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Phytochemicals And Antioxidant Of Methanol Extract Of Gracilaria Salicornia, Halimeda Gracilis, Halimeda Macroloba, And Hypnea Asperi From Tidung Island Coastal Region

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Abstract

The research aimed to find out the content of active compound of simplicia and the ability of methanol extract of Gracilaria salicornia, Halimeda gracilis, Halimeda macroloba, and Hypnea asperi as the antioxidant. Phytochemical testing of macro algae simplicia was conducted qualitatively to determine the presence of the active compound, i.e. phenolic, flavonoids, tanin, saponin, steroid, triterpenoid, alkaloid, and hidroquinon. The test method of antioxidant activity is the capture of free radical 1,1-Diphenyl-2-Picrylhidrazyl (DPPH). The active compounds which belongs to macro algae that are tested are flavonoids, steroids, and saponins. The results of examination of antioxidant activity showed the highest percentage of free radical capture of DPPH methanol extract of G. salicornia was 7,228%. The result of univariate ANOVA in antioxidant activity, indicating that there was an effect of concentration on free radical capture of DPPH ($p \leq 0,05$) and did not show the interaction between species of macro algae and concentration. The highest percentage of interception DPPH free radical of methanol extracts of each macro algae tested i.e. G. salicornia, H. asperi, H. gracilis, and H. Macroloba respectively

Keywords: Antioxidant, Macro algae, Methanol extract, Phytochemicals.

1. INTRODUCTION

Worldwide interest in finding new and safe antioxidants from natural sources such as to minimize oxidative damage to living cells (Shanab et al, 2012) or can counteract free radical-induced and other oxidative stress processes, and in so doing decrease the incidence of human diseases (Vijayavel & Martinez, 2010). Marine is one source of active ingredients that are valuable in nutrition and pharmaceuticals, such as antioxidants. Macro algae is one of marine organisms which is considered to have a source of active ingredients. Many research were reported that macro algae are rich in various phytochemicals and antioxidant which has potential in human health. Content of phytochemicals in macro algae and their antioxidants activity are sometime interrelated.

Macro algae produce bioactive compounds, including polyphenols, alkaloids, terpenes, phycocyanins, carotenoids, chlorophylls, flavonoid, phenolic compounds, (terpenes, flavonoids), etc (Maharana et al, 2015; Osuna-Ruiz et al, 2016). Activity of antioxidant from

methanol and diethyl ether extract of eight edible species macro algae at North Borneo Malaysia have reported by Matanjun et al (2008). Activity of antioxidant of 30 species of Hawaiian marine algae reported by Kelman et al (2012). Research in bioactive compounds present in ethanolic extracts from 18 macroalgae of the Portuguese coast reported by Andrade et al (2013). Antioxidant activities and phenolic contents of three red seaweeds (Division: Rhodophyta) harvested from the Gulf of Mannar of Peninsular India reported by Chakraborty et al (2015). Antioxidant screening and phenolic content of ethanol extracts of 17 macro algae at Baja California Peninsula reported by Tenorio-Rodriguez et al (2017).

In Indonesia, some research about phytochemicals and antioxidant activity from macro algae were reported by Lantah et al (2017) about phytochemical content and antioxidant activity of methanol extract of seaweed *Kappaphycus alvarezii*. Extracted Phytochemical screening and antioxidant activity from ethanol extract of *Eucheuma spinosum* reported by Sari et al (2015). Exploration of Bioactive Compound to Brown Algae *Sargassum* sp. as antioxidant from West of Aceh Coastal reported by Gazali et al (2018).

Tidung is one of the island in the Seribu islands, which is located in the north of Java Island, Indonesia with geographical position east longitude 106º47'83'' – 106º51'15'' and south latitude 5º79'33'' – 5º80'25''. Tidung Island has beautiful sea shore with high diversity of species at its coastal region, making Tidung Island become one of marine tourism area. Handayani and Widowati (2016) reported at Tidung Island coastal region, there were 21 species of macro algae, i.e. Chlorophyta 48% (10 species); Phaeophyta 28% (6 species); and Rhodophyta 24% (5 species). Although there are many species of macro algae that exist, not many people have explored their use in various fields of health.

Widowati et al (2019) had find out the ability of methanol extract of *G. salicornia, H. gracilis, H. macroloba*, and *H. asperi* as antibacterial in inhibiting *E. coli* (ATCC 8739), *S. aureus* (ATCC 6539), and *P. aeruginosa* (ATCC 9027). To continues the research, the aimed of the research were to find out the content of active compound of 4 macro algae simplicia and the ability of methanol extract of 4 macro algae as antioxidant. The macro algae are *Gracilaria salicornia, Halimeda gracilis, Halimeda macroloba, Hypnea asperi* which were collected from Tidung Island coastal region.

2. RESEARCH METHODS

A. Study area

The research area were phytochemical of some macro algae and antioxidant activity of macro algae methanol extract. The research was done at IPB Biopharmaceutical Study Center, Bogor.

3. MATERIALS RESEARCH

1. Tools

Micropippette 100-1000 μL, 200 μL pipette tips, 1 mL pipette tips, 1.5 mL microcentrifuge tubes, burner, alumunium foil, stir bar, Biosafety Cabinet (BSC), funnel, exicator, Beaker, hot plate, measuring flask 5,0 mL, analytical balance, autoclave, and oven.

2. Materials

Macro algae (*G. salicornia, H. gracilis, H. macroloba, H. asperi)*; Methanol, Distilled Water; Vitamin C; 1,1-Diphenyl-2-Picrylhidrazyl (DPPH); Ethanol p.a. Aquadest, HCl, ethyl

alkohol, amyl alkohol, $FeCl_3$, diethyl ether, H_2SO_4 2 M, CH_3COOH anhydrous, NH_3, $CHCl_3$, Dragendorf's reagent, Mayer reagent, Wagner reagent, 10% NaOH, parafilm.

Procedures

1. Preparation of G. salicornia, H. gracilis, H. macroloba, and H. asperi Simplicia

Simplicia was done by quick drying in low temperature. There were seven stages in the preparation of simplicia, namely wet sorting, chopping, drying, dry sorting, packing and storage, and quality inspection (Prasetya and Inoriah, 2013). The preparation of simplicia was done according Widowati et al. (2019).

2. Phytochemical Screening of G. salicornia, H. gracilis, H. macroloba, and H. asperi simplicia

Phytochemical screening of macro algae simplicia was conducted qualitatively to determine the presence of the active compound. Phytochemical screening of macro algae simplicia refers to Harbone (1998). The color intensity or the precipitate formation was used as analytical responses to these tests.

2.1 Phenolic

Phenolic tests (flavonoids, tannins, and saponins), simplicia were treated equally until they become filtrate, before further testing according to the type of each phenolic compound. The same treatment shall be administered in the following ways; 5 g simplicia of macro algae were executed into the beaker glass, then 20 mL of aquadest were added into it. The mixture were heated, and filtered. The filtrate of the filtration were tested further according to the type of each phenolic compound then performed in the following ways:

2.1.1 Flavonoids

The filtrate was added Mg, HCl : ethyl alkohol = (1: 1), and amyl alkohol. The filtrate was positive containing flavonoids, characterized by the color of amyl alcohol layer was orange.

2.1.2 Tanins

The filtrate was added 3 drops of $FeCl_3$. The filtrate was positive containing tannin, characterized by the solution was greenish black.

2.1.3 Saponins

The filtrate was shaken strongly to form a foam. The filtrate was positive containing saponins, characterized by a stable foam was formed.

2.2 Steroids and triterpenoids

One gram simplicia of macro algae was mixed with 10 mL hot ethyl alcohol, and then filtered. The filtrate were heated to dry. The dried filtrate were added 1 mL of diethyl ether, then homogenized. After the mixture was homogeneous, 1 drop of concentrated H_2SO_4 and CH_3COOH anhydrous was added. Positive results containing triterpenoid, characterized by red or purple color was formed. Positive result containing steroids, characterized by a green or blue color was formed.

2.3 Alkaloids

One g of macro algae and a few drops of NH_3 were mixed. The mixture was added 5 mL of $CHCl_3$, then filtered. The filtrate was added H_2SO_4 2 M and shaken regularly, then set aside to form 3 layers. The first layer was added by Dragendorf's reagent. Positive result contains

an alkaloid, it will form an orange precipitate. The first layer added Mayer reagent. A positive result contains alkaloids, will form white precipitate. The first layer added Wagner reagent. A positive result contains an alkaloid, it would form a brown precipitate.

2.4 Hidrokuinons

One g of macro algae simplicia was added methanol, then heated and filtered. The filtrate of the filtration was then added with 3 drops of 10% NaOH. A positive result contains hydroquinone, it would form a red color.

3. Preparation of G. salicornia, H. gracilis, H. macroloba and H. asperi Methanol Extract

The method used in this research for the making of macro algae methanol extract was maseration method at room temperature which refers to Senja et al (2014). The making of methanol extract was carried out in the following ways; the ready-mixed macro algae simplicia was inserted and immersed in a maserator using 80% methanol at room temperature with a simplicia ratio: solvent, i.e 0.5 kg: 1 L methanol. The simplicia were soaked for 15 hours and for further dissolve of active substances contained in the macro algae simplicia. It was shuffled using shaker for 3 X 24 hours. The maseration results were filtered by filter paper, then the maserate was transferred into an impermeable bottle. The filtered sediment was immersed in 1 L of methanol at room temperature for 15 hours, then re-filter. The remaining sediments are soaked again in the same way. The maserate of the overall immersion result were adjoined. The maserate was evaporated in a waterbath at 40 °C. The evaporated maserate was then pressured by evaporating the solvent using a vaccum rotary evaporator at low pressure in 70°C to obtain a viscous extract. Furthermore, the condensed extract was carried out freeze drying to obtain dry extract.

4. The Examination of Antioxidant Activity of G. salicornia, H. gracilis, H. macroloba, and H. asperi Methanol Extract

The antioxidant test in this research used the capture free radical of DPPH microplate method referring to Salazar-Aranda et al (2011). The antioxidant test of methanol extract *G. salicornia*, *H. gracilis*, *H. macroloba*, and *H. asperi* were done as follows: the samples of macro algae extract (100 ppm, 200 ppm, 300 ppm, 500 ppm, and 700 ppm) and vitamin C (20 ppm, 10 ppm, 5 ppm, 2.5 ppm, and 1.25 ppm) as much 100 µL were inserted into well in microplate. The samples of macro algae extract and vitamin C replication twice and the second well were added 100 µL DPPH. For blank controls only ethanol 100 µL was added. The mixture were incubated at room temperature under dark conditions for 30 minutes. The blanks in replication 1 and 2 contain only 100 µL ethanol and 100 µL DPPH were added, whereas for negative control, it contains only 200 µL ethanol. After it was being incubated, then measured using a dynex microplate reader at 517 nm wavelength. The absorbation was recorded and calculated of the percentage of DPPH free radical capture. Antioxidant activity was calculated based on DPPH radical uptake resistance by calculating percentage of DPPH free radical capture, i.e. % free radical capture DPPH = [(absorbance of blank solution - absorbance of sample solution of macro algae methanol extract) / absorbance of blank solution)] X100%.

Data Analysis

Explanation of the analysis model applied to interpretation data, especially was analysis using statistical models. The selection of data analysis in this research refers to Seltman (2015). The

data of antioxidant activity test of vitamin C and methanol extract of *G. salicornia*, *H. gracilis*, *H. macroloba*, and *H. asperi* on DPPH free radical capture were done by determining IC50 value using probit test using IBM SPSS Statistics 22 software. The result of antioxidant activity test which showed the percentage of DPPH free radical capture more than 50%, then tested ANOVA univariate statistic using IBM SPSS Statistics 22 software and the results showed significant difference then continued test (Post Hoc). If the results show a significant difference, the Tukey test was followed.

4. RESULTS AND DISCUSSION

1. Phytochemical Test

The result of phytochemical examination showed that simplicia of *G. salicornia, H. gracilis, H. macroloba,* and *H. asperi* have active compound. The active compounds contained in the macro algae simplicia which were tested in this research are flavonoids, saponins, and steroids. The content of active compounds in macro algae simplicia is not the same in each type. This is shown in table 1 below:

Table 1. The Result of Phytochemical Test of Macro Algae Simplicia

No	Active compunds	Simplicia of Macro Algae			
		G. salicornia	*H. asperi*	*H. gracilis*	*H. macroloba*
1	Flavonoid	+	–	–	+
2	Alkaloid	–	–	–	–
3	Tannin	–	–	–	–
4	Saponin	+	+	+	–
5	Hydroquinone	–	–	–	–
6	Steroid	+	–	+	+
7	Triterpenoid	–	–	–	–

Description: + = there are active compounds, – = there is no active compounds

The flavonoid content is belonged to two macro algae, which are *H. macroloba* and *G. salicornia*. Flavonoids are a class of phenolic compounds that are rows of C6-C3-C6 compounds (a carbon skeleton comprising two C6 (benzene rings) connected by a three-carbon aliphatic chain), consisting of one aromatic ring A, one aromatic ring B, and the middle ring heterocyclic containing oxygen (Redha, 2010; Silvikasari, 2011).The role of flavonoids in the body are various, such as, it can increase antioxidant activity, promote normal cell and tissue development, and renew the entire inner part of the body. Flavonoids have been shown to be anti-inflammatory, allergic, antiviral, anti-aging, and anticancer. The improvement of antioxidant activity by flavonoids contributes to the treatment of cardiovascular disease by inhibition of cyclooxygenase activity, platelet lipooksigenase, and macrophages (Kumar, 2014).

Another content which found in the simplicia of macro algae-phytochemicals-tested is saponins. Saponin is belonged to three macro algae, which are, *H. asperi, H. gracilis,* and *G. salicornia*. Saponins is a fine froth used in intracellular staining by histochemistry, in order to make the access of antibodies in intracellular proteins acceptable (Santhi and Sengottuvel, 2016). Saponins, such as soaps or detergents (natural surfactants) in the macro algae are glycosides composed of sugars binding to aglycons (sapogenins), which are compounds having structures comprising triterpenoid or steroid chains and are non-polar (Fahrunnida and

Pratiwi, 2015). Several studies mentioned that saponins have antibacterial activity (Rosyidah et al, 2010). In addition, saponins also have antitussive and expectorant effects that can be used as cough medicine, and antiinflammatory characteristics to cure edema (Fahrunnida and Pratiwi, 2015).

The other ingredients which found in the macro algae simplicia that are tested for phytochemicals are steroids. Steroids are owned by the three macro algae simplicia, namely *H. gracilis, H. macroloba,* and *G. salicornia*. Steroids are include in class of triterpenoid compounds, containing a cyclopentane perhidrofenantren nucleus (Harborne, 1998). Steroids play an important role in the medical and pharmaceutical field, some of the roles are as inhibitors of ovulation (estrogen - the sex hormone steroids), as a preventative of miscarriage and pregnancy test (synthetic progestin), as antiinflammatory, antiallergic, antipyretic, and antihypertensive drugs (glucocorticoid), and as a diuretic and heart-reinforcing drug (cardiac glycoside steroids) (Pramana and Saleh, 2013).

The results of this research, shows differences with the research that was conducted by Govindasamy et al (2011), *H. macroloba* extract contains more active compounds, namely alkaloids, flavonoids, saponins, steroids, terpenoids, and tannins. Another research which is conducted by Paramsivam et al. (2016), showed that *G. salicornia* extract contained tannins, falvonoid, phenol, steroids. The difference of the content of active compounds in macro algae in this research and other research may be caused by the type of material used in this research is in the form of simplicia.

2. The Antioxidant Activity Test

The results of antioxidant examination of DPPH method showed that methanol extract of *G. salicornia*, *H. gracilis*, *H. macroloba*, and *H. asperi* have different antioxidant activity from each other. The antioxidant activity of methanol extract of *H. gracilis* and *G. salicornia* on the percentage of DPPH free radical capture increased along with the determined of increasing concentration. The highest of methanol extract antioxidant activity of *H. gracilis* and *G. salicornia* at 700 ppm are 6.590% and 7.288% respectively. The percentage of DPPH free radical capture from each macro algae methanol extract will be shown in table 2 below:

Table 2. Antioxidant Activity of Methanol Macro Algae Extracts Tested on DPPH Free Radical Capture

No	Species of Macro Algae	Concentration of Macro Algae Extracts (ppm)	Average Percentage of Free Radical DPPH Arrest (%)
1	*H. asperi*	100	2.9325510
		200	2.9325510
		300	4.1055715
		500	5.8651030
		700	5.5718480
2	*H. gracilis*	100	2.4355300
		200	2.4355300
		300	3.5816620
		500	6.1604585
		700	6.5902580
3	*H. macroloba*	100	1.7699115

		200	3.0120480
		300	5.7228915
		500	3.9156625
		700	3.6144580
4	*G. salicornia*	100	2.1084340
		200	3.1626505
		300	5.4216870
		500	5.7228915
		700	7.2289155

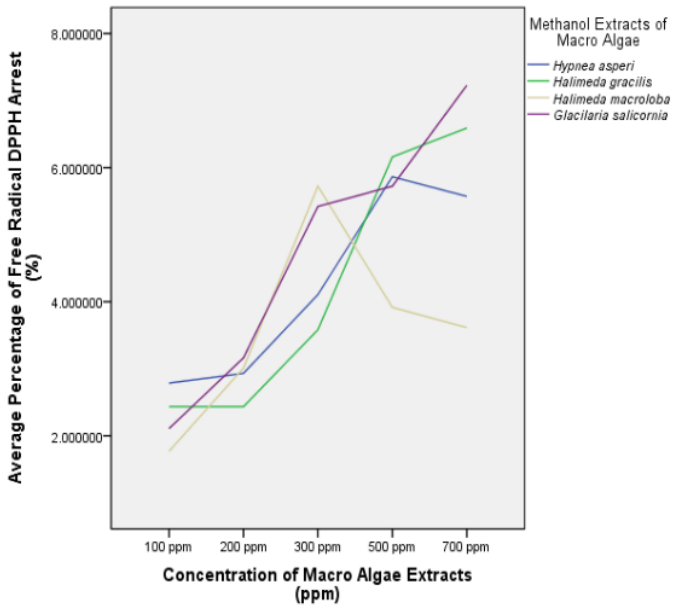

Figure 1. Graph of Antioxidant Activity of Macro Algae Methanol Extract Tested on DPPH Free Radical Capture

According to (Lailiyah et al, 2014), the instability of these outcomes is likely to occur when antioxidant activity begins to weaken at large concentrations because the compounds contained in the macro algae extract are prooxidant. In contrast to antioxidant compounds that will react with free radical DPPH through the mechanism of donor protons or hydrogen atoms from antioxidant compounds to free radicals, thus breaking the free radical chain into a form of non-radical compounds marked by the decay of color DPPH from purple to yellow. The color decay is directly proportional to the antioxidant activity of DPPH free radical capture (Lailiyah et al, 2014; Marraskuranto et al, 2008). According to Sandrasari (2008) in Lailiyah et al (2014), antioxidant compounds are very strong, if free radical capture is more than 80%, it is pretty strong, if free radical capture is 50-80%, and it is weak, if free radical capture is less than 50%. In this study, macro algae methanol extract is classified as a weak antioxidant.

Data from antioxidant test results on DPPH free radical capture percentage showed that antioxidant activity of methanol macro algae extract tested was lower compared to antioxidant activity of vitamin C as antioxidant control which also tested, although the concentration of extract was determined to 700 ppm. The results of examination of vitamin C antioxidant activity on DPPH free radical capture showed that IC_{50} was at concentration 3.595099 ppm.

The results of the research are different from the research conducted by Lailiyah*et al*. (2014) showed that the antioxidant activity of crude extract of methanol and n-hexane *Sargassum cristaefolium* on the percentage of DPPH free radical capture at concentration of 400 ppm, reached 80.78% and 63.37% respectively. Another study conducted by Rumengan and Mantiri (2015), showed that the antioxidant activity of ethanol extract *Caulerpa racemosa* on the percentage of free radical capture DPPH at a concentration of 500 ppm, which is 70%. Therefore, the antioxidant activity of macro algae tested one of them is determined by the type of solvent.

The result of ANOVA univariat on antioxidant activity of macro algae methanol extract tested show there is an effect of macro algae methanol extract concentration on DPPH free radical capture and there is significant difference of influence at concentration ($p \leq 0.05$). However, Tukey's test results on differences in antioxidant activity of methanol macro algae extract tested at the same concentration show that there is no significant effect difference ($p \leq 0.05$). Tukey's results showed a significant difference in concentration, indicating that the concentration of 100 ppm is significantly different with 300 ppm, 500 ppm and 700 ppm. At 200 ppm concentrations is significantly different with concentrations of 500 and 700 ppm. The difference in the effect of macro algae methanol extract concentration on DPPH free radical capture is shown in table 3 below:

Table 3. Differences in the Effect Of Macro Algae Methanol Extract Concentration Tested On DPPH Free Radical Capture

No	Concentrations Methanol Extract of Macro Algae (ppm)	Subset		
		1	2	3
1	100	2.27494975		
2	200	2.88569488	2.88569488	
3	300		4.70795300	4.70795300
4	500			5.41602887
5	700			5.75136988
	Sig.	0.930	0.166	0.662

The results of antioxidant examination on the percentage of DPPH free radical capture show that the antioxidant activity of two macro algae extracts of algae are stable, with active compound content in each macro algae, *salicornia* (flavonoid, steroid, and saponin) and *H. gracilis* (steroids and saponins). Two macro algae extracts show unstable antioxidant activity, with active compound content in each macro algae, *H. macroloba* (flavonoid and steroid) and *H. asperi* (saponin). The results of antioxidant examination of each macro algae methanol extract on the percentage of DPPH free radical capture at the same concentration show no significant effect difference. According to Fidriyani *et al*. (2015), flavonoid compounds have the potential as antioxidants. *G. salicornia* and *H. macroloba* methanol

extracts containing flavonoid compounds have antioxidant activity. Various research results showed that flavonoid compounds have antioxidant role (Redha, 2010). According to Redha (2010), the ability of flavonoids as antioxidants is donating by hydrogen atoms, by means of metal, in the form of glucoside, or in free form (aglikon). The results of a study conducted by Redha (2010), showed that the bound flavonoid had significant correlation coefficient on total antioxidant activity when compared with flavonoid in free form. Flavonoids also help prevent tissue damage by superoxide radicals with released neutrophil cells, protect the cell structure, increase the effectiveness of vitamin C, antiinflammation, prevent bone loss and as an antibiotic (Asfar, 2015; Firdiyani et al, 2015).

The steroids contained in the methanol extract of *G. salicornia*, *H. gracilis*, and *H. macroloba* has antioxidant activity that serves as a compound that can donate hydrogen atoms to free radicals. Steroid compounds containing many -OH groups (sterols), in the presence of substituents of hydroxyl groups attached to hydrocarbon chains tend to donate hydrogen atoms to free radicals (Lailiyah et al., 2014).

Secondary metabolites are compounds in plants is known as a radical scavenger sources, namely the phenol compounds (e.g. a flavonol, flavonon, flavones), phenyl propanoid, antakuinon, or lignan (alkaloids, saponins, and flavonoids) (Firdiyani *et al*., 2015). Saponin is a phenolic compound classes are beneficial as one source of antioxidants in inhibiting free radicals (Firdiyani et at, 2015; Latief et al, 2013). In this study, the methanol extracts of *G. salicornia*, *H. asperi*, and *H. gracilis* contain saponins which have antioxidant activity.

Antioxidant compounds are very beneficial for health, to prevent disease such as cancer and tumors, blood vessel constriction, and premature aging, as well as antioxidant compounds also plays an important role in maintaining the quality of food products, such as preventing rancidity, discoloration and aroma, as well as physical damage to the product (Rumengan dan Mantiri, 2015). The content of vitamin in the macro algae, such as vitamin B12, Vitamin C, and vitamin E are also useful as natural antioxidants. Specifically, vitamin B12 useful as to prevent aging (antiaging), Chronic Fatique Syndrome (CFS), and treat anemia. Vitamin C is beneficial as the immune system, increasing the activity of iron absorption in the intestine, and control the formation of tissue and bone matrix. Levels of vitamin C in macro green algae can reach 500-3000 mg/kg dry weight and a red macro algae reaching 100-800 mg/kg. The content of vitamin E on the macro algae can inhibit the formation of oxidation of Low Density Lipoprotein (LDL) (Suparmi and Sahri, 2009).

5. CONCLUSIONS

Phytochemical examination results show that the active compounds simplicia owned of each macro algae tested, namely *G. salicornia* (flavonoids, saponins, and steroids), *H. asperi* (steroids), *H. gracilis* (saponins and steroid), *H. macroloba* (flavonoids and steroids).

The highest percentage of interception DPPH free radical of methanol extracts of each macro algae tested .e. *G. salicornia*, *H. asperi*, *H. gracilis*, and *H. Macroloba* respectively. In this study, macro algae methanol extract was classified as a weak antioxidant.

The univariate ANOVA in antioxidant activity, indicating that there was an effect of concentration on free radical capture of DPPH ($p \leq 0{,}05$) and did not show the interaction between species of macro algae and concentration.

Phytochemical testing and antioxidant activity of *G. salicornia, H. gracilis, H. macroloba* and *H. asperi* extracts is needed, to reach IC50, using extract solvents other methanol, such as ethanol, n-hexane, and ether, as a comparison of the results with methanol extract.

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