Antiproliferative and apoptotic activity of sulfated polysaccharide isolated from Hypnea valentiae red seaweed in human skin malignant melanoma cells

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ABSTRACT

Objective: Malignant melanoma is a highly metastatic cutaneous cancer. Deregulated apoptosis has been identified as a major cause of cancer drug resistance. The objective of the study is to evaluate antiproliferative activity of Hypnea valentiae extract in human skin malignant melanoma (SK-MEL) cells.

Methods: In this study, sulfated polysaccharide fraction was precipitated from aqueous extract obtained from H. valentiae. MTT assay was used to determine the cell viability of the crude sulfated polysaccharide against SK-MEL cells and normal L6 cell line (Rat skeletal muscle). Acridine orange (AO) and Ethidium bromide (EB) staining method was applied to study induction of apoptosis in SK-MEL cells.

Results: Dose-dependent reduction in cell viability was observed with an IC50 of 30 μg/ml in SK-MEL cancer cells. The sulfated polysaccharide treated SK-MEL cells followed by AO, EB staining, showed typical early apoptotic, and late apoptotic morphological changes.

Conclusion: The isolated crude sulfated polysaccharide from H. valentiae produced potent growth inhibition and induction of apoptosis in SK-MEL cells but caused no cytotoxicity in normal L6 skeletal muscle cells.

Keywords: Antiproliferative, Cell viability, Cytotoxicity, Human skin malignant melanoma cells, L6 (Rat skeletal muscle cells).

INTRODUCTION

Cancer is one of the leading causes for death throughout the world and the current treatment with chemotherapeutic drugs has a major disadvantage which causes various toxic side effects [1,7,10]. Malignant melanoma is aggressive with poor survival rate and is known to have the highest mutational load of all cancers. Several small molecule inhibitors, including vemurafenib and dabrafenib, are successful but long-term treatment has shown that the tumors often become resistant to such inhibitors. Surgery, immunotherapy (α-IFN or interleukin-2) is the limited treatment available for use [3,6]. Seaweed, a macroalgae seen in various forms, color and occurs along the coastline line with its biochemical components such as carbohydrates, proteins, vitamins, fat, and minerals [6,8,9]. Various bioactive compounds are isolated from seaweeds with antioxidant, antiviral, anti-inflammatory, and anticancer activity [2,5,25,26]. Recent studies have shown that they possess health promoting nutrients and phytochemicals that are good in antioxidant activities and cholesterol reducing effects [15]. Furthermore, seaweeds are highly potential in secondary metabolites and sources of dietary fiber that differ chemically and physiochemically, and therefore, they perform different physiological effects on humans [4,23]. These metabolites will be useful in the development of new pharmaceutical agents [23]. Thus, the secondary metabolites have potential medicinal applications including antibacterial, antiviral, antitumor, and antifungal activities [16,26]. Further the bioactivities of seaweed extracted from red seaweed had opened opportunities in various fields such as pharmaceuticals, cosmeceuticals, nutraceuticals, and functional foods [12,14,17]. The present study was performed to examine the effects of sulfated crude polysaccharide on tumor growth inhibition and induction of apoptosis in human skin malignant melanoma cells (SK-MEL), and to determine the cytotoxicity effects in SK-MEL cells in vivo.

METHODS

Algal collection and sample preparation
The red seaweed (Hypnea valentiae) from Cuddalore coast Tamil Nadu, India. The collected sample was dried for a week until no moisture content was left behind [21,24]. The dried sample was completely homogenized (Fig. 1).

Extraction of polysaccharide from seaweed
Three extracts 10-g powdered samples were dissolved in 500 ml of distilled water. The boiled extracts of the batches were then cooled down at room temperature and filtered completely (countryman and Whatman filter paper). The pH of the filtrates was adjusted to 7 to increase the yield of extraction. About 4% trichloroacetic acid (TCA) was added to the extract and incubated overnight at 4°C. TCA precipitates the proteins in the extract hence followed by the overnight incubation, the precipitated proteins were removed through centrifugation and the resulting supernatant was collected. To the supernatant of the extract, 1% calcium chloride was added and incubated overnight at 4°C. This calcium chloride precipitates the alginic acid present in the extracts and hence they are removed through centrifugation. Followed by this, thrice the volume of absolute ethanol (100%) was added to the supernatants of the extract and incubated overnight at 4°C. The precipitated polysaccharides collected through centrifugation. The pellet was completely air dried in the hot air oven [11].

Characterization of seaweed extract by Fourier transform infrared (FTIR) spectroscopy
The nano spray dried seaweed extract was analyzed using Fourier transform infrared spectroscopy (Shimadzu, Japan) with wavelengths in the range of 4000-400 cm\(^{-1}\). The resulting spectra directly correspond to the functional groups present in the structures of given sample [12,13] (Fig. 2).
Cytotoxicity screening by MTT assay

**Cell lines and maintenance**

L6 cell line (Rat skeletal muscle) was procured from National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM-HiMedia), supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic cocktail containing Penicillin (100 U/ml), Streptomycin (100 µg/ml), and Amphotericin B (2.5 µg/ml). The cell containing TC flasks (25cm²) were incubated at 37°C at 5% CO₂ environment with humidity in a cell culture incubator (Galaxy® 170 Eppendorf, Germany). The viability of test sample treated cells was evaluated by direct observation of cells by Inverted phase contrast microscope and further quantified by MTT assay method [27].

**SK-MEL Cell lines and maintenance**

SK-MEL cell line (Human Skin cancer) was procured from NCCS, Pune, India. The cells were cultured in DMEM-HiMedia, supplemented with 10% heat inactivated FBS and 1% antibiotic cocktail containing Penicillin (100 U/ml), Streptomycin (100 µg/ml), and Amphotericin B (2.5 µg/ml). The cells containing TC flasks (25cm²) were incubated at 37°C at 5% CO₂ environment with humidity in a cell culture incubator (Galaxy® 170 Eppendorf, Germany). The viability of test sample treated cells was evaluated by direct observation of cells by Inverted phase contrast microscope. The viability of the treated cells was further quantified by MTT assay method [27].

**In vitro MTT cytotoxicity assay**

The monolayer of cells grown in TC flask was exposed to Trypsin/EDTA solution (0.025% trypsin and 0.01% EDTA in Phosphate Buffered Saline [PBS]). The trypsinized cells were diluted in the cell culture media at a concentration of 5 x 10⁵ cells/well (in 100 µl). The 96 well plates were seeded with cells and incubated for 3–4 days at the cell culture incubator. The test samples were prepared in DMEM media (100 mg/ml) and filtered using 0.2 µm Millipore syringe filters. The samples were further diluted in DMEM media and seeded to the wells containing cultured cells at final concentrations of 6.25 µg, 12.5 µg, 25 µg, 50 µg, and 100 µg, respectively. Untreated wells were kept as control. All the experiments were done in triplicate and average values were taken to minimize errors. After treatment with the test samples, the plates were further incubated for 24 h [27].

**Microscopic cytotoxicity observation**

After sample addition, the treated as well as the control wells were observed at regular intervals up to 24 h in an inverted phase contrast tissue culture microscope (Labomed TCM-400 with MICA PATM HD camera) and the observations were photographed. Any detectable changes in the morphology of the cells, such as rounding, shrinking of cells, granulation, and vacuolation in the cytoplasm were considered as indicators of cytotoxicity [19,27].

**Determination of apoptosis by acridine orange (AO) and ethidium bromide (EB) double staining**

DNA-binding dyes AO and EB (Sigma, USA) were used for the detection of apoptotic and necrotic cells [Zhang et al, 1998]. AO is taken up by both viable and non-viable cells, which emit green fluorescence when they intercalate into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells, which emits red fluorescence by intercalation into DNA. After treatment with different concentrations of the cells were washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10 min. The stained cells were washed twice with 1× PBS and observed by a fluorescence microscope in a fluorescent microscope (Olympus CKX41 with Optika Pr05 camera) [18]. Thus, the cells were divided into four categories as living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation), and necrotic cells (uniformly orange stained cell nuclei) [22].

**RESULTS AND DISCUSSION**

**Extraction of crude sulfated polysaccharide from seaweed**

The seaweed was dried and then powdered and the aqueous extraction and polysaccharide precipitation. The obtained crude extract was spray dried for three batches.

**Characterization of crude sulfated polysaccharide by FTIR spectroscopy**

The dried sample was analyzed using ATR Fourier transform infrared spectroscopy (Shimadzu, Japan) with wavelengths in the range of 4000–4000 cm⁻¹. Similarly, the band at 2974 cm⁻¹ of extract corresponds to the C-H stretching. Further, the peak at 1318 cm⁻¹ of extract corresponds to the C-O-C stretching of glycosidic bonds. The band at 926 cm⁻¹ of extract corresponds to the C-O stretching signifying the presence of aromatic esters. The peak at 906 cm⁻¹ of extract signifies the presence of bending vibration of C-O-S stretching. Hence, from the spectra, it was characterized that the above functional groups are present in the extract.

| Functional Group | Peak Value cm⁻¹ | Presence/Interpretation |
|------------------|-----------------|-------------------------|
| CH Stretching    | 2974            | CH Stretching of sugars  |
| C=O             | 1629            | Carboxylic group         |
| S=O             | 1392,1041       | Sulfate groups connected to sugars |
| C-O-C           | 1138            | Stretching of glycosidic bond |
| C-O-S           | 906             | Bending vibrations of C-O-S |

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Effect of crude sulfated polysaccharide on cell viability

**Effect of sulfated polysaccharide on L6 skeletal muscle cell viability**

The cytotoxicity was absent in L6 rat skeletal muscle cells subjected to the administration of different concentrations of the extract (Fig. 3).

**Effect of sulfated polysaccharide on SK-MEL 28 cell viability**

The dose-dependent reduction in cell viability was observed in SK-MEL cancer cells with the administration of different concentrations of the extract with an IC$_{50}$ of 32.66 µg/ml (Fig. 4).

Microscopic cytotoxicity observation

No cytotoxicity was observed in L6 skeletal muscle cells and cells did not show any morphological changes on treatment with crude sulfated polysaccharide (Fig. 5). Cytotoxicity and morphological changes observed in SK-MEL cells on treatment with sulfated polysaccharide on phase contrast microscopic observation (Fig. 6).

Determination of apoptosis by AO and EB double staining

AO is a vital dye which stains both live and dead cells. EB will stain only the cells that lost membrane integrity, that is, EB will permeate only cells which have lost membrane integrity. Live cells will appear uniformly green. Early apoptotic cells will stain green where the nucleus contains bright green dots because of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate EB which stains orange but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin. The cells were divided into four categories as follows: Living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation), and necrotic cells (uniformly orange-stained cell nuclei) (Fig. 7).
CONCLUSION
The polysaccharide fraction was extracted from *H. valentiae*. The crude extract was characterized using FTIR. The spectra obtained show the characteristic peaks of sulfated polysaccharide along with additional peaks signifying the presence of other monosaccharides. The anti-proliferative activity of extract was identified using MTT cytotoxicity assay in SK-MEL cells. AO and EB staining confirmed apoptosis in SK-MEL cells after treatment with extract. From the cytotoxicity assay, it is confirmed that the extract is not cytotoxic and does not affect the cell viability of L6 skeletal muscle cell. AO and EB staining confirmed apoptosis in SK-MEL cells after treatment with extract. The AO/EB staining procedure the normal live cells take up the green fluorescence and the IC50 dose shows early and late apoptosis. The crude extract must be purified and characterized to determine the structure of bioactive constituent. Further studies on cell cycle arrest with flow cytometry must be performed. We would like to conclude the crude sulfated polysaccharide from *H. valentiae* shows antiproliferative potential against SK-MEL cells.

AUTHOR CONTRIBUTION
Negha Rajendran carried out experiment of this research like extraction and FTIR and contributed to manuscript draft preparation and Ramya Ravichandran carried out experiments in L6 and SK-MEL MTT mammalian cell-based assays and Veeramuthu Sheneshwari Veerikethy participated in Fluorescent AO and EB ideation and conceptualization, methodology planning, research supervision and data analysis of results and manuscript preparation.

CONFLICTS OF INTEREST
We declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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