Algal Research 89 (2025) 104054

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Enhancing lutein concentration in an indigenous microalgal strain through salinity, light intensity and nutrient concentrations and evaluation of its anticancer potential

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ARTICLE INFO

Keywords:
Bioprospecting
Mediterranean
Chlorella
Cancerogenic
Autochthonous microalgae

ABSTRACT

The search for new approaches for treating cancer is an urgent need and microalgae have recently shown to be an underestimated source of biocompounds. In this work, the anticancer potential of an indigenous *Chlorella*-like microalgal strain was investigated by optimizing its lutein content using a Design of Experiment (DoE) approach. Several parameters were assessed such as NaCl, phosphate and nitrate concentration in combination with light intensity. Sodium chloride and nitrate concentrations showed antagonist effects on lutein accumulation. The crude methanolic extracts of the obtained microalgal cultures, containing 2.15 ± 0.12, 1.70 ± 0.17 and of 0.68 ± 0.09 μg mg^{-1} of lutein, were tested on cancer cell lines, revealing a dosage-dependent antiproliferative effect on melanoma A2058 cells. The extracts' fractions containing lutein exhibited similar effects, likely due to a concerted action of multiple substances, with lutein being one of the main contributors. The described approach, which combined bioprocess optimization and assessment of anticancer proprieties, showed a significant potential in the discovery of new bioactives for pharmaceutics.

1. Introduction

Cancer is one of the most deadly diseases all over the world, with 1,958,310 new cancer cases and 609,820 cancer deaths in only United States within 2023 [1]. The search for new drugs to treat cancer is a pressing need, as the current cures mainly rely on chemotherapy and radiotherapy, which cause several collateral effects [2]. The application of natural compounds in pharmaceutics, in particular as anticancer agent, is consolidated since antiquity, with several molecules today identified as anticancerogenic [3]. In particular, the bioprospecting of marine living beings is a research field of growing interest, accounting for several microorganisms identified as potential sources of pharmaceuticals. Among them, microalgae have been recognised as an interesting source of bioactives [4]. Microalgae constitute a polyphyletic group of mainly aquatic microorganisms spread across the eukaryotic tree of life, with the sole exception of prokaryotic class Cyanophyta [5]. Due to their complex evolutionary history, microalgae account for wide-ranging characteristics, allowing their application in different technological fields, including cosmetic, nutraceuticals, wastewater treatment and bioenergy [6,7]. The characteristics of microalgal species may reflect the environment in which they live, and algae isolated from the Mediterranean Sea reflect the different conditions therein and show a broad taxonomic diversity [8]. In particular, algae isolated from saltern pond may accumulate various high-value compounds as an adaptative response to the extreme conditions in which they live [9]. It is well known that each species of microalgae produces specific compounds depending on its genetics. As an example, *Dunaliella salina* is well known for the production of beta-carotene, *Haematococcus pluvialis* of astaxanthin and *Nannochloropsis gaditana* for fatty acids of the omega-3 series. Nevertheless, it has also been observed that the microalgal composition may vary depending on other factors, such as cultivation conditions. Several research studies explored methods to increase the content of

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https://doi.org/10.1016/j.algal.2025.104054
Received 5 September 2024; Received in revised form 26 February 2025; Accepted 16 April 2025
Available online 17 April 2025

bioactives in microalgae, with also some reviews on the topic [10]. A common trigger for lipid accumulation, for example, is nutrient limitation, particularly nitrogen. Salinity also plays a significant role in influencing microalgal growth and can lead to carotenoid and other bioactive accumulation, together with the increase of temperature [11,12]. The delivery of light to microalgal cultures is also extensively studied as a trigger to modify the biochemical composition of the biomass, in terms of both light intensity [13] or timing of light distribution (i.e. flashing lights [14]). Few attempts have been made to simultaneously study these parameters until now, likely due to the multitude of potential combinations coming from their integration. In this work, we employed a Design of Experiment (DoE) approach to optimize the bioprocess parameters for the bioactive content in an indigenous microalgal strain. After the initial isolation from saltern ponds of Trapani, Italy, and the molecular characterization of the algae, the concentration of nitrate, phosphate, NaCl and light provided to the culture were optimized in order to obtain an increase in lutein, chosen as output for being a well-known anticancerogenic compound [15]. The selected culture conditions were scaled-up and the final microalgal extracts and derived fractions were tested for their anticancerogenic potential on both normal and cancer cell lines. This work serves as a benchmark for its innovative approach in researching growth conditions tailored to induce bioactive compounds, thereby contributing to increase the knowledge on marine microalgal potential for biodrug discovery.

2. Materials and methods

2.1. Microalgae culturing

The microalga used in this study was harvested and isolated in the "Saline di Trapani e Paceco" during summer (September 2017), (GPS coordinates 37.966845, 12.505649), in Sicily, Italy. After the isolation, the alga was maintained in modified F/2 without silicates [16] with some modifications, as reported in supplementary information.

In all the experiments, a Delta Ohm-HD 9021 quantometer equipped with a Photosynthetic Active Radiation (PAR) probe (Delta Ohm LP 9021 PAR) was used to measure light intensity.

For the DoE experiments (see next paragraph), a sterile flat glass reactor was employed, with an internal thickness of 4 mm and a volume of 125 mL, illuminated using a LED panel with adjustable light intensity and placed inside an incubator with a fixed temperature of 25 °C. The medium was modified in each experiment as explained in the next paragraph. The initial concentration of microalgae was maintained within a range of 0.06–0.13 g L^{-1}. A constant air flow of 0.7 L min^{-1} was provided to the reactor, inducing turbulence. After 7 days of cultivation, the biomass was harvested by centrifugation, washed freeze-dried in a bench lyophilizator (Alpha 1–2 LDplus, Christ, DE) before further analysis.

2.2. Experimental design through DoE methodology

In order to assess the best conditions for increasing bioactive content in microalgae, a Design of Experiments (DoE) approach-based experimental design was carried out with lutein as the main output. Through DoE, experiments are designed to establish relationships between considered factors and the system's output response, using minimal trials. Design Expert 13 software was used for this purpose. This software aids in study design and facilitates understanding of results through Response Surface Modelling (RSM). Four key factors were optimized in a specific range: light intensity (from 2 to 400 μmol m^{-2} s^{-1}), salinity as NaCl concentration (from 0 to 42.5 g L^{-1}), nitrate concentration, NO_3^- (from 0 to 6 g L^{-1}) and phosphate concentration, PO_4^{3-} (from 0 to 0.3 g L^{-1}). The other components of the growth medium were those of the modified F/2 receipt. 27 tests were carried out.

2.3. Scale-up

The results obtained by the first 27 tests carried out in minimal trials allowed to select the optimal conditions to be scaled-up. The selected conditions were the following: MAX (maximizing lutein production with low nitrate concentration and high salinity), with light intensity 100 μmol m^{-2} s^{-1}, nitrate concentration 0.1 g L^{-1}, phosphate concentration 0.01 g L^{-1}, NaCl concentration 30 g L^{-1}; MAX_2 (maximizing lutein production with high nitrate concentration and low salinity) with light intensity 100 μmol m^{-2} s^{-1}, nitrate concentration 4 g L^{-1}, phosphate concentration 0.01 g L^{-1}, NaCl concentration 5 g L^{-1}; MIN (minimizing lutein concentration) with light intensity 300 μmol m^{-2} s^{-1}, nitrate concentration 0.9 g L^{-1}, phosphate concentration 0.1 g L^{-1}, NaCl concentration 6.5 g L^{-1}. These conditions were applied to 2.5 L cultures, for 8 days. All experiments were performed in triplicate. Sterile flasks were maintained for 8 days with constant illumination for 24 h/day, temperature between 24 and 26 °C, agitation provided through a magnetic stirrer. The initial concentration was between 0.05 and 0.07 g L^{-1} of biomass. During the experiment, a 0.7 L min^{-1} flow of air enriched with 5 % of carbon dioxide was provided.

2.4. Analysis on lutein content

The lutein content in various obtained samples was analyzed using HPLC (Agilent Technologies, 1220 Infinity LC) on total extracts as previously shown [11]. 3–6 mg of lyophilized and ground microalgal biomass were placed, together with 0.2 g of glass beads and 1 mL of methanol. The sample was mixed by vortexing for 30 s and centrifuged for 20 min at 12000 rpm, then the supernatant was analyzed.

2.5. DNA extraction

Total DNA extraction was performed after 7 days of microalgal growth in 500 mL flasks (inoculum of 5000 cell mL^{-1} and final concentration of about 10^5 cell mL^{-1}). Briefly, after harvesting 50 mL of cellular suspension by centrifugation (3000 rpm at 4 °C for 15 min), the DNA was extracted from the pellet according to Ruocco et al., 2018 [17], with the addition of a lysis buffer and incubation for 45′ at 65 °C with vortexing every 15 min. After two steps of precipitation with a solution of chloroform/isoamyl alcohol (24:1), the supernatant was collected, and a volume of ice-cold isopropanol (100 %) was added. After incubation at −20 °C for 1 h, the DNA was precipitated by centrifugation and washed with 75 % EtOH before solubilization in 20 μL of sterile water was used for pellet resuspension. Quality and quantity check of DNA was performed using Nanodrop One (Thermo Fisher) and by agarose gel electrophoresis.

2.6. Genetic identification

For the genetic identification of the algal strain, we amplified the hypervariable V4 region of the 18S rRNA and the D1-D3 subunit of 28S rRNA according to Kooistra et al. and Shaked and de Vargas [18,19], respectively. The amplicons were sequenced in both forward and reverse directions, and the taxonomic assignation was determined with three different approaches: 1) an initial blastn search against the nucleotide database of NCBI to find the best match with published sequences; 2) a blastn search against the curated SILVA database in the SINA ACT tool ([20] https://www.arb-silva.de/aligner, accessed on 25 November 2024); 3) a blastn search and a phylogenetic tree reconstruction using the 18S sequences of the phylogenetic study on *Chlorella* and *Chlorella*-like species by Heeg and Wolf, 2015 [21]. This multi-step approach was required to limit the impact of taxonomic mis-assignations of sequences belonging to this complex algal group in our analyses. For the latter analysis, the sequences of the work by Heeg and Wolf (2015) were downloaded from GenBank and aligned to the 18S sequence of our strain using the ClustalW algorithm [22] implemented in the software BioEdit

v7.2.6 [23]. Introns, regions longer than our query sequence, and identical sequences resulting from the latter operations were removed from the alignment. The best model of nucleotide substitution was calculated in jModelTest v2.1.10 [24] using the corrected Akaike information criterion [25] and then a Bayesian tree was inferred in MrBayes v3.2 [26] under the substitution model resulting from the jModelTest analysis and with the following parameters: two replica runs and four chains for 1,000,000 generations and sampling chains every 1000 steps. Convergence and effective sample sizes (ESS) for all parameters were investigated in Tracer v.1.7 [27], and considered acceptable when ESS was >200. The first 10 % of the samples was discarded as burn-in. The majority-rule consensus trees were visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 25 November 2024). The sequences of *Chlorella saccharophila* (FM946000) and *Chloroidium ellipsoideum* (FM946015) were chosen as outgroup following Heeg and Wolf, 2015 [21].

2.7. Chemical extraction and fractionation

The lyophilized biomass of our microalgal strain was extracted with 100 % methanol (1:5, w/v), for 1 h, followed by agitation, sonication with three bursts of 30 s with a pause of 10s each. After recovering by centrifugation, the organic phase was removed with a rotavapor, and the extract was stored at −80 °C until use. Fractionation of 20 mg of each extract was performed as described in Cutignano et al. [28]. Briefly, an adsorption cartridge was loaded with the extract and gradually eluted with multiple solvents. The elution gradient was performed using different solvent conditions to obtain five fractions named A, B, C, D, E. The cartridge was activated with 6 mL of methanol and 12 mL of water. The elution gradient was as follows: i.18 mL of distilled water for the extraction of amino acid and saccharides (Fraction A); ii. 24 mL of methanol/water (50:50) for the extraction of nucleosides (Fraction B); iii. 18 mL acetonitrile/water (70:30) for the extraction of glycolipids and phospholipids (Fraction C); iv. 18 mL 100 % acetonitrile for the extraction of free fatty acid and sterols (Fraction D); v. 18 mL dichloromethane/methanol (90:10) for the extraction of triglycerides (Fraction E).

2.8. Cell culture

Human malignant melanoma cell (A2058; ATCC® CRL-11147™) and human immortalized normal keratinocytes (HaCaT; CEINGE Biotecnologie Avanzate S.c.ar.l.) were cultured in DMEM (Corning®) supplemented with 10 % Fetal Bovine Serum (FBS), 1 % L-glutamine, 1 % Pen-Strep solution in a humidified incubator at 37 °C and 5 % CO_2. Human Multiple Myeloma (RPMI 8226) cells were cultured in RPMI 1940 medium supplemented by 10 % FBS, 1 % L-glutamine and 1 % Pen-Strep solution in a humidified incubator at 37 °C and 5 % CO_2. Human plasma cell leukemia (JJN-3) cells were cultured in 40 % ISCOVE's DMEM and 40 % DMEM with 20 % FBS, 1 % L-glutamine; 1 % Pen-Strep solution in a humidified incubator at 37 °C and 5 % CO_2.

2.9. Antiproliferative assays

To estimate the in vitro cytotoxic and anti-proliferative effects of the total extracts and fractions, the HaCaT, A2058, RPMI 8226, JJN-3 cell lines were seeded and incubated for 24 h before the replacement of a medium containing growing extract or fraction concentrations (1 μg mL^{-1}, 10 μg mL^{-1}, and 100 μg mL^{-1}) dissolved in dimethyl sulfoxide (DMSO), and further incubated for 48 or 72 h, respectively, as described in Lauritano et al., 2020 [29]. Each concentration was tested at least in triplicate. For HaCaT and A2058 cells, MTT cytotoxic test was used as described by Baudelet et al., 2013 and Martínez et al., 2022 [30,31]. For RPMI 8226 and JJN-3 cell lines, WST-8 antiproliferative assay was used.

Cell survival was calculated as a percentage of viable cells with tested samples, relative to untreated controls. Extracts and fractions with cell viability above 50 % were not considered active. Results are reported as the mean ± SD of triplicate. Mean, standard deviation and statistical analysis were estimated on biological triplicates using GraphPad Prism8 software (GraphPad, San Diego, CA, USA).

2.10. H_2DCFDA assay

Antioxidant activity of total extracts of the microalga was detected with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), a non-fluorescent compound permeable to the cell membrane, which can be oxidized by reactive oxygen species (ROS) giving a highly fluorescent 2′,7′-dichlorofluorescein (DCF). HaCaT cells were seeded at density of 1×10^4 in 96 well plate and pretreated for 4 h with three concentrations of total extracts (1, 10, 100 μg mL^{-1}). The H_2DCFDA was added to the cells for 45 min. After the incubation time, the media with H2DCFDA was removed and 1 mM of H_2O_2 was added for 30, 60 and 90 min in order to induce oxidative stress. The fluorescence emitted from the cells treated with H_2DCFDA was compared to the untreated cells and 1.5 mM of Trolox was used as a positive control. Statistical analysis was performed by two-way ANOVA using GraphPad Prism software, version 8.0 (GraphPad, San Diego, CA, USA).

3. Results and discussion

3.1. Molecular identification

The blastn analysis of our V4—18S rRNA amplicon (deposited to GenBank with the accession number PQ640034) against the NCBI nucleotide database returned 57 hits with identical scores in terms of coverage (100 %) and similarity (100 %); these hits belonged to strains of different genera like *Chlorella*, *Dicloster*, *Dictyosphaerium*, *Masaia*, and *Parachlorella*. Similarly, the blastn search in SILVA and the classification based on the lowest common ancestor (LCA) implemented in the SINA ACT webtool returned an assignation at Class level (Trebouxiophyceae). On the contrary, the blastn search against the 18S sequences of Hegg and Wolf., 2015 [21] found a single best match with *Dicloster acuatus* (acc. n. FM205848, 99.68 %), which the authors provisionally include in *Parachlorella*, but not excluding a possible separate group. In the V4—18S Bayesian consensus tree (Fig. S1, Supplementary Material), the sequences of our algal strain and *Dicloster acuatus* are outside the highly supported (posterior probability, p.p. = 1) *Parachlorella* clade but, in turn, not included into the *Chlorella* clade. This could be a consequence of the short fragment utilized in this study, but a real phylogenetic signal cannot be excluded. Indeed, from a cladistic point of view, our algal strain and *Dicloster acuatus* share character states with both *Clorella* and *Parachlorella* clades (Fig. S2, Supplementary Material). Our fragment of 28S rRNA (deposited to GenBank with the accession number PQ640035) provided instead a best match with *Micractinium reisseri* (*Chlorella* clade) in both the blastn searches against NCBI nucleotide (98.05 % similarity, 99 % coverage) and the dataset by Hegg and Wolf, 2015 [21] (91.10 % similarity, 100 % coverage); the SILVA analysis returned again a poorly conclusive attribution as Chlorophyta at 85.3 % of similarity. Taken together, all these data prevent us from assigning our algal strain to not only any known species, but also genus or more inclusive clade. In addition, they highlight the controversial taxonomic status of this green algal group and call for further molecular analyses utilizing different molecular markers and supported by morphologically-characterized strains to shed light on this difficult group. As a consequence, in this paper we refer to our algal strain as *Chlorella*-like because of its spherical cell shape and light green coloration visible under light microscopy (Fig. 1).

3.2. Lutein content optimization and modelling of results

According to the experimental design obtained through the Design Expert software, a series of 27 trials was performed by varying the

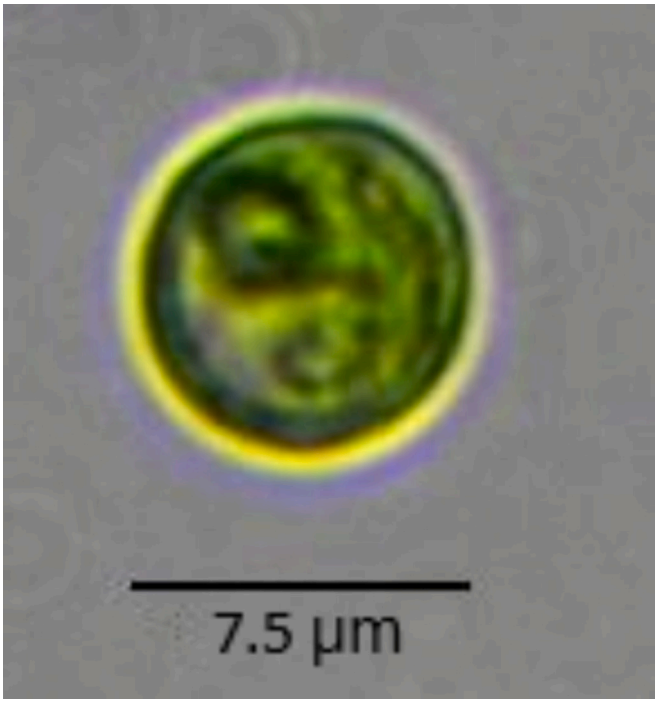

Fig. 1. 100× picture of the isolated microalga. The bar measures 7.5 μm.

applied lighting condition, nitrate and phosphate concentration and salinity in terms of NaCl concentration (Table 1). The biomass content of lutein served as experimental output, as indicated in Table 1. The data were then analyzed by employing a polynomial model to fit the experimental points. A reduced quartic model yielded the best fitting, with an F-value of 290.92 and a *p*-value <0.0001, indicating the model's significance, with an R^2 of 0.998. The employed factors were: A- Light Intensity, B-NO_3^-, C-PO_4^{3-} and D- NaCl. *P*-values <0.1 indicate model terms are significant. In this case the all the factors employed by the model were significant.

The coded equation which gives the concentration of lutein is:

$$\begin{aligned}\text{Lutein} = {} & 0.0715 - 0.139^{*}\,A - 0.128^{*}C + 0.0750^{*}D + 0.0568^{*}AC \\ & - 0.0389^{*}AD - 0.252^{*}BD + 0.0863^{*}A^2 + 0.154^{*}B^2 \\ & + 0.113^{*}C^2 + 0.0788^{*}D^2 - 0.0734^{*}ABC - 0.0411^{*}ABD \\ & + 0.0170^{*}ACD + 0.0828^{*}BCD + 0.0195^{*}A^2B - 0.0251^{*}A^2D \\ & - 0.0783^{*}ABCD + 0.0418^{*}A^2B^2 - 0.0281A^3B\end{aligned} \quad (1)$$

Table 2 presents variables, coefficients and the relevant F and *p* values. Analysis of coefficients indicated that the variable BD gave the most pronounced antagonistic effect on the final output, while B^2 showed the strongest cooperative effect.

When plotting the predicted values against the actual data points, a notable agreement between predicted and experimental data is observed (Fig. 2 a). The line in the figure represents perfect model performance.

Fig. 2 b reports the response surface of the model as lutein concentration (μg mg of biomass^{-1}), shown in z-axis, vs concentration of NO_3^- (g L^{-1}) and of NaCl (g L^{-1}), represented on the x and y axes respectively, PO_4^{3-} concentration (g L^{-1}) and light intensity (μmol s^{-1} m^{-2}) are indicated as parameters. The surface labelled as A displays a PO_4^{3-} concentration of 0.01 g L^{-1} and a light intensity of 100 μmol s^{-1} m^{-2}. If either one or both of these parameters are increased to 0.2 and 300, respectively, the overall response surface retains its shape but shifts downwards, indicating a reduction in lutein production (B surface).

From both graphs, it is evident that two conditions enhance the production of lutein. One occurs at low salinity and high nitrate concentrations, while the other at high salinity and low nitrate concentrations. This observation suggests an antagonistic relationship between these two factors regarding lutein production. Concerning light intensity and phosphate concentration, lutein production is favoured at the lowest values within the specified range.

Literature reports how the lutein content of microalgae may be modified when changing the culture conditions [15]. The nitrogen source is one of the main studied parameters; it is reported that nitrogen repletion is necessary for inducing lutein accumulation. Salinity has been reported as an increasing factor for lutein accumulation in microalgae [32]. In this work, we observed an antagonistic effect between nitrate concentration and salinity given by NaCl concentration which was never been observed before.

Table 1
Experimental design applied for the optimization of lutein production in *Chlorella* and results of the experimental trials.

Trial	Light Intensity [μmol m^{-2} s^{-1}]	NO_3^- [g L^{-1}]	PO_4^{3-} [g L^{-1}]	NaCl [g L^{-1}]	Lutein [μg mg biomass^{-1}]
1	100	0.1	0.01	5.0	0.424
2	300	0.1	0.01	5.0	0.126
3	100	4.0	0.01	5.0	1.124
4	300	4.0	0.01	5.0	0.859
5	100	0.1	0.2	5.0	0.253
6	300	0.1	0.2	5.0	0.095
7	100	4.0	0.2	5.0	0.586
8	300	4.0	0.2	5.0	0.500
9	100	0.1	0.01	30.0	1.358
10	300	0.1	0.01	30.0	0.688
11	100	4.0	0.01	30.0	0.571
12	300	4.0	0.01	30.0	0.231
13	100	0.1	0.2	30.0	0.517
14	300	0.1	0.2	30.0	0.749
15	100	4.0	0.2	30.0	0.651
16	300	4.0	0.2	30.0	0
17	2	2.0	0.1	17.5	0.688
18	400	2.0	0.1	17.5	0.146
19	200	0.0	0.1	17.5	0.234
20	200	6.0	0.1	17.5	0.714
21	200	2.0	0	17.5	0.364
22	200	2.0	0.3	17.5	0.283
23	200	2.0	0.1	0.0	0.119
24	200	2.0	0.1	42.5	0.557
25	200	2.0	0.1	17.5	0.080
26	200	2.0	0.1	17.5	0.105
27	200	2.0	0.1	17.5	0.053

Table 2
Estimation and statistic of coefficients.

Source	Coefficient	F-value	p-value	
Model		290.92	< 0.0001	significant
Intercept	0.0715			
A-Light	−0.1391	843.39	< 0.0001	
C-PO4–	−0.1281	547.61	< 0.0001	
D-NaCl	0.0750	44.50	0.0003	
AC	0.0568	94.55	< 0.0001	
AD	−0.0389	44.19	0.0003	
BD	−0.2519	1854.94	< 0.0001	
A^2	0.0863	281.33	< 0.0001	
B^2	0.1539	564.12	< 0.0001	
C^2	0.1134	278.63	< 0.0001	
D^2	0.0788	115.32	< 0.0001	
ABC	−0.0734	157.41	< 0.0001	
ABD	−0.0411	49.45	0.0002	
ACD	0.0170	8.45	0.0228	
BCD	0.0828	200.21	< 0.0001	
A^2B	0.0195	11.12	0.0125	
A^2D	−0.0251	3.92	0.0882	
ABCD	−0.0783	179.03	< 0.0001	
A^2B^2	0.0418	11.26	0.0122	
A^3B	−0.0281	23.12	0.0019	
Lack of Fit		0.7327	0.6635	not significant

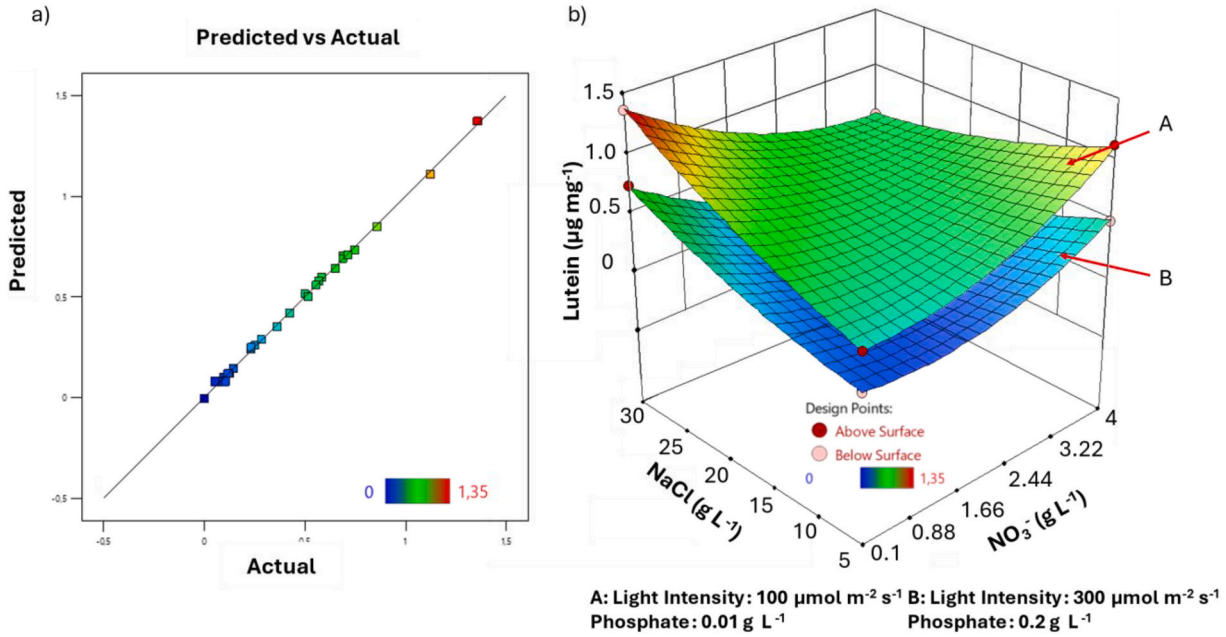

Fig. 2. (a) Model prediction vs results (Predicted vs actual data) of the experiment expressed as lutein content (µg mg^{-1}), (b) response surface of the lutein content as a function of NaCl and NO_3^-concentrations; in A light intensity was 100 µmol s^{-1} m^{-2} and PO_4^{3-}0.01 g L^{-1}; in B light intensity was 300 µmol s^{-1} m^{-2} and PO_4^{3-}0.2 g L^{-1}.

Our findings regarding light intensity confirm what previously reported in literature, namely that generally high light intensities lead to a decrease in lutein accumulation [33,34]. Similarly, phosphate concentration was previously reported to have an antagonistic effect on lutein accumulation in microalgae [35], as shown also in this work where a lower phosphate concentration caused a raise in lutein content.

3.3. Scale-up

Three growth conditions were chosen to be reproduced on a larger scale of 2,5 L for 8-days batch cultivation in 3 L Erlenmeyer flasks. Two of these conditions aimed at maximizing lutein production; we chose to maintain constant phosphate concentration (0,01 g L^{-1}) and light intensity (100 µmol m^{-2} s^{-1}), while varying salinity and nitrate concentration to obtain extreme and opposing conditions. The third condition was chosen to minimize lutein production (as indicated in Section 2.3). Each trial was conducted in triplicate, and values are reported as arithmetic means. MAX condition gave a lutein content of 2.15 ± 0.12 µg mg^{-1}, MAX_2 of 1.70 ± 0.17 µg mg^{-1} and MIN of 0.68 ± 0.09 µg mg^{-1} (Fig. 3), meaning that the stress condition characterized by low nitrogen concentration and high salinity has the most significant influence on the production of lutein by this microalgal strain. This finding is remarkable as it differs from the prevailing findings reported in the literature so far.

3.4. Anti-proliferative activity of raw extract

The organic phases obtained from three freeze-dried pellets of *Chlorella*-like grown in three different conditions named MIN, MAX, and MAX_2 were tested for bioactivity on human normal (Fig. 4 a) and cancer cell lines (Fig. 4b-d), using MTT assay for HaCaT and A2058 adherent cells and WST-8 assay for RPMI 8226 and JJN3 suspension cell lines. Both MTT and WST-8 were used as standard assays to measure the cellular metabolic activity as indication of cell viability, cytotoxicity, and proliferations [36].

Each of the three total extracts of *Chlorella* showed the highest antiproliferative activity against the human malignant melanoma cancer A2058 cells after 72 h treatment (Fig. 4 b) reducing cell viability to

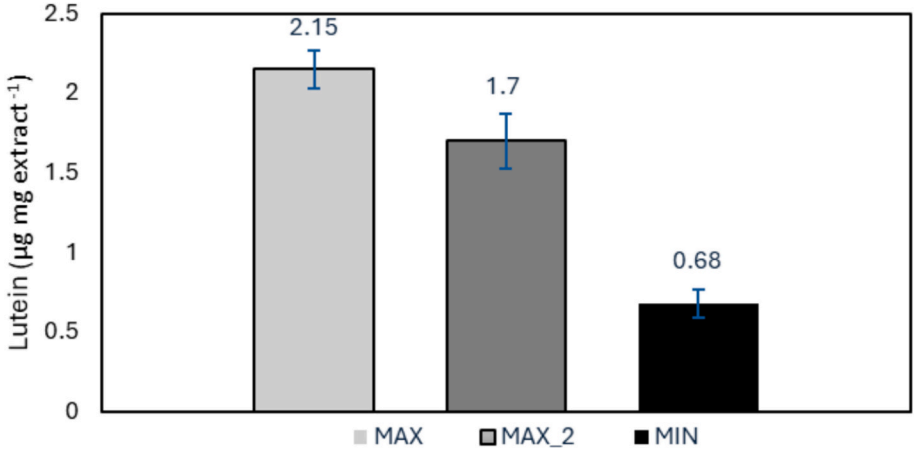

Fig. 3. Lutein content in total extract of the cultures MAX, MAX_2 and MIN.

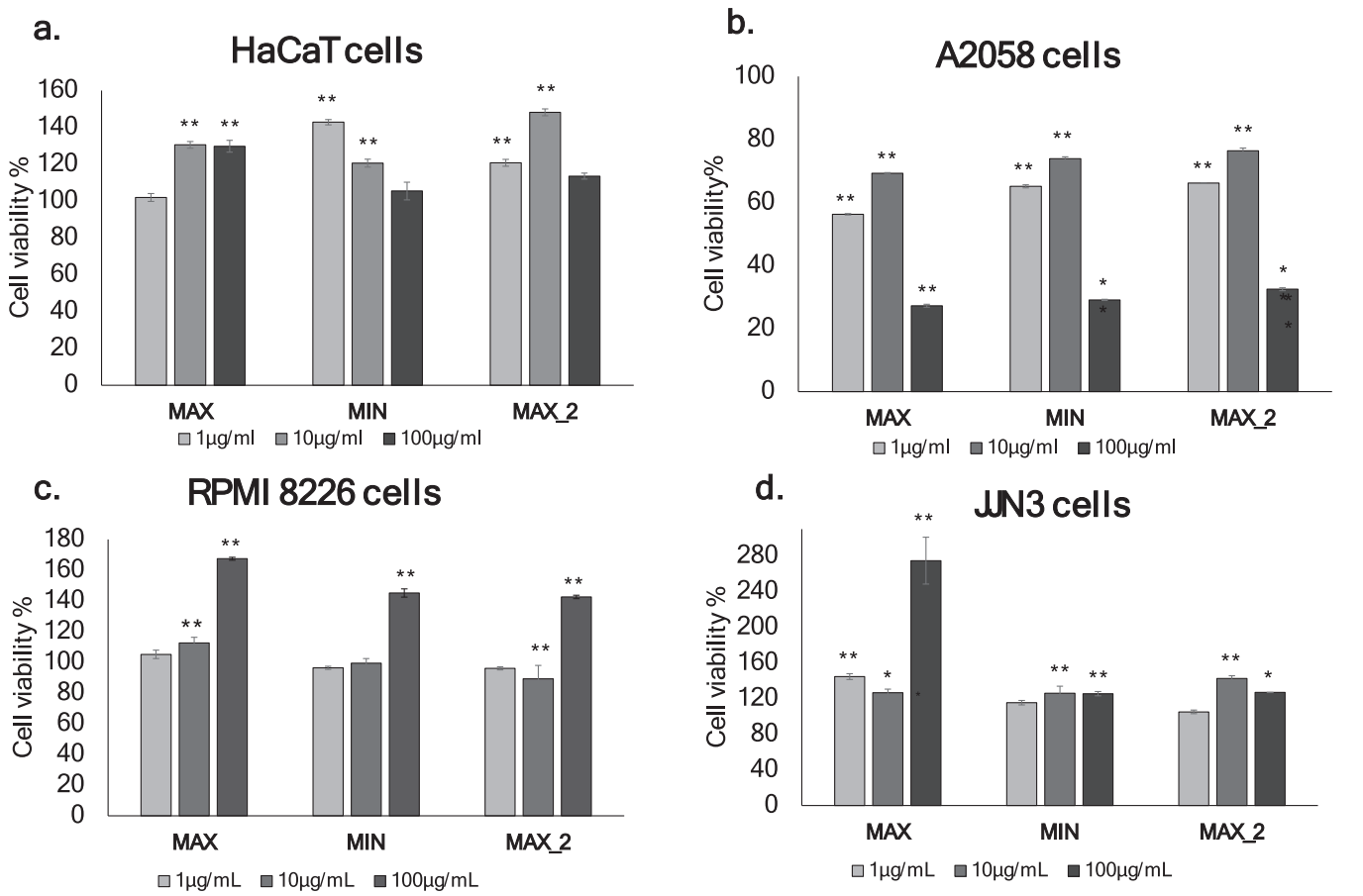

Fig. 4. Antiproliferative effects of *Chlorella*, raw extracts on a, HaCaT, b, A2058 cell lines assessed by MTT Assay and on c, RPMI8226, d, JJN3, cell lines assessed by WST-8 Assay, Results are expressed as percentage of cell survival after 72 h exposure ($n = 3$), Cell growth was expressed as the percentage cell viability with respect to cells incubated only with the vehicle (DMSO) for each concentration (1 µg mL^{-1}, 10 µg mL^{-1}, and 100 µg mL^{-1}) $^{**}p < 0.01$ vs untreated cells.

about 30 %, while cytotoxic effects were not observed for normal keratinocytes HaCaT (Fig. 4 a). At concentration of 100 µg mL^{-1} a significative reduction of cell proliferation was observed, equal to 27 % for MAX total extract, 29 % for MIN total extract and 30 % MAX_2 total extract. When testing the other two concentrations (1 µg mL^{-1} and 10 µg mL^{-1}) we also observed a moderate reduction of cell proliferation ranging from 70 to 80 % for 10 µg mL^{-1} and from 60 to 70 % for the 1 µg mL^{-1} (Fig. 4 b). After 48 h of exposure, the extracts exhibited antiproliferative activity on melanoma cells, although this effect was less pronounced than at 72 h. The MAX_2 condition showed greater activity, reducing cell viability in a dose-dependent manner to around 60 % (see Supplementary Materials, Fig. S3).

Fig. 4 c and d show the cell viability percentage of other cancer cell lines (RPMI 8226 and JJN3, respectively) treated with the three different total extracts of *Chlorella* (MAX, MIN, and MAX_2, respectively) at three different concentrations for 72 h. In general, no cytotoxic effects were observed in these tumour cell lines. In particular, in the RPMI 8226 multiple myeloma cancer cells, there was not significant arrest of proliferation at all the tested conditions. At concentration of 100 µg mL^{-1} it was observed a pro-proliferative activity (Fig. 4 c). Similar effect was observed in the leukemia cell JJN3 (Fig. 4 d). No significant antiproliferative effects were observed for MIN and MAX total extract. For these reasons, further antiproliferative investigations were conducted only on A2058 cell line. Literature reports that in other cases total extract of *Chlorella* sp. showed antitumoral effects against different cancer cells. For example, Sawasdee et al. reported that an ethanolic extract of *Chlorella* had antiproliferative effects on lung cancer (A549 cells), cervical cancer (Hela cells), breast cancer (MCF7 cells), hepatocellular carcinoma (Huh7 cells), and cholangiocarcinoma (CCA cells; KKU213A cells) [15]. In another work, El-fayoumy et al. showed that the total extract of *Chlorella vulgaris* had an antiproliferative effect on HeLa cells [37].

3.5. Antioxidant activity of raw extracts

The antioxidant activity of the MIN, MAX and MAX 2 total extracts of *Chlorella*-like was evaluated on HaCaT cells using the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay to measure the reactive oxygen species levels in the cells. H_2O_2 was used to induce the production of ROS in the cells. Trolox, a vitamin E analogue, was used as positive control for its known antioxidant activity [38]. All the results were compared to the control condition consisting in cells with media and vehicle (DMSO 0.5 %) used for diluting the extracts. Cells were pretreated for 4 h with each extract at concentration of 1, 10, 100 µg mL^{-1}.

As showed in Fig. 5, a reduction of intracellular ROS was observed with MAX _2 total extract (Fig. 5 c) that reduced the ROS production in a dose-dependent manner with the highest activity at 100 µg mL^{-1} compared to the control and to the basal condition. The basal condition is referred to as the measurement of fluorescence at time zero of treatment with H_2O_2. A moderate activity was observed also for the MIN and MAX extracts (Fig. 5 a and b) but lower compared to MAX_2.

3.6. Antiproliferative activity of the fractions

Given that the effects of MAX and MAX_2 total extracts were comparable, only the total extract of MAX_2 together with that of MIN microalgal cells were fractionated as described in Section 2.7. Fractions

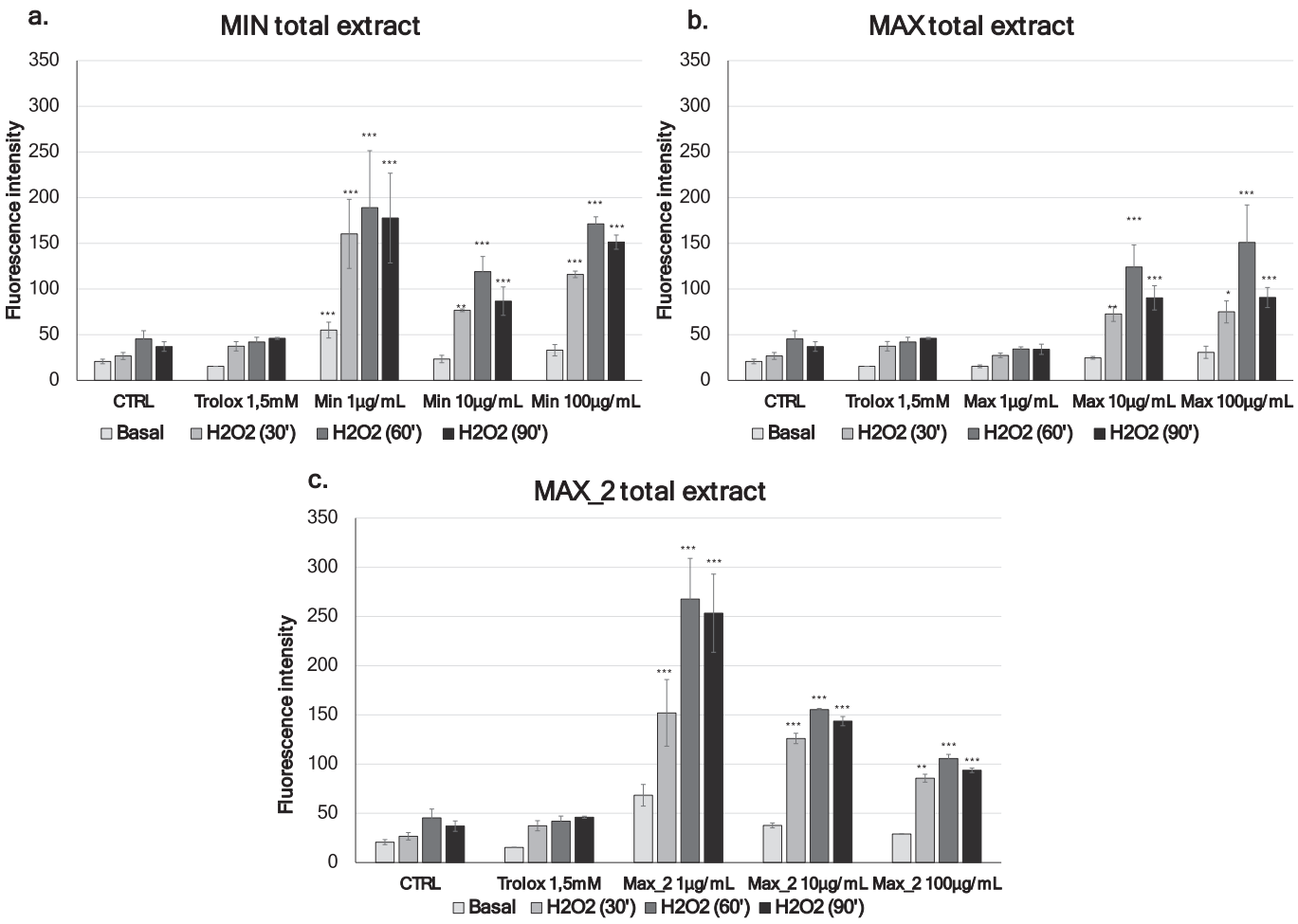

Fig. 5. H_2DCFDA antioxidant assay on HaCaT keratinocyte.

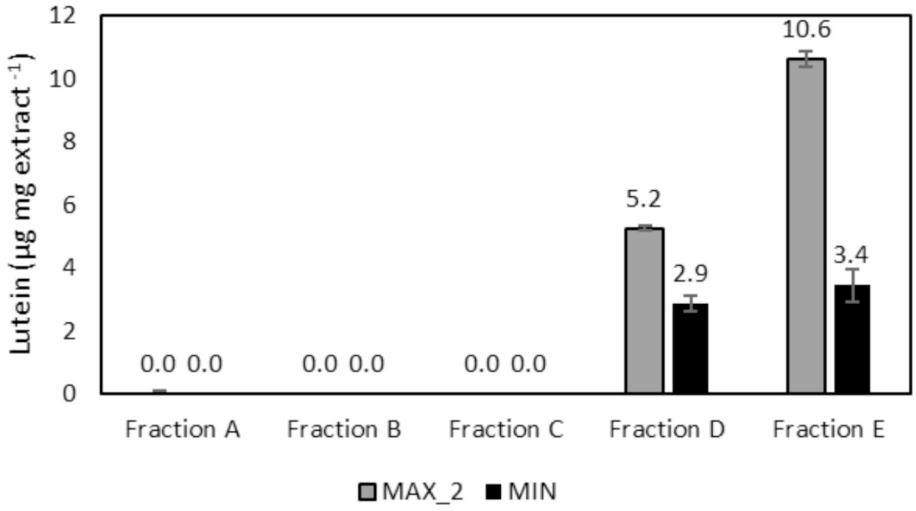

Fig. 6. Analysis of fractions derived from total extract of MIN and MAX_2 cells.

were analyzed for lutein content, revealing that the pigment was present only in fractions D and E, due to its lipophilic nature.

Its concentration in fractions derived from MAX_2 extract was higher than those from MIN extract, as expected (Fig. 6). The fractions were tested in A2058 cells, where the raw extracts showed significative antiproliferative activities, and in HaCaT cells as a control, for 48 h at three different concentrations (1 µg mL^{-1}, 10 µg mL^{-1}, and 100 µg mL^{-1}).

Some fractions exhibited a cytotoxic effect against the cell line HaCaT (Fig. 7). Fractions derived from the total MIN extract demonstrated an increased cytotoxicity towards normal cells, with the exception of fraction B at 1 µg mL^{-1}, fraction C at 1 and 10 µg mL^{-1}, fraction D at 1 and 10 µg mL^{-1} and fraction E at 1 µg mL^{-1} (Fig. 7 a). Conversely, fractions obtained from the extract MAX_2 did not show a cytotoxic

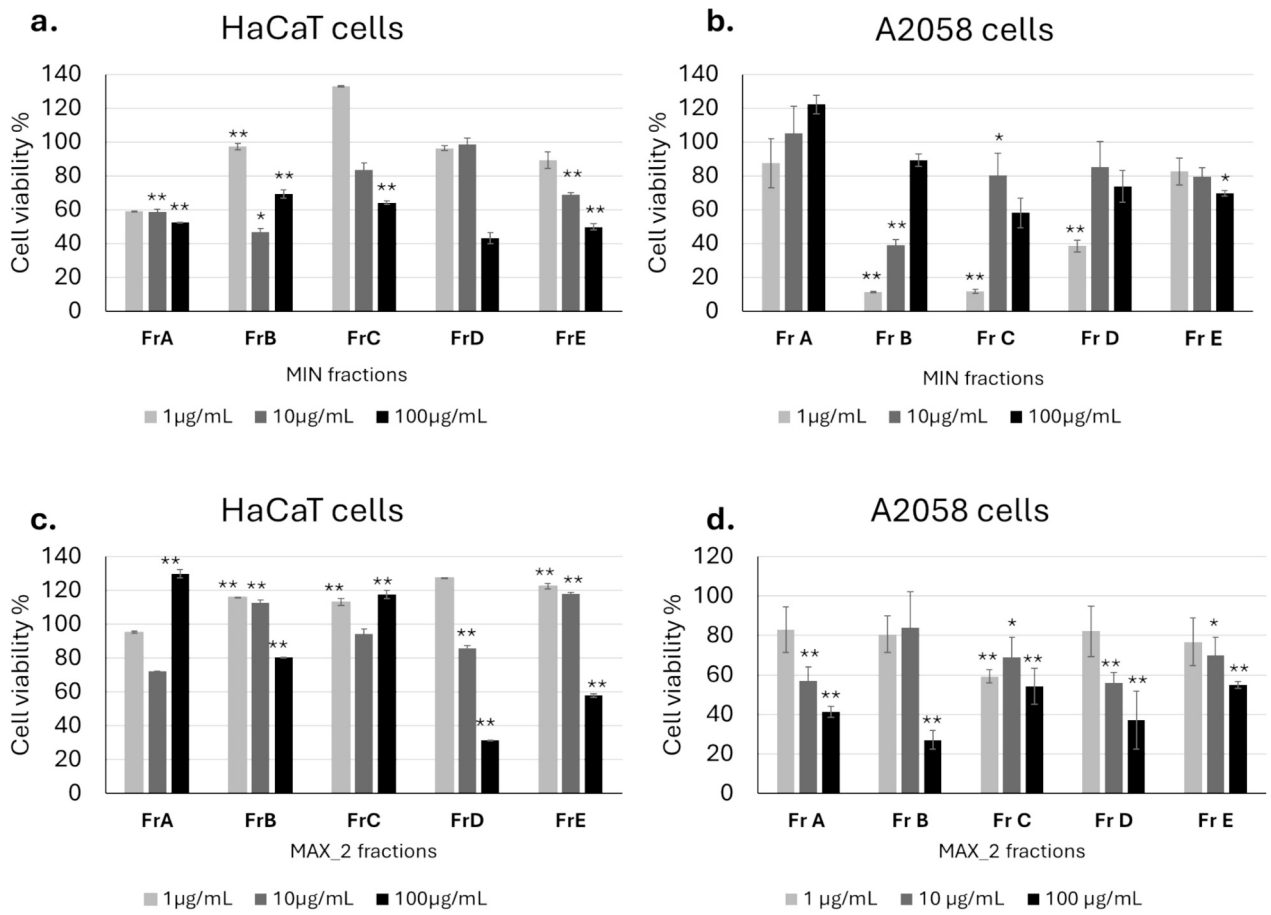

Fig. 7. Antiproliferative effect of *Chlorella*, fractions on HaCaT and A2058 cells after treatment for 48 h with a-b fractions of MIN extract, c-d fractions of MAX_2 extract, Results are expressed as percentage of cell survival after 48 h exposure ($n = 3$), Cell growth was expressed as the percentage cell viability with respect to the cells incubated only with the vehicle (DMSO) for each concentration (1 µg mL^{-1}, 10 µg mL^{-1}, and 100 µg mL^{-1}) $^{**}p < 0.01$, $^{*}p < 0.05$ vs, untreated cells.

trend for almost all fractions, except for fractions D and E tested at 100 µg mL^{-1} which reduced the viability under the 60 % (Fig. 7 b). These two fractions are the ones containing lutein, as already observed.

MIN fractions displayed a significant antiproliferative activity on melanoma cells, particularly fraction B and fraction C at 1 µg mL^{-1} which reduced cell viability below 20 % (Fig. 7 d). This effect is unlikely due to carotenoid content, as this class of compounds is only present in fractions E and D. Fractions A, D and E obtained from MAX_2 extract displayed a dose-dependent activity against melanoma cells (Fig. 7 e). Fraction B also showed a significative reduction in cell proliferation at around 30 % when tested at 100 µg m^{-1}. The two fractions containing lutein, D and E, exhibited a dose-dependent antiproliferative effect both on normal and on melanoma cells. Similar dose-dependent antiproliferative effects on melanoma cells were previously observed for fractions D and E from other algae rich in carotenoids [39]. While the effect of pure lutein on A2058 cell line is not reported in literature, studies on other melanoma cell lines, such as A375 cells, showed no antiproliferative effect [40]. This result is therefore significant, although it is likely due to a combination of bioactives rather than just lutein.

While lutein has a protective effect for human keratinocytes with oxidative damage [41], previous studies have attributed an antiproliferative effect on healthy HaCaT cells at concentration above 15 µM, equivalent to 8.5 µg mL^{-1} [42]. In the present study, the concentration of lutein in the fractions was, at worst (MAX_2 100 µg mL^{-1}), around 1 µg mL^{-1}. However, lutein was not the only compound present in the fraction, which contained other carotenoids and bioactives, the characterization of which was beyond the scope of this work. Additionally, it was also demonstrated that other carotenoids such as β-carotene and cantaxanthin reduced the proliferation of human keratinocytes [43]. Therefore, it is reasonable to assume that the final effect is a concerted action of multiple substances, with lutein being one of the main contributors to the antiproliferative effect on both HaCaT and melanoma cells.

Overall, the fractions deriving from MAX_2 extract showed greater potential as they exhibited antiproliferative activity while demonstrating lower cytotoxicity on normal cells. The described approach, which combined bioprocess optimization by adjusting the growth conditions of an autochthonous strain and assessment of the anticancer properties, is promising for the discovery of new bioactives to be used in pharmaceutics.

4. Conclusions

The lutein production of an indigenous microalgal strain was optimized by using a DoE approach, which revealed for the first time that salt and nitrate concentrations had antagonist effect. In particular, the conditions which increase lutein concentration are at low salinity and high nitrate concentration and at high salinity and low nitrate concentrations. The crude methanolic extracts of the cultured microalgae which showed antiproliferative effect against A2058 melanoma cell line were fractionated. Lutein-containing fractions had a dose-dependent antiproliferative effect both on melanoma and on keratinocyte cells, which is likely due to a concerted action of multiple substances, with lutein being one of the main contributors. This approach, involving a bioprocessing perspective with evaluation of the anticancerogenic potential, has potential in the production of new pharmaceuticals from

microalgae.

CRediT authorship contribution statement

Arima Marchese: Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Serena Lima:** Writing – review & editing, Writing – original draft, Resources, Methodology, Conceptualization. **Valeria Villanova:** Resources. **Eleonora Montuori:** Validation, Investigation, Formal analysis. **Daniele De Luca:** Investigation, Formal analysis. **Chiara Lauritano:** Writing – review & editing, Supervision, Methodology. **Francesca Scargiali:** Writing – review & editing, Funding acquisition.

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.

Acknowledgements

The authors would like to thank the WWF for permission to work in the study area. This work was supported by the Italian Ministry of Education, University and Research, PRIN2022, named "PHOTO-CONTROL- A knowledge-based approach to automatic control and optimisation of photosynthetic bioprocesses", (PRIN 0228WNXKX_002).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104054.

Data availability

Data will be made available on request.

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