LC-MS based identification of secondary metabolites from marine antagonistic endophytic bacteria

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Genet. Mol. Res. 17 (1): gmr16039857

Received October 14, 2017

Accepted November 08, 2017

Published December 24, 2017

DOI http://dx.doi.org/10.4238/gmr16039857

ABSTRACT. Halophytes occupy coastal and sub-coastal area of marine environment. They potential candidates for search of novel and new bacterial flora that have immense potential to yield novel therapeutic agents. Six different endophytic bacteria have been isolated from pneumatophores and roots of three halophytes (Salsola imbricata, *Avicennia marina* and *Haplopeplis perfoliata*) collected from western coastal area of Jeddah, Saudi Arabia. After testing against five fungal pathogens all were active against oomycetes fungal pathogens, *Phytophthora capsici* and *Pythium ultimum*. Molecular identification of the bacteria was done on the basis of *16S rRNA* gene sequences which revealed 95.9–99.4% sequence identity to related type strains and were placed in four major genera and two major classes: Actinobacteria (*Streptomyces* and *Nocardioides*) and α-Proteobacteria (*Inquilinus* and *Labrezia*). Active metabolites of these six bacterial endophytes including EA61, EA83, EA85, EA87, EA97 and EA220 were identified by subjecting to chemical analyses using liquid chromatography-mass spectrometry (LC-MS). LC-MS analyses showed presence of different active compounds in the culture extracts of these isolates. Some of these metabolites are already reported as synthetic molecules and has diverse biological functions as antimicrobial, anti-inflammatory and anthelmintic compounds such as such as Sulfamethoxypyridazine, Sulfamonomethoxine, Sulfamerazine and Dimetridazole, Sulfadiazin.

Nalidixic acid and Oxibendazole. This study provides an insight into potential bacterial flora of halophytes producing bioactive metabolites of medical significance.

Key words: Halophytes; Antagonistic bacteria; 16S rRRNA gene sequence; LC-MS analyses; Metabolites identification

INTRODUCTION

The rise in resistant microorganisms to antibiotics is one of the risks in health sector and rate of death is high worldwide due to infectious diseases (Nascimento et al., 2000). Therefore, there is need for discovery of new drugs from different sources to combat against these infectious diseases. Halophytes are salt tolerant plants that inhabit in saline environment such as sand dunes and rocky coastal area. Under these unfavorable conditions of salinity, anaerobic conditions, tides, winds, and high temperatures favor different types of physiological traits to develop and help to withstand in harsh conditions. This habitat enables halophytes under these stressful conditions to include unique and novel microflora with diverse secondary metabolites and biological functions. This microflora of marine plants may be useful in finding the effective and useful biomolecules and drugs for the treatment of human diseases (Haefner, 2003).

Marine flora especially bacteria yielded secondary metabolites that have anti- inflammatory, anticancer, and antimicrobial properties. Halophytes contain different types of active metabolites in their culture extract with antimicrobial activities (Bandaranayake, 2002). Extracts from halophytes have been reported to show biological activities such as antibacterial, antifungal, cytotoxic, neurotoxic and antiviral (Chandrasekaran et al., 2009; Premanathan et al., 2009). As a potential source for such active secondary metabolites halophytes are an ideal source for investigation of associated microorganisms and their bioactive compounds. Microflora of halophytes comprises both rhizospheric and endophytic bacteria which play important role for the host survival and well-being. Halophyte associated microflora produce secondary metabolites and provides several beneficial effects including resistance against plant pathogens (Chung et al., 2003). Halophytes associated bacterial communities are beneficial for the host and perform different functions inside and outside of host by yielding useful enzymes and antibiotics (Roy et al., 2002; Thatoi et al., 2013). Endophytic marine bacteria from halophytes always possess a broad spectrum of antimicrobial activities and help in survival of host against different b bacterial and fungal pathogens (Hu et al., 2010; Jose et al., 2013). Despite of their importance endophytes from halophytes are least studied. There are also few studies from coastal areas of the Red sea and for halophytes associated endophytic bacterial flora.

Recently, for identification of secondary metabolites metabolomics approach has been used to identify metabolites (Rochfort, 2005). For identification of complex metabolites liquid chromatography-mass spectrometry (LC-MS) is used to identify unknown compounds from complex samples. This technique is high-throughput and highly sensitive to for detection and identification of unknown compounds present in biological samples (Villas-Bôase et al., 2005; Lee et al., 2011).

Therefore, we designed a study for identification of the selected six endophytic bacteria isolated from three different halophytes (*Salsola imbricata, Avicennia marina, Haplopeplis perfoliata*) using 16S rDNA sequencing and further identification of metabolites using LC-MS technique. Different bioactive compounds have been identified from culture extract of these bacteria such as Sulfamonomethoxine, Metronidazole-oh, Ibuprofen, Sulfadiazin, Sulfacetamide, Diazepam and Oxibendazole.

MATERIALS AND METHODS

Sample collection and isolation of endophytic bacteria from halophytes

Six different bacterial strains have been isolated in a study (unpublished) from three different halophytes specimens (*Salsola imbricata*, *Avicennia marina* and *Haplopeplis perfoliata*) were collected from coast of Thuwal region in Jeddah, Saudi Arabia. These six bacterial strains were isolated from sterilized roots and pneumatophores after washing with disinfectants as described previously (Bibi et al., 2017). After sterilization of roots leaves and pneumatophores segments, small pieces of sterilized roots, leaves and pneumatophores segments were ground in FAS using sterile mortar and pestle. Aliquots were further serially diluted (10-3, 10-4 and 10-5) and plated in triplicate on half strength R2A (½ R2A) and starch-casein agar (Himedia) in sea water supplemented with cycloheximide and nystatin 50 µg/ml) and plates were incubated at 25°C for 2 weeks for bacterial growth. Pure bacterial strains were further stabbed and stored in 15% (v/v) glycerol stock of strains at -70°C in King Fahd Medical Research Centre and given lab number (Table 1).

Screening for antifungal activity and identification*16S rRNA* gene sequencing

These six bacterial strain were tested against five different fungal pathogens; *Phytophthora capsici* (*P. capsici*), *Pythium ultimum* (*Py. ultimum*), *Magnaporthe grisea* (obtained in this laboratory) *Altenaria malli* (KCTC 6972) and *Fusarium moniliforme* (KCTC 6149) obtained from Korean type culture collection centre (KCTC). Antagonistic activity against fungal pathogens was determined by using cross streak method and identified using *16S rRNA* gene sequencing as described previously (Bibi et al., 2017).

Bacterial DNA extraction of and *16S rRNA* gene sequencing

Genomic DNA was extracted from the selected antagonistic bacterial isolates using a DNA extraction kit (Thermo Scientific, Waltham, USA). To identify antagonistic bacteria, *16S rRNA* gene sequencing was performed. Using bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT -3'), the *16S rRNA* gene fragment was amplified under following PCR conditions: one cycle of 95°C for 5 min followed by 28 cycles of 95°C for 1 min, and annealing at 58°C for 50s with extension at 72°C for 50s, and a final extension step at 72°C for 10 min. PCR products were purified using PCR purification kit (Thermo Scientific, Waltham, USA), and sequenced commercially (Macrogen, South Korea). *16S rRNA* gene sequences obtained were blast using the EzTaxon server (http://eztaxon-e.ezbiocloud.net) (Kim et al., 2012) to identify antagonistic bacteria. Phylogenetic positions of the antagonistic bacteria were confirmed using CLUSTALX (Thompson et al., 1997) multiple alignments of the bacterial sequences were performed and BioEdit software (Hall, 1999) was used to edit the gaps. The neighbour-joining method in the MEGA6 Programme was used for construction of the phylogenetic tree based on the *16S rRNA* gene sequences (Tamura et al., 2013).

Optimization of bacterial culture condition for production of antifungal activity

To optimize culture conditions of selected bacterial strains for the production of antifungal activities, an appropriate medium for culturing was selected. Four different media i.e., ½ R2A broth, ½ TSB, ½NB in sea water and Marine broth in distilled were used for culturing. After every 24 h optical density (OD) was checked and antifungal activity was assessed against *P. capsici*, *Py. ultimum* using disc diffusion method. The effect of temperature was checked at different ranges of temperatures (20°C to 40°C) in ½ R2A broths. For pH optimization, different ranges of pH values (5–12) were used for the growth and antifungal compound production in ½ R2A broth.

LC-MS analysis of bacterial culture

5 ml bacterial culture was placed on -80°C for 5 min, and then transfer to 37°C water bath for 5 min and repeats this procedure 5 times. Centrifuge at 15000 g for 10 min and transfer 3 ml supernatant to tube and add 12 ml acetonitrile and vortex for 30 sec. Centrifuge again at 15000 g for 10 min and 300 µl supernatant was taken for LC-MS metabolomics analysis. Injection volume was 3 µl and samples are analyzed on Agilent 6540 B TOF/Q-TOF Mass Spectrometer coupled with Agilent 1290 UPLC and Dual AJS ESI ion source. An ACQUITY UPLC HSS T3 (100 × 2.1 mm, 1.8 µm) column and pre-column (Phenomenex Security Guard™) is used to separate sample. Column temperature was set to 45°C and flow rate was 0.5 ml/min. Acquisition range was from 50 m/z to 1500 m/z and scan rate was 1.00 spec/sec. MS parameters was set as follow: capillary voltage 3500 V, nebulizer pressure 35 psi, drying gas 10L/min, gas temperature 325°C, vaporizer 200V, voltage charge 1000 V; negative-ion mode capillary voltage 3500 V, corona negative 15.0 V, fragmentor 175 V, skimmer1 65.0 V, octopole RF Peak 750 V; positive ion mode capillary voltage 3500 V, corona positive 4.0 V, fragmentor 175 V, skimmer1 65.0 V and octopole RF Peak 750 V. Raw data was imported to Agilent Mass Hunter Qualitative Analysis B.06.00 software. Metabolites were identified by in-house database.

RESULTS

Isolation and screening of endophytic bacteria from halophytes

In this study, three halophytes samples were collected from western coastal area of Jeddah and endophytic bacteria were isolated from roots and pneumatophores of halophytes. These six bacterial endophytes were further screened for their antagonistic activity against five pathogenic fungi i.e., *Pythium ultimum*, *Phytophthora capsici*, *Magnaporthe grisea*, *Altenaria mali* and *Fusarium oxysporum*. Six endophytes showed activity against both *Py. ultimum* and *P. capsici* and some were not active against other fungal pathogens tested. Strain EA61 showed activity against four fungal pathogens and were negative against *F. oxysporum*. Similarly strain EA97 and EA220 were positive against *Py. ultimum*, *P. capsici* and *M. grisea* while negative for other two while strain EA83, EA85 and EA87 were only active against *Py. ultimum* and *P. capsici* oomycetes fungi (Table 1).

Identification of antagonistic bacteria based on *16S rRNA* gene sequence

Six antagonistic bacteria were identified by using *16S rRNA* gene sequence analysis. Four of them, EA61, EA83, EA85 and EA87 belong to Actinobacteria. While strain EA97 and strain EA220 belong to α-Proteobacteria (Table 1). Sequence identity of antagonistic bacteria was from 95.9% to 99.4% (Table 1). The phylogenetic tree inferred using *16S rRNA* gene data showed that branching patterns remained constant. High bootstrap values were recorded in the phylogenetic tree using *16S rRNA* gene sequences data (Figure 1). Two different clusters have been generated for isolates of class Actinobacteria. Antagonistic strains of class Actinobacteria were placed in a separate cluster recovered with higher bootstrap values of 99% to 100%.

Table 1. Taxonomic identification, antifungal activity and enzymes production of rhizo and endophytic bacteria from halophytes.

Lab no	[a]Closely related type strain	Accession number	[b] % identity	Class	*Py. ultimum*	*P. capsici*	*M. grisea*	*A. mali*	*F. oxysporum*
	Salsola imbricata								
	Roots								
EA61	*Streptomyces enissocaesilis* NBRC 100763^T	KY436434	99.4	**Actinobacteria**	+++	+	+	+++	-
	Avicennia marina								
	Pneumatophores								
EA83	*Nocardioides aromaticivorans* H-1^T	KY436456	99.4	**Actinobacteria**	+	+	-	-	-
EA85	*Streptomyces spectabilis* NBRC 13424 ^{T}	KY436458	95.9	**Actinobacteria**	+	++	-	-	-
EA87	*Nocardioides albus* KCTC 9186 ^{T}	KY436460	99.1	**Actinobacteria**	++	+	-	-	-
	Roots								
EA97	*Inquilinus limosus* DSM 16000 ^{T}	KY436470	96.4	**Alphaproteobacteria**	+++	+	+	-	-
	Haplopeplis perfoliata								
	Roots								
EA220	*Inquilinus alexandrii* DFL-11^T	KY234242	98.1	**Alphaproteobacteria**	+	+	+	-	-

[a]Identification based on partial *16S rRNA* gene sequence analyses of all antagonistic bacteria; [b]% similarity with closely related type strain; [c]Antagonistic activity of all bacteria isolated in this study. The activity was measured after 3-5 days incubation at 28°C by measuring the clear zone of mycelial growth inhibition: -, Negative; +, 3 mm; ++, between 4 mm to 6 mm; +++, between 7 to 9 mm.

Antagonistic bacteria in Actinobacteria mainly belonged to the genera *Nocardioides* and *Streptomyces.* Representative isolates in this class belong to four different genera i.e., *Nocardioides*, *Arthrobacter*, *Streptomyces* and *Mycobacterium*. Two strains of α-Proteobacteria were palced in two separate clusters also showing high bootstrap values (91% to 100%).

The representative strains of α-Proteobacteria belong to two different genera i.e., *Labrenzia* and *Inquilinus*. Two strains EA85 and EA97 were novel and new antagonistic endophytic bacterial strains showing low *16S rRNA* gene sequence similarity (<97%) (Table 1). In phylogenetic analysis *Bacillus subtiils* was used as an out group.

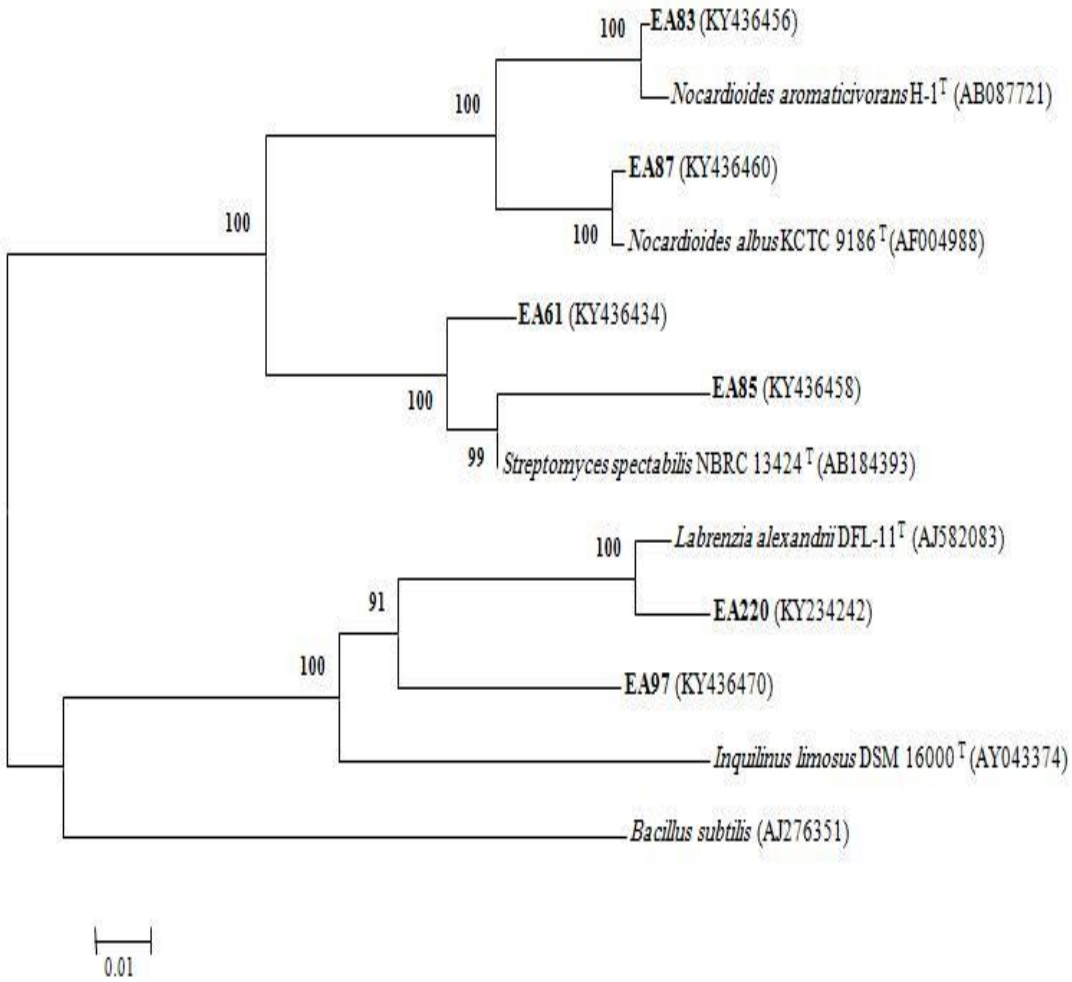

Figure 1. Phylogenetic distribution of endophytic antagonistic bacteria isolated from halophytes on the basis of *16S rRNA* gene sequences obtained from bacteria and closely related sequences of the type strains of other species. The phylogenetic relationships were inferred from the *16S rRNA* gene by using the neighbour-joining method from distances computed with the Jukes-Cantor algorithm. Bootstrap values (1,000 replicates) are shown next to the branches. GenBank accession numbers for each sequence are shown in parentheses. Bar 0.01 accumulated changes per nucleotide. Isolates selected for bioactive metabolites identification are highlighted.

Culture condition optimization and identification of metabolites by LC-MS

Culture media tested for optimization of bacterial growth ½ R2A broths was found to be the best culturing media. Selected strains showed best growth at pH 7.5 and 28°C in the shaking incubator (140 rpm). After optimization of conditions, selected bacterial strains were grown in 5 ml ½ R2A broths in sea water for 36-48 h until OD600 reached 0.9. LC-MS analyses of culture of these six strains identified different chemical constituents (Figures 2a and 2b and Table 2).

LC-MS analysis was performed to identify known and unknown metabolites from culture extract. LC-MS analysis showed presence of various active metabolites in culture extract of all six bacteria strains. All of these compounds although are not new but are known as synthetic molecule and not have their origin from bacteria. Identification of metabolites was determined by LC-MS analysis and comparing results from NIST database. Strain of *Streptomyces* sp. (EA61) showed peaks for only four secondary metabolites in both the positive- and negative-ion mode (Figures 2a and 2b).

Table 2. Secondary metabolites detected in crude extract of bacteria isolated from halophytes.

S. No.	Ion mode	ID Source	Name	Formula	RT	Precursor	Mass	Score	Relative Reference Mass difference	Area
	Strain EA61									
1	Negative	DBSearch	Sulfamonomethoxine	C11 H12 N4 O3 S	1.12	279.0569	280.0643	87.35	-4.7	209272
2	Negative	DBSearch	Metronidazole-oh	C6 H9 N3 O4	2.692	232.0571	187.059	96.71	1.67	633292
3	Negative	DBSearch	Ibuprofen	C13 H18 O2	17.591	265.1451	206.1309	89.31	-1.02	898447
4	Positive	DBSearch	Dimetridazole	C5 H7 N3 O2	4.061	159.0879	141.0531	75.73	5.19	1044068
	Strain EA83									
5	Negative	DBSearch	Sulfamonomethoxine	C11 H12 N4 O3 S	1.112	279.0555	280.0629	76.23	0.46	128199
6	Negative	DBSearch	Metronidazole-oh	C6 H9 N3 O4	2.661	232.0568	187.0586	95.49	3.59	673424
7	Negative	DBSearch	Sulfadiazin	C10 H10 N4 O2 S	2.673	249.0463	250.0538	85.56	-5.4	225564
8	Negative	DBSearch	Sulfaethoxypyridazine	C12 H14 N4 O3 S	3.158	293.0718	294.0794	90.29	-2.59	173652
9	Negative	DBSearch	Ibuprofen	C13 H18 O2	17.346	265.1448	206.1304	86.87	1.19	514480
10	Negative	DBSearch	Gemfibrozil	C15 H22 O3	18.289	309.1704	250.1565	83.38	1.68	204221
11	Positive	DBSearch	Dimetridazole	C5 H7 N3 O2	4.061	159.0878	141.0539	47.56	-0.69	897920
12	Positive	DBSearch	Nalidixic acid	C12 H12 N2 O3	9.72	233.0923	232.085	86.9	-0.87	567512
	Strain EA85									
13	Negative	DBSearch	Sulfamonomethoxine	C11 H12 N4 O3 S	1.115	279.0565	280.064	88.74	-3.53	328203
14	Negative	DBSearch	Sulfadiazin	C10 H10 N4 O2 S	2.677	249.0463	250.0538	85.59	-5.31	290107
15	Negative	DBSearch	Ibuprofen	C13 H18 O2	17.599	265.1449	206.1307	87.71	-0.05	699256
16	Positive	DBSearch	Metronidazole-oh	C6 H9 N3 O4	4.06	188.066	187.0587	91.77	3.32	17067054
	Strain EA87									
17	Negative	DBSearch	Allopurinol	C5 H4 N4 O	1.067	135.0308	136.0381	85.77	2.83	623417
18	Negative	DBSearch	Nalidixic acid	C12 H12 N2 O3	2.501	277.0835	232.0849	87.24	-0.56	1163972
19	Negative	DBSearch	Ibuprofen	C13 H18 O2	17.595	265.1452	206.131	88.46	-1.5	790367
20	Positive	DBSearch	Sulfacetamide	C8 H10 N2 O3 S	1.807	215.0478	214.0405	75.53	3.14	1181016
21	Positive	DBSearch	Metronidazole-oh	C6 H9 N3 O4	4.063	188.0667	187.0596	94.09	-1.36	21182800
	Strain EA97									
22	Negative	DBSearch	Nalidixic acid	C12 H12 N2 O3	2.494	277.0833	232.0848	87.87	-0.03	1819479
23	Negative	DBSearch	Diazepam	C16 H13 Cl N2 O	2.526	283.0655	284.0731	61.02	-4.99	337634
24	Negativ	DBSearc	Ibuprofen	C13 H18 O2	17.58	265.1453	206.131	86.0	-2.1	815052

	e	h			8		1	8		
25	Positive	DBSearch	Sulfacetamide	C8 H10 N2 O3 S	1.809	215.0485	214.0414	93.75	-0.72	1278958
26	Positive	DBSearch	Metronidazole-oh	C6 H9 N3 O4	4.066	188.0673	187.06	89.3	-3.79	21453990
	Strain EA220									
27	Negative	DBSearch	Allopurinol	C5 H4 N4 O	1.057	135.0308	136.0381	85.64	2.92	491705
28	Negative	DBSearch	Diazepam	C16 H13 Cl N2 O	2.02	283.0658	284.0731	61.81	-5.31	293775
29	Positive	DBSearch	Oxibendazole	C12 H15 N3 O3	3.513	250.1195	249.1122	95.63	-3.57	483436

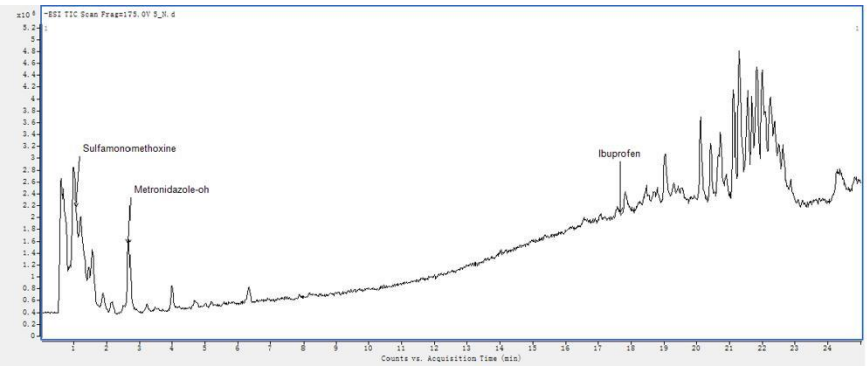

Figure 2a. Negative mode LC/MS analysis of *Streptomyces sp.* (EA61)

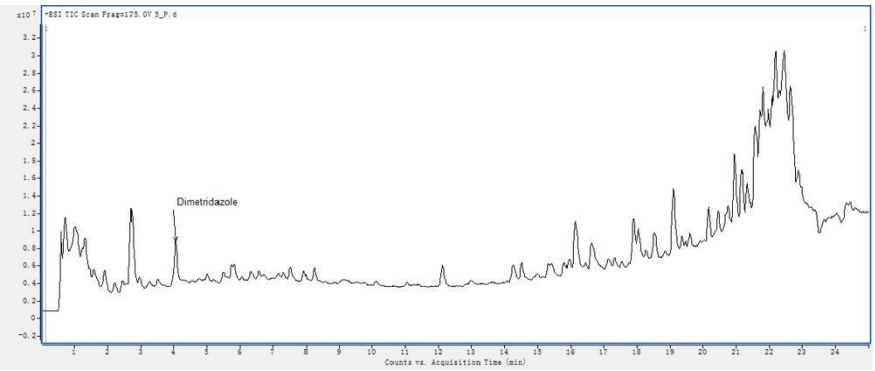

Figure 2b. Positive mode LC/MS analysis.

These compopunds include Sulfamonomethoxine, Metronidazole-oh, Ibuprofen and Dimetridazole. For non-*Streptomyces, Nocardioides* sp. (EA83), eight different peaks were identified for active compounds including Sulfamonomethoxine, Metronidazole-oh, Sulfadiazin, Sulfaethoxypyridazine, Ibuprofen, Gemfibrozil, Dimetridazole and Nalidixic acid were detected in the both positive and negative-ion mode (Figures 3a and 3b).

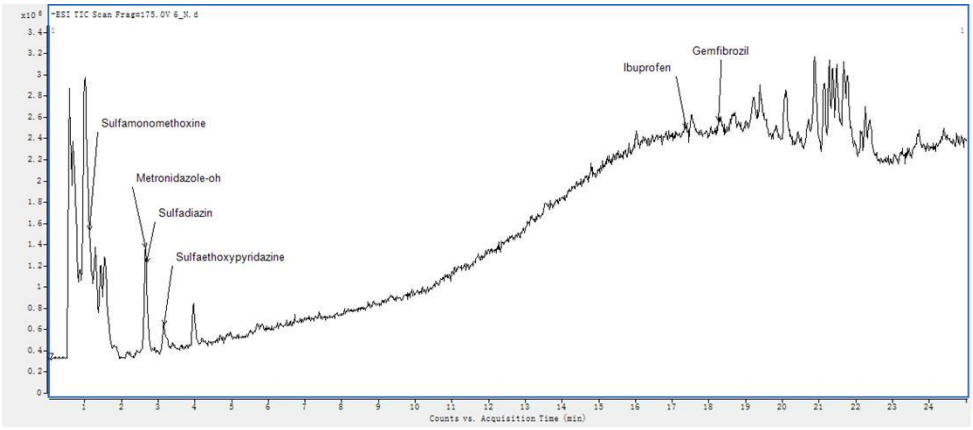

Figure 3a. Negative mode LC/MS analysis of *Nocardioides sp.* (EA83)

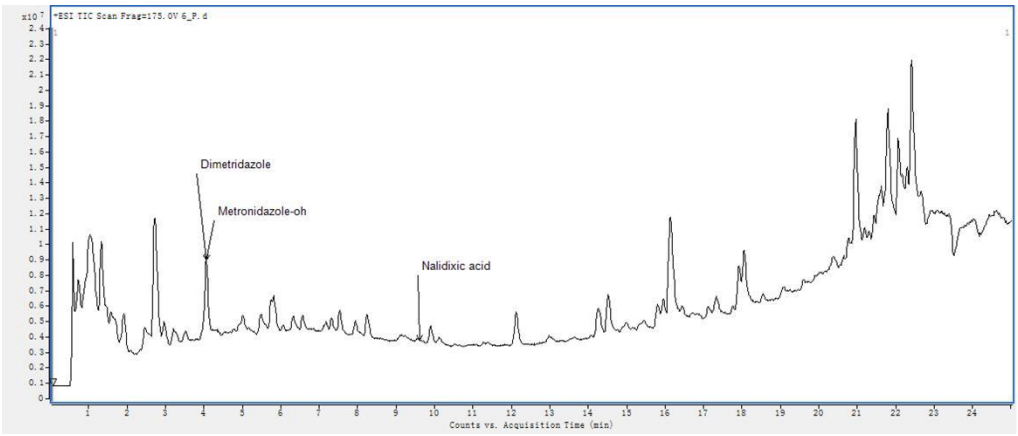

Figure 3b. Positive mode LC/MS analysis.

Strain of *Streptomyces* (EA85) showed the presence of 4 bioactive compounds in their culture extract i.e., Sulfamonomethoxine, Sulfadiazin, Ibuprofen, and Metronidazole-oh (Figures 4a and 4b).

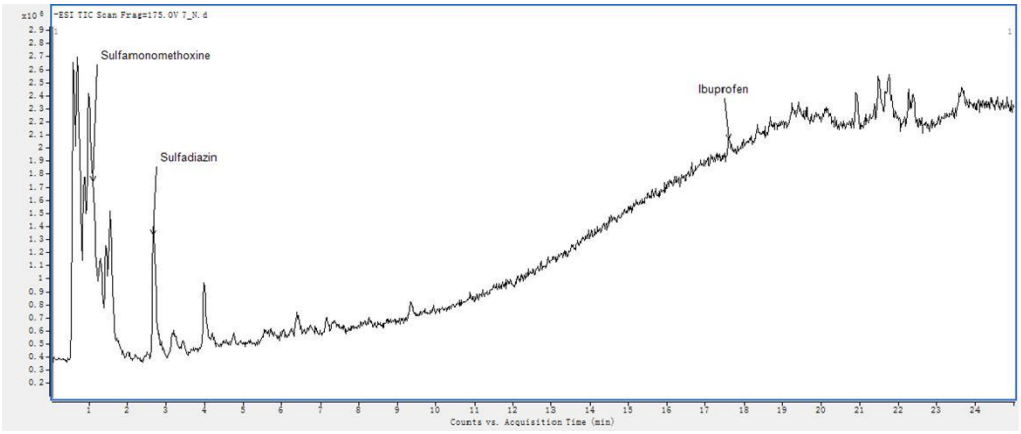

Figure 4a. Negative mode LC/MS analysis of *Streptomyces sp.* (EA85)

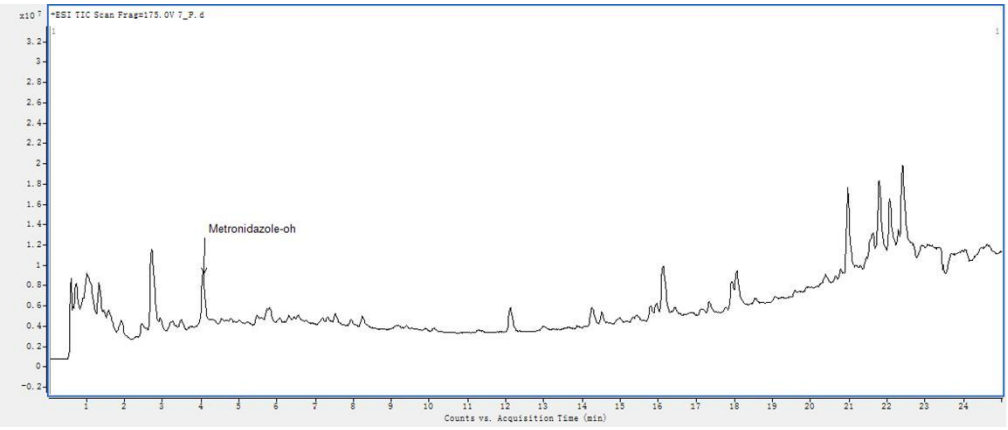

Figure 4b. Positive mode LC/MS analysis.

Another non-*streptomyces*, *Nocardioides* sp. (EA87) showed peaks of only five active metabolites in both positive- and negative-ion mode have been detected among hundred others. These bioactive metabolites include Allopurinol, Nalidixic acid, Ibuprofen, Sulfacetamide and Metronidazole-oh (Figures 5a and 5b).

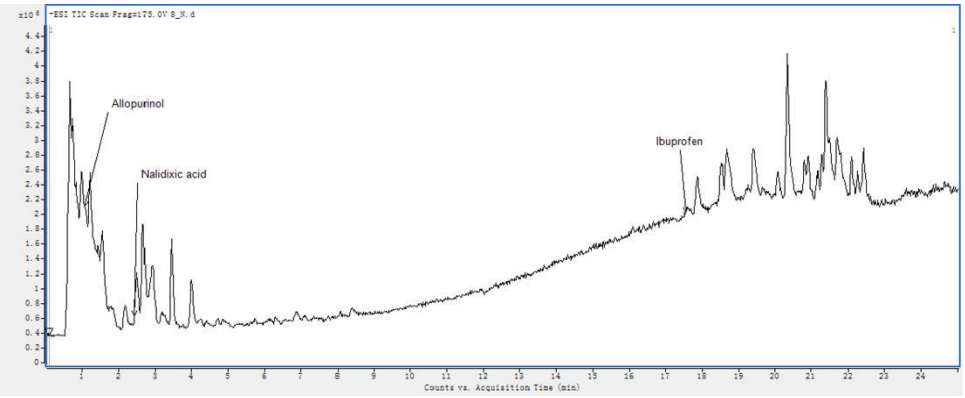

Figure 5a. Negative mode LC/MS analysis of *Nocardioides sp.* (EA87)

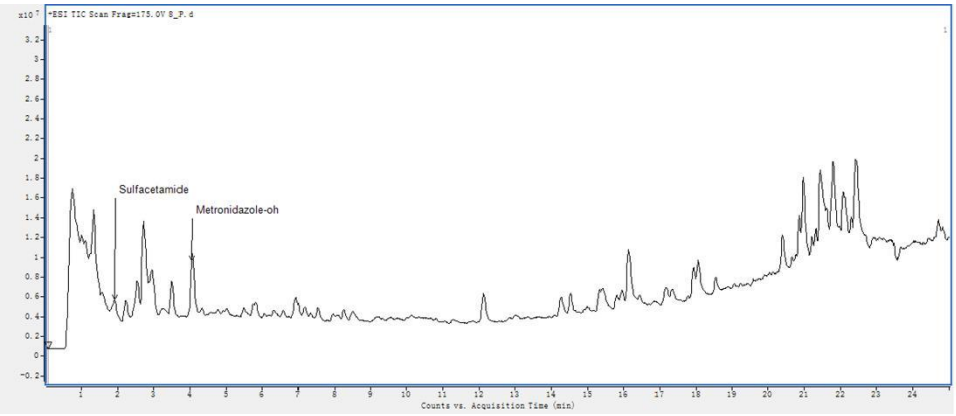

Figure 5b. Positive mode LC/MS analysis.

For strain EA97 closely related to *Inquilinus limosus*, four active compounds have been detected among hundreds of peaks for different metabolites. These four compounds include Nalidixic acid, Diazepam, Ibuprofen, Sulfacetamide and Metronidazole-oh (Figures 6a and 6b).

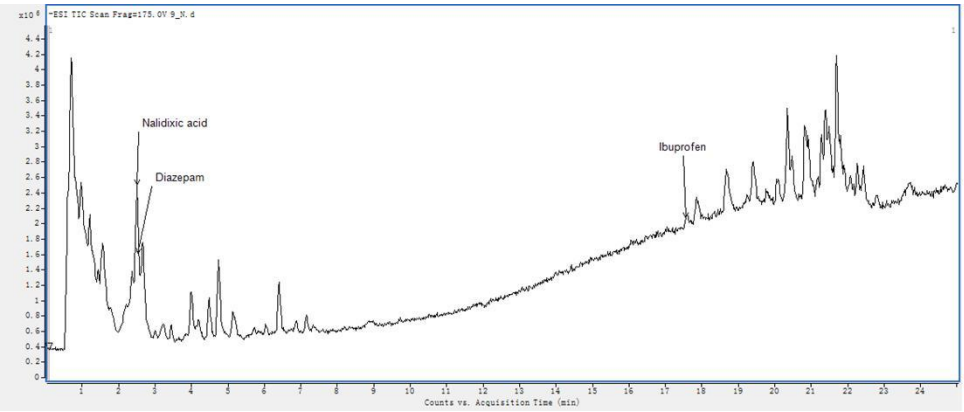

Figure 6a. Negative mode LC/MS analysis of *Inquilinus sp.* (EA97)

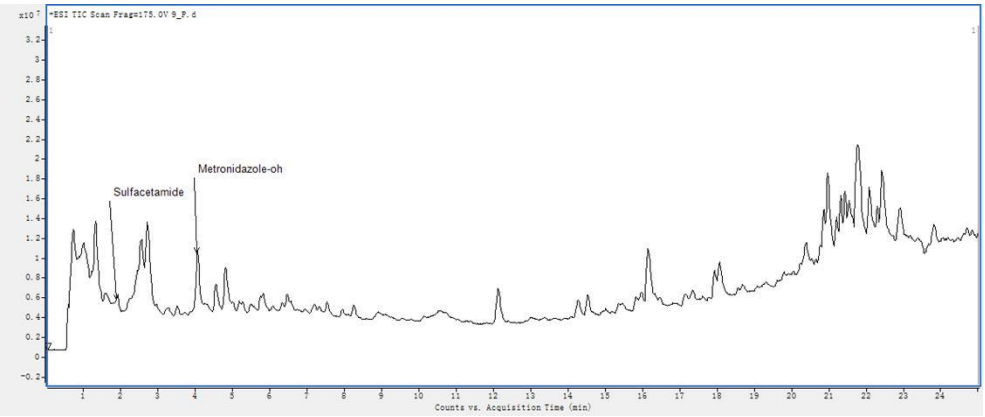

Figure 6b. Positive mode LC/MS analysis.

LC-MS analysis of strain EA220, *Labrenzia alexandrii* showed the presence of three bioactive compounds among several compounds detected. These bioactive compounds include Allopurinol, Diazepam and Oxibendazole (Figures 7a and 7b). These bioactive secondary metabolites are already known for their activities.

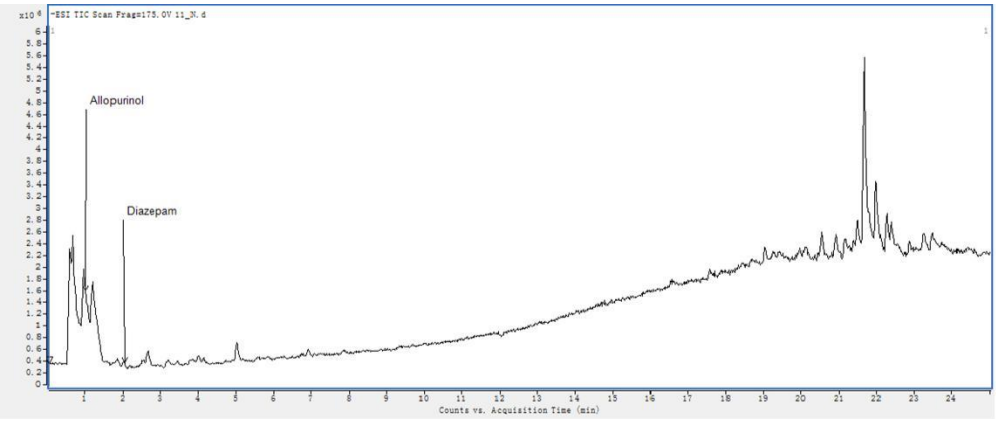

Figure 7a. Negative mode LC/MS analysis of *Labrenzia sp.* (EA220)

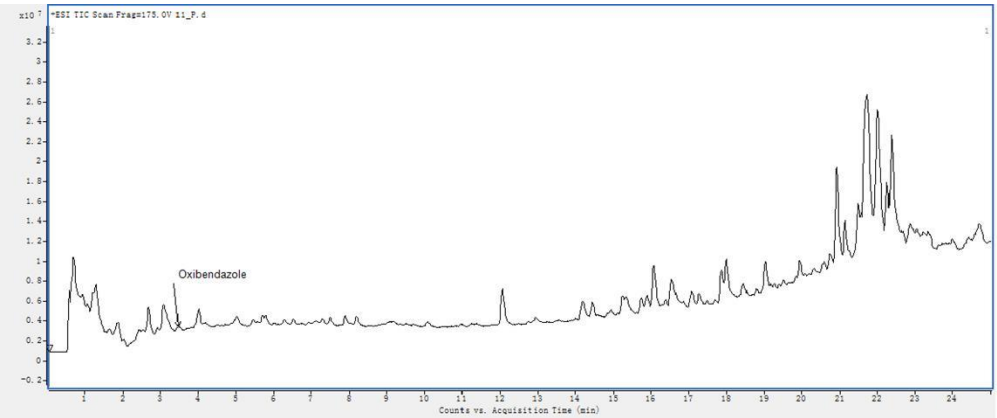

Figure 7b. Positive mode LC/MS analysis.

DISCUSSION

Due to emergence of resistant bacteria to different antibiotics there is need of discovering new drugs to combat different infectious diseases. Currently, marine environment especially halophytes under extreme environmental conditions become a source for discovery of new therapeutic agents (Malve, 2016). These harsh conditions lead them to produce certain metabolites enabling them to survive under these harsh conditions (De Carvalho and Fernandes, 2010). In this study, we have selected six different antagonistic bacteria active against fungal pathogens. These endophytic bacteria were isolated from roots and Pneumatophores of different halophytes mentioned in Table 1. Secondary metabolite production by endophytic bacteria is one of the phennomenon used by bacteria to defend host against different pathogens and predators (Coombs et al., 2004). Halophytes associated bacterial flora has potential to produce different bioactive metabolites (Hu et al., 2010). These bacterial endophytes belong to two different classes of Actinobacteria and α-Proteobacteria. Four strains, EA61, EA83, EA85 and EA87 belong to two different genera *Streptomyces* and *Nocardioides* placed in Actinobacteria i.e., Class α-Proteobacteria also comprises of two different strains EA97 and EA225 belong to genera *Inquilinus* and *Labrenzia*.

Marine actinomycetes are major source for discovery of new and novel natural products. Marine Actinobacteria produce novel antimicrobial and anticancer compounds such as salinosporamides, potential anticancer agent isolated from species of *Salinispora* and are in clinical trials for use as anticancer agents (Fenical et al., 2006). Previously, different strains of Actinobacteria isolated from halophytes showed antimicrobial activity against different human pathogenic bacteria (Lee et al., 2014). Halophytes growing near coastal areas are potential source for isolation of microbial flora especially Actinobacteria because of chemodiversity in environmental factors of that habitat. Presence of major numbers of *Streptomyces* strains amongst antagonists from halophyte are in accordance previous studies (Eccleston et al., 2008; Ravikumar et al., 2012).

From marine sources different strains of α-Proteobacteria were reported to be in symbiosis, nitrogen fixers and capable of producing antibiotics (Wagner-Döbler et al., 2002; Castro et al., 2014). Antibiotic production by *Streptomycetes* is significant feature and new bioactive compounds have been isolated from different environment (Fiedler et al., 2005). Most of the endophytic bacteria in our study belong to Actinobacteria and were recovered from roots and pneumatophores. Several studies reported diverse bioactive compounds including steroids, peptides, alkaloids, terpenoids, quinines, flavonoids and phenols from halophytes associated endophytic bacteria (Newman and Cragg, 2007). Inside halophyte, endophytic *Streptomycetes* are important niche, taking nutrients from host and in turn provide protection against different pathogens. These endophytic bacteria produce metabolites that are not toxic to host plant and are important bioactive metabolites in drug discovery (Castillo et al., 2007; Moyer, 2009).

Production of antifungal metabolite was enhanced by culturing in R2A as culture media using optimum culture conditions. The maximum antifungal activity was observed after 48hrs of growth at 28°C with pH 7.5. We used LC-MS technique mainly focus on polar metabolites (especially phosphate-containing compounds), most of which cannot be analyzed using GC-MS. LC-MS confirms presence of various active compounds although not novel but already known for their bioactivity. Two strains of endophytic *Streptomyces* (EA61 and EA83) produce Sulfamonomethoxine, Sulfadiazin, Metronidazole-oh, Ibuprofen and Dimetridazole. These compounds are known for antimicrobial, antiphytopathogenic and as biocontrol agents. These antibacterial sulfonamides are

synthetic antimicrobial agents and also used as antibiotic in different inflammatory diseases (Vicente and Pérez-Trallero, 2010). Two endophytic bacteria belong to genus *Nocardioides* showed spectra of ten different active compounds including antibacterial, antifungal and antiprotozoal compounds (Fig. 3a, b and 5a,b). Both these strains EA83 and EA87 showed presence of Sulfamonomethoxine, Metronidazole-oh, Sulfadiazin, Sulfaethoxypyridazine, Ibuprofen, Gemfibrozil, Dimetridazole and Nalidixic acid. While in strain EA87 Allopurinol and Sulfacetamide also detected (Table 2). These antagonistic marine Actinobacteria contain variety of bioactive compounds and showed wide range of activities including cytotoxicity, antibacterial, antifungal and anti-angiogenesis. As Gesheva and Vasileva-Tonkova (2012) reported production of different antimicrobial compounds by marine species of *Nocardioides*. Strain EA83 showed close similarity of 99.4% on the basis of 16S rDNA to type strain of *Nocardioides* aromaticivorans H-1T that was previously isolated from contaminated river in Japan and ability to degrade both dibenzofuran and carbazole (Kubota et al., 2005). No antimicrobial compound yet reported from this strain as detected in our strain EA85. Similarly other strain EA87 has close relatedness to *Nocardioides albus* KCTC 9186 T. This strain of *Nocardioides* is already known for production of antimicrobial compounds such as rodaplutin (Dellweg et al., 1988). Bioactive compounds detected in strain EA87 are different and not reported before. Such as sulfacetamide is synthetic antibiotic effective against both gram-positive and gram-negative bacteria no reported before from any natural source? Two endophytic strains of α-Proteobacteria, EA97 and EA220 were effective against oomycetes fungi as well as against M. grisea. These both endophytic bacteria related to type strains of *Inquilinus limosus* DSM 16000 T and Labrezia alexandrii DFL-11T respectively. These strain showed spectra for seven different bioactive compounds among hundred others present in their culture extract. These compounds are mainly Nalidixic acid, Diazepam, Ibuprofen, Sulfacetamide, Metronidazole-oh, Allopurinol and Oxibendazole. These bioactive metabolites from our study have medicinal uses due to antimicrobial, anti-inflammatory and anthelmintic activities. No such compound has been reported before in strains of Inquilinus and Labrezia. According to LC-MS analyses, all compounds detected in these six selected endophytic bacteria have their pharmaceutical and medicinal use and reported as anti-inflammatory, anthelmintic, antibacterial and antifungal compounds. Antifungal activities detected in these six strains are due to production of these bioactive metabolites secreted by antagonistic endophytic bacterial strains.

CONCLUSION

In this study, six antagonistic endophytic bacteria inhabiting pneumatophores and roots of halophytes from coastal area of Saudi Arabia have been screened against pathogenic fungi and their potential active metabolites have been identified. Strains exhibited spectra of different bioactive compounds including known antibiotics and pharmaceutical compounds of synthetic nature not reported from natural source such as bacteria. These results suggest that marine coastal plants are reservoir of antagonistic bacterial flora which are potential source of bioactive metabolites and can be used in medicine and as biocontrol agent as well.

ACKNOWLEDGMENT

This work was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant No. (1433/141/430). The author, therefore, acknowledge with thanks DSR technical and financial support.

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