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Original Article

Green Algae *Halimeda macroloba* in Spermonde Archipelago: Phytochemical and *In Vitro* Antibacterial Studies

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ABSTRACT

Background: Green algae *Halimeda macroloba* compounds active against human, fish, and shrimp pathogenic bacteria. It is one of the marine natural organisms (MNO) which is a diverse source of secondary metabolites. **Objective:** We have set our goal towards determining the antimicrobial potential of crude extracts of green algae *H. macroloba*. **Materials and Methods:** Three crude mixtures of Marine Natural Product (MNP) were obtained from macroalgae *Halimeda macroloba* (Lae-Lae island, Spermonde Archipelago) by extraction (n-hexane, ethyl acetate, and methanol were used as solvents). **Results:** Theses mixtures (phytochemical tests showed they contained steroids, terpenoids, and alkaloids) were screened for their activity against shrimp pathogenic bacteria (*Vibrio harveyi* (M-120), *Aeromonas hydrophilla*, and *Vibrio parahaemolyticus* (T-170)). The obtained results confirmed weak antibacterial activity of studied extracts of *H. macroloba*. The ethyl acetate extract was the most potent antimicrobial agent at a concentration of 4 µg/25 µl. The inhibition zones for the growth of *A. hydrophilla* (the most susceptible microorganism) and *V. harveyi* were at 8.27 mm and 8.23 mm, respectively (inhibition zone was 15.2 mm for ciprofloxacin which was used as a positive control). **Conclusion:** They might be even used in the future as alternatives to conventional drugs in aquaculture.

Key words: *Halimeda macroloba*, Antibacterial Activity, Shrimp Pathogenic Bacteria, Phytochemical screening

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INTRODUCTION

Green algae, known as green seaweed, emerged between 900 and 500 billion years ago. These algae belong to the eukaryotic photosynthetic group. One of them is Halimeda from the Udoteaceae family. This plant lives in habitats associated with coral reefs and contains high amounts of calcium carbonate. Therefore, Halimeda is classified as calcified or calcareous algae.[1,2] According to several studies, Chlorophyta or green algae compounds active against human, fish, and shrimp pathogenic bacteria. It is one of the marine natural organisms (MNO) which is a diverse source of secondary metabolites. Natural products produced by various species of the genus Halimeda, including *Halimeda macroloba, H. opuntia*, *H. macrophysa*, *H. gracilis*, *H. tuna*, and *H. renschi* have been tested as potential antibacterial agent.[3-7] For example, 4 new diterpenoids, halimedatrial-tipe compounds (structures 1,3,4,5) (Figure 1) which are potential as antibacterial agents[8], have been isolated from several Halimeda species. Halimedatrial has antibacterial activity against a number of marine microorganisms, including *Vibrio splendida, V. leiognathi, V. harveyi, Bacillus subtilis*, and *Staphylococcus aureus*.[9,10]

Halimeda macroloba in Lae-Lae Island, Spermonde Archipelago, grows in complex environmental conditions (relatively high and changing salinity of seawater, high heavy metal content, and susceptibility to the surrounding organisms). Environmental factors such as temperature, humidity, light intensity, water supply, minerals, and CO_2, affect the growth and production of secondary metabolites in a plant.[11] In order to survive, as a response to mentioned complex environmental factors, seaweed developed the ability to produce distinctive secondary metabolites.

A preliminary screening of the antimicrobial activity of crude algae extracts can help in the search of new antimicrobial seaweed metabolites. Such results can direct further phytochemical investigation and result in the isolation and structural elucidation of new natural products. For that reason, we have set our goal towards determining the antimicrobial potential of crude extracts of green algae *Halimeda macroloba* that grows in challenging environmental conditions of Spermonde Archipelago.

MATERIALS AND METHODS

Collection of sample

Green algae samples (*Halimeda macroloba*) were collected by hand (Snorkeling) from Lae-Lae Island, Makassar City, South Sulawesi Province, Indonesia. Samples were washed with seawater, freshwater, and distilled water to clean seaweed from salt, epiphytes, and other impurities. Then the wet samples (1 kg) were dried using a freeze drier. The sample was identified by the Productivity and Water Quality Laboratory, Faculty of Marine Science and Fisheries, Hasanuddin University, South Sulawesi Province, Indonesia.

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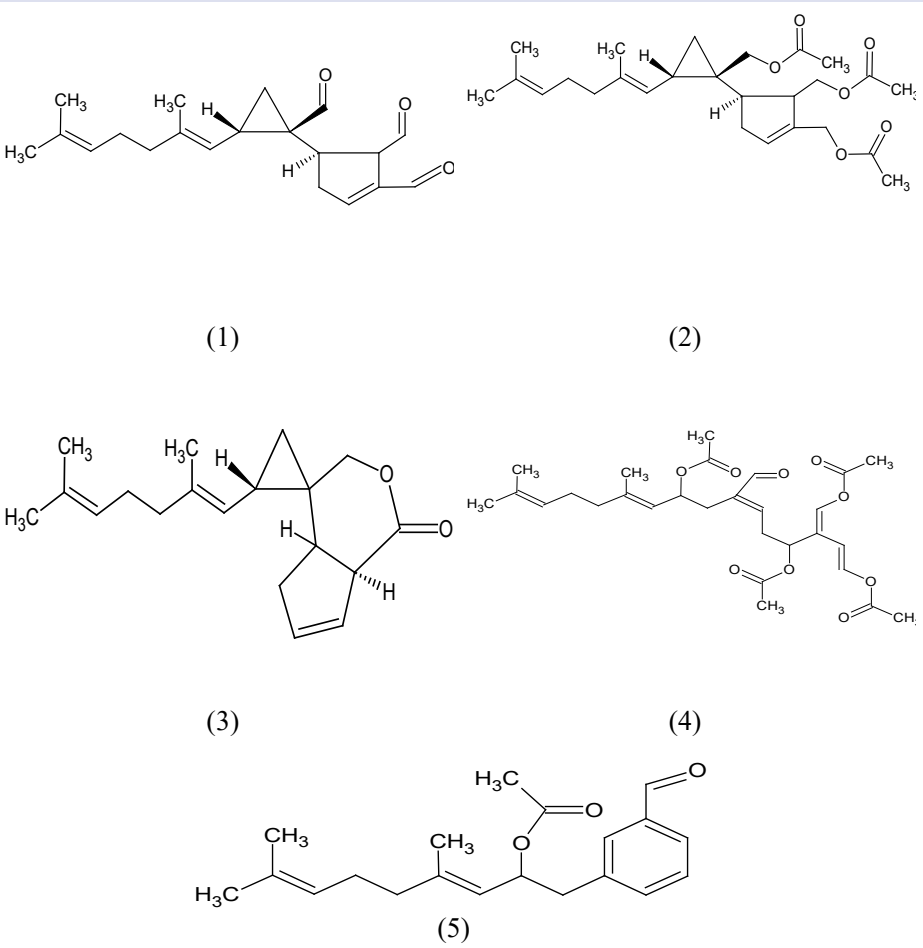

Figure 1: Chemical structures of compound isolated from *Halimeda* sp.: Halimedatrial (1), halomedatriol triacetate (2), halimedalactone (3), halimedatetraacetate (4), and bis-nor dianpenoid (5).

Instrumentation

Buchner suction flasks and funnels, vacuum pumps, Buchi Rotavapor R-200, n-hexane, ethyl acetate, methanol (technical grade), and dimethyl sulfoxide (99% purity Merck, Germany) for preparation of extracts.

Extractions

Samples were extracted in various solvents (a selection was based on the difference in their polarity). The extraction of secondary metabolites was carried out using n-hexane, ethyl acetate, and methanol; a ratio of powder extract to solvent was set 1:8 (w:v).[12]

Antibacterial analysis

Bacterial species (*Vibrio harveyi* (M-120), *Aeromonas hydrophilla*, and *Vibrio parahaemolyticus* (T-170) (shrimp pathogenic bacteria)) were donated by The Center for Brackish Water Aquaculture, Takalar, and Maros, South Sulawesi Province, Indonesia. The turbidity of the suspension was standardized against 0.5 McFarland using a spectrophotometer at a wavelength of 600 nm.[13] The bacterial inoculum was 10^8 cfu/ml. Antibacterial activity tests included positive, negative control and antibacterial activity tests for seaweed extract. The positive control test was carried out using ciprofloxacin antibiotics, and the negative control test used solvents (DMSO). The antimicrobial activity was determined by using slightly modified agar diffusion method.[14-15] Sterile paper discs with a diameter of 6 mm were soaked in extracts with various concentrations. The concentrations were 4 µg, 3 µg, and 2 µg (25 µl /disc). The samples were incubated for 24 hours at 30°C. The distinct zone around the paper disc is a sign of bacterial activity. Every experiment was conducted in triplicate. Inhibition zones >15 mm were declared as strong, from 8 to 15 mm as moderate, and 1 to 8 mm as weak activities.[16]

Phytochemical test

A phytochemical identification test was used to determine the chemical content in a material qualitatively. Slightly modified previously described tests were used to determine the presence of alkaloid, flavonoid, steroid, and terpenoid compounds in the extracts.[17]

RESULTS AND DISCUSSION

Results of the phytochemical tests (Table 1) showed that the crude extracts have several classes of compounds. They were dominated by steroid/terpenoid compounds. A positive Wegner test suggested the presence of alkaloids. Although, the outcome of two other diagnostic tests for alkaloids (Meyer and Dragendorf) was negative. These results are consistent with the results of the previous studies, which stated that steroids and terpenoids are found in *Halimeda* sp.[5,8]

Many representatives of these classes of compounds are strong antimicrobials. Thus, it was reasonable to expect that herein studied Halimeda extract will have at least some antimicrobial potential. The result of the antibacterial activity screening of four crude extracts against Gram-negative bacteria is summarized in Table 2. Of all studied samples, the crude ethyl acetate extract of *Halimeda macroloba* showed

Table 1: Results of the phytochemical test.

No	Phytochemicals	Extracts		
		N-hexane	Ethyl acetate	Methanol
1	Wegner	+	+	+
2	Meyer	-	-	-
3	Dragendorf	-	-	-
4	FeCl3	-	-	-
5	Lieberman-Buchard	++	++	++

(-)Negative, (+)Weak positive, (++)Strong positive

Table 2: Average zone of inhibition for crude extracts *Halimeda macroloba*.

Test samples	Concentration (µg/25µl)	Inhibitory Zone (mm)		
		Ah[a]	Vp[b]	Vh[c]
N-hexane	4	6.0	6.0	6.0
	3	6.0	6.0	6.0
	2	6.0	6.0	6.0
Ciprofloxacin	40 ppm	14.2	13.0	11.8
Ethyl acetate	4	8.27	6.0	8.23
	3	7.39	6.0	7.83
	2	7.04	6.0	7.17
Ciprofloxacin	40 ppm	15.2	10.6	12.3
Methanol	4	6.0	6.0	6.0
	3	6.0	6.0	6.0
	2	6.0	6.0	6.0
Ciprofloxacin	40 ppm	14.7	14.5	14.0

[a]*Aeromonas hydrophila*, [b]*Vibrio parahaemolyticus*, [c]*Vibrio harveyi*

the highest antimicrobial activity. It inhibited the growth of two Gram-negative bacteria. *Aeromonas hydrophilla* with the inhibition zone of 8.27 mm at a concentration of 4 µg, was the most susceptible organism. Inhibition of the growth of *Vibrio harveyi* was slightly weaker at a concentration of 4 µg. It was 8.23 mm. The value of the inhibition zones belonged to the category of weak.[16] As previously shown, metabolites of green algae *Halimeda macroloba* exhibit a broad spectrum of antibacterial activity.[3] *Halimeda* sp. showed to inhibit the growth of Gram-positive bacteria *Bacillus cereus* and *Staphylococcus aureus*.[7] While *Halimeda opuntia* metabolites are active against *Escherichia coli* and *Staphylococcus aureus* bacteria. The results are inhibition of 21 mm and 19 mm using 70% ethanol solvent.[6]

Other related studies to Halimeda, showed that methanolic extracts of *H. macrophysa, H. gracilis, H. opuntia*, and *H. renschi* inhibited growth of four types of bacteria.[5] Extracts of another species *H. tuna* were tested against 10 types of human pathogenic bacterial strains. The results of this study showed that methanolic extract had higher antimicrobial potential, compared to ethanol and chloroform ones.[4]

The detected antibacterial activity of Halimeda extracts might be the outcome of the presence of terpenoid compounds. For example, compounds halimedatrial (1), halomedatriol triacetate (2), halimedalactone (3), halimedatetraacetate (4), and Bis-nor-Dianpenoid (5) have been isolated from several species of *Halimeda* sp. (Figure 1). These species have activity against bacteria, i.e. *Vibrio* sp., *Bacillus subtilis*, and *Staphylococcus aureus.*[10] Halimedatrial (diterpenoid trialdehyde derivative), and halimedatetraacetate (tetraacetate diterpenoid derivative), secondary metabolites that are mostly found in Halimeda algae, are reported as antibacterial agents against some marine microorganisms.[9]

Although crude extracts of *Halimeda macroloba* show only weak bacterial growth inhibition, it does confirm that some of its constituents are potential strong antimicrobials. Isolation and structure elucidation of pure constituents and their subsequent antimicrobial screening is needed to locate exact carriers of the detected activity. Thus, here presented data can be used as a starting point and illustrates potential of secondary metabolites from macroalgae in Lae-Lae Island as potential antibacterial agents against shrimp pathogenic bacteria. They might be even used in the future as alternatives to conventional antibiotics in aquaculture.

CONCLUSION

Halimeda macroloba crude extracts (3 different solvents) showed weak antibacterial activities against the tested organisms (3 Gram-negative bacteria). Ethyl acetate crude extract had the highest activity (disc diffusion method). The fast phytochemical test was used to determine the presence of certain classes of compound in the studied extracts and suggested that the extracts contained steroids, terpenoids, and alkaloids. However, further studies (isolation and structural elucidation) are needed to locate exact carriers of the observed antimicrobial activity.

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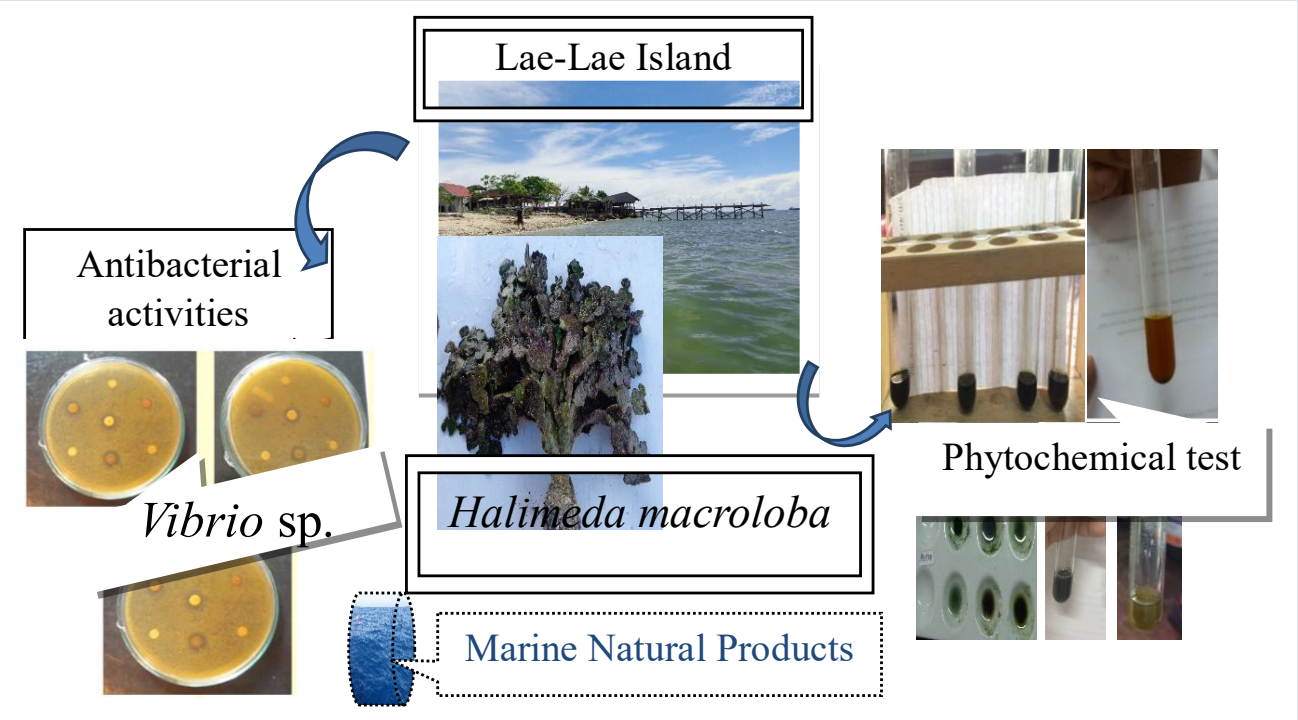
GRAPHICAL ABSTRACT



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