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Molecular Identification, Isolation and Evaluation of Persian Gulf Actinomycetes as Candidates of Cytotoxic Metabolites Against Breast Cancer

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Abstract

Introduction: Marine actinomycetes have a great potential to produce unique bioactive compounds due to their special adaptation in the harsh ocean environment. The current study aimed to isolate anti-cancer compounds producing actinomycetes from sediments of Harra forests of the Persian Gulf and investigate their potential as anti-breast cancer metabolites.

Methods: In the current study, 40 sediment samples of Harra forests of the Persian Gulf were collected. Samples were diluted and cultured in a starch casein agar selective medium. The strains were isolated and purified, using morphological and microscopic methods. Forty strains were cultured in a starch casein broth and the metabolites were extracted using ethyl acetate. The produced metabolites were extracted from active strains and their cytocidal activities were evaluated against the breast cancer cell line. Finally, effective-metabolites-producing bacteria were identified using the molecular method.

Results: Of the sediments, 186 strains were isolated and identified. Results showed that the isolates had cytocidal activities against the breast cancer cell line. The results also revealed that the 2HP and 4HP strains showed more anti-cancer activities than the others. Results of this study showed that sediments of Harra forests of the Persian Gulf were rich in active actinomycetes, which can be used in the production of new anti-cancer compounds.

Conclusions: The obtained results give evidence that it is essential to scrutinize these marine microorganisms, which have a great potential to be used in the pharmaceutical fields, in search for new drugs.

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INTRODUCTION

Actinomycetes are filamentous, Gram-positive, free-living, and saprophyte bacteria that sometimes are symbiotic with plants. These bacteria can be isolated from all ecosystems including soil, water, marine sediments, and even hot waters [1]. Actinomycetes form about 10% of the population of bacteria in the seabed sediment. Microorganisms of marine habitats are considered as amazing sources of strong bioactive materials [2]. Actinomycetes have a great potential to produce secondary metabolites such as antibiotics, enzymes, herbicides, anti-cancer materials, and other useful compounds [3, 4]. About 23000 kinds of active secondary metabolites are produced by microorganisms, and actinomycetes produce more than 75% of different antibiotics and antimicrobial agents known as new active sources [5]. In recent years, due to the need for new drugs, marine microorganisms, with their potential to produce unique metabolites, are considered as new sources [6]. Cancer is one of the most serious issues that threaten human life and breast cancer is the second leading cause of death in women. Many marine natural products with anti-cancer effects are derived from marine *Actinobacteria*. These metabolites play an important role in identifying drug compounds. At present, few studies are conducted on

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the anti-cancer role of bioactive compounds derived from marine *Actinobacteria* [7]. In the past half century, thousands of antibiotics are successfully used to treat microbial diseases. Therefore, it is no surprise that a large number of bacteria and tumor cells show resistance against antimicrobial agents. In the past, the solution to this problem was to use new anti-cancer drugs and antimicrobial sources. However, in recent years, the use of these antibiotics is drastically reduced due to the exhaustion of conventional resources. Hence, scarce resources such as marine *Actinobacteria* raised attention. These bacteria are capable of producing anti-cancer bioactive metabolites which are isolated from the marine environment [8]. In the current study, actinomycetes were isolated from the mangrove ecosystem of the Naiband Bay in Assaluyeh located in the Persian Gulf, and their metabolites' cytocidal effects on breast cancer were evaluated.

METHODS

Sampling and Isolation of Actinomycetes

To isolate actinomycetes in the current study, geographic coordinates of the Naiband Bay located in Assaluyeh, Bushehr province, were obtained using the GPS in June 2015. Forty points were determined with 500 meters distance from each other. Coordinates of each sample are shown in Table 1.
Collected samples were put in sampling containers and transported to the laboratory in the shortest time. Then, samples were incubated for 1 hour at 55°C to reduce vegetative forms of actinomycetes and other bacteria [9]. The serial dilution method was used to isolate actinomycetes and various dilutions obtained from sediment samples were cultured for 7 days in a starch-casein agar medium (including 15 g of agar, 1 g of potassium phosphate, 2 g of sodium chloride, 2 g of potassium nitrate, 0.3 g of casein, 0.05 g of magnesium sulfate 7-water, 0.01 g of ferrous sulfate 7-water, and 0.02 g of calcium carbonate in a total volume of 1 liter of filtered sea water to increase the isolation rate of actinomycetes) in 7.5 pH, and were incubated at 28°C. To prevent the growth of fungi and bacteria, 25 µg/L nystatin and 10 µg/L nalidixic acid were respectively added to the culture medium. After 7 days, colonies of actinomycetes were selected and isolated based on the specific characteristics [10].

Extraction of Metabolites from Actinomycetes Isolates

To extract the anti-cancer metabolites, active isolates of actinomycetes were inoculated in starch-casein broth liquid medium. To produce anti-cancer compounds, Erlenmeyer flasks were put for 7 days at 28°C in a shaking incubator at 180 rpm [3]. To isolate the mycelia from the liquid phase, the culture medium containing metabolites was passed through syringe filters (45 µm size) and centrifuged at 5000 rpm for 10 minutes. Due to the solubility of antibiotic compounds in organic solvents, an ethyl acetate organic solvent was used to extract the anti-cancer metabolites. Ethyl acetate was added by an equal volume of supernatant obtained from each isolate. The organic phase, containing antibiotic compounds, was isolated by a decanter and condensed by heat. The extracted metabolites were used to investigate the anti-cancer activity of the isolates on the breast cancer cell line [11, 12].

Table 1: Coordinates of Sampling Stations

Station	East longitude	North latitude
1	3039567	662318
2	3039446	662457
3	3039326	662651
4	3039204	662762
5	3039082	663232
6	3038964	663232
7	3038850	664498
8	3038735	664498
9	3038617	664911
10	3038499	665270
11	3038378	665436
12	3038256	665548
13	3038135	665659
14	3039444	662265
15	3039322	662403
16	3039201	662570
17	3040926	662657
18	3040805	662768
19	3038837	662959
20	3038817	663180
21	3038598	663484
22	3038598	664035
23	3038366	664530
24	3038265	666179
25	3038129	665248
26	3038008	665386
27	3037887	665553
28	3037766	665692
29	3039196	662185
30	3040923	662382
31	3038956	662628
32	3038835	662822
33	3038715	663016
34	3038594	663154
35	3038472	663293
36	3038353	663597
37	3038233	663846
38	3038112	664012
39	3037992	664178
40	3037837	664510

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Cell Culture

Cell line 4T1 was purchased from the Pasteur Institute of Iran and cultured in the medium RPMI 1640 (Biosera, England) enriched with 10% fetal bovine serum and streptomycin (100 µg/mL) and penicillin (100 U/mL). Cells were incubated at 37°C, 95% humidity, and 5% carbon dioxide [13].

Investigation of Cytocidal Features

To evaluate the anti-cancer properties of metabolites, cells were cultured for 24 hours in 96-well plates. For this purpose, a 90-mL culture medium containing 2×10^4 cells and 10 microliters of isolated metabolites (concentration of 1 mg/mL) were added to each well. The test for the positive control samples was conducted adding Triton X-100, negative control (no metabolites), and ethyl acetate control in 24 hours of incubation. To assess cytotoxicity, the MTT assay was used. This method is based on reviving solution dimethyl diphenyl tetrazolium bromide color to an insoluble product of purple formazan by the mitochondrial reductase enzyme activity in the living cells, and the number of living cells can be detected from the nonliving ones. The MTT solution at a concentration of 5 mg/mL was prepared in a RPMI medium. The MTT assay was used to investigate the cytocidal activity of metabolites on cell lines, and the results were read by the enzyme-linked immunosorbent assay (ELISA) reader device at 430 and 692 nm wavelengths [14]. Isolated metabolites, with the highest cytotoxicity, were selected and their cytotoxicity effects were evaluated in different concentrations (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) within 24 and 48 hours [14, 15].

Observing Cytopathic Effects

To view the cytopathic effects of metabolites on the breast cancer cell line, imaging was performed using an inverted microscope.

Molecular Identification of Actinomycetes Isolates Using 16S rDNA

DNA extraction was conducted using a bacterial DNA extraction kit according to the manufacturer's instructions (Yekta Teb Tajhiz, Iran). Polymerase chain reaction (PCR) was performed in a total volume of 25 µL containing 1mM $MgCl_2$, 2 U Taq polymerase (Cinnagen, Iran), 75 µM of each dNTP, 1 µL of each primer (with concentration of 10 pmol) containing forward primer (5'AGAGTTTGATCCTGGCTCAG 3') 27f and reverse primer (5' GGTTACCTTGTTACGACTT 3') 1492r, 12.5 µL 10 X PCR (Cinnagen, Iran), and 1 µL of template DNA. In this technique, to begin the amplification process, a thermal cycler PCR machine (BioRAD) was set at 94°C for 1 minute. Then, 35 cycles of PCR were amplified at 94°C for 1 minute, 52°C for 30 seconds, and 72°C for 1 minute. Finally, elongation was done for 4 minutes at 72°C. Then, to ensure the amplification of the 16S rDNA gene, electrophoresis was conducted on 1% agarose gel, containing Tris/Borate/EDTA (TBE) 1X buffer for 60 minutes at 90 V. Finally, a UV transilluminator was used to observe the results. At last, the PCR final product was sent to the South Korean Macrogen Co. for sequencing [16].

Statistical Analysis

One-way ANOVA with post hoc test was employed to compare numeric data among different concentrations.

RESULTS

Isolation of Actinomycetes from Marine Sediments

Among 40 sediment samples, 186 marine actinomycetes were isolated based on the colony, morphological, and microscopic characteristics. The morphological characteristics of actinomycetes are rough and dry looking, and due to their closeness to fungi, their appearance is similar to them (Figure 1).

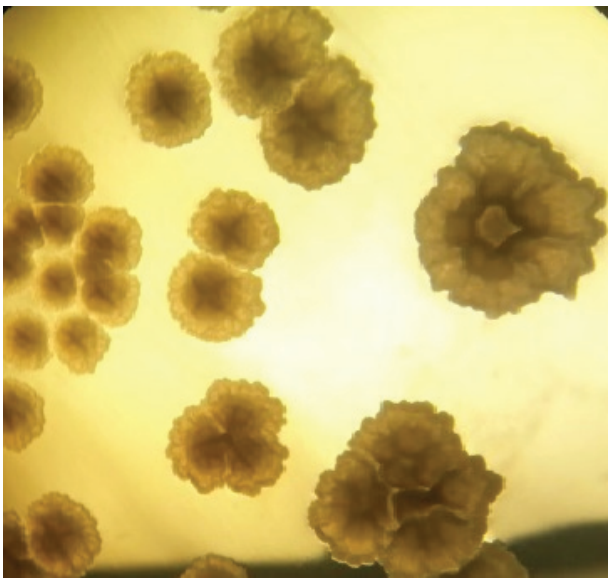

Figure 1: Colonies of the Isolated Actinomycetes

Identification of Actinomycetes with Highest Percentage of Cytotoxicity

At this stage, metabolites of 186 isolates at a concentration of 1 mg/mL were treated on the breast cancer cell lines. The highest cytotoxic effects were observed in samples of 2HP (34.27%) and 4HP (33.50%). Results of the metabolites extracted from samples at different concentrations showed that increased concentration of 2HP and 4HP led to decrease survival of cancer cells; hence, in the 2HP sample, the survival rate of cancer cells was decreased from 99.2% in 0.0001 mg/mL concentration to 65.66% in 100 mg/mL, within 48 hours. In addition, in the 4HP sample, the cell survival rate was decreased from 99.98% in 0.0001 mg/mL concentration to 63.8% in 100 mg/mL (Figure 2).

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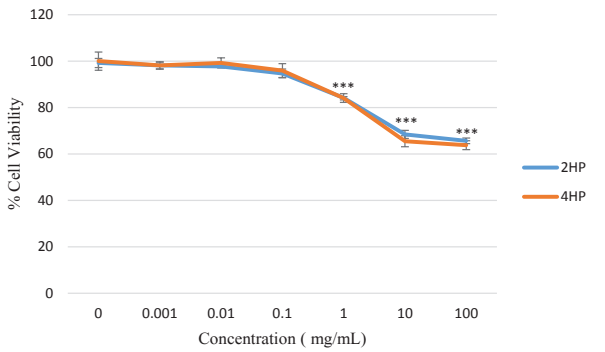

Figure 2: Comparison of the Diagram of Changes of the Samples (2HP and 4HP) at Different Concentrations at 48 Hours.
The diagram showed that survival of cancer cells significantly decreased at the concentrations of 1, 10, and 100 mg/mL. ***: P < 0.001

Imaging by an Inverted Microscope

Cytopathic effects of 2HP and 4HP actinomycetes metabolites were observed using an inverted microscope, and imaged after 48 hours. The results showed that metabolites caused morphological changes associated with apoptosis in 4T1 cells; therefore, in the treated cells, the structure of the spindle-shaped cells changed and it was observed as a spherical or collapsed form (Figure 3).

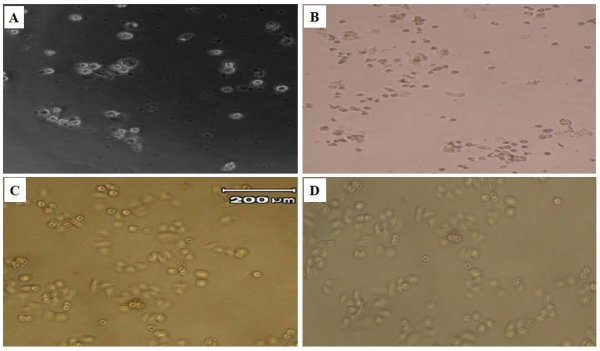

Figure 3: Cytopathic Effects of 2HP and 4HP Isolated Metabolites, Compared with the Negative and Ethyl Acetate Controls on the Breast Cancer Cell Line after 48 Hours. A) Negative Control, B) 2HP Isolate, C) 4HP Isolate, D) Ethyl Acetate Control

16S rDNA Sequencing of the Selected Strains

The 16S rDNA gene of the selected strains was sequenced. The partial sequence of the 16S rDNA gene was amplified using the BLAST software and compared with the other bacteria. The results showed that the sequence of the selected 2HP and 4HP isolates obtained based on the sequence homology of 98% had a genetic similarity with the streptomyces strains.

DISCUSSION

It is important to find new antitumor agents to treat diseases and cancers. Streptomyces are one of the most important microorganism producing antitumor agents. Wide biodiversity in the seas and oceans is a sign of the diversity of chemicals in marine environments. Since marine organisms, especially actinomycetes, have the largest metabolic and genomic diversity, there are efforts to discover marine actinomycetes as new drug sources. With limited screenings dedicated to marine actinomycetes until today, the discovery of new secondary metabolites produced by the marine actinomycetes of the soil type became higher. According to the increasing trend of resistance against antibiotics in the bacteria and chemotherapy drug problems, the use of marine natural compounds attracted the scientists` attention [1]. Therefore, oceans are a huge library of natural unique compounds and products, and promising and surprising bioactive materials that can be never found on the earth. Recently, it is demonstrated that the ocean floor is an ecosystem with unique forms of actinomycetes in itself. It seems that actinomycetes are distributed in all over the oceans; therefore, they are found in tidal regions, ocean sediments, seawater, mangroves, fishes, jellyfishes, seaweeds, and sponges. Since the marine environmental conditions are totally different from that of drought, it seems that marine actinomycetes should also be very different from their soil forms. Hence, marine actinomycetes are able to produce new antibiotics and bioactive compounds [16, 17]. In the present study, Persian Gulf was selected as a marine ecosystem to evaluate the anti-cancer activity of actinomycetes in the sediment samples which can be cultured using above mentioned methods. Mangrove areas located in the Naiband Bay in Assaluyeh were selected as sampling stations. Sampling was mostly done close to the roots of mangrove trees to increase bacterial isolation, and significant colonies of actinomycetes were isolated from them, and the primary identification was performed based on the morphological features of the colony. In the current study, 186 actinomycete species were isolated from the Naiband. Ravi Kumar *et al.*, (2012) collected actinomycete isolates from the mangrove ecosystem, and reported that the mangrove ecosystem was a good place to isolate actinomycetes [14]. In the present study, the effects of purified cytocidal metabolites were examined at a concentration of 1 mg/mL for 48 hours and the results indicated the inhibition of cancer cells proliferation. Kumar *et al.*, (2013) isolated different actinomycete strains from the Indian Ocean, and reported that the isolated strains had inhibitory effects on the growth of breast cancer cell lines [18]. Moreover, Valliappan *et al.*, (2013) reported that the actinomycetes extract showed a cytocidal effect on cancer cells and breast cancer cell lines [19], consistent with the results of the present study indicating the cytotoxic effect of actinomycetes extract on the breast cancer cell line. Jayamadhuri and Krishna (2013) isolated actinomycete strains from a tidal zone, and reported that actinomycete extracts had cytotoxic effects on the growth of breast cancer cell lines [20]. Fedorov conducted a study on marine actinomycetes in 2013 and

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reported that actinomycetes had a great potential in the treatment of cancer consistent with the result of the present study [21]. Ravikumar *et al.*, (2012) reported that the anti-cancer property of cell-free extracts from actinomycete isolates might be due to the presence of the active secondary metabolites such as alkaloids and quinines [14]. Manivasagan *et al.*, (2014) reported that the marine environmental conditions are extremely different from that of the terrestrial ones. The marine microbes have different characteristics, and therefore, might produce different types of bioactive compounds in their challenging living conditions [22]. According to above mentioned effects of actinomycete extracts, further studies seem to be necessary to identify possible mechanisms of apoptosis induction by the metabolites extracted from the actinomycetes on the cancer cell lines. Studies on the effects of actinomycete metabolites on human normal cells need to be fully understood. Moreover, the susceptibility of actinomycete metabolites should be investigated *in vivo*.

In general, according to drug resistance and problems caused by the side effects of chemotherapy in patients with cancer, identification of new effective drugs including metabolites from actinomycetes may play an important role in further studies on cancer treatment. It is hoped that with a greater focus on marine natural resources, cancer and other diseases can be controlled.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

ETHICS APPROVAL

Not applicable.

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