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Cytotoxic Activities of Actinobacteria Isolated from the Saudi Habitats by the Brine Shrimp Lethality Assay

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The recent studies have increased our interest in the cytotoxic activities of actinobacteria. In the present study, ten actinobacterial isolates were randomly selected from 56 isolated recovered from the Saudi habitats to be screened for their cytotoxic potential by the brine shrimp lethality assay. The isolates were subjected to fermentation for production of their secondary metabolites and then extracted by ethyl acetate. The crude extracts dissolved in methanol were used for the screening. The brine shrimp lethality test was conducted on each of the extracts at five different concentrations 5, 10, 25, 50 and 100 μg/ml. The extracts of the actinobacteria isolates showed IC 50 in the range from 4.56 to 88.38ug/ml. Based on their characters; four isolates were found to belong to genus *Streptomyces*, three isolates to genus *Micromonospora*, one isolate to genus *Nonomurea*, one isolate to genus *Actinomadura* and one isolate could not be assigned to a genus. This work reveals that actinobacteria from the Saudi habitats have high potential anti-cancer activities and can provide a definite source for novel drugs.

Key words: Actinobacteria, cytotoxic activity, brine shrimp lethality assay, characterization.

Actinobacteria are Gram positive bacteria frequently filamentous and sporulating organisms with DNA rich in G+C from 57-75% [1]. Among the well-characterized pharmaceutically relevant microorganisms, actinobacteria remain a major source of novel therapeutically relevant natural products [2]. Their physiological and biochemical activities are varied depending on ecological and geographical conditions. Therefore, exploration of new soils and habitats for screening actinobacteria may lead to the discovery of strains producing novel bioactive compounds useful for pharmaceutical application [3].

Cancer still represents one of the most serious human health problems despite the great progress in understanding its biology and pharmacology [4]. Discovering novel anticancer agents both synthetically and naturally have become increasingly important as many cancers do not respond to current treatments [5]. Approximately, 60% of the approved chemotherapeutic drugs are derived from natural compounds [6] among those, 50% of the natural antibiotics are produced by actinobacteria.

Brine shrimp lethality assay is known to be an efficient, rapid and inexpensive test for a broad range of biological activities and it requires only small amount of sample. This bioassay has a good correlation with cytotoxic activities [3]. Therefore, the aim of this work was to employ the brine shrimp lethality test to screen for the cytotoxic potential of actinobacteria isolated from the Saudi habitats.

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MATERIALS AND METHODS

Isolation of the strains

Isolation of the actinobacterial isolates was done using the MM medium recommended by Hozzein et al. [7] by the spread plate technique of soil samples collected from different regions of Saudi Arabia. The isolation plates were incubated at 28°C for 7- 28 days and colonies appeared on the plates were purified, maintained in MM slants and ten of the isolated strains were randomly selected for further studies.

Fermentation of the selected strains

A loopful of each of the ten selected actinobacteria strains was inoculated separately into 50 ml of ISP2 medium [8] in 250 ml of Erlenmeyer flasks and incubated for 2 days in shaking incubator at 150 rpm and 28ºC. Ten percent (v/v) of the seed inoculums were transferred into 200 ml of starch nitrate [9] as a production medium in 1 L Erlenmeyer flasks. The inoculated cultures in the production medium were incubated for 6 days on a shaker at 150 rpm and 28ºC.

Extraction of bioactive metabolites

After incubation, the culture broth was filtered through Whatmann No. 1 filter paper to get cell free extract. The extracellular metabolites in the cell free filtrate were extracted twice by liquid-liquid extraction with equal volume (1:1) of ethyl acetate which was added and mixed with the filtrate by vigorous shaking. The organic phase was then collected and evaporated to dryness in a rotary evaporator.

Sample preparation

The crude extracts were dissolved in methanol to obtain stock solutions of 1 mg/ml concentrations. Different concentrations of the extracts were prepared by dilution with methanol to obtain five different concentrations 5, 10, 25, 50 and 100µg/ml.

Brine shrimp lethality assay

The brine shrimp lethality test was conducted according to the method of Kekuda *et al.* [10] to determine cytotoxic nature of crude extract. Dried cysts of brine shrimp, *Artemia salina*, were hatched in a container containing artificial sea water which was prepared with 10g of a commercial salt mixture and 500 ml of distilled water. The container was well aerated with the aid of an air pump and incubated at 28-30°C and a proper light source for 48 hrs. After hatching, the active nauplii free from egg shells were collected from the container by a pipette to be used for the assay.

The test was done in three replicates where ten nauplii added into each concentration of each extract in 96 well plate. Methanol instead of extract was used as a control and then the plates were incubated at 28-30°C. After 24 hrs, dead shrimp was counted using a microscope. Larvae did not exhibit any internal or external movement was calculated as dead and the percentage of mortality of each concentration was calculated as compared with control. The data were transferred to the software program Origin (Version 6.1052; Origin Lab Corp Northampton, MA 01060, USA) which was used to calculate the IC50 values for each extract.

Morphological characteristics of the isolates

Morphological characteristics of the actinobacterial isolates, notably the spore bearing hyphae and the spore chains, were examined under light microscope by using the cover-slip technique [11] after incubation of the cultures at 28°C for 14 days on ISP 2 agar [8].

Chemotaxonomic characterization of the isolates

Whole cell hydrolysate was used to analyze the cell wall amino acids and sugars. Diaminopimelic acid (DAP) isomers were determined by the method of Hasegawa et al. [12] and whole-cell sugars were analyzed according to the method of Staneck and Roberts [13].

RESULTS AND DISCUSSION

Cancer is still a major public health threat worldwide. In USA, cancer has become the number one killing disease since 1999 [14]. The usual therapeutic methods for cancer treatment are surgery, radiotherapy, immunotherapy and chemotherapy[5].

Although heavily studied over the past six decades, actinobacteria continue to prove themselves as reliable sources of novel bioactive compounds. Antitumor antibiotics produced by actinomycetes are among the most important cancer chemotherapeutic agents including members of the anthracycline, bleomycin, actinomycin, mitomycin and aureolic acid families[15].

The brine shrimp lethality assay was proposed by Michael *et al.* [16], and later developed by Vanhaecke *et al.* [17], and Sleet and Brendel [18].

This procedure is considered to be very useful in determining various biological activities [19]. This method is rapid, inexpensive, reliable and simple which has been used for over thirty years in toxicological studies. The assay correlates in most cases reasonably well with cytotoxic and antitumor properties. This method needs no special equipment and no aseptic technique. It utilizes a large number of organisms for validation, relatively small amount of sample and does not require animal serum as needed for other methods of cytotoxicity testing [20]. Therefore, the brine shrimp lethality assay has been proved to be a convenient system for monitoring biological activities of natural products [3].

In the course of screening for cytotoxic activities of actinobacteria, 56 isolates were obtained from soil samples collected from different Saudi regions. Out of the 56 isolates, ten isolates were randomly selected for cytotoxic activities screening by the brine shrimp lethality assay.

The selected isolates were cultivated and their metabolites were extracted. Brine shrimp lethality test was conducted on each of the extracts at five different concentrations 5, 10, 25, 50 and 100µg/ml. The cytotoxic activity of different concentrations of each extract, in terms of mortality of brine shrimps, is shown in Fig. 1. No mortality was found in the methanol negative control group. It was found that the degree of lethality of each extract was directly proportional to its concentration, to increase with increasing the concentration of the sample. Similarly, it was observed in previous studies that the mortality of brine shrimp larvae by *Streptomyces* extracts was concentration dependent [21].

In the present study, highest lethal effect was observed at 100µg/ml extract concentration at which the mortality was 100% in the extracts of GD13, GO23, GO61 and GO87. Among the highly active extracts, only GO23 gave 100% mortality starting from 25µg/ml, and then GO61 and GO87 showed 100% mortality starting from 50µg/ml.

The extracts studied in this work showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to detect antitumor compounds. The IC 50

Table 1. The IC 50 values (µg/ml) for extracts of the selected actinobacterial isolates

Strain ID	IC 50 value µg/ml
GD10	39.527
GD13	24.234
GD15	44.737
GO23	14.696
GO32	65.038
GO55	88.383
GO57	65.039
GO61	4.564
GO63	47.523
GO87	17.11

Table 2. Characteristics of the selected actinobacterial isolates and their tentative classification.

Isolate	Morphological appearance	Chemotaxonomical characteristics	Tentative classification
GO23, GO32, GO55, GO57	Extensively branched mycelia with long mainly spiral spore chains	*LL*-DAP, Glucose	*Streptomyces*
GO61, GO63, GO87	Single spherical to ellipsoidal spores along branching mycelium on short sporophores and darkcolored spores occur in older cultures	*meso*-DAP, Arabinose and xylose	*Micromonospora*
GD10	Rod-shaped to branched short filaments	*meso*-DAP, Glucose	ND
GD13	Extensively branched substrate mycelium that differentiates into short straight chains of spores	*meso*-DAP, Madurose	*Nonomurea*
GD15	Short chains of conidia on the aerial mycelium.	*meso*-DAP, Madurose	*Actinomadura*

results of the ten actinomycetes evaluated in this screening are listed in Table 1. Meyer *et al.* [22] reported that, if the brine shrimp lethality assay displayed LC 50 < 1000µg/ml of natural derived products was known to contain physiologically active principles. The ethyl acetate extracts of the ten isolates showed high levels of toxicity with the IC 50 in < 100µg/ml. The actinobacteria isolates showed IC 50 in the range from 4.56 to 88.38ug/ml. Therefore, these extracts can be regarded as promising candidates for containing potential anticancer drugs as suggested by Sivasankar *et al.* [23]. However, further and more specific bioassays are necessary in order to confirm this conclusion. The actinobacterial isolates were characterized on the basis of morphological and chemotaxonomical characteristics. The characteristics and tentative classification of the isolates are shown in Table 2. Based on their characters, the ten isolates were found to belong to four genera. The isolates GO23, GO32, GO55 and GO57 belong to genus *Streptomyces*; isolates GO61, GO63 and GO87

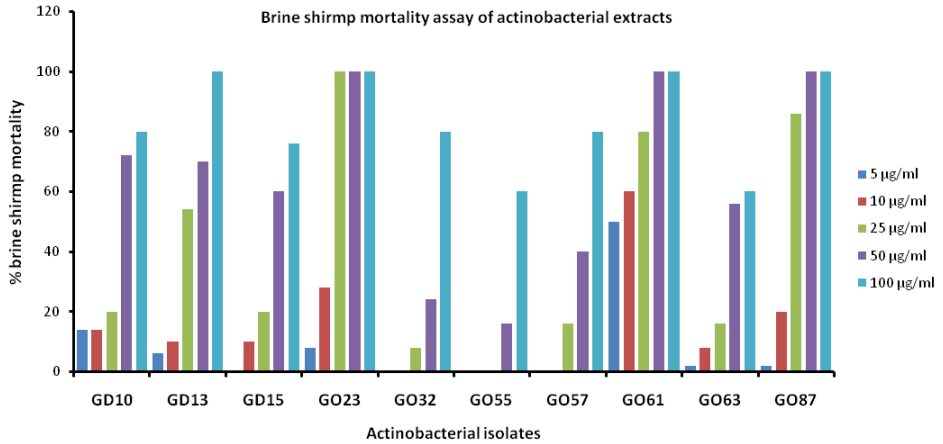

Fig. 1. Brine shrimp lethality assay of different concentrations of actinobacterial extracts

belong to genus *Micromonospora*; isolate GD13 belong to genus *Nonomurea*; isolate GD15 belong to genus *Actinomadura* and isolate GD10 could not be assigned to a genus based on the available data.

A number of studies have been carried out on cytotoxic activities of actinobacterial extracts using brine shrimp assay. From ethyl acetate extract of a *Streptomyces* strain, Sultan *et al*. [24] isolated three active metabolites which were found to cause a marked lethal effect on brine shrimp. Similarly, Ripa *et al.* [25] showed potent lethal effect of ethyl acetate extract and a purified compound of *Streptomyces rajshahiensis* against brine shrimp.

In conclusion, the cytotoxicity exhibited by the tested actinobacterial extracts was promising and indicates the presence of potent antitumor compounds. Further studies on purification and identification of the active principals in the highly active extracts should be carried out. The results of this study suggested that actinobacteria from the unexplored Saudi habitats could provide lead compounds of medical value.

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