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ISOLATION, IDENTIFICATION AND BIOACTIVITY TEST OF NON POLAR COMPOUNDS ON n-HEXANE EXTRACT OF *Haliclona (Reniera) fascigera* FROM SAMALONA ISLAND-SPERMONDE ARCHIPELAGO

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ABSTRACT

Isolation, identification and bioactivity test of nonpolar compunds has been conducted on n-hexane extract sponge *Haliclona (Reniera) fascigera* of the island Samalona, Makassar, South Sulawesi. Isolation techniques consist of maceration, partition, fractionation using 2 steps using vacuum column chromatography and flash column chromatography and also purification step. Identification of isolate was analyzed with TLC, FTIR, GC-MS, and ^{1}H NMR. The research result was obtained isolate as much as 4 mg and clear solid form. FTIR analysis indicate functional group OH, CH_2, CH_3, C=O, =CH and C-O. Identification of spot TLC under UV light and spotting solvent show that the spot was active on UV long and short waves and dark brown with cerium sulphate. The results show that isolate A contain aliphatic and alcohol compounds. Bioactivity test results indicate that these isolate A be able to inhibit the growth of bacteria *Staphylococus aureus* with inhibition zone diameter were 11.60 mm (100 ppm); 10.40 mm (50 ppm); 9.50 mm (25 ppm); 6.50 mm (10 ppm); 6,60 mm (negative control); 17.40 mm (positive control) and against bacteria *Escherichia coli* with inhibition zone diameter were 6.70 mm (100 ppm); 6,10 mm (50 ppm); 5,90 mm (25 ppm); 5.85 mm (10 ppm); 5,20 mm (negative control); 18.60 mm (positive control).

Keywords: Isolation, Sponge *Haliclona (Reniera) fascigera*, Antibacterial

1. INTRODUCTION

As one of the world's largest archipelago nation, Indonesia has the resources are very abundant biodiversity that includes plants, animals, and microorganisms, either living on land and at sea. Given the waters is the biggest part of the territory of Indonesia, it is certain that the natural resources of the sea is very potential to be developed and utilized in order to improve the welfare and prosperity of the people.

Sponges, as part of the diversity of marine natural resources, also produces a wide variety of molecules results of the process of metabolism. Metabolism sponges produce both primary and secondary metabolites. Primary metabolites are molecules produced by an organism to the process of growth and development needs. Furthermore, secondary metabolites are compounds produced by organisms for self-defense from extreme environmental conditions as well as against other organisms.[5] The formation of secondary metabolites strongly influenced by the environment, resulting in a different environment, the organism will produce different secondary metabolites. Initially, secondary metabolites are only as a waste product of metabolism process. Recently, the role of secondary metabolites was very important and strategic. Benefits and roles, among others as a chemical defense to protect marine life from extreme environments, to survive from predators, as a mediator in the competition, antifouling, as a facilitator of reproduction, and protects from ultraviolet radiation. Another benefit to

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humans, secondary metabolites are also potential as a source of bioactive compounds for pharmaceuticals, health foods, and cosmetics.[6]

Some metabolites bioactivity have been isolated and identified from the sponge. Discodermolide compound is a secondary metabolite of *Discodermia dissoluta* sponge known active as anticancer. Halichondrin B compound of sponge Halichondria okadai proven active against leukemia.[3] Furthermore, Jaspamida compound from sponge *Stylissa flabeliformis* was potential as anticancer.[1] Based on the research of Fajarningsih et al.,[2] that secondary metabolites Sponges *Crella papilata* was potential as antitumor compounds.

Sponges *Haliclona (Reniera) fascigera* is one sponge species belonging to the class Demospongiae. This species is also part of the genus haliclona and the family of Chalinidae. As the sponge in general, *Haliclona (Reniera) fascigera* has a porous body, pink, tubular and hard surface like a rock. The sponge species can also absorb oxygen from the water through the process of diffusion. Research Handayani et al.,[4] showed that the ethyl acetate extract sponge haliclona fascigera have potential as antimicrobial activity. On n-hexane fraction of *Haliclona (Reniera) fascigera* have not more conducted research to explore typical organic compound and its bioactivity on nonpolar fraction.

The purpose of this study, namely to isolate and identify organic compounds nonpolar of n-hexane fraction at *Haliclona (Reniera) fascigera* sponge and to test the antibacterial activity by using a diffusion method.

2. METHOD

Materials Research

Research materials are sponge *Haliclona (Reniera) fascigera*, methanol grade, chloroform grade, ethyl acetate grade, n-hexane grade, TLC, adsorbent column chromatography such as silica G 60 (70-230 Mesh), G 60 F_{254} 0,2-0,5 mm (Merck), $Ce(SO_4)_2$, Media Muller Hinton Agar (MHA), Escherichia coli, *Staphylococcus aureus*, and the Liebermann-Buchard reagent.

Research Tools

The tools used in this study include glass tools, Buchner funnel, ovens, chamber for KLT containers, for penotol capillary tube, vacuum column chromatography (VCC) and flash column chromatography (FCC) to fractionate the extract of the maceration process, some instrumentation equipment such as, UV lamp 254-365 nm, analytical balance Ohaus AP-110, evaporator RUDOLF Rotavapor R-200, melting point determination apparatus Electrothermal Melting point apparatus, FTIR spectroscopy Prestige-21 Shimadzu, Bruker 300 MHz NMR, and GC-MS QP2010 Ultra Shimadzu.

Extraction

Sponges *Haliclona (Reniera) fascigera* which has been cut macerated with methanol. Results maceration then collected and evaporated using rotary evaporator. Methanol crude extract was further partitioned with n-hexane and analyzed by TLC using appropriate eluent. The right eluent composition on TLC was applicated to vacuum column chromatographic (VCC) and flash column chromatography (FCC).

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Isolation

N-hexane fraction was fractionated using vacuum column chromatographic techniques (VCC) and flash column chromatography (FCC) with a suitable eluent. Furthermore, result fractionation analyzed by TLC using appropriate eluent and the same spot pattern of fractions are combined and evaporated. The good separation on TLC is marked by formation stains separated. The next step was flash column chroatography to isolate until found single spot isolate.

Identification

Isolates was tested purity by TLC analysis using the two kinds of systems eluent and measuring the melting point. Determination of organic compound group of isolates carried through the test group as phytochemicals test using Liebermann-Buchard reagent. Identification compound of isolat were supported by FTIR analysis, GC-MS, and ^{1}H-NMR.

Antibacterial Activity Test

Antibacterial activity test performed using agar diffusion method, using media Mueller Hinton Agar (MHA). MHA Media bacteria spread on a petri dish test. Furthermore, sterile filter paper with a diameter of 5 mm is dipped into the sample and then shaken until no liquid sample dripping. Control treatment (positive control; Amoxiline and negative controls, solvents) treated as a sample. Filter paper containing the sample, the positive control, and negative media placed on a surface. Then incubated in an incubator at 37 ^{0}C for 1x24 hours. Measurements were made on the size of sona clear that formed around the filter paper discs using a sliding bar.

3. RESULTS AND DISCUSSION

Isolation of organic compound nonpolar of n-hexane extract on haliclona (Reniera) fascigera sponge

Sponges *Haliclona (Reniera) fascigera* which has been cut as much as 424.5 g macerated using methanol for 5 times to 24 hours. Results maceration is filtered and the filtrate evaporated to maserat produce methanol condensed as much as 14.07 g. Then maserat condensed methanol partitioned with n-hexane using a separating funnel. Before partitioned condensed maserat is suspended prior to the methanol-water mixture with a ratio of 8:2 in order to obtain good separation and apparent occurred two phases at separating funnel. The partitioning process performed 5 times and each process partition left for about 1 hour so that the two phases are well separated. When the process is underway, the solvent n-hexane is at the top because it has a density smaller than water and methanol. Results the partition process with n-hexane solvent evaporated and n-hexane extract obtained viscous brownish yellow as much as 857.5 mg.

Furthermore, n-hexane extract was fractionated through two phases: vacuum column chromatography and flash column chromatography. Phase separation using VCC done to obtain a group of simpler compounds. The grouping is designed and based on a comparison between the eluent n-hexane and ethyl acetate. Comparison eluent used on VCC this stage, is 100: 0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 0: 100 and 100 mL of methanol as washers that can pull the rest of the components of the sample in the vacuum column chromatography. At this stage it obtained 11 fractions, fractions that have stains that are similar or virtually

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identical on TLC plate combined. So that when analyzed by TLC isolate obtained one or a group of fractions weighing 511 mg which is the combined result of fractions 1-4.

Isolates with yellow amorphous form from VCC as much as 511 mg, further purified using flash column chromatography techniques (FCC) with n-hexane and ethyl acetate as the eluent. On the implementation of the summit technique begins with the type of solvent is in the form of n-hexane alone, then shifted polarity level by mixing with the solvent ethyl acetate. Mixing is done by comparison varied gradually, until terminated by simply using ethyl acetate alone. Comparison eluent system used in this technique, which is 300: 0, 190: 10 180: 20 170: 30 160: 40, 140: 60 and 0: 100. Fractions result of press column chromatographic techniques can be seen in Table1 1

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Table 1. Results Combined Fraction of Flash Column Chromatography.

Fraction code	Factions combined	Spot profile on TLC		
		UV254 nm	UV365 nm	Cerium Sulfate
1	HAL-FH-G 1-4	-	-	√
2	HAL-FH-G 5-13	-	-	√
3	HAL-FH-G 14-21	√	-	√
4	HAL-FH-G 22-27	√	-	√
5	HAL-FH-G 28-60	-	-	-
6	HAL-FH-G 61-64	√	√	√
7	HAL-FH-G 65-67	√	√	√
8	HAL-FH-G 68-73	√	-	√
9	HAL-FH-G 75-85	√	√	√
10	HAL-FH-G 86-116	√	-	√
11	HAL-FH-G117-137	√	-	√
12	HAL-FH-G138-144	√	-	√
13	HAL-FH-G145-169	-	-	√
14	HAL-FH-G170-205	√	-	√
15	HAL-FH-G206-210	√	-	√

Information : √ = Spot light
- = Spot no light

Furthermore, the combined fractions 9th shaped somewhat oily precipitate a yellowish as much as 43.7 mg treated by preparative TLC using eluent 90% n-hexane and 10% ethyl acetate. Based on the analysis of TLC chromatogram obtained some stains, but only one chromatogram that looks at two wavelengths is labeled F. In purification processes isolates F was separated three times using TLC preparative (silica gel 60, 20x20 aluminum plate). Based on analysis of TLC using the eluent ethyl acetate and

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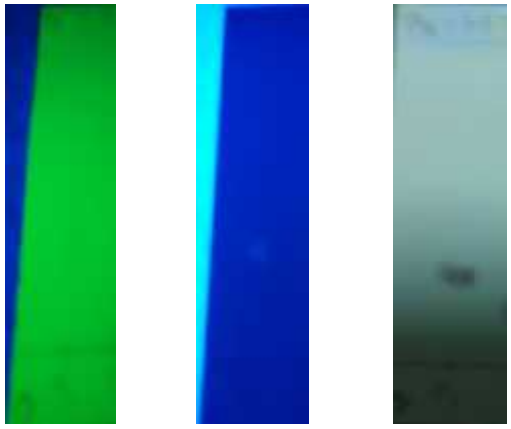
n-hexane in the ratio of 9: 1 isolat F results showed preparative third single spot visible in UV light-shortwave and longwave UV-with Rf value is 0.3 cm. Isolates preparative results are expressed as compound A, translucent solid form attached to the wall by 4 mg vial. TLC analysis results on compound A can be seen in Figure 1.



UV-254 nm **UV-365 nm** **$Ce(SO_4)_2$**

Figure 1. The results of TLC on the compound A

Analysis Data of Fourier Transform Infrared (FTIR) Spectroscopy

The IR spectrum is specific for each compound in which each compound will show the composition and pattern of absorption bands that differ primarily in the pattern of absorption fingerprint (finger print). The limited ability of FTIR which can only identify the type of functional groups contained in a compound, the FTIR alone can not be used to determine the molecular structure without the support of other instruments. However, FTIR is helpful to identify the types of functional groups of a compound and a compound resemblance to the standard compound. Some of the functional groups that stand out and are often identified is CH2, CH3, = CH, ≡CH, O-H, N-H, C = O, C-O, C = C, C≡C, C≡N, and NO2, C-O-C and C-C-O. In Figure 2 is shown absorption pattern of functional groups on the FTIR of compound A.

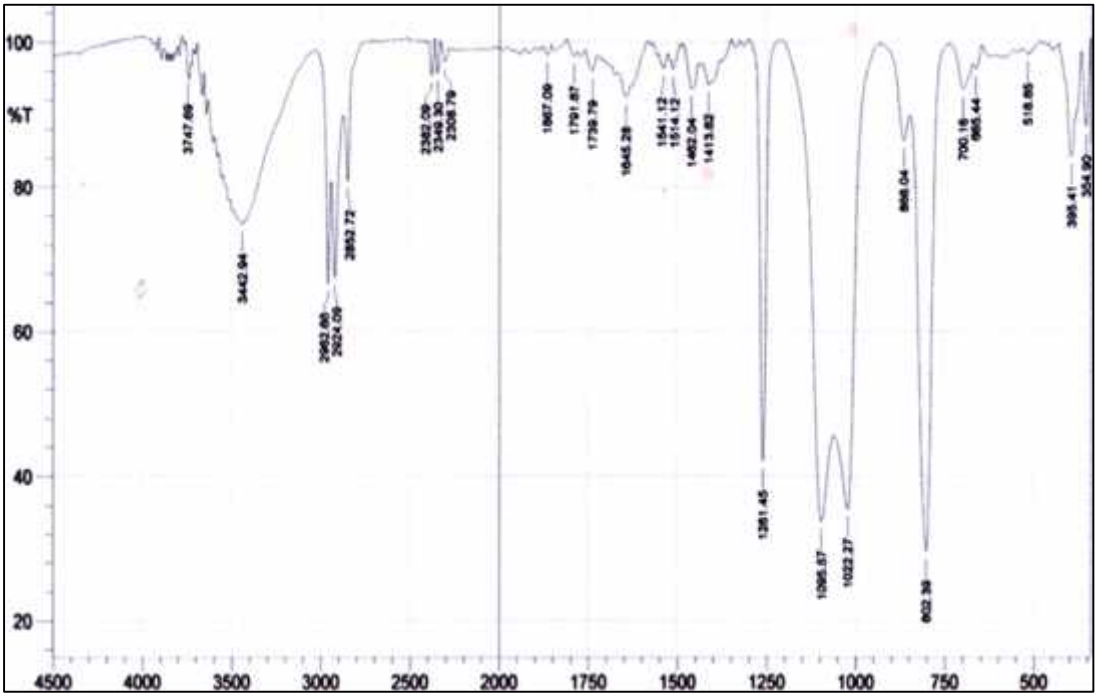


Figure 2. The FTIR spectrum of compound A

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Summary FTIR data of compound A:

No	Absorption band (cm^{-1})	Functional Groups
1.	3442,94	Stretching O-H
2.	2852,72 and 2962,66; 2924,09	Stretching CH_3 and CH_2
3.	1739.79	Stretching C=O(weak)
4.	1645.26	Stretching C=C
6.	1462.04 and 413.62	Bending CH_2 and CH_3
7.	1095.57, 1022.27 and 1201.45	Stretching C-O
8.	802.39	Bend =C-H
9.	700.16	Bending rocking CH_2

Analysis Data of Gas Chromatography Mass Spectrometri Isolate A

Gas Chromatography-Mass (GC-MS) Spectrometry is one method analysis of organic compounds using two methods of analysis of compounds which gas chromatography (GC) to analyze the amount of compound qualitatively and quantitatively as well mass spectrometry (MS) to analyze molecular mass of each peaks on GC chromatogram. The result of GC-MS analysis for isolate A were showed in Figure 3.

Figure 3. Kromatogram GC-MS

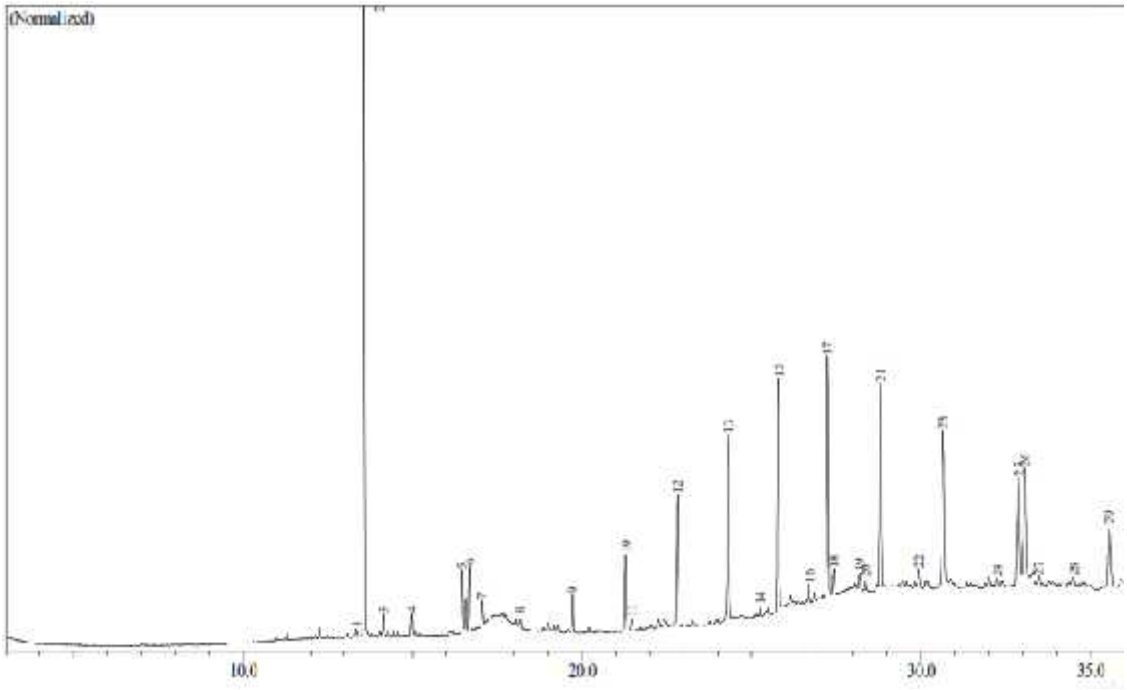

Table 2. Components of Compound A Based analysis GC-MS

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No	Retention Time (t_R)	Area (%)	Molecular Weight	Molecular Formula	Compound Name	SI
1	13,327	0,36	212	$C_{14}H_{28}O$	E-7-Tetradecen-1-ol	91
2	13,575	19,18	242	$C_{16}H_{34}O$	1-Hexadecanol	96
3	14,132	0,61	270	$C_{17}H_{34}O_2$	Hexadecanoid Acid, Methyl Ester	96
4	14,974	0,65	228	$C_{15}H_{32}O$	1-Pentadecanol	96
5	16,459	2,06	270	$C_{18}H_{38}O$	1-Octadecanol	96
6	16,665	2,01	296	$C_{19}H_{36}O_2$	9-Octadecenoic Acid, Methyl Ester	94
7	17,050	0,77	298	$C_{19}H_{38}O_2$	Octadecanoic acid, methyl ester	91
8	18,188	0,38	282	$C_{20}H_{42}$	Eicosane	90
9	19,730	1,23	366	$C_{26}H_{54}$	Xexacosane	96
10	21,276	2,60	562	$C_{40}H_{82}$	Tetracontane	96
11	21,466	0,57	210	$C_{14}H_{26}O$	9-Tetradecenal, (Z)-	70
12	22,808	4,68	562	$C_{40}H_{82}$	Tetracontane	96
13	24,319	6,88	506	$C_{36}H_{74}$	Hexatriacontane	96
14	25,244	0,34	506	$C_{36}H_{74}$	Hexatriacontane	92
15	25,796	8,40	562	$C_{40}H_{82}$	Tetracontane	96
16	26,697	0,61	758	$C_{54}H_{110}$	Tetrapentacontane	89
17	27,240	8,91	758	$C_{54}H_{110}$	Tetrapentacontane	96
18	27,426	1,00	410	$C_{30}H_{50}$	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	95
19	28,191	0,60	506	$C_{36}H_{74}$	Hexatriacontane	90
20	28,361	0,45	268	$C_{19}H_{40}$	3-Methyloctadecane	82
21	28,813	9,29	506	$C_{36}H_{74}$	Hexatriacontane	96
22	29.924	0,83	758	$C_{54}H_{110}$	Tetrapentacontane	94
23	30,658	8,07	758	$C_{54}H_{110}$	Tetrapentacontane	95
24	32,242	0,35	758	$C_{54}H_{110}$	Tetrapentacontane	86
25	32,874	6,72	758	$C_{54}H_{110}$	Tetrapentacontane	96
26	33,059	7,18	194	$C_{12}H_{18}O_2$	10,10-Dimethyl-3-Oxatricyclo[7.1.10]Undecan-4-one	82
27	33,478	0,35	262	$C_{19}H_{34}$	E,Z-1,3,12-Nonadecatriene	81
28	34,486	0,40	842	$C_{60}H_{122}$	Hexacontane	68
29	35,567	4,57	758	$C_{54}H_{110}$	Tetrapentacontane	96

The results of GC-MS analysis showed that the data obtained 29 A compound chromatogram peak abundance of compounds with the greatest demonstrated by compounds 2nd peak is with an area of 19.18% with a retention time **(t_R)** 13.575. MS spectrum of compound A at t R 13.575 indicates that the molecular mass of the compound at t R 242. The result is fragmentation with the main fragment m/z = 242 shows the similarity of the fragmentation pattern with

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the compound in the standard library that is 1-Hexadecanol with molecular formula C 16 H 34 O and the similarity index is 96%.

Analysis data of H-NMR

Spectrum H-NMR of isolate A (Figure 4) shows that there are five signals that appear in the spectrum. The all signal indicate representation of proton type in isolate A (CH_3, CH_2, CH or OH). At the signal (triplet) at chemical shift, δ 3.6 ppm, indicate methylene proton (-CH_2-) was bounded to electronegative atoms (OH). The typical of broad singlet signal at chemical shift, δ 1.7 ppm, was hydroxyl proton (OH). Furthermore, the multiplet signal at chemical shift, δ 1.5 ppm, indicates methyne signal (-CH-) bound to the methyl or methylene groups (-CH_2- or –CH_3) and (OH). The multiplet signals at chemical shift, δ 1.3, were overlapping signals of -CH_2- and signals at δ 0.8 ppm as signal triplet of -CH_3 most shielding and bound to methylene group.

Antibacterial Activity Test

Measuring the diameter of the barriers some variations in the concentration of the compound A and B isolated from n-hexane extract sponge *Haliclona (Reniera) fascigera* against bacteria E. Coli and S. aureus after incubation 1x24 hours obtained the results listed in Table 3.

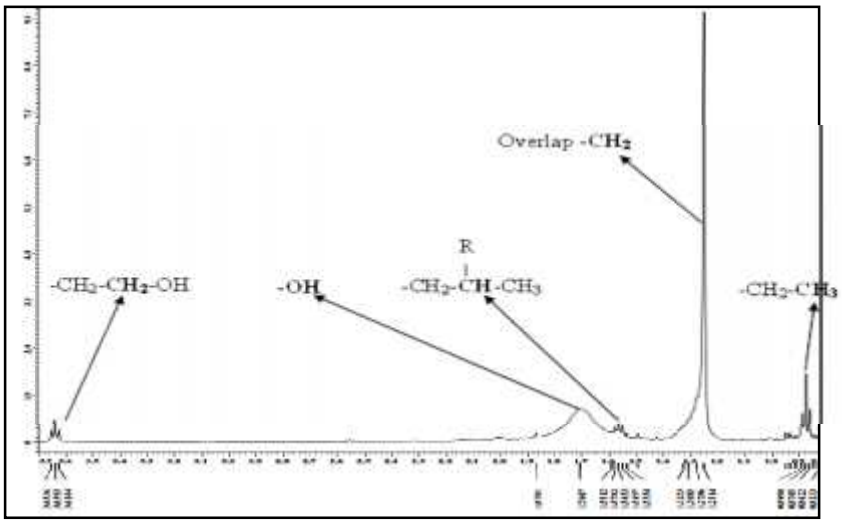

Figure 4. H-NMR spectrum of isolate A

The results of measuring the diameter of zone of inhibition against test bacteria *E. Coli* and *S. aureus* showed that the compound A greatest bioactivity looked after incubated for 24 hours at a concentration of 100 ppm, which generate barriers diameter of 6.70 mm at a test bacterium *E. Coli* and 11.60 mm in test bacteria *S. aureus*. Furthermore consecutive bioactivity of the compound A with a concentration of 50 ppm, 25 ppm, 10 ppm and has a diameter of inhibition zone respectively 6.10 mm, 5.90 mm, and 5.85 mm in the test baktei *E. Coli* bacteria test while in *S. aureus* respectively 10.40 mm, 9.50 mm, and 6.50 mm.

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Table 3. Measurement of the inhibition of the sample, the positive control, and negative control against test bacteria

Sample code	Concentration compound (ppm)	diameter zona hambatan (mm)	
		E. Coli	*S. aureus*
A	10	5.85	6.50
B	25	5.90	9.50
C	50	6.10	10.40
D	100	**6.70**	**11.60**
E	Kontrol (-)	5.20	6.60
F	Kontrol (+)	18.60	17.40

Figure 4. Power inhibition of compound A to the test bacteria *E. Coli* (left) and *S. aureus* (right) at 1x24 hours incubation

5. CONCLUSION

Isolate GB9-P3F1 from n-hexane extract of *Haliclona (Reniera) fascigera* sponge were clear solid, active in UV short, UV long wave and cerium sulphate. Isolate showed single spot on TLC silica G60 F254. Identification of isolate A using GC-MS showed that isolate A consist of 29 compounds as aliphatic compounds and supported by functional groups data of FTIR and H-NMR. Bioactiviry test of isolate A to bacterium *E. coli* and *S. aureus* using diffusion method, show that isolate A active as antibacterial at a concentration of 100 ppm with zone inhibition 6.70 mm in the bacterium *E. coli* and 11.60 mm in bacteria *S. aureus.*

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