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 spectrometry, liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS), and isolation of active compounds were conducted in this study.

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. Identification of the active compounds contained in the active fractions using LC–MS/MS revealed that the compounds were cyclic bis-1,3-dialkylpyridinium, cyclic bis-1,3-dialkylpyridinium, and hydrogenhaliclonacyclamine A.

Conclusion: This result supported the idea 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.
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 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 based 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 spectrometry/mass spectrometry (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 Bar. 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 17.10⁻¹, and it was active against Sa (di: 15 mm) and Bs (di: 10.7 mm). Isolated strain UJ 17.10⁻¹ was selected for further investigation. Sequencing of 16S rDNA for UJ 17.10⁻¹ 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.

| Sample (50 µg) | *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 |
| Fraction 3 | 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*](image-url)
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 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].

| 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                    |

This statement supported the ideas that the production of secondary metabolite in bacterial occurred in the membrane cell.

LC–MS/MS spectrum at [M-Na]⁺:703 confirmed that the potential active compound derived from fraction 1 was halistanol sulfate C. Previous study reported that this anti-HSV-1 compound was isolated from marine sponge Petromica citrina (Demospongiae) [4], *Haliclona* sp. [4], *Epipolaxis* sp. [17], and *Petromica clociopoytide* [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 50.21 mM [10].

**CONCLUSION**
Considering the result and discussion indicated that marine Actinomycyes *A. tropicus* isolated from *Haliclona*'s sp. collected from Untung Java 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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AUTHOR’S CONTRIBUTIONS
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CONFLICTS OF INTEREST
The authors declared that they have no conflicts of interest regarding the publications of this paper.

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