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The diversity and antimicrobial activity of endophyte fungi isolated from the sponge at NGHE Island, Ha Tien Sea, Kien Giang Province, Vietnam

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ABSTRACT

The diversity and biological activities of fungi associated with the sponges, were investigated by using a culture-dependent method followed by an analysis of the fungal rDNA-ITS sequences. A total of 79 fungal isolates were isolated from 36 sponge samples at NGHE island in Ha Tien Sea, Kien Giang province of the Mekong Delta of Vietnam, Colonies grew on agar medium during 24 h and mycelia developed in the center of colonies with the growth of brown spores. There were 63/79 isolates had the abilities of antibacterial activity when using agar surface diffusion method to investigate the antibacterial activity with 4 reference bacterial strains including *Bacillus cereus; Escherichia coli; Salmonella typhimunum* and *Staphylococcus aureus*; however, there were 14 isolates that made halos (sterile zones) with diameter >2 mm and against 2 – 3 reference bacteria were chosen to be identied based on the nucleotide sequence of the internal transcribed spacer (ITS) rDNA. The sequences from the selected fungal isolates showed high degrees of similarity to those of the GenBank reference strains (99% - 100%), all of them were identified as various species of *Aspergillus.* Five bioactive compounds were identified in the organic solvent ethyl-acetate. The identification of bioactive chemical compounds are based on the peak area, retention time, molecular weight and molecular formular. GC-MS analysis of *Aspergillus fumigatus* HN11a strain revealed the existence of the Phthalic acid, di-(1-hexen-5-yl) ester, 3-Nitropropionic acid, 2,6-Dihydroxybenzoic acid 3TMS, and Propanamide, Methylpent-4-enylamine.

Key words : *18S-rDNA-ITS, Antibacterial ability, Aspergillus, Fungal diversity, Marine sponge*

Introduction

Sponges (Phylum: Porifera) are evolutionarily ancient metazoans that have existed for 700–800 million years. They not only populate in tropical oceans with great abundance but also occur in temperate waters and even in freshwater (Hentschel *et al*., 2002; Radjasa *et al.,* 2007). Marine sponges are widely distributed from intertidal zones to thousands of meters deep in the ocean (Fusetani and Matsunaga, 1993). The sponge was known as the largest producer of bioactive compounds among other marine invertebrates, so that it has great potential to become a lead compound to be developed. Taylor has reported in the last decade as many as 50 percent of the bioactive compounds found in marine invertebrates derived from sponges. Antibacterial, antifungal, antitumor and antiviral are some potentials that have been discovered and developed from sponges (Taylor *et al.,* 2007). The sponge is also known as a very fertile host for a variety of microorganisms symbionts. Microbial symbionts can be either bacteria or fungi which contribute over 40-60% of the animal biomass (Handayani and Rustini, 2016).

Natural products from fungi are considered an important source for novel antibacterial and antifungal compounds due to their abundannce in fungal species diversity, their rich in secondary metabolites and their improvements in the genetic breeding and fermentation processes. The antimicrobial activities of an increasing number of fungi living in distinctive environments are being investigated for the discovery of new antibacterial and antifungal compounds, for example the endophytic fungi from wild plants and marine fungi. In the last decade, many novel bioactive natural products extracted from marine fungi have been found to have the cytotoxic, anticancer, antiviral, antibacterial or antifungal activities (Rateb and Ebel, 2011; Singh, *et al.,* 2015; Cheung *et al*., 2014; Wang *et al*., 2013; Mayer *et al*., 2013; Thomas *et al*., 2010).

The antibacterial and antifungal compounds produced from marine fungi have been quickly increased since 2010, and the marine fungi have been an important source of antibacterial and antifungal compounds (Xu *et al*., 2015).

The discovery of penicillin, a natural product from *Penicillium notatum* in 1929 marked the beginning of age of bioactive compound exploitation for the treatment of a variety of human health problems (Wistreich and Lechtman, 1976). Polyketides (PKs) are a group of natural secondary metabolite compounds produced by microorganisms and plants. PKs have been regarded as one of the richest ''drug gold mine'' groups (Borchardt, 1999) since many of them are used as the therapeutic drugs. These include anti-bacterial (tetracycline, griseofulvin) and anti-tumor agents (enediyne), immunosuppressants (rapamycin), and cholesterol-lowering agents (lovastatin, compactin). PKs are extremely diverse in both structure and biological activity.

Polyketide synthases (PKSs) are major enzymes in plants, fungi, and bacteria that are responsible for polyketide biosynthesis (Hertweck, 2009). Fungal PKSs are generally large multidomain systems that comprise a minimal set of ketosynthase, acyl-transferase and acyl carrier protein domains. Type I PKSs elongate their polyketide products iteratively. In addition, ketoreductase, dehydratase and enoyl reductase domains may catalyse optional β-keto processing reactions and optional accessory domains provide cyclase activity and methyl transferase activity (Cox, 2007). Type I fungal PKSs are grouped into three classes based on their optional processing domains: non-reducing (e.g., melanin, aatoxin), partially reducing (e.g., msas, calicheamicin), and highly reducing PKSs (e.g., T-toxin, fumonisin) (Kim and Knudsen, 2011; Chiang *et al.,* 2010).

Fungal type I polyketide (PK) compounds are highly valuable for medical treatment and are extremely diverse in structure, partly because of the enzymatic activities of reducing domains in polyketide synthases (PKSs).

Most of the 105 marine fungi having antibacterial or antifungal compounds were identified, and approximately 50% of them were identified based on their DNA sequence. The dominant genera in the marine fungi producing antibacterial and antimicrobial compounds were the *Aspergillus* genus (31 strains) and the *Penicillium* genus (16 strains) (Xu *et al.,* 2015).

Polyketides and nonribosomal peptides have been immensely concerned over the past decades, and numbers of various novel polyketide and nonribosomal peptide compounds have been found from marine-derived microbes, most of which showed different biological activities and ecological functions (Gao *et al.,* 2008). The polyketides are a large and structurally diverse group of natural products that have found widespread used in human and animal medicine (antibacterials, e.g. erythromycin and tetracycline; immunosupressors, e.g. rapamycin; antitumorals, e.g. daunorubicin and doxorubicin; anticholesteremic, e.g. statins; antiparasitics, e.g. avermictin) and also used in agriculture and the chemical industries, some of them had a high commercial value (Cox, 2007; Hertweck, 2009). Polyketides have been isolated from bacteria, fungi and plants, yet relatively few polyketides have been described from endophytic fungi. The detection of polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) genes in sponge-associated fungi could suggest their potential roles in the chemical defense process of sponge host.

In the past few years, Gas chromatography Mass spectrometry (GC-MS) have been used as one of the technological platform for finger print analysis of secondary metabolites in both plant and non-plant species (Kell *et al*., 2005), and in bacteria as *Pseudomonas aeruginosa* (Altaee *et al*., 2016).

This research was studied to investigate the cultutivable diversity of fungi from sponges of the Ha Tien Sea, Vietnam and to analyse the genes coding of PKS-I, PKS-II, and NRPS fungi as well as to explore the potential use of these newly sponge-associated fungi as a novel source of bioactives and

elucidation of secondary metabolites from the cultures broth of fungi analyzed by GC-MS technique.

Materials and Methods

Sponge-Associated Fungal Isolation

In this study, marine sponges were collected from NGHE islands in Ha Tien Sea in April, 2016, and sponge samples were taken at a depth of 0.5 – 1.0 m by hand. Samples were then kept in sterile polythene bags and stored in plastic box with ice and salt to keep at temperature (- 5°C).

Samples were transferred to the laboratory at Can Tho University within day, samples were then stored in – 20 °C freezer until use. Under sterile conditions, 5 g sponge tissue was excised, was washed in 70% ethanol and immediately immersed in sterile seawater, filled seawater and ground using a sterile mortar. The homogenate was diluted in sterile natural seawater (10^{-1} to 10^{-3}), 100 µl of each dilution was plated in quadruplicate onto Glucose Peptone Yeast Extract Agar (GPY) (1.0 g glucose, 0.1 g yeast extract, 0.5 g peptone, 15 g agar and per liter seawater, pH 8.0), Gause 1 (starch 20 g, KNO_3 1.0 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4$ 0.01 g, 15 g agar, pH 7.2-7.4) and Luria-Bertani (LB) agar plates (Li and Wang, 2009) containing the antibiotics as ampicillin and streptomycin (100 mg/ml for each one). The plates were incubated at room temperature for 1-3 weeks until the morphology of fungi could be distinguished.

Screening assays for antibacterial activity

The search for bioactivity was performed with isolated fungal isolates from sponges with the method of agar blocks (Nedialkova and Naidenova, 2005) with pathogenic bacteria namely *Escherichia coli* (E): ATCC 25922, *Bacillus cereus* (B): ATCC 11778, *Salmonella typhimurium* (S): ATCC 14028, *Staphylococus aureus* (St): TCC 25923 were obtained from the National Agro Forestry Fisheries Quality Assurance Department - Branch 6, 386C Cach Mang Thang Tam street, Bui Huu Nghia ward, Binh Thuy district, Can Tho city, Vietnam).

Molecular Analysis of Fungi

Fungal genomic DNA extraction was conducted using the method of Skory *et al.* (1992) from the pure culture in GPY broth at 30 °C for a week. The rDNA-ITS fragments were amplified using the primers ITS1 and ITS4 (White *et al.*, 1990). PCR amplication was performed in a thermo cycler C1000 (Bio-RAD) with an initial denaturation at 94 °C for 10 min followed by 30 cycles with the temperature profile, 94 °C for 50 s, 55 °C for 50 s, and 72 °C for 1 min, and a final extention of 10 min at 72 °C. In the best isolate(s) (high ability of antibacterial activity), 18 isolates of 4 sites were chosen to sequence and the results were compared to sequences of GenBank based on partial 18S rDNA ITS sequences to show relationships between fungal endophytic strains (Tamura *et al.*, 2011) and phylogenetic tree were constructed by the neighbor-joining method using the MEGA software version 7.0 based on 1000 bootstraps.

Screening of PKS-I, PKS-II, NRPS genes

Screening of PKS-I, PKS-II, NRPS genes was carried out using specific primers mentioned in Table 1. The PCR condition was set to follow the condition in the barcoding stage.

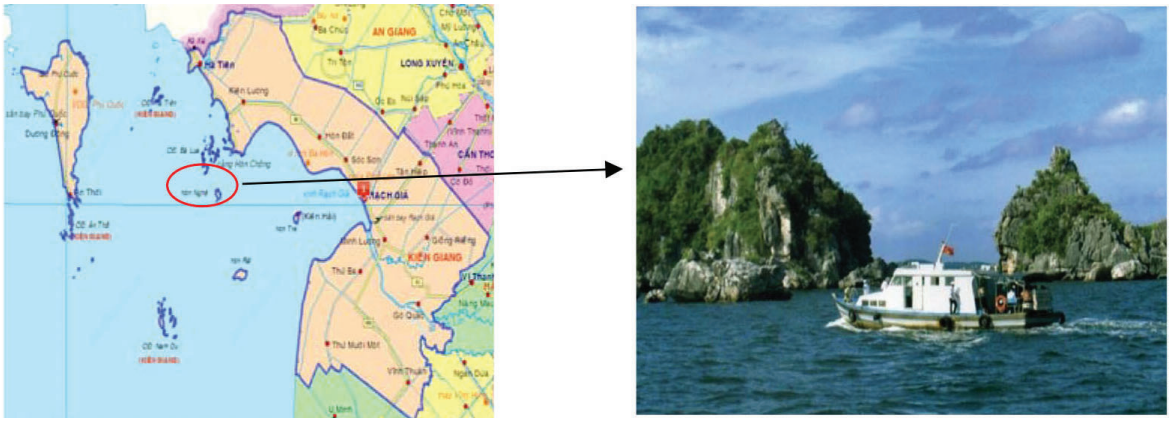


Fig. 1. Sponge samples were collected at NGHE island in Ha Tien Sea

Table 1. The primers were used in this study

Primer's name	Oligonucleotides	Target	References
ITS 1	5′-TCC GTA GGT GAA CCT GCG G-3′	Fungal barcoding	Sibero *et al.* (2018)
ITS 4	5′-TCC TCC GCT TAT TGA TAT GC-3′		
A2gam Forward	5′-AAG GCN GGC GSB GCS TAY STG CC-3′	NRPS gene detection	Radjasa *et al.* (2005)
A3gamReverse	5′-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3′		
KAF1	5′-GAR KSI CAY GGI ACI GGI AC-3′	PKS-I gene detection	Amnuaykan-janasin *et al.* (2005)
KAR1	5′-CCA YTG IGC ICC RTG ICC IGA RAA-3′		
IIPF6	5′-TSG CST GCT TCG AYG CSA TC-3′	PKS-II gene detection	Sun *et al.*, (2012)
IIPR6	5′-TGG AAN CCG CCG AAB CCG CT-3′		

Fungi were cultured in 250 ml flasks at 30°C for 24 hours with shaking at 150 rpm. Fermentation was carried out in 100 l fermenter with 50 l medium 2216 and 10% bacterial inoculum at 30°C for 52 hours. The neutral pH was maintained automatically by NaOH or HCl 1N. The obtained culture broth (50 l) was extracted with ethyl acetate (25 l × 3 times). The combined organic solutions were then decanted, filtered and concentrated under reduced pressure to yield 5.2 g of crude extract which was chromatographed on a silica gel column using a gradient of 1 - 100% acetone in hexane to afford three fractions F1-3, after that it was continuously chromatographed on a silica gel column using a gradient of 1 - 100% acetone in methanol to afford three fractions F4-6. Therefore, six fractions were received from 2 kinds of organic solvent (hexane – acetone and acetone – methanol).

GC/MS analysis

The samples were analysed by the GC/MS belonging to the Environment Laboratoty, Department of Environment, College of Environment and Natural Resources, Can Tho University. The GC-MS analysis of samples was carried out using Shimadzu Thermo with TG-SQC column; (15m x 0.25mm x 0.25µm). Helium was used as the carrier gas and the temperature programming was set as follows:

	Speed (°C/min)	Temperature (°C)	Keep (min)
Initial		50	1.00
Ramp 1	2.00	70	2.00
Ramp 2	10.00	150	2.00
Ramp 3	10.00	250	10.00
Total time		43 minutes	

10 µL sample was injected with split less mode. Mass spectra were recorded over 35-400 amu range with electron impact ionization energy of 70 eV, total running time for one sample was 43 min. Quantitative determination was made by relating respective peak areas to TIC areas from GC-MS.

Results and Discussion

The sponge samples collected from NGHE island (Ha Tien Sea) were *Leucosolenia* sp. (Class Calcinea) and *Hexactinosa* sp. (Class Hexactiella).

From 36 sponge samples, 79 fungal isolates were isolated from three kinds of medium (GPY, Gauss and LB). Almost their colonies have round-shaped; diameter size of these colonies varied from 0.2 to 3.0 mm (Fig. 2).

As the observation under microscope, mycelia had septum and conidiophore with diameter about

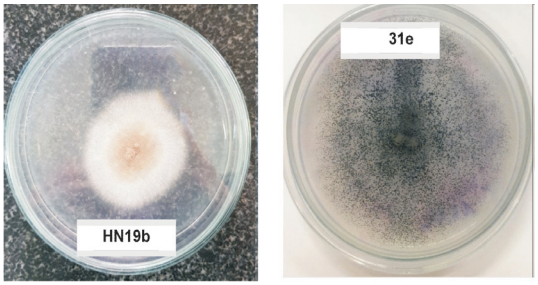


Fig. 2. Colonies of two fungal isolates developed on agar medium

40 mm and many spores had brown color (Fig. 3).

After 48 h incubation, there were 58/79 fungal isolates had halos [produced sterile zones] (antimicrobial activity); however, 14/61 isolates had big

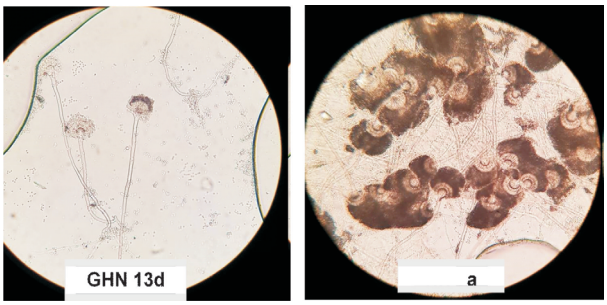


Fig. 3. Mycelium and spogiangium of 2 fungal isolates

halos [showed higher inhibition zones] (>2 mm) against 3 pathogenic bacterial strains as *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* (Fig. 5 and Table 3).

Fourteen fungal isolates were chosen to identify and all the rDNA-ITS sequences of the fungi from the sponges had an affiliation (Table 3) with the strains that belonged to Ascomycota, order *Eurotiales* and genus *Aspergillus*.

An UPGMA phylogenetic tree (Fig. 4) of these isolates described the two clusters. Cluster A had 13 strains including 2 smaller clusters as cluster A1 with 9 strains (*Aspergillus tubingensis* HN19c, *A. fumigatus* HN19a, *A. tereus* HN18b and A. HN11a had the close relationships) together with *A.clavatonanicus* HN13c, *A. fumigatus* HN31e and *A. fumigatus* GHN23b) and *A. nomius* GHN11a and *A. fumigatus* HN37c while cluster A2 with 4 strains: *Aspergillus niger* HN31b, *Aspergillus* sp. HN22b, *A. tereus* HN20c and *A. tereus* HN7b. Cluster B only had 1 strain (*Aspergillus melinus* HN28c).

The presence of the biological gene cluster (BGC) such as NRPS, PKS-I and PKS-II was detected in this study (Fig. 5). The presence of biosynthetic gene clusters (BGC) was screened to understand the ability of the prospective fungi in producing bioactive polyketides and non-ribosomal peptides. Some studies have demonstrated that the presence of the biosynthetic gene cluster (BGC) such as polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) genes in microorganisms have been correlated to their capability to inhibit the growth of other microorganisms (Passari *et al.*, 2016; Bo¨hringer *et al.*, 2017; Samak *et al.*, 2018).

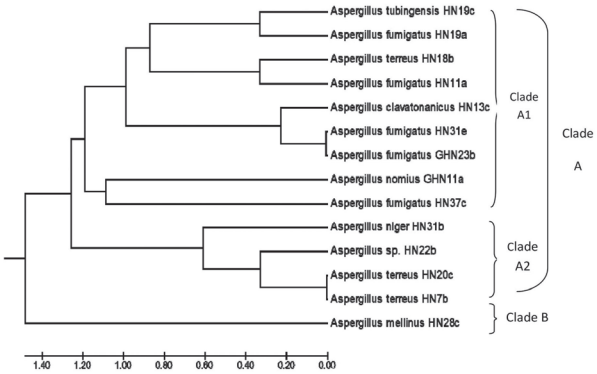


Fig. 4. Phylogenetic tree showing the relative position of fungal strains by the UPGMA method of complete ITS sequence (ITS1 primer). Numbers in the figure refers to percentage bootstrap values which were calculated for 1000 replicates. Bar, 0.02 was per nucleotide position.

Most of these fungi PKS genes have been identified were in the Ascomycota including the imperfect fungi in Deuteromycotina. However, there were 6/14 positive isolates contained only a single PKS gene using all of these sets of primers. Using our newly designed KA-series primers (KAF1/KAR1 and IIPF6-IIPR6) we have identified nine unique PKS genes from the species of *Aspergillus.* These primer pairs also amplified 13 PKS gene fragments.

The numbering in the figure refers to the codes of fungal isolates.

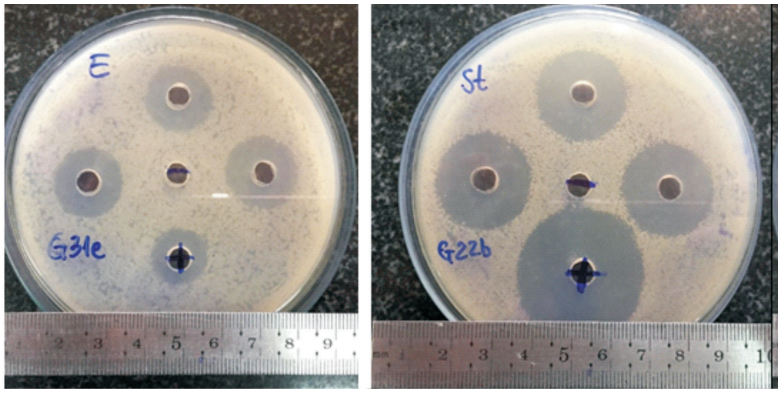


Fig. 4. Halos of several fungi isolates against to pathogenic bacteria

Table 2. Antimicrobial activity of sponge-derived fungi

No	Fungal isolate	Diameter of halos [D = d1 - d2] (mm)			
		Bacillus cereus	*Escherichia coli*	*Sal. typhymurium*	*Sta. aureus*
01	GHN1a*	ND	ND	8.33 c	ND
02	GHN2b	ND	04.33 k	ND	10.33 mno
03	GHN11a	12.67 hij	08.33 hi	ND	21.67 g
04	GHN11b	18.33 bc	ND	ND	28.00 cd
05	GHN12a	10.33 lmn	ND	ND	16.33 i
06	GHN21a	ND	24,67 b	ND	ND
07	GHN1b	ND	09,00 fghi	ND	ND
08	GHN22a	14.33 fg	ND	ND	ND
09	GHN22b	13.00 hij	ND	ND	23.67 f
10	GHN23a	ND	ND	ND	12.00 l
11	GHN23b	14.00 gh	05,33 k	ND	15.67 ij
12	GHN23c	10.67 klm	ND	ND	33.33 b
13	GHN23d	19.33 ab	ND	ND	26.33 e
14	GHN24a	10.67 klm	ND	ND	14.33 jk
15	GHN31a	ND	10.33 efg	ND	ND
16	GHN32a	ND	ND	26.67 a	09.33 nop
17	GHN36a	ND	ND	ND	11.33 lm
18	HN1a**	09.33 mnop	19.67 c	ND	ND
19	HN3a	19.33 ab	ND	ND	39.33 a
20	HN7a	ND	ND	ND	12.00 l
21	HN7b	06.00 rs	04.67 k	ND	16.33 i
22	HN11a	20.33 a	14.33 d	ND	33.67 b
23	HN11b	ND	05.00 k	ND	13.67 k
24	HN12a	ND	ND	ND	19.33 h
25	HN12b	19.33 ab	ND	ND	17.00 i
26	HN13b	ND	07.33 ij	15.67 cd	ND
27	HN13c	14.00 gh	10.67 ef	ND	28.33 c
28	HN13d	ND	ND	ND	24.67 f
29	HN14a	04.00 t	ND	16.67 c	ND
30	HN14c	11.33 jkl	ND	ND	16.67 i
31	HN16a	09.67 mno	ND	ND	14.00 k
32	HN16b	06.00 rs	ND	ND	11.67 lm
33	HN17a	07.33 qr	ND	ND	11.67 lm
34	HN17b	05.33 st	ND	14.33 de	ND
35	HN18b	09.00 nop	14.33 d	ND	11.67 lm
36	HN19a	09.33 mnop	11.33 e	ND	06.33 qr
37	HN19b	06.00 rs	08.67 ghi	ND	15.67 ij
38	HN19c	10.33 lmn	ND	15.67 cd	11.67 lm
39	HN20c	09.33 mnop	04.33 k	ND	14.00 k
40	HN21a	ND	ND	ND	13.67 k
41	HN21b	09.00 nop	ND	ND	14.33 jk
42	HN22b	16.33 de	09.33 fgh	ND	23.67 f
43	HN23a	11.33 ijk	ND	ND	10.67 lmn
44	HN24a	06.00 rs	ND	ND	14.33 jk
45	HN24c	15.67 ef	ND	ND	04.00 t
46	HN25a	04.00 t	ND	ND	13.67 k
47	HN27a	04.33 t	09.33 fgh	ND	14.33 jk
48	HN28c	16.33 de	08.67 hij	ND	28.00 cd
49	HN30c	ND	ND	ND	26.67 de
50	HN31b	08.33 nop	08.67 ghi	ND	14.00 k
51	HN31c	ND	ND	ND	14.67 jk
52	HN31e	17.33 cd	13.67 d	ND	17.00 i

Table 2. *Continued ...*

No	Fungal isolate	Diameter of halos [D = d1 - d2] (mm)			
		Bacillus cereus	*Escherichia coli*	*Sal. typhymurium*	*Sta. aureus*
53	HN32g	10.00 lmn	ND	ND	09.33 nop
54	HN33c	ND	ND	ND	20.00 h
55	HN36d	08.00 opq	ND	ND	08.67 op
56	HN37c	12.33 ij	13.67 d	ND	16.33 i
57	HN37e	06.00 rs	06.00 jk	ND	09.00 nop
58	HN37f	09.00 mno	ND	ND	20.33 gh
	Control	10.00 lmn	09.00 f	07.00 l	06.00 r
		Streptomycin	Tetracyclin	Tetracyclin	Tetracyclin

*isolates were isolated on Gauss medium; ** isolates were isolated on GYP
T D = diameter of inhibition zone of isolates, d1 = diameter of inhibition zone, d2 = diameter of well, ND: not detected he numbers followed by the same word not different at $p<0.01$

M=ladder; 1: control; 2:HN20c, 3:HN31e, 4: HN19c 5:HN22b; 6:HN31b, 7:GHN23b,

8:: HN19a 9: HN28c; 10:HN13c: 11:HN7b, 12:HN18b; 13&14:control;

15:GHN11a; 16: HN37c; 17: HN11a

M 1 3 14 15 16 17

M 1 2 3 4 5 6 7 8 9 10 11 12

250 bp

These indicated that our KAF1/KAR1 and KAF1/KAR2 primer pairs were able to identify a variety of genes encoding different reducing PKSs for HR-type PK compounds.

Among the BCGs that were screened, only the NPRS genes showed the positive results. That was

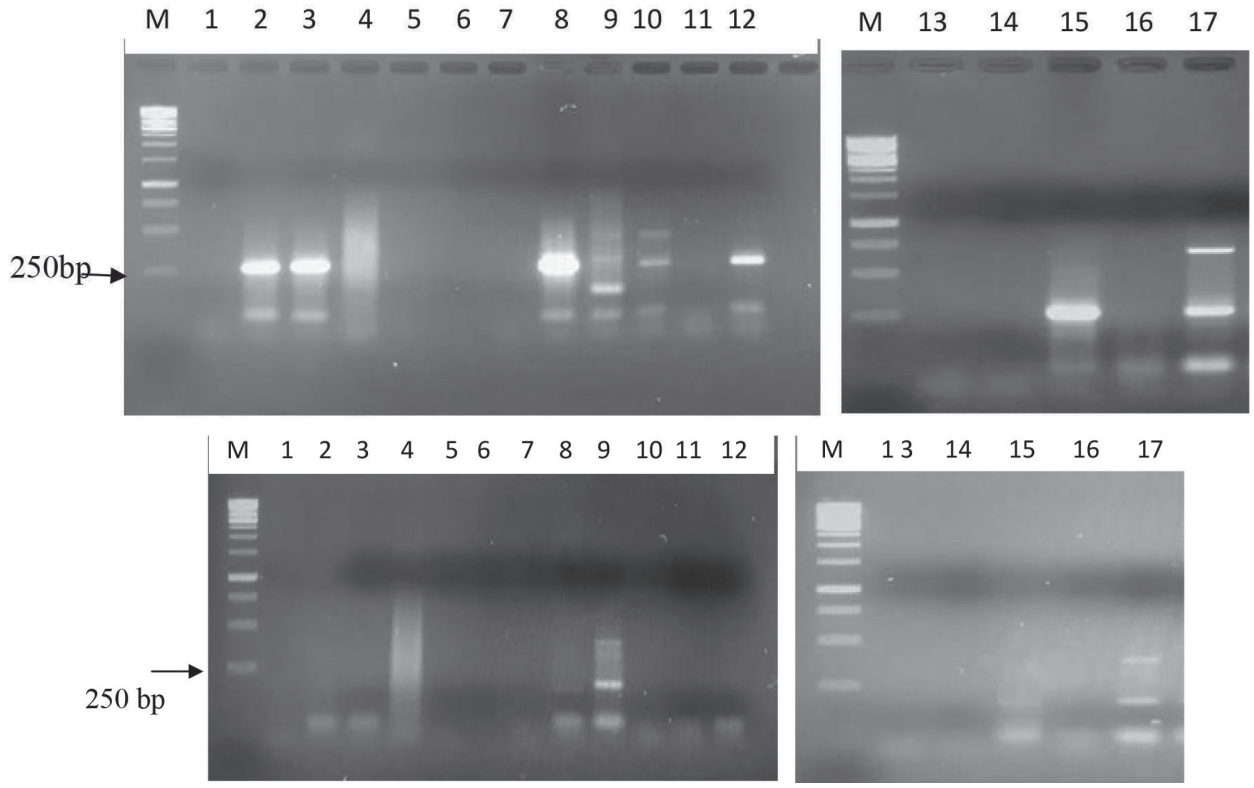


Fig. 5. Agarose gel electrophoresis of PCR products from DNA isolated from representative fungi strains: (A) Selective amplification of 250 bp fragments using primers A2gamForward/ A3gamReverse specified for NRPS adenylation sequences; (B) Selective amplification of the 680-700 bp fragments using primers KAF1/KAR1 for PKS-I; (C) Specific primers of 120-150 bp fragments using primers IIPF6-IIPR6 for PKS-II

proved by the presenses of the clear bands in the gel electrophoresis under UV illumination. Fig. 5 showed the strains HN20c, HN31e, HN19a, HN18b, GHN11a and HN11a had gene around 250 bp. The PKS-I gene presented bands in the gel at 700 bp with two strains HN13c and HN11a while the PKS-II showed the strains HN31e, HN28c, HN13c, GHN11a, HN37c and HN11a had gene around 120-150 bp.

PCR-based screening revealed that the sponge-associated fungi *Aspergillus clavatonanicus* HN13c and *Aspergillus fumigatus* strain HN11a have capabilities to produce secondary metabolites, in particular a nonribosomal polypeptides. As indicated in Figure 5, fungal strain HN11a possesses the NRPS gene as represented by the occurrence of a single DNA band of approximately 250 bp similar to the positive control on the agarose gel. Further inhibitory interaction test showed that the strain HN11a inhibited the growth of *Escherichia coli* (Table 2) and this strain also inhibited the growth of positive-gram bacteria.

The presence of biosynthetic gene clusters (BGC) was screened to understand the ability of the prospective fungi to produce bioactive polyketides and non-ribosomal peptides. Some studies have demonstrated that the presence of the biosynthetic gene cluster (BGC) such as polyketide synthase (PKS) and

Table 3. Phylogenetic affiliation of isolates on the basis of 18S rDNA-ITS gene sequences by using the BLAST programme in the GenBank database based on sequence similarity

Taxonomic group and strain	Closest species relative	Similarity (%)	Overlap(nt)
HN20c	*Aspergillus terreus (*MT316343.1)	100	571
HN31e	*Aspergillus fumigatus* (MG659682.1)	99	558
HN19c	*Aspergillus tubingensis* (KT310979.1)	100	570
HN22b	*Aspergillus sp.* (MK054270.1)	100	561
HN31b	*Aspergillus niger* (*MK886749.1)*	100	564
GHN23b	*Aspergillus fumigatus* (MH540722.1)	99	559
HN19a	*Aspergillus fumigatus* (KY827337.1)	99	559
HN28c	*Aspergillus mellinus* (MH865256.1)	98	533
HN13c	*Aspergillus clavatonanicus* (MH625699.1)	99	560
HN7b	*Aspergillus terreus* (MG647853.1s)	99	571
HN18b	*Aspergillus terreus* (KT310979.1)	100	568
GHN11a	*Aspergillus nomius* (MN700028.1)	99	572
HN37c	*Aspergillus fumigatus* (MH345848.1)	100	555
HN11a	*Aspergillus fumigatus* (MG659682.1)	100	558

Table 3. Distribution of NRPS, PKS-I, and PKS-II in *Aspergillus*

No	Taxa	NPRS A2-A3gamForward	PKS-I KAF1-KAR1	NPS-II IIPF6-IIPR6
01	*Aspergillus terreus* HN20C	-	—	-
02	*Aspergillus fumigatus* HN31e	+	-	+
03	*Aspergillus tubingensis* HN19c	+	-	-
04	*Aspergillus* sp. HN22b	-	-	-
05	*Aspergillus niger* HN31b	-	-	-
06	*Aspergillus fumigatus* GHN23b	-	-	-
07	*Aspergillus fumigatus* HN19a	-	-	-
08	*Aspergillus mellinus* HN28c	+	-	+
09	*Aspergillus clavatonanicus* HN13c	+	+	+
10	*Aspergillus terreus* HN7b	-	-	-
11	*Aspergillus terreus* HN18b	-	-	-
12	*Aspergillus nomius* GHN11a	+	-	+
13	*Aspergillus fumigatus* HN37c	-	-	+
14	*Aspergillus fumigatus* HN11a	+	+	+

non-ribosomal peptide synthase (NRPS) genes in microorganisms is correlated to their capability to inhibit the growth of other microorganisms (Passari *et al.*, 2016; Bo¨hringer *et al.*, 2017; Samak *et al.*, 2018). We detected the existences of the PKS-I, PKS-II and NRPS genes in 7 species of the genus *Aspergillus*-associated sponge.

Particularly, the PKS or NRPS genes were detected in the fungal isolates with antimicrobial activity, for example the isolates HN31e, HN19c, HN28c, HN13c, GHN11a, HN37c, and HN11a implying their potentials in the production of antimicrobial PKS or NRPS compounds. However, there were some isolates that did not have PKS or NRPS genes, for example HN20c, HN22b, HN31b, GHN23b, HN7b, HN19a and HN18b which also showed antimicrobial activity, which may resulted from other secondary metabolites instead of PKS/NRPS compounds.

Interestingly, the members of the fungal genus *Aspergillus*, which are ubiquitous in terrestrial, are also the principle sources of bioactive compounds in marine sponges. Out of more than 680 fungal strains isolated worldwide from 16 sponge species, major-

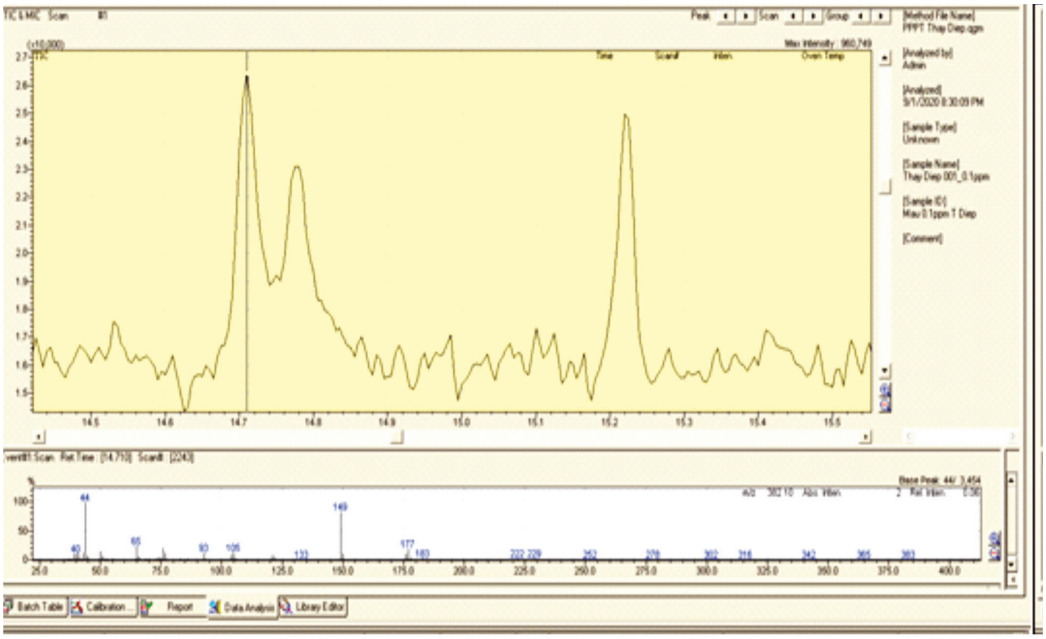


Fig. 6a. Identification of Bioactive Constituents by GC-MS (peak 1) [Phthalic acid, di-(1-hexen-5-yl) ester]

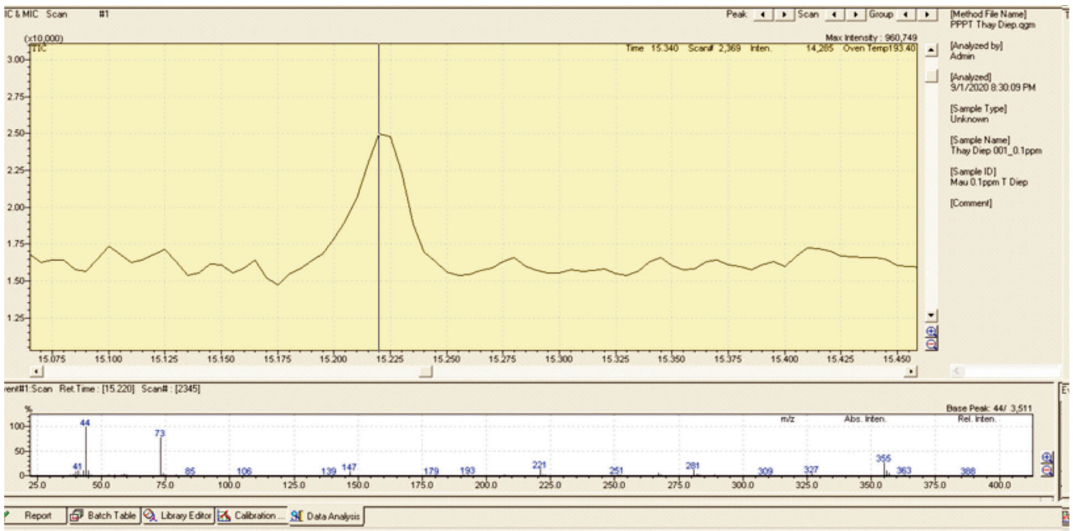


Fig. 6b. Identification of Bioactive Constituents by GC-MS (peak 2) [3-Nitropropionic acid]

ity belong to the genera *Aspergillus* and *Penicillium* (Höller *et al.*, 2000). The detected PKS and NRPS genes suggested the potentials of sponge-associated fungi in the biosynthesis of related bioactive metabolites with chemical defense role for sponge at the gene level for some extents (Zhou *et al.*, 2011).

Among 14 strains, the *Aspergillus fumigatus* strain HN11a was selected to be identified by GC-MC because it inhibited three in four bacterial reference strains and it was detected 3 genes: PKS-I, PKS-II and NPKS clearly.

GC-MS Analysis

The components present in the ethyl acetate of *Aspergillus fumigatus* strain HN11a were identified by GC-MS. The chromatogram is shown in Fig. 6.

The active principles with their retention times (RT) molecular formula, molecular weights (MW) and the therapeutic use in the ethyl acetate of *Aspergillus fumigatus* HN11a strain are presented in Table 4.

Using the GC-MS, Sridevi *et al.* (2013) analysed the *Pergularia Daemia* and they found five bioactive constituents which were identified namely of among Phthalic acid, di-(1-hexen-5-yl) ester, one of five bioactive compounds, and it has the ability of fungal infection with medically bioactive compounds. Lu *et al.* (2015) showed that 3-Nitropropionic acid (3-NPA) was found in extracts of several strains of endophytic fungi in *Phomopsis* sp., it is known as a mitochondrial inhibitor which induces a cellular en-

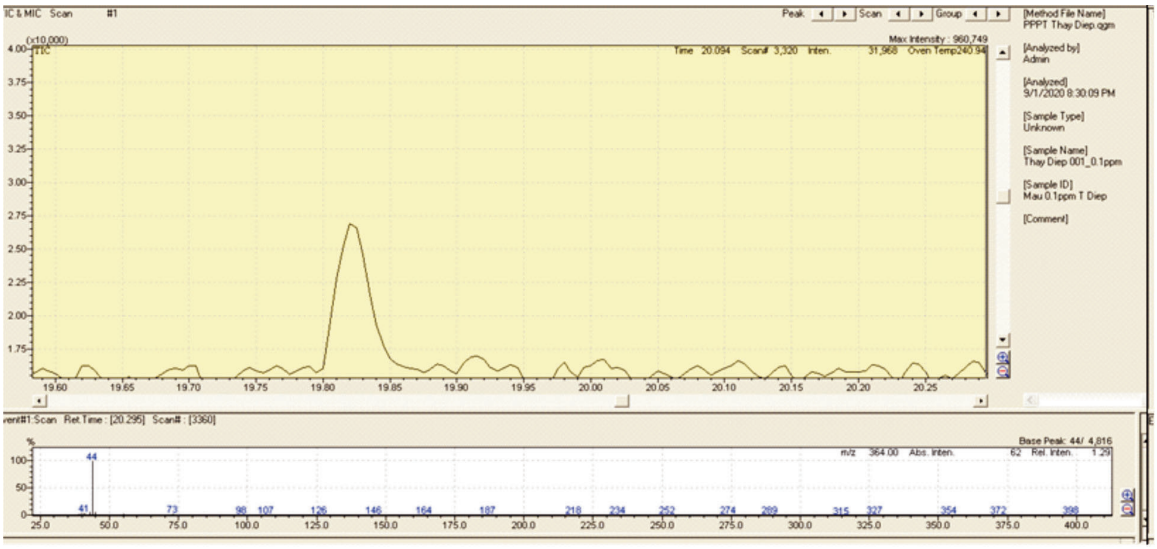


Fig. 6c. Identification of Bioactive Constituents by GC-MS (peak 5) (2,6-DIHYDROXYBENZOIC ACID 3TMS)

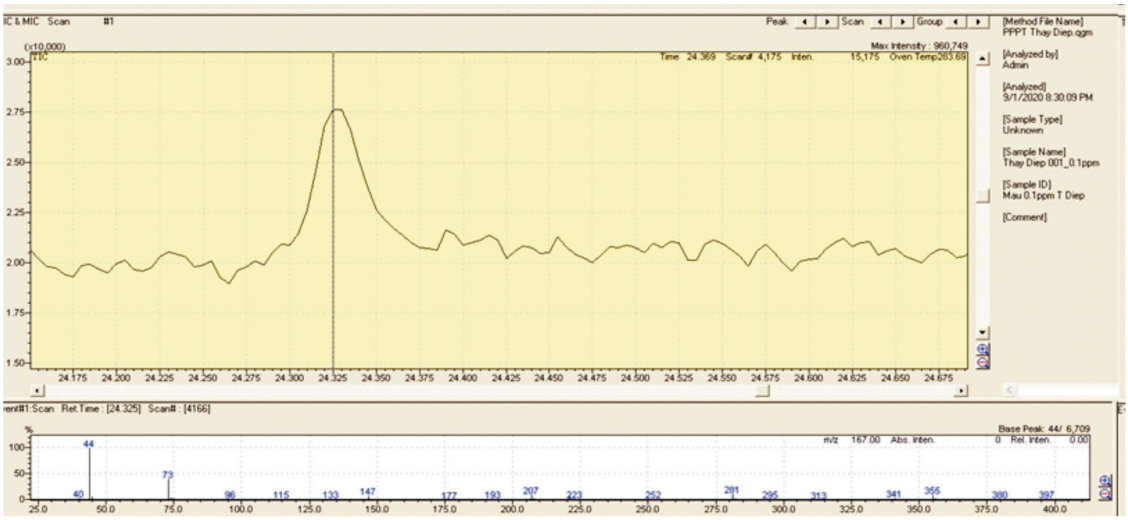


Fig. 6d. Identification of Bioactive Constituents by GC-MS (peak 9) [Propanamide]

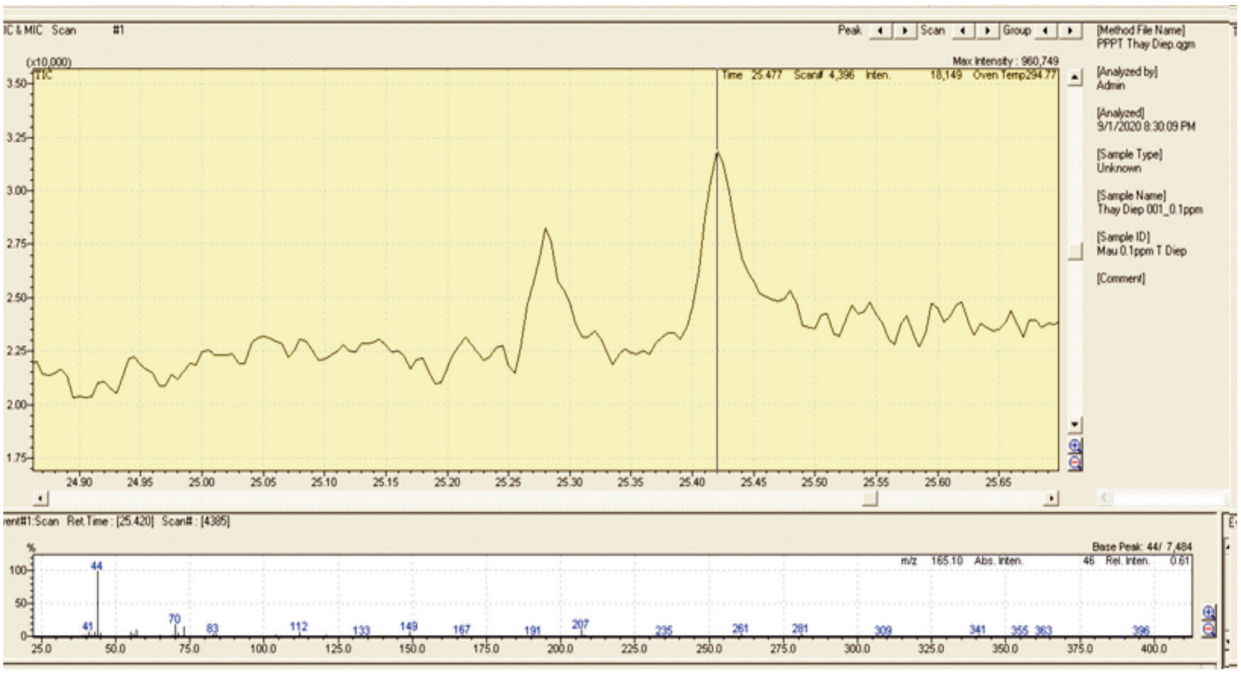
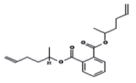
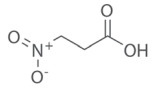
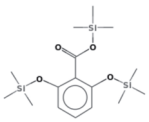
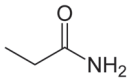
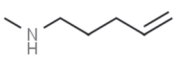


Fig. 6e. Identification of Bioactive Constituents by GC-MS (peak 10) [Methylpent-4-enylam]

ergy deficit (Coles *et al.*, 1979). The bioactivities of 3-NPA such as antimycobacterial activity, antibacterial activity, antifungal activity and insecticidal activity have been proved by researchers (Chomcheon *et al.*, 2005; Flores *et al.*, 2013). From twenty-eight major compounds which were identified in *Moringa oleifera* fruit extracted by GC-MS analysis- contained of which the maximum quantum was 2,6-dihydroxybenzoic acid, Dihydroxybenzoic acids which are used as intermediates for pharmaceuticals, especially for antipyretic analgesic, ant rheumatism and antimicrobial activity (Juurlink *et al.*, 2014). The GC-MS analysis revealed different peaks of ten compounds in *Jasmine* flower, the results

Table 4. Bioactive compounds in *Asperigillus fumigatus* HN11a strain identified by Gas Chromatography Mass Spectrometry (GCMS)

Peak	RT (min)	Name of the compound	Cmpound nature	Molecular formula	Molecular weight	Therapeutic use
1	14.715	Phthalic acid, di-(1-hexen-5-yl) ester	Organic acid compound	$C_{20}H_{26}O_4$		Anti-Fungal
2	15.220	3-Nitropropionic acid	Organic Acid	$C_3H_5NO_4$		Antimicrobial
5	19.820	2,6-Dihy-droxybenzoic acid 3TMS	Benzoic acid compound	$C_{16}H_{30}O_4$		Antimicrobial
9	24.325	Propanamide	Amide (Volatile compound) and antifungal	C_3H_7NO		Antimicrobial. Antioxidant, anticancer
10	25.420	Methylpent-4-enylamine	Amine	$C_6H_{13}N$		Antimicrobial

showed that the main compounds were 2-Phenylthiolane (57.31%), Cyclohexene, 3-ethenyl- (25.91%), Acetaldehyde (12.70%), N Methylallylamine (9.99%), Propanamide (6.79%) and Phthalic acid, bis (7-methyloctyl) ester (5.21%) and Propanamide have antioxidant and antimicrobial properties and Phthalic acid together with Propanamide exhibit antioxidant, cancer preventive, pesticide, hypocholesterolemic, dermatitigenic and anemiagenic properties (Rassem *et al.*, 2015). Sathya *et al.* (2016) analysed the root exudation in chilli (*Capsicum annuum* L.) cv. K2 30 day old seedlings using GC-MS, and received 15 volatile compounds in which the Methylpent-4-enylamine is one of those fifteen compounds and this amine is an antibacterial agent.

It was concluded that five bioactive compounds in the *Asperigillus fumigatus* HN11a strain identified by Gas Chromatography Mass Spectrometry (GCMS) were antimicrobials, this result also coincided with the result of antimicrobial activity of *Asperigillus fumigatus* HN11a strain (it went againsts positve-gram bacteria and negative-gram bacteria).

Conclusion

In conclusion, the cultuvable diversity of sponge-associated fungi from the Ha Tien Sea was been established. Genus *Aspergillus* were found as the predominant strains showing the antibacterial activity. Besides, the *Asperigillus fumigatus* acts as a rare fungus which showed the antimicrobial activity. It is indicated that the marine sponge were the potent sources for the endophytic fungi which have a wide biological activity against pathogenic bacteria as well as Gram-positive and Gram-negative bacteria. This makes it becomes a promising application for such newly functional sponge-associated fungi as a novel source of bioactives. The biological activities of each of the identified components range from antimicrobial, antioxidant and antitumoral activities. The research findings have shown that they are extensively rich in secondary metabolites and they have been reported as the bioactive compounds which have been used in the world.

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