in ultrasonic bath for 30 minutes, 60 minutes, and 120 minutes are presented in Table 3.

Table 3. Total monomeric anthocyanin content of *Syzygium oleana* leaves and fruit

<i>S. oleana</i>	Total monomeric anthocyanin (mg/L)		
	30 minutes	60 minutes	120 minutes
Leaves	1.56±0.19	2.56±0.25	5.34±0.59
Fruit	63.62±11.04	147.34±4.13	171.1±6.28

120 minutes is the optimal duration trialed for extraction of anthocyanin both in leaves and fruit with the anthocyanin content increased with increasing of extraction time. Table 4 shows the antioxidant activity of the extract from *S. oleana* leaves and fruit using a 120 minutes extraction time. The fruit has a higher monomeric anthocyanin content than leaves suggesting that they are a more promising source of colorant for food than leaves. Beverage processing can use the fruit extract for coloring since anthocyanin is stable compound with refrigeration and acidification are effective in maintaining anthocyanin quality (Howard et al. 2016). The mean anthocyanin content of berry cultivated in Chile was 335.5 mg/mL, which have the same color as *S. oleana* fruit (Guerrero et al. 2010).

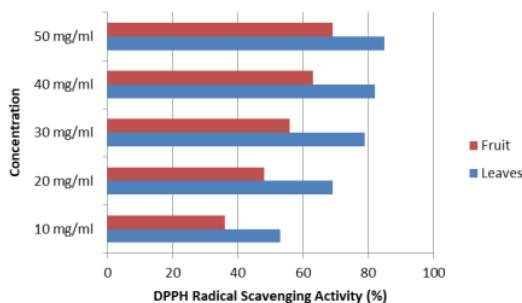
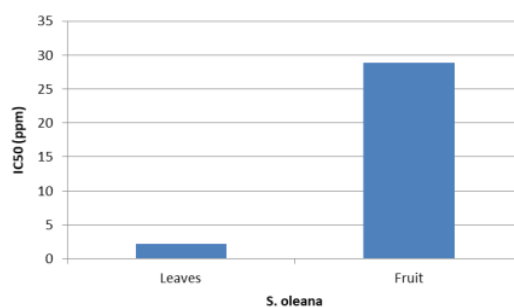
S. oleana leaves have a red color, and Table 4 showed the low amount of anthocyanin, while fruit with deep purple extract has higher anthocyanin content. So, the fruit is a richer source of colorant than the leaves.

DPPH radical scavenging activity and IC₅₀

DPPH radical scavenging activity of *S. oleana* leaf and fruit extracts can be seen in Figure 5. Anthocyanin are radical scavengers. Figure 5 shows that the DPPH radical scavenging activity is higher in leaf extract than fruit even though the anthocyanin content is higher in the fruit (Table 3). Free radicals can be reduced due to anthocyanin's ability to donate hydrogen. There was a positive correlation between DPPH radical scavenging activity with concentration, as was found in a similar study by Ge and Ma (2013). It can be concluded that the antioxidant properties in leaves are due to another antioxidant, not anthocyanin, and identification needs further research.

For food coloring, the fruit is clearly more effective than leaves, but leaves of *S. oleana* could be the source of a more potent antioxidant and could be processed as a food additive to increase the antioxidant ability rather than to provide color for food.

Figure 6 shows the IC₅₀ of *S. oleana* fruit and leaf extract after 120 min extraction in ultrasonic bath. In accordance with the results of the DPPH scavenging activity value, the value of IC₅₀ showed similar results. Both leaves and fruit of *S. oleana* are the active antioxidants but leaves have higher antioxidant activity than fruit and higher activity as radical scavengers than fruit suggesting the antioxidant properties of the leaves are not due to anthocyanin. Polyphenols, including anthocyanins, are components of antioxidants. But the results of this study showed that might be other types of colorless polyphenols that also play a role in antioxidants in leaves.

**Figure 5.** DPPH radical scavenging activity (%) of *Syzygium oleana***Figure 6.** IC₅₀ of *Syzygium oleana*

In comparison with *S. oleana*, the leaves of *Cissus sicyoides* has been used as traditional medicine, and the berries have been shown to exhibit a value of IC₅₀ 0.99 mg/ml (Barnaby et al. 2018). A berry cultivar, *Sideroxylon obtusifolium* has purple skin color and anthocyanin content of 236.5 mg/100g and showed potential free radical activity higher than butylated hydroxytoluene (Figueiredo and Lima 2015). The ethanolic extract of *Vaccinium corymbosum* (blueberry) has 73.25% DPPH radical scavenging activity at 3 mg/ml and IC₅₀ values of 2.5 mg/ml (Samad et al. 2014).

To conclude, our finding indicates *S. oleana* may provide a promising alternative for based colorants. As the fruit contains a higher anthocyanin content, it is suggested that fruit may be a better source than the leaves for the development of food colorant. The leaf content is higher in antioxidants than the fruit so could be used to supplement antioxidant activity in manufactured food products.

The previously unidentified peonidin is of particular interest due to the generally non-toxic and stable nature of this group of anthocyanin. Also, as some peonidin have been also found to be powerful radical scavengers and effective suppressors of inflammatory gene expression, the properties of this new peonidin warrants further research.

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