Anti-mycobacterium compound derived from *Erythrobacter* sp. isolated from *Callyspongia aurizusa*

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Abstract. Pathogenic bacteria, especially Mycobacterium, to be one of the causes of disease in society. Sponge bacterial symbionts are one of the potential sources for producing new antibacterial compounds. This study was carried out for investigating the antimycobacterial compounds derived from the *Callyspongia aurizusa*’s bacterial symbiont. Antimycobacterial compound isolation was done by growing selected bacteria SP.PP.3 in marine broth then continued for active compound separation. The separation of an active compound was carried out using High Performance Liquid Chromatography (HPLC), whereas the antibacterial activity was tested using agar diffusion method against *Mycobacterium smegmatis*. The compounds were identified by spectroscopy method (LC-MS). An antimycobacterial compound was detected in the HPLC fraction with code FH8. LC-MS and 1HNMR analysis predicted that there was a bromophycolide compound that contained active fractions. 16S rRNA molecular identification indicated that the strain SP.PP.3 was closely related to *Erythrobacter* sp. with 99% homology similarity.

1. Introduction

Sponges are sessile marine invertebrates which are reported as the largest source of marine secondary metabolites [1]. Secondary metabolites are produced as an adaptation mechanism such as self-defense against predators and stressful conditions in the surrounding environment. Most of the secondary metabolites produced are reported to have a pharmacological activity such as antiviral, antibiotic, antitumor, and anti-inflammatory [2]. Several studies have reported that the active compounds derived from sponges, to be considered produced by associate microorganisms [3][4]. The number of sponge’s associate microbes are very large, mostly reach 60% of the sponge dry mass [2] and 40% of them are bacteria [5].

The discovery of secondary metabolites derived from microbes isolated from sponge tissue was increasing since some paper reported there is a biosynthetic correlation of promising compounds among the host and symbiont. The potential compounds derived from sponge’s bacteria symbiost needs to be explored considering that Indonesia is in the coral triangle area that very well known as a hotspot of marine mega-diversity [6]. Since biological diversity correlated to the chemical diversity, the investigating of Indonesian marine macro & microorganisms as a source of bioactive compounds were very important. In this study, we investigated the bioactive compounds derived from bacteria that isolated from *Callyspongia aurizusa* collected from Seribu Island. *Callyspongia aurizusa* has previously been reported to produce callyaerin with anti-tuberculosis activity and cytotoxic effects [7]. Meanwhile, antibacterial compounds isolated from the bacteria that are associated with *Callyspongia aurizusa* have not been widely reported.
Concerning to the potential of sponge’s associate bacteria and the high incidence of pathogenic bacterial infectious diseases, we tried to find the new source of the antibiotic producer. One of the pathogenic mycobacterial infection diseases with a high incidence rate is tuberculosis (TB). TB was also one of the highest causes of death in the world [8]. This data encourages immediate research to find anti-tuberculosis or anti-mycobacterium compounds. This research was done with several steps such as isolation of potent microbial, screening the potential strain, separation of the active compound and preliminary identification of active compounds.

2. Method

2.1. Bacterial strain selection

The bacterial strain used in the research was taken from Research Center for Oceanography culture collections. The previous work reported, among 106 strains that screened for antibacterial activity, the bacteria strain SP.PP.13.2016 was strongest against *Escherichia coli*, *Bacillus subtilis*, *Vibrio eltor* and *Staphylococcus aureus* [9]. This strain was isolated from marine sponge *Callyspongia aerizusa* collected from Pari Island water, in 2016. Approximately 100μL of each glycerol stock was put into 800μL of Difco TM 2216 marine broth media. The bacterial culture was incubated during 24 hours in shaker incubator (29°C, 150 rpm). Then, bacteria was inoculated into marine agar medium. Since the strain was grown, about 10 mL of marine broth of cultivation was carried out, during 72 hours in shaker incubator (29°C, 150rpm). After harvesting, the bacterial broth was extracted using ethyl acetate and centrifuged. The extract was directly checked for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Mycobacterium smegmatis*. Antibacterial activity test was carried out by the diffusion method. The concentration of bacterial extract was 0.2 mg/ml.

2.2. Bacterial strain characterization

The characterization of bacteria was done with macroscopic, microscopic and molecular analysis. The macroscopic morphology of the colony was observed directly when grown in the petri dish; while the microscopical morphology of cells was observed using a microscope. Gram staining was tested using HIMEDIA® K001-1KT Gram Stains-Kit. Molecularly analysis was done by sequencing the 16S rRNA gene, comparing to the BLAST database, and reconstructing the phylogeny tree. Sequencing was done by sending the sample to PT. Indonesian Genetics Science. The phylogeny reconstruction was carried out using the Neighbor-Joining tree model, the Maximum Likelihood statistical method, and the bootstrap phylogeny analysis method with 500 repetitions.

2.3. Active compound separation and identification

Semi large-scale (15 mL) of bacterial cultivation was done using 10% marine broth medium. About 1.5 L bacterial preculture was poured into 15 L of total volume, incubated in 30°C shaker incubator during 72 hours at 110 rpm. After harvesting, the bacteria broth was extracted using ethyl acetate 1:1. Separation of compounds was done using HPLC Hitachi chromataster. Approximately, 50 mg of ethyl acetate extract was separated using isocratic solvent system of 60% methanol-water, flow rate 2 mL/min, 250 mm x 10 ID ODS column chromatography. UV-VIS detector at λ 220 nm was applied to detect the peaks [10]. Anti-mycobacteria activity test was applied for HPLC fractions. The concentration of fraction was 0.5 μg/μL, while the positive control (rifampicin) concentration was 0.25 μg/μL. Identification of active compounds was done using LC-MS and 1H-NMR analysis. LC-MS was conducted by the DKI Jakarta Provincial Health Laboratory, while 1H-NMR was conducted at the University of Flor.

3. Result and discussions

3.1. Bacterial characterization

The observations of the macroscopic morphological characteristics of SP.PP.13.131016.10-1.6 strain bacteria were grown on marine media so that it was an orange colony, circular in shape, convex margin, and a smooth, lominous surface. While the bacterial cell characteristic was, rod-shaped. In Gram staining
test, this bacterial strain showed Gram negative group, marked by red sticking to the cell wall. The sequence alignment of 16S-rRNA gene cluster showed 100% similarity to the overlapping area in both sequences. After that, the similarity of sequences in GenBank was searched for consensus (contig) sequences through NCBI BLAST. From the results of BLAST (Figure 1), it is known that SP.PP.13.131016.10-1.63 isolates belong to the *Erythrobacter* genus because it has a 99% homology with many strains of *Erythrobacter* sp. *Erythrobacter* genus were widely isolated from marine environment such as sea water [11][12], sediment [13][14][15][16], Coral mucus [17][18]. Most of them produce active compounds such as cytotoxic [14][15].

![Figure 1. The phylogeny tree of SP.PP.13.131016.10-1.6 strain.](image)

The phylogeny tree of selected strain SP.PP.13.131016.10-1.6 significantly closed to the *Erythrobacter* sp. This data indicated that the strain belongs to the *Erythrobacter* genus. *Erythrobacter* sp. produced heme peroxidases that play an important role in anti-*Mycobacterium smegmatis* activity [18][19].

### 3.2. Bacterial extraction, chemical separation, and antibacterial assay.

The ethyl acetate extract from 15L bacterial broth was resulting of 934.4 mg. Furthermore, this yield needs to be optimizing in the future work. The antibacterial activity tests of the extract against *E. coli* was 10.80 mm, *S. aureus* 11.5 mm and *M. smegmatis* 11 mm. Base to this activity, this strain was selected to further separation. About 50 mg of extract was applied to HPLC, C18 Column and resulting of 21 fractions. Figure 2 was the profile of extract separation using HPLC. While the data on anti-mycobacteria activity of the 21 fractions was shown in Table 1.
Figure 2. The HPLC chromatogram of *Erythrobacter* sp. extract at λ, 220.

Separation profile of substances contained in bacterial extract (Figure 2) indicated there were more than 20 peaks/fractions separated in this method. The activity was checked as described in Table 2.

| Fraction no (0.2μg/μL) | Diameter inhibition (mm) |
|------------------------|--------------------------|
| 1                      | 15.858                   |
| 2                      | 14.988                   |
| 3                      | 11.929                   |
| 4                      | 15.766                   |
| 5                      | 15.669                   |
| 6                      | 14.005                   |
| 7                      | 16.093                   |
| 8                      | 19.115                   |
| 9                      | 16.642                   |
| 10                     | 15.717                   |
| 11                     | 15.236                   |
| 12                     | -                        |
| 13                     | 18.068                   |
| 14                     | -                        |
| 15                     | 14.478                   |
| 16                     | 9.479                    |
| 17                     | 16.278                   |
| 18                     | 8.826                    |
| 19                     | 16.141                   |
| 20                     | 14.531                   |
| 21                     | 16.507                   |
| **Rifampicin**         | **37.357**               |

Most of the fractions have moderate activity against *Mycobacterium smegmatis*. The highest activity was shown by fraction 8, while fractions 12 and 14 didn’t show any activity. The activity of extract & fractions were less than positive control rifampicin, because rifampicin was a pure active compound and already through a detailed studies for optimizing the activity. *Mycobacterium smegmatis* susceptibility
test of antibiotics erythromycin, streptomycin and tetracyclin on the concentration of 0.1-1 μg/μL showed the inhibition zone in a range 0-6 mm for erythromycin and streptomycin and 6-9.5 mm for tetracyclin [20]. Its mean that the activity of the hplc’s fractions were stronger than antibiotic erythromycin, streptomycin and tetracyclin. Study of synergetic among substances in each fraction was needed to optimizing the activity.

3.3. Chemical structure determination
The LC-MS analysis of extract indicated molecular mass (m/z) at 701.89 gr/mol. The fragmentation of this mass spectrum describes in figure 3.

The previous research reported that the active antifungal compound derived from marine algae Callphycus serratus bromophylcolide B has molecular weight [M+Cl]⁺; 701 [21]. The structure of this compound have bromine element (halogen group). Considering result of the 1H NMR spectrum (Table 2.), there was halogen signal at 4.13-4.31 ppm, aromatic signal at 6.6-7.71 ppm and alkyl at 0.9-2.35 ppm.

| No. | δ 1H (ppm) | Multiplicity, J (Hz) | δ 1H (ppm) Bromophylcolide B[25] |
|-----|------------|----------------------|----------------------------------|
| 1   | 7.71       | dd                   | 7.78 (dd)                        |
| 2   | 7.63       | d                    | 7.68 (d)                         |
| 3   | 6.60       | d, 2.45              | 6.79 (d)                         |
| 4   | 5.30       | s                    | 5.52 (s)                         |
| 5   | 4.31       | m                    | 4.47 (m)                         |
| 6   | 4.13       | d                    | 3.46 (d)                         |
| 7   | 3.60       | dd                   | 3.60 (dd)                        |
| 8   | 2.29       | s                    | 2.23 (s)                         |
| 9   | 2.01       | m                    | 2.03 (m)                         |
| 10  | 1.99       | m                    | 1.99 (mm)                        |
| 11  | 1.89       | m                    | 1.84 (m)                         |
| 12  | 1.69       | s                    | 1.69 (s)                         |
| 13  | 1.64       | dd                   | 1.66 (dd)                        |
| 14  | 1.42       | m                    | 1.45 (m)                         |
| 15  | 1.43       | s                    | 1.47 (s)                         |
| 16  | 1.28       | s                    | 1.25 (s)                         |

m: multiplet, t: triplet, d: doublet, s: singlet
Proton number 6 that have chemical shift at 4.1 ppm indicated the methine proton that attached to halogenate compound [-CH-Br] and proton number 7 indicated methylene protons that attached to bromine [-CH2-Br] [22]. Proton chemical shift data in table 2 similar with the known bromophycolide B that isolated by Kubanek team [23]. Data in table 2 explain the chemical shifts of protons contain in active substance compare to the previous finding by Kubanek team. The predicted active compound contained in *Erythrobacter* sp extract was bromophycolide B. The further analysis was needed to validate the chemical structure.

### 4. Conclusion

*Callyspongia aerizusa* symbionic bacteria, *Erythrobacter* sp (SP.PP.313.131016.-10-1.6) collected from The Thousand Islands (SP.PP.13.131016.10-1.6) has the potential to produce anti-mycobacteria compounds. The SP.PP.13.131016.10-1.6 strain was Gram negative bacterium that has an orange colony with a smooth surface and is closely related to *Erythrobacter* sp. with 99% homology. The predicted active anti-mycobacteria compound separated with HPLC reverse phase was bromophycolide analog. The LC-MS and 1HNMR data supported this prediction. Further analysis such as 12C.NMR, 2D.NMR was needed to validate this preliminary structural determination.

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