

In vitro anti-cancer potency of *Mortierella elongata* lipids against MCF 7 cells through induction of apoptosis and cell cycle arrest

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ABSTRACT

Globally, cancer remains the second-whacking cause of mortality. Several studies, like *in vitro* cell studies, clinical trials, *in vivo* studies, and cohort studies, have authenticated the anticancer potency of omega-3 polyunsaturated fatty acids (PUFA) against various cancer types. The present study reports the *in vitro* anticancer potency of PUFA produced by *Mortierella elongata* (Accession No. OK402027) against Michigan Cancer Foundation-7 (MCF-7) breast cancer cell lines. The biocompatibility and cytotoxicity of *M. elongata* lipids were evaluated against human embryonic kidney and MCF-7 cells, respectively. The anti-proliferative activity of *M. elongata* lipids was examined at three different concentrations based on the inhibitory concentration (IC₅₀) value. The apoptotic activity of *M. elongata* lipids was analyzed by fluorescence microscopy by implementing three different staining methods, such as acridine orange/ethidium bromide, 4,6-diamidino-2-phenylindole, and propidium iodide. Further, the biological activity of *M. elongata* on apoptosis induction, cell cycle progression, mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) generation were evaluated. The MTT assay revealed the anti-proliferative activity of *M. elongata* lipids, and the IC₅₀ value of *M. elongata* lipids at 24 h was found to be 28 ± 1.3 µg/mL. A significant decrease in the percentage of live cells with 57.36% of apoptotic and 14.10% of necrotic cells was revealed in cells treated with 100 µg/mL of *M. elongata* lipids. Anti-proliferative activity is associated with increased ROS generation and the loss of MMP. The lipids of *M. elongata* induced a dose-dependent G₁ arrest and were found to be more effective at 100 µg/ml, accumulating 54.49% of MCF-7 cells in the G₁ phase. Taken together, the present study has confirmed the *in vitro* anticancer potency of *M. elongata* lipids via apoptosis and cell cycle arrest.

1. INTRODUCTION

Globally, cancer ranks as the second-leading cause of mortality. Approximately 1.8 million people were diagnosed with cancer, and around 9.6 million people lost their lives in 2018. This burden keeps increasing, and it's expected to escalate by two-fold in another two decades (World Health Organization, 2020).

Several investigations, including *in vitro* cell studies, clinical trials, animal models, and cohort studies, have authentically reported that omega-3 polyunsaturated fatty acids (PUFA) hold anticancer properties against various types of cancer. Omega 3 and omega 6 endocannabinoids (such as arachidonyl ethanolamide, docosahexaenoyl ethanolamide [DHEA], and eicosapentaenoyl ethanolamide [EPEA]) acting as agonists activate cannabinoid and other receptors, resulting in several signaling cascades. These signals are known to intervene in almost all-

important stages of cancer development and establish anticancer effects by preventing the commencement of tumors, inhibiting and suppressing the rapid growth of tumors, and restraining the metastasis [1]. The antagonist effect of endocannabinoids was antiproliferative (inhibition of cancer cell growth) and was attained by either a combined process of autophagy [2], apoptosis, or arresting the cell cycle. Normally, both autophagy and cell cycle arrest will end with the induction of apoptosis (programmed cell death). Endocannabinoids were reported to exhibit anti-angiogenesis (preventing the formation of new blood vessels), anti-invasive, and anti-metastatic (inhibiting metastasis) properties [1]. These endocannabinoids do not elicit autophagy or pro-apoptotic activity in normal cells [2], which authenticates the use of omega-3 PUFA and their derived endocannabinoids as preventive and therapeutic agents as well as adjuvants in cancer therapy. Several studies have suggested that DHEA, EPEA, DHA, and EPA possess adjuvant potency in breast cancer therapy [3-5]. An *in vitro* study using Michigan Cancer Foundation-7 (MCF-7) breast cancer cell lines has demonstrated the ability of DHEA, EPEA, DHA, and EPA to elicit the expression of the PRAR₁ receptor and attenuate the AKI-mTOR pathway, resulting in inhibiting the proliferation of cancer cells by promoting autophagy [2]. Regular dosages of dietary DHA (120 mg) and EPA (180 mg) during

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chemotherapy in breast cancer patients have increased the antioxidant level, superoxide dismutase, glutathione reductase, and catalase activity in the patients' blood profiles. Thus, PUFA acts as an adjuvant in battling post-effect chemotherapy [5].

Therefore, the *in vitro* anticancer potency of *M. elongata* lipids containing both omega 6 and omega 3 essential fatty acids such as gamma-linolenic acid (GLA), arachidonic acid (ARA), EPA, and DHA was investigated against MCF-7 cells.

2. MATERIALS AND METHODS

2.1. Isolation of Oleaginous Fungi

The oleaginous fungal isolate, *Mortierella elongata* (accession no. 402027), was isolated from the terrestrial soil of Nilgiris Hill, Western Ghats of Tamil Nadu, at 11.4007°N and 76.7358°E. In our earlier study, the total lipids were extracted using a low-toxicity solvent system with 3:2 v/v of n-hexane and isopropyl alcohol. The extracted total lipids were esterified, and fatty acid profiles were determined. The oleaginous fungus *M. elongata* was found to be a potential source of PUFA and was noted to produce the most essential fatty acids like omega 6 and omega 3, such as 0.79% of GLA, 1.24% of ARA, 1.24% of EPA, and 6.83% of DHA.

2.2. Anti-cancer Study

2.2.1. Cell culture

Human embryonic kidney cells (HEK) and human breast cancer cells (MCF-7) were procured from the National Centre for Cell Sciences, Pune, India. HEK and MCF-7 cells were seeded separately in T-25 flasks containing Dulbecco's Modified Eagle's Medium (DMEM) augmented with Balanced Salt Solution, l-glutamine (2 mM), and made up to contain 4-(2-hydroxyethyl)-piperazine ethane sulfonic acid (HEPES, 10 mM), sodium pyruvate (1 mM), fetal bovine serum (10% FBS, GIBCO, USA), and non-essential amino acids. Antibiotics such as penicillin and streptomycin of concentration (100 IU/100 µg) adjusted to 1 mL/l were used. Cells were incubated at 37°C in a 5% CO₂ humidified incubator.

2.2.2. Evaluation of cytotoxicity

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Hi-Media) analysis was carried out to evaluate the inhibitory concentration (IC₅₀). Cells of density 1×10^4 cells/well were cultured in a 96-well plate and incubated for 48 h to attain 80% confluence. The spent medium was replaced with fresh medium without disturbing the monolayer cells. The cells were treated with various concentrations of *M. elongata* samples and incubated for 48 h at 37°C. After incubation, the spent medium was again removed with intense care without disturbing the adhered cells. An aliquot of 100 µL of DMEM and 100 µL of MTT dye were added to each well and allowed to be incubated for 4 h at 37°C. After the treatment, the supernatant was removed. To make formazan crystals soluble, 50 µL of DMSO was added and incubated for 10 min. Cells without treatment were taken as controls. The ELIZA multi-well plate reader (ROBONIK, India) was utilized to determine the optical density at 620 nm. The percentage viability of cells was calculated with the obtained OD value using the formula:

$$\% \text{ of viability} = \left[\frac{(\text{OD value of experimental sample})}{(\text{OD value of experimental control})} \right] \times 100$$

2.2.3. Morphological study

The opted breast cancer cells of density 1×10^5 cells/coverslip were fixed on the coverslip with 3:1 v/v of ethanol a acetic acid.

Subsequently, the cells were treated with different concentrations of the test sample based on the IC₅₀ values. Then the coverslips were mounted on the glass slide to determine the morphology of the treated and untreated cells. The morphological changes of the three monolayer cells were visualized and micro-graphed using a Nikon bright field microscope (Japan) at 20× magnification.

2.2.4. Analysis of apoptotic cell death by fluorescence microscopy

A nuclear staining dye blend was prepared by adding an equal proportion of 100 mg/mL of Acridine Orange (AO) and 100 mg/mL of Ethidium Bromide (EtBr) in deionized water. An aliquot of 1 µL of blended dye was mixed with 0.9 mL of cell suspension at a density of 1×10^5 cells/mL grown on the coverslip attached to a 6-well culture plate [6]. The cancer cells treated with various concentrations of *M. elongata* sample were processed by washing with PBS. Immediately, the cells were stained with 10 µL of AO/EtBr and incubated for 3 min. Subsequently, the cells were again washed with PBS and visualized under a fluorescence microscope (Nikon Eclipse, Inc., Japan) with an excitation filter of 480 nm. Besides, the cells were placed on a glass coverslip in six-well culture plates and treated with different concentrations of *M. elongata* samples for 24 h. After treatment, the cells were fixed with methanol and acetic acid (3:1) v/v. The fixed cells were washed with PBS and subsequently stained with 10 µL of 4,6-diamidino-2-phenylindole (DAPI) and incubated for 20 min in the dark. After incubation, the stained cells were observed under a fluorescence microscope (Nikon Eclipse, Inc., Japan) with an excitation filter of 480 nm [7]. The MCF-7 cells were placed on a glass coverslip in six-well culture plates and treated with different concentrations of *M. elongata* samples. After 24 h of treatment, the cells were washed 3 times with PBS. Then the cells were stained with 50 µL of propidium iodide (PI) at a concentration of 5 mg/mL and incubated for 10 min. After incubation, the stained cells were mounted and observed under a fluorescence microscope (Nikon Eclipse, Inc., Japan) with an excitation filter of 480 nm [8].

2.2.5. Determination of mitochondrial membrane potential (MMP)

The MCF-7 cells were placed on a glass coverslip in a six-well culture plate and treated with different concentrations of *M. elongata* samples. After 24 h of treatment, the cells were washed 3 times with PBS. Then the cells were stained with 2 µM of JC-1 dye and incubated at 37°C at 5% CO₂ for 15–30 min. After incubation, the stained cells were washed and observed under a fluorescence microscope (Nikon Eclipse, Inc., Japan) [9].

2.2.6. Detection of apoptosis by annexin-V/fluorescein isothiocyanate (FITC) flow cytometer

MCF-7 cells at a density of 1×10^5 cells/ml were grown in a six-well culture plate. After 24 h of incubation in a 5% CO₂ atmospheric incubator at 37°C, MCF-7 cells were treated with different concentrations of *M. elongata* samples for 24 h. Cells without treatment are considered control. After 24 h of treatment, the cells were harvested using trypsin, washed twice, and immediately stained using annexin V-FITC and PI. It was then incubated in the dark for 16 min at RT. Subsequently, binding buffer was added, and flow cytometry was carried out in duplicate with the BD FACS verse flow cytometer. Around 10,000 events were collected for all the experimental samples and controls. Fluorescent signal intensity was recorded and determined by Cell Quest and Modifit.

2.2.7. Determination of cell cycle

MCF7 cells at a density of 1×10^5 cells/ml were cultured in a six-well cell culture plate for 24 h at 37°C in a 5% CO₂ atmospheric incubator. The cells were then treated with different concentrations of *M. elongata*

samples. The untreated cells were considered a control. The treatment was then carried out for 24 h of incubation. After treatment, trypsin was used to harvest the cells, and 70% ethanol was used for fixation. Post-fixation, the cells were incubated at -20°C for 1 h. Subsequently, the cells were suspended in 0.5 mL of PBS supplemented with 50 $\mu\text{g/mL}$ of PI and 100 $\mu\text{g/mL}$ RNase. It is once again incubated for 30 min. A BD-FACS flow cytometer was used to analyze the cell cycle. The flow cytometer was done in duplicate. Around 10,000 events were collected, and fluorescent signals were recorded and determined by cell quest and modifit.

2.2.8. Reactive oxygen species assay

MCF-7 cells at a cell density of 1×10^5 cells/mL were seeded and grown in a six-well culture plate for 24 h at 37°C in a 5% CO_2 atmospheric incubator. After 24 h of incubation, the spent medium was replaced with fresh medium containing DMSO (0.25%, v/v) and different concentrations of *M. elongata* samples. It is then incubated for 6 h. Then, the cells were stained with 10 μM of DCFH-DA (2,7-dichlorofluorescein diacetate, Hi-Media). It was further incubated for 20 min at 37°C . The cells were then harvested and washed with medium without serum. The production of ROS was measured by flow cytometry for all the samples.

2.2.9. Statistics

All the *in vitro* cell culture studies were done in triplicate, and the statistical analysis was performed by SPSS software version 17.0. $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

The isolate, *M. elongata* (Accession No. OK402027), was isolated from the terrestrial soil of Nilgiris Hill, Western Ghats of Tamil Nadu, and was found to be a potent PUFA-producing oleaginous fungus. Several studies have reported the accumulation of PUFAs in *Mortierella* species, with special attention to *M. elongata* [10-12], as well as the anti-cancer activity of PUFAs such as ARA, gamma-linolenic acid, and EPA [13-18]. Several authors have specifically reported the anti-cancerous activity of PUFAs produced from *Mortierella* spp. [19-21].

3.1. Biocompatibility and Cytotoxic Effect of *M. elongata* Lipid

The biocompatibility and cytotoxic effects of the PUFA-enriched lipids extracted from *M. elongata* were investigated on normal HEK and MCF-7, respectively. The HEK cells were treated with various concentrations of total lipids. The MTT assay showed that *M. elongata* lipids had no cytotoxic effect on normal HEK cells. Thus, lipids from *M. elongata* were found to be compatible with human cell lines. The European Food Safety Authority (EFSA, 2008) has reported the safety of fungal oil from *Mortierella* spp.; it is not known to produce mycotoxin and is not pathogenic to mankind [22]. The cancerous cell line, MCF-7 cells, was treated with different concentrations ranging from 20, 40, 60, 80, and 100 $\mu\text{g/mL}$ of *M. elongata* lipids and the reference drug, doxorubicin (Dox). The MTT assay indicated a significant and concentration-dependent cytotoxic effect against MCF-7 cells. The percentage inhibition of cell proliferation of the reference drug and lipids of *M. elongata* is graphically represented [Figure 1]. Based on the dose-dependent curve, the IC_{50} values of Dox and *M. elongata* lipids at 24 h were found to be 18 ± 0.4 and 28 ± 1.3 $\mu\text{g/mL}$, respectively. The National Cancer Institute, US, has recommended that the cytotoxicity of a compound be highly significant if the IC_{50} value is <20 $\mu\text{g/mL}$. Several studies have reported the anti-proliferative activity of PUFA-rich oil from various sources [23,24]. Palakurthi *et al.*, have evaluated the IC_{50} value of EPA on 15 various cancer cell lines and reported 43.3

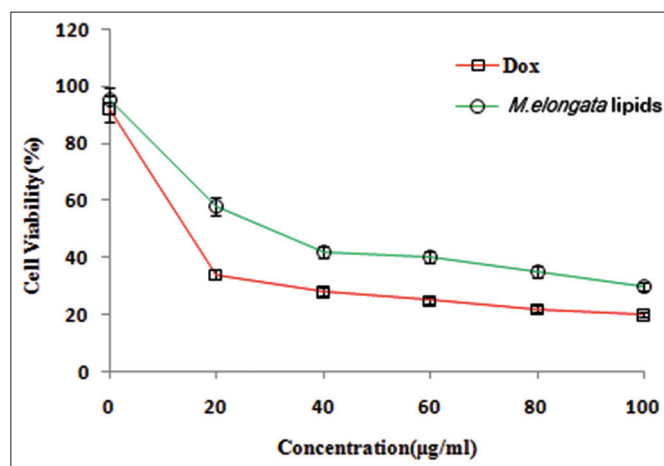


Figure 1: Anti-Proliferative activity of *Mortierella elongata* lipids on Michigan Cancer Foundation-7 cells.

± 3.6 μM against MCF-7 cells [25], and So *et al.*, have reported the IC_{50} of DHA and EPA against LA-N-1 cells at 48 h to be 18 ± 1 μM and 35 ± 2 μM [26], which were found to be in line with the present study. Therefore, the following concentrations of 25, 50, and 100 $\mu\text{g/mL}$ of *M. elongata* lipids were chosen for the experimental studies.

3.2. The Morphological Variations of MCF-7

The anti-proliferative activity was further examined for morphological changes in the treated and untreated MCF-7 cells using an inverted light microscope. The most common variations, like cell detachment, cell shrinkage, and circular-shaped apoptotic bodies, were detected [Figure 2]. When compared to control cells, a decrease in the number of cells and an increase in the number of apoptotic bodies were observed in treated cells in a dose-dependent manner. The maximum number of apoptotic bodies were found in cells treated with 100 $\mu\text{g/mL}$ of *M. elongata* lipids. Thus, the presence of these morphological changes confirmed the apoptotic activity of lipids from *M. elongata*. Similar morphological features of apoptosis were induced by *Thamnidium elegans* and *Nannochloropsis salina* lipid-derived salts against MCF-7 cells, as reported by Sayegh *et al.* [19].

3.3. Analysis of Apoptotic Cell Death by AO/EtBr, DAPI, and PI

AO/EtBr, a fluorescent staining technique, was performed to distinguish the live, apoptotic, and necrotic cells in MCF-7 cells treated with *M. elongata* lipids. The fluorescence micrograph of AO/EtBr-stained and untreated cells is represented in Figure 3. The untreated MCF-7 cells were largely and uniformly stained green in color. Among the treated cells, significant and adverse changes were observed in a dose-dependent manner. Based on the stages of apoptosis, the cells were found to get stained in a varied color pattern. The cells in the initial stages of apoptosis were stained pale green in color, and the cells in the later stages of apoptosis were found to get stained nonuniformly with both pale orange and very mild green color [Figure 3b and c]. The necrotic cells were completely stained orange in color, which was observed in cells treated with 100 $\mu\text{g/mL}$ of *M. elongata* lipids [Figure 3d]. This staining is evidence of apoptosis and is in line with the work reported by Chatterjee *et al.* [27] and Roy *et al.*, [28], where the AO/EtBr staining of the ER + MCF-7 cells revealed the presence of apoptotic signals that were induced by the treatment with alpha-linolenic acid.

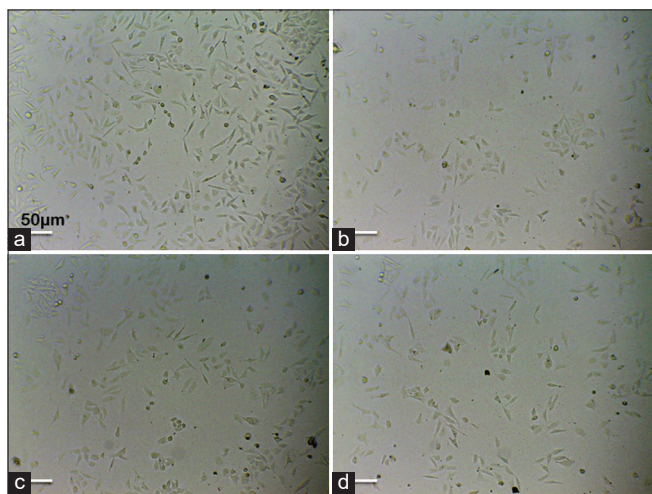


Figure 2: Effects of *Mortierella elongata* lipids on cell morphology. The Michigan Cancer Foundation-7 cells treated with various concentration of *M. elongata* lipids for 24 h. (a) untreated, (b) 25 µg/mL, and (c) 50 µg/mL, (d) 100 µg/mL.

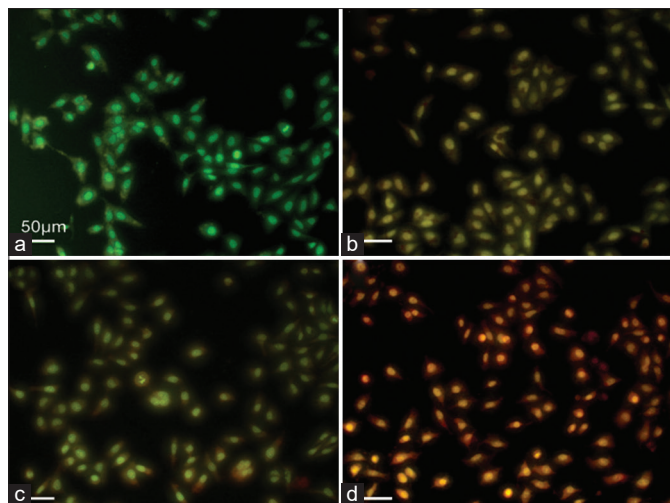


Figure 3: Induction of apoptosis by *Mortierella elongata* Lipids. Acridine orange/ethidium bromide staining of Michigan Cancer Foundation-7 cells treated with various concentration of *M. elongata* lipids for 24 h. (a) untreated, (b) 25 µg/mL, and (c) 50 µg/mL, (d) 100 µg/mL.

DAPI, a nuclear-specific fluorescent staining technique, was carried out to elucidate the nuclear morphology of both untreated and 24-h-treated MCF-7 cells. Figure 4 represents the micrograph of DAPI-stained control and treated cells of MCF-7 cells. The untreated cells indicated a weak blue fluorescence with an intact nucleus. The treated cells have indicated a strong blue fluorescence with an increased number of nuclear fragmentations with irregular edges around the nucleus. The intensity of fluorescence was found to be higher in cells treated with 100 µg/mL of *M. elongata* lipids [Figure 4d]. This DAPI staining result is similar to the activity of leishmanial lipid (pLLD) on the growth of leukemic cell lines [27] and oleic acid against hepatocellular carcinoma [29].

PI, a DNA-specific fluorescent staining technique, was performed to elucidate the nuclear morphology of apoptotic cells. Figure 5 represents the micrograph of PI-stained control and treated cells of MCF-7 cells. The untreated cells showed a weak red fluorescence with

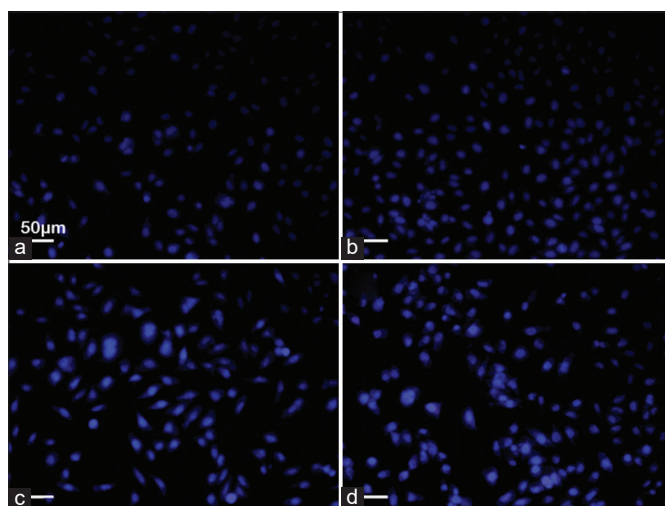


Figure 4: Induction of apoptosis by *Mortierella elongata* Lipids. DAPI staining of Michigan Cancer Foundation-7 cells treated with various concentration of *M. elongata* lipids for 24 h. (a) untreated, (b) 25 µg/mL, and (c) 50 µg/mL, (d) 100 µg/mL.

an intact nucleus. The treated cells showed a strong red fluorescence with an increased number of nuclear fragmentations with irregular edges around the nucleus. The intensity of fluorescence was found to be higher in cells treated with 100 µg/mL of *M. elongata* lipids [Figure 5d]. Thus, the apoptotic morphological changes elucidated by PI staining in this study are similar to the results observed in THP-I cells (Human Monocyte Leukemia cells) treated with oxidized DHA [30] and MCF-7 cells treated with punicic acid, an omega-5 fatty acid [31].

3.4. Determination of MMP

The JC-1 stain enters and accumulates in the mitochondria of healthy cells and forms J aggregates immediately due to energized and negatively charged mitochondria. In the case of apoptotic cells, the J aggregates are not formed due to a loss of electrochemical potential, and hence the JC-1 stain remains green in color. Figure 6 represents the assessment of mitochondrial depolarization using JC-1 dye. The control cells showed strong green-colored fluorescence [Figure 6a]. The treated cells revealed a weak fluorescence [Figure 6d]. A decrease in the green (530 nm) fluorescence intensity by exposure to lipids from *M. elongata* indicates the depolarization or disruption of the mitochondrial membrane. Thus, JC-1 acts as an indicator of MMP and indicates the loss of membrane potential after treatment with *M. elongata* lipids. This mitochondrial dysfunction by *M. elongata* is in line with the activity of PUFA-rich lipids from various sources [27,28].

3.5. Quantification of Apoptotic Stages by Annexin V-FITC

The mode of cell death by apoptosis was further confirmed, and their apoptotic phases were quantified by flow cytometric assay using Annexin V-FITC and the PI staining method. The flow cytometric results are represented in Figure 7. The treatment of different doses of lipids from *M. elongata* on MCF-7 cancer cells for 24 h has resulted in a significant decrease in the percentage of live cells, while the percentage of apoptotic and necrotic cells was found to be increased when compared to the untreated cells. The MCF-7 cells treated with 25 µg/mL of *M. elongata* lipids indicated 31.73% of apoptotic cells and 5.98% of necrotic cells. Treatment with 50 µg/mL has resulted in 43.60% and 13.10% of apoptotic and necrotic cells, whereas 100 µg/mL has resulted in 57.36% of apoptotic and 14.10% of necrotic

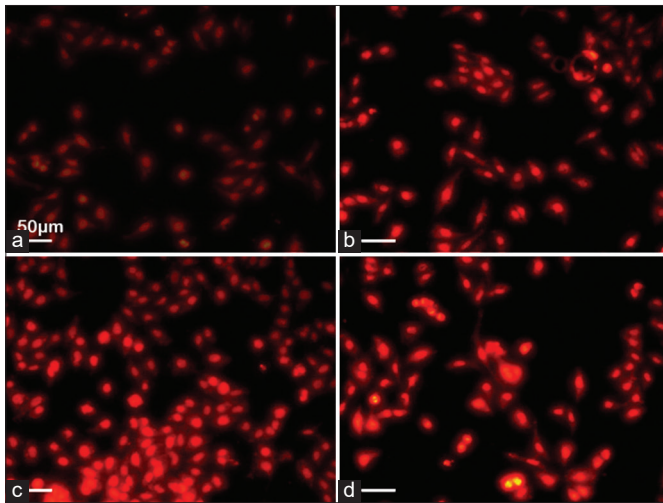


Figure 5: Induction of apoptosis by *Mortierella elongata* lipids. Propidium iodide Staining of Michigan Cancer Foundation-7 cells treated with various concentration of *M. elongata* lipids for 24 h. (a) untreated, (b) 25 µg/mL, (c) 50 µg/mL, and (d) 100 µg/mL.

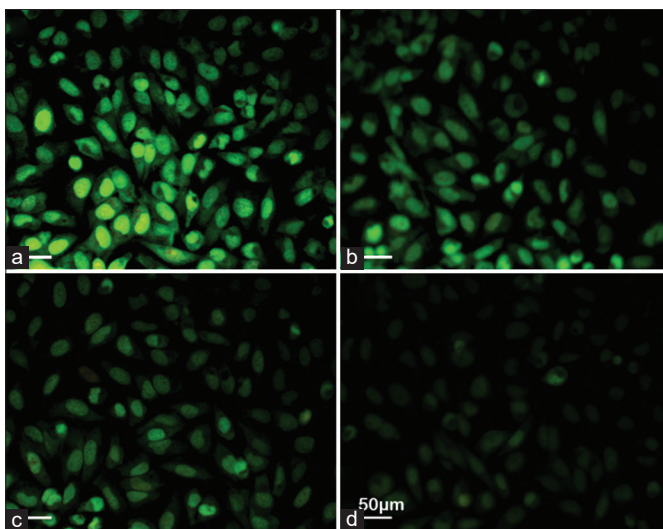


Figure 6: Effects of *Mortierella elongata* lipids on mitochondrial membrane potential. The Michigan Cancer Foundation-7 cells treated with various concentration of *M. elongata* lipids for 24 h. (a) untreated, (b) 25 µg/mL, (c) 50 µg/mL, and (d) 100 µg/mL.

cells. Thus, the antiproliferative effect of *M. elongata* lipids was found to be dose-dependent. The induction of apoptosis by *M. elongata* lipids is consistent with the previous reports, which revealed the anti-cancer potency of PUFA-rich lipids from different sources against various cancer cell lines. Several researchers have quantified the apoptotic cells induced by the treatment with PUFA using annexin V-FITC and PI stains, and their results were in line with the present findings [26,32,33].

3.6. Effect of *M. elongata* Lipids on Cell Cycle Regulation

The involvement of PUFA-rich lipids of *M. elongata* in arresting the cell cycle progression was evaluated by flow cytometry using PI stain. The flow cytometric results are represented in Figure 8. The percentage of cell population in MCF-7 treated with different concentrations of *M. elongata* lipids was found to accumulate more in the G₁ phase and

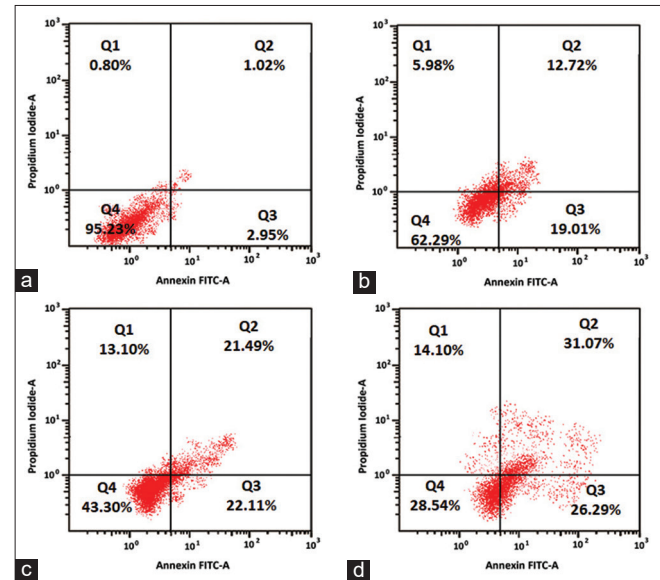


Figure 7: Analysis of apoptosis through Annexin V –fluorescein isothiocyanate flow cytometry. Michigan Cancer Foundation-7 cells treated with various concentration of *Mortierella elongata* lipids for 24 h. (a) untreated, (b) 25 µg/mL, (c) 50 µg/mL, and (d) 100 µg/mL.

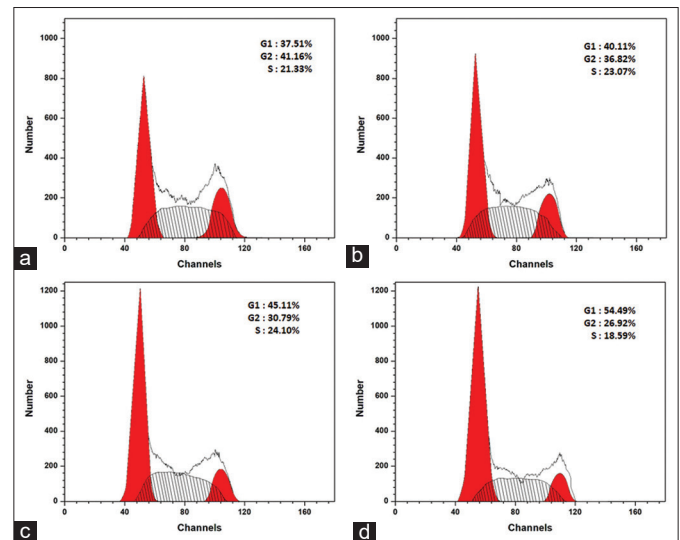


Figure 8: Cell cycle analysis by flow cytometry. The Michigan Cancer Foundation-7 cells treated with various concentration of *Mortierella elongata* lipids treated for 24 h. (a). untreated, (b) 25 µg/mL, (c) 50 µg/mL, and (d) 100µg/mL.

decrease in the M phase when compared to the control [Figure 9]. The cell population in the G₁ phase was found to be 40.11% in 25 µg/mL of treated cells, 45.11% in 50 µg/mL of treated cells, and 54.49% in 100 µg/mL of treated cells. The percentage of cell population in the G₁ phase increases with respect to the increase in concentration of lipids. The percentage of cells in S phase was found to be slightly increased in 25 and 50 µg/mL treated cells in comparison with control, whereas S phase was significantly decreased to 18.59% in the cells treated with 100 µg/mL. Thus, the flow cytometric method suggested that *M. elongata* lipids trigger the G₁ cell cycle arrest in a concentration-dependent manner, accompanied by a decrease in the percentage of cells at the S phase. Several reports have revealed that PUFA-rich

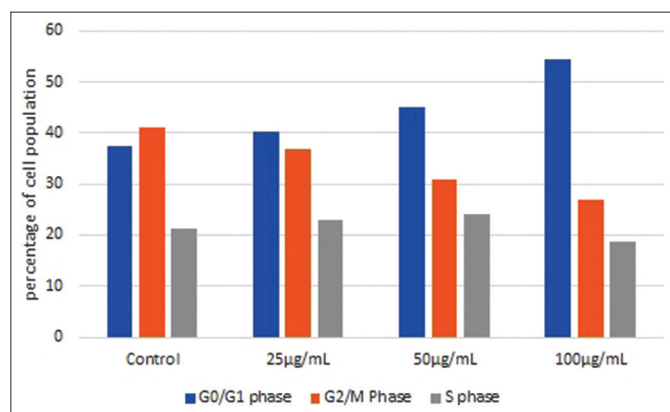


Figure 9: Effect of *Mortierella elongata* lipids on cell cycle progression.

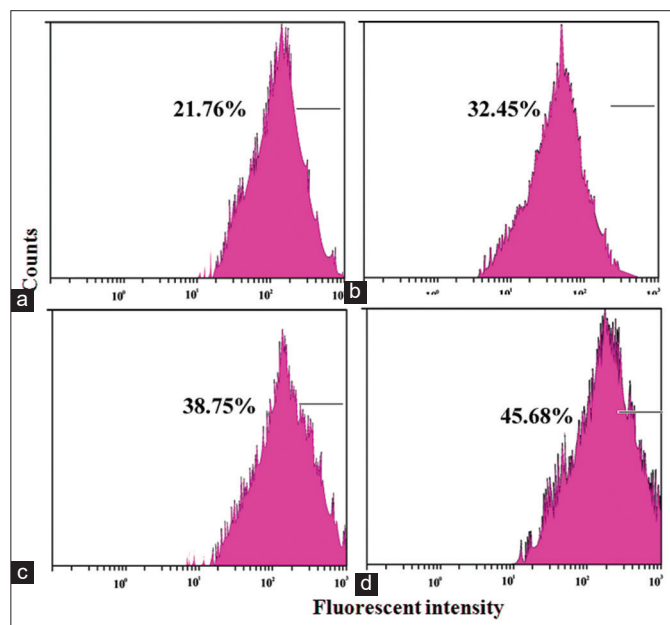


Figure 10: Effect of *Mortierella elongata* lipids on reactive oxygen species generation. The Michigan Cancer Foundation-7 cells treated with various concentration of *M. elongata* lipids treated for 24 h. (a) untreated, (b) 25 µg/mL, (c) 50 µg/mL, and (d) 100 µg/mL.

lipids from varied sources could deploy their anti-cancer activity against various cancer cell lines through triggering cell cycle arrest at the G₁ phase in a concentration- and time-dependent manner [25-27]. EPA and DHA were known to down-regulate the levels of cyclin-dependent kinase-2, cyclin E, cyclin D1, and cyclin G1 proteins in a concentration-dependent manner, which could possibly be attributed to their ability to trigger the cell cycle arrest at the G₁ phase [25,26].

3.7. Effect of *M. Elongata* Lipids on the Generation of ROS

ROS are well known for their involvement in apoptosis-mediated cell death. Therefore, the production of ROS was determined in the MCF-7 cells treated with three different concentrations of *M. elongata* lipids for 24 h. The non-fluorescent dye DCF-DA got oxidized in the presence of ROS and resulted in the formation of DCF, a fluorescent molecule that was measured at 530 nm. The generation of intracellular ROS was determined by flow cytometry. The histogram of cytometric analysis is represented in Figure 10, which indicates the significant difference in the levels of intracellular ROS generation in MCF-7 cells

treated with *M. elongata* lipids compared to the untreated cells. A gradual increase in ROS generation in the treated MCF-7 cells was observed with an increase in the concentration of *M. elongata* lipids. The percentage of DCF-positive cells in the MCF-7-treated cells was 32.45, 38.75, and 45.68% at 25, 50, and 100 µg/mL concentrations, respectively, which was significantly higher than the control with 21.76%. The effect of *M. elongata* lipids on ROS generation was found to be dose-dependent. The increased generation of ROS in cancer cells mediates apoptosis, and the results were in line with previous studies [27,34,35]. Oono *et al.*, (2020) revealed the anticancer effect of EPA on PC3 cells through increased production of ROS [35], and Chatterjee *et al.*, (2015) have demonstrated that pLLD induces apoptosis via increased generation of ROS by regulating the MAPK pathway [27]. Thus, flow cytometric analysis using DCF-DA has confirmed the anti-cancerous activity of *M. elongata* lipids via apoptosis.

4. CONCLUSION

The present study has clearly revealed the anti-proliferative potency of *M. elongata* lipids against MCF-7 cells. The anti-proliferative activity was found to be associated with the induction of apoptosis and cell cycle arrest, along with other hallmarks of apoptosis such as increased generation of ROS and loss of MMP. The anticancer activity of *M. elongata* lipids was found to be more significant in a concentration-dependent manner. The anti-proliferative activity of the sample was directly proportional to its concentration. Thus, *M. elongata* lipids inhibit proliferation through apoptosis and cell cycle arrest at the G₁ phase. This *in vitro* anti-cancer study could be further taken for *in vivo* studies in the future.

5. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

6. FUNDING

There is no funding to report.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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