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## [biodiv] Editor Decision

3 pesan

Agustina Putri <smujo.id@gmail.com>

8 Juni 2021 pukul 13.06

Kepada: Wendy Alexander Tanod <wendytanod@stplpalu.ac.id>, Anton <antonius\_rumengan@unsrat.ac.id>

Wendy Alexander Tanod, Anton:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North Sulawesi, Indonesia".

Our decision is: Revisions Required

"Note: Kindly send your revised paper to a professional English proofreader prior to resubmission"

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[Biodiversitas Journal of Biological Diversity](#)



**U-Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North .doc**

2464K

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Agustina Putri <smujo.id@gmail.com>

8 Juni 2021 pukul 13.21

Kepada: Wendy Alexander Tanod <wendytanod@stplpalu.ac.id>, Anton <antonius\_rumengan@unsrat.ac.id>

Wendy Alexander Tanod, Anton:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North Sulawesi, Indonesia".

Our decision is: Revisions Required

"Note: Kindly send your revised paper to a professional English proofreader prior to resubmission"

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Reviewer E:

The authors assessed the pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves in Indonesia using HPLC.

I do have several suggestions that need to be addressed by major revision before publication. Below are some specific comments to be taken into consideration by the authors:

### General comments

- Definitely, the article has a great novelty to publish in the journal.
- The manuscript presents interesting data with innovative findings and falls into the scope of the journal.
- The introduction is weakly written. Re-write it with proper background, justification, and hypotheses.
- The methodology is sound enough to interpret the data and draw a final and complete conclusion.
- The authors presented adequate information in the methodology to allow replication of the estimated methods properly.
- There are a lot of grammatical mistakes throughout the whole MS. These should be addressed well by an English Expert.
- The discussion needs to be improved with explanation and citing appropriate references.

### Specific comments

Title: It should be changed to "Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves in Indonesia"

**Abstract**

**Line 9-11:** The identification of pigment profiles were detected chlorophyll a (68.61%), chlorophyll b (27.69%), lutein (29.94%), beta-carotene (14.05%), pheophytin a (8.72), violaxanthin (5.19%), and neoxanthin (3.65%). Meaningless sentence. Rephrase it.

**Line 16:** The authors didn't study any economic analysis. Hence, delete the word "economic".

**Introduction**

The authors studied leaf pigments. They should emphasize the importance, justification, hypothesis of leaf pigments in the Introduction. Please avoid too many unnecessary references.

**Line 29:** Add relevant and recent references to support the sentence "----enzymatic and non-enzymatic antioxidants (doi.10.1186/s12870-018-1484-1; doi.10.1371/journal.pone.0206388; doi.10.3389/fpls.2020.559876)

**Line 31:** Delete the word "economically".

**Line 31:** Add the sentences "Literature has reported that leaves are the natural sources of pigments such as chlorophyll a, chlorophyll b, betacyanins, betaxanthins, betalains (<https://www.wfpublisher.com/Abstract/5378>; doi.10.5424/sjar/2015132-6843; doi.10.5958/0975-6906.2017.00025.6; doi.10.2298/GENSR1501085S). These are potential sources of carotenoids pigments such as  $\beta$ -carotene,  $\alpha$ -carotene, xanthophylls including lutein, neoxanthin, zeaxanthin, and violaxanthin, etc. (Doi.10.1038/s41598-021-91157-8). In addition, leaves also had other pigments such as phenolics and different groups of flavonoids, including flavonols, flavones, flavanols, flavanones, etc. (doi.10.1038/s41598-020-71727-y; doi.10.1186/s12870-020-02700-0; doi.10.1038/s41598-019-52033-8). All these pigments exhibited high antioxidant capacity (doi.10.1080/09064710.2017.1367029." "just before "From literature studies,----."

**Line 32-34:** One of the mangroves has the potential to use its pigment content, namely *Rhizophora mucronata* (Pringgenies et al. 2018). Prabhu and Bhute (2015) reported that the brown pigment from the stem of *R. mucronata* has been used as a textile dye. Grammatical mistake. Rephrase it.

**Line 34-35:** Change "Previous studies have reported *R. mucronata* leaves to contain chlorophyll and carotenoid pigments" to "Previous studies have reported that *R. mucronata* leaves contain chlorophyll and carotenoid pigments".

**Materials and methods**

Write details extraction procedure of pigments. Write a subsection namely "Solvents and reagents" and enlist them accordingly. Write another subsection name "quantification of pigments using HPLC" and describe how they quantified the different pigments using HPLC".

**Line 51:** -----ratio of 1:3 (name the solvents) ---.

**Results and discussion**

-Please avoid too many unnecessary references. Explain the role of pigments by citing recent and relevant references as suggested.

-Provide the LOQ and LOD values in a Table

**Line 99:** Change "chlorophyll-a ---" to "chlorophyll a ---". Follow this style for whole MS where it exists. Example line 100, 103, 104, 107, 108, 109, 127, 130 and 132 (pheophytin a), 150, 164, 165, 202,

**Line 100:** Change "chlorophyll-b ---" to "chlorophyll b ---". Follow this style for the whole MS where it exists. Example line 102, 103, 104, 107, 109, 110, 151, 164, 165, 203,

**Line 114:** Add a sentence "However, amaranth contains good macular pigment lutein in the leaves (Doi.10.1038/s41598-021-91157-8)." just before the sentence "Until now, there has been--."

**Line 119:** Add sentences "Furthermore, carotenoids including  $\beta$ -carotene acts as an antioxidant. Previous studies have shown that carotenoids have strong DPPH and ABTS antioxidant activity in different amaranth species such as drought-tolerant amaranth (doi.10.1186/s12870-020-02780-y), *A. gangeticus* (doi.10.3389/fnut.2020.587257), *A. hypochondriacus* (doi.10.1038/s41598-020-71714-3), stem amaranth (doi.10.1038/s41598-020-60252-7), *A. blitum* (doi.10.1038/s41598-020-59848-w), green amaranth (doi.10.1038/s41598-020-57687-3), weedy amaranth (doi.10.1038/s41598-019-50977-5), and red amaranth (doi.10.1371/journal.pone.0222517). Carotenoids including  $\beta$ -carotene protect the photosynthetic tissue through direct quenching of triplet chlorophyll, preventing the generation of singlet oxygen and protecting from oxidative damage in abiotic stress like salinity and drought (doi:10.3389/fpls.2020.559876). It also detoxifies various forms of reactive oxygen species ROS (doi.10.1038/s41598-018-34944-0; doi.10.1007/s12010-018-2784-5) through increasing it's concentration under abiotic stress like drought and salinity (doi.10.1038/s41598-018-30897-6; doi.10.1002/jsfa.9423; doi.10.1016/j.foodchem.2018.01.097)." just before the sentence "Beta-carotene is a pigment ----."

**Line 139:** Change "----and xanthophylls ---" to "----of xanthophylls group---".

**Line 147:** If the  $Pa > 0.7$ , ----. Add spaces before and after the symbol ">". Follow this style for the whole MS where it exists.

**Line 148:** If the value is  $0.3 \leq Pa \leq 0.7$ , ----. Add spaces before and after the symbol " $\leq$ ". Follow this style for the whole MS where it exists.

**Line 163:** Add more references to support the statement "literature studies ---- antioxidant properties (doi.10.2298/GENSR1801209S; doi.10.1016/S2095-3119(17)61778-7; doi.10.3906/tar-1601-24, İnanç 2011).

**Line 242:** Check the spelling of "picrile".

**Line 243:** ----Dewanto, et al. 2019 ). Delete the space after "2019".

**Line 254:** Check the spelling of "probit".

**Line 255-256:** Add spaces before and after the symbol “≤”, “>” and “<”.

**Line 284:** Add more references to support the sentence “---antioxidant substances useful for the self-defense mechanism of ROS (doi.10.1007/s12010-018-2784-5).”

Recommendation: Revisions Required

Reviewer S:

The manuscript is well-written and very informative. However, there are a few questions from me regarding the experiment.

1. The English of the whole article is acceptable. However, it has to be checked and corrected for better understanding.
2. In the discussion, focus on the outcome of your optimization method and how it can be applied, instead of simply describing previous literatures.
3. Herbarium voucher number for plant identification should be mentioned.
4. Mention the model and brand of Instruments used in the study.
5. The Mangrove samples were extracted with ethanol (Merck) in a ratio of 1:3 based on the methodology described by Dewanto et al. (2018). The detail methodology should be mentioned. Collection (summer or rainy), drying, Extraction method, temperature, time and solvent concentration.
6. Why authors used for ethanol for pigment extraction. Acetone is good solvent for the extraction of pigments... Explain
7. The detailed extraction methodology for HPLC analysis should be mentioned.
8. How the standards are selected for HPLC analysis?
9. Change IC50 as IC<sub>50</sub>
10. Why authors used Vitamin C as reference compound in DPPH radical inhibition assay. The manuscript mainly focuses on pigment analysis from *Rhizophora mucronata* It should be better the results expressed in any one of the pigment compound like Neoxanthin, Violaxanthin, Lutein and etc.,
11. As it is research work, it is not clear why the authors present the previous reports (Jeffrey et al. 1997; Zapata et al. 2000) in Table 1. In my opinion the table should be removed.
12. Authors should insert the peak name (compound name) in the Figure 2
13. The manuscript mainly focuses on pigment analysis. It does not mention the pigments biological activity and its implications for biochemical or toxicological science in the introduction of the paper.
14. The antioxidant activity of ethanolic extract of *Rhizophora mucronata* leaves was reported earlier by Thirunavukkarasu et al. (2017), Adhikari et al. (2016) and Haryoto and Novan Visia Farmasari, (2019). What is the novelty of your study?.

Thirunavukkarasu, S. Asha, T. Ramanathan, D. Kannan, N. Sudhakar, phytochemical analysis of mangrove derived crude plant extract- *Rhizophora mucronata*, J. Global Trends Pharm Sci, 2017; 8(2): 3813 – 3820.

Adhikari A, Ray M, Das AK, Sur TK. Antidiabetic and antioxidant activity of *Rhizophora mucronata* leaves (Indian sundarban mangrove): An *in vitro* and *in vivo* study. Ayu. 2016; 37(1):76-81.

Haryoto and Novan Visia Farmasari, 2019. Antioxidant Activity of Extract Ethanol from the Leaves *Rhizophora mucronata* and *Rhizophora apiculata*. Journal of Engineering and Applied Sciences, 14: 6119-6121.

Recommendation: Revisions Required

[Kutipan teks disembunyikan]

 **U-Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North .doc**  
2464K

Agustina Putri <smujo.id@gmail.com>

8 Juni 2021 pukul 14.44

Kepada: Wendy Alexander Tanod <wendytanod@stplpalu.ac.id>, Anton <antonius\_rumengan@unsrat.ac.id>

Wendy Alexander Tanod, Anton:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North Sulawesi, Indonesia".

Our decision is: Revisions Required

"Note: Kindly send your revised paper to a professional English proofreader prior to resubmission"

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Reviewer A:

After evaluating the manuscript Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North Sulawesi, Indonesia, I recommend acceptance of the manuscript after a minor revision. The manuscript is well written and justified. The introduction is concise and informative. Materials and methods, although simplified by the authors, clearly indicate the references of the methods used. RESULTS AND DISCUSSION are complete, as well as figures and tables are clear to the reader. Considerations:

Line 51: Describe the extractor used together with ethanol in a 1:3 ratio.

Line 59: The Pigment analysis was based on the method by Hegazi et al. 1998.

Line 57-64: Describe the chromatographic column.

Line 173: Replace Fig 2 to Fig 3.

[Kutipan teks disembunyikan]



**U-Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North .doc**

2464K

# Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North Sulawesi, Indonesia

ANTONIUS PETRUS RUMENGAN<sup>1,✉</sup>, ELVI SISKA MANDIANGAN<sup>2</sup>, WENDY ALEXANDER TANOD<sup>3</sup>,  
DARUS SAADAH JOHANIS PARANSA<sup>1</sup>, CAROLUS PAULUS PARUNTU<sup>1</sup>, DESY MARIA HELENA MANTIRI<sup>1</sup>

<sup>1</sup>Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Sam Ratulangi. Jl. Kampus UNSRAT Bahu, Manado 95115, North Sulawesi, Indonesia. Tel./fax.: +62-852-4027-3030, ✉email: antonius\_rumengan@unsrat.ac.id

<sup>2</sup>Research and Development Agency of North Sulawesi Province. Jl. 17 Agustus, Bumi Beringin, Wenang, Manado 95113, North Sulawesi, Indonesia

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Manuscript received: 23 May 2021. Revision accepted: 21 June 2021.

**Abstract.** Rumengan AP, Mandiangan ES, Tanod WA, Paransa DSJ, Paruntu CP, Mantiri DMH. 2021. Identification of pigment profiles and antioxidant activity of *Rhizophora mucronata* mangrove leaves origin Lembeh, North Sulawesi, Indonesia. *Biodiversitas* 22: 2805-2816. Mangroves plants contain unique pigments which can serve both nutraceutical and pharmaceutical purpose. Therefore, this preliminary study aims to identify the pigment profiles of *Rhizophora mucronata* mangrove leaves (HPLC method) and to computationally evaluate the antioxidant mechanism of the pigments (PASS and STITCH analysis). Furthermore, it evaluated the antioxidant capacity of *R. mucronata* leaves extracts (DPPH method), and the pigment profiles detected using HPLC were chlorophyll a (68.61%), chlorophyll b (27.69%), lutein (29.94%), beta-carotene (14.05%), pheophytin a (8.72), violaxanthin (5.19%), and neoxanthin (3.65%). Beta-carotene, lutein, neoxanthin, and violaxanthin were predicted to have potential as antioxidants properties using PASS analysis. While neoxanthin and violaxanthin were predicted as free radical scavengers, beta-carotene was an Nrf-2 stimulant. The STITCH analysis showed that the pigments contained in the leaves interacted synergistically by activating as antioxidant enzymes and inhibiting the expression of oxidative stress proteins. The ethanol extract of *R. mucronata* leaves may be a potent antioxidant with an IC<sub>50</sub> 20.99 ± 0.33 µg/mL. Therefore, the pigment contained in *R. mucronata* leaves is a potential source of antioxidants.

**Keywords:** Beta-carotene, chlorophyll, lutein, neoxanthin, violaxanthin

## INTRODUCTION

Mangrove plants live in low tide areas, and they play a critical role in the coastal ecosystem by supporting fisheries, tourism, and a genetic reservoir (Liu 2012). It functions as a protector of the coastline, preventing seawater abrasion, the habitat of various aquatic biota, and acts best carbon storage ecosystem (Kepel et al. 2017; Rumengan et al. 2018; Saptiani et al. 2018; Tidore et al. 2018). Furthermore, coastal communities have also used mangroves for house construction, charcoal making materials (Dahdouh-Guebas et al. 2000), functional foodstuffs (Jariyah et al. 2014; Kardiman et al. 2017; Analuddin et al. 2019), textile dyes (Pringgenies et al. 2017), and traditional medicine (Revathi et al. 2014; Rout and Basak 2014; Alhaddad et al. 2019; Andriani et al. 2020). These plants have a self-defense mechanism because they live in areas with fluctuations in salinity and temperature (Dewanto et al. 2018). The mechanism designed consists of enzymatic and non-enzymatic antioxidants defense (Sarker et al. 2018a; Sarker and Oba 2018a; 2020a). Pigments in mangroves significantly affect photosynthetic reactions, stress avoidance, and defense mechanisms (Croft and Chen 2017).

Previous studies reported that leaves are the natural sources of pigments such as chlorophyll a, chlorophyll b, betacyanins, betaxanthins, betalains (Sarker et al. 2014;

2015a; 2015b; 2017). These are potential sources of carotenoids pigments such as beta-carotene, alpha-carotene, xanthophylls including lutein, neoxanthin, zeaxanthin, and violaxanthin (Sarker and Oba 2021). In addition, leaves consist of other pigments such as phenolics and different groups of flavonoids, including flavonols, flavones, flavanones (Sarker and Oba 2019a; 2020b; 2020c), and they all exhibit high antioxidant capacity (Sarker et al. 2018b).

Previous studies also showed that mangroves contain unique pigments which may serve nutraceutical and pharmaceutical functions (Banerjee et al. 2017). One of the mangroves has the potential to use its pigment content such as *Rhizophora mucronata* (Pringgenies et al. 2018). Prabhu and Bhute (2015) reported that the brown pigment from the stem of *R. mucronata* was used as a textile dye. Furthermore, it was previously reported that the leaves contain chlorophyll and carotenoid pigments (Flores-de-Santiago et al. 2016; Ridlo et al. 2017). However, no study has reported the complete profile of the pigments in the mangrove leaves of *R. mucronata*. The pigments can be used as a source of functional and natural food coloring (Mapari et al. 2005).

The mangrove *Rhizophora mucronata* showed medicinal potential for coastal communities. Meanwhile, the fruit, leaves, bark, and flowers of *R. mucronata* were used to treat various diseases, such as cognitive function

(Suganthy and Pandima Devi 2016), diabetes (Bandaranayake 1998; Aljaghtmi et al. 2017), diarrhea (Puspitasar et al. 2012), hepatitis (Ravikumar and Gnanadesigan 2012), and inflammation (Rohini and Das 2009). The antioxidant capacity of the leaves obtained from India and Asia was widely reported (Imdadul et al. 2011). This is a preliminary study to identify the pigment profiles of *Rhizophora mucronata* mangrove leaves using the high-performance liquid chromatography method and to computationally evaluate the antioxidant mechanism of the pigments. Furthermore, it evaluated the antioxidant capacity of *R. mucronata* leaves extracts using the DPPH method.

## MATERIALS AND METHODS

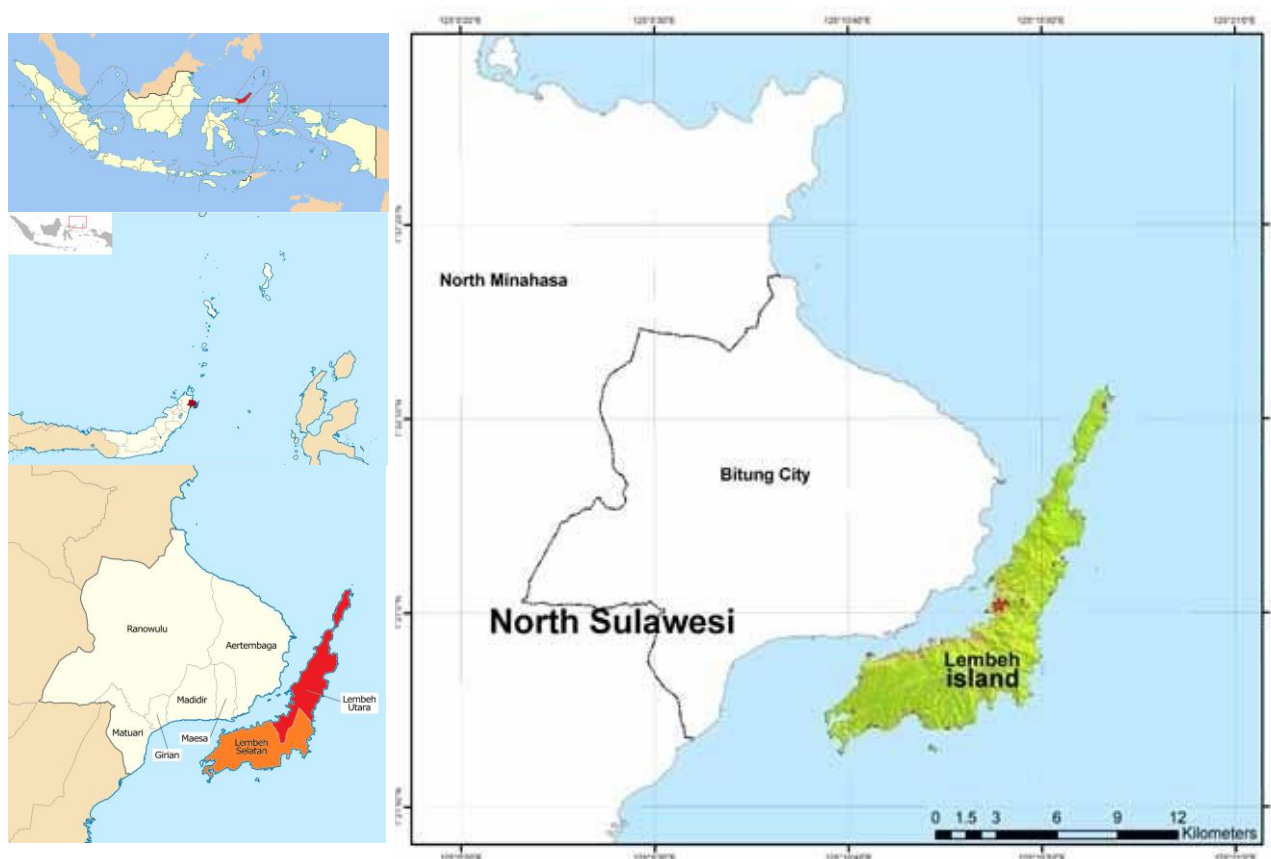
### Sampling and extraction

Mangrove leaves sample (1000 g) was obtained from Lembbeh Island, North Sulawesi, Indonesia. *Rhizophora mucronata* leaves were documented and put in a cool temperature container. They were collected in summer with hot sunny conditions (in July 2019) and were taken to the laboratory for extraction. The leaves were finely mashed to a powder, and the extraction was performed three times with ethanol pure grade (Merck) solvent. In addition, the *R. mucronata* leaves powder (100 g) was macerated using ethanol (1:3 w/v) for 48 hours, with occasional shaking. The resultant maceration product was filtered through filter

paper to separate the filtrate from the residue. Furthermore, the filtrate was evaporated (rotary vacuum evaporator Buchi R-300) at 40°C during 4-6 hours to obtain a crude extract (Dewanto et al. 2018). According to Lichtenthaler (1987), the chlorophyll and carotenoid groups have a phytol chain attached to the porphyrin ring system. The possession of the phytol side chain, which is esterified to the ring carboxyl group, gives chlorophyll and carotenoids their lipid character. Therefore, ethanol is one of the polar solvents used to extract fat-soluble pigments from living plant tissues containing water. Figure 1 showed the location of mangrove leaves sampling.

### Identification of pigment profiles

The identification of mangrove leaves pigments was conducted on a High-Performance Liquid Chromatography (HPLC-20AD-Shimadzu) with SPD-M20A photodiode array detector (PDA). The Pigment analysis was based on the method by Hegazi et al. (1998), and the Shimadzu UV-1800 spectrophotometer was used in determining the wavelength. Meanwhile, the detection of mangrove leaves pigments was conducted by HPLC at 450 nm (carotenoids detection) and 660 nm (chlorophyll detection) respectively. The analytical column was a LiChroCART 250×4 mm I.D. packed with Lichrospher 100, RP-18e (5µm spherical particles), and the precolumn was an ODS-hypersil (C18) with a diameter of 5µm, 20×4 mm.



**Figure 1.** Location of *Rhizophora mucronata* mangrove leaves sampling in Lembbeh Island, North Sulawesi, Indonesia (1° 27' 8.78" N and 125° 14' 41.90" E)

Dry pigment crude extract was dissolved in 5 mL acetone (Paransa et al. 2014) and filtered using a filter membrane (0.2 µm), then 20 µL was injected into HPLC. The pigment elution was conducted at a 1 mL/min flow rate at 30 °C using a gradient elution system from a mixture of methanol, acetone, and ammonium acetate (1 M) solutions. Acetone was used in the pigment extraction because of its amphipathic nature of having a polar and a nonpolar end. Also, it has a significant partial negative and positive charge on the oxygen and carbonyl atom with two nonpolar alpha carbons. However, it is less polar than water and ethanol and can dissolve nonpolar substances. The nature of its small polarity allows it to dissolve polar substances and has fewer properties than water and ethanol. Therefore, acetone is an appropriate solvent, which allows more excellent resolution for detecting pigments using chromatography. It breaks chlorophyll lipid bonds to plant thylakoid structures and suspends pigments in solution (Henriques et al. 2007).

### Computational analysis

The pigment detected with HPLC predicted biological activity-related antioxidants using the PASS (prediction of activity spectra for substances) analysis <http://www.pharmaexpert.ru/passonline/index.ph>. PASS is a tool used for predicting the biological activity of compounds (Riyadi et al. 2020). The predicted activity requires a structural formula in the form of canonical SMILE obtained from the National Center for Biotechnology Information <https://pubchem.ncbi.nlm.nih.gov/>. The pigments were analyzed for their interactions with the STITCH database (search tool for interactions of chemicals) <http://stitch.embl.de/>.

### Antioxidant assay

Measurement of the antioxidant capacity of mangrove leaves was carried out using the DPPH method (Oke and Hamburger 2002), and the assays were conducted using 96 well plate microplates. The ethanol extract of *Rhizophora mucronata* leaves was prepared in series concentrations of 10, 20, 40, 80 µg/mL in methanol solution (Merck). Then, 160 µL of extract from each concentration series was fed into the microplate well. Furthermore, 40 µL of DPPH (Merck) 0.76 mM solution was added to each well that contained a sample. A comparison control used vitamin C with a concentration series of 4, 6, 8, 10 µg/mL. As a control sample, each series of dilutions (160 µL) was added to the microplate well before adding 40 µL of methanol. A negative control (without extract) was made by adding 160 µL methanol with 40 µL DPPH, and 200 µL methanol was blank. The microplate was incubated in a dark room at 25–28 °C for 30 minutes, and after that, the absorbance of each well was measured with the Multiskan GO Microplate Spectrophotometer (Thermo Scientific) at a wavelength of 517 nm. The IC<sub>50</sub> determination was measured from the inhibition percentage data in units of µg/mL, and a probit analysis was used to determine the IC<sub>50</sub> value. The

following equation was used to determine the percentage of DPPH inhibition:

$$\% \text{ Inhibition} = \frac{(C - D) - (A - B)}{(C - D)} \times 100\%$$

Where :

A: Sample absorbance

B: Absorbance control sample

C: Absorbance of negative control

D: Absorbance blank

## RESULTS AND DISCUSSION

The dry extract of *Rhizophora mucronata* mangrove leaves was detected for pigment profiles by HPLC at a wavelength of 450 and 660 nm, with a retention time of 20 to 40 minutes. Table 1 and Figure 2 showed the pigment profiles, and the chromatogram for the leaves extract.

Table 1 showed that the *R. mucronata* leaves extract detected seven pigments dominated by chlorophyll a (68.61%) and chlorophyll b (27.69%). Chlorophyll is a unique green pigment in almost every green part of plants, such as leaves (İnanç 2011). Chlorophyll a is a pigment with a chlorine ring, where magnesium is surrounded by four nitrogen atoms (Taiz et al. 2006). Meanwhile, in chlorophyll b, the –CHO group replaces –CH<sub>3</sub> on the C7 atom (Pareek et al. 2017). There is also chlorophyll a and b epimer at 11.69% and 5.19%, and Limantara and Heriyanto (2012) stated that they always accompany the presence of chlorophyll. Chlorophyll a and b are common and dominant pigments in the leaves of green plants, such as mangroves (Liu 2012; Dou et al. 2018). Ridlo et al. (2017) reported *R. mucronata* leaves to contain chlorophyll and carotenoid pigments. Furthermore, Flores-de-Santiago et al. (2016) also reported that *Rhizophora mangle* leaves contain chlorophyll a and b. Their levels depend on the season and physiological conditions of the mangroves. Bohn and Walczyk (2004) detected chlorophyll a and the epimer at the wavelength spectrum of 429, 664 nm, while chlorophyll b and the epimer were discovered at 456, 648 nm. Kusmita et al. (2015) detected chlorophyll-b at the wavelength spectrum of 456, 596, 645 nm.

Lutein is a member of the xanthophyll and carotenoid family (Al-ali et al. 2020), and it has a yellowish-orange color, called macular pigment, which is not in abundance (Landrum and Bone 2001; Aruldass et al. 2018). Ngginak et al. (2017) reported detecting lutein on the wavelength spectrum of 417, 443, and 472 nm, while Kurniawan et al. (2020) reported detected it at 447, 451, 472 nm. Also, Sarker and Oba (2021) reported that leaves contain good macular pigment lutein. Previously, no study has detected lutein pigment in mangrove leaves. The detection was conducted using random sampling of old mangrove leaves that were yellowish in color.

**Table 1.** Identification of pigment profiles from *Rhizophora mucronata* leaves with HPLC

Wavelength detection (nm)	Retention time (min)	Area (%)	Wavelength spectrum (nm)	Pigment profiles	References (Jeffrey et al. 1997; Zapata et al. 2000)	
					Retention time (min)	Wavelength spectrum (nm)
450	20.06	3.65	415, 437, 465	Neoxanthin	–	415.1, 438.5, 467.1
450	21.72	5.19	417, 442, 470	Violaxanthin	21.32	416, 440, 470
450	29.29	24.94	421, 447, 475	Lutein	27.65	422, 446, 475
450	33.91	20.65	462, 598, 647	Chlorophyll b	31.62	462, 599, 648
660		7.04				
450	34.20	5.19	461, 599, 648	Chlorophyll b epimer	31.87	462, 599, 650
450	35.53	11.30	430, 617, 663	Chlorophyll a	33.15	431, 617, 662
660	35.53	57.31				
450	35.89	2.08	430, 617, 663	Chlorophyll a epimer	33.48	430, 615, 664
660	35.89	9.61	430, 616, 663			
660	37.63	8.72	409, 505, 535, 608, 664	Pheophytin a	–	409.5, 505.3, 534.7, 608.9, 665.5
450	38.39	14.05	453, 479	Beta-carotene	35.95	426, 452, 477

Beta-carotene is one of the red-yellow, orange or red-orange carotenoids in natural plants that carry out photosynthesis (Kusbandari and Susanti 2017). It may be fat-soluble, insoluble in water, easily damaged, unstable at high temperatures, and precursor of vitamin A (Strobel et al. 2007). Furthermore, carotenoids including beta-carotene act as an antioxidant. Previous studies showed that they have strong DPPH and ABTS antioxidant activity in different amaranth species such as drought-tolerant amaranth (Sarker and Oba 2020d), *A. gangeticus* (Sarker et al. 2020a), *A. hypochondriacus* (Sarker and Oba 2020e), stem amaranth (Sarker et al. 2020b), *A. blitum* (Sarker and Oba 2020f), green amaranth (Sarker et al. 2020c), weedy amaranth (Sarker and Oba 2019b), and red amaranth (Sarker and Oba 2019c). Furthermore, carotenoids including beta-carotene protect the photosynthetic tissue through direct quenching of triplet chlorophyll. This prevents the generation of singlet oxygen from oxidative damage in abiotic stress like salinity and drought (Sarker and Oba 2020a). It also detoxifies various forms of reactive oxygen species (ROS) (Sarker and Oba 2018b; 2018c) through increasing beta-carotene concentration (Sarker and Oba 2018d; 2018e; 2019d). Beta-carotene is a pigment synthesized by plants (Bogacz-Radomska and Harasym 2018). Radu et al. (2012) reported detecting this pigment on a wavelength spectrum of 445, 472, 498 nm. Meanwhile, it was detected by Mangunsong et al. (2019) at a wavelength spectrum of 460 nm. Beta-carotene was also detected on the wavelength spectrum of 450.20 and 477.60 nm (Kusbandari and Susanti 2017). The literature showed that this pigment was detected in the leaves and roots of mangroves *Avicennia officinalis*, *Excoecaria agallocha*, *Kandelia candel*, and *Rhizophora mucronata* (Ravindran et al. 2012). In addition, it was also detected in the mangroves of *Bruguiera gymnorrhiza*, *Sonneratia alba*, and *Xylocarpus granatum* (Analuddin et al. 2019).

Pheophytin a pigment is a chlorophyll a without  $Mg^{2+}$  ion, and it is dominant in fresh green leaves, which is degraded due to heating and storage processes (Hsu et al. 2013). It is produced naturally by plant leaves and acts as an intermediary for the first electron carrier in the transfer

pathway for photosystem II plants (Eijkelhoff and Dekker 1997). Bohn and Walczyk (2004) detected pheophytin-a on the wavelength spectrum of 405, 661 nm while Kusmita et al. (2015) were on wavelength spectrum of 408, 505, 535, 609, 665 nm.

Violaxanthin is a natural xanthophyll pigment with an orange color found in various plants (Giossi et al. 2020). It is biosynthesized from zeaxanthin by an epoxidation reaction and has a 5,6-epoxy double group found in orange fruits, green vegetables, and microalgae (Melendez-Martinez et al. 2008). Furthermore, it plays a role in photoprotection mechanisms such as the ability of plants to adapt to contrasting light environments (Bowen-O'Connor et al. 2013). Wang et al. (2018) detected violaxanthin pigments at the wavelength spectrum of 417.6, 440.9, and 470.1 nm while Ruban et al. (2001) detection was at 470 nm. These pigments were also reported in mangroves with high salinity (Falqueto et al. 2008).

Neoxanthin pigments are carotenoids of xanthophylls groups. It acts as an intermediary for the biosynthesis of the hormone abscisic acid in plants (Perreau et al. 2020). Furthermore, it serves as a protection against photooxidative stress (Dall'Osto et al. 2007). Neoxanthin is the primary xanthophyll pigment found in green plants (Giossi et al. 2020). Chandrika et al. (2005) detected neoxanthin in the wavelength spectrum of 413, 436, 465 nm, and was detected by Gupta et al. (2015) at 415, 437, 465 nm. In mangroves, the neoxanthin pigment was found in mangroves *Avicennia alba* (Sasamoto et al. 2020).

The pigment detected in *R. mucronata* leaves extract predicted its probability to be active (Pa) value as an antioxidant. Bioactivity prediction was conducted using PASS analysis (Filimonov et al. 2014). The Pa value describes the potential activity of a compound. When the  $Pa > 0.7$ , it is estimated to have a high bioactivity potential, both for computational and laboratory assays. Meanwhile, when the value is  $0.3 \leq Pa \leq 0.7$ , then the compound has the computational ability as an antioxidant, but it has not been proven in the laboratory. Also, when the  $Pa < 0.3$ , it is predicted that the compound has a low bioactivity potential (Aisiah et al. 2020; Riyadi et al. 2021). However, the



bioactivity of chlorophyll a and b may not be predicted by PASS analysis because they have a metal element in their structure. The pigments beta-carotene, lutein, neoxanthin, pheophytin-a, and violaxanthin have their potential bioactivity as antioxidants, free radical scavengers, and NF-E2-related factor (Nrf-2) stimulant. Figure 3 showed the probability to be active as the antioxidant of the pigment in the *R. mucronata* leaves extract.

Figure 3 showed that beta-carotene, lutein, neoxanthin, and violaxanthin are predicted to have potential as general antioxidants. Neoxanthin and violaxanthin were also predicted to be free radical scavengers. Meanwhile, beta-carotene was predicted as an Nrf-2 stimulant, which regulates antioxidant protein as protection from oxidative damage (Ma 2013; Cui et al. 2016; Riyadi et al. 2019). Chlorophyll cannot be predicted by PASS analysis as an antioxidant, even though it has antioxidant properties as predicted by previous studies (İnanç 2011; Keleş et al. 2016; Sarker et al. 2018c; 2018d). Durga et al. (2015)

reported chlorophyll a and b from medicinal plants with high potential antioxidants. Pérez-gálvez et al. (2020) stated that chlorophyll b showed higher antioxidant activity than chlorophyll a.

Kurniawan et al. (2020) stated that lutein can act as an antioxidant and maintaining organs such as the eyes, brain, and skin. The content in marigold plant (*Tagetes* spp.) was reported to have a DPPH radical inhibition ability of 89.90% (Ingkasupart et al. 2015). Furthermore, it was reported to work as an antioxidant in the photo-stressed retina (Kamoshita et al. 2016)

Beta-carotene is also reported to be an antioxidant and an anti-carcinogen (Paolini et al. 2003). Berti et al. (2014) stated that it is effective as a radioprotective agent and acts as an antioxidant. Mueller and Boehm (2011) reported that beta-carotene and its derivatives showed antioxidant properties measured by the  $\alpha$ TEAC, chemiluminescence (CL), and ferric reducing activity (FRAP) methods.

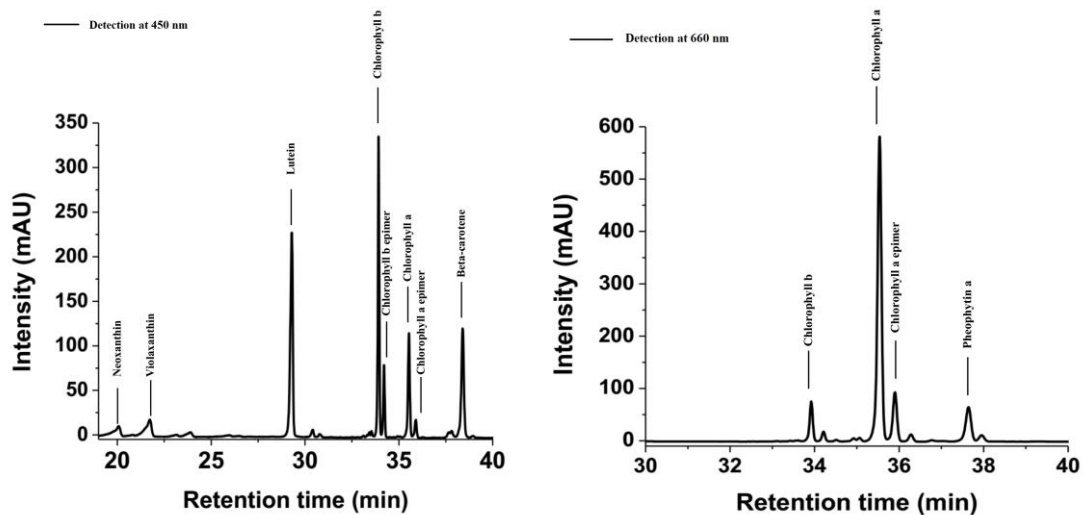


Figure 2. HPLC Chromatogram of *Rhizophora mucronata* leaves ethanol extract

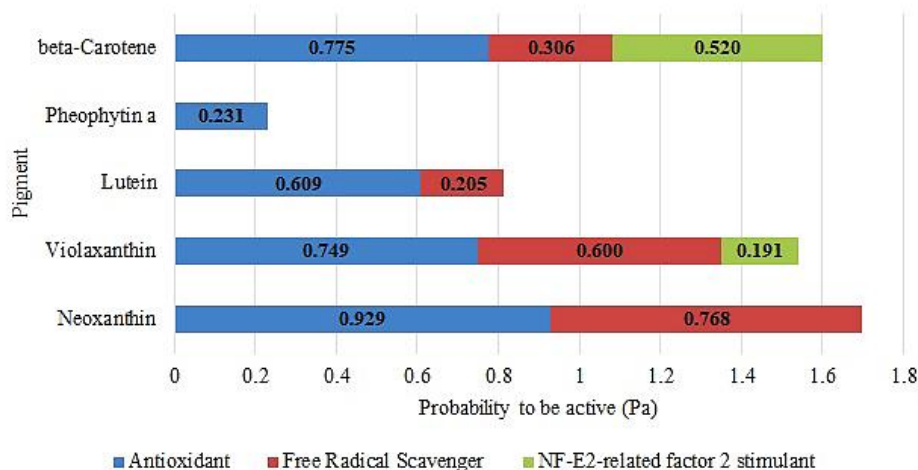


Figure 3. Probability to be active of pigments contain in *Rhizophora mucronata* leaves as an antioxidant with PASS analysis

Furthermore, Figure 3 showed the prediction of beta-carotene bioactivity as an Nrf-2 stimulant. The literature showed beta-carotene can activate the Nrf2-ARE (antioxidant response element) pathway to provide a neuroprotective effect from traumatic brain injury (Chen et al. 2019). Ben-dor et al. (2005) reported that beta-carotene stimulates Nrf-2 in the leukemia promyelocytic core body and regulates phase II enzyme expression (associated with cancer-preventing gene activation).

Dall'Osto et al. (2007) stated that neoxanthin acts as an antioxidant in the photosystem II supercomplex in plant thylakoid to protect membrane lipids photooxidation. In addition, Giossi et al. (2020) reported that neoxanthin was directly involved in photoprotection as an antioxidant to increase the activity of ROS scavenge under extreme light conditions. Sarker and Oba (2020g) reported that the neoxanthin contained in *Amaranthus tricolor* showed DPPH radical scavenging activity. Neoxanthin reduces oxidative-induced DNA base damage by less than 50%. In lower concentrations than lutein, it is a better inhibitor of oxidative-induced DNA damage (Şahin et al. 2020).

Dall'Osto et al. (2007) also reported the antioxidant properties of violaxanthin as photoprotection, even though it is lower than neoxanthin. The literature also reports the antioxidant properties of violaxanthin and its derivatives isolated from mangoes, with strong lipid peroxidation inhibition capabilities (Araki et al. 2016). Furthermore, violaxanthin isolated from microalgae *Eustigmatos cf. polyphem* was also reported to have radical scavenger capabilities with 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2-azobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical assays (Wang et al. 2018). However, Figure 2 showed that pheophytin has a low Pa value as an antioxidant, and it prevents oxidative DNA damage and lipid peroxidation. It works by reducing reactive oxygen species, such as DPPH, and by chelation of metal ions, such as Fe (II) (Hsu et al. 2013). Kusmita et al. (2015) also reported that pheophytin contained in green tea has an antioxidant capacity (DPPH method) with  $IC_{50} = 573 \pm 0.23$  mg/L.

The mechanism of antioxidant action of the pigments in *R. mucronata* leaves extract should be properly understood. Furthermore, the pigments in Table 1 were evaluated by STITCH. This is a tool for integrating information about the interactions of metabolic pathways, crystal structure, binding experiments, and relationships between chemicals (Kuhn et al. 2008). Figure 4 showed the interactions between the pigments.

Figure 4 showed the interaction between the working mechanism of the pigments in the *R. mucronata* leaves extract. Chlorophyll a, chlorophyll b, neoxanthin, and violaxanthin support the performance of lutein and beta-carotene. Furthermore, Beta-carotene pigments activate and catalyze the transcriptional regulation of the BCMO1 protein (beta, beta-Carotene 15,15'-monooxygenase-1), which is a key enzyme in vitamin A metabolism (Lietz et al. 2010). BCMO1 was reported to be expressed in intestinal tissue and plays a role in lipid metabolism (Lietz et al. 2012). It affects the RBP2 protein (Retinol-binding protein 2), which plays a role in the absorption and

metabolism of intracellular vitamin A (Blaner et al. 2020). Figure 4 showed that lutein affects the expression of BCO2 (Beta, beta-carotene 9', 10'-oxygenase) to catalyze beta carotene. In addition, Lietz et al. (2012) also reported that BCO2 contributes to the formation of vitamin A to improve antioxidant performance and immune function (Zhang et al. 2019).

Figure 4 also showed that lutein affects the PPARA gene (Peroxisome proliferator-activated receptor alpha), which strengthens antioxidant and anti-inflammatory defense systems (Moran et al. 2014) by increasing regulation of the enzyme expression and inhibiting the activity of NF- $\kappa$ B (nuclear factor kappa b) (Gao and Li 2012). PPARA activates BCMO1 to metabolize vitamin A. It also regulates the expression of SCARB1 (scavenger receptor class B type 1), which is an HDL (high-density lipoprotein) receptor protein that mediates carotenoid uptake (Hoekstra and Sorci-Thomas 2017). Soran et al. (2015) reported that HDL showed an antioxidant effect in protecting oxidative stress due to increased LDL (low-density lipoprotein). Figure 4 showed that lutein affects UCP1 (uncoupling protein 1) expression directly or through activation by PPARA. UCP1 gene reduces the production of ROS (reactive oxygen species) in mitochondria (Oelkrug et al. 2014) to prevent oxidative stress in adipose tissue (Shabalina et al. 2006).

Figure 4 showed beta-carotene affects BDNF (Brain-derived neurotrophic factor) expression and it is a gene in the brain that promotes the survival of nerve cells (neurons) by playing a role in the growth, maturation (differentiation), and maintenance of neuron cells. Wu et al. (2016) reported that BDNF increases superoxide dismutases and glutathione reductase expression. It also reduces the oxidative protein damage index (Lee et al. 2009), reduces antioxidant protein expression (Wu et al. 2012), restores reduced mitochondrial electron-coupling capacity, and increases mitochondrial uncoupling protein 2 (UCP2). It acts as an antioxidant by reducing the production of superoxide anions (Chan et al. 2010). Figure 4 also showed that beta-carotene and lutein inhibit the activation of the protein MMP9 (Matrix Metalloproteinase-9) through increased BDNF expression. Furthermore, the MMP9 gene regulates the tissue remodeling process by activating cytokines and chemokines, causing inflammation and fibrosis in the tissue (Yabluchanskiy et al. 2013). Figure 4 showed that beta-carotene activates FN1 (Fibronectin 1) action to inhibit the enzyme lipoxigenases (LOX) and MMP9 expression. LOX enzyme oxidizes fatty acids and causes inflammation in tissues (Ratnasari et al. 2017). Meanwhile, FN1 is activated through the Nrf-2 pathway (Prestigiacomo and Suter-Dick 2018) to bind LOX. Oxidation and inflammation in the tissue can be inhibited (Fogelgren et al. 2005). In summary, the pigments in the leaves of *R. mucronata* work synergistically by activating antioxidant enzymes and inhibiting the expression of oxidative stress proteins.

The extract of *R. mucronata* leaves was evaluated for its antioxidant capacity as a free radical scavenger using the DPPH method. Meanwhile, DPPH is a stable free radical which accepts electrons or hydrogen atoms to form

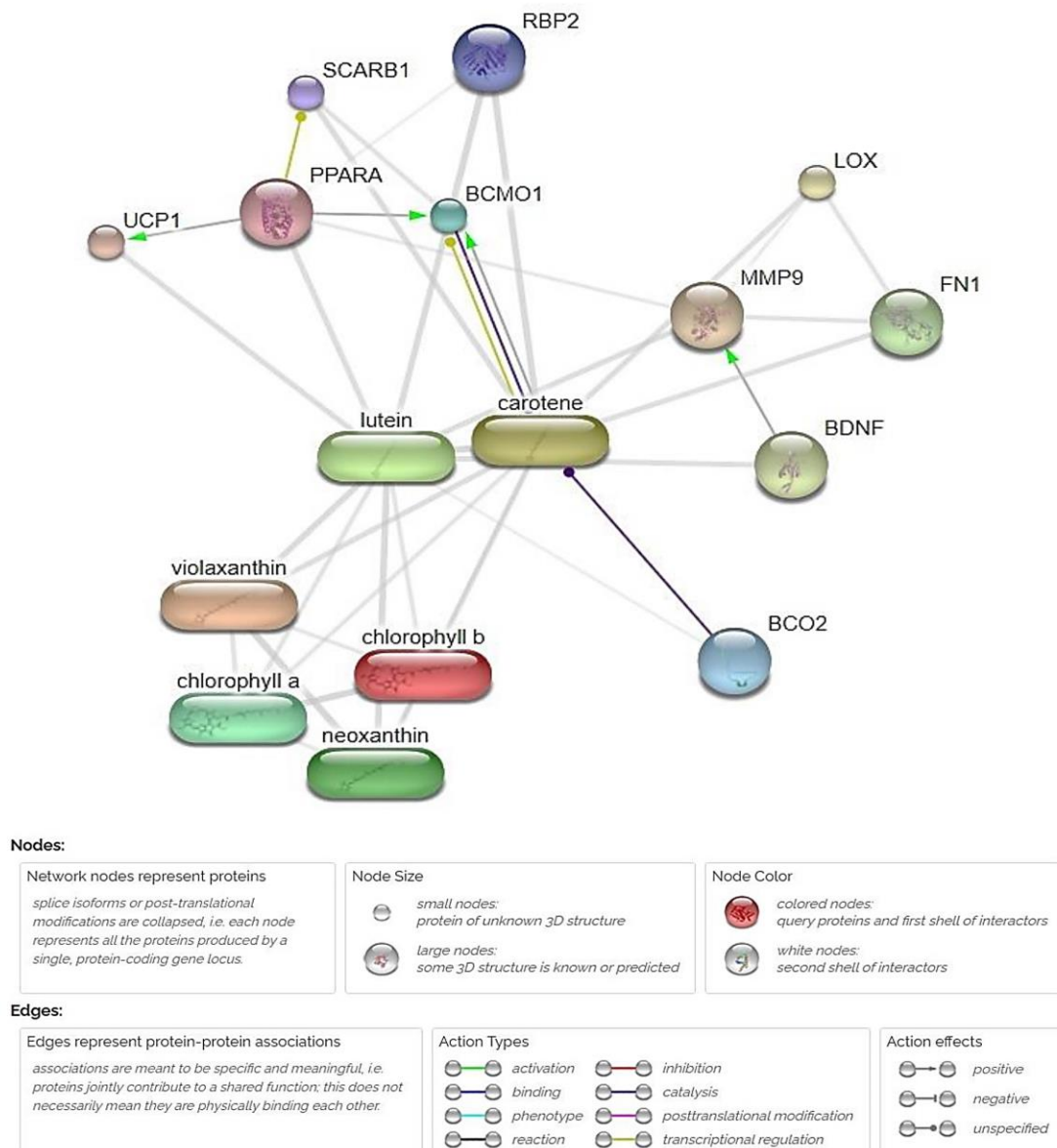
stable diamagnetic molecules (Tanod et al. 2019a). The antioxidant capacity was evaluated by calculating DPPH purple light intensity level proportional to the decrease in DPPH concentration. This reduction was caused by the reaction of the 2,2-diphenyl-1-picrylhydrazyl molecule with the hydrogen atoms released by the components of the sample molecule. It formed hydrazone diphenyl picril compound and caused DPPH to change color from purple to yellow (Tanod et al. 2019b). The reactivity of the *R. mucronata* leaves extract with stable free radicals was also evaluated, and the antioxidant capacity was compared with vitamin C (Figure 5). In addition, Vitamin C is commonly used to compare assaying antioxidant activity because it is cheaper and easier to obtain (Lung and Destiani 2014).

Figure 5 showed that the *R. mucronata* leaves extract has antioxidant activity because hydrogen atoms or electrons were donated to react with DPPH radicals.

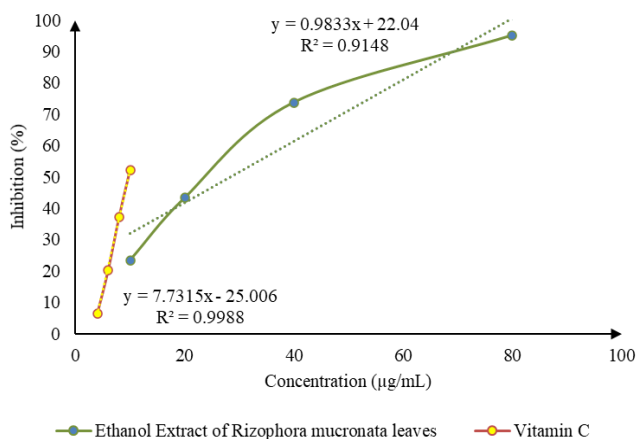
Increased concentration of the extract also increased the percentage of DPPH free radical inhibition. Table 2 showed that the inhibition percentage was evaluated for  $IC_{50}$  determination by probit analysis. According to the Blois (1958) category, antioxidant activity can be categorized as very strong ( $IC_{50} < 50 \mu\text{g/mL}$ ), strong ( $50 \leq IC_{50} \leq 100 \mu\text{g/mL}$ ), moderate ( $100 \leq IC_{50} \leq 150 \mu\text{g/mL}$ ), weak ( $150 \leq IC_{50} \leq 200 \mu\text{g/mL}$ ), and very weak ( $IC_{50} > 200 \mu\text{g/mL}$ ).

**Table 2.** The  $IC_{50}$  value of the *Rhizophora mucronata* leaves extract using the DPPH method was compared with vitamin C

Sample	$IC_{50}$ ( $\mu\text{g/mL}$ )
<i>R. mucronata</i> leaves	$20.99 \pm 0.33$
Vitamin C	$9.62 \pm 0.09$



**Figure 4.** Mechanism action as an antioxidant of pigments in *Rhizophora mucronata* leaves with STITCH analysis



**Figure 5.** DPPH radical inhibition from the *Rhizophora mucronata* leaves extract compared to vitamin C

Table 2 showed that the *R. mucronata* leaves extract and vitamin C had very strong antioxidant activity, with a DPPH concentration of 0.76 mM. Literature studies showed that the  $IC_{50}$  of the same sample can vary depending on the DPPH concentration, sample origin, conditions, and the solvent used (Dewanto et al. 2019). Furthermore, fractionation and crude extract of *R. mucronata* mature leaves from Penunggul, East Java, Indonesia showed  $IC_{50}$  from  $82.97 \pm 51.15$  to  $491.78 \pm 427.59$   $\mu\text{g/mL}$ , while the  $IC_{50}$  ascorbic acid (vitamin C) was  $12.36$   $\mu\text{g/mL}$ , with a DPPH concentration of 0.4 mM (Sasmito et al. 2016). The ethanol extract obtained from Sunderban, India, was reported to show DPPH radical scavenging ( $IC_{50}$ )  $6.65 \pm 0.10$   $\mu\text{g/mL}$ , with a DPPH concentration of 0.135 mM (Adhikari et al. 2016). Two compounds isolated from the methanol extract of the leaves from Vallarpadam, India, showed  $IC_{50}$  of 0.76-0.84 mg/mL, with DPPH 0.1 mM (Chakraborty and Raola 2017). Furthermore, the leaves extract from Tugurejo, Central Java, Indonesia, was reported to have  $IC_{50}$  of 113.41 ppm (methanol), 151.13 ppm (n-hexane), 184.78 ppm (ethyl acetate), with a DPPH concentration of 0.1 mM (Ridlo et al. 2017). The ethanol extract obtained from the coast of Palu Bay showed an  $IC_{50}$  of  $103.95 \pm 0.38$   $\mu\text{g/mL}$ , with a DPPH concentration of 50  $\mu\text{M}$  (Dewanto et al. 2018). Furthermore, stick balm preparations from methanol extract of *R. mucronata* showed  $IC_{50}$  of 57.40, 57.14, and 77.32 ppm, with a DPPH concentration of 0.004% (Faiqoh et al. 2020).

Literature studies reported varying  $IC_{50}$  values of vitamin C with different DPPH concentrations. The  $IC_{50}$  of vitamin C with a DPPH concentration of 0.1 mM, was 3.90  $\mu\text{g/mL}$  (Pratiwi et al. 2013) and 4.76  $\mu\text{g/mL}$  (Widyowati et al. 2014). Isnindar et al. (2016) reported an  $IC_{50}$  with a DPPH concentration of 0.4 mM to be 1.83  $\mu\text{g/mL}$ . Aliyu et al. (2017) reported an  $IC_{50}$  with a DPPH concentration of 0.2 mM to be 13.89  $\mu\text{g/mL}$ . Meanwhile, Johnson et al. (2017) reported an  $IC_{50}$  with a DPPH concentration of 0.004% (w/v) to be 37.50  $\mu\text{g/mL}$ . Najihudin et al. (2017)

reported an  $IC_{50}$  with a DPPH concentration of 0.01% (w/v) to be 4.71  $\mu\text{g/mL}$ . Dewanto et al. (2019) reported an  $IC_{50}$  of vitamin C with a DPPH concentration of 50  $\mu\text{M}$  to be  $31.35 \pm 0.81$   $\mu\text{g/mL}$ .

Mangroves are plants that live in tidal areas, and they experience variations in environmental pressure. In addition, variations in ecological pressure trigger reactive oxygen species (ROS) production and interfere with mangrove growth (Dasgupta et al. 2012). The pigments contained in mangroves produce antioxidant substances useful for the self-defense mechanism of ROS (Thatoi et al. 2014; Sarker and Oba 2018c; Dewanto et al. 2021). Therefore, it can be concluded that chlorophyll a, chlorophyll b, beta-carotene, lutein, neoxanthin, pheophytin a, and violaxanthin are the pigment profiles in *Rhizophora mucronata* mangrove leaves. All pigments detected have strong potential as antioxidants, especially as free radical scavengers and Nrf-2 stimulants. They interact with each other to inactivate antioxidant enzymes and inhibit the expression of oxidative stress proteins. This study showed that the pigment in *R. mucronata* leaves is a potential source of antioxidants for human health. Furthermore, it was developed by isolating the pigments from *Rhizophora mucronata* leaves and comparing the antioxidant power with standards for nutraceuticals and pharmaceuticals preparation.

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