

Linoelaidic acid exhibits antitumor activity towards MDA-MB-231 cancer cell-induced tumor mice model through modulation of glutathione level and activation of Caspase family

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ABSTRACT

Novel therapeutic approaches are desperately needed because cancer continues to be a major health burden. The purpose of this study was to examine the anticancer potential of linoelaidic acid in a model of tumor-induced mice. This study aims to highlight the vital significance of marine sources of lipids, particularly bioactive polyunsaturated fatty acids, as sustainable substitutes for conventional sources. It also highlights their potential to address global nutritional challenges and support the development of next-generation functional products that promote health through their potential to prevent cancer. Here we studied how well linoelaidic acid inhibits tumor development and induces apoptosis in a cancer model in mice (C57BL/6 J). Compound linoelaidic acid (Treatment- I, II and III) was administered to animals for 56 days after MDA-MB-231 cells were injected into C57BL/6 J mice to create tumor-induced mouse models. Tumor development, apoptosis, and biochemical parameters were evaluated by checking the P53 activation, DNA fragmentation. With a maximal inhibition of 73.13% at 0.014 mg/kg body weight/day (5 μ M), linoelaidic acid dramatically decreased tumor weight and volume. The TUNEL assay showed that treated mice had higher levels of p53 and caspase-3 along with enhanced apoptosis. Improved antioxidant status and decreased lipid peroxidation were revealed by biochemical analysis. Linoelaidic acid can significantly cause apoptosis in tumors injected with MDA-MB-231 in C57BL/6 J mice model. In this MDA-MB-231-inoculated tumor, caspase and p53 activation preferentially mediates the production of apoptosis. These findings necessitate more research to determine whether linoelaidic acid extracted from tapra fish (*Opisthopterus tardoore*) oil can be used as a dietary supplement to prevent and treat breast cancer.

Introduction

Metabolic reprogramming is one of the key indicators that sets tumor cells apart from healthy cells. Among these, the reprogramming of fatty acid metabolism provides tumor cells with selective growth advantages to withstand hostile microenvironments while also supporting the fuel supply for energy and meeting the demands of fast biosynthesis. Early study revealed that the mechanisms of fatty acid metabolic reprogramming in tumor cells and the series of responses (such as active

ferroptosis signalling), analyse the potential impacts on various cancers and focus on the significance of targeting fatty acid metabolic pathways and dietary control in cancer treatment (Roopashree et al., 2024). The use of radiotherapy, chemotherapy, and surgery as therapeutic approaches in the treatment of cancer is associated with several adverse consequences. The omega-6 FAs, widely available in the daily diet, are essential cellular components that play important roles in diverse physio-pathological processes (Amin et al., 2010). Marine organisms like fishes, algae, sea weeds represents significant sources of unique

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omega 3, 6 & 9 fatty acid profiles with significant anti-inflammatory, Cardioprotective and anticancer properties. Tapra fish (*Opisthopterus tardoore*), which are mostly available at southern coastal part of West Bengal (Digha Mohana, Sankarpur, Tajpur, etc.), contain many kinds of fatty acids, including 9-Octadecenoic acid, 9-cis,11-transoctadecadienoate, Linoelaidic acid, trans-9-Octadecenoic acid, pentyl ester, etc (Pradhan et al., 2020). Linoelaidic acid, (a trans-isomer of linoleic acid, 18:2 n-6 trans) found in different marine resources including *Opisthopterus tardoore* oil which showed anticancer potentiality against MCF 7 breast cancer cell line (Dutta et al., 2023). Recent challenges including sustainability concerns, climate change impacts on marine ecosystems, and scalability of production are addressed alongside future perspectives for technological innovations in extraction methods, compounds isolation, study about bioprospecting of marine fish derived fatty acids as well as food integration strategies. It is difficult to create an optimal animal model of the tumor microenvironment that most closely resembles a human tumor. There are currently a number of mouse models, although they offer some advantages, such as patient-derived tumor xenografts and genetically modified mice models. The development and metastasis of MDA-MB-231 tumors are similar to those of human breast cancer (Bonanni et al., 2012). Tumor initiation, promotion, progression, and metastasis are the stages that often define the process in a mouse model of growing tumors. Research using animal models has demonstrated that the development of breast cancer is often influenced by mutations in genes such as TP53, Bax, caspase-3, 8, 9, and others (Malekian et al., 2020). The aimed of this study to underscores the critical importance of marine source of lipids mainly bioactive polyunsaturated fatty acid i.e., linoelaidic acid as sustainable alternatives to conventional sources while highlighting their potential to address global nutritional challenges and support the development of next-generation health-promoting functional products (John et al., 2025).

Materials and methods

Materials

3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium (MTT) and Fetal bovine serum (FBS), Hi-cDNA synthesis kit were procured from Hi-Media. Treated compound Linoelaidic acid, Tris-HCl, Triton X-100, Sodium dodecyl sulfate (SDS), ethanol, needed for western blot were procured from Sigma Aldrich. Collagenase type 4 (Himedia; TC-214), 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich; D9542), bovine serum albumin (BSA; Himedia; TC548), All primers (p53, caspase-3, 8, 9 & Bax) from Bio-serve, Acridine orange (AO; Himedia; MB116); Ethidium bromide (EtBr; Himedia; MB071); TUNEL (Elabscience, E-CK-A322, AF488), Antibodies (P53, Caspase-3, 9 & Bcl-2) from proteintech. All other chemicals were of molecular biology grade and procured from Hi-Media, India.

Cell culture and maintenance: Depending on the medium and laboratory procedures, MDA-MB-231 cells collected from the human mammary gland was purchased from NCCS, Pune, with passage number-43, and kept in a conventional culture environment, usually at 37°C, without CO₂. They are frequently cultivated in media such as Leibovitz's L-15 medium supplemented with FBS, glutamine, and antibiotics, and are frequently sub cultured using trypsin/EDTA when 70–80% confluent.

Ethics review: We confirmed that all methods were performed in accordance with the relevant guidelines and regulations. The research was carried out in compliance with the ARRIVE guidelines. The animal protocols and procedures for experiments were approved by the Institutional Animal Ethical Committee of Midnapore City College vide Ref No. (2256/PO/Re/S/23/CCSEA) MCC/IAEC-SP/10/23-24 Dated. 15.04.2024, and all experiments were conducted according to the ethical and safety guidelines of the Institute.

Animal care, handling and group selection: Centre for Laboratory Animal Research and Training (CLART), Kalyani, West Bengal, provided

the female C57BL/6 J wild-type mice. Every animal used in this investigation was 8–12 weeks old. They were kept under standard conditions at the Midnapore City College, and they were fed commercial mouse food that had a 12-hour light-dark cycle. Throughout the experiment, the animals were closely monitored for indications of discomfort and extreme stress, including decreased feeding or drinking, decreased activity, and weight loss.

T cell development was stopped early after the mice were acquired. Due to their well-characterised, fully sequenced genome, C57BL/6 J mice were chosen as the standard model for immune-oncology because they exhibit extreme robustness and severity. To deplete T cells, inject 0.5 mg of Anti-CD4 (CD4 Monoclonal Antibody GK1.5, eBioscience) antibody into each mouse. It was injected for three days in a row, then every three days for maintenance (Ray et al., 2007).

After random assignment mice were distributed into four groups (n = 6), served with a standard diet with water ad libitum and continued for 14th day for acclimatization and at the day of 15, MDA-MB-231 was inoculated at a single dose by subcutaneous fat pad (5×10^6 /ml) to all group of animals (Le Naour et al., 2020). The Control group which was fed with normal diet and inoculated with mammary tumor cells and received no treatment. The Treatment I group; mammary tumor were treated with Linoelaidic Acid 0.006 mg/kg body weight/day (2 μ M). Treatment II group, Mice with mammary tumor were treated with Linoelaidic Acid 0.014 mg/kg body weight/day (5 μ M). Treatment III group, Mice with mammary tumor were treated with Linoelaidic Acid 0.28 mg/kg body weight/day (10 μ M) body weight/day. All these treatments were ongoing up to 55 days. Primary tumor samples were collected and frozen in liquid nitrogen after sacrifice on 56th day. Mice were examined daily for the appearance of tumor by palpation, and the first day of tumor detection was recorded. Mice were anesthetized using chloroform 15 days after the first appearance of tumor. A blood specimen was collected by cardiac puncture, and the tumor was dissected out, measured, and weighed. Blood and tumor specimens were stored at -70°C. A portion of the tumor tissue was embedded in OCT (optimal cutting temperature) compound for immunohistochemistry of ER, PR, and HER-2 expression, and for histological evaluation by hematoxylin and eosin (H&E) stain. The following formula was used to determine the tumor volume: tumor volume (mm³) = length (mm) $\times$ width (mm) $\times$ width (mm) $\times$ 1/2. This grouping allows evaluation of linoelaidic acid's efficacy in inhibiting tumor growth and inducing apoptosis, compared to untreated control group.

Single cell preparation from tumor tissue: After washing 50 mg of fresh tumor tissue samples with phosphate-buffered saline (PBS), 0.5 ml (2 mg/ml) collagenase (Cat. No # TC211, HiMedia, India) was added to the PBS mixture, and the tissue was minced with a sharp knife. After that, samples were filtered through a nylon filter mesh (20 μ m) (Garcia-Flores et al., 2023). The cells were then used for further experiments.

Cell viability assay (MTT): The effect of linoelaidic acid on the cell viability of MDA-MB-231 injected tumor cell was examined by the MTT assay kit method EZcount™ MTT cell assay kit. As previously mentioned, the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay was carried out³. In summary, cells were cultivated in a 96-well plate at a density of 1×10^6 cells/ml following single cell preparation. The cells were treated with 10 μ l of MTT (5 mg/ml) after 1 day. After that, the cultures were re-incubated for a further 4 h. An ELISA Reader was used to measure the absorbance at 490 nm and 630 nm after the supernatant was removed and 100 μ l of solubilisation was added to each well to fully dissolve the crystals (Dash et al., 2014).

Lactate dehydrogenase assay (LDH): The CCK0366 LDH Cytotoxicity Assay Kit was used to quantify LDH activity in accordance with the manufacturer's protocol (HiMedia). In summary, each sample yielded 50 μ l of conditioned media (CM). 50 μ l of conditioned media were extracted from each sample after the cells were lysed for 45 min at 37 °C using 10 μ l of the lysis buffer included in the assay kit. The CM and samples were subjected to the LDH test. A multimode reader was used to

detect absorbance at 450 and 600 nm (Dutta et al., 2023).

Determination of reduced glutathione (GSH): Quantization of GSH estimation in cell lysate (1×10^6 cells/ml) was performed according to the standard method (Chakraborty et al., 2011). About 25 mg of tumor tissue were homogenized in 0.2 ml of 25% metaphosphoric acid with 0.75 ml of 0.1 M sodium phosphate 5 mM EDTA buffer (precise pH 8.0) in order to measure GSH. Proteins were precipitated by centrifuging the whole homogenate at 10,500xg for 10 min at 4°C. 0.4 ml of the phosphate-EDTA buffer was added to dilute 0.1 ml of the supernatant. 0.1 ml of the diluted supernatant, 2.8 ml of phosphate-EDTA buffer, and 0.1 ml of TCA (Tri-Chloro-Acetic acid) solution (1 mg/ml of reagent-grade absolute methanol, prepared shortly previous to use) made up the final assay combination (3 ml). The sample was put into a quartz cuvette after being mixed and allowed to sit at room temperature for 15 min. The LABMAN UV spectrophotometer (LMSP-UV 1900) was used to measure the GSH intensity at 405 nm. The amount of GSH was calculated by using a standard curve produced by known level of GSH at various concentration using the absorbance of 405 nm.

Determination of oxidized glutathione (GSSG): After the derivatization of GSH with 2-vinyl pyridine, the GSSG level was measured according to the method (Mahapatra et al., 2009). It was crucial to quickly prepare the samples according to GSH sample preparation in order to reduce GSH oxidation. As a result, shortly after preparation, 2-vinylpyridine was used to derivatize the supernatant for 60 min at room temperature. In a typical experiment, 0.5 ml of undiluted 2-vinylpyridine (stored at -20°C) was added to 0.15 ml of supernatant in a plastic tube under a hood. To settle the protein precipitation, the sample was centrifuged for 10 min at 1200 rpm after being deionized with 4% sulphosalicylic acid. The supernatant as collected and the GSSG was estimated with the reaction of DTNB at the absorption of 412 nm. Plotting known levels of GSSG at various concentrations using the absorbance of 412 nm produced a standard curve from which the amount of GSSG present was calculated.

Total nitric oxide assay (NO): The EZAssay TM Nitric Oxide Estimation Kit (CCK061–200) was used to measure the nitric oxide assay (Hunter et al., 2013). As previously mentioned, NO production was calculated by adding the quantities of NO₂ and NO₃ in the culture supernatant. In a nutshell, a 96-well microplate was filled with 80 µl of culture supernatant and 80 µl of the Griess reagent. The mixture was then allowed to sit at room temperature for ten minutes. An ELISA automatic micro plate reader (LABSYSTEM, Ldx R1, and R2108014) was used to detect the optical density at 570 nm. A standard curve built with known concentrations of NaNO₂ and NaNO₃ was used to calculate the amounts of NO₂ and NO₃. Since D-MEM had extremely low NO₃ (<1 mM), each sample's background could be disregarded.

Reverse transcription and polymerase chain reaction: TRIzol (Life Technologies) was used to extract the total RNA from the tumor tissues of mice. The Nanodrop 2000c Bio spectrophotometer was utilised to measure the amount of mRNA. One microgram of total RNA was reverse-transcribed into cDNA for every RNA sample using a High-capacity cDNA synthesis kit (HI media, Hi GenoMB, Maharashtra, India). Using semi-q-PCR analysis, target gene expression for p53, Caspase–3, 8, and 9, Bax and Bcl2 was examined, displayed in Table 1. The specific gene was evaluated compared with GAPDH (Dutta et al., 2023) by using agarose gel electrophoresis.

Densitometry analysis: Software tools were used to perform densitometry measurements and estimate the corresponding mRNA expression after the DNA bands on the gel were recorded using a gel documentation system. The agarose gels' band densities were compared using ImageJ software. The mean area values of the bands were shown together with the rectangular area of each individual band. By choosing the same region for every band, the band densities on an agarose gel were compared. The mean density value of each band was shown in Excel after the housekeeping gene (GAPDH) was separated from the genes of interest (p53, Caspase–3, 8, and 9). After analysing all of the data using ANOVA, a Tukey's multiple comparison test was conducted, with significance criteria set at $p < 0.05$ ($n = 3$).

Immunoblotting analysis of p53, Caspase 3, 9 & Bcl–2: The MDA-MB–231 induced tumor tissue treated with the linoelaidic acid were lysed with radioimmunoprecipitation assay (RIPA) buffer, and the protein content was quantified by the Lowry method. 50 µg protein samples were placed in loading buffer and boiled for 5 min, then electrophoresed by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Model No. 165.8033FC). The membrane was then blocked with 4% bovine serum albumin (BSA) and incubated with primary antibody overnight at 4°C. The primary antibody used in this study included β-actin, p53, IL–10, IL1Ra, caspase 3, 9, and Bax at a 1:1000 ratio. The membrane was then washed three times with phosphate buffer saline with tween–20 (PBST) and incubated with horseradish peroxidase (HRP) labelled anti-mouse secondary antibody 1:7500 (Abgenex) for 1 h at 4°C. Specific protein bands were detected using 3,3',4,4'-Tetraaminobiphenyl tetrahydrochloride (DAB). When inhibitors were employed, cells were pre-treated for 8 h with inhibitors before the addition of linoelaidic acid.⁹ Immunoblotting assay was done by using Bio-Rad Western blot apparatus (serial no: 041BR183625) funded by ICMR (File no: 5/9/1223/2019-Nut. Dated–25.07.2019). ImageJ software was used to measure protein immunoreactivity.

Detection of apoptotic morphological changes by immunohistochemical staining using Acridine orange-ethidium bromide staining: Fresh samples of tumor tissue weighing 50 mg were washed with PBS. The tissue was then chopped with a sharp knife after 0.5 ml (2 mg/ml) collagenase (Cat. No # TC211, HiMedia, India) was added to the PBS mixture. A nylon filter mesh with a pore size of 20 µm was then used to filter the samples. 20 µL of the samples were then put onto a grease-free slide and let to dry in a laminar air flow. The slide was fixed with ice-chilled methanol and subsequently cleaned with 4% paraformaldehyde (Cat. No. TC703, HiMedia, India). A fluorescent microscope (Olympus, model no. BX43F, cellSens Standard) was used to visualize the changes in morphology of cell using AO/Et-Br stain (1:1) (Mitra et al., 2025). Quantification of apoptosis measured by using the ImageJ software.

DAPI staining for nuclear morphological evaluation: PBS was used to wash 50 mg of fresh tumor tissue samples. Next, 0.5 ml (2 mg/ml) collagenase (Cat. No # TC211, HiMedia, India) was added to the PBS mixture, and the tissue was minced using a sharp knife. After that, samples were filtered using a 20 µm nylon filter mesh. Following that, 20 µL of the samples were applied to a slide free of grease and allowed to dry in a laminar air flow. The slide was then cleaned with 4% paraformaldehyde (Cat. No. TC703, HiMedia, India) after being fixed with

Table 1

List of genes used for checking anti-cancer potentiality on MDA-MB–231 induced tumor.

Gene Name	Sense	Anti- Sense
GAPDH	GGTGAAGGTCGAGTCAACG	GTGAAGACGCCAGTGGACTC
P53	TGCGTGTGGAGTATTGGATG	TGGTACAGTCAGAGCCAACCTC
Caspase–3	GTGGAAGTACGATGATATGGC	CGCAAAGTGACTGGATGAACC
Caspase–8	ACAATGCCAGATTCTCCCTAC	CAGACAGTTCCTCCGAGTTTG
Caspase–9	CGCCACCATCTTCTCCCTG	CCAAGCTCTCTTCTCTCTC
Bax	CGGCGAATTGGAGATGAAGTGG	CTAGCAAAGTAGAAGAGGGCAACC
Bcl2	TGTGGATGACTGACTACCTGAACC	CAGCCAGGAGAAATCAACAGAGG

ice-chilled methanol. The alterations in nuclear morphology were seen using DAPI stain (1:1000), which was then visualized using a fluorescent microscope (Olympus, model no. BX43F, cellSens Standard) (Mitra et al., 2025). Quantification of live nuclei was done by using the ImageJ software.

ROS levels in linoelaidic acid treated MDA-MB-231 induced tumor cell: The intracellular ROS concentration was determined using active oxygen sensing. Using a fluorescence microscope, H2DCFDA was deacetylated intracellularly by a nonspecific esterase to produce the fluorescent compound 2,7-dichlorofluorescein (DCF). Linoelaidic acid was administered to MDA-MB-231 induce tumor in mice model for 0.006 mg/kg body weight and 0.014 mg/kg body weight. PBS was used to clean the single cells before 1 mg/ml of H2DCFDA was applied for 30 min at 37°C (Loo, 2002). DCF presence was determined using a fluorescence microscope (Olympus, Model No. BX43F). Quantitative result was obtained by a multimode microplate reader at 480 and 530 nm. Calculate the fold change by following the equation: Fold change = Mean normalized treated value/Mean normalized control value.

DNA fragmentation checked by TUNEL assay: DNA fragmentation is the term used to describe apoptosis signals. Terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) is a method for detecting DNA fragments and has been applied to evaluate the induced apoptotic effects of treatments with linoelaidic acid. The MDA-MB-231 was examined for signs of apoptosis using TUNEL analysis. TUNEL staining was performed using an in-situ cell death detection kit (Elabscience, E-CK-A321) in compliance with the manufacturer's instructions (Shirata, 2017 #185) (Loo, 2002). Percentage of TUNEL positive cell quantified using ImageJ software.

Statistical analysis: There was a minimum of three repetitions of each parameter. The results were displayed as mean $\pm$ SEM, $n = 6$. The means of the treated and control groups was compared using multiple comparison t -tests at $p < 0.05$ as a limit of significance after a one-way ANOVA test (using Graph Pad PRISM).

Result

Tumor growth inhibition and measurement using linoelaidic acid on MDA-MB-231-induced C57BL/6 J mice model

The MDA-MB-231 induced tumor volume was measured in the C57BL/6 J mice. The MDA-MB-231 tumors in the Control group ($n = 3$) had an average volume of 1500.01 ± 350.11 mm³, but the tumors in the Treatment-I, II and III groups treated with linoelaidic acid had an average volume of 1100 ± 71 mm³, 448 ± 51 mm³, and 810 ± 63 mm³ (< 0.05) after 56th days (Fig. 1A). Breast cancer cells MDA-MB-231 obtained from the tumor of MDA-MB-231-bearing C57BL/6 J mice, were treated with linoelaidic acid for up to 56 days, and the weight of these tumors decrease simultaneously by increasing dose of linoelaidic acid, from the selected doses Treatment- II (0.014 mg/kg body weight/day) is more effective for decreasing the tumor weight (Fig. 1B).

The MTT assay measures the cell viability of MDA-MB-231 induced tumor cell on C57BL/6 J mice model

In a model of tumor-induced mice, the MTT test is used to objectively analyze the viability and proliferation of collected tumor cells, mainly to determine the effectiveness of an anti-cancer treatment. Interestingly, 31.28%, 73.13%, and 47.9% of cell death were observed following treatment with linoelaidic acid at Treatment-I, II and III groups, respectively. According to that analysis, 100% of the viable cells at the start of the trial were significantly ($p < 0.05$) reduced after treatment with linoelaidic acid (Fig. 2A).

Cytotoxicity assessment of linoelaidic acid on MDA-MB-231 induced tumor cell on C57BL/6 J mice model by LDH assay

Glycolysis, the main energy source for many cancer cells, depends on LDH. When LDH is inhibited, less ATP is produced, which depletes energy and causes cell death. Pro-apoptotic pathways may be triggered by LDH inhibition, resulting in programmed cell death. It was found that LDH-A expression had no relationship to other clinic pathological factors and significantly linked with tumor size. According to this study, linoelaidic acid lowers the MDA-MB-231 injected tumor cell lactate

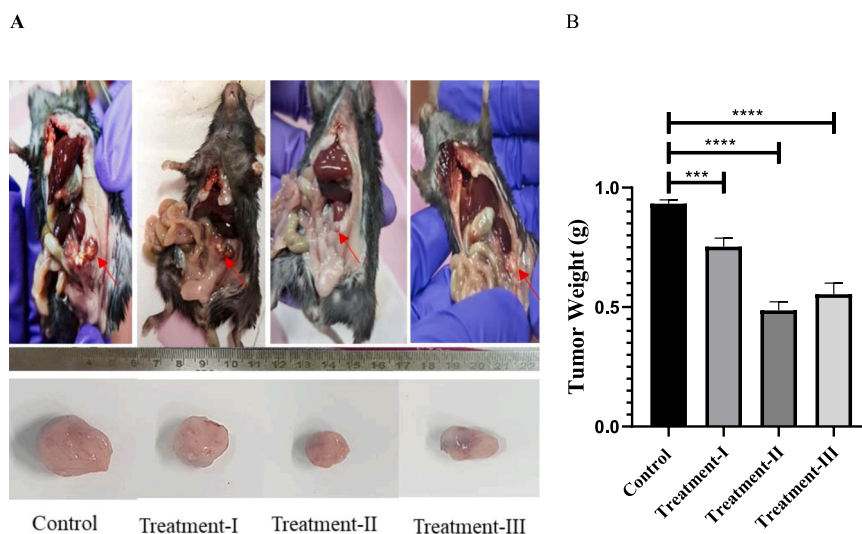


Fig. 1. (A) *In Vivo* anti-tumour activity of Linoelaidic Acid from Marine Tapra Fish (*Opisthopterus tardoore*) Oil was assessed in MDA-MB-231 Cancer Cell-induced Mice (C57BL/6 J). The groups are as follows: Control: Untreated control, Treatment I: Linoelaidic acid with 0.006 mg/kg, Treatment II: Linoelaidic acid with 0.014 mg/kg, Treatment III: Linoelaidic acid with 0.28 mg/kg. (B) Tumor weights of MDA-MB-231-induced tumors were depicted diagrammatically following linoelaidic acid treatment. The results are expressed as mean $\pm$ SE ($n = 6$). Statistical significance (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) was determined using a one-way ANOVA multiple comparison test, indicating significant differences in tumor growth between treatment groups and the untreated control group.

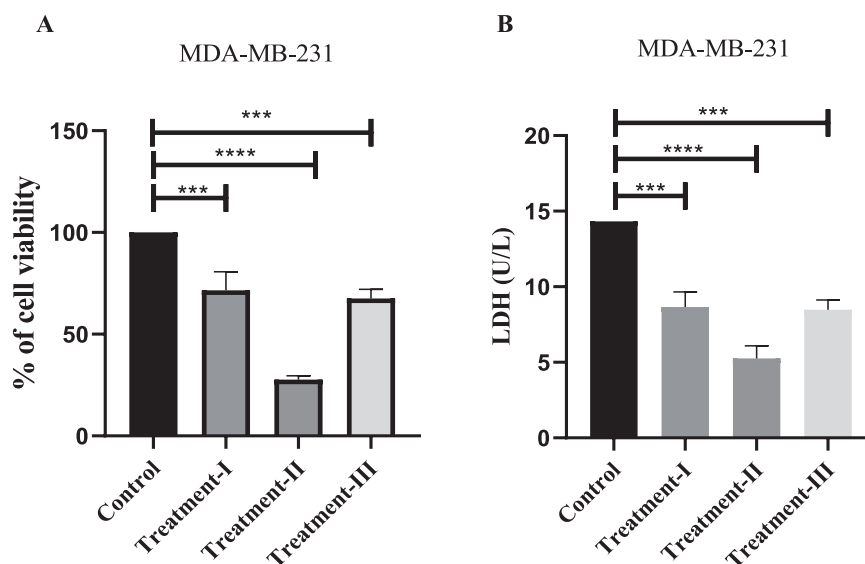


Fig. 2. Effect of Linoelaidic Acid on MDA-MB-231-Induced Tumor Cells (1×10^6 /ml). The groups are as follows: Control: Untreated control, Treatment I: Linoelaidic acid with 0.006 mg/kg, Treatment II: Linoelaidic acid with 0.014 mg/kg, Treatment III: Linoelaidic acid with 0.28 mg/kg. **(A)** Cell Viability Assay: Cell death rates were measured using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay, as described in the Materials and Methods section. The results show the percentage of viable cells remaining after treatment with Linoelaidic Acid. **(B)** Cytotoxicity Assay: Cytotoxicity levels were assessed using the LDH (Lactate Dehydrogenase) assay, as described in the Materials and Methods section. Values are expressed as mean $\pm$ SE (n = 6). Superscripts indicate significant differences (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05) compared to the control group.

dehydrogenase level, indicating a symptom of cell damage (Fig. 2B). The mean LDH level in the control group was 14.33 (U/L), and decreased accordingly to 8.650 (U/L), 5.260 (U/L), and 8.480 (U/L), in Treatment I, II and III groups.

Evaluation of intracellular redox balance (GSH, GSSG, NO) of MDA-MB-231 induced tumor cell on C57BL/6 J mice model by applying linoelaidic acid

Lowering glutathione (GSH) levels in mice can cause cell death

through a number of mechanisms, such as increasing oxidative stress that damages cellular components, promoting apoptosis by interfering with mitochondrial function and activating caspases, and causing ferroptosis due to the accumulation of lipid peroxides. It was discovered that Treatment-II group of linoelaidic acid was more effective than the other dose at lowering the GSH level in MDA-MB-231 cells (Fig. 3A). In mouse models, elevated amounts of oxidized glutathione (GSSG) cause cell death mainly by overpowering the antioxidant defenses of the cell, changing the intracellular environment to an oxidized state (oxidative stress), and initiating particular necrotic or apoptotic signaling cascades.

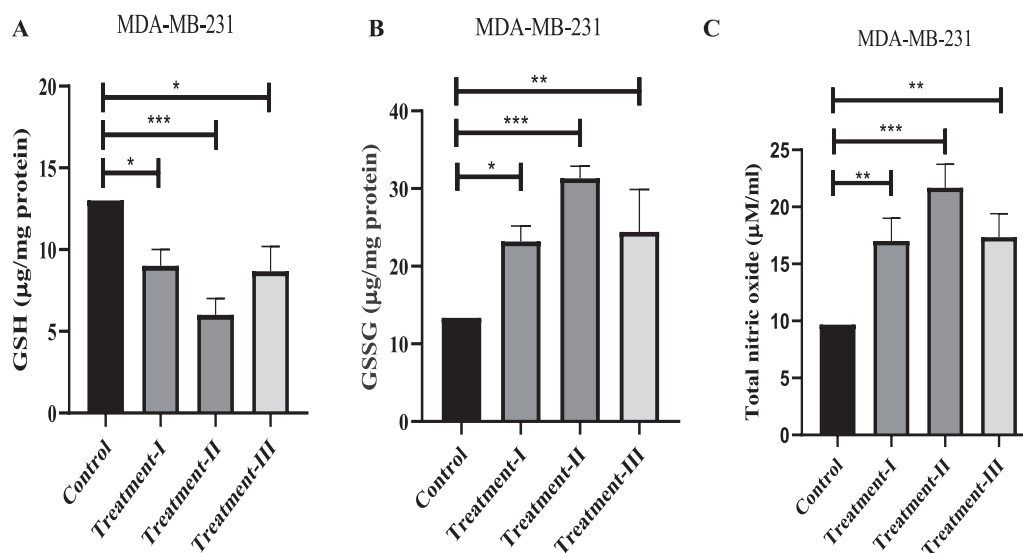


Fig. 3. Effect of Linoelaidic Acid on Glutathione Levels and Cell Viability in MDA-MB-231-Induced Tumor Cells (1×10^6 /ml). The groups are as follows: Control: Untreated control, Treatment I: Linoelaidic acid with 0.006 mg/kg, Treatment II: Linoelaidic acid with 0.014 mg/kg, Treatment III: Linoelaidic acid with 0.28 mg/kg. **(A)** The level of reduced glutathione (GSH) was measured in linoelaidic acid-treated tumor cells using GSH assay, as described in the Materials and Methods section. **(B)** The level of oxidized glutathione (GSSG) was measured in linoelaidic acid-treated tumor cells using GSSG assay, as described in the Materials and Methods section. **(C)** The effect of linoelaidic acid on MDA-MB-231-induced tumor cells was evaluated by assessing Nitric Oxide assay, as described in the Materials and Methods section. Values are expressed as mean $\pm$ SE (n = 6). Superscripts indicate significant differences ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 compared to the control group.

In addition, GSSG levels were raised significantly ($p < 0.05$) following treatment of MDA-MB-231 injected tumor cell to linoelaidic acid. As in the previous tests, it was discovered that the higher dose (5 μ M) of linoelaidic acid had a greater effect than the lower dose (Treatment-I) (Fig. 3B). These findings quickly showed that linoelaidic acid affected the redox equilibrium in MDA-MB-231, causing cell death and hence

averting cancer.

In a mouse model, elevated nitric oxide (NO) can cause cell death in a number of ways, chiefly by damaging DNA, interfering with energy metabolism, and triggering apoptotic pathways. Furthermore, NO and superoxide can combine to create peroxynitrite, a highly reactive chemical that harms DNA and proteins and aids in cell death. Further

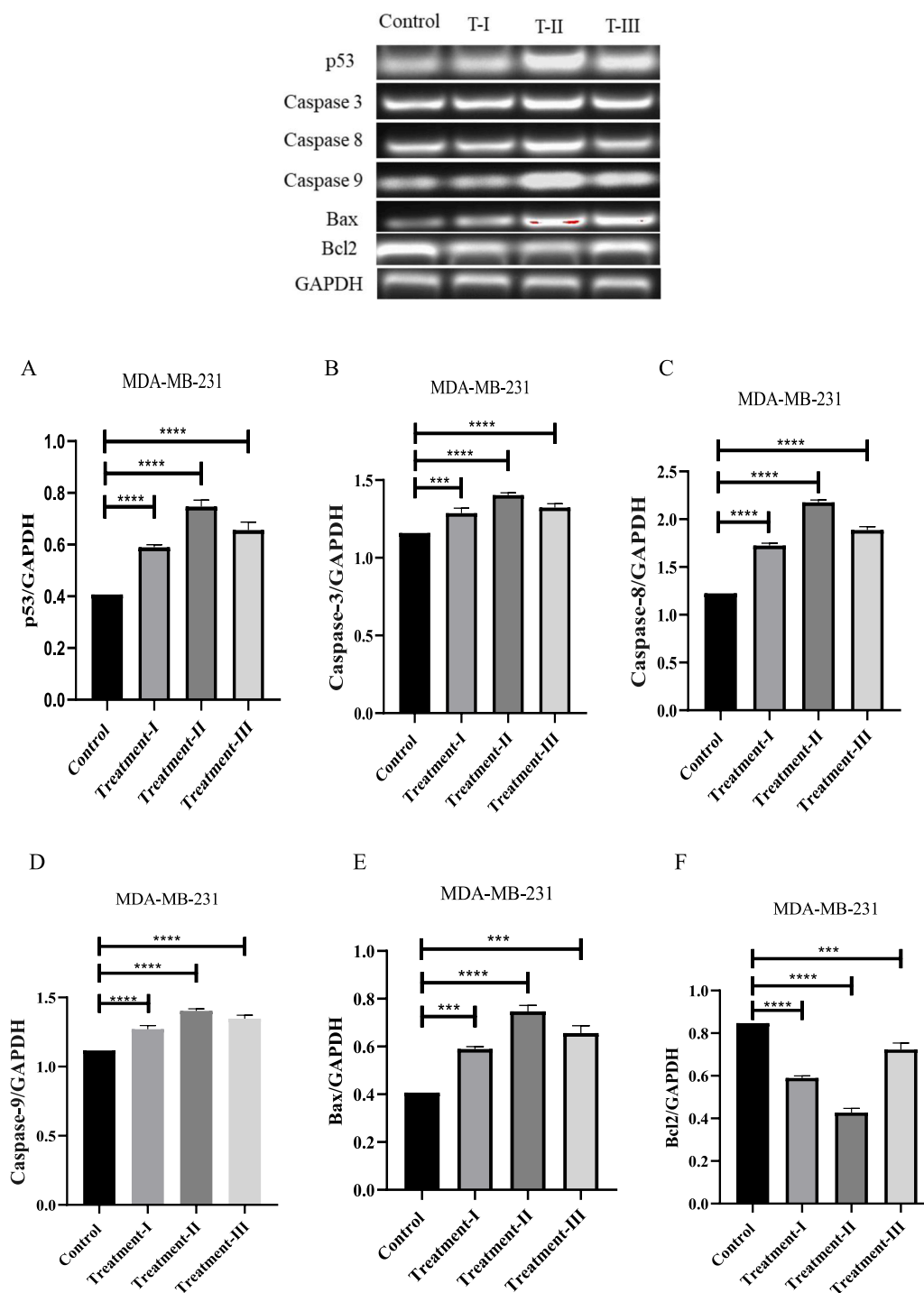


Fig. 4. Relative mRNA Expression of Apoptosis-Related Genes in MDA-MB-231-Induced Tumor Tissue: The mRNA expression levels of key apoptosis-related genes were measured by semi quantitative PCR (semi-qPCR) in tumor tissues from MDA-MB-231-induced mice treated with different doses of Linoelaidic Acid. (A) p53 (B) Caspase-3 (C) Caspase-8 (D) Caspase-9: Initiator caspase involved in the intrinsic pathway of apoptosis (E) Bax (F) Bcl2: Anti-apoptotic gene involved in inhibiting mitochondrial outer membrane permeabilization in MDA-MB-231 induced tumor tissue from untreated control group, Treatment I (Normal diet + Linoelaidic acid at 0.006 mg/kg body weight/day), Treatment II (Normal diet + Linoelaidic acid at 0.014 mg/kg body weight/day), Treatment III (Normal diet + Linoelaidic acid at 0.24 mg/kg body weight/day). Statistical analysis was denoted as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ using one-way ANOVA multiple comparison test.

evidence of cell injury was provided by our results, which showed that MDA-MB-231 injected tumor cell treated with linoelaidic acid produced higher NO (Fig. 3C) generation.

Linoelaidic acid modulates gene expressions of apoptotic genes (p53, Caspase-3,8,9, Bax and Bcl2) of MDA-MB-231 induced tumor cell on C57BL/6 J mice model

Further stability of reference genes was validated under different experimental series with the dose-dependent exposure with linoelaidic acid (2 μ M, 5 μ M, 10 μ M) on MDA-MB-231 injected tumor cell. Apoptotic death induction in nascent neoplastic cells was regarded as the principal mechanism by which p53 prevents tumor development and our results was strongly agreed with that which shown in Fig. 4A. In this context, increased p53 after Linoelaidic acid exposure restricted the MDA-MB-231 injected tumor cell proliferation via modulating genes related to the cell cycle and apoptosis. The capacity of anticancer drugs to initiate caspase activation seems to be a crucial factor in determining susceptibility or resistance to cytotoxic therapy, considering the significant role that caspases play as effector molecules in a variety of cell death processes, including drug-induced apoptosis. In this study the caspase 3, 8, 9 is mostly activated (Figs. 4B, 4C & 4D) after linoelaidic acid treatment. Figs. 4E and 4F showed that the incubation of MDA-MB-231 tumor mice model with 2 μ M linoelaidic acid significantly reduced Bcl2 expression levels, while 5 μ M significantly decreased them more than 50% when compared to untreated viable cells. Similarly, the incubation of MDA-MB-231 induced tumor mice model with 2 μ M linoelaidic acid increased the expression levels of Bax but 5 μ M dose is more effective when compared to control breast cancer cells.

Immunoblotting assay of MDA-MB-231 induced tumor cell

To further investigate the mechanism regarding apoptosis,

immunoblotting assay of p53, caspase 3, caspase 9, and Bcl-2 were assessed. Immunoblotting of target protein expression revealed that the expression of the p53 protein, Bcl-2, caspase 3, caspase 9 considerably enhanced, following linoelaidic acid treatments (Fig. 5). To investigate whether the changes in p53, caspase 3, caspase 9 and Bcl-2 mRNA levels are associated with alteration of protein levels, it was validated by the expression of p53 which slightly increased in Treatment-I group. In MDA-MB-231 induced tumor cells, linoelaidic acid significantly increased the expression of caspase 3 by 1.11 and 1.27-fold at Treatment-I&II group in comparison to untreated control group (Fig. 5B). This Figure illustrated that caspase 3 protein expression increased by 1.14-fold at the Treatment-II group compared to the Treatment-I group. Caspase 9 Similar to this, MDA-MB-231 cells treated with Treatment-I and II group exhibited considerably higher levels of caspase 9 expression by 1.24 and 1.34-fold, respectively, than untreated cells (Fig. 5C). Caspase 9 protein expression increased by 1.07-fold at Treatment-II group compared to Treatment-I group. Fig. 5B & Fig. 5C demonstrate how linoelaidic acid caused caspase 3 and caspase 9 to cleave, resulting in a marked increase in their active forms. We also investigated the effect of linoelaidic acid treatment on gene expression of apoptotic marker. Our data proved that linoelaidic acid decrease the Bcl-2 expression in MDA-MB-231 induced tumor cell (Fig. 5D). Downregulation of Bcl-2 is frequently associated to cytotoxicity by inducing the opening of the mitochondrial voltage-dependent anion channel. Our finding confirmed that MDA-MB-231 induced tumor cell treated with linoelaidic acid can induce apoptosis process as well as it protects our body from inflammation which can prevent cell proliferation. Linoelaidic acid dramatically raised the levels of caspase 3 and caspase 9 protein expression, demonstrating that it can induce apoptosis in MDA-MB-231 induced tumor cells. The maximum activation rates of caspase 3 and caspase 9 were noticed in highest concentration of linoelaidic acid treated MDA-MB-231 induced tumor cells i.e., Treatment-II (Fig. 5D).

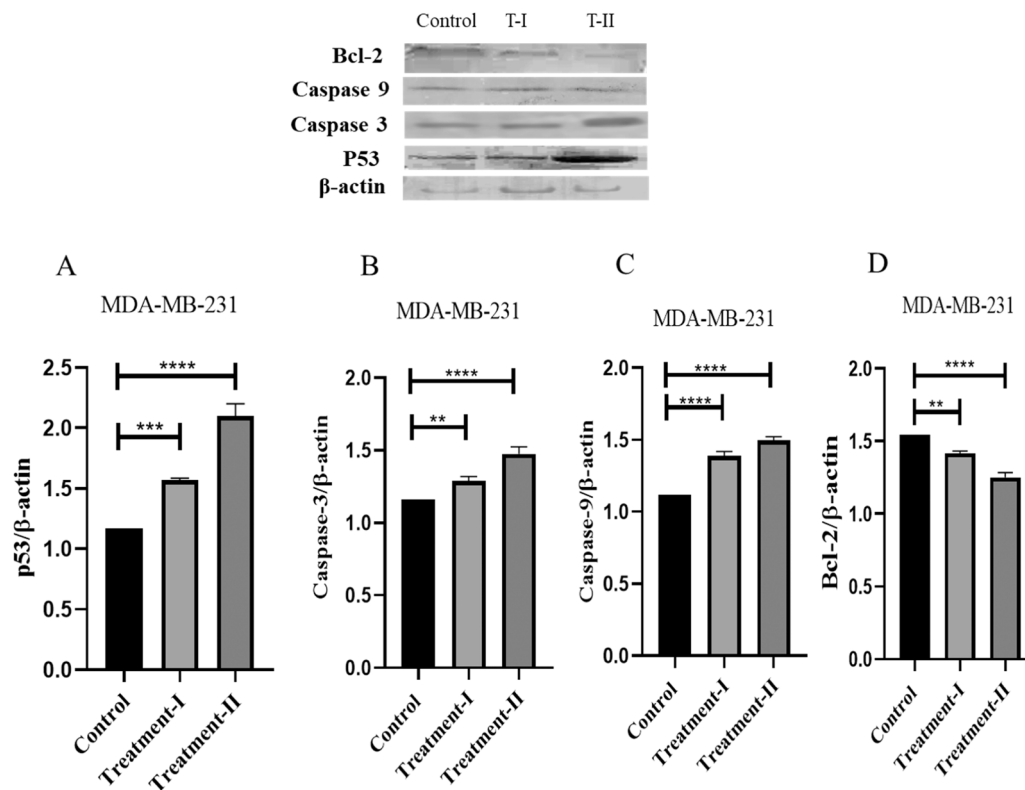


Fig. 5. Immunoblotting assay of linoelaidic acid treated MDA-MB-231 induced tumor. The groups are as follows: Control: Untreated control, Treatment I: Linoelaidic acid with 0.006 mg/kg, Treatment II: Linoelaidic acid with 0.014 mg/kg. Values are expressed as mean $\pm$ SE (n = 6) of three experiments compared with the control group. Statistical analysis was denoted as ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 using one-way ANOVA multiple comparison test.

Apoptosis detection in linoelaidic acid treated MDA-MB-231 induced tumor cell on C57BL/6 J mice model

The AO/Et-Br staining was used to investigate morphological changes with a dose-dependent effect on breast cancer cells. After treatment with linoelaidic acid the cytotoxicity was increased at Treatment-II group (Fig. 6C). Fig. 6D confirmed the quantification of apoptotic cells using AO/EtBr staining. Hereafter, treatment with linoelaidic acid treatment-II group is visible for a more effective dose to increase apoptotic percentage. The red channel mainly indicates the higher apoptotic percentage, and the diagram of Fig. 6D & 6E confirmed the red channel are mostly visible at Treatment-II group when analyzed

with image J software.

Linoelaidic acid induces intracellular ROS generation in MDA-MB-231 induced tumor cells

ROS formation was analyzed on the basis of the study of DCF signals. Fluorescence intensity generated by H2DCFDA was increased in a dose-dependent manner in linoelaidic acid-treated MDA-MB-231 induced tumor cells and was much greater than that of the untreated control group (Fig. 7A & B). At the group of Treatment-II, linoelaidic acid triggered substantial generation of ROS in cancer cells. These results demonstrated a dose-dependent increase in the degree of apoptosis. Our

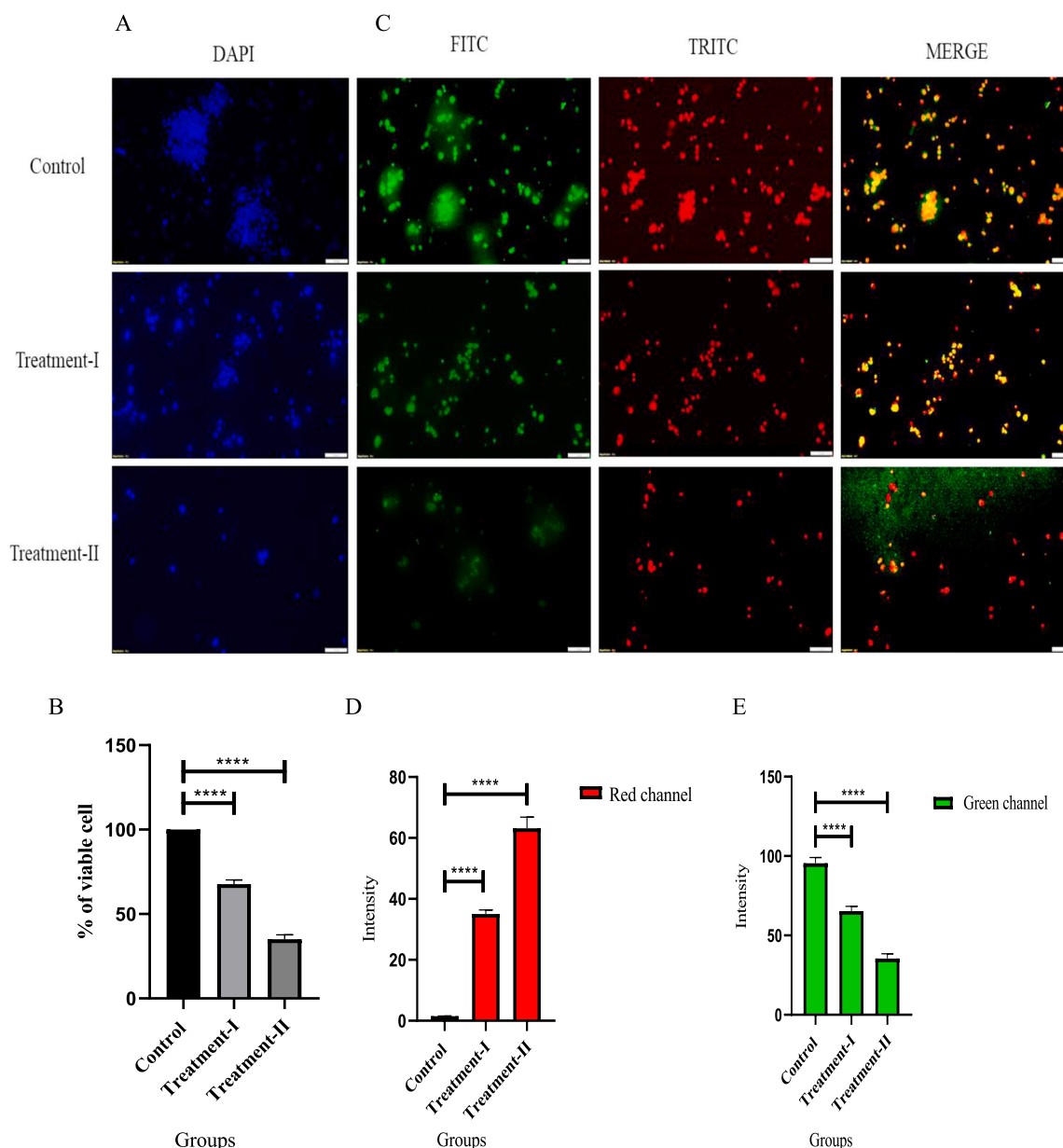


Fig. 6. Nuclear Morphology and Apoptosis Detection in Linoelaidic Acid-Treated MDA-MB-231-Induced Tumor Cells. The groups are as follows Control: Untreated control, Treatment I: Linoelaidic acid with 0.006 mg/kg, Treatment II: Linoelaidic acid with 0.014 mg/kg. (A) Nuclear Morphology study by DAPI staining. Fluorescence microscopic images were recorded at 10x magnification, showing changes in nuclear structure and morphology indicative of apoptosis. (B) quantification of live nuclei. (C) Apoptosis Detection by Acridine orange and ethidium bromide (AO/Et-Br) staining was used to detect apoptosis in linoelaidic acid-treated MDA-MB-231-induced tumor cells (1×10^6 /ml). Fluorescence microscopic images were recorded at 10x magnification using an Olympus fluorescence microscope (BX43F). The images showed representative results from three independent experiments. The scale bar correspondence to 100 μ m. (D & E) Bar diagram represent death rate (Red channel) and viability (Green channel) of tumor cell by AO/Et-Br staining. Statistical analysis was denoted as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ using one-way ANOVA multiple comparison test.

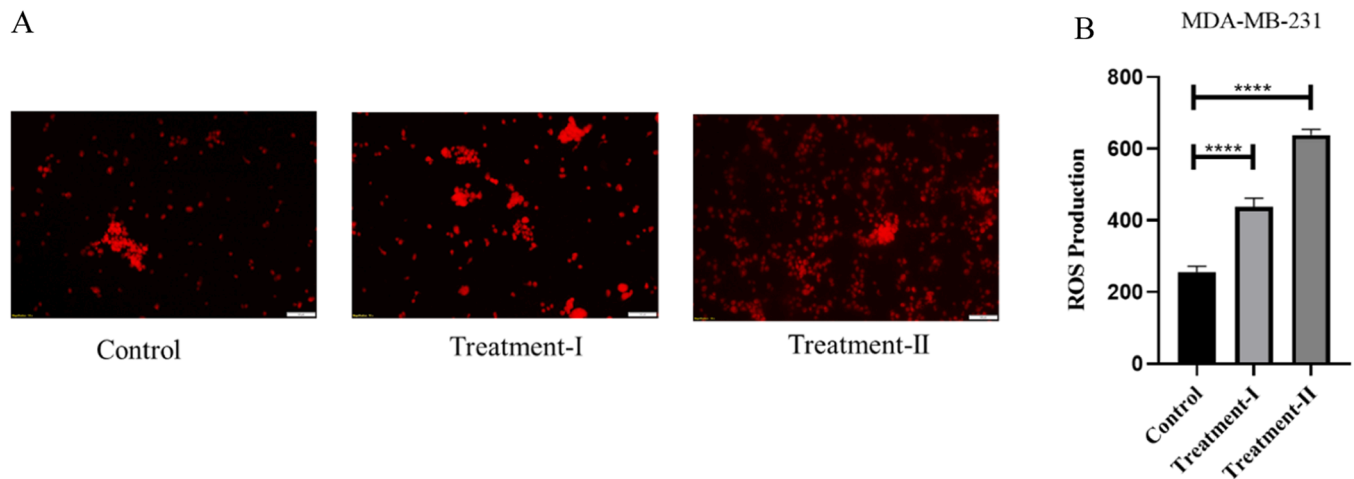


Fig. 7. Intracellular reactive oxygen species measurement was done from linoelaidic acid-treated MDA-MB-231 induced tumor cell. The groups are as follows: Control: Untreated control, Treatment I: Linoelaidic acid with 0.006 mg/kg, Treatment II: Linoelaidic acid with 0.014 mg/kg. (A) The levels of ROS were considered as DCF fluorescence intensity and recorded by Fluorescence microscopic images here at 10x magnification. The scale bar correspondence to 100 μ m. (B) ROS production level was measured by multimode reader. Statistical analysis was denoted as **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05 using one-way ANOVA multiple comparison test.

research revealed that linoelaidic acid massively increased cellular ROS levels, which in turn caused apoptosis. It appears that the situation of oxidative damage is connected to the rise in cell death. Based on the results, we put forth the hypothesis that linoelaidic acid might cause the MDA-MB-231 induced tumor model to undergo oxidative stress-induced apoptosis. The variations in ROS production between the treatment group and the untreated control group are displayed in Fig. 7B. In comparison to the untreated control group, the production of ROS rose concurrently by 1.32 and 2.09 times in Treatment-I and Treatment-II, respectively. We attempted to clarify the role of ROS in the development of linoelaidic acid-induced apoptosis in this investigation.

Decoding nuclear morphology with DAPI after getting linoelaidic acid in MDA-MB-231 induced tumor cell on C57BL/6 J mice model

A fluorescent Neuclic stain called DAPI (4',6-diamidino-2-phenylindole) is frequently used in mouse models to visualize nuclei in both preserved and living cells as well as in tissue slices. It was shown that more stains are visible in control group means live nuclei are mostly

visualize in control group but in this result, it had found out that treatment with linoelaidic acid helps to decrease the live nuclei in MDA-MB-231 breast cancer cell isolated from MDA-MB-231 injected C57BL/6 J mice model (Fig. 6A). These live nuclei count was significant in comparison to the untreated control cells done by ImageJ software (Fig. 6B).

Detecting DNA strand breaks of linoelaidic acid treated MDA-MB-231 induced tumor cell on C57BL/6 L mice model with TUNEL assay

Apoptosis, or programmed cell death, is found in mouse cancer tissues using the TUNEL assay where a fluorescent or colored tag to the 3'-hydroxyl ends of broken DNA, it detects DNA fragmentation, a sign of apoptosis. This makes it possible to see and measure apoptotic cells in tissue slices, which aids in understanding how different linoelaidic acid treatments affect tumor cells. After linoelaidic acid treatment fragmented DNA is mostly visualize in fluorescence microscopy (10x) (Fig. 8A). Fig. 8B indicates the % of TUNEL-positive cells, done by ImageJ software. The Treatment-II group is more effective for create the

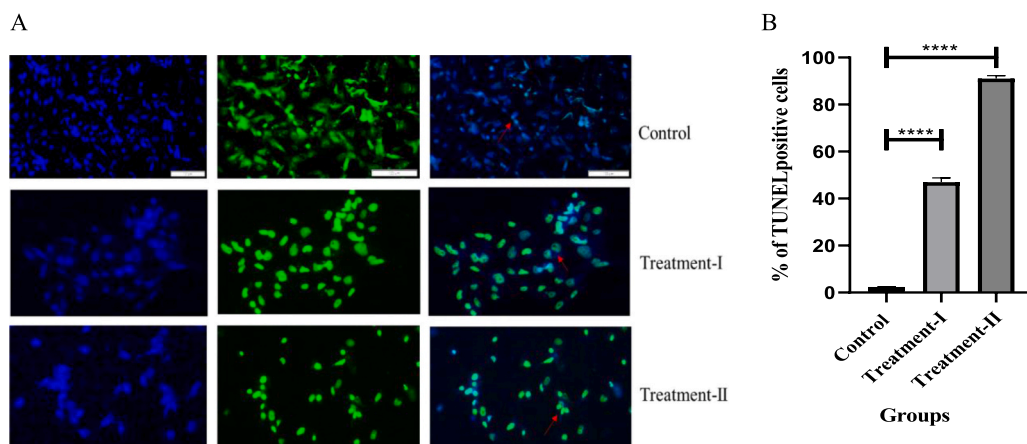


Fig. 8. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was used to detect DNA fragmentation in linoelaidic acid-treated MDA-MB-231-induced tumor cells. The groups are as follows: Control: Untreated control, Treatment I: Linoelaidic acid with 0.006 mg/kg, Treatment II: Linoelaidic acid with 0.014 mg/kg. (A) The images show intact nuclear DNA (Blue fluorescence) and fragmented nuclear DNA (Green fluorescence), indicating apoptotic cells. Fluorescence microscopic images were recorded at 10x magnification using an Olympus fluorescence microscope (BX43F). The scale bar correspondence to 100 μ m. (B) Bar diagram represents % of TUNEL-positive cells. Statistical analysis was denoted as **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05 using one-way ANOVA multiple comparison test.

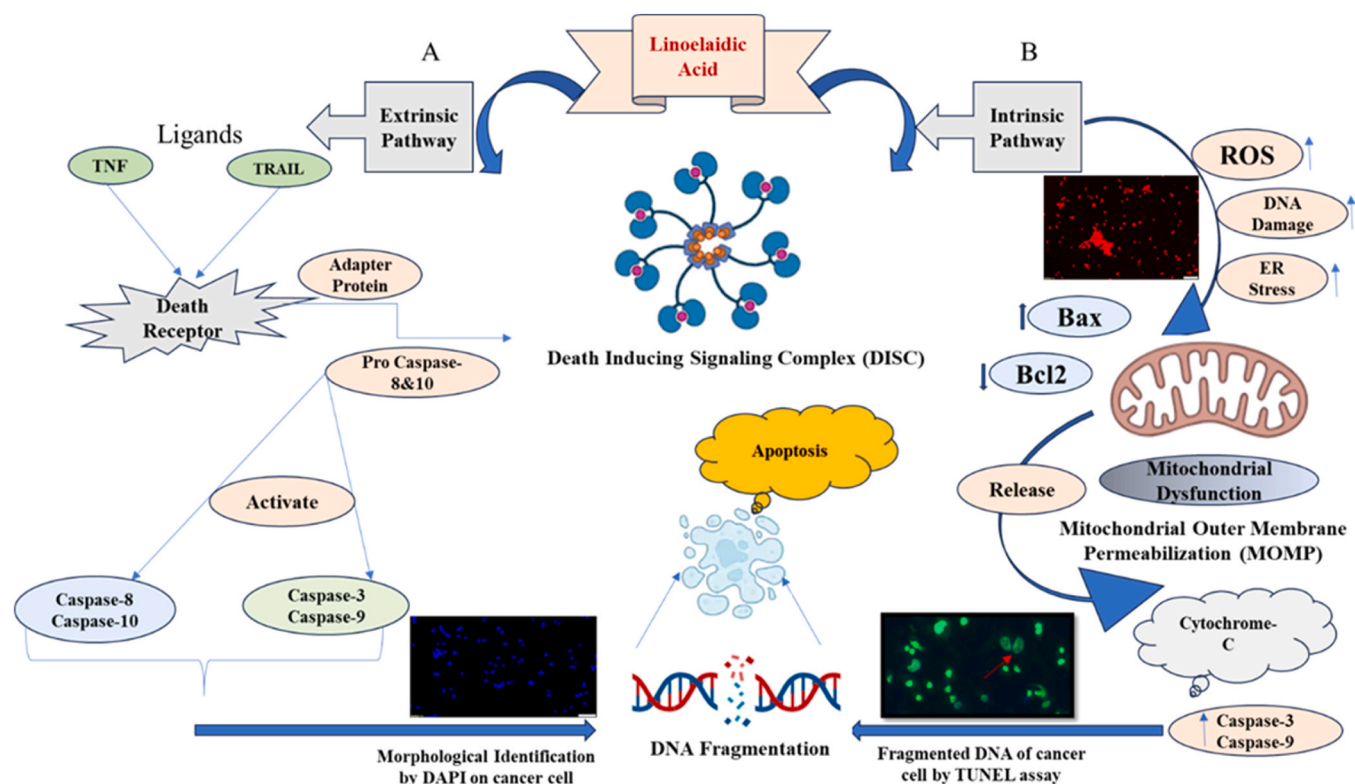


Fig. 9. Possible mechanism for apoptosis by Caspase activation and ROS generation using Linoelaidic acid.

DNA fragmentation compared to the untreated control group. It was found that the percentage of fragmented DNA was 2.17%, 46.67% and 90.27%, respectively, in the Control, Treatment-I and Treatment-II groups (Fig. 8B).

Discussion

In the present study, the anti-cancer effect of linoelaidic acid derived from marine *O. tardoore* oil is demonstrated via altered redox balance and inducing apoptosis as well as by morphological observation via fluorescence microscope. Earlier, GC-MS analysis was used to identify the fatty acids present in *O. tardoore* oil and found linoelaidic acid as one of them (Pradhan et al., 2020). Recently, natural products are a promising complement to the toxic anti-cancer medications now on the market, but their efficacy and bioavailability present significant challenges to their practical application as therapeutic or preventive agents. Using nutritional combinations with synergistic effects is one way to solve these issues (Saw, and Swamy et al., 2010, 2008). Linoelaidic acids are ω^6 trans fatty acids under the group of linoleic acid (Suresh et al., 2015). Conjugated linoleic acid (CLA), a fatty acid having two conjugated cis/trans double bonds and eighteen carbon atoms, has demonstrated anticarcinogenic properties on DMBA-induced skin cancer in mice (Salminen et al., 1998). Cis/trans fatty acids mainly activates the wild p53, which helps to activate apoptosis or regulate the uncontrolled cell growth (Hirata et al., 2021). Linoelaidic acid acts as a cis/trans fatty acid of linoleic acid but it has no cytotoxic effect on human peripheral blood lymphocytes (PBLs) (Figure. S1).

Compared to synthesized medications, this fatty acid has a comparatively low risk of harmful side effects and are readily available, affordable and sustainable resource. The novel findings of this study to determine the synergistic effects of Linoelaidic acid, an isomer of omega-6 polyunsaturated fatty acid found in fish oil, on the development of breast cancer. Each group of mice, tumor volume is determined independently in order to address the key angiogenesis factors at various stages of tumor growth (Fig. 1). Marine edible fish are one of the

numerous biological sources that are valuable natural resources for the creation of novel drugs as they do not pose a threat to human health. After being exposed to linoelaidic acid, the vitality of MDA-MB-231-injected tumor cells declined in a dose-dependent manner (Fig. 2A). Lactate dehydrogenase (LDH) is an enzyme found in nearly all living cells. LDH catalyzes the conversion of pyruvate to lactate in the same way as it catalyzes the conversion of NAD⁺ to NADH and vice versa. A dehydrogenase is an enzyme that transfers a hydride from one molecule to another. LDH enzymes belong to four distinct classes.

This article specifically addressed LDHs that either use D-lactate or rely on cytochrome c are D-lactate dehydrogenase (cytochrome) and L-lactate dehydrogenase (cytochrome). LDH is extensively expressed in physiological tissues such as blood cells and heart muscle. because tissue injury causes it to be released. It was found that LDH-A expression was independent of other clinical pathological variables and had a strong correlation with tumor growth. Inhibition of cancer cell proliferation, increased intracellular oxidative stress, and stimulation of mitochondrial pathway apoptosis were the outcomes of low amount of LDH-A (Fig. 2B). Because tumor cells may adjust to a variety of micro environmental features and cytotoxic treatments, their metabolism is likewise quite adaptable. The GSH levels in MDA-MB-231 injected tumor cell considerably ($p < 0.05$) dropped following treatment with two distinct dosages of linoelaidic acid, according to the results. It was discovered that Treatment-II group of linoelaidic acid was more effective than the other dose at lowering the GSH level in MDA-MB-231 injected tumor cell (Fig. 3A). The primary cause of this impact is an increase in GSSG levels. The rise in blood GSSG could be attributed to two factors: (a) an increase in peroxide production by the tumor, which can cause GSH oxidation in red blood cells in addition to altering glutathione-related and antioxidant enzyme activities; and (b) an increase in the release of GSSG into the blood from various tissues. The overproduction of reactive oxygen species (ROS) in the face of inadequate or malfunctioning antioxidant defense systems is a sign of oxidative stress. Among different established biomarkers for illnesses involving oxidative stress is becoming more and more popular (Zitka

et al., 2012). It is commonly known that GSSG is harmful to cells and that the quantity of GSH balances it out. The cellular stress following linoelaidic acid treatment was validated by the elevated GSSG level and decreased GSH level in MDA-MB-231 (Fig. 3B). Glutathione is an important cellular component and involves in the protection against different cellular peroxides, free radicals, and toxic compounds in the tumor tissue (Valko et al., 2016).

Our results also indicated the enhanced NO production in linoelaidic acid-treated MDA-MB-231 injected tumor cell (Fig. 3C). NO (nmol/L) dramatically increased total protein synthesis in MDA-MB-231 induced mice model and also increased cell proliferation. NO specifically increased the translation of cyclin D1 and ornithine decarboxylase (ODC) without altering their mRNA levels or half-lives. NO was targeted in ER + breast cancer cells by specific NO depletion and NOS2 silencing and atmosphere formation capacity, stem cell markers and tamoxifen sensitivity were analyzed. In this study the parameters were made to analyze the association of NOS2 gene expression with the survival of ER-breast cancer implanted C57BL/6J mice model treated with Linoelaidic acid. Hence, linoelaidic acid may promote nitrite generation in MDA-MB-231 induced tumor tissue which played a crucial role in cancer cell growth arrest.

In this manuscript, we examined mRNA expression level changes of p53, caspase, Bcl-2 and the results showed that p53 expression increased in a dose-dependent way (Fig. 4A). By controlling processes including cell cycle arrest, DNA repair, and apoptosis (programmed cell death) in response to cellular stress, the p53 protein functions as a "guardian of the genome," preventing the development of tumors. When it works, it stops damaged cells from multiplying by either stopping the cell cycle for repair or, in the event that the damage is irreversible, initiating cell death (Fei et al., 2002). Linoelaidic acid helps to increase the activation of p53 in MDA-MB-231 induced tumor mice model. The caspases are the primary mediators of apoptosis, despite the involvement of several genes. Aspartate-specific cysteine proteases, or caspases, cleave their substrates on the carboxyl side of the aspartate residue (3, 4). Two thirds of the at least 14 distinct caspases that are currently known to exist are involved in apoptosis. The two primary categories of caspases implicated in apoptosis are the downstream effector caspases (e.g., caspases 2, 3, 6, and 7) and the initiator caspases (e.g., caspases 8, 9, and 10). The latter group's members are in charge of the morphological alterations that occur during apoptosis as well as the degradation of certain cell proteins (Fan et al., 2005). In addition it was found that due to the activation of Caspase family apoptosis rate increase after linoelaidic acid treatment (Figs. 4B, 4C, 4D). Bax, a key proapoptotic member of the Bcl-2 family and a protein necessary for cell death, is widely expressed in both in vitro and in vivo models of breast cancer, indicating that Bax could be a target for breast cancer therapy. The proapoptotic role of Bax is probably activated by a number of interrelated processes, including its translocation from the cytosol to the mitochondria, oligomerization, and insertion into mitochondrial membranes during cellular stress (Fig. 4E). The main role of Bcl-2 in cancer is anti-apoptotic, or preventing programmed cell death. Cancer cells survive as a result, which encourages tumor growth and renders them immune to treatments that depend on cell death. In our study the amount of Bcl2 production is downregulated (Fig. 4F), it means the linoelaidic acid may help to increase the cell death in tumor induce mice model.

TP53/p53, a tumour suppressor gene, is mutated in around 50% of all human malignancies. Further, tumor suppression gene, p53 induced cell death well documented and also have significant impact on both malignant and non-transformed cells react to various anticancer therapies. Cell cycle arrest, cell senescence, DNA repair, metabolic adaptability, and cell death are just a few of the biological activities that p53 reportedly controls by directly regulating about 500 target genes in a homotetrameric transcription factor (Aubrey et al., 2019). To examine the effect of linoelaidic acid on p53 protein expression, we performed the Western blotting and the expression level of p53 was found to be

increased in a dose-dependent manner (Fig. 5). In this context, increased p53 after linoelaidic acid exposure restricted the MDA-MB-231 induced tumor cell proliferation via modulating genes related to the cell cycle and apoptosis (Kemp et al., 2003). Our results revealed that linoelaidic acid mediated apoptosis was initiated via the expression of mitochondrial Bax and P53 among the different biological origins. Caspase-3, an executioner caspase that is broadly distributed and an effector protein in the apoptotic process, is the most significant regulator of apoptosis in the caspase family (McIlwain et al., 2014 & Pu et al., 2017). Caspase-3 is an inactive dimer that is activated by cleaving a specific subunit with a protease. To activate caspase-3, several proteases cleave the caspase-3 zymogen (cleaved-caspase-3). Cleaved-caspase-3 continues to cut other substrates, which causes the protease cascade to grow and ultimately result in cell death. Protein function changes brought on by the cleaved substrates result in apoptotic cellular changes (Crawford and Wells, 2011). Thus, activation of caspase-3 indicates that cells are being induced to undergo apoptosis. Caspases are synthesized as pro-caspases, an inactive form which cleaved into their active form during apoptosis. Hence, determination of caspase activity is considered a gold standard for apoptosis detection (Brauchle et al., 2014; Elmore, 2007). Several caspases, may be activated during apoptosis connected to apoptosis are brought about by activated caspase-9, which mobilizes a series of effector caspases, such as caspase-3. Our results clearly shown with consistent that linoelaidic acid exerted an antitumor effect on MDA-MB-231 induced tumor cells via caspase activation (Fig. 5).

In apoptotic cells, dual AO/Et-Br fluorescence labeling can identify fundamental morphological alterations. It also showed the differentiation of necrotic cells, early and late apoptotic cells, and normal cells. A qualitative and quantitative approach to identifying apoptosis examined via AO/EB staining (Laha et al., 2015). We hypothesize that AO entered intact-membrane healthy and early apoptotic cells and fluoresced green when bound to DNA. When linked to concentrated DNA fragments or apoptotic entities, EB only entered cells with damaged membranes, such as late apoptotic and dead cells, displaying orange-red fluorescence. The AO/EB approach enhances the detection of apoptosis and can differentiate between late apoptotic and dead cells as compared to AO labeling (Fig. 6C & D). DAPI (4',6-diamidino-2-phenylindole) is a common fluorescent dye used in research and diagnostics to detect and examine cancer cells, although it is not a cancer treatment in and of itself. Its main purpose is to visualize the cellular DNA content and nuclear shape, which are frequently changed in cancer cells. Findings from this study shown that the damaged cells were identified with this staining and Treatment-II group of linoelaidic acid is the effective dose. Apoptotic cells can be identified by DAPI staining because they display morphological alterations such as nuclear condensation, fragmentation, and blabbing. These alterations can be used to evaluate the effectiveness of medications or therapies that cause apoptosis since they are suggestive of programmed cell death (Fig. 6A & B). There are numerous cells signaling mechanisms in cancer cells, especially those associated with ROS generation and scavenging. Although ROS are by products of regular metabolism, their effects on cells depend on their concentration. In intracellular signaling, ROS work as "redox messengers" at low concentrations, but at high concentrations, they can trigger oxidative alteration of macromolecules, which can impair cellular functions and hasten apoptosis. It was verified that the mechanisms by which linoelaidic acid promotes apoptosis involves significant increase in ROS production. Results confirmed that linoelaidic acid at 0.014 mg/kg body weight significantly increased ROS generation (Fig. 7A & 7B). In addition, we conducted TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) staining to identify apoptosis, or programmed cell death, in tissue samples, including malignancies from mice. It functions by identifying DNA fragments that are indicative of apoptotic cells such that they may be seen under a microscope. Terminal deoxynucleotidyl transferase (TdT), an enzyme that can add nucleotides to the 3' end of DNA breaks, is used in TUNEL labeling. These fractures are caused by DNA fragmentation in apoptotic cells. TUNEL labeling can be

used to assess how treatments like chemotherapy or targeted medicines affect tumor cells in mice. Researchers can ascertain whether a treatment causes apoptosis, which is a desired result in cancer therapy, by looking at TUNEL staining patterns in tumor tissue sections (Weylandt et al., 2011). In consistent with other morphological examination, linoelaidic acid showed ability to breakdown of the DNA and create apoptosis rate highly in tumor tissue collected from MDA-MB-231 injected C57BL/6 J mice model (Fig. 8 A & B) which supports linoelaidic acid induced cellular apoptosis. The limitation of this present study is to select the specific xenograft mice for tumor induction for model selection due to infrastructural constrain which might be addressed in our future study.

Conclusions

It concluded that Linoelaidic acid have some health-beneficial effect on cancer. It plays an essential role in cell signaling and in the cell structure and fluidity of membranes, however, oily fish and fish oil may contain anti-cancer activity. Linoelaidic acid is an Omega 6 Trans fatty acids and is a geometric isomer of linoleic acid. Omega-3, 6, and 9 fatty acids—which people are unable to create on their own—are among the many species and ecosystems that make up marine bioresources, which are crucial to preserving the existing order and providing humans with the resources they need to thrive. Fatty acids with anti-inflammatory and anti-carcinogenic properties can be found in sea fish. There is currently little information available regarding the possible anti-cancer properties of the fish (*Opisthopterus tardoore*) from which linoelaidic acid was extracted. *Opisthopterus tardoore* fish can be used as an adjuvant therapy for triple-negative breast cancer patients or mitigate cancer related fatigue due to its well-characterized poly unsaturated and monounsaturated fatty acids and can be used as sustainable bioresource for health promotion.

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CRedit authorship contribution statement

Sibani Raut: Investigation, Formal analysis. **Manisha Phoujdar:** Software, Methodology. **Titli Panchali:** Software, Resources. **Pipika Das:** Software, Formal analysis, Data curation. **Riya Kar:** Software, Resources. **Ananya Dutta:** Writing – original draft, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **PRADHAN SHRABANI:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Kuntal Ghosh:** Writing – review & editing, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Approval for animal experiments

The animal study was approved by the Institutional Animal Ethics

Committee (2256/PO/Re/S/23/CCSEA) MCC/IAEC-SP/10/23–24).

Clinical Perspective

According to data from epidemiological studies, omega-3 and omega-6 fatty acids have a positive impact on a number of cancers, including breast cancer, including overall survival, a favourable prognosis, and a decrease in specific mortality (more than 25% in patients with lung cancer), as well as pancreatic ductal adenocarcinoma. As we have seen in this study, the isomer of linoleic acid, known as linoelaidic acid, has anticancer potentiality. A few in vivo investigations have also noted similar effects of linoleic acid on breast and other malignancies.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.sesb.2026.100013.

Data availability

Data will be made available on request.

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