ANTIOXIDANT AND TOXICITY ACTIVITIES of *Gracilaria gracilis* METHANOL EXTRACT BASED ON DIFFERENT EXTRACTION METHODS

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Abstract

Gracilaria gracilis (red macroalgae) was collected from Sayang Heulang Beach, Garut, West Java. It has a number of secondary metabolites that potentially serve as a source of natural antioxidants. The objective of this study was to compare the optimal extraction method in particular time variation for producing the highest percent of yield, antioxidant activity, and toxicity of *G. gracilis* methanol extract. This study was conducted experimentally, including macroalgae sampling and preparation, phytochemical screening, extraction of *G. gracilis* by maceration and UAE methods, antioxidant activity testing based on the DPPH test (*2.2 diphenyl-1-picrylhydrazyl*) and toxicity testing using the BSLT (*Brine Shrimp Lethality*) method. The results indicated that the methanol extract of *G. gracilis* contains flavonoids and steroids, with yield percentages of 12.93% for maceration and 12.1% for UAE. The antioxidant activity (IC_{50}) of maceration was 86.46 ppm, whereas the UAE was 59.01 ppm. Then, the toxicity test (LC_{50}) for maceration was 28.35 ppm whereas the UAE was 27.76 ppm. Macerated methanol extract and UAE *G. gracilis* have the potential to be powerful antioxidants based on their IC_{50} values. Then, macerated methanol extract and UAE are included in the highly toxic category.

***Keywords**: antioxidant, BSLT, DPPH, Gracilaria gracilis*

Introduction

Reactive oxygen species (ROS) are a significant example of free radicals (Sukweenadhi et al., 2020). Oxidative stress, which is induced by endogenous factors such as reactive oxygen species (ROS), causes an imbalance between free radicals and antioxidants in the body, which is triggered by an overabundance of free radicals and a shortage of antioxidants (González-Palma et al., 2016; Sukweenadhi et al., 2020). *G. gracilis* as an antioxidant by capturing reactive oxygen species (ROS), prevent regeneration by reactive oxygen species (ROS), and increasing the activity of cellular enzyme antioxidants (Bhernama et al., 2021).

Antioxidants are chemical substances with the ability to neutralize free radicals (Hidayati et al., 2017). This substance inhibits oxidative pathways that lead to degenerative illnesses by giving electrons to achieve a stable form (Hidayati et al., 2017). In addition to the antioxidant activity test, it is also possible to conduct toxicity testing. The BSLT method is used to determine the toxicity of plant extracts and seeks to predict the toxicity of a sample (natural material). If the extract belongs to the non-toxic group, it may be able to expand its use, for example as a food supplement or cosmetic raw material, whereas it may be possible to expand its usage for medicinal raw materials if the extract contains harmful compounds. This approach has the advantages of being faster, cheaper, simpler, not requiring aseptic conditions, and being trustworthy.

Numerous research have developed antioxidant compounds from natural

ingredients, particularly marine biota such as macroalgae, prior to the present (Yudharini et al., 2016). *G. gracilis* (red macroalgae) contains secondary metabolites such as terpenoids, carotenoids, vitamins, xanthophylls, chlorophyll, a number of antioxidant chemicals (Widowaty et al., 2020), tannins (Kamoda et al., 2021), saponins (Febrianto et al., 2019; Purwaningsih & Deskawati, 2021), phenols (Febrianto et al., 2019; Purwaningsih & Deskawati, 2021; Widowaty et al., 2020), alkaloids (Febrianto et al., 2019; Kamoda et al., 2021; Kurmukov, 2013; Purwaningsih & Deskawati, 2021), and flavonoids (Febrianto et al., 2019; Kamoda et al., 2021; Kurmukov, 2013; Purwaningsih & Deskawati, 2021; Widowaty et al., 2020).

For the extraction of bioactive substances, maceration is a common conventional extraction process. This approach has the drawback of using a greater quantity of solvent and requiring a lengthy extraction time (Prasetyaningrum et al., 2022). Examples of other modern extraction methods are Ultrasound-assisted extraction (UAE), Microwave-assisted extraction (MAE), Ultrasound-microwave assisted extraction (UMAE), Hydrothermal-assisted extraction (HAE), and High-pressure assisted extraction (HPAE) (Dai & Mumper, 2010).

More frequently, ultrasound-assisted extraction (UAE) method is utilized to extract natural compounds. It can boost the extraction mass transfer rate resulting from macroalgal cavitation (Prasetyaningrum et al., 2022). The extraction procedure is brief because the solubility of the analyte in the extraction medium improves as surface tension and solvent viscosity decrease, hence enhancing extraction efficiency (Prasetyaningrum et al., 2022). The destruction of the cell wall polymer structure permits more bioactive compounds from macroalgae to enter the liquid extraction phase (Prasetyaningrum et al., 2022).

Based on the preceding explanation, the purpose of this study was to determine the most effective extraction method for increasing the percent yield and total content of phytochemical compounds, as well as to evaluate the antioxidant activity and toxicity of the methanol extract of *G. gracilis*.

Research Methods

Materials and *Tools*

The materials used in this study were *G. gracilis*, methanol (Merck, Germany) solvent, ascorbic acid, aquadest, DPPH *(2,2-diphenyl-1-picrylhydrazil*) reagent, *Artemia salina* Leach larvae, and iodized salt.

Then, the tools used include analytical balance (Boeco Germany); a rotary evaporator, Erlenmeyer (Iwaki), beaker (Iwaki), volumetric flask, filter paper, aluminum foil, sonicator, and UV-Vis spectrophotometer (Genesys).

Procedure

1) Sample Preparation

G. gracilis was collected from Sayang Heulang Beach in Pameungpeuk District, Garut Regency, West Java Province in the morning (08.30-10.00 a.m (Western Indonesia Time). The macroalgae were washed by sea water to remove contaminants (like sand, epiphytes, and so on) and stored at cooler box for transporting from beach to laboratory. In the laboratory, macroalgae were rinsed by tap water and dried by air drying at room temperature and then placed in an oven at 60°C for 90 minutes (Panjaitan & Natalia, 2021).

This seaweed species was identified by Oceanographic Research Center – BRIN, East Ancol, Jakarta. The dried macroalgae were then ground to yield macroalgae powder (18 mesh particle size). It was then weighed, placed in a plastic bag, and stored at room temperature in preparation for extraction (Panjaitan & Natalia, 2021).

2) Maceration Method

30 grams of *G. gracilis* powder were macerated with methanol (p.a) (300 mL) using three different times (24, 48, and 72 hours) and subjected to rotary evaporation and further concentrated using waterbath. The yield percentage was calculated (Sari & Suharyanto, 2021). Finally, the crude extracts were stored at -20^{0}C for futher analysis.

3) Ultrasound Assisted Extraction (UAE) Method

30 grams of *G. gracilis* powder were mixed with 300 mL of methanolic solutions (1:10; (w/v)) with three different extractions (10, 20 and 30 minutes). The UAE treatments were performed using an ultrasonic water bath (Bransonic) at 42 kHz and 50% of ultrasonic amplitude. All the extraction procedures were performed in triplicate. The filtrate is evaporated using a rotary evaporator at ± 40°C and then weighed to determine its yield (Sasongko et al., 2018).

4) Phytochemical Screening

Phytochemical screening of *G. gracilis* conducted several tests for alkaloids, flavonoids, saponins, tannins or polyphenols, terpenoids, and steroids (Harborne, 1987). This aimed to identify the secondary metabolite compounds contained in methanol extract of *G. gracilis.*

5) Antioxidant Activity

The *2,2-diphenyl-2-picrylhydrazil* (DPPH) method was performed according to the methodology previously reported in (Gazali et al., 2018) . After an incubation of 30 min in darkness at room temperature, absorbance at 516 nm was measured (Ery Al Ridho, Rafika Sari, 2013). The RSA (Radical scavenging activity) of the DPPH radical was calculated using the following equation (1). The IC_{50} was calculated by linear regression by plotting concentration of each extract or reference compound with their corresponding (Coulombier, 2020).

$$\% \text{ inhibition} = \frac{A_{control} - A_{sample}}{A_{\text{control}}} \times 100\% \quad (1)$$

6) BSLT Assay

The hatching of 38 grams of *Artemia salina* Leach eggs in 1000 mL of aquadest to obtain 38 parts per thousand (ppt) of sanitation is conducted in insulated hatcheries (light and dark) that are illuminated with light (25 watts) to warm the hatchery's temperature. 1 gram of *Artemia salina* Leach eggs was dissolved by 2 liters of artificial seawater (38 ppt) to the vessel. After 24 hours, 0.06% yeast are added to avoid larva death. 48 hours later. Ten larvae of *Artemia salina* Leach (aged 48 hours) were put into the test solution with concentrations of 1000, 100, and 10 µg/mL and were carried out on the blank as a control. Then, this was observed for 24 hours.(Wulandari, 2014).

Results and Discussion

The identification results showed that the macroalgae used was *Gracilaria gracilis* (Stackhouse) Steenoft, L.M. Irvine & Farnham, 1995.

Methanol extract of G. gracilis (Maceration Method)

The results obtained from maceration with time variations can affect the % yield of the extract. % The highest yield of maceration treatment at 72 hours was 12,93% (Table 1). During maceration process the color of the macerated methanol extract of *G. gracilis* changed from light green to dark green. In addition, the longer the extraction time, the higher the yield produced so that it reaches the optimum extraction point, after reaching the optimum point the yield will decrease (Kristanti et al., 2019).

Methanol extract of G. gracilis (UAE method)

UAE extraction yields were higher than the yields obtained from maceration (Table 1). Based on Table 1, the peak of % yield (UAE method) is 12.1 % at 30 minutes. If compared to maceration result where the optimum time is 72 hours which yielded 12.93%, UAE method has better time efficiency. Moreover, it can be concluded that % yield obtained is categorized as good because the yield is > 10%. In addition, the extraction time can affect the percentage of yield extraction. For further analysis, only the optimum yield of each extraction time was analyzed for antioxidant and toxicity test.

In short period time, the dissolution of compound is not optimal, therefore the material has not been extracted efficiently, and vice versa. The longer the extraction time will increase % yield because the contact between the solvent and sample will be longer, so that the dissolution process of compounds will continue and stop until the solvent is dissolved saturated (Kristanti et al., 2019).

Table 1. %Yield of methanol extract of *G. gracilis*

Maceration Method	Time	Yield (%)
Maceration	24 hours	6,26%
	48 hours	9,16%
	72 hours	12,93%
UAE	10 minutes	3,86%
	20 minutes	8,8%
	30 minutes	12,1%

The advantages of UAE usage are faster extraction rate, its simplicity, increased yield as well as reduced cost and processing time (Hanjabam, 2019). Besides, UAE efficiency is dependent on various factors, such as ultrasound power, temperature, time, solvents to solids ratio and characteristics of the compounds to be extracted, (Dobrincic et al., 2020).

The more solvents, the more compounds extracted, and the longer the extraction, the longer the contact time between the material and the solvent so as to obtain a high % yield (Kristanti et al., 2019).

Phytochemical screening

The results of the phytochemical screening of *G. gracilis* (both from the maceration method and the UAE) showed the presence of flavonoid and steroid compounds. The extraction of flavonoid content using methanol produces a red color on the amyl alcohol layer. The identification of steroids/triterpenoids in the methanol extract of *G. gracilis* was marked by the formation of a green ring using chloroform reagent ($CHCl_3$). (Habibi et al., 2018).

Table 2. Phytochemical screening of methanolic extract of *G. gracilis*

Compound	UAE (30 minutes)	Maceration (72 hours)	Purba et al., (2019)	Kamoda et al., (2021)
Flavonoids	+	+	+	+
Alkaloids	-	-	+	+
Tanins	-	-	-	+
Saponins	-	-	+	-
Quinons	-	-	-	-
Steroids	+	+	-	-
Triterpenoids	-	-	+	-

Several factors which influence phytochemical compounds of macroalgae such as different types or species, locations, seasons and so on. Julyasih and Putu (2020) reported that phytochemical compounds contents are influenced by various factors, namely species, varieties, growing conditions, seasonal variations, processing and storage methods.

In addition, environmental factors, such as cultivation location, altitude, temperature, time of sun exposure, rainfall, climate, and soil can affect the primary and secondary metabolites of a plant (Artika et al., 2018).

Antioxidant activity

In the present study, the antioxidant activity of methanol extract of *G. Gracilis* was assessed through the DPPH radical scavenging (Table 3). Generally, the discoloration (from deep purple to pale yellow) acts as an indicator of antioxidant activity where the changing color is measured at 516 nm. Moreover, the neutralisation DPPH test is based on donating electrons from the antioxidants in order to neutralise the DPPH radical (Munteanu and Apetrei, 2021). In addition, the doses or the concentration of extract that can reduce intensity of 50% radical absorption is expressed as IC_{50} value (Hidayati, 2016).

Antioxidant activities in this present study were presented in Table 3, 4, and 5. Futhermore, IC_{50} value of macerated and UAE methanol extract of *G. Gracilis* were 59.01 ppm and 86.46 ppm, respectively. Ascorbic acid as a positive control had antioxidant activity 1.55 ppm.

Table 3. Antioxidant activity of macerated methanol extract of macroalgae *G. gracilis*

Test Concentration (ppm)	Absorbance (nm)			Average	% Inhibition
	1	2	3		
0	1.687	1.687	1.687	1.687	0
20	0.943	1.026	0.909	0.96	43.13
40	0.869	0.938	0.733	0.85	49.81
60	0.978	0.969	0.612	0.85	49.44
80	0.85	0.834	0.716	0.8	52.58

Table 4. Antioxidant activity of UAE methanol extract of macroalgae *G. gracilis*

Test Concentration (ppm)	Absorbance (nm)			Average	% Inhibition
	1	2	3		
0	1.687	1.687	1.687	1.687	0
20	0.942	0.986	1.121	1.016	39.75
40	1.008	0.937	0.905	0.950	43.69
60	1.152	0.735	0.969	0.952	43.57
80	0.869	0.917	0.734	0.84	50.21

Table 5. Antioxidant activity of ascorbic acid

Test Concentration (ppm)	Absorbance (nm)			Average	% Inhibition
	1	2	3		
0	1,687	1,687	1,687	1,687	0
2	0,426	0,722	0,539	0,562	66,67
4	0,289	0,509	0,677	0,492	70,86
6	0.327	0,225	0,411	0,321	80,97
8	0,145	0,164	0,136	0,148	91,21

Based on (Molyneux, 2004), a compound is classified as very strong when the IC_{50} value is <50 ppm; strong when the IC_{50} value is 50–100 ppm; moderate when the IC50 value is 101–150 ppm; and weak antioxidants when the IC_{50} value is >150 ppm. It can be concluded that macerated and UAE methanol extract of *G. Gracilis* showed strong antioxidant activity. If compared to ascorbic acid (positive control), there is a significant difference since ascorbic acid is a pure compound while the methanol extract of *G. gracilis* is still a raw extract (Fidrianny & Darmawati, 2014).

Results of this study suggest that macerated and UAE methanol extract of *G. Gracilis* contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage like flavonoid. Naturally, flavonoid is occuring in plants. The best-described antioxidant property of flavonoids derives from its ability to directly scavenge the reactive oxygen species. Flavonoids can immediately chelate free radicals by donating a hydrogen atom or by single-electron transfer (Banjarnahor, 2014).

These results are different from the research of Widowaty et al., (2020) *Gracilaria sp.* which is 221.76 ppm where this is due to factors that influence it such as seasonal variations, processing and storage methods. In addition, antioxidant activity of *G. verrucosa*, collected from Pok Tunggal Beach are 188.53 ppm. (Febrianto et al., 2019).

Generally steroid compounds are used as basic ingredients for making drugs while flavonoids which have antioxidant mechanisms stemming from their ability to transfer an electron to free radical compounds and also form complexes with metals and metal ions such as Cu and Fe, which can catalyze reactions that eventually produce radicals. free (Rachmatiah et al., 2022).

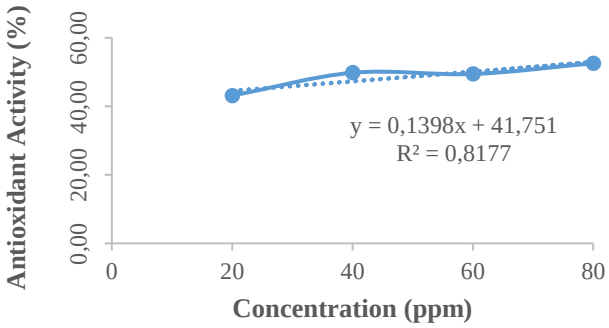

Figure 1. Antioxidant activity of macerated methanol extract of *G. gracilis*

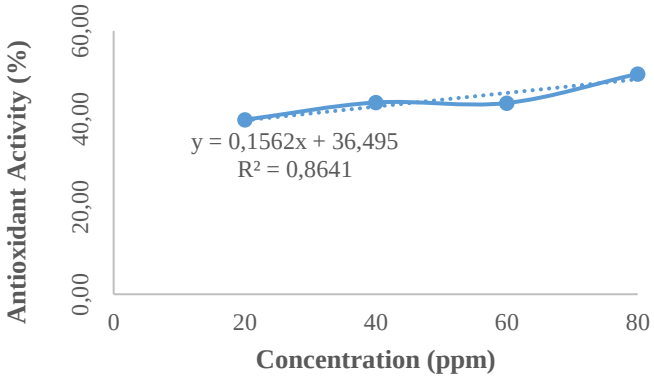

Figure 2. Antioxidant activity of UAE methanol extract of *G. gracilis*

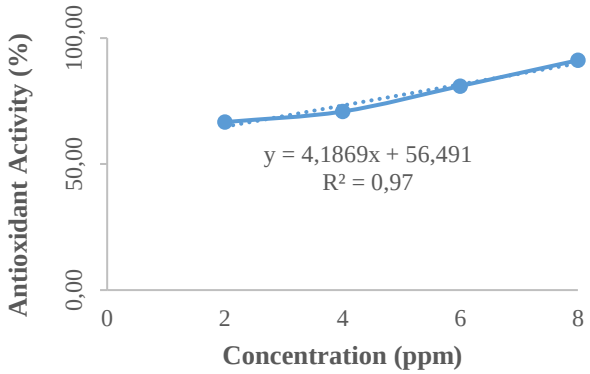

Figure 3. Antioxidant activity of ascorbic acid

Toxicity of methanol extract of G. gracilis

In this study, we used probit analysis in order to obtain a straight line curve so that the determination of the LC_{50} value is more precise. The results of the toxicity test of *G. gracilis* extract using the BSLT method will be shown in Table 6 and 7.

Table 6. The toxicity of macerated methanol extract of *G. gracilis* (72 hours) using BSLT method

Concentration	Repeat					Average	% Died	Probit value of % Mortality
	1	2	3	4	5			
1000 ppm	7	7	7	8	7	7.2	72	5.58
100 ppm	5	4	4	3	3	3.8	38	4.69
10 ppm	0	1	0	2	2	1	10	3.72
Blanko	0	0	0	0	0	0	0	0

Table 7. The toxicity of UAE methanol extract of *G. gracilis* (30 minutes) using BSLT method

Concentration	Repeat					Average	% Died	Probit value of % Mortality
	1	2	3	4	5			
1000 ppm	6	6	7	6	7	6.4	64	5.36
100 ppm	3	5	2	4	3	3.4	34	4.85
10 ppm	1	0	0	2	1	0.8	8	3.59
Blanko	0	0	0	0	0	0	0	0

The percentage of mortality obtained can be displayed in the form of a curve based on the ratio of the percentage of mortality of *Artemia salina* L. larvae to the concentration of extracts from all research samples. The larvae of each sample are shown in Figure 5 and Figure 6.

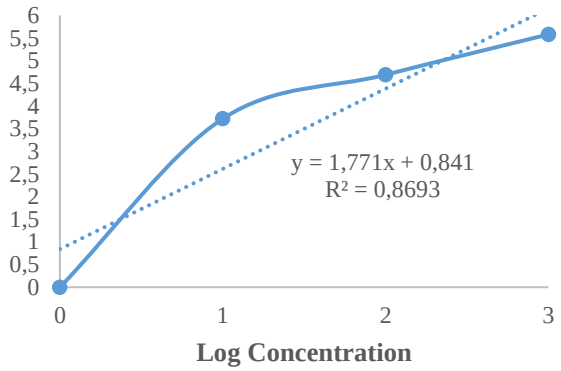

Figure 5. Probit curve LC_{50} analysis of macerated methanol extract of *G. gracilis* (72 hours)

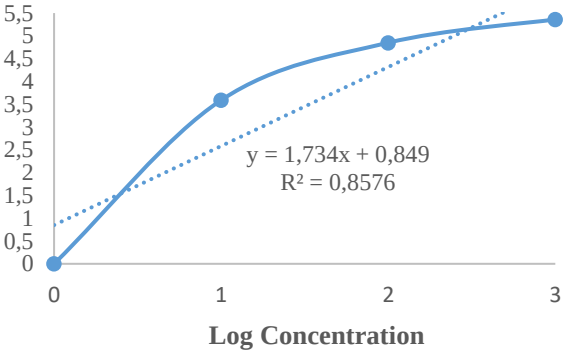

Figure 6. Probit curve LC_{50} analysis of macerated methanol extract of *G. gracilis* (72 hours)

Based on the calculation of the first probit analysis, the Maceration method as can be seen in Figure after being analyzed the results show the value of LC_{50} < 1000 which is 27.76 ppm in the very Toxic category, the equation y = 1.771x − 0.841 and the correlation coefficient on the graph is obtained of R^2 at 0.8693 (86.9%).

Furthermore, in the figure using the UAE method, the LC_{50} value is 28.35 ppm with the category of Very Toxic Toxicity, the equation y = 1.734x + 0.849 with a correlation coefficient based on the graph of 0.8576 (85.8%) (Silitonga et al., 2022). The mechanism of steroid and flavonoid compounds has an anticancer effect mechanism that functions as stomach poisoning. Therefore, if it enters the larva's body, its digestive system will be disturbed. In addition, these compounds will inhibit taste receptors in the mouth area of the larvae.

This resulted in the larvae not getting a taste stimulus so they were unable to recognize their food, resulting in starvation. (Surya & Darmawan, 2018). Based on the results of this study, it was found that the extraction results from both methods were highly toxic because *G. gracilis* had flavonoid and steroid.

Conclusions

The highest %yields in both methods were both for a long time (72 hours (maceration) and 30 minutes (UAE)). Both methods of methanol extraction *G. gracilis* is strong, although the IC_{50} value in the UAE is 86.46 ppm higher than maceration at 59.01 ppm. Meanwhile, the two methanol extraction methods for *G. gracilis* are toxic, although the LC_{50} value in the UAE is 28.35 ppm higher than 27.76 ppm for maceration.

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