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Article

Chemical characterization of the lichen-symbiont microalga *Asterochloris erici* and study of its cytostatic effect on the L929 murine fibrosarcoma cell line

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Abstract: New resources of food, pharmaceuticals or biotechnological products are needed. The huge biodiversity of aero-terrestrial lichen-symbiont microalgae remains unexplored. Viability of these for human consumption demands the demonstration of the absence of toxic effects. In vitro biocompatibility of crude homogenates of axenic microalga *Asterochloris erici*, symbiotic in the lichen *Cladonia cristatella*, was analyzed after treatment of cultured L929 fibroblasts with different doses of microalgal homogenates. The results show that crude homogenates of *A. erici* do not induce fibroblast cytotoxicity but seem to have some cytostatic effect inducing slight cell cycle alterations and intracellular reactive oxygen species (ROS) increase at the highest dose. Carotenoid analysis demonstrates high content of lutein, a xanthophyll with antioxidant and cytostatic properties in vivo. These findings confirm that *Asterochloris erici* can be considered suitable for the development of alimentary or pharmaceutical applications. The cytostatic effects should be further investigated for antitumor agents.

Keywords: bioactivity; phytochemistry; cytostatic, cytotoxicity; microalga

1. Introduction

Lichen thalli are the outcome (holobiont) of close and cyclical symbiotic associations involving, at least, two different organisms, a heterotrophic fungus (mycobiont) and photoautotrophic partners (photobionts) [1,2]. Such photobionts being unicellular green algae (phycobionts, chlorobionts) or/and cyanobacteria (cyanobiont). Lichenization allows the symbionts to colonize diverse terrestrial habitats from the seashores to the high mountains; reaching a large distribution from the tropics to the Polar Regions [3,4]. Their slow growth, long life and adaptation to extreme environmental conditions are supported by the production of numerous protective compounds against different physical and biological stresses [5]. Isolation and culture methods of lichen phycobionts, have been greatly improved in the last decades [6–8].

Green algae, or Chlorophyta, are a huge and diverse phylum of eukaryotic microorganisms. These eukaryotic photoautotrophs should not be confused with the prokaryotic cyanobacteria, also known as blue-green algae. Microalgal biotechnology has developed for various commercial applications. As photosynthetic organisms, algae contain pigments such as chlorophylls together

with high contents of proteins, vitamins and polysaccharides which can be applied for nutrient supplements, cosmetic purposes or human or animal consumption [9–12].

Some species of microalgae and lichens are natural sources of bioactive compounds such as antibiotics, antioxidants or toxins with remarkable biotechnological potential [13–15]. Moreover, many of these microalgal compounds may act as antiviral, antitumor, anti-inflammatory and antimicrobial agents with a marked selectivity in a variety of molecular targets, making them attractive for the pharmaceutical industry [16–18]. They have also garnered attention for their potential in the food industry as antioxidants and antimicrobial additives in a scenario where additives such as parabenes or alkylphenols are in question due to the concern on their endocrine disruption potential [19]. Several of the aforementioned compounds are polysaccharides, carotenoids or unsaturated long chain fatty acids bearing chemical structures not found elsewhere – or which are present at much higher concentrations than in other natural sources [20]. In particular, the biotechnological importance of lipid compounds is beginning to attract strong attention in applied research (i.e. biofuels) [21]. Likewise, it has being established that some lichen microalgae show original and differential combinations of polysaccharides in their cell walls and extracellular polymers with immunostimulating capabilities of biotechnological interest [18,22].

However, the huge biodiversity of microalgae remains, to date, largely unexplored and unexploited [23], so they represent a unique opportunity to obtain novel or known metabolites at low cost. At present, the industrial biotechnological potential of free-living aquatic algae as *Chlorella, Chlamydomonas* and *Dunaliella* spp. is being developed [24]. Nevertheless, the potential of using aero-terrestrial algae associated with lichens (known as phycobionts) has never been studied. *Asterochloris erici* (formerly known as *Trebouxia erici* Ahmadjian, 1960) is a phycobiont isolated from the north American endemic lichen *Cladonia cristatella* Tuck., belonging to the class Trebouxiophyceae (Chlorophyta) that has been molecularly and phylogenetically well described by Škaloud and Peksa [25].

The possible use of this species for biomedical, cosmetic and alimentary purposes requires the development of biocompatibility studies in order to demonstrate the absence of toxic or injurious effects on mammalian cells. The toxicity of a new agent can be analyzed by in vitro tests with cell cultures, by in vivo experiments with animal models, and by clinical studies in humans. In vitro studies with different cell types are the first step performed to evaluate the possible damage induced by a new compound in contact with cells. These in vitro biocompatibility assays involve maximum standardization and control of the experimental conditions while reducing costs and ethical concerns.

The objective of this study is to determine the nutritional properties, the biocompatibility and the absence of induced toxicity in vitro of one strain (SAG 32.85 = UTEX 911) of the lichen microalga *Asterochloris erici* (Ahmadjian) Škaloud and Peksa, as a preliminary step for animal testing.

2. Experimental Section

2.1. Instruments

A Qiagen tissuelyser was used for the homogenization of microalgae suspensions. Optical microscopy was performed with a Leitz Labovert FS inverted microscope, equipped with a Leica DC 300 digital camera. Cells were examined by a LEICA SP2 confocal laser scanning microscope. A FACScalibur Becton Dickinson flow cytometer was used for Intracellular reactive oxygen species (ROS) content and cell viability. Cell cycle analysis and apoptosis detection were done with a LSR Becton Dickinson flow cytometer and calculations done with the CellQuest Program of Becton Dickinson. Chromatographic analysis of pigments was performed by ultra-high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) with photodiode-array detection (UHPLC-PDA). A Rotofix 32 centrifuge (Hettich zentrifugen, Germany) was used for the extraction procedure and a Cary 60 UV-Vis spectrophotometer (Agilent, Spain) was used for all spectrophotometrical methods.

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2.2. Chemicals and biochemicals

Dulbecco's Modified Eagle's Medium (DMEM) and propidium iodide (PI) were bought from Sigma Chemical Company (St. Louis, MO, USA). Fetal Bovine Serum (FBS) was purchased to Gibco (BRL). L-glutamine, penicillin and streptomycin were from BioWhittaker Europe (Belgium). FITC-phalloidin and DAPI (4'-6-diamidino-2'-phenylindole) were from Molecular Probes while Hoechst 33258 was purchased from PolySciences, Inc. (Warrington, PA). 2′,7′-dichlorofluorescein diacetate (DCFH2-DA) was obtained from Serva, Heidelberg/Germany. Carotenoid and chlorophyll standards were purchased from DHI LASB Products (Hørsholm, Denmark). Acetonitrile and methanol HPLC grade were from Fisher Scientific (New Hampshire, USA). Gallic acid, Folin Ciocalteu Reagent (FCR), 2,2-diphenyl-1-picrylhydrazyl (DPPH•), and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Madrid, Spain). Ethanol and methanol were purchased from Scharlau (Barcelone, Spain), while sodium carbonate was obtained from Panreac (Barcelone, Spain). Water (resistance 18.2 MΩ cm) was obtained from a Millipore Milli-Q-System (Billerica, MA, USA). Other chemicals used were of analytical grade.

2.3. Strain maintenance, culture conditions and preparation of microalgae homogenates

An axenic strain of the lichen photobiont *Asterochloris erici* (Ahmadjian) Škaloud and Peksa (SAG 32.85 = UTEX 911) was used for this study. In order to obtain material for in vitro biocompatibility studies, microalgal cultures were carried out for 15 days in BBM3N as previously described [26]. The cultures were then centrifuged at 15 557g for 10 min and the cell pellets were frozen and stored at -20ºC. The day before biocompatibility testing, microalgae were homogenated at 1 mg/mL in complete DMEM (DMEM supplemented with 10% FBS, 1 mM L-glutamine, 200 µg/mL of penicillin and 200 µg/mL of streptomycin) with a tissuelyser (1 mm glass beads, 40 Hz, 15 min), sterlised in an autoclave and subsequent frozen-stored. Working dilutions in complete DMEM containing 0.1, 0.01 and 0.001 mg/mL were prepared just before their application to mammalian cells.

2.4. Proximate chemical composition determination

Proteins, ash, moisture and nitrogen contents in fresh *Asterochloris erici* (Ahmadjian) Škaloud and Peksa were determined by standard AOAC [27] methods. The moisture content was determined by oven method at 105ºC until a constant weight was obtained. The ash content was gravimetrically determined after heating at 550ºC for 24 h in a muffle furnace. Protein content was calculated by converting the nitrogen content, determined by Kjeldahl method (6.25 x N). Total lipids were extracted with chloroform/methanol (2:1, v/v) according to Sánchez-Machado, López-Cervantes, López-Hernandez & Paseiro-Losada, [28]. Total carbohydrate content was determined as the weight difference using protein, lipid, moisture and ash content data. All measurements were performed in triplicate.

2.5. Antioxidant activity and total phenolic compounds

2.5.1. Extraction procedure

Extraction procedure was accomplished from freeze-dried microalgae, according to Goiris et al. [29] with some modifications. Extractions were performed in the dark at room temperature. 100 mg of biomass were weighed by triplicate in falcon tubes where the extraction process took place. For this purpose, 2 mL of the extraction solvent (ethanol/water, 3/1, v/v) were added and the mixture was crushed in a mortar, to break the cell walls, and stirred for 20 min. Then, samples were centrifuged at 6000 rpm for 10 min. After that, a second extraction of the resulting pellets was completed using another 2 mL of ethanol/water mixture, and the combined supernatants for each sample were filtered through nylon membrane filters (0.45 µm pore size) and, finally, maintained at -18 °C until the beginning of the analysis.

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2.5.2. Radical Scavenging Activity (RSA)

The free radical DPPH• scavenging activity of extracts was evaluated by a slightly modified colorimetric method proposed by Brand-Williams, Cuvelier, & Berset [30]. In order to estimate the RSA, firstly, a DPPH• solution (40 ppm, w/v) was freshly prepared in methanol. Then, 3.9 mL of this DPPH• solution were mixed with 0.1 mL of the extract sample or 0.1 mL of extraction solvent (blank sample) and the mixture was shaken in a vortex. The reaction mixture was left for 60 min at room temperature in the dark. After an incubation period, the absorbance was measured at 517 nm to determine the concentration of remaining DPPH• radical. Therefore, RSA (%) was calculated using the following equation:

$$RSA\ (\%) = \frac{(A_{DPPH} - A_{sample})}{A_{DPPH}} \times 100 \quad (1)$$

where ADPPH is the absorbance of the DPPH• radical in blank sample and Asample is the absorbance of the DPPH• radical in the biomass extract (sample). A standard calibration curve was prepared with Trolox at a concentration of 0.5 - 400 ppm (w/v). Finally, RSA of the samples was expressed as Trolox equivalents (TE) in μmol / g of sample (dry weight basis, DW).

2.5.3. Total phenolic content

The concentration of total phenolics compounds (TPC) in extracts was determined according to the Folin-Ciocalteu method [31] with some modifications. A 0.75 μL aliquot of the sample extract or extraction solvent (blank sample) was mixed with 645 μL of Milli-Q water and 30 μL of FCR. Next, 75 μL of 20% (w/v) sodium carbonate and 675 μL of Milli-Q water were added and the total solution was mixed briefly in a vortex. The mixture was incubated for 60 min at room temperature in darkness. At the end of the incubation period, absorbance was measured at 725 nm in a a UV-vis spectrophotometer. A standard calibration curve was prepared with gallic acid at a concentration of 10 - 500 ppm (w/v). The results were expressed as mg gallic acid equivalents (GAE) / g of sample (DW).

2.6. Carotenoids and chlorophylls extraction and chromatographic analysis

Microalgae lyophilized powder was frozen with liquid nitrogen and then homogenized in a tissuelyser with stainless steel beads (2 min, 40 Hz). Pigments were extracted from 5 mg of microalgae powder using 1 mL of acetone (95%). Extraction was performed in a ultrasonic bath for 5 min, twice or up to complete extraction of pigments. After extraction, samples were centrifuged at 16 200 g for 5 minutes and then the extracts were filtered through nylon membrane filters (0.22 μm pore size).

Carotenoid composition was determined according to García-Plazaola & Becerril (2001) [32] with some modifications. The separation was achieved on an Agilent 46 x 10 mm, 2.7 mm on a 150 mm x 4.6 mm, 5 μm particle size, Poroshell HPH-C18 column (Tecknokroma, Barcelona, Spain). The mobile phase consisted of two components: solvent A, acetonitrile:methanol:tris buffer (0.1 M, pH 8) (83:2:15, v/v/v); and solvent B, methanol:ethyl acetate (68:32, v/v). The pigments were eluted using a linear gradient from 100% A to 100% B for the first 7 min, followed by an isocratic elution with 100% B for the next 4 min. The mobile phase flow rate was 1 mL/min. The injection volume was 10 μL. The on-line UV-vis spectra was recorded from 350 to 800 nm. Absorbance was meausured at 445 nm for all carotenoids (with exception of astaxantin measured at 477 nm) and at 631 nm and 664 nm for chlorophyll b and a, respectively. Carotenoids (β-carotene, neoxanthin, fucoxanthin, violaxanthin, anteraxanthin, astaxanthin, zeaxanthin and lutein), chlorophyll a and chlorophyll b were quantified in the the microalgae extract using molar absorptivity. The results were expressed in μg / g of sample (DW) and all measurements were performed in triplicate.

2.7 Cell culture for the in vitro biocompatibility studies

L929 mouse fibroblasts were seeded on 6 well culture plates at a density of 105 cells/mL in complete DMEM under a CO_2 (5%) atmosphere and at 37 °C for 24 h. To evaluate the effect of *A. erici*

 doi:10.20944/preprints202105.0459.v1

on fibroblasts, different doses (0.1, 0.01 and 0.001 mg/mL) of the lichen microalga crude homogenate were added to the culture medium and cells were maintained in the presence of these homogenates for 24 h. After each treatment, the attached fibroblasts were washed with PBS and harvested using 0.25% trypsin-EDTA solution for 15 min. The reaction was stopped with culture medium and cells were counted with a Neubauer hemocytometer for the analysis of cell proliferation, centrifuged at 310 g for 10 min and resuspended in fresh medium for the analysis of viability, cell cycle, apoptosis and intracellular ROS content by flow cytometry. Controls with cultures without material extracts were carried out in parallel.

2.8. Cell morphology studies

2.8.1. Optical microscopy

Optical microscopy studies were carried out on L929 mouse fibroblasts after 24 h culture in the presence of different doses (0.1, 0.01 and 0.001 mg/mL) of *A. erici*. Cells were examined with a inverted microscope equipped with a digital camera. Controls without material were carried out in parallel.

2.8.2. Confocal microscopy

Confocal microscopy studies were performed as described elsewhere [33]. Briefly, L929 fibroblasts were cultured for 24 h on glass coverslips in the presence of different doses (0.1, 0.01 and 0.001 mg/mL) of *A. erici* crude homogenates. Controls without extracts of microalgal homogenates were carried out in parallel. After fixation with 3.7% paraformaldehyde in PBS for 10 min, samples were washed with PBS and permeabilized with 0.1% Triton X-100 for 3 to 5 min. The samples were then washed with PBS and preincubated with PBS containing 1% BSA for 20 to 30 min. Then, cells were incubated for 20 min with FITC-phalloidin (1:40, v/v) to stain F-actin filaments. Samples were then washed with PBS and the cell nuclei were stained with 3 μM DAPI in PBS. After staining and washing with PBS, cells were examined by a confocal laser scanning microscope. The fluorescence of FITC was excited at 488 nm and the emitted fluorescence was measured at 491-586 nm. DAPI fluorescence was excited at 405 nm and measured at 420–480 nm.

2.9. Cell cycle analysis and apoptosis detection

Cell cycle analysis and apoptosis detection studies were performed as described previously by Matesanz et al. (2013). Briefly, cells were incubated with Hoechst 33258 (5 μg/mL), ethanol (30%), and BSA (1%) in PBS, used as a nucleic acid stain, for 30 min at room temperature in darkness. The fluorescence of Hoechst was excited at 350 nm and the emission was measured at 450 nm in a flow cytometer. The cell percentage in each cycle phase: G0/G1, S and G2/M was calculated with the CellQuest Program of Becton Dickinson and the SubG1 fraction was used as indicative of apoptosis.

2.10. Intracellular reactive oxygen species (ROS) content and cell viability

The assessment of intracellular ROS content and cell viability studies have been described in detail by Matesanz et al. (2013). Briefly, cells were incubated at 37ºC for 30 min with 100 μM DCFH2-DA. To measure the intracellular ROS, the fluorescence of DCF was excited by a 15 mW laser tuning to 488 nm and the emitted fluorescence was measured with a 530/30 band pass filter in a flow cytometer. Cell viability was determined by PI exclusion test and flow cytometry after addition of PI (0.005% in PBS) to stain the DNA of dead cells.

2.11. Statistics

Data are expressed as means ± standard deviations of a representative of three experiments carried out in triplicate. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 19 software. Statistical comparisons were made by analysis of variance (ANOVA). Scheffé test was used for post hoc evaluations of differences among groups. In all the statistical evaluations, $p < 0.05$ was considered as statistically significant.

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3. Results

3.1. Proximate composition, antioxidant activity and total phenolic compounds

The proximate chemical composition of fresh *A. erici* is shown in Table 1. The moisture content was 75 ± 1% WW (wet weight). The ash content was 5 ± 1 % DW, value of the same order of magnitud as those reported for the green microalgae chlorella (5,8%) and *Botryococcus braunii* (5,4%) [34]. Nevertheless, it is important to point out that the low ash content found in *A. erici* is similar to that of land plants (5-10%) (Rupérez, 2002) but interestingly it seems very low when compared to the data reported for some red macroalgae like seaweed *Gracilaria changii* (42% WW) [35]. The protein content was 37 ± 1 % DW, being higher than in the green microalgae such as *Chlorella vulgaris* and *Haematococcus pluvialis*, and several seaweeds as *Sargassum vulgare* or *Gracilaria changii* [35,36], but in the same order of magnitude as those reported for the microalgae spirulina, *Tetraselmis chuii* and *Chlorella* sp. by other authors [34]. This protein content was similar than other proteinic vegetable foods, such as soybeans, indicating that *A. erici* may become a potential source of this nutrient. The total lipid content was 10 ± 2 % DW, that is higher than these values observed for other algae, in general always less that 6% (Herbreteau, Coiffard, Derrien & De Roeck-Holtzhauer, 1997; Sánchez-Machado, López-Cervantes, López-Hernandez & Paseiro-Losada, 2004; Gómez-Ordoñez, Jiménez-Escrig & Ruperez, 2010; Peinado, Girón, Koutsidis & Ames, 2014; Chan & Matanjun, 2017). However, this lipid value can be considered low, taking into account that some microalgae have lipid content near to 41% WW (*Haematococcus pluvialis*) or 49% WW (*Nannochloropsis granulata*). Finally, the total carbohydrate content (determined as the weight difference using protein, lipid, moisture and ash content data) was 48% DW, that is in the range of 4 – 76% (carbohydrate plus fiber) reported for various authors for different seaweed species (Marinho-Soriano, Fonseca, Carneiro & Moreira, 2006).

Table 1. The proximate composition of *Asterochloris erici*.

Composition	Concentration (% dry weight)
Moisture	75 ± 1
Ash	5 ± 1
Protein	37 ± 1
Lipids	10 ± 2

Data are the mean of three independent samples ± standard error.

The TPC of ethanolic extracts *A. erici* (see section 2.5.1) was estimated and 7.5 ± 0.5 mg GAE/g DW were found. The value of TPC was high in comparison with other green microalgae such as chlorella, between 0.75 – 2.21 mg GAE/g DW [29] measured in a similar extraction procedure. The antioxidant activity was analyzed by the DPPH method, that measures free radical scavenging ability. High DPPH values might be attributed to high levels of phenolic compounds. In this sense, the antioxidant values exhibited by *A. erici* in the present study (10.6 ± 0.6 µmol TE/g WW) show a good correlation with TPC value clearly indicate that the antioxidant activity of *A. erici* was due to its phenolic content.

3.2. Carotenoid and chlorophyll content

The values of chlorophyll a and b in *A. erici* are shown in Table 2. The value of chlorophyll b is higher than that of chlorophyll a. In relation with carotenoids, neoxanthin, violaxanthin, antheraxanthin and lutein were detected with a clear predominance of lutein (Table 2). The quantification of this pigment rendered values of 1.2 mg/g WW, in the same order of magnitude as other chlorophytes with commercial interest for cancer and retinal degeneration prevention [37]. This predominance of lutein has also been observed in *Chlorella vulgaris* [38] where it has been found to be bioavailable when administered as whole algal dietary supplements, increasing erythrocyte lutein content [39].

 doi:10.20944/preprints202105.0459.v1

Table 2. Chlorophyll and carotenoid composition of *Asterochloris erici*.

Pigment	Concentration (μg / g microalga dry weight)
Chlorophyll a	507 ± 41
Chlorophyll b	6105 ± 580
β- Carotene	268 ± 20
Neoxanthin	165± 12
Fucoxanthin	n.d.
Violaxanthin	267 ± 16
Antheraxanthin	57 ± 6
Astaxanthin	106 ± 9
Zeaxanthin	n.d.
Lutein	1211 ± 119

Data are the mean of three independent samples ± standard error. N.d. under detection limit.

3.3 Cellular cytocompatibility

Cell viability, morphology, proliferation, cell cycle, apoptosis and intracellular free radicals of L929 mouse fibroblasts cultured for 24 h in the presence of different doses of *A. erici* crude homogenates were analyzed in order to evaluate the absence of toxic effects on mammalian cells.

Figure 1 shows optical microscopy images of L929 cells after 24 h culture with 0.1 mg/mL of *A. erici* homogenate. Due to mechanical resistance of the microalgal cell walls, the envelopes of some algae can still be seen on the L929 monolayer. Fibroblast membranes seemed intact and no signs of necrosis were seen in cultures. In addition, cell counts were performed to confirm these observations. Percent viability of cells was the same with respect to untreated control regardless of applied dose (Figure 2B). Instead, a dose dependent effect was observed when studying the cell proliferation and the number of cells was progressively reduced becoming a significant effect at higher doses (Figure 2A). In summary, these studies revealed that the crude homogenates of *A. erici* do not affect cell viability, but they have a significant effect on cell proliferation as higher doses of it are applied.

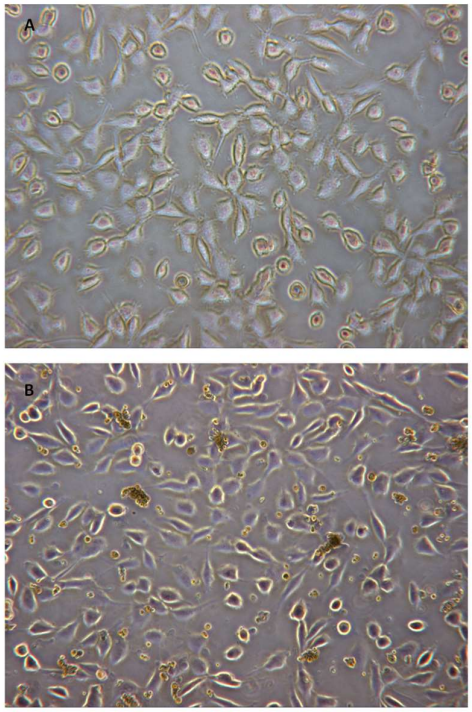

Figure 1. Morphology evaluation of L929 fibroblasts. A) Control. B) Treated with 0.1 mg/ml of the lichen microalgal crude homogenates for 24 h. Cells were seeded at a density of 2 x 105 cells/ml. Magnification 200X. Bright field.

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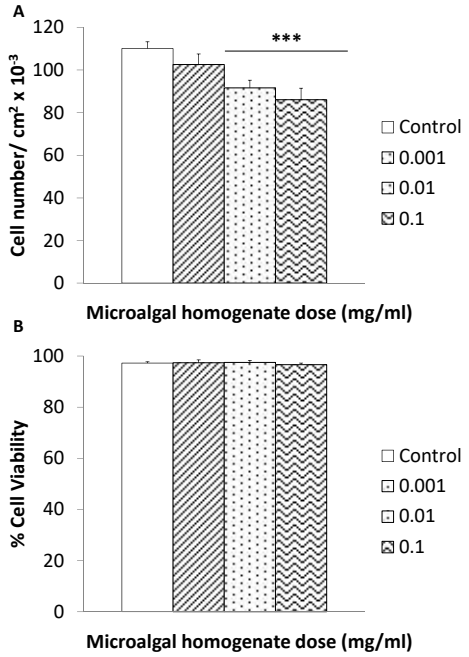

Figure 2. Cytotoxicity. L929 fibroblasts treated with different doses of the lichen microalgal crude homogenates (mg/ml) for 24 h. A) Cell proliferation. B) Cell viability. Data are expressed as means + standard deviations. Statistical significance ***p<0.005.

To assess the potential cytotoxicity of *A. erici*, intracellular free radicals were measured by flow cytometry in L929 cells from cultures treated with different doses of microalgal crude homogenates. The results are shown in Figure 3 where a significant increase in free radicals with the highest dose applied is observed.

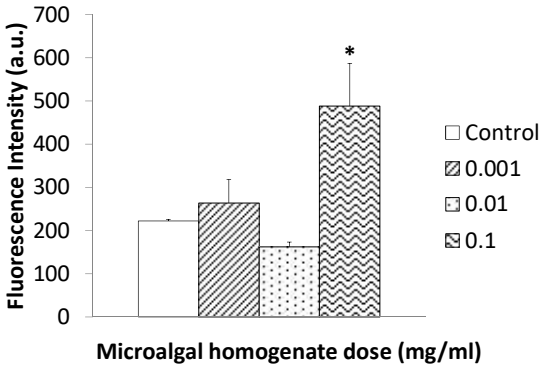

Figure 3. Intracellular ROS. Level of intracellular reactive oxygen species (ROS) in L929 fibroblasts treated with different doses of the lichen microalgal crude homogenates (mg/ml) for 24 h. Data are expressed as means + standard deviations. In each sample, 10 000 cells were analyzed. Statistical significance *p <0.05.

The studies of cell cycle and apoptosis detection (Figure 4) showed that the levels of L929 fibroblasts in G0/G1 phase increased very slightly, but the differences are not significant. However,

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the levels of cells in S phase decreased with all the concentrations of crude microalgae homogenate, the lowest dose achieving a statistically significant decrease. Levels of cells in subG1 fraction, usually taken as an apoptosis indicator, are slightly increased in a dose dependent manner.

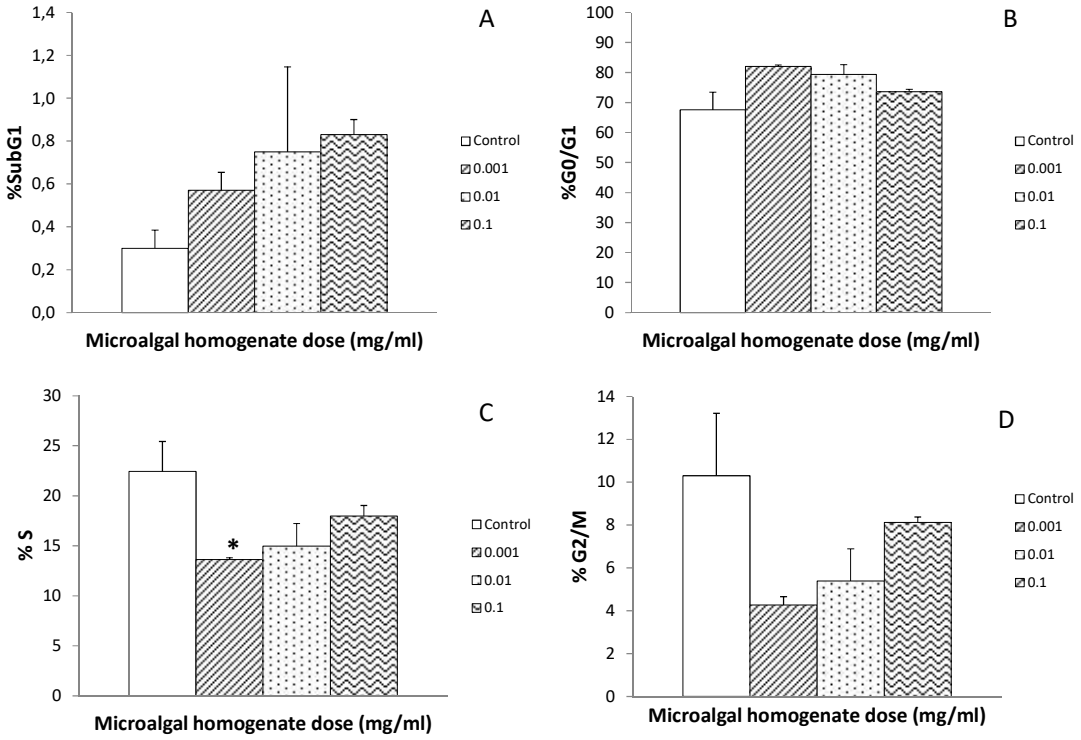

Figure 4. Cell cycle and apoptosis. Cell cycle analysis of L929 fibroblasts treated with different doses of the lichen microalgal crude homogenates (mg/ml) for 24 h. A) SubG1 fraction, indicative of apoptosis, B) G0/G1, C) S and D) G2/M phases of a representative of three repetitive experiments. In each sample, 10 000 cells were analyzed. Statistical significance *p <0.05.

An assessment of the cell morphology and actin cytoskeleton architecture was performed by confocal microscopy (Figure 5). The photographs show that there is no apparent alteration of nuclear chromatin or actin filaments of the cytoskeleton at any of the concentrations studied (Figure 5B, C and D) compared with controls (Figure 5A). At higher concentrations the red autofluorescence of chlorophyll can be observed (Figure 5C and D).

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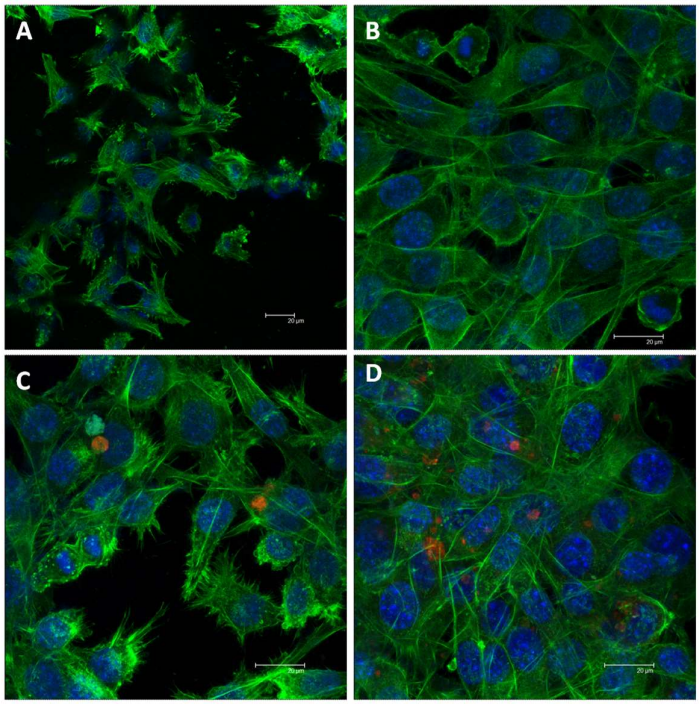

Figure 5. Cytoskeleton ultrastructure. Confocal microscopy images of L929 fibroblasts cultured for 24 h in the presence of different doses of *Asterochloris erici* crude homogenates. Cells were stained with DAPI for the visualization of the cell nuclei in blue and FITC-phalloidin for cytoplasmic F-actin filaments in green. Red emission corresponds to microalgal chlorophyll autofluorescence. Overlay of the three fluorescence channels. A) Control fibroblasts. B), C) and D) fibroblasts cultured with 0.001 mg/ml, 0.01 mg/ml 0.1 mg/ml of microalgal homogenates, respectively (magnification: see magnitude bar).

4. Discussion

To our best knowledge this is the first study on the biocompatibility of lichen microalgae on mammalian cells using a systematic standardized in vitro approach. Our results demonstrate that whole lichen microalgal homogenates do not induce cytotoxicity or morphological alterations. However, they induce an intracellular ROS increase at the highest dose, and they can slow down the rate of cell proliferation preventing their entrance in S phase and therefore the fraction of cells attaining G2/M phases. Chlorella carotenoids, and more specifically xanthophylls, have been found to inhibit cancer cells proliferation [38,40,41]. In order to learn if this effect could be related with a possible high xanthophyll content, a chromatographic analysis of *A. erici* carotenoid composition was carried out, and results revealed the presence of an important amount of lutein (Table 2). Xanthophylls are a group of carotenoids containing at least one O atom known to play a crucial role in photoprotection of photosystem II (PSII) from ROS under excess light. Lutein is a tetraterpenoid ($C_{40}H_{56}O_2$) alternating single and double carbon-carbon bonds with attached methyl side groups with a predominant function in the deactivation of 3Chl* [42]. The presence of a hydroxyl group at both ends of the molecule distinguishes lutein (and its stereoisomer zeaxanthin) from other carotenoids and is responsible for its high chemical reactivity [43]. Since xanthophyll biosynthesis does not occur in animal cells, their primary intake depends on diet (or supplementation), being lutein intake from dietary sources strongly associated with plasma concentrations [reviewed in 44].

Apart from its best-known pivotal role against oxidative damage of retinal tissues, lutein has anti-inflammatory, immunomodulatory, neuroprotective, and antiangiogenic properties. It exerts several antioxidant activities from upregulation of antioxidant enzymes to direct antioxidant action

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such as inhibition of membrane lipids peroxidation. In lipopolysaccharide- (LPS-) stimulated macrophages, lutein has been found to decrease intracellular hydrogen peroxide (H2O2) accumulation by scavenging superoxide anion and H_2O_2. The administration of lutein affords neuroprotective effects against cerebral ischemic injury increasing reduced/oxidized GSH ratio as well as the enzymatic activities of superoxide dismutase, GSH-peroxidase and catalase. It has been shown to inhibit the expression of proinflammatory genes by suppressing nuclear factor NF-κB translocation and reducing the secretion of cytokines (TNF, IL-1) and arachidonic acid metabolites [45].

Cladonia lichens present an important biotechnological potential [46] and thus Cladonia's phycobiont *Asterochloris erici* is also likely to possess important bioactivity. This species has been relatively well characterized at the genetic and physiological levels and is readily available from various international collections (Škaloud and Peksa, 2010). Although experiments with algal-free mycobionts have demonstrated that many lichen secondary metabolites are synthesized mainly by the fungal component, phycobionts belonging to specific species of green microalgae may contribute to a lichen's secondary metabolite profile. Furthermore, lichenized fungi produce biologically active metabolites only in association with suitable algal partners [47]. These substances show bioactive properties being considered as promising sources of antibiotic, antioxidant and anticancer drugs [17,18,48,49].

Symbiont eukaryotic microalgae have been shown to produce a high number of new substances with high biological activity on various mammalian cells. For example, maitotoxin causes the release of neurotransmitters from pheochromocytoma cells, or muscle contraction in a Ca -dependent manner [50,51]. Zooxanthellatoxins derived from Symbiodinium, coral phycobionts, have been shown to cause platelet aggregation and aorta contraction through the regulation of Ca^{2+} levels and permeability and the enhancement of tyrosine phosphorylation of p42 mitogen-activated protein kinase [52,53]. Albeit the best studied symbiotic microalgae are freshwater and marine organisms, all symbiotic organisms seemingly share certain biological traits such as their ability to grow in low nutrient habitats and to exchange substances with other organisms very distant phylogenetically as animals, bacteria or archaea [54,55] rendering them exceptional sources of bioactive products.

The results presented here indicate, for the first time, that a lichen microalgal species is not toxic or induce acute damage on mammalian cells in culture although they induce slight cytostatic effect together with a moderate increase of intracellular free radicals at the highest dose applied. Microalgal compounds such as antioxidants and polysaccharides have been demonstrated to modulate carcinogenesis in the gastrointestinal tract [56] and augment antitumor resistance [57]. Alcoholic extracts of *Chlorella sorokiniana* decreased cell viability, induced apoptosis and oxidative stress in a human hepatoma cell line [40]. Polysaccharides from *Chlorella pyrenoidosa* presented significant antitumor activity against A549 human lung adenocarcinoma cells [58]. Therefore, this slight cytostatic activity should be further studied in tumor cells in order to test algal components potential as preventive nutraceuticals or even for anticancer drugs.

These preliminary results indicate that the lichen microalga *Asterochloris erici* is a potential candidate for various biotechnological applications. Perhaps the most immediate ones could be the use of lichen microalgae in dietary supplements. The food supplements containing microalgae known as Blue-Green Algae Supplements (BGAS), are mainly derived from prokaryotic organisms such as spirulina (reclassified as *Arthrospira platensis*), *Arthrospira maxima* and *Aphanizomenon flos-aquae* species (cyanobacteria). Whereas spirulina is "Generally Recognized As Safe (GRAS)" by the Food and Drug Administration of the USA, both spirulina and *A. flos-aquae* are listed in Annex B of EFSA "Compendium of botanicals" reported to contain naturally occurring substances of possible concern. In natural environment (e.g. lakes) they can coexist with other potentially toxic strains of cyanobacteria, such as *Microcystis* sp. which share the same habitat, causing BGAS product contamination as evidenced in different countries [59]. In addition, *A. flos-aquae* has been found to produce neurotoxins [60].

On the other hand, studies on microalgae and microalgal compounds safety have been frequently performed in vivo using murine models. There is a growing social and ethical concern for

the use and sacrifice of animals for biological tests and it is mandatory to find and apply alternative methods in order to limit animal experimentation to a minimum. An in vitro approach is in better accordance with this new scenario since it avoids animal testing and constitutes a rapid, reliable and cost-effective method for the assessment of natural products bioactivity providing, at the same time, insights into the mechanisms of action at the cellular level.

5. Conclusions

These results, altogether, present the first experimental evidence that the crude homogenates of the lichen microalga *Asterochloris erici* do not induce acute toxicity in mammalian cells rendering them suitable for whole animal testing. This microalga seems to have a slight cytostatic effect, delaying cell proliferation. The high content of the xanthophyll lutein, a well-known pigment with potent antioxidant and cancer prevention properties, could be involved in this cytostatic effect as demonstrated for other chlorophytes. In addition, *Asterochloris erici* has appreciable protein content and low total lipid content, making it an important low-fat functional ingredient in the food industry.

Author Contributions: M. T. Portolés, E. Barreno and M. Catalá designed the study. M. T. Portolés and E. Barreno were responsible for funding acquisition. E. Barreno, M. Villa and M. Catala obtained microalgal biomass. I. Sierra and S. Morante-Zarcero designed and performed the analytical and nutrient studies of *A. erici* . C. Matesanz and J. Linares performed the cellular assays, cytometric and confocal studies under the direction and supervision of M. T. Portolés. Manuscript was written and revised by all the authors

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