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

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Abstract. This study 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 ¹H NMR spectroscopy was used to characterize the compositional profile of extracts (comprising carbohydrates, organic acids, amino acids, and main phenolic compounds) was characterized by ¹H 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 ¹H 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 showed higher quantification levels of metabolites, especially specifically carbohydrates and amino acids, than 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 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 obtained from different stages of maturity, utilizing locations, using combined NMR approach. The study spectroscopy, particularly to identify antioxidant components. Additionally, it effectively showeases 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.

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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 within 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 2472 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.

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Antioxidant analysis

Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2-diphenyl picrylhydrazyl) radical scavenging activity was adapted from the research conducted 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 sample (2 mL) was combined 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 at a wavelength of 516 nm was measured using a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystem 10 Series, USA). Moreover, free radical scavenging activity was also quantified in terms of $\mu\text{mol Trolox}$ (Sigma-Aldrich, Germany) equivalents per unit of dry weight (DW) ($\mu\text{mol TE/g}$).

Sample preparation for $^1\text{H-NMR}$

The production of the $^1\text{H-NMR}$ samples was done using were prepared through a slightly altered methodology from the previously published studies (Gogna et al., 2015; Mishra et al., 2019; Kim et al., 2010). Approximately 25 g of the sample's crude extract was 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 mixture was subjected to vortexing and sonication for 1 min. Using a microcentrifuge, the solution underwent, followed by homogenization followed by 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 $^1\text{H-NMR}$.

$^1\text{H-NMR}$ spectroscopy

The $^1\text{H-NMR}$ analysis was conducted using with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at a temperature of 25°C. 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 60°, and a pre-saturation mode set at 4.27 ppm. Additionally, an internal lock was established using the deuterated solvent. The spectral width was quantified measured within the range of 0 to 10 ppm.

Data analysis

The mean values and standard deviations (mean \pm 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 $^1\text{H-NMR}$ 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 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, 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 to that of the TMSP signal, which served serving as an internal reference. The Subsequently, the $^1\text{H-NMR}$ 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 environment equilibrium was attained (Harbone, 1987).

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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 attenuationscavenging activity of the sample (Molyneux, 2004; Sayuti & Yenrina, 2015).

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Purwakusumah et al. (2016) state that random results in emphasized the measurementsignificance 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 troloxTrolox 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 an analog of water-soluble vitamin E or α -tocopherol analog. The. This study aimed to compare antioxidant capacity expressedcalculated in troloxTrolox 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 contrastHowever, 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.

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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 μ mol TE/g to 2795.33± 9.07 μ mol TE/g (Table 1). An analysis of varianceThe conducted ANOVA followed by an LSD analysis of the ten samples revealed 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 fruitSpecifically, 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 μ mol TE/g and Batola (Banjar Regency locations of) and 2795.33±9.07 μ mol TE/g. The (Batola Regency). Conversely, the lowest yield was found values were observed in purple fruits, with 260.58±0.91 μ mol TE/g and 364.05±3.82 μ mol 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 μ mol TE/g, but reported by Lai et al. (2015). These appeared to be higher than the antioxidant capacity of, 8.79-92.60 μ mol TE/g, identified in grapes, blueberries, blackberries, bananas, oranges, mangoes, kiwifruitkiwifruits, and apples in by Wu et al.'s (2004) study, which is 8.79-92.60 μ mol TE/g. This study demonstrates that suggested the high potential of the purple fruitfruits and other portions of *R. tomentosa* have a high potential as an antioxidant sourcesources.

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Maskam et al. (2014) 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* has had the lowest least DPPH-reducing ability. This indicates showed that the ability of flavonoids to function as potent antioxidants antioxidant and free radical scavengers will depend depended on the location of the hydroxyl group and other structural characteristics of their chemical structure. Wu et al. (2004) found that fruit extracts from measured substantial antioxidant activity in the extract of *R. tomentosa* with fruits 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 the current results obtained for leaves and fruits of *R. tomentosa* in this study, where compounds that act as antioxidants are possible, namely comprising flavonoids and phenols, which have a similar mechanism for functioning as antioxidants antioxidant mechanisms.

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There is a 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 capacity these 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. Flavonoids Furthermore, flavonoids are most majorly prevalent among phenolics and are present in all plant parts. Anti Apart 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* leaf leaves and fruit extracts is closely related to their fruits with potent antioxidant activity.

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

Furthermore, the chemical properties of *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 ¹H NMR spectroscopy. This technique significantly benefits analyzing complex mixtures, such as food extracts. Notably, this method is This non-destructive and requires a relatively spectroscopy method, requiring a simple sample preparation process within a and short duration. Figure 2 depicts the, is significantly advantageous for analyzing complex mixtures, such as food extracts. The representative ¹H NMR spectra of the examined samples are depicted in Figure 2.

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¹H 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 employed used to identify metabolite types of metabolites due to its capability of generating to generate a particular and distinctive spectrum for each compound. The results were A study assessed results based on the quantity of compounds identified, as opposed compared to the signals observed during the NMR analysis (Leiss et al., 2011). Compound identification has been becomes simplified through the widespread application of NMR metabolomics techniques methods. This can be accomplished by comparing the sample signals generated by the samples to those generated by the same compounds in prior reports using CD₃OD-D₂O 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 used serves as a common extraction solvent because it can extract 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 research papers multiple publications, were consulted to conduct a comparative analysis of potentially identifiable signal changes. The, with the coupling constant was a crucial parameter utilized in this study used to authenticate the correspondence between the signals in the data and the references (Kim et al., 2010).

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The ¹H-NMR spectra were separated divided into three regions based on their chemical shifts (δ), namely the aliphatic compounds (amino and organic acids and including terpenoids), carbohydrates, and aromatic compounds. Aliphatic compounds (organic and amino acids) were detected within the chemical shifts 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 ¹H-NMR spectra of leaf extract of leaves and fruit extracts fruits obtained from two locations were analyzed and compared.

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The findings derived from the ¹H-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 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 producerproducers 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 leafleaves and fruit offruits from both locations. The intensity is higherHigher intensities were recorded in young leaves and green fruits than incompared to older leaves and red and purple fruits. This is, consistent with the obtained antioxidant activity.

The ¹H 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 noticeableThe important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, and syringic acid (Table 2). The ¹H-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 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 ¹H 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 disparitydisparities 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 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 ¹H 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 ¹H 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 ¹H-NMR have been demonstrated in diverse in metabolomics studies. Nevertheless, the utilization of ¹H-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 ¹H-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 ¹H 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 indicateshowed that the content of compounds in red and purple fruits has acontained higher quantificationquantities of metabolites, especially specifically 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 necessary would be needed to propose their determine the role played in nutritional applications.

ACKNOWLEDGMENTS

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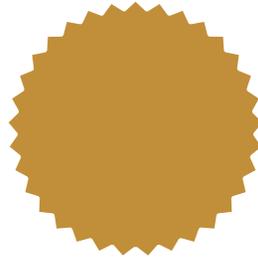
The authors express their gratitudeare grateful to the Indonesian Ministry of Research, Technology, and Higher Education for the Republic of Indonesia for providing financial support for this research endeavor (provided (Grant Number: 130/E5/PG.02.00.PL/2023).

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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



PT. Internasional Translasi Edukasi, Jakarta

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



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

[biodiv] Submission Acknowledgement

Ahmad Dwi Setyawan via SMUJO <support@smujo.com>
Reply-To: Ahmad Dwi Setyawan <editors@smujo.id>
To: Evi <evimintowati@ulm.ac.id>

Tue, Nov 28, 2023 at 12:29 AM

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

Regards,

Dr. Evi Mintowati Kuntorini

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

Author(s) name:

EVI MINTOWATI KUNTORINI¹✉, LILING TRIYASMONO²✉, and MARIA DEWI ASTUTI³

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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 ¹H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

EVI MINTOWATI KUNTORINI¹♥, LILING TRIYASMONO²♥, AND MARIA DEWI ASTUTI³

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³Department of Chemistry, Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, Jl. A. Yani Km. 36, Banjar Baru 70713, South Kalimantan, Indonesia.

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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, 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,

53 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA
54 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022)
55 reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at
56 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
57 leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

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

66 MATERIALS AND METHODS

67 Plant materials

68 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
69 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
70 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
71 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
72 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
73 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.



74
75 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

76 Procedures

77 Crude ethanol extract preparation

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

84 Antioxidant analysis

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

93 Sample preparation for ¹H-NMR

94 The ¹H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015;
95 Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at
96 2 mL with methanol-d₄ solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).

97 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
98 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
99 NMR.

100
101 ¹H NMR spectroscopy

102 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
103 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-
104 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
105 width was measured within the range of 0 to 10 ppm.

106 Data analysis

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

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

120 RESULTS AND DISCUSSION

121 Yield and antioxidant capacity of ethanol extracts

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

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

131
132 **Table 1.** Yield (% w/w) and DPPH antioxidant scavenging capacity of *R. tomentosa* leaves and fruits ethanol extract
133

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

149
150 Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging
151 healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread
152 DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl
153 compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

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

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

163 *R. tomentosa* leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58 ± 0.91
164 $\mu\text{mol TE/g}$ to $2795.33 \pm 9.07 \mu\text{mol TE/g}$ (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten
165 samples statistically indicated significant differences ($P < 0.05$). The findings displayed ethanol extracts possessed similar
166 antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical
167 scavenging capacity, with respective values at $2360.35 \pm 6.86 \mu\text{mol TE/g}$ (Banjar Regency) and $2795.33 \pm 9.07 \mu\text{mol TE/g}$
168 (Batola Regency). Conversely, the lowest values were observed in purple fruits, with $260.58 \pm 0.91 \mu\text{mol TE/g}$ and
169 $364.05 \pm 3.82 \mu\text{mol TE/g}$, respectively, which were below the $431.17 \pm 14.5 \mu\text{mol TE/g}$ reported by Lai et al. (2015). These
170 appeared to be higher than antioxidant capacity, 8.79 - $92.60 \mu\text{mol TE/g}$, identified in grapes, blueberries, blackberries,
171 bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the
172 purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
173 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
174 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
175 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50} : $6.27 \pm 0.25 \text{ g/mL}$)
176 (Cui et al. 2013).

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

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

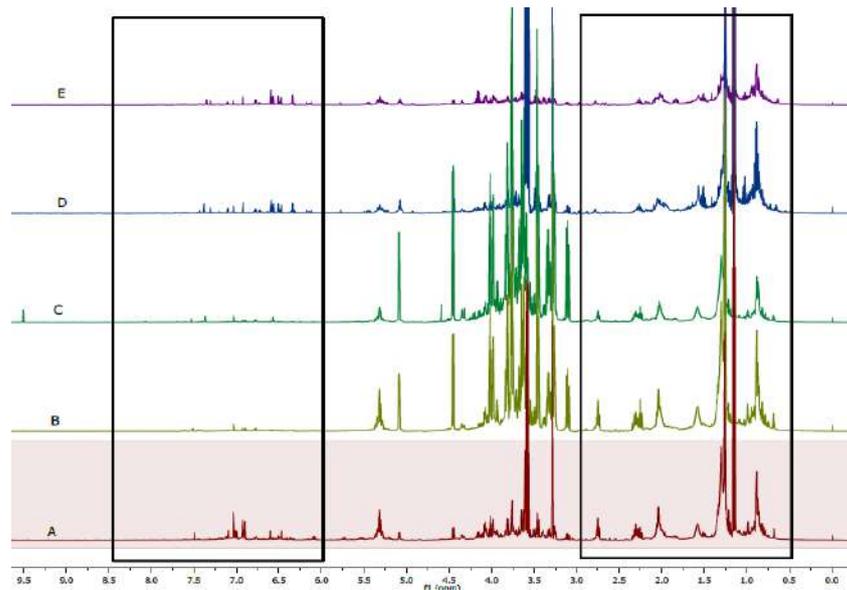
188 The existence of a direct correlation between antioxidant activity and total phenolic content that serves as an indicator
189 has been proven by Idris et al (2022). The presence of total flavonoids (TFC) and total phenolics (TPC) has an important
190 role as electron donors, chain breakers and free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

201 202 **Compounds identification in ^1H NMR spectra of *R. tomentosa* leaves and fruits**

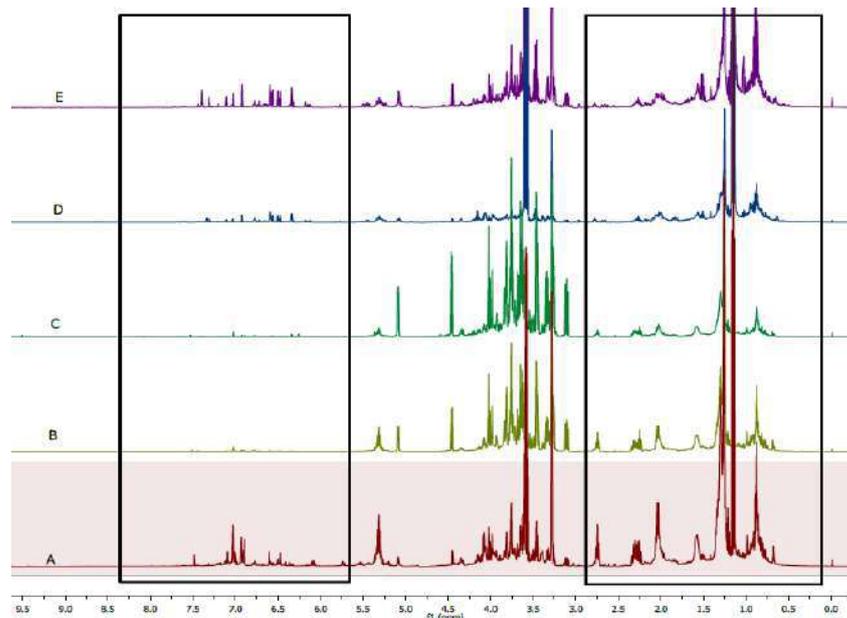
203 The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were characterized for antioxidant
204 activity by ^1H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation
205 process and short duration, is significantly advantageous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

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Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract 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; Mishra 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 $\text{CD}_3\text{OD-D}_2\text{O}$ 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).

226 The ¹H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino
 227 and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of
 228 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
 229 various developmental phases of the ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

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

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

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

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

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

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

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

261 quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al.
262 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and
263 amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test
264 results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample
265 locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011)
266 reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based
267 on the ¹H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually
268 as fruits ripen while accumulating more amino acids and sugars.

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

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

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370

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Thank you for the kindness of the editors.

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



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 ^1H 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,
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U-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa.doc

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9. Reviewer 2's response to reviewer invitation (3-1-2024)
- Manuscript review



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

Wed, Jan 3, 2024 at 5:04 PM

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. tomentos* 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 ¹H 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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, diarrhoea, 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?

50 al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
51 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA
52 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022)
53 reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at
54 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
55 leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

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

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64 MATERIALS AND METHODS

65 Plant materials

66 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
67 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
68 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
69 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
70 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
71 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.



72 |
73 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

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74 Procedures

75 Crude ethanol extract preparation

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

82 Antioxidant analysis

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

91 Sample preparation for ¹H-NMR

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

94 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
 95 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
 96 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
 97 NMR.

98
 99 ¹H NMR spectroscopy

100 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
 101 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-
 102 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
 103 width was measured within the range of 0 to 10 ppm.

104 **Data analysis**

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

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

118 **RESULTS AND DISCUSSION**

119 **Yield and antioxidant capacity of ethanol extracts**

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

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

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129
 130 **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

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

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

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

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

161 *R. tomentosa* leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91
162 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten
163 samples statistically indicated significant differences (P<0.05). The findings displayed ethanol extracts possessed similar
164 antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical
165 scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g
166 (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and
167 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These
168 appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries,
169 bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the
170 purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
171 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
172 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
173 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC₅₀: 6.27±0.25 g/mL)
174 (Cui et al. 2013).

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

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

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

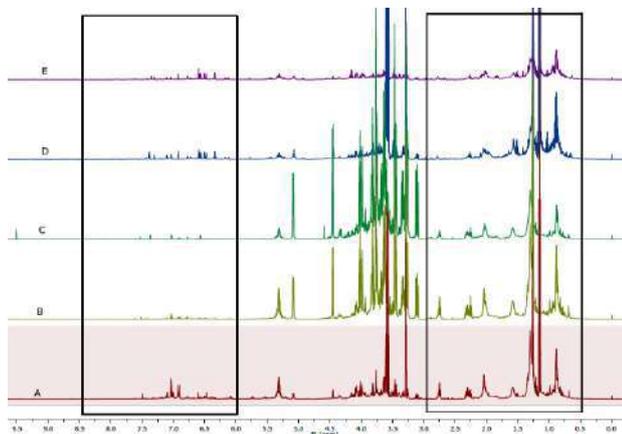
200 Compounds identification in ¹H NMR spectra of *R. tomentosa* leaves and fruits

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

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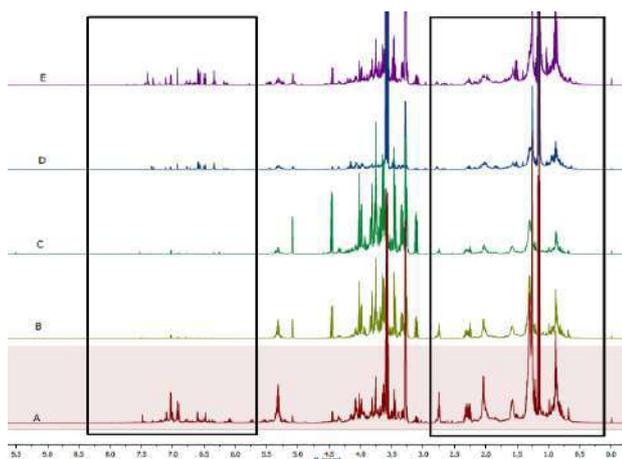


Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in Batola of *Rhodomomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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; Misra 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 $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 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 ¹H 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 ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

The ¹H-NMR analysis 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 ¹H 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-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. 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. ¹H 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- rhamnopyranoside	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 ¹H 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

259 quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al.
260 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and
261 amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test
262 results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample
263 locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011)
264 reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based
265 on the ¹H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually
266 as fruits ripen while accumulating more amino acids and sugars.

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

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

286
287 Note : In the discussion section, the author has to provide a brief description of the conditions found in the regions of
288 Banjar and Batola, including details about the soil, water/climate, and the author's aim for collecting samples from these
289 areas. Additionally, the author can discuss and compare the results of the antioxidant activity observed in organs like
290 leaves and fruit obtained from both locations.

291 ACKNOWLEDGMENTS

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

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



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 <i>R. tomentos</i> 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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3 attachments

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 **Rev-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa (Reviewer 2).doc**
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 **Rev-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa (Revision 2).doc**
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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 ¹H 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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, diarrhoea, 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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50 (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, 52 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA 53 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) 54 previously reported the most significant antioxidant activity in the green fruits from Banjarbaru, with values of DPPH as 55 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 56 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.

57 [The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a 58 lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is 59 considered a pest due to the rapid growth rate possessed. This study is a continuation of the previous one as part of a series 60 of research roadmaps, there has been no comprehensive study on the metabolic and antioxidant profiles of leaves and fruits 61 at various stages of maturity, originating from two different locations. No comprehensive investigation has been conducted 62 on the metabolic and antioxidant profiles of leaves and fruits at various maturation stages, originating from two separate 63 locations. This examination represents inaugural systematic analysis of metabolites contained in *R. tomentosa* leaves as 64 well as fruits obtained from different locations, using combined NMR spectroscopy, particularly to identify antioxidant 65 components. Additionally, it effectively shows the suitability and effectiveness of the NMR method in analyzing the 66 metabolites of plants

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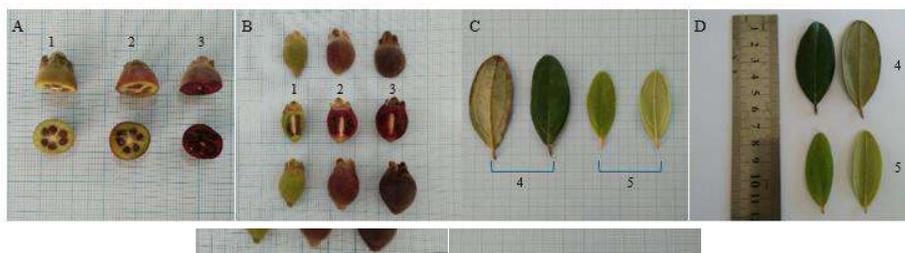
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67 MATERIALS AND METHODS

68 Plant materials

69 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple, 70 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue 71 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S, 72 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023. 73 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed 74 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.



75 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. **A.** Fruits from Batola, **B.** Fruits from Banjar, **C.** Leaves from Batola 76 **D.** Leaves from Banjar. 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves. 77 78

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79 Procedures

80 Crude ethanol extract preparation

81 Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in 82 an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in 83 ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini 84 et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a 85 rotary evaporator until dried, then stored in a refrigeration unit for further analysis.

86 Antioxidant analysis

87 Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2-diphenyl 88 picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the 89 appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as 90 well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm 91 was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystem 10 Series, USA). Moreover, 92

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

96 Sample preparation for $^1\text{H-NMR}$

97 The ^1H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015;
98 Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at
99 2 mL with methanol- d_4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
100 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
101 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ^1H
102 NMR.

104 ^1H NMR spectroscopy

105 The ^1H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
106 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-
107 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
108 width was measured within the range of 0 to 10 ppm.

109 Data analysis

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

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

123 RESULTS AND DISCUSSION

124 Yield and antioxidant capacity of ethanol extracts

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

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

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

141 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 \pm 3.43 ^d	1429.53 \pm 9.07 ^d
Old leaves	12.296	11.49	836.20 \pm 9.07 ^c	881.24 \pm 5.94 ^c

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Green fruits	5.63	6.05	2360.35 ± 6.86 ^a	2795.33 ± 9.07 ^a
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.

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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 μ mol TE/g to 2795.33± 9.07 μ mol 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 μ mol TE/g (Banjar Regency) and 2795.33±9.07 μ mol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 μ mol TE/g and 364.05±3.82 μ mol TE/g, respectively, which were below the 431.17±14.5 μ mol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 μ mol 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 (IC₅₀: 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. tomentosa* plants around the Banjarbaru area, continuing the results of previous studies.

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

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

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

227 **Compounds identification in ¹H NMR spectra of *R. tomentosa* leaves and fruits**

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

232 aromatic area carbohydrate area amino acids area

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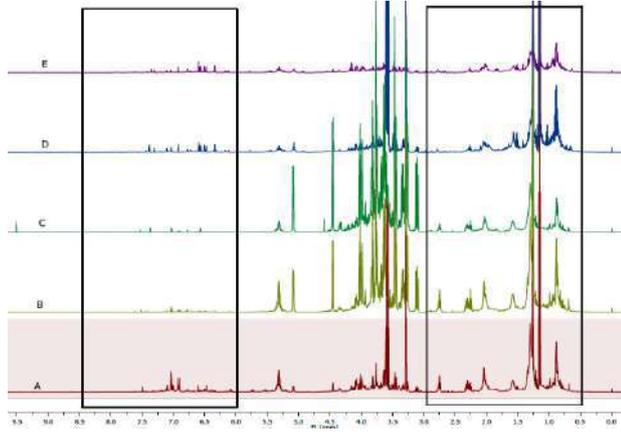
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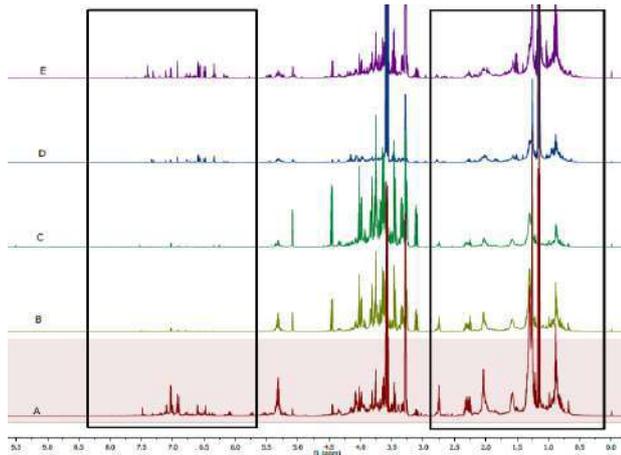


Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in Batola of *Rhodomomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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; Misra 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 $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 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).

252 The ¹H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino
 253 and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of
 254 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
 255 various developmental phases of the ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

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

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

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

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

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

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

279
 280 A comparison of the ¹H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical
 281 signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits
 282 showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher
 283 signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits

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284 contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic
285 compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral
286 signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and
287 quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al.
288 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and
289 amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test
290 results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample
291 locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011)
292 reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based
293 on the ¹H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually
294 as fruits ripen while accumulating more amino acids and sugars.

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

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

314
315 Note : In the discussion section, the author has to provide a brief description of the conditions found in the regions of
316 Banjar and Batola, including details about the soil, water/climate, and the author's aim for collecting samples from these
317 areas. Additionally, the author can discuss and compare the results of the antioxidant activity observed in organs like
318 leaves and fruit obtained from both locations.

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321 Indonesia for providing financial support for this research endeavor (Number: 130/E5/PG.02.00.PL/2023).

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Antioxidant activity and ¹H 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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, diarrhoea, 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

50 al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
51 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA
52 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022)
53 [previously](#) reported the most significant antioxidant activity in the green fruits [from Banjarbaru](#), with values of DPPH as
54 well as FRAP at 1419.75 ± 3.48 as well as 1367.59 ± 9.12 $\mu\text{mol TE/g DW}$. Ethanol extracts showed the highest values of
55 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 .

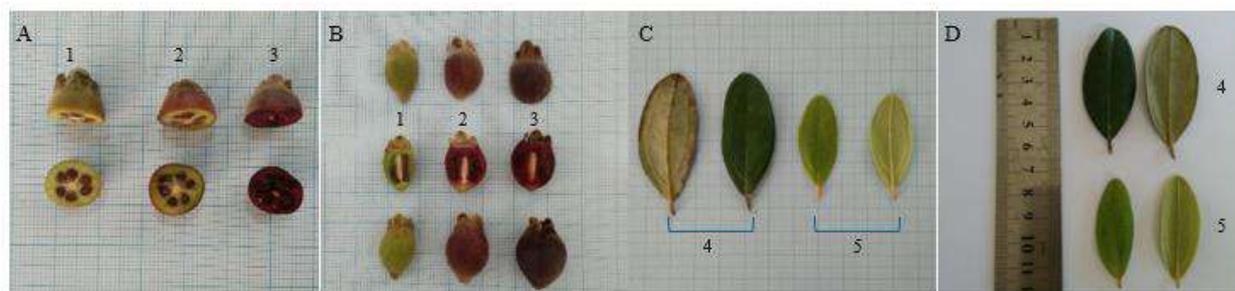
56 The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a
57 lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is
58 considered a pest due to the rapid growth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

64

MATERIALS AND METHODS

Plant materials

65 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
66 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
67 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
68 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
69 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
70 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.
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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

76 Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in
77 an oven, followed by grinding at ambient temperature. Approximately 500 g of each ground material was macerated in
78 ethanol at 1000 mL (SmartLab, Indonesia) for 72 h by replacing the solvent every 24 h (Nurcholis et al. 2021; Kuntorini
79 et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a
80 rotary evaporator until dried, then stored in a refrigeration unit for further analysis.
81
82

Antioxidant analysis

83 Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2-diphenyl
84 picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the
85 appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as
86 well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm
87 was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesystem 10 Series, USA). Moreover,
88 the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (μmol
89 TE/g), using Trolox from Sigma-Aldrich, Germany.
90
91

Sample preparation for ¹H-NMR

92 The ¹H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015;
93 Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at
94
95

96 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
97 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
98 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
99 NMR.

100 101 ¹H NMR spectroscopy

102 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
103 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-
104 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
105 width was measured within the range of 0 to 10 ppm.

106 **Data analysis**

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

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

120 **RESULTS AND DISCUSSION**

121 **Yield and antioxidant capacity of ethanol extracts**

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

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

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

137
138 **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

151 Note: The presented data consists of the mean ± standard deviation. Data were evaluated using one-way ANOVA and the LSD test with =
152 0.05. Numbers followed by various superscript letters in the same column produce significantly different outcomes.
153

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

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

167 *R. Tomentosa* leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58 ± 0.91
168 $\mu\text{mol TE/g}$ to $2795.33 \pm 9.07 \mu\text{mol TE/g}$ (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten
169 samples statistically indicated significant differences ($P < 0.05$). The findings displayed ethanol extracts possessed similar
170 antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical
171 scavenging capacity, with respective values at $2360.35 \pm 6.86 \mu\text{mol TE/g}$ (Banjar Regency) and $2795.33 \pm 9.07 \mu\text{mol TE/g}$
172 (Batola Regency). Conversely, the lowest values were observed in purple fruits, with $260.58 \pm 0.91 \mu\text{mol TE/g}$ and
173 $364.05 \pm 3.82 \mu\text{mol TE/g}$, respectively, which were below the $431.17 \pm 14.5 \mu\text{mol TE/g}$ reported by Lai et al. (2015). These
174 appeared to be higher than antioxidant capacity, $8.79\text{-}92.60 \mu\text{mol TE/g}$, identified in grapes, blueberries, blackberries,
175 bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the
176 purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
177 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
178 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
179 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50} : $6.27 \pm 0.25 \text{ g/mL}$)
180 (Cui et al. 2013).

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

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

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

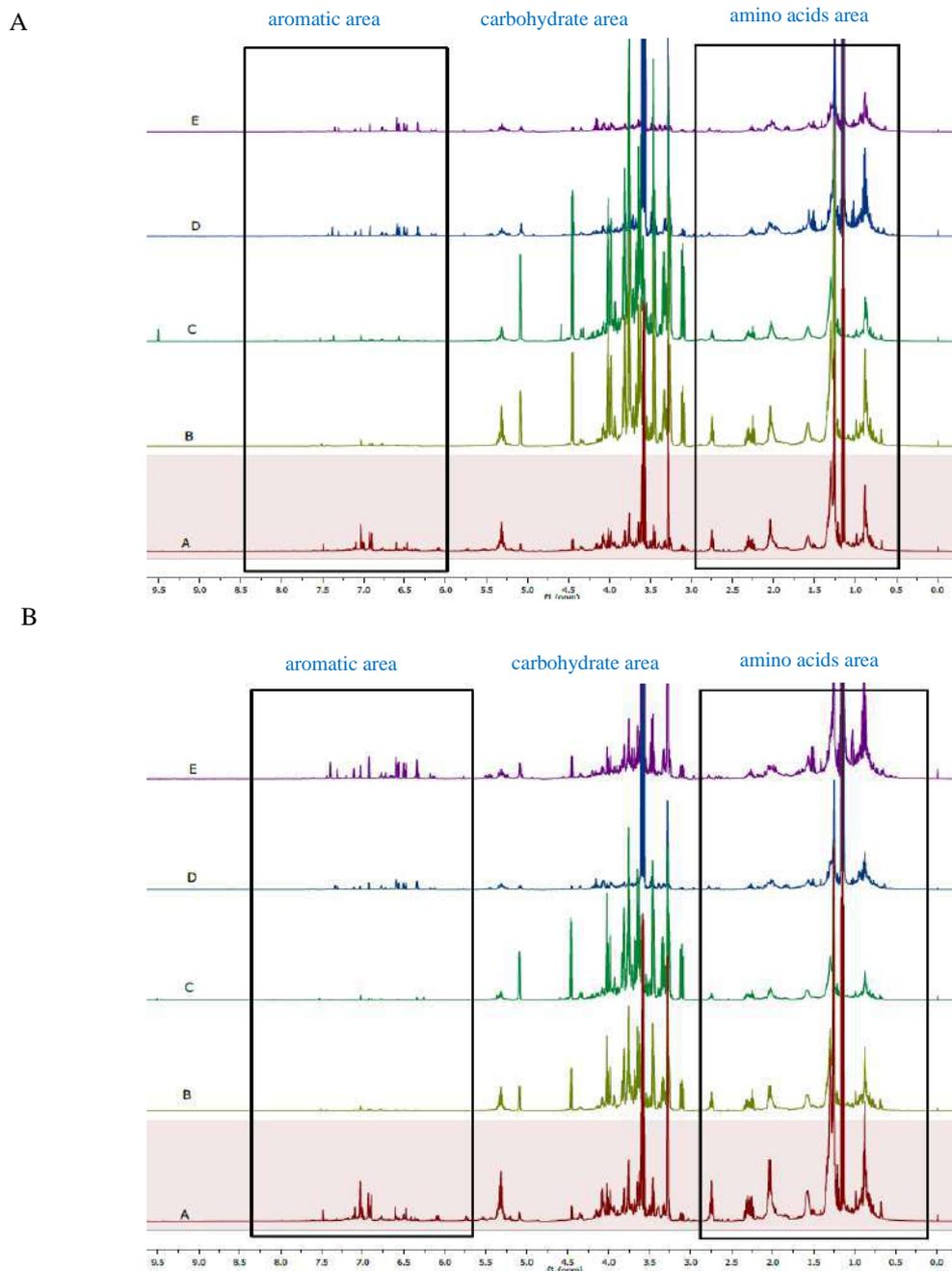
199 The study by Ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased
200 antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron*
201 *heudelotii*, with the lowest phenolic and vitamin C content,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

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

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

221 Compounds identification in ¹H NMR spectra of *R. tomentosa* leaves and fruits

222 The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were characterized for antioxidant
223 activity by ¹H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation
224 process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The
225 representative ¹H NMR spectra of the examined samples are depicted in Figure 2.
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Figure 2. Representative ¹H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

232 NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic
 233 fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate
 234 a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for
 235 metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and
 236 reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds
 237 identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes
 238 simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample
 239 signals to those generated by the same compounds in prior reports using CD₃OD-D₂O as the solvent (Kim et al. 2010; Ali
 240 et al. 2011; Nuringtyas et al. 2012; Gogna et al. 2015; Cerulli. 2018; Mishra et al. 2019). Aqueous methanol serves as a
 241 common extraction solvent capable of extracting a broad spectrum of polar and non-polar compounds. Various solvents
 242 can impact the chemical shift of substances in NMR. Therefore, multiple publications were consulted to conduct a
 243 comparative analysis of potentially identifiable signal changes, with the coupling constant as a crucial parameter used to
 244 authenticate correspondence between signals in the data and references (Kim et al. 2010).

245 The ¹H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino
 246 and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of
 247 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
 248 various developmental phases of the ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

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

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

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

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

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

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

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

A comparison of the ¹H 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 ¹H 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 ¹H-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 ¹H 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-rhamnopyranoside, 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 ¹H 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-rhamnopyranoside, 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)



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

Wed, Mar 27, 2024 at 10:07 AM

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)



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

Invitation to review for manuscript

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>
To: "Dr. Whika Febria Dewatisari , S.Si., M.Si" <whika@ecampus.ut.ac.id>

Wed, Mar 27, 2024 at 11:18 AM

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)



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

Invitation to review for manuscript

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>
To: siti hamidatul aliyah <sitihamidatula@gmail.com>

Mon, Feb 12, 2024 at 11:29 AM

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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 **U-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa..doc**
4098K

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



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 ¹H 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]



U-Antioxidant activity and 1H NMR profiling *Rhodomyrtus tomentosa*..doc
4098K

Antioxidant activity and ¹H 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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, 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

50 al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
51 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA
52 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022)
53 reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at
54 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
55 leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

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

64 MATERIALS AND METHODS

65 Plant materials

66 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
67 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
68 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
69 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
70 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
71 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.



72
73 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

74 Procedures

75 Crude ethanol extract preparation

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

82 Antioxidant analysis

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

91 Sample preparation for ¹H-NMR

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

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?

94 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
95 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
96 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
97 NMR.

98 ¹H NMR spectroscopy

99 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
100 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-
101 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
102 width was measured within the range of 0 to 10 ppm.
103

104 Data analysis

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

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

118 RESULTS AND DISCUSSION

119 Yield and antioxidant capacity of ethanol extracts

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

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

129
130 **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

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

147
148 Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging
149 healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread
150 DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl
151 compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

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

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

161 *R. tomentosa* leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58 ± 0.91
162 $\mu\text{mol TE/g}$ to $2795.33 \pm 9.07 \mu\text{mol TE/g}$ (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten
163 samples statistically indicated significant differences ($P < 0.05$). The findings displayed ethanol extracts possessed similar
164 antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical
165 scavenging capacity, with respective values at $2360.35 \pm 6.86 \mu\text{mol TE/g}$ (Banjar Regency) and $2795.33 \pm 9.07 \mu\text{mol TE/g}$
166 (Batola Regency). Conversely, the lowest values were observed in purple fruits, with $260.58 \pm 0.91 \mu\text{mol TE/g}$ and
167 $364.05 \pm 3.82 \mu\text{mol TE/g}$, respectively, which were below the $431.17 \pm 14.5 \mu\text{mol TE/g}$ reported by Lai et al. (2015). These
168 appeared to be higher than antioxidant capacity, 8.79 - $92.60 \mu\text{mol TE/g}$, identified in grapes, blueberries, blackberries,
169 bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the
170 purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
171 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
172 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
173 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50} : $6.27 \pm 0.25 \text{ g/mL}$)
174 (Cui et al. 2013).

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

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

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

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

200 The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were characterized for antioxidant
201 activity by ^1H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation
202 process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The
203 representative ^1H 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?

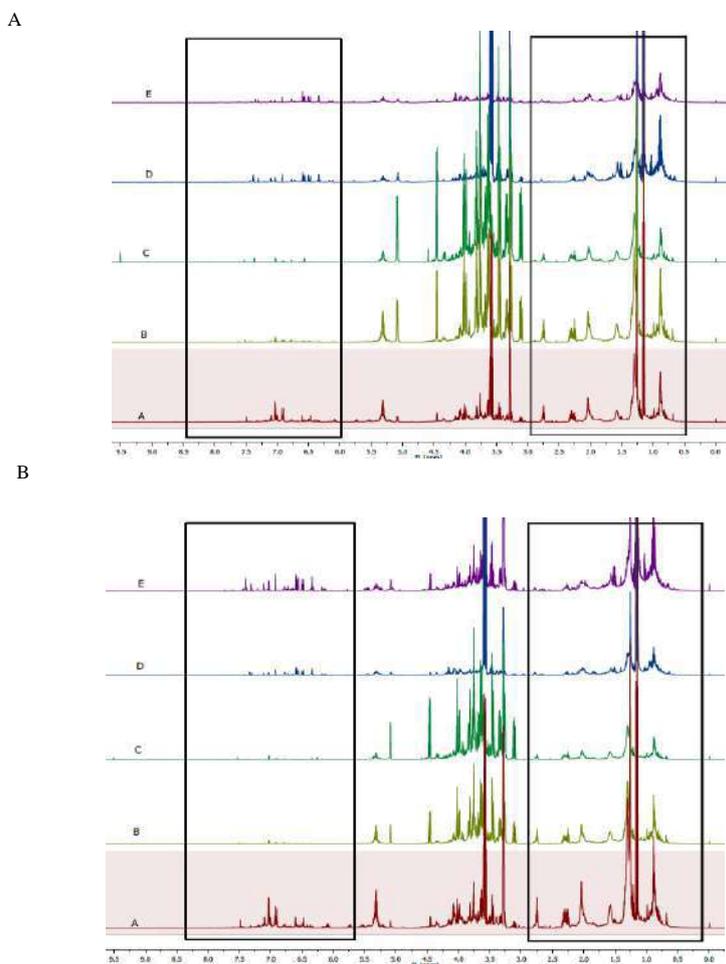


Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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; Misra 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 $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 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).

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

224 The ¹H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino
 225 and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of
 226 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
 227 various developmental phases of the ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

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

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

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

248 **Table 2.** ¹H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and
 249 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

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

251
 252 A comparison of the ¹H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical
 253 signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits
 254 showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher
 255 signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits
 256 contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic
 257 compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral
 258 signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

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?

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*?

259 quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al.
260 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and
261 amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test
262 results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample
263 locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011)
264 reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based
265 on the ¹H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually
266 as fruits ripen while accumulating more amino acids and sugars.

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

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

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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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367 phenolic profiles during wine aging. Food Res Int 106:568-579. DOI: 10.1016/j.foodres.2017.12.054
368

15. Third Revised submission for reviewer 3th (25-3-2024)
 - Cover Letter revision Journal Biodiversitas
 - Revised manuscript



Evi M Kuntorini ULM <evimintowati1169@gmail.com>

Manuscript Review Feedback

Evi M Kuntorini ULM <evimintowati1169@gmail.com>
 Kepada: siti hamidatul aliyah <sitihamidatula@gmail.com>

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 <i>R. tomentosa</i> 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 <i>R. tomentosa</i> 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 <i>R. tomentosa</i> extracts, and how could this contribute to the understanding of its health benefits?	Thank you for the comment. The antioxidant properties of <i>R. tomentosa</i> 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,

		<p>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.</p>
5	<p>Could you explain in more detail what the numbers at the bottom indicate and what their relationship is to the peaks?</p>	<p>Thank you for the suggestion. We have added a caption to the figure of ^1H NMR spectra divided into three regions based on chemical shifts (figure 2) and the explanation on page 6 lines 232-248.</p>
6	<p>What differences were observed in the aromatic region of the NMR spectra between young and old leaves, as well as green and mature fruits?</p>	<p>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 (Table 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.</p>
7	<p>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 <i>Rhodomyrtus tomentosa</i>?</p>	<p>Thank you for your question. The implication of the difference in spectral signal intensity among green, red, and purple fruits in ^1HNMR 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).</p>
8	<p>Based on the results, do all parts of the plant have the potential as antioxidants or which one is the most potential?</p>	<p>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 <i>R.</i></p>

	<i>tomentosa</i> is a wild plant and it is difficult to obtain ripe fruits in large quantities as antioxidant material (page 7 line 286-291).
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Again thank you for your kind support and consideration to our manuscript.

Regards,

Dr. Evi Mintowati Kuntorini

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Lambung Mangkurat University

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[Kutipan teks disembunyikan]

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 **U-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa. (Reviewer 3).doc**
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 **U-Antioxidant activity and 1H NMR profiling Rhodomyrtus tomentosa. (Revision 3).doc**
4085K

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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 ¹H 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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, 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

50 al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
51 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA
52 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022)
53 reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at
54 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
55 leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

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

64 MATERIALS AND METHODS

65 Plant materials

66 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
67 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
68 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
69 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
70 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
71 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.



72
73 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

74 Procedures

75 Crude ethanol extract preparation

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

82 Antioxidant analysis

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

91 Sample preparation for ¹H-NMR

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

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?

94 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
95 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
96 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
97 NMR.

98 ¹H NMR spectroscopy

99 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
100 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-
101 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
102 width was measured within the range of 0 to 10 ppm.
103

104 Data analysis

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

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

118 RESULTS AND DISCUSSION

119 Yield and antioxidant capacity of ethanol extracts

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

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

129
130 **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

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

147
148 Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging
149 healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread
150 DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl
151 compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

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

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

161 *R. tomentosa* leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91
162 $\mu\text{mol TE/g}$ to 2795.33± 9.07 $\mu\text{mol TE/g}$ (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten
163 samples statistically indicated significant differences ($P < 0.05$). The findings displayed ethanol extracts possessed similar
164 antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical
165 scavenging capacity, with respective values at 2360.35±6.86 $\mu\text{mol TE/g}$ (Banjar Regency) and 2795.33±9.07 $\mu\text{mol TE/g}$
166 (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 $\mu\text{mol TE/g}$ and
167 364.05±3.82 $\mu\text{mol TE/g}$, respectively, which were below the 431.17±14.5 $\mu\text{mol TE/g}$ reported by Lai et al. (2015). These
168 appeared to be higher than antioxidant capacity, 8.79-92.60 $\mu\text{mol TE/g}$, identified in grapes, blueberries, blackberries,
169 bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the
170 purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
171 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
172 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
173 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50} : 6.27±0.25 g/mL)
174 (Cui et al. 2013).

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

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

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

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

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

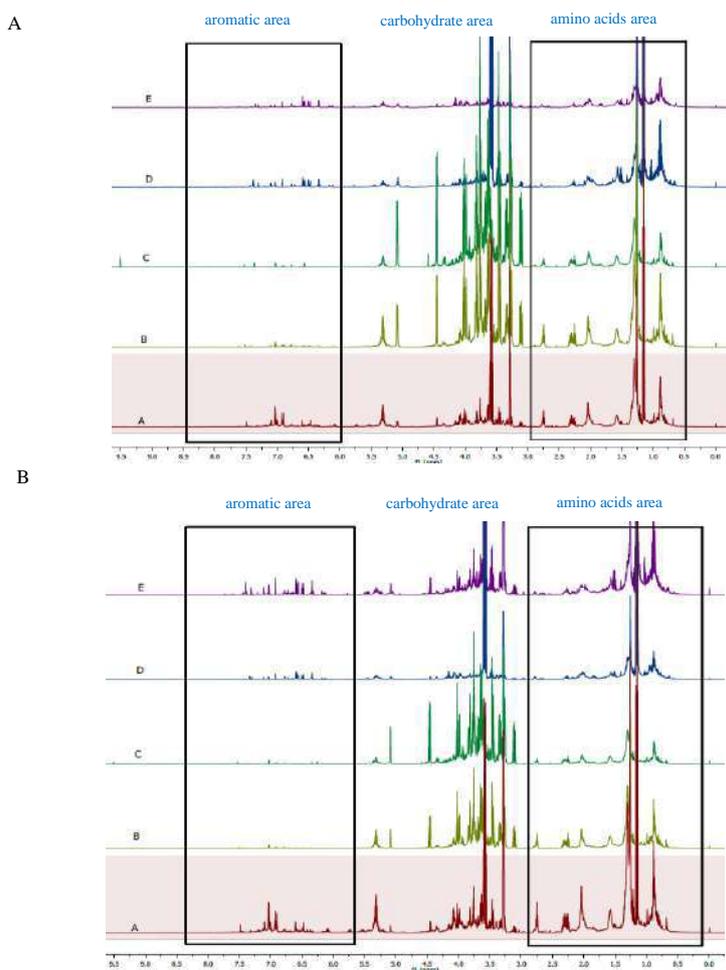
208 The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were characterized for antioxidant
209 activity by ^1H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation
210

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

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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?

211 process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The
212 representative ^1H NMR spectra of the examined samples are depicted in Figure 2.
213



214

215 **Figure 2.** Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in
216 Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.
217
218

219 NMR spectroscopy, which ascertains the magnetic resonance of molecular nuclei in the presence of external magnetic
220 fields (Hatada and Kitayama, 2004), has been widely used to identify types of metabolites due to its capability to generate
221 a particular and distinctive spectrum for each compound. Additionally, NMR spectroscopy is widely used as a method for
222 metabolomic investigations in a variety of organisms, due to its non-invasive and quantitative character, robustness and
223 reproducibility (Deborde et al. 2017; Misrha et al. 2019). A study assessed results based on the quantity of compounds
224 identified, compared to the signals observed during NMR analysis (Leiss et al. 2011). Compound identification becomes
225 simplified through the widespread application of NMR metabolomics methods. This is accomplished by comparing sample
226 signals to those generated by the same compounds in prior reports using $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ as the solvent (Kim et al. 2010; Ali

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

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 ¹H 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 ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

The ¹H-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 ¹H 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-rhamnopyranoside, 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.

Table 2. ¹H 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- rhamnopyranoside	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

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?

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

A comparison of the ¹H 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 ¹H 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 ¹H-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 ¹H 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 ¹H 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

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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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?

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376

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



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)



Evi M Kuntorini ULM <evimintowati1169@gmail.com>

Manuscript Review Feedback

Evi M Kuntorini ULM <evimintowati1169@gmail.com>
Kepada: siti hamidatul aliyah <sitihamidatula@gmail.com>

25 Maret 2024 pukul 18.26

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,

Dr. Evi Mintowati Kuntorini

Associate Professor

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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



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

[biodiv] Editor Decision

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>
To: Smujo Editors <editors@smujo.id>

Tue, Apr 2, 2024 at 12:39 AM

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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E-mail : evimintowati@ulm.ac.id

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.

Reviewer 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 <i>R. tomentos</i> 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 <i>R tomentosa</i>	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 <i>R. tomentosa</i> 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 <i>R. tomentosa</i> 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 <i>R. tomentosa</i> extracts, and how could this contribute to the understanding of its health benefits?	Thank you for the comment. The antioxidant properties of <i>R. tomentosa</i> 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-rhamnopyranoside, 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 (Table 1). Important flavonoid compounds identified included gallic acid, myricetin, myricetin 3-O-rhamnopyranoside, 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 <i>Rhodomyrtus tomentosa</i> ?	among green, red, and purple fruits in ^1H NMR 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-rhamnopyranoside, 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 <i>R. tomentosa</i> 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 ¹H 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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, diarrhoea, 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?

50 al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
51 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA
52 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022)
53 reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at
54 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
55 leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

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

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64 MATERIALS AND METHODS

65 Plant materials

66 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
67 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
68 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
69 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
70 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
71 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.



72 |
73 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

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74 Procedures

75 Crude ethanol extract preparation

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

81

82 Antioxidant analysis

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

90

91 Sample preparation for ¹H-NMR

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

94 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
 95 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
 96 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
 97 NMR.

98
 99 ¹H NMR spectroscopy

100 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
 101 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-
 102 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
 103 width was measured within the range of 0 to 10 ppm.

104 **Data analysis**

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

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

118 **RESULTS AND DISCUSSION**

119 **Yield and antioxidant capacity of ethanol extracts**

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

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

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129
 130 **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

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

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

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

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

161 *R. tomentosa* leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91
162 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten
163 samples statistically indicated significant differences (P<0.05). The findings displayed ethanol extracts possessed similar
164 antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical
165 scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g
166 (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and
167 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These
168 appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries,
169 bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the
170 purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
171 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
172 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
173 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC₅₀: 6.27±0.25 g/mL)
174 (Cui et al. 2013).

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

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

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

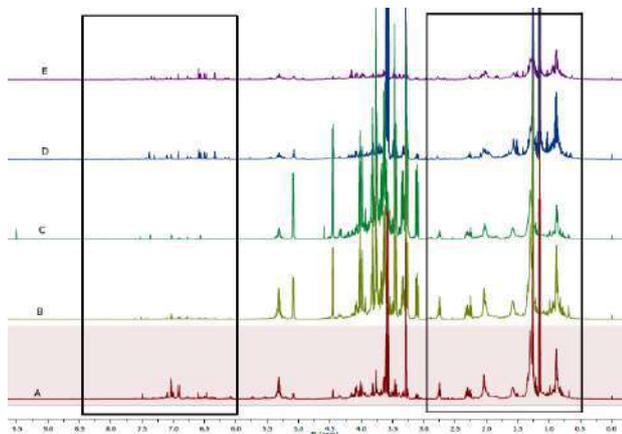
200 Compounds identification in ¹H NMR spectra of *R. tomentosa* leaves and fruits

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

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A



B

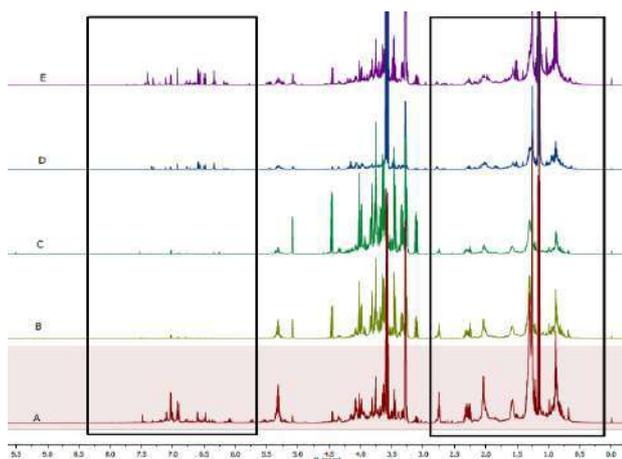


Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in Batola of *Rhodomomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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; Misra 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 $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 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 ¹H 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 ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

The ¹H-NMR analysis 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 ¹H 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. ¹H 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 ¹H 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

259 quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al.
260 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and
261 amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test
262 results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample
263 locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011)
264 reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based
265 on the ¹H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually
266 as fruits ripen while accumulating more amino acids and sugars.

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

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

286 Note : In the discussion section, the author has to provide a brief description of the conditions found in the regions of
287 Banjar and Batola, including details about the soil, water/climate, and the author's aim for collecting samples from these
288 areas. Additionally, the author can discuss and compare the results of the antioxidant activity observed in organs like
289 leaves and fruit obtained from both locations.
290

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373

Antioxidant activity and ¹H 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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, 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

50 al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
51 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA
52 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022)
53 reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at
54 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
55 leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

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

64 MATERIALS AND METHODS

65 Plant materials

66 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
67 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
68 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
69 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
70 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
71 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.



72
73 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

74 Procedures

75 Crude ethanol extract preparation

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

82 Antioxidant analysis

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

91 Sample preparation for ¹H-NMR

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

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?

94 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
95 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
96 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
97 NMR.

98 ¹H NMR spectroscopy

99 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
100 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-
101 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
102 width was measured within the range of 0 to 10 ppm.
103

104 Data analysis

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

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

118 RESULTS AND DISCUSSION

119 Yield and antioxidant capacity of ethanol extracts

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

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

129
130 **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

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

147
148 Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging
149 healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread
150 DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl
151 compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

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

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

161 *R. tomentosa* leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91
162 $\mu\text{mol TE/g}$ to 2795.33± 9.07 $\mu\text{mol TE/g}$ (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten
163 samples statistically indicated significant differences ($P < 0.05$). The findings displayed ethanol extracts possessed similar
164 antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical
165 scavenging capacity, with respective values at 2360.35±6.86 $\mu\text{mol TE/g}$ (Banjar Regency) and 2795.33±9.07 $\mu\text{mol TE/g}$
166 (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 $\mu\text{mol TE/g}$ and
167 364.05±3.82 $\mu\text{mol TE/g}$, respectively, which were below the 431.17±14.5 $\mu\text{mol TE/g}$ reported by Lai et al. (2015). These
168 appeared to be higher than antioxidant capacity, 8.79-92.60 $\mu\text{mol TE/g}$, identified in grapes, blueberries, blackberries,
169 bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the
170 purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
171 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
172 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
173 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50} : 6.27±0.25 g/mL)
174 (Cui et al. 2013).

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

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

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

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

200 The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were characterized for antioxidant
201 activity by ^1H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation
202 process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The
203 representative ^1H 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?

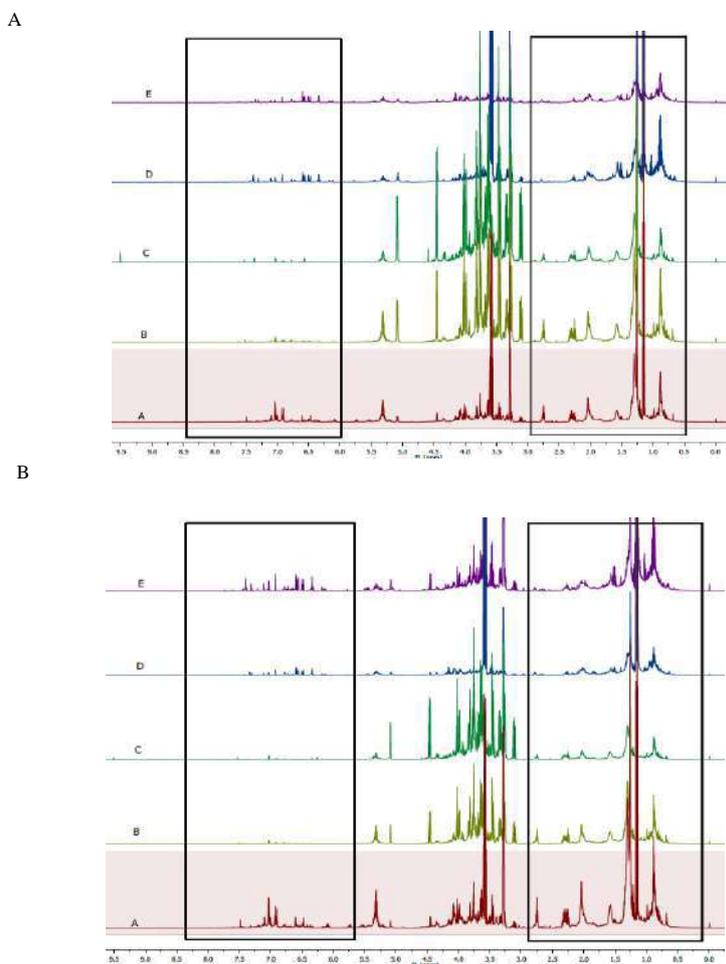


Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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; Misra 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 $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 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).

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

224 The ¹H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino
 225 and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of
 226 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
 227 various developmental phases of the ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

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

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

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

248 **Table 2.** ¹H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and
 249 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

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

251
 252 A comparison of the ¹H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical
 253 signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits
 254 showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher
 255 signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits
 256 contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic
 257 compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral
 258 signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

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?

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*?

259 quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al.
260 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and
261 amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test
262 results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample
263 locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011)
264 reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based
265 on the ¹H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually
266 as fruits ripen while accumulating more amino acids and sugars.

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

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

286 ACKNOWLEDGMENTS

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288 Indonesia for providing financial support for this research endeavor (Number: 130/E5/PG.02.00.PL/2023).

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368

Antioxidant activity and ¹H 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. ¹H 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 ¹H NMR spectra of young and old leaves extract identified key 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 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, 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

50 al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
51 flowers, and stems due to the exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as the DNA
52 damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022)
53 reported the most significant antioxidant activity in the green fruits, with values of DPPH as well as FRAP at
54 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
55 leaves as well as green fruits, measuring 96.375±3.96 as well as 95.731±5.42 mg QE/g DW.

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

64 MATERIALS AND METHODS

65 Plant materials

66 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
67 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
68 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
69 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
70 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
71 samples with certificate number of 1007/IPH.1.01/If.07/IX/2023.



72
73 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk.

74 Procedures

75 Crude ethanol extract preparation

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

82 Antioxidant analysis

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

91 Sample preparation for ¹H-NMR

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

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?

94 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
95 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
96 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
97 NMR.

98 ¹H NMR spectroscopy

99 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
100 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-
101 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
102 width was measured within the range of 0 to 10 ppm.
103

104 Data analysis

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

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

118 RESULTS AND DISCUSSION

119 Yield and antioxidant capacity of ethanol extracts

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

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

129
130 **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

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

147
148 Antioxidants can bind to free radicals and thus prevent many diseases, including various types of NDs, from damaging
149 healthy cells. Its activity can be measured by several in vitro experiments. One of the most simple, rapid, and widespread
150 DPPH measurements (Rondevaldova et al. 2022). The ethanol extracts of *R. tomentosa* samples, rich in hydroxyl
151 compounds, reduced DPPH to DPPH-H through hydrogen donation. An increase in 1,1-diphenyl-2-picryl-hydrazyn level

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

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

161 *R. tomentosa* leaves and fruits show antioxidant capacity as calculated by DPPH method ranged from 260.58±0.91
162 µmol TE/g to 2795.33± 9.07 µmol TE/g (Table 1.). The conducted ANOVA followed by an LSD analysis of the ten
163 samples statistically indicated significant differences (P<0.05). The findings displayed ethanol extracts possessed similar
164 antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH radical
165 scavenging capacity, with respective values at 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g
166 (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 µmol TE/g and
167 364.05±3.82 µmol TE/g, respectively, which were below the 431.17±14.5 µmol TE/g reported by Lai et al. (2015). These
168 appeared to be higher than antioxidant capacity, 8.79-92.60 µmol TE/g, identified in grapes, blueberries, blackberries,
169 bananas, oranges, mangoes, kiwifruits, and apples by Wu et al. (2015). This study suggested the high potential of the
170 purple fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
171 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
172 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
173 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC₅₀: 6.27±0.25 g/mL)
174 (Cui et al. 2013).

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

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

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

199 **Compounds identification in ¹H NMR spectra of *R. tomentosa* leaves and fruits**

200 The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were characterized for antioxidant
201 activity by ¹H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation
202 process and short duration, is significantly advantageous for analyzing complex mixtures, such as food extracts. The
203 representative ¹H 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?

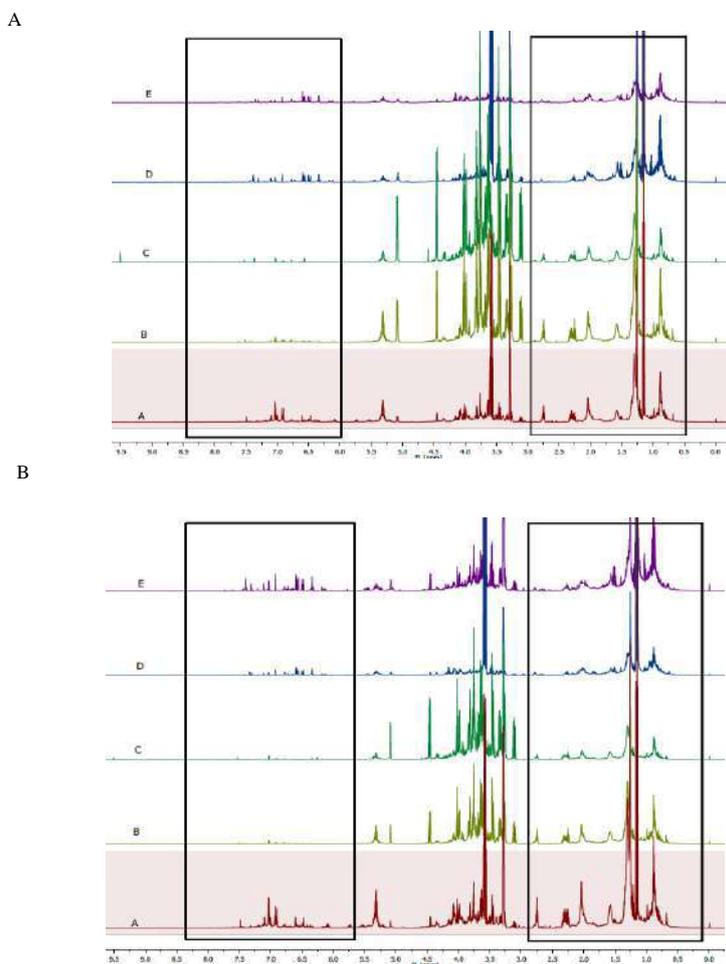


Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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; Misra 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 $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 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).

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

224 The ¹H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino
 225 and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of
 226 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
 227 various developmental phases of the ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

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

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

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

248 **Table 2.** ¹H NMR chemical shifts (δ) and coupling constants (Hz) of putative metabolites of *R. tomentosa* leaves and
 249 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

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

251
 252 A comparison of the ¹H NMR spectral analysis of extracts from purple, red, and green fruits showed nearly identical
 253 signal diversity among the three samples (Figure 2). However, in terms of signal intensity or integral, red and purple fruits
 254 showed more pronounced results for carbohydrates and amino acids compared to green fruits, which showed a higher
 255 signal intensity for aromatic compounds. The disparities in spectral signal intensities suggested that red and purple fruits
 256 contained more metabolites, particularly carbohydrates and amino acids, while green fruits were richer in aromatic
 257 compounds. According to Lacy et al. (2014), the concentration of metabolites present could affect the intensity of spectral
 258 signals measured with NMR. One of the advantages of the NMR method is its ability to concurrently yield qualitative and

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?

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*?

259 quantitative data, as the signal intensity is directly proportional to the molar concentration of the compound (Pauli et al.
260 2005). In this study, the ethanol extracts of green fruits, despite containing lower concentrations of carbohydrates and
261 amino acids than red and purple fruits, showed a higher concentration of aromatic compounds. Antioxidant capacity test
262 results (Table. 1) showed a greater antioxidant capacity in green fruits compared to red and purple fruits at both sample
263 locations, with Batola showing superior antioxidant capacity and spectral signal intensity than Banjar. Ali et al. (2011)
264 reported similar consistent metabolite profiles in various stages of fruit development in grape cultivars (*Vitis* spp.) based
265 on the ¹H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually
266 as fruits ripen while accumulating more amino acids and sugars.

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

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

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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 ¹H 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 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. ¹H 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 ¹H NMR spectra of young and old leaves extract leaf extracts identified key 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 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, 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 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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50 polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential
51 (Lai et al. 2013; Lai et al. 2015). Besides the health benefits, it is used in the production of wines and beverages (Yin et
52 al., 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
53 flowers, and stems due to their exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as their
54 DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al.
55 (2022) previously reported the most significant antioxidant activity in the green fruits from Banjarbaru, with values of
56 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
57 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.

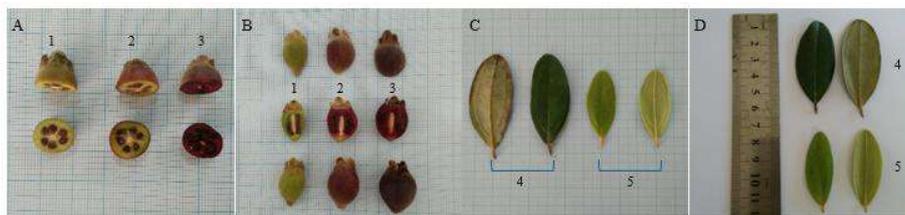
58 The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a
59 lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is
60 considered a pest due to the rapid growth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

66

MATERIALS AND METHODS

67 Plant materials

68 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple,
69 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue color
70 (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.
71 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
72 samples with certificate number of-1007/IPH.1.01/If.07/IX/2023.
73



74 **Figure 1.** Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola
75 D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.
76
77

78 Procedures

79 Crude ethanol extract preparation

80 Leaves of different ages were selectively harvested from apical branches, and fruits were subjected to drying at 40°C in
81 an oven, followed by grinding at ambient temperature. Approximately 500-g of each ground material was macerated in
82 ethanol at 1000 mL (SmartLab, Indonesia) for 72_h by replacing the solvent every 24_h (Nurcholis et al. 2021; Kuntorini
83 et al. 2022). Extracts from identical samples were homogenized, filtrated to release cellular debris, as well as utilizing a
84 rotary evaporator until dried, then stored in a refrigeration unit for further analysis.
85

86 Antioxidant analysis

87 Following the methods of Purwakusumah et al. (2016) and Kuntorini et al. (2022), DPPH (2,2-diphenyl
88 picrylhydrazyl) radical scavenging activity was assessed by diluting ethanol extracts in absolute methanol to attain the
89 appropriate concentration. The sample (2 mL) was combined with 0.17 mM DPPH at 2 mL (Sigma-Aldrich, Germany) as
90 well as incubated in a light-free environment for 30 mins at ambient temperature. Absorbance at a wavelength of 516 nm
91 was measured utilizing a UV/Vis spectrophotometer (UV/Vis Spectrophotometer, Genesys 10 Series, USA). Moreover,
92 the free radical scavenging activity was quantified in micromoles of Trolox equivalents per unit of dry weight (DW) (µmol
93 TE/g), using Trolox from Sigma-Aldrich, Germany.
94

95 Sample preparation for ¹H-NMR

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96 The ¹H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015;
97 Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tube at
98 2 mL with methanol-d4 solution at 1 mL containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich).
99 The combination was subjected to vortexing and sonication for 1 min, followed by homogenization and centrifugation for
100 1 min at 10,000 rpm. The supernatant was compiled and transferred to the NMR tube for subsequent examination using ¹H
101 NMR.

102 ¹H NMR spectroscopy

103 The ¹H NMR analysis was conducted with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The set of
104 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-
105 saturation mode set at 4.27 ppm. Additionally, an internal lock was established using deuterated solvent, and the spectral
106 width was measured within the range of 0 to 10 ppm.
107

108 Data analysis

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

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

122 RESULTS AND DISCUSSION

123 Yield and antioxidant capacity of ethanol extracts

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

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

133 Extraction using solvents is one way to withdraw the active ingredients of an extract. The success of the extraction
134 process is very closely related to the yield, quality and content of active compounds produced. The higher the yield value
135 produced indicates the more value of the extract produced. Ethanol was effective to extract in extracting sterol, flavonoid,
136 phenolic, and alkaloid (Wardani et al. 2019). The yield indicates the amount number of chemical compounds contained in
137 the extract. The results of the yield in the samples in the two regions with antioxidant capacity were not subjected to
138 statistical analysis. Differences in yield values were found between leaf and fruit samples, but showed no different values
139 in the two regions.
140

141 **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 ^e	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. ~~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-picrylhydrazyl 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 ranging from 260.58±0.91 μ mol TE/g to 2795.33± 9.07 μ mol 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 μ mol TE/g (Banjar Regency) and 2795.33±9.07 μ mol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with 260.58±0.91 μ mol TE/g and 364.05±3.82 μ mol TE/g, respectively, which were below the 431.17±14.5 μ mol TE/g reported by Lai et al. (2015). These appeared to be higher than antioxidant capacity, 8.79-92.60 μ mol 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 (IC₅₀: 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. 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, *Riciodendron 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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215 ~~amount of~~ flavonoid content of each plant is directly proportional to its antioxidant activity. Likewise, the presence of
216 phenolic compounds ~~owned by~~ each plant also correlates with its antioxidant activity. [This is due to the redox properties
217 that have the potential as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016)]. These
218 compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et
219 al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins)
220 and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely
221 found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants, phenolic
222 compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and antiallergic
223 activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high flavonoid and
224 phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.
225

226 Compounds identification in ¹H NMR spectra of *R. tomentosa* leaves and fruits

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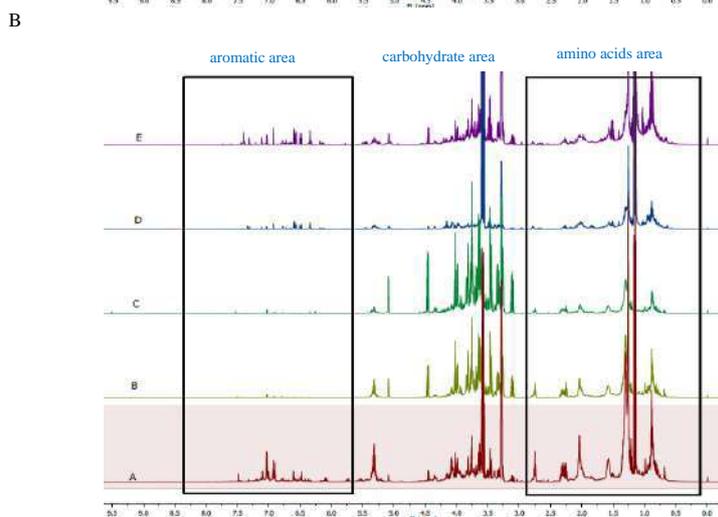
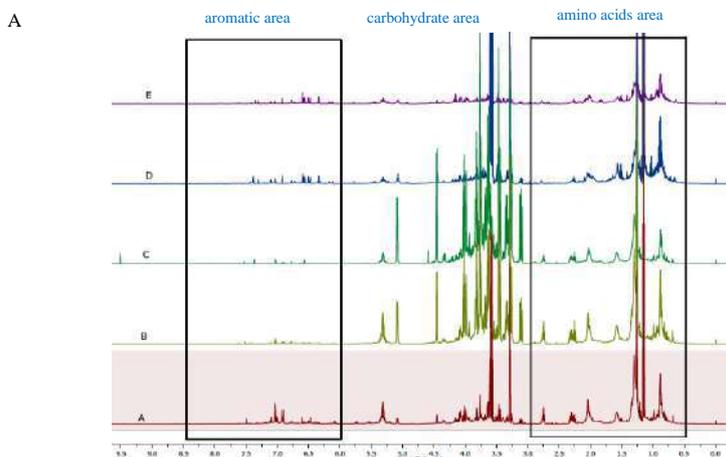


Figure 2. Representative ¹H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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 CD₃OD-D₂O 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 ¹H 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 ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

The ¹H-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 ¹H 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-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. 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. ¹H 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
<i>Amino Acids</i>		
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
<i>Organic Acids</i>		
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
<i>Sugars</i>		
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
<i>Aromatics Compounds</i>		
	7.03 (s)	Ali et al. 2010

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

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

A comparison of the ¹H 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 ¹H 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 ¹H-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 ¹H 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 ¹H 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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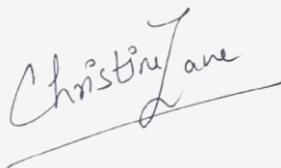
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Antioxidant activity and ¹H NMR profiling *Rhodomyrtus tomentosa* (Ait.) Hassk leaves and fruits from South Borneo

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 either old and young, whereas there were three stages of fruit maturity, namely green, red and purple. ¹H 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 ¹H NMR spectra of young and old leaf extracts identified key 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 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.,

50 2021). Presently, studies related to *R. tomentosa* primarily examine the phytochemical components found in leaves,
51 flowers, and stems due to their exceptional antioxidant, anti-bacterial, and anti-inflammatory attributes, as well as their
52 DNA damage-alleviating ability (Wu et al. 2015; Vo and Ngo, 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al.
53 (2022) **Previously reported that green fruits from Banjarbaru showed the most significant antioxidant activity**, with values
54 of DPPH as well as FRAP at 1419.75 ± 3.48 as well as 1367.59 ± 9.12 $\mu\text{mol TE/g DW}$. Ethanol extracts showed the highest
55 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 .

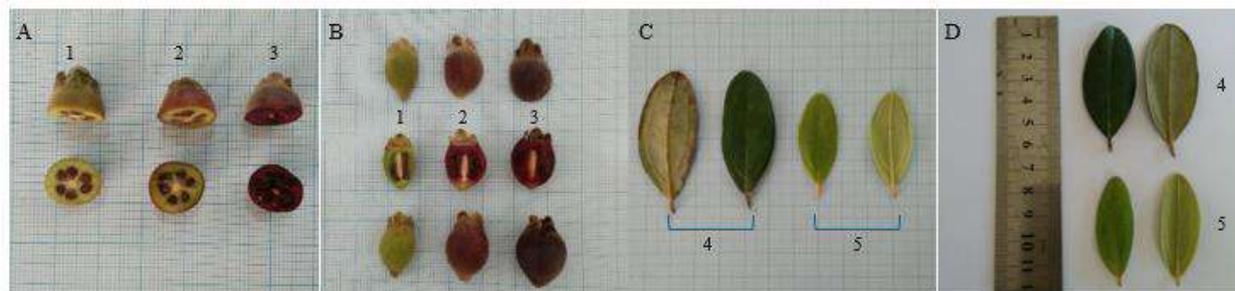
56 The limited use of *R. tomentosa* in South Kalimantan, particularly its leaves and fruits, until recently, is attributed to a
57 lack of knowledge about the extraction methods and potential benefits. Despite possessing several advantages, this plant is
58 considered a pest due to the rapid growth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

64

MATERIALS AND METHODS

Plant materials

65 Samples of younger and older leaves (2nd - 6th and 7th - 12th order from the shoot) of *R. tomentosa* as well as red, purple
66 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
67 and green fruits were collected as presented in Figure 1. As a reference, Munsell Color Charts were applied for plant tissue
68 color (Wilde, 1977). These were obtained from natural habitats in Martapura, Banjar District (3°26'00.6"S,
69 114°51'30.5"E), and Marabahan, Batola District (3°09'16.0"S, 114°37'19.8"E), South Kalimantan in June - July 2023.
70 Moreover, the Herbarium Bogoriense, located at the Indonesian Institute of Sciences, Bogor identified and confirmed
71 samples with certificate number 1007/IPH.1.01/If.07/IX/2023.



72

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

75

Procedures

Crude ethanol extract preparation

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

81

Antioxidant analysis

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

89

Sample preparation for ¹H-NMR

90 The ¹H NMR samples were prepared through a modified methodology from previous studies (Gogna et al. 2015;
91 Mishra et al. 2019; Kim et al. 2010). Approximately 25 g of crude extracts of the samples were set into Eppendorf tubes at
92

93

94

95

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 ¹H NMR.

¹H NMR spectroscopy

The ¹H 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 ¹H 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 ¹H 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.

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

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

167 *R. Tomentosa* leaves and fruits show antioxidant capacity as calculated by the DPPH method ranging from
168 $260.58 \pm 0.91 \mu\text{mol TE/g}$ to $2795.33 \pm 9.07 \mu\text{mol TE/g}$ (Table 1). The conducted ANOVA followed by an LSD analysis of
169 the ten samples indicated statistically significant differences ($P < 0.05$). The findings showed that ethanol extracts possessed
170 similar antioxidant capacity at both sample locations. Specifically, green fruits' ethanol extracts showed the highest DPPH
171 radical scavenging capacity, with respective values at $2360.35 \pm 6.86 \mu\text{mol TE/g}$ (Banjar Regency) and $2795.33 \pm 9.07 \mu\text{mol}$
172 TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, with $260.58 \pm 0.91 \mu\text{mol TE/g}$ and
173 $364.05 \pm 3.82 \mu\text{mol TE/g}$, respectively, which were below the $431.17 \pm 14.5 \mu\text{mol TE/g}$ reported by Lai et al. (2015). These
174 appeared to be higher than antioxidant capacity, $8.79\text{-}92.60 \mu\text{mol TE/g}$, identified in grapes, blueberries, blackberries,
175 bananas, oranges, mangoes, kiwifruits and apples by Wu et al. (2015). This study suggested the high potential of the purple
176 fruits and other portions of *R. tomentosa* as antioxidant sources. Maskam et al. (2014) stated that the extract of *R.*
177 *tomentosa* fruits possessed potent antioxidant properties. In addition, it was suggested that the antioxidant activity of fruit
178 extracts of *R. tomentosa* was due to phenol compounds. In fact, purified extracts of anthocyanin from *R. tomentosa* fruits
179 were found to have a strong antioxidant effect, including the ability to eliminate DPPH radicals (IC_{50} : $6.27 \pm 0.25 \text{ g/mL}$)
180 (Cui et al. 2013).

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

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

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

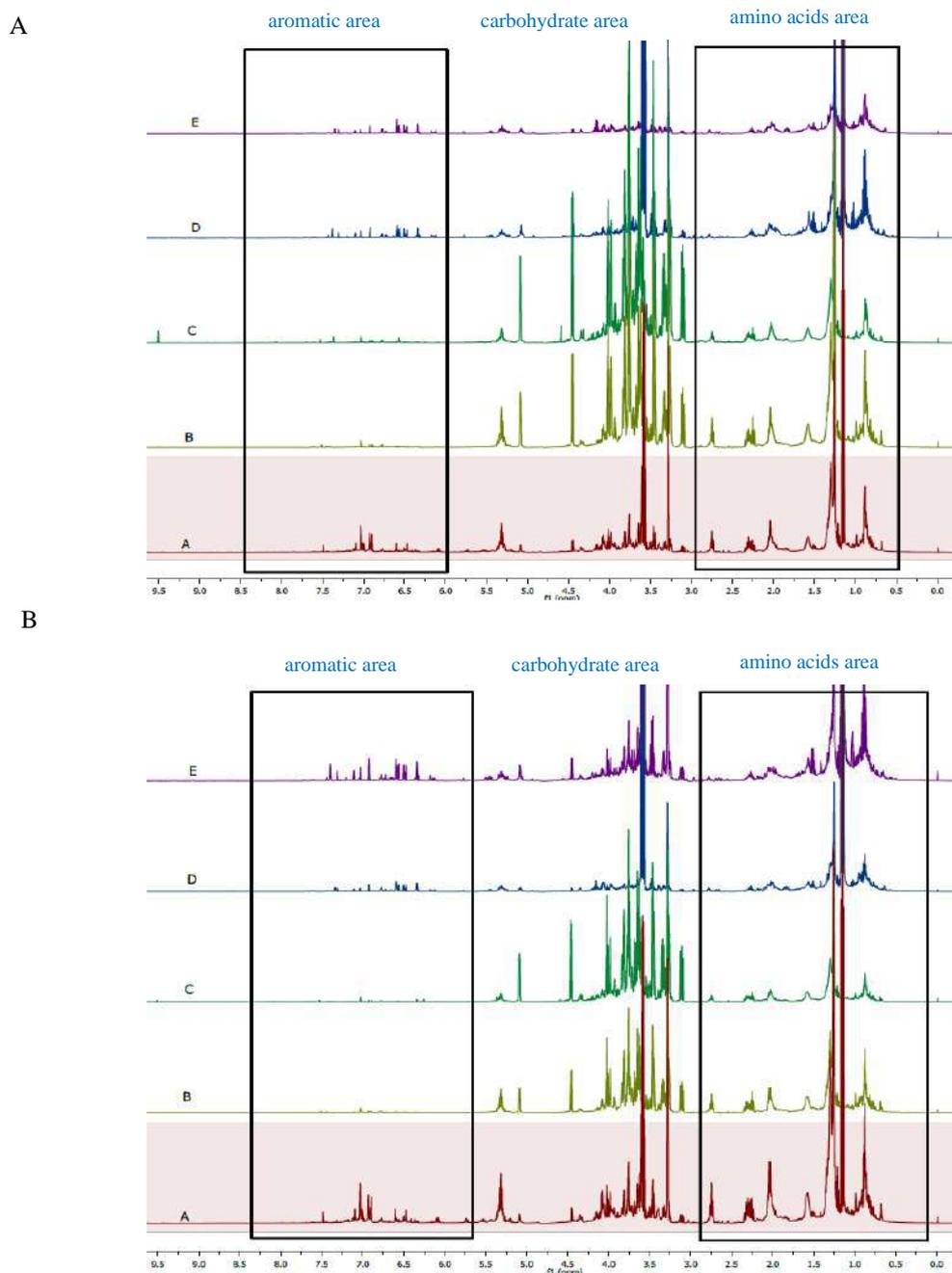
199 The study by ene-Obong et al. (2018) on *Monodora myristica*, using DPPH as a parameter, had an increased
200 antioxidant activity because of a high concentration of flavonoids, phenolics, and vitamin C. Conversely, *Ricinodendron*
201 *heudelotii*, with the lowest phenolic and vitamin C content,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

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

214 These compounds can be divided into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids
215 (Lin et al. 2016). Several studies have shown that phenolic compounds such as flavonoids (especially flavonols and
216 anthocyanins) and hydroxycinnamic acids are the most widespread and diverse polyphenols. In addition, flavonoids are the
217 most widely found phenolic compounds and are found in all parts of the plant. In addition to acting as antioxidants,
218 phenolic compounds provide health benefits such as antimicrobial, anti-inflammatory, cytotoxic, antitumor, and
219 antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al. 2021). Zhao et al. (2019) associated high
220 flavonoid and phenol content in the extract of *R. tomentosa* leaves as well as fruits with potent antioxidant activity.
221

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

223 The chemical properties of *R. tomentosa* ethanol extract in leaves as well as fruits were analyzed for antioxidant
224 activity by ^1H NMR spectroscopy. This non-destructive spectroscopy method, requiring a simple sample preparation
225 process and short duration, is significantly advantageous for analyzing complex mixtures such as food extracts. The
226 representative ^1H NMR spectra of the examined samples are depicted in Figure 2.
227



229 **Figure 2.** Representative ^1H NMR spectra of (a) fruits and leaves extract location in Banjar and (b) fruits and leaves extract location in
230 Batola of *Rhodomyrtus tomentosa* (Ait.) Hassk. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.
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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; Misra 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 CD₃OD-D₂O 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 ¹H 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 ¹H NMR spectra of extract of leaves and fruits obtained from two locations.

The ¹H-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.

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Table 2. ¹H 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		
<i>Aspartate</i>	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2021
<i>Glutamic acid</i>	2.07 (m); 2.36 (m)	Kim et al. 2010
<i>Leucine</i>	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
<i>Methionine</i>	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2010
Organic Acids		
<i>Fumaric acid</i>	6.56 (s)	Kim et al. 2010
<i>Malic acid</i>	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
<i>Succinic acid</i>	2.56 (s)	Kim et al. 2010
Sugars		
<i>Mannitol</i>	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
<i>β-glucose</i>	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
<i>α-glucose</i>	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
<i>Sucrose</i>	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
Aromatics Compounds		
<i>Gallic acid</i>	7.03 (s)	Ali et al. 2010
<i>Myricetin</i>	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2010
<i>Myricetin 3-O- rhamnpyranoside</i>	6.98 (s)	Cerulli et al. 2018
<i>Quercetin-3-O- glucoside</i>	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
<i>Quercetin</i>	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
<i>Syringic acid</i>	3.89 (s)	Ali et al. 2010
Other compounds		
<i>α-Linolenic acid</i>	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
<i>Choline</i>	3.20 (s)	Ali et al. 2010
<i>Sterol</i>	0.68 (s)	Liu et al. 2017

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

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The ¹H 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 ¹H 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 ¹H 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 ¹H-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 ¹H 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 ¹H 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)
- Manuscript review



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[biodiv] Editor Decision

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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". Complete your revision with a Table of Responses containing your answers to reviewer comments (for multiple comments) or enable Track Changes.

Our decision is: **Revisions Required**-----
Reviewer A:Recommendation: Revisions Required

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

[biodiv] Editor Decision

Smujo Editors via SMUJO <support@smujo.com>

Sat, May 11, 2024 at 4:44 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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1 ANTIOXIDANT ACTIVITY AND ¹H NMR PROFILING OF LEAVES AND FRUITS OF RHODOMYRTUS
2 TOMENTOSA (AIT.) HASSK FROM SOUTH KALIMANTAN, INDONESIA

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

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

23 INTRODUCTION

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

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

44 Recent studies have focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for
45 pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal
46 properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols,
47 polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential
48 (Lai et al. 2013; Lai et al. 2015). It is used to produce wines and beverages (Yin et al., 2021). Currently, *R. tomentosa* is
49 mainly studied for the phytochemical components found in leaves, flowers, and stems due to their potent antioxidant, anti-
50 bacterial, and anti-inflammatory activities, as well as the ability to reduce DNA damage (Wu et al. 2015; Vo and Ngo,
51 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 $\mu\text{mol TE/g DW}$. 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.

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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.

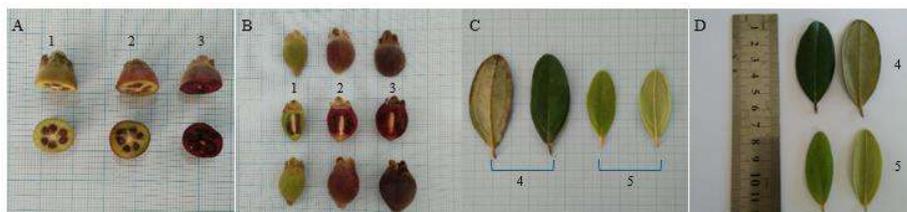


Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.

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, filtered to release cellular debris and dried utilizing a rotary evaporator, then stored in a refrigeration unit for further analysis.

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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, Genesystem 10 Series, USA). The free radical scavenging activity was quantified in micromoles of Trolox (Sigma-Aldrich, Germany) equivalents per unit of dry weight (DW) ($\mu\text{mol TE/g}$).

Sample preparation for ¹H-NMR

The ¹H 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% TMS⁺ (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 ¹H NMR.

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¹H NMR spectroscopy

The ¹H 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 ¹H 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 ¹H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

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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 simplicia powder was mixed with an ethanol solution. The simplicia 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.

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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.

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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 chroman-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.

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The antioxidant capacity of *R. tomentosa* leaves and fruits ranges from 260.58±0.91 μ mol TE/g to 2795.33± 9.07 μ mol 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 μ mol TE/g (Banjar Regency) and 2795.33±9.07 μ mol TE/g (Batola Regency). Conversely, the lowest values were observed in purple fruits, i.e., 260.58±0.91 μ mol TE/g and 364.05±3.82 μ mol TE/g, respectively, which were lower than those reported by Lai et al. 2015, i.e., 431.17±14.5 μ mol TE/g. These appeared to be higher than antioxidant capacity, 8.79-92.60 μ mol 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).

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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).

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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.

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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.

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

The chemical properties of *R. tomentosa* ethanol extract in leaves and fruits were analyzed for antioxidant activity by 1H 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 1H NMR spectra of the samples are presented in Figure 2.

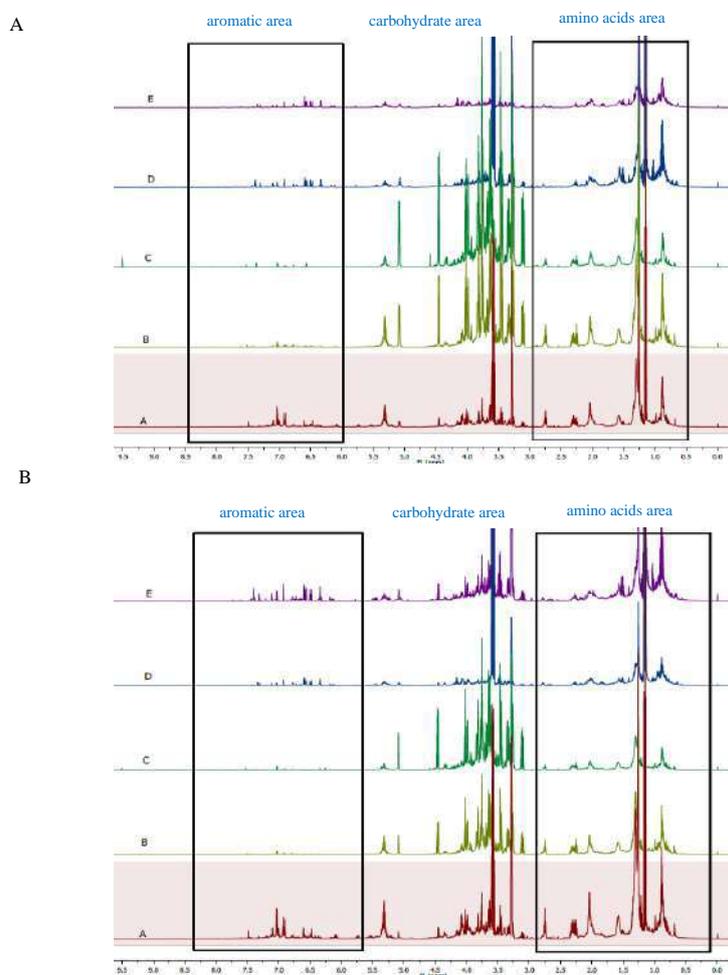


Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. extracts were collected from Banjar, and (b) fruits and leaves were collected from Batola. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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; Mishra 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 $\text{CD}_3\text{OD-D}_2\text{O}$ 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 ¹H 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 ¹H NMR spectra of leaves and fruit extracts from two locations.

The ¹H-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. ¹H 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		
Galic 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- rhamnopyranoside	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 ¹H 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 ¹H 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

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

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

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

298 ACKNOWLEDGMENTS

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



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

[biodiv] Editor Decision

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>
To: Smujo Editors <editors@smujo.id>

Sat, May 11, 2024 at 6:31 PM

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 ₂ PO 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?? 1 st 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 $\mu\text{mol TE/g}$, 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 <i>R. tomentosa</i> leaves and fruits with potent antioxidant activity.	Thank you for the suggestion. Revised accordingly : As in <i>R. tomentosa</i> 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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1 **Antioxidant activity and ¹H NMR profiling of leaves and fruits of *Rhodomyrtus tomentosa* (ait.) Hassk**
2 **from South Kalimantan, Indonesia**

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

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

23 **INTRODUCTION**

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

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

43 Recent studies have focused on exploring the functional characteristics of *R. tomentosa* extracts, renowned for
44 pharmacological properties and traditional applications in treating various ailments (Yang et al., 2023). The medicinal
45 properties of this plant and its components have been extensively investigated. Fruits are rich in flavonoids, phenols,
46 polysaccharides, and other bioactive compounds, including piceatannol, a stilbene component with anti-leukemia potential
47 (Lai et al. 2013; Lai et al. 2015). It is used to produce wines and beverages (Yin et al., 2021). Currently, *R. tomentosa* is
48 mainly studied for the phytochemical components found in leaves, flowers, and stems due to their potent antioxidant, anti-
49 bacterial, and anti-inflammatory activities, as well as the ability to reduce DNA damage (Wu et al. 2015; Vo and Ngo,
50 2019, Ridlo et al. 2020; Hu et al. 2022). Kuntorini et al. (2022) reported previously that green fruits of *R. tomentosa* from
51 Banjarbaru showed potent antioxidant activity, with IC₅₀ 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.

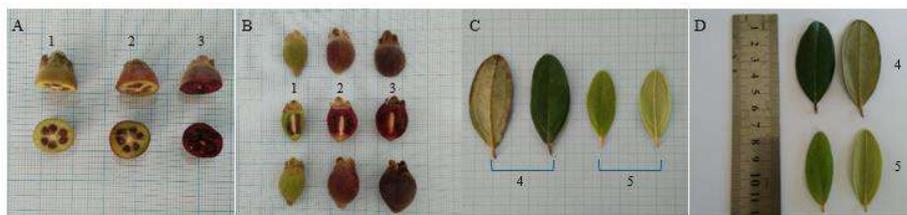


Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola, B. Fruits from Banjar, C. Leaves from Batola D. Leaves from Banjar, 1. Green fruits, 2. Red fruits, 3. Purple fruits, 4. Old leaves, 5. Young leaves.

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, Genesystem 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 ¹H-NMR

The ¹H 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 KH₂PO₄ 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 ¹H NMR.

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¹H NMR spectroscopy

The ¹H 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 ¹H 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 ¹H NMR signals were adjusted to total intensity to produce data suitable for multivariate analysis.

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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.](#)

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.

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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

156 The antioxidant capacity of *R. tomentosa* leaves and fruits ranges from 260.58±0.91 µmol TE/g to 2795.33± 9.07 µmol
157 TE/g (Table 1). The findings showed that ethanol extracts possessed similar antioxidant capacity from samples from
158 different locations. The ethanol extract of green fruits' had the highest DPPH radical scavenging capacity, i.e., with
159 respective values of 2360.35±6.86 µmol TE/g (Banjar Regency) and 2795.33±9.07 µmol TE/g (Batola Regency).
160 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,
161 respectively, which were lower than those reported by Lai et al. 2015, i.e., 431.17±14.5 µmol TE/g. [These results of
162 DPPH radical scavenging capacity were higher than that of grapes, blueberries, blackberries, bananas, oranges, mangoes,
163 kiwis, and apples \(8.79-92.60 µmol TE/g\) \(Wu et al. 2015\).](#) This study suggested the high antioxidant potential of the
164 purple fruits and other plant parts of *R. tomentosa*. Maskam et al. (2014) stated that the fruit extract of *R. tomentosa* had
165 potent antioxidant properties due to phenol compounds. Purified extracts of anthocyanin from *R. tomentosa* fruits have
166 strong antioxidant activity, including the ability to scavenge DPPH radicals (IC₅₀: 6.27±0.25 g/mL) (Cui et al. 2013).

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

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

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

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

190 There is a direct correlation between antioxidant activity and total phenolic content (Idris et al. 2022). Total flavonoids
191 (TFC) and total phenolics (TPC) play important roles as electron donors, chain breakers, and free radical catchers in the
192 antioxidant mechanism. Therefore, the flavonoid content in the plant is directly proportional to its antioxidant activity.
193 Likewise, the presence of phenolic compounds also correlates with their antioxidant activity due to their redox properties
194 as hydrogen donors and reducing agents that can capture free radicals (Hung, 2016). Phenolic compounds can be divided
195 into simple phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (Lin et al. 2016). Several studies
196 have shown that phenolic compounds such as flavonoids (especially flavonols and anthocyanins) and hydroxycinnamic
197 acids are the most widespread and diverse polyphenols. In addition, flavonoids are the most widely found phenolic
198 compounds in all parts of the plant. Phenolic compounds also provide other health benefits such as antimicrobial, anti-
199 inflammatory, cytotoxic, antitumor, and antiallergic activities (Limmongkon et al. 2017; Zhang et al. 2018; Yin et al.
200 2021). [As in leaf and fruit extracts of *R. tomentosa*, the high content of flavonoids and phenols is directly proportional to its
201 antioxidant activity \(Zhao et al. 2019\).](#)

202 **Compounds identification of *R. tomentosa* leaves and fruits by ¹H NMR spectra**

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

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aromatic area

carbohydrate area

amino acids area

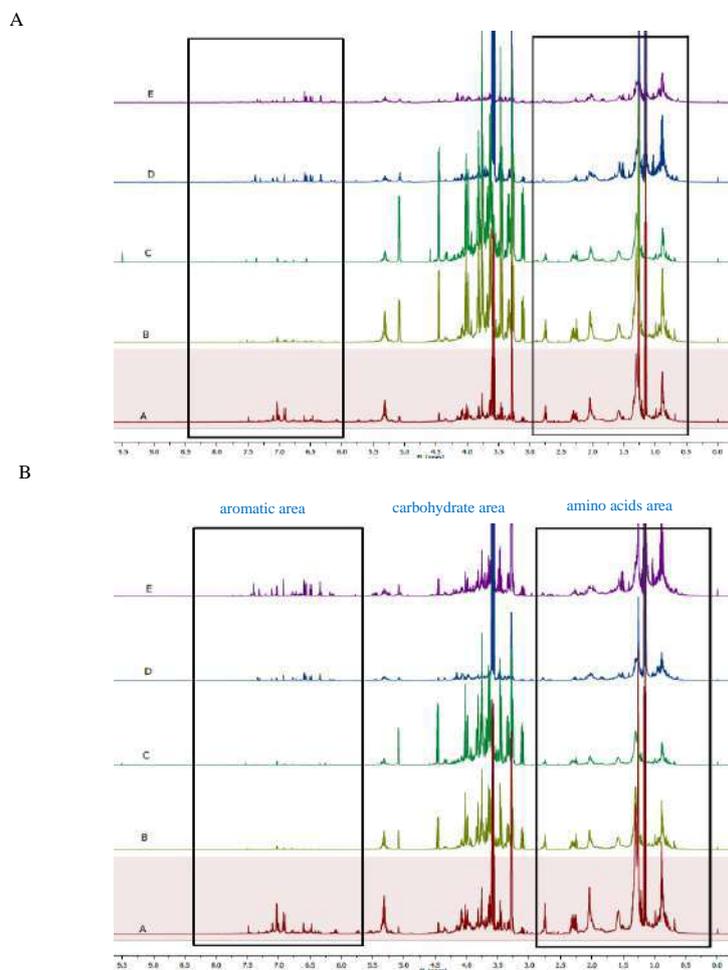


Figure 2. Representative ^1H NMR spectra of (a) fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. extracts were collected from Banjar, and (b) fruits and leaves were collected from Batola. A: Young leaves, B: Old leaves, C: Purple fruits, D: Red fruits, E: Green fruits.

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; Misra 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 $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 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).

231 The ¹H NMR spectra were divided into three regions based on chemical shifts (δ), namely aliphatic compounds (amino
 232 and organic acids including terpenoids), carbohydrates, and aromatic compounds detected within the chemical shifts of
 233 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
 234 various developmental phases of the ¹H NMR spectra of leaves and fruit extracts from two locations.

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

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

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

Compound	Chemical shifts δ (ppm) and coupling constants (Hz)	Reference
<i>Amino Acids</i>		
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
<i>Organic Acids</i>		
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
<i>Sugars</i>		
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
<i>Aromatics Compounds</i>		
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- rhamnopyranoside	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
<i>Other compounds</i>		
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

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

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

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

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

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

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

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21. Uncorrected proof from Biodiversitas Journal (14-5-2024)



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22. Submitted corrected proof to Biodiversitas
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[biodiv] New notification from Biodiversitas Journal of Biological Diversity

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

Tue, May 14, 2024 at 9:08 PM

To: Aditya Triyanto <aditya@smujo.id>, Ahmad Dwi Setyawan <editors@smujo.id>

Dear Mr. Ahmad Dwi Setyawan

Thank you for the great news!
I have submitted an improved manuscript.

We are pleased that our paper has been officially accepted for publication.
Again thank you for your kind support and consideration to our manuscript.

Regards,

Dr. Evi Mintowati Kuntorini

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**16606-Article Text-1117766-1-18-20240514 (Corrected proof).doc**

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Antioxidant activity and ¹H 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 ¹H NMR profiling of leaves and fruits of *Rhodomyrtus tomentosa* (ait.) Hassk from South Kalimantan, Indonesia. *Biodiversitas* 25: xxx. *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. ¹H 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 ¹H 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 IC₅₀ 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.

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 ¹H-NMR

The ¹H 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 KH₂PO₄ 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 ¹H NMR.

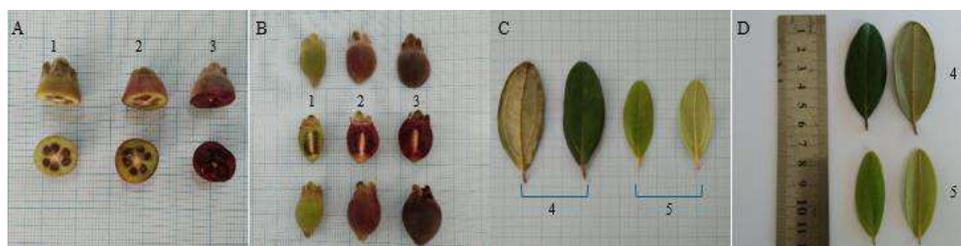


Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. A. Fruits from Batola; B. Fruits from Banjar; C. Leaves from Batola; D. Leaves from Banjar; 1. Green fruits; 2. Red fruits; 3. Purple fruits; 4. Old leaves; 5. Young leaves

¹H NMR spectroscopy

The ¹H 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 ¹H 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 ¹H 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-picrylhydrazyl 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 chroman-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 (IC₅₀: 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 ($\mu\text{mol TE/g DW}$)	
	Banjar	Batola	Banjar	Batola
Young leaves	9.66	8.064	1143.01 \pm 3.43 ^d	1429.53 \pm 9.07 ^d
Old leaves	12.296	11.49	836.20 \pm 9.07 ^c	881.24 \pm 5.94 ^c
Green fruits	5.63	6.05	2360.35 \pm 6.86 ^e	2795.33 \pm 9.07 ^e
Red fruits	9.85	7.76	415.06 \pm 2.99 ^b	443.47 \pm 2.06 ^b
Purple fruits	14.35	15.24	260.58 \pm 0.91 ^a	364.05 \pm 3.82 ^a

Note: The data represent the mean \pm standard deviation. Data were evaluated using one-way ANOVA and the LSD test with $\alpha = 0.05$. 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 anti-allergic 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 ¹H NMR spectra

The chemical properties of *R. tomentosa* ethanol extract in leaves and fruits were analyzed for antioxidant activity by ¹H 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 ¹H 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 CD₃OD-D₂O 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. ¹H 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
<i>Amino acids</i>		
<i>Aspartate</i>	2.68 (dd, J= 3.0; 17.0 Hz)	Cerulli et al. 2018
<i>Glutamic acid</i>	2.07 (m); 2.36 (m)	Kim et al. 2010
<i>Leucine</i>	0.96 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)	Leyva et al. 2021
<i>Methionine</i>	2.15 m ; 2.65 (t, J= 6.08; 6.08 Hz)	Ali et al. 2011
<i>Organic acids</i>		
<i>Fumaric acid</i>	6.56 (s)	Kim et al. 2010
<i>Malic acid</i>	4.34 (dd, J= 6.6 ; 4.7 Hz)	Kim et al. 2010
<i>Succinic acid</i>	2.56 (s)	Kim et al. 2010
<i>Sugars</i>		
<i>Mannitol</i>	3.77 (d, J= 3.28 Hz)	Nuringtyas et al. 2012
<i>β-glucose</i>	4.48 (d, J= 7.79 Hz)	Nuringtyas et al. 2012
<i>α-glucose</i>	5.11 (d, J= 3.84 Hz)	Nuringtyas et al. 2012
<i>Sucrose</i>	5.39 (d, J= 3.91 Hz)	Nuringtyas et al. 2012
<i>Aromatics compounds</i>		
<i>Gallic acid</i>	7.03 (s)	Ali et al. 2011
<i>Myricetin</i>	6.28 (d, J= 1.99 Hz); 7.32 (s)	Ali et al. 2011
<i>Myricetin 3-O-rhamnopyranoside</i>	6.98 (s)	Cerulli et al. 2018
<i>Quercetin-3-O-glucoside</i>	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)	Cerulli et al. 2018
<i>Quercetin</i>	6.25 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)	Liu et al. 2017
<i>Syringic acid</i>	3.89 (s)	Ali et al. 2011
<i>Other compounds</i>		
<i>α-Linolenic acid</i>	1.16 (t, J=7.04 ; 7.04); 1.29 (m)	Rizzuti et al. 2013
<i>Choline</i>	3.20 (s)	Ali et al. 2011
<i>Sterol</i>	0.68 (s)	Liu et al. 2017

Note: s: Singlet; d: Doublet, dd: Double doublet; t: Triplet; m: Multiplet

The ¹H 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 ¹H NMR spectra of leaves and fruit extracts from two locations.

The ¹H-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 ¹H 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 ¹H 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 ¹H NMR spectra obtained. The green stage comprises the highest phenol concentrations, which decrease gradually as fruits ripen while accumulating more amino acids and sugars.

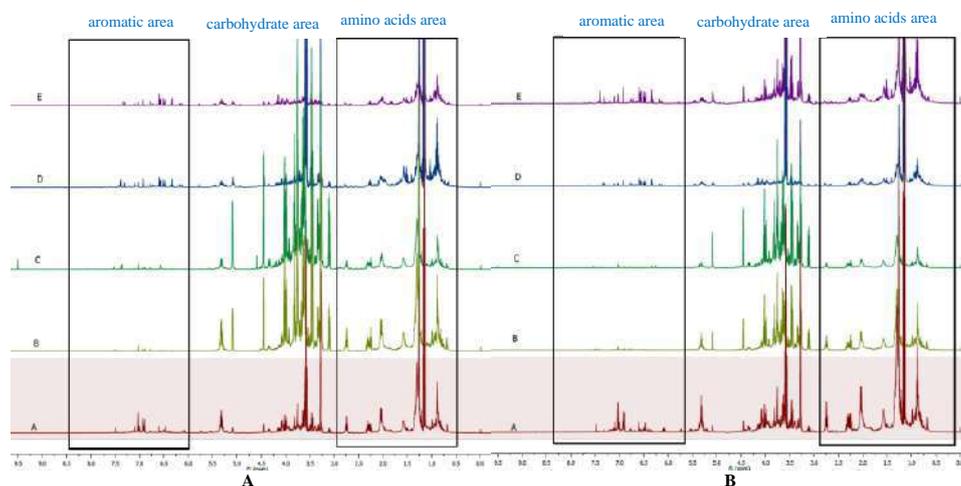


Figure 2. Representative ^1H -NMR spectra of: A. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. extracts were collected from Banjar; and B. Fruits and leaves were collected from Batola. A: Young leaves; B: Old leaves; C: Purple fruits; D: Red fruits; E: Green fruits

Despite the benefits of applying ^1H -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 ^1H -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-rhamnopyranoside, 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 ^1H -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-rhamnopyranoside, 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.

ACKNOWLEDGMENTS

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EVI MINTOWATI KUNTORINI, LILING TRIYASMONO, MARIA DEWI ASTUTI:

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27. Published manuscript

Antioxidant activity and ¹H NMR profiling of leaves and fruits of *Rhodomyrtus tomentosa* from South Kalimantan, Indonesia

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Manuscript received: 27 November 2023. Revision accepted: 12 May 2024.

Abstract. Kuntorini EM, Triyasmono L, Astuti MD. 2024. Antioxidant activity and ¹H 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. ¹H 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 ¹H 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 IC₅₀ 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.

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, Genesystem 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 ¹H-NMR

The ¹H 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 KH₂PO₄ 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 ¹H NMR.

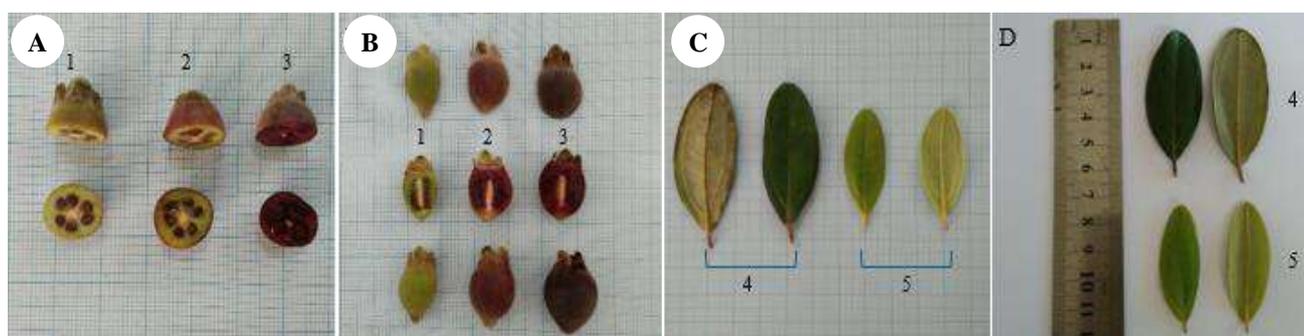


Figure 1. Fruits and leaves of *Rhodomyrtus tomentosa*. A. Fruits from Batola; B. Fruits from Banjar; C. Leaves from Batola; D. Leaves from Banjar; 1. Green fruits; 2. Red fruits; 3. Purple fruits; 4. Old leaves; 5. Young leaves

¹H NMR spectroscopy

The ¹H 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 ¹H 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 ¹H 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-picrylhydrazyn 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 (IC₅₀: 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 $\alpha = 0.05$. 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 ¹H NMR spectra

The chemical properties of *R. tomentosa* ethanol extract in leaves and fruits were analyzed for antioxidant activity by ¹H 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 ¹H 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 CD₃OD-D₂O 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. ¹H 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- rhamnopyranoside	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 ¹H 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 ¹H NMR spectra of leaves and fruit extracts from two locations.

The ¹H-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 ¹H 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 ¹H 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).

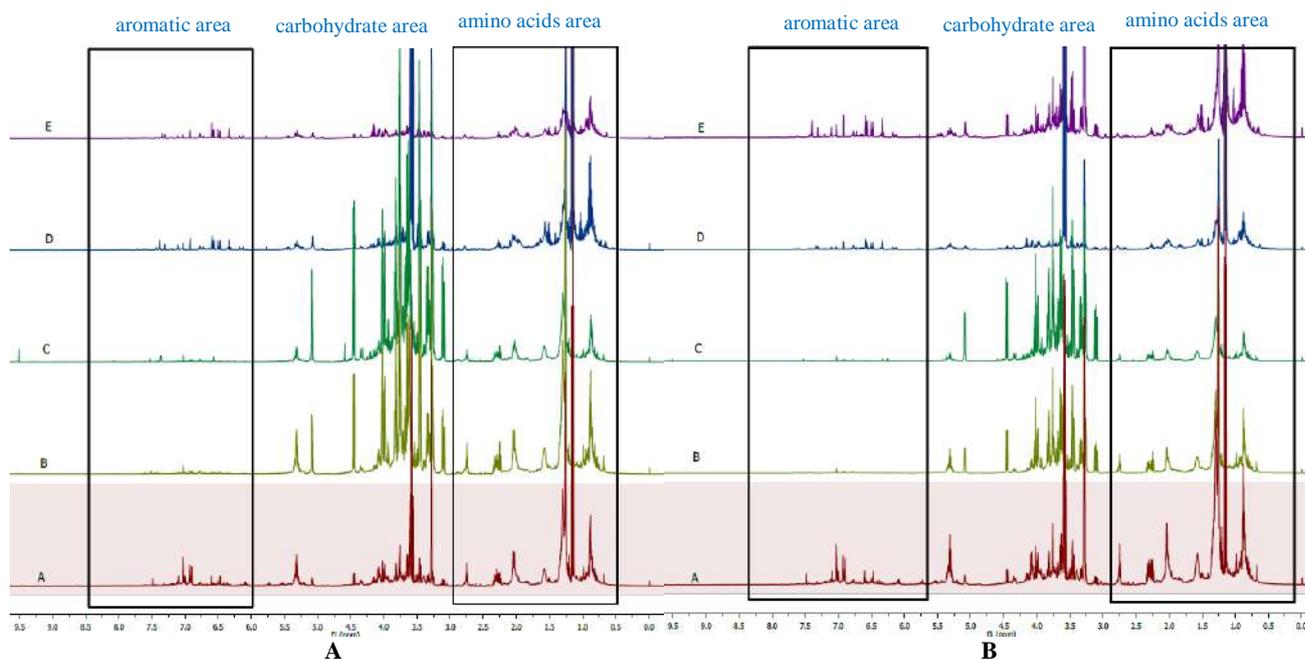


Figure 2. Representative ¹H NMR spectra of: A. Fruits and leaves of *Rhodomyrtus tomentosa* (Ait.) Hassk. extracts were collected from Banjar; and B. Fruits and leaves were collected from Batola. A: Young leaves; B: Old leaves; C: Purple fruits; D: Red fruits; E: Green fruits

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 ¹H 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 ¹H-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 ¹H 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 ¹H 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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