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Investigating the Cytotoxic Effects of Persian Gulf Marine Actinomycetes Protease on Blood Cancer Cell Line

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ABSTRACT

Background: Considering their specific adaption to marine milieus, marine actinomycetes constitute an excellent source for producing unique bioactive substances. They are also important sources of antibiotics and extracellular enzymes, geosmin, and enzymes with potent antibacterial and anticarcinogenic properties and low toxicity. The objective of this study is to isolate actinomycetes producing antitumor substances from the sediments of Hara forests in the Persian Gulf and investigate the positive in vitro effects of these microorganisms through protease production on blood cancer cell lines.

Methods: In this study, 30 samples of sediments were collected from Hara forests. After the strains were isolated, the samples were identified using morphologic characteristics. The protease was extracted and its cytocidal activity on the Aacute lymphoblastic leukemia (ALL) was studied.

Results: The results indicate that out of the 30 actinomycetal isolates, 15 specimens were capable of producing protease, of which 2 affected the malignant cell line. In addition, statistical analysis revealed that the protease exerted greater cytocidal effect on the blood cancer cell line at higher concentrations. The cytopathic property of the protease was observed as cell rupture.

Conclusions: Our findings indicate that sediments of the Hara forests in the Persian Gulf are rich in active actinomycetes producing novel anticarcinogenic substances, warranting identification and purification. These results suggest the need for studies on marine microbes as a new potential for pharmaceutical research.

Keywords: Protease, Marine actinomycetes, Cytotoxic, Blood cancer

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Introduction

Microorganisms and naturally occurring substances are gaining attention for use in treatment of diseases. Natural bacterial products constitute a rich source of novel substances with diverse biologic effects, manifesting specific properties in different biologic systems[1]. Microorganisms, especially marine microbes, are widely recognized and studied as antagonist and biocontrol agents. Actinomycetes account for some 10% of the bacterial population in seabed sediments. Marine microorganisms are a marvelous source for potent bioactive substances. Actinomycetes are among bacteria that occur abundantly in various conditions. It has also been shown that 75% of antibiotics isolated from actinomycetes are produced by the genus Streptomyces. This genus has gained extensive interest due to its protease production ability.

Blood cancer is a group of neoplastic disorders, comprising 8% of all human malignancies[2]. As a result of their high prevalence in childhood, high mortality rate, substantial costs, and lengthy hospital stay, blood cancers greatly influence parents and families[3]. Any treatment modality used for acute and chronic lymphoid and myeloid blood cancer is not only expensive, but also painful and risky in many cases. Considering the prevalence and refractoriness of blood cancer to chemotherapy, novel treatment agents are required to mitigate risks of treatment.

Methods

Screening and isolation of actinomycetes

In order to isolate the actinomycetes, the geographic coordinates of Nayband Gulf in Asaluyeh County, Bushehr Province, were determined using the Global Positioning System (GPS). A total of 30 spots, distances at 500 meters from each other, were selected. The coordinates of each station are given in **Table 1**. A total of 30 samples were collected in special sampling vessels from the designated stations, and transferred to the laboratory as soon as possible. Then, the samples were preserved at 55ºC for one hour to suppress the growing forms of actinomycetes and other bacteria. Serial dilutions were then prepared. For this purpose, 1 mL of the sample was mixed with 9 mL physiologic serum. Then, 1 mL of the supernatant was mixed with 9 mL physiologic serum. The samples were diluted to a concentration of 10-4[4].

Bacterial isolation and identification

In this study, the diluted samples from Hara forests were cultured on starch casein agar (SCA) medium. In order to inhibit other microorganisms, 10 µg nalidixic acid, and to inhibit fungi, 25 µg/L nystatin were added to the culture medium. Subsequently, the culture plates were incubated at 28ºC for 7-12 days. Bacterial identification was accomplished using phenotypic characteristics of the colony and Gram staining[5].

Protease activity assay

Actinomycetal isolated were studied for enzyme assay. For this purpose, 0.5 McFarland was used for equal turbidity of all samples. Then, one loop of actinomycetes was inoculated on tryptic soy broth (TSB) medium to assess growth. Subsequently, the selected samples were cultured on skim milk agar. After incubation at 27ºC for 48 hours, the halo size was measured using a ruler[6].

Enzyme extraction

In order to extract protease from actinomycetes, one full loop of actinomycetal colony was cultured on 25 mL liquid modified glucose nutrient agar (containing 5 g peptone, 3 g yeast extract, 10 g glucose, 3 g sodium chloride, 3 g beef extract, 3 g calcium carbonate) and incubated at 30oC for 3 days in shaking incubator. After 3 days, 10% of the medium was taken and incubated at 30ºC for 3 days in shaking incubator with 100 mL casein broth containing 5% sodium chloride and pH 9. After 3 days, 1 µL of

the medium was taken and incubated for 12 hours under similar conditions with 100 mL casein broth. In the last step, the sample was centrifuged for 25 minutes at 12,000 rpm and 4°C in refrigerated centrifuge. The supernatant was taken as crude enzyme and filtrated with 0.22 µm syringe filter. The purified enzyme was preserved in microtube at -20°C[7].

Protease purification

To further purify the enzyme, precipitation with ammonium sulfate and dialysis bag was used. All steps of purification were performed at 4°C. To achieve this, ammonium sulfate powder was added to the obtained enzyme solution to yield 55% saturation. The solution was centrifuged at 12,000 rpm and 4°C for 15 minutes. The resulting precipitate was slowly dissolved in 0.01 M phosphate buffer with pH 7.5. Dialysis was performed to remove ammonium sulfate. The dialysis membrane (Sigma-Aldrich Co), 45 mm in width and 12 kDa pores, was cut to the size of 15 cm (measured with a ruler) and placed in the previously prepared phosphate buffer. After a few minutes, a string was attached to one end, and 6 mL of the enzyme-containing solution was slowly transferred onto it with sampler. The other end was blocked with some free space in the bag. Subsequently, the dialysis bag was placed in a clean beaker filled to one-third of its volume with distilled water. It was transferred on a magnet plate to a cold environment as slowly as possible, and the water inside the beaker was changed every 6 hours. After completion of dialysis, the enzyme-containing solution was transferred to microtube and enzyme activity was assessed at 280 nm wavelength[8]. The concentration of the purified enzyme was evaluated using nanodrop.

Protease activity assay

One enzyme unit is the amount of enzyme that may free soluble fragments equivalent to 0.001 absorption increase in 30 minutes under standard conditions. In order to assess enzyme activity, 0.6 mL of 1.5% casein was dissolved in 20 mM Tris-HCl buffer (pH 8.5) and 100 µL of the isolated enzymatic solution was added to it. The reaction mixture was

Table 1: Coordinates of sampling stations

Northern latitude	Eastern longitude	Station
662318	3039567	1
662457	3039446	2
662651	3039326	3
662762	3039204	4
663232	3039082	5
663232	3038964	6
664498	3038850	7
664498	3038735	8
664911	3038617	9
665270	3038499	10
665436	3038378	11
665548	3038256	12
665659	3038135	13
662265	3039444	14
662403	3039322	15
662570	3039201	16
662657	3040926	17
662768	3040805	18
662959	3038837	19
663180	3038817	20
663484	3038598	21
664035	3038598	22
664530	3038366	23
666179	3038265	24
665248	3038129	25
665386	3038008	26
665553	3037887	27
665692	3037766	28
662185	3039196	29
662382	3040923	30

incubated at 37ºC for 30 minutes. Then, the reaction was stopped with addition of 0.64 mL TCA buffer (containing 0.11 M trichloric acid, 0.22 M sodium acetate, 0.33 M acetic acid) and incubation at 37ºC for 10 minutes. The resulting mixture was centrifuged at 12,000 rpm for 10 minutes and the absorption of the supernatant at 280 nm was measured using nanodrop. The activity was reported as enzyme unit per milliliter[9].

Blood cell line culture

In this study, we used the KG1 human blood cell line. The cell line, NCBI code C119, of human origin, bone marrow tissue with acute myeloid leukemia, was obtained from the cell line collection of Iranian Pasteur Institute. The cells were cultured on RPMI 1640 medium containing 10% bovine serum albumin, 2 mM L-glutamine, 100 **c**g/mL streptomycin, and 100 U/mL penicillin. The percentage of viable cells was determined using trypan blue. The toxicity of isolated enzymes was studied based on cell proliferation using the MTT assay (3-(4 5-dimethyl-2-thiazolyl)-2 5-diphenyl-2h-tetrazolium bromide). The assay was performed in 96-well plates, each well containing 90 **µ**L cells (10,000) and 10 **µ**L enzyme. The isolated enzymes at a concentration of 1 mg/mL were used to treat the blood cancer cells in the 96-well plate in triple. After the results were obtained, enzymes with the greatest toxicity were selected and used to treat the cancer cells at concentrations of 0.01 to 0.00001 mg/mL. In addition, throughout the experiment, a negative control (without enzyme) was used. It is worth mentioning that the plated were incubated at 37ºC with 5% CO_2. After the incubation period, 10 **µ**L of the MTT solution was added to all wells and the plates were placed in the oven for 3 hours, followed by centrifugation at 18,000 rpm for 10 minutes. Subsequently, the supernatant was discarded and the cellular precipitates were dried in the oven at 37ºC for a few minutes. Finally, the formazan crystals deposited in cell cytoplasm were dissolved with addition of dimethyl sulfoxide to each well and shaken for 15 minutes in the dark. Color intensity was recorded using an ELISA reader at 570 nm of wavelength[7].

Effect of actinomycetal protease on blood cancer cell line

In order to analyze the results of cell growth and proliferation at each dilution of the enzyme, the following equations were used:

Percentage of viable cell = 100 x mean absorption of viable cells / mean absorption of negative control

Percentage of dead cells = 100 – percentage of viable cells[7].

Observing and photographing the cytopathic effects using reverse microscopy

In order to observe the cytopathic effects of protease on blood cancer cell line, photography was performed using a reverse microscope.

Results

Frequency of actinomycetal colonies in hara samples

Out of 30 samples studied, a total of 186 colonies were isolated. Based on morphology and Gram staining, 30 isolated were selected as being actinomycetes. Evaluation of gross appearance and microscopic characteristics revealed that protease-producing bacteria create colonies resembling dried plaster of different colors. All colonies were covered with air revealed Gram-positive filamentous bacilli (**Figures 1 & 2**).

Statistical analysis of frequency of actinomycetal colonies from hara samples was performed using T-test and ANOVA, on SPSS version 19 (**Diagram 1**).

Protease activity assay

After screening on skim milk agar medium,

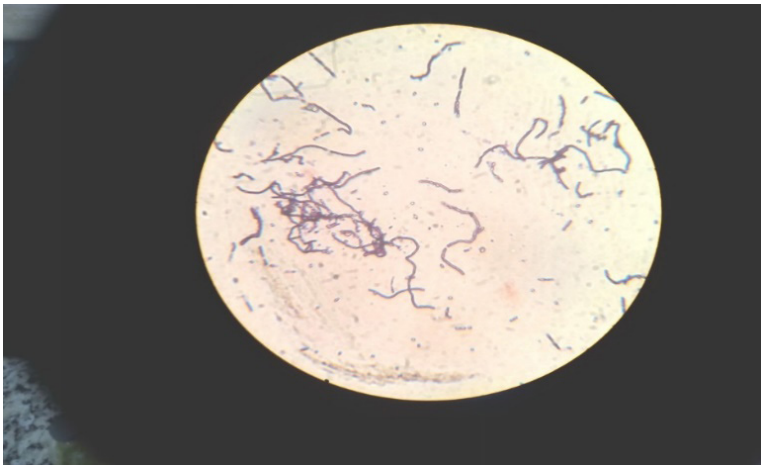

Figure 1: Gram staining of actinomycetes colony under light microscopy at 100x magnification

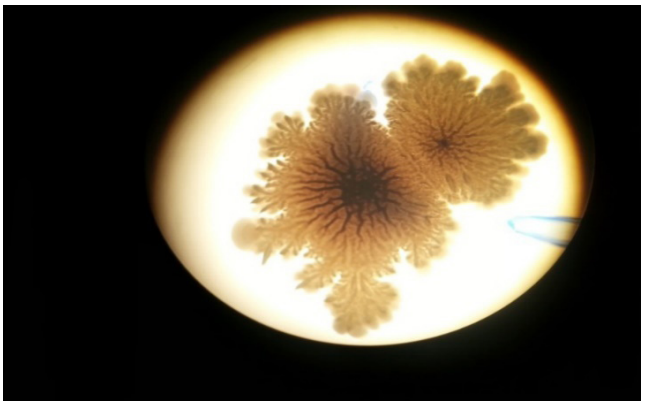

Figure 2: Color and format of fat and stiff colony such as Actinomycetes grown in SCA medium and microscopic image of mycelia observed by microscopy.

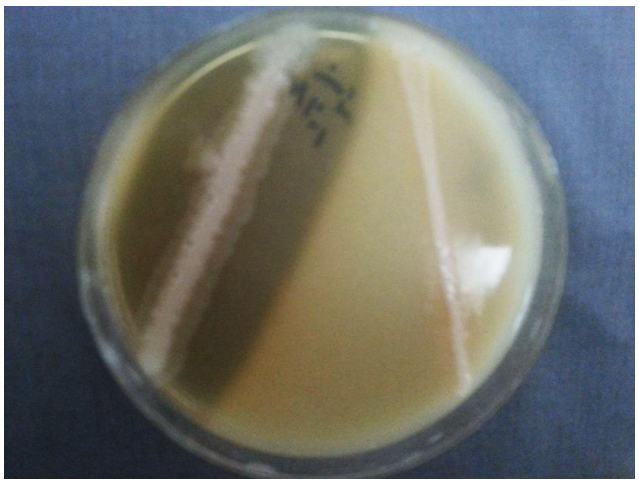

Figure 3: The activity of Proteolytic Actinomycetes Leading to create of halo the colony which shows digestion of Casein By bacteria (left) and lack of Casein digestion leading to lack of halo existence (right)

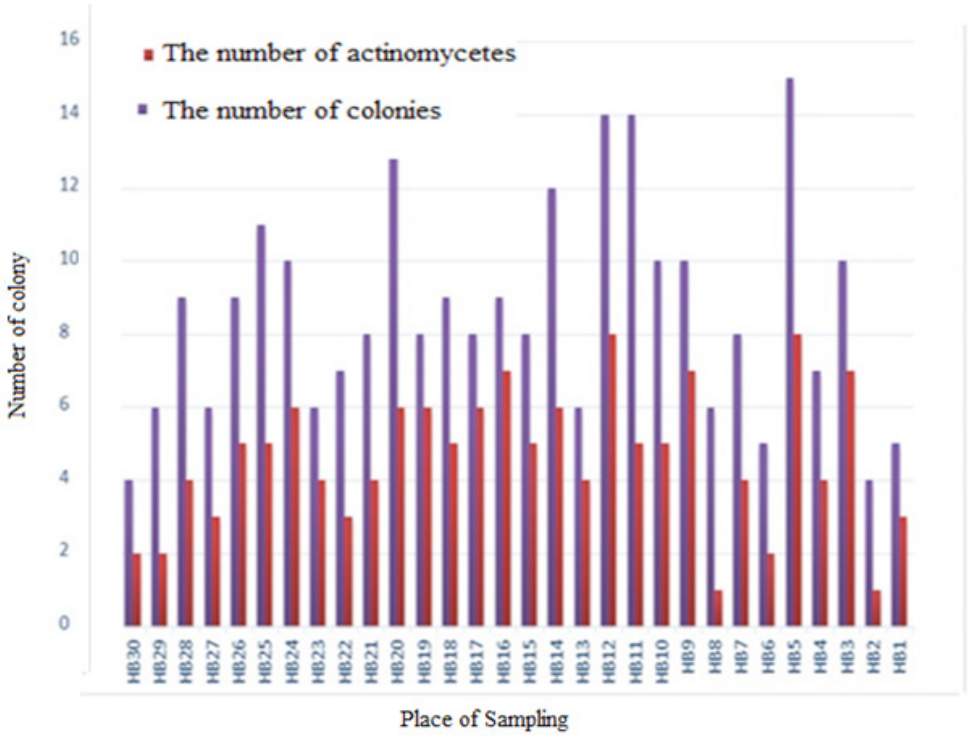

Diagram 1: Frequency of actinomycetes colonies in Persian Gulf hara deposits

proteolytic activity was assessed, identifying 15 out of 30 isolates with protease activity (**Figure 3**).

Protease enzyme activity

Protease enzyme activity is defined as the amount of enzyme that may yield 1µg of the product tyrosine for each mL of the substrate casein in a period of 1 minute. In this study, we found the enzyme activity (expressed in international units) to be equal to 0.421 U/mL for sample 20, and 0.433 U/mL for sample 30.

Effect of actinomycetal protease on blood cancer cell line

The cytotoxic effect of the 15 isolated enzymes was first exerted at a concentration of 5 mg/mL (**Diagram 2**). All cells treated with protease were photographed using reverse phase microscope. Cytotoxic effects were evaluated at concentrations from 0.01 to 0.00001 mg/mL. The findings indicate that the greatest cytotoxicity was observed at 0.0001 mg/mL actinomyces 20 (29%) and the smallest cytotoxicity was observed at 0.001 mg/mL actinomyces 30 (26%) (**Diagram 3**).

Identification of actinomycetes based on colony morphology

Figure (1, 2, and 3).

Photographing cytopathic effects using reverse microscopy

The cytopathic effects protease from actinomycetal isolates 20 and 30 were observed by reverse microscopy and photographed after 48 hours. The results indicated that the protease causes significant morphologic changes, leading to changes in cell

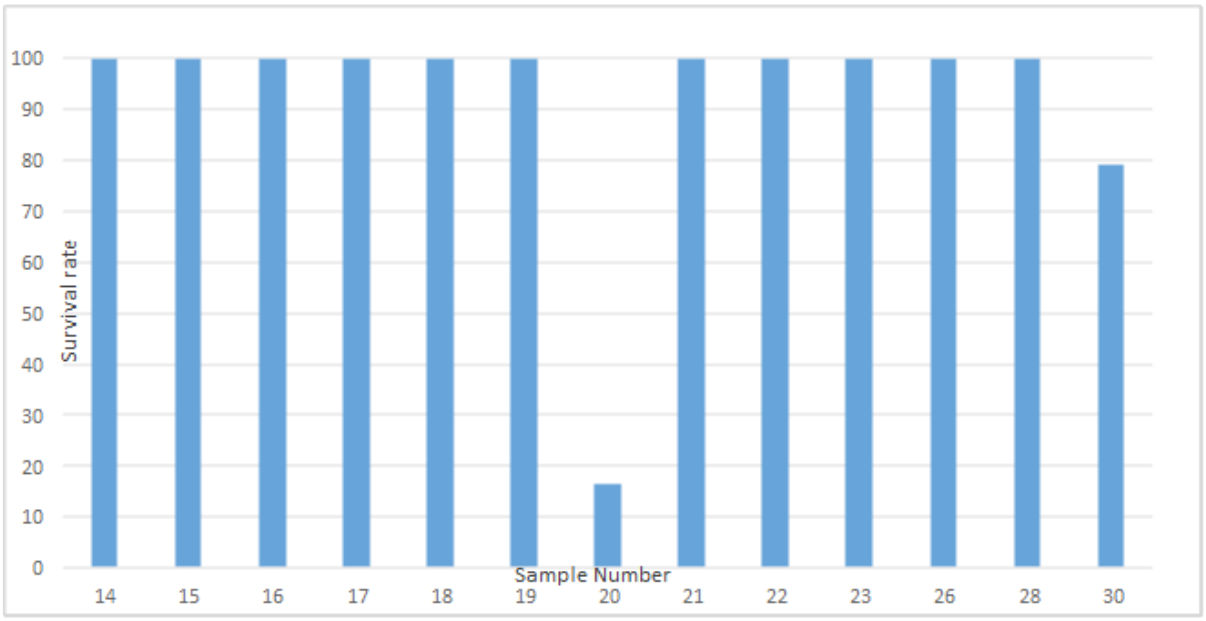

Diagram 2: Survival rate of cancerous cells at 5 mg/mL after 48 hours

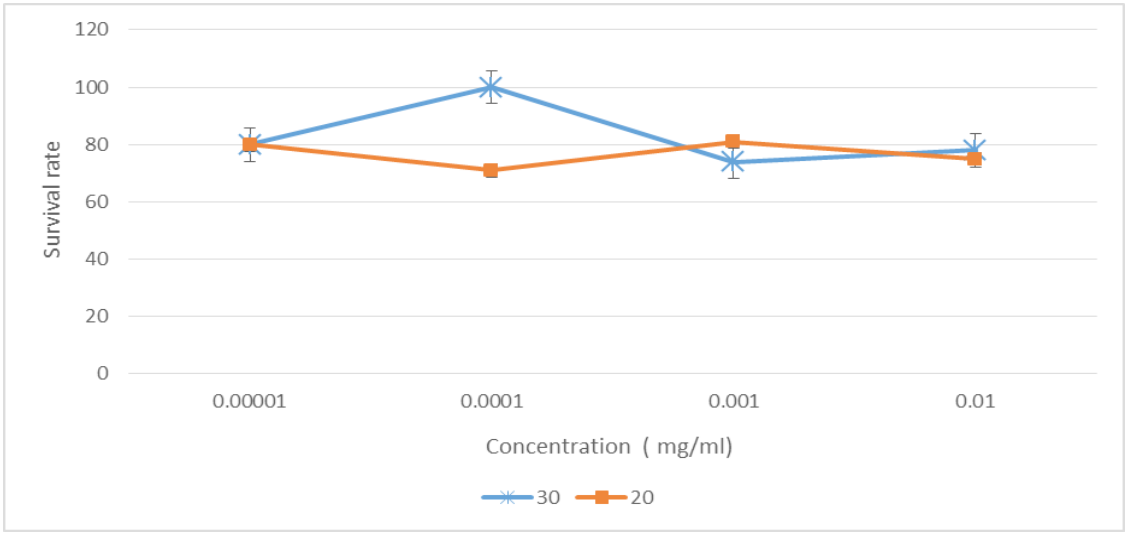

Diagram 3: Treatment of blood cancer cell line with protease from two selected isolates at different concentrations

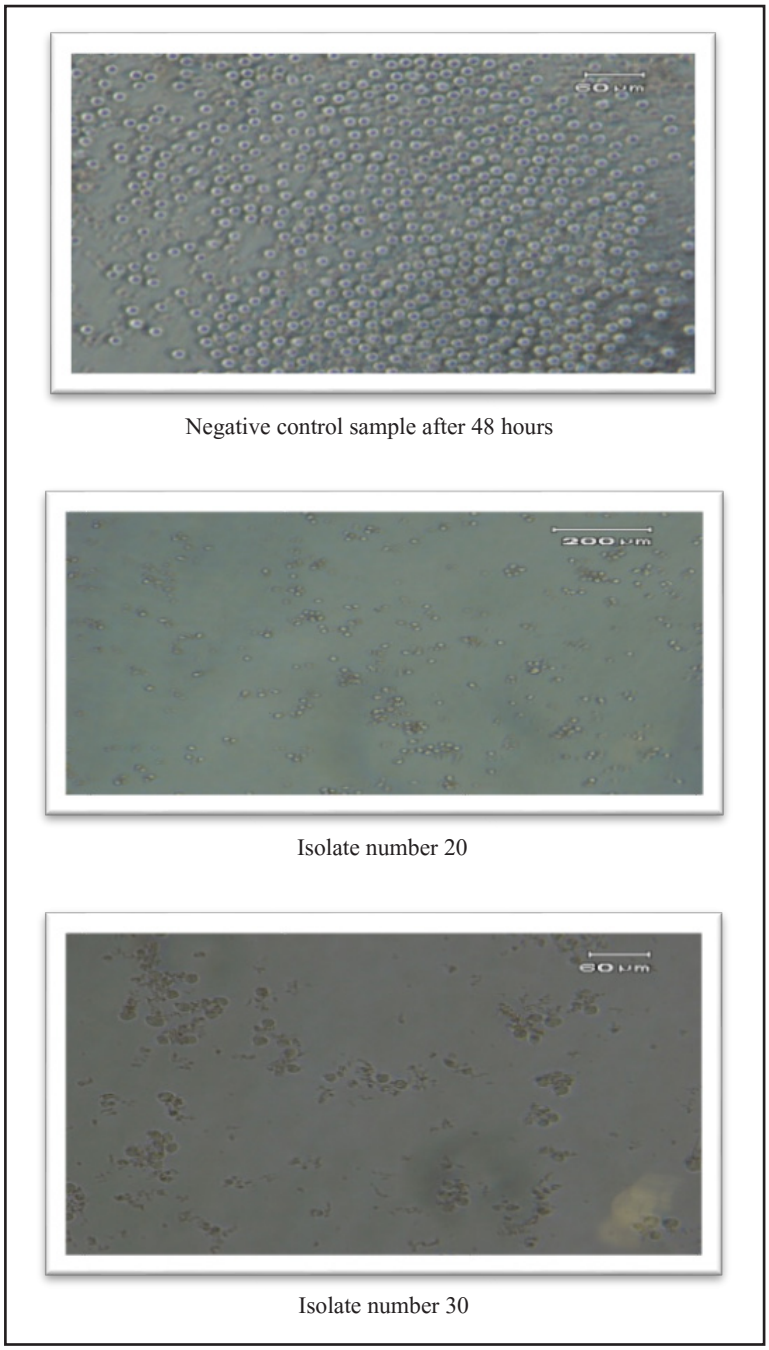

Figure 4: Morphologic changes of blood cancer cells in isolates 20 and 30. The enzyme concentration is 0.01 mg/mL

skeleton and cell fragmentation. The density of cancerous cells was 16.54 for actinomycetal isolate 20, and 15.79 for actinomycetal isolate 30, indicating maximal anticarcinogenic activity for these isolates in the cell line studied (**Figure 4**).

Photography with reverse microscopy

The results of this section are presented in Figure 4.

Discussion

Actinomycetes are among effective bacteria used for antibiotic and enzyme production for the purpose of treating diseases. Novel anticarcinogenic compounds derived from natural sources, such as actinomycetes, have gained much interest due to their chemical diversity and high efficiency[10]. Moreover, the members of this genus, particularly the Streptomyces, produce more than half of bioactive metabolite, including antitumor substances[11].

A simple analysis of data from previous studies reveals that the search for drugs from seas has surged by an annual 13%, and continues to rise. It may be said that the oceans harbor a vast library of unique and promising bioactive agents and compounds, which cannot be found on terrestrial environments. It has been recently proven that the floor of the ocean, as an ecosystem, is home to unique forms of actinomycetes. It appears that the actinomycetes are distributed throughout the oceans, found in tidal areas, sea water, fish, jellyfish, sea moss, mangroves, sponges, and ocean sediments. Given the very different conditions of the marine milieu compared to the land, it appears that marine actinomycetes will differ substantially from their counterparts in soil. Thus, they may be able to produce novel bioactive compounds and antibiotics[12].

In this study, we chose the Persian Gulf as a marine ecosystem to capture actinomycetes from its sediments, which may be cultured with the methods employed in this study. The Persian Gulf is one of the largest shelters for marine creatures, including coral, small aquarium fish, edible and inedible fish, scallops, snails, mollusks, anemones, sponges, jellyfish, tortoises, dolphins, sharks, and a plethora of marine microorganisms[13].

The sampling site was selected in the hara forests in the Naybad Gulf, Asaluyeh. Samples were taken preferentially from the proximity of hara roots to improve bacterial isolation. Considerable colonies of actinomycetes were isolated and preliminary differentiation was made using colony morphology. In this study, we obtained 186 actinomycetal isolates from the Nayband Gulf, Asaluyeh. In 2012, Ravikumar et al. collected actinomycetal isolates from the mangrove ecosystem and reported that the mangrove ecosystem is a suitable site for actinomycetal isolation[14].

In 2007, Kathiresan and Manivannan isolated an alkaline protease with antibacterial properties from a Streptomyces species[15]. Awad et al., in 2013, reported maximal activity of alkaline protease in Streptomyces pseudogrisiolus NRC-15, which is consistent with findings of the present study demonstrating the ability of actinomycetes producing protease[16]. Other studies have indicated that most actinomycetal genera are aerobic bacteria and exhibit greatest growth at an aeration rate of 205-255 round per minute. Increased aeration rate will increase the cellular biomass and with greater numbers of microorganisms per unit volume, more alkaline protease will be yielded[17]. Abdelwahed et al. in 2014 studied Streptomyces ambofaciens and reported maximal alkaline protease production at 50oC. They concluded that this bacterium is capable of producing large amounts of the protease[18]. Furthermore, in 2013, Mane et al. reported that actinomycetes, especially Streptomyces, are capable of producing protease. They found the maximal activity of the alkaline protease from Streptomyces D1 to be at pH 10, indicating that different genera of actinomycetes may produce protease at different conditions and in different

amounts[19]. The results of the present study also indicate that out of 30 actinomycetal samples, only 2 bacteria showed maximal protease activity which may be due to differences in bacterial genus, thermal and environmental conditions, as well as the specific characteristics of the bacterium in producing protease. This probably indicates that each genus of actinomycetes has the potential to produce maximal protease in specific growth conditions; therefore, certain growth conditions are optimal for actinomycetes, as suggested by findings of previous studies.

In addition, in a study for identification of protease-producing actinomycetes conducted by Suthindhiran et al. in 2014, it was reported that these bacteria are able to produce proteases such as chymotrypsin serine protease, widely used in industries[20]. Moreover, regarding the anticarcinogenic properties of actinomycetes, Jeong et al. in 2010 reported that the members of this genus, especially Streptomyces, produce more than half of all bioactive metabolites, including anticarcinogenic compounds[21].

In 2009, Kharat et al. observed anticarcinogenic effects of different compounds derived from Streptomyces bacteria on various cell lines, including that of chronic myeloid leukemia (K562). Regarding purification and characterization of protease from actinomycetes and its cytotoxicity on cancerous cell line A549[22], Balachandran et al. in 2012 reported that the strains ERIA-31 (50.04 **µ**g/mL) and ERIA-33 (55.07 **µ**g/mL) possess protease and cytotoxic effects on cancerous cell lines, indicating that actinomycetes have anticarcinogenic properties at different concentrations which is consistent with findings of previous studies[7].

In 2013, Mosavi et al. studied the effects of growth inhibition and differentiation of metabolites soluble in ethyl acetate native bacterium, Streptomyces calvus, on human myeloid leukemia cell line K562, and reported that considering the growth inhibiting and differentiating effects if the metabolite derived from Streptomyces calvus on cell line K562, this metabolite may be used in future studies on patients suffering from CML[23]. Bressolier et al. who studied the Streptomyces genus with protease activity found out that this bacterium may be used in different stages of treatment[24]. In 2012, Balachandran et al. investigated the proteases extracted from actinomycetes and concluded that these proteases may inhibit the growth of human adenocarcinoma (A549) cancerous cells to a considerable extent[7], which is consistent with our findings indicating the ability of protease-producing bacteria to treat cancerous cell lines. In a study by Hames-Cocabas et al. in 2007, it was reported that proteases in the diet may reduce the risk of cancer[25]. Kumar et al. in 1999 reported that proteases are capable of hydrolyzing proteins of the cell wall[26]. It is also possible that in the present study, the protease activity of the actinomycetes has hydrolyzed proteins in cells walls of cancerous cells, leading to their necrosis and reduced survival. In 2009, Olano et al. stated that actinomycetes possess the ability to produce secondary metabolites with biologic activity, including proteases with antitumor properties which inhibit the growth of cancer cells through signal inhibition in cellular metabolism[27]. The studies published by Attaway et al. in 1993 dealing with the issue of cytotoxic and antitumor compounds derived from marine bacteria defines cytotoxicity as the toxic effect against cancer cells in cell culture milieu, and states that marine bacteria, including actinomycetes, exert toxic effects (necrosis, cell tumescence, and apoptosis induction) against cancer cells[28]. It is possible in the present study that the protease has caused necrosis, tumescence, and necrosis in cancerous cell lines, indicating its therapeutic properties.

Conclusions

Proteases are produced by a wide range of

microorganisms. Nevertheless, bacteria are the most popular source of industrial protease production. Considering the prevalence of cancer and its refractoriness against chemotherapy, novel treatment options are strongly needed. In this scenario, biologic agents extracted from living creatures constitute a major source for these novel antitumor compounds.

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