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Polyunsaturated Fatty Acids from *Thamnidium elegans* and *Mortierella alpina* Suppress Prostate Cancer Cell Proliferation and Migration

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Abstract: *Thamnidium elegans* and *Mortierella alpina* are two oleaginous fungi that belong to Mucoromycota that synthesize polyunsaturated fatty acids, which are credited with multiple health benefits and possible anticancer properties. These fungi were cultivated on culture media, with glucose or glycerol as a carbon source. After extracting the lipids, we transformed them into fatty acid lithium salts (FALSs), which are water-soluble and absorbable mammalian cells, including DU-145 and PC-3 cancer cells. The two cell lines, both long-established prostate cancer models, were treated with FALSs and indicated increased susceptibility to the lipid derivatives. The viability and proliferation rates were significantly reduced, as well as their migratory capabilities, which were significantly impaired compared to olive oil-derived FALS, which was used as a control substance. We conclude that the FALS derivatives of microbial lipids from these organisms exhibit anticancer effects, by suppressing the proliferation and migration of human prostate cancer cell lines.

Keywords: oleaginous fungi; gamma-linolenic acid; di-homo-gamma-linolenic acid; arachidonic acid; fatty acid lithium salts; anticancer; prostate cancer; single cell oils; proliferation; migration

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1. Introduction

Polyunsaturated fatty acids (PUFAs) have attracted the interest of the biotechnology sector, due to their beneficial properties for human health. PUFAs are taken up by the cell and used as an energy source or, under certain circumstances, for functional and structural purposes. The incorporation of PUFAs carrying *cis* double bonds in cell membranes is crucial for functionality, since saturation is inversely correlated with membrane fluidity [1]. Additionally, their absorbance by cells may increase the cell's susceptibility to oxidative stress damage, since the aliphatic chains containing double bonds act as substrates for reactive oxygen species (ROS)-generating lipid hydroperoxides [2]. This phenomenon, also known as lipid peroxidation, propagates exponentially, when not suppressed by the cell's antioxidant mechanisms, and results in excessive oxidative damage, the loss of membrane integrity, and finally cell death [3]. Flooding the cells' catabolic pathways with unsaturated lipids drastically affects their oxidative stress levels, damages vulnerable intracellular

structures and biomolecules, and significantly induces cell death-related pathways, such as ferroptosis and apoptosis [4,5].

Gamma-linolenic acid (GLA, 18:3n-6), arachidonic acid (ARA, 20:4n-6), and di-homo-gamma-linolenic acid (DGLA, 20:3n-6), as members of the omega-6 PUFAs group, are known for their systemic roles as cytoprotective/neuroprotective, anti-inflammatory, and anticancer agents, justifying the ever-increasing interest in them by the research community and industry [6–12]. They are assumed to act through the modulation of the cell metabolism, the generation of reactive oxygen species, the alteration of the membranes' fluidity, and interference with intracellular signal transduction cascades [13–15]. At the level of the cell, these fatty acids are believed to possess high cytotoxic potential compared to other members of the group, as has been shown by various in vitro studies [5,11,15–18]. However, a significant limiting factor in regard to their extensive study and adoption is their scarcity. Since most food oils that contain significant amounts of them are mostly exploited as nutritional ingredients, their price remains high due to their low abundance and the cost of their isolation and purification. Recently, advances in microbial biotechnology have allowed the production of microbial oils containing PUFAs of high pharmaceutical value, using various species that belong to Mucoromycota, especially *Mortierella* and *Thamnidium*. These species are known for their ability to grow on cost-effective substrates, such as food and agricultural industry byproducts (glycerol, pomace, etc.), and accumulate lipids that contain fatty acids like GLA, DGLA, and ARA in significant quantities, while the isolation and purification process is relatively simple [19–21]. Additionally, the whole process can be scaled up to an industrial level to produce great amounts of food- and even pharmaceutical-grade microbial lipids, with potential therapeutic benefits. Currently, these fatty acids are commercially available mostly as dietary supplements and can be found as components of plant-derived oils (like borage and evening primrose oils) that contain GLA and DGL, or green algae-derived single cell oils, in the case of ARA.

The exploitation of single cell oils, as substances of high added value from a pharmaceutical perspective, has occurred in the past, investigating the anticancer and antimicrobial potential of lipids [22–24]. In these articles, the lipids were not administered as free fatty acids, as they are characterized by poor solubility; nonetheless, they were transformed into fatty acid lithium/potassium salts (FALSs/FAPSs). These are liquid, water-soluble compounds that do not require further transformations to be assimilable (e.g., the formation of liposomes or micelles) and they have already been found to cause cell death, by causing oxidative damage and inducing apoptosis [22–24]. Given the variety of different fatty acids that can be produced through fermentation, it is important to recognize both the molecules possessing increased medicinal interest, as well as to invest in the optimization of the culture process to achieve better yields and find more cost-effective strategies.

This investigation aimed to produce microbial oils from the fungi *Mortierella alpina* CBS 343.66 and *Thamnidium elegans* CCF 1465, containing ARA, DGLA, and GLA, respectively, and examine their effects on the DU-145 and the PC-3 prostate cancer cell lines. The oils were administered as FALSs in the cell cultures and key cellular functions were monitored to investigate any potential anticancer roles. The purpose of this study was to investigate whether the lipid extracts of these organisms, which are (bibliographically) credited with the aforementioned beneficial properties, can exhibit anticancer actions. These lipids are relatively expensive in purified form, which is a major drawback that affects their implementation as nutraceutical products. Since recent advances in biotechnology now allow the production of vast quantities of many fatty acids that were previously considered "rare", in the form of accumulated fats in oleaginous microorganisms, a question arises as to whether these mixtures could have some benefits as nutraceutical products. As limited evidence exists on this field and only a negligible number of studies investigate the potential benefits of microbial lipids, this article focused on these two Mucoromycota species and their lipid extracts are assessed for their basic anticancer potential [22–25]. To set a context for this study, since microbial lipids are a rather novel notion in cancer research, two commercially available lipid formulations are used as reference points, namely

olive oil (which contains the relatively inert oleic acid) and fish oil (which contains the well-established cytotoxic EPA in large quantities), as has also been performed in similar experiments [24].

2. Materials and Methods

2.1. Biological Material and Culture Conditions

The fungi *Mortierella alpina* CBS 343.66 and *Thamnidium elegans* CCF 1465 were kept for maintenance purposes on potato dextrose agar (Condalab, Madrid, Spain) at 7 ± 1 °C and the master cultures were regularly sub-cultured. The growth medium of *M. alpina* CBS 343.66 contained glucose as a carbon and energy source at 30 g/L, while *T. elegans* CCF 1465 was provided with glycerol at 30 g/L. Both growth media were supplemented with minerals and their full compositions are presented in Appendix A (Table A1). Both media were nitrogen limited, with a yeast extract at 2.0 g/L being the only nitrogen source, while in the case of *M. alpina*, it was also deployed as a source of magnesium and iron. A medium pH of 6.5 ± 0.5 was achieved after sterilization at 121 °C for 20 min and remained practically stable during cultivation, due to the high buffer capacity of the media containing Na_2HPO_4 and KH_2PO_4.

Submerged cultures were performed in 250 mL Erlenmeyer flasks containing 50 mL of the growth medium. The flasks were aseptically inoculated with 1 mL of spore suspension, containing 10^6 fungal spores, in laminar flow apparatus. Incubation lasted for approximately 6 days in an orbital shaker (ZHICHENG ZHWY 211C, Shanghai, China), equipped with a ventilation system, at 28 ± 1 °C and an agitation rate of 180 ± 5 rpm.

2.2. Biomass Determination

Fungal mycelia were collected using vacuum filtration, through Whatman No. 1 paper. The harvested biomass was washed with distilled water in triplicate and then dried at 80 °C, until a constant weight was achieved. Finally, the total biomass (x, g/L) was gravimetrically determined.

2.3. Extraction and Purification of Mycelial Lipids

The total mycelial lipids were extracted in chloroform, methanol 2:1 (vol/vol) (Fisher Chemical, Hampton, NH, USA), as described elsewhere [26]. The extracts were filtered through Whatman No. 1 paper and washed with a 0.88% (wt/vol) KCl (Honeywell, Charlotte, NC, USA) solution to remove non-lipid components. Subsequently, the samples were evaporated under a vacuum, using a Rotavapor R-20 device (BUCHI, Flawil, Switzerland). The total cellular lipid quantities (L, g/L) were gravimetrically determined and expressed as a percentage in regard to the respective dry cell mass (L/x%).

2.4. Fatty Acid Composition of Cellular Lipids

To determine the fatty acid composition of all the samples used, the total lipids were converted into their fatty acid methyl esters (FAMEs) in a two-step reaction, according to the AFNOR method [27]. The produced FAMEs were analyzed with a gas chromatography (GC) (Agilent 7890A device, Agilent Technologies, Shanghai, China) device. The GC device was equipped with a flame ionization detector at 280 °C, using a HP-88 (J&W Scientific, Folsom, CA, USA) column (60 m × 0.32 mm) for the analyses. The carrier gas was helium, at a flow rate of 1 mL/min, and the analysis was run at 200 °C. The peaks of the FAMEs were identified through a comparison with authentic standards.

2.5. Preparation of Fatty Acid Lithium Salt (FALS) Solution pH 7

Saponification in 10 mL KOH (Carlo Erba, Emmendingen, Germany) 1 N ethanol (Fisher Chemical, Hampton, NH, USA) solution (95%), under reflux for 1 h and 45 min, of 1 g of lipids, occurred for the cleavage of the glycerides. Acidification of the mixture occurred with a 10 mL HCl (Sigma-Aldrich, Saint Louis, MO, USA) 4 N solution and the free FAs were extracted with 5 mL hexane (Sigma-Aldrich, Saint Louis, MO, USA) in triplicate.

Following this, the organic phase was washed with distilled water until neutrality (pH 7) and dried over Na_2SO_4 (Honeywell, Charlotte, NC, USA). The organic phase was removed under a vacuum.

For the preparation of the FALSs, 1 g of free fatty acids (FFAs) was diluted in 10 mL ethanol/diethyl ether (1:1; Sigma-Aldrich, Saint Louis, MO, USA) and then LiOH (Sigma-Aldrich, Saint Louis, MO, USA) 1 N was added, until pH 9 was reached. Thereafter, the solvents were removed under a vacuum at T = 50 °C and H_3PO_4 (Honeywell, Charlotte, NC, USA) 0.2 N was progressively added to the soap solution under stirring, until neutrality was reached. Finally, distilled water was added to a final volume of 10 mL, resulting in a 10% wt/vol fatty acid–lithium salt aqueous solution.

2.6. Cell Lines and Culture

The DU-145 and the PC-3 cell lines were from ATCC and were used as models of human prostate cancer. The cells were cultured in the RPMI 1640 cell culture medium (Biowest, Nuaillé, France), supplemented with 10% vol/vol fetal bovine serum (FBS) (Biowest, Nuaillé, France) and 1% vol/vol penicillin-streptomycin (Biowest, Nuaillé, France), in a CO_2 incubator at 37 °C. All the cell culture expendables (100 mm dishes, 24-well microplates, 6-well microplates, Boyden–Transwell chambers) were purchased from Greiner Bio-One (Kremsmünster, Austria).

2.7. Proliferation Assays

Equal numbers of cells were seeded in 24-well plates and left to attach and grow for 24 h. After this interval, the media were aspirated and replaced with fresh RPMI 1640 and supplemented with 10% FBS and the designated concentrations of FALSs (0–90 µg/mL for *T. elegans* FALSs; 0–110 µg/mL for *M. alpina* FALSs; 0–54 µg/mL for E-EPA FALSs; and 0–250 µg/mL for olive oil FALSs). Following 48 h of incubation, the viable cells were fixed using a 4% formaldehyde solution and, subsequently, stained using a crystal violet solution. The cell-bound stain was eluted using a 30% acetic acid solution and the optical density (O.D.) of the crystal violet solution was determined using a spectrophotometer at 595 nm. The O.D. values were converted into cell numbers using standard curves, as described in previous studies [23].

2.8. Wound Healing–Scratch Test Assay

The wound healing assay was used to study the cells' wound healing capability following the treatment with FALSs [28]. The cells were seeded in 6-well plates and cultured until the formation of a full monolayer. Scratches were made using P200 pipette tips and the old medium and debris were aspirated. RPMI 1640 was supplemented with 10% FBS and selected doses of FALSs were added and photographs of the wounds were taken. The cells were incubated for 72 h and, every 24 h, photographs were taken using a camera mounted on an inverted microscope.

2.9. Boyden Chamber Assay

To assess cell migration, equal cell numbers were placed in 8 µm inserts containing the serum-free RPMI 1640 medium and designated doses of FALSs. The inserts were then submerged in 24-well plates containing the serum-supplemented medium. The cells were left to migrate for 24 h and, subsequently, the non-migrated cells were scraped away, while the migrated cells were fixed, stained, photographed, and counted, as described in previous studies by our team [23].

2.10. Statistical Analysis

All the experiments were conducted in triplicate and the resulting values were analyzed using Prism 8 software (GraphPad, La Jolla, CA, USA). The proliferation data were analyzed using built-in models in Prism 8 for IC_{50} determination and the results are presented as scatter plots with a model-calculated fitting line. The statistical differences

between the IC_{50} values were calculated using multiple independent *t*-tests. The wound-healing photographs were analyzed using the Wound Healing Tool for ImageJ and the resulting values were analyzed in Prism 8 [29]. Image analysis was performed using FIJI (v.2.9.0) software [30]. A two-way analysis of variance (ANOVA) was used to compare the effects of the treatments during the time-course experiments and report statistically significant differences. The Boyden/Transwell chamber photographs were analyzed using the Cell Counter plug-in in FIJI (v.2.9.0) and the data were plotted using Prism 8. A one-way ANOVA was used to calculate the statistical significance between the various treatment groups. Statistical significance was set to 0.05 for all the tests.

3. Results

3.1. Biomass Production, Lipid Accumulation, and Fatty Acid Composition

M. alpina CBS 343.66 and *T. elegans* CCF 1465 were selected for this study due to their ability to accumulate PUFA-containing lipids. *T. elegans* cultivated on glycerol produced a high amount of biomass (x = 12.0 g/L), which was rich in lipids (L/x% = 49.0%, wt/wt). The most dominant fatty acid produced was oleic (OA) (18:1n-9) (29.7%, wt/wt), followed by palmitic acid (PA) (16:0) (23.3%, wt/wt) and linoleic acid (LA) (18:2n-6) (22.1%, wt/wt). Most importantly, the lipids contained GLA (i.e., 19.4%, wt/wt) in significant quantities as well, highlighting the suitability of *T. elegans* as a GLA producer. On the other hand, *M. alpina* was cultivated in a mineral medium, with glucose as the sole carbon and energy source, and produced 4.1 g/L of dry biomass, which contained satisfactory levels of cellular lipids (i.e., L/x% = 14.6%, wt/wt) (Table 1).

Table 1. Fatty acid composition of the methyl ester mixtures used in the fatty acid lithium salts. Experiments were performed in duplicate.

Source of Lipids	x (g/L)	L/x% (wt/wt)	Fatty Acid Composition (%, wt/wt)													
			14:0	16:0	16:1 n-7	18:0	18:1 n-9	18:2 n-6	18:3 n-6	18:3 n-3	18:4 n-3	20:1 n-9	20:3 n-6	20:4 n-6	20:5 n-3	Others
Thamnidium elegans **CCF 1465**	12.0	49.0	5.5	23.3	ND	ND	29.7	22.1	19.4	ND	ND	ND	ND	ND	ND	ND
Mortierella alpina **CBS 343.66**	4.1	14.6	1.0	32.2	0.2	8.4	18.0	8.3	2.9	0.9	ND	19.8	4.5	3.5	ND	0.2
E-EPA	-	-	ND	0.5	0.4	3.1	9.2	0.6	0.5	2.3	1.7	4.4	ND	ND	75	2.3
Olive oil	-	-	ND	12.0	2.0	2.6	74.5	7.6	ND	0.7	ND	ND	ND	ND	ND	0.6

The main cellular fatty acids produced were 16:0, representing 32.2% of the total FAs, followed by gondoic acid (20:1n-9) (19.8%, wt/wt) and OA (18.0%, wt/wt) (Table 1). Regarding the important polyunsaturated fatty acids produced and accumulated by this fungus, considerable amounts of DGLA (20:3n-6) (4.5%, wt/wt), ARA (3.5%, wt/wt), and GLA (2.9%, wt/wt) were recorded. Finally, a lower percentage of alpha-linolenic acid (18:3n-3) was found. The aforementioned amounts account for total PUFAs that exceed 10.0% (wt/wt) of the fatty acids of the total lipids. Lastly, the fatty acid compositions of the negative control (olive oil) and positive control (eicosapentaenoic acid, EPA 20:5n-3, concentrate in the commercial form E-EPA), in the following experiments, are also presented in Table 2.

Table 2. The IC_{50} value of PC-3 and DU-145 following 48 h of incubation with a range of FALS concentrations.

Cell Line	*Thamnidium elegans* CCF 1465	*Mortierella alpina* CBS 343.66	E-EPA	Olive Oil
PC-3				
Mean (μg/mL)	60.97	43.01	22.10	91.25
Std. Deviation	1.94	2.20	2.65	3.92
Std. Error of Mean	1.12	1.27	1.53	2.26
DU-145				
Mean (μg/mL)	61.20	43.28	21.12	90.97
Std. Deviation	1.92	3.02	4.21	4.19
Std. Error of Mean	1.11	1.74	2.43	2.42

3.2. Effects on Cell Viability and Proliferation

Following 48 h of exposure of DU-145 and PC-3 cells to various concentrations of FALSs from *T. elegans*, *M. alpina*, the EPA concentrate (in the commercial form E-EPA, used as a positive control) [16], and olive oil (as a negative control), the IC_{50} values were calculated (Figure 1, Table 2). Olive oil and fish oil (in the form of EPA concentrate) were chosen as controls, due to their high oleic acid and EPA content, respectively [24]. Both are commercially available lipids, incorporated into various nutraceutical and dietary products. Olive oil (mainly composed of oleic acid) indicated the lowest cytotoxicity among all the fatty acid formulations. On the other hand, the EPA concentrate FALSs exhibited the most evident cytotoxic and anti-proliferative actions, which were attributed to its high EPA content, whose acute cytotoxic effects are well-established. These observations justified the use of these two (non-fungal) lipids as controls, with the rationale behind this selection being their availability. Olive oil and E-EPA FALSs, in this study, serve as two reference points, creating a range within which the actions of the fungal lipids can be empirically compared.

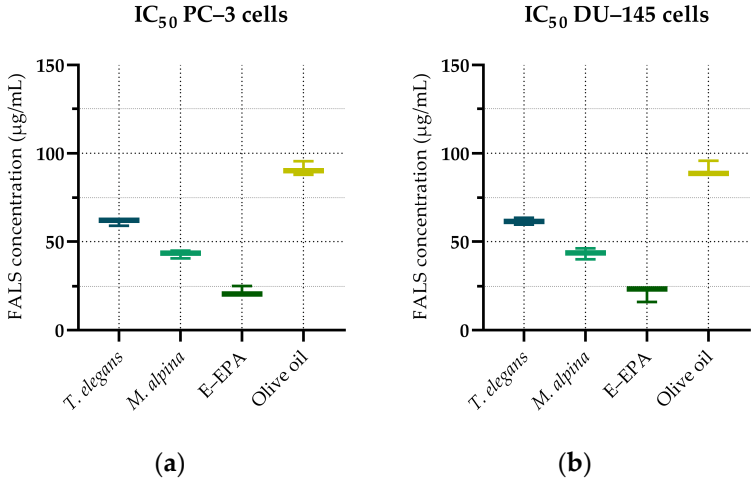


Figure 1. Fatty acid lithium salts IC_{50} determination. Following 48 h of incubation with increasing concentrations of FALSs, the number of remaining live cells was determined using the crystal violet assay. The data were analyzed using the built-in equations in the Prism 8 software. Each bar corresponds to the mean value of three repetitive IC_{50} calculations of the different lipid formulations ± the standard error of the mean (SEM). (**a**) PC-3 or (**b**) DU-145 cell IC_{50} values resulting from three independent experiments, showing the different degrees of cytotoxicity between the tested FALSs. One-way ANOVA indicated statistically significant differences among all the treatment groups for both PC-3 ($F[3, 8] = 332.1$, p-value < 0.0001) and DU-145 cells ($F[3, 8] = 217.4$, p-value < 0.0001).

The microbial lipid FALSs fell in between the two controls, justifying their use and indicating PUFA-related dose-dependent cytotoxicity. The *T. elegans* lipids, containing about 19.4% wt/wt GLA and in total 41.5% unsaturated fatty acids, exhibited an IC_{50} of ~60 μg/mL in both DU-145 cells and PC-3 cells, following 48 h of incubation (Figure A1a). The *M. alpina* lipids, containing DGLA (4.5%, wt/wt), ARA (3.5%, wt/wt), and GLA (2.9%, wt/wt), exhibited an IC_{50} of ~43 μg/mL in both cell lines (Figure A1b). The *M. alpina* extract was more potent compared to *T. elegans* FALSs; however, it was less cytotoxic than E-EPA FALSs (Figure 1).

The cell morphology after treatment with FALSs was significantly altered, as shown in the figures, with the formation of lipid droplets being evident, as well as visible signs of cell swelling, dark spots in the cytoplasmic area, and membrane blebbing (Figure 2). All these irregularities are signs of cell death, either by necrosis or ferroptosis (due to the uncontrolled accumulation of excessive amounts of lipids, which leads to toxicity and peroxidation), or apoptosis (due to the activation of stress-related pathways) [31–33]. FALSs from *Mortierella alpina* demonstrated more severe cytotoxic effects, which were credited to the presence of DGLA, with an increased concentration of 4.5% wt/wt, and that of ARA, with a concentration of 3.5% wt/wt [11,15].

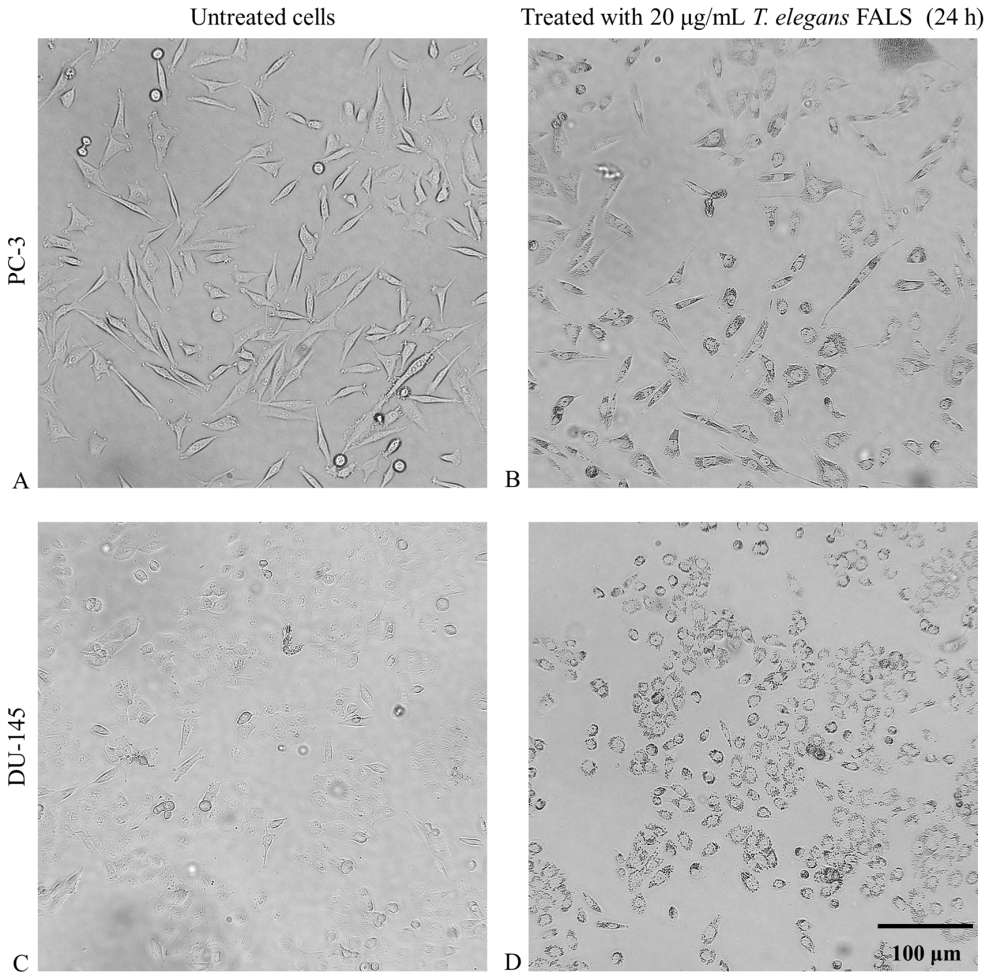


Figure 2. Cell morphology under a phase-contrast microscope. Following incubation with 20 μg/mL FALSs for 24 h, cells grown on glass coverslips were visualized under a phase-contrast microscope. All photographs were taken at ×200 magnification. (**A**) Untreated PC-3 cells; (**B**) PC-3 treated with *T. elegans* FALSs; (**C**) untreated DU-145 cells; and (**D**) DU-145 cells treated with *T. elegans* FALSs. Both cell lines exhibited darker coloration, increased and abnormal sizes, and visible granulation.

3.3. Effects on Wound Healing Ability

The effects of *T. elegans* and *M. alpina* on wound healing ability were also assessed. We performed dose–response experiments using various FALS concentrations (since every formulation had a significantly different IC_{50} value) and to compare them, we selected three main doses (10, 20, and 40 μg/mL) that were related to one another and, in regard to this spectrum, differences regarding the FALS effects on cell migration could be documented (without significant cytotoxicity) (Figure 3).

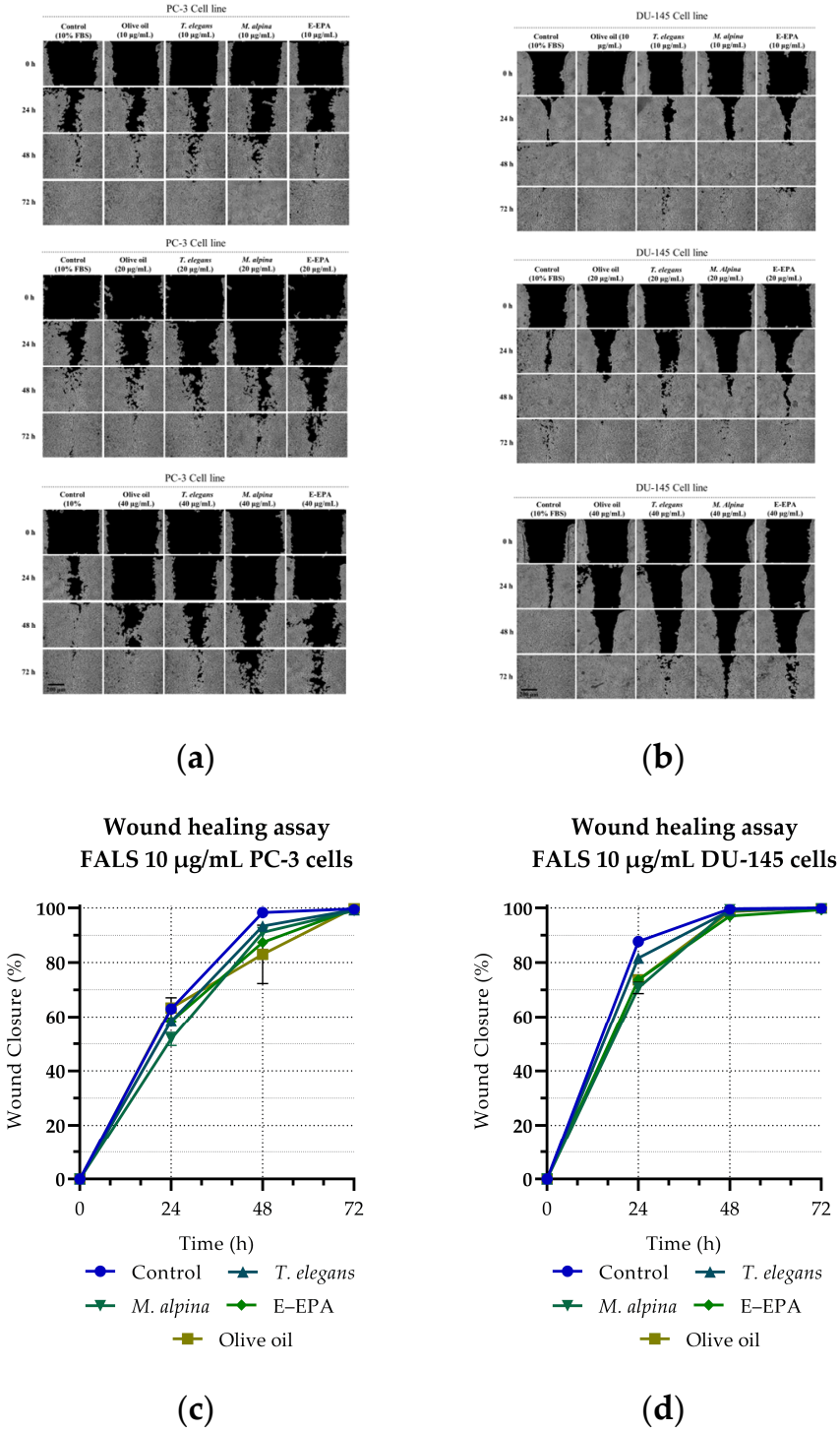


Figure 3. *Cont.*

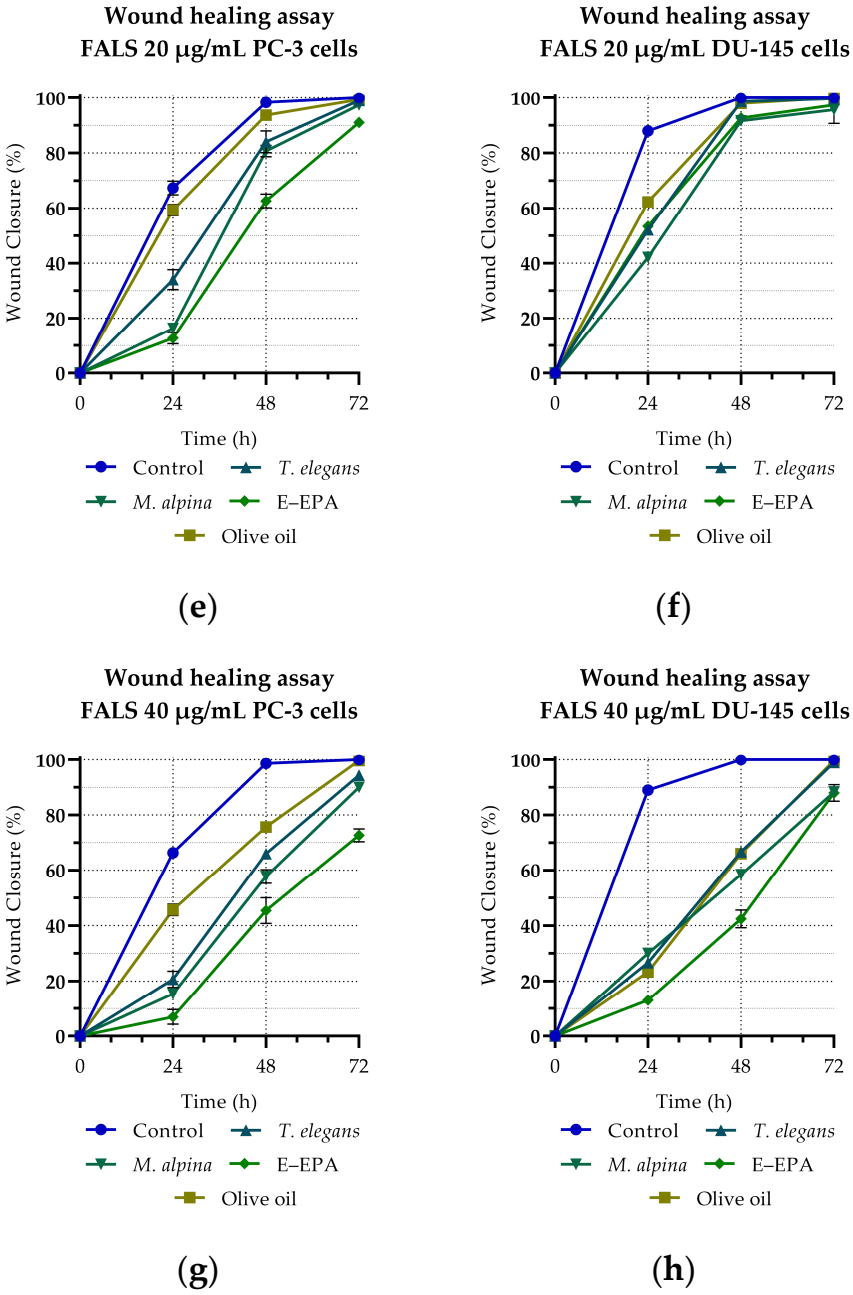


Figure 3. Wound healing assay. Using the scratch test, the effects of the various FALS formulations on the wound healing ability of (**a**) PC-3 and (**b**) DU-145 cells were assessed. Three different doses were administered (10, 20, and 40 µg/mL) after the wound formation and photographs were taken at the key time points of 0, 24, 48, and 72 h. Cells supplemented only with 10% FBS were used as control samples. The data analysis was performed using the Wound Healing macro tool in the FIJI software and all the graphs were designed using Prism 8. Statistical differences were indicated by a two-way ANOVA. The plots present the effects of the different doses on the two cell lines in regard to the wound closure percentage during the key intervals. (**c**,**d**) PC-3 ($F[12, 30] = 2.301$, p-value = 0.0318) and DU-145 cells treated with 10 µg/mL FALSs ($F[12, 30] = 75.37$, p-value < 0.0001); (**e**,**f**) PC-3 ($F[12, 30] = 123.2$, p-value < 0.0001), and DU-145 treated with 20 µg/mL FALS ($F[12, 30] = 133.0$, p-value < 0.0001); and (**g**,**h**) PC-3 ($F[12, 30] = 205.6$, p-value < 0.0001) and DU-145 cells treated with 40 µg/mL FALSs ($F[12, 30] = 857.2$, p-value < 0.0001). Each line represents a different FALS formulation and each point is the mean wound closure percentage extracted from photographs of three different wells. The error bars correspond to the standard error of the mean (SEM).

Even at the lowest concentration, the EPA-treated cell's healing ability indicated significant impairment in both PC-3 (F[12, 30] = 2.301, *p*-value = 0.0318) and DU-145 cells (F[12, 30] = 75.37, *p*-value < 0.0001), justifying its selection as a positive control. The medium dose of 20 μg/mL led to significant migratory impairment by all formulations in both PC-3 (F[12, 30] = 123.2, *p*-value < 0.0001) and DU-145 cells (F[12, 30] = 133.0, *p*-value < 0,0001), with the most active being the EPA concentrate, followed by the *M. alpina* FALSs and, to a lesser (but significant) extent, the *T. elegans*-derived FALSs. At this dose, the olive oil-derived FALSs did not indicate significant wound healing impairment, also justifying the dose and control selection. Finally, the high dose of 40 μg/mL led to a greater effect by inhibiting the final wound closure, even within 72 h, in both PC-3 (F[12, 30] = 205.6, *p*-value < 0.0001) and DU-145 cells (F[12, 30] = 857.2, *p*-value < 0.0001). As a control for the assay, cells treated only with 10% serum-containing medium were used, which had a consistent pattern and rapid rate of wound healing.

3.4. Effects on Cancer Cell Migration

Regarding migration, a similar experimental design to the wound healing experiments was followed, by treating PC-3 and DU-145 cells with a medium FALS concentration (20 μg/mL) for 24 h. This dose was selected using the wound healing experiments, where at 20 μg/mL the olive oil-treated cells had a similar response to the untreated cells; the *T. elegans*-derived FALSs impaired migration to a greater extent; the *M. alpina*-derived FALSs caused more intense migration inhibition; and, finally, the EPA-treated cells had the greatest migratory impairment. Regarding *T. elegans* FALSs, treatment for 24 h caused a 40% decrease in the migration of PC-3 cells and a 50% decrease in that of DU-145 cells (Figure 4).

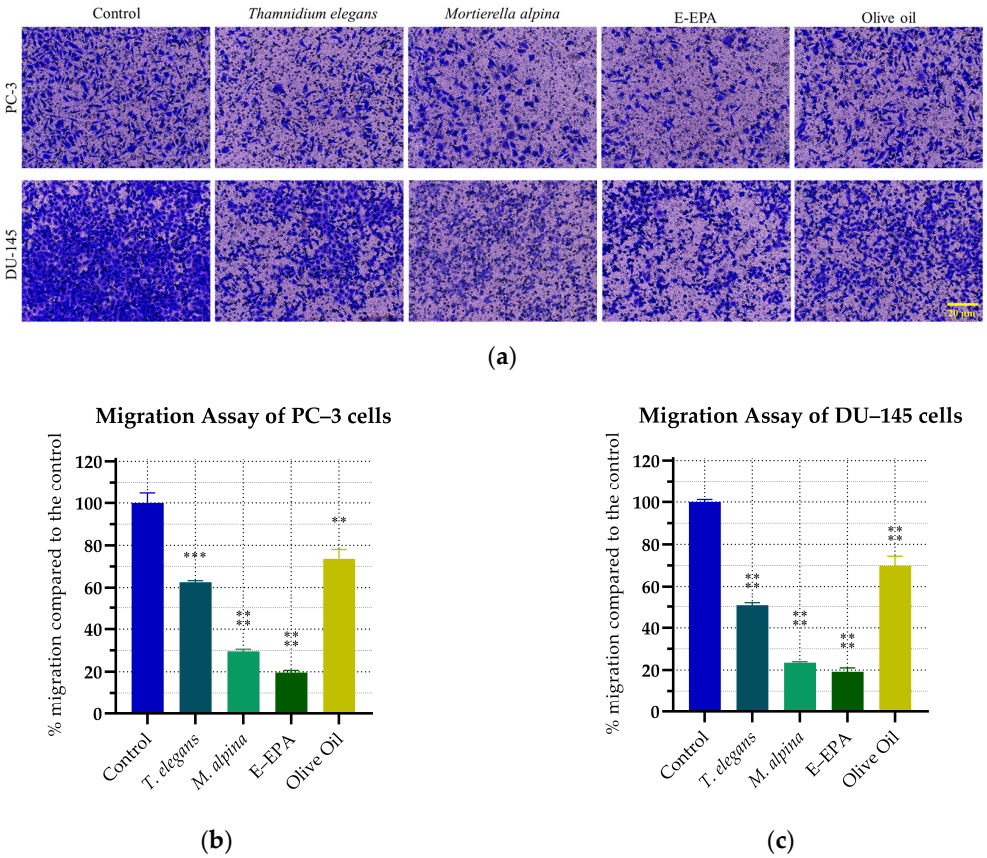


Figure 4. Migration assay. Using Boyden chambers, the effects of 20 μg/mL FALSs on the migratory capability of PC-3 and DU-145 cells, after 24 h of incubation, were assessed. To study cell migration,

FALSs were present in the cell medium (inside the insert), while the chemoattractant medium (10% FBS) did not contain FALSs. (**a**) After 24 h, the cells crossing the filter were fixed and stained with crystal violet, and photographs were taken using a photonic microscope. (**b**,**c**) The images were analyzed using the built-in tool cell counter in FIJI. The data analysis was performed on Prism 8 and the migration rate of (**b**) PC-3 and (**c**) DU-145 cells are presented as the mean percentage of migration compared to the control (no FALSs). Three independent experiments were performed to extract the mean values and the error bars correspond to the standard error of the mean (SEM). Multiple independent *t*-tests were used to investigate the statistical significance of the differences in the mean migration rates: ** correspond to a p-value < 0.01, *** correspond to a p-value < 0.001, and **** correspond to a p-value < 0.0001.

M. alpina FALSs reduced the migration levels of PC-3 cells by 70% and by 75% in DU-145 cells. The effects of the EPA concentrate were even greater, causing an 80% reduction in cell migration, while the use of olive oil FALSs at the same concentration only reduced migration by 40–50% in both cell lines, which was significantly lower compared to the microbial-derived FALSs. These observations emphasize the potential antitumor activity GLA-, DGLA-, and ARA-rich lipids can exhibit and support the further use of lithium salts (FALSs) as a way to administer them, as has already been shown by previous studies [22–24].

4. Discussion

In this study, lipids from *T. elegans* and *M. alpina* were administered for the first time in regard to human prostate cancer cell lines and an assessment of the cells' ability to proliferate and migrate was performed. Lipids from both microorganisms were transformed into FALSs, a bio-assimilable form of insoluble long-chain PUFAs that has previously been used for in vitro assays by both our research group [22–24] and other researchers [34,35]. The PC-3 cells and the DU-145 cells, which were used as models in this study, are two of the most studied prostate adenocarcinoma cell lines, since the 1970s [36,37]. They have been used by numerous scientists and are credited with reproducibility and accurate modelling ability of cells with high metastatic potential. The results from both cell lines were consistent and our study is one of the first of its kind, where microbial lipids are assessed for their application in prostate cancer [23]. In this study, it is not the mechanism underlying the observed effects which is being questioned; this article focuses on how *T. elegans* and *M. alpina* lipids can affect cancer cells, compared to lipids already present in the human diet. Of course, all lipids were transformed into the form of PUFAs, thus being quite novel in terms of delivery system selection; however, a main aspect of this study is the potential of turning a cost-effective substrate, like glucose or industrial-grade glycerol, into products with high nutraceutical added value.

T. elegans CCF 1465 cultivated on glycerol produced significant amounts of biomass, which is in accordance with previous studies, confirming herein that this fungus is an exception to the general rule that considers glycerol to be a not very appropriate carbon source for Mucorales, due to the low uptake rates that have been recorded [19,38,39]. More importantly, this fungus accumulated lipids rich in GLA, a PUFA of increased significance regarding its potential anticancer properties, which justified the selection of this strain. In vitro data have provided much evidence on the suppressive role of GLA on cancer cell proliferation and migration in many cancer types, among which also lies prostate cancer [22,23,34,35,40,41]. Previous studies have shown that *T. elegans* CCF 1465 lipids in the form of fatty acid potassium salts (FAPSs) exhibit anticancer properties against the breast cancer MCF-7 cell line [24], and the current study greatly expands and verifies these results, since cytotoxicity against prostate cancer cells is demonstrated, as well as inhibitory effects on cell migration. All these in vitro data, as well as our study, significantly support experiments conducted in 2006, where Pham et al. showed that the incorporation of GLA-rich foods in rat diets reduced the size of prostate tumors [42], a discovery that truly questions whether more research should be conducted in this direction. GLA is not

a common fatty acid in human nutrition and it mainly reaches us in trace amounts, due to the limited organisms that are able to synthesize it. However, some oleaginous fungi produce it and it is noteworthy that many of them can be grown in laboratory- and/or industrial-scale settings. Examples of such producers are *Cunninghamella echinulata* and *Cunninghamella elegans*, except for *Thamnidium elegans*, all of which have been found to grow on glucose or even glycerol as a carbon source and their fatty acids possess anticancer properties, as indicated by in vitro experiments. Overall, growing *T. elegans* on glycerol, which is a byproduct of agricultural and food industries, in combination with the successful accumulation of GLA-rich lipids, is a great example of a cost-effective method and substrate that can lead to the production of high-added value substances. On the other hand, *M. alpina* CBS 343.66 was selected due to its ability to synthesize GLA, DGLA, and ARA. Contrary to *T. elegans*, however, glucose is a more favorable carbon source for lipid biosynthesis for *M. alpina* [20,43]. In this respect, since glycerol is a more economical option for large-scale applications, it should be noted that when the Mucoromycota *Umbelopsis isabellina* was cultured in blends of glucose and glycerol, the authors came to the conclusion that glycerol, even in high concentrations, when provided during the late oleaginous phase, contributed to suppress lipid degradation without altering the fatty acid composition [38]. Thus, there are indications that under the appropriate culture conditions, this fungus can be utilized for industrial purposes. GLA is a precursor to the synthesis of DGLA, which is an intermediate of ARA biosynthesis [44]. Intriguingly, Wynn and Ratledge considered the elongation of GLA to DGLA as the rate-limiting step in ARA production as, according to the authors, an enhancement of the elongase activity could lead to an increase in ARA production [45]. In general, DGLA is much less abundant in the human diet and it is only present in some seed oils that are relatively uncommon; it has been credited with strong tumor-suppressive properties, following in vitro experiments on human cancer cells [11,12,18]. ARA is a relatively common fatty acid, available in many meat and dairy foods, which may possess strong tumoricidal properties [15]. *M. alpina* CBS 343.66 might not produce large quantities of ARA, however its ARA content could be improved by growing the fungus at a lower temperature (i.e., T = 18 °C), as Lindberg and Molin (1993) proposed for this strain [46].

M. alpina FALSs were found to be significantly more cytotoxic compared to *T. elegans* lipids, an observation that may be a result of the combined action of DGLA and ARA in cancer cells. Tallima et al., in 2023, concluded that the cytotoxicity observed on numerous cell lines, following incubation with ARA, was a result of changes in the cell membrane, which possibly activate contact inhibition, as well as the activation of cell surface membrane-associated neutral sphingomyelinase (nSMase) that led to an accumulation of Cer that ultimately suppressed cell proliferation [15]. Bae et al., in 2020, also observed inhibitory effects of ARA on the human colon cancer cell line, HT-29 [17]. They proposed that the cells in the presence of ARA were unable to synthesize the necessary fatty acids needed for mitotic activity, as a result of the ARA-imposed inhibition of lipogenesis. The induction of apoptosis was also explained as a result of endoplasmic reticulum (ER) stress, which is a direct consequence of the incorporation of vast quantities of unsaturated fatty acids in ER membranes [17,47]. This observation is in accordance with the altered morphology of PC-3 and DU-145 cells we observed (visible membrane blebbing and abnormal/atypical cell sizes), both of which showed signs of lipid droplet formation inside their ER, which is a sign of increased lipid assimilation. The induction of apoptotic death has also been reported in the past in human chronic myeloid leukemia cells [48], indicating a potential universal mode of action against multiple cancer types. The excessive extracellular concentrations of fatty acids and the subsequent increased absorbance in the ER, is also known to provoke a condition known as peroxidation, since the unsaturated bonds of these fatty acids can act as substrates for the propagation of free radical reactions. The redox imbalance, the subsequent organelle dysfunction, and the signaling cascade dysregulation, are major cell death pathway activators that can lead the cell to an apoptotic, ferroptotic, or necrotic death [49–52]. The swelling observed in many cells, as a direct consequence of lipid

accumulation and lipid droplet formation in their interior, serves as another marker of their distressed metabolism, which is another trigger mechanism for cell death pathways [52].

Moreover, DGLA, the other major PUFA found in *M. alpina* FALSs, also contributes to the observed suppression of cell proliferation and migration. DGLA has been reported to cause oxidative damage to cancer cells, thus leading to excessive peroxidation, which ultimately provokes apoptosis and ferroptosis [11,12,18,53]. In general, at the biochemical level, the cytotoxic actions of DGLA are considered similar (if not equal) to those of GLA. Both fatty acids are metabolic precursors of high importance in the class I prostaglandin pathway (PGE1). This class of prostaglandins and their various derivatives (e.g., leukotrienes), in addition to their hormone-like roles, are also credited with cytoprotective roles and decreases in thrombogenicity [54]. In 2020, Perez et al. showed that the dietary consumption of DGLA by the nematode *Caenorhabditis elegans* led to germline cell ferroptosis [55]. They also monitored the effects of DGLA administration on the human fibrosarcoma cell line HT-1080 and confirmed that ferroptosis was also being induced, leading to the cancer cells' death.

In addition to the fatty acids *per se*, the method used for their solubilization and administration and their saponification with lithium cations also play an important role in their anticancer potential [56–59]. The delivery of fatty acids is a major issue in both in vitro and in vivo settings. The administration of lithium salts combines all the effects the fatty acids can have on the cellular mechanisms, but also provides the therapeutic benefits of lithium. Lithium (in the form of LiCl) is a long-established therapy for psychiatric disorders; however, recent evidence suggests that anticancer properties can be attributed to lithium [56–59]. Our study does not separately study the actions of lithium and those of fatty acids. The FALSs were administrated as a novel substance, in the same way as other studies where other lithium salts were used as anticancer agents (LiCl and Li_2CO_3) [58,60–64]. Of course, compared to other metal ions (like potassium, which has been used in previous studies [24]), lithium has the drawback of being relatively rare in the human organism, since a normal human adult has only about 7.0 mg of lithium [65]. Excessive lithium could lead to lithium toxicity and this should be considered in future studies, where in vivo data from FALSs may be presented [65]. This toxicity also begs the question whether therapy with FALSs could be conjugated with state-of-the-art drug delivery systems to specifically target cancer cells. Targeting cancer cells could significantly improve patient outcomes, since FALSs could be used as adjuvant means to improve the efficiency of conventional chemotherapies [66]. Chen et al. (2022) described a method to induce ferroptosis in drug-resistant prostate cancer, by using a biomimetic lipid nanoparticle containing DGLA and siDECR1 [67]. Their method indicated both increased targeting and tumoricidal activity [67]. Additionally, targeting would also protect normal cells and tissues from exposure to high concentrations of cytotoxic agents and would allow us to exploit different routes for their administration, in addition to the conventional means of insertion through the human diet [68,69]. Regarding FALS mechanisms of action, Kalampounias et al., in 2024, showed that *Cunninghamella elegans* FALSs act by increasing intracellular ROS levels [23]. Similar mechanisms have also been proposed by other researchers; however, more detailed studies in the future could uncover the exact pathways participating in the phenomena, allowing us to amplify the effects and weaponize them against cancer. Hence, these observations, combined with the fact that many chemotherapies act by modulating oxidative stress levels, could mean that in the future, FALSs from (microbial) lipids could be studied for their potential implementation in medication regimens.

This study aimed to explore the anticancer potential of microbially produced lipids and set a context for their further investigation. Since only limited studies exist that assess FALSs, FAPSs, or single cell oils in general [22–24,34,35], the reference points for this study were commercially available lipids, already present in human nutrition. Of course, different diets include diverse lipid sources, including multiple plant and animal sources. Two of the most beneficial diets are Mediterranean and pescetarian (more specifically fat-rich fish products like sardines, cod liver oil, etc.) diets [70–72]. The Mediterranean diet uses olive oil

as the main culinary fat, while a major component of the pescatarian diet is omega-3 PFAs and, most notably, EPA. Therefore, since elements from both diets have been correlated with anticancer effects and the health effects from these nutrient schemes are undisputable, we used olive oil and fish oil as "controls" in our study [70,72,73]. Olive oil contains the mostly inert fatty acid (compared to PUFAs), oleic acid (~75% wt/wt), while lipids like GLA, DGLA, and ARA are mostly absent. Therefore, it was used as a reference point for low cytotoxicity, since the aforementioned fatty acids are far more cytotoxic than oleic acid and linolenic acid. On the other hand, fish oils rich in EPA and other omega-3 fatty acids contain increased concentrations of cytotoxic PUFAs. EPA accounts for 75% wt/wt of the total mass, while ARA accounts for around ~4.4% wt/wt. Thus, it is credited with high cytotoxic potential in many in vitro studies and was exploited as a positive control in this study, as was also the case in similar articles [24]. Our study categorized all four formulations from less cytotoxic to more cytotoxic, providing a context for the comparisons that can be made in the future. All these data together indicate that the lipid formulations produced by selected Mucoromycota can possess significant anticancer properties and can lead to the significant suppression of tumor cell proliferation and migration. It is likely that all of the analyzed mechanisms interact in complex ways and their combination leads to tumoricidal effects; therefore, more research is warranted in order to extrapolate the findings. FALSs have been repeatedly used during the last 25 years as fatty acid carriers for in vitro studies and, given the positive outcomes produced by those studies, it may be a good idea to employ more complex and accurate tumor models, like organoids, 3D cultures, and ultimately organism models. Oleaginous microorganisms seem to have great pharmaceutical potential, being a "convenient" producer of rare lipids, which can be implemented in future studies regarding drug research. This notion is expected to provide stronger and more application-oriented evidence to support the wider exploitation of single cell oils as important nutraceuticals.

5. Conclusions

This study highlights the use of the filamentous fungi, *T. elegans* CCF 1465 and *M. alpina* CBS 343.66, as producers of single cell oils (SCO) with high anticancer potential. After their conversion to the drastic form of FALSs, the effects of fatty acids derived from the abovementioned microorganisms on cell proliferation and migration were studied, and significant inhibitory effects were documented regarding both parameters. These results fortify the use of FALSs as a novel way to administer fatty acids to cells in in vitro conditions and validate the suspected anticancer properties of GLA, DGLA, and ARA, encouraging further research regarding their mechanism of action and possible utilization. Additionally, the exploitation of industrial-grade glycerol as a substrate for *T. elegans* growth highlights the possibility of exploiting low-value carbon sources, often treated as byproducts or even waste, to produce pharmaceutical substances. *T. elegans* was found to accumulate slightly less cytotoxic PUFA compared to *M. alpina*, which mostly accumulated DGLA and ARA.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. The composition of the two synthetic growth media that were used for the cultures performed in this study.

Compound	Supplier	*Thamnidium elegans* CCF 1465	*Mortierella alpina* CBS 343.66
		Concentration (g/L)	
Glycerol (99.0% analytical grade)	Sigma-Aldrich (Darmstadt, Germany)	30.00	-
Glucose	AppliChem (Darmstadt, Germany)	-	30.00
Yeast Extract	Condalab (Madrid, Spain)	2.00	2.00
$(NH_4)_2SO_4$	Sigma-Aldrich	2.00	-
KH_2PO_4	AppliChem	7.00	12.00
Na_2HPO_4	AppliChem	2.50	12.00
$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich	1.50	-
$CuSO_4 \cdot 5H_2O$	BDH (Poole, UK)	-	0.0001
$Co(NO_3) \cdot 6H_2O$	Merck (Darmstadt, Germany)	-	0.0001
$FeCl_3 \cdot 6H_2O$	BDH	0.15	0.08
$ZnSO_4 \cdot 7H_2O$	Merck	0.02	0.001
$MnSO_4 \cdot 5H_2O$	Honeywell (Charlotte, NC, USA)	0.06	0.0001
$CaCl_2 \cdot H_2O$	PENTA (Prague, Czech Republic)	0.15	0.10

Appendix B

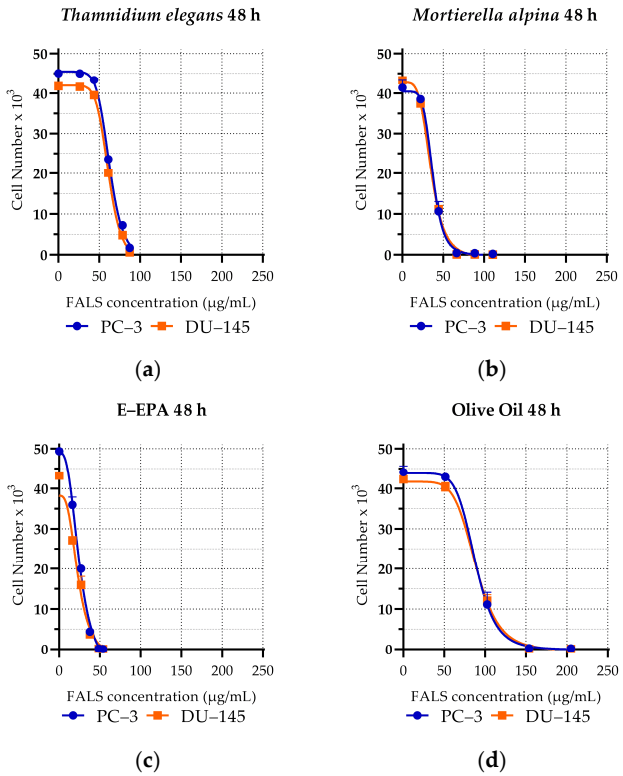


Figure A1. Proliferation/viability curves for PC-3 and DU-145 cells. Following 48 h of incubation with increasing concentrations of FALSs, the number of remaining live cells was determined using

the crystal violet assay. The data were analyzed using the built-in equations in the Prism 8 software. The curves of PC-3 cells are annotated using the blue line and the blue dots, and those of DU-145 cells are indicated using the orange line and orange squares. Proliferation curve following incubation with FALSs for: (**a**) *Thamnidium elegans*; (**b**) *Mortierella alpina*; (**c**) E-EPA; and (**d**) olive oil. All the experiments were conducted in triplicate and every point represents the average cell number ± standard error of the mean (SEM) values.

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