6.0. CHAPTER 4

SCREENING OF ANTICANCER ACTIVITY OF *S. POLYCYSTUM*

SODIUM ALGINATE THROUGH MTT ASSAY

6.1. INTRODUCTION

Cancer is known to be one of the diseases that most badly threaten to human’s life. Cancer is globally the second most life threatening disease, whose mortality follows immediately after that of cardiovascular disease. Research and development of drugs against cancer and its complications have been receiving increasing attention. Unfortunately drugs, which are used for cancer therapy are toxic and affect not only cancer cells but also normal cells and tissues. Surgery and chemotherapy are the mainstream therapeutic methods followed for cancer throughout the world, but the existing chemotherapeutic drugs have a number of limitations, such as adverse effects, limited efficacy and high rates of secondary failure. Therefore, the research and development of anticancer drugs with high efficiency and low toxicity, particularly the drugs extracted from natural resources with anticancer activity and no side effects, is of greater importance (Efferth *et al.*, 2007).

In the last decades, much attention has focused on the therapeutic properties of polysaccharides and their chemical derivatives, especially sulfated derivatives (Lee *et al.*, 2003; Han *et al.*, 2005; Yang *et al.*, 2005; Xing *et al.*, 2005). The role of sulfated polysaccharides in biological systems is involved in several cellular processes, such as molecular recognition, cell development and differentiation, and cell–cell interaction.
Many sulfated polysaccharides exerted are potent antioxidant, anticoagulant, antithrombotic and antiviral activities (Soeda et al., 1993; Urbinati et al., 2004; Han et al., 2005; Xing et al., 2005). They are also known to inhibit some tumor development (Peng et al., 2005; Yang et al., 2005).

Recently marine brown algae attract much attention, because they represent a rich and easily regenerated source of polysaccharides like alginic acids, laminarans and fucoidans with various structures and biological activities. The sulphated polysaccharides (fucoidans), which mainly build up of α-l-fucopyranose (α-l-Fuco) residues, have wide variety of biological activities, including anticoagulant, antivirus, immunomodulating and antitumor activities, in particular, the antitumor activity has attracted considerable attention (Khotimchenko, 2010; Wijesekara et al., 2011). Several investigations have been reported that fucoidans effectively inhibited proliferation and colonies formation of cancer cells in vitro (Jiang et al., 2010; Ermakova et al., 2011), as well as inhibitory activity of tumours growth in vivo (Ye et al., 2008). Antitumor activity of fucoidan from seaweed U. pinnatifida in PC-3, HeLa, A549 and HepG2 cancer cells in similar pattern to that of commercial fucoidan was reported (Synytsya et al., 2010). Teruya et al. (2007) reported the anti-proliferative activity of over sulfated fucoidan from commercially cultured Cladosiphon okamuranus TOKIDA in U937 cancer cells. Fucose rich sulfated polysaccharide from Ecklonia cava has antiproliferative effects on murine colon carcinoma (CT-26), human leukemic monocyte lymphoma (U-937), human promyelocytic leukemia (HL-60), and mouse melanoma (B-16) cell lines (Athukorala et al., 2009). Fucoidan was found to inhibit
proliferation and induce apoptosis in human lymphoma HS-Sultan cell lines (Aisa et al., 2004). Considering the importance of the above, the present study was undertaken to screen the anticancer activity of *S. polycystum* sodium alginate against mouth carcinoma (HEP-2), liver carcinoma (HepG2), breast carcinoma (MCF7) and cervix carcinoma (HeLa) cell lines by MTT assay method with the following objectives.

**Objectives**

1. To prepare different concentrations of sodium alginate for MTT assay.

2. To screen the anticancer activity of sodium alginate against mouth carcinoma (HEP-2), liver carcinoma (HepG2), breast carcinoma (MCF7) and cervix carcinoma (HeLa) cell lines by MTT assay.
6.2. MATERIALS AND METHODS

6.2.1. Assay of antitumor activity of *S. polycystum* sodium alginate against cancer cell lines

6.2.1.1. Maintenance of cell lines

The antitumor assay was performed against four different carcinoma cell lines such as mouth (HEP-2), liver (HepG2), breast (MCF7) and cervix (HeLa) cell lines. These cell lines were obtained from National Centre for Cell Science, Pune, India. The individual cell lines were further maintained in Cell Culture Laboratory, Pondicherry Centre for Biological Sciences, Pondicherry.

6.2.1.2. MTT assay

The viability of cells were determined by MTT assay method using HEP-2, HepG2, MCF7 and HeLa cell lines by the method described by Mosmann (1983).

6.2.1.2.1. Drug preparation

Four different concentrations (25, 50, 100 and 250µg/ml) of *S. polycystum* sodium alginate were prepared and they were individually dissolved in 10% Dimethysulfoxide (DMSO) to give a final concentration of DMSO not more than 0.5% and did not affect cell survival.

6.2.1.2.2. Principle

The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able
to take up the tetrazolium salt. The enzyme (mitochondrial dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed and dissolved in DMSO solution. The colour developed is then determined in an ELISA reader at 570 nm.

6.2.1.2.3. Reagents

(i) MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide): 0.5mg MTT/ml of serum-free Dulbecco Modified Essential Medium (DMEM).

(ii) Solubilizing solution: Dimethylsulfoxide

(iii) Phosphate buffered saline (PBS) (pH 7.4): As described under cell culture reagents.

6.2.1.2.4. Procedure

The HEP-2, HepG2, MCF7 and HeLa cells were plated separately in 96 well microplates at a concentration of $1 \times 10^5$ cells/well. After 24 h, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37°C. After starvation, cells were treated with different concentrations of sodium alginate (25-250µg/ml) for 48 h. During the treatment period, the medium was aspirated for 24 and 48h and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37°C in a CO$_2$ incubator.

The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and
this was mixed properly by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software. Control was also maintained throughout the experiment (untreated cells). The assay was performed in triplicate for each cell lines. The results were compared with positive control (Cyclophasphamide treated cell lines).

6.2.2. Statistical analysis

The data obtained in the present study were expressed as Mean ± SD and were analyzed using one way ANOVA at 5% level of significance. Further a multiple comparison test (Tukey’s) was conducted to compare the significant differences among the parameters using computer software Statistica 6.0 (Statosoft, Bedford, UK). The inhibitory concentration 50% (IC 50) value of the test product sodium alginate against the cancer cell lines was determined through probit analysis by using the software (EPA probit analysis software, USA).
6.3. RESULTS

The anticancer activity of *S. polycystum* sodium alginate was screened by MTT assay method. The results obtained are given below.

6.3.1. Anticancer activity on mouth carcinoma (HEP-2) cell line

The anticancer activity of sodium alginate on mouth carcinoma (HEP-2) cell line is given in the Table 6.1. The control group of HEP-2 cell lines showed 100% cell viability on 24 and 48h of incubation. But the percentage inhibition of cancer cell was increased with increasing concentrations of sodium alginate as well as increasing duration of incubation. Accordingly, the inhibition rate of mouth cancer HEP-2 cells observed was 11.76, 23.52, 32.02 and 43.13% during 24h of incubation. Further it increased to 15.20, 28.52, 37.65 and 48.94% during 48h of incubation, respectively in 25, 50, 100 and 250 µg/ml concentrations of sodium alginate treatment, whereas in the standard drug Cyclophasphamide-20 treated mouth carcinoma infected cells, the inhibition rate observed was 83.76 and 86.11% on 24 and 48h of incubation, respectively. The IC 50 value of *S. polycystum* sodium alginate against HEP-2 carcinoma cell line was determined as 238.161 µg/ml (Plate 6.1a). The one way ANOVA test revealed that the cell viability of HEP-2 cell line as a function of variation between control and different concentrations of sodium alginate treatment during 24th and 48thh of experimental durations was statistically more significant (F= 1016.91 and 1676.398; P< 0.0001) (Table 6.1a).
6.3.2. Anticancer activity on liver carcinoma (HepG2) cell line

The anticancer activity of sodium alginate on liver carcinoma (HepG2) cell line showed good effect. The result inferred that the inhibition rate of liver cancer infected cells was increased with increasing concentrations of sodium alginate as well as increasing duration of experiment. It was 30.19, 38.37, 47.61 and 59.11% during 24h of incubation, but it increased to 36.04, 45.34, 53.03 and 68.94% during 48h of incubation in 25, 50, 100 and 250 µg/ml concentrations of sodium alginate treatments, respectively. Similarly, the standard drug Cyclophosphamide-20 treated cell line showed the inhibition rate of 79.60 and 85.91% respectively during 24th and 48th h of incubation. In control group, no inhibition of cells was found during the respective time intervals of incubation. The IC 50 value of *S. polycystum* sodium alginate against HepG2 carcinoma cell line was observed as 70.164 µg/ml (Table 6.2) (Plate 6.1b). The statistical one way ANOVA test conducted for the data on cell viability of HepG2 cell line as a function of variation between control and different concentrations of sodium alginate treatment during 24 and 48h of experimental period was highly significant (F= 1893.086 and 1543.041; P< 0.0001) (Table 6.2a).

6.3.3. Anticancer activity on breast cancer (MCF7) cell line

The result on anticancer activity of sodium alginate against breast carcinoma (MCF7) cell line is given in the Table 6.3. The control group displayed 100% cell viability during 24th and 48th h of incubation. But in the experimental groups treated with different concentrations (25, 50, 100 and 250µg/ml) of sodium alginate displayed
the inhibition rate of 18.71, 31.25, 44.32 and 48.26% during 24\textsuperscript{th} h of incubation, whereas the inhibition rate increased to 24.66, 36.49, 54.94 and 63.61% during 48\textsuperscript{th} h of incubation, respectively. In standard drug Cyclophosphamide-20 treated breast carcinoma infected cells, the inhibition rate observed was 85.18 during 24h of incubation and 87.35% during 48\textsuperscript{th} h of incubation. The IC 50 value of \textit{S. polycystum} sodium alginate against MCF7 carcinoma cell line was calculated as 100.541 µg/ml (plate 6.1c). The one way ANOVA test revealed that the cell viability of MCF7 cell line as a function of variation between control and different concentrations of sodium alginate treatment during 24\textsuperscript{th} and 48\textsuperscript{th} h of incubation was statistically more significant (F= 1486.648 and 1667.711; P< 0.0001) (Table 6.3a).

6.3.4. Anticancer activity on cervix carcinoma (HeLa) cell line

The anticancer activity of sodium alginate against cervix carcinoma (HeLa) cell line is given in the Table 6.4. The cell viability of control group was 100% during 24\textsuperscript{th} and 48\textsuperscript{th} h of incubation. But the inhibition rate of HeLa cells was increased with increasing concentrations of sodium alginate and incubation duration, accordingly it was 13.08, 30.71, 49.60 and 58.04% during 24\textsuperscript{th} h of incubation, but the inhibition rate increased to 22.77, 36.78, 55.29 and 60.98% during 48\textsuperscript{th} h of incubation in 25, 50, 100 and 250µg/ml concentrations of sodium alginate treatment, respectively. However, in the standard drug Cyclophosphamide-20 treated cervix carcinoma infected cells, the inhibition rate observed was 81.51 and 85.22 %, respectively during 24\textsuperscript{th}and 48\textsuperscript{th} h of incubation. The IC 50 value of \textit{S. polycystum} sodium alginate against HeLa carcinoma cell line was determined as 107.251 µg/ml (Plate 6.1d). The statistical one way
ANOVA test conducted for the data on cell viability of HeLa cell line as a function of variation between control and different concentrations of sodium alginate treatment during 24th and 48th h of incubation was highly significant (F= 1621.022 and 1624.194; P< 0.0001) (Table 6.4a).
6.4. DISCUSSION

Seaweed polysaccharides have higher antiviral, anti-cancer, anti-proliferation, and anticoagulant activities than any other plant based natural polysaccharides in vivo and in vitro (Gavrilova et al., 1997; Teruya et al., 2007; Hayakawa and Nagamine, 2009; Trinchero et al., 2009). Many polysaccharides obtained from natural sources are considered to be biological response modifiers and have been shown to enhance various immune responses and anticancer activity (Percival and McDowell, 1967; Painter, 1983). Among the polysaccharides, seaweed based polysaccharides such as fucoidans, alginates and laminarins are having biological activities including anticancer activities (Choi and Kim, 2013). In the present study, the anticancer activity of S. polycystum sodium alginate was determined against four different carcinoma cell lines viz mouth (HEP-2), liver (HepG2), breast (MCF7) and cervix (HeLa) by MTT assay method.

The cell viability of control group of HEP-2, HepG2, MCF7 and HeLa cell lines was 100% each during 24th and 48th h of experiment. But the inhibition rate of all the tested cell lines was significantly (P< 0.05) increased with increasing concentrations of sodium alginate and experimental duration. The results revealed that the cell inhibition rate was 43.13 & 48.94%, 59.11 & 68.94%, 48.26 & 63.61% and 58.04 & 60.98% during 24th and 48th h of incubation, respectively in HEP-2, HepG2, MCF7 and HeLa cancer cell lines treated with the highest concentration (250µg/ml) of sodium alginate. Similarly, Choi and Kim (2013) have studied the anticancer activities of fucoidan of Fucus vesiculosus against AGS, MCF7 and HepG2 cancer cell lines for 24h of
incubation. They observed that the native fucoidan (un irradiated) at the concentration of 4 mg/ml displayed the cytotoxicity against MCF7 was 46% during 24h of incubation. The cytotoxicity of the degraded fucoidan increased depending on its concentration. Furthermore, when fucoidan was irradiated at the absorbed doses of 100 kGy, the cytotoxicity of the degraded fucoidan appeared to be increased. For instance at a lower concentration of irradiated fucoidan (0.5mg/ml), the cytotoxicity was not significantly varied between native and degraded samples. However, the cytotoxicity of native fucoidan significantly increased to 50% and 66% in 2mg/ml irradiated fucoidan at 50 kGy and 100 kGy, respectively. When the concentration level increased to 4 mg/ml, the difference in cytotoxicity became more pronounced. When the fucoidan irradiated at 30 kGy, the cytotoxicity was also statistically increased because of the possible dose effect. Simultaneously, the cytotoxicity of native fucoidan against HepG-2 cell line was 35% and that of irradiated fucoidan at 100 kGy increased up to 57%. When fucoidan was irradiated at a dose higher than 30 kGy, the cytotoxicity of irradiated fucoidan significantly (P< 0.05) increased.

Karnjanapratum and You (2011) reported the cytotoxic effect of sulfated polysaccharides of Monostroma nitidum against human cervical cell line (HeLa). They observed the anticancer activities of the crude and fractionated polysaccharides at different concentrations (125, 250, and 500µg/ml) expressed as a percentage of growth inhibition of HeLa cancer cell lines. Both the crude and fractionated polysaccharides exhibited the anticancer activity of 40.0% on HeLa cells at 500g/ml concentration. Ye et al. (2008) have studied the antitumor activity of polysaccharides of Sargassum
*pallidum* (SP) against cancer cell lines. They reported that the fractions of polysaccharides SP-3-1 and SP-3-2 presented significantly higher antitumor activity against the HepG2 cells, A549 cells and MGC-803 cells *in vitro* than control groups, and the inhibition ability was dose-dependent. At 1.0 mg/ml, the inhibition rate of fraction SP-3-1 on the HepG2 cells, A549 cells, and MGC-803 cells was 62.2%, 64.8% and 79.6%, respectively. But the SP-3-2 fraction only exhibited higher (81.4%) antitumor activity against the HepG2 cells *in vitro* than a blank control at 1.0mg/ml, dose-dependently fractions. SP-1-1 and SP-2-2 were also showed obvious antitumor activity against the HepG2 cells (59.7% and 63.0%, respectively) at 1.0mg/ml. However, 1.0mg/ml concentration of SP-2-1 exhibited 50% of clear antitumor activity against the A549 cells. These differences in antitumor activities may be attributed to their difference on molecular weights, charge characteristics and monosaccharide distributions (Dias *et al.*, 2005). It has been reported that the polysaccharide bioactivities of brown seaweed are closely related to several structural parameters, such as the degree of sulfation, the molecular weight, the sulfation position, type of sugar and glycosidic branching (Duarte *et al.*, 2001).

From the present findings, it could be confirmed that the *S. polycystum*- sodium alginate has higher antitumor activity against the HEP-2, HepG2, MCF7 and HeLa cell lines *in vitro*, which may be related to their glucuronic acid, mannuronic acid and sulfate contents. This study highlights the potential achievable efficacy of *S. polycystum*-sodium alginate is an alternative chemotherapeutic agent in cancer treatment.
6.5. SUMMARY

The present investigation was carried out to study the *in vitro* anticancer activities of *S. polycystum* sodium alginate against mouth carcinoma cell line (HEP-2), liver carcinoma cell line (HepG2), breast carcinoma cell line (MCF7) and cervix carcinoma cell line (HeLa) by cytotoxic assay (MTT) method. The highlight of the study is summarized below.

- The cell viability of control group of HEP-2, HepG2, MCF7 and HeLa cell lines was 100% each during 24\textsuperscript{th} and 48\textsuperscript{th} h of experiment. But the inhibition rate of all the tested cell lines was significantly (P< 0.05) increased with increasing concentrations (25 to 250µg/ml) of sodium alginate and experimental duration.

- During 24\textsuperscript{th} h of incubation, the cell inhibition recorded as 11.76 to 43.13% against HEP-2 cell line; 30.19 to 59.11% against HepG2 cell line; 18.71 to 48.26% against MCF7 cell line and 13.08 to 58.04% against HeLa cell line, respectively in 25 to 250µg/ml concentrations of *S. polycystum* sodium alginate treatment.

- Similarly, during 48\textsuperscript{th} h of incubation, the cell inhibition recorded as 15.20 to 48.94% against HEP-2 cell line; 36.04 to 68.94% against HepG2 cell line; 24.66 to 63.61% against MCF7 cell line and 22.77 to 60.98% against HeLa cell line, respectively in 25 to 250µg/ml concentrations of *S. polycystum* sodium alginate treatment.
The IC 50 values of *S. polycystum* sodium alginate determined against the tested carcinoma cell lines such as HEP-2, HepG2, MCF7 and HeLa were 238.161, 70.164, 100.541 and 107.251 µg/ml, respectively.