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A study on the ethanolic extracts of red seaweed- *gracilaria corticata*(j.agardh) j. Agardh, to assess the antiproliferative activity and morphological characterization of apoptosis on hela cell lines

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Abstract

Marine algae are excellent source of bioactive compounds that can be used as an alternative source for finding novel anti-cancer molecules. Gracilaria corticata, red algae from Surathkal beach, Karnataka were studied for their anti-proliferative activities and their morphological characterization on He La cells were assessed. Cytotoxicity of the algal ethanolic extracts on HeLa cells were assayed by 3-(4,5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) reduction method. Morphological assessment was done by examinations with Hoechst staining and acid vacuoles were determined using acridine orange. Induction of apoptosis was studied using caspase activity.Based on IC_{50}value, further morphological assessment was carried out and apoptosis was confirmed using Hoechst 33342 staining and acridine orange staining respectively. Treated cells became round with blebbings with condensed nuclei. Acidic lysosomal vacuoles formation occurred in treated cells.These red algae were able to suppress proliferation and promote apoptosis-- mediated cell death with induction of initial stages of apoptosis in HeLa cell lines. Thus, this seaweed can be a potent candidate for isolating new green drug anticancer molecules. However, further characterization at the molecular and structural levels are required.

Keywords: HeLa, MTT, Acridine orange, Hoechst 33342, Gracilaria corticata

Introduction

Cancer is one of the most serious threats in human diseases and carcinoma of the uterine cervix is the second most common female tumor worldwide, surpassed only by breast cancer. Its incidence is disproportionately high (>80%) in the developing world and its treatment of disease is usually palliative, aimed only at symptom control [1]. Presently, there is a considerable scientific discovery of new anti-cancer agents from natural products. Due to the resistance of cancer cells to antitumor drugs, finding new effective anticancer compounds with minimal side effects has been an extraordinary challenge for many scientists.

Marine organisms are vital and promising resources in cancer research and a number of compounds have undergone clinical trials as antitumor agents. Seaweed is a marine macro alga. They are classified as red algae (Rhodophyta), brown algae (Phaeophyta) or green algae (Chlorophyta), based on the pigmentation and depending on their nutrient and chemical composition. They have been used as foodstuff that has been historically consumed around the globe but is only consumed in appreciable amounts in certain areas of the world today. Certain algae have long been used in traditional Chinese herbal medicine in the treatment of cancer [2]. While chemical analysis would suggest a number of nutritional benefits of seaweed for their richness in polysaccharides and terpenoids, they are considered as promising bioactive molecules with anticancer activity, that seaweeds and their extracts have attracted great interest in the pharmaceutical industry as a source of bioactive compounds. From 1998 to2006, so far 592 marine natural products were investigated for its activity in pharmacology studies [3].It has been long time that human beings have known about marine algae, its rich source of pharmacologically active metabolites with antineoplastic, antimicrobial and antiviral effects [4,5].

From the past few decades, there has been an upsurge in the search for new plant-derived drugs. Marine compounds are however under-represented in the current pharmacology boom. It is anticipated that the marine environment will become a valuable

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source of novel compounds in the near future, as it represents 95% of the biosphere. Recent studies have shown that several bioactive compounds obtained from algae induced tumor cell death by apoptotic mechanism [6-8]. Substances isolated from algae, such as fucoidan, laminaran and terpenoids have activity against cancer cell strains [9,10]. Extensive researches is being carried out on the cellular and molecular basis of the carcinogenesis cascade that provides a targeted approach for cancer chemoprevention, which also aims to halt or reverse the development and progression of precancerous cells through use of non- cytotoxic doses of nutrients and/or pharmacological agents [11,12].

Natural marine products evidencing apoptotic activity has dragged attention as new leads for anticancer alternative and complementary preventive or therapeutic agents [13]. Nowadays, seaweed-related products are used widely, not only as health foods, but also in clinical drugs for the prevention and treatment cancer. The protective effects of dietary component in Asian communities, kelps and other red and green seaweed against mammary [14], skin [15] and intestinal cancer [16] are supported by epidemiological data [17] and rodent model studies [18]. As a need for a more evidence relating anticancer activity, the present study was conducted on HeLa cell lines.

Chemicals and Reagents

HeLa cancer cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India. RPMI 1640 medium (#AL199A, Himedia), Fetal Bovine Serum (#RM10432, Himedia), MTT Reagent (5 mg/ml) (# 4060 Himedia),DMSO (#PHR1309, Sigma), Berberine (# B3251, Sigma), D-PBS (#TL1006, Himedia), 96-well plate for culturing the cells (From Corning,USA),T25 flask (# 12556009, Biolite - Thermo), Paraformaldehyde(PFA) (HiMedia), Acridineorange(Sigma),EDTA, Aceticacid (Merck),

Methodology and Experimental Design

Collection of seaweed sample

Seaweeds were collected from the rocks of Surathkal beach (13 00`34.1" N lat. and 74 47`16.1" E long.), Dakshina Kannada district, Karnataka. Samples were washed with freshwater to remove adhering debris and identified as *Gracilaria* sp. by Dr. C. R. K Reddy, CSIR-Central Salt and Marine Chemicals Research Institute, Baroda, Gujarat. The collected samples were transferred to the laboratory in a polythene bag, shade dried and powdered.

Seaweed extraction

The powdered seaweed was extracted successively using Soxhlet extractor sequentially with different solvents of increasing polarity namely: chloroform, acetone, methanol, ethanol, and water until the extract was clear. The resulting pasty extracts were stored in a refrigerator at 4 C for future use.

Culturing of HeLa Cells

Human cervical cancer (HeLa) cell lines obtained from (NCCS) Pune were maintained in RPMI-1640 supplemented with 10% FBS(full form), penicillin (100 U/ml) in a humidified atmosphere of 50 μg/ml CO_2at 37 C.

Cell viability assay

In vitro assay for cytotoxic activity: Cytotoxicity of the algal ethanolic extracts on HeLa cells were assayed by 3-(4,5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) reduction assay [19].The anticancer activity of the seaweed extracts was observed at 24hours, 48 hours and 72 hours, in which ethanol extracts of *G. corticata* showed a greater activity with an IC_{50} value of 244.7μg/ml at 48hours. However, the drug was found to be inactive at 72hours.

Morphological examinations were conducted by Hoechst 33342 and acridine orange(AO) staining and were performed when the cells reached 70–80% of confluence.

Apoptosis detection assay with Hoechst 33342 (Sigma B-2262)

HeLa cells were stained with Hoechst 33342 to study cellular and nuclear morphology. H33342 binds preferentially to A-T base-pairs in DNA. The dye internalizing in the cells is an active transport process which requires physiologic conditions and does not require permeabilization for labeling. The condition typically varies among various cell types. The procedure for staining and analyzing5x10^6cells/coverslip approximately was seeded in buffered media, pH 7.2. After overnight incubation, the cells were treated with drugs of IC_{50}values and incubated for 48 hours, then fixedin 4%PFA. Homogenously aspirated and spent media was removed and 1 ml of saline was added and centrifuged at 1500 rpm for 10 minutes. Supernatant was discarded and 2μg/ml of Hoechst stain was added to pellet. Cells were then incubated at 37°C for 1 hour. Apoptosis was analyzed under fluorescent microscope after incubation. Washing is not recommended.

Morphological studies

2μg/ml of Hoechst 33342 staining was used to study cellular and nuclear morphology, to stain the nuclei for 15 minutes in dark at room temperature. Acridine orange staining was done to study the formation of acidic vacuoles. After 48 hours of ethanolic extract treatment, cells were additionally incubated in fresh media containing acridine orange (1μg/ml) for 15 minutes without fixation.

After the staining procedures, extra stain was drained out, washed briefly in phosphate buffered saline (PBS)and mounted in PBS containing 10% glycerol, 2% N-propylgallate. Cells were visualized under both bright field and fluorescence for Hoechst(excitation356nm,emission 465nmand excitation 488nm, emission 530 (green) ,650(red) for acridine orange in a fluorescence microscope(Olympus).

Caspase Assay

The protease activity of caspases-3, -8,and -9 in HeLa cells was performed using colorimetric assay kit(GenScript kit,) that is based on spectrophotometric detection of the caspase enzymes after cleavage from the labeled substrate. About 2 10^6HeLa cells were treated with ethanolic extract at IC_{50} and incubated for 48hours, while untreated cells incubated for 48 hours acted as control. Then, the cells were centrifuged for 5 min at 2000 rpm to remove the media. Followed by wash with PBS twice and centrifuged at 2000 rpm for 5minutes. The cell pellets were later lysed by the addition of 50μL cold prepared lysis buffer containing 0.5μL dithiothreitol mixed well, and incubated on ice for exactly 1 hour. The tubes were vortexed. The resulting cell lysate was centrifuged for 1minute at 10000 rpm at 4°C, and the supernatant was collected. Then, 50μL 2x reaction buffer containing 0.5μL dithiothreitol were added to 50μL supernatant containing 200μg protein in each tube, to which 5μL caspase substrate was added, transferred to 96-well plate, wrapped, and incubated at 37°C for 2 hours away from light. At the end of the incubation period, the samples were read at 405 nm in ELISA microplate reader. Data was presented as optical density (OD) (405 nm; mean SD) and graph was plotted. Active caspase cleaves the peptide and releases the chromophore pNA that can be detected spectrophotometrically at optical density 405 nm. Theoretically, the apoptotic cell lysates containing active tested caspases should yield a considerable emission compared with the non-apoptotic cell lysates.

Results and Discussion

The results of the cytotoxicity tested by observing the cellular morphological change showed that ethanolic extracts in dose- and time dependently inhibited the proliferation of the HeLa cancer cell lines. Induction of apoptosis is a useful approach in cancer therapies. Kerr & Wyllie (1972) were the first authors that described morphological features such as morphology of cells and induction of cell death [20]. Several species of algae have been found to be sources of metabolites with anti-tumoral and immune- stimulant activities [21].

Apoptosis is a normal physiologic process that plays a vital role in homeostasis and growth of the normal and cancer cells, dys regulation of apoptosis is usually considered as a major cancer property[22,23].The nuclei with condensed chromatin and apoptotic bodies of various sizes containing well-preserved but compacted cytoplasmic organelles and/or nuclear fragments, which are the typical characteristics of apoptosis [24].Several cellular and molecular, biological features in apoptotic cells, such as cell shrinkage, DNA fragmentations, and activation of the caspase cascade are exhibited. The series of photographs in figures 3A,3B & 3C illustrates some of the possible morphologies seen with inverted microscopy, Hoechst 33342 nuclear staining and AO staining and fluorescence microscopy. Thus, apoptotic characteristics such as cell membrane blebbing, shrinkage of cells /reduction, and separated apoptotic bodies were observed and the results also suggested that there should be active substances interacting with special cancer-associated receptors or cancer cell special molecule, thus triggering some mechanisms that cause death of cancer cell.

Cytotoxicity of marine algal extracts was evaluated by growth inhibition assay (Figure 1).When the growth inhibited cells were stained with AO and Hoechst 33342 apoptotic cell death was observed in time- and dose-dependent manner in all cultures (Figure 2). These results suggested that ethanolic extract caused irreversible cell damages in cultured cells. Intact membranes allow the uptake of AO, which binds to double-stranded DNA and fluorescence green under 488 nm excitation. In apoptotic cells, condensed chromatin material resulted in clumps of intense green fluorescent spots within the cell while untreated cells show a diffuse green fluorescence. The characteristic condensation patterns were observed.

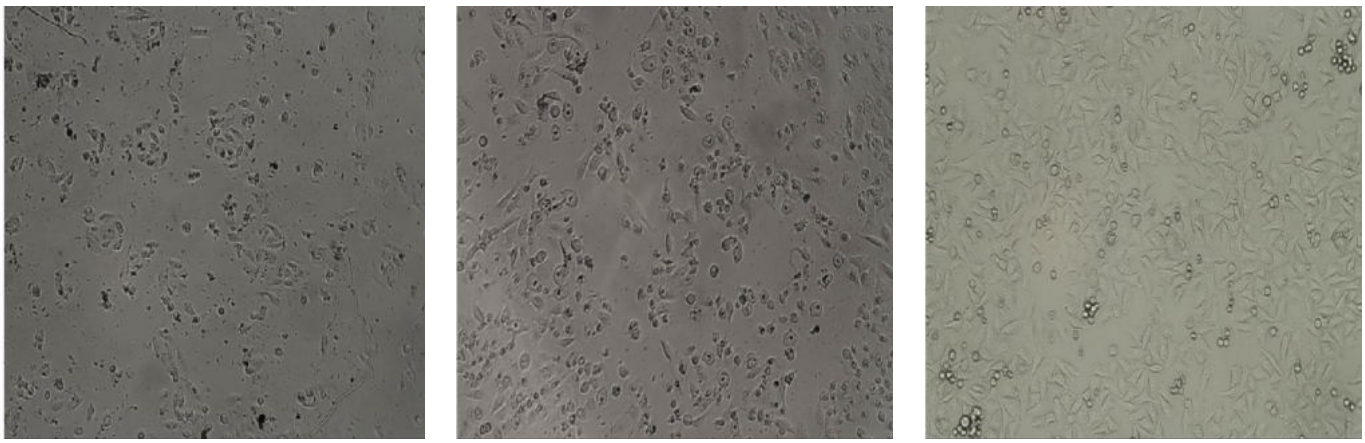
Acridine orange stains both cytoplasm and nucleic acids in uncharged form, which fluoresce bright green, whereas in protonated form, it accumulates in the lysosomal acidic vacuoles, forms aggregates and fluoresce bright red. When studied under a fluorescence microscope, it was observed that in the non-treated and control sets, a minimal red fluorescence was found, whereas, increased red fluorescence were observed in the treated cells. In ethanolic extract treated cells, almost the whole cytoplasm be came red (Figure 3) indicating the merging of all the acidic vacuoles.

The induction of apoptosis essentially needs the activation of caspases. These enzymes are the group of intracellular proteases which are responsible for planning the cell into apoptotic bodies during apoptosis. Caspases are present as inactive proenzymes form, that are activated by proteolytic cleavage. Caspases-8,-9, and -3 are located at key junctions in apoptosis pathways.

As shown in Figure 4, ethanolic extracts increased the activity of caspases-8, -9, and -3 in a time-dependent manner. According to the studies concerning caspase activities, several distinct pathways exist resulting in the induction of apoptosis by seaweed. In this work, we noted that ethanolic extracts activate caspase-3 in a time-response fashion (Figure 4).

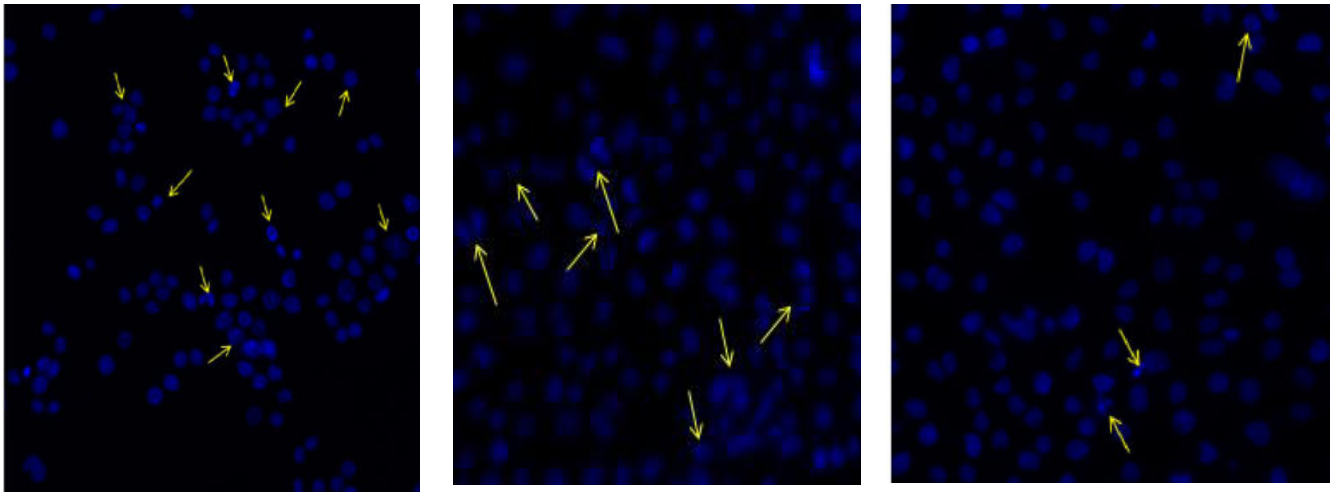
Marine algae contain many unidentified bioactive components that are physiologically active substances. Several researchers have reported the studies on bioactivity of marine algae against cancer cell lines where their findings have brought great promise to the

development of cancer treatment activities [25, 26].These algae can be potent candidates for isolating new lead anticancer molecules. Similar studies using apoptotic parameters revealed apoptotic mechanisms with total methanolic extract of red algae, *Gracilaria tenuistipitata* [27]. Antiproliferative activity of algal extracts in cancer cell lines, may be due to water-soluble polysaccharides, such as laminarans and fucoidans, which are representative anticancer substances extracted from seaweeds [28]. Induction of apoptosis by seaweed via the activation of caspase-3 was reported previously to be mediated through a mitochondrial pathway [29]. Previously the cold water extract of red alga, *Gracilaria corticata*, possessing biological activity against tumor cells replication was reported by Zandi *et al*[30]. However, there is a need for further characterization at both molecular and structural levels.



a b c

Figure:1 Morphological changes of HeLa cells under inverted microscope a) Treated with ethanolic extract b) Berberine c) Untreated



a b c

Figure: 2-HeLa cells grown on sterile coverslips were treated with IC_{50}doses of the ethanolic extracts for 48 hours. Then, they were stained with Hoechst 33342 to study the nuclear morphology under fluorescence microscope. a) 5-Flurouracil b) Ethanolic extract c) Untreated. (arrows show nuclei with deformity)

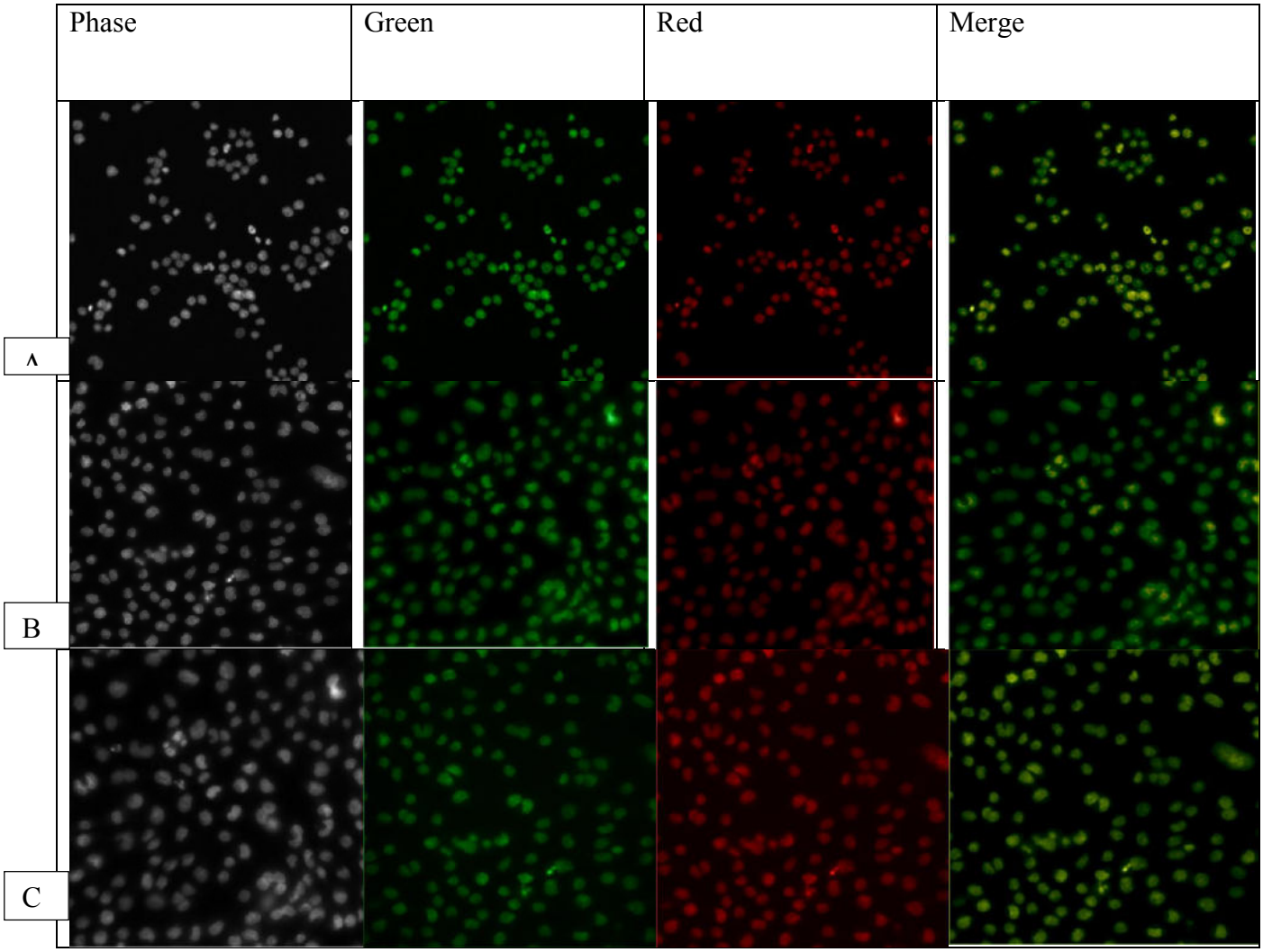


Figure:3 HeLa cells grown on sterile coverslips were treated with IC_{50} doses of ethanolic extracts for 48 hours. Then they were stained with acridine orange(1µg/ml) to study the formation of acidic vacuoles.

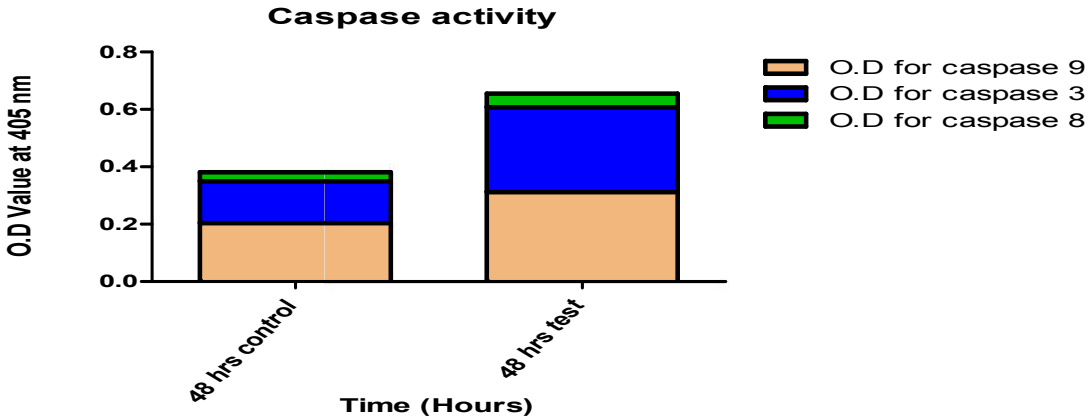


Figure: 4 Activity of caspases-3, -8, and -9.The total cell lysates from He La cells treated with ethanolic extracts for 48 hours was analyzed using caspase colorimetric protease assay kits

Conclusion

Our *in vitro* study demonstrated that ethanolic extracts of *Gracilaria corticata* (J. Agardh) J. Agardhhas cytotoxic activity on HeLa human cervical cancer cell line. Its ability to induce apoptosis was also understood with increased caspase activity on human cervical cancer cell line. Thus, this can emerge as a novel candidate as a potential natural anti-cancer agent. However, further molecular and structural level characterization has to be studied.

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