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Characterization and Identification of Antibacterial Compound from *Pseudoalteromonas piscicida* Associated with *Chromodoris lochi*

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Abstract. The present study was focused on characterization and analyzed the active compound against human pathogenic bacteria. Thirty-three isolates bacteria associated *C. lochi* from Saparua Island, were screened as antibacterial activity against strain bacteria of Multi-Drug Resistant (MDR). Screening antibacterial activity was done by the overlay method; the largest clear zone of activity was chosen for further analysis. Identified species of the best candidate was done using molecular work (16s rDNA genes with specific primer). Extraction of bio compound was done with three different solvents (methanol, ethyl acetate, hexane) into paper disk plate assay. The compound was successfully characterized by LCMS/MS spectroscopic data. The isolate SM-N-3-7 showed inhibitory activity against MDR pathogens viz., *Klebsiella pneumonia*, *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*. The fraction active was successfully isolation from the ethyl acetate fraction. The structure compound extracted from SM-N-3-7 was identified as (E)-Hexadecyl-ferulate and Bis (2-ethylhexyl) phthalate. Based on molecular identification, the isolate SM-N-3-7 was closely related to 99% with *Pseudolateromonas piscicida*. We concluded that *P. piscida* associated *C. lochi* has great potential for the antimicrobial agent.

INTRODUCTION

Antibiotic resistance is one of the major problems for public health; imported case in human bacterial infection is increasing admission over the latest six years. The case was impacted by healthcare-associated infections (HCAIs), which cause considerable morbidity and mortality [1]. Among them, *Klebsiella pneumonia*, *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, have been the best common bacterial infection. Several efforts are raised to treat infection such us the use of bacteriocins, essential oil (Eos), antibodies and phage therapy [2]. The increase of the infection case implies having fewer antibacterial agents to treat infections. However, discovering natural product from the marine is still necessary to investigate.

A marine invertebrate is well known as a source of drug discovery, several marine organisms especially marine invertebrate has potential as antimicrobial, antioxidant, anticancer and antiviral [3-4]. As one of marine invertebrate, there is still poorly report about the chemical potential of nudibranch. There are about 3000 described species of opisthobranch, and at least 40% of these have been found exclusive on the Indo-Pacific tropics, and still many species of them are unknown. Therefore, many works that should be done and explore nudibranch. Chromodoris nudibranchs are the widest genera of nudibranch (16 genera, 300+ species) in tropical coral reef and subtropical

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coastal waters. Chromodoris is one of the most diverse heterobranch classes. They are highly colouration and brightly that invited predators for prey on [5]. The presence of colour in the nudibranch is an indication of the existence of chemical weapons that will react when attacked by predators. The chemical compounds are mostly located in the mantle of nudibranch. Previously studied has been reported that Chromodoris are trophic specialists that derive terpenoids from the sponges they eat [6]. An environmental condition will highly affect the biological function of the nudibranch in response to adaptation. Therefore, this condition is the cause of the nudibranch in evolving its environment.

Accumulation of bioactive metabolites in nudibranch is promising for further research to develop the potential of chemical compound [7]. The present study aimed to explore the potential of a biochemical compound of bacteria associated nudibranch against human pathogen bacteria and to characterize the compound.

EXPERIMENTAL DETAILS

Specimen Collection and Isolation of Bacteria Associated with Nudibranch

Three nudibranch specimens were collected from Itawaka (30 29.9730'S, 1280 93.0930'E) and Nusa Laut (80 38.7470'S, 1280 48.7090'E) Ambon Sea, Indonesia by SCUBA diving in depth between 5 and 20 m below sea surface on September 2018. Documentation of nudibranch samples was taken using underwater housed digital camera Canon Power Shot 110 and Canon G7X Mark II. Each sample was preserved in zipper-lock plastic filled by sterile seawater to keep the sample fresh for the next processed immediately after return to shore. Collected Nudibranch was split by two different anatomies part which is body and stomach part using a sterile scalpel blade, and right after that, samples have been mash using sterilized laboratory mortar and pestle (Haldenwanger-Morgan Advanced Materials, England). In order to growing the associated bacteria from nudibranch tissue, mashed tissue part of nudibranch were diluted serially (10-3, 10-4, and 10-5) in glass tube contains 9ml sterilized sea water and spread on Disposable Petri Dish (Biologix sterile plastic petri dish, China) contains of Nutrient Agar medium (Lab-Lemco 0.0006%, Yeast Extract 0.0013%, Peptone 0.003%, Sodium Chloride 0.003%, Agar 0.01%) and incubated for 2 days in the room temperature (27-32 ºC) [8]. The preliminary identification was using morphological features of bacterial growth. Selected bacteria were transferred using streak method into a new Nutrient agar medium to be purified [9].

Antibacterial Activity Assay

The purified bacteria from nudibranch were tested using the overlay method to gain screened potential of bacterial activity against MDR pathogens viz., *Klebsiella pneumonia*, *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*. The pathogen bacteria were collected from Kariadi Hospital, Semarang. Two replicate of associate bacteria were tested during this assay. The antibacterial activity was monitored by measuring the clear inhibition zone. The potential bacteria were classified and reconfirmation by using Paper disk antibacterial method.

DNA Extraction, Amplification and Sequencing

The DNA extraction was prepared by using the following procedure described in Kristiana *et al.* [4]. The PCR product mixture using specific primer according to bacterial 16S rDNA contains primer 27F, and 1492 R. Resulting PCR product were inputted in Genetika Science to following the sequenced process with the 27F and 1492R primer. Total one sequences were gained. The sequence was trimming using MEGA 7.0.26 program to align the pure DNA pair base and then cluster using ClustalX aligned for grouping the bacteria similarity and to construct Neighbor-joining tree. Each of bacteria specimen sequence was identified the closest similarity hit in GenBank. In most cases, were using BLAST that provided by NCBI.

Extraction of Crude Extract and Fractionation

Crude extract collected by growing the bacteria using nutrient broth medium. Cultured bacteria were incubated in Erlenmeyer for five days with the addition of shake element using scientific shaker (Thermo scientific). Bacteria

were grown in the nutrient broth media were separated from the polar element using ethyl acetate and proceed to the rotary evaporator process to gain the crude extract.

Stability of Antibacterial Compound

The antibacterial activity was evaluated using the Disc Diffusion Method [10], according to the Clinical and Laboratory Standards Institute [11]. *S. aureus* was streaked on to Muller-Hinton agar medium (Sigma-Aldrich) with the total amount of standard (0.5 Mc Farland). The paper disk (ϕ 6 mm; Advantec. Japan) containing 15 µl of crude extract was placed on the surface of the agar plate culture. The concentrations of fraction were 50 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml. Ethyl acetate and methanol were used as the negative control. Vancomycin was chosen as a positive control (Sigma-Aldrich). The culture plates were incubated overnight at 37 °C. Active isolates were shown by the clear zone around the disk [12].

Characterization of Compound

LCMS/MS was performed using a UNIFI chromatographic instrument. The column used for the identification was acuity UPLC® HSS T3 1.8µ (2.1 x 100 mm). Mobile phase A was 0.1%formic acid/water, and mobile phase B was acetonitrile + 0.1 formic acid with gradient A/B = 95/5, 60/40, 0/100 and 95/5 in 10 min.

RESULT AND DISCUSSION

Identification of Species

Three species of *Chromodoris lochi* (species code: SMD, SMH and SMN) were collected around sponges and tunicate in Saparua Island, Ambon. Its size 40 mm, the species has been identified based on nudibranch and sea slugs identification book [15]. The morphology is blue with a dark blue submarginal band and middorsal line; rhinophores and gill range from yellow to pink. On the walls on the outside of fringing and barrier reefs where it feeds on sponges. The first identification was in the western and central Pacific Ocean since 1982.

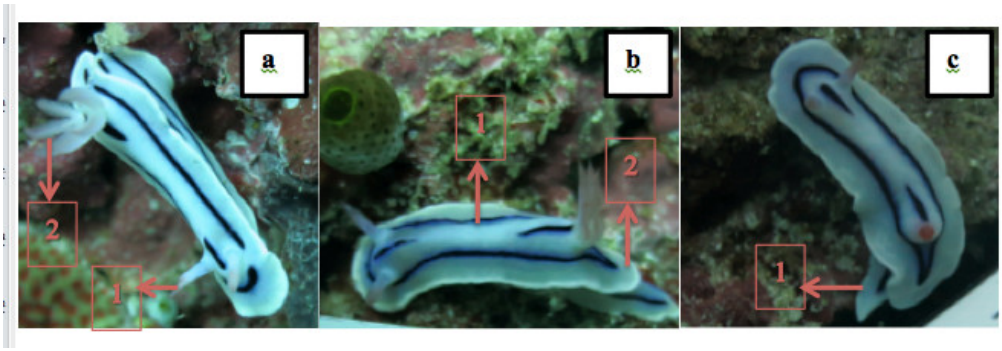

FIGURE 1. The morphological of *C. lochi*. Photo of living individuals (a) 1. Rhinophore, 2. gill (b) 1. mantle, 2. parapodium (c) 1. tail

Antibacterial Activity Assay

The total of associate bacteria gained from nudibranch *C. lochi* were 33 isolates. They were purified and screened based on colour, shape and elevation (Figure 2). The isolate that shown the highest activity to inhibit related pathogen *Klebsiella pneumonia, Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus*, and *Escherichia coli* were from isolate SMN-3-7. This isolate was tested by the overlay method and shown clear zone inhibition 15 mm from the centered agar plate (Fig. 2). Many other bacteria associated with marine organism were shown an anti-bacterial potential against pathogens. Diverse and complexity of marine environment (e.g., varying salt concentration, nutrient richness) were stimulated the microbial metabolism and deliver to produce a diverse chemical composition in microorganism [3]. Exploring a new anti-pathogen potential of associated bacteria from

marine invertebrate was quite famous and well proofed by several studies [13-14]. A microorganism that associates in marine invertebrate such as Nudibranch could be a new way to discover more potential anti-pathogen microorganism. Isolate SMN-3-7 was a discovery to the gained the potential of a new anti-pathogen source.

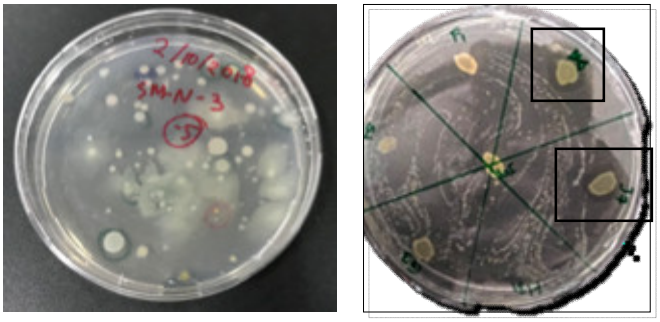

FIGURE 2. Morphology isolates symbiont nudibranch, and square line indicate its activity against human pathogen bacteria.

Molecular Analysis and Phylogenic Studies

Associate bacteria sequence SMN-3-7 that shown the most-antibacterial activity was analyzed through BLAST and Neighborhood-Joining tree were constructed by MEGA 7. Shown more over 99% query cover hit, Isolate SMN-3-7 was 99% closely related with *Pseudoalteromonas piscicida*. Phylogenetic tree analysis were shown a number of bootstrapping cover from SMN-3-7 isolate between other related bacteria. Isolate SMN-3-7 revealed the taxonomic position by showing phylogenetically related close to *P. piscicida*. The number of evolution time was shown in Figure 3 is not many DNA pairbase were evolving further. In the group of Gene *Pseudoalteromonas sp.,* isolate SMN-3-7 still shown similarity distance between the other species. This main isolate that gives more strong anti-bacterial activity was identified as Gammaproteobacteria.

Antibacterial Activity of Fraction

Antibacterial activity was shown in Table 1. EtAc fraction had activity against *K. pneumonia, B. subtilis* and *E. coli* at concentration 250 µg/ml. MeOH fraction active against *S. aureus* at concentration 250 µg/ml. The ethyl acetate fraction has strong activity against gram-negative and gram-positive bacteria than methanol fraction. So, we focused on the characterization of ethyl acetate fraction with determined the mass molecule by LCMS. The source of the fraction is identified as *Pseudoaltermonas piscicida*. Previous studies reported that *P. piscicida* produced Pseudocelin A as anticonvulsant activity [13].

TABLE 1. Antibacterial activity of fraction from *P. piscicida*

Pathogenic bacteria	**Partition (Concentration fraction µg/ml)**							
	Methanol				**Ethyl acetate**			
	50	250	500	1000	50	250	500	1000
Klebsiella pneumonia	-	-	-	-	-	+	+	+
Staphylococcus aureus	-	+	+	+	-	-	-	-
Bacillus subtilis	-	-	-	-	-	+	+	+
Eschericia coli	-	-	-	-	-	+	+	+

Noted. (+) The presence of activity (-) no activity

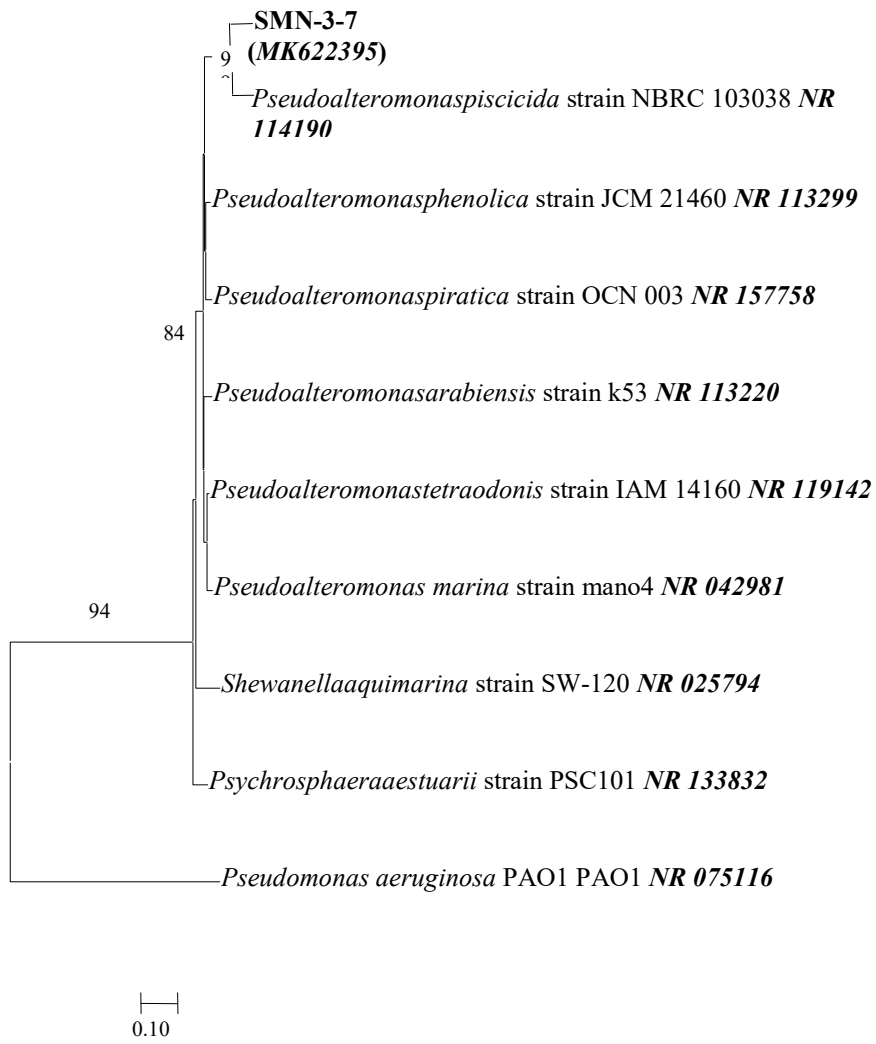

FIGURE 3. Phylogenetic tree of bacteria associated with Nudibranch *C. lochi* located in Nusa Laut, Saparua, Indonesia.

Phylogenetic trees were build using maximum like hood processing and the number shown of a bootstrap replicate of 1000 data with MEGA 7.0.26 bioinformatics software. The tree represents phylogenetic diversity between SMN-3-7 isolate and other bacteria species related to *Pseudoalteromonas sp*. The outer group was chosen to determine the different group of genus *Pseudomonas aeruginosa.* The accession number of each species was written in bold and italic style right after the species name — the scale bar in the bottom of the figure represented the distance of evolutionary sequence.

Characterization of Compound

We identified two dominant compounds from the ethyl acetate fraction in LC-MS/MS (Figure 4). These compound are (E)-Hexadecyl-ferulate observed ions at m/z 419.31 and Bis (2-ethylhexyl) phthalate observed ions at m/z 413.26.

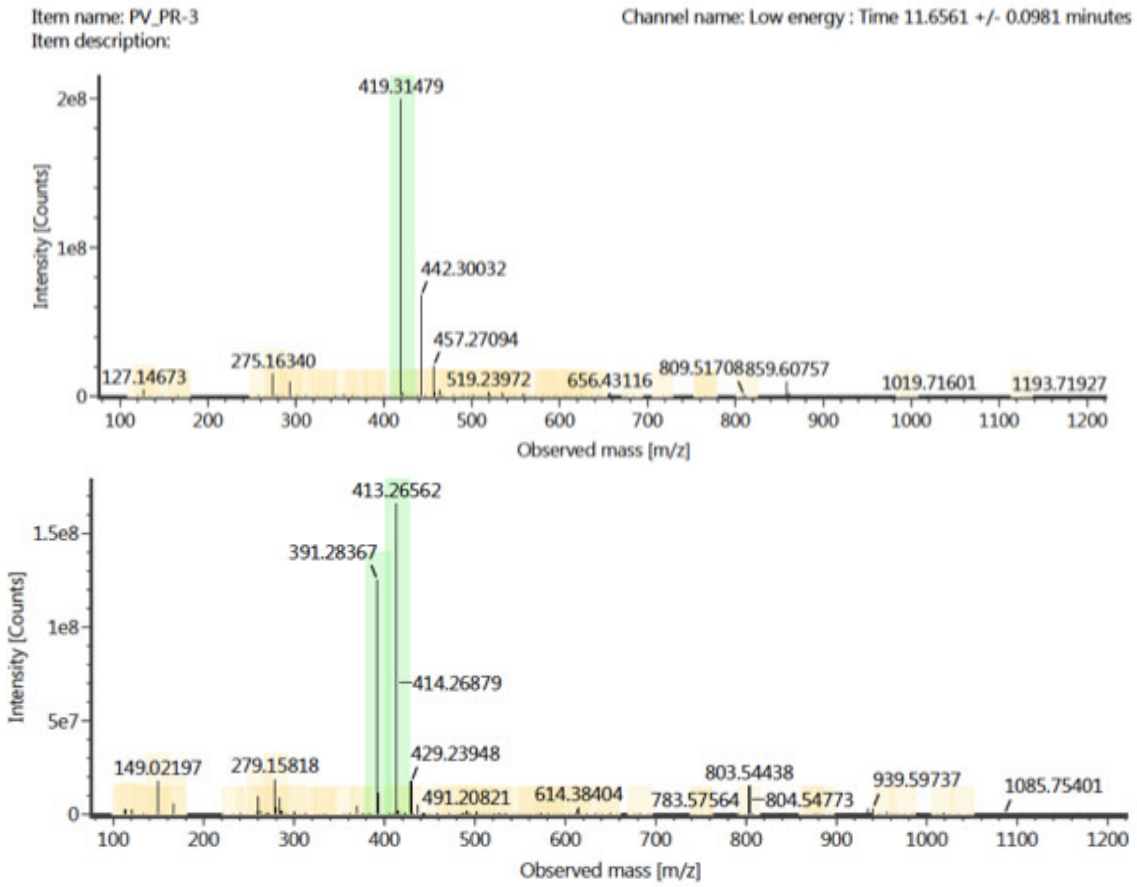

FIGURE 4. LC-MS report of ethyl acetate extract. The green colour is determined by the main mass molecule of the compound. (E)-Hexadecyl-ferulate observed ions at *m/z* 419.31 and Bi s(2-ethylhexyl) phthalate observed ions at *m/z* 413.26.

SUMMARY

This study reported the potential of bacteria symbiont nudibranch that has antibacterial activity against human pathogen bacteria. The ethyl acetate fraction has strong activity against gram-negative and gram-positive bacteria than methanol fraction against *K. pneumonia*, *B. subtilis* and *E. coli* at concentration 250 µg/ml. Further analysis by LCMS indicated that the dominant compounds are (E)-Hexadecyl-ferulate and Bis(2-ethylhexyl) phthalate. Further study is necessary to purify the compound and find the minimum inhibition concentration of the active compound.

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