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Antimicrobial activities of five bacterial isolates associated with two Red Sea Sponges and their potential against multidrug resistant bacterial pathogens

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ABSTRACT

Sponges are sessile animals and important filter feeders belonging to the phylum Porifera. These organisms have developed efficient defense mechanisms, harboring bacterial communities capable of producing bioactive compounds. These compounds may aid in antibiotic production research since they were shown to be active against bacteria of medical importance. The current study aimed at the isolation and identification of some sponge-associated bacteria and to evaluate their potential as antimicrobial producers. Five Gram-positive bacterial isolates were isolated from the two sponge species; *Ircinia echinata and Amphimedon* sp., collected from the Egyptian coast of the Red Sea on Egypt (Hurghada).The 5 bacterial isolates were coded as HA1, HA2, HA3, HA4 and HA5, and identified by 16S rDNA analysis as *Bacillus* sp., *Lysinibacillus boronitolerans*, *Planomicrobium flavidum*, *Bacillus safensis* and *Bacillus pumilus*; respectively. The bacterial isolates were evaluated for their antimicrobial activity as well as multidrug resistant pathogens. The results revealed that three of the isolates had strong antimicrobial potential towards multidrug resistant bacteria. Meanwhile, two isolates showed no vast antimicrobial potential. These findings suggested that the marine bacteria may represent a promising source of antimicrobial agents, as an important strategy for developing alternative therapies for treating infectious diseases caused by multidrug-resistant bacteria.

INTRODUCTION

Oceans represent more than 70% of the earth's surface, however being to a large extent unexplored. The improvement about modern applied sciences that facilitate sub-sea sampling yet harvesting has widened the accessibility after areas under ocean level (Synnes, 2007). This leaded to isolation concerning bioactive compounds from marine organisms against viral, bacterial, parasitic and fungal diseases (Mohamed Shreadah *et al*., 2018; Abdelmohsen *et al*., 2017 and Anjum *et al*., 2016).

Marine natural products are superb within theirs structural/chemical features or t herefore of their pharmacological properties than the herbal products (Ibrahim *et al*.,

2016 and Kiuru *et al*., 2016). Marine sponges, belong to the phylum Porifera (Andersen, 2017 and Anjum *et al*., 2016), attracted researchers and industrial sectors due to their ability to produce a variety of bioactive secondary metabolites that have many applications including drug discovery (Mioso *et al*., 2017; Kobayashi, 2016 and Garcia-Vilas *et al*., 2015). About 5000 compounds to date were isolated from sponges worldwide, accounting for about 30% of all compounds obtained from the marine environment so far. Approximately, more than two hundred discovered bioactive compounds derived from sponges are reported yearly since the last decade (Hu *et al*., 2011).

It is hypothesized that marine sponges followed different metabolic pathways in order to produce such unique and diverse bioactive components to support sponges' survival in the marine environment. This develops defense against microbial infections towards the competitive diversity of microorganisms in the surrounding environment (Mehbub *et al*., 2014 and Roue *et al*., 2012). Many medications derived from sponges are presented in the market and others are in clinical trials including Eribulin Mesylate, Cytarabine, and Vidarabine (Martins *et al*., 2014).

The unique geographical position of the Red sea in the desert attached with its unique physical (such as depth and relatively high surface temperature) and chemical (high salinity) features present great biological diversity not existing elsewhere (Behzad *et al*., 2016). These features of the Red Sea offer the chance for bioprospecting of natural products with medicinal and nutritional importance. For example, a prior study that was carried out on extracts from five sponge species has shown encouraging antimicrobial activity against a wide range of pathogenic microbes (Perveen *et al*., 2002). Metabolites: sipholenol A and 2,10-dibromo-3-chloro-7-chamigrene that was isolated from Red Sea *Siphonochalina siphonella* extracts showed potent antifouling effects (Al-Lihaibi *et al*., 2015). Moreover, chemical agents isolated from marine sponges showed cytotoxic effect against many cancer cell lines including HepG2, MCF-7, and Vero as well as their hepatoprotective potential (Aboul Ela *et al*., 2012; Abdel-Lateff *et al*., 2016; Alarif *et al*., 2016; Abdel Monein *et al*., 2017 and Shreadah *et al*., 2019).

Marine bacteria are source of secondary metabolites in the harsh oceanic conditions. Brominated biphenyl compound was isolated from *P. phenolica* that inhibit the methicillin resistant *S. aureus* strains Isnansetyo and Kamei, (2003) and Franks *et al*. (2005) stated that tambjamine like alkaloid of *Pseudoalteromonas tunicata* has anti-fungal potential (Isnansetyo and Kamei 2003 and Franks *et al*., 2005). These compounds have been isolated from marine invertebrates which were possessed antimicrobial, immunosuppressive and anti-proliferative activities (Lindquist and Fenical 1991). Evidence indicates that the colonizing bacterium at the surface of higher organisms is the source of these compounds (Konig *et al*., 2006). This has been indicated by Burke and colleagues by elucidation of YP1 biosynthetic pathway in *P. tunicate* (Burkholder *et al*., 1966). The colonizing bacterium at the surface of higher organisms is the origin of these compounds (Konig *et al*., 2006). This has been indicated by Burke and colleagues by elucidation of YP1 biosynthetic pathway in P. tunicate (Burkholder *et al*., 1966).

It is extensively accepted that culture-based techniques are insufficient for studying bacterial diversity from environmental samples, as many bacteria cannot be cultured and/or effectively identified using current and traditional techniques (Amann *et al*., 1995). The use of molecular approaches to define microbial diversity has significantly enhanced the knowledge about natural microbial communities (Amann *et al*., 1995; Friedrich *et al*., 2001 and Webster *et al*., 2001). Yet, microbial

cultivation is crucial to search for new bioactive compound producing strains. In this study, in this study, five sponge associated bacterial isolates were tested for their antibacterial potential against Gram positive and Gram negative bacteria and also the MDR bacteria.

MATERIALS AND METHODS

Sponge collection

Two Sponge samples (Figure 1) were collected by SCUBA diving from the Egyptian Red Sea region. One of them was collected from El-Gouna region at depth 1.5 m (GPS: N: 27 22 39.98, E: 33 40 58.95) and the other one was collected from the National Institute of Oceanography and Fisheries station, Hurghada at depth 2m (GPS: N: 27 17 07.45, E: 33 46 26.50). (Figure 2).

Fig. 1: Sponge samples 1 and 2

Fig. 2: Map of Egypt showing the two sampling sites on the Red Sea coast; El-Gouna and Hurghada.

Samples were cut from the sponge with a dive knife while wearing latex gloves and individual pieces were transferred to separate plastic sample collection bags, brought to the surface, maintained at ambient seawater temperature and transported to the laboratory in the same day of collection.

Sponges were designated Sample 1 and Sample 2. Both sponges Samples 1 and 2 were taxonomically identified by Prof. Michele Kelly in National Institute of Water and Atmospheric Research (NIWA) Ltd., Auckland, New Zealand. Both Sponge Samples have been deposited in the NIWA Museum, under registration numbers (NIWAKD 6671 and NIWAKD 6672; respectively).

Sponge processing

Immediately after collection, Sponge samples were transferred to plastic bags containing sea-water and transported to the laboratory. Sponge specimens were rinsed in sterile seawater, cut into pieces with sterile scalpels of ca. 1 cm and then thoroughly homogenized in a sterile mortar with 10 volumes of sterile sea water. The supernatant was diluted in ten-fold series (10^{-1}, 10^{-2}, and 10^{-3}) using autoclaved environmental water. Fifty microliter aliquots of each dilution were plated on various agar plates' solid media.

Isolation of Sponge Associated Bacteria

Two growth media were prepared to isolate a wide range of the sponge associated bacteria; Marine Agar (Weiner *et al*., 1985) and ISP medium 2 (Shirling and Gottlieb 1966) served as general rich media to grow many heterotrophic marine bacteria. All media were supplemented with 0.2 μm pore size filtered cycloheximide (100 μg/mL), nystatin (25 μg/mL) and nalidixic acid (25 μg/mL). Cycloheximide and nystatin inhibit fungal growth, while nalidixic acid inhibits many fast-growing Gram-negative bacteria [34]. All media contained Difco Bacto agar (18 g/L) and were prepared in 1 L artificial sea water (NaCl 234.7 g, $MgCl_2 \cdot 6H_2O$ 106.4 g, Na_2SO_4 39.2 g, $CaCl_2$ 11.0 g, $NaHCO_3$ 1.92 g, KCl 6.64 g, KBr 0.96 g, H_3BO_3 0.26 g, $SrCl_2$ 0.24 g, NaF 0.03 g and ddH_2O to 10.0 L) (Lyman and Fleming 1940). The inoculated plates were incu-bated at 28°C for 5 - 10 days. Distinct colony morpho-types were picked and re-streaked until visually free of contaminants. Isolates were inoculated into liquid media ISP medium 2. The isolates were maintained on plates for short-term storage and long-term strain collections were set up in medium supplemented with 30% glycerol at −80°C (Sambrook *et al*., 2001).

16S rRNA gene bacterial identification

The isolated bacteria were first identified to the species level by PCR amplification of the 16S rRNA gene, BLAST analysis, and comparison with sequences in the GenBank nucleotide database. Specifically, the 16S rRNA gene from the strain was amplified using universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GTTACCTTGTTACGACTT-3'). The PCR conditions was designed with 95° C for 5 min as initial denaturing step followed by 30 cycles of denaturing at 95° C for 1 min and primer annealed at 54 1C for 1 min and elongation at 72° C for 90 s. Finally, extension step at 72° C for 10 min (Kamke and Schmitt 2010). Gel documentation system (Geldoc-it, UVP, England) was applied for data analysis using Totallab analysis software (ww.totallab.com, Ver.1.0.1). Positive amplicons with 1500 bp were eluted from agarose gel. Resultant PCR products were purified with Micro spin filters and quantities spectrophotometrically. Sequence analysis was employed using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp. Korea).

Screening of antimicrobial activity

After 14 days of incubation culture on marine broth medium at 28°C with agitation of 120 rpm, cells were harvested via filtration through 0.44 μm Millipore filters. Then, centrifuged at 10,000 rpm for 5 min. Disk diffusion assay was performed for bacterial supernatant against different gram positive and negative bacterial strains. 6 mm diameter paper discs were immerged with 100 μL volumes of sponge bacterial supernatant. Mueller-Hinton agar plates seeded with microorganisms and discs and incubated at 37°C for 24 h. Clear circular zone around the sample impregnated disc after incubation was evaluated as activity indicator. All the assays were carried out in triplicate measurements (Ghuman *et al*., 2016).

The filtrate of marine sponge bacterial isolates were assayed for their activity against gram positive bacterial strains of *Staphylococcus aureus*, gram negative *Escherichia coli* strains, Multidrug resistant *Pseudomonas aeruginosa*, Extended Spectrum Beta-Lactamase (ESBL) Producing *klebsiella pneumoniae*, and Methicillin resistant *Staphylococcus aureus.*

RESULTS AND DISCUSSION

In this study, two sponge samples were collected from Red Sea and were taxonomically identified as *Ircinia echinata* and *Amphimedon sp*. Five bacterial isolates were isolated from the sponge samples. The isolates were coded as HA1, HA2, HA3, HA4 and HA5, among which the first three isolates were isolated from the sponge *Ircinia echinata* and the last two were from the sponge *Amphimedon sp*. Initially, 16S rRNA sequence analysis was employed as molecular marker to identify the sponge symbiotic bacterial isolates (Thakur *et al*., 2003). The tested isolates belonged to the firmicutes. Interestingly, the firmicutes have been reported from diverse marine sponges irrespective of their taxonomic identity, geo-graphic location, or natural products profile (Hentschel *et al*., 2001; Webster and Hill 2001). The isolates coded numerically as HA1, HA2, HA3, HA4 and HA5 were identified as *Bacillus sp.*, *Lysinibacillus boronitolerans*, *Planomicrobium flavidum*, *Bacillus safensis* and *Bacillus pumilus;* respectively with 100%, 91.93%, 92.95%, 98.62% and 96.65% identity (Table 1) comparing with National Center for Biotechnology Information sequences database (Figure 3).

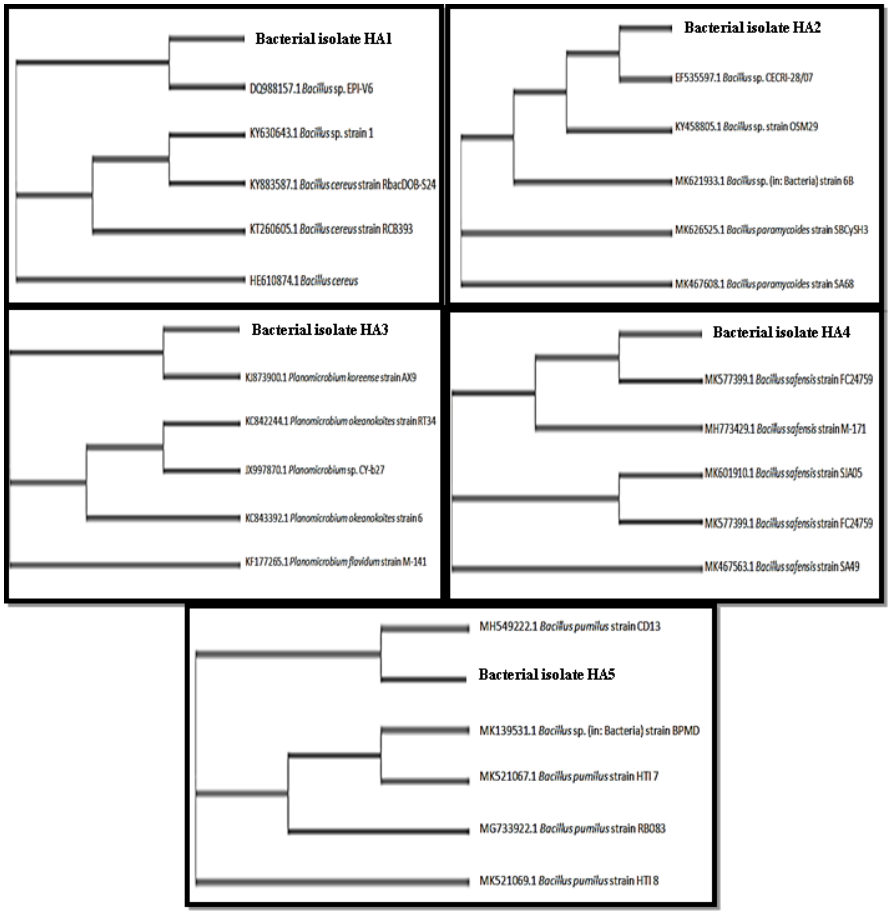

Figure 3: phylogenetic trees of the five marine sponge symbiotic bacteria based on 16S ribosomal RNA sequence analysis.

Table 1: The five marine sponge symbiotic bacterial isolates identified based on 16S rRNA.

Bacterial isolate code	Sponge source	Bacterial taxonomic similarity	Accession number	Identity %
HA1	***Ircinia echinata***	*Bacillus* sp. strain OSM29 16S ribosomal RNA gene, partial sequence	KY458805.1	100
HA2		*Lysinibacillus boronitolerans* strain AM-6/07 16S ribosomal RNAgene, partial sequence	EF516988.1	91.93
HA3		*Planomicrobium flavidum* strain 10 16S ribosomal RNA gene, partial sequence	KC843389.1	92.95
HA4	***Amphimedon sp.***	*Bacillus safensis* strain APT43 16S ribosomal RNA gene, partialsequence	KC519415.1	98.62
HA5		*Bacillus pumilus* strain AL3 16S ribosomal RNA gene, partialsequence	KF410589.1	96.65

The bacterial filtrates were evaluated as antimicrobial secondary metabolites products against positive and negative gram bacterial and *Candida albicans*.

Interestingly, the tested bacterial isolates were evaluated for their antimicrobial activity (Table 2). To achieve this goal, broth culture filtrates were tested against Gram positive and Gram negative human pathogenic bacteria, as well as *candida albicans*. Data revealed that the bacterial isolates HA1, HA2, HA3, HA4 and HA5 reflected significant antimicrobial activity except second and third marine sponge bacterial which were identified as *Lysinibacillus boronitolerans* and *Planomicrobium flavidum*. Filtrate of *Bacillus sp.* showed high activity against *Staphylococcus aureus* and moderate activity against *E. coli,* and weak against *Candida albicans*.

Table 2: Antimicrobial activity of marine sponge bacterial filtrates against gram-positive bacterial strains

Marine sponge bacterial isolates	***Staphylococcus aureus***	***E. coli***	***Candida albicans***
Bacillus sp.	+++	++	+
Lysinibacillus boronitolerans	-	-	-
Planomicrobium flavidum	-	-	-
Bacillus safensis	+++	++	++
Bacillus pumilus	++	+++	++

(-): with No activity; (+): Weak activity (<5 mm zone of inhibition); (++): Moderate activity (>5 mm zone); (+++): Strong activity (>10 mm zone).

On the other hand as shown in Table 2; HA4 bacterial isolate filtrate (identified as *Bacillus safensis*) reflected high activity against *Staphylococcus aureus,* and moderate activities were detected for *E. coli* and *Candida albicans. On the same manner, HA5 which was identified as Bacillus pumilus* expressed moderate activity against the *Staphylococcus aureus and Candida albicans*, and high activities were detected against *E. coli*.

The isolated bacteria were also evaluated against MDR *Pseudomonas aeruginosa*, ESBL producing *Klebsiella pneumoniae*, and Methicillin resistant *Staphylococcus aureus*. Among which, only *Bacillus sp., Bacillus safensis*, and

Bacillus pumilus bacterial filtrates showed significant activity against MDR bacteria. Generally, First filtrate of *Bacillus sp.* showed high activity against *Pseudomonas aeruginosa*, moderate against methicillin resistant *Staphylococcus aureus*, and weak activity against ESBL producing *Klebsiella pneumoniae.* On the other hand, The fourth sponge marine symbiotic bacterial filtrate (HA4; *Bacillus safensis*) showed moderate activity against *Pseudomonas aeruginosa*, and ESBL *Klebsiella pneumoniae*, and weak activity against *Staphylococcus aureus*. Whereas, HA5; *Bacillus pumilus* showed high antimicrobial activity against MDR *Pseudomonas aeruginosa*, moderate activity against Methicillin resistant *Staphylococcus aureus*, and weak activity against ESBL *Klebsiella pneumoniae.* (Table 3).

Table 3: Antibacterial activity of filtrate of marine bacterial isolate against Multi-drug-resistant (MDR) bacteria.

Marine sponge bacterial isolates	MDR *Pseudomonas* sp.	ESBL *Klebsiella Pneumoniae*	Methicillin resistant *Staphylococcus aureus*
Bacillus sp.	+++	+	++
Lysinibacillus boronitolerans	-	-	-
Planomicrobium flavidum	-	-	+
Bacillus safensis	++	++	+
Bacillus pumilus	+++	+	++

(-): with No activity; (+): Weak activity (<5 mm zone of inhibition); (++): Moderate activity (>5 mm zone); (+++): Strong activity (>10 mm zone).

The discovery of 5 strains of marine sponge associated bacteria exhibited good antimicrobial activity . The discovery of new classes of antibiotics has become necessary due to the increased incidence of multiple drug resistance among pathogenic microorganisms to drugs that are currently in clinical use (Manikandan *et al*., 2011).The strain HA1 was specified as Bacillus sp. and it was essentially found in all sponges. Similarly, Pabel *et al*., (2003) also reported marine Bacillus species in sponges. The current study revealed that Bacillus species was found in all sponges. These findings support that in many cases symbioses between host sponges and their associated bacteria are mutual, i.e., not only beneficial to the bacteria but also to the sponge. Lemos *et al*. (1985) reported that the production of compounds by microbes which are found on the surfaces of organisms would be highly advantageous. They also demonstrated that bioactive compounds may be produced when competitors or predators attack the sponges (Lemos *et al*., 1985). However, Thacker *et al*., (1998) found no clue that the production of secondary metabolites was enhanced by the presence of such competitor; somewhat they suggested that the persistent threat of predators might preserve high concentrations of the compound. The variety of antibiotic producing marine bacteria isolated in the present study proposes that sponges are rich sources of unique bacteria.

The bacterial community observed in the current study may be only a fraction of the total diversity of associated bacteria. Antibacterial potential among the marine bacteria is well-known and has been described in a number of studies (Uzair *et al*., 2006). Primary results are encouraging that the isolated strains exhibit antibacterial potential and the current study also assured that Bacillus sp. is widely known as rich sources of antimicrobial compounds as reported by Minkwitz and Berg (2001) and Gebhardt *et al*., (2002). The strain Bacillus sp. showed antimicrobial activity

against both Gram-negative and Gram- positive bacteria as well as MDR resistant bacteria. The high frequency of activity against Gram- positive bacteria was expected, since Gram-negative bacteria are generally less susceptible to antimicrobials than Gram-positive bacteria because of the presence of an outer membrane and Lipopolysaccharide (LPS) which together act as an efficient barrier against hydrophobic and lipophilic molecules (Snyder and McIntosh 2000).

The strains HA4 and HA5 were effective against fungi. Their filtrates inhibited the activity against *Candida albicans* which causes human genital infections, e.g., vaginitis and oral infection of infants and AIDS patients (Egusa *et al*., 2008). Secondary metabolite of marine bacterial isolates HA1, HA4 and HA5 showed large inhibitory activity against pathogenic bacteria and fungi. Inhibitory activity was observed against several bacteria, including vital pathogenic species such as methicillin resistant Staphylococcus aureus and ESBL producing *Klebsiella* sp. And MDR *Pseudomonas* sp. and that opens up interesting issues in the search for new compounds against multidrug-resistant pathogenic bacteria. The current study revealed that marine bacterial metabolites may be potential against new anti- MDR strains.

CONCLUSIONS

Despite there is a very little information is available on the production of antimicrobial agents and phylogenetic identification of sponge symbiotic bacteria found off the coastline of the Red Sea, which is known to have rich biodiversity that still to be studied extensively. From this study it can be concluded that the Red Sea sponge-associated bacterial strains along high potent because of producing antimicrobial components which are active against multidrug-resistant bacteria.

Therefore, the isolates which confirmed activity towards these testedmicro-organisms are also valuable for further study. The results of the present investigation clearly revealed that the marine bacteria from the sponges are potential sources of novel antibiotics.

Conflict of Interest

The authors declare that there is no any economic interest or any conflict of interest exists.

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