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Exploring Marine Invertebrate-Associated Bacteria for Novel Antibiotics Discovery

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ABSTRACT

Marine bacteria associated with marine invertebrates are interesting source to explore compounds with potential bioactivities. In this study we screened bacteria associated with various samples (n=16) from Pulau Pari, Kepulauan Seribu, Jakarta for their antibacterial activities. The aim of this study is to obtain potential bacterial strains that can produce novel antibiotics. Isolation of bacteria from the 14 marine invertebrates and 2 of other marine samples was performed in four media: Marine Agar, ISP2, YMA, and MS. A total of 97 bacterial strains were selected to test their antibacterial activity against Gram-positive (*Mycobacterium smegmatis* NCTC 8159, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6051) and Gram-negative bacteria (*Eschericia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853). The result showed a total of 19 bacterial strains were active. Furthermore, one strain (MS.P.013.2) exhibited potent antibacterial activity against *M. smegmatis.* Partial identification of the strain using 16S rRNA sequencing revealed 99.58% sequence similarity to *Micrococcus luteus* NCTC 2665^T. Chemical analysis of the MS.P.013.2 methanol extracts using GC-MS showed the majority of volatile compounds were 9,12-Octadecadienoic acid, methyl ester; 9,12-Octadecadienoic acid (*Z,Z*); Octadecanoic acid; 2-(Dimethylamino)ethyl vaccenoate; and Cyclopropane,1,1-dichloro-2,2,3,3-tetramethyl-. These compounds were previously reported to have putative antibacterial activity, emphasize the prospect of targeting this bacterial strain for further exploration, especially to isolate and to characterize novel antibiotics.

Keywords: actinobacteria, antibiotic, marine invertebrates, Indonesia

INTRODUCTION

Indonesia, recognized as the world's largest archipelagic nation, boasts an impressive array of about 13,558 islands (Andréfouët *et al*., 2022). This exceptional geographical characteristic provides a wealth of marine life diversity, establishing Indonesia as a hub of mega-biodiversity concerning marine organisms (Riandy and Handayani, 2015). Comparable to other life forms, marine species biosynthesize metabolites, encompassing both primary and secondary compounds, to sustain their biological functions. Residing within an extreme habitat frequently prompts these organisms to produce multiple secondary metabolites characterized by distinct chemical attributes (Jiménez, 2018). In serving as a component of their defence mechanism, these metabolites have demonstrated a spectrum of diverse biological activities, thus holding significant relevance in the realms of drug discovery and advancement (Dewi *et al*., 2008). Bacteria associated with marine invertebrates have been reported to produce antimicrobial substances dating back to the 1950s (Schinke *et al*., 2017; W. Wang *et al*., 2020). In recent times, there has been a growing interest towards investigating marine invertebrate-associated bacteria as a viable reservoir to search for innovative antibiotics with either novel structures or work mechanism. This inclination arises due to the abundant biological and chemical diversity reported in marine organisms, bacteria included, rendering them auspicious subjects for finding novel pharmaceutical leads (El Samak *et al*., 2018; El-Hossary *et al*., 2017; Wibowo *et al*., 2021).

Marine invertebrates have been recognized as a rich source of bacteria that are potential producers of unique and novel antibiotics (Boufridi *et al*., 2024). Recently, there has been a significant increase in the discovery of new antibiotics from marine microbes due to advancements in structure characterization and sampling technologies. Marine invertebrate-associated bacteria are of particular interest in the search for novel antibiotics due to their potential to produce unique and novel bioactive compounds (Srinivasan *et al*., 2021). Furthermore, the vast diversity of microorganisms in marine niches continues to yield many novel bioactive compounds, thus emphasizing the untapped potential of bacteria associated with invertebrates (Utermann *et al*., 2020). Several indications suggest that bacteria associated with marine invertebrates are significant contributors to a diverse array of bioactive compounds (Santos *et al*., 2020). Consequently, these bacteria offer a viable alternative for the exploration of biologically active substances, therefore it is worth to documenting these microorganisms. As these bacteria also represent a significantly underexplored domain, with only approximately 1% of pure cultures successfully cultivated thus far, it is believed that there are still a few parts of unexplored marine invertebrates-associated bacteria as potential producers of antibiotic substances. Therefore, in this study, we aimed to explore the antibacterial potential and chemical constituents of marine invertebrate-associated bacteria that we collected from Pulau Pari, Kepulauan Seribu, Jakarta.

MATERIALS AND METHODS

Collection of marine invertebrates and bacteria

Marine invertebrates were collected from four sites near Pari Island, Kepulauan Seribu, Jakarta by SCUBA diving. Sampling sites were placed on the north and south of Pari Island; the majority of the island's people lived near the local harbour in the southern portion of the island (Figure 1) (Supplement Table I). An underwater photograph of each sample invertebrate colony was taken for identification purposes. Pictures of all samples were stored in Google Drive (https://docs.google.com/document/d/10CJmRpxMmhtIALSEfuwQEpt5NslWfaZ0/edit). Identification of marine invertebrates follows de Voogd *et al* (2023) based on the morphological features.

Approximately 5-10 cm^3 of the marine samples were cut from the colonies and placed in zip-lock bags. The samples were stored in an icebox and transported within 12 h to the nearest facility for bacteria isolation. At the facility, 1 cm^3 of each sample were rinsed using sterile sea water to remove the weakly attached bacteria. Samples were homogenized using sterile mortar and stamper, followed by serial dilution with sterile sea water up to 10^{-5} with a factor of 10. Diluted samples (100 µL) were streaked onto agar plates with appropriate isolation media. Incubation at room temperature (± 28°C) was carried out for 14 days, with daily observations of colony development. Colonies morphologically suspected as Actinobacteria and exhibiting non-fast growth were selected for further analysis.

The isolation media used in this study were Marine Agar (HiMedia), MS Agar (20 g mannitol (Merck), 20 g soya flour (Health Paradise)), YM Agar (10 g malt extract (HiMedia), 5 g yeast extract (HiMedia, and 4 g glucose (Sigma-Aldrich)),

ISP 2 (4 g yeast extract (HiMedia), 10 g malt extract (HiMedia), and 4 g dextrose (Sigma-Aldrich)). Antifungal nystatin 25 µg/mL (Sigma Life Science) and agar 16 g/L (Oxoid) were added to all media type. All the media was prepared in 1 L of seawater.

Bacterial Cultivation and Antibacterial Screening

Each type of isolation media was used to recultivating the marine bacteria, as well as to obtain a pure and a single bacterial strain. The isolated single-strain bacteria were then transferred to marine agar and this isolate was incubated at room temperature for 21 days to conduct antibacterial assays using the agar plug diffusion method as has been done previously in the preliminary screening by Wibowo *et al*. (2019). Briefly, Agar plugs were obtained using a sterile cork-borer of 8 mm diameter. The plugs were transferred onto Mueller Hinton Agar (MHA) previously streaked with bacterial test organisms. The bacterial strains used for the assay were Gram-positive bacteria *M. smegmatis* NCTC 8159, *S. aureus* ATCC 25923, *Bacillus subtilis* ATCC 6051 and Gram-negative bacteria *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853.

Figure 1. Map of sampling sites in Pari Island.

Each test bacteria were incubated in 10 mL of Mueller Hinton Broth at room temperature overnight, and 100 µL of the resulting solution was streaked onto MHA. Agar plugs from the isolated bacteria were then positioned on the agar plates, followed by a 24-hour incubation period at room temperature. The presence of a clear zone around the agar plug indicated the zone of inhibition from the isolated bacteria. The inhibition zone area was measured in mm after reduced with the agar plug diameter (7 mm).

Partial identification using 16S rRNA

Genomic DNA of the selected isolate (MS.P.013.2) was extracted using the phenol-chloroform method (Franco-Correa *et al*., 2010). The extracted gDNA was used as a template to amplify the 16S rRNA gene using 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R primer (5'-TACGGYTACCTTGTTACGAC-3'). The PCR was carried out using MyTaq HS Red mix according to the manufacturer guide (Bioline, BIO-25047). The amplified 16S rRNA gene was sequenced using third party service from 1st Base (https://base-asia.com). The 16S rRNA gene nucleotide sequences were then trimmed and identified for the closely related species using EzBioCloud web server (Yoon *et al*., 2017). The MS.P.013.2 nucleotide sequence and the closely related species were further used for phylogenetic tree reconstruction, carried in the MEGA7 (Kumar *et al*., 2016), using the Neighbor-joining method (Saitou & Nei, 1987) and Kimura 2-parameter to calculate the evolutionary distances (Kimura, 1980).

Extraction of antibacterial compounds

For chemical analysis, agar medium from active bacterium MS.P.013.2 was subjected to extraction. Agar medium was cut into small pieces and then soaked with 10 ml methanol (pro analysis, Merck) in a 50 mL Falcon tube. The tube was placed in a shaker with a rotation of 120 rpm for 30 minutes. The extraction process was repeated three times.

Chemical analysis using GCMS

Methanol extract of the bacterium MS.P.013.2 was analysed using GCMS-QP2010 SE (Shimadzu Corporation). The GC utilized RTX5-MS column (30 m, 0.25 mm ID, 0.25 µm) with the oven temperature program 60°C stationary for 2 minutes and the gradually increased to 300°C with the rate 15°C per minute. The temperature was then stationary again at 30°C for 6 minutes. Injection temperature was 230°C. Sample with the volume of 2 µL was injected with the split ratio 10:1. Helium was utilized as an inert gas carrier with linear velocity of 39 cm/sec and total flow of 27 mL/min. The ion from 50 – 550 *m/z* were scanned from minute 4 – 25 with 0.3 sec event time. The GC-MS analysis in this study was performed by interpretation of the spectra to the references in the National Institute Standards and Technology database using NIST 20 mass spectral library (NIST20R.lib).

Table I. Number of bacterial strains isolated from each marine invertebrates in each culture medium.

Invertebrates	Location	Number of isolated bacteria				Total bacterial isolates
		MA	ISP2	MS	YMA	
Aaptos suberiotoides (P013)	Site 1	1	-	3	1	5
Clathria reinwardti (P043)	Site 4	1	-	1	-	2
Dasychalina fragilis (P017)	Site 1	-	4	-	-	4
Dasychalina fragilis (P040)	Site 4	2	9	-	-	11
Didemnum sp. (P061)	Site 2	2	-	1	-	3
Dysidea sp. (P015)	Site 1	-	1	2	1	4
Haliclona sp. (P058)	Site 3	1	-	-	-	1
Ircinia ramose (P045)	Site 4	3	-	-	3	6
Lamellodysidea sp. (P035)	Site 2	4	3	8	1	16
Opheodesoma sp. (P006)	Site 1	-	2	2	2	6
Petrosia nigricans (P039)	Site 2	-	14	-	-	14
Stylissa cartery (P057)	Site 3	-	-	1	-	1
Stylissa sp. (P001)	Site 1	1	2	9	1	13
Unidentified (P020)	Site 1	-	-	1	-	1
Unidentified (P027)	Site 2	-	-	4	1	5
Xestopsongia sp. (P022)	Site 1	-	-	5	-	5
TOTAL		15	35	37	10	97

RESULTS AND DISCUSSION

Isolation of the bacteria

We collected a total of 16 marine invertebrates from 4 sites in Pari Island, Kepulauan Seribu, Jakarta. The sites are situated within zone impacted by anthropogenic activities, such as tourism, as Pari Island was one of the most visited locations during holidays by Jakarta residents. The increases volume of tourism not only influence the loss of habitat quality but also might be harm to the marine invertebrate. As reported by Rizzi et.al. (2023), the accumulated contaminants from pharmaceuticals, personal care products, and sunscreen by tourists could potentially pose a hazard to marine invertebrates, such as increases in enzyme production involved in the cell detoxification process. However, the marine invertebrates in the vicinity of Pari Island are noteworthy for their uniqueness, given its proximity to Jakarta Bay. Despite of residing in an environment with relatively high environmental pressure, these organisms are continuing to flourish and thrive exceptionally well.

We meticulously selected and isolated a total of 97 bacterial strains, from 14 marine invertebrates and 2 other marine samples, using four kind of isolation media namely MA, ISP2, MS, and YMA (Table I). These media types were pecifically chosen for their efficacy in the isolation of actinomycetes, particularly marine rare actinomycetes (Subramani & Sipkema, 2019). Medium ISP2 and MS became the isolation media with the high number of picked bacterial isolate due to the high number of colonies that grow well in those media.

Antibacterial activity of the isolated bacteria

Only 19.6% out of 97 marine bacterial strains in this study demonstrated activity against at least one of the pathogenic test strains (Table II). Surprisingly, none of the isolates inhibited the growth of *B. subtilis*, although 10 strains exhibited inhibitory effects against *S. aureus*. Notably, six isolates demonstrated the ability to inhibit the growth of *M. smegmatis*. Additionally, two isolates displayed inhibitory effects against *E. coli.*

The examination of inhibition zone diameters revealed that the isolated bacteria formed distinct zones surrounding the agar plug region. The majority displayed diameters ranging from 2 to 5 mm. Remarkably, one bacterium, specifically isolate MS.P.013.2 (no. 51) demonstrated a substantial clear zone around the agar plug, with diameter of inhibition 14 mm (Figure 2). This bacterium was subsequently identified using the 16S rRNA Sanger sequencing.

Table II. Number of active bacterial strains that showed inhibition against tested pathogenic bacterial strains

Test pathogenic bacteria	Number of active strains
B. subtilis	-
E. coli	2
M. smegmatis	6
P. aeruginosa	1
S. aureus	10
Total active strains	19

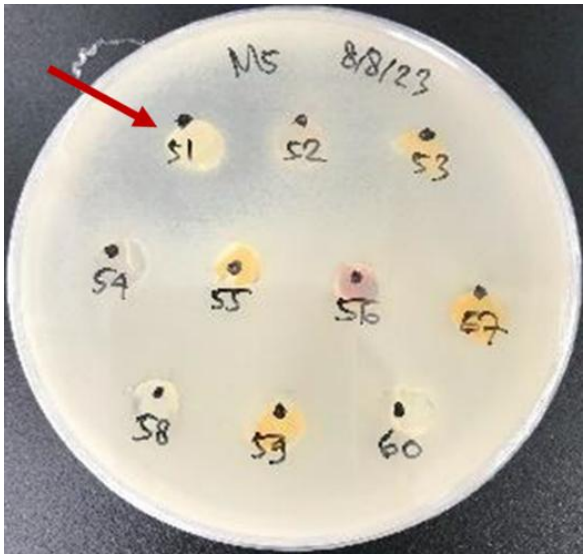


Figure 2. The red arrow pointed out MS.P.013.2 (no.51) showed the widest inhibition zone against *Mycobacterium smegmatis* NCTC 8159.

Potential bacterium MS.P.013.2 was obtained from the sponge *Aaptos suberitoides* using MS isolation medium. The sponge is known to produce cytotoxic alkaloid compounds such as aaptamines, suberitines, aaptodines (Liu *et al*., 2012; Pham *et al*., 2013; Wang *et al*., 2020). The compounds aaptamines also showed potential antibacterial activity against *Mycobacterium smegmatis* (Nadar *et al*., 2022). Previous research on the bacterial communities associated with *A. suberitoides* has provided detailed characterization of the microbial communities (Cahyani *et al*., 2023; Hayami *et al*., 2023). Therefore, this study paves the way for more extensive exploration of the cultivable bacteria derived from *A. suberitoides*, highlighting their potential for the source of bioactive compounds.

Identification of the bacterium MS.P.013.2 using the 16S rRNA Sanger sequencing

Based on the 16S rRNA gene sequencing, the bacterium MS.P.013.2 has a highest similarity with *M. luteus* and belong to the genus *Micrococcus* (Supplementary Table II). Phylogenetic tree reconstruction was carried out to delineate the isolate MS.P.013.2. The Neighbor-joining tree reconstruction showed the isolate MS.P.013.2 in the clade together with the *M. luteus* supported with the bootstrap value 82% (Figure 3). Therefore, the isolate MS.P.013.2 in this study was identified as *M. luteus*.

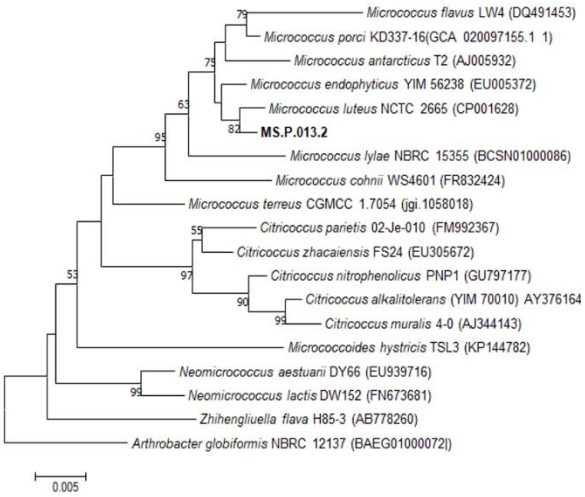


Figure 3. Phylogenetic tree reconstruction of the isolate MS.P.013.2 (shown in bold) and eighteen relatively closest species (accession number in parentheses) with a total of 1196 base pairs for each sequence. Tree was constructed using the Neighbour-joining method and the evolutionary distances were computed using the Kimura 2-parameter method. The bootstrap value >50% was shown in the node.

M. luteus was known as cosmopolitan bacteria which can be found in the soil (Tuleva *et al*., 2009), painting (Wieser *et al*., 2002), marine environments (Mohanrasu *et al*., 2021), and marine invertebrates (Bultel-Poncé *et al*., 1998) with various potencies such as antimicrobe, biosurfactant, and producer of biodegradable polyhydroxybutyrate. Studies reported marine *M. luteus* exhibited a potent antibacterial activity and also producers of anti-biofilm due to its exopolysaccharide and pigment against test bacteria that isolated from wound, namely, *Staphylococcus* sp., *Klebsiella* sp., *Pseudomonas* sp. (Nisha *et al*., 2020; Umadevi & Krishnaveni, 2013).

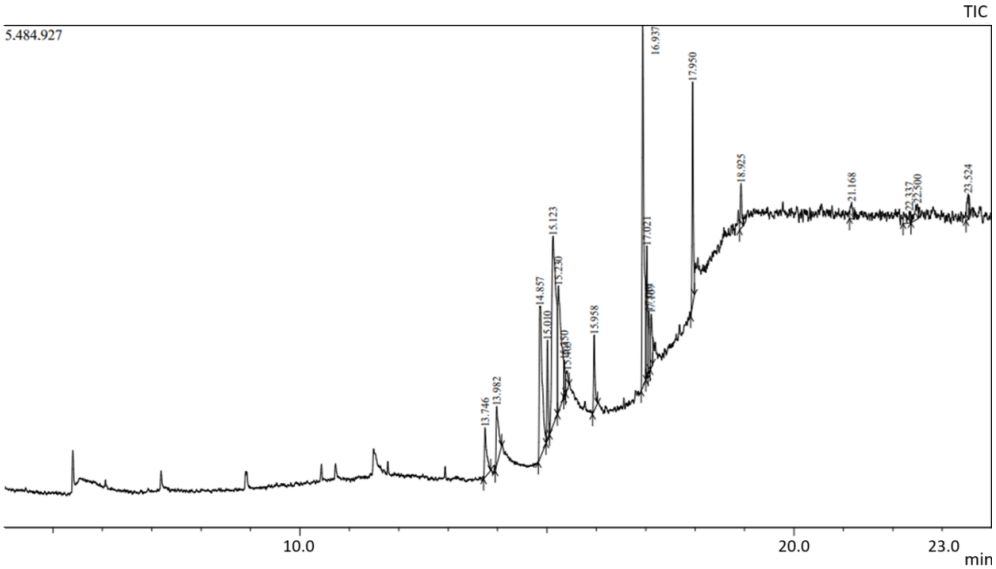


Figure 4. Total ion chromatogram (TIC) of the extract from bacterium MS.P.013.2.

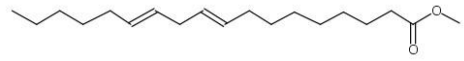
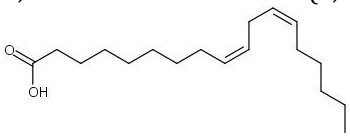
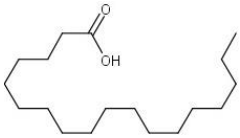
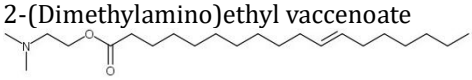
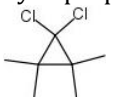
Chemical analysis of the extracts using GC-MS

The chromatogram of methanol extracts of bacterium MS.P.013.2 (Figure 4) indicated the major compounds 9,12-Octadecadienoic acid (*Z,Z*)- (20.96%), 2-(Dimethylamino)ethyl vaccenoate (18.23%), Octadecanoic acid (11.75%), 9,12-Octadecadienoic acid, methyl ester (11.65%) and Cyclopropane, 1,1-dichloro-2,2,3,3-tetramethyl- (6.76%) (Table III). Our study indicated that fatty acid octadecanoic acids constitutes are the most abundant compounds in the methanol extract of bacterium MS.P.013.2. This finding aligns with study of Parsons *et al*. (2012) who reported similar results in extracts of brown algae (*C. compressa*) and marine sponge (*S. officinalis*), emphasizing the pivotal role of fatty acids octadecanoic and hexadecanoic acids, as primary components with function as anionic surfactants under low pH conditions. These fatty acids were also reported to target the disruption of bacterial cell walls and membranes (Abou-Elela *et al*., 2009).

Fatty acid compound 9,12-Octadecadienoic acid, methyl ester was detected in a blue green algae *M. aeruginosa* extract which showed antibacterial activity against foodborne pathogenic bacteria such as *E. coli* 0157 H7 (ATCC 51659), *K. pneumoniae* (LMD 7726), *P. aeruginosa* (NRRL B-272), and *S. aureus* (ATCC 13565). Additionally, this compound also showed potent anticancer activity against colon cancer cell lines with IC_{50} of 28.73 µg/ml (Abdel-Rahman *et al*., 2020). On the other hand, fatty acid compound 9,12-Octadecadienoic acid (*Z,Z*) has been reported from a medicinal plant *P. alatum* (Wall. ex-Wight & Arn.) Swingle (Parthipan *et al*., 2015). Meanwhile, fatty acid octadecanoic acid was isolated from an aquatic plant *S. auriculata* exhibited antibacterial activity against various *S. aureus* isolated from bovine mastitis (Purgato *et al*., 2021). A cyclo-alkane compound Cyclopropane,1,1-dichloro-2,2,3,3-tetramethyl- was detected in the extract of a fungi *P italicum* which inhibited the growth of a soil-borne fungal pathogen, *M phaseolina* (Khan & Javaid, 2022). Interestingly, there is no prior report on the bioactivity of a nitrogen containing compound 2-(Dimethylamino)ethyl vaccenoate. Therefore, this study contributes novel insight to suggest the present of potential compounds 2-(Dimethylamino)ethyl vaccenoate from the extract of sponge-associated bacterium *M. luteus* that may contribute to the activity of the extract against *M. smegmatis*. However, further study needed to isolate and to test the compound for proving the hypothesis.

The phylum Actinobacteria has long been recognized as a dynamic source of numerous bioactive secondary metabolites. The potential bacterium MS.P.013.2 in this study was identified as belonging to the phylum actinobacteria. Our analysis of the methanol extract from bacterium MS.P.013.2 employed using GC-MS revealed a total of 19 compounds present in the extract (Table III).

Table III. GC-MS analysis results showed volatile compounds in the extract of MS.P.013.2

No.	Compound name	RT (min)	% Area	Chemical formula	Molecular weight
1.	Hexadecanoic acid, methyl ester	13.746	2.90	$C_{17}H_{34}O_2$	270
2.	n-Hexadecanoic acid	13.982	3.93	$C_{16}H_{32}O_2$	256
3.	9,12-Octadecadienoic acid, methyl ester 	14.857	11.65	$C_{19}H_{34}O_2$	294
4.	Methyl stearate	15.010	3.23	$C_{19}H_{38}O_2$	298
5.	9,12-Octadecadienoic acid (Z,Z) 	15.123	20.96	$C_{18}H_{32}O_2$	280
6.	Octadecanoic acid 	15.230	11.75	$C_{18}H_{36}O_2$	284
7.	Geldanamycin, 18,21-didehydro-6,17-didemethoxy-18,21-dideoxo-18,21-dihydroxy-15-meth	15.350	1.11	$C_{30}H_{44}N_2O_8$	560
8.	Methyl 5,9-heptadecadienoate	15.405	0.99	$C_{18}H_{32}O_2$	280
9.	Dimethylaminoethyl palmitate	15.958	2.83	$C_{20}H_{41}NO_2$	327
10.	2-(Dimethylamino)ethyl vaccenoate 	16.937	18.23	$C_{22}H_{43}NO_2$	353
11.	1-Monopalmitin, 2TMS derivative	17.021	4.55	$C_{25}H_{54}O_4Si_2$	474
12.	Dimethylaminoethyl oleate	17.061	2.24	$C_{22}H_{43}NO_2$	353
13.	Glycyl-L-tryptophylglycine	17.107	2.30	$C_{15}H_{18}N_4O_4$	318
14.	Cyclopropane, 1,1-dichloro-2,2,3,3-tetramethyl- 	17.950	6.76	$C_7H_{12}Cl_2$	166
15.	2-Methyl-4-phenyl-1,2,4-triaza-spiro[4.5]decane-3-thione	18.925	1.71	$C_{14}H_{19}N_3S$	261
16.	.beta.-Sitosterol acetate	21.168	1.03	$C_{31}H_{52}O_2$	456
17.	Ethyl homovanillate, TMS derivative	22.337	1.29	$C_{14}H_{22}O_4Si$	282
18.	Silicic acid, diethyl bis(trimethylsilyl) ester	22.500	1.37	$C_{10}H_{28}O_4Si_3$	296
19.	Tris(tert-butyldimethylsilyloxy)arsane	23.524	1.19	$C_{18}H_{45}AsO_3Si_3$	468

CONCLUSION

Our investigation uncovered notable antibacterial properties among a total 19 bacterial strains associated with marine invertebrates collected from Pulau Pari, Kepulauan Seribu, Jakarta. One strain (MS.P.013.2) exhibited potent activity against *M. smegmatis* and identified as *M. luteus*. The chemical analysis of the extract MS.P.013.2 highlight major compounds, including fatty acids 9,12-Octadecadienoic acid, methyl ester; 9,12-Octadecadienoic acid (*Z,Z*); Octadecanoic acid; a nitrogen containing compound 2-(Dimethylamino)ethyl vaccenoate; and a cylco alkane Cyclopropane,1,1-dichloro-2,2,3,3-tetramethyl-, present in the extract. These compounds were previously reported to have antibacterial activity, thus suggesting that the isolate MS.P.013.2 might be a potential producer of promising antibiotic. However, none of those compounds have been reported for their antibacterial activity against *M. smegmatis*, underscores the significance of marine bacteria in

antibiotic discovery. This study contributes valuable insights into the bioactive potential of bacteria associated with marine invertebrates, that is crucial for combating bacterial infections. Further study to isolate and to test the bioactive compounds from the isolate MS.P.013.2 is needed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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