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Research Article

In Vitro Pharmacological Evaluation of the Keratosid Sponge *Spongia officinalis* var. Ceylonensis (Dendy) for Anti-Cancer Activity

Winson Sam*, Sudhakaran Nair C R, Rajesh Ramachandran

College of Pharmaceutical Sciences, Govt. Medical College, Thiruvananthapuram

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ABSTRACT

Marine sponges belonging to the phylum porifera have been subject to anti-cancer studies which have yielded anticancer agents like spongothymidine and spongouridine. The present work is the *in vitro* pharmacological evaluation of the extract of the keratosid sponge *Spongia officinalis* var. Ceylonensis (Dendy) for anti-cancer activity. Purified fractions of *Spongia officinalis* induced anti-proliferative effects on A549, lung carcinoma cells with an IC 50 values of 1.24µg/µl, whereas it was relatively nontoxic to normal cell lines L929 at the mentioned concentrations. Flow cytometric analysis depicts inhibition at G2/M phase which can be considered significant. Overall results suggests potent anticancer effect of the extract which can find applications in therapeutic interventions. The flow cytometric analysis was performed using the MUSE cell cycle kit on Muse™ flow cytometer (Millipore, USA). The extract showed cytotoxicity on lung cancer cells by causing cell cycle arrest at G2/M phase.

Keywords: Marine Sponges, Anti-cancer, MTT assay, Flow cytometry, Porifera.

INTRODUCTION

Nature has consistently been the source of pharmaceuticals to alleviate human diseases from time immemorial. Even today the search is on. The relatively untapped marine environment making up 71% of the biosphere[1] is the current frontier. Marine sponges living in this environment have yielded the highest diversity of novel molecules with pharmacological activity ranging from anti-feedant to anticancer activity.

Sponges are sessile, benthic, multicellular invertebrate animals belonging to the phylum porifera. The property that characterizes sponges is the ability to imbibe large quantities of fluid, it is, perhaps this property that has given these poriferons the generic name 'SPONGE'. A recent paper has quantified the number of sponges described scientifically to be 8553 species[2].

Sponges have been subject to detailed molecular studies which have led to their classification into four classes[3]within the phylum porifera namely:

Calcarea which comprise calcareous or calcisponges which typically have calcite spicules.

Hexactinellida or glass sponges) which have triaxonic silica spicules.

Demospongiae which have silica spicules which are monaxonic, tetraxonic, and/or polyaxonic and/or spongin fibres derived from collagen (Order Keratosida to which the sponge under this study belongs shows a lack of spicules). This class has the maximum number among the described sponges.

Homoscleromorpha have small spicules and are mostly encrusting.

As they are sessile in the adult life they lack the ability to move away from threats or move towards better locations. This has led them to evolve chemical defence strategies. One of the best described of this strategies is symbiotic relationships with bacteria and fungi which in turn produce chemicals that act as deterrent to competitors and predators alike. The other important strategy is to produce secondary metabolites, which in turn are products of primary metabolism, having the same effect.

It is these chemicals that are sought for potential pharmacological use. Drug discovery from sponges was put into high gear with the isolation and characterization of the nucleosides, spongothymidine and spongouridine, of the marine sponge *Cryptotethya crypta*[4]. These were used as templates for the synthesis of Ara-C, an agent used against lymphoma, and the antiviral drug Ara-A used clinically. Subsequently isolated secondary metabolites from sponges showing anti-inflammatory, anti-tumour, immunosuppressive neurosuppressive, antiviral, antimalarial, antibiotic, antifouling and other activities have been extensively reviewed[5].

Previously studying sponges from the east coast of India Dr Sudhakaran Nair and Winson Sam[6], the first two authors of this work, reported *in vitro* anticancer activity in the marine sponge *Axinella donnani*.

Cancer is a disease characterized by uncontrolled proliferation of cells that has an origin in the interplay of genetic and environmental factors. The disease has alarming statistics in terms of morbidity and mortality. Patients coping with the disease have a significant decrease in the quality of life. Among the cancers Lung cancer is associated with the highest mortality worldwide. This

**Author for Correspondence: assistant.professor@live.com*

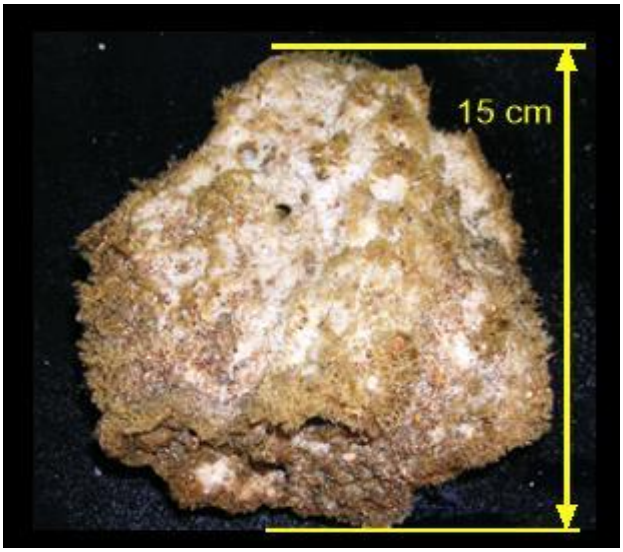

Figure 1: The figure is the photograph of the marine sponge Spongia officinalis var. ceylonensis Dendy. The wet weight was 900g. The photograph was taken using a 10 MP CCD digital camera.

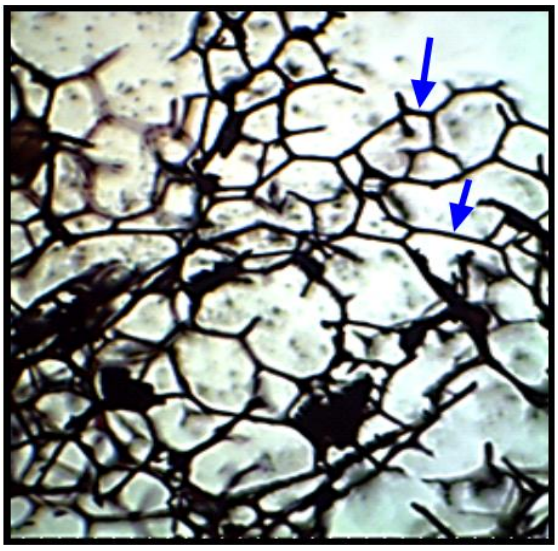

Figure 2: The figure is a photograph of a section of the marine sponge spongia officinalis var. ceylonensis Dendy stained with safranin under 10X magnification. The arrows point to the spongin fibres. The image was captured using TS view software from a generic camera connected to a compound microscope.

Table 1: Taxonomy of *Spongia officinalis* variety Ceylonensis (Dendy).

Kingdom	Animalia
Phylum	Porifera
Class	Demospongiae
Order	Keratosida Grant
Family	Spongiidae Gray
Genus	Spongia

Table 2: Data of flow cyometric cell cycle analysis.

	G0/G1	S	G2/M
% Gated	74	10.2	10.8
Mean	3398.1	5211.1	6133.3
% CV	12.2	6.2	5.5

Table 3: Data of flow cyometric cell cycle analysis.

	G0/G1	S	G2/M
% Gated	62.8	9.7	13.9
Mean	3711.9	5121.9	6412.5
% CV	9.4	5.9	5.8

suggests that the interventions for this cancer at this point in time are wanting. The present work is an effort towards discovering a new intervention for lung cancer. The work summarizes the in vitro anti-cancer study on lung cancer cells using MTT assay and flow cytometry using the extracts of the marine sponge *Spongia officinalis* var. Ceylonesis (Dendy), a member of keratosid sponges, which have yielded chemicals with novel structures.

MATERIALS AND METHODS

Sponge material

The sponge material was collected from the coast of Tamilnadu state in India off the fishing hamlet of Arokyapuram approximately 8°07'04.6"N 77°34'26.2"E. The sponge was identified by Dr P.A.Thomas, PhD, DSc (Taxonomy & Biodiversity), Emeritus Scientist, ICAR (Indian Council for Agricultural Research).

The taxonomic detail of this sponge is shown in table 1.

The sponges were obtained as a by catch in the nets of fishermen fishing the high seas, which was immediately transferred to the laboratory on ice, washed and lyophilized.

Preparation of the sponge extract

100 g of the identified freeze dried sponge was weighed and extracted with 100 ml of a 1:1 dichloromethane methanol mixture. The extracts was dried to free solvent and subjected to lyophilization, and used for further studies.

Cell line

Non-small cell lung cancer derived cell line A549 was sourced from the National Centre for Cell Sciences, Pune, India. The cells were maintained in DMEM (Invitrogen) with 10% Fetal bovine serum (FBS) in an atmosphere containing 5% CO_2 at 37ºC.

MTT assay

This assay was carried out according to the procedure followed by Arung and others[7].

MTT is a colorimetric assay that measures the ability of mitochondrial succinate dehydrogenase to reduce the yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to a purple coloured formazan product which accumulates within the cell. Solubilizing the cells with Dimethyl sulfoxide releases the formazan product which is measured spectrophotometrically at 540 nm. Briefly 5 X 10^3 cells were seeded on to 96 well culture plates (Nunc, USA) and grown to 60% confluency. The cells were treated with different concentration of extracts such as 5 and 10µg. After 24 hours incubation the media

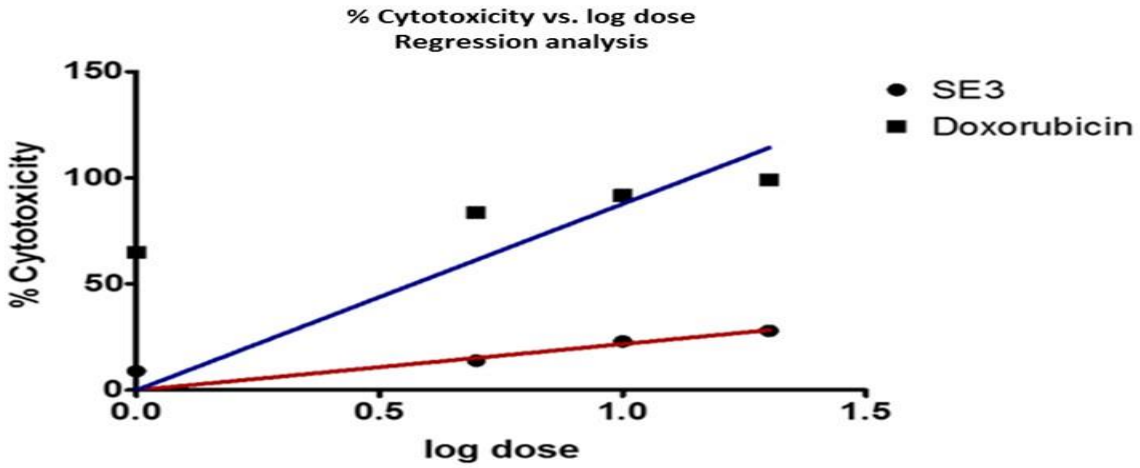

Figure 3: The figure is the result of the MTT assay showing the linear regression of the Log Dose vs. % Cytotoxicity of the extract of *Spongia officinalis* var. ceylonensis (Dendy) on fibroblast cell line L929 (control). The graph was generated using GraphPad Prism version 6.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

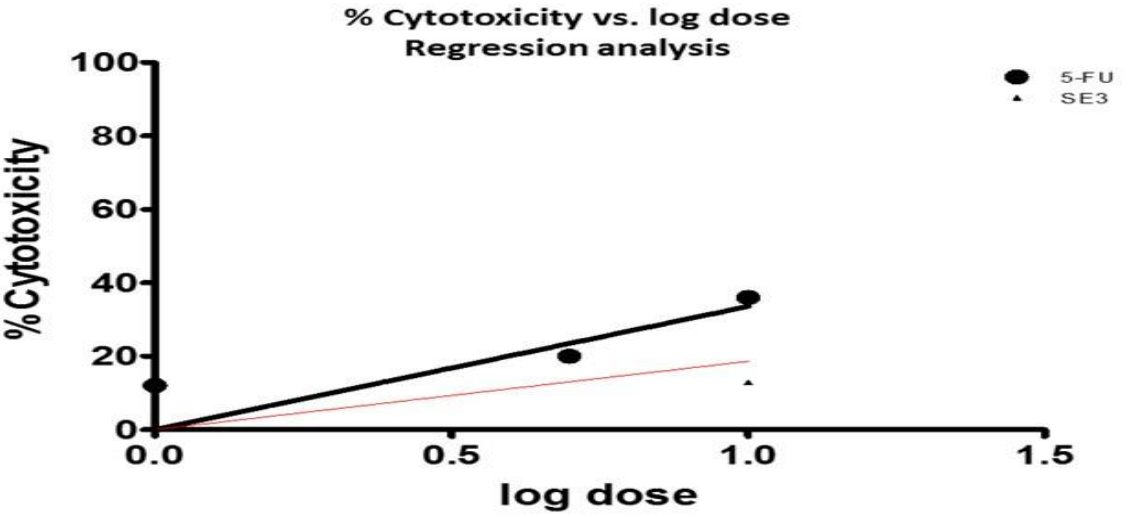

Figure 4: The figure is a graph showing the linear regression of the log Dose vs. % Cytotoxicity of the extract of *Spongia officinalis* var. ceylonensis (Dendy) on lung cancer cell line A549 determined using the MTT assay. The calculation and graph was generated using GraphPad Prism version 6.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

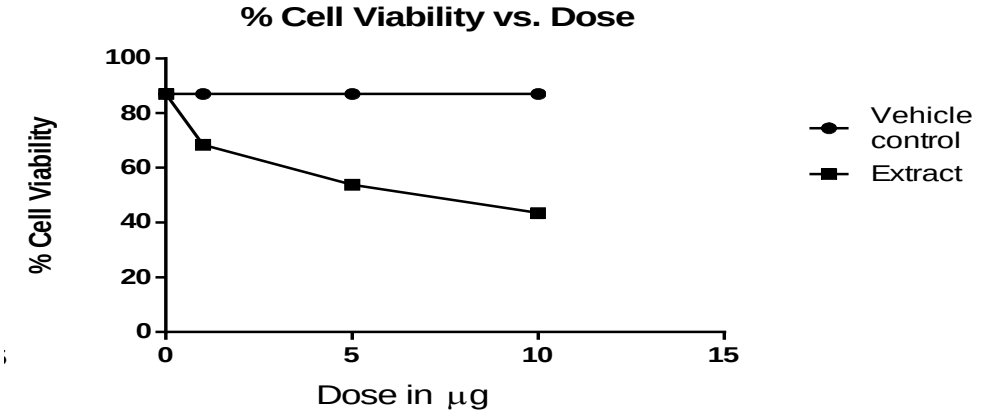

Figure 5: This figure shows the dose dependant decrease in viability of the extract of Spongia officinalis var. ceylonensis Dendy on cultured A549 (Lung cancer) cells.

was replaced with new media to which 50ul of MTT was added and incubated for 3 hours. The reduced purple formazan crystals were solubilized with DMSO for 30 minutes and read spectrophotometrically.

Ethidum bromide/ Acridine orange live dead assay

DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells[8]. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. After treatment with different concentrations of DMSC the cells were washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at

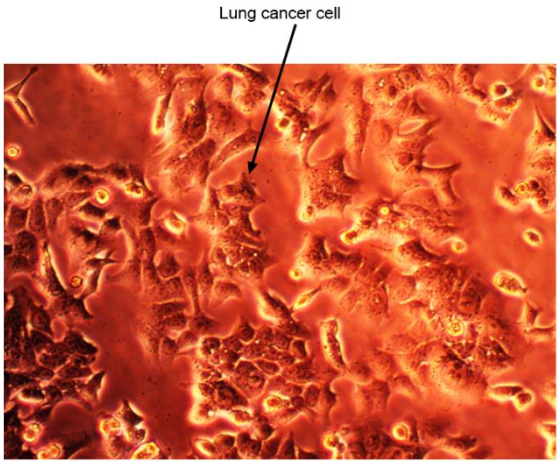

Figure 6: This figure shows the morphology of the lung cancer cells that served as control (A549). The arrows points to a representative lung cancer cell in the culture.

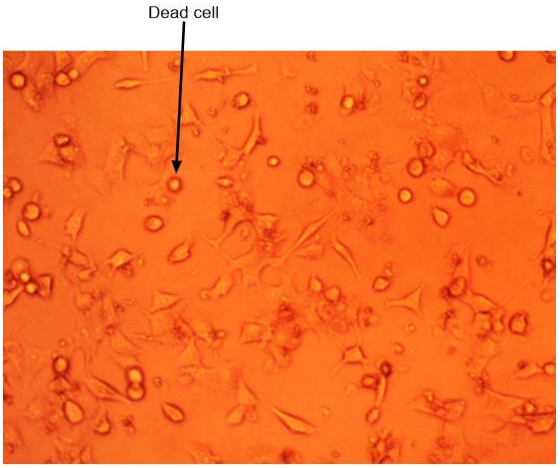

Figure 7: This figure shows the lung cancer cell (A549) which were treated with 5 µg of the extract of Spongia officinalis var. ceylonensis (Dendy). A representative dead cell is pointed out by the arrows.

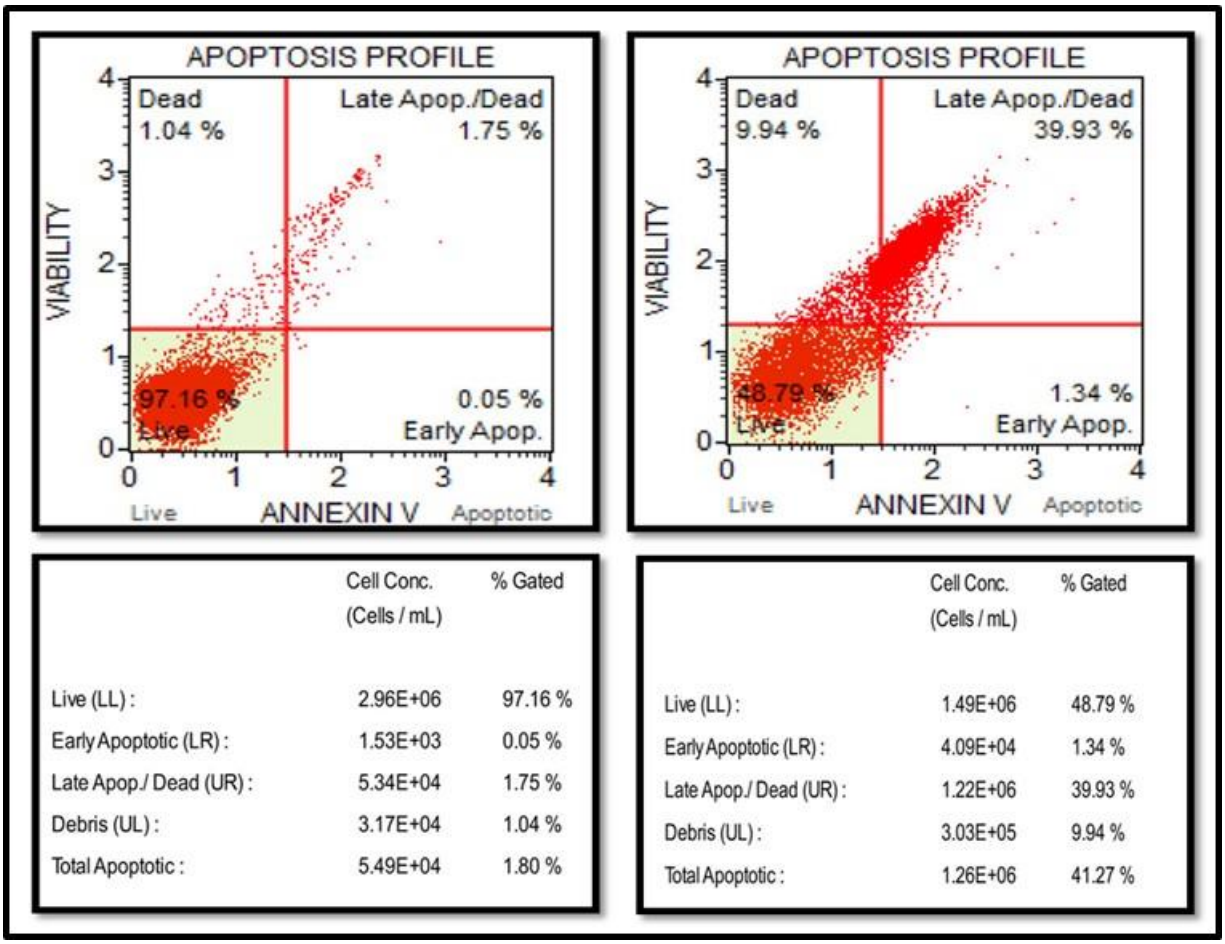

Figure 8: Result of the Ethidum bromide/ Acridine orange live dead assay.

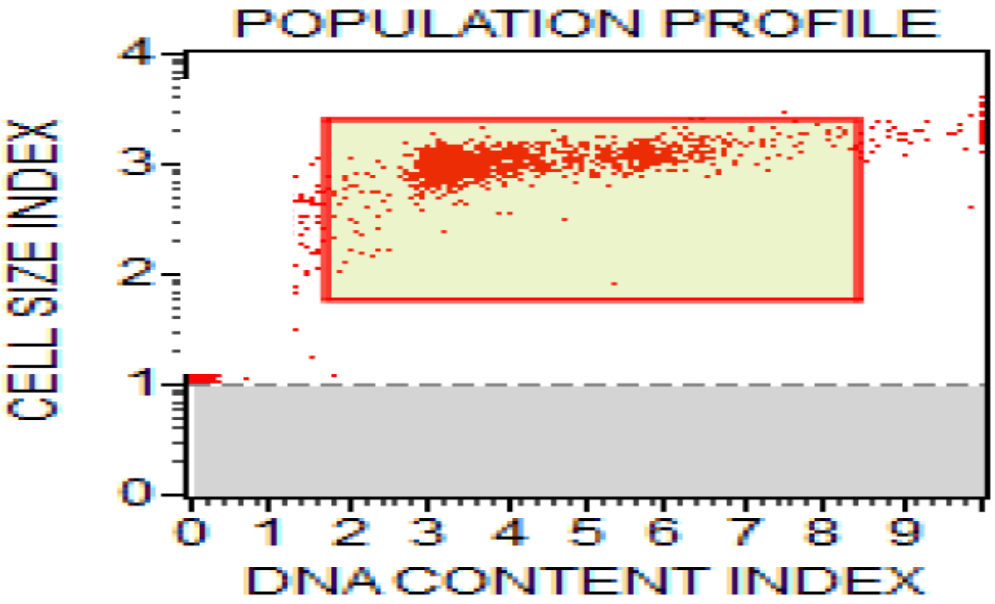

Figure 9: The figure shows the distribution pattern of the control cells (lung cancer, A549) on forward scatter in flow cytometric analysis. The rectangle shows the gated population subjected to DNA content analysis.

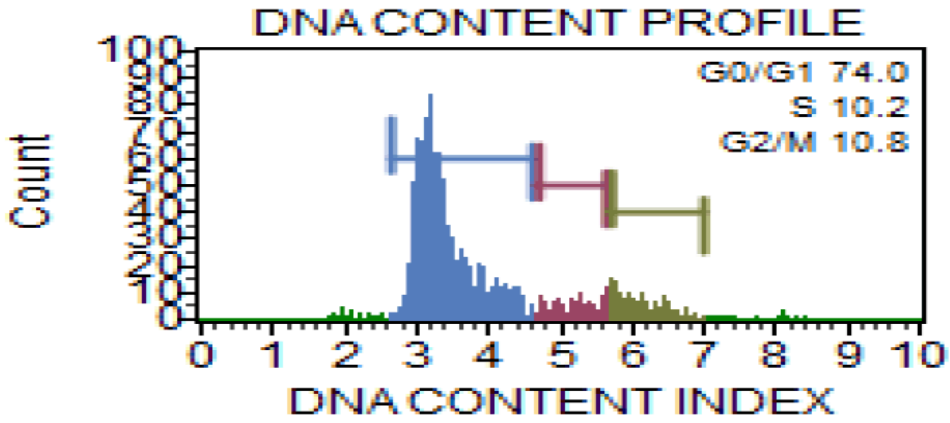

Figure 10: The figure shows the typical cell-cycle profile (control) for the mammalian A549 (lung cancer cells) control gated cell population obtained during flow cytometric analysis by plotting cell count versus DNA content.

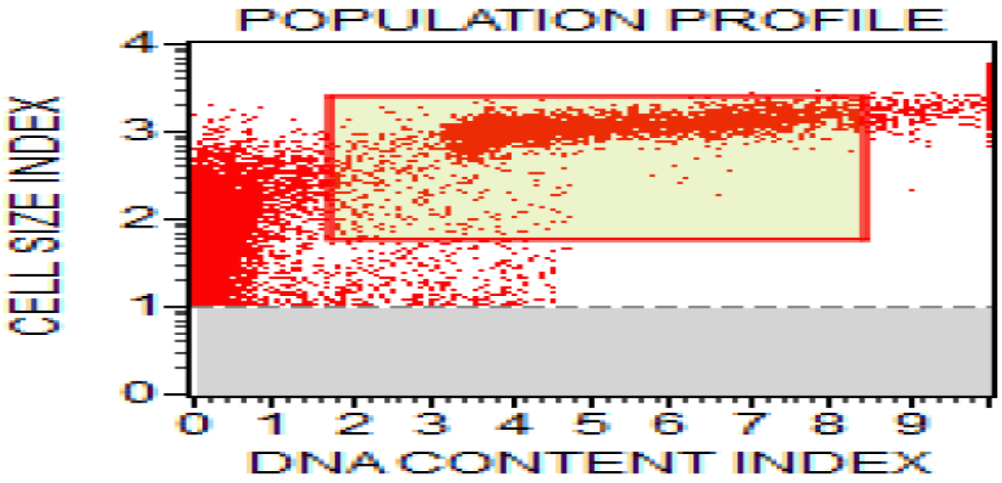

Figure 11: The figure shows the distribution pattern of the treated cells (lung cancer, A549) on forward scatter in flow cytometric analysis. The rectangle shows the gated population subjected to DNA content analysis.

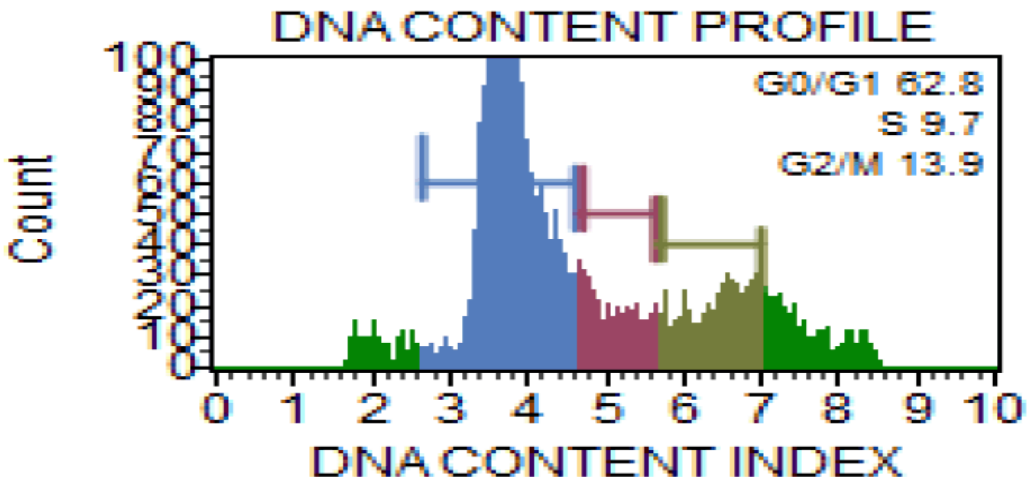

Figure 12: The figure shows the cell-cycle profile for the mammalian A549 (lung cancer cells) treated cell population obtained during flow cytometric analysis by plotting cell count versus DNA content.

room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera). . The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).

Cell cycle analysis

Cell cycle analysis using flow cytometry: In this method the nuclear DNA content of the cell is measured by flow cytometry at high speeds. For this propidium iodide, a dye which stoichimetrically binds DNA, is used. Viable cells exclude this dye therefore the cells to be analysed are first permeabilised and fixed before adding the dye. The flow cytometric analysis were done using the MUSE cell cycle kit on Muse™ flow cytometer (Millipore, USA). The analysis was done following the manufacturers protocols (Cat; No: MCH100106). In brief cells were cultured, treated with the extract, harvested and diluted to as per standard cell culture protocols with appropriate controls. Subsequently the cells were washed with PBS, harvested, diluted to 1 X 10^7 cells/mL using growth medium. The cells were then centrifuged and fixed with ethanol, and stained with propidium iodide (PI). After staining, the cells were analysed in the flow cytometer.

RESULTS

The sponge in Figure 1.was collected and identified as *Spongia officinalis* var. ceylonensis (Dendy). A section of the sponge stained with safranin is shown in Figure 2. Figure 3 shows the regression analysis of the MTT assay of the extract and the standard drug on lung fibroblast cells, which are non-cancerous. It is seen that the IC50 for the extract is 0.2 µg/ml and that for the standard drug used, doxorubicin, is 3.7 x 10^{-3} µg/ml. Figure 4 shows the regression analysis of the MTT assay of the extract and 5-FU, used as the standard drug, The IC50 was calculated to be 0.023mg/ml for SE3 and 0.005 mg/ml for 5-FU. Figure 5 shows the dose dependant reduction in viability of the A549 cells when treated with the extract. Figure 6 shows the result of the Ethidum bromide/ Acridine orange live dead assay. It is seen that the extract causes apoptosis of the A549 cells. The flow cytometric analysis of the sponge extract shows cell cycle arrest at the G2/M phase of the cell cycle. The data of the control cells is shown in Figure 7, Figure 8 and table 2. The data of the treated cells is shown in Figure 9, Figure 10 and table 3. The dose of the extract used for flow cytometric analysis was 20µg.

DISCUSSION

The results show the anti-cancer activity of the extract of the sponge *Spongia officinalis* var. ceylonensis (Dendy) on lung cancer cells (A549). The mechanism of action of the extract is by bringing about cell arrest in the G2/M phase of the cell cycle.

Lung cancer cells are very hardy cells and there exists very few known drugs for the treatment of the same. This makes the development of drugs for the treatment of this cancer a niche area of therapeutic need. The present study is a step in this direction. The evidence shows great potential for further development.

ACKNOWLEDGEMENT

The authors wish to thank Dr P.A.Thomas, Emeritus Scientist, ICAR (Indian Council for Agricultural Research) for identifying the sponge.

ABBREVIATIONS USED

DMEM FBS (Foetal Bovine Serum), MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), AO (Acridine orange), EB (Ethidium bromide), 5-FU (5-fluorouracil)

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