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Research Article

THE ANTIBACTERIAL EVALUATION OF HALICLONA ASSOCIATED BACTERIA AND THE RELATING COMPOUNDS DERIVED FROM THE HOST

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ABSTRACT

Objective: The objective of this study was to investigate the active compounds derived from *Haliclona* sp. associated bacteria collected from Untung Jawa Island, Jakarta.

Methods: The bacterial isolation, screening of antibacterial activity, purification, and prediction of potential compounds using liquid chromatography–mass spectroscopy/mass spectroscopy (LC–MS/MS) as well as 16S rDNA bacterial characterization were conducted.

Results: The potential extract of bacterial strain UJ.17.10^{-4} showed the highest anti-Gram-positive bacteria. The 16S rDNA gene of this strain had 99% similarity with the actinobacteria *Agromyces tropicus*. Chemical separation of supernatant extract yielded 13 potent fractions. Identification of antibacterial compounds contained in active fractions using LC-MS/MS were halistanol sulphate C([M+H]$^+$:703), cyclic bis-1,3-dialkylpyridinium([M+H]$^+$: 213.72). In previous research reported that these compounds were isolated from *Haliclona* sponge and showed anticancer activity.

Conclusion: This result supported the ideas that *A. tropicus* plays an important role in synthesizing, halistanol sulfate C, haliclorensin, and cyclic bis-1,3-dialkylpyridinium metabolite derived from the host.

Keywords: Antibacterial, Active compounds, *Haliclona (gellius)* sp., *Agromyces tropicus.*

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INTRODUCTION

Halichondrida, Poecilosclerida, and Dictyoceratida are reported producing bioactive compounds [2]. Especially, *Haliclona* sp. is one of the marine sponges that produce several potential active pharmacological compounds. At least more than 190 metabolites were isolated from this genus. *Haliclona* (*Gellius*) sp. was very potential as antimicrobial and anticancer compounds producer such as cyclic bis-1,3-dialkylpyridinium [6], anti-multidrugs resistance compound kendariamide [15], antimycobacterial, hydroxyhaliclonacyclamine A [3]. Difficulties in cultivation have become one of the obstacles to develop drug from sponges.

Previous studies reported that there was a collaboration between sponge and associate microorganisms for synthesizing secondary metabolite. Associated microorganism produces secondary metabolism which is structurally related to their host. The most of the therapeutic compounds producers are marine actinomyces and fungi [11]. Some of the therapeutics active compounds derived from callyspongia associated bacteria such as *Pseudomonas taiwanese* and *Lysinibacillus sphaericus* were reported very potential actives against pathogenic bacteria, fungi, and *Mycobacterium tuberculosis* H7Rv strain [20].

Haliclona was one of marine sponges reported the largest associate microorganisms diversity. α-, β-, γ-, δ-, and ε-*Proteobacteria, Cyanobacteria, Planctomycetes, Firmicutes, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Fusobacteria, Nitrospirae, and Verrucomicrobia* were detected associated with *Haliclona* (*Gellius*) [8]. Based on the NRPS and PKS genes, analysis of *Haliclona simulans* associated microbiota showed that the most producing active compounds are actinobacteria, sulfitobacter, and pseudovibrio [10]. The investigation of secondary metabolite produced by *Haliclona* associated microorganism was essential to know the true producer of active compound, as well as to find the cultivable source.

Several compounds that first reported derived from higher marine biota, finally consider being produced by the associate microorganism. First, swinholide A was isolated from *Theonella swinholide*, later confirmed that this compound was produced by the filamentous heterotrophic bacteria. Polybrominated biphenyl ethers antibiotic compound was derived from *Dysidea herbacea* that was also produced by endosymbiotic cyanobacteria *Escillatoria spongeliae* [13,16]. Several active compounds that originally derived from marine associate microorganism were benzoic acid that derived from *Streptomyces cheonanensis* VUK-A [19], 2-Mercaptobenzothiazole derived from *Micrococcus* sp., and a bacterial isolated from Tedania sponge already in synthesize stage [21].

The purpose of this research was to find the cultivable resource of active compounds as well as to study the related active compounds derived from *Haliclona* and its associated microorganisms.

METHODS

A voucher of *Haliclona* sp., used in this study was collected from the Seribu Island using scuba diving, in June 2014, at the location S.05°58.422'; E.106°42.189. The solvent used for extraction and fractionation were ethyl acetate (Merck), methanol (Merck), acetone (Merck), and n-hexane (Merck). Microorganism media for bacterial isolation were 10% SYP that contained 0.5 g peptone, 0.1 g yeast extract, and 16 g agar in 1 L seawater.

Bacterial isolation and antibacterial analysis

Approximately 1 cm^3 square sponge slices were washed using sea water sterile and immersed in 10 mL sterile sea water. The dilution was adjusted for 1:10, 1:100, 1:1000, and 1:10,000. About 100 µL sample solution in each concentration (10^{-1}–10^{-4}) was dropped in marine agar media. About 1–2 weeks of incubation, the appearing bacterial colony was counted and isolated until obtained single colony.

All of the isolated strains were cultured on 10 mL of 10% marine broth and incubated during 72 h in a shaker incubator at 28°C, 100 rpm. After harvesting, the bacterial solution was centrifuged (6000 rpm, 4°C,

15 min) and extracted using organic solvent ethyl acetate for supernatant and acetone for the pellet. All of the extracts were tested for antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* (Sa) and *Bacillus subtilis* (Bs) using agar diffusion method [1]. About 20 µL of the sample that contained 100 µg of the bacterial extract was dropped on paper disk and laid on Mueller-Hinton Agar media after bacterial inoculation. Approximately 10 µg of ampicillin was used for positive control and the organic solvent for negative control. After overnight incubation, the zone of bacterial growth inhibition was measured.

Characterization of potent bacterial extract

The single isolated colony of the potent antibacterial strain was characterized using a molecular method by 16S rDNA sequencing. The amino acid pattern compared to the existing online database using BLAST method. The molecular analysis was done in INACC Lab. Research Center for Biology Indonesian Institute of Sciences.

Semi-large fermentation and chemical separation of the potent antibacterial fraction

The potent of bacterial strain UJ 17.10^{-4} was cultured in 15 L of 100% SYP broth medium in a shaker incubator at 100 rpm, 28°C. Harvesting of bacterial broth was done after 72 h incubation. The bacterial broth was centrifuged at 6000 rpm at 4°C for 15 min. The supernatant and pellet were separated and extracted using the organic solvent. The supernatant was partitioned using ethyl acetate (1/1), while pellet was using methanol.

After evaporation, both of extracts were assayed against *Staphylococcus aureus* (Sa) and Bs. The highest zone inhibition among both of extracts was continued for open column chromatography separation.

The supernatant extract was chromatographed using silica gel G (230–400 mesh) and eluted with n-hexane/ethyl acetate gradient.

Each fraction was grouped base on the similarity of thin-layer chromatography spot. All of the collected fractions were evaluated for antibacterial activity.

Secondary metabolite analysis

Secondary metabolite analysis of potent fraction was done using liquid chromatography–mass spectroscopy/mass spectroscopy (LC–MS/MS) chromatography. The solvent used for LC–MS/MS was the methanol-water gradient, flow 0.2 mL, column temperature: 40°C, and max pressure 300 Bat. Analysis of LC–MS was done at the Jakarta Medical Laboratory.

RESULTS AND DISCUSSION

Isolation of *Haliclona'*s associated bacterial using direct plating resulted in exactly 31 strains. The evaluation of antibacterial against Gram-positive Sa, in general, showed that the most of bacterial extracts have diameter inhibition at the range of 10–15 mm. Fig. 1 was described the recapitulation of antibacterial evaluation of all of the isolated strains.

The supernatant extracts were moderately active against Sa, about 95.3% of them showed diameter inhibition (di) in a range of 10–15 mm. Approximately 61.2% of pellet extracts showed weak activity against Sa with diameter inhibition lower than 10 mm. The supernatant extracts showed more susceptible to Sa than pellet extracts. The activity of supernatant and pellet extracts against Bs seems to be equal, with the total active strains inhibition zone around 10–15 mm, was 61% and weak activity with inhibition zone <10 mm was 38.7%.

One of the potent extracts was showed by strain UJ 17.10^{-4}.11, and it was active against Sa (di: 15 mm) and Bs (di: 10.7 mm). Isolated strain UJ 17.10^{-4}.11 was selected for further investigation. Sequencing of 16S rDNA for UJ 17.10^{-4}.11 indicated 99.9% similar to *Agromyces tropicus*.

Separation of the potent fraction

About 2.0434 g of supernatant extracts was obtained from 15 L fermentation broth. Separation of this extract was done using silica gel column chromatography and n-hexane-ethyl acetate eluent. This separation resulted in 13 fractions; the antibacterial potency was described in Table 1.

Table 1: The antibacterial assay of *A. tropicus* fractions

Sample (50 µg)	Diameter inhibition (mm) against	
	Staphylococcus aureus	*Bacillus subtilis*
Ampicillin 10 µg	33.50	22.05
Supernatant extract	8.34	20.78
Pellet extract	6.40	14.88
Fraction 1	13.40	21.70
Fraction 2	7.88	19.08
Fraction3	14.55	21.43
Fraction 4	8.85	18.00
Fraction 5	12.93	21.48
Fraction 6	9.15	13.70
Fraction 7	8.25	17.85
Fraction 8	5.15	18.45
Fraction 9	5.88	14.10
Fraction 10	4.90	15.68
Fraction 11	9.13	16.33
Fraction 12	11.35	18.25
Fraction 13	7.70	19.53

A. tropicus: Agromyces tropicus

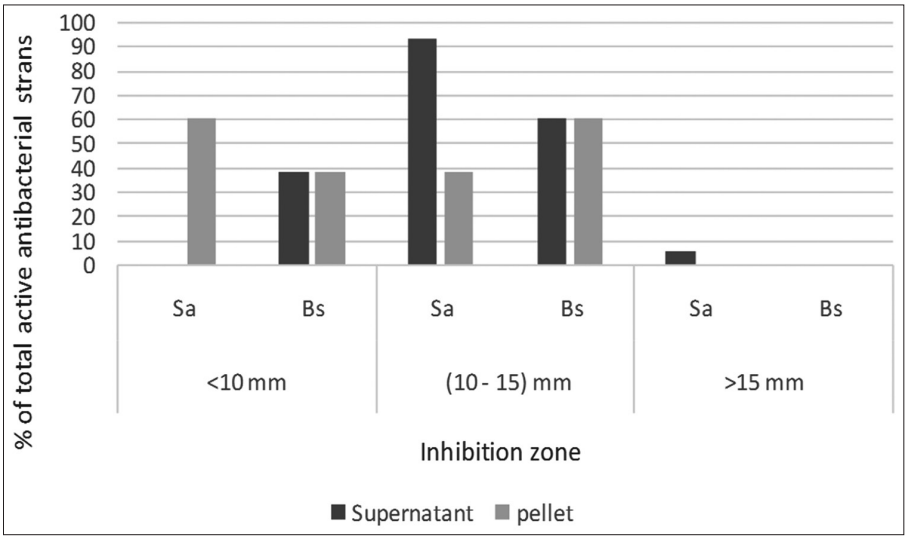

Fig. 1: The potency of bacterial extracts against *Staphylococcus aureus* and *Bacillus subtilis*

Almost all of the fractions were very potent against Bs with the diameter inhibition more than 14.10 mm**.** The potent fraction against Sa was fraction 1, 3, 5, and 12. These fractions were analyzed for the secondary metabolite.

The result of LC–MS/MS analysis

The LC–MS/MS analysis of fraction 3 indicated the peaks at retention time 3.66, 4.68, 5.88, 6.73, 14.40, 15.59, 16.27, and 18.65 min. The molecular weight respective to those retention time was 860.61, 449.42, 1371.44, 1833.32, 1957.93, 936.00, 1187.01, and 701.14 g/mol. The major compound appeared at retention time 4.68 min. The retention time, molecular weight, and predicted compounds are listed in Table 2.

The LC–MS/MS data in Table 2 showed that several compounds contained in the potential fraction of *A. tropicus* were halistanol sulfate C, triacylglycerol, *cyclic bis-1,3-dialkylpyridinium,* haliclorensin, chlorothiazide, lysergide, and L-saccharopine.

DISCUSSION

The potency of bacteria isolated from *Haliclona* sp. indicated that the most strain has moderate anti-Gram-positive bacteria with the diameter inhibition around 10–15 mm at the concentration extract was 100 µg/20 µL.

The supernatant extract of the most bacterial strain showed stronger active against anti-Gram-positive bacterial than pellet extracts. Those data informed that the secondary metabolites were excreted out from the cell or extracellular. The maintenance of membrane cell was essential for bacteria, especially under the stressful condition such as high pressure, nutrient starvation, and high salinity in the marine environment. The bacterial response to environment adaptive will change in metabolite composition such as protein, sterol, hopanoid, carotenoid, and mostly changing in membrane composition [7,14]. This statement supported the ideas that the production of secondary metabolite in bacterial occurred in the membrane cell.

LC–MS/MS spectrum at $[M\text{-}Na]^+$:703 confirmed that the potential active compound derived from fraction 1 was halistanol sulfate C. Previous study reported that this anti-HSV-1compound was isolated from marine sponge *Petromica citrina* (Demospongiae) [4], *Haliclona* sp. [4], *Epipolaris* sp. [17], and *Petromica ciocalyptoides* [5].

Compound indicating in the most active antibacterial activity of faction 3 was cyclic bis-1,3-dialkylpyridinium with $[M+H]^+$: 449.992 m/z. Cyclic bis-1,3-dialkylpyridinium was compound possess cytotoxic and antimicrobial that isolated from *Haliclona* sp. [6]. The bis-1,3-dialkylpyridinium carbon skeletons were variety found contained in *Haliclona* sp. [9].

The other alkaloid haliclorensin was found in fraction 4 $[M+H]^+$: 213.72. The first time, this compound was isolated from *Haliclona* sp. [10]. The revised structure was reported by Koren *et al*. (1998) [22]. This alkaloid was cytotoxic with the LD value of 50 2.1 mM [10].

CONCLUSION

Considering the result and discussion indicated that marine *Actinomyces A. tropicus* isolated from *Haliclona's* sp. collected from Untung Jawa contained several active compounds that previously isolated from the marine sponge *Haliclona* sp. This finding has supported the ideas that associate microorganisms play a role in producing secondary metabolite.

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Table 2: The LC–MS/MS data and estimated compounds contained in the potential fractions

Fraction no	Retention time (min)	The molecular weight (m/z)	Possibility compounds
1	1.45	1495.40	Ni
	20.87	703.00	Halistanol sulfate C [7]
	22.23	701.07	Ni
2	1.45	1743.71	Ni
	18.66	1124.93	Ni
	20.87	1967.64	Ni
	21.72	700.88	Ni
3	3.66	860.61	triacylglycerol [12]
	4.68	449.42	Cyclic bis-1,3-dialkylpyridinium/ glycochenodeoxycholate [6]
	5.88	1371.44	Ni
	6.73	1833.32	Ni
	14.40	1957.93	Ni
	15.59	936.00	Ni
	16.27	1187.01	Ni
	18.65	701.14	Ni
4	0.77	213.72	Haliclorensin [10]
	3.49	295.93	Chlorothiazide
	4.34	681.77	Ni
	4.86	323.20	Lysergide
5	0.94	574.73	Ni
	2.47	1273.60	Ni
	3.15	906.99	Ni
	4.51	1366.49	Ni
8	1.45	276.50	L-saccharopine [18]
	9.29	695.86	Ni
	16.27	704.10	Ni
	20.87	1028.44	Ni
13	1.45	947.97	Ni
	17.29	927.12	Ni
	20.87	1157.87	Ni
	21.72	1207.15	Ni

LC–MS/MS: Liquid chromatography–mass spectroscopy/mass spectroscopy

AUTHOR'S CONTRIBUTIONS

The complete research work and manuscript preparation were done by Tutik Murniasih, MasteriaYunovilsa Putra for structural analysis, and Tri Aryono Hadi for sponge taxonomy and manuscript preparation.

CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest regarding the publications of this paper.

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