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Evaluation of anti-proliferative effect of sardine oil emulsion on A549 and HCT 15 cancer cell lines

Chinnasamy Arulvasu[1*], Devadoss Dinesh[1], Ramar Manikandan[2a], Mohan Pandi[3], Pappu Srinivasan [2b], Nagarthnam Radakrishnan[4], Subramanian Sellamuthu[1], Durai Prabhu[1] and Gajendran Babu[1]

[1]Department of Zoology, University of Madras, Guindy Campus, Chennai-600 025, India.

[2a]Department of Animal Health and Management, [b]Department of Bioinformatics, Alagappa University, Karaikudi - 630 003, India.

[3]Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai-600 025, India.

[4]Signal transduction Lab, Department of Biotechnology, Indian Institute of Technology Madras,Chennai – 600036, India.

Corres.author: arulvasu_zoo@rediffmail.com
Tele - +91-44-22202836, Fax - +91- 44- 22301003

Abstract: Sardine oil emulsion prepared from *Sardinella longiceps* (Indian oil sardine) was investigated to examine the anticancer properties against human cancer cell lines. The fatty acid profile of sardine oil emulsion was analyzed by gas chomatography (GC). It has significant amount of n-3 polyunsaturated fatty acids (PUFA), which effectively inhibit the cancer cell growth. For cell viability assessment human lung adenocarcinoma epithelial cell line (A549) and human colon cancer line (HCT 15) were treated with different concentrations (2, 4, 6, 8 and 10 mg/ml) of sardine oil emulsion for 48 h incubation. Proliferation of both cancer cells were significantly affected with increase the concentration of sardine oil emulsion. The IC_{50} value of sardine oil emulsion on A549 was 4 mg/ml whereas in HCT 15 it is 8 mg/ml. DNA fragmentation analysis also revealed that sardine oil induced apoptotic cell death of both cancer cell lines.
Keywords: Sardine oil, fish oil, A549, HCT15 and n-3 PUFA

Introduction

Cancer is a still major health problem in both developing and developed countries.[1] It is widely held that 80-90% of human cancers may be attributable to environmental and life style factors.[2] According to World Health Organization (2007) 7.9 million deaths (13% of all deaths) were occurred due to cancer around the world. In which 1.3 million deaths were occurred due to lung cancer and 639,000 deaths occurred due to colon cancer. Tobacco usage is the single most risk factor for lung cancer and also diets as well as environmental factors are important risk factors for colon cancer. The concept of cancer prevention using naturally occurring substances that could be included in the human diet is gaining attention.[3]

Fish oils rich in n-3 fatty acids have attracted attention because of their chemopreventive effects on carcinogenesis for decades. Epidemiological studies have shown that consumption of fish is correlated with lower incidence of cancer.[4, 5] Human populations consuming high amount of n-3 fatty acids have lower incidence of breast, prostate and colon cancers than those who consume low amount of n-3 fatty acids.[6,7] Eicosapentaenoic acid (EPA- 20:5, n-3) and

Docosahexaenoic acid (DHA- 22: 6, n-3) are the important n-3 PUFA readily oxidized in the presence of free radicals because they have additional carbon-carbon double bonds compared with other fatty acids.[8] n-3 PUFAs also have some special biological effects, such as decreasing superoxide generation in neutrophils, inhibiting cancer cell growth, increasing lipid peroxidation, regulating vasorelaxation by a prostaglandin-dependent pathway and inducing apoptosis.[9] In the present study, we attempt to examine the anti-proliferative effect of sardine oil emulsion against A549 (human lung adenocarcinoma epithelial cell line) and HCT 15 (human colon cancer line) cancer cell lines.

Materials and methods

Extraction of sardine oil

The sardine oil was prepared by solvent extraction method described as Immanuel et al.[10] Briefly, the grained fish was continuously agitated with acetone (Hi Media Laboratories, Mumbai) for 24 h. The solvent phase was collected and evaporated for 6-8 h at 35 °C. After complete the evaporation sardine oil was collected at bottom of the flask. It was filtered for purification and stored at -20°C in dark container.

Preparation of Sardine oil emulsion

Sardine oil emulsion was prepared by adopting the method of Tamaru et al.[11]. 20 ml of sardine oil was heated at 30°C for 5 min. To this 1ml of egg-yolk (emulsifier) and 20 ml of Milli Q water added. The mixer was homogenized using cyclo-mixer (Remi equipments) for 20 min. The oil emulsion was observed under a microscope to check homogeneity in distribution and the emulsion was sterilized using membrane filters (0.22 µm). The peroxidation value of the emulsion was determined by the AOAC Official method[12] prior to use in cell line treatment.

Fatty acid analysis

Fatty acid composition of sardine oil emulsion was analyzed and standardized in our laboratory by Arulvasu and Munuswamy[13].100 mg of sardine oil was saponified with 2 ml of sodium methoxide in methanol (0.5N) and incubated 10 min in a boiling water bath. The solution was cooled at room temperature and to this 2 ml of boron trifluoride/methanol complex (14%) was added. The solution was then heated for 20 min in boiling water bath (80°C). After cooling, 1 ml of hexane was added, and the mixture was heated for another 2 min and cooled at room temperature. To this 1.25 ml of saturated sodium chloride was added. The mixture was shaken vigorously and after phase separation, the organic layer was separated and transferred to a 2 ml vial containing 1 mg of anhydrous sodium sulfate. The fatty acid methyl esters were analyzed using a Hewlett Packard 5890 gas chomatograph equipped with a fused silica capillary column (12 m long × 0.22 mm inner diameter) coated with BPX70. The oven temperature was 180 °C and helium was used as the carrier gas at 2 ml/min. Integrated peak areas of the fatty acid metyl esters were identified by comparison with known standards. Results are expressed as the percent (w/w) of total fatty acids detected.

Cell culture

Cancer cell lines A549 and HCT 15 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% CO_2 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 subcultured every 6 days.

Assessment of cell viability

Cell viability was determined using MTT (3-(4, 5- dimethylthiazol-2yl)-2, 5- diphenyltetrazolium bromide) assay according to a previously described protocol Mosmann.[14] In order to detect the cytotoxicity of sardine oil emulsion, A549 and HCT 15 cells were treated with sardine oil emulsion at the concentration of 2, 4, 6, 8 and 10 mg/ml for 48 h incubation time. The intensity of formazan, reduced product of MTT (HiMedia Laboratories, Mumbai, India) after reaction with active mitochondria of live cells, was determined by measuring the absorbance in 96 well microplate reader (Bio-Tek, Powerwave XS, USA) at a wavelength of 570 nm. Results were expressed as percentage inhibition considering absorbance control cells as 100% viable.

Cell morphological studies

General morphological structure of cell was examined to the effect of sardine oil emulsion on both A549 and HCT 15 cell lines, for that, cells were cultured in 100 mm dishes. After treated with sardine oil emulsion (48 h incubation) cells were photographed under inverted light microscope (Nikon, Sclipse TS 100) at 40X magnification.

Propidium iodide staining method

For the propidium iodide staining both A549 and HCT 15 cells were plated at 5×10^4 cells/well into a six well chamber plate. The cells were treated with sardine oil emulsion for 48 h. The cells were washed with PBS and fixed in methanol:acetic acid (3:1 v/v) for 10 min and stained with 50 µg/ml of propidium iodide (HiMedia

Laboratories, Mumbai, India) for 20 min. After staining, the cells were visualized immediately under the fluorescence microscope (Axioskope2, Carl Zeiss) at 10X magnification.

DNA fragmentation analysis

Fragmentation of chomatin to units of single or multiple nucleosomes that form the nucleosomal DNA ladder in agarose gel is an established hallmark of programmed cell death or apoptosis. Briefly, the cells were cultured in 100 mm dishes, treated with sardine oil for 48 h. Following this treatment, the cells were washed with PBS (pH 7.5), harvested and pelleted by centrifugation (12000 rpm) at 4 ˚C. The pellet was incubated with DNA lysis buffer [10 nM Tris pH (7.5), 400 mM NaCl, 1mM EDTA and1% Triton X-100] for 30 min on ice and then centrifuged at 12000 rpm. The supernatant that was obtained was incubated overnight with RNase (0.2mg/ml) at room temperature and then with proteinase K (0.1mg/ml) for 2 h at 37 ˚C. DNA was extracted using phenol:choloroform:isoamylalcohol (25:24:1) mixture and precipitated with 0.1M of sodium acetate and 2 volume of absolute ethanol. Equal amount of DNA samples (20 µg) were electrophoresed on a 1.5% agarose gel in Tris-borate EDTA buffer and visualized by ethidium bromide staining.[15]

Statistical analysis

Statistical analyses were performed using the SPSS commercial statistical package (SPSS, version 16.0 for windows, SPSS Inc., Chicago, USA) and the significant means were compared using the Duncan test.

Result and Discussion

Fatty acid composition of sardine oil emulsion

Sardine oil extracted with solvent from the fish was 5.46 g/100 g wet weight. GC analysis showed the fatty acid composition of sardine oil emulsion was listed in Table 1. There is mixture of saturated, monounsaturated and polyunsaturated fatty acids are present. The amount of PUFAs are 49%, among PUFAs 45% are alone n-3 PUFAs.

Sardine oil on cell viability

In order for a therapeutic agent to be truly effective, it should be toxic to tumor cells with out harming normal cells. From the literature, it seems as though fatty acids fulfill this criterion. To assess the effect of sardine oil emulsion on viability of the cells, different concentrations were tested on both cell lines. In A549 cells the IC_{50} value was observed in 4 mg/ml of sardine oil emulsion concentration at 48 h incubation, where as in HCT 15 it is 8 mg/ml of sardine oil emulsion concentration (Fig. 1). It indicates sardine oil emulsion has mild effect on human colon cancer cell line when compare with human lung adenocarcinoma cell line. According to Ueda et al.[16] the IC_{50} value of sardine oil emulsion on human breast cancer cell line MCF-7 was 1mg/ml. interestingly there was very mild effect on human hepatocarcinoma cell line HepG2. The exact mechanisms are not identified why this kind of effect variation among different cancer cell lines. The cytotoxicity effect of sardine oil may due to the presence of n-3PUFA. Because n-3 PUFA have the cytotoxicity effect via the oxidative stress due to its free carbon-carbon bond.[16]

Morphological variation of cells

Sardine oil emulsion induced changes in cellular morphology were documented by means of inverted microscopy. Microscopically the normal A549 and HCT 15 cells (Fig. 2a and 2b) appear healthily, polygonal in shape and attached to the well plate. After 48 h treatment with sardine oil emulsion, noticeable changes in the morphology and density of A549 and HCT 15 cells were observed (Fig.2c and 2d). Morphological assessment of treated A549 (4 mg/ml) and HCT 15 (8 mg/ml) cells clearly indicated the play of apoptotic mechanisms leading to death. Morphological changes including cell shrinkage and loss of colony formation ability were observed. The treated cells appeared rounded off, shrunken and detached. Siddiqui et al.[17] recorded DHA induced apoptosis of cancer cell line on membrane-originated processes. Our sardine oil emulsion has significant amount of DHA it may act on cell membranes of A549 and HCT 15 to made shinkages on the cells.

Propidium iodide staining method

Cell death of A549 and HCT 15 cell lines upon sardine oil emulsion treatment was detected using Propidium iodide (PI) staining method. In this method relies upon the fact that PI is impermeable to cell with an intact plasma membrane, however when cell integrity becomes compromised it gains access to the nucleus where it complexes with DNA rendering the nucleus highly fluorescent.[18] Figure 3a and 3d shows the sardine oil emulsion untreated control cells of A549 and HCT15 cell lines. Here PI very negligible amount of PI positive cells were present. Figure 3b and 3e shows the moderate amount of PI positive cells of A549 and HCT 15 cell lines treated with 4 mg/ml and 8 mg/ml of sardine oil emulsion respectively. In both the cancer cell lines increased in the concentration of sardine oil emulsion number of PI positive cells were increased significantly (Fig. 3c and 3f). This may due to the presence of EPA and DHA in the sardine oil emulsion. Because EPA and DHA are readily oxidized in the presence of free radicals because they have

additional carbon-carbon double bonds compared with other fatty acids.[8]

Effect of sardine oil on cellular DNA:

DNA fragmentation analysis is a typical assay to find out the drug induced apoptosis cell death.[19] 48 h incubation of A549 and HCT 15 cells with sardine oil emulsion induced fragmentation of cellular DNA in a concentration and time depend manner (fig. 4). In case of control in both the cell lines there is no fragmentation was appeared in both cell lines. DNA fragmentations were appeared at 4 mg/ml and 8 mg/ml concentrations of sardine oil emulsion in A549 and HCT 15 cells respectively. When concentration was increased in both the cell lines the number of fragmentations was more. Similar effect was recorded by Yonezawa et al.[19] on cancer cell lines using EPA and DHA an n-3 PUFAs. Our sardine oil has significant amount of these n-3 PUFAs, it may induced apoptotic cell death.

Based on our results we summarized sardine oil emulsion has significant amount of n-3 PUFA, which are able to inhibit the proliferation of cancer cells. The inhibitory effect of sardine oil emulsion on A549 and HCT 15 cell lines are dose and time dependent manner. A possible explanation for the inhibitory/cytotoxicity of PUFA would be the surfactant nature of the fatty acid molecules. Because, PUFA interact with cellular membranes and modulate membrane bound enzymes, there by inhibiting a sequence of events leading to apoptosis. This possibility should also be studied further.

Table 1: Selected Fatty acid composition (percentage of total fatty acids) of sardine oil emulsion

Fatty Acids	Common name	Composition (%)
14:0	Myristic acid	3.90
16:0	Palmitic acid	16.51
18:0	Stearic acid	2.41
18:1n-9	Oleic acid	13.90
18:2n-6	Linoleic acid	18.21
18:3n-3	Linolenic acid	7.24
20:5n-3	Eicosapentaenoic acid	6.01
22:6n-3	Docosahexaenoic acid	8.92
	Others	22.90

Figure 1: Effect of sardine oil emulsion on the growth of A549 and HCT 15 cells.
MTT assay were performed with sardine oil A549 (diamond) and HCT 15 (square) cells. Results are expressed as a percentage of the control without treatment of sardine oil. Each point represents the mean ± S.D. of 5 independent experiments. $^{*}P< 0.05$ significant with control

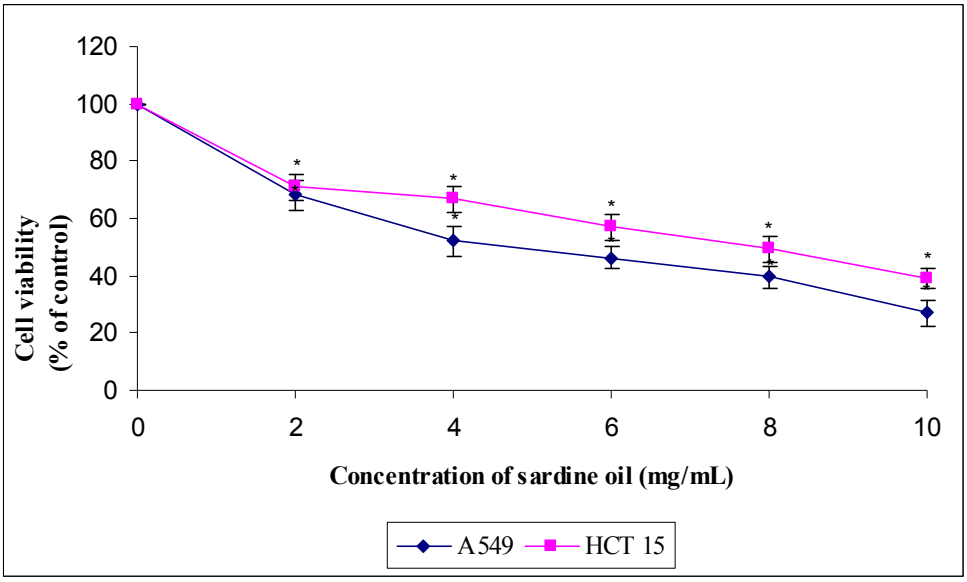


Figure 2: Morphological variations of cells.
Control A549 cells **& B-** Control HCT 15 cells **C-** Sardine oil emulsion treated A549 cells **& D-** Sardine oil emulsion treated HCT 15 cells. In both the cases the control cells appeared healthy and polygonal in nature but in sardine oil treated group cells shrinkage takes place.

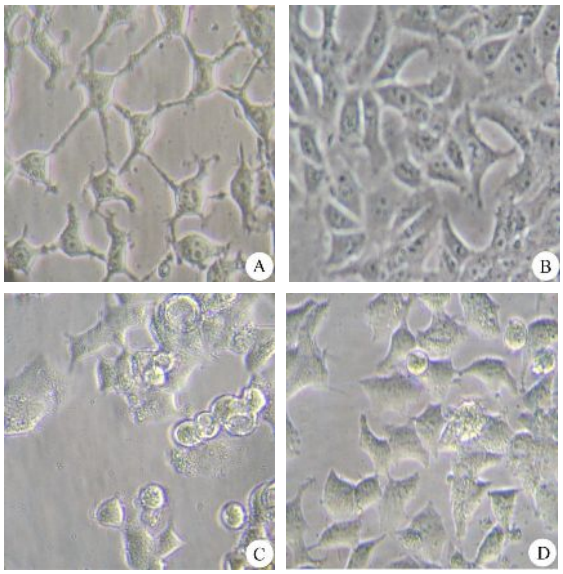


Figure 3: Propidium iodide stained cells.
A-C showed the A549 cells coupled with PI & D-F showed the HCT 15 cells coupled with PI. In both the cell lines there was negligible amount of PI positive cells are recorded in control cells. The number PI positive cells were increased when concentration of sardine oil emulsion was increased.

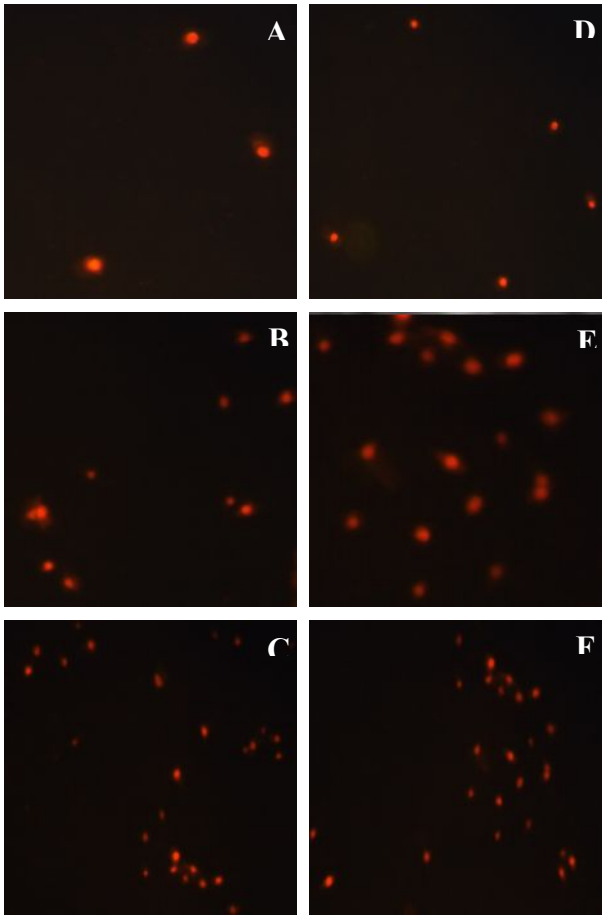
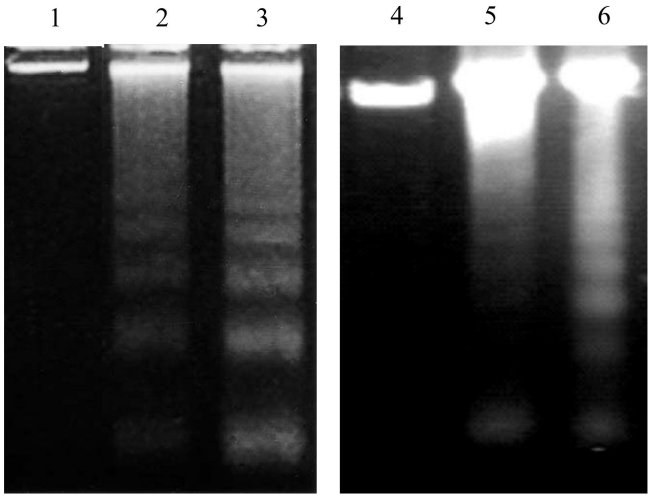


Figure 4: Sardine oil emulsion induced DNA fragmentation.
Lane 1: control cells of A549; Lane 2: cells treated with 4 mg/ml of sardine oil emulsion; Lane 3: cells treated with 10 mg/ml of sardine oil emulsion; Lane 4: control cells of HCT 15; Lane 5: cells treated with 8 mg/ml of sardine oil emulsion; Lane 6: cells treated with 10 mg/L of sardine oil emulsion.

Figure. 4



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