Physical treatments to induce the antibacterial and antioxidant activities of green algae Halimeda sp. from Seribu Islands, North Jakarta, Indonesia

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Abstract. Seaweeds have ecological functions as primary producers in marine waters. Most of them have an important economic value as a producer of hydrocolloids (alginate, agar and carrageenan) that are used in various industries of food and pharmaceuticals. Secondary metabolites produced in seaweed are usually for self-defense from algae predators such as fish, echinodermata, crustaceans or mollusca. This study aimed to determine the antibacterial and antioxidant activities of green algae Halimeda gracilis and Halimeda macroloba, and to determine the effect of physical treatment to antibacterial and antioxidant activities. The yield of crude extract of H. gracilis obtained was about 18.55-23.94% while the extract of H. macroloba was just about 13.49-17.88%. Antibacterial activity of crude extract of H. gracilis was higher as compared to that of H. macroloba against both tested bacteria. Induction of bacteria (Vibrio harveyi and Stenotrophomonas maltophilia) on H. gracilis led to a reduction in the antibacterial activity against Escherichia coli. Induction of bacteria in H. gracilis did not show antibacterial activity against Staphylococcus aureus. Antibacterial activity of crude extracts of H. macroloba on cutting and bacterial induction treatments were higher in both test bacteria S. aureus and E. coli.

Keywords: antimicrobial; extract; induction; metabolites; seaweed.

1. Introduction
Seaweed is one of the primary producers in the marine waters alongside with phytoplankton, seagrass, and mangrove. In the medical field, seaweed can be utilized as a medication to treat various diseases. Bioactive compounds potencies of seaweed have been studied in some researches, among them are seaweed as an antioxidant [1], antibacterial [2], anticancer [3], and antiinflammation agent [4]. Bioactive compounds that can be found in seaweed are including groups of polysaccharides, fats and fatty acids, pigments, and other secondary metabolites such as phenol, alkaloid, terpenoid, and lectin [5].

There are more than 300 secondary metabolites have been identified from green algae and less than half of the green algae are order Bryopsidales [6]. Orders Udoteaceae, Caulerpaceae, and Halimedaceae produce more 85% bioactive compounds than Bryopsidales. Some other research
studying Halimedaceae bioactive compound potency are *Halimeda macroloba* [7] and *H. tuna* [8] as an antimicrobial agent. Seaweed from order Halimedaceae also has potency as an antioxidant and anticancer agent [9]. Seaweed produces secondary metabolite for its self-defense from algae predators such as fish, Echinodermata, crustacea, or molluscs. There were changes of halimedatrial and halimedatetraacetate content in *Halimeda* sp. which is suspected as the effect of its self-defense mechanism [10].

Metabolism responds to produce secondary metabolites as self-defense from physical disruption and environmental conditions can be used to increase algae bioactive compound production. There was a difference in algae metabolism due to the treatment given [10, 11]. However, there is not much information available about the effect of physical treatment and environmental conditions on the antibacterial and antioxidant activities of algae. This study aims to determine the antibacterial and antioxidant activities of physically treated and bacteria-induced green algae *H. macroloba* and *H. gracilis*, physical treatment, and bacteria induction is given as a way to increase the antibacterial and antioxidant activity.

2. Method

Green algae *H. gracilis* and *H. macroloba* were collected from Karya Island, Seribu Islands, North Jakarta, Indonesia. Samples *H. gracilis* and *H. macroloba* were given physical treatment (intact, cut, and ground) and induced with two different bacteria (*Vibrio* harveyi and *Stenotrophomonas* maltophilia). The sample was ground using a mortar and cut by its edge using scissors. The cut sample was then soaked in seawater for two hours. Bacteria induction was done by soaking samples for six hours in 500 mL of seawater which has been suspended with each 500 mL of *V. harveyi* and *S. maltophilia* (1:10).

All of the samples were stored inside liquid nitrogen with the temperature at -80 °C and dried using the freeze-drying method in freeze dryer at 0 °C. The dried samples were extracted using the maceration method in methanol solvent with ratio 1:10 w/v for 24 h. Maceration was done repeatedly for three times or until the filtrates look clear then they were evaporated using rotary evaporator. Nutrient agar was used to grow the bacteria *S. aureus* and *V. harveyi* for 24 hours of incubation at 37 °C. Grown bacteria were suspended in sterilized nutrient broth and incubated under the same condition. Bacteria was ready to be assayed when the optical density (OD) level reached 0.5-0.8, each OD level of *E. coli* and *S. aureus* were 0.8 and 0.7.

As much as 20 µL of tested bacteria was added into 20 mL of Müller Hinton agar in a sterilized petri dish, eight holes of diffusion well made when the agar media was already solid. Every two holes of the well were filled with 20 µL of solution containing 2 mg of extract, 20 µL of solution containing 1 mg of extract, and 20 µL of solution containing 0.5 mg of extract while the other two holes were filled with each methanol as negative control and 300 µL of chloramphenicol as positive control. To let the extract diffused, the petri dish was stored inside a refrigerator for two hours and then incubated for 24 hours at 37 °C, the growth of bacteria observed every three hours. Antioxidant activity assayed using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) with three different extract concentrations (100, 200, and 400 ppm). Each concentration of 4.5 mL of extract was added into a test tube with 0.5 mL of DPPH and homogenized using vortex.

Samples of extract and DPPH were incubated for 30 minutes at 37 °C then measured for their absorbance values using a spectrophotometer with the wavelength at 517 nm. A blank solution was made to measure its absorbance value by adding 4.5 mL of methanol solvent and 0.5 mL of DPPH solution. Antioxidant activity is shown by IC50 which is the concentration needed to catch 50% of DPPH free radical.

3. Result

3.1. *Halimeda* sp. Extract Yield

Maceration is one of traditional cold extraction methods used to solve the bioactive compound conveniently. The maceration method was chosen to avoid the use of high temperature that will lead to damage for some compounds which are not tolerant of heat. Antioxidant bioactive compound
flavonoids from medicinal plant could be damaged by high temperature in the heat extraction [12]. The filtrate from maceration was then concentrated using a rotary evaporator to evaporate all of the methanol solvent left in the sample until the crude extract obtained. Crude extract yield of *Halimeda* sp. was gained from sample initial weight after the freeze-drying process and its weight after the extraction (Table 1).

**Table 1.** Crude extract yield (%) of *H. gracilis* and *H. macroloba*.

| Treatment                | Sample          | *H. gracilis*   | *H. macroloba* |
|--------------------------|-----------------|-----------------|----------------|
| Intact                   |                 | 19.83±1.74      | 17.88±1.25     |
| Ground                   |                 | 20.24±2.18      | 14.79±4.45     |
| Cut                      |                 | 23.94±3.39      | 15.55±0.58     |
| *V. harveyi* induction   |                 | 21.77±2.22      | 13.49±1.22     |
| *S. maltophilia* induction|                 | 18.55±1.58      | 15.25±1.63     |

According to Table 1, it is known that the crude extract yield of *H. gracilis* is on the range 18.55-23.94% and the crude extract yield of *H. macroloba* is on 13.49-17.88%. Based on this research, green algae *H. gracilis* produced more yield than *H. macroloba* at each treatment. The highest yield of *H. gracilis* crude extract was obtained by the cut ones meanwhile the highest yield of *H. macroloba* crude extract was obtained by the intact ones.

3.2. Antimicrobial Activity of *Halimeda* sp. Crude Extract

The result showed that both samples have antimicrobial activity. The 2 mg concentration used gave better results than 0.5 mg concentration on both control samples with clear zone diameter 6.00±0.00 mm and 3.50±1.71 mm against *E. coli* and 10.00±0.00 mm and 5.50±1.71 mm against *S. aureus* (Figure 1).

**Figure 1.** Antibacterial activity against tested bacteria; (G) *H. gracilis*, (M) *H. macroloba*; (1) intact, (2) cut, (3) ground, (4) *V. harveyi* induction, (5) *S. maltophilia* induction.

Green algae *H. gracilis* and *H. macroloba* could inhibit Gram-positive bacteria *S. aureus* growth better than Gram-negative bacteria *E. coli*. Antimicrobial activity of *H. gracilis* could be categorized as a moderate level meanwhile antimicrobial activity of *H. macroloba* could be categorized as a low level. Antimicrobial activity is categorized as a low level if the inhibition zone was less than 5 mm and for moderate level is 5-10 mm. Physical treatment and bacteria induction gave a significant difference in both samples. Crude extracts G1, G2, and G3 showed higher antimicrobial activity than crude extract M1, M2, and M3. Bacteria induction on G4 and G5 decreased the antimicrobial activity of *H. gracilis* against *E. coli* and no antibacterial activity at all against *S. aureus* meanwhile M4 and M5 antibacterial activity were increased against both tested bacteria.
3.3. Antioxidant of Halimeda sp. Crude Extract

Crude extracts of *H. gracilis* and *H. macroloba* were proven to have antioxidant activity on all treatment. Green algae *H. gracilis* has a higher antioxidant activity level than *H. macroloba*. Physical treatment (cut and ground) and bacteria induction (*V. harveyi* and *S. maltophilia*) decreased the antioxidant activity of both crude extracts (Table 2).

| Sample     | Treatment                  | IC$_{50}$ Value (ppm) |
|------------|----------------------------|------------------------|
| *H. gracilis* | Intact                    | 290.49                 |
|            | Cut                       | 470.49                 |
|            | Ground                    | 442.73                 |
| V. *harveyi* induction |                           | 440.39                 |
| S. *maltophilia* induction |                            | 585.76                 |
| *H. macroloba* | Intact                    | 1,147.14               |
|            | Cut                       | 820.51                 |
|            | Ground                    | 1,047.59               |
| V. *harveyi* induction |                           | 712.23                 |
| S. *maltophilia* induction |                            | 596.93                 |

Green algae *H. gracilis* inducted by bacteria *S. maltophilia* also intact and ground *H. macroloba* have the lowest antioxidant activity amongst all treatment given. A very high-level antioxidant has IC$_{50}$ <50 ppm and IC$_{50}$ of a high, moderate, and low-level antioxidant are 50-100 ppm, 101-150 ppm, and >150 ppm [13]. All of the samples have a very low antioxidant activity.

4. Discussions

All the extractions in this research are done under the same condition. The differences in the yield between two species of green algae were due to the active compound content of each sample. Methanol was chosen for its ability to extract all of the active compounds at all level of polarity. Crude extract yield of *H. gracilis* was more than *H. macroloba* crude extract yield because the metabolite compound containing pigment inside *H. gracilis* was more extracted. The crude extract of *H. gracilis* on cutting could reach the highest yield because of the physical treatment stimulates the metabolite activity meanwhile physical treatment and bacteria induction on *H. macroloba* led to the decrease of the crude extract yield. Halimadatrial content of *Halimeda* sp. was increased around 25% because of the physical treatment meanwhile there was an increase of halimedatetraacetate from 35% to around 5% on control *Halimeda* sp. crude extract [10].

The increase of antibacterial activity of cut *H. gracilis* against bacteria *S. aureus* and ground *H. gracilis* against bacteria *E. coli* might happen because of the self defense of the green algae as suspected. A secondary metabolite was produced as a self defense mechanism which leads to the increase of antibacterial activity where physical treatment also led to the increase of secondary metabolite production quantity. Based on this research, the more crude extract yield obtained the higher its antibacterial activity. Environmental conditions and the presence of predation are affecting the metabolism of red algae *Gracilaria vermiculophylla* and its chemical compound production for self defense [11]. The decrease of antibacterial activity of *H. gracilis* inducted by Gram-negative bacteria *V. harveyi* and *S. maltophilia* was because of the sample *H. gracilis* have first produced metabolite to survive in the new environment with the inducted bacteria inside. Thus, the production of secondary metabolite in *H. gracilis* was less targeted to Gram-positive bacteria like *S. aureus*.

The crude extract of *H. gracilis* still has antibacterial activity against *E. coli* despite it appeared that the activity was decreasing compared to the intact sample. The decrease of its antibacterial activity might happen because of other uncontrolled factors such as the biologic condition of the green algae when collected from the waters. The increase of antibacterial activity of *H. macroloba* inducted by *V. harveyi* against *S. aureus* and *S. maltophilia* against *E. coli* is because of the self defense mechanism stimulated the production of secondary metabolite. Bacteria *V. harveyi* and *S. maltophilia* were both isolated from the waters where *S. maltophilia* is specifically isolated from algae with the
ice-ice disease. Induction of bacteria *S. maltophilia* increased the antibacterial activity because the green algae recognized it as pathogen bacteria. The two samples of green algae might have different self defense mechanism and respond to physical interference or environmental conditions. Secondary metabolite content as a self defense mechanism depends on the extracted part of the plant, its habitat, and the numbers of algae predators around its environment [10].

Antioxidant activity of *H. gracilis* and *H. macroloba* were related to the secondary metabolite content obtained. It appeared that physical treatment and bacteria induction decreased the antioxidant activity of *H. gracilis* from 290.49 ppm to a higher value of IC$_{50}$ during the preparation of the sample. The value of secondary metabolite which has antibacterial activity is suspected to increase because of the physical treatment and bacteria induction meanwhile the secondary metabolite which has antioxidant activity was decreased. Green algae *H. macroloba* responds to physical treatment and bacteria induction by producing antioxidant secondary metabolite. Intact *H. macroloba* extract has IC$_{50}$ of 1147.14 ppm and its antioxidant activity was increased on the cut, ground, and bacteria induced treatment with each value of IC$_{50}$ 820.51 ppm, 1047.59 ppm, 712.23 ppm, and 596.93 ppm. Dried algae have lower antioxidant activity than the undried ones [14]. The extract of green, brown, and red algae collected from The Hawaiian Islands by different depths was reported to have a significant difference in antioxidant activity [15]. Extract from algae collected from shallow waters (0-3 m) has higher antioxidant activity than the ones collected from the depth around 70 m.

5. Conclusions

Physical treatment and bacteria induction gave a significant difference to the antibacterial and antioxidant activity of *H. gracilis* and *H. macroloba*. The physical treatment given to *H. gracilis* increased the antibacterial activity meanwhile bacteria induction decreased the activity, the antibacterial activity is categorized as a moderate level. Contrarily, the physical treatment decreased the antibacterial activity of *H. macroloba* yet bacteria induction increased the activity, the antibacterial activity is categorized as a low level. The antioxidant activity of *H. gracilis* showed a decreasing toward physical treatment and bacteria induction but the antioxidant activity of *H. macroloba* was increasing. The antioxidant activity of both samples is categorized as a low level.

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