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CYTOTOXIC EFFECT OF METHANOL EXTRACTS OF SEAWEEDS

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ABSTRACT

Anticancer activity of marine macro algae (seaweeds) *Ulothrixflacca, Ulva fasciata* and *Caulerpataxifolia* against an invitroswiss albino neuroblastoma model (N2a) cell line was studied. During the present investigation crude methanol extracts of seaweeds were prepared using soxhlet apparatus. Crude extracts were dissolved in DMSO. In vitro anticancer activity of seaweeds at various concentrations (10–5000 ng/ml) was studied against the chosen cell lines using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole). All the three extracts showed anticancer activity with excess LDH release and ROS production which activated structural injury to mitochondrial membrane. Of which Methanolic extract of *Caulerpataxifolia* (275 ng/ml) has shown greater IC50 value than *Ulothrixflacca*and *Ulva fasciata*which showed 500ng/ml and 400ng/ml respectively, indicating greater anticancer activity. The present study indicates that the methanolic seaweed extracts were effective in the production of ROS-dependent mitochondrial damage-induced cytotoxicity in N2a cells.

KEYWORDS: ***Anticancer activity, methanol extracts, seaweeds, Neuroblastoma (N2a) cell line.***

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INTRODUCTION

In India seaweeds are mainly exploited by industries for phycocolloids but very poorly explored for their beneficial application in pharmacology[1]. Seaweeds are marine plants because they use the sun's energy to produce carbohydrates from carbon dioxide and water. Marine organisms produce pharmacologically important diverse group of natural products [2, 3] that include algae, which produce novel and unexplored sources of potentially useful bioactive compounds that might represent useful leads in the development of new pharmaceutical agents[4]. The seaweeds are also bestowed with varied sources of bioactive natural products that exhibits antimicrobial properties against plant pathogens [5, 6, 7, 8]. In addition, seaweeds are associated with antioxidant, antiviral, antibacterial, anthelmintic and antifungal activities as well as cytotoxicity, suggesting many potential applications in the pharmaceutical, nutraceutical and agricultural fields [9, 10, 11, 12]. In recent years, an increasing number of marine natural products have been reported to display antimicrobial activities[13] and anti-tumor compounds have been isolated from sponges, tunicates, algae and other organisms[14]. Discovery of anticancer drugs that must kill or disable only tumor cells without undue toxicity is an extraordinary challenge[15]. Toxicity of the plant or microbial material is considered as the presence of antitumor compounds[16].The evaluation of the anti-cancer potential of crude extracts from different sea organisms has been carried out by in vitro cytotoxicity tests in malignant cell cultures[17]. Isolation of cytotoxic anti-tumor substances from marine organisms has been reported in several references during the last 40 years[18]. Hundreds of potential anti-tumor agents have been isolated from marine origin especially from marine algae[19, 20]. Recently, much attention has been paid to the anticancer activity of seaweed constituents. Several investigators have reported that crude seaweeds or their organic extracts have anti-proliferative activity in human cancer cell lines in vitro, as well as inhibiting activity in tumors growing in mice in vivo[21], and antigenotoxic effect in human lymphocyte cultures in vitro. During the past decade, the search of novel substances with considerable potential for chemo-sensitization was advantageous in revealing the compounds of natural origin possessing the ability of enhancing the cytotoxic activity of chemotherapeutic. For example, phytochemicals such as flavonoids, polyphenol-rich extracts and isolated phlorotannin components have been shown to inhibit proliferation of cancer cells and to influence anti-inflammatory responses[22, 23]. Several marine algal polysaccharides, fucoidans, ulvan in particular have been found to induced apoptosis in cancer cells[24]. Recently, the polysaccharides and peptides, isolated from seaweeds have become a matter of great interest for cancer therapy. The mechanisms of their anticancer activity are related to their ability to suppress the growth of cancer cells. Cytotoxic or cytostatic effects, to enhance the immune responses, and to inhibited tumor angiogenesis[25]. According to the studies of biosorption of basic dyes using ulothrixspp, the results suggest that the physical interactions between sorbent particles and sorbate ions play an important role for the adsorption of methylene blue onto the biosorbent[26]. Another species of ulothrix, U. tenuissima was found to be non-genotoxic but caused sterility at higher concentrations due to oxidative stress[27]. As per best of our knowledge very less studies have been conducted regarding its medicinal activities. The effect of red (Gracillariacorticata), green (Ulva fasciata) and brown (Sargassumilicifolium) seaweeds alcoholic extract, against five important human cancer cell lines (MCF-7, MDA-MB-231, HeLa, HepG2, and HT-29) proliferation, apoptosis and cell cycle arrest were evaluated. Algal extract reducing activities were as follows: G. corticata > S. ilicifolium > U. fasciata[28]. In vitro cytotoxic potential of a methanolic extract of U . fasciata Delile (MEUF) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against human colon carcinoma (HT-29), human hepatocyte carcinoma (Hep-G2), and human breast carcinoma (MCF-7) cell lines was investigated. Maximum cytotoxic activity of MEUF was established for Hep-G2 with lowest OD or percent cell survival; highest percent cell inhibition with significant difference ($p > 0.05$) was compared to HT-29 and MCF-7[29]. Three sulfated polysaccharides(Ulva fasciata (UFP),Gloiopeltisfurcata (GFP), Sargassumhenslouianum (SHP))were isolated from three algae including green alga Ulva fasciata, red alga Gloiopeltisfurcata and brown alga Sargassumhenslouianum by ultrasonic extraction and radial flow chromatography. Results indicated that the in vitro antitumor and antioxidant activities of the three polysaccharides may be related to combined effects of sulfate content and uronic acid content[30]. In a T47D cell-based reporter assay, the Caulerpa spp. algal pigment caulerpin (1) inhibited hypoxia-induced as well as 1,10-phenanthroline-induced HIF-1 activation. Under hypoxic conditions, it is proposed that 1 may disrupt mitochondrial ROS-regulated HIF-1 activation and HIF-1 downstream target gene expression by inhibiting the transport or delivery of electrons to complex III[31].Neuroblastoma is a malignant pediatric cancer of the postganglionic sympathetic nervous system and derived from the neural crest cells during embryonic development. Initially it develops in the adrenal gland and metastasizes to liver, bone, bone marrow, lymph nodes, neck and chest. It is the most common cancer in babies younger than one and second most common tumors in children[32]. According to Cancer Facts and Figures (2013) and Atlanta, GA: American Cancer Society, (2013), 7% childhood cancers are of neuroblastoma and is responsible for 15% of all cancer deaths in children younger than 15 years. About 30%–50% of children with high-risk neuroblastoma experience long-term survival. Neuroblastoma tumor comprises of various heterogeneous population of cells which differ at morphological, biochemical and genetic levels was revealed from few investigations[33, 34]. The treatments available are not specific for this particular type of cancer and the drugs that are in routine use as defined by the American cancer society includes vincristine, neostar

and other nitrogenous mustard compounds. Neuroblastoma has heterogeneous population of cells of different genetic background[35]. Increasing evidence supports that molecular and genetic factors such as N-myc oncogene amplification, deletion of short arm of chromosome 1 and high expression of neurotrophin receptors (TrkA and TrkB) are associated with malignant transformation and progression of neuroblastoma. Despite targeting new molecular targets, and the use of multimodal therapy which includes surgery, radiotherapy in conjunction with chemotherapy and monoclonal antibody based immunotherapy, approximately 40% of children with high-risk neuroblastoma remain incurable. The statement was in agreement with various studies[36, 37, 38]. Hence, the identification and development of new therapeutic compounds with less toxicity are urgently needed. In this context an attempt has been made to identify the anticancer activity of three chlorophyceae members (*Ulothrixflacca, Ulva fasciata* and *Caulerpataxifolia*) methanolic extracts using an invitroswiss albino Neuroblastoma model (N2a) cell line.

MATERIALS AND METHODS

Seaweed collection

*Ulothrixflacca, Ulva fasciata*and *Caulerpataxifolia* are the three seeweeds collected from the rocky platforms at Tenneti Park located at Vishakhapatnam coastal banks. Species identification was done by referring the printed journal (Y. Sarojini, P.Santharao, B. Sujatha K.Lakshminarayana Distribution and diversity of marine macro algae in relation to environmental factors at Visakhapatnam coast. Seaweed Res. Utiln., 35 (1&2): 55-64, 2013 and authentified at Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh. The three seaweed species were collected during the months of July and August of the year 2013. They were carefully picked from the submerged rocks of Vishakhapatnam coastal banks and washed with fresh water thoroughly to remove the cell debris, sand and other particles. They were then kept in the ice bags and transferred to the laboratory. In laboratory, seaweeds were again washed with distilled water carefully and kept for shade dry. After drying, the seaweeds were stored in desiccator for further use.

Preparation of extracts

The dried seaweeds were weighed to 10gms and immersed in 100ml of methanol solvent and placed on orbital shaker for 24 hrs. After 24 hrs, the supernatant was collected and the solvent was evaporated using vacuum dryer to make the final volume one fourth of the original volume[39] and stored in airtight bottles.

Cell culture condition and maintenance

The Neuroblastoma (N2a) cell line was obtained from the National Centre for Cell Science (NCCS), India. The Cells were seeded into plates and flasks in 1:1, DMEM supplemented with 10% FBS, antibiotic and antimycotic solution (Sigma, St. Louis, MO, USA) in a conditions of 5% CO_2 and 95% air at 37 °C. The media was changed on alternate days and treated with different concentrations (5-85 µg/ mL) of seaweed extract dissolved in DMSO and water (1: 9 v/v).

Cytotoxicity of seaweed extracts (MTT assay)

Cell viability was defined as the ability of cells to metabolically reduce the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a purple formazan dye[40]. Cells were seeded into individual 96-well plates and incubated under the above conditions. After a day of incubation, cells were treated with various concentrations of three seaweed extracts ranging from 10-5000ng/ml. In order to obtain IC_{50} values absorbance was measured at 570 nm in an ELISA multiplate reader. The percentage inhibition of growth was calculated using the formula

% Cell viability = 100-[100× (A_c- A_t)/ A_c]
Where, A_t = absorbance value of test compound,
A_c = Absorbance value of control.

Measurement of cytotoxicity by lactate dehydrogenase (LDH) assay

The cytotoxicity was quantified in terms of plasma membrane damage by measuring the amount of LDH released by means of a LDH-estimation kit (Agappe-11407002) according to the manufacturer's instructions. LDH activity was measured through the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD^+) at a wavelength of 340 nm. The rate of increase in enzyme activity due to the formation of reduced nicotinamide adenine dinucleotide (NADH) is directly proportional to the LDH activity in the sample. The N2a cells were plated at a density of 5×10^4 cells/well in 24-well plate for 24 h and cells were treated with various concentrations of plant extracts (10–5000ng/ mL) for different time intervals (3–24 h). The cells were precipitated by centrifugation at 2,500 rpm for 5 min at 4 ˚C and the supernatant (100 µL) was mixed with 1000 µL reaction mixture and the percent activity was calculated. The total LDH activity was measured by lysis (2% Triton X-100) of untreated cells. The cells were seeded in petri dishes (1×10^6 cells) and then treated with different concentrations (10–5000ng/ mL) of extract for 24 h.

Measurement of intracellular ROS

The intracellular ROS was estimated to measure oxidative stress induced by the toxins using oxidation-sensitive dye DCFH-DA (dichloro-dihydro-fluorescein diacetate) . The assay is based on the principle that the non-fluorescent fluorescin DCFH-DA derivatives will emit fluorescence after being oxidized by the radicals generated by the toxins. The nonionic, nonpolar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH-DA which is oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of reactive oxygen species (ROS). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress generated in the cells. The cells

were plated at a density of 5 ×10^4 cells/well in 24-well plate for 24 h and cells were treated with various concentrations of plant extracts (10–5000ng/ mL). Cells were then treated with 20 µM DCFH-DA for 30 min and intracellular ROS was measured by the fluorimetric detection of DCF oxidation at an excitation wavelength of 485 nm and an emission wavelength of 522 nm. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. The fluorescent intensity percentage was measured using florescent microscope (ZEES).

Measurement of mitochondrial membrane potential (MMP)

The electrical potential across the inner mitochondrial membrane was measured using the fluorescent dye rhodamine 123 to estimate the mitochondrial membrane integrity. The cells werecultured in 24 well plates and treated as mentioned earlier. After treatment rhodamine 123 (10 µg/ml) was added to the cells and incubated for 60 min at 37 ˚C. Then the cells were collected after washing twice with PBS and the fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Hidex plate chameleon™ V (Finland).

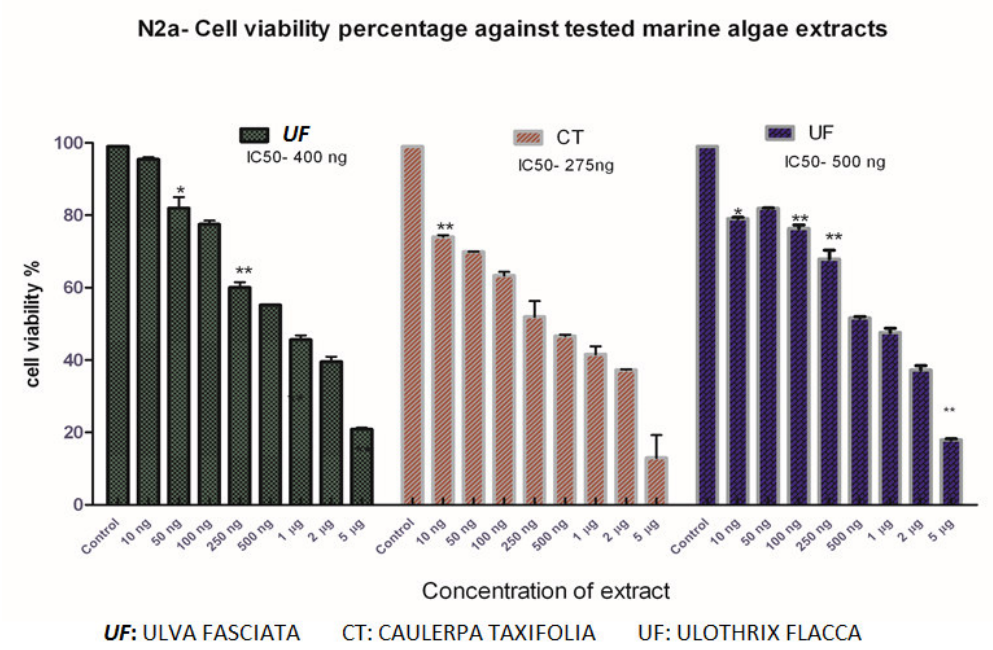
RESULTS

Cytotoxic potential of seaweed extracts on N2a cells MTT assay

The cytotoxic effect of *Ulothrixflacca, Ulva fasciata* and *Caulerpataxifolia* extracts were investigated on N2a cells (Neuroblastoma cell line from albino mice)by MTT assay with various concentrations of seaweed extracts (10 to 5000 ng/mL). As the concentration of extracts in the culture increased, the cell viability decreased significantly as shown in the figure. After 24 h of extract treatment all cells exhibited significant decrease in cell viability even at lower concentration.

GRAPH 1

Cytotoxic effect of Ulothrixflacca, Ulva fasciataand Caulerpataxifolia extracts on Neuroblastoma cell line by MTT assay at increasing concentrations (10-5000ng/ml)

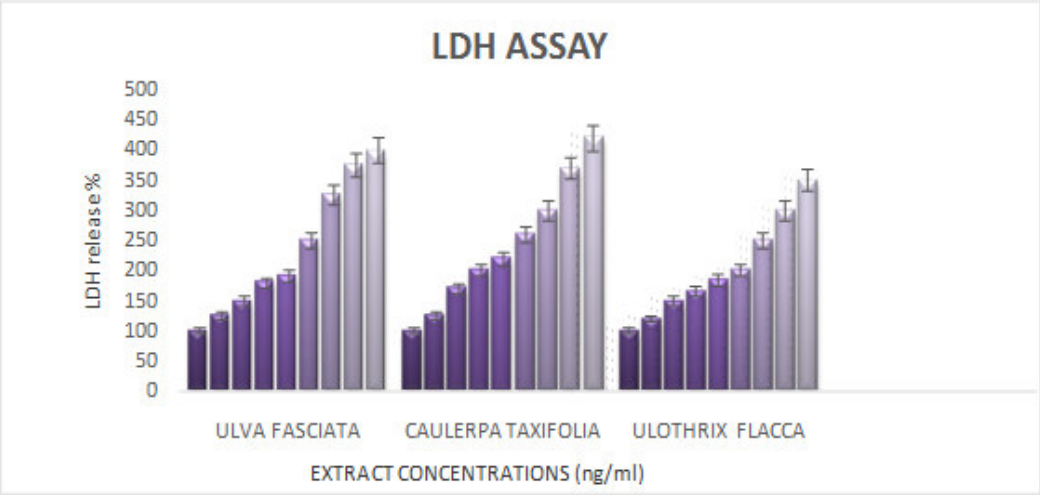


LDH assay

Cytotoxicity of *Ulva fasciataCaulerpataxifolia* and *Ulothrixflacca*extracts were also confirmed by LDH leakage assay. The level of extra cellular LDH increased with increase in the number of dead cells depending on the concentration of the extracts. Results represent the ± SD of 4 replicates. And the percentage LDH release and concentration of seaweed extracts are shown in the figure below.

GRAPH 2

Effects of Ulva fasciata,Caulerpataxifoliaand Ulothrixflaccamethanolic extract on the plasma membrane damage of N2a cells

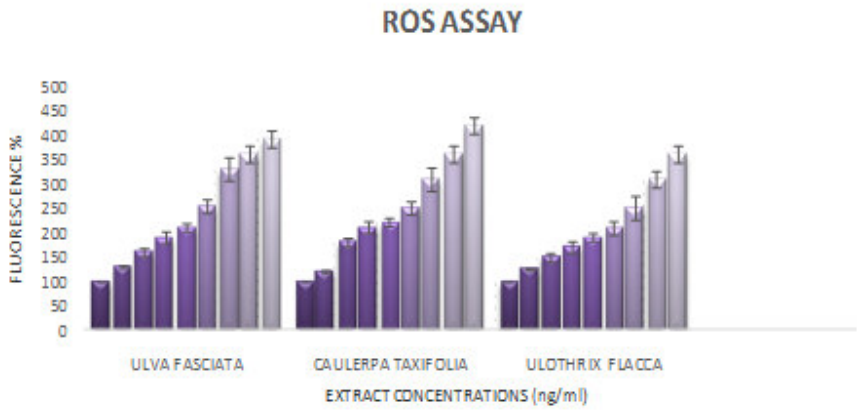


Determination of Reactive Oxygen Species

To examine the extend of cell damage by the production of reactive oxygen species and the loss of mitochondrial membrane potential caused by the methanol extracts of *Ulva fasciata, Caulerpataxifolia* and *Ulothrixflacca* over the N2a cells. It was found that the generation of ROS increased in a dose dependent manner with increase in concentration of seaweed extracts and it is represented in the figure below.

GRAPH 3

Effects of Ulva fasciata,Caulerpataxifolia and Ulothrixflaccamethanolic extracts on the ROS generation in N2a cells



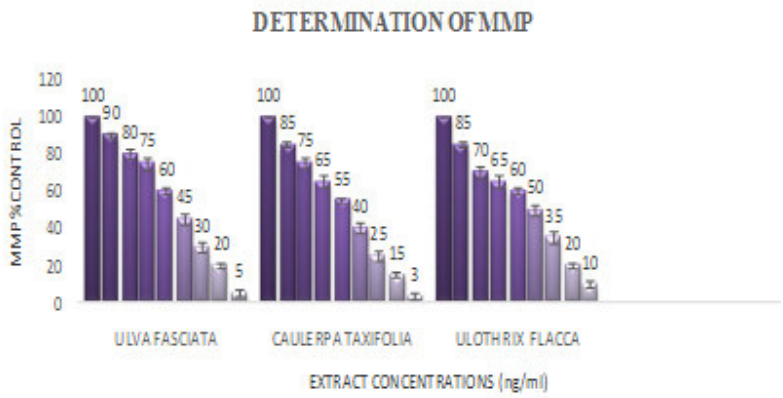
Increase in the release of reactive oxygen species in a dose dependent manner. Results represent the ± SD of 4 replicates.

Determination of mitochondrial membrane potential

The concentration of extract and percentage loss in mitochondrial membrane potential is shown in the figure below.

GRAPH 4

Effects of Ulva fasciata,Caulerpataxifolia and Ulothrixflaccamethanolic extracts over the depletion of mitochondrial membrane potential



The loss in mitochondrial membrane potential with respect to the increasing concentration of plant extracts. Results represent the ± SD of 4 replicates.

DISCUSSION

In this study, anticancer activity in vitro from three different marine algae samples (*Ulva fasiata, Caulerpataxifolia* and *Ulothrixflaccca*), were determined.Neuroblastoma is a malignant pediatric cancer of the postganglionic sympathetic nervous system and derived from the neural crest cells during embryonic development. Initially it develops in the adrenal gland and metastasizes to liver, bone, bone marrow, lymph nodes, neck and chest. It is the most common cancer in babies younger than one and second most common tumors in children. It is crucial to find new therapeutic agents that can exhibit anti-proliferative effects on neuroblastoma cells irrespective of their genetic abnormalities. Considering the above results the chlorophyceae members were efficient anticancerous materials. Increase in the leakage of lactate dehydrogenase with respect to damage of membrane in a dose dependent manner. A stable cytosolic enzyme of lactate dehydrogenase (LDH) catalyzes the oxidation of L-lactate to pyruvate. Upon membrane damage in cells, LDH enzyme is released into the culture medium, suggesting the loss of membrane integrity[41]. Thus the fixation of inhibitory concentration requires the LDH test and the result suggest nearly same concentration as exerted earlier in MTT and hence, the IC50 values were fixed nearer to the values of 400ng/ml, 275ng/ml and 500ng/ml for *Ulva fasciata, Caulerpataxifolia* and *Ulothrixflacca* respectively. Oxidative stress occurs due to the increase in the intracellular ROS level and that serves as a major cause for the various metabolic impairments and even for the death of the cell[42]. The increase in the release of ROS should correlate with the decrease in mitochondrial membrane potential and the results were found to be very appropriate. The live cells shows increased mitochondrial membrane potential[43] and when the cells were treated with varying concentrations of seaweed extracts the mitochondrial membrane potential decreased significantly in a dose dependent manner. The potential concentration at which the 50%of loss in membrane potential served as a best known concentration to act as a cytotoxic substance and it was found that the 400ng/ml, 275ng/ml and 500ng/ml of extracts of *Ulva fasciata,Caulerpataxifolia* and *Ulothrixflacca* respectively were best noted for the generation of ROS and for decreasing the mitochondrial membrane potential of the cells. At 400ng of *Ulva fasciata* extract, 275ng of *Caulerpataxifolia* extract and 500ng of the *Ulothrixflacca* extract decreased the cell viability to 50%. And these concentrations were fixed to be the IC50 concentrations and was further confirmed with determining the LDH assay.The concentrations of *Ulva fasciata, Caulerpataxifolia*and *Ulothrixflacca* extracts at which there was 50% death was termed to be the IC50 value and it was found to be 400ng/ml, 275ng/ml and 500ng/ml respectively.The free radical raise and oxidative stress can lead to activation of mitochondria mediated induction of intrinsic apoptotic pathway[44]. Thus the current study revealed the potential of extracts to induce apoptosis by generating ROS. During the process of apoptosis there will be a great loss in mitochondrial membrane potential due to the formation of pores and this determination of mitochondrial membrane potential would help in serving as an indication for the initiation of apoptosis. And it must be in correlation with the generation of ROS and the results were more appropriate to the ROS assay and further the apoptosis initiation can be confirmed by extending the study to pathway analysis. These results were complementary to the results produced by Fischel et al., [45]. The other two seaweeds *Ulothrixflacca*and *Ulva fasciata* also showed effective cytotoxic effects against the neuroblastoma cell line. It is clearly evident that *Ulothrixflacca*and *Ulva fasciata*also shows anticancer activity, yet the structural elucidation of compounds of interest had to be performed using mass spectrometry.

The MTT assay was confirmed using LDH assay, ROS assay and MMP assays. All the three assays gave supportive results for the MTT assay.

CONCLUSION

Therefore in conclusion, the present study showed a decrease in cancer cell count as a confirmatory evidence for protection against Neuroblastoma (N2a). Consequently the increased levels of LDH (lactate dehydrogenase) enzyme and ROS with increased concentrations of seaweed extracts confirmed the anticancer activity of methanol extracts of the three seaweeds. All the three seaweeds showed promising anticancer activity. Thus, from the above observations and other parameters it was concluded that the seaweeds *Ulva fasciata, Caulerpataxifolia* and *Ulothrixflacca* possesses anticancer activity against neuroblastoma cell line.

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