ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 09, Issue, 03, pp.26384-26388, March, 2019

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

ANTIMICROBIAL ACTIVITY OF BACTERIA ASSOCIATED WITH SPONGE *Xestospongiatestudinaria* IN VIETNAM

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ARTICLE INFO

Article History:
Received 24th December, 2018
Received in revised form
28th January, 2019
Accepted 26th February, 2019
Published online 31st March, 2019

Key Words:
Antimicrobial activity, Cultivable bacteria, Marine sponge, Sponge-Associated bacteria, *Xestospongiatestudinaria*

ABSTRACT

Drug-resistant bacteria is spreading globally and threatening to human health. However, the number of novel antibiotics is decreasing gradually. Therefore, the strategies and studies for the discovery of novel antibiotics are essential to develop. Microorganisms associated with sponges are highly diverse and are one of the rich sources of bioactive compounds with different bioactive activity. In this study, we isolated 104 bacterial strains associated with the sponge *Xestospongiatestudinaria*, 20 of which exhibited antimicrobial activity against at least one of indicator microorganisms *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25923, *Bacillus subtilis* ATCC 27212, *Staphylococcus aureus* ATCC 12222, *Candida albicans* ATCC 7754. Identification of antimicrobial strains based on the 16S rRNA gene sequence showed the activity strains belonged to six different genera, including *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*, *Vibrio*, *Pseudovibri*. Our study revealed that sponge-associated bacteria from sponge are a potential source of antimicrobial compounds.

Citation: Nguyen Thị Kim Cuc, Tran Thi Hong, Tran Thi Kim Dung, Nguyen Phuong Hoa and Pham Viet Cuong 2019. "Antimicrobial activity of bacteria associated with sponge Xestospongiatestudinaria in Vietnam", *International Journal of Development Research*, 09, (03), 26384-26388.

INTRODUCTION

Marine sponges are the oldest sessile invertebrates living on the Earth (Maloof *et al*., 2010). The sponge body has structural complexities with different cell layers (Hentschel, *et al*., 2012) that provide a unique ecological niche for a wide range of different microbes (Li*et al*., 2011; Hentschel *et al*., 2006; Taylor *et al*., 2007). Among these, especially sponge-associated bacteria have become an important target for microbiological research, revealing new bacterial species and phyla with unprecedented metabolic properties. Until now, already more than 47 bacterial phyla have been detected in sponges (Reveillaud *et al*., 2014). Apart from the microbial diversity, the potential bioactive compounds isolated from the sponges and their associated microbes haveled to an additional focus on sponge-associated microbes (Alex and Antunes, 2015). Many novel compounds from sponge-associated microbes were reported in recent years (Indranagat *et al*., 2016). These include, for example, antiviral compounds such as vidarabine (Sagar *et al*., 2010); anticancer compounds such as discodermolide, halichondrin B and bryostatin 1 (Dunlap *et al*., 2007); antimicrobial compounds such as tirandamycins, thiopeptide TP-1161, reveromycins, phenazines, aplysinamisin, aerophobin, isofistularin-3 (Engelhardt *et al*., 2010; Fremlin*et al*., 2011, Sacristán-Soriano *et al*., 2011; Schneemann *et al*., 2011; Micheal& Peter, 2015); and antimalarial compounds such as depsipeptides (Fotie & Morgan, 2008), swinholide A (Andrianasolo *et al*., 2005), onnamide A (Piel *et al*., 2004), psymberin (Fischbach and Walsh, 2006), amycofuran, amycocyclopiazonic acid, amycolactam (Kwon *et al*., 2014), similanpyrone C, similanamide and pyripyropene T (Chadaporn *et al*., 2015), protease inhibitors (Hong *et al*., 2018a, b, c). The increase in antimicrobial resistance is an important public health threat. Therefore, research and discovery are needed in order to find novel antibiotics. The previous studies reveal that Vietnam has a high diversity of marine sponge and their associated microbes (Cuc *et al*., 2017; Dat *et al*., 2018a, b, c). Different biological activities from bacteria associated with Vietnamese sponge are also reported such as antimicrobial, protease inhibitory activity (Hong *et al*., 2018 a, b, c; Dat *et al*., 2018d). In this study, we isolated and screened antimicrobial bacteria from the sponge *Xestospongiatestudinaria* in Vietnam.

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MATERIALS AND METHODS

Collection of sponge sample: The sponge samle was collected by SCUBA diving in the depth of 15 - 20m in the marine region of VinhMoc, Quang Tri (107°07'01.4"E; 17°05'08.6"N). Samples were stored in containers with sea water and kept at -20°C for isolation of bacteria. The sponge was identified based on analysis of the 18S rRNA and COI genes.

Isolation of bacteria from the sponge samle: The sponge specimen of ~1 cm^3 was rinsed in sterile sea water and then homogenized thoroughly in 10 volumes of sterile sea water in a sterile mortar. The supernatant was 10-fold serially diluted till 10^{-6} and subsequently plated onto six different media: OLIGO (0.05 % yeast extract, 0.05%tryptone, 0.01% sodium glycerolphosphate, 1.5 % agar), M1 (1 % starch, 0.4 %, 0.2 %peptone, 1.5 % agar), SCA (0.5 % starch, 0.002 % casein, 0.1 % KNO_3,0.1 % NaCl, 0.1 % K_2HPO_4,0.5 mL/L $MgSO_4$100mM, 0.5mL/L $FeSO_4$100mM, 0.5 mL/L $CaCO_3$100mM, 1.5 % agar), AIA (0.01 % peptone, 0.001% L-asparagine,0.4 % sodium propionate, 0.005% K_2HPO_4, 0.001 % MgSO4, 0.001 g/L $FeSO_4$, 1 mL/L glycerol, 1.5 % agar), R2A (0.5 g/L yeast extract, 0.5 g/L glucose, 0.5 g/L peptone, 0.5 g/L casein hydrolysate, 0.5 g/L starch, 0.3 g/L sodium pyruvate, 0.3 g/L K_2HPO_4, 0.05 g/L $MgSO_4$, 1.5 % agar), MA (0.5 % peptone, 0.1 % yeast extract, 1.5 % agar). All culture media were prepared with natural sea water at pH 7 and all plates, produced in triplicate, were incubated for 3 - 5 d at 30°C. Representative microbial isolates with different colony morphotypes and microscopic appearance were selected, pure cultured, and stored with 20% glycerin (v/v) at -80°C.

Screening for antimicrobial producing bacteria: Testing for potential antimicrobial activities of isolates was performed against five typical pathogenicorganisms: *Escherichia coli* ATCC25922, *Pseudomonasaeruginosa* ATCC 25923, *Bacillussubtilis* ATCC 27212, *Staphylococcus aureus* ATCC 12222, *Candida albicans* ATCC 7754.The pure culture of isolates was incubated in 10 mL liquid marine agar (1 g of yeast extract/L, 5 g of tryptone/L, 0.01 g of $FePO_4$/L, and 1 000 mL natural seawater) at 30°C for 24 h. After reaching the log phase, a 1 mL portion of each culture was transferred to an Eppendorf tube and centrifuged at 10 000 rpm for 10 min. The supernatant of all isolated strains was then used to saturate sterilized paper discs (Whatman, 6 mm), which were then placed on the surfaces of agar plates presaturated with the indicator microorganisms. The dishes were then incubated under a temperature of 30°C for 48 h. Antimicrobial activity of isolates was determined based on the formation of inhibition zones around the discs.

Identification of the isolates by 16S rRNA analysis: The most potential antimicrobial isolates were identified using 16S rRNA gene sequencing. The genomic DNA of strains was isolated according to Rainey *et al*. (1996) and the 16S rRNA gene was amplified with universal primers: 27f (5′-AGAGTTTGATCCTGGCT CAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane *et al*., 1985). The PCR cycling parameters: an initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 50 seconds, amplification at 72°C for 1.5 minutes and a final extension at 72°C for 7 minutes. The 16S rRNA gene sequencing was carried by DNA Analyzer (ABI PRISM 3100, Applied Bioscience).

The 16S rRNA sequences of isolates were compared to available sequences in the Gen Bank database using the Blast search programme (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Isolation of bacteria from the sponge *X. testudinaria:* A total of 104 strains with distinct colony characteristics were isolated from the sponge *X. testudinaria*. The number of strains isolated from different media was different. The highest number of strains were isolated from medium M1 (29), followed by media such MA (26), R2A (18), SCA (17), AIA (9) and OLIGO (5). It was observed that nutrient-poor media (e.g., M1, MA, R2A, SCA) resulted in the isolation of a highernumber of strains compared to nutrient-poor mediasuchas AIA and OLIGO.

Antimicrobial activity of cultivable bacteria: Antimicrobial assay showed that 20 out of 104 bacterial strains isolated from the sponge *X. testudinaria* displayed antimicrobial activity against at least one indicator microorganisms with inhibition diameters from 9 to 21 mm (Table 1). Eight isolates showed antimicrobial activity against one indicator microorganisms, 10 isolates showed antimicrobial activity against 2 indicator microorganisms and one isolate showed antimicrobial activity against 3 indicator microorganisms. There is no any isolate that showed antimicrobial activity against 4 or 5 indicator microorganisms. Among five indicator microorganisms, *P. aeruginosa* ATCC 25923 was inhibited least by isolated strains (4 isolates), followed by *S. aureus* (6 isolates), *E. coli* ATCC 25922 (7 isolates), *C. albicans* ATCC 7754 (7 isolates), and *B. subtilis* ATCC 27212 (8 isolates). These results showed that bacteria associated with the sponge *X. testudinaria* as a source of antimicrobial compounds.

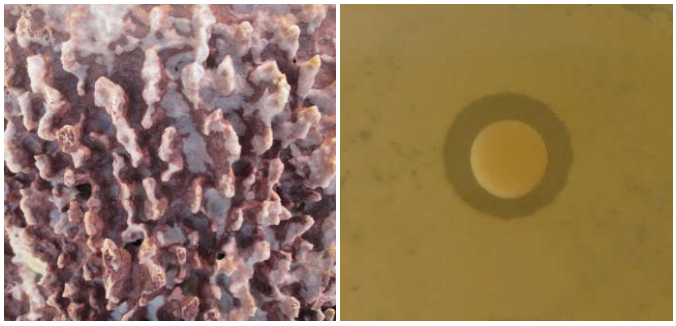

Figure 1.The surface of the sponge *X. testudinaria* (left) and inhibition zone of antimicrobial isolate (right)

The previous studies show that cultivable bacteria from sponge are capable of inhibiting different pathogenic microorganisms. Manikandan *et al.* (2014) isolated 10 bacterial strains from sponge in the Gulf of Mannar in which four strains exhibited antimicrobial activity. The strain *Stenotrophomonas* sp. SMAKK001 exhibited strong activity against *S.aureus* and exhibited moderate activity against *S.mutans*, *E. coli*, *V. cholerae*, *C. albicans* and *A. niger*. The strain *B. subtilis* SMAKK002 exhibited strong activity against *S. aureus* and moderate activity against *S. mutans* and *V. cholerae* whereas it exhibited weak activity against *P. aeruginosa*, *C. albicans* and *A. niger*. The strains *Bacillus* sp.SMAKK003 exhibited moderate activity against *S. mutans*, *S. aureus*, *V. cholera*, *C. albicans*, and weak activity against *A. niger*. Especially, the strain SMAKK001 exhibited moderate activity against all the multiple drug resistant (MDR) strains tested.

Table 1. Antimicrobial activity of isolated bacteria from the sponge *X. testudinaria* (D = mm)

No	Isolate	*E. coli* ATCC25922	*P. aeruginosa* ATCC 25923	*S. aureus* ATCC 12222	*B. subtilis* ATCC 27212	*C. albicans* ATCC 7754
1	XT01	-	-	9	12	-
2	XT03	-	20	20	-	-
3	XT06	20	-	-	-	10
4	XT10	20	-	-	15	-
5	XT13	-	14	-	-	15
6	XT17	-	-	-	10	-
7	XT19	15	-	17	-	-
8	XT25	-	-	-	10	-
9	XT28	10	11	-	-	18
10	XT32	-	-	19	-	-
11	XT34	10	-	-		21
12	XT35	-	-	-	15	-
13	XT39	20	-	-	-	17
14	XT41	-	-	18	-	-
15	XT43	-	-	-	15	-
16	XT47	-	22	-	-	14
17	XT50	-	-	-	10	-
18	XT52	13	-	-	-	13
19	XT55	-	-	10	-	-
20	XT59	-	-	-	20	-

Table 2. Identification of antimicrobial bacteria from the sponge *X. testudinaria*

No	Strains	Closest strain in GenBank (Accession number)	% identity
1	XT01	*Bacillus subtilis* JCM 1465 (AB598736)	100
2	XT03	*Paenibacillus terrae* AM141 (AF391124)	99.5
3	XT06	*Pseudomonas* sp. CK57 (EU686687)	99.7
4	XT10	*Paenibacillusamylolyticus* JCM 9906 (D85396)	98.8
5	XT13	*Bacillus megaterium* NBRC 15308 (AB271751)	99.4
6	XT17	*Streptomyces* sp. CHR3 (AF026080)	99.5
7	XT19	*Bacillus licheniformis*BCRC 11702 (NR_116023)	100
8	XT25	*Bacillus subtilis BCRC* 10255 (NR_116017)	100
9	XT28	*Bacillus subtilis*SBMP4 (NR_118383)	100
10	XT32	*Bacillus amyloliquefaciens*BCRC 11601 (NR_116022)	99.3
11	XT34	*Pseudomonas fluvialis*ASS-1 (NR_159318)	100
12	XT35	*Vibrio panuliri*LBS2 (NR_136876)	100
13	XT39	*Pseudovibriostylochi*UST20140214-052 (NR_149237)	99.6
14	XT41	*Streptomyces ascomycinicus*DSM 40822 (NR_116222)	100
15	XT43	*Vibrio maritimus*R-40493 (NR_117551)	99.6
16	XT47	*Pseudovibrioaxinellae*Ad2 (NR_118255)	99.1
17	XT50	*Streptomyces glebosus*NRRL B-3248 (NR_116221)	99.3
18	XT52	*Streptomyces* sp. A-29 (EU430264)	100
19	XT55	*Streptomyces* sp. EF-73 (AF076309)	100
20	XT59	*Pseudovibrio japonicas* WSF2 (NR_041391)	99.2

The strain SMAKK002 exhibited moderate activity against MDR *Pseudomonas* sp., MDR *Staphylococcus* sp., and exhibited weak activity against MDR *Klebsiella* sp. The filtrate of strain SMAKK003 exhibited moderate activity against only MDR *Staphylococcus* sp. and exhibited weak activity against the MDR *Pseudomonas* sp. and MDR *Klebsiella*s sp.. Cita *et al*. (2017) isolated 15 bacterial strains from the sponge *X. testudinaria*in Tanjung Kasuari, Sorong, Papua. Six out of 15 isolated strains exhibited antimicrobial activity against at least one of indicator microorganisms *E. coli*, *B. subtilis*, *K. pneumonia*. In another study, Matobole *et al*. (2017) isolated 415 bacterial strains from the sponges *Isodictyacompressa* and *Higginsiabidentifera*, thirty-five isolates of which showed antibacterial activity against at least one of indicator bacteria *M.smegmatis* LR222, *B. cereus* ATCC10702, *S.epidermidis* ATCC14990, *P.putida* ATCC27853and the multidrug resistant *E. coli* 1699. Interestingly, twelve strains exhibited activity against the multi-drug resistant *E. coli* 1699. Similarity, Dat *et al.* (2018d) isolated 96 bacterial strains from sponge in Da Nang, Vietnam, 31 of which exhibited antimicrobial activity against at least one of five indicator microorganisms *E. coli* ATCC 25922, *P. aeruginosa* ATCC 25923, *B. subtilis* ATCC 27212, *S. aureus* ATCC 12222, *C. albicans* ATCC 7754. In addition, the compound Macrolactin A isolated from the strain with highest activity (V08DN1) exhibited antimicrobial activity against *E. coli*, *S. aureus*, *B. subtilis*, and *P. aeruginosa* with MIC values of 32, 8, 64, 8 µg/mL, respectively.

Identification of antimicrobial isolates: Identification of the antimicrobial strains based on the 16S rRNA gene sequence (Table 2) showed that activity isolates belonged to six different genera, including *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*, *Vibrio*, *Pseudovibrio*. The identities ranged from 98.8% to 100% identity compared to sequences from GenBank. The antimicrobial isolates were predominant by genus *Bacillus* (6 strains), followed by *Streptomyces* (5 strains), *Pseudovibrio* (3 strains), *Paenibacillus* (2 strains), *Pseudomonas* (2 strains), and *Vibrio* (2 strains). As expected, the genera are well-known for their antimicrobial activities (Indranagat et al., 2016; Matobole et al., 2017; Hoai et al., 2018). In a recent review paper, Indranagat et al (2016) revised antimicrobial microbes associated with sponge and showed that at least 35 bacterial and 12 fungal genera were capable of producing antimicrobials, of which *Streptomyces*, *Pseudovibrio*, *Bacillus*, *Aspergillus* and *Penicillium* were the

prominent producers of antimicrobial compounds. In the line previous studies, our study revealed that sponge-associated bacteria from sponge are a potential source of antimicrobial compounds.

Conclusion

In this study, we isolated 104 bacterial strains associated with the sponge *X. testudinaria*, 20 of which exhibited antimicrobial activity against at least one of indicator microorganisms *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC 25923, *Bacillus subtilis* ATCC 27212, *Staphylococcus aureus* ATCC 12222, *Candida albicans* ATCC 7754. Identification of antimicrobial strains based on the 16S rRNA gene sequence showed the activity strains belonged to six different genera, including *Bacillus, Paenibacillus*, *Pseudomonas*, *Streptomyces*, *Vibrio*, *Pseudovibrio*. Our study revealed that sponge-associated bacteria from sponge are a potential source of antimicrobial compounds.

Acknowledgement

This work was financially supported by Vietnamese Ministry of Science and Technology under grant number: ĐTĐLCN.17/14.

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