

The anti-bacterial effect of phenolic compounds from three species of marine macroalgae

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Abstract. Scania AE, Chasani AR. 2021. The anti-bacterial effect of phenolic compounds from three species of marine macroalgae. *Biodiversitas* 22: 3412-3417. Bacterial infection is still a serious problem in human health due to many bacteria that are resistant to synthetic antibiotics. Natural antibiotics are needed for substituting synthetic antibiotics such as antibiotics derived from marine macroalgae. Marine macroalgae respond to extreme environmental conditions by producing secondary metabolites containing bioactive substances. Bioactive substances such as phenolic compounds in marine macroalgae may have an antibacterial effect on bacteria. Our research was aimed to detect the presence of phenolic compounds in three species of marine macroalgae (*Ulva lactuca* (Chlorophyta), *Sargassum polycystum* (Phaeophyta), *Palmaria palmata* (Rhodophyta)), and also to determine the most effective inhibition of phenols-contained extracts of marine macroalgae to the growth of *Escherichia coli* and *Salmonella typhi*. Our research was conducted through field sampling for collecting samples and laboratory work for identifying and extracting samples including qualitative testing of phenolic compounds, rejuvenating bacteria, preparing the tested bacteria, and determine the inhibition index of extracts against *E.coli* and *S.typhi*. Our results indicated that the extract of *U. lactuca*, *S. polycystum*, and *P.palmata* contained phenolic compounds. Phenols-contained extract of *Sargassum polycystum* is the most effective in inhibiting the growth of *E. coli* and *S. typhi*. The incubation temperature, the type of bacteria influence the antibacterial activity of marine macroalgae.

Keywords: anti-bacteria, marine macroalgae, phenolic compounds, *Sargassum polycystum*, qualitative test

INTRODUCTION

Technological advances coincide with the emergence of various diseases in all societies. Bacterial infection is one of the causes of disease which is still a serious problem in human life. There have been many synthetic antibiotic products that have been used as antibacterial, but there are still many bacteria that are resistant to these synthetic antibiotics. According to the Ministry of Health (2011), the high number of infections, both endemic and epidemic, and the use of antibiotics are suspected to be the cause of bacterial resistance. So, that natural antibiotic is needed as a substitute including marine macroalgae (Bleakley and Hayes 2017).

Marine macroalgae in Indonesia are very abundant and have a highly important value that can be used as food, cosmetics, fuel, and medicines (Luning 1990). Marine macroalgae (seaweeds) are non-vascular benthic thallophytes that the plant body can not be distinguished between roots, stems, and leaves. Three major phyla of marine macroalgae are often found in the intertidal zone, i.e. Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae). Marine macroalgae grow attached to the seabed and are mostly anchored to a fairly sturdy hard substrate such as corals or rocks (Diaz-Pulido and McCook 2008).

According to Choi et al. (2010), extreme environmental conditions such as high salinity levels and periodic exposure to direct sunlight and air are responded to by marine macroalgae by producing secondary metabolites.

These secondary metabolites have bioactive substances that can be used to protect macroalgae from predatory threats (Masduqi et al. 2014). Bioactive substances such as phenolic compounds in marine macroalgae may have an antibacterial effect on human pathogenic bacteria. Several previous studies have detected the presence of phenolic compounds in marine macroalgae i.e. *Ulva lactuca* (Arbi et al. 2016), *Sargassum polycystum* (Masduqi et al. 2014), and *Palmaria palmata* (Holdt and Kraan 2011). Therefore, our study was conducted not only to detect the presence of phenolic compounds in different marine macroalgae but also to determine the most effective inhibition of phenol-contained extracts of marine macroalgae to the growth of *Escherichia coli* and *Salmonella typhi*.

MATERIALS AND METHODS

Marine algae that used in this research were *Ulva lactuca* (Chlorophyta), *Sargassum polycystum* (Phaeophyta), *Palmaria palmata* (Rhodophyta). *Escherichia coli* isolate, and *Salmonella typhi* isolate was obtained from human digestive tract. Other chemical compounds were Nutrient Agar (NA), 70% ethanol, ethyl acetate, methanol, formic acid, and ferric chloride. The tools used in our research were ziplock plastic, icebox, blender, analytical scale, Erlenmeyer, measuring cup, measuring pipette, hotplate/stirrer, filter paper (Oxoid), disc paper, evaporator, ose, incubator, laminar airflow

(LAF), Petri dishes, sterile swabs, silica gel plate, chamber, and UV-vis spectrophotometer.

Marine macroalgae sampling and identification

Marine macroalgae samples were collected from Sepanjang Coast in Gunungkidul, Yogyakarta, Indonesia by hand-picking during the lowest tide every month from July to November 2019. Using zip lock plastic filled with seawater, samples were put in the icebox for temporary storage before being taken to the laboratory. The samples were cleaned using freshwater and stored as wet herbaria voucher specimens in Plant Systematics Laboratory, Faculty of Biology, Gadjah Mada University.

Marine macroalgae herbaria were identified using the identification book of Dawes (1981), the ITIS Standard Report Page (2021), and the FAO Species Identification Guide for Fishery Purposes: The Living Marine Resources of The Western Central Pacific, Volume 1 (Seaweeds, Corals, Bivalves and Gastropods) (1998). Identification was done by observing the morphology of marine macroalgae such as habitus or life form, the color of the thallus, the shape of the thallus, and the type of branching of the thallus. Then, the observation data were adjusted to the diagnosis characters in the identification book to ensure the identity of marine macroalgae samples.

Extraction of macroalgae samples

After oven-dried at 45°C for 3 hours, the dried samples were powdered using a blender. Fifty grams of samples were put into Erlenmeyer tube, then macerated using 100 ml 70% ethanol and macerated for two days. After two days, the filtrate was filtered using filter paper and dried in an incubator at 37°C for 4 days to get thick extracts.

Qualitative test for phenolic compounds

Qualitative assay for phenolic compounds was done by *Thin Layer Chromatography* (TLC) method. Fifty mg of marine macroalgae extracts added with 1 ml of ethyl acetate were vortexed for 2 minutes and centrifuged for 3 minutes. The resulting liquid phase was taken and dropped as much as 10 µl on the silica gel plate. The plate was inserted into the saturated chamber of the mobile phase of 8:2 methanol and formic acid ratio then smeared until the limit. The plates were dried and observed under ultraviolet light. The results obtained were sprayed with a ferric chloride reagent. The presence of phenolic compounds was marked by the appearance of a blue-gray color.

Phenol qualitative test was carried out using the TLC method with a stationary phase of a 60 F₂₅₄ silica gel plate (Merck) and a mobile phase of methanol: formic acid with a ratio of 8: 2. A UV lamp with a wavelength of 254 nm and 366nm was used and a solution of FeCl₃ as a spray reagent. The 254 nm wavelength was adjusted to the fluorescence indicator on the TLC plate so that the plate will glow and the target compound will appear as a dark stain. UV with a wavelength of 366 nm was used as a comparison during observation. Light and fluorescence from organic compounds can appear at 254 nm and 366 nm (Bobbitt et al. 1968).

Bacteria rejuvenation

One ose of pure culture of *E. coli* and *S. typhi* was taken and inoculated by the streak method on sloping Nutrient Agar (NA) medium. Bacterial culture on each agar slant was incubated at 37°C for 24 hours.

Preparation of test bacteria

Escherichia coli and *S. typhi* isolates were taken each one ose and inoculated into NA media with streak plate method.

Test of inhibition

In vitro testing was carried out using the disk diffusion method (Oxoid paper disk). The sterilized NA medium was cooled at a temperature of 40 - 45°C and poured into a petri dish in *laminar airflow* aseptically. After the NA medium on the petri dish has solidified, the bacterial suspension of *E. coli* and *S. typhi* were spread evenly on the surface of the medium using a sterile swab. Phenol-contained extracts of marine macroalgae were taken as much as 20 µL and dripped on disc paper. The disc paper was affixed to the surface of the NA medium which had been smeared with the bacterial suspension. After incubated at 37°C for 24 and 48 hours, the diameter of bacterial growth and the zone of inhibition around the disc paper were measured. The inhibitory activity of macroalgae extracts was calculated from the formation of a clear zone around the disc paper.

Data analysis

The inhibition index (IP) of macroalgae extract against bacterial growth was measured by the following calculation formula:

$$IP = \frac{\text{Clear Zone Diameter} - \text{Disc Paper Diameter}}{\text{Disc Paper Diameter}}$$

RESULTS AND DISCUSSION

Marine macroalgae

Three species of marine macroalgae were sampled due to their abundance, commonly and easily found in the intertidal zone of Gunungkidul coastal areas, and they contain phenol (Arbi et al. 2016; Masduqi et al. 2014; and Holdt and Kraan 2011). The three marine macroalgae were *Ulva lactuca* Linn., *Sargassum polycystum* C. Agardh., and *Palmaria palmate* (Linnaeus) F. Weber & D. Mohr (Figure 1).

Ulva lactuca (sea lettuce) belongs to green algae (Chlorophyta), characterized by bright green to dark green thallus. The size of the thallus is about 7 cm in length and 2-3 cm in width. The thallus expands into a thin, wide, shiny sheet with a punched or wavy edge. The body structure consists of blades and holdfast. The blade is a flat and slippery sheet with sympodial branching, while holdfast is like a basalt disc or basalt plate. This sympodial branching is not visible because the types cannot be distinguished (Handayani 2016).

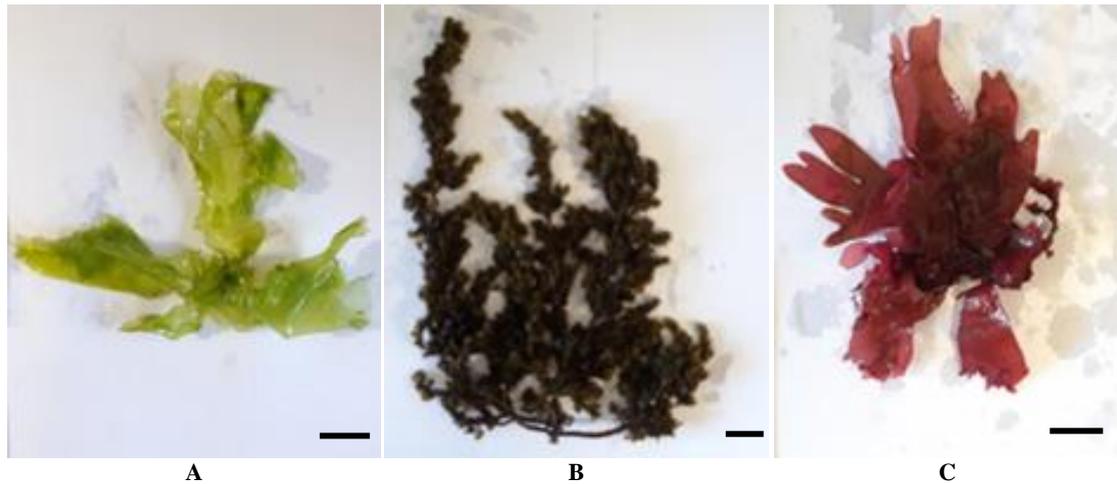


Figure 1. Marine macroalgae collected from Sepanjang Coast in Gunungkidul, Yogyakarta, Indonesia: A. *U. lactuca*, B. *S. polycystum*, C. *P. palmata*. Bar = 2 cm

Sargassum polycystum (Phaeophyta) grows in rocky habitats in shallow water and is attached to coral reefs or rocks. *S. polycystum* is characterized by dark brown to yellowish-brown thallus. The length of the thallus is about 25-30 cm with the frond size around 1-3 cm in length and 0.5-2 cm in width. According to Widyartini et al. (2015), *S. polycystum* has cylindrical and vesicle-shaped thallus. This leaf-shaped thallus generally has jagged edges and is tapered or rounded and longitudinal. Cryptostomatas are scattered on the leaf surface. The vesicles are round. The main branches grow lush at the ends. The primary branches are terete, while the bearing secondary branches are alternately unorganized with much simple proliferation and are shaped like the letter Y.

Palmaria palmata (dulse) is characterized by a red-brown thallus with a thin membrane, belong to red algae (Rhodophyta). They grow on broken coral or sand substrate that is inundated by seawater. Their thallus size around 14 cm in length and 8-10 cm in width. Their body structure consists of a blade which is a thin sheet, slippery and shaped like a ribbon, and a holdfast that resembles a basalt disc or basalt plate. The type of branching is dichotomous or regular bifurcation (Sofiyana 2016).

Phenol qualitative test

The selection of FeCl_3 as staining reagent was done based on the chemical content in the sample and based on Alen et al (2017), the research mentioned FeCl_3 reagent is a typical reagent for detection of phenolic compounds. The results of the chromatogram pattern were presented in Figure 2. In Figure 2. It showed there a change in color to dark green or blackish blue indicates a positive result of phenolic compounds which reduce Fe^{3+} to Fe^{2+} . According to Hanani (2015), the presence of phenolic compounds in the sample is indicated by changing color to a strong blue or black after being sprayed with FeCl_3 solution.

Antibacterial activity test

The growth inhibition of *E. coli* and *S. typhi* by phenols-contained extracts of *U. lactuca*, *S. polycystum*,

and *P. palmata* was detected by the clear zone that formed around the disc paper. Comparison of inhibitory zone diameter of extracts, positive control, and negative control was the basis for determining the effectiveness of the antibacterial activity. Amoxicillin was used as a positive control, while distilled water was used as a negative control. The growth inhibition of *E. coli* and *S. typhi* caused by positive and negative control were presented in Figure 3 and Figure 4.

Based on the results in Fig. 3 and 4, it can be seen that the negative control distilled water has no clear zone as an indication of bacterial growth inhibition. Positive control amoxicillin results in the formation of a clear zone around the disc paper that indicated the growth inhibition of *E. coli* and *S. typhi*. According to Maida and Lestari (2019), Amoxicillin is a semi-synthetic penicillin derivative compound. Amoxicillin is a broad-spectrum β -lactam antibiotic and is often used as an effective antibiotic to terminate Gram-positive and Gram-negative bacteria. The mechanism of action of amoxicillin is by inhibiting the mucopeptide that is formed for the synthesis of the bacterial cell wall. The disruption of the bacterial cell wall results in the inability of the bacteria to overcome the pressure difference of osmosis inside and outside the cell that can cause the death of bacteria (Wiedemann 1997).

The growth inhibition of three marine algae (*U. lactuca*, *S. polycystum*, and *P. palmata*) against *E. coli* and *S. typhi* were presented in Figures 5 and 6.

The zone of inhibition produced by the extracts indicated that the phenol-contained extract of the three macroalgae has antibacterial activity. Bacterial growth inhibition was classified based on the average inhibition zone diameter as follows: weak or less inhibition when the average inhibition zone diameter is < than 5 mm, moderate inhibition when the average of the inhibition zone diameter is between 5-10 mm, strong inhibition when the average of the inhibition zone diameter is between 10-20 mm, and very strong inhibition when the average diameter of the inhibition zone is more than 20 mm (David and Stout 1971). The diameter of the inhibitory zone of treatments was presented in Tables 1 and 2.

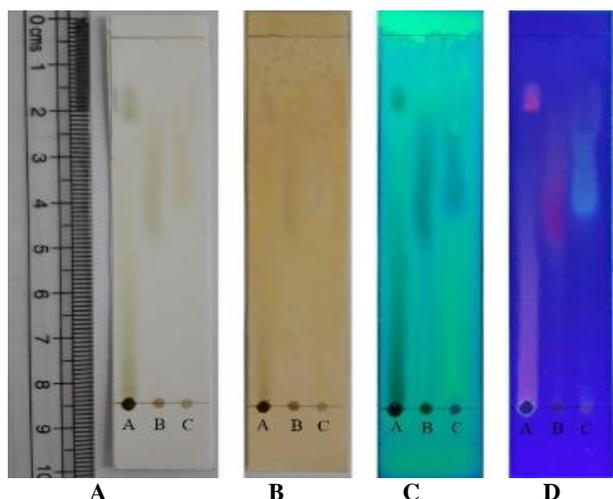


Figure 2. TLC profile: A. Before spraying with FeCl₃, B. After spraying with FeCl₃, C. After spraying with FeCl₃ and observed under UV light of 254 nm, D. After spraying with FeCl₃ and observed under UV light of 366 nm. A: *U. lactuca* extract, B: *S. polycystum* extract, C: *P. palmata* extract

The comparison of the inhibition index of treatment to the growth of the tested bacteria at 24 hours incubation and 48 hours incubation was in Figure 7.

Sargassum polycystum extract had the highest inhibition index (Figure 7). *S. polycystum* extract has the highest antibacterial activity that might be caused by the presence of flavonoids (Manteu et al. 2018). The flavonoid compounds can denature bacterial cell proteins which are lipophilic and damage cell membranes. Flavonoids are formed a complex with bacterial cell proteins through hydrogen bonds. The presence of hydrogen bonds with flavonoids causes the protein structure of bacteria was damage. Therefore, the cell wall structure and bacterial cytoplasmic membrane which contains proteins become unstable and lose their biological activity. Bacterial cell death occurs because the permeability function of bacterial cells was disrupted and lysis (Harborne 1985).

Figure 7. shows the changes in the inhibitory index after 24 hours and 48 hours of incubation. The inhibition index decreased after 48 hours of incubation. The inhibitory index of *U. lactuca* extract against *E. coli* decreased from 1,2 to 1,1 and against *S. typhi* decreased from 1,2 to 1,0 after 48 hours of incubation. The inhibition zone diameter of *S. polycystum* extract against *E. coli* decreased from 1,3 to 1,2 and *S. typhi* decreased from 1,4 to 1,3. While The inhibition zone diameter of *P. palmata* against *E. coli* decreased from 0,9 to 0,8 and *S. typhi* decreased from 0,5 to 0,4.

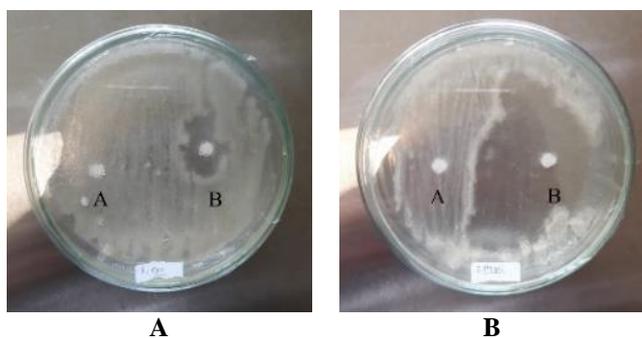


Figure 3. The growth inhibition of positive control amoxicillin and negative control against (a) *Escherichia coli* (b) *Salmonella typhi* after 24-hours of incubation on NA medium at 37°C. A: negative control, B: positive control

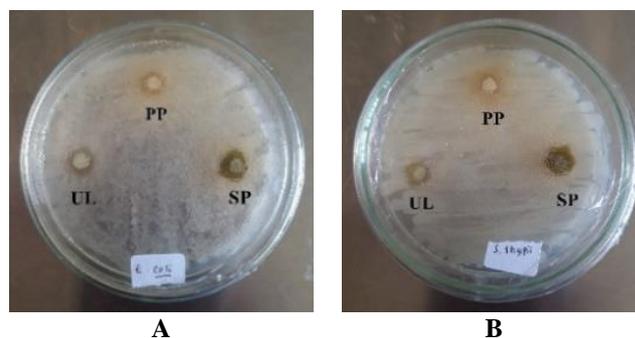


Figure 5. The growth inhibition of phenol-contained extracts of marine macroalgae against (a) *E. coli* and (b) *S. typhi* after 24 hours of incubation on NA medium incubation at 37°C. *U. lactuca*, SP: *S. polycystum*, PP: *P. palmata*

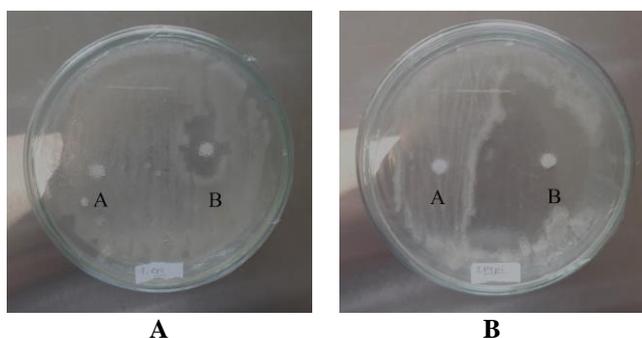


Figure 4. The growth inhibition of positive control amoxicillin and negative control against (a) *Escherichia coli* (b) *Salmonella typhi* after 48 hours of incubation on NA medium at 37°C. A: negative control, B: positive control

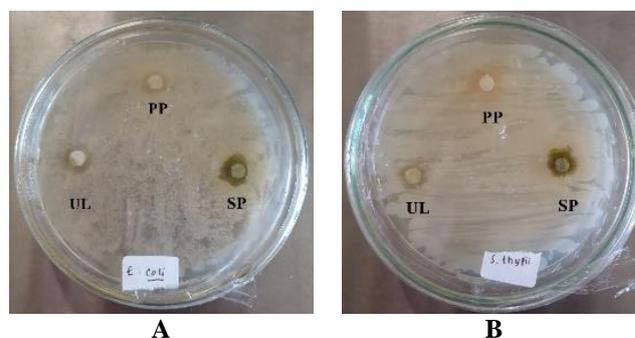


Figure 6. The growth inhibition of phenol-contained extracts of marine macroalgae against (a) *E. coli* and (b) *S. typhi* after 48 hours of at 37°C. UL: *U. lactuca*, SP: *S. polycystum*, PP: *P. palmata*

Table 1. The Inhibition index (IP) and inhibition category after 24 hours of incubation at 37°C

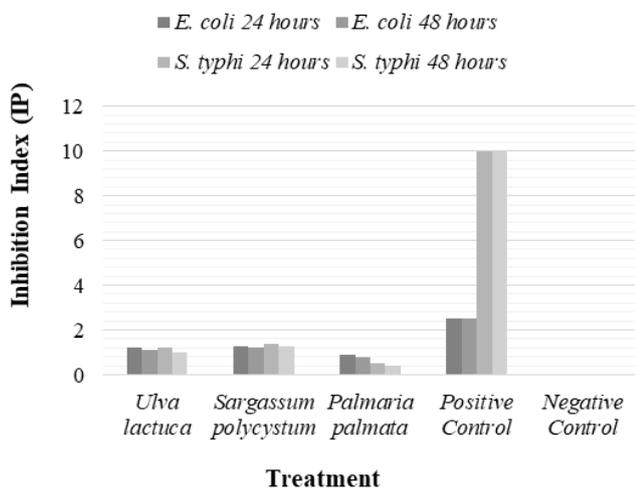
Treatments	Bacteria	IP	D (mm)	Category
<i>Ulva lactuca</i>	<i>E. coli</i>	1,2	11	Strong
	<i>S. typhi</i>	1,2	11	Strong
<i>Sargassum polycystum</i>	<i>E. coli</i>	1,3	11,5	Strong
	<i>S. typhi</i>	1,4	12	Strong
<i>Palmaria palmata</i>	<i>E. coli</i>	0,9	9,5	Moderate
	<i>S. typhi</i>	0,5	7,5	Moderate
Positive control	<i>E. coli</i>	2,5	17,5	Strong
	<i>S. typhi</i>	10	55	Very strong
Negative control	<i>E. coli</i>	0	0	None
	<i>S. typhi</i>	0	0	None

Note: IP: Inhibition Index, D: Diameter of inhibitory zone

Table 2. The Inhibition index (IP) and inhibition category after 48 hours of incubation at 37°C

Treatments	Bacteria	IP	d (mm)	Category
<i>Ulva lactuca</i>	<i>E. coli</i>	1,1	10,5	Strong
	<i>S. typhi</i>	1	10	Strong
<i>Sargassum polycystum</i>	<i>E. coli</i>	1,2	11	Strong
	<i>S. typhi</i>	1,3	11,5	Strong
<i>Palmaria palmata</i>	<i>E. coli</i>	0,8	9	Moderate
	<i>S. typhi</i>	0,4	7	Moderate
Positive control	<i>E. coli</i>	2,5	17,5	Strong
	<i>S. typhi</i>	10	55	Very strong
Negative control	<i>E. coli</i>	0	0	None
	<i>S. typhi</i>	0	0	None

Note: IP: Inhibition Index (IP), d: Diameter of inhibitory zone

**Figure 7.** Inhibition index of phenol-contained marine macroalgae extracts against *E. coli* and *S. typhi* after 24 hours 48 hours of incubation at 37°C incubation

According to Trisia et al. (2018), antibacterials are categorized into two types, i.e., bacteriocides and bacteriostatic. Substances that can terminate bacterial growth are bacteriocides characterized by an increase in the diameter of the inhibition zone, while substances that

inhibit bacterial growth are bacteriostatic. This compound can terminate bacteria by stopping their physiological activity (Mycek 2001). The results in this study showed the reduction of inhibition zone size due to the inhibitory ability by the phenol-contained extract of marine macroalgae decreases over time. Eventually, bacteria will grow and reproduce again when the inhibitory ability runs out. It is because the longer a material is used, the less diffused substance will be so that the antimicrobial activity in inhibiting bacterial growth will be lower. The extract of *U. lactuca*, *S. polycystum*, and *P. palmata* have bacteriostatic activity because it cannot stop the growth of bacteria or even terminate the bacteria but only inhibit the growth of bacteria. The Minimum Inhibitory Concentration (MIC) value was not calculated or was not obtained because of the low concentration and because the antibacterial activity was bacteriostatic or only inhibited, the Minimum Bactericidal Concentration (MBC) value was not found.

Furthermore, the anti-bacterial effect is influenced by several factors such as incubation temperature and the type of bacteria (Zeniusa et al. 2019). Although bacteria will grow optimally when incubated at 35°C but our study showed that bacteria grow well at 37 °C. Bacteria that have high permeability, hydrophilic porin, and nonpolar lipid layer cause bioactive compounds that could not enter into bacterial cells so that the antibacterial effect of phenolic compounds is also not optimal. Bacterial suspension turbidity also impacts anti-bacterial effects (Kumar et al. 2013). Turbidity means the optical density of bacterial cells. Bacteria divide and grow, populate the medium, and hence it looks turbid. When it is added with something that kills the bacteria, cells burst open then hence the solution looks clear again. So, the more turbid the bacterial suspension is used, the less anti-bacterial effect it has and vice versa.

In conclusion, our results indicated that the extract of *U. lactuca*, *S. polycystum*, and *P. palmata* contained phenolic compounds. Phenols-contained extract of *Sargassum polycystum* is the most effective inhibitor to the growth of *E. coli* and *S. typhi*. The incubation temperature and type of bacteria influence the differences in the anti-bacterial effect of marine macroalgae

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