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The Morphoanatomy, Histochemistry, and Phytochemistry of the Leaves and Fruits of *Rhodomyrtus tomentosa*

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Abstract. ~~.~~ *Rhodomyrtus tomentosa* (Ait.) Hassk is a native ~~to~~plant in Southeast Asia~~. The entire plant~~, and it has been used as an ingredient in traditional Vietnamese, Chinese, and Malaysian medicine for a long ~~time. Bioactive substances are abundant in~~period. Its ripe fruits~~. However,~~ also contain several bioactive substances, but only a few studies have examined the phytochemical changes and distribution ~~throughout~~during the leaf and fruit growth. ~~This~~Therefore, this study ~~evaluated~~aims to assess the morphoanatomy, histochemical, and phytochemical properties of *R. tomentosa* leaves and fruits. The cross-sectional anatomical structure of its leaves ~~included~~includes the epidermis, mesophyll, and carrier bundle, while the fruit ~~included~~consists of a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. ~~Flavonoids~~The results also showed the presence of flavonoids, tannins, terpenoids, and alkaloids~~ were found~~ in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves ~~contained~~had the highest total flavonoid concentration ~~(~~of 196 ± 1.77 mg QE/g~~),~~, while green fruit had the highest total phenol concentration ~~(~~of 97.70 ± 18.15 mg GAE/g~~),~~ ~~—~~. The results also revealed that the old leaves ~~contained~~had the highest total alkaloid ~~concentration (~~content of 13.22 ± 0.98~~%), and~~%, while the red fruit had the highest total tannin ~~concentration (~~content of 1.66 ± 0.15 mg GAE/g~~).~~.

Keywords: Karamunting, histochemical, flavonoid, leaves, fruits,

Running title: The Morphoanatomy, Histochemistry, and Phytochemistry of the Leaves and Fruits of *Rhodomyrtus tomentosa*

INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk~~.~~. is a prolific Southeast Asian evergreen shrub in the Myrtaceae family~~, has~~ with rose-pink blooms and dark purple edible bell-shaped fruits. This plant favors natural light and adapts quickly to soil conditions. ~~Moreover~~Furthermore, it requires little maintenance and is rarely affected by pests and ~~illnesses. Wounds~~diseases. *R. tomentosa* has also been used to treat wounds, colic diarrhea, heartburn, abscesses, discomfort, and gynecopathy ~~have been treated with *R. tomentosa*. It can also relieve discomfort.~~. In China, ~~they use~~ it is often used as medicine ~~to treat~~ for the treatment of urinary tract infections (Hamid et al. 2017). Modern pharmacological studies ~~discovered~~revealed that ~~*R. tomentosa*~~the plant has antibacterial, anticancer, anti-inflammatory, and antioxidant effects. In Indonesia, ~~*R. tomentosa*~~its roots and leaves can be used to treat diarrhea, ~~stomachaches~~stomach aches, and postpartum recovery. Most of these effects ~~match~~are similar to those of the typical *R. tomentosa* ~~usage~~ (Zhao et al. 2019). Epidemiological studies have shown that eating fruits and vegetables ~~may protect~~has protective effects against chronic diseases, such as cardiovascular disease, type 2 diabetes, and cancer, as well as other health benefits. Phytochemicals, particularly phenolics, are responsible for ~~fruit and vegetable~~these health benefits~~. According to recent findings,~~ obtained from fruits and vegetables. Recent studies revealed that *R. tomentosa* berries contain ~~over~~more than 19 phenolics, including anthocyanins, phenolic acids, flavonols, ellagitannins, and stilbenes. ~~Due~~They are also a regional favorite due to their nutritional value and taste~~, *R. tomentosa* berries are a regional favorite~~ (Zhao et al. 2017).

Histochemical analyses of fruit development can ~~help~~ explain how metabolites affecting color and flavor are synthesized and accumulated during plant organ development. Histological studies ~~can~~are often used to assess the morphological and anatomical ~~outcome~~structure of *R. tomentosa* leaves and fruits. However, most of them did not address the secondary metabolite localization. ~~Particular~~Some staining ~~and~~ procedures can reveal metabolites, including pectin, lipids, lignin, proteins, phenolic compounds, tannins, starch grains, essential oils, rubber, and terpenoids (Vio-Michaelis et al., 2020). Unequivocal botanical characterizations ~~are~~also needed ~~to identify~~ for the proper identification of medicinal plants ~~correctly. Then, the present~~. Therefore, this study ~~evaluated~~aims to assess the ~~leaves' and fruits'~~ morphoanatomy, histochemistry, and phytochemistry of *R. ~~tomentosa~~tomentosa*'s fruits and leaves.

Style Definition ...

MATERIALS AND METHODS

Plant materials

The 2nd and 10th leaves from the stem tip ~~and~~as well as the green, red, and purple fruits of *R. tomentosa* were collected from wild plants in Banjarbaru, South Kalimantan, Indonesia ~~(~~with coordinates of 3°29′0″S, 114°52′0″E~~)~~ ~~(~~, as shown in Figure 1~~)~~. Three replicate samples of leaves and fruit were collected from three different plants. Furthermore, identification of the samples was carried out at the Herbarium Bogoriense at the Indonesian Institute of Sciences in Bogor, Indonesia~~, identified the plant specimen~~.

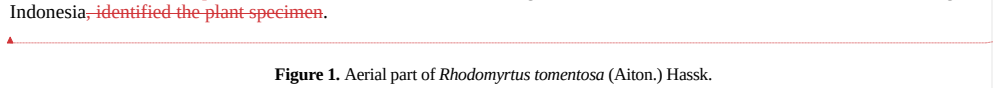

Figure 1. Aerial part of *Rhodomyrtus tomentosa* (Aiton.) Hassk.

Procedures

Morphoanatomy slides preparation

Ruzin (1999) ~~described~~reported that the transverse sections of leaves and fruit were made using paraffin embedding with safranin staining. Minor adjustments were made to the immersion duration ~~in~~during dehydration and dealcoholization. ~~Three~~Furthermore, three replicates of fruits and leaves from three different plants were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70% 5:5:90) solution for 24 hours. ~~These replicates~~They were then dehydrated, stored in 70% ethanol, and embedded in paraffin. Sections with a thickness of 8 to 10 µm ~~thick sections~~ were obtained ~~on~~with a rotatory microtome, after which they were dyed with Safranin, and mounted in Entelan to serve as permanent slides.

Histochemical test

Fresh~~,~~ and fully developed leaves ~~(~~from the central nerve region and ~~the~~ blade~~)~~ were cross-sectioned and ~~subjected to~~used for the histochemical test. ~~Hand~~Furthermore, hand-cut sections were treated with a dilute 10% aqueous acidic $FeCl_3$ (Sigma–Aldrich, Germany) solution containing a small quantity of Na_2CO_3 (Sigma–Aldrich, Germany) ~~solution~~, mounted in clove oil for phenolic compounds, which were identified as dark green or black color in tissues (Dhale, 2011). ~~Flavonoids~~The flavonoids in the samples were identified using Wilson's reagent. Each segment was soaked in citric acid:boric (Prolabo) (5:5 w/w~~)~~), which was placed in 100 mL absolute ethanol for 15 minutes~~,~~. The samples were then mounted in glycerine–~~:~~water~~,~~ and ~~inspected~~examined under a light microscope (Olympus, Tokyo, Japan). The yellow color ~~signified~~indicates the presence of flavonoids (Dai et al. 1996). The presence of these compounds in cells or tissues was also confirmed when the 5% Copper acetate ($Cu_2(CH_3COO)_4$) stains terpenoids yellow-brown~~, indicating their presence in cells or tissues~~ (Harborne, 1987; Martin et al. 2002). $FeCl_3$ (0.5% - 1% $FeCl_3$ in 0.1 N HCL) stained tannins blackish green, ~~suggesting~~which indicates their ~~existence~~presence (Dhale, 2011). Wagner's reagent ~~detected~~was used to detect alkaloids, ~~and~~while red-brown tissues or cells ~~indicates~~show alkaloid compounds (Furr & Mahlberg, 1981). The material was then photographed using OptiLab and a light microscope (Olympus, Tokyo, Japan).

Crude extract preparation

The 2nd –and 6th young ~~and~~as well as 7th –and 12th old leaves were picked from the tip shoot of *R. tomentosa*, while the green, red, and purple fruits were dried in an oven at 40°C before being ~~powdered~~converted to powder at room temperature. Furthermore, 500 g of each powdered ~~material~~sample was macerated for 24 hours in ~~1000~~1,000 mL of ethanol (SmartLab, Indonesia). After 24 hours, the solvent was discarded and replenished thrice (Kusuma et al. 2016). ~~Finally,~~The extracts from the same samples were pooled, mixed, filtered, and dried using a rotary evaporator. The extract yield was calculated ~~by this~~using the equation: (weight of ethanol extract/weight of dried leaves/fruits) × 100%.

Total phenolic compounds

Folin-Ciocalteu colorimetry ~~measured~~was used to measure the triplicates' total phenolic content (TPC) (Roy et al. 2018). The sample's extract absorbance was measured ~~with~~using a UV/Vis spectrophotometer (spectrophotometer UV 1800–Shimadzu, Japan) at 760 nm~~, and~~. Subsequently, the total phenol content was calculated ~~using~~with the standard gallate acid (Merck, Germany) at mg GAE/g dry weight.

Total flavonoids content

Total flavonoid content was determined using AlCl colorimetry~~.~~, where 1 mL of sample extract and 4 mL of distilled water were mixed in a test tube with 0.3 mL of 5% sodium nitrite (Sigma–Aldrich, Germany). After 5 minutes, 0.3 mL of 10% ~~aluminum~~aluminium chloride (Sigma–Aldrich, Germany) was added ~~and stirred. After 5 minutes,~~ to the mixture, followed by stirring. A total of 2 ml of sodium hydroxide (1 M) was also added~~, and~~ after 5 minutes, followed by distilled water ~~was added~~ until the volume was precisely 10 mL. The ~~identical~~ method used for the ~~earlier~~previous measurement was also used to ~~gauge~~assess the sample's extract absorbance at 510 nm. ~~Finally, the~~The total flavonoid content was determined using a Quercetin standard curve (Sigma–Aldrich, Germany) at mg QE/g dry weight (Roy et al. 2018).

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Total tannin content

The Folin-Ciocalteu method was used to calculate the total tannin~~. The following ingredients were added to 0.1 mL of sample extract: 7.5 mL of~~ in the samples. A total of 7.5 mL distilled water, 0.5 mL of 10% u F-C reagent, and 1 mL of 35% sodium carbonate solution were added to 0.1 mL of the extract (Sigma–Aldrich, Germany). ~~Then, using distilled water~~Subsequently, the volume of the mixture was increased to 10 mL~~. After being mixed, the mixture was~~ using distilled water and then left at room temperature for 30 minutes. The sample's extract absorbance was determined at a wavelength of 725 nm ~~using~~with the same method ~~as~~used for the ~~earlier~~previous measurement. ~~Finally, the~~The total tannin content was ~~determined~~evaluated using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight (Roy et al. 2018).

Total alkaloids content

A total of 2.5 g of the sample extract was diluted in 50 mL of 10% acetic acid (in ethanol). ~~After being~~ The solution was shaken with a magnetic stirrer for 4 hours~~, the solution was~~ and then filtered. ~~After that~~Subsequently, the filtrate ~~is~~was evaporated~~. Then,~~ and ammonium hydroxide (Sigma-Aldrich, Germany) was drip-applied until an alkaloid precipitate was formed. The precipitate-filtering filter paper was ~~weighted~~weighed before ~~usage. After that,~~it was used for the process. The residue was filtered and then cleaned with ~~an~~a 1% ammonium hydroxide ~~1%~~ solution. The filter paper containing the precipitate was oven dried for 30 minutes at 60°C. After the residue ~~had~~was cooled, it was weighed until a constant weight was obtained. The weight of the alkaloid precipitate ~~acquired~~recorded from the sample's initial weighing was used to calculate ~~the alkaloid~~its yield (Alasa et al. 2017).

Data analysis

The results of three replications were used to calculate the means and standard deviations (mean ± SD). ~~Using~~ The data obtained in this study were analyzed using the IBM SPSS statistics 21 and Microsoft Excel~~®, the~~®. The quantitative data were ~~statistically~~ evaluated using a one-way analysis of variance, with significant differences set at $p < 0.05$. When a different result was observed, a variance study was ~~followed by~~carried out with an LSD post hoc test.

RESULTS AND DISCUSSION

Morphoanatomy leaves and fruits *R. tomentosa*

Young and old *R. tomentosa* leaves have similar epidermal, mesophyll, and transport bundle structures. The epidermis consists of two parts, namely the adaxial and abaxial epidermis ~~comprises the epidermis. Compared to the abaxial epidermis,~~, where the adaxial is larger. The mesophyll ~~comprises palisade tissue and~~ contains spongy~~,~~ and single-layer palisade ~~tissue~~ tissues that are grouped compactly. The leaves are dorsiventral in shape~~, having~~ with palisade tissue on the adaxial side. The leaf stalk's carrier bundle network ~~is made up~~consists of the phloem and xylem. The leaf bone has parenchyma tissue as filling ~~and~~as well as collenchyma under the epidermis as reinforcement. Trichomes cover the leaves' lower epidermis, while a thin cuticle covers the upper ~~epidermis (~~part, as shown in Figure 2~~).~~.

The fruit grows from fertilization~~. The growth of fruit~~ along with its components~~,~~ such as ~~fruit~~ skin (pericarp), seed coat, endosperm, and embryo ~~follow fruit development~~. Bacca/buni fruit of *R. tomentosa* has exocarp, mesocarp, and endocarp layers. The cross-section of green, red, and purple fruit ~~displays~~shows a ~~fruit~~ pericarp layer with an outer exocarp~~/outer layer~~, middle mesocarp, and inner endocarp~~. (Fig.~~, as shown in Figure 3~~).~~. The stiff exocarp has a polyhedral epidermis and several sclerenchyma layers~~. The~~, while the edible mesocarp has big~~,~~ and watery parenchyma cells. The endocarp cells are thin-walled~~. The~~, and the 1.~~5mm~~5 mm seeds have 6 pseudo-locules with thin false septa (Hermanto et al. 2013; Hamid et al. 2017).

Histochemical

~~-~~Histochemical tests are often used to measure the secondary metabolites of plants. Furthermore, the ~~plant. Histochemical~~ tests ~~use~~used qualitative analysis to detect compounds without measuring their concentration (Dhale, 2011). Secondary metabolite components~~,~~ such as phenols, flavonoids, tannins, terpenoids, and alkaloids ~~are~~were found in tomentosa's leaves and fruits at specific locations or nearly everywhere. Secondary metabolites can be detected histochemically~~. After providing the reagent, the identified compound will color change~~ based on their reaction color with some reagent.

Histochemical tests on young and old leaves, as well as green, red, and purple fruit ~~indicated~~showed favorable reactions to the flavonoid compounds (4f-4j) and phenols (Figure 4k-4o). Young and old leaves have phenolic~~,~~ and flavonoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory

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space, and trichomes. ~~Fruit phenolic and flavonoid compounds~~They were ~~found~~also present in the exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds of the fruit.

Figure 4p-4t shows alkaloid compounds in ~~young and old leaves in~~ the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes~~. of the young and old leaves.~~ In green and red fruit, ~~alkaloids~~they were found in the endosperm, exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

~~Figure 4u-4y shows tannin compounds in young and old leaves~~Based on Figure 4u-4y, there were tannin compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals of both leaf samples. They were also present in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichome, and seeds of the fruits, except the purple fruit.

Figure 4z-4d1 shows the terpenoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals~~. Tannin compounds~~ of the young and old leaves. They were also found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, ~~trichome~~trichomes, and seeds~~, but~~ of the fruits. However, terpenoid compounds were not present in the endocarp of purple fruit.

~~Figure 4z-4d1 shows terpenoid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Terpenoid compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Terpenoid compounds was not found in the endocarp of purple fruit.~~

Phytochemistry

The ~~measurement results of the~~ highest total flavonoid levels ~~in~~ of 196.0 mg QE/g were recorded in the young leaves ~~were 196.0 mg QE/g,~~ followed by green fruit and old leaves, as well as red and purple fruits, namely 164.625 mg QE/g, ~~and~~ 134.875 mg QE/g, ~~followed by flavonoid levels in red and purple fruits as many as~~ 84.625 mg QE/g, and 55.75 mg QE/g. ~~The results of the measurement of~~, respectively. Furthermore, the highest total phenol content ~~were~~of 97.70 mg/mL was recorded in the green fruit, followed by old leaves at 59.30 mg/mL, young leaves at 54.40 mg/mL, red fruit at 19.40 mg/mL, and the lowest was purple fruit ~~as much as~~with 17.23mg/mL. The ~~measurement results of the~~ highest total alkaloid content of 13.226% was found in the old leaves ~~were 13.226%, ,~~ while values of 9.276%, 7.798%, 7.046%, and 5.64% were obtained from the green ~~fruit at 9.276%, ,~~ red ~~fruit at 7.798%, ,~~ and purple ~~fruit at 7.046%, and~~ fruits, as well as young leaves ~~at the lowest at 5.64%. The measurement results of~~. The result also revealed that red fruit had the highest total tannin content ~~in red fruit were~~of 1.658 mg GAE/g, followed by purple fruit ~~,~~ young and old leaves at 1.402 mg GAE/g, ~~young leaves~~ 0.943 mg GAE/g, ~~old leaves~~and 0.880 mg GAE/g, respectively. The lowest of 0.804 mg GAE/g was recorded in the ~~lowest tannin content in~~ green fruit ~~was~~, as ~~much as 0.804 mg GAE/g. (~~shown in Table 1~~).~~

Table 1. Total amount of compounds in ethanolic extract of leaves and fruits of R. tomentosa

Sample	Total Flavonoid (mgQE/g)	Total phenolic (mgGAE/g)	Total alkaloid (%)	Total tanin (mgGAE/g)
Young leaves	**196 ± 1.77^{e}**	**54.40 ± 0.09^{b}**	**5.64 ± 0.20^{a}**	**0.94 ± 0.74^{a}**
Old leaves	**134.88 ± 6.89^{c}**	**59.30 ± 16.73^{b}**	**13.22 ± 0.98^{d}**	**0.88 ± 0.01^{a}**
Green fruits	**164.63 ± 2.65^{d}**	**97.70 ± 18.15^{c}**	**9.28 ± 0.33^{c}**	**0.80 ± 0.30^{a}**
Red fruits	**84.63 ± 2.30^{b}**	**19.40 ± 0.47^{a}**	**7.80 ± 0.97^{bc}**	**1.66 ± 0.15^{c}**
Purple fruits	**55.75 ± 0.71^{a}**	**17.23 ± 4.20^{a}**	**7.05 ± 0.41^{ab}**	**1.40 ± 1.20^{b}**

Note: The data presented are the mean ± standard deviation. One-Way ANOVA and LSD test with = 0.05 were used to evaluate data. Numbers followed by different superscript letters in the same column show significantly different results.

Discussion

~~According to research by~~ Al-Edany and Al-Saadi (2012) ~~on~~who studied the anatomical characteristics of several Myrtaceae species~~,~~ revealed that the upper surface of ~~the~~ epidermal tissue on ~~the~~their leaves ~~of the Myrtaceae family is~~was covered by a variable-thickness cuticle layer. ~~Young~~Furthermore, young and old Karamunting leaves have one layer of epidermal tissue on the upper surface (adaxial). The epidermal tissue's cells are square in shape and are closely packed. Al-Edany and Al-Saadi (2012) ~~found~~reported that Myrtaceae leaf epidermal tissue is square or rectangular and uniseriate. ~~Epidermal tissue~~They protects plant organs by minimizing the rate of transpiration with ~~its~~their water-resistant cuticle and wax (Fahn, 1991).

Kantachot et al. (2007) examined the leaves of 28 species in five genera of the Myrtaceae family and found that *R. tomentosa* had dorsiventral leaves~~, which have~~ with palisade tissue on only ~~on~~ one side. The cells in the upper epidermis are thicker than those in the lower epidermis~~. The leaves of *R. tomentosa* do not~~, and they have ~~a~~no hypodermis. The lower epidermis ~~of the leaves~~ has unicellular trichomes and anomocytic stomata. In the cross-section of the leaf stalk and petiole,

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vascular tissue ~~forms~~formed a U-shaped pattern~~. Drusen~~, while drusen-shaped Ca oxalate crystals ~~are seen~~can be observed in the leaf bone maternal parenchyma tissue.

Karamunting leaves have palisade/pole and spongy mesophyll tissue. ~~The~~ Furthermore, the palisade parenchyma ~~is~~can be found beneath the upper epidermis~~. The~~, and its cells ~~that comprise the palisade parenchyma tissue~~ are ~~formed like pillars, appear~~ tightly packed, ~~and are just~~ one layer thick~~. There are many~~ and appear like a pillar. It also contains several chloroplasts ~~in this palisade parenchyma tissue. The~~, and the spongy tissue is located beneath the palisade ~~tissue~~. The cells ~~that make up~~in the spongy tissue ~~are formed~~appear like branches, ~~appear~~ loosely organized, and ~~are~~ uneven. ~~Spongy~~The spongy parenchyma ~~tissue~~ contains lesser number of chloroplasts~~, although less than palisade/pillar tissue. According~~ compared to the palisade. Al-Edany and Al-Saadi (2012~~),~~) revealed that Myrtaceae leaves' mesophyll tissue has 1 to 2 layers of palisade ~~tissue and~~. It also has several rows of up to 15 layers of compact and loose spongy tissue or 2 layers of palisade and spongy tissue on the adaxial ~~surface~~ and ~~spongy tissue on the~~ abaxial surface, respectively.

Karamunting leaves have bicollateral vascular bundles consisting of xylem bundles flanked by phloem. Ferreira et al. (2011) ~~describe~~revealed that the bundle of vessels in *Psidium guineense* Sw. (family Myrtaceae) leaves include xylem flanked by phloem. ~~The xylem and phloem~~ These components function as a delivery system for vascular plants~~. The~~, where the primary function of the xylem is to transport water and solutes, while ~~the primary function of the~~ phloem ~~is to distribute~~distributes photosynthesis products (Fahn, 1991).

~~Karamunting leaf~~ The epidermal tissue on the abaxial ~~side~~surface of the leaf sample had unicellular trichomes. Furthermore, Kantachot et al. (2007) identified abundant unicellular and uniseriate trichomes on the leaves of Myrtaceae species, such as *Decaspermum parviflorum*, *Melaleuca cajuputi*, *Psidium guajava*, *Rhodamnia dumetorum*, *Tristaniopsis burmanica* var. *rufescens*, and *R. tomentosa*. Trichomes from modified epidermal cells reduce evaporation~~, stimulate,~~ and minimize animal disturbances (Nugroho et al. 2006).

Retamales et al. (2014) ~~found~~ revealed that the fruit anatomy of *Myrceugenia rufa* (Myrtaceae) ~~fruit anatomy~~ was similar to that of *R. tomentosa*. The ripe fruit's pericarp has three zones~~:~~, namely exocarp, mesocarp, and endocarp. The exocarp cells are irregular and plano-convex~~. The~~, while the cuticle is thin and covered ~~in~~with hair. The mesocarp comprises 7-8 layers of large and isodiametric parenchyma cells with thin walls~~. Multiple~~, as well as multiple and large secretory cavities ~~are observed throughout this tissue~~. The endocarp is a delicate tissue that surrounds the seed.

Histochemical test results by Agustina et al. (2016) ~~stated~~revealed that flavonoids and phenols ~~are found~~were present in the stem, leaf, and flower-fruit-seed organs of *Acalypha indica*. Phenol in *Acalypha wilkesiana*, ~~is~~was also distributed in the xylem ~~in~~of the stem organs, leaf mesophyll, and ~~in the~~ flower-fruit seed.

Flavonoids can be found in every plant organ, including the leaves, roots, wood, flowers, nectar, and seeds. ~~Flavonoids can be~~These compounds are often stored in cell walls, vacuoles, and glandular trichomes. Lipophilic compounds generated by glandular trichomes include terpenoids, lipids, waxes, and flavonoid aglycones. Flavonoids ~~concentrate~~were concentrated in the cells ~~and are not present~~, but were absent in the intercellular space, ~~resulting in~~and this led to a symplast distribution (Petrussa et al., 2013).

~~According to~~ Cartea et al. (2011~~),~~) stated that phenolic compounds are widespread phytochemicals. ~~Structure-wise~~Based on their structure, they are simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. ~~Flavonoids~~Furthermore, flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids are ~~plants'~~the most abundant and diversified polyphenols~~. Flavonoids are the most prevalent~~ in plants. The results also revealed that flavonoids were the dominant phenolics and ~~are~~were found ~~throughout~~in all ~~plants. Flavonoids in~~ parts of the plant. In the leaf and fruit epidermis ~~protect~~, they have a protective effect against UV radiation and pigment, stimulate nitrogen-fixing nodules, and ~~fight disease~~prevent diseases.

The ~~same~~ study also found positive reactions in leaf mesophyll cells, pith parenchyma, and stem cortical parenchyma cells of *Adhatoda zeylanica* (Acanthaceae), *Ruta graveolence* Linn. (Rutaceae), and *Vitex negundo* Linn. (Verbenaceae) (Dhale, 2011). Alkaloids are heterocyclic nitrogen-containing chemicals ~~utilized in medicine~~, which are often used as medicinal ingredient.

~~Alkaloids~~These compounds protect plants from insects and herbivores, detoxify toxins, and store nitrogen. ~~Much human consumption contains alkaloids~~They are also present in several foods consumed by humans, such as coffee, tea, cocoa, tobacco, areca nut, and marijuana. Alkaloids have neuroprotective, blood pressure-lowering, pain-relieving, antibacterial, anticancer, and antimalarial effects (Carqueijeiro et al., 2013). ~~Alkaloids in~~In *R. tomentosa's* leaves and fruits, they support its historic medicinal usage in Singapore. ~~In China, the~~The leaves were used as a painkiller~~,~~ in China, while the roots ~~for~~was developed for the treatment of stomach ulcers, and the seeds for digestion and snake bites. In Indonesia, ~~the leaves~~they were used to ~~cure wounds. In traditional Vietnamese medicine, the~~facilitate the wound healing process. The plant's raw fruit ~~treats~~was used to treat diarrhea and dysentery in Vietnam, while the ripe ~~ones~~variant can boost the immune system (Zhao et al., 2019).

~~According to Ferreira et al. (2011), histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves indicated positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. These tannins in *R. tomentosa* leaves and fruits help treat diarrhea and dysentery. Ferreira et al. (2011) added that Myrtaceae leaves are used to treat diarrhea and intestinal infections due to their astringent tannin content. This plant also fights bacteria, tumors, and malaria. Tannins protect plants, which is why epidermal cells have them. Myrtaceae plants have tannins in their vascular systems also.~~

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Ferreira et al. (2011) who carried out histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves reported positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. The presence of these compounds in *R. tomentosa* leaves and fruits is responsible for its ability to treat diarrhea and dysentery. Ferreira et al. (2011) also revealed that Myrtaceae leaves were used to treat diarrhea and intestinal infections due to their astringent tannin content. This plant also fights bacteria, tumors, and malaria. Epidermal cells have high levels of tannins due to their ability to protect plant. Previous studies revealed that Myrtaceae plants also have these compound in their vascular system.

R. tomentosa meroterpenoids ~~are~~have both antibacterial and antifungal. ~~Myrtaceae~~Furthermore, the secondary metabolites ~~like~~ of Myrtaceae, such as meroterpenoids have ~~been~~ extensively been explored as antibiotics. ~~Thus,~~This shows that *R. tomentosa* ~~could~~can prevent microbial deterioration and extend food shelf life as a biocontrol agent and natural preservative (Zhao et al., 2019). Meroterpenoid compounds have been isolated and identified from various Myrtaceae species, including *Eucalyptus tereticornis* (Liu et al., 2018) and *Leptospermum brachyandrum* (Zou et al., 2018).

Belwal et al. (2019) ~~found~~reported that flavonoids reduced ~~throughout~~during fruit ripening in *R. ellipticus*, *M. esculenta*, *Prunus persica*, blueberry (*Vaccinium corymbosum*), and blackberry fruit~~, as did~~ . A similar reduction pattern was also observed by Lin et al. (2020) in blueberries. Flavonoid levels vary by organ type~~.~~, and this finding is consistent with Hou et al. (2021) ~~found~~ that phenols and flavonoids ~~drop~~decreased from 5.38 mg RE/g in young citrus fruits to 0.69 mg RE/g in ripe ~~fruit~~variants. The same trend was ~~seen~~also recorded in grapes and lemons (Zhu et al., 2020). This phenomenon ~~may~~can be produced by ~~many~~several enzymes involved in flavonoid synthesis, such as 1,2-rhamnosyltransferase and flavanone-7-O-glucosides, which accelerate biosynthesis in the early growth period. Insufficient flavonoid aglycones ~~may~~can explain the decrease in flavonoids during citrus fruit ripening.

Lai et al. (2013) ~~found~~observed a decrease in total phenol levels during *R. tomentosa* fruit ripening. The same pattern of declining phenol levels during ~~fruit~~ ripening ~~occurs~~also occurred in Myrtaceae plants, such as Brazilian cherry *Eugenia uniflora* L. (Celli et al., 2011). Belwal et al. (2019) ~~further explain~~reported that the accumulation and degradation of distinct phenolic compounds during ~~fruit ripening connected to~~the process is associated with their biosynthesis pathways, which are mainly regulated by enzyme expression ~~and~~as well as various genetic and environmental variables. Elmastaş et al. (2017) ~~added~~stated that phenol compounds are intermediate products of phenylpropanoid metabolic pathway ~~intermediates. Enzymes~~. Furthermore, enzymes convert aromatic amino acids into polyphenols~~. Enzyme~~, and their level of activity affects the production of these molecules. Genetic diversity, pH, light, temperature, and climatic change ~~affect enzyme activity.~~are influential factors of enzymatic functions. Fruit enzyme activity ~~might vary depending~~varies based on the level of plant growth and ripening.

The cross-sectional anatomical structure of leaves ~~included~~include the epidermis, mesophyll, carrier bundle, secretory cavity, and trichomes~~, while~~. Meanwhile, the fruit ~~included~~consists of a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. Flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves ~~contained~~had the highest total flavonoid concentration ~~(~~of 196 ± 1.77 mg QE/g~~)~~, while green fruit had the highest total phenol concentration ~~(~~of 97.70 ± 18.15 mg GAE/g~~), old leaves contained the~~. The highest total alkaloid concentration ~~(~~of 13.22 ± 0.98~~%), and~~% was obtained from old leaves, while red fruit had the highest total tannin concentration ~~(~~of 1.66 ± 0.15 mg GAE/g~~).~~.

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Manuscript Title

The Morphoanatomy, Histochemistry, and Phytochemistry of the Leaves and Fruits of *Rhodomyrtus tomentosa*

Author(s)

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[biodiv] Submission Acknowledgement

Ahmad Dwi Setyawan <smujo.id@gmail.com> Thu, Oct 20, 2022 at 12:03 AM
To: Evi <evimintowati@ulm.ac.id>

Evi:

Thank you for submitting the manuscript, "The Morphoanatomy, Histochemistry, and Phytochemistry of the Leaves and Fruits of Rhodomyrtus tomentosa " to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/12627
Username: evimintowati

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ahmad Dwi Setyawan

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Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>

[biodiv] Editor Decision

Nor Liza <smujo.id@gmail.com> Thu, Oct 20, 2022 at 10:54 AM
To: EVI MINTOWATI KUNTORINI <evimintowati@ulm.ac.id>

EVI MINTOWATI KUNTORINI:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "The Morphoanatomy, Histochemistry, and Phytochemistry of the Leaves and Fruits of Rhodomyrtus tomentosa ".

Our decision is: Revisions Required

Reviewer A:

Dear author,

Here are some comments for the paper titled 'The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa' :*

- This paper has too brief in the 'Introduction' part (it should consist of about 600-700 words)

- This manuscript has outdated references to be published in Biodiversitas Journal. Reference list should consist of at least 20 citations which 80% of international scientific journals published in the last 10 years (2012-2022), and a maximum of 10% references from national publication

- Please follow the guidance for reference writing (https://smujo.id/biodiv/guidance-for-author)

Best regards

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

4. First revised submission (20-10-2022)

- Manuscript Biodiversitas_Revision

The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

Abstract. *Rhodomyrtus tomentosa* (Ait.) Hassk is native to Southeast Asia. The entire plant has been used in traditional Vietnamese, Chinese, and Malaysian medicine for a long time. Bioactive substances are abundant in ripe fruits. However, few studies have examined phytochemical changes and distribution throughout leaf and fruit growth. This study evaluated the morphoanatomy, histochemical, and phytochemical properties of *R. tomentosa* leaves and fruits. The cross-sectional anatomical structures observed in the leaves included the epidermis, mesophyll, and carrier bundle, while the fruit structures observed included a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. Flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavities, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavities, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

Keywords: Karamunting, histochemistry, flavonoids, leaves, fruits, *Rhodomyrtus tomentosa*

Running title: The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk., a prolific evergreen shrub in the Myrtaceae family in southern and southeastern Asia, from India, east through southern China, the Philippines, and south to Malaysia and Sulawesi, has rose-pink blooms and dark purple edible bell-shaped fruits. In the Indonesian language it is known as “karamunting”, while in English it is most commonly called “rose myrtle”. This plant favors natural open light and adapts quickly to a range of soil conditions. Moreover, it requires little maintenance and is rarely affected by pests and illnesses. Most parts of this plant (leaves, roots, buds and fruits) have been used in traditional medicines in Vietnam, China and Malaysia for a long time. In traditional medicine of Vietnam, the unripe fruits of the plant are utilized to treat diarrhea or dysentery and ripe ones are used to stimulate the immune system (Zhao et al. 2019).Wounds, heartburn, abscesses, and gynecopathy have been treated with *R. tomentosa*. In China, they use it as a medicine to treat urinary tract infections (Hamid et al. 2017a). Modern pharmacological studies discovered that *R. tomentosa* has antibacterial (Zhao et al. 2018), anticancer (Zhang et al. 2016), anti-inflammatory (Na-Phatthalung et al. 2018), and antioxidant (Hamid et al. 2017b) effects. In Indonesia, *R. tomentosa* roots and leaves treat diarrhea, stomachaches, and postpartum recovery. Most of these effects match typical *R. tomentosa* usage (Zhao et al. 2019). Epidemiological studies have shown that eating fruits and vegetables may protect against chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer, as well as other health benefits (Del Rio et al. 2013). Phytochemicals, particularly phenolics, are responsible for fruit and vegetable health benefits. According to recent findings, *R. tomentosa* berries contain over 19 phenolics, including anthocyanins, phenolic acids, flavonols, ellagitannins, and stilbenes. Due to their nutritional value and taste, *R. tomentosa* berries are a regional favorite (Zhao et al. 2017).

Anatomical studies are essential taxonomic indicators, including determining where compounds are secreted and accumulated in a medicinal species' vegetative and generative organs, and important in the quality control of medicinal plants. Pharmacognosy studies of macroscopic and microscopic characteristics are needed for medicinal raw material authentication and for quality assessment in drug standardization. Among the various alternative plant medicinal sources, accurate plant authentication is required (Raghvendra et al. 2021). Various taxa of Myrtaceae have been the subject of anatomical studies (Ferreira et al. 2011; Al-Edany et al. 2012); however, there has been no histochemical anatomical study of the leaves and fruit of *R. tomentosa*.

Histochemical studies are needed to reveal the typical pattern of distribution of secondary metabolites in plant tissues. Secondary metabolites are distributed in plant tissues based on their functionality. At the cellular level, secondary metabolites can be located in specific secretory tissues or are distributed throughout the tissue's cells, including in the parenchyma of the transport bundle (Nugroho 2017).

Histochemical analyses of fruit development can help explain how metabolites affecting color and flavor are synthesized and accumulated during plant organ development. Histological studies can assess the morphological and anatomical details of *R. tomentosa* leaves and fruits. However, studies have not usually addressed secondary metabolite localization in this species. Particular staining and procedures can reveal metabolites, including pectin, lipids, lignin, proteins, phenolic compounds, tannins, starch grains, essential oils, rubber, and terpenoids (Vio-Michaelis et al. 2020). Unequivocal botanical characterizations are needed to identify medicinal plants correctly. Thus, the objective of this present study was to evaluate the leaves' and fruits' morphoanatomy, histochemistry, and phytochemistry of a sample of identified karamunting (*R. tomentosa*) plants collected in South Kalimantan, Indonesia.

MATERIALS AND METHODS

Plant materials

The 2nd and 10th leaves down from the stem tip and the green, red, and purple fruits of *R. tomentosa* were collected from wild plants in Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) (Figure 1). Three replicate samples of leaves and fruit were collected from three different plants. Herbarium Bogoriense at the Indonesian Institute of Sciences in Bogor, Indonesia, identified the plant specimen.

Figure 1. Aerial part of *Rhodomyrtus tomentosa* (Aiton.) Hassk.

Procedures

Morphoanatomy slides preparation

Retamales et al. (2014) described the transverse sections of leaves and fruit using paraffin embedding with safranin staining. In the present study, m~~M~~inor adjustments were made to the immersion duration in dehydration and dealcoholization. Three replicates of fruits and leaves from three different plants were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70%, 5:5:90) solution for 24 hours. These replicates were then dehydrated, stored in 70% ethanol, and embedded in paraffin. Sections 8 to 10 µm thick were obtained on a rotatory microtome, dyed with Safranin, and mounted in Entelan to serve as permanent slides.

Histochemical test

Fresh, fully developed leaves (from the central nerve region and the blade) were cross-sectioned and subjected to histochemical test. Hand-cut sections were treated with a dilute 10% aqueous acidic $FeCl_3$ (Sigma–Aldrich, Germany) solution containing a small quantity of Na_2CO_3 (Sigma–Aldrich, Germany) solution mounted in clove oil for phenolic compounds, which were identified as dark green or black color in tissues (Trimanto et al. 2018). Flavonoids were identified using Wilson's reagent. Each segment was soaked in citric acid:boric (Prolabo) (5:5 w/w) in 100 mL absolute ethanol for 15 minutes, mounted in glycerine–water, and inspected under a light microscope (Olympus, Tokyo, Japan). ~~The~~ A yellow color signified the presence of flavonoids (Pratiwi et al. 2020). With 5% copper acetate ($Cu_2(CH_3COO)_4$), terpenoids stained yellow-brown, indicating their presence in cells or tissues (Pratiwi et al. 2020). $FeCl_3$ (0.5% - 1% $FeCl_3$ in 0.1 N HCL) stained tannins blackish green, suggesting their existence (Robil and Tolentino 2015). Wagner's reagent was used to detect alkaloids, and any red-brown staining of tissues or cells indicated the presence of alkaloid compounds (Pratiwi et al. 2020). The material was photographed using OptiLab and a light microscope (Olympus, Tokyo, Japan).

Crude extract preparation

Young leaves ~~(~~2nd – 6th down from the tip) and old leaves (7th – 12th down from the tip) were picked from the shoot of *R. tomentosa* plants, while harvested green, red, and purple fruits were dried in an oven at 40°C before being powdered at

room temperature. A sample of 500 g of each powdered material was macerated for 24 hours in 1000 mL of ethanol (SmartLab, Indonesia). After 24 hours, the solvent was discarded and replenished thrice (Kusuma et al. 2016). Finally, extracts from the same samples were pooled, mixed, filtered, and dried using a rotary evaporator. The extract yield was calculated by this equation: (weight of ethanol extract/weight of dried leaves or fruits) × 100%.

Total phenolic compounds

Folin-Ciocalteu colorimetry was used to determine the triplicate samples' total phenolic contents (TPC) (Roy et al. 2018). The samples' extract absorbances were measured with a UV-Vis spectrophotometer (spectrophotometer UV 1800–Shimadzu, Japan) at 760 nm, and the total phenol content was calculated using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight.

Total flavonoids content

Total flavonoid content was determined using AlCl colorimetry. A 1 mL sample of extract was mixed in a test tube with 4 mL of distilled water and with 0.3 mL of 5% sodium nitrite (Sigma–Aldrich, Germany). After 5 minutes, 0.3 mL of 10% aluminum chloride (Sigma–Aldrich, Germany) was added and stirred. After 5 minutes, 2 ml of sodium hydroxide (1 M) was added, and distilled water was added until the volume was precisely 10 mL. The identical method used for the earlier measurement was used to gauge the sample's extract absorbance at 510 nm. Finally, the total flavonoid content was determined using a quercetin standard curve (Sigma–Aldrich, Germany) at mg QE/g dry weight (Roy et al. 2018).

Total tannin content

The Folin-Ciocalteu method was used to calculate the total tannin content. The following ingredients were added to 0.1 mL of sample extract: 7.5 mL of distilled water, 0.5 mL of 10% F-C reagent, and 1 mL of 35% sodium carbonate solution (Sigma–Aldrich, Germany). Then, using distilled water, the volume was increased to 10 mL. After being mixed, the mixture was left at room temperature for 30 minutes. The sample's extract absorbance was determined at a wavelength of 725 nm. Finally, the total tannin content was determined using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight (Roy et al. 2018).

Total alkaloids content

2.5 g of the sample extract was diluted in 50 mL of 10% acetic acid (in ethanol). After being shaken with a magnetic stirrer for 4 hours, the solution was filtered. After that, the filtrate was evaporated. Then, ammonium hydroxide (Sigma-Aldrich, Germany) was drip-applied until an alkaloid precipitate formed. The precipitate-filtering filter paper was weighed before usage. After that, the residue was filtered and cleaned with an ammonium hydroxide 1% solution. The filter paper containing the precipitate was oven dried for 30 minutes at 60°C. After the residue had cooled, it was weighed until a constant weight was obtained. The alkaloid yield was calculated by the weight percentage of the precipitated alkaloid obtained against the initial weight of the extract. (Alasa et al. 2017).

Data analysis

The results from three replications were used to calculate the means and standard deviations (mean ± SD). Using IBM SPSS statistics 21 and Microsoft Excel®, the quantitative data were statistically evaluated using a one-way analysis of variance, with significant differences set at $p < 0.05$. When a statistically significant difference was detected, the analysis of variance was followed by an LSD post hoc test.

RESULTS AND DISCUSSION

Morphoanatomy of *R. tomentosa* leaves and fruits

Young and old *R. tomentosa* leaves have similar epidermal, mesophyll, and transport bundle structures. The adaxial and abaxial epidermis comprises the epidermis. Compared to the abaxial epidermis, the adaxial is larger. The mesophyll consists of palisade tissue and spongy, single-layer palisade tissue grouped compactly. The leaves are dorsiventral in shape, having palisade tissue on the adaxial side. The leaf stalk's carrier bundle network is made up of phloem and xylem. The leaf bone has parenchyma tissue as filling and collenchyma under the epidermis as reinforcement. Trichomes cover the leave's lower epidermis, while a thin cuticle covers the upper epidermis (Figure 2).

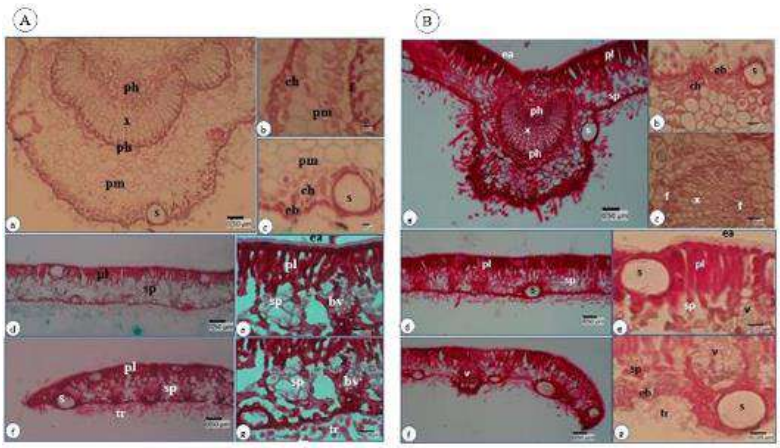

Figure 2. Cross-section of (A) young leaves, (B) old leaves *of R. tomentosa;* palisade mesophyll (pl), sponge mesophyll (sp), parenchyma midrib (pm), secretory cavity (sc), trichomes (tr), abaxial epidermis (eb), adaxial epidermis (ea), collenchyma (ch), vascular bundle (v), phloem (f), xylem (x)

The fruit grows from fertilization. The growth of fruit components, such as fruit skin (pericarp), seed coat, endosperm, and embryo, follows fruit development. *R. tomentosa* fruit is a bacca/buni 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 (Hamid et al. 2017a). The cross-section of green, red, and purple fruit displays a fruit pericarp layer with an exocarp/outer layer, middle mesocarp, and endocarp. (Fig. 3). The stiff exocarp has a polyhedral epidermis and several sclerenchyma layers. The edible mesocarp has big, watery parenchyma cells. The endocarp cells are thin-walled. The 1.5 mm seeds have 6 pseudo-locules with thin false septa (Hermanto et al. 2013; Hamid et al. 2017a).

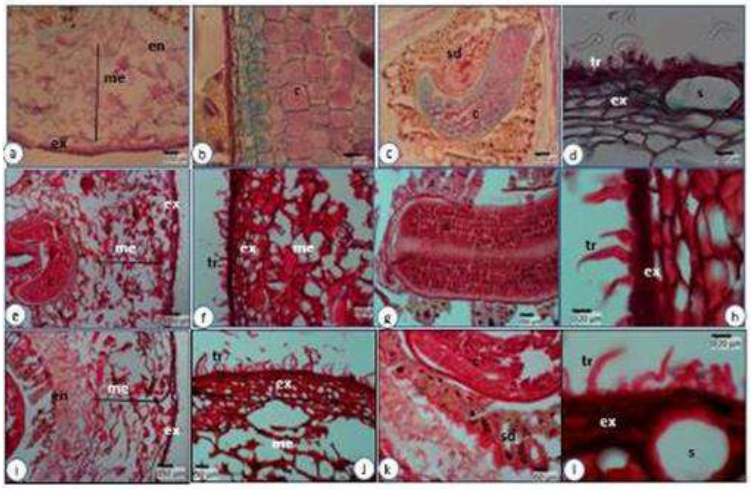

Figure 3. Cross-section of green fruit (a-d), red fruit (e-h) and purple fruit (i-l) of *R. tomentosa;* exocarp (ex) mesocarp (me), endocarp (en), seed (sd), secretory cavity (s), trichomes (tr)

Histochemical tests

Histochemical tests measure the secondary metabolites of the plant. Histochemical tests use qualitative analysis to detect compounds without measuring their concentration (Dhale 2011). Secondary metabolite components such as phenols, flavonoids, tannins, terpenoids, and alkaloids were found in *R. tomentosa*'s leaves and fruits at specific locations or more generally in the tissues. The secondary metabolites were detected histochemically. After application of reagents, the identified compounds showed specific color changes (Figure 4).

Histochemical tests on young and old leaves, green, red, and purple fruit indicated positive reactions to flavonoid compounds (4f-4j) and phenols (Figure 4k-4o). Young and old leaves had phenolic, and flavonoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. Fruit phenolic and flavonoid compounds were found in the exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4p-4t shows alkaloid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. In green and red fruit, alkaloids were found in the endosperm, exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4u-4y shows tannin compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Tannin compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichome, and seeds, in the green and red fruit but not in the purple fruit.

Figure 4z-4d1 shows terpenoid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Terpenoid compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Terpenoid compounds were not found in the endocarp of purple fruit.

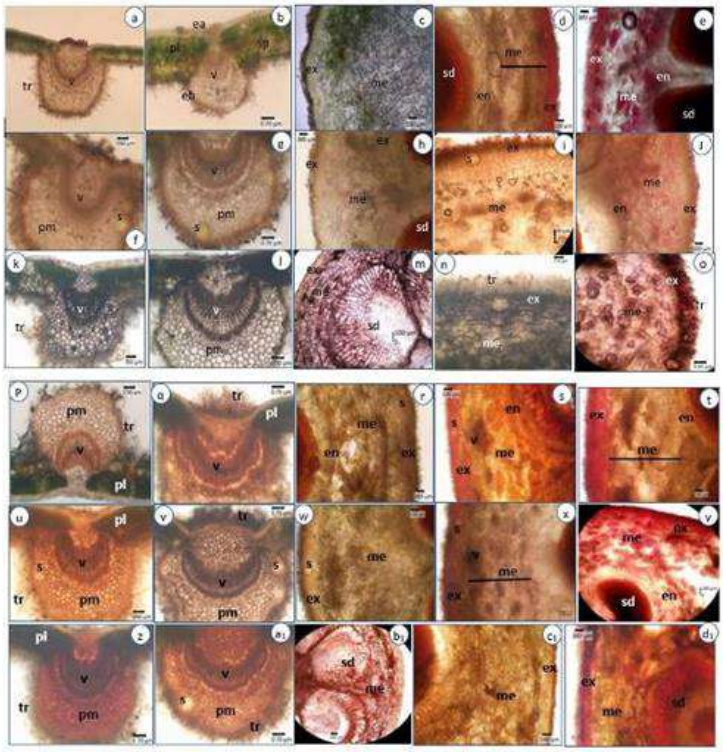

Figure 4. Transverse sections of histochemically stained leaves and fruits of *R. Tomentosa.* (a-e) Free-hand section of a young leaf without staining; (f-j) the positive reaction to Wilson's reagent indicating the presence of flavonoid in leaves and fruits; (k-o) the positive reaction to ferric chloride indicating the presence of phenolics in leaves and fruits: (p-t) the positive reaction to Wagner's reagent

indicating the presence of alkaloid in leaves and fruits; (u-y) the positive reaction to $FeCl_3$ indicating the presence of tannin in leaves and fruits; (z-d_1) the positive reaction to $Cu_2(CH_3COO)_4$ 5% indicating the presence of terpenoid in leaves and fruits: palisade mesophyll (pm), sponge mesophyll (sm), parenchyma midrib (pm), secretory cavity (s), trichomes (tr), druse crystal (dc), abaxial epidermis (eb), adaxial epidermis (ea) exocarp (ex) mesocarp (me), endocarp (en), seed (sd) vascular bundle (v).

Phytochemistry

The results of phytochemical measurements showed that the highest total flavonoid levels were in young leaves at 196.0 mg QE/g, followed by green fruit and old leaves, 164.625 mg QE/g, and 134.875 mg QE/g, followed by flavonoid levels in red and purple fruits at 84.625 mg QE/g and 55.75 mg QE/g, respectively. The highest measured total phenol content was 97.70 mg/mL recorded in green fruit, followed by old leaves at 59.30 mg/mL, young leaves at 54.40 mg/mL, red fruit at 19.40 mg/mL, and the lowest, in purple fruit at 17.23mg/mL. The highest total alkaloid content was recorded in old leaves at 13.23%, green fruit at 9.28%, red fruit at 7.80%, purple fruit at 7.05%, and young leaves, the lowest at 5.64%. For total tannin content, the measurements showed the highest value in red fruit at 1.658 mg GAE/g, followed by purple fruit at 1.402 mg GAE/g, young leaves at 0.943 mg GAE/g, old leaves at 0.880 mg GAE/g, and the lowest tannin content in green fruit at 0.804 mg GAE/g. (Table 1).

Table 1. Total amount of compounds in ethanolic extracts of leaves and fruits of *R. tomentosa*

Sample	Total flavonoids (mgQE/g)	Total phenolics (mgGAE/g)	Total alkaloids (%)	Total tannins (mgGAE/g)
Young leaves	196 ± 1.77^{e}	54.40 ± 0.09^{b}	5.64 ± 0.20^{a}	0.94 ± 0.74^{a}
Old leaves	134.88 ± 6.89^{c}	59.30 ± 16.73^{b}	13.22 ± 0.98^{d}	0.88 ± 0.01^{a}
Green fruits	164.63 ± 2.65^{d}	97.70 ± 18.15^{c}	9.28 ± 0.33^{c}	0.80 ± 0.30^{a}
Red fruits	84.63 ± 2.30^{b}	19.40 ± 0.47^{a}	$7.80 \pm 0.97b^{c}$	1.66 ± 0.15^{c}
Purple fruits	55.75 ± 0.71^{a}	17.23 ± 4.20^{a}	$7.05 \pm 0.41a^{b}$	1.40 ± 1.20^{b}

Note: The data presented are the mean ± standard deviation. One-Way ANOVA and LSD test with = 0.05 were used to evaluate data. Numbers followed by different superscript letters in the same column show significantly different results.

Discussion

According to research by Al-Edany and Al-Saadi (2012) on the anatomical characteristics of several Myrtaceae species, the upper surface of the epidermal tissue on the leaves of the Myrtaceae family is covered by a variable-thickness cuticle layer. Young and old leaves of karamunting (*Rhodomyrtus tomentosa*) have one layer of epidermal tissue on the upper surface (adaxial). The epidermal tissue's cells are square and closely packed. Al-Edany and Al-Saadi (2012) found that the cells of Myrtaceae leaf epidermal tissue are square or rectangular and uniseriate.

Kantachot et al. (2007) examined the leaves of 28 species in 12 genera of the Myrtaceae family and found that *R. tomentosa* had dorsiventral leaves, which have palisade tissue only on one side. The cells in the upper epidermis are thicker than those in the lower epidermis. The leaves of *R. tomentosa* do not have a hypodermis. The lower epidermis of the leaves has unicellular trichomes and anomocytic stomata. In the cross-section of the leaf stalk and petiole, vascular tissue forms a U-shaped pattern. Drusen-shaped Ca oxalate crystals are seen in leaf bone maternal parenchyma tissue.

The leaves of karamunting have palisade/pole and spongy mesophyll tissue. The palisade parenchyma is found beneath the upper epidermis. The cells that comprise the palisade parenchyma tissue are formed like pillars, appear tightly packed, and are just one layer thick. There are many chloroplasts in this palisade parenchyma tissue. The spongy tissue is located beneath the palisade tissue. The cells that make up the spongy tissue are formed like branches, appear loosely organized, and are uneven. Spongy parenchyma tissue contains chloroplasts, although less than palisade/pillar tissue. According to Al-Edany and Al-Saadi (2012), Myrtaceae leaves' mesophyll tissue has 1 to 2 layers of palisade tissue and several rows of up to 15 layers of compact and loose spongy tissue or 2 layers of palisade tissue on the adaxial surface, and spongy tissue on the abaxial surface.

Karamunting leaves have bicollateral vascular bundles consisting of xylem bundles flanked by phloem. Ferreira et al. (2011) describe the bundle of vessels in *Psidium guineense* Sw. (family Myrtaceae) leaves that include xylem flanked by phloem. The xylem and phloem function as a delivery system for vascular plants. The primary function of the xylem is to transport water and solutes, while the primary function of the phloem is to distribute photosynthesis products.

Karamunting leaf epidermal tissue on the abaxial side has unicellular trichomes. Kantachot et al. (2007) identified abundant unicellular and uniseriate trichomes on the leaves of Myrtaceae species such as *Decaspermum parviflorum*, *Melaleuca cajuputi*, *Psidium guajava*, *Rhodamnia dumetorum*, *Tristaniopsis burmanica* var. *rufescens*, and *R. tomentosa*. Trichomes from modified epidermal cells reduce evaporation from the leaves, and minimize animal disturbances (Nugroho 2017).

Retamales et al. (2014) found *Myrceugenia rufa* (Myrtaceae) fruit anatomy similar to *R. tomentosa*. The ripe fruit's pericarp has three zones: exocarp, mesocarp, and endocarp. The exocarp cells are irregular and plano-convex. The cuticle is thin and covered in hair. The mesocarp comprises 7-8 layers of large and isodiametric parenchyma cells with thin walls.

Multiple and large secretory cavities are observed throughout this tissue. The endocarp is a delicate tissue that surrounds the seed.

Histochemical test results by Agustina et al. (2016) recorded flavonoids and other phenolics in the stem, leaf, and flower-fruit-seed organs of *Acalypha indica*. Phenols in *Acalypha wilkesiana* are also distributed in the xylem in stem organs, leaf mesophyll, and in the flower-fruit seed.

Flavonoids can be found in every plant organ, including the leaves, roots, wood, flowers, nectar, and seeds. Flavonoids can be stored in cell walls, vacuoles, and glandular trichomes. Lipophilic compounds generated by glandular trichomes include terpenoids, lipids, waxes, and flavonoid aglycones. Flavonoids concentrate in cells and are not present in the intercellular space, resulting in a symplastic distribution (Petrussa et al. 2013).

According to Cartea et al. (2011), phenolic compounds are widespread phytochemicals. Structure-wise, they are simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. Flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids are plants' most abundant and diversified polyphenols. Flavonoids are the most prevalent phenolics and are found throughout all plants. Flavonoids in the leaf and fruit epidermis protect against UV radiation and pigment, stimulate nitrogen-fixing nodules, and fight disease.

The same study found positive reactions to reagents that detect phenolics, in leaf mesophyll cells, pith parenchyma, and stem cortical parenchyma cells of *Adhatoda zeylanica* (Acanthaceae), *Ruta graveolence* Linn. (Rutaceae), and *Vitex negundo* Linn. (Verbenaceae) (Dhale 2011).

Alkaloids are heterocyclic nitrogen-containing chemicals utilized in medicine. Alkaloids protect plants from insects and herbivores, detoxify toxins, and store nitrogen. Many products consumed by humans contains alkaloids, such as coffee, tea, cocoa, tobacco, areca nut, and marijuana. Alkaloids have neuroprotective, blood pressure-lowering, pain-relieving, antibacterial, anticancer, and antimalarial effects (Carqueijeiro et al. 2013). Alkaloids detected in *R. tomentosa*'s leaves and fruits support its historic medicinal usage in Singapore. In China, the leaves were traditionally used as a painkiller, the roots for stomach ulcers, and the seeds for improved digestion and for snake bites. In Indonesia, the leaves were used to cure wounds. In traditional Vietnamese medicine, the plant's raw fruit treat diarrhea and dysentery, while the ripe ones boost the immune system (Zhao et al. 2019).

According to Ferreira et al. (2011), histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves indicated positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. These tannins in *R. tomentosa* leaves and fruits help treat diarrhea and dysentery. Ferreira et al. (2011) added that Myrtaceae leaves are used to treat diarrhea and intestinal infections due to their astringent tannin content. Tannins protect plants, which is why epidermal cells have them. Myrtaceae plants have tannins in their vascular systems also.

The meroterpenoids of *R. tomentosa* are antibacterial and antifungal. Myrtaceae secondary metabolites like meroterpenoids have been extensively explored as antibiotics. Thus, *R. tomentosa* could prevent microbial deterioration and extend food shelf life as a biocontrol agent and natural preservative (Zhao et al. 2019). Meroterpenoid compounds have been isolated and identified from various other Myrtaceae species, including *Eucalyptus tereticornis* (Liu et al. 2018) and *Leptospermum brachyandrum* (Zou et al. 2018).

Belwal et al. (2019) reported that flavonoid content declined throughout fruit ripening in *Rubus ellipticus*, *Myrica esculenta*, *Prunus persica*, blueberry (*Vaccinium corymbosum*), and blackberry fruit, as did Lin et al. (2020) in blueberries. Flavonoid levels vary by organ type. Hou et al. (2021) found that phenols and flavonoids drop from 5.38 mg RE/g in young citrus fruits to 0.69 mg RE/g in ripe fruit. The same trend was seen in grapes and lemons (Zhu et al.~~,~~ 2020). This phenomenon may be produced by many enzymes involved in flavonoid synthesis, such as 1,2-rhamnosyltransferase and flavanone-7-O-glucosides, which accelerate biosynthesis in the early growth period. Insufficient flavonoid aglycones may explain the decrease in flavonoids during citrus fruit ripening.

Lai et al. (2013) found a decrease in total phenol levels during *R. tomentosa* fruit ripening. The same pattern of declining phenol levels during fruit ripening occurs in other Myrtaceae plants such as Brazilian cherry *Eugenia uniflora* L. (Celli et al. 2011). Belwal et al. (2019) further explain the accumulation and degradation of distinct phenolic compounds during fruit ripening connected to their biosynthesis pathways, which are mainly regulated by enzyme expression and various genetic and environmental variables. Elmastaş et al. (2017) added that phenol compounds are phenylpropanoid metabolic pathway intermediates. Enzymes convert aromatic amino acids into polyphenols. Enzyme activity affects the production of these molecules. Genetic diversity, pH, light, temperature, and climatic change affect enzyme activity. Fruit enzyme activity might vary depending on plant growth and ripening.

The anatomical characteristics reported in this study contribute to the identification of *R. tomentosa* and support quality control tests. Some features may ~~be~~ stand out, such as as hairy leaves with curved edges; epidermal cells with ripples and wall projections: and the secretory cavities with dimensions that go beyond the height of the palisade parenchyma. Characters that can contribute to accuracy in taxonomic classification include cross-sectional anatomical structure of leaves including the epidermis, mesophyll, carrier bundle, secretory cavity, and trichomes, while fruit characteristics include a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. In this study, flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the

highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).s

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Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>

[biodiv] Editor Decision

Smujo Editors <support@mail.smujo.id> Wed, Dec 7, 2022 at 5:51 PM
To: EVI MINTOWATI KUNTORINI <evimintowati@ulm.ac.id>

EVI MINTOWATI KUNTORINI:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of Rhodomyrtus tomentosa".

Our decision is: Revisions Required

Reviewer A:

This is a well-written and clear account of a study into the pharmacological properties of a wayside plant ("karamunting", *Rhodomyrtus tomentosa*) found in Indonesia and many other parts of the Indo-Pacific. The anatomical and chemical studies appear to have been carefully executed and the results obtained have been placed in the context of studies by other teams who have worked on the pharmacology of the Myrtaceae. The Figures and Tables are detailed and present well the findings of the study The Discussion section draws together well the results of other workers and summarises the contribution made by this particular study.

I have attached here a track-changed editing of the manuscript sent to me. I recommend changes for the authors to consider. Thank you for giving me the opportunity to learn about your research.

Recommendation: Revisions Required

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The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

Abstract. *Rhodomyrtus tomentosa* (Ait.) Hassk is native to Southeast Asia. The entire plant has been used in traditional Vietnamese, Chinese, and Malaysian medicine for a long time. Bioactive substances are abundant in ripe fruits. However, few studies have examined phytochemical changes and distribution throughout leaf and fruit growth. This study evaluated the morphoanatomy, histochemical, and phytochemical properties of *R. tomentosa* leaves and fruits. The cross-sectional anatomical structures of observed in the leaves leaves included the epidermis, mesophyll, and carrier bundle, while the fruit structures observed included a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. Flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavities, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavities, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

Keywords: Karamunting, histochemistrycal, flavonoids, leaves, fruits, *Rhodomyrtus tomentosa*

Running title: The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk., a prolific evergreen shrub in the Myrtaceae family in southern and southeastern Asia, from India, east through southern China, the Philippines, and south to Malaysia and Sulawesi, has rose-pink blooms and dark purple edible bell-shaped fruits. In the Indonesian language it is known as "kKaramunting", while in English it is often calledmost commonly called "rRose mMyrtle". This plant favors natural open light and adapts quickly to a range of soil conditions. Moreover, it requires little maintenance and is rarely affected by pests and illnesses. The whol Most e parts of this plant (leaves, roots, buds and fruits) have been used in traditional medicines in Vietnammese, Chinanese and Malaysian medicine for a long time. In traditional medicine of Vietnam, the unripe fruits of the plant are utilized to treat diarrhea or dysentery and ripe ones are used to stimulate the immune system (Zhao et al. 2019).Wounds, heartburn, abscesses, and gynecopathy have been treated with *R. tomentosa*. It can also relieve discomfort. In China, they use it as a medicine to treat urinary tract infections (Hamid et al. 2017). Modern pharmacological studies discovered that *R. tomentosa* has antibacterial, anticancer, anti-inflammatory, and antioxidant effects. In Indonesia, *R. tomentosa* roots and leaves treat diarrhea, stomachaches, and postpartum recovery. Most of these effects match typical *R. tomentosa* usage (Zhao et al. 2019). Epidemiological studies have shown that eating fruits and vegetables may protect against chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer, as well as other health benefits. Phytochemicals, particularly phenolics, are responsible for fruit and vegetable health benefits. According to recent findings, *R. tomentosa* berries contain over 19 phenolics, including anthocyanins, phenolic acids, flavonols, ellagitannins, and stilbenes. Due to their nutritional value and taste, *R. tomentosa* berries are a regional favorite (Zhao et al. 2017).

Anatomical studies are essential in taxonomic parametersindicators, including determining where compounds are secreted and accumulated in a medicinal species' vegetative and generative organs, and important in the quality control of medicinal plants. Pharmacognosy studies of macroscopic and microscopic characteristics are needed for medicinal raw material authentication and for quality assessment for in drug standardization. Among the various alternative plant medicinal sources, the authenticity of accurate plant sources authentication is required (Raghvendra et al., 2021). Various taxa of Myrtaceae have been the subject of anatomical studies (Ferreira et al. 2011; Al-Edany et al. 2012); however, there has been no histochemical anatomical study of the leaves and fruit of *R. tomentosa*.

HThe histochemical studiesy has not yetare needed to reveal ed a the typical pattern for of distribution ofing secondary metabolites in plant tissues. Secondary metabolites are distributed in plant tissues based on their functionality. At the cellular level, secondary metabolites can be located are deposited in cells, andin specific secretory tissues or are distributed throughout the tissue's cells, including in the parenchyma of the transport bundle (Nugroho, 2017).

Commented [A1]: Because you refer to *Rhodomyrtus tomentosa* by the name Karamunting in several place in this paper it is important at the beginning of the paper to tell the readers that this is the Indonesian common name for the species.

Commented [A2]: This sentence is rather vague and perhaps should be deleted.

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Histochemical analyses of fruit development can help explain how metabolites affecting color and flavor are synthesized and accumulated during plant organ development. Histological studies can assess the morphological and anatomical ~~outcome~~ details of *R. tomentosa* leaves and fruits. However, ~~most~~ studies ~~did~~ have not usually addressed secondary metabolite localization in this species. Particular staining and procedures can reveal metabolites, including pectin, lipids, lignin, proteins, phenolic compounds, tannins, starch grains, essential oils, rubber, and terpenoids (Vio-Michaelis et al.~~,~~ 2020). Unequivocal botanical characterizations are needed to identify medicinal plants correctly. ~~Then~~Thus, the objective of ~~,~~ this~~e~~ present study was to evaluate~~d~~ the leaves' and fruits' morphoanatomy, histochemistry, and phytochemistry of a sample of identified karamunting (*R. tomentosa*) plants collected in South Kalimantan, Indonesia.

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MATERIALS AND METHODS

Plant materials

The 2nd and 10th leaves from the stem tip and the green, red, and purple fruits of *R. tomentosa* were collected from wild plants in Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) (Figure 1). Three replicate samples of leaves and fruit were collected from three different plants. Herbarium Bogoriense at the Indonesian Institute of Sciences in Bogor, Indonesia, identified the plant specimen.

Figure 1. Aerial part of *Rhodomyrtus tomentosa* (Aiton.) Hassk.

Procedures

Morphoanatomy slides preparation

Retamales et al. (2014) described ~~that~~ the transverse sections of leaves and fruit ~~were made~~ using ~~paraffin~~paraffin embedding with safranin staining. Minor adjustments were made to the immersion duration in dehydration and dealcoholization. Three replicates of fruits and leaves from three different plants were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70%, 5:5:90) solution for 24 hours. These replicates were then dehydrated, stored in 70% ethanol, and embedded in paraffin. Sections 8 to 10 µm thick ~~sections~~ were obtained on a rotatory microtome, dyed with Safranin, and mounted in Entelan to serve as permanent slides.

Histochemical test

Fresh, fully developed leaves (from the central nerve region and the blade) were cross-sectioned and subjected to histochemical test. Hand-cut sections were treated with a dilute 10% aqueous acidic $FeCl_3$ (Sigma–Aldrich, Germany) solution containing a small quantity of Na_2CO_3 (Sigma–Aldrich, Germany) solution mounted in clove oil for phenolic compounds, which were identified as dark green or black color in tissues (Trimanto et al.~~,~~ 2018). Flavonoids were identified using Wilson's reagent. Each segment was soaked in citric acid:boric (Prolabo) (5:5 w/w) in 100 mL absolute ethanol for 15 minutes, mounted in glycerine–water, and inspected under a light microscope (Olympus, Tokyo, Japan). The yellow color signified the presence of flavonoids (Pratiwi~~,~~ et al.~~,~~ 2020). With 5% ~~5% c~~Copper acetate ($Cu_2(CH_3COO)_4$), ~~stains~~ terpenoids stained yellow-brown, indicating their presence in cells or tissues (Pratiwi~~,~~ et al.~~,~~ 2020). $FeCl_3$ (0.5% - 1% $FeCl_3$ in 0.1 N HCL) stain~~ed~~ed tannins blackish green, suggesting their existence (Robil and Tolentino 2015). Wagner's reagent was used to detect~~ed~~ alkaloids, and any red-brown staining of tissues or cells indicated the presence of alkaloid compounds (Pratiwi~~,~~ et al.~~,~~ 2020). The material was photographed using OptiLab and a light microscope (Olympus, Tokyo, Japan).

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Crude extract preparation

Young leaves ~~The~~ (2nd – 6th down ~~young~~ from the tip) and old leaves (7th – 12th ~~old leaves~~down from the tip) were picked from the ~~tip~~ shoot of *R. ~~T~~omentosa* plants, while harvested ~~the~~ green, red, and purple fruits were dried in an oven at 40°C before being powdered at room temperature. A sample of 500 g of each powdered material was macerated for 24 hours in 1000 mL of ethanol (SmartLab, Indonesia). After 24 hours, the solvent was discarded and replenished thrice (Kusuma et al. 2016). Finally, extracts from the same samples were pooled, mixed, filtered, and dried using a rotary evaporator. The extract yield was calculated by this equation: (weight of ethanol extract/weight of dried leaves or ~~/~~fruits) × 100%.

Total phenolic compounds

Folin-Ciocalteu colorimetry was used to ~~measured~~ the triplicate samples'~~s'~~ total phenolic contents (TPC) (Roy et al. 2018). The sample~~'~~s' extract absorbance ~~was~~ were measured with a UV-~~/~~Vis spectrophotometer (spectrophotometer UV 1800–Shimadzu, Japan) at 760 nm, and the total phenol content was calculated using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight.

Total flavonoids content

Total flavonoid content was determined using AlCl colorimetry. A 1 mL ~~of~~ sample of extract ~~and~~ was mixed in a test tube with 4 mL of distilled water ~~were mixed in a test tube~~and with 0.3 mL of 5% sodium nitrite (Sigma–Aldrich, Germany). After 5 minutes, 0.3 mL of 10% aluminum chloride (Sigma–Aldrich, Germany) was added and stirred. After 5 minutes, 2 ml of sodium hydroxide (1 M) was added, and distilled water was added until the volume was precisely 10 mL. The identical method used for the earlier measurement was used to gauge the sample's extract absorbance at 510 nm. Finally, the total flavonoid content was determined using a q~~Q~~uercetin standard curve (Sigma–Aldrich, Germany) at mg QE/g dry weight (Roy et al. 2018).

Total tannin content

The Folin-Ciocalteu method was used to calculate the total tannin content. The following ingredients were added to 0.1 mL of sample extract: 7.5 mL of distilled water, 0.5 mL of 10% ~~u~~ F-C reagent, and 1 mL of 35% sodium carbonate solution (Sigma–Aldrich, Germany). Then, using distilled water, the volume was increased to 10 mL. After being mixed, the mixture was left at room temperature for 30 minutes. The sample's extract absorbance was determined at a wavelength of 725 nm using the same method as the earlier measurement. Finally, the total tannin content was determined using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight (Roy et al. 2018).

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Total alkaloids content

2.5 g of the sample extract was diluted in 50 mL of 10% acetic acid (in ethanol). After being shaken with a magnetic stirrer for 4 hours, the solution was filtered. After that, the filtrate ~~is~~ was evaporated. Then, ammonium hydroxide (Sigma-Aldrich, Germany) was drip-applied until an alkaloid precipitate formed. The precipitate-filtering filter paper was weigh~~t~~ed before usage. After that, the residue was filtered and cleaned with an ammonium hydroxide 1% solution. The filter paper containing the precipitate was oven dried for 30 minutes at 60°C. After the residue had cooled, it was weighed until a constant weight was obtained. The weight of the alkaloid precipitate acquired from the sample's initial weighing was used to calculate the alkaloid yield (Alasa et al. 2017).

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Data analysis

The results ~~of~~from three replications were used to calculate the means and standard deviations (mean ± SD). Using IBM SPSS statistics 21 and Microsoft Excel®, the quantitative data were statistically evaluated using a one-way analysis of variance, with significant differences set at $p < 0.05$. When a statistically significant differenc~~e~~t ~~result~~ was ~~observed~~detected, ~~a variance~~ the analysis of variance ~~study~~ was followed by an LSD post hoc test.

RESULTS AND DISCUSSION

Morphoanatomy of *R. tomentosa* leaves and fruits ~~*R. tomentosa*~~

Young and old *R. tomentosa* leaves have similar epidermal, mesophyll, and transport bundle structures. The adaxial and abaxial epidermis comprises the epidermis. Compared to the abaxial epidermis, the adaxial is larger. The mesophyll ~~comprises~~ consists of palisade tissue and spongy, single-layer palisade tissue grouped compactly. The leaves are dorsiventral in shape, having palisade tissue on the adaxial side. The leaf stalk's carrier bundle network is made up of phloem and xylem. The leaf bone has parenchyma tissue as filling and collenchyma under the epidermis as reinforcement. Trichomes cover the leave's' lower epidermis, while a thin cuticle covers the upper epidermis (Figure 2).

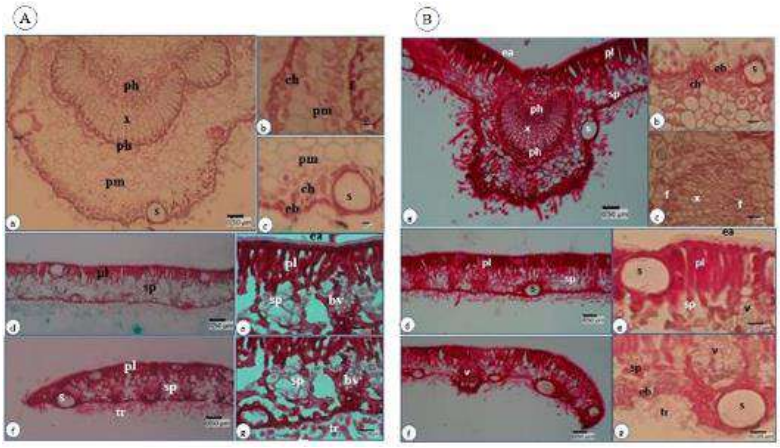

Figure 2. Cross-section of (A) young leaves, (B) old leaves *of R. tomentosa*~~:~~ palisade mesophyll (pl), sponge mesophyll (sp), parenchyma midrib (pm), secretory cavity (sc), trichomes (tr), abaxial epidermis (eb), adaxial epidermis (ea), collenchyma (ch), vascular bundle (v), phloem (f), xylem (x)

The fruit grows from fertilization. The growth of fruit components, such as fruit skin (pericarp), seed coat, endosperm, and embryo, follows fruit development. Bacca/buni fruit of *R. tomentosa* has exocarp, mesocarp, and endocarp layers. The cross-section of green, red, and purple fruit displays a fruit pericarp layer with an exocarp/outer layer, middle mesocarp, and endocarp. (Fig. 3). The stiff exocarp has a polyhedral epidermis and several sclerenchyma layers. The edible mesocarp has big, watery parenchyma cells. The endocarp cells are thin-walled. The 1.5 mm seeds have 6 pseudo-locules with thin false septa (Hermanto et al. 2013; Hamid et al. 2017).

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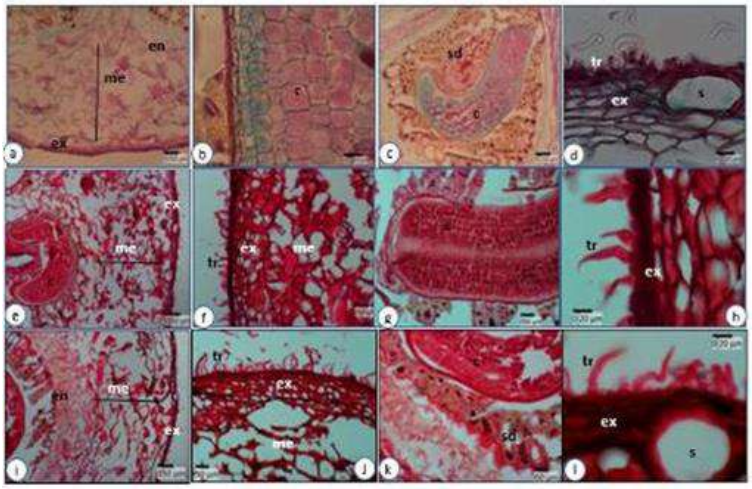

Figure 3. Cross-section of green fruit (a-d), red fruit (e-h) and purple fruit (i-l) of *R. ~~tomentos~~tomentosa*~~;a.~~ exocarp (ex) mesocarp (me), endocarp (en), seed (sd), secretory cavity (s), trichomes (tr)

~~Histochemical~~ Histochemical t~~T~~estsstry

Histochemical tests measure the secondary metabolites of the plant. Histochemical tests use qualitative analysis to detect compounds without measuring their concentration (Dhale~~,~~ 2011). Secondary metabolite components such as phenols, flavonoids, tannins, terpenoids, and alkaloids ~~are~~ were found in *R. tomentosa*'s leaves and fruits at specific locations or ~~nearly everywhere~~more generally in the tissues. The sSecondary metabolites ~~can be~~were detected histochemically. After ~~providing~~ application of ~~the~~ reagents, the identified compounds ~~will~~ showed specific color changes (Figure 4).

Histochemical tests on young and old leaves, green, red, and purple fruit indicated ~~favorable~~ positive reactions to flavonoid compounds (4f-4j) and phenols (Figure 4k-4o). Young and old leaves ~~have~~ had phenolic, and flavonoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. Fruit phenolic and flavonoid compounds were found in the exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4p-4t shows alkaloid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. In green and red fruit, alkaloids were found in the endosperm, exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4u-4y shows tannin compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Tannin compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichome, and seeds, in the green and red fruit but not in the purple fruit.

Figure 4z-4d1 shows terpenoid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Terpenoid compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Terpenoid compounds ~~was~~ were not found in the endocarp of purple fruit.

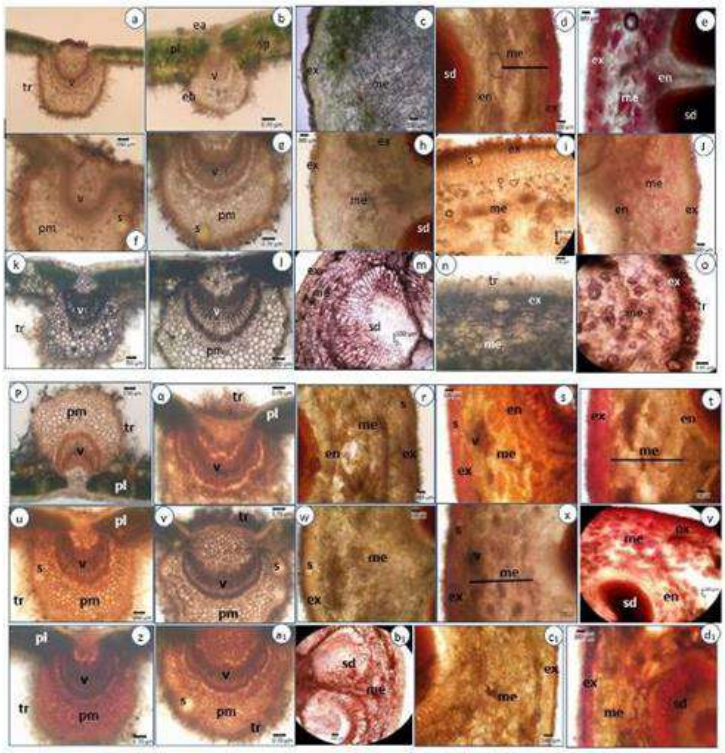

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Figure 4. Transverse sections of histochemically stained leaves and fruits of *R. Tomentosa*,~~.~~ (a-e) Free-hand section of a young leaf without staining;~~.~~ (f-j) the positive reaction ~~of~~ to Wilson's reagent indicating the presence of flavonoid in leaves and fruits;~~.~~ (k-o) the positive reaction ~~of~~ to ferric chloride indicating the presence of phenolics in leaves and fruits;~~.~~ (p-t) the positive reaction to ~~of reagent~~ Wagner's reagent indicating the presence of alkaloid in leaves and fruits;~~.~~ (u-y) the positive reaction ~~of~~ to $FeCl_3$ indicating the presence of tannin in leaves and fruits;~~.~~ (z-d_1) the positive reaction ~~of~~ to $Cu_2(CH_3COO)_4$ 5% indicating the presence of terpenoid in leaves and fruits;~~.~~ palisade mesophyll (pm), sponge mesophyll (sm), parenchyma midrib (pm), secretory cavity (s), trichomes (tr), druse crystal (dc), abaxial epidermis (eb), adaxial epidermis (ea) exocarp (ex) mesocarp (me), endocarp (en), seed (sd) vascular bundle (v).

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Phytochemistry

The results of phytochemical measurements ~~results of~~showed that the highest total flavonoid levels were in young leaves at ~~were~~ 196.0 mg QE/g, followed by green fruit and old leaves, 164.625 mg QE/g, and 134.875 mg QE/g, followed by flavonoid levels in red and purple fruits ~~as many as~~at 84.625 mg QE/g and 55.75 mg QE/g, respectively. The ~~results of the measurement of the~~ highest measured total phenol content w~~as~~ere 97.70 mg/mL recorded in green fruit, followed by old leaves at 59.30 mg/mL, young leaves at 54.40 mg/mL, red fruit at 19.40 mg/mL, and the lowest, ~~was~~ in purple fruit ~~as much as~~at 17.23mg/mL. ~~The measurement results of T~~the highest total alkaloid content ~~of~~ was recorded in old leaves at ~~were~~ 13.2~~326~~%, green fruit at 9.2~~876~~%, red fruit at 7.~~80798~~%, purple fruit at 7.0~~546~~%, and young leaves, ~~at~~ the lowest at 5.64%. ~~The measurement results of the highest~~For total tannin content, the measurements showed the highest value in red fruit ~~were~~ at 1.658 mg GAE/g, followed by purple fruit at 1.402 mg GAE/g, young leaves at 0.943 mg GAE/g, old leaves at 0.880 mg GAE/g, and the lowest tannin content in green fruit ~~was as much as~~at 0.804 mg GAE/g. (Table 1).

Table 1. Total amount of compounds in ethanolic extracts of leaves and fruits of *R. tomentosa*

Sample	Total ~~f~~Flavonoids (mgQE/g)	Total phenolics (mgGAE/g)	Total alkaloids (%)	Total tannins (mgGAE/g)
Young leaves	196 ± 1.77e	54.40 ± 0.09b	5.64 ± 0.20a	0.94 ± 0.74a
Old leaves	134.88 ± 6.89c	59.30 ± 16.73b	13.22 ± 0.98d	0.88 ± 0.01a
Green fruits	164.63 ± 2.65d	97.70 ± 18.15c	9.28 ± 0.33c	0.80 ± 0.30a
Red fruits	84.63 ± 2.30b	19.40 ± 0.47a	7.80 ± 0.97bc	1.66 ± 0.15c
Purple fruits	55.75 ± 0.71a	17.23 ± 4.20a	7.05 ± 0.41ab	1.40 ± 1.20b

Note: The data presented are the mean ± standard deviation. One-Way ANOVA and LSD test with = 0.05 were used to evaluate data. Numbers followed by different superscript letters in the same column show significantly different results.

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Discussion

According to research by Al-Edany and Al-Saadi (2012) on the anatomical characteristics of several Myrtaceae species, the upper surface of the epidermal tissue on the leaves of the Myrtaceae family is covered by a variable-thickness cuticle layer. Young and old leaves of k~~K~~aramunting (*Rhodomyrtus tomentosa*) ~~leaves~~ have one layer of epidermal tissue on the upper surface (adaxial). The epidermal tissue's cells are square and closely packed. Al-Edany and Al-Saadi (2012) found that the cells of Myrtaceae leaf epidermal tissue ~~is~~ are square or rectangular and uniser~~r~~iate.

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Kantachot et al. (2007) examined the leaves of 28 species in 12 ~~five~~ genera of the Myrtaceae family and found that *R. tomentosa* had dorsiventral leaves, which have palisade tissue only on one side. The cells in the upper epidermis are thicker than those in the lower epidermis. The leaves of *R. tomentosa* do not have a hypodermis. The lower epidermis of the leaves has unicellular trichomes and anomocytic stomata. In the cross-section of the leaf stalk and petiole, vascular tissue forms a U-shaped pattern. Drusen-shaped Ca oxalate crystals are seen in leaf bone maternal parenchyma tissue.

~~Karamunting~~ The leaves of karamunting ~~leaves~~ have palisade/pole and spongy mesophyll tissue. The palisade parenchyma is found beneath the upper epidermis. The cells that comprise the palisade parenchyma tissue are formed like pillars, appear tightly packed, and are just one layer thick. There are many chloroplasts in this palisade parenchyma tissue. The spongy tissue is located beneath the palisade tissue. The cells that make up the spongy tissue are formed like branches, appear loosely organized, and are uneven. Spongy parenchyma tissue contains chloroplasts, although less than palisade/pillar tissue. According to Al-Edany and Al-Saadi (2012), Myrtaceae leaves' mesophyll tissue has 1 to 2 layers of palisade tissue and several rows of up to 15 layers of compact and loose spongy tissue or 2 layers of palisade tissue on the adaxial surface, and spongy tissue on the abaxial surface.

Karamunting leaves have bicollateral vascular bundles consisting of xylem bundles flanked by phloem. Ferreira et al. (2011) describe the bundle of vessels in *Psidium guineense* Sw. (family Myrtaceae) leaves that include xylem flanked by phloem. The xylem and phloem function as a delivery system for vascular plants. The primary function of the xylem is to transport water and solutes, while the primary function of the phloem is to distribute photosynthesis products.

Karamunting leaf epidermal tissue on the abaxial side ~~had~~ has unicellular trichomes. Kantachot et al. (2007) identified abundant unicellular and uniseriate trichomes on the leaves of Myrtaceae species such as *Decaspermum parviflorum*, *Melaleuca cajuputi*, *Psidium guajava*, *Rhodamnia dumetorum*, *Tristaniopsis burmanica* var. *rufescens*, and *R. tomentosa*. Trichomes from modified epidermal cells reduce evaporation from the leaves, ~~stimulate,~~ and minimize animal disturbances (Nugroho~~,~~ 2017).

Retamales et al. (2014) found *Myrceugenia rufa* (Myrtaceae) fruit anatomy similar to *R. tomentosa*. The ripe fruit's pericarp has three zones: exocarp, mesocarp, and endocarp. The exocarp cells are irregular and plano-convex. The cuticle is thin and covered in hair. The mesocarp comprises 7-8 layers of large and isodiametric parenchyma cells with thin walls. Multiple and large secretory cavities are observed throughout this tissue. The endocarp is a delicate tissue that surrounds the seed.

Histochemical test results by Agustina et al. (2016) ~~stated that~~recorded flavonoids and other phenolics ~~are found~~ in the stem, leaf, and flower-fruit-seed organs of *Acalypha indica*. Phenols in *Acalypha wilkesiana* ~~is~~ are also distributed in the xylem in stem organs, leaf mesophyll, and in the flower-fruit seed.

Flavonoids can be found in every plant organ, including the leaves, roots, wood, flowers, nectar, and seeds. Flavonoids can be stored in cell walls, vacuoles, and glandular trichomes. Lipophilic compounds generated by glandular trichomes include terpenoids, lipids, waxes, and flavonoid aglycones. Flavonoids concentrate in cells and are not present in the intercellular space, resulting in a symplastic distribution (Petrussa et al.~~,~~ 2013).

According to Cartea et al. (2011), phenolic compounds are widespread phytochemicals. Structure-wise, they are simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. Flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids are plants' most abundant and diversified polyphenols. Flavonoids are the most prevalent phenolics and are found throughout all plants. Flavonoids in the leaf and fruit epidermis protect against UV radiation and pigment, stimulate nitrogen-fixing nodules, and fight disease.

The same study found positive reactions to reagents that detect phenolics, in leaf mesophyll cells, pith parenchyma, and stem cortical parenchyma cells of *Adhatoda zeylanica* (Acanthaceae), *Ruta graveolence* Linn. (Rutaceae), and *Vitex negundo* Linn. (Verbenaceae) (Dhale~~,~~ 2011).

Alkaloids are heterocyclic nitrogen-containing chemicals utilized in medicine.

Alkaloids protect plants from insects and herbivores, detoxify toxins, and store nitrogen. ~~Much~~ Many products ~~human~~ consumed by humans ~~umption~~ contains alkaloids, such as coffee, tea, cocoa, tobacco, areca nut, and marijuana. Alkaloids have neuroprotective, blood pressure-lowering, pain-relieving, antibacterial, anticancer, and antimalarial effects (Carqueijeiro et al.~~,~~ 2013). Alkaloids detected in *R. tomentosa's* leaves and fruits support its historic medicinal usage in Singapore. In China, the leaves were traditionally used as a painkiller, the roots for stomach ulcers, and the seeds for improved digestion and for snake bites. In Indonesia, the leaves were used to cure wounds. In traditional Vietnamese medicine, the plant's raw fruit treat~~s~~ diarrhea and dysentery, while the ripe ones boost the immune system (Zhao et al.~~,~~ 2019).

According to Ferreira et al. (2011), histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves indicated positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. These tannins in *R. tomentosa* leaves and fruits help treat diarrhea and dysentery. Ferreira et al. (2011) added that Myrtaceae leaves are used to treat diarrhea and intestinal infections due to their astringent tannin content. This plant also fights bacteria, tumors, and malaria. Tannins protect plants, which is why epidermal cells have them. Myrtaceae plants have tannins in their vascular systems also.

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~~*R. tomentosa*~~ The meroterpenoids of *R. tomentosa* are antibacterial and antifungal. Myrtaceae secondary metabolites like meroterpenoids have been extensively explored as antibiotics. Thus, *R. tomentosa* could prevent microbial deterioration and extend food shelf life as a biocontrol agent and natural preservative (Zhao et al.~~,~~ 2019). Meroterpenoid compounds have been isolated and identified from various other Myrtaceae species, including *Eucalyptus tereticornis* (Liu et al.~~,~~ 2018) and *Leptospermum brachyandrum* (Zou et al.~~,~~ 2018).

Belwal et al. (2019) ~~found~~ reported that flavonoid~~s~~ content ~~reduced~~ declined throughout fruit ripening in *Rubus~~,~~ ellipticus*, *Myrica~~,~~ esculenta*, *Prunus persica*, blueberry (*Vaccinium corymbosum*), and blackberry fruit, as did Lin et al. (2020) in blueberries. Flavonoid levels vary by organ type. Hou et al. (2021) found that phenols and flavonoids drop from 5.38 mg RE/g in young citrus fruits to 0.69 mg RE/g in ripe fruit. The same trend was seen in grapes and lemons (Zhu et al., 2020). This phenomenon may be produced by many enzymes involved in flavonoid synthesis, such as 1,2-rhamnosyltransferase and flavanone-7-O-glucosides, which accelerate biosynthesis in the early growth period. Insufficient flavonoid aglycones may explain the decrease in flavonoids during citrus fruit ripening.

Lai et al. (2013) found a decrease in total phenol levels during *R. tomentosa* fruit ripening. The same pattern of declining phenol levels during fruit ripening occurs in Myrtaceae plants as Brazilian cherry *Eugenia uniflora* L. (Celli et al.~~,~~ 2011). Belwal et al. (2019) further explain the accumulation and degradation of distinct phenolic compounds during fruit ripening connected to their biosynthesis pathways, which are mainly regulated by enzyme expression and various genetic and environmental variables. Elmastaş et al. (2017) added that phenol compounds are phenylpropanoid metabolic pathway intermediates. Enzymes convert aromatic amino acids into polyphenols. Enzyme activity affects the production of these molecules. Genetic diversity, pH, light, temperature, and climatic change affect enzyme activity. Fruit enzyme activity might vary depending on plant growth and ripening.

The anatomical characteristics reported in this study contribute to the identification of ~~*this species*~~*R. tomentosa* and support quality control tests. Some features may be stand~~ed~~ out, such as as hairy leaves with curved edges; epidermal cells with ripples and wall projections; and~~,~~ ~~in addition,~~ the secretory cavities with dimensions that go beyond the height of the palisade parenchyma. ~~The~~ Characters that can contribute to accuracy in taxonomic classification include cross-sectional anatomical structure of leaves includ~~ed~~ the epidermis, mesophyll, carrier bundle, secretory cavity, and trichomes,

while fruit characteristics included a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. In this study, flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

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6. Second revised submission (7-12-2022)
- Manuscript Biodiversitas_Revision

The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

Abstract. *Rhodomyrtus tomentosa* (Ait.) Hassk is native to Southeast Asia. The entire plant has been used in traditional Vietnamese, Chinese, and Malaysian medicine for a long time. Bioactive substances are abundant in ripe fruits. However, few studies have examined phytochemical changes and distribution throughout leaf and fruit growth. This study evaluated the morphoanatomy, histochemical, and phytochemical properties of *R. tomentosa* leaves and fruits. The cross-sectional anatomical structures observed in the leaves included the epidermis, mesophyll, and carrier bundle, while the fruit structures observed included a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. Flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavities, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavities, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

Keywords: Karamunting, histochemistry, flavonoids, leaves, fruits, *Rhodomyrtus tomentosa*

Running title: The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk., a prolific evergreen shrub in the Myrtaceae family in southern and southeastern Asia, from India, east through southern China, the Philippines, and south to Malaysia and Sulawesi, has rose-pink blooms and dark purple edible bell-shaped fruits. In the Indonesian language it is known as “karamunting”, while in English it is most commonly called “rose myrtle”. This plant favors natural open light and adapts quickly to a range of soil conditions. Moreover, it requires little maintenance and is rarely affected by pests and illnesses. Most parts of this plant (leaves, roots, buds and fruits) have been used in traditional medicines in Vietnam, China and Malaysia for a long time. In traditional medicine of Vietnam, the unripe fruits of the plant are utilized to treat diarrhea or dysentery and ripe ones are used to stimulate the immune system (Zhao et al. 2019).Wounds, heartburn, abscesses, and gynecopathy have been treated with *R. tomentosa*. In China, they use it as a medicine to treat urinary tract infections (Hamid et al. 2017a). Modern pharmacological studies discovered that *R. tomentosa* has antibacterial (Zhao et al. 2018), anticancer (Zhang et al. 2016), anti-inflammatory (Na-Phatthalung et al. 2018), and antioxidant (Hamid et al. 2017b) effects. In Indonesia, *R. tomentosa* roots and leaves treat diarrhea, stomachaches, and postpartum recovery. Most of these effects match typical *R. tomentosa* usage (Zhao et al. 2019). Epidemiological studies have shown that eating fruits and vegetables may protect against chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer, as well as other health benefits (Del Rio et al. 2013). Phytochemicals, particularly phenolics, are responsible for fruit and vegetable health benefits. According to recent findings, *R. tomentosa* berries contain over 19 phenolics, including anthocyanins, phenolic acids, flavonols, ellagitannins, and stilbenes. Due to their nutritional value and taste, *R. tomentosa* berries are a regional favorite (Zhao et al. 2017).

Anatomical studies are essential taxonomic indicators, including determining where compounds are secreted and accumulated in a medicinal species' vegetative and generative organs, and important in the quality control of medicinal plants. Pharmacognosy studies of macroscopic and microscopic characteristics are needed for medicinal raw material authentication and for quality assessment in drug standardization. Among the various alternative plant medicinal sources, accurate plant authentication is required (Raghvendra et al. 2021). Various taxa of Myrtaceae have been the subject of anatomical studies (Ferreira et al. 2011; Al-Edany et al. 2012); however, there has been no histochemical anatomical study of the leaves and fruit of *R. tomentosa*.

Histochemical studies are needed to reveal the typical pattern of distribution of secondary metabolites in plant tissues. Secondary metabolites are distributed in plant tissues based on their functionality. At the cellular level, secondary metabolites can be located in specific secretory tissues or are distributed throughout the tissue's cells, including in the parenchyma of the transport bundle (Nugroho 2017).

Histochemical analyses of fruit development can help explain how metabolites affecting color and flavor are synthesized and accumulated during plant organ development. Histological studies can assess the morphological and anatomical details of *R. tomentosa* leaves and fruits. However, studies have not usually addressed secondary metabolite localization in this species. Particular staining and procedures can reveal metabolites, including pectin, lipids, lignin, proteins, phenolic compounds, tannins, starch grains, essential oils, rubber, and terpenoids (Vio-Michaelis et al. 2020). Unequivocal botanical characterizations are needed to identify medicinal plants correctly. Thus, the objective of this present study was to evaluate the leaves' and fruits' morphoanatomy, histochemistry, and phytochemistry of a sample of identified karamunting (*R. tomentosa*) plants collected in South Kalimantan, Indonesia.

MATERIALS AND METHODS

Plant materials

The 2nd and 10th leaves from the stem tip and the green, red, and purple fruits of *R. tomentosa* were collected from wild plants in Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) (Figure 1). Three replicate samples of leaves and fruit were collected from three different plants. Herbarium Bogoriense at the Indonesian Institute of Sciences in Bogor, Indonesia, identified the plant specimen.

Figure 1. Aerial part of *Rhodomyrtus tomentosa* (Aiton.) Hassk.

Procedures

Morphoanatomy slides preparation

Retamales et al. (2014) described the transverse sections of leaves and fruit using paraffin embedding with safranin staining. Minor adjustments were made to the immersion duration in dehydration and dealcoholization. Three replicates of fruits and leaves from three different plants were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70%, 5:5:90) solution for 24 hours. These replicates were then dehydrated, stored in 70% ethanol, and embedded in paraffin. Sections 8 to 10 µm thick were obtained on a rotatory microtome, dyed with Safranin, and mounted in Entelan to serve as permanent slides.

Histochemical test

Fresh, fully developed leaves (from the central nerve region and the blade) were cross-sectioned and subjected to histochemical test. Hand-cut sections were treated with a dilute 10% aqueous acidic $FeCl_3$ (Sigma–Aldrich, Germany) solution containing a small quantity of Na_2CO_3 (Sigma–Aldrich, Germany) solution mounted in clove oil for phenolic compounds, which were identified as dark green or black color in tissues (Trimanto et al. 2018). Flavonoids were identified using Wilson's reagent. Each segment was soaked in citric acid:boric (Prolabo) (5:5 w/w) in 100 mL absolute ethanol for 15 minutes, mounted in glycerine–water, and inspected under a light microscope (Olympus, Tokyo, Japan). The yellow color signified the presence of flavonoids (Pratiwi et al. 2020). With 5% copper acetate ($Cu_2(CH_3COO)_4$), terpenoids stained yellow-brown, indicating their presence in cells or tissues (Pratiwi et al. 2020). $FeCl_3$ (0.5% - 1% $FeCl_3$ in 0.1 N HCL) stained tannins blackish green, suggesting their existence (Robil and Tolentino 2015). Wagner's reagent was used to detect alkaloids, and any red-brown staining of tissues or cells indicated the presence of alkaloid compounds (Pratiwi et al. 2020). The material was photographed using OptiLab and a light microscope (Olympus, Tokyo, Japan).

Crude extract preparation

Young leaves (2nd – 6th down from the tip) and old leaves (7th – 12th down from the tip) were picked from the shoot of *R. tomentosa* plants, while harvested green, red, and purple fruits were dried in an oven at 40°C before being powdered at

room temperature. A sample of 500 g of each powdered material was macerated for 24 hours in 1000 mL of ethanol (SmartLab, Indonesia). After 24 hours, the solvent was discarded and replenished thrice (Kusuma et al. 2016). Finally, extracts from the same samples were pooled, mixed, filtered, and dried using a rotary evaporator. The extract yield was calculated by this equation: (weight of ethanol extract/weight of dried leaves or fruits) × 100%.

Total phenolic compounds

Folin-Ciocalteu colorimetry was used to the triplicate samples' total phenolic contents (TPC) (Roy et al. 2018). The samples' extract absorbance were measured with a UV-Vis spectrophotometer (spectrophotometer UV 1800–Shimadzu, Japan) at 760 nm, and the total phenol content was calculated using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight.

Total flavonoids content

Total flavonoid content was determined using AlCl colorimetry. A 1 mL sample of extract was mixed in a test tube with 4 mL of distilled water and with 0.3 mL of 5% sodium nitrite (Sigma–Aldrich, Germany). After 5 minutes, 0.3 mL of 10% aluminum chloride (Sigma–Aldrich, Germany) was added and stirred. After 5 minutes, 2 ml of sodium hydroxide (1 M) was added, and distilled water was added until the volume was precisely 10 mL. The identical method used for the earlier measurement was used to gauge the sample's extract absorbance at 510 nm. Finally, the total flavonoid content was determined using a quercetin standard curve (Sigma–Aldrich, Germany) at mg QE/g dry weight (Roy et al. 2018).

Total tannin content

The Folin-Ciocalteu method was used to calculate the total tannin content. The following ingredients were added to 0.1 mL of sample extract: 7.5 mL of distilled water, 0.5 mL of 10% F-C reagent, and 1 mL of 35% sodium carbonate solution (Sigma–Aldrich, Germany). Then, using distilled water, the volume was increased to 10 mL. After being mixed, the mixture was left at room temperature for 30 minutes. The sample's extract absorbance was determined at a wavelength of 725 nm. Finally, the total tannin content was determined using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight (Roy et al. 2018).

Total alkaloids content

2.5 g of the sample extract was diluted in 50 mL of 10% acetic acid (in ethanol). After being shaken with a magnetic stirrer for 4 hours, the solution was filtered. After that, the filtrate was evaporated. Then, ammonium hydroxide (Sigma-Aldrich, Germany) was drip-applied until an alkaloid precipitate formed. The precipitate-filtering filter paper was weighed before usage. After that, the residue was filtered and cleaned with an ammonium hydroxide 1% solution. The filter paper containing the precipitate was oven dried for 30 minutes at 60°C. After the residue had cooled, it was weighed until a constant weight was obtained. The alkaloid yield was calculated by the weight percentage of the precipitated alkaloid obtained against the initial weight of the extract. (Alasa et al. 2017).

Data analysis

The resultsfrom three replications were used to calculate the means and standard deviations (mean ± SD). Using IBM SPSS statistics 21 and Microsoft Excel®, the quantitative data were statistically evaluated using a one-way analysis of variance, with significant differences set at $p < 0.05$. When a statistically significant difference was detected, the analysis of variance was followed by an LSD post hoc test.

RESULTS AND DISCUSSION

Morphoanatomy of *R. tomentosa* leaves and fruits

Young and old *R. tomentosa* leaves have similar epidermal, mesophyll, and transport bundle structures. The adaxial and abaxial epidermis comprises the epidermis. Compared to the abaxial epidermis, the adaxial is larger. The mesophyll consists of palisade tissue and spongy, single-layer palisade tissue grouped compactly. The leaves are dorsiventral in shape, having palisade tissue on the adaxial side. The leaf stalk's carrier bundle network is made up of phloem and xylem. The leaf bone has parenchyma tissue as filling and collenchyma under the epidermis as reinforcement. Trichomes cover the leave's lower epidermis, while a thin cuticle covers the upper epidermis (Figure 2).

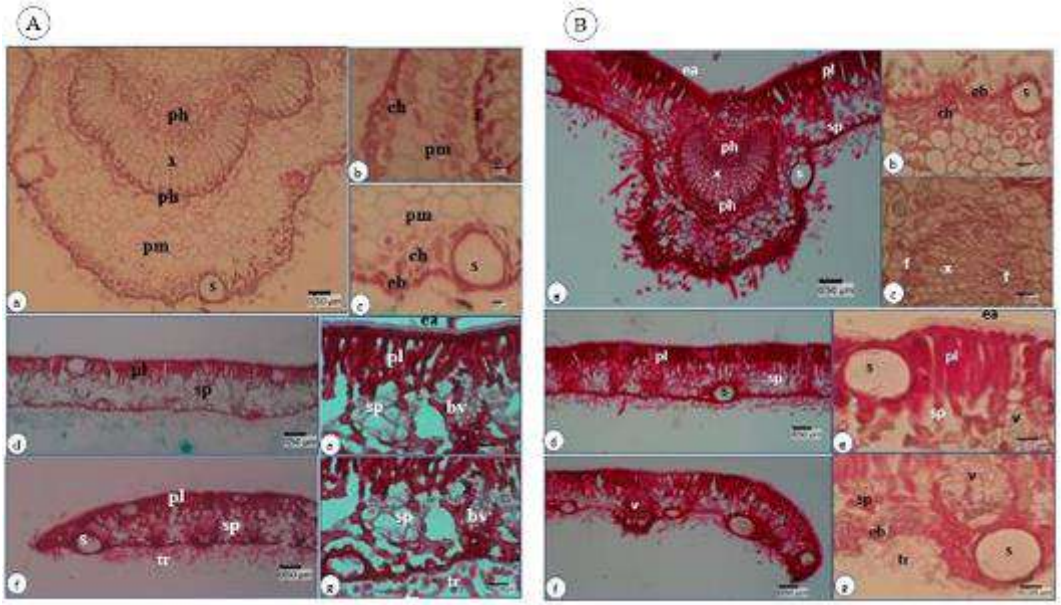

Figure 2. Cross-section of (A) young leaves, (B) old leaves *of R. tomentosa;* palisade mesophyll (pl), sponge mesophyll (sp), parenchyma midrib (pm), secretory cavity (sc), trichomes (tr), abaxial epidermis (eb), adaxial epidermis (ea), collenchyma (ch), vascular bundle (v), phloem (f), xylem (x)

The fruit grows from fertilization. The growth of fruit components, such as fruit skin (pericarp), seed coat, endosperm, and embryo, follows fruit development. *R. tomentosa* fruit is a bacca/buni 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 (Hamid et al. 2017a). The cross-section of green, red, and purple fruit displays a fruit pericarp layer with an exocarp/outer layer, middle mesocarp, and endocarp. (Fig. 3). The stiff exocarp has a polyhedral epidermis and several sclerenchyma layers. The edible mesocarp has big, watery parenchyma cells. The endocarp cells are thin-walled. The 1.5 mm seeds have 6 pseudo-locules with thin false septa (Hermanto et al. 2013; Hamid et al. 2017).

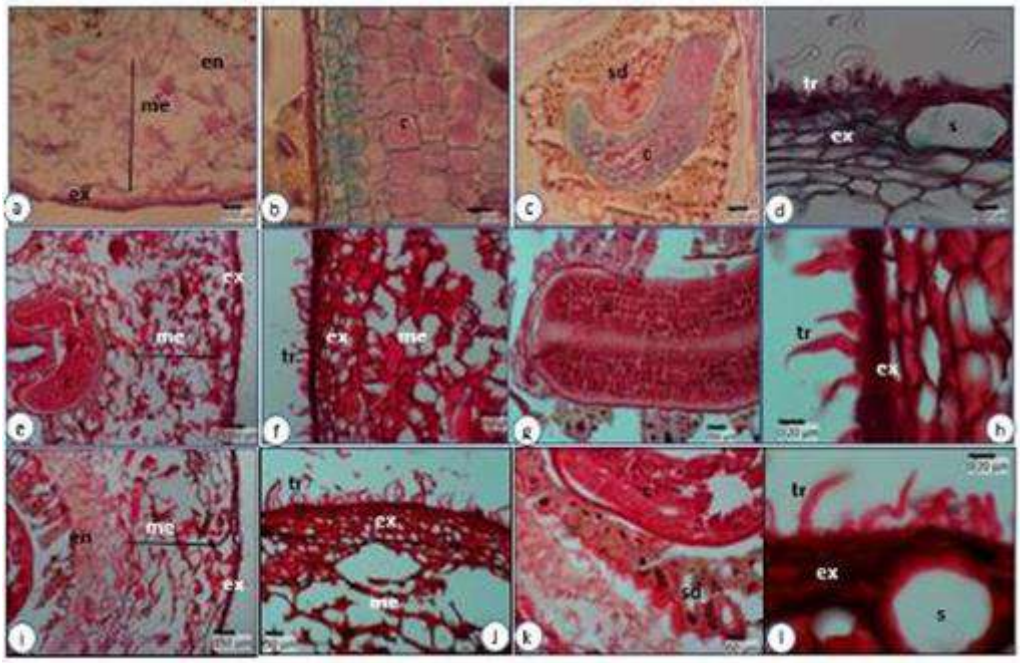

Figure 3. Cross-section of green fruit (a-d), red fruit (e-h) and purple fruit (i-l) of *R. tomentosa;* exocarp (ex) mesocarp (me), endocarp (en), seed (sd), secretory cavity (s), trichomes (tr)

Histochemical tests

Histochemical tests measure the secondary metabolites of the plant. Histochemical tests use qualitative analysis to detect compounds without measuring their concentration (Dhale 2011). Secondary metabolite components such as phenols, flavonoids, tannins, terpenoids, and alkaloids were found in *R. tomentosa*'s leaves and fruits at specific locations or more generally in the tissues. The secondary metabolites were detected histochemically. After application of reagents, the identified compounds showed specific color changes (Figure 4).

Histochemical tests on young and old leaves, green, red, and purple fruit indicated positive reactions to flavonoid compounds (4f-4j) and phenols (Figure 4k-4o). Young and old leaves had phenolic, and flavonoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. Fruit phenolic and flavonoid compounds were found in the exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4p-4t shows alkaloid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. In green and red fruit, alkaloids were found in the endosperm, exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4u-4y shows tannin compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Tannin compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichome, and seeds, in the green and red fruit but not in the purple fruit.

Figure 4z-4d1 shows terpenoid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Terpenoid compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Terpenoid compounds were not found in the endocarp of purple fruit.

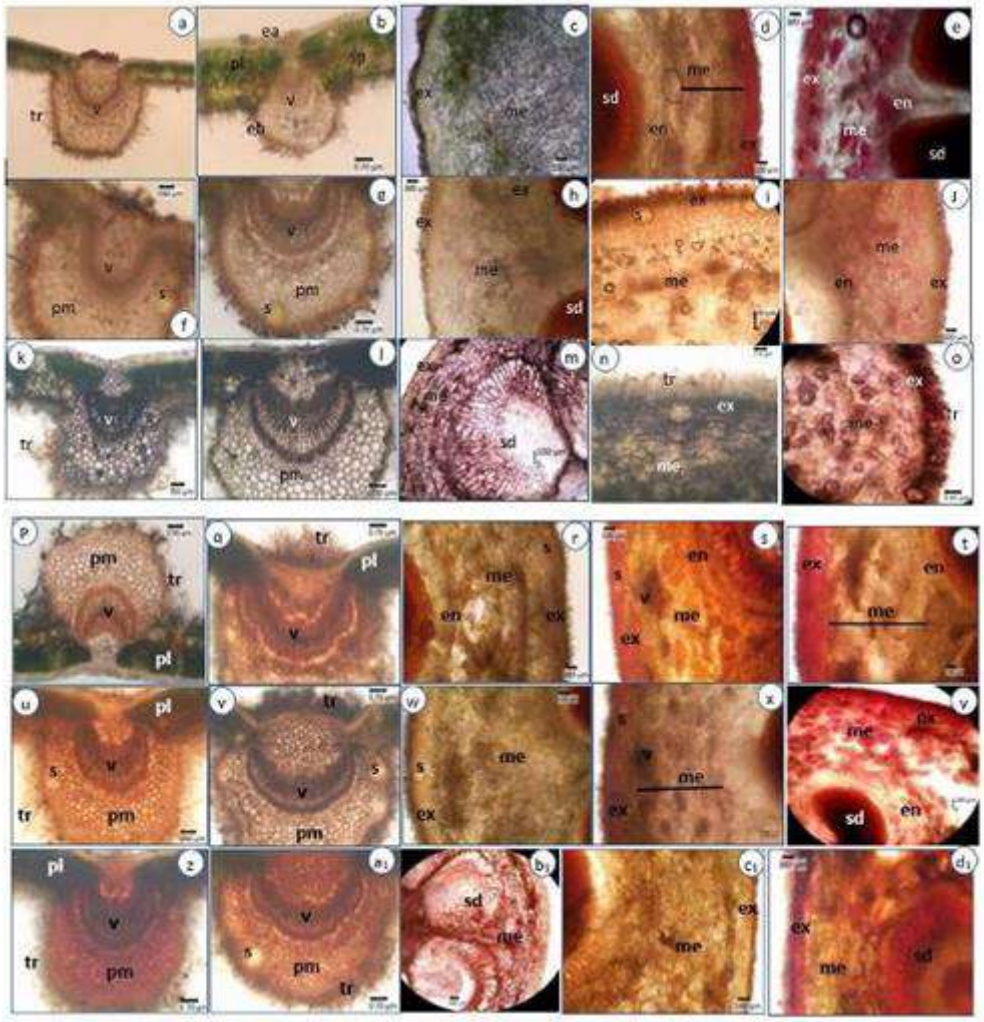

Figure 4. Transverse sections of histochemically stained leaves and fruits of *R. Tomentosa*. (a-e) Free-hand section of a young leaf without staining; (f-j) the positive reaction to Wilson's reagent indicating the presence of flavonoid in leaves and fruits; (k-o) the positive reaction to ferric chloride indicating the presence of phenolics in leaves and fruits: (p-t) the positive reaction to Wagner's reagent

indicating the presence of alkaloid in leaves and fruits; (u-y) the positive reaction to $FeCl_3$ indicating the presence of tannin in leaves and fruits; (z-d_1) the positive reaction to $Cu_2(CH_3COO)_4$ 5% indicating the presence of terpenoid in leaves and fruits: palisade mesophyll (pm), sponge mesophyll (sm), parenchyma midrib (pm), secretory cavity (s), trichomes (tr), druse crystal (dc), abaxial epidermis (eb), adaxial epidermis (ea) exocarp (ex) mesocarp (me), endocarp (en), seed (sd) vascular bundle (v).

Phytochemistry

The results of phytochemical measurements showed that the highest total flavonoid levels were in young leaves at 196.0 mg QE/g, followed by green fruit and old leaves, 164.625 mg QE/g, and 134.875 mg QE/g, followed by flavonoid levels in red and purple fruits at 84.625 mg QE/g and 55.75 mg QE/g, respectively. The highest measured total phenol content was 97.70 mg/mL recorded in green fruit, followed by old leaves at 59.30 mg/mL, young leaves at 54.40 mg/mL, red fruit at 19.40 mg/mL, and the lowest, in purple fruit at 17.23mg/mL. The highest total alkaloid content was recorded in old leaves at 13.23%, green fruit at 9.28%, red fruit at 7.80%, purple fruit at 7.05%, and young leaves, the lowest at 5.64%. For total tannin content, the measurements showed the highest value in red fruit at 1.658 mg GAE/g, followed by purple fruit at 1.402 mg GAE/g, young leaves at 0.943 mg GAE/g, old leaves at 0.880 mg GAE/g, and the lowest tannin content in green fruit at 0.804 mg GAE/g. (Table 1).

Table 1. Total amount of compounds in ethanolic extracts of leaves and fruits of *R. tomentosa*

Sample	Total flavonoids (mgQE/g)	Total phenolics (mgGAE/g)	Total alkaloids (%)	Total tannins (mgGAE/g)
Young leaves	196 ± 1.77^{e}	54.40 ± 0.09^{b}	5.64 ± 0.20^{a}	0.94 ± 0.74^{a}
Old leaves	134.88 ± 6.89^{c}	59.30 ± 16.73^{b}	13.22 ± 0.98^{d}	0.88 ± 0.01^{a}
Green fruits	164.63 ± 2.65^{d}	97.70 ± 18.15^{c}	9.28 ± 0.33^{c}	0.80 ± 0.30^{a}
Red fruits	84.63 ± 2.30^{b}	19.40 ± 0.47^{a}	$7.80 \pm 0.97b^{c}$	1.66 ± 0.15^{c}
Purple fruits	55.75 ± 0.71^{a}	17.23 ± 4.20^{a}	$7.05 \pm 0.41a^{b}$	1.40 ± 1.20^{b}

Note: The data presented are the mean ± standard deviation. One-Way ANOVA and LSD test with = 0.05 were used to evaluate data. Numbers followed by different superscript letters in the same column show significantly different results.

Discussion

According to research by Al-Edany and Al-Saadi (2012) on the anatomical characteristics of several Myrtaceae species, the upper surface of the epidermal tissue on the leaves of the Myrtaceae family is covered by a variable-thickness cuticle layer. Young and old leaves of karamunting (*Rhodomyrtus tomentosa*) have one layer of epidermal tissue on the upper surface (adaxial). The epidermal tissue's cells are square and closely packed. Al-Edany and Al-Saadi (2012) found that the cells of Myrtaceae leaf epidermal tissue are square or rectangular and uniseriate.

Kantachot et al. (2007) examined the leaves of 28 species in 12 genera of the Myrtaceae family and found that *R. tomentosa* had dorsiventral leaves, which have palisade tissue only on one side. The cells in the upper epidermis are thicker than those in the lower epidermis. The leaves of *R. tomentosa* do not have a hypodermis. The lower epidermis of the leaves has unicellular trichomes and anomocytic stomata. In the cross-section of the leaf stalk and petiole, vascular tissue forms a U-shaped pattern. Drusen-shaped Ca oxalate crystals are seen in leaf bone maternal parenchyma tissue.

The leaves of karamunting have palisade/pole and spongy mesophyll tissue. The palisade parenchyma is found beneath the upper epidermis. The cells that comprise the palisade parenchyma tissue are formed like pillars, appear tightly packed, and are just one layer thick. There are many chloroplasts in this palisade parenchyma tissue. The spongy tissue is located beneath the palisade tissue. The cells that make up the spongy tissue are formed like branches, appear loosely organized, and are uneven. Spongy parenchyma tissue contains chloroplasts, although less than palisade/pillar tissue. According to Al-Edany and Al-Saadi (2012), Myrtaceae leaves' mesophyll tissue has 1 to 2 layers of palisade tissue and several rows of up to 15 layers of compact and loose spongy tissue or 2 layers of palisade tissue on the adaxial surface, and spongy tissue on the abaxial surface.

Karamunting leaves have bicollateral vascular bundles consisting of xylem bundles flanked by phloem. Ferreira et al. (2011) describe the bundle of vessels in *Psidium guineense* Sw. (family Myrtaceae) leaves that include xylem flanked by phloem. The xylem and phloem function as a delivery system for vascular plants. The primary function of the xylem is to transport water and solutes, while the primary function of the phloem is to distribute photosynthesis products.

Karamunting leaf epidermal tissue on the abaxial side has unicellular trichomes. Kantachot et al. (2007) identified abundant unicellular and uniseriate trichomes on the leaves of Myrtaceae species such as *Decaspermum parviflorum*, *Melaleuca cajuputi*, *Psidium guajava*, *Rhodamnia dumetorum*, *Tristaniopsis burmanica* var. *rufescens*, and *R. tomentosa*. Trichomes from modified epidermal cells reduce evaporation from the leaves, and minimize animal disturbances (Nugroho 2017).

Retamales et al. (2014) found *Myrceugenia rufa* (Myrtaceae) fruit anatomy similar to *R. tomentosa*. The ripe fruit's pericarp has three zones: exocarp, mesocarp, and endocarp. The exocarp cells are irregular and plano-convex. The cuticle is thin and covered in hair. The mesocarp comprises 7-8 layers of large and isodiametric parenchyma cells with thin walls.

Multiple and large secretory cavities are observed throughout this tissue. The endocarp is a delicate tissue that surrounds the seed.

Histochemical test results by Agustina et al. (2016) recorded flavonoids and other phenolics in the stem, leaf, and flower-fruit-seed organs of *Acalypha indica*. Phenols in *Acalypha wilkesiana* are also distributed in the xylem in stem organs, leaf mesophyll, and in the flower-fruit seed.

Flavonoids can be found in every plant organ, including the leaves, roots, wood, flowers, nectar, and seeds. Flavonoids can be stored in cell walls, vacuoles, and glandular trichomes. Lipophilic compounds generated by glandular trichomes include terpenoids, lipids, waxes, and flavonoid aglycones. Flavonoids concentrate in cells and are not present in the intercellular space, resulting in a symplastic distribution (Petrussa et al. 2013).

According to Cartea et al. (2011), phenolic compounds are widespread phytochemicals. Structure-wise, they are simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. Flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids are plants' most abundant and diversified polyphenols. Flavonoids are the most prevalent phenolics and are found throughout all plants. Flavonoids in the leaf and fruit epidermis protect against UV radiation and pigment, stimulate nitrogen-fixing nodules, and fight disease.

The same study found positive reactions to reagents that detect phenolics, in leaf mesophyll cells, pith parenchyma, and stem cortical parenchyma cells of *Adhatoda zeylanica* (Acanthaceae), *Ruta graveolence* Linn. (Rutaceae), and *Vitex negundo* Linn. (Verbenaceae) (Dhale 2011).

Alkaloids are heterocyclic nitrogen-containing chemicals utilized in medicine. Alkaloids protect plants from insects and herbivores, detoxify toxins, and store nitrogen. Many products consumed by humans contains alkaloids, such as coffee, tea, cocoa, tobacco, areca nut, and marijuana. Alkaloids have neuroprotective, blood pressure-lowering, pain-relieving, antibacterial, anticancer, and antimalarial effects (Carqueijeiro et al. 2013). Alkaloids detected in *R. tomentosa's* leaves and fruits support its historic medicinal usage in Singapore. In China, the leaves were traditionally used as a painkiller, the roots for stomach ulcers, and the seeds for improved digestion and for snake bites. In Indonesia, the leaves were used to cure wounds. In traditional Vietnamese medicine, the plant's raw fruit treat diarrhea and dysentery, while the ripe ones boost the immune system (Zhao et al. 2019).

According to Ferreira et al. (2011), histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves indicated positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. These tannins in *R. tomentosa* leaves and fruits help treat diarrhea and dysentery. Ferreira et al. (2011) added that Myrtaceae leaves are used to treat diarrhea and intestinal infections due to their astringent tannin content. Tannins protect plants, which is why epidermal cells have them. Myrtaceae plants have tannins in their vascular systems also.

The meroterpenoids of *R. tomentosa* are antibacterial and antifungal. Myrtaceae secondary metabolites like meroterpenoids have been extensively explored as antibiotics. Thus, *R. tomentosa* could prevent microbial deterioration and extend food shelf life as a biocontrol agent and natural preservative (Zhao et al. 2019). Meroterpenoid compounds have been isolated and identified from various other Myrtaceae species, including *Eucalyptus tereticornis* (Liu et al. 2018) and *Leptospermum brachyandrum* (Zou et al. 2018).

Belwal et al. (2019) reported that flavonoid content declined throughout fruit ripening in *Rubus ellipticus*, *Myrica esculenta*, *Prunus persica*, blueberry (*Vaccinium corymbosum*), and blackberry fruit, as did Lin et al. (2020) in blueberries. Flavonoid levels vary by organ type. Hou et al. (2021) found that phenols and flavonoids drop from 5.38 mg RE/g in young citrus fruits to 0.69 mg RE/g in ripe fruit. The same trend was seen in grapes and lemons (Zhu et al., 2020). This phenomenon may be produced by many enzymes involved in flavonoid synthesis, such as 1,2-rhamnosyltransferase and flavanone-7-O-glucosides, which accelerate biosynthesis in the early growth period. Insufficient flavonoid aglycones may explain the decrease in flavonoids during citrus fruit ripening.

Lai et al. (2013) found a decrease in total phenol levels during *R. tomentosa* fruit ripening. The same pattern of declining phenol levels during fruit ripening occurs in Myrtaceae plants as Brazilian cherry *Eugenia uniflora* L. (Celli et al. 2011). Belwal et al. (2019) further explain the accumulation and degradation of distinct phenolic compounds during fruit ripening connected to their biosynthesis pathways, which are mainly regulated by enzyme expression and various genetic and environmental variables. Elmastaş et al. (2017) added that phenol compounds are phenylpropanoid metabolic pathway intermediates. Enzymes convert aromatic amino acids into polyphenols. Enzyme activity affects the production of these molecules. Genetic diversity, pH, light, temperature, and climatic change affect enzyme activity. Fruit enzyme activity might vary depending on plant growth and ripening.

The anatomical characteristics reported in this study contribute to the identification of *R. tomentosa* and support quality control tests. Some features may be stand out, such as as hairy leaves with curved edges; epidermal cells with ripples and wall projections: and the secretory cavities with dimensions that go beyond the height of the palisade parenchyma. Characters that can contribute to accuracy in taxonomic classification include cross-sectional anatomical structure of leaves including the epidermis, mesophyll, carrier bundle, secretory cavity, and trichomes, while fruit characteristics include a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. In this study, flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the

highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

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[biodiv] Editor Decision

Smujo Editors <support@mail.smujo.id> Tue, Dec 20, 2022 at 6:07 PM
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EVI MINTOWATI KUNTORINI:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of Rhodomyrtus tomentosa".

Our decision is: Revisions Required

Reviewer A:

I have reviewed this revised version of the paper and find it to be well done. With some very minor changes (mainly in the REFERENCES section) the paper should be ready for publication. I have attached here my tacked changes editing of this revised version to help the authors and editors locate where the minor changes are needed. Thank you for allowing me to learn of your work.

Recommendation: Revisions Required

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The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

Abstract. *Rhodomyrtus tomentosa* (Ait.) Hassk is native to Southeast Asia. The entire plant has been used in traditional Vietnamese, Chinese, and Malaysian medicine for a long time. Bioactive substances are abundant in ripe fruits. However, few studies have examined phytochemical changes and distribution throughout leaf and fruit growth. This study evaluated the morphoanatomy, histochemical, and phytochemical properties of *R. tomentosa* leaves and fruits. The cross-sectional anatomical structures observed in the leaves included the epidermis, mesophyll, and carrier bundle, while the fruit structures observed included a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. Flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavities, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavities, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

Keywords: Karamunting, histochemistry, flavonoids, leaves, fruits, *Rhodomyrtus tomentosa*

Running title: The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk., a prolific evergreen shrub in the Myrtaceae family in southern and southeastern Asia, from India, east through southern China, the Philippines, and south to Malaysia and Sulawesi, has rose-pink blooms and dark purple edible bell-shaped fruits. In the Indonesian language it is known as “karamunting”, while in English it is most commonly called “rose myrtle”. This plant favors natural open light and adapts quickly to a range of soil conditions. Moreover, it requires little maintenance and is rarely affected by pests and illnesses. Most parts of this plant (leaves, roots, buds and fruits) have been used in traditional medicines in Vietnam, China and Malaysia for a long time. In traditional medicine of Vietnam, the unripe fruits of the plant are utilized to treat diarrhea or dysentery and ripe ones are used to stimulate the immune system (Zhao et al. 2019).Wounds, heartburn, abscesses, and gynecopathy have been treated with *R. tomentosa*. In China, they use it as a medicine to treat urinary tract infections (Hamid et al. 2017a). Modern pharmacological studies discovered that *R. tomentosa* has antibacterial (Zhao et al. 2018), anticancer (Zhang et al. 2016), anti-inflammatory (Na-Phatthalung et al. 2018), and antioxidant (Hamid et al. 2017b) effects. In Indonesia, *R. tomentosa* roots and leaves treat diarrhea, stomachaches, and postpartum recovery. Most of these effects match typical *R. tomentosa* usage (Zhao et al. 2019). Epidemiological studies have shown that eating fruits and vegetables may protect against chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer, as well as other health benefits (Del Rio et al. 2013). Phytochemicals, particularly phenolics, are responsible for fruit and vegetable health benefits. According to recent findings, *R. tomentosa* berries contain over 19 phenolics, including anthocyanins, phenolic acids, flavonols, ellagitannins, and stilbenes. Due to their nutritional value and taste, *R. tomentosa* berries are a regional favorite (Zhao et al. 2017).

Anatomical studies are essential taxonomic indicators, including determining where compounds are secreted and accumulated in a medicinal species' vegetative and generative organs, and important in the quality control of medicinal plants. Pharmacognosy studies of macroscopic and microscopic characteristics are needed for medicinal raw material authentication and for quality assessment in drug standardization. Among the various alternative plant medicinal sources, accurate plant authentication is required (Raghvendra et al. 2021). Various taxa of Myrtaceae have been the subject of anatomical studies (Ferreira et al. 2011; Al-Edany et al. 2012); however, there has been no histochemical anatomical study of the leaves and fruit of *R. tomentosa*.

Histochemical studies are needed to reveal the typical pattern of distribution of secondary metabolites in plant tissues. Secondary metabolites are distributed in plant tissues based on their functionality. At the cellular level, secondary metabolites can be located in specific secretory tissues or are distributed throughout the tissue's cells, including in the parenchyma of the transport bundle (Nugroho 2017).

Histochemical analyses of fruit development can help explain how metabolites affecting color and flavor are synthesized and accumulated during plant organ development. Histological studies can assess the morphological and anatomical details of *R. tomentosa* leaves and fruits. However, studies have not usually addressed secondary metabolite localization in this species. Particular staining and procedures can reveal metabolites, including pectin, lipids, lignin, proteins, phenolic compounds, tannins, starch grains, essential oils, rubber, and terpenoids (Vio-Michaelis et al. 2020). Unequivocal botanical characterizations are needed to identify medicinal plants correctly. Thus, the objective of this present study was to evaluate the leaves' and fruits' morphoanatomy, histochemistry, and phytochemistry of a sample of identified karamunting (*R. tomentosa*) plants collected in South Kalimantan, Indonesia.

MATERIALS AND METHODS

Plant materials

The 2nd and 10th leaves down from the stem tip and the green, red, and purple fruits of *R. tomentosa* were collected from wild plants in Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) (Figure 1). Three replicate samples of leaves and fruit were collected from three different plants. Herbarium Bogoriense at the Indonesian Institute of Sciences in Bogor, Indonesia, identified the plant specimen.

Figure 1. Aerial part of *Rhodomyrtus tomentosa* (Aiton.) Hassk.

Procedures

Morphoanatomy slides preparation

Retamales et al. (2014) described the transverse sections of leaves and fruit using paraffin embedding with safranin staining. In the present study, m~~M~~inor adjustments were made to the immersion duration in dehydration and dealcoholization. Three replicates of fruits and leaves from three different plants were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70%, 5:5:90) solution for 24 hours. These replicates were then dehydrated, stored in 70% ethanol, and embedded in paraffin. Sections 8 to 10 µm thick were obtained on a rotatory microtome, dyed with Safranin, and mounted in Entelan to serve as permanent slides.

Histochemical test

Fresh, fully developed leaves (from the central nerve region and the blade) were cross-sectioned and subjected to histochemical test. Hand-cut sections were treated with a dilute 10% aqueous acidic $FeCl_3$ (Sigma–Aldrich, Germany) solution containing a small quantity of Na_2CO_3 (Sigma–Aldrich, Germany) solution mounted in clove oil for phenolic compounds, which were identified as dark green or black color in tissues (Trimanto et al. 2018). Flavonoids were identified using Wilson's reagent. Each segment was soaked in citric acid:boric (Prolabo) (5:5 w/w) in 100 mL absolute ethanol for 15 minutes, mounted in glycerine–water, and inspected under a light microscope (Olympus, Tokyo, Japan). ~~The~~ A yellow color signified the presence of flavonoids (Pratiwi et al. 2020). With 5% copper acetate ($Cu_2(CH_3COO)_4$), terpenoids stained yellow-brown, indicating their presence in cells or tissues (Pratiwi et al. 2020). $FeCl_3$ (0.5% - 1% $FeCl_3$ in 0.1 N HCL) stained tannins blackish green, suggesting their existence (Robil and Tolentino 2015). Wagner's reagent was used to detect alkaloids, and any red-brown staining of tissues or cells indicated the presence of alkaloid compounds (Pratiwi et al. 2020). The material was photographed using OptiLab and a light microscope (Olympus, Tokyo, Japan).

Crude extract preparation

Young leaves ~~(~~2nd – 6th down from the tip) and old leaves (7th – 12th down from the tip) were picked from the shoot of *R. tomentosa* plants, while harvested green, red, and purple fruits were dried in an oven at 40°C before being powdered at

room temperature. A sample of 500 g of each powdered material was macerated for 24 hours in 1000 mL of ethanol (SmartLab, Indonesia). After 24 hours, the solvent was discarded and replenished thrice (Kusuma et al. 2016). Finally, extracts from the same samples were pooled, mixed, filtered, and dried using a rotary evaporator. The extract yield was calculated by this equation: (weight of ethanol extract/weight of dried leaves or fruits) × 100%.

Total phenolic compounds

Folin-Ciocalteu colorimetry was used to determine the triplicate samples' total phenolic contents (TPC) (Roy et al. 2018). The samples' extract absorbances were measured with a UV-Vis spectrophotometer (spectrophotometer UV 1800–Shimadzu, Japan) at 760 nm, and the total phenol content was calculated using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight.

Total flavonoids content

Total flavonoid content was determined using AlCl colorimetry. A 1 mL sample of extract was mixed in a test tube with 4 mL of distilled water and with 0.3 mL of 5% sodium nitrite (Sigma–Aldrich, Germany). After 5 minutes, 0.3 mL of 10% aluminum chloride (Sigma–Aldrich, Germany) was added and stirred. After 5 minutes, 2 ml of sodium hydroxide (1 M) was added, and distilled water was added until the volume was precisely 10 mL. The identical method used for the earlier measurement was used to gauge the sample's extract absorbance at 510 nm. Finally, the total flavonoid content was determined using a quercetin standard curve (Sigma–Aldrich, Germany) at mg QE/g dry weight (Roy et al. 2018).

Total tannin content

The Folin-Ciocalteu method was used to calculate the total tannin content. The following ingredients were added to 0.1 mL of sample extract: 7.5 mL of distilled water, 0.5 mL of 10% F-C reagent, and 1 mL of 35% sodium carbonate solution (Sigma–Aldrich, Germany). Then, using distilled water, the volume was increased to 10 mL. After being mixed, the mixture was left at room temperature for 30 minutes. The sample's extract absorbance was determined at a wavelength of 725 nm. Finally, the total tannin content was determined using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight (Roy et al. 2018).

Total alkaloids content

2.5 g of the sample extract was diluted in 50 mL of 10% acetic acid (in ethanol). After being shaken with a magnetic stirrer for 4 hours, the solution was filtered. After that, the filtrate was evaporated. Then, ammonium hydroxide (Sigma-Aldrich, Germany) was drip-applied until an alkaloid precipitate formed. The precipitate-filtering filter paper was weighed before usage. After that, the residue was filtered and cleaned with an ammonium hydroxide 1% solution. The filter paper containing the precipitate was oven dried for 30 minutes at 60°C. After the residue had cooled, it was weighed until a constant weight was obtained. The alkaloid yield was calculated by the weight percentage of the precipitated alkaloid obtained against the initial weight of the extract. (Alasa et al. 2017).

Data analysis

The results from three replications were used to calculate the means and standard deviations (mean ± SD). Using IBM SPSS statistics 21 and Microsoft Excel®, the quantitative data were statistically evaluated using a one-way analysis of variance, with significant differences set at $p < 0.05$. When a statistically significant difference was detected, the analysis of variance was followed by an LSD post hoc test.

RESULTS AND DISCUSSION

Morphoanatomy of *R. tomentosa* leaves and fruits

Young and old *R. tomentosa* leaves have similar epidermal, mesophyll, and transport bundle structures. The adaxial and abaxial epidermis comprises the epidermis. Compared to the abaxial epidermis, the adaxial is larger. The mesophyll consists of palisade tissue and spongy, single-layer palisade tissue grouped compactly. The leaves are dorsiventral in shape, having palisade tissue on the adaxial side. The leaf stalk's carrier bundle network is made up of phloem and xylem. The leaf bone has parenchyma tissue as filling and collenchyma under the epidermis as reinforcement. Trichomes cover the leave's lower epidermis, while a thin cuticle covers the upper epidermis (Figure 2).

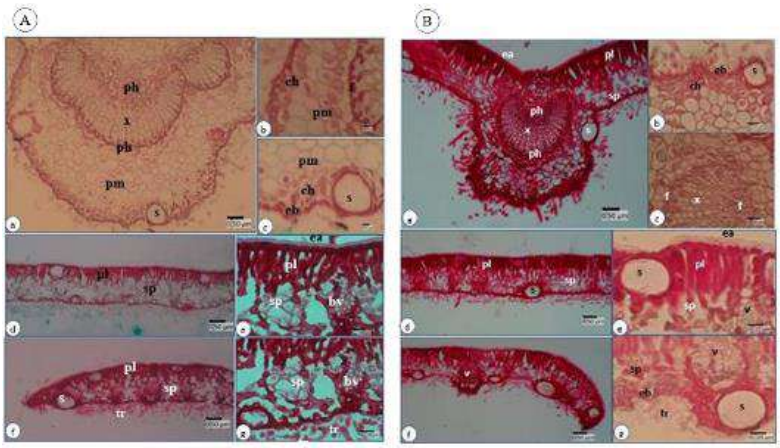

Figure 2. Cross-section of (A) young leaves, (B) old leaves *of R. tomentosa;* palisade mesophyll (pl), sponge mesophyll (sp), parenchyma midrib (pm), secretory cavity (sc), trichomes (tr), abaxial epidermis (eb), adaxial epidermis (ea), collenchyma (ch), vascular bundle (v), phloem (f), xylem (x)

The fruit grows from fertilization. The growth of fruit components, such as fruit skin (pericarp), seed coat, endosperm, and embryo, follows fruit development. *R. tomentosa* fruit is a bacca/buni 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 (Hamid et al. 2017a). The cross-section of green, red, and purple fruit displays a fruit pericarp layer with an exocarp/outer layer, middle mesocarp, and endocarp. (Fig. 3). The stiff exocarp has a polyhedral epidermis and several sclerenchyma layers. The edible mesocarp has big, watery parenchyma cells. The endocarp cells are thin-walled. The 1.5 mm seeds have 6 pseudo-locules with thin false septa (Hermanto et al. 2013; Hamid et al. 2017a).

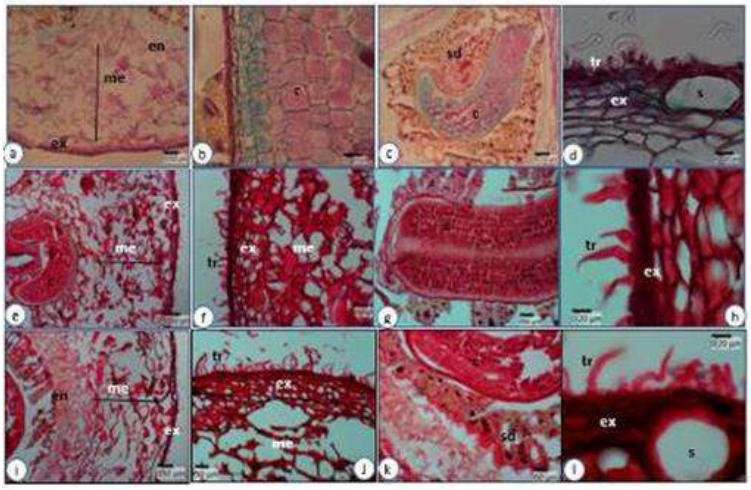

Figure 3. Cross-section of green fruit (a-d), red fruit (e-h) and purple fruit (i-l) of *R. tomentosa;* exocarp (ex) mesocarp (me), endocarp (en), seed (sd), secretory cavity (s), trichomes (tr)

Histochemical tests

Histochemical tests measure the secondary metabolites of the plant. Histochemical tests use qualitative analysis to detect compounds without measuring their concentration (Dhale 2011). Secondary metabolite components such as phenols, flavonoids, tannins, terpenoids, and alkaloids were found in *R. tomentosa*'s leaves and fruits at specific locations or more generally in the tissues. The secondary metabolites were detected histochemically. After application of reagents, the identified compounds showed specific color changes (Figure 4).

Histochemical tests on young and old leaves, green, red, and purple fruit indicated positive reactions to flavonoid compounds (4f-4j) and phenols (Figure 4k-4o). Young and old leaves had phenolic, and flavonoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. Fruit phenolic and flavonoid compounds were found in the exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4p-4t shows alkaloid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. In green and red fruit, alkaloids were found in the endosperm, exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4u-4y shows tannin compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Tannin compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichome, and seeds, in the green and red fruit but not in the purple fruit.

Figure 4z-4d1 shows terpenoid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Terpenoid compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Terpenoid compounds were not found in the endocarp of purple fruit.

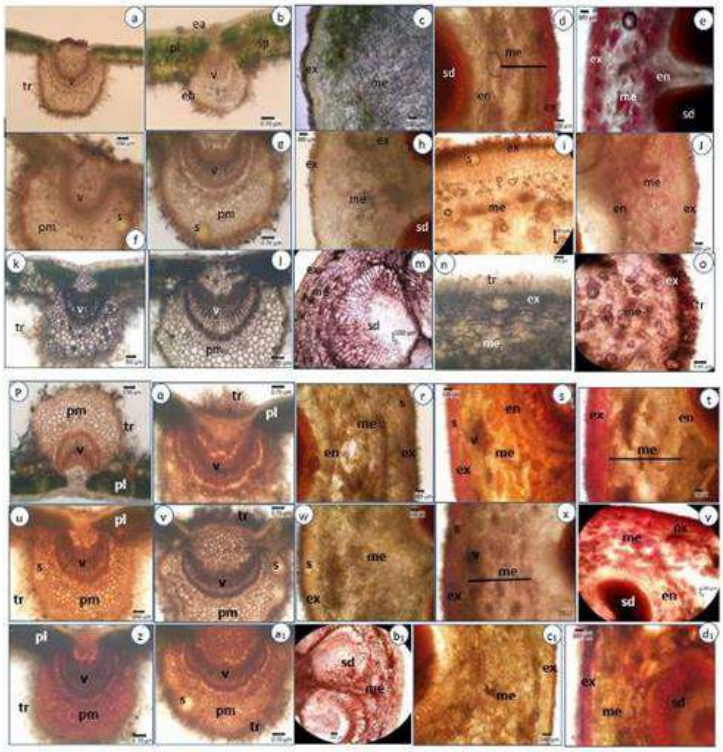

Figure 4. Transverse sections of histochemically stained leaves and fruits of *R. Tomentosa.* (a-e) Free-hand section of a young leaf without staining; (f-j) the positive reaction to Wilson's reagent indicating the presence of flavonoid in leaves and fruits; (k-o) the positive reaction to ferric chloride indicating the presence of phenolics in leaves and fruits: (p-t) the positive reaction to Wagner's reagent

indicating the presence of alkaloid in leaves and fruits; (u-y) the positive reaction to $FeCl_3$ indicating the presence of tannin in leaves and fruits; (z-d_1) the positive reaction to $Cu_2(CH_3COO)_4$ 5% indicating the presence of terpenoid in leaves and fruits: palisade mesophyll (pm), sponge mesophyll (sm), parenchyma midrib (pm), secretory cavity (s), trichomes (tr), druse crystal (dc), abaxial epidermis (eb), adaxial epidermis (ea) exocarp (ex) mesocarp (me), endocarp (en), seed (sd) vascular bundle (v).

Phytochemistry

The results of phytochemical measurements showed that the highest total flavonoid levels were in young leaves at 196.0 mg QE/g, followed by green fruit and old leaves, 164.625 mg QE/g, and 134.875 mg QE/g, followed by flavonoid levels in red and purple fruits at 84.625 mg QE/g and 55.75 mg QE/g, respectively. The highest measured total phenol content was 97.70 mg/mL recorded in green fruit, followed by old leaves at 59.30 mg/mL, young leaves at 54.40 mg/mL, red fruit at 19.40 mg/mL, and the lowest, in purple fruit at 17.23mg/mL. The highest total alkaloid content was recorded in old leaves at 13.23%, green fruit at 9.28%, red fruit at 7.80%, purple fruit at 7.05%, and young leaves, the lowest at 5.64%. For total tannin content, the measurements showed the highest value in red fruit at 1.658 mg GAE/g, followed by purple fruit at 1.402 mg GAE/g, young leaves at 0.943 mg GAE/g, old leaves at 0.880 mg GAE/g, and the lowest tannin content in green fruit at 0.804 mg GAE/g. (Table 1).

Table 1. Total amount of compounds in ethanolic extracts of leaves and fruits of *R. tomentosa*

Sample	Total flavonoids (mgQE/g)	Total phenolics (mgGAE/g)	Total alkaloids (%)	Total tannins (mgGAE/g)
Young leaves	196 ± 1.77^{e}	54.40 ± 0.09^{b}	5.64 ± 0.20^{a}	0.94 ± 0.74^{a}
Old leaves	134.88 ± 6.89^{c}	59.30 ± 16.73^{b}	13.22 ± 0.98^{d}	0.88 ± 0.01^{a}
Green fruits	164.63 ± 2.65^{d}	97.70 ± 18.15^{c}	9.28 ± 0.33^{c}	0.80 ± 0.30^{a}
Red fruits	84.63 ± 2.30^{b}	19.40 ± 0.47^{a}	$7.80 \pm 0.97b^{c}$	1.66 ± 0.15^{c}
Purple fruits	55.75 ± 0.71^{a}	17.23 ± 4.20^{a}	$7.05 \pm 0.41a^{b}$	1.40 ± 1.20^{b}

Note: The data presented are the mean ± standard deviation. One-Way ANOVA and LSD test with = 0.05 were used to evaluate data. Numbers followed by different superscript letters in the same column show significantly different results.

Discussion

According to research by Al-Edany and Al-Saadi (2012) on the anatomical characteristics of several Myrtaceae species, the upper surface of the epidermal tissue on the leaves of the Myrtaceae family is covered by a variable-thickness cuticle layer. Young and old leaves of karamunting (*Rhodomyrtus tomentosa*) have one layer of epidermal tissue on the upper surface (adaxial). The epidermal tissue's cells are square and closely packed. Al-Edany and Al-Saadi (2012) found that the cells of Myrtaceae leaf epidermal tissue are square or rectangular and uniseriate.

Kantachot et al. (2007) examined the leaves of 28 species in 12 genera of the Myrtaceae family and found that *R. tomentosa* had dorsiventral leaves, which have palisade tissue only on one side. The cells in the upper epidermis are thicker than those in the lower epidermis. The leaves of *R. tomentosa* do not have a hypodermis. The lower epidermis of the leaves has unicellular trichomes and anomocytic stomata. In the cross-section of the leaf stalk and petiole, vascular tissue forms a U-shaped pattern. Drusen-shaped Ca oxalate crystals are seen in leaf bone maternal parenchyma tissue.

The leaves of karamunting have palisade/pole and spongy mesophyll tissue. The palisade parenchyma is found beneath the upper epidermis. The cells that comprise the palisade parenchyma tissue are formed like pillars, appear tightly packed, and are just one layer thick. There are many chloroplasts in this palisade parenchyma tissue. The spongy tissue is located beneath the palisade tissue. The cells that make up the spongy tissue are formed like branches, appear loosely organized, and are uneven. Spongy parenchyma tissue contains chloroplasts, although less than palisade/pillar tissue. According to Al-Edany and Al-Saadi (2012), Myrtaceae leaves' mesophyll tissue has 1 to 2 layers of palisade tissue and several rows of up to 15 layers of compact and loose spongy tissue or 2 layers of palisade tissue on the adaxial surface, and spongy tissue on the abaxial surface.

Karamunting leaves have bicollateral vascular bundles consisting of xylem bundles flanked by phloem. Ferreira et al. (2011) describe the bundle of vessels in *Psidium guineense* Sw. (family Myrtaceae) leaves that include xylem flanked by phloem. The xylem and phloem function as a delivery system for vascular plants. The primary function of the xylem is to transport water and solutes, while the primary function of the phloem is to distribute photosynthesis products.

Karamunting leaf epidermal tissue on the abaxial side has unicellular trichomes. Kantachot et al. (2007) identified abundant unicellular and uniseriate trichomes on the leaves of Myrtaceae species such as *Decaspermum parviflorum*, *Melaleuca cajuputi*, *Psidium guajava*, *Rhodamnia dumetorum*, *Tristaniopsis burmanica* var. *rufescens*, and *R. tomentosa*. Trichomes from modified epidermal cells reduce evaporation from the leaves, and minimize animal disturbances (Nugroho 2017).

Retamales et al. (2014) found *Myrceugenia rufa* (Myrtaceae) fruit anatomy similar to *R. tomentosa*. The ripe fruit's pericarp has three zones: exocarp, mesocarp, and endocarp. The exocarp cells are irregular and plano-convex. The cuticle is thin and covered in hair. The mesocarp comprises 7-8 layers of large and isodiametric parenchyma cells with thin walls.

Multiple and large secretory cavities are observed throughout this tissue. The endocarp is a delicate tissue that surrounds the seed.

Histochemical test results by Agustina et al. (2016) recorded flavonoids and other phenolics in the stem, leaf, and flower-fruit-seed organs of *Acalypha indica*. Phenols in *Acalypha wilkesiana* are also distributed in the xylem in stem organs, leaf mesophyll, and in the flower-fruit seed.

Flavonoids can be found in every plant organ, including the leaves, roots, wood, flowers, nectar, and seeds. Flavonoids can be stored in cell walls, vacuoles, and glandular trichomes. Lipophilic compounds generated by glandular trichomes include terpenoids, lipids, waxes, and flavonoid aglycones. Flavonoids concentrate in cells and are not present in the intercellular space, resulting in a symplastic distribution (Petrussa et al. 2013).

According to Cartea et al. (2011), phenolic compounds are widespread phytochemicals. Structure-wise, they are simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. Flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids are plants' most abundant and diversified polyphenols. Flavonoids are the most prevalent phenolics and are found throughout all plants. Flavonoids in the leaf and fruit epidermis protect against UV radiation and pigment, stimulate nitrogen-fixing nodules, and fight disease.

The same study found positive reactions to reagents that detect phenolics, in leaf mesophyll cells, pith parenchyma, and stem cortical parenchyma cells of *Adhatoda zeylanica* (Acanthaceae), *Ruta graveolence* Linn. (Rutaceae), and *Vitex negundo* Linn. (Verbenaceae) (Dhale 2011).

Alkaloids are heterocyclic nitrogen-containing chemicals utilized in medicine. Alkaloids protect plants from insects and herbivores, detoxify toxins, and store nitrogen. Many products consumed by humans contains alkaloids, such as coffee, tea, cocoa, tobacco, areca nut, and marijuana. Alkaloids have neuroprotective, blood pressure-lowering, pain-relieving, antibacterial, anticancer, and antimalarial effects (Carqueijeiro et al. 2013). Alkaloids detected in *R. tomentosa*'s leaves and fruits support its historic medicinal usage in Singapore. In China, the leaves were traditionally used as a painkiller, the roots for stomach ulcers, and the seeds for improved digestion and for snake bites. In Indonesia, the leaves were used to cure wounds. In traditional Vietnamese medicine, the plant's raw fruit treat diarrhea and dysentery, while the ripe ones boost the immune system (Zhao et al. 2019).

According to Ferreira et al. (2011), histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves indicated positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. These tannins in *R. tomentosa* leaves and fruits help treat diarrhea and dysentery. Ferreira et al. (2011) added that Myrtaceae leaves are used to treat diarrhea and intestinal infections due to their astringent tannin content. Tannins protect plants, which is why epidermal cells have them. Myrtaceae plants have tannins in their vascular systems also.

The meroterpenoids of *R. tomentosa* are antibacterial and antifungal. Myrtaceae secondary metabolites like meroterpenoids have been extensively explored as antibiotics. Thus, *R. tomentosa* could prevent microbial deterioration and extend food shelf life as a biocontrol agent and natural preservative (Zhao et al. 2019). Meroterpenoid compounds have been isolated and identified from various other Myrtaceae species, including *Eucalyptus tereticornis* (Liu et al. 2018) and *Leptospermum brachyandrum* (Zou et al. 2018).

Belwal et al. (2019) reported that flavonoid content declined throughout fruit ripening in *Rubus ellipticus*, *Myrica esculenta*, *Prunus persica*, blueberry (*Vaccinium corymbosum*), and blackberry fruit, as did Lin et al. (2020) in blueberries. Flavonoid levels vary by organ type. Hou et al. (2021) found that phenols and flavonoids drop from 5.38 mg RE/g in young citrus fruits to 0.69 mg RE/g in ripe fruit. The same trend was seen in grapes and lemons (Zhu et al.~~,~~ 2020). This phenomenon may be produced by many enzymes involved in flavonoid synthesis, such as 1,2-rhamnosyltransferase and flavanone-7-O-glucosides, which accelerate biosynthesis in the early growth period. Insufficient flavonoid aglycones may explain the decrease in flavonoids during citrus fruit ripening.

Lai et al. (2013) found a decrease in total phenol levels during *R. tomentosa* fruit ripening. The same pattern of declining phenol levels during fruit ripening occurs in other Myrtaceae plants such as Brazilian cherry *Eugenia uniflora* L. (Celli et al. 2011). Belwal et al. (2019) further explain the accumulation and degradation of distinct phenolic compounds during fruit ripening connected to their biosynthesis pathways, which are mainly regulated by enzyme expression and various genetic and environmental variables. Elmastaş et al. (2017) added that phenol compounds are phenylpropanoid metabolic pathway intermediates. Enzymes convert aromatic amino acids into polyphenols. Enzyme activity affects the production of these molecules. Genetic diversity, pH, light, temperature, and climatic change affect enzyme activity. Fruit enzyme activity might vary depending on plant growth and ripening.

The anatomical characteristics reported in this study contribute to the identification of *R. tomentosa* and support quality control tests. Some features may ~~be~~ stand out, such as as hairy leaves with curved edges; epidermal cells with ripples and wall projections: and the secretory cavities with dimensions that go beyond the height of the palisade parenchyma. Characters that can contribute to accuracy in taxonomic classification include cross-sectional anatomical structure of leaves including the epidermis, mesophyll, carrier bundle, secretory cavity, and trichomes, while fruit characteristics include a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. In this study, flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the

highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).s

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The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

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Abstract. *Rhodomyrtus tomentosa* (Ait.) Hassk is native to Southeast Asia. The entire plant has been used in traditional Vietnamese, Chinese, and Malaysian medicine for a long time. Bioactive substances are abundant in ripe fruits. However, few studies have examined phytochemical changes and distribution throughout leaf and fruit growth. This study evaluated the morphoanatomy, histochemical, and phytochemical properties of *R. tomentosa* leaves and fruits. The cross-sectional anatomical structures observed in the leaves included the epidermis, mesophyll, and carrier bundle, while the fruit structures observed included a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. Flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavities, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavities, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

Keywords: Karamunting, histochemistry, flavonoids, leaves, fruits, *Rhodomyrtus tomentosa*

Running title: The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk., a prolific evergreen shrub in the Myrtaceae family in southern and southeastern Asia, from India, east through southern China, the Philippines, and south to Malaysia and Sulawesi, has rose-pink blooms and dark purple edible bell-shaped fruits. In the Indonesian language it is known as “karamunting”, while in English it is most commonly called “rose myrtle”. This plant favors natural open light and adapts quickly to a range of soil conditions. Moreover, it requires little maintenance and is rarely affected by pests and illnesses. Most parts of this plant (leaves, roots, buds and fruits) have been used in traditional medicines in Vietnam, China and Malaysia for a long time. In traditional medicine of Vietnam, the unripe fruits of the plant are utilized to treat diarrhea or dysentery and ripe ones are used to stimulate the immune system (Zhao et al. 2019).Wounds, heartburn, abscesses, and gynecopathy have been treated with *R. tomentosa*. In China, they use it as a medicine to treat urinary tract infections (Hamid et al. 2017a). Modern pharmacological studies discovered that *R. tomentosa* has antibacterial (Zhao et al. 2018), anticancer (Zhang et al. 2016), anti-inflammatory (Na-Phatthalung et al. 2018), and antioxidant (Hamid et al. 2017b) effects. In Indonesia, *R. tomentosa* roots and leaves treat diarrhea, stomachaches, and postpartum recovery. Most of these effects match typical *R. tomentosa* usage (Zhao et al. 2019). Epidemiological studies have shown that eating fruits and vegetables may protect against chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer, as well as other health benefits (Del Rio et al. 2013). Phytochemicals, particularly phenolics, are responsible for fruit and vegetable health benefits. According to recent findings, *R. tomentosa* berries contain over 19 phenolics, including anthocyanins, phenolic acids, flavonols, ellagitannins, and stilbenes. Due to their nutritional value and taste, *R. tomentosa* berries are a regional favorite (Zhao et al. 2017).

Anatomical studies are essential taxonomic indicators, including determining where compounds are secreted and accumulated in a medicinal species' vegetative and generative organs, and important in the quality control of medicinal plants. Pharmacognosy studies of macroscopic and microscopic characteristics are needed for medicinal raw material authentication and for quality assessment in drug standardization. Among the various alternative plant medicinal sources, accurate plant authentication is required (Raghvendra et al. 2021). Various taxa of Myrtaceae have been the subject of anatomical studies (Ferreira et al. 2011; Al-Edany et al. 2012); however, there has been no histochemical anatomical study of the leaves and fruit of *R. tomentosa*.

Histochemical studies are needed to reveal the typical pattern of distribution of secondary metabolites in plant tissues. Secondary metabolites are distributed in plant tissues based on their functionality. At the cellular level, secondary

metabolites can be located in specific secretory tissues or are distributed throughout the tissue's cells, including in the parenchyma of the transport bundle (Nugroho 2017).

Histochemical analyses of fruit development can help explain how metabolites affecting color and flavor are synthesized and accumulated during plant organ development. Histological studies can assess the morphological and anatomical details of *R. tomentosa* leaves and fruits. However, studies have not usually addressed secondary metabolite localization in this species. Particular staining and procedures can reveal metabolites, including pectin, lipids, lignin, proteins, phenolic compounds, tannins, starch grains, essential oils, rubber, and terpenoids (Vio-Michaelis et al. 2020). Unequivocal botanical characterizations are needed to identify medicinal plants correctly. Thus, the objective of this present study was to evaluate the leaves' and fruits' morphoanatomy, histochemistry, and phytochemistry of a sample of identified karamunting (*R. tomentosa*) plants collected in South Kalimantan, Indonesia.

MATERIALS AND METHODS

Plant materials

The 2nd and 10th leaves down from the stem tip and the green, red, and purple fruits of *R. tomentosa* were collected from wild plants in Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) (Figure 1). Three replicate samples of leaves and fruit were collected from three different plants. Herbarium Bogoriense at the Indonesian Institute of Sciences in Bogor, Indonesia, identified the plant specimen.

Figure 1. Aerial part of *Rhodomyrtus tomentosa* (Aiton.) Hassk.

Procedures

Morphoanatomy slides preparation

Retamales et al. (2014) described the transverse sections of leaves and fruit using paraffin embedding with safranin staining. In the present study, minor adjustments were made to the immersion duration in dehydration and dealcoholization. Three replicates of fruits and leaves from three different plants were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70%, 5:5:90) solution for 24 hours. These replicates were then dehydrated, stored in 70% ethanol, and embedded in paraffin. Sections 8 to 10 µm thick were obtained on a rotatory microtome, dyed with Safranin, and mounted in Entelan to serve as permanent slides.

Histochemical test

Fresh, fully developed leaves (from the central nerve region and the blade) were cross-sectioned and subjected to histochemical test. Hand-cut sections were treated with a dilute 10% aqueous acidic $FeCl_3$ (Sigma–Aldrich, Germany) solution containing a small quantity of Na_2CO_3 (Sigma–Aldrich, Germany) solution mounted in clove oil for phenolic compounds, which were identified as dark green or black color in tissues (Trimanto et al. 2018). Flavonoids were identified using Wilson's reagent. Each segment was soaked in citric acid:boric (Prolabo) (5:5 w/w) in 100 mL absolute ethanol for 15 minutes, mounted in glycerine–water, and inspected under a light microscope (Olympus, Tokyo, Japan). A yellow color signified the presence of flavonoids (Pratiwi et al. 2020). With 5% copper acetate ($Cu_2(CH_3COO)_4$), terpenoids stained yellow-brown, indicating their presence in cells or tissues (Pratiwi et al. 2020). $FeCl_3$ (0.5% - 1% $FeCl_3$ in 0.1 N HCL) stained tannins blackish green, suggesting their existence (Robil and Tolentino 2015). Wagner's reagent was used to detect alkaloids, and any red-brown staining of tissues or cells indicated the presence of alkaloid compounds (Pratiwi et al. 2020). The material was photographed using OptiLab and a light microscope (Olympus, Tokyo, Japan).

Crude extract preparation

Young leaves (2^{nd} – 6^{th} down from the tip) and old leaves (7^{th} – 12^{th} down from the tip) were picked from the shoot of *R. tomentosa* plants, while harvested green, red, and purple fruits were dried in an oven at 40°C before being powdered at room temperature. A sample of 500 g of each powdered material was macerated for 24 hours in 1000 mL of ethanol (SmartLab, Indonesia). After 24 hours, the solvent was discarded and replenished thrice (Kusuma et al. 2016). Finally, extracts from the same samples were pooled, mixed, filtered, and dried using a rotary evaporator. The extract yield was calculated by this equation: (weight of ethanol extract/weight of dried leaves or fruits) × 100%.

Total phenolic compounds

Folin-Ciocalteu colorimetry was used to determine the triplicate samples' total phenolic contents (TPC) (Roy et al. 2018). The samples' extract absorbances were measured with a UV-Vis spectrophotometer (spectrophotometer UV 1800–Shimadzu, Japan) at 760 nm, and the total phenol content was calculated using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight.

Total flavonoids content

Total flavonoid content was determined using AlCl colorimetry. A 1 mL sample of extract was mixed in a test tube with 4 mL of distilled water and with 0.3 mL of 5% sodium nitrite (Sigma–Aldrich, Germany). After 5 minutes, 0.3 mL of 10% aluminum chloride (Sigma–Aldrich, Germany) was added and stirred. After 5 minutes, 2 ml of sodium hydroxide (1 M) was added, and distilled water was added until the volume was precisely 10 mL. The identical method used for the earlier measurement was used to gauge the sample's extract absorbance at 510 nm. Finally, the total flavonoid content was determined using a quercetin standard curve (Sigma–Aldrich, Germany) at mg QE/g dry weight (Roy et al. 2018).

Total tannin content

The Folin-Ciocalteu method was used to calculate the total tannin content. The following ingredients were added to 0.1 mL of sample extract: 7.5 mL of distilled water, 0.5 mL of 10% F-C reagent, and 1 mL of 35% sodium carbonate solution (Sigma–Aldrich, Germany). Then, using distilled water, the volume was increased to 10 mL. After being mixed, the mixture was left at room temperature for 30 minutes. The sample's extract absorbance was determined at a wavelength of 725 nm. Finally, the total tannin content was determined using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight (Roy et al. 2018).

Total alkaloids content

2.5 g of the sample extract was diluted in 50 mL of 10% acetic acid (in ethanol). After being shaken with a magnetic stirrer for 4 hours, the solution was filtered. After that, the filtrate was evaporated. Then, ammonium hydroxide (Sigma-Aldrich, Germany) was drip-applied until an alkaloid precipitate formed. The precipitate-filtering filter paper was weighed before usage. After that, the residue was filtered and cleaned with an ammonium hydroxide 1% solution. The filter paper containing the precipitate was oven dried for 30 minutes at 60°C. After the residue had cooled, it was weighed until a constant weight was obtained. The alkaloid yield was calculated by the weight percentage of the precipitated alkaloid obtained against the initial weight of the extract. (Alasa et al. 2017).

Data analysis

The results from three replications were used to calculate the means and standard deviations (mean ± SD). Using IBM SPSS statistics 21 and Microsoft Excel®, the quantitative data were statistically evaluated using a one-way analysis of variance, with significant differences set at $p < 0.05$. When a statistically significant difference was detected, the analysis of variance was followed by an LSD post hoc test.

RESULTS AND DISCUSSION

Morphoanatomy of *R. tomentosa* leaves and fruits

Young and old *R. tomentosa* leaves have similar epidermal, mesophyll, and transport bundle structures. The adaxial and abaxial epidermis comprises the epidermis. Compared to the abaxial epidermis, the adaxial is larger. The mesophyll consists of palisade tissue and spongy, single-layer palisade tissue grouped compactly. The leaves are dorsiventral in shape, having palisade tissue on the adaxial side. The leaf stalk's carrier bundle network is made up of phloem and xylem. The leaf bone has parenchyma tissue as filling and collenchyma under the epidermis as reinforcement. Trichomes cover the leave's lower epidermis, while a thin cuticle covers the upper epidermis (Figure 2).

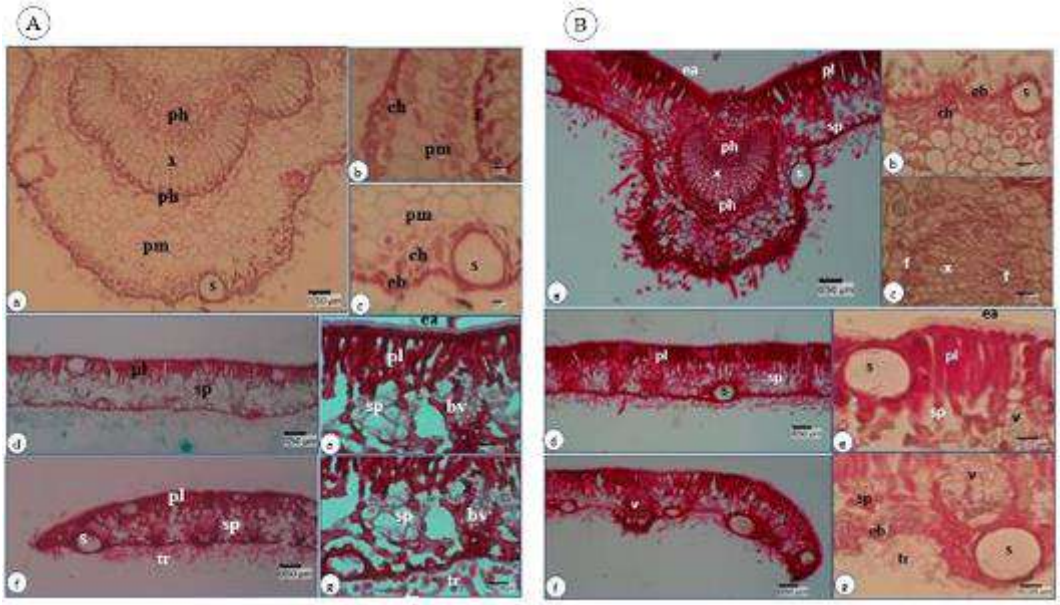

Figure 2. Cross-section of (A) young leaves, (B) old leaves *of R. tomentosa;* palisade mesophyll (pl), sponge mesophyll (sp), parenchyma midrib (pm), secretory cavity (sc), trichomes (tr), abaxial epidermis (eb), adaxial epidermis (ea), collenchyma (ch), vascular bundle (v), phloem (f), xylem (x)

The fruit grows from fertilization. The growth of fruit components, such as fruit skin (pericarp), seed coat, endosperm, and embryo, follows fruit development. *R. tomentosa* fruit is a bacca/buni 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 (Hamid et al. 2017a). The cross-section of green, red, and purple fruit displays a fruit pericarp layer with an exocarp/outer layer, middle mesocarp, and endocarp. (Fig. 3). The stiff exocarp has a polyhedral epidermis and several sclerenchyma layers. The edible mesocarp has big, watery parenchyma cells. The endocarp cells are thin-walled. The 1.5 mm seeds have 6 pseudo-locules with thin false septa (Hermanto et al. 2013; Hamid et al. 2017a).

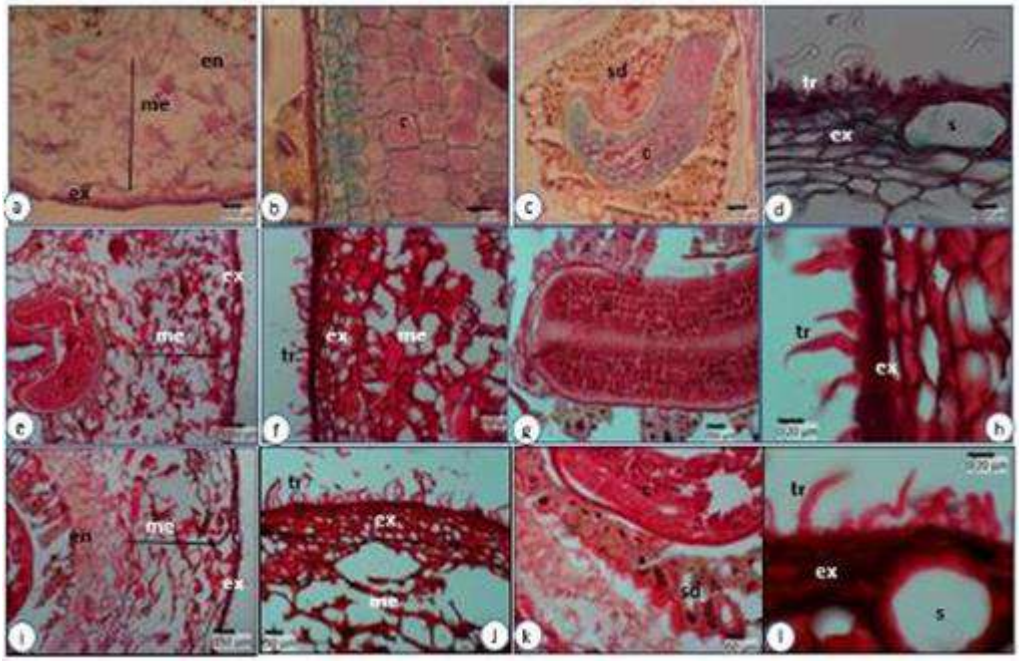

Figure 3. Cross-section of green fruit (a-d), red fruit (e-h) and purple fruit (i-l) of *R. tomentosa;* exocarp (ex) mesocarp (me), endocarp (en), seed (sd), secretory cavity (s), trichomes (tr)

Histochemical tests

Histochemical tests measure the secondary metabolites of the plant. Histochemical tests use qualitative analysis to detect compounds without measuring their concentration (Dhale 2011). Secondary metabolite components such as phenols, flavonoids, tannins, terpenoids, and alkaloids were found in *R. tomentosa*'s leaves and fruits at specific locations or more generally in the tissues. The secondary metabolites were detected histochemically. After application of reagents, the identified compounds showed specific color changes (Figure 4).

Histochemical tests on young and old leaves, green, red, and purple fruit indicated positive reactions to flavonoid compounds (4f-4j) and phenols (Figure 4k-4o). Young and old leaves had phenolic, and flavonoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. Fruit phenolic and flavonoid compounds were found in the exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4p-4t shows alkaloid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. In green and red fruit, alkaloids were found in the endosperm, exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4u-4y shows tannin compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Tannin compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichome, and seeds, in the green and red fruit but not in the purple fruit.

Figure 4z-4d1 shows terpenoid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Terpenoid compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Terpenoid compounds were not found in the endocarp of purple fruit.

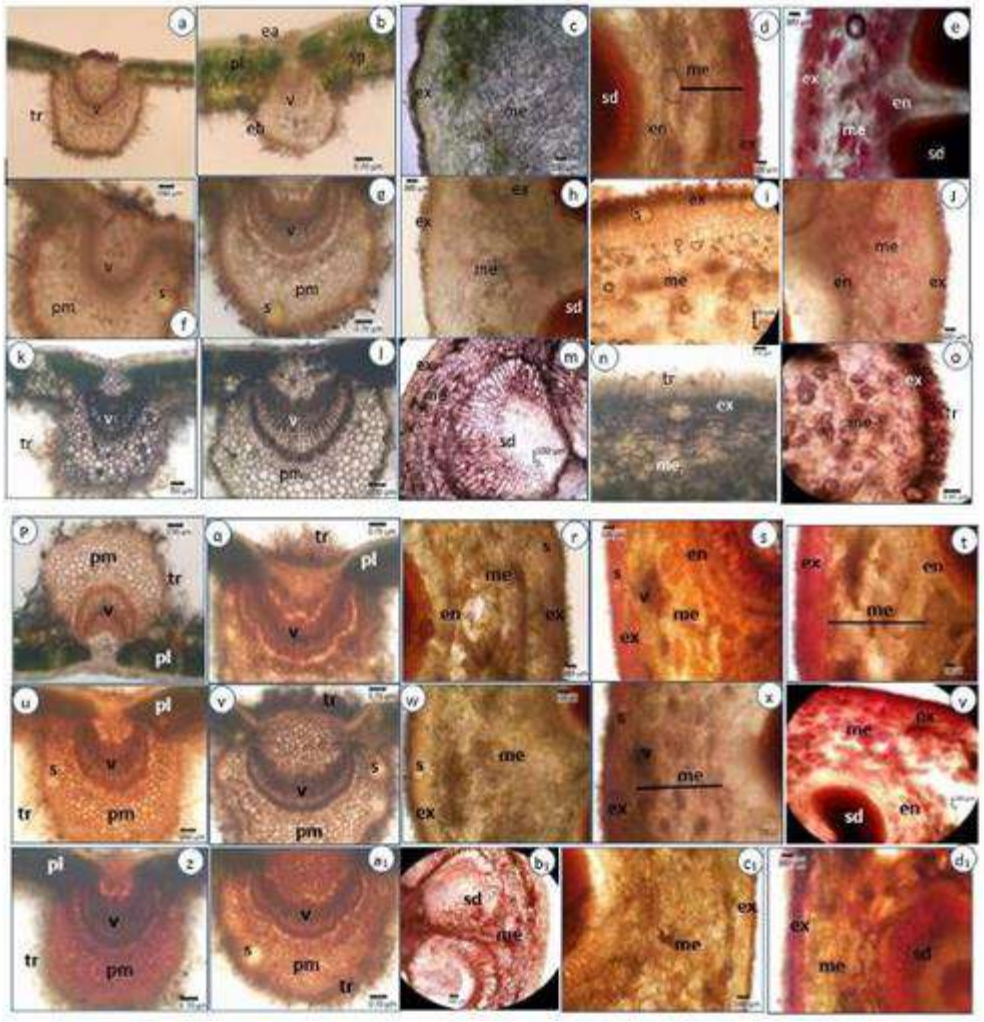

Figure 4. Transverse sections of histochemically stained leaves and fruits of *R. Tomentosa*. (a-e) Free-hand section of a young leaf without staining; (f-j) the positive reaction to Wilson's reagent indicating the presence of flavonoid in leaves and fruits; (k-o) the positive reaction to ferric chloride indicating the presence of phenolics in leaves and fruits: (p-t) the positive reaction to Wagner's reagent

indicating the presence of alkaloid in leaves and fruits; (u-y) the positive reaction to $FeCl_3$ indicating the presence of tannin in leaves and fruits; (z-d_1) the positive reaction to $Cu_2(CH_3COO)_4$ 5% indicating the presence of terpenoid in leaves and fruits: palisade mesophyll (pm), sponge mesophyll (sm), parenchyma midrib (pm), secretory cavity (s), trichomes (tr), druse crystal (dc), abaxial epidermis (eb), adaxial epidermis (ea) exocarp (ex) mesocarp (me), endocarp (en), seed (sd) vascular bundle (v).

Phytochemistry

The results of phytochemical measurements showed that the highest total flavonoid levels were in young leaves at 196.0 mg QE/g, followed by green fruit and old leaves, 164.625 mg QE/g, and 134.875 mg QE/g, followed by flavonoid levels in red and purple fruits at 84.625 mg QE/g and 55.75 mg QE/g, respectively. The highest measured total phenol content was 97.70 mg/mL recorded in green fruit, followed by old leaves at 59.30 mg/mL, young leaves at 54.40 mg/mL, red fruit at 19.40 mg/mL, and the lowest, in purple fruit at 17.23mg/mL. The highest total alkaloid content was recorded in old leaves at 13.23%, green fruit at 9.28%, red fruit at 7.80%, purple fruit at 7.05%, and young leaves, the lowest at 5.64%. For total tannin content, the measurements showed the highest value in red fruit at 1.658 mg GAE/g, followed by purple fruit at 1.402 mg GAE/g, young leaves at 0.943 mg GAE/g, old leaves at 0.880 mg GAE/g, and the lowest tannin content in green fruit at 0.804 mg GAE/g. (Table 1).

Table 1. Total amount of compounds in ethanolic extracts of leaves and fruits of *R. tomentosa*

Sample	Total flavonoids (mgQE/g)	Total phenolics (mgGAE/g)	Total alkaloids (%)	Total tannins (mgGAE/g)
Young leaves	196 ± 1.77^{e}	54.40 ± 0.09^{b}	5.64 ± 0.20^{a}	0.94 ± 0.74^{a}
Old leaves	134.88 ± 6.89^{c}	59.30 ± 16.73^{b}	13.22 ± 0.98^{d}	0.88 ± 0.01^{a}
Green fruits	164.63 ± 2.65^{d}	97.70 ± 18.15^{c}	9.28 ± 0.33^{c}	0.80 ± 0.30^{a}
Red fruits	84.63 ± 2.30^{b}	19.40 ± 0.47^{a}	$7.80 \pm 0.97b^{c}$	1.66 ± 0.15^{c}
Purple fruits	55.75 ± 0.71^{a}	17.23 ± 4.20^{a}	$7.05 \pm 0.41a^{b}$	1.40 ± 1.20^{b}

Note: The data presented are the mean ± standard deviation. One-Way ANOVA and LSD test with = 0.05 were used to evaluate data. Numbers followed by different superscript letters in the same column show significantly different results.

Discussion

According to research by Al-Edany and Al-Saadi (2012) on the anatomical characteristics of several Myrtaceae species, the upper surface of the epidermal tissue on the leaves of the Myrtaceae family is covered by a variable-thickness cuticle layer. Young and old leaves of karamunting (*Rhodomyrtus tomentosa*) have one layer of epidermal tissue on the upper surface (adaxial). The epidermal tissue's cells are square and closely packed. Al-Edany and Al-Saadi (2012) found that the cells of Myrtaceae leaf epidermal tissue are square or rectangular and uniseriate.

Kantachot et al. (2007) examined the leaves of 28 species in 12 genera of the Myrtaceae family and found that *R. tomentosa* had dorsiventral leaves, which have palisade tissue only on one side. The cells in the upper epidermis are thicker than those in the lower epidermis. The leaves of *R. tomentosa* do not have a hypodermis. The lower epidermis of the leaves has unicellular trichomes and anomocytic stomata. In the cross-section of the leaf stalk and petiole, vascular tissue forms a U-shaped pattern. Drusen-shaped Ca oxalate crystals are seen in leaf bone maternal parenchyma tissue.

The leaves of karamunting have palisade/pole and spongy mesophyll tissue. The palisade parenchyma is found beneath the upper epidermis. The cells that comprise the palisade parenchyma tissue are formed like pillars, appear tightly packed, and are just one layer thick. There are many chloroplasts in this palisade parenchyma tissue. The spongy tissue is located beneath the palisade tissue. The cells that make up the spongy tissue are formed like branches, appear loosely organized, and are uneven. Spongy parenchyma tissue contains chloroplasts, although less than palisade/pillar tissue. According to Al-Edany and Al-Saadi (2012), Myrtaceae leaves' mesophyll tissue has 1 to 2 layers of palisade tissue and several rows of up to 15 layers of compact and loose spongy tissue or 2 layers of palisade tissue on the adaxial surface, and spongy tissue on the abaxial surface.

Karamunting leaves have bicollateral vascular bundles consisting of xylem bundles flanked by phloem. Ferreira et al. (2011) describe the bundle of vessels in *Psidium guineense* Sw. (family Myrtaceae) leaves that include xylem flanked by phloem. The xylem and phloem function as a delivery system for vascular plants. The primary function of the xylem is to transport water and solutes, while the primary function of the phloem is to distribute photosynthesis products.

Karamunting leaf epidermal tissue on the abaxial side has unicellular trichomes. Kantachot et al. (2007) identified abundant unicellular and uniseriate trichomes on the leaves of Myrtaceae species such as *Decaspermum parviflorum*, *Melaleuca cajuputi*, *Psidium guajava*, *Rhodamnia dumetorum*, *Tristaniopsis burmanica* var. *rufescens*, and *R. tomentosa*. Trichomes from modified epidermal cells reduce evaporation from the leaves, and minimize animal disturbances (Nugroho 2017).

Retamales et al. (2014) found *Myrceugenia rufa* (Myrtaceae) fruit anatomy similar to *R. tomentosa*. The ripe fruit's pericarp has three zones: exocarp, mesocarp, and endocarp. The exocarp cells are irregular and plano-convex. The cuticle is thin and covered in hair. The mesocarp comprises 7-8 layers of large and isodiametric parenchyma cells with thin walls.

Multiple and large secretory cavities are observed throughout this tissue. The endocarp is a delicate tissue that surrounds the seed.

Histochemical test results by Agustina et al. (2016) recorded flavonoids and other phenolics in the stem, leaf, and flower-fruit-seed organs of *Acalypha indica*. Phenols in *Acalypha wilkesiana* are also distributed in the xylem in stem organs, leaf mesophyll, and in the flower-fruit seed.

Flavonoids can be found in every plant organ, including the leaves, roots, wood, flowers, nectar, and seeds. Flavonoids can be stored in cell walls, vacuoles, and glandular trichomes. Lipophilic compounds generated by glandular trichomes include terpenoids, lipids, waxes, and flavonoid aglycones. Flavonoids concentrate in cells and are not present in the intercellular space, resulting in a symplastic distribution (Petrussa et al. 2013).

According to Cartea et al. (2011), phenolic compounds are widespread phytochemicals. Structure-wise, they are simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. Flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids are plants' most abundant and diversified polyphenols. Flavonoids are the most prevalent phenolics and are found throughout all plants. Flavonoids in the leaf and fruit epidermis protect against UV radiation and pigment, stimulate nitrogen-fixing nodules, and fight disease.

The same study found positive reactions to reagents that detect phenolics, in leaf mesophyll cells, pith parenchyma, and stem cortical parenchyma cells of *Adhatoda zeylanica* (Acanthaceae), *Ruta graveolence* Linn. (Rutaceae), and *Vitex negundo* Linn. (Verbenaceae) (Dhale 2011).

Alkaloids are heterocyclic nitrogen-containing chemicals utilized in medicine. Alkaloids protect plants from insects and herbivores, detoxify toxins, and store nitrogen. Many products consumed by humans contains alkaloids, such as coffee, tea, cocoa, tobacco, areca nut, and marijuana. Alkaloids have neuroprotective, blood pressure-lowering, pain-relieving, antibacterial, anticancer, and antimalarial effects (Carqueijeiro et al. 2013). Alkaloids detected in *R. tomentosa's* leaves and fruits support its historic medicinal usage in Singapore. In China, the leaves were traditionally used as a painkiller, the roots for stomach ulcers, and the seeds for improved digestion and for snake bites. In Indonesia, the leaves were used to cure wounds. In traditional Vietnamese medicine, the plant's raw fruit treat diarrhea and dysentery, while the ripe ones boost the immune system (Zhao et al. 2019).

According to Ferreira et al. (2011), histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves indicated positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. These tannins in *R. tomentosa* leaves and fruits help treat diarrhea and dysentery. Ferreira et al. (2011) added that Myrtaceae leaves are used to treat diarrhea and intestinal infections due to their astringent tannin content. Tannins protect plants, which is why epidermal cells have them. Myrtaceae plants have tannins in their vascular systems also.

The meroterpenoids of *R. tomentosa* are antibacterial and antifungal. Myrtaceae secondary metabolites like meroterpenoids have been extensively explored as antibiotics. Thus, *R. tomentosa* could prevent microbial deterioration and extend food shelf life as a biocontrol agent and natural preservative (Zhao et al. 2019). Meroterpenoid compounds have been isolated and identified from various other Myrtaceae species, including *Eucalyptus tereticornis* (Liu et al. 2018) and *Leptospermum brachyandrum* (Zou et al. 2018).

Belwal et al. (2019) reported that flavonoid content declined throughout fruit ripening in *Rubus ellipticus*, *Myrica esculenta*, *Prunus persica*, blueberry (*Vaccinium corymbosum*), and blackberry fruit, as did Lin et al. (2020) in blueberries. Flavonoid levels vary by organ type. Hou et al. (2021) found that phenols and flavonoids drop from 5.38 mg RE/g in young citrus fruits to 0.69 mg RE/g in ripe fruit. The same trend was seen in grapes and lemons (Zhu et al. 2020). This phenomenon may be produced by many enzymes involved in flavonoid synthesis, such as 1,2-rhamnosyltransferase and flavanone-7-O-glucosides, which accelerate biosynthesis in the early growth period. Insufficient flavonoid aglycones may explain the decrease in flavonoids during citrus fruit ripening.

Lai et al. (2013) found a decrease in total phenol levels during *R. tomentosa* fruit ripening. The same pattern of declining phenol levels during fruit ripening occurs in other Myrtaceae plants such as Brazilian cherry *Eugenia uniflora* L. (Celli et al. 2011). Belwal et al. (2019) further explain the accumulation and degradation of distinct phenolic compounds during fruit ripening connected to their biosynthesis pathways, which are mainly regulated by enzyme expression and various genetic and environmental variables. Elmastaş et al. (2017) added that phenol compounds are phenylpropanoid metabolic pathway intermediates. Enzymes convert aromatic amino acids into polyphenols. Enzyme activity affects the production of these molecules. Genetic diversity, pH, light, temperature, and climatic change affect enzyme activity. Fruit enzyme activity might vary depending on plant growth and ripening.

The anatomical characteristics reported in this study contribute to the identification of *R. tomentosa* and support quality control tests. Some features may stand out, such as as hairy leaves with curved edges; epidermal cells with ripples and wall projections: and the secretory cavities with dimensions that go beyond the height of the palisade parenchyma. Characters that can contribute to accuracy in taxonomic classification include cross-sectional anatomical structure of leaves including the epidermis, mesophyll, carrier bundle, secretory cavity, and trichomes, while fruit characteristics include a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. In this study, flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the

highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).s

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The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

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Abstract. *Kuntorini EM, Sari SG, Fariani R. 2023. The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of* Rhodomyrtus tomentosa*. Biodiversitas 24: 98-105. Rhodomyrtus tomentosa* (Ait.) Hassk is native to Southeast Asia. The entire plant has been used in traditional Vietnamese, Chinese, and Malaysian medicine for a long time. Bioactive substances are abundant in ripe fruits. However, few studies have examined phytochemical changes and distribution throughout leaf and fruit growth. This study evaluated the morphoanatomy, histochemical, and phytochemical properties of *R. tomentosa* leaves and fruits. The cross-sectional anatomical structures observed in the leaves included the epidermis, mesophyll, and carrier bundle, while the fruit structures observed included a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. Flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavities, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavities, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

Keywords: Flavonoids, fruits, histochemistry, Karamunting, leaves, *Rhodomyrtus tomentosa*

INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk., a prolific evergreen shrub in the Myrtaceae family in southern and southeastern Asia, from India, east through southern China, the Philippines, and south to Malaysia and Sulawesi, has rose-pink blooms and dark purple edible bell-shaped fruits. In the Indonesian language, it is known as "*karamunting*", while in English, it is most commonly called "rose myrtle". This plant favors natural open light and adapts quickly to a range of soil conditions. Moreover, it requires little maintenance and is rarely affected by pests and illnesses. Most parts of this plant (leaves, roots, buds and fruits) have been used in traditional medicines in Vietnam, China and Malaysia for a long time. In the traditional medicine of Vietnam, the unripe fruits of the plant are utilized to treat diarrhea or dysentery and ripe ones are used to stimulate the immune system (Zhao et al. 2019). Wounds, heartburn, abscesses, and gynecopathy have been treated with *R. tomentosa*. In China, they use it as a medicine to treat urinary tract infections (Hamid et al. 2017a). Modern pharmacological studies discovered that *R. tomentosa* has antibacterial (Zhao et al. 2018), anticancer (Zhang et al. 2016), anti-inflammatory (Na-Phatthalung et al. 2018), and antioxidant (Hamid et al. 2017b) effects. In Indonesia, *R. tomentosa* roots and leaves treat diarrhea, stomachaches, and postpartum recovery. Most of these effects match typical *R. tomentosa* usage (Zhao et al. 2019). Epidemiological studies have shown that eating fruits and vegetables may protect against chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer, as well as other health benefits (Del Rio et al. 2013). Phytochemicals, particularly phenolics, are responsible for fruit and vegetable health benefits. According to recent findings, *R. tomentosa* berries contain over 19 phenolics, including anthocyanins, phenolic acids, flavonols, ellagitannins, and stilbenes. Due to their nutritional value and taste, *R. tomentosa* berries are a regional favorite (Zhao et al. 2017).

Anatomical studies are essential taxonomic indicators, including determining where compounds are secreted and accumulated in a medicinal species' vegetative and generative organs and are important in the quality control of medicinal plants. Pharmacognosy studies of macroscopic and microscopic characteristics are needed for medicinal raw material authentication and for quality assessment in drug standardization. Among the various alternative plant medicinal sources, accurate plant authentication is required (Raghvendra et al. 2021). Various taxa of Myrtaceae have been the subject of anatomical studies (Ferreira et al. 2011; Al-Edany et al. 2012); however, there has been no histochemical anatomical study of the leaves and fruit of *R. tomentosa*.

Histochemical studies are needed to reveal the typical pattern of distribution of secondary metabolites in plant tissues. Secondary metabolites are distributed in plant tissues based on their functionality. At the cellular level, secondary metabolites can be located in specific secretory tissues or are distributed throughout the tissue's cells,

including in the parenchyma of the transport bundle (Nugroho 2017).

Histochemical analyses of fruit development can help explain how metabolites affecting color and flavor are synthesized and accumulated during plant organ development. Histological studies can assess the morphological and anatomical details of *R. tomentosa* leaves and fruits. However, studies have not usually addressed secondary metabolite localization in this species. Particular staining and procedures can reveal metabolites, including pectin, lipids, lignin, proteins, phenolic compounds, tannins, starch grains, essential oils, rubber, and terpenoids (Vio-Michaelis et al. 2020). Unequivocal botanical characterizations are needed to identify medicinal plants correctly. Thus, the objective of this present study was to evaluate the leaves' and fruits' morphoanatomy, histochemistry, and phytochemistry of a sample of identified *R. tomentosa* collected in South Kalimantan, Indonesia.

MATERIALS AND METHODS

Plant materials

The 2nd and 10th leaves down from the stem tip and the green, red, and purple fruits of *R. tomentosa* were collected from wild plants in Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) (Figure 1). Three replicate samples of leaves and fruit were collected from three different plants. Herbarium Bogoriense at the Indonesian Institute of Sciences in Bogor, Indonesia, identified the plant specimen.

Procedures

Morphoanatomy slides preparation

Retamales et al. (2014) described the transverse sections of leaves and fruit using paraffin embedding with safranin staining. In the present study, minor adjustments were made to the immersion duration in dehydration and dealcoholization. Three replicates of fruits and leaves from three different plants were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70%, 5:5:90) solution for 24 hours. These replicates were then dehydrated, stored in 70% ethanol, and embedded in paraffin. Sections 8 to 10 µm thick were obtained on a rotatory microtome, dyed with Safranin, and mounted in Entelan to serve as permanent slides.

Histochemical test

Fresh, fully developed leaves (from the central nerve region and the blade) were cross-sectioned and subjected to the histochemical test. Hand-cut sections were treated with a dilute 10% aqueous acidic $FeCl_3$ (Sigma-Aldrich, Germany) solution containing a small quantity of Na_2CO_3 (Sigma-Aldrich, Germany) solution mounted in clove oil for phenolic compounds, which were identified as dark green or black color in tissues (Trimanto et al. 2018). Flavonoids were identified using Wilson's reagent. Each segment was soaked in citric acid:boric (Prolabo) (5:5 w/w) in 100 mL absolute ethanol for 15 minutes, mounted in glycerine water and inspected under a light microscope (Olympus, Tokyo, Japan). A yellow color signifies the presence of flavonoids (Pratiwi et al. 2020). With 5% copper acetate ($Cu_2(CH_3COO)_4$), terpenoids stained yellow-brown, indicating their presence in cells or tissues (Pratiwi et al. 2020). $FeCl_3$ (0.5% - 1% $FeCl_3$ in 0.1 N HCL) stained tannins blackish green, suggesting their existence (Robil and Tolentino 2015). Wagner's reagent was used to detect alkaloids, and any red-brown staining of tissues or cells indicated the presence of alkaloid compounds (Pratiwi et al. 2020). The material was photographed using OptiLab and a light microscope (Olympus, Tokyo, Japan).

Crude extract preparation

Young leaves (2nd - 6th down from the tip) and old leaves (7th - 12th down from the tip) were picked from the shoot of *R. tomentosa* plants, while harvested green, red, and purple fruits were dried in an oven at 40°C before being powdered at room temperature. A sample of 500 g of each powdered material was macerated for 24 hours in 1000 mL of ethanol (SmartLab, Indonesia). After 24 hours, the solvent was discarded and replenished thrice (Kusuma et al. 2016). Finally, extracts from the same samples were pooled, mixed, filtered, and dried using a rotary evaporator. The extract yield was calculated by this equation: (weight of ethanol extract/weight of dried leaves or fruits) × 100%.

Total phenolic compounds

Folin-Ciocalteu colorimetry was used to determine the triplicate samples' total phenolic contents (TPC) (Roy et al. 2018). The samples' extract absorbances were measured with a UV-Vis spectrophotometer (spectrophotometer UV 1800-Shimadzu, Japan) at 760 nm, and the total phenol content was calculated using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight.

Figure 1. Aerial part of *Rhodomyrtus tomentosa* (Aiton.) Hassk

Total flavonoids content

Total flavonoid content was determined using AlCl colorimetry. A 1 mL sample of the extract was mixed in a test tube with 4 mL of distilled water and with 0.3 mL of 5% sodium nitrite (Sigma-Aldrich, Germany). After 5 minutes, 0.3 mL of 10% aluminum chloride (Sigma-Aldrich, Germany) was added and stirred. After 5 minutes, 2 ml of sodium hydroxide (1 M) was added, and distilled water was added until the volume was precisely 10 mL. The identical method used for the earlier measurement was used to gauge the sample's extract absorbance at 510 nm. Finally, the total flavonoid content was determined using a quercetin standard curve (Sigma-Aldrich, Germany) at mg QE/g dry weight (Roy et al. 2018).

Total tannin content

The Folin-Ciocalteu method was used to calculate the total tannin content. The following ingredients were added to 0.1 mL of sample extract: 7.5 mL of distilled water, 0.5 mL of 10% F-C reagent, and 1 mL of 35% sodium carbonate solution (Sigma-Aldrich, Germany). Then, using distilled water, the volume was increased to 10 mL. After being mixed, the mixture was left at room temperature for 30 minutes. The sample's extract absorbance was determined at a wavelength of 725 nm. Finally, the total tannin content was determined using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight (Roy et al. 2018).

Total alkaloids content

2.5 g of the sample extract was diluted in 50 mL of 10% acetic acid (in ethanol). After being shaken with a magnetic stirrer for 4 hours, the solution was filtered. After that, the filtrate was evaporated. Then, ammonium hydroxide (Sigma-Aldrich, Germany) was drip-applied until an alkaloid precipitate formed. The precipitate-filtering filter paper was weighed before usage. After that, the residue was filtered and cleaned with an ammonium hydroxide 1% solution. The filter paper containing the precipitate was oven dried for 30 minutes at 60°C. After the residue had cooled, it was weighed until a constant weight was obtained. The alkaloid yield was calculated by the weight percentage of the precipitated alkaloid obtained against the initial weight of the extract. (Alasa et al. 2017).

Data analysis

The results from three replications were used to calculate the means and standard deviations (mean ± SD). Using IBM SPSS statistics 21 and Microsoft Excel®, the quantitative data were statistically evaluated using a one-way analysis of variance, with significant differences set at $p < 0.05$. When a statistically significant difference was detected, the analysis of variance was followed by an LSD post hoc test.

RESULTS AND DISCUSSION

Morphoanatomy of *R. tomentosa* leaves and fruits

Young and old *R. tomentosa* leaves have similar epidermal, mesophyll, and transport bundle structures. The adaxial and abaxial epidermis comprises the epidermis. Compared to the abaxial epidermis, the adaxial is larger. The mesophyll consists of palisade tissue and spongy, single-layer palisade tissue grouped compactly. The leaves are dorsiventral in shape, having palisade tissue on the adaxial side. The leaf stalk's carrier bundle network is made up of phloem and xylem. The leaf bone has parenchyma tissue as filling and collenchyma under the epidermis as reinforcement. Trichomes cover the leave's lower epidermis, while a thin cuticle covers the upper epidermis (Figure 2).

The fruit grows from fertilization. The growth of fruit components, such as fruit skin (pericarp), seed coat, endosperm, and embryo, follows fruit development. *R. tomentosa* fruit is a bacca/buni 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 (Hamid et al. 2017a). The cross-section of green, red, and purple fruit displays a fruit pericarp layer with an exocarp/outer layer, middle mesocarp, and endocarp. (Figure 3). The stiff exocarp has a polyhedral epidermis and several sclerenchyma layers. The edible mesocarp has big, watery parenchyma cells. The endocarp cells are thin-walled. The 1.5 mm seeds have 6 pseudo-locules with thin false septa (Hermanto et al. 2013; Hamid et al. 2017a).

Histochemical tests

Histochemical tests measure the secondary metabolites of the plant. Histochemical tests use qualitative analysis to detect compounds without measuring their concentration (Dhale 2011). Secondary metabolite components such as phenols, flavonoids, tannins, terpenoids, and alkaloids were found in *R. tomentosa*'s leaves and fruits at specific locations or, more generally, in the tissues. The secondary metabolites were detected histochemically. After the application of reagents, the identified compounds showed specific color changes (Figure 4).

Histochemical tests on young and old leaves, green, red, and purple fruit indicated positive reactions to flavonoid compounds (4f-4j) and phenols (Figure 4k-4o). Young and old leaves had phenolic, and flavonoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. Fruit phenolic and flavonoid compounds were found in the exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4p-4t shows alkaloid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. In green and red fruit, alkaloids were found in the endosperm, exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

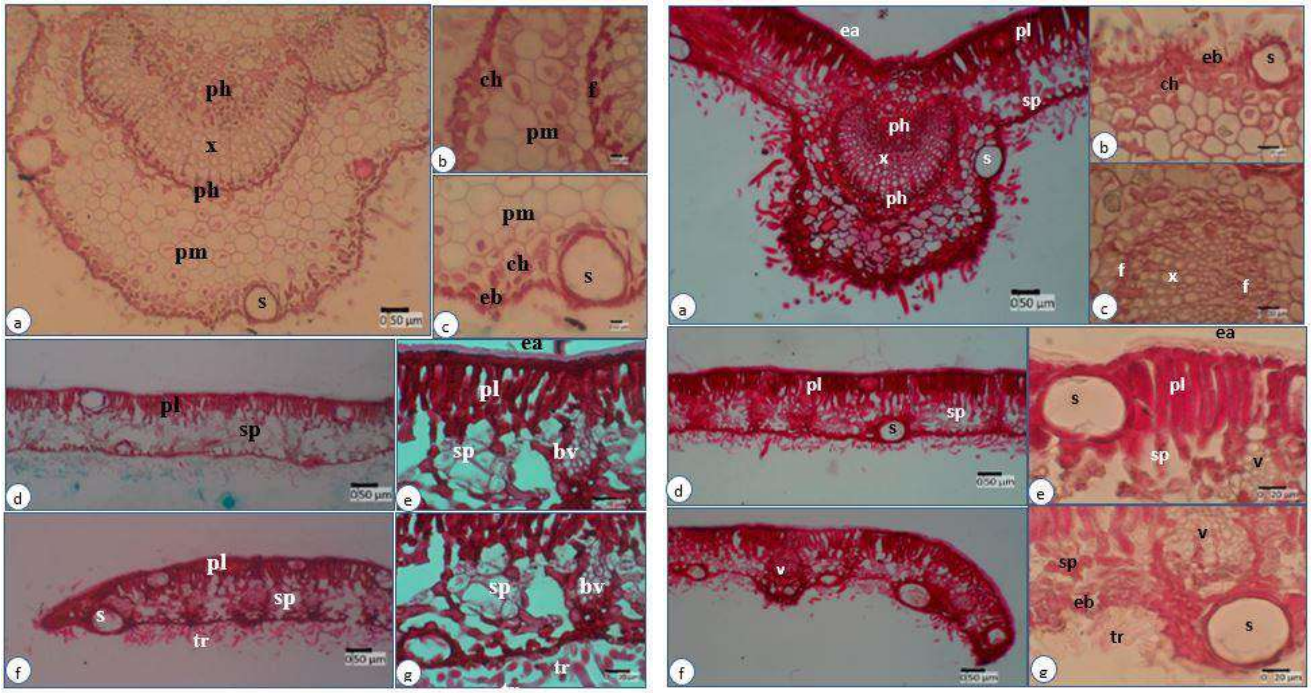

Figure 2. Cross-section of (A) young leaves, (B) old leaves *of R. tomentosa;* palisade mesophyll (pl), sponge mesophyll (sp), parenchyma midrib (pm), secretory cavity (sc), trichomes (tr), abaxial epidermis (eb), adaxial epidermis (ea), collenchyma (ch), vascular bundle (v), phloem (f), xylem (x)

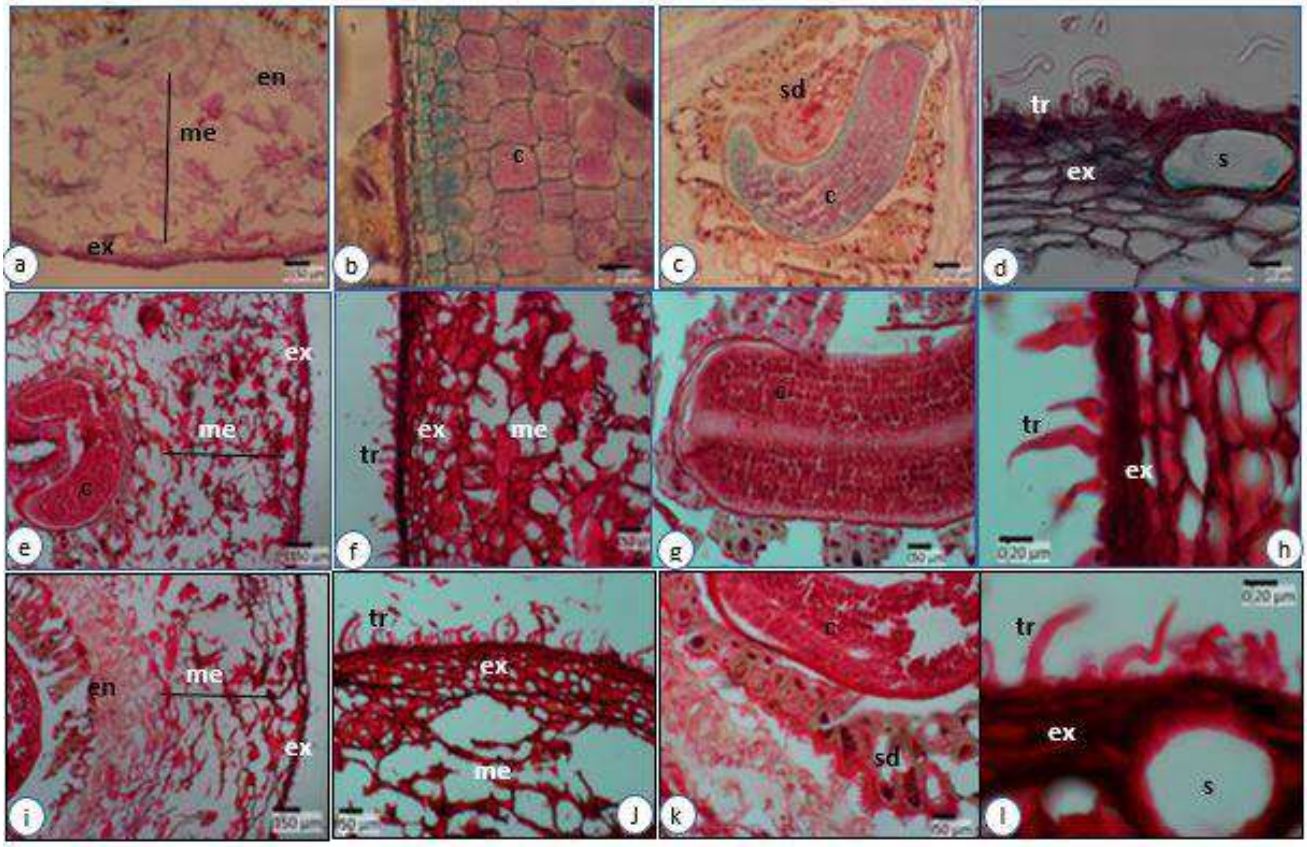

Figure 3. Cross-section of green fruit (a-d), red fruit (e-h) and purple fruit (i-l) of *R. tomentosa;* exocarp (ex) mesocarp (me), endocarp (en), seed (sd), secretory cavity (s), trichomes (tr)

Figure 4u-4y shows tannin compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Tannin compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichome, and seeds in the green and red fruit but not in the purple fruit.

Figure 4z-4d1 shows terpenoid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Terpenoid compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Terpenoid compounds were not found in the endocarp of purple fruit.

Phytochemistry

The results of phytochemical measurements showed that the highest total flavonoid levels were in young leaves at 196.0 mg QE/g, followed by green fruit and old leaves, 164.625 mg QE/g, and 134.875 mg QE/g, followed by flavonoid levels in red and purple fruits at 84.625 mg QE/g and 55.75 mg QE/g, respectively. The highest measured total phenol content was 97.70 mg/mL recorded in green fruit, followed by old leaves at 59.30 mg/mL, young leaves at 54.40 mg/mL, red fruit at 19.40 mg/mL, and the lowest, in purple fruit at 17.23mg/mL. The highest total alkaloid content was recorded in old leaves at 13.23%, green fruit at 9.28%, red fruit at 7.80%, purple fruit at 7.05%, and young leaves, the lowest at 5.64%. For total tannin content, the measurements showed the highest value in red fruit at 1.658 mg GAE/g, followed by purple fruit at 1.402 mg GAE/g, young leaves at 0.943 mg GAE/g, old leaves at 0.880 mg GAE/g, and the lowest tannin content in green fruit at 0.804 mg GAE/g. (Table 1).

Discussion

According to research by Al-Edany and Al-Saadi (2012) on the anatomical characteristics of several Myrtaceae species, the upper surface of the epidermal tissue on the leaves of the Myrtaceae family is covered by a variable-thickness cuticle layer. Young and old leaves of *R. tomentosa* have one layer of epidermal tissue on the upper surface (adaxial). The epidermal tissue's cells are square and closely packed. Al-Edany and Al-Saadi (2012) found that the cells of Myrtaceae leaf epidermal tissue are square or rectangular and uniseriate.

Kantachot et al. (2007) examined the leaves of 28 species in 12 genera of the Myrtaceae family and found that *R. tomentosa* had dorsiventral leaves, which have palisade tissue only on one side. The cells in the upper epidermis are thicker than those in the lower epidermis. The leaves of *R. tomentosa* do not have a hypodermis. The lower epidermis of the leaves has unicellular trichomes and anomocytic stomata. In the cross-section of the leaf stalk and petiole, vascular tissue forms a U-shaped pattern. Drusen-shaped Ca oxalate crystals are seen in leaf bone maternal parenchyma tissue.

The leaves of *R. tomentosa* have palisade/pole and spongy mesophyll tissue. The palisade parenchyma is found beneath the upper epidermis. The cells that comprise the palisade parenchyma tissue are formed like pillars, appear tightly packed, and are just one layer thick. There are many chloroplasts in this palisade parenchyma tissue. The spongy tissue is located beneath the palisade tissue. The cells that make up the spongy tissue are formed like branches, appear loosely organized, and are uneven. Spongy parenchyma tissue contains chloroplasts, although less than palisade/pillar tissue. According to Al-Edany and Al-Saadi (2012), Myrtaceae leaves' mesophyll tissue has 1 to 2 layers of palisade tissue and several rows of up to 15 layers of compact and loose spongy tissue or 2 layers of palisade tissue on the adaxial surface, and spongy tissue on the abaxial surface.

Rhodomyrtus tomentosa leaves have bicollateral vascular bundles consisting of xylem bundles flanked by phloem. Ferreira et al. (2011) describe the bundle of vessels in *Psidium guineense* Sw. (family Myrtaceae) leaves that include xylem flanked by phloem. The xylem and phloem function as a delivery system for vascular plants. The primary function of the xylem is to transport water and solutes, while the primary function of the phloem is to distribute photosynthesis products.

Rhodomyrtus tomentosa leaf epidermal tissue on the abaxial side has unicellular trichomes. Kantachot et al. (2007) identified abundant unicellular and uniseriate trichomes on the leaves of Myrtaceae species such as *Decaspermum parviflorum*, *Melaleuca cajuputi*, *Psidium guajava*, *Rhodamnia dumetorum*, *Tristaniopsis burmanica* var. *rufescens*, and *R. tomentosa*. Trichomes from modified epidermal cells reduce evaporation from the leaves and minimize animal disturbances (Nugroho 2017).

Table 1. Total amount of compounds in ethanolic extracts of leaves and fruits of *R. tomentosa*

Sample	Total flavonoids (mgQE/g)	Total phenolics (mgGAE/g)	Total alkaloids (%)	Total tannins (mgGAE/g)
Young leaves	196 ± 1.77^{e}	54.40 ± 0.09^{b}	5.64 ± 0.20^{a}	0.94 ± 0.74^{a}
Old leaves	134.88 ± 6.89^{c}	59.30 ± 16.73^{b}	13.22 ± 0.98^{d}	0.88 ± 0.01^{a}
Green fruits	164.63 ± 2.65^{d}	97.70 ± 18.15^{c}	9.28 ± 0.33^{c}	0.80 ± 0.30^{a}
Red fruits	84.63 ± 2.30^{b}	19.40 ± 0.47^{a}	$7.80 \pm 0.97b^{c}$	1.66 ± 0.15^{c}
Purple fruits	55.75 ± 0.71^{a}	17.23 ± 4.20^{a}	$7.05 \pm 0.41a^{b}$	1.40 ± 1.20^{b}

Note: The data presented are the mean ± standard deviation. One-Way ANOVA and LSD test with = 0.05 were used to evaluate data. Numbers followed by different superscript letters in the same column show significantly different results

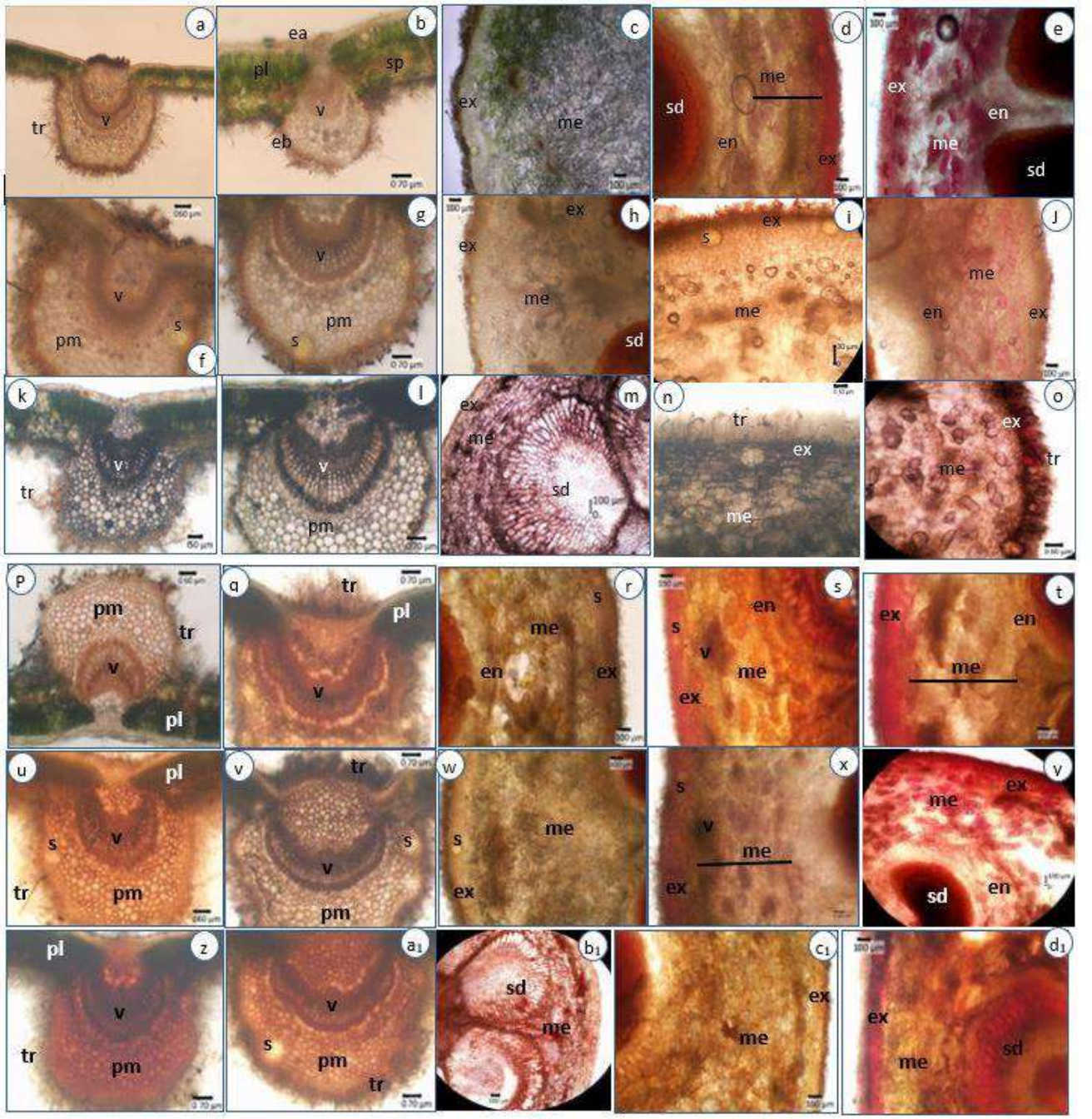

Figure 4. Transverse sections of histochemically stained leaves and fruits of *R. Tomentosa*. (a-e) Free-hand section of a young leaf without staining; (f-j) the positive reaction to Wilson's reagent indicating the presence of flavonoid in leaves and fruits; (k-o) the positive reaction to ferric chloride indicating the presence of phenolics in leaves and fruits: (p-t) the positive reaction to Wagner's reagent indicating the presence of alkaloid in leaves and fruits; (u-y) the positive reaction to $FeCl_3$ indicating the presence of tannin in leaves and fruits; (z-d_1) the positive reaction to $Cu_2(CH_3COO)_4$ 5% indicating the presence of terpenoid in leaves and fruits: palisade mesophyll (pm), sponge mesophyll (sm), parenchyma midrib (pm), secretory cavity (s), trichomes (tr), druse crystal (dc), abaxial epidermis (eb), adaxial epidermis (ea) exocarp (ex) mesocarp (me), endocarp (en), seed (sd) vascular bundle (v)

Retamales et al. (2014) found *Myrceugenia rufa* (Myrtaceae) fruit anatomy similar to *R. tomentosa*. The ripe fruit's pericarp has three zones: exocarp, mesocarp, and endocarp. The exocarp cells are irregular and plano-convex. The cuticle is thin and covered in hair. The mesocarp comprises 7-8 layers of large and isodiametric parenchyma cells with thin walls. Multiple and large secretory cavities are observed throughout this tissue. The endocarp is a delicate tissue that surrounds the seed.

Histochemical test results by Agustina et al. (2016) recorded flavonoids and other phenolics in the stem, leaf, and flower-fruit-seed organs of *Acalypha indica*. Phenols in

Acalypha wilkesiana are also distributed in the xylem in stem organs, leaf mesophyll, and in the flower-fruit seed.

Flavonoids can be found in every plant organ, including the leaves, roots, wood, flowers, nectar, and seeds. Flavonoids can be stored in cell walls, vacuoles, and glandular trichomes. Lipophilic compounds generated by glandular trichomes include terpenoids, lipids, waxes, and flavonoid aglycones. Flavonoids concentrate in cells and are not present in the intercellular space, resulting in a symplastic distribution (Petrussa et al. 2013).

According to Cartea et al. (2011), phenolic compounds are widespread phytochemicals. Structure-wise, they are simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. Flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids are plants' most abundant and diversified polyphenols. Flavonoids are the most prevalent phenolics and are found throughout all plants. Flavonoids in the leaf and fruit epidermis protect against UV radiation and pigment, stimulate nitrogen-fixing nodules, and fight disease.

The same study found positive reactions to reagents that detect phenolics in leaf mesophyll cells, pith parenchyma, and stem cortical parenchyma cells of *Adhatoda zeylanica* (Acanthaceae), *Ruta graveolence* Linn. (Rutaceae), and *Vitex negundo* Linn. (Verbenaceae) (Dhale 2011).

Alkaloids are heterocyclic nitrogen-containing chemicals utilized in medicine. Alkaloids protect plants from insects and herbivores, detoxify toxins, and store nitrogen. Many products consumed by humans contain alkaloids, such as coffee, tea, cocoa, tobacco, areca nut, and marijuana. Alkaloids have neuroprotective, blood pressure-lowering, pain-relieving, antibacterial, anticancer, and antimalarial effects (Carqueijeiro et al. 2013). Alkaloids detected in *R. tomentosa's* leaves and fruits support its historic medicinal usage in Singapore. In China, the leaves were traditionally used as a painkiller, the roots for stomach ulcers, and the seeds for improved digestion and for snake bites. In Indonesia, the leaves were used to cure wounds. In traditional Vietnamese medicine, the plant's raw fruit treats diarrhea and dysentery, while the ripe ones boost the immune system (Zhao et al. 2019).

According to Ferreira et al. (2011), histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves indicated positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. These tannins in *R. tomentosa* leaves and fruits help treat diarrhea and dysentery. Ferreira et al. (2011) added that Myrtaceae leaves are used to treat diarrhea and intestinal infections due to their astringent tannin content. Tannins protect plants, which is why epidermal cells have them. Myrtaceae plants have tannins in their vascular systems also.

The meroterpenoids of *R. tomentosa* are antibacterial and antifungal. Myrtaceae secondary metabolites like meroterpenoids have been extensively explored as antibiotics. Thus, *R. tomentosa* could prevent microbial deterioration and extend food shelf life as a biocontrol agent and natural preservative (Zhao et al. 2019). Meroterpenoid compounds have been isolated and identified from various other Myrtaceae species, including *Eucalyptus tereticornis* (Liu et al. 2018) and *Leptospermum brachyandrum* (Zou et al. 2018).

Belwal et al. (2019) reported that flavonoid content declined throughout fruit ripening in *Rubus ellipticus*, *Myrica esculenta*, *Prunus persica*, blueberry (*Vaccinium corymbosum*), and blackberry fruit, as did Lin et al. (2020) in blueberries. Flavonoid levels vary by organ type. Hou et al. (2021) found that phenols and flavonoids drop from 5.38 mg RE/g in young citrus fruits to 0.69 mg RE/g in ripe fruit. The same trend was seen in grapes and lemons (Zhu et al. 2020). This phenomenon may be produced by many enzymes involved in flavonoid synthesis, such as 1,2-rhamnosyltransferase and flavanone-7-O-glucosides, which accelerate biosynthesis in the early growth period. Insufficient flavonoid aglycones may explain the decrease in flavonoids during citrus fruit ripening.

Lai et al. (2013) found a decrease in total phenol levels during *R. tomentosa* fruit ripening. The same pattern of declining phenol levels during fruit ripening occurs in other Myrtaceae plants, such as Brazilian cherry *Eugenia uniflora* L. (Celli et al. 2011). Belwal et al. (2019) further explain the accumulation and degradation of distinct phenolic compounds during fruit ripening connected to their biosynthesis pathways, which are mainly regulated by enzyme expression and various genetic and environmental variables. Elmastaş et al. (2017) added that phenol compounds are phenylpropanoid metabolic pathway intermediates. Enzymes convert aromatic amino acids into polyphenols. Enzyme activity affects the production of these molecules. Genetic diversity, pH, light, temperature, and climatic change affect enzyme activity. Fruit enzyme activity might vary depending on plant growth and ripening.

The anatomical characteristics reported in this study contribute to the identification of *R. tomentosa* and support quality control tests. Some features may stand out, such as hairy leaves with curved edges; epidermal cells with ripples and wall projections: and secretory cavities with dimensions that go beyond the height of the palisade parenchyma. Characters that can contribute to accuracy in taxonomic classification include the cross-sectional anatomical structure of leaves, including the epidermis, mesophyll, carrier bundle, secretory cavity, and trichomes, while fruit characteristics include a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. In this study, flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

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The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of *Rhodomyrtus tomentosa*

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Abstract. *Kuntorini EM, Sari SG, Fariani R. 2023. The morphoanatomy, histochemistry, and phytochemistry of the leaves and fruits of* Rhodomyrtus tomentosa. *Biodiversitas 24: 98-105. Rhodomyrtus tomentosa* (Ait.) Hassk is native to Southeast Asia. The entire plant has been used in traditional Vietnamese, Chinese, and Malaysian medicine for a long time. Bioactive substances are abundant in ripe fruits. However, few studies have examined phytochemical changes and distribution throughout leaf and fruit growth. This study evaluated the morphoanatomy, histochemical, and phytochemical properties of *R. tomentosa* leaves and fruits. The cross-sectional anatomical structures observed in the leaves included the epidermis, mesophyll, and carrier bundle, while the fruit structures observed included a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. Flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavities, and trichomes. They were also distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavities, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

Keywords: Flavonoids, fruits, histochemistry, Karamunting, leaves, *Rhodomyrtus tomentosa*

INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk., a prolific evergreen shrub in the Myrtaceae family in southern and southeastern Asia, from India, east through southern China, the Philippines, and south to Malaysia and Sulawesi, has rose-pink blooms and dark purple edible bell-shaped fruits. In the Indonesian language, it is known as "*karamunting*", while in English, it is most commonly called "rose myrtle". This plant favors natural open light and adapts quickly to a range of soil conditions. Moreover, it requires little maintenance and is rarely affected by pests and illnesses. Most parts of this plant (leaves, roots, buds and fruits) have been used in traditional medicines in Vietnam, China and Malaysia for a long time. In the traditional medicine of Vietnam, the unripe fruits of the plant are utilized to treat diarrhea or dysentery and ripe ones are used to stimulate the immune system (Zhao et al. 2019). Wounds, heartburn, abscesses, and gynecopathy have been treated with *R. tomentosa*. In China, they use it as a medicine to treat urinary tract infections (Hamid et al. 2017a). Modern pharmacological studies discovered that *R. tomentosa* has antibacterial (Zhao et al. 2018), anticancer (Zhang et al. 2016), anti-inflammatory (Na-Phatthalung et al. 2018), and antioxidant (Hamid et al. 2017b) effects. In Indonesia, *R. tomentosa* roots and leaves treat diarrhea, stomachaches, and postpartum recovery. Most of these effects match typical *R. tomentosa* usage (Zhao et al. 2019). Epidemiological studies have shown that eating fruits and vegetables may protect against chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer, as well as other health benefits (Del Rio et al. 2013). Phytochemicals, particularly phenolics, are responsible for fruit and vegetable health benefits. According to recent findings, *R. tomentosa* berries contain over 19 phenolics, including anthocyanins, phenolic acids, flavonols, ellagitannins, and stilbenes. Due to their nutritional value and taste, *R. tomentosa* berries are a regional favorite (Zhao et al. 2017).

Anatomical studies are essential taxonomic indicators, including determining where compounds are secreted and accumulated in a medicinal species' vegetative and generative organs and are important in the quality control of medicinal plants. Pharmacognosy studies of macroscopic and microscopic characteristics are needed for medicinal raw material authentication and for quality assessment in drug standardization. Among the various alternative plant medicinal sources, accurate plant authentication is required (Raghvendra et al. 2021). Various taxa of Myrtaceae have been the subject of anatomical studies (Ferreira et al. 2011); however, there has been no histochemical anatomical study of the leaves and fruit of *R. tomentosa*.

Histochemical studies are needed to reveal the typical pattern of distribution of secondary metabolites in plant tissues. Secondary metabolites are distributed in plant tissues based on their functionality. At the cellular level, secondary metabolites can be located in specific secretory tissues or are distributed throughout the tissue's cells,

including in the parenchyma of the transport bundle (Nugroho 2017).

Histochemical analyses of fruit development can help explain how metabolites affecting color and flavor are synthesized and accumulated during plant organ development. Histological studies can assess the morphological and anatomical details of *R. tomentosa* leaves and fruits. However, studies have not usually addressed secondary metabolite localization in this species. Particular staining and procedures can reveal metabolites, including pectin, lipids, lignin, proteins, phenolic compounds, tannins, starch grains, essential oils, rubber, and terpenoids (Vio-Michaelis et al. 2020). Unequivocal botanical characterizations are needed to identify medicinal plants correctly. Thus, the objective of this present study was to evaluate the leaves' and fruits' morphoanatomy, histochemistry, and phytochemistry of a sample of identified *R. tomentosa* collected in South Kalimantan, Indonesia.

MATERIALS AND METHODS

Plant materials

The 2nd and 10th leaves down from the stem tip and the green, red, and purple fruits of *R. tomentosa* were collected from wild plants in Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) (Figure 1). Three replicate samples of leaves and fruit were collected from three different plants. Herbarium Bogoriense at the Indonesian Institute of Sciences in Bogor, Indonesia, identified the plant specimen.

Procedures

Morphoanatomy slides preparation

Retamales et al. (2014) described the transverse sections of leaves and fruit using paraffin embedding with safranin staining. In the present study, minor adjustments were made to the immersion duration in dehydration and dealcoholization. Three replicates of fruits and leaves from three different plants were fixed in FAA (Formalin 40%: Glacial acetic acid: Alcohol 70%, 5:5:90) solution for 24 hours. These replicates were then dehydrated, stored in 70% ethanol, and embedded in paraffin. Sections 8 to 10 µm thick were obtained on a rotatory microtome, dyed with Safranin, and mounted in Entelan to serve as permanent slides.

Histochemical test

Fresh, fully developed leaves (from the central nerve region and the blade) were cross-sectioned and subjected to the histochemical test. Hand-cut sections were treated with a dilute 10% aqueous acidic $FeCl_3$ (Sigma-Aldrich, Germany) solution containing a small quantity of Na_2CO_3 (Sigma-Aldrich, Germany) solution mounted in clove oil for phenolic compounds, which were identified as dark green or black color in tissues (Trimanto et al. 2018). Flavonoids were identified using Wilson's reagent. Each segment was soaked in citric acid:boric (Prolabo) (5:5 w/w) in 100 mL absolute ethanol for 15 minutes, mounted in glycerine water and inspected under a light microscope (Olympus, Tokyo, Japan). A yellow color signifies the presence of flavonoids (Pratiwi et al. 2020). With 5% copper acetate ($Cu_2(CH_3COO)_4$), terpenoids stained yellow-brown, indicating their presence in cells or tissues (Pratiwi et al. 2020). $FeCl_3$ (0.5% - 1% $FeCl_3$ in 0.1 N HCL) stained tannins blackish green, suggesting their existence (Robil and Tolentino 2015). Wagner's reagent was used to detect alkaloids, and any red-brown staining of tissues or cells indicated the presence of alkaloid compounds (Pratiwi et al. 2020). The material was photographed using OptiLab and a light microscope (Olympus, Tokyo, Japan).

Crude extract preparation

Young leaves (2nd - 6th down from the tip) and old leaves (7th - 12th down from the tip) were picked from the shoot of *R. tomentosa* plants, while harvested green, red, and purple fruits were dried in an oven at 40°C before being powdered at room temperature. A sample of 500 g of each powdered material was macerated for 24 hours in 1000 mL of ethanol (SmartLab, Indonesia). After 24 hours, the solvent was discarded and replenished thrice (Kusuma et al. 2016). Finally, extracts from the same samples were pooled, mixed, filtered, and dried using a rotary evaporator. The extract yield was calculated by this equation: (weight of ethanol extract/weight of dried leaves or fruits) × 100%.

Total phenolic compounds

Folin-Ciocalteu colorimetry was used to determine the triplicate samples' total phenolic contents (TPC) (Roy et al. 2018). The samples' extract absorbances were measured with a UV-Vis spectrophotometer (spectrophotometer UV 1800-Shimadzu, Japan) at 760 nm, and the total phenol content was calculated using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight.

Figure 1. Aerial part of *Rhodomyrtus tomentosa* (Aiton.) Hassk

Total flavonoids content

Total flavonoid content was determined using AlCl colorimetry. A 1 mL sample of the extract was mixed in a test tube with 4 mL of distilled water and with 0.3 mL of 5% sodium nitrite (Sigma-Aldrich, Germany). After 5 minutes, 0.3 mL of 10% aluminum chloride (Sigma-Aldrich, Germany) was added and stirred. After 5 minutes, 2 ml of sodium hydroxide (1 M) was added, and distilled water was added until the volume was precisely 10 mL. The identical method used for the earlier measurement was used to gauge the sample's extract absorbance at 510 nm. Finally, the total flavonoid content was determined using a quercetin standard curve (Sigma-Aldrich, Germany) at mg QE/g dry weight (Roy et al. 2018).

Total tannin content

The Folin-Ciocalteu method was used to calculate the total tannin content. The following ingredients were added to 0.1 mL of sample extract: 7.5 mL of distilled water, 0.5 mL of 10% F-C reagent, and 1 mL of 35% sodium carbonate solution (Sigma-Aldrich, Germany). Then, using distilled water, the volume was increased to 10 mL. After being mixed, the mixture was left at room temperature for 30 minutes. The sample's extract absorbance was determined at a wavelength of 725 nm. Finally, the total tannin content was determined using the standard gallate acid (Merck, Germany) at mg GAE/g dry weight (Roy et al. 2018).

Total alkaloids content

2.5 g of the sample extract was diluted in 50 mL of 10% acetic acid (in ethanol). After being shaken with a magnetic stirrer for 4 hours, the solution was filtered. After that, the filtrate was evaporated. Then, ammonium hydroxide (Sigma-Aldrich, Germany) was drip-applied until an alkaloid precipitate formed. The precipitate-filtering filter paper was weighed before usage. After that, the residue was filtered and cleaned with an ammonium hydroxide 1% solution. The filter paper containing the precipitate was oven dried for 30 minutes at 60°C. After the residue had cooled, it was weighed until a constant weight was obtained. The alkaloid yield was calculated by the weight percentage of the precipitated alkaloid obtained against the initial weight of the extract. (Alasa et al. 2017).

Data analysis

The results from three replications were used to calculate the means and standard deviations (mean ± SD). Using IBM SPSS statistics 21 and Microsoft Excel®, the quantitative data were statistically evaluated using a one-way analysis of variance, with significant differences set at $p < 0.05$. When a statistically significant difference was detected, the analysis of variance was followed by an LSD post hoc test.

RESULTS AND DISCUSSION

Morphoanatomy of *R. tomentosa* leaves and fruits

Young and old *R. tomentosa* leaves have similar epidermal, mesophyll, and transport bundle structures. The adaxial and abaxial epidermis comprises the epidermis. Compared to the abaxial epidermis, the adaxial is larger. The mesophyll consists of palisade tissue and spongy, single-layer palisade tissue grouped compactly. The leaves are dorsiventral in shape, having palisade tissue on the adaxial side. The leaf stalk's carrier bundle network is made up of phloem and xylem. The leaf bone has parenchyma tissue as filling and collenchyma under the epidermis as reinforcement. Trichomes cover the leave's lower epidermis, while a thin cuticle covers the upper epidermis (Figure 2).

The fruit grows from fertilization. The growth of fruit components, such as fruit skin (pericarp), seed coat, endosperm, and embryo, follows fruit development. *R. tomentosa* fruit is a bacca/buni 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 (Hamid et al. 2017a). The cross-section of green, red, and purple fruit displays a fruit pericarp layer with an exocarp/outer layer, middle mesocarp, and endocarp. (Figure 3). The stiff exocarp has a polyhedral epidermis and several sclerenchyma layers. The edible mesocarp has big, watery parenchyma cells. The endocarp cells are thin-walled. The 1.5 mm seeds have 6 pseudo-locules with thin false septa (Hermanto et al. 2013; Hamid et al. 2017a).

Histochemical tests

Histochemical tests measure the secondary metabolites of the plant. Histochemical tests use qualitative analysis to detect compounds without measuring their concentration (Dhale 2011). Secondary metabolite components such as phenols, flavonoids, tannins, terpenoids, and alkaloids were found in *R. tomentosa*'s leaves and fruits at specific locations or, more generally, in the tissues. The secondary metabolites were detected histochemically. After the application of reagents, the identified compounds showed specific color changes (Figure 4).

Histochemical tests on young and old leaves, green, red, and purple fruit indicated positive reactions to flavonoid compounds (4f-4j) and phenols (Figure 4k-4o). Young and old leaves had phenolic, and flavonoid compounds in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. Fruit phenolic and flavonoid compounds were found in the exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

Figure 4p-4t shows alkaloid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory space, and trichomes. In green and red fruit, alkaloids were found in the endosperm, exocarp, mesocarp, endocarp, secretory space, xylem, phloem, trichomes, and seeds.

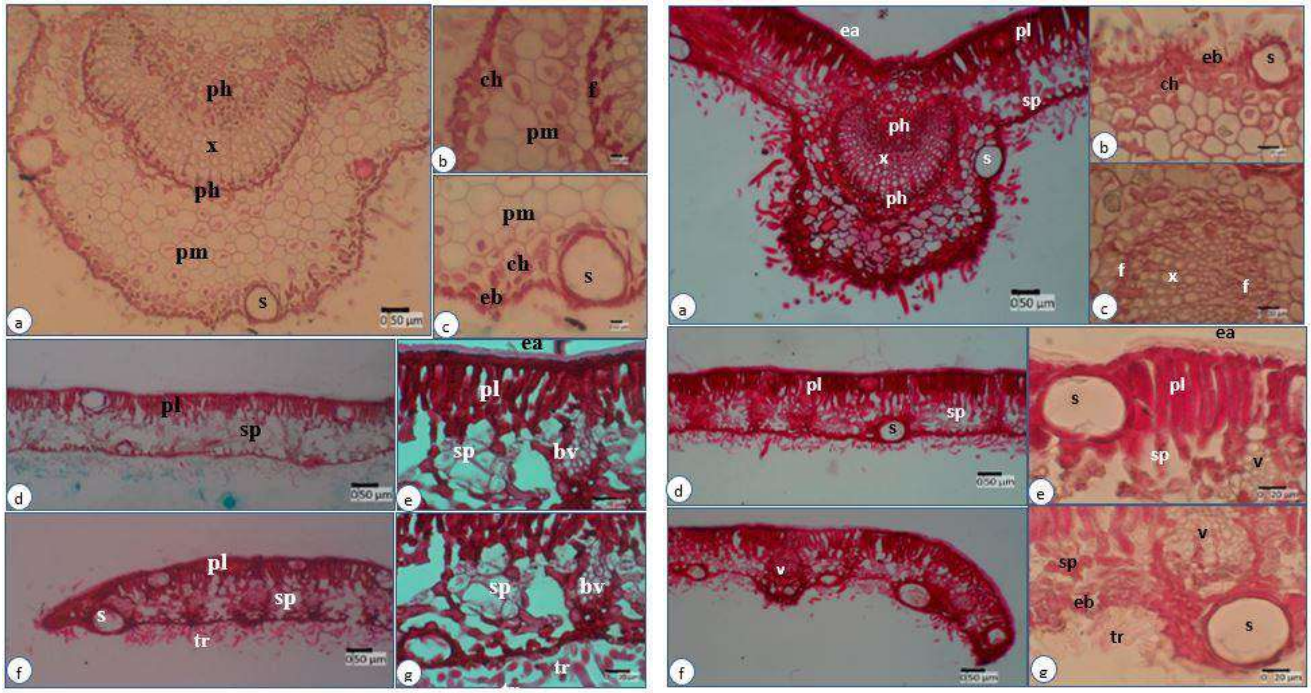

Figure 2. Cross-section of (A) young leaves, (B) old leaves *of R. tomentosa;* palisade mesophyll (pl), sponge mesophyll (sp), parenchyma midrib (pm), secretory cavity (sc), trichomes (tr), abaxial epidermis (eb), adaxial epidermis (ea), collenchyma (ch), vascular bundle (v), phloem (f), xylem (x)

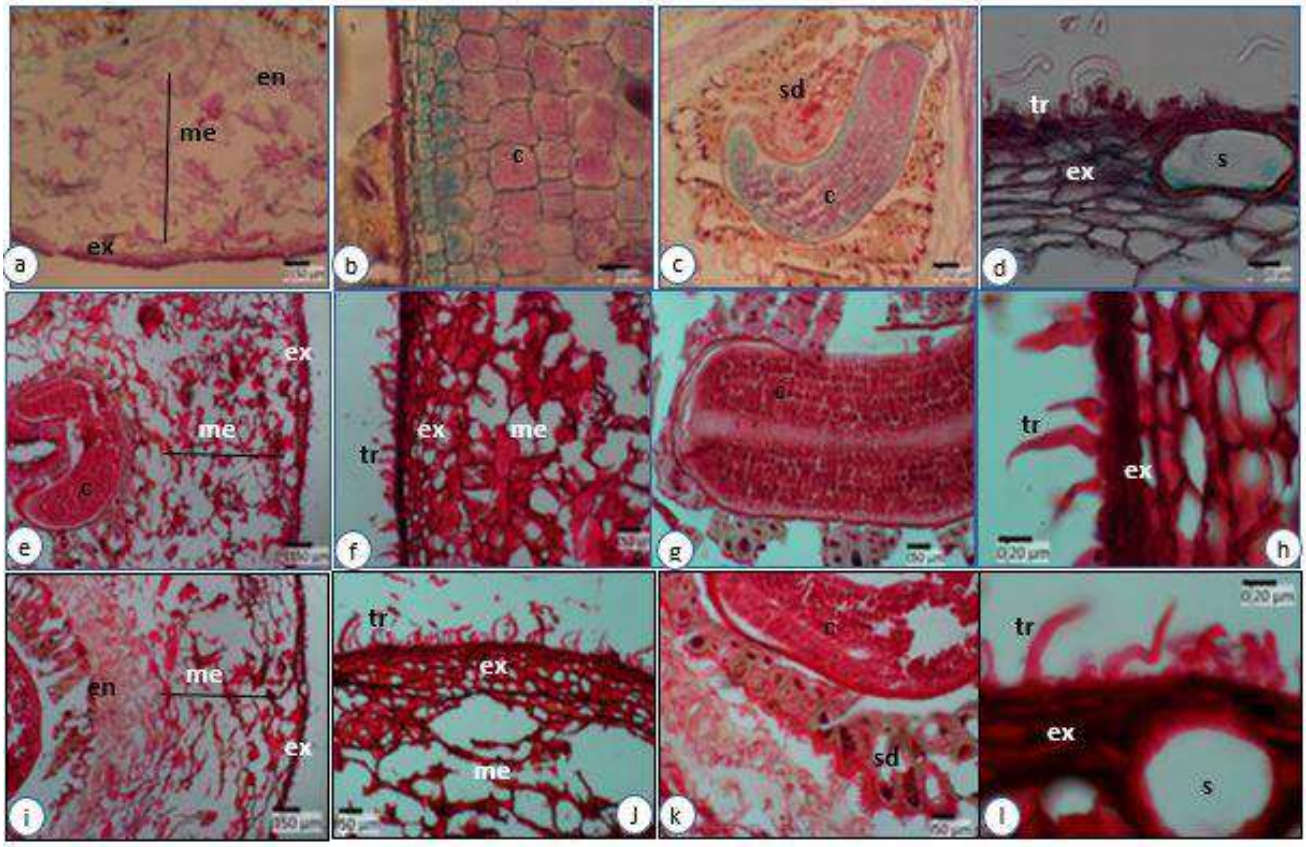

Figure 3. Cross-section of green fruit (a-d), red fruit (e-h) and purple fruit (i-l) of *R. tomentosa;* exocarp (ex) mesocarp (me), endocarp (en), seed (sd), secretory cavity (s), trichomes (tr)

Figure 4u-4y shows tannin compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Tannin compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichome, and seeds in the green and red fruit but not in the purple fruit.

Figure 4z-4d1 shows terpenoid compounds in young and old leaves in the adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, trichomes, and druse crystals. Terpenoid compounds were found in the exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Terpenoid compounds were not found in the endocarp of purple fruit.

Phytochemistry

The results of phytochemical measurements showed that the highest total flavonoid levels were in young leaves at 196.0 mg QE/g, followed by green fruit and old leaves, 164.625 mg QE/g, and 134.875 mg QE/g, followed by flavonoid levels in red and purple fruits at 84.625 mg QE/g and 55.75 mg QE/g, respectively. The highest measured total phenol content was 97.70 mg/mL recorded in green fruit, followed by old leaves at 59.30 mg/mL, young leaves at 54.40 mg/mL, red fruit at 19.40 mg/mL, and the lowest, in purple fruit at 17.23mg/mL. The highest total alkaloid content was recorded in old leaves at 13.23%, green fruit at 9.28%, red fruit at 7.80%, purple fruit at 7.05%, and young leaves, the lowest at 5.64%. For total tannin content, the measurements showed the highest value in red fruit at 1.658 mg GAE/g, followed by purple fruit at 1.402 mg GAE/g, young leaves at 0.943 mg GAE/g, old leaves at 0.880 mg GAE/g, and the lowest tannin content in green fruit at 0.804 mg GAE/g. (Table 1).

Discussion

According to research by Al-Edany and Al-Saadi (2012) on the anatomical characteristics of several Myrtaceae species, the upper surface of the epidermal tissue on the leaves of the Myrtaceae family is covered by a variable-thickness cuticle layer. Young and old leaves of *R. tomentosa* have one layer of epidermal tissue on the upper surface (adaxial). The epidermal tissue's cells are square and closely packed. Al-Edany and Al-Saadi (2012) found that the cells of Myrtaceae leaf epidermal tissue are square or rectangular and uniseriate.

Kantachot et al. (2007) examined the leaves of 28 species in 12 genera of the Myrtaceae family and found that *R. tomentosa* had dorsiventral leaves, which have palisade tissue only on one side. The cells in the upper epidermis are thicker than those in the lower epidermis. The leaves of *R. tomentosa* do not have a hypodermis. The lower epidermis of the leaves has unicellular trichomes and anomocytic stomata. In the cross-section of the leaf stalk and petiole, vascular tissue forms a U-shaped pattern. Drusen-shaped Ca oxalate crystals are seen in leaf bone maternal parenchyma tissue.

The leaves of *R. tomentosa* have palisade/pole and spongy mesophyll tissue. The palisade parenchyma is found beneath the upper epidermis. The cells that comprise the palisade parenchyma tissue are formed like pillars, appear tightly packed, and are just one layer thick. There are many chloroplasts in this palisade parenchyma tissue. The spongy tissue is located beneath the palisade tissue. The cells that make up the spongy tissue are formed like branches, appear loosely organized, and are uneven. Spongy parenchyma tissue contains chloroplasts, although less than palisade/pillar tissue. According to Al-Edany and Al-Saadi (2012), Myrtaceae leaves' mesophyll tissue has 1 to 2 layers of palisade tissue and several rows of up to 15 layers of compact and loose spongy tissue or 2 layers of palisade tissue on the adaxial surface, and spongy tissue on the abaxial surface.

Rhodomyrtus tomentosa leaves have bicollateral vascular bundles consisting of xylem bundles flanked by phloem. Ferreira et al. (2011) describe the bundle of vessels in *Psidium guineense* Sw. (family Myrtaceae) leaves that include xylem flanked by phloem. The xylem and phloem function as a delivery system for vascular plants. The primary function of the xylem is to transport water and solutes, while the primary function of the phloem is to distribute photosynthesis products.

Rhodomyrtus tomentosa leaf epidermal tissue on the abaxial side has unicellular trichomes. Kantachot et al. (2007) identified abundant unicellular and uniseriate trichomes on the leaves of Myrtaceae species such as *Decaspermum parviflorum*, *Melaleuca cajuputi*, *Psidium guajava*, *Rhodamnia dumetorum*, *Tristaniopsis burmanica* var. *rufescens*, and *R. tomentosa*. Trichomes from modified epidermal cells reduce evaporation from the leaves and minimize animal disturbances (Nugroho 2017).

Table 1. Total amount of compounds in ethanolic extracts of leaves and fruits of *R. tomentosa*

Sample	Total flavonoids (mgQE/g)	Total phenolics (mgGAE/g)	Total alkaloids (%)	Total tannins (mgGAE/g)
Young leaves	196 ± 1.77^{e}	54.40 ± 0.09^{b}	5.64 ± 0.20^{a}	0.94 ± 0.74^{a}
Old leaves	134.88 ± 6.89^{c}	59.30 ± 16.73^{b}	13.22 ± 0.98^{d}	0.88 ± 0.01^{a}
Green fruits	164.63 ± 2.65^{d}	97.70 ± 18.15^{c}	9.28 ± 0.33^{c}	0.80 ± 0.30^{a}
Red fruits	84.63 ± 2.30^{b}	19.40 ± 0.47^{a}	$7.80 \pm 0.97b^{c}$	1.66 ± 0.15^{c}
Purple fruits	55.75 ± 0.71^{a}	17.23 ± 4.20^{a}	$7.05 \pm 0.41a^{b}$	1.40 ± 1.20^{b}

Note: The data presented are the mean ± standard deviation. One-Way ANOVA and LSD test with = 0.05 were used to evaluate data. Numbers followed by different superscript letters in the same column show significantly different results

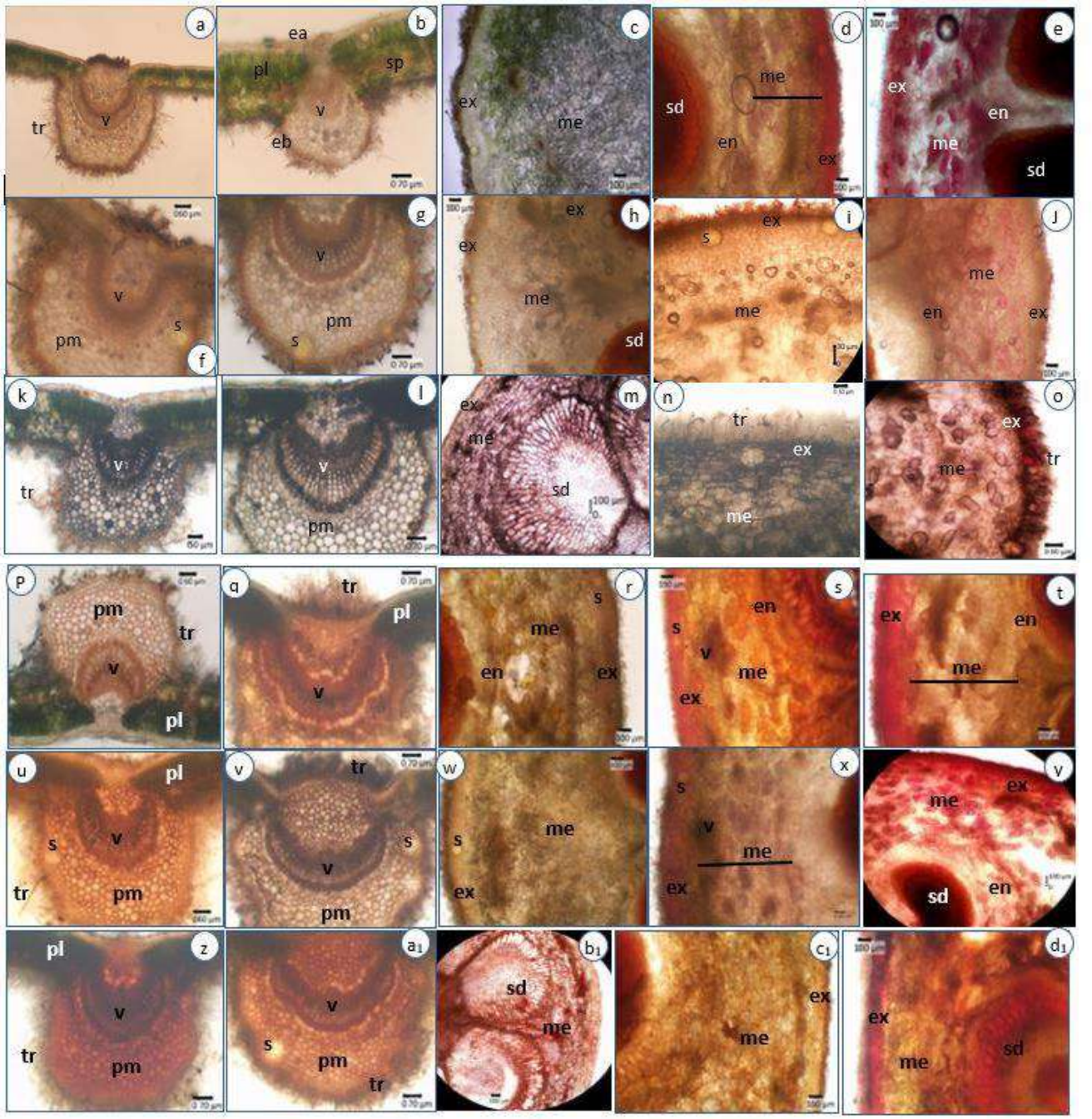

Figure 4. Transverse sections of histochemically stained leaves and fruits of *R. Tomentosa*. (a-e) Free-hand section of a young leaf without staining; (f-j) the positive reaction to Wilson's reagent indicating the presence of flavonoid in leaves and fruits; (k-o) the positive reaction to ferric chloride indicating the presence of phenolics in leaves and fruits: (p-t) the positive reaction to Wagner's reagent indicating the presence of alkaloid in leaves and fruits; (u-y) the positive reaction to $FeCl_3$ indicating the presence of tannin in leaves and fruits; (z-d_1) the positive reaction to $Cu_2(CH_3COO)_4$ 5% indicating the presence of terpenoid in leaves and fruits: palisade mesophyll (pm), sponge mesophyll (sm), parenchyma midrib (pm), secretory cavity (s), trichomes (tr), druse crystal (dc), abaxial epidermis (eb), adaxial epidermis (ea) exocarp (ex) mesocarp (me), endocarp (en), seed (sd) vascular bundle (v)

Retamales et al. (2014) found *Myrceugenia rufa* (Myrtaceae) fruit anatomy similar to *R. tomentosa*. The ripe fruit's pericarp has three zones: exocarp, mesocarp, and endocarp. The exocarp cells are irregular and plano-convex. The cuticle is thin and covered in hair. The mesocarp comprises 7-8 layers of large and isodiametric parenchyma cells with thin walls. Multiple and large secretory cavities are observed throughout this tissue. The endocarp is a delicate tissue that surrounds the seed.

Histochemical test results by Agustina et al. (2016) recorded flavonoids and other phenolics in the stem, leaf, and flower-fruit-seed organs of *Acalypha indica*. Phenols in

Acalypha wilkesiana are also distributed in the xylem in stem organs, leaf mesophyll, and in the flower-fruit seed.

Flavonoids can be found in every plant organ, including the leaves, roots, wood, flowers, nectar, and seeds. Flavonoids can be stored in cell walls, vacuoles, and glandular trichomes. Lipophilic compounds generated by glandular trichomes include terpenoids, lipids, waxes, and flavonoid aglycones. Flavonoids concentrate in cells and are not present in the intercellular space, resulting in a symplastic distribution (Petrussa et al. 2013).

According to Cartea et al. (2011), phenolic compounds are widespread phytochemicals. Structure-wise, they are simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. Flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids are plants' most abundant and diversified polyphenols. Flavonoids are the most prevalent phenolics and are found throughout all plants. Flavonoids in the leaf and fruit epidermis protect against UV radiation and pigment, stimulate nitrogen-fixing nodules, and fight disease.

The same study found positive reactions to reagents that detect phenolics in leaf mesophyll cells, pith parenchyma, and stem cortical parenchyma cells of *Adhatoda zeylanica* (Acanthaceae), *Ruta graveolence* Linn. (Rutaceae), and *Vitex negundo* Linn. (Verbenaceae) (Dhale 2011).

Alkaloids are heterocyclic nitrogen-containing chemicals utilized in medicine. Alkaloids protect plants from insects and herbivores, detoxify toxins, and store nitrogen. Many products consumed by humans contain alkaloids, such as coffee, tea, cocoa, tobacco, areca nut, and marijuana. Alkaloids have neuroprotective, blood pressure-lowering, pain-relieving, antibacterial, anticancer, and antimalarial effects (Carqueijeiro et al. 2013). Alkaloids detected in *R. tomentosa's* leaves and fruits support its historic medicinal usage in Singapore. In China, the leaves were traditionally used as a painkiller, the roots for stomach ulcers, and the seeds for improved digestion and for snake bites. In Indonesia, the leaves were used to cure wounds. In traditional Vietnamese medicine, the plant's raw fruit treats diarrhea and dysentery, while the ripe ones boost the immune system (Zhao et al. 2019).

According to Ferreira et al. (2011), histochemical tannin assays on *Psidium guineense* Sw (Myrtaceae) leaves indicated positive results in the epidermis, parenchyma, vascular bundles, and secretory cavities. These tannins in *R. tomentosa* leaves and fruits help treat diarrhea and dysentery. Ferreira et al. (2011) added that Myrtaceae leaves are used to treat diarrhea and intestinal infections due to their astringent tannin content. Tannins protect plants, which is why epidermal cells have them. Myrtaceae plants have tannins in their vascular systems also.

The meroterpenoids of *R. tomentosa* are antibacterial and antifungal. Myrtaceae secondary metabolites like meroterpenoids have been extensively explored as antibiotics. Thus, *R. tomentosa* could prevent microbial deterioration and extend food shelf life as a biocontrol agent and natural preservative (Zhao et al. 2019). Meroterpenoid compounds have been isolated and identified from various other Myrtaceae species, including *Eucalyptus tereticornis* (Liu et al. 2018) and *Leptospermum brachyandrum* (Zou et al. 2018).

Belwal et al. (2019) reported that flavonoid content declined throughout fruit ripening in *Rubus ellipticus*, *Myrica esculenta*, *Prunus persica*, blueberry (*Vaccinium corymbosum*), and blackberry fruit, as did Lin et al. (2020) in blueberries. Flavonoid levels vary by organ type. Hou et al. (2021) found that phenols and flavonoids drop from 5.38 mg RE/g in young citrus fruits to 0.69 mg RE/g in ripe fruit. The same trend was seen in grapes and lemons (Zhu et al. 2020). This phenomenon may be produced by many enzymes involved in flavonoid synthesis, such as 1,2-rhamnosyltransferase and flavanone-7-O-glucosides, which accelerate biosynthesis in the early growth period. Insufficient flavonoid aglycones may explain the decrease in flavonoids during citrus fruit ripening.

Lai et al. (2013) found a decrease in total phenol levels during *R. tomentosa* fruit ripening. The same pattern of declining phenol levels during fruit ripening occurs in other Myrtaceae plants, such as Brazilian cherry *Eugenia uniflora* L. (Celli et al. 2011). Belwal et al. (2019) further explain the accumulation and degradation of distinct phenolic compounds during fruit ripening connected to their biosynthesis pathways, which are mainly regulated by enzyme expression and various genetic and environmental variables. Elmastaş et al. (2017) added that phenol compounds are phenylpropanoid metabolic pathway intermediates. Enzymes convert aromatic amino acids into polyphenols. Enzyme activity affects the production of these molecules. Genetic diversity, pH, light, temperature, and climatic change affect enzyme activity. Fruit enzyme activity might vary depending on plant growth and ripening.

The anatomical characteristics reported in this study contribute to the identification of *R. tomentosa* and support quality control tests. Some features may stand out, such as hairy leaves with curved edges; epidermal cells with ripples and wall projections: and secretory cavities with dimensions that go beyond the height of the palisade parenchyma. Characters that can contribute to accuracy in taxonomic classification include the cross-sectional anatomical structure of leaves, including the epidermis, mesophyll, carrier bundle, secretory cavity, and trichomes, while fruit characteristics include a pericarp layer with an exocarp/outer layer, mesocarp, and endocarp. In this study, flavonoids, tannins, terpenoids, and alkaloids were found in the leaf's adaxial and abaxial epidermis, mesophyll, xylem, phloem, midrib parenchyma, secretory cavity, and trichomes. They were distributed throughout the fruit's exocarp, mesocarp, endocarp, secretory cavity, xylem, phloem, trichomes, and seeds. Young leaves contained the highest total flavonoid concentration (196 ± 1.77 mg QE/g), green fruit had the highest total phenol concentration (97.70 ± 18.15 mg GAE/g), old leaves contained the highest total alkaloid concentration (13.22 ± 0.98%), and red fruit had the highest total tannin concentration (1.66 ± 0.15 mg GAE/g).

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