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Effect of Heating Coarse Extract of Brown Macroalgae (*Padina australis*) from Tial Waters, Salahutu District, Central Maluku Regency on Antioxidant Activity

Nurani Hasanela*, Fensia Analda Souhoka

Department of Chemistry, Faculty Mathematics and Natural Sciences, Pattimura University, Jl. Ir. M. Putuhena, Ambon, 97233, Indonesia

*Corresponding Author: hasanela.nurani2@gmail.com

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Abstract

Physical and chemical factors influence antioxidant activity. One of the physical factors that can affect antioxidant activity is heat. This study aims to determine the phytochemical content and the effect of heating the crude extract of marine macroalgae *Padina australis* on antioxidant activity. The research method used, namely maceration using methanol as a solvent to obtain a crude extract, then evaporation of the solvent at a temperature of 45 °C. Furthermore, phytochemical tests were carried out, characterization using TLC and UV-Vis spectrophotometer, and determination of free radical scavenging activity of DPPH. The results of the phytochemical test showed that the crude extract of *Padina australis* was positive for bioactive compounds, namely saponins, alkaloids, terpenoids, steroids, and flavonoids. Characterization by TLC and UV-Vis spectrophotometer showed the presence of secondary metabolites, namely chlorophyll a and carotenoids. The use of temperature in the solvent evaporation process affects the stability of bioactive compounds and secondary metabolites. The results of the antioxidant activity test (IC_{50}) of the crude extract of *Padina australis* against DPPH obtained 163 ppm, so it is classified as an antioxidant in the medium category.

Keywords: Antioxidant activity, biopigments, crude extracts, free radicals, Padina australis

INTRODUCTION

Free radicals are atoms, molecules, or compounds with unpaired electrons, so they are reactive and easy to react (Winarti, 2010). Several free radicals such as hydroxyl radicals (OH*), alkoxyl radicals (RO*), superoxide anions (O_2*), nitric oxide (NO*), peroxy nitrite (OONO*), and hydrogen peroxide (H_2O_2) in the body are formed naturally. Continuously through normal cell metabolic processes (Sayuti & Yenrina, 2015). Free radicals outside the body come from gamma, ultraviolet (UV) radiation, environmental pollution, and cigarette smoke (Wijaya, 1996). Diseases that the influence of free radicals can cause are cancer, heart disease, Alzheimer's, Parkinson's, arthritis, and immune system disorders (Seyoum, Asres, & El-Fiky, 2006).

Antioxidants are components that can fight or reduce a compound's oxidation rate (Wanasundara & Shahidi, 2005). Antioxidants can work effectively because they can donate an electron to pair with free radicals to eliminate the reactive nature of radicals. Antioxidants that form new radicals have lower reactivity. This is because antioxidants have a structure with a conjugated system, which can delocalize free electrons in its structure. These new radicals can then be neutralized with other antioxidants or mechanisms to restore them to their original form (Lü, Lin, Yao, & Chen, 2010). One of the antioxidant compounds that can be produced is from marine macroalgae.

Indonesia has vast waters and is rich in biodiversity, one of which is macroalgae. Macroalgae or algae are aquatic organisms with high economic value and play an ecological role as producers and food chains. Macroalgae have many benefits that can be used in industry, medicine, food, and energy sources. Macroalgae or marine algae (seaweed) produce chemical compounds from primary metabolites known as hydrocolloids. Several sources of hydrocolloid are known to come from marine algae, which are used as industrial raw materials such as raw materials for making agar, carrageenan, and alginate.

Macroalgae generally consist of 3 divisions, namely *Rhodophyta* (red algae), *Chlorophyta* (green algae), and *Phaeophyta* (brown algae) (Hoek, 1998). *Phaeophyta* is autotrophic organisms because they can carry out photosynthesis. One of the macroalgae

of the *Phaeophyta* division (brown algae), abundant in Tial waters, Salahutu District, Central Maluku, is *Padina australis*. *Padina australis* macroalgae have the potential to be used as antioxidants, biogas, and bioethanol in addition to being used as food products. This is because *Padina australis* contains many organic chemicals and secondary metabolites. Secondary metabolite compounds contained in *Padina australis* can be isolated using an organic solvent called crude extract.

The potential activity of *Padina australis* as an antioxidant can be attributed to its ability to interact with free radicals. Antioxidant activity is strongly influenced by bioactive compounds in marine macroalgae *Padina australis*. Physical and chemical factors strongly influence bioactive compounds found in marine macroalgae. Heat is one of the physical factors that affect bioactive compounds' stability.

METHODOLOGY

Materials and Instrumentals

The materials used were marine macroalgae *Padina australis*, distilled water, filter paper, chemicals with p.a. quality, namely methanol, hydrochloric acid, Mayer reagent, $FeCl_3$, anhydrous acetic acid, concentrated sulfuric acid, Mg powder, ethyl acetate, 1,1-diphenyl-2-picryhydrazil (DPPH), and quercetin.

The tools used in this study, namely a set of glassware (pyrex), analytical balance (Denver Instrument XP-3000), blender (Miyako BL-152F), shaker (GFL), rotary evaporator (rotavapor R-215 Buchii), hot plate (Cimarec 2), TLC plate, capillary tube, and UV-Vis spectrophotometer 20^+ series.

Methods

Sampling

The macroalgae Padina australis was taken from Tial Waters, Salahutu District, Central Maluku Regency. The part of the sample taken is the thallus. Samples were fresh and brought to the Laboratory of Basic Chemistry, University of Pattimura.

Sample Extraction

The marine macroalgae of *Padina australis* were cleaned of impurities such as sand, stone, and other types of macroalgae. The macroalgae were washed with running water until clean and then weighed. The sample extract was made by the maceration method using methanol as solvent. The cleaned macroalgae sample was put into an Erlenmeyer, and 150 mL of methanol was added until the sample was submerged. Then the sample was shaken for 24 hours at room temperature. The extraction results were filtered, then the filtrate was evaporated using a rotary evaporator at 45 °C. The extracts obtained were then used for phytochemical tests.

Phytochemical Test

Saponin test

As much as 2 mL of the extract was put into a test tube, and then 2 mL of distilled water was added and shaken until homogeneous. Then, the extract is heated for 2-3 minutes. Then cooled and shaken vigorously. Test positive when foaming.

Alkaloid test

Macroalgae sample extract as much as 2 mL of the extract was put into a test tube, and then 5 mL of 2 M HCl and three drops of Mayer's reagent were added. The formation of a yellow or red-brown precipitate on the tube indicates the presence of alkaloids.

Phenolic Test

As much as 2 mL of the extract was put into a test tube, and two drops of 1% $FeCl_3$ were added and then shaken. The test is positive if it produces a deep blue color.

Steroid test

As much as 2 mL of the extract was put into a test tube, and then 10 drops of anhydrous acetic acid and 3 drops of concentrated sulfuric acid were added. The test is positive if it produces a blue or green color.

Flavonoid test

As much as 2 mL of the extract was put into a test tube and added a little Mg powder and 1 mL of 1% HCl. The test is positive if it produces foam and orange color.

Characterization of the crude extract of *Padina australis* by Thin Layer Chromatography (TLC)

TLC characterization was carried out to determine the amount and type of secondary metabolism in the form of photosynthetic pigments that had been successfully extracted. A 25 L of crude macroalgae extract was spotted on a 2×10 cm silica plate. The eluent in the form of ethyl acetate was prepared in a vessel, and then the TLC plate was eluted in a TLC vessel.

Characterization of bio pigments with UV-Vis spectrophotometer

Chlorophyll and carotenoid compounds contained in the photosynthetic pigment fraction were characterized using a UV-Vis spectrophotometer. The crude extract of the sample was placed on a UV-Vis spectrophotometer, and an absorption scan was performed with a wavelength from 380-800 nm.

Preparation of 40 ppm DPPH solution

A total of 0.01 g of DPPH was put into a 250 mL volumetric flask, and then methanol was added to the mark. The solution is used immediately, kept at a low temperature, and protected from light.

Determination of the maximum wavelength of DPPH

Using a methanol blank, a 5 mL of 40 ppm DPPH solution was observed for absorption in the wavelength range of 400-600 nm.

Determination of DPPH free radical scavenging activity

A total of 0.1 g of extract was made into a solution of 1000 ppm, and then diluted to concentrations of 5; 10; 15; 20; 25; 50; and 100 ppm. A total of 1 mL was taken from each sample solution that had been made, put into a test tube and added to 1 mL of 40 ppm DPPH solution, then allowed to stand for 30 minutes at room temperature. Absorbance measurements were carried out at the maximum wavelength (514 nm) using a UV-Vis spectrophotometer. The test was carried out with two measures. The same procedure was also performed on standard quercetin. The percentage of inhibition or inhibition of the DPPH radical from each concentration of the sample solution can be calculated using the formula (Equation 1).

$$I = \frac{A0-A1}{A0} \times 100\% \quad (1)$$

I: Percentage of Inhibition/Inhibitor (%), A0: Absorbance Blank (solvent + DPPH), A1: Sample Absorbance (solvent+DPPH+sample). After obtaining the percentage of inhibition from each concentration, the antioxidant activity was determined using the line equation of % inhibition as the Y axis and the sample concentration as the X axis (µg/mL). Antioxidant activity expressed by IC_{50} is calculated by entering the value of 50 into the line equation as Y and then calculating the value of X as the concentration of IC_{50}.

RESULTS AND DISCUSSION

***Padina australis* Marine Macroalgae**

Marine macroalgae *Padina australis* is a brown alga that lives in the waters of Tial Village, Salahutu District, Central Maluku Regency. The characteristics of the marine macroalgae *Padina australis* are elephant ear-shaped and pale brown in color. *Padina australis* lives attached to rocks at a depth of about 10-30 cm. The morphology of the marine macroalgae *Padina australis* is shown in Figure 1.

Figure 1. *Padina australis* Marine Macroalgae

Sample Extraction

The marine macroalgae *Padina australis* was cleaned of impurities so as not to be contaminated during the extraction process. Then 150 g were weighed and put into a 500 mL Erlenmeyer. The extraction process is carried out by maceration. The organic solvent used is methanol. Furthermore, the sample was shaken for 24 hours at room temperature to extract the marine macroalgae perfectly.

Organic solvents have different abilities in dissolving bioactive compounds. To dissolve bioactive compounds, methanol can dissolve polar compounds such as alkaloids, flavonoids, saponins, and carbohydrates (Kuppusamy, Yusoff, Parine, & Govindan, 2015). The stability of bioactive compounds is strongly influenced by physical and chemical factors such as temperature, pH, oxygen, and storage time (Hasanela, Gaspersz, Silaban, & Sohilait, 2020). The extraction of bioactive compounds was successfully characterized by the rupture of *Padina australis* cells resulting in a dark green extract with a distinctive marine odor and is referred to as crude extract.

The resulting crude extract is then evaporated with an evaporator. In this study, the temperature used is 45 °C (Rohmat, Ibrahim, & Riyadi, 2014). This is intended to determine the effect of heat on

bioactive compounds and the ability of antioxidant activity contained in marine macroalgae *Padina australis*. The crude extract of marine macroalgae *Padina australis* before and after the evaporator showed differences in the color of blackish green and fresh green. The evaporation process with heat causes the degradation of bio pigment compounds and bioactive compounds. The crude extract of the marine macroalga *Padina australis* is shown in Figure 2.

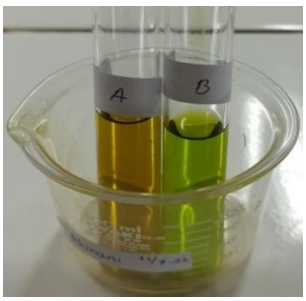

Figure 2. Crude extract of marine macroalgae *Padina australis* (a) Crude extract after evaporation with heat (b) Fresh crude extract without heating

***Padina australis* Phytochemical Test**

The screening was carried out to identify bioactive compounds from marine macroalgae *Padina australis*, which act as secondary metabolites in the antioxidant process by phytochemical tests. Phytochemical testing of marine macroalgae *Padina australis* using crude extract. The results of the phytochemical test of marine macroalgae *Padina australis* showed that there were bioactive compounds with positive effects, namely saponins, alkaloids, steroids, terpenoids, flavonoids and negative results for phenolics. The results of the phytochemical test of marine macroalgae *Padina australis* are shown in Figure 3.

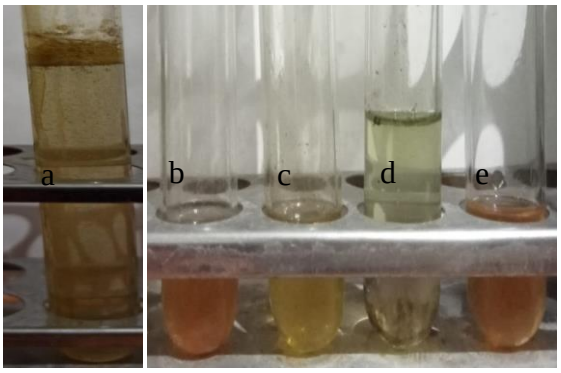

Figure 3. Color changes that occur in the phytochemical test (a) saponin test, (b) alkaloid test, (c) phenolic test, (d) steroid test, (e) flavonoid

The saponin test was characterized by the formation of foam after shaking. This is because saponins have active functional groups (polar and nonpolar) that can form micelles (Fajriaty, Ih, Andres, & Setyaningrum, 2018; Robinson, 1995). The foam appearing in the saponin test also shows the presence of glycosides that can create foam in water so that it is hydrolyzed into glucose and other compounds (Marliana, Suryanti, & Suyono, 2005).

A change indicated positive results from the alkaloid test in color to reddish, but no precipitate was formed. This is because the alkaloids in plant tissues are found to be around only 1% (Kristanti, Aminah, Tanjung, & Kurniadi, 2008). Alkaloids in marine macroalgae *Padina australis* showed positive results but were present in small amounts.

Steroid and terpenoid tests showed positive results, indicated by the formation of brownish and blue-green rings. This test is based on the ability of the compound to form a color with concentrated H_2SO_4 in an anhydrous acetic acid solvent (Ergina, Nuryanti, & Pursitasari, 2017; Septiadi, Pringgenies, & Radjasa, 2013).

A color change indicates a positive test for flavonoids to slightly reddish. Flavonoids have various types and are aglycones (free form) or bound as glycosides. Polymethoxy and polyhydroxy aglycones are nonpolar and semipolar, while flavonoid glycosides are polar because they contain several hydroxy groups and sugars solvent (Ergina, Nuryanti, & Pursitasari, 2017; Septiadi, Pringgenies, & Radjasa, 2013). This causes the flavonoid group to be extracted by methanol. The results of phytochemical testing of marine macroalgae *Padina australis* are presented in Table 1.

Table 1. Phytochemical test of marine macroalgae *Padina autralis*

Phytochemical Test	Positive Results according to the Library	Test Result		
		Discoloration	+	-
Saponin	Stable foam	Stable foam	√	-
Alkaloid	A red precipitate is formed	Reddish orange	√	-
Fenol	Green/dark green	Yellow	-	√
Steroid	Green/blue	Green	√	-
Terpenoid	Red/purple	Purplish red	√	-
Flavonoid	Dark red, yellow/orange	Red	√	-

Characterization of *Padina australis* Coarse Extract by TLC

Thin Layer Chromatography (TLC) can be used as a preliminary test to determine the antioxidant potential of the marine macroalgae *Padina australis*. TLC characterization chose the amount and type of bio pigment contained in the crude extract of marine macroalgae *Padina australis* (Figure 4). The bio pigments were eluted on a TLC plate composed of a silica matrix using ethyl acetate as the eluent. Based on the qualitative test with the TLC plate, it was seen that there were two pale and thin bands identified, namely green color suspected of chlorophyll and brownish yellow color suspected of carotenoids. The presence of carotenoid pigments causes the marine macroalga *Padina australis* to brown (Kailola, 2012).

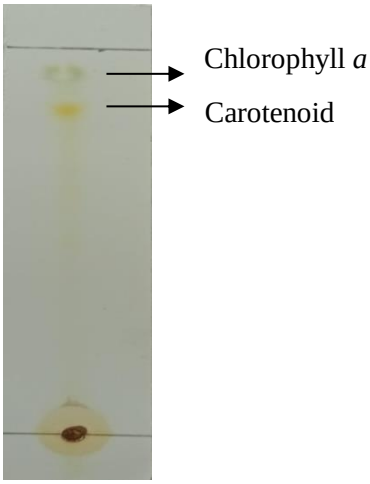

Figure 4. Chromatogram of crude extract of marine macroalgae *Padina australis*

Biopigments such as chlorophyll and carotenoids are secondary metabolite bioactive compounds that act as antioxidants. The antioxidant activity shown by the crude extract of marine macroalgae *Padina australis* is not only given by a mixture of bio pigments but also compounds soluble in methanol solvents. The antioxidant activity of biopigments is due to many conjugated double bonds. Heating in the evaporation process at a temperature of 45 °C makes the chromatogram on TLC pale and thin. This is because bio pigments such as chlorophyll and carotenoids are very sensitive to temperature, sunlight, and oxygen, so they will be easily degraded into their derived molecules (Arrohmah, 2007).

Characterization of *Padina australis* Coarse Extract with UV-Vis

UV-Vis spectrophotometric analysis was conducted to determine the bio pigment spectrum contained in the crude extract of marine macroalgae *Padina australis*. Biopigments generally have chromophore groups that absorb energy at a reasonably high wavelength (380-800 nm). This allows for an electronic transition to a higher orbital level. The number of heteroatoms and conjugated double bonds in the bio pigment structure will determine the characteristic absorption area so that it can be used as a quantitative analysis of bio pigments (Rodriguez-Amaya & Kimura, 2004).

The absorption pattern showed the presence of bio pigments, namely chlorophyll a, which was identified at a typical sharp absorption of 430 and 665.5 nm, while carotenoid compounds were identified at 480 nm. Analysis with UV-Vis spectrophotometry can answer the initial test using TLC. To determine the content and type of bio pigment from marine macroalgae *Padina australis*, purification can be performed, and then the purification results can be re-tested with UV-Vis spectrophotometry. The effects of the absorption spectrum of the crude marine macroalgae extract *Padina australis* are shown in Figure 5.

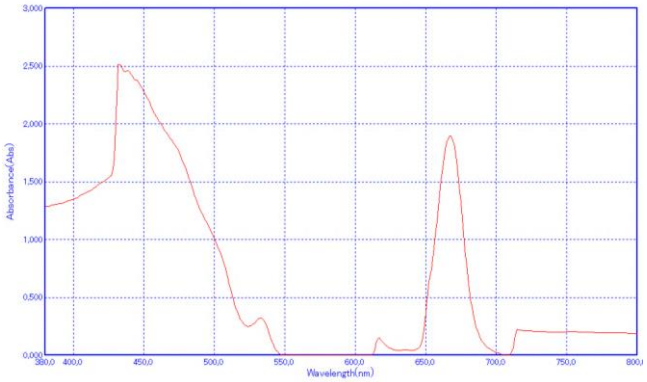

Figure 5. Spectrum of crude extract of marine macroalgae *Padina australis*

Determination of Maximum Wavelength DPPH

The antioxidant activity was determined using the electron transfer method with DPPH as a free radical. The purpose of using DPPH is because the measurement is simple, fast, and does not require a lot of reagents (Sayuti & Yenrina, 2015). Determination of the maximum wavelength of DPPH is carried out to obtain the absorbance value with the highest measurement sensitivity.

The maximum absorbance value of DPPH was achieved at a wavelength of 514 nm. The maximum absorbance of DPPH is between the wavelength of 510-520 nm (Miliauskas, Venskutonis, & van Beek, 2004). Furthermore, the maximum wavelength of DPPH was used to determine antioxidant activity.

The standard curve for the DPPH wavelength is shown in Figure 6.

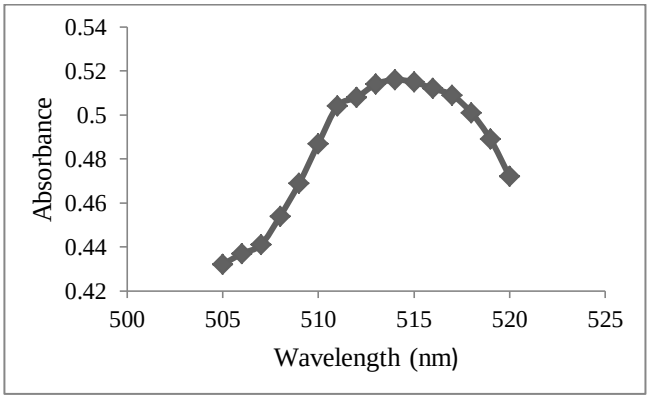

Figure 6. DPPH wavelength

Determination of DPPH Free Radical Antidote Activity

Determination of the free radical scavenging activity of DPPH was expressed by the IC_{50} value using a control, quercetin. The concentration of the standard quercetin solution was 2.5; 5; 7.5; and 10 ppm. The results of the quercetin standard curve are obtained y = 5.576x + 42.67 with a linear regression value of R^2 = 0.958. Based on this data, the IC_{50} value for the quercetin standard was 1.31 ppm. Quercetin (2-(3,4-dihydroyphenyl)-3,5,7-trihydroxy 4 Hchromen-4-one) is a flavonoid of the flavonol group with a keto group at C-4 and has a hydroxyl group at C-3 or C-atoms 5, which are neighbors of flavones and flavonols.

Flavonoid compounds have potential as antioxidants because they have a hydroxyl group bound to the carbon of the aromatic ring to ward off free radicals (Dewi, Puspawati, Swantara, Asih, & Rita, 2014). The reaction mechanism between antioxidants and DPPH radicals depends on the structural conformation of antioxidants (Wanasundara & Shahidi, 2005). The advantage of DPPH is that it can react with weak antioxidants (Aruna, Rigelhof, & Miller, 2001) and can be used to test hydrophilic or lipophilic antioxidants (Prior, Wu, & Schaich, 2005). The standard curve of DPPH free radical scavenging activity with quercetin is shown in Figure 7. The antioxidant activity produced in the crude extract of marine macroalgae *Padina australis* is not only obtained from secondary metabolites of bio pigments but also from the solvent extraction process in producing bioactive compounds. Antioxidant activity was determined by calculating the difference in the decrease in the absorbance value of the extract mixture with DPPH. The results showed that the higher the concentration of the extract, the higher the level of inhibition.

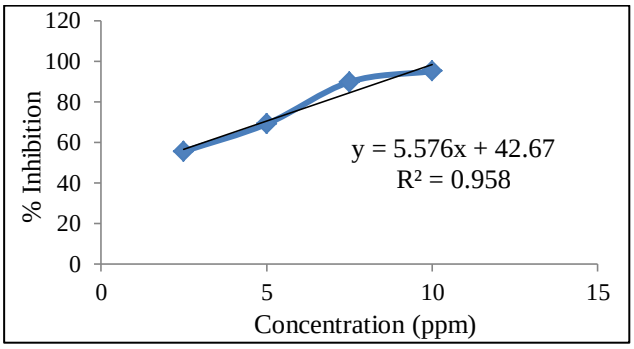

Figure 7. Standard curve of free radical scavenging activity with quercetin

This is because the activity of antioxidant compounds is improving at inhibiting free radicals (Rohmat et al., 2014). Based on the calculation results, the free radical scavenging activity of DPPH crude extract of marine macroalgae *Padina australis* with the regression equation y = 0.231x + 12.25 has an IC_{50} value of 163 ppm and is classified as a moderate antioxidant.

The antioxidant ability of the crude extract of marine macroalgae *Padina australis* decreased due to the influence of the heating factor during solvent evaporation. According to Husni (Husni, Putra, & Lelana, 2014), marine macroalga *Padina australis* in fresh conditions has an IC_{50} value of 37.68 ppm and is classified as a potent antioxidant. The state of the marine macroalgae extracts that undergoes a heating process will affect the ability of antioxidant activity. This is because secondary metabolites such as bio pigments and bioactive compounds are very sensitive to high temperatures. The antioxidant activity of marine macroalgae *Padina australis* is shown in Figure 8.

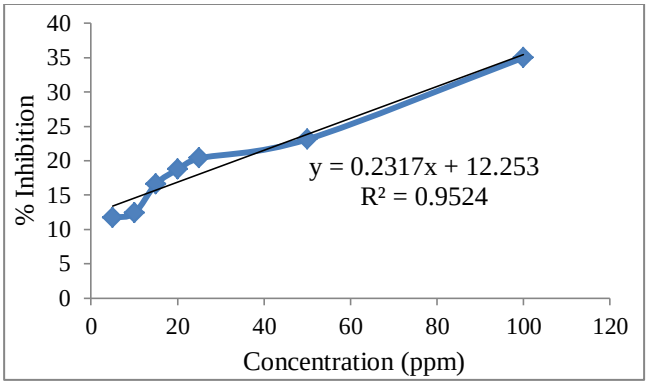

Figure 8. Antioxidant Aktivity of marine macroalgae *Padina australis*

The antioxidant activity of the crude extract is estimated to be relatively low due to the low concentration of components that contain antioxidant activity. In addition, natural extracts may also include components that can interfere with the electron transfer reaction between antioxidants and free radicals, such as weak acid groups. The effect of the solvent on the extraction process can also affect the antioxidant activity. The methanol solvent used can only dissolve polar compounds and cannot dissolve non-polar and semi-polar compounds. The best antioxidant activity was achieved in the extraction process using ethyl acetate solvent, while the n-hexane and methanol extracts were classified as weak antioxidants (Hidayati, Yudiati, Pringgenies, Arifin, & OktaviyantI, 2019).

CONCLUSION

Phytochemical tests on marine macroalgae *Padina australis* showed positive results for saponins, alkaloids, terpenoids, steroids, and flavonoids and a negative result for phenolics. The use of temperature in the solvent evaporation process affects the stability of bioactive compounds and secondary metabolites. The results of the antioxidant activity test on marine macroalgae *Padina australis* were in the medium category with an IC_{50} value of 163 ppm.

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