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IN VITRO CYTOTOXICITY OF MARINE SPONGE *SPONGIA TOSTA* IN A-549, HepG2, HT-29 AND Vero CANCER CELL LINES.

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ABSTRACT

Cancer is a multigenic disease causing about one fifth of the deaths in each year worldwide. Cancer is caused mainly due to mutation or malfunction of cell cycle controlling machineries. Standard treatment for curing cancer including chemotherapies, coupled with radiation therapies are available but sometime they are associated with severe side effects, as for example, radiation therapies partially disrupts patient's normal immune systems. Nausea, vomiting, loss of appetite are also common side effects of those standard treatment. Drug development using marine bioresources is limited even though the ocean occupies about 70% of the earth and contains a large number of biological materials. In this study, the silver nano particles synthesized methanolic extract of *Marine sponge spongia Tosta* (AgNPs -MEMSST) was examined for anticancer activity against lung cancer cell line, colon cancer cell line, liver carcinoma, kidney-African green monkey cell line. The sponge extract dose dependently inhibited viability in this cell line. Our results indicated that methanolic extract of sponge suppressed cell growth in these cell lines, hence the extract possess excellent anticancer potential that may be used for therapeutic purpose of cancer treatment with proper evaluation procedures.

KEYWARDS: Cancer cell line, Anticancer, *In vitro*, Marine sponge - spongia Tosta.

INTRODUCTION

Cancer is the general denomination for a series of diseases associated with regulatory abnormalities in cell growth and homeostasis. Overall, more than 100 distinct type of cancer have been described (Hanahan & Weinberg, 2000). According to World Health organization, the second leading cause of death in developed countries is cancer and it is also the three leading causes of death for adults in developing countries (Gmbh, 2009). Cancer can be treated by surgery, radiation, chemotherapy, hormones and immunotherapy (American cancer society 2011). However, there is no potent medicine in the existing cancer treatments as many of these drugs can cause side effects in many circumstances.

Various studies have been carried out to look for alternative treatment for cancer researchers have been focusing on the discovery of new compounds derived from the natural products which have potential anticancer properties. Antioxidant is one of the main compounds which act to protect against damages by free radicals and other reactive oxygen species. It was proven that there is a positive correlation between the amount of antioxidants in the food diet and the lowering of cancer mortality in an individual (Boopathy and Kathiresan, 2010). The possible explanation for this is that the antioxidant can cause regression of premalignant lesions and thus, inhibit the development of these lesions into cancer.

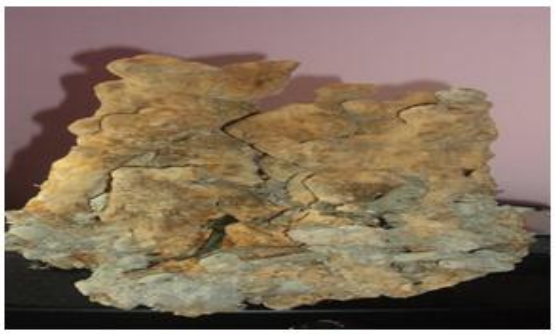
Natural products produced by living organisms have been widely exploited by people for the development of various products including food, fragrances, insecticides, pigments and therapeutic drugs (Carte, 1996). Apart from using plant derivatives and microbial products as a source to produce medicine, researchers on marine organisms have been increased drastically over the years to discover new pharmaceutical agents. It has been found that the isolated bioactive compounds from the marine organisms have much health beneficial effects as their biological activities have the potential to giving better efficacy and specificity of drugs against certain diseases. These newly isolated compounds from the marine environment have the ability to withstand extreme conditions in terms of pressure, salinity and temperature as well as having unique structural and functional features compared to the terrestrial organisms (Boopathy and Kathiresan, 2010).

In this, methanolic extract of *Marine sponge spongia Tosta* has been used to treat various cancer cells *in vitro*. In the cytotoxicity test, AgNPs -MEMSST was evaluated using methyl thiazole tetrazoluim(MTT) assay. It is a simple, economic and reliable method to determine the viability of the cells after being treated with certain drugs (Shetty et al., 1996). It involves the ability of the living cells to reduce the MTT compound which results in colour changes. The present study aims to determine the anticancer and cytotoxicity potential of the crude extracts from AgNPs -MEMSST.

MATERIALS AND METHODS

Collection of Sample

The sponge sample was collected as entangled specimens form a bottom trawl fish new operated off Manoli and mare islands of Mandapam group of islands, Gulf of Mannar at Rameshwaram. It was collected by bicatching method. The samples were placed inside sterile ethyl propylene bags under water and transferred to the lab aseptically in ice boxes.



PREPARATION OF SPONGE EXTRACTS

Prior to the extraction, samples were washed with water cleaned air dried Iyophilized and powdered. They were stored for further use for the extraction of crude bioactives, 100g of powdered material was exhaustively extracted with 200ml of methanol using soxlet apparatus and concentrated in a rotary evaporated at reduced pressure.

IN VITRO ANTICANCER ACTIVITY

Chemicals

3 – (4,5 – dimethyl thiazol – 2 – yl) – 5 – diphenyl tetrazoluim bromide (MTT). Fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's modified eagles medium (DMEM) and Trypsin were obtained from sigma Aldrich co, St. Louis, USA EDTA, Glucose and Antibiotics from Hi-media laboratories Ltd., Mumbai. Dimethyl surfoxide (DMSO) and propane from E. Merck ltd., Mumbai India all other reagents and chemicals used in the study were of analytical grade.

CELL LINES AND CULTURE MEDIUM

A-549(Lung carcinoma cell line), HT-29(Colon carcinoma cell line), Vero (kidney-African green monkey) and HepG2 (Liver carcinoma cell line) were procured from National Centre from Cell Sciences (NCCS) Pune. India stock cells are cultured in DMEM supplemented with 10% penicillin (100IU/ml), streptomycin (100µg/ml) and amphotericin B (5µg/ml) in an humidified atomsophere of 5% Co_2 at 37^0C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% Glucose in PBS). The stock cultures were grown in $25cm^2$ culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd.), Kolkata, India).

PREPARATIONS OF TEST SOLUTIONS

For cytotoxicity studies, each weighed MEMSST drug was dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

DETERMINATION OF CELL VIABILITY BY MTT ASSAY

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. This assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved in the cleavage of tetrazolium salt 3-(4,5 dimethyl thiazole-2-yl)- 2, 5, diphenyl tetrazoluim bromide (MTT) into a blue colored product. (Formazan) by mitochondrial enzyme succinate dehydrogenase The number of cells was found to be proportional to the extent of formazan production by the cells used (Francis and Rita 1986).

The monolayer cell culture was trypsinized and the cell count was adjusted and the cell count was adjusted to 1.0 x 10^5 cells / ml using DMEM containing 10% FBS to each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off washed the monolayer once with medium and 100µl of different test concentrations of test drugs were added to the partial monolayer in microtitre plates. The plates were then incubated in 37^0C for 3 days in 5% Co_2 atmosphere, and microscopic examination was carried out and observations were noted every 24h interval. After 72 h, the drug solutions in the wells were discarded and 50µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3h at 37^0C in 5% Co_2 atmosphere. The supernatant was removed and 100µl propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm (Francis and Rita 1986). The percentage growth inhibition was calculated using the following formula and concentration of the test drug needed to inhibit cell growth by 50% (CTC 50) values is generated from the dose- response curves for each cell line.

$$\% \text{ Growth inhibition} = 100 - \left(\frac{\text{Mean OD of Individual test group}}{\text{Mean OD of control group}} \right) \times 100$$

RESULTS

The cytotoxic effects of methanolic extract of *Marine Sponge Spongia Tosta* are shown is table 1 and figure 1. In the presence of investigation *in vitro* anticancer activity of the AgNPs -MEMSST was evaluated against the cancer cell lines viz. A-549(Lung carcinoma cell line), HT-29(Colon carcinoma cell line),Vero (kidney-African green monkey) and HepG2(Liver carcinoma cell line). The anticancer activity displayed by methanolic extract of this *Maine Sponge Spongia Tosta* was bound to be 77. 55± 2.3% at 100µg/ml concentration against the A-549(Lung carcinoma cell line) Cell line and CTC_{50} value was recorded as 166. 67±1.1µg/ml. Table 2 and figure 1 another result 78. 72± 0.8% at 100µg/ml concentration against the HT-29(Colon carcinoma cell line) and the CTC_{50} value was recorded as 176. 67±0.6µg/ml, Table 3: 71. 85± 0.3% at 100µg/ml concentration against the vero cell line and CTC_{50} value was recorded as 390.00 ±1.1µg/ml. Table 4 and figure 4 another finall reulst 78. 86± 0.4% at 100µg/ml concentration against the HepG2 cell line and CTC_{50} value was recorded as 150.00 ±1.1µg/ml.

Table 1: Cytotoxic properties of AgNPs synthesized MEMSST against Lung cancer cell line.

Sl. No	Name of Test sample	Test Concn. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	AgNPs-MEMSST	1000 500 250 125 62.5	77.55±2.3 60.13±0.7 57.32±1.1 48.05±0.2 44.17±1.2	166.67±1.1

Table 2: Cytotoxic properties of Methanolic extract of MSST against HT-29 cell line

Sl. No	Name of Test sample	Test Concn. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	AgNPs-MEMSST	1000 500 250 125 62.5	78.72±0.8 66.62±0.7 59.77±0.5 44.54±0.5 38.32±0.6	176.67±0.6

Table 3: Cytotoxic properties of AgNPs synthesized MEMSST against Vero cell line

Sl. No	Name of Test sample	Test Concn. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	AgNPs-MEMSST	1000 500 250 125 62.5	71.85±0.3 55.74±1.5 42.97±2.3 26.97±1.3 16.05±2.8	390.00±1.6

Table 4: Cytotoxic properties of AgNPs synthesized MEMSST against HepG2 cell line

Sl. No	Name of Test sample	Test Concn. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	AgNPs-MEMSST	1000 500 250 125 62.5	78.86±0.4 71.28±0.7 57.73±0.4 48.57±0.4 41.58±0.2	150.00±0.4

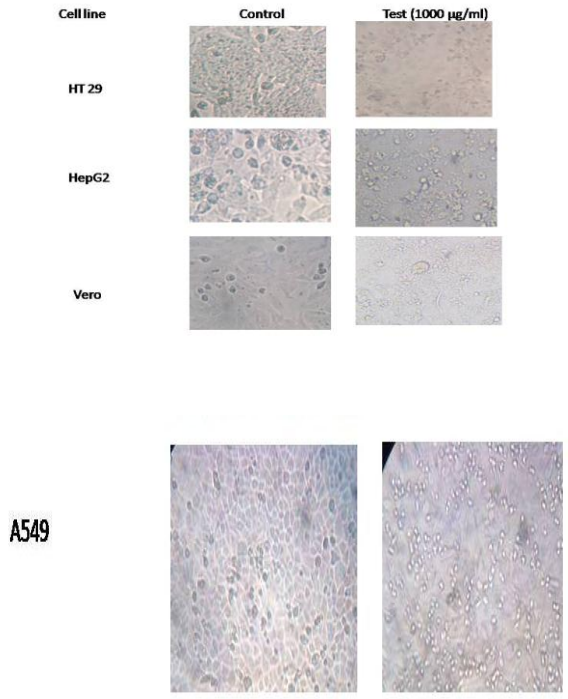


Figure:1 Anticancer potential of AgNPs synthesized MEMSST against HT-29, HepG2, Vero and A-549 cancer cell lines.

DISCUSSION

Based on this study, the *in vitro* cytotoxicity of MSST was determined using 3-(4,5-dimethyl thiazol-2-yl)- 2-4-diphenyl tetrazolium bromide (MTT) assay. It is a colorimetric test which commonly used to measure cell viability based on the ability of mitochondria in the viable cells to produce dehydrogenase enzyme (Yedjou and Tihonwou, 2007). MTT is a water soluble tetrazolium salt which turns yellowish when dissolves in solution. When MTT was added to the cells, the dehydrogenase enzyme reduces the soluble yellow, tetrazolium salt to insoluble purple formazan crystals. Crystals can be solubilised using organic solvent like dimethyl sulfoxide (DMSO) that was used in this study and then to be quantifated spectrometrically using ELISA reader. Thus, there is a linear relationship between the concentrations of purple crystals formed in the number of cells that are metabolically active. The cytotoxicity of MSST can be studied when comparison was made between the amount of formazan crystals produced in cells treated with MSST extracts and the untreated control cells. This enables the effectiveness of the sea crude extract to be determined using a dose – response curve (Taskin et al., 2010). Mitochondira are the site where intracellular reduction of tetrazoluim salts by metabolically active dehydrogenase enzymes are carried out. During this reaction, reducing agents such as NADH and NADPH are also generated at the same time (Selvi et al., 2011). However, dead cells do not have the ability to cleave the tetrazoluim ring. Hence change does not occur. This is because dehydrogenase enzyme only present in the mitochondria of the live cells, not dead cells. In this study, untreated cells were used as negative control where as positive control was prepared by using 50% DMSO to kill the cancer cells.

Cancer has a source on the human population for many years. Although numerous advances have been made in prevention, diagnosis and treatment of the disease, it still continues to torment mankind (Hanahan and Weinberg, 2000). There are limited research published for anticancer affect of AgNPs synthesized MEMSST, but the potential of other species as an antitumor, anti-inflammatory have been well known.

CONCLUSION

The *in vitro* cytotoxicity assays offers quick, simple and cost-efficient way of testing the toxicity and forms an important tool for high through put screening of AgNPs - MEMSST. From the present findings, it can be concluded that the studied extracts shows moderate toxicity against these cell lines irrespective of their origin. So, the MEMSST is a natural marine sponge that could be a good candidate for cancer treatments.

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