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FISH OIL INDUCES APOPTOSIS IN OSTEOSARCOMA CELLS THROUGH ROS GENERATION AND INCREASE OF CASPASE 3 AND 7 ACTIVITY

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ABSTRACT

Omega-3 Polyunsaturated fatty acids (PUFAs), mainly present in fish oil, are part of the human diet. Fish oil has received particular attention for its anti-inflammatory, antiproliferative, proapoptotic, antiangiogenetic, anti-invasion, and antimetastatic properties. MG-63 cells were cultured and treated with different concentrations (10-100 µg/ml) of fish oil. Cells viability analysis by MTT assay, intracellular ROS generation, nuclear condensation, JC-1 for MMP, caspase 3 and 7 and annexin FITC assay for apoptosis were carried out. Results of cells viability data showed that fish oil changes the characteristic morphology of cells and also decreased the cell number in a concentration-dependent manner. The results from fluorescent microscopic data of nuclear condensation, apoptosis and intracellular ROS generation revealed that fish oil content significantly induced the level of ROS and led to nuclear apoptosis.

KEYWORDS*:* Osteosarcoma Cell (MG-63), ROS, JC-1, Caspase 3 and 7, Annexin FITC.

Cancer is known as major health problem worldwide; thus it is increasing regardless of intensive and including for research and drug development. Among cancer, bone cancer is one of the third most common malignancies in women globally, although it is more prevalent in the developing countries (Kumar et al., 2006). Osteosarcoma is a bone cancer typically develops in the tibia near the knee, the thighbone (femur) or the upper arm bone (humerus) near the shoulder. It is the most common type of bone cancer in children. Osteosarcoma tends to develop during growth spurts in early adolescence. During this period of rapid bone growth it may increases risk of tumours in children, the average age of diagnosis is 15. Bone cancer can be seen in adults over the age of 60 and it can also be seen in people who have undergone radiation for cancer treatment. Individuals who have a family history of cancers and who have retinoblastoma, a cancer of the retina of the eye, have a higher incidence of sarcoma. The incidence rates of primary osteosarcoma and of osteosarcomas that occurred as a second or later cancer were highest among blacks. The annual incidence of osteosarcoma reported in African Americans is 5.2 cases per million populations and in Caucasians are 4.6 cases per million populations younger than 20 years (Mirabello et al., 2009). The incidence of bone cancer having Paget's disease was highest in whites in the oldest age group. The average male to female ratio of osteosarcoma was 1.22:1.00. Dietary fish oil (FO) has been shown to have beneficial effects on some chronic degenerative diseases such as cardiovascular disease (Xin et al*.,* 2012; S. Car and R. Webel., 2012) rheumatoid arthritis (E. A. Miles and P. C. Calder, 2012).diabetes (I. Rudkowska., 2010) other autoimmune diseases (R. S. Chapkin et al*.*, 2009; P. C. Calder., 2007), and cancer (V. C. Vaughan, et al*.,* 2013; J. Cockbain et al., 2012). The beneficial effects of Fish oil seem to be due to its high content of the omega-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). EPA is a long-chain omega-3 PUFA that has 20 carbon atoms and 5 double bonds (20:5); DHA has a longer chain, 22 carbon atoms and 6 double bounds (22:6). Both agents are essential fatty acids (FAs) that cannot be synthesized by mammals and thus must be obtained from dietary sources. The potential use of fish oil for the enhancement of the efficacy of anticancer treatments in relation to its ability to enhance the uptake of anticancer drugs, regulate the oxidative status of tumour cells, and inhibit tumour cell invasion and metastasis

MATERIALS AND METHODS

Culture of Human Osteosarcoma Cell Line

Human osteosarcoma MG-63 cell line were obtained from cell repository-National Centre for Cell Sciences, Pune, India. Fish oil was purchased from market. MG-63 cells were cultured in EMEM medium (Himedia with 2.0 mM L-glutamine, 1.0% penicillin and streptomycin solution adjusted to contain 1.5 g/l NaHCO3 and 10% fetal bovine serum (Himedia). Cells were grown at 37ºC, 5% CO_2 in a humidified air.

Cell Proliferation/ Cytotoxicity Assay

This assay is based on the enzymatic reduction phenomenon of 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT) dye and provides a direct relationship between the viable cells and absorbance. To evaluate the antiproliferative effect of fish oil thus MTT assay was performed with some

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modification. In brief, MG-63 cells were trypsinized and 2×10^3 cells were seeded per well in 96-well culture-plate in 100 µl required complete media. Cells were incubated overnight at 37°C and 5% CO2. Cells were then treated with fish oil treatments prepared in required medium at different concentrations for 24h. After each treatment period, 10 µl of MTT reagent was added to each well and the plate was reincubated at 37°C for 3 h until formazan blue crystals were developed. Supernatant was discarded from each well and 100 µl of DMSO was added to dissolve the dark blue formazan crystals at 37°C for 10 min. The absorbance was recorded at 540 nm by a microplate reader (BIORAD-680). The percentage cell viability was calculated by using the formula:

% Cell viability = [(OD of treated) / (OD of control)] × 100

The cellular morphology was observed after 48 h of total lipid treatment under trinocular inverted phase contrast microscopy (Nikon ECLIPSE T*i*-S, Japan).

Measurement of ROS Production

Bone cancer cell line MG-63 were seeded in 96-well plate and exposed to Fish oil for 6 h. Microscopic fluorescence imaging was used to study ROS generation in MG-63 cells after exposure to different concentrations of fish oil (Ahmad et. al., 2015). Cells were incubated with 10 mM 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min at 37°C. The reaction mixture was aspirated and replaced by 200 µl of PBS in each well. The plate was kept on a shaker for 10 min at room temperature in the dark. An inverted fluorescent microscope was used to visualize intracellular fluorescence of cells and images were taken. For quantitative ROS analysis, PC-3 cells (1×10^4 per well) MG-63 cells were treated with different concentrations of fish oil for 6 h. After exposure, cells were incubated with 10 mM DCFH-DA for 30 min at 37°C. Fluorescence intensity was measured by multi well micro-plate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek) at excitation wavelength of 485 nm and emission wavelength of 528 nm. Values were expressed as the percentage of fluorescence intensity wells.

DAPI Staining for Apoptosis Analysis

The apoptotic effects of Fish oil were analyzed by using 4′, 6′-diamidino-2 phenylindole (DAPI) fluorescent nuclear dye (Kaleem et al., 2015). The cells were fixed in 4% paraformaldehyde for 10 min after washing in PBS. The cells were permealized with buffer (3% paraformaldehyde and 0.5% Triton X -100) and stained with DAPI dye. The images were taken and number of cells was quantified using a fluorescent microscope.

Assessment of Mitochondrial Membrane Potential

The mitochondrial membrane potential decreases during apoptosis, Flouroprobe 5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidazol-carbocyanine iodide (JC-1) is a cationic and liophilic dye, due to its cationic nature this dye has been extensively used to study the mitochondrial membrane potential (Kapoor and Kakkar, 2012). Normal or untreated cells have high membrane potential (polarized mitochondria) this dye concentrates in the matrix of mitochondria, and forms red fluorescent aggregates (J-aggregates) in mitochondrial matrix. Any event that dissipated the mitochondrial membrane potential (depolarized mitochondria) prevents the accumulation of the JC-1 dye in the mitochondrial matrix or in mitochondria and thus, the dye is distributed throughout the entire cell leading to a shift from red (J-aggregates) to green fluorescence (JC-1 monomers). A decrease in red/green ratio is indicative of apoptosis. For this assay MG-63 cells were grown in 96-well plate and treated with two different concentration of fish oil (25µg/ml and 50µg/ml). After 24 h exposure, the treated cells were washed with PBS and stained with 2 mg/ml of JC-1 dye in required media without at 37°C in dark for 30 min. The photographs were then taken by inverted fluorescent phase contrast microscope and the mitochondrial depolarization patterns of cells for cells quantification were examined by using imaging software NIS-Elements F 4.00.00.

Annexin V-FITC/PI Stained Fluorescence Microscopy

This is an apoptosis detection technique, which was performed by using Annexin V-FITC/PI following previously described protocol with some modification (Looi et al., 2013). For *in vitro* fluorescent staining, $1x10^4$cells per well MG-63 cells were seeded in 96 well-plate overnight. MG-63 cells with different treatments at two different concentrations (25µg/ml and 50µg/ml) for 24 hour. Live cells were stained with FITC-annexin V (BD Biosciences, San Jose, CA) for 15 min in 1x biding buffer and then 5 µl of PI (Himedia) for 15 min at room temperature in the dark. Cells were washed three times with PBS and the fluorescent images were acquired using inverted fluorescence microscope with a camera (Nikon ECLIPSE Ti-S, Japan).

Analysis of Caspase-3 and 7 Activity

The caspase-3 and 7 activity was assayed using Caspase-3 and 7 Colorimetric Assay Kit Catalog No:V35118 (treated and untreated) of cells were resuspended with chilled lysis buffer. The cell lysate was incubated on ice for 10 min prior to centrifugation (10,0006g for 1 min). The reaction buffer with 10 mM DTT was added to the supernatant of cell lysate and incubated further for 30 min on ice. The cell lysate (50 ml) was aliquoted into wells of 96-well microplate and 50 ml of reaction buffer containing 10 mM DTT was then added to the lysate. About 5 ml of 4mM DEVD-pNA substrate was added in each wells and incubated at 37^{O}C for 2h.Absorbance at 405 nm was then read in a microplate reader. The absorbance of treated cells was compared with untreated control to find the change in caspase-3 and 7 activity.

Statistical Analysis

All the data were presented as the means±SEM of results achieved after experiment from three or four cultures. Statistical analysis was performed by ANOVA method followed by the Newman–Keuls test of significance using Graph Pad Prism 5 software. Probability values of $p \leq 0.05$ was considered to be statistically significant.

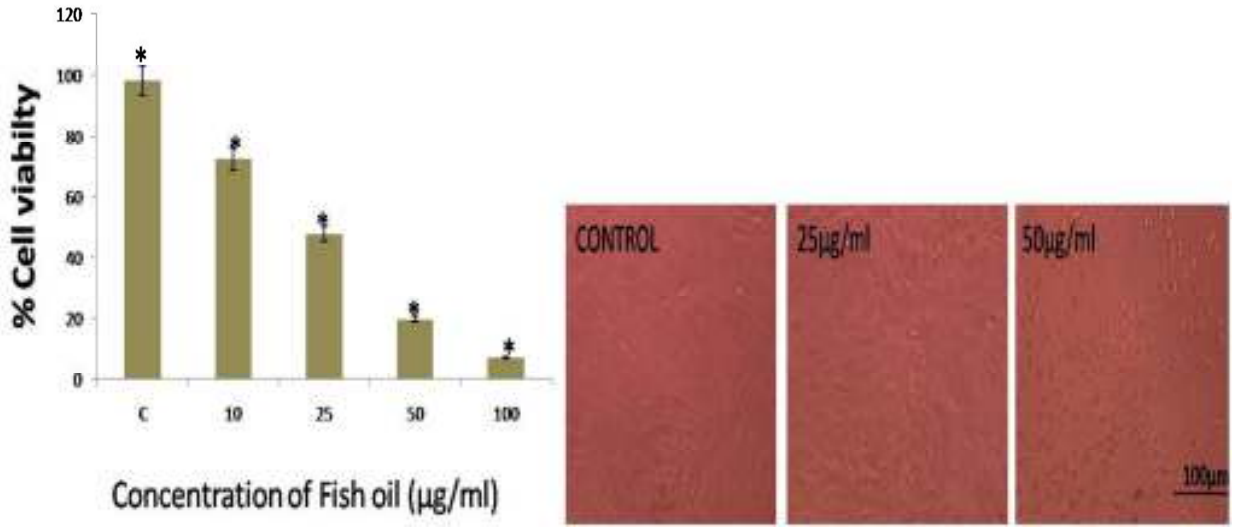
RESULTS AND DISCUSSION

Effect of Fish Oil in MG-63 Cells.

To find out the experimental doses of fish oil, cytotoxic tests were performed on MG-63 cells using MTT assay and cell morphology assessment. At higher concentration of 50µg/ml dose induces cell death and changes cell morphology, cell death was observed at 50µg/ml concentration of fish oil.

ROS Generation in MG-63 Cells

Excessive ROS production caused to oxidation of macromolecules resulted in cellular damage of MG-63 cells. The treatment of MG-63 cells with fish oil for 6 h resulted in a dose-dependent enhancement of ROS generation by increasing the intensity of DCF fluorescence as compared with the untreated cells (Fig. 2A). The fish oil treatment at concentrations of 25 µg/ml and 50 µg/ml increased ROS level. This result suggested that fish oil induced the cell death by oxidative stress as shown in Fig.2.



(A) **(B)**

Figure 1: MTT assay of fish oil against MG-63 cells. Fish oil induces Morphological changes and inhibits cell viability of MG-63 cells. (A) Morphological view of live and dead cells of MG-63 cell line treated with 25µg/ml to 50µg/ml concentrations of fish oil. Photomicrographs were taken by inverted phase contrast microscope. (B) The percent cell viability of MG-63 cells measured by a MTT assay at 24 h as described in the experimental section. Values are expressed as means ± SEM of at least three independent experiments as compared with their respective control.

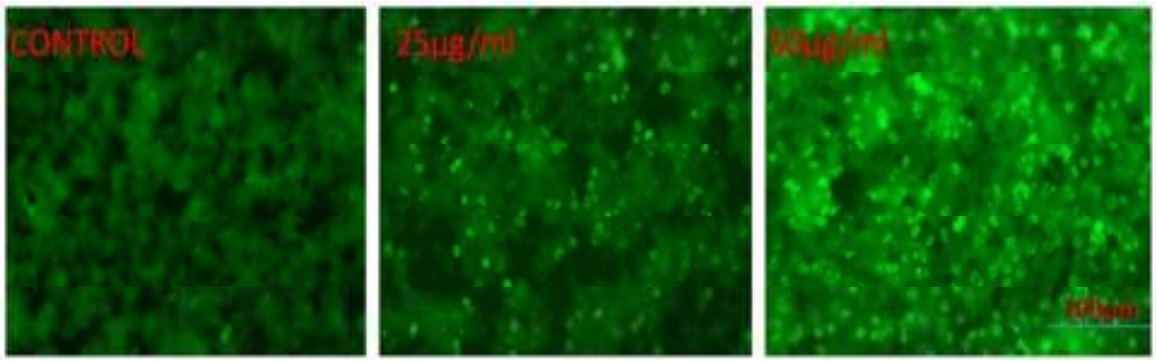


Figure 2: ROS generation assay of fish oil against MG-63 cells. (A) Photomicrographs showing intracellular ROS generation induced by 25µg/ml and 50µg/ml of fish oil in MG-63 cell line. Photomicrographs were taken by florescence phase contrast microscope. (B) Quantitative data were expressed as the percentage of fluorescence intensity relative to the control in MG-63 cells. Values are expressed as means ± SEM of at least three independent experiments, $^{*}p < 0.05$ as compared with their respective control.

Nuclear Change with Apoptosis in MG-63 Cells

Fish oil was tested to ascertain the DNA damage as following exposure of various concentration of compound to MG-63 cells. A dose dependent nuclear condensation in MG-63 cells induced by the fish oil was recorded as shown in Fig. 3. The maximum chromatin condensation was observed in the cells treated at 50 µg/ml of fish oil.

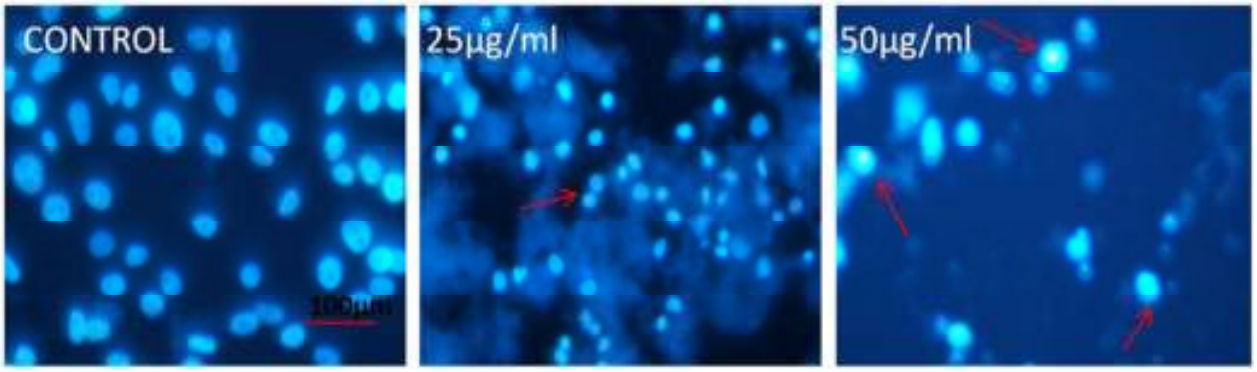


Figure 3: Chromatin condensation of MG-63 cells stained with DAPI after fish oil treatment. Cells were treated with 25µg/ml and 50µg/ml of fish oil. Photomicrographs were taken by fluorescence phase contrast microscope that showed nuclear fragmented in cells.

Fish Oil Modulates Mitochondrial Membrane Potential of MG-63 Cells

The increased and decreased florescent intensity of red and green florescent caused by JC-1 indicates the change in mitochondrial membrane potential. Result indicated that cells treated with 25µg/ml and 50µg/ml of Fish oil induced a strong green and low red fluorescence is generated at a dose dependent manner in Fig. 4.

Annexin FITC Staining for Apoptosis

Our results indicated that cell treated with 25µg/ml and 50µg/ml of fish oil induced the green and red fluorescence which lead to increase the cell death. maximum fuorescence green and red occur at 50µg/ml dose as compared to their control as shown in Fig. 5 thus it leading to apoptotic cell death.

Fish oil Induces Caspase-3 and 7 Activity in MG-63 Treated Cells

The activity of caspase (cysteine-dependent aspartate-specific proteinase), an important biochemical feature in apoptotic signaling, was further investigated to determine whether the apoptosis was induced by fish oil. Caspase-3 and 7 is the main downstream effecter caspase in apoptotic pathway and hence we compared its activity in treated and untreated control cells. The result showed that caspase-3 and 7 was significantly increased the apoptosis with increased the concentration of fish oil (Fig. 6).

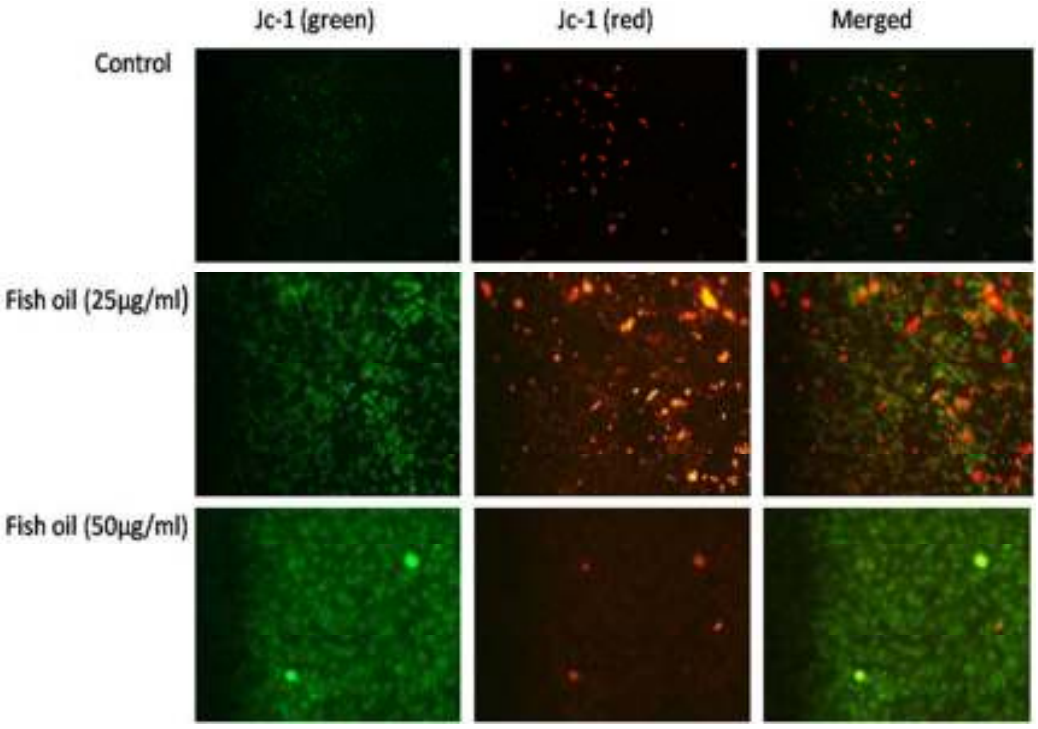


Figure 4: Photograph showing JC-1 red, JC-1 green and merge image. The JC-1 green fluorescence indicates a decrease in mitochondrial membrane potential, an early event in apoptosis. Increased concentrations of fish oil attenuated the loss of mitochondrial membrane potential.

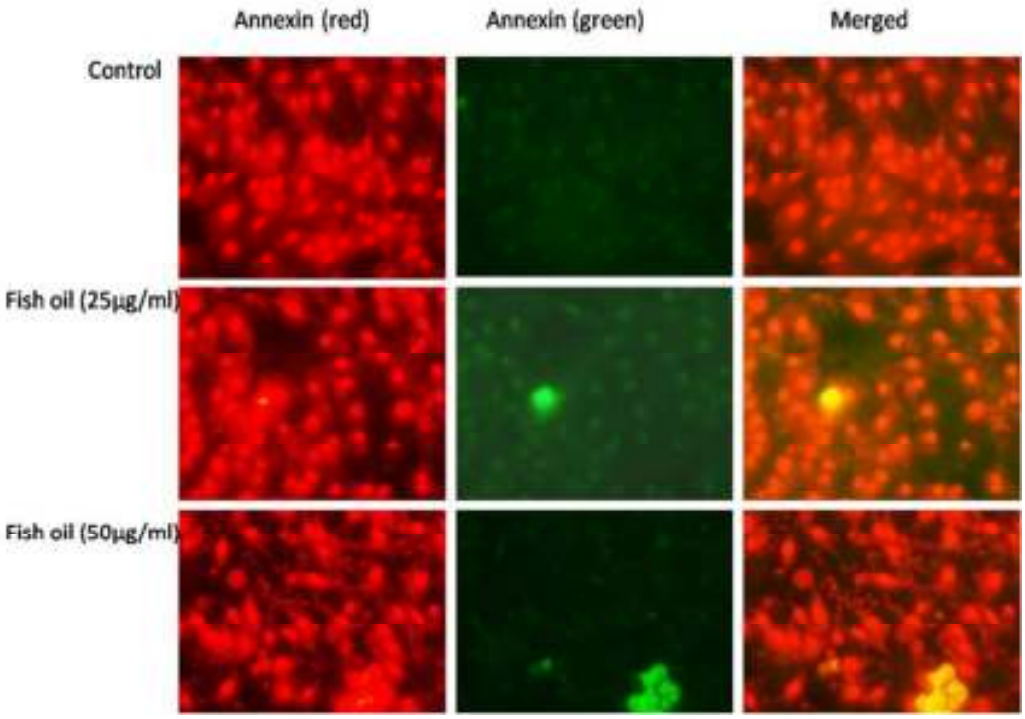


Figure 5: Photomicrographs of fish oil treatment showing extensive ANNEXIN FITC staining technique for apoptosis.

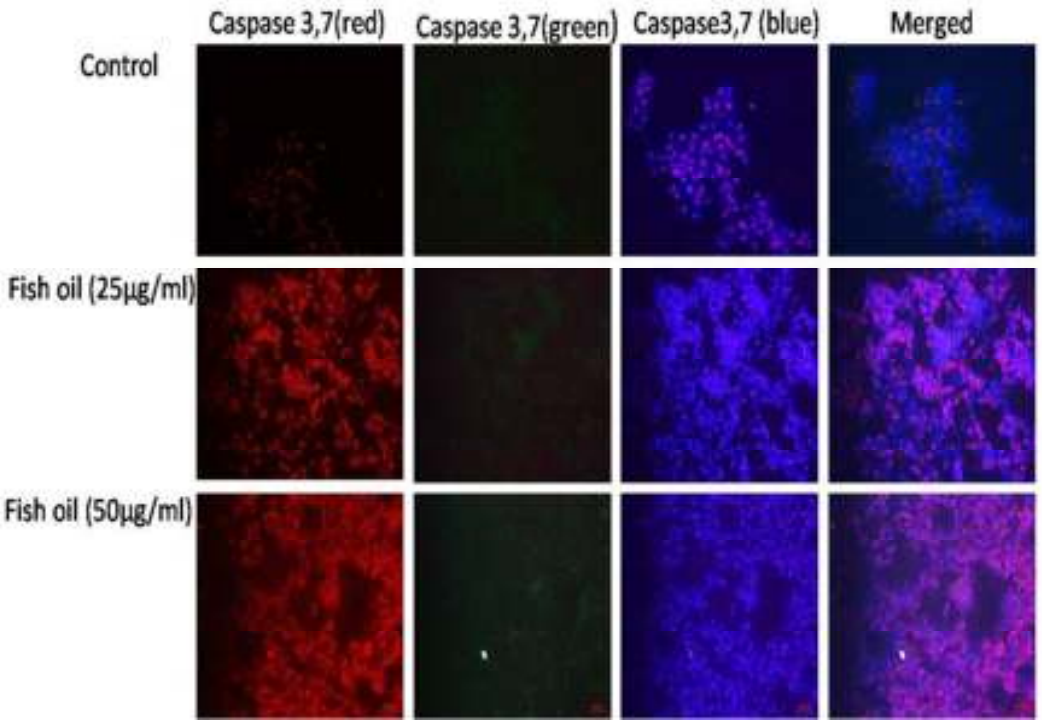


Figure 6: Photomicrographs of fish oil treatment showing extensive Caspase 3, 7 staining technique for apoptosis.

CONCLUSION

Fish oil has been demonstrated significant potential as anticancer therapeutic agents due to their ability to inhibit tumor growth, angiogenesis, and metastasis without many side effect (Cragg, et al.,1997). The MG-63 cancer cell line showed a significant reduction in cell number following treatment with fish oil. In the present study resulted from both the inhibition of cell growth and variation in cells characteristics because of induction of apoptosis. Apoptosis is the characteristic feature of nuclear condensation, protrusion of cell membrane and fragmentation of cells.

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