Inhibition effectivity of *Halimeda macroloba* seaweed extract against fish indigenous bacteria for safety fisheries product

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**Abstract.** Concerns about the potential for residual antibiotics can cause adverse health effects for consumers. The use of inappropriate antibiotics is feared to lead to antimicrobial resistance by pathogens. New sources of antibacterial can be obtained from bioactive compounds from seaweed, one of which is a *H. macroloba* type. The research objective was to determine the effectiveness of *H. macroloba* seaweed extract inhibition against indigenous bacteria in fish. *H. macroloba* samples taken from the waters of Pane Island. The extraction process was carried out by maceration method with methanol p.a solvent. The secondary metabolites were obtained with phytochemical test. Inhibition test with the Kirby-Bauer agar diffusion method through experimental by adding DMSO. *H. macroloba* have secondary metabolite compounds, there are alkaloids and flavonoids which a function as an inhibitory force for bacteria. Different treatment of *H. macroloba* extract resulted in a significant effect on *Vibrio parahaemolyticus* with an inhibitory value of 10.73 mm. Meanwhile, different treatment on *Aeromonas hydrophyla* and *Aeromonas salmonicida* was stated to have no significant effect in increasing the inhibition zone diameter. However, it was directly proportional to the increasing effectiveness of the inhibition zone diameter, the most effective treatment was at a concentration of 100%.

1. Introduction
The safety of fishery products is still an important issue to be addressed immediately. The safety of a product needs to be controlled from raw materials, processing to the final product. Currently fishery products made from raw materials from the aquaculture industry still need to undertake antibiotic residue tests, especially products to be exported. This indicates that there are fish cultivators who still use chemicals in the form of antibiotics to treat fish diseases caused by bacteria. The use of antibiotics that are not permitted in aquaculture production activities is the main cause of chemical contamination and rejection of fishery products.

There are two major groups of pathogenic bacteria that can contaminate aquaculture products during capture, namely indigenous bacteria and non-indigenous bacteria. Indigenous bacteria are bacteria that normally or accidentally enter the aquatic environment, these bacteria are referred to as native microflora, for example, among others, *Aeromonas hydrophyla*, *Clostridium botulinum*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Vibrio vulnificus* and *Listeria monocytogenes*. Meanwhile, non-
indigenous bacteria found in fish are bacteria that are present due to contamination from water or processing. The types are *Salmonella* sp., *Shigella* sp., *Escherichia coli*, *Edwardsiella* sp. and *Staphylococcus aureus*.

Indonesia has a good potential to develop and utilize marine resources, including seaweed. Seaweed contains primary and secondary metabolites. The potential of seaweed species growing in the intertidal zone of Pane Island is quite varied, there are 3 divisions of seaweed found, namely the Paeophyceae, Chlorophyceae and Rhodophyceae divisions. The Paeophyceae division is dominated by *Sargassum* sp., This is confirmed by [1] that *Sargassum* sp, from the Paeophyceae division can grow well on Pane Island due to the suitable substrate characteristics, namely dead coral, sand and a little mud. Apart from these divisions, there is also a Chlorophyceae division which also grows a lot in these waters, the type that grows is *Halimeda macroloba*. *H. macroloba* is a type of calcareous seaweed which is often found in coral reef areas with calm coastal conditions, somewhat protected and living in colonies [2]. In their natural habitat, the *Halimeda* sp. has the ability to excrete secondary metabolites in its metabolic process to defend itself from predators and pests. [3] The active ingredients released by *Halimeda* are very effective in preventing predators and bacteria.

Based on this background, it is very necessary to conduct a preliminary test to find out how effective the inhibitory power of *H. macroloba* seaweed extract can inhibit the bacteria that have often caused mass death in aquaculture production, in order to support the safety of food quality from fishery products.

2. Materials and methods

2.1. Collection and preparation of seaweed

Collection of *H. macroloba* from the intertidal zone of Pane island waters was done in June 2020 during the lowest tide and dry season. *H. macroloba* were collected directly while it is still firmly attached in that substrate. Initial sample preparation in the field was washing first the seaweed samples with seawater to remove the epiphytes and extraneous matter that were still attached, then it was hung on to reduce that moisture content. The collected macroalgae were transported to the laboratory in a cool box. Then second wash with fresh water in the laboratory. The seaweed is dried at room temperature for 5 days, after which it is mashed and becomes a fine powder.

2.2. Extraction of bioactives and phytochemical analysis

*H. macroloba* extraction using the maceration method for 2x24 hours. Extraction was carried out by inserting 500 grams of fine seaweed powder into the Erlenmeyer flask and adding 5 liters of methanol pa, then transferred to ten 200 mL volume bottles so that it is easy to stir the stirrer. After being macerated for 2 days the extract solution was filtered using filter paper, then evaporated using a rotary evaporator at a temperature of 44 °C. The resulting seaweed extract filtrate was stored in a closed vial bottle. Phytochemical analysis refers to [4] with the compounds tested including alkaloids, flavonoids, saponins, tannins, and steroids.

2.3. Extract concentration

The concentration of *H. macroloba* extract was obtained by suction using a micropipette. Determination of the total volume of the extract and solvent was 10 mL with a concentration of 20%, 40%, 60%, 80% and 100%. The solvent used to dilute the extract was DMSO (dimethyl sulfoxide) analyzer solvent. DMSO can dissolve almost all compounds, both polar and nonpolar, and does not have bactericidal activity so it does not interfere with the results of observations of antibacterial activity testing [5]. The comparison between the extract and DMSO can be seen in Table 1.
Table 1. Making concentration

| Treatment of concentration | H. macroloba extract (mL) | DMSO solvent volume (mL) |
|----------------------------|---------------------------|--------------------------|
| 20%                        | 2                         | 8                        |
| 40%                        | 4                         | 6                        |
| 60%                        | 6                         | 4                        |
| 80%                        | 8                         | 2                        |
| 100%                       | 10                        | 0                        |
| Control (+)                | Chloramphenicol           |                          |
| Control (-)                | Water                     |                          |

2.4. Indigenous bacteria strains
The bacteria used were derived from pure cultures with different strains. These indigenous bacteria include *Vibrio parahaemolyticus* (ATCC 17802), *Aeromonas salmonicida* (ATCC 33658), and *Aeromonas hydrophyla* (ATCC 35654). All of them were collected from the stock of Fish Quarantine and Inspection Agency Regional Surabaya. One ose of the bacteria from pure cultures was taken and then grown by inoculating it onto the surface of NA (Nutrient Agar) media. Pure cultures wrapped in plastic warp were then stored in an incubator for 1x24 hours at 37°C for *V. parahaemolyticus* and 30°C for *A. salmonicida* and *A. hydrophyla*. There was storage with this temperature aims to adjust the nature of pathogenic bacteria being tested. Food destroying microbes are usually classified as mesophyll microbes which grow well at an optimum temperature of 37°C.

2.5. Inhibition assay
The method was used the agar diffusion method according to [6]. Disc paper measuring 6 mm is placed on agar media that has been cultured by *V. parahaemolyticus, A. salmonicida* and *A. hydrophyla* bacteria. Then, the disc paper was dripped with extract concentrations of 20%, 40%, 60%, 80% and 100% on each petri dish. The petri dishes were wrapped using plastic wraps and stored in an incubator at 37°C for 1x24 hours. The inhibition of microorganism growth by antibacterial was seen as a clear area around the disc paper. Measurement of the resistance zone diameter was carried out using a caliper and was repeated 3 times.

2.6. Data analysis
Data were obtained through experimental laboratories with the one-way Anova method, then followed by the DNMRT test (Duncan New Multiple Range Test) to determine whether each treatment given had a significant effect on the test parameters.

3. Results and discussion
The main objective of this study was to evaluate the ability of the *H. macroloba* macroalgae species from Pane Island to produce bioactive compounds that have therapeutic potential. The analysis was done because the effectiveness of the antibacterial activity is considered to be an indicator seaweed ability to synthesize bioactive secondary metabolites.

3.1. Bioactive of seaweeds extracts
The bioactive compounds contained in *H. macroloba* were tested qualitatively based on changes in color or deposits formed in response to a given reagent. The presence of bioactive components in *H. macroloba* extract is presented in Table 2.

Based on the results of the phytochemical test, *H. macroloba* extract showed positive results against alkaloid compounds in Mayer, Wagner and Drangendoff reagents and flavonoid compounds. The active component is thought to have an antibacterial role in *H. macroloba*. This is in accordance with [7] that alkaloid and flavonoid compounds are also found in the *H. opuntia* type of Halimedaceae seaweed extract. Alkaloids function as antibiotics and anti-inflammatory which can reduce pain, improve blood
circulation, restore stamina after childbirth and prevent infection [8]. Although the number of flavonoids was not assessed in this study, previous results found the high flavonoid content in plants indicates high antibacterial activity [9].

Table 2. Test results of the secondary metabolite components of H. macroloba extract

| No. | Bioactive Compound | Observation results | Conclusion | Standard (color) |
|-----|--------------------|---------------------|------------|------------------|
| 1.  | Alkaloid           | Orange sediment formed | (+)        | Orange sediment formed (Dragendorff reagent) |
|     |                    |                     |            | Brown sediment formed (Wegner reagent)        |
|     |                    |                     |            | Yellow precipitate formed (Mayer reagent)     |
| 2.  | Flavonoids         | Yellow fluorescence | (+)        | 2 Intensive yellow fluorescence at UV366     |
| 3.  | Saponins           | Non-formed          | (-)        | The presence of foam that lasts <10 minutes as high as 1-10cm and the foam does not disappear after adding one drop of HCL 2N |
| 4.  | Tannin             | Non-formed          | (-)        | Dark blue/greenish black                     |
| 5.  | Steroids           | Non-formed          | (-)        | Greenish blue ring was formed                |

Description: (+) detected, (-) not detected

3.2. Effectiveness of extract inhibition

Bioactive compounds obtained from the extraction process are tested for them against bacteria. The results of the study were to see the antibacterial properties, incubation was carried out for 1x24 hours so that a clear zone was formed from each treatment of H. macroloba extract with the tested bacteria (Figure 1).

Description: A. Extract treatment against Vibrio parahaemolyticus bacteria, B. extract treatment against Aeromonas hydrophyla bacteria, C. extract treatment against Aeromonas salmonicida bacteria.

Figure 1. Inhibition zone of H. macroloba extract treatment against tested bacteria.

Based on the research that has been done, it shows that H. macroloba extract has inhibitory activity against V. parahaemolyticus (Figure 1A), A. hydrophyla (Figure 1B) and A. salmonicida bacteria (Figure 1C) with moderate to strong response. This response can be seen from the diameter value of the inhibitory power of H. macroloba extract against the tested bacteria. Referring to [10] that bacterial growth based on the diameter of the clear zone can be classified into four response groups, namely weak response with a diameter of ≤ 5 mm, moderate response with a diameter of 5-10 mm, strong response with a diameter of 10-20 mm, and very strong response with a diameter of ≥ 20 mm. The effectiveness of the inhibition of H. macroloba extract can be seen in Table 3.
3.2.1. The inhibitory effectiveness of H. macroloba extract against V. parahaemolyticus. According to Table 3 on V. parahaemolyticus bacteria, it was found that giving H. macroloba extract had a significant effect in increasing the clear zone in the growth of these bacteria (P < 0.05). Based on the results of the effectiveness test of increasing the diameter of the inhibition zone in V. parahaemolyticus bacteria in 20%, 40%, 60% and 80% treatment, it is included in the medium response category with diameter values of each treatment of 6.46 mm, 7.33 mm, 8.13 mm and 9.26 mm. However, in treatment 100% has a diameter of the inhibition zone with a strong response value of 10.73 mm. The results of this study were also supported by [11] who conducted an antibacterial study of the green algae species showing that H. macroloba extract with ethanol: n-hexane solvent was active against gram-positive E. coli bacteria with a diameter of 19 mm. In addition, according to [12] the factors affecting the size of the inhibition area are the sensitivity of the organism, the culture medium, the incubation conditions, and the agar diffusion rate. Moreover, other factors that influence agar diffusion include the concentration of microorganisms, media composition, incubation temperature, and incubation time [13].

The ability of H. macroloba extract to increase the inhibition zone diameter in V. parahaemolyticus bacteria is due to the ability of secondary metabolites in the form of phenolic compounds (alkaloids and flavonoids) that are owned by this type of macroalgae for a natural antibacterial potential. The formation of bacterial walls can be inhibited by secondary metabolites found in the extract, leading to the increase of the inhibition zone diameter. In line with [14], these studies have demonstrated that macroalga shows a positive relationship between antimicrobial activity potential and the number of phenolic compounds of the crude extract including flavonoids. Therefore, substances obtained from seaweeds are presumed as a source of bioactive compounds and they have been isolated substances have bacteriostatic and bactericidal properties [15].

3.2.2. The inhibitory effectiveness of H. macroloba extract against A. hydrophyla. Based on the results of the study, it was found that the addition of H. macroloba extract had no significant effect in increasing the inhibition zone on the growth of A. hydrophyla bacteria (P > 0.05). However, the addition of the given treatment concentration can also increase the diameter of the inhibition zone in the process of inhibiting the growth of the tested bacteria. These results indicate the existence of inhibitory activity of bacteria from H. macroloba extract. The treatment of H. macroloba extract with methanol solvent gave the highest effectiveness (100%). The evidence showed the formation of the highest inhibition zone diameter compared to other concentrations in inhibiting the growth of A. hydrophyla with an average value of 7.43 mm. The lowest effectiveness resulted from the treatment of the extract concentration of 20% with an average inhibition zone diameter of 7.03 mm. Based on this observation, the inhibitory value of H. macroloba extract against A. hydrophyla bacteria was classified in the moderate response category.

The finding result in this study is similar with the previous research [16]. The authors stated use of Halimeda gracilis extract with a concentration of 2 mg in Staphylococcus aureus bacteria formed an inhibition zone of 6.00 mm. According to [17] differences in the inhibition zone are caused by various

### Table 3. The effectiveness of the inhibition of H. macroloba extract against the tested bacteria.

| Treatment     | Replication | V. parahaemolyticus | A. hydrophyla | A. salmonicida |
|---------------|-------------|---------------------|---------------|---------------|
| C- (water)    | 3           | 0.00                | 0.00          | 0.00          |
| 20%           | 3           | 6.46b               | 7.03b         | 6.16b         |
| 40%           | 3           | 7.33bc              | 7.23b         | 6.90b         |
| 60%           | 3           | 8.13bc              | 7.37b         | 6.93b         |
| 80%           | 3           | 9.27cd              | 7.40b         | 7.13b         |
| 100%          | 3           | 10.73d              | 7.43b         | 7.30b         |
| C+ (Chlorampenicol) | 3       | 34.20a              | 26.00c        | 51.14c        |

Note: Numbers followed by the same letter in a column indicate insignificance.
factors, one of which is the composition of the cell wall of each bacterium. *S. aureus* bacteria are gram-positive whose cell walls consist of teichoic acid, theikoronic acid and several polysaccharide molecules, while bacteria gram negative cell wall consists of three main components: the outer membrane lipoproteins containing protein molecules called porins and lipopolysaccharides. Likewise, [18] reported that the arrangement of the cell walls of each type of bacteria also affects the diameter of the inhibition zone formed. *A. hydrophyla* is classified as a gram-negative bacterium, [19] the cell wall of gram-negative bacteria consists of multiple layers, making it difficult to penetrate, while gram-positive bacteria have simpler cell walls.

Other study [11] on an antibacterial study of the green algae species found that *H. macroloba* with ethanol: n-hexane solvent, was active against *E. coli*, which was shown by the formation of an inhibition zone with 19 mm in diameter. The slight difference result was presumably due to the use of different solvents. The inhibition zone reflects to the bioactive compounds contained in the seaweed extract. Others [20] stated that antibacterial activity is influenced by the concentration of the extract, the content of antibacterial compounds, the diffusion power of the extract, and the type of bacteria that is inhibited.

3.2.3. The inhibitory effectiveness of *H. macroloba* extract against *A. salmonicida*. The results showed that the addition of *H. macroloba* extract had no significant effect in increasing the zone of inhibition on the growth of *A. salmonicida* bacteria (P > 0.05). However, the addition of treatment concentrations was directly proportional to the increase in the inhibition zone during the bacterial growth process. These results prove the potential for antibacterial activity in *H. macroloba* extract. The most effective concentration of *H. macroloba* extract treatment in inhibiting bacterial growth was 100% treatment, with an inhibition zone value of 7.30 mm. While the concentration of the extract treatment which had the lowest inhibition zone was 20% treatment, with an inhibition zone value of 6.16 mm. The value of the effectiveness of the inhibition zone can be categorized as moderate response.

The inhibition zone value is still lower than the other results of [21] which used polysaccharides extract from green alga *Ulva fasciata* against *A. salmonicida* bacteria, where the resulting inhibition zone value was 13 mm. According to [20] the synthesis of antimicrobial compounds by seaweeds may depend on several factors, including geographical location, environmental factors and alga physiological conditions (active growth or sexual maturity).

4. Conclusions

Our results extend previous observations in macroalgae especially on the inhibition effectivity of green macroalgae species *H. macroloba* against indigenous bacteria of fish. Methanol extract from bioactive compounds of *H. macroloba* showed the potential for inhibition of indigenous bacteria in fish. The most extensive inhibitory effectiveness with strong response from *H. macroloba* extract was shown by *V. parahaemolyticus* bacteria than the other 2 test bacteria (*A. hydrophyla* and *A. salmonicida*). However, the addition of the concentration of *H. macroloba* extract against *A. hydrophyla* and *A. salmonicida* bacteria showed an increase in the area of inhibition. Our findings suggest that *H. macroloba* methanol extract represents a source of novel antibacterial compounds against *V. parahaemolyticus, A. hydrophyla* and *A. salmonicida* in fish. As an alternative compound for natural antibiotics as control of fish infection, further research can also be made regarding the body's response to the infected fish. Exploitation of macroalgae biomass as a source of antibacterial medicine will increase the economic resources of coastal communities.

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