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Exploration of marine genus *Chroococcidiopsis* sp.: a valuable source for antioxidant industry?

Joana Assunção[1,2] · Helena M. Amaro[1] · Graciliana Lopes[1,3] · Tânia Tavares[2] · F. Xavier Malcata[2,4] · A. Catarina Guedes[1]

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Abstract

A cyanobacterium of the genus *Chroococcidiopsis* has been recently unveiled to go to Mars, owing to its unique ability to stand harsh environmental conditions. Nevertheless, it has been little characterized in terms of other biotechnological applications. The aim of this study was thus to explore the biotechnological potential of *Chroococcidiopsis* sp. LEGE 06174, using GRAS (generally recognized as safe) solvents for the combined extraction of bioactive compounds. Due to its mucilaginous sheath, sulfuric acid, NaOH, PBS, DMSO 20%, DMSO 100%, and acetone were tested as preliminary treatment—in attempts to enhance pigment extraction. Extracts were then obtained by several series of successive extractions: phosphate-buffered saline (PBS) (serie A), ethanol-PBS (serie B), acetone-PBS (serie C), methanol-PBS (serie D), and acetone-methanol-PBS (serie E). Such extracts were screened for antioxidant capacity, cytotoxicity, and physicochemical characterization in terms of polysaccharides, pigments, and total phenolic compounds. Pre-treatment with PBS proved the most effective toward overall pigment extraction and dissolution of the said mucilaginous sheath. *Chroococcidiopsis* sp. extracts have unfolded some interesting bioactive features, particularly upon pre-treatment with PBS—i.e., high antioxidant capacity and high content in polysaccharides, scytonemin, phycobiliproteins, and phenolic compounds. The highest total pigment content was obtained in serie D, particularly carotenoids, whereas the highest level of phenolic compounds was found in serie B. All in all, *Chroococcidiopsis* sp. appears to constitute a significant biotechnological resource worthy of further exploitation in terms of high added-value compounds.

Keywords Cyanobacteria · Successive extraction · Polysaccharides · Carotenoids · Phycobiliproteins · Phenolic compounds · Biotechnological value

Introduction

Chroococcidiopsis is one of the most primitive genera of photosynthetic and extremophile cyanobacteria, able to survive in extreme conditions (i.e., extreme temperatures, long-term desiccation, nutrient deprivation, extreme pH, or high radiation) (Billi and Caiola 1996, 2010; Cockell et al. 2005). Its outstanding resistance apparently derives from a unique morphology—with cells preferentially organized as aggregates, with spherical, oval, or irregular forms, and protected by an exocellular sheath made of polysaccharides (or mucilaginous envelope) (Billi et al. 2001; Komárek and Johansen 2015).

The aforementioned resistance has made this cyanobacterium worthy of consideration as an outstanding platform for the development of technologies meant for arid areas (i.e., deserts), including survival in extraterrestrial environments, namely on the neighboring planet Mars (Caiola and Billi 2007; Billi 2018). In addition to the interesting features pertaining to Mars, *Chroococcidiopsis* appears to bear a significant biotechnological potential on Earth—yet experimentation with its biomass for extraction of value-added compounds, with possible use as food, feed, cosmetics, or pharmaceuticals, has lagged far behind (Das et al. 2018).

✉ A. Catarina Guedes
acatarinaguedes@gmail.com

1 CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, Novo Edificio do Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n 4450-208, Matosinhos, Portugal

2 LEPABE – Laboratory for Process Engineering, Environment, Biotechnology and Energy, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

3 Department of Biology, FCUP – Faculty of Sciences of University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

4 FEUP – Faculty of Engineering of University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

Cyanobacteria have for some time proven interesting in industrial production as they are extremely resistant and can grow on minimal amounts of nutrients due to distinctive metabolic pathways, which also unveils novel and unique molecules not yet explored for commercial purposes. *Chroococcidiopsis* sp., in particular, is considered to be a self-settler, meaning that it possesses the ability to spontaneously aggregate and self-flocculate in culture (Das et al. 2018). Such a feature is advantageous for large-scale production since it will reduce the amount of energy incurred in biomass harvesting which may, in some cases, account for 30% of the whole microalga processing costs (Barros et al. 2015).

The prospect of finding different bioactive compounds from nonconventional microbial sources from marine environments, especially cyanobacteria, has attracted attention in recent years (Mandal et al. 2020). *Chroococcidiopsis*, in particular, is a likely source of several added-value bioactive compounds—namely, polysaccharides, pigments (chlorophylls, carotenoids, phycobiliproteins, and scytonemin), phenolic compounds, lipids, vitamins, and proteins—which open up a number of possible applications in food, feed, and pharmaceuticals for human and animal welfare, besides cosmetics and dyes (Hayashi et al. 1997; Řezanka et al. 2003; Antonopoulou et al. 2005; Das et al. 2018).

Chroococcidiopsis is a well-known synthesizer of polysaccharides containing sulfur in their mucilaginous sheath (Billi et al. 2001) that can be interesting for use as a nutraceutical additive, therapeutic agent, or cosmetic ingredient (Raposo et al. 2013, 2015). Some of the reported bioactivities exhibited by such type of polysaccharides include antitumor, antiviral, anti-inflammatory, antioxidant, anticoagulant, antimicrobial, immunomodulation, and bio-adhesive properties (Raposo et al. 2013, 2015).

Pigments such as carotenoids (i.e., β-carotene, lycopene, and zeaxanthin) are photosynthetic accessory pigments, with a lipophilic nature that carries a number of benefits for human health, including being powerful antioxidant agents. They can also act as anticancer or anti-immunomodulatory agents (Singh et al. 2017b; Saini et al. 2018; Demay et al. 2019). Furthermore, carotenoids can be used as pharmaceuticals in the form of provitamin A (retinol), important to prevent eye disease (Namitha and Negi 2010), or in aquaculture and poultry production, for pigmentation, and as immunosupplementation (Yaakob et al. 2014). In addition, they can be utilized as dyes and in cosmetics, like shampoo or soaps, with the extra advantage of being non-toxic nor carcinogenic (Rizwan et al. 2018; Sathasivam et al. 2019), and also eco-friendly (Guedes et al. 2011a; Alam 2019; Sathasivam et al. 2019). On the other hand, water-soluble phycobiliproteins pigments like phycocyanin, allophycocyanin, and phycoerythrin, commonly found in cyanobacteria and certain eukaryotic red algae (MacColl 1998), have been claimed to exhibit remarkable antioxidant, anti-inflammatory, neuroprotective, anticancer, and hepatoprotective actions (Pagels et al. 2019). The bright colors of blue (phycocyanin and allophycocyanin) and red (phycoerythrin) make these substances easily applicable as dyes for textiles, beverages, foods, and cosmetics, and they can be used as a fluorescent tool for research or medical diagnosis (Chu 2012; Alam 2019).

Phenolic compounds are found in cyanobacteria, bearing relevant antioxidant properties that make them suitable for both human and animal diet formulations (Kepekçi and Saygideger 2012; Freile-Pelegrín and Robledo 2013; Jerez-Martel et al. 2017; Lindner and Pleissner 2019); they also protect the cardiovascular and nervous systems, further to their preventive effects against cancer (Freile-Pelegrín and Robledo 2013).

Chroococcidiopsis biomass provides an excellent opportunity to explore different solvent-mediated extraction strategies, since optimization of metabolite recovery and combination of green methodologies are important issues within the biotechnological field. Use and reutilization of biomass as per a biorefinery concept may support economically more feasible processes of extraction, while resorting to GRAS (generally recognized as safe) solvents has been gaining importance for being a green and safer extraction methodology.

In view of the above arguments, *Chroococcidiopsis* is likely to possess a biotechnological potential—but only a few studies have tackled its antioxidant activity and inventory of added-value compounds (Das et al. 2018; Montero-Lobato et al. 2020). Therefore, this study aimed at shedding light on the bioactivity potential of the strain *Chroococcidiopsis* sp. LEGE 06174, exploiting the most promising compounds, particularly focusing on polysaccharides, pigments, and phenolic compounds. With the ultimate goal of a biorefinery approach, extraction methodologies were optimized regarding the relevant compounds, having in mind that this cyanobacterium has an intricate mucilaginous sheath composition.

Materials and methods

Microorganism and biomass production

The marine cyanobacterium *Chroococcidiopsis* sp. LEGE 06174 was obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) (http://lege.ciimar.up.pt/).

Biomass of *Chroococcidiopsis* sp. was produced in sterilized, 5-L glass balloons (with a working volume of 4.8 L), in batch mode, using Z8 as culture medium (Kotai 1972) with 25 $g.L^{-1}$ Tropical Marin® sea salt at pH 7.2 ± 0.1. The cultures were kept in an acclimatized chamber at 25 °C and a light intensity of 100 $\mu mol_{photons}.m^{-2}.s^{-1}$ was provided by fluorescent lamps (Biolux, Osram) under a light/dark cycle of 16 h:8 h. Agitation was provided by air passed through a 0.20 μm membrane filter, at an airflow of 0.75 L_{air}

$L_{culture}^{-1}$ min^{-1} sufficient enough to maintain the culture in suspension. The inoculum was set with an initial optical density of 0.1 (OD = λ_{680} nm – λ_{750} nm), and biomass production was monitored over time in order to determine the most suitable day of harvesting (right before reaching the stationary phase). Culture samples were collected regularly and their dry weight (DW) determined, based on the linear relationship between OD and DW (DW = 2×10^7 OD - 10^6, $r^2 = 0.9798$). DW was ascertained by filtering a known volume of culture through preconditioned GF/C glass microfiber filters (Whatman), and further drying at 100 °C until constant weight. When the growth phase was close to an end, the biomass was harvested by centrifugation (1400×*g* for 20 min). The wet biomass was then freeze-dried and kept in a vacuum desiccator for further use.

Biomass pre-treatment

A preliminary study was performed aimed at improving the experimental procedure in terms of total recovery of pigment via selection of the most adequate pre-treatment to remove its polysaccharide envelope. Hence, six different pre-chemical treatments were tested in *Chroococcidiopsis* sp. biomass: sulfuric acid (0.025 M), sodium hydroxide (NaOH; 0.20 M), phosphate-buffered saline (PBS; 20 mM, pH = 7.4), dimethyl sulfoxide (DMSO; 100%), DMSO (20% *v/v*), and acetone (p.a). The pre-treatments were assayed using 85 mg of freeze-dried biomass (in triplicate), 270 ± 20 mg of glass beads, and 5 mL of solvent tested. Cells were crushed in a bead beater Precellys Homogenizer (Bertin Technologies, France), using three cycles (6 series of 8000 rpm for 30 s, with a pause for 40 s).

The effectiveness of the pre-treatment was assessed by measuring pigment recovery and performing a successive extraction with 100% of methanol (20 mM, pH = 7.4) for chlorophyll *a* (chl *a*) and carotenoid extraction, or phosphate buffer for phycobiliprotein extraction. The pre-treatment that allowed the best extraction of pigments was selected to further proceed investigating antioxidant potential and chemical characterizing the metabolites.

Pigment-targeted extraction

After having selected the most adequate pre-treatment to improve total pigment content extraction, *Chroococcidiopsis* sp. biomass was subjected to five different series of extractions, using several GRAS (generally recognized as safe) solvents: acetone (A), ethanol (E), methanol (M), and phosphate-buffered saline (PBS) (20 mM, pH 7.4). All extractions were performed using 85 mg of freeze-dried biomass (in triplicate), 270 ± 20 mg of glass beads and 5 mL of the aforementioned solvents, in combination with a bead beater homogenizer as described previously. After pre-treatment several successive extractions (consecutive extractions with the same biomass sample) were grouped in different series of extraction as follows: PBS (serie A), ethanol-PBS (serie B), acetone-PBS (serie C), methanol-PBS (serie D), and acetone-methanol-PBS (serie E). The extraction with PBS was performed by vortex stirring (Velp Scientifica) for 10 s to avoid foam formation. All extracts were prepared and handled under dimmed light, transferred to 5-mL Eppendorfs, and centrifuged at 2744×*g* for 3 min.

Antioxidant capacity assessment

Evaluation of the antioxidant capacity of the extracts was performed via two distinct radical scavenging assays: $ABTS^{\bullet+}$ and $NO^{\bullet-}$. Both resorted to spectrophotometry using a Multiskan Go (Thermo Fisher) microplate spectrophotometer. Organic extracts were prepared in DMSO/PBS (1:4, *v/v*), to a final concentration of 5 mg mL^{-1} and a dilution series of 0.16, 0.31, 0.63, 1.25, 2.5, and 5 mg mL^{-1}. The aqueous (PBS) extracts were freeze-dried and resuspended in DMSO:PBS (1:4, *v/v*) to final concentrations ranging from 0.02 to 2.08 mg mL^{-1}. All assays were conducted with three biological and three chemical replicates.

$ABTS^{\bullet+}$ assay

The $ABTS^{\bullet+}$ assay was used to assess the total radical-scavenging capacity of *Chroococcidiopsis* extracts (Guedes et al. 2013). Briefly, ABTS radical cation was produced via the reaction of potassium persulfate (Sigma) (0.66 mg mL^{-1}) and ABTS (Sigma) (3.84 mg mL^{-1}). For the assay, 63 μL of extract samples (dilution series 0.16 to 5 mg mL^{-1}) was combined with 180 μL of ABTS radical cation solution, with absorption of 0.70 ± 0.02. All samples were incubated over 6 min, and then absorbance was read at $\lambda = 734$ nm.

$NO^{\bullet-}$ assay

The $NO^{\bullet-}$ assay was used to evaluate the specific antiradical capacity of *Chroococcidiopsis* extracts, by the procedure by Kumar et al. (2008) adapted to spectrophotometer plaque by Pinho and Sousa (2011). In brief, 50 μL of sample extracts (concentrations ranging from 0.16 to 5 mg mL^{-1}) was added with 50 μL of sodium nitroprusside (SNP) at 5 mM, prepared with phosphate-buffered saline (20 mM, pH = 7.4). The reaction mixture was incubated at room temperature under continuous light at an intensity of 23 ± 2 $\mu mol_{photons}$ m^{-2} s^{-1} for 1 h. The reaction mixture was further added with 50 μL Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediaminedihydrochloride in 2% (*v/v*) phosphoric acid). After 10 min, absorbance was read at 562 nm.

The percent inhibition in both assays was calculated as follows:

$$\%\text{inhibition} = \left[\left(\text{Abs}_{\text{sample}}-\text{Abs}_{\text{blank}}\right)-\text{Abs}_{\text{control}}\right]/\text{Abs}_{\text{control}} \times 100$$

where $\text{Abs}_{\text{sample}}$ denotes reacting extract absorbance, $\text{Abs}_{\text{blank}}$ is extract absorbance with the reaction solvent, and $\text{Abs}_{\text{control}}$ is absorbance of DMSO/PBS (1:4 *v/v*). Results were plotted in dose-response curves, and the values of IC_{25} and IC_{50} were calculated using software GraphPad Prism 8—and expressed in $\mu g_{\text{extract}}\ mL^{-1}$.

Cytotoxicity assessment (MTT assay)

All obtained extracts were analyzed for its cytotoxicity/cell viability, using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The rationale of this method is based on the reduction of MTT by the NAD(P)H-dependent cellular oxidoreductase enzymes, present in the mitochondria (and thus only working viable cells), to formazan salts; this reduction leads to the development of a violet/blue color on living cells, measured at 550 nm by optical density (Berridge and Tan 1993; Capasso et al. 2003). The assay used human cardiac microvascular endothelial (hCMED) cells obtained from American Type Culture Collection (ATCC). In brief, cells were seeded in a 96-well plate with a final concentration of 10×10^4 cells mL^{-1} with Dulbecco's Modified Eagle Medium (DMEM) for 24 h. The cells were incubated for 24 and 48 h with DMEM and the extracts of *Chroococcidiopsis* sp. at two different concentrations (100 and 200 $\mu g.mL^{-1}$) to a final volume of 100 μL. Cell medium with 1 and 20% of DMSO was added to the multiplate as negative and positive control (maintenance of cell viability and cell death), respectively, for the organic extracts. For the aqueous extracts, the negative control (growth) was DMEM, because the samples were dissolved in water. To assess cell viability, 20 μL of MTT solution (1 mg mL^{-1}) was added to the well plate and incubated at 37 °C for 3.5 h. In a final step, DMSO was added to the wells to dissolve the formazan salts and absorbance was read at 550 nm.

Extract chemical characterization

Polysaccharide content determination

The polysaccharide content in the mucilaginous sheath extracted with the pre-treatment and in the aqueous extracts with PBS was determined according to Dubois et al. (1951). In short, 1 mg mL^{-1} of mass extract was added to 4 mL of sulfuric acid (1 M) to bring about hydrolysis of the saccharides. After 1-h incubation at 100 °C, 100 μL of each sample was homogenized and 300 μL of deionized water (dH_2O), 400 μL of phenol (5%, *w/v*), and 2 mL sulfuric acid (98%, *v/v*) were added. The blank was obtained by adding 100 μL of sulfuric acid (1 M) to the dH_2O, phenol, and concentrated sulfuric acid. The mixtures were maintained at 27 °C in a water bath for 30 min. The absorbance was then read at 490 nm using a Shimadzu UV-1800 spectrophotometer. A standard curve was prepared with glucose (Alfa Aesar) dissolved in water, ranging within 0–500 $\mu g\ mL^{-1}$. The content in total polysaccharides in the extracts was expressed as μg of glucose equivalents (GUE) per gram of dried biomass, by resorting to linear model: absorbance = 0.002 × total content polysaccharides ($\mu g_{GUE}/g_{DW}$) + 0.0349 ($R^2 = 0.985$).

Chlorophyll a and carotenoid content and profile

To select the best pre-treatment solvent, methanolic extracts were spectrophotometrically quantified for their total content in chlorophyll *a* (chl *a*) and carotenoids, according to Lichtenthaler and Buschmann (2001). Absorption was read at λ_{470}, λ_{652}, and λ_{665} nm and the content calculated as follows:

$$\text{Chl } a\ \left(\mu g.mL^{-1}\right) = 16.72A_{665}-9.16A_{652}$$
$$\text{Total carotenoids}\ \left(\mu g.mL^{-1}\right) = 1000A_{470}-1.63\ \text{Chl } a/221$$

The final results were expressed in mg per gram of dry weight.

For the successive organic extraction series, with ethanol, acetone, and methanol, pigments were identified and quantified in the extracts, via high-performance liquid chromatograph (HPLC) with photodiode array (PDA) detection (Waters Alliance 2695, USA) following a method described elsewhere (Guedes et al. 2011a).

The extracts were resuspended in acetone:acetonitrile (9:1) at a concentration of 10 mg mL^{-1} and an internal standard, β-apo-carotenol (170 mg L^{-1}; Sigma Aldrich) was added. A column heater (Waters, USA) was used during the analyses in order to maintain a constant temperature of 25 °C.

The stationary phase consisted of a 4 × 250 mm Purospher STAR RP-18e (5 μm) column (Merck), and the mobile phase of ethyl acetate (solvent A) and acetonitrile:water at 9:1 (*v/v*) (solvent B). A flow rate of 1 mL min^{-1} was set with a gradient using solvents A and B with the following elution profile: 0–31 min (0–60% A); 31–36 min (60% A); 36–38 min (60–100% A); 38–43 min (100% A); 43–50 min (100–0% A); and 50–55 min (0% A). Spectra from all peaks were collected in the range 250–750 nm. The compounds were identified by comparing their retention times and the UV-visible spectrum with the true chromatographic standards and quantified via calibration curves. Standards (HPLC grade) were purchased from Extrasynthese (lutein, zeaxanthin, and β-carotene) and Sigma (echinenone).

Phycobiliprotein content The total phycobiliprotein (PBP) content, including phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) in *Chroococcidiopsis* sp. aqueous extracts, pre-treatment, and PBS extracts was

determined according to Bennett and Bogorad (1973). All samples were measured in a spectrophotometer Shimadzu UV-1800, at wavelengths of λ_{562}, λ_{615}, and λ_{652}, and quantification was as follows:

$$\mathrm{PC}\left(\mathrm{mg.mL^{-1}}\right) = \mathrm{A_{615}}-0.474\ \mathrm{A_{652}}$$
$$\mathrm{APC}\left(\mathrm{mg.mL^{-1}}\right) = \left(\mathrm{A_{652}}-0.208\ \mathrm{A_{652}}\right)/5.09$$
$$\mathrm{PE}\left(\mathrm{mg.mL^{-1}}\right) = \left[\mathrm{A_{562}}-2.41(\mathrm{PC})-0.849\ (\mathrm{APC})\right]/9.62$$

The results were expressed in mg per milligram of dry weight.

Scytonemin content

Scytonemin is an external pigment present in the mucilaginous sheath, and its content was determined only in the pre-treatment extract. Therefore, after extraction of 1 mg of pre-treated dry extract with 1 mL of acetone, the scytonemin content was measured spectrophotometrically according to Garcia-Pichel and Castenholz (1991), at wavelengths λ_{384}, λ_{665}, λ_{640}, through the following equation:

$$\mathrm{Scytonemin}\ \left(\mathrm{mg\ mg_{DW^{-1}}}\right) = 1.04\mathrm{A_{384}}-0.79\mathrm{A_{665}}-0.27\mathrm{A_{490.}}$$

Scytonemin content was expressed in milligram of dry weight (mg mg_{DW}^{-1}) using the extinction coefficient for scytonemin in acetone (112.6 L g^{-1} cm^{-1}) (Garcia-Pichel et al. 1992)

Total phenolic determination

The total phenolic content in the extracts was determined via Folin-Ciocalteu colorimetric method (Folin and Ciocalteu 1927), adapted by Maadane et al. (2015), using gallic acid for standard. In brief, 50 μL of extract samples (at a concentration of 5 mg mL^{-1}) was added to a well plate with 125 μL of deionized water and 25 μL of Folin-Ciocalteu reagent and kept in the dark for 5 min. Seventy-five microliters of sodium carbonate (6.60 wt%) was added to the mixture and kept at room temperature over 60 min. The absorbance of the samples was then measured at $\lambda = 760$ nm using a microplate spectrophotometer (Multiskan Go, ThermoFisher) and gallic acid (Acros Organics) as standard (calibration curve: ranging from 0 to 200 μg mL^{-1} (DMSO/PBS (1:4 *v/v*)). The total content of phenolic compounds in the extracts was expressed in mg of gallic acid equivalent (GAE) per gram of dried biomass (mg_{GAE} g_{DW}^{-1}), using the equation fitted by linear regression: absorbance = 0.0058 × total phenolic content (mg_{GAE} g_{DW}^{-1}) + 0.0498 ($R^2 = 0.999$).

Statistical analysis

The experimental data were analyzed with the software GraphPad Prism 8. A diagnostic revealed a non-normal distribution of the data. Hence, a one-way ANOVA, with Tukey's multiple comparison test, was used to assess variances between total content in chlorophylls, carotenoids, PBPs, phenolic compounds, and antioxidant capacity. Student's *t* tests were performed to ascertain significant differences ($p < 0.05$) among the results of the cytotoxicity tests.

Results

Chroococcidiopsis sp. biomass production

Biomass production by *Chroococcidiopsis* sp. LEGE 01764 was monitored over time. During the cultivation, cyanobacteria can accumulate several compounds with high antioxidant capacity (i.e., carotenoids) (Amaro et al. 2015). For that reason, the growth curve of *Chroococcidiopsis* sp. was produced and the late exponential phase, reached on day 21 (Fig. 1) was set to harvest biomass.

Cyanobacterial pre-treatment selection

The results of biomass pre-treatment are depicted in Fig. 2. The pre-treatment allowing a higher recovery of pigments, namely, chl *a* and PBPs, was PBS. The PBS pre-treatment allowed to double the extraction of chl *a* (1.94 ± 0.02 mg g_{DW}^{-1}), when compared with the biomass without pre-treatment (1.02 ± 0.02 mg g_{DW}^{-1}), and also helped increase the PBP concentration, thus achieving a value of 3.15 ± 0.15 mg g_{DW}^{-1} (against 3.02 ± 0.05, without pre-treatment), while carotenoid content did not undergo a significant increase (0.51 ± 04 mg g_{DW}^{-1}, when compared to that with pre-treatment (0.45 ± 0.00 mg g_{DW}^{-1})).

On the other hand, DMSO (20% *v/v*) doubled chl *a* and increased the carotenoid content by 1.2-fold, but PBP content was reduced about 1.6 times when compared to the extracts without pre-treatment. Overall, DMSO (20%, *v/v*) only increased the

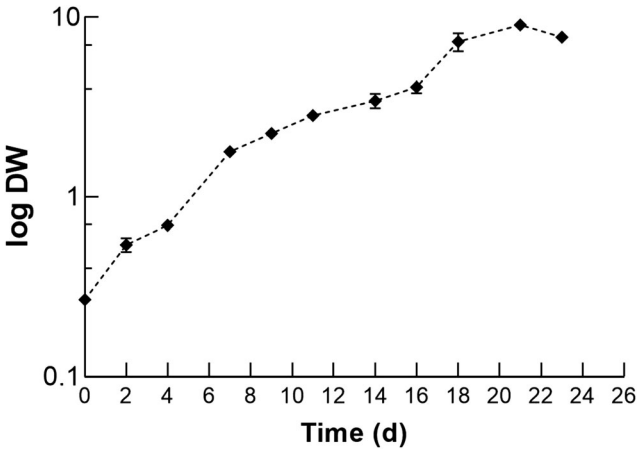

Fig. 1 Growth curve of *Chroococcidiopsis* sp. LEGE 06174, expressed in natural logarithm of dry weight (log DW ( ). The data points show mean ± SD, $n = 3$

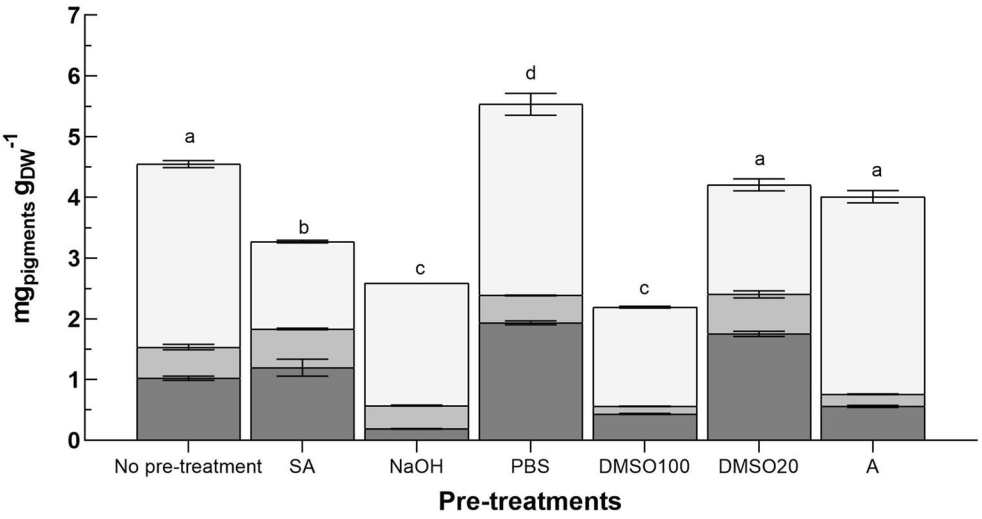

Fig. 2 Influence of six different pre-treatments with SA-sulfuric acid (0.025 M), NaOH (0.20 M), PBS (20 mM, pH = 7.4), DMSO 100%, DMSO 20% (*v*/*v*), and A-acetone upon contents of total chl *a* (■) (■), carotenoids (□), and total phycobiliprotein content (□), after a methanolic extraction and successive PBS extraction upon *Chroococcidiopsis* sp. biomass. The control is represented with no pre-treatment. The results are reported as mean ± SD ($n = 3$) and expressed in $mg_{pigments}.g_{DW}^{-1}$. Different letters mean that they are statistically different ($p < 0.05$) (one-way ANOVA, Tukey's multiple comparisons test)

pigment content by 6%, so PBS appeared as the best pre-treatment for increasing overall pigment extraction up to 20% (when compared to the extracts without pre-treatment). It was therefore selected to be used as solvent for biomass pre-treatment.

Extract antioxidant capacity

Antioxidant capacity of all extracts of *Chroococcidiopsis* was evaluated by two different methods, $ABTS^{\bullet+}$ and $NO^{\bullet-}$, and results compared by the IC_{50} and IC_{25} values.

Regarding the $ABTS^{\bullet+}$ assay (Table 1), the IC_{50} values of aqueous extracts confirm its high antioxidant capacity, in particular PBS extract of the pre-treatment ($IC_{50} = 50.71 \pm 3.40$ $\mu g_{extract}$ mL^{-1}), followed by the aqueous extract of serie E ($IC_{50} = 161 \pm 17$ $\mu g_{extract}.mL^{-1}$). No statistical differences were found among aqueous extracts of serie C ($IC_{50} = 310.13 \pm 6.19$ $\mu g_{extract}$ mL^{-1}) and serie D ($IC_{50} = 325.10 \pm 11.51$ $\mu g_{extract}$ mL^{-1}). Within the organic extracts, the best IC_{50} was obtained in the acetonic extract of series C (and E) (284.68 ± 17.04 $\mu g_{extract}$ mL^{-1}).

Regarding the $NO^{\bullet-}$ results (Table 1), the lowest IC_{50} values were again found to be ones with PBS, either in pre-treatment ($IC_{50} = 199.60 \pm 43.46$ $\mu g_{extract}$ mL^{-1}) and in aqueous extract of serie D ($IC_{50} = 216.20 \pm 9.41$ $\mu g_{extract}$ mL^{-1}). with no significant differences between them ($p > 0.05$). Within the organic extracts, the best IC_{50} was obtained in methanolic extract (serie D), i.e., 429.04 ± 30.61 $\mu g_{extract}$ mL^{-1}.

Extract cytotoxicity

All extracts, both organic and aqueous, including the pre-treatment were tested for their cytotoxicity via the MTT assay at two concentrations: 100 and 200 μg mL^{-1}, for 24 and 48 h. The choice of concentrations was based on the lowest IC_{50} values found for the aqueous extracts, i.e., those that have higher antioxidant capacity.

The organic extracts did not have any adverse effects upon cell viability (when compared to the control, DMSO, 1%), except for the methanolic extract of serie E (at both concentrations tested) (Fig. 3a).

The aqueous extracts showed toxicity at 100 and 200 μg mL^{-1}. In the latter, the cell viability was only maintained over the 24-h exposure. The pre-treatment extract was the only one showing cytotoxicity at both concentrations and both times of exposure, with significant differences versus the control (DMEM media) ($p < 0.05$) (Fig. 3b).

Extract chemical characterization

Total polysaccharide content

The total content in polysaccharides was determined for the aqueous (PBS) extracts, including pre-treatment (Fig. 4). Pre-treatment extracted the highest content in polysaccharides (115.18 ± 4.63 μg_{GUE} $g_{extract}^{-1}$) followed by the serie A (38.97 ± 5.36 μg_{GUE} $g_{extract}^{-1}$) and C (26.18 ± 3.88 μg_{GUE} $g_{extract}^{-1}$). Use of several organic extractions, serie E, was not favorable to increase polysaccharide content ($10.68\ 97 \pm 1.13$ μg_{GUE} $g_{extract}^{-1}$).

Extract pigment profile and content

The pigment profile of the successive extractions of *Chroococcidiopsis* with antioxidant potential, of the tested series

Table 1 Antioxidant capacity represented described by IC_{25} and IC_{50} values ($\mu g_{extract}\ mL^{-1}$), obtained with $ABTS^{\bullet+}$ and $NO^{\bullet-}$ assays for each extraction series, including pre-treatment of biomass of *Chroococcidiopsis* sp. LEGE 06174. The results are expressed as mean ± SD ($n = 3$). For each column, different letters mean $p < 0.05$ (two-way ANOVA; Tukey's multiple comparisons test)

Series of extraction	Extracts ($\mu g_{extract}.mL^{-1}$)	$ABTS^{\bullet+}$ IC_{50}	$NO^{\bullet-}$ IC_{50}
Pre-treatment	PBS	50.71 ± 3.40^{a}	199.60 ± 43.46^{a}
Serie A	PBS	430.58 ± 30.61^{b}	275.46 ± 1.77^{b}
Serie B	Ethanol	358.50 ± 16.55^{b}	620.13 ± 18.35^{c}
	Phosphate buffer	500.03 ± 30.01^{c}	418.72 ± 1.40^{d}
Serie C	Acetone	284.68 ± 17.04^{d}	n.d.
	Phosphate buffer	310.13 ± 6.19^{d}	426.56 ± 20.94^{d}
Serie D	Methanol	427.54 ± 23.96^{c}	429.04 ± 30.61^{d}
	Phosphate buffer	325.10 ± 11.51^{d}	216.20 ± 9.41^{a}
Serie E	Acetone	284.68 ± 17.04^{d}	n.d.
	Methanol	905.40 ± 30.75^{f}	640.04 ± 34.38^{c}
	Phosphate buffer	160.71 ± 16.72^{g}	326.55 ± 17.03^{e}

n.d. not determined

A, B, C, D, and E, was assessed. The total chl *a* and total carotenoids were estimated in the organic extracts by HPLC, whereas the PBPs (namely PC, APC, and PE) were evaluated in the aqueous solvent by spectrophotometry.

The pigment content of the organic and aqueous extracts is shown in Table 2. Concerning the first ones, the methanolic extract of serie D stood out with the highest content of both chl *a* (5.06 ± 0.34 mg g_{DW}^{-1}) and total carotenoids ($1.72 \pm$

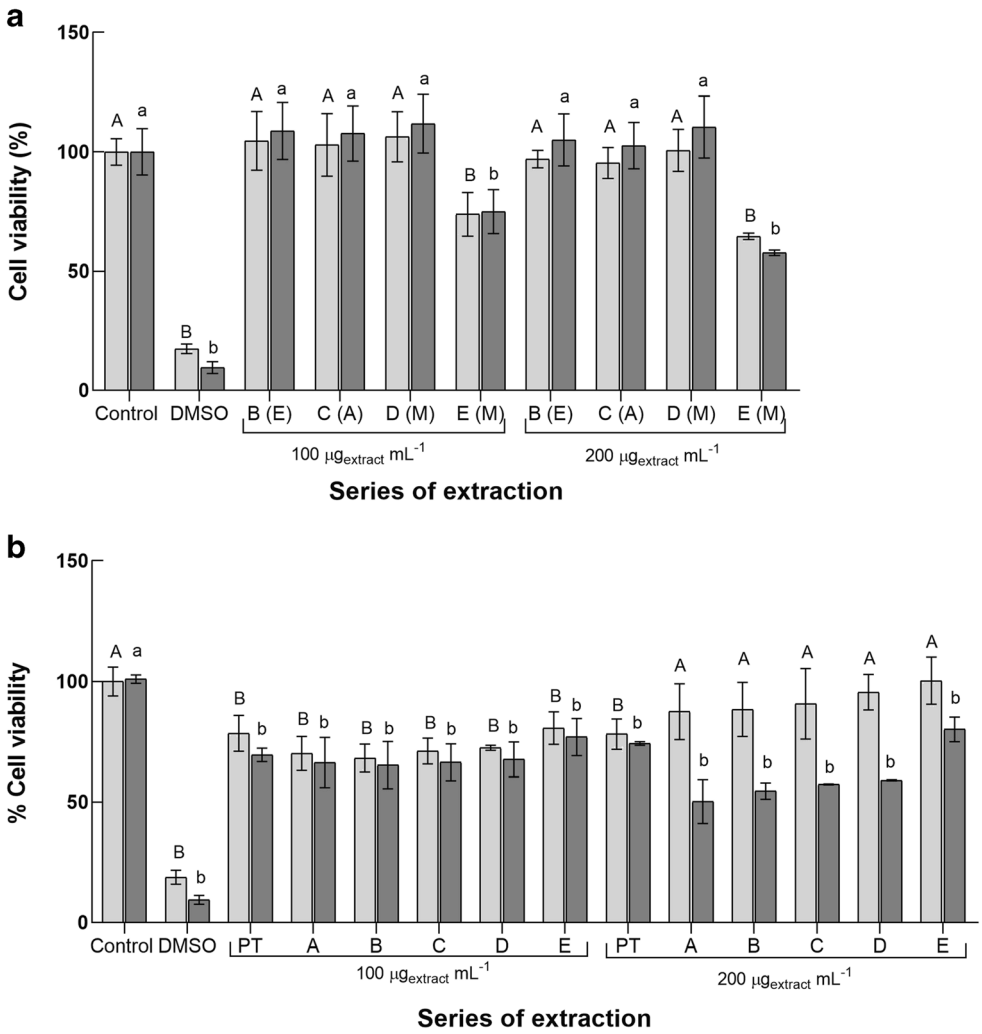

Fig. 3 Cytotoxicity effects of the successive *Chroococcidiopsis* sp. LEGE 06174 extracts serie A (PT-PBS), B (PT-ethanol-PBS), C (PT-acetone-PBS), D (PT-methanol-PT), and E (PT-acetone-methanol-PBS) for the organic (**a**) and aqueous (**b**) extracts against hCMED cells. Organic extracts are represented as follows: B (E) – serie B, ethanolic; C(A) – serie C, acetonic; D(M), serie D, methanolic; and E(M) – serie E, methanolic. In aqueous extracts, each capital letter refers to each series, adding the pre-treatment (PT) with PBS. The control (no cell death) was tested with DMSO 1%; the negative control (DMSO) (cell death) was tested with DMSO 20%. The extracts were exposed for 24 (■) and 48 h (▩), at 100 $\mu g.mL^{-1}$ and at 200 $\mu g.mL^{-1}$ Results are reported as mean ± SD ($n = 4$) and expressed in $\mu g_{extract}.mL^{-1}$. Different letters mean $p < 0.05$ (Student's *t* test)

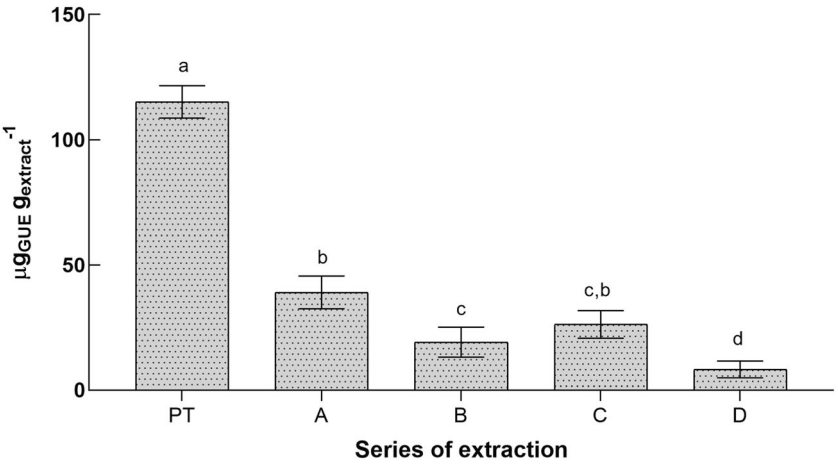

Fig. 4 Total polysaccharide content at each aqueous series of extraction serie A (PT-PBS), B (PT-ethanol-PBS), C (PT-acetone-PBS), D (PT-methanol-PT), and E (PT-acetone-methanol-PBS), including pre-treatment (PT) with PSB solvent. The results are reported as mean ± SD ($n = 3$ and expressed in $\mu g_{GUE}.g_{extract}^{-1}$. Different letters mean $p < 0.05$ (one-way ANOVA, Tukey's multiple comparisons test)

0.05 mg g_{DW}^{-1}). The combination with acetonic extract (2.41 ± 0.15 g g_{DW}^{-1}) of serie E was significantly higher, in terms of chl *a* content, than the acetonic extraction (serie C) ($p < 0.05$) alone. Furthermore, the acetonic extract (serie C) seems more selective to extract carotenoids than chl *a*.

The results concerning the aqueous extracts and PBP content, in particular PC, APC, and PE, are shown in Table 2. In general, pre-treatment with PBS extracts led to the highest amount of the three pigments under scrutiny, with 2.54 ± 0.19 mg g_{DW}^{-1} for PC, 2.57 ± 0.17 mg g_{DW}^{-1} for APC, and 1.62 ± 0.05 mg g_{DW}^{-1} for PE, with a total pigment content in PBPs of 6.74 ± 0.49 mg g_{DW}^{-1}. However, serie A extraction attained the highest result for APC, with 2.09 ± 0.07 mg g_{DW}^{-1}, followed by PC with 1.08 ± 0.00 mg g_{DW}^{-1} and PE with 1.01 ± 0.17 mg g_{DW}^{-1}. The total PBP content seemed to be affected when successive extraction occurs with organic solvents—as their content is drastically reduced about 2 times, when compared to the PBS extraction of serie A.

In general, the overall content in pigments (chl *a* and carotenoids) is higher in serie D, including the PBPs extracted in the pre-treatment.

The carotenoid and chl *a* profiles were studied in the various organic extracts (Table 3). The chl *a* and derivatives were the most abundant pigments found in all extracts studied, followed by several xanthophylls, like zeaxanthin and echinenone. Carotenes like β-carotene and α-carotene derivatives were also found. The profiles of the acetonic (series C/E) (see Fig. 5) and methanolic (serie D) extracts were found similar (except for the presence of pheophytin (pheo)), yet the content in each pigment was very different. Such extracts also presented two zeaxanthin derivatives that were not found in the other extracts. In the case of the ethanolic extract (serie B), two chl derivatives (including pheo) were not extracted, nor the two zeaxanthin derivatives. Lycopene was also detected in all extracts, except for the methanolic extract of serie E. In this case, neither two zeaxanthin derivatives were present, nor was pheo (Table 3).

The ethanolic extract (serie B) contains echinenone (445.84 ± 28.22 mg g_{DW}^{-1}) and lycopene (49.09 ± 2.58 μg g_{DW}^{-1}) as the major carotenoids extracted, besides chl *a* (3766.85 ± 207.12 μg g_{DW}^{-1}). The acetone extract of serie C contained also echinenone as major carotenoid (169.27 ± 4.27 μg g_{DW}^{-1}), followed by lycopene (29.23 ± 3.21 μg g_{DW}^{-1}) and a high content of a particular chl *a* derivative (944.98 ± 49.50 μg g_{DW}^{-1}). This extract also contains pheo (230.62 ± 13.41 μg g_{DW}^{-1}), which did not show up in any other extract profiles.

Serie D accounted for the extract with the highest content in all carotenoids, especially xanthophyll and echinenone (581.54 ± 20.40 μg g_{DW}^{-1}) and β-carotene (313.28 ± 11.66 μg g_{DW}^{-1}) and α-carotene (254.33 ± 2.32 μg g_{DW}^{-1}). Chl attained the highest value in the form of chl *a* derivative (2239.78 ± 115.05 μg g_{DW}^{-1}). The methanolic extract of serie E was more selective to extract echinenone (75.70 ± 3.68 μg g_{DW}^{-1}), followed by zeaxanthin (2.64 ± 0.05 μg g_{DW}^{-1}). In terms of chlorophylls, chl *a* derivative (562.22 ± 49.85 μg g_{DW}^{-1}) exhibited the highest content.

Scytonemin was quantified only in the extract of the pre-treatment. Its content was 0.127 mg g_{DW}^{-1} (corresponding to 0.01%$_{DW}$).

Total phenolic compound content

The TPC content of the various successive extractions of *Chroococcidiopsis* was evaluated by the Folin-Ciocalteu method (Fig. 6).

The PBS solvent proved to be the most effective in extracting phenolic compounds, and the pre-treatment performed before all extraction series was able to extract a high percentage of the more polar TPC—reaching 1.64 ± 0.02 mg_{GAE} g_{DW}^{-1}. Serie B cumulatively achieved the highest level of extraction of TPC, with a total of 1.98 ± 0.19 mg_{GAE} g_{DW}^{-1}, while the ethanolic fraction attained 1.47 ± 0.19 mg_{GAE} g_{DW}^{-1} (corresponding to the fraction of TPC with more apolar nature). Series A and E contain the lower content in phenolic compounds, with 0.27 ± 0.01 mg_{GAE} g_{DW}^{-1} and 0.22 ± 0.01 mg_{GAE} g_{DW}^{-1}, respectively, with no significant differences between them ($p > 0.05$).

Table 2 Pigment composition of *Chroococcidiopsis* sp. LEGE 06174. extracts, in terms of total chlorophyll *a*, total carotenoids, and total phycobiliproteins, including phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE), and total pigment content in the different series of extraction (series A, B, C, D and E), including pre-treatment. The results are reported as mean ± SD ($n = 3$). For each column, different letters mean $p < 0.05$ (one-way ANOVA, Tukey's multiple comparisons test)

Series of extraction	Extracts	Total chlorophyll *a* (mg g_{DW}^{-1})	Total carotenoids (mg g_{DW}^{-1})	Phycobiliproteins (mg g_{DW}^{-1})			Total PBP (mg.g_{DW}^{-1})	Total pigment content** (mg.g_{DW}^{-1})
				PC (mg.g_{DW}^{-1})	APC (mg.g_{DW}^{-1})	PE (mg.g_{DW}^{-1})		
Pre-treatment*	PBS	-	-	2.54 ± 0.19^{a}	2.57 ± 0.17^{a}	1.62 ± 0.05^{a}	6.74 ± 0.49^{a}	6.74 ± 0.49^{a}
Serie A	PBS	-	-	1.08 ± 0.06^{b}	2.09 ± 0.07^{b}	1.01 ± 0.17^{b}	4.90 ± 0.17^{b}	4.90 ± 0.17^{b}
Serie B	Ethanol	4.48 ± 0.21^{a}	0.62 ± 0.01^{a}	-	-	-		7.11 ± 0.24^{a}
	Phosphate buffer	-	-	0.67 ± 0.03^{c}	0.92 ± 0.04^{c}	0.42 ± 0.01^{c}	2.01 ± 0.09^{c}	
Serie C	Acetone	2.41 ± 0.15^{b}	1.07 ± 0.05^{a}					5.49 ± 0.09^{b}
	Phosphate buffer	-	-	0.76 ± 0.03^{c}	0.87 ± 0.06^{c}	0.39 ± 0.01^{c}	2.02 ± 0.09^{c}	
Serie D	Methanol	5.06 ± 0.34^{c}	1.72 ± 0.05^{b}	-	-	-		9.68 ± 0.47^{c}
	Phosphate buffer	-	-	0.99 ± 0.04^{d}	1.31 ± 0.05^{d}	0.60 ± 0.01^{c}	$2.90 \pm 0.10^{c,d}$	
Serie E	Acetone	2.41 ± 0.15^{b}	1.07 ± 0.05^{a}	-	-	-		
	Methanol	0.81 ± 0.11^{d}	0.11 ± 0.000^{c}	-	-	-		
	***$\Sigma(A+M)$	3.22 ± 0.14^{e}	$1.18 \pm 0.05^{a,d}$					
	Phosphate buffer	-	-	$0.81 \pm 0.02^{c,d}$	0.91 ± 0.06^{c}	0.50 ± 0.04^{c}	2.23 ± 0.10^{c}	

*The step of pre-treatment with PBS was employed in all extraction series

**Total pigment content of the extraction series does not include the pigment content obtained in the pre-treatment

***Σ denotes the sum of pigments in the successive acetonic and the methanolic extracts of serie E

Table 3 Chlorophyll and carotenoid profile and content of *Chroococcidiopsis* sp. LEGE 01674 extracts in the different organic extraction series (serie B, C, D, and E). The results are reported as mean ± SD ($n = 3$) and are expressed in μg g_{DW}^{-1}. For each row, different letters mean $p < 0.05$ (one-way ANOVA, Tukey's multiple comparisons test)

Identified pigments	Concentration of pigment ($\mu g_{pigment}\ g_{DW}^{-1}$)			
	Serie B (ethanol)	Serie C/E (acetone)	Serie D (methanol)	Serie E (methanol)
Carotenoids				
Carotenes				
α-carotene*	27.91±0.56[a]	20.27±1.86[a]	254.33±2.32[b]	10.81±0.27[c]
α-carotene derivative*	35.10±1.66[a]	17.09±0.39[b]	306.49±3.38[c]	11.88±0.08[b]
β-carotene	40.78±3.17[a]	25.13±0.89[b]	313.28±11.66[c]	11.25±0.41[d]
Lycopene*	49.09±2.58[a]	29.23±3.21[b]	60.09±4.33[c]	n.d.
Xanthophylls				
Echinenone	445.845±28.22[a]	169.27±4.27[b]	581.54±20.40[c]	562.22±49.85[c]
Zeaxanthin	9.57±0.88[a]	6.36±0.66[a]	50.91±4.07[b]	2.64±0.05[c]
Zeaxanthin derivative**	n.d.	7.42±0.62[a]	15.21±0.54[b]	n.d.
Zeaxanthin derivative**	n.d.	7.11±0.65[a]	13.96±0.91[b]	n.d.
Chl a and derivatives				
Chlorophyll *a*	3766.85±207.12[a]	909.84±69.54[b]	2123.95±162.34[c]	146.49±32.91[d]
Chlorophyll *a* derivative***	n.d.	944.98±49.50[a]	2239.78±115.05[b]	562.22±49.85[c]
Chlorophyll *a* derivative***	714±0.00[a]	284.24±35.84[b]	693.47±75.84[c]	131.91±8.61[d]
Pheophytin***	n.d.	230.62±13.41	n.d.	n.d.

*Concentration in β-carotene equivalent; **concentration in zeaxanthin equivalent; ***concentration in chl *a* equivalent.

Serie C reached the highest content of TPC with polar nature, with its PBS fraction possessing a total of 1.20 ± 0.11 $mg_{GAE}\ g_{DW}^{-1}$. Serie D, including extraction with methanol, was not so effective when compared to the other series, with a total of 0.68 ± 0.04 $mg_{GAE}\ g_{DW}^{-1}$—of which the organic fraction contributed 0.08 ± 0.00 $mg_{GAE}\ g_{DW}^{-1}$ and the aqueous fraction 0.19 ± 0.02 $mg_{GAE}\ g_{DW}^{-1}$.

Correlation between antioxidant capacity and chemical characterized compounds

The correlation between the antioxidant capacity (IC_{50} value) and the compounds here studied—and described as having antioxidant capacity in the literature (i.e., Amaro et al. 2015; Singh et al. 2017b; Pagels et al. 2019)—was determined in both aqueous and

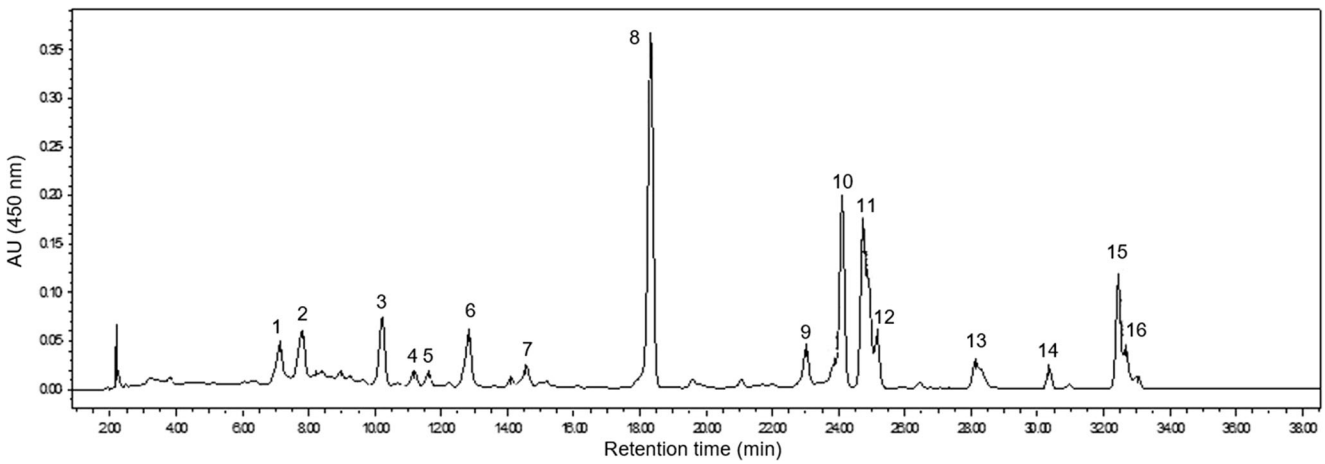

Fig. 5 Chl and carotenoid HPLC-profile of acetonic successive extracts (serie C/E) of *Chroococcidiopsis* sp. LEGE 06174. HPLC-PDA recorded at 450 nm. Lycopene (1), zeaxanthin derivatives (2,3), zeaxanthin (6), unidentified carotenoids (4,5,7,13,14), chl *a* derivatives (9,10,12), equinenone (11), β-carotene (15), α-carotene (16), and internal standard (trans-β-Apo-8′-carotenal) (8)

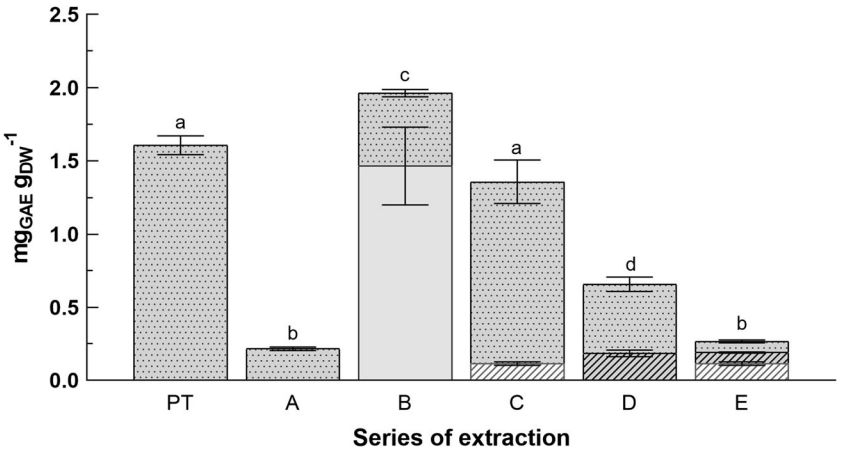

Fig. 6 Total phenolic compound (TPC) content at each series of extraction, A (PT-PBS), B (PT-ethanol-PBS), C (PT-acetone-PBS), D (PT-methanol-PT), and E (PT-acetone-methanol-PBS), and corresponding solvents, PBS ⬚, including PT (pre-treatment), ethanol ▨, acetone ▨ and methanol ▨. The results are reported as mean ± SD (n=3). Different letters mean p<0.05 (one-way ANOVA, Tukey's multiple comparisons test)

organic extracts and is depicted in Table 4. For the aqueous extracts, the studied compounds—PBPs, polysaccharides, and phenolic content—had significant influence on the antioxidant capacity for $ABTS^{\bullet+}$ assay ($p < 0.05$), having polysaccharides a more preponderant role. The negative correlation [$r\,(x,y) = -0.71$] observed describes a high antioxidant capacity with the lower IC_{50} and with the increment of content in such compounds. In $NO^{\bullet-}$ assay, PBPs and polysaccharides were seen to contribute to the major bioactivity of the extracts ($p < 0.05$).

Regarding the organic extracts, chl and the carotenoids showed to have a significant role concerning the bioactivity in the $ABTS^{\bullet+}$ assay ($p < 0.05$), while for the $NO^{\bullet-}$, none of the studied compounds was observed to contribute directly to the antioxidant capacity.

Discussion

From a biotechnological perspective, *Chroococcidiopsis* sp. holds potential as a new source of metabolites bearing antioxidant properties, with eventual application in food, nutraceuticals, or cosmetics. Its mucilaginous envelope, rich in polysaccharides, is likely to make extraction of certain intracellular compounds (i.e., pigments) more difficult—so there is room to improve and optimize extraction methodologies, always resorting to GRAS solvents; this guarantees their acceptance by the food industry, abiding the biorefinery concept. Therefore, this study tested different combinations of extraction methodologies with GRAS solvents, with extracts screened for antioxidant potential (and cytotoxicity) and targeted at potential antioxidant compounds, namely pigments and phenolic compounds, in view of their expected biotechnological applications.

Finding strategies to remove the complex polysaccharide in the envelope of *Chroococcidiopsis* sp. would aid toward higher recovery of total pigments (chl *a*, carotenoids, and PBPs). A preliminary study encompassing a number of chemical pretreatments (with H_2SO_4, NaOH, PBS, acetone, and DMSO 20% and 100% (*v/v*)) of *Chroococcidiopsis* sp. biomass was performed, and their influence on pigment content

Table 4 Correlation between the antioxidant capacity (IC_{50}) of $ABTS^{\bullet+}$ and $NO^{\bullet-}$ assays and the content of the chemical characterized compounds in both aqueous and organic extracts, by using Pearson product-moment correlation. The variables in bold have a statistical relationship ($p < 0.05$) with the antioxidant capacity

Aquous extracts (n=9)					Organic extracts (n=17)				
$X_{variable}$	$Y_{variable}$	$r\,(x,y)$	r^2	p value	$X_{variable}$	$Y_{variable}$	$r\,(x,y)$	r^2	p value
$ABTS^{\bullet+}$ IC_{50}	PBP content	−0.57	0.33	**$1.54\times10-3$**	$ABTS^{\bullet+}$ IC_{50}	Chl content	−0.85	0.72	**$3.06\times10-2$**
	Polysaccharide content	−0.71	0.45	**$1.55\times10-3$**		Total carotenoid content	−0.95	0.92	**$2.62\times10-3$**
	Phenolic compound content	−0.59	0.46	**$1.22\times10-2$**		Phenolic compound content	0.67	0.45	1.421×10^{-1}
$NO^{\bullet-}$ IC_{50}	PBP content	−0.68	0.35	**$2.68\times10-3$**	$NO^{\bullet-}$ IC_{50}	Chl content	−0.44	0.20	3.795×10^{-1}
	Polysaccharide content	−0.56	0.32	**$1.86\times10-2$**		Total carotenoid content	−0.50	0.25	3.10×10^{-1}
	Phenolic compound content	−0.01	0.00	9.55×10^{-1}		Phenolic compound content	0.49	0.24	3.21×10^{-1}

was ascertained after successive extraction with methanol (to extract chls and carotenoids) and phosphate saline solution (to extract PBPs). The best pre-treatment was with PBS, which increased the overall pigment extraction (up to 20%), followed by the DMSO 20%, the control, and the acetone, while the lowest overall pigment extraction was noticed in the pre-treated biomass with DMSO (100%) and NaOH. The results support the hypothesis that PBS solvent helped remove the mucilaginous envelope, because it doubled the content of chl *a* and also improved the PBP content (compared to the control), thus making such pigments more bioavailable and easier to be extracted. The mucilaginous envelope is mainly composed of polysaccharides and an S-layer (encompassing acid-, sulfate-, and beta-linked polysaccharides) (Billi et al. 2001; Budel 2011), which accounts for high polarity that turns this structure soluble in aqueous solvents (Bazaka et al. 2011). Owing to the presence of multi-OH groups, it highly favors the dissolution of such polymers and a strong interaction molecule–molecule and molecule–water is established via hydrogen bonding. Thus, the most polar solvents used in this study DMSO 20% or even methanol by itself (i.e., control) also seemed to have good pigment extraction.

Alkaline pre-treatment seemed to be ineffective in removing the polysaccharides present at the cell walls, contrary to the acid pre-treatment with sulfuric acid. In fact, several laboratory methods employ acid hydrolysis to dissolve polysaccharides (Challouf et al. 2011). For some polysaccharides, the acidic environment tends to protonate the amino groups and the level in water solubility increases (Guo et al. 2017) which probably helped in the mucilage dissolution. On the other hand, DMSO (100%) had a lower apparent pigment extraction yield. DMSO is an organic and potent solvent known to dissolve both polar and non-polar compounds which suggest an easy penetration of the barrier sheath and promotion of the extraction of a big portion of intracellular metabolites, inclusively chlorophylls and carotenoids (Capriotti and Capriotti 2012). This is supported by the deep green dark color of this pre-treated extract (data not shown). However, this solvent was not selected for the subsequent work as our goal was to take advantage of the polarity of different GRAS solvents to obtain the most relevant classes of bioactive and antioxidant compounds (in more purified form). Therefore, PBS was the chosen solvent to apply in the biomass as pre-treatment.

Regarding antioxidant capacity, assessed via both $ABTS^{\bullet+}$ and $NO^{\bullet-}$ assays, organic and aqueous successive extracts (including the one with pre-treatment) unfolded a good bioactive potential of the cyanobacterium, here exploited. ABTS is one of the most common methods to assess general antioxidant capacity—since the free radical cation ABTS ($ABTS^{\bullet+}$) can be scavenged by both lipophilic and water-soluble compounds present in the samples. This method has been claimed as more effective and accurate than others, e.g., DPPH (Singh et al. 2017a). On the other hand, the radical anion nitric oxide ($NO^{\bullet-}$) is a biological molecule occurring naturally in the human body where it is free to react with superoxide and form peroxynitrite ($ONOO^{\bullet}$) that can interfere with the regular molecular and physiological function of cells. It has been hypothesized to trigger several human diseases, including immune and neurodegenerative disorders and may also play a deleterious role in cancer development (Knott and Bossy-Wetzel 2009; Salimian Rizi et al. 2017; Smallwood et al. 2018). This is why such an assay can provide meaningful insight, from a biotechnological perspective, as long as free radical $NO^{\bullet-}$ possesses biological interest toward the development of relevant human diseases.

In both assays, the pre-treated extract was found to be the most bioactive, in view of its lowest IC_{50} values. Such behavior may be rationalized by the high content in PBPs in combination with the high concentrations in cyanobacterial polysaccharides. The latter can be associated with external pigment scytonemin and were both found as constituents of the mucilaginous sheath of *Chroococcidiopsis* sp. cells. They have been reported in several cyanobacteria and are claimed to possess important antioxidant properties (Abed et al. 2009; Parwani et al. 2014; Cruz et al. 2020; Stoyneva-Gärtner et al. 2020). Although this study have quantified scytonemin, this was not considered to be correlated with antioxidant capacity because its content was considered marginal ($0.01\%_{DW}$). In nature, this pigment is usually present in cyanobacterial mucilaginous matrices under environmental stress conditions. Those are meant to protect cells against environmental stressors (i.e., low PAR/UV-radiation, desiccation) (Garcia-Pichel and Castenholz 1991; Rastogi et al. 2014). The mucilaginous sheath of *Chroococcidiopsis* is associated with the production of the external pigment scytonemin known to function as part of an effective cellular defense mechanism against the production of reactive oxygen species and damage to DNA (Matsui et al. 2012; Richa Pathak et al. 2018).

The organic extracts with higher biotechnological potential were the acetonic one (serie C) and the methanolic one (serie D), as assayed by $ABTS^{\bullet-}$ and $NO^{\bullet-}$, respectively. Carotenoids and phenolic compound have been widely described as major contributors to the antioxidant capacity of microalgae and cyanobacteria (Sakata et al. 1994; Miranda et al. 1998; Goiris et al. 2012) and are probably responsible by the aforementioned bioactivity to a great extent. This is in agreement with the high content of carotenoids in the methanolic extract (serie D), which stood out not only for its high content in carotenes (i.e., β-carotene, α-carotene and derivatives, lycopene), but also for its high content in xanthophylls (i.e., echinenone, zeaxanthin, and derivatives). Carotenoids such as lycopene have even a higher antioxidant activity than other natural antioxidants (i.e., β-carotene, zeaxanthin, α-tocopherol) (Amarowicz 2011). Despite the acetonic extract (serie C) exhibiting low IC_{50} value (ABTS), it just ranked second in terms of total carotenoid content, along with a low

TPC suggesting that other types of metabolites with bioactive properties may be involved. Note that some studies are controversial regarding the direct correlation between the contents of carotenoids and phenolic compounds versus antioxidant capacity (Goh et al. 2010; Maadane et al. 2015). Li et al. (2007), when evaluating the phenolic profile and the total carotenoid content of fractionated extracts from several types of microalgae and cyanobacteria, have reported a poor correlation between those parameters. It should be highlighted that cyanobacterial and microalgal matrices possess other types of antioxidant compounds, e.g., polyunsaturated fatty acids and vitamins (i.e., α-tocopherol) (Miranda et al. 1998; El-Baky et al. 2003; Guedes et al. 2011a; Safafar et al. 2015) which can be indirectly extracted, and thus, synergistically and antagonistically influence the antioxidant capacity of the said extracts. On the other hand, chls and their derivatives (i.e., pheo) can also show bioactivity and free radical scavenging capacity (Hsu et al. 2013), which justify the results pertaining to the bioactivity of the organic extracts. Interestingly, pheo was found in the lowest (organic) IC_{50} of the acetonic extract (serie C); this pigment is considered one of the strongest antioxidants among the chls (Hsu et al. 2013).

A particular remark goes to the $NO^{\bullet-}$ results, in that the aqueous extracts exhibited the highest scavenging capacity. These results arise once again, from the presence of content high in polysaccharides and PBPs (in particular PC and APC). For instance, PC has been reported as having important antioxidant properties against such reactive oxygen species as hydroxyl and peroxyl (Mysliwa-Kurdziel and Solymosi 2016), as well as peroxynitrite ($ONOO^-$) (Bhat and Madyastha 2001) and nitric oxide (Thangam et al. 2013). In addition, the scavenging ability of PC (and its chromophore, phycocyanobilin) had been reported before by Bhat and Madyastha (2001), specifically against peroxynitrite in *Arthrospira platensis*. This may help explain their affinity with nitric oxide products, along with the best performance of the aqueous extracts (Bhat and Madyastha 2001). Although no relationship was established between the content in phenolic compounds and the bioactivity in $NO^{\bullet-}$ (Table 4), such compounds have been described as having high antioxidant capacities (Freile-Pelegrín and Robledo 2013) and can establish potential synergetic or antagonistic effects with other uncharacterized bioactive compounds present in the complex cyanobacterial matrices.

In addition, the different extracts possess different antioxidant compounds, which are able to scavenge different types of free radicals ($ABTS^{\bullet+}$ and the $NO^{\bullet-}$ in this case). This is consistent with the studies by Goh et al. (2010), who showed an antioxidant capacity in both non-polar and polar solvents of the marine diatom *Navicula clavata* and the green microalgae *Chlorella marina* and *Dunaliella salina.* Furthermore, comparing the antioxidant capacity data of *Chroococcidiopsis* sp. with other cyanobacterial and microalgal extracts (e.g., Amaro et al. 2015; Pagels et al. 2020), the IC_{50} values obtained in the $ABTS^{\bullet+}$ and $NO^{\bullet-}$ revealed to be slightly higher. For instance, lower IC_{50} values (2–4-fold than our strain) in organic extracts of cyanobacterium *Cyanobium* sp., the same species, displayed higher antioxidant capacity in its aqueous extracts—3-5 fold lower compared to our study (except for the pre-treatment). Similar trends were shown in the $NO^{\bullet-}$ assay, when compared to the cyanobacterium *Gloethece* sp. and *Scenedesmus obliquus* (Amaro et al. 2015), *Nannochloropsis oculata*, and *Gracilaria gracilis* (Ebrahimzadeh et al. 2017). Yet, it is worth mentioning that in the latter studies (e.g., Amaro et al. 2015; Pagels et al. 2020) bioactive compounds production was optimized, while in this study, *Chroococcidiopsis* cultivation conditions were not improved, in order to enhance the maximum production of antioxidant compounds. For this reason, *Chroococcidiopsis* sp. still has important antioxidant properties that are worth to be deeply explored.

Assessment of antioxidant capacity in our cyanobacterial extracts is important to evaluate their potential bioactivity and thus eventual use in the food or pharmaceutical industries. However, cytotoxicity is also an important factor to take into account, so as to guarantee that there is no risk to human or animal health. The assay performed here resorted to hCMED cells. This is a broadly used model, being representative of the endothelial tissues widely spread in the human body. Such endothelial cells are associated with tissues that promote peripheral vascularization and maintenance of such vital organs as the heart, kidneys, liver, and skin (Rajendran et al. 2013).

The results demonstrated that pre-treated extract with the highest antioxidant potential also displayed some degree of cytotoxicity (in both concentrations tested).

In fact, most aqueous extracts showed loss of cellular viability (up to 36–50%, at both tested concentrations). These results were somewhat unexpected because these aqueous extracts are rich in natural and non-toxic PBPs. For instance, PBP-aqueous-rich extracts, obtained by successive extractions in cyanobacterium *Cyanobium* sp. did not possess cytotoxicity against human liver cell line HepG2 (Pagels et al. 2020). Besides, this strain is not reported to produce aqueous toxins (Ramos et al. 2018).

The most plausible explanation for such results is the presence of salts in the mass of extract (carried by the phosphate saline buffer) for its putative unfavorable negative effect upon cell viability. Cytotoxicity associated with PBS was described previously for other cell lines (Schuerer et al. 2017). Moreover, dissolution of the lyophilized PBS mass in neutral pH water can induce pH alteration of the originally alkaline cell medium (acidification), apparent in its orange/yellowish coloration (data not shown). This means that the nature of the extracts might slightly affect the pH of the media where cells grow, and thus affect cell viability. The same effect was shown in the 200 μg mL^{-1} concentration tested, but for the extracts exposed to solvent for over 48 h. Upon increase in the concentration of the (aqueous) extract and time of the

exposure, the cell viability is lost possibly due to pH alteration, as mentioned above.

On the other hand, the successive organic extracts did not show any signs of cytotoxicity, except the methanolic extract of serie E. This extract showed a significant loss of cell viability (up to 30%) at both concentrations tested. Upon extraction with acetone, certain uncharacterized toxic compounds are apparently unveiled in the next extraction. Interestingly, a study including five different cyanobacterial orders (Synechococcales, Oscillatoriales, Chroococcales, Chroococcidiopsidales, and Nostocales) found that organic extracts were more potent by inducing loss of cell viability, than aqueous extracts (Shishido et al. 2020). However, the cell lines (i.e., cancer cell lines) tested were different from those here represented. Each algal extract has variable patterns of cell inhibition intrinsically dependent on the species, extraction processing (i.e., single, successive extraction), the type of extract produced (i.e., more polar or apolar), respective composition, and concentrations (i.e., Bechelli et al. 2011; Maadane et al. 2015).

Pigments and phenolic compounds of microalgae and cyanobacteria have been reported to possess important antioxidant, anti-inflammatory, neuroprotective, or antitumor properties (Goiris et al. 2012; Stramarkou et al. 2016; da Silva and Sant'Anna 2017; Park et al. 2018; Haoujar et al. 2019). They have also been explored by the food dye industry and for their advantages when incorporated in animal feed. The cost of their production process is expected to reduce based on optimization of methods of metabolite recovery (greener and safer), coupled with reuse of biomass within a biorefinery concept. This study accordingly focused on the use of GRAS solvents, combined with extraction to increase the content of a particular group of compounds (i.e., pigments and phenolic compounds) and successive extracts have indeed been demonstrated elsewhere to be more efficient toward metabolite recovery than single steps (Trivedi et al. 2016; Pagels et al. 2020).

In terms of pigment extraction, the results have shown that the best overall extraction serie was D, especially with regard to chl *a* and carotenoids. Interestingly, when compared to serie E that resorts to two organic solvents in a row (acetone and methanol), the latter did enhance neither carotenoids nor chl content. In many cases, acetone is extensively used to extract photosynthetic pigments from microalgae and cyanobacteria, with a wide range of polarity (Guedes et al. 2011b; Pasquet et al. 2011; Amaro et al. 2015). However, solvents with polarity closer to that of water, such as methanol (0.762), stood out in terms of recovery of such compounds when compared to other methanolic extracts of different species of cyanobacteria, e.g., *Pseudanabaena* sp., *Spirulina* sp., and *Lyngbya* sp., with total carotenoid contents of c.a. 0.92, 1.05, and 0.51 mg g_{DW}^{-1}, respectively (Paliwal et al. 2015); chl *a* ranged between 0.70 and 4.80 mg.g_{DW}^{-1}, with the highest value attained by *Spirulina* sp. When compared to this study, *Chroococcidiopsis* sp. achieved better results regarding both carotenoids (1.72 ± 0.05 mg g_{DW}^{-1}) and chl (5.06 ± 0.34 mg g_{DW}^{-1}).

The pigment profile of organic extracts revealed that, depending on the extraction successive processes, both content and profile can be affected. Serie D, which attained the highest content in carotenoids (ca. up to 18% of the total pigment recovered), was shown to extract high quantities of carotenes (α- and β-carotene, and lycopene), up to 66.2% of the total analyzed carotenoid (profile), as well as xanthophyll, zeaxanthin, and some of their derivatives. On the other hand, series B (ethanol) and C (acetone) were selective toward extraction of echinenone (xanthophyll) and lycopene (carotene). Such xanthophylls as zeaxanthin and echinenone are found in cyanobacteria associated with chlorophyll-binding polypeptides in the photosynthetic apparatus (Grossman et al. 1995; Guedes et al. 2011b). This might explain their relative polarity in more polar solvents like ethanol or methanol. On the other hand, carotenes tend to be more soluble in more apolar solvents (i.e., acetone), because they are associated to protein complexes present in the lipids that constitute both the cell membrane and the cell wall (Hirschberg and Chamovitz 1994; Amaro et al. 2015). The results are not consistent, at least those pertaining to the extraction of carotenes with the more polar used solvent (methanol), and also to selective extraction of lycopene in the ethanolic extract. However, extraction efficiencies can vary depending on the way the solvent penetrates the cell walls and membranes (Hirschberg and Chamovitz 1994). Pre-treatment with PBS could have influenced such a degree of penetration and contributed to the bioavailability of certain classes of carotenoids in the biomass extracts of *Chroococcidiopsis* sp.

Note that the most abundant pigments in the organic extracts, besides chl *a* and derivatives, included not only xanthophylls, echinenone, and zeaxanthin, but also carotenes such as lycopene, β-carotene, and α-carotene derivatives. It is known that β-carotene and zeaxanthin are among the prevailing carotenoids present in cyanobacteria, yet their content varies across species or strains (Takaichi and Mochimaru 2007). Other types of ketocarotenoids, e.g., echinenone or canthaxanthin, are also commonly found in cyanobacteria, as well glycosylated carotenoid mixoxantophyll (Hirschberg and Chamovitz 1994; Takaichi and Mochimaru 2007; Lopes et al. 2020). The results of *Chroococcidiopsis* sp. extracts are in agreement with the presence of β-carotene, zeaxanthin, and echinenone, but myxoxanthophyll or canthaxanthin was not detected in any extract. Cyanobacteria possess a great diversity of metabolic pathways and production of several metabolites, including pigments, thereby are regulated by a wide range of enzymes and genes with specific functions (Takaichi and Mochimaru 2007). Presence (or absence) of certain carotenoids (and the associated different profiles) is thus dictated by the evolution of those metabolic routes in

cyanobacteria, in order to adapt to the ecological niches where they are inserted.

Concerning the results on pertaining to the total PBP content and profile, all successive extracts were able to recover PC, APC, and PE with the extract of the pre-treatment being the best in extracting all those pigments at once. When organic solvents are employed in the successive extraction, the content of PBP is significantly reduced to a half when compared to the PBS extraction in serie A. Organic solvents can influence the PBP bioavailability by solvation process (Khabiri et al. 2013). Such interaction between the (organic) solvent and the PBPs can lead to the disruption of certain non-covalent forces that may alter their conformation and ultimately, lead to protein denaturation. This alteration in the protein conformation reveals significant alterations in their absorption spectrum (it lowers) (Taylor and Feller 2002), which is concomitant with lower concentrations of non-altered PBPs available in the successive PBS extracts. It should be highlighted that successive extraction with methanol (serie D) seems to keep the PBP in at slightly higher concentrations, possibly because methanol has a more polar nature (closer to that of water) and more a higher affinity to the PBPs, than acetone or ethanol.

In addition, PBPs appear to have a significant contribution to the total pigment content of *Chroococcidiopsis*. In serie A, together with pre-treatment, PBPs accounted for 14% of DW. In fact, PBPs can account for up to 20% of cyanobacterial DW, besides contributing to 40–60% of the total proteins in the whole cell (Bogorad 1975).

When compared to other studies available in the literature, *Chroococcidiopsis* sp. has a low content of PBPs. For instance, Hossain et al. (2016) studying different types of cyanobacteria, including *Oscillatoria* sp., *Lyngbya* sp., *Microcystis* sp., and *Spirulina* sp., reported ca. 78, 127, 75, and 19 mg g_{DW}^{-1}, respectively. The low PBP content found in this study was confirmed upon comparison with the data of Pumas et al. (2011), who attempted to determine the thermostability of PBPs in four cyanobacterial strains. They reported the highest total PBP content as 181.63 mg g_{DW}^{-1} in *Leptolyngbya* sp. KC45, followed by *Phormidium* sp. PD40-1, *Cyanosarcina* sp. SK40, and *Scytonema* sp. TP40 which attained a PBP total content of 165.47, 72.55, and 37.50 mg g_{DW}^{-1}, respectively. Despite the low results in this study, culture conditions can be optimized in order to improve the production of such pigments, thus turning this cyanobacterium into a potential valuable source of PBPs.

Another note goes to the scytonemin quantified in the pre-treatment extract. In this study, scytonemin reached a maximum value of 0.127 mg g_{DW}^{-1} (0.01%$_{DW}$). This extracellular pigment can attain up to 5% of the DW in cyanobacterial cultures, but in nature, its content may be much higher (Rastogi et al. 2014). It is usually produced under stress conditions, in particular high levels of light intensity, as so is considered a photoprotector pigment of cyanobacterial cells, its synthesis is particularly triggered by UV radiation balanced with light PAR at a suitable UV/PAR ratio, and it is able to exert a strong free radical scavenging activity (Wada et al. 2013).

Series D entailed the best extraction series in terms of recovery of pigments in *Chroococcidiopsis* sp., including PBPs extracted in the pre-treatment. However, the total content in pigments does not lie in the same range of those discussed elsewhere (Pumas et al. 2011; Paliwal et al. 2015; Hossain et al. 2016), except for the study by Paliwal et al. (2015) regarding carotenoid and chl contents. Such a comparison should take into account that different solvents, as well as different extraction methods (and duration of extraction), can influence total pigment contents. For instance, Lopes et al. (2020) studied pigments of five different terrestrial and aquatic cyanobacteria in both acetone and ethanol and concluded that acetone was more effective in extracting pigments (chls and carotenoids) in quantity, when compared to those obtained via ethanol. Moreover, the conditions under which cyanobacteria are exposed during growth (i.e., light, temperature, pH, nutrients) and their inherent genetic capacity to produce certain pigments (species- or strain-dependent) also interfere with the concentrations and ratios of the various pigments (El-Baky et al. 2003; Saleh et al. 2011; Maadane et al. 2015; Paliwal et al. 2015).

The results on TPC have shown that the phenolic compounds present in *Chroococcidiopsis* sp. biomass possess different polar characteristics. This is found throughout the various series, in particular that presenting the highest results (serie B) (1.47 ± 0.19 mg_{GAE} g_{DW}^{-1}) in which ethanolic and PBS fractions accounted cumulatively for the highest content of such compounds (1.98 ± 0.19 mg_{GAE} g_{DW}^{-1}). It should be highlighted that the extract with pre-treatment includes also a major portion of TPCs of polar nature (1.64 ± 0.02 mg_{GAE} g_{DW}^{-1}). The results produced by serie D, in particular the methanolic fraction, were not so good when compared to those from the other series (0.68 ± 0.04 mg_{GAE} g_{DW}^{-1}). Several studies indicate that methanol is one of the best solvents to extract phenolic compounds (in particular those with strong antioxidant activity) from different cyanobacterial and microalgae species (e.g., *Spirulina* sp., *Chlorella sorokiniana, Chlorella marina, Dunaliella salina*, and *Desmodesmus)* (Miranda et al. 1998; Manivannan et al. 2012; Hemalatha et al. 2013; Safafar et al. 2015). Such a solvent is claimed as one of the best in terms of disintegration of cell membranes (Klein et al. 2012). When compared to other studies, the methanolic fraction (serie D) of our study showed low concentrations of TPC. Ijaz and Hasnain (2016) found a value of TPC of 4.32 mg_{GAE} g_{DW}^{-1} for *Chroococcidiopsis* sp. SI-ST when using 70% methanol. In the same study, the authors reported the highest value of TCP for *Leptolyngbya* sp. SI-SM, with 12.6 mg_{GAE} g_{DW}^{-1}, as well as 12.4 mg_{GAE} g_{DW}^{-1} for *Calothrix* sp. SI-SV. On the other hand, Hossain et al. (2016) found a high concentration of TPC in *Lyngbya* sp., ca. 5.02 ± 0.20 mg_{GAE} g_{DW}^{-1} in aqueous

extracts, followed by *Oscillatoria* sp. with 2.96 ± 0.14 mg_{GAE} g_{DW}^{-1}, *Microcystis* sp. with 2.65 ± 0.01 mg_{GAE} g_{DW}^{-1}, and *Spirulina* sp. with 1.78 ± 0.07 mg_{GAE} g_{DW}^{-1} (Hossain et al. 2016). Further comparisons should consider that TPC depends on the intrinsic characteristic of the microorganism studied and its growth conditions (i.e., light, nutrients, stress exposure), in addition to the extraction method and the solvents used (Li et al. 2007; Maadane et al. 2015; Safafar et al. 2015).

In brief, *Chroococcidiopsis* sp. demonstrated a high biotechnological potential. The results provide some clues about the putative applications of its extracts, in particular for food, feed, nutraceuticals, or cosmetics, as they can exert antioxidant properties and are rich in polysaccharides and in a number of pigments like chls, carotenoids, PBPs, or scytonemin, and in phenolic compounds. The high content of some of such compounds (in particular polysaccharides, PBPs, and scytonemin) was reflected in a superior free radical scavenging capacity of the pre-treated extract which makes it quite appealing to the functional food/feed industries, as well as cosmeceutical (i.e., topical formulations with antioxidant properties) and dye industry. Despite the signs of cytotoxicity, further studies are suggested with different models of cells, in order to assess in more detail the possible risk to human and animal health.

The organic extracts also proved promising, in particular the methanolic extract (serie D) owing to its high antioxidant capacity, absence of harm to human endothelial cells, and richness in chls, carotenoids, and phenolic compounds. The profile of carotenoids included high quantities of carotenes (α- and β-carotenes, lycopene) and xanthophylls (echinenone, zeaxanthin). Those are non-toxic and biodegradable pigments, with likely application in the dyes and feed/food/nutraceutical fields, as part of product formulation to be included in human or animal diets and meant to help prevent diseases related to oxidative stress.

The ethanolic extract (serie B) also showed a high potential in terms of phenolic compounds—and the extra advantage of being non-cytotoxic; hence, potential applications in feed/food/nutraceuticals or cosmetics are envisaged.

In a view of the biorefinery concept and the goal of a safer and greener process, serie D was shown to be the best in recovering the major content in terms of pigments, and B was found to best in recovering phenolic compounds.

Finally, *Chroococcidiopsis* sp. is a potential source of bioactive (antioxidant) compounds, including pigments and such other antioxidant compounds as polysaccharides and phenolics, to be applied as dyes, or else in the nutraceutical, cosmetic, and food/feed fields. At the same time, successive extractions permitted optimization of metabolite recovery, toward a safer and greener process methodology, to be integrated with biorefinery concept so as to minimize processing costs.

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Declarations

Conflict of interest The authors declare no competing interests.

References

Abed RMM, Dobretsov S, Sudesh K (2009) Applications of cyanobacteria in biotechnology. J Appl Microbiol 26:1–12

Alam T (2019) Extraction of natural colors from marine algae. J Agric Mar Sci 23:81–91

Amaro HM, Fernandes F, Valentão P, Andrade PB, Sousa-Pinto I, Malcata FX, Gudes AC (2015) Effect of solvent system on extractability of lipidic components of *Scenedesmus obliquus* (M2-1) and *Gloeothece* sp. on antioxidant scavenging capacity thereof. Mar Drugs 13:6453–6471

Amarowicz R (2011) Lycopene as a natural antioxidant. Eur J Lipid Sci Technol 113:675–677

Antonopoulou S, Karantonis HC, Nomikos T, Oikonomou A, Fragopoulou E, Pantazidou A (2005) Bioactive polar lipids from *Chroococcidiopsis* sp. (Cyanobacteria). Comp Biochem Physiol B 142:269–282

Barros AI, Gonçalves AL, Simões M, Pires JCM (2015) Harvesting techniques applied to microalgae: a review. Renew Sust Energ Rev 41:1489–1500

Bazaka K, Crawford RJ, Nazarenko EL, Ivanova EP (2011) Bacterial extracellular polysaccharides. Adv Exp Med Biol 715:213–226

Bechelli J, Coppage M, Rosell K, Liesveld J (2011) Cytotoxicity of algae extracts on normal and malignant cells. Leuk Res Treatment 2011:1–7

Bennett A, Bogorad L (1973) Complementary chromatic adaptation in a filamentous blue-green alga. J Cell Biol 58:419–435

Berridge MV, Tan AS (1993) Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 303:474–482

Bhat VB, Madyastha KM (2001) Scavenging of peroxynitrite by phycocyanin and phycocyanobilin from *Spirulina platensis*: protection against oxidative damage to DNA. Biochem Biophys Res Commun 285:262–266

Billi D (2018) Desert cyanobacteria under space and planetary simulations: a tool for searching for life beyond Earth and supporting human space exploration. Int J Astrobiol 18:1–7

Billi D, Caiola MG (1996) Effects of nitrogen and phosphorus deprivation on *Chroococcidiopsis* sp. (Chroococcales). Algol Stud 83:93–105

Billi D, Caiola MG (2010) Effects on of nitrogen limitation and starvation *Chroococcidiopsis*. New Phytol 133:563–571

Billi D, Friedmann EI, Helm RF, Potts M (2001) Gene transfer to the desiccation-tolerant cyanobacterium *Chroococcidiopsis*. J Bacteriol 183:2298–2305

Bogorad L (1975) Phycobiliproteins and complementary chromatic adaptation. Annu Rev Plant Physiol 26:369–401

Budel B (2011) Cyanobacteria: habitats and species. In: Lüttge U (ed) Plant Dessication Tolerance. Springer, Berlin, pp 11–21

Caiola MG, Billi D (2007) *Chroococcidiopsis* from desert to Mars. In: Seckbach J (ed) Algae and cyanobacteria in extreme enviornments. Springer, Dordrecht, pp 553–568

Capasso JM, Cossio BR, Berl T, Rivard CJ, Jiménez C (2003) A colorimetric assay for determination of cell viability in algal cultures. Biomol Eng 20:133–138

Capriotti K, Capriotti JA (2012) Dimethyl sulfoxide: History, chemistry, and clinical utility in dermatology. J Clin Aesthet Dermatol 5:24–26

Challouf R, Trabelsi L, Ben Dhieb R, Abed OE, Ghozzi K, Ammar JB, Omran H, Ouada HB (2011) Evaluation of cytotoxicity and biological activities in extracellular polysaccharides released by cyanobacterium *Arthrospira platensis*. Braz Arch Biol Technol 54:831–838

Chu W (2012) Biotechnological applications of microalgae. Int e-J Sci Med Educ 6:24–37

Cockell CS, Schuerger AC, Billi D, Friedmann EI, Panitz C (2005) Effects of a simulated Martian UV Flux on the cyanobacterium, *Chroococcidiopsis* sp. 029. Astrobiology 5:127–140

Cruz D, Vasconcelos V, Pierre G, Michaud P, Dellatre C (2020) Exopolysaccharides from cyanobacteria: strategies for bioprocess development. Appl Sci 10:1–20

da Silva FV, Sant'Anna C (2017) Impact of culture conditions on the chlorophyll content of microalgae for biotechnological applications. World J Microbiol Biotechnol 33:1–20

Das P, Quadir MA, Chaudhary AK, Thaher MI, Khan S, Alghazal G, Al-Jabri H (2018) Outdoor continuous cultivation of self-settling marine cyanobacterium *Chroococcidiopsis* sp. Ind Biotechnol 14:45–53

Demay J, Bernard C, Reinhardt A, Marie B (2019) Natural products from cyanobacteria: focus on beneficial activities. Mar Drugs 17:1–49

Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F (1951) A colorimetric method for the determination of sugars. Nature 168:167

Ebrahimzadeh MA, Khalili M, Dehpour AA (2017) Antioxidant activity of ethyl acetate and methanolic extracts of two marine algae, *Nannochloropsis oculata* and *Gracilaria gracilis* – an in vitro assay. Braz J Pharm Sci 54:e17280

El-Baky HHA, El Baz FK, El-Baroty GS (2003) *Spirulina* species as a source of carotenoids and α-tocopherol and its anticarcinoma factors. Biotechnology (Faisalabad) 2:222–240

Folin O, Ciocalteu V (1927) Tyrosine and tryptophane in proteins. J Biol Chem 73:627–650

Freile-Pelegrín Y, Robledo D (2013) Bioactive phenolic compounds from algae. In: Hernández-Ledesma B, Herrero M (eds) Bioactive compounds from marine foods: plant and animal sources. John Wiley & Sons, London, pp 113–129

Garcia-Pichel F, Castenholz RW (1991) Characterization and biolgical implications of scytonemin, a cyanobacterial sheath pigment. J Phycol 27:395–409

Garcia-Pichel F, Sherry ND, Castenholz RW (1992) Evidence for an ultraviolet sunscreen role of extracellular pigment for an ultraviolet sunscreen role of the extracellular pigment scytonemin in the terrestrial cyanobacterium *Chlorogloeopsis* sp. Photochem Photobiol 56: 17–23

Goh S-H, Yusoff FM, Loh SP (2010) A comparison of the antioxidant properties and total phenolic content in a diatom, *Chaetoceros* sp. and a green microalga, *Nannochloropsis* sp. J Agric Sci 2:123–130

Goiris K, Muylaert K, Fraeye I (2012) Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. J Appl Phycol 24:1477–1486

Grossman AR, Bhaya D, Apt KE, Kehoe DM (1995) Light-harvesting complexes in oxygenic photosynthesis: diversity, control, and evolution. Annu Rev Genet 29:231–288

Guedes AC, Amaro HM, Barbosa CR, Pereira RD, Malcata FX (2011a) Fatty acid composition of several wild microalgae and cyanobacteria, with a focus on eicosapentaenoic, docosahexaenoic and α-linolenic acids for eventual dietary uses. Food Res Int 44: 2721–2729

Guedes AC, Amaro HM, Malcata FX (2011b) Microalgae as sources of carotenoids. Mar Drugs 9:625–644

Guedes AC, Amaro HM, Gião MS, Malcata FX (2013) Optimization of ABTS radical cation assay specifically for determination of antioxidant capacity of intracellular extracts of microalgae and cyanobacteria. Food Chem 138:638–643

Guo MQ, Hu X, Wang C, Ai L (2017) Polysaccharides: structure and solubility. In: Xu Z (ed) Solubility of Polysaccharides. IntechOpen, Rijeka, pp 7–22

Haoujar I, Cacciola F, Abrini J, Mangraviti D, Giuffrida D, Majdoub YOE, Kounnoun A, MIceli N, Taviano AF, Mondello L, Rigano F, Senhaji NS (2019) The contribution of carotenoids, phenolic compounds, and flavonoids to the antioxidative properties of marine microalgae isolated from Mediterranean Morocco. Molecules 24:1–17

Hayashi NR, Terazono K, Hasegawa N, Kodama T, Igarashi Y (1997) Identification and characterization of phycobiliprotein from a thermophilic cyanobacterium, *Chroococcidiopsis* sp. strain TS-821. J Ferment Bioeng 84:475–477

Hemalatha A, Girija K, Parthiban C, Saranya C, Anantharaman P (2013) Antioxidant properties and total phenolic content of a marine diatom, *Navicula clavata* and green microalgae, *Chlorella marina* and *Dunaliella salina*. Pelagia Res Libr 4:151–157

Hirschberg J, Chamovitz D (1994) Carotenoids in cyanobacteria. In: Bryant DA (ed) The Molecular Biology of Cyanobacteria. Springer, Dordrecht, pp 559–579

Hossain MF, Ratnayake RR, Meerajini K, Wasantha Kumara KL (2016) Antioxidant properties in some selected cyanobacteria isolated from fresh water bodies of Sri Lanka. Food Sci Nutr 4:753–758

Hsu CY, Chao PY, Hu SP, Yang CM (2013) The antioxidant and free radical scavenging activities of chlorophylls and pheophytins. Food Nutr Sci 04:1–8

Ijaz S, Hasnain S (2016) Antioxidant potential of indigenous cyanobacterial strains in relation with their phenolic and flavonoid contents. Nat Prod Res 30:1297–1300

Jerez-Martel I, García-Poza S, Rodríguez-Martel G, Rico M, Afonso-Olivares C, Gómez-Pinchetti L (2017) Phenolic profile and antioxidant activity of crude extracts from microalgae and cyanobacteria strains. J Food Qual 2017:2924508

Kepekçi RA, Saygideger SD (2012) Enhancement of phenolic compound production in *Spirulina platensis* by two-step batch mode cultivation. J Appl Phycol 24:897–905

Khabiri M, Minofar B, Brezovský J, Damborský ER (2013) Interaction of organic solvents with protein structures at protein-solvent interface. J Mol Model 19:4701–4711

Klein BC, Walter C, Lange HA, Buchholz R (2012) Microalgae as natural sources for antioxidative compounds. J Appl Phycol 24:1133–1139

Knott AB, Bossy-Wetzel E (2009) Nitric oxide in health and disease of the nervous system. Antioxid Redox Signal 11:541–553

Komárek J, Johansen JR (2015) Coccoid cyanobacteria. In: Wehr JD, Sheath RG, Kociolek JP (eds) Freshwater algae of North America

- ecology and classification., 2nd edn. Elsevier, Amsterdam, pp 75–133

Kotai J (1972) Instructions for preparation of modified nutrient solution Z8 for algae. Publication 8-11169. Norwegian Institute for Water Research, Blindern, Norway

Kumar S, Kumar D, Saroha K, Singh N, Vashishta B (2008) Antioxidant and free radical scavenging potential of *Citrullus colocynthis* (L.) Schrad. methanolic fruit extract. Acta Pharma 58:215–220

Li HB, Cheng KW, Wong CC, Fan KW, Chen F, Jiang Y (2007) Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chem 102:771–776

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids measurement and UV-VIS characterization. Curr Protoc Food Anal Chem F4.3.1-F4:1–8

Lindner AV, Pleissner D (2019) Utilization of phenolic compounds by microalgae. Algal Res 42:101602

Lopes G, Clarinha D, Vasconcelos V (2020) Carotenoids from cyanobacteria: a biotechnological approach for the topical treatment of psoriasis. Microorganisms 8:1–19

Maadane A, Merghoub N, Ainane T, Arroussi HE, Benhima R, Amzazi S, Bkri Y, Wahby I (2015) Antioxidant activity of some Moroccan marine microalgae: pufa profiles, carotenoids and phenolic content. J Biotechnol 215:13–19

MacColl R (1998) Cyanobacterial phycobilisomes. J Struct Biol 124: 311–334

Mandal MK, Chanu NK, Chaurasia N (2020) Cyanobacterial pigments and their fluorescence characteristics: applications in research and industry. In: Singh PK, Kumar A, Singh VK, Shrivastava AK (eds) Advances in cyanobacterial biology. Academic Press, New York, pp 55–72

Manivannan K, Anantharaman P, Balasubramanian T (2012) Evaluation of antioxidant properties of marine microalga *Chlorella marina* (Butcher, 1952). Asian Pac J Trop Biomed 2:S342–S346

Matsui K, Nazifi E, Hirai Y, Wada N, Matsugo S, Sakamoto T (2012) The cyanobacterial UV-absorbing pigment scytonemin displays radical-scavenging activity. J Gen Appl Microbiol 58:137–144

Miranda MS, Cintra RG, Barros SBM, Mancini-Filho J (1998) Antioxidant activity of the microalga *Spirulina maxima*. Braz J Med Biol Res 31:1075–1079

Montero-Lobato Z, Fuentes JL, Garbayo I, Ascaso C, Wierzchos J, Vega JM, Vílchez C (2020) Identification, biochemical composition and phycobiliproteins production of *Chroococcidiopsis* sp. from arid environment. Process Biochem 97:112–120

Mysliwa-Kurdziel B, Solymosi K (2016) Phycobilins and phycobiliproteins used in food industry and medicine. Mini-Rev Med Chem 17:1173–1193

Namitha KK, Negi PS (2010) Chemistry and biotechnology of carotenoids. Crit Rev Food Sci Nutr 50:1040–8398

Pagels F, Guedes AC, Amaro HM, Kijjoa A, Vasconcelos V (2019) Phycobiliproteins from cyanobacteria: chemistry and biotechnological applications. Biotechnol Adv 37:1–22

Pagels F, Salvaterra D, Amaro HM, Lopes G, Sousa-Pinto I, Vasconcelos V, Guedes AC (2020) Bioactive potential of *Cyanobium* sp. pigment-rich extracts. J Appl Phycol 32:3031–3040

Paliwal C, Ghosh T, Bhayani K, Maurya R, Mishra S (2015) Mar Drugs 13:5384–5401

Park WS, Kim HJ, Li M, Lim DH, Kim J, Kwak SS, Kang CM, Ferruzzi MG, Ahn MJ (2018) Two classes of pigments, carotenoids and c-phycocyanin, in *Spirulina* powder and their antioxidant activities. Molecules 23:23082065

Parwani L, Bhatnagar M, Bhatnagar A, Sharma V (2014) Antioxidant and iron-chelating activities of cyanobacterial exopolymers with potential for wound healing. J Appl Phycol 26:1473–1482

Pasquet V, Chérouvrier JR, Farhat F, Thiéri V, Piot JM, Bérard JB, Kaas R, Sieve B, Patrice T, Cadoret JP, Picot L (2011) Study on the microalgal pigments extraction process: performance of microwave assisted extraction. Process Biochem 46:59–67

Pinho R, Sousa C (2011) Is nitric oxide decrease observed with naphthoquinones in LPS stimulated RAW 264.7 macrophages a beneficial property? PLoS One 6:e24098

Pumas C, Vacharapiyasophon P, Peerapornpisal Y, Leelapornpisid P, Boonchum W, Ishii M, Khanongnuch C (2011) Thermostablility of phycobiliproteins and antioxidant activity from four thermotolerant cyanobacteria. Phycol Res 59:166–174

Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y (2013) The vascular endothelium and human diseases. Int J Biol Sci 9:1057–1106

Ramos V, Morais J, Castelo-Branco R, Pinheiro Â, Martins J, Regueiras A, Pereira AL, Lopes V, Frazão B, Gomes D, Moreira C, Osswald J, Leão PN, Vasconcelos VM (2018) Cyanobacterial diversity held in mBRCs as a biotechnological asset: the case study of the newly established LEGE culture collection. J Appl Phycol 30:1437–1451

Raposo MFJ, Morais RMSC, Morais AMMB (2013) Bioactivity and applications of sulphated polysaccharides from marine microalgae. Mar Drugs 11:233–252

Raposo MFJ, Morais AMB, Morais RMSC (2015) Marine polysaccharides from algae with potential biomedical applications. Mar Drugs 13:2967–3028

Rastogi RP, Madamwar D, Incharoensakdi A (2014) Multiple defense systems in cyanobacteria in response to solar UV radiation. In: Douglas D (ed) Cyanobacteria. Nova Science Pub, New York, pp 125–158

Řezanka T, Víden I, Go JV, Dor I, Dembitsky VM (2003) Polar lipids and fatty acids of three wild cyanobacterial strains of the genus *Chroococcidiopsis*. Folia Microbiol (Praha) 48:781–786

Richa Pathak J, Sonker AS, Singh V, Sinha RP (2018) Potential applications of natural bioactive cyanobacterial UV-protective compounds. In: La Barre S, Bates S (eds) Blue Biotechnology: production and use of marine molecules. Wiley-VCH, Weinheim, pp 683–707

Rizwan M, Mujtaba G, Memon SA, Lee K, Rashid N (2018) Exploring the potential of microalgae for new biotechnology applications and beyond: a review. Renew Sust Energ Rev 92:394–404

Safafar H, Wagenen J, Van Møller P, Jacobsen C (2015) Carotenoids, phenolic compounds and tocopherols contribute to the antioxidative properties of some microalgae species grown on industrial wastewater. Mar Drugs 13:7339–7356

Saini DK, Pabbi S, Shukla P (2018) Cyanobacterial pigments: perspectives and biotechnological approaches. Food Chem Toxicol 120: 616–624

Sakata K, Yamamoto K, Watanabe N (1994) Antioxidative compounds from marine organisms. In: Hiramatsu M, Yoshikawa T, Inoue M (eds) Food and Free Radicals. Springer, Boston, pp 164–182

Saleh M, Dhar DW, Singh PK (2011) Comparative pigmente profiles of different *Spirulina* strains. Res Biotechnol 2:67–74

Salimian Rizi B, Achreja A, Nagrath D (2017) Nitric oxide: the forgotten child of tumor metabolism. Trends Cancer 3:659–672

Sathasivam R, Radhakrishnan R, Hashem A, Elsayed AH, Abd Allah F (2019) Microalgae metabolites: a rich source for food and medicine. Saudi J Biol Sci 26:709–722

Schuerer N, Stein E, Inic-kanada A, Purcher M, Hohenadl C, Bintner N, Montanaro J, Barisani-Asenbauer T (2017) Implications for ophthalmic formulations: ocular buffers show varied cytotoxic impact on human corneal-limbal and human conjuctival epithelial cells. Cornea 36:712–718

Shishido TK, Popin RV, Jokela J, Wahlsten M, Fiore MF, Fewer DP, Herfindal L, Sivonen K (2020) Dereplication of natural products with antimicrobial and anticancer activity from Brazilian cyanobacteria. Toxins 12:12

Singh DP, Prabha R, Verma S, Meena KK (2017a) Antioxidant properties and polyphenolic content in terrestrial cyanobacteria. 3Biotech 7:1–14

Singh R, Parihar P, Singh M, Bajguz A, Kumar J, Singh S, Singh VP, Pracasi SM (2017b) Uncovering potential applications of cyanobacteria and algal metabolites in biology, agriculture and medicine: current status and future prospects. Front Microbiol 8:1–37

Smallwood MJ, Nissim A, Knight AR, Whiteman M, Haigh R, Wyniard PG (2018) Oxidative stress in autoimmune rheumatic diseases. Free Radic Biol Med 125:3–14

Stoyneva-Gärtner M, Uzunov B, Gärtner G (2020) Enigmatic microalgae from aeroterrestrial and extreme habitats in cosmetics: the potential of the untapped natural sources. Cosmetics 7:27

Stramarkou M, Papadaki S, Kyriakopoulou K, Krokida M (2016) Recovery of functional pigments from four different species of microalgae. IOSR J Environ Sci Toxicol Food Technol 10:26–30

Takaichi S, Mochimaru M (2007) Carotenoids and carotenogenesis in cyanobacteria: unique ketocarotenoids and carotenoid glycosides. Cell Mol Life Sci 64:2607–2619

Taylor ATS, Feller SE (2002) Structural studies of phycobiliproteins from *Spirulina*: combining spectroscopy, thermodynamics, and molecular modeling in an undergraduate biochemistry experiment. J Chem Educ 79:1467–1470

Thangam R, Suresh V, Princy WA, Rajkumar M, SenthiKumar N, Gunasekaran P, Rengasamy R, Anbazhagan C, Karevi K, Kannan S (2013) C-Phycocyanin from *Oscillatoria tenuis* exhibited an antioxidant and in vitro antiproliferative activity through induction of apoptosis and G0/G1 cell cycle arrest. Food Chem 140:262–272

Trivedi N, Baghel RS, Bothwell J, Gupta V, Reddy CRK, Lali AM, Jha B (2016) An integrated process for the extraction of fuel and chemicals from marine macroalgal biomass. Sci Rep 6:1–8

Wada N, Sakamoto T, Matsugo S (2013) Multiple roles of photosynthetic and sunscreen pigments in cyanobacteria focusing on the oxidative stress. Metabolites 3:463–483

Yaakob Z, Ali E, Zainal A, Mohamed M, Takrif MS (2014) An overview: biomolecules from microalgae for animal feed and aquaculture. J Biol Res 21:1–10