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Anti- cancer compounds from actinomycetes inducing DNA damage in cancer cell lines

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ABSTRACT

DNA damaging activity of anticancer drugs produced by Actinomycetes is directly linked to the cell cycle arrest leading to apoptosis and other secondary responses. Major signaling pathways are involved for permeability of mitochondrial membranes and the binding of receptor proteins leading to cell death. Extracellular compounds from Actinomycetes cells expressed higher levels of the anti- tumour enzyme (290µg/ml/hr) which induces considerable damage to cancer cell lines. L-asparaginase activity reported is higher than those expressed by the microbes currently used for commercial production. A very low percentage of viability was noted for the cancer cells when compared to the normal controls. DNA breaks are observed in the tetrads of cancer cells when treated with the exudates but the normal cells have not been disturbed. Studies on the exudates have identified novel compounds which have proven anti- tumour property. The 16srRNA sequencing confirmed the strains involved to be of Actinomycetes, including three novel strains. The DNA cleaving properties of the Actinomycete exudates have opened up possibilities to limit cell proliferation in Cancer cell lines.

KEYWORDS

L-asparaginase;
Chromosomal breaks;
Tumour cells;
Anti- cancer compounds;
UV spectrum.

INTRODUCTION

The search for novel drugs is still a priority goal for cancer therapy, due to rapid development of resistance to multiple chemotherapeutic drugs. In addition, the high toxicity of chemotherapy drugs and their undesirable side-effects increased the demand for novel anti- cancer drugs[1]. As evidenced, Actinomycetes produce innumerable number of potent compounds such as anti- tumour antibiotics, bioactive peptides, enzymes etc. All such compounds have known to improve the life cycle and viability of normal cells. Various bioactive compounds produced by the Actinomycetes are antibiotics, enzymes, IAA, L- Asparaginase etc. The anti- tumour activity exhibited by the strains producing L-asparaginase and other bioactive compounds are promising. Hence an important aspect of this part of the study was to analyze the anti- tumour effect of Actinomycete extracts and their

impact on human chromosomes. L-Asparaginase suppresses the nutrient uptake of cancer cells by depleting the asparagines which are the essential amino acids for the proliferation of cancer cells.

DNA damage is linked to failure of cytokinesis and mitotic death. DNA damage triggers mitotic chromatic decondensation and histone phosphorylation associated with apoptosis. Anti- cancer drugs exert their effect by causing DNA damage and subsequent apoptosis induction[2]. DNA damage response pathways can activate cell cycle checkpoints to arrest the cell either transiently or permanently. DNA damage response is thought to protect against cancer progression and the therapeutic rationale for specifically targeting members of DNA damage response pathways[3].

DNA damage responses can be linked to variety of biological mechanisms including apoptosis and Fanconi anaemia. Apoptosis is linked to p53 response and DNA damage and can trigger other secondary responses. Defective damage repair also plays a critical role in Fanconi anaemia through BRCA DNA damage repair pathway inducing stress by alkylating agents and even influences drug response in cancer treatment[4,5]. The cell death is triggered by damage of DNA strands stimulating permeability of mitochondrial membranes by the binding of death receptor proteins.

MATERIALS AND METHOD

Isolation and characterization of actinomycetes

Actinomycete strains were isolated from the holdfast region of seaweed samples (rocky shores of Kanyakumari region) by serial dilution procedures and by pour plating them onto Glycerol Asparagine Agar. The plates were added with antibiotics (acitidione and nalidixic acid) to inhibit other intruding microbes. The colonies were isolated based on their colouration and pigmentation properties. Strains with better pigmentation properties and antagonistic effects against pathogenic bacteria were chosen for this study. Six potent strains were selected and stored. Both the classical approach (biochemical studies) and molecular characterization (16srRNA studies) were used for the taxonomical confirmation. Biochemical assays such as Gram staining, Catalase Test, Voges Proskauer Test, Methyl Red Test, Triple sugar ion test, Citrate utilization Test, Production of amylase, sensitivity of the strains to antibiotics etc. were performed for confirming the strains upto the genus level. Antimicrobial activities of the obtained Actinomycetes were performed against four different strains of pathogenic bacteria (*E.coli, Staphylococcus, Bacillus spp., Klebsiella and Pseudomonas)* by the disc diffusion method.

Determination of L-asparaginase activity by the strains

L-asparaginase is an important anti- cancer enzyme used for leukemias. Assay of the strains for L-asparaginase activity depended on the spectrophotometric measurement of ammonia released into the culture medium by the action of the enzyme on Asparagine. Standard Nezzlerization method was followed for estimating the amount of L-asparaginase produced by the Actinomycete strains. In this assay, the rate of hydrolysis of L-asparagine was determined by measuring the ammonia released using Nessler's reaction. A mixture of 0.1ml of enzyme extract, 0.2ml of 0.05M Tris-HCl buffer (pH 8.6), and 1.7ml of 0.01M L-asparagine was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid. After centrifugation at 10000 rpm, 0.5 ml of the supernatant was diluted to 7ml with distilled water and treated with 1ml of Nessler's reagent. The colour reaction was allowed to develop for 10 minutes and the absorbance was read at 480 nm with a spectrophotometer. The ammonia liberated was extrapolated from a curve derived with ammonium sulphate. One unit (IU) of L-asparaginase was defined as that amount of enzymes which liberates 1µmol of ammonia per minute under the assay conditions[6].

The species of fresh strains of Actinomycetes were cultured separately in test tubes containing Actinomycetes broth with 1% Asparagine. The colour change obtained after treating with Nessler's reagent was measured using a spectrophotometer at 450 nm. Controls were maintained with Actinomycete strains cultured without the addition of Asparagin substrate and the OD values of control assays were used for calculating the L-asparaginase production.

L-asparaginase production was assessed by varying the pH[4-8] and Temperature ranges (30- 39°C). The optimum pH and temperature for the higher production of L-asparaginase was noted.

UV/Vis spectroscopic analysis of the exudates

The methanolic extracts of the exuded peptides from the culture media of Actinomycetes were used for the study. Methanol was used as the blank. The band width

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was set at a range from 190 to 380 nm. The experiment was carried out by varying the wavelength 5nm each time. The graph showing the spectrum was plotted with the band width on the X-axis and absorbance on the Y axis.

16srRNA analysis

DNA was extracted from mass cultured Actinomycetes by C-TAB method. Universal primer was used for PCR amplification. Purified PCR products were resolved on agarose gels, eluted and purified. Sequencing was done by fluorescent labeled dideoxy nucleotide mediated chain termination method. BLAST and CLUSTALW was used to compare the similarity sequences and to construct the phylogenetic tree. The obtained sequences were submitted to Genbank it for obtaining the accession numbers from NCBI database.

MTT assay

The cytotoxic activity of cell exudates was measured by MTT assay protocol[7]. The cells used for the study were normal lines of Human Breast Carcinoma cells (HBC) for control and Human Colon Tumor cell lines (HCT) as the target cells. The cells were seeded in a 96 well plate at a seeding density of 5000 cells/ well. MTT was added at a concentration of 0.5 mg/ml in DMSO at a concentration of 100µl/well. The optical density of the wells was read at 570 nm. Different concentrations of the drug were tested in the ratios such as 1:500, 1: 1000 and 1: 1500 (µg/ml)) in DMSO. Positive controls were maintained with cell lines not added with the Actinomycete extracts and the negative controls with heat inactivated supernatant solutions. Percentage cell viability of different samples was calculated by standard methods.

Effect of actinomycete extracts on chromosomes of tumor cells

Strains from the above tests were used to test their efficiency in cleaving the DNA of cancer cells. Both the cellular mass and the filtrate were separately treated with hexane a number of times and the extracts were concentrated. The test was performed for both normal cell lines and cancer cells. The treated chromosomes were mounted on glass slides. The slides were stained with the giemsa staining solution for 10 minutes. The slide was then passed through xylene and was mounted on DPX and observed under oil immersion objective. The chromatid breaks, dicentrics, rings etc., were noted. The same procedure was repeated for all the samples. The chromatid breaks were scored and expressed as mean break/ cell value. Any individual expression less than 0.8 were considered hyposensitive, individual expression in break/ cell of 0.8 and 1 was sensitive and individual expression more than one was considered hypersensitive.

RESULTS AND DISCUSSION

All the isolated Actinomycete strains from the marine seaweeds exhibited powerful pigmentation and antagonistic properties. Six of the most potent strains were chosen for the production of anti- cancer bioactive compounds. All the strains were confirmed for their taxonomic positions both by classical biochemical procedures and by 16srRNA sequence analysis. Among the six strains sequenced for taxonomical confirmations, produced three novel strains (PKP1-*Streptomyces sp*. Strain with accession number EU714295, PKP4-*Gordonia sp*. Strain with accession number HM352835 and PKP6-*Rhodococcus sp*. Strain with accession number HM246707, in NCBI. Two of the sequences (PKP2 and PKP3) exhibited 100% similarities to strains of Streptomycetes ie., *S. noursei* and *S. coelicolor*. One of them (PKP5) was *Actinomyces georgia*.

Bacterial L-asparaginase is used therapeutically to treat cancer. L-asparaginase acts by depleting the availability of asparagine which is highly essential for the cancer cells[8]. UV/Vis spectrophotometry confirmed the presence of the enzyme such as L-Asparaginase and Indolocarbazoles. Bioassay proved that L-asparaginase was produced at high levels, as represented in Figure 1. ie., 290µg/ml/hr which is a higher expression level when compared to other bacteria which are commercially used for the production of this anti- cancer compound. This higher concentration of the enzyme was produced at a pH range of 7.5 and a temperature of 32°C.

The exudates produced by the Actinomycetes were solvent extracted to higher purities and they are plentiful producers of polyketides, bioactive peptides, isoprenoides, indolocarbazoles and many other compounds important for pharmacological applications. Around 35 types of anticancer compounds have been reported from Actinomycetes so far which are cytotoxic against cancer cell lines[9].

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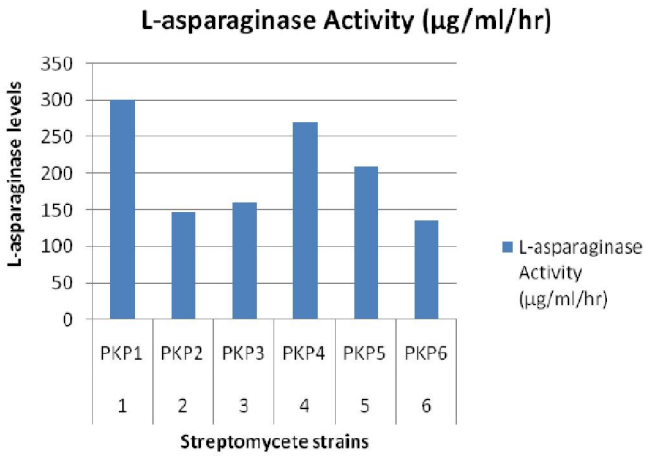

Figure 1 : Different levels of L-asparaginase activities exhibited by the six actinomycete strains chosen for the studies at µg/ml/hr

Analyses of exudates in the culture media showed peaks pertaining to the presence of cyclic compounds and enzymatic components similar to L-asparaginase. Peaks were obtained at 203 to 240nms which corresponds to the groups like R-CH=CH-CH=CH-R and R-CH=CH-CH=O. Benzene and quinines are also exhibited in this particular region. The chemical structures of anti- cancer compounds, such as Indolocarbazoles, isoprenoides and L-asparaginase are built up by these functional groups. The details are represented in Figure 2a and 2b. Indolocarbazoles constitute a separate type of anti- cancer drugs with several mechanisms of action including DNA damage targeting on topoisomerases I and II and inhibition of protein kinases, including serine/ threonine and tyrosine kinases[10].

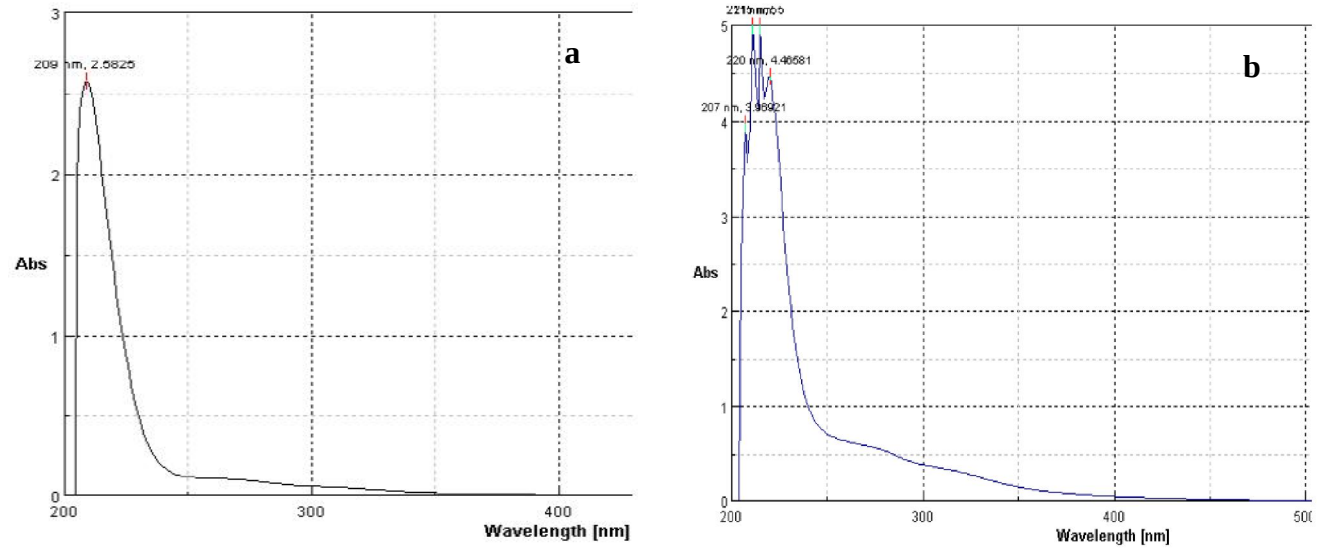

Figure 2 : a and b UV spectral analysis for extruded compounds from the actinomycete cultures taken by a UV- visual spectrophotometer.

The solvent extracts used for the MTT assay were purely extracellular exudates. Thus, the data obtained clearly depicts that the bioactive compounds released into the culture medium as proven by the UV and IR spectral studies has effectively acted against the cancer cell lines in order to inactivate only those cells with tumorigenic properties. Compounds produced by Actinomycetes are safe for normal cells in the human body as they cause no damage, i.e., even up to minor levels. The details of the anti- cancer agents with respect to the percentage cell viability of the cancer and normal cell lines are depicted in the Figures 3a to 3f. The enzymes released by the Actinomycetes such as L-asparaginase, which has proven anti- tumour property could also be considered for the effect. Hence the study explains that the extracellular Actinomycete extracts are active against the tumor cell lines preferably due to the production of useful anti- tumour agents such as L-asparaginase. L-Asparaginase destroys asparagines outside the cells forcing the cells to rely completely on what they can produce on their own. The compounds purified to the extreme conditions could be used as an alternative for currently used L-Asparaginase in the treatment of malignant tumors as they cause no ill effect to normal cells but can effectively act upon cancerous cells[11]. The anti- tumour compounds belonging to different structural classes such as Streptokodin, Chartreusin and other Indolocarbazoles exert anti- tumour activity by inducing DNA damage through DNA cleavage mediated by topoisomerase I or II inhibition[12].

The extracts from the Actinomycete sample tested were found to be effective in damaging the DNA

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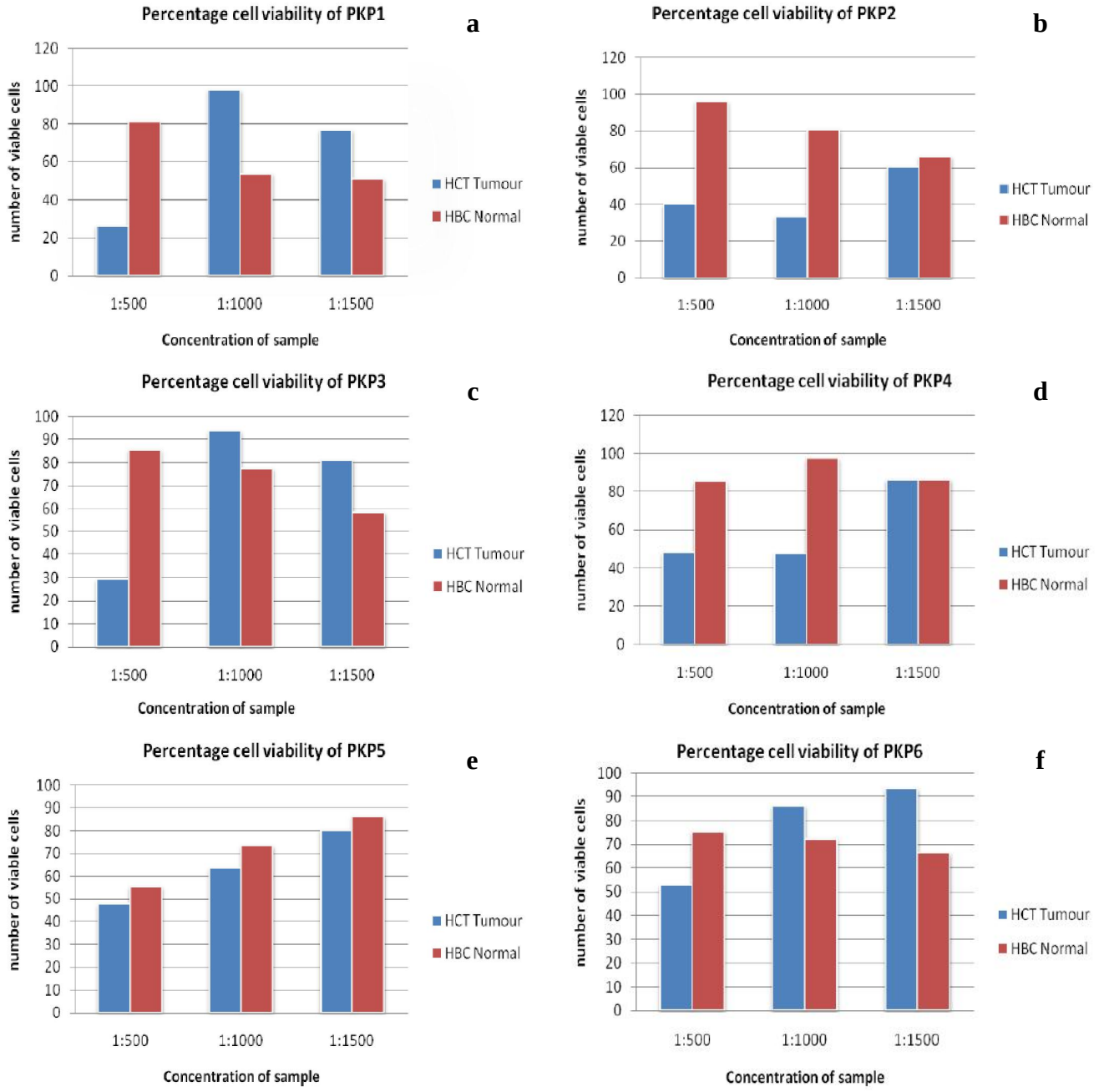

Figure 3 : a-f Anti- tumour effects by solvent extracts of actinomycete samples. Treatment done on normal HBC cell lines and HCT tumour cell lines.

strands of cancer cells. Complex pathways involving numerous molecules have evolved to perform such damage. Moreover, higher concentrations of the Actinomycete extracts were found to be more reactive than the compounds at lower concentrations which again produced more number of breaks. The mitotic index of the samples as well as metaphase of the chromosomes were unaffected by the drug in case of normal cells as observed by the chromosome morphology. The cell cycle as well as the normal regulatory mechanisms are in no way affected or altered by the drug, in case of normal cell cultures, proving the drug to be safe enough for further chemotherapeutic administrations. The above details are depicted in Figures 4a to 4f and 5a, 5b. The results corroborated with other scientists which supports the view on Streptokordin which is a methylpyridine compound produced by *Streptomyces sp*., isolated from deep sea sediments at Ayu trough. It exhibited significant anti- tumour activity against human breast cancer[13]. It is an accepted fact that activation of checkpoints in response to DNA damage leads to cell cycle arrest.

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Inhibition of lymphocyte proliferation is by the activation of p53 checkpoint signals. Many of the anticancer agents act through the Mevalonate pathways (MP) which includes the MP modulators and inhibitors. These agents increased the cytotoxicity of anticancer drugs and inhibited the proliferation of lymphocytes[14].

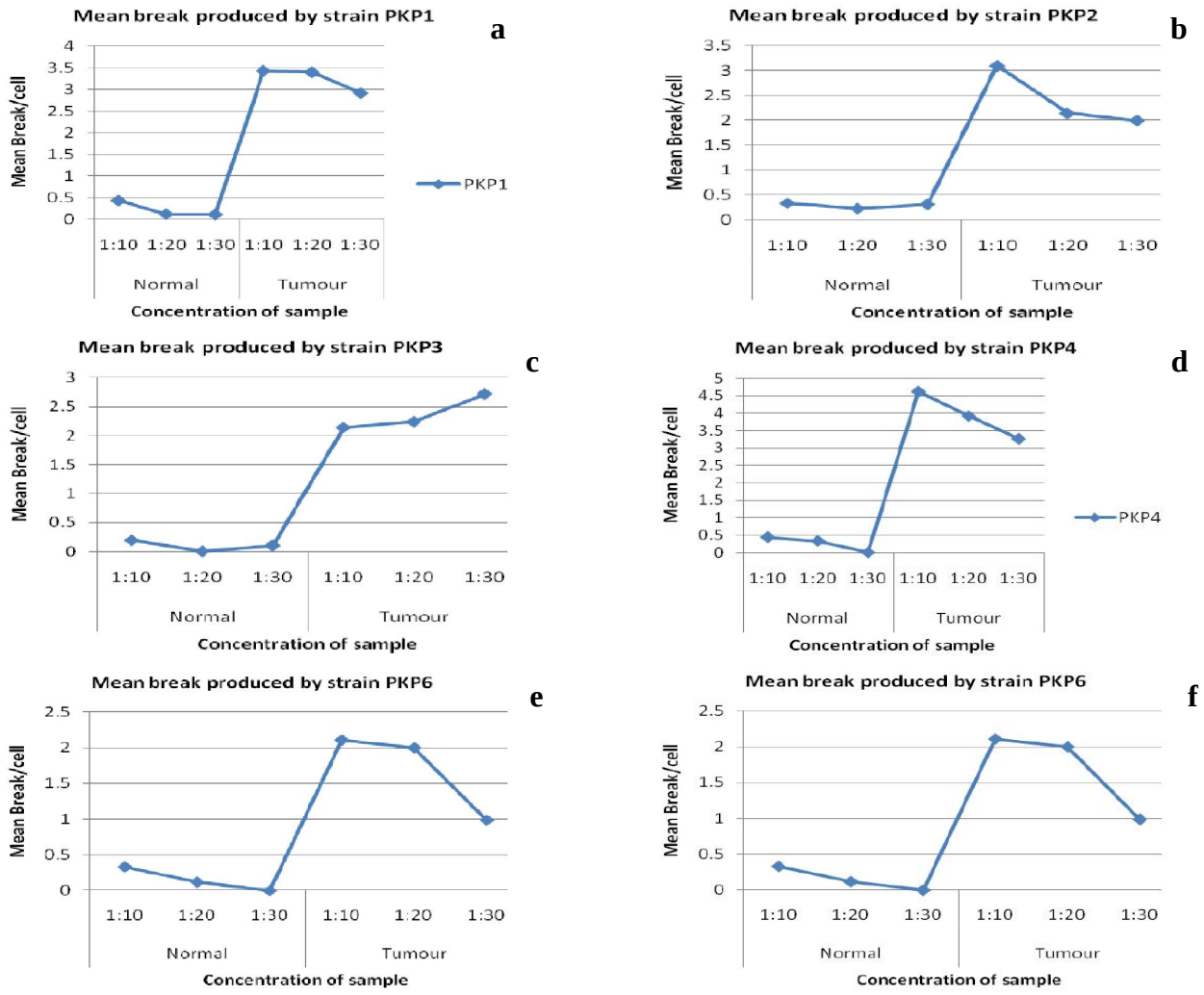

Figure 4 : a-f Results of cytogenetic analysis of lymphocyte chromosomes after treatment with the exudates of actinomycetes

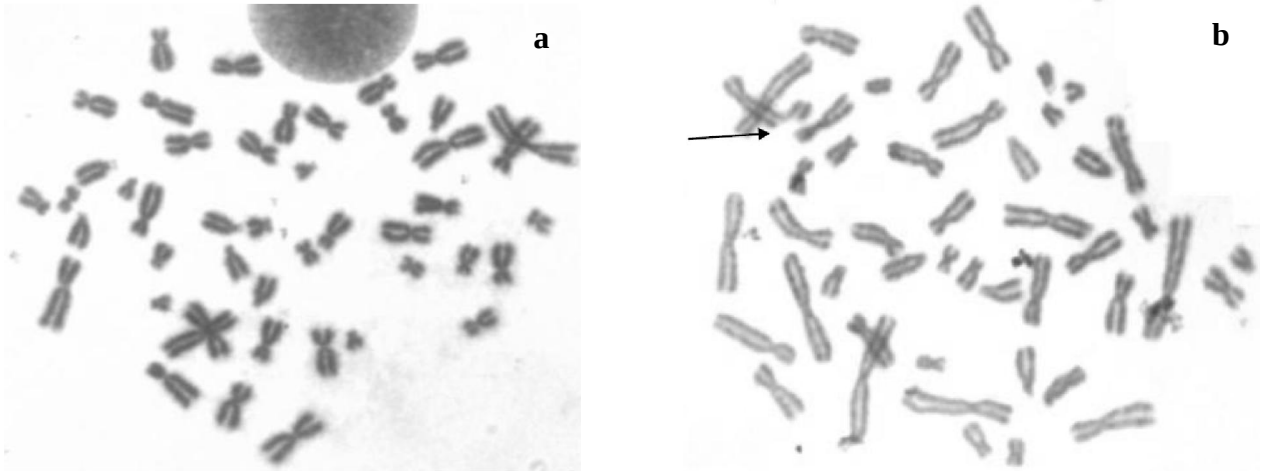

Figure 5 : a and b Normal chromosomes treated with anti- tumour compound. No breaks are observed at magnifications at 4000x (oil immersion); Chromosomes from tumour cell lines after treatment with the sample. Magnification at 4000x (oil immersion). Breaks are shown with arrows.

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SUMMARY AND CONCLUSIONS

All the strains tested, proved to belong to the Actinomycete strains based upon their 16srRNA analysis and phylogenetic grouping. L-asparaginase and other anti- cancer compounds produced by the strains are effective in controlling the proliferation of the cancer cells. Cancer cell lines were rapidly and preferentially killed by some of the Actinomycete exudates. Higher concentrations of the Actinomycete extracts killed more of the cancer cells proving to be cytotoxic to the cancer cells tested. Both higher as well as lower concentrations of the extracellular solvent extracts proved to be harmless to the normal cells tested. Thus, Actinomycete samples are potent producers of anti tumor agents and there is no cytotoxicity against normal human lymphocytes and brought in no changes to the cell cycle. The toxicity exhibited by Actinomycete exudates indicate clearly that many such anti- cancer agents especially, antileukemic compounds are produced by the strains as proved by earlier works which reveal that Rebeccamycin, produced by Actinomycetes, inhibits the growth of human lung adenocarcinoma cells and produces single-strand breaks in the DNA of these cells[9].

The ocean without any doubt is keeping a multitude of new anti- cancer compounds providing novel structural and physiological diversity to be discovered and used. Continuous effort for unravel the biosynthesis of already known compounds and the isolation and characterization of their biosynthesis gene clusters will lead to the development of new anti- tumour compounds, hopefully with improved therapeutic properties by using combinatorial biosynthesis approaches.

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