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Antioxidant ~~activity~~Activity and ^{1}H NMR ~~profiling~~Profiling of ~~South Borneo~~ *Rhodomyrtus tomentosa* (Ait.) Hassk ~~leaves~~Leaves and ~~fruits~~Fruits from South Borneo

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Abstract. ~~In this~~This study~~, the~~ aimed to evaluate and compare antioxidant activity of ethanol extracts from ~~leaves and fruits of~~ *~~R.~~R. tomentosa* (Ait.) Hassk ~~was evaluated~~leaves and ~~compared~~fruits in two different ~~location samples.~~ locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were ~~classified into~~ categorized as young and old ~~categories~~, while fruits were ~~divided~~classified into three maturity stages~~:~~, including green, red, and purple. ~~The wild-grown fruits and leaves were gathered from Banjar and Batola, South Kalimantan Province, Indonesia. The~~ ^{1}H NMR spectroscopy was used to characterize the compositional profile of extracts ~~(~~, comprising carbohydrates, organic acids, amino acids, and main phenolic compounds~~) was characterized by ^{1}H NMR spectroscopy, and~~. Furthermore, antioxidant capacity was ~~analyzed using~~ assessed with DPPH~~. Intensity~~, showing intensity differences ~~were also detected~~ in ~~the~~ aromatic ~~shift~~shifts in leaves and fruits ~~and leaves~~ for both regions~~, Banjar and Batola.~~. Young leaves and green fruits had greater intensity ~~than the~~compared to old leaves, as well as red and purple fruits~~. This result correlates~~, correlating with ~~the~~ antioxidant capacity ~~obtained, and in the~~ results. Analysis of ^{1}H NMR spectra of ~~extracts of~~ young and old leaves ~~regional chemical shift 10.0 - 6.0 ppm aromatic compound area,~~extract identified key metabolites, including gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid~~. The difference~~, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity ~~indicates that the content of compounds in red and purple fruits has a~~showed higher ~~quantification~~levels of metabolites, ~~especially~~specifically, carbohydrates and amino acids, ~~than in~~in red and purple fruits compared to green fruits~~. In comparison, the content~~ which only had greater quantities of aromatic compounds ~~in green fruits has a higher quantification than others~~.

Keywords: DPPH, myricetin, ~~metabolite~~metabolites profiling, *R. tomentosa*.

INTRODUCTION

~~Antioxidants are~~ An antioxidant is a bioactive ~~compounds that can block~~compound capable of counteracting the harmful effects of free radicals and ~~substrate oxidation. Free radicals induce~~ oxidative stress induced by reactive mechanisms ~~that lead to~~which cause cellular damage~~, cellular~~ and degeneration, ~~and~~along with the development of chronic degenerative diseases ~~such as~~, including cancer, diabetes, cardiovascular disorders, and neurovascular diseases (Masisi et al~~.~~., 2016). ~~Generally, the body's need for antioxidants is fulfilled by using~~ The reliance on synthetic ~~antioxidants~~antioxidant, such as 4-hexyl-resorcinol~~. Nevertheless, multiple studies have proved that synthetic antioxidants have led to~~, to meet body needs has raised concerns due to the associated detrimental health ~~consequences~~effects and toxicological implications. ~~Therefore,~~ Consequently, there is an emphasis on the increasing importance of applying natural ~~antioxidants is becoming increasingly important and sought after. The~~antioxidant derived from bioactive plant components ~~found in plants can minimize the presence of free radicals~~ (Saeed et al~~.~~., 2012; Ismandari et al~~.~~., 2020).

Rhodomyrtus tomentosa (Ait.) Hassk, commonly known as Rose Myrtle, is ~~a~~an evergreen perennial shrub with ~~evergreen characteristics and thrives in its natural habitat. This plant species may be valuable for researching and developing new,~~inherent potential for discovering structurally diverse~~,~~ and biologically active compounds (Zhao et al~~.~~., 2019). ~~Recently, there has been a notable emphasis on~~ Recent studies focused on exploring the functional characteristics of ~~extracts derived from~~ *~~R.~~R. tomentosa* ~~in various studies. These extracts have historically been applied to mitigate a range of ailments while exhibiting~~ extracts, renowned for pharmacological properties ~~that safeguard the plant against biotic and abiotic challenges~~ and traditional applications in treating various ailments (Yang et al~~.~~., 2023). ~~Many researchers have extensively investigated the~~ The medicinal properties of *~~R. tomentosa~~* ~~and have explored the~~

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~~therapeutic potential of various plant~~ this plant and its components~~. The fruit exhibits a high content of~~ have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive ~~components (Lai et al. 2015). The fruit contained~~ compounds, including piceatannol, a stilbene component ~~promising to boost health and exhibit~~ with anti-leukemia potential (Lai et al.~~.~~., 2013~~). Furthermore,~~; 2015). Besides the ~~fruit has been~~health benefits, it is used ~~to make delicious~~in the production of wines and beverages (Yin et al.~~.~~., 2021). Presently, studies related to *R. tomentosa* primarily ~~focus on examining~~examine the phytochemical components found in ~~its~~ leaves, flowers, and stems~~. This emphasis is attributed~~ due to the ~~remarkable~~exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the ~~potential for~~ DNA damage~~-~~alleviating ~~DNA damage exhibited by these plant parts~~ability (Dachriyanus et al.~~.~~., 2002; Lavanya et al.~~.~~., 2012; Wu et al.~~.~~., 2015; Ridlo et al.~~.~~., 2020; Hu et al.~~.~~., 2022). ~~Additionally, it has been noted by~~ Kuntorini et al. (2022) ~~that the green fruit of rose myrtle had~~reported the most significant antioxidant activity~~,~~ in the green fruits, with DPPH and FRAP values of 1419.75±3.48 and 1367.59±9.12 µmol TE/g DW, respectively. The ethanol ~~extract exhibited~~extracts showed the highest TFC values in the young leaves and green fruits, measuring 96.375±3.96 and 95.731±5.42 mg QE/g DW, respectively.

~~However, the~~The limited use of ~~rose myrtle plants~~ *R. tomentosa* in South Kalimantan, ~~mainly~~particularly its ~~fruit~~leaves and ~~foliage, has been very scarce~~fruits, until ~~now. This~~recently, is ~~due~~attributed to a lack of knowledge ~~regarding~~about the ~~utilized technique~~extraction methods and ~~the plant's~~potential benefits. ~~The rose myrtle~~Despite possessing several advantages, this plant ~~has not been utilized to its full potential and~~ is considered a pest due to ~~its~~the rapid growth rate~~. The advantages of the rose myrtle shrub, namely its fruit and leaves, are not commonly recognized. A thorough~~ possessed. No comprehensive investigation ~~into~~has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations~~, has not been conducted thus far. Hence, the primary objective of this work is to examine the metabolites present in *R. tomentosa* taken from different locations, with a specific focus on the fruits and leaves. Metabolites were identified directly from the samples using NMR spectroscopy. A metabolic examination was performed on the fruits and leaves of *R. tomentosa* from different locations at different maturation phases. The objective was to identify the specific components that exhibited substantial contributions as antioxidants.~~. This study represents the inaugural systematic ~~investigation~~examination of metabolites contained in *R. tomentosa* leaves and fruits ~~and leaves at~~obtained from different ~~stages of maturity, utilizing a~~locations, using combined NMR ~~approach. The study~~ spectroscopy, particularly to identify antioxidant components. Additionally, it effectively ~~showcases~~shows the suitability and effectiveness of the NMR-based ~~approach~~method in analyzing plant metabolites.

MATERIALS AND METHODS

Plant materials

~~The leaf samples were chosen from the~~ Samples of younger ~~leaves (2nd - 6th order from the shoot)~~ and ~~the~~ older leaves (2nd - 6th and 7th -- 12th order from the shoot) of *R. tomentosa*~~. The Munsell Color Charts were employed~~, as ~~a reference for color in plant tissues. The fruit samples included in this study consisted of~~well as green, red, and purple fruits (Wilde, 1977) ~~(~~were collected as presented in Figure 1~~). The specimens~~. These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), ~~located in~~ South Kalimantan~~, Indonesia, during~~ in June - July 2023. ~~The~~Munsell Color Charts were applied as a reference for plant tissue color. Moreover, the Herbarium Bogoriense, Indonesian Institute of Sciences in Bogor~~, Indonesia,~~ identified ~~the samples. The identification was~~and confirmed samples with certificate number 1007/IPH.1.01/If.07/IX/2023.

Procedures

Crude ethanol extract preparation

~~The leaves~~Leaves of ~~varying~~different ages~~, including both young and old leaves,~~ were selectively harvested from ~~the~~ apical ~~branch of *R. tomentosa*. The~~ branches, and fruits were subjected to ~~a~~ drying ~~process~~ at ~~a temperature of~~ 40°C ~~with~~in an oven, followed by grinding at room temperature. Approximately 500 g of each ground material was macerated in 1000 mL of ethanol (SmartLab, Indonesia) for ~~24~~72 h~~. The~~ by replacing the solvent ~~was dumped and replaced~~ every 24 h~~, and this procedure was iterated three times~~ (Nurcholis et al.~~.~~., 2021; Kuntorini et al.~~.~~., 2022). ~~The extracts obtained~~Extracts from ~~the~~ identical samples were ~~mixed,~~ homogenized, filtrated to remove cellular debris, and dried using a rotary evaporator~~. The resulting extract was~~, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

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~~The examination~~Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. ~~the~~(2022), DPPH (2,2-diphenyl picrylhydrazyl) radical scavenging activity was ~~adapted from the research conducted~~assessed by ~~Purwakusumah et al. (2016) and Kuntorini et al. (2022). The~~ ethanol extracts ~~were diluted~~ in absolute methanol to attain the appropriate concentration ~~using absolute methanol (SmartLab, Indonesia). Subsequently, 2 mL of the~~. The sample (2 mL) was ~~combined~~mixed with 2 mL of 0.17 mM DPPH (Sigma-Aldrich, Germany~~). The solution was~~) and incubated in a light-free environment for 30 mins at ambient temperature. ~~During this period, the absorbance~~Absorbance at a wavelength of 516 nm was measured using a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). ~~The quantification of~~Moreover, free radical scavenging activity was ~~also performed~~quantified in ~~terms of~~ µmol Trolox (Sigma-Aldrich, Germany) equivalents per unit of dry weight (DW~~)~~() (µmol TE/g).

Sample preparation for ^{1}H- NMR

The ~~production of the~~ ^{1}H- NMR samples ~~was done using~~were prepared through a ~~slightly altered~~modified methodology from ~~the previously published~~previous studies (Gogna et al~~.~~., 2015; Mishra et al~~.~~., 2019; Kim et al~~.~~., 2010). Approximately 25 g of ~~the sample's~~ crude ~~extract was~~ extracts of the samples were placed into a 2 mL Eppendorf tube with 1 mL of methanol-d4 solution containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The ~~combination underwent~~mixture was subjected to vortexing and sonication for 1 min~~. Using a microcentrifuge, the solution underwent~~ , followed by homogenization ~~followed by~~and centrifugation ~~for 1 min~~ at ~~a rotational speed of~~ 10,000 rpm for 1 min. The supernatant ~~of the sample~~ was ~~gathered,~~collected and transferred into the NMR tube~~, and afterward readied~~ for subsequent analysis using ^{1}H- NMR.

^{1}H NMR spectroscopy

The ^{1}H- NMR analysis was conducted ~~using~~ with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at ~~a temperature of~~ 25°C. ~~A~~The set of parameters ~~was employed, encompassing~~ applied included 128 scans over 10 mins~~. These parameters included~~ , a relaxation delay of 1.5 mins, an X_angle of 60º, and a pre-saturation mode set at 4.27 ppm. ~~The~~ Additionally, an internal lock was established using ~~the~~ deuterated solvent~~. The~~, and the spectral width was ~~quantified~~measured within the range of 0 to 10 ppm.

Data analysis

~~The mean~~Mean values and standard ~~deviation~~deviations (mean ± SD) were calculated ~~using~~from three replications. ~~The~~ Statistical analysis of quantitative data ~~underwent statistical analysis using~~was performed with a one-way analysis of variance~~.~~ (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at ~~a significance level of~~ $p < 0.05$~~. The software programs Microsoft Excel® and IBM SPSS Statistics 21 were utilized for this analysis. When a~~ , and in case of divergent ~~outcome was detected, a~~results, post hoc ~~test~~ testing was conducted using the LSD method ~~followed the variance analysis~~.

The ^{1}H- NMR spectra were analyzed using ~~the~~ MestReNova software~~. In addition, the spectra underwent~~, including processes of manual phasing, baseline modification, and calibration to internal standard solution signals (TMSP) ~~located~~ at ~~the~~a chemical shift of 0.0 ppm. The observed ~~NMR resonances'~~ multiplicities of NMR resonances were designated ~~per~~according to the established convention. The symbols used in this context ~~are~~were s = singlet, d = doublet, dd = doublet of doublets, t = triplet, and m = multiplet (Mishra et al~~.~~., 2019). ~~In addition~~Additionally, the identification of metabolites was conducted by a comparative analysis of information ~~included within a metabolite~~found in a database derived from prior studies (Kim et al~~.~~., 2010; Ali et al~~.~~., 2011; Nuringtyas et al~~.~~., 2012; Gogna et al~~.~~., 2015; Cerulli~~.~~., 2018; Mishra et al~~.~~., 2019). ~~The~~ Semi-quantitative examination of the signals ~~were examined semi-quantitatively~~was performed by comparing ~~the signal's area~~their areas to that of the TMSP signal, ~~which served~~serving as an internal reference. ~~The~~ Subsequently, the ^{1}H- NMR signals were normalized to total intensity to ~~generate~~produce data suitable for multivariate ~~data~~ analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The ~~findings of the~~ extraction process ~~demonstrate a diverse range of~~ yielded varying extract weights across ~~several~~ samples collected from both locations, as ~~depicted~~presented in Table 1. The ethanol extracts from purple fruits ~~exhibited~~showed the most significant output~~, measuring~~ at 15.24% w/w~~. Conversely, the~~ , while green fruits ~~yielded~~produced the lowest ~~amount~~yield, precisely 5.63% w/w.

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Maceration ~~is,~~ a non-thermal extraction ~~technique employed~~method, was used to prevent the degradation of secondary metabolite compounds by ~~avoiding the application of~~circumventing high temperatures. ~~The~~ During the experiment, coarse ~~powder of~~ simplisia ~~is combined~~powder was mixed with an aqueous solution. ~~The active compounds are extracted by immersing the~~ The simplisia powder ~~in a suitable liquid~~was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and ~~ensuring protection from~~ avoiding light. ~~The entry of the liquid~~ exposure. Liquid entered into the ~~cell will occur~~cells through diffusion, causing the ~~cell wall. The~~ dissolution of cellular contents ~~occurs~~ due to ~~the disparity in~~ concentration disparities between the ~~extracellular solution~~extra and ~~the~~ intracellular fluid. ~~The liquid with a higher concentration will undergo diffusion, resulting in its displacement by the liquid with a lower concentration. The occurrence persists~~ until ~~an equilibrium of a~~ solute concentration ~~is achieved between the extracellular and intracellular environments~~equilibrium was attained (Harbone, 1987).

This study ~~used the~~applied DPPH method ~~because it is the most~~ due to its common ~~method~~use for measuring in vitro antioxidant activity in medicinal plants (Zhou & Yu, 2004). ~~The hydrogen donation of hydroxyl compounds in the~~ The ethanol ~~extract~~extracts of *R. tomentosa* samples ~~is responsible for reducing, rich in hydroxyl compounds, reduced~~ DPPH to DPPH-H. ~~through hydrogen donation.~~ An increase in ~~the amount of~~ 1,1-diphenyl-2-picryl-hydrazyn ~~will result~~level resulted in a color change ~~in color~~ from dark purple to pastel pink or yellow, which ~~can be~~was observed with a spectrophotometer ~~in order~~ to determine the free radical ~~attenuation~~scavenging activity of the sample (Molyneux, 2004; Sayuti & Yenrina, 2015).

Purwakusumah et al. (2016) ~~state that random results in~~emphasized the ~~measurement~~significance of standardizing antioxidant capacity ~~using various methodologies are a significant obstacle in~~measurements to enhance comparability and ~~may result in~~mitigate discrepancies. ~~Therefore, the~~Expressing antioxidant capacity ~~method is expressed~~ as millimolar ~~trolox~~Trolox equivalents per gram of sample extract. ~~Trolox, also known as~~ provides a more descriptive and meaningful representation than percentage inhibition. Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic~~.)~~) is ~~a~~an analog of water-soluble vitamin E or α-tocopherol ~~analog. The~~. This study aimed to compare antioxidant capacity, ~~expressed~~calculated in ~~trolox~~Trolox equivalents ~~is more meaningful and descriptive than in percent inhibition. The study aims to compare the antioxidant capacity, represented as the trolox equivalent, disregarding the utilization of~~ without using diverse methods. ~~In contrast~~However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage ~~of~~ inhibition exerted by antioxidant chemicals against radicals or metal radicals.

The antioxidant capacity of ~~the~~*R. Tomentosa* leaves and fruits ~~of *R. tomentosa*, as~~ measured by ~~the~~ DPPH method ~~varied~~ ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). ~~An analysis of variance~~The conducted ANOVA followed by an LSD analysis of the ten samples ~~revealed a~~showed statistically significant ~~difference~~differences (P<0.05~~) between samples.~~). The results ~~of this study demonstrated~~showed that the ethanol extracts ~~of leaves and fruits had~~ possessed similar antioxidant ~~properties in their~~ capacity ~~to capture free radicals~~ at both sample locations. ~~This can be seen in green fruit~~Specifically, ethanol ~~extract, having~~extracts of green fruits had the highest DPPH radical scavenging capacity, with respective values of ~~Banjar Regency locations of~~ 2360.35±6.86 µmol TE/g ~~and Batola~~(Banjar Regency ~~locations of~~) and 2795.33±9.07 µmol TE/g. ~~The~~ (Batola Regency). Conversely, the lowest ~~yield was found~~values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively. ~~The antioxidant capacity of purple fruit in this study was lower than the capacity reported by Lai et al. (2015).~~, which ~~is~~ were below the 431.17±14.5 µmol TE/g, ~~but~~ reported by Lai et al. (2015). These appeared to be higher than ~~the~~ antioxidant capacity ~~of~~, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, ~~kiwifruit~~kiwifruits, and apples ~~in~~by Wu et al.~~'s~~ (2004~~) study, which is 8.79-92.60 µmol TE/g.~~). This study ~~demonstrates that~~suggested the high potential of the purple ~~fruit~~fruits and other portions of *R. tomentosa* ~~have a high potential~~ as ~~an~~ antioxidant ~~source~~sources.

Maskam et al. (2014) ~~state~~stated that the extract of *R. tomentosa* ~~fruit extract possesses~~fruits possessed potent antioxidant properties. ~~The results of~~Additionally, Lavanya et al. (2012) ~~also demonstrated that leaf extracts possess a substantial inhibiting ability, which is~~detected 2.7 and 3.0 times greater ~~than that of~~inhibiting ability in leaves extract than in gallic and ellagic acids, respectively. ~~Therefore, the extract demonstrates~~, signifying excellent antioxidant activity.

In general, the ~~results of~~ differences observed in antioxidant activity ~~in~~between the two locations ~~differed, with the antioxidant capacity of Batola Regency being more significant than that of Banjar Regency. Compared~~were attributed to ~~Banjar regency, the high antioxidant capacity of Batola regency is due to its high~~ variations in total phenolic and ~~total~~ flavonoid content. ~~The~~, as Batola Regency samples showed a higher antioxidant ~~content of plants has a linear correlation~~capacity than those from Banjar Regency, which was consistent with the ~~phenolic and flavonoid content of samples, as~~ results reported by Zargoosh et al. (2019).

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The study by Ene-Obong et al.'s (2018) study on *Monodora myristica*, using DPPH as a parameter also demonstrated a, showed high level of antioxidant activity. In addition due to having a high concentration of flavonoids and, phenolics, it also has the highest concentration of and vitamin C, making it a potent antioxidant. With the . Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, *Ricinodendron heudelotii* hashad the lowestleast DPPH-reducing ability. This indicatesshowed that the ability of flavonoids to function as potent antioxidantsantioxidant and free radical scavengers will dependdepended on the location of the hydroxyl group and other structural characteristics of their chemical structure.. Wu et al. (2004) found that fruit extracts frommeasured substantial antioxidant activity in the extract of *R. tomentosa* withfruits containing high flavonoid concentrations exhibited high antioxidant activity as measured by, through various methods including DPPH, FRAP, inhibition of lipid peroxidation activity, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the antioxidant capacity of thecurrent results obtained for leaves and fruits of *R. tomentosa* in this study, where compounds that act as antioxidants are possible, namelycomprising flavonoids and phenols, which have a similar mechanism for functioning as antioxidantsantioxidant mechanisms.

There is aA direct correlation exists between antioxidant activity and total phenolic content and antioxidant activity,, which serves as it is commonly believed that plants with a higher phenolic content have more significant antioxidant activity. Nonetheless,reliable indicator, but some reports contradict this. The phenolic content of plants can serve as a reliable indicator of their antioxidant capacitythese expressions (Chanda & Dave, 2009). According to Cartea et al. (2011), phenolic compounds constitute a large category of phytochemicals abundant in plants. Based on its chemical structure, it is divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids., based on their chemical structures. Flavonoids (notably flavonols and anthocyanins) and hydroxycinnamic acids are plants'the most extensive and diverse polyphenols. FlavonoidsFurthermore, flavonoids are mostmajorly prevalent among phenolics and are present in all plant parts. AntiApart from acting as antioxidant, phenolic compounds provide health benefits including anti-inflammatory, antimicrobial, antiallergic, and cytotoxic antitumor activity all play a role in phenolic compounds' ability to improve human health, but antioxidant activity is the phenolic compound's most vital function. According to. Zhao et al. (2019), the presence of) associated high flavonoid and phenol content in the extract of *R. tomentosa* leafleaves and fruit extracts is closely related to their fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits
Furthermore, the chemical properties of *R.* ***R. tomentosa*** **leaves and fruits**

The chemical properties of the ethanol extracts tested extract of *R. tomentosa* leaves and fruits were characterized for antioxidant activity were characterized by ^{1}H NMR spectroscopy. This technique significantly benefits analyzing complex mixtures, such as food extracts. Notably, this method is This non-destructive and requires a relativelyspectroscopy method, requiring a simple sample preparation process, within aand short duration. Figure 2 depicts the, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

NMR spectroscopy was used to ascertain, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004). Additionally, NMR), has been widely employedused to identify metabolite types of metabolites due to its capability of generatingto generate a particular and distinctive spectrum for each compound. The results wereA study assessed results based on the quantity of compounds identified, as opposedcompared to the signals observed during the NMR analysis (Leiss et al., 2011). Compound identification has beenbecomes simplified through the widespread application of NMR metabolomics techniquesmethods. This can beis accomplished by comparing thesample signals generated by the samples to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al., 2010; Ali et al., 2011; Nuringtyas et al., 2012; Gogna et al., 2015; Cerulli., 2018; Mishra et al., 2019). Aqueous methanol is frequently usedserves as ana common extraction solvent because it can extractcapable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, Multiple research papersmultiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes. The, with the coupling constant wasas a crucial parameter utilized in this studyused to authenticate the correspondence between the signals in the data and the references (Kim et al., 2010).

The ^{1}H-NMR spectra were separateddivided into three regions based on their chemical shiftshifts (δ), namely the aliphatic compounds (amino and organic acids andincluding terpenoids,), carbohydrates, and aromatic compounds. Aliphatic compounds (organic and amino acids) were detected within the chemical shiftshifts of 0.5–3.0 ppm, carbohydrates within 3.1–6.0 ppm, and aromatic compounds in > 6 ppm, respectively (Kim et al., 2010). In addition, as shown in Figure 2, the depicts the analysis and comparison of various developmental phases of the ^{1}H-NMR spectra of leafextract of leaves and fruit extractsfruits obtained from two locations were analyzed and compared.

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The ~~findings derived from the~~ ^{1}H-NMR analysis ~~of the putative compounds suggested~~results showed the presence of primary and secondary metabolite compounds. ~~In particular, the primary metabolites consisted of~~, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm~~), while~~) classified as primary and aromatic compounds (chemical shift > 6 ppm) ~~were classified~~ as secondary ~~metabolites~~. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra ~~throughout~~all through the maturation phases of leaves and fruits. ~~The~~ Young leaves and green fruit samples showed higher signal intensities ~~observed~~ in the aromatic region ~~of the young leaves and green fruit samples collected from two different locations (Banjar and Batola) were found to be higher compared to those observed in the~~compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits ~~and leaves exhibit distinct attributes~~were observed in both Batola and Banjar, particularly ~~within~~in the 5.0-3.0 ppm chemical shift range. The chemical shift ~~is predominantly~~was more substantial in ~~the fruit~~fruits than in ~~the leaves. This result indicates that fruit is~~leaves, suggesting fruits as the primary ~~producer~~producers of the anomeric content of glucose, α-glucose, and fructose. ~~Particularly~~ Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose~~), intensity and diversity varied between fruit and leaf samples.~~

~~).~~ Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) ~~observed~~detected in ~~the leaf~~leaves and ~~fruit of~~fruits from both locations. ~~The intensity is higher~~Higher intensities were recorded in young leaves and green fruits ~~than in~~compared to older leaves and red and purple fruits. ~~This is~~, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves ~~detected~~showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. ~~Particularly noticeable~~The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). ~~The ^{1}H NMR spectrum reveals~~Young leaves showed a significant concentration ~~level in young leaves, which corresponds~~of these compounds, corresponding to the comparatively high antioxidant capacity observed ~~in young leaves in contrast to old leaves.~~. This was consistent with the similar trend reported by Gogna et al. (2015) ~~conducted similar studies on~~regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.~~). Using.~~) using NMR spectroscopy~~, they determined that the total phenol content was highest in young leaves, which is consistent with their antioxidant activity and phenol and flavonoid content~~.

A comparison of the ^{1}H NMR spectral analysis ~~conducted on~~ of extracts from purple, red, and green ~~fruit extracts revealed that the spectral~~ fruits showed nearly identical signal diversity ~~of all~~among the three samples ~~was nearly identical~~ (Figure 2). However, ~~regarding~~in terms of signal intensity or integral, ~~the~~ red and purple fruits showed more pronounced results for carbohydrates and amino acids ~~were more pronounced in red and purple fruit than in green fruit. However, green fruit exhibited~~compared to green fruits, which showed a higher signal intensity for aromatic compounds ~~than red and purple fruit.~~. The ~~disparity~~disparities in spectral signal intensities ~~suggests~~suggested that red and purple fruits ~~contain~~contained more ~~compounds containing~~ metabolites, particularly carbohydrates and amino acids, ~~than~~while green fruits~~. Conversely,~~ were richer in aromatic compounds ~~are more abundant in green fruits than other fruits.~~. According to Lacy et al. (2014), the concentration of metabolites present ~~can~~could affect the intensity of spectral signals measured ~~at~~with NMR. One of the ~~merits~~advantages of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al., 2005). ~~It can be deduced that~~In this study, the ethanol ~~extract~~extracts of green ~~fruit contains a~~fruits, despite containing lower ~~concentration~~concentrations of carbohydrates and amino acids ~~compared to the extracts of~~than red and purple ~~fruit. However, it contains~~fruits, showed a higher concentration of aromatic compounds. ~~The findings from the antioxidant~~Antioxidant capacity test results (Table. 1) ~~indicate that green fruits exhibit~~showed a greater antioxidant capacity ~~than~~in green fruits compared to red and purple fruits at ~~two specific~~both sample locations~~. Specifically, the sample from the~~, with Batola ~~site demonstrates~~showing superior antioxidant capacity and spectral signal intensity ~~compared to the~~ than Banjar ~~site sample~~. Ali et al. (2011) ~~conducted research comparing ^{1}H NMR spectra of~~reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.~~). Their findings indicate that metabolite profiles exhibit a consistent pattern throughout all stages of fruit development.~~) based on the ^{1}H NMR spectra obtained. The green stage ~~of fruit development is characterized by~~comprises the highest ~~concentration of phenols~~phenol concentrations, which ~~decreases~~decrease gradually as ~~the fruit ripens. However, as the grapes mature, they accumulate~~fruits ripen while accumulating more amino acids and sugars.

~~The~~Despite the benefits of ~~employing~~applying ^{1}H-NMR ~~have been demonstrated in diverse~~ in metabolomics studies~~. Nevertheless, the utilization of ^{1}H NMR spectroscopy posed significant problems in the~~ , challenges arise in chemical identification ~~process owing~~due to overlapping signals across various regions, ~~most notably~~

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withinspecifically in the range of 5.0-3.0 ppm, which corresponded range corresponding to sugar compounds. Consequently, the failure to discernThis study faced difficulties in discerning signals withinin the sugar region, except for glucose and sucrose, resulted in a diminished limiting the detection of certain substances within the scope of this investigation. The findings additionally demonstrated the detection of . However, 20 potential compounds were identified through analysis of the ^{1}H- NMR spectra, detailed analysis, as presented in Table 2. The detected amino acid region exhibitedshowed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed withinin the 3.0 - 2.0 ppm range. The commonly detected Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were also observed withincommonly detected in the chemical shift of 5.00 - 3.50 ppm. In theThe less crowded regions, at 10.0 - 6.0 ppm showed several phenolics were identified, including, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid (chemical shifts 10.0 - 6.0 ppm). The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study showed that Intensity identified intensity differences were also detected in the aromatic shift (6-7.5 ppm) in of leaves and fruits and leaves for from both regions. Young leaves and green fruits had greater intensity than the old leaves, red and purple fruits. This correlates, correlating with the obtained antioxidant capacity obtained. In the . The ^{1}H NMR spectra of extracts of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm , particularly in the aromatic compound area, identifiedshowed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity indicatesshowed that the content of compounds in red and purple fruits has acontained higher quantificationquantities of metabolites, especiallyspecifically carbohydrates and amino acids, thanwhile green fruits, while the content of comprised more aromatic compounds in green fruits has a higher quantification than others. Therefore, . This signified the potential of *R. tomentosa* leaves and fruits could be considered as promising as an antioxidant agents, even thoughalthough further studies are necessarywould be needed to propose their determine the role played in nutritional applications.

ACKNOWLEDGMENTS

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Manuscript Title

Antioxidant Activity and 1H NMR Profiling of *Rhodomyrtus tomentosa*(Ait.) Hassk Leaves and Fruits from South Borneo

Author(s)

Evi Mintowati Kuntorini, Liling Triyasmono and Maria Dewi Astuti

Date Issued

November 20, 2023

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2. Submitted to the journal “BIODIVERSITAS” (28-11-2023)

UNIVERSITAS LAMBUNG MANGKURAT

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

[biodiv] Submission Acknowledgement

Ahmad Dwi Setyawan via SMUJO <support@smujo.com> Tue, Nov 28, 2023 at 12:29 AM
Reply-To: Ahmad Dwi Setyawan <editors@smujo.id>
To: Evi <evimintowati@ulm.ac.id>

Evi:

Thank you for submitting the manuscript, "Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa (Ait.) Hassk leaves and fruits from South Borneo" 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:

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Smujo Editors via SMUJO <support@smujo.com> Wed, Nov 29, 2023 at 4:22 PM
Reply-To: Smujo Editors <editors@smujo.id>
To: EVI MINTOWATI KUNTORINI <evimintowati@ulm.ac.id>, LILING TRIYASMONO <liling.triyasmono@ulm.ac.id>, MARIA DEWI ASTUTI <mdastuti@ulm.ac.id>

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Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Fri, Dec 1, 2023 at 10:31 AM
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Regards,

Dr. Evi Mintowati Kuntorini

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Sincerely,

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Author(s) name:

EVI MINTOWATI KUNTORINI[1♥], LILING TRIYASMONO[2♥], and MARIA DEWI ASTUTI[3]

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In this study, NMR was used to identify the content of secondary metabolites in the leaves and fruit of *R. tomentosa*. Our research shows that green fruit and young leaves contain higher levels of flavonoid compounds than ripe fruit (purple fruit).

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

EVI MINTOWATI KUNTORINI[1♥], LILING TRIYASMONO[2♥], AND MARIA DEWI ASTUTI[3]

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Manuscript received: DD MM 2016 (Date of abstract/manuscript submission). Revision accepted: ..... 2016. (8 pt)

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, myricetin, metabolite profiling, *R. tomentosa*.

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds. In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of R. tomentosa. The main components of R. tomentosa include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,

flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 μmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. No comprehensive investigation has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2^{nd} - 6^{th} and 7^{th} - 12^{th} order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (μmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).

The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an aqueous solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^d	1429.53 ± 9.07^d
Old leaves	12.296	11.49	836.20 ± 9.07^c	881.24 ± 5.94^c
Green fruits	5.63	6.05	2360.35 ± 6.86^e	2795.33 ± 9.07^e
Red fruits	9.85	7.76	415.06 ± 2.99^b	443.47 ± 2.06^b
Purple fruits	14.35	15.24	260.58 ± 0.91^a	364.05 ± 3.82^a

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the differences observed in antioxidant activity between the two locations were attributed to variations in total phenolic and flavonoid content, as Batola Regency samples showed a higher antioxidant capacity than those from Banjar Regency, which was in line with Zargoosh et al. (2019).

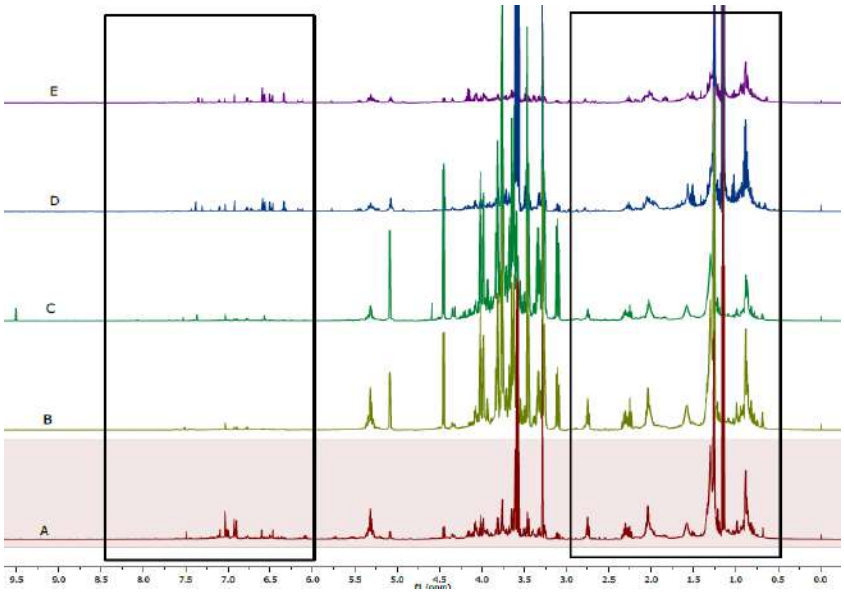
The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

The chemical properties of R. tomentosa ethanol extract *in* leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

A



B

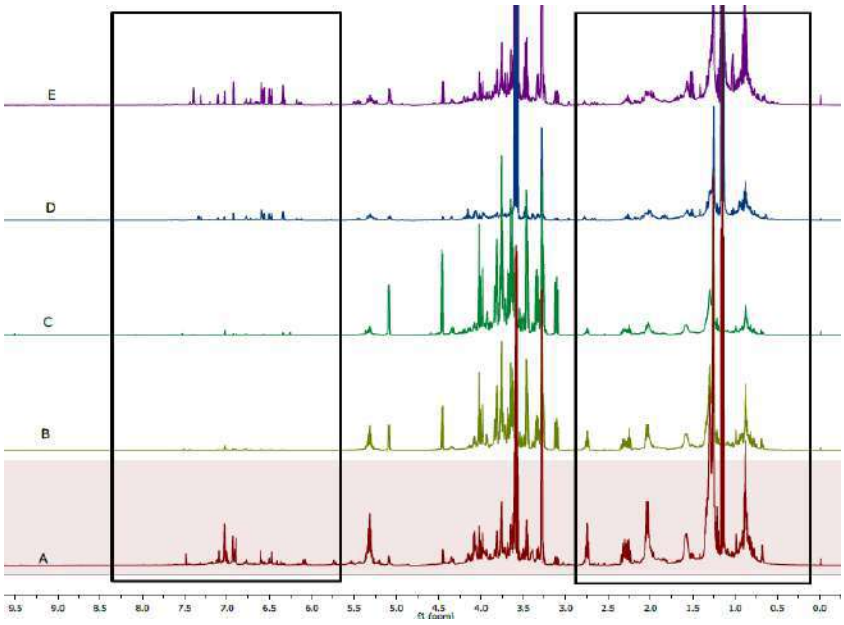


Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

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5. Submitted through OJS (1-12-2023)

UNIVERSITAS LAMBUNG MANGKURAT

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

[biodiv] Editor Decision

Managing Editor <unsjournals@gmail.com> Fri, Dec 1, 2023 at 10:51 AM
To: Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>

Kindly send your paper through OJS and do not need to CC this email.

Thank you,
Regards,

Ahmad Dwi Setyawan
Managing Editor,

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6. Decline submission (1-1-2024)

UNIVERSITAS LAMBUNG MANGKURAT

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

[biodiv] Editor Decision

Smujo Editors via SMUJO <support@smujo.com> Mon, Jan 1, 2024 at 7:13 AM
Reply-To: Smujo Editors <editors@smujo.id>
To: EVI MINTOWATI KUNTORINI <evimintowati@ulm.ac.id>, LILING TRIYASMONO <liling.triyasmono@ulm.ac.id>, MARIA DEWI ASTUTI <mdastuti@ulm.ac.id>

EVI MINTOWATI KUNTORINI, LILING TRIYASMONO , MARIA DEWI ASTUTI:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa (Ait.) Hassk leaves and fruits from South Borneo".

Our decision is to: Decline Submission

Note: We have invited c. 20 experts but no one wants to review. So, please make your "own-review" by sending your paper to at least two reviewers, and one professional language proofreader; then providing us with the following 5 documents, i.e.: 1. paper commented by reviewer-1 (include: name and email address), 2. paper commented by reviewer-2 (include: name and email address), 3. table of response, 4. certificate of proofreading, and 5. final revised paper after proofreading. Reviewers should come from different universities/institutions with the author and have a Scopus ID.

Biodiversitas Journal of Biological Diversity

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7. Author's response (4-1-2024)

UNIVERSITAS LAMBUNG MANGKURAT

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

[biodiv] Editor Decision

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Thu, Jan 4, 2024 at 7:50 AM
To: Smujo Editors <editors@smujo.id>

Dear Smujo Editors

Thank you for the kindness of the editors.

I will send the revised manuscript immediately after review by 2 reviewers and proofreading.

Regards,

Dr. Evi Mintowati Kuntorini

Associate Professor
Laboratory of Plant Structure and Development, Faculty of Mathematics and Natural Science,
Lambung Mangkurat University
Jl. A.Yani Km 36 Banjarbaru, South Kalimantan, 70714, Indonesia
E-mail : evimintowati@ulm.ac.id

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8. Invitation to review for manuscript reviewer 2 (2 jan 2024)

UNIVERSITAS LAMBUNG MANGKURAT

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

Invitation to review for manuscript

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Tue, Jan 2, 2024 at 11:41 AM
To: whika@ecampus.ut.ac.id

Manuscript Number: 16606

Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo.

Dear Dr. Dewatisari

I would like to invite you to review the above referenced manuscript submitted, as I believe it falls within your expertise and interest. The abstract and this manuscript is included below.

Please respond to this invitation at your earliest opportunity.
I hope you will be able to review this manuscript.

Thank you in advance for your contribution and time.

Regards,

Dr. Evi Mintowati Kuntorini

Associate Professor
Laboratory of Plant Structure and Development, Faculty of Mathematics and Natural Science,
Lambung Mangkurat University
Jl. A.Yani Km 36 Banjarbaru, South Kalimantan, 70714, Indonesia
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9. Reviewer 2's response to reviewer invitation (3-1-2024)
- Manuscript review

UNIVERSITAS LAMBUNG MANGKURAT

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

Invitation to review for manuscript

Dr. Whika Febria Dewatisari , S.Si., M.Si <whika@ecampus.ut.ac.id> Wed, Jan 3, 2024 at 5:04 PM
To: Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>

Dear Dr. Evi Mintowati Kuntorini,

I hope this email finds you well. I am writing to inform you that I have reviewed your manuscript titled "Antioxidant activity and 1H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo". (Manuscript Number: 16606). I was impressed with the clarity of your research objectives, methodology, and the significance of your findings. However, there are a few minor revisions that need to be addressed. Below, I have outlined the specific revisions required:

1. The authors are requested to provide some context for their choice of focusing on leaves and fruits rather than the roots or stems of flowers.

2. It is important to clarify the number of *R. tomentosas* present in Kalimantan. Is this plant abundant? This information is relevant as it indicates the potential for its utilization in the development of natural medications.

3. In the discussion section, it is necessary for the author to provide a brief description of the conditions present in the regions of Banjar and Batola, including details about the soil, water/climate, and the author's objective behind collecting samples from these areas. Additionally, the author can compare and discuss the results of antioxidant activity observed in organs such as leaves, and fruit obtained from both locations.

4. In the results section, it should be clarified whether the results from the table and the DPPH analysis correspond with each other. If there are any discrepancies, they should be explained.

5. Both the results and discussion sections should be structured in a way that each paragraph contains complete elements, including multiple explanatory sentences, and each paragraph should focus on a single topic.

Please refer to the comments in the manuscript for further feedback from the reviewer (attached manuscript with the reviewer's comments)

If you have any questions or require any further clarification, please do not hesitate to reach out.

Best regards,

Whika Febria Dewatisari

Dari: Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>
Dikirim: Selasa, 02 Januari 2024 10.41
Kepada: Dr. Whika Febria Dewatisari , S.Si., M.Si <whika@ecampus.ut.ac.id>
Subjek: Invitation to review for manuscript

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds (……..). In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of *R. tomentosa*. The main components of R. tomentosa include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et

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al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. No comprehensive investigation has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants

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MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

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Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at

2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an aqueous solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

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Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^d	1429.53 ± 9.07^d
Old leaves	12.296	11.49	836.20 ± 9.07^c	881.24 ± 5.94^c
Green fruits	5.63	6.05	2360.35 ± 6.86^e	2795.33 ± 9.07^e
Red fruits	9.85	7.76	415.06 ± 2.99^b	443.47 ± 2.06^b
Purple fruits	14.35	15.24	260.58 ± 0.91^a	364.05 ± 3.82^a

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

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Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the differences observed in antioxidant activity between the two locations were attributed to variations in total phenolic and flavonoid content, as Batola Regency samples showed a higher antioxidant capacity than those from Banjar Regency, which was in line with Zargoosh et al. (2019).

The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

The chemical properties of *R. tomentosa* ethanol extract *in* leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

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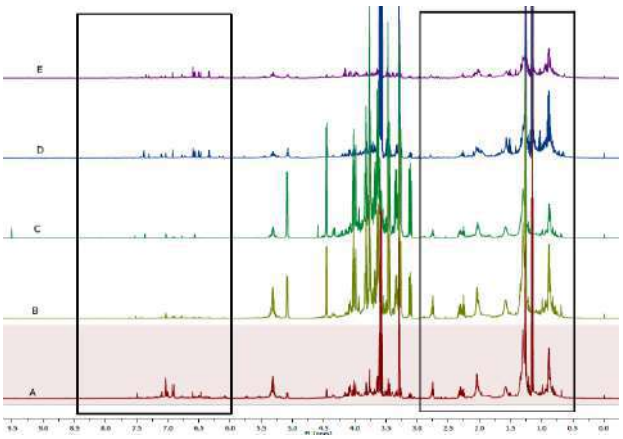

B

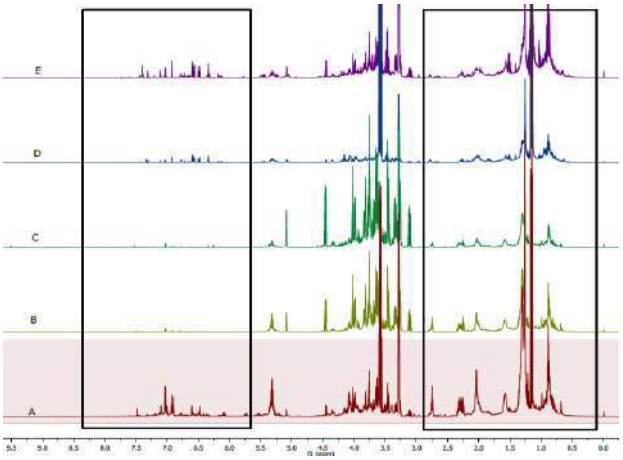

Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

Note : In the discussion section, the author has to provide a brief description of the conditions found in the regions of Banjar and Batola, including details about the soil, water/climate, and the author's aim for collecting samples from these areas. Additionally, the author can discuss and compare the results of the antioxidant activity observed in organs like leaves and fruit obtained from both locations.

ACKNOWLEDGMENTS

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10. Second revised submission to reviewer 2nd (26-3-2024)
    - Cover Letter Revision Journal Biodiversitas
    - Revised manuscript

UNIVERSITAS LAMBUNG MANGKURAT

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

Invitation to review for manuscript

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Tue, Mar 26, 2024 at 1:39 PM
To: "Dr. Whika Febria Dewatisari , S.Si., M.Si" <whika@ecampus.ut.ac.id>

Dr. Whika Febria Dewatisari, M.Sc.

Thank you for reviewing our manuscript. We are glad to receive the positive comments from the reviewer and we believe that these will improve the quality of our manuscript. Below we provide the point-by-point responses. We present the revised results of the reviewer comments (attached). All modifications in the manuscript have been highlighted in blue.

No	Suggestion Reviewer 2	Response from author
1	The authors required provide some context for why the authors chose to concentrate the research research on leaves and fruits? not roots or stems of flowers?	Thanks for your comment. This study is a continuation of the previous one as part of a series of research roadmaps, there has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity, originating from two different locations. We have added and explained in page 2, line 52-60.
2	Add citation	Thank you for your nice reminder. Revised accordingly
3	How many *R. tomentosas* are there in Kalimantan? Is it plentiful? For this reason, there is a chance that this plant will be utilized in the creation of natural medication.	This study wanted to find parts of the karamunting plant as antioxidants other than the ripe fruit. This is because the karamunting plant is a wild plant, the existence of this plant was once widely found, but now it is increasingly difficult to find because of the conversion of bush land into housing and offices in South Kalimantan, as well as the many obstacles to obtaining ripe karamunting fruit. The next study of our research roadmap is the utilization of the most potential parts as antioxidants for nutraceutical products.
4	The author needs to describe the image, including how old the fruits and leaves are. derived from batola or banjar?	Thank you for your nice reminder. Sample criteria are described on page 2 line 68-70, as a reference for fruit samples, Munsell Color Charts for plant tissue color were used (Wilde, 1977). Revised accordingly
5	Is the ethanol solution meant here?	Thank you for your correction. Revised accordingly
6	Does this table's yield results and DPPH results correspond with each other? If any, they are explainable.	Thank you for the comment. The yield shows the amount of chemical compounds contained in the extract. The yield results in the samples in both regions with antioxidant capacity were not subjected to statistical analysis. Differences in yield values existed between leaf and fruit samples, but showed no different values in the

		two regions. the explanation on page 3 lines 131-136.
7	Each paragraph should have a complete element, such as numerous explanation sentences, and each paragraph should only have one topic phrase.	Thank you for the suggestion. We have added discussion as suggested by the reviewer in the highlighted manuscript. These can be found in the page 3, Line 181-188.
8	Font italic for R tomentosa	Thank you for your nice reminder. Revised accordingly
9	Note : In the discussion section, the author has to provide a brief description of the conditions found in the regions of Banjar and Batola, including details about the soil, water/climate, and the author's aim for collecting samples from these areas. Additionally, the author can discuss and compare the results of the antioxidant activity observed in organs like leaves and fruit obtained from both locations.	Thank you for the suggestion. We have added discussion as suggested by the reviewer in the highlighted manuscript. These can be found in the page 3 line 189-198 and page 6 line 258-290

Again thank you for your kind support and consideration to our manuscript.

Regards,

Dr. Evi Mintowati Kuntorini

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Again thank you for your kind support and consideration to our manuscript.

Sincerely,

Evi Mintowati Kuntorini
Email : evimintowati@ulm.ac.id

Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds (Hamid et al., ...2017). In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of *R. tomentosa*. The main components of *R. tomentosa* include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential

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(Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) previously reported the most significant antioxidant activity in the green fruits from Banjarbaru, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. This study is a continuation of the previous one as part of a series of research roadmaps, there has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity, originating from two different locations. ~~No comprehensive investigation has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations.~~ This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

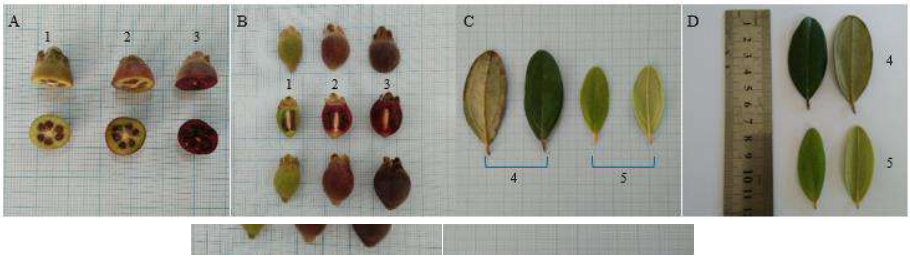

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover,

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the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an ethanol ~~aqueous~~ solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Extraction using solvents is one way to withdraw the active ingredients of an extract. The success of the extraction process is very closely related to the yield, quality and content of active compounds produced. The higher the yield value produced indicates the more value of the extract produced. Ethanol was effective to extract sterol, flavonoid, phenolic, and alkaloid (Wardani et al. 2019). The yield indicates the amount of chemical compounds contained in the extract. The results of the yield in the samples in the two regions with antioxidant capacity were not subjected to statistical analysis. Differences in yield values were found between leaf and fruit samples, but showed no different values in the two regions.

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^{d}	1429.53 ± 9.07^{d}
Old leaves	12.296	11.49	836.20 ± 9.07^{c}	881.24 ± 5.94^{c}

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Green fruits	5.63	6.05	2360.35 ± 6.86^{e}	2795.33 ± 9.07^{e}
Red fruits	9.85	7.76	415.06 ± 2.99^{b}	443.47 ± 2.06^{b}
Purple fruits	14.35	15.24	260.58 ± 0.91^{a}	364.05 ± 3.82^{a}

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the antioxidant capacity results in the two locations were different, where the antioxidant capacity of Batola district was more significant than that of Banjar district. Compared to Banjar District, the high antioxidant capacity of Batola District is due to the higher content of phenolics and flavonoids (aromatic compounds) based on the NMR analysis results in Figures 2A and 2B. Ethanol extracts of green fruits and young leaves showed higher relative concentrations of aromatic compounds. Antioxidant capacity test results (Table. 1) showed greater antioxidant capacity in green fruits and young leaves compared to red and purple fruits at both sample sites, with Batola showing higher antioxidant capacity and spectral signal intensity compared to Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples, as reported by Zargoosh et al. (2019).

The difference in the results of antioxidant capacity in the two regions shows that there are factors that affect the content of compounds in plants. The environmental conditions of the two areas are relatively the same such as temperature, soil pH and humidity. The selection of Batola and Banjar regions to find alternative *R. tometosa* plants around the Banjarbaru area, continuing the results of previous studies.

The effect of habitat on the amount of secondary metabolites in different herbs has been studied previously. the role of habitat has been emphasized as a factor affecting the quantity and accumulation of secondary metabolites. The location a plant grows can affect the process of producing effective substances due to temperature and humidity changes. The mechanisms underlying environmental effects on the accumulation of secondary metabolites is not properly understood. However, the environment influences the type and number of chemical reactions through its effect on the process of metabolite production and factors associated with the production process (eg. enzymes) (Zargoosh et al. (2019).

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~~In general, the differences observed in antioxidant activity between the two locations were attributed to variations in total phenolic and flavonoid content, as Batola Regency samples showed a higher antioxidant capacity than those from Banjar Regency, which was in line with Zargoosh et al. (2019).~~

The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

The chemical properties of *R. tomentosa* ethanol extract *in* leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

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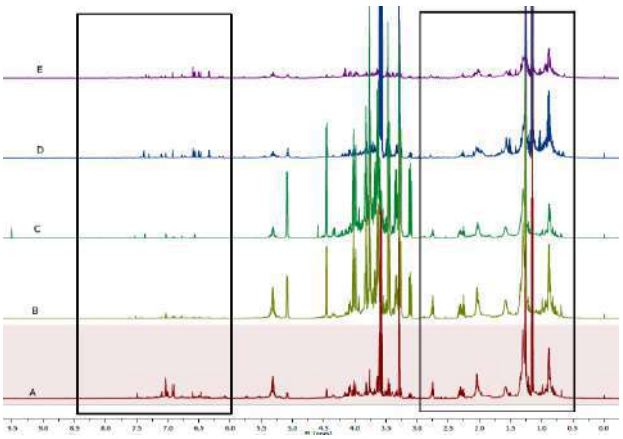

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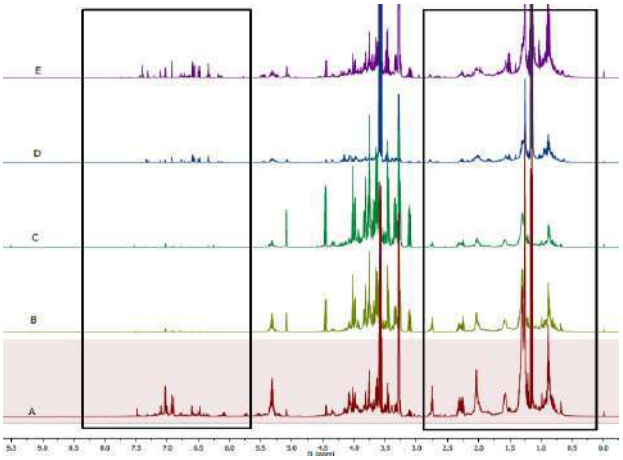

Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Amino Acids		
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Organic Acids		
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Sugars		
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics Compounds		
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
Other compounds		
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits

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contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

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ACKNOWLEDGMENTS

The authors express their gratitude to the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia for providing financial support for this research endeavor (Number: 130/E5/PG.02.00.PL/2023).

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds (Hamid et al., 2017). In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of *R. tomentosa*. The main components of *R. tomentosa* include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et

al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) previously reported the most significant antioxidant activity in the green fruits from Banjarbaru, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. This study is a continuation of the previous one as part of a series of research roadmaps, there has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity, originating from two different locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

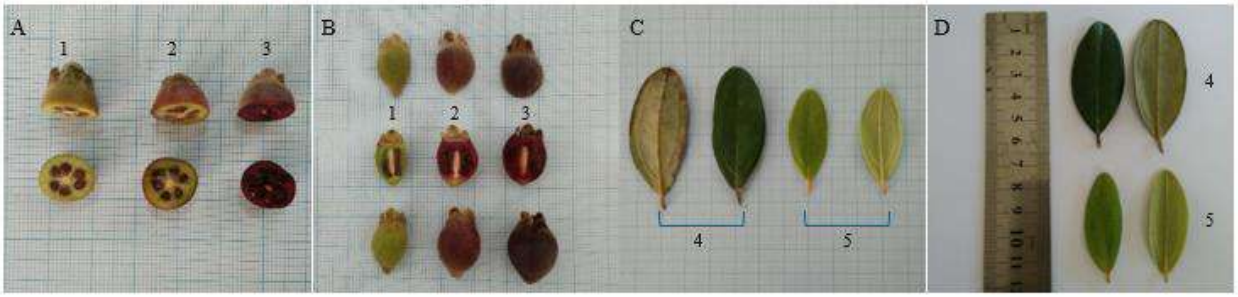

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at

2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an ethanol solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Extraction using solvents is one way to withdraw the active ingredients of an extract. The success of the extraction process is very closely related to the yield, quality and content of active compounds produced. The higher the yield value produced indicates the more value of the extract produced. Ethanol was effective to extract sterol, flavonoid, phenolic, and alkaloid (Wardani et al. 2019). The yield indicates the amount of chemical compounds contained in the extract. The results of the yield in the samples in the two regions with antioxidant capacity were not subjected to statistical analysis. Differences in yield values were found between leaf and fruit samples, but showed no different values in the two regions.

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^{d}	1429.53 ± 9.07^{d}
Old leaves	12.296	11.49	836.20 ± 9.07^{c}	881.24 ± 5.94^{c}
Green fruits	5.63	6.05	2360.35 ± 6.86^{e}	2795.33 ± 9.07^{e}
Red fruits	9.85	7.76	415.06 ± 2.99^{b}	443.47 ± 2.06^{b}
Purple fruits	14.35	15.24	260.58 ± 0.91^{a}	364.05 ± 3.82^{a}

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the antioxidant capacity results in the two locations were different, where the antioxidant capacity of Batola district was more significant than that of Banjar district. Compared to Banjar District, the high antioxidant capacity of Batola District is due to the higher content of phenolics and flavonoids (aromatic compounds) based on the NMR analysis results in Figures 2A and 2B. Ethanol extracts of green fruits and young leaves showed higher relative concentrations of aromatic compounds. Antioxidant capacity test results (Table. 1) showed greater antioxidant capacity in green fruits and young leaves compared to red and purple fruits at both sample sites, with Batola showing higher antioxidant capacity and spectral signal intensity compared to Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples, as reported by Zargoosh et al. (2019).

The difference in the results of antioxidant capacity in the two regions shows that there are factors that affect the content of compounds in plants. The environmental conditions of the two areas are relatively the same such as temperature, soil pH and humidity. The selection of Batola and Banjar regions to find alternative *R. tometosa* plants around the Banjarbaru area, continuing the results of previous studies.

The effect of habitat on the amount of secondary metabolites in different herbs has been studied previously. the role of habitat has been emphasized as a factor affecting the quantity and accumulation of secondary metabolites. The location a plant grows can affect the process of producing effective substances due to temperature and humidity changes. The mechanisms underlying environmental effects on the accumulation of secondary metabolites is not properly understood. However, the environment influences the type and number of chemical reactions through its effect on the process of metabolite production and factors associated with the production process (eg. enzymes) (Zargoosh et al. (2019).

The study by Ene-Obong et al. (2018) on *Monodora myristica,* using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii,* with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et

al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

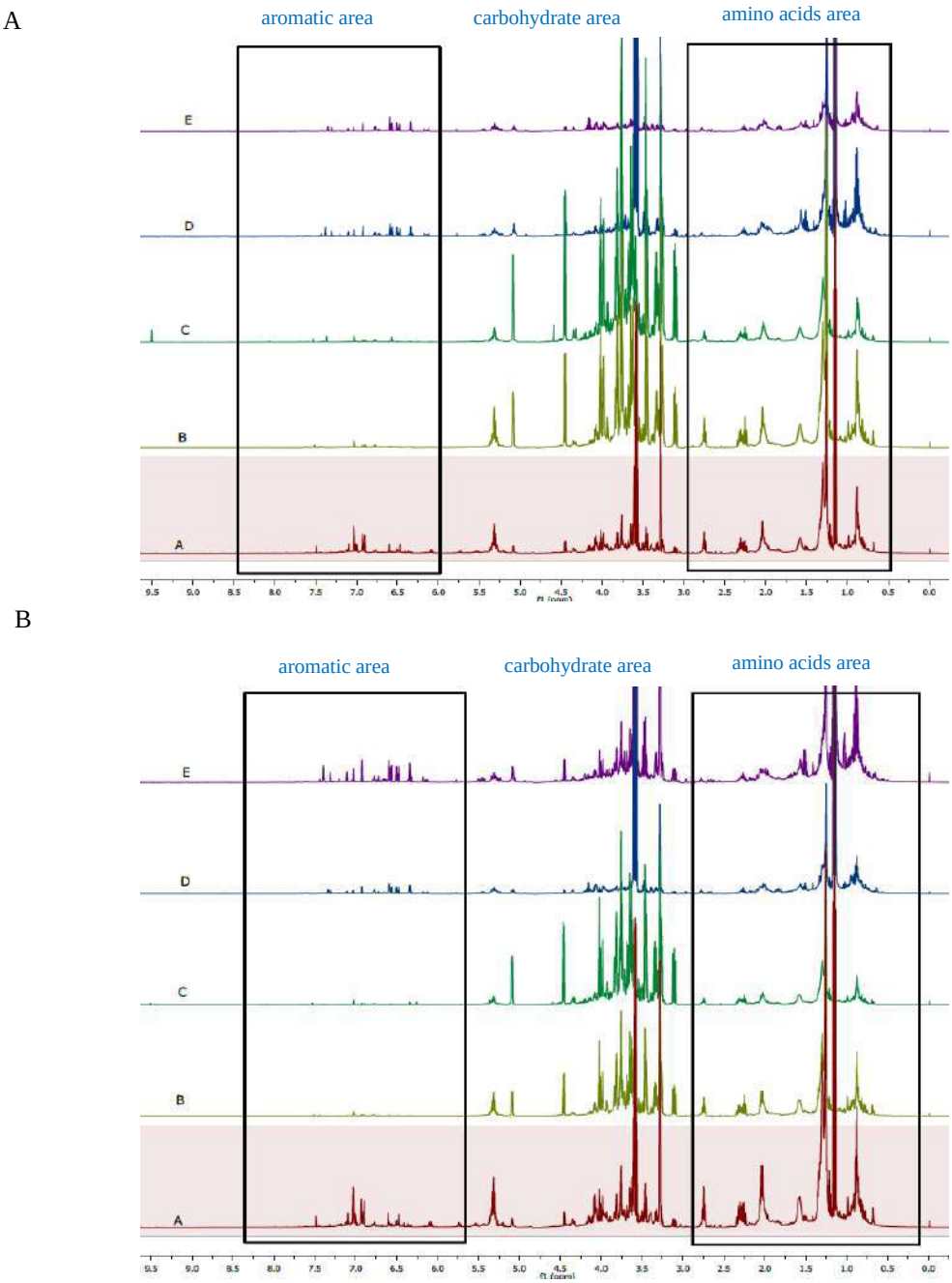

Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Amino Acids		
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Organic Acids		
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Sugars		
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics Compounds		
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018

Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
Other compounds		
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

ACKNOWLEDGMENTS

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11. Reviewer 2's response to the revised manuscript (27-3-2024)

UNIVERSITAS LAMBUNG MANGKURAT

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

Invitation to review for manuscript

Dr. Whika Febria Dewatisari , S.Si., M.Si <whika@ecampus.ut.ac.id> Wed, Mar 27, 2024 at 10:07 AM
To: Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>

Dear Dr. Evi Mintowati Kuntorini,

I hope this email finds you well. I am pleased to inform you that the revisions made have effectively addressed the concerns and suggestions I provided as a reviewer.

I would like to express my appreciation for your receptiveness to my suggestions and your prompt action in implementing the necessary changes. The manuscript is well written and attested.

Thank you for the opportunity to review your manuscript, and I wish you the best of luck with its submission.

Regards,

Whika Febria Dewatisari

Dari: Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>
Dikirim: Selasa, 26 Maret 2024 12.39
Kepada: Dr. Whika Febria Dewatisari , S.Si., M.Si <whika@ecampus.ut.ac.id>
Subjek: Re: Invitation to review for manuscript

[Quoted text hidden]

12. Author's response for reviewer 2nd (27-3-2024)

UNIVERSITAS LAMBUNG MANGKURAT

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

Invitation to review for manuscript

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Wed, Mar 27, 2024 at 11:18 AM
To: "Dr. Whika Febria Dewatisari , S.Si., M.Si" <whika@ecampus.ut.ac.id>

Dear Dr Whika Febria Dewatisari, M.Sc.

I would like to acknowledge the invaluable contributions in improving our manuscript.
Thank you very much for your time and feedback.

Regards,

Dr. Evi Mintowati Kuntorini

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13. Invitation to review for manuscript reviewer 3th (12-2-2024)

UNIVERSITAS LAMBUNG MANGKURAT

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

Invitation to review for manuscript

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Mon, Feb 12, 2024 at 11:29 AM
To: siti hamidatul aliyah <sitihamidatula@gmail.com>

Dear Dr. Siti Hamidatul 'Aliyah

My submission to Biodiversitas Journal of Biological Diversity, "Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa (Ait.) Hassk leaves and fruits from South Borneo" need "own-review" by sending my paper to at least two reviewers.

I would like to invite you to review the above referenced manuscript submitted, as I believe it falls within your expertise and interest. The abstract and this manuscript is included below.

Please respond to this invitation at your earliest opportunity.
I hope you will be able to review this manuscript.

Thank you in advance for your contribution and time.

Regards,

Dr. Evi Mintowati Kuntorini

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14. Reviewer 3's response to reviewer invitation (14-2-2024)
    - Manuscript review

Gmail

Evi M Kuntorini ULM <evimintowati1169@gmail.com>

Manuscript Review Feedback

siti hamidatul aliyah <sitihamidatula@gmail.com> 14 Februari 2024 pukul 11.02
Kepada: evimintowati1169@gmail.com

Dear Dr Evi Mintowati K,

I trust this email finds you well. I am writing to provide feedback on the recent review of the manuscript titled "**Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo**". I appreciate the opportunity to contribute to the peer review process and commend the authors on their valuable research.

Please find attached the detailed feedback document containing my comments and suggestions for improvement. I believe these insights will contribute to enhancing the overall quality and clarity of the manuscript.

If you have any questions or require further clarification on any of the points raised, please feel free to reach out. I am more than willing to discuss any aspect of my review.

Thank you for your attention to this matter, and I look forward to the continued progress of this manuscript.

Best regards,

[Siti Hamidatul 'Aliyah]
[Sekolah Tinggi Ilmu Kesehatan Harapan Ibu, Jambi]
[Department of Pharmacy]

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds. In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of R. tomentosa. The main components of R. tomentosa include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et

al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. No comprehensive investigation has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

Commented [SA1]: The reason for choosing NMR for identification is its ability to provide detailed information about the chemical structure of compounds present in the sample?

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at

2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an aqueous solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^d	1429.53 ± 9.07^d
Old leaves	12.296	11.49	836.20 ± 9.07^c	881.24 ± 5.94^c
Green fruits	5.63	6.05	2360.35 ± 6.86^e	2795.33 ± 9.07^e
Red fruits	9.85	7.76	415.06 ± 2.99^b	443.47 ± 2.06^b
Purple fruits	14.35	15.24	260.58 ± 0.91^a	364.05 ± 3.82^a

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the differences observed in antioxidant activity between the two locations were attributed to variations in total phenolic and flavonoid content, as Batola Regency samples showed a higher antioxidant capacity than those from Banjar Regency, which was in line with Zargoosh et al. (2019).

The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

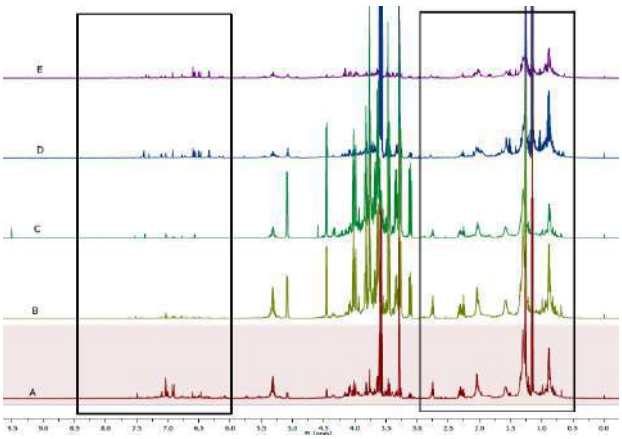
The chemical properties of R. tomentosa ethanol extract *in* leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

Commented [SA2]: How can the differences in antioxidant activity among R. tomentosa samples from both locations be interpreted, and what factors may influence them?

Commented [SA3]: What is the relationship between phenolic and flavonoid content and the antioxidant activity of R. tomentosa extracts, based on the provided results?

Commented [SA4]: Is there potential for further research to better understand the antioxidant properties of R. tomentosa extracts, and how could this contribute to the understanding of its health benefits?

A



B

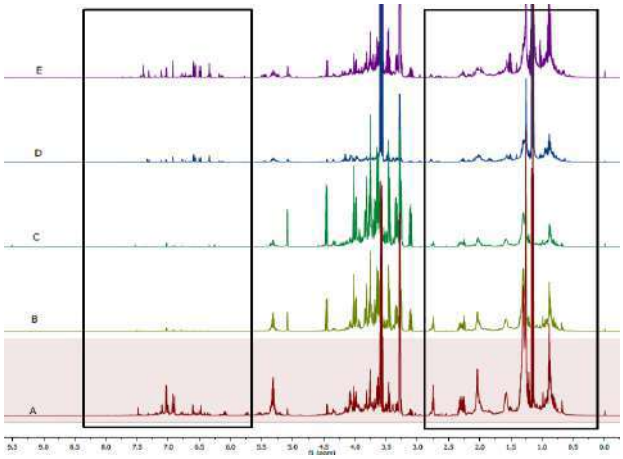


Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

Commented [SA5]: Could you explain in more detail what the numbers at the bottom indicate and what their relationship is to the peaks?

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Commented [SA6]: What differences were observed in the aromatic region of the NMR spectra between young and old leaves, as well as green and mature fruits?

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

Commented [SA7]: What are the implications of the differences in spectral signal intensity among green, red, and purple fruits in the 1H NMR analysis regarding the antioxidant potential of Rhodomyrtus tomentosa?

quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

Commented [SA8]: Based on the results, do all parts of the plant have the potential as antioxidants or which one is the most potential?

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25 Maret 2024 pukul 09.23

Dear Dr. Siti Hamidatul ‘Aliyah, M.Sc.

Thank you for reviewing our manuscript. We are glad to receive the positive comments from the reviewer and we believe that these will improve the quality of our manuscript. Below we provide the point-by-point responses. We present the revised results of the reviewer comments (attached). All modifications in the manuscript have been highlighted in blue.

No	Suggestion Reviewer 2	Response from author
1	The reason for choosing NMR for identification is its ability to provide detailed information about the chemical structure of compounds present in the sample?	Thank you for your question. The reason for choosing NMR is to identify compounds that function as antioxidants in the sample, because NMR methods effectively show the suitability and effectiveness in analyzing plant metabolite profiles. Compounds that are antioxidants in the sample are identified based on NMR spectra. We have explained in page 2, line 61-62.
2	How can the differences in antioxidant activity among *R. tomentosa* samples from both locations be interpreted, and what factors may influence them?	Thank you for the comment. We have added discussion as suggested by the reviewer in the highlighted manuscript. (page 4, line 175-182 and page 7 line 266-274)
3	What is the relationship between phenolic and flavonoid content and the antioxidant activity of *R. tomentosa* extracts, based on the provided results?	Thank you for the comment. We have added discussion as suggested by the reviewer in the highlighted manuscript. (page 4, line 175-182 and page 7 line 266-274)
4	Is there potential for further research to better understand the antioxidant properties of R. tomentosa extracts, and how could this contribute to the understanding of its health benefits?	Thank you for the comment. The antioxidant properties of *R. tomentosa* fruits and leaves extracts with different maturities can be seen from the results of compound profiles through NMR analysis. NMR method is able to produce qualitative and quantitative data simultaneously, because the signal intensity is directly proportional to the molar concentration of the compound (page 7 line 266-271). Variations in intensity were identified in the aromatic shift (6.5-7.5 ppm) detected in leaves and fruits from both sites. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple colored fruits (Figure 2), consistent with the antioxidant activity obtained (Table 1). Important flavonoid compounds identified including gallic acid,

		myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2) were antioxidant in nature. Young leaves and green fruits showed significant relative concentrations of these compounds (Figure 2), corresponding to high antioxidant capacity. This makes green fruits and young leaves usable as antioxidant material in addition to red fruits due to their flavonoid content, which we explain on page 6 lines 245-263. Further research from our research roadmap is the utilization of the most potential parts as antioxidants for nutraceutical products.
5	Could you explain in more detail what the numbers at the bottom indicate and what their relationship is to the peaks?	Thank you for the suggestion. We have added a caption to the figure of ^{1}H NMR spectra divided into three regions based on chemical shifts (figure 2) and the explanation on page 6 lines 232-248.
6	What differences were observed in the aromatic region of the NMR spectra between young and old leaves, as well as green and mature fruits?	Thank you for your question. Intensity variations were identified in the aromatic shift (6.5-7.5 ppm) detected in the leaves and fruits from both sites. Higher intensities were recorded in young leaves and green colored fruits compared to older leaves and red and purple colored fruits (Figure 2), consistent with the antioxidant activity obtained (Tabel 1). Important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). the explanation on page 6 lines 245-253.
7	What are the implications of the differences in spectral signal intensity among green, red, and purple fruits in the 1H NMR analysis regarding the antioxidant potential of Rhodomyrtus tomentosa?	Thank you for your question. The implication of the difference in spectral signal intensity among green, red, and purple fruits in ^{1}HNMR analysis shows that green fruits have higher spectral peak intensity in the aromatic area which indicates high relative concentration of flavonoids and phenol compounds (gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid) and correlates with high antioxidant capacity values in green fruits (page 5 line 238-240). Purple and red fruits had higher peak intensities in the carbohydrate and amino acid areas. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars (page 7 line 260-274).
8	Based on the results, do all parts of the plant have the potential as antioxidants or which one is the most potential?	These results indicate that leaves and fruits have potential as antioxidants, but young leaves and green fruits have the most potential compared to other parts, considering that *R.*

		tomentosa is a wild plant and it is difficult to obtain ripe fruits in large quantities as antioxidant material (page 7 line 286-291).

Again thank you for your kind support and consideration to our manuscript.

Regards,

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Again thank you for your kind support and consideration to our manuscript.

Sincerely,

Evi Mintowati Kuntorini
Email : evimintowati@ulm.ac.id

Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds. In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of R. tomentosa. The main components of R. tomentosa include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et

al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. No comprehensive investigation has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. The study effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

Commented [SA1]: The reason for choosing NMR for identification is its ability to provide detailed information about the chemical structure of compounds present in the sample?

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at

2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an aqueous solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^d	1429.53 ± 9.07^d
Old leaves	12.296	11.49	836.20 ± 9.07^c	881.24 ± 5.94^c
Green fruits	5.63	6.05	2360.35 ± 6.86^e	2795.33 ± 9.07^e
Red fruits	9.85	7.76	415.06 ± 2.99^b	443.47 ± 2.06^b
Purple fruits	14.35	15.24	260.58 ± 0.91^a	364.05 ± 3.82^a

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the antioxidant capacity results in the two locations were different, where the antioxidant capacity of Batola district was more significant than that of Banjar district. Compared to Banjar District, the high antioxidant capacity of Batola District is due to the higher content of phenolics and flavonoids (aromatic compounds) based on the NMR analysis results in Figures 2A and 2B. Ethanol extracts of green fruits and young leaves showed higher relative concentrations of aromatic compounds. Antioxidant capacity test results (Table. 1) showed greater antioxidant capacity in green fruits and young leaves compared to red and purple fruits at both sample sites, with Batola showing higher antioxidant capacity and spectral signal intensity compared to Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples, as reported by Zargoosh et al. (2019).

~~In general, the differences observed in antioxidant activity between the two locations were attributed to variations in total phenolic and flavonoid content, as Batola Regency samples showed a higher antioxidant capacity than those from Banjar Regency, which was in line with Zargoosh et al.~~ (2019).

The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

The chemical properties of R. tomentosa ethanol extract *in* leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation

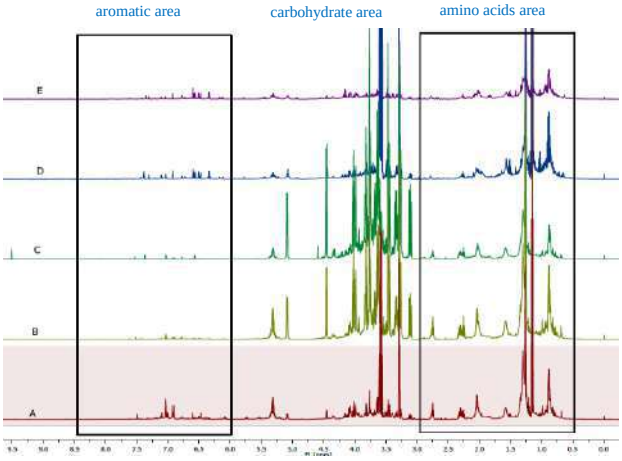
Commented [SA2]: How can the differences in antioxidant activity among R. tomentosa samples from both locations be interpreted, and what factors may influence them?

Commented [SA3]: What is the relationship between phenolic and flavonoid content and the antioxidant activity of R. tomentosa extracts, based on the provided results?

Commented [SA4]: Is there potential for further research to better understand the antioxidant properties of R. tomentosa extracts, and how could this contribute to the understanding of its health benefits?

process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

A



B

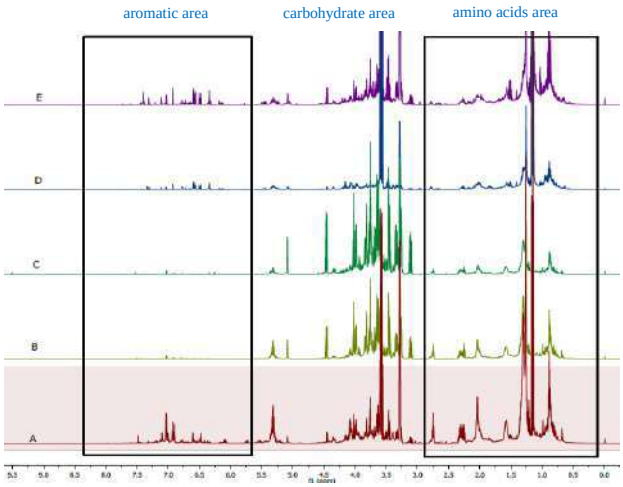


Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

Commented [SA5]: Could you explain in more detail what the numbers at the bottom indicate and what their relationship is to the peaks?

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali

et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves and green fruits showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Commented [SA6]: What differences were observed in the aromatic region of the NMR spectra between young and old leaves, as well as green and mature fruits?

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Amino Acids		
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Organic Acids		
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Sugars		
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics Compounds		
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
Other compounds		
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

Commented [SA7]: What are the implications of the differences in spectral signal intensity among green, red, and purple fruits in the 1H NMR analysis regarding the antioxidant potential of Rhodomyrtus tomentosa?

Commented [SA8]: Based on the results, do all parts of the plant have the potential as antioxidants or which one is the most potential?

ACKNOWLEDGMENTS

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16. Reviewer 3's response to the revised manuscript (25-3-2024)

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Evi M Kuntorini ULM <evimintowati1169@gmail.com>

Manuscript Review Feedback

siti hamidatul aliyah <sitihamidatula@gmail.com> 25 Maret 2024 pukul 14.19
Kepada: Evi M Kuntorini ULM <evimintowati1169@gmail.com>

Dear Dr. Evi M Kuntorini, M.Si,

I have read the manuscript that has been revised, and there is nothing else to be fixed. Thank you for your trust.
May you always be safe and healthy

Best regard,
Siti Hamidatul 'Aliyah
[Kutipan teks disembunyikan]

17. Author's response for reviewer 3th (25-3-2024)

Gmail **Evi M Kuntorini ULM <evimintowati1169@gmail.com>**

Manuscript Review Feedback

Evi M Kuntorini ULM <evimintowati1169@gmail.com> 25 Maret 2024 pukul 18.26
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Dear Dr. Siti Hamidatul 'Aliyah, M.Sc.

We would like to acknowledge the invaluable contributions in improving our manuscript.
Thank you very much for your time and feedback.

Regards,

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[Kutipan teks disembunyikan]

18. Revision Resubmitted to Biodiversitas Journal (2-4-2024)

- Cover Letter Revision
- Manuscript commented by reviewer-2 (include: name and email address),
- Manuscript commented by reviewer-3 (include: name and email address),
- Manuscript proofreading tracked
- certificate of proofreading
- final revised manuscript after proofreading

UNIVERSITAS LAMBUNG MANGKURAT

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

[biodiv] Editor Decision

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Tue, Apr 2, 2024 at 12:39 AM
To: Smujo Editors <editors@smujo.id>

Dear Editors Biodiversitas Journal of Biological Diversity

I would like to submit the review results of the 2 reviewers as well as the necessary documents as requested by the Smujo editors. The following 5 documents i.e.:
1. paper commented by reviewer-1 (include: name and email address),
2. paper commented by reviewer-2 (include: name and email address),
3. table of response
4. certificate of proofreading
5. final revised paper after proofreading.

Regards,

Dr. Evi Mintowati Kuntorini

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On Mon, Jan 1, 2024 at 7:13 AM Smujo Editors via SMUJO <support@smujo.com> wrote:
[Quoted text hidden]

5 attachments

1. U-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa (Commented Reviewer 1).doc
4088K

2. U-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa. (Commented Reviewer 2).doc
4100K

3. Cover Letter Revision (Table of response).docx
20K

4. Certificate proofreading.pdf
75K

5. final revised paper after proofreading_U-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa_Revision 2.doc
652K

Dear Editors Biodiversitas Journal of Biological Diversity

I would like to express my appreciation for the constructive comments and direction we received during the peer review process. We have carefully addressed all of the reviewers' concerns and suggestions, and we feel the revised work has been greatly enhanced as a result. We are glad to receive the positive comments from the reviewer and we believe that these will improve the quality of our manuscript.

Please find below our responses to the comments and recommendations given by both reviewers. We believe that these changes have substantially improved the quality and rigor of our paper. We respectfully request that our revised article be considered for publication. Please do not hesitate to advise us of any necessary adjustments or clarifications, and we will promptly address them.

Reviwer 1 : Dr. Whika Febria Dewatisari, M.Sc.
Email : whika@ecampus.ut.ac.id

No	Suggestion Reviewer 1	Response from author
1	The authors required provide some context for why the authors chose to concentrate the research research on leaves and fruits? not roots or stems of flowers?	Thanks for your comment. This study is a continuation of the previous one as part of a series of research roadmaps, there has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity, originating from two different locations. We have added and explained in page 2, line 52-60.
2	Add citation	Thank you for your nice reminder. Revised accordingly
3	How many *R. tomentosas* are there in Kalimantan? Is it plentiful? For this reason, there is a chance that this plant will be utilized in the creation of natural medication.	This study wanted to find parts of the karamunting plant as antioxidants other than the ripe fruit. This is because the karamunting plant is a wild plant, the existence of this plant was once widely found, but now it is increasingly difficult to find because of the conversion of bush land into housing and offices in South Kalimantan, as well as the many obstacles to obtaining ripe karamunting fruit. The next study of our research roadmap is the utilization of the most potential parts as antioxidants for nutraceutical products.
4	The author needs to describe the image, including how old the fruits and leaves are. derived from batola or banjar?	Thank you for your nice reminder. Sample criteria are described on page 2 line 68-70, as a reference for fruit samples, Munsell Color Charts for plant tissue color were used (Wilde, 1977). Revised accordingly
5	Is the ethanol solution meant here?	Thank you for your correction. Revised accordingly
6	Does this table's yield results and DPPH results correspond with each other? If any, they are explainable.	Thank you for the comment. The yield shows the amount of chemical compounds contained in the extract. The yield results in

		the samples in both regions with antioxidant capacity were not subjected to statistical analysis. Differences in yield values existed between leaf and fruit samples, but showed no different values in the two regions. the explanation on page 3 lines 131-136.
7	Each paragraph should have a complete element, such as numerous explanation sentences, and each paragraph should only have one topic phrase.	Thank you for the suggestion. We have added discussion as suggested by the reviewer in the highlighted manuscript. These can be found in the page 3, Line 181-188.
8	Font italic for R tomentosa	Thank you for your nice reminder. Revised accordingly
9	Note : In the discussion section, the author has to provide a brief description of the conditions found in the regions of Banjar and Batola, including details about the soil, water/climate, and the author's aim for collecting samples from these areas. Additionally, the author can discuss and compare the results of the antioxidant activity observed in organs like leaves and fruit obtained from both locations.	Thank you for the suggestion. We have added discussion as suggested by the reviewer in the highlighted manuscript. These can be found in the page 3 line 189-198 and page 6 line 258-290

Reviwer 2 : Dr. Siti Hamidatul ‘Aliyah, M.Sc.
Email : sitihamidatula@stikes-hi.ac.id

No	Suggestion Reviewer 2	Response from author
1	The reason for choosing NMR for identification is its ability to provide detailed information about the chemical structure of compounds present in the sample?	Thank you for your question. The reason for choosing NMR is to identify compounds that function as antioxidants in the sample, because NMR methods effectively show the suitability and effectiveness in analyzing plant metabolite profiles. Compounds that are antioxidants in the sample are identified based on NMR spectra. We have explained in page 2, line 61-62.
2	How can the differences in antioxidant activity among *R. tomentosa* samples from both locations be interpreted, and what factors may influence them?	Thank you for the comment. We have added discussion as suggested by the reviewer in the highlighted manuscript. (page 4, line 175-182 and page 7 line 266-274)
3	What is the relationship between phenolic and flavonoid content and the antioxidant activity of *R. tomentosa* extracts, based on the provided results?	Thank you for the comment. We have added discussion as suggested by the reviewer in the highlighted manuscript. (page 4, line 175-182 and page 7 line 266-274)

4	Is there potential for further research to better understand the antioxidant properties of *R. tomentosa* extracts, and how could this contribute to the understanding of its health benefits?	Thank you for the comment. The antioxidant properties of *R. tomentosa* fruits and leaves extracts with different maturities can be seen from the results of compound profiles through NMR analysis. NMR method is able to produce qualitative and quantitative data simultaneously, because the signal intensity is directly proportional to the molar concentration of the compound (page 7 line 266-271). Variations in intensity were identified in the aromatic shift (6.5-7.5 ppm) detected in leaves and fruits from both sites. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple colored fruits (Figure 2), consistent with the antioxidant activity obtained (Table 1). Important flavonoid compounds identified including gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2) were antioxidant in nature. Young leaves and green fruits showed significant relative concentrations of these compounds (Figure 2), corresponding to high antioxidant capacity. This makes green fruits and young leaves usable as antioxidant material in addition to red fruits due to their flavonoid content, which we explain on page 6 lines 245-263. Further research from our research roadmap is the utilization of the most potential parts as antioxidants for nutraceutical products.
5	Could you explain in more detail what the numbers at the bottom indicate and what their relationship is to the peaks?	Thank you for the suggestion. We have added a caption to the figure of ^{1}H NMR spectra divided into three regions based on chemical shifts (figure 2) and the explanation on page 6 lines 232-248.
6	What differences were observed in the aromatic region of the NMR spectra between young and old leaves, as well as green and mature fruits?	Thank you for your question. Intensity variations were identified in the aromatic shift (6.5-7.5 ppm) detected in the leaves and fruits from both sites. Higher intensities were recorded in young leaves and green colored fruits compared to older leaves and red and purple colored fruits (Figure 2), consistent with the antioxidant activity obtained (Tabel 1). Important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). the explanation on page 6 lines 245-253.
7	What are the implications of the differences in spectral signal intensity among green, red,	Thank you for your question. The implication of the difference in spectral signal intensity

	and purple fruits in the 1H NMR analysis regarding the antioxidant potential of *Rhodomyrtus tomentosa?*	among green, red, and purple fruits in ^{1}HNMR analysis shows that green fruits have higher spectral peak intensity in the aromatic area which indicates high relative concentration of flavonoids and phenol compounds (gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid) and correlates with high antioxidant capacity values in green fruits (page 5 line 238-240). Purple and red fruits had higher peak intensities in the carbohydrate and amino acid areas. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars (page 7 line 260-274).
8	Based on the results, do all parts of the plant have the potential as antioxidants or which one is the most potential?	These results indicate that leaves and fruits have potential as antioxidants, but young leaves and green fruits have the most potential compared to other parts, considering that *R. tomentosa* is a wild plant and it is difficult to obtain ripe fruits in large quantities as antioxidant material (page 7 line 286-291).

We would also like to acknowledge the invaluable contributions of the reviewers in improving our manuscript. Their feedback has been instrumental in refining our work, and we appreciate the time and effort they dedicated to the review process.

Thank you for your time and consideration. We look forward to hearing from you regarding the status of our manuscript.

Sincerely,

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds (……..). In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of *R. tomentosa*. The main components of R. tomentosa include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et

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Commented [DD2]: the authors required provide some context for why the authors chose to concentrate the research research on leaves and fruits? not roots or stems of flowers?

al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. No comprehensive investigation has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants

Commented [DD3]: How many R. tomentosas are there in Kalimantan? Is it plentiful? For this reason, there is a chance that this plant will be utilized in the creation of natural medication.

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

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Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at

2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an aqueous solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Commented [DD5]: Is the ethanol solution meant here?

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^d	1429.53 ± 9.07^d
Old leaves	12.296	11.49	836.20 ± 9.07^c	881.24 ± 5.94^c
Green fruits	5.63	6.05	2360.35 ± 6.86^e	2795.33 ± 9.07^e
Red fruits	9.85	7.76	415.06 ± 2.99^b	443.47 ± 2.06^b
Purple fruits	14.35	15.24	260.58 ± 0.91^a	364.05 ± 3.82^a

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

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Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the differences observed in antioxidant activity between the two locations were attributed to variations in total phenolic and flavonoid content, as Batola Regency samples showed a higher antioxidant capacity than those from Banjar Regency, which was in line with Zargoosh et al. (2019).

The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

The chemical properties of *R. tomentosa* ethanol extract *in* leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

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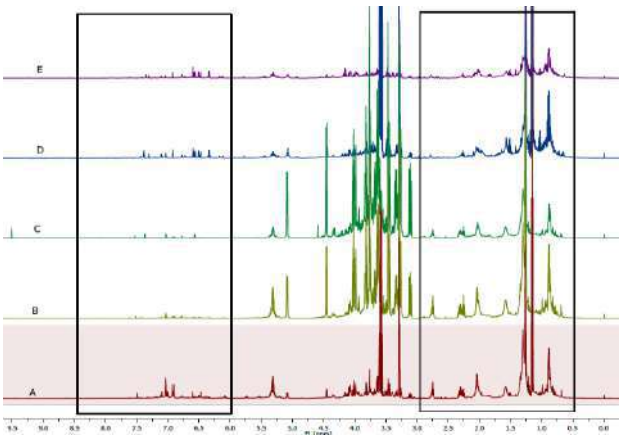
A



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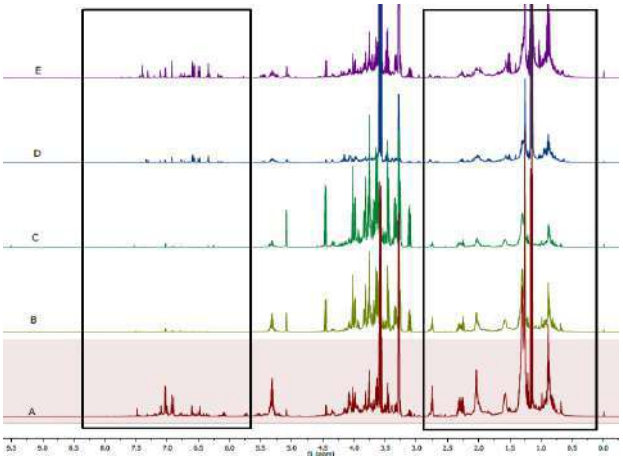

Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

Note : In the discussion section, the author has to provide a brief description of the conditions found in the regions of Banjar and Batola, including details about the soil, water/climate, and the author's aim for collecting samples from these areas. Additionally, the author can discuss and compare the results of the antioxidant activity observed in organs like leaves and fruit obtained from both locations.

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds. In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of R. tomentosa. The main components of R. tomentosa include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et

al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. No comprehensive investigation has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

Commented [SA1]: The reason for choosing NMR for identification is its ability to provide detailed information about the chemical structure of compounds present in the sample?

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at

2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an aqueous solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^d	1429.53 ± 9.07^d
Old leaves	12.296	11.49	836.20 ± 9.07^c	881.24 ± 5.94^c
Green fruits	5.63	6.05	2360.35 ± 6.86^e	2795.33 ± 9.07^e
Red fruits	9.85	7.76	415.06 ± 2.99^b	443.47 ± 2.06^b
Purple fruits	14.35	15.24	260.58 ± 0.91^a	364.05 ± 3.82^a

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the differences observed in antioxidant activity between the two locations were attributed to variations in total phenolic and flavonoid content, as Batola Regency samples showed a higher antioxidant capacity than those from Banjar Regency, which was in line with Zargoosh et al. (2019).

The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

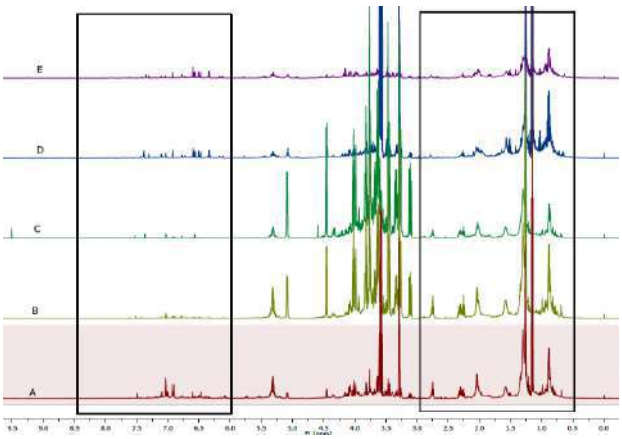
The chemical properties of R. tomentosa ethanol extract *in* leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

Commented [SA2]: How can the differences in antioxidant activity among R. tomentosa samples from both locations be interpreted, and what factors may influence them?

Commented [SA3]: What is the relationship between phenolic and flavonoid content and the antioxidant activity of R. tomentosa extracts, based on the provided results?

Commented [SA4]: Is there potential for further research to better understand the antioxidant properties of R. tomentosa extracts, and how could this contribute to the understanding of its health benefits?

A



B

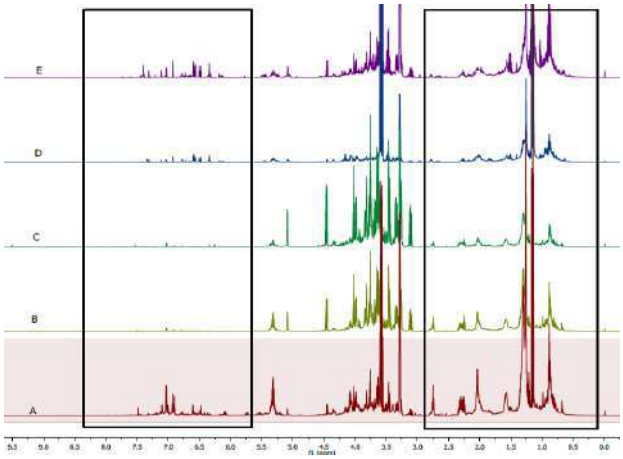


Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

Commented [SA5]: Could you explain in more detail what the numbers at the bottom indicate and what their relationship is to the peaks?

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Commented [SA6]: What differences were observed in the aromatic region of the NMR spectra between young and old leaves, as well as green and mature fruits?

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

Commented [SA7]: What are the implications of the differences in spectral signal intensity among green, red, and purple fruits in the 1H NMR analysis regarding the antioxidant potential of Rhodomyrtus tomentosa?

quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

Commented [SA8]: Based on the results, do all parts of the plant have the potential as antioxidants or which one is the most potential?

ACKNOWLEDGMENTS

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aimed to evaluate and compare antioxidant activity of ethanol extracts from *R. tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaves extract identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidant, such as 4-hexyl-resorcinol, to meet body needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidant derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. It contains phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity to treat various diseases induced by free radicals. As a result, it can become an effective preventive defence strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, diarrhea, stomach pain, gynaecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds. In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-diadrenal medication (Vo and Ngo, 2019). Most of these effects correspond to those observed in traditional uses of R. tomentosa. The main components of R. tomentosa include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et

al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. No comprehensive investigation has been conducted on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

Commented [SA1]: The reason for choosing NMR for identification is its ability to provide detailed information about the chemical structure of compounds present in the sample?

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at

2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations, as presented in Table 1. The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, and green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an aqueous solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered into the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^d	1429.53 ± 9.07^d
Old leaves	12.296	11.49	836.20 ± 9.07^c	881.24 ± 5.94^c
Green fruits	5.63	6.05	2360.35 ± 6.86^e	2795.33 ± 9.07^e
Red fruits	9.85	7.76	415.06 ± 2.99^b	443.47 ± 2.06^b
Purple fruits	14.35	15.24	260.58 ± 0.91^a	364.05 ± 3.82^a

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings displayed ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from R. tomentosa fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the differences observed in antioxidant activity between the two locations were attributed to variations in total phenolic and flavonoid content, as Batola Regency samples showed a higher antioxidant capacity than those from Banjar Regency, which was in line with Zargoosh et al. (2019).

The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidant and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the amount of flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

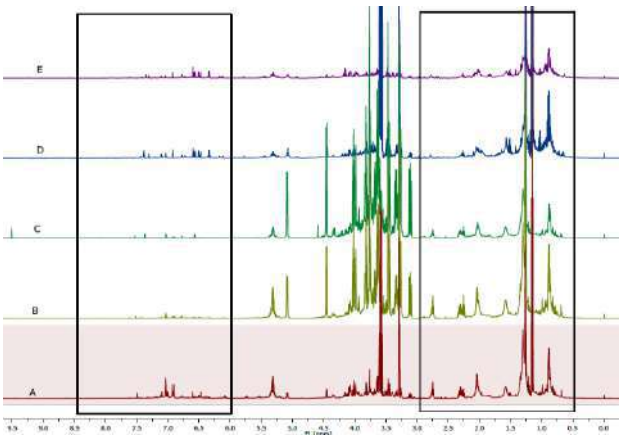
The chemical properties of R. tomentosa ethanol extract *in* leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

Commented [SA2]: How can the differences in antioxidant activity among R. tomentosa samples from both locations be interpreted, and what factors may influence them?

Commented [SA3]: What is the relationship between phenolic and flavonoid content and the antioxidant activity of R. tomentosa extracts, based on the provided results?

Commented [SA4]: Is there potential for further research to better understand the antioxidant properties of R. tomentosa extracts, and how could this contribute to the understanding of its health benefits?

A



B

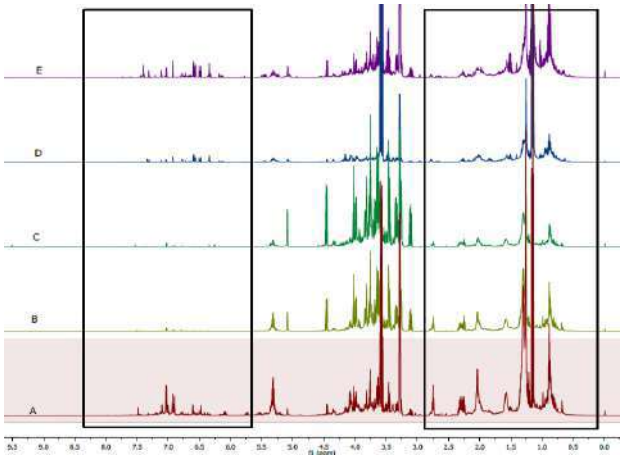


Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

Commented [SA5]: Could you explain in more detail what the numbers at the bottom indicate and what their relationship is to the peaks?

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red, and purple leaves, as depicted in Figures 2A and 2B.

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Commented [SA6]: What differences were observed in the aromatic region of the NMR spectra between young and old leaves, as well as green and mature fruits?

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

Commented [SA7]: What are the implications of the differences in spectral signal intensity among green, red, and purple fruits in the 1H NMR analysis regarding the antioxidant potential of Rhodomyrtus tomentosa?

quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis, as presented in Table 2. The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6-7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies would be needed to determine the role played in nutritional applications.

Commented [SA8]: Based on the results, do all parts of the plant have the potential as antioxidants or which one is the most potential?

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study ~~aimed~~ aims to evaluate and compare the antioxidant activity of ethanol extracts from *R. Tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as old and young, while there were three stages of fruit maturity, including green, red, as well as purple. ^{1}H NMR spectroscopy was used to characterize the extracts' compositional profile, comprising Torganic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, showing Dintensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old ~~leaves extract~~ leaf extracts identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 –_ 6.0 ppm aromatic compound area. The differences in spectral signal intensity showed higher levels of metabolites, specifically carbohydrates and amino acids, in red and purple fruits compared to green fruits which only had greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms which cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidants, such as 4-hexyl-resorcinol, to meet ~~body~~ bodily needs has raised concerns due to the associated detrimental health effects and toxicological implications. Consequently, there is an emphasis on the increasing importance of applying natural antioxidants derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. ~~It contains~~ They may contain phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity ~~to~~ which may treat various diseases induced by free radicals. As a result, ~~it can become~~ they may become part of an effective preventive ~~defence~~ defense strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, ~~diarrhea,~~ stomach pain, gyn~~a~~ecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds (Hamid et al., 2017). In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-~~d~~iadrenal medication (Vo and Ngo, 2019). Most of these effects correspond ~~to~~ with those observed in traditional uses of *R. tomentosa*. The main components of *R. tomentosa* include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

Recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols,

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polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to their exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as their DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) previously reported the most significant antioxidant activity in the green fruits from Banjarbaru, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. This study is a continuation of a ~~the~~ previous one as part of a series of research roadmaps. ~~,~~ ~~T~~there has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity, originating from two different locations. This examination represents an inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. The study effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number ~~of~~ 1007/IPH.1.01/If.07/IX/2023.

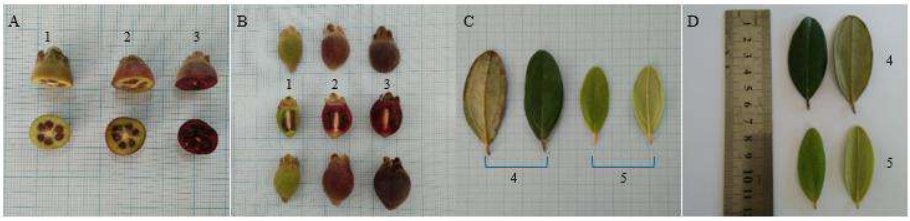

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

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The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations~~, as presented in~~ (Table 1). The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, ~~and~~ whereas green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an ethanol solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid ~~entered into~~entered the cells through diffusion~~,~~ causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Extraction using solvents is one way to withdraw the active ingredients of an extract. The success of the extraction process is very closely related to the yield, quality and content of active compounds produced. The higher the yield value produced indicates the more value of the extract produced. Ethanol was effective ~~to extract~~ in extracting sterol, flavonoid, phenolic~~,~~ and alkaloid (Wardani et al. 2019). The yield indicates the ~~amount~~ number of chemical compounds contained in the extract. The results of the yield in the samples in the two regions with antioxidant capacity were not subjected to statistical analysis. Differences in yield values were found between leaf and fruit samples~~,~~ but showed no different values in the two regions.

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^{d}	1429.53 ± 9.07^{d}
Old leaves	12.296	11.49	836.20 ± 9.07^{c}	881.24 ± 5.94^{c}
Green fruits	5.63	6.05	2360.35 ± 6.86^{e}	2795.33 ± 9.07^{e}
Red fruits	9.85	7.76	415.06 ± 2.99^{b}	443.47 ± 2.06^{b}
Purple fruits	14.35	15.24	260.58 ± 0.91^{a}	364.05 ± 3.82^{a}

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Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments , O— one of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level resulted in a color change from dark purple to pastel pink or yellow, which was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by the DPPH method rang~~ed~~ing from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1~~.~~). The conducted ANOVA followed by an LSD analysis of the ten samples statistically indicated significant differences ($P<0.05$). The findings ~~displayed~~ showed that ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits~~,~~ and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the antioxidant capacity results in the two locations were different, ~~where~~ with the antioxidant capacity of the Batola district ~~was~~ being more significant than that of the Banjar district. Compared to Banjar District, the high antioxidant capacity of Batola District is due to the higher content of phenolics and flavonoids (aromatic compounds) based on the NMR analysis results in Figures 2A and 2B. Ethanol extracts of green fruits and young leaves showed higher relative concentrations of aromatic compounds. Antioxidant capacity test results (Table~~.~~ 1) showed greater antioxidant capacity in green fruits and young leaves compared to red and purple fruits at both sample sites, with Batola showing higher antioxidant capacity and spectral signal intensity compared to Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples, as reported by Zargoosh et al. (2019).

The difference in the results of antioxidant capacity in the two regions shows that there are factors that affect the content of compounds in plants. The environmental conditions of the two areas are relatively the same such as temperature, soil pH and humidity. The selection of Batola and Banjar regions to find alternative *R. tometosa* plants around the Banjarbaru area, continuing the results of previous studies.

The effect of habitat on the amount of secondary metabolites in different herbs has been studied previously.~~ T~~the role of habitat has been emphasized as a factor affecting the quantity and accumulation of secondary metabolites. The location in which a plant grows can affect the process of producing effective substances due to temperature and humidity changes. The mechanisms underlying environmental effects on the accumulation of secondary metabolites ~~is~~are not properly understood. However, the environment influences the type and number of chemical reactions through its effect on the process of metabolite production and factors associated with the production process (eg. Enzymes) (Zargoosh et al. (2019).

The study by ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidants and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. Tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al. (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the

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~~amount of~~ flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were characterized for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures~~,~~ such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

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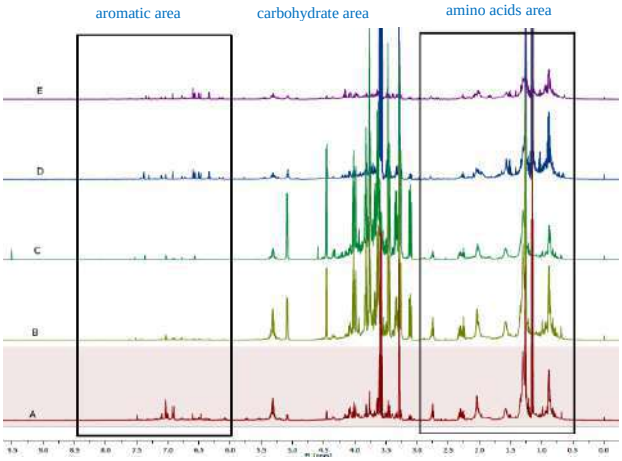

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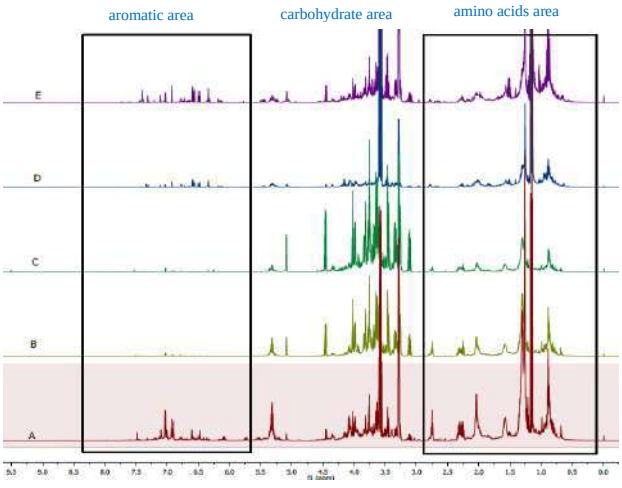

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Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used ~~as a method~~ for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red~~,~~ and purple leaves~~, as depicted in~~ (Figures 2A and 2B).

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Amino Acids		
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Organic Acids		
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Sugars		
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics Compounds	7.03 (s)	Ali et al. 2010

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Gallic acid		
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
Other compounds		
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0 - 3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region, except for glucose and sucrose, limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis~~, as presented in~~ (Table 2). The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6 - 7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaves extract for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites, specifically carbohydrates and amino acids, while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies ~~would be~~ may be needed to determine the role played in nutritional applications.

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Antioxidant activity and ^{1}H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aims to evaluate and compare the antioxidant activity of ethanol extracts from *R. Tomentosa* (Ait.) Hassk leaves and fruits in two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. Leaves were categorized as eitther old and young, whereas there were three stages of fruit maturity, namely green, red and purple. ^{1}H NMR spectroscopy was used to identify the extracts' compositional profiles, which comprised organic acids, carbohydrates, amino acids, as well as main phenolic compounds. Furthermore, antioxidant capacity was assessed with DPPH, which showed intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had greater intensity compared to old leaves, as well as red and purple fruits, correlating with antioxidant capacity results. Analysis of ^{1}H NMR spectra of young and old leaf extracts identified key metabolites, such as gallic acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm aromatic compound area. The differences in spectral signal intensity revealed higher levels of metabolite levels, specifically carbohydrates and amino acids, in red and purple fruits. This is in contrast to green fruits which displayed only greater quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms. These mechanisms cause cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, as well as neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidants, such as 4-hexyl-resorcinol, to meet bodily needs has roused concern due to the associated detrimental health effects and toxicological implications. Consequently, there is an increasing emphasis on the importance of applying natural antioxidants derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. They may contain phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids and alkaloids with considerable antioxidant activity and suitable for health maintenance. Researchers have shown that plant chemical compounds possess antioxidant activity which may treat various diseases induced by free radicals. As a result, they may become part of an effective preventive defense strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with inherent potential for discovering structurally diverse and biologically active compounds (Zhao et al. 2019). In traditional Chinese, Vietnamese and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, stomach pain, gynecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and traditional treatments for women after birth. The locals of Indonesia use the crushed leaves of *R. tomentosa* to treat wounds (Hamid et al., 2017). In Thailand, *R. tomentosa* is used as an anti-inflammatory, anti-diarrheal and anti-dysentery medication (Vo and Ngo, 2019). Most of these effects correspond with those observed in traditional uses of *R. tomentosa*. The main components of *R. tomentosa* include triterpenoids, flavonoids, meroterpenoids of phenols and microelements (Zhao et al. 2019).

There have been recent studies focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). Similar the health benefits, it is used in the production of wines and beverages (Yin et al.,

2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves, flowers, and stems due to their exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as their DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) Previously reported that green fruits from Banjarbaru showed the most significant antioxidant activity, with values of DPPH as well as FRAP at 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is considered a pest due to the rapid growth rate possessed. This study is a continuation of a previous one as part of a series of research roadmaps. There has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity, originating from two different locations. This examination represents an inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant components. The study effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

MATERIALS AND METHODS

Plant materials

Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed samples with certificate number 1007/IPH.1.01/If.07/IX/2023.

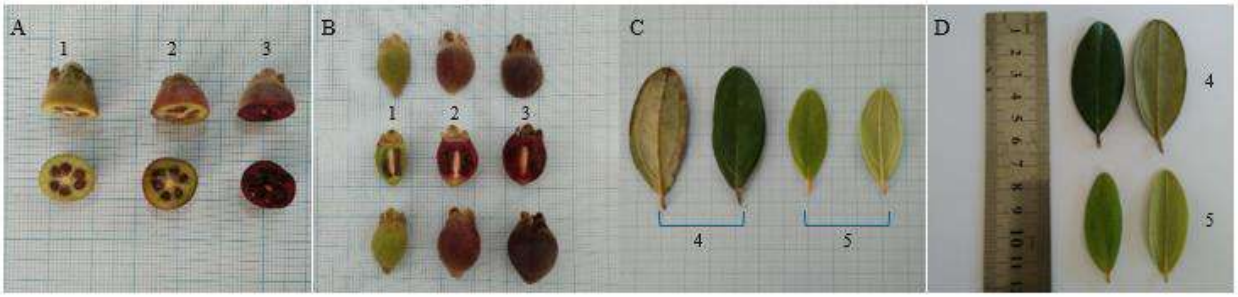

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.

Procedures

Crude ethanol extract preparation

Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500g of each ground material was macerated in ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris as well as dried utilizing a rotary evaporator, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). Moreover, the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol TE/g), using Trolox from Sigma-Aldrich, Germany.

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tubes at

2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, as well as a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral width was measured within the range of 0 to 10 ppm.

Data analysis

Mean values as well as standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and in case of divergent results, post hoc testing was conducted using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, as well as calibration to signals of internal standard solution (TMSP) at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, as well as m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by a comparative analysis of information found in a database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative examination of the signals was performed by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extraction process yielded varying extract weights across samples collected from both locations (Table 1). The ethanol extracts from purple fruits showed the most significant output at 15.24% w/w, whereas green fruits produced the lowest yield, precisely 5.63% w/w.

Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds by circumventing high temperatures. During the experiment, coarse simplisia powder was mixed with an ethanol solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure. Liquid entered the cells through diffusion causing the dissolution of cellular contents due to concentration disparities between the extra and intracellular fluid until a solute concentration equilibrium was attained (Harbone, 1987).

Extraction using solvents is one way to obtain the active ingredients of an extract. The success of the extraction process is very closely related to the yield, quality and content of active compounds produced. The higher the yield value produced indicates the more value of the extract produced. Ethanol was effective in extracting sterol, flavonoid, phenolic and alkaloid (Wardani et al. 2019). The yield indicates the number of chemical compounds contained in the extract. The results of the yield in the samples in the two regions with antioxidant capacity were not subjected to statistical analysis. Differences in yield values were found between leaf and fruit samples but showed no different values in the two regions.

Table 1. Yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^d	1429.53 ± 9.07^d
Old leaves	12.296	11.49	836.20 ± 9.07^c	881.24 ± 5.94^c
Green fruits	5.63	6.05	2360.35 ± 6.86^e	2795.33 ± 9.07^e
Red fruits	9.85	7.76	415.06 ± 2.99^b	443.47 ± 2.06^b
Purple fruits	14.35	15.24	260.58 ± 0.91^a	364.05 ± 3.82^a

Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.

Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments — one of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level resulted in a color change from dark purple to pastel pink or yellow. This was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition**.** Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods. However, the assessment of antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition exerted by antioxidant chemicals against radicals or metal radicals.

R. Tomentosa leaves and fruits show antioxidant capacity as calculated by the DPPH method ranging from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1). The conducted ANOVA followed by an LSD analysis of the ten samples indicated statistically significant differences ($P<0.05$). The findings showed that ethanol extracts possessed similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R. tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

In general, the antioxidant capacity results in the two locations were different, with the antioxidant capacity of the Batola district being more significant than that of the Banjar district. Compared to Banjar District, the high antioxidant capacity of Batola District is due to the higher content of phenolics and flavonoids (aromatic compounds) based on the NMR analysis results in Figures 2A and 2B. Ethanol extracts of green fruits and young leaves showed higher relative concentrations of aromatic compounds. Antioxidant capacity test results (Table 1) showed greater antioxidant capacity in green fruits and young leaves compared to red and purple fruits at both sample sites, with Batola showing higher antioxidant capacity and spectral signal intensity compared to Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples, as reported by Zargoosh et al. (2019).

The difference in the results of antioxidant capacity in the two regions shows that there are factors that affect the content of plant compounds. The environmental conditions of the two areas are relatively the same such as temperature, soil pH and humidity. The selection of Batola and Banjar regions to find alternative *R. tometosa* plants around the Banjarbaru area, continuing the results of previous studies.

The effect of habitat on the amount of secondary metabolites in different herbs has been studied previously. The role of habitat has been emphasized as a factor affecting the quantity and accumulation of secondary metabolites. The location in which a plant grows can affect the process of producing effective substances due to temperature and humidity changes. The mechanisms underlying environmental effects on the accumulation of secondary metabolites are not properly understood. However, the environment influences the type and number of chemical reactions through its effect on the process of metabolite production and factors associated with the production process (eg. Enzymes) (Zargoosh et al. (2019).

The study by ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. This showed that the ability of flavonoids to function as potent antioxidants and free radical scavengers depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. Tomentosa* fruit extract containing high flavonoid concentrations, through various methods including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results obtained for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator has been proven by Idris et al. (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important role as electron donors, chain breakers and free radical catchers in the antioxidant process mechanism. Therefore, the flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds contained by owned by each plant also correlates with its antioxidant activity. This is due to the redox properties that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016).

These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.

Compounds identification in ^{1}H NMR spectra of *R. tomentosa* leaves and fruits

The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were analyzed for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation process and short duration, is significantly advantageous for analyzing complex mixtures such as food extracts. The representative ^{1}H NMR spectra of the examined samples are depicted in Figure 2.

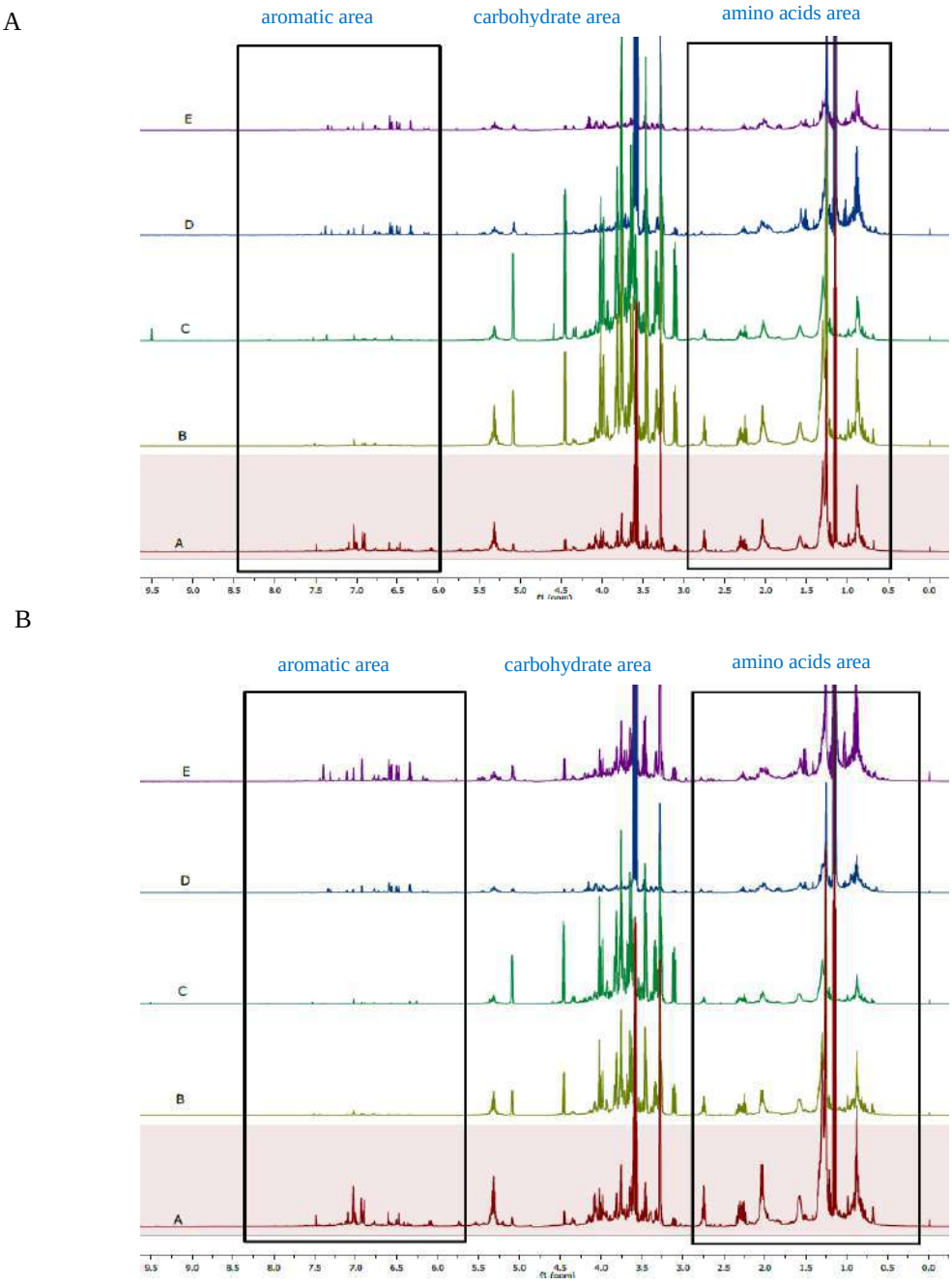

Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves exctract location in Banjar and (b) fruits and leaves exctract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites. This is due to its capability to generate a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used for metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of extract of leaves and fruits obtained from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary and aromatic compounds (chemical shift > 6 ppm) as secondary. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region compared to old, red and purple leaves (Figures 2A and 2B).

Distinct attributes in NMR spectra derived from leaves and fruits were observed in both Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with the obtained antioxidant activity.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Amino Acids		
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Organic Acids		
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Sugars		
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics Compounds		
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
Other compounds		
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. This was consistent with the similar trend reported by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity determined in young and old leaves as well as papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test results (Table 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0 - 3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region (except for glucose and sucrose), limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis (Table 2). The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Moreover, sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the chemical shift of 5.00 - 3.50 ppm. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in the aromatic shift (6 - 7.5 ppm) of leaves and fruits from both regions. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaf extracts for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of metabolites (specifically carbohydrates and amino acids), while green fruits comprised more aromatic compounds. This signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies may be needed to determine the role played in nutritional applications.

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19. Editor Decision : Revision Required from reviewer 4th (22-4-2024)

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ANTIOXIDANT ACTIVITY AND ^{1}H NMR PROFILING OF LEAVES AND FRUITS OF *RHODOMYRTUS TOMENTOSA* (AIT.) HASSK FROM SOUTH KALIMANTAN, INDONESIA

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aims to evaluate and compare the antioxidant activity of leaves and fruits ethanol extracts of *R. tomentosa* (Ait.) Hassk from two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. The samples included old and young leaves and three stages of fruit maturity, namely green, red, and purple. ^{1}H NMR spectroscopy was used to identify the chemical profile of extracts, which comprised organic acids, carbohydrates, amino acids, and phenolic compounds. Antioxidant capacity was assessed with DPPH d, which showed intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had higher intensity than old leaves and red and purple fruits, correlating with antioxidant capacity. Analysis of ^{1}H NMR spectra of young and old leaf extracts identified vital metabolites, such as gallic acid, myricetin 3-O-rhamnopyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm as aromatic compound area. The differences in spectral signal intensity revealed higher metabolite levels, specifically carbohydrates and amino acids, in red and purple fruits. However, green fruits had higher quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms. The oxidative stress causes cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, and neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidants, such as 4-hexyl-resorcinol, to meet the daily requirement of antioxidants has roused concern due to the associated detrimental health effects and toxicological implications. Consequently, there is an increasing emphasis on applying natural antioxidants derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. They may contain phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids, and alkaloids, which have considerable antioxidant activity for maintaining health. Researchers have shown that plant chemical compounds possess antioxidant activity to prevent diseases induced by free radicals. As a result, they may become part of an effective preventive defense strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with diverse structural compounds and biological activities (Zhao et al. 2019). In traditional Chinese, Vietnamese, and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, stomach pain, gynecology, and wound healing. Parts of the roots and trunks are used for stomach diseases and after childbirth. Local Indonesians use the crushed leaves of *R. tomentosa* to treat wounds (Hamid et al., 2017). *R. tomentosa* is an anti-inflammatory, anti-diarrheal, and anti-dysentery medication in Thailand (Vo and Ngo, 2019). ~~Most of these effects correspond with those observed in traditional uses of *R. tomentosa*.~~ The main components of *R. tomentosa* include triterpenoids, flavonoids, meroterpenoids of phenols, and microelements (Zhao et al. 2019).

Recent studies have focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). It is used to produce wines and beverages (Yin et al., 2021). Currently, *R. tomentosa* is mainly studied for the phytochemical components found in leaves, flowers, and stems due to their potent antioxidant, anti-bacterial, and anti-inflammatory activities, as well as the ability to reduce DNA damage (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported previously that green fruits of *R.tomentosa* from

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Banjarbaru showed potent antioxidant activity, with IC_{50} values against DPPH and FRAP were 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves and green fruits, namely 96.375±3.96 and 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite several advantages, this plant is a pest due to its rapid growth. This study continues a previous one as part of a series of research roadmaps. There has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity, ~~originating from two different locations~~. This examination represents an inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves and fruits obtained from two locations, using combined NMR spectroscopy, particularly to identify antioxidant components. The study effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

MATERIALS AND METHODS

Plant materials

Samples of young and mature leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* were collected. Red, purple, and green fruits were also collected (Figure 1). Munsell Color Charts were used as a reference for plant tissue color (Wilde, 1977). The samples were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. The samples were identified and authenticated in the Herbarium Bogoriense, National Research and Innovation Agency, with certificate number 1007/IPH.1.01/If.07/IX/2023.

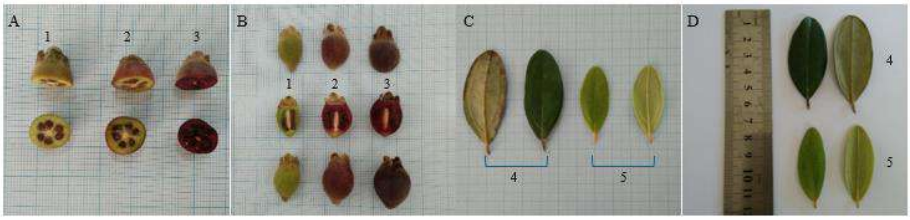

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.

Procedures

Crude ethanol extract preparation

Different ages of leaves were selectively harvested from apical branches, and also fruits were dried at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500g of each ground material was macerated in 1000 ml ethanol (SmartLab, Indonesia) for 72 h. The solvent was replaced every 24 hours (Nurcholis et al. 2021; Kuntorini et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris and dried utilizing a rotary evaporator, then stored in a refrigeration unit for further analysis.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to obtain the appropriate concentration. The sample (2 mL) was added with 2 mL of 0.17 mM DPPH (Sigma-Aldrich, Germany) and then incubated in the dark for 30 mins at ambient temperature. The absorbance of the samples was measured at a wavelength of 516 nm utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). The free radical scavenging activity was quantified in micromoles of Trolox (Sigma-Aldrich, Germany) equivalents per unit of dry weight (DW) (µmol TE/g).

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared using a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tubes at 2 mL with the methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The mixture was vortexed and sonication for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was transferred to the NMR tube for subsequent examination using ^{1}H NMR.

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^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, and a pre-saturation mode set at 4.27 ppm. An internal lock was also established using deuterated solvent, and the spectral width was measured from 0 to 10 ppm.

Data analysis

Mean values and standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and significant differences among treatments were analyzed further using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, and calibration to internal standard solution (TMSP) signals at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, and m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by comparing with database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative signals were examined by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extract yields were varied (Table 1): the highest was obtained from purple fruits (15.24% w/w), whereas green fruits produced the lowest yield (5.63% w/w). Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds without heating. ~~During the experiment, coarse simplisia powder was mixed with an ethanol solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure~~. In the maceration process, the solvent entered the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extracellular and intracellular fluid until a solute concentration equilibrium was obtained (Harbone, 1987).

The solvent used in the extraction process is to extract the active ingredients. The higher the yield produced indicates, the more value of the extract produced. Ethanol effectively extracted sterol, flavonoid, phenolic, and alkaloid (Wardani et al. 2019). The yield indicates the amount of extracted chemical compounds. The yield results in the samples in the two regions with antioxidant capacity were not subjected to statistical analysis. Differences in yield were found between leaf and fruit samples but showed no different values in the two areas.

Table 1. Extract yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Extract Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^{d}	1429.53 ± 9.07^{d}
Old leaves	12.296	11.49	836.20 ± 9.07^{c}	881.24 ± 5.94^{c}
Green fruits	5.63	6.05	2360.35 ± 6.86^{e}	2795.33 ± 9.07^{e}
Red fruits	9.85	7.76	415.06 ± 2.99^{b}	443.47 ± 2.06^{b}
Purple fruits	14.35	15.24	260.58 ± 0.91^{a}	364.05 ± 3.82^{a}

Note: The data represent the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by different superscript letters in the same column are significantly different.

Antioxidants bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging healthy cells. Its activity can be measured by several in vitro experiments — one of the most simple, rapid, and widespread DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples were rich in hydroxyl compounds that can reduce DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level resulted in a color change from dark purple to pink or yellow. This was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per

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gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition. Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. ~~This study aimed to compare antioxidant capacity calculated in Trolox equivalents without using diverse methods.~~ However, assessing antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition of antioxidant compounds against radicals or metal radicals.

The antioxidant capacity of *R. tomentosa* leaves and fruits ranges from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1). ~~The conducted ANOVA followed by an LSD analysis of the ten samples indicated statistically significant differences (P<0.05).~~ The findings showed that although from different locations, ethanol extracts possessed similar antioxidant capacity. The ethanol extract of green fruits' had the highest DPPH radical scavenging capacity, i.e., with respective values of 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, i.e., 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were lower than those reported by Lai et al. 2015, i.e., 431.17±14.5 µmol TE/g. These appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the fruit extract of *R. tomentosa* had potent antioxidant properties due to phenol compounds. Purified extracts of anthocyanin from *R. tomentosa* fruits have strong antioxidant activity, including the ability to scavenge DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

The antioxidant capacity of *R.tomentosa* extract from the two locations differed, with the extract's antioxidant capacity from the Batola district being higher than that of the Banjar district that was due to the higher content of phenolics and flavonoids (aromatic compounds) (Figures 2A and 2B). Ethanol extracts of green fruits and young leaves had higher concentrations of aromatic compounds. Table 1 showed that green fruits and young leaves had higher antioxidant capacity than red and purple fruits at both sample sites, with extract from Batola having higher antioxidant capacity and spectral signal intensity than Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples (Zargoosh et al. 2019).

The difference in the antioxidant capacity of the extracts from the two regions was due to factors affecting plant compound content. The environmental conditions of the two areas are relatively the same in terms of temperature, soil pH, and humidity. The sample collection from the Batola and Banjar regions around the Banjarbaru area continues previous studies.

The effect of habitat on the amount of secondary metabolites in different herbs has been studied previously. Habitat could be a factor affecting the quantity and accumulation of secondary metabolites. The location where a plant grows can influence the process of producing chemical compounds due to its temperature and humidity. The mechanisms underlying environmental effects on accumulating secondary metabolites are not adequately understood. However, the environment influences the type and number of chemical reactions through its impact on metabolite production and factors associated with the production process (e.g., Enzymes) (Zargoosh et al. 2019).

The study by ene-Obong et al. (2018) on *Monodora myristica* showed increased antioxidant activity because of increasing flavonoids, phenolics, and vitamin C content. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. It showed that the antioxidant activity and free radical scavenger depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoids through various methods, including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. This observation is consistent with the current results for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms.

There is a direct correlation between antioxidant activity and total phenolic content (Idris et al. 2022). Total flavonoids (TFC) and total phenolics (TPC) play important roles as electron donors, chain breakers, and free radical catchers in the antioxidant mechanism. Therefore, the flavonoid content in the plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds also correlates with their antioxidant activity due to their redox properties as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). Phenolic compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds in all parts of the plant. Phenolic compounds also provide other health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves and fruits with potent antioxidant activity.

Compounds identification of *R. tomentosa* leaves and fruits by ^{1}H NMR spectra

The chemical properties of *R. tomentosa* ethanol extract in leaves and fruits were analyzed for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method requires a simple sample preparation process and short duration, which is significantly advantageous for analyzing complex mixtures such as food extracts. The ^{1}H NMR spectra of the samples are presented in Figure 2.

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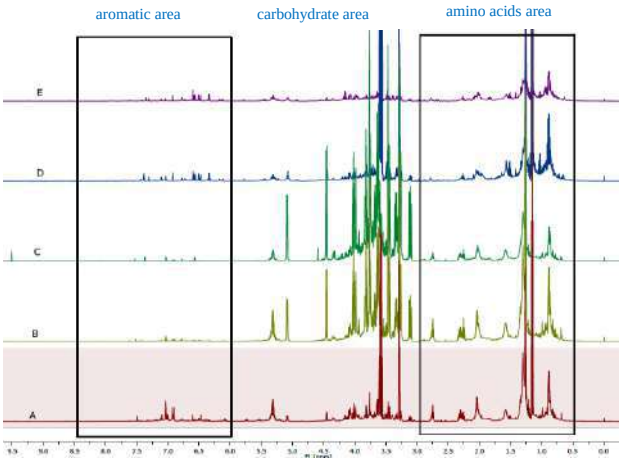

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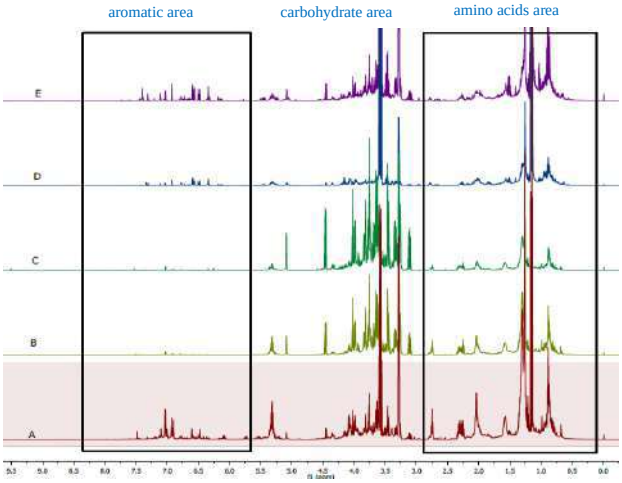

Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. extracts were collected from Banjar, and (b) fruits and leaves were collected from Batola. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify secondary metabolites due to its capability to generate a particular and distinctive spectrum for each compound. NMR spectroscopy is widely used for metabolomic investigations in various organisms due to its non-invasive and quantitative character, robustness, and reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). The NMR metabolomics methods simplify compound identification by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol is a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling

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constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of leaves and fruit extracts from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary metabolites and aromatic compounds (chemical shift > 6 ppm) as secondary metabolites. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region than old, red, and purple leaves (Figures 2A and 2B).

Distinct attributes in NMR spectra derived from leaves and fruits were observed in samples from Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with their antioxidant activity.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Amino Acids		
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Organic Acids		
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Sugars		
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics Compounds		
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
Other compounds		
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed the presence of aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The identified flavonoid compounds included gallic acid, myricetin, myricetin 3-O-rhamnopyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. It was consistent with the study by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity in young and old leaves and papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed almost identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids than green fruits, which showed a higher signal

intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits had higher aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites could affect the intensity of spectral signals in NMR analysis. The advantage of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, the ethanol extracts of green fruits showed a higher concentration of aromatic compounds. The results of antioxidant capacity (Table 1) showed a higher antioxidant capacity in green fruits than in red and purple fruits at both sample locations. Batola had better antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0 - 3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region (except for glucose and sucrose), limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis (Table 2). The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the 5.00 - 3.50 ppm chemical shift. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in both regions' aromatic shift (6 - 7.5 ppm) of leaves and fruits. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaf extracts for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of primary metabolites (specifically carbohydrates and amino acids), while green fruits comprised more aromatic compounds. It signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies may be needed to determine their role in nutritional applications.

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20. Fourth revision resubmitted (11-5-2024)
    - Cover Letter revision Journal Biodiversitas
    - Revised manuscript

UNIVERSITAS LAMBUNG MANGKURAT

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

[biodiv] Editor Decision

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Sat, May 11, 2024 at 6:31 PM
To: Smujo Editors <editors@smujo.id>

Dear Editors Biodiversitas Journal of Biological Diversity

Thank you for the notice
I have sent the latest revision and response table of my revised manuscript, because in the system I sent the wrong revised manuscript file.

Regards,

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Dear Editors Biodiversitas Journal of Biological Diversity

We appreciate the reviewers for their valuable time in reviewing our paper and providing valuable comments. It was your valuable and insightful comments that led to improvements in the current version. The authors have carefully considered the comments and tried their best to address each comment. Thank you for the sentence corrections provided by the reviewers to our manuscript. We hope that this manuscript after careful revision can meet your high standards.

Below we provide the point-by-point responses. All modifications in the manuscript have been highlighted in blue.

No	Suggestion Reviewer	Response from author
1	Gynecology??	Thank you for your correction. Revised accordingly : gynecopathy (page 1 line 38).
2	Could be explained in the Material and Methods	Thank you for your correction. Revised accordingly (page 2 line 67-68).
3	Re-phrase : Extracts from identical samples were homogenized, filtrated to release cellular debris and dried utilizing a rotary evaporator, then stored in a refrigeration unit for further analysis	Thank you for the suggestion. Revised accordingly : Finally, extracts from the same samples were pooled, thoroughly mixed, filtered to remove cell debris, and then dried using a rotary evaporator, then stored in a refrigeration unit for further analysis. The yield of extract was calculated by this equation, namely (weight of ethanol extract/weight of dried leaves/fruits) × 100%. (page 2 line 82-83)
4	please check the weight of the sample	Thank you for your correction. Revised accordingly : 25 mg (page 2 line 96).
	Re-phrase : the samples were set into Eppendorf tubes at 2 mL with the methanol-d4 solution at 1 mL containing 0.001% TMSP	Thank you for the suggestion. Revised accordingly : 25 mg of crude extracts was placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent, comprising 0.5 mL of methanol-d4, 0.5 mL of KH_2PO_4 buffer, and pH 6.0 containing 0.001% TMSP (page 2 line 95-96)
5	Should be in the method : During the experiment, coarse simplisia powder was mixed with an ethanol solution. The simplisia powder was immersed for several days to allow the extraction of active compounds while maintaining a room temperature environment and avoiding light exposure	Thank you for the suggestion. Revised accordingly (page 2 line 80-82).
6	Re-phrase : The higher the yield produced indicates, the more value of the extract produced.	Thank you for the suggestion. Revised accordingly : The yield shows the amount of

		chemical compounds contain in the extract. (page 3 line 126-127).
7	Why ? : were not subjected to statistical analysis	Thank you for your question. We had problems in collecting a large number of samples due to wild plants, especially fruit samples, so it was not possible to do replications and statistical tests. The sample extract resulted from several time extraction processes and then we pooled and mixed them thoroughly. The pooled extract then was used for many analysis activities in this research
8	Re-phrase : Differences in yield were found between leaf and fruit samples but showed no different values in the two areas.	Thank you for the suggestion. Revised accordingly : The yield of leaf and fruit samples did not show different values in the two regions. (page 3 line129-130).
9	Stand for?? 1st mentioned : NDs	Thank you for your correction. Revised accordingly : non-communicable diseases (NCDs) (page 3 line 148-149).
10	Re-phrase : This was observed with a spectrophotometer to decide the free radical scavenging activity of the sample (Sayuti & Yenrina, 2015; Ismandari et al. 2020).	Thank you for the suggestion. Revised accordingly : This can be observed using a spectrophotometer so that the free radical scavenging activity of the sample can be known (Sayuti & Yenrina, 2015; Ismandari et al. 2020). (page 3 line153-154).
11	Re-phrase : Purwakusumah et al. (2016) emphasized the significance of standardizing antioxidant capacity measurements to enhance comparability and mitigate discrepancies. Expressing antioxidant capacity as millimolar Trolox equivalents per gram of sample extract provides a more descriptive and meaningful representation than percentage inhibition.	Thank you for the suggestion. Revised accordingly : Purwakusumah et al. (2016) Antioxidant capacity expressed as trolox equivalents will be more meaningful and descriptive than that expressed as percent inhibition. (page 3 line155-156).
12	Re-phrase : These appeared to be higher than antioxidant capacity, 8.79-92.60 μmol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015).	Thank you for the suggestion. Revised accordingly : These results had higher than the antioxidant (page 4 line 164-166).
13	What do you mean by oher portions?	Thank you for your correction. Revised accordingly : other parts (page 4 line 168).
14	Re-phrase : However, the environment influences the type and number of chemical reactions through its impact on metabolite production and factors associated with the production process (e.g., Enzymes) (Zargoosh et al. 2019).	Thank you for the suggestion. Revised accordingly : Zargoosh et al (2019) showed that the environment strongly influences the production of metabolites, including enzymes. (page 4 line184-185).

15	Re-phrase : This observation is consistent with the current results for leaves and fruits comprising flavonoids and phenols, which have similar antioxidant mechanisms	Thank you for the suggestion. Revised accordingly : As shown in the results obtained, leaves and fruits containing flavonoids and phenols have similar antioxidant mechanisms. (page 4 line 191-192).
16	Re-phrase : Zhao et al. (2019) associated high flavonoid and phenol content in the extract of *R. tomentosa* leaves and fruits with potent antioxidant activity.	Thank you for the suggestion. Revised accordingly : As in *R. tementosa* leaf and fruit extracts, the high content of flavonoids and phenols is directly proportional to its antioxidant activity (Zhao et al. 2019) (page 4 line 203-204).
17	Re-phrase : A study assessed results based on the quantity of compounds identified, compared to the signals observed during NMR analysis (Leiss et al. 2011).	Thank you for the suggestion. Revised accordingly : (page 5 line 226-227).

We would also like to acknowledge the invaluable contributions of the reviewers in improving our manuscript. Their feedback has been instrumental in refining our work, and we appreciate the time and effort they dedicated to the review process.

Thank you for your time and consideration. We look forward to hearing from you regarding the status of our manuscript.

Sincerely,

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Antioxidant activity and ^{1}H NMR profiling of leaves and fruits of *Rhodomyrtus tomentosa* (ait.) Hassk from South Kalimantan, Indonesia

Abstract. *Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aims to evaluate and compare the antioxidant activity of leaves and fruits ethanol extracts of *R. tomentosa* (Ait.) Hassk from two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. The samples included old and young leaves and three stages of fruit maturity, namely green, red, and purple. ^{1}H NMR spectroscopy was used to identify the chemical profile of extracts, which comprised organic acids, carbohydrates, amino acids, and phenolic compounds. Antioxidant capacity was assessed with DPPH, which showed intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had higher intensity than old leaves and red and purple fruits, correlating with antioxidant capacity. Analysis of ^{1}H NMR spectra of young and old leaf extracts identified vital metabolites, such as gallic acid, myricetin 3-O-rhamnopyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0 - 6.0 ppm as aromatic compound area. The differences in spectral signal intensity revealed higher metabolite levels, specifically carbohydrates and amino acids, in red and purple fruits. However, green fruits had higher quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms. The oxidative stress causes cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, and neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidants, such as 4-hexyl-resorcinol, to meet the daily requirement of antioxidants has roused concern due to the associated detrimental health effects and toxicological implications. Consequently, there is an increasing emphasis on applying natural antioxidants derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. They may contain phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids, and alkaloids, which have considerable antioxidant activity for maintaining health. Researchers have shown that plant chemical compounds possess antioxidant activity to prevent diseases induced by free radicals. As a result, they may become part of an effective preventive defense strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (Rhodomyrtus tomentosa (Ait.) Hassk) is an evergreen perennial shrub with diverse structural compounds and biological activities (Zhao et al. 2019). In traditional Chinese, Vietnamese, and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, stomach pain, gynecopathy, and wound healing. Parts of the roots and trunks are used for stomach diseases and after childbirth. Local Indonesians use the crushed leaves of *R. tomentosa* to treat wounds (Hamid et al., 2017). *R. tomentosa* is an anti-inflammatory, anti-diarrheal, and anti-dysentery medication in Thailand (Vo and Ngo, 2019). The main components of *R. tomentosa* include triterpenoids, flavonoids, meroterpenoids of phenols, and microelements (Zhao et al. 2019).

Recent studies have focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013; Lai et al. 2015). It is used to produce wines and beverages (Yin et al., 2021). Currently, *R. tomentosa* is mainly studied for the phytochemical components found in leaves, flowers, and stems due to their potent antioxidant, anti-bacterial, and anti-inflammatory activities, as well as the ability to reduce DNA damage (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported previously that green fruits of *R.tomentosa* from Banjarbaru showed potent antioxidant activity, with IC_{50} values against DPPH and FRAP were 1419.75±3.48 as well as

1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves and green fruits, namely 96.375±3.96 and 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite several advantages, this plant is a pest due to its rapid growth. This study continues a previous one as part of a series of research roadmaps. There has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity. This examination represents an inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves and fruits obtained from two locations, using combined NMR spectroscopy, particularly to identify antioxidant components. The study effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

MATERIALS AND METHODS

Plant materials

Samples of young and mature leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* were collected. Red, purple, and green fruits were also collected (Figure 1). Munsell Color Charts were used as a reference for plant tissue color (Wilde, 1977). The samples were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June - July 2023. The samples were identified and authenticated in the Herbarium Bogoriense, National Research and Innovation Agency, with certificate number 1007/IPH.1.01/If.07/IX/2023.

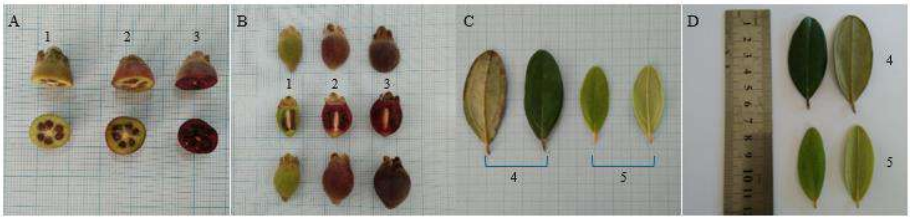


Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.

Procedures

Crude ethanol extract preparation

Fruits and different leaf ages were selectively harvested from apical branches and dried at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500g of each ground material was macerated in 1000 ml ethanol (SmartLab, Indonesia) for 72 h. The solvent was replaced every 24 hours (Nurcholis et al. 2021; Kuntorini et al. 2022). Finally, extracts from the same samples were pooled, thoroughly mixed, filtered to remove cell debris, dried using a rotary evaporator, and stored in refrigeration for further analysis. The extract yield was calculated by weight of ethanol extract/weight of dried leaves/fruits) × 100%.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2–diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to obtain the appropriate concentration. The sample (2 mL) was added with 2 mL of 0.17 mM DPPH (Sigma-Aldrich, Germany) and then incubated in the dark for 30 mins at ambient temperature. The absorbance of the samples was measured at a wavelength of 516 nm utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). The free radical scavenging activity was quantified in micromoles of Trolox (Sigma-Aldrich, Germany) equivalents per unit of dry weight (DW) (µmol TE/g).

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared using a modified methodology from previous studies (Gogna et al. 2015; Mishra et al. 2019; Kim et al. 2010). Approximately 25 mg of crude extract was placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent, comprising 0.5 mL of methanol-d4, 0.5 mL of KH_2PO_4 buffer, and pH 6.0 containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The mixture was vortexed and sonicated for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was transferred to the NMR tube for subsequent examination using ^{1}H NMR.

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, and a pre-saturation mode set at 4.27 ppm. An internal lock was also established using deuterated solvent, and the spectral width was measured from 0 to 10 ppm.

Data analysis

Mean values and standard deviations (mean ± SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a one-way analysis of variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p < 0.05$, and significant differences among treatments were analyzed further using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, and calibration to internal standard solution (TMSP) signals at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, and m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by comparing with database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Semi-quantitative signals were examined by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extract yields were varied (Table 1); the highest was obtained from purple fruits (15.24% w/w), whereas green fruits produced the lowest yield (5.63% w/w). Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds without heating. In the maceration process, the solvent entered the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extracellular and intracellular fluid until a solute concentration equilibrium was obtained (Harbone, 1987).

The solvent used in the extraction process is to extract the active ingredients. Ethanol effectively extracted sterol, flavonoid, phenolic, and alkaloid (Wardani et al. 2019). The yield of extract indicates the amount of extracted chemical compounds. The yields of the samples from two regions with antioxidant capacity were not subjected to statistical analysis. The extract yield of leaf and fruit from the two areas was not significantly different.

Commented [u1]: Why??

Commented [B32R1]: Thank you for your question. We had problems in collecting a large number of samples due to wild plants, especially fruit samples, so it was not possible to do replications and statistical tests. The sample extract resulted from several time extraction processes and then we pooled and mixed them thoroughly. The pooled extract then was used for many analysis activities in this research

Table 1. Extract yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Extract Yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 ± 3.43^{d}	1429.53 ± 9.07^{d}
Old leaves	12.296	11.49	836.20 ± 9.07^{c}	881.24 ± 5.94^{c}
Green fruits	5.63	6.05	2360.35 ± 6.86^{e}	2795.33 ± 9.07^{e}
Red fruits	9.85	7.76	415.06 ± 2.99^{b}	443.47 ± 2.06^{b}
Purple fruits	14.35	15.24	260.58 ± 0.91^{a}	364.05 ± 3.82^{a}

Note: The data represent the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by different superscript letters in the same column are significantly different.

Antioxidants bind to free radicals and thus prevent many diseases, including various non-communicable diseases (NCDs), from damaging healthy cells. Its activity can be measured by several in vitro experiments — one of the most simple, rapid, and widespread is DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples were rich in hydroxyl compounds that can reduce DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level resulted in a color change from dark purple to pink or yellow. It can be observed using a spectrophotometer so that the free radical scavenging activity of the sample can be quantified (Sayuti & Yenrina, 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) stated that antioxidant capacity expressed as trolox equivalents are more meaningful and descriptive than that expressed as percent inhibition. Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. However, assessing antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition of antioxidant compounds against radicals or metal radicals.

The antioxidant capacity of *R. tomentosa* leaves and fruits ranges from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1). The findings showed that ethanol extracts possessed similar antioxidant capacity from samples from different locations. The ethanol extract of green fruits' had the highest DPPH radical scavenging capacity, i.e., with respective values of 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, i.e., 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were lower than those reported by Lai et al. 2015, i.e., 431.17±14.5 µmol TE/g. These results of DPPH radical scavenging capacity were higher than that of grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwis, and apples (8.79-92.60 µmol TE/g) (Wu et al. 2015). This study suggested the high antioxidant potential of the purple fruits and other plant parts of *R. tomentosa*. Maskam et al. (2014) stated that the fruit extract of *R. tomentosa* had potent antioxidant properties due to phenol compounds. Purified extracts of anthocyanin from *R. tomentosa* fruits have strong antioxidant activity, including the ability to scavenge DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

The antioxidant capacity of *R.tomentosa* extract from the two locations differed, with the extract's antioxidant capacity from the Batola district being higher than that of the Banjar district that was due to the higher content of phenolics and flavonoids (aromatic compounds) (Figures 2A and 2B). Ethanol extracts of green fruits and young leaves had higher concentrations of aromatic compounds. Table 1 showed that green fruits and young leaves had higher antioxidant capacity than red and purple fruits at both sample sites, with extract from Batola having higher antioxidant capacity and spectral signal intensity than Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples (Zargoosh et al. 2019).

The difference in the antioxidant capacity of the extracts from the two regions was due to factors affecting plant compound content. The environmental conditions of the two areas are relatively the same in terms of temperature, soil pH, and humidity. The sample collection from the Batola and Banjar regions around the Banjarbaru area continues previous studies.

The effect of habitat on the amount of secondary metabolites in different herbs has been studied previously. Habitat could be a factor affecting the quantity and accumulation of secondary metabolites. The location where a plant grows can influence the process of producing chemical compounds due to its temperature and humidity. The mechanisms underlying environmental effects on accumulating secondary metabolites are not adequately understood. Zargoosh et al. (2019) showed that the environment strongly influences the production of metabolites, including enzymes.

The study by ene-Obong et al. (2018) on *Monodora myristica* showed increased antioxidant activity because of increasing flavonoids, phenolics, and vitamin C content. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. It showed that the antioxidant activity and free radical scavenger depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoids through various methods, including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. The results show that leaves and fruits containing flavonoids and phenols have similar antioxidant activity.

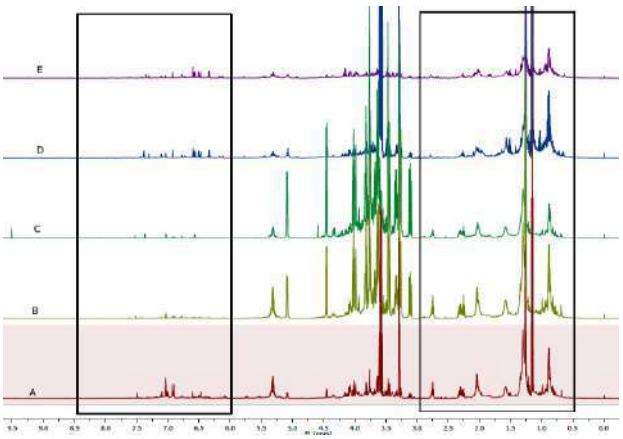
There is a direct correlation between antioxidant activity and total phenolic content (Idris et al. 2022). Total flavonoids (TFC) and total phenolics (TPC) play important roles as electron donors, chain breakers, and free radical catchers in the antioxidant mechanism. Therefore, the flavonoid content in the plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds also correlates with their antioxidant activity due to their redox properties as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). Phenolic compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds in all parts of the plant. Phenolic compounds also provide other health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). As in leaf and fruit extracts of *R.tomentosa*, the high content of flavonoids and phenols is directly proportional to its antioxidant activity (Zhao et al. 2019).

Compounds identification of *R. tomentosa* leaves and fruits by ^{1}H NMR spectra

The chemical properties of *R. tomentosa* ethanol extract in leaves and fruits were analyzed for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method requires a simple sample preparation process and short duration, which is significantly advantageous for analyzing complex mixtures such as food extracts. The ^{1}H NMR spectra of the samples are presented in Figure 2.

aromatic area carbohydrate area amino acids area

A



B

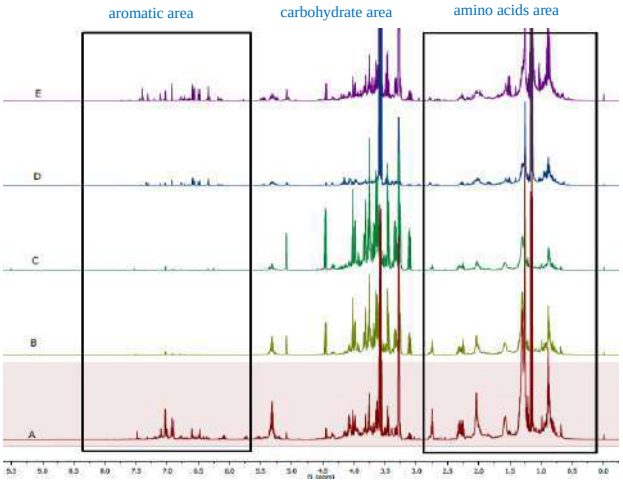


Figure 2. Representative ^{1}H NMR spectra of (a) fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. extracts were collected from Banjar, and (b) fruits and leaves were collected from Batola. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama, 2004), has been widely used to identify secondary metabolites due to its capability to generate a particular and distinctive spectrum for each compound. NMR spectroscopy is widely used for metabolomic investigations in various organisms due to its non-invasive and quantitative character, robustness, and reproducibility (Deborde et al. 2017; Misrha et al. 2019). Furthermore, the number of identified compounds can be correlated with the number and type of NMR signals (Leiss et al. 2011). The NMR metabolomics methods simplify compound identification by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol is a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5–3.0 ppm, 3.1–6.0 ppm, and > 6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of leaves and fruit extracts from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary metabolites and aromatic compounds (chemical shift > 6 ppm) as secondary metabolites. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region than old, red, and purple leaves (Figures 2A and 2B).

Distinct attributes in NMR spectra derived from leaves and fruits were observed in samples from Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves and red and purple fruits, consistent with their antioxidant activity.

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4.

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Amino Acids		
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Organic Acids		
Fumaric acid	6.56 (s)	Kim et al. 2010
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Sugars		
Mannitol	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics Compounds		
Gallic acid	7.03 (s)	Ali et al. 2010
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
Myricetin 3-O- rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O- glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2010
Other compounds		
α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
Choline	3.20 (s)	Ali et al. 2010
Sterol	0.68 (s)	Liu et al. 2017

Note : s= singlet, d= doublet, dd= double doublet, t= triplet, m= multiplet

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed the presence of aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The identified flavonoid compounds included gallic acid, myricetin, myricetin 3-O-rhamnopyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. It was consistent with the study by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity in young and old leaves and papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed almost identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids than green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits had higher aromatic

compounds. According to Lacy et al. (2014), the concentration of metabolites could affect the intensity of spectral signals in NMR analysis. The advantage of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, the ethanol extracts of green fruits showed a higher concentration of aromatic compounds. The results of antioxidant capacity (Table 1) showed a higher antioxidant capacity in green fruits than in red and purple fruits at both sample locations. Batola had better antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the ^{1}H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0 - 3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region (except for glucose and sucrose), limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis (Table 2). The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0 - 2.0 ppm range. Sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the 5.00 - 3.50 ppm chemical shift. The less crowded regions at 10.0 - 6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in both regions' aromatic shift (6 - 7.5 ppm) of leaves and fruits. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaf extracts for the regional chemical shift 10.0 - 6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of primary metabolites (specifically carbohydrates and amino acids), while green fruits comprised more aromatic compounds. It signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies may be needed to determine their role in nutritional applications.

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Antioxidant activity and ^{1}H NMR profiling of leaves and fruits of *Rhodomyrtus tomentosa* (ait.) Hassk from South Kalimantan, Indonesia

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Abstract. *Kuntorini EM, Triyasmono L, Astuti MD. 2024. Antioxidant activity and ^{1}H NMR profiling of leaves and fruits of* Rhodomyrtus tomentosa *(ait.) Hassk from South Kalimantan, Indonesia. Biodiversitas 25: xxxx. Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aims to evaluate and compare the antioxidant activity of leaves and fruits ethanol extracts of *R. tomentosa* (Ait.) Hassk from two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. The samples included old and young leaves and three stages of fruit maturity, namely green, red, and purple. ^{1}H NMR spectroscopy was used to identify the chemical profile of extracts, which comprised organic acids, carbohydrates, amino acids, and phenolic compounds. Antioxidant capacity was assessed with DPPH, which showed intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had higher intensity than old leaves and red and purple fruits, correlating with antioxidant capacity. Analysis of ^{1}H NMR spectra of young and old leaf extracts identified vital metabolites, such as gallic acid, myricetin 3-O-rhamnopyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0-6.0 ppm as aromatic compound area. The differences in spectral signal intensity revealed higher metabolite levels, specifically carbohydrates and amino acids, in red and purple fruits. However, green fruits had higher quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *R. tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms. The oxidative stress causes cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, and neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidants, such as 4-hexyl-resorcinol, to meet the daily requirement of antioxidants has roused concern due to the associated detrimental health effects and toxicological implications. Consequently, there is an increasing emphasis on applying natural antioxidants derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. They may contain phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids, and alkaloids, which have considerable antioxidant activity for maintaining health. Researchers have shown that plant chemical compounds possess antioxidant activity to prevent diseases induced by free radicals. As a result, they may become part of an effective preventive defense strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (*Rhodomyrtus tomentosa* (Ait.) Hassk) is an evergreen perennial shrub with diverse structural compounds and biological activities (Zhao et al. 2019). In traditional Chinese, Vietnamese, and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, stomach pain, gynecopathy, and wound healing. Parts of the roots and trunks are used for stomach diseases and after childbirth. Local Indonesians use the crushed leaves of *R. tomentosa* to treat wounds (Hamid et al. 2017). *Rhodomyrtus tomentosa* is an anti-inflammatory, anti-diarrheal, and anti-dysentery medication in Thailand (Vo and Ngo 2019). The main components of *R. tomentosa* include triterpenoids, flavonoids, meroterpenoids of phenols, and microelements (Zhao et al. 2019).

Recent studies have focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al. 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013, 2015). It is used to produce wines and beverages (Yin et al. 2021). Currently, *R. tomentosa* is mainly studied for the phytochemical components found in leaves, flowers, and stems due to

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their potent antioxidant, anti-bacterial, and anti-inflammatory activities, as well as the ability to reduce DNA damage (Wu et al. 2015; Vo and Ngo 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported previously that green fruits of *R. tomentosa* from Banjarbaru showed potent antioxidant activity, with IC_{50} values against DPPH and FRAP were 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves and green fruits, namely 96.375±3.96 and 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite several advantages, this plant is a pest due to its rapid growth. This study continues a previous one as part of a series of research roadmaps. There has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity. This examination represents an inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves and fruits obtained from two locations, using combined NMR spectroscopy, particularly to identify antioxidant components. The study effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

MATERIALS AND METHODS

Plant materials

Samples of young and mature leaves (2^{nd}-6^{th} and 7^{th}-12^{th} order from the shoot) of *R. tomentosa* were collected. Red, purple, and green fruits were also collected (Figure 1). Munsell Color Charts were used as a reference for plant tissue color (Wilde 1977). The samples were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June-July 2023. The samples were identified and authenticated in the Herbarium Bogoriense, National Research and Innovation Agency, with certificate number 1007/IPH.1.01/If.07/IX/2023.

Procedures

Crude ethanol extract preparation

Fruits and different leaf ages were selectively harvested from apical branches and dried at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500g of each ground material was macerated in 1000 ml ethanol (SmartLab, Indonesia) for 72 h. The solvent was replaced every 24 hours (Nurcholis et al. 2021; Kuntorini et al. 2022). Finally, extracts from the same samples were pooled, thoroughly mixed, filtered to remove cell debris, dried using a rotary evaporator, and stored in refrigeration for further analysis. The extract yield was calculated by weight of ethanol extract/weight of dried leaves/fruits) × 100%.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2-diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to obtain the appropriate concentration. The sample (2 mL) was added with 2 mL of 0.17 mM DPPH (Sigma-Aldrich, Germany) and then incubated in the dark for 30 mins at ambient temperature. The absorbance of the samples was measured at a wavelength of 516 nm utilizing a UV/Vis Spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). The free radical scavenging activity was quantified in micromoles of Trolox (Sigma-Aldrich, Germany) equivalents per unit of Dry Weight (DW) (µmol TE/g).

Sample preparation for ^{1}H-NMR

The ^{1}H NMR samples were prepared using a modified methodology from previous studies (Kim et al. 2010; Gogna et al. 2015; Mishra et al. 2019). Approximately 25 mg of crude extract was placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent, comprising 0.5 mL of methanol-d4, 0.5 mL of KH_2PO_4 buffer, and pH 6.0 containing 0.001% TMSP (Trimethyl Silypropionic acid sodium, Sigma-Aldrich). The mixture was vortexed and sonicated for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was transferred to the NMR tube for subsequent examination using ^{1}H NMR.

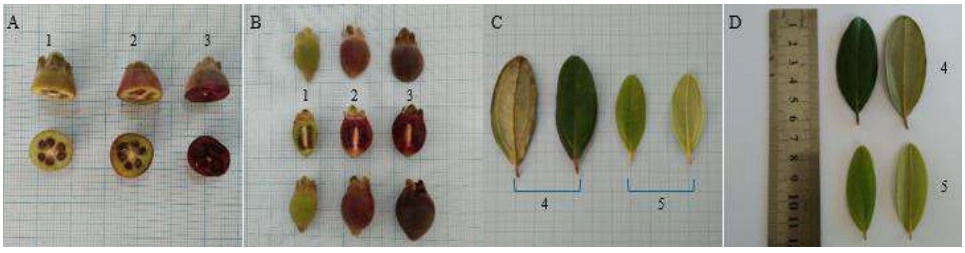

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola; B. Fruits from Banjar; C. Leaves from Batola; D. Leaves from Banjar; 1. Green fruits; 2. Red fruits; 3. Purple fruits; 4. Old leaves; 5. Young leaves

[1]H NMR spectroscopy

The [1]H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, and a pre-saturation mode set at 4.27 ppm. An internal lock was also established using deuterated solvent, and the spectral width was measured from 0 to 10 ppm.

Data analysis

Mean values and standard deviations (mean±SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a One-Way Analysis of Variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p<0.05$, and significant differences among treatments were analyzed further using the LSD method.

The [1]H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, and calibration to internal standard solution (TMSP) signals at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s = singlet, dd = doublet of doublets, d = doublet, t = triplet, and m = multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by comparing with database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli 2018; Mishra et al. 2019). Semi-quantitative signals were examined by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the [1]H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extract yields were varied (Table 1); the highest was obtained from purple fruits (15.24% w/w), whereas green fruits produced the lowest yield (5.63% w/w). Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds without heating. In the maceration process, the solvent entered the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extracellular and intracellular fluid until a solute concentration equilibrium was obtained (Harbone 1987).

The solvent used in the extraction process is to extract the active ingredients. Ethanol effectively extracted sterol, flavonoid, phenolic, and alkaloid (Wardani et al. 2019). The yield of extract indicates the amount of extracted chemical compounds. The yields of the samples from two regions with antioxidant capacity were not subjected to statistical analysis. The extract yield of leaf and fruit from the two areas was not significantly different.

Antioxidants bind to free radicals and thus prevent many diseases, including various Non-Communicable Diseases (NCDs), from damaging healthy cells. Its activity can be measured by several in vitro experiments — one of the most simple, rapid, and widespread is DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples were rich in hydroxyl compounds that can reduce DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level resulted in a color change from dark purple to pink or yellow. It can be observed using a spectrophotometer so that the free radical scavenging activity of the sample can be quantified (Sayuti and Yenrina 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) stated that antioxidant capacity expressed as trolox equivalents are more meaningful and descriptive than that expressed as percent inhibition. Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. However, assessing antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition of antioxidant compounds against radicals or metal radicals.

The antioxidant capacity of *R. tomentosa* leaves and fruits ranges from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1). The findings showed that ethanol extracts possessed similar antioxidant capacity from samples from different locations. The ethanol extract of green fruits' had the highest DPPH radical scavenging capacity, i.e., with respective values of 2360.35±6.86 µmol TE/g (Banjar District) and 2795.33±9.07 µmol TE/g (Batola District). Conversely, the lowest values were observed in purple fruits, i.e., 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were lower than those reported by Lai et al. (2015), i.e., 431.17±14.5 µmol TE/g. These results of DPPH radical scavenging capacity were higher than that of grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwis, and apples (8.79-92.60 µmol TE/g) (Wu et al. 2015). This study suggested the high antioxidant potential of the purple fruits and other plant parts of *R. tomentosa*. Maskam et al. (2014) stated that the fruit extract of *R. tomentosa* had potent antioxidant properties due to phenol compounds. Purified extracts of anthocyanin from *R. tomentosa* fruits have strong antioxidant activity, including the ability to scavenge DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

The antioxidant capacity of *R. tomentosa* extract from the two locations differed, with the extract's antioxidant capacity from the Batola district being higher than that of the Banjar district that was due to the higher content of phenolics and flavonoids (aromatic compounds) (Figures 2.A and 2.B). Ethanol extracts of green fruits and young leaves had higher concentrations of aromatic compounds. Table 1 showed that green fruits and young leaves had higher antioxidant capacity than red and purple fruits at both sample sites, with extract from Batola having higher antioxidant capacity and spectral signal intensity than Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples (Zargoosh et al. 2019).

Table 1. Extract yield (%, w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract

Sample	Extract yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01±3.43^{d}	1429.53±9.07^{d}
Old leaves	12.296	11.49	836.20±9.07^{c}	881.24±5.94^{c}
Green fruits	5.63	6.05	2360.35±6.86^{e}	2795.33±9.07^{e}
Red fruits	9.85	7.76	415.06±2.99^{b}	443.47±2.06^{b}
Purple fruits	14.35	15.24	260.58±0.91^{a}	364.05±3.82^{a}

Note: The data represent the mean±standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by different superscript letters in the same column are significantly different

The difference in the antioxidant capacity of the extracts from the two regions was due to factors affecting plant compound content. The environmental conditions of the two areas are relatively the same in terms of temperature, soil pH, and humidity. The sample collection from the Batola and Banjar regions around the Banjarbaru area continues previous studies.

The effect of habitat on the amount of secondary metabolites in different herbs has been studied previously. Habitat could be a factor affecting the quantity and accumulation of secondary metabolites. The location where a plant grows can influence the process of producing chemical compounds due to its temperature and humidity. The mechanisms underlying environmental effects on accumulating secondary metabolites are not adequately understood. Zargoosh et al. (2019) showed that the environment strongly influences the production of metabolites, including enzymes.

The study by Ene-Obong et al. (2018) on *Monodora myristica* showed increased antioxidant activity because of increasing flavonoids, phenolics, and vitamin C content. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. It showed that the antioxidant activity and free radical scavenger depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoids through various methods, including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. The results show that leaves and fruits containing flavonoids and phenols have similar antioxidant activity.

There is a direct correlation between antioxidant activity and total phenolic content (Idris et al. 2022). Total Flavonoids Contents (TFC) and Total Phenolics Contents (TPC) play important roles as electron donors, chain breakers, and free radical catchers in the antioxidant mechanism. Therefore, the flavonoid content in the plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds also correlates with their antioxidant activity due to their redox properties as hydrogen donors and reducing agents that can capture free radicals (Van Hung 2016). Phenolic compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds in all parts of the plant. Phenolic compounds also provide other health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). As in leaf and fruit extracts of *R.tomentosa*, the high content of flavonoids and phenols is directly proportional to its antioxidant activity (Zhao et al. 2019).

Compounds identification of *R. tomentosa* leaves and fruits by ^{1}H NMR spectra

The chemical properties of *R. tomentosa* ethanol extract in leaves and fruits were analyzed for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method requires a simple sample preparation process and short duration, which is significantly advantageous for analyzing complex mixtures such as food extracts. The ^{1}H NMR spectra of the samples are presented in Figure 2.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama 2004), has been widely used to identify secondary metabolites due to its capability to generate a particular and distinctive spectrum for each compound. NMR spectroscopy is widely used for metabolomic investigations in various organisms due to its non-invasive and quantitative character, robustness, and reproducibility (Deborde et al. 2017; Mishra et al. 2019). Furthermore, the number of identified compounds can be correlated with the number and type of NMR signals (Leiss et al. 2011). The NMR metabolomics methods simplify compound identification by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli 2018; Mishra et al. 2019). Aqueous methanol is a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

Table 2. [1]H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and fruits extracts in MeOH-d4

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
Amino acids		
Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2018
Glutamic acid	2.07 (m); 2.36 (m)	Kim et al. 2010
Leucine	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
Methionine	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2011
Organic acids	6.56 (s)	Kim et al. 2010
Fumaric acid		
Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
Succinic acid	2.56 (s)	Kim et al. 2010
Sugars	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
Mannitol		
β-glucose	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
α-glucose	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
Sucrose	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics compounds	7.03 (s)	Ali et al. 2011
Gallic acid		
Myricetin	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2011
Myricetin 3-O-rhamnpyranoside	6.98 (s)	Cerulli et al. 2018
Quercetin-3-O-glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
Quercetin	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
Syringic acid	3.89 (s)	Ali et al. 2011
Other compounds	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
α -Linolenic acid		
Choline	3.20 (s)	Ali et al. 2011
Sterol	0.68 (s)	Liu et al. 2017

Note: s: Singlet; d: Doublet, dd: Double doublet; t: Triplet; m: Multiplet

The [1]H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5-3.0 ppm, 3.1-6.0 ppm, and >6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the [1]H NMR spectra of leaves and fruit extracts from two locations.

The [1]H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary metabolites and aromatic compounds (chemical shift >6 ppm) as secondary metabolites. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region than old, red, and purple leaves (Figures 2.A and 2.B).

Distinct attributes in NMR spectra derived from leaves and fruits were observed in samples from Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves, red and purple fruits, consistent with their antioxidant activity.

The [1]H NMR spectra of ethanol extracts from young and old leaves showed the presence of aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The identified flavonoid compounds included gallic acid, myricetin, myricetin 3-O-rhamnopyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. It was consistent with the study by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity in young and old leaves and papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

A comparison of the [1]H NMR spectral analysis of extracts from purple, red, and green fruits showed almost identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids than green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits had higher aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites could affect the intensity of spectral signals in NMR analysis. The advantage of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005). In this study, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, the ethanol extracts of green fruits showed a higher concentration of aromatic compounds. The results of antioxidant capacity (Table 1) showed a higher antioxidant capacity in green fruits than in red and purple fruits at both sample locations. Batola had better antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the [1]H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

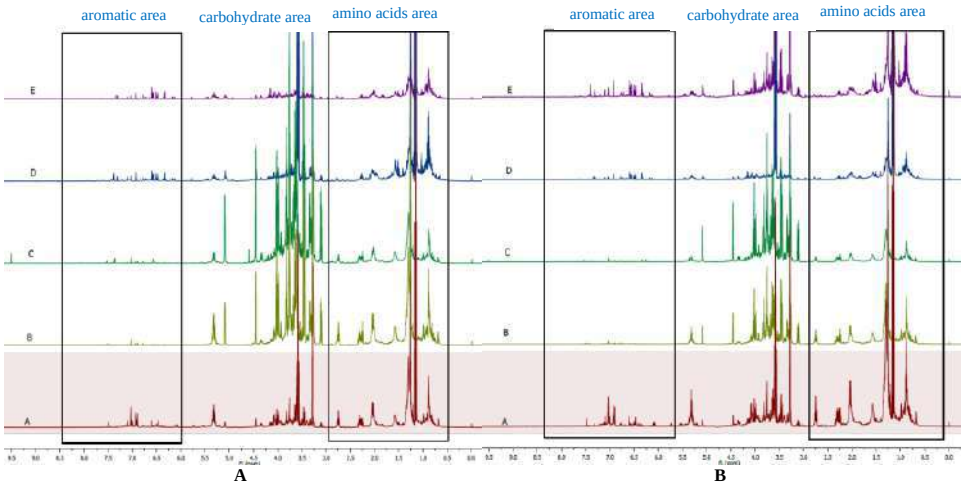

Figure 2. Representative ^{1}H NMR spectra of: A. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. extracts were collected from Banjar; and B. Fruits and leaves were collected from Batola. A: Young leaves; B: Old leaves; C: Purple fruits; D: Red fruits; E: Green fruits

Despite the benefits of applying ^{1}H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region (except for glucose and sucrose), limiting the detection of certain substances. However, 20 potential compounds were identified through the ^{1}H NMR spectra analysis (Table 2). The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0-2.0 ppm range. Sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the 5.00-3.50 ppm chemical shift. The less crowded regions at 10.0-6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in both regions' aromatic shift (6-7.5 ppm) of leaves and fruits. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The ^{1}H NMR spectra of young and old leaf extracts for the regional chemical shift 10.0-6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of primary metabolites (specifically carbohydrates and amino acids), while green fruits comprised more aromatic compounds. It signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies may be needed to determine their role in nutritional applications.

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Antioxidant activity and ^{1}H NMR profiling of leaves and fruits of *Rhodomyrtus tomentosa* from South Kalimantan, Indonesia

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Abstract. *Kuntorini EM, Triyasmono L, Astuti MD. 2024. Antioxidant activity and ^{1}H NMR profiling of leaves and fruits of* Rhodomyrtus tomentosa *from South Kalimantan, Indonesia. Biodiversitas 25: 2020-2027. Rhodomyrtus tomentosa* (Aiton) Hassk is a flowering plant belonging to Myrtaceae, native to southern and southeastern Asia. This study aims to evaluate and compare the antioxidant activity of leaves and fruits ethanol extracts of *R. tomentosa* (Ait.) Hassk from two locations, namely Banjar and Batola, South Kalimantan Province, Indonesia. The samples included old and young leaves and three stages of fruit maturity, namely green, red, and purple. ^{1}H NMR spectroscopy was used to identify the chemical profile of extracts, which comprised organic acids, carbohydrates, amino acids, and phenolic compounds. Antioxidant capacity was assessed with DPPH, which showed intensity differences in aromatic shifts in leaves and fruits for both regions. Young leaves and green fruits had higher intensity than old leaves and red and purple fruits, correlating with antioxidant capacity. Analysis of ^{1}H NMR spectra of young and old leaf extracts identified vital metabolites, such as gallic acid, myricetin 3-O-rhamnopyranoside, myricetin, quercetin, quercetin-3-O-glucoside, as well as syringic acid, in regional chemical shifts of 10.0-6.0 ppm as aromatic compound area. The differences in spectral signal intensity revealed higher metabolite levels, specifically carbohydrates and amino acids, in red and purple fruits. However, green fruits had higher quantities of aromatic compounds.

Keywords: DPPH, metabolite profiling, myricetin, *Rhodomyrtus tomentosa*

INTRODUCTION

An antioxidant is a bioactive compound capable of counteracting the harmful effects of free radicals and oxidative stress induced by reactive mechanisms. The oxidative stress causes cellular damage and degeneration, along with chronic degenerative diseases development, including diabetes, cancer, cardiovascular diseases, and neurovascular diseases (Masisi et al. 2016). The reliance on synthetic antioxidants, such as 4-hexyl-resorcinol, to meet the daily requirement of antioxidants has roused concern due to the associated detrimental health effects and toxicological implications. Consequently, there is an increasing emphasis on applying natural antioxidants derived from bioactive plant components (Maskam et al. 2014; Ismandari et al. 2020). Medicinal plants are the most potent sources of natural antioxidants. They may contain phytochemical compounds such as flavonoids, phenolic acids, tannins, terpenoids, and alkaloids, which have considerable antioxidant activity for maintaining health. Researchers have shown that plant chemical compounds possess antioxidant activity to prevent diseases induced by free radicals. As a result, they may become part of an effective preventive defense strategy against various human diseases. Natural antioxidants extracted from medicinal plants are inexpensive, safe, and do not have toxic effects compared to synthetic antioxidants (Maskam et al. 2014).

Rose Myrtle (*Rhodomyrtus tomentosa* (Ait.) Hassk) is an evergreen perennial shrub with diverse structural compounds and biological activities (Zhao et al. 2019). In traditional Chinese, Vietnamese, and Malaysian medicine, all parts of the plant (leaves, roots, flowers, fruits) have long been used to treat diarrhea, stomach pain, gynecopathy, and wound healing. Parts of the roots and trunks are used for stomach diseases and after childbirth. Local Indonesians use the crushed leaves of *R. tomentosa* to treat wounds (Hamid et al. 2017). *Rhodomyrtus tomentosa* is an anti-inflammatory, anti-diarrheal, anti-dysentery medication in Thailand (Vo and Ngo 2019). The main components of *R. tomentosa* include triterpenoids, flavonoids, meroterpenoids of phenols, and microelements (Kusuma et al. 2016; Zhao et al. 2019).

Recent studies have focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for pharmacological properties and traditional applications in treating various ailments (Yang et al. 2023). The medicinal properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols, polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential (Lai et al. 2013, 2015). It is used to produce wines and beverages (Yin et al. 2021). Currently, *R. tomentosa* is mainly studied for the phytochemical components found in leaves, flowers, and stems due to their

potent antioxidant, anti-bacterial, and anti-inflammatory activities, as well as the ability to reduce DNA damage (Wu et al. 2015; Kusuma et al. 2016; Vo and Ngo 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported previously that green fruits of *R. tomentosa* from Banjarbaru showed potent antioxidant activity, with IC_{50} values against DPPH and FRAP were 1419.75±3.48 as well as 1367.59±9.12 µmol TE/g DW. Ethanol extracts showed the highest values of TFC in the young leaves and green fruits, namely 96.375±3.96 and 95.731±5.42 mg QE/g DW.

The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, is attributed to a lack of knowledge about the extraction methods and potential benefits. Despite several advantages, this plant is a pest due to its rapid growth. This study continues a previous one as part of a series of research roadmaps. There has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits at various stages of maturity. This examination represents an inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves and fruits obtained from two locations, using combined NMR spectroscopy, particularly to identify antioxidant components. The study effectively shows the suitability and effectiveness of the NMR method in analyzing the metabolites of plants.

MATERIALS AND METHODS

Plant materials

Samples of young and mature leaves (2^{nd}-6^{th} and 7^{th}-12^{th} order from the shoot) of *R. tomentosa* were collected. Red, purple, and green fruits were also collected (Figure 1). Munsell Color Charts were used as a reference for plant tissue color (Wilde 1977). The samples were obtained from natural habitats in Martapura, Banjar District (3°26′00.6″S, 114°51′30.5″E), and Marabahan, Batola District (3°09′16.0″S, 114°37′19.8″E), South Kalimantan in June-July 2023. The samples were identified and authenticated in the Herbarium Bogoriense, National Research and Innovation Agency, with certificate number 1007/IPH.1.01/If.07/IX/2023.

Procedures

Crude ethanol extract preparation

Fruits and different leaf ages were selectively harvested from apical branches and dried at 40°C in an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in 1000 mL ethanol (SmartLab, Indonesia) for 72 h. The solvent was replaced every 24 hours (Nurcholis et al. 2021; Kuntorini et al. 2022). Finally, extracts from the same samples were pooled, thoroughly mixed, filtered to remove cell debris, dried using a rotary evaporator, and stored in refrigeration for further analysis. The extract yield was calculated by weight of ethanol extract/weight of dried leaves/fruits) × 100%.

Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2-diphenyl picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to obtain the appropriate concentration. The sample (2 mL) was added with 2 mL of 0.17 mM DPPH (Sigma-Aldrich, Germany) and then incubated in the dark for 30 mins at ambient temperature. The absorbance of the samples was measured at wavelength of 516 nm utilizing UV/Vis Spectrophotometer (UV/Vis Spectrophotometer, Genesystm 10 Series, USA). The free radical scavenging activity was quantified in micromoles of Trolox (Sigma-Aldrich, Germany) equivalents per unit of Dry Weight (DW) (µmol TE/g).

Sample preparation for [1]H-NMR

The ^{1}H NMR samples were prepared using a modified methodology from previous studies (Kim et al. 2010; Gogna et al. 2015; Mishra et al. 2019). Approximately 25 mg of crude extract was placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent, comprising 0.5 mL of methanol-d4, 0.5 mL of KH_2PO_4 buffer, and pH 6.0 containing 0.001% TMSP (Trimethyl Silypropionic acid sodium, Sigma-Aldrich). The mixture was vortexed and sonicated for 1 min, followed by homogenization and centrifugation for 1 min at 10,000 rpm. The supernatant was transferred to the NMR tube for subsequent examination using ^{1}H NMR.

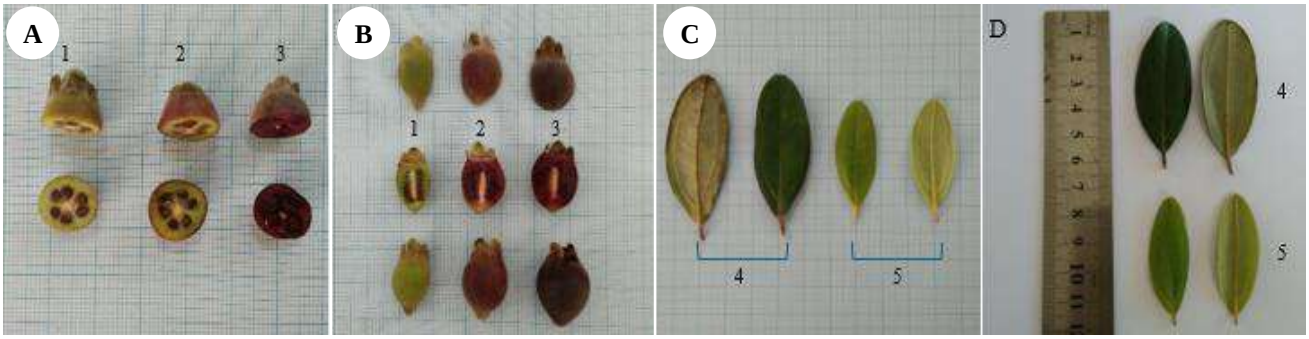

Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa*. A. Fruits from Batola; B. Fruits from Banjar; C. Leaves from Batola; D. Leaves from Banjar; 1. Green fruits; 2. Red fruits; 3. Purple fruits; 4. Old leaves; 5. Young leaves

^{1}H NMR spectroscopy

The ^{1}H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The parameters applied included 128 scans over 10 mins, a relaxation delay of 1.5 mins, an X_angle of 60°, and a pre-saturation mode set at 4.27 ppm. An internal lock was also established using deuterated solvent, and the spectral width was measured from 0 to 10 ppm.

Data analysis

Mean values and standard deviations (mean±SD) were calculated based on three replications. Quantitative data underwent statistical analysis using a One-Way Analysis of Variance (ANOVA) using Microsoft Excel® and IBM SPSS Statistics 21 software. Significant differences were determined at $p<0.05$, and significant differences among treatments were analyzed further using the LSD method.

The ^{1}H NMR spectra were analyzed using MestReNova software, including processes of manual phasing, baseline modification, and calibration to internal standard solution (TMSP) signals at a chemical shift of 0.0 ppm. The observed NMR resonance multiplicities were designated in line with the established convention. The symbols used in this context were s: singlet, dd: doublet of doublets, d: doublet, t: triplet, and m: multiplet (Mishra et al. 2019). Additionally, the identification of metabolites was conducted by comparing with database derived from prior studies (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli 2018; Mishra et al. 2019). Semi-quantitative signals were examined by comparing their areas to the TMSP signal, serving as an internal reference. Subsequently, the ^{1}H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

RESULTS AND DISCUSSION

Yield and antioxidant capacity of ethanol extracts

The extract yields were varied (Table 1); the highest was obtained from purple fruits (15.24% w/w), whereas green fruits produced the lowest yield (5.63% w/w). Maceration, a non-thermal extraction method, was used to prevent the degradation of secondary metabolite compounds without heating. In the maceration process, the solvent entered the cells through diffusion, causing the dissolution of cellular contents due to concentration disparities between the extracellular and intracellular fluid until a solute concentration equilibrium was obtained (Harbone 1987).

The solvent used in the extraction process is to extract the active ingredients. Ethanol effectively extracted sterol, flavonoid, phenolic, and alkaloid (Wardani et al. 2019). The yield of extract indicates the amount of extracted chemical compounds. The yields of the samples from two regions with antioxidant capacity were not subjected to statistical analysis. The extract yield of leaf and fruit from the two areas was not significantly different.

Antioxidants bind to free radicals and thus prevent many diseases, including various Non-Communicable Diseases (NCDs), from damaging healthy cells. Its activity can be measured by several in vitro experiments - one of the most simple, rapid, and widespread is DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples were rich in hydroxyl compounds that can reduce DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level resulted in a color change from dark purple to pink or yellow. It can be observed using a spectrophotometer so that the free radical scavenging activity of the sample can be quantified (Sayuti and Yenrina 2015; Ismandari et al. 2020).

Purwakusumah et al. (2016) stated that antioxidant capacity expressed as trolox equivalents are more meaningful and descriptive than that expressed as percent inhibition. Trolox (6-hydroxy-2,5,7,8 tetramethyl croman-2-carboxylic) is an analog of water-soluble vitamin E or α-tocopherol. However, assessing antioxidant activity based on percent inhibition focuses solely on the minimal percentage inhibition of antioxidant compounds against radicals or metal radicals.

The antioxidant capacity of *R. tomentosa* leaves and fruits ranges from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1). The findings showed that ethanol extracts possessed similar antioxidant capacity from samples from different locations. The ethanol extract of green fruits' had the highest DPPH radical scavenging capacity, i.e., with respective values of 2360.35±6.86 µmol TE/g (Banjar District) and 2795.33±9.07 µmol TE/g (Batola District). Conversely, the lowest values were observed in purple fruits, i.e., 260.58±0.91 µmol TE/g and 364.05±3.82 µmol TE/g, respectively, which were lower than those reported by Lai et al. (2015), i.e., 431.17±14.5 µmol TE/g. These results of DPPH radical scavenging capacity were higher than that of grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwis, and apples (8.79-92.60 µmol TE/g) (Wu et al. 2015). This study suggested the high antioxidant potential of the purple fruits and other plant parts of *R. tomentosa*. Maskam et al. (2014) stated that the fruit extract of *R. tomentosa* had potent antioxidant properties due to phenol compounds. Purified extracts of anthocyanin from *R. tomentosa* fruits have strong antioxidant activity, including the ability to scavenge DPPH radicals (IC_{50}: 6.27±0.25 g/mL) (Cui et al. 2013).

The antioxidant capacity of *R. tomentosa* extract from the two locations differed, with the extract's antioxidant capacity from the Batola district being higher than that of the Banjar district that was due to the higher content of phenolics and flavonoids (aromatic compounds) (Figures 2.A and 2.B). Ethanol extracts of green fruits and young leaves had higher concentrations of aromatic compounds. Table 1 showed that green fruits and young leaves had higher antioxidant capacity than red and purple fruits at both sample sites, with extract from Batola having higher antioxidant capacity and spectral signal intensity than Banjar (Figure 2). The antioxidant content of plants has a linear correlation with the phenolic and flavonoid content of the samples (Zargoosh et al. 2019).

Table 1. Extract yield (%, w/w) and DPPH antioxidant scavenging capacity of *Rhodomyrtus tomentosa* leaves and fruits ethanol extract

Sample	Extract yield (%, w/w)		DPPH radical scavenging capacity (µmol TE/g DW)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01±3.43^d	1429.53±9.07^d
Old leaves	12.296	11.49	836.20±9.07^c	881.24±5.94^c
Green fruits	5.63	6.05	2360.35±6.86^e	2795.33±9.07^e
Red fruits	9.85	7.76	415.06±2.99^b	443.47±2.06^b
Purple fruits	14.35	15.24	260.58±0.91^a	364.05±3.82^a

Note: The data represent the mean±standard deviation. Data were evaluated using one-way ANOVA and the LSD test with = 0.05. Numbers followed by different superscript letters in the same column are significantly different

The difference in the antioxidant capacity of the extracts from the two regions was due to factors affecting plant compound content. The environmental conditions of the two areas are relatively the same in terms of temperature, soil pH, and humidity. The sample collection from the Batola and Banjar regions around the Banjarbaru area continues previous studies.

The effect of habitat on the amount of secondary metabolites in different herbs has been studied previously. Habitat could be a factor affecting the quantity and accumulation of secondary metabolites. The location where a plant grows can influence the process of producing chemical compounds due to its temperature and humidity. The mechanisms underlying environmental effects on accumulating secondary metabolites are not adequately understood. Zargoosh et al. (2019) showed that the environment strongly influences the production of metabolites, including enzymes.

The study by Ene-Obong et al. (2018) on *Monodora myristica* showed increased antioxidant activity because of increasing flavonoids, phenolics, and vitamin C content. Conversely, *Ricinodendron heudelotii*, with the lowest phenolic and vitamin C content, had the least DPPH-reducing ability. It showed that the antioxidant activity and free radical scavenger depended on the location of the hydroxyl group and other structural characteristics. Wu et al. (2015) measured substantial antioxidant activity in *R. tomentosa* fruit extract containing high flavonoids through various methods, including DPPH, FRAP, inhibition of lipid peroxidation, superoxide anion radical activity, and hydroxyl radicals. The results show that leaves and fruits containing flavonoids and phenols have similar antioxidant activity.

There is a direct correlation between antioxidant activity and total phenolic content (Idris et al. 2022). Total Flavonoids Contents (TFC) and Total Phenolics Contents (TPC) play important roles as electron donors, chain breakers, and free radical catchers in the antioxidant mechanism. Therefore, the flavonoid content in the plant is directly proportional to its antioxidant activity. Likewise, the presence of phenolic compounds also correlates with their antioxidant activity due to their redox properties as hydrogen donors and reducing agents that can capture free radicals (Van Hung 2016). Phenolic compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic compounds in all parts of the plant. Phenolic compounds also provide other health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). As in leaf and fruit extracts of *R. tomentosa*, the high content of flavonoids and phenols is directly proportional to its antioxidant activity (Zhao et al. 2019).

Compounds identification of *R. tomentosa* leaves and fruits by ^{1}H NMR spectra

The chemical properties of *R. tomentosa* ethanol extract in leaves and fruits were analyzed for antioxidant activity by ^{1}H NMR spectroscopy. This non-destructive spectroscopy method requires a simple sample preparation process and short duration, which is significantly advantageous for analyzing complex mixtures such as food extracts. The ^{1}H NMR spectra of the samples are presented in Figure 2.

NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic fields (Hatada and Kitayama 2004), has been widely used to identify secondary metabolites due to its capability to generate a particular and distinctive spectrum for each compound. NMR spectroscopy is widely used for metabolomic investigations in various organisms due to its non-invasive and quantitative character, robustness, and reproducibility (Deborde et al. 2017; Mishra et al. 2019). Furthermore, the number of identified compounds can be correlated with the number and type of NMR signals (Leiss et al. 2011). The NMR metabolomics methods simplify compound identification by comparing sample signals to those generated by the same compounds in prior reports using CD_3OD-D_2O as the solvent (Kim et al. 2010; Ali et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli 2018; Mishra et al. 2019). Aqueous methanol is a common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to authenticate correspondence between signals in the data and references (Kim et al. 2010).

Table 2. ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *Rhodomyrtus tomentosa* leaves and fruits extracts in MeOH-d4

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)		Reference
Amino acids			
Aspartate	2.68	(dd, J= 3.0; 17.0 Hz)	Cerulli et al. (2018)
Glutamic acid	2.07	(m); 2.36 (m)	Kim et al. (2010)
Leucine	0.96	(d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. (2021)
Methionine	2.15 m; 2.65	(t, J= 6.08; 6.08 Hz)	Ali et al. (2011)
Organic acids			
Fumaric acid	6.56	(s)	Kim et al. (2010)
Malic acid	4.34	(dd, J= 6.6 ; 4.7 Hz)	Kim et al. (2010)
Succinic acid	2.56	(s)	Kim et al. (2010)
Sugars			
Mannitol	3.77	(d, J= 3.28 Hz)	Nuringtyas et al. (2012)
β-glucose	4.48	(d, J= 7.79 Hz)	Nuringtyas et al. (2012)
α-glucose	5.11	(d, J= 3.84 Hz)	Nuringtyas et al. (2012)
Sucrose	5.39	(d, J= 3.91 Hz)	Nuringtyas et al. (2012)
Aromatics compounds			
Gallic acid	7.03	(s)	Ali et al. (2011)
Myricetin	6.28	(d, J= 1.99 Hz); 7.32 (s)	Ali et al. (2011)
Myricetin 3-O- rhamnpyranoside	6.98	(s)	Cerulli et al. (2018)
Quercetin-3-O- glucoside	6.18	(d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. (2018)
Quercetin	6.25	(s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. (2017)
Syringic acid	3.89	(s)	Ali et al. (2011)
Other compounds			
α -Linolenic acid	1.16	(t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. (2013)
Choline	3.20	(s)	Ali et al. (2011)
Sterol	0.68	(s)	Liu et al. (2017)

Note: s: Singlet; d: Doublet, dd: Double doublet; t: Triplet; m: Multiplet

The ^{1}H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of 0.5-3.0 ppm, 3.1-6.0 ppm, and >6 ppm, respectively (Kim et al. 2010). Figure 2 depicts the analysis and comparison of various developmental phases of the ^{1}H NMR spectra of leaves and fruit extracts from two locations.

The ^{1}H-NMR analysis results showed the presence of primary and secondary metabolite compounds, with amino acids (chemical shift of 2.0-0.5 ppm) and carbohydrates (chemical shift of 5.0-3.0 ppm) classified as primary metabolites and aromatic compounds (chemical shift >6 ppm) as secondary metabolites. A gradual decline in phenolic content was observed in the aromatic regions of the NMR spectra all through the maturation phases of leaves and fruits. Young leaves and green fruit samples showed higher signal intensities in the aromatic region than old, red, and purple leaves (Figures 2.A and 2.B).

Distinct attributes in NMR spectra derived from leaves and fruits were observed in samples from Batola and Banjar, particularly in the 5.0-3.0 ppm chemical shift range. The chemical shift was more substantial in fruits than in leaves, suggesting fruits as the primary producers of the anomeric content of glucose, α-glucose, and fructose. Intensity and diversity varied between leaves and fruit samples for chemical shifts of approximately 5.31 ppm (sucrose). Intensity variations were also identified in the aromatic shifts (6.5-7.5 ppm) detected in leaves and fruits from both locations. Higher intensities were recorded in young leaves and green fruits compared to older leaves, red and purple fruits, consistent with their antioxidant activity.

The ^{1}H NMR spectra of ethanol extracts from young and old leaves showed the presence of aromatic compounds with a regional chemical shift ranging from 10.0 to 6.0 ppm. The identified flavonoid compounds included gallic acid, myricetin, myricetin 3-O-rhamnopyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). Young leaves showed a significant concentration of these compounds, corresponding to the comparatively high antioxidant capacity observed. It was consistent with the study by Gogna et al. (2015) regarding total phenol content, flavonoid, and antioxidant activity in young and old leaves and papaya seeds (*Carica papaya* L.) using NMR spectroscopy.

A comparison of the ^{1}H NMR spectral analysis of extracts from purple, red, and green fruits showed almost identical signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits showed more pronounced results for carbohydrates and amino acids than green fruits, which showed a higher signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits contained more metabolites, particularly carbohydrates and amino acids, while green fruits had higher aromatic compounds. According to Lacy et al. (2014), the concentration of metabolites could affect the intensity of spectral signals in NMR analysis. The advantage of the NMR method is its ability to concurrently yield qualitative and quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al. 2005).

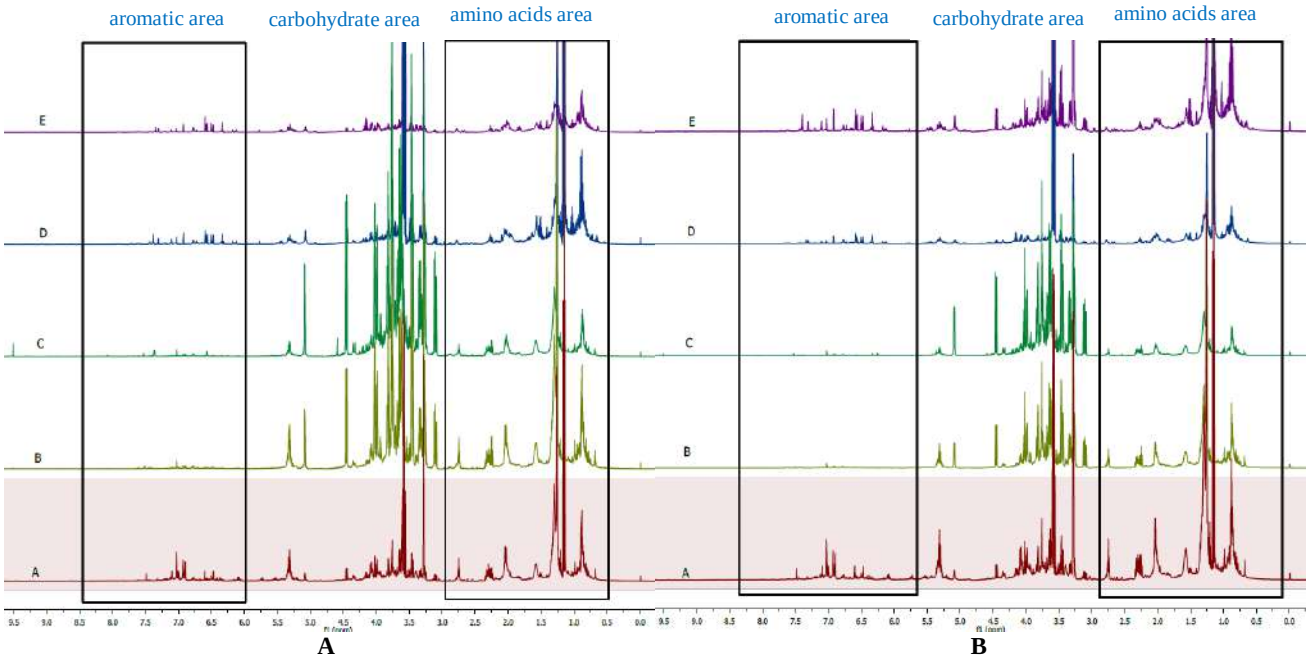

Figure 2. Representative [1]H NMR spectra of: A. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. extracts were collected from Banjar; and B. Fruits and leaves were collected from Batola. A: Young leaves; B: Old leaves; C: Purple fruits; D: Red fruits; E: Green fruits

In this study, despite containing lower concentrations of carbohydrates and amino acids than red and purple fruits, the ethanol extracts of green fruits showed a higher concentration of aromatic compounds. The results of antioxidant capacity (Table 1) showed a higher antioxidant capacity in green fruits than in red and purple fruits at both sample locations. Batola had better antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011) reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based on the [1]H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

Despite the benefits of applying [1]H-NMR in metabolomics studies, challenges arise in chemical identification due to overlapping signals across various regions, specifically in the 5.0-3.0 ppm range corresponding to sugar compounds. This study faced difficulties in discerning signals in the sugar region (except for glucose and sucrose), limiting the detection of certain substances. However, 20 potential compounds were identified through the [1]H NMR spectra analysis (Table 2). The detected amino acid region showed distinct signals corresponding to leucine, glutamate, methionine, and aspartate, characterized by chemical shifts from 3.0 to 0.5 ppm. The chemical shifts of organic acid compounds, including malic, fumaric, and succinic acids, were observed in the 3.0-2.0 ppm range. Sugars, such as mannitol, β-glucose, α-glucose, and sucrose, were commonly detected in the 5.00-3.50 ppm chemical shift. The less crowded regions at 10.0-6.0 ppm showed several phenolics, namely gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The remaining compounds identified were α-linolenic acid, choline, and sterols.

In conclusion, this study identified intensity differences in both regions' aromatic shift (6-7.5 ppm) of leaves and fruits. Young leaves and green fruits had greater intensity, correlating with the obtained antioxidant capacity. The [1]H NMR spectra of young and old leaf extracts for the regional chemical shift 10.0-6.0 ppm, particularly in the aromatic compound area, showed gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid. The difference in spectral signal intensity showed that red and purple fruits contained higher quantities of primary metabolites (specifically carbohydrates and amino acids), while green fruits comprised more aromatic compounds. It signified the potential of *R. tomentosa* leaves and fruits as promising antioxidant agents, although further studies may be needed to determine their role in nutritional applications.

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