CHAPTER VIII

Anticancerous activity of the sulphated polysaccharide

8.1 INTRODUCTION

A genetic basis for human carcinogenesis has been established through biochemical and molecular analysis of the disease. Many different types of human cancer have been caused by occupational exposure while other has been attributed to environmental exposure to chemical and/or viral agents. Cancer has been the focus of a massive research effort for decades, and the treatments like chemotherapy and radiation lack the specificity needed to kill cancer cells without simultaneously damaging normal cells, as evidenced by the side effects accompanied by these treatments. In chemotherapy, drugs like cisplatin, carboplatin, cyclophosphamide, doxorubicin, melphalan, mictomycin, gemcitabine etc have been used for the treatment of cancer. (Black and Livingston, 1992) Strategies for cancer control rely on knowledge of incidence and mortality rate for individual tumours as well as information on their specific risk factors.

Many anticarcinogens were immunosuppressive agents. They repress tumour growth; meanwhile, they are adverse to immune system of organism. It has become an important aim of research in immunopharmacology and oncotherapy to discover and identify new anti-tumour drugs which can potentialize the immune function. Algal polysaccharides are the main source of native sulphated polysaccharides which is shown to have effective anti-
tumour activities by attacking the cancer cell directly or enhancing the host's immune function.

There are more than 200 kinds of anti-tumour polysaccharides extracted from the algae *Lentinus edodes, Ganoderma lucidum, Grifola frondosa, Agaricus baize, Dictyophora indusiata,* etc. Low-molecular weight fucoidan isolated from *Ascophyllum nodosum* shows an anti-proliferative effect on both normal and malignant cells, including fibroblasts (Hamster Kidney Fibroblast CCL39), sigmoid colon adenocarcinoma cells (COLO320 DM), and smooth muscle cells (Vischer and Buddecke, 1991). Fucoidans exhibit anti-tumour, anticancer, antimetastatic, and fibrinolytic properties in mice (Coombe *et al.*, 1987). A red algal polysaccharide designated as \( \lambda \)-carrageenan can inhibit tumour growth via the activation of natural killer (NK) cells and promote lymphocyte proliferation. The funoran polysaccharide from *Gloiopeltis tenax* has been shown the ability of increasing spleen weight and augmenting T-helper, T-cytotoxic and NK cells on tumour-bearing mice. Aisa *et al.*, (2005) reported that fucoidan from *F. Vesiculosus* inhibited the proliferation and induced apoptosis in human lymphoma HS-Sultan cell lines. They reported the fucoidan-induced apoptosis through a mitochondrial pathway as the mitochondrial potential in HS-Sultan cells was decreased 24 h after treatment with fucoidan. Alekseyenko *et al.*, (2007) studied the anti-tumour and anti-metastatic activities of fucoidan isolated from *Fucus evanescens*. Kim *et al.*, (2006) investigated the anti-apoptotic activity of laminaran polysaccharides isolated from the *Laminaria japonica*. The authors carried out a detailed pharmacological investigation on the laminaran polysaccharides and reported that it suppressed mouse thymocyte apoptosis and at the same time significantly induced the up regulation of 33 immunomodulatory genes from
a total of 7410 genes which were examined using a cDNA microarray. Alginates from brown seaweeds have also been reported to possess anti-tumour activity. de Sousa et al., (2007a); de Souza et al., (2007b) investigated the in vivo anti-tumour activity of two alginates (Sargassum vulgare high viscosity and S. vulgare low viscosity).

Present chapter deals with the study of anti-tumour activity of the polysaccharide isolated from Ulva fasciata. Studies in mice cell lines, human cell lines and cell cycle analysis are included in the chapter.

8.2 RESULTS

8.2.1 Cytotoxicity screening by trypan blue exclusion method

The result for trypan blue exclusion method for DLA cell lines is shown in figure VIII-1. Polysaccharide showed marked cytotoxicity for DLA cell lines. The concentration of the polysaccharide required for 50% cell death IC$_{50}$ was found to be 220 µg.

Figure VIII-1: Effect of polysaccharide on Dalton’s Lymphoma Ascites (DLA)

Data are mean ± SD of three values
The result for trypan blue exclusion method for EAC cell lines is shown in figure VIII-2. Polysaccharide showed marked cytotoxicity for EAC cell lines. The concentration of the polysaccharide required for 50% cell death IC$_{50}$ was found to be 120 µg.

**Figure VIII- 2: Effect of polysaccharide on Ehrlich’s Ascites Carcinoma (EAC)**

![Bar chart showing effect of polysaccharide concentration on EAC cell death](image)

Data are mean ± SD of three values

**8.2.2 In vivo anti-tumour activity**

The anti-tumour activity of polysaccharide was studied and the results are given in figure VIII-3. The polysaccharide showed significant anti-tumour activity against solid tumour induced by DLA cell lines. The polysaccharide when administered at concentrations 20 mg, 50 mg and 100 mg/kg bodyweight reduced the tumour volume significantly when compared to control.
Figure VIII- 3: Effect of polysaccharide on average solid tumour volume in DLA induced mice.

Percentage reduction in tumour volume for each tested concentrations were calculated and the results are shown in figure VIII-4.

Figure VIII- 4: Effect of polysaccharide on percentage reduction in solid tumour volume in DLA induced mice.
8.2.3 Effect of Polysaccharide on average life span of ascites tumour bearing mice:

Anti-tumour activity of polysaccharide for EAC cell line was studied and the results are given in figure VIII-5. The polysaccharide showed significant anti-tumour activity against tumour induced by EAC cell lines. The polysaccharide when administered at concentrations 20 mg, 50 mg and 100 mg/Kg bodyweight after implantation of tumour cell increases the life span of the treated groups significantly when compared to control.

Figure VIII-5: Effect of polysaccharide on EAC bearing mice

8.2.4 Cytotoxicity screening by MTT assay:

8.2.4.1 OVCAR Cell line

The result of MTT assay for OVCAR cell line is shown in Figure VIII-6. There was a decrease in cell viability in a dose dependent manner. Fifty
percentage cell death was observed for the drug concentration 355 µg for 48 hours of incubation.

**Figure VIII-6: Effect of various concentrations of polysaccharide on percentage viability of OVCAR cell lines**

Data are mean ± SD of three values

8.2.4.2 PC3 Cell line

The result of MTT assay for PC3 Cell line is shown in Figure VIII-7. The result did not support the decrease in cell viability in a dose dependent manner. So the fifty percentage cell death could not be determined.

**Figure VIII-7: Effect of various concentrations of polysaccharide on percentage viability of PC3 cell line**

Data are mean ± SD of three values
8.2.4.3 HepG2 Cell line

The result of MTT assay for HepG2 Cell line is shown in Figure VIII-8. The result did not support the decrease in cell viability in a dose dependent manner. So the fifty percentage cell death could not be determined.

Figure VIII-8: Effect of various concentrations of polysaccharide on percentage viability of HepG2 cell line

[Graph showing the effect of various concentrations of polysaccharide on percentage viability of HepG2 cell line.]

Data are mean ± SD of three values

8.2.5 Determination of nuclear morphology

Nuclear morphological studies are shown in Plate VIII-1 (a, b, c, d, e). The changes in chromatin organization after treatment with various concentrations of polysaccharide were determined microscopically by assessing staining with Hoechst 33258. Cells were examined by fluorescence microscopy (360/40 nm excitation and 460/50 nm emission filters). The apoptotic cells were identified by the presence of highly condensed chromatin or fragmented nuclei.
Plate VIII-1: Hoechst staining using OVCAR cells

A- Untreated
B- Treated (50 µg/mL), C- Treated (100 µg/mL).
D- Treated (200 µg/mL), E- Treated (400 µg/mL).
Percentages of apoptotic cells induced by polysaccharide on OVCAR cells were calculated and the results are given in Table VIII-1.

**Table VIII-1: Percentage of apoptotic cells induced by polysaccharide on OVCAR cell lines by Hoechst staining**

<table>
<thead>
<tr>
<th>Polysaccharide concentration (µg/mL)</th>
<th>% of apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>18 ± 0.23</td>
</tr>
<tr>
<td>100</td>
<td>33 ± 0.45</td>
</tr>
<tr>
<td>200</td>
<td>47 ± 0.34</td>
</tr>
<tr>
<td>400</td>
<td>69 ± 0.27</td>
</tr>
</tbody>
</table>

Data are mean ± SD of three values

**8.2.6 Cell cycle analysis:**

The results of the cell cycle analysis are given in figure VIII-9 (A,B,C,D,E). Fluorescence-activated cell sorter (FACS) analysis was performed to determine all cell cycle kinetics. Cell cycle analysis was conducted for OVCAR cell lines treated with various concentrations of polysaccharide (50, 100, 200 & 400 µg/mL). The cells were then analysed by a fluorescence-activated flow cytometer (FACScan, Becton Dickinson, USA equipped with CellQuest™ Pro software). The kinetics of apoptosis induced by polysaccharide for various time intervals was checked and apoptotic cells were determined by quantitating the sub-G1 peak using the FACS analysis software.
Figure VIII-9: Cell cycle analysis for OVCAR cell lines

A- Treated (50 µg/mL)
B- Treated (100 µg/mL)
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C- Treated (200 µg/mL)
The percentage apoptosis induced by the polysaccharide on OVCAR cell line was found out and the result is given figure VIII-10.
Figure VIII-10: Percentage of apoptosis induced by the polysaccharide on OVCAR cell lines.

8.3 DISCUSSION

In the present study the anti-tumour activity of polysaccharide isolated from Ulva was determined. The results of present investigation revealed that polysaccharide with wide spectrum of bioactivities have cytotoxicity against DLA and EAC cell lines. The polysaccharide exhibited a concentration dependent cytotoxicity against mice cell lines. Cytotoxicity is one of the chemotherapeutic targets of anti-tumour activity (Suffness and Pezzuto, 1991).

The anti-tumour activity was evaluated in solid tumour model induced by DLA cell lines. Polysaccharide showed significant inhibition in tumour volume and the inhibition was in a dose dependent manner. The purified polysaccharide fraction showed profound antitumour effects against EAC cells in mice model as evidenced by the increase in life span of the animals
and the activity was in a dose dependent manner. Ehrlich ascites tumour is a rapidly growing carcinoma with very aggressive behaviour. The reliable criteria for judging the value for any anticancer drug is the prolongation of life span (Clarkson and Burchenal., 1965). No toxic symptoms were observed for all tested doses during the period of study.

The EAC implantation induces a local inflammatory reaction, with increasing vascular permeability, which results in an Oedema formation, cellular migration and a progressive acites fluid formation (Fecchio et al., 1990). The ascites fluid is essential for tumour growth since it constitutes a direct nutritional source for tumour cells (Shimzu et al., 2004). The anti-tumour activity of the polysaccharide can be correlated with its anti-inflammatory and antioxidant activity which were studied in the earlier chapters. It has been reported that the underlying mechanisms for the treatment of cancer by polysaccharide involve a direct cytotoxic effect on cancer cells and a cell-mediated immune response induced by this agent (Zhu, 2007).

The MTT assay for human cell lines treated with various concentration of polysaccharide revealed that the polysaccharide showed cytotoxicity against OVCAR cell lines at higher concentration. The other two cell lines, PC3 and HepG2 showed no inhibition by the polysaccharide.

The apoptogenic potential of the polysaccharide was estimated by the changes in chromatin organisation after treating the OVCAR cell lines with polysaccharide and then staining with Hoechst 33258. Hoechst staining revealed the apoptosis inducing capacity of the polysaccharide. There was a concentration-dependent increase in the percentage of apoptotic cells after 48 hours of incubation. The apoptotic cells were identified by the presence of
highly condensed chromatin or fragmented nuclei. Apoptosis gave some clues about effective anticancer effect and many chemotherapeutic agents were reported to exert their anti-tumour effect by inducing apoptosis in cancer cells. Induction of apoptosis seems to be a reliable marker for the evaluation of potential agents for cancer therapy (Syamsudin et al., 2010).

The cell cycle analysis showed that the polysaccharide at higher concentration could inhibit the cancer cells at their sub-G0 phase itself. This is represented by an increased quantity of DNA in the sub-G0 phase. Cell cycle analysis is a method in cell biology that employs flow cytometry to distinguish cells in different phases of the cell cycle. The cells are permeabilised and treated with a fluorescent dye that stains DNA quantitatively, usually propidium iodide (PI). The fluorescence intensity of the stained cells at certain wavelengths will therefore correlate with the amount of DNA they contain. As the DNA content of cells duplicates during the S phase of the cell cycle, the relative amount of cells in the G0 phase and G1 phase (before S phase), in the S phase, and in the G2 phase and M phase (after S phase) can be determined, as the fluorescence of cells in the G2/M phase will be twice as high as that of cells in the G0/G1 phase.

All the results revealed the promising activity of the polysaccharide as a natural drug for cancer therapy. Moreover the study conducted for venous thrombosis in earlier chapter showed that the polysaccharide could be developed as a drug for thrombosis, as venous thrombosis is the major side effect caused by the chemotherapeutic drugs. The association between cancer and venous thromboembolism (VTE) has been recognised for almost 150 years (Trousseau, 1877). In cancer patients VTE complications are common and the second leading cause of death (Haddad and Greeno, 2006).
incidence of deep venous thrombosis (DVT) was markedly higher in patients with malignant disorders than in patients with other, non-malignant diseases. An increased risk of venous thromboembolism (VTE) has been suggested by the high incidence of pulmonary embolism and subclinical activation of the coagulation system in non-surgical patients with cancer. The relationship between cancer and thrombosis is further supported by the greater risk of patients with idiopathic VTE developing overt malignancy than patients whose thrombotic episode is associated with a well recognized risk factor. The antithrombotic effect of the sulphated polysaccharide together with anticancer effect makes the molecule a suitable natural drug for the treatment of cancer.