Poly-unsaturated Fatty Acids from *Thamnidium elegans* and *Mortierella alpina* Suppress Prostate Cancer Cells Proliferation and Migration

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Abstract

Thamnidium elegans and *Mortierella alpina* are two oleaginous fungi that belong to Mucoromycota that synthesize gamma-linolenic acid (GLA), di-homo-gamma-linolenic acid (DHGLA) and arachidonic acid (ARA), respectively, all of which are credited with multiple health benefits and possible anticancer properties. The fungi were grown on culture media with glucose or glycerol as a carbon source. After extracting the lipids, we transformed them into fatty acid lithium salts (FALS), which are water-soluble and absorbable by DU-145 and PC-3 cancer cells. The two cell lines, both long-established prostate cancer models, were treated with FALS 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 were 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;

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 in cell membranes is crucial for functionality since saturation is invertedly correlated to membrane fluidity (Mukerjee *et al.*, 2021). The incorporation of PUFAs carrying *cis* double bonds in the cell membranes 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. This phenomenon, also known as lipid peroxidation, propagates exponentially if not suppressed by the cell's antioxidant mechanisms and results in excessive oxidative damage, loss of membrane integrity, and finally cell death (Ayala, Muñoz & Argüelles, 2014). 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 (Marmunti & Catalá, 2007; Ge *et al.*, 2009).

Gamma-linolenic acid (GLA, C18:3 n-6), arachidonic acid (ARA, C20:4 n-6) and di-homo-gamma-linolenic acid (DHGLA, C20:3 n-6) are three fatty acids belonging to the omega-6 PUFAs known for their cytoprotective/neuroprotective, anti-inflammatory, and anticancer properties, justifying the ever-increasing interest in research and industry (Kapoor & Huang, 2006; Chang *et al.*, 2010; Wang, Lin & Gu, 2012; Xu & Qian, 2014; Tallima & El Ridi, 2017; Wang *et al.*, 2021; Pereira *et al.*, 2024). They are assumed to act through modulation of the cell metabolism, generation of reactive oxygen species, alteration of the membranes' fluidity, and interfering with the intracellular signal transduction cascades (Das, 2020; Ponnampalam, Sinclair & Holman, 2021; Tallima & El Ridi, 2023). These fatty acids are believed to possess a high cytotoxic potential compared to other members of the group (Colquhoun & Schumacher, 2001; Ge *et al.*, 2009; Wang, Lin & Gu, 2012; Bae *et al.*, 2020; Sarparast *et al.*, 2023; Tallima & El Ridi, 2023). However, a significant limiting factor in their extensive study and adoption was their scarcity. Since most food oils that contain significant amounts

of them are mostly exploited as nutritional ingredients, their price remained high due to their low abundance and the cost of 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, DHGLA, and ARA in significant quantities, while the isolation and purification process is relatively simple (Bellou *et al.*, 2012; Hao *et al.*, 2015; Fazili *et al.*, 2022). Additionally, the whole process can be scaled up to an industrial level to produce great amounts of food- or even pharmaceutical-grade microbial lipids with potential therapeutic benefits.

Similar approaches, exploiting single-cell oils as substances of high added value from a pharmaceutical perspective, have been made in the past, investigating the lipids' anticancer and antimicrobial potential (Alakhras *et al.*, 2015; Sayegh *et al.*, 2016; Kalampounias *et al.*, 2024). 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 (FALS/FAPS). This form is a liquid, water-soluble compound that does not require further transformations to be assimilable (e.g. the formation of liposomes or micelles) and has already been found to cause cell death by causing oxidative damage, and inducting apoptosis (Alakhras *et al.*, 2015; Sayegh *et al.*, 2016; Kalampounias *et al.*, 2024). 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 use 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 significant percentages of ARA, DHGLA, and GLA, respectively, and examine their effects on the DU-145 and the PC-3 prostate cancer cell lines. The oils were administered as FALS in the cell cultures, and key cellular functions were monitored to investigate any potential anticancer role.

Experimental Procedures

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 master cultures were regularly sub-cultured. The growth medium of *M. alpina* CBS 343.66 contained as a carbon and energy source glucose 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 Table 1. Both media were nitrogen-limited, with 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 ferrum. Medium pH was 6.5 ± 0.5 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 growth medium. Flasks were aseptically inoculated with 1 mL of spore suspension containing 10^6 fungal spores in a laminar flow apparatus. Incubation was held 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.

Analytical methods

Biomass determination

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

Extraction and purification of mycelial lipids

Total mycelial lipids were extracted in chloroform: methanol 2:1 (vol/vol) (Fisher Chemical, Hampton, NH, USA) as described elsewhere (Folch, Lees & Sloane, 1957). 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 the respective dry cell mass (L/x%).

Fatty acid composition of cellular lipids

To determine the fatty acid composition of all the samples used, total lipids were converted into their fatty acid methyl esters (FAMEs) in a two-step reaction, according to the AFNOR method (AFNOR, 1984). 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 an HP-88 (J&W Scientific, Folsom, CF, USA) column (60 m × 0.32 mm) for the analyses. Carrier gas was helium at a flow rate of 1 mL/min and the analysis was run at 200 ◦C. Peaks of FAMEs were identified through comparison to authentic standards.

Preparation of fatty acid lithium salts (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 glycerides cleavage. Acidification of the mixture was held with 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, 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 FALS preparation, 1 g of 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. Thereafter, the solvents were removed under 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. Finally, distilled water was added to a final volume of 10 mL, resulting in a 10% wt/vol fatty acids-lithium salt aqueous solution.

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 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 ºC. All cell culture expendables (100 mm dishes, 24-well microplates, 6-well microplates, Boyden-Transwell Chambers) were purchased from Greiner Bio-One (Kremsmünster, Austria).

Biological Functions Assays

Proliferation Assays

Equal numbers of cells were seeded inside 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, supplemented with 10% FBS and the designated concentrations of FALS. 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 to cell numbers using standard curves as described in previous studies (Kalampounias *et al.*, 2024).

Wound Healing-Scratch Test Assay

The wound healing assay was used to study the cells' wound healing capability following treatment with FALS (Rodriguez, Wu & Guan, 2005). Cells were seeded inside 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 supplemented with 10% FBS and selected doses of FALS 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.

168 ***Boyden Chamber Assay***

169 To assess migration, equal cell numbers were placed in 8 μm inserts containing serum-free RPMI
170 1640 medium and designated doses of FALS. The inserts were then submerged in 24-well plates
171 containing serum-supplemented medium. The cells were left to migrate for 24 h, and subsequently,
172 the non-migrated cells were scrapped away while the migrated cells were fixed, stained,
173 photographed, and counted as described in previous studies from our team (Kalampounias *et al.*,
174 2024).

175 **Statistical Analysis**

176 All experiments were conducted in triplicate and the resulting values were analyzed using the Prism
177 8 software (GraphPad, La Jolla, CF, USA). The proliferation data were analyzed using built-in models
178 in Prism 8 for IC_{50} determination and the results are presented as scatter plots with a model-calculated
179 fitting line. The wound-healing photographs were analyzed using the Wound Healing Tool for ImageJ
180 and the resulting values were analyzed in Prism 8 (Suarez-Arnedo *et al.*, 2020). The
181 Boyden/Transwell chamber photographs were analyzed using the Cell Counter plug-in for ImageJ
182 and the data were plotted using Prism 8.

183

184 **Results & Discussion**

185 **Biomass production, lipid accumulation, and fatty acid composition**

186 *M. alpina* CBS 343.66 and *T. elegans* CCF 1465 were selected for this study due to their ability to
187 accumulate PUFA-containing lipids. *T. elegans* cultivated on glycerol produced a significant amount
188 of biomass (i.e., 12.0 g/L), confirming herein that it is an exception to the general rule that considers
189 glycerol as a not very appropriate carbon source for Mucorales due to the low uptake rates that have
190 been recorded (Bellou *et al.*, 2012; Dritsas & Aggelis, 2023). Additionally, it must be pointed out that
191 the produced biomass was rich in lipids, containing L/x% = 49.0 % (wt/wt). In this case, the most
192 dominant fatty acid produced was C18:1 n-9 (29.7%, wt/wt), followed by C16:0 (23.3 %, wt/wt) and
193 C18:2 n-6 (22.1 %, wt/wt). Most importantly, though, significant quantities of C18:3 n-6 (i.e., 19.4

194 %, wt/wt) were synthesized as well, highlighting the suitability of *T. elegans* as a GLA producer. On
195 the other hand, *M. alpina* was cultivated in a mineral medium with glucose as the sole carbon and
196 energy source and produced 4.1 g/L of dry biomass containing satisfying levels of cellular lipids (i.e.,
197 L/x% = 14.6%, wt/wt) (Table 2). The main cellular fatty acids produced were palmitic (C16:0),
198 representing 32.2% of total FA, followed by gondoic (C20:1 n-9) (19.8%, wt/wt), and oleic (C18:1
199 n-9) (18.0%, wt/wt) (Table 2). Regarding the important polyunsaturated fatty acids produced and
200 accumulated by this fungus, considerable amounts of dihomo-GLA (C20:3 n-6) (4.5%, wt/wt), ARA
201 (C20:4 n-6) (3.5%, wt/wt) and GLA (C18:3 n-6, 2.9%, wt/wt) were recorded. Finally, a lower
202 percentage of alpha-linolenic acid (C18:3 n-3) was found. The aforementioned account for a total of
203 PUFAs that exceeds 10.0% (wt/wt) of the fatty acids of total lipids. However, it must be noted that
204 lipid synthesis and its content in ARA could be improved by growing the fungus at a lower
205 temperature (i.e., T = 18°C), as Lindberg and Molin (1993) proposed for *M. alpina* CBS 343.66
206 (Lindberg & Molin, 1993). Lastly, the fatty acid compositions of the negative control (olive oil) and
207 positive control (eicosapentaenoic acid, EPA 20:5 n-3, concentrate in the commercial form of E-EPA)
208 of the following experiments are also presented in Table 2.

209 **Effects on Cell Viability and Proliferation**

210 Following a 48-hour exposure of DU-145 and PC-3 cells to various concentrations of FALS from *T.*
211 *elegans*, *M. alpina*, EPA concentrate (in the commercial form of E-EPA, used as a positive control)
212 (Colquhoun & Schumacher, 2001), and olive oil (as a negative control), the IC_{50} values were
213 calculated (Figure 1, Table 3). Among the four FALS formulations, the EPA-concentrate FALS
214 exhibited the most evident cytotoxic and anti-proliferative action, and olive-oil-derived FALS was
215 the mildest. The microbial lipid FALS fell in between the two controls, justifying their use and
216 indicating PUFA-related dose-dependent cytotoxicity. The *T. elegans* lipids, containing about 19.4%
217 wt/wt GLA and in total 41.5% unsaturated fatty acids, exhibited an IC_{50} of ~60 μg/mL in both DU-
218 145 cells and PC-3 cells following 48 hours of incubation (Figure 1). The cell morphology after
219 treatment with FALS had 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, and apoptotic characteristics (Figure 2). FALS from *Mortierella alpina* demonstrated more severe cytotoxic effects, which were credited to the presence of DHGLA in the increased concentration of 4.5% wt/wt, and that of ARA in the concentration of 3.5% wt/wt (Wang, Lin & Gu, 2012; Tallima & El Ridi, 2023). *M. alpina* produced a considerable amount of DHGLA, which is a metabolic precursor of high importance in the class I prostaglandin pathway (PGE1). This class of prostaglandins and their various derivatives (e.g. leukotrienes) besides their hormone-like roles, are also credited with cytoprotective roles and decreases in thrombogenicity (Sinzinger *et al.*, 1996). This speculation highlights the potential multiple health benefits resulting from the lipids embedded into human nutrition.

Effects on Cell Migration

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 this spectrum, differences regarding the FALS effects on cell migration could be documented (without significant cytotoxicity) (Figure 3). Even at the lowest concentration, the EPA-treated cell's healing ability indicated significant impairment, justifying its selection as a positive control. The medium dose of 20 µg/mL led to significant migratory impairment by all formulations, with the most active being the EPA concentrate, followed by the *M. alpina* FALS and, to a lesser (but significant) point, the *T. elegans*-derived FALS. At this dose, the olive oil-derived FALS 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. 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.

Migration

Regarding migration, a similar experimental design to 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 untreated cells; the *T. elegans*-derived FALS impaired migration to a greater point; *M. alpina*-FALS imposed a more intense migration inhibition; and finally, the EPA-treated cells had the greater degree of migratory ability impairment. Regarding *T. elegans* FALS, 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, respectively (Figure 4). *M. alpina* FALS reduced migration levels of PC-3 cells by 70% in PC-3 cells and by 75% in DU-145. The effects of EPA concentrate were even greater, causing an 80% reduction in cell migration, while the use of olive oil FALS at the same concentration only reduced migration by 40-50% in both cell lines, which was significantly lower compared to the microbial-derived FALS. These observations, emphasize the potential antitumor activity GLA-, DHGLA-, and ARA-rich lipids can exhibit and support the further use of lithium salts (FALS) as a way to administer them as has already been shown by previous studies (Alakhras *et al.*, 2015; Sayegh *et al.*, 2016; Kalampounias *et al.*, 2024).

Conclusions

This study highlights the use of the filamentous fungi *Thamnidium elegans* and *Mortierella alpina* as producers of SCOs with high anticancer potential. After conversion to the drastic form of FALS, the effects of lipids from the two microorganisms on cell proliferation and migration were studied, and serious inhibitory effects were documented regarding both parameters. These results fortify the use of FALS as a novel way to administer fatty acids to cells in in-vitro conditions and validate the suspected anticancer properties of GLA, DHGLA, 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 PUFAs compared to *M. alpina*, which mostly accumulated DHGLA and ARA.

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Authorship

Conceptualization, P.K., G.A. and S.P.; methodology, G.K., P.D., D.K, T.A. and C.G.; software, G.K., P.D., D.K., T.A.; validation, G.K., P.D., and D.K.; formal analysis, G.K., P.D., D.K. and T.A.; investigation, G.K., P.D. and D.K.; resources, C.G., S.P., G.A. and P.K.; data curation, G.K., P.D., D.K.; writing—original draft preparation, G.K, P.D. and D.K. writing—review and editing, S.P., G.A and P.K.; visualization, G.K., and T.A.; supervision, P.K.; project administration, S.P.; funding acquisition, S.P..

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Tables

Table **1** The composition of the two synthetic growth media that were used for the cultures performed in this study.

Compound	**Supplier**	***Mortierella alpina CBS 343.66***	***Thamnidium elegans CCF 1465***
		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	12.00	7.00
Na_2HPO_4	Applichem	12.00	2.50
$MgSO_4.7H_2O$	Sigma-Aldrich	-	1.50
$CuSO_4.5H_2O$	BDH (Poole, England)	0.0001	-
$Co(NO_3).6H_2O$	Merck (Darmstadt, Germany)	0.0001	-
$FeCl_3.6H_2O$	BDH	0.08	0.15
$ZnSO_4.7H_2O$	Merck	0.001	0.02
$MnSO_4.5H_2O$	Honeywell (Charlotte, North Carolina, USA)	0.0001	0.06
$CaCl_2.H_2O$	PENTA (Prague, Czechia)	0.10	0.15

377 Table 2 Fatty acid composition of the methyl ester mixtures used in the fatty acid lithium salts. Experiments were performed in duplicate.

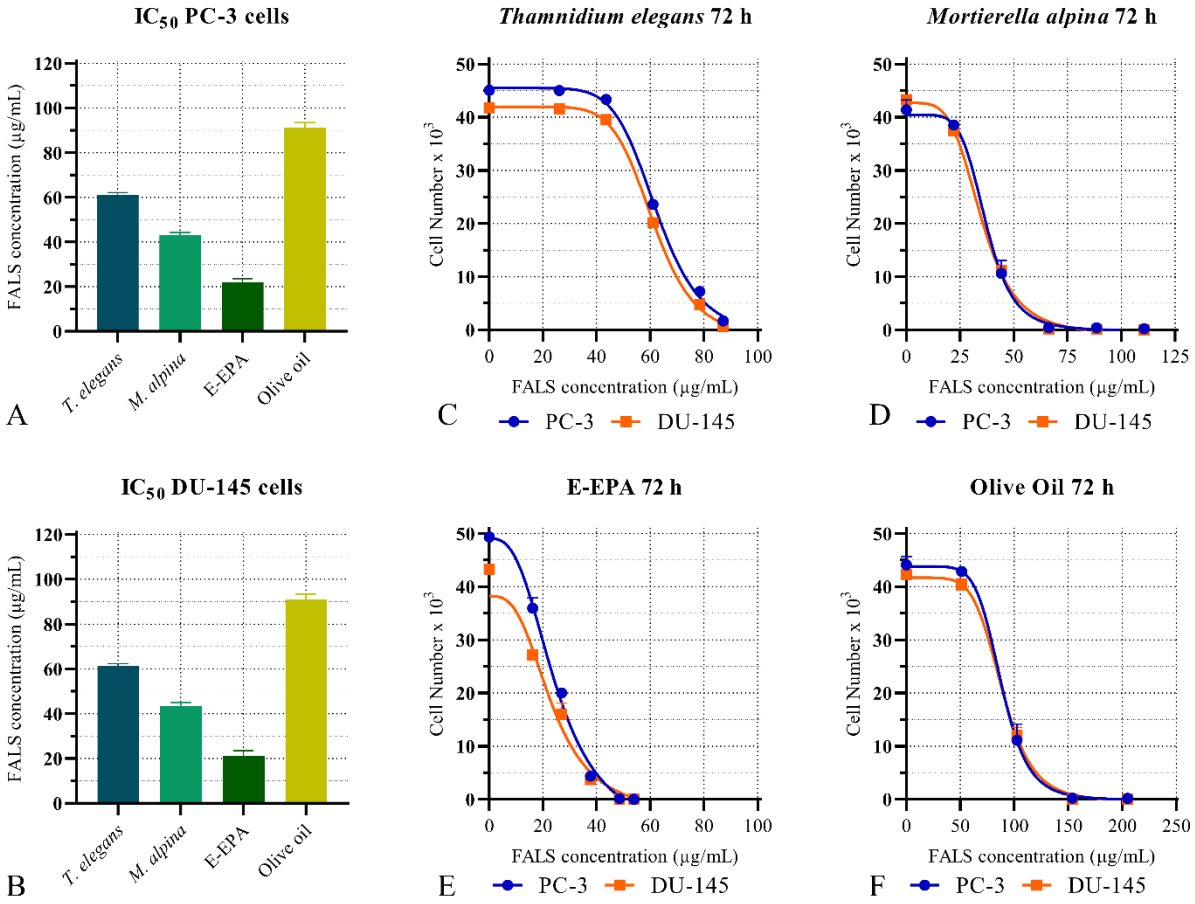
			Fatty acid composition (%, wt/wt)													
Source of lipid	**x (g/L)**	**L/x% (wt/wt)**	**C14:0**	**C16:0**	**C16:1 n-7**	**C18:0**	**C18:1 n-9**	**C18:2 n-6**	**C18:3 n-6**	**C18:3 n-3**	**C18:4 n-3**	**C20:1 n-9**	**C20:3 n-6**	**C20:4 n-6**	**C20:5 n-3**	**Others**
***Mortierella alpina* CBS 343.66**	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
***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
Olive oil	-	-	ND	12.0	2.0	2.6	74.5	7.6	ND	0.7	ND	ND	ND	ND	ND	0.6
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

378 **Abbreviations:** ND: Not-Detected

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

Cell line	*M. alpina* *CBS 343.66*	*T. elegans* *CCF 1465*	E-EPA	Olive oil
PC-3				
Mean	43.01	60.97	22.10	91.25
Std. Deviation	2.20	1.94	2.65	3.92
Std. Error of Mean	1.27	1.12	1.53	2.26
DU-145				
Mean	43.28	61.20	21.12	90.97
Std. Deviation	3.02	1.92	4.21	4.19
Std. Error of Mean	1.74	1.11	2.43	2.42

382 **Figures**



383

384 **Figure 1 IC_{50} determination and proliferation curves of PC-3 and DU-145 cells.** Following 48 h
385 of incubation with increasing concentrations of FALS, the number of remaining alive cells was
386 determined using the crystal violet assay. The data were analyzed using the built-in equations of the
387 Prism 8 software. Each bar corresponds to the mean value of three repetitive IC_{50} calculations of the
388 different lipid formulations ± the standard error of the mean (SEM). (A) PC-3 or (B) DU-145 cell
389 IC50 values resulting from three independent experiments showing the different degrees of
390 cytotoxicity between the tested FALS. The proliferation curves of PC-3 cells are annotated using the
391 blue line and the blue dots, and those of DU-145 cells are indicated using the orange line and orange
392 squares. Proliferation curve following incubation with FALS from: (C) *Thamnidium elegans*; (D)
393 *Mortierella alpina*; (E) E-EPA; (F) Olive oil; All experiments were conducted in triplicate, and every
394 point represents the average cell number ± standard error of the mean (SEM) values.

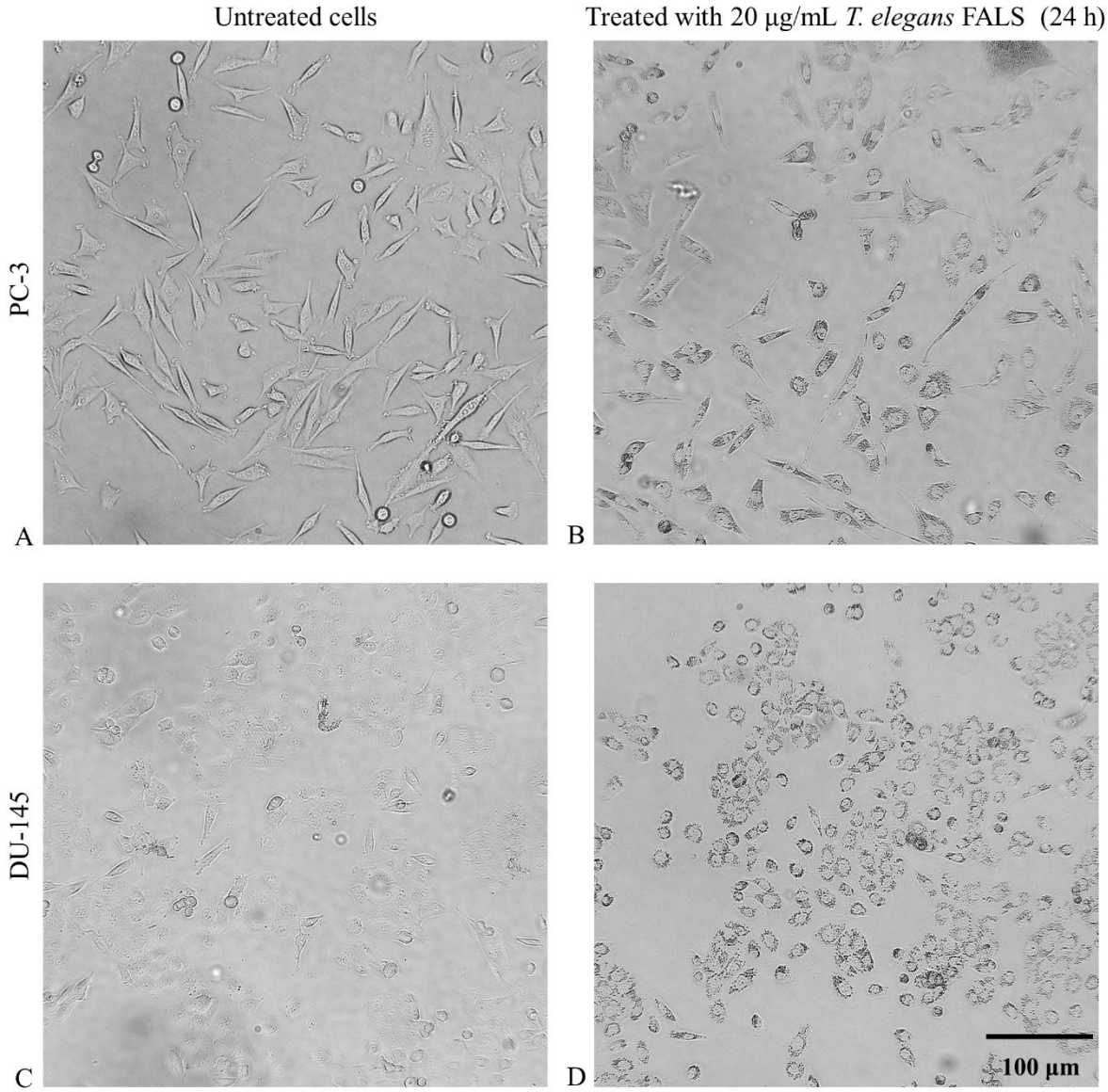
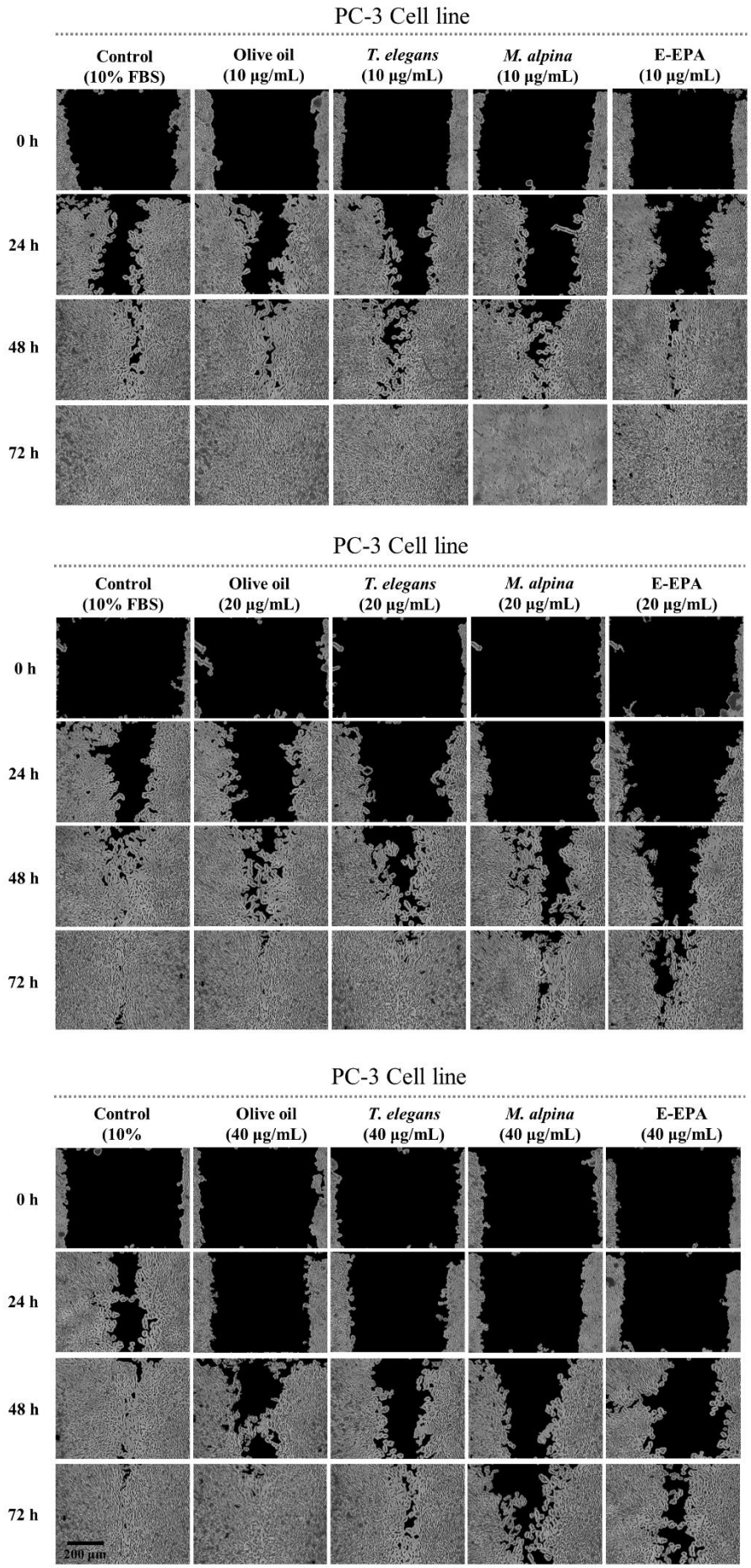
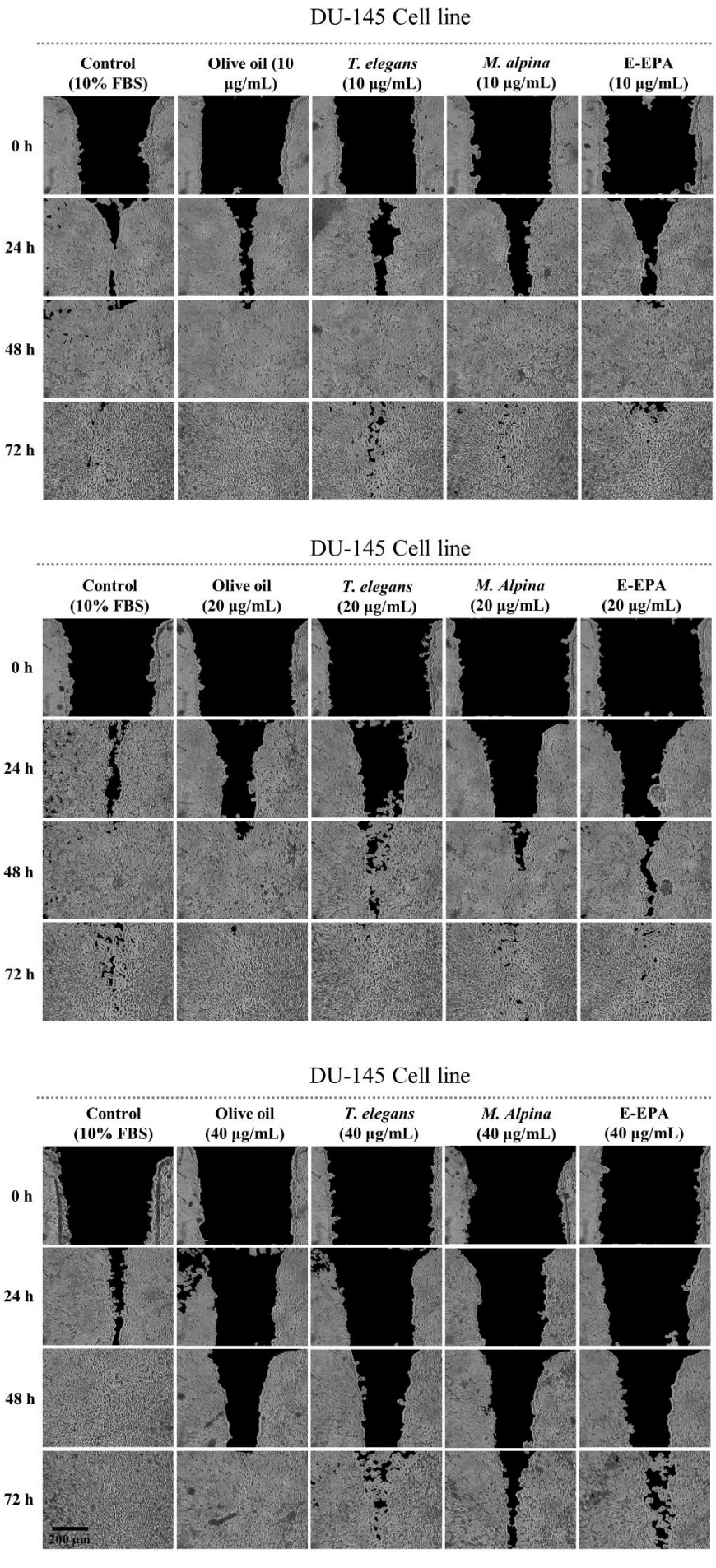


Figure 2 Cell morphology under a phase-contrast microscope. Following incubation with 20 µg/mL FALS for 24 h, cells grown on glass coverslips were visualized under a phase contrast microscope. All photographs were taken at ×200 magnification. (A) Untreated PC-3 cells; (B) PC-3 treated with *T. elegans*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


PC-3 Cell line
Control (10% FBS)
Olive oil (10 µg/mL)
T. elegans (10 µg/mL)
M. alpina (10 µg/mL)
E-EPA (10 µg/mL)
0 h
24 h
48 h
72 h
PC-3 Cell line
Control (10% FBS)
Olive oil (20 µg/mL)
T. elegans (20 µg/mL)
M. alpina (20 µg/mL)
E-EPA (20 µg/mL)
0 h
24 h
48 h
72 h
PC-3 Cell line
Control (10%
Olive oil (40 µg/mL)
T. elegans (40 µg/mL)
M. alpina (40 µg/mL)
E-EPA (40 µg/mL)
0 h
24 h
48 h
72 h
200 µm

A


DU-145 Cell line
Control (10% FBS)
Olive oil (10 µg/mL)
T. elegans (10 µg/mL)
M. alpina (10 µg/mL)
E-EPA (10 µg/mL)
0 h
24 h
48 h
72 h
DU-145 Cell line
Control (10% FBS)
Olive oil (20 µg/mL)
T. elegans (20 µg/mL)
M. Alpina (20 µg/mL)
E-EPA (20 µg/mL)
0 h
24 h
48 h
72 h
DU-145 Cell line
Control (10% FBS)
Olive oil (40 µg/mL)
T. elegans (40 µg/mL)
M. Alpina (40 µg/mL)
E-EPA (40 µg/mL)
0 h
24 h
48 h
72 h
200 µm

B

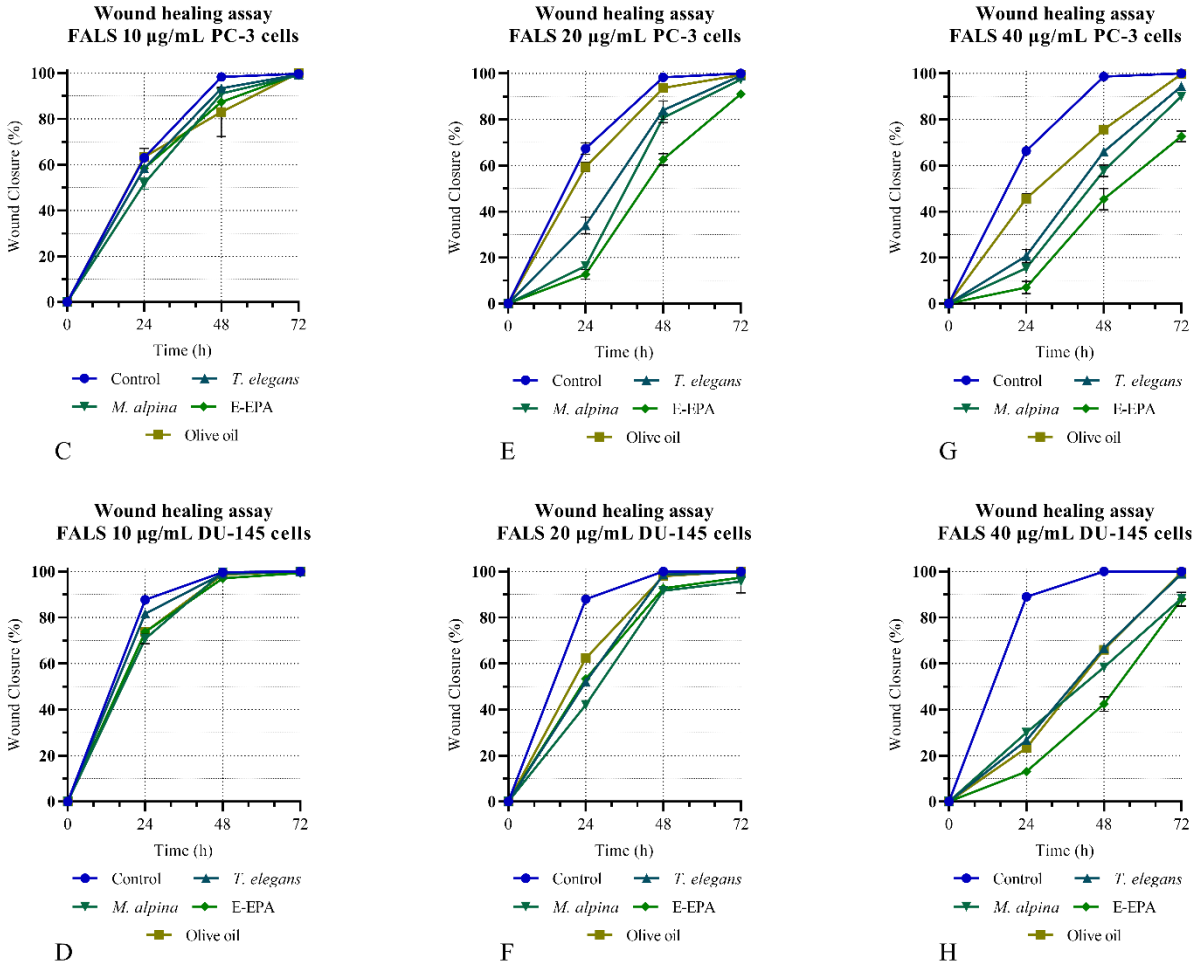


Figure 3 Wound healing assay. Using the scratch test, the effects of the various FALS formulations on the wound healing ability of (A) PC-3 and (B) DU-145 cells were assessed. Three different doses were administered (10, 20, and 40 µg/mL) after the wound formation, and photographs were taken at the key 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 ImageJ software, and all the graphs were designed using Prism 8. The plots present the effects of the different doses on the two cell lines in the wound closure percentage during the key intervals. (C, D) PC-3 and DU-145 treated with 10 µg/mL FALS; (E, F) PC-3 and DU-145 treated with 20 µg/mL FALS; and (G, H) PC-3 and DU-145 treated with 40 µg/mL FALS; Each line represents a different FALS formulation, and each point is the mean percentage extracted from three photographs. The error bars correspond to the standard error of the mean (SEM).

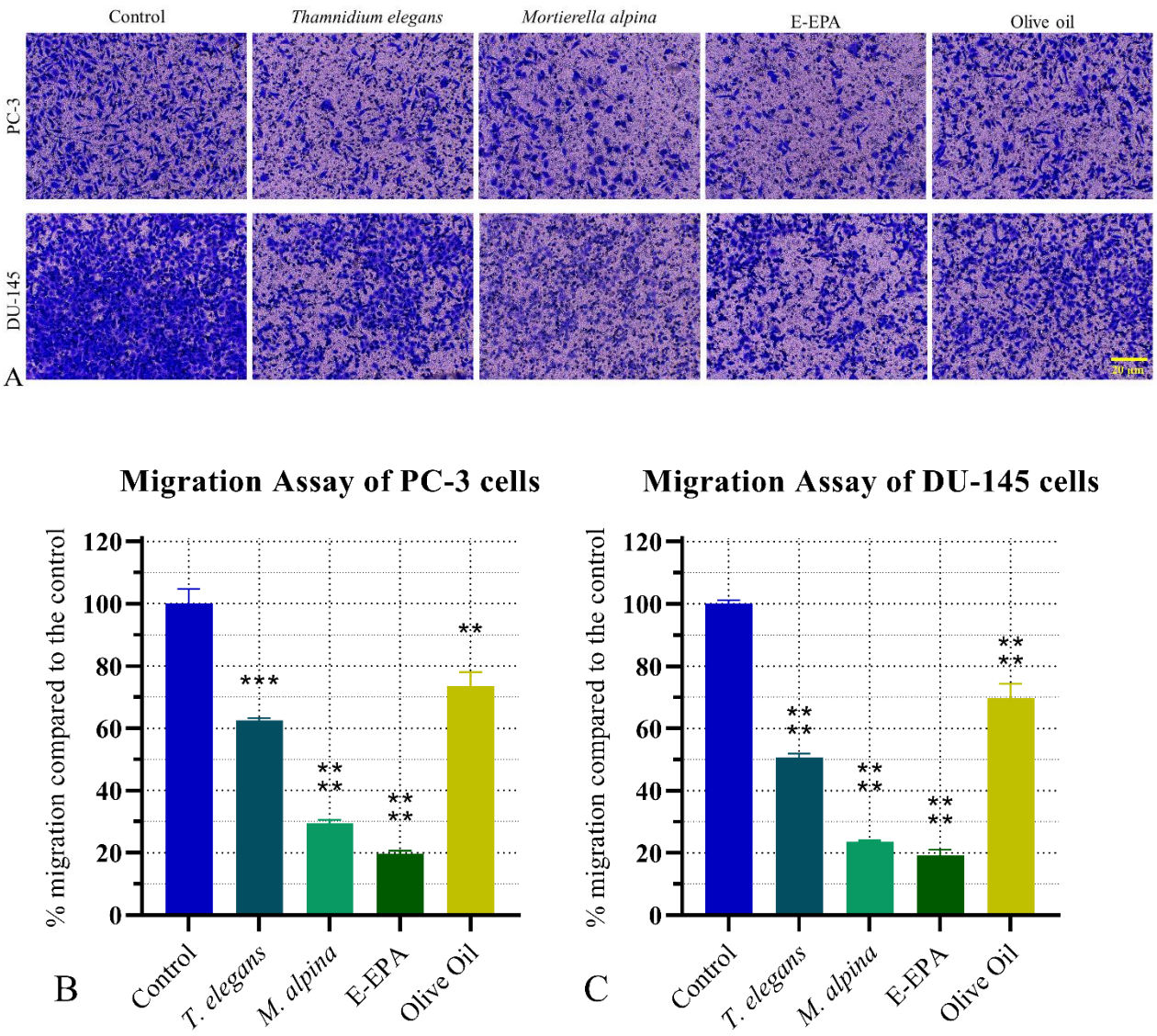


Figure 4 Migration assay. Using Boyden chambers, the effects of 20 µg/mL FALS on the migratory capability of PC-3 and DU-145 cells after a 24-hour incubation were assessed. To study the migration, FALS were present in the cell medium (inside the insert) while the chemoattractant medium (10% FBS did not contain FALS. (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 by ImageJ. The data analysis was performed on Prism 8 and the migration rate of (B) PC-3, and (C) DU-145 cells is presented as mean percentages of migration compared to the control (no FALS). Three independent experiments were performed to extract the mean values and the error bars correspond to the standard error of the mean (SEM).