ASSAY OF GENOTOXIC AND CYTOTOXIC POTENTIAL OF A COMPOUND EXTRACTED FROM MARINE STREPTOMYCES

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Summary

Actinomycetes are a group of Gram positive bacteria with high G+C Content. The presence and distribution of actinomycetes have been demonstrated to be associated with their various ecological habitats, including beach sand and sea water. Marine actinomycetes are capable of producing different types of novel secondary metabolites. Many of these metabolites possess biological activities and have the potential to be developed as therapeutic agents. Marine actinomycetes are still a prolific but underexploited source for the discovery of novel secondary metabolites. The aim of the present study was to evaluate the cytotoxic and genotoxic effect of a compound previously extracted from marine *Streptomyces* sp. The strain was isolated from sediment samples collected at the Marakkanam coast of Bay of Bengal, India. Cytotoxicity of the extracted compound was assessed on MDA-MB-435S (Breast cancer cell line) and WRL-68 (Liver cell line). Genotoxicity was tested by assay of chromosomal aberrations and micronucleus test. The compound was found to be more toxic to MDA-MB-435S cell line when compared to WRL-68 cell line. The compound showed the Ic_{50} value of 65µg/ml against MDA-MB-435S cells and against WRL-68 cell line it showed the Ic_{50} value of 250 µg/ml. The exposure of compound on chromosomes did not show any aberrations, gaps and chromatid breaks. Based on the results it can be concluded that the extracted compound possess less cytotoxicity and genotoxicity on normal cells.

Keywords: Marine actinomycetes, *Streptomyces*, Cytotoxicity, Genotoxicity

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Introduction

Actinomycetes capable of producing many types of secondary metabolites are a group of prokaryotic organisms which are gram-positive bacteria that grow extensively in soils with rich organic matter. The presence and distribution of actinomycetes have been demonstrated to be associated with their various ecological habitats, including beach sand and sea water. Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites (1) notably antibiotics, antitumor agents, immunosuppressive agents and enzymes. Because of the excellent track record of actinomycetes in this regard, a significant amount of effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (2). Thus, it is crucial that new groups of actinomycetes from unexplored or underexploited habitats be pursued as sources of novel bioactive secondary metabolites. The diversity of life in the terrestrial environment is extraordinary, but in oceans even more greateer biodiversity occurs (3). More than 70% of our planet's surface is covered by oceans and life on earth originated from the sea. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than in the tropical rainforests. As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds.

Over the past 75 years, natural product derived compounds have led to the discovery of many drugs to treat numerous human diseases (4). Natural products are chemical compounds derived from living organisms e.g. plants, animals and microorganisms. They can be defined as chemical compounds isolated or derived from organisms as primary or secondary metabolites. By employing sophisticated techniques under various screening programs, the rate of discovery of natural compounds exceeded 1 million so far. Out of which 22,500 biologically active compounds that have been extracted are from microbes, 45% are produced by actinobacteria, 38% by fungi and 17% by unicellular bacteria (5). Little is known about the microbial diversity of marine sediments, which is an inexhaustible resource that has not been fully exploited. Marine extremophiles serves as valuable natural resource for novel products such as antibiotics, antitumor agents, and other therapeutic substances (6). Microbial secondary metabolites have been known as one of the immense reservoir of natural chemical diversity with potent biological activity (7). Most bacterial secondary metabolites are generated through a unique, multi-step biosynthetic process with specific enzymes for each complex structure formation. Actinomycetes especially *Streptomyces* species have received considerable attention as biocontrol agents. *Streptomyces* is the largest genus of actinobacteria belongs to the family streptomycetaceae. *Streptomyces,* the most predominant genera (more than 500 species) of the soil actinobacteria found to be one of the major sources (75-80%) for commercially available antibiotics. *Streptomyces* are gram positive and have genomes with high G-C content. Streptomycetes are characterised by a complex secondary metabolism. They produce over two-thirds of the clinically

useful antibiotics of natural origin. *Streptomyces* produces an extensive branching substrate and aerial mycelium (8). Since the first antibiotic discovery in 1942, there have been continued efforts towards screening compounds from the genus *Streptomyces* (9) which is known to be the largest antibiotic-producing genus. In fact, about 60% of the antibiotics developed for agriculture and horticulture have been isolated from *Streptomyces* species (10). Actinomycetes represent one of the most studied and exploited classes of bacteria for their ability to make a wide range of biologically active metabolites (11). Actinobacteria plays an important role among the marine bacterial communities, because of its diversity and ability to produce novel chemical compounds of high commercial value (12, 6). Hence a study was planned to evaluate the cytotoxic and genotoxic effect of previously extracted compound from marine *Streptomyces* species.

Materials and Methods

Isolation and characterization of the strain

The strain *Streptomyces* species was previously isolated from the sediment samples collected at the saltterns of Marrakanam coast of Bay of Bengal, southern India (13). The strain was selectively isolated using Starch casein agar, ISP No.1 medium and the nutritional and cultural conditions for the growth were optimized (13).

Extraction and purification of the compound

Well grown slant culture of the potential isolate was used for preparation of seed culture. The seed culture was inoculated in 50 ml medium containing the optimized production medium prepared with sea water 50 %, distilled water 50 %, pH 8.2 and incubated for 2 days in rotary shaker (200 rpm) at 30°CThe inoculums (10%) were transferred into 200ml production medium in 1 liter Erlenmeyer flasks and kept for fermentation for a week. After fermentation, the broth was centrifuged at 4000 rpm for 10 min at 10°C and the supernatant was separated and filtered in 0.2 µ membrane filter. The supernatant was extracted twice with n- Butanol (400 ml) and washed with 500 ml water. After separation, the organic phase was dried over Na_2SO_4 (anhydrous). The extract was then concentrated in rotary vacuum and lyophilized using a freeze drier (Thermo, USA) at 5°C for 5 hours. The crude extracts were stored at -20 °C. The butanol layer was concentrated and the residual suspension. The crude extracts were stored at -20°C. The butanol layer was concentrated and the residual suspension (750 mg) was chromoto-graphed over silica gel column and eluted with chroloform:MeOH. The active fractions were collected and further separated by preparative TLC on silica gel with chroloform:MeOH (8:2).

Cell lines

The cell lines MDA-MB-435S and WRL-68 cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India. The cell lines were maintained in L-15 medium (Himedia, India) and supplemented with 10 % PBS (v/v), 100 mg/l streptomycin 100 IU/ml penicillin (Himedia) at 37 °C along with 5 % CO_2 supply in a CO_2 incubator.

Assay of cytotoxicity

The cytotoxic effect of the compound (20 to 100µg/ml) was tested on MDA-MB-435S and WRL-68 cell lines by XTT cell proliferation assay. XTT (2, 3 bis (2 methoxy - 4 nitro 5- sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) assay was performed on MDA-MB-435S and WRL-68 cell lines maintained in L-15 medium. Approximately $6x10^3$cells (of each cell type) were seeded on a 96- well plate and were supplemented with 200µL of culture media for a period of 24 hrs. The media were then removed, and 200µL of fresh media containing varying concentration of the compound (20, 40, 60, 80 and 100 µg) was added to the cells in the exponential growing phase and the plate was incubated in a CO_2 incubator with 5 % CO_2 supply for 24 hrs. At the end of the incubation period, the media were removed and the fresh media added.50µL XTT reagent prepared in medium containing 25µmol L^{-1} of phenazine methosulfate was then added and the plates were wrapped in an aluminum foil and incubated in a humidified atmosphere at 37°C for 4 hrs (14). After the incubation period the orange colored complex formed was read at 450 nm using a dynex opsys MR^{TM} microplate reader using a reference filter of 630 nm. Wells containing only media and XTT reagent were used as blank and wells containing cells grown on media and no compound treatments were served as control. The percentage cytotoxicity was calculated by using the formula,

$$\text{Cytotoxicity (\%)} = [(Ac - As)/Ac] \times 100$$

Where, Ac – control absorbance and As – sample absorbance

Assay of chromosomal aberrations

The effect of the compound on healthy human chromosome was studied to evaluate chromosomal aberrations if any. The donors (volunteers) who have no record of smoking and any history of chemical or radiation exposure was included in this study. Venous blood was drawn in a sterile container after obtaining informal consent from the volunteers. Tests performed by adding 0.6 ml of heparinized blood to 6.0 ml of RPMI 1640 medium (Himedia, India) supplemented with 1.2 ml of fetal bovine serum, lymphocytes were stimulated with 4% phytohemagglutinin and the cultures were incubated at 37°C for 72 hours (15). Varying concentration of the compound (50- 200 µg/ml) was added in G2 phase of the cell cycle. One hour prior to harvest, 0.4 mg/ml of colchicine was added to arrest the cells at metaphase and subjected to G-banding. The slide containing metaphase spread was stained with giemsa, dried and observed under light microscope in 40X and in 100 X magnification (Carl Zeiss, Germany) for any aberrations, gaps and chromatid breaks.

Micronucleus test

To test the effect of the compound on chromosome breakage and formation of micronuclei in the cell cytosol, a micronuclei test was carried out according to the method of Capriglione et al. (16). After 42 hrs of incubation of a mixture, cytochalasin B was added and processed for slide preparation. A slide was prepared and stained with giemsa, dried and observed under light microscope in 40X and in 100 X magnification (Carl Zeiss, Germany) for any micronucleus formation.

Results and discussion

The cytotoxic effect of different concentrations of the compound on MDA-MB-435S cell line and WRL-68 cell line were evaluated by XTT assay in 96 well plates. This assay is often used to measure viable cell where XTT reduced to orange colour formazan in viable cell. For determination of IC_{50} value, cells were assessed for proliferation inhibition and the IC_{50} values were calculated as 65µg/ml for MDA-MB-435S cell lines (Figure 1). However, 100 µg of a compound showed only 21 % inhibition over WRL-68 cell line. Different concentrations of the compound showed concentration and time dependent inhibition on tested cells. The observed IC_{50} value indicates that the compound was less toxic to normal cells when compared to the malignant cells. The assessment of cytotoxicity is important and crucial step in the assessment and development of new therapeutic drugs for clinical application. Secondary metabolites produced from marine actinomycetes have distinct chemical structures, which may form the basis for the synthesis of new drugs.

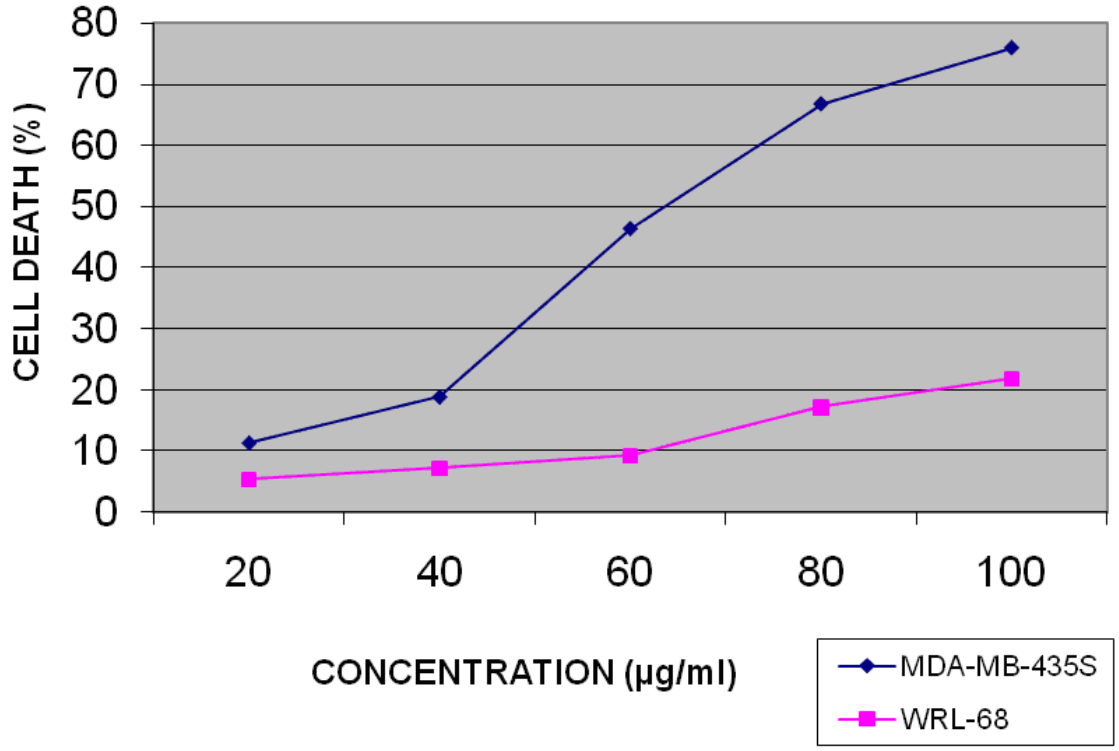
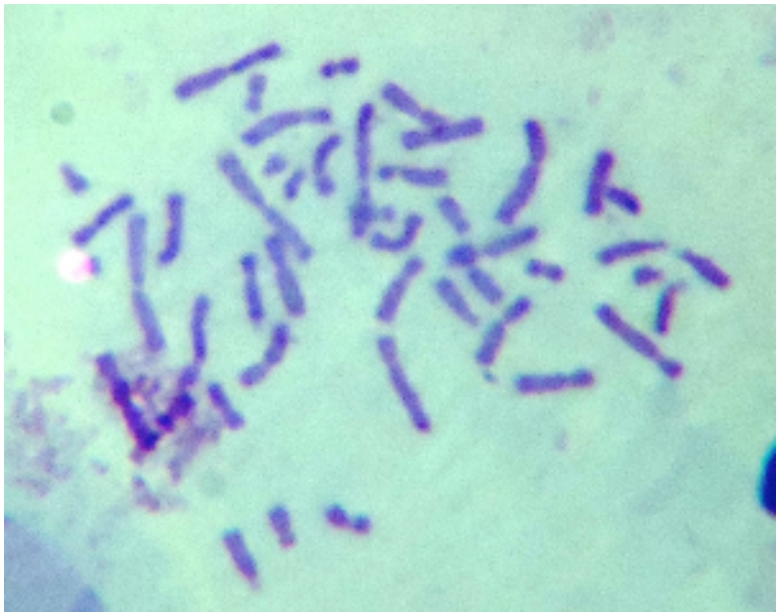


Figure 1. Effect of the compound on MDA-MB-435S and WRL-68 cell lines determined by XTT assay. Cells treated with the compound at different concentration (20µg to 100µg/ml). Values are mean of three independent experiments

The effect of the compound on human chromosomes was tested with or without compound and observed for aberrations. The compound (50µg/ml) did not show any

chromosomal aberrations (Figure 2 B) when compared to untreated control (Figure 2 A). This shows that the compound (50µg/ml) is non toxic to cells.

A



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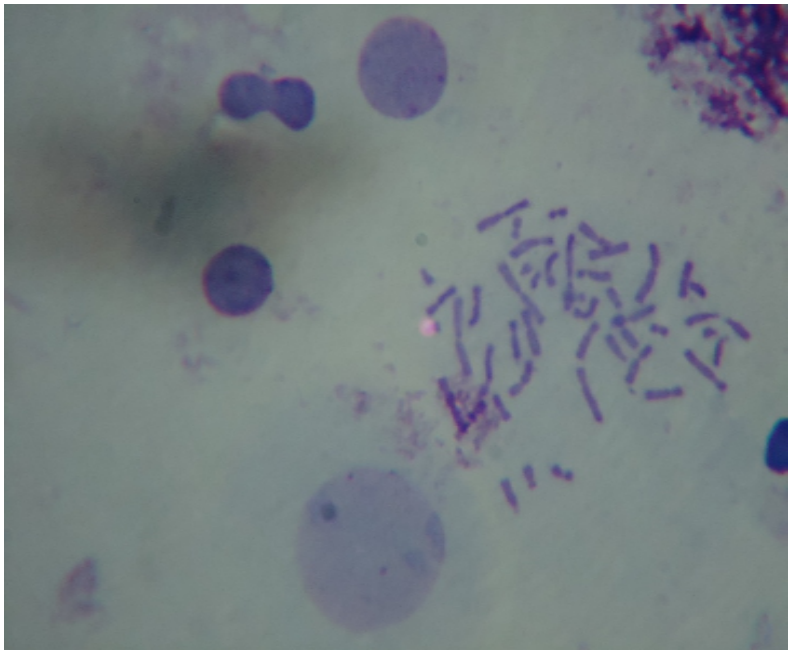
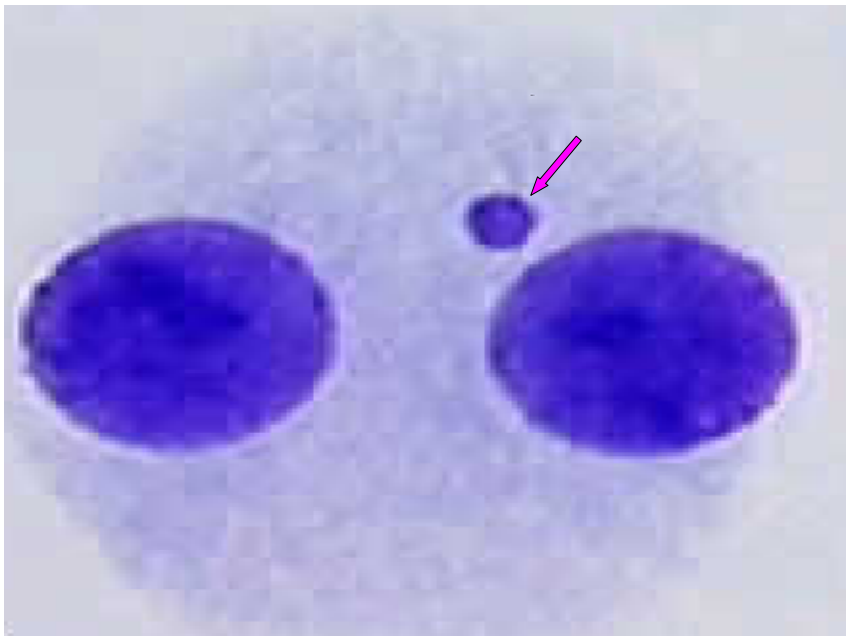


Figure 2. Effect of the compound on human chromosomal aberrations. A) Metaphase spread for the untreated sample. B) Metaphase spread chromosome treated with compound (50µg/ml) Addition of the compound did not show any chromosomal aberrations

A



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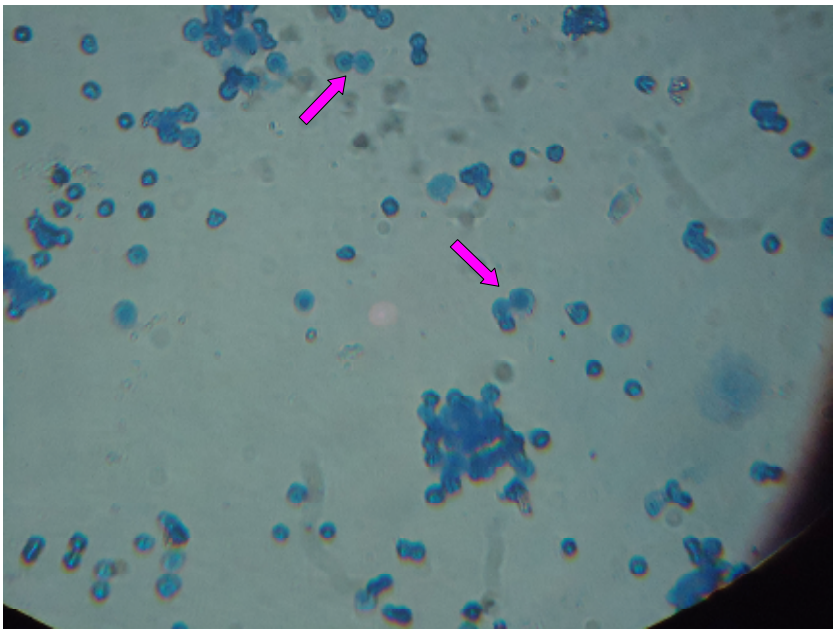

Figure 3. Effect of the compound on micronucleus formation. A) Micronucleus was seen for untreated sample. B) Binucleated cells were seen in sample treated with compound (100µg/ml)

Cytogenetic analysis of chromosomal aberrations has been suggested to be a useful tool to determine the safe maximum allowable concentration (MAC) of any drug (17). The

MAC of a chemical compound is defined as the maximum concentration which does not have any adverse affect on human health on exposure. Chromosomal aberrations in human peripheral lymphocytes are recognized as a valuable biomarker to study the effect of the drug.

Micronuclei separated from and in addition to the main nucleus of a cell are the results of acentric fragments or lagging chromosomes that fail to incorporate into either of the daughter nuclei during telophase of the mitotic cell division. The effect of compound on MN test was measured in the case of untreated sample (Figure 3 A) micronucleus was seen and in the case of compound (100µg/ml)treated, a binucleated cells were seen (Figure 3 B). A micronucleus test is a test used in toxicological screening for potential genotoxic compounds. There are two major versions of this test, one is *in vivo* and the other is *in vitro*. The *in vivo* test normally uses mouse bone marrow or mouse peripheral blood. An *ex vivo* / *in vitro* analysis of lymphocytes in the presence of cytochalasin-B, an inhibitor of actins, allows to distinguish easily between mononucleated cells which did not divide and binucleated cells which completed nuclear division during *in vitro* culture. Indeed, in these conditions the frequencies of mononucleated cells provide an indication of the background level of chromosome/genome mutations accumulated *in vivo* and the frequencies of binucleated cells with MN a measure of the damage accumulated before cultivation plus mutations expressed during the first *in vitro* mitosis cell division.

Conclusions

Based on the results obtained it can be concluded that the active compound isolated from *Streptomyces* species is less toxic to cells. The compound (100µg) was selectively cytotoxic (76% cell death) to MDA-MB-435S cancer cell line. Whereas it shows low low cytotoxicity (21% cell death) at 100µg on WRL-68.The compound also not show any chromosomal aberrations indicate that it is not having any genotoxic property. The micronucleus test with the compound also showed the formation of binucleated cells which indicate that it is not toxic to cells.

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