ANTIBACTERIAL ACTIVITY

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ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACT OF *GRACILARIA SALICORNIA*, *HALIMEDA GRACILIS*, *HALIMEDA MACROLOBA*, AND *HYPNEA ASPERI* FROM INDONESIA

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***Abstract*— The research aimed to find out the ability of methanol extract of G. salicornia, H. gracilis, H. macroloba, and H. asperi from Indonesia as antibacterial in inhibiting E. coli (ATCC 8739), S. aureus (ATCC 6539), and P. aeruginosa (ATCC 9027). The research design is quasi experimental by using statistic group comparison. The test method for antibacterial activity is well diffusion which refers to CLSI document M02-A11. The antibacterial activity test showed the highest inhibitory zone to E. coli, only in methanol extract of G. salicornia 20%, the highest inhibitory zone to S. aureus is in methanol extract of H. asperi 20%, and the highest inhibitory zone to P. aeruginosa is in methanol extract of H. gracilis 20%. The differences in the effect of macro algae methanol extract which tested at the same concentration on S. aureus showed that significant effect on methanol extract of H. asperi and G. salicornia at concentration 20% ($p \leq 0,05$) and to P. aeruginosa did not showed significant difference of influence, and comparison of methanol macro algae extract effect at same concentration between S. aureus and P. aeruginosa did not show significant effect difference ($p > 0,05$).**

***Keywords*— Antibacterial; Macro algae; Methanol extract**

1. INTRODUCTION

Algae belongs to the Thallophyta division, which distinguished by dominant pigments, is divided into seven divisions, namely: *Cyanophyta, Chlorophyta, Euglenophyta, Crysophyta, Bacillariophyta, Phaeophyta,* and *Rhodophyta*. Among the seven algae divisions, only three benthic divisions are known as macro algae, namely *Chlorophyta* (green algae), *Phaeophyta* (brown algae), and *Rhodophyta* (red algae). Approximately 8.6% of the total marine biota in Indonesia is macro algae and the area of macro algae habitat reaches 1.2 million hectares. It is the largest area in the world for macro algae habitats [1]. Macro algae or seaweed are scattered throughout Indonesian marine waters [2]. The diversity of macro algae in Indonesia are *Rhodophyta* ranks most of the growing number of species in Indonesian marine waters (about 452 species), followed by *Chlorophyta* (about 196 species), and *Phaeophyta* (about 134 species) [1]. Macro algae can be used as a widely used food source in Asian countries and as a source of techno functional polysaccharides (jelly, carrageen, and alginate) in Western countries, as well as

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medicinal ingredients, as an alternative to dietary protein sources, and as source of potential bioactive protein components in the pharmaceutical industry [3].

Pulau Tidung, Kepulauan Seribu, Indonesia, is a marine tourism destination that has the beauty of white sand, coral reefs, and various marine life. One of the marine biota that stands out in the waters of Tidung Island is macro algae. The results of research conducted by [4] on the diversity of macro algae in the water of Pulau Tidung, Kepulauan Seribu there are 21 types of macro algae which have composition of *Chlorophyta* 48% (10 species), *Phaeophyta* 28% (six species), and *Rhodophyta* 24% five types).

Macro algae have enormous potential in pharmacological fields, such as antioxidants and antibacterial. Research on macro algae antibacterial activity, has been done, among others by [5-8]. Research on phytochemical and antibacterial activity of macro algae was conducted by [9-12]. Research on antioxidant and macro algae antibacterial activity among others was performed by [13]. As for the utilization of potential of macro algae in the waters of Tidung Island has not been explored in depth.

Motivated from aforementioned explanation, the purpose of this research is to discover the antibacterial activity of methanol extract of *G. salicornia, H. gracilis, H. macroloba,* and *H. asperi* in inhibiting *E. coli, S. aureus,* and *P. aeruginosa*.

The rest of this paper is organized as follow. Section 2 presents research design. Section 3 presents proposed method. Section 4 presents obtained results and following by discussion. Finally, Section 5 concludes this work.

2. RESEARCH DESIGN

The extraction of macro algae was done at Bogor Agriculture Institute, Biopharmaceutical Study Center, Indonesia. The research on antibacterial activity was conducted in the Research Laboratory at Universitas Nasional Indonesia from September 2016 to December 2016.

2.1. TOOLS

One mL pipette tips, 1.5 mL microcentrifuge tubes, 200 μL pipette tips, burner, aluminum foil, stir bar, biosafety cabinet (BSC), Petri dishes, cork borer, funnel, exicator, glassware, hot plate, measuring flask 5,0 mL, micropippette 100-1000 μL, micropippette 10-100 μL, swab, analytical balance, round ose, parafilm, autoclave, and oven.

2.2. MATERIALS

Macro algae (*Gracilaria salicornia, Halimeda gracilis, Halimeda macroloba, Hypnea asperi),* bacteria (*Escherichiacoli* ATCC 8739, *S. aureus* ATCC 6539, *P. aeruginosa* ATCC 9027), $BaCl_2$ p.a., H_2SO_4 p.a., Mueller Hinton Agar (MHA) CM0337 (OXOID), Nutrient Agar (NA) CM0337 (OXOID), methanol p.a., Dimethyl Sulfoxide (DMSO) p.a., NaCl p.a., alcohol 70%, antibiotics, doxycycline and ciprofloxacin, distilled water, ethanol p.a., Mc. Farland 0.5 standard aqueous.

2.3. STERILIZATION

Glasswares were sterilized using oven at 180 ºC for 120 minutes. NaCl 0.85%, and the media were sterilized using a 121 ºC temperature autoclave for 15 minutes. Petri dishes that have been sterilized, then performed irradiation by using Ultra Violet (UV) rays for ± 1 hour in BSC.

2.4. PREPARATION OF *G. SALICORNIA*, *H. GRACILIS*, *H. MACROLOBA*, AND *H. ASPERI SIMPLICIA*

Simplicia was done by quick drying in low temperature. There were seven stages in the preparation of simplicia, namely wet sorting, chopping, drying, dry sorting, packing and

storage, and quality inspection [14]. The preparation of simplicia is done in the following ways; the macro algae was collected by taking all the parts of macro algae. Then, macro algae cleared from susbtrat, dirt, or foreign objects. Macro algae were washed with flowing fresh water. Macro algae were dried by drying them in the sun for 3-7 days. Macro algae that had been dried, then cut into pieces using scissors. The result of cutting was spread evenly on the tray to be dried naturally (dried in the sun indirectly). After the macro algae were being dried and became dry, then the macro algae were referred to as simplicia macro algae. The macro algae simplicia was dried remove from any foreign impurities or materials left behind.

2.5. PREPARATION OF NUTRIENT AGAR (NA) MEDIUM

Nutrient agar 3.6 g dissolved with 150 mL aquadest in a 250 mL erlenmeyer flask. The mixture was heated and stirred until all dissolved. After it was being dissolved, the NA was sterilized by autoclave for 15 minutes at 121 °C. To have sterile nutrients, it was transferred into a Petri dish of 100 mm diameter by 20-30 mL (for cultivation). Next, to have sterile nutrients, 6 mL of nutrients were transferred into the test tube. Then, the test tube was tilted (for the storage of pure cultures as stock). The nutrient agar in a Petri dish and the tilted tube was cooled down until it was solid in room temperature with closed cup and closed tube. If it was not used on the same day, NA is reversed to avoid water droplets that are formed and stored in refrigerators at 2–8 ºC.

2.6. PREPARATION OF MUELLER HINTON AGAR (MHA) MEDIUM

The Clinical Laboratory Standard Institute (CLSI) sets the standard for the disk antibacterial discontinuity test in Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard-Eleventh Edition (CLSI document M02-A11) [15]. In the document, it was arranged of the media preparation in order to antibacterial sensitivity test, namely Mueller Hinton Agar (MHA). Mueller Hinton agar 38g dissolved in 1L of aquadest and heated until dissolved. The MHA solution was sterilized by autoclave for 15 minutes at 121 °C. To have a sterile Mueller Hinton, 20-30 mL of it is poured into a 100 mm diameter Petri dish. The MHA agar in the Petri dish was cooled until it became solid at room temperature with a closed cup. Mueller Hinton to be reversed to avoid water droplets formed. If it was not in use on the same day, the MHA was stored in refrigerator at 2-8°C.

2.7. INOCULATION OF THE BACTERIA

Bacteria cultured and made an equivalent suspension to the Mc. Farland 0.5 standard. The suspension of test bacteria was cultured on NA medium by streak plate method using a spherical ose to separate the colonies. Nutrient agar medium was incubated for 18-24 hours at 37 °C. Two test tubes are labeled according to the test bacteria for the suspension. Five mL of NaCl 0.85% sterile were each transferred to a different sterile reaction tube, then the bacteria colony were added using a spherical ose by rubbing a spherical ose containing test bacteria to the sterile reaction tube wall near the surface of NaCl 0.85% sterile, then mixed with 0.85% NaCl sterile until homogeneous. When it was becoming homogeneous, the suspension of the test bacteria was shaken for 15 seconds until mixed to all parts of 0.85% NaCl sterile. The suspension of the test bacteria was compared with Mc. Farland 0.5 turbidity standard solution. The turbidity of bacteria suspension test was measured by using a spectrophotometer at 625 nm wavelength. The turbidity suspension with 0.08-0.13 absorbance is equivalent to 1-2 × 108 CFU/mL.

2.8. PREPARATION OF *E. COLI*, *S. AUREUS*, AND *P. AERUGINOSA* SUSPENSION

Two test tubes were labeled according to the test bacteria for the suspension. Five mL of NaCl 0.85% sterile were each transferred to a different sterile reaction tube, then the bacteria colonies were added using a spherical ose by rubbing a spherical ose containing test bacteria to the sterile reaction tube wall near the surface of NaCl 0.85% sterile, then mixed with 0.85% NaCl sterile until homogeneous. When they became homogeneous, the suspension of the test bacteria was shaken for 15 seconds until mixed to all parts of 0.85% NaCl sterile. The suspension of the test bacteria was compared with Mc. Farland 0.5 turbidity standard solution. The turbidity of bacterial suspension test was measured by using a spectrophotometer at 625 nm wavelength. The turbidity suspension with 0.08-0.13 absorbance is equivalent to 1-2 X 108 CFU/mL.

2.9. PREPARATION OF 600 PPM DOXYCYCLINE AND 100 PPM CIPROFLOXACIN SOLUTION

Clinical Laboratory Standard Institute (CLSI) established a standard control for antibacterial sensitivity testing in Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement (CLSI M100-S21document) [16]. In the document, it was regulated that antibiotics that were used as a control according to the test bacteria The antibiotics which was used as a control of antibiotics in this research were doxycycline for the test bacteria, which were *E. coli* and *S. aureus,* and ciprofloxacin for the test bacteria *P. aeruginosa*. The making of 600 ppm doxycycline and 100 ppm ciprofloxacin was done by the following ways; 3 mg and 0.5 mg of doxycycline, were dissolved with DMSO in 5.0 mL of different measuring flask. Those doxycycline and ciprofloxacin were shaken until they were homogeneous.

2.10. PREPARATION OF *G. SALICORNIA*, *H. GRACILIS*, *H. MACROLOBA* AND *H. ASPERI* METHANOL EXTRACT

The method used in this research for the making of macro algae methanol extract was maseration method at room temperature. The making of methanol extract was carried out in the following ways; the ready mixed macro algae simplicia was inserted and immersed in a maserator using 80% methanol at room temperature with a simplicia ratio: solvent, i.e 0.5 kg: 1 L methanol. The soaking simplicia took 15 hours and for further dissolve of active substances contained in the macro algae simplicia, it was shuffled using shaker for 3 X 24 hours. The maseration results were filtered using filter paper, then the maserate was transferred into an impermeable bottle. The filtered sediment was then immersed in 1 L of methanol at room temperature for 15 hours, then re-filter. The remaining sediments are soaked again in the same way. The maserate of the overall immersion result were adjoined. The maserate was evaporated in a waterbath at 40 °C. The evaporated maserate was then pressured by evaporating the solvent using a vacuum rotary evaporator with a low pressure at 70 °C to obtain a viscous extract. Furthermore, the condensed extract was carried out freeze drying to obtain dry extract.

2.11. PREPARATION OF *G. SALICORNIA*, *H. GRACILIS*, *H. MACROLOBA*, AND *H. ASPERI* 4%, 8%, 12%, 16 %, AND 20% METHANOL EXTRACT

A sterile of 5.0 mL measuring flask was prepared as much 4 pieces, each flask was labeled 4%, 8%, 12%, 16%, and 20%. Each macro algae methanol extract of 0.2 g, 0.4 g, 0.6 g, 0.8 g, and 1 g were inserted into the labeled flasks. The macro algae methanol extract was diluted by DMSO to the point of volume the measuring flask. Each measuring flask was shaken until the methanol extract dissolves homogeneously.

3. PROPOSED METHOD

3.1. RESEARCH DESIGN

This section presents research design of antibacterial activity of *G. salicornia*, *H. gracilis*, *H. macroloba*, and *H. asperi* methanol extract against *E. coli*, *S. aureus*, and *P. aeruginosa*. Before testing of antibacterial activity, it was calculated minimum number of replication. In this research, the minimum replication with the following calculations:

$$(r-1)\,(t-1) \geq 15$$
$$(r-1)\,(20-1) \geq 15$$
$$19r-19 \geq 15$$
$$19r \geq 15+19$$
$$19r \geq 34$$
$$r \geq 1{,}789 \approx 2$$

r = replication
t = treatment
15 = degrees of general freedom

Four treatments with five levels of treatment in this study, required at least two replications. However, the researcher sets four replications. Thus, 36 units of experiments were obtained, plus one antibiotic control as much four replications and one solvent control four times replications. The number of units in this research were 44 experimental units for one type of test bacteria. So that, the total units for the three types of test bacteria in this study were 132 experimental units. After calculating the minimum number of replications, then it was conducted a randomize of the treatment order.

The method of testing the antibacterial activity of macro algae methanol extract which were tested against *E. coli, S. aureus* and *P. aeruginosa* in this study, used a well diffusion method on the agar medium. The steps below show how to test macro algae methanol extract which were tested against *E. coli, S. aureus* and *P. aeruginosa* are; the bottom of the Petri dish were divided into 4 sections using permanent markers and rulers, then each Petri dish was labeled, Positive Control, and Negative Control. The suspension of *E. coli, S. aureus,* and *P. aeruginosa* was flattened by the spread plate method using sterile cotton swabs to the entire surface of agar MHA. Each part of the Petri dish was perforated using cork borer of 4.60 mm diameter. The methanol macro algae extract solution of 4%, 8%, 12%, 18%, and 20%, doxycycline, ciprofloxacin and DMSO (negative control) were taken, by using pipette as much 50 µL, into each hole with the MHA labeled on the Petri dish. The Petri dish was incubated for 18 hours at 37°C without reversing. The inhibitory zone in the form of clear zone which was formed, observed and measured by using a sliding range.

3.2. DATA ANALYSIS

The selection of data analysis in this research refers to [17]. The antibacterial activity of macro algae methanol extract which tested to *E. coli*, *S. aureus*, and *P. aeruginosa* was analyzed using IBM SPSS Statistics 22 software and was conducted ANOVA univariate test. The results which showed a significant difference, was then conducted a Tukey test. The effect of macro algae methanol extract which were tested at the same concentration, previously were made variable combinations between the species of macro algae, macro algae extract concentration, and bacteria type, then were analyzed using IBM SPSS Statistics 22 software and one-way ANOVA test. If the results showed a significant difference, then the Tukey test was followed [17].

4. RESULT AND DISCUSSION

The average of the methanol extract zone diameter drags from the macro algae tested are shown in Table I and Figure 1 below:

Table I. The average diameter of inhibitory zone of macro algae methanol extracts which tested toward *E. coli*, *S. aureus*, and *P. aeruginosa*

No	Methanol extracts of macro algae	Concentrations methanol extract of macro algae	Average ± SD Inhibitory Zone Diameter (mm)		
			E. coli	*S. aureus*	*P. aeruginosa*
1	*Hypnea asperi*	4%	6.40 ± 0.00	7.40 ± 0.84	6.40 ± 0.00
		8%	6.40 ± 0.00	7.85 ± 1.15	6.40 ± 0.00
		12%	6.40 ± 0.00	8.42 ± 0.23	6.61 ± 0.42
		16%	6.40 ± 0.00	8.45 ± 1.18	7.10 ± 0.80
		20%	6.40 ± 0.00	11.92 ± 2.45	8.30 ± 1.94
2	*Halimeda gracilis*	4%	6.40 ± 0.00	7.05 ± 0.78	8.30 ± 0.18
		8%	6.40 ± 0.00	7.76 ± 0.93	8.85 ± 0.31
		12%	6.40 ± 0.00	8.82 ± 0.58	9.30 ± 2.13
		16%	6.40 ± 0.00	8.87 ± 0.61	9.87 ± 2.02
		20%	6.40 ± 0.00	9.82 ± 3.27	10.62 ± 4.02
3	*Halimeda macroloba*	4%	6.40 ± 0.00	7.86 ± 0.72	6.92 ± 0.60
		8%	6.40 ± 0.00	8.66 ± 0.52	7.45 ± 1.21
		12%	6.40 ± 0.00	8.67 ± 0.45	7.86 ± 1.30
		16%	6.40 ± 0.00	8.72 ± 0.45	7.96 ± 1.06
		20%	6.40 ± 0.00	10.83 ± 1.07	8.98 ± 1.88
4	*Gracilaria salicornia*	4%	6.40 ± 0.00	6.55 ± 0.30	6.40 ± 0.00
		8%	6.40 ± 0.00	6.95 ± 0.61	6.40 ± 0.00
		12%	6.40 ± 0.00	7.43 ± 0.61	6.61 ± 0.42
		16%	6.40 ± 0.00	7.58 ± 0.88	6.72 ± 6.50
		20%	7.00 ± 1.20	8.17 ± 1.64	6.40 ± 0.00

Note: The diameter of well is 6.40 mm

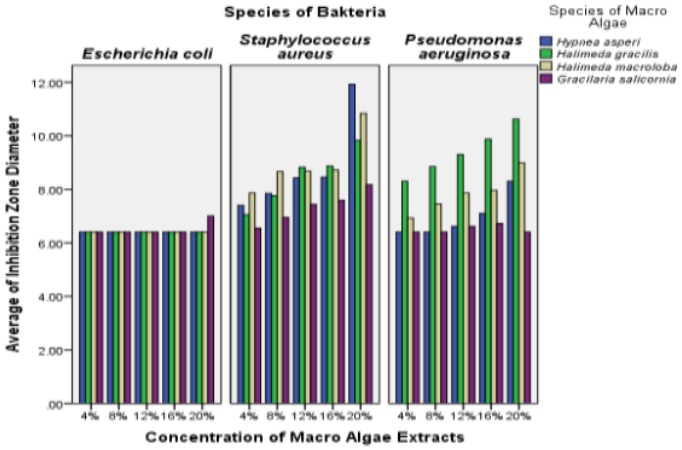


Fig 1 The graphic of antibacterial activity of macro algae methanol extracts which tested toward *E. coli*, *S. aureus* and *P. aeruginosa*

From primary data, the results of testing the antibacterial activity of the methanol extracts of *H. gracilis, H. macroloba* and *H. asperi* at concentrations of 4%, 8%, 12%, 16%, and 20%, as well as *G. salicornia* with concentrations of 4%, 8%, 12%, 16% against *E. coli*, showed no activity antibacterial. Methanol extracts of *G. salicornia* with a concentration of 20%, shows an average of antibacterial activity of (7.00 ± 1.20) mm. In contrast to the results of the antibacterial activity of the methanol extracts of macro algae that are tested against *E. coli*, the antibacterial activity of the methanol extracts of macro algae that are tested against *S. aureus* indicate variations in the diameter of the zones of drag. The antibacterial activity of the methanol extracts of macro algae that are tested against *S. aureus* increases along with the increasing concentration of each macro algae extract. Methanol extracts of *G. salicornia*, *H. gracilis*, *H. macroloba*, and *H. asperi* shows the result of the diameter of the zones of highest inhibitory at concentrations of 20%, with an average of each registration (8.17 ± 1.64) mm, (9.82 ± 3.27) mm, (10.83 ± 1.07) mm and (11.92 ± 2.45) mm. Result of antibacterial activity of macro algae methanol extracts that are tested against *P. aeruginosa* also showed an increase in line with the greater concentration. The methanol extract of macro algae *G. salicornia*, *H. asperi*, *H. macroloba*, and *H. gracilis* results shows the diameter of the zones of highest inhibitory at concentrations of 20%, with an average of each registration (6.50 ± 6.72) mm (8.30 ± 1.94) mm (8.98 ± 1.88), and (10.62 ± 4.02) mm.

The results in this study contrasts with research conducted by [6], Disk diffusion method of antibacterial activity by charging extract 30 μL on Whatman filter paper disk no. 1 and 6.00 mm in diameter indicates that methanol extracts, *H. macroloba* can inhibit *E. coli* drag zone diameter of 3.00 mm but cannot inhibit *S. aureus* [6], while research conducted by [18], disk diffusion method of antibacterial activity by charging extract 30 μL at 6.00 mm in diameter sterile disk and extract content of as much as 60 μg, indicating that *H. macroloba* can inhibit *E. coli* and *S. aureus* with each diameter drag zones of 16 ± 0.51 mm and 18 ± 0.95 mm. Many factors lead to affordable antibacterial activity of macro algae, which are the habitat of macro algae, species of macro algae, harvest difference stages of growth, methods of extraction methods, experimentation, and solvent used in the extraction [6].

The results of the univariate ANOVA on the antibacterial activity of the methanol extracts of macro algae that are tested against *E. coli*, *S. aureus*, *P. aeruginosa* and shows that there is the influence of the type of macro algae, the concentration of methanol extracts of macro algae, the different types of bacteria, the interaction of this type of macro algae with a type of bacteria, and the interaction of methanol extract concentration of macro algae with the bacterial species ($p \leq 0.05$). The results of the Tukey about differences influence the type of macro algae, shows that there are significant differences between the methanol extracts of *G. salicornia* methanol extracts with the three macro algae and other *H. asperi* by *H. gracilis* ($p \leq 0.05$). The difference of type's macro algae against *E. coli*, *S. aureus*, *P. aeruginosa* and shown in Table II. There is the Tukey result regarding the influence of the concentration, indicating that there is a significant difference in the 20% concentration of methanol extracts of macro algae with concentration of 4%, 8%, 12%, and 16% ($p \leq 0.05$) (Table III).

Table II. The different influence of macro algae methanol extract toward inhibitory zone diameter of *E. coli, S. aureus*, dan *P. aeruginosa*

No	Species of Macro Algae	Subset		
		1	2	3
1	*Gracilaria salicornia*	6.7892		
2	*Hypnea asperi*		7.3908	
3	*Halimeda macroloba*		7.7300	7.7300
4	*Halimeda gracilis*			8.0858
	Sig.	1.000	0.310	0.269

Table III. The different influence concentration of macro algae methanol extract which tested toward inhibitory zone diameter of *E. coli*, *S. aureus*, *P. aeruginosa*

No	Concentrations methanol extract of macro algae	Subset		
		1	2	3
1	4%	6.8740		
2	8%	7.1604	7.1604	
3	12%	7.4458	7.4458	
4	16%		7.5750	
5	20%			8.4396
	Sig.	0.072	0.324	1.000

The results of the univariate ANOVA on the antibacterial activity of the methanol extracts of macro algae that are tested against *S. aureus*, shows there is the influence of the type of macro algae and extract the methanol concentration of macro algae ($p \leq 0.05$). The results of Tukey about differences influence the type of macro algae, shows that there are significant differences between *G. salicornia* with *H. asperi*, *H. gracilis*, and *H. macroloba* ($p \leq 0.05$). Tukey results regarding the influence of the concentration, indicating that there is a significant difference in the concentration of methanol extracts of macro algae with a concentration of 20% methanol extract of macro algae 4%, 8%, 12%, and 16% ($p \leq 0.05$). The difference of influence of type of macro algae and extract the methanol concentration of macro algae against *S. aureus* is shown in Table IV and the difference in the influence of the concentration of methanol extracts of macro algae can be seen in Table V.

Table IV. The different influence of macro algae methanol extract which tested toward inhibitory zone diameter of *S. aureus*

No	Species of macro algae	Subset	
		1	2
1	*Gracilaria salicornia*	7.3400	
2	*Halimeda gracilis*		8.4675
3	*Hypnea asperi*		8.8100
4	*Halimeda macroloba*		8.9525
	Sig.	1.000	0.585

Table V. The different influence concentration of macro algae methanol extract which tested toward inhibitory zone diameter of *S. aureus* and *P. aeruginosa*

No	Concentrations methanol extract of macro algae	Subset	
		1	2
1	4%	7.2156	
2	8%	7.8063	
3	12%	8.3406	
4	16%	8.4094	
5	20%		10.1906
	Sig.	0.052	1.000

The results of the univariate ANOVA on the antibacterial activity of the methanol extracts of macro algae tested against *P. aeruginosa*, showed that there is influent of the type macro algae and extract the methanol concentration of macro algae ($p \leq 0.05$). The results of the Tukey about differences influence the type of macro algae, shows that there are significant differences between *G. salicornia* with *H. asperi* and *H. macroloba*, *H. gracilis* with *G. salicornia*, *H. asperi*, and *H. macroloba* ($p \leq 0.05$). The results of Tukey regarding the influence of the concentration, indicating that there is asignificant difference between the concentration of methanol extracts of macro algae with a concentration of 20% methanol extract of macro algae 4% ($p \leq 0.05$). The difference influence of type macro algae and differences influence the concentration of methanol extracts of macro algae against *P. aeruginosa* shown in Table VI and the differences influence the concentration of methanol extracts of macro algae can be seen in Table VII.

Table VI. The different influence of macro algae methanol extract which tested toward inhibitory zone diameter of *P. aeruginosa*

No	Methanol Extracts of Macro Algae	Subset		
		1	2	3
1	*Gracilaria salicornia*	6.5075		
2	*Hypnea asperi*	6.9625	6.9625	
3	*Halimeda macroloba*		7.8375	
4	*Halimeda gracilis*			9.3900
	Sig.	0.728	0.201	1.000

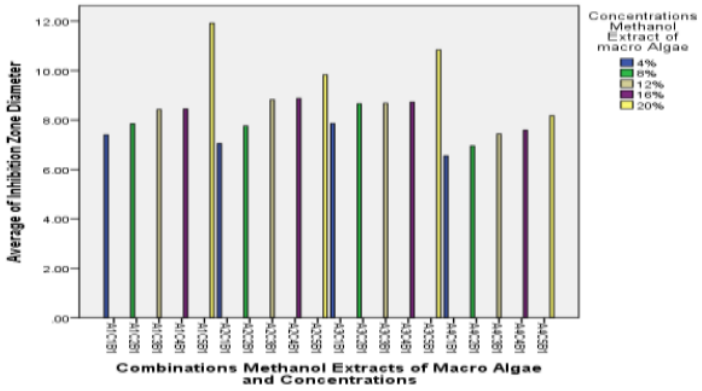
Table VII. The different influence concentration of macro algae methanol extract which tested toward inhibitory zone of *P. aeruginosa*

No	Concentrations Methanol Extract of Macro Algae	Subset	
		1	2
1	4%	7.0063	
2	8%	7.2750	7.2750
3	12%	7.5969	7.5969
4	16%	7.9156	7.9156
5	20%		8.5781
	Sig.	0.352	0.073

One of ANOVA results each macro algae and extract methanol concentration of methanol extracts on the antibacterial activity tested against *S. aureus*, shows that there is influence difference of methanol extracts of macro algae tested against *S. aureus* in the same concentration ($p \leq 0.05$). Tukey results regarding the influence of the methanol extract of macro algae tested against *S. aureus* in the same concentration ($p \leq 0.05$), indicating that there is a different significant influence of methanol extract of *H. asperi* and *G. salicornia* on concentration of extracts macro algae 20% methanol ($p \leq 0.05$). The difference of influence of type of macro algae against *S. aureus* on the same concentration shown in Table VIII and Figure 2.

Table VIII. The different influence of each macro algae methanol extracts which tested toward *S. aeureus* on the same concentration

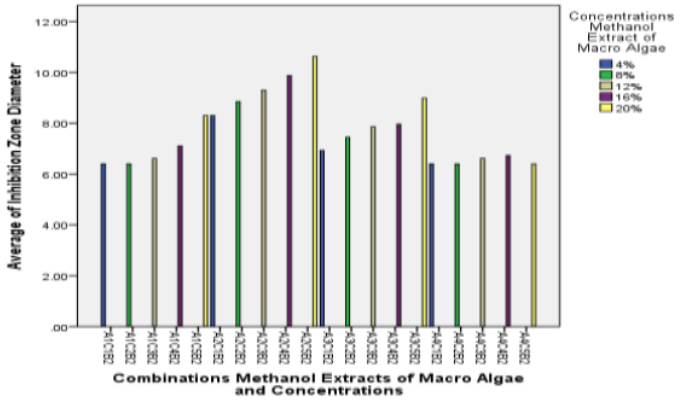
No	Methanol extracts of and concentration of macro algae	Comparison methanol extracts and concentration of macro algae		Sig.
1	*Hypnea asperi* 4%	*Halimeda gracilis*	4%	1.000
		Halimeda macroloba	4%	1.000
		Gracilaria salicornia	4%	1.000
2	*Hypnea asperi* 8%	*Halimeda gracilis*	8%	1.000
		Halimeda macroloba	8%	1.000
		Gracilaria salicornia	8%	1.000
3	*Hypnea asperi* 12%	*Halimeda gracilis*	12%	1.000
		Halimeda macroloba	12%	1.000
		Gracilaria salicornia	12%	1.000
4	*Hypnea asperi* 16%	*Halimeda gracilis*	16%	1.000
		Halimeda macroloba	16%	1.000
		Gracilaria salicornia	16%	1.000
5	*Hypnea asperi* 20%	*Halimeda gracilis*	20%	0.616
		Halimeda macroloba	20%	0.999
		Gracilaria salicornia	20%	0.006



Notes: A1 = *H. asperi*, A2 = *H. gracilis*, H3 = *H. macroloba*, H4 = *G. salicornia* C1= 4%, C2 = 8%, C3 = 12%, C4 = 16%, C5 = 20%

Fig. 2 The graphic of each comparison of macro algae methanol extract toward inhibitory diameter zone toward *S. aureus*

The results of the Tukey regarding the influence of the methanol extract of macro algae that are tested against *P. aeruginosa* in the same concentration, indicating that there is no difference significant influence on any macro algae extract methanol is tested ($p > 0.05$). The influence of type of macro algae against *P. aeruginosa* in the same concentrations shown in Figure 3 below:



Notes : A1 = *H. asperi*, A2 = *H. gracilis*, H3 = *H. macroloba*, H4 = *G. salicornia* C1= 4%, C2 = 8%, C3 = 12%, C4 = 16%, C5 = 20%

Fig. 3 Graph of comparison of each macro algae methanol extract with inhibitory zone diameter against P. aeruginosa

The results of the Tukey regarding the influence of the methanol extract of macro algae tested against *S. aureus* and *P. aeruginosa* in the same concentration, indicating that there is no difference significant influence on any macro algae extract methanol is tested ($p > 0.05$). Primary test results data control antibiotic doxycycline 600 ppm against *E. coli* and *S. aureus*, as well as ciprofloxacin 100 ppm shows that the results of these controls in accordance with the documents specified in CLSI M100-S21.

Control of antibiotics (doxycycline 600 ppm) as positive controls tested against *E. coli* and *S. aureus* and ciprofloxacin 100 ppm as the positive control tested against *P. aeruginosa*, fit into the category of sensitive, based on interpretation standard for Quality Control ranges CLSI document M100-S21. The average diameter of the inhibitory zones produced by each of the antibiotics of 20.81 mm (*E. coli*) 31.14 mm (*S. aureus*) and 30.44 mm (bacteria test *P. aeruginosa*). Working principles of antibacterial are bacteriostatic and bactericidal functions. Principle of antibacterial compound is bacteriostatic only able to inhibit bacterial growth if the granting of the compound continues to be done, however if stopped or run out, then the growth and reproduction of bacteria will be active again marked with the reduced diameter of the inhibitory zones. Working principles of antibacterial works is bactericidal if diameter zone barriers increase caused by the antibacterial compound can kill and stop the physiological activity of bacteria, although the granting of the antibacterial compound is discontinued [19].

The principle of action of doxycycline is bacteriostatic. Doxycycline inhibits bacterial growth and cell division. When the bacteria are exposed to antibiotics, it will cause the cleavage's dismissal. However, after the antibiotic is missing, then the cell will be divided again. Doxycycline interfere with protein synthesis by binding the 30S subunit ribosome. And block the attachment transfer RNA (tRNA), so the new amino acid cannot be added to protein's chain that causes protein synthesis is hampered. The working principle of ciprofloxacin is bactericidal. Ciprofloxacin entered in the fluoroquinolones that work by disrupting DNA gyrase enzyme by forming a complex bond, thus causing DNA damaged that ultimately causes cell death [20]. Referring to the antibiotic doxycycline control range 30 μg and ciprofloxacin 5 μg, primary data the results of testing the antibacterial activity of the methanol extracts of *H. gracilis*, *H. macroloba*, and *H. asperi* against *E. coli*, *S. aureus*, *P. aeruginosa* and indicates that all the results of the diameter of the zones of drag at any concentration still fall into the category of resistant. In general, the activity of antibacterial agents against Gram positive bacteria and Gram

negative is very similar. The working principle of antibacterial is bacteriostatic (inhibits the reproduction of the bacteria, however it does not kill the bacteria) and bactericidal (kills bacteria). Some antibacterial can work as bacteriostatic in low concentrations and can work as bactericidal in high concentrations, as well as bacteriostatic can work bactericidal on a high concentration [21]. Antibacterial agents are classified on the basis of specific targets against bacterial cells, such as disrupting cell wall synthesis, inhibits the synthesis of proteins, disrupt the synthesis of nucleic acids or inhibit the metabolic pathways [20]. According to [22], the content of the active compounds in an organism in drag power against antibacterial.

In this study, the antibacterial activity of macro algae methanol extract that was tested showed that the diameter of drag zones of each macro algae better on inhibiting *S. aureus* and *P. aeruginosa* than *E. coli*. The difference sensitivity of Gram positive bacteria and Gram negative antibacterial can be caused by a few things, namely the difference in substance and structure of the bacterial cell, constituent of the cell wall, peptidoglycan, total amount of lipids, cross ties and activities enzymes (which determines penetration), binding and antimicrobial activity. *Escherichia coli* and *P. aeruginosa* has three layers of cell wall with high lipid content percentage (11-22%) consisting of lipoproteins, outer membrane phospholipids and lipopolysaccharide. Outer membrane phospholipids such active compounds which are antibacterial hard penetrates the cell wall of *E. coli*, while in *S. aureus*, single layered cell wall structure with the percentage content of lipids [23, 24, 25].

Other factors that cause discrepancies' result of antibacterial activity of a material nature against different type of bacteria, which is solvent and concentration used in the extraction. According to [26], each different solvent will dissolve the polarity nature of bioactive of different components. In this study, the possibility of concentration of methanol extracts of macro algae that are set too low for the active compounds that are antibacterial work optimally [26]. According to [27], sub-optimal antibacterial activity can lead to the formation of protein complexes with antibacterial compounds formed through weak ties and immediately undergo decomposition, so antibacterial compounds cannot be coagulating of protein and lyses the cells of *E. coli*.

According to [22], environmental conditions affect the biochemical reactions in the body of organisms including macro algae, such as primary metabolites and metabolite biosynthesis. The influence of sampling location zone (the influence of biotic and abiotic component based on gradient of environmental factors) is very influential on the antibacterial activity. Environmental factors that affect the stability of the active ingredients, which are temperature, radiation of light, air (oxygen, carbon dioxide and water vapor) and moisture, while the factors that affect stability, such as pH, nature of the water, the condition of the biotic, and the presence of a chemical that becomes contaminant [22]. Antibacterial properties are classified based on its ability to inhibit or kill the bacteria and type of bacteria. Broad spectrum antibacterial properties if antibacterial inhibits or kills Gram positive and Gram-negative bacteria. Narrow spectrum antibacterial properties if antibacterial only inhibit or kill the bacteria Gram positive or Gram negative. Spectrum antibacterial properties is limited (limited spectrum) if effective against certain bacterial species [21]. In this study, the macro algae methanol extract examined showed broad spectrum antibacterial properties because the macro algae methanol extracts can inhibit *S. aureus* and *P. aeruginosa*.

5. CONCLUSION

This work has presented a study on antibacterial activity of methanol extract of *Gracilaria salicornia*, *Halimeda gracilis*, *Halimeda macroloba*, and *Hypnea asperi* from Indonesia. Based on the research results, they can be drawn conclusions as foolow: (1) Antibacterial test results against *E. coli* showed the average diameter zone of the

methanol extract of *G. salicornia* on 20% concentration, *i.e.*, of 7.00 ± 1.20 mm; (2) Antibacterial test results against *S. aureus* indicates that the average diameter of highest inhibitory zone of methanol extracts of macro algae is tested on 20 % concentrations i.e. *H. asperi*, *H. macroloba*, *H. gracilis*, and *G. salicornia* respectively; (3) Antibacterial test results against *P. aeruginosa* shows the average diameter of highest inhibitory zone of the methanol extracts of macro algae is tested on 20 % concentrations i.e. *H. gracilis*, *H. macroloba*, *H. asperi*, and *G. salicornia* respectively; (4) The antibacterial activity of macro algae methanol extracts tested on the same concentration against *S. aureus* indicate that there is a different significant influence between the methanol extract of *H. asperi* and *G. salicornia* on 20% concentrations; (5) The antibacterial activity of macro algae methanol extracts that are tested on the same concentration against *P. aeruginosa* showed no difference significant influence; and (6) The comparison antibacterial activity of macro algae methanol extracts which tested on the same concentrate between *S. aureus* and *P. aeruginosa*s howed no difference significant influence.

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