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Identification and Evaluation of Antibacterial Compounds from the *Vibrio* sp. associated with the Ascidian *Pycnoclavella diminuta*

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ABSTRACT

This study aims to obtain secondary metabolites that have antibacterial activity from bacteria endophytic of ascidian *Pycnoclavella diminuta* collected from the coast of Bitung, North Sulawesi, Indonesia. The research was started with screening antibacterial activity of six bacteria isolated from *P. diminuta*. The active bacteria were selected for identification using standard biological molecular method and further fermentation to produce secondary metabolites. The isolation of secondary metabolites was conducted by various chromatography method and then selected fraction was based on the antibacterial activity from bioautography results. The result showed that the active isolate by coding M2.Tnk.Bt 5.10^{-3}.2 was identified as *Vibrio* sp. The potential active fraction was further sub-fractionated by HPLC semi-preparative and each of these sub-fractions was tested against *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Eschericia coli*, and *Vibrio cholerae*. The test results showed that all 17 sub-fractions were active against Gram-positive bacteria *S. aureus* and *B. cereus*, and only one sub-fraction (SFr 2) showed antibacterial activity against *E. faecalis*. Whereas in Gram-negative bacteria, 2 sub-fractions (SFr 12 and SFr 13) showed antibacterial activity against *E. coli* and 12 sub-fractions active against *V. cholerae*. GC-MS analysis of the SFr 2 sub-fraction showed the presence of fifteen phytochemical constituents with a major compound Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3- (2 methylpropyl).

Keywords: *Vibrio sp.; Pycnoclavella diminuta; antibacterial compounds*

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INTRODUCTION

Infectious diseases caused by microorganisms continue to increase every year and become a challenge faced by the global community (Smith et al., 2014). The rising number of infectious diseases is caused by the evolution of microbial pathogens that are resistant to existing antibiotics (Lupetti et al., 2002). The emergence of widespread resistant bacteria has led researchers to explore new alternative antibacterial agents from natural sources to be developed as new drugs (Walsh, 2003; Viejo-Diaz et al., 2005).

Recently, marine ecosystems have attracted attention as a potential new source for natural bioactive compounds (Kjer et al., 2010). The oceans, which cover more than 70% of our planet's surface with all its uniqueness and biodiversity, consist of abundant types of microbes (Bugni & Ireland, 2004; König et al., 2006). Marine microorganisms have demonstrated the diversity of secondary metabolites that can be produced. Many new secondary metabolites have been reported with a wide range of biological activity. The extreme conditions resulted from competition between bacteria in the marine environment arise due to harsh chemical and physical environmental conditions, adaptation to different habitats and environments with high levels of stress, such as cold temperature, high pressure and little/no light, that cause these microorganisms naturally required to synthesize secondary metabolites that can be used as a weapon of defense and competition (Nikapitiya, 2012; Zheng et al., 2005). Therefore, marine creatures are prospective to produce a variety of new compounds with unique structural and chemical properties with higher biological activity than terrestrial creatures, so that they have a huge potential for the pharmaceutical industry (Nikapitiya, 2012).

Ascidian or tunicata is of one of the marine invertebrates from which thousands of metabolites have been successfully isolated and it produces many bioactive compounds with various bioactivity. Several studies have proven that some secondary metabolites isolated from ascidian are of symbiotic microorganism origin

(Davidson, 1993; Schmidt & Donia, 2010). Several findings on the secondary metabolites have shown a wide range of biological activity from ascidian-associated marine microorganisms including the activity as an antibacterial agent (Wyche et al., 2012; Ellis et al., 2014).

The marine biodiversity off the coastlines of Indonesia's many islands offers a tremendous opportunity to investigate potential antimicrobial compounds from both the marine invertebrates and the microorganisms that they carry (Putra et al., 2016a; Putra et al., 2016b). Nonetheless, information on pharmacological activities from Indonesian ascidians and associated microorganisms is still limited. Here, we report a study on secondary metabolites produced from a species of *Vibrio* that is associated with the ascidian *Pycnoclavella diminuta* collected from the coast of Bitung in North Sulawesi, Indonesia.

METHODS

Sample Collection

Strain M2.Tnk.Bt 5.10^{-3}.2 was isolated from ascidian *Pycnoclavella diminuta* collected from the coast of Bitung, North Sulawesi, Indonesia at a depth of 5-10 m by SCUBA diving. It has been deposited in the strain collection of Research Center for Oceanography, Indonesian Institute of Sciences, Indonesia.

Identification of the Bacteria Strain

Strain identification was carried out by molecular taxonomy using 16S rRNA gene sequencing. A single colony was sub-cultured in Marine Broth at 29 °C for 48 h. Genomic DNA was extracted following the protocol described by Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd). Amplification of the 16S rRNA was performed using MyTaq Red Mix (Bioline) with specific primers 27F: AGAGTTTGATCMTGGCTCAG and 1492R: TACGGYTACCTTGTTACGACTT. Polymerase chain reaction was performed in a 25 µL mix reaction containing 9.5 µL dd H_2O, 12.5 µL 2x MyTaq Red Mix, 20 µmol/µL 27F Primer, 20 µmol/µL 1492R Primer and DNA Template. The PCR condition as follows 35 cycles of 95 °C for 15 s (denaturation), 52 °C for 15 s (annealing), 68 °C for 45 s (extension). PCR products purification with Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The resulting sequence was then analyzed using BLAST and compared with nucleotide on the National Center for Biotechnology Information (NCBI) database.

Cultivation, Extraction, and Isolation

A seed culture of the strain M2.Tnk.Bt 5.10^{-3}.2 was prepared by inoculation of 50 mL medium (starch 20 g, glucose (Merck) 10 g, peptone 5 g, yeast extract 5 g and $CaCO_3$ (Merck) 5 g in 1 liter seawater) in a 100 mL Erlenmeyer flask in a shaker (29 °C/150 rpm) for 24 h. This seed culture (10 mL each) was then transferred to ten 2 L Erlenmeyer flask containing 1 L with same medium and cultured with shaking (150 rpm) for 4 days at 29 °C. The fermentation broth was extracted using ethyl acetate and the extract was concentrated by evaporation. The ethyl acetate extract was fractionated by vacuum liquid chromatography using on a silica gel column with elution performed on a gradient system of increasing polarity from n-hexane to EtOAc to Acetone to MeOH. Each fraction was collected and evaporated for bioautography assay. The active fraction was further subjected to HPLC semi-preparative using isocratic elution with acetonitrile:H_2O (35:65) (flow rate 2 mL/min, Agilent C18 (10 x 250 mm), UV-Vis at 254 nm). The different fractions were collected and concentrated by evaporation. The concentrated fractions were tested for antibacterial assay to identify the active fraction.

Antibacterial Activity Assay

Bioautography

Antibacterial activity of the fraction was carried out using the bioautography technique as previously described by Valle Jr et al. (2016) with slight modifications. The fractions were spotted on the GF_{254} silica gel TLC plate and eluted with three different eluent systems as follows: FA (n-heksan:$CHCl_3$; 3,5:1,5), FB ($CHCl_3$:Acetone; 4:1), FC and FD ($CHCl_3$:MeOH; 4,5:1,5). Then, the TLC plate was observed under UV light at 254 wavelengths to determine the separation profile of each fraction. Furthermore, the TLC plate was placed aseptically to the media which had previously been inoculated with the bacteria tested (*Staphylococcus aureus* ATCC 6538 and *Eschericia coli* ATCC 8739) for 60 minutes so that the active compound on the TLC plate was transferred by diffusion into the media. Then, the TLC plate was removed and the agar plate was incubated at 37 °C for 18-24 h. The growth inhibition zones were observed and compared with TLC plate R_f value result.

Agar diffusion method

The sub-fraction results were conducted using agar diffusion method (CLSI, 2012). The bacteria tested (*Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 11778, *Enterococcus faecalis, Eschericia coli* ATCC 8739 and *Vibrio cholerae*) were prepared by turbidimetry using UV-Vis spectrophotometer at a wavelength of 625 nm to obtain absorbance between 0.08-0.12, which is equivalent to the standard 0.5 McFarland solution, and inoculated in sterile petri dish by streaking the cotton swab sterile on the surface of the MHA (*Mueller-Hinton Agar*) media. The procedure was repeated by rotating the petri dish up to 60° to the bacterial suspension evenly on the entire surface of the Mueller-Hinton agar medium. After 3-5 min, aliquots of

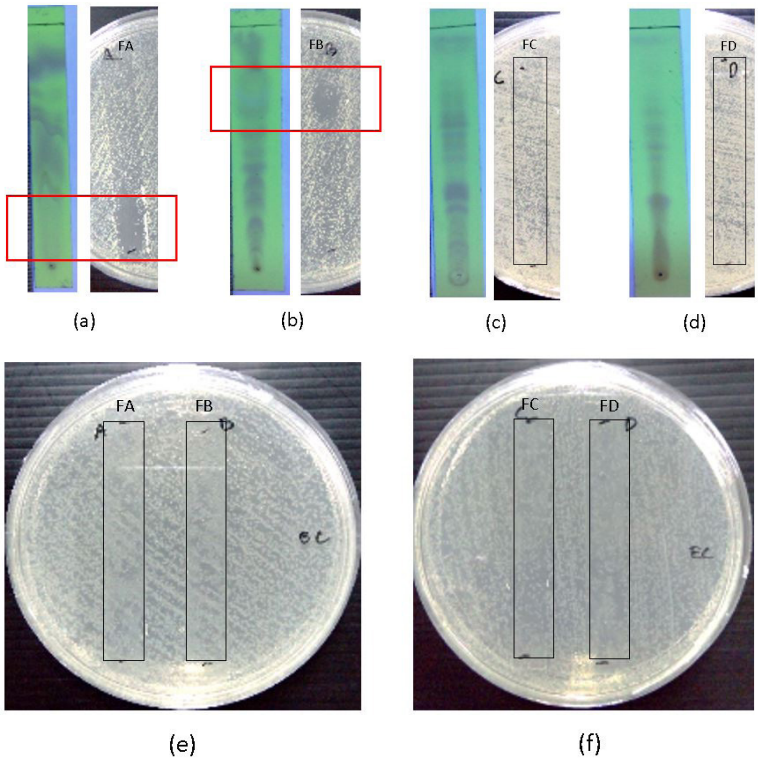

Figure 1. Bioautography results; (a) Fraction A against *S. aureus*; (b) Fraction B against *S. aureus*; (c) Fraction C against *S. aureus*; (d) Fraction D against *S. aureus*; (e) Fractions A and B against *E. coli*; (f) Fractions C and D against *E. coli*

20 µL of each sub-fraction samples were pipetted on to filter paper disc of 6 mm diameter that had been placed on the surface of the media. Then they were incubated at 37 °C for 18-24 h. The chloramphenicol disc 30 µg/disc (Oxoid) was used as a positive control and solvent (DMSO 10%) was used as a negative control.

GC-MS analysis of subfraction

Active sub-fraction based on antibacterial activity testing was characterized by GC-MS analysis by injecting 5 µL sample into HP Ultra 2 Capillary Column (30 m x 0.2 mm i.d, 0.11 µm film thickness) of GC-MS (Agilent Technologies 7890 Gas Chromatograph with autosampler and 5975 Mass Selective Detector and Chemstation Data System), with helium (1.2 mL/min) used as a carrier gas. A temperature gradient program was applied with the initial temperature at 80 °C hold for 0 minute, rising at 3 °C/min to 150 °C, hold for 1 minute and finally rising 20 °C/min to 280 °C, hold for 26 min. The resulting *m/z* peaks (mass to charge ratio), which were characteristic of active sub-fractions, were compared to the spectrum library of corresponding organic compounds.

RESULTS AND DISCUSSION

Identification of the Bacteria Strain

The isolate of M2.Tnk.Bt 5.10^{-3}.2 showed high homology to *Vibrio* sp. M-137-19 (99% sequence similarity). Identification result that was carried out on M2.Tnk. Bt 5.10^{-3}.2 confirmed several previous studies, which showed the predominance of Gram-negative bacteria in the marine environment and also reported 36% of Gram-negative bacteria as an antibiotic producer (Fenical, 1993; Bernen, 1997). *Vibrio* sp. bacteria show its dominance in marine ecosystems and some previous studies have shown this type of species to produce various types of secondary metabolites, which have antibacterial activity (Okada et al., 2005; Al-Zereini et al., 2010; Liaw et al., 2015).

Antibacterial Activity

The fractionation process by vacuum liquid chromatography produced 13 fractions, which were then monitored by Thin Layer Chromatography (TLC). Based on the same TLC separation pattern, 4 fraction combinations were obtained (FA, FB, FC and FD).

Table 1. Antibacterial activity of sub-fractions

Sample	Concentration (µg/disk)	Zone of Inhibition (mm)				
		SA	BC	EF	EC	VC
SFr 1	18	7.65 ± 0.35	8.35 ± 0.35	0	0	0
SFr 2	150	10.85 ± 0.77	8.75 ± 0.92	8.85 ± 0.49	0	10.3 ± 1.13
SFr 3	38	10.95 ± 2.05	7.85 ± 1.20	0	0	10.55 ± 0.63
SFr 4	10	9.6 ± 0.28	7.05 ± 0.21	0	0	11.9 ± 1.13
SFr 5	24	10.7 ± 1.13	10 ± 0.14	0	0	11.9 ± 2.54
SFr 6	20	7.45 ± 1.06	7.1 ± 0.42	0	0	0
SFr 7	22	9.2 ± 2.26	8.85 ± 2.75	0	0	11.15 ± 0.35
SFr 8	10	8.95 ± 0.35	8.55 ± 1.48	0	0	0
SFr 9	14	9.35 ± 0.92	9.8 ± 1.27	0	0	9.55 ± 1.20
SFr 10	28	9.1 ± 1.41	10.55 ± 0.21	0	0	0
SFr 11	16	9.55 ± 0.49	9.9 ± 0.42	0	0	9.75 ± 0.77
SFr 12	18	8.75 ± 0.92	11.25 ± 0.63	0	6.95 ± 0.07	10.1 ± 0.56
SFr 13	12	9.2 ± 0.28	9.15 ± 1.06	0	6.75 ± 0.35	8.35 ± 1.34
SFr 14	10	8.25 ± 1.62	7.2 ± 0.56	0	0	9.85 ± 2.47
SFr 15	18	7.7 ± 0.14	7.8 ± 0.70	0	0	9.45 ± 0.35
SFr 15A	10	7.95 ± 0.49	8.5 ± 0.42	0	0	0
SFr 16	12	8.9 ± 1.98	10.15 ± 2.33	0	0	11.15 ± 0.49
CHL	30	30.45 ± 1.48	21.35 ± 0.70	18.4 ± 0	23.05 ± 1.48	39.1 ± 2.40

Results (shown as mean ± SD), CHL (Chloramphenicol), SA (*S. aureus*), BC (*B. cereus*), EF (*E. faecalis*), EC (*E. coli*), VC (*V. cholerae*)

These fractions were further tested for antibacterial activity using bioautography (Fig 1). Bioautography is applied as the preliminary antibacterial assay in order to determine the presence or absence of activity (Choma & GrZelak, 2011). In the preliminary screening, test strains (*Staphylococcus aureus* and *Eschericia coli*) were chosen to represent each group of Gram-positive bacteria and Gram-negative bacteria, respectively. The results showed the fraction B (FB) have antibacterial activity against *S. aureus* at R_f value (0.75). The fraction A (FA) also formed an inhibition zone against the *S. aureus*, but the R_f value could not be determined due to the separation pattern was not good enough on the TLC plate. Whereas against *E. coli,* no inhibition zone was formed for all fraction.

Based on the bioautography results, FB was chosen to be sub-fractionated further using HPLC semi-preparative. The separation process was achieved for 25 min and it obtained 17 sub-fractions (SFr 1 - SFr 16). The fractions obtained were evaluated to determine antibacterial activity using agar diffusion method (Table 1). The results of the antibacterial activity of 17 sub-fractions against Gram-positive bacteria showed the antibacterial activity that characterized by the formation of inhibitory zones in both *S. aureus* and *B. cereus*, but only sub-fraction of SFr 2 showed the inhibitory zone against *E. faecalis*. In the antibacterial activity assay of 17 sub-fractions against Gram-negative bacteria, not all sub-fractions showed activity against Gram-negative bacteria. Only sub-fraction of SFr 12 and SFr 13 showed inhibition activity against *E. coli* and 12 sub-fractions (SFr 2, SFr 3, SFr 4, SFr 5, SFr 7, SFr 9, SFr 11, SFr 12, SFr 13, SFr 14, SFr 15 and SFr 16) showed activity against *V. cholerae*.

The sub-fractions obtained were more active against Gram-positive bacteria than Gram-negative bacteria. This difference could be due to the cell wall structure of Gram-negative bacteria, which is more complex than that of Gram-positive bacteria, the membrane accumulation mechanisms in Gram-negative bacteria, or the mechanism of destruction of molecules introduced from the outside, that is caused by the presence of enzymes in the periplasmic space (Motamedi et al., 2010). Besides that, the difference results could be caused by different concentration from each sub-fractions.

GC-MS Analysis

The identification of sub-fraction SFr 2 was achieved by GC-MS to determine the type of compounds that produced antibacterial activity. SFr 2 was selected based on the yield that was more representative for analyzing further than other sub-fractions. Figure 2

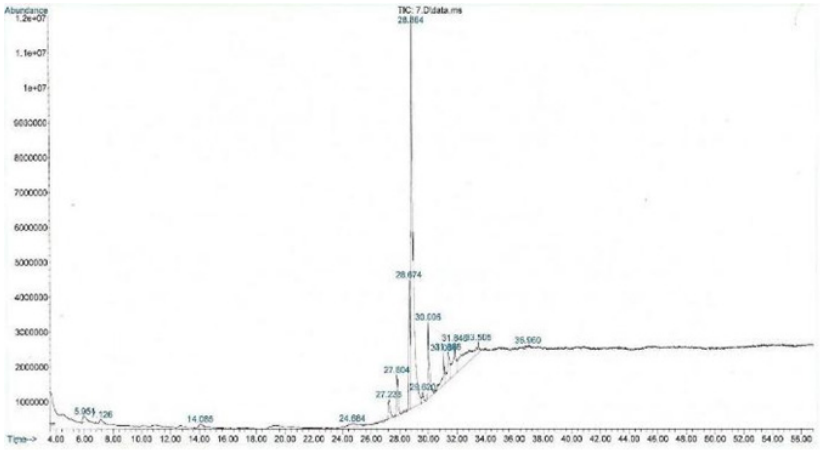

Figure 2. Gas chromatogram of SFr 2 profile

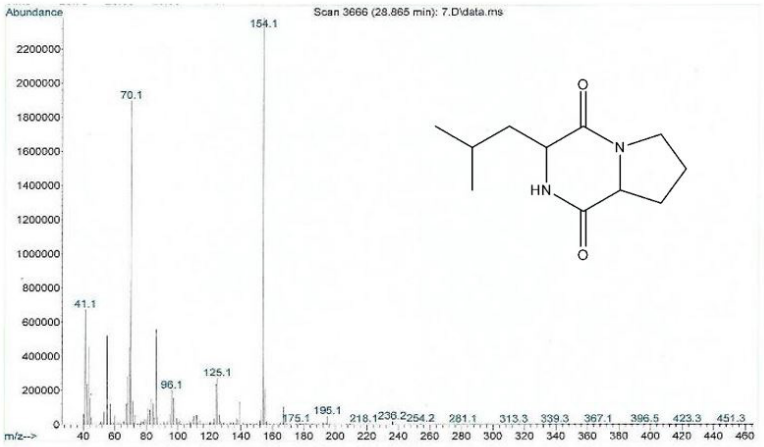

Figure 3. Chemical Structure and EI-MS spectrum of Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2 methylpropyl)

showed the chromatogram of SFr 2 with the dominant peak with the highest abundance at retention time of 28.864 min (peak area 48.34%). Based on the GC-MS library record at that retention, it was confirmed that the molecular weight of the compound was 210, and the molecular formula was determined as $C_{11}H_{18}N_2O_2$, which was identified as Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3- (2-methylpropyl) (PPDHMP). The EI-MS spectrum of SFr 2 indicated ions at 70 (M-$C_7H_{10}NO_2$) and 154 (M-C_4H_8) (Fig 3). The fragmentation pattern of the spectrum has similarities to the EI-MS spectrum obtained in the previous study by Guo et al. (2015).

Based on the results, the PPDHMP was suggested as the main constituent of SFr 2 that could contribute to the antibacterial activity. This compound was previously isolated from *Vibrio parahaemolyticus* and from endophytic fungi *Penicillium* sp. (Fdhila et al., 2003; Pandey et al., 2010; Devi & Wahab, 2012). Other chemical compounds detected by GC-MS were identified (Table 2). Some of the fatty acids with small quantities were tetradecanoic acid (1.26%) and oleic acid (6.71%), which have been reported to have antibacterial activity (Huang et al., 2010; Dilika et al., 2000; Yoon et al., 2018).

CONCLUSION

The secondary metabolite from *Vibrio* sp. shows promise as a potential source of the antibacterial compound. GC-MS analysis of the SFr 2 sub-fraction revealed the presence of fifteen phytochemical constituents with a major compound Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3- (2 methylpropyl). Isolation of pure compounds and optimization of the production of secondary metabolites should be carried out further.

ACKNOWLEDGMENT

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Table 2. Phytochemicals of the SFr 2 sub-fraction

Compound Identified	Retention Time (min)	Peak Area (%)
Phenol, 2-methoxy	5.952	1.18
Propane, 2-chloro	7.124	0.82
Phenol, 2,6-dimethoxy	14.088	0.75
Benzoid acid, 4-hidroxy-3-methoxy	24.686	2.02
Tetradecanoic acid	27.238	1.26
2-isopropyl-5-methylcyclohexamine	27.803	3.87
Prolylleucyl anhydride	28.672	9.79
Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-metylpropyl)	28.865	48.34
9-octadecenoic acid (z)	29.623	0.95
Oleic acid	30.010	6.71
9-octadecenoic acid (z)	31.085	3.31
8-hexadecenal, 14-methyl-, (z)	31.389	4.44
1, 8, 15, 22-tetraaza-2, 7, 16, 21-cy-clooctacosanetetrone	31.851	4.85
Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)	33.505	10.59
2-hydroxy-1-(hyroxymethyl) ethyl (9z)-9-octadecenoate	36.960	1.12

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