Isolation and characterization of marine-derived actinomycetes with cytotoxic activity from the Red Sea coast

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ABSTRACT

Objective: To isolate and evaluate the cytotoxic activity of different actinomycetes species isolated from the Red Sea coast in Sharm el-Sheikh, Egypt.

Methods: Forty actinomycetes strains were isolated from different sediments and seawater samples collected from the Red Sea coast in Egypt. Actinomycetes were recognized by morphological and microscopic examinations. Cell viability and cytotoxicity induced by the crude extracts on breast cancer cell lines MDA-MB-231 were assessed using methylene blue assay. The strains with promising cytotoxic activity were identified by sequencing and amplifying the 16S rRNA genes. The antibacterial activities of the crude extracts were performed using Kirby–Bauer disc diffusion method.

Results: The results indicated that five ethyl acetate extracts exhibited cytotoxicity towards breast cancer cell lines MDA-MB-231. The highest cytotoxic activity was found for the ethyl acetate extracts of EGY2 and EGY39. The isolate EGY3 was identified as a new Streptomyces species, while the actinomycete EGY22 was found to be a member of the genus Nocardiopsis sp. The crude extract of the isolate EGY8 showed slightly high antimicrobial activity against different test microorganisms.

Conclusions: The results of the present study reveal that marine sediments of the Red Sea are a potent source of novel species of actinomycetes. The isolates may be useful in discovery of novel bioactive compounds and an important step in the development of microbial natural product research.

1. Introduction

Breast cancer is one of the leading causes of cancer death among women in worldwide [1]. Incidence rates are high in more developed countries and low in less developed countries. In Egypt, breast cancer is the most common cancer among women and representing 18.9% of total cancer cases [2]. As per literature, the natural products derived from microorganisms provide an excellent source of cancer medication [3]. Among such microorganisms, Gram-positive actinomycete bacteria are of special interest. They are known to produce chemically diverse compounds with a wide range of biological activities [4–8]. From the ecological point of view, several species of actinomycetes (i.e., Streptomyces and Nocardiopsis) are frequently distributed in marine environments such as oceans, rivers and seas. They are dwelled in sponges, marine sediments, sea sands and water [9].

The Red Sea is one of the most spectacular coastal and marine environments in Egypt and has a rich biodiversity. The sea has a number of unique marine habitats including sea-grass beds, salt-pans, mangroves, coral reefs, salt marshes, numerous...
fish species and different microbial communities [10,11]. According to best of our knowledge, little studies have been conducted to isolate actinomycetes from the Red Sea’s seawater and sediments [12,13]. This study aimed to isolate different actinomycetes from seawater and marine sediments of the Red Sea and evaluate the cytotoxic activity of their crude extracts against breast cancer cell line (MDA-MB-231). Additionally, the extracts were assayed against different microbial pathogens. The strains that have cytotoxic activity were phylogenetically characterized based on the 16S rRNA gene sequencing.

2. Materials and methods

2.1. Samples collection

Twenty two sediments and sea water samples were collected from different parts of the Red Sea in Sharm el-Sheikh, South Sinai, Egypt. The collection sites were Sharks Bay, Umm Marikha Bay, Ras Mohammed and Ras Um Sid. Samples were collected at ~10 m depths below the water surface and kept at 4 °C for further working up.

2.2. Isolation of actinomycetes

From each sample, 1 g was dispersed in 9 mL of sterilized water and vortexed for 2 min. The samples were subjected to heat treatment in a water bath at 60 °C for 10 min to eliminate non-sporulating bacteria. Following serial dilution (10–1, 10–2 and 10–3) of the suspension with sterile water, a 100-μL aliquot was spread on humic acid-vitamin agar [14] and starch-casein agar [15]. All media were prepared in 50% sea water and supplemented with nalidixic acid (75 μg/mL) and cycloheximide (50 μg/mL). The plates were incubated at 30 °C for 7–30 days until the colonies appeared. Forty actinomycete strains were picked up and took a voucher numbers (EGY1–EGY40).

2.3. Morphological characterization

Characterization of the isolated strains was performed by morphological methods [16].

2.4. Fermentation and extraction of metabolites

To prepare the cultures, chunks of well-grown agar plate of each strain were used to inoculate 2 × 100 cm² Erlenmeyer flasks each containing 100 mL of Waksman medium with 50% sea water. The Waksman liquid medium is consisting of glucose (2.0 g/100 mL), meat extract (0.5 g/100 mL), peptone (0.5 g/100 mL), dried yeast (0.3 g/100 mL), NaCl (0.5 g/100 mL) and CaCO₃ (0.3 g/100 mL). The cultures were grown at 28 °C for 3–4 days with orbital shaking at 200 r/min. To construct a library of crude extracts, the culture broths of different strains were extracted consecutively with ethyl acetate. The cell pellets were extracted three times with methanol. The solvents from culture broth and mycelia were evaporated under vacuum and collected together in a small glass vial and stored at ~20 °C for further use. Each vial took a serial number identical to the number of its own bacterial strain. Each crude extract was dissolved in dimethyl sulfoxide for further investigation.

2.5. Cell culture

Human breast cancer cell line MDA-MB-231 was obtained from the Institut National de la Santé et de la Recherche Médicale, Dijon, France. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/L of glucose, 4 mmol/L of L-glutamine, and 10% heat-inactivated fetal calf serum.

2.6. Cell viability assay

MDA-MB-231 cells were seeded in 96-well plates (4 × 10^4 cells per well) with 100 μL of Dulbecco’s modified Eagle’s medium. Cells were incubated at 37 °C in a 5% CO₂ incubator for 24 h. Crude extracts at different doses were added to each well. After 24 h incubation, the culture media was removed and the cells were washed with 100 μL phosphate-buffered saline. Cells were fixed by adding 100 μL of 70% ethanol and incubated at room temperature for 15 min. After removal of ethanol, 100 μL of methylene blue dye was added. The plates were incubated at room temperature for 15 min. To remove the excess of dye, the plate washed three times with tap water and then incubated for 2 h at 37 °C. Dye was eluted from the attached cells by adding 100 μL of 0.1 mol/L HCl in each well and then incubated for 5 min at room temperature. The developed blue color was measured using a microplate reader at 630 nm. Controls referred to wells containing only cells and medium with and without 10% dimethyl sulfoxide.

2.7. Antimicrobial activity

Two Gram-positive bacteria [Streptococcus pyogenes (S. pyogenes) and Staphylococcus haemolyticus (S. haemolyticus)], two Gram-negative bacteria [Salmonella typhi (S. typhi) and Pseudomonas aeruginosa (P. aeruginosa)] and fungal yeast [Candida tropicalis (C. tropicalis)] were used in this study. The microbial strains were obtained and confirmed at the Department of Botany and Microbiology, Faculty of Science, Helwan University. The concentrations of the bacterial crude extracts were adjusted to 50 mg/mL. The antimicrobial activities were performed by using disk diffusion method [17]. Chloramphenicol (10 μg/disc) and ampicillin (5 μg/disc) were used as positive controls. The activity was determined by measuring the diameter of the inhibition zones in millimeters.

2.8. Isolation of genomic DNA of actinomycetes cultures

The actinomycete strains (EGY2, EGY3, EGY22, EGY27, and EGY39) were grown in 100 mL of tryptic soy broth for 24 h at 28 °C. The cells were centrifuged at 10000 r/min and washed two times with Tris–EDTA buffer. The DNA was extracted from actinomycetes strains using gene JET™ genomic DNA purification kit (Thermo Scientific Fermentas, Vilnius, Lithuania).

2.9. Partial 16S rRNA gene amplification, DNA sequencing and phylogenetic analysis

The gene coding 16S rRNA was amplified by PCR from the isolated genomic DNA using the forward (5’TACCGGAGTGTTCATCG-3’) and the reverse (5’TGCAGGTGCTGCGCAGTGTTCAT-3’) primers. The PCR conditions consisted of
3. Results

3.1. Isolation and fermentation of actinomycetes

Twelve sediments and ten water samples were collected from different bays of Sharm el-Sheikh (~10 m deeps) on the Red Sea coast at South Sinai, Egypt. A total of forty actinomycete strains were isolated based on colony morphology and microscopic appearance. The highest number of actinomycetes were obtained from Ras Um Sid (i.e. 16 strains) followed by Ras Mohammed (i.e. 11 strains), Sharks Bay (i.e. 9 strains) and then Um Marikha Bay (i.e. 3 strains). Several collected water samples didn’t produce actinomycetes, even after 6 weeks of cultivation. Only three strains were isolated from ten sea water samples. These strains were observed in starch casein agar medium after 4 weeks. The location, direction and the total number of actinomycetes isolated from different samples are summarized in Table 1. The isolated strains were cultivated in Erlenmeyer flasks each containing 100 mL of liquid Waksman medium [18]. Fermentation was carried out at 28 °C for 5 days while shaking at 200 rpm. After extraction and evaporation, different crude extracts were produced and took a number similar to the number of its own actinomycete strain.

3.2. Cytotoxic activity of the crude extracts

The cytotoxic activity of forty crude extracts (EGY1–EGY40) was evaluated against MDA-MB-231 breast cancer cell line using doxorubicin as a positive control. Doxorubicin is known to induce apoptosis in several cancer cells [19]. Different concentrations of the crude extracts (25, 50, 100, 200 and 300 μg/mL) were incubated with the cells for 24 h. Only five extracts corresponding to EGY2, EGY3, EGY22, EGY27 and EGY39 revealed a significant concentration dependent decrease in the cell viability of MDA-MB-231 cancer cell line (Figure 1). The highest cytotoxic activity were found for the crude extracts of EGY2 and EGY39, as it lowered IC50 values to (19.50 ± 0.03) μg/mL and (29.6 ± 0.43) μg/mL, respectively.

The morphological changes of the breast cancer cell line (MDA-MB-231) after treatment with the crude extracts of the Streptomyces sp. EGY2 and EGY39 were evaluated under inverted microscope (Figure 2). The microscopic observations revealed the both extracts have outstanding effect on treated MDA-MB-231 cells compared to the untreated cells. The numbers of the apoptotic cells in case of treatment with the crude extract of Streptomyces sp. EGY2 (25 μg/mL) were more than EGY39. The cells showed signs of detachment from the surface of the wells, which denoted cell death. Chemical screening of the most active crude extract of Streptomyces sp. EGY2 showed on thin layer chromatography several yellow bands (RF 0.5–0.9). They appeared as an orange spots under UV light (365 nm) and gave a reddish-brown color upon spraying with anisaldehyde/H2SO4 reagent. This and the blue color reaction with 2 mol/L sodium hydroxide pointed to the presence of anthraquinone derivatives in the crude extract [6,8].

3.3. Partial characterization of the isolated strains

The actinomycetes that have cytotoxic activity (EGY2, EGY3, EGY22, EGY27 and EGY39) were selected for morphological and molecular characterization. Morphological observation revealed that both aerial and vegetative hyphae were abundant and well developed (Table 2). The color of aerial and substrate mycelium of most strains were varied from white to yellow. Some of the isolated strains produced yellow to orange pigments, while the isolates EGY22 and EGY39 were non-pigmented.

Molecular identification was carried on the basis of 16S rRNA gene sequences [20]. The 16S rRNA genes were sequenced and sequences were blasted against the NCBI GenBank database. From blast analysis, the isolates EGY2, EGY3, EGY27 and EGY39 belonged to the genus Streptomyces sp. The bacterial strain EGY3 was identified as a new Streptomyces species based on sequence similarities < 98.2%. A maximum-likelihood tree [21], based on 16S rRNA gene sequences, was constructed for the five isolates to show the relationships.
between the strains and some other related actinomycetes species (Figure 3). Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1000 resampling [22]. From the tree, the high similarity and high bootstrap values suggested that the isolates EGY2, EGY3, EGY27 and EGY39 represented the same species. Interestingly, isolate EGY22 form distinct clade and falls within the genus Nocardia.

3.4. Antimicrobial screening of crude extracts

The forty isolates were also screened for their inhibitory activity against different human pathogenic bacteria S. pyogenes, S. haemolyticus, S. typhi, P. aeruginosa and C. tropicalis (Table 3). The bacterial growth inhibition was determined as the diameter of inhibition zones around the discs. The crude extracts corresponding to EGY1, EGY4, EGY22, EGY39 and EGY40 showed activities against some of the tested microorganisms, while the other extracts didn’t show any activity. The crude extract of the isolate EGY8 showed slightly high antimicrobial activity against different test microorganisms. The extracts EGY16 and EGY27 showed only antibacterial activity against P. aeruginosa.

Table 2
Morphological characteristics of selected actinomycetes strains.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Aerial mycelium</th>
<th>Substrate mycelium</th>
<th>Diffusible pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGY2</td>
<td>White</td>
<td>Light yellow</td>
<td>Orange</td>
</tr>
<tr>
<td>EGY3</td>
<td>White</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>EGY22</td>
<td>Yellow</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>EGY27</td>
<td>White</td>
<td>Yellow</td>
<td>None</td>
</tr>
<tr>
<td>EGY39</td>
<td>White</td>
<td>Light Yellow</td>
<td>None</td>
</tr>
</tbody>
</table>

Figure 1. Effects of different crude extracts of EGY2(A), EGY3 (B), EGY22 (C), EGY27 (D), EGY39 (E) on breast cancer cell line (MDA-MB-231). Cells were seeded in a 96-well culture plate (4 × 10^3 cells per well) for 24 h and then treated with indicated concentrations of crude extracts.

Figure 2. Morphological changes of the breast cancer cell line (MDA-MB-231) after treatment with the crude extracts of the Streptomyces sp. EGY2 and EGY39 for 24 h (100x magnification).
4. Discussion

The seas and oceans cover more than 70% of the Earth's surface and considered a valuable source for microorganisms and bioactive secondary metabolites [23,24]. The Red Sea is a unique marine ecosystem with high salinity, high water temperature and high microbial diversity in comparison with the other tropical seas [25,26]. However, few studies reported the isolation of actinomycetes from Red Sea sediments and water. In this study, twenty two sediments and sea water samples were collected from the Red Sea at different depths. The actinobacterial community of the samples was investigated by primary culturing on humic acid and starch casein agar media by serial dilution method. Twenty seven actinomycetes strains were isolated from twelve sediment

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Table 3
Antimicrobial activities of the isolated strains (mm).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>S. pyogenes</th>
<th>S. haemolyticus</th>
<th>S. typhi</th>
<th>P. aeruginosa</th>
<th>C. tropicalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGY1</td>
<td>10</td>
<td>10</td>
<td>13</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>EGY4</td>
<td>7</td>
<td>–</td>
<td>10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EGY8</td>
<td>15</td>
<td>13</td>
<td>27</td>
<td>25</td>
<td>–</td>
</tr>
<tr>
<td>EGY16</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>EGY22</td>
<td>12</td>
<td>13</td>
<td>25</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>EGY27</td>
<td>–</td>
<td>–</td>
<td>8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EGY39</td>
<td>12</td>
<td>5</td>
<td>25</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>EGY40</td>
<td>14</td>
<td>10</td>
<td>18</td>
<td>13</td>
<td>23</td>
</tr>
</tbody>
</table>

*: Inactive. Chloramphenicol (10 μg/disc) and ampicillin (5 μg/disc) were used as a positive controls.
samples. As per literature, marine sediments are a valuable source for the isolation of novel actinomycetes with the potential to produce new drugs [27]. Of the ten seawater samples, only three actinomycetes were grown on starch casein agar medium after 30 days of incubation. On humic acid cultivating media, no colonies appeared till 60 days. Our results indicate that the bacterial community of the Red Sea water is poor in actinobacteria. According to Mustafa et al., the main bacterial phyla along the Red Sea water were predominantly proteobacteria, firmicutes, fusobacteria, bacteroidetes, spirochetes and different human pathogens [11]. Ngugi et al. reported the presence of prochlorococcus related to the high-light-adapted (HL2) ecotype in the water of the Red Sea [26].

The results of the cytotoxic activity of crude extracts of the isolates EGY2, EGY3, EGY22, EGY27 and EGY39 may be due to the presence of some interesting cytotoxic compounds. The crude extract of EGY2 showed the highest cytotoxic activity against MDA-MB-231 breast cancer cell line due to the presence of several anthraquinone type compounds. It was reported that anthraquinones isolated from terrestrial and marine actinomycetes exhibited potent cytotoxic activity against a variety of cancer cells [28,29]. Microscopic examination of the breast cancer cell line (MDA-MB-231) treated with crude extracts EGY2 and EGY39 showed characteristic morphological features. The apoptotic cells have some aspects such as condensation of nuclear heterochromatin, partition of cytoplasmic and nucleus into membrane-bound vesicles [30]. Our results also showed some extracts had antimicrobial activities against several pathogenic bacteria. As per literature, several marine-derived actinomycetes exhibited antimicrobial activities against different microorganisms [31]. The isolated actinomycetes with cytotoxic activity were identified based on microscopic and morphological examination of the colonies [32]. The aerial and substrate mycelia of the isolates were grown well in Waksman agar and this may be due to sufficient amount of nutrients included in the media. The isolates EGY2, EGY3 and EGY27 have the ability to produce orange and yellow pigments. Actinomycetes are characterized by the production of various types of pigments such as anthraquinones [6] and phenazines [18]. The pigments may be dissolved into the media or it may be retained in the mycelium. Amplification and sequence of the DNA genomes extracted from the isolated strains (i.e. EGY2, EGY3, EGY22, EGY27 and EGY39) revealed that the majority of them belong to the genus Streptomyces sp. It has the ability to produce bioactive secondary metabolites such as antimicrobial and anticancer drugs [33]. Only the isolate EGY22 was found to be a member of the genus Nocardia sp. The genus Nocardia is commonly obtained from marine environments, including marine sponges [34].

In conclusion, our study suggests that Red Sea sediments are a source of a wide variety of interesting actinomycetes species. The crude extracts of EGY2 and EGY39 were found to possess anticancer properties and it could be used as potent drug candidate in pharmaceutical preparations. Further studies concerning purification, characterization and identification of the active compounds are recommended.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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References


