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CELL VIABILITY AND CYTOTOXIC EFFECT OF *ACANTHOPHORA SPICIFERA* (RED SEAWEED) ON HT29 COLON CANCER CELL LINE

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Keywords:

Red Seaweed, Comet assay, *Acanthophora spicifera*, Cell viability, Cell mortality, Cytotoxicity

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ABSTRACT: *Acanthophora Spicifera* is a species of marine red seaweed and in the family Rhodomelaceae. They were freshly collected from Mandapam Coastal Area, Rameswaram Tamil Nadu, India and rinsed in seawater and packed in aseptic bags for further proceedings to the laboratory. Seaweeds are potential renewable resources in the marine environment. It has been used as antioxidant and antimutagen. The results of the present study revealed the reduction of cell viability after the treatment of methanolic extract of *Acanthophora spicifera* (10-200 µg/mL). In DNA Fragmentation by comet assay showed the methanolic extract of *Acanthophora spicifera*, the presence of the tail reflect the DNA damage and the potent cell mortality was shown at the concentration 200 µg/ml then decreased significantly with the decreased concentration of extract. Our study also demonstrated the revealed the protective effect of *Acanthophora spicifera* in HT-29 cell line treated with the methanolic extract of *Acanthophora spicifera*. Therefore, we concluded that *Acanthophora spicifera* might be used as a rich source of phytoconstituents and natural antioxidants.

INTRODUCTION: Cancer is a dreadful human disease, increasing with changing lifestyle, nutrition, and global warming. Cancer treatments do not have potent medicine as the currently available drugs are causing side effects in some instances. In this context, a variety of ingredients of traditional medicines and herbs are widely investigated in several parts of the world to analyze their potential as therapeutic agents [1-3]. Seaweeds are important sources of protein, iodine, vitamins, and minerals and hence, their metabolites have shown promising activities against cancer incidences [4]. Medicinal plants have been used by humans for centuries in folklore medicine [5].

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Medicinal plants are also incorporated into the historical medicine of virtually all human cultures. The plants are a rich source of secondary metabolites with interesting biological activities. Therefore, these secondary metabolites have an important source with a variety of structural arrangements and properties [6]. Macroalgae act as allelopathic, antimicrobial, antifouling and herbivore deterrents, or as ultraviolet screening agents [7]. They are also used by the pharmaceutical industry in drug development to treat diseases like cancer, Acquired Immune-Deficiency Syndrome (AIDS), inflammation, pain, arthritis, infection for virus, bacteria and fungus [8]. Currently, algae represent about 9% of biomedical compounds obtained from sea [9].

Among the different compounds with functional properties, antioxidants are the most widely studied. Antioxidants are the substances, which can defend human severe diseases including melanoma, cardiac disorders, diabetes, cancer, inflammatory

that explain their potential use in increasing shelf life of food and as medicine [10].

However, the seaweeds are of particular interest given the anticancer property, but till date, there is limited and incomplete research has been performed on this traditional plant. Moreover, screening of crude extract or phytochemical potential to induce apoptosis has not been properly elucidated in the past as it is the best strategy for developing a novel anticancer drug. Hence, the objective of the present study is to investigate the methanolic extract of *Acanthophora spicifera* potential for their antiproliferative and apoptosis-inducing activities on human adenocarcinoma colorectal (HT-29) cell line.

MATERIALS AND METHODS:

Collection of Seaweeds: *Acanthophora spicifera* were collected from Gulf of Mannar, Rameswaram, Tamil Nadu, India. The collected samples were cleaned well with seawater to remove all the extraneous matter such as epiphytes, sand particles, pebbles, and shells and brought to the laboratory in sterile bags. Then the samples were washed with tap water and distilled water and spread in the darkroom for drying, after which the dried samples were powdered and subsequently stored at 4 °C.

Cell Lines: Cell lines Human Colon cancer cell adenocarcinoma (HT-29 cell line) was obtained from National Centre for Cell Sciences (NCCS), Pune, India.

Culture Medium: Stock cells of HT-29 cell line were cultured in Dulbecco's modified eagle medium (DMEM) and supplemented with 10% inactivated Fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37 ºC until confluent. The cells were dissociated with Trypsin phosphate versene glucose (TPVG) solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). 25 cm culture flasks were used to grow the stock cultures and all experiments were carried out in 96 microtitre well plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions: The seaweed extract were separately dissolved in distilled Dimethyl sulfoxide (DMSO) and the volume was made up with Dulbecco's modified eagle medium (DMEM) supplemented with 2% inactivated FBS to obtain a stock solution of 1000 µg/ml concentration and sterilized by filtration. From this stock solution, four different lower dilutions (200, 100, 50, 25, 10 µg/ml) were prepared.

Reagents:

- Dulbecco's modified eagle medium (DMEM)
- 10% Fetal bovine serum (FBS)
- Propanol
- Phosphate-buffered saline (PBS)
- MTT Reagent
- Cytotoxic kit: Calcein A (Component A), two vials, 40 µL each, 4 mM in anhydrous DMSO• Ethidium homodimer-1 (Component B), two vials, 200 µL each, 2 mM in DMSO/H_2 O 1:4 (v/v)
- Phosphate-buffered saline (PBS)
- Triton X-100
- Distilled water
- Dimethyl sulfoxide (DMSO)
- Fetal bovine serum (FBS)
- Hydrogen peroxide
- Lysis solution

Cell Viability by MTT Assay:

Procedure: The monolayer cell culture was trypsinized, and the cell count was adjusted to 1.0 × 10 using DMEM containing 10% FBS. 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added to each well of the 96 well microtitre well plates. After 24 h, when a partial monolayer was formed, the supernatant was flicked off and washed the monolayer once with medium. To this, 100 µl of different test concentrations of seaweed extract were added respectively on to the partial monolayer in microtitre well plates. The plates were then incubated at 37 ºC ± 2 ºC for 2 days in 5% CO_2 atmosphere, and microscopic examination was carried out, and observations were noted every 24 h interval.

After 48 h, the test substances in the wells were discarded, and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37 ºC in a 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added. All the plates were gently

shaken to solubilize the formed formazan. Then the absorbance was measured using a microplate reader at a wavelength of 540 nm using UV-visible spectrophotometer. The optical density of the sample was compared to that of control to obtain the percentage viability as follow [11].

Cell Fragmentation Study by Comet Assay:

Procedure: The DNA fragmentation effects of methanolic extract of *Acanthophora spicifera* on HT-29 cell lines were evaluated by using alkaline single cell gel electrophoresis assay (comet assay) according to Singh *et al*., a method with minor modifications. The HT-29 cells were seeded onto 6-well cell culture plates (approximately 2 × 10cells per well) with cell culture medium and incubated at 37 °C in 5% CO_2 for 24 h for cell establishment.

After 24 h, below IC50 concentrations of methanolic extract of *Acanthophora Spicifera* (200 µmol/L in 1% DMSO) were added to the cells and incubated for another 24 h at 37 °C. DMSO (1%) was used as a negative control, and 50 µmol/L H_2O_2 was used as a positive control. After incubation, the cells were washed with PBS, harvested using trypsin/ ethylenediaminetetraacetic acid (EDTA) and collected for centrifugation at 1750 rpm/min for 5 min at 4 °C.

DNA was allowed to unwind for 40 min in freshly prepared alkaline electrophoresis buffer containing 300 mmol/L NaOH and 10 mmol/L Na_2EDTA (pH 13.0). After unwinding, electrophoresis was run at 300 mA for 25 min at 4 °C under minimal illumination to prevent further DNA damage. The slides were washed three times with a neutralization buffer (0.4 mol/L Tris, pH 7.5) for 5 min at 4 °C and then treated with ethanol for another 5 min before staining. Dried microscope slides were stained with EB (2 µg/mL in distilled H_2O; 70 µL/slide) covered with a coverslip and analyzed using a fluorescence microscope (Leica DM 1000, Solms, Germany) at a 200x magnification with epifluorescence equipped with a rhodamine filter (with an excitation wavelength of 546 nm; and a barrier of 580 nm). A hundred cells were randomly scored by eye in each sample on a scale of 0-4 based on fluorescence beyond the nucleus. Thus, the total visual score could range from 0 (all undamaged) to 400 (all maximally damaged) arbitrary units (AU), as reported. All experiments were repeated in triplicate [12].

Cell Cytotoxicity Assay:

Procedure: Two sets of replicates for each condition were used: one high control and one for the actual assay. Replicates of 100 µl of media without cells were used to serve as the blank for that condition. After 24 h the cells and blanks were treated with 100 µl of their respective seaweed extract at predetermined concentrations in serum-free media. On the day of the assay, the cytotoxicity reagents were prepared according to the manufacturer's protocol. One non-sterile clean, clear, flat bottom 96-well plate was set aside and labeled as an assay plate for each experimental plate used. 4 µl (2% total volume) of Triton X-100 was added to each of the high control wells and mixed thoroughly using a multichannel pipette to ensure the cells membranes were properly degraded. The plate was then centrifuged for 5 min at 1000 rpm.

A multichannel pipette was used to transfer 100 µl of supernatant from the top of all the wells of the experimental culture plate to the assay plate. Care was taken not to disturb the cells or draw up any debris. 100 µl of the mixed detection kit reagent was then added to each of the assay wells on top of the supernatant in rapid succession. The total volume in each well was 200 µl. The assay plates were then incubated at room temperature in the dark for twenty minutes. After which they were read using a standard plate reader with a reference wavelength of 490 nm.

RESULTS AND DISCUSSION:

Cell Viability by MTT Assay: The results of cell viability by MTT assay was shown in **Table 1** and **Fig. 1**. The induction of cytotoxicity or inhibition of cell viability in tumoral cells is an important property for the application of seaweed as chemotherapeutic agents. The MTT assay constitutes an important indicator of mitochondrial activity and it has been applied to the measurement of cell viability. The reduction of cell viability after the treatment of methanolic extract of *Acanthophora spicifera* (10-200 µg/mL), HT-29 untreated cells and cyclophosphamide as standard (200 ug/mL) on HT-29 cell lines in a dose-dependent manner.

Cytotoxicity can be defined as the degree to which an agent or chemical compound possesses a specific destructive action on certain cells. We examined the cytotoxic effect of methanolic extract of *Acanthophra spicifera* on adenocarcinoma colorectal cancer (HT-29) cells. According to the Suffness and Pezzuto (2009) [13], a crude extract can be considered as cytotoxic against carcinoma cells *in-vitro* and can be used for anticancer drug development if the standard IC_{50} value is less than 30 µg/mL. Based on the results, *Cladophora* spp were considered as highly cytotoxic against HT29 cell lines. This lack of cytotoxic activity might be due to masking of biological activity by the presence of some inhibitory compounds in the crude extract.

TABLE 1: EFFECT OF DIFFERENT CONCENTRATION OF METHANOLIC EXTRACT OF *ACANTHOPHORA SPICIFERA* ON HT-29 CELL VIABILITY

S. no.	Treatment	Conc. (µg/ml)	Absorbance@ 540nm	% Cell viability
1	HT29 untreated cells	-	0.318	100 ± 5.9
2	MESW treated	10	0.267	83.9 ± 7.1*
3	MESW treated	25	0.221	69.4 ± 5.8*
4	MESW treated	50	0.205	64.4 ± 4.1 *
5	MESW treated	100	0.187	58.8 ± 5.2 *
6	MESW treated	200	0.119	37.4 ± 3.5 *
7	Cyclophosphamide(STD)	200	0.097	30.5 ± 2.1*

Values are mean ± SD expressed as (n=3); *P<0.001, as compared with HT-29 incubated cells.
MESW - Methanolic Extract of *Acanthophora spicifera*

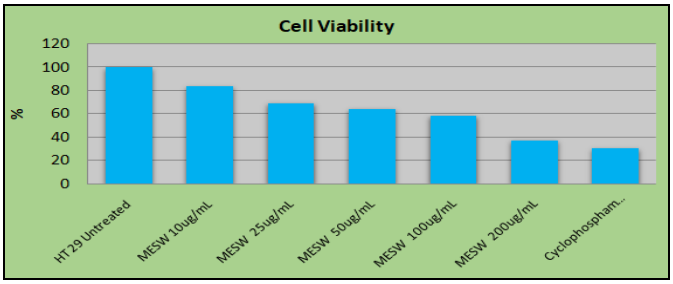

FIG. 1: CELL VIABILITY STUDY BY MTT ASSAY

Finding from our study demonstrated that methanolic extract of *Acanthophra spicifera* reduced cell viability of HT-29 cells as shown in the MTT assay **Fig. 1**. We have demonstrated that there was a dose-dependent response with treated cells and untreated cells to adenocarcinoma colorectal cancer (HT-29) cells. Untreated cells shows 100% viability and methanolic extract of *Acanthophora spicifera* treated cells showed 10 µg/mL (83.9 ± 7.1%), 25 µg/mL (69.4 ± 5.8%), 50 µg/mL (64.4 ± 4.1%), 100 µg/mL (58.8 ± 5.2%), 200 µg/mL (37.4 ± 3.5%) and Standard drug cyclophosphamide showed 200 µg/mL (30.5 ± 2.1%). Considering the previous study, the algae extracts *P. gymnospora* did not present cytotoxicity in the concentration tested of 312.15 μgmL^{-1} with 99.40% (dp11.39) viability for L929. The positive cell proliferation activity could also be demonstrated for methanolic extract of these algae. Also, the methanolic extract of *P. pavonia*, brown algae, demonstrated low cytotoxicity for normal lung human cells MRC-5 of IC_{50}>200 μgmL^{-1} [14]. *Sargassum* sp. evaluated in a concentration of 312.15 μgmL^{-1} also had no cytotoxicity. However, *Sargassum angustifolium* presented cytotoxicity effect against T47D and HT 29 cell lines with an IC_{50} of 166.42 ± 26.7 and 190.24 ± 52.8 μgmL^{-1}. However, alginate isolated from *Sargassum fulvellum* demonstrated antitumor effect against murine tumor Sarcoma 180 (no cystic and solid form), and Erlich carcinoma [15].

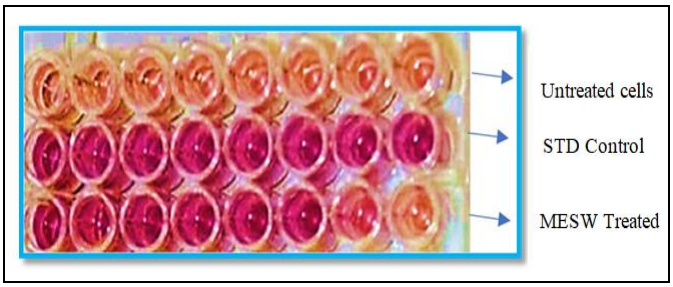

FIG. 2: MTT ASSAY MICROPLATE IMAGE

Cytotoxic Effect on HT29 Colon Cancer Cell Line: The extract showed a cytotoxic effect on the HT-29 cell line. As shown in **Fig. 4** the potent cell mortality was at the concentration of 200 µg/ml then decreased significantly with the decreased concentration of extract. Singh *et al.*, (2014) [16] reported that the methanolic extract of *F. hispida* in an artificially created wound in animals had caused excellent wound healing process activities and proposed that *F. hispida* are responsible for the upregulation of collagen in the wound site causing healing Property.

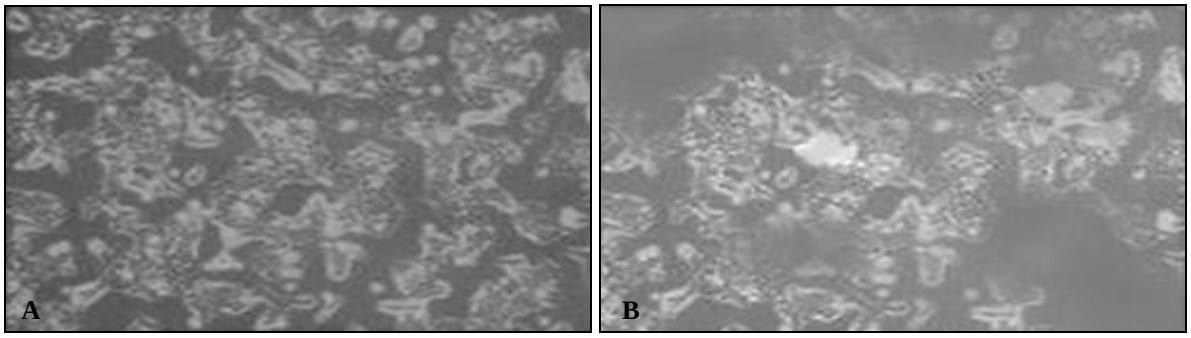

FIG. 3: CYTOTOXIC EFFECT OF *ACANTHOPHORA SPICIFERA* ON HT29 COLON CANCER CELL LINE. A - HT-29 UNTREATED CELLS, B - METHANOLIC EXTRACT OF *ACANTHOPHORA SPICIFERA* TREATED CELLS

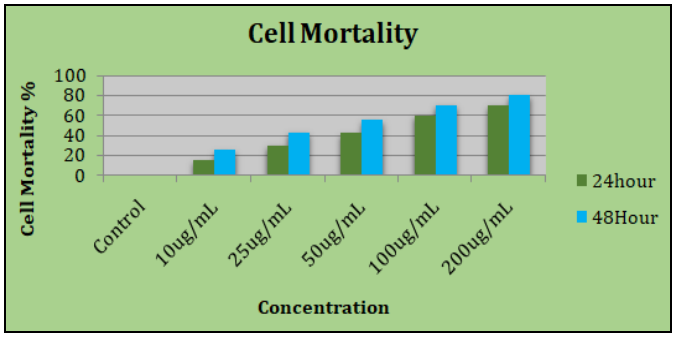

FIG. 4: CELL MORTALITY OF *ACANTHOPHORA SPICIFERA*

Cell Fragmentation Study by Comet Assay: Photographic pictures **Fig. 5** of DNA fragment migration patterns by alkaline comet assay evaluated with a fluorescence microscope (with excitation filter 420-490 nm). (A) Intact cells (control), most of DNA is located in the head of the comet. (B) Cell lines treated by methanolic extract of *Acanthophora spicifera*, the presence of the tail reflect the DNA damage.

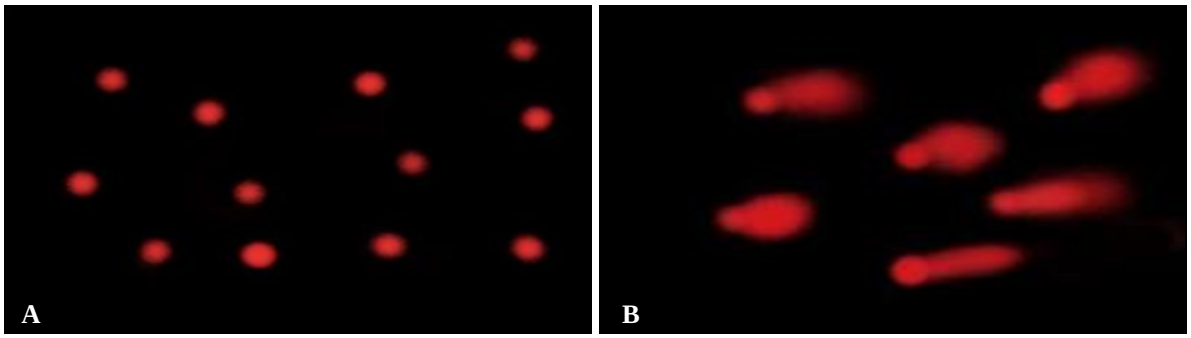

FIG. 5: DNA DAMAGING EFFECTS OF METHANOLIC EXTRACT OF *ACANTHOPHORA SPICIFERA* BY COMET ASSAY. A - NEGATIVE CONTROL, B - METHANOLIC EXTRACT OF *ACANTHOPHORA SPICIFERA* TREATED (200 µm/l)

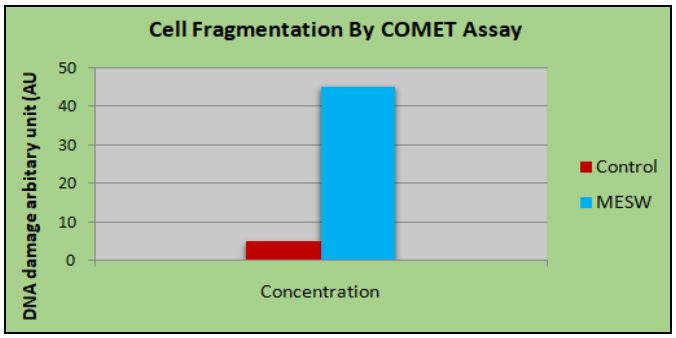

FIG. 6: THE COMET ASSAY OF METHANOLIC EXTRACT OF *ACANTHOPHORA SPICIFERA* ON HT-29 CELL LINES. Values are shown as mean ± SD which are three separate experiments performed in triplicate. Negative control $P<0.05$ statistically significant as compared with control

Evaluating the DNA damaging effects of methanolic extract of *Acanthophora spicifera* by comet assay. DNA damage of HT-29 cells without any treatment (A) and treated with 65 µg/ml methanolic extract of *Acanthophora spicifera* for 72 h, respectively. The percentages of damaged DNA, 72 h after treatments are compared with untreated and control groups in HT-29 cells.

The majority of the cells had longer DNA tail length and showed extensive DNA damage. The features characteristic of methanolic extract of *Acantho-phora spicifera* induced DNA damage was observed at a 65 µg/mL concentration **Fig. 6**. Aboutwerat *et al*., (2003) [17] revealed that Reactive Oxygen Species (ROS) are involved in some degenerative diseases such as atherosclerosis, cancer, liver cirrhosis, and diabetes mellitus. ROS can cause oxidative damage and interact with cellular macromolecules such as DNA, proteins, fatty acids and carbohydrates. It is known that the damage to macromolecules, especially DNA, leads to many degenerative diseases like cancer [18].

Morphology of Colon Cancer Cells Observed by Inverted Microscopy (A – 100x; B-200x):

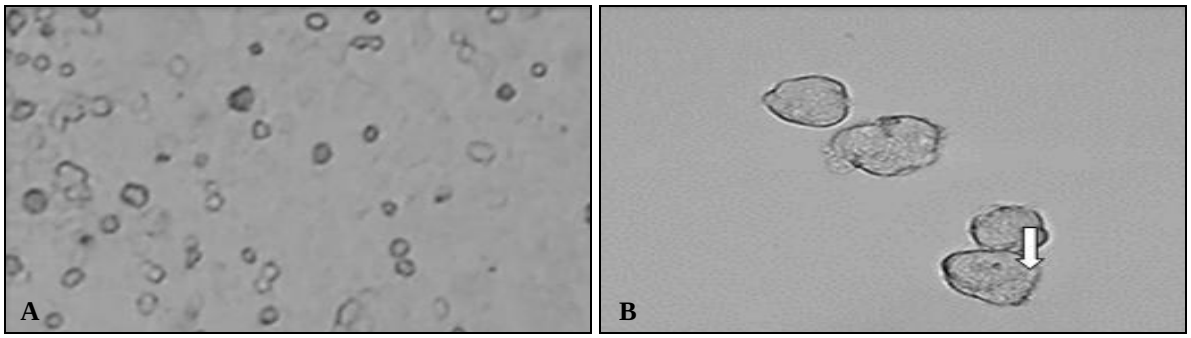

FIG. 7: MORPHOLOGY OF COLON CANCER CELLS OBSERVED BY INVERTED MICROSCOPY. A – HT-29 CELLS (100x), B – HT-29 CELL LINES + METHANOLIC EXTRACT OF *ACANTHOPHORA SPICIFERA* TREATED (200x)

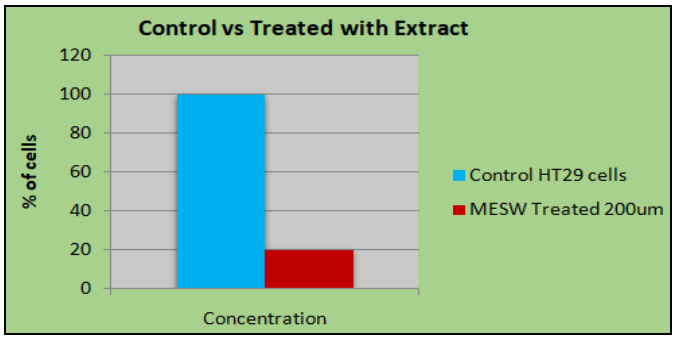

FIG. 8: PERCENTAGE OF APOPTOTIC CELLS IN CONTROL *vs*. TREATED WITH METHANOLIC EXTRACT OF *ACANTHOPHORA SPICIFERA*. Values are shown as mean ± SD which are three separate experiments performed in triplicate. Negative control P<0.05 statistically significant as compared with control

Study of control cells using an inverted microscope indicated that these cells had normal morphological specifications and maintained these specifications until the completion of treatment steps.

The Y-axis is the % of cells affected by the methanolic extract of *Acanthophora spicifera* treatment compared to the total cells treated with vehicle control. The error bars are the estimated 95% confidence interval around the mean. Results here are representative of 3 independent experiments. Apoptotic cells which had lost their membrane integrity was appeared as orange cells and showed morphological features of apoptosis. Cells were identified as apoptotic cells based on specific morphological criteria, including condensation and fragmentation of chromatin, also the formation of apoptotic bodies.

The results of our study were by Jayakumar *et al.*, (2012) [19] who have reported the methanolic extract of Acanthophora could significantly protect DNA damage even at low concentrations. Reactive hydroxyl radicals produced from H_2O_2 by the Fenton reaction, can bind to DNA at metal binding sites and induce strand breaks associated with DNA damage, mutations and genetic instability which could lead to carcinogenesis.

From the above findings, we revealed the protective effect of *Acanthophora spicifera* in HT-29 cell line treated with the methanolic extract of *Acanthophora spicifera*.

CONCLUSION: Our present investigation has demonstrated the protective effect of *Acanthophora spicifera* (Red seaweed) in HT29 cell line treated with a methanolic extract of *Acanthophora spicifera* collected from the Mandapam Coastal Area, Rameswaram, Tamil Nadu, India.

The findings of the present study revealed the use of *Acanthophora spicifera* as a natural antioxidant.

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