9. ANTI-CANCER ACTIVITY OF PURIFIED BIOACTIVE FRACTION PG3 EXTRACTED FROM *PADINA GYMNOSPORA*

1. Introduction

Cancer is a debilitating disease which has afflicted a noticeable proportion of the entire population of the world in all generations (Atawodi, 2011). The development of resistance to chemotherapy is considered as a major hindrance to treatment of various cancers, as a notable proportion of tumors relapses and develops resistance, eventually resulting in multidrug resistance following exposure to multiple anti-cancer drugs with prevalent structure and mechanisms of action (Perez, 2009). Furthermore, ideally anti-cancer agents should act exclusively against tumor cells; however, numerous chemotherapeutic drugs which are presently being used for cancer patients exhibit considerable adverse side effects on the human body, namely, bleeding, hair loss, diarrhea, and immunosuppression (Kranz and Dobbelstein, 2012). Hence, discovery of new natural products and metabolites isolated from microorganisms, animals, and plants possessing high efficacy against tumor cells without any toxicity on normal cells is a big leap in scientific researches. Apoptosis as a highly regulated programmed cell death has become a matter of great interest in cancer therapy and oncology because of the high potential of various chemotherapeutic agents in inducing apoptosis in a variety of cancer cells (Elmore, 2007). Thus, screening for natural products capable of inducing apoptosis in cancer cells that can be used alone or in combination with other chemotherapeutic drugs has now been in progress in order to elevate the therapeutic effects and reduce the side effects in cancer therapy (Ji et al., 2009).
The growing body of experimental and epidemiological evidence supporting the preventive role of marine products in controlling chronic diseases such as cancer has stimulated significant scientific interest in characterizing the active ingredients of marine products. Marine algae as part of diets and traditional remedies in the Eastern Hemisphere are still underexploited plant resources. Due to their unique living environment, algae are rich in bioactive constituents such as phycocyanin, terpenes, fucosterol, and polysaccharides (Yang et al., 2010). Extensive beneficial health effects of marine algae have highlighted their role as a source of functional ingredients in recent years (Pangestuti and Kim, 2011). Hence, it is clearly documented that in vivo and in vitro studies with marine algae compounds continue to be extremely active in recent history (Mayer and Gustafson, 2003).

Brown algae or Phaeophyceae characterized by their natural pigments form an important group of marine algae (Khan et al., 2010). Different types of brown algae including wakame (Undaria pinnatifida), kombu (Laminaria japonica), and hijiki (Sargassum fusiforme) are staples in East Asians diet, especially Japan and Korea (Asai et al., 2004). Viscous components including gepsin, porphyran, alginic acid, and oligosaccharide protecting the seaweed from invasion of bacteria that are involved in different health benefits of brown algae are clear illustrations of the importance and diversity of the constituents isolated from Phaeophyceae (Kwon and Nam, 2006).

Carotenoids as a group of natural pigments with more than 600 members possess a variety of biological activities including radical scavenging, immunomodulation, singlet oxygen quenching activity, and other pharmacological effects (van Poppel and van den Berg, 1997). Carotenoids include two main subclasses of nonpolar hydrocarbon carotenes
and polar compounds named xanthophylls. One well-known example of xanthophylls for anti-cancer activity is fucoxanthin (Das et al., 2005). Fucoxanthin (3′-acetoxy-5,6-epoxy-3,5′-dihydroxy-6′7′-didehydro-5,6,7,8,5′,6′-hexahydro-ββ-caroten-8-on) with a unique carotenoid structure including an allenic band and a 5,6-monoepoxide is a major nonprovitamin A carotenoid isolated from brown seaweed (Asai, 2004). This orange-colored pigment carotenoid contributes more than 10% of the total carotenoids in nature, particularly in the marine ecosystem, albeit, besides brown algae, fucoxanthin was also isolated from diatoms (Bacillariophyta) (Ishikawa et al., 2008).

The extensive research on the crude extracts of various brown algae isolated from different marine environments against different cancer cell lines shows promising anti-cancer potential. Several reports on the potent antioxidant property of fucoxanthin and its metabolites are available (Sangeetha et al., 2008; 2009; Chandini et al., 2008; Sachindra et al., 2007; Nomura et al., 1997). While its antioxidant property was initially thought to be the main reason behind its anti-carcinogenic effect, it is now established that the realm of fucoxanthin’s effect is wider and involves several other biological processes as well. Moreover, some studies have reported the pro-oxidant effect of fucoxanthin on cancer cells with the production of free radicals and have proposed this to be one of the mechanisms by which it protects against cancer cells (Sangeetha et al., 2013). Besides direct anti-cancer activity, the in vitro and ex vivo investigations of fucoxanthin also revealed the antiangiogenic effect of this carotenoid as a complementary potential for cancer prevention (Moghadamtousi et al., 2014).

The bioactive pigment fraction PG3 of P. gymnospora suspected to be fucoxanthin is treated against cancer cell lines (MCF7 and A549) and also normal cell
line (VERO) to study the anti-cancer properties of PG3 through cell line treatment for morphological changes, cytotoxicity activity by MTT assay for IC₅₀ value and DNA fragmentation for DNA damage/apoptosis.

2. Materials and Methods

2.1. Anti-cancer activity

2.1.1. Cell line and culture

Human breast adenocarcinoma cell line (MCF7), Human lung carcinoma cell line (A549) and VERO cell lines were obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U ml⁻¹), and streptomycin (100 μg ml⁻¹) in a humidified atmosphere of 50 μg ml⁻¹ CO₂ at 37°C.

2.1.2. Cell treatment

The monolayer cells were detached with trypsin-ethylene diaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a haemocytometer and diluted with medium supplemented with 5% FBS to give final destiny of 10,000 cells well⁻¹ and incubated to allow for cell attachment at 37°C, 5% CO₂, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in DMSO except the sample ‘positive control’, which is dissolved in phosphate buffer saline (PBS). The samples were then diluted with serum free medium to obtain the desired eight test sample concentrations. Aliquots of 100 μl of these different sample dilutions were added to the appropriate wells already containing 100 μl of medium resulted the required final sample
concentrations as 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 μg ml\(^{-1}\). The plates were incubated for additional 48 h at 37°C, 5% CO\(_2\), 95% air and 100% relative humidity. Medium without sample serve as control. Triplicate was maintained for all concentration.

2.1.3. In vitro cytotoxicity assay (MTT assay)

The anti-cancer activity of active fraction PG3 on MCF7, A549 and VERO cell lines were determined by the MTT assay (Mosmann et al., 1983). Cells (1 × 10\(^5\) well\(^{-1}\)) were plated in 0.2 ml of medium/well in 96-well plates and incubated at 5% CO\(_2\) incubator for 72 h. Various concentrations of active fraction PG3 were added to 0.1% DMSO for 24 h at 5% CO\(_2\) incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20 μl well\(^{-1}\) (5 mg ml\(^{-1}\)) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4 h incubation, 1 ml of DMSO was added. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC\(_{50}\)) was determined graphically. The effect of the samples on the proliferation of MCF7, A549 and VERO cells were expressed as the % cell viability, and were calculated.

2.1.4. DNA fragmentation

Cells (1 × 10\(^5\)/well) were plated in 1 ml of medium/well in 24 well plates. Incubate at 5% CO\(_2\) incubator for 72 h. Then, add various concentrations of active fraction PG3 in 0.1% DMSO for 24 h at 5% CO\(_2\) incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), Add 500 μl of TPVG (trypsinization) and discard that solution.
Isolation of DNA

Centrifuge 2 ml of cells at 3000 rpm for 5 min. The pellet obtained was suspended in 200 µL of 1X TE Buffer and 100 µL of 10% SDS was added and mixed well, incubated at 50°C for 20 min. Add 300 µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1) mixed well, and centrifuge at 10,000 rpm for 10 min. Transfer the supernatant to new 1.5 mL eppendorf tube. 1000 µL of Isopropanol was added to the supernatant, mixed by inverting the tube (4-5 times), centrifuged at 1000 rpm for 10 min supernatant was discarded. 500 µL of 70% ethanol was added and centrifuged at 10,000 rpm for 10 min, supernatant was discarded. The pellets are air dried till there are no traces of ethanol. The pellet is resuspended in 20 µL of 1XTE Buffer. The extracted DNA is electrophoresed on 1.2% agarose gel.

Agarose gel electrophoresis

Agarose gel electrophoresis is carried out in a horizontal submarine electrophoresis unit.1.2% Agarose gel was prepared by adding 0.72 gm of Agarose in 60 mL of diluted 1X TBE buffer and dissolved by heating the content to get up to clear solution. Allow the solution to cool at room temperature, 5 µL of Ethidium bromide was added and mixed, the agarose is poured into the casting system with combs. The gel is allowed to solidify, and the casting system is disassembled carefully without disturbing the wells. The gel is transferred to 1X TBE buffer filled electrophoresis tank. 5 µL of gel loading dye was added to 20µL of sample DNA, mixed well, and loaded to gel. 10 µl of 1 kb DNA marker was also added near to the well. The power card terminals were connected at respective positions and run the gel at 50 V till the gel loading dye migrates
more than half of the length of gel. The separated DNA bands were visualized under UV Transilluminator.

3. Results

3.1. Anti-cancer activity

Human breast adenocarcinoma cell line (MCF7), Human lung carcinoma cell line (A549) and VERO cell lines were seeded at a density of $1 \times 10^5$ cells per well onto 96-well plate. After incubation of the samples for 24 h, the cells were washed, and visualized for morphological changes. The control plate did not show any morphological changes (Fig. 17) and were irregular confluent aggregates with rounded and polygonal cell morphology. PG3 active fraction on cell treatment with MCF7, A549 and VERO cells inhibited the proliferation of cells dose dependently (Figs. 18 - 20).

3.1.1. Cytotoxicity assay

Percentage of cell viability of MCF7, A549 and VERO cell lines were assessed by treating the active fraction PG3 with different concentrations (1-1000 μg ml$^{-1}$) after 24 h of incubation (Table 3). A significant decrease was seen in the cell viability at different concentration of the active fraction PG3 of *P. gymnospora* (Fig. 21). The IC$_{50}$ value of PG3 active fraction on MCF7, A549 and VERO cells were calculated and presented in Table 4.

3.1.2. DNA fragmentation

DNA was isolated from MCF7 and A549cell lines treated with PG3 and also from untreated MCF7 and A549 cell line as control. DNA marker of 1kb is also loaded in agarose gel and electrophoresis is carried out. The results indicated that there is clear
DNA damage to MCF7 and A549 cells after treatment with PG3 (Fig. 22). MCF7 showed higher damage than A549. Bands in lane 5 (MCF7 lane) are much more clear and sharp compared lane 2 (A549 lane). This confirms better anti-cancer activity of PG3 against MCF7 cell lines.

4. Discussion

In the previous chapter, the bioactive pigment in active fraction PG3 of P. gymnospora, partially characterized was suspected to be fucoxanthin. Fucoxanthin is a marine carotenoid exhibiting several health benefits. The anti-cancer effect of fucoxanthin and its deacetylated metabolite, fucoxanthinol, is well documented. In view of its potent anti-carcinogenic activity, the need to understand the underlying mechanisms has gained prominence. Towards achieving this goal, several researchers have carried out studies in various cell lines and in vivo and have deciphered that fucoxanthin exerts its anti-proliferative and cancer preventing influence via different molecules and pathways including the Bcl-2 proteins, MAPK, NFkB, Caspases, GADD45, and several other molecules that are involved in either cell cycle arrest, apoptosis, or metastasis. Thus, in addition to decreasing the frequency of occurrence and growth of tumours, fucoxanthin has a cytotoxic effect on cancer cells. Some studies show that this effect is selective, i.e., fucoxanthin has the capability to target cancer cells only, leaving normal physiological cells unaffected/less affected. Hence, fucoxanthin and its metabolites show great promise as chemotherapeutic agents in cancer. Fucoxanthin influences a multitude of molecular and cellular processes. It exerts strong effects on cancer cells and shows synergistic
activity in combination with established cytotoxic drugs. This raises the possibility that it could become an interesting anti-cancer compound in various types of cancer.

Fucoxanthin has a unique structure including an unusual allenic bond and 5, 6-monoepoxide in its molecule. The structure of carotenoids may be important in reducing growth and in inducing apoptosis in cancer cells. Fucoxanthin may also regulate the redox signals, and then facilitate the progression of apoptosis through Bel-2 protein suppression and caspase-dependent and -independent pathways (Kotake-Nara et al., 2005).

In the present study, MCF7, A549 and VERO cell lines were treated with bioactive pigment fraction PG3 which may be fucoxanthin. After incubation the cells were visualized for morphological changes. The control plate did not show any morphological changes. The treated cells showed cell damage, reduction in cell volume and apoptotic bodies which are dose dependent. VERO cells showed minimal or no damage in lower concentrations. This shows that the active fraction PG3 is also selective towards carcinogenic cells.

Similarly, Yamamoto et al., (2011) have concluded that the effect of fucoxanthinol (deacetylated metabolite of fucoxanthin) was more potent than fucoxanthin and PEL cells were more susceptible to the effects of fucoxanthin and fucoxanthinol than HeLa cells. Kotake-Nara et al., (2001) have reported a dose dependent reduction in cell viability in prostate cancer cell lines exposed to fucoxanthin along with morphological changes such as rounding up, detachment and reduction in cell volume and apoptotic bodies. The DNA fragmentation observed in cells treated with fucoxanthin suggested that apoptosis was the cause of suppression of cell viability. In a separate study on the effect
of neoxanthin and fucoxanthin on PC-3 prostate cancer cells, Kotake-Nara et al., (2005) have reported decreased cell viability, rounding up, reduced cell volume, chromatin condensation, nuclei fragmentation, formation of apoptotic bodies in addition to the apoptotic DNA ladder indicating apoptosis in the cells. In a recent study by Ganesan et al., (2011) on 11 carotenoids, two marine carotenoids, siphonaxanthin and fucoxanthin were found to possess potent growth inhibitory and apoptosis inducing effect in HL-60 leukemia cells. Cell viability was reduced with fucoxanthin treatment and apoptosis was characterized by DNA fragmentation and chromatin condensation. Jaswir et al., (2011) have reported the dose dependent growth inhibition of H1299 (lung cancer) cells and morphological changes, such as decrease in cell size and nuclear condensation.

In the current study, MTT assay was used to study the cytotoxicity effect of bioactive pigment fraction PG3 on MCF7, A549 and VERO cell lines and found that IC$_{50}$ value of MCF7 is 7.8 $\mu$g ml$^{-1}$, A549 is 12 $\mu$g ml$^{-1}$ and VERO is 15.6 $\mu$g ml$^{-1}$. These values clearly support the statement that fucoxanthin is selective in its cytotoxicity. VERO cells showing IC$_{50}$ value nearly twice that of MCF7 shows that it is more effective on MCF7 than A549.

Similar to the current study, The cytotoxicity of fucoxanthin isolated from U. pinnatifida was investigated in three lines of human prostate cancer cells, namely, PC-3, DU145, and LNCaP by Kotake-Nara et al., 2001. According to their results, 72 h treatment with fucoxanthin (20 $\mu$mol/L) significantly reduced cell viability to 9.8% for LNCaP, 5.0% for DU 145, and 14.9% for PC-3. Induction of DNA fragmentation by
fucoxanthin detected by TUNEL assay suggested that apoptosis is responsible for the reduction in cell viability of human prostate cancer cells.

Kim et al., (1998) studied on chemopreventive activity of fucoxanthin isolated from 
*Hijikia fusiforme* on the development of putativepreneoplastic aberrant crypt foci (ACF) in the colon of B6C3F1 male mice induced by 1,2-dimethylhydrazinedihydrochloride proved the potential of fucoxanthin as a chemopreventive agent against colon carcinogenesis. Treatment with fucoxanthin (0.01%) in the drinking water of the mice for 7 weeks significantly decreased the ACF/mouse from 63.3 for the control group to 47.1 value. These promising antitumor results shown by fucoxanthin warranted the various detailed investigations for determining the exact mechanisms underlying such a strong antitumor potential. Further, studies by Hosokawa et al., (1999) on antitumor activity of fucoxanthin isolated from *U. pinnatifida* against human leukemic HL-60 cells showed significant inhibitory effect on HL-60 proliferation in a dose-dependent manner. Treatment of HL-60 with 11.3 μΜ and 45.2 μΜ of fucoxanthin for 24 h reduced the viability to 46.0% and 17.3% compared to the control value, respectively. Induction of DNA fragmentation by fucoxanthin implied apoptosis for reduced proliferation of HL-60 cells.

The results obtained by Konishi et al., (2006) show the dose and time dependent anti-proliferative effect of fucoxanthinol and fucoxanthin in HL-60, MCF-7, and Caco-2 cells. In their studies, they found the anti-proliferative effects of fucoxanthinol to be greater than fucoxanthin. Their experiments involved studying the effect of metabolites like fucoxanthinol and halocynthiaxanthin and comparing it with fucoxanthin. They have concluded that both the metabolites showed superior anti-proliferative activity and have
speculated that one of the factors that may contribute to this may be the presence of hydroxyl functional group in place of the acetyl group of fucoxanthin, a feature common to both metabolites, in addition to unique structures such as 5,6-epoxide, acetylenic and allenic bonds. Zhang et al., (2008) have reported the anti-proliferative effect of fucoxanthin on EJ-1 (urinary bladder cancer) cells. Apoptosis characterized by condensed chromatin, nuclear fragmentation, and apoptotic bodies in addition to the DNA ladder was observed.

In the present study, DNA fragmentation was examined through agarose gel electrophoresis. 5 lanes that contain DNA of A549 control cells, A549 cells treated with bioactive fraction PG3 (may be fucoxanthin), marker of 1 Kb, MCF7 control cells, MCF7 cells treated with fucoxanthin were used respectively. DNA ladder characteristic of DNA damage/apoptosis was seen in lane 2 (A549 treated cells) and lane 5 (MCF7 treated cells). DNA ladder in lane 5 is clearer that shows that MCF7 cell lines are highly damaged than A549 cell lines.

Similarly, Hosokawa et al., (1999) have reported DNA fragmentation and the DNA ladder characteristic of apoptosis in HL-60 cells treated with fucoxanthin. In a separate study, fucoxanthin was found to induce cellular DNA fragmentation/internucleosomal DNA degradation by activation of endogenous nucleases in a dose dependent manner unlike β-carotene and astaxanthin. The authors have hypothesized that since fucoxanthin is converted to fucoxanthinol prior to uptake and also fucoxanthinol shows greater inhibition of growth, the superior anti-proliferative effect of fucoxanthin may be due to its metabolites Hosokawa et al., (2004). DNA fragmentation typical of apoptotic cells was observed in NSCLC-N6 (human non-small cell
bronchopulmonary carcinoma) cells treated with fucoxanthin along with typical morphological changes such as rounding up, reduction in cell volume, chromatin condensation, nuclei fragmentation, and formation of apoptotic bodies Moreau et al., (2006). However, in the same study, no apoptosis was observed in SRA (human lens epithelial cells) indicating the specific action of fucoxanthin against carcinogenic cells.

The results showed that bioactive fraction PG3 of *P. gymnospora* had cytotoxic activity against MCF7 and A549 cell lines. The cytotoxic activity increased after concentration of the sample. Analysis of DNA fragmentation of MCF7 and A549 cells showed that the suspected bioactive pigment fucoxanthin fragmented DNA as a feature of apoptosis. These anti-cancer properties may be related to fucoxanthin content although this hypothesis need to be further investigated.
Table 4: Cytotoxicity effect of purified active fraction PG3 of *Padina gymnospora* on MC7, A549 and VERO cell lines

<table>
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<tr>
<th>S. No</th>
<th>Concentration µg/ml</th>
<th>Dilution</th>
<th>MCF7 Absorbance 540 nm</th>
<th>% cell Viability</th>
<th>A549 Absorbance 540 nm</th>
<th>% cell Viability</th>
<th>VERO Absorbance 540 nm</th>
<th>% cell Viability</th>
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Table 5: IC$_{50}$ value of active fraction PG3 on MC7, A549 and VERO cell lines

<table>
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<th>S. No</th>
<th>Cell line</th>
<th>IC$_{50}$ (µg ml$^{-1}$)</th>
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<tr>
<td>1</td>
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<tr>
<td>3</td>
<td>VERO</td>
<td>15.6</td>
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Fig. 17: MCF7 (A), A549 (B) and VERO (C) control cell lines
Fig. 18: Morphological changes in MCF7 cell line on treatment with active fraction PG3 (A-1000 μg, B-500 μg, C-250 μg, D-125 μg, E-62.5 μg, F-31.2 μg, G-15.6 μg and H-7.8 μg)
Fig. 19: Morphological changes in A549 cell line on treatment with active fraction PG3 (A-1000 µg, B-500 µg, C-250 µg, D-125 µg, E-62.5 µg, F-31.2 µg, G-15.6 µg and H-7.8 µg)
Fig. 20: Morphological changes in VERO cell line on treatment with active fraction PG3 (A-1000 µg, B-500 µg, C-250 µg, D-125 µg, E-62.5 µg, F-31.2 µg, G-15.6 µg and H-7.8 µg)
**Fig. 21:** Cytotoxicity of active fraction PG3 against MCF7, A549 and VERO cell lines

**Fig. 22:** DNA fragmentation of MCF7 and A549