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COMPARISON OF ABTS (2,2-AZINOBIS(3-ETHYLBENZOTHIAZOLINE)-6-SULFONIC ACID) AND DPPH (1,1-DIPHENYL-2-PICRYLHYDRAZYL) ASSAYS TO MEASURE THE ANTIOXIDANT ACTIVITY OF ETANOL EXTRACT OF RED ALGA *(Gelidium sp)*

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Abstract

Seaweeds have an ecological function as primary producers in marine waters. It also has an important economic value as a producer of hydrocolloids (alginate, agar, and carrageenan) used in various food and pharmaceutical industries. This study aims to determine the antioxidant activity of red algae *(Gelidium sp).* Extraction was done using the maceration method using 96% ethanol and concentrated by rotary evaporation. The antioxidant activity of the extract was tested using ABTS and DPPH methods. Antioxidant activity was seen from the IC_{50}. The results showed that IC_{50} from ethanol extract of red algae *(Gelidium sp)* with the DPPH method is 3,9154 ppm. Then, using the ABTS method, IC_{50} from ethanol extract of red algae *(Gelidium sp)* is 9,1178 ppm. This result shows that the ethanol extract from red algae *(Gelidium sp)* has very strong antioxidant activity (<50 ppm).

Keywords: ABTS, antioxidant, DPPH, red algae

Introduction

Indonesia's coastline is approximately 99,093 kilometers long, and its waters cover an area of approximately 3.2 million square kilometers. This area is rich in biological and non-biological natural resources, including minerals.[1] Seaweed is one of the fishery commodities that can be used as one of the mainstays of the economy in Indonesia. Indonesia's strategic location and abundant seaweed production make it the second-largest producer after China.[2] The three main groups of seaweed are red algae (*Rhodophyta*), brown algae (*Phaeophyta*), and green algae (*Chlorophyta*), based on their classification.[3] Seaweeds are cultivated for nutraceutical or functional foods but are largely used to produce compounds and gelling components in the pharmaceuticals and food industries.[4] Various studies have shown that seaweed

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contains bioactive compounds such as carotenoids, phenol compounds, sulfate derivatives, polysaccharides, and vitamins. These compounds have benefits, namely as antioxidants that can prevent free radicals.[5,6]

Antioxidant activity methods can determine the characteristics of different antioxidants in a sample. Various methods can be used to measure the total characteristics of antioxidants,[7] however, there is no single method that is considered to be the most ideal. Different activity methods may result in different antioxidant mechanisms of action.[8] Some methods that can be used to analyze antioxidant activity include 2,2-Azinobis(3-Ethylbenzothiazoline)-6-Sulfonic Acid (ABTS) and 1,1-Diphenyl-2-Picrylhydrazyl (DPPH). Both methods use the same principle: antioxidant compounds' ability to reduce free radicals or free radical radical oxidizers. The difference is in the free radical compounds used, namely ABTS and DPPH.[9]

In connection with the content of active ingredient compounds contained in seaweed that are beneficial for health and beauty, an observation or review of the antioxidant activity of ethanol extracts of red algae (*Gelidium sp*) using the ABTS and DPPH methods was conducted. Thus, the objective of this study was to evaluate the comparison of antioxidant activity measurements obtained by ABTS and DPPH assays from the ethanol extract of *Gelidium sp*. Analyses of seaweeds as samples are currently rare. Therefore, information regarding comparative testing of different antioxidant capacities is not available for various seaweeds commonly used in Indonesia.

Methods

Tools

UV-Vis spectrophotometer (Shimadzu®), analytical balance (Acis®), hot plate (IKA®), micropipette, pipette, filter paper, beaker glass (Pyrex®), measuring cup (Pyrex®), beaker (Pyrex®), rotary evaporator (Buchi®) and blender (Philips®).

Materials

The materials used in this study include *Gelidium sp* red algae samples from Pameungpeuk, West Java, Indonesia, ethanol 96% (Brataco®), methanol p.a (Brataco®), ethanol p.a (Brataco®), DPPH (Sigma Aldrich®), ABTS (Sigma Aldrich®), potassium persulfate (Emsure®), aquadest.

Experimental Procedure

Sample Preparation

Red algae (*Gelidium sp*) samples were sorted, washed, and dried. Red seaweed is traditionally cleaned, dried under the sun, and collected at night. The procedure is repeated for seven cycles each day, totaling seven days. The dried samples were pulverized using a blender and then stored in a dry, tightly closed place.

Preparation of Ethanol Extract of *Gelidium sp*

A 480 g sample was macerated using 96% ethanol for 3x24 hours with occasional stirring and then filtered. The filters were obtained and thus concentrated using a rotary evaporator to get a thick extract. The extract obtained was computed as a percentage yield using the equation:

$$\% \text{ Rendement} = \frac{weight\ extract}{Ssimplicia\ weight} \text{ x } 100\%$$

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Antioxidant Activity Assay by ABTS Method
Preparation of ABTS 7 mM Stock Solution

The procedure follows the previous report with minor modifications.[10] 20 mg of ABTS was dissolved with 5 mL of distilled water and then incubated at room temperature for 12-18 hours. Then, 3.5 mg of potassium persulfate was dissolved with 5 mL of distilled water and incubated at room temperature for 12-18 hours. Then, the ABTS and potassium persulfate solutions were mixed and diluted with 25 mL of ethanol p.a in a glass volumetric flask.

Preparation of 1000 ppm Vitamin C Stock Solution

50 mg of vitamin C was weighed and then dissolved to 25 mL with ethanol p.a in a glass volumetric flask.

Preparation of 1000 ppm *Gelidium sp* Ethanol Extract Stock Solution

50 mg of *Gelidium sp* ethanol extract was weighed and then dissolved with 50 mL of ethanol p.a in a glass volumetric flask.

Blank Absorbance Measurement

3 mL of ABTS stock solution was taken, and the wavelength was read with a UV-VIS spectrophotometer at 400-800 nm wavelength. The absorbance was then measured so that the maximum wavelength was obtained.

ABTS Binding Activity Assay with Vitamin C

This procedure was carried out by pipetting 20, 40, 60, 80, and 100 mL of vitamin C stock solution and then filling each of them up to the volume of a 10 mL volumetric flask with ethanol p.a so that a solution with a concentration of 2, 4, 6, 8, and 10 ppm was obtained. From the solution, 1 mL of solution was taken, and 2 mL of ABTS stock solution was added. The solution was incubated for 15 minutes, and the absorbance of the sample was measured using a UV-VIS spectrophotometer at a maximum wavelength of 737 nm.

Analysis of ABTS Binding Activity with *Gelidium sp* Extracts

Sampling was done by pipetting 20, 40, 60, 80, and 100 mL of the *Gelidium sp* ethanol extract stock solution and then filling each to a volume of 10 mL in a volumetric flask with ethanol p.a so that a solution with a concentration of 2, 4, 6, 8, and 10 ppm was obtained. From the solution, 1 mL of solution was taken, and 2 mL of ABTS stock solution was added. The solution was incubated for 15 minutes, and the absorbance of the sample was measured using a UV-VIS spectrophotometer at a maximum wavelength of 737 nm.

Antioxidant Activity Assay with DPPH Method
Preparation of 50 ppm DPPH Stock Solution

Antioxidant testing with the DPPH method as done in previous studies.[11] 5 mg of DPPH was weighed and then dissolved with 100 mL of methanol p.a in a glass volumetric flask.

Preparation of 1000 ppm Vitamin C Stock Solution

We weighed 50 mg of vitamin C and then dissolved it with 50 mL of methanol p.a in a glass volumetric flask.

Preparation of 1000 ppm *Gelidium sp* Extract Stock Solution

50 mg of ethanol extract of *Gelidium sp* was weighed and then dissolved with 50 mL of methanol p.a in a glass volumetric flask.

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Blank Absorbance Measurement

3 mL of DPPH stock solution was taken, then wavelength scanning was carried out with a UV-VIS spectrophotometer at a wavelength of 400-800 nm. The absorbance was measured using a UV-VIS spectrophotometer at the maximum wavelength obtained.

DPPH Binding Activity Assay with Vitamin C

The measurements were carried out by pipetting 20, 30, 40, 50, and 60 µL respectively, from the vitamin C stock solution and then filling each up to a volume of 10 mL volumetric flask with methanol p.a so that a solution with a concentration of 2, 3, 4, 5, and 6 ppm was obtained. 1 mL of solution was taken from the solution, and 2 mL of DPPH stock solution was added. The solution was incubated for 30 minutes, and the absorbance of the sample was then measured using a UV-VIS spectrophotometer at a maximum wavelength of 516 nm.

Analysis of DPPH Binding Activity with *Gelidium sp* Extracts

The evaluation was carried out by pipetting 20, 40, 60, 80, 100, and 120 mL, respectively, from the extract stock solution and sufficed each up to a volume of 10 mL volumetric flask using methanol p.a to obtain a solution with a concentration of 2, 4, 6, 8, 10, and 12 ppm. 1 mL of solution was taken from the solution, and 2 mL of DPPH stock solution was added. The solution was incubated for 30 minutes, and the absorbance of the sample was measured using a UV-VIS spectrophotometer at a maximum wavelength of 516 nm.

Data Analysis

Antioxidant activity can be known by obtaining % inhibition data and $IC_{50,}$ which states the concentration that can reduce free radicals by 50%. The use of the calculation below can determine the percent inhibition value:

$$\% \text{ inhibition} = \frac{Blanko\ Absorbance - Sample\ Absorbance}{Blanko\ Absorbance} \times 100\%$$

Result

Table 1. Rendement

Simplicia Weight (g)	Extract Weight (g)	Rendement (%)
3.1535	3.1531	0.8298

Figure 1. Red algae (A) wet (B) after seven days of drying under sunlight.

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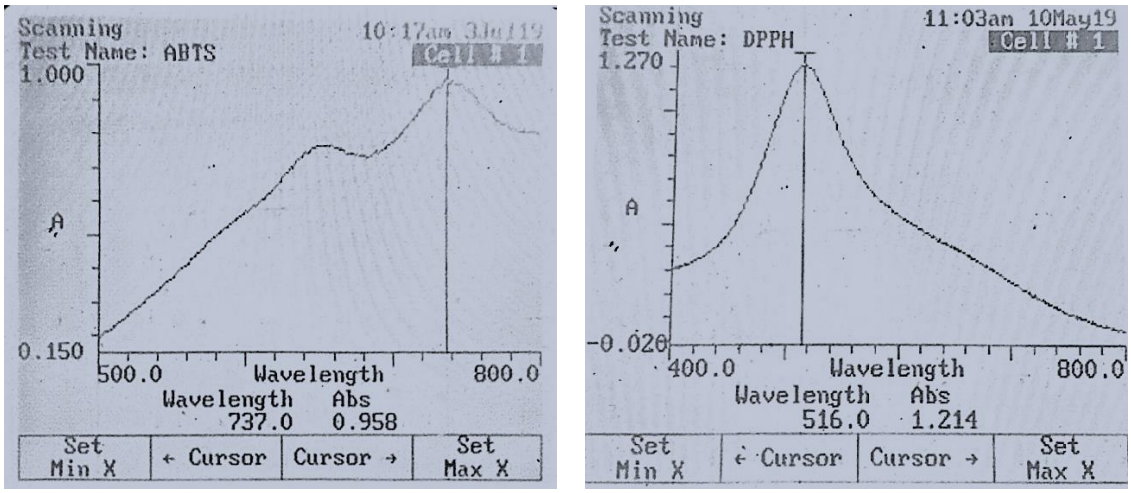

(A) (B)

Figure 2. Scanning results of ABTS (A) and DPPH (B) wavelengths

Table 2. Antioxidant Assay Results Using the ABTS Method

Sample Solution	C (ppm)	Absorbance (nm)		% Inhibition	IC_{50} (ppm)
		$\bar{x}$	$\bar{x} \pm SD$		
Vitamin C	2	0.7230	0.7230±0.0008	28.8386	5.6396
	4	0.6120	0.6120±0.0008	39.7638	
	6	0.4870	0.4870±0.0008	52.0669	
	8	0.3607	0.3607±0.0005	64.5013	
	10	0.2493	0.2493±0.0005	75.4593	
***Gelidium sp* ethanol extract**	2	0.5853	0.5853±0.0009	38.9005	9.1178
	4	0.5510	0.5510±0.0008	42.4843	
	6	0.5237	0.5237±0.0005	45.3375	
	8	0.4947	0.4947±0.0017	48.3646	
	10	0.4677	0.4677±0.0005	51.1830	

Table 3. Antioxidant Assay Results Using the DPPH Method

Sample Solution	C (ppm)	Absorbance (nm)		% Inhibition	IC_{50} (ppm)
		$\bar{x}$	$\bar{x} \pm SD$		
Vitamin C	2	0.6557	0.6557±0.0012	45.9912	2.4451
	3	0.5557	0.5557±0.0005	54.3932	
	4	0.4257	0.4257±0.0012	64.6897	
	5	0.3257	0.3257±0.0005	73.2565	
	6	0.2257	0.2257±0.0005	81.4113	
***Gelidium sp* ethanol extract**	2	0.6977	0.6977±0.0012	42.5316	3.9154
	4	0.6020	0.6020±0.0024	50.4119	

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Table 3. (Extension)

Sample Solution	C (ppm)	Absorbance (nm)		% Inhibition	IC_{50} (ppm)
		$\bar{x}$	$\bar{x}$ ± SD		
	6	0.5130	0.5130±0.0008	57.743	
	8	0.4080	0.4080±0.0008	66.3921	
	10	0.3020	0.3020±0.0008	75.1236	
	12	0.2133	0.2133±0.0005	82.4272	

Discussion

The result of the extraction obtained was that the yield of ethanol extract of *Gelidium sp* amounted to 0.8298%. This result is smaller than the previous report, which amounted to 0.9%. The extraction yield depends on the solubility of the bioactive components.[12]

The antioxidant activity of *Gelidium sp* ethanol extract by ABTS and DPPH methods using vitamin C as a positive control obtained wavelengths of 737 and 516 nm, respectively. This comparison of antioxidant tests is expected to determine a reliable way of reading results and formulate practical guidelines for selecting and confirming the assay uniformity and comparison of results. Multiple evaluations rely on the same methodology, evaluating the sample's capacity to absorb radicals or decrease redox-active substances observed by a spectrophotometer. Furthermore, two categories of tests exist. One strategy uses electron transfer to reduce colored oxidants, such as those used in DPPH and ABTS assays. This study reports that the ethanol extract of *Gelidium sp* has an IC_{50} value of 9.1178 ppm from the results with the ABTS method, while the results with the DPPH method are 3.9154 ppm. Both results were compared with vitamin C (IC_{50} of 5.6396 ppm by ABTS method and IC_{50} of 2.4451 ppm by DPPH method). Although the antioxidant activity of both methods is still low compared to vitamin C, it is still included in the category of very strong antioxidants (IC_{50}<50 ppm). This finding is in line with the previous report, where the ethanol extract of *Gelidium sp* has very strong antioxidant activity with an IC_{50} value of 22.15±1.63.[13] This represents the antiradical activity that can reduce 50% of free radical activity by an antioxidant agent.[14] According to the specifications, the examination for DPPH is constructed on the reduction of purple DPPH to 1,1-diphenyl-2-picryl hydrazine. In contrast, the ABTS assay is based on creating blue or green $ABTS^{-+}$ that antioxidants can reduce. Both assay methods are widely used, but their use of non-physiological radicals is limited.[15]

Conclusion

Overall, the results of this study indicate that the antioxidant activity test with the DPPH method produces higher antioxidant activity than the ABTS method on *Gelidium sp* ethanol extract, where the IC_{50} value of the DPPH method test results is lower than the ABTS method.

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