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THE EFFECTIVENESS OF GREEN SEAWEED (*Ulva reticulate*) ANTIBACTERIAL COMPOUND EXTRACT ON THE BACTERIA *Vibrio parahaemolyticus*

Efektivitas Senyawa Antibakteri Ekstrak Rumput Laut Hijau (*Ulva reticulata*) Untuk Menghambat Pertumbuhan Bakteri *Vibrio parahaemolyticus*

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ABSTRACT

Green seaweed (*Ulva reticulata*) is found in many parts of Indonesia. The potential of green seaweed is not widely known to the public, even though it is considered a parasite on other types of seaweed. The purpose of this study was to determine the antibacterial compounds contained in green seaweed and their effectiveness against Vibrio parahaemolyticus bacteria. Green seaweed was extracted by maceration method and then qualitative and quantitative tests were carried out. After that, the minimum inhibitory concentration test was carried out and continued with the minimum bactericidal concentration test and the inhibition test with 5 treatments and 3 replications. The data from the qualitative and quantitative test of seaweed, the minimum inhibitory concentration and the minimum bactericidal were analyzed descriptively, while the data from the inhibition zone test was analyzed statistically by ANOVA. The results of qualitative and quantitative tests showed that green seaweed contained flavonoids (6.4909 mg/g), tannins (70.7500 mg/g) and saponins (443.7286 mg/g). The results of the minimum inhibitory concentration test showed that the concentration of 3.125% was the minimum concentration to inhibit bacterial growth. In the inhibition test, it can be seen that P4 (6%) is the best treatment. The results of statistical analysis show that the treatments given are significantly different, except that P4 (6%) and P5 (7,5%) are not significantly different. From this study it can be concluded that green seaweed extract is effective in inhibiting the growth of Vibrio parahaemolyticus bacteria.

Keywords: Natural antibiotics; minimum inhibitory concentration; vibriosis; inhibition zone

ABSTRAK

Rumput laut hijau (*Ulva reticulata*) banyak ditemukan di berbagai wilayah Indonesia. Potensi rumput laut hijau belum banyak diketahui masyarakat, bahkan dianggap sebagai benalu pada rumput laut jenis lain. Tujuan dari penelitian ini adalah mengevaluasi senyawa antibakteri yang terkandung dalam rumput laut hijau dan efektivitasnya terhadap bakteri *Vibrio parahaemolyticus*. Rumput laut hijau diekstraksi dengan metode maserasi kemudian dilakukan analisis fitokimianya baik secara kualitatif maupun kuantitatif . Setelah itu, dilakukan uji

konsentrasi hambat minimum dan dilanjutkan uji konsentrasi bakterisidal minimum serta uji daya hambat dengan 5 perlakuan dan 3 ulangan. Data hasil uji kualitatif dan kuantitatif rumput laut, konsentrasi hambat minimum dan bakterisidal minimum dianalisis secara deskriptif, sedangkan data hasil uji zona hambat dianalisis secara statistik dengan ANOVA. Hasil uji kualitatif dan kuantitatif menunjukkan bahwa rumput laut hijau mengandung senyawa flavonoid (6,4909 mg/g), tanin (70,7500 mg/g) dan saponin (443,7286 mg/g). Hasil uji konsentrasi hambat minimum menunjukkan bahwa konsentrasi 3,125% merupakan konsentrasi minimum untuk menghambat pertumbuhan bakteri. Pada uji daya hambat dapat diketahuibahwa P4 (6%) merupakan perlakuan terbaik, Hasil analisis statistik menunjukkan bahwa perlakuan yang diberikan berbeda signifikan, kecuali pada P4 (6%) dan P5 (7,5%) tidak berbeda signifikan. Dari penelitian ini dapat disimpulkan bahwa ekstrak rumput laut hijau efektif dalam menghambat pertumbuhan bakteri *Vibrio parahaemolyticus*.

Kata Kunci: Antibiotik alami; konsentrasi hambat minimum; vibriosis; daya hambat;

INTRODUCTION

Vibrio parahaemolyticus naturally inhabits estuaries and coastal areas in tropical zones. This bacterium possesses a plasmid (pAP1) of 70 kbp containing two genes, tdh and trh, which produce toxins similar to the insecticidal toxins (Photorhabdus insect-related / Pir), namely PirA and PirB. The toxins from these genes cause damage to the hepatopancreas, leading to shrimp mortality (Gomez *et al.*, 2014) and are identified as causative agents of vibriosis diseases, including AHPND (Acute Hepatopancreatic Necrosis Disease) (Sirikharin *et al.*, 2015).

Various efforts to control diseases related to vibriosis have been widely implemented. These control measures often involve the use of synthetic antibiotics or specific drugs (Reantaso & Arthur, 2018). However, prolonged use of antibiotics can lead to new problems, such as increased resistance of pathogenic bacteria to antibiotics, unintended killing of non-target organisms, and environmental pollution (Sengupta & Chattopadhyay, 2012). Therefore, to reduce the impact of synthetic antibiotics, natural antibiotics are needed that can be effectively used over the long term, combat pathogenic bacteria, and are environmentally friendly.

Seaweed can serve as a natural antibiotic. According to Ravikumar *et al.*, (2016) *Ulva reticulata* is a type of algae containing compounds with antimicrobial properties. Green seaweed (*Ulva reticulata*) is commonly found in Banten, Maluku, South Sulawesi, and East Sumba (Huyyirnah, 2016; Tarigan, 2020). In previous studies, green seaweed extracted with methanol + H_2O exhibited antibacterial activity, forming the largest inhibition zone of 22.67 mm, with no toxic effects observed on *Artemia salina* nauplii in toxicity tests (Mutalib & Khartiono, 2018). Green seaweed is much cheaper than other types of seaweed, as its market demand is low. Moreover, the potential of green seaweed has not been extensively studied, especially regarding disease management in aquaculture. Therefore, this study aims to examine the effectiveness of the antibacterial compounds in green seaweed (*Ulva reticulata*) against *Vibrio parahaemolyticus* bacteria.

RESEARCH METHODS

Materials

This research was conducted on June – July at Fisheries Laboratory of University Muhammadiyah Malang. The materials used in this study include tannic acid, tetracycline antibiotic, *Vibrio parahaemolyticus* bacteria, *Ulva reticulata*, 70% ethanol, paper discs, McFarland I, 0.9% physiological Na, NB (Nutrient Broth), BCG reagent, Dragendorff reagent, Folin-Ciocalteu reagent, Liebermann reagent, Mayer reagent, and TSA.

The equipment used includes an automatic colony counter, Memmert incubator, Abl LAF 120, HS-digital magnetic stirrer, petri dishes, ZZKD rotary evaporator, and UV-Vis spectrophotometer.

Fresh *U. reticulata* (sourced from the waters around Madura) was sun-dried. Once dried, it was blended into a fine powder (simplicia). The simplicia was then weighed to 600 grams and macerated using 1.2 liters of 70% ethanol. Maceration was conducted for 3 x 24 hours at room temperature. The maceration results were separated into filtrate and residue by filtering with a filter cloth. The filtrate was evaporated using a rotary evaporator at 60°C for 30 minutes until the solvent stopped flowing, yielding a thick extract.

Qualitative and Quantitative Phytochemical Tests of Green Seaweed (*Ulva reticulata*)

In the qualitative test, several compounds were examined, including alkaloids, flavonoids, saponins, and tannins. For the alkaloid test, the extract was added with Dragendorff and Mayer reagents; if orange and white precipitates formed, it indicated the presence of alkaloids. For the flavonoid test, 0.1 g of Mg and concentrated HCl were added to the extract; if the solution turned yellow to red, it indicated the presence of flavonoids. For the saponin test, the extract was mixed with Liebermann-Burchard reagent (LB) and shaken vigorously; the formation of a brown or blue-green ring and stable foam indicated the presence of saponins. For the tannin test, the extract was added with 10% FeCl3; a greenish-black color indicated the presence of tannins. Quantitative tests for alkaloids, flavonoids, saponins, and tannins were conducted using UV-Vis spectrophotometry at wavelengths of 430–760 nm, following Dewi's (2020) method.

Minimum Inhibitory Concentration Test Using the Turbidimetric Method

The minimum inhibitory concentration test was performed using the turbidimetric method (Munira & Nasir, 2023). The principle of the turbidimetric method is to visually assess sample turbidity, with the sample then tested using a spectrophotometer to determine turbidity accurately. The extract concentrations used were 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.39%, and 0.195% (Wulandari *et al.*, 2021). *Vibrio parahaemolyticus* bacterial isolates were obtained from the Fisheries Laboratory at Brawijaya University. *Vibrio parahaemolyticus* bacterial suspension was standardized with McFarland I solution (10^6 CFU/ml). A volume of 1 ml of the bacterial suspension was added to each test tube. The absorbance of each treatment was measured at a wavelength of 426 nm, then incubated for 24 hours at 37°C. After incubation, the turbidity in each test tube was visually observed, and the absorbance of each sample was measured again at the same wavelength.

Minimum Bactericidal Concentration Test Using the TPC (Total Plate Count) Method

After the minimum inhibitory concentration test, the minimum bactericidal concentration test was conducted with the same extract concentrations. Serial dilution was then performed with 0.9% physiological Na solution. The last three dilutions were plated in petri dishes with 0.1 ml of each and added to warm TSA + 2% NaCl medium. The plates were homogenized by forming a figure-eight motion. The plates were then incubated at 37°C for 24 hours. After 24 hours, bacterial colonies were counted using the formula according to Larry (2001) as referenced in Nurjannah et al. (2017).

$$\frac{\text{Number of colonies}}{(1 \times n1) + (0{,}1 \times n2) + (0{,}01 \times n3) +} \times d$$

Description:

n1 = number of bacterial colonies on the first countable plate
n2 = number of bacterial colonies on the second countable plate

n3 = number of bacterial colonies on the third countable plate
d = dilution factor at which the colonies can first be counted

Inhibition Zone Test

Place 100 µl (with a density of 10^6 CFU/ml) of *Vibrio parahaemolyticus* bacteria on TSA medium and spread evenly using a triangle spreader. Then, place a 6 mm diameter paper disc that has been dripped with 30 µl of green seaweed extract on the medium. The extract concentrations used are 1.5%, 3%, 4.5%, 6%, and 7.5%, with each treatment repeated three times. The plates are then incubated for 24 hours at 37°C. After incubation, the clear zones formed are observed and measured. The area of the inhibition zone is calculated using the formula (Rohmana, 2015):

$$Lz = Lav - Ld$$

Description:
Lz = Diameter of the inhibition zone (mm)
Lav = Diameter of the inhibition zone with the paper disc (mm)
Ld = Diameter of the paper disc (mm)

Data Analysis

The design was used a Completely Randomized Design (CRD). Data from the minimum inhibitory concentration test, minimum bactericidal concentration, and the qualitative and quantitative tests of green seaweed were analyzed descriptively. The inhibition zone data were statistically analyzed using Microsoft Excel with a one-way ANOVA test. If the ANOVA results show P > 0.05, then a further LSD 0.01 test is conducted to determine the differences among treatments.

RESULTS

Qualitative and Quantitative Phytochemical Tests of Green Seaweed (*Ulva reticulata*)

The results of the qualitative and quantitative phytochemical tests of green seaweed are shown in Table 1.

Tabel 1. Results of qualitative and quantitative phytochemical analysis of ethanolic extract of green seaweed

Phtochemical compounds	Amount
Flavonoids	6.4909 mg/g
Tannins	70.7500 mg/g
Saponins	443.7286 mg/g

Based on the results of qualitative and quantitative tests of antibacterial compounds in green seaweed from Table 1, it was found that secondary metabolites in the green seaweed extract included flavonoids at 6.4909 mg, saponins at 443.7286 mg, and tannins at 70.7500 mg. The largest content in the green seaweed extract was saponins, while flavonoids had the smallest content.

Minimum Inhibitory Concentration (MIC) Test

The results of the minimum inhibitory concentration test using the turbidimetric method are shown in Table 2.

Table 2. Minimum Inhibitory Concentration Test Results

Concentration	OD value before incubation	OD value after incubation	ΔOD	Description
100%	3.677	3.558	-119	decrease
50%	3.428	3.326	-102	decrease
25%	2.959	2.877	-82	decrease
12.5%	2.070	2.001	-69	decrease
6.25%	1.378	1.288	-90	decrease
3.125%	1.016	0.743	-1.015,257	decrease
1.56%	0.827	1.516	1.515,173	increase
0.78%	0.721	1.664	1.663,279	increase
0.39%	0.677	1.216	1.215,323	increase
0.195%	0.469	1.613	1.612,531	increase
K +	1.088	1.124		increase
K -	1.858	1.716		decrease

*K+ = *Vibrio parahaemolyticus* bacteria equivalent to McFarland I
K- = 100% *Ulva reticulata* green seaweed extract

Based on Table 2, the minimum concentration to inhibit the growth of *Vibrio parahaemolyticus* was found to be at 3.125%. The minimum inhibitory concentration can be determined by observing the lowest concentration that provides the smallest OD difference.

Minimum Bactericidal Concentration (MBC) Test

The results of the minimum bactericidal concentration test using the TPC (Total Plate Count) method are shown in Table 3.

Table 3. Minimum Bactericidal Concentration Test Results

Concentration	Bacterial count	Description
100%	-	decrease
50%	0.019x 10^3 CFU/mL	decrease
25%	100x 10^3 CFU/mL	decrease
12.5%	105.22x 10^3 CFU/mL	decrease
6.25%	117.65x 10^3 CFU/mL	decrease
3.125%	96.52x 10^3 CFU/mL	decrease
1.56%	185.66x 10^3 CFU/mL	increase
0.78%	1633.87x 10^3 CFU/mL	increase
0.39%	26506.27x 10^3 CFU/mL	increase
0.195%	181383.29x 10^3 CFU/mL	increase
K +	175329.11x 10^3 CFU/mL	increase
K -	-	decrease

*K+ = *Vibrio parahaemolyticus* bacteria equivalent to McFarland I
K- = Tetracycline antibiotic 30 ppm

Table 3 shows that no minimum bactericidal concentration was found, and the green seaweed extract was bacteriostatic, meaning it inhibited bacterial growth but did not kill *Vibrio parahaemolyticus*. According to Soelama *et al.* (2015), an antimicrobial compound is bacteriostatic if it inhibits bacterial growth only while the compound is present; once it is removed, bacterial growth resumes.

Inhibition Zone

The results of the inhibition zone test for green seaweed extract against *Vibrio parahaemolyticus* bacteria can be seen in Figure 1.

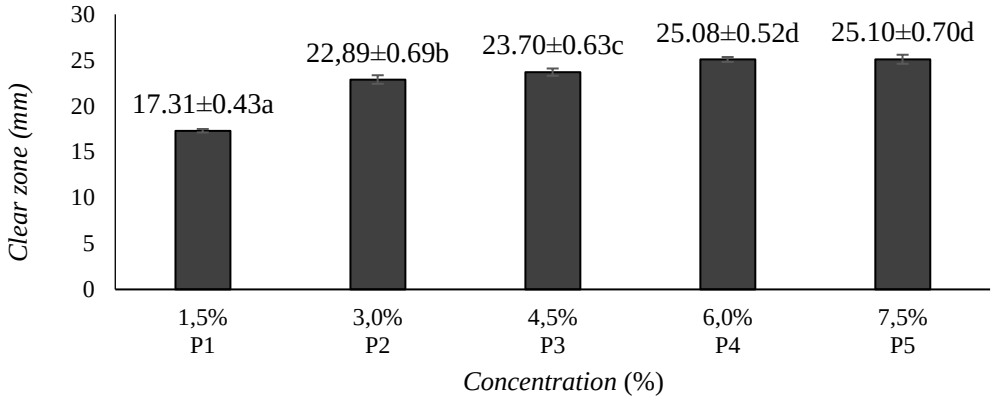

Figure 1. Graph of clear zones of green seaweed extract

Based on Figure 1, it can be seen that at P1, with a 1.5% seaweed extract concentration, the average clear zone was 17.31 mm. At P2, with a 3% extract concentration, the average clear zone was 22.56 mm. P3, with a 4.5% extract concentration, had an average clear zone of 23.36 mm. P4, with a 6% extract concentration, had an average clear zone of 24.98 mm, and P5, with a 7.5% extract concentration, had an average clear zone of 25.10 mm. The inhibition zone data were analyzed using a one-way ANOVA, which showed significant differences between the treatments, as the P-value was greater than the F-critical value of 0.05. After determining that the treatments were significantly different, a BNT test was performed to identify the differences between each treatment. The results of the BNT test indicated significant differences between treatments, except for P4 and P5, which were not significantly different. This suggests that although the 7.5% dose showed the highest result, statistically, it did not differ from the 6% dose.

DISCUSSION

Qualitative and Quantitative Phytochemical Tests of Green Seaweed (*Ulva reticulata*)

The qualitative and quantitative content of primary and secondary metabolites in plants is significantly influenced by various environmental factors, including temperature, altitude, climate, cultivation location, soil, and sunlight intensity. These factors can induce stress responses in plants, leading to alterations in metabolite profiles, which are crucial for their survival and adaptation (Riwanti, 2019). The secondary metabolite compounds, including phenolics and terpenoids, are primarily involved in plant defense and can be upregulated in response to environmental stressors (Salam *et al.*, 2023).

In previous studies, only qualitative tests of green seaweed extract (*Ulva reticulata*) were conducted, with varying results. For example, Lukman *et al.*, (2015) mentioned that green seaweed extract contains alkaloids, flavonoids, and triterpenoid saponins. Meanwhile, Ndahawali *et al.*, (2021) found that the extract contains alkaloids and phenolic compounds. However, these studies did not specify the quantities of the compounds present in the green seaweed extract. Ate *et al.*, (2017) stated that factors influencing the phytochemical content of seaweed include species, environmental growth conditions, methods of processing and storage, seasonal variations, and varieties.

Minimum Inhibitory Concentration (MIC) Turbidimetric Method

A positive OD difference indicates an increase in bacterial population, while a negative OD difference indicates a decrease in bacterial growth (Munfaati *et al.*, 2015). In Table 2, the bacterial population is inversely related to concentration used. The higher the concentration, the greater the antibacterial compounds contained within, thereby effectively inhibiting bacterial population growth.

Minimum Bactericidal Concentration (MBC) using Total Plate Count Method

The results of the minimum bactericidal concentration test indicate that no minimum bactericidal concentration was found, and the green seaweed extract is only bacteriostatic, meaning it can inhibit bacterial growth but is not bactericidal and does not kill *Vibrio parahaemolyticus*. According to Soelama *et al.*, (2015) an antimicrobial compound is considered bacteriostatic if it inhibits bacterial growth only when the compound is administered. However, if the administration of the compound is stopped or depleted, bacterial growth will increase and form colonies. In contrast, a bactericidal compound is characterized by the absence of bacterial growth and colonies, resulting in a clear zone that continues to increase during the incubation period. Compounds with bactericidal properties can halt bacterial growth and physiological activity even after the compound is no longer present.

Inhibition Zone

Based on the results of the inhibition zone test, it can be observed that the clear zone produced shows a continuous increase. However, P4 (6%) and P5 (7.5%) did not show significant differences statistically, so P4 was chosen as the maximum dose for further application. Determining the optimal dose of phytochemicals is a crucial step in the development of aquaculture products based on natural ingredients. This is because if the dose is too low, the phytochemicals may not be effective enough to provide the expected benefits, such as improving growth, boosting immunity, or inhibiting pathogen growth. On the other hand, a dose that is too high may cause toxic effects on fish, such as organ damage, decreased appetite, or even death (Engalycheva & Subaev, 2023; Suvorov, 2024). Additionally, from an economic efficiency standpoint, the P4 (6%) dose, when applied in vivo, has lower costs compared to P7 (7.5%).

The clear zone formed in the inhibition zone test becomes larger as the concentration increases (Rastina, 2015). The size of the clear zone produced is related to the antibacterial activity of the compounds in the green seaweed extract, including flavonoids, tannins, and saponins. According to Rahmawatiani *et al.*, (2020) secondary metabolites such as flavonoids, saponins, tannins, and phenolics are antibacterial compounds that are effective against both Gram-positive and Gram-negative bacteria. The role of flavonoids as antibacterial agents can be divided into three main actions: inhibiting nucleic acid synthesis, disrupting cell membrane function by forming complexes with extracellular proteins, thereby damaging the bacterial cell membrane and causing intracellular substances to leak out, and inhibiting energy metabolism in bacterial cells (Hakim & Editia, 2018). The tannin content in the green seaweed extract is 70.7500 mg/g.

Tannins are known to help bacterial cells adhere by assisting enzymes, and they interfere with transport proteins inside the bacterial cell. Tannins specifically target the polypeptide component of bacterial cell walls, preventing the wall from forming properly. Additionally, tannins inhibit bacterial cell formation by interfering with DNA topoisomerase and reverse transcriptase enzymes (Rijayanti, 2014). Furthermore, another antibacterial agent in green seaweed extract is saponin, which has the highest concentration in the extract at 443.7286 mg/g. Saponins disrupt bacterial cell permeability. This disruption damages the

bacterial cell membrane and causes important components, such as nucleic acids, nucleotides, and proteins, to leak out of the cell (Sapara, 2016).

CONCLUSION

The green seaweed extract (Ulva reticulata) can inhibit the growth of *Vibrio parahaemolyticus* at a minimum inhibitory concentration of 3.125%. The recommended maximum dose of green seaweed extract is P4 (6%). The secondary metabolites contained in the green seaweed extract (*Ulva reticulata*) include flavonoids (6.4909 mg/g), tannins (70.7500 mg/g), and saponins (443.7286 mg/g).

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