Chapter 6

Anti-cancer studies of PUFA extracts from 
*Sardinella longiceps* and *Sardinella fimbriata*: 
Breast and Prostate cancer

6.1 Introduction

Human epidemiologic studies have shown that dietary intake of fish oil may protect against the development of certain cancers including breast, colon, and prostate (Hursting *et al.* 1990, Sasaki *et al.* 1993, Caygill *et al.* 1996, Gago-Dominguez *et al.* 2003). Reported benefits of n-3 PUFA dietary supplements given before or during cancer therapy include reversing tumor cell drug resistance (Das *et al.* 1998); reducing the gastrointestinal, hematological, or cardiac side effects of various chemotherapeutic treatments (Hardman *et al.* 1999, Germain *et al.* 1999, Shao *et al.* 1997); decreasing cancer cachexia (Karmali *et al.* 1996, Tisdale *et al.* 1993, Barbet *et al.* 1999); and protecting from alopecia (Takahata *et al.* 1999). The results of *in vitro* studies have shown that a small amount of either EPA or of DHA added to cell culture medium can cause tumour cell death but not kill
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Cultured normal cells (de Vries et al. 1992, Begin et al. 1986, Price & Tisdale 1998, de Salis and Meckling-Gill 1995). Thus, it is thought that one or both of these n-3 fatty acids is responsible for the beneficial effects of fish oil against tumor growth.

6.1.1 Breast Cancer

In 2012, 1.5 million people worldwide will be told they have breast cancer. Nearly 1 in 4 women with cancer in the world have breast cancer, and half of them live in developed countries. According to an Indian health news report, one in 22 women in India is likely to suffer from breast cancer during her lifetime, while the figure is definitely more in the US with one in eight being a victim. Hence, biomedical research and application of well known and readily available sources of anti-cancerous compounds for creation of breast cancer drugs is an urgent worldwide need.

There is growing interest in the use of n-3 PUFAs, like EPA and DHA, as an agent against the growth of breast cancer to retard the growth of tumorigenic cells or xenografts (Rose 1997, Bougnoux 1999, Das 1999). The addition of fish oil to the diet of nude mice bearing human tumor xenografts increased the efficacy of the cancer chemotherapy drugs including edelfosine against MDA-MB 231 human breast cancer tumors (Hardman et al. 1997), irinotecan (CPT-11) against MCF-7 human breast tumors (Hardman et al. 1999), epirubicin against rat mammary tumors (Germain et al. 1999), and cyclophosphamide (Shao et al. 1997) or mitomycin against MX-1 human mammary tumors (Shao et al. 1995). Marine fatty acids, particularly the long-chain EPA and DHA, have been
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Consistently shown to inhibit the proliferation of breast cancer cell lines in vitro and to reduce the risk and progression of these tumours in animal experiments (Rose and Connolly 1999, Rose and Connolly 2000). Results suggested that PUFA of the linoleic group (n-6 PUFA) stimulate mammary tumor development (Welsch 1994, Bartsch et al. 1999), whereas PUFA of the Linolenic group (n-3 PUFA) and especially those from marine origin (EPA and DHA) inhibit tumor growth in mice (Connolly et al. 1999).

6.1.2 Prostate Cancer

Prostate Cancer is the most common non-skin cancer in the world, affecting 1 in 6 men in the United States. A non-smoking man is more likely to develop prostate cancer than he is to develop colon, bladder, melanoma, lymphoma and kidney cancers combined. In fact, a man is 35% more likely to be diagnosed with prostate cancer than a woman is to be diagnosed with breast cancer. In 2009, more than 192,000 men have been diagnosed with prostate cancer, and more than 27,000 men died from the disease (http://www.prostatecancerfoundation.org). In the late 80s and early 90s great attention was given to screening asymptomatic men by measuring concentration of prostate specific antigen (PSA), which eventually led to a significant increase in the detection of clinically insignificant tumours. Though the exact pathogenesis is not clear, epidemiological evidence supports a relationship between prostate cancer and serum levels of testosterone (Ross et al. 1992). Other risk factors include advanced age, family history, African-American ethnicity, poor diet and cadmium exposure (Pienta and Esper 1993). The frequency of prostate cancer increases exponentially with advanced age and the natural progression to
prostate cancer tends to be more aggressive in younger men and those with a family history of the disease. Hence, it is imperative that more research is expended in finding excellent sources of naturally available anti-cancerous compounds which can aid drugs against prostate cancer.

Experimental studies show that prostate tumor growth is inhibited by long chain omega-3 PUFA such as EPA and DHA (Connolly JM 1997, Rose 1997, Rose and Connolly 1991, Karmali et al. 1987). There is epidemiological support for a protective influence of omega-3 fattyacids against prostate cancer (Godley 1996, Lanier et al. 1996). Consumption of oily fish and other foods rich in omega-3 fattyacids may help prevent the spread of carcinoma prostate (Brown et al 2006). The omega-3 fattyacids interfere with functions of omega-6, which cancer cells may use as a source of energy and prevent them from spreading beyond the prostate. Both EPA and DHA can inhibit the biological activity of eicosanoids and androgens (Faust et al., 1989, Zaccheo et al. 1998, Liang and Liao 1992), which are both known to have a stimulating effect on prostate cancer cell growth (Rose 1997, Ghosh and Myers 1997). In animal models and in human prostate cancer cell lines, EPA and DHA are known to suppress cell growth (Conolly et al. 1997). The concentrations of EPA and DHA are high in fish oils and they consistently inhibit tumor cell growth in animal models and in cell lines from human prostate tumors (Rose and Connolly 1999).

Most cancer studies on PUFA or fish oil has not distinguished EPA and DHA separately and most studies believed that both have similar biochemical effects. Some studies reported a similar, but slightly subdued
effect, of DHA as compared to EPA and hypothesized that this could be partly due to retro-conversion to EPA (Price and Tisdale 1998).

The objective of this study was to determine the cytotoxicity on breast cancer cells (MCF-7) of PUFA extracts from two different species of sardines, viz. *S. fimbriata* and *S. longiceps*, found in the same area with in their ranges. Comparison of their respective PUFA profile with the degree of cytotoxicity is also attempted.

**6.2 Materials and Methods**

**6.2.1 Extract Preparation and Determination of PUFA Composition**

Freshly caught samples of the fishes were subjected to the procedure documented in Chapter 2 to obtain a mixture of substantially pure PUFA. The composition of PUFAs in the above mixture was directly analysed by Gas Chromatography (GC) adopting the fattyacid methyl ester (FAME) method mentioned in Chapter 3 (3.3) and individual fatty acids were expressed as a percentage of total fatty acids.

**6.2.2 Cell Culture**

Cancer cell lines MCF-7 and DU-145 were obtained from National Centre for Cell Science (NCCS), Pune, India and cell culture supplies were purchased from HiMedia Laboratories (Mumbai, India). Cells were maintained at 37°C under 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml
penicillin and 100 U/ml streptomycin. The culture medium was changed every 2 days and the cells were sub-cultured every 6 days.

6.2.3 Assessment of Cell Viability

Cell viability was determined using MTT (3- (4, 5- dimethylthiazol-2yl)-2, 5- diphenyltetrazolium bromide) assay as described in Mosmann (1983). In order to detect the cytotoxicity of the fish oil extracts, both cell lines were treated with the extract at different concentrations for a specified incubation time. The intensity of formazan, reduced product of MTT after reaction with active mitochondria of live cells, was determined by measuring the absorbance in a 96 well microplate reader (Bio-Tek, Powerwave XS, USA). Results were expressed as percentage inhibition considering absorbance control cells as 100% viable.

MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue impermeable formazan crystals (Mosmann 1983). Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals. The number of surviving cells is directly proportional to the level of the formazan product created. The detailed procedure is as follows.

Seeded MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines at a density of 5000 cells/well (100µl each) in a 96 well microplate and grown in a 5% CO₂ incubator at 37º C. When cells have adhered and are about 50% confluent, serially diluted concentrations of the cytotoxin (100µl
each) is added. Control wells were devoid of toxin and replaced with 100µl of DMEM.

Stock Concentration:

- *S. fimbriata*: 0.02g/ml of DMSO
- *S. longiceps*: 0.02g/ml of DMSO

Working Concentrations:

100, 200, 400, 600, 800, 1000 µg/ml of 10% DMEM (Dulbecco’s Modified Eagles Medium) in triplicates. After 72 hrs incubation in CO₂ incubator (37º C), aspirated out the 200µl of solution without disturbing the cells and 100µl of MTT is added at a concentration of 1mg/ml of 10% DMEM. This is incubated in dark for 2 hrs in CO₂ incubator at 37º C. 100µl lysis buffer is added to dissolve the formazan crystals and incubated in dark for another 4 hrs. The plate is read on ELISA reader at a wavelength of 570 nm to measure optical density (OD). Calculated percentage of cytotoxicity as per the below formula

\[
\text{Cytotoxicity} = 100 - \frac{\text{treated OD}}{\text{control OD}} \times 100.
\]

6.2.4 Statistical Analysis

The obtained results were analyzed using 1-way ANOVA (Zar *et al.* 1984) against the control followed by Fisher’s LSD test and p<0.01 was considered as significant.

6.3 Results

The respective cytotoxicity profiles of the two species for MCF-7 are illustrated in Figure 26 and values indicated in Table 12 and for DU-145 are
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illustrated in Figure 27 and Table 13. Statistically the means for each of the concentrations were found to be significant with \( p < 0.01 \) (See Appendix).

Table 13 tabulates the IC50 values of the two extracts against both cell lines, along with peak values and corresponding concentrations.

Table 12: Activity of PUFA extracts of *S. longiceps* and *S. fimbriata* on MCF-7 at different concentrations

<table>
<thead>
<tr>
<th>Conc.</th>
<th><em>Sardinella longiceps</em> OD Mean±SD</th>
<th>Cytotoxicity</th>
<th><em>Sardinella fimbriata</em> OD Mean±SD</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.77±0.07</td>
<td>0.00</td>
<td>2.77±0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>100</td>
<td>1.06±0.06</td>
<td>61.50</td>
<td>2.38±0.03</td>
<td>14.02</td>
</tr>
<tr>
<td>200</td>
<td>0.38±0.01</td>
<td>86.20</td>
<td>1.18±0.03</td>
<td>57.21</td>
</tr>
<tr>
<td>400</td>
<td>0.41±0.03</td>
<td>85.19</td>
<td>0.38±0.03</td>
<td>86.24</td>
</tr>
<tr>
<td>600</td>
<td>0.44±0.02</td>
<td>83.79</td>
<td>0.38±0.01</td>
<td>86.20</td>
</tr>
<tr>
<td>800</td>
<td>0.54±0.03</td>
<td>80.47</td>
<td>0.4±0.01</td>
<td>85.24</td>
</tr>
<tr>
<td>1000</td>
<td>0.58±0.03</td>
<td>78.92</td>
<td>0.38±0.01</td>
<td>85.94</td>
</tr>
</tbody>
</table>

It can be seen that the peak effects for both species is around 86% for MCF-7 and around 90% for DU-145. For extracts from *S. longiceps*, the peak effect happens at a concentration of 200µg/ml for both MCF-7 and
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DU-145. At 200µg/ml, extracts from *S. fimbriata* show peak activity for DU-145 while a concentration of 400µg/ml is required for the same extract to show peak effect for MCF-7.

Table 14: Summary of the cytotoxic activity of PUFA extracts on MCF-7 and DU-145 cell lines

<table>
<thead>
<tr>
<th>Parameters</th>
<th><em>S. fimbriata</em></th>
<th><em>S. longiceps</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (MCF-7)</td>
<td>180.01 µg/ml</td>
<td>81.17 µg/ml</td>
</tr>
<tr>
<td>IC50 (DU-145)</td>
<td>67.64 µg/ml</td>
<td>53.07 µg/ml</td>
</tr>
<tr>
<td>Peak Cytotoxicity (MCF-7)</td>
<td>86.28 %</td>
<td>86.30 %</td>
</tr>
<tr>
<td>Peak Cytotoxicity (DU-145)</td>
<td>90.41%</td>
<td>89.73%</td>
</tr>
<tr>
<td>Concentration at Peak (MCF-7)</td>
<td>400 µg/ml</td>
<td>200 µg/ml</td>
</tr>
<tr>
<td>Concentration at Peak (DU-145)</td>
<td>200 µg/ml</td>
<td>200 µg/ml</td>
</tr>
<tr>
<td>% EPA</td>
<td>32.52%</td>
<td>55.54%</td>
</tr>
<tr>
<td>% DHA</td>
<td>65.82%</td>
<td>24.02%</td>
</tr>
<tr>
<td>EPA:DHA Ratio</td>
<td>3:8</td>
<td>3:2</td>
</tr>
</tbody>
</table>

Fig. 26: Cytotoxic Activity of PUFA extracts from *S. longiceps* and *S. fimbriata* on MCF-7 cell line
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Fig. 17: Cytotoxic activity of PUFA extracts from S. longiceps and S. fimbriata on DU-145 cell line

The PUFA extracts were analyzed by GC to identify the fatty acids present in the extract. The major compounds identified were unsaturated fatty acids ranging from C20 to C24 with a preponderance of C20:5 (EPA) and C22:6 (DHA) PUFA. GC analyses of the PUFA from the fish S. longiceps showed an EPA content of 55.54% and a DHA presence of 32.52%. The GC analyses of the PUFA from the fish S. fimbriata gave a much lower EPA content of 24.02% but a correspondingly higher DHA value of 65.82%. To correlate with the concentrations of EPA and DHA in both the extracts, values obtained from Gas Chromatography are also included in Table 14.

6.4 Discussion

The results showed that both the fish oil extracts have a great degree of cytotoxic effects on MCF-7 and DU-145 cells of the order of 86-90% at
the peak – activity itself being dose dependent at lower concentrations. The IC\textsubscript{50} value of sardine oil emulsion on human breast cancer cell line MCF-7 was as high as 1000 µg/ml (Ueda \textit{et al.} 2007), while it is evident that substantially pure PUFA from our experiments have a much stronger cytotoxic effect which is an order less than prior studies – 180 µg/ml for \textit{S. fimbriata} and 81 µg/ml for \textit{S. longiceps}. It can also be concluded that the EPA-rich \textit{S. longiceps} extracts have a greater cytotoxic effect on MCF-7 cells at lower concentrations as compared to the DHA-rich \textit{S. fimbriata} as evident from the IC\textsubscript{50} values. This is also true to lesser extent for DU-145 cells too.

Though there are no established studies proving EPA to be more toxic against MCF-7 and DU-145 as compared to DHA, it was believed until recently that the action of DHA is mostly by retro-converting to EPA. Hence, the action of DHA on the annihilation of cancer cells could be delayed until the conversion occurs (Price and Tisdale 1998). However, recent evidences indicate that the action of both EPA and DHA could be very different and differs with different cell lines.

Inhibition of cell growth can be accomplished by either a decrease in cell proliferation or an increase in apoptosis or both. Apoptosis is therefore an important cellular mechanism for growth regulation. In humans, the production of Arachidonic acid (AA) from linoleic acid is suppressed by n-3 fatty acids (Hague and Christoffersen 1984). Suppression of AA production by n-3 fatty acids also suppresses the production of AA-derived eicosanoids. Cyclooxygenase (COX) and lipoxygenase (LOX) act on 20-carbon fatty acids to produce cell-signaling molecules. COX activity on AA or EPA
produces prostaglandins or thromboxanes; LOX activity on AA or EPA produces the leukotrienes. The 2-series prostaglandins produced from AA tend to be pro-inflammatory and pro-proliferative in most tissues. The 3-series prostaglandins produced from EPA tend to be less promotional for inflammation and proliferation; thus, EPA-derived prostaglandins are less favourable for the development and the growth of cancer cells. COX has 2 isozymes: COX 1 and COX 2. COX 1 is constitutively produced by most cell types, and COX 2 is produced as part of the inflammatory response. Incorporation of n-3 fatty acids has been shown to suppress the production of COX 2 (Singh et al. 1999, Obata et al. 1999) and can reduce the inflammatory response (Needleman et al. 1979) by changing the types of eicosanoids that are produced.

Several mechanisms have been proposed for suppression of tumor cell growth by n-3 fatty acids, and new mechanisms are frequently reported as additional knowledge is gained of the regulation of gene expression by fatty acids. It is likely that suppression of tumor cell growth by n-3 fatty acids is due to the combination of these mechanisms rather than to a single, unique activity that is the sole mechanism of action (Hardman 2004). The formation of cytostatic and cytotoxic compounds after peroxidation of long chain PUFAs have been proposed as the primary mechanism for the activity of n-3 PUFAs against cancers (Gonzalez et al. 1991, Gonzalez 1995, Das 1990). Other mechanisms proposed include the alteration in prostaglandin synthesis (Rose et al. 1994), alteration in gene transcription (Jump & Clarke 1999), suppression of n-6 fatty acid transport (Sauer et al. 2000), and modulation of anti oxidant enzymes (AOE) and of apoptosis (Fernandez et al. 1996).
It was reported earlier that while omega-3 fatty acids could selectively inhibit tumor cell proliferation, they were not cytotoxic in normal cells (Begin et al. 1986, Hardman et al. 1997). It is also reported that the anticancer effect of DHA and EPA in different human cancer cell lines (such as MCF-7, MDA-MB-231 and MDA-MB-435s) was also markedly different (Kang et al. 2010). Several studies have suggested that the anticancer property of DHA is attributable to its ability to induce apoptosis (Chamras et al. 2002, Sun et al. 2008, Calviello et al. 1999, Connolly et al. 1999, Siddiqui et al. 2001). It was also reported that treatment of HL-60 cells with EPA results in caspases 3, 6, 8 and 9 activation, bid cleavage, and cytochrome c release (Arita et al. 2001). It is speculated that omega-3 FAs may preferentially increase Reactive Oxygen Species (ROS) accumulation in the plasma membrane lipid rafts where the assembly of the death-inducing signaling complex (DISC) and the subsequent activation of caspase 8 takes place (Gajate et al. 2009).

Androgens play an important role in proliferation, differentiation, maintenance and function of the prostate (Lee 1996). Evidence shows that androgens are also involved in the development and progression of prostate cancer (Ross et al. 1992). The androgen receptor (AR), is a ligand dependent transcription factor belonging to the nuclear steroid hormone receptor super family (Evans 1988), and is the essential mediator for androgen action. In addition to its physiological functions, the AR plays a critical role in the development of prostate cancer. The DU-145 cell line is a well established androgen responsive prostate cancer cell line. DU-145 cells express the AR and a number of androgen inducible genes such as prostate specific antigen (PSA) and hK2 (Horoszewicz et al. 1983, Montgomery...
DHA decreases androgen simulated DU-145 cell growth. Furthermore, androgen induction of five androgen regulated genes were significantly repressed by DHA at steady state MRA levels. Similarly EPA was able to reduce both the translational and transcriptional levels of PSA and hK2 genes. Hence, it is suggested EPA and DHA treatment inhibit androgen action including cell growth response (Chung et al. 2001).

Fatty acids may modulate prostate carcinogenesis through numerous processes, such as modification of membrane phospholipid composition (Stubbs & Smith 1984), alteration of cell signaling and receptor activity (Sebokova et al. 1988, Kubota et al. 1998, Novak et al. 2003), lipid peroxidation (North et al. 1994), cyclooxygenase inhibition (Ringbom et al. 2001), cytokine production (Jolly et al. 1997), and interference with androgen activity (Liang & Liao 1992). Another possibility is that EPA and DHA have numerous anti-inflammatory properties that have been linked with decreased cancer risk (Healy et al. 2000).

It may be noted that in both extracts, the cytotoxicity marginally decreases after attaining the peak value at a specific concentration – though this phenomenon is unknown among normal drugs, it is displayed by several natural compounds (Scheim 2009).

6.5 Conclusion

In conclusion, widely available marine fishes like sardines serve as a rich source of DHA and EPA that are known to reduce the risk of breast and prostate cancer. These fish oils also have the ability to control the proliferation of cancer cells and hence are potential drug sources for
oncologic pharmaceuticals. It is also clear that the extracts from *S. longiceps* which have a higher content of EPA than DHA, and also more widely available in comparison, seems to possess greater annihilation power as compared to *S. fimbriata*. Mechanism of action of these two compounds is still subject to further study and can vary between cancer cell lines. Such an in-depth study probably will give more insights into the right combination of these fatty acids to be applied for faster impact on different cancer cell lines. This will also mould how a pharmaceutical industry will convert these widely available raw materials into drugs which can be applied for different kinds of cancer conditions.