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ORIGINAL ARTICLE

# Production of polyunsaturated single cell oils possessing antimicrobial and anticancer properties

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**Abstract** Natural bioactive agents, with antimicrobial and/or anticancer activity, such as polyunsaturated fatty acids (PUFAs), are at the forefront of biotechnological research due to the high interest in these compounds in pharmacy. This paper reports production of polyunsaturated lipids by the fungus *Thamnidium elegans* cultivated on raw glycerol, and the microalga *Nannochloropsis salina* cultivated under autotrophic conditions. Fungal lipids, containing gamma linolenic acid, consisted of 82 % neutral lipids. Algal lipids, containing eicosapentaenoic acid (EPA), consisted of 52.7 % glycolipids plus sphingolipids, 28.5 % neutral lipids and 18.8 % phospholipids. EPA was located mainly in polar lipids. Fatty acid potassium salts (FAPS) produced from the above lipids effectively inhibited the growth of many Gram negative bacteria [such as *Serratia* sp., *Neisseria gonorrhoeae*, *Shigella sonnei*, *Proteus mirabilis*, *P. vulgaris*, *Escherichia coli* ATCC BAA1001 *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Salmonella typhimurium* but not *E. coli* ATCC 11229 and NCTC 12241 (ATCC 25922) and *Moraxella catarrhalis*], Gram positive bacteria (i.e. *Micrococcus luteus*, *Staphylococcus aureus* ATCC 25923 and ATCC 43330, *S. epidermidis*, *S. saprophyticus*, *Enterococcus faecalis* but not *S. aureus* ATCC 29213 and *Streptococcus agalactiae*) and *Candida albicans* and provoked a marked lethality on an MCF-7 cancer cell line, even at low concentrations. It was concluded that FAPS derived from polyunsaturated microbial lipids could be the basis for the development of new therapeutic agents.

**Keywords** *Thamnidium elegans* · *Nannochloropsis salina* · Gamma linolenic acid · Eicosapentaenoic acid · Fatty acid potassium salts · Anti-microbial · Anti-cancer

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## Introduction

Increased microbial resistance to traditional antibiotic drugs leads to undesirable side effects and potentially increased mortality (Adwan and Abu-Hasan 1998; Chitemerere and Mukanganyama 2014). For this reason, researchers are encouraged to develop new strategies including the use of natural antimicrobial agents such as essential oils, bacteriocins etc., to control resistant human pathogens (El-Sheekh et al. 2006; Negi 2012; Chitemerere and Mukanganyama 2014; Yang et al. 2014a; Elbeshehy et al. 2015).

In this context, polyunsaturated fatty acids (PUFAs) are attracting attention as potential antimicrobial agents due to their safety, high efficiency, wide spectrum of activity and the lack of resistance mechanisms (Huang et al. 2010; Desbois and Lawlor 2013). Moreover, several PUFAs, such as

dihomo-γ-linolenic (DGLA), arachidonic (AA), and eicosapentaenoic (EPA) acids, give rise to certain compounds such as prostaglandins, leukotrienes, lipoxins and resolvins that have potent anti-inflammatory activities (Das 2006; Bellou et al. 2016). Several long chain fatty acids, especially PUFAs, also possess anti-cancer properties and therefore their use as antimicrobial agents provides additional benefit (Borowitzka 1995; Das 2006; Desbois and Smith 2010).

A review by Desbois and Smith (2010) concluded that the anti-bacterial properties of long chain fatty acids are due mainly to disruption of electron transport chain and to their interference with oxidative phosphorylation, leading mostly to cell lysis. Indeed, long-chain PUFAs have been reported to inhibit bacterial enoyl-acyl carrier protein reductase—an essential component of bacterial fatty acid synthesis (Zheng et al. 2005). The anti-cancer properties of PUFAs have been attributed primarily to the disturbance provoked on gene expression and protein activity by disrupting cell cycle progression and inducing apoptosis (Xu and Qian 2014). Additionally, PUFAs undergo lipid peroxidation, yielding free radicals that they react with reactive oxygen species (ROS) produced by certain anticancer drugs, harming various cell targets (Menéndez et al. 2001; Conklin 2002; Xu and Qian 2014; Alakhras et al. 2015). The anti-cancer activity of PUFAs has also been attributed to the fluidity of cell membranes, which is enhanced by the presence of PUFAs in the structures of membrane lipids facilitating the entry of anticancer drugs into the cell (Menéndez et al. 2001).

Although PUFAs with antimicrobial and/or anticancer activities, such as γ-linolenic acid (GLA; C18:3n-6) (Huang et al. 2010; Alakhras et al. 2015), DGLA (C20:3n-6) (Wang et al. 2012), AA (C20:4n-6) (Huang et al. 2010), EPA (C20:5n-3) (Borowitzka 1995; Ward and Singh 2005; Bellou et al. 2014a), and docosahexaenoic acid (DHA; C22:6n-3) (Mayer and Hamann 2004; Ward and Singh 2005; Bellou et al. 2014a), are present in various foods, commercially exploitable sources are very limited. Among them, microalgae belonging to the genera *Chlamydomonas*, *Crypthecodinium*, *Gyrodinium*, *Nannochloropsis*, *Pavlova*, *Porphyridum*, *Schizochytrium*, etc. are known to synthesize AA, EPA and DHA (Li et al. 2005; Makri et al. 2011; Bellou and Aggelis 2012; Bellou et al. 2014a, 2016). Species of *Nannochloropsis* may also synthesize EPA in percentages up to 38 % in total lipids (Hoffmann et al. 2010; Bellou and Aggelis 2012; Van Wagenen et al. 2012). Fungi belonging to Zygomycetes have the ability to synthesize PUFAs in considerable quantities, e.g. GLA, AA, EPA and so on (Fakas et al. 2008, 2009; Sakuradani et al. 2009; Economou et al. 2011a, b; Bellou et al. 2012, 2014b, 2016). GLA is produced principally by *Cunninghamella echinulata*, *Mortierella ramanniana*, *M. isabellina*, *M. alpina*, *Mucor* sp., *Thamnidium elegans* and *Zygorhynchus moelleri*, which accumulate storage lipids containing GLA up to 19 % w/w in their total lipids (Bellou et al. 2012, 2014b, 2016). Interestingly, Zygomycetes are able to grow on agro-industrial residues such as the crude glycerol arising during biodiesel manufacture, which enhances the biotechnological perspective of these organisms as a source of PUFAs (Chatzifragkou et al. 2011; Bellou et al. 2012).

The aim of this study was the production and characterization of polyunsaturated lipids synthesized by the fungus *Thamnidium elegans* and the microalga *Nannochloropsis salina* cultivated in bioreactors. Specifically, *T. elegans* was cultivated in media containing raw glycerol as the sole carbon source, which is a substrate of high biotechnological interest. The lipids produced, containing either GLA or EPA, were converted into water-soluble fatty acid potassium salts (FAPS) and tested in vitro against numerous human pathogenic microorganisms and the MCF-7 cancer cell line. It is concluded that FAPS derived from the lipids of the above organisms effectively inhibit the growth of many Gram positive and Gram negative strains and exhibit a marked lethality on breast cancer cells, even at low concentrations.

## Materials and methods

### Microorganisms and culture conditions

*Thamnidium elegans* CCF 1465 was maintained on potato dextrose agar (PDA, Himedia, India) at 6±1 °C. *Nannochloropsis salina*, provided by BlueBioTech Int. (Kollmar, Germany), was maintained in 250 cc flasks containing artificial seawater (ASW) as described by Bellou and Aggelis (2012).

*T. elegans* was cultivated in a laboratory-scale bioreactor (New Brunswick, BioFlo/ CelliGen 115) of total volume 3 L and working volume 1.5 L operating under batch cultivation mode. The medium was prepared according to Bellou et al. (2012). Raw glycerol (containing monoglycerides, diglycerides and free fatty acids, 2 %; NaCl, 3 %; methanol<0.1 %; water 3 %), discharged after a biodiesel production process (Pettas Industrial and Commercial S.A., Patras, Greece) was used as the sole carbon source at 35 g/L, while $(NH_4)_2SO_4$(Merck) at 1 g/L and yeast extract at 2 g/L were used as nitrogen sources. The bioreactor vessel, containing the medium, was sterilized at 121 °C for 60 min and kept at room temperature for 48 h to ensure medium sterility. Spore suspension (80 mL, prepared as described in Bellou et al. 2012) was added in the medium as inoculum and the culture was grown for 120 h. The culture was performed aerobically (dissolved oxygen concentration>10 % on saturation) by supplying air at 1.5 vvm. The agitation rate varied from 300 to 400 rpm. Incoming air passed through a bacteriological filter with 0.2 μm pore size (Whatman, Kent, England). The pH was adjusted automatically to 4±0.1 by adding 1 M NaOH (Merck,

Darmstadt, Germany) and the temperature was controlled at 28±0.1 °C.

Cultures of *N. salina* were uni-algal but non-aseptic, and were performed in ASW medium in a laboratory-scale home-made glass photobioreactor (resembling an open pond), with a total volume of 8.7 L and working volume of 5 L. A description of the photobioreactor and detailed culture conditions are given in Bellou and Aggelis (2012). After inoculation, the culture was carried out for a period of 25 days.

## Analytical methods

Fungal biomass was harvested by filtration under vacuum through Whatman no. 1 paper and washed with cold distilled water and 1–3 mL ethanol. Microalgal biomass was collected by centrifugation (15,000 rpm, 15 min, 4 °C, Heraeus, Biofuge Stratos, Osterode, Germany) and washed twice with cold distilled water. Mycelia and algal cells were dried at 80 °C until constant weight, and determined gravimetrically.

Total lipids were extracted according to Folch et al. (1957) in chloroform (Fluka): methanol (Sigma) (2:1, v/v), for 24 h at 25 °C. The lipid extract was collected by filtration through Whatman no. 1 paper, and the solvent evaporated under vacuum. Lipids were determined quantitatively both gravimetrically and by gas chromatography (GC) using an internal standard (see below).

Fractionation and determination of fatty acid composition of lipids was performed as described by Bellou et al. (2012). Briefly, microalgal and fungal lipids were fractionated by using a column (15 mm x 400 mm) containing 1 g silicic acid (Fluka), activated by heating overnight at 80 °C, per 100 mg lipids. The lipid fractions, i.e. neutral lipids (NL), glycolipids plus sphingolipids (G+S) and phospholipids (P), were eluted successively with dichloromethane (Sigma) 100 mL, acetone (Fluka) 100 mL and methanol (Sigma) 50 mL, respectively (Bellou et al. 2012). Lipids and the individual lipid fractions were quantified by using known quantities of margaric acid (C17:0) (Sigma), added to the lipids as an internal standard before trans-methylation. Preparation of fatty acid methyl esters and GC were performed as described by Bellou et al. (2012) using an Agilent 7890 A device (Agilent Technologies, Shanghai, China) equipped with an HP-88 (J&W Scientific, Folsom, CA) column (60 m×0.32 mm), and a flame ionisation detector (FID) detector at 280 °C, while helium was used as carrier gas (at a flow rate 1 mL/min). The analysis was run at 200 °C. Peaks of methyl esters were identified by reference to authentic standards.

### Preparation of FAPS solutions pH 7

For cleavage of glycerides, around 1 g lipids derived from *T. elegans* or *N. salina* and commercial oils used as controls, i.e. olive oil (Hellenic Fine Oils, Athens, Greece) containing oleic acid in high concentrations, evening primrose oil (Solgar, Herts, UK) containing GLA and fish oil rich in EPA (Midsona Finland Oy, Espoo, Finland), was saponified in 10 mL KOH 1 *N* alcohol solution (95 %) at reflux for 1 h and 45 min. The produced free fatty acids were then separated from glycerol and other hydrophilic compounds after acidification of the mixture with 10 mL 4 *N* HCl solution and extracted three times with 5 mL hexane. The organic phase was washed repeatedly with water until neutrality and dried over anhydrous $Na_2SO_4$ (Sigma, St Louis, MO). Finally, 0.6 and 0.4 g free fatty acids of *T. elegans* and *N. salina*, respectively, were obtained after hexane removal under vacuum and stored under reduced nitrogen atmosphere, in the dark at T=8 °C. Respectively, 0.6 g free fatty acids derived from each of the commercial oils was produced.

For FAPS preparation, the amount of free fatty acids obtained was diluted in ethanol/diethyl ether (1:1) (Sigma) and then 1 *N* KOH was added until pH 9. Solvents were then removed under vacuum at *T*=50 °C and 0.2 *N* $H_3PO_4$ was added gradually to the soap solution under stirring, until neutrality (pH 7). Lastly, distilled water was added to a final volume of 10 mL, resulting in FAPS aqueous solutions containing 6 % and 4 %, w/v free fatty acids, for *T. elegans* and *N. salina*, respectively. Similarly, FAPS aqueous solutions containing 6 % free fatty acids were produced from the above mentioned commercial oils.

Fatty acid composition of FAPS preparations was determined after acidification of the FAPS solution with 4 *N* HCl, followed by extraction in triplicate with chloroform. The fatty acid mixtures were dried and, after trans-methylation, their composition was determined by GC as above.

### Test organisms

The FAPS were tested for their bactericidal activity by the agar well-diffusion method against eight Gram positive strains (i.e. *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 49732, *Streptococcus agalactiae* ATCC 12386, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43330, *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Staphylococcus aureus* subsp. *aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus saprophyticus* ATCC 15305), 13 Gram-negative strains (i.e. *Enterobacter cloacae* ATCC 23355, *Escherichia coli* ATCC 11229, *Escherichia coli* ATCC BAA1001, *Escherichia coli* NCTC 12241 (ATCC 25922), *Klebsiella oxytoca* ATCC 49131, *Klebsiella pneumonia* ATCC 700603, *Moraxella catarrhalis* ATCC 25238, *Neisseria gonorrhoeae* ATCC 49226, *Proteus mirabilis* ATCC 14153, *Proteus vulgaris* ATCC 49132, *Salmonella typhimurium* ATCC 14028, *Serratia* sp. ATCC 39006 and *Shigella sonnei* ATCC 25931)

and a pathogenic fungus (i.e. *Candida albicans* ATCC 10231). All bacterial strains were maintained on Mueller Hinton Agar medium and *C. albicans* was maintained on Sabouraud agar at 4 °C in the laboratories of the Department of Biology, King Abdulaziz University. Overnight nutrient broth subculture of the test organisms was done before use.

### Agar well diffusion method

The antimicrobial activity of FAPS was tested by the well diffusion method. FAPS were dissolved in dimethyl sulfoxide (DMSO) to a final concentration 1 mg/mL, sterilized by filtration using sintered glass filter, and stored at 4 °C. The appropriate solidified medium (i.e. Mueller Hinton Agar for bacterial strains and PDA for fungal strain) was inoculated with 50 μL actively growing 16-h-old microbial inocula (containing $10^5$–$10^6$ CFU/mL) that was spread onto the agar surface of the plates using sterile cotton swabs in order to obtain uniform microbial growth on both control and test plates. After absorption of inocula into the agar medium, 4-mm diameter wells were cut from the agar using a sterile borer and 60 μL FAPS solution containing fatty acids 40 μg/mL was transferred into each well. In each plate one well loaded with DMSO was used as a control. All plates inoculated with bacterial strains were incubated aerobically at 35 °C for 18–24 h while plates inoculated with *C. albicans* were incubated at 28 °C for 48 h. After incubation, the diameter of inhibition zone of bacterial growth around the agar wells was measured from the outside edge of the well to the area of visible bacterial growth.

### MCF-7 cell line culture and treatment

MCF-7 cells ($30\times10^3$ cells/well) were grown in a culture medium as described by Elkady et al. (2012) and treated with the various doses of FAPS for 24 h. At the end of the treatment the cells were harvested and cell viability was determined using the trypan blue dye exclusion assay, as previously described in Elkady et al. (2012). Pictures of MCF-7 cells were taken directly from culture plates using a phase microscope 10x.

### Statistical analysis

The experimental data were treated statistically using Origin version 9. One-way analysis of variance (ANOVA) was used to examine significant differences at $P<0.05$. The hypothesis of a dose–response effect of FAPS on cancer cells was tested using linear regression analysis.

## Results

### Production and characterization of microbial lipids

*Thamidium elegans* produced 5.6 g/L biomass containing 21.3 %, w/w lipids in dry mycelia after 120 h of cultivation; 17.2 g/L of glycerol were left unconsumed resulting in a yield of total biomass on glycerol consumed equal to 31 %, w/w. Concerning *Nannochloropsis salina*, 0.5 g/L biomass was produced with a lipid content of 14.7 %, w/w.

The fatty acid compositions of intracellular lipids and individual lipid fractions synthesized by *T. elegans* and *N. salina* under the conditions described above, as well as the fatty acid composition of the FAPS preparations, are shown in Table 1 (see also Fig. S1). Oleic acid (C18:1) was the major fatty acid in the lipids of *T. elegans* (found at 43.9 % in total lipids), followed by linoleic (C18:2) and stearic (C18:0) acids. GLA (C18:3γ) was found at 6.5 % w/w in total lipids. Lipid fractionation showed that neutral lipids (NL) were the major lipid fraction (81.7 % w/w in total lipids), whereas polar lipids (i.e. glycolipids plus sphingolipis—G+S, phospholipids—P) were detected in lower amounts, i.e. 12.1 % and 6.2 %, for G+S and P, respectively. PUFAs, and especially GLA, were found in higher amounts in polar lipids, and mainly P, than in NL (Table 1). In *N. salina* total lipids, palmitoleic acid (C16:1) was predominant and found at higher percentages, up to 30.1 % w/w in total lipids, while EPA was also found at high percentages, i.e. 26.3 %. When total lipids were analyzed in the respective lipid fractions, the data obtained showed that G+S (52.7 % w/w in total lipids) predominated over NL (28.5 %) and P (18.8 %). Additionally, PUFAs were esterified principally in polar lipids, especially in G+S, whereas saturated fatty acids were located mainly in NL.

FAPS derived from commercial oils (see above) were used as controls. The fatty acid composition of these oils and that of the respective FAPS preparations is shown in Table 1. Olive and fish oils consisted mainly of oleic acid and EPA (around 75 % w/w in total lipids), respectively, whereas evening primrose oil contained 11.3 % GLA and significant amounts of linoleic acid (up to 71.9 % w/w in total lipids).

### Antimicrobial activity of the FAPS derived from *T. elegans* and *N. salina* lipids

The algal and fungal oils were converted into FAPS and tested against various human pathogens for their antimicrobial efficacy, which resulted in the formation of a variable diameter zone of inhibition (Table 2) (see also Fig. S2). DMSO (used as control) did not affect the growth of any of the tested pathogens. Except for *Escherichia coli* strains ATCC 11229 and NCTC 12241 (ATCC 25922), *Klebiella oxycota*, *Moraxella catarrhalis*, *Staphylococcus aureus* ATCC 29213 and *Streptococcus agalactiae*, the algal FAPS significantly inhibited the

**Table 1** Fatty acid composition of lipids of *Thamnidium elegans* and *Nannochloropsis salina*; and those of commercial olive, evening primrose and fish oils. TL total lipids, NL neutral lipids, G+S glycolipids plus sphingolipids, P phospholipids, FAPS fatty acid potassium salts

| Microorganisms | Lipids | C14:0 | C14:1 | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | C18:3γ | C20:5 | Others[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. elegans* | FAPS | 0.6±0.1 | 0.1±0 | 19.0±1.3 | 1.6±0.1 | 10.3±0.5 | 44.3±1.7 | 16.3±0.5 | 6.8±0.2 | – | 1.0±0 |
| | TL | 0.6±0 | 0.1±0 | 19.6±1.2 | 1.7±0 | 11.4±1.2 | 43.9±0.5 | 15.5±1.4 | 6.5±0.3 | – | 0.7±0 |
| | NL | 0.9±0 | 0.2±0 | 23.4±0.7 | 1.3±0 | 12.0±0 | 38.4±2.1 | 16.5±0.9 | 5.9±0.1 | – | 1.4±0 |
| | G+S | 0.4±0 | 0.3±0 | 24.5±0.9 | 1.2±0 | 11.1±0.3 | 39.8±1.6 | 15.8±0.4 | 5.6±0.2 | – | 1.3±0 |
| | P | 0.5±0 | 0.2±0 | 22.0±0.9 | 1.0±0 | 10.4±0.3 | 37.8±1.6 | 17.5±0.1 | 7.7±0.3 | – | 2.9±0 |
| *N. salina* | FAPS | 6.7±0.2 | 3.4±0.1 | 20.0±1.0 | 29.5±0.7 | 0.5±0.1 | 6.5±1.0 | 2.8±0.1 | tr[b] | 29.7±1.1 | 0.9±0 |
| | TL | 8.0±0.7 | 4.0±0.1 | 20.5±1.3 | 30.1±1.7 | 0.4±0 | 5.9±0.5 | 3.0±0.1 | 0.8±0 | 26.3±0.9 | 1.0±0.1 |
| | NL | 5.1±0.5 | 3.0±0.1 | 29.3±1.1 | 35.1±1.0 | 4.9±0 | 9.8±0.3 | 2.6±0.1 | 0.2±0 | 6.9±0.5 | 3.1±0 |
| | G+S | 6.1±0.2 | 1.1±0.1 | 17.3±1.3 | 26.1±1.7 | 0.2±0 | 3.8±0.2 | 2.5±0.1 | 0.8±0 | 30.6±0.9 | 1.0±0.1 |
| | P | 5.0±0.7 | 4.6±0.1 | 19.5±1.3 | 25.7±1.7 | 1.2±0 | 4.9±0.5 | 5.1±0.1 | 1.9±0 | 27.0±0.9 | 4.1±0.1 |
| Commercial oil | | | | | | | | | | | |
| Olive oil | FAPS | – | – | 12.5±1.1 | 2.2±0.1 | 2.5±0 | 75.5±1.5 | 7.3±0.2 | – | – | – |
| | TL | – | – | 12.0±0.7 | 2.1±0.1 | 2.8±0.3 | 74.2±2.7 | 8.0±0.1 | – | – | 0.9±0 |
| Evening primrose oil | FAPS | – | – | 6.4±0.3 | – | 2.2±0.1 | 8.1±0.4 | 71.9±1.7 | 11.4±0.7 | – | – |
| | TL | – | – | 6.5±0.6 | – | 2.2±0 | 8.1±0.2 | 70.1±2.6 | 13.1±0.5 | – | – |
| Fish oil | FAPS | 0.1 | 0.1 | 0.6±0 | 0.3±0 | 5.0±0 | 10.1±0.9 | 0.7±0 | – | 73.8±1.7 | 9.5±0.2 |
| | TL | 0.1 | 0.1 | 0.4±0 | 0.3±0 | 4.6±0.2 | 9.5±0.7 | 1.4±0 | – | 75.3±2.0 | 8.5±0.7 |

[a] Others: mainly C10:0 and C12:0

[b] Traces

growth of the tested pathogens. Significantly higher inhibition was observed in the case of the Gram negative strains *Serratia* sp., *Neisseria gonorrhoeae*, *Shigella sonnei*, *Proteus mirabilis* and in the Gram positive *Micrococcus luteus*. With the exception of *Klebsiella oxytoca*, the algal-derived FAPS were proved much more effective than the respective fungal preparation against both Gram negative and Gram positive strains, and in most cases the differences in inhibition, as estimated by the diameter of inhibition zone, were statistically significant. Interestingly, the same strains that were highly resistant to algal FAPS were also resistant to fungal FAPS. The most sensitive strains to fungal FAPS were *Serratia* sp., *Shigella sonnei*, *Neisseria gonorrhoeae*, *Proteus mirabilis*, *P. vulgaris* and *Enterobacter cloacae*. Both algal and fungal FAPS showed antifungal activity against *Candida albicans* but algal FAPS proved superior.

Olive oil derived FAPS did not affect the growth of any of the tested pathogens. However, FAPS derived of fish oil significantly affected the growth of the Gram negative bacteria *Escherichia coli* ATCC BAA1001, *Moraxella catarrhalis* and *Neisseria gonorrhoeae*, and the Gram positive *Enterococcus faecalis*, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermis*. Similarly, evening primrose FAPS inhibited the growth of *Escherichia coli* ATCC BAA1001, *Moraxella catarrhalis*, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermis* (Table 2).

## Anticancer activity of the fatty acid potassium salts derived from *T. elegans* and *N. salina* lipids

Cells of the MCF-7 human breast cancer cell line were incubated for 24 h in media containing FAPS derived from either microbial oils (i.e. *T. elegans* or *N. salina*) or commercial oils (i.e. olive, evening primrose or fish oil) in increasing concentrations, before being harvested and assayed for cell viability by trypan blue dye exclusion assay. The results are summarized in Figs. 1 and 2. The preparations containing PUFAs consistently exhibited a dose-dependent lethal effect on MCF-7 cells, significantly higher than that observed when olive-oil-derived FAPS were applied. The number of the viable cells after 24 h treatment was linearly negatively correlated with dose, presenting a coefficient of linear regression ($R^2$) of 0.99, 0.94, 0.995,0.97 and 0.83, for *N. salina*, *T. elegans*, fish, evening primrose and olive oils, respectively. In addition, the anti-proliferative potentiality of the *T. elegans* FAPS was stronger than that of *N.* salina, since the IC50 value (i.e. the FAPS concentration that destroys cells by 50 %) of the *T. elegans* FAPS was 0.3 μg/mL, significantly lower than that of *N. salina* (=0.45 μg/mL). FAPS derived from evening primrose and fish oils exhibited IC50 values of around 0.4 μg/mL.

Morphological features of apoptosis induced by *N. salina*- and *T. elegans*-derived FAPS were observed in MCF-7 cells (Fig. 3).

**Table 2** Antimicrobial activity against human pathogens according to the agar diffusion method of fatty acid potassium salts (FAPS) derived from lipids extracted from *T. elegans* and *N. salina*; and those derived from commercial evening primrose and fish oils. FAPS were used at concentration 40 μg/mL. Values represent the mean±standard error ($n$= 3) of the inhibition zone (in millimetres excluded the diameter of the well)

| Human pathogens | Inhibition zone (in mm) provoked by FAPS derived from lipids of: | |
|---|---|---|
| | *T. elegans* | *N. salina* |
| Gram negative | | |
| *Enterobacter cloacae* ATCC 23355 | 11.51±0.23 | 14.10±0.38 |
| *Escherichia coli* ATCC 11229 | 0.00±0.00 | 0.00±0.00 |
| *Escherichia coli* ATCC BAA1001 | 9.68±0.22 | 14.80±0.44 |
| *Escherichia coli* NCTC 12241 (ATCC 25922) | 0.00±0.00 | 0.00±0.00 |
| *Klebsiella oxytoca* ATCC 49131 | 6.95±0.19 | 0.00±0.00 |
| *Klebsiella pneumoniae* ATCC 700603 | 10.89±0.21 | 13.63±0.07 |
| *Moraxella catarrhalis* ATCC 25238 | 0.00±0.00 | 0.00±0.00 |
| *Neisseria gonorrhoeae* ATCC 49226 | 13.05±0.15 | 19.20±0.38 |
| *Proteus mirabilis* ATCC 14153 | 12.96±0.29 | 17.77±0.23 |
| *Proteus vulgaris* ATCC 49132 | 11.55±0.24 | 14.57±0.22 |
| *Salmonella typhimurium* ATCC 14028 | 10.71±0.25 | 11.00±0.00 |
| *Serratia* sp. ATCC 39006 | 16.79±0.15 | 22.80±0.42 |
| *Shigella sonnei* ATCC 25931 | 13.86±0.38 | 19.00±0.51 |
| Gram positive | | |
| *Enterococcus faecalis* ATCC 29212 | 7.52±0.62 | 12.53±0.33 |
| *Micrococcus luteus* ATCC 49732 | 7.70±0.12 | 17.27±0.15 |
| *Staphylococcus aureus* (MRSA) ATCC 43330 | 11.75±0.36 | 13.43±0.22 |
| *S. aureus* subsp. *aureus* ATCC 25923 | 8.25±0.19 | 15.07±0.23 |
| *S. aureus* subsp. *aureus* ATCC 29213 | 0.00±0.00 | 0.00±0.00 |
| *Staphylococcus epidermidis* ATCC 12228 | 9.17±0.07 | 15.27±0.37 |
| *Staphylococcus saprophyticus* ATCC 15305 | 9.24±0.33 | 12.13±0.19 |
| *Streptococcus agalactiae* ATCC 12386 | 0.00±0.00 | 0.00±0.00 |
| Fungus | | |
| *Candida albicans* ATCC 10231 | 7.92±0.00 | 11.37±0.19 |
| | Evening primrose oil | Fish oil |
| Gram negative | | |
| *Escherichia coli* ATCC BAA1001 | 12.33±1.45 | 22.33±1.45 |
| *Klebsiella oxytoca* ATCC 49131 | 0.00±0.00 | 0.00±0.00 |
| *Klebsiella pneumoniae* ATCC 700603 | 0.00±0.00 | 0.00±0.00 |
| *Moraxella catarrhalis* ATCC 25238 | 20.67±0.67 | 23.33±1.67 |
| *Neisseria gonorrhoeae* ATCC 49226 | 0.00±0.00 | 18.00±3.51 |
| Gram positive | | |
| *Enterococcus faecalis* ATCC 29212 | 0.00±0.00 | 11.67±0.33 |
| *Staphylococcus aureus* (MRSA) ATCC 43330 | 0.00±0.00 | 0.00±0.00 |
| *S. aureus* subsp. *aureus* ATCC 25923 | 12.00±1.15 | 14.33±1.20 |
| *Staphylococcus epidermidis* ATCC 12228 | 10.22±0.33 | 14.00±1.00 |

## Discussion

The production of natural bioactive compounds is at the forefront of biotechnological research due to the high interest in these compounds in pharmacy and medicine for the development of new therapeutic agents. Numerous microorganisms, especially fungi and microalgae, are among the organisms known for their ability to produce a wide variety of bioactive secondary metabolites and are, therefore, potential sources of novel drugs.

Microbial lipids have gained much attention due to the ability of oleaginous fungi to produce PUFA rich lipids in large amounts (Certik and Shimizu 1999; Ward and Singh 2005; Papanikolaou et al. 2010; Bellou et al. 2012, 2014b).

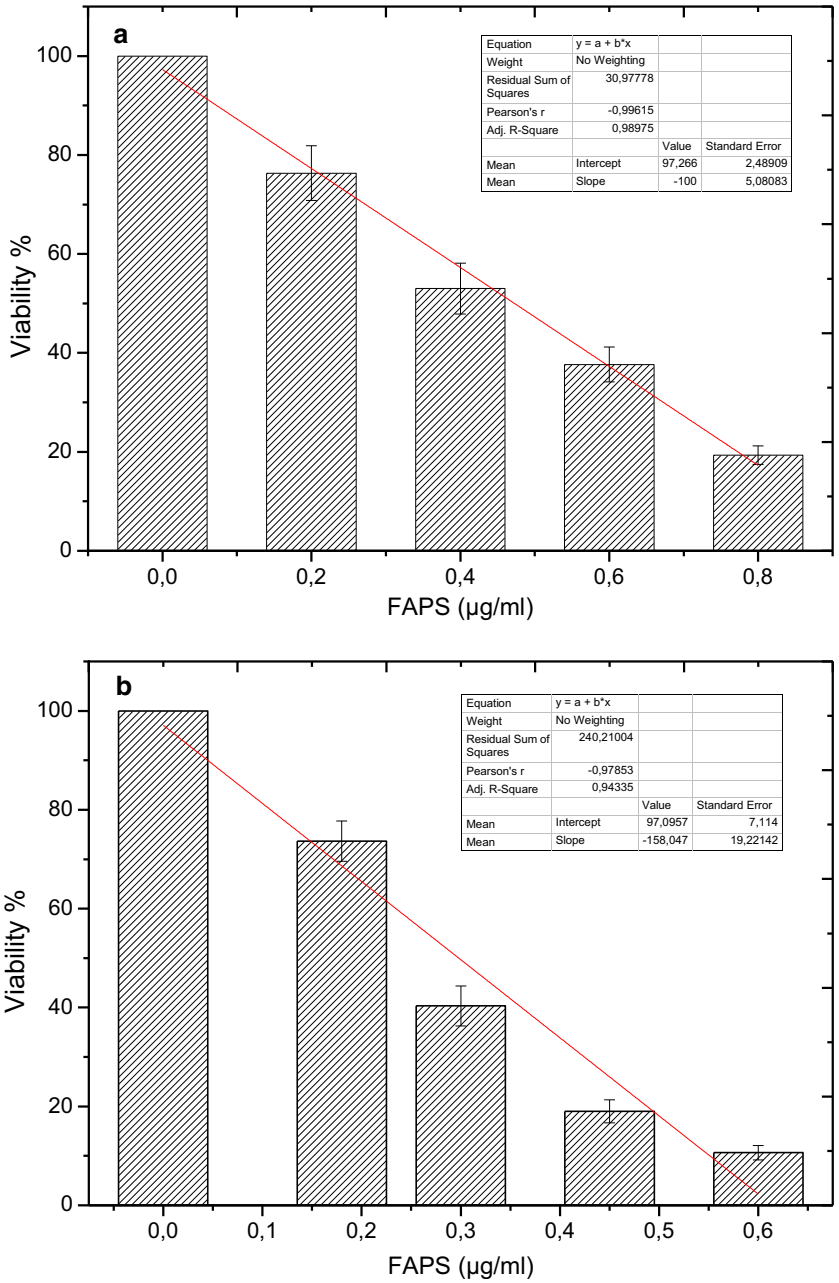


**Fig. 1** Viability (% on the blank) of human breast cancer cell line MCF-7 in the presence of fatty acid potassium salts (FAPS) derived from lipids of **a** *Nannochloropsis salina* and **b** *Thamnidium elegans*. The experiments were repeated two times in triplicate, and the values reported are mean±SE. For details see text

This biochemical feature, in combination with the capacity of oleaginous fungi to grow and accumulate lipids using a wide range of low- or zero-cost substrates derived from industrial and agro-industrial processes (Chatzifragkou et al. 2010, 2011; Bellou et al. 2012, 2014b), is of high biotechnological interest. In the current study, the fungus *T. elegans* cultivated on glycerol was used as a source of GLA, which is a fatty acid commonly produced by Zygomycetes (Fakas et al. 2008, 2009; Taha et al. 2010; Bellou et al. 2012, 2014b). Biomass yield described in this paper is in accordance with that obtained with various Zygomycetes (i.e. *C. echinulata*, *M. isabellina*, *M. ramanniana*) cultivated on glycerol and other agro-industrial residues, but lipid accumulation was markedly higher (Papanikolaou et al. 2004; Fakas et al. 2008, 2009; Bellou et al. 2012, 2014b; Klempova et al. 2013). Concerning GLA production, the results obtained are similar to those mentioned in the literature for *T. elegans*, but lower than values reported for other Zygomycetes strains when cultivated on glucose, glycerol and some other carbon sources (Fakas et al. 2009; Bellou et al. 2012, 2014b; Klempova et al. 2013; Zikou et al. 2013). Certainly, the biomass and lipid yields, as well as the fatty acid composition, which confers specific antimicrobial and anticancer properties on the lipids produced, are influenced strongly by the culture conditions and the

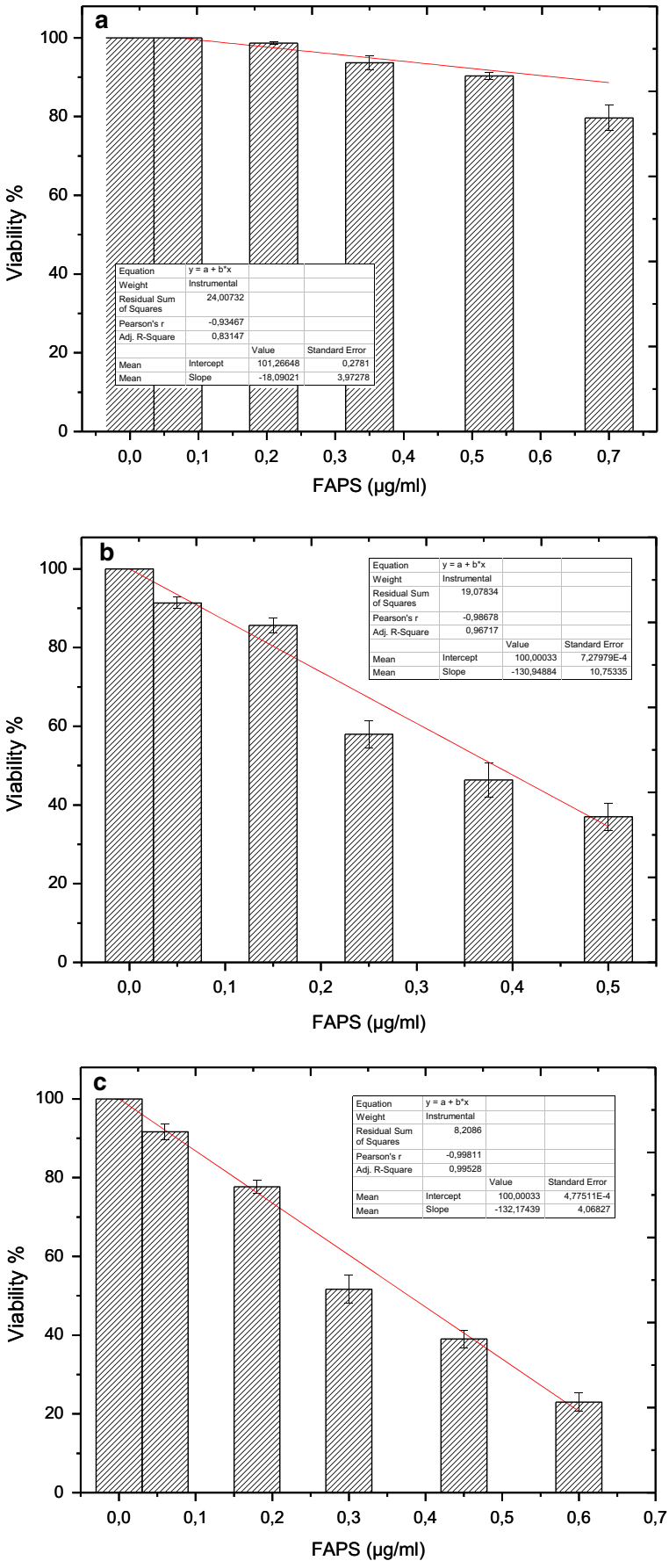


**Fig. 2** Viability (% on the blank) of human breast cancer cell line MCF-7 in the presence of FAPS derived from **a** olive, **b** fish, and **c** evening primrose oil. The experiments were repeated two times in triplicate, and the values are reported as mean±SE. For details see text

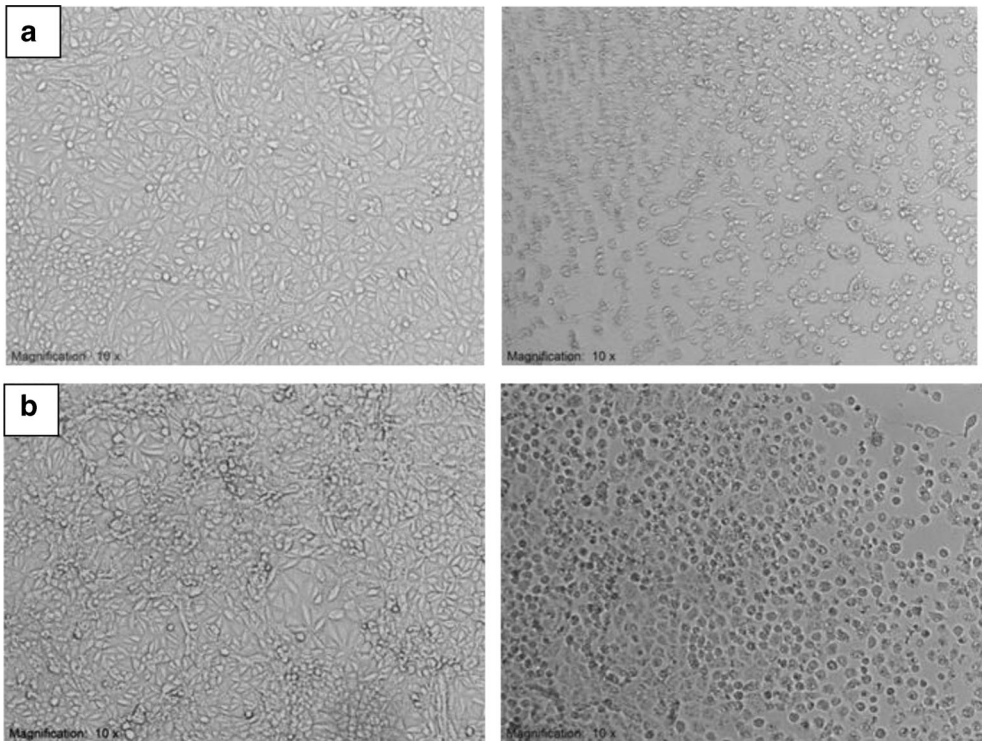


**Fig. 3** Microphotographs showing morphological features of apoptosis in MCF-7 cells induced by FAPS derived by lipids of *N. salina* at **a** 0.8 μg/mL and **b** *Thamnidium elegans* at 0.6 μg/mL. *Left panels* Untreated cells, *right panels* Cells treated with FAPS for 24 h. The results depicted are representative of independent experiments. The photographs were taken directly from culture plates using a phase microscope 10×

composition of the growth medium, especially the nature of both the carbon and nitrogen sources, and therefore these parameters should be optimized from the perspective of a large-scale application.

EPA is produced by various oleaginous microorganisms such as fungi (e.g. *Mortieralla alpina*) (Bajpai et al. 1991; Sakuradani et al. 2009; Vadivelan and Venkateswaran 2014) and microalgae (e.g. *Nannochloropsis* strains) (Ward and Singh 2005; Bellou et al. 2014a, and references therein). In the current investigation, the microalga *N. salina* was used as source of EPA. Biomass yield by *N. salina* was low, which is typical for autotrophic cultures in which the air provided is not enriched with carbon dioxide (Volkman et al. 1993). However, autotrophic cultures are nowadays considered the most promising, being associated with very low cost (Bellou et al. 2014a). Lipid accumulation could be considered low when compared to findings reported in literature (Hoffmann et al. 2010). However, it should be noted that estimation of lipid content was performed according to Bellou and Aggelis (2012) using an internal standard in order to exclude, other than lipids, lipophilic molecules. This method may underestimate the lipid content since the polar head group of polar lipids is not taken into account. EPA content reported here was similar to, or even higher than, those reported for *N. salina* elsewhere (Hoffmann et al. 2010; Bellou and Aggelis 2012; Van Wagenen et al. 2012). In the current investigation, α-linolenic acid was found in traces, which is common in *Nannochloropsis* strains (Bellou et al. 2014a).

Among the various natural compounds described in the international literature, PUFAs have been identified to possess interesting biological activities. Specifically, n-3 PUFAs have been associated with many beneficial effects on human health, while n-6 PUFAs have been proved to be implicated in many pathological situations such as cancer, cardiovascular diseases, etc. (Russo 2009; Xu and Qian 2014). Calder and Yaqoob (2009) reported that EPA and DHA improve the physical nature of cell membranes and membrane protein-mediated responses, eicosanoid generation, cell signaling and gene expression in many different cell types. GLA is of significant pharmaceutical interest since it is effective against tumor cells without harming normal cells (Fan and Chapkin 1998; Kenny et al. 2000; Menéndez et al. 2001; Alakhras et al. 2015), whereas it also proved efficient against rheumatoid arthritis (Belch and Muir 1998), and the development of atherosclerosis (Fan and Chapkin 1998). The bactericidal activity of PUFAs has been reported by Shin et al. (2007), Desbois and Smith (2010) and Al-Saif et al. (2014). Certain fatty acids produced by algae exert inhibitory effects on a variety of human pathogens (Desbois and Smith 2010; Ermakova et al. 2013). Galbraith and Miller (1973) have shown that fatty acids with a chain length more than ten carbon atoms induced lysis of bacterial protoplasts. Accordingly, Wu et al. (2006) suggested that the fatty acids primarily affect the plasma membranes. Zheng et al. (2005) reported that unsaturated fatty acids inhibit fatty acid synthesis in bacteria. According to Giamarellos-Bourboulis et al. (1998) a direct effect of n-6

PUFAs on Gram negative bacteria is prone to peroxidation ending in free radicals capable of attacking bacterial outer membranes and facilitating the action of antimicrobials.

In general, Gram-positive bacteria are more sensitive than the Gram-negative bacteria probably due to the low permeability of the outer membrane of the latter. According to Shin et al. (2007), the outer membrane acts as an effective barrier against many hydrophobic substances, including long-chain fatty acids. For instance, *E. coli* resists long-chain unsaturated fatty acids such as linoleic acid (Sun et al. 2003). Therefore, decreasing the hydrophobicity of long-chain fatty acids by using FAPS instead of free fatty acids may improve fatty acid uptake, leading to increased sensitivity of Gram negative bacteria. The presence of double bonds on the aliphatic chain also affects molecular polarity and therefore PUFAs are expected to be more effective than their saturated homologues. Indeed, the use of FAPS derived from olive oil (containing mainly oleic acid and less than 8 % PUFAs) proved to have no inhibitive action against either Gram positive or negative bacteria. Interestingly, in the present study, *N. salina*- and *T. elegans*-derived FAPS, used indicatively at a concentration 40 μg/mL, which has been found in preliminary experiments to be effective against several human pathogens, showed a significant antibacterial activity against Gram negative bacteria that is similar or even higher than that recorded against Gram positive bacteria. Except against *Klebsiella oxycota*, *N. salina*-derived FAPS proved more effective than *T. elegans*-derived FAPS, probably due to the presence of EPA in *N. salina* lipids. The presence of high amounts of EPA could be attributed to the fact that fish FAPS were more effective in some cases (i.e. *E. coli*, *M. catarrhalis* and *N. gonorrhoeae*) when compared to both microbial and evening primrose FAPS. Notably, divergences were found in the effectiveness of FAPS containing GLA against the various pathogens, since an inhibitive action of fungal FAPS against *Klebsiella* strains, *N. gonorrhoeae*, *E. faecilis* and *S. aureus* ATCC 43330 was observed, whereas the growth of these strains was not inhibited when evening primrose FAPS were applied. This difference could be attributed to the presence of significant amounts of linoleic acid in evening primrose, which may reduce the activity of GLA, but this hypothesis needs verification. Various PUFAs, including EPA and GLA, proved effective against the Gram-positive bacteria *Propionibacterium acnes* and *Staphylococcus aureus* (Desbois and Lawlor 2013). However, important differences concerning sensitivity were recorded even among the members of the Gram negative bacteria. Specifically regarding *E. coli* strains, two of the three strains tested proved highly resistant to both *N. salina*- and *T. elegans*-derived FAPS. According to Bergsson et al. (2002), *E. coli* has a very selective hydrophilic surface because of the side chains of lipopolysaccharides, and thus hydrophobic molecules, such as aliphatic chains, have difficulty in entering the bilayer. Other studies, however, report that *E. coli* is sensitive to long-chain unsaturated fatty acids (see, for instance, Al-Saif et al. 2014) and therefore it seems that the observed resistance is rather strain-specific.

Both *N. salina*- and *T. elegans*-derived FAPS suppressed growth of human breast cancer cell line MCF-7 in a dose-dependent manner. These findings deserve attention knowing that current chemotherapy for breast cancer is unsatisfactory (Normanno et al. 2005; Rahman et al. 2007). The activity of *T. elegans* -and *N. salina*-derived FAPS against human breast cancer cells may be attributed to the presence of GLA and EPA in these preparations. Indeed, significant tumor suppression was observed when FAPS derived from commercial oils containing PUFAs (i.e. evening primrose or fish oils) were used, compared to the findings obtained when FAPS derived from olive oil (consisting mainly of olive oil and less than 7.5 % PUFAs) were applied. Actually, both GLA and EPA were found to induce apoptosis in HL-60 cells (Gillis et al. 2002). Fatty acid lithium salts derived from *Cunninghamella echinulata* lipids (containing GLA) were also effective against HL-60 cells (Alakhras et al. 2015). Treatment of breast cancer MDA-MB-231 cells with GLA resulted in significant growth inhibition (Menéndez et al. 2001). Similarly, GLA lithium soap was confirmed as a growth inhibitor of pancreatic cancer cells (Ravichandran et al. 1998a, b), and of human glioblastoma cell lines (Ilc et al. 1999). EPA has also proved effective against a diverse panel of human cancer cells (Palakurthi et al. 2000), whereas it inhibited the proliferation of human non-small cell lung cancer A549 and H1299 cells (Yang et al. 2014b). However, in the above mentioned studies, with the exception of Alakhras et al. (2015), pure fatty acids were used, which is a major drawback considering the high purification cost of GLA and EPA, in particular when the starting oil contains, besides these fatty acids, other PUFAs that make the purification process difficult. Therefore, in the present investigation, FAPS derived from total lipids of both *T. elegans* and *N. salina* were used instead of pure GLA and EPA. *T. elegans*-derived FAPS proved more effective than those from *N. salina*, and this finding may suggest that GLA is more effective, even at low concentration, than EPA.

In conclusion lipids containing PUFAs (i.e. EPA and GLA) may be produced efficiently by *N. salina* and *T. elegans* and converted into water-soluble FAPS. These preparations proved effective against several important human pathogens, including both Gram negative and Gram positive strains. The sensitivity of Gram negative strains to FAPS needs attention as these bacteria are often resistant to various antibiotics. Besides, both *N. salina* -and *T. elegans*-derived FAPS suppressed growth of the MCF-7 cancer cell line. Between them, *T. elegans* FAPS proved more effective even when compared with FAPS derived from commercial oils containing high amounts of PUFAs (i.e. evening primrose or fish oil FAPS). The oleaginous microorganisms, having the capacity to produce PUFA-rich lipids economically, can be considered as

potential sources of PUFA-based preparations suitable for the treatment of major diseases.

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**Compliance with ethical standards**

**Ethical standards** The manuscript does not contain clinical studies or patient data.

**Conflict of interest** The authors declare that they have no conflict of interest.

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