

Cytoprotective Effects of Microalgal Extracts on H₂O₂-Induced Oxidative Stress in Human Lung Adenocarcinoma A549 Cell Line

Helen Herbert

helen.herbert@research.atu.ie

Atlantic Technological University

Nicolas Touzet

Atlantic Technological University

Eva Campion

Atlantic Technological University

Research Article

Keywords: Microalgae, A549 cell line, antioxidants, recovery, oxidative stress, gene expression

Posted Date: May 31st, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4423050/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

The body's innate defences often fall short in combating oxidative stress in cells, highlighting the importance of integrating antioxidants from natural sources for preventative healthcare and nutrition. Microalgae-derived compounds like carotenoids and EPA-rich fatty acids play a critical role in protecting cells from oxidative stress. This study evaluated the antioxidant and cytoprotective properties of diethyl ether (Et₂O) extracts prepared from the biomass of the microalgae *Cyanophora paradoxa*, *Tetraselmis chuii* and *Phaeodactylum tricornutum*, which were supplemented to human lung cancer cells (A549) experiencing oxidative stress.

The extracts of *T. chuii* and *C. paradoxa* exhibited higher antioxidant content (123 and 150 µmol Trolox equivalent mg/Recovered Dried Material (RDM)) as compared to *P. tricornutum* when analysed using the FC assay. All extracts (150 µg mL⁻¹) demonstrated protective effects by attenuating H₂O₂-induced oxidative stress in A549 cells, as confirmed by the MTS (≤ 105% increase in cell growth) and DCF-DA assays (≤ 58% decrease in ROS production). RT-qPCR analysis revealed significant changes in the expression of apoptosis regulatory genes, with a decrease in the pro-apoptotic BAX gene (< 50%) and an increase in the anti-apoptotic Bcl-2 gene expression (> 200%), when cells were treated with extracts compared to cells treated with medium only after exposure to H₂O₂.

These findings suggest that the microalgal extracts successfully mitigated the H₂O₂-induced oxidative stress in the A459 cells, likely attributable to their inherent antioxidative and apoptosis regulating properties. These attributes, which appear to restore homeostasis, present promising avenues for use in cosmetics and therapeutic contexts.

Introduction

The overproduction of free radicals and reactive oxygen species (ROS) during cellular respiration can induce oxidative stress, potentially resulting in cellular or tissue damage (Snezhkina et al. 2019). Oxidative stress is associated with several diseases in humans (e.g. cancer, diabetes, hypertension and neurodegenerative diseases), underscoring the critical need to maintain cellular redox balance for continuous homeostasis (Snezhkina et al. 2019). Endogenous and exogenous antioxidants play a crucial role in counteracting the deleterious effects of free radicals in cells (Janciauskiene 2020). However, some synthetic exogenous antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are primarily used to maintain the nutritional quality of processed foods, may pose potential health risks, including toxicity at elevated doses (Bouayed and Bohn 2010). Consequently, there has been considerable scientific interest in identifying safe and natural exogenous antioxidant alternatives (Wang et al. 2023).

For some time, terrestrial plants have been recognised as sources rich in antioxidants such as carotenoids, phenols and vitamins (Ferdous and Yusof 2021; Wang et al. 2023). More recently, microalgae have emerged as potent and sustainable alternative sources with the capacity to produce

diverse bioactive metabolites (Zhong and Shahidi 2015; Wang et al. 2023). Several microalgal species such as *Chlorella* sp., *Arthrospira* sp. or *Dunaliella* sp. are currently cultivated commercially for the refinement of their biomass and the extraction of valuable components like carotenoids, fatty acids and proteins, which are used within the nutrition, cosmetics and health industries (Khavari et al. 2021). These compounds have also shown the potential to reduce oxidative stress, to enhance immune regulation and to suppress inflammatory responses in human cells (Bhalamurugan et al. 2018).

Certain microalgae are rich in carotenoids such as lutein, zeaxanthin, astaxanthin, peridinin or β -carotene, which have all been recognised for their antioxidant properties (Gong and Bassi 2016). Beyond these, microalgae can also produce value-added metabolites such as long-chain polyunsaturated fatty acids (PUFAs), which are also known for their health benefits (Kumar et al. 2019). The omega-3 fatty acids Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA), in particular, are produced in significant amounts by some microalgae species and constitute important PUFAs with documented anti-inflammatory attributes (Kumar et al. 2019). These properties can help promote healing by reducing swelling and pain in damaged cells and tissues (Calder 2017; Singh et al. 2019). However, the exact mechanisms underlying the protective functions and specific molecular targets of carotenoids and fatty acids *in vivo* and *in vitro* are still poorly understood (Fiedor and Burda 2014).

Several studies have investigated the effects of microalgal extracts on gene expression related to inflammation, oxidative stress and cell proliferation in mammalian cells (Nigjeh et al. 2013; Robertson et al. 2015; Suh et al. 2017). For instance, the protective potential of microalgal extracts has been investigated using *Tetraselmis suecicia* (Kylin) Butcher in a study that examined gene expression in human lung cancer cells (A549) (Sansone et al. 2017). Targeting gene expression is imperative for elucidating the role microalgal compounds may exert in cellular defence against oxidative stress, providing insights into apoptosis regulation, tumour suppression, copper homeostasis or molecular chaperone functions. This knowledge has potential applications in the development of protective strategies against oxidative damage across a range of disorders, highlighting the therapeutic and health-supporting potential of microalgae-derived compounds in combating oxidative stress (Sablina et al. 2005; Itoh et al. 2008; Sinthupibulyakit et al. 2010; Zuehlke et al. 2015; Alam et al. 2022).

Considering the diverse biochemical composition of microalgae, they hold significant prospects as sources of natural antioxidants (Ferdous and Yusof 2021). As such, this study aimed to evaluate the antioxidant capacity as well as pigment and fatty acid signatures of three microalgae species from distinct lineages, the Glaucophyte *Cyanophora paradoxa* Korshikov CCAP 981/1, the Chlorophyte *Tetraselmis chuii* Butcher CCAP 66/21B and the Diatom *Phaeodactylum tricornutum* Bohlin CCAP 1052/1. The extracts from these microalgae were then evaluated for their ability to reduce oxidative stress induced by hydrogen peroxide (H_2O_2) in human lung carcinoma (A549) cells.

Materials and methods

Microalgae Cultivation Setup

The marine chlorophyte *Tetraselmis chuii* Butcher CCAP 66/21B and diatom *Phaeodactylum tricornutum* Bohlin CCAP 1052/1 were cultured in 5 L glass flasks, each containing 4 L of filtered-sterilised seawater. The seawater was enriched with Guillard f/2 medium (Guillard 1975; Mc Gee et al. 2018) and supplemented, only for *P. tricornutum* Bohlin, with silicate (30 mg L^{-1}). The freshwater glaucophyte *Cyanophora paradoxa* Korshikov CCAP 981/1 was grown in a 5 L glass bioreactor filled with 4 L of sterile Bold Basal Medium and Vitamins (BBM) (Bold 1949; Mc Gee et al. 2018). The cultures were maintained at a temperature of 20°C under a 14:10 light/dark cycle, with an illumination of $80 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided by cool white fluorescent LEDs. Upon reaching the end of the exponential growth phase (30 days), the biomass was harvested by centrifugation ($2000\times g$, 3 min) using an Eppendorf Centrifuge 5702. Desalting of the marine microalgal biomass was performed using 0.5 M ammonium formate. Subsequently, all biomass samples were freeze-dried and stored at -20°C until required for further processing.

Preparation of Microalgal Extract for Bioactivity Analysis

The microalgal extracts were prepared by extracting 100 mg of freeze-dried biomass in 30 mL of diethyl ether (Et_2O) (Mc Gee et al. 2020). Samples were stored at -6°C for 24 h before centrifugation ($2000\times g$, for 5 min) using an Eppendorf Centrifuge 5702. The supernatant was recovered and passed through a $0.22 \mu\text{m}$ PTFE syringe filter (Fisher Scientific). The supernatants of the extracts obtained were then placed in pre-weighed vials and evaporated to dryness. The dried extracts were resuspended in $100 \mu\text{L}$ of dimethyl sulfoxide (DMSO) and stored at -20°C until needed. On the day of analysis, fresh working stocks were prepared using cell culture medium to achieve a DMSO concentration below 1%.

HPLC-UV analysis of Pigment Content

Pigments in the reconstituted extracts were separated at a constant room temperature on a Shimadzu Nexera LC-20AD system coupled with a Prominence Diode Array Detector SPD-M20A. The extracts were injected in a $20 \mu\text{L}$ loop and separated using a Phenomenex Onyx C18 $100 \times 4.6\text{mm}$ ID monolithic column. The gradient applied was that of Mc Gee et al. (2018), which made use of a stepped gradient solvent program involving a 10% B initial isocratic run for 0.10 min, a linear gradient to 65% B from 0.10–2.00 min, an isocratic hold at 65% B for 2 min, a linear gradient to 90% B for 1 min followed by a hold at 90% B for 1 min and a final re-equilibration step to the initial conditions from 6.01–7.50 min. The mobile phase was prepared using two solvent mixtures: A, consisting of 80:20 (v/v) methanol/0.5M ammonium acetate, and B, including 70:30 (v/v) acetone/acetonitrile. Pigment identification was attained by the comparison between the retention times and peak absorption spectrum of standards for zeaxanthin, chlorophyll *a*, chlorophyll *b*, β -carotene, lutein and fucoxanthin standards (Sigma and DHI).

Gas chromatography analysis (GCMS) of Fatty Acid content

FAME analysis of the reconstituted extracts was carried out by gas chromatography (GC) (Agilent Technologies 7890A GC System) coupled to mass spectrometry (MS) (Agilent Technologies 5975C inert Mass Selective Detector with Triple-Axis Detector) equipped with a DB-WAX 30 m column (0.25 mm internal diameter). Injections of extracts derivatised using TMSH were carried out in a splitless mode.

The program was set up with an inlet temperature of 250°C and helium carrier gas at 20.9 psi. The oven gradient temperature was as follows: an initial hold at 50°C for 1 min followed by a 5°C/min ramp to 200°C and finally a 3°C/min ramp to 230°C for 18 min with a run time of 59 min. The mass spectrometry (MS) solvent delay was set to 5 min. Fatty acids were identified by comparing retention times to the Supelco® 37 Component FAME standards Mix and cross-referenced to the NIST MS Search 2.0 instrument's internal library.

Antioxidant Capacity of the Microalgal Extracts

The antioxidant activity of the microalgal extracts at a concentration of 0.25 mg mL⁻¹ was determined using the Folin-Ciocalteu (FC) and Trolox Equivalent Antioxidant Capacity (TEAC) assays as previously described in Barone et al. (2021).

Determination of the Total Phenolic Content - Folin-Ciocalteu (FC) assay

The FC working solution (1:10 dilution) was generated by adding 1.5 mL of Folin-Ciocalteu reagent to 13.5 mL of ultrapure water. Sodium bicarbonate (Na₂CO₃) was prepared at a concentration of 37.5 g L⁻¹ in ultrapure water. Trolox, a water-soluble vitamin E analogue was used at a stock concentration of 1500 µM to generate a 10-point standard calibration curve in the range 0–30 µM. To determine the total phenolic content, 500 µL FC working stock and 400 µL Na₂CO₃ were added to 100 µL of microalgal extract or Trolox standard, and the absorbance was measured at 760 nm.

Evaluation of the antioxidant capacity - Trolox equivalent antioxidant capacity (TEAC) assay

For the TEAC assay, a radical cation solution was prepared using 7 mM of ABTS combined with 2.45 mM potassium persulfate. The ABTS + solution was diluted with ethanol (50%) to reach an absorbance of 0.700 ± 0.050 at 734 nm. For the analysis, 100 µL of the microalgal extract was mixed with 900 µL of diluted ABTS + solution and the samples were incubated for 15 min at room temperature. The absorbance of samples, standards and controls was immediately recorded at 734 nm.

Preparation of Human A549 Cells

The human lung adenocarcinoma cell line A549 (Sigma-Aldrich, UK) was grown in Ham's F-12 Nutrient Mix (BioSciences) supplemented with 10% fetal bovine serum (FBS) (Scientific Laboratory Supplies) and 1% Penicillin-Streptomycin (Pen-Strep) (Cytiva) in a 5% CO₂ atmosphere at 37°C based on Panja et al. (2016). Exponential phase cells were sub-cultured every two days, ensuring the use of actively proliferating cells throughout the experiments. Cell viability was determined using 0.4% trypan blue exclusion as described in Cooper et al. (2016).

Cell Viability Analysis – MTS assay

The effect of H₂O₂ and the extracts on cell viability was determined using the tetrazolium compound-based CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay (Promega) (Herbert et al. 2024). Initially, A549 lung cells (2×10⁴ cells mL⁻¹) seeded in 96-well plates were treated with H₂O₂ (0.1 mM – 0.8 mM) for 24 h to induce oxidative stress, based on Yilmazer (2018). Prior to the microalgal extract treatment, A549 cells underwent a 24 h stress induction with H₂O₂ (0.5 mM). Subsequently, the H₂O₂ was removed, and cells were washed with PBS and treated with 200 µL of freshly prepared extract (150, 100 and 50 µg mL⁻¹) or medium only. DMSO (< 1%) treated cells and non-treated cells were included as vehicle and negative controls, respectively. Doxorubicin (5 µg mL⁻¹) was used as a positive control for cytotoxicity. Following treatment, an MTS cytotoxicity assay was performed in accordance with the manufacturer's instructions. Briefly, the MTS reagent was added to each well and cells were incubated at 37°C for 3 h before measuring absorbance at 490 nm using a FLUOstar OPTIMA microplate reader. The absorbance recorded was analysed and the results were expressed as a percentage of cell viability of the extract-treated cells (at various concentrations) compared to the untreated control cells (without microalgal extract). Values were expressed as mean ± SD from three separate experiments, each performed in triplicate and samples were normalised using the vehicle control. The percentage of viable cells was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{Abs}(\text{sample}) - \text{Abs}(\text{background})}{\text{Abs}(\text{control}) - \text{Abs}(\text{background})} \times 100$$

Detection of Intracellular Levels of Reactive Oxygen Species (ROS)

The DCF-DA assay was used to evaluate the capacity of microalgal extracts to generate intracellular levels of reactive oxygen species (ROS) in the A549 lung cell line. The assay is based on the conversion rate of 2', 7'-dichloro-dihydro-fluorescein diacetate (H₂DCF-DA) into a fluorescent form 2', 7'-dichlorofluorescein (DCF), as previously described in Nizioł-Lukaszewska et al. (2018) and Bujak et al. (2021). Cells were seeded in 96-well plates at a density of 5×10⁴ cells mL⁻¹ and initially cultured for 24 h. Following this, 100 µL of H₂DCF-DA (Cayman, VWR) at a concentration of 20 µM in serum-free medium (Ham's F12) was added to the wells and the cells were incubated for 45 min at 37°C. The H₂DCF-DA solution was then carefully removed and cells were washed twice with PBS before treatment with the stressor, hydrogen peroxide (H₂O₂), at a concentration of 0.5 mM for 24 h. After this incubation, cells were washed twice with PBS followed by the addition of varying concentrations (150, 100 and 50 µg mL⁻¹) of microalgal extracts for 24 and 48 h treatment periods. Once the incubation period was completed, the magnitude of fluorescence was measured using fluorescence spectroscopy with a microplate reader (FLUOstar OPTIMA) (excitation and emission wavelength 490/520nm, respectively). Values were expressed as mean ± SD of three separate experiments. Each experiment was performed in triplicate. The results were calculated as a percentage of untreated control against the extract-treated cells and

were normalised with the vehicle control. The formula used for the percentage of reactive oxygen species (ROS) is as follows:

$$\% \text{Reactive Oxygen Species} = \frac{\text{Fluo}(\text{sample}) - \text{Fluo}(\text{background})}{\text{Fluo}(\text{control}) - \text{Fluo}(\text{background})} \times 100$$

RNA extraction using Trizol reagent

A549 lung cells (3×10^5 cells mL^{-1}) were seeded in 6-well plates and incubated overnight before treatment with 0.5 mM H_2O_2 for 24 h to induce oxidative stress based on the method of Sansone et al. (2017). Subsequently, cells were treated with Et_2O extracts at a range of concentrations (150, 100 and 50 $\mu\text{g mL}^{-1}$) for 24 and 48 h. After incubation, RNA was extracted using 400 μL Trizol reagent (Invitrogen ThermoFisher) per 1×10^7 cultured cells. This was followed by a 5 min room temperature incubation to permit complete dissociation of the nucleoprotein complex. Then 80 μL of chloroform was added to facilitate phase separation. The sample was then shaken gently for 15 s to create an emulsion and incubated at room temperature for 3 min. Followed by centrifugation at 12,000x g at 4°C for 15 min (Hettich Mikro 200R). During centrifugation, three distinct layers were formed: a lower red phenol-chloroform layer, an interphase and an upper colourless aqueous phase. The RNA-containing aqueous phase was transferred to a fresh tube and RNA precipitation was achieved with the addition of 200 μL of ice-cold isopropanol, followed by a 10 min incubation at room temperature. The tubes were then centrifuged at 12,000x g at 4°C for 10 min. The RNA precipitate was seen as a white gel-like pellet and the supernatant was carefully discarded. The RNA pellet was washed with 400 μL of 75% ethanol. The sample was vortexed and then centrifuged at 7500x g at 4°C for 5 min. After discarding the supernatant, the RNA pellet was air-dried for 5–10 min and then re-suspended in 20 μL of RNase-free water. This was followed by heating at 60°C and gentle mixing to ensure homogeneity before aliquoting. RNA was quantified using microvolume spectrophotometry (DeNovix DS-11). The purity of RNA was assessed using the 260 nm/280nm ratio, which was consistently within the range of 1.8-2.0, indicative of high purity. Following purification, the RNA samples were stored at -80°C until required for analysis.

Reverse Transcription – Qualitative PCR analyses (RT-qPCR)

cDNA was prepared using 200 ng of each RNA sample using the QuantiTech® Reverse Transcription Kit (Qiagen) and following the manufacturing instructions. Subsequently, qPCR for amplification of cDNA was performed in a 10 μL reaction volume using the QuantiNova™ SYBR® Green PCR kit (Qiagen, Cat. No. 208052) on the StepOnePlus Real-Time PCR System (Applied Biosystem) and based on the protocol of Sansone et al. (2017). The amplification program applied included an initial heat activation step at 95°C for 10 min followed by 35 cycles at 95°C for 15 s (denaturation) and 60°C for 60 s (annealing/extension). Melting curves were performed to verify the specificity of the PCR assays (Table 1). Results were normalised to the endogenous control gene glyceraldehyde 3-phosphate dehydrogenase GADPH (F: 5'-GTCTCCTCTGACTTCAACAGCG-3' and R: 5'-ACCACCCTGTTGCTGTAGCCAA-3') (Zhang et al.

2015). The relative gene expression level was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Cells treated with < 1% DMSO were used as a vehicle reference.

Statistical Analysis

Analysis of variance (ANOVA) was performed to identify significant differences between the antioxidant activity of microalgae extracts, cell viability, ROS production and gene expression in human lung cancer cells. The data matrix underwent principal component analysis (PCA) to evaluate the relationships between the various bioactivity variables measured for the three microalgal species. All statistical analyses were performed using the IBM SPSS Statistics 26 package.

Results

Pigment Profiling of Microalgal Extracts by High-Performance Liquid Chromatography (HPLC)

Pigment analysis of the extracts obtained from *C. paradoxa* Korshikov CCAP 981/1, *T. chuii* Butcher CCAP 66/21B and *P. tricornutum* Bohlin CCAP 1052/1 strains were performed using HPLC-UV. The profiles generated demonstrated the presence of chlorophylls, xanthophylls and carotenes (Fig. 1, Table 2). Specifically, β -carotene was present in all three species while fucoxanthin were only identified in *P. tricornutum* Bohlin. Lutein and chlorophyll *b* were found in *T. chuii* Butcher with zeaxanthin only being detected in *C. paradoxa* Korshikov.

Table 1 Primer sequences used for Reverse Transcription Qualitative PCR

Pathway/Response	Gene Name and Accession number	Primer Sequence (5'- 3')
Apoptosis	P53 (NM_000546) ^{ac}	Forward 5'-CCTCAGCATCTTATCCGAGTGG-3' Reverse 5'-TGGATGGTGGTACAGTCAGAGC-3'
Apoptosis	BAX (NM_004324) ^{ac}	Forward 5'-TCAGGATGCGTCCACCAAGAAG-3' Reverse 5'-TGTGTCCACGGCGGCAATCATC-3'
Apoptosis	Bcl-2 (NM_000633) ^{ac}	Forward 5'-ATCGCCCTGTGGATGACTGAGT-3' Reverse 5'-GCCAGGAGAAATCAAACAGAGGC-3'
Apoptosis	Caspase 3 (NM_004346) ^c	Forward 5'-GGAAGCGAATCAATGGACTCTGG-3' Reverse 5'-GCATCGACATCTGTACCAGACC-3'
Cellular stress response	HSP90AA1 (NM_005348) ^d	Forward 5'-TCTGCCTCTGGTGATGAGATGG-3' Reverse 5'-CGTTCCACAAAGGCTGAGTTAGC-3'
Antioxidant response	ATOX1 (NM_004045) ^b	Forward 5'-TCTGAGCACAGCATGGACACTC-3' Reverse 5'-TCTGGAAGCCAGCGGGAGGAT-3'
Reference gene	GAPDH (NM_002046) ^e	Forward 5'-GTCTCCTCTGACTTCAACAGCG-3' Reverse

Reference ^a Yuce et al. 2023, ^b Tsybal et al. 2023, ^c Gao et al. 2020, ^d Zhou et al. 2023, ^e Jin et al. 2023.

Table 2 HPLC pigment peak identification present in extracts derived from *Tetraselmis chuii* Butcher CCAP 66/21B, *Cyanophora paradoxa* Korshikov CCAP 981/1 and *Phaeodactylum tricornutum* Bohlin CCAP 1052/1

RT (min)	Pigment	Abbreviation	$\lambda_{\max}$ nm	Published $\lambda_{\max}$ nm
1.416	Fucoxanthin (s)	Fuco	452	451 ^b
2.448	Lutein (s)	Lut	425,448,475	425, 448,475 ^a
2.412	Zeaxanthin (s)	Zea	422, 454, 479	424,452,478 ^a
3.129	Chlorophyll <i>b</i> (s)	Chl <i>b</i>	462,599,646	463,599,648 ^a
5.482	β -carotene (s)	β -car	455,480	454,479 ^b
(s) Identified using a standard.				
Reference ^a Mc Gee et al. 2018, ^b Egeland et al. 2011				

Fatty Acid Profiling of Microalgal Extracts by Gas Chromatography Analysis (GCMS)

The analysis of fatty acids in the extracts of strains *C. paradoxa* Korshikov, *T. chuii* Butcher and *P. tricornutum* Bohlin using GC/MS revealed a composition mostly containing C16:0, C18:0, C20:4 and C20:5 (Fig. 2). Variations in the FAME proportions among the extracts were observed with C16:0 being the predominant fatty acid, accounting for 46% – 68% overall. There were also differences in the proportions of C18:0 between species, with proportions ranging from 22–32%. C20:4 (7.6%) was found exclusively in *C. paradoxa* Korshikov and extracts from both *C. paradoxa* Korshikov and *P. tricornutum* Bohlin also contained EPA (C20:5) (14% and 31.3%, respectively).

Antioxidant Capacity of Microalgal Extracts

To evaluate the antioxidant capacity of the microalgal extracts, both the FC and TEAC assays were undertaken. Figure 3 outlines the antioxidant activities observed for the extracts of *T. chuii* Butcher CCAP 66/21B, *C. paradoxa* Korshikov CCAP 981/1 and *P. tricornutum* Bohlin CCAP 1052/1. The FC assay revealed a significantly higher antioxidant potential in the *T. chuii* Butcher extract (212 μmol Trolox eq mg/ Recovered Dried Material (RDM)) compared to the *C. paradoxa* Korshikov and *P. tricornutum* Bohlin Et₂O extracts (135 and 119 μmol Trolox eq mg/RDM) (ANOVA, $n = 9$, $p < 0.001$). The antioxidant activity

observed for the TEAC assay was significantly lower in the *P. tricornutum* Bohlin extract (44 μmol Trolox equivalent mg/RDM) compared to those of *T. chuii* Butcher and *C. paradoxa* Korshikov (95 and 117 μmol Trolox eq/mg RDM, respectively) (ANOVA, $n = 9$, $p < 0.001$).

Cell Recovery and Viability of Human A549 Cells using Microalgal Extracts

The cytoprotective effect of microalgal extracts at a cellular level was measured by the MTS assay. Initially, A549 lung cells were treated with hydrogen peroxide (H_2O_2) at a range of concentrations (0.1, 0.3, 0.5, 0.6, 0.8 mM) to induce oxidative stress. From this analysis, a treatment concentration of 0.5 mM H_2O_2 was then selected for these experiments. The concentration resulted in 64% viability (Fig. 4) and thus allowed the study of the effects of moderate oxidative stress on cells. Treatment of the stressed cells with solely medium led to a non-significant increase in cell viability (63 to $\leq 74\%$). However, the addition of all the microalgal extracts induced a significant proliferation of A549 lung cells, with cell viability increasing at 24 and 48 h in a dose-response manner ($150 \mu\text{g mL}^{-1}$ and $100 \mu\text{g mL}^{-1}$) compared to cells treated with medium only. Specifically, the *T. chuii* Butcher CCAP 66/21B extract ($150 \mu\text{g mL}^{-1}$) significantly increased cell viability after 24 and 48 h (63 to 129% and 71 to 146%), respectively (ANOVA, $n = 24$, $p < 0.001$). Similarly, treatment with *C. paradoxa* Korshikov CCAP 981/1 extract ($150 \mu\text{g mL}^{-1}$) showed significant increases in cell viability (72 to 132% and 73 to 135% after 24 and 48 h, respectively) (ANOVA, $n = 24$, $p < 0.001$). Treatment with the *P. tricornutum* Bohlin CCAP 1052/1 extract increased cell recovery after 24 and 48 h treatment (63 to 111% and 74 to 127%, respectively) (ANOVA, $n = 24$, $p < 0.001$). Additionally, treatment with both *C. paradoxa* Korshikov CCAP 981/1 and *P. tricornutum* Bohlin CCAP 1052/1 extracts ($100 \mu\text{g mL}^{-1}$) demonstrated increases in cell viability after 48 h treatment (74 to 98% and 74 to 115%, respectively).

Impact of Microalgal Extracts on Reactive Oxygen Species Levels

The impact of microalgal-derived extracts on the modulation of intracellular ROS generation in A549 lung cells following H_2O_2 treatment was measured by the DCF-DA assay. As shown in Fig. 5, H_2O_2 -treated cells (0.5 mM, 24 h) showed a significant reduction in ROS production upon the application of the higher concentration of extract ($150 \mu\text{g mL}^{-1}$) compared to cells treated with medium only (24 h) (ANOVA, $n = 60$, $p < 0.001$). However, no notable decrease in ROS levels was observed following treatment with the extract at the lower concentration ($50 \mu\text{g mL}^{-1}$) when compared to control cells or following 48 h treatment. The results indicate that *T. chuii* Butcher, *C. paradoxa* Korshikov and *P. tricornutum* Bohlin extracts used at a concentration of $150 \mu\text{g mL}^{-1}$ may be effective in diminishing intracellular ROS generation within 24 h of treatment.

Impact of Microalgal Extracts on Gene Expression

Gene expression analyses focusing on genes related to apoptosis regulation, cellular stress response and antioxidant mechanisms were investigated using RT-qPCR (Fig. 6). Following pre-treatment with 0.5 mM H₂O₂ to induce oxidative stress, treatment for 48 h with microalgal extracts from *T. chuii* Butcher, *C. paradoxa* Korshikov and *P. tricornutum* Bohlin led to a significant decrease in the BAX gene expression (47%, 41% and 49%, respectively) and an increase in Bcl-2 gene expression (191%, 236% and 206%, respectively) compared to cells subjected to oxidative stress and treated with medium only (t-test, $p < 0.03$). In addition, treatment with the extract from *T. chuii* Butcher significantly upregulated P53 expression (63%) after 24 h (t-test, $p < 0.023$) compared to cells treated with medium only. Furthermore, a notable difference in the expression of Antioxidant-1 (ATOX1) was observed after 24 h, with an upregulation in cells treated with medium only (t-test, $p = 0.002$) and a downregulation following treatment with extracts derived from *C. paradoxa* Korshikov, *T. chuii* Butcher and *P. tricornutum* Bohlin (39%, 37% and 40%, respectively) (t-test, $p < 0.003$). All extracts from *T. chuii* Butcher (t-test, $p < 0.001$), *C. paradoxa* Korshikov (t-test, $p < 0.019$), and *P. tricornutum* Bohlin (t-test, $p < 0.001$) significantly decreased expression of HSP90AA1 (55%, 52%, 59%, respectively) when compared to the medium only treatment after 48 h.

Principal Component Analysis (PCA)

A principal component analysis (PCA) was carried out to examine patterns of bioactivity in the microalgal extracts obtained from *T. chuii* Butcher, *C. paradoxa* Korshikov and *P. tricornutum* Bohlin. The PCA revealed two components accounting for 100% of the variance (Fig. 7). Component 1 (CP1) was positively related to bioactive variables such as antioxidant activity, cell viability and gene expression in A549 cells subjected to H₂O₂ stress and treated with *C. paradox* Korshikov extracts at a concentration of 150 µg mL⁻¹ for 24 h. In addition, the ROS activity of extract (150 µg mL⁻¹) in A549 was closely associated with the negative domain of CP1 in relation to *T. chuii* Butcher.

The extracts from the three microalgae in this study were projected in different locations of the PCA plane. In particular, *P. tricornutum* Bohlin was firmly associated with the negative axes of CP1 while *T. chuii* Butcher and *C. paradoxa* Korshikov were instead located along the positive domain of CP1 and CP2, respectively.

Discussion

Microalgae hold significant potential as sources of high-value metabolites that can be exploited across several sectors (Sathasivam and Ki 2018). These organisms can produce a range of health-promoting metabolites, which can positively influence cell homeostasis by preventing oxidative stress and subsequent cellular necrosis or apoptosis (Sathasivam and Ki 2018). As such, the present study focused on evaluating the effects of extracts from three microalgal species on human lung cancer cells subjected to H₂O₂ induced stress.

Antioxidant Potential of the Microalgal Extracts

The microalgal extracts examined here contained important carotenoids such as β -carotene, lutein and fucoxanthin, which are widely recognised for their antioxidant properties in the nutraceutical and pharmaceutical industries (Gammone et al. 2015). The current study revealed that extracts from *T. chuii* Butcher and *C. paradoxa* Korshikov demonstrated superior antioxidant capabilities compared to those from *P. tricornutum* Bohlin. Notably, lutein, which is found in extracts derived from *T. chuii* Butcher, is recognised for its beneficial health effects due to its antioxidative properties (Becerra et al. 2020). Studies have demonstrated that lutein offers a cytoprotective effect in the human colon adenocarcinoma cell line HT-29 against deoxynivalenol-induced oxidative stress, while also preventing apoptosis (Krishnaswamy et al. 2010). Similarly, fucoxanthin, which was present in the *P. tricornutum* Bohlin extracts, has been found to protect against H_2O_2 -induced oxidative damage in hepatocellular carcinoma (L02) cells (Wang et al. 2018). Research has highlighted that the antioxidant capacity and effectiveness of these substances extend beyond their concentration and are also determined by factors such as bioavailability, compound interactions and the body's absorption and distribution processes, as well as the specific conditions of oxidative stress (Neto et al. 2017).

Cytoprotection of Microalgal extracts

H_2O_2 is known to induce damage to lipids, proteins and nucleic acids by generating reactive oxygen species and is often used in *in vitro* models to induce cellular-level oxidative stress (Gille and Joenje 1992; Upadhyay et al. 2018; Peng et al. 2020). Here, the extracts of *T. chuii* Butcher, *C. paradoxa* Korshikov and *P. tricornutum* Bohlin exhibited a protective effect against oxidative stress in human cancer cells by enhancing cell proliferation and reducing ROS levels. This protective effect, e.g. antioxidant activity, is likely the result of synergistic effects among various bioactive compounds present in the microalgal extracts (Barkia et al. 2019, Pagels et al. 2022). Similarly, research on the green microalga *Hindakia tetrachotoma* ME03 (Printz) C. Bock, Pröschold and Krienitz has shown a significant reduction in both ROS production and in the incidence of apoptotic and necrotic pathways in MCF-7 cells (Bulut et al. 2023). These findings further indicate that microalgae species from several distinct lineages do possess potent antioxidant-associated cytoprotective potential.

Modulation of Gene Expression by Microalgal Extracts

To characterise putative cytoprotective mechanisms of the microalgal extracts at a molecular level, genes related to apoptosis, antioxidant and cellular stress response were targeted. The findings revealed that the extracts led to a reduction in BAX expression while simultaneously elevating Bcl-2 expression. This alteration may indicate a biological shift towards suppressing apoptotic processes and enhancing cellular survival, serving as a protective response against oxidative stress (Qian 2022, Fulda 2010). This study also did not reveal any significant increase in the expression of the caspase 3 gene. This observation aligns with prior research indicating that heightened levels of Bcl-2 expression can inhibit caspase enzymes 3, 6, 7 and 9 (Qian 2022). These caspases play crucial roles in the execution phase of apoptosis, so this activity could thereby extend the lifespan of cells by inhibiting apoptosis (Boice 2020). Treatment with *T. chuii* Butcher extract upregulated the expression of the tumor suppressor gene P53

after 24 h. This increase in P53 expression may indicate a potential activation of DNA repair mechanisms, providing additional protection against oxidative damage (Liu et al. 2020).

Furthermore, a significant decrease in heat shock protein (HSP90AA1) gene expression was observed after 48 h of treatment with the microalgal extracts. Considering that HSP90AA1 is a chaperone protein crucial for protein folding and stress response (Xu et al. 2023), this reduction in expression may suggest a potential attenuation of cellular stress, implying a reduced reliance on this chaperone system in response to decreased oxidative conditions. HSP90AA1 has been shown to protect against various stresses (including oxidative stress) and possesses dual roles in apoptosis regulation, exhibiting both proapoptotic and antiapoptotic effects (Fulda et al. 2010, Kennedy et al. 2014). Its reduced expression has been correlated with enhanced survival outcomes in patients with lung cancer (Gallegos Ruiz et al. 2008). Previous research has highlighted the complex role of HSP90AA1 in cellular stress responses. Specifically, the overexpression of the HSP90AA1 gene was found to increase the rate of apoptosis in the U937 cell line following exposure to apoptosis-inducing agents such as tumor necrosis factor (TNF) and cycloheximide (Galea-Lauri et al. 1996; Garrido et al. 2001). This may help explain the upregulation of the HSP90AA1 gene seen here following treatment with medium only, a seemingly pro-apoptotic response, triggering cell death following stress stimuli. This increase in HSP90AA1 during oxidative stress may underline the cell's attempt to mitigate damage and underscore the dual activity of this protein. In scenarios where stress is sustained or damage is irreparable, this elevation in expression may pivot towards apoptotic pathways, acting as a safeguard to prevent further damage and to maintain cellular homeostasis (Peng et al. 2022).

Another noteworthy finding was the downregulation of the Antioxidant 1 (ATOX1) gene after treatment with microalgal extracts, despite its known protective roles as an antioxidant and as a copper chaperone (Kim et al. 2018). This observation underscores the complex and multifaceted nature of cellular defence against oxidative stress. The observed downregulation in response to microalgal extract treatment may suggest the activation of alternative antioxidant pathways or mechanisms of cytoprotection that circumvent the need for an ATOX-1-mediated response. Indeed, research has shown that cells possess multiple antioxidant defence systems beyond ATOX1, such as superoxide dismutase, catalase, glutathione peroxidase and various non-enzymatic antioxidants like vitamin C and E (Chaudhary et al. 2023). Contrasting findings using the green microalga *Tetraselmis suecica* (Kylin) Butcher revealed that ethanol/water extracts could reduce oxidative stress by upregulating the ATOX1 gene in A549 cells pretreated with H₂O₂ (30 mM) and subsequently exposed to various concentrations of extracts (100, 200 and 400 µg mL⁻¹) (Sansone et al. 2017). It is likely that the specific chemical makeup of *Tetraselmis suecica* (Kylin) Butcher extracts may have activated cellular pathways that led to ATOX1 upregulation, a response that might not be triggered with extracts derived from other species, such as the Et₂O extracts obtained in this study. Here, an upregulation of the ATOX1 gene was observed in cells treated with medium only, indicating a response to oxidative stress and an attempt to mount a defence to potential cellular damage. The microalgal extracts modulated the antioxidant response differently as compared to the response seen following treatment with medium only, having significantly influenced gene

expression profiles related to apoptosis, stress response and antioxidant mechanisms. This includes the modulation of both pro-apoptotic and anti-apoptotic as well as tumour suppressor genes, indicating their potential as protective agents against oxidative stress at the molecular level.

4.6 Conclusion

Extracts of the microalgae, *Cyanophora paradoxa* Korshikov CCAP 981/1, *Tetraselmis chuii* Butcher CCAP 66/21B and *Phaeodactylum tricornutum* Bohlin CCAP 1052/1 demonstrated significant cytoprotective effects in human cancer cell line (A549) by mitigating H₂O₂-induced oxidative stress. In particular, the apoptotic genes BAX and Bcl-2 exhibited significant downregulation and upregulation, respectively, highlighting potential pathways towards the elucidation of the molecular mechanisms underlying the cytoprotective abilities of these extracts. The observed reduction in oxidative stress in A459 cells following treatment with the microalgal extracts constitutes a promising avenue for exploitation in cosmetic or therapeutic contexts for mitigating cellular damage.

Declarations

Acknowledgements

The authors wish to express their sincere gratitude and thanks to the Department of Science, ATU Sligo, especially Siobhan O'Connor, Róisín Atcheson and Una McCarty for their support.

Author's Contributions:

H.H. wrote the original draft. H.H., E.C. and N.T. established the concept and developed the experimental design. H.H. conducted all experiments, and analyses. E.C. provided support with PCR analysis. E.C. and N.T. supervised, reviewed and edited the manuscript. All authors reviewed the final manuscript.

Ethical Approval

Not Applicable

Funding

The Authors would like to acknowledge the financial support provided by ATU Sligo President's Bursary Fund.

Availability of data and materials

Data and materials will be made available on request.

Competing Interests

The authors declare that they have no conflict of interest in the publication.

References

1. Alam M, Alam S, Shamsi A, Adnan M, Elsbali AM, Al-Soud WA, Alreshidi M, Hawsawi YM, Tippana A, Pasupuleti VR, Hassan MI (2022) Bax/Bcl-2 cascade is regulated by the EGFR pathway: therapeutic targeting of non-small cell lung cancer. *Front Oncol* 12:869672
2. Barkia I, Saari N, Manning SR (2019) Microalgae for high-value products towards human health and nutrition. *Mar Drugs* 17:304.
3. Barone ME, Parkes R, Herbert H, McDonnell A, Conlon T, Aranyos A, Fierli D, Fleming GT, Touzet N (2021) Comparative response of marine microalgae to H₂O₂-induced oxidative stress. *Appl Biochem Biotechnol* 193: 4052–4067
4. Becerra MO, Contreras LM, Lo MH, Díaz JM, Herrera GC (2020) Lutein as a functional food ingredient: stability and bioavailability. *J Funct Foods*, 66:103771
5. Bhalamurugan GL, Valerie O, Mark L (2018) Valuable bioproducts obtained from microalgal biomass and their commercial applications: a review. *Environ Eng Res* 23:229–241
6. Boice A, Bouchier-Hayes L (2020) Targeting apoptotic caspases in cancer. *Biochim Biophys Acta (BBA)-Molecular Cell Res* 1867:118688
7. Bold HC (1949) 'The Morphology of *Chlamydomonas chlamydogama*, Sp. Nov.', *Bulletin of the Torrey Botanical Club* 1:101–108
8. Bouayed J, Bohn T (2010) Exogenous antioxidants - Double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid Med Cell Longev* 3:228–237
9. Bujak T, Zagórska-Dziok M, Ziemiańska A, Nizioł-Łukaszewska Z, Wasilewski T, Hordyjewicz-Baran Z (2021) Antioxidant and cytoprotective properties of plant extract from dry flowers as functional dyes for cosmetic products. *Molecules* 26:2809
10. Bulut O, Sönmez Ç, Öktem HA (2023) *Hindakia tetrachotoma* ME03 (Chlorophyta) has high phenolic content, antioxidant capacity, and attenuates H₂O₂-induced oxidative stress and apoptosis in human cells. *Phycologia* 62:293–302
11. Calder PC (2017) Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochem Soc Trans* 45:1105–1115
12. Chaudhary P, Janmeda P, Docea AO, Yeskaliyeva B, Abdull Razis AF, Modu B, Calina D, Sharifi-Rad J (2023) Oxidative stress, free radicals and antioxidants: Potential crosstalk in the pathophysiology of human diseases. *Front Chem* 11:1158198
13. Cooper JR, Abdullatif MB, Burnett EC, Kempseil KE, Conforti F, Tolley H, Collins JE, Davies DE (2016) Long term culture of the A549 cancer cell line promotes multilamellar body formation and differentiation towards an alveolar type II pneumocyte phenotype. *PLOS One* 11:e0164438
14. Egeland ES, Garrido JL, Clementson L, Andersen K, Thomas CS, Zapata M, Airs R, Llewellyn CA, Newman GL, Rodríguez F, Roy S (2011) Part VII data sheets aiding identification of phytoplankton

- carotenoids and chlorophylls. In: Roy S, Ljewellyn CA (eds) *Phytoplankton Pigments*. Cambridge University Press, Cambridge, pp 665–674
15. Ferdous UT, Yusof ZN (2021) Medicinal prospects of antioxidants from algal sources in cancer therapy. *Front Pharmacol* 12:157
 16. Fiedor J, Burda K (2014) Potential role of carotenoids as antioxidants in human health and disease. *Nutrients* 6:466–488
 17. Fulda S, Gorman AM, Hori O, Samali A (2010) Cellular stress responses: cell survival and cell death. *Inter J Cell Biol* 2010:1–23
 18. Galea-Lauri J, Richardson AJ, Latchman DS, Katz DR (1996) Increased heat shock protein 90 (hsp90) expression leads to increased apoptosis in the monoblastoid cell line U937 following induction with TNF-alpha and cycloheximide: a possible role in immunopathology. *J Immunol* 157:4109–4118
 19. Gallegos Ruiz MI, Floor K, Roepman P, Rodriguez JA, Meijer GA, Mooi WJ, Jassem E, Niklinski J, Muley T, van Zandwijk N, Smit EF (2008) Integration of gene dosage and gene expression in non-small cell lung cancer, identification of HSP90 as potential target. *PLoS One* 3: 1–8
 20. Gammone MA, Riccioni G, D'Orazio N (2015) Marine carotenoids against oxidative stress: effects on human health. *Mar Drugs* 13:6226–6246
 21. Gao M, Liu T, Li J, Guan Q, Wang H, Yan S, Li Z, Zuo D, Zhang W, Wu Y (2020) YAN, a novel microtubule inhibitor, inhibits P-gp and MRP1 function and induces mitotic slippage followed by apoptosis in multidrug-resistant A549/Taxol cells. *Toxicol Vitro* 69:104971
 22. Garrido C, Gurbuxani S, Ravagnan L, Kroemer G (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem Biophys Res Commun* 286:433–442
 23. Gille JJ, Joenje H (1992) Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia. *Mut Res* 1:405–414
 24. Gong M, Bassi A (2016) Carotenoids from microalgae: A review of recent developments. *Biotechnol Adv* 34:1396–1412
 25. Guillard RR (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) *Culture of Marine Invertebrate Animals*. Boston, MA, Springer US, pp. 29–60
 26. Herbert H, Parkes R, Barone ME, Picciotto S, Adamo G, Paterna A, Manno M, Bongiovanni A, Campion E, Touzet N (2024) Effects of cultivation stress on the Glaucophyte *Cyanophora paradoxa* and bioactive potential in human cancer cell lines. *Phycologia*. 63:278-289
 27. Itoh S, Kim HW, Nakagawa O, Ozumi K, Lessner SM, Aoki H, Akram K, McKinney RD, Ushio-Fukai M, Fukai T (2008) Novel role of antioxidant-1 (Atox1) as a copper-dependent transcription factor involved in cell proliferation. *J Biol Chem* 283:9157–9167
 28. Janciauskiene S (2020) The beneficial effects of antioxidants in health and diseases. *Chron Obstr Pulm Dis: J COPD Found* 7:182–202.

29. Jin Y, Zhang D, Deng K, Wu P, Yang D, Xie Z, Qiu W, Yu G (2023) Role of the cAMP-PKA-NF- κ B pathway in Mucin1 over-expression in A549 cells during respiratory syncytial virus infection. BMC Infect Dis 23:845
30. Kennedy D, Jäger R, Mosser DD, Samali A (2014) Regulation of apoptosis by heat shock proteins. IUBMB Life 66:327–338
31. Khavari F, Saidijam M, Taheri M, Nouri F (2021) Microalgae: therapeutic potentials and applications. Mol Biol Rep 48:4757–4765
32. Kim DW, Shin MJ, Choi YJ, Kwon HJ, Lee SH, Lee S, Park J, Han KH, Eum WS, Choi SY (2018) Tat-ATOX1 inhibits inflammatory responses via regulation of MAPK and NF- κ B pathways. BMB Rep 51:654–659
33. Krishnaswamy R, Devaraj SN, Padma VV (2010) Lutein protects HT-29 cells against Deoxynivalenol-induced oxidative stress and apoptosis: prevention of NF- κ B nuclear localization and down regulation of NF- κ B and Cyclo-Oxygenase - 2 expression. Free Radic Biol Med 49:50–60
34. Kumar BR, Deviram G, Mathimani T, Duc PA, Pugazhendhi A (2019) Microalgae as rich source of polyunsaturated fatty acids. Biocatal Agric Biotechnol 17:583–588
35. Liu X, Fan L, Lu C, Yin S, Hu H (2020) Functional role of p53 in the regulation of chemical-induced oxidative stress. Oxid Med Cell Longev 2020:1–10
36. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.
37. Mc Gee D, Archer L, Paskuliakova A, Mc Coy GR, Fleming GT, Gillespie E, Touzet N (2018) Rapid chemotaxonomic profiling for the identification of high-value carotenoids in microalgae. J Appl Phycol 30:385–399
38. Mc Gee D, Archer L, Smyth TJ, Fleming GT, Touzet N (2020) Bioprospecting and LED-based spectral enhancement of antimicrobial activity of microalgae isolated from the west of Ireland. Algal Res 45:101704
39. Neto JJ, de Almeida TS, de Medeiros JL, Vieira LR, Moreira TB, Maia AI, Ribeiro PR, de Brito ES, Farias DF, Carvalho AF (2017) Impact of bioaccessibility and bioavailability of phenolic compounds in biological systems upon the antioxidant activity of the ethanolic extract of *Triplaris gardneriana* seeds. Biomed Pharmacother 88:999–1007
40. Nigjeh ES, Yusoff FM, Mohamed Alitheen NB, Rasoli M, Keong YS, Omar AR (2013) Cytotoxic effect of ethanol extract of microalga, *Chaetoceros calcitrans*, and its mechanisms in inducing apoptosis in human breast cancer cell line. BioMed Res Int 2013:1–9
41. Nizioł-Łukaszewska Z, Furman-Toczek D, Zagórska-Dziok M (2018) Antioxidant activity and cytotoxicity of Jerusalem artichoke tubers and leaves extract on HaCaT and BJ fibroblast cells. Lipids in Health Dis 17:280
42. Pagels F, Amaro HM, Tavares TG, Amil BF, Guedes AC (2022) Potential of microalgae extracts for food and feed supplementation—A promising source of antioxidant and anti-inflammatory compounds. Life 12:1901.

43. Panja S, Ghate NB, Mandal N (2016) A microalga, *Euglena tuba* induces apoptosis and suppresses metastasis in human lung and breast carcinoma cells through ROS-mediated regulation of MAPKs. *Cancer Cell Int* 16:1–3
44. Peng C, Zhao F, Li H, Li L, Yang Y, Liu F (2022) HSP90 mediates the connection of multiple programmed cell death in diseases. *Cell Death Dis* 13:929.
45. Peng X, Hu T, Zhang Y, Zhao A, Natarajan B, Wei J, Yan H, Chen H, Lin C (2020) Synthesis of caffeic acid sulphonamide derivatives and their protective effect against H₂O₂ induced oxidative damage in A549 cells. *RSC Adv* 10:9924–9933
46. Qian S, Wei Z, Yang W, Huang J, Yang Y, Wang J (2022) The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. *Front Oncol* 12:985363.
47. Robertson RC, Guihéneuf F, Bahar B, Schmid M, Stengel DB, Fitzgerald GF, Ross RP, Stanton C (2015) The anti-inflammatory effect of algae-derived lipid extracts on lipopolysaccharide (LPS)-stimulated human THP-1 macrophages. *Mar Drugs* 13:5402–5424
48. Sablina AA, Budanov AV, Ilyinskaya GV, Agapova LS, Kravchenko JE, Chumakov PM (2005) The antioxidant function of the p53 tumor suppressor. *Nat Med* 11:1306–1313
49. Sansone C, Galasso C, Orefice I, Nuzzo G, Luongo E, Cutignano A, Romano G, Brunet C, Fontana A, Esposito F, Ianora A (2017) The green microalga *Tetraselmis suecica* reduces oxidative stress and induces repairing mechanisms in human cells. *Sci Rep* 7:1–12
50. Sathasivam R, Ki JS (2018) A review of the biological activities of microalgal carotenoids and their potential use in healthcare and cosmetic industries. *Mar Drugs* 16:28
51. Singh N, Baby D, Rajguru JP, Patil PB, Thakkannavar SS, Pujari VB (2019) Inflammation and cancer. *Ann Afr Med* 18:121–126
52. Sinthupibulyakit C, Ittarat W, St Clair WH, St Clair DK (2010) p53 protects lung cancer cells against metabolic stress. *Int J Oncol* 37:1575–1581
53. Snezhkina AV, Kudryavtseva AV, Kardymon OL, Savvateeva MV, Melnikova NV, Krasnov GS, Dmitriev AA (2019) ROS generation and antioxidant defense systems in normal and malignant cells. *Oxid Med Cell Longev* 1–17
54. Suh SS, Yang EJ, Lee SG, Youn UJ, Han SJ, Kim IC, Kim S (2017) Bioactivities of ethanol extract from the Antarctic freshwater microalga, *Chloromonas* sp. *Int J Med Sci* 14:560–569
55. Tsymbal S, Refeld A, Zatsepin V, Kuchur O (2023) The p53 protein is a suppressor of Atox1 copper chaperon in tumor cells under genotoxic effects. *PLoS One* 18:e0295944
56. Upadhyay S, Vaish S, Dhiman M (2018) Hydrogen peroxide-induced oxidative stress and its impact on innate immune responses in lung carcinoma A549 cells. *Mol Cell Biochem* 450:135–147
57. Wang N, Pei H, Xiang W, Li T, Lin S, Wu J, Chen Z, Wu H, Li C, Wu H (2023) Rapid screening of microalgae as potential sources of natural antioxidants. *Foods* 12:2652
58. Wang X, Cui YJ, Qi J, Zhu MM, Zhang TL, Cheng M, Liu SM, Wang GC (2018) Fucoxanthin exerts cytoprotective effects against hydrogen peroxide-induced oxidative damage in L02 Cells. *BioMed*

59. Xu Y, Zou Y, Zhou S, Niu MM, Zhang Y, Li J, Xu Z, Yang L (2023) Discovery of potent heat shock protein 90 (Hsp90) inhibitors: structure-based virtual screening, molecular dynamics simulation, and biological evaluation. *J Enzyme Inhibit Med Chem* 38:2220558.
60. Yilmazer A (2018) Cancer cell lines involving cancer stem cell populations respond to oxidative stress. *Biotechnol Rep* 17:24–30
61. Yuce M, Gumuskaptan C, Con AH, Yazici F (2023) Conjugated linoleic acid strengthens the apoptotic effect of cisplatin in A549 cells. *Prostaglandins Other Lipid Mediat* 166:1098–8823
62. Zhang JY, Zhang F, Hong CQ, Giuliano AE, Cui XJ, Zhou GJ, Zhang GJ, Cui YK (2015) Critical protein GAPDH and its regulatory mechanisms in cancer cells *Cancer Biol Med* 12:10–22
63. Zhong Y, Shahidi F (2015) Methods for the assessment of antioxidant activity in foods. In: *Handbook of Antioxidants for Food Preservation*. Shahidi F (ed) Woodhead Publishing, Cambridge, UK 287–333
64. Zhou C, Yu T, Zhu R, Lu J, Ouyang X, Zhang Z, Chen Q, Li J, Cui J, Jiang F, Jin KY (2023) Timosaponin AIII promotes non-small-cell lung cancer ferroptosis through targeting and facilitating HSP90 mediated GPX4 ubiquitination and degradation. *Int. J. Biol. Sci* 19:1471–1489
65. Zuehlke AD, Beebe K, Neckers L, Prince T (2015) Regulation and function of the human HSP90AA1 gene. *Gene* 570: 8–16

Figures

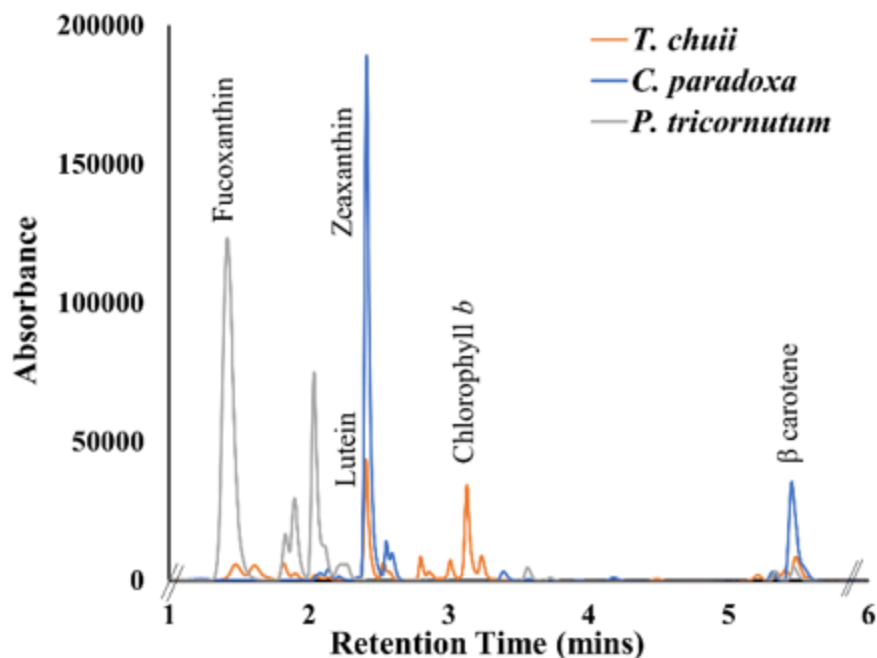


Figure 1

Overlaid HPLC-UV chromatograms showing the main pigments present in the extracts derived from *Tetraselmis chuii* Butcher CCAP 66/21B, *Cyanophora paradoxa* Korshikov CCAP 981/1 and *Phaeodactylum tricornutum* Bohlin CCAP 1052/1

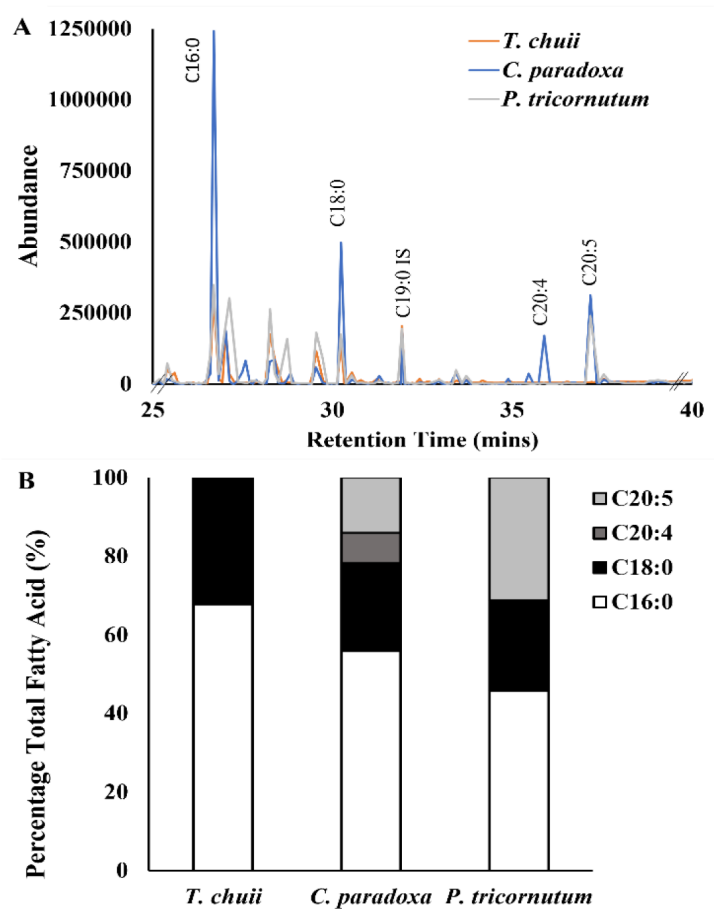


Fig. 2 Overlaid GC-MS chromatogram of FAMES (a) and their relative proportions (%) (b) in the extracts derived from *Tetraselmis chuii* Butcher CCAP 66/21B, *Cyanophora paradoxa* Korshikov CCAP 981/1 and *Phaeodactylum tricornutum* Bohlin CCAP 1052/1

Figure 2

See image above for figure legend

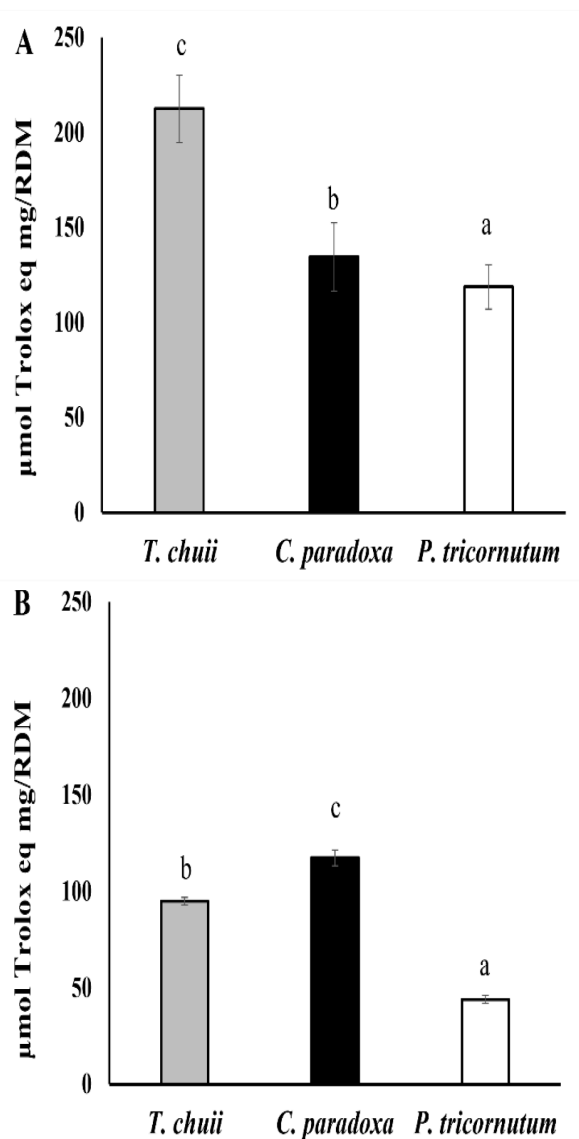


Fig. 3 Antioxidant potential of the extracts of *Tetraselmis chuii* Butcher CCAP 66/21B, *Cyanophora paradoxa* Korshikov CCAP 981/1 and *Phaeodactylum tricornutum* Bohlin CCAP 1052/1 analysed with the FC (a) and TEAC (b) assays. Letters show homogenous subsets that indicate a significant difference ($p < 0.001$) and error bars represent standard deviation ($n = 3$)

Figure 3

See image above for figure legend

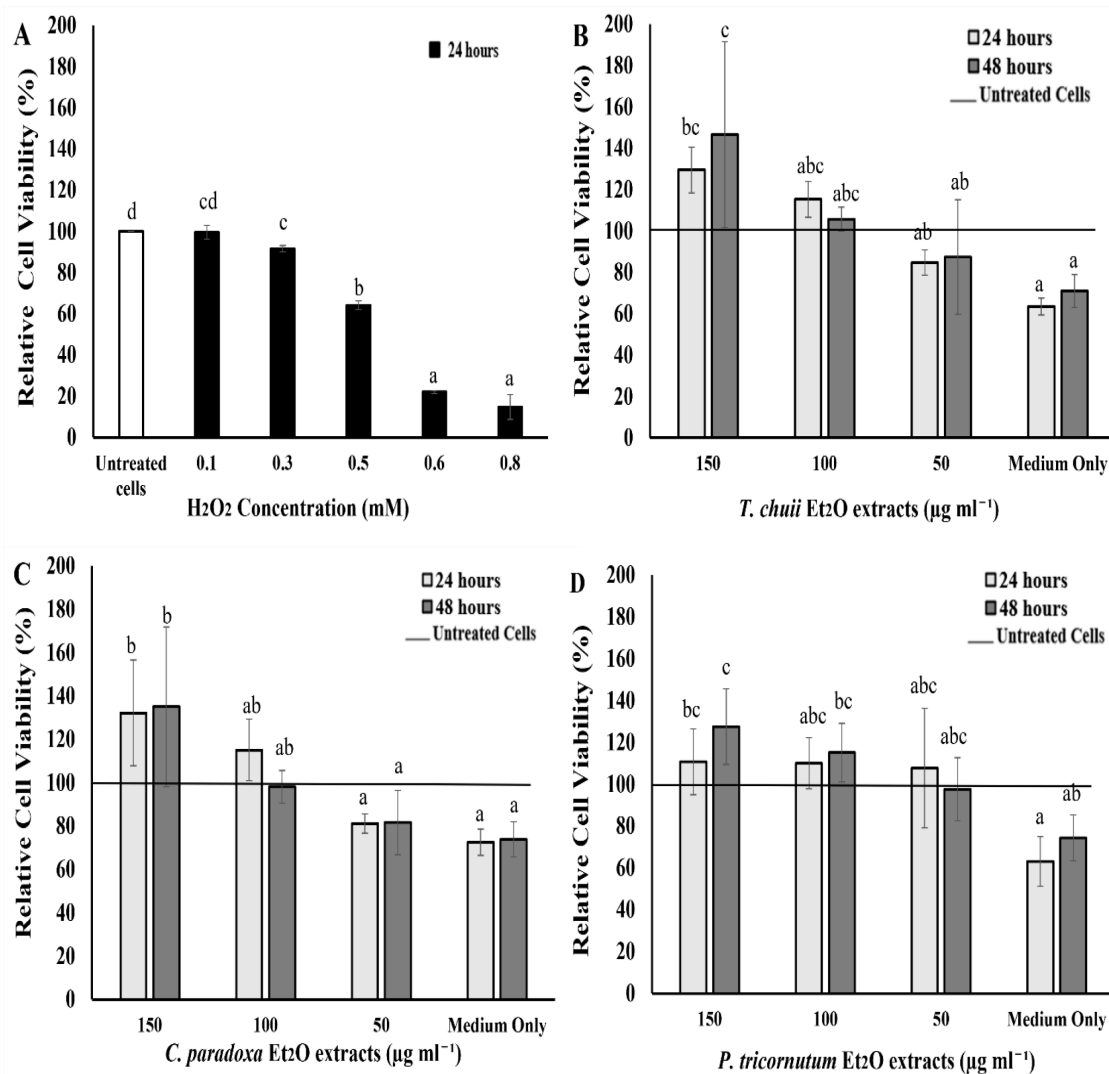


Fig. 4 The range of H₂O₂ concentrations used to induce oxidative stress in A549 cells (a) and panels (b) to (d) illustrate the *in vitro* cytoprotective activity of the extracts from *Tetraselmis chuii* Butcher CCAP 66/21B (b), *Cyanophora paradoxa* Korshikov CCAP 981/1 (c) and *Phaeodactylum tricornutum* Bohlin CCAP 1052/1 (d) at 24 and 48 h in A549 cells pre-treated with 0.5 mM H₂O₂. Three independent assays were performed in triplicate; data is shown as mean ± SD. Letters show homogenous subsets that indicate a significant difference ($p < 0.001$)

Figure 4

See image above for figure legend

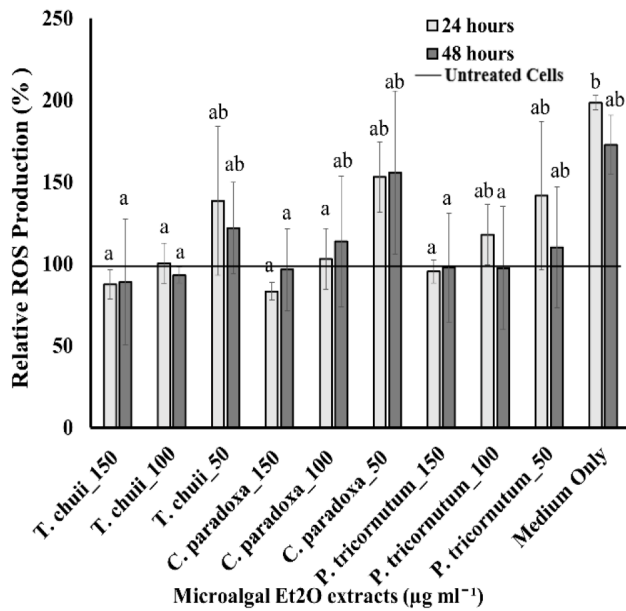


Fig. 5 Effects of the extracts from *Tetraselmis chuii* Butcher CCAP 66/21B, *Cyanophora paradoxa* Korshikov CCAP 981/1 and *Phaeodactylum tricornutum* Bohlin CCAP 1052/1 on the production of Reactive Oxygen Species in human lung adenocarcinoma cells (A549) following 24 h pre-treatment with 0.5 mM H₂O₂. Three independent assays were performed in triplicate; data is shown as mean ± SD. Letters show homogenous subsets that indicate a significant difference ($p < 0.001$)

Figure 5

See image above for figure legend

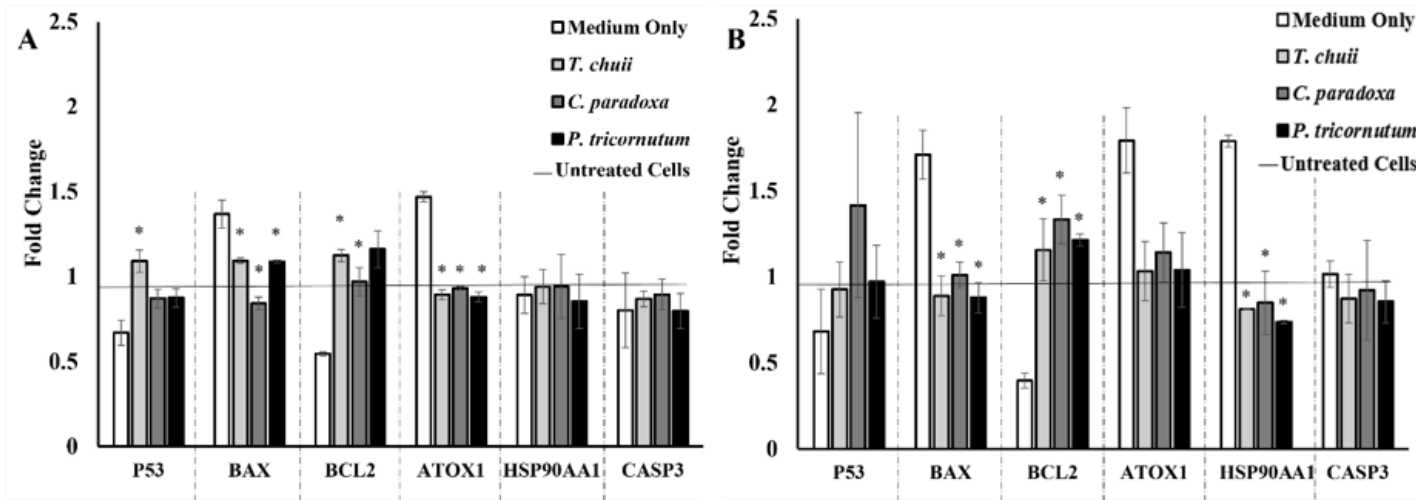


Figure 6

Changes in gene expression in A549 lung cells after 24 h exposure to H₂O₂ and subsequent 24 (a) and 48 (b) h treatment with microalgal extracts (150 µg mL⁻¹). Data were obtained from two independent experiments, each performed in triplicate. Stars indicate significant differences relative to the medium only

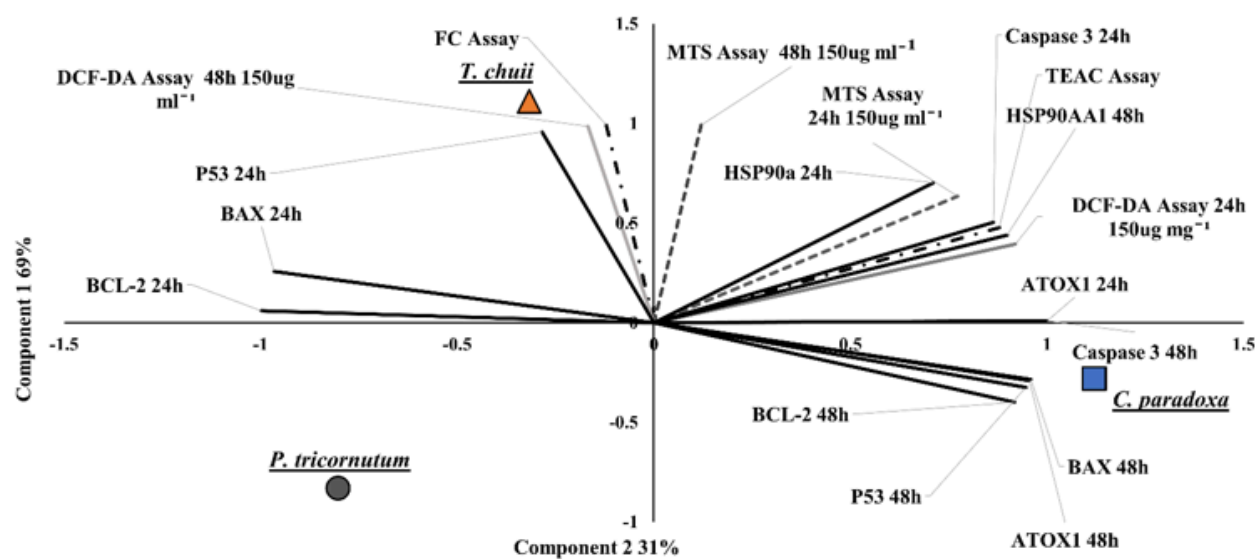


Fig. 7 PCA analysis indicating the correlations between microalgae species and the evaluated bioactive variables. The analysis includes data from Antioxidant Assay — , MTS Assay -----, DCF-DA Assay ————— and Gene Expression studies —————

Figure 7

See image above for figure legend