Original Research Article

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Lipid extract of microalgae *Chlorella vulgaris* and *Arthrospira platensis* as a bioactive ingredient

Purificación García-Segovia[1], Zaida Natalia Uribe-Wandurraga[1], Marta Igual[1], José Luís Mullor[2] and Javier Martínez-Monzó[1]

Abstract

A growing market for seaweed products has emerged in the last 20 years, with microalgae and their extracts representing a source of high-value chemicals. In this study, antioxidative and anti-inflammatory activities of lipid fraction extract from *Chlorella vulgaris* (Chlorella) and *Arthrospira platensis* (Spirulina) on Normal Human Dermal Fibroblasts (NHDF cells) were evaluated. Treatments with Chlorella and Spirulina lipid extracts in NHDF cells at 0.1, 0.5, and 1% significantly protect UVA-induced damage by decreasing oxidative stress in the form of reactive oxygen species (ROS) but do not display any significant protection from HEV-induced damage. Chlorella or Spirulina lipid extracts at 0.1 and 0.5% display significant anti-inflammatory effects by inhibiting the 2-propanol-induced IL-6 expression on NHDF cells *in vitro* but showed no decrease in TNF-α expression. Chlorella and Spirulina lipid extracts present antioxidant and anti-inflammatory activity on NHDF cells.

Keywords

Chlorella, Spirulina, lipid extract, antioxidant, anti-inflammatory

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INTRODUCTION

Microalgae and their extracts represent a comprehensive novel source of compounds with biological activity (Jacob-Lopes et al., 2019). Several species are grown on a large scale. They are used as nutritional supplements, including *Chlorella vulgaris* (Chlorella) and *Arthrospira platensis* (Spirulina) extracts of β-carotene, astaxanthin, and long-chain polyunsaturated fatty acids (Nazih and Bard, 2018; Raposo and De Morais, 2015; Rodriguez-Garcia and Guil-Guerrero, 2008; Vo et al., 2015). Chlorella is a popular microalga commercially cultivated worldwide and rich in bioactive compounds, including carotenes, astaxanthin, lutein, and fucoxanthin. In addition, authors also report that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), bioactive compounds of functional values, are also present (Guedes et al., 2011). The composition and characterization of the lipid profile of Chlorella is well known (El-Chaghaby et al., 2019; Sijil et al., 2023; Tokuşoglu and Ünal, 2003). The lipid fraction of freeze-dried Chlorella is around 13.3%, being the primary fatty acids; oleic acid (33.14%), docosahexaenoic acid (20.9%), palmitic acid (15.4%), linoleic acid (9.7%), stearic acid (6.2%), EPA (3.2%) docosapentaenoic acid (3.1%), and alpha-linolenic acid (1.9%) (Tokuşoglu and Ünal, 2003). The monounsaturated (MUFA), polyunsaturated (PUFA), and saturated (SFA) fatty acid contents of Chlorella are 35.4%, 38.9%, and 22.2%, respectively (Tokuşoglu and Ünal, 2003). Chlorella is also identified as a direct scavenger of free radicals causing oxidative stress; according to

[1]Food Technology Department, Universitat Politècnica de València, Valencia, Spain
[2]Bionos Biotech, Biopolo La Fe, Valencia, Spain

Corresponding author:
Javier Martínez-Monzó, Food Technology Department, Universitat Politècnica de València, Camino de Vera s/n, Valencia 46022, Spain.
Email: xmartine@tal.upv.es

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Rodriguez-Garcia and Guil-Guerrero (2008), the microalga has higher antioxidant power than synthetic antioxidants commonly used in foods and animal production. The microalga is also a natural growth promoter, immune booster, and tissue rebuilder (Conde et al., 2021, 2022; Katiyar and Arora, 2020; Matos et al., 2020; Tabarzad et al., 2020; Wu et al., 2021). Spirulina is rich in protein with high quality and almost all essential amino acids. In addition, it is a rich source of minerals, vitamins, and antioxidants including phycocyanin, carotenoids, tocopherols, and phenolic compounds (Batista et al., 2013). Spirulina contains around 7% lipid, mostly composed of essential poly-unsaturated fatty acids like γ-linolenic acid, α-linoleic acid, EPA, and DHA. The lipid fraction of freeze-dried Spirulina was composed by oleic acid (34%), palmitic acid (27%), linoleic acid (12%), stearic acid (7%), g-linolenic acid (4.6%), docosahexaenoic acid (3%), EPA (2.5%), and palmitoleic acid (2.1%) (Tokuşoglu and Ünal, 2003). The monounsaturated (MUFA), polyunsaturated (PUFA), and saturated (SFA) fatty acid contents of Spirulina are 38%, 25%, and 35%, respectively (Tokuşoglu and Ünal, 2003). Thanks to its nutrient content, Spirulina has been represented as a nutraceutical food, as it has been proposed as an effective supplement, improving antioxidant status and protecting cancer incidence (Conde et al., 2021, 2022; Guldas et al., 2021; Katiyar and Arora, 2020; Matos et al., 2020; Tabarzad et al., 2020; Wu et al., 2021).

Oxidative stress and inflammation contribute to cardiovascular disease pathogenesis, including atherosclerosis, cardiac hypertrophy, heart failure, and hypertension. Overproduction of reactive oxygen species (ROS) indicates oxidative stress has been observed in those cardiovascular disease conditions. ROS also contributes to vascular dysfunction and remodelling through oxidative damage in endothelial cells. In addition, evidence indicates that low-density lipoprotein oxidation is essential for atherogenesis. However, the microenvironment present within the atherosclerotic lesion is pro-inflammatory. Besides being a lipid metabolism disorder, atherosclerosis is now recognised as a chronic inflammatory disease. Accumulating evidence shows that excessive inflammation within the arterial wall is a risk factor for cardiovascular diseases and can promote atherogenesis. Agents with antioxidant and anti-inflammatory activity may be beneficial in combating cardiovascular diseases (Deng and Chow, 2010).

Ultraviolet radiation (UVR) has received much attention in research because of ozone depletion in the stratosphere. UVR is classified into three, based on wavelength: ultraviolet A (UVA; 400–320 nm), ultraviolet B (320–290 nm), and ultraviolet C (290–200 nm). UVA has been reported to be hemotoxic, enzyme-disrupting, embryotoxic, and genotoxic while also destructive to the immune system (Osman et al., 2019).

Blue light or high-energy visible light (HEV) refers to wavelengths between 390 and 500 nm. It is emitted from many artificial light sources, such as blue LED and direct sunlight. Because of its high energy, HEV has a greater dispersion than other wavelengths on the visible spectrum and can penetrate animal tissues. Studies have shown that irradiation of mammalian cells with visible light induces cellular damage primarily via ROS (Devasagayam et al., 2004).

Inflammation is an important host defence mechanism characterised by a complex interaction between mediators of inflammation and inflammatory cells. Uncontrolled inflammation can lead to tissue injury and chronic diseases (Sibi and Rabina, 2016). Cytokines are a family of proteins and glycoproteins that regulate the inflammatory and immune response. These cytokines are soluble molecules acting as chemical mediators released during the process, which helps to intensify and propagate the inflammatory response, frequently including tumour necrosis factor-alpha (TNF-α), monocyte chemoattractant protein (CCL2/MCP-1), C-reactive protein (C-RP), interleukin-1 (IL-1), interleukin-6 (IL-6), interferon-γ (INF-γ), dual oxidase 1 (DUOX1), chemokine receptor 4 (CXCR4), interleukin-8 (IL-8), histamine, interleukin-10 (IL-10), transforming growth factor β (TGF-β), cyclooxygenase 2 (COX-2), and prostaglandin E2 (PGE2) (Guzmán et al., 2001; Raposo and De Morais, 2015; Subramoniam et al., 2012). Although their role in the inflammatory process is complex, these molecules modulate the activity and function of other cells to coordinate and control the inflammatory response.

For these reasons, new anti-inflammatory and antioxidant agents, from natural sources, with fewer adverse effects, are alternatives that could be developed for long-term administration. Microalgae are natural sources that could be a sustainable source of bioactive compounds with antioxidant and anti-inflammatory activity (Al-Qahtani and Binobead, 2019; Conde et al., 2021, 2022; Guldas et al., 2021; Ramos-Romero et al., 2021; Sijil et al., 2023; Tabarzad et al., 2020; Wu et al., 2021).

The purpose of this study was to investigate the cytotoxic, antioxidative, and anti-inflammatory activities of lipid fraction extracts from Chlorella and Spirulina on Normal Human Dermal Fibroblasts (NHDF cells), with the aim of identifying natural compounds with functional properties.

MATERIALS AND METHODS

Raw materials

Freeze-dried *Arthrospira platensis* (Spirulina) and *Chlorella vulgaris* (Chlorella) were supplied by Alga Energy (S.A., Madrid, Spain). The microalgae were processed to extract the lipid fraction following the protocol detailed by Axelsson and Gentili (2014). Eight millilitres of a 2:1 chloroform/methanol (v/v) (Sigma-Aldrich Chemie Gmbh, Munich, Germany) mixture was added to

dry algal biomass (200 mg) in a glass centrifuge tube. The biomass was manually suspended by vigorously shaking the tube for a few seconds and 2 mL of a 0.73% NaCl (Sigma-Aldrich Chemie Gmbh, Munich, Germany) water solution was added to produce a 2:1:0.8 system of chloroform/methanol/water (v/v/v) in a total 10 mL solution. Phase separation was facilitated by 2 min of centrifugation at 350 × g with the lower (heavy) phase recovered for analysis. Extracts were stored at 4 °C to avoid alteration. Dilutions were freshly prepared each time.

Cell culture and cytotoxicity assay

Normal Human Dermal Fibroblasts (NHDF cells) (Bionos, Valencia, Spain) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich Chemie Gmbh, Munich, Germany), 100 units/mL of penicillin G, 0.1 mg/mL streptomycin, and 2 mM L-glutamine (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA), at 37 °C in a humidified incubator (Heracell VIOS 160i, Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 5% CO_2. To determine cell numbers in each plate (as a quality control of live cells), cell viability was first assayed using Trypan-Blue Solution (Bio-Rad Laboratories, S.A., Alcobendas, Madrid, Spain) (the blue compound can only penetrate dead cells). Live cells were counted in a Bürker chamber under the microscope.

Cell viability was then assessed with an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay, described by Monti et al. (2015).

For the MTT assay, NHDF cells were cultured overnight at a 10,000 cells per well density in a 96-well plate, in growth media supplied with 20% FBS. After 24 h, the lipid microalgae extracts were solved in DMEM and added x2 to obtain final concentrations of 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039%. Following 24 h incubation, the medium was removed, and wells were washed with phosphate buffered saline solution (PBS) (Sigma-Aldrich Chemie Gmbh, Munich, Germany) to eliminate any residual medium, a solution of MTT solution 1:11 was added to each well. Plates were incubated in the humidified incubator (Heracell VIOS 160i, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37 °C for 3 h. Reactive MTT was removed, and 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemie Gmbh, Munich, Germany) was added to each well to solubilise formazan crystals before absorbance measurements at 550 nm. Absorbance values lower than the control cells indicated a reduction in the rate of cell proliferation. In contrast, a higher absorbance rate indicated an increase in cell viability and proliferation. Cell survival was expressed as the percentage of viable cells in the presence of extract compared to the control samples. One biological replicate with eight technical replicates per concentration and sixteen technical replicates for the control were used.

Oxidative stress assessment

Antioxidant and protective effects of Chlorella and Spirulina extracts were analysed by means HEV-UVA induced oxidative stress. For oxidative stress assessment, NHDF cells were cultured as described in 2.2. section. NHDF cells were cultured overnight at 10,000 cells per well of density in a 96-well black plate, in growth media. 24 h later, the lipid fraction extract of Chlorella and Spirulina was solved in DMEM and added x2 to obtain the final concentrations (0.1, 0.5, and 1%). After 24 h of incubation with the products, culture medium of all wells was replaced by PBS, and ROS master mix (Sigma-Aldrich Chemie Gmbh, Munich, Germany) was added to all cultured wells, immediately before the UVA or HEV radiation.

Four control wells without cells were included in the assay, to obtain the blank control, and four control wells irradiated without cells were included in the assay, to obtain the irradiated blank control. For assays with HEV irradiation, cells were exposed to 24 J/cm^2 of HEV irradiation. Six technical replicates per condition were assessed for ROS accumulation after exposure to HEV light (Potency = 6.7 mW/cm^2, 60 min, total energy = 24 J/cm^2). In assays with UVA irradiation, cells were exposed to 7.85 J/cm^2 UVA irradiation. Six technical replicates per condition were assessed for ROS accumulation after exposure to UVA light (Potency = 4.08 mW/cm^2, 30 min, total energy = 7.85 J/cm^2).

Non-irradiated controls were incubated at 37 °C for 60 min for HEV irradiated experiences and 30 min for UVA irradiated experiences in dark conditions. After 2 h ROS master mix addition, fluorescence was measured at $\lambda_{ex} = 490$ and $\lambda_{em} = 525$ in all samples to give ROS accumulation. The intracellular ROS accumulated reacted with a fluorogenic sensor localised in the cytoplasm, resulting in a fluorometric product proportional to the amount of ROS present. The mean of blank controls was subtracted from all samples data, after, all the data were normalised to the control and represented as mean ± standard error of the mean, analysed statistically comparing treated vs control and irradiated vs non-irradiated samples.

Anti-inflammatory response

NHDF cells were cultured at a 200,000 cells per good density in a 6-well plate, in growth medium. After 24 h, the medium was removed and Chlorella or Spirulina extracts (0.1, 0.5, and 1%) were added to cells and left for 24 h, then 3% 2-propanol (Sigma-Aldrich Chemie Gmbh, Munich, Germany) was added to induce the inflammatory response. The culture was incubated for 20 h, after,

cells were washed with PBS buffer and collected in a lysis buffer to proceed with RNA extraction. Total RNA was extracted using a RNeasy kit (Qiagen, Las Matas, Madrid, Spain) and treated with DNAse-I (Qiagen, Las Matas, Madrid, Spain) to remove any contamination from genomic DNA. RNA quality and quantity were checked in a NanoDrop spectrophotometer (NanoDrop 8000, Thermo Fisher Scientific SL, Madrid, Spain) and 1 μg of total RNA was used to synthesise cDNA, using a First-strand Synthesis kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, France). The suitability of each primer pair used in this study for RT-qPCR, TNF-α, IL-6, and ACT (Sigma-Aldrich Chemie Gmbh, Munich, Germany) was previously evaluated to determine melting curves, efficiency of amplification, and specificity of the primers. Finally, quantitative PCR (qPCR) was performed in a real-time PCR machine (QuantStudio 5, Applied BioSystem, Thermo Fisher Scientific SL. Madrid, Spain). To perform raw data analysis, we used the Pfaffl method to calculate the gene relative expression ratio to ACT (internal control-housekeeping gene) (Pfaffl, 2001). The mathematical model of relative expression ratio in real-time PCR is shown in Equation 1.

$$ratio = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}} \quad (1)$$

where E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP_{target} is the CP deviation control-sample of the target gene transcript; ΔCP_{ref} = CP deviation of control-sample of reference gene transcript.

Statistical analyses

Analysis of variance (ANOVA), with a confidence level of 95% ($p < 0.05$), using XLSTAT Software, version 2020.1.2., was applied to evaluate the differences among samples (Addinsoft, 2020). Dunnett's Test was used to compare means from several experimental groups against control groups mean values, to identify the pairs with significant differences.

RESULTS AND DISCUSSION

Cell viability

Tissue homeostasis in multicellular organisms is attained by a balance between the rate of cell proliferation and cell death. The competition for limiting amounts of exogenous factors has been shown to regulate cell proliferation, growth, and survival, and this competition has been proposed as a mechanism to determine cell viability. The study of cell viability and proliferation is key for evaluating cell population's responses to external factors, such as growth factors, antibiotics, and anti-cancer drugs. MTT assays reflect the effects produced by a substance or treatment upon cell viability, which may be interpreted as toxic effects (cytotoxicity) if cell viability is compromised or stimulating effects (proliferation) if cell viability increases, comparing the treatments with the untreated control group. Cell viability after treatment with Chlorella and Spirulina lipid extracts was assessed in Normal Human Dermal Fibroblasts (NHDF). These results determined the ability to increase cell viability and proliferation, plus the effective concentrations. Figure 1 shows results of cell viability of NHDF cells treated with Chlorella lipid extracts at different concentrations showing treatment with Chlorella lipid extracts was not significant ($p > 0.05$) and there was no remarkable cytotoxicity at concentrations < 5% than the untreated control. Statistical cytotoxicity at concentration 0.039% is considered irrelevant. Likewise, results indicated that treatment with Spirulina lipid extracts (Figure 2) did not show any relevant cytotoxic effect ($p > 0.05$), compared to the untreated control. Cytotoxicity at concentrations 1.25% is considered irrelevant as shown in Figure 2. These results indicate that Chlorella and Spirulina lipid extracts do not present any significant cytotoxicity for NHDF cells after 24 h treatment at concentrations ≤ 5%.

Antioxidant effects of lipid *Chlorella* and *spirulina* extracts on NHDF cells

Oxidative stress is one of the main causes of the ageing process. The production of ROS (reactive oxygen species) has various intrinsic and extrinsic sources, and both affect the main structures of the skin. The role of free radicals in tissue damage has been the subject of several studies. In this assay, NHDF cells were cultured over 24 h with lipid fractions of Chlorella and Spirulina extracts and were exposed to UVA irradiation to investigate the response of the cells to the UVA induced oxidative stress, testing the potential of Chlorella and Spirulina in counteracting ROS induction. Figure 3 shows ROS accumulation in samples treated with Chlorella and Spirulina lipid extracts at 0.1, 0.5, and 1%, irradiated with UVA, normalised to the irradiated group. Results show 7.85 J/cm^2 of UVA irradiation significantly induces ROS levels in NHDF cells by 332.7 ± 35.8%, compared to the control group. The treatment with Chlorella lipid extract at 1% and Spirulina lipid extracts at 0.1, 0.5, and 1% significantly decreased ($p < 0.05$) ROS levels by 22.3 ± 9.3, 24.9 ± 9.7, 21.7 ± 9.5, and 26.9 ± 8.5%, respectively, compared to the control + UVA. Still, the treatment with Chlorella at 0.1 and 0.5% decreased ROS levels by 12.8 ± 10.0 and 18.6 ± 11.7%, respectively, even though results were not statistically significant ($p > 0.05$) when compared to the untreated control. Guzmán et al. (2001) in a pharmacological study of hydro soluble and lipid extracts of *Chlorella stigmatophora* indicated that hydro soluble

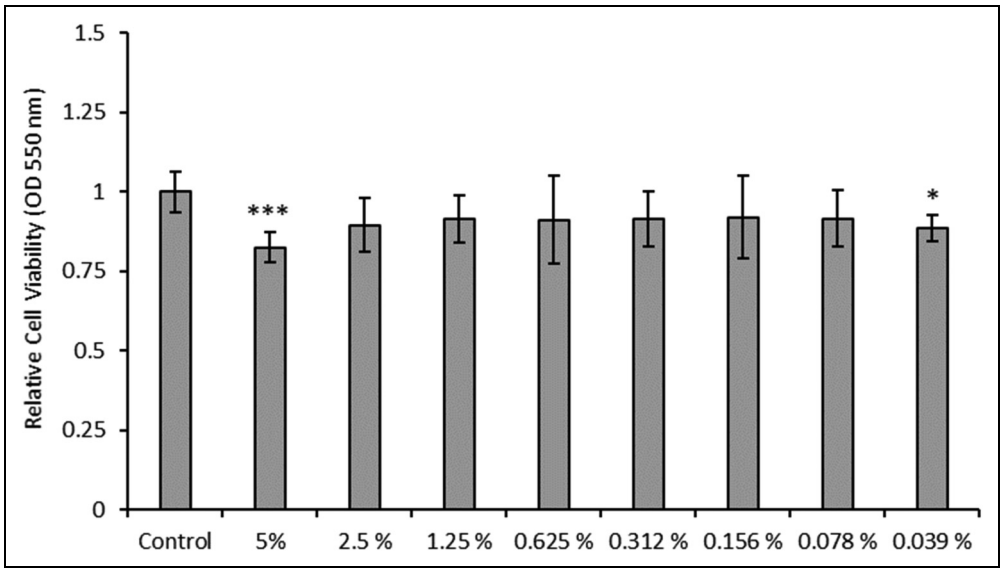

Figure 1. Graphical representation of the results showing cell viability after treatment with Chlorella at 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039%. * Represents statistical significance with $p < 0.05$. *** Represents statistical significance with $p < 0.001$.

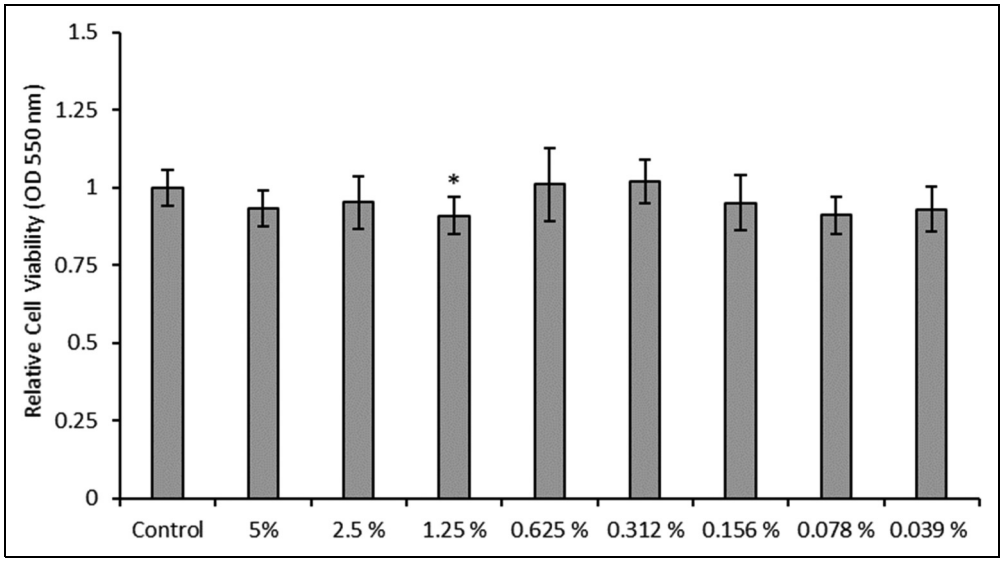

Figure 2. Graphical representation of the results showing cell viability after treatment with Spirulina at 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039%. * Represents statistical significance with $p < 0.05$.

components of both species show significant anti-inflammatory, analgesic, and free radical scavenging activity. However, these activities were not detected in the liposoluble fractions (Guzmán et al., 2001). Shown in Figure 3, 1% of Chlorella lipid extracts have a significant effect on ROS-UVA induced reduction; this can be related with the content of astaxanthin of Chlorella (Guedes et al., 2011). Chlorella had a total pigment content of 1.2%, with astaxanthin, lutein, and zeaxanthin the main carotenoids and both chlorophyll a and b (Batista et al., 2013). Carotenoids have been proposed as good antioxidants that can act as free radical scavengers, owing to their chemical structure. Here, Chlorella lipid extract can have antioxidant activity; however, the effect of Spirulina on ROS-UVA induced reduction was more evident (Figure 3) and significant ($p < 0.05$) at all concentrations studied. Spirulina contains several active ingredients, notably phycocyanin and β-carotene that have potent antioxidant activities (Deng

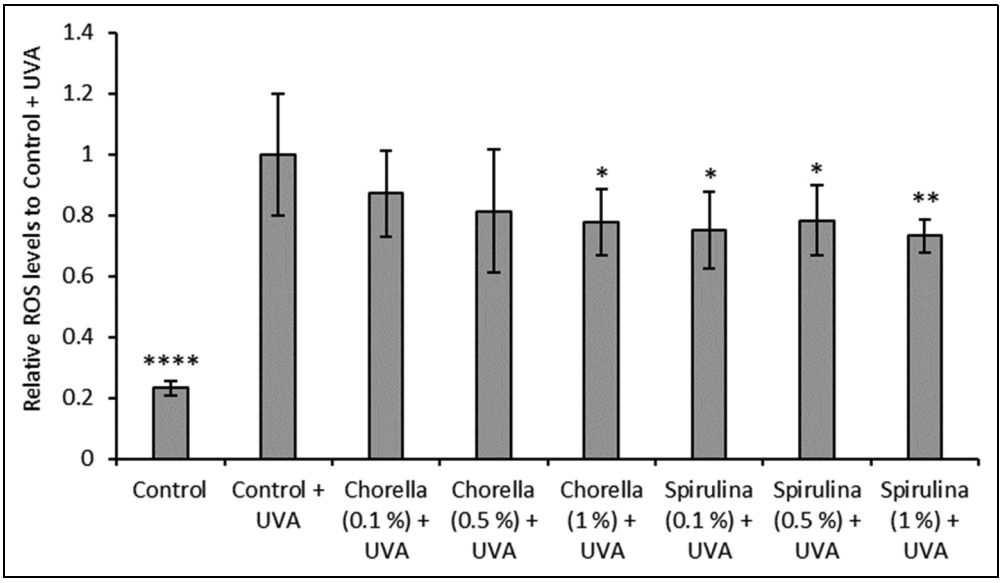

Figure 3. ROS accumulation in samples treated with Chlorella and Spirulina at 0.1, 0.5, and 1%, irradiated with UVA, normalised to the irradiated group. * Represent statistical significance with $p < 0.05$. ** Represent statistical significance with $p < 0.01$. **** Represent statistical significance with $p < 0.0001$.

and Chow, 2010). Several studies have reported the antioxidant and / or anti-inflammatory activities of Spirulina or its extracts *in vitro* (Dartsch, 2008). The antioxidant effect was suggested to be associated with β-carotene and tocopherol (Vo et al., 2015). Notably, the Chlorella and Spirulina lipid extracts can efficiently protect NHDF cells from the deleterious effects of UVA irradiation when tested at concentrations compatible with cell viability, i.e., not associated to any cytotoxicity.

Figure 4 shows ROS accumulation in samples treated with Chlorella and Spirulina lipid extracts at 0.1, 0.5, and 1%, irradiated with high-energy visible light (HEV), and normalised to the irradiated group. Results showed that 24 J/cm^2 of HEV irradiation significantly induced ($p < 0.05$) ROS levels in NHDF cells by $439.0 \pm 49.6\%$, compared to the control group. The treatments with Chlorella lipid extract at 0.1, 0.5, and 1% decreased ROS levels by 17.1 ± 18.4, 18.3 ± 23.4, and $12.5 \pm 18.4\%$, respectively; although results were not statistically significant ($p > 0.05$), compared to the irradiated control. In contrast, the treatment with Spirulina lipid extracts at 0.1% significantly increased ($p < 0.05$) ROS levels by $76.2 \pm 36.1\%$, while treatments at 0.5 and 1% did not show any significant effect ($p < 0.05$) compared to the irradiated control. Results obtained regarding ROS-HEV induction showed that both extracts were ineffective as antioxidants. Blue light or HEV refers to wavelengths between 390 and 500 nm. These wavelengths correspond with carotenoids wavelengths absorption (Ambati et al., 2014; Yahia and Ornelas-Paz, 2010). The conjugated double-bond system constitutes the light-absorbing chromophore that gives the carotenoids their colour. Being highly unsaturated, carotenoids are prone to isomerisation and oxidation. HEV can promote isomerisation of all *trans* carotenoids, their usual configuration, to the *cis* forms, resulting in losses of activity, reducing their antioxidant capacity.

Anti-inflammatory effects of lipid *Chlorella* and *spirulina* extracts on NHDF cells

Cytokines are a family of proteins and glycoproteins that regulate the inflammatory and immune response. These cytokines are soluble molecules acting as chemical mediators, which help to intensify and propagate the inflammatory response; including IL-6 and tumour necrosis factor-alpha (TNF-α) (Dallos et al., 2006; Neurath and Finotto, 2011). To assess the anti-inflammatory potential of Chlorella and Spirulina lipid extracts on the expression of IL-6 and TNF-α genes in NHDF cells, qPCR *in vitro* was used (Filion, 2012). The inflammatory response was induced by treatment with 2-propanol, (Vandebriel et al., 2010) while IL-6 and TNF-α expression were quantified by RT-qPCR mRNA. TNF-α, IL-6, and ACTIN (housekeeping, reference gene) were amplified using four technical replicates of cDNAs. Figure 5 shows IL-6 expression after treating NHDF cells with Chlorella or Spirulina lipid extracts at 0.1 and 0.5%, and 3% 2-propanol, compared to a control + 2-propanol. The treatment with 2-propanol significantly increased ($p < 0.05$) IL-6 gene expression by $81.6 \pm 4.1\%$, showing that 2-propanol was activating the inflammatory response through this metabolic pathway. When cells were incubated with Chlorella and Spirulina lipid extracts at 0.1 and 0.5%, IL- 6 expression significantly reduced ($p < 0.05$) by 42.1 ± 6.2 and

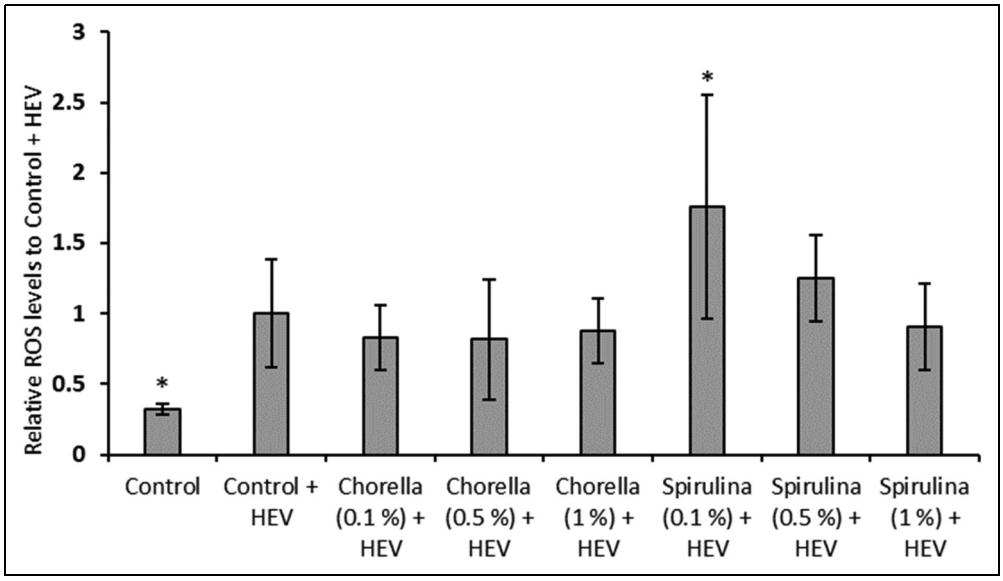

Figure 4. ROS accumulation in samples treated with Chlorella and Spirulina at 0.1, 0.5, and 1%, irradiated with HEV, normalised to the irradiated group. * Represent statistical significance with $p < 0.05$.

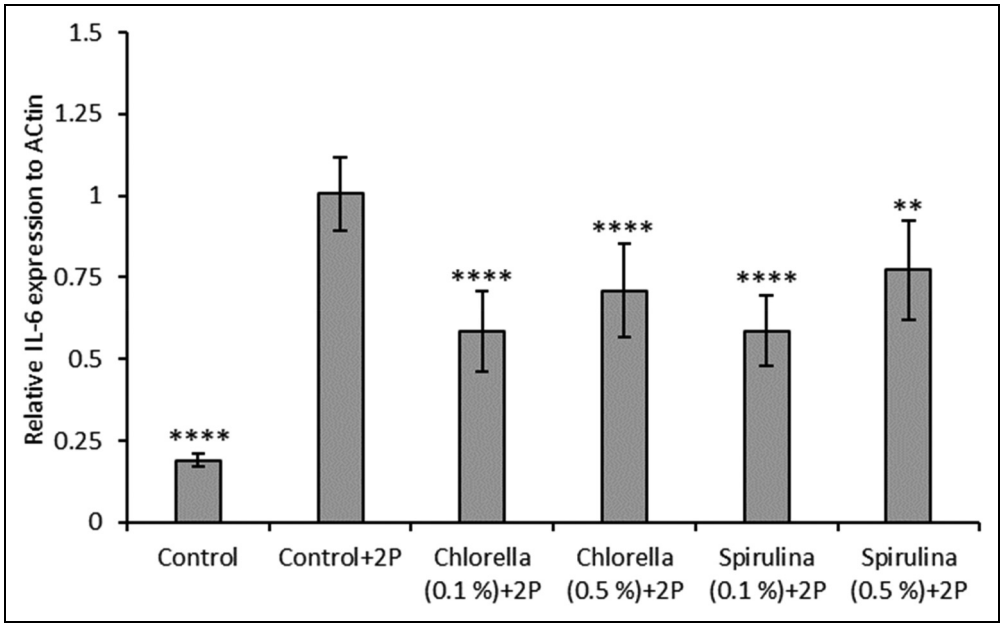

Figure 5. IL-6 expression results after treating NHDF cells with Chlorella and Spirulina at 0.1 and 0.5%, 2-propanol at 3%, compared to the control + 2-propanol. ** Represents statistical significance with $p < 0.01$. **** Represents statistical significance with $p < 0.0001$.

$29.7 \pm 6.6\%$, and 42.0 ± 5.6 and $23.4 \pm 6.7\%$, respectively, compared to the control + 2-propanol. In parallel, the treatment with 3% 2-propanol (Figure 6) increased TNF-α by $33.8 \pm 8.4\%$, showing that 2-propanol was activating the inflammatory response through this metabolic pathway, even though results were not statistically significant ($p > 0.05$). The treatment with Chlorella lipid extract at 0.5% decreased TNF-α expression by $16.1 \pm 11.1\%$, even though results were not statistically significant ($p > 0.05$), compared to the control + 2-propanol. In contrast, the treatment with Chlorella lipid extract at 0.1% and Spirulina lipid extracts at 0.1 and 0.5% did not show any decrease in TNF-α expression.

Al-Qahtani and Binobead (2019), using the aqueous extract of *Spirulina platensis* (SP) against D-galactosamine, induced hepatotoxicity in rats, demonstrating that all doses

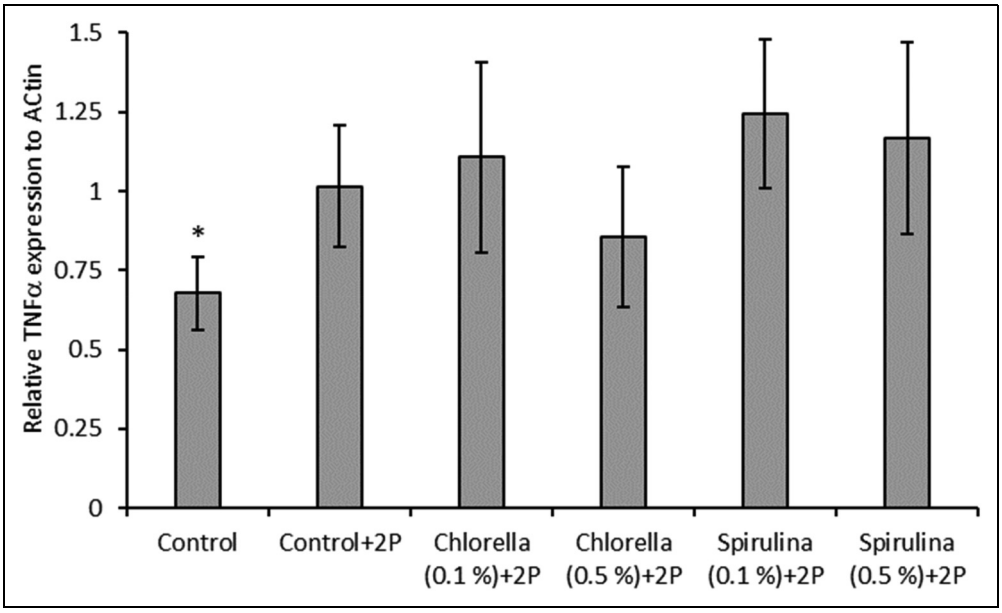

Figure 6. TNF-α expression results after treating NHDF cells with Chlorella and Spirulina at 0.1 and 0.5%, 2-propanol at 3%, compared to the control + 2-propanol. * Represent statistical significance with $p < 0.05$.

of SP can significantly reduce the secretion of IL-6. Choi et al. (2019) showed that the Spirulina extract from a non-thermal ultrasonic process enhanced anti-inflammation activities in mice microglial cells, associated to a significant down-regulation of mRNA expression of the pro-inflammatory cytokines IL-6. The high anti-inflammatory effects of the extracts closely correlated with the high amounts of chlorophylls, which have strong antioxidant activities, obtained only through a non-thermal ultrasonic process. Since Spirulina has diverse active substances, such as C-phycocyanin, beta-carotene, chlorophyll, and functional fatty acids, they have been reported to have excellent anti-inflammatory effects (Soni et al., 2017). Sibi and Rabina (2016) using 80% methanolic extract of *C. vulgaris* found that their extracts inhibit the inflammatory response, by reducing the production of IL-6 in LPS activated RAW 264.7 cells *in vitro*. Chlorella contains higher amounts of total chlorophylls and carotenoids compared to Spirulina (Hynstova et al., 2018). According to our results (Figure 5) the effect of lipid extracts (Chlorella or Spirulina) at concentrations assayed on anti-inflammatory activity evaluated as IL-6 expression on NHDF cells was significant ($p < 0.05$) and similar for both extracts.

Several authors evaluated the anti-inflammatory effects of Chlorella and Spirulina by TNF-a expression (Al-Qahtani and Binobead, 2019; Chen et al., 2012; Cherng et al., 2010; Guzmán et al., 2001; Habashy et al., 2018; Kwak et al., 2012; Rezaei et al., 2018; Shih et al., 2009; Sibi, 2015). Sibi (2015) evaluated the anti-inflammatory activity of lipid extracts of six Chlorella species by *in vitro* screening, based on inhibition of pro-inflammatory cytokines, TNF-α was produced by human peripheral blood mononuclear cells. In this study, the stimulatory role of *Propionibacterium acnes* on pro-inflammatory cytokine (TNF-α) production was demonstrated followed by an inhibitory action by the *C. vulgaris* extracts where 73.46% inhibition was observed. Cherng et al. (2010) analysed Chlorella-11 peptide (Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe) in preventing inflammation progression *in vitro*. The levels on TNF-α production after lipopolysaccharides (LPS) activation were inhibited by the Chlorella-11 peptide. Guzmán et al. (2001) studied hydro soluble and liposoluble extracts of *Chlorella stigmatophora* and *Phaeodactylum tricornutum*, their results showed tests of the methanol fractions did not reveal significant anti-inflammatory activity even at the highest concentration, indicating that this activity is restricted to the aqueous extracts. Chen et al. (2012) investigated the effects of Spirulina water extract and C-phycocyanin on BV-2 microglial cell cytotoxicity and inflammatory gene expression, showing that the components significantly reduced expression of inflammation-related genes TNF-α mRNAs. Other authors, using a carrageenan-induced thermal hyperalgesia model also show the effect of C-phycocyanin (C-PC) from SP attenuation of TNF-α formation (Shih et al., 2009). In this study the results shown (Figure 6) that both lipid extracts (Chlorella or Spirulina) at concentrations assayed, have no significant anti-inflammatory ($p > 0.05$) activity evaluated as TNF-α expression in NHDF cells. This activity of Spirulina can be associated with phycocyanin in the hydrophilic phase but not in the lipid extract. For Chlorella the anti-inflammatory effect related to TNF-α expression was associated with peptides in the hydrophilic phase or the

role of fatty acids, chlorophylls, and carotenoids, however, this effect was only detected at concentrations of 0.5% and results were not statistically significant.

CONCLUSIONS

In this study the cytotoxic, antioxidative, and anti-inflammatory activities of lipid fraction extracts from *Chlorella vulgaris* and *Arthrospira platensis* on Normal Human Dermal Fibroblasts cells was investigated. Lipid extracts of Chlorella and Spirulina do not present any significant cytotoxicity for Normal Human Dermal Fibroblasts (NHDF cells) after 24 h treatment at concentrations $\leq 5\%$. Treatments with Chlorella and Spirulina lipid extracts in NHDF cells significantly protects against UVA induced damage, by decreasing oxidative stress in the form of reactive oxygen species (ROS), but do not display any significant protection against HEV induced damage. In contrast, treatment during 24 h with Chlorella or Spirulina lipid extracts at 0.1 and 0.5% displays significant anti-inflammatory effects through inhibition of the 2-propanol-induced IL-6 expression in NHDF cells *in vitro*. The treatment with Chlorella or Spirulina lipid extracts at 0.1 and 0.5% did not show any decrease in TNF-α expression.

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AUTHOR CONTRIBUTIONS

P. G. and J. M.: designed the study; M. I., Z. U., and L. M. conducted the study; P. G. and J. M. performed statistical analysis; J. M. wrote the manuscript and had primary responsibility for the final content. All authors have read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

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ORCID iDs

Purificación García-Segovia https://orcid.org/0000-0002-4968-5050

Javier Martínez-Monzó https://orcid.org/0000-0002-1123-2304

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