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RESEARCH ARTICLE

BACTERIA EXTRACT ACTIVITY ASSOCIATED WITH SPONGES *Haliclona* sp.2 AND *Axinellid* sp. AS ANTIBACTERIAL.

Endang Sulistyarini Gultom¹, Dwi Suryanto², Erman Munir² and Diky Setya Diningrat¹.

1. Biology Department, FMIPA, Medan State University. Jl. Willem Iskandar Pasar V, Medan 20221, Indonesia.
2. Biology Department, FMIPA, University of North Sumatera. Jl. Bioteknologi 1, Medan 20155, Indonesia.

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Abstract

Isolation of bacteria for antibacterial activity was conducted on sponge-associated marine bacteria, *Haliclona* sp.2 and *Axinellid* sp. from Ngge Island, Sibolga. Seventeen isolates were obtained from the two sponges. Based on antibacterial test, 11 isolates (64%) were potential as antibacterial, whereas 6 isolates (35%) were not indicated as antibacterial. Isolate H2, A1, and A2 with broad spectra of antibacterial activity were further separated for extraction of secondary metabolites. Based on screening results of isolates, ethyl acetate extract of isolate H2, A1, A2 and n-hexane extract of isolate A2 displayed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, whereas methanol extract A2 was only able to inhibit the growth of *Escherichia coli*. For bioactive compound test, EH2, EA1 and EA2 were positive for alkaloid, whereas HA2 and MA2 were positive for alkaloid and saponin. Based on purification of secondary metabolite using Thin Layer Chromatography (TLC), there were 22 fractions with solvents of different polarities. Screening of antibacterial activity for isolate extract fractions showed that MA2 of fraction 2 had a strong inhibitive effect against *E. coli* and *S. Aureus* about 10,15 mm and 9,95 mm consecutively. The antagonistic activity of EH2 of fraction 3 against *S. aureus* and *E. coli* were about 7,05 mm and 7,45 mm consecutively, whereas EA1 of fraction 3 was about 6,55 mm against *P. Aeruginosa*. The results of phytochemical secondary metabolite of isolate extract fractions showed that MA2 of fraction 2 and MA2 of fraction 3 were positive for saponin, while EH2 fraction 3 and EA1 fraction 3 were positive for alkaloid. Bacteria associated with sponges *Haliclona* sp.2 and *Axinellid* sp. had activity as antibacterial against bacteria *S. aureus*, *E. coli* and *P. aeruginosa*.

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Introduction:-

Pathogenic microbe has been considered as a crucial problem in human healthy and can cause various diseases. The use of chemical compounds that acts as antibacterial such as irrational antibiotic to treat various diseases caused by pathogen, could make a negative side effect even it could cause bacteria becomes resistance against specific

Corresponding Author:-Diky Setya Diningrat.

Address:-Biology Department, FMIPA, Medan State University. Jl. Willem Iskandar Pasar V, Medan 20221, Indonesia.

antibacterial. In order to overcome this issue, it is important to explore antibacterial originated from bioactive compounds of plants, animals and microorganisms.

Sponge is one of corral reef components that has a potential bioactive but not has been explored. Exploration of sponges as bioactive source has been reported in various researches. However, the use of secondary metabolites produced by bacteria associated with sponges as antibacterial source is more beneficial as compared to isolation from its host. The excessive exploration of sponges to produce new bioactive could cause overfishing that could damage marine ecology. In addition to the growth of sponges that is relatively slow will inhibit the biomass availability as source of secondary metabolites. Therefore, the use of bacteria associated with sponges will be efficient because the bacteria could be cultured in laboratory and could be multiplied in relatively fast (Abubakar et al., 2011).

Capability of sponges in producing bioactive compounds due to its symbiosis with bacteria. The relationship sponges with bacteria will produce secondary metabolite as a form of defense against natural enemies such as predators, pathogenic microorganisms, competitor and fouling organisms (Taylor et al., 2007). This relationship includes a generous supply of nutrients through metabolism translocation such as nitrification, nitrogen fixation, photosynthesis, and an enhancement of chemical defenses. Due to the roles, symbiotic bacteria with sponges is estimated has a great potential on the production of bioactive compounds isolated from sponges (Kennedy et al., 2008).

Some researchers of marine explore a new bioactive compounds from sponge-symbiotic bacteria. Peptide was isolated from both sponge *Hyatella* sp. and an associated *Vibrio* sp. that has antibacterial activity (Taylor et al., 2007). New metabolites from sponge-symbiotic bacteria contribute to has antibacterial and antiplasmodial activity. Extract of n-hexane and ethyl acetate from 4 (four) potential species of bacteria isolated from sponges have antibacterial activity (Dash et al., 2009; Inbaneson et al., 2012). One of antibacterial compounds was isolated from bacteria *Pseudovibrio* spp. associated with sponges *Axinella dissimilis* (Halloran et al., 2011).

Bacteria associated with sponges include representative of the Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (Taylor et al., 2007). Selvin et al., (2009) reported that potential microbe which produce active compounds are Cyanobacteria, Fungi and Actinomycetes. The active compound resulted by sponge *Dendrilla nigra* associated with Actinomycetes *Nocardioopsis dassonvillei* MAD08 was as antimicrobial compound against gram-negative bacteria (*Staphylococcus aureus* MTCC 2940, *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *Rhodococcus rhodochrous* MTCC 265) and gram-positive bacteria (*Vibrio cholerae* PC6, *Pseudomonas aeruginosa* MTCC 2453 and *Escherichia coli* MTCC 2939). Some bacteria isolates associated with sponges *Jaspis* sp. are antibacterial because of inhibiting the growth of *Staphylococcus aureus*, *Vibrio harveyi*, *Escherichia coli*, *Pseudomonas aeruginosa* and EPEC K-11 (Abubakar et al., 2011). Correlation between production of antibacterial compound by bacterial symbionts with sponges has been investigated by Narsinha and Anil (2000), that antibacterial compound produced by bacterial symbionts such as *proteobacterium* MBIC 3368, *Idiomarina* sp and *Pseudomonas* sp. were very affected by protein recombinant of *Suberites domuncula* as host. This research confirmed that there is a collaboration in biosynthesis of secondary metabolite between symbiont microbe and sponges.

Based on various results research, an investigation for sponge-associated bacteria as antibacterial which easy to be cultured, was needed to be conducted. The isolation results will be tested for antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*.

Materials and Methods:-

2.1 Sample collection

The marine sponges were collected off from Ngge Island, Sibolga by SCUBA diving at 2-10 m. Temperature, salinity, and pH were determined at the moment of sample collection. Sponges samples were collected as needed then put into plastic bag filled marine water and oxygen then put into cool box. Sponges were identified at Laboratory of Marine Biology of Fishery Faculty of Riau University.

2.2 Extraction of Secondary Metabolite from Potential Bacterial Isolate

Suspension was made with turbidity justified with Standard of McFarland 10^8 CFU/ml, then spreaded with cotton swab on the surface of NA media. Some suspensions were made on petri disk and incubated for 5-6 days at 25°C. Bacterial cultures which obtained were cut into small pieces then put into erlemeyer 500 ml then it was added \pm 150 ml solvent n-hexane, macerated for 3 x 24 hours. It was filtered with Whatman 42, thus resulted macerat and residual. Residual was reextracted with ethyl acetate and filtered. With the same way, residual was extracted with methanol. Each macerat was centrifuged with 3000 rpm for 15 minutes, thus result supernatant. Then the

supernatant was separated with each their solvent by using rotary evaporator at 45°C. It was obtained extract n-hexana, ethyl acetate and methanol secondary of potential bacterial isolate(Nofiani *et al.*, 2009). The series levels of extraction was shown in appendix 4a.

2.3 Screening Bacterial Extract for Antibacterial Activity

Bacterial suspension of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* were as tested bacteria. For extract activity test, suspension of test bacterial culture was prepared by inoculation each them into nutrient broth media and incubated for 24 hours at 25°C. Activity test conducted by inoculated 200µl suspension of bacterial test that was rejuveniled into agar media and spread out with hockey stick thus test bacterial suspension mix on media surface. Then it was kept for a few moment to let the bacterial test fuse with the media. Then it was put into oxoid disk that was dropped with 10 µl of extract –n- hexana secondary metabolite potential bacteria into agar media that was inoculated with *Staphylococcus Aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Then incubated for 24 hours at 25oC and investigated inhibition zone formed with vernier caliper. The same treatment was applied against extract ethyl acetate and methanol of secondary metabolites of potential bacteria.

2.4 Identification of Chemical Compound in Potential Isolate Extract n-Hexana, Ethyl acetate and Methanol.

Preliminary test of secondary metabolite was carried out on each potential isolate extract. 5 ml aquadest and chloroform were added into the extract then mixed strongly and let it for a few minute until two layers formed. Water layer was used for metabolite secondary assay of flavonoid, fenolik, and saponin whereas Chloroform layer was used for terpenoid and steroid (Harbone, 2006).

2.5 Identification and Isolation Secondary Metabolite Potential Isolate Extract by Preparative Thin Layer Chromatography.

Chamber was saturated with raising solvent by covered chamber with filter paper, then raising solvent was put into chamber until all filter paper throughly wet by the solvent. Extract solvent contained secondary metabolite of potential isolate spotted on preparative TLC with 20 x 20 cm until the plate saturated by extract solvent (1.5 cm from under line), plate was let until dry for ±15 minutes. Previous plate was marked upper and down limit. Then plate put into chamber that was saturated and eluted until the solvent reached upper limit line. Node formed was checked under UV light and pattern formed of node was drawn with pencil. Then the pattern was scraped with spatula and each node was separated into vial furthermore was washed and separated from its silica. Compounds that were separated from the silica, the solvents was volatiled and antimicrobe test was conducted.

2.6 Screening of Antibacteria Activity Fraction of n-hexana, Etil Acetate and Methanol of Potential Bacteria Isolate.

Bacterial suspensions of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* were as bacterial test. For screening, the suspensions were inoculated nutrient broth media and was incubated for 24 hours at 25°C. Identification of antibacterial activity was used disc diffusion assay (Bauer *et al.*, 1996). 200 µl suspensions that already rejuveniled were inoculated into nutrient agar media and spreaded out with hockey stick, thus bacterial suspension test was mixed throughly on surface media.

Then it was waited for a few minute until the bacterial suspension fused with media. Then it placed on the empty oxoid disk that already spotted with 10µl fraction n- hexana secondary metabolite of potential bacteria into media NA that was inoculated with bacteria *S. aureus*, *P. aeruginosa* and *E. coli*. The culture was incubated for 24 hours at 25^o C and inhibition zone formed was observed with vernier caliper. The same treatment was applied on fraction ethyl acetate and methanol of secondary metabolite of potential bacteria. Furthermore, result of screening antibacterial activity was tested for phytochemical identification of potential isolate extract fraction.

Results and Discussions:-

3.1 Sponges Characteristic

Sponges were taken from Ngge Island, Sibolga from 2- 10 m with purposive sampling method by scuba diving. Determination of pH with pH – meter shown that pH of marine water of sponge sampling was 7.5; salinity 30‰ and temperature 29°C. Collecting sponges was located based on GPS that were N. 01. 39. 001. E. 098. 47. 661. N. 01. 34. 379. E. 098. 45. 251. N. 01. 34. 361. E. 098. 45. 252. N. 01. 35. 419. E. 098. 41. 831. Measurement of sampling location condition was to create suitable environment for symbion bacterial test thus symbiont bacteria was able survive to live. Sponges sample were taken as needed then put into plastic bag that filled with marine water and oxygen and then put into cool box. The sponges from Ngge Sibolga were *Haliclona* sp.2 and *Axinellid* sp.

(Figure4.1).In addition, *Haliclona* sp.2 has oscula on each branch and blue colour, whereas *Axinellid* sp.has a shape like fan,yellowness, and oscula on whole surface (Gosliner *et al*, 1996)



Figure 4.1. Sponges (a) *Haliclona* sp.2 and (b) *Axinellid* sp.

3.2 Isolation and Purification of Symbiont Bacteria

Based on isolation results, there were 17 isolates from mesophyl of the two sponges that consisted of 8 isolates of *Haliclona* sp.2 and 9 isolates of *Axinellid* sp. (Appendix2b). Bacteria in mesophyl was bacteria that has a similarity secondary metabolite with the sponges. Thiel *et al.* (2007) had discovered bacteria in large amount on sponges mesophyl *Chondrilla nucula*. The bacteria was isolated based on morphology, size and shape of colony. Seventeen isolates were isolated based on color and colony shape (Table 1). It showed possibility many differences of activity for each isolate that used for further test. Isolation and purification of symbiont bacteria could be shown as follows:

Table 1. Characterization morphology of bacterial isolate that was isolated based on colour and shape on sponges *Haliclona* sp.2 and *Axinellid* sp.

Species	Isolate Code	Colony form	Color	Elevation	The edge of the colony
<i>Haliclona</i> sp.2	H1	Round	Yellow	Flat	Entire
	H2	Round	Beige	Flat	Entire
	H3	Bobbin	Beige	Raised panel	Round
	H4	Round	Yellow	flat	Wave
	H5	Round	Beige	Flat	Entire
	H6	Bobbin	Yellowish beige	Arise	Entire
	H7	Round	Beige	Flat	Irregular
	H8	Irregular	Beige	Flat	Curly
<i>Axinellid</i> sp.	A1	Round	Beige	Flat	Wave
	A2	Bobbin	Yellow	Arised panel	Entire
	A3	Irregular	Beige	Panel	Curly
	A4	Round	Beige	Flat	Entire
	A5	Round	slightly yellow	Flat	Entire
	A6	Round	Beige	curve	Entire
	A7	Root like	Beige	Flat	Curly
	A8	Irregular	Beige	Flat	Jagged
	A9	Irregular	Beige	Flat	Blade

Various microorganisms associated with sponges. For instance, unicellular algae, Cyanobacteria, dinoflagellates and Archaea. Among these microorganisms, bacteria are the most dominant that constituted up to 40% of the biomass or 60% of the tissue volume of certain sponge species (Lee *et al*, 2006). According to Vasanthabharathi *et al.*, (2012), bacterial density that associated with sponges *H. cribriformis*, *S. carnosa*, *C. diffusa* and *S. officinalis* Var. *ceylonensis* was in the range was 13×10^3 - 1.6×10^7 CFU/g, 6.77×10^3 - 1.5×10^7 CFU/g, 7.68×10^3 - 1.1×10^7 CFU/g and 2.69×10^3 - 1.4×10^7 CFU/g. In Taylor *et al.* (2007) described that associations between sponges and microorganisms can be maintained by two ways that is filter feeding which microbes can be recruited from the surrounding water to the sponges (horizontal transmission) or by reproductive stages (vertical transmission). In vertical transmission, oocyte from the adult mesohyl of sponge can phagocytosis to the microbial cells so bacterial symbiont could be found on embryo/ sponges larva.

Differentiation of diversity and number of bacterial isolates of each kind of sponges might be due to isolation technique and growth media that used for isolation bacteria associated with sponges. Nutrient contents of culture media would affect bacterial growth. According to Haloran *et al.* (2011), there were 73 from the marine sponges

Axinella dissimilis, *Polymastia boletiformis* and *Haliclona simulans* and identified as being *Pseudovibrio spp.* by 16S rRNA genesequencing and phylogenetic analysis. Radjasa *et al.* (2008) reported, there were 32 bacterial isolates from sponges *Haliclona sp.* in North Java Sea. Murniasih and Rasyid (2010) also reported that there were 75 bacterial isolates from sponges *Theonella sp.*, *Aaptos sp.*, *Melophlus sarassinorum.*, *Callyspongia sp.*, *Ircinia sp.*, *Stylissa flabeliformes*, *Lisoclinum sp.*, and *Clarithria sp.* by direct plating. Abdullah (2006) also had obtained 7 bacterial isolates associated with sponges *Axinella sp.* by using marine broth agar media.

Symbiont bacteria produce a wide array of secondary metabolites antibacterial (Lee *et al.*, 2001). Although the mechanism of association between sponges and bacteria is not known well, the association could produce secondary metabolite. Secondary metabolite production by sponges has similarity with microorganisms associated with sponges itself, include bacteria (Radjasa *et al.*, 2007). In addition, bacteria associated with organisms have probability an interaction with the host organisms (Lee *et al.*, 2001). The biochemical interaction make a possibility that symbiont bacteria produces the same bioactive compound with its host. In Taylor *et al.* (2007), was explained that bacteria symbiosis with sponges are able to photosynthesis, oxidize methane, nitrification, nitrogen fixation, sulphat reduction and dehalogenase.

3.3 Extraction of Secondary Metabolite from Potential Bacteria

Extraction of secondary metabolite was carried out by maceration (Appendix 4b). In Nofiani *et al.* (2009) was described that maceration can produce intracellular antimicrobe compound thus bacterial cell need to be broken to obtain antibacterial compounds. Different polarity of solvents in maceration were n-hexane (nonpolar), ethyl acetate (semipolar) and methanol (polar). According to Dash *et al.* (2009) polarity differences on solvent used in order to isolate secondary metabolite and identification of following stage.

Extraction of secondary metabolites with different polarity of solvent was conducted serially. The isolates for this stage were determined based on the ability to inhibit some bacteria tests, that were H2, A1 and A2. Then, results of secondary metabolite extraction were extract of n-hexane, ethyl acetate and methanol of potential bacteria isolates. Taylor *et al.* (2007) described that, secondary metabolites produced by symbiont bacteria is potential as precursor of biosynthesis of sponges metabolite.

3.4 Screening Activity of Extract Antibacteria of Isolate Potential Bacteria

Results of screening for antibacteria activity of potential bacteria isolate extract were each isolates of extract ethyl acetate (EH2, EA1 and EA2) and extract n-hexane A2 showed antagonistic capability against bacteria test *S. aureus* and *E. coli*.

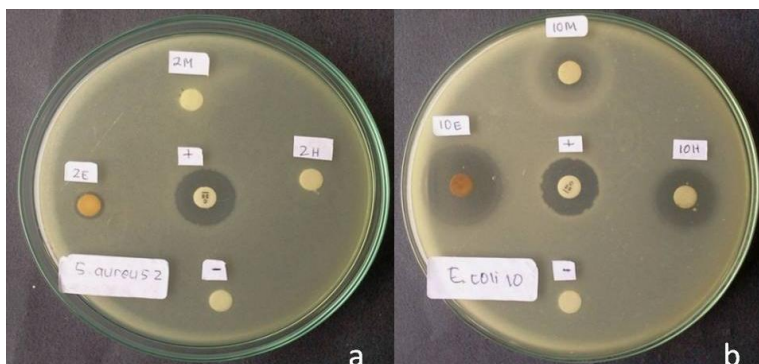


Figure 3. Sampling of antibacterial activity (a) extract ethyl acetate of isolate H2 (EH2) against *S. Aureus* and (b) Extract n-hexane, ethyl acetate and methanol of isolate A2 (HA2, EA2 and MA2) against *E. coli*.

Based on screening results of antibacteria activity, H2 and A1 were able to inhibit growth of *P. Aeruginosa*, while screening results of secondary metabolite extract showed that both isolates did not perform antibacterial activity against *P. aeruginosa* (Table 4.3). Extract hexane and ethyl acetate of isolate A2 (HA2) resulted inhibition zone against *S. aureus* and *E. coli* (Table 4.3) but not for *P. Aeruginosa*. For antagonist test of isolate showed that isolate A2 was capable of inhibiting growth of both bacteria *S. aureus* and *E. coli* (Table 4.3). According to Tinambunan *et al.* (2012) extract of bacteria associated with sponges with concentration 10% showed largest zone inhibition of antibacterial activity against *S. aureus* and *E. coli*. Extract ethyl acetate ethyl acetate of isolate A2 (EA2) had broad spectrum activity because it was capable of inhibiting gram positive and gram negative bacteria and according to David and Stout (1971) it was included as active compound with strong inhibition capacity (diameter of inhibition zone against *S. aureus* and *E. coli* were 20,05 mm and 23,00 mm consecutively).

Extracts of three isolates (H2, A1 and A2) showed negative results against bacteria test *P. aeruginosa*. While extract MA2 only capable of inhibiting growth of bacteria test *E. coli* (Table 4.3). This might due to MA2 was suggested contain secondary metabolite that was facilitated to disturb permeability cell membrane of gram negative bacteria thus cell bacteria lysis. In Nofiani et al. (2009) explained that there are some suggestions that caused bacterial isolates did not show antibacterial activity namely (1) isolat has gene code formation secondary metabolite compound but it is not expressed at normal condition and will be expressed when it is induced previously; (2) symbiont bacteria produces antimicrobe compound intracellularly but it does not have antibacteria activity against bacteria test. Radjasa *et al.* (2007) stated that the production of secondary metabolite from the sponges has similar with microorganisms associated with sponges. It indicate that the production of secondary metabolite from simbiotic bacteria is affected by host sponge. Thus when simbiotic bacteria is isolated from host sponge, it will affect secondary metabolite as antibacteri.

3.5 Test Chemical Compound of Extract n-Hexana, Ethyl Acetate and Methanol of Potential Isolate

Identification of secondary metabolite content is an important initial step in searching new bioactive compound from natural material and as precursor for synthesis new medicine or as a medicine prototype with certain activity (Harborne, 2006). Capability of antagonistic activity against bacteria test is due to presence of secondary metabolite produced by isolate of symbiont bacteria. Test component of extract chemical compounds, n-hexana, ethylacetate and methanol of isolate potential bacteria was conducted as initial test to investigate the characteristic of secondary metabolite. Alkaloid was positive for each extract ethylacetate isolate (H2, A1 and A2) by used dragendorff reagent and resulted red sediment (Appendix 5b). Furthermore, extract n-hexana and methanol of isolate A2 were positive contained alkaloid and sapoin (Table 4.4). Alkaloid and sapoin indicated antagonist ability against bacteria test (Tabel 4.3) whereas identification other secondary metabolite such as steroid, terpenoid, fenolic and flavonoid showed negative result.

3.6 Identification of Secondary Metabolites in Potential Bacteria Isolates by using Thin Layer Chromatography (TLC) Preparative

Identification secondary metabolite compounds of symbiont bacteria was conducted with TLC (Penesyana et al., 2011). Based on preparative TLC results were found 22 fractions observed under UV from five extracts (EH2, EA1, EA2, HA2 and MA2) with different polarity. Movement phase was used to eluted the samples and varied based on polarity gradient (Yuhernita, 2011). Movement phase drained through static phase and carried components of extract potential bacteria isolate. Different component will move with different rate. Based on preparative chromatography results, separation of compounds in extracts of bacteria isolates into pure substances were obtained 6 fractions in HA2 (nonpolar) while EH2, EA1, EA2 and MA2 (semipolar and polar) obtained 4 fractions for each. (Figure 4).

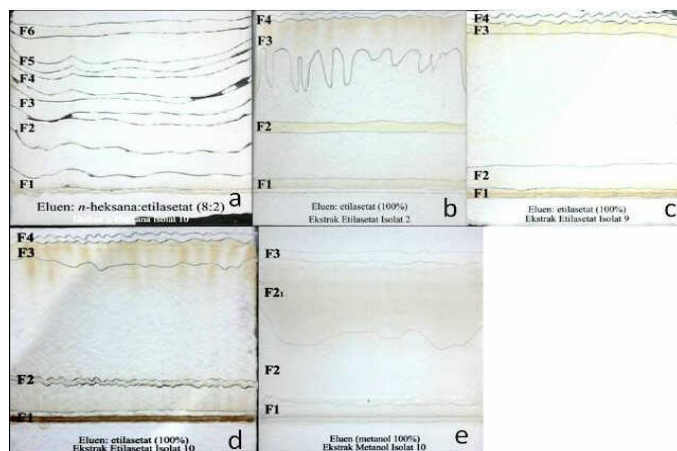


Figure 4. Results of *Thin Layer Chromatography (TLC) preparative* for secondary metabolite of extract potential bacteria isolate. (a) HA2; (b) EH2; (c) EA1; (d) EA2; (e) MA2.

3.7 Screening Antibacteria Activity on Extract Fraction of Potential Bacterial Isolate

Secondary metabolite fraction of extract potential bacterial isolate showed antagonist ability against bacteria test. Based on screening results of antibacterial activity, the largest antagonist ability of secondary metabolite extract

fraction was MA2F2 against *E. coli* and *S. aureus* bacteria with diameter 10,15 mm and 9,95 mm consecutively (Fig.5). MA2 was obtained from sponges *Axinellid sp.* that contained saponin.

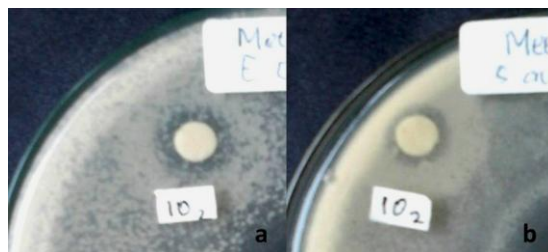


Figure.5. Examples of antibacteria activity of extract methanol of Isolates A2 fraction 2 (MA2F2) against bacteria (a) *E.coli* and (b) *S. Aureus*

Antibacteria activity was also found on EH2F3 against bacteria *S. aureus* and *E. coli*. Isolate H2 was obtained from sponges *Haliclona sp.2* with secondary metabolite alkaloid. Antagonist ability was indicated with the formation clear zone against bacteria *S. aureus* and *E. coli* with diameter 7,05 mm and 7,45 mm consecutively. Research about antibacteria activity of sponges genus *Haliclona* was also conducted previously. Radjasa et al. (2007) stated that symbiont bacteria of sponges *Haliclona sp.* showed antibacteria activity against some bacteria test, one of them was *S. aureus* with diameter > 9 mm.

Screening results of antibacteria activity of extract fraction of Isolate EA1F3 showed clear zone with diameter 6.55mm against *P.aeruginosa*. This result was different from antibacteria activity screening of isolate extract, where extract isolate EA1 did not have antibacteria activity against *P. Aeruginosa*. Extract fraction screening result of isolate MA2 was also showed differences from extract screening of bacterial isolate. For initial screening, isolate MA2 was only showed antagonistic ability against bacteria *E. coli* whereas extract fraction screening of bacterial isolate MA2F2 and MA2F3 showed antagonistic ability against *E. coli* and *S. Aureus*.

A research conducted by Nofiani et al. (2009) stated that extract methanol of bacteria associated with sponges showed antibacteria activity but based on minimum inhibition concentration value, antimicrobe activity still categorized as weak. Both extract fractions of n-hexana and ethyl acetate of isolate A2 did not show presence of antibacterial activity. This might due to the presence of other secondary metabolites that associated in each other and this indication has not detected thus capable of producing antibacterial activity against bacteria test. Other factors was the amount of secondary metabolite fraction obtained was small thus it was not capable of inhibiting the bacteria test growth.

3.8 Chemical Compound Assay of Extract Fraction n-Hexana, Ethyl Acetate and Methanol of Potential Bacterial Isolate

Based on screening results of antibacterial activity extract fraction of EH2F3, EA1F3, MA2F2, and MA2F3 have antagonist ability against bacteria test. Furthermore, test of chemical compound for secondary metabolite was conducted. It showed that MA2F2 and MA2F3 were positive contained saponin while EH2F3 and EA1F3 was positive contained alkaloid. Saponin for MA2F2 and MA2F3 and alkaloid for EH2F3 and EA1F3, have the same result with previous test of secondary metabolite compound. Secondary metabolite such as alkaloid and saponin are potential as antibacterial. According to Zheng (2005), secondary metabolite alkaloid of bacteria associated with sponges *Himeniacidon parleve* also showed antibacterial activity against *S. aureus*. Antibacteria activity produced by symbiont bacteria of sponges *Haliclona sp.2* and *Axinellid sp* are enable competition occur in the sea ecosystem. According to Dash et al (2009), symbiont bacteria which produces antibacterial compound is enable to form colony on the sponges surface. Saponin, tannin and flavonoid are secondary metabolites that have antibacterial activity. Saponin is bioactive compound to increase membran permeability thus cell hemolysis occur, when saponin interacts with bacterial cell, the bacterial cell will be broken or lysis. This could be made as opportunity to develop secondary metabolite for further research (Ganiswarna, 1995).

Conclusions:-

Based on research results, the conclusion of this research were as this following:

1. Extracts of EH2, EA1, EA2, HA2 and MA2 have antibacterial activity on bacteria *S. Aureus*, *E. Coli* and *P. Aeruginosa*.
2. Extract of EH2 was very potential against *S. aureus* and *E. Coli* compared with extracts EH2, EA1, HA2 and MA2.

3. It was found 4 (four) extract fractions of secondary metabolites (EH2F3, EA1F3, MA2F2 and MA2F3) which have an activity against *S. aureus*, *E. coli* and *P. Aeruginosa*.
4. Based on phytochemical screening of secondary metabolite fractions (EH2F3, EA1F3, MA2F2 and MA2F3) that has antibacterial activity were alkaloid and saponin.

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