**PRESSURIZED LIQUID EXTRACTION OF NEOCHLORIS OLEOABUNDANS
FOR THE RECOVERY OF BIOACTIVE CAROTENOIDS WITH ANTI-
PROLIFERATIVE ACTIVITY AGAINST HUMAN COLON CANCER CELLS**

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Abstract

In recent years, the green microalgae *Neochloris oleoabundans* have demonstrated to be an interesting natural source of carotenoids that could be used as potential food additive. In this work, different *N. oleoabundans* extracts obtained by pressurized liquid extraction (PLE) have been analyzed in depth to evaluate the influence of different culture conditions (effect of nitrogen, light intensity or carbon supplied) not only on the total carotenoid content but also on the carotenoid composition produced by this microalgae. Regardless of the cultivation conditions, lutein and carotenoids monoesters were the most abundant carotenoids representing more than 60 % of the total content in all extracts. Afterwards, the effect of the different *N. oleoabundans* extracts and the dose-effect of the most potent algae extracts (namely, N9, PS and CO₂ (-)) on the proliferation of human colon cancer cells lines (HT-29 and SW480) and a cell line established from a primary colon cancer cell culture (HGUE-C-1) was evaluated by an MTT assay whereas a stepwise multiple regression analysis was performed to get additional evidences on the relationship between carotenoid content and the antiproliferative activity. Results revealed that, as a general trend, those extracts with high total carotenoid content showed comparably antiproliferative activity being possible to establish a high correlation between the cell proliferation values and the carotenoid constituents. Monoesters showed the highest contribution to cell proliferation inhibition whereas lutein and violaxanthin showed negative correlation and diesters and zeaxanthin showed a positive significant contribution to cell proliferation.

Keywords: *Neochloris oleoabundans*, Carotenoids, Pressurized Liquid Extraction, Colon cancer, Antiproliferative effects.

1. Introduction

Nowadays, cancer constitutes one of the leading causes of death. Cancer research has been mainly focused on the search for curative treatments, and few studies have aimed to develop preventive strategies that can be useful in the long period of carcinogenesis of the tumors. This period is suitable for employing chemopreventive strategies based on the use of natural, synthetic or biological substances to reduce the risk of developing a cancer (Talero et al., 2015). In this regard, the exploration of compounds from natural sources with health effects with the aim of developing a new functional food or nutraceutical is an intense field of research.

Marine environment, which contains a vast array of organisms (bacteria, cyanobacteria, fungi, algae, microalgae or small invertebrates with unique biological properties), represents a huge and underutilized natural source to isolate bioactive compounds to be used in the food and pharmaceutical industries. Among all the marine sources, microalgae have raised an enormous interest. These microorganisms may be used as natural bioreactors since depending on the cultivation conditions they are able to stimulate the synthesis of compounds with health effects, including carotenoids (Herrero, Mendiola, Castro-Puyana, & Ibáñez, 2012; Ibáñez & Cifuentes, 2013). In fact, contents up to 10 % carotenoids (w/w) have been described in some microalgae species and may be of high interest as source of pure carotenoids (Guedes, Amaro, & Malcata, 2011). The beneficial effects of carotenoids as antioxidant compounds are well documented. Moreover, they have been also associated with relevant bioactivities such as lower risk of coronary heart diseases and prevention of cancer. Several studies have demonstrated the antiproliferative activity of carotenoids, such as violaxanthin, zeaxanthin, lutein or fucoxanthin, isolated from microalgae against different cancer cells (Guedes, Amaro, & Malcata, 2011; Christaki, Bonos, Giannenas, & Florou-Paneri,

2013; Pasquet et al., 2011; Cha, Koo, & Lee, 2008; Moghadamtousi et al., 2014). A key point to carry out these analyses is obviously the extraction of carotenoids from the microalgae. From the viewpoint of the extraction processes, the new challenges involve the development of green and sustainable processes which enable a fast, selective and efficient extraction and isolation of bioactive compounds from natural matrices (Herrero & Ibáñez, 2015; Roselló-Soto et al., 2015; Barba, Grimi, & Vorobiev, 2015). In this regard, advanced extraction techniques based on the use of compressed fluids (Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE)) (Herrero, Castro-Puyana, Mendiola, & Ibáñez, 2013) and the employment of new strategies based on ultrasound-assisted extraction (Parkianov et al., 2015a) and pulsed electric field assisted extraction (Parkianov et al., 2015b, Parkianov et al., 2015c) are among the most promising techniques. These techniques are considered alternative processes to extract nutritionally valuable compounds from microalgae while complying with green chemistry principles and sustainability. Among them, PLE (technique based on the extraction at high temperatures (usually above the boiling point of the solvent) and pressures that maintain the solvent in the liquid state during the extraction) has demonstrated being an interesting alternative to extract carotenoids from different microalgae such as *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella vulgaris*, *Spirulina platensis*, etc. (Jaime et al, 2010; Denery, Dragull, Tang, & Li, 2004; Herrero, Jaime, Martín-Álvarez, Cifuentes, & Ibáñez, 2006; Plaza et al., 2012; Cha et al., 2010; Jaime et al., 2005). Recently, PLE has also been successfully applied to the extraction of carotenoids from *Neochloris oleoabundans* (Castro-Puyana et al., 2013). Besides the production of lipids, *N. oleoabundans* has recently demonstrated its potential to accumulate relevant amounts of carotenoids when it is grown under certain

conditions so that it can be considered as a novel source of natural carotenoids (Chue et al., 2012; Goiris et al., 2012; Castro-Puyana et al., 2013; Urreta et al., 2014).

Considering that microalgae structure has a marked influence in cell disruption and thus in the degree of extraction of intracellular valuable compounds, extraction conditions should be optimized targeting the compounds of interest. In this sense, it is important to focus not only on the family of compounds of interest, for instance carotenoids, but also on the specific compounds responsible for a described bioactivity. Therefore, it is crucial also to perform an in depth chemical characterization, using potent analytical techniques, of the compounds present in the bioactive extracts and to correlate them with the associated bioactivity.

Therefore, the aim of this work was to study the composition and content of carotenoids in different *N. oleoabundans* extracts obtained by PLE in order to evaluate the differences in the type and amount of carotenoids produced when different culture conditions (effect of nitrogen, light intensity or carbon supplied) are employed. Furthermore, the activity of the different *N. oleoabundans* extracts against two colon cancer cell lines and one primary colon cancer cells was also investigated to establish correlations between not only the carotenoid content but also carotenoid constituents and their antiproliferative activity.

2. Material and methods

2.1 Chemicals and reagents

Ethanol was supplied by Panreac Quimica (Barcelona, Spain). Methanol, methyl *tert*-butyl ether (MTBE), hexane, and acetone were obtained from LabScan (Gliwice, Poland). Sea sand was from VWR (Leuven, Belgium). Standard samples of β -carotene, lutein, chlorophyll *a* (from *Anacystis nidulans algae*), and chlorophyll *b* (from spinach)

were obtained from Sigma-Aldrich (Madrid, Spain), whereas astaxanthin monopalmitate and astaxanthin dipalmitate were purchased from CaroteNature GmbH (Lupsingen, Switzerland).

2.2 Samples

Neochloris oleoabundans (UTEX#1185) was obtained from the culture collection of algae at the University of Texas (Austin, TX, USA). Modified Bold's Basal Medium (BBM) was used as medium for cultivation (Andersen, Berges, Harrison, & Watanabe, 2005). Cultures were established in BBM containing 9 mM KNO₃ until cell reached optical density (OD 660 nm) of 5.0. Aliquots of 200 ml were centrifuged (520 x g during 5 min), washed with distilled water once and used as inoculum in three consecutive experiments aimed to study the effect of nitrogen, light and CO₂ in the carotenoid and chlorophyll cellular content.

All the experiments were carried out in a culture chamber at 24 ± 2 °C, under 16-h photoperiod. Cultures were grown in batch mode, using 9-cm wide glass reactors containing a working volume of 1 L and subjected to continuous stirring by bubbling air at a constant flow rate.

To evaluate the effect of nitrogen, cultures were grown in BBM supplemented with KNO₃ (used as nitrogen source) at concentrations of 3, 6 and 9 mM (samples N3, N6 and N9, respectively). Pure CO₂ was supplied automatically to the bubbling air for 30 s every 10 min to maintain the pH below 8 and ensure carbon sufficiency. The incident light intensity on the reactor surface was 400 μmol photons m⁻² s⁻¹ (Philips TLD 58 W).

To determine the effect of light intensity, cultures were grown in BBM supplemented with 3 mM KNO₃ and subjected to two different incident light intensities on the reactor surface: 240 μmol photons m⁻² s⁻¹ (sample LL) and of 400 μmol photons m⁻² s⁻¹ (sample HL). Pure CO₂ was automatically supplied as is described above.

The effect of carbon supply was investigated in cultures grown in BBM supplemented with 3 mM KNO₃ and under an incident light intensity of 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ which were cultivated suppressing the CO₂ addition during cultivation (sample CO₂ (-)) or supplementing CO₂ automatically as is described above (sample CO₂ (+)).

In all of the above experiments, cells were harvested by centrifugation (520 x g, 5 min) 6 days after reaching the stationary phase of growth, which was induced by the depletion of the initially supplemented nitrogen, as described in Urreta et al. (Urreta et al., 2014). The obtained algal biomass was pre-frozen at -20 °C, lyophilized and stored under dry and dark conditions until further use.

In addition to those samples described above, other two samples were included in this study. The first of them was obtained from cultures grown in MBB medium at 3 mM KNO₃, using an incident light intensity of 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and with automatic injection of CO₂ (therefore under similar culture conditions than those used for samples N3, HL and CO₂ (+)). In this case, cells were harvested 10 days after reaching the stationary phase of growth instead of after 6 days (sample D10).

The last sample (sample PS) was collected from a culture operated at pilot plant scale which was used to reproduce the culture conditions corresponding to sample N3, therefore grown with 3 mM KNO₃, 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of light intensity and automatic supply of CO₂, until 6-days after reaching the stationary phase of growth. This culture was grown in a 30-cm wide tubular photo bioreactor with a working volume of 40 liters.

2.3 Pressurized Liquid Extraction (PLE)

Before PLE, between 1.2 and 2.5 g of lyophilized algae samples were treated by 3 cycles of cryogenic grinding using a Mixer Mill CryoMill (Retsch, Hann, Germany) to break down the cell wall to obtain the highest extraction yield. Three steps were carried

out in each cycle: precooling (frequency 1/s = 5 during 2 min), grinding (frequency 1/s = 20 during 3 min) and intermediate cooling (frequency 1/s = 5 during 1 min), as previously described (Castro-Puyana et al., 2013). PLE extractions of pre-treated samples were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA). Extractions were carried out using ethanol as solvent at 100 °C and 20 min as static extraction time, as suggested in Castro-Puyana et al., 2013. An extraction cell heating-up was applied during 5 min prior to each extraction (time fixed by the system). All extractions were done (in duplicate) using 11 ml extraction cells at 1500 psi containing 0.6 g of algae mixed homogenously with 2 g of sea sand. In addition, these samples were introduced inside the cell between sand layers in a sandwich-like format. The extracts obtained were protected from light and stored under refrigeration until HPLC analysis.

To carry out the antiproliferative activity assay, the extracts were evaporated with a Rotavapor R-210 (Büchi Labortechnik AG, Flawil, Switzerland). Dry extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at 125 mg/mL, and stored as aliquots at -80 °C until use.

2.4 Quantification of carotenoids and chlorophylls by LC-DAD

Carotenoid and chlorophylls content in the extracts was determined by HPLC employing an Agilent HP 1100 series liquid chromatograph (Agilent Technologies, CA, USA) equipped with a DAD, and using a YMC-C30 reversed-phase column (250 mm × 4.6 mm id, 5 µm particle size, YMC Europe, Schermbeck, Germany). The mobile phase was a mixture of MeOH: MTBE: water (90:7:3 v/v/v) (A) and MeOH: MTBE (10:90 v/v) (B) eluted according to the following gradient: 0 min, 0% B; 20 min, 30% B; 35 min, 50% B; 45 min, 80% B; 50 min, 100% B; 52 min, 0% B. Flow rate was 0.8 mL/min, the injection volume was 10 µL, and detection was at 450 and 660 nm

(recorded spectra from 240 to 770 nm by DAD). For the calibration curve, standard solutions of β -carotene (ranging from 0.025 to 1 mg/mL), lutein (from 1.25×10^{-3} to 0.04 mg/mL), astaxanthin monopalmitate and dipalmitate (both ranging from 6.25×10^{-4} to 0.04 mg/mL), chlorophyll *a* and chlorophyll *b* (both from 3.13×10^{-3} to 0.2 mg/mL) were prepared by appropriate dilution of the stocks and injected into the LC-DAD instrument. Each standard was dissolved from a stock solution (1-2 mg/mL) with different solvents, i.e. lutein, chlorophyll *a*, and chlorophyll *b* were dissolved in ethanol, astaxanthin monopalmitate and astaxanthin dipalmitate in hexane: acetone (1:1 v/v), and β -carotene in hexane. The linear regression equations for each standard curve were obtained by plotting the amount of standard compound injected against the peak area.

2.5 Cell culture conditions

Colon adenocarcinoma HT-29 and SW480 cells were obtained from the IMIM (Institut Municipal d'Investigació Mèdica, Barcelona, Spain) and ATCC (American Type Culture Collection, LGC Promochem, UK), respectively, and HGUE-C-1 was an established cell line derived from a primary colon cancer cell line of a single primary human colon carcinoma at the Hospital General Universitario de Elche (Alicante, Spain). The cells were grown in DMEM supplemented with 5% heat inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin G, and 50 μ g/mL streptomycin at 37 °C in a humidified atmosphere with 5 % CO₂. The cells were trypsinized every three days according to the manufacturer's instructions, and they were seeded in 96-well plates.

2.6 Antiproliferative activity assay

To study the effect of *N. oleoabundans* extracts on the proliferation of HT-29, SW480 and HGUE-C-1 cell lines, the cells were seeded at a density of 5×10^3 cells/well, permitted to adhere overnight at 37 °C, and exposed to algae extracts of 27, 83 or 250

µg/mL for 24 h. After incubations in the presence of the extract, the cells were washed with PBS and incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 3-4 h at 37 °C and 5% CO₂. MTT is reduced to formazan by the succinate dehydrogenase system of active mitochondria, and hence, specifically used to assay for the viable cells as measurement of cell proliferation. The medium was removed, and 100 µL of DMSO per well was added to dissolve the formazan crystals. The plates were shaken for 15 min and analyzed by using a microplate reader (SPECTROstar Omega, BMG LabTech GmbH, Offenburg, Germany) at 570 nm.

2.7 Statistical analysis

Data sets obtained from chemical analysis, as well as those obtained from the antiproliferative activity assay were analysed with one-way analysis of variance (ANOVA) and Tukey's post hoc analysis at the ($p < 0.05$) significance level. To get additional evidences on the relationship between carotenoid content and the antiproliferative activity, a stepwise multiple regression analysis was applied. This approach was applied to find predictive equations for the antiproliferative activity with the carotenoids concentrations as the predictor variables. Regression (Beta) coefficients were analyzed to evaluate key variables for the prediction of the antiproliferative activity. The magnitude of Beta coefficients allows comparing the relative contribution of each independent variable in the prediction of the dependent variable. All the statistical analyses were performed using statistical package Statistica 7.1 (StatSoft Inc., OK, USA).

3. Results and discussion

3.1 Analysis and quantification of carotenoids

Previous works have shown that *N. oleoabundans* can be considered as a novel source of natural carotenoids when grown under certain conditions (Castro-Puyana et al., 2013). Considering that different cultivation conditions (such as nitrogen concentration, light intensity and carbon supply) have an important impact on the accumulation of carotenoids (Urreta et al., 2014), it is relevant a deeper analysis to know how these conditions affect the type and amount of carotenoids produced.

Nine extracts marked as N3, N6, N9, LL, HL, CO₂ (-), CO₂ (+), D10, and PS, were obtained from cultures of *N. oleoabundans* grown under different controlled conditions (see section 2.2) by PLE. **Table 1** shows the values of extraction yield (determined as extract dry weight/initial dry weight expressed as a percentage) obtained for each algae extract, which ranged from 23.4 to 35.5 %.

To establish differences in the carotenoid and chlorophyll composition of each *N. oleoabundans* extract and to evaluate the influence of the different nutritional and environmental factors in their production, it is essential to carry out the chemical characterization of each extract and an accurate quantification of each component.

The application of the LC-(APCI)-MS methodology previously described (Castro-Puyana et al., 2013) provided information on the main pigments (carotenoids and chlorophylls) found in the PLE extracts. **Figure 1** depicts, as example, the chromatographic profiles (at a wavelength equal to 450 nm) obtained for three different *N. oleoabundans* extracts (PS, N9, and CO₂ (-)). In spite all extracts have a similar chromatographic profile, important differences were obtained in terms of the amount of carotenoids and chlorophylls produced by *N. oleoabundans* at the different cultivation conditions (**Table 1**).

The quantitation of carotenoids whose standard were no commercially available was performed using as standards those with higher similarity in terms of spectra and

structure. In addition, a molecular-weight-correction factor (determined by dividing the molecular weight of the carotenoid to be quantified by that of the standard) was applied to consider the difference in detector response (Dhooghe et al., 2008; Chandra, Rana, & Li, 2001). Thus, violaxanthin (peak 1), lutein (peak 3), canthaxanthin (peak 5), canthaxanthin related-compound (peak 7) and echinenone (peak 8) were quantified using the calibration curve of lutein; zeaxanthin (peak 4), β -carotene (peak 13) and carotenoids monoester others than astaxanthin (peaks 9 and 11) were quantified using the calibration curve of β -carotene; astaxanthin monoesters (peaks 10 and 12) and diesters (peaks 14-16) were quantified using the calibration curves of astaxanthin monopalmitate and astaxanthin dipalmitate, respectively. On the other hand, chlorophyll *b* (peak 2) and chlorophyll *a* (peak 6) were quantified using the calibration curves of their respective standard solutions.

As mentioned, **Table 1** shows the total carotenoids and chlorophylls content along with the amount of the main carotenoids (violaxanthin, lutein, zeaxanthin, cantaxanthin, monoesters, β -carotene, and diesters) obtained from the different *N. oleoabundans* cultures. As it can be observed, regardless of the culture conditions employed, all the extract analyzed contained between 3 and 19-times higher amount of carotenoids than chlorophylls. However, there is a considerable range of variation in the content of both types of pigments among the different culture conditions employed.

In general, there was an inverse relationship between the amount of nitrogen supplemented to the cultures and the pigment levels in the extracts. One-way ANOVA showed significant increase in the total carotenoids content in the extracts obtained from cultures supplemented with the two highest amounts of nitrogen (samples N6 and N9) in relation to those obtained from cultures with the lowest nitrogen concentration

(sample N3). This inverse relationship between nitrogen and pigment levels was even more evident to the chlorophyll content.

The effects of carbon availability and light were assayed in cultures supplemented with the lowest nitrogen concentration. Under this conditions, lack of carbon gave rise to a significant increase in the carotenoid content compared to cultures grown with CO₂ supply (from 62.6 to 113.8 mg/g extract), as well as to an increase in chlorophyll concentration (from 14.1 to 38.9 mg/g extract). Contrarily, the variation of the light intensity from 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (sample HL) to 240 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (sample LL), did not imply a significant change in the pigments content compared to the above factors.

Compared to any other analyzed extract, sample D10 exhibited a significant reduction both in the carotenoid concentration as in the chlorophyll content (45.2 and 2.3 mg/g extract, respectively). This sample was obtained from a culture grown under similar conditions than N3, CO₂ (+) and HL, but subjected to a longer culture period (4 more days).

Considering the above results, it can be noted that firstly, pigment levels were mainly affected by nutritional factors (nitrogen and carbon) as well as by the culture time and secondly, that both types of pigments vary similarly with respect to the culture conditions. Sample PS is an exception to the above, as it exhibited high levels of carotenoids and a very low level of chlorophyll. This sample comes from cultures set at a pilot scale using similar culture conditions to those of samples N3, CO₂ (+) or HL. From our point of view, the differences observed among the above samples are attributed to the impossibility of strictly reproduce the culture conditions established at laboratory scale when cultures are set outdoor (for instance, to the temperature

fluctuations in outdoor cultures). However, these differences support the importance of culture factors on the pigment content of microalgae.

The ability of microalgae to adapt their pigment content to environmental changes is well documented and it is considered as a cellular mechanism to quickly optimize the photosynthetic efficiency or even as a survival mechanism to face long-term unfavorable environmental conditions. Among these unfavorable conditions, nutrient limitation, and especially nitrogen starvation, is a well-known factor affecting microalgae pigment content (Solovchenko, Khozin-Goldberg, Didi-Cohen, Cohen, & Merzlyak, 2008; Li, Horsman, Wang, Wu, & Lan, 2008; Del Campo et al., 2000; Jin, Polle, Lee, Hyund, & Chang, 2003). Several authors indicates that when nitrogen availability become limiting for growth, cellular nitrogen-rich compounds (mainly photosynthetic proteins and chlorophylls), are degraded and used as an internal source of nitrogen to face its unavailability in the surrounding media (Li, Horsman, Wang, Wu, & Lan, 2008; Pruvost, Vooren, Cogne, & Legrand, 2009; Rismani-Yazdi, Haznedaroglu, Hsin, & Peccia1, 2012). This process of degradation can explain the inverse relationship observed between the nitrogen supplied to the cultures and chlorophyll content in the analyzed samples. Furthermore, as chlorophylls, those known as primary carotenoids are closely bound to photosynthetic proteins, so degradation of photosynthetic structures inevitably would lead to a concomitant reduction of carotenoid content (Solovchenko, Khozin-Goldberg, Didi-Cohen, Cohen, & Merzlyak, 2008). This justifies the observed similar pattern for the content of both types of pigments in relation to culture conditions.

It must be noted that, regardless of the specific culture conditions applied, all the extracts analyzed in this work come from cultures maintained for at least 6 days in stationary growth-phase and that the stationary growth-phase was triggered by the

complete depletion of the nitrogen initially supplied to the medium (whatever the others culture factors applied). Therefore, there is an additive effect to nitrogen starvation exerted over the other applied culture factors. In this context, higher amounts of nitrogen could provide to the cells higher reserve of nitrogen-rich compounds to face the nitrogen unavailability, thus leading to a lesser pigment reduction under the same starvation time. Likewise, by removing the CO₂ supply, cells are subjected to a lesser imbalance between the availability of carbon and nitrogen, which would reduce the internal nitrogen demand and the degradation of photosynthetic pigment in the sample CO₂ (-) (Urreta et al., 2014). In an opposite way, long-term nitrogen-starvation would give rise to further pigment degradation, which would explain the found lowest chlorophyll content in the sample named D10.

Culture conditions not only affected the total carotenoid content, but also the carotenoid composition. This can be observed in **Table 1**, which shows the concentration of specific carotenoids in the extracts (as well as in **Figure 2** which displays their relative content). Regardless of the cultivation conditions, lutein and carotenoid monoesters were the most abundant carotenoids, representing more than 60% of the total content in all extracts. However, the relative content of these two compounds displayed an opposite pattern regarding the culture conditions. For instance, lutein account for the largest fraction of the total carotenoids in the extract obtained from cultures with the highest nitrogen dosage (N9) and from those lacking of carbon (CO₂ (-)), 48.4 % and 54.7 % respectively, which also show the lowest percentage of monoesters (12.6 % and 7.9 %, respectively). It is worth mentioning that these same samples showed the highest pigment values (chlorophylls and carotenoids). On the contrary, the highest fraction of monoesters, along with the lowest of lutein, was found in the extract D10 (that exhibited the lowest pigment content) and in the extract PS. Therefore, the latter extract stands out

both for its high content of total carotenoids as for a significant fraction of monoesters. In spite of the pattern above described, the absolute value of lutein and monoesters in the extract differs from the percentage due to the differences in the total carotenoid content among samples from different culture conditions. This makes that sample D10 displays a monoester content halved compared to PS (22.6 and 53.1 mg/g extract, respectively), despite having a similar percentage (close to 45% of the total carotenoids).

Among monoesters produced by *N. oleabundans*, we identified two specific esters of astaxanthin by applying the LC-(APCI)-MS methodology. Astaxanthin (which is one of the most valuable microalgae carotenoid due to its antioxidative properties) is synthesized and massively accumulated in the cytoplasmatic oil bodies by certain microalgae under some stressful environmental conditions (Jin, Polle, Lee, Hyund, & Chang, 2003). For instance, under stressful culture conditions, esters of astaxanthin can account for more than 95% of total carotenoids in *Haematococcus pluvialis*, where it seems to act for preventing photo-oxidative damage of cellular lipids (Solovchenko, Chivkunova, & Maslova, 2011). In this sense, astaxanthin play a very different role compared to lutein, which is a structural and functional component of the photosynthetic apparatus of most of green microalgae.

Among the other major carotenoids identified in the extracts, only violaxanthin displayed a clear pattern regarding to the culture conditions. Violaxanthin content was directly proportional to that of lutein, so the highest absolute content of this carotenoid was found in samples N9, N6 and CO₂ (-).

3.2 Anti-proliferative activity

In previous studies, HT-29 cell line has shown to be more refractory to the anti-proliferative activity of natural compounds than other established colon cancer cell lines

(Valdés et al., 2013). Besides, this cell line constitutes a paradigm of the colon carcinogenesis pathway proposed by Fearon and Volgestein (1990). Thus, in the present work, we screened the algae extracts on HT-29 line as a previous step in order to select the most active extract. To attain this, HT-29 cells were incubated with 250 µg/mL of the different algae extracts for 24 h, and cell proliferation was analyzed by the MTT assay. In general, most of the extracts reduced the proliferation of HT-29 human colon cells at an initial concentration of 250 µg/mL after 24 h treatment (**Figure 3**). As a general trend, those extracts with high total carotenoid content showed comparably high antiproliferative activity. An exception to this observation was N6 extract that showed the highest carotenoid concentration, but only inhibited proliferation by 16 %. A possible explanation for the low inhibitory potency observed with this extract is that the presence of certain carotenoids or other constituents in the extract may have potential contrasting effects. Then, the contribution of each carotenoid to the antiproliferative activity was evaluated using a stepwise multiple regression model. This analysis showed that when six variables (violaxanthin, lutein, zeaxanthin, monoesters, b-carotene and diesters) were fitted to the cell proliferation data, the values of the adjusted coefficient of multiple determination (adjusted R^2) was 0.981 ($p < 0.05$). This means that 98% of the variation in the cell proliferation data was explained by the six variables. It was found that monoesters showed the highest contribution to inhibit cell proliferation (Beta = -1.83, $p < 0.01$). Likewise, lutein and violaxanthin showed negative correlation (Beta = -1.39, $p < 0.05$; Beta = -0.83, $p < 0.05$; respectively) with cell proliferation. In contrast, diesters and zeaxanthin showed positive significant contribution (Beta = 1.41, $p < 0.05$; Beta = 1.06, $p < 0.01$) to cell proliferation. The antiproliferative effect of violaxanthin has been previously demonstrated on MCF-7 human mammary cancer cells, in which a concentration as low as 1 µg/mL of a violaxanthin-

enriched fraction obtained from *Dunaliella tertiolecta* microalgae exerted significant inhibition on cell proliferation after 24 h exposure (Pasquet et al., 2011). In agreement with this, two of the most potent extracts tested in the present study contained violaxanthin at concentrations (~1.8 and ~2.8 µg/mL in N9 and CO₂ (-), respectively) that were close to that previously reported. In another report, violaxanthin was found as possibly involved in the antiproliferative and pro-apoptotic activity of *Chlorella ellipsoidea* extracts on HCT-116 human colon cancer cells (Cha, Koo, & Lee, 2008). On the other side, low-micromolar concentration (10 µM) of lutein has been shown to protect HT-29 cells against deoxynivalenol-induced oxidative stress and apoptosis (Krishnaswamy, Devaraj, & Padma, 2010). In our present study, the assayed lutein concentrations (~15 µg/mL) in two of the most potent extracts (N9 and CO₂ (-)) were higher than 10 µM (~5.6 µg/mL) and exerted significant inhibition (~35%) of HT-29 cell proliferation. These contrasting results compared with those reported in the literature support the idea that factors including the lutein concentration, the presence of other constituents in the extracts, or cell culture conditions such as cell density might influence the outcome of the anti-proliferative studies with these compounds. In agreement with other reports, our data suggested that the presence of zeaxanthin did not enhance the growth inhibitory activity of the extracts on cancer cells (Kotake-Nara et al., 2001). To further characterize the anti-proliferative effect of the most active algae extracts (N9, PS, and CO₂ (-)), the effective concentration range of the extracts was determined on different colon cancer cell lines exhibiting different drug resistance patterns. To that aim, the dose-effect of the most potent algae extracts (N9, PS, and CO₂ (-)) on proliferation of primary colon cancer cells (HGUE-C-1) and two cell lines (HT-29 and SW480) was evaluated after exposure of the cells to different concentrations of each extract by MTT assay. As it can be observed in **Figure 4**, in general, the treatments

with algae extracts resulted in a reduction of cell proliferation in dose-dependent manner. However, cells showed different sensitivity to the extracts. In particular, SW480 line (**Figure 4 B**) was the most sensitive to the three extracts exhibiting significant decrease on cell proliferation after exposure to the lowest assayed concentration (i.e., 27 µg/mL) of the extracts. By contrast, HGUE-C-1 cells (**Figure 4 C**) were more resistant to the effect of the extracts, showing to be especially refractory to PS extract that, in comparison with its effect on the other cell lines, exhibited negligible activity at the highest assayed concentration (250 µg/mL). In the same cell line, N9 extract at highest concentration inhibited the cell proliferation 36.4 % compared to control (**Figure 4 C**). The strongest resistance of HGUE-C-1 cell line could be in correlation to the higher presence of a mesenchymal phenotype which is associated to the invasion and metastasis linked to cancer stem cells in numerous carcinomas (Grasso et al., 2015).

Besides the cytoprotective attributes of carotenoids, in recent years, rising evidence suggest that these compounds may inhibit cancer cell proliferation and, in some cases, induce cell death. Although several mechanisms on their mode of action have been suggested, the exact molecular mechanisms by which carotenoids exert their anticancer effects are still unclear.

4. Conclusions

The study of *N. oleoabundans* extracts obtained by PLE has enabled to demonstrate that under appropriate culture conditions, these green microalgae are able to produce considerable amounts of carotenoids that provide to the biomass an added value. Lutein, carotenoids monoesters and violaxanthin were the most abundant carotenoids, regardless the cultivation conditions. In addition to evaluate the composition and total

carotenoid content of each *N. oleoabundans* PLE extract, the activity of these extracts against colon cancer cell lines has been also investigated. The results obtained demonstrated that those extracts with high carotenoid content showed comparably high antiproliferative activity. In this way, it was possible to correlate the cell proliferation values and the carotenoid constituents. Thus, while carotenoids monoesters showed the highest contribution to inhibit cell proliferation, lutein and violaxanthin showed negative correlation and diesters and zeaxanthin showed a positive significant contribution to cell proliferation. This work opens new possibilities in the use of carotenoids from *N. oleoabundans* as a functional food ingredient or nutraceutical that can help in the prevention of colon cancer development. However, more in depth studies are needed in order to scientifically demonstrate the observed effects *in-vivo*.

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Figure captions

Figure 1. LC-UV chromatograms (450 nm) of three different algae extract; PS, N9, and CO₂ -. PLE conditions: cryogenic grinding pretreatment; temperature, 100 °C; static extraction time, 20 min; extraction pressure, 1,500 psi. Chromatographic conditions: YMC-C30 reversed-phase column (250 mm×4.6- mm inner diameter, 5-µm particle size); mobile phase, methanol-MTBE-water (90:7:3 v/v/v) (solvent A) and methanol-MTBE (10:90 v/v) (solvent B) eluted according to the following gradient: 0 min, 0 % solvent B; 20 min, 30 % solvent B; 35 min, 50 % solvent B; 45 min, 80 % solvent B; 50 min, 100 % solvent B; 52 min, 0 % solvent B; flow rate, 0.8 mL/min; injection volume, 10 µL. Peak identification: 1, violaxanthin; 2, chlorophyll *b*; 3, lutein; 4, zeaxanthin; 5, canthaxanthin; 6, chlorophyll *a*; 7, canthaxanthin-related; 8, echinenone, 9-12, monoesters; 13, β-carotene; 14-16, diesters.

Figure 2. Relative content of the carotenoids obtained from the different *N. oleoabundans* cultures analyzed.

Figure 3. Effect of 250 µg mL⁻¹ of the different algae extracts on HT-29 cell proliferation upon treatment for 24 h (black bars). Error bars are given as the 95% confidence interval of three independent experiments each performed in triplicate. Total carotenoids (mg/g extract) (grey bars). The data are given as the mean ± SD.

Figure 4. Dose-dependent effect of the algae extracts PS, N9 and CO₂ - on HT-29 (A), SW-480 (B) and HGUE-C-1 (C) cell proliferation upon treatment for 24 h. The data are given as the mean ± SD. One-way analysis of variance (ANOVA) and statistical comparisons of the different treatments were performed using Tukey's test. ***p<0.001 versus control group.

Table 1. Extraction yield, total amount of carotenoid and chlorophylls, and content of the main carotenoids identify in *N. oleoabundans* PLE extracts cultivated under different grown conditions.

Sample	Extraction yield (%)	Total carotenoids mg /g extract	Violaxanthin mg/g extract (peak 2)	Lutein mg/g extract (peak 5)	Zeaxanthin mg/g extract (peak 7)	Cantaxanthin mg/g extract (peak 8)	Monoesteres mg/g extract (peaks 13-16)	β -caroteno mg/g extract (peak 17)	Diesteres mg/g extract (peaks 18-20)	Total chlorophylls mg /g extract
N9	29.7 $\pm$ 0.7	129.4 ^{d,e,h}	11.0 ^d	62.6 ^{d,g}	6.0 ^{a,b,d,e}	13.6 ^{d,g,h}	16.3 ^{a,c,d,f}	17.4 ^{d,e,g,h}	2.0 ^{d,g,h}	42.7 ^d
N6	24.4 $\pm$ 1.0	142.8 ^{d,e}	6.8 ^{e,g}	46.3 ^e	6.1 ^{a,b,d,e}	17.3 ^{e,g}	40.3 ^e	20.9 ^{d,e,h}	3.0 ^e	24.9 ^e
N3	35.5 $\pm$ 0.8	76.7 ^{b,c}	2.7 ^{a,b,c,f,h}	21.2 ^{b,c,h}	5.5 ^{a,b,d,e,h}	5.3 ^{a,b,f,i}	27.9 ^b	11.4 ^{a,b,c,f,g}	1.2 ^{a,b,c,g,i}	10.7 ^{a,b,c,h}
HL	33.6 $\pm$ 0.7	62.0 ^{a,c,f}	2.0 ^{a,b,c,f,h,i}	17.2 ^a	6.3 ^{a,b,d,e}	5.9 ^{a,b,c,f,i}	20.0 ^{a,c,d,i}	8.1 ^{a,b,c,i}	1.0 ^{a,b,c,i}	7.6 ^{a,b,c,h}
LL	30.0 $\pm$ 0.7	66.1 ^{a,b,c,f}	3.1 ^{a,b,c,f,h}	23.1 ^{b,c,h}	2.8 ^{c,g,h,i}	7.6 ^{a,c,f}	17.5 ^{a,c,d}	10.4 ^{a,b,c,f,g}	1.1 ^{a,b,c,i}	11.8 ^{a,b,c,f,h}
CO ₂ (+)	26.6 $\pm$ 0.7	62.6 ^{a,c,f}	3.5 ^{a,b,c,f,h}	26.9 ^{f,h}	12.8 ^f	6.0 ^{a,b,c,f,i}	12.7 ^{c,d,f,g}		0.3 ^f	14.1 ^{c,f}
CO ₂ (-)	26.3 $\pm$ 1.0	113.8 ^{g,h}	7.0 ^{e,g}	62.2 ^{d,g}	3.0 ^{g,h,i}	16.2 ^{d,e,g}	9.0 ^{f,g}	13.5 ^{b,c,d,f,g,h}	1.7 ^{b,d,g,h}	38.9 ^g
PS	23.4 $\pm$ 0.7	118.6 ^h	2.9 ^{a,b,c,f,h}	24.7 ^{b,c,f,h}	3.3 ^{b,c,g,h,i}	13.1 ^{d,h}	53.1 ^h	16.9 ^{d,e,f,g,h}	2.2 ^{d,g,h}	10.9 ^{a,b,c,h}
D10	32.8 $\pm$ 0.8	45.2 ⁱ	0.8 ^{a,i}	6.7 ⁱ	2.0 ^{c,g,h,i}	4.7 ^{a,b,f,i}	22.6 ^{a,i}	6.1 ^{a,i}	0.9 ^{a,b,c,i}	2.3 ⁱ

^{a-i} Means in a column without a common superscrit letter differ (p<0.05), as analyzed by one-way ANOVA