

ANTICANCER EFFECT OF POLYUNSATURATED FATTY ACID EXTRACTS FROM SARDINES ON HUMAN CANCER CELL LINES

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ABSTRACT

Aim: Investigation and comparison of cytotoxic activity of polyunsaturated fatty acid (PUFA) extracts from two species of marine fishes - *Sardinella longiceps* and *Sardinella fimbriata* - abundant in the west coast of India.

Methods: Polyunsaturated fatty acid (PUFA) isolated from the fishes were subjected to Gas Chromatography for its quantitative analysis. PUFA isolate from the tissue extracts were used to conduct the study. Cytotoxicity of the PUFA extracts on Cell lines MCF-7 (breast cancer) and DU-145 (prostate cancer) were analyzed. Both cell lines were subjected to MTT Assay with extracts at working concentrations of 100, 200, 400, 800, 1000 µg/ml. **Results:** Both extracts showed significant cytotoxic effects against the two cell lines and a peak cytotoxicity of 85-90% was apparent. IC-50 values were calculated from the graphs and it was found that *S. longiceps* extracts had a slightly lower IC-50 value indicating that it is cytotoxic to cancer cells invitro even at a lower concentration as compared to extracts from *S. fimbriata*. **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. 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 with *S. fimbriata*, seems to possess greater annihilation effect on both cancer cell lines.

KEY WORDS

Anticancer; PUFA, DHA, EPA, Cytotoxic, *Sardinella fimbriata*, *Sardinella longiceps*

INTRODUCTION

Breast cancer and prostate cancer are the most prevalent malignancies seen in mankind worldwide. 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. Prostate Cancer is the most common non-skin cancer in the world. The frequency of prostate cancer increases exponentially with advancing 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. Despite the recent developments in cancer therapy, traditional cancer therapies have multiple limitations, leading to treatment failure, cancer relapse and long term adverse effects [1]. These limitations are mainly related to drug resistance by tumor cells and high regeneration potential of cancer stem cells. Hence, it is imperative that more research is expended in finding sources of naturally available anti-cancerous compounds which can aid drugs against breast and prostate cancer with little or no toxicity on normal cells

Nutrition influences cancer etiology in about 35% of cancer cases. Human epidemiologic studies have shown that dietary intake of fish oil may protect against the development of certain cancers including breast, colon, and prostate [2]. Reported benefits of n-3 PUFA dietary supplements given before or during cancer therapy include reversing tumor cell drug resistance [3]; reducing the gastrointestinal, hematological, or cardiac side effects of various chemotherapeutic treatments [4]. 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 tumor cell death but not kill cultured normal cells [5-8]. It is therefore believed that one or both of these n-3 fatty acids is responsible for the beneficial effects of fish oil against tumor growth.

Most cancer studies on PUFA or fish oil have not distinguished between EPA and DHA s assuming 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 [7]. 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 within their ranges. Comparison of their respective PUFA profile with the degree of cytotoxicity is also attempted.

MATERIALS AND METHODS

Fish samples

Fresh samples of *S. longiceps* and *S. fimbriata*, were collected from the Kaalamukku landing centre (9°58'57"N, 76°14'33"E) at Kochi. Samples were washed in sterile water and brought to the laboratory in an ice box within 30 minutes after collection.

Preparation of extracts

The internal organs were removed and the meat sliced. Slices were blended and centrifuged at 10,000 rpm for 15 minutes. Post centrifugation, the oil phase was separated and saponified for converting the triglycerides to free fatty acids. The fatty acid mixture was subjected to Urea Complexing [9] and subsequently to low temperature fractional crystallization [10] to obtain a mixture of substantially pure PUFA.

Determination of Fatty acid composition

The composition of the PUFA in the above mixture was directly analysed by Gas Chromatography (GC) using

fatty acid methyl ester (FAME) method [11]. The fatty acids were separated by gas liquid chromatography (Thermo Trace GC Ultra) equipped with a capillary column (30m long and 0.54mm diameter) and a flame ionization detector in the presence of hydrogen and air. Nitrogen was used as the carrier gas at a flow rate of 0.8ml/min. Initial temperature was set at 70°C and was increased at a rate of 3°C/min until peak temperature of 250°C was reached. Injector and detector temperatures were kept at 260°C and 275°C respectively. Fatty acids separated were identified by the comparison of retention times with those obtained by the separation of a mixture of standard fatty acids. Measurement of peak areas and data processing were carried out by Thermo Chrom card software. Individual fatty acids were expressed as a percentage of total fatty acids.

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 Hi Media 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.

Assessment of Cell Viability

Cell viability was determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay [12]. 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. 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 37°C. When cells had adhered and were about 50% confluent, the serially diluted concentrations of the cytotoxin (100µl each) were added to the wells to get the final concentration of 100, 200, 400, 600, 800, 1000 µg/ml. Control wells were devoid of toxin and replaced with DMSO to get the final concentration of 1%.

After 72 hrs incubation in CO₂ incubator at 37° C, aspirated out 200µl of solution from the wells without disturbing the cells and added 100µl of MTT at a concentration of 1mg/ml of 10% DMEM. Incubated in dark for 2 hrs in CO₂ incubator at 37° C. Added 100µl lysis buffer to dissolve the formazan crystals and incubated in dark for another 4 hrs. Read the plate on ELISA reader at a wavelength of 570 nm to measure optical density (OD).

Calculated percentage of cytotoxicity as per the below formula

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

Statistical Analysis

The obtained results were analyzed using 1-way ANOVA [13] against the control followed by Fisher's LSD test and p<0.01 was considered as significant.

RESULTS & DISCUSSION

The respective cytotoxicity profiles of the two species for MCF-7 are illustrated in Fig: 1 and values indicated in Table: 1; and for DU-145 are illustrated in Fig: 2 and Table: 2; statistically, the means for each of the concentrations were found to be significant with p < 0.01.

Table: 3 tabulates the IC-50 values of the two extracts against both cell lines, along with peak values and corresponding concentrations. 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 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 1: Activity of PUFA extracts of *S. longiceps* and *S. fimbriata* on MCF-7 at different concentrations

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

Table 2: Activity of PUFA extracts of *S. longiceps* and *S. fimbriata* on DU-145 at different concentrations

Conc.	<i>Sardinella longiceps</i>		<i>Sardinella fimbriata</i>	
	OD Mean±SD	Cytotoxicity	OD Mean±SD	Cytotoxicity
0	2.18±0.08	0.00	2.18±0.08	0.00
100	0.25±0.08	88.11	0.54±0.55	74.83
200	0.22±0.01	89.73	0.2±0.04	90.41
400	0.25±0	88.35	0.21±0	90.18
600	0.32±0.04	85.09	0.22±0.01	89.93
800	0.35±0.01	83.80	0.23±0.03	89.13
1000	0.37±0.01	82.83	0.33±0.11	84.52

Table 3: Summary of the cytotoxic activity of PUFA extracts on MCF-7 and DU-145 cell lines

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

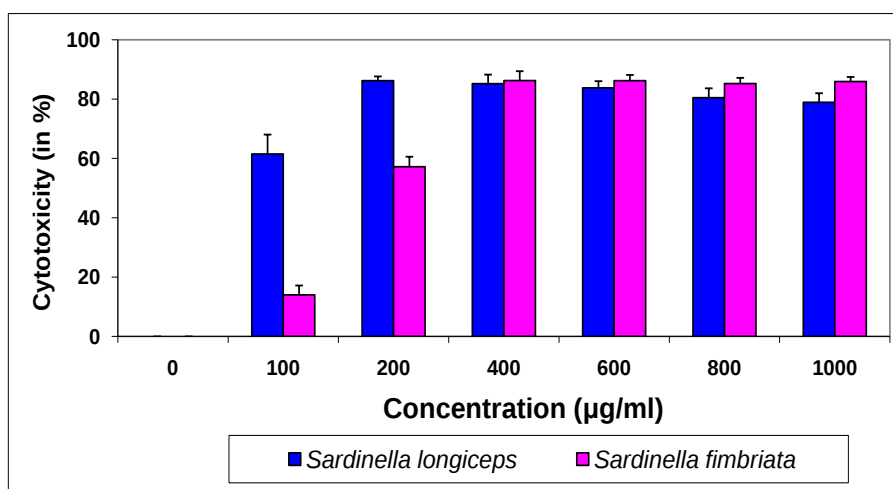


Fig 1: Cytotoxic Activity of PUFA extracts from *S. longiceps* and *S. fimbriata* on MCF-7 cell line

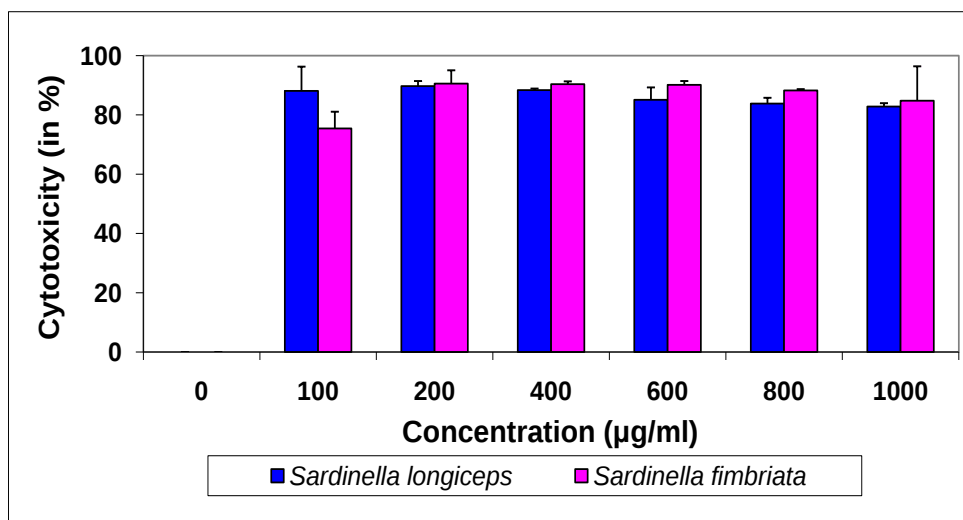


Fig 2: Cytotoxic activity of PUFA extracts from *S. longiceps* and *S. fimbriata* on DU-145 cell line

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 presence 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 figure 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: 3.

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 – the activity itself being dose dependent at lower concentrations. The IC₅₀ value of sardine oil emulsion on human breast cancer cell line MCF-7 was as high as 1000 µg/ml [14],

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 *S. fimbriata* and 81 µg/ml for *S. longiceps*. It can also be concluded that the EPA-rich *S. longiceps* extracts have a greater cytotoxic effect on MCF-7 cells at lower concentrations as compared to the DHA-rich *S. fimbriata* as evident from the IC₅₀ 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 happens [7]. However, recent evidences indicate that the action of both EPA and DHA could be very different and differs with different cell lines.

Many 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 a single, unique activity that is the sole mechanism of action [15]. The formation of cytostatic and cytotoxic compounds after peroxidation of long chain PUFAs has been proposed as the primary mechanism for the activity of n-3 PUFAs against cancers [16-18]. Other mechanisms proposed include the alteration in prostaglandin synthesis [19], alteration in gene transcription [20], suppression of n-6 fatty acid transport [21], and modulation of antioxidant enzymes(AOE) and of apoptosis [22]. EPA inhibits NF- κ B activation and IL-6 production in cancer cells, and there by induce apoptosis [23]. These effects of EPA might have benefitted in achieving improved cytotoxic potential by the PUFA extract from *S. longiceps* as revealed by our study. It may also 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 [24].

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 with *S. fimbriata*, seems to possess greater annihilation power. Mechanism of action of these two compounds is still subject of 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 pharmaceutical industry will convert these widely available raw materials as drug sources and also as adjuvants, to improve the efficacy and tolerability of traditional anticancer therapies.

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