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Bioactive Crude Extracts from Four Bacterial Isolates of Marine Sediments from Red Sea, Gulf of Aqaba, Jordan

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Abstract

Escalation in number of resistant pathogens and diseases enhanced scientists to explore the unconventional habitats for bioactive compounds. Marine microorganisms represent a promising source for natural products due to the incredible diversity of chemical compounds that were isolated. Due to distinctive features and biodiversity of Red Sea biota, it was selected as a resource for isolation of bacteria with interesting bioactivity. Detection of bioactivity was performed using antimicrobial and antioxidant assays. Four bacterial isolates were found to have bioactivity at least in one assay. Crude extract of *Brevibacterium* sp. has weak antibacterial and moderate antioxidant effects, *Moraxella* sp. with weak antibacterial crude extract, and *Corynebacterium* sp. with potent antioxidant activity. Therefore, sediment marine bacteria represent an interesting source for antimicrobial and antioxidant secondary metabolites.

Keywords: Marine Bacteria, Antimicrobial, Antioxidant, Red Sea, Gulf of Aqaba.

1. Introduction

Despite the large number and diversity of bioactive compounds isolated from terrestrial microorganisms, since the penicillin era, new infectious diseases and resistant pathogens still represent a serious problem for human life (Cragg *et al.,* 1997; Desriac *et al.,* 2013). Therefore, the exploration of new and under-explored sources becomes extremely important in finding compounds with interesting bioactivities that can be used as new antibiotics (Penesyan *et al.,* 2011).

The oceans cover almost 70% of the earth's surface and contain a variety of species, many of which have no terrestrial counterparts (Whitehead, 1999). Marine bacteria as other marine biota produce novel compounds with unique structures (Boobathy *et al.,* 2009; Gram et al., 2010). They occupy different niches in the ocean, either planktonic, associated with inert or biotic surfaces, or they inhabit the sediments (Jayanth *et al.,* 2002). Approximately, 230 structurally characterized bioactive marine natural products were reported from 2009-2011, of which 102 compounds have antimicrobial activities (Mayer *et al.,* 2013).

The Red Sea has distinctive features such as unique coral reef systems, high level of available marine biota and great seasonal fluctuation of air and water temperature (Temraz *et al.,* 2006). Thus, it represents a valuable environmental source for organisms with promising bioactive metabolites. To date, few studies were performed on the Red Sea bacteria from the Jordanian side of the Gulf of Aqaba. They are represented by a publication on bioactivity of secondary metabolites isolated from a bacterium *Vibrio* sp. associated with the soft coral *Sinularia polydactyla* (Al-Zereini *et al.,* 2010), and master theses on bacterial communities associated with reef corals (Al Khateeb, 2011; Jaber, 2012; Khalfa, 2013).

In an ongoing research, the author is interested in screening natural crude extracts and isolation of biologically active compounds from marine organisms. Herein, isolation, cultivation, and extraction of antimicrobial and antioxidant crude extracts from four Red Sea sediment bacteria are described.

2. Materials and Methods

2.1. Collection of Marine Sediments

Sediment samples were collected in April 2008 in sterile plastic bags from different locations in front of the Marine Science Station (MSS)/Gulf of Aqaba (29°27 latitude and 34°58 longitude), at 10 m depth by SCUBA diving (Figure 1). Collection sites are characterized by presence of fringing reefs in some locations and sea grass meadows and sandy bottoms in other locations. These samples were transported in refrigerated containers (4 °C) to the laboratory in Mutah University.

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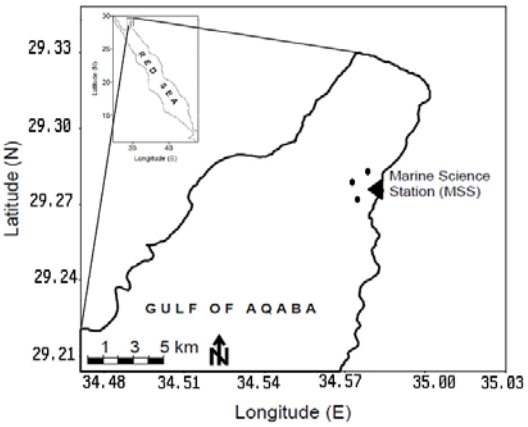

Figure 1. Collection sites in front of Marine Science Station on the Jordanian coast of Gulf of Aqaba.

2.2. Isolation, Characterization and Identification of Bacterial Samples

One gram of each sand sample was suspended in 10 ml sterile filtered sea water. The suspensions were then serially diluted (10^{-2} - 10^{-6}) with sterile filtered sea water. 50 µl from each tube was spread on modified Luria-Bertani (mLB) agar plates (0.5% tryptone, 0.5% yeast extract, 1% NaCl, 1.8% agar in a half strength marine sea water, pH 7.2) supplemented with cycloheximide (50 mg/l) and nystatin (50 mg/l) to inhibit the growth of yeasts and fungi. The plates were monitored for bacterial growth. New colonies were streaked on new agar plates for purification. This process was repeated several times till pure culture plates were obtained. 2 ml from culture of each pure bacteria isolate were conserved in 80% glycerol (1:1) and stored at – 20 °C till the date of screening.

Strains that show activity at least in one assay were identified using either RapID^{TM} ONE system (Remel, USA) and RapID^{TM} NF plus system (Remel, USA) for oxidase negative Gram negative bacteria, and oxidase positive Gram negative bacteria respectively or RapID^{TM} CB plus system (Remel, USA) for Gram positive bacteria.

2.3. Bacterial Cultivation and Extraction of Fluid Cultures

Bacterial strains were cultured in 1L Erlenmeyer flasks containing 500 ml of mLB medium on an orbital shaker (120 rpm, Forma Orbital Shaker, Thermo electron cooperation, USA) at 25 °C. During the fermentation process, 10 ml sample and thereafter daily samples were taken to monitor the bacterial growth. The growth was followed by OD measurements of 10 fold-diluted samples at 600 nm (UV/Vis Spectrometer, Lambda 16, Perkin-Elmer, Langen), and by changes in pH value (pH 523, WTW, Germany). As the OD ceased, the culture fluid was separated from the bacterial cells by centrifugation (5000g, 15 minutes, Beckman GS-6, Beckman coulter/USA). The supernatant was adjusted to pH 4 and extracted with an equal volume of ethyl acetate. The organic phase was dried over Na_2SO_4, concentrated *in vacuo* at 45 °C and the resulting residue was dissolved in methanol to a final concentration of 10 mg/ml.

2.4. In Vitro Antimicrobial Activity

The antibacterial activity was determined by agar diffusion test and the minimum inhibitory concentration (MIC) was determined by serial dilution assay according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004) with some modifications. The test microorganisms used in this study were *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922**,** *Klebsiella pneumoniae* ATCC 13883, and *Bacillus subtilis* ATCC 6633, seeded on LB agar plates (0.5% tryptone, 0.5% yeast extract, 1% NaCl, 1.8% agar).

Briefly, Muller-Hinton agar plates were seeded with 10^6 cell/ml of overnight grown test bacterial strains and were used for agar diffusion test. Six millimeter sterile filter paper discs impregnated with 300 µg/disc of the test samples were placed on the surface of the plates. All the plates were incubated at 37 °C for 24–48 hr. Antimicrobial activity was calculated by measuring the diameter of the inhibition zones. Minimum inhibitory concentration (MIC) was determined using serial dilutions of test samples and positive control (Chloramphenicol) starting from a final concentration of 1 mg/ml and 100 µg/ml, respectively. Test samples (100 µl) were diluted serially in 96 well plates and each microbial strain suspension (100 µl of 2×10^6 cell /ml) was added in each well. All prepared cultures were incubated at 37 °C for 24 hr. The MIC was determined as the minimum concentration of test sample that inhibits growth of microorganism and the OD_{600nm} of the culture is near to or equal zero. The experiment was performed in triplicate.

2.5. Antioxidant Assay

Antioxidant activity was measured in terms of the radical scavenging ability and decolorization of both used radicals, 2,20-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH).

The antioxidant capacity assay was carried out using the improved $ABTS^{\bullet+}$ assay according to Re *et al*. (1999). Briefly, $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate (final concentration in distilled water) and incubation at room temperature in darkness for 16 hr. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.005 at 734 nm UV/VIS Spectrophotometer. Crude extracts were concentrated in 20 mg/ml methanol, such that to give a maximum of 80% inhibition of the blank absorbance with 10 µl of sample. To 2 ml of diluted $ABTS^{\bullet+}$, 10 µl of each extract solution was added and mixed vigorously. The reactive mixture was allowed to stand at room temperature for 6 min and the absorbance was recorded immediately at 734 nm. Trolox standard solutions (concentrations from 0 to 20 µM) in ethanol were prepared and assayed using the same conditions. As blanks the same volume of solvents was run in each assay. The percentage of inhibition of absorbance at 734 nm was calculated and the results were expressed as a function of concentration of trolox for the standard reference data. Results were expressed in terms of trolox equivalent antioxidant capacity (TEAC), i.e., µM trolox/mg crude extract of coral or µM trolox/mg crude extract of bacteria culture. Assay was performed in

triplicate for each sample and each concentration of standard.

Scavenging ability of DPPH radical was estimated according to the method of Brand- Williams *et al*. (1995) with minor modification. 12.5 µl of different extracts was added to 2.5 ml methanolic solution of 0.1 mM DPPH. The mixture was shaken vigorously and left to stand at room temperature for 30 min. The decrease in absorbance of the resulting solution was measured at 517 nm by UV/Vis spectrophotometer. Methanol (99.5%) was used as a blank. Trolox standard (concentrations from 0 to 20 µM) in ethanol was prepared and assayed using the same conditions. The scavenging effect of the DPPH radical by the sample was calculated according to the formula

$$Scavenging\ effect\ (\%) = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

Results were expressed in terms of mean value of trolox equivalent antioxidant capacity (TEAC). Assay was performed in triplicate for each sample and each concentration of standard.

2.6. *Statistical Analysis*

Means and standard deviations were deduced using *Excel* software.

3. Results and Discussion

In this work, twenty six bacterial isolates were obtained from collected samples. They were selected based on differences on morphological and biochemical tests. Twenty of these isolates (77%) are Gram positive bacteria while the Gram negative ones are represented by six isolates (23%).

During screening, two bacterial isolates showed activity in agar diffusion test, a Gram positive (*Brevibacterium* sp.) and Gram negative (*Moraxella* sp.) bacteria. They were isolated from sand samples in the sea grass area and their bioactivities against the tested microorganisms are summarized in tables 1 and 2.

Table 1. The inhibition zones (mm) caused by active bacterial isolates against tested microorganisms in agar diffusion test.

Test microorganism				
Name of isolates	***K. pneumoniae***	***E. coli***	***S. aureus***	***B. subtilis***
	Inhibition zone(mm ±SD[a]) 300 µg/disc			
Brevibacterium sp.	-	-	-	8 ± 0.6
Moraxella sp.	-	-	8 ± 0.6	-

[(a)] Standard deviation

The crude extracts of interesting isolates were weakly active against Gram positive test strains (*S. aureus* and *B. subtilis*), while Gram negative bacteria were resistant to the applied substances. This resistance could be attributed to low permeability of the cell wall of these strains to the bioactive ingredients in the extracts.

Table 2. Minimum inhibitory concentration (MIC) of the active bacterial crude extracts against susceptible microorganisms in serial dilution assay.

Name of isolates	**MIC (µg/ml)**	
	S. aureus	***B. subtilis***
Brevibacterium sp.	1000S[a]	500C
Moraxella sp.	500C[b]	1000S
Chloramphenicol	<8	<8

[(a)]: biostatic[(b)]: biocidal

To exclude the solubility problem of the extracts in agar, serial dilution assay was performed and the minimum inhibitory concentrations of the applied test substance were deduced. The results obtained in this test coincided with the agar diffusion test, where the extracts were weakly active against the susceptible Gram positive bacterial strains with MIC between 500-1000 µg/ml.

To date, *Brevibacterium* species are described as terrestrial representatives producing antimicrobial compounds only against Gram positive bacteria (Motta and Brandelli, 2002). Nevertheless, it was reported as a marine trait that inhibits Gram-negative pathogens, including *Klebsiella pneumoniae* and multi-drug resistant *E. coli* (Wietz, 2011). While secondary metabolites of *Moraxella* sp. was reported to exhibit various biological activity depending on medium and condition of bacterial cultivation (Nofiani *et al.*, 2012).

Moreover, *Brevibacteriun* sp. and *Corynebacterium* sp., isolated from the sand sample collected from sea grass area, were found to have antioxidant activity (Table 3) in ABTS assay. DPPH and ABTS procedures are commonly used in antioxidant activity assays in biological systems. They have a similar mechanism in that the absorption spectra of the stable free radical changes when the molecule is reduced by an antioxidant or a free radical species (Teow *et al.*, 2007).

ABTS assay was more predictive for antioxidant capacity than DPPH for extracts of marine bacteria. The $ABTS^{\bullet+}$ radical scavenging activity of 100 µg/ml of bacterial crude extracts ranged between 54%-63% (TEAC 119-138 µM Trolox/mg crude extract) with *Corynebacterium* sp. produce metabolites with most potent antioxidant activity. The higher values obtained in ABTS compared to DPPH could be attribute to the fact that ABTS detect antioxidant capacity of hydrophobic and hydrophilic extracts while DPPH predict antioxidant capacity for hydrophobic antioxidants. Similar observation was noticed by Rivero-pérze and his colleague in the antioxidant profile of red wine (Rivero-pérze *et al.*, 2007). In addition, ABTS is soluble in both aqueous and organic solvents and reacts relatively rapidly compared to DPPH.

Table 3. The antioxidant capacity of bacterial crude extracts measured as DPPH and ABTS. TEAC (µM Trolox/mg crude extract)

Name of isolates	DPPH (±SD[a])		ABTS (±SD)	
	TEAC	% inhibition	TEAC	% inhibition
Brevibacterium sp.	124.9 (±51.2)	32.29 (±13.8)	119.14 (±29.1)	54.22 (±13.66)
Corynebacterium sp.	115.73 (±20.75)	29.82 (±5.61)	138.31 (±6.09)	63.29 (±2.86)

(a)Standard deviation

The antimicrobial activity has been extensively reported for extracts of various groups of marine organisms (Blunt *et al.*, 2012 and the previous reports in this series). Higher percentages of microbial biologically active compounds were isolated from symbiotic bacteria associated with marine macroorganisms than from free living bacteria (Shnit-Orland and Kushmaro 2008). Recently, numerous compounds were isolated from Red Sea bacteria with antimicrobial as well as cytotoxic activities (Al-Zereini *et al*., 2010; Shaaban *et al*., 2013).

Nowadays, there is a great interest in evaluating the protective activity of natural antioxidants. Marine bacteria provided a resource of novel antioxidants with potential application in biomedicine, in food and feed, and in cosmetics or related products (Dunlap *et al.*, 2003). Bacterial species associated with seaweed from the Red Sea were found to give extracts with higher scavenging effect on DPPH radical (Abdel-Wahab *et al.*, 2013). Microorganisms, especially in marine niches where there is high reactive oxygen species (ROS) at electron rich areas due to metabolic and photosynthetic activity, are exposed to high level of oxidative stress through a combination of photosynthesis, symbiont oxygen production, and intense sunlight intensities leading to UV-induced free radical production (Townsend, 2008). Thus, as a protective mechanism, they produce metabolites that reduce the effect of the resulting oxidative radicals.

4. Conclusion

Marine sediment bacteria are promising sources for biologically active metabolites. In the present study, the crude extracts of interesting bacterial isolates exhibited antimicrobial and antioxidant activities. Therefore, further studies are required to isolate and purify the individual compounds behind such activities and elucidate their structures.

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