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Antimicrobial Activities of *Eucheuma cottonii* Extract: Marine Cultivation in Sumenep, East Java, Indonesia

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ABSTRACT

Eucheuma cottonii is a valuable marine algae species widely cultivated in Sumenep, Madura. Recent research on the biochemical characterization of various marine algae species has identified active anti-microbial polyphenols and their derivatives, such as flavonoids. These compounds possess anti-inflammatory and anti-microbial properties. Organisms with sufficient levels of polyphenols and flavonoids tend to have an enhanced immunity against bacterial and viral infections. As a result, *Eucheuma cottonii* has the potential to serve as a natural source for new medicines derived from the marine resources of Madura Island. This study aimed to optimize the use of marine biological resources, specifically *Eucheuma cottonii*, as the main ingredient for anti-microbial drugs. The research was conducted between May and November 2021 at the Integrated Laboratory of the University of Trunojoyo Madura. The stages of the research included sampling *Eucheuma cottonii*, extracting its active compounds, and testing the anti-microbial activity against *Aeromonas hydrophila*. The results showed that the average yield of *Eucheuma cottonii* extract ranged from 2.978% to 3.296%. The extract demonstrated anti-microbial properties, as evidenced by the formation of inhibition zones in *Aeromonas hydrophila* colonies.

INTRODUCTION

The public is familiar with land-based medicinal plants such as the red betel (*Piper ornatum*), red ginger (*Zingiber officinale* Linn. var. *rubrum*), chili herbs (*Piper retrofractum*), and aloe vera (*Aloe vera*), while the benefits of sea algae are less well known **(Sartika *et al.*, 2013)**. *Eucheuma cottonii* is the leading commodity marine algae in Madura which has been widely cultivated in Sumenep. People have known *Eucheuma cottonii* as a raw material for gelatin and carrageenan. In fact, *Eucheuma cottonii* cells

contain active compounds resulting from primary and secondary metabolism. These active compounds are very useful for health supplements and medicines. Triterpenoids, steroids and phenolics are active anti-microbial compounds in a wide spectrum **(Sari *et al*., 2018)**.

The results of a recent study on the biochemical characterization of several species of algae found that *Eucheuma cottonii* contains active compounds of polyphenols and flavonoids **(Fajarningsih *et al*., 2018)**. Polyphenols and flavonoids possess anti-inflammatory and anti-microbial properties. Anti-microbials have the ability to destroy bacteria, viruses, fungi, and protozoa in organisms, as well as microbes on the surfaces of objects such as tables, chairs, doorknobs, and stairs **(Hughes & Mcloughlin, 2019)**. Anti-microbial contains biocidal compounds that can destroy the bacteria, viruses and germs. Polyphenols and flavonoids are able to destruct cell walls and membranes, so that they can disrupt the permeability of bacterial and viral cells. Organisms that already contain sufficient polyphenols and flavonoids can have immunity against bacterial and viral infections.

Polyphenols and flavonoids make *Eucheuma cottonii* a promising source of new natural medicines derived from the marine biological resources of Madura. The vast sea area around Madura holds enormous potential for medicinal algae **(Arisandi *et al*., 2017)**. Various marine algae species thrive along the coast and the sea of Madura Island. The abundance of seaweed on Madura Island presents a valuable source of secondary metabolites, which have great potential as raw materials for the pharmaceutical industry. The bioactive compounds produced by seaweed are primarily used to protect the algae from pests and diseases. Extracts from *Eucheuma cottonii* have been shown to inhibit the proliferation of both Gram-positive and Gram-negative bacteria **(Fajarningsih *et al*., 2018)**. However, none of the algae species cultivated in Madura have yet been utilized as raw materials for medicine. Based on this background, the current research focuses on testing the anti-microbial compounds derived from seaweed cultivated in Sumenep.

MATERIALS AND METHODS

The research was conducted from May to November 2021 at the University of Trunojoyo Madura Integrated Laboratory. *Eucheuma cottonii* were taken from the seaweed cultivation center in Saronggi Village, Bluto District, Sumenep Regency. The research utilized various equipment for collecting and cleaning *Eucheuma cottonii* samples, extraction and formulation of antiseptics, and anti-microbial testing. The materials used in the study included 20kg of *Eucheuma cottonii*, extractive agents and antiseptic formulations, as well as *Aeromonas hydrophila* and bacterial culture materials.

Extraction of *Eucheuma cottonii*

The extraction process began by preparing the necessary equipment and materials. The *Eucheuma cottonii* samples were thoroughly washed using clean water. Once

cleaned, the samples were cut into approximately 1cm pieces and dried in an oven at 50°C. After drying, the samples were ground into a fine powder, which was then macerated using ethanol, methanol, or a 1:1 mixture of ethanol and methanol for 48 hours. The maceration results were filtered through filter paper to separate impurities. The filtrate containing the Eucheuma cottonii extract was collected in an Erlenmeyer flask. The extract was then evaporated using a rotary evaporator at 45°C. Finally, the evaporated extract was heated in an oven at 50°C for around three hours to ensure the complete removal of any solvent trapped in the active compounds **(Fajarningsih *et al.*, 2015)**.

Testing of anti-microbial compounds

The testing of anti-microbial compounds involved preparing an anti-microbial solution for the disc diffusion test, with concentrations of 0ppm (control) and 500ppm. A liquid culture of *Aeromonas hydrophila* was then distributed evenly over the surface of the agar medium in a petri dish. After allowing 15-30 minutes for the culture to settle, disc paper impregnated with the anti-microbial compound was carefully placed on the agar surface. The plates were incubated at 35°C for 24 hours, after which the diameter of the inhibition zone around the disc was measured using a caliper. The incubation was extended to 48 hours to assess the properties of the antimicrobial compounds. If the inhibition zone remained clear after 48 hours, the compound was determined to be bactericidal. If the area became overgrown with bacteria, the compound was classified as bacteriostatic **(Walidah *et al.*, 2014)**.

Research parameters

a) The yield of *Eucheuma cottonii*

The percentage by weight of anti-microbial compounds contained in *Eucheuma cottonii* was calculated using the formula:

$$\text{Anti-microbial compound (\%)} = \frac{Extract\ Weight(gr)}{Dried\ Seaweed\ Weight\ (gr)} \text{x } 100\%$$

b) Anti-microbial activity

Anti-microbial activity test aims to prove that the active compounds contained in *Eucheuma cottonii* extract have anti-microbial activity. The results of the anti-microbial activity test were an inhibition zone for bacterial growth which had a diameter of several millimeters (mm). The diameter of the inhibition zone was measured using a caliper. The inhibition zone formed indicated that the tested *Eucheuma cottonii* extract contained an antimicrobial activity. The effectiveness of antimicrobial compounds can be determined by calculating the rate of formation of the inhibition zone for bacterial growth using the formula:

$$ADZ = \sqrt[t]{\left\{\frac{dt}{do} - 1\right\}} \text{ x } 100\%$$

description: *ADZ* = average daily inhibition zone diameter
do = initial diameter (mm)
dt = final diameter (mm)
t = observation time (day)

RESULTS

Yield of anti-microbial compounds

The yield of *Eucheuma cottonii* extract was obtained from samples extracted using 100% methanol. The first step involved calculating the water content to ensure it met the Indonesian National Standard (SNI). If the moisture content of the *Eucheuma cottonii* sample was within the acceptable range, the sample could be used as the main ingredient for the extract.

According to the Indonesian National Standard 2690, high-quality *Eucheuma cottonii* samples should have a maximum water content of 30% **(Nosa *et al.*, 2020)**. The moisture content of the *Eucheuma cottonii* samples collected from the seaweed cultivation center in Saronggi Village, Saronggi District, Sumenep Regency was 19%, which complies with the SNI for dried seaweed. This moisture content is notably lower compared to the 76.15% reported in the study by **Maharany *et al.* (2017)**. The variation in moisture content is significantly influenced by factors such as the quality of the material, environmental conditions, storage methods, temperature, and humidity.

The average yield of the *Eucheuma cottonii* extract, when using three types of solvents (ethanol, methanol, and a 1:1 mixture of ethanol and methanol), ranged from 2.978 to 3.296% (Table 1).

Table 1. Extract yield of *Eucheuma cottonii*

No	Solvent	Extract weight (gr)	Yield (%)
			Repetition 1
1	Ethanol	1.754	1.816
2	Methanol	1.774	1.980
3	Ethanol : Methanol (1:1)	1.509	1.636
	Average 1	**1.679**	**1.811**
			Repetition 2
1	Ethanol	1.218	1.308
2	Methanol	4.141	4.647
3	Ethanol : Methanol (1:1)	7.468	8.389
	Average 2	**4.276**	**4.781**
	Average 1 & 2	**2.978**	**3.296**

Activity of anti-microbial compounds

The results of observations concerning the bacterial colonies previously given *Eucheuma cottonii* extract for 48 hours showed that there was an activity of anti-microbial compounds in the form of a clear zone around the bacterial colonies. The clear zone formed proves that *Eucheuma cottonii* extract produces chemical compounds that can inhibit the colonization of *Aeromonas hydrophila*. These chemical substances have anti-microbial activity which can be in the form of antibiotics, pigments, toxins, and inhibitory enzymes (Fig. 2) **(Kausalya & Rao, 2015)**. The increase in the diameter of the anti-bacterial inhibition zone formed by the extract of the red algae (*Eucheuma cottonii*) proved the strong inhibition of the growth of the test bacterial colonies **(Bhuyar *et al.*, 2020)**. The inhibitory of *Eucheuma cottonii* extract is closely related to the process of bacterial colonization, namely the ability to adhere, motility, and chemotaxis of bacteria to nutrients and organic matter. **Hamijaya *et al.* (2014)** confirmed that the inhibition of the growth of bacterial colonies by anti-microbial compounds can occur because there is a process of cell destruction, namely inhibiting the formation of cell walls, damaging the permeability of the cytoplasmic membrane, changing protein molecules and nucleic acids.

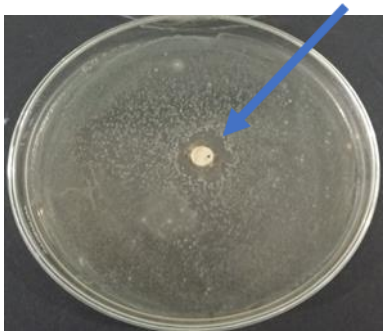

Fig. 1. Inhibition zone around *Aeromonas hydrophila* colonies

Based on Fig. (1), the test results for the activity of anti-microbial compounds vary. This variation is due to the differing abilities of bacteria to resist the effects of these compounds, which are influenced by the thickness and composition of their cell walls. *Aeromonas hydrophila* is a Gram-negative bacterium with a cell wall primarily composed of porin and lipopolysaccharide **(Sinurat *et al.*, 2019)**. The porin in the outer membrane of Gram-negative bacteria has hydrophilic properties, making it more difficult for the hydrophobic molecules of ***Eucheuma cottonii*** extract to penetrate into the bacterial cell wall (**Bhuyar *et al.*, 2020)**. The hydrophobic nature of the extract contrasts with the hydrophilic porin, creating a barrier to penetration. Additionally, the multi-layered arrangement of the Gram-negative bacterial cell wall can influence the size of the inhibition zone, as it makes the wall more challenging to penetrate (Table 2).

Table 2. The results of testing the activity of anti-microbial compounds

No	Repetition	Bacteria	Solvent		
			Ethanol	**Methanol**	**Ethanol: Methanol**
	24 hours				
	1.	*A. hydrophila*	(+)	(+)	(+)
	2.	*A. hydrophila*	(+)	(+)	(+)
	48 hours				
	1	*A. hydrophila*	(-)	(+)	(+)
	2	*A. hydrophila*	(+)	(+)	(-)

DISCUSSION

Table (1) shows that the average yield obtained from the first repetition was 1.679 grams (1.811%), while the second repetition yielded an average of 4.276 grams (4.781%). The average yield of *Eucheuma cottonii* extract in this study is relatively lower compared to the results reported by **Maharany *et al.* (2017)**, who obtained an average yield of 6.6% using methanol as a solvent. The differences in extraction results are likely due to variations in the characteristics of *Eucheuma cottonii* samples from different locations, as environmental factors can influence metabolic processes in the organisms.

The extracts obtained in this study were dark green, paste-like, and had a relatively mild odor. The texture resulted from maceration with methanol, followed by distillation using a rotary evaporator and a 5-hour evaporation process at 40°C in an oven. The extraction process was carefully conducted to maximize the separation of the solvent from the pure extract. The extraction method used aligns with that of **Maharany *et al.* (2017)**, resulting in a paste-like form of the extract with a relatively small yield. Despite the small yield, the extract has a relatively high selling price. The demand for *Eucheuma cottonii* extract as an anti-microbial product is expected to be high once it is marketed (Fig. 2).

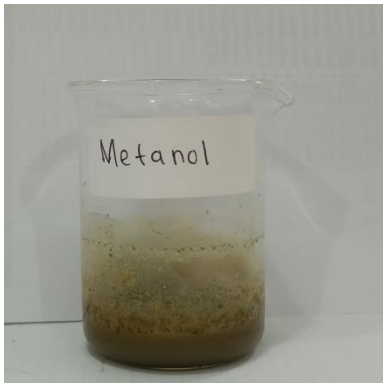

Fig. 2. Extract of *Eucheuma cottonii*

The activity of anti-microbial compounds was indicated by the formation of an inhibition zone in the bacterial culture medium. The inhibition zone was formed because bacteria were unable to grow in that area due to the presence of anti-microbial compounds produced by *Eucheuma cottonii* extract. Observation of the activity of anti-microbial compounds derived from *Eucheuma cottonii* extract for 48 hours showed an increase in the diameter of the inhibition zone. This means that *Eucheuma cottonii* extract was relatively effective in inhibiting the growth of the bacterial population, as indicated by the increase in the diameter of the bacterial inhibition zone (Table 3).

The increase in the population of *Aeromonas hydrophila* was indicated by the increasing width and thickness of the colony area. If a certain part of the culture media does not contain a population of bacteria, it indicates that there are compounds that inhibit growth and development. This is indicated by the part of the bacterial culture media that has been given *Eucheuma cottonii* extract. Bacteria cannot grow and the diameter of the inhibition zone increases within 48 hours. The increase in the diameter of the inhibition zone proved that *Eucheuma cottonii* extract was proven to be effective in destroying bacteria, so it could be used as a medicine for diseases caused by bacteria. When compared to the research conducted by **Sari *et al.* (2018)**, the data from this study show a different range of inhibition zone diameters for *Eucheuma cottonii* extract. **Sari *et al.* (2018)** reported that the inhibition zone diameters against *Bacillus cereus* were 5.82 mm at 100% concentration, 3.9mm at 75% concentration, and 8.6mm at 50% concentration. In contrast, the inhibition zone diameter observed in this study for a 50% concentration was 4.5mm, which is greater than the 3.9mm reported by **Sari *et al.* (2018)** for the same concentration. This indicates that the *Eucheuma cottonii* extract in this study exhibited a relatively higher anti-microbial activity compared to the previous research.

Table 3. Results of testing the effectiveness of *Eucheuma cottonii* extract against *Aeromonas hydrophilla*

Concentration	1x24 hours	Category	2x48 hours	Category
0%	0	x	0	x
0%	0	x	0	x
0%	0	x	0	x
50%	1,5	L	2	L
50%	1,5	L	4,5	L
50%	2,5	L	3,5	L
75%	3	L	3,5	L
75%	3,5	L	5,5	S
75%	5,5	S	5,5	S
100%	8	S	10	S
100%	8,5	S	10	S
100%	8,5	S	9,5	S

Bacterial zone of inhibition category:
(x) there is no inhibition zone; (L) <5 Weak; (S) 5-10 Moderate; (K) 10-20 Strong; (SK) >20 Very Strong.

According to **Zaraswati and Eva (2012)**, extracts containing anti-microbial compounds can have either bacteriostatic or bactericidal properties. Bacteriostatic properties are characterized by the ability of the extract to inhibit bacterial growth without destroying the bacteria. In contrast, bactericidal properties are evident when the extract can actively destroy bacteria. During the 2 x 48-hour incubation process, if the diameter of the inhibition zone decreases, the extract is considered to have bacteriostatic properties. Conversely, if the diameter of the inhibition zone increases on the second day, it indicates that the extract has bactericidal properties, as the anti-microbial compounds have effectively destroyed the bacteria.

The zone of inhibition observed after applying *Eucheuma cottonii* extract indicates that the extract contains phytochemicals capable of inhibiting the growth of *Aeromonas hydrophila*. These anti-microbial bioactive compounds, which include phenols, flavonoids, tannins, and saponins, play a crucial role in inhibiting bacterial population growth and killing bacteria. Flavonoid compounds, in particular, act by forming complex protein compounds outside the bacterial cell wall that are soluble in water. This action damages cell membranes and disrupts intracellular components **(Nganjow *et al*., 2013)**. According to **Hughes and McLoughlin (2019)**, the mechanism of anti-microbial compounds involves damaging bacterial cell walls, which alters cell permeability and affects protein and nucleic acid molecules. Additionally, these compounds inhibit enzyme activity and the synthesis of nucleic acids and proteins, further contributing to bacterial cell destruction.

CONCLUSION

The average yield of *Eucheuma cottonii* extract was 2.978 - 3.296%. *Eucheuma cottonii* extract was proven to be effective in inhibiting the population growth of *Aeromonas hydrophila*.

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