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Antibacterial Activity and Identification of Active Compounds of Seaweed Extract *Sargassum* sp., *Halimeda opuntia* and *Halymenia* sp. from Lae-Lae Island of South Sulawesi

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Abstract**—***In addition to containing primary metabolites, seaweed also contains secondary metabolites in the form of active compounds that function as antimicrobial and anticancer. Some species of seaweed include brown seaweed from the genus of Sargassum, green seaweed from the genus of Halimeda and red seaweed from the genus of Halymenia. The waters around Makassar City found species of Sargassum sp., Halimeda opuntia and Halymenia sp., which is abundant especially on Lae-Lae Island, but studies on its potential bioactivity are still very limited. This research aims to determine the bioactivity and identification of the active compound groups of the extracts of Sargassum sp., Halimeda opuntia and Halymenia sp. The research was carried out in December – April 2021. Samples were extracted using the meseration method. Antibacterial activity test used agar diffusion method and phytochemical test used Thin Layer Chromatography (TLC). The yield of methanol extract and seaweed hexane of Sargassum sp. respectively 2.4 and 2.3% and Halimeda opuntia of 0.53 and 1.15% and Halymenia sp. of 2.42 and 0.89%. The six seaweed extracts had no activity against E. coli bacteria. Hexane extract of Sargassum sp. had the highest activity against the bacteria of S. typhi with an average diameter of 25.67 mm and the highest level against the bacteria of A. hydrophila with an average diameter of 18.2 mm with methanol as solvent. The same activity was shown by methanol extracts of Sargassum sp. and Halymenia sp. against the bacteria of V. harveyi with an average zone diameter of 18.04 mm. However, only the methanol extract of Halimeda opuntia had activity against the bacteria of P. aeruginosa with an average diameter of 8.63 mm. An important finding that was found from the results of this research was the extract of Sargassum sp. showed the highest activity against S. typhi, A. hydrophila and V. harveyi with a higher diameter inhibition zone than the commercial antibiotic activity of ciprofloxacin as a positive comparison. This shows that the extract of Sargassum sp. has a great potential as a source of new antibiotics, especially against S. typhi. The extracts of methanol and hexane of Halimeda opuntia and methanol of Sargassum sp. contains 5 active compounds namely, alkaloids, flavonoids, tannins, triterpenonids and saponins. While the seaweed hexane extracts of Sargassum sp., only contains 3 active compounds namely, alkaloids, tannins and saponins. The methanol extracts of Halimeda opuntia contains 4 active compounds namely, flavonoids, tannins, triterpenonids and saponins, while the hexane extracts contain alkalaoids, flavonoids, tannins and saponins.*

Keywords**—*** ***Seaweed extract, anti-bacterial, Sargassum sp., Halimeda opuntia, Halymenia sp.***

I. INTRODUCTION

Aquaculture production increased by 3.26% from 2016 to 2017 [1]. However, the increase in production and demand for freshwater fish commodities, both consumption and non-consumption (ornamental fish) in Indonesia will carry the risk of being attacked by fish pests and diseases that

have the potential to damage the sustainability of the biological resources of fisheries. Fish and shrimp disease is one of the serious problems faced by aquaculture farmers because it has the potential to cause huge losses due to increased fish and shrimp mortality. Seeing these problems, other alternatives are needed as a solution to prevent infection by pathogenic organisms generally using antibiotics. However, the usage of antibiotics causes microbial resistance. The emergence of resistance and infection of antibacterial pathogenicity *E. coli*, *P. aeruginosa*, S. *tyhpi*, *V. harveyi*, *A. hydrophila* makes scientists seek to find drugs as new antibacterials. The alternative way is the use of seaweed.

In addition to containing primary metabolites, seaweed also contains secondary metabolites in the form of bioactive compounds that have the potential as antibacterial compounds [2]. This is in line with the research of Kasmiati et al., 2018 [3] which reported that several active compounds in seaweed function as antimicrobials to inhibit the growth of other competitive microorganisms and are potential for new secondary metabolites. Seaweed has been widely studied to have potential as an antibacterial [4], one of which is brown seaweed from the genus of *Sargassum*. *Sargassum polycystum* is rich in secondary metabolites, such as phenols, flavonoids, tannins, sterols, terpenoids, saponins, alkaloids and glycosides [5]. Red seaweed of the genus of *Halymenia* apart from being a source of natural pigments, also contains active components that have been widely reported as antioxidants, antibacterials, antimalarials, and antivirals [6]; [7].

The waters around Makassar City found species of *Sargassum* sp., *Halimeda opuntia* and *Halymenia* sp., which is abundant especially on Lae-Lae Island, but studies on its potential bioactivity are still very limited. This research aims to determine the active compound content and antibacterial activity of *Sargassum* sp., *Halimeda opuntia* and *Halymenia* sp. found on Lae-Lae Island of South Sulawesi.

II. MATERIAL AND METHODS

2.1 Materials

The materials used were seaweed of *Sargassum* sp., *Halimeda opuntia* and *Halymenia* sp., methanol and hexane, eule solution (methanol-chloroform (2:1)), 4% of H2SO4 solution, aluminum plate of KLT Silica Gel 60 F254, DMSO and ciprofloxacin commercial antibiotics. Test bacteria of *Escherichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa* were obtained from Faculty of Medicine of Hasanuddin University, while *Aeromonas hydrophila* and *Vibrio harveyi* were obtained from the Brackish Water Aquaculture Center (BPBAP) of Takalar. Growth media for bacteria are Nutrient Broth (NB), Nutrient Agar (NA) and Tryptone Soya Agar (TSA). Disc paper in diameter of 6 mm, filter paper of Whatman No. 01. The equipments used are a blender, autoclave, oven, rotary vacuum evaporator, laminar air flow, vacuum pump, analytical scale, chamber, micropipette, incubator, magnetic plate, stirrer, and glassware.

2.2 Collection and Preparation of Seaweed

Seaweed samples of *Sargassum* sp., *Halimeda opuntia* and *Halymenia* sp. collected from the waters of Lae-Lae Island using snorkeling equipment. The sampling location was reached by motorboat. Samples were taken by removing the sand substrate and then washing it with seawater to clean it from adhering dirt such as sand, shellfish, and mud. Each sample was put in a plastic sample and stored in a cold box containing ice as a cooling medium to maintain the freshness of the seaweed during the trip to the laboratory. After arriving at the laboratory, each seaweed sample was washed with fresh running water to clean it from seawater and remaining dirt, then drained and weighed to determine the wet weight of the sample. The samples were dried in the shade for 7 - 10 days to obtain dried seaweed which was then weighed to determine the weight of the dry sample. Each species of seaweed is mashed using a blender to produce seaweed flour and then stored in the refrigerator in an airtight container.

2.3 Seaweed Extraction

Sample extraction using methanol and hexane solvent with maceration method refers to El Shafay et al., 2016 [8] which has been modified. A total of 150 g of each species of seaweed was put into a baker and then added with each solvent of methanol and hexane of 600 ml (1:4, w/v) and allowed to stand at room temperature for 3 days with stirring using a magnetic stirrer. The homogenate was filtered using filter paper of Whatman No.1 in a vacuum to separate the dregs and the filtrate. The filtrate was evaporated using a rotary vacuum evaporator to evaporate the solvent to obtain a crude extract of methanol and hexane in the form of a concentrate from each species of seaweed, thereby obtaining 2 crude extracts for each species of seaweed so that the total extracts of the three species of seaweed were 6 extracts. The crude extract was then weighed to determine its weight. The extract was stored in a glass container with a lid and stored in a refrigerator before being used in the test. Before the testing, the instrument was sterilized. Instrument sterilization is carried out by wrapping glassware using paper and then placing it in the oven and sterilizing it at 180°C for 2 hours.

2.4 Antibacterial activity

2.4.1 Preparation of Media and Test Bacteria

NB and NA media were prepared by dissolving separately 13 g of NB and 23 g of NA in 1 L of distilled water and then heated until dissolved. Both media were sterilized in an autoclave at 121ºC for 15 minutes. Similarly, TSA media were prepared in the same way as NA media. The agar medium was cooled to about 50ºC before being poured into the petridis. The test bacteria were rejuvenated on sterile sloping media and incubated at 37ºC for 24 hours. A total of 1 ose of bacteria on slanted agar was inoculated on NB media, homogenized using a vortex and then incubated at 37ºC for 24 hours to achieve viability of 108 colonies/ml.

2.4.2 Test of Antibacterial Activity

The antibacterial activity test of crude extracts of methanol and hexane refers to Bauer at al. (1996) [9] and Christobel at al. (2011) [10] with the modified agar diffusion method. A total of 1 ml of each bacterial culture was inoculated on NA media (except *A. hydrophila* and *V. harveyi* on TSA media) in petri dishes. Each extract of methanol and hexane was taken as much as 1000 g and dissolved in 50 µl of the solvent until completely dissolved. 10 µl was taken and applied to paper disk in a diameter of 6 mm at a dose of 200 µg/disk. After the solvent evaporates, each disk is dripped with 5 µl of DMSO and then placed on agar media which already contains the test bacteria. As a positive control used ciprofloxacin commercial antibiotic of 5 µg [8] while the negative control used DMSO of 5 µl. Petri dishes containing the test samples were wrapped in plastic wrap and incubated at 37ºC for 24 hours. The clear zone formed around the disc indicated the inhibition of bacterial activity by the extract which was expressed in millimeters as the average value of three replications.

2.5 Identification of Active Compounds

2.5.1 Phytochemical Test

Identification of the group of active compounds contained in the methanol and hexane extracts of *Sargassum* sp., *Halimeda opuntia* and *Halymenia* sp. carried out through a phytochemical test using the method of Thin Layer Chromatography (TLC) refers to Harbone (1984) [11] by observing the presence of spots on the extract that has been sprayed on the TLC plate. Aluminum Plate of TLC Silica Gel 60 F254 was cut to a size of 1 x 5 cm for each test carried out. Then each extract to be tested was spotted on the prepared plate. Furthermore, the eluent solution of Methanol - Chloroform (2:1) was prepared and put into the chamber. After that, the TLC plate that has been stained with extract is put into a chamber that already contains eluent for the elution process. After completion of the elution process, the TLC plate was sprayed with certain reagents to see the color changes that occurred.

Flavonoid compounds were identified by spraying reagent solution of AlCl3 10% on a TLC plate that had gone through the elution process. Positive results were seen from the change in the color of the stain to light yellow-green. Triterpenoids/steroids were identified by spraying reagent solution of H2SO4 10%. A positive result was seen from the change in the color of the stain to brownish pink. Tannins were identified by spraying reagent solution of FeCl3 5%. Positive results were seen from the change in the color of the stain to dark blue-black.

Alkaloid compounds were identified by means of ± 2 mg of extract put into a mortar then 10 ml of chloroform was added and dissolved. Added 5 ml of chloroform-ammonia 0.05 M, filtered into a test tube. To the filtrate, 10 - 20 drops of sulfuric acid 2 N were added and then gently shaken for 2 - 3 minutes and allowed to form 2 layers. The top layer was taken and put into 2 test tubes and tested with Mayer and Dragendorff reagents. The formation of a white precipitate against the reagent of Mayer and an orange-red precipitate with the reagent of Dragendorff's showed a positive result of the alkaloid test. Saponins were identified by means of 1 ml of water fraction inserted into a test tube. The tube is shaken for 1 - 2 minutes. The formation of a permanent foam (not disappear for 5 minutes) indicates the presence of saponins.

III. RESULT AND DISCUSSION

3.1 Yield

Sample extraction was carried out in stages which included homogenization and partitioning of each sample in methanol and hexane. Homogenization of the sample with each solvent methanol and hexane at room temperature for three consecutive days aims to maximize the uptake of the active compound components. The concentrated homogenate was partitioned in methanol and hexane, respectively, so that crude extracts of methanol and hexane were obtained. The extracted yield of *Sargassum* sp. obtained from the waters of Lae-Lae island and it can be seen in Table 1.

Table 1. Yield of sample extraction

Sample	Gross Weight (BB) (g)	Simple Weight (BS) (g)	Extract	Extract Weight (BE) (g)	% Yield = BE/BS x 100%
Sargassum sp.	5650	300	methanol	7,2	2,4
			hexane	6,9	2,3
Halimeda opuntia	5350	300	methanol	7,27	0,53
			hexane	2,68	1,15
Halymenia sp.	3560	300	methanol	1,6	2,42
			hexane	3,45	0,89

Yield can be an important parameter to determine the total components that can be extracted and the effectiveness of an extract that can be utilized. The results after the extraction process are complete, the yield sequentially obtained is *Sargassum* sp. (methanol), *Sargassum* sp. (hexane), *Halimeda opuntia* (methanol), *Halimeda opuntia* (hexane), *Halymenia* sp. (methanol) and *Halymenia* sp. (hexane) of 2.4; 2.3; 0.53; 1.15; 2.42 and 0.89%. The three highest yields obtained were the extracts of methanol and hexane of *Sargassum* sp. and methanol extract of *Halymenia* sp. by 2.3%: 2.4% and 2.42%.

The extracts of *Sargassum* sp. and *Halymenia* sp. which used methanol as a solvent resulted in a higher yield when compared to hexane solvent. This is due to the dissolved bioactive components in nonpolar solvents are relatively small. The difference in yield is influenced by the type of solvent used. The yield of the resulting extract is influenced by several factors, including the selection of the type of solvent, the ratio of the number of samples to the solvent, the extraction temperature, the particle size of the sample and the extraction time [12], [13] dan [14].

Methanol is a form of alcohol with the chemical formula CH3OH (Araya et al., 2020). The chemical structure of methanol consists of a hydroxyl group (polar) and a carbon group (nonpolar) so that methanol is polar. Extraction with methanol produces more extracts, because the highly polar nature of methanol is thought to be able to extract more bioactive components that are highly polar and slightly nonpolar [15].

3.3 Antibacterial Activity

The antibacterial activity test in this research used the bacteria of *E. coli*, *P. aeruginosa*, *S. thypi*, *A. hydrophila* and *V. harveyi*. The use of these five bacteria aims to see the activity of seaweed sample extracts that can inhibit bacterial growth. The method used in this research was the agar diffusion method with a dose of each extract was 200 µg/disk. The positive control used the ciprofloxacin antibiotic of 5 µg/disk and the negative control used DMSO of 5 µg/disk. The results showed that the antibacterial activity of each sample extract against five bacteria was indicated by the formation of a halo zone around the paper disc. The results of the measurement of the inhibition zone formed can be seen in (Fig.1).

The research results, the six seaweed extracts namely *Sargassum* sp. (Methanol and hexane), *Halimeda opuntia* (methanol and hexane) and *Halymenia* sp. (methanol and hexane) had no activity against the bacteria *E. coli*. The hexane extract of *Sargassum* sp. had the highest activity against the bacteria of *S. typhi* with an average diameter of the inhibition zone of 25.67 mm and the highest against the bacteria of *A. hydrophila* with an average diameter of 18.2 mm with methanol as solvent. The same activity was shown by the methanol extract of *Sargassum* sp. and *Halymenia* sp. against the bacteria of *V. harveyi* with an average diameter of the inhibition zone 18.04 mm. However, only the methanol extract of *Halimeda opuntia* had activity against the bacteria of *P. aeruginosa* with an average inhibition zone diameter of 8.63 mm. This shows a wider activity as an antibacterial because it has inhibitory activity against all test bacteria except for *E. coli* (Fig. 2).

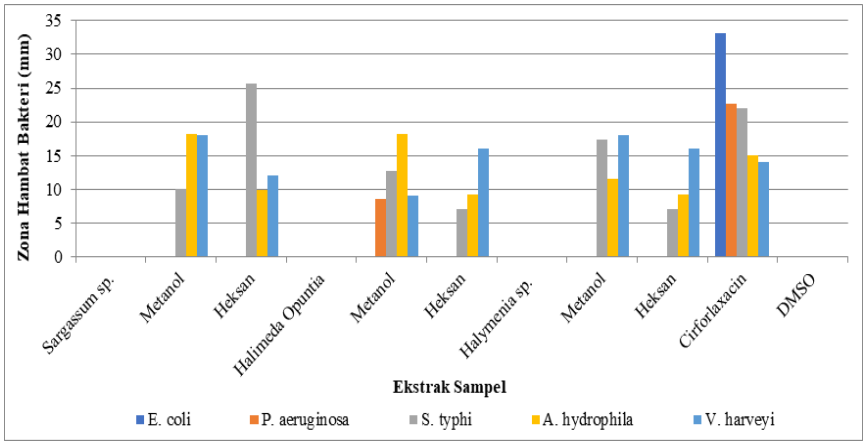

Fig.1. Diagram of antibacterial activity of seaweed extract samples of Sargassum sp., Halimeda opuntia and Halymenia sp. against the bacteria: E. coli, P. aeruginosa, S. thypi, A. hydrophila and V. harveyi, (+) Ciprofloxacin and (-) DMSO

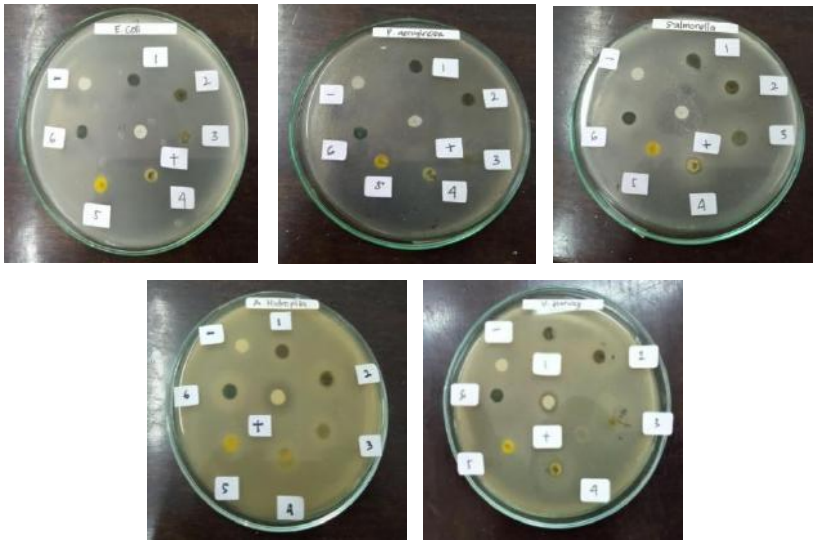

Fig.2. The results of antibacterial activities of the extracts of Sargassum sp., Halimeda opuntia and Halymenia sp. against the bacteria: E. coli, P. aeruginosa, S. thypi, A. hydrophila and V. harveyi, (+) Ciprofloxacin and (-) DMSO

The important finding that was found from the results of this research was the extract of *Sargassum* sp. showed the highest activity against *S. typhi*, *A. hydrophila* and *V. harveyi* with a larger inhibitory diameter than the commercial antibiotic of ciprofloxacin as a positive comparison (Figure 2). This indicates that the extract of *Sargassum* sp. has great potential as a source of new antibiotics, especially against the *S. typhi*. While the positive control of ciprofloxacin showed activity against all types of the test bacteria with an inhibition zone diameter range of 14.1 – 33.12 mm and the negative control of DMSO showed no activity against all the test bacteria.

The antibacterial ability of the six sample extracts was due to the active compounds contained in the seaweed extract. The inhibition mechanism of the bacteria by steroid compounds is thought to be damaging the bacterial cell membrane. Based on how it works, antibacterials are divided into bactericidal and bacteriostatic [16]. Bacteriostatic antibacterials are substances that work to inhibit bacterial growth [17], while bactericidal antibacterials are substances that work to kill bacteria [18].

The diameter of the inhibition zone in each bacterium showed differences, this could be due to several factors. The first factor at the time of 24-hours incubation of bacteria experienced a logarithmic phase where the growth of bacteria was twice that of the lag phase. The second factor is resistance by the bacteria by decreasing permeability so that it is difficult for antibacterials to enter cells, forming shortcuts to avoid the inhibited stages and increasing the production of enzymes that are inhibited by antibacterials.

The positive control of ciprofloxacin showed activity against all species of the test bacteria with a range of

diameter of the inhibition zone from 14.1 to 33.12 mm. According to Mpila et al. (2012) [19] Ciprofloxacin is effective against bacteria that are resistant to other antibiotics such as penicillins, aminoglycosides, cephalosporins and tetracyclines and is effective against gram-negative and gram-positive bacteria. While the average diameter of the inhibition zone in the negative control treatment for the five bacteria was 0 mm. According to Handayani et al. (2009) [20] negative control treatment using dimethylsulfoxide (DMSO) is a solvent that dissolves almost all polar and non-polar compounds, besides that DMSO does not inhibit bacterial growth in the antibacterial activity test using the agar diffusion method. According to Bansemir (2006) [21], there are three categories of the ability of the test material to inhibit test bacteria which is characterized by the formation of a clear zone around the paper disc, namely the size of the inhibition zone >15 mm is classified as strong, from 8 to 15 mm is classified as moderate and from 1-8 mm is classified as weak activity, when the results of the research were compared with the positive control of ciprofloxacin, the antibacterial extracts of methanol and hexane of *Sargassum sp.* and methanol extract of *Halymenia sp.* against the bacteria of *A.hydropilla, S.typhi and V. harveyi* had a strong inhibition zone category. Methanol extract of *Halimeda opuntia* has a strong zone of inhibition against *A. hydropilla, S.typhi, P. aeruginosa and V. harveyi*. The hexane extract ability of *Halimeda opuntia* and *Halymenia sp.* categorized as weak against *S. typhi*, moderate against *A. hydrophila* and strong against *V. harveyi*.

3.2 Identification of Active Compounds

Phytochemical tests were carried out with the aim of looking at the class of active compounds contained in each sample extract which was marked by a color change that occurred after the reagent was added. This phytochemical test is a simple way to detect the presence of secondary metabolites in sample extracts.

Table 2. Results of screening for the bioactive compounds of seaweed extracts of Sargassum sp., Halimeda opuntia dan Halymenia sp.

No.	Sample	Test Types				
		Alkaloid	**Flavonoid**	**Tanin**	**Triterpenoid**	**Saponin**
1.	*Sargassum*sp. (methanol)	+	+	+	+	+
2.	*Sargassum*sp. (hexane)	+	-	+	-	+
3.	*Halimeda opuntia* (methanol)	-	+	+	+	+
4.	*Halimeda opuntia* (hexane)	+	+	+	-	+
5.	*Halymenia* sp. (methanol)	+	+	+	+	+
6.	*Halymenia* sp. (hexane)	+	+	+	+	+

Information:: (+) there are active compounds; (-) no active compounds

The data of research results (Table 2) shows the methanol extract of *Sargassum* sp. and *Halymenia* sp. with methanol and hexane as solvents containing 5 active compounds namely, alkaloids, flavonoids, tannins, triterpenonids and saponins. While the seaweed hexane extract of *Sargassum* sp. contains only 3 active compounds namely, alkaloids, tannins and saponins. The seaweed extract of *Halimeda opuntia* contains 4 active compounds namely, flavonoids, tannins, triterpenonides and saponins in the methanol extract and alkaloids, flavonoids, tannins and saponins in the hexane extract.

This is presumably due to the types of the solvent of methanol which is a universal solvent which has a polar group (-OH) and a nonpolar group (-CH3) so that it can attract polar analytes ([22]; [23]; [24]) and nonpolar [25] and [26]. Some secondary metabolites that can be dissolved by methanol such as flavonoids, alkaloids, saponins, tannins [27] and [28]. Meanwhile, hexane is a non-polar solvent [29]. Compounds that can be dissolved by this solvent are alkaloids, tannins and saponins [30] and [31].

The term of alkaloid comes from the Arabic, alkali meaning soda and from the Greek, eidos meaning appearance. This compound was introduced by W. Meisner in the early 19th century to denote a natural substance that reacts like a base. The biological activity of alkaloid compounds is due to the presence of a nitrogen-containing base group [32]. Its ability as an antibacterial is done by disrupting the peptidoglycan constituent components in bacterial cells, so that the bacterial cell layer is not fully formed and causes cell death in the bacteria.

Flavonoids are one of the secondary metabolites which are phenolic and play a role in pharmacology. Flavonoids have

the ability to inhibit bacterial growth by several different mechanisms, including flavonoids causing damage to the permeability of bacterial cell walls [33], microsomes and lysosomes as a result of interactions between flavonoids and the DNA of bacteria [34], Different mechanisms were proposed by Di Carlo et al. (1999) and Estrela et al. (1995) in Sabir (2005) [35] which states that the hydroxyl group contained in the structure of flavonoid compounds causes changes in organic components and nutrient transport which will eventually lead to toxic effects on bacteria.

Saponin comes from the Latin word of Sapo which means foam producer [36]. The presence of saponins in the methanol extract is due to the presence of active glycoside bonds. Ganiswarna (1995) in Darsana et al. (2012) [37] stated that saponins work as antibacterial by inhibiting the synthesis of bacterial cell walls, interfering with the permeability of bacterial cells by binding to the outer membrane, resulting in damaged or destroyed cell walls (Arabsky et al., 2009), binding cholesterol in bacterial cells so that bacteria become lysed stability of bacterial cell membranes [38].

Tannins or commonly called tannic acid [39] are secondary compounds from abundant plants that are effective against bacteria. (The presence of tannins in methanol solvents is due to tannins are polyphenols which have an OH group [40] so that they are easily soluble in water, ethanol, acetone, or methanol [41]. Work mechanisms of tannins as an antimicrobial by shrinking the cell wall or cell membrane so that it interferes with the permeability of the bacterial cell wall itself.

Triterpenoid compounds from the extraction result are secondary metabolites that have the potential as antibacterial because they can lock the performance of enzymes that can bind DNA. In accordance with the opinion of Nurjanah et al. (2011) [42], triterpenoids can inhibit enzyme performance by binding to the active site of the enzyme which will bind to DNA and split it, so that the enzyme becomes locked and cannot bind DNA.

IV. CONCLUSION

Seaweed methanol extracts of *Sargassum* sp., *Halimeda opuntia* and *Halymenia* sp. had strong antibacterial activity against the test bacteria namely, *S. typhi*, *A. hydrophila* and *V. harveyi*, but was not active against the bacteria of *E. coli*. The methanol extract of *Halimeda opuntia* had wider antibacterial activity as indicated by the inhibition zone formed on four bacteria namely, *P. aeruginosa*, *S. typhi*, *A. hydrophila* and *V. harveyi.*

The extracts of methanol and hexane of *Halymenia* sp. and methanol of *Sargassum* sp. contains 5 active compounds namely, alkaloids, flavonoids, tannins, triterpenonids and saponins. Meanwhile, the hexane extract of *Sargassum sp.* only contains 3 active compounds namely, alkaloids, tannins and saponins. The methanol extract of *Halimeda opuntia* contains 4 active compounds namely, flavonoids, tannins, triterpenonids and saponins, while the hexane extract contains alkalaoids, flavonoids, tannins and saponins.

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