1. Submitted to the journal “Records of Natural Products Journal” (26-7-2023)
2. Editor Decision: Accepted with Revisions Required (9-9-2023)
3. First revised submission (18-9-2023)
   - Cover Letter Revision
   - Manuscript Revision
4. Editor Decision: Minor Revisions (19-9-2023)
5. Second revised submission_Minor Revised (27-9-2023)
   - Cover Letter Revision
   - Manuscript Revision
   - Manuscript tracking proofreading
   -Certificate proofreading
6. Editor Decision: Accepted for publication RNP-2307-2853 (29-8-2023)
7. Accepted response RNP-2307-2853 (1-10-2023)
8. Invoice for RNP-2307-2853 (29-9-2023)
9. Final Proof for RNP-2307-2853 (5-10-2023)
10. submission final Proof for RNP-2307-2853 (5-10-2023)
   - Manuscript final proof
11. Published manuscript

1. Submitted to the journal “Records of Natural Products Journal” (26-7-2023)

UNIVERSITAS LAMBUNG MANGKURAT

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

Your submission has been received

ACG Publications <info@acgpubs.org> Wed, Jul 26, 2023 at 10:00 PM
To: Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>

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Records of Natural Products
RNP-2307-2853
Metabolomic profiling of Rhodomyrtus tomentosa (Ait.) Hassk. leaves and fruits using 1H NMR
Evi Mintowati Kuntorini,Laurentius Hartanto Nugroho,Maryani Maryani,Tri Rini Nuringtyas

Dear Professor Evi Mintowati Kuntorini,

Thank you for your recent e-mail containing the submission of your manuscript to be published in Records of Natural Products. The reference number of your manuscript is RNP-2307-2853. Please visit to author Article Management System (PAMS) to follow the status of your manuscript on the website of journal.

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Editorial Office

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2. Editor Decision: Accepted with Revisions Required (9-9-2023)

UNIVERSITAS LAMBUNG MANGKURAT

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

Decision is available for your submission RNP-2307-2853

ACG Publications <info@acgpubs.org> Sat, Sep 9, 2023 at 4:23 PM
To: Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>

ACG PUBLICATIONS

Records of Natural Products
RNP-2307-2853
Metabolomic profiling of Rhodomyrtus tomentosa (Ait.) Hassk. leaves and fruits using 1H NMR
Evi Mintowati Kuntorini,Laurentius Hartanto Nugroho,Maryani Maryani,Tri Rini Nuringtyas

Dear Professor Evi Mintowati Kuntorini,

I am writing to inform you that your manuscript entitled "**Metabolomic profiling of Rhodomyrtus tomentosa (Ait.) Hassk. leaves and fruits using 1H NMR** " has now been reviewed. I shall be grateful If you could kindly revise your manuscript based on the comments of reviewers and resubmit your modified manuscript as soon as possible. Please prepare a response to reviewer letter, answer the questions or comments item by item and highlighted the corrections with red color on the manuscript. If you do not send your revised manuscript within 21 days we will stop the process and your manuscript will be rejected automatically. Please login to the PAMS to see the further comments on your submission.

****To get access to your account go to PAMS (http://www.acgpubs.org/login) system and please click recover your password (on the right side) and follow the instructions**

Please note, the revised manuscript is also subject to additional review. I look forward to receiving your revised manuscript.

Sincerely yours,

Ahmet C. Gören

Records of Natural Products

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COMMENTS from REVIEWERS

Reviewer-1

The manuscript "Metabolomic profiling of Rhodomyrtus tomentosa (Ait.) Hassk. leaves and fruits using 1H NMR" by E M Kuntorini et al is a study of the metabolic profile of R. tomentosa fruits and leaves at various

maturity stages and evaluating their phytomedicinal values, using 1H NMR and multivariate statistics. The leaves were classified as young and old, while the fruits were divided into green, red and purple maturity stages. The results of the study are interesting and I deem the manuscript suitable for publication in Rec. Nat. Prod., provided the authors address the following concerns:

1-More details should be given about the ripening stages of the fruits - in terms of days after flowering etc. The classification of the leave as young or old (in terms of order from the shoot) is not clear and should be explained better.

2-The authors should include a discussion on the solvent extraction method used for NMR sample preparation and how it affects the chemical shifts of the identified secondary metabolites.

3-The deuterated solvent methanol-d4 was used, however most reference chemical shifts of metabolites given in standard databases from studies using D2O as the solvent. Was there any peak mismatch that the authors discovered in their experiments due to this.

4-The References Mishra et al Natural Product Research, doi.org/10.1080/14786419.2020.1762190 (2020), and Mishra et al, Environmental and Experimental Botany 164 (2019) 58-70 should be included, which are more recent NMR metabolomic studies of plants using 1D and 2D NMR methods.

5- Were any 2D NMR experiments performed to quantify metabolites that could not be properly identified in the crowded 1D 1H spectra?

6- While there is a discussion about the phenolic and antioxidant metabolite concentration in young and old leaves and hence their relative utility for phytomedicine, this discussion is missing for the unripe,ripe and mature fruits. It is evident and expected that the sucrose content will increase, but what effect does it have on the medicinal value of the fruits, since the authors mention at several places about usage of R. tomentosa in traditional medicines.

Reviewer-2

1. Researchers have given only the literature number for metabolite detection and determination. However, the materials and methods should also briefly explain how they do this, and which databases and programs they use.

2. Researchers must explain why they did not use 2D NMR methods (HSQC, TOCSY) to verify metabolites. Because The peaks can overlap in a 500 MHz device, 2D NMR verification for Metabolite determination has become a common scientific opinion today.

3. First revised submission (18-9-2023)
- Cover Letter Revision
- Manuscript Revision

UNIVERSITAS LAMBUNG MANGKURAT

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

Decision is available for your submission RNP-2307-2853

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Mon, Sep 18, 2023 at 11:40 AM
To: ACG Publications <info@acgpubs.org>

Dear Prof. Dr. Ahmet C. Gören

Co-Editor-in-Chief
Records of Natural Products Journal

I am writing in response to the comments and suggestions made by the reviewers of our work, "Metabolomic profiling of *Rhodomyrtus tomentosa* (Ait.) Hassk. leaves and fruits using ^{1}H NMR," which was submitted for publication in Records of Natural Products Journal. 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.

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.

This is our explain and responses to the comments Reviewer 1.

Suggestion 1 :

More details should be given about the ripening stages of the fruits - in terms of days after flowering etc. The classification of the leave as young or old (in terms of order from the shoot) is not clear and should be explained better.

Response :

Thank you for the suggestion. The *R. tomentosa* plants used in this study were wild-growing specimens. Consequently, the selection of leaf and fruit sample criteria is based on the Munsell Color Charts for Plant tissues color guide (Wilde, 1977). We have included this revision in the Figure 1 and Page 2; Lines 82-89 to the revised paper.

Suggestion 2 :

The authors should include a discussion on the solvent extraction method used for NMR sample preparation and how it affects the chemical shifts of the identified secondary metabolites.

Response :

Thank you for the comment. We have added discussion as suggested by the reviewer in the highlighted manuscript (page 4 line 157-165.

Suggestion 3 :

The deuterated solvent methanol-d4 was used, however most reference chemical shifts of metabolites given in standard databases from studies using D2O as the solvent. Was there any peak mismatch that the authors discovered in their experiments due to this.

Response :

We appreciate your feedback. However, it is important to clarify that the sources cited did not only utilize D2O as the solvent for their experiments. The solvents employed in the cited references 13 to 18 were CD3OD – D2O. We would also want to highlight that solvents were employed in accordance with the approach outlined by Kim et al. [15] in order to ensure the pH stability and potential changes of the NMR signals. The updated and detailed description of the solvent employed in this investigation may be seen on page 3, namely lines 112 to 116.

Suggestion 4 :

The References Mishra et al Natural Product Research, doi.org/10.1080/14786419.2020.1762190 (2020), and Mishra et al, Environmental and Experimental Botany 164

(2019) 58-70 should be included, which are more recent NMR metabolomic studies of plants using 1D and 2D NMR methods.

Response :

We have added a discussion according to the reviewer's suggestions and included the References Mishra et al (2020), and Mishra et al, (2019). These can be found in the Page 4; Line 128-136 and 139 - 143; Page 10 ; Line 333-342.

Suggestion 5 :

Were any 2D NMR experiments performed to quantify metabolites that could not be properly identified in the crowded 1D 1H spectra?

Response :

We appreciate your suggestions. We did not conduct the 2D NMR. We concur that this is one of the limitations of this research. We understood this, which is why we did not utilize signals in crowded areas, such as the sugar regions. Therefore, we also carefully examine the coupling constant of each signal to the references in order to limit the potential of identification error. As may be seen in Table 1's heading, we have also included the word "putative" before the metabolites.

Suggestion 6 :

While there is a discussion about the phenolic and antioxidant metabolite concentration in young and old leaves and hence their relative utility for phytomedicine, this discussion is missing for the unripe,ripe and mature fruits. It is evident and expected that the sucrose content will increase, but what effect does it have on the medicinal value of the fruits, since the authors mention at several places about usage of *R. tomentosa* in traditional medicines.

Response :

Thank you for the suggestion. We have added a discussion according to the reviewer's suggestions on the medicinal value of the fruits for the unripe, ripe and mature fruits in the highlighted manuscript (page 10 line 363-374 and page 10 -11; line 375-385).

This is our explain and responses to the comments Reviewer 2.

Suggestion 1 :

Researchers have given only the literature number for metabolite detection and determination. However, the materials and methods should also briefly explain how they do this, and which databases and programs they use.

Response :

Thank you for the comments. We have added how we process the NMR spectra as well as the programs that we used to process the spectra. The explanation can be found in page 4 line 128 – 136.

Suggestion 2 :

Researchers must explain why they did not use 2D NMR methods (HSQC, TOCSY) to verify metabolites. Because The peaks can overlap in a 500 MHz device, 2D NMR verification for Metabolite determination has become a common scientific opinion today.

Response :

We appreciate your suggestions. We did not conduct the 2D NMR. We concur that this is one of the limitations of this research. We understood this, which is why we did not utilize signals in crowded areas, such as the sugar regions. Therefore, we also carefully examine the coupling constant of each signal to the references in order to limit the potential of identification error. As may be seen in Table 1's heading, we have also included the word "putative" before the metabolites.

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.

Regards,

Dr. Evi Mintowati Kuntorini

Associate Professor
Laboratory of Plant Structure and Development, Faculty of Mathematics and Natural Science,
Lambung Mangkurat University
Jl. A.Yani Km 36 Banjarbaru, South Kalimantan, 70714, Indonesia
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2 attachments

Manuscript ACG-Pubs-OA-RNP Revision (submitted).doc
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Cover letter revision manuscript ACG publication-1 (submitted).docx
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Dear Mr. Ahmet C. Gören
Co-Editor-in-Chief
Records of Natural Products Journal

I am writing in response to the comments and suggestions made by the reviewers of our work, "Metabolomic profiling of *Rhodomyrtus tomentosa* (Ait.) Hassk. leaves and fruits using ^{1}H NMR," which was submitted for publication in Records of Natural Products Journal. 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.

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		
No	**Suggestion**	**Response from author**
1	More details should be given about the ripening stages of the fruits - in terms of days after flowering etc. The classification of the leave as young or old (in terms of order from the shoot) is not clear and should be explained better	Thank you for the suggestion. The *R. tomentosa* plants used in this study were wild-growing specimens. Consequently, the selection of leaf and fruit sample criteria is based on the Munsell Color Charts for Plant tissues color guide (Wilde, 1977). We have included this revision in the Figure 1 and Page 2; Lines 82-89 to the revised paper.
2	The authors should include a discussion on the solvent extraction method used for NMR sample preparation and how it affects the chemical shifts of the identified secondary metabolites.	Thank you for the comment. We have added discussion as suggested by the reviewer in the highlighted manuscript (page 4 line 157-165
3	The deuterated solvent methanol-d4 was used, however most reference chemical shifts of metabolites given in standard databases from studies using D_2O as the solvent. Was there any peak mismatch that the authors discovered in their experiments due to this.	We appreciate your feedback. However, it is important to clarify that the sources cited did not only utilize D_2O as the solvent for their experiments. The solvents employed in the cited references 13 to 18 were CD_3OD – D_2O. We would also want to highlight that solvents were employed in accordance with the approach outlined by Kim et al. [15] in order to ensure the pH stability and potential changes of the NMR signals. The updated and detailed description of the solvent employed in this investigation may be seen on page 3, namely lines 112 to 116.
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	metabolomic studies of plants using 1D and 2D NMR methods.	
5	Were any 2D NMR experiments performed to quantify metabolites that could not be properly identified in the crowded 1D 1H spectra?	We appreciate your suggestions. We did not conduct the 2D NMR. We concur that this is one of the limitations of this research. We understood this, which is why we did not utilize signals in crowded areas, such as the sugar regions. Therefore, we also carefully examine the coupling constant of each signal to the references in order to limit the potential of identification error. As may be seen in Table 1's heading, we have also included the word "putative" before the metabolites.
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Reviewer 2		
No	**Suggestion**	**Response from author**
1	Researchers have given only the literature number for metabolite detection and determination. However, the materials and methods should also briefly explain how they do this, and which databases and programs they use.	Thank you for the comments. We have added how we process the NMR spectra as well as the programs that we used to process the spectra. The explanation can be found in page 4 line 128 – 136.
2	Researchers must explain why they did not use 2D NMR methods (HSQC, TOCSY) to verify metabolites. Because The peaks can overlap in a 500 MHz device, 2D NMR verification for Metabolite determination has become a common scientific opinion today.	We appreciate your suggestions. We did not conduct the 2D NMR. We concur that this is one of the limitations of this research. We understood this, which is why we did not utilize signals in crowded areas, such as the sugar regions. Therefore, we also carefully examine the coupling constant of each signal to the references in order to limit the potential of identification error. As may be seen in Table 1's heading, we have also included the word "putative" before the metabolites.

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

Sincerely,

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

ORIGINAL ARTICLE

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Rec. Nat. Prod. X:X (202X) XX-XX

records of natural products

Metabolomic profiling of *Rhodomyrtus tomentosa* (Ait.) Hassk. leaves and fruits using ^{1}H NMR

Evi Mintowati Kuntorini [1,2*], Laurentius Hartanto Nugroho [1], Maryani [1] and Tri Rini Nuringtyas [1,3*]

[1]Faculty of Biology, Universitas Gadjah Mada. Teknika Selatan Street, 55281, Yogyakarta, Indonesia

[2]*Departement of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat. A. Yani Km. 36 Street, Banjarbaru City, 70714, South Kalimantan, Indonesia*

[3]*Research Center for Biotechnology, Universitas Gadjah Mada, Teknika Utara stree, 55281, Yogyakarta, Indonesia*

(Received Month Day, 201X; Revised Month Day, 201X; Accepted Month Day, 201X)

Abstract: Several studies have extensively documented the presence of metabolites with antibacterial, anticancer, antioxidant, and anti-inflammatory properties in extracts derived from *Rhodomyrtus tomentosa* fruits and leaves. Therefore, this study is aimed at evaluating the metabolite profile of *R. tomentosa* fruits and leaves at various maturity stages, as well as determining their phytomedicinal values. ^{1}H NMR and chemometric analysis were used to conduct a metabolomics study to compare the metabolite profile and phytomedicinal values of different plant organs at varying ages. The leaves were classified into young and old categories, while the fruits were divided into three maturity stages, namely green, red, and purple. The wild-grown fruits and leaves of *R. tomentosa* (Ait.) Hassk were gathered in Banjarbaru, South Kalimantan Province, Indonesia. The multivariate analysis showed that choline, methionine, mannitol, and β-glucose compounds were three times higher in the fruits compared to the leaves. Meanwhile, the concentration of aspartate, myricetin, and quercetin compounds was three times higher in the leaves compared to the fruits. Secondary metabolites, including flavonoids, were identified in higher quantities in young leaves and green fruits compared to old leaves, as well as red and purple fruits.

Keywords: *Rhodomyrtus tomentosa*, flavonoid, ^{1}H-NMR, multivariate statistical analysis

1. Introduction

Plants play a vital role in people's daily lives by providing essential resources such as medicine, food, fiber for clothing, and wood for building. Furthermore, plants used for the treatment of different medical conditions are considered the most valuable among various natural resources. At present, individuals without access to modern healthcare still place great value on traditional medical practices. Several studies have shown that the majority of traditional medicines are produced from plant-based materials. A recent report reveals that about 80% of the global population relies on plants to treat a

* Corresponding author: E-Mail: evimintowati@ulm.ac.id ; Phone: +85228952178 : tririni@ugm.ac.id ; Phone : +82110117807

The article was published by ACG Publications

http://www.acgpubs.org/journal/records-of-natural-products Month-Month 202x EISSN:1307-6167

DOI: http://doi.org/10.25135/rnp.18.17.06.108

Available online: X/X/202X

wide range of ailments, highlighting their widespread usage. Moreover, herbal medicines have become integral components of modern therapy, with 25% of medications available around the world being derived from plants [1].

Rose myrtle, scientifically known as *Rhodomyrtus tomentosa* (Ait.) Hassk., is a blossoming plant that falls under the Myrtaceae family. Based on previous reports, it is indigenous to southern and southeastern Asia, with its natural habitat spanning from India, southern China, the Philippines, and Malaysia, to Sulawesi. This versatile plant exhibits a remarkable ability to grow in various environments, ranging from sea level to elevations of 2400 m. Furthermore, it thrives in diverse locations, such as natural forests, beaches, wetlands, riparian zones, moist and wet woods, as well as bog borders, requiring intense sunlight and minimal soil conditions [2,3,4]. In tropical and subtropical gardens, *R. tomentosa* is a well-liked ornamental plant, cultivated for its abundant blooms and delectable, edible fruits. The fruits are often used for several culinary applications, such as pies, salads, and jams. In Vietnam and China, they are processed into wine, jellies, or canned fruit [2]. Modern pharmacological studies have shown that *R. tomentosa* components demonstrate a diverse array of pharmacological properties, namely antibacterial [5], anticancer [6], anti-inflammatory [7], as well as antioxidant [8]. Traditional Malaysian, Chinese, and Vietnamese medicine have employed its leaves, roots, buds, and fruits for medicinal purposes [4]. For example, the Dayak and the Paser local tribes in East Kalimantan, Indonesia, utilize *R. tomentosa* roots for the treatment of diarrhea and stomachaches, as well as a postpartum tonic [9]. The crushed leaves serve as external poultices, and the tar obtained from its wood is used for eyebrow darkening. The majority of these effects are similar to those observed in the traditional applications of *R. tomentosa*. Several phytochemical studies showed that the plant contains flavonoids, triterpenoids, phenols, microelements, and meroterpenoids [2]. Among the various compounds, rhodomyrtone stands out as the most prominent compound, possessing numerous potential pharmacological properties [10], while piceatannol is the main and most effective phenolic component [3].

A previous study explored the total phenolic content, antioxidant capacity, total flavonoid content, as well as compound distribution in *R. tomentosa* using histochemical analysis [11]. The limitation of this report was that it did not examine the specific antioxidant profile of the fruits and leaves at various stages of development. To address this gap, ^{1}H-NMR was utilized to analyze the metabolite profile in the leaves and fruits. Therefore, this study is aimed at analyzing the secondary and primary metabolites of various parts of *R. tomentosa*, specifically the fruits and leaves. The existence of these compounds was then correlated with the previously reported bioactivity and phytomedicinal qualities. NMR spectroscopy and multivariate data analysis without any chromatographic separation were used to identify metabolites directly from the samples. A metabolic analysis was conducted on *R. tomentosa* fruits and leaves at various stages of maturation and multivariate statistics were used to determine the compounds that significantly contributed to the variations between both parts. Based on the findings, this is the first systematic study of *R. tomentosa* fruits and leaves at various stages of maturity employing a combined NMR and multivariate statistical approach, and it demonstrates the applicability of the NMR-based approach in plant metabolomics.

2. Materials and Methods

2.1. Plant materials

Rhodomyrtus tomentosa plant in this study grows in the wild. The samples were gathered in the wild in Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) in August-October 2020. Using Munsell Color Charts for Plant tissues as a guide for color the samples of leaves and fruits [12]. The leaf samples were selected from young leaves (2nd – 6th order from the shoot [Munsell Color Carts guide: 5GY (7/8-6/8)]) and old leaves (7th -12th order from the shoot [Munsell Color Carts guide: 2.5G (4/4-3/4)]). Furthermore, fruit samples used are green [Munsell Color Carts guide: 2.5GY (7/8-6/10)], red [Munsell Color Carts:10R (6/10-5/10)], and purple [Munsell Color Carts:5RP (4/4-3/2)] with a color guide using Munsell Color Charts for Plant tissues (Figure 1A.).Three replicates of each leaf and fruit were analyzed and identified by the Herbarium Bogoriense, Indonesian Institute of Sciences, located in Bogor, Indonesia, under certificate number 1007/IPH.1.01/If.07/IX/2020.

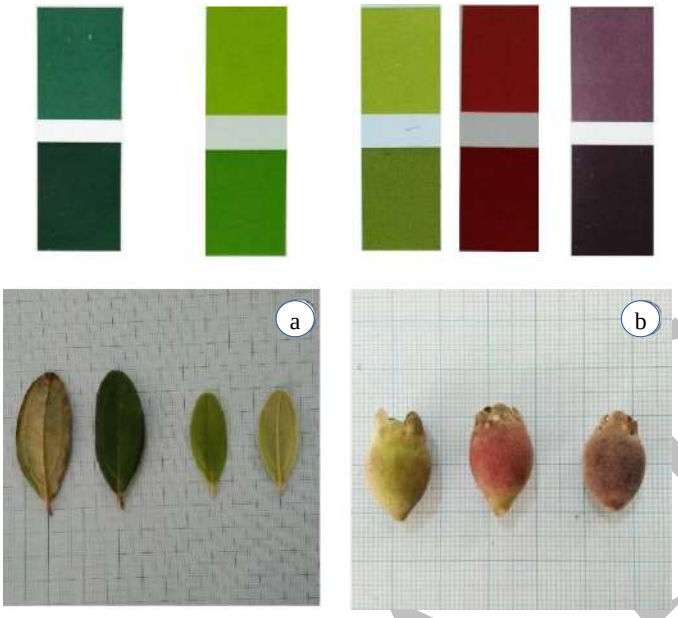

Figure 1A. *Rhodomyrtus tomentosa* (Ait.) Hassk. a: leaves, b. fruits

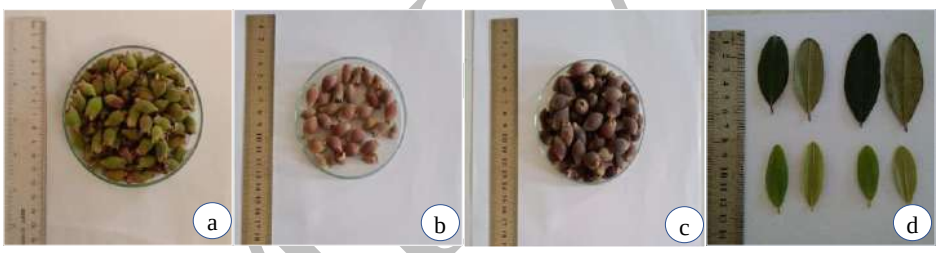

Figure 1B. *Rhodomyrtus tomentosa* (Ait.) Hassk. a: green fruits, b: red fruits, c: purple fruits,d: young and old leaves.

2.2. Crude extract preparation and sample preparation for ^{1}H-NMR

The old and young leaves were carefully selected from the tip shoots of *R. tomentosa*, and the fruits were dried in an oven at 40°C and subsequently ground at room temperature. Each grounded material of 500 g was macerated in ethanol of 1000 mL (SmartLab, Indonesia) for a duration of 24 h. The solvent was then discarded and changed every 24 h, and this process was repeated three times [9,11]. Furthermore, extracts from the same samples were combined, properly mixed, filtered to eliminate cell debris, and dried using a rotary evaporator.

^{1}H-NMR sample preparation was carried out using a slightly modified version of the sample extraction methods by [15]. A total of 25 mg of the crude extract were placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent consisting of 0.5 mL of methanol-d4 and 0.5 mL of KH_2PO_4 buffer, pH 6.0 containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The mixture was then vortexed and sonicated for 1 min. The solution was homogenized and centrifuged for 1 min at 10,000 rpm using a microcentrifuge. The supernatant was collected, placed in the NMR tube, and prepared for ^{1}H-NMR analysis.

2.3. NMR experiments

^{1}H-NMR was carried out with a 500 MHz spectroscopy (JEOL JNM ECZ500R) at 25°C. The following parameters were used for a total of 128 scans lasting for 10 min, namely a relaxation delay

of 1.5 seconds, X_angle 60°, and a pre-saturation mode of 4.27 ppm. The deuterated solvent was set as the internal lock, and the spectral width was then measured from 0 to 10 ppm.

2.4. Data analysis

The ^{1}H-NMR spectra were analyzed using MestReNova analysis. Furthermore, the spectra were processed using manual phasing, baseline adjustment, and calibration to internal standard solution signals (TMSP) positioned at the chemical shift of 0.0 ppm. Multiplicities in the observed NMR resonances were labeled according to the convention: s = singlet; d = doublet; dd = doublet of doublets; t = triplet; and m = multiplet [14]. Furthermore, metabolites were identified by comparing the information in a metabolite database from previous studies [13,14,15,16,17,18]. Signal analysis was carried out semi-quantitatively by comparing the area of a signal to that of the TMSP signal as an internal standard. All ^{1}H-NMR signals were normalized to total intensity for developing data for multivariate data analysis. MetaboAnalyst software (MetaboAnalyst.ca) was used to analyze multivariate data using Principal Component Analysis (PCA), Partial Least-Squares Discriminant Analysis (PLS-DA), and hierarchical clustering heat map analysis. The spectra were centered and scaled with autoscaling. The data was initially processed using the PCA to evaluate the natural clustering characters of the data. When the data showed quite clear grouping, then the data was subjected to PLS-DA. PLS-DA was used to maximize covariance between measured data (NMR peak intensities) and the response variable (predictive classifications). The PLS-DA coefficient loading plot can be used to find significant metabolites contributing to the separation between the two classes [14]. The model's predictive ability (Q^2) was measured using cross-validation, and the statistical significance was determined using a permutation test. The model's important compounds were ranked using variable importance of projection (VIP) scores. A VIP score of >1 indicated that the variable contributed to the variation of the sample. The t-test analysis was used to determine significant differences in metabolites between all samples with p-values ≤ 0.01.

3. Results and Discussion

3.1. Visual analysis of ^{1}H-NMR spectra

NMR spectroscopy was a technique for determining the magnetic resonance of molecular nuclei interacting with external magnetic fields [19]. NMR could produce a distinct and specific spectrum for each compound and was often used to determine the type of metabolite. The quality of the results was determined by the number of compounds identified rather than the number of signals observed during the NMR analysis [20]. The NMR metabolomics approaches have been used widely, thus determining the compounds less complicated and can be done by comparing the signal produced by the samples to those produced by the same compound in previous reports utilizing the same solvent CD_3OD-D_2O [13,14,15,16,17,18]. As mentioned by Kim et al. [15], aqueous methanol is commonly used as an extraction solvent alone as methanol is a universal solvent with a wide range of compounds extracted, both non-polar and polar. The chemical shift of substances in NMR can be influenced by various solvents. Multiple reference papers were utilized to conduct a comparative analysis of potential signal changes that may be identified. In this work, the coupling constant was utilized as an important parameter to validate the matching signals in our data with the references.

The ^{1}H-NMR spectra were commonly separated into three regions based on their chemical shift (δ), namely the amino acids and terpenoids, carbohydrates, and aromatic compounds. Aliphatic compounds (organic and amino acids) were found in the chemical shift 0.5-3.0 ppm, carbohydrates in 3.1 – 6.0 ppm, and aromatic compounds in > 6 ppm. The ^{1}H-NMR spectra of the leaf and fruit extracts were analyzed and compared. The various developmental stages of leaves and fruits were analyzed, contrasted, and depicted in Figure 2.

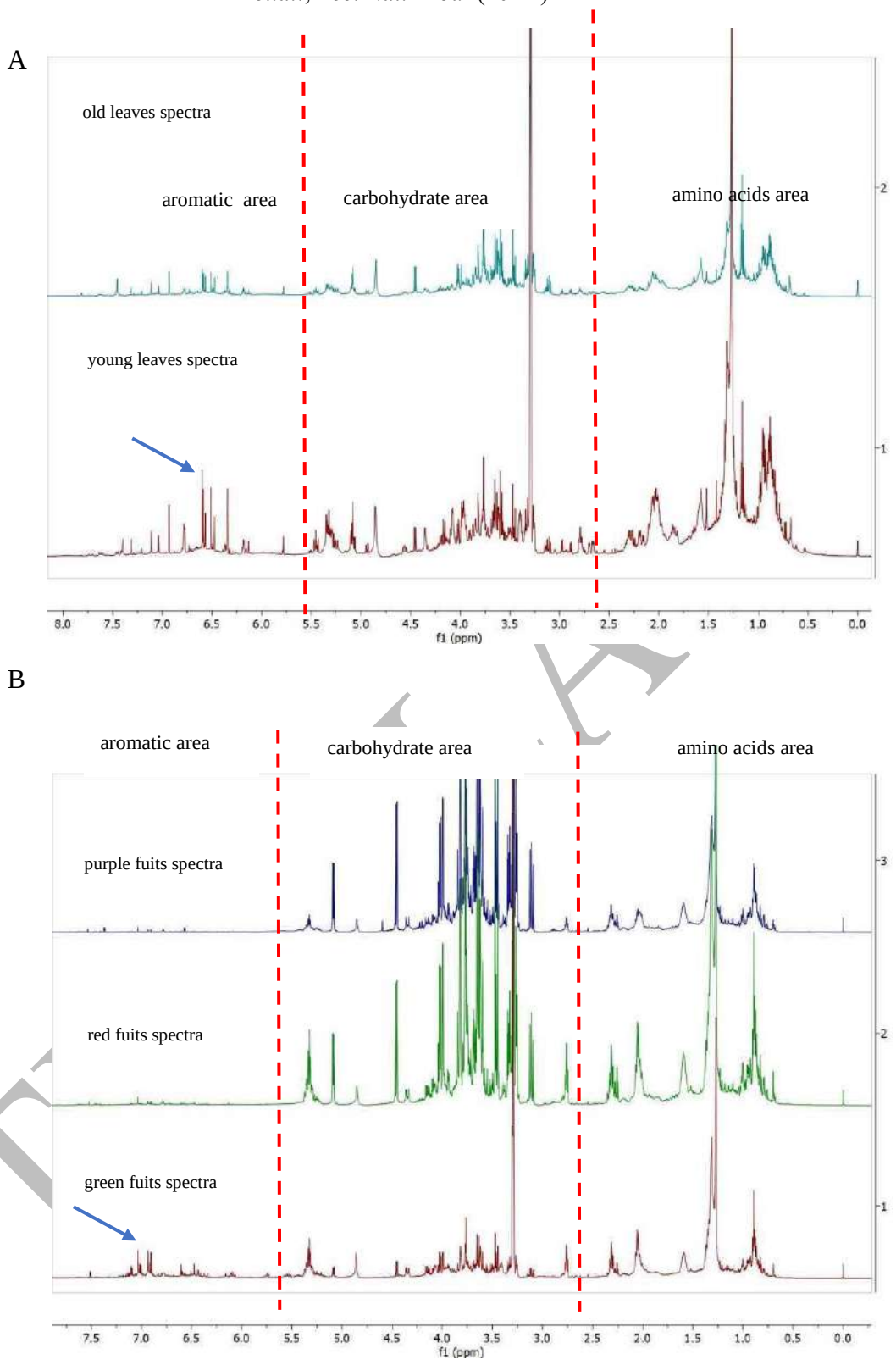

Figure 2. The comparison of the ^{1}H-NMR spectrum (δ 0.00–8.00 ppm) indicates signals of *R. tomentosa* from (A) leaves and (B) fruits of *R. tomentosa.* The arrow show the aromatics regions which observed different in signal intensities.

The results of the putative compounds identified by ^{1}H-NMR showed the presence of primary and secondary metabolite compounds. The primary metabolites included amino acids (chemical shift 2.0 -0.5 ppm) and carbohydrates (chemical shift 5.0-3.0 ppm), with aromatic compounds (chemical shift > 6 ppm) being the secondary metabolites. A gradual decrease in phenolic content was observed during the leaf and fruit growth in the aromatic regions of the NMR spectra. Specifically, the intensities of signals in the aromatic area in the young leaves and green fruit samples were higher than in the old, red and purple leaves (Figures 2A and B).

3.2. Identification of metabolites / Assignment of ^{1}H-NMR signals

Despite its various advantages in metabolomics study, the use of ^{1}H-NMR presented a significant challenge in chemical identification due to overlapping signals in multiple regions, particularly in the 5.0-3.0 ppm region, which corresponds to sugar compounds. Therefore, the signals in the sugar region were not picked as the particular identifying signals unless for the very general sugars such as glucose and sucrose. This may decrease the number of substances that can be detected in this investigation. This study revealed the identification of 20 putative compounds based on the ^{1}H-NMR spectra, as presented in Table 1. In the amnio acid region we identified the specific signals of leucine, glutamate, methionine, and aspartate, with chemical shifts ranging from 3.0-0.5 ppm. The organic acid compounds, such as malic acid, fumaric acid, and succinic acid were observed in the chemical shifts of between 3.0 - 2.0 ppm. The frequently identified sugars including mannitol, β-glucose, α-glucose, and sucrose could be observed in the chemical shifts of 5.00 - 3.50 ppm. The aromatic regions which observed to be less crowded, several phenolics can be identified, including gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid (chemical shifts 10.0 - 6.0 ppm). The rest of compounds identified were α-Linolenic acid, choline, and sterols.

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

No	*Compound*	*Chemical shifts δ (ppm) and coupling constants (Hz)*
	Amino Acids	
1	*Aspartate*	2.68 (dd, J= 3.0; 17.0 Hz)
2	*Glutamic acid*	2.06 (m); 2.34 (m)
3	*Leucine*	0.93 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)
4	*Methionine*	2.16 m ; 2.79 (t, J= 6.08; 6.08 Hz)
	Organic Acids	
5	*Fumaric acid*	6.51 (s)
6	*Malic acid*	4.34 (dd, J= 6.6 ; 4.7 Hz)
7	*Succinic acid*	2.54 (s)
	Sugars	
8	*Mannitol*	3.77 (d, J= 3.28 Hz)
9	*β-glucose*	4.45 (d, J= 7.79 Hz)
10	*α-glucose*	5.09 (d, J= 3.84 Hz)
11	*Sucrose*	5.35 (d, J= 3.91 Hz)
	Aromatics Compounds	
12	*Gallic acid*	7.03 (s)
13	*Myricetin*	6.34 (d, J= 1.99 Hz); 7.32 (s)
14	*Myricetin 3-O-rhamnpyranoside*	6.93 (s)
15	*Quercetin-3-O-glucoside*	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)
16	*Quercetin*	6.13 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)
17	*Syringic acid*	3.88 (s)
	Other compounds	
18	*α -Linolenic acid*	1.16 (t, J=7.04 ; 7.04); 1.29 (m)
19	*Choline*	3.25 (s)
20	*Sterol*	0.70 (s)

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

3.2. Multivariate data analysis

A multivariate PCA was performed to assess the variations of compounds present in the fruits and leaves of *R. tomentosa*. The PCA score plot was used to demonstrate the separation of classes, while the correlation between variables was interpreted in the loading plot [21]. MetaboAnalyst 5.0 automatically generated five PCs representing 87.6% of all observed variants. The 2D score diagram derived from PC1 and PC2 clearly distinguished fruit and leaf samples. Figure 3A illustrates that the Q^2 cumulative of PC1 and PC2 yielded 57.6% of variants, already above 50%, indicating a reliable model. PLS-DA has been implemented to the multivariate analysis to enhance separation. PC1 and PC2 accounted for 35% and 21.7% of the observed variants, respectively, for a total of 56.7% Q^2 in the 2D score plot. On the PLS-DA score plot, samples of leaves and fruits were separated. The fruits were positioned in the negative region of PC1, while the leaves were placed in the positive region (Figure 3B).

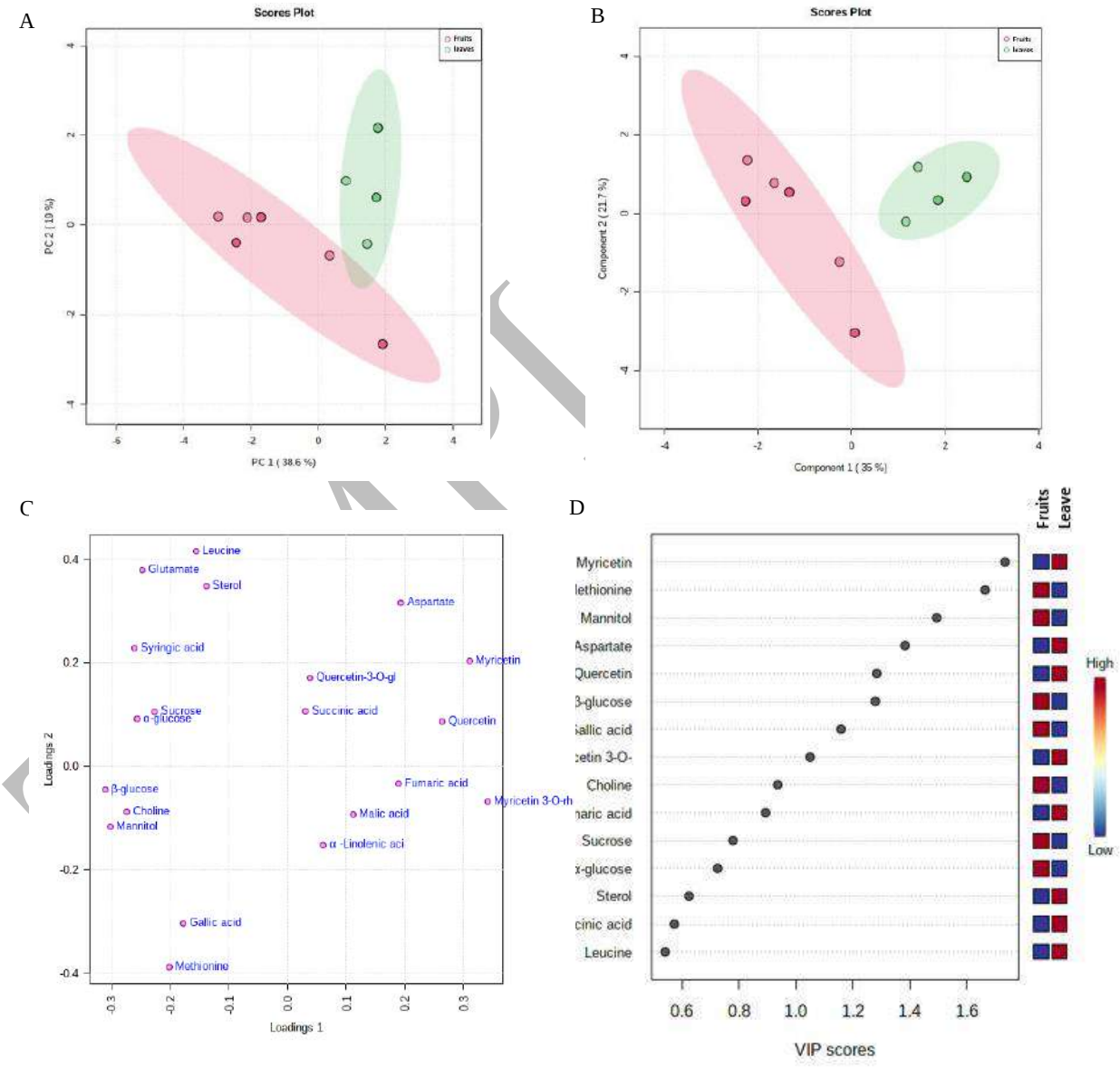

Figure 3. Multivariate data analysis of *R. tomentosa* leaf and fruit samples (A). PCA Score Plot; (B). PLS-DA score plot; (C). PLS-DA loading plot analysis; (D). Variables importants in projection (VIP) based on PLS-DA.

Cross-validation was used to determine the Q_2 to assess the predictability of the PLS-DA model. When $R^2 = 1$ and $Q^2 = 1$, the model could precisely describe and predict the data[22]. In this study, PLS-DA demonstrated distinct separation ($R^2 = 1$) and excellent predictability ($Q^2 = 0.9$). The PLS-DA model with a CV-ANOVA less than 0.05 was determined to be the most accurate through 20 permutation tests that validated the results. Based on these results, the model was reliable [20].

After a separation between the leaves and fruits of *R. tomentosa* has been observed, a loading plot will be used to identify the compounds that distinguish the two groups. Among the 20 compounds observed, Figure 3C revealed that there were five distinct components in the leaf profile with fruit (Table 1). Observed in the positive region of PC1 were fumaric acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin, and aspartate.

When PLS-DA is implemented, the VIP score is readily available. The VIP reflected the significance of the model's variables and was recognized as a valuable instrument for identifying the variables that contributed the most to the examined variants. Moreover, a VIP score greater than one was frequently used as a selection criterion [23]. As shown in Figure 3D, the score graph revealed that nine metabolites, including myricetin, myricetin 3-O-rhamnpyranoside, quercetin, aspartate, choline, gallic acid, methionine, mannitol, and -glucose, had values greater than 1.

These results suggested that more research was required beyond VIP score compounds. to determine if there were notable variations in the chemical composition of fruits and leaves of *R. tomentosa* at the compound level. The concentration was determined using semi-quantitative examination of the compound signal obtained from the internal signal of the TMSP standard. The analysis of signal integration findings was conducted using independent t-tests.

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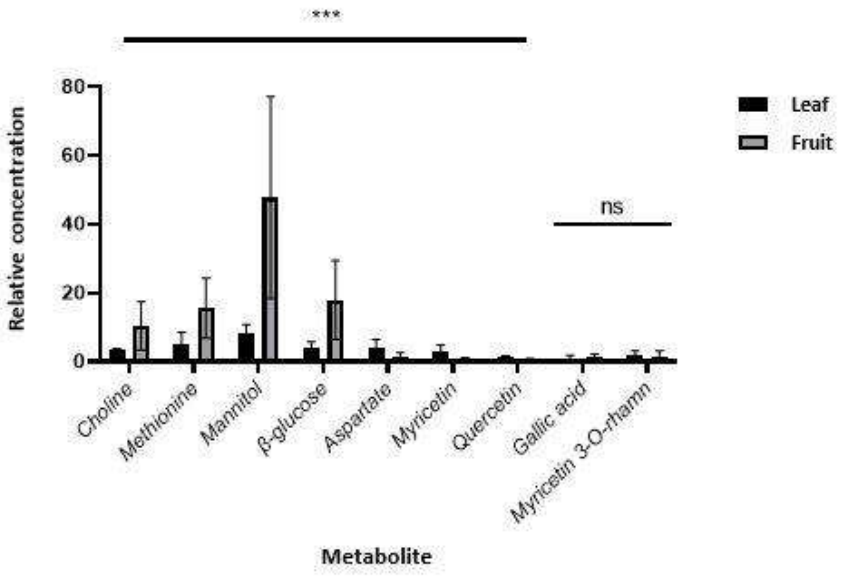

Figure 4. Histogram comparison of metabolite compound concentrations as important contributors to the leaves and fruits of *R. tomentosa*.

Heatmap was used to further assess differences in the diversity of compound content between the fruits and leaves. Furthermore, the concentration of compounds found in the fruits and leaves of *R. tomentosa* was visualized through cluster analysis [24]. Heatmap was a form of visualization of the distribution of data depicted in the form of color changes. The relative concentrations of compounds in the fruits and leaves of *R. tomentosa* served as the data for heatmap analysis. The data were then presented based on the groups of samples. The heatmap analysis results showed that compounds found in the leaves and fruits demonstrated high diversity and varied in concentration, as shown in Figure 5.

Young leaves and green fruits appeared in the same cluster on the heatmap, and certain compounds had higher concentrations compared to others. These include malic acid, α linolenic acid, aspartate, quercetin 3-O glucoside, fumaric acid, myricetin 3-O-rhamnpyranoside, myricetin,

quercetin, methionine, and gallic acid, which were all indicated by a dark brown color. Meanwhile, old leaves, as well as red and purple fruits had a lower concentration, as indicated by light brown to dark blue colors. Quercetin 3-O glucoside, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, as well as gallic acid compounds were members of the flavonoid group. This is in accordance with the total flavonoid content and the value of the antioxidant capacity of green fruit and young leaves which are higher than old leaves, red and purple fruits in the results of previous research[11], namely the total flavonoid content of green fruit is 95.731±5.42 mg QE/g DW and the value of antioxidant capacity 1419.75±3.48 µmol TE/g DW and young leaves with total flavonoid content 96.375±3.96 mg QE/g DW and antioxidant capacity value 1069.38±6.57 µmol TE/g DW, while total flavonoid content and antioxidant capacity value old leaves 70.311±5.22 mg QE/g DW and 844.91±5.72 µmol TE/g DW, red fruit 88.125±2.72 mg QE/g DW and 263.93±1.60 µmol TE/g DW and purple fruit 67.115±2.57 mg QE/g DW and 127.49±0.57 µmol TE/g DW [11].

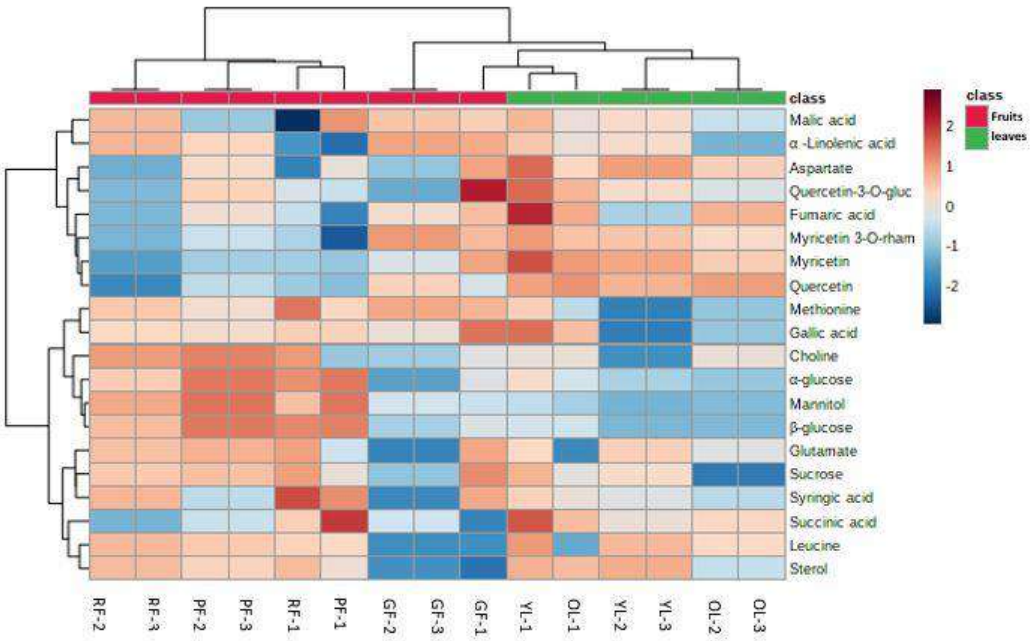

Figure 5. Heatmap of the leaf and fruit of *R. tomentosa*. The blue color represents low concentration and the brown color represents high concentration. Note: YL (young leaves), OL (old leaves), GF (green fruit), RF (red fruit), PF (purple fruit)

These findings were consistent with those of Ali et al. [16] and Gogna et al. [13], who also acquired comparable results. The study revealed that the number of hydroxycinnamates, caftaric, coutaric acid, and quercetin glucoside compounds, which belong to the phenol and flavonoids families, increased in grapes (*Vitis* spp.) during the later stages of green fruit development and declined abruptly after ripening [16]. Young leaves had the highest phenol and flavonoid content and antioxidant activity, followed by mature leaves and seeds. Furthermore Belwal et al. [25] reported that immature fruits contain significant quantities of polyphenols, including flavonoids. This finding indicated that the pre-ripening period served as a defense mechanism for fruit against various congenital diseases. The presence of higher amounts of antioxidants in the unripe fruits of *Rubus ellipticus* and *Myrica esculenta* was consistent with this finding. According to these findings, as the fruit ripened, phenols and flavonoids oxidized and participated in the biosynthesis of anthocyanins, which accumulated during maturation, thereby decreasing the flavonoid concentration.

This current study is infact explained what we have reported in our previous study focusing on the antioxidant antioxidant capacity, total flavonoid content, as well as compound distribution in *R. tomentosa* using histochemical analysis. The antioxidant activity evaluation using FRAP and DPPH exhibited a comparable proportion, especially in the green fruits ethanol extracts which exhibited the

highest FRAP value of 1367.59±9.12 mol TE/g DW and DPPH radical scavenging ability value of 1419.75±3.48 mol TE/g DW. The lowest antioxidant activity was observed in the purple fruits with FRAP value of 138.38±1.13 mol TE/g DW and DPPH value of 127.49±0.57. As a comparison the DPPH value of the purple fruits were almost four times lower than the activity reported by Lai et al. [4] of 431.17±14.5 μmol TE/g DW and higher than those reported by Wu et al. [26] of 8.79-92.60 μmol TE/g DW, which were measured in fruits such as grape, blueberries, blackberries, kiwifruit, oranges, apples, mangoes, and bananas [27].

NMR experiments were used to identify and confirm the presence of a wide variety of metabolites in all three samples (seed, skin, and pericarp) obtained from *Momordica charantia* fruits as reported by Mishra et al. [28]. To identify the metabolic differences between the seed, skin, and pericarp samples, multivariate statistical analysis was used. Different parts of the fruit had significantly different concentrations of important metabolites. The highest total flavonoid and phenolic contents were found in seeds and pericarp, followed by skin. Flavonoid metabolites that are synthesized from naringenin and have been identified in their study include luteolin, catechin, kaempferol, quercetin and myricetin. Based on metabolic analysis, the fruit's pericarp and seeds more antioxidants activities than the skin does. The scavenging effects of methanol extracts of ripe fruits measured by DPPH assay were in the order: seed > pericarp > skin [28].

According to the heatmap, red and purple fruits belonged to the same cluster, as indicated in Figure 5. The results demonstrated that the choline, mannitol, β-glucose, α-glucose, and sucrose compounds had a higher concentration, as indicated by a dark brown color. Meanwhile, young leaf and green fruits had a lower concentration, which was shown by light blue to dark blue colors. Mannitol, β-glucose, α-glucose, and sucrose were carbohydrates (sugars) compounds. According to Ali et al. [16], the concentration of glucose and fructose increased during the ripening stage of grapes (*Vitis* spp.).

The growth of grapefruit as reported by Ali et al. [16], was similar to that of *R. tomentosa* fruit, as they both underwent a complex series of biochemical and physical changes, such as variations in composition, size, color, taste, texture, as well as pathogen resistance. The development of grapes could be separated into three phases. During the initial phase (phase I), the fruit grew quickly, primarily due to cell division and expansion. During this phase, the biosynthesis of various compounds, including malic acid, tartaric acid, hydroxycinnamates, and tannins, occurred and reached a maximum concentration approximately 60 days after flowering. Phase II referred to the growth lag phase, which was often observed 7–10 weeks after flowering, and was characterized by the accumulation of sugar. In Phase III (ripening), the berries experienced significant changes in morphology and composition. Moreover, during this phase, the berry's size doubled, indicating the onset of color development (associated with anthocyanin accumulation in red wine), along with an increase in sweetness (particularly in fructose and glucose levels), and a concurrent decrease in acidity.

The sugar content of fruits is frequently employed as an indicator for assessing their level of ripeness and determining the optimal time for harvest. Carbohydrates, particularly sucrose, are produced by the process of photosynthesis in grapevine leaves. The carbohydrates were delivered to the fruits via the phloem. The sugar content underwent alteration after the transfer as a result of the loss of water. Furthermore, sugar was utilized not just as a source of carbon and energy, but also as a means to modulate the regulation of gene expression. The accumulation of fructose and glucose started during the second phase of fruit growth and persisted thereafter. The transportation of monosaccharides via transporters facilitates the delivery of sugars to cellular organelles [16]. Wang et al. (29) assert that sugar plays a crucial role in facilitating plant development and providing energy. Fructose and glucose play a crucial role in the synthesis of sucrose and serve as precursors for the formation of organic acids and pyruvate. Throughout the developmental process, there was a notable and substantial rise in the concentrations of fructose and glucose.

The dark violet, bell-shaped edible berries of *R. tomentosa*, as described by Salni et al. [30], have been traditionally employed as folk medicine to address issues such as dysentery, diarrhea, and traumatic hemorrhage. Additionally, these berries have played a role in crafting a renowned fermented beverage called "Ruou sim" on Phu Quoc Island in southern Vietnam. As they mature, the fruits acquire a deep purple color and possess an astringent taste [4,31,3]. Within China, the berries are

transformed into delectable pies, jams, and salad additions. Additionally, these fruits play a key role in the creation of traditional wines, beverages, jellies, and freshly canned syrups for human consumption. Notably, the berries of *R. tomentosa* harbor a rich assortment of chemical constituents including sugars, minerals, vitamins phenols, flavonoid glycosides, organic acids, amino acids, quinones, and polysaccharides. Furthermore, this plant thrives in subtropical and tropical regions, serving as a valuable source for cultivating novel ingredients that contribute to promoting health benefits [26].

4. Conclusion

R. tomentosa appeared to have played a significant and holistic role in the daily lives of ancient societies, providing medical benefits. Multiple biological activities of this plant, including antifungal, antimicrobial, antimalarial, antioxidant, anti-inflammatory, and osteogenic properties, have been documented. Therefore, it was essential to comprehend the various parts of *R. tomentosa*, such as the leaves and fruits at various phases of maturation, and the metabolic fate of its various classes of compounds. This study demonstrated that the combination of ^{1}H-NMR and multivariate data analysis enabled the detection of significant differences between the various developmental stages of the leaves and fruits used in this investigation. At various stages of development, the samples contained substantially different amounts of sugar, aromatic compounds, and phenolic compounds. Green fruits and young leaves contained substantial concentrations of phenolics, including quercetin 3-O glucoside, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, and gallic acid. During the final phases, choline, mannitol, α-glucose, β-glucose, and sucrose concentrations increased. The approach of this study was useful for analyzing a variety of compounds within the *R. tomentosa* metabolome; however, further research with more sensitive analytical instruments may be desirable to provide a thorough examination of the metabolome transformation and metabolism of the fruit and leaf of *R. tomentosa* at different stages of development.

Acknowledgments

The authors are grateful to Gadjah Mada University in Yogyakarta, Indonesia, for their generous support of this study through the RTA Grant 2022.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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4. Editor Decision: Revisions Required reviewer 2 (19-9-2023)

UNIVERSITAS LAMBUNG MANGKURAT

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

Decision is available for your submission RNP-2307-2853

ACG Publications <info@acgpubs.org> Tue, Sep 19, 2023 at 1:58 PM
To: Evi Mintowati Kuntorini <evimintowati@ulm.ac.id>

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Records of Natural Products
RNP-2307-2853
Metabolomic profiling of Rhodomyrtus tomentosa (Ait.) Hassk. leaves and fruits using 1H NMR
Evi Mintowati Kuntorini,Laurentius Hartanto Nugroho,Maryani Maryani,Tri Rini Nuringtyas

Dear Professor Evi Mintowati Kuntorini,

I am writing to inform you that your manuscript entitled "**Metabolomic profiling of Rhodomyrtus tomentosa (Ait.) Hassk. leaves and fruits using 1H NMR** " has now been reviewed. I shall be grateful If you could kindly revise your manuscript based on the comments of reviewers and resubmit your modified manuscript as soon as possible. Please prepare a response to reviewer letter, answer the questions or comments item by item and highlighted the corrections with red color on the manuscript. If you do not send your revised manuscript within 21 days we will stop the process and your manuscript will be rejected automatically. Please login to the PAMS to see the further comments on your submission.

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Please note, the revised manuscript is also subject to additional review. I look forward to receiving your revised manuscript.

Sincerely yours,

Ahmet C. Gören

Records of Natural Products

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COMMENTS from REVIEWERS

1) Please correct the references according to the style of the journal.

2) Language of the text must be improved. I strongly recommend getting professional aid to the authors.

3) FID data of the NMR spectra must be provided in Supporting information. Some of the further studies and checks might be possible in this way in the future.

Yenikent Mahallesi, Fırat Caddesi, 1/3, Gebze, Kocaeli, Türkiye
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5. Second revised submission_Minor Revised (27-9-2023)

- Cover Letter Revision
- Manuscript Revision
- Manuscript tracking proofreading
- Certificate proofreading

UNIVERSITAS LAMBUNG MANGKURAT

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

Decision is available for your submission RNP-2307-2853

Evi Mintowati Kuntorini <evimintowati@ulm.ac.id> Wed, Sep 27, 2023 at 8:04 AM
To: ACG Publications <info@acgpubs.org>

Dear Prof. Dr. Ahmet C. Gören

Co-Editor-in-Chief
Records of Natural Products Journal

We would like to thank you for the letter dated 19/09/2023, and the opportunity to resubmit a minor revised copy of this manuscript. We would also like to take this opportunity to express our thanks to the reviewers for the positive feedback and helpful comments for correction or modification.

We believe have resulted in an improved revised manuscript, which you will find uploaded alongside this document. The manuscript has been revised to address the reviewer comments, which are appended alongside our responses to this letter.

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

Some minor revisions for the authors to consider:

[Comment 1] Please correct the references according to the style of the journal.
Response: Thank you very much for the reminder. We have made revisions accordingly.

[Comment 1] Language of the text must be improved. I strongly recommend getting professional aid to the authors.
Response: Revised accordingly. We have been revised to include a proofreading certificate.

[Comment 1] FID data of the NMR spectra must be provided in Supporting information. Some of the further studies and checks might be possible in this way in the future.
Response: Thank you for the suggestion, the raw data submitted and available in Supporting information and email.

We very much hope the revised manuscript is accepted for publication in Records of Natural Products Journal.

Regards,

Dr. Evi Mintowati Kuntorini

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

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

ORIGINAL ARTICLE

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Metabolomics Profiling of *Rhodomyrtus tomentosa* (Ait.) Hassk. Leaves and Fruits using ^{1}H NMR

Evi Mintowati Kuntorini [1,2*], Laurentius Hartanto Nugroho [1], Maryani [1] and Tri Rini Nuringtyas [1,3*]

[1]*Faculty of Biology, Universitas Gadjah Mada. Teknika Selatan Street, 55281, Yogyakarta, Indonesia*

[2]*Departement of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat. A. Yani Km. 36 Street, Banjarbaru City, 70714, South Kalimantan, Indonesia*

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(Received Month Day, 201X; Revised Month Day, 201X; Accepted Month Day, 201X)

Abstract: Several investigations are extensively documenting the presence of metabolites with antibacterial, anticancer, antioxidant, and anti-inflammatory properties in extracts derived from *Rhodomyrtus tomentosa* fruits and leaves. Therefore, this study aimed to evaluate the metabolite profile of *R. tomentosa* fruits and leaves at various maturity stages and determine their phytomedicinal values. ^{1}H NMR and chemometric analyses were used to conduct a metabolomics study for comparing the metabolite profile and phytomedicinal values of different plant organs at varying ages. Leaves were classified into young and old categories, while fruits were divided into three maturity stages, namely green, red, and purple. The wild-grown fruits and leaves of *R. tomentosa* (Ait.) Hassk were gathered in Banjarbaru, South Kalimantan Province, Indonesia. The results of multivariate analysis showed that choline, methionine, mannitol, and β-glucose compounds were three times higher in fruits compared to leaves. Meanwhile, the concentration of aspartate, myricetin, and quercetin compounds was three times higher in leaves compared to fruits. The quantities of secondary metabolites, including flavonoids, were higher in young leaves and green fruits than in old, red, and purple fruits.

Keywords: *Rhodomyrtus tomentosa*, flavonoid, ^{1}H-NMR, multivariate statistical analysis

1. Introduction

Plants are significantly important in the daily lives of people by providing essential resources such as medicine, food, fiber for clothing, and wood for building. Among these resources, plants used for treating different medical conditions are considered the most valuable. Individuals without access to modern healthcare still place great value on traditional medical practices. Several studies have shown that the majority of traditional medicines are produced from plant-based materials. A recent report stated that approximately 80% of the global population relies on plants to treat a wide range of ailments, indicating their widespread usage. Consequently, herbal medicines have become integral

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The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products Month-Month 202x EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.18.17.06.108
Available online: X/X/202X

components of modern therapy, with 25% of medications available worldwide originating from plants [1].

Rose myrtle, scientifically known as *Rhodomyrtus tomentosa*/*R. tomentosa* (Ait.) Hassk. is a blossoming plant belonging to the Myrtaceae family. According to previous reports, *R. tomentosa* is indigenous to southern and southeastern Asia, with its natural habitat spanning from India, southern China, the Philippines, and Malaysia, to Sulawesi. This plant has an exceptional ability to grow in various environments, ranging from sea level to elevations of 2400 m. Furthermore, it thrives in diverse locations, including natural forests, beaches, wetlands, riparian zones, moist, wet woods, and bog borders, requiring intense sunlight and minimal soil conditions [2-4]. In tropical and subtropical gardens, *R. tomentosa* is a well-liked ornamental plant, cultivated for its abundant blooms, delectable, and edible fruits. These fruits are often used for several culinary applications, such as pies, salads, and jams, including additional processing into wine, jellies, or canned fruits in Vietnam and China [2]. Modern pharmacological studies have shown that *R. tomentosa* components possess diverse properties, namely antibacterial [5], anticancer [6], anti-inflammatory [7], and antioxidant [8]. Traditional Malaysian, Chinese, and Vietnamese medicine has used its leaves, roots, buds, and fruits for medicinal purposes [4]. For example, the Dayak and the Paser local tribes in East Kalimantan, Indonesia, use the roots to treat diarrhea and stomachaches, and as a postpartum tonic [9]. The crushed leaves serve as external poultices and the tar obtained from its wood is used for eyebrow darkening. These traditional applications are in line with the effects observed in modern pharmacological studies. Several phytochemical reports showed that the plant contains flavonoids, triterpenoids, phenols, microelements, and meroterpenoids [2]. Among these compounds, rhodomyrtone is the most prominent, possessing numerous potential pharmacological properties [10], while piceatannol serves as the main and highly effective phenolic component [3].

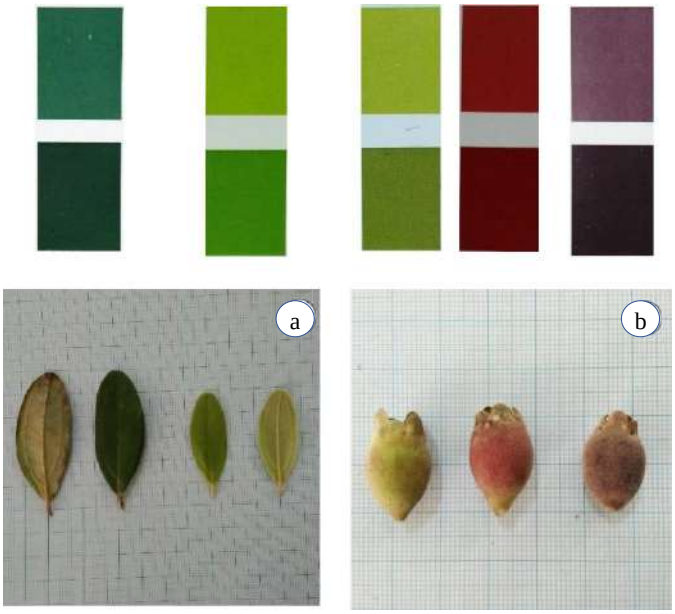
In a previous study, the total phenolic content, antioxidant capacity, total flavonoids, and compound distribution in *R. tomentosa* were explored using histochemical analysis [11]. This report did not examine the specific antioxidant profile of fruits and leaves at various stages of development. To address the limitation, ^{1}H-NMR was used to analyze the metabolite profile in leaves and fruits. Therefore, this study aimed to analyze the secondary and primary metabolites of various parts of *R. tomentosa*, specifically fruits and leaves. The existence of these compounds was correlated with the previously reported bioactivity and phytomedicinal qualities. NMR spectroscopy and multivariate data analysis without chromatographic separation were used to identify metabolites directly from the samples. A metabolic analysis was also conducted on *R. tomentosa* fruits and leaves at various stages of maturation. Subsequently, multivariate statistics were used to determine the compounds significantly contributing to the variations between both parts. This study is the first systematic examination of *R. tomentosa* fruits and leaves at various stages of maturity using a combined NMR and multivariate statistical method. Analysis showed the applicability of the NMR-based method in plant metabolomics.

2. Materials and Methods

2.1. Plant materials

In this study, *R. tomentosa* plant was obtained from its natural habitat in the wild. The samples were collected from Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) in August-October 2020. To accurately characterize the plant tissues, Munsell Color Charts were used as a reference guide for samples of leaves and fruits [12]. Leaves samples were selected from young leaves (2^{nd} – 6^{th} order from the shoot [Munsell Color Carts guide: 5GY (7/8-6/8)]) and old leaves (7^{th} -12^{th} order from the shoot [Munsell Color Carts guide: 2.5G (4/4-3/4)]). Furthermore, fruit samples used were green [Munsell Color Carts guide: 2.5GY (7/8-6/10)], red [Munsell Color Carts:10R (6/10-5/10)], and purple [Munsell Color Carts:5RP (4/4-3/2)] with a color guide using Munsell Color Charts for Plant tissues, as illustrated in Figure 1A. Subsequently, three replicates of each leaves and fruits were analyzed, followed by identification using the Herbarium Bogoriense, Indonesian Institute of Sciences, located in Bogor, Indonesia, under certificate number 1007/IPH.1.01/If.07/IX/2020.

A



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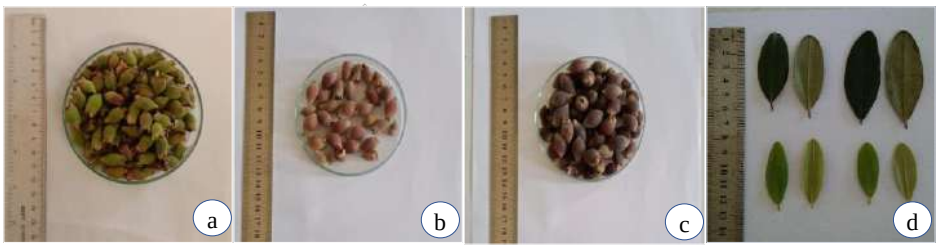


Figure 1. *Rhodomyrtus tomentosa* (Ait.) Hassk. (A) a: leaves, b. fruits, with a color guide using Munsell Color Charts for Plant tissues (B) a: green fruits, b: red fruits, c: purple fruits, d: young and old leaves.

2.2. Crude extract preparation and sample preparation for ^{1}H-NMR

The old and young leaves were carefully selected from the tip shoots of *R. tomentosa*, and fruits were dried in an oven at 40 °C, and ground at room temperature. Each grounded material of 500 g was macerated in ethanol of 1000 mL (SmartLab, Indonesia) for 24 h. The solvent was discarded and changed every 24 h, with this process being repeated three times [9,11]. Furthermore, extracts from the same samples were combined, properly mixed, filtered to eliminate cell debris, and dried using a rotary evaporator.

^{1}H-NMR sample preparation was carried out using a slightly modified version of the sample extraction of previously reported methods [15]. Precisely, 25 mg of the crude extract was placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent, comprising 0.5 mL of methanol-d4, 0.5 mL of KH_2PO_4 buffer, and pH 6.0 containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The mixture was vortexed, sonicated for 1 min, homogenized, and centrifuged for 1 min at 10,000 rpm using a microcentrifuge. The supernatant was collected, placed in the NMR tube, and prepared for ^{1}H-NMR analysis.

2.3. NMR experiments

^{1}H-NMR was carried out using a 500 MHz spectroscopy (JEOL JNM ECZ500R) at a temperature of 25 °C. The parameters used for a total of 128 scans lasting for 10 min included a

relaxation delay of 1.5 seconds, X_angle 60°, and a pre-saturation mode of 4.27 ppm. Subsequently, the deuterated solvent was set as the internal lock, and spectral width was measured from 0 to 10 ppm.

2.4. Data analysis

The ^{1}H-NMR spectra were analyzed using MestReNova analysis, followed by processing with manual phasing, baseline adjustment, and calibration to internal standard solution signals (TMSP) positioned at the chemical shift of 0.0 ppm. Multiplicities in the observed NMR resonances were labeled according to the convention: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, and m = multiplet [14]. Furthermore, metabolites were identified by comparing the information in the database from previous studies [13-18]. For semi-quantitative analysis, the area of a signal analysis was compared to that of the TMSP signal as an internal standard. All ^{1}H-NMR signals were normalized to total intensity to develop data for multivariate analysis. MetaboAnalyst software (MetaboAnalyst.ca) was used to analyze multivariate data using Principal Component Analysis (PCA), Partial Least-Squares Discriminant Analysis (PLS-DA), and hierarchical clustering heat map analyses. The spectra were centered and scaled with autoscaling, followed by initial data processing using PCA to evaluate the natural clustering characters. After a clear grouping was observed, the data were subjected to PLS-DA to maximize covariance between measured data (NMR peak intensities) and the response variable (predictive classifications). The PLS-DA coefficient loading plot was used to determine significant metabolites contributing to the separation between the two classes [14]. The model's predictive ability (Q^2) was measured using cross-validation, while statistical significance was determined using a permutation test. This was followed by the ranking of the important compounds in the model using variable importance of projection (VIP) scores. A VIP score of >1 indicated that the variable contributed to the variation of the sample. The t-test analysis was used to determine significant differences in metabolites between all samples with p-values ≤ 0.01.

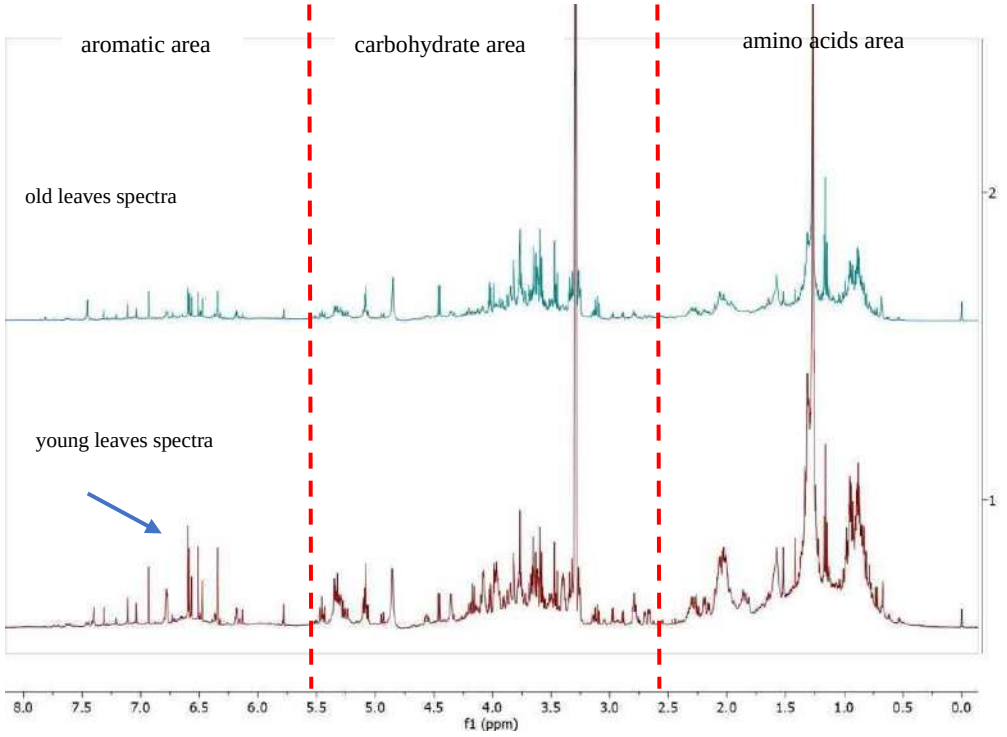
3. Results and Discussion

3.1. Visual analysis of ^{1}H-NMR spectra

NMR spectroscopy was used as a method to determine the magnetic resonance of molecular nuclei interacting with external magnetic fields [19]. Moreover, NMR could produce a distinct and specific spectrum for each compound, leading to its frequent use in determining the type of metabolite. The quality of the results was determined by the number of compounds identified, rather than signals observed during the NMR analysis [20]. The NMR metabolomics methods have been used widely, making compound identification less complicated. This can be achieved by comparing the signal produced by the samples to those generated by the same compounds in previous reports using CD_3OD-D_2O as the solvent [13-18]. Aqueous methanol is commonly used as an extraction solvent alone due to its ability to extract a wide range of compounds, both non-polar and polar. The chemical shift of substances in NMR can be influenced by various solvents. Consequently, multiple reference papers were used to conduct a comparative analysis of potential signal changes that could be identified. In this study, the coupling constant was used as an important parameter to validate the matching signals in the data with the references [15].

The ^{1}H-NMR spectra were separated into three regions based on their chemical shift (δ), namely the amino acids and terpenoids, carbohydrates, and aromatic compounds. Aliphatic compounds (organic and amino acids) were found in the chemical shift 0.5-3.0 ppm, carbohydrates in 3.1 – 6.0 ppm, and aromatic compounds in > 6 ppm. Furthermore, the various developmental stages of ^{1}H-NMR spectra of leaves and fruits extracts were analyzed and compared, as shown in Figure 2.

A



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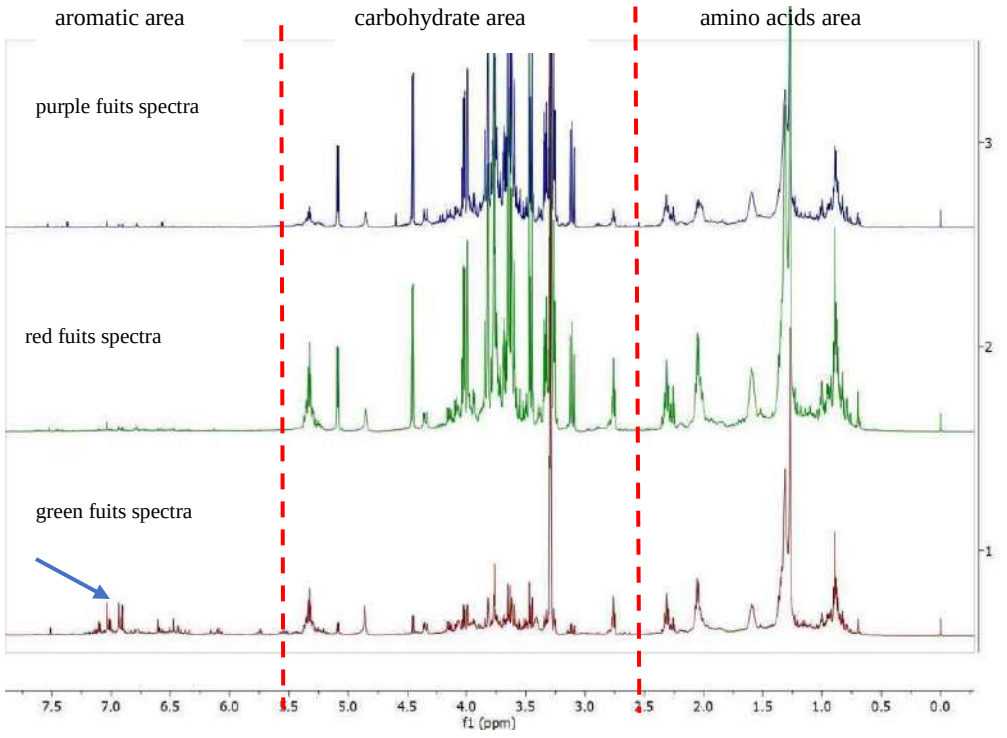

Figure 2. The comparison of the ^{1}H-NMR spectrum (δ 0.00–8.00 ppm) indicates signals of *R. tomentosa* from (A) leaves and (B) fruits of *Rhodomyrtus tomentosa*. The arrow shows the aromatics regions which observed differences in signal intensities.

The results of the putative compounds identified by ^{1}H-NMR showed the presence of primary and secondary metabolite compounds. Specifically, the primary metabolites included amino acids (chemical shift 2.0-0.5 ppm) and carbohydrates (chemical shift 5.0-3.0 ppm), with aromatic compounds (chemical shift > 6 ppm) being the secondary metabolites. In the aromatic regions of the NMR spectra, a gradual decrease in phenolic content was observed during leaves and fruits growth. The intensities of signals in the aromatic area in the young leaves and green fruits samples were higher than in the old, red, and purple leaves, as illustrated in Figures 2A and B.

3.2. Identification of metabolites/Assignment of ^{1}H-NMR signals

The advantages of using ^{1}H-NMR have been shown in various metabolomics studies. However, ^{1}H-NMR presented a significant challenge in chemical identification due to overlapping signals in multiple regions, particularly at 5.0-3.0 ppm, which corresponded to sugar compounds. This caused the inability to identify signals in the sugar region, except for glucose and sucrose, leading to a decrease in the number of substances detected in this study. The results also showed the identification of 20 putative compounds based on the ^{1}H-NMR spectra, as presented in Table 1. In the amnio acid region, the specific signals of leucine, glutamate, methionine, and aspartate were identified, with chemical shifts ranging from 3.0-0.5 ppm. The organic acid compounds, such as malic, fumaric, and succinic acids were observed in the chemical shifts of between 3.0 - 2.0 ppm. The frequently identified sugars, including mannitol, β-glucose, α-glucose, and sucrose were also observed at 5.00 - 3.50 ppm. In the less crowded regions, several phenolics were identified, including 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.

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

No	*Compound*	*Chemical shifts δ (ppm) and coupling constants (Hz)*
	Amino Acids	
1	*Aspartate*	2.68 (dd, J= 3.0; 17.0 Hz)
2	*Glutamic acid*	2.06 (m); 2.34 (m)
3	*Leucine*	0.93 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)
4	*Methionine*	2.16 m ; 2.79 (t, J= 6.08; 6.08 Hz)
	Organic Acids	
5	*Fumaric acid*	6.51 (s)
6	*Malic acid*	4.34 (dd, J= 6.6 ; 4.7 Hz)
7	*Succinic acid*	2.54 (s)
	Sugars	
8	*Mannitol*	3.77 (d, J= 3.28 Hz)
9	*β-glucose*	4.45 (d, J= 7.79 Hz)
10	*α-glucose*	5.09 (d, J= 3.84 Hz)
11	*Sucrose*	5.35 (d, J= 3.91 Hz)
	Aromatics Compounds	
12	*Gallic acid*	7.03 (s)
13	*Myricetin*	6.34 (d, J= 1.99 Hz); 7.32 (s)
14	*Myricetin 3-O-rhamnpyranoside*	6.93 (s)
15	*Quercetin-3-O-glucoside*	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)
16	*Quercetin*	6.13 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)
17	*Syringic acid*	3.88 (s)
	Other compounds	
18	*α -Linolenic acid*	1.16 (t, J=7.04 ; 7.04); 1.29 (m)
19	*Choline*	3.25 (s)
20	*Sterol*	0.70 (s)

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

3.2. Multivariate data analysis

Multivariate PCA was performed to assess the variations of compounds present in fruits and leaves of *R. tomentosa*. The PCA score plot was used to show the separation of classes, while the correlation between variables was interpreted in the loading plot [21]. MetaboAnalyst 5.0 automatically generated five PCs representing 87.6% of all observed variants. Subsequently, the 2D score diagram derived from PC1 and PC2 distinguished fruits and leaves samples. As shown in Figure 3A, the Q^2 cumulative of PC1 and PC2 yielded 57.6% of variants, which was above 50%, indicating a reliable model. PLS-DA was implemented in multivariate analysis to enhance separation, where PC1 and PC2 accounted for 35% and 21.7% of the observed variants, respectively, for a total of 56.7% Q^2 in the 2D score plot. On the PLS-DA score plot presented in Figure 3B, samples of leaves and fruits were positioned in the negative and positive regions of PC1, respectively,

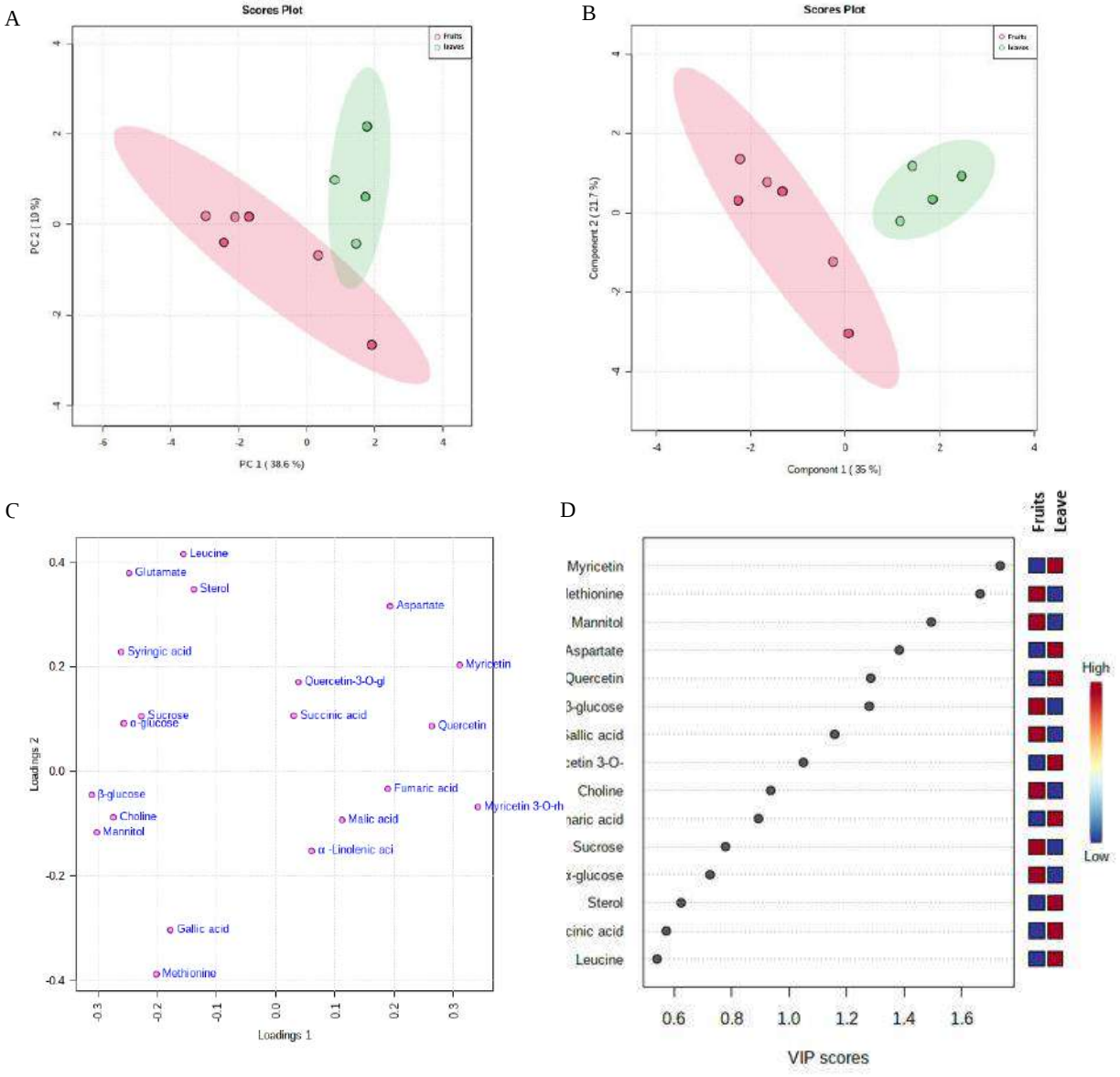

Figure 3. Multivariate data analysis of *Rhodomyrtus tomentosa* leaves and fruits samples (A). PCA Score Plot; (B). PLS-DA score plot; (C). PLS-DA loading plot analysis; (D). Variables important in projection (VIP) based on PLS-DA.

Cross-validation was used to determine the Q^2, which assessed the predictability of the PLS-DA model. When $R^2 = 1$ and $Q^2 = 1$, the model could precisely describe and predict the data [22]. In this

study, PLS-DA showed distinct separation (R^2 = 1) and excellent predictability (Q^2 = 0.9). The PLS-DA model with a CV-ANOVA less than 0.05 was determined to be the most accurate through 20 permutation tests that validated the results, indicating the reliability of the model [20].

After a separation between leaves and fruits of *R. tomentosa* was observed, a loading plot was used to identify the compounds that distinguished the two groups. Among the 20 compounds observed, Figure 3C showed that there were five distinct components in leaves profile with fruits, as illustrated in Table 1. Compounds observed in the positive region of PC1 were fumaric acid, myricetin, myricetin 3-*O*-rhamnpyranoside, quercetin, and aspartate.

During the implementation of PLS-DA, the VIP score was readily available, reflecting the significance of the model's variables. The VIP was recognized as an instrument for identifying the variables that contributed mainly to the examined variants. Moreover, a VIP score greater than one was frequently used as a selection criterion [23]. As shown in Figure 3D, the score graph indicated that nine metabolites, including myricetin, myricetin 3-*O*-rhamnpyranoside, quercetin, aspartate, choline, gallic acid, methionine, mannitol, and β-glucose, had values greater than 1.

These results suggested the need for further study beyond VIP score compounds to determine when there were notable variations in the chemical composition of fruits and leaves of *R. tomentosa* at the compound level. Subsequently, concentration determination was carried out using a semi-quantitative examination of the compound signal obtained from the internal signal of the TMSP standard. Analysis of signal integration results was conducted using independent t-tests.

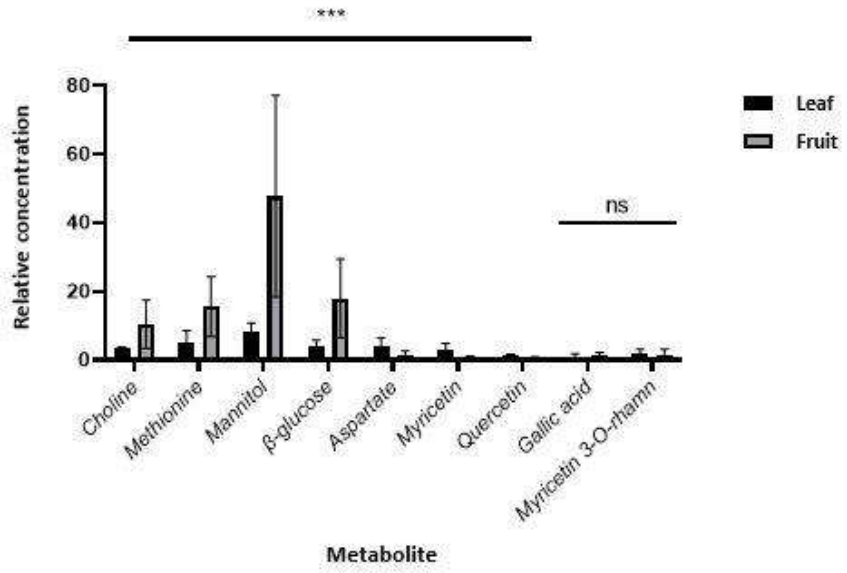

Figure 4. Comparison of metabolite concentrations as major contributors to differences between the leaves and the fruits of *Rhodomyrtus tomentosa*.

A heatmap was used to assess further differences in the diversity of compound content between fruits and leaves. The concentration of compounds found in fruits and leaves of *R. tomentosa* was visualized through cluster analysis [24]. Heatmap was a form of visualization method that represented data distribution through color changes. In this study, the relative concentrations of compounds in fruits and leaves of *R. tomentosa* served as the data for heatmap analysis, as presented based on the groups of samples. The results showed that the compounds identified showed high diversity and varied concentration, as shown in Figure 5.

Young leaves and green fruits appeared in the same cluster on the heatmap, while some compounds had higher concentrations compared to others. Compounds such as malic acid, α linolenic acid, aspartate, quercetin 3-*O*-glucoside, fumaric acid, myricetin 3-*O*-rhamnpyranoside, myricetin, quercetin, methionine, and gallic acid were indicated by a dark brown color. Meanwhile, old leaves, red, and purple fruits had a lower concentration, as indicated by light brown to dark blue colors.

Quercetin 3-*O*-glucoside, myricetin 3-*O*-rhamnpyranoside, myricetin, quercetin, and gallic acid compounds were members of the flavonoids group. This was in accordance with the total flavonoids content and the value of the antioxidant capacity of green fruits and young leaves, which were higher than old leaves, red, and purple fruits in a previous study [11]. Based on the results, the total flavonoid content of green fruits was 95.731±5.42 mg QE/g DW and the value of antioxidant capacity was 1419.75±3.48 µmol TE/g DW. The young leaves had a total flavonoid content of 96.375±3.96 mg QE/g DW and an antioxidant capacity value of 1069.38±6.57 µmol TE/g DW. The total flavonoid and antioxidant capacity values of old leaves were 70.311±5.22 mg QE/g DW and 844.91±5.72 µmol TE/g DW, red fruits had 88.125±2.72 mg QE/g DW and 263.93±1.60 µmol TE/g DW, while purple fruits 67.115±2.57 mg QE/g DW and 127.49±0.57 µmol TE/g DW, respectively [11].

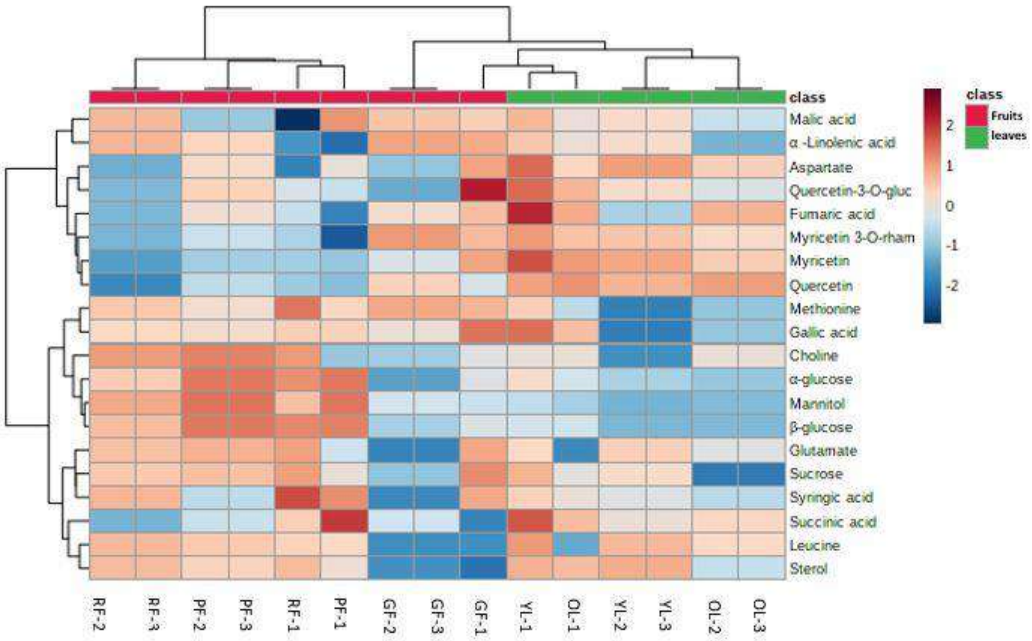

Figure 5. Heatmap of leaves and fruits of *Rhodomyrtus tomentosa*. The blue color represents low concentration and the brown color represents high concentration. Note: YL (young leaves), OL (old leaves), GF (green fruits), RF (red fruits), PF (purple fruits)

The number of hydroxycinnamates, caftaric, coutaric acid, and quercetin glucoside compounds in the phenol and flavonoid families increased in grapes (*Vitis* spp.) during the final stages of green fruits development and significantly declined after ripening 16]. Young leaves in *Carica papaya* L. had the highest phenol, flavonoid, and antioxidant activity, followed by mature leaves and seeds [13]. Furthermore, immature wild edible fruits contained significant quantities of polyphenols, including flavonoid [25], indicating that the pre-ripening period served as a defense mechanism for fruits against various congenital diseases. The presence of higher amounts of antioxidants in the unripe fruits of *Rubus ellipticus* and *Myrica esculenta* was consistent with this observation. Based on these results, as fruits ripened, phenols and flavonoids oxidized, participating in the biosynthesis of anthocyanins, which accumulated during maturation, leading to a decrease in flavonoid concentration.

This study elaborated on a previous study focusing on the antioxidant capacity, total flavonoid content, and compound distribution in *R. tomentosa* using histochemical analysis. The antioxidant activity evaluation using FRAP and DPPH showed a comparable proportion, particularly in the green fruits ethanol extracts, indicating the highest FRAP value of 1367.59±9.12 µmol TE/g DW and DPPH radical scavenging ability value of 1419.75±3.48 µmol TE/g DW. The lowest antioxidant activity was observed in the purple fruits with FRAP value of 138.38±1.13 µmol TE/g DW and DPPH of 127.49±0.57 µmol TE/g DW. As a comparison, the DPPH value of the purple fruits was almost four times lower than the activity antioxidant ORAC value of 431.17±14.5 µmol TE/g DW [4] but higher

than another study [26], which was measured in a variety of consumed fruits such as grape, kiwifruit, oranges, apples, mangoes, blueberries, bananas, and blackberries 8.79–92.60 µmol TE/g DW [27].

NMR experiments were carried out to identify and confirm the presence of a wide variety of metabolites in all three samples, namely seed, skin, and pericarp, obtained from *Momordica charantia* fruits [28]. To identify the metabolic differences between these samples, a multivariate statistical analysis was conducted. Different parts of fruits showed significantly varying concentrations of important metabolites, where the highest total flavonoid and phenolic contents were found in seeds and pericarp, followed by skin. Flavonoids metabolites synthesized from naringenin and identified included luteolin, catechin, kaempferol, quercetin, and myricetin. Based on metabolic analysis, the pericarp and seeds contained higher antioxidant activities compared to the skin. The scavenging effects of methanol extracts of ripe fruits measured by DPPH assay were arranged as seed > pericarp > skin [28].

According to the heatmap presented in Figure 5, red and purple fruits belonged to the same cluster. The results showed that the choline, mannitol, β-glucose, α-glucose, and sucrose compounds had a higher concentration, as indicated by a dark brown color. Meanwhile, young leaves and green fruits had a lower concentration, which was shown by the light-blue to dark-blue colors. Mannitol, β-glucose, α-glucose, and sucrose were carbohydrates (sugars) compounds. Ali et al. [16] reported that the concentration of glucose and fructose increased during the ripening stage of grapes (*Vitis* spp.).

The growth of grapefruits was similar to *R. tomentosa* fruits, as both passed through a complex series of biochemical and physical changes, including variations in composition, size, color, taste, texture, and pathogen resistance. Generally, the development of grapes could be separated into three phases. During the initial phase (phase I), fruits grew rapidly due to cell division and expansion, accompanied by the biosynthesis of various compounds, including malic, tartaric, hydroxycinnamates, and tannins. These compounds reached a maximum concentration approximately 60 days after flowering. Phase II referred to the growth lag phase, which was often observed 7 to 10 weeks after flowering, characterized by sugar accumulation. In Phase III (ripening), the berries experienced significant changes in morphology and composition, doubling in size. This indicated the onset of color development associated with anthocyanin accumulation in red wine as well as an increased sweetness, particularly in fructose and glucose levels, followed by decreased acidity [16].

The sugar content of fruits was used as an indicator for assessing the level of ripeness and determining the optimal time for harvest. Carbohydrates, particularly sucrose, were produced by the process of photosynthesis in grapevine leaves and transported to fruits through the phloem. During this process, the sugar content passed through alteration after the transfer due to the loss of water. Furthermore, sugar was used as a source of carbon, energy, and a means of modulating the regulation of gene expression. The accumulation of fructose and glucose started during the second phase of fruits growth, followed by a persistent process, incorporating the transportation of monosaccharides through transporters to facilitate the delivery of sugars to cellular organelles [16]. Sugar plays a crucial role in facilitating plant development, providing energy, where fructose and glucose synthesize sucrose as precursors for the formation of organic acids and pyruvate. Throughout the developmental process, there was a significant rise in the concentrations of fructose and glucose [29].

The dark violet, bell-shaped edible berries of *R. tomentosa*, have been traditionally used as folk medicine to treat issues such as dysentery, diarrhea, and traumatic hemorrhage [30]. Additionally, these berries were used in crafting a renowned fermented beverage called "Ruou sim" on Phu Quoc Island in southern Vietnam, maturing to acquire a deep purple color and an astringent taste [3,4,31]. In China, these berries are transformed into delectable pies, jams, and salad additions, playing a significant role in the creation of traditional wines, beverages, jellies, and freshly canned syrups for human consumption. The berries of *R. tomentosa* contain a rich assortment of chemical constituents, including sugars, minerals, vitamins, phenols, flavonoid glycosides, organic acids, amino acids, quinones, and polysaccharides. Furthermore, this plant thrives in subtropical and tropical regions, serving as a valuable source for cultivating novel ingredients that promote health benefits [26].

4. Conclusion

Rhodomyrtus tomentosa appeared to have played a significant and holistic role in the daily lives of ancient societies, providing medical benefits. Multiple biological activities of this plant, including antifungal, antimicrobial, antimalarial, antioxidant, anti-inflammatory, and osteogenic properties, have been documented. Therefore, it was essential to comprehend the various parts of *R. tomentosa*, such as the leaves and fruits at various phases of maturation, and the metabolic fate of its various classes of compounds. This study demonstrated that the combination of ^{1}H-NMR and multivariate data analysis enabled the detection of significant differences between the various developmental stages of the leaves and fruits used in this investigation. At various stages of development, the samples contained substantially different amounts of sugar, aromatic compounds, and phenolic compounds. Green fruits and young leaves contained substantial concentrations of phenolics, including quercetin 3-O glucoside, myricetin 3-*O*-rhamnpyranoside, myricetin, quercetin, and gallic acid. During the final phases, choline, mannitol, α-glucose, β-glucose, and sucrose concentrations increased. The approach of this study was useful for analyzing a variety of compounds within the *R. tomentosa* metabolome; however, further research with more sensitive analytical instruments may be desirable to provide a thorough examination of the metabolome transformation and metabolism of the fruit and leaf of *R. tomentosa* at different stages of development.

Acknowledgments

The authors are grateful to Gadjah Mada University in Yogyakarta, Indonesia, for supporting this study through the RTA Grant 2022.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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~~Metabolomic profiling~~Metabolomics Profiling of *Rhodomyrtus tomentosa* (Ait.) Hassk. ~~leaves~~Leaves and ~~fruits~~Fruits using ^{1}H NMR

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(Received Month Day, 201X; Revised Month Day, 201X; Accepted Month Day, 201X)

Abstract: ~~—~~Several ~~studies have~~investigations are extensively ~~documented~~documenting the presence of metabolites with antibacterial, anticancer, antioxidant, and anti-inflammatory properties in extracts derived from *Rhodomyrtus tomentosa* fruits and leaves. Therefore, this study ~~is~~ aimed ~~at evaluating~~to evaluate the metabolite profile of *R. tomentosa* fruits ~~and~~as well as leaves at various maturity stages~~, as well as determining~~ and determine their phytomedicinal values. ^{1}H NMR and chemometric ~~analysis~~analyses were used to conduct ~~a~~ metabolomics study ~~to compare~~for comparing the metabolite profile and phytomedicinal values of different plant organs at varying ages. ~~The leaves~~Leaves were classified into young and old categories, while ~~the~~ fruits were divided into three maturity stages, namely green, red, and purple. The wild-grown fruits and leaves of *R. tomentosa* (Ait.) Hassk were gathered in Banjarbaru, South Kalimantan Province, Indonesia. The results of multivariate analysis showed that choline, methionine, mannitol, and β-glucose compounds were three times higher in ~~the~~ fruits compared to ~~the~~ leaves. Meanwhile, the concentration of aspartate, myricetin, and quercetin compounds was three times higher in ~~the~~ leaves compared to ~~the~~ fruits. ~~Secondary~~The quantities of secondary metabolites, including ~~flavonoids~~flavonoid, were ~~identified in~~found to be higher ~~quantities~~ in young leaves and green fruits ~~compared to~~than in old leaves, ~~as well as~~ red, and purple fruits.

Keywords: *Rhodomyrtus tomentosa*, flavonoid, ^{1}H-NMR, multivariate statistical analysis

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EISSN:1307-6167

~~DOI: http://doi.org/10.25135/rnp.18.17.06.108~~

DOI: http://doi.org/10.25135/rnp.18.17.06.108

Available online: X/X/202X

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1. Introduction

Plants play a vital roleare significantly important in people'sthe daily lives of people by providing essential resources such as medicine, food, fiber for clothing, and wood for building. FurthermoreAmong these resources, plants used for the treatment oftreating different medical conditions are considered the most valuable among various natural resources. At present, individuals, Individuals without access to modern healthcare still place great value on traditional medical practices. Several studies have shown that the majority of traditional medicines are produced from plant-based materials. A recent report revealsstated that aboutapproximately 80% of the global population relies on plants to treat a wide range of ailments, highlightingindicating their widespread usage. MoreoverConsequently, herbal medicines have become integral components of modern therapy, with 25% of medications available around the world being derivedworldwide originating from plants [1].

Rose myrtle, scientifically known as *Rhodomyrtus tomentosa*/*R. tomentosa* (Ait.) Hassk.,, is a blossoming plant that falls underbelonging to the Myrtaceae family. Based onAccording to previous reports, it*R. tomentosa* is indigenous to southern and southeastern Asia, with its natural habitat spanning from India, southern China, the Philippines, and Malaysia, to Sulawesi. This versatile plant exhibits a remarkablehas an exceptional ability to grow in various environments, ranging from sea level to elevations of 2400 m. Furthermore, it thrives in diverse locations, such asincluding natural forests, beaches, wetlands, riparian zones, moist and, wet woods, as well asand bog borders, requiring intense sunlight and minimal soil conditions [2-4]. In tropical and subtropical gardens, *R. tomentosa* is a well-liked ornamental plant, cultivated for its abundant blooms and, delectable, and edible fruits. TheThese fruits are often used for several culinary applications, such as pies, salads, and jams. In Vietnam and China, they are processed, including additional processing into wine, jellies, or canned fruitfruits in Vietnam and China [2]. Modern pharmacological studies have shown that *R. tomentosa* components demonstrate apossess diverse array of pharmacological properties, namely antibacterial [5], anticancer [6], anti-inflammatory [7], as well asand antioxidant [8]. Traditional Malaysian, Chinese, and Vietnamese medicine have employedhas used its leaves, roots, buds, and fruits for medicinal purposes [4]. For example, the Dayak and the Paser local tribes in East Kalimantan, Indonesia, utilize *R. tomentosa* use the roots for the treatment of diarrhea, and stomachaches, as wellland as a postpartum tonic [9]. The crushed leaves serve as external poultices, and the tar obtained from its wood is used for eyebrow darkening. The majority of these effects are similar to those observed in theseThese traditional applications of *R. tomentosa*.are in line with the effects observed in modern pharmacological studies. Several phytochemical studiesreports showed that the plant contains flavonoidsflavonoid, triterpenoids, phenols, microelements, and meroterpenoids [2]. Among the variousthese compounds, rhodomyrtone stands out asis the most prominent compound, possessing numerous potential pharmacological properties [10], while piceatannol issserves as the main and mosthighly effective phenolic component [3].

AIn a previous study explored, the total phenolic content, antioxidant capacity, total flavonoid content, as well as , and compound distribution in *R. tomentosa* were explored using histochemical analysis [11]. The limitation of thisThis report was that it did not examine the specific antioxidant profile of the fruits and leaves at various stages of development. To address this gapthe limitation, ^{1}H-NMR was utilizedused to analyze the metabolite profile in the leaves and fruits. Therefore, this study is aimed at analyzingto analyze the secondary and primary metabolites of various parts of *R. tomentosa*, specifically the fruits and leaves. The existence of these compounds was then correlated with the previously reported bioactivity and phytomedicinal qualities. NMR spectroscopy and multivariate data analysis without any chromatographic separation were used to identify metabolites directly from the samples. A metabolic analysis was also conducted on *R. tomentosa* fruits and leaves at various stages of maturation and. Subsequently, multivariate statistics were used to determine the compounds that significantly contributed to the variations between both parts. Based on the findings, this This study is the first systematic studyexamination of *R. tomentosa* fruits and leaves at various stages of maturity employingusing a combined NMR and multivariate statistical approach, and it

demonstratesmethod. Analysis showed the applicability of the NMR-based approachmethod in plant metabolomics.

2. Materials and Methods

2.1. Plant materials

*Rhodomyrtus*In this study, *R. tomentosa* plant in this study growswas obtained from its natural habitat in the wild. The samples were gathered in the wild incollected from Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) in August-October 2020. UsingTo accurately characterize the plant tissues, Munsell Color Charts for Plant tissueswere used as a reference guide for color the samples of leaves and fruits [12]. The leafLeaves samples were selected from young leaves (2^{nd} – 6^{th} order from the shoot [Munsell Color Carts guide: 5GY (7/8-6/8)]) and old leaves (7^{th} -12^{th} order from the shoot [Munsell Color Carts guide: 2.5G (4/4-3/4)]). Furthermore, fruitfruits samples used arewere green [Munsell Color Carts guide: 2.5GY (7/8-6/10)], red [Munsell Color Carts:10R (6/10-5/10)], and purple [Munsell Color Carts:5RP (4/4-3/2)] with a color guide using Munsell Color Charts for Plant tissues (, as illustrated in Figure 1A.).Three. Subsequently, three replicates of each leafleaves and fruitfruits were analyzed and identified, followed by identification using the Herbarium Bogoriense, Indonesian Institute of Sciences, located in Bogor, Indonesia, under certificate number 1007/IPH.1.01/If.07/IX/2020.

Figure 1A. *Rhodomyrtus*R. *tomentosa* (Ait.) Hassk. a: leaves, b. fruits

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Figure 1B. ~~*Rhodomyrtus*~~*R. tomentosa* (Ait.) Hassk. a: green fruits, b: red fruits, c: purple fruits, d: young and old leaves.

2.2. Crude extract preparation and sample preparation for ^{1}H-NMR

The old and young leaves were carefully selected from the tip shoots of *R. tomentosa*, and ~~the~~ fruits were dried in an oven at 40°C, and ~~subsequently~~ ground at room temperature. Each grounded material of 500 g was macerated in ethanol of 1000 mL (SmartLab, Indonesia) for ~~a duration of~~ 24 h. The solvent was ~~then~~ discarded and changed every 24 h, ~~and~~with this process ~~was~~being repeated three times [9,11]. Furthermore, extracts from the same samples were combined, properly mixed, filtered to eliminate cell debris, and dried using a rotary evaporator.

^{1}H-NMR sample preparation was carried out using a slightly modified version of the sample extraction of previously reported methods ~~by~~ [15]. ~~A total of~~ Specifically, 25 mg of the crude extract ~~were~~was placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent ~~consisting of,~~ comprising 0.5 mL of methanol-d4 ~~and,~~ 0.5 mL of KH_2PO_4 buffer, and pH 6.0 containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The mixture was ~~then~~ vortexed ~~and,~~ sonicated for 1 min~~. The solution was~~, homogenized, and centrifuged for 1 min at 10,000 rpm using a microcentrifuge. The supernatant was collected, placed in the NMR tube, and prepared for ^{1}H-NMR analysis.

2.3. NMR experiments

^{1}H-NMR was carried out ~~with~~using a 500 MHz spectroscopy (JEOL JNM ECZ500R) at a temperature of 25°C. The ~~following~~ parameters ~~were~~ used for a total of 128 scans lasting for 10 min~~,~~ ~~namely~~ included a relaxation delay of 1.5 seconds, X_angle 60º, and a pre-saturation mode of 4.27 ppm. ~~The~~Subsequently, the deuterated solvent was set as the internal lock, and ~~the~~ spectral width was ~~then~~ measured from 0 to 10 ppm.

2.4. Data analysis

The ^{1}H-NMR spectra were analyzed using MestReNova analysis~~. Furthermore, the spectra were processed using~~, followed by processing with manual phasing, baseline adjustment, and calibration to internal standard solution signals (TMSP) positioned at the chemical shift of 0.0 ppm. Multiplicities in the observed NMR resonances were labeled according to the convention: s = singlet~~;~~, d = doublet~~;~~, dd = doublet of doublets~~;~~, t = triplet~~;~~, and m = multiplet [14]. Furthermore, metabolites were identified by comparing the information in ~~a metabolite~~the database from previous studies [13-18]. ~~Signal analysis was carried out~~For semi-~~quantitatively by comparing~~quantitative analysis, the area of a signal analysis was compared to that of the TMSP signal as an internal standard. All ^{1}H-NMR signals were normalized to total intensity ~~for developing~~to develop data for multivariate ~~data~~ analysis. MetaboAnalyst software (MetaboAnalyst.ca) was used to analyze multivariate data using Principal Component Analysis (PCA), Partial Least-Squares Discriminant Analysis (PLS-DA), and hierarchical clustering heat map ~~analysis~~analyses. The spectra were centered and scaled with autoscaling~~. The~~, followed by initial data ~~was initially processed~~processing using ~~the~~ PCA to evaluate the natural clustering characters~~ of the data. When the data showed quite~~. After a clear grouping~~, then~~ was observed, the data ~~was~~were subjected to PLS-DA~~. PLS-DA was used~~ to maximize covariance between measured data (NMR peak intensities) and the response variable (predictive classifications). The PLS-DA coefficient loading plot ~~can be~~was used to ~~find~~determine significant metabolites contributing to the separation between the two classes [14]. The model's predictive ability (Q^2) was measured using

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cross-validation, ~~and the~~while statistical significance was determined using a permutation test. ~~The model's~~This was followed by the ranking of the important compounds ~~were ranked~~in the model using variable importance of projection (VIP) scores. A VIP score of >1 indicated that the variable contributed to the variation of the sample. The t-test analysis was used to determine significant differences in metabolites between all samples with p-values ≤ 0.01.

3. Results and Discussion

3.1. Visual analysis of ^{1}H-NMR spectra

NMR spectroscopy was used as a ~~technique for determining~~method to determine the magnetic resonance of molecular nuclei interacting with external magnetic fields [19]. Moreover, NMR could produce a distinct and specific spectrum for each compound ~~and was often used to determine~~, leading to its frequent use in determining the type of metabolite. The quality of the results was determined by the number of compounds identified, rather than ~~the number of~~ signals observed during the NMR analysis [20]. The NMR metabolomics ~~approaches~~methods have been used widely, ~~thus determining the compounds~~making compound identification less complicated ~~and~~. This can be ~~done~~achieved by comparing the signal produced by the samples to those ~~produced~~generated by the same compound in previous reports ~~utilizing the same solvent~~ using CD_3OD-D_2O, as the solvent [13-18]. Aqueous methanol is commonly used as an extraction solvent alone ~~as methanol is a universal solvent with~~due to its ability to extract a wide range of compounds ~~extracted~~, both non-polar and polar. The chemical shift of substances in NMR can be influenced by various solvents. ~~Multiple~~Consequently, multiple reference papers were ~~utilized~~used to conduct a comparative analysis of potential signal changes that ~~may~~could be identified. In this ~~work~~study, the coupling constant was ~~utilized~~used as an important parameter to validate the matching signals in ~~our~~the data with the references [15].

The ^{1}H-NMR spectra were ~~commonly~~ separated into three regions based on their chemical shift (δ), namely the amino acids and terpenoids, carbohydrates, and aromatic compounds. Aliphatic compounds (organic and amino acids) were found in the chemical shift 0.5-3.0 ppm, carbohydrates in 3.1 – 6.0 ppm, and aromatic compounds in > 6 ppm. ~~The ^{1}H-NMR spectra of~~ Furthermore, the ~~leaf and fruit extracts were analyzed and compared. The~~ various developmental stages of ^{1}H-NMR spectra of leaves and fruits extracts were analyzed~~, contrasted,~~ and ~~depicted~~compared, as shown in Figure 2.

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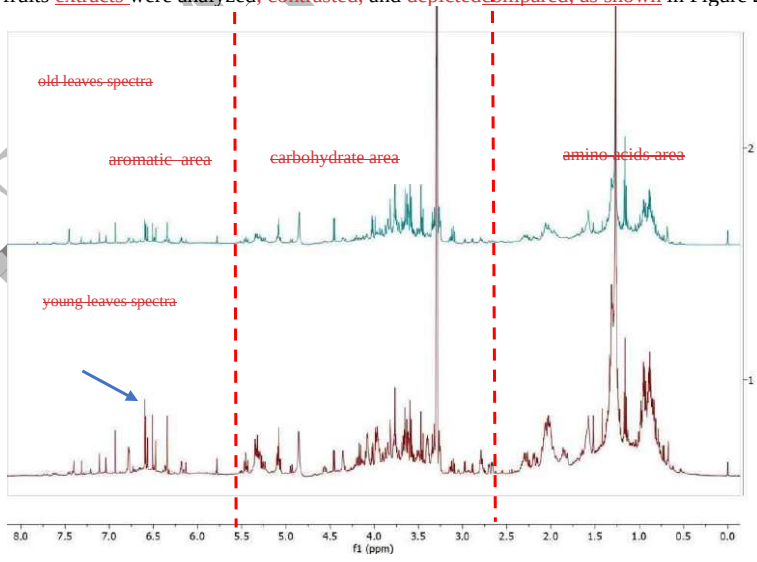

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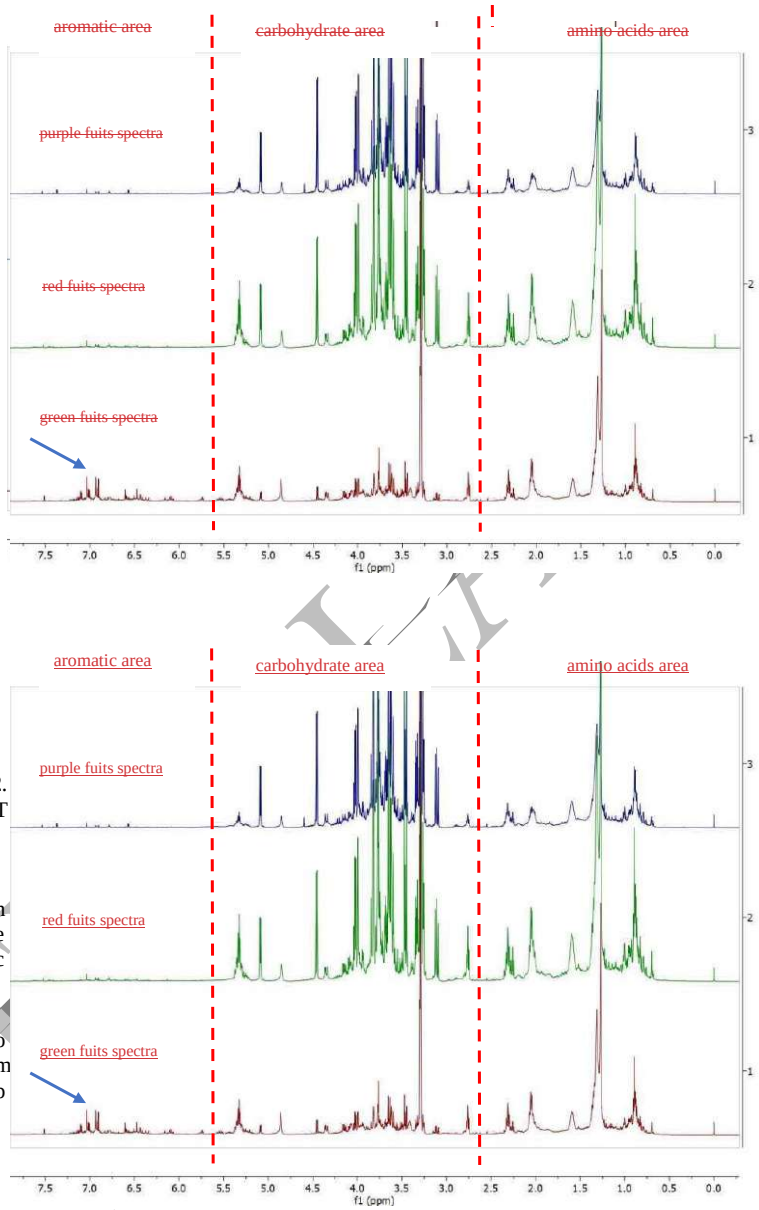

Figure 2. Comparison of the ^{1}H-NMR spectrum (δ 0.00–8.00 ppm) indicates signals of *R. tomentosa* from (A) leaves and (B) fruits of *R. tomentosa*. The arrow ~~show~~shows the aromatics regions which observed ~~different~~differences in signal intensities.

The results of the putative compounds identified by ^{1}H-NMR showed the presence of primary and secondary metabolite compounds. ~~The~~Specifically, the primary metabolites included amino acids (chemical shift 2.0 -0.5 ppm) and carbohydrates (chemical shift 5.0-3.0 ppm), with aromatic compounds (chemical shift > 6 ppm) being the secondary metabolites. ~~A~~In the aromatic regions of the NMR spectra, a gradual decrease in phenolic content was observed during ~~the leaf~~leaves and ~~fruit~~fruits growth ~~in the aromatic regions of the NMR spectra. Specifically, the~~. The intensities of signals in the aromatic area in the young leaves and green ~~fruit~~fruits samples were higher than in the old, red, and purple leaves ~~(~~, as illustrated in Figures 2A and B~~).~~.

3.2. Identification of metabolites ~~/~~/Assignment of ^{1}H-NMR signals

~~Despite its various~~ The advantages of using ^{1}H-NMR have been shown in various metabolomics ~~study, the use of~~studies. However, ^{1}H-NMR presented a significant challenge in chemical identification due to overlapping signals in multiple regions, particularly ~~in the~~at 5.0-3.0 ppm ~~region~~, which ~~corresponds~~corresponded to sugar compounds. ~~Therefore, the~~This caused the inability to identify signals in the sugar region ~~were not picked as the particular identifying signals unless~~, except for ~~the very general sugars such as~~ glucose and sucrose~~. This may~~, leading to a decrease in the number of substances ~~that can be~~ detected in this ~~investigation. This~~ study ~~revealed~~. The results also showed the identification of 20 putative compounds based on the ^{1}H-NMR spectra, as presented in Table 1. In the amnio acid region ~~we identified~~, the specific signals of leucine, glutamate, methionine, and aspartate were identified, with chemical shifts ranging from 3.0-0.5 ppm. The organic acid compounds, such as malic ~~acid~~, fumaric ~~acid~~, and succinic ~~acid~~acids were observed in the chemical shifts of between 3.0 - 2.0 ppm. The frequently identified sugars including mannitol, β-glucose, α-glucose, and sucrose ~~could be~~were also observed ~~in the chemical shifts of~~at 5.00 - 3.50 ppm. ~~The aromatic regions which observed to be~~ In the less crowded regions, several phenolics ~~can be~~were identified, including gallic acid, myricetin, myricetin 3-O-rhamnpyranoside, quercetin-3-O-glucoside, quercetin, and syringic acid (chemical shifts 10.0 - 6.0 ppm). The rest of the compounds identified were α-Linolenic acid, choline, and sterols.

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

No	*Compound*	*Chemical shifts δ (ppm) and coupling constants (Hz)*
	Amino Acids	
1	*Aspartate*	2.68 (dd, J= 3.0; 17.0 Hz)
2	*Glutamic acid*	2.06 (m); 2.34 (m)
3	*Leucine*	0.93 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)
4	*Methionine*	2.16 m ; 2.79 (t, J= 6.08; 6.08 Hz)
	Organic Acids	
5	*Fumaric acid*	6.51 (s)
6	*Malic acid*	4.34 (dd, J= 6.6 ; 4.7 Hz)
7	*Succinic acid*	2.54 (s)
	Sugars	
8	*Mannitol*	3.77 (d, J= 3.28 Hz)
9	*β-glucose*	4.45 (d, J= 7.79 Hz)
10	*α-glucose*	5.09 (d, J= 3.84 Hz)
11	*Sucrose*	5.35 (d, J= 3.91 Hz)
	Aromatics Compounds	
12	*Gallic acid*	7.03 (s)
13	*Myricetin*	6.34 (d, J= 1.99 Hz); 7.32 (s)
14	*Myricetin 3-O-rhamnpyranoside*	6.93 (s)
15	*Quercetin-3-O-glucoside*	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)
16	*Quercetin*	6.13 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)
17	*Syringic acid*	3.88 (s)
	Other compounds	
18	*α -Linolenic acid*	1.16 (t, J=7.04 ; 7.04); 1.29 (m)

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19	*Choline*	3.25 (s)
20	*Sterol*	0.70 (s)

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

3.2. Multivariate data analysis

~~A multivariate~~Multivariate PCA was performed to assess the variations of compounds present in ~~the~~ fruits and leaves of *R. tomentosa*. The PCA score plot was used to ~~demonstrate~~show the separation of classes, while the correlation between variables was interpreted in the loading plot [21]. MetaboAnalyst 5.0 automatically generated five PCs representing 87.6% of all observed variants. ~~The~~ Subsequently, the 2D score diagram derived from PC1 and PC2 ~~clearly~~ distinguished ~~fruit~~fruits and ~~leaf~~leaves samples. As shown in Figure 3A ~~illustrates that~~, the Q^2 cumulative of PC1 and PC2 yielded 57.6% of variants, ~~already~~which was above 50%, indicating a reliable model. PLS-DA ~~has been~~was implemented ~~to the~~in multivariate analysis to enhance separation~~.~~, where PC1 and PC2 accounted for 35% and 21.7% of the observed variants, respectively, for a total of 56.7% Q^2 in the 2D score plot. On the PLS-DA score plot presented in Figure 3B, samples of leaves and fruits were ~~separated. The fruits were~~ positioned in the negative ~~region of PC1, while the leaves were placed in the~~and positive ~~region (Figure 3B).~~regions of PC1, respectively.

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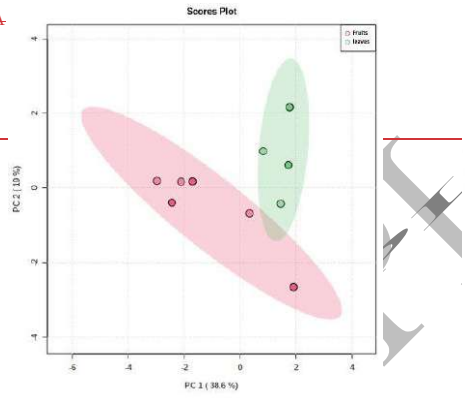

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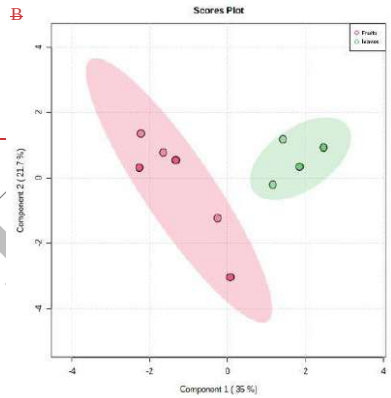

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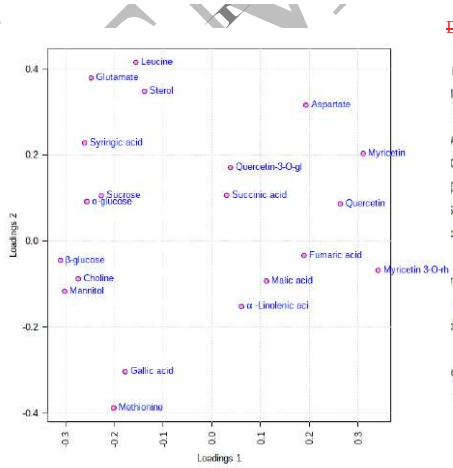

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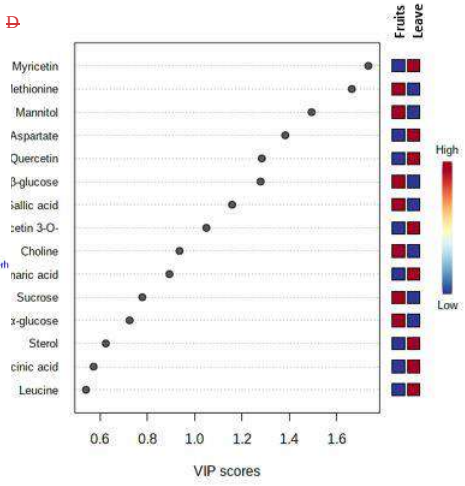

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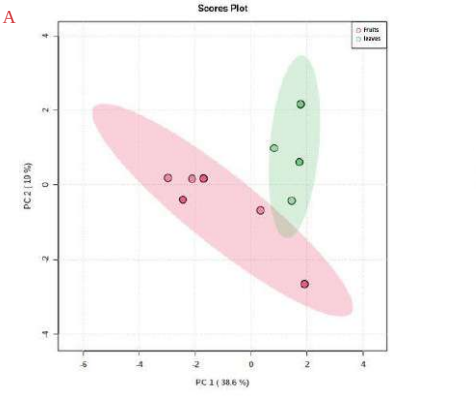

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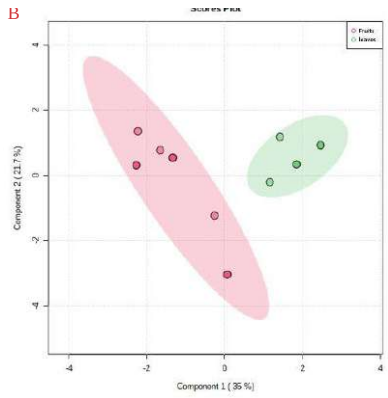

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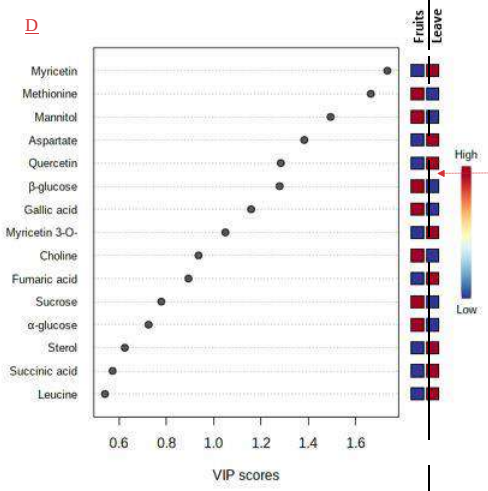

Figure 3. Multivariate data analysis of *R. tomer*
Score Plot; (B). PLS-DA score plot; (C
~~important~~important in projection (VIP)

Cross-validation was used to determine the Q_2 PLS-DA model. When $R^2 = 1$ and $Q^2 = 1$, the model In this study, PLS-DA ~~demonstrated~~showed distinc ($Q^2 = 0.9$). The PLS-DA model with a CV-ANOV accurate through 20 permutation tests that validated reliability of the model ~~was reliable~~ [20].

After a separation between ~~the~~ leaves and loading plot ~~will be~~was used to identify the compou Among the 20 compounds observed, Figure 3C components in ~~the leaf~~leaves profile with ~~fruit (~~fruits observed in the positive region of PC1 were fumaric quercetin, and aspartate.

~~When~~ During the implementation of PLS-D available. ~~The VIP reflected,~~ reflecting the significance of the model's variables ~~and~~. The VIP was recognized as ~~a valuable~~an instrument for identifying the variables that contributed ~~the most~~mostly to the examined variants. Moreover, a VIP score greater than one was frequently used as a selection criterion [23]. As shown in Figure 3D, the score graph ~~revealed~~indicated that nine metabolites, including myricetin, myricetin 3-O-rhamnpyranoside, quercetin, aspartate, choline, gallic acid, methionine, mannitol, and β-glucose, had values greater than 1.

These results suggested ~~that more research was required~~the need for further study beyond VIP score compounds~~.~~ to determine ~~if~~when there were notable variations in the chemical composition of fruits and leaves of *R. tomentosa* at the compound level. ~~The~~Subsequently, concentration determination was ~~determined~~carried out using a semi-quantitative examination of the compound signal obtained from the internal signal of the TMSP standard. ~~The analysis~~Analysis of signal integration ~~findings~~results was conducted using independent t-tests.

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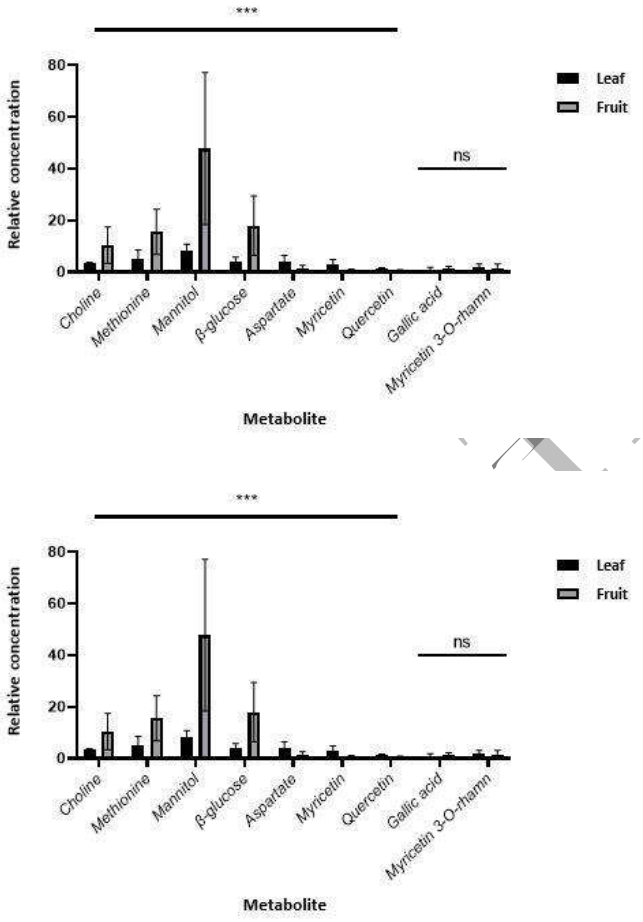

Figure 4. Histogram comparison of metabolite compound concentrations as important contributors to ~~the~~ leaves and fruits of *R. tomentosa*.

~~Heatmap~~A heatmap was used to further assess differences in the diversity of compound content between ~~the~~ fruits and leaves. ~~Furthermore, the~~The concentration of compounds found in ~~the~~ fruits and leaves of *R. tomentosa* was visualized through cluster analysis [24]. Heatmap was a form of visualization ~~of the~~ method that represented data distribution ~~of data depicted in the form of~~through color changes. ~~The~~ In this study, the relative concentrations of compounds in ~~the~~ fruits and leaves of *R. tomentosa* served as the data for heatmap analysis~~. The data were then~~, as presented based on the groups of samples. The ~~heatmap analysis~~ results showed that the compounds ~~found in the leaves and fruits demonstrated~~identified showed high diversity and varied ~~in~~ concentration, as shown in Figure 5.

Young leaves and green fruits appeared in the same cluster on the heatmap, ~~and certain~~while some compounds had higher concentrations compared to others. ~~These include~~Compounds such as malic acid, α linolenic acid, aspartate, quercetin 3-O glucoside, fumaric acid, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, methionine, and gallic acid~~, which~~ were ~~all~~ indicated by a dark brown color. Meanwhile, old leaves, ~~as well as~~ red, and purple fruits had a lower concentration, as

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indicated by light brown to dark blue colors. Quercetin 3-O glucoside, myricetin 3-O-rhamnpyranoside, myricetin, quercetin, ~~as well as~~and gallic acid compounds were members of ~~the~~ flavonoid group. This ~~is~~was in accordance with the total flavonoid content and the value of the antioxidant capacity of green ~~fruit~~fruits and young leaves, which ~~are~~were higher than old leaves, red, and purple fruits in ~~the results of~~a previous ~~research~~study [11~~], namely~~]. Based on the results, the total flavonoid content of green ~~fruit is~~fruits was 95.731±5.42 mg QE/g DW and the value of antioxidant capacity ~~1419~~was1419.75±3.48 µmol TE/g DW ~~and~~, The young leaves ~~with~~had a total flavonoid content of 96.375±3.96 mg QE/g DW and an antioxidant capacity value of 1069.38±6.57 µmol TE/g DW~~, while~~. The total flavonoid ~~content~~ and antioxidant capacity ~~value~~values of old leaves were 70.311±5.22 mg QE/g DW and 844.91±5.72 µmol TE/g DW, red ~~fruit~~fruits had 88.125±2.72 mg QE/g DW and 263.93±1.60 µmol TE/g DW ~~and~~, while purple ~~fruit~~fruits 67.115±2.57 mg QE/g DW and 127.49±0.57 µmol TE/g DW, respectively [11].

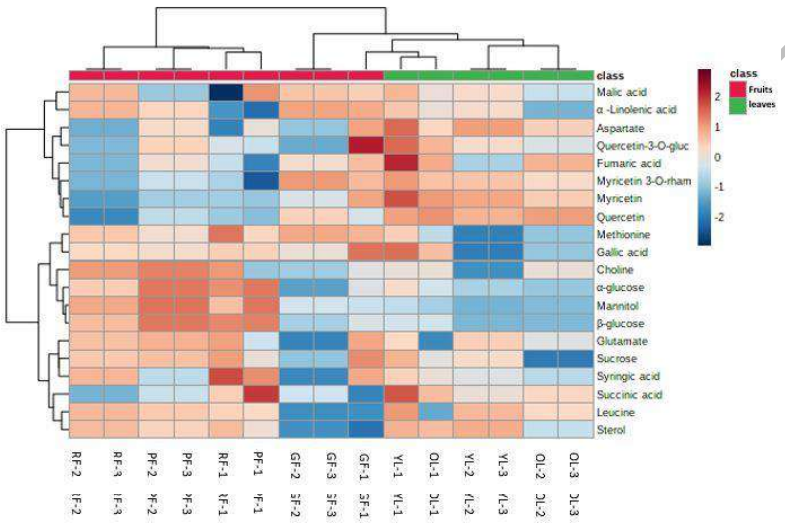

Figure 5. Heatmap of ~~the leaf~~leaves and ~~fruit~~fruits of *R. tomentosa*. The blue color represents low concentration and the brown color represents high concentration. Note: YL (young leaves), OL (old leaves), GF (green ~~fruit~~fruits), RF (red ~~fruit~~fruits), PF (purple ~~fruit~~fruits)

The number of hydroxycinnamates, caftaric, coutaric acid, and quercetin glucoside compounds~~, which belong to~~ in the phenol and ~~flavonoids~~flavonoid families~~,~~ increased in grapes (*Vitis* spp.) during the ~~later~~final stages of green ~~fruit~~fruits development and significantly declined ~~abruptly~~ after ripening 16]. Young leaves had the highest phenol ~~and~~, flavonoid ~~content~~, and antioxidant activity, followed by mature leaves and seeds [13]. ~~Immature~~Furthermore, immature fruits ~~contain~~contained significant quantities of polyphenols, including ~~flavonoids~~flavonoid [25~~]. This finding indicated~~, indicating that the pre-ripening period served as a defense mechanism for ~~fruit~~fruits against various congenital diseases. The presence of higher amounts of antioxidants in the unripe fruits of *Rubus ellipticus* and *Myrica esculenta* was consistent with this ~~finding. According to~~ observation. Based on these ~~findings~~results, as ~~the fruit~~fruits ripened, phenols and ~~flavonoids~~flavonoid oxidized ~~and participated~~, participating in the biosynthesis of anthocyanins, which accumulated during maturation, ~~thereby decreasing the~~leading to a decrease in flavonoid concentration.

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This ~~current~~ study ~~is infact explained what we have reported in our~~elaborated on a previous study focusing on the antioxidant capacity, total flavonoid content, ~~as well as~~and compound distribution in *R. tomentosa* using histochemical analysis. The antioxidant activity evaluation using FRAP and DPPH ~~exhibited~~showed a comparable proportion, ~~especially~~particularly in the green fruits ethanol extracts ~~which exhibited~~, indicating the highest FRAP value of 1367.59±9.12 mol TE/g DW and DPPH radical scavenging ability value of 1419.75±3.48 mol TE/g DW. The lowest antioxidant activity was observed in the purple fruits with FRAP value of 138.38±1.13 mol TE/g DW and DPPH ~~value~~ of 127.49±0.57. As a comparison, the DPPH value of the purple fruits ~~were~~was almost four times lower than the activity antioxidant ORAC value of 431.17±14.5 µmol TE/g DW [4~~] and another~~]. Another study showed a higher value of 8.79-92.60 µmol TE/g DW [26], which were measured in fruits such as grape, blueberries, blackberries, ~~kiwifruit~~kiwifruits, oranges, apples, mangoes, and bananas [27].

NMR ~~also~~ experiments were ~~used~~carried out to identify and confirm the presence of a wide variety of metabolites in all three samples ~~(~~, namely seed, skin, and pericarp~~)~~, obtained from *Momordica charantia* fruits [28]. To identify the metabolic differences between ~~the seed, skin, and pericarp~~these samples, a multivariate statistical analysis was ~~used~~conducted. Different parts of ~~the fruit had~~fruits showed significantly ~~different~~varying concentrations of ~~–~~important metabolites. ~~The~~, where the highest total flavonoid and phenolic contents were found in seeds and pericarp, followed by skin. Flavonoid metabolites that ~~are~~were synthesized from naringenin and ~~have been~~ identified ~~in their study include~~included luteolin, catechin, kaempferol, quercetin, and myricetin. Based on metabolic analysis, the ~~fruit's~~ pericarp and seeds ~~more antioxidant~~contained higher antioxidant activities ~~than~~compared to the skin ~~does~~. The scavenging effects of methanol extracts of ripe fruits measured by DPPH assay were ~~in the order:~~ arranged as seed > pericarp > skin [28].

~~–~~According to the heatmap presented in Figure 5, red and purple fruits belonged to the same cluster~~, as indicated in Figure 5.~~. The results ~~demonstrated~~showed that the choline, mannitol, β-glucose, α-glucose, and sucrose compounds had a higher concentration, as indicated by a dark brown color. Meanwhile, young ~~leaf~~leaves and green fruits had a lower concentration, which was shown by the light~~–~~blue to dark~~–~~blue colors. Mannitol, β-glucose, α-glucose, and sucrose were carbohydrates (sugars) compounds. ~~According to~~ Ali et al. [16~~,~~] reported that the concentration of glucose and fructose increased during the ripening stage of grapes (*Vitis* spp.).

~~–~~The growth of ~~grapefruit~~grapefruits was similar to ~~that of~~ *R. tomentosa* ~~fruit~~fruits, as ~~they~~ both ~~underwent~~passed through a complex series of biochemical and physical changes, ~~such as~~including variations in composition, size, color, taste, texture, ~~as well as~~and pathogen resistance. ~~The~~Generally, the development of grapes could be separated into three phases. During the initial phase (phase I), ~~the fruit~~fruits grew ~~quickly, primarily~~rapidly due to cell division and expansion~~. During this phase,~~ accompanied by the biosynthesis of various compounds, including malic~~ acid~~, tartaric~~ acid~~, hydroxycinnamates, and tannins~~, occurred and~~. These compounds reached a maximum concentration approximately 60 days after flowering. Phase II referred to the growth lag phase, which was often observed 7~~–~~ to 10 weeks after flowering~~, and was~~ characterized by the accumulation of sugar. In Phase III (ripening), the berries experienced significant changes in morphology and composition~~. Moreover, during this phase, the berry's~~ , doubling in size ~~doubled, indicating~~. This indicated the onset of color development ~~(~~associated with anthocyanin accumulation in red wine~~)~~, ~~along with~~ as well as an ~~increase in~~increased sweetness ~~(~~, particularly in fructose and glucose levels~~), and a concurrent decrease in~~, followed by decreased acidity [16].

The sugar content of fruits ~~is frequently employed~~was used as an indicator for assessing ~~their~~the level of ripeness and determining the optimal time for harvest. Carbohydrates, particularly sucrose, ~~are~~were produced by the process of photosynthesis in grapevine leaves~~. The carbohydrates were delivered~~ and transported to ~~the~~ fruits ~~via~~through the phloem. ~~The~~ During this process, the sugar content ~~underwent~~passed through alteration after the transfer ~~as a result of~~ due to the loss of water. Furthermore, sugar was ~~utilized not just~~used as a source of carbon ~~and~~, energy, ~~but also as~~and a means ~~to modulate~~of modulating the regulation of gene expression. The accumulation of fructose and glucose started during the second phase of ~~fruit~~fruits growth ~~and persisted thereafter. The~~, followed by a persistent process, incorporating the transportation of monosaccharides ~~via~~through transporters ~~facilitates~~to facilitate the delivery of sugars to cellular organelles [16]. Sugar plays a crucial role in

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facilitating plant development~~ and~~, providing energy~~. Fructose~~, where fructose and glucose ~~play a crucial role in the synthesis of~~synthesize sucrose~~ and serve~~ as precursors for the formation of organic acids and pyruvate. Throughout the developmental process, there was a ~~notable and substantial~~significant rise in the concentrations of fructose and glucose [29].

The dark violet, bell-shaped edible berries of *R. tomentosa*, have been traditionally ~~employed~~used as folk medicine to ~~address~~treat issues such as dysentery, diarrhea, and traumatic hemorrhage [30]. Additionally, these berries ~~have played a role~~were used in crafting a renowned fermented beverage called "Ruou sim" on Phu Quoc Island in southern Vietnam~~. As they mature, the fruits~~, maturing to acquire a deep purple color and ~~possess~~ an astringent taste [3,4,31]. ~~Within~~In China, ~~the~~these berries are transformed into delectable pies, jams, and salad additions~~. Additionally, these fruits play a key~~, playing a significant role in the creation of traditional wines, beverages, jellies, and freshly canned syrups for human consumption. ~~Notably, the~~The berries of *R. tomentosa* ~~harbor~~contain a rich assortment of chemical constituents, including sugars, minerals,~~–~~ vitamins phenols, flavonoid glycosides, organic acids, amino acids, quinones, and polysaccharides. Furthermore, this plant thrives in subtropical and tropical regions, serving as a valuable source for cultivating novel ingredients that ~~contribute to promoting~~promote health benefits [26].

4. Conclusion

In conclusion, this study showed that *R. tomentosa*~~ appeared to have~~ played a significant ~~and holistic~~ role in the daily lives of ancient societies, providing medical benefits. ~~Multiple~~Several biological activities ~~of this plant~~have been documented regarding *R. tomentosa*, including antifungal, antimicrobial, antimalarial, antioxidant, anti-inflammatory, and osteogenic properties~~, have been documented. Therefore, it was essential~~. This showed the need to comprehend ~~the~~ various parts of ~~*R. tomentosa*~~*the plant*, such as ~~the~~ leaves and fruits at ~~various~~different phases of maturation, and the metabolic ~~fate~~transformation of ~~its various classes of~~associated compounds. ~~This~~In this study ~~demonstrated that~~, the combination of ^{1}H-NMR and multivariate data analysis enabled the detection of significant differences between the various developmental stages of ~~the~~ leaves and fruits ~~used in this investigation. At various stages of development~~. The results showed that the samples contained substantially different amounts of sugar, aromatic compounds, and phenolic compounds~~. Green~~ at various developmental stages. Furthermore, green fruits and young leaves contained substantial concentrations of phenolics, including quercetin 3-O glucoside, myricetin 3-O-rhamnpyranoside, myricetin, quercetin and gallic acid. During the final phases, choline, mannitol, α-glucose, β-glucose, and sucrose concentrations increased. ~~The approach of this study was useful~~This method showed promising potential for analyzing a variety of compounds within the *R. tomentosa* metabolome~~; however~~. However, further ~~research~~study with more sensitive analytical instruments ~~may be desirable~~is recommended to provide a thorough examination of the metabolome transformation ~~and~~ as well as the metabolism of ~~the fruit~~fruits and ~~leaf~~leaves of *R. tomentosa* at different developmental stages~~ of development~~.

Acknowledgments

The authors are grateful to Gadjah Mada University in Yogyakarta, Indonesia, for ~~their generous support of~~supporting this study through the RTA Grant 2022.

Supporting Information

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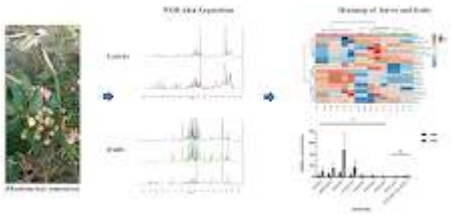

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Metabolomics Profiling of *Rhodomyrtus tomentosa* (Ait.) Hassk. Leaves and Fruits using ^{1}H NMR

Evi Mintowati Kuntorini [1,2*], Laurentius Hartanto Nugroho [1], Maryani Maryani [1] and Tri Rini Nuringtyas [1,3*]

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(Received July 26, 2023; Revised September 27, 2023; Accepted September 29, 2023)

Abstract: Several investigations are extensively documenting the presence of metabolites with antibacterial, anticancer, antioxidant, and anti-inflammatory properties in extracts derived from *Rhodomyrtus tomentosa* fruits and leaves. Therefore, this study aimed to evaluate the metabolite profile of *R. tomentosa* fruits and leaves at various maturity stages and determine their phytomedicinal values. ^{1}H NMR and chemometric analyses were used to conduct a metabolomics study for comparing the metabolite profile and phytomedicinal values of different plant organs at varying ages. Leaves were classified into young and old categories, while fruits were divided into three maturity stages, namely green, red, and purple. The wild-grown fruits and leaves of *R. tomentosa* (Ait.) Hassk were gathered in Banjarbaru, South Kalimantan Province, Indonesia. The results of multivariate analysis showed that choline, methionine, mannitol, and β-glucose compounds were three times higher in fruits compared to leaves. Meanwhile, the concentration of aspartate, myricetin, and quercetin compounds was three times higher in leaves compared to fruits. The quantities of secondary metabolites, including flavonoids, were higher in young leaves and green fruits than in old, red, and purple fruits.

Keywords: *Rhodomyrtus tomentosa*, flavonoid, ^{1}H-NMR, multivariate statistical analysis

1. Introduction

Plants are significantly important in the daily lives of people by providing essential resources such as medicine, food, fiber for clothing, and wood for building. Among these resources, plants used for treating different medical conditions are considered the most valuable. Individuals without access to modern healthcare still place great value on traditional medical practices. Several studies have shown that the majority of traditional medicines are produced from plant-based materials. A recent report stated that approximately 80% of the global population relies on plants to treat a wide range of ailments, indicating their widespread usage. Consequently, herbal medicines have become integral

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components of modern therapy, with 25% of medications available worldwide originating from plants [1].

Rose myrtle, scientifically known as *Rhodomyrtus tomentosa*/*R. tomentosa* (Ait.) Hassk. is a blossoming plant belonging to the Myrtaceae family. According to previous reports, *R. tomentosa* is indigenous to southern and southeastern Asia, with its natural habitat spanning from India, southern China, the Philippines, and Malaysia, to Sulawesi. This plant has an exceptional ability to grow in various environments, ranging from sea level to elevations of 2400 m. Furthermore, it thrives in diverse locations, including natural forests, beaches, wetlands, riparian zones, moist, wet woods, and bog borders, requiring intense sunlight and minimal soil conditions [2-4]. In tropical and subtropical gardens, *R. tomentosa* is a well-liked ornamental plant, cultivated for its abundant blooms, delectable, and edible fruits. These fruits are often used for several culinary applications, such as pies, salads, and jams, including additional processing into wine, jellies, or canned fruits in Vietnam and China [2]. Modern pharmacological studies have shown that *R. tomentosa* components possess diverse properties, namely antibacterial [5], anticancer [6], anti-inflammatory [7], and antioxidant [8]. Traditional Malaysian, Chinese, and Vietnamese medicine has used its leaves, roots, buds, and fruits for medicinal purposes [4]. For example, the Dayak and the Paser local tribes in East Kalimantan, Indonesia, use the roots to treat diarrhea and stomachaches, and as a postpartum tonic [9]. The crushed leaves serve as external poultices and the tar obtained from its wood is used for eyebrow darkening. These traditional applications are in line with the effects observed in modern pharmacological studies. Several phytochemical reports showed that the plant contains flavonoids, triterpenoids, phenols, microelements, and meroterpenoids [2]. Among these compounds, rhodomyrtone is the most prominent, possessing numerous potential pharmacological properties [10], while piceatannol serves as the main and highly effective phenolic component [3].

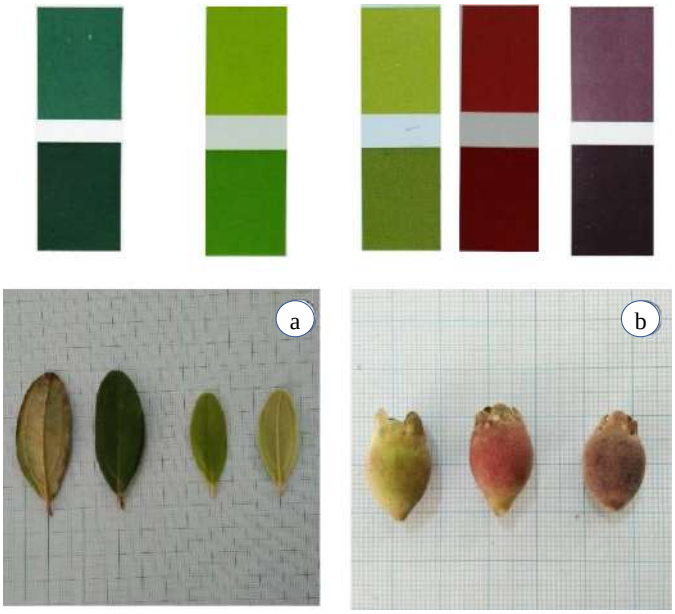
In a previous study, the total phenolic content, antioxidant capacity, total flavonoids, and compound distribution in *R. tomentosa* were explored using histochemical analysis [11]. This report did not examine the specific antioxidant profile of fruits and leaves at various stages of development. To address the limitation, ^{1}H-NMR was used to analyze the metabolite profile in leaves and fruits. Therefore, this study aimed to analyze the secondary and primary metabolites of various parts of *R. tomentosa*, specifically fruits and leaves. The existence of these compounds was correlated with the previously reported bioactivity and phytomedicinal qualities. NMR spectroscopy and multivariate data analysis without chromatographic separation were used to identify metabolites directly from the samples. A metabolic analysis was also conducted on *R. tomentosa* fruits and leaves at various stages of maturation. Subsequently, multivariate statistics were used to determine the compounds significantly contributing to the variations between both parts. This study is the first systematic examination of *R. tomentosa* fruits and leaves at various stages of maturity using a combined NMR and multivariate statistical method. Analysis showed the applicability of the NMR-based method in plant metabolomics.

2. Materials and Methods

2.1. Plant materials

In this study, *R. tomentosa* plant was obtained from its natural habitat in the wild. The samples were collected from Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) in August-October 2020. To accurately characterize the plant tissues, Munsell Color Charts were used as a reference guide for samples of leaves and fruits [12]. Leaves samples were selected from young leaves (2^{nd} – 6^{th} order from the shoot [Munsell Color Carts guide: 5GY (7/8-6/8)]) and old leaves (7^{th} -12^{th} order from the shoot [Munsell Color Carts guide: 2.5G (4/4-3/4)]). Furthermore, fruit samples used were green [Munsell Color Carts guide: 2.5GY (7/8-6/10)], red [Munsell Color Carts:10R (6/10-5/10)], and purple [Munsell Color Carts:5RP (4/4-3/2)] with a color guide using Munsell Color Charts for Plant tissues, as illustrated in Figure 1A. Subsequently, three replicates of each leaves and fruits were analyzed, followed by identification using the Herbarium Bogoriense, Indonesian Institute of Sciences, located in Bogor, Indonesia, under certificate number 1007/IPH.1.01/If.07/IX/2020.

A



B

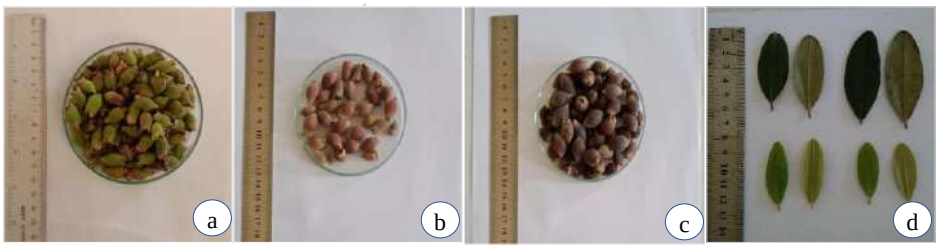


Figure 1. *Rhodomyrtus tomentosa* (Ait.) Hassk. (A) a: leaves, b. fruits, with a color guide using Munsell Color Charts for Plant tissues (B) a: green fruits, b: red fruits, c: purple fruits, d: young and old leaves.

2.2. Crude extract preparation and sample preparation for ^{1}H-NMR

The old and young leaves were carefully selected from the tip shoots of *R. tomentosa*, and fruits were dried in an oven at 40 °C, and ground at room temperature. Each grounded material of 500 g was macerated in ethanol of 1000 mL (SmartLab, Indonesia) for 24 h. The solvent was discarded and changed every 24 h, with this process being repeated three times [9,11]. Furthermore, extracts from the same samples were combined, properly mixed, filtered to eliminate cell debris, and dried using a rotary evaporator.

^{1}H-NMR sample preparation was carried out using a slightly modified version of the sample extraction of previously reported methods [15]. Precisely, 25 mg of the crude extract was placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent, comprising 0.5 mL of methanol-d4, 0.5 mL of KH_2PO_4 buffer, and pH 6.0 containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The mixture was vortexed, sonicated for 1 min, homogenized, and centrifuged for 1 min at 10,000 rpm using a microcentrifuge. The supernatant was collected, placed in the NMR tube, and prepared for ^{1}H-NMR analysis.

2.3. NMR experiments

^{1}H-NMR was carried out using a 500 MHz spectroscopy (JEOL JNM ECZ500R) at a temperature of 25 °C. The parameters used for a total of 128 scans lasting for 10 min included a

relaxation delay of 1.5 seconds, X_angle 60°, and a pre-saturation mode of 4.27 ppm. Subsequently, the deuterated solvent was set as the internal lock, and spectral width was measured from 0 to 10 ppm.

2.4. Data analysis

The ^{1}H-NMR spectra were analyzed using MestReNova analysis, followed by processing with manual phasing, baseline adjustment, and calibration to internal standard solution signals (TMSP) positioned at the chemical shift of 0.0 ppm. Multiplicities in the observed NMR resonances were labeled according to the convention: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, and m = multiplet [14]. Furthermore, metabolites were identified by comparing the information in the database from previous studies [13-18]. For semi-quantitative analysis, the area of a signal analysis was compared to that of the TMSP signal as an internal standard. All ^{1}H-NMR signals were normalized to total intensity to develop data for multivariate analysis. MetaboAnalyst software (MetaboAnalyst.ca) was used to analyze multivariate data using Principal Component Analysis (PCA), Partial Least-Squares Discriminant Analysis (PLS-DA), and hierarchical clustering heat map analyses. The spectra were centered and scaled with autoscaling, followed by initial data processing using PCA to evaluate the natural clustering characters. After a clear grouping was observed, the data were subjected to PLS-DA to maximize covariance between measured data (NMR peak intensities) and the response variable (predictive classifications). The PLS-DA coefficient loading plot was used to determine significant metabolites contributing to the separation between the two classes [14]. The model's predictive ability (Q^2) was measured using cross-validation, while statistical significance was determined using a permutation test. This was followed by the ranking of the important compounds in the model using variable importance of projection (VIP) scores. A VIP score of >1 indicated that the variable contributed to the variation of the sample. The t-test analysis was used to determine significant differences in metabolites between all samples with p-values ≤ 0.01.

3. Results and Discussion

3.1. Visual analysis of ^{1}H-NMR spectra

NMR spectroscopy was used as a method to determine the magnetic resonance of molecular nuclei interacting with external magnetic fields [19]. Moreover, NMR could produce a distinct and specific spectrum for each compound, leading to its frequent use in determining the type of metabolite. The quality of the results was determined by the number of compounds identified, rather than signals observed during the NMR analysis [20]. The NMR metabolomics methods have been used widely, making compound identification less complicated. This can be achieved by comparing the signal produced by the samples to those generated by the same compounds in previous reports using CD_3OD-D_2O as the solvent [13-18]. Aqueous methanol is commonly used as an extraction solvent alone due to its ability to extract a wide range of compounds, both non-polar and polar. The chemical shift of substances in NMR can be influenced by various solvents. Consequently, multiple reference papers were used to conduct a comparative analysis of potential signal changes that could be identified. In this study, the coupling constant was used as an important parameter to validate the matching signals in the data with the references [15].

The ^{1}H-NMR spectra were separated into three regions based on their chemical shift (δ), namely the amino acids and terpenoids, carbohydrates, and aromatic compounds. Aliphatic compounds (organic and amino acids) were found in the chemical shift 0.5-3.0 ppm, carbohydrates in 3.1 – 6.0 ppm, and aromatic compounds in > 6 ppm. Furthermore, the various developmental stages of ^{1}H-NMR spectra of leaves and fruits extracts were analyzed and compared, as shown in Figure 2.

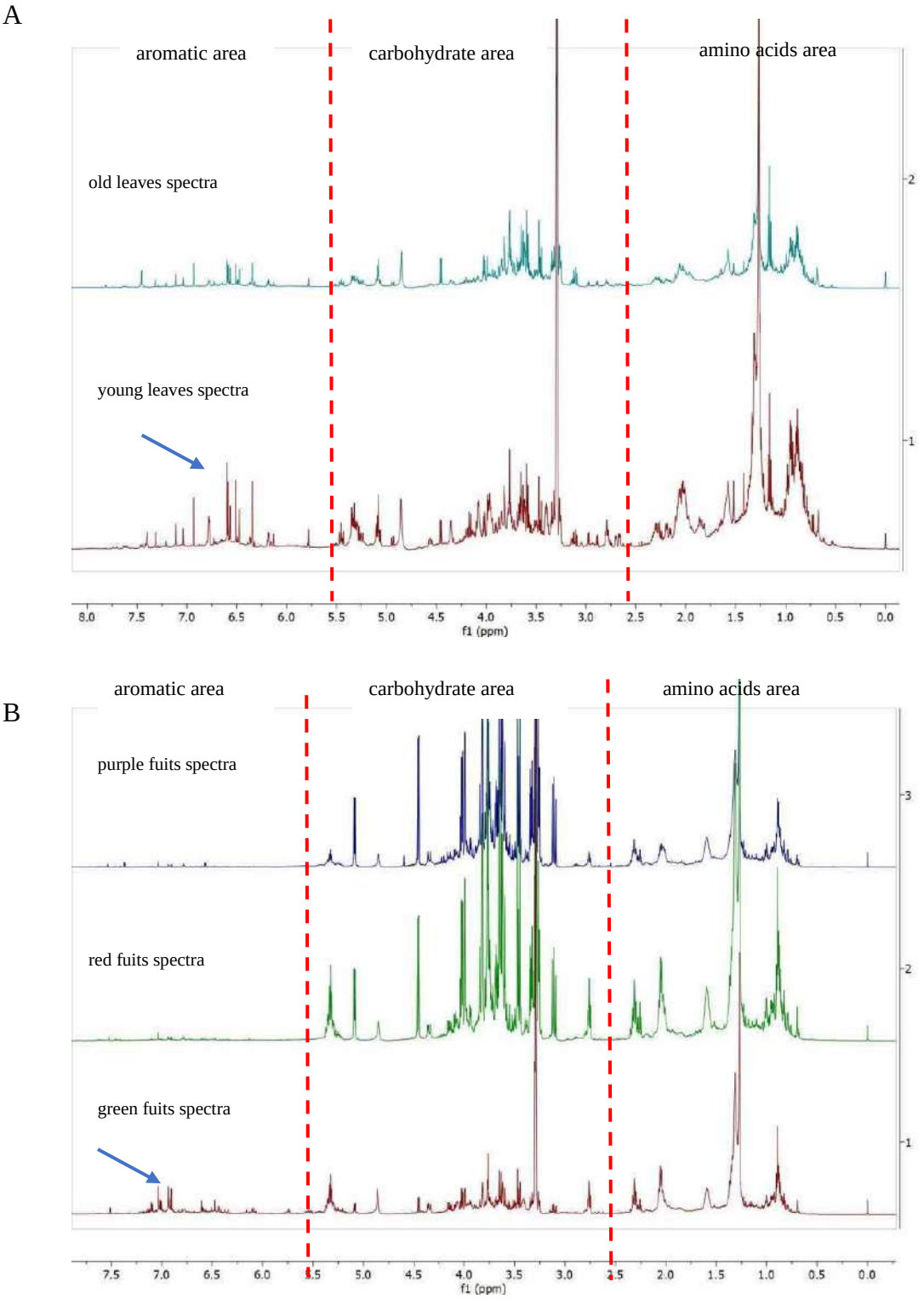


Figure 2. The comparison of the ^{1}H-NMR spectrum (δ 0.00–8.00 ppm) indicates signals of *R. tomentosa* from (A) leaves and (B) fruits of *Rhodomyrtus tomentosa*. The arrow shows the aromatics regions which observed differences in signal intensities.

The results of the putative compounds identified by ^{1}H-NMR showed the presence of primary and secondary metabolite compounds. Specifically, the primary metabolites included amino acids (chemical shift 2.0-0.5 ppm) and carbohydrates (chemical shift 5.0-3.0 ppm), with aromatic compounds (chemical shift > 6 ppm) being the secondary metabolites. In the aromatic regions of the NMR spectra, a gradual decrease in phenolic content was observed during leaves and fruits growth. The intensities of signals in the aromatic area in the young leaves and green fruits samples were higher than in the old, red, and purple leaves, as illustrated in Figures 2A and B.

3.2. Identification of metabolites/Assignment of ^{1}H-NMR signals

The advantages of using ^{1}H-NMR have been shown in various metabolomics studies. However, ^{1}H-NMR presented a significant challenge in chemical identification due to overlapping signals in multiple regions, particularly at 5.0-3.0 ppm, which corresponded to sugar compounds. This caused the inability to identify signals in the sugar region, except for glucose and sucrose, leading to a decrease in the number of substances detected in this study. The results also showed the identification of 20 putative compounds based on the ^{1}H-NMR spectra, as presented in Table 1. In the amnio acid region, the specific signals of leucine, glutamate, methionine, and aspartate were identified, with chemical shifts ranging from 3.0-0.5 ppm. The organic acid compounds, such as malic, fumaric, and succinic acids were observed in the chemical shifts of between 3.0 - 2.0 ppm. The frequently identified sugars, including mannitol, β-glucose, α-glucose, and sucrose were also observed at 5.00 - 3.50 ppm. In the less crowded regions, several phenolics were identified, including 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.

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

No	***Compound***	***Chemical shifts δ (ppm) and coupling constants (Hz)***
	Amino Acids	
1	*Aspartate*	2.68 (dd, J= 3.0; 17.0 Hz)
2	*Glutamic acid*	2.06 (m); 2.34 (m)
3	*Leucine*	0.93 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)
4	*Methionine*	2.16 m ; 2.79 (t, J= 6.08; 6.08 Hz)
	Organic Acids	
5	*Fumaric acid*	6.51 (s)
6	*Malic acid*	4.34 (dd, J= 6.6 ; 4.7 Hz)
7	*Succinic acid*	2.54 (s)
	Sugars	
8	*Mannitol*	3.77 (d, J= 3.28 Hz)
9	*β-glucose*	4.45 (d, J= 7.79 Hz)
10	*α-glucose*	5.09 (d, J= 3.84 Hz)
11	*Sucrose*	5.35 (d, J= 3.91 Hz)
	Aromatics Compounds	
12	*Gallic acid*	7.03 (s)
13	*Myricetin*	6.34 (d, J= 1.99 Hz); 7.32 (s)
14	*Myricetin 3-O-rhamnpyranoside*	6.93 (s)
15	*Quercetin-3-O-glucoside*	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)
16	*Quercetin*	6.13 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)
17	*Syringic acid*	3.88 (s)
	Other compounds	
18	*α -Linolenic acid*	1.16 (t, J=7.04 ; 7.04); 1.29 (m)
19	*Choline*	3.25 (s)
20	*Sterol*	0.70 (s)

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

3.2. Multivariate data analysis

Multivariate PCA was performed to assess the variations of compounds present in fruits and leaves of *R. tomentosa*. The PCA score plot was used to show the separation of classes, while the correlation between variables was interpreted in the loading plot [21]. MetaboAnalyst 5.0 automatically generated five PCs representing 87.6% of all observed variants. Subsequently, the 2D score diagram derived from PC1 and PC2 distinguished fruits and leaves samples. As shown in Figure 3A, the Q^2 cumulative of PC1 and PC2 yielded 57.6% of variants, which was above 50%, indicating a reliable model. PLS-DA was implemented in multivariate analysis to enhance separation, where PC1 and PC2 accounted for 35% and 21.7% of the observed variants, respectively, for a total of 56.7% Q^2 in the 2D score plot. On the PLS-DA score plot presented in Figure 3B, samples of leaves and fruits were positioned in the negative and positive regions of PC1, respectively,

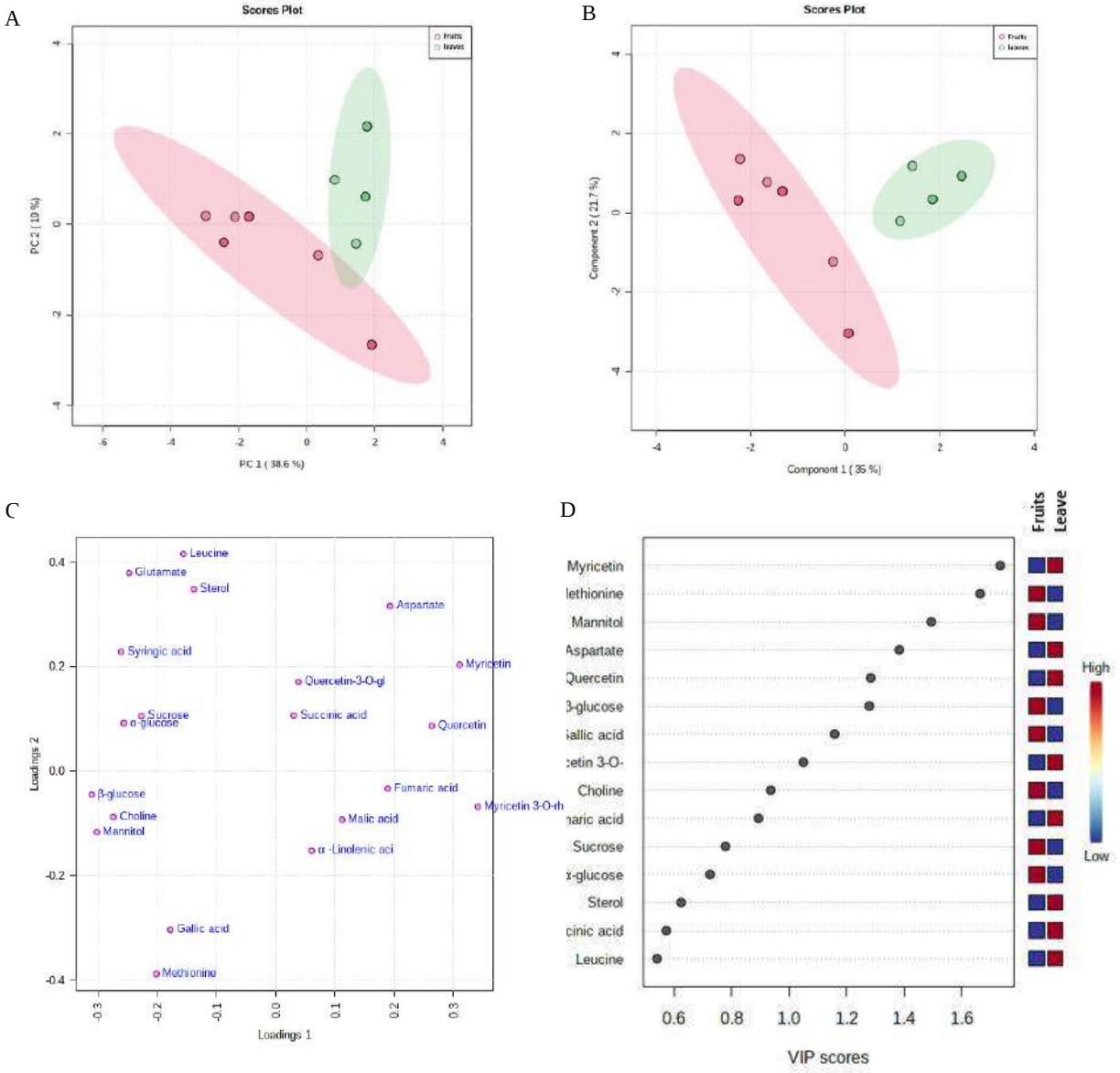


Figure 3. Multivariate data analysis of *Rhodomyrtus tomentosa* leaves and fruits samples (A). PCA Score Plot; (B). PLS-DA score plot; (C). PLS-DA loading plot analysis; (D). Variables important in projection (VIP) based on PLS-DA.

Cross-validation was used to determine the Q^2, which assessed the predictability of the PLS-DA model. When $R^2 = 1$ and $Q^2 = 1$, the model could precisely describe and predict the data [22]. In this

study, PLS-DA showed distinct separation (R^2 = 1) and excellent predictability (Q^2 = 0.9). The PLS-DA model with a CV-ANOVA less than 0.05 was determined to be the most accurate through 20 permutation tests that validated the results, indicating the reliability of the model [20].

After a separation between leaves and fruits of *R. tomentosa* was observed, a loading plot was used to identify the compounds that distinguished the two groups. Among the 20 compounds observed, Figure 3C showed that there were five distinct components in leaves profile with fruits, as illustrated in Table 1. Compounds observed in the positive region of PC1 were fumaric acid, myricetin, myricetin 3-*O*-rhamnpyranoside, quercetin, and aspartate.

During the implementation of PLS-DA, the VIP score was readily available, reflecting the significance of the model's variables. The VIP was recognized as an instrument for identifying the variables that contributed mainly to the examined variants. Moreover, a VIP score greater than one was frequently used as a selection criterion [23]. As shown in Figure 3D, the score graph indicated that nine metabolites, including myricetin, myricetin 3-*O*-rhamnpyranoside, quercetin, aspartate, choline, gallic acid, methionine, mannitol, and β-glucose, had values greater than 1.

These results suggested the need for further study beyond VIP score compounds to determine when there were notable variations in the chemical composition of fruits and leaves of *R. tomentosa* at the compound level. Subsequently, concentration determination was carried out using a semi-quantitative examination of the compound signal obtained from the internal signal of the TMSP standard. Analysis of signal integration results was conducted using independent t-tests.

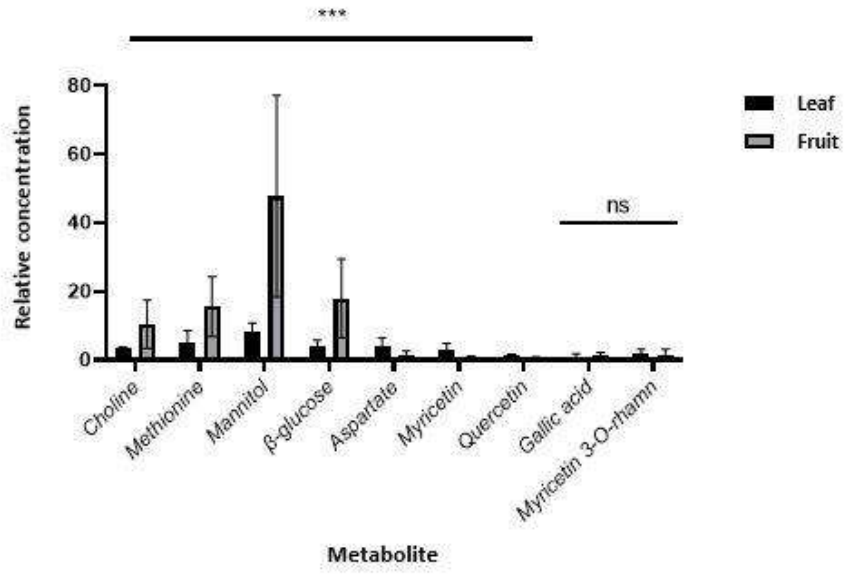


Figure 4. Comparison of metabolite concentrations as major contributors to differences between the leaves and the fruits of *Rhodomyrtus tomentosa*.

A heatmap was used to assess further differences in the diversity of compound content between fruits and leaves. The concentration of compounds found in fruits and leaves of *R. tomentosa* was visualized through cluster analysis [24]. Heatmap was a form of visualization method that represented data distribution through color changes. In this study, the relative concentrations of compounds in fruits and leaves of *R. tomentosa* served as the data for heatmap analysis, as presented based on the groups of samples. The results showed that the compounds identified showed high diversity and varied concentration, as shown in Figure 5.

Young leaves and green fruits appeared in the same cluster on the heatmap, while some compounds had higher concentrations compared to others. Compounds such as malic acid, α linolenic acid, aspartate, quercetin 3-*O*-glucoside, fumaric acid, myricetin 3-*O*-rhamnpyranoside, myricetin, quercetin, methionine, and gallic acid were indicated by a dark brown color. Meanwhile, old leaves, red, and purple fruits had a lower concentration, as indicated by light brown to dark blue colors.

Quercetin 3-*O*-glucoside, myricetin 3-*O*-rhamnpyranoside, myricetin, quercetin, and gallic acid compounds were members of the flavonoids group. This was in accordance with the total flavonoids content and the value of the antioxidant capacity of green fruits and young leaves, which were higher than old leaves, red, and purple fruits in a previous study [11]. Based on the results, the total flavonoid content of green fruits was 95.731±5.42 mg QE/g DW and the value of antioxidant capacity was 1419.75±3.48 µmol TE/g DW. The young leaves had a total flavonoid content of 96.375±3.96 mg QE/g DW and an antioxidant capacity value of 1069.38±6.57 µmol TE/g DW. The total flavonoid and antioxidant capacity values of old leaves were 70.311±5.22 mg QE/g DW and 844.91±5.72 µmol TE/g DW, red fruits had 88.125±2.72 mg QE/g DW and 263.93±1.60 µmol TE/g DW, while purple fruits 67.115±2.57 mg QE/g DW and 127.49±0.57 µmol TE/g DW, respectively [11].

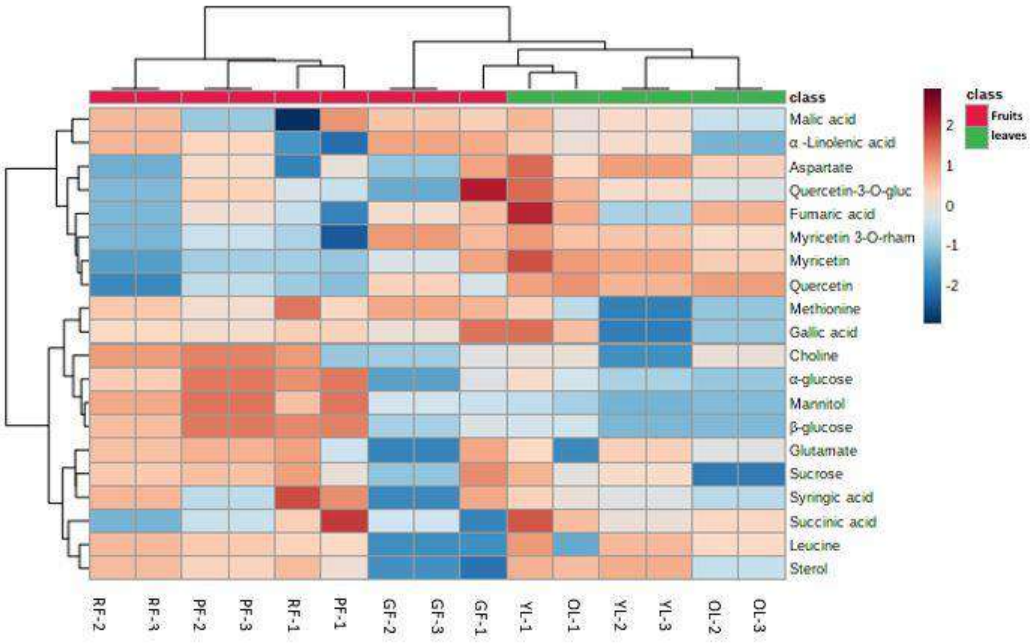


Figure 5. Heatmap of leaves and fruits of *Rhodomyrtus tomentosa*. The blue color represents low concentration and the brown color represents high concentration. Note: YL (young leaves), OL (old leaves), GF (green fruits), RF (red fruits), PF (purple fruits)

The number of hydroxycinnamates, caftaric, coutaric acid, and quercetin glucoside compounds in the phenol and flavonoid families increased in grapes (*Vitis* spp.) during the final stages of green fruits development and significantly declined after ripening 16]. Young leaves in *Carica papaya* L. had the highest phenol, flavonoid, and antioxidant activity, followed by mature leaves and seeds [13]. Furthermore, immature wild edible fruits contained significant quantities of polyphenols, including flavonoid [25], indicating that the pre-ripening period served as a defense mechanism for fruits against various congenital diseases. The presence of higher amounts of antioxidants in the unripe fruits of *Rubus ellipticus* and *Myrica esculenta* was consistent with this observation. Based on these results, as fruits ripened, phenols and flavonoids oxidized, participating in the biosynthesis of anthocyanins, which accumulated during maturation, leading to a decrease in flavonoid concentration.

This study elaborated on a previous study focusing on the antioxidant capacity, total flavonoid content, and compound distribution in *R. tomentosa* using histochemical analysis. The antioxidant activity evaluation using FRAP and DPPH showed a comparable proportion, particularly in the green fruits ethanol extracts, indicating the highest FRAP value of 1367.59±9.12 µmol TE/g DW and DPPH radical scavenging ability value of 1419.75±3.48 µmol TE/g DW. The lowest antioxidant activity was observed in the purple fruits with FRAP value of 138.38±1.13 µmol TE/g DW and DPPH of 127.49±0.57 µmol TE/g DW. As a comparison, the DPPH value of the purple fruits was almost four times lower than the activity antioxidant ORAC value of 431.17±14.5 µmol TE/g DW [4] but higher

than another study [26], which was measured in a variety of consumed fruits such as grape, kiwifruit, oranges, apples, mangoes, blueberries, bananas, and blackberries 8.79–92.60 μmol TE/g DW [27].

NMR experiments were carried out to identify and confirm the presence of a wide variety of metabolites in all three samples, namely seed, skin, and pericarp, obtained from *Momordica charantia* fruits [28]. To identify the metabolic differences between these samples, a multivariate statistical analysis was conducted. Different parts of fruits showed significantly varying concentrations of important metabolites, where the highest total flavonoid and phenolic contents were found in seeds and pericarp, followed by skin. Flavonoids metabolites synthesized from naringenin and identified included luteolin, catechin, kaempferol, quercetin, and myricetin. Based on metabolic analysis, the pericarp and seeds contained higher antioxidant activities compared to the skin. The scavenging effects of methanol extracts of ripe fruits measured by DPPH assay were arranged as seed > pericarp > skin [28].

According to the heatmap presented in Figure 5, red and purple fruits belonged to the same cluster. The results showed that the choline, mannitol, β-glucose, α-glucose, and sucrose compounds had a higher concentration, as indicated by a dark brown color. Meanwhile, young leaves and green fruits had a lower concentration, which was shown by the light-blue to dark-blue colors. Mannitol, β-glucose, α-glucose, and sucrose were carbohydrates (sugars) compounds. Ali et al. [16] reported that the concentration of glucose and fructose increased during the ripening stage of grapes (*Vitis* spp.).

The growth of grapefruits was similar to *R. tomentosa* fruits, as both passed through a complex series of biochemical and physical changes, including variations in composition, size, color, taste, texture, and pathogen resistance. Generally, the development of grapes could be separated into three phases. During the initial phase (phase I), fruits grew rapidly due to cell division and expansion, accompanied by the biosynthesis of various compounds, including malic, tartaric, hydroxycinnamates, and tannins. These compounds reached a maximum concentration approximately 60 days after flowering. Phase II referred to the growth lag phase, which was often observed 7 to 10 weeks after flowering, characterized by sugar accumulation. In Phase III (ripening), the berries experienced significant changes in morphology and composition, doubling in size. This indicated the onset of color development associated with anthocyanin accumulation in red wine as well as an increased sweetness, particularly in fructose and glucose levels, followed by decreased acidity [16].

The sugar content of fruits was used as an indicator for assessing the level of ripeness and determining the optimal time for harvest. Carbohydrates, particularly sucrose, were produced by the process of photosynthesis in grapevine leaves and transported to fruits through the phloem. During this process, the sugar content passed through alteration after the transfer due to the loss of water. Furthermore, sugar was used as a source of carbon, energy, and a means of modulating the regulation of gene expression. The accumulation of fructose and glucose started during the second phase of fruits growth, followed by a persistent process, incorporating the transportation of monosaccharides through transporters to facilitate the delivery of sugars to cellular organelles [16]. Sugar plays a crucial role in facilitating plant development, providing energy, where fructose and glucose synthesize sucrose as precursors for the formation of organic acids and pyruvate. Throughout the developmental process, there was a significant rise in the concentrations of fructose and glucose [29].

The dark violet, bell-shaped edible berries of *R. tomentosa*, have been traditionally used as folk medicine to treat issues such as dysentery, diarrhea, and traumatic hemorrhage [30]. Additionally, these berries were used in crafting a renowned fermented beverage called "Ruou sim" on Phu Quoc Island in southern Vietnam, maturing to acquire a deep purple color and an astringent taste [3,4,31]. In China, these berries are transformed into delectable pies, jams, and salad additions, playing a significant role in the creation of traditional wines, beverages, jellies, and freshly canned syrups for human consumption. The berries of *R. tomentosa* contain a rich assortment of chemical constituents, including sugars, minerals, vitamins, phenols, flavonoid glycosides, organic acids, amino acids, quinones, and polysaccharides. Furthermore, this plant thrives in subtropical and tropical regions, serving as a valuable source for cultivating novel ingredients that promote health benefits [26].

4. Conclusion

Rhodomyrtus tomentosa appeared to have played a significant and holistic role in the daily lives of ancient societies, providing medical benefits. Multiple biological activities of this plant, including antifungal, antimicrobial, antimalarial, antioxidant, anti-inflammatory, and osteogenic properties, have been documented. Therefore, it was essential to comprehend the various parts of *R. tomentosa*, such as the leaves and fruits at various phases of maturation, and the metabolic fate of its various classes of compounds. This study demonstrated that the combination of ^{1}H-NMR and multivariate data analysis enabled the detection of significant differences between the various developmental stages of the leaves and fruits used in this investigation. At various stages of development, the samples contained substantially different amounts of sugar, aromatic compounds, and phenolic compounds. Green fruits and young leaves contained substantial concentrations of phenolics, including quercetin 3-O glucoside, myricetin 3-*O*-rhamnpyranoside, myricetin, quercetin, and gallic acid. During the final phases, choline, mannitol, α-glucose, β-glucose, and sucrose concentrations increased. The approach of this study was useful for analyzing a variety of compounds within the *R. tomentosa* metabolome; however, further research with more sensitive analytical instruments may be desirable to provide a thorough examination of the metabolome transformation and metabolism of the fruit and leaf of *R. tomentosa* at different stages of development.

Acknowledgments

The authors are grateful to Gadjah Mada University in Yogyakarta, Indonesia, for supporting this study through the RTA Grant 2022.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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11. Published manuscript

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Metabolomics Profiling of *Rhodomyrtus tomentosa* (Ait.) Hassk. Leaves and Fruits Using ^{1}H NMR Spectroscopy

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(Received July 26, 2023; Revised September 27, 2023; Accepted September 29, 2023)

Abstract: Several investigations are extensively documenting the presence of metabolites with antibacterial, anticancer, antioxidant, and anti-inflammatory properties in extracts derived from *Rhodomyrtus tomentosa* fruits and leaves. Therefore, this study aimed to evaluate the metabolite profile of *R. tomentosa* fruits and leaves at various maturity stages and determine their phytomedicinal values. ^{1}H NMR and chemometric analyses were used to conduct a metabolomics study for comparing the metabolite profile and phytomedicinal values of different plant organs at varying ages. Leaves were classified into young and old categories, while fruits were divided into three maturity stages, namely green, red, and purple. The wild-grown fruits and leaves of *R. tomentosa* (Ait.) Hassk were gathered in Banjarbaru, South Kalimantan Province, Indonesia. The results of multivariate analysis showed that choline, methionine, mannitol, and β-glucose compounds were three times higher in fruits compared to leaves. Meanwhile, the concentration of aspartate, myricetin, and quercetin compounds was three times higher in leaves compared to fruits. The quantities of secondary metabolites, including flavonoids, were higher in young leaves and green fruits than in old, red, and purple fruits.

Keywords: *Rhodomyrtus tomentosa*; flavonoid; ^{1}H-NMR; multivariate statistical analysis.

1. Introduction

Plants are significantly important in the daily lives of people by providing essential resources such as medicine, food, fiber for clothing, and wood for building. Among these resources, plants used for treating different medical conditions are considered the most valuable. Individuals without access to modern healthcare still place great value on traditional medical practices. Several studies have shown that the majority of traditional medicines are produced from plant-based materials. A recent report stated that approximately 80% of the global population relies on plants to treat a wide range of ailments, indicating their widespread usage. Consequently, herbal medicines have become integral components of modern therapy, with 25% of medications available worldwide originating from plants [1].

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The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products November-December 2023 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.418.2307.2853
Available online: October 10,2023

Rose myrtle, scientifically known as *Rhodomyrtus tomentosa*/*R. tomentosa* (Ait.) Hassk. is a blossoming plant belonging to the Myrtaceae family. According to previous reports, *R. tomentosa* is indigenous to southern and southeastern Asia, with its natural habitat spanning from India, southern China, the Philippines, and Malaysia, to Sulawesi. This plant has an exceptional ability to grow in various environments, ranging from sea level to elevations of 2400 m. Furthermore, it thrives in diverse locations, including natural forests, beaches, wetlands, riparian zones, moist, wet woods, and bog borders, requiring intense sunlight and minimal soil conditions [2-4]. In tropical and subtropical gardens, *R. tomentosa* is a well-liked ornamental plant, cultivated for its abundant blooms, delectable, and edible fruits. These fruits are often used for several culinary applications, such as pies, salads, and jams, including additional processing into wine, jellies, or canned fruits in Vietnam and China [2]. Modern pharmacological studies have shown that *R. tomentosa* components possess diverse properties, namely antibacterial [5], anticancer [6], anti-inflammatory [7], and antioxidant [8]. Traditional Malaysian, Chinese, and Vietnamese medicine has used its leaves, roots, buds, and fruits for medicinal purposes [4]. For example, the Dayak and the Paser local tribes in East Kalimantan, Indonesia, use the roots to treat diarrhea and stomachaches, and as a postpartum tonic [9]. The crushed leaves serve as external poultices and the tar obtained from its wood is used for eyebrow darkening. These traditional applications are in line with the effects observed in modern pharmacological studies. Several phytochemical reports showed that the plant contains flavonoids, triterpenoids, phenols, microelements, and meroterpenoids [2]. Among these compounds, rhodomyrtone is the most prominent, possessing numerous potential pharmacological properties [10], while piceatannol serves as the main and highly effective phenolic component [3].

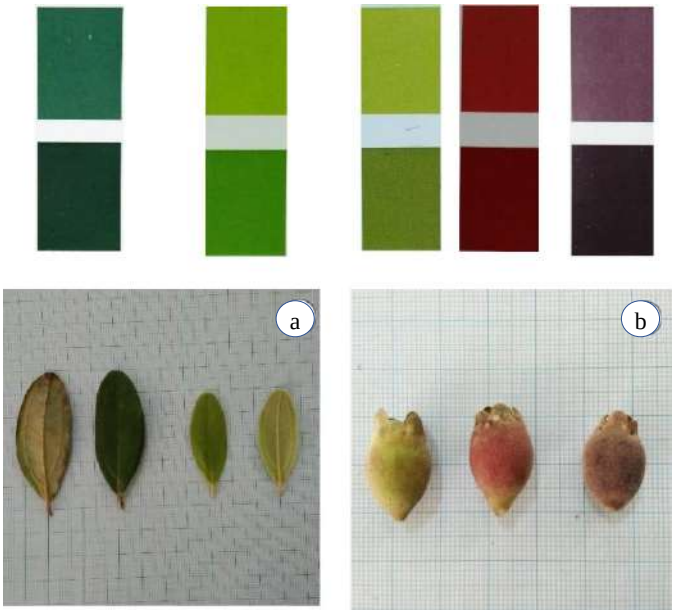
In a previous study, the total phenolic content, antioxidant capacity, total flavonoids, and compound distribution in *R. tomentosa* were explored using histochemical analysis [11]. This report did not examine the specific antioxidant profile of fruits and leaves at various stages of development. To address the limitation, ^{1}H-NMR was used to analyze the metabolite profile in leaves and fruits. Therefore, this study aimed to analyze the secondary and primary metabolites of various parts of *R. tomentosa*, specifically fruits and leaves. The existence of these compounds was correlated with the previously reported bioactivity and phytomedicinal qualities. NMR spectroscopy and multivariate data analysis without chromatographic separation were used to identify metabolites directly from the samples. A metabolic analysis was also conducted on *R. tomentosa* fruits and leaves at various stages of maturation. Subsequently, multivariate statistics were used to determine the compounds significantly contributing to the variations between both parts. This study is the first systematic examination of *R. tomentosa* fruits and leaves at various stages of maturity using a combined NMR and multivariate statistical method. Analysis showed the applicability of the NMR-based method in plant metabolomics.

2. Materials and Methods

2.1. Plant Materials

In this study, *R. tomentosa* plant was obtained from its natural habitat in the wild. The samples were collected from Banjarbaru, South Kalimantan, Indonesia (3°29′0″S, 114°52′0″E) in August-October 2020. To accurately characterize the plant tissues, Munsell Color Charts were used as a reference guide for samples of leaves and fruits [12]. Leaves samples were selected from young leaves (2^{nd} – 6^{th} order from the shoot [Munsell Color Carts guide: 5GY (7/8-6/8)]) and old leaves (7^{th} -12^{th} order from the shoot [Munsell Color Carts guide: 2.5G (4/4-3/4)]). Furthermore, fruit samples used were green [Munsell Color Carts guide: 2.5GY (7/8-6/10)], red [Munsell Color Carts:10R (6/10-5/10)], and purple [Munsell Color Carts:5RP (4/4-3/2)] with a color guide using Munsell Color Charts for Plant tissues, as illustrated in Figure 1A. Subsequently, three replicates of each leaves and fruits were analyzed, followed by identification using the Herbarium Bogoriense, Indonesian Institute of Sciences, located in Bogor, Indonesia, under certificate number 1007/IPH.1.01/If.07/IX/2020.

A



B

Figure 1. *Rhodomyrtus tomentosa* (Ait.) Hassk. (**A**) a: leaves, b. fruits, with a color guide using Munsell Color Charts for Plant tissues (**B**) a: green fruits, b: red fruits, c: purple fruits, d: young and old leaves.

2.2. Crude Extract Preparation and Sample Preparation for ^{1}H-NMR

The old and young leaves were carefully selected from the tip shoots of *R. tomentosa*, and fruits were dried in an oven at 40 °C, and ground at room temperature. Each grounded material of 500 g was macerated in ethanol of 1000 mL (SmartLab, Indonesia) for 24 h. The solvent was discarded and changed every 24 h, with this process being repeated three times [9,11]. Furthermore, extracts from the same samples were combined, properly mixed, filtered to eliminate cell debris, and dried using a rotary evaporator.

^{1}H-NMR sample preparation was carried out using a slightly modified version of the sample extraction of previously reported methods [15]. Precisely, 25 mg of the crude extract was placed into a 2 mL Eppendorf tube along with 1 mL of NMR solvent, comprising 0.5 mL of methanol-d4, 0.5 mL of KH_2PO_4 buffer, and pH 6.0 containing 0.001% TMSP (trimethyl silypropionic acid sodium, Sigma-Aldrich). The mixture was vortexed, sonicated for 1 min, homogenized, and centrifuged for 1 min at 10,000 rpm using a microcentrifuge. The supernatant was collected, placed in the NMR tube, and prepared for ^{1}H-NMR analysis.

2.3. NMR Experiments

^{1}H-NMR was carried out using a 500 MHz spectroscopy (JEOL JNM ECZ500R) at a temperature of 25 °C. The parameters used for a total of 128 scans lasting for 10 min included a relaxation delay of 1.5 seconds, X_angle 60^o, and a pre-saturation mode of 4.27 ppm. Subsequently, the deuterated solvent was set as the internal lock, and spectral width was measured from 0 to 10 ppm.

2.4. Data Analysis

The ^{1}H-NMR spectra were analyzed using MestReNova analysis, followed by processing with manual phasing, baseline adjustment, and calibration to internal standard solution signals (TMSP) positioned at the chemical shift of 0.0 ppm. Multiplicities in the observed NMR resonances were labeled according to the convention: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, and m = multiplet [14]. Furthermore, metabolites were identified by comparing the information in the database from previous studies [13-18]. For semi-quantitative analysis, the area of a signal analysis was compared to that of the TMSP signal as an internal standard. All ^{1}H-NMR signals were normalized to total intensity to develop data for multivariate analysis. MetaboAnalyst software (MetaboAnalyst.ca) was used to analyze multivariate data using Principal Component Analysis (PCA), Partial Least-Squares Discriminant Analysis (PLS-DA), and hierarchical clustering heat map analyses. The spectra were centered and scaled with autoscaling, followed by initial data processing using PCA to evaluate the natural clustering characters. After a clear grouping was observed, the data were subjected to PLS-DA to maximize covariance between measured data (NMR peak intensities) and the response variable (predictive classifications). The PLS-DA coefficient loading plot was used to determine significant metabolites contributing to the separation between the two classes [14]. The model's predictive ability (Q^2) was measured using cross-validation, while statistical significance was determined using a permutation test. This was followed by the ranking of the important compounds in the model using variable importance of projection (VIP) scores. A VIP score of >1 indicated that the variable contributed to the variation of the sample. The t-test analysis was used to determine significant differences in metabolites between all samples with p-values ≤ 0.01.

3. Results and Discussion

3.1. Visual Analysis of ^{1}H-NMR Spectra

NMR spectroscopy was used as a method to determine the magnetic resonance of molecular nuclei interacting with external magnetic fields [19]. Moreover, NMR could produce a distinct and specific spectrum for each compound, leading to its frequent use in determining the type of metabolite. The quality of the results was determined by the number of compounds identified, rather than signals observed during the NMR analysis [20]. The NMR metabolomics methods have been used widely, making compound identification less complicated. This can be achieved by comparing the signal produced by the samples to those generated by the same compounds in previous reports using CD_3OD-D_2O as the solvent [13-18]. Aqueous methanol is commonly used as an extraction solvent alone due to its ability to extract a wide range of compounds, both non-polar and polar. The chemical shift of substances in NMR can be influenced by various solvents. Consequently, multiple reference papers were used to conduct a comparative analysis of potential signal changes that could be identified. In this study, the coupling constant was used as an important parameter to validate the matching signals in the data with the references [15].

The ^{1}H-NMR spectra were separated into three regions based on their chemical shift (δ), namely the amino acids and terpenoids, carbohydrates, and aromatic compounds. Aliphatic compounds (organic and amino acids) were found in the chemical shift 0.5-3.0 ppm, carbohydrates in 3.1 – 6.0 ppm, and aromatic compounds in > 6 ppm. Furthermore, the various developmental stages of ^{1}H-NMR spectra of leaves and fruits extracts were analyzed and compared, as shown in Figure 2.

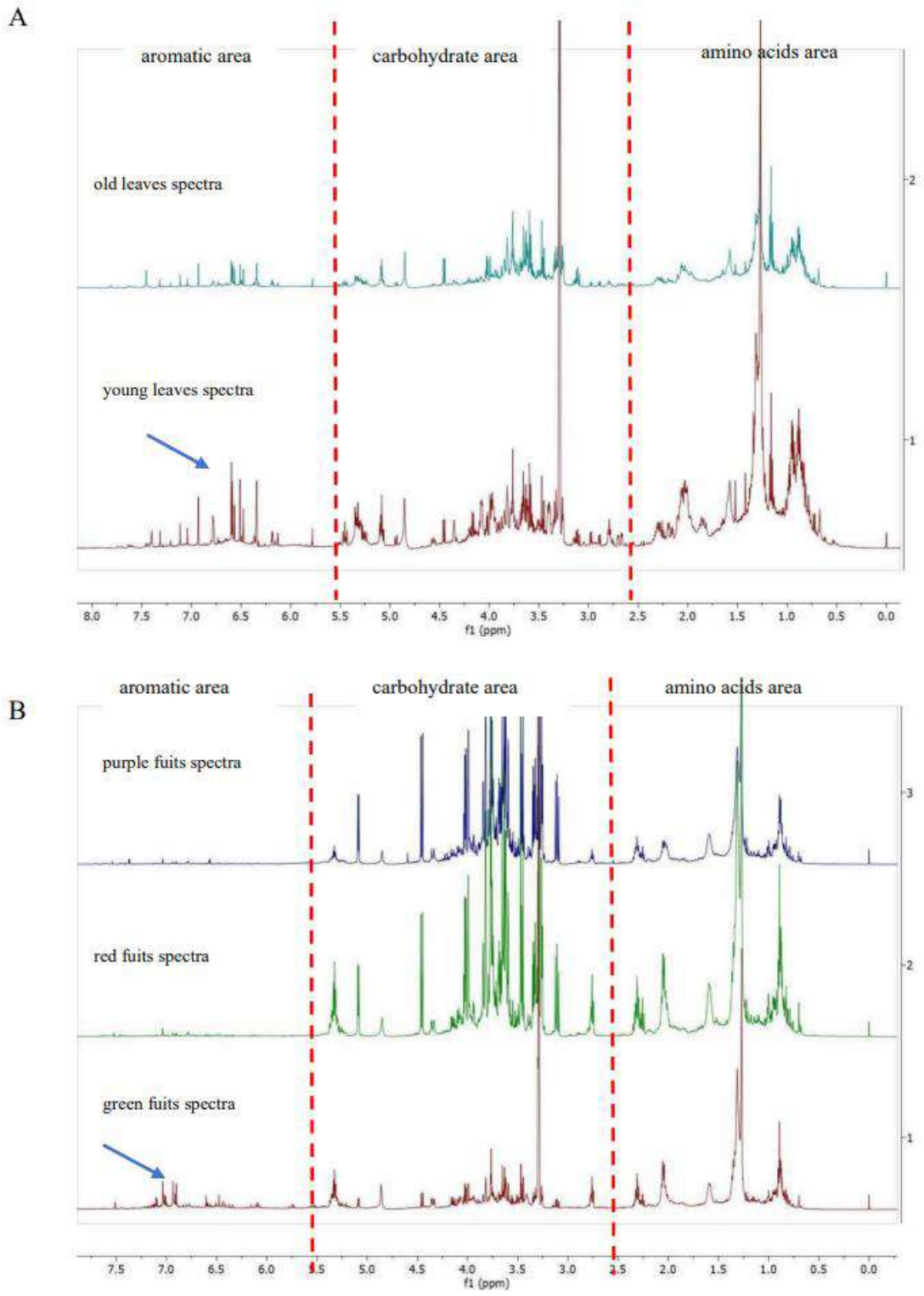


Figure 2. The comparison of the ^{1}H-NMR spectrum (δ 0.00–8.00 ppm) indicates signals of *Rhodomyrtus tomentosa* from (**A**) leaves and (**B**) fruits of *R. tomentosa*. The arrow shows the aromatics regions which observed differences in signal intensities.

The results of the putative compounds identified by ^{1}H-NMR showed the presence of primary and secondary metabolite compounds. Specifically, the primary metabolites included amino acids (chemical shift 2.0-0.5 ppm) and carbohydrates (chemical shift 5.0-3.0 ppm), with aromatic compounds (chemical shift > 6 ppm) being the secondary metabolites. In the aromatic regions of the NMR spectra, a gradual decrease in phenolic content was observed during leaves and fruits growth. The intensities of signals in the aromatic area in the young leaves and green fruits samples were higher than in the old, red, and purple leaves, as illustrated in Figures 2A and B.

3.2. Identification of Metabolites/Assignment of ^{1}H-NMR Signals

The advantages of using ^{1}H-NMR have been shown in various metabolomics studies. However, ^{1}H-NMR presented a significant challenge in chemical identification due to overlapping signals in multiple regions, particularly at 5.0-3.0 ppm, which corresponded to sugar compounds. This caused the inability to identify signals in the sugar region, except for glucose and sucrose, leading to a decrease in the number of substances detected in this study. The results also showed the identification of 20 putative compounds based on the ^{1}H-NMR spectra, as presented in Table 1. In the amnio acid region, the specific signals of leucine, glutamate, methionine, and aspartate were identified, with chemical shifts ranging from 3.0-0.5 ppm. The organic acid compounds, such as malic, fumaric, and succinic acids were observed in the chemical shifts of between 3.0 - 2.0 ppm. The frequently identified sugars, including mannitol, β-glucose, α-glucose, and sucrose were also observed at 5.00 - 3.50 ppm. In the less crowded regions, several phenolics were identified, including 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.

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

No	Compound	Chemical shifts δ (ppm) and coupling constants (Hz)
	Amino Acids	
1	Aspartate	2.68 (dd, J= 3.0; 17.0 Hz)
2	Glutamic acid	2.06 (m); 2.34 (m)
3	Leucine	0.93 (d, J= 6.85 Hz); 0.98 (d, J= 5.92 Hz)
4	Methionine	2.16 m ; 2.79 (t, J= 6.08; 6.08 Hz)
	Organic Acids	
5	Fumaric acid	6.51 (s)
6	Malic acid	4.34 (dd, J= 6.6 ; 4.7 Hz)
7	Succinic acid	2.54 (s)
	Sugars	
8	Mannitol	3.77 (d, J= 3.28 Hz)
9	β-glucose	4.45 (d, J= 7.79 Hz)
10	α-glucose	5.09 (d, J= 3.84 Hz)
11	Sucrose	5.35 (d, J= 3.91 Hz)
	Aromatics Compounds	
12	Gallic acid	7.03 (s)
13	Myricetin	6.34 (d, J= 1.99 Hz); 7.32 (s)
14	Myricetin 3-O-rhamnpyranoside	6.93 (s)
15	Quercetin-3-O-glucoside	6.18 (d, J= 2.08 Hz); 6.37 (d, J= 2.04 Hz)
16	Quercetin	6.13 (s); 7.63 (d, J= 2.22 Hz); 7.51 (s)
17	Syringic acid	3.88 (s)
	Other compounds	
18	α -Linolenic acid	1.16 (t, J=7.04 ; 7.04); 1.29 (m)
19	Choline	3.25 (s)
20	Sterol	0.70 (s)

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

3.2. Multivariate Data Analysis

Multivariate PCA was performed to assess the variations of compounds present in fruits and leaves of *R. tomentosa*. The PCA score plot was used to show the separation of classes, while the correlation between variables was interpreted in the loading plot [21]. MetaboAnalyst 5.0 automatically generated five PCs representing 87.6% of all observed variants. Subsequently, the 2D score diagram derived from PC1 and PC2 distinguished fruits and leaves samples. As shown in Figure 3A, the Q^2 cumulative of PC1 and PC2 yielded 57.6% of variants, which was above 50%, indicating a reliable model. PLS-DA was implemented in multivariate analysis to enhance separation, where PC1 and PC2 accounted for 35% and 21.7% of the observed variants, respectively, for a total of 56.7% Q^2 in the 2D score plot. On the PLS-DA score plot presented in Figure 3B, samples of leaves and fruits were positioned in the negative and positive regions of PC1, respectively,

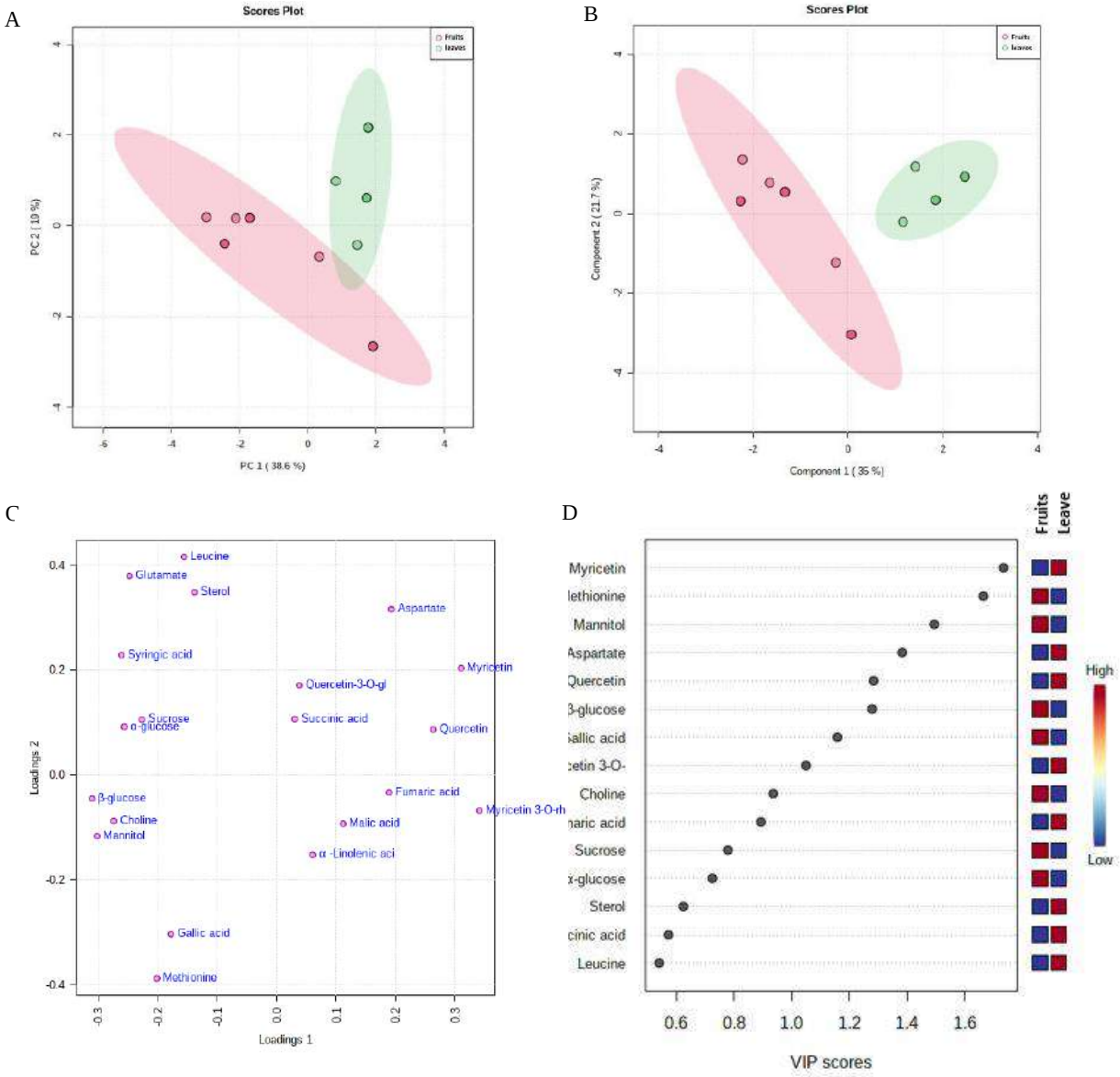


Figure 3. Multivariate data analysis of *Rhodomyrtus tomentosa* leaves and fruits samples (**A**). PCA Score Plot; (**B**). PLS-DA score plot; (**C**). PLS-DA loading plot analysis; (**D**). Variables important in projection (VIP) based on PLS-DA.

Cross-validation was used to determine the Q^2, which assessed the predictability of the PLS-DA model. When $R^2 = 1$ and $Q^2 = 1$, the model could precisely describe and predict the data [22]. In this study, PLS-DA showed distinct separation ($R^2 = 1$) and excellent predictability ($Q^2 = 0.9$). The PLS-DA model with a CV-ANOVA less than 0.05 was determined to be the most accurate through 20 permutation tests that validated the results, indicating the reliability of the model [20].

After a separation between leaves and fruits of *R. tomentosa* was observed, a loading plot was used to identify the compounds that distinguished the two groups. Among the 20 compounds observed, Figure 3C showed that there were five distinct components in leaves profile with fruits, as illustrated in Table 1. Compounds observed in the positive region of PC1 were fumaric acid, myricetin, myricetin 3-*O*-rhamnpyranoside, quercetin, and aspartate.

During the implementation of PLS-DA, the VIP score was readily available, reflecting the significance of the model's variables. The VIP was recognized as an instrument for identifying the variables that contributed mainly to the examined variants. Moreover, a VIP score greater than one was frequently used as a selection criterion [23]. As shown in Figure 3D, the score graph indicated that nine metabolites, including myricetin, myricetin 3-*O*-rhamnpyranoside, quercetin, aspartate, choline, gallic acid, methionine, mannitol, and β-glucose, had values greater than 1.

These results suggested the need for further study beyond VIP score compounds to determine when there were notable variations in the chemical composition of fruits and leaves of *R. tomentosa* at the compound level. Subsequently, concentration determination was carried out using a semi-quantitative examination of the compound signal obtained from the internal signal of the TMSP standard. Analysis of signal integration results was conducted using independent t-tests.

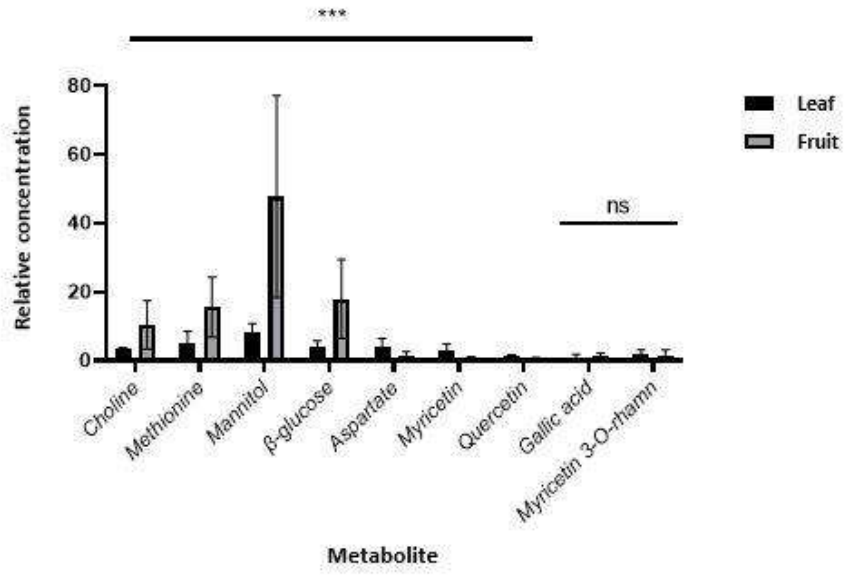


Figure 4. Comparison of metabolite concentrations as major contributors to differences between the leaves and the fruits of *Rhodomyrtus tomentosa*

A heatmap was used to assess further differences in the diversity of compound content between fruits and leaves. The concentration of compounds found in fruits and leaves of *R. tomentosa* was visualized through cluster analysis [24]. Heatmap was a form of visualization method that represented data distribution through color changes. In this study, the relative concentrations of compounds in fruits and leaves of *R. tomentosa* served as the data for heatmap analysis, as presented based on the groups of samples. The results showed that the compounds identified showed high diversity and varied concentration, as shown in Figure 5.

Young leaves and green fruits appeared in the same cluster on the heatmap, while some compounds had higher concentrations compared to others. Compounds such as malic acid, α linolenic acid, aspartate, quercetin 3-*O*-glucoside, fumaric acid, myricetin 3-*O*-rhamnpyranoside, myricetin,

quercetin, methionine, and gallic acid were indicated by a dark brown color. Meanwhile, old leaves, red, and purple fruits had a lower concentration, as indicated by light brown to dark blue colors. Quercetin 3-*O*-glucoside, myricetin 3-*O*-rhamnpyranoside, myricetin, quercetin, and gallic acid compounds were members of the flavonoids group. This was in accordance with the total flavonoids content and the value of the antioxidant capacity of green fruits and young leaves, which were higher than old leaves, red, and purple fruits in a previous study [11]. Based on the results, the total flavonoid content of green fruits was 95.731±5.42 mg QE/g DW and the value of antioxidant capacity was 1419.75±3.48 µmol TE/g DW. The young leaves had a total flavonoid content of 96.375±3.96 mg QE/g DW and an antioxidant capacity value of 1069.38±6.57 µmol TE/g DW. The total flavonoid and antioxidant capacity values of old leaves were 70.311±5.22 mg QE/g DW and 844.91±5.72 µmol TE/g DW, red fruits had 88.125±2.72 mg QE/g DW and 263.93±1.60 µmol TE/g DW, while purple fruits 67.115±2.57 mg QE/g DW and 127.49±0.57 µmol TE/g DW, respectively [11].

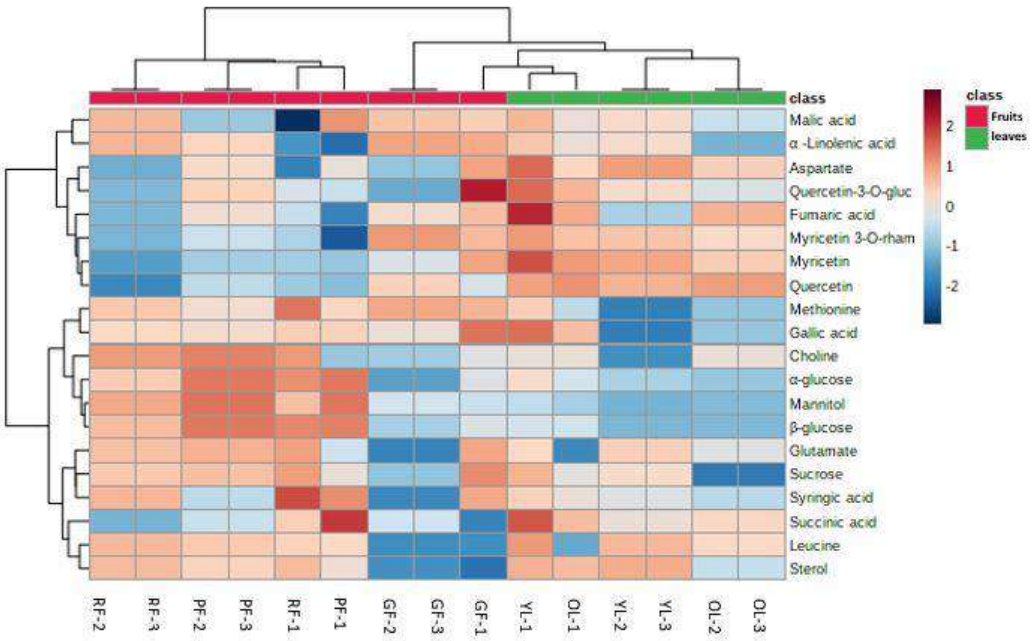


Figure 5. Heatmap of leaves and fruits of *Rhodomyrtus tomentosa*. The blue color represents low concentration and the brown color represents high concentration. Note: YL (young leaves), OL (old leaves), GF (green fruits), RF (red fruits), PF (purple fruits)

The number of hydroxycinnamates, caftaric, coutaric acid, and quercetin glucoside compounds in the phenol and flavonoid families increased in grapes (*Vitis* spp.) during the final stages of green fruits development and significantly declined after ripening 16]. Young leaves in *Carica papaya* L. had the highest phenol, flavonoid, and antioxidant activity, followed by mature leaves and seeds [13]. Furthermore, immature wild edible fruits contained significant quantities of polyphenols, including flavonoid [25], indicating that the pre-ripening period served as a defense mechanism for fruits against various congenital diseases. The presence of higher amounts of antioxidants in the unripe fruits of *Rubus ellipticus* and *Myrica esculenta* was consistent with this observation. Based on these results, as fruits ripened, phenols and flavonoids oxidized, participating in the biosynthesis of anthocyanins, which accumulated during maturation, leading to a decrease in flavonoid concentration.

This study elaborated on a previous study focusing on the antioxidant capacity, total flavonoid content, and compound distribution in *R. tomentosa* using histochemical analysis. The antioxidant activity evaluation using FRAP and DPPH showed a comparable proportion, particularly in the green fruits ethanol extracts, indicating the highest FRAP value of 1367.59±9.12 µmol TE/g DW and DPPH radical scavenging ability value of 1419.75±3.48 µmol TE/g DW. The lowest antioxidant activity was observed in the purple fruits with FRAP value of 138.38±1.13 µmol TE/g DW and DPPH of

127.49±0.57 μmol TE/g DW. As a comparison, the DPPH value of the purple fruits was almost four times lower than the activity antioxidant ORAC value of 431.17±14.5 μmol TE/g DW [4] but higher than another study [26], which was measured in a variety of consumed fruits such as grape, kiwifruit, oranges, apples, mangoes, blueberries, bananas, and blackberries 8.79–92.60 μmol TE/g DW [27].

NMR experiments were carried out to identify and confirm the presence of a wide variety of metabolites in all three samples, namely seed, skin, and pericarp, obtained from *Momordica charantia* fruits [28]. To identify the metabolic differences between these samples, a multivariate statistical analysis was conducted. Different parts of fruits showed significantly varying concentrations of important metabolites, where the highest total flavonoid and phenolic contents were found in seeds and pericarp, followed by skin. Flavonoids metabolites synthesized from naringenin and identified included luteolin, catechin, kaempferol, quercetin, and myricetin. Based on metabolic analysis, the pericarp and seeds contained higher antioxidant activities compared to the skin. The scavenging effects of methanol extracts of ripe fruits measured by DPPH assay were arranged as seed > pericarp > skin [28].

According to the heatmap presented in Figure 5, red and purple fruits belonged to the same cluster. The results showed that the choline, mannitol, β-glucose, α-glucose, and sucrose compounds had a higher concentration, as indicated by a dark brown color. Meanwhile, young leaves and green fruits had a lower concentration, which was shown by the light-blue to dark-blue colors. Mannitol, β-glucose, α-glucose, and sucrose were carbohydrates (sugars) compounds. Ali et al. [16] reported that the concentration of glucose and fructose increased during the ripening stage of grapes (*Vitis* spp.).

The growth of grapefruits was similar to *R. tomentosa* fruits, as both passed through a complex series of biochemical and physical changes, including variations in composition, size, color, taste, texture, and pathogen resistance. Generally, the development of grapes could be separated into three phases. During the initial phase (phase I), fruits grew rapidly due to cell division and expansion, accompanied by the biosynthesis of various compounds, including malic, tartaric, hydroxycinnamates, and tannins. These compounds reached a maximum concentration approximately 60 days after flowering. Phase II referred to the growth lag phase, which was often observed 7 to 10 weeks after flowering, characterized by sugar accumulation. In Phase III (ripening), the berries experienced significant changes in morphology and composition, doubling in size. This indicated the onset of color development associated with anthocyanin accumulation in red wine as well as an increased sweetness, particularly in fructose and glucose levels, followed by decreased acidity [16].

The sugar content of fruits was used as an indicator for assessing the level of ripeness and determining the optimal time for harvest. Carbohydrates, particularly sucrose, were produced by the process of photosynthesis in grapevine leaves and transported to fruits through the phloem. During this process, the sugar content passed through alteration after the transfer due to the loss of water. Furthermore, sugar was used as a source of carbon, energy, and a means of modulating the regulation of gene expression. The accumulation of fructose and glucose started during the second phase of fruits growth, followed by a persistent process, incorporating the transportation of monosaccharides through transporters to facilitate the delivery of sugars to cellular organelles [16]. Sugar plays a crucial role in facilitating plant development, providing energy, where fructose and glucose synthesize sucrose as precursors for the formation of organic acids and pyruvate. Throughout the developmental process, there was a significant rise in the concentrations of fructose and glucose [29].

The dark violet, bell-shaped edible berries of *R. tomentosa*, have been traditionally used as folk medicine to treat issues such as dysentery, diarrhea, and traumatic hemorrhage [30]. Additionally, these berries were used in crafting a renowned fermented beverage called "Ruou sim" on Phu Quoc Island in southern Vietnam, maturing to acquire a deep purple color and an astringent taste [3,4,31]. In China, these berries are transformed into delectable pies, jams, and salad additions, playing a significant role in the creation of traditional wines, beverages, jellies, and freshly canned syrups for human consumption. The berries of *R. tomentosa* contain a rich assortment of chemical constituents, including sugars, minerals, vitamins, phenols, flavonoid glycosides, organic acids, amino acids, quinones, and polysaccharides. Furthermore, this plant thrives in subtropical and tropical regions, serving as a valuable source for cultivating novel ingredients that promote health benefits [26].

4. Conclusion

Rhodomyrtus tomentosa appeared to have played a significant and holistic role in the daily lives of ancient societies, providing medical benefits. Multiple biological activities of this plant, including antifungal, antimicrobial, antimalarial, antioxidant, anti-inflammatory, and osteogenic properties, have been documented. Therefore, it was essential to comprehend the various parts of *R. tomentosa*, such as the leaves and fruits at various phases of maturation, and the metabolic fate of its various classes of compounds. This study demonstrated that the combination of ^{1}H-NMR and multivariate data analysis enabled the detection of significant differences between the various developmental stages of the leaves and fruits used in this investigation. At various stages of development, the samples contained substantially different amounts of sugar, aromatic compounds, and phenolic compounds. Green fruits and young leaves contained substantial concentrations of phenolics, including quercetin 3-O glucoside, myricetin 3-*O*-rhamnpyranoside, myricetin, quercetin, and gallic acid. During the final phases, choline, mannitol, α-glucose, β-glucose, and sucrose concentrations increased. The approach of this study was useful for analyzing a variety of compounds within the *R. tomentosa* metabolome; however, further research with more sensitive analytical instruments may be desirable to provide a thorough examination of the metabolome transformation and metabolism of the fruit and leaf of *R. tomentosa* at different stages of development.

Acknowledgments

The authors are grateful to Gadjah Mada University in Yogyakarta, Indonesia, for supporting this study through the RTA Grant 2022.

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