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Biological activities and variation of symbiotic fungi isolated from Coral reefs collected from Red Sea in Egypt

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ABSTRACT

Ten specimens of coral reefs were collected from the Red Sea in the Ein El-Sukhna region. Fungal isolation was done using two media, Dextrose Yeast Extract Agar (DYA) and Rose Bengal Agar (RBA). The morphological traits identified 18 fungal isolates belonging to the phyla Ascomycota, Mucoromycota and Deuteromycota. Five genera in three orders have been isolated: Eutrotiales (*Aspergillus, Penicillium* and *Byssochlamys*), Mucorales (*Rhizopus*) and Moniliales (*Curvularia*). The heat mapping clustering of the isolated fungi declared that *Aspergillus* and *Penicillium* were the most frequently isolate fungi in coral reefs. It was found that *A. fumigatus* colonised eight coral samples with 80% colonisation rate. Moreover, about 50% of the isolated fungal species were specific to one coral reef only such as *A.candidus* and *A.carneus* isolated from *Isophyllastrea rigida* only, *A.japonicus* and *A.ochraceopetaliformis* from *Glaxaea fascicularis, A.niger van Tieghem* from *Porites astreoides, A.sydowii, A.terreus* and *P.waksmanii* from *Cladocora arbuscula, P.janthinellum* from *Pterogorgia guadalupensis* and *Curvularia tuberculata, Byssochlamys spectabilis* and *Rhizopus oryzae* from *Acropora humilis*. Biological activities (antimicrobial, antioxidant antiradical and cytotoxicity) of the most predominant fungal species were investigated. The antimicrobial activity of coral fungal filtrates were investigated against six pathogenic bacteria including *Escherichia coli* ATCC11775, *Neisseria gonorrhoeae* ATCC19424, *Pseudomonas aeruginosa* ATCC10145, *Streptococcus faecalis* ATCC19433, *Staphylococcus aureus* subsp. *aureus* ATCC25923, *Bacillus subtilis* subsp. *spizizenii* ATCC6633 and two pathogenic yeast including *Candida albicans* ATCC7102 and *Candida parapsilosis* ATCC22019. Most of these fungal filtrates exhibited moderate to high antibacterial activities against both gram positive and gram negative bacteria, however it showed relatively low bioactivity towards the pathogenic *Candida* species. Investigating the free radical scavenging activity using DPPH reagent showed low to moderate bioactivities. The highest cytotoxic activity against liver cancer cell line Hep-G2 with an IC_{50} values of 18.8 µg/ml was exhibited by *Aspergillus ochraceopetaliformis* MN083316 and a metabolomics study was done on the ethyl acetate extract of this strain using LC-ESI-MS fingerprints leading to the isolation and purification of compound **1**. Using 1D and 2D NMR techniques compound **1** was identified as ditryptophenaline. Compound **1** exhibited a strong antimicrobial, antioxidant activities as well as cytotoxic activities against MCF-7 and HEPG2 with IC_{50} values of 5.8 and 7.6 mmole, respectively.

The objective of this study, isolation of Coral-reef associated fungi and studying their biological activities to produce the most active secondary metabolite which might possess a novel biological activity.

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Coral reefs; Red Sea; fungal variability; antimicrobial activity; free radical scavenging activity; cytotoxic activity

Introduction

More than 70% of the earth's surface occupying by the oceans that support large habitats of living organisms which are considered as a source of a vast groups of structurally unique natural products. These natural products are mainly isolated from invertebrates common to ecosystems of coral reefs, such as tunicates, sponges, soft corals, molluscs and bryozoans (Putri et al. 2015).

Approximately 70,000 fungal species have been described as symbiotic microbes (John et al. 2009), among them 1500 species of marine-derived fungi were mentioned largely from coastal ecosystems (John et al. 2009). They are associated with abundant forms of marine organisms including sponges (Harvell et al. 1999), scleractinian corals and gorgonians.

Although the microbial communities (especially fungi) associated with coral reefs possessed a little

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attention, coral reefs are known as one of the most varied ecosystems on earth. These microbial communities are recognised to be very important members of many ecosystems especially that of coral reefs (Balser et al. 2006; Gutnecht et al. 2006; Schimel et al. 2007). Actually, the coral reef supporting broad microbial diversity facilitating structurally and environmentally complex array of habitats. This microbial diversity affects both host physiology and ultimately ecosystem processes (Ainsworth et al. 2009). Novel bioactive natural products that are not found in terrestrial strains have been produced by marine microbes (Jensen and Fenical 2002; Konig et al. 2006; Blunt et al. 2008). The actual producers or participants in the biosynthesis of secondary metabolites isolated from the marine hosts are the symbiotic microorganisms (Proksch et al. 2003; Li 2009). The symbiotic microbes in corals are believed to be a promising source of new drugs that are still unexplored (Radjasa 2004; Bhatnagar and Kim 2010).

Marine-derived fungi have been known as a source of structurally novel metabolites of potent bioactivity (Bugni and Ireland 2004; Blunt et al. 2006; Saleem et al. 2007). The secondary metabolites produced by coral-associated fungi are of great interest (Putri et al. 2015). The extract produced by many *Aspergillus* and *Penicillium* species are known to produce a large variety of active compounds, such as: *Aspergillus versicolor* isolated from soft coral *Xestospongia exigua* was found to be rich source of novel polyketides (Putri et al. 2015).

Marine-derived fungi (MDF) live in a symbiotic relationship with marine invertebrates and produce a huge numbers of novel bioactive metabolites including antibiotics, antioxidants, antitumors, antifungals, antialgals, antiinsects and acetylcholine esterase inhibitors (Kjer et al. 2010; Lee et al. 2010; Almeida et al. 2011; Chu et al. 2011; Thirunavukkarasu et al. 2012). Marine derived *Aspergillus* species produce many non-ribosomal peptides, polyketides, lipopeptides and isoprenoids of pharmaceutical importance (Mayer and Hamann 2004). *Aspergillus versicolor* produce a novel lipopeptide (Lee et al. 2010) and *Aspergillus niger* synthesises seven new diterpenoides (Hiort et al. 2004).

In the present work, the culturable diversity of fungi isolated from coral reefs, collected from Ein El-Sukhna region, Red Sea, Egypt were investigated. The antimicrobial, free radical scavenging and cytotoxic activities of fungal symbionts were also assayed. Moreover, the LC-MS fingerprints of the ethyl acetate extract of the most potent fungus, *Aspergillus ochraceopetaliformis* MN083316 were studied.

Materials and methods

Collection of coral reef materials

Ten specimens of coral reefs belonging to nine families (*Acroporide, Faviidae, Euphylliidae, Scleractinia incertae sedis, Gorgoniidae, Mussidae, Poritidae, Pocilloporidae* and *Agariciidae*) were collected from Ein El-Sukhna-Zafarana Rd 65 KM, Red Sea, Egypt at depth of 2–5 m by SCUBA diving and preserved under aseptic conditions until transported to the laboratory in ice box.

Culture media

Dextrose yeast agar (DYA) (g/l): [dextrose (10), yeast extract (10), agar (20)] and **Rose bengal agar (RBA) (g/l)**:[peptone (5), glucose (10), KH_2PO_4 (1), $MgSO_4.7H_2O$ (0.5), Rose Bengal (0.33), Agar (20)] were prepared using sea water supplied with the antibiotic benzyl penicillin (150 mg/l) to prevent bacterial growth.

Isolation of coral reef symbiotic fungi

Coral reef samples were rinsed with sterile distilled water (3 times) prior to isolation processes.

Impression method

A small pieces of approximately 1 cm^3 and 2 cm^3 of the inner tissues of each coral species (soft and hard, respectively) were excised under sterile conditions with scalp and forceps and directly spread onto petri plates containing different culture media (Koh et al. 2000; Cao et al. 2015).

Dilution method

Pieces of soft coral (1 cm^3) were crushed using a sterile morter and pestle. The homogenised tissues were serially diluted (10^{-2} and 10^{-3}) with sea water. One ml/dish from each dilution were plated on petri dishes containing different culture media (Putri et al. 2015).

Blender method

The specimens of stony coral were cut into small pieces and homogenised using a blender containing 20 ml sterile sea water under aseptic conditions. 100 µl of the resulting homogenate were directly plated onto petri dishes containing different culture media (Wang et al. 2011).

The plates were incubated at 25–27 °C for one week. Each developed fungal isolate was individually picked and transferred onto a new fresh medium and incubated at 25–27 °C for one week for purification and identification purposes. Stock cultures of the purified fungal isolates were sub-cultured on slants and preserved in refrigerator. The identification of fungal species takes place using the available identification references and molecular identification while the identification of coral reef species was performed with the aid of Marine Species Identification Portal. They were found to be *Acropora humilis, Favia speciosa, Glaxaea fascicularis, Acropora cervicornis, Cladocora arbuscula, Pterogorgia guadalupensis, Isophyllastrea rigida, Porites astreoides, Stylophora pistillata* and *Povona clavus*.

Extraction of the secondary metabolites from isolated symbiotic fungi

Pure fungal species were inoculated into 500 ml Erlenmeyer flasks containing 200 ml of both DY and RB broths. After incubation at 27–30 °C under shaking at 120 rpm for 12 days, the mycelia were separated from the culture filtrate by filtration. The supernatants were extracted with ethyl acetate (EtOAc) three successive times. The EtOAc phase was evaporated under vacuum using rotary evaporator to give solid or oily extract. On the other hand, mycelia were extracted with EtOAc three times to yield EtOAc extract. The obtained extracts were lyophilised and stored freeze dry to be used for bioassays (Wang et al. 2011).

Biological activity of the isolated symbiotic fungi

Assay of antimicrobial activity

Agar disc diffusion method was used. Paper discs were impregnated with 20 mg of the extracts dissolved in 1 ml DMSO. The discs were placed upon agar plates inoculated with bacterial and fungal pathogens including *Streptococcus faecalis* ATCC19433, *Staphylococcus aureus* subsp. *aureus* ATCC25923, *Escherichia coli* ATCC11775, *Neisseria gonorrhoeae* ATCC19424, *Pseudomonas aeruginosa* ATCC10145, *Bacillus subtilis* subsp. *spizizenii* ATCC6633 and two pathogenic fungi including *Candida albicans* ATCC7102 and *Candida parapsilosis* ATCC22019. Plates incubated at 37 °C and after 24 hr incubation, the inhibition zone diameters (mm) around discs were measured. Negative controls were only treated with DMSO. Relative activity was determined in response to the standard antibiotics used. Augmentin and fluconazole were used as positive controls for antibacterial and antifungal, respectively. Relative activity of the test extract = (x-y/z-y) × 100 (Gaurav et al. 2010). Where, (x): total area of inhibition of the test extract, (y): total area of inhibition of the solvent, (z):total area of inhibition of the standard drug.

Assay of free radical scavenging activity

It was performed using free radical scavenging (FRS) model according to (Hamed 2009). One mg of EtOAc extract of each of the fungal species were dissolved in 1 ml DMSO to prepare stock solution of 1000 µg/ml. 0.0035 g DPPH (2,2-diphenyl-1-picrylhydrazyl radical) was dissolved in 100 ml of methanol HPLC grade to prepare 0.0035% solution and stored in dark until use. 0.1 ml of stock solution was added to 0.9 ml of methanolic DPPH solution to reach the maximum concentration of tested samples 100 µg/ml. The reaction mixture was incubated for 30 min, then measured at wave length 540 nm. Blank using DMSO was measured. The free radicals scavenging activity of fungal extracts were calculated from the following equation:

$$\%\text{Scavengingactivity} = \left\{ \left(A_{blank} - A_{sample}\right) / A_{blank} \right\} \times 100$$

Where, A_{blank} (Absorbance of reaction mixture without test sample "DPPH" only).

A_{sample} (Absorbance of reaction mixture in presence of test samples).

Assay of cytotoxic activity

Assay of cytotoxicity of symbiotic fungal extracts against human cancer cell lines

Human liver carcinoma (HEPG 2) and breast cancer (MCF7) cell lines used in this study were obtained from the American Type Culture Collection (ATCC,

Minnesota, USA). Different concentrations of the extract were prepared (5, 12.5, 25, 50 µg/ml) using DMSO. Cells were seeded in 96-well microtiter plates at initial concentration of 3×10^3 cell/well in 150 µl fresh medium and left for 24 hr to attach to the plates. Different concentrations 0, 5, 12.5, 25, 50 µg/ml of sample were added. For each sample concentration 3 wells were used. The plates were incubated for 48 hr. The cells were fixed with 50 µl cold trichloroacetic acid 10% for 1 hr at 4 C. Then the plates were washed with distilled water using automatic washer Tecan, Germany and stained with 50 µl 0.4% SRB dissolved in 1 % acetic acid for 30 min at room temperature and washed with 1 % acetic acid and air-dried. The dye was solubilised with 100 µl/well of 10 M tris base (pH 10.5) and optical density (O.D.) of each well was measured spectrophotometrically at 570 nm with an ELISA microplate reader (Sunrise Tecan reader, Germany). The mean background absorbance was automatically subtracted and mean values of each sample concentration was calculated.

Calculation

The percentage of cell survival was calculated as follows:

Surviving fraction = O.D. (treated cells)/O.D. (control cells).

The IC_{50} values (the concentrations of resveratrol required to produce 50% inhibition of cell growth) were also calculated.

The LC-MS fingerprints of the A. ochraceopetaliformis EtOAc extract

The fungual species, *A. ochraceopetaliformis* MN083316 was cultured on RBA medium (2 L, 250 mL medium/500 mL Erlenmeyer flask) at 120 rpm, 27°C for 12 days. The culture filtrate was extracted thrice with EtOAc and the resulting EtOAc extract was evaporated under vacuum to give EtOAc extract for future analysis.

LC-ESI-MS spectrum was measured with an Agilent Technologies (Santa Clara, USA) HPLC1260 series coupled to Agilent Technologies (Santa Clara, USA) 6420 Triple quad LC-MS ion trap mass spectrometer fitted with an ESI source (Toyama University, Toyama, Japan). The samples were dissolved in methanol for HPLC (1 mg/mL) for injection into the HPLC-ESI-MS system. Separation through LC-column was performed using gradient separation from 20-100% methanol/water over a period of 20 min. with a flow rate 1 ml/min and monitored by absorption at 254 and 210 nm with a photodiode array detector. Positive and negative ionisation modes were detected with mass scan range 50–1500 amu. NMR spectra were recorded at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) on a JEOL ECA500II spectrometer with chemical shift values expressed in δ (ppm) downfield from TMS, as an internal standard. Column chromatography was performed with Cosmosil 5C18-Ar-II column, Nacalai Tesque INC., 10 × 250 mm, Agilent, USA).

Statistical analysis

Was performed by SPSS 15.0 for windows evaluation version. (SPSS Inc. Released 2007. SPSS for Windows, Version 15.0. Chicago, SPSS Inc.)

Results and discussion

Variability of coral reef symbiotic fungi

Three techniques and two nutrient media were used for isolation of coral reef associated fungi. A total of 137 fungal isolates constituting 18 species were screened from the ten coral reef samples. Hard corals attained 80 isolates with frequency 58.4% while soft corals recorded 57 isolates with frequency 41.6% of the total isolates (Table 1). Impression and blender techniques were used for hard corals. Impression technique revealed 16 isolates representing 20% of the total taxa, whereas blender technique recorded 64 isolates representing 80% of the total count of hard coral. Dilution and impression techniques were used for soft corals. The dilution technique recorded higher count than the impression technique where 52 isolates with frequency 91.2 % while 5 isolates with 8.8 % frequency were detected, respectively (Table 1).

Medium DYA proved to be the most suitable in blender technique for isolation of symbiotic fungi from hard coral reef with frequency 41.3 % (Table 2). Meanwhile, medium RBA was the most suitable in soft coral using dilution technique with frequency 87.7%. Consequently the former media and techniques were used in the next experiments.

Table 1. Total isolates and frequency percentage of coral Reefs symbiotic fungi isolated on four cultural techniques.

Coral species	Isolation techniques					
	Impression		Blender			
Hard coral	TI (cfu/plate)	Freq. %	TI (cfu/plate)	Freq. %	Total isolates/survay	Freq. %
Acropora humilis	3	18.75	14	21.88	17	21.25
Favia speciosa	4	25.00	1	1.56	5	6.25
Glaxaea fascicularis	2	12.50	6	9.40	8	10.00
Acropora cervicornis	2	12.50	8	12.50	10	12.50
Cladocora arbuscula	1	6.25	11	17.20	12	15.00
Stylophora pistillata	2	12.50	1	1.56	3	3.75
Povona clavus	2	12.50	23	36.00	25	31.25
Total	**16**	**20.00**	**64**	**80.00**	**80**	**58.40**
	Impression		**Dilution**			
Soft coral	**TI (cfu/ plate)**	**Freq. %**	**TI (cfu/ plate)**	**Freq. %**	**Total isolates/survay**	**Freq. %**
Pterogorgia guadalupensis	1	20.00	5	9.60	6	10.53
Isophyllastrea rigida	2	40.00	45	86.54	47	82.46
Porites astreoides	2	40.00	2	3.85	4	7.02
Total	**5**	**8.77**	**52**	**91.23**	**57**	**41.60**
- TI: Total isolates (cfu/coral specimens plated), Freq. % = No. of isolated species/Total isolates × 100					**137**	**100**

Table 2. Fungal isolates screened from ten coral species collected from Red Sea, El-Ein El- Soukhna region using two growth media.

	Isolation techniques			
	Impression		Blender	
Hard coral	Media		Media	
Coral species	DYA	RBA	DYA	RBA
Acropora humilis	2	1	11	3
Favia speciosa	4	-	-	1
Glaxaea fascicularis	1	1	-	6
Acropora cervicornis	-	2	8	-
Cladocora arbuscula	1	-	10	1
Stylophora pistillata	1	1	1	-
Povona clavus	2	-	3	20
Total Isolates (CFU)	**11**	**5**	**33**	**31**
Freq. %	**13.75**	**6.30**	**41.30**	**38.80**
	Impression		**Dilution**	
	Media		**Media**	
Soft coral	DYA	RBA	DYA	RBA
Pterogorgia guadalupensis	1	-	1	4
Isophyllastrea rigida	-	2	1	44
Porites astreoides	2	-	-	2
Total Isolates (CFU)	**3**	**2**	**2**	**50**
Freq. %	**5.30**	**3.50**	**3.50**	**87.70**
Total Isolates (CFU) in both Hard and Soft corals	**DYA 49**		**RMA 88**	**137**
Freq. %	**35.70**		**64.30**	**100**

DYA: Dextrose Yeast Extract Agar, RBA: Rose Bengal Agar.

Venn diagram (Figure 1), showed that a number of overlapped fungal species were isolated from both hard and soft coral samples when cultured on DYA and RBA media. On the DYA medium 33 fungal isolates were overlapped between both coral reef species, while 15 and 1 isolates were specific to hard and soft coral species, respectively. Also in RBA medium 34 fungal isolates overlapped between both coral reef species, whereas a large number of fungal isolates were specific to hard (11 isolates) and soft (43 isolates) coral reef.

The heat map clustering (Figure 2(a,b)) of total fungal count declared that Aspergilli were the most frequent genera in both hard and soft corals. *Aspergillus aculeatus, A. parasiticus, A. carneus* and *A. fumigatus* being the highly colonised species to coral reefs. Genus *Penicillium* followed in density of occurrence in coral reefs. It was found that two infrequent genera, *Curvularia tuberculata* and *Rhizopus oryzae*, were detected once with 2 and 1 colonies from coral tissues, respectively.

Concerning the variation in coral reefs, heat map Figure 2(a,b) indicates that the soft coral reef species *Isophyllastrea rigida* was highly colonised with fungal isolates representing 34.31% of total isolates in the ten samples. Followed by the hard coral reefs *Povona clavus* and *Acropora humilis* representing 19% and 12.4% of the total fungal count, respectively.

In close relation, limited information is known about the microbial diversity associated with marine coral reefs, despite the vital role that microorganisms may play in coral reef ecosystems (Wang et al. 2011). Fifty three isolates belong to 18 fungal species were isolated from the tissue of the gorgonian coral *Echinogorgia rebekka* from the Weizhou coral reef in the South China Sea. They are all belongs to the family Ascomycota and were distributed among seven

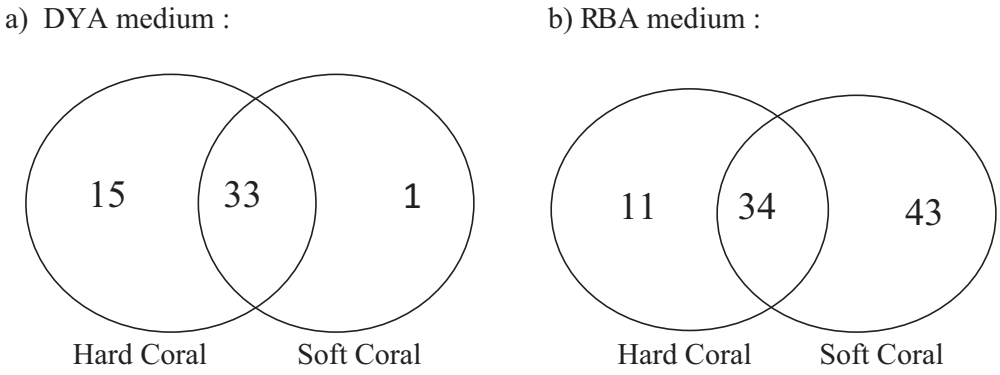

Figure 1. Venn diagram of the total number and the overlap fungal species isolated from coral reefs using different growth media (a) DYA and (b) RBA.

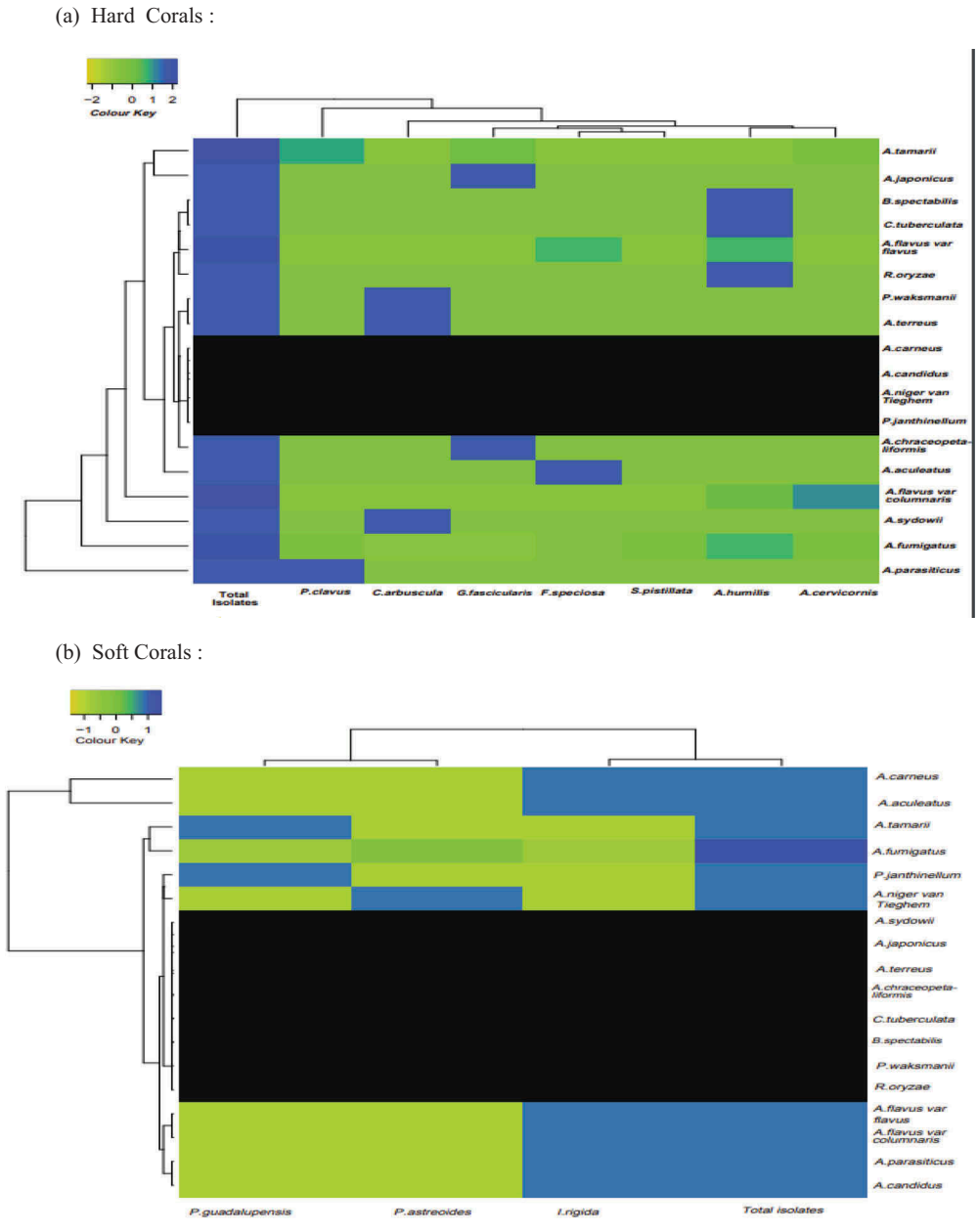

Figure 2. (a) and (b) Heatmap clustering of the total number of symbiotic fungal species isolated from Coral reefs.

genera in five orders Eurotiales (*Aspergillus, Penicillium*), Capnodiales (*Cladosporium*), Trichosphaerial (*Nigrospora*), Pleosporales (*Alternaria*) and Hypocreales (*Hypocrea* and *Nectria*) (Wang et al. 2011). The diversity of coral reef associated microorganisms has been poorly investigated in remote geographical areas like Red Sea (Strobel and Daisy 2003; Huang et al. 2007). Meanwhile (Koh et al. 2000) isolated 51 isolates of deuteromycetes from 18 fungal taxa, 2 types of yeasts and several isolates of sterile filamentous fungi. *Penicillium* spp. from the *P. janthinellum* series appear to be ubiquitous to gorgonian coral reef species.

Biological activities of coral reef symbiotic fungi

The biological activities were carried out on the all of the isolated symbiotic fungi then the most active and frequent fungal genera present were chosen, *Aspergillus ochraceopetaliformis* MN083316, *A. aculeatus, A. fumigatus, A. carneus, A. parasiticus* and *B. spectabilis* MN093943.

Antimicrobial activity of coral reef symbiotic fungi

Antimicrobial metabolites in coral reef fungal extracts are one of the most promising resources due to the increasing demand to novel antibiotic drugs with new mechanism of action to overcome bacterial drug resistance. A large number of symbiotic microorganisms in corals are considered to be a promising source of these novel drugs which have been largely unexplored (Radjasa 2004; Bhatnagar and Kim 2010).

The present data (Figure 3) declared high antimicrobial potentiality of most of the fungal filtrate extracts against the tested pathogenic bacteria and yeast. The most active fungal filtrate extract was *Aspergillus ochraceopetaliformis* MN083316 grown on both media DYA and RBA. It exhibited antimicrobial activity against pathogenic bacteria and yeast with relative activity ranging (37–110%) and (25-30%) in DYA medium, respectively. While in RBA medium, the antimicrobial activities of *A. ochraceopetaliformis* MN083316, *A. fumigatus* and *B. spectabilis* were much higher than the other fungal species. *A. ochraceopetaliformis* MN083316 showed relative activity ranging from 39–85% against bacteria and from 25 – 35% against yeast, *A. fumigatus* exhibited relative activity from 30- 70% against bacteria and from 25 – 28% against yeast while, *B. spectabilis* has relative activity ranging from 30–70% against bacteria and from 22 – 45% against yeast.

Candida albicans and *C. parapsilosis* showed high resistance to the fungal filtrate extracts compared to bacterial pathogens, where the fungal isolates cultured on DYA media showed no activity against both candida except in cased *A. ochraceopetaliformis* MN083316 and *B. spectabilis* with relative activity 23-30% and 21-25%, respectively. Among bacteria, *E. coli* ATCC11775 and *S. feacalis* ATCC19433 were the most resistant pathogens treated with fungal filtrate extracts (DYA medium) as 3 only of the 6 fungal extracts showed activity. The highly susceptible pathogen was *B. subtilis* ATCC6633 treated with *A. ochraceopetaliformis* MN083316 (DYA medium) as it exhibited the highest relative activity (110%) in the assay compared to other pathogens used.

Similar results were obtained by Wang et al (Wang et al. 2011) who claimed that out of 18 fungal strains isolated from Gorgonian coral *Echinogorgia rebekka* collected from the Weizhou coral reef in the South China Sea, 12 were found to show moderate to high level of antibacterial activities against *S. aureus*, while 9 had moderate to very high activities against *M. tetragenus*. (Wang et al. 2017) further reported that the compounds isolated from coral-derived *Aspergillus tritici* which isolated from the coral *Galaxea fascicularis* collected at Port Dickson, Malaysia, showed high antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA), *Vibrio vulnificus, Vibrio rotiferianus* and *Vibrio campbellii*. (Sabdaningsih et al. 2016) tested the crude extracts of marine derived fungi associated with soft corals isolated from Panjang Island against MDR-*Staphylococcus haemolyticus* and found that it exhibited strong antibacterial activity. (Abd El-Hady et al. 2014) examined the supernatant and mycelial extracts from the static culture of *Emericella unguis* isolated from the soft coral *Sinularia* sp. collected from Hurghada coast, Red Sea, Egypt, and found that it showed high antimicrobial activity against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans*.

As a result, we can assume that there is a symbiotic relationship between coral reef and its associated fungi enabling fungi to be an alternative to produce antibacterial compounds to overcome the multidrug resistant pathogens by producing novel secondary metabolites, Mabrouk et al (Mabrouk et al. 2008).

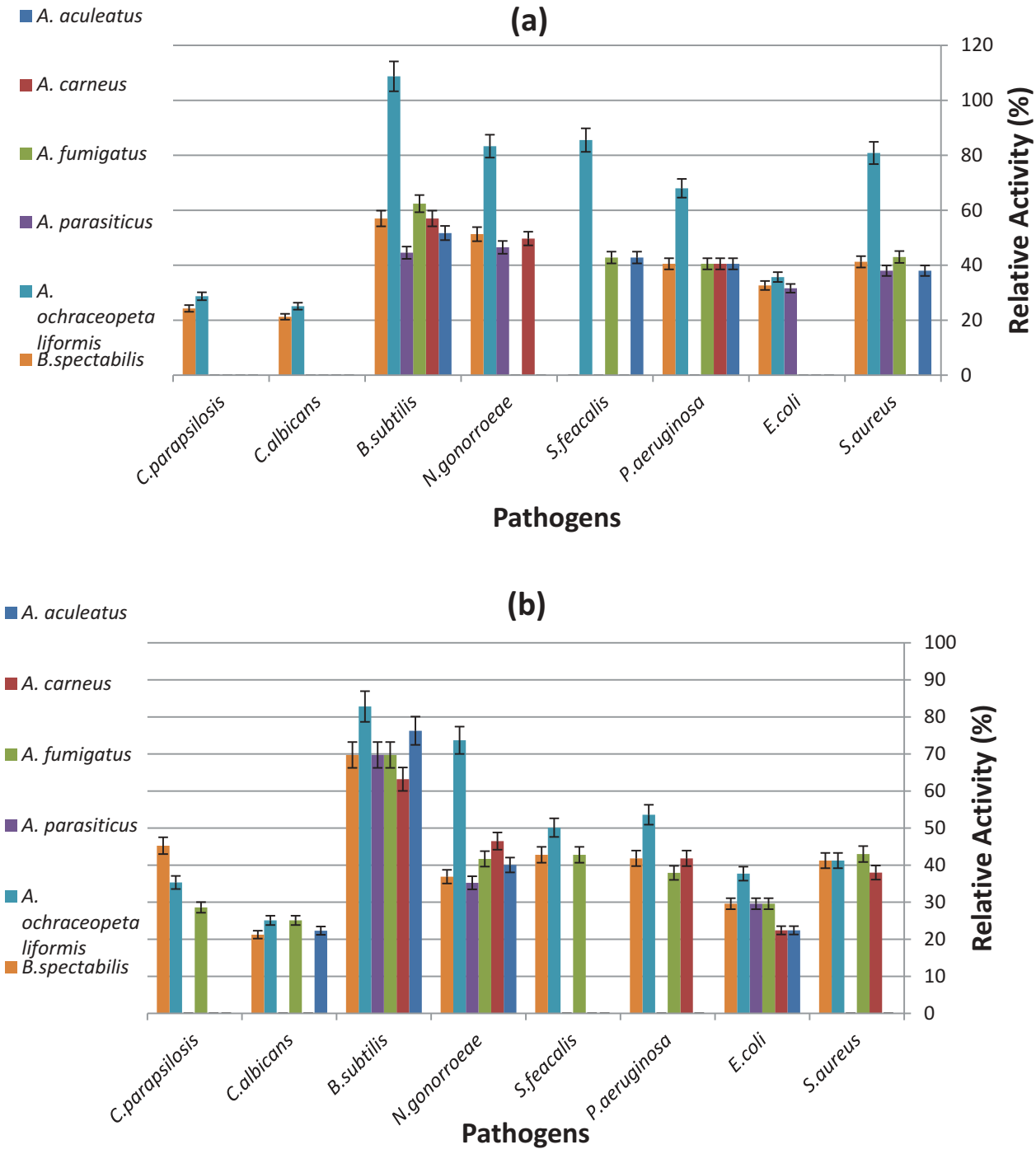

Figure 3. Antimicrobial activity of the most frequent Coral reefs symbiotic fungal species (a) Cultured on DYA, (b) Cultured on RBA.

Free radical scavenging activity of coral reef associated fungi

According to results shown in Figure 4, the growth medium have a role in the antioxidant activity of the coral reef symbiotic fungi. *A. aculeatus* and *A. parasiticus* grown on DYA showed higher antioxidant activity than those grown on RBA. Whereas *A. carneus, A. fumigatus, A. ochraceopetaliformis* MN083316 and *B. spectabilis* exhibited higher antioxidant activities when grown on RBA than on DYA. The descending arrangement of the most potent antioxidant fungal species grown on DYA was *A. ochraceopetaliformis* (19.5%) > *A. aculeatus* (15.6%) > *B. spectabilis* (14.2%) > *A. carneus* (11.3%) > *A. parasiticus* (11%) > *A. fumigatus* (10%). However,

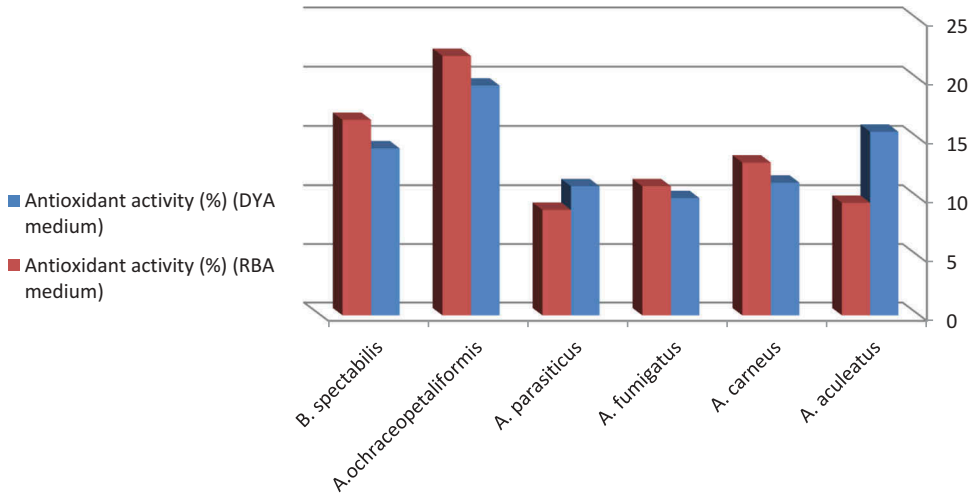

Figure 4. Antioxidant activity of the filtrate extract of the most frequent fungal species isolated from Red Sea Coral reefs.

A. ochraceopetaliformis (22%) > *B. spectabilis* (16.6%) > *A. carneus* (13%) > *A. fumigatus* (11%) > *A. aculeatus* (9.6%) > *A. parasiticus* (9%) were descendly arranged in case of RBA. From the previous results, we declare that *A. ochraceopetaliformis* MN083316 is the most active antioxidant isolate when grown on both media DYA and RBA. Similar results were obtained by (Mabrouk et al. 2008), who reported that fungi associated with the soft coral *Sinularia* sp. collected from Hurghada coast, Red Sea, Egypt, showed antioxidant and antimicrobial activities.

Cytotoxicity of coral reef symbiotic fungi against tumour cell line

The cytotoxic activity was assayed in the filtrate extract of the most active coral reef symbiotic fungal species (*A. ochraceopetaliformis* MN083316) which exhibited also the highest antimicrobial and antioxidant activities (Figure 5). The fungal species showed high cytotoxic activity against human liver carcinoma cell line (HEPG-2) with IC_{50} value 18.8 µg/ml. In close relation, the culture filtrate extract of *A. tritici* isolated from the coral *Galaxea fascicularis* collected at Port Dickson, Malaysia, showed cytotoxic and antimicrobial activities (Sabdaningsih et al. 2016). (Hou et al. 2017) isolated cytotoxic compound Aspersymmetide A from *A. versicolor* isolated from a Gorgonian *Carijoa* sp. collected from Weizhou coral reefs in South China Sea. This compound displayed cytotoxic activity against NCI-H292 and A431 cell lines. Chondrosterins A isolated from soft coral associated fungus *Chondrostereum* sp.

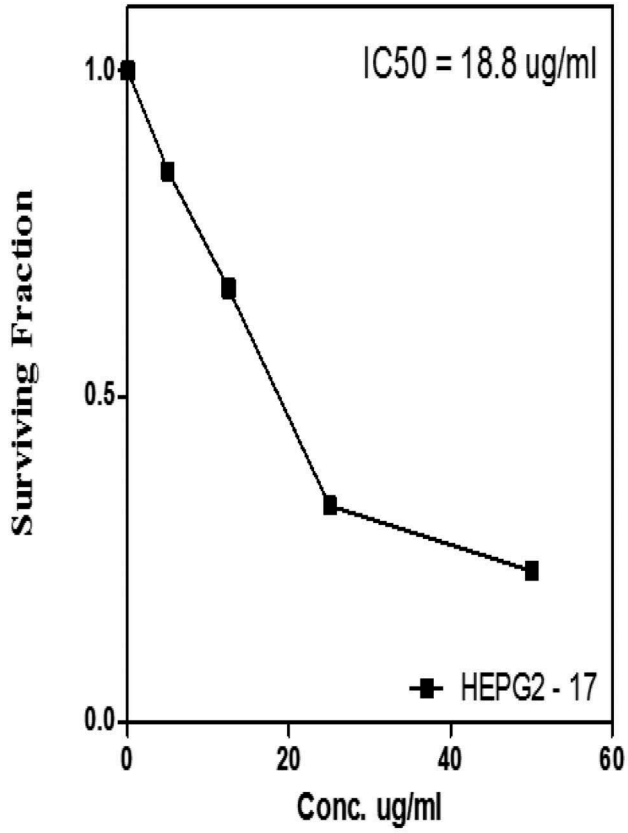

Figure 5. Cytotoxic activity of the filtrate extract of *Aspergillus ochraceopetaliformis* MN083316 isolated from Red Sea Coral species against human liver carcinoma cell line (HEPG-2).

showed potent cytotoxicity against human lung cancer cell line A549, human nasopharyngeal carcinoma cell line CNE2 and human colon cancer cell line Lovo (Li et al. 2012).

The LC-MS fingerprints of the A. ochraceopetaliformis EtOAc extract

In order to correlate the promising biological activities to the active constituents of the ethyl acetate extract of *A. ochraceopetaliformis* MN083316 grown on RBA medium, the LC–MS of the ethyl acetate extract revealed the presence of several metabolites. From the ESI mass spectrum (Figure 6), we could expect the mass of compound **1** appearing at retention time 17.75 min to be 692 deduced from the two molecular ion peaks $[M]^+$ at *m/z* 693 and $[M + Na]^+$ at m/z 715 (Figure 7). Isolation and purification of compound **1** from the crude EtOAc extract was done with RP-HPLC using 70% MeOH/water for 60 min at flow rate 2 ml/min at UV 254 nm to afford compound **1** (6.3 mg). From ^1H (Figure 8), ^{13}C (Figure 9), COSY and HMQC NMR spectra and comparison of this data with similarly isolated compounds we could deduce the structure of **1** to be the diketopiperazine compound ditryptophenaline (Figure 10).

To confirm that compound **1** is the responsible for the promising biological activities of the EtOAc extract of *A. ochraceopetaliformis* MN083316. The antimicrobial, antioxidant and cytotoxic activities of compound **1** was done.

Compound **1**, ditryptophenaline, showed a strong antimicrobial activity against *E. coli, B. subtilis* and *C. parapsilosis* with inhibition zone ranging from 8 to 9 mm at concentration 50 mmole. Also, **1** showed a strong antioxidant activity of about 14.3% at concentration 5 mmole. The cytotoxic activity of **1** against human breast carcinoma (MCF-7) and human liver carcinoma (HEPG2) cell lines was with IC_{50} values of 5.8 and 7.6 mmole, respectively.

Ditryptophenaline was previously isolated for the first time from *Aspergillus flavus* var. *columnaris* (Maes et al. 1986). (Shaaban et al. 2014) tested the crude extract isolated from *A. oryzae* MMAO1 for their antimicrobial and cytotoxic activities and related the antimicrobial activity to ditryptophenaline with other compounds (as constituents of the extract). The extract showed antimicrobial activity against a set of microorganisms include *Bacillus subtilis, Staphylococcus aureus, Streptomyces viridochromogenes* (Tü 57), *Escherichia coli, Candida albicans* and *Mucor miehi*, also showed cytotoxic activity against Brine shrimp.

(Yang et al. 2013b) reported that ditryptophenaline was isolated from mangrove endophytic fungus No.Gx-3a in the sea of South China exhibited strong inhibitory activity on KB and KBv200 cell lines with LD_{50} values of 8.0 and 12.0 μm, respectively.

In this paper, we report the heat map of symbiotic fungal species in relation to their variability in the Red sea coral reefs. The antimicrobial, antioxidant and

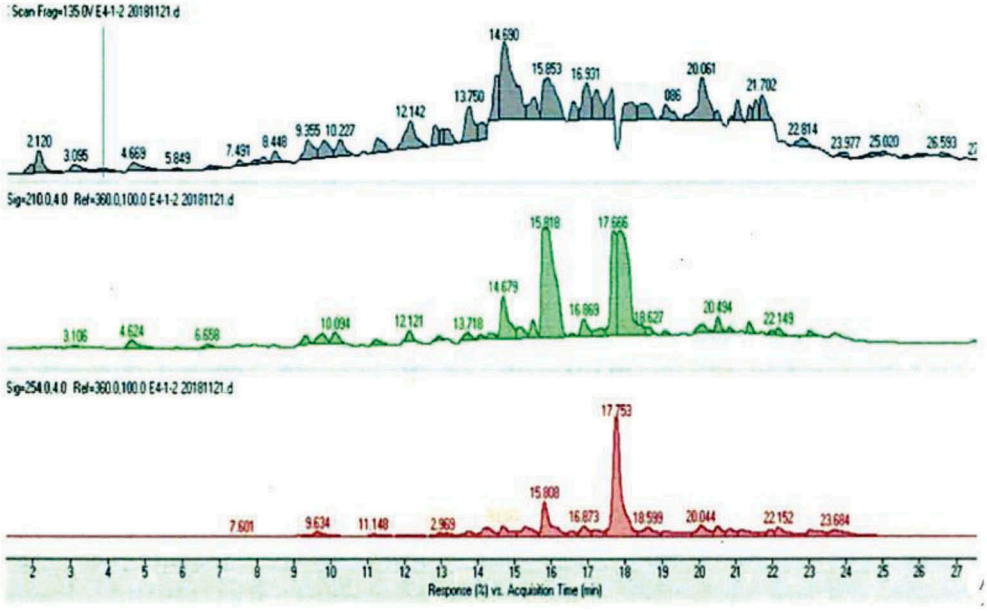

Figure 6. Metabolomic profiling using LC-MS, chromatogram of ethyl acetate extract of *A.ochraceopetaliformis* showing (a) Total ion chromatogram, (b) UV 210 nm and c) UV 254 nm.

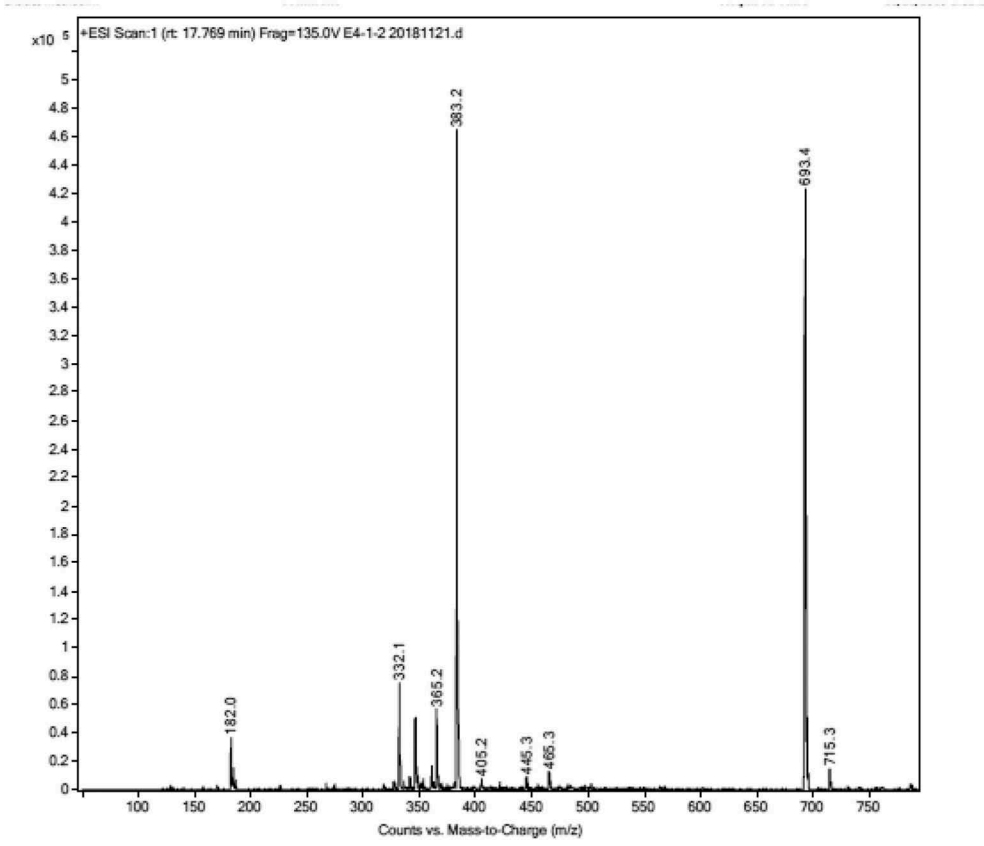

Figure 7. ESI-MS of ditryptophenaline 1.

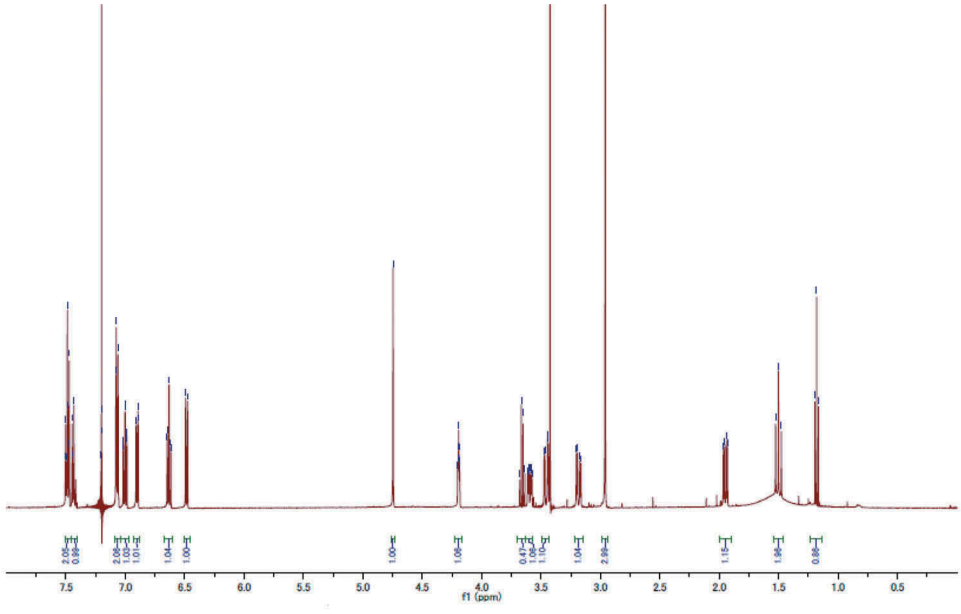

Figure 8. ^{1}H NMR spectrum of 1 in $CDCl_3$ 500 MHz.

cytotoxic activities of the culture filtrates of the isolated fungi were studied. The major compound ditryptophenaline was isolated from the most active fungus, *A. ochraceopetaliformis* MN083316 and found to highly contribute to the biological activities observed.

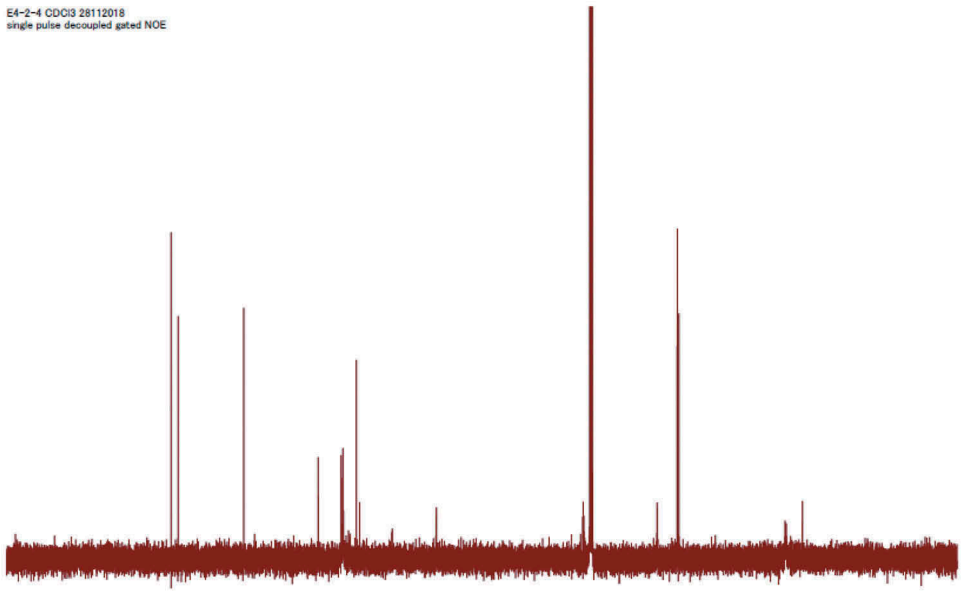

Figure 9. ^{13}C NMR spectrum of 1 in $CDCl_3$ 500 MHz.

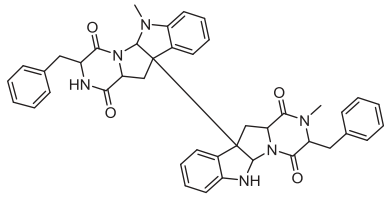

Figure 10. Chemical structure of 1 ditryptophenaline.

Conclusion

Coral reefs either hard or soft represent a host for different groups of microorganisms with biological activities such as antimicrobial, antioxidant and anticancer. These microorganisms can produce active secondary metabolites which might represent a new skeleton or possess a new biological activity which face no resistance from pathogens.

Disclosure statement

No potential conflict of interest was reported by the authors.

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