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BAX and P53 Over-Expression Mediated by the Marine Alga *Sargassum Myriocystum* leads to MCF-7, Hepg2 and Hela Cancer Cells Apoptosis and Induces *In-Ovo* Anti-Angiogenesis Effects

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Abstract

As marine algae are well known for their biological activities, in the present work, the brown marine alga *Sargassum myriocystum* was analysed for its anti-cancer potentials on *in-vitro* cancer cell lines. MTT assay was performed to screen for its cytotoxicity in HepG2, MCF-7 and HeLa cancer cells, followed by bioactivity guided fractionation through thin layer chromatography (TLC). The mechanism of anti-cancer action of the bioactive fraction (SF6) was evaluated by DNA fragmentation analysis, LDH activity, caspase 3,7, 10 assay, cell cycle study by flow cytometry and study of gene expression by qRT-PCR methods. The anti-angiogenic potential was checked on chick embryos by CAM assay. The characterization of bioactive compound was carried out by HPLC, ESI-MS and GC-MS studies. Significant cytotoxicity and apoptosis induction in HepG2, MCF-7 and HeLa cancer cells by SF6 were confirmed as per MTT assay results, while no toxicity was observed on normal lymphocytes. The up regulation of apoptosis regulatory genes, p53 and Bax in cancer cells by SF6 was indicated by qRT-PCR results. The anti-angiogenesis property was clearly evidenced on chick embryos through CAM assay, with significant inhibition of blood vessel formation (66%) and branching growth. Characterization of SF6 through HPLC, ESI-MS, followed by GC-MS analysis indicated the presence of imidazole carboxamide and ellagic acid, which are listed in the anti-cancer database. These compounds along with some unidentified compounds in the partially purified *S. myriocystum* fraction appear to be majorly responsible for the anti-cancer activity of this brown alga. It can be concluded from the study results that *S. myriocystum* has potent antiproliferative, apoptogenic and angiogenesis inhibitory properties towards cancer cells and has the potential towards the development of anti-cancer drug molecule, after complete characterization of the bioactive compound and *in-vivo* validation studies.

Keywords: Anti-angiogenesis, BAX, Brown algae, Caspase, DNA fragmentation, p53, *Sargassum myriocystum*

1. Introduction

Cancer is a complex disease characterized by uncontrolled proliferation of affected cells that invade the cells around and metastasize to other tissues; it is the second major cause of death worldwide. It is one of the major health problems of people at the global level (Rashan *et al*., 2018). Cancer can be a result of inherited genes or can result from mutations in the chromosome of a healthy cell; otherwise, it can be triggered by external factors such as chemicals, alcohol, infectious agents, tobacco and radiation (Zong *et al*., 2012). Cancer cells grow by evading one's immune responses, cell cycle regulatory mechanisms, suppressing pro-apoptotic genes and by neo-angiogenesis (Hanahan and Weinberg, 2011). The advancements in the technology for its diagnosis and treatment have miserably failed to reduce the mortality rate. Chemotherapeutic drugs of synthetic origin come with their own set of side effects. Hence, compounds from natural sources which can upregulate the pro-apoptotic genes, have anti-angiogenesis properties and do not have any side effects to the normal cells are most sought-after goals in cancer therapy research.

Marine environment constitutes 71% of the earth's surface. Among the marine organisms, Algae have gained special interest owing to their diverse application potential, being rich in minerals, vitamins, fatty acids, amino acids, fibers and bioactive metabolites comprising sulfated polysaccharides, alginates, phlorotannins and carotenoids (Mac Artain and Gill, 2007; Chandini *et al*., 2008; Cerna, 2011; Misurcova *et al*., 2012). Compounds from macro algae have a variety of biological, therapeutic activities including anti-cancer, antifouling, antibiotics, anti-inflammatory, antiviral, antimitotic and cytotoxic (Kim and Ta, 2011; Smit, 2004; Gupta and Ghannam, 2011). However, only a smaller fraction of algae has been analysed for their anti-cancer activity.

Sargassum is a brown alga, normally seen in the intertidal region at the bottom of the sea water. *Sargassum* group of brown algae are found in tropical areas of the world and are most obviously found near coral reefs. *Sargassum* is the largest brown seaweed and mainly grows on coral bubbles and the rocky shores (Agardh, 1848). The brown colour of algae is due to the presence of

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fucoxanthin (Willstatter and Page, 1914). Mostly, *Sargassum* is used as food, animal feed, fertilizer for plants and for commercial production of alginate in industries. Many species of *Sargassum* are used for medicinal purposes also (Gade *et al*., 2013). *Sargassum* can be consumed as food, either cooked or raw, and has diverse therapeutic applications. It has high iodine content that prevents goitre. It additionally contains algin, laminarin and fucoidan, compounds that can prevent stroke and heart diseases. Algin has the capability to remove toxic components like radioactivity and lead from human body. Tea prepared from *Sargassum* promotes weight loss. The basal part of this seaweed, which contains high amount of algin, can be used to dress wounds and skin burns, after drying (Novaczek, 2001). *S. latifolium* has E1–E4 (Polysaccharides), that inhibit cytochrome P450 1A, which is a carcinogen activator, while selective cytotoxicity was exhibited by E3 towards lymphoblastic leukemia cells (Gamal-Eldeen *et al*., 2009). An extract from the brown seaweed *S. thunbergii* has shown antitumor activity (Zhuang *et al*., 1995) and inhibited rat mammary adenocarcinoma cell metastasis (Coombe *et al*., 1987). The brown marine macroalga, *S. myriocystum,* was reported to have potential radical scavenging activity but has not been explored for its anticancer and anti angiogenesis properties (Badrinathan *et al*., 2012). Hence, the present research work was mainly focused to evaluate the cytotoxic potential of *S. myriocystum* against different cancer cell lines and also to identify the bioactive compound(s) responsible for this activity, mainly to find a natural anti-cancer compound that would be highly efficient in combating cancer.

2. Materials and Methods

2.1. Collection of the algal sample and extraction of the metabolites

The brown marine macroalga, *Sargassum myriocystum,* was picked up from the Gulf of Mannar, south east coast of India, Rameshwaram district in Tamilnadu. The sample was authenticated by the scientist (Dr. Karuppanan Eswaran), at the Marine Algal Research Station (MARS). The collected samples were rinsed 3- 5 times with water and dried under the shade for 7 days. The dried sample was powdered in a mixer and was extracted using methanol in a Soxhlet apparatus. The methanol extract of *S. myriocystum* was filtered and concentrated in a rotary evaporator at 40 ºC. The concentrated extracts were evaporated to dryness (methanol was completely evaporated) and the dried extract was stored in a refrigerator at 4ºC until use. A stock solution of 1 mg/ml in DMSO was used for the assays.

2.2. Cancer cell lines used

The cell lines for the current research were procured from NCCS (National Center for Cell sciences), Pune in India. The HepG2 (liver), HeLa (cervical) and MCF-7 (breast) cancer cell lines, authenticated and mycoplasma free, were used, which were maintained under controlled conditions in a humidified CO_2 incubator (with 5%CO_2) and at 37ºC temperature. The media used for culturing the cells was MEM (from HIMEDIA) augmented with 10% Serum (Fetal Bovine), streptomycin (100µg/ml) and Penicillin (1000 U/ ml).

2.3. Screening for cytotoxicity

After subculturing the cells at suitable concentrations (1 x 10^6 cells/mL) to 96 well plates for 24 h, various concentrations (200, 100, 50, 10 and 1 µg/mL) of the dried methanol extracts of the algae, dissolved in DMSO, were added for different time intervals (24h, 48h, 72h and 96 h). Diluted DMSO served as the vehicle control. Each experiment was performed in triplicates. After the treatment period, 10 µL of MTT dye was added and the 96 well plates were kept at 37°C in a CO2 incubator for 3 hrs under dark conditions. Following this, DMSO was added (100 µL) to all the cells in the multiwell plates and the optical density was taken in an ELISA reader at a wavelength of 540 nm. The percentage viability was calculated by the following formula:

% viability = Absorbance value of treated samples/Absorbance value of control sample x 100 (Mosmann, 1983).

2.4. Purification by thin layer chromatography (TLC) and bioassay

S. myriocystum extract was purified partially, by performing TLC fractionation using thin layer chromatography sheets (silica 50×20 cm size, Merck), as per standard protocols (Kirchner, 1974). The TLC sheets were cut into pieces of appropriate sizes (6 cm x 7 cm) and placed in the TLC chamber inside the hot air over at 110º C for 45 min to activation of TLC sheets. The sample diluted with methanol was prepared for spotting the samples on TLC sheet. The sheet was kept inside the beaker containing a prepared solvent combination of Acetonitrile: Chloroform: Dichloromethane: Toluene in at 1:2:2:1 ratio. The beaker was covered with a lid to prevent solvent evaporation. Once the samples reached the top, the sheet was removed using forceps and immediately the solvent line was marked with a pencil. The fractions were marked by viewing under day light and under UV light (254 nm and 366 nm). Preparative TLC was carried out by scraping each band separately and dissolving in methanol. Six fractions (SF1, SF2, SF3, SF4, SF5 and SF6) were obtained. These samples were centrifuged at 5000 rpm for 15 min. The supernatant was carefully decanted to a vial, and the methanol was allowed to evaporate at room temperature, and the dry powder representing each fraction was weighed separately. These fractions were tested for anti-cancer activity using MTT assay in order to select the best fraction for further experiments.

2.5. HPLC and ESI -MS analysis

Further purification of *S. myriocystum* fraction was carried out by allowing the sample to run in a semi-preparative HPLC (Shimadzu make) LC-20. The C18 analytical column was injected with 2 µL of the TLC-purified SF6 fraction at 0.5 mL/min flow rate and the resultant fractions were eluted out at different retention times. By default, water was used in pump A and methanol in pump B to pass the pressurized liquid solvent carrying the sample. Afterwards, C18 preparatory column was used to collect the different fractions separated out. 100 microlitres of sample was injected into the preparatory column, the flow rate being 1.2 mL/min and was run for about 25 minutes. The fractions eluted from HPLC column were analyzed through the Mass spectrometer (Impact HD QTOF Mass spectrometer, Bruker, USA).

2.6. GC MS analysis

At the GC MS facility of SITRA (South Indian Textile Research Association), Coimbatore, the fraction (SF6) was analyzed to identify the compounds. The system (Thermo Scientific Trace DSQ GC-MS) uses Helium as the carrier gas. The SF6 fraction of *S. myriocystum* was injected to the GC-MS system (1µl), the initial temperature was 70°C for 3 min, which was gradually increased to 260°C at 6°C /min. The sample gets vaporised and separated into different components, based on the mass-to-charge ratio (m/z). The components produce different peaks at different retention times. With the help of library search and search in anticancer compound database (http://data-analysis.charite.de/care/), we identified and quantified the unknown compounds present in the sample.

2.7. Trypan blue dye exclusion assay

The cell concentration and viability were measured by trypan blue staining method. Cancer cells (1x 10^6cells/ml) were treated at the IC_{50} concentration of SF6 for 48 hrs, while the control cells received DMSO (0.4%). After 48 hrs, the cells from both control and treated flasks were treated with trypsin, detached cells were collected and centrifuged for 10 min (1000rpm). The cells thus collected were suspended in phosphate buffer, mixed with trypan blue dye (0.4% in PBS) and the viability of the cells was determined by manually counting the cells in a haemocytometer and observing under the microscope. The cell viability and cell count were calculated (Strober, 2001).

2.8. Lactate dehydrogenase (LDH) cytotoxicity assay

The cytotoxicity mediated by *S. myriocystum* fraction (SF6) on the HepG2, MCF-7 and HeLa cancer cell lines was analysed by LDH assay using a commercially available kit (G-Biosciences, India) as per the standard methodology provided by the manufacturer of the kit (Weyermann *et al*., 2005). To the cultured cells, 50 µg/mL of SF6 was added and the cells were incubated for 48 hours. After this, the supernatant was collected from the treated flasks, where LDH would have been released from damaged cells. As a negative control, medium containing serum was used. The DMSO treated (control) cells were treated with 150 µL of lysis buffer. The cells were freezed and thawed for 3-4 times until the cells got lysed. The cells were centrifuged for 30 min at 10,000rpm. The supernatant was collected by aspiration and was the positive control used in this assay.

In a micro-titre plate, 50 µL of treated sample, positive and negative controls were added to different wells in triplicates. The LDH substrate (50 µL) was treated to all the samples and the reaction mixture was incubated in a CO2 incubator at 37º C for 20 min. To stop the reaction, stop solution (200 µL) was added, and the optical density was recorded in an Elisa reader at 490 nm.

The cytotoxicity was calculated as a percentage as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental } OD_{490} - \text{Blank} OD_{490}}{\text{Control } OD_{490}} \times 100$$

2.9. DNA fragmentation

Treated (48 h) cancer cells (MCF-7, HeLa and HepG2), growing exponentially in 25 cm^2 tissue culture flasks were harvested, and genomic DNA was isolated. The assay was performed as per the instructions given in the mammalian genomic DNA isolation kit manual (Bangalore Genei). Cells (2 x 10^6 cells/mL) were sub-cultured and allowed to adhere for 24 h. The most effective concentration (50 µg/mL) of SF6 was added to adherent cancer cells and incubated at 37 ºC for 48 hours in a CO2 incubator. Trypsinization and centrifugation were used to collect the extract-treated or untreated cells. The cells were processed with RNase (20 mg/mL), 20 µL of proteinase K (20 mg/mL), followed by lysis buffer. Finally, 200 µL of chilled ethanol was added to the sample mix and stored overnight at -20 ºC. The precipitated DNA was collected by centrifugation, discarding the supernatant and drying. The completely dry DNA was stored at -20º C until further use in TE buffer (Tris-EDTA buffer). The DNA samples were visualized after gel electrophoresis in a 0.8% agarose gel (Sambrook et al., 1989).

2.10. Caspase enzyme activity

Caspase enzyme (caspase-3,7,10) activity was checked using the Caspase Assay Kit procured from G Biosciences (kit 786-205A) in MCF-7, HeLa and HepG2 cancer cells treated with SF6 fraction (50 µg /mL) for a duration 48 h, while the control cells were treated with DMSO (0.4%) for the same duration. All these cells were processed (as per the instructions in kit manual), and the collected cells were treated with 150 µL of lysis buffer. The cells were frozen and thawed for 3-4times, centrifuged (10,000rpm for 30 min), and the supernatant thus obtained from the treated and untreated samples were used for the caspase assay. 50 µL of treated and untreated samples were taken in different wells of a microtitre plate. The samples were treated with 50 µL of 5mM DTT in 1 ml of 2X caspase assay buffer and 50 µL of AFC-conjugated substrate mix. The wells where only reagents were added served as the blank. At 0 minute (t=0), the optical density of the samples was noted down in an ELISA reader at 405 nm. The samples were kept at 37 ºC in a CO2 incubator for about 90-120 minutes, and at every 15 minutes the optical density was measured until a significant difference in the readings were recorded.

2.11. Flow cytometry

The bioactive fraction SF6 (50 µg/mL) was treated to MCF-7, HepG2 and HeLa cancer cells (2 x 10^6 cells/mL) for 48 h and after this duration, flow cytometry was used to quantify DNA in the treated cells to analyse the cell cycle stages (G2/M, S and G0/G1 phases) (Pazaroski and Darzynkiewicz, 2004). The treated cells were harvested after trypsinizing and to these cells, 2 mL of ice-cold buffer (PBS) was added to make a uniform cell suspension. The cell suspension was transferred to tubes pre-coated with 2% FBS (Fetal bovine serum) and kept overnight. The cells were fixed with 9 mL of 70% chilled ethanol and stored at -20ºC until further use. The cell suspension was centrifuged at 4ºC for 10 min at 1000 rpm and the pellet was washed with 3mL of ice-cold PBS (two times) and at 4ºC for 10 minutes centrifuged at 1000 rpm. The cells were stained with 500 µ L of PI/Triton X -100 staining solution (1 mg Rnase, 0.02 mg/mL PI and 0.1% Triton X-100) in a dark condition and incubated at 37ºC in a CO2 incubator for 15 min. The cells were scored by a BD FACS verse (488 nm, at IISC Bengaluru) flow

cytometer and the data was analysed by BD FACS Diva™ software (v 6.0)

2.12. p53 and Bax mRNA levels through qRTPCR

After treating the breast cancer (MCF-7) cells (wild type p53) with 50 µg/mL concentration of SF6 fraction for a period of 48 h, RNA was extracted with the help of RNAiso Plus (Takara, Japan), from the treated MCF-7 cells, as per the methodology given in the instruction manual of the kit for extraction of total RNA (Cat. #9108/9109). Following RNA isolation, cDNA was synthesized as per the instruction in the manual of the cDNA synthesizer kit (Thermo Scientific, #K1622). Real time PCR (RT-PCR) was carried out for the isolated RNA sample using SYBR Green Chemistry kit from Bioline, USA (Vapo-protect PCR system, Eppendorf). The endogenous control was β-actin gene to compare the levels of expression of p53 and Bax. The condition for the PCR to run the sample was as follows i) 10 min denaturation at 95ºC, ii) 30 s annealing at 60ºC and iii) 30 s extension at 72ºC. The primers used for the genes are shown in Table 1. The results were analysed with the help of gene expression formula. The results are expressed as relative fold changes in the sample treated cell gene expression as compared to that of the control cell.

Table 1 Sequences of Reverse and Forward Primers used for the assay

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
p53	AGAGTCTATAGGCCCACCCC	GCTCGACGCTAGGATCTGAC
Bax	TTTGCTTCAGGGTTTCATCC	CAGTTGAAGTTGCCGTCAGA
β - actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG

2.13. Anti-angiogeneis assay on chick embryos

Anti-angiogenic property of the bioactive fraction SF6 was analysed by following a modified method of the chick embryo assay (Hardeep *et al*., 2014). The study was performed at the Institute of Animal Health and Veterinary Biologicals (IAH &VB), Bengaluru. Fertilized chicken eggs were purchased from the department of poultry sciences, Veterinary College and maintained at 37°C in an incubator at 60% humidity. Egg candler was used to mark on the eggs where the extract needs to be injected. On 8th day after fertilization, the eggs were pricked on the mark made on the Chorioallantoic membrane (CAM) layer using the egg puncher. Under aseptic conditions, 100 µl of *S. myriocystum* extract at different concentrations (2.0, 1.0 and 0.5 mg /mL) was injected to the CAM layer. 100 µl of DMSO diluted using phosphate buffer (1:9) and eggs without injection were used as controls. The eggs were then sealed with parafilm wax to prevent contamination and returned to the incubator for 72 hrs. After 72 hrs of incubation, the eggs were opened and the blood vessels (bv) were counted manually in the treated and control groups. The anti-angiogenesis effect of the algal sample was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Number of bv (C)} - \text{Number of bv (T)}}{\text{Number of blood vessels formation (C)}} \times 100$$

bv - blood vessel; C-control: T-treated

2.14. Histopathological examination (HPE) of the blood vessels

From chick embryos treated with varying concentrations (2.0, 1.0 and 0.5 mg /mL) of *S. myriocystum* extract for 72 hrs, as well as from DMSO treated control embryos, slides for histopathological examination *(*HPE) were prepared from various layers of the embryos. Hematoxylin and Eosin stain were used for this purpose. The stained slides were observed under an Olympus microscope (binocular, 5X 541). Slides were screened for: (1) Structure of blood vessel, mainly endothelial cells (2) Blood vessel number and frequency (3) Integrity of the membrane.

2.15. Statistical analysis

All the experimental procedures were performed in triplicate samples and the results being expressed in terms of mean ± standard error. One-way analysis of variance (ANOVA) and Duncan‘s multiple range test (DMRT) were performed to analyse the experimental data with Graph Pad Prism 6.0 software. A p value of < 0.05 was considered as significant.

3. Results

3.1. Anti-proliferative effect of the methanol extract of S. myriocystum as per MTT assay

As shown in Figure 1, the results of MTT assay revealed significant cytotoxicity caused by *S. myriocystum* extract to MCF-7, HeLa and HepG2 cells with the most effective response being observed at 200µg/mL concentration of treatment. The viability of 100 µg/mL of extract treated HeLa cells was 50.14%, HepG2 cells was 47.03 % and MCF-7 was 45.76% and that of 200 µg/mL treated HeLa cells was 44.67%, HepG2 was 39.7% and MCF-7 cells was 35.4 %, for the treatment period of 96 h. The IC_{50} value of *S. myriocystum* was calculated from the dose response as, 115µg/mL for HeLa, 80 µg/mL for MCF-7 and 60 µg/mL for HepG2 cells for 96h.

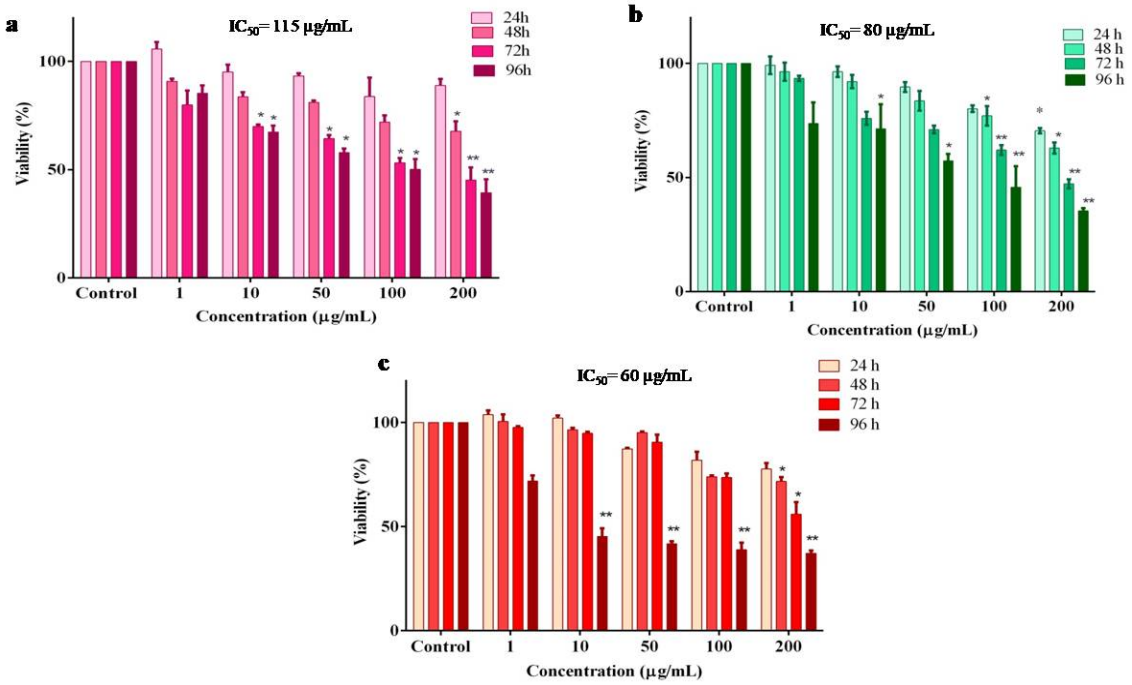

Figure 1. Effect of *S. myriocystum* methanol extract as per MTT assay. a) HeLa; b) MCF-7; c) HepG2 cancer cells post treatment with *S. myriocystum* for 24, 48, 72 and 96h. The values are expressed as mean±SE, with n=4. * $p<0.05$ and ** $p<0.01$.

3.2. Identification of SF6 as the bioactive fraction by TLC and bioassay

The methanol extract of *S. myriocystum* was fractionated by thin layer chromatography using different combinations of polar and non-polar solvents. Out of the several solvent combinations used on the TLC sheets, the solvent combination of Acetonitrile: Chloroform: Dichloromethane: Toluenein 1:2:2:1 ratio gave best separation of the components with six different fractions (Figure 2).

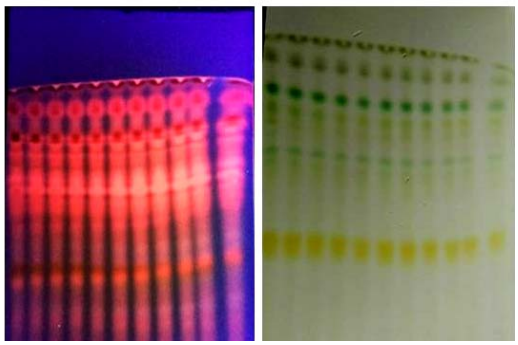

Figure 2. TLC fractionation of the methanol extract of SM under visible and UV light

These fractions were again tested on cancer cells through MTT assay to determine their cytotoxicity. Promising anti-proliferative effects were demonstrated by fraction 6 (SF6) as compared to other fractions against MCF-7 cancer cell line at all treatment concentrations (1, 10, 25 & 50 µg/mL) and at 48 h of treatment (Table 2). This SF6 fraction was considered as the bioactive fraction and was tested on HepG2, MCF-7 and HeLa cancer cells for 24, 48, 72 and 96 h. The results (Figure 3) were found to be highly significant at all treatment concentrations ($p<0.05$) with 39.34 % viability in HeLa, 39.76 % viability in HepG2 and 40.22 % in MCF-7 cells at 100 µg/mL concentration of SF6 and at 96 h of treatment. The cytotoxicity to the cancer cells was directly proportional to the dose and time of treatments. No cytotoxicity was observed on the treated normal lymphocytes at the tested concentrations.

Table. 2 Screening different fractions of *S. myriocystum* on MCF-7 cell line by MTT assay at 48h. Values are represented as mean ± SE. n=3.

S. myriocystum fractions	Concentration (µg/mL)			
	1	10	25	50
	Cell Viability (%)			
SF1	100.68 ± 1.11	95.89 ± 1.30	66.13 ± 1.41	74.21 ± 0.96
SF2	119.69 ± 1.93	97.60 ±1.40	73.39 ± 1.49	65.58 ± 1.48
SF3	127.32 ± 0.88	118.32 ± 1.79	81.26 ± 1.11	60.30 ±0.71
SF4	82.05 ± 0.67	99.31 ± 1.94	71.43 ± 1.07	51.74 ± 0.44
SF5	85.75 ±0.88	76.67 ± 1.69	62.91 ± 1.34	57.26 ± 0.52
SF6	67.12 ±0.86	62.08 ± 1.22	51.30 ± 1.08	47.84 ± 0.83

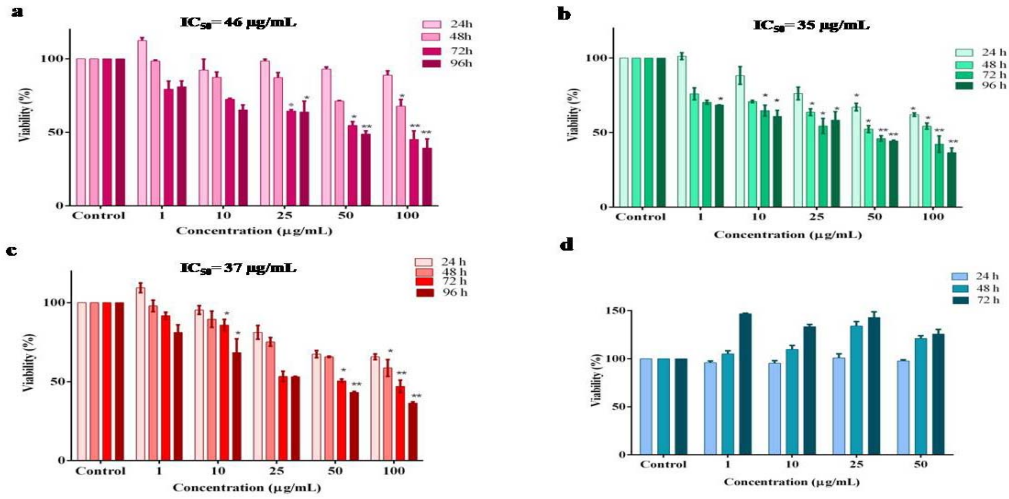

Figure 3. Effect of SF6 extract as per MTT assay on a) HeLa b) MCF-7 c) HepG2 d) Normal Lymphocytes post treatment with fraction SF6 for 24, 48, 72 and 96h. The values are expressed as mean±SE, and n=4. * $p<0.05$ and ** $p<0.01$. IC_{50} values are shown in the graphs for each cancer cell line.

3.3. HPLC and ESI-MS analysis of SF6 indicates molecular fragments of 182.1 and 302.1 m/z values

The partially purified fraction (SF6) obtained from TLC was again purified by the HPLC method. The fractions were eluted at different retention times (RT). The fraction eluted at 8.8 RT (largest peak) was collected and ESI-MS analysis was carried out. The obtained mass spectra revealed presence of a fragment having an m/z value of 182.1 and another one with 302.1 which were taken and compared with the GC-MS result (Figure 4).

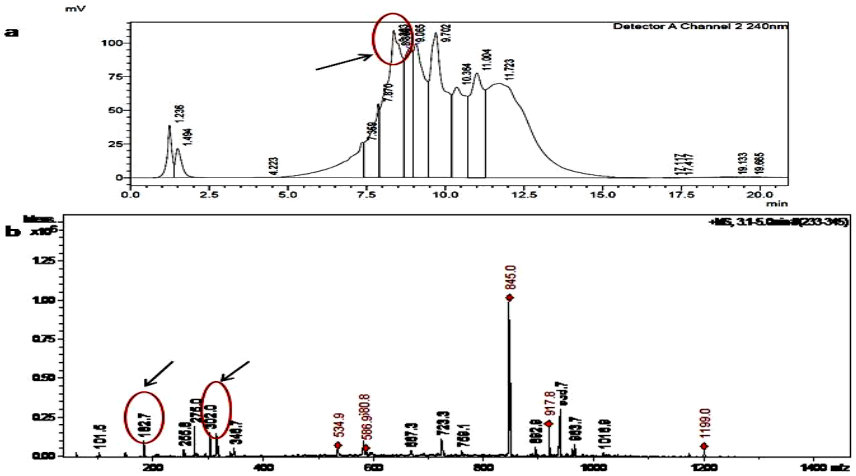

Figure 4. HPLC analysis of SF6 fraction (a) Chromatogram after HPLC of SF6 (b) ESI-MS of the fraction eluted at RT 8.8 min, showing spectral intensity of 182.7 and 302.1 Arrows indicate the peaks which resulted in showing m/z values of significant anti-cancer compounds.

3.4. GC-MS analysis indicates presence of dacarbazine and ellagic acid in SF6

The bioactive fraction SF6 was analysed by GC -MS method for characterizing the compounds in it. Through the library search (provided by SITRA in Coimbatore), dacarbazine (m/z 182.19) (Chen *et al*., 2008) and ellagic acid (302.19) reported as anti-cancer compounds in the anti-cancer database (http://data-analysis.charite.de/care/) were found to be present in SF6 through GC-MS results (Figure 5).

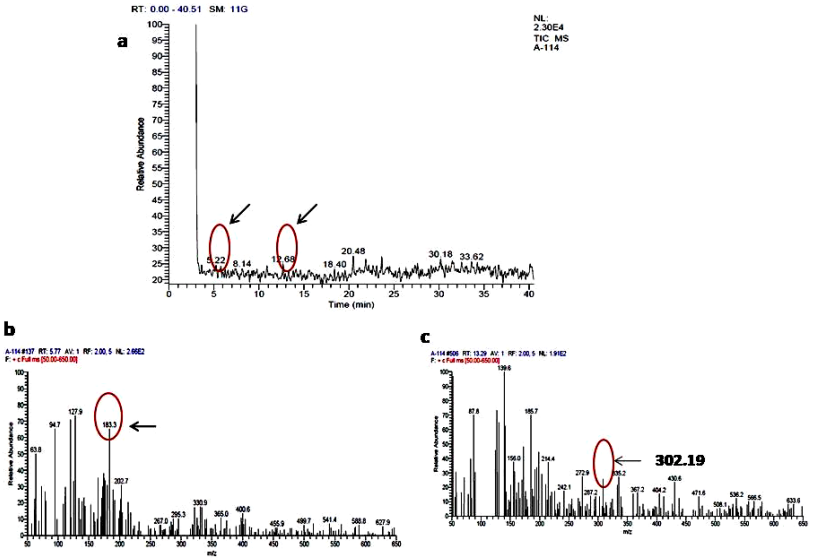

Figure 5. GC MS results of SF6 fraction (a). Chromatogram of SF6 (b) Mass spectrum showing a fragment of 183 m/z (peak of RT 5.77 min) c) Mass spectrum showing a fragment of 302.19 m/z (peak of RT 13. 29 min).

3.5. SF6 treatment decreases cell viability as tested by trypan blue assay

Through trypan blue assay, we found that the number of dead cells increased along with a decrease in viable cell count, in the SF6 (50 µg/mL) treated HepG2, MCF-7 and HeLa cancer cells after 48 h, while majority of them were viable (97- 99%) in the control group (**Table 3**).

Table 3 Assessment of total cell count and viability by trypan blue method.

Cell lines	**Cell count ($1x10^6$ cells/ml)**					
	Control			**SF6 (50 µg/mL) Treated**		
	Viable Cells	**Non-viable Cells**	**Viability (%)**	**Viable cells**	**Non-viable cells**	**Viability (%)**
HeLa	5.76	0.07	98.79	2.83	2.22	56.03
MCF-7	2.53	0.07	97.30	1.48	1.59	48.20
HepG2	3.12	0.02	99.36	1.60	1.14	58.39
Lymphocytes	1.32	0.03	97.77	0.99	0.02	98.01

3.6. Direct cytotoxicity caused by SF6 as per LDH assay

LDH assay is used to determine the release of LDH enzyme into the medium when the cells are undergoing lysis. The percentage cytotoxicity of HeLa, Hep G2 and MCF-7 cells treated for 48 h with 50 µg/mL of SF6 are 27.1%, 18% and 35% respectively, while no cytotoxic effect was seen on normal lymphocytes (Figure 6)

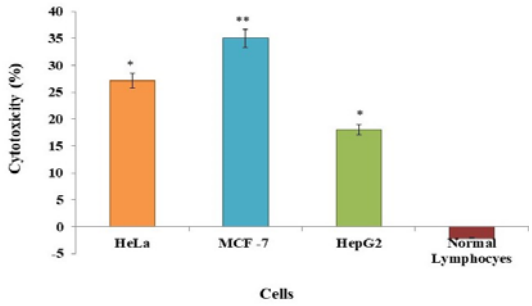

Figure 6. Cytotoxicity as per LDH assay on HeLa, MCF-7 cells, HepG2 cells and Normal Lymphocytes treated with SF6. * $p<0.05$, ** $p<0.01$.

3.7. SF6 induces caspase-3, 7, 10 activation in cancer cells

To evaluate the apoptogenic property of the algal fraction, caspase activity assay was performed on HepG2, MCF-7 and HeLa cancer cells after treating with SF6 at 50 µg/mL concentration for 48 h duration. In our analysis, we found an increase in caspase-3, 7, 10 activity of the SF6 treated cancer cells as compared to the negative controls. The increase in the caspase activity from initial (0 min) to final (90 min) was 23.11% in HepG2 cells, 15.53% in HeLa and 23.0% in MCF -7 cells, whereas in the control cells there was hardly any increase (Table 4).

Table 4. Activity (%) of caspase in HepG2, MCF-7 and HeLa cells treated with SF6

Time (min)	MCF-7		HeLa		HepG2	
	Control	Treated	Control	Treated	Control	Treated
0	7.17	17.92	1.36	6.84	2.19	13.73
30	7.21	13.18	1.06	7.32	2.1	24.7
60	6.04	25.27	1.71	15.15	2.9	37.08
90	7.96	41.03	1.82	22.37	3.02	37.45

3.8. DNA fragmentation mediated by SF6

DNA fragmentation is an indication of ongoing apoptotic process in cells. From Figure 7, it is clear that DNA was smeared in HepG2, MCF-7 and HeLa cancer cells treated with SF6, suggesting that treated cells were undergoing apoptosis as compared to control cells which are viable.

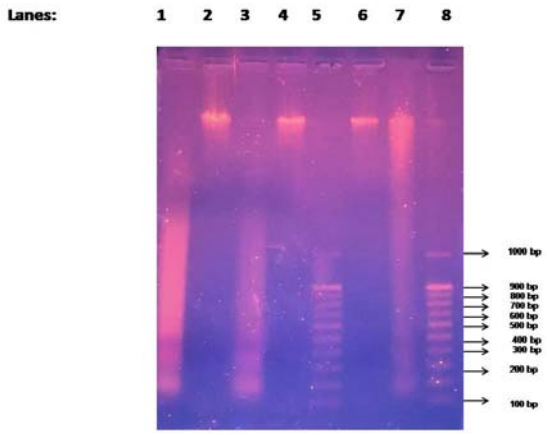

Figure 7. Photographs of agarose gel showing DNA isolated from SF6 treated and control cancer cells. Lanes: 1- treated HeLa; 2-control HeLa; 3- treated MCF-7; 4-control MCF-7; 5-Ladder DNA; 6-treated HepG2; 7-control HepG2; 8-laddere DNA.

3.9. SF6 induces altered cell populations in different phases of the cell cycle

Cell cycle stages of SF6 treated (48 h duration) cancer cells were analysed by flow cytometry with Propidium Iodide staining method. Propidium iodide stains the dead cells and emits fluorescence. The distribution of DNA in each of the phases (G0/G1, G2, S, M) of cell cycle in the treated and control groups was analysed with BD bioscience software. As shown in Figure 8, more cells were accumulated in the sub G1 phase with about 33.9% in HeLa, 56% in HepG2 and 66% in MCF-7 cells. Sub-G_1 phase represents the apoptotic cells. Simultaneous to these changes, there was a decline in the percentage of cells in other phases such as G1, S and M, as shown in the DNA histogram.

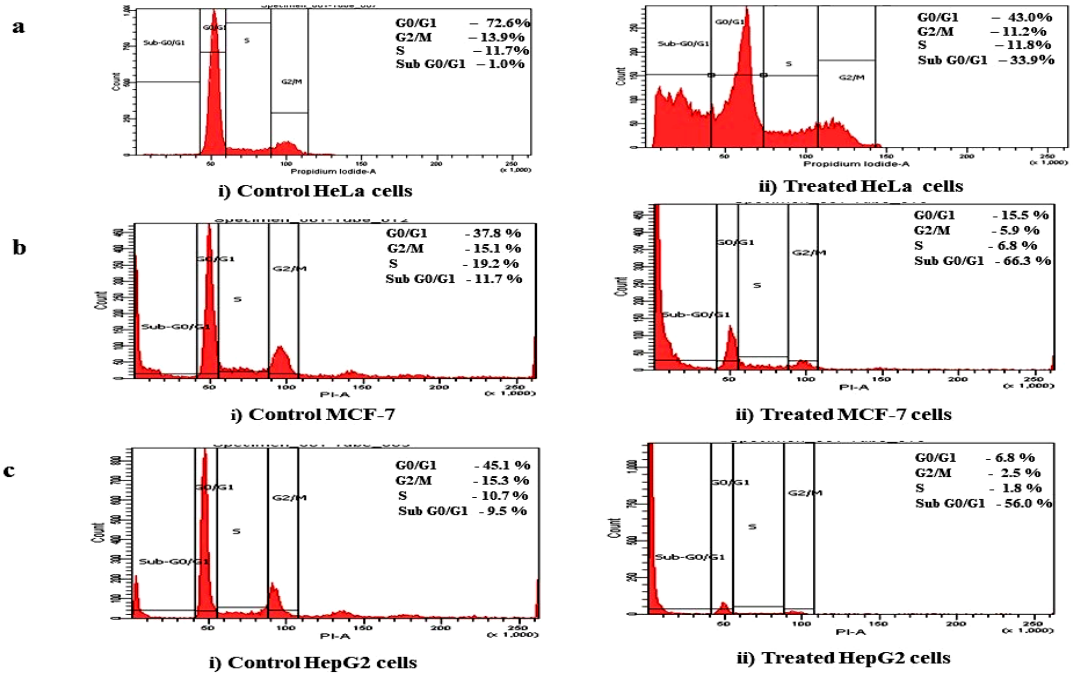

Figure 8. Cell cycle analysis of SF6 treated cancer cell lines through flow cytometry

3.10. Enhanced p53 and BAX gene expressions mediated by SF6 in MCF-7 cancer cells

The level of p53 and Bax gene expressions in MCF-7 cells was measured after 48 hrs of administration of SF6 fraction through qRT PCR. The levels of p53 and Bax genes were set to 1 in untreated control MCF-7 cancer cells and their relative expression is shown in Figure 9. Both p53 and Bax mRNAs were increased in the cells treated with SF6 in comparison with their levels in the negative controls (baseline).

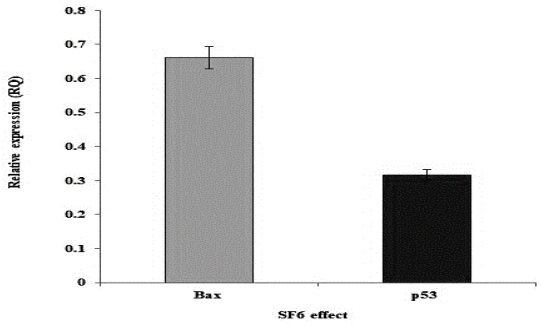

Figure 9. Expression of genes regulating apoptosis in the cells treated with SF6

3.11. S. myriocystum induces anti- angiogenesis effects in chick embryos

Various doses (2.0, 1.0 and 0.5 mg/mL) of extract from *S. myriocystum* were chosen for injecting into the 8th day fertilized chicken eggs for CAM assay. Blood vessel formation was checked after 72hrs of treatment. The main indication of anti-angiogenesis process is the inhibition of secondary and tertiary blood vessel branching regions along with lesser number of blood vessels, which were prominently observed in our experiments (Figure10). Significant inhibition of blood vessel formation and branching growth were observed at all treatment concentrations, with the effect being directly proportional to the increasing dosages of the algal extract. At 0.5 mg/mL concentration 43%, 1.0mg/mL 46% and at 2 mg/mL of treatment concentration 66% of inhibition in blood vessels was seen in comparison with the control eggs (Table 5).

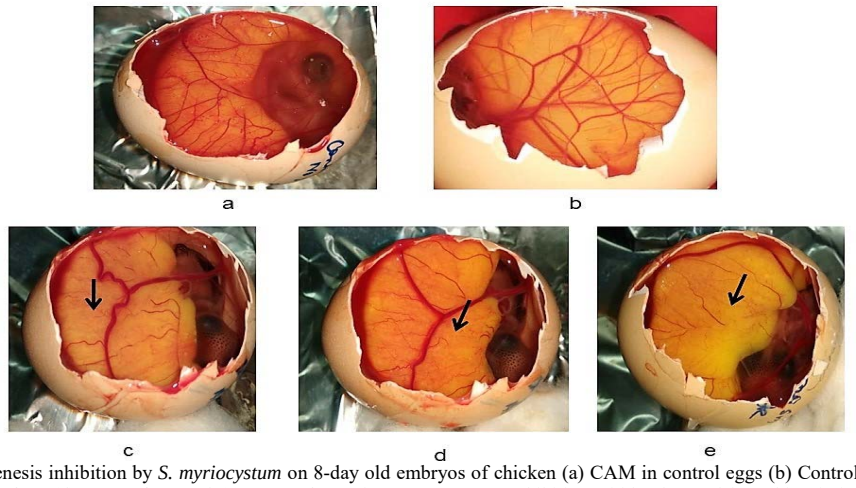

Figure 10. Angiogenesis inhibition by *S. myriocystum* on 8-day old embryos of chicken (a) CAM in control eggs (b) Controls with DMSO treatment (c) 0.5 mg/mL (d) 1.0 mg/mL and (e) 2.0 mg/mL of SF6 treatment. Inhibition of blood vessel branching is indicated by the arrows.

Table 5 Inhibition (%) of blood vessels in chick embryos injected with different concentrations of SF6 as per CAM assay.

Concentration of SF6 (mg/mL)	Blood vessels (Number) in control group	Blood vessels (Number) in treated group	Inhibition in blood vessel formation (%)
0.5	30 ± 5	17 ± 1	43
1.0	30 ± 5	16 ± 1	46
2.0	30 ± 5	10 ± 1.5	66.66

3.12. Obliteration of endothelial lining of blood vessels mediated by SF6 as per histopathological observations

The effects of *S. myriocystum* on angiogenesis were analyzed by histochemistry after staining with H&E. In control embryos, more endothelial cells and higher number of large and small blood vessels were seen, while in the treated embryos lesser number of large and small blood vessels were observed, and the effect was dose dependent. Obliteration of endothelial lining of large blood vessel was clearly seen on embryos treated with 2 mg/mL of algal extract. Loss of ectodermal and mesodermal integrity was also noted (Figure11).

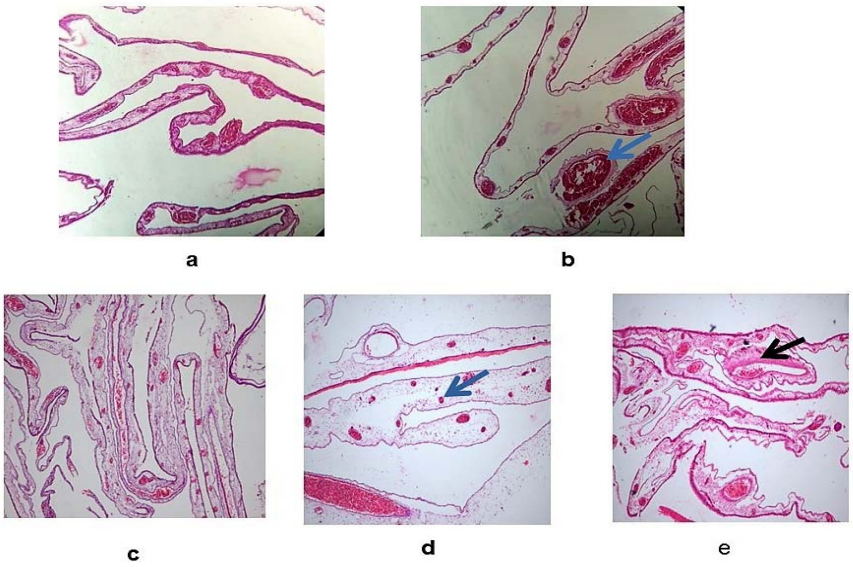

Figure11. Histopathological examination of CAM layer in chick embryos (40 X) (a) Untreated control (b) DMSO-vehicle control (c) 0.5 mg/mL of SF6 (d) 1.0 mg/mL of SF6 (e) 2.0 mg/mL of SF6 treated groups. Blue arrow shows large and small blood vessels. Black arrows show the destruction of endothelial wall lining of large blood vessels. Scale bar (A- E) - 160X

4. Discussion

Natural compounds show promise towards the treatment of multiple diseases such as cancer. Since ancient times, many countries including India and China have used medicinal plants, microorganisms and marine resources for healing purposes (Liu *et al*., 2012). Marine macroalgae were found to possess innumerable structurally diverse secondary metabolites with various bioactivities, which are under exploited for therapeutic purposes (Hong *et al.,* 2009). In this regard, the present work was initiated to explore the marine sea weeds, which are unreported for any economic significance, for finding their anti-cancer properties. The focus of the current research was to analyse the marine brown alga *Sargassum myriocystum* for anti-cancer activity, to identify the bioactive compound and analyse its mechanism of action on cancer cells.

The methanol extract of *S. myriocystum* (SM) and the bioactive fraction (SF6) were checked for their anti-cancer effects through MTT assay on cervical, breast and liver cancer cell lines. The results demonstrated that both crude and the SF6 fraction have anti-proliferative activity against cancer cells and the effect was dose and time dependent, with increasing concentrations causing decreased cell viabilities of all cancer cell lines. The IC_{50} value of the TLC purified fraction SF6 was 35µg/mL for MCF-7, 46µg/mL for HeLa and 37 µg/mL for HepG2 cells. A similar study on another species, *S. oligocystum*, reported its anti-cancer effects with IC_{50} values as 500 and 400 µg/mL against Daudi and K562 cell lines, respectively (Zandi et al., 2010). The IC_{50} value of *S. myriocystum* in the present work is lower when compared to this report. Trypan blue assay results showed higher number of non-viable cells in SF6-treated (50µg/mL) cancer cells (Table 3), with $1.60x10^6$ cells/mL, $1.48x10^6$ cells/mL and $2.83x10^6$ cells/mL for HepG2, MCF-7 and HeLa cells respectively (control cell concentrations were $3.12x10^6$, $2.53\ x10^6$ and $5.76\ x10^6$ cells/mL respectively in HepG2, MCF-7 and HeLa). The anticancer activity of a fucoidan from *Sargassum* sp. was reported as at 1000 µg/ml concentration (Ale et al., 2011) and the viability of Lewis Lung Carcinoma cells (LLC) was 40±7% and that of melanoma B16 cells (MC) was 56% at 100 and 200 µg/mL treatment concentrations. Compared to these reports, the treatment of bioactive fraction SF6 from *S. myriocystum* in the present study (Figure 3) has resulted in lower cell viabilities of 48% in HeLa, 43% in MCF-7 and 44% in HepG2 cells at much lesser concentration (50 µg/mL), thus proving its higher cytotoxicity towards the cancer cells. The same concentration of SF6 indicated its safety to normal healthy lymphocytes, with their viability being 99-100% at all of the time intervals.

Further evidence for the direct cytotoxicity caused by SF6 to cancer cells was provided by LDH assay. LDH release is used to assess the extent of toxicity and damage in cells and tissues due to traumatic exposures (Stoddart, 2011). The assay is based on the principle that, an increase in the production of formazan in the culture supernatant is directly proportional to the extent of cytotoxicity caused to the cells by any external agent. The current study results showed 18%, 35% and 27.1% cytotoxicity to HepG2, MCF-7 and HeLa cells treated with SF6 respectively, thus indicating the direct cytotoxic effect of SF6, apart from its anti-proliferative effects. The LDH mediated cytotoxicity was negative on normal lymphocytes (Figure 6), indicating the safety of SF6 to non-cancerous cells.

Apoptosis is a key indicator of programmed cell death resulting in changes in the morphological features such as chromatin condensation, membrane blebbing, DNA fragmentation, poly (ADPribose) polymerase (PARP) cleavage and increased caspase enzyme activity (Smyth *et al*., 2002). The mechanism of cell death in the SF6-treated cancer cells in the current study was indeed due to apoptosis, as proven by the results of DNA fragmentation pattern on agarose gel (Figure 7) and increased caspase 3,7,10 activities (Table 4) in HepG2, MCF-7 and HeLa cancer cells (caspases being the key enzymes regulating apoptosis in cells). Apoptosis induction in the SF6 -treated cancer cells was further supported by the gene expression

studies through qRT-PCR method in MCF-7 cells. As MCF-7 cancer cell line was found to be highly susceptible to SF6 treatment by most of the assay results such as higher caspase activation, greater fragmentation of DNA, higher LDH cytotoxicity, higher percentage of apoptotic cells (66%) in subG1 phase than both HeLa and HepG2 cells, we chose to perform gene expression studies in this cell line. Both p53 and Bax mRNA levels were elevated in MCF-7 cells in comparison with the untreated control cells (Figure 9). It is well known that p53 is mutated in more than 50% of all cancers in humans, and p53 tightly regulates the progression of cells through the DNA replication phase (Bell et al., 2002). Elevated p53 in the present study might be triggering the death of the SF6-treated cancer cells. Bax is a pro-apoptotic gene, which was reported to be down regulated in tumour cells, by which they escape apoptosis and are able to grow (Finucane et al., 1999). The enhanced expression of Bax by SF6 treatment in the current study proves the apoptotic and anticancer potential of *S. myriocystum* fraction.

Analysis of cell cycle stages is an important approach for understanding the effect of drug treatment to cancer cells. In the present study, when the cell cycle stages of MCF-7, HepG2 and HeLa cells were assessed by flow cytometry, it was observed that higher percentage of cells were accumulated in the sub-G1 phase in MCF-7 and HepG2 cells and thereby lead to the death of these cells (Figure 8). Sub-G1 phase indicates that the population of cells accumulated in the apoptotic phase (Grana and Reddy, 1995). In HeLa cells, higher percentage of cells were found in G0/G1 phase, and thereby suggesting that G0/G1 phase arrest led to the apoptosis of HeLa cells. Thus, the results confirm SF6 to be a strong inducer of apoptosis in cancer cells.

Cancer cells get nourishment for their uncontrolled growth by neo- angiogenesis. An ideal anti-cancer agent should be able to inhibit the formation of new blood vessels under *in-vivo* conditions. Chick embryos grow by forming newer blood vessels every day and could be considered suitable for demonstrating anti-angiogenesis experiments. In the present study, the fraction SF6 from *S. myriocystum* demonstrated potent anti-angiogenesis effects on chick embryos (Figures 10 &11), proving to be efficient anti-cancer compound.

The promising anti-cancer traits of SF6 prompted us to characterize this fraction through GC-MS. As per this GC-MS study, it was found that dacarbazine (imidazole carboxamide) and ellagic acid are present in *S. myriocystum* fraction. Dacarbazine (imidazole carboxamide organic compound) is used as an anti-tumor drug for metastatic melanoma, sarcoma, Hodgkin lymphoma and effectively killing the cancer cells by adding alkyl groups to the DNA (Chen et al 2008; Sarkar *et al*., 2013). Imidazole compounds are widely found in marine resources especially marine sponges (Ahond *et al*., 1988; Dunbar *et al*., 2000). Imidazole based anti-cancer drugs have special properties to destroy the cancer cells by first inhibiting DNA synthesis and stopping cell growth and division. Apart from dacarbazine, ellagic acid is also one of the compounds observed in the bioactive fraction SF6. Ellagic acid is a polyphenol compound, belonging to the ellagitannin family and derivative of gallic acid (Milivojevic *et al*., 2011; Ribeiro *et al*., 2007; Cai *et al*., 2017). Ellagic acid is reported mainly from fruits and vegetables and has been reported as antioxidant, anti-microbial and anti-cancer compound (Shahidi *et al*., 1992; Sanchez *et al*., 1999). Polyphenol group of compounds are abundantly found in seaweeds (Arguelles, 2020).

Molecular ion fragments related to dacarbazine and ellagic acid found from the ESI-MS results were observed even among the compounds obtained from GC-MS results. Further we found that neither the extract nor the bioactive fraction (SF6) from *S. myriocystum* have any toxicity to normal blood lymphocytes. We assume that the presence of dacarbazine and ellagic acid in *S. myriocystum* along with other unidentified compounds might be responsible for its anti-cancer activity and they might be acting synergistically towards inhibiting cancer cell proliferation.

S. myriocystum can be used for edible purposes also, as per reports (Kaliaperumal *et al*., 1995; Ogawa, *et al*., 2004; Shynu *et al.,* 2013), and has the potential for the development of an anti-cancer therapeutic in the future, as shown through the results of the current study. Other species of *Sargassum* were earlier reported for various biological activities including anti-cancer activity (Khanavi *et al*., 2010; Ye *et al*., 2008; Chen *et al*., 2012). But with respect to *S. myriocystum*, the current research work reports for the first time, as far as our knowledge and available literature, its anti-cancer potential along with its mechanism of anti-cancer activity. As *Sargassum* group of marine algae are edible with nutritional and medicinal properties, through the current study results it can be concluded that *S. myriocystum* shows great promise towards anti-cancer drug developmental studies in the future.

5. Conclusions

Based on the current study results, it can be concluded that the brown marine alga, *S.myriocystum,* has promising anti-cancer potential as demonstrated by anti-angiogenesis and apoptogenic properties and hence can be taken up for further characterization, pre-clinical and clinical studies towards drug development. The therapeutic potential of this edible sea weed opens up further avenues for its commercial exploitation towards a safer alternative therapy for cancer through future research in this regard.

6. Declarations

Funding

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Conflicts of interest/Competing interests

The authors disclose that they do not have any conflicts of interest

Ethics approval

Not applicable

Consent to participate

The written consent from healthy human volunteers was documented for isolation of lymphocytes.

Consent for publication

Not applicable

Availability of data and material

All data generated during this study are either included in this published article or available from the corresponding author on request.

Code availability

Not applicable

Authors' contributions

SP and VKN designed the experiments. SP performed all experiments and interpreted the data and wrote the first draft of the manuscript. SP and VKN contributed substantially to the revisions. Both authors read and approved the final manuscript.

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