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Induction of apoptosis in Hela cells by ethanolic extract of *Skeletonema costatum*

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ABSTRACT

Marine organisms are rich sources of new, biologically active compounds. Phytoplanktons have traditionally been used as foods. Algal powder as a food supplement for prevention or therapy of bacterial fish disease. Recently, much attention has been paid to the anticancer activity of Phytoplankton. Thus, we have screened organic extracts of Phytoplankton for their anticancer activity against human cancer cell line, and selected *Skeletonema costatum* as a candidate for use in treatment. The ethanolic extracts of *Skeletonema costatum* (EESC) showed cytotoxic activity against human cervical adenocarcinoma cell line, (HeLa). The IC_{50} of EESC against the HeLa cells was 250 µg/ml. Treatment of HeLa cells with various concentrations of EESC resulted in growth inhibition and induction of apoptosis in a dose-dependent manner. These results strongly suggest that EESC may be a potential candidate in the field of anticancer drug discovery.

Introduction

Due to their tremendous biodiversity, marine organisms are attractive sources of novel, biologically-active compounds. Over the past two decades, about 3000 new compounds have been discovered and isolated from various marine organisms; some of these compounds have been employed in clinical therapies [1]. Diatoms are among the most important photoautotrophic organisms, driving food web dynamics in some of the most productive marine systems, particularly in areas of nutrient upwelling. They are found in marine and freshwater ecosystems as well as brackish water [2]. They are economically important in industry while also having a major role in biological and chemical processes. Phytoplankton species exhibit antibacterial, antifungal, anticancer and anti inflammatory properties and some forms are used as a source of antibiotics, food additives and cosmetic production. Some of the micro algal strains are recognized as an excellent source of proteins, carbohydrates, lipids and vitamins, which are used as food and feed additives [3]. The brown algae in particular, the majority of the compounds described are diterpenoids. Some of them are known for their anti tumor effects (e.g.: Cyclic diterpenes from Dictyotaceae species) [4, 5]. Thus, we have surveyed organic extracts of phytoplankton for anticancer activity against human cell lines; we selected *Skeletonema costatum* for anticancer drugs. *Skeletonema costatum* was originally described by Greville (1866) [6] as *Melosira costata*, based on light microscopy (LM) of material from Hong Kong Bay[7]. The diatom is commonly found in almost all sort of seawater.

Induction of apoptosis is a useful approach in cancer therapies. Apoptosis, a major process of programmed cell death, plays an important role in the regulation of tissue Development and homeostasis [8, 9, 10]. Cancer is a disease state caused by the disruption of cellular homeostasis between cell death and cell proliferation [11]. As compounds which can induce apoptosis are considered to have potential as antitumour agents [12], many efforts have been made to discover new drugs through the isolation of apoptosis inducing agents from natural products.

In this work, we evaluated the anticancer activity of the crude ethanolic extracts of *Skeletonema costatum* against human cervical adenocarcinoma cell line, (HeLa line) via induction of apoptosis.

Materials and Methods

Skeletonema costatum collected from the coastline of parangipettai, Cuddalore district , Tamil Nadu, South East Coast of India. About 50 g of powdered *Skeletonema costatum* was taken in a round bottom flask and add ethanol and macerated for 7 days. During maceration the whole content was warmed 2 times a day at an interval of six hours. At the end of 7th day the extract was filtered through muslin cloth while hot and the extract was concentrated to a semisolid mass and dried in a desiccators. This extract was used for various experimental purposes.

Cell line and cell culture

Human cervical adenocarcinoma cell line, HeLa, used in this work was purchased from the National Centre for Cell Science (NCCS), Pune, India. HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 0.1% gentamycin, in a humidified incubator containing 5% CO_2 at 37^0 C. When necessary, 0.5% DMSO, containing an appropriate concentration of EESC was added.

Cytotoxicity and antiproliferative activity

Cytotoxicity was determined by the MTT assay, as previously described [13]. In brief, cells were plated in 96-well plates at an initial density of 1x 10^4 cells per well. After incubation for 24 h at 37^0 C, cells were treated with various concentrations of EESC and incubated for 24 h. MTT solution was added to each well and further incubated for 4 h at 37^0 C. The optical density was read with an ELISA reader at 550 nm.

Antiproliferative activity was determined by a trypan blue exclusion assay [14]. For the trypan blue exclusion assay, cells

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were cultured in a 35 mm dish and exposed to various concentrations of EESC for 7 days. The cells were trypsinised, washed with phosphate buffered saline (PBS), and trypan blue dye solution was then added to the cell suspension. Viable cells were counted with a haemocytometer.

DAPI staining

Cells were washed with PBS and fixed with 3.7% Para formaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 4, 6- diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The cells were washed twice with PBS and analyzed under a fluorescence microscope.

TUNEL assay

TUNEL assay was also used for determination of apoptosis through the detection of DNA fragmentation. TUNEL assay was performed using the Deadend^{TM} Colorimetric Apoptosis Detection System (Promega KK,Tokyo, Japan). In brief, cells were plated on poly-L-lysine-coated slides. The cells were then air-dried in a tissue culture hood for 1 h. After two washes with PBS, the cells were fixed with 4% (w/v) paraformaldehyde in PBS in a Coplin jar for 25 min at room temperature and rinsed twice with PBS. Then, the cells were immersed in 0.2% (v/v) Triton X-100 solution for 5 min and rinsed with PBS. Control and positive control cells were treated with DMSO and DNase I, respectively. The cells were equilibrated with equilibration buffer at room temperature for 5 min. TdT (Terminal deoxynucleotidyl transferase) enzyme reaction mixture and biotinylated nucleotide mixture were subsequently added to the cells, and the cells were then covered With cover slips and incubated for 1 h at 37^0C. The reaction was terminated by immersion of the slides in 2 x SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 15 min. The slides were washed with PBS and treated with 0.3% (v/v) hydrogen peroxide for 5 min. The slides were then incubated with streptavidin HRP solution in PBS for 30 min, rinsed with PBS, and finally incubated with DAB components until a light brown background developed. The stained cells were immediately examined under a light microscope.

Results and Discussion

Cytotoxic activity of EESC on HeLa cells

The cytotoxic effect of EESC on HeLa cells was determined. Cells were exposed to various concentrations of EESC (0–500µg/ml) for 24 h. Cells treated with 0.1% DMSO were used as control. The IC_{50} value of EESC on HeLa cells was 250µg/ml after treatment for 24 h. To investigate the effect of EESC on growth inhibition of HeLa cells, cells were treated with EESC (50, 100, 150 or 200µg/ml) for 7 days and counted at 2 day intervals by the Trypan blue exclusion method. Compared to control cells, cells treated with EESC were significantly inhibited by 33.1 ± 4.5% at 50µg/ml EESC concentration for 1day of incubation. When the EESC concentration was increased to 50, 100, 150 and 200µg/ml, the inhibitory rate was increased to 39.2 ± 3.9%, 79.8 ± 4.5%, 94.4 ± 2.0% and 98.5 ± 1.8%, respectively, after 7 days incubation. These dose and time-dependent effects of EESC on growth inhibition of HeLa cells are shown in Fig.1.

Induction of apoptosis by EESC on HeLa cell

In order to determine whether the growth inhibitory effect of EESC was due to apoptosis, HeLa cells were treated with EESC for 48 h, and nuclear DAPI staining was performed. As shown in Fig.2, nuclei with condensed chromatin and apoptotic bodies, typical characteristics of apoptosis, were observed in HeLa cells incubated with EESC, and the number of apoptotic cells increased as the EESC concentration increased (data not shown). Because DNA fragmentation is another characteristic of apoptosis [15], DNA fragmentation assay was also carried out by a TUNEL assay using the Deadend^{TM} Colorimetric TUNEL System (Promega). As shown in Fig. 3, the nuclei of cells treated with EESC were stained dark brown, but no stained nucleus was observed in control cells.

Recently, much attention has been paid to marine organisms for the screening of biologically active compounds [1]. Apoptosis is essential for normal growth and development of multicellular organisms, including metazoans and higher plants. Many morphological criteria of apoptotic cell death were met, including an increase in chromatin margination, degradation of the nucleus, and DNA fragmentation. Biochemical assays of the activities of cell death-associated proteases, caspases, measured using highly specific fluorogenic substrates, increased with time in darkness and paralleled the morphological changes. Antibodies raised against mammalian caspases cross-reacted with specific proteins in the alga. The pattern of expression of these immunologic ally reactive proteins was correlated with the onset of cell death. The occurrence of key components of apoptosis, and particularly a caspase-mediated cell death cascade in a relatively ancient linage of eukaryotic photoautotroph, argues against current theories that cell death evolved in multicellular organisms [16]. The harmful effects of PUAs have been demonstrated at the organism level as inducers of apoptosis in sea urchin embryos [17].We have observed *Skeletonema costatum* pretreatment significantly increased apoptotic morphological changes in HeLa cells. Apoptosis is essential for normal growth and development of multicellular organisms, including metazoans and higher plants. Although cell death processes have been reported in unicellular organisms, key elements of apoptotic pathways have not been identified [16].

In this work, we found that the crude ethanolic extracts of *S. costatum* (EESC) had antiproliferative activity on human cervical adenocarcinoma cell line. In apoptotic cells, several cellular and molecular biological features, such as cell shrinkage, DNA fragmentations, and activation of the caspase cascade, are exhibited [18]. In our results, we also observed cell shrinkage and DNA fragmentation, as determined by DAPI staining and TUNEL assay, respectively. Activation of the caspase cascade is a wellknown molecular mechanism for the induction of apoptosis. In the early stage of activation of the caspase cascade, inactive initiator caspases, such as procaspase-8 and -9, are turned to active caspase-8 and -9 by self-processing. Activated initiator caspases then cleave inactive executioner procaspase-3 into active caspase-3, which acts downstream in the cleavage of specific intracellular substrates, such as poly (ADP-ribose) polymerase (PARP). Mitochondria also play an important role in the induction of apoptosis. The release of cytochrome C from the mitochondria into the cytosol is a characteristic of a mitochondria-dependent pathway [19]. We reported here that EESC can activate caspase-8 and -3, cleave PARP, and mediate the release of cytochrome C. These results suggest that EESC -induced apoptosis may be mediated via a mitochondria-dependent pathway.

The microscopical observation showed a typical morphology of apoptosis i.e., cell pyknosis, chromosomal condensation and nuclear fragmentation in *Skeletonema costatum* treated cells. Apoptosis has been shown to play an important role in determining cellular cytotoxicity [20].

Apoptosis has been shown to be a significant mode of cell death after cytotoxic drug treatment [21]. Apoptosis, also called programmed cell death, is the mechanism complementary to proliferation. When cells undergo apoptosis, several classical phenomena can occur, including membrane blebbing, condensation of cytoplasm and the activation of end nuclease/ specific proteases. Later on, the genome will be fractured, and smaller apoptotic bodies will be formed [22]. Macrophages will phagocytose the apoptotic cell. Through the process of apoptosis, unwanted or dysfunctional cells can be eliminated. Two major apoptosis pathways have been demonstrated. One is the mitochondrial/cytochrome C (intrinsic) pathway, which activates Caspase-9 and Caspase-3. The second pathway is extrinsic pathway, in which extrinsic signals activate Caspase-8 and Caspase-3. Caspase-3 is activated in most of the process of apoptosis [23]. Caspase-3 is the most important protein in the process of apoptosis. Caspase-3 will finally induce the apoptotic morphologies, including DNA fragmentation and cell shrinkage [24]. The increased ROS levels and subsequent oxidative DNA damage might be the reason for increased apoptotic morphological changes in the *Skeletonema costatum* treated cells. ROS is implicated in disruption of mitochondrial membrane potential and release of cytochrome c in the cytosol which after binding with Apaf-1 formed apoptosome complex. This complex initiated the caspase cascade by activation of caspase 9. ROS generation is also involved in p53 and in Fas-induced apoptosis [25, 26]. Moreover tumor suppressor protein p53 and p38, MAPK play a prominent role in the CAPE induced apoptotic cell death which might contribute to the antitumor effects of CAPE in c6 glioma cells [27].

Conclusion

In conclusion, this study showed that EESC could inhibit the growth of cancer cells and could induce apoptosis via a mitochondria-dependent pathway in human cervical adenocarcinoma HeLa cells. According to these results, it is suggested that the ethanolic extracts of *Skeletonema Costatum* are valuable for the development of anticancer drugs.

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Cytotoxic activity

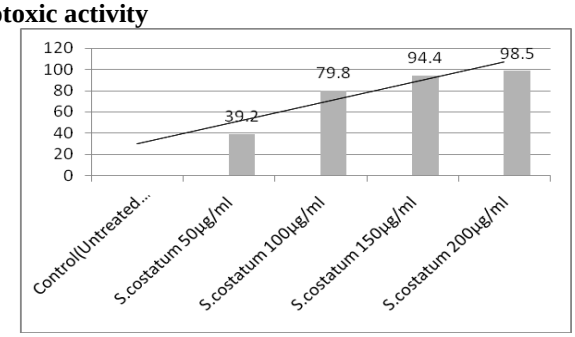
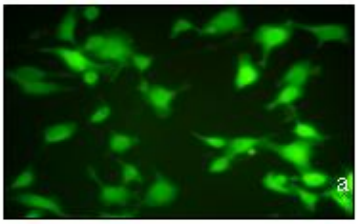
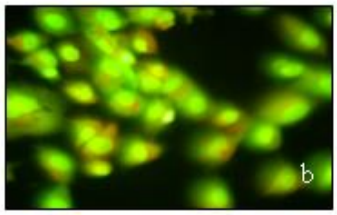


Fig. 1. The growth inhibition effects of EESC on HeLa cells. Cells were treated with EESC ranging in dose from 0µg/ml to200 µg/ml for 7 days. The growth inhibition effects were determined by Trypan blue exclusion assay. Cells treated with 0.1% DMSO were used as control. All experiments were conducted in triplicate, and the values and bars represent mean and SD, respectively.

Induction of apoptosis by EESC on HeLa cell



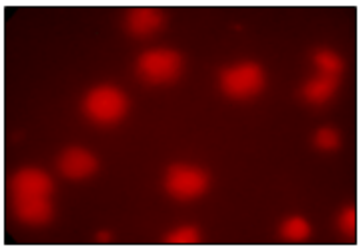
(a) Control



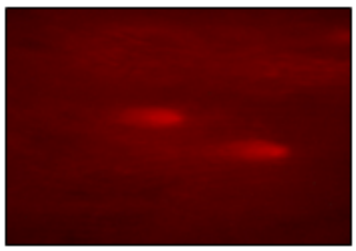
(b) *S. costatum* (150 µg/ml)

Fig. 2. Morphological changes of HeLa cells after EESC treatment for 48 h followed by DAPI staining. (a) Fluorescence microscope photographs of control cells treated with 0.1% DMSO and (b) cells treated with 150 µg/ml EESC.

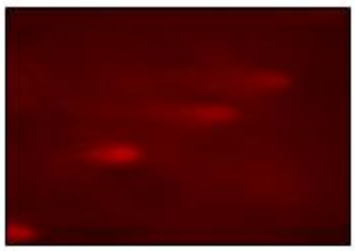
TUNEL assay



(a) Negative Control



(b) *S.costatum* (150 µg/ml)



(c) positive control after Treated with EESC

Fig.3.TUNEL assay of HeLa cells after treatment with 0.1% DMSO (a – negative control), EESC (b – 150 µg/ml), or DNase I (c – positive control) for 48 h. Dark brown staining of the nuclei of the HeLa cells was observed after cells were treated with EESC and DNase I, whereas no staining of the nuclei of DMSO-treated control cells was detected. a, b, c: Magnification x100.

EESC = Ethanolic Extracts of *Skeletonema costatum*

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