Preliminary study of cultivated algae from South Sulawesi as antibacterial agent against fish pathogenic bacteria

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Abstract. The way to prevent or treating bacterial diseases outbreaks with drugs or chemicals is the one of main solutions to solve the problems in aquaculture. The use of antibacterial agents has increased significantly in aquaculture practices, since the antibiotics used in both human as well as veterinary medicines have been tried experimentally to treat bacterial infections of fish. However, the evolving resistance of fish pathogenic bacteria to existing antibiotics has necessitated development of new alternatives. In the last three decades the discovery of metabolites with biological activities from algae has increased significantly. In this study, sixteen extracts from four algae of genus Eucheuma were tested by in-vitro against five pathogenic bacteria (Aeromonas salmonicida, Aeromonas hydrophila, Pseudomonas anguilliseptica, Vibrio anguillarum and Yersinia ruckeri). The algae were extracted in Soxhlet apparatus using solvents with increased polarity (hexane, dichloromethane, methanol and water) for 24 h. The extract solutions were then evaporated and lyophilized before using for the antibacterial test with agar diffusion method. The results revealed that three of four extracts (hexane, dichloromethane and water) were active against all pathogens, while methanol did not. The highest activity was shown by water extract, followed by the hexane and the dichloromethane extracts with lower activities. The water extract of Eucheuma spinosum had a broad activity since it was active against four of five pathogenic strains. Vibrio anguillarum and Pseudomonas anguillaseptica were the two most susceptible pathogens, while the most resistant was presented by Aeromonas hydrophila. The screening results confirm that algae were potential to be developed as a source of antibacterial compounds or as a health-promoting food for aquaculture.

1. Introduction
The intensification of the system in aquaculture has had a significant impact on the spread of disease by pathogenic bacteria. This infectious disease can be due to high population density which results in poor water quality and horizontal transmission of pathogens among dense populations. Prevention efforts can be made by maintaining water quality and enhancing the immune system of cultivated organisms by administering supplements such as probiotics. The problem is, precautions do not always go as planned. Declining water quality can result in a weakening of the immune system of the cultivating organism, which makes it easier for pathogenic bacterial pathogens, which have been living together without any effect on their host, turning to attack their weak host.
Usually a pathogen infection and rapid transmission in a population can only be treated with the use of antibiotics [1]. The method with the use of antibiotics is the only way to overcome the infection of cultivated organisms. However, due to its easy use and farmers' lack of knowledge of its effects, the use of antibiotics is often carried out excessively, not only as a propylaxis effort, but has been used as a prevention effort by giving it through feed. This results in the accumulation of synthetic antibiotics, which are not recycled in the body of cultivated organisms, and accumulates in the area of aquaculture ponds. This problem will certainly become a major problem in the effort to intensify the cultivation system.

The sea occupies a vast area on the surface of the earth, which covers 2/3 of the earth's surface. Besides having a large area, the sea also has a diversity of biological resources that is very high compared to the mainland. About 80% of species on earth are found in the sea. But its potential has not been much studied because of obstacles in getting its source at sea. Scuba equipment and underwater survey vessels are still very limited, so research on the potential of underwater organisms is still experiencing problems.

One type of marine organism whose potential has long been known since ancient times is seaweed. Seaweed has been used for a long time as a treatment tool for various diseases due to its diverse secondary metabolite content. Because it is easily obtained and cultivated, seaweed has long been used as food from the sea, especially for people who live in coastal areas. Its content is rich in vitamins and minerals which has the potential to be used as nutritional supplements in human food and feed, as well as to work to improve the immune system of cultivated organisms. This advantage makes seaweed very potential to be used as a natural antibiotic substitute for synthetic antibiotics that have been used by farmers. Natural antibiotics are very low in side effects, even non-existent, because they are easily recycled in the body of both cultivated organisms and the environment.

Seaweed belongs to a group of macro-sized algae, so it is known as macroalgae. Seaweed or macroalgae consists of 3 classes, namely Chlorophyceae, Rhodophyceae and Phaeophyceae classes. One of the genera of commercial seaweed that has been widely cultivated to produce phycocolloid content that is needed in the world for the food, beverage, pharmacy and cosmetics industries is seaweed from the genus *Eucheuma*. The genus *Eucheuma* belongs to the group of red seaweed (Rhodophyceae) which produces carraginan type phycocolloid. The species used in this study are from the genus *Eucheuma* that has been cultivated in South Sulawesi, namely *Eucheuma cottonii* or better known as *Kappaphycus alvarezii*, *Eucheuma denticulatum*, *Eucheuma spinosum*, and *Eucheuma edule*.

2. Material and Methods

2.1. Seaweed Preparation

Seaweed samples used in this study consisted of 5 species of the genus *Eucheuma*, which were taken from different locations. Sample of *Eucheuma cottonii* are from Jeneponto, *Eucheuma edule* from Bone, *Eucheuma denticulatum* from Takalar, *Eucheuma spinosum* from Jeneponto. Seaweed samples were obtained in dry form. In the laboratory the sample was then washed again using tap water to remove salt and then rinsed with distilled water. Samples that have been cleaned of impurities and salts were lyophilized until dry and then mashed into powder using a grinder.

2.2. Seaweed Extraction

Sample extraction was done sequentially using soxhlets and solvents of different polarity, starting with hexane, followed by DCM, Methanol and finally with water. The extraction time is 24 hours. After extraction, the extract solution was then filtered using Whatman filter paper No. 1, the filtrate solution was evaporated using a rotary evaporator. The residue was left overnight and then used for extraction with dichloromethane (DCM). The same extraction process was carried out up to the water solvent. Crude extract was put in a vial and stored in cold temperatures until used. For water extract, it was first evaporated with a rotary evaporator, then followed by lyophilization.

2.3. Pathogenic Bacteria Test
The pathogenic species used in this test consisted of 5 species of fish pathogenic bacteria, such as *Aeromonas salmonicida, Aeromonas hydrophila, Pseudomonas anguilliseptica, Vibrio anguillarum*, and *Yersinia ruckeri*. These pathogenic bacteria come from the collection of test cultures of Ernst Moritz Arndt University (EMAU), Greifswald, Germany.

### 2.4. Antibacterial Activity Test

The antibacterial activity test was carried out using the diffusion method which had been done previously by Zainuddin [2–4]. A total of 2 mg of crude extract was redissolved in 50 µL of each extraction solvent, then dropped on a paper disk with a diameter of 6 mm aseptically, and then left until the solvent dries in a laminar box. Test bacteria were prepared by rejuvenating isolates one day before use. The test bacteria were incubated at 28ºC for 24 hours and then used to test the antibacterial activity by the agar diffusion method. A total of 1 bacterial loop was taken from a Petri dish containing a rejuvenated isolate, and then dissolved in 2 mL of 0.9% NaCl physiological solution. The isolate solution was then taken as much as 200 µL and then placed in a glass baker containing 20 mL of warm TSA media. After mixing evenly, the agar solution contains test bacteria, then poured into a 9 mm diameter petri dish. Petri dishes containing agar solution and bacteria were allowed to cool before being stored in a reversed position in an incubator at 28ºC for 24 hours. As a positive control, 20 µg / disk of oxytetracyclin was used. For negative controls, 50 µL / disk of each solvent was used. The level of antibacterial activity is categorized as follows: very high ≥20 mm, high 15-<20 mm, moderate 10-<15 mm, low> 6-<10 mm, no activity = 6 mm

### 3. Results

Seaweed samples used in this study were taken from several seaweed cultivation locations in South Sulawesi, pictures of *Eucheuma* species were performed in the following figure (Figure 1).

![Eucheuma edule (EE)](image1)
![Eucheuma denticulatum (ED)](image2)
![Eucheuma spinosum (ES)](image3)

**Figure 1.** *Eucheuma* species commercially cultivated in South Sulawesi
In addition to seaweed sample, bacteria that were used in this study are pathogenic bacteria which cause various diseases in cultivation organisms, as shown in the figure below (Table 1).

**Table 1.** Fish diseases caused by pathogenic bacteria and their symptoms

| Pathogens                        | Diseases          | Symptoms                        |
|----------------------------------|-------------------|---------------------------------|
| *Vibrio anguillarum*             | Vibrosis          | haemorrhage erythema ulcer      |
| *Pseudomonas anguilliseptica*    | Red spot          | dark skin haemorrhage ulcer septicemia |
| *Aeromonas salmonicida*          | Furunculosis      | haemorrhage lesion ulcer        |
| *Aeromonas hydrophila*           | MAS (Motile Aeromonas Septicemia) | Haemorrhage Septicemia ulcer fin necrosis exophthalmus |
| *Yersinia ruckeri*               | Redmouth disease  | Hemorrhages on oral cavity discoloration anorexia |

From the results of antibacterial activity tests on 16 extracts obtained from 4 species of the genus *Eucheuma*, it was seen that very high activity was shown by water extracts from *E. spinosum* against *Pseudomonas anguilliseptica* with inhibition zone diameter of 34.73 mm (Figure 2). Also very high activity was demonstrated by water extracts from *E. cottoni* with inhibition zone diameter of 26.37 mm.
The other two extracts, each hexane extract from *E. edule* and *E. denticulatum*, have only low activity, with inhibition zone diameters of 8.0 mm and 8.5 mm respectively.

![Figure 2. Antibacterial activity of seaweed extracts against pathogenic bacteria *Pseudomonas anguilliseptica* (EC = Eucheuma cottonii; EE = Eucheuma edule; ES = Eucheuma spinosum; ED = Eucheuma denticulatum; Hex = n-hexane extract; H$_2$O = water extract; N = 3, Value = average +SD)](image)

The seaweed extract activity test on *Aeromonas salmonicida* showed very high activity by water extract of *E. denticulatum* with a diameter of inhibition zone of 29.37 mm. A very high activity was also shown by water extracts from *E. edule* with inhibition zone diameter of 21.17 mm and high level activity was shown by water extracts from *E. spinosum* with 16.77 mm (Figure 3). Only low activity was demonstrated by DCM extracts from *E. edule* and *E. denticulatum* with inhibition zone diameters of 8.50 and 8.20 mm, respectively.

![Figure 3. Antibacterial activity of seaweed extracts against pathogenic bacteria *Aeromonas salmonicida* (EE = Eucheuma edule; ES = Eucheuma spinosum; ED = Eucheuma denticulatum; DCM = dichloromethane extract; H$_2$O = water extract; N = 3, Value = average +SD)](image)
The inhibition assay of *Eucheuma* extract on the bacterium *Yersinia ruckeri* showed a very high activity on the water extract from *E. denticulatum* with inhibition zone diameter of 25.83 mm. Followed by high activity of water extract from *E. spinosum* with inhibition zone diameter of 17.0 mm. The water extract of *E. edule* showed a moderate activity with a diameter of inhibition zone of 12.50 mm. Both dichloromethane extracts of *E. edule* and *E. denticulatum* only showed low activities with each inhibition zone diameters of <10 mm (Figure 4.).

![Figure 4](image-url)  
**Figure 4.** Antibacterial activity of seaweed extracts against pathogenic bacteria *Yersinia ruckeri* (EE = *Eucheuma edule*; ES = *Eucheuma spinosum*; ED = *Eucheuma denticulatum*; DCM = dichloromethane extract; H2O = water extract; N = 3, Value = average +SD)

A moderate activity against *Vibrio anguillarum* was shown by water extract of *E. spinosum* with inhibition zone diameter of 15.9 mm, followed by hexane extract from *E. denticulatum* with a low activity (8.4 mm diameter), and hexane extract of *E. edule* with diameter of 7.0 mm (Figure 5).

![Figure 5](image-url)  
**Figure 5.** Antibacterial activity of seaweed extracts against pathogenic bacteria *Vibrio anguillarum* (EE = *Eucheuma edule*; ES = *Eucheuma spinosum*; ED = *Eucheuma denticulatum*; Hex = n-hexane extract; H2O = water extract. N = 3, Value = average +SD)
4. Discussion

*Eucheuma* seaweed is commercial seaweed cultured in tropical climates to supply the world's carrageenans needs. The genus *Eucheuma* is a member of the red algae group or Rhodyphyceae which is easy to cultivate and has economic value for countries such as Indonesia, the Philippines, Malaysia, Tanzania and Madagascar [5]. In addition to its benefits to produce carrageenan, *Eucheuma* seaweed has also been widely studied for its antimicrobial potential, especially as an antibacterial agent [6].

The content of carrageenan phycocolloid produced by the red algae group of the genus *Eucheuma* is suspected to be an anti-infective agent against pathogenic bacteria. From the results of this study it was seen that generally polar water extracts, which have high activity in inhibiting all test pathogenic bacteria except *Aeromonas hydrophila*. Carrageenan is a polysaccharide that has sulfate bonds, dissolves in water and easily binds water at room temperature. In this study *E. cottoni* which has the kappa-carrageenan shows very high activity only to one pathogenic bacterium *Pseudomonas anguillaseptica*. Utilization of k-carrageenan as an antibacterial revealed the damage of bacterial cell wall and cytoplasmic membrane and suppress the growth of both Gram-positive and Gram-negative bacteria [7].

Very high activity of water extracts of various species of *Eucheuma* against *Pseudomonas angilaseptica*, *Aeromonas salmonicida* and *Yersinia ruckeri*, indicates that secondary metabolites that have antibacterial activity found in water extracts are a group of very polar metabolites. Unfortunately in our study no activity was found from all species of the genus *Aeromonas*, except *A. hydrophila*. This is consistent with the results of Sulistyaningisih et al [8] who reported that ethanolic extract of *E. cottoni* was active against *S. aureus*, *B. subtilis* and *S. epidermidis* at a concentration of 30, 40, 50 and 60% (w/v). Ethanolic extract of *E. spinosum* at concentrations of 1%; 2%; 5%, and 10% (v / v) resulting in inhibition zones of 1.98; 4.14; 7.42; and 10.27 mm, respectively. The MIC value of *E. spinosum* extract against *S. aureus* was 10%.
The low potential of n-hexane and DCM extracts from the genus *Eucheuma* in our study, is in accordance with the results of Fattah [20], where hexane extract of *E. spinosum* did not inhibit *Vibrio cholerae* and *Staphylococcus aureus*. Methanol and ethanol extracts had activity in concentrations of 0.4%, 4% and 40% against *Vibrio cholerae* and *Staphylococcus aureus*. A higher inhibition zone was found in methanolic extract with a concentration of 40% against *Staphylococcus aureus* [20]. The inhibition zone of methanolic extract of *E. spinosum* against *Staphylococcus aureus* and *Escherichia coli* at concentrations of 80 mg/mL was 4 mm and 3 mm, respectively. Phytochemical analysis of methanol extract of *E. spinosum* revealed flavonoids, triterpenoids and ascobic acid as main compounds [21]. Siregar et al. [22] also reported that methanol extract from *Euchema* sp. contains alkaloid compounds, while n-hexane extract contains steroid compounds.

Activities from *Eucheuma serra* which was collected from Warambadi seashore of Sumba Island were also reported by Anggadiredja [23]. *E. serra* produced a variety of compounds with antibacterial activities which consist of 3 fatty acids, 3 steroids, and 2 aldehydes. All compounds were active against Gram-positive bacteria *Bacillus subtilis* and 2 compounds showed active against Gram-negative *Escherichia coli*. No activity was detected in all compounds against *Pseudomonas aeruginosa* and *Salmonella typhimurium*.

Overall, the content of bioactive compounds in macroalgae has the potential to be developed into antibiotic drugs, because these compounds can inhibit the growth of pathogenic microorganisms [24]. Steroids or triterpenoids also have the ability to inhibit bacterial growth through the mechanism of inhibiting protein synthesis that results in changes in the components of the bacterial cell [22]. Terpenoid compounds which tend to dissolve in lipids will more easily penetrate the cell walls of Gram positive and negative bacteria [25]. Phenolic compounds are compounds that are known to have antimicrobial, anti-inflammatory, antiviral and anticancer activities [26,27]. Saponin has been exploited biologically as an antioxidant, anti diabetic and antiobesity, antibacterial and anticancer [28].

### 5. Conclusion

Besides having a high commercial value of the phycocoloid content, especially the carrageenan, red seaweed of the genus *Eucheuma* has also been proven to be potentially used as a natural antibiotic. The application can be by immersion method or oral administration as a dietary supplement for cultivation organisms.

Its activity is very high in polar extracts or water extracts, very beneficial and easy for farmers to use in the field. Water is a safe solvent and does not cause side effects like other extracts, especially in its application in the field. In addition, the cost is cheap when compared to the price of synthetic antibiotic drugs or the price of organic solvents. To use it in the form of natural antibiotic preparations, further research is needed to isolate the secondary metabolite compounds by guided isolation method and identification of the pure compounds with various methods of chromatography and NMR.

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### References

[1] Thakur A B, Vaidya R B and Suryawanshi S A 2003 Pathogenicity and antibiotic susceptibility of Vibrio species isolated from moribund shrimps *Indian J. Mar. Sci.* 32 71–5

[2] Zainuddin E N 2006 *Chemical and biological investigations of selected cyanobacteria (Blue-Green Algae)* (PhD Thesis). Greifswald (DE): University Greifswald

[3] Zainuddin E N, Anshary H, Huyyirnah H, Hiola R and Baxa D V 2019 Antibacterial activity of Caulerpa racemosa against pathogenic bacteria promoting “ice-ice” disease in the red alga
Gracilaria verrucosa J. Appl. Phycol. 31 3201–12

[4] Zainuddin E N 2010 Antibacterial potential of marine algae collected from South Sulawesi coast against human pathogens Proceedings of International Conference and Talkshow on Medicinal Plants (Jakarta, Indonesia)

[5] Campbell R and Hotchkiss S 2017 Carrageenan industry market overview Tropical seaweed farming trends, problems and opportunities (Springer) pp 193–205

[6] Wiyanto D B 2010 Uji aktivitas antibakteri ekstrak rumput laut Kappaphycus alvarezii dan Eucheuma denticulatum terhadap bakteri Aeromonas hydrophila dan Vibrio harveyii J. Kelaut. Indones. J. Mar. Sci. Technol. 3 1–17

[7] Zhu M, Ge L, Lyu Y, Zi Y, Li X, Li D and Mu C 2017 Preparation, characterization and antibacterial activity of oxidized κ-carrageenan Carbohydr. Polym. 174 1051–8

[8] Kandhasamy M and Arunachalam K D 2008 Evaluation of in vitro antibacterial property of seaweeds of southeast coast of India African J. Biotechnol. 7

[9] Kim I H and Lee J-H 2008 Antimicrobial activities against methicillin-resistant Staphylococcus aureus from macroalgae J. Ind. Eng. Chem. 14 568–72

[10] Singh M, Manikandan S and Kumaraguru A K 2012 In vitro antibacterial activity of selected brown marine macroalgae extracts collected from the Pudumadam Coast of “Gulf of Mannar” region against fish pathogens Int J Hum. Genet Med Biotechnol Microbiol Stud. ISSN 1732–2319

[11] Kolanjinathan K and Stella D 2009 Antibacterial activity of marine macro algae against human pathogens Recent Res. Sci. Technol. 1 20–2

[12] Karthikaidevi G, Manivannan K, Thirumaran G, Anantharaman P and Balasubramanian T 2009 Antibacterial properties of selected green seaweeds from Vedalai coastal waters; Gulf of Mannar marine biosphere reserve Glob. J. Pharmacol. 3 107–12

[13] Thirunavukkarasu R, Pandiyan P, Balaraman D, Subaramaniyan K, Edward Gnana Johi G E, Manikkm S and Sadaiyappan B 2013 Isolation of bioactive compound from marine seaweeds against fish pathogenic bacteria Vibrio alginolyticus (VA09) and characterisation by FTIR J. Coast. Life Med. 1 26–33

[14] Mattulada I K, Trilaksana A C and Abduh D A 2018 Efektivitas antibakteri ekstrak alga merah (Eucheuma spinosum) untuk menghambat pertumbuhan bakteri Porphyromonas gingivalis (Antibacterial Effectivity of red algae extract (Euchema spinosum) to inhibit the growth of bacteri Porphyromonas gingivalis) MDJ (Makassar Dent. Journal) 7 40–5

[15] Soelama H J J, Kepel B J and Siagian K V 2015 Uji minimum inhibitory concentration (MIC) ekstrak rumput laut (Eucheuma cottonii) sebagai antibakteri terhadap Streptococcus mutans e-GiGi 3

[16] Choudhury S, Sree A, Mukherjee S C, Pattnaik P and Bapuji M 2005 In vitro antibacterial activity of extracts of selected marine algae and mangroves against fish pathogens Asian Fish. Sci. 18 285–94

[17] Hanin N N F and Pratiwi R 2017 Kandungan Fenolik, Flavonoid dan Aktivitas Antioksidan Ekstrak Daun Paku Laut (Acrostichum aureum L.) Fertil dan Steril di Kawasan Mangrove Kulon Progo, Yogyakarta J. Trop. Biodivers. Biotechnol. 2 51–6

[18] Iskandar Y, Halimah E, Shifa N, Fushiilla Z, Silvana G and Zuhrotun 2019 Activity of Red Algae (Eucheuma Cottonii) against some Bacteria and Fungi J. Pharm. Sci. Res. 11 2362–6

[19] Safitri A, Srihardyastuti A, Roosdiana A and Sutrisno S 2018 Antibacterial Activity and Phytochemical Analysis of Edible Seaweed Eucheuma spinosum Against Staphylococcus aureus J. Pure Appl. Chem. Res. 7 308–15

[20] Fattah A 2013 Affectivity of Red Alga Eucheuma spinosum as Pathogenic Antibacterial in Coastal Organisms and Human (Hasanuddin University)

[21] Hanapi A, Fasya A G, Mardiyah U and Miftahurrahmah M 2013 Uji aktivitas antioksidan dan antibakteri ekstrak metanol alga merah Eucheuma Spinosum dari Perairan Wongsorejo Banyuwangi Alchemy 2 126–37
[22] Siregar A F, Sabdono A and Pringgenies D 2012 Potensi antibakteri ekstrak rumput laut terhadap bakteri penyakit kulit Pseudomonas aeruginosa, Staphylococcus epidermidis, dan Micrococcus luteus J. Mar. Res. 1 152–60

[23] Anggadiredja J T 2011 Diversity of antibacterial compounds from Eucheuma serra, Halimeda opuntia, and Hydroclathrus clathratus J. Teknol. Lingkung. 12 131–42

[24] Salosso Y and Jasmanindar Y 2018 Diversity of brown macroalgae in Kupang Bay waters and alginate content potential and its phytochemistry Aquac. Aquarium, Conserv. Legis. 11 598–605

[25] Rosyidah K, Nurmuhaimina S A, Komari N and Astuti M D 2012 Aktivitas antibakteri fraksi saponin dari kulit batang tumbuhan kasturi (Mangifera casturi) Alchemy

[26] Aliyu A B, Musa A M, Sallau M S and Oyewale A O 2009 Proximate composition, mineral elements and anti-nutritional factors of Anisopus mannii NE Br.(Asclepiadaceae). Trends Appl. Sci. Res. 4 68–72

[27] Setyorini H B and Maria E 2020 Analisis kandungan fitokimia pada berbagai jenis makroalga di Pantai Jungwok, Kabupaten Gunungkidul, Yogyakarta. Saintek Perikanan Saintek Perikan. Indones. J. Fish. Sci. Technol. 16 15–21

[28] Feroz B 2018 Saponins from Marine Macroalgae: A Review J Mar Sci Res Dev 8