BIODIVERSITAS
Volume 20, Number 12, December 2019
Pages: 3619-3625

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d201221

Anatomical structure, flavonoid content, and antioxidant activity of *Rhodomyrtus tomentosa* leaves and fruits on different age and maturity level

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Manuscript received: 4 October 2019. Revision accepted: 20 November 2019.

Abstract. *Kuntorini EM, Nugroho LH, Maryani, Nuringtyas TR. 2019. Anatomical structure, flavonoid content, and antioxidant activity of* Rhodomyrtus tomentosa *leaves and fruits on different age and maturity level. Biodiversitas 20: 3619-3625.* Karamunting (*Rhodomyrtus tomentosa* (Aiton.) Hassk.) is a native plant to southeast Asian countries, and is a well-known medicinal plant used to treat colic diarrhea, wounds, heartburn, abscesses, gynecopathy, and as a pain killer. However, the use of *R. tomentosa* has not been optimized. This research aimed to observe the anatomical structure, the location, and distribution of flavonoid and to find out the antioxidant activity based on the leaves age and the fruit maturity. Anatomical slides preparation of leaves and fruits were made using the paraffin embedding method with safranin staining. The distribution of flavonoid was analyzed by histochemical test and antioxidant activity was done with DPPH (1,1-diphenyl-2-picrylhydrazyl) method. Leaf anatomical structure shows that the leaf bifacial (dorsiventral) consisted of upper epidermis, mesophyll (palisade and sponge), collateral vascular bundle, parenchyma midrib, abaxial epidermis, globular oil cavity, and non-glandular trichome. Transverse section of green fruit consists of exocarp (thin outer layer), mesocarp (thick inner layer, soft and runny) and endocarp (thin false septa) layers. The histochemical test showed that flavonoid was observed in the leaf epidermis, mesophyll, vascular bundles, secretory cavity, parenchyma and in all fruit types exocarp, mesocarp, endocarp. Antioxidant activity showed that the extract of the young leaves (IC_{50} = 14.67 ppm) was stronger than the old leaves (IC_{50} = 19.86 ppm). The antioxidant activity of the purple fruits extract (IC_{50} = 12.98 ppm) was stronger than the red fruits (IC_{50} = 28.63 ppm) and the green fruits (IC_{50} = 48.36 ppm) but it was weaker than quercetin (IC_{50} = 1.29 ppm). The purple fruit had the highest antioxidant activity compared to other extracts. This information will be useful for developing karamunting as a potential resource of natural antioxidants for functional foods and health products.

Keywords: Anatomical structure, antioxidant activity, histochemical content, *Rhodomyrtus tomentosa*

INTRODUCTION

Karamunting *(Rhodomyrtus tomentosa* (Aiton) Hassk.), also named as Rose Myrtle, is an evergreen shrub in the family Myrtaceae and mainly distributed in southeast Asian countries, especially southern China, Japan and Thailand (Wu et al. 2015). *R. tomentosa* plants have long been used as medicines for the majority of people in Vietnam, China, Malaysia, and Indonesia in Kalimantan by Dayaks and Pasers (Kusuma et al. 2016; Hamid et al. 2017). The edible berries of *R. tomentosa* are of dark violet color, bell shape and have been historically used as a folk medicine to treat diarrhea, dysentery and traumatic hemorrhage (Geetha et al. 2010), while its leaves by Chinese society are used to cure diarrhea, dysentery, and bleeding (Wu et al. 2015). All parts of this plant (leaves, roots, buds, and fruits) have been used traditionally in Vietnamese, Chinese, and Malaysian medicine. The leaves have traditionally been used to treat colic, dysentery, abscesses, and tuberculosis (Hamid et al. 2017).

The chemical constituents of *R. tomentosa* have been reported and consisted of hydrolyzable tannins, flavones, triterpenes, phenols, amino acids, organic acids, quinones, polysaccharide, steroids and other chemical constituents (Tung et al. 2009; Wu et al. 2015). Flavonoids are a large group of plant polyphenol secondary metabolites that were widely found in the leaves, seeds, bark, and flowers of plants. Depending on their structural characteristics, flavonoids are divided into six classes, including flavanones, flavones, flavonols, isoflavonoids, anthocyanins and flavans (Wu et al. 2015). It is already well recognized that flavonoids possess antilipoperoxidant, antitumoral, antiplatelet, antiischemic, antiallergic, antinflammatory, antibacterial and antioxidant activities. In addition, flavonoids exhibit strong antioxidant capacities through scavenging oxygen free radicals, promote antioxidase or inhibit oxidative enzymes (Sayuti and Yenrina 2015). Therefore, the biochemistry and medicinal aspects of flavonoids have received increasing attention recently.

Distribution of secondary metabolite compounds at the level of plant tissue with histochemical analysis has not been a common pattern. Secondary metabolites are distributed in tissues according to their function in plants.

Secondary metabolites are deposited in cells, certain secretory tissues or spread in tissues even in vascular bundles (Nugroho 2017). Flavonoid compounds at the cellular level are found in leaf and flower epidermal cells, with variations in flowers being higher than in leaves. At the subcellular level, flavonoids and anthocyanins are often found in vacuoles (Wink 2010). Anatomical studies for various genera in Myrtaceae have been carried out (Kantachot et al. 2007; Ferreira at al. 2011; Al-Edany et al. 2012), but no histochemical anatomical studies have been carried out on the leaves and fruits of *R. tomentosa*.

Previous studies on this plant showed antibacterial activity of ethanolic leaves extract against *Streptococcus pyogenes* (Limsuwan et al. 2011; Mordmuang et al. 2015). *R. tomentosa* extract possesses potential antiinflammatory and antiulcer activity, and can serve as a potent antioxidant (Lavanya et al. 2012; Jeong et al. 2013). Studies conducted on *R.tomentosa* fruits showed antioxidant properties due to the presence of phenolic compounds (Cui et al. 2013; Lai et al. 2013; Lai et al. 2015). Several other studies have previously been conducted on the antioxidant activity test on *R. tomentosa* leaves and fruits extracts. However, there is no record that explained the histochemical tests on anatomical structures which are then used to discover the location of flavonoids accumulation as well as the test of the antioxidant activity of the leaves and fruits of *R. tomentosa* based on the leaves age and the fruit maturity. Therefore, it is necessary to conduct a study to find out the antioxidant activity, anatomical structure and histochemical test for flavonoids of *R. tomentosa* leaves and fruit-based on the leaves age and the maturity of the fruit.

MATERIALS AND METHODS

Plant materials

The materials used were the 2nd and 10th leaves from the tip stem, green fruits, red fruits and purple fruits of *R. tomentosa* collected from wild plants in Banua Botanic Gardens, Banjarbaru, South Kalimantan, Indonesia. Three replicate samples of leaves and fruit were taken from three different individual plants. The sample plant identification was done by Herbarium Bogoriense, Indonesian Institute of Sciences, Bogor, Indonesia.

Anatomy slides preparation

For permanent anatomical slides, the leaves transverse sections were prepared using paraffin embedding method, while transverse sections of fruits were obtained using freehand section with safranin staining according to the method by Ruzin (1999) with slight modifications in the time length of immersion process in dehydration and dealcoholization. Three replicates from three different plants of the fruits (27 samples) and leaves (18 samples) were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70% 5:5:90) solution for 24 hours. Dehydration was done by soaking the samples in the alcohol with 70%, 80%, 95% concentrations, for 30 minutes respectively. De-alcoholization was done by immersing the samples in alcohol: xylol with a ratio of 3:1, 1:1, 1:3, each xylol 100% I and xylol 100% II was for 30 min. The sample was immersed in a solution of xylol II, an infiltration process was then performed with paraffin: xylol (9:1) at 57°C for 24 hours. The paraffin mixture: xylol was replaced with pure paraffin at a fixed 57°C for 24 hours. The sample was blocked in pure paraffin. The paraffin which contains sample was then sliced using a rotary microtome with 10 µm thickness. The sample sections were stained using safranin.

Flavonoid content

Histochemical test enables the identification and localization of specific substances within tissues. The methods depend on chemical reactions between the substance to be identified and localized in a tissue section, and one or more reagents in which the tissue section is incubated. The histochemical tries to arrange matters so that the end product of the chemical reaction is both colored and insoluble, and therefore easily visible on microscopy (Bancrof 1975).

Table 1. Flavonoid with histochemical method on *R. tomentosa* leaf

Sample	Compounds dispersion location				
	Epidermal	Mesophyll	Vascular bundles	Parenchyma	Trichome
Young leaf	+	+	+	+	-
Old leaf	+	+	+	+	-

Note: (+) positive; (-) negative

Table 2. Flavonoid with histochemical method on *R. tomentosa* Fruit

Sample	Compounds dispersion location			
	Exocarp	Mesocarp	Endocarp	Seed
Green fruit	+	+	+	+
Red fruit	+	+	+	-
Purple fruit	+	+	+	-

Note: (+) positive; (-) negative

The cellular distribution of flavonoids in plants can be observed using a histochemical method, according to Dai et al. (1996) with slight modifications in the ratio of Wilson's reagent concentrations. The leaves and fruits samples were thinly sliced using a hand microtome and razor blades without size thickness. The thin transverse sections of leaves and fruit were then immersed for 15 minutes in citric acid: Boric (5:5 w/w) in 100 mL of absolute ethanol (Wilson's reagent) and were rinsed with aquadest (distilled water). The sample sections were then covered with glycerol and observed under a microscope. Yellow color formation indicated positive results for flavonoid compound.

Antioxidant activity

Antioxidant activity of methanolic extract of leaves and fruits was carried out using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method by Maulina (2014) with slight modifications in DPPH concentration. 50 grams of each kind of powdered material was macerated using 250 mL methanol solvent for 24 hours, and the methanol solvent was discarded and replaced with the new one for every 24 hours (3 times) (Harborne 1987). Antioxidant activity test was done by weighing 10 mg of concentrated extract, and was dissolved in 10 mL methanol. From 1000 ppm concentration of the main solution, the solution was diluted for 10, 20, 30, 40 and 50 ppm concentrations in a 10 mL measuring flask. One mL of 0.4 mM DPPH solution was added on each 4 mL concentration of the extract sample solution. The sample was then mixed using vortex and left for 26 minutes in a dark place at room temperature. The absorbance was then measured with UV-VIS spectrophotometer at λ_{max} = 516 nm. Quercetin was used as a positive control (1, 2, 3, 4 and 5 ppm concentration) which treated with the same treatment as the methanol extract.

Data analysis

The qualitative anatomical and flavonoid content data obtained were analyzed descriptively. Antioxidant activity test was analyzed by using a linear regression equation (y= ax+b) in order to obtain the IC_{50} score. The analysis of antioxidant activity which was determined by the magnitude of the DPPH radical uptake resistance was performed through the calculation of the DPPH uptake inhibition percentage by using the formula (Charisma et al. 2018) as follows:

$$\% \text{ Inhibition} = \frac{A_{blanco} - A_{exctarct} \times 100\%}{A_{blanco}}$$

Where:

Blanco A: 0.4 mM DPPH radical uptake in methanol at 515 nm wavelength

Sample A: 0.4 mM DPPH radical uptake which given the sample treatment in methanol at 515 nm wavelength

RESULTS AND DISCUSSION

Anatomical structure

The cross-section structures of young and old leaves showed almost the same in anatomical bifacial (dorsiventral) structure which composed of one-upper layered epidermis, the mesophyll (palisade and sponge), the xylem and phloem transport vessels, one layer of abaxial epidermis, globular oil cavity and non-glandular trichome (Figure 1.A). The young leaves have more trichomes than the old leaves. The outer cell wall of upper epidermis cells was covered with a thin cuticle. The epidermis cells are square-shaped and arranged tightly.

Leaf mesophyll tissue consisted of one layer of palisade and few layers of sponge tissues. Palisade is located beneath the upper epidermal tissue with columnar-shaped cells compactly arranged, and contains many chloroplasts. The sponge tissue lies beneath the palisade tissue, with shaped like branches cells, appearing to be disorganized, and irregular. The sponge tissue chloroplasts are fewer than those of palisade tissue. Vascular bundles are collateral type and bundle sheath extension in collateral bundle of lamina. In abaxial epidermis, many single-shaped, unicellular and uniseriate non-glandular trichomes were found. Kantachot et al. (2007) reported that *R. tomentosa* has bifacial leaf type where the leaves have one side palisade tissue. Adaxial–epidermal cell size is greater than those of abaxial epidermis. Hypodermis was not observed in leaf. There are unicellular, uniseriate, non-glandular trichomes and anomocytic type stomata in the abaxial epidermis. Midrib has a U-shaped pattern. There is crystal dress in the midrib parenchyma tissue. Oil cavities located close to both surfaces, globular, and lined with epithelial-like cells.

Anatomical studies are critical to know the structure of the organ, the cells, and the tissues that are possible to synthesize secondary metabolite compounds (Sharma et al. 2017). Each organ has its characteristic, which indicates the location of secondary metabolites synthesis. Nugroho (2017) stated that organs that accumulate secondary metabolites are not always the organs that synthesize them, even groups of the same compounds are synthesized in different organs in different plants. The skin or pericarpium of purple fruit of fruit *R. tomentosa* was detected as accumulation tissue of anthocyanin substance. Anthocyanin is a flavonoid group that may give red, blue and purple pigment in plants (Nugroho 2017)

Secretory cavity is spread on both sides of the leaf surface, on the inner side of the upper and abaxial epidermis tissue. Nugroho (2017) reported that lipophilic-producing secretory spaces are found in various families, such as Myrtaceae, Rutaceae, and Leguminosae. Kantachot et al. (2007) reported all species Myrtaceae have globular oil cavities in mesophyll that are underneath both epidermises.

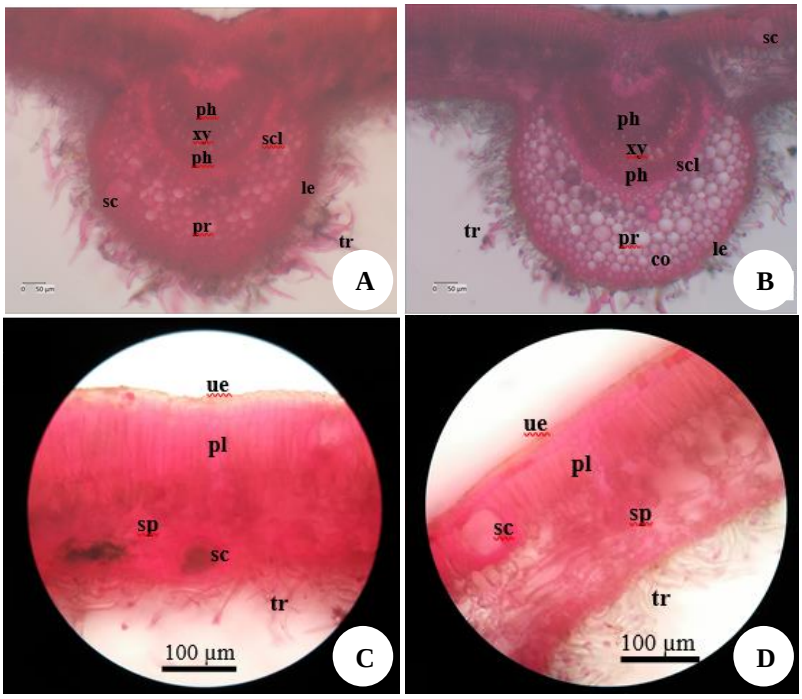

Figure 1. A. Cross-section of midrib young *R. tomentosa* leaves, B. cross-section of green *R. tomentosa* fruit, C. Cross-section of lamina young *R. tomentosa* leaves D. Cross-section of lamina old *R. tomentosa* leaves: upper epidermis (ue), palisade (pl), sponge (sp), phloem (ph), xylem (xy), sclerenchyma (scl), parenchyma (pr), lower epidermis (le), secretory cavity (sc), collenchyma (co), non glandular trichome (tr)

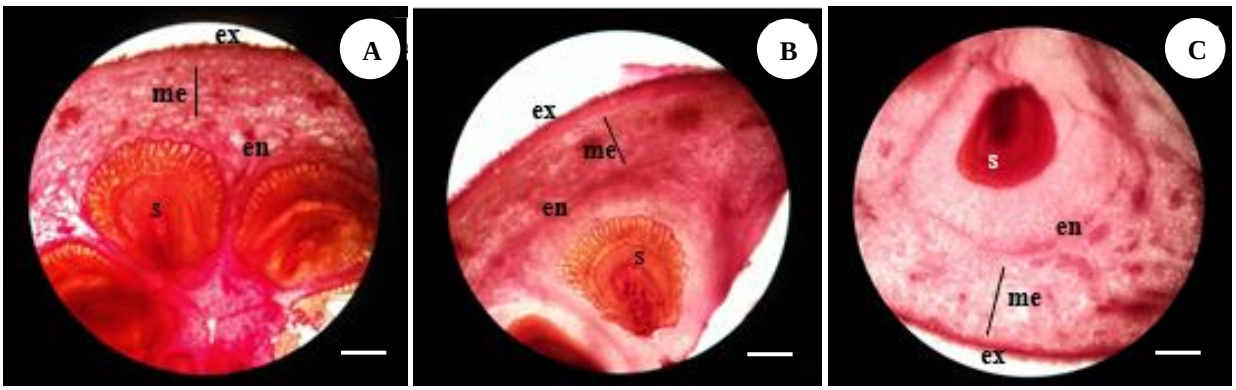

Figure 2. A. Cross-section of green fruit, B. Cross-section of red fruit, C. Cross-section of purple fruit: exocarp (ex) Mesocarp (me), Endocarp (en), seed (s). Scale bars = 600 µm

Table 3. The IC_{50} scores of the leaves and fruits of *R. tomentosa* methanol extract and quercetin

Test material name	IC_{50} (ppm)	Antioxidant activity
Young Leaf	14.67	Very strong
Old Leaf	19.86	Very strong
Green Fruit	48.36	Very strong
Red fruit	28.63	Very strong
Purple Fruit	12.98	Very strong
Quercetin	1.29	Very strong

Note: Molyneux (2004). IC50< 50 ppm: Very Strong, IC50 100-150 ppm: Medium, IC50 50-100 ppm: Strong, IC_{50} 151-200 ppm: weak

Transverse section of the green, red, and purple fruits shows similar anatomical structures. The cross-section structure of the fruits consists of pericarp fruit layer that can be divided into: exocarp/outer layer, mesocarp, and endocarp (Figure 2.A-C). *R tomentosa* fruit is a *bacca* fruit, which has three layers (exocarp, mesocarp, and endocarp). The thin outer layer (exocarp) is rather stiff and the thick inner layer (mesocarp), soft and runny, is the edible part. The seeds are 1.5 mm in diameter, at 6 pseudolocules separated by thin false septa (endocarp) (Hermanto et al. 2013; Hamid et al. 2017).

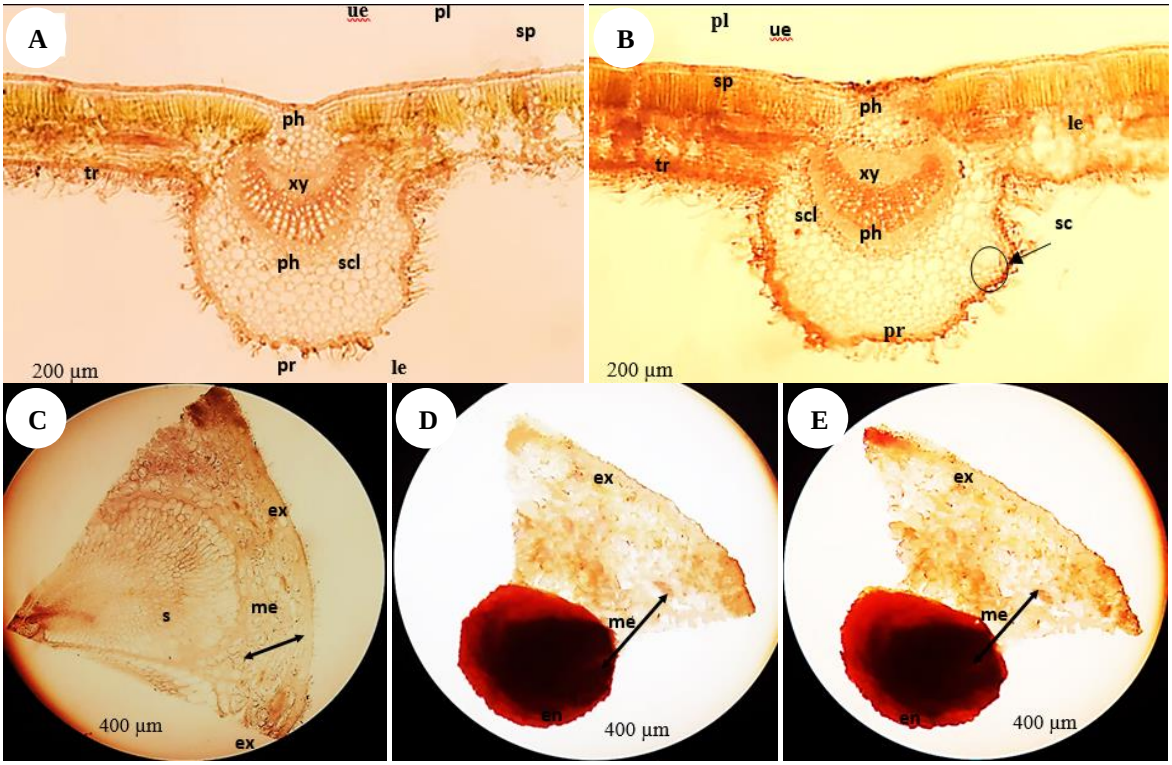

Figure 3. **A**. Cross-section of midrib young leaves**, B.** Cross-section of midrib old leaves, C. cross-section of green fruit, D. Cross-section of red fruit, E. Cross-section of purple fruit: upper epidermis (ue), palisade (pl), sponge (sp), phloem (ph), xylem (xy), sclerenchyma (scl), parenchyma (pr) lower epidermis (le), secretory cavity (sc), nonglandular trichome (tr), exocarp (ex) mesocarp (me), endocarp (en), seed (s)

Retamales et al. (2014) reported that anatomy of *Myrceugenia rufa* (Myrtaceae) fruit is almost the same with fruit of *R. tomentosa*. The pericarp of mature fruits is differentiated into three zones: exocarp, mesocarp, and endocarp. The exocarp is a unicellular layer, composed of irregular and plano-convex cells. A thin cuticle and numerous hairs cover the surface of this tissue. The mesocarp is composed of 7-8 layers of parenchymous cells, which are large and isodiametric in shape, with thin walls. Abundant and large secretory cavities are observed throughout this tissue. The endocarp is a thin tissue surrounding the seeds with sclerified cell walls.

Flavonoid content

The content of chemical compounds in plants can be histochemically observed. Histochemical tests showed that flavonoids gave yellow color when stained with Wilson's reagent (Dai et al. 1996). Flavonoid in leaves of *R. tomentosa* was found in both epidermis, mesophyll, vascular bundles, secretory cavity, and parenchymal midrib. Flavonoid in green, red and purple fruit was found in exocarp, mesocarp, and endocarp, and only green fruit seeds contain flavonoid (Figure 3.A-E ; Tables 1 and 2). Agustina et al. (2016) reported that flavonoids were stored in vacuole leaves mesophyll, glandular trichomes, exocarp, mesocarp and endocarp fruits of *Acalypha indica* L. and *Acalypha wilkesiana* Muell. Arg. Ferreira et al. (2011) also reported the histochemical evaluations of *Psidium guineense* Sw. leaves that performed the presence of compounds with proven therapeutic actions (such as flavonoids) distributed through epidermis, parenchyma and secretory cavities. Flavonoids are antioxidants that can efficiently capture oxygen-reactive substances as well as reduce and chelate ferric ions that catalyze lipid peroxidation.

Antioxidant Activity

Antioxidant activity of methanol extract of sample was carried out using the DPPH method, and measured using a UV-Vis spectrophotometer. DPPH free radical scavenger method was chosen due to its simplicity, ease, speed, sensitivity and the little amount of samples needed. The test sample ability to inhibit DPPH as free radical in methanol solution with the IC_{50} score (concentration of test extract capable of inhibiting free radical by 50%) was used as a parameter in determining the antioxidant activity of the extract tested. DPPH is a stable free radical which possesses purple color will turn to yellow compound when it reacts with antioxidant compounds. In the reaction, antioxidants release electrons to DPPH (Molyneux 2004).

The antioxidant activity of the young leaf methanol extract (IC_{50} = 14.67 ppm) is stronger than the old leaf (IC_{50} = 19.86 ppm) (Table 3). Young and old leaves were detected to be contained flavonoid compounds (Table 1),

but have different antioxidant activity. Rauf et al. (2017) reported that the powder tea from the younger avocado (*Persea americana* Mill.) leaves consisted of the higher the antioxidant activity compared to the old leaves powder tea. This is because young leaves have higher levels of total phenols and flavonoids than older leaves. Antioxidant activity increases with increasing levels of total phenols and flavonoids which are bioactive compounds that act as antioxidants. According to Ramadan (2015), the young leaves *Stelechocarpus burahol* have higher activity of PAL (*phenylalanine ammonia-lyase*) enzyme used in phenylalanine synthesis as precursors of more phenolic compounds at the beginning of growth, but as the leaf age in plants increase, PAL enzyme activity will decrease significantly.

The antioxidant activity of methanol extract of the purple fruit (IC_{50} = 12.98 ppm) is stronger than the red fruit (IC_{50} = 28.63 ppm) and the green fruit (IC_{50} = 48.36 ppm) When it compared with the positive control of quercetin (IC_{50} = 1.29 ppm), it showed that the antioxidant activity of leaf and fruit showed weaker antioxidant than those of quercetin. Cui et al. (2013); Liu et al. (2012); Wu et al. (2015) reported there is anthocyanin substance in skin and purple fruit *R. tomentosa*. Anthocyanin is a flavonoid group that may give red, blue and purple pigment in plants (Nugroho 2017). Anthocyanins have antioxidant activity, so this is an important group of compound to prevent many diseases related to oxidative stress (Agustina et al. 2016). Compared to other fruits and vegetables, the extract from *R. tomentosa* berries was rich in flavonoids by possessing more than 20 times of total flavonoids content than those in cranberry and even higher amounts than those in other berries and vegetables (Wu et al. 2015). Flavonoids act as an antioxidant because it is a good reductor so it can prevent oxidative stress (Pereira et al. 2009; Agustina et al. 2016).

In conclusion, the leaf bifacial (dorsiventral) consisted of upper epidermis, mesophyll (palisade and sponge), collateral vascular bundle, parenchyma midrib, abaxial epidermis, globular oil cavity and nonglandular trichome The histochemical test showed flavonoid could be found in the leaves and fruits of *R. tomentosa* and distributed in epidermis, mesophyll, vascular bundles, globular oil cavity, parenchymal midrib, exocarp, mesocarp, and endocarp. Extract of purple fruit had the highest antioxidant activity compared to other extract and young leaves extract had higher antioxidant activity than old leaves.

ACKNOWLEDGEMENTS

We very gratefully thank the reviewers for their valuable comments and suggestion for improving this manuscript. The authors would like to especially thank BUDI DN (*Beasiswa Unggulan Dosen Indonesia*) scholarship from LPDP (*Lembaga Pengelola Dana Pendidikan*) and Ministry of Research, Technology & Higher Education, the Republic of Indonesia for financially supporting this research. Thanks also address to Maria D. Astuti and Syamsiah for helping the research.

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