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Chemical characterization and antioxidant potential of *Arthrospira* sp., *Thalassiosira* sp., and *Raphidonema* sp.

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ABSTRACT

Microalgae are emerging as valuable sources of bioactive compounds. This study evaluates hexane extracts from *Thalassiosira* sp. and *Raphidonema* sp., and *Arthrospira* sp., for their bioactive potential. Saturated fatty acids predominated in *Arthrospira* sp. and *Thalassiosira* sp. while *Raphidonema* was rich in polyunsaturated fatty acids. Carotenoids, such as carotenes and xanthophylls, were abundant in *Arthrospira* sp., while *Thalassiosira* sp. contained chlorophylls and fucoxanthin derivatives, and *Raphidonema* sp. showed high levels of chlorophylls and xanthophylls. Antioxidant assays revealed up to 70 % radical scavenging activity, 60 % iron chelation, and up to 67 (μM) ferric-reducing power. Dose-dependent protective effects were observed in *Schizosaccharomyces pombe* and HepG2 cells, with viability improvements up to 50 %, indicating their potential as antioxidant-rich ingredients for functional foods, promoting health and disease prevention. This study enhances our understanding of *Thalassiosira* sp. and *Raphidonema* sp. while underscoring the promising applications of microalgae extracts in functional foods.

1. Introduction

Microalgae are considered a natural and valuable source of molecules such as lipids, with valuable polyunsaturated fatty acids (PUFAs), essential vitamins, proteins, polysaccharides, and pigments, such as carotenes, with a broad spectrum of bioactivities (Chen et al., 2020; El-Baz et al., 2020; Gürlek et al., 2020; Monteiro et al., 2020). Therefore, microalgae have been recognized as a high-value, untapped, sustainable

Abbreviations: (DPPH), 1,1-diphenyl-2-picrylhydrazyl; (TPTZ), 2,4,6-tris(2-pyridyl)-*s*-triazine; (H2DCF), 2′,7′-dichlorofluorescein; (H_2DCFDA), 2′,7′-dichlorofluorescein diacetate; (ferrozine), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate; (MTT), 3-[4,5- dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide; (NBT), 4-nitro blue tetrazolium chloride; (ATCC), American Type Culture Collection; (AE), *Arthrospira* sp. extract; (CFU), colony-forming unit; (DMSO), dimethyl sulfoxide; (DPPH•), DPPH radical; (DW), dry weight; (EDTA), ethylenediamine tetraacetic acid; (FA), fatty acid; (FAMEs), fatty acid methyl esters; (FRAP), ferric reducing antioxidant power; (FBS), fetal bovine serum; (DMEM), gas chromatography with flame ionization detector (GC-FID, high glucose Dulbecco's Modified Eagle's Medium; (HPLC-DAD), High performance liquid chromatography coupled to a diode array detector; (ICA), iron chelating activity; (MUFAs), monounsaturated fatty acids; (PMS), *N*-methylphenazonium methosulfate; (PBS), phosphate-buffered saline; (PUFAs), polyunsaturated fatty acids; (PI), propidium iodide; (RE), *Raphidonema* sp. extract; (ROS), reactive oxygen species; (SFA), saturated fatty acids; (t-BOOH), *tert*-butyl hydroperoxide; (TE)., *Thalassiosira* sp. extract.

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resource, offering a reservoir of exceptional bioactive ingredients for research in biomedicine, food, pharmaceuticals, and the chemical industries. Among the strategies to harness these compounds, the choice of solvent is critical for efficient extraction. Hexane, a nonpolar solvent, is widely used for extracting lipids and pigments from microalgae due to its high efficiency in solubilizing hydrophobic compounds, cost-effectiveness, and compatibility with large-scale operations (Adam et al., 2012; Ranjith Kumar et al., 2015; Taher et al., 2014).

Oxidative stress refers to the imbalance between reactive oxygen species (ROS) production and the effectiveness of the antioxidant defense system. ROS, including superoxide, hydroxyl radicals, and hydrogen peroxide, function as signaling molecules at lower concentrations but can cause cellular damage when accumulated excessively (Bai et al., 2022; Galaris et al., 2019; Jelic et al., 2021; Luo et al., 2020). Living organisms possess an intrinsic antioxidant defense system, involving scavenging, metal chelation, and enzymatic processes, to mitigate ROS-induced damage. Additionally, exogenous antioxidants from dietary sources, such as vitamin C and carotenes, prevent ROS-related harm (Badr et al., 2019; Chaudhary et al., 2023; Galaris et al., 2019).

Recent epidemiological evidence consistently demonstrates the correlation between consuming antioxidant-rich foods and positive health outcomes (Grosso, 2018). Moreover, there is a growing consumer interest in diverse diets and novel products, particularly those based on microalgae. This trend signifies a desire to prioritize health, sustainability, and safety in dietary choices (Ferreira de Oliveira & Bragotto, 2022). Recent studies reveal the significant potential of microalgae-derived antioxidants, including carotenoids and polyphenols, in mitigating oxidative stress and enhancing health outcomes (Pereira et al., 2024). Microalgae, with their inherent biological properties, emerge as promising candidates for integration into future food production systems (Ferreira de Oliveira & Bragotto, 2022).

Microalgae from different classes, such as *Thalassiosira* sp. (Mediophyceae), *Raphidonema* sp. (Trebouxiophyceae), and *Arthrospira* sp. (Cyanophyceae), exhibit diverse biochemical profiles, which can vary not only between species but also among strains within the same species. These variations are critical for determining their specific applications in food, pharmaceuticals, and cosmetics (Joseph et al., 2017; Leya et al., 2009; Park et al., 2018). *Arthrospira*, commonly known as Spirulina, is a filamentous cyanobacterium recognized for its safe use as a functional food (Wan et al., 2016). The potential health benefits of *Arthrospira* can be attributed to its diverse chemical composition, encompassing proteins, carbohydrates, essential amino acids, minerals, essential fatty acids, vitamins, and pigments (Nikolova et al., 2024; Wan et al., 2016). *Thalassiosira* and *Raphidonema*, as all microalgae play crucial roles in aquatic ecosystems, due to their contribution to primary production and nutrient cycling (Naselli-Flores & Padisák, 2023). Furthermore, they are nutrient-rich, offering essential fatty acids, proteins, carotenoids, and other bioactive compounds with versatile applications such as in food supplements, feeds, pharmaceutical ingredients, and cosmetics (Saxena, Tiwari, et al., 2021; Yi et al., 2017). However, these genera lack EU approval for food use, possibly due to production costs, low demand, or regulatory constraints. Reiterating the point, it has been consistently emphasized that the microalgae and microalgal products approved in the EU constitute a minimal number of species compared to the high number found in nature (Guiry, 2012). Despite limited research, exploring *Thalassiosira* and *Raphidonema* within the broader context of microalgae could unveil their potential as sources of functional foods and nutraceuticals for human health and disease prevention.

Therefore, the present study aims to assess three different genera of microalgae, namely *Arthrospira* sp., *Thalassiosira* sp., and *Raphidonema* sp., as sources of bioactive compounds that can be used to develop functional food ingredients. This involves characterizing the chemical composition of hexane microalgae extracts, focusing on their content in fatty acids and pigments to understand their potential bioactivity. Furthermore, the antioxidant potential of these microalgae extracts was evaluated using two cell models, *Schizosaccharomyces pombe* and human hepatocytes, providing a comprehensive analysis.

2. Material and methods

2.1. Chemicals

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (ferrozine), 1,1-diphenyl-2-picrylhydrazyl (DPPH), iron sulfate ($FeSO_4$), β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), H_2O_2, and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). *N*-methylphenazonium methosulfate (PMS), adenine (99 %) and (+)-lysine monohydrochloride (99 %) were purchased from ACROS Organics (Belgium). 4-Nitro blue tetrazolium chloride (NBT) was purchased from Roche Diagnostics (Germany). Agar and glucose monohydrated were purchased from LabChem/Laborspirit (Lisbon, Portugal), yeast extract for microbiology was obtained from Panreac Aplichem (Germany). Uracil (≥ 99 %), L-leucine (≥ 98 %), propidium iodide, high glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA solution, antibiotic–antimycotic solution, *tert*-butyl hydroperoxide (*t*-BOOH), 3-[4,5- dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide, ≥ 97.8 % purity (MTT), adenine, L-histidine, 2′,7′-dichlorofluorescein diacetate (H_2DCFDA), fucoxanthin (HPLC grade), lutein (HPLC grade), neoxanthin (HPLC grade), chlorophyll *a* (HPLC grade) and β-carotene (HPLC grade) standards were purchased from Sigma (Darmstadt, Germany).

2.2. Microalgae material and extraction procedure

Arthrospira sp., *Raphidonema* sp., and *Thalassiosira* sp. were produced by A4F, SA - Algae for Future (Lisbon Portugal) at pilot scale in a semi-continuous regime (dilutions of 80–90 %), only using solar irradiance. While *Arthrospira* sp. was cultivated in an open cascade raceway, *Raphidonema* sp. and *Thalassiosira* sp. were produced in multilayer horizontal tubular photobioreactors. *Arthrospira* sp. was cultivated using A4F's alkali industrial medium with an average pH of 10 and a maximum temperature of around 25 ± 2 °C. Cryophilic *Raphidonema* sp. was produced using A4F's freshwater industrial medium at a maximum temperature of 10 °C, maintaining a pH between 7 and 8. For the production of *Thalassiosira* sp., A4F's artificial saltwater industrial medium was used with h pH between 7 and 8 and a maximum temperature of around 25 ± 2 °C. The biomass of each strain was harvested by centrifugation (5000 rpm × 10 min × 3, Eppendorf 5804R) and spray-dried.

Microalgae extracts were prepared by overnight extraction of 20 g of dry-weighted biomass with 2 × 150 mL hexane. The process was completed with heat (40 °C) for 30 min, and sonication (130 W, 40 KHz) (Branson 2510, Germany) for 60 min. Samples were centrifuged (5000 rpm × 10 min, Eppendorf 5804R), the supernatant was filtered (Whatman filter paper 1), concentrated under a vacuum evaporator (Buchi, RE121 + 461, Flawil, Switzerland), and then lyophilized (Bioblock Scientific, Christ Alpha 2–4 LD Plus, Osterode am Harz, Germany). The obtained residues were resuspended in DMSO and stored at 4 °C, in the dark, until further use.

2.3. Microalgae fatty acid composition by GC-FID

The fatty acid (FA) composition of the microalgae extracts was assessed through gas chromatography coupled to a flame ionization detector (GC-FID). The lipid-rich extracts obtained by the extraction procedure (Section 2.2) were subjected to a derivatization procedure using sulfuric acid in methanol (2 %) to obtain volatile fatty acid methyl esters (FAMEs) as previously described (Cassani et al., 2022). Fatty acid identification was conducted using external standards, and their calibration curve was used to determine FA composition. Results were

reported as a percentage of total FA content.

2.4. Chromatographic analysis of microalgal pigments by HPLC-DAD

Microalgal pigments were detected and quantified by high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD) as previously described (Garcia-Perez et al., 2022). Pigments were spectrophotometrically detected by DAD at a wavelength of 440 nm. The identification was conducted by direct comparison of the chromatogram with commercial standards or by comparing the UV–Vis absorption spectra of each peak. For quantification, five standards were used to obtain the corresponding calibration curves: chlorophyll *a*, fucoxanthin, neoxanthin, lutein, and β-carotene, with a purity ≥95 % (HPLC grade).

The identified compounds were categorized into three broad families based on their biosynthetic origin: chlorophylls, xanthophylls, and carotenes. Thus, the chlorophylls family included chlorophyll *a*, chlorophyll *a* epimer, chlorophyll *a* allomer, chlorophyll *b*, chlorophyll *b* epimer, and pheophorbide *a*, which were quantified according to chlorophyll *a* calibration curve ($y = 97{,}847x - 56.16$; $R^2 = 0.993$;). The xanthophylls family included, violaxanthin, zeaxanthin, zeaxanthin derivative, and several unidentified xanthophylls were quantified according to the neoxanthin calibration curve ($y = 74{,}421x - 125.82$; $R^2 = 0.992$). Fucoxanthin and fucoxanthin derivatives were quantified according to the fucoxanthin calibration curve ($y = 3^{8x} - 187{,}769$; $R^2 = 0.9896$). Lutein was quantified according to the lutein calibration curve ($y = 387{,}526x - 460.85$; $R^2 = 0.996$). Finally, the carotene family was only represented by β-carotene and quantified according to its calibration curve ($y = 1.131x - 1.372$; $R^2 = 0.998$). The results from chlorophylls and xanthophylls were expressed in micrograms per gram of microalgae dry weight (μg/g DW), and the results from carotenes were expressed in milligrams per gram of microalgae dry weight (mg/g DW).

2.5. Antioxidant profile of microalgae extracts determined by biochemical methods

2.5.1. $DPPH^{\bullet}$ scavenging activity

The ability of the microalgae extracts to scavenge DPPH radical (DPPH•) was determined as previously described with some modifications (Silva et al., 2008). Briefly, 400 μM $DPPH^{\bullet}$ in methanol 100 % (*v*/v) was prepared and the absorbance at 515 nm was adjusted between 0.9 and 1 in a microplate reader (SpectraMaxPlus). Twenty microliters of microalgae extracts (at a final concentration of 0.5 mg/mL) were plated with 130 μL of $DPPH^{\bullet}$ solution and the absorbance at 515 nm was measured after 60 min. Results were expressed in terms of the percentage of scavenging of $DPPH^{\bullet}$, compared to the control (without sample) calculated as Eq. (1).

$$\%DPPH\bullet scavenging = \frac{Abs\ control - (Abs\ sample - Abs\ Blank\ Sample)}{Abs\ control} x\,100 \quad (1)$$

2.5.2. Iron chelating activity (ICA)

The ability of microalgae extracts to chelate Fe^{2+} was determined as previously described with several modifications (Russo et al., 2005). Briefly, 50 μL of microalgae extracts (at a final concentration of 0.5 mg/mL) were plated with 50 μL of $FeSO_4$ solution (0.12 mM in ultrapure water) and 50 μL of ferrozine solution (0.6 mM in ultrapure water). After 15 min of incubation in the dark, at room temperature, absorbance was read at 515 nm. Results were expressed in terms of the percentage of ICA to represent the inhibition of the ferrozine–Fe^{2+} complex, compared to the control (without sample) calculated according to Eq. (2).

$$\%ICA = \frac{Abs\ control - (Abs\ sample - Abs\ Blank\ Sample)}{Abs\ control} x\,100 \quad (2)$$

2.5.3. Superoxide radical ($O_2^{\bullet-}$) scavenging activity

The $O_2^{\bullet-}$ scavenging activity was assessed spectrophotometrically, using the PMS-NADH nonenzymatic assay as described (Oliveira et al., 2009). Briefly, 25 μL of microalgae extracts (at a final concentration of 0.5 mg/mL) were plated with 25 μL of NADH (166 μM in phosphate buffer pH 7.4), and 75 μL of NBT solution (43 μM in phosphate buffer pH 7.4). The reaction started by adding 25 μL of PMS solution (2.7 μM, phosphate buffer pH 7.4) and after 10 min of incubation in the dark at room temperature, the absorbance was read at 560 nm. Results of $O_2^{\bullet-}$ scavenging activity were expressed as a percentage of inhibition of the production of NBT relative to the control (without sample), according to Eq. (3).

$$\%INBT = \frac{Abs\ control - (Abs\ sample - Abs\ Blank\ Sample)}{Abs\ control} x\,100 \quad (3)$$

2.5.4. Ferric reducing antioxidant power (FRAP)

FRAP assay was evaluated according to a procedure described previously (Tachakittirungrod et al., 2007). The working solution was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ (40 mM in 40 mM HCL), and ferric chloride (20 mM in water) in a ratio of 10:1:1 (*v:v:v*). This solution was prepared daily and warmed at 37 °C for 10 min before use. Microalgae extracts were diluted in acetate buffer and 10 μL of each sample (at a final concentration of 0.5 mg/mL) were mixed with 75 μL of working solution. After 4 min of incubation in the dark, at 37 °C, absorbance was read at 593 nm. Results of FRAP were expressed as equivalents of Fe^{2+} directly taken from the $FeSO_4$ linear regression line.

2.6. Microalgae effects in Schizosaccharomyces pombe

2.6.1. Yeast strain and culture media

The fission yeast *S. pombe*, strain 972 h^-, was selected as the eukaryotic cell model organism to study intracellular oxidation. Yeast cultures were maintained on YES medium containing 0.5 % (*w/v*) yeast extract BD Bacto™, 3 % (*w/v*) glucose, 225 mg/L adenine, L-histidine, L-leucine, uracil and L-lysine hydrochloride, at 30 °C. Agar at 2 % (*w/v*) was added for solid media. Cultures were incubated at 30 °C and 200 revolutions per min (rpm) were applied for liquid media agitation. Liquid cultures were monitored by optical density at 595 nm (OD_{595}).

2.6.2. Cell viability assays

The protective effect of the marine microalgae extracts against H_2O_2-induced damage was evaluated through viability assays, using *S. pombe* as previously described (Špačková et al., 2020). An exponentially growing culture of strain 972 h^- was divided into aliquots and treated with 25 mM H_2O_2 or 1 % (*v/v*) methanol (solvent of the extracts), as the negative control, followed by incubation at 30 °C and 200 rpm. Aliquots of 100 μL were collected at 0, 20, 60, 90, and 120 min of treatment, serially diluted in sterile deionized water to 10^{-4}, and 40 μL of the highest dilution suspension were spread on YES solid medium. The plates were then incubated for 3 days at 30 °C and the colonies were counted. Viability was calculated as a percentage of the colony-forming unit (CFU), taking 0 min treatment as 100 % viability.

2.6.3. Determination of intracellular oxidation and cell viability by flow cytometry

The determination of intracellular oxidation and cell viability was based on the method as previously described with some modifications (Špačková et al., 2020). The *S. pombe* strain was cultured overnight and then diluted to $OD_{595} = 0.1$. The culture was grown until $OD_{595} = 0.4$ and then the cells were centrifugated (3 min, 5000 rpm, 4 °C, Eppendorf 5804R), washed twice with phosphate-buffered saline (PBS, pH 7.4), and suspended in PBS to $OD_{595} = 0.04$. From this suspension, 500 μL was taken and stored at 4 °C for autofluorescence measurement. Propidium iodide (PI) and 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) were added to final concentrations of 3 μg/mL and 50 μM, respectively and

the suspension was incubated for 1 h in the dark at 30 °C and 200 rpm. Aliquots were prepared for the different treatments: negative control (only yeast cells in PBS), solvent control (DMSO 1 %), positive control (yeast cells and 25 mM H_2O_2), and the treatment (coincubation of yeast cells, 25 mM H_2O_2 and 750 μg/mL *Raphidonema* sp., *Thalassiosira* sp., or *Arthrospira* sp. extract). The treatments were incubated for 20 min at 30 °C, 200 rpm, and subsequently analyzed by flow cytometry in the cytometer CytoFLEX V2-B2-R2, Beckman for MOE. At least 20,000 events were processed and the Software CytExpert 2.4 was used to analyze the results.

2.7. Microalgae effect in HepG2 cell line

2.7.1. HepG2 cell culture

HepG2 cells (human hepatocytes) from the American Type Culture Collection (ATCC HB-8065) were maintained in culture in 25 cm^2 polystyrene flasks with complete DMEM (supplemented with 10 % FBS, 2 mM L-glutamine, 1 % antibiotic–antimycotic solution, and 1.5 g/L sodium bicarbonate) at 37 °C in a humidified incubator containing 5 % CO_2. Cells were sub-cultured weekly using 0.25 % trypsin-EDTA and seeded at 1.5×10^6 cells/mL density.

2.7.2. Cytotoxicity assay

To evaluate the potential cytotoxicity of microalgae extracts, HepG2 cells were seeded in 96-well culture plates at a density of 1.5×10^6 cells/mL (200 μL/well) in complete DMEM. Twenty-four hours after plating, the medium was discarded and the cells were incubated with the microalgae extracts at different concentrations, ranging from 12.5 to 500 μg/mL, for 4 h of incubation. Cells just in DMEM (without the extract) or with 10 % DMSO in DMEM were used as normal growth and death controls, respectively, and solvent control (DMSO 1 %) was also performed. Cell metabolic activity (viability) was determined using the MTT assay as described before (Oliveira et al., 2018). The cell viability was expressed as a percentage of the control cells.

2.7.3. Cytoprotective effect of microalgae extracts against tert-butyl hydroperoxide (t-BOOH)-induced oxidative damage

Microalgae extracts' ability to protect HepG2 cells against *t*-BOOH-induced oxidative damage was evaluated using a co-incubation regimen. HepG2 cells were co-incubated with microalgae extract at 1.25, 12.5, or 125 μg/mL and *t*-BOOH (0.5 mM) for 4 h, and cell viability was determined by MTT assay (Oliveira et al., 2018). The cell viability was expressed as a percentage of the control cells.

2.8. Statistical analysis

All results are presented as mean ± standard deviation (mean ± SD) from at least three independent experiments. Data was analyzed in GraphPad Prism v. 8.0 Software (San Diego, CA, USA). Results were analyzed by One-Way ANOVA Multiple Comparisons Test followed by Tukey post-tests for multiple comparisons. Significant differences were considered when $p < 0.05$ and different letters mean statistical differences between conditions.

3. Results and discussion

3.1. Chemical characterization of microalgae extracts

3.1.1. Fatty Acid (FA) content

To evaluate potential biological activities, extracts of *Arthrospira* sp., *Thalassiosira* sp., and *Raphidonema* sp. were characterized chemically to identify metabolites. Regarding the relative FA profile, expressed as a percentage of the total FA content, differences between all studied microalgae were observed (Table 1).

Extracts from the three microalgae differed in the content of the type of fatty acids. *Arthrospira* sp. extract (AE) and *Thalassiosira* sp. extract (TE) were richer in saturated FA (SFA) while *Raphidonema* sp. extract (RE) contained more polyunsaturated FA (PUFAs) than monounsaturated FA (MUFAs) or SFAs. Specifically, AE and TE displayed higher content in saturated SFA, 57.59 % and 42.88 %, respectively, while RE showed the lowest content (28.96 %). Palmitic acid (C16:0) was by far the most abundant SFA, AE being the richest in this compound (54.59 %) when compared to TE (26.91 %) and RE (22.76 %). In line with these results, *Arthrospira platensis* revealed a high content in SFA (67.4 %), with a prevalence of palmitic acid (37.6 %; (Matos et al., 2020), and *Thalassiosira* sp. also showed high content in SFA with palmitic acid as the most abundant (54.41 %; (Joseph et al., 2017). Although there are very few studies with *Raphidonema* sp. (synonym of *Koliella*) regarding lipid content, the predominance of SFA (8–13 %) and palmitic acid have also been reported in *Koliella antarctica* (Lima et al., 2021).

Considering MUFAs, TE and RE presented the highest contents (32.21 % and 30.72 % respectively) compared to AE (13.15 %). Oleic acid (C18:1 cis; 22.05 %) was the most abundant MUFAs in RE while palmitoleic acid (C16:1) and cis-10-pentadecenoic acid (C15:1; 18.41 % and 10.79 % respectively) were the most abundant MUFAs in TE. These results agree with reports on high MUFAs content in *Thalassiosira* sp. (33.24 %; (Saxena, Marella, et al., 2021) and the prevalence of C18:1n-9 (oleic acid; 9–43 %) among MUFAs in *Koliella antarctica* (Lima et al., 2021).

A different pattern was found regarding PUFAs content when compared with MUFAs. RE displayed higher content in PUFAs (40 %), followed by AE (29 %) and TE (25 %). The predominant PUFAs in RE was linoleic acid (C18:3 ω3; 22 %), in AE were γ-linolenic acid (C18:3 ω6; 17 %) and linoleic acid (C18:2 cis; 13 %), and in TE cis-13,16-docosadienoic acid (C22:2; 18 %). In general terms, these results are in agreement with the literature regarding the high PUFAs content in *A. platensis* (33.28 %; (Tudor et al., 2021) and *Thalassiosira weissflogii* TRG10-p105 (57.8–62.4 %; (Katayama et al., 2022) and the prevalence of linoleic (3–13 %) and α-linolenic (10–34 %) acids in *K. antarctica* (Lima et al., 2021). Dietary intake of ω6-FA and ω3-FA has been described as having several health benefits such as lowering oxidative stress and blood pressure, improvement of immune response and insulin resistance, and prevention of heart disease and other chronic diseases (Pekkoh et al., 2022). Our results indicate that AE and RE are rich in ω6-FA and ω3-FA, respectively, suggesting significant potential for these microalgae to contribute to human health as functional food ingredients or supplements.

3.1.2. Pigments composition

The identification and quantification of pigment content, conducted using high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD), is depicted in Table 2 (the chromatographic analysis is provided in supplementary material Fig. S1). Concerning carotenes, β-carotene was the unique compound detected and it was found in all extracts, with higher prevalence observed in AE (663.2 mg/g) and TE (651.8 mg/g). *Raphidonema* sp. showed a remarkably lower concentration (6.69 mg/g) similar to what has been reported in the literature (34–39 μg/g freeze-dried dry mass; (Leya et al., 2009).

Regarding the group of chlorophylls, pheophorbide *a* was the only one found in the three extracts, AE and TE (658.6 and 835 μg/g, respectively) presenting higher content than RE (61 μg/g, Table 2). Pheophorbide *a* has gathered considerable attention in recent years as a non-invasive and highly selective strategy for cancer treatment. It has demonstrated efficacy as a potent photosensitizer and is frequently employed in combination with photodynamic therapy (Saide et al., 2020). Other studies proved that pheophorbide *a* also revealed anti-inflammatory and antioxidant properties (Lanfer-Marquez et al., 2005; Lauritano et al., 2020). However, RE was the only one presenting other pigments than pheophorbide *a*, some of them with considerably high concentrations such as chlorophyll *b* epimer (263.9 μg/g), chlorophyll *a* epimer (164.2 μg/g) and chlorophyll *b* (143.7 μg/g). Chlorophyll and its

Table 1
Fatty acid (FA) content (expressed as a percentage of total FA) of microalgae extracts. ND – Not Detected.

FA		*Arthrospira* sp.	*Thalassiosira* sp.	*Raphidonema* sp.
Saturated fatty acid (SFA)				
C14:0	Myristic acid	ND	9.80	1.08
C15:0	Pentadecanoic acid	ND	1.26	ND
C16:0	Palmitic acid	57.59	26.91	22.76
C17:0	Heptadecanoic acid	ND	0.93	1.44
C18:0	Stearic acid	ND	3.21	3.68
C24:0	Lignoceric acid	ND	0.78	ND
Total SFAs		57.59	42.88	28.96
Monounsaturated fatty acid (MUFA)				
C14:1	Myristoleic acid	ND	0.27	ND
C15:1	cis-10-Pentadecenoic acid	13.15	10.79	6.14
C16:1	Palmitoleic acid	ND	18.41	1.35
C17:1	cis-10-Heptadecanoic acid	ND	1.13	ND
C18:1 cis	Oleic acid	ND	ND	22.05
C20:1	cis-11-Eicosenoic acid	ND	1.61	1.18
Total MUFAs		13.15	32.21	30.72
Polyunsaturated fatty acid (PUFA)				
C18:2 cis	Linoleic acid	12.73	1.80	5.31
C18:3 ω6	γ-Linolenic acid	16.54	ND	ND
C18:3 ω3	Linolenic acid	ND	ND	21.78
C20:3 ω3 + C22:1	cis-11,14,17-Eicosatrienoic acid + Erucate acid	ND	ND	3.11
C22:2	cis-13,16-Docosadienoic acid	ND	17.86	10.12
C22:6	cis-4,7,10,13,16,19-Docosahexaenoic acid	ND	5.26	ND
Total PUFAs		29.26	24.91	40.32

derivative products are recognized for their health-promoting attributes, serving not only as colorants but also exhibiting antioxidant properties and holding potential therapeutic benefits (Christaki et al., 2015). Per our observations, chlorophyll *a* and *b* were also found in the pigments produced by *Raphidonema* spp. (Leya et al., 2009). We observed lower amounts of chlorophyll *a* and *b* compared to the epimer and allomer derivatives. These data suggest chlorophylls in this extract presented low stability and RE is more susceptible to structural transformations.

For the group of xanthophylls, AE was the richest extract (5460.5 μg/g), being considerably higher than TE (12.6 μg/g) and RE (554.6 μg/g). Notably, a high amount of zeaxanthin (3245.2 μg/g) was evidenced in AE. Following our work, zeaxanthin was also one of the pigments with the highest concentration in *A. platensis* (Park et al., 2018). However, the reported contents were lower than in our study with concentration ranges of xanthophylls and zeaxanthin (250–470 mg/100 g and 125–200 mg/100 g, respectively; (Thomas, 2010). Unlike zeaxanthin, fucoxanthin (12.6 μg/g) and fucoxanthin derivative (12.6 μg/g) were in higher concentration in the extract of TE, and the compounds violaxanthin (355 μg/g) and lutein (250.5 μg/g) were in higher amount in RE. High concentrations of these two pigments were also found in *Raphidonema* sp. (strains 011–99 and 112–00; (Leya et al., 2009) in which the former presented 631 and 551 μg/g and the latter 180 and 116 μg/g of lutein and violaxanthin, respectively. Possibly the high concentration of zeaxanthin in the AE may result from the conversion of β-carotene to produce zeaxanthin via the non-heme-containing *β*-carotene hydroxylase enzyme while in the case of the RE, the conversion of zeaxanthin to violaxanthin may have occurred by the action of the enzyme zeaxanthin epoxidase (Velmurugan & Kodiveri Muthukaliannan, 2022). These results enhance our comprehension of the potential applications of these microalgae species, particularly in areas like antioxidant activity and cancer treatment as the chemical composition of the extracts has revealed compounds with reported relevant biological activities that can be further explored in future research.

Table 2
Analysis and quantification of pigment composition in microalgae extracts detected by HPLC-DAD. ND – Not Detected.

Pigments	*Arthrospira* sp.	*Thalassiosira* sp.	*Raphidonema* sp.
Carotenes (mg/g DW)			
β-carotene	663.2	651.8	6.69
Total	663.2	651.8	6.69
Chlorophylls (μg/g DW)			
Chlorophyll *a* allomer	ND	ND	117.5
Chlorophyll *a*	ND	ND	64.8
Chlorophyll *a* epimer	ND	ND	164.2
Chlorophyll *b*	ND	ND	143.7
Chlorophyll *b* epimer	ND	ND	263.9
Pheophorbide *a*	658.6	835.0	61.0
Total	658.6	835.0	815.0
Xanthophylls (μg/g DW)			
Fucoxanthin	ND	12.6	ND
Fucoxanthin derivative	25.1	12.6	ND
Violaxanthin	ND	ND	355.0
Zeaxanthin	3245.2	ND	ND
Zeaxanthin derivative	218.5	ND	ND
Lutein	94.2	ND	250.5
Unidentified xanthophyll	ND	ND	144.9
Unidentified xanthophyll	ND	ND	159.2
Unidentified xanthophyll	728.1	ND	ND
Unidentified xanthophyll	469.5	ND	ND
Unidentified xanthophyll	404.2	ND	ND
Total	5460.5	25.2	909.7

3.2. Antioxidant profile of microalgae extracts

As shown in Table 3, the antioxidant profile of the microalgae extracts was evaluated using four different methodologies: DPPH scavenging, ICA, $O_2^{\bullet-}$ scavenging through NBT assay, and FRAP. With these

Table 3
Antioxidant activity of microalgae extracts determined by biochemical methods. Extracts of *Arthrospira* sp., *Thalassiosira* sp., and *Raphidonema* sp. were tested at a final concentration of 0.5 mg/mL. Data represent mean ± SD for three independent experiments. Significance was assessed with the One-Way ANOVA Multiple Comparisons Test between all the values in the same column. Different letters mean significant differences ($p < 0.05$).

Extracts	DPPH (% of inhibition)	ICA (% of iron chelation)	$O_2^{\bullet-}$ (% of inhibition)	FRAP (Equivalents Fe^{2+} μM)
Arthrospira sp.	68.4 ± 6.0^{a}	22.0 ± 6.7^{a}	38.3 ± 10.3^{a}	6.0 ± 1.6^{a}
Thalassiosira sp.	16.8 ± 7.7^{b}	58.8 ± 4.3^{b}	47.3 ± 7.7^{a}	38.3 ± 2.7^{b}
Raphidonema sp.	20.0 ± 3.7^{b}	43.0 ± 5.9^{c}	14.8 ± 4.0^{b}	67.3 ± 5.8^{c}

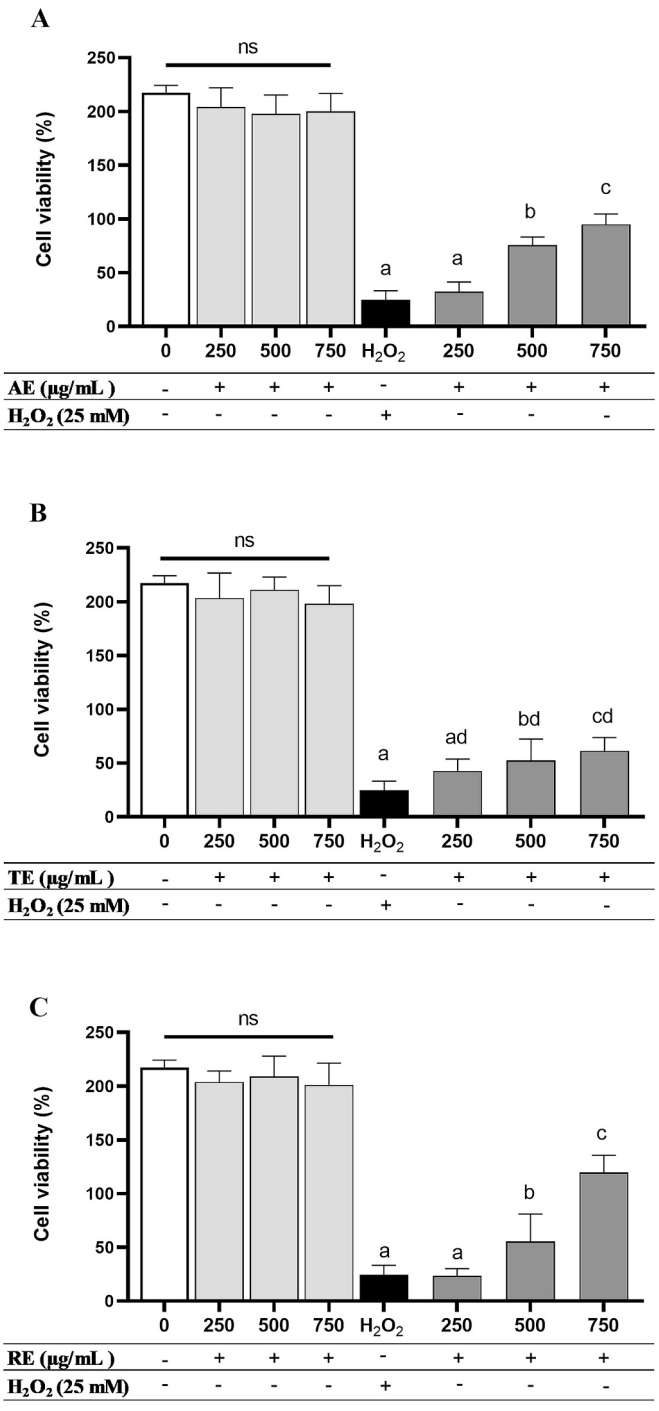


Fig. 1. Viability of *Schizosaccharomyces pombe* upon exposure 250, 500 or 750 µg/mL *Arthrospira* sp. (A), *Thalassiosira* sp. (B) or *Raphidonema* sp. (C) extracts with or without 25 mM H_2O_2. Cells from exponentially growing cultures were incubated with H_2O_2 and respective microalga extract for 120 min at 30 °C, 200 rpm, and aliquots were harvested, serially diluted to 10^{-4}, and spread on YES solid medium. Besides solvent (same volume as the highest concentration of extract), the negative control contained *S. pombe* cells (white bars) and the positive control contained cells with 25 mM H_2O_2 (black bars). Upon incubation at 30 °C for 48 h, colonies were counted, and viability was calculated, taking 0 min as 100 % viability. Data is presented as mean ± SD of three independent experiments. Statistical significance was investigated with One-way ANOVA followed by Tukey's post-hoc test, ns means no significant differences between treatments with just extracts (light grey bars) compared to control cells, and different letters mean significant differences between treatments with extracts and H_2O_2 (grey bars) compared to positive control.

approaches, it would be possible to assess the chemical basis of the antioxidant potential of the extracts. Among all samples, AE showed the highest DPPH$^{\bullet}$ scavenging activity (68.4 ± 1.9 %) followed by TE and RE (around 16–20 % each; Table 3). This indicates that AE has a strong ability to neutralize free radicals compared to the other extracts tested. The apolar nature of the solvent seems to be pivotal to extracting radical scavenging compounds from AE as it has been shown that aqueous and ethanolic extracts of *A. platensis* did not scavenge DPPH$^{\bullet}$ radicals (Matos et al., 2020). Considerable values of DPPH$^{\bullet}$ scavenging activity were also found with a *Thalassiosira* sp. methanolic extract (20 mg/mL; 36.54 ± 0.49 % inhibition; (Peraman & Nachimuthu, 2019) and although studies with *Raphidonema* sp. extracts in this context are scarce, the reported production of α-tocopherol (Leya et al., 2009) makes this microalga a candidate for radical scavenging activity as suggested by our results (Table 3).

Iron is an essential element for life; however, when present in excess biological systems can be harmed since, under redox-active form, it catalyzes the generation of ROS, such as $^{\bullet}$OH and SO$^{\bullet}$, leading to the oxidation of unsaturated lipids and increase in oxidative damage in proteins and nucleic acids (Carrasco-Castilla et al., 2012; Maioli et al., 2010). Hence, the chelation of metal ions plays an important role in balancing the antioxidant state (Saiga et al., 2003). Results showed that TE has the highest ICA (58.8 ± 1.3 %) followed by RE (43.0 ± 2.0 %) and AE (22.0 % ± 2.0 %). This activity has been shown also in aqueous extracts of *Crypthecodinium cohnii* and *Schizochytrium* sp. with 63.13 % and 70.59 %, respectively (Yu et al., 2017). Chelation properties of *Arthrospira* spp. were additionally demonstrated in a peptide fraction of *Arthrospira maxima*, being even more efficient than the reference chelator Na_2EDTA (Montalvo et al., 2019).

The scavenging activity of $O_2^{\bullet-}$ by AE, TE, and RE was investigated with the NBT assay. TE and AE displayed more $O_2^{\bullet-}$ scavenging activity (47.3 ± 3.1 % and 38.3 ± 4.2 % respectively) than RE (14.8 ± 2.0 %). To the best of our knowledge, this study is the first one that investigated $O_2^{\bullet-}$ scavenging activity in TE and RE extracts. Previous studies showed that 5.0 mg/mL of *Chlorella* sp. polysaccharide extract inhibited NBT production at 32.10 ± 1.20 % (Song et al., 2018). Regarding *Arthospira* sp., previous investigations have demonstrated the antioxidant potential of the GM15 peptide from *A. platensis* by elimination of superoxide and hydroxyl radicals, besides influencing intracellular oxidative stress (Song et al., 2018). In addition, the steroid fraction from *Arthospira maxima* was also effective in eliminating $O_2^{\bullet-}$ (El Baroty et al., 2020). However, polar compounds from *Arthrospira* sp. do not seem active against this radical since water extracts from *A. platensis* did not have a detectable activity (Kaga et al., 2021). Hence, superoxide scavenging activity is evident in other microalgae species and extracts obtained through different solvents. This observation suggests that microalgae have different bioactive compounds with the capacity for superoxide scavenging.

The antioxidant activity by electron transfer was assessed by the FRAP assay. As shown in Table 3, FRAP activity was higher in RE (67.3 ± 1.9 µM Fe^{2+}) followed by TE (38.3 ± 0.9 µM Fe^{2+}) and AE (6.0 ± 0.5 µM Fe^{2+}, $p < 0.05$). These values are considerably lower than water and ethanolic *A. platensis* extracts and ethanolic extract of *Skeletonema* sp. (1.5 mM Fe^{2+}) even considering the differences in concentrations used in the assays (50 mg/mL; (Cardoso et al., 2020; Matos et al., 2020) with the present work (0.5 mg/mL). Overall, the results indicate that the three studied microalgae extracts possess varying levels of antioxidant activities and mechanisms of action. AE showed strong DPPH scavenging activity, suggesting high radical scavenging activity, TE demonstrated high iron chelating and superoxide scavenging activities, while RE exhibited the highest FRAP activity, suggesting reducing activity by electron transfer. These findings contribute to understanding the antioxidant potential of these genera and highlight their potential applications in various fields, such as pharmaceuticals, cosmetics, functional foods, and supplements.

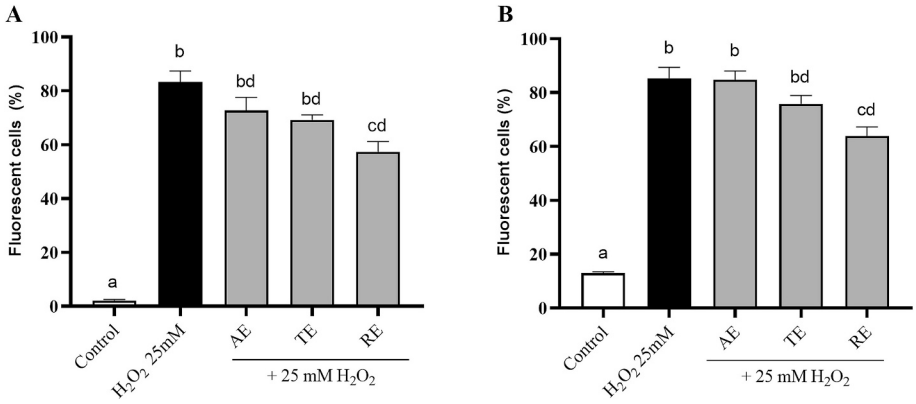


Fig. 2. Protective effect of *Arthrospira* sp. (AE), *Thalassiosira* sp. (TE), and *Raphidonema* sp. (RE) hexane extracts on intracellular oxidation and viability of *Schizosaccharomyces pombe* cells challenged with 25 mM H_2O_2. Cells previously incubated with propidium iodide (PI; A) and 2′,7′-dichlorofluorescein diacetate (H_2DCFDA; B) were treated with 25 mM H_2O_2 or 25 mM H_2O_2 and 750 μg/mL of each extract for 20 min at 30 °C, 200 rpm. Besides the solvent of the extracts (DMSO 1 %, data not shown), the negative control contained only *S. pombe* cells (white bars) and the positive control contained cells with 25 mM H_2O_2 (black bars). Viability was assessed by detection of fluorescence emitted by PI (A) and intracellular oxidation was measured by detection of fluorescence emitted by H_2DCFDA (B), by flow cytometry. Data represents the mean of three independent experiments. Statistical significance was investigated with One-way ANOVA Multiple Comparisons Test followed by Tukey's post-hoc test, different letters mean significant differences between treatments ($p < 0.05$).

3.3. Protective role of microalgae extracts against H_2O_2-induced oxidative damage in Schizosaccharomyces pombe cells

To assess the protective role of microalgae extracts as antioxidant agents in a eukaryotic cell context, *Schizosaccharomyces pombe* was used as the experimental model. Cells treated with microalgae extract (250, 500, or 750 μg/mL) and untreated cells were exposed for 0, 20, 60, 90 (data not shown), and 120 min to oxidative stress (25 mM H_2O_2) and cell viability was assessed (Fig. 1).

Schizosaccharomyces pombe exposed to AE, TE, and RE (250, 500, or 750 μg/mL) revealed no considerable loss of viability compared to the negative control, indicating that both concentrations would not induce toxicity. Oxidative stress induced by 25 mM H_2O_2 led to a reduction of cell viability of approximately 200 percentage points compared to the negative control (cells without H_2O_2 nor microalgae extracts). Nevertheless, the administration of microalgae extracts revealed a dose-dependent protective effect. In fact, viability increased with increasing extract concentrations in the three extracts tested with significant differences when concentrations of 500 μg/mL or 750 μg/mL were used. Regarding the protective effect of microalgae extracts, which corresponds to the difference in cell viability between treated cells (exposed to extracts and H_2O_2) and the positive control (exposed only to H_2O_2), the highest concentration (750 μg/mL) provided a significant protective effect of 71, 37, and 95 percentage points, in the presence of AE (Fig. 1A), TE (Fig. 1B) and RE (Fig. 1C) respectively, which indicates the amelioration of oxidative damage induced by H_2O_2. At lower concentrations (500 μg/mL), a significant protective effect was also observed, with 51, 28, and 31 percentage points for AE (Fig. 1A), TE (Fig. 1B) and RE (Fig. 1C), respectively.

Antioxidant activity of extracts or fractions from *Arthrospira* sp., *Thalassiosira* sp., and *Raphidonema* sp. are scarce. One example is a peptide isolated from *A. platensis* which can reduce ROS levels caused by H_2O_2 exposure in kidney cells (MDCK cells; (Velayutham et al., 2021). Antioxidant activity has been found in different microalgae as, for instance, an ethanol/water extract of *Tetraselmis suecica* induced a significant recovery effect on H_2O_2 stressed human lung adenocarcinoma cells, in which several factors implicated in oxidative damage repairing pathways were expressed and activated (Sansone et al., 2017). This strong antioxidant activity could be related to the high levels of carotenoids such as the xanthophylls lutein, violaxanthin, neoxanthin, antheraxanthin and loroxanthin esters identified in the extract (Sansone et al., 2017), some of them found in *Arthrospira* sp., *Thalassiosira* sp. and *Raphidonema* sp. hexane extracts (Table 2). To the best of our knowledge, this is the first study evaluating the protective effects of microalgae extracts against H_2O_2-induced oxidative damage in *S. pombe*, and the first one to highlight the ability of TE and RE to increase the viability of cells exposed to an oxidative damage inducer.

The protective effect of microalgae extracts against H_2O_2-induced oxidative stress on cellular viability was further investigated for plasma membrane integrity with PI by flow cytometry (Fig. 2A). Propidium iodide is a fluorochrome that crosses damaged or compromised plasma membranes, typically occurring in necrotic cells, and binds to nucleic acids to emit a red fluorescence, which has been used to measure the degree of cell membrane integrity and hence infer of viability (Zhao et al., 2021).

The percentage of events negative for PI was notably high (>95 %; data not shown) for all tested microalgae extracts, indicating that the extracts did not affect cell viability. However, exposure to 25 mM H_2O_2 increased the proportion of cells displaying red fluorescence (83.28 ± 5.8 %; Fig. 2A) compared to the negative control (2.12 ± 0.56 %), indicating that the stressor compromises cell membrane integrity and viability. On the other hand, exposure to 25 mM H_2O_2 in the presence of RE significantly decreased the proportion of fluorescent cells (57.4 ± 5.3 %). These results suggest that this extract protects *S. pombe* cells against H_2O_2 toxicity, which are in line with the viability assays assessed as CFU (Fig. 1C), the radical scavenging activity (DPPH and $O_2^{\bullet}$) and iron chelating activity (Table 3). Despite the reproducible lower proportion of PI-positive cells in the AE- and TE-treated groups when compared with the control (Fig. 2A and B), the lack of significance is not in accordance with the viability results obtained with CFU (Fig. 1A and B). The shorter exposure of cells to the treatments in the cytometry experiments (20 min) relative to the experiment of viability assessment by CFU (120 min) might account for such differences, however, as reported elsewhere (Davey & Hexley, 2011), PI may not always reflect lack of viability of cells as they can still be viable under a certain degree of membrane disturbance, which would be enough for being permeable to the fluorochrome.

The antioxidant activity of the extracts was further investigated with the redox-sensitive fluorochrome H_2DCFDA. The diacetate form of 2′,7′-dichlorofluorescein can easily pass through the plasma membrane, once inside the cell, esterases break down H_2DCFDA to form 2′,7′-dichlorofluorescein (H_2DCF), which accumulates inside the cell because it cannot cross cell membranes. The oxidized form of H_2DCF is fluorescent, making it a useful indicator of intracellular oxidation when measuring the overall fluorescence of the cell (Marques et al., 2011) and enabling the investigation of ROS production upon an insult in the absence or the

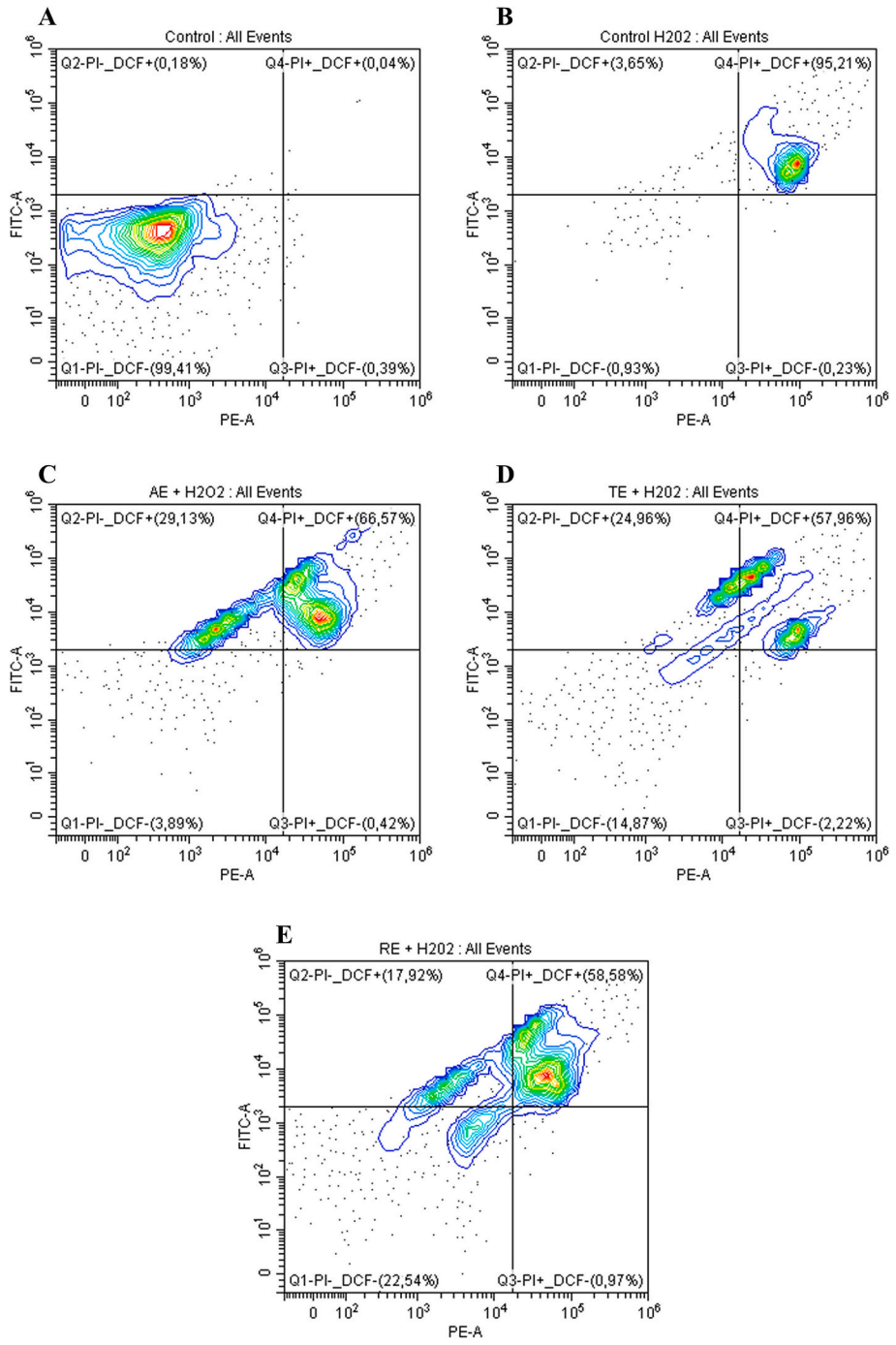


Fig. 3. Contour dot plots representative of 3 replicates of the percentage of *Schizosaccharomyces pombe* cells in response to staining with propidium iodide (PI; PE-A channel) and 2′,7′-dichlorofluorescein diacetate (H_2DCFDA and represented as DCF in the dot plots; FITC-A channel) analyzed by flow cytometry (see Fig. 3 for details). Besides the solvent of the extracts (DMSO 1 %, data not shown), *A* represents control cells without any treatment; *B*, positive control (25 mM H_2O_2); *C*, 750 μg/mL *Arthrospira* sp. extract treatment with 25 mM H_2O_2; *D*, 750 μg/mL *Thalassiosira* sp. extract treatment with 25 mM H_2O_2; *E*, 750 μg/mL *Raphidonema* sp. treatment with 25 mM H_2O_2. Contour dot plots were divided into four quadrants according to the double staining: quadrant 1 (Q1), PI-_DCF-; quadrant 2 (Q2), PI-_DCF+; quadrant 3 (Q3), PI+_DCF-; and quadrant 4 (Q4), PI+_DCF+).

presence of an antioxidant. Cell suspensions only treated with AE, TE, or RE emitted fluorescence similar to the negative control (no treatment; data not shown), which suggests no effect on intracellular oxidation. As expected, when cells were exposed to 25 mM H_2O_2, the proportion of fluorescent cells was significantly higher (85.37 ± 5.69 % of the population; Fig. 2B). Interestingly, the group treated with 25 mM H_2O_2 in the presence of RE showed a significantly lower percentage of fluorescent cells (63.95 ± 4.7 %; Fig. 2B), indicating an inhibition of ROS production in cells exposed to H_2O_2. This result correlates with the iron chelating activity (Table 3), since the strong reducing power of antioxidants may also affect metal ions by blocking the Fenton reaction between H_2O_2 and Fe^{2+} and lowering the formation of highly reactive hydroxyl radical (Bouayed & Bohn, 2010) and the viability results assessed as CFU (Fig. 1C) and PI fluorescence (Fig. 2A). Although there was a consistent lower proportion of DCF-positive cells observed in the AE- and TE-treated groups compared to the control group (as shown in Fig. 2B), the lack of statistical significance does not align with the viability results obtained from CFU assays (as depicted in Fig. 1A and B).

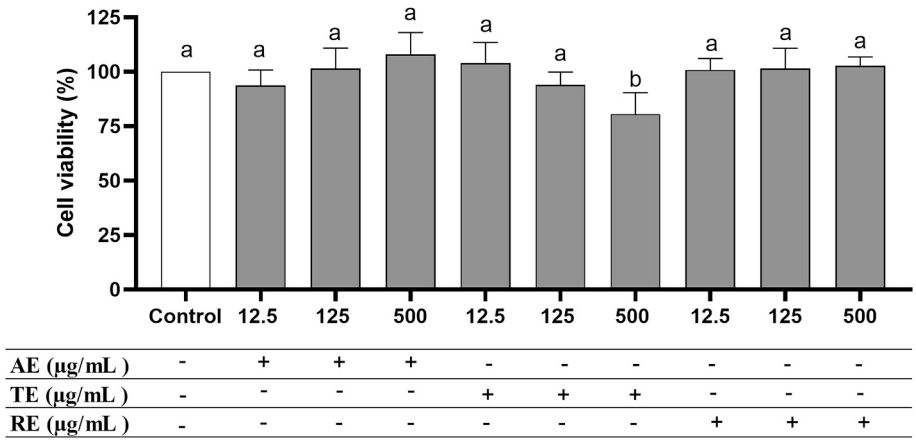


Fig. 4. Cytotoxicity of *Arthrospira* sp. (AE), *Thalassiosira* sp. (TE) and *Raphidonema* sp. (RE) hexane extracts on HepG2 cells. Besides the solvent of the extracts (DMSO 1 %, data not shown), cells were incubated with microalgae extracts (12.5, 125 or 500 µg/mL) for 4 h, and viability was assessed with the MTT assay. Cell viability is expressed relative to the control cells (100 % cell viability). Data is presented as mean ± SD of three independent experiments. Statistical significance was investigated with One-way ANOVA Multiple Comparisons Test followed by Tukey's post-hoc test, different letters mean significant differences ($p < 0.05$).

As discussed above, the lower exposure time to the treatments in the cytometry assays may have contributed to the lack of significance.

Our findings offer valuable insights into the protective effects of microalgae extracts against H_2O_2-induced oxidative cellular damage. Many chronic diseases are believed to be caused by oxidative stress, which causes cell damage. To prevent this, one effective approach is to use natural products like microalgae, which have antioxidant properties that can protect cells from harm (Vahdati et al., 2022). The ability of microalgae extracts to protect against intracellular oxidative stress has already been studied. *Chlorella* sp. (Vahdati et al., 2022), *Asterarcys quadricellulare* (Saadaoui et al., 2023), and *Chlorella vulgaris* (Makpol et al., 2009) are relevant examples. In addition, some xanthophylls extracted from microalgae, such as fucoxanthin (Wang, Cui, et al., 2018), identified in AE and TE (Table 2), violaxanthin (Wang, Huang, et al., 2018), identified in RE (Table 2), zeaxanthin (Pistelli et al., 2021), identified in AE (Table 2), and lutein (Pistelli et al., 2021) identified in AE and RE (Table 2), have also been studied to prevent oxidative stress. Furthermore, other studies revealed that β-carotene, also identified in all microalgae extracts used in this study (Table 2), can scavenge ROS and showed photochemopreventive and anti-cancer properties in murine melanoma cell model (B16-F0) and HaCaT cell line after UV-induced injury (Pistelli et al., 2021).

To further support the previous findings regarding the antioxidant activity of microalgae extracts, we analyzed the combined staining of the dyes PI and H_2DCFDA within a cellular context. The cytogram of untreated *S. pombe* control cells (cell culture without any treatment; Fig. 3A) revealed that nearly all yeast cells (99.41 %) marked negatively to PI and H_2DCFDA indicating an absence of loss of viability and intracellular oxidation.

As anticipated, treatment with 25 mM of H_2O_2 (Fig. 3B) increased fluorescence in both channels, indicating concomitant intracellular oxidation and loss of viability. Interestingly, exposure to H_2O_2 in the presence of AE, TE, or RE significantly increased the percentage of PI- and DCF-negative cells compared to the positive control (0.93 %, Fig. 3B), suggesting attenuation of the oxidative stress and increased viability (3.89 %, 14.87 %, and 22.54 %; Fig. 3C, D and E respectively). Similarly, these treatments elevated the percentage of PI-negative and DCF-positive cells (29.13 %, 24.96 %, and 17.92 % Fig. 3C, D and E respectively) compared to the positive control (3.65 %, Fig. 3B) and reduced the percentage of PI-positive and DCF-negative cells (0.42 %, 2.22 %, and 0.97 % Fig. 3C, D and E respectively) compared to the H_2O_2 positive control (0.23 %; Fig. 3B). Likewise, AE, TE, and RE lowered the percentage of PI- and DCF-positive cells (66.57 %, 57.96 %, and 58.58 % Fig. 3C, D and E respectively), compared to the H_2O_2 positive control (95.21 %; Fig. 3B). Collectively, these findings indicate that microalgae extracts possess antioxidant properties by mitigating cell permeabilization and intracellular oxidation, thereby shielding cells from oxidative damage induced by H_2O_2.

3.4. Protective effects of microalgae extracts against tert-butyl hydroperoxide (t-BOOH)-induced oxidative damage in a human hepatocyte cell line (HepG2)

To investigate the potential application in human health, the protective activity against oxidative stress of the extracts was assessed in the human cell line HepG2, a well-established cell model for toxicity and stress protection studies. To evaluate toxicity, cells were incubated with the microalgae extracts for 4 h, at several concentrations (up to 500 µg/mL), mimicking acute cell exposure. In general, the incubation of HepG2 cells with microalgae extracts did not induce any toxicity except for 500 µg/mL TE which revealed a significant decrease in viability (Fig. 4). Cytotoxicity of microalgae extracts has been previously reported in HepG2 with *C. vulgaris*, *Nannochloropsis oculata*, and *T. weissflogii* methanolic extracts (Venkatraman et al., 2022). The reported IC_{50} values for 24 h treatment was in the range of 402.4–488.6 µg/mL, which suggests stronger toxicity even though the incubation time is longer. Despite the fact of the different biological origins of the extracts, the solvent used in the extractions might be influencing cytotoxicity. In fact, unlike our hexane AE, an ethanolic extract from *A. platensis* revealed strong cytotoxic activity (IC_{50} 20.56 ± 1.7 µg/mL) for a 24 h incubation period (Gheda et al., 2023), suggesting that extracts obtained with apolar solvents from microalgae may exhibit lower toxicity compared to extracts made with polar solvents. To our knowledge, there are no published studies with *Raphidonema* sp. extracts addressing cytotoxicity however, studies with *Dunaliella salina* (El-Baz et al., 2018), which belong to the same phylum (Chlorophyta) as *Raphidonema* sp., revealed that carotenoid and polar fractions are slightly cytotoxic on HepG2 cells. It is possible that these microalgae share similar metabolites but the differences in polarity of the solvents most likely contributed to the disparity of cytotoxicity.

In cells and tissues, *t*-BOOH is commonly used as a stressor to evaluate mechanisms of oxidative stress-induced cellular damage (Kučera et al., 2014). In hepatocytes, *t*-BOOH specifically depletes cellular antioxidant defenses, leading to the generation of radicals that initiate lipid peroxidation, thereby simulating a state of oxidative stress (Kim et al., 1998; Kučera et al., 2014). This phenomenon can aid in understanding how microalgae extracts exert their antioxidant activity.

According to cytotoxicity results (Fig. 4), concentrations of 1.25,

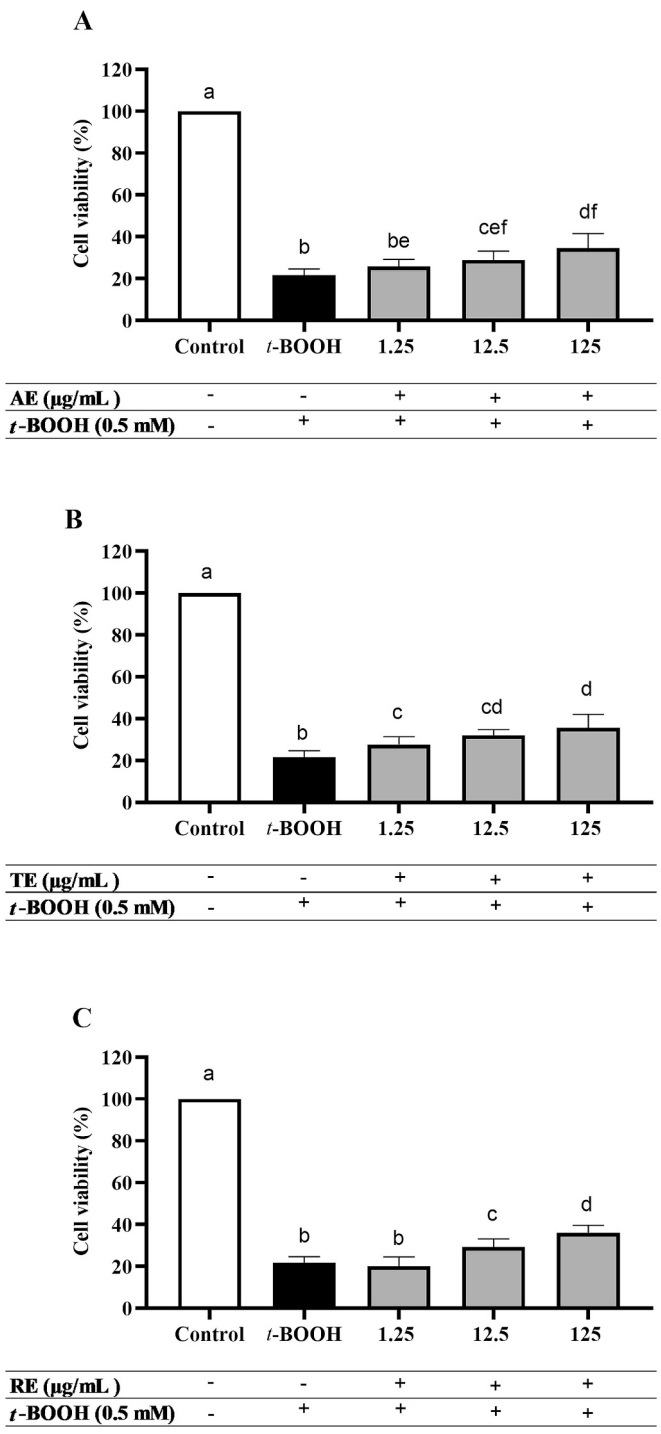


Fig. 5. Cytoprotection of *Arthrospira* sp. (AE; A), *Thalassiosira* sp. (TE; B), and *Raphidonema* sp. (RE; C) hexane extracts on HepG2 cells against *t*-BOOH. Cells were co-incubated with 1.25, 12.5, or 125 µg/mL microalgae extract and 0.5 mM *t*-BOOH for 4 h, and cell viability was determined by the MTT assay. Besides the solvent of the extracts (DMSO 1 %, data not shown), control contained HepG2 cells without treatment (white bars) and the positive control contained HepG2 cells and 0.5 mM *t*-BOOH (black bars). Data is presented as mean ± SD of three independent experiments. Statistical significance was investigated with One-way ANOVA Multiple Comparisons Test followed by Tukey's post-hoc test, different letters mean significant differences between treatments ($p < 0.05$).

12.5, and 125 µg/mL of microalgae extracts were selected to avoid toxicity. So, AE, TE, and RE were used to evaluate their cytoprotective effect against acute exposure, using *t*-BOOH-induced oxidative stress (0.5 mM) in a co-incubation regimen during 4 h (Fig. 5). All extracts tested protected HepG2 cells against *t*-BOOH-induced oxidative stress in a dose-dependent manner. Notably, TE significantly protected cells at the lowest concentration tested (1.25 µg/mL; Fig. 5B), indicating that this is the most potent of the tested extracts. With 12.5 µg/mL and 125 µg/mL, all extracts significantly increased cell viability under *t*-BOOH insult, suggesting that AE, TE, and RE have antioxidant activity in human cells.

As discussed, the antioxidant activity of microalgae extracts has been reported before in literature. While studies specifically focusing on *Arthrospira* sp., *Thalassiosira* sp., and *Raphidonema* sp. extracts remain scarce existing research highlights the bioactive potential of these and other microalgae. For instance, Spirulina is well-known for its antioxidant properties, with several studies demonstrating its ability to neutralize oxidative stress and protect cells from damage (López-Rodríguez et al., 2021). Recent findings have shown that Spirulina extracts possess significant antioxidant and anti-inflammatory effects, underscoring their promise as functional food ingredients (Gómez et al., 2023). While there is limited data on *Thalassiosira* and *Raphidonema* species, other microalgae, such as *Chlorella* sp. water extracts that showed protective effects against H_2O_2-induced oxidative damage in INS-1 (832/13) rat insulinoma cell line, by amelioration of cellular oxidative stress and apoptosis (Lin et al., 2014). Interestingly, antioxidant compounds from microalgae either polar or apolar, as water extracts and hexane extracts from this study show antioxidant properties in human cells, which is a distinct trend compared with the cytotoxicity discussed above. Moreover, *Thalassiosira* species, such as *Thalassiosira rotula*, have demonstrated significant antioxidant activity, further supporting the potential of this genus in protecting against oxidative damage (Frleta Matas et al., 2023). Previous studies also reported that antioxidant peptides of *Navicula incerta* at 100 µM have protective effects against ethanol-induced oxidative cytotoxicity in HepG2/CYP2E1 cells (Kang et al., 2012). Similarly, an antioxidant peptide purified from enzymatic hydrolysates of *Isochrysis zhanjiangensis* at 50 and 100 µM showed a protective effect against ethanol-induced oxidative stress in HepG2 cells (Chen et al., 2019). Protective effects of microalgae extracts were also shown in vivo in which post-oral administration of methanol extract of *Euglena tuba* reduced the toxic levels of iron in iron-overloaded mice, protecting the liver from oxidative stress and fibrosis (Panja et al., 2014).

4. Conclusions

Overall, this study provides evidence on chemical composition, antioxidant potential, absence of relevant cytotoxicity, and cytoprotective effects of extracts from *Arthrospira* sp., *Thalassiosira* sp., and *Raphidonema* sp. Specifically, the results indicate that *Arthrospira* sp. and *Raphidonema* sp. extracts are rich in essential fatty acids (ω6-FA and ω3-FA, respectively), which could play a role in supporting human health, particularly as functional food ingredients and supplement formulations. The antioxidant assays demonstrated that all extracts exhibited notable antioxidant activity, and the protective effects observed in the HepG2 cell model further highlight their potential as agents for combating oxidative stress. Importantly, *Arthrospira* sp. and *Raphidonema* sp. extracts showed a dose-dependent cytoprotective effect, suggesting their applicability in mitigating oxidative damage in human cells.

Despite the promising results, further research is needed, especially concerning the underexplored *Raphidonema* sp., to better understand the bioactive components of these microalgae and their potential mechanisms of action. The chemical profiling of these extracts revealed compounds with known bioactivity, particularly in terms of antioxidant and anti-inflammatory effects, which could be leveraged for future applications in the development of functional foods, pharmaceuticals, and cosmetics.

By addressing key research objectives encompassing chemical

characterization, antioxidant evaluation of microalgae extracts using two-cell models, and the need for further research and development of microalgae-based functional food products, this work contributes to the broader goal of expanding the range of approved microalgae species for food use in the EU. Furthermore, it leverages their chemical and bioactive properties for human health and disease prevention. Ultimately, this study serves as an initial step toward bridging the gap between scientific inquiry and regulatory approval, paving the way for commercialization of innovative microalgae-based products with promising health benefits.

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CRediT authorship contribution statement

Vera Castro: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Conceptualization. **Ana Teixeira:** Writing – review & editing, Investigation, Formal analysis. **Luara Simões:** Writing – review & editing, Investigation, Formal analysis. **Franklin Chamorro:** Validation, Investigation, Formal analysis. **Catarina Lourenço-Lopes:** Validation, Investigation, Formal analysis. **Celina Parreira:** Writing – review & editing, Resources, Project administration. **Sara M. Badenes:** Writing – review & editing, Resources, Project administration. **Luís Costa:** Writing – review & editing, Resources, Project administration. **Miguel A. Prieto:** Validation, Resources, Methodology. **Rui Oliveira:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization. **Alberto C.P. Dias:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.142554.

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