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# Antioxidant Activity Identification of Crude Extract of *Halimeda* sp. From the Seribu Islands Using Different Testing Methods (DPPH, CUPRAC, and FRAP)

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**ABSTRACT**

The green seaweed from the genus *Halimeda* is a marine biological resource that holds promise as a source of bioactive compounds, such as antioxidant compounds. The antioxidant activity may differ based on the assay technique since each technique relies on a unique reaction mechanism. This research intended to assess the antioxidant properties of crude *Halimeda* sp. extract from the Seribu Islands employing three techniques: DPPH, CUPRAC, and FRAP. The sample was obtained through maceration with methanol and subsequently assessed for free radical scavenging activity and metal ion reduction capability. The findings indicated that the CUPRAC method yielded the greatest antioxidant activity, scoring 147.91 µmol Trolox/g extract, while the FRAP method followed with 83.61 µmol Trolox/g extract. The DPPH method generated an $IC_{50}$ value of 226.09 mg/L. These varying values suggest that the chemical properties of the compounds in the extract react differently to each testing principle. Consequently, employing several antioxidant assays is essential for a more thorough assessment of the antioxidant capacity of *Halimeda* sp. seaweed extract.

## INTRODUCTION

Indonesia, being an archipelagic country, has remarkably high marine biodiversity, which includes macroalgae or seaweed. Seaweed possesses economic significance as a food source and is also acknowledged for its potential bioactive compounds, such as antioxidants (Susanto *et al.,* 2017). Antioxidant substances are essential in counteracting free radicals, which are reactive entities that cause oxidative stress and several degenerative illnesses like cancer, diabetes, and cardiovascular conditions (López-Alarcón & Denicola, 2013). Exploration of natural antioxidant sources from the sea, such as seaweed, has become essential as a safer alternative compared to synthetic antioxidants.

The genus *Halimeda* sp. is a type of green algae (*Chlorophyta*) widely found in

Indonesian waters, including the Seribu Islands region. *Halimeda* sp. is known to contain various secondary metabolites such as flavonoids, phenolics, terpenoids, and tannins, which are strongly suspected to contribute to its antioxidant activity (Mulyani *et al.,* 2018). However, the antioxidant potential of *Halimeda* sp. from the Seribu Islands has not been comprehensively explored. The measurement of antioxidant activity in natural products is highly dependent on the analytical method used. Each method is based on different reaction mechanisms, thus potentially yielding inconsistent values for the same sample (Munteanu & Apetrei, 2021).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) technique is frequently utilized, relying on the hydrogen atom transfer (HAT) process. The FRAP (Ferric Reducing Antioxidant Power) technique assesses the capability to convert ferric ions ($Fe^{3+}$) into ferrous ($Fe^{2+}$), while the CUPRAC (Cupric Reducing Antioxidant Capacity) technique is based on the reduction of cupric ions ($Cu^{2+}$) to cuprous ($Cu^{+}$) (Apak *et al.,* 2016). These differences in fundamental principles cause each method to have selectivity toward specific antioxidant compounds. For instance, phenolic compounds that are acidic may respond differently to neutral pH-based methods (DPPH and CUPRAC) compared to acid pH-based methods (FRAP) (Thaipong *et al.,* 2006).

Several previous studies have reported comparisons of antioxidant testing methods on various types of seaweed (Wati *et al.,* 2020; Zeb, 2020), but specific studies simultaneously comparing the three methods (DPPH, CUPRAC, and FRAP) on *Halimeda* sp. extracts remain limited. Variations in results due to different testing methods can cause confusion in data interpretation and comparison of potential across studies. Based on this background, this study was conducted to examine and compare the antioxidant activity of *Halimeda* sp. seaweed extract from the Seribu Islands using three testing methods: DPPH, CUPRAC, and FRAP. This research is expected to determine the best testing method for antioxidant activity and provide information regarding the antioxidant activity content of *Halimeda* sp. seaweed from the Seribu Islands.

## METHODS

### Place and Time

The study was conducted in September 2025 at the Aquatic Productivity Laboratory. Antioxidant activity analyses were performed at the Marine Biotechnology Laboratory, Faculty of Fisheries and Marine Science, Jenderal Soedirman University, Indonesia.

### Tools and Materials

The instruments utilized in this research comprised analytical devices like erlenmeyer flasks, graduated cylinders, micropipettes, dropping pipettes, shakers, spectrophotometers, test tubes, analytical balances, vortex mixers, and various other analytical equipment. The main substance utilized in the research was *Halimeda* sp. seaweed gathered from Pramuka Island in the Thousand Islands (Kepulauan Seribu), along with the necessary chemical reagents for analysis.

### Sample Extraction

Samples of *Halimeda* sp. seaweed were collected from the waters surrounding the Seribu Islands. The gathered samples were purged of debris, sand, epiphytes, and substrate leftovers clinging to the thallus. The samples were then dried and crushed into powder to enhance the contact surface area between the material and the solvent. Extraction utilized the maceration technique with 99.9% ethanol as the solvent. The powder sample was immersed in the solvent at ambient temperature for a predetermined duration, then filtered to isolate

the liquid from the solid remains. The filtrate obtained was subsequently evaporated with a rotary vacuum evaporator to yield crude seaweed extract as a paste. The extract served as the sample for testing antioxidant activity through FRAP, CUPRAC, and DPPH methods. The extraction process adhered to the approach of Lee *et al.* (2017) with modifications tailored to the sample type and analytical needs.

**Antioxidant Activity Using the FRAP Method**

The FRAP assay was conducted to determine the extract's ability to reduce $Fe^{3+}$ ions to $Fe^{2+}$. The FRAP reagent was prepared from a mixture of acetate buffer, TPTZ solution, and $FeCl_3$. The extract solution was mixed with the FRAP reagent and then incubated until the reaction occurred. After incubation, the absorbance of the solution was measured using a spectrophotometer at a wavelength of 593 nm. Antioxidant activity values were calculated based on a Trolox standard curve and expressed as µmol Trolox/g extract. This method followed the principle developed by Benzie and Strain (1996) with modifications.

**Antioxidant Activity Using the CUPRAC Method**

The CUPRAC method was used to measure the extract's ability to reduce $Cu^{2+}$ ions to $Cu^{+}$. The assay was performed by mixing $CuCl_2$ solution, neocuproine, ammonium acetate buffer, and the sample solution. The reaction mixture was then incubated until a colored complex formed, and the absorbance was measured at a wavelength of 450 nm. Antioxidant activity was calculated based on a Trolox standard curve and expressed in units of µmol Trolox/g extract. This testing procedure followed Apak *et al. (*2008) with adjustments to the volume and concentration of the test solutions.

**Antioxidant Activity Using the DPPH Method**

The capability of the extract to neutralize DPPH free radicals was assessed using the DPPH assay. The extract solution was made at different concentrations, and each concentration was combined with DPPH solution. The blend was kept in the dark to avoid the breakdown of reagents caused by light. Once the incubation period ended, the absorbance was recorded at a wavelength of 517 nm. The percentage of inhibition was determined by examining the reduction in absorbance of the test solution relative to the control. The $IC_{50}$ value was derived from the regression equation relating extract concentration to inhibition percentage. A reduced $IC_{50}$ value signifies more potent antioxidant activity (Molyneux, 2004). The percentage of inhibition was determined using this formula:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \text{ x } 100\%$$

Once the inhibition percentage was determined for each concentration, the sample concentration and % inhibition was graphed into a linear regression equation y = ax + b. This equation was utilized to calculate the $IC_{50}$ value for every sample. The $IC_{50}$ value represents the concentration of the sample that can eliminate 50% of the original DPPH concentration. A lower $IC_{50}$ value signifies greater antioxidant activity. A compound is deemed to have very strong antioxidant activity when the $IC_{50}$ value is under 50 ppm, strong if the $IC_{50}$ value falls between 50-100 ppm, moderate if the $IC_{50}$ value is between 100-150 ppm, and weak if the $IC_{50}$ value is between 150-200 ppm.

## RESULTS

**Antioxidant Activity of *Halimeda* sp. Extract**

The crude extract of *Halimeda* sp. exhibited antioxidant activity when evaluated using the FRAP, CUPRAC, and DPPH assays. The antioxidant values obtained differed among the

three analytical methods. The CUPRAC assay produced the highest antioxidant capacity, with a value of 147.91 µmol Trolox/g extract, followed by the FRAP assay at 83.61 µmol Trolox/g extract. In contrast, the DPPH assay yielded an $IC_{50}$ value of 226.09 ppm (Table 1).

Table 1. Antioxidant Activity of *Halimeda* sp.

| Sample | FRAP (µmol troloks/g extract) | CUPRAC (µmol troloks/g extract) | $IC_{50}$ DPPH (ppm) |
|---|---|---|---|
| *Halimeda* sp. | 83.61 | 147.91 | 226.09 |
| *Halimeda gracilis*[*] | - | - | 290.49 – 375.50 |
| *Halimeda opuntia*[**] | 175.93 | 212.43 | 95.91 |
| *Halimeda macroloba*[***] | - | - | 259.34 (n-hexane) and 1567.27 (ethyl acetate) |

Notes: [*]Basir *et al.* (2017), [**]Nufus *et al.* (2017), [***]Muzaki *et al.* (2018)

Based on the $IC_{50}$ classification, the antioxidant activity of the crude extract against DPPH radicals was categorized as weak. The relationship between extract concentration and DPPH radical inhibition showed a linear trend (Figure 1). The regression equation obtained from the inhibition curve was used to determine the $IC_{50}$ value of the extract

**Absorbance Values of Crude Extract from *Halimeda* sp.**

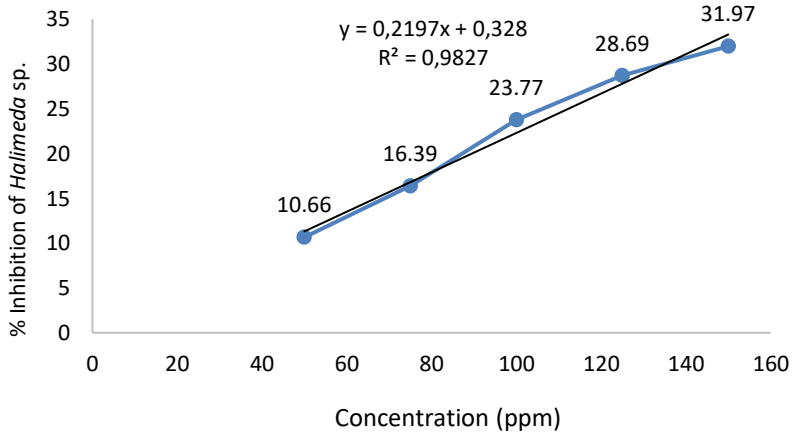


Figure 1. Absorbance Values of Crude Extract from *Halimeda* sp.

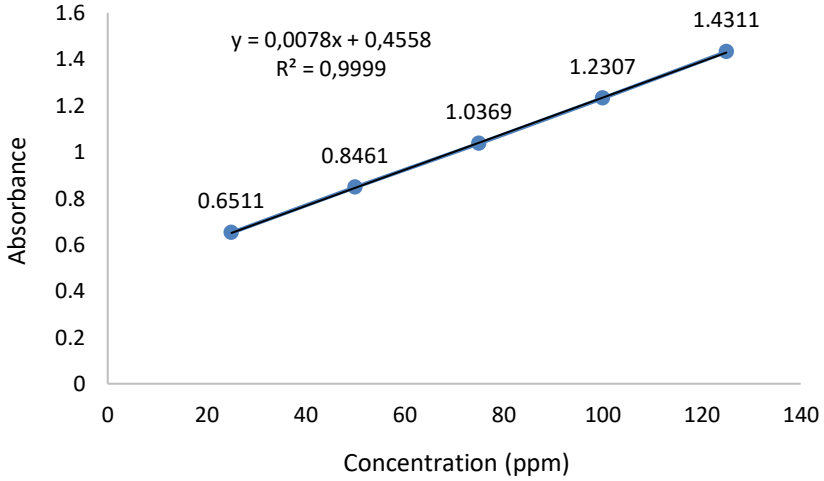


Figure 2. Absorbance Values of the Trolox Standard Solution

For the FRAP and CUPRAC assays, antioxidant activity was calculated using the Trolox standard calibration curve. The regression equation obtained from the Trolox standard showed good linearity (Figure 2), which was subsequently used to determine antioxidant capacity in µmol Trolox equivalents per gram of extract.

## DISCUSSION

The crude extract of *Halimeda* sp. seaweed showed antioxidant activity across all three methods used. The antioxidant activity values obtained differed between the FRAP, CUPRAC, and DPPH methods. This difference indicates that the compounds in the extract responded differently to the different testing mechanisms. The results showed that the CUPRAC value for crude *Halimeda* sp. extract was 147.91 µmol Trolox/g extract. This value was higher than the FRAP result, which reached 83.61 µmol Trolox/g extract. Meanwhile, the DPPH assay produced an $IC_{50}$ of 226.09 ppm (Table 1). Based on the classification of antioxidant activity using $IC_{50}$ values, extracts with $IC_{50}$ values greater than 200 ppm are categorized as having weak antioxidant activity. Thus, the crude *Halimeda* sp. extract in this study demonstrated relatively low DPPH radical scavenging ability.

These outcomes correspond with the conclusions of Nufus et al. (2017), who indicated that Halimeda opuntia extract exhibited notably distinct activity values, namely 175.93 µmol Trolox/g (FRAP), 212.43 µmol Trolox/g (CUPRAC), and an $IC_{50}$ of 95.91 ppm (DPPH). Basir *et al.* (2017) reported that *Halimeda gracilis* extract exhibited relatively weak antioxidant activity, as indicated by $IC_{50}$ values of 290.49 ppm (methanol) and 375.50 ppm (ethyl acetate). Research by Muzaki *et al.* (2018) further reinforced evidence that the polarity of the extraction solvent affects the $IC_{50}$ value, as seen in ethyl acetate and n-hexane extracts of *Halimeda macroloba*, which had values of 1567.27 ppm and 259.34 ppm, respectively.

The differences in values clearly indicate that the mechanisms behind each testing method significantly influence the final results (Prior *et al.,* 2005). The DPPH method, in principle, measures radical scavenging activity, with results expressed in $IC_{50}$ units. The relationship between these two variables is inverse, meaning that the smaller the $IC_{50}$ value obtained, the higher the antioxidant potential of the test sample. The determination of the $IC_{50}$ value was performed using a linear regression equation representing the correlation between the extract concentration series and the percentage inhibition of DPPH radicals (Figure 1). The $IC_{50}$ value of the crude *Halimeda* sp. extract, which reached 226.09 ppm, indicates very weak antioxidant activity. Molyneux (2004) states that a compound is regarded as a very potent antioxidant if the $IC_{50}$ value is under 50 ppm, strong if it falls between 50-100 ppm, moderate if it is between 100-150 ppm, and weak if it lies in the range of 150-200 ppm.

Unlike the DPPH method, which measures radical scavenging activity, the FRAP and CUPRAC methods measure the total reducing capacity of the test sample. The results of both methods are expressed in equivalent values; in principle, the larger the measured number, the higher the antioxidant activity of the sample. The standard solution used as a comparator in both methods is Trolox (vitamin E). The selection of Trolox was based on its role as a secondary antioxidant, which works through free radical scavenging mechanisms while inhibiting the propagation of chain reactions. The regression results of concentration (x) against absorbance values (y) of the Trolox standard solution yielded the equation $y = 0.0078x + 0.4561$ with a coefficient of determination ($R^2$) of 0.9999. The antioxidant activity values of the *Halimeda* sp. extract were then calculated by substituting the measured sample absorbance values into the regression equation (Figure 2).

The higher value obtained using the CUPRAC method compared to FRAP indicates that the antioxidant compounds in the crude *Halimeda* sp. extract possess excellent affinity and ability to reduce copper ions ($Cu^{2+}$) compared to iron ions ($Fe^{3+}$) (Apak *et al.,* 2008). This is consistent with the statement that the CUPRAC method has more universal sensitivity toward various classes of antioxidants, such as ascorbic acid, α-tocopherol, flavonoids, and other phenolic compounds, compared to iron reduction methods like FRAP (Özyürek *et al.,* 2011). Benzie and Strain (1996) stated that the FRAP method is commonly used to test the antioxidant capacity of plants, which in principle involves electron transfer reactions from antioxidants to $Fe^{3+}$-TPTZ compounds. The $Fe^{3+}$-TPTZ compound itself represents oxidizing compounds that may be present in the material.

The composition of compounds in the crude *Halimeda* sp. extract is suspected to be the main cause of this difference. Phenolic and flavonoid compounds, which have been reported to be abundant in green algae, are generally good electron donors (Perez *et al.,* 2016). Crude *Halimeda* sp. extract tends to be more easily detected by metal reduction-based assays (CUPRAC and FRAP) than radical scavenging assays like DPPH, especially if the DPPH radical has steric hindrance that inhibits interaction with certain antioxidant molecules (Prior *et al.,* 2005). The high CUPRAC value may also indicate the presence of specific antioxidant compounds, such as ascorbic acid or flavonoids with ortho-dihydroxy groups, which have highly efficient $Cu^{2+}$ reduction capabilities (Apak *et al.,* 2004). Thus, this study confirms that *Halimeda* sp. extract from the Seribu Islands is rich in antioxidant compounds with strong reduction mechanisms, and these results align with the findings of Magalhães *et al.* (2008) on other algae, which also showed variations in results between testing methods.

## CONCLUSION

The crude extract of *Halimeda* sp. seaweed from the Seribu Islands showed antioxidant activity through three testing methods: DPPH, CUPRAC, and FRAP. The CUPRAC method yielded the highest activity value of 147.91 µmol Trolox/g extract, while the FRAP method followed with 83.61 µmol Trolox/g extract. The DPPH method yielded an $IC_{50}$ value of 226.09 ppm, suggesting low free radical scavenging activity. The variations in outcomes among methods indicate that the antioxidant activity of *Halimeda* sp. extract is affected by the testing reaction principles employed. Thus, employing various methods at the same time is essential for acquiring a more complete understanding of antioxidant activity.

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