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Sponge-associated actinobacteria Kien Giang sea exhibiting antimicrobial activity against human pathogenic *Salmonella typhimurium*

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Abstract – The Kien Giang sea located on Thailand Bay is mostly unexplored where is anticipated to be able to provide a rich source of Actinobacteria, the prolific producers of antimicrobial secondary metabolites. Total 198 actinobacterial isolates were isolated from 63 sponge samples from 7 different sites in the Kien Giang Sea, Vietnam. Based on the ability of antimicrobial activity, 73/198 had against *Salmonella typhimurium* in which there were seven isolates with strong, 51 medium, and 15 weak resistances. Six best isolates (diameter of sterile ring >21 mm) were selected to identify by PCR 16S rDNA gene analysis and sequencing. The result showed that five strains characterized as *Streptomyces* spp., one strain belonged to genus *Rhodococcus* and *Rhodococcus rhodochrous* H2.6c strain was the highest antimicrobial activity to *Salmonella typhimurium.*

Keywords: antimicrobial activity, Kien Giang sea, *Salmonella typhimurium*, sponge, *Streptomyces*.

I. INTRODUCTION

According to the World Health Organization, plants can provide different drug varieties for low-income nations to cope with their primary health care needs [1]. *Salmonella* is a genus of the family Enterobacteriaceae with rod-shaped (bacillus) Gram-negative bacteria. The two species of *Salmonella* are *Salmonella enterica* and *S. bongori. S. enterica* is one species divided into six subspecies, including over 2,600 serotypes. *Salmonella* was named after Daniel Elmer Salmon (1850–1914), an American veterinarian. Salmonellosis is the most important foodborne illness worldwide [2]. The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) estimated that *Salmonella enterocolitis* occasioned 95.1 million cases and 50,771 diseases in 2017 [3]. *Salmonella Enteritidis* and *Typhimurium* are the agents associated with non-typhoidal *Salmonella enterica* outbreaks and usually cause self-limiting gastrointestinal infections in healthy adults [4]. However, children, the elderly, pregnant, and HIV-infected individuals form the high-risk groups for *Salmonella* systemic disease [3]. Salmonellosis during gestation constitutes the cause of fetal death in domestic livestock and fetal and maternal mortality in mice [5-8]. Multiple case reports demonstrate an association between *Salmonella* infections and stillbirth, preterm birth, chorioamnionitis, and miscarriage in humans [9-11].

In recent years, the importance of originated foods from fresh vegetables as the potential vehicles of enteropathogens, such as *Salmonella*, has been reported [12]. Some outbreaks of human *Salmonella* infection linked to fresh vegetables have been announced in developed countries [13].

Vegetables contaminated with many pathogens via direct or indirect contact with humans, rodents, reptiles, manure, and irrigation water ([13-15]. In Southeast Asian countries, including Vietnam, people usually have a habit of consuming raw vegetables sold in the wet markets.

Furthermore, the antibiotic resistance of *Salmonella* has become a severe problem in public health. A few reports regarding the resistance of *Salmonella* isolated from vegetables in Southeast Asian countries were published [16-18].

The plants are medicinally important due to the presence of biologically active secondary metabolites such as alkaloids, flavonoids, steroids, saponins, and terpenoids, which exert their effects by interacting with human physiology. However, medical plants change active secondary metabolites depending on plant age plants grow in good fertility soil, parts of the tree as flowers, branches, young tree?

Microorganisms have several mechanisms for survival depending on the respective environment: One of the most common mechanisms for inhibition or elimination of competition in the production of antimicrobial compounds, including antibacterial and antifungal. These compounds are often toxic to the encompassing community, providing a selective advantage for nutrients, carbon, and space [19-20]. We have been used the products from antimicrobial compounds, including antibacterials and antifungals in the mainland. However, one among the foremost diverse biomes on our planet has yet to be examined for antimicrobial production: the marine deep subsurface biosphere.

Marine biodiversity contains an array of secondary metabolites synthesized by marine microfauna and microflora that scientific research focused. However, there is a scarcity of research on the ecological importance of antimicrobial compounds within natural habitats. This study researched the marine microfauna and microflora near the shore and depth from 0.5 to 1.0 m.

The Kien Giang sea located on Thailand Bay is mostly unexplored. Therefore, this location can provide a rich source of Actinobacteria, the prolific producers of antimicrobial secondary metabolites. To our knowledge, no studies have reported the range and antimicrobial activities of Actinobacteria from this sea. Therefore, there is a high possibility to spot novel Actinobacteria and find out valuable antimicrobial secondary metabolites. This study aimed to isolate and identify the Actinobacteria and discover potential sources of antimicrobial secondary metabolites to human pathogenic bacteria, especially *Salmonella typhymurium*.

II. MATERIALS AND METHODS

Materials

The Sponge samples collected at Kien Giang sea that used to isolate actinobacteria. The *Samonella typhimurium* (ATCC 14028) used for testing the agent of antibacterial isolates..

Isolation of actinobacteria

Starch Casein Agar medium was used for the isolation of sponge-associated actinobacteria. It was supplemented with aginalxic (0.5 mg/L) and nystatin (0.5 mg/L) to inhibit fungi and Gram-negative bacteria. Sponge samples were rinsed with sterile natural seawater to remove the microbes loosely attached to the surface. Subsequently, a couple of tissue cubes were excised from different sections (including cortex and endosome) of the sponge samples. They were dug pieces and aseptically ground using sterilized pestles and mortars. Actinobacteria were isolated by means of serial dilution and plating techniques. The inoculated plates were incubated at 28 ºC for 3–6 weeks. The colonies bearing distinct morphological characteristics were picked up and transferred to freshly prepared media until pure cultures were obtained [21].

Screening assays for antibacterial activity

The liquid cultures were grown with shaking at 150 rpm for one day at 30°C. The broth was centrifuged at 5,000 rpm, 15 minutes. The supernatant was stored at 4°C. The bacterial test organisms were plated in the LB medium. The antimicrobial extract was added to the wells, the plates were incubated at 4°C for 2h for the diffusion of antimicrobial extract and observed for the zones of inhibition at 28°C for 48h.

The agar well diffusion method

The active isolates were cultured by the method given in the previous step. The supernatants were used for testing extracellular antimicrobial activity by the agar well diffusion method. By using a sterile cork borer, wells were punctured in the appropriate agar medium previously seeded with one of the test organisms. One hundred microliters of the culture supernatants were added to each well. The plates were then incubated at 4^{o}C for at least 2 h to allow the diffusion of crude extracts followed by incubation for 24 h at 37^{o}C for bacteria and 48 h at 28^{o}C for yeast. The diameters of inhibition zones were monitored and measured [22] and positive control was nystatin.

Screening of isolated microorganisms had for inhibitory activity. The isolates screened for antibacterial metabolite production using the agar well diffusion method that inocula were prepared by growing the varied test organisms on separate agar plates. The colonies from plates were transferred with inoculating loop into 3 mL of normal saline in a test tube. The density of these suspensions adjusted to 0.5 McFarland standards.

By means of a sterile cork borer wells (8 mm in diameter) were made in the agar and filled with 0.2 ml of 72 h culture of the isolated microorganism. Two replicates of the experiment were done and the plates were incubated at

37°C for 18 h. The diameters of the zone of growth-inhibition produced were measured and the mean values calculated.

Genomic DNA extraction

Bacterial cells from these cultures were collected by centrifugation, and genomic DNA was extracted [23].

16S rDNA Gene Amplification and Sequencing

The PCR was performed in a final volume of 25 µl which was composed of about 50ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 ρM of Actinomycetes specific primers S-C-Act-0235-a-S-20 (5'-CGCGGCCTATCAGCTTGTTG-3') and S-C-Act-0878-a-A-19 (5'-CCGTACTCCCCAGGCGGGG-3') [24] and 1U of Taq polymerase with the appropriate reaction buffer under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 50s, annealing at 52°C for 50s, and 72°C for 90s. The amplified products were separated by gel electrophoresis in 1.2% agarose gels which were stained with Safeview dye.

Sequence analysis

The 16S rRNA gene sequences compared with those from the type strains available in NCBI (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) [25].

For phylogenetic analysis, multiple sequence alignment performed using CLUSTALX, version 1.81. The Phylogenetic tree constructed using Mega 7.0. The consistency of the trees was verified by bootstrapping (1000 replicates) for the UPGMA method.

Statistical analysis

The experimental results analyzed the ANOVA with the isolates and levels of diameters of inhibition zones. All analyses conducted using the program MSTATC, Minitab 16. The data were considered significantly different at $P<0.01$. Duncan's test at $P = 0.01$ using to differentiate.

III. RESULTS AND DISCUSSION

Isolation of actinobacteria

From 63 sponge samples collected from 7 sites of Kien Giang sea: (1) Nui Den, Phao Dai ward, Ha Tien city; (2) Kien Vang island, (3) Re Lon island, (4) Re Nho island, Binh An village, Kien Luong district; (5) Ba Hon Dam island, (6) Heo island and (7) Nghe Island, Son Hai village, Kien Luong district isolated 198 actinobacterial isolates on SCA agar medium. Almost colonies have round-shaped, milky, white clear and yellow, entire or loabate margin, diameter size of these colonies varied from 0.2 to 3.0 mm (Figure 1). All of them have Gram-positive.

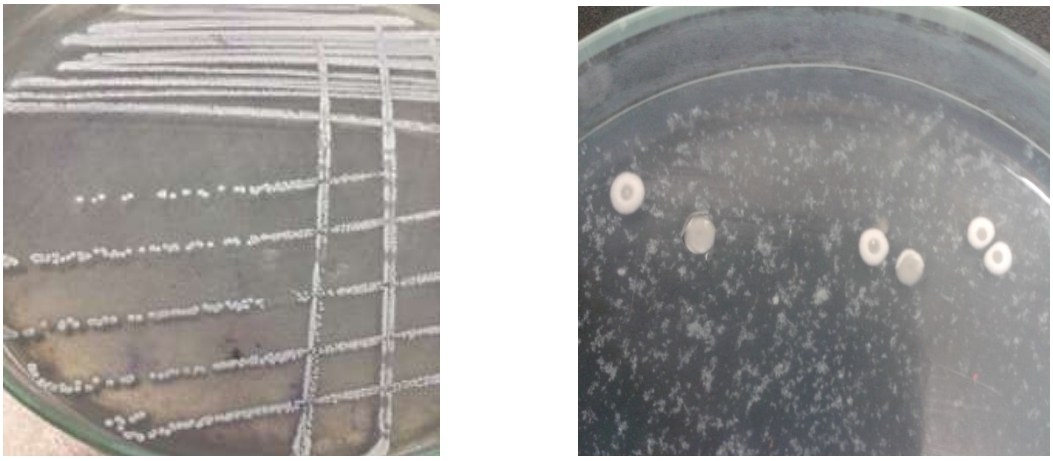

Figure 1. Shapes and size of colonies of actinobacterial isolates

Screening assays for antibacterial activity

Seventy-two of 198 tested isolates could produce antimicrobial active metabolites inhibiting at least one of the test pathogens. However there were 73/198 isolates were actively against *Salmonella typhimurium*, among 7/73 isolates had strong resistance [+++] (9.6%), 51/73 moderate resistance (66.9%) [++] and 15/73 resistance (20.5%) [+] [22] (Table 1, Figure 2, Figure 3).

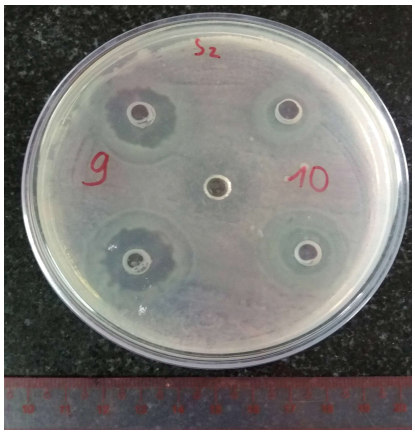

Figure 2. Sterile ring around colonies applied actinobacterial isolates against Salmonella typhimurium

Table 1. Antimicrobial activity of 73 actinobacterial isolates to *Salmonella typhymurium*

No	Bacterial isolates	Inhibition zone	Antibacterial Level [22]	No	Bacterial isolates	Inhibition zone	Antibacterial Level [22]
01	ND1.1b	20.0 e	+++	38	HD1.3e	15.0 h	++
02	ND1.3a	15.0 h	++	39	HD1.4b	05.0 s	+
03	ND1.3b	14.0 i	++	40	HD1.4d	14.0 i	++
04	ND1.4b	14.0 i	++	41	HD1.4e	14.0 i	++
05	ND1.5a	11.0 m	++	42	HD1.5a	07.0 q	++
06	ND1.5d	14.0 i	++	43	HD1.5c	22.0 c	+++
07	ND1.6b	07.0 q	++	44	HD1.5d	05.0 s	+
08	ND1.7a	12.0 l	++	45	HD1.6a	04.0 t	+
09	ND2.4	18.0 g	++	46	HD1.6c	05.0 s	+
10	ND2.5a	12.0 l	++	47	HD2.1a	19.0 f	++
11	ND2.5b	06.0 r	++	48	HD2.1f	11.0 m	++
12	ND2.6a	12.0 l	++	49	HD2.2a	21.0 d	+++
13	ND2.6b	11.0 m	++	50	HD2.2b	22.0 c	+++
14	ND2.6c	14.0 i	++	51	HD2.3a	06.0 r	++
15	ND2.6d	07.0 q	++	52	HD2.3b	15.0 h	++
16	ND2.7a	04.0 t	+	53	HD2.3c	09.0 o	++
17	ND2.7c	12.0 l	++	54	HD2.3d	23.0 b	+++
18	ND2.7d	07.0 q	++	55	HD2.4a	09.0 o	++
19	ND2.8a	06.0 r	++	56	HD2.5a	14.0 i	++
20	ND2.8b	02.0 u	+	57	HD2.5b	05.0 s	+
21	ND2.8c	07.0 q	++	58	HD2.5d	14.0 i	++
22	RL1a	07.0 q	++	59	HD2.6b	06.0 r	++
23	RL1b	04.0 t	+	60	HD2.6c	24.0 a	+++
24	RL1e	07.0 q	++	61	HD2.7a	14.0 i	++
25	RL2b	06.0 r	++	62	HD2.7d	13.0 k	++
26	RL2c	02.0 u	+	63	HD2.8a	04.0 t	+
27	RL3a	07.0 q	++	64	HD2.8b	09.0 o	++
28	RN1c	07.0 q	++	65	HD2.8p	10.0 n	++
29	RN4b	07.0 q	++	66	HD2.8r	04.0 t	+
30	RN5a	06.0 r	++	67	HD2.8s	04.0 t	+
31	RN5b	02.0 u	+	68	HD2.9a	12.0 l	++
32	RN5c	07.0 q	++	69	HD2.9c	22.0 c	+++
33	HD1.2a	04.0 t	+	70	HD2.9d	09.0 o	++
34	HD1.2b	12.0 l	++	71	H1a	08.0 p	++
35	HD1.2c	10.0 n	++	72	N3b	04.0 t	+
36	HD1.3b	12.0 l	++	73	N10f	07.0 q	++
37	HD1.3d	19.0 f	++		Positive Control (tetracycline)	08.0 p	
	CV (%) = 2.75%						

In Means within a column followed by the same letter/s are not significantly different at $p < 0.01$
Inhibition zone : diameter [$D = d_1 - d_2$] (mm) ;

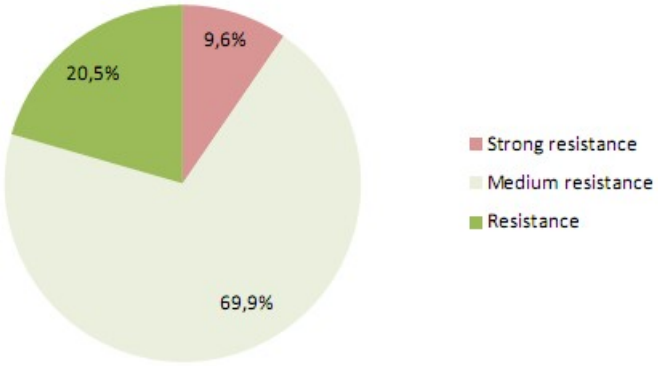

Figure 3. Microbial activity of 73 actinobacterial isolates to *Salmonella typhymurium*

Based on [22] evaluation, the isolates as HD2.6c, HD2.3d, HD1.5c, HD2.2b, HD2.9c, and HD2.2a were the best (+++) with a diameter of sterile ring >21 mm differed from the others statistically chosen to identify by PCR 16S-rDNA gene technique and sequencing. (Table 2).

Identify actinobacterial isolates

The result from Table 3 showed that 5/6 strains belonged to *Streptomyces,* and one strain was *Rhodococcus*.

Table 2. Phylogenetic affiliation of 6 actinobacterial isolates basis on 16S rDNA gene sequences by using BLAST program in the GenBank database based on sequence similarity

No	Actinobacterial isolates	Closest species relative	Similarity (%)
	Actinomycetaceae		
1	HD1.5c	*Streptomyces tateyamensis* strain 18I (MH919315)	99.83
		Streptomyces chumphonensis strain AM-4 (MG009024)	
2	HD2.2a	*Streptomyces qinglanensis* strain 172205 (MT568572)	99.01
		Streptomyces ramulosus strain NIOT_MBCT7 (MN175624)	99.01
3	HD2.2b	*Streptomyces flaveolus* strain ADIP1 (KF732809)	99.34
		Streptomyces ambofaciens strain M (MK929483)	99.34
4	HD2.3d	*Streptomyces ambofaciens* strain I (MK929479)	99.51
		Streptomyces coelicolor strain DSM 40233 (KY820720)	99.51
5	HD2.9c	*Streptomyces griseoaurantiacus* strain XY173 (MH432690)	99.50
		Streptomyces albidoflavus strain HQA017 (KT758349)	99.50
	Nocardiaceae		
6	HD2.6c	*Rhodococcus rhodochrous* strain ATCC BAA870 (CP032675)	99.67
		Rhodococcus pyridinivorans strain S5-TSA-30 (MN179918)	99.67

The UPGMA phylogenetic tree (Figure 3) of these isolates described in the two clusters, Cluster A had five strains, in which *Rhodococus rhodochrous* HD2.6c strain had a high relationship *Streptomyces griseoaurantiacus* HD2.9c. Both related to *Streptomyces flaveolus* HD2.2b; *Streptomyces tateyamensis* HD1.5c and *Streptomyces qinglanensis* HD2.2a, while cluster B only had 1 *Streptomyces ambofaciens* HD2.3d.

Even though sponge samples collected at seven various sites of Kien Giang sea with more than 180 actinobacterial isolates, the actinobacteria isolates had the high antimicrobial activity concentrated to Ba Hon Dam island, perhaps this site is pristine or wildly (nobody lives on this island) in comparison to other sites having people live crowdedly.

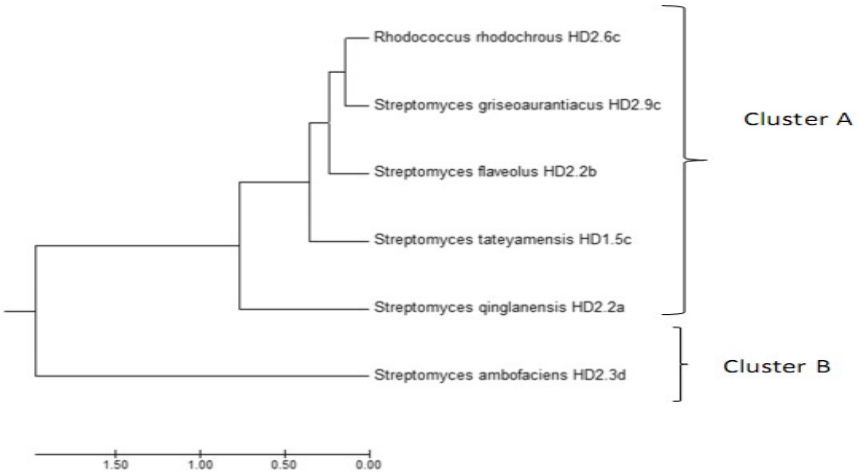

Figure 4. The UPGMA phylogenetic tree of partial 16S rRNA gene sequences of actinobacteria isolated from sponges of the Kien Giang Sea and closely related type strains. Numbers in the figure refer to percentage bootstrap values calculated for 1000 replicates. Bar, 0.02 was per nucleotide position.

In recent years, the importance of foods originating from fresh vegetables because the potential vehicles of enteropathogens like *Salmonella* reported [12]. Some outbreaks of human *Salmonell*a infection linked to fresh vegetables have been announced in developed countries [13].

Vegetables are easily contaminated with many pathogens via direct or indirect contact with humans, rodents, reptiles, manure, and irrigation water [13-15]. In Southeast Asian countries including Vietnam, people usually have a habit of consuming raw vegetables sold within the wet markets. Furthermore, the antibiotic resistance of *Salmonella* has become a severe problem in publicly health.

The emergence of communicable diseases and multidrug-resistant pathogens represents a worldwide threat and has increased many folds over a previous few decades. The infections are caused by antibiotic-resistant bacteria, which kill around 700,000 people anunually worldwide [26-27]. Natural products are contemplated as a linchpin of medical treatments. The chemical compounds produced in nature account for quite 65% of the entire number of approved drugs by the Food and Drug Administration (FDA) [28]. There are about 50,000 natural products discovered from microorganisms, and about 10,000 of them are of therapeutic importance. These products are in use as antitumor agents, antibiotics, enzyme inhibitors, agrochemicals, anticoagulants, cardioactive agents, anticancer agents, and anti-inflammatory agents [29]. The unexploited environments have more feasibility of isolating new species and perhaps explored for bioactive metabolites with unique chemical structures and activity [30]. Marine microbial communities are the richest and most diversified source of low relative molecular mass biologically active compounds. A good range of bio-prospecting techniques has been untilized in utilizing the bioactive potential of marine organisms [31]. Among the microorganisms, phylum actinobacteria represent an eminent and noteworthy source of therapeutically and commercially important products. Approximately 85% of the known antibiotics are produced by actinobacteria, predominantly by the genus *Streptomyces*, therefore, considered as most precious and economical prokaryotes [32].

Lee [33] isolated Actinobacteria at four sites of Tropical Mangrove Sediments in Malaysia, 87 isolates isolated and identified ten genera *Streptomyces, Mycobacterium, Leifsonia, Microbacterium, Sinomonas, Nocardia, Terrabacter, Streptacidiphilus, Micromonospora, Gordonia*, and *Nocardioides. And n*ine *Streptomyces* sp isolates were producing potent antimicrobial secondary metabolites, indicating that *Streptomyce*s isolates providing high quality metabolites for drug discovery purposes. The identification of *Streptomyces* because the most bioactive genus during this study is in line with other researchers, as *Streptomyces* can catabolize a good range of compounds and produce secondary metabolites with diverse biological activities and chemical structure [34]. *Streptomyces* is the largest genus of the Actinobacteria over two-thirds of all-natural antibiotics derived from this group of bacteria. *Streptomyces* features a huge biosynthetic potential that is still unchallenged among other microbial groups. That proven as some *Streptomyces* species whose biosynthetic repertoire was only three to five secondary metabolites. It possesses 20 genomic regions encoding known or predicted biosynthetic pathways [33, 35] recognized three isolates (MUSC 56, MUSC135, and MUSC164) exhibited a broad spectrum of antibacterial activity, with MUSC 135 being the strongest of inhibition effects against MRSA (inhibition zone of 12 mm), *Bacillus cereus* (4 mm), *Acinetobacter*

calcoaceticus (4 mm), and *Salmonella typhi* (4 mm). Our results showed that 5/6 strains were *Streptomyces* having high inhibitory activities to *Salmonella typhi.*

Sengupta [36] isolated 9/54 bioactive actinomycetes strains capable of producing antimicrobial-secondary metabolite from Sundarbans mangrove ecosystem, and therefore the best strain was determined *Streptomyces albogriseolus* NRRL B-1305T isolates showed antimicrobial activity against both bacterial and fungal test organisms among this strain against *Salmonella typhymurium* strongly (+++).

In recent years, Sholkamy [37] evaluated the antagonistic activity of the bioactive metabolites against various bacterial, fungal, and nematode pathogens from *Streptomyces* species. Among the three, the isolate SA4 exhibited significant antimicrobial and anti-nematicidal activity towards selected microbial pathogens. The antibacterial activity of compounds extracted from *S. cuspidosporus* SA4 reported effective against *S. aureus* (16.00 mm) *S. typhi* (20.00 mm) compared with streptomycin …., Gas Chromatography-Mass Spectrometry (GC-MS) analysis of strain SA4 bioactive extract publicized the existence of 1, 2- Benzenedicarboxylic acid, bis(2-Methylpropyl) ester compound.

Even though the structure of antimicrobial compounds has not been analyzed yet, the scientists studied these compounds, and they recognized that they had high effectiveness on *Salmonella typhymurium* and it is hoped these compounds that discovered to prosses high activity on *Salmonella typhymurium*, especially genus *Streptomyces.*

V. CONCLUSION

One hundred and ninety-eight isolates were isolated from 63 sponge samples at seven sites of Kien Giang Sea. There were 73/198 isolates having an antibacterial activity to *Salmonella typhymurium* among 7/73 strains had strong resistance (+++) (9.6%), 55/73 isolates had moderate resistance (++) (77.9%), and 15/73 isolates had resistance (+) (20.5%) during which *Rhodococcus rhodochrous* H2.6c strain had the highest antimicrobial activity.

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