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Synechocystis salina: potential bioactivity and combined extraction of added-value metabolites

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Abstract

Few studies have focused on the biotechnological potential of the marine cyanobacterium *Synechocystis salina*, in particular its bioactive metabolites. Hence, an opportunity exists to explore various solvent combinations in attempts to improve extraction of cellular contents of metabolites with antioxidant features as a part of a structured attempt to determine whether *S. salina* LEGE 06,155 holds a promise for industrial exploitation. Extracts were obtained by single extractions with four GRAS solvents: hexane (H), acetone (A), ethanol (E), and buffer phosphate (PBS) (series A) and successive extractions: H-E-PBS (series B), H-A-PBS (series C), and H-A-E-PBS (series D). The yields, antioxidant capacity, and cytotoxicity of the extracts, as well their content in pigments, lipids, and phenolic compounds were assessed. The combination of solvents of series B produced the highest yield of extraction, of 33.56%. Aqueous extracts for single (series A) and successive extractions (series B and C) presented the highest antioxidant capacity, for $ABTS^{\bullet+}$ and $NO^{\bullet-}$, respectively, and had a high content of phycobiliproteins (PBPs). The acetonic (series A) and ethanolic (series B) extracts also showed high antioxidant capacity, in particular for $DPPH^{\bullet}$ and $O_2^{\bullet-}$, respectively. Overall, organic extracts did not show signs of cytotoxicity. Series B extraction enhanced general pigment content, yet single extraction with ethanol (series A) produced the highest content in carotenoids, in particular echinenone and β-carotene, and PBS (series A) improved PBPs content. The lipid content was enhanced in series A (ethanol), while the total phenolic content was improved in series B. *Synechocystis* has accordingly demonstrated potential for eventual exploitation as biotechnological resource, in terms value-added compounds with antioxidant features.

Keywords Cyanobacteria · Successive extraction · Antioxidant · Pigments · Carotenoids · Phycobiliproteins · Phenolic compounds

Introduction

Nowadays, industry is trying to reinvent itself via search for natural molecules and value-added compounds with interesting and useful bioactive properties toward expansion of the portfolio of natural products available for human health to enrich human diets as nutraceuticals or use as additives in pharmaceutical, cosmetics, or animal feed industries (Abed et al. 2009; Rizwan et al. 2018).

Cyanobacteria are a group of diverse photosynthetic microorganisms which possess adaptation strategies, metabolic plasticity, and ability to synthesize chemically diverse molecules (i.e., alkaloids, polyketides, indoles, fatty acids, amines) (Abed et al. 2009; Guedes et al. 2011a; Assunção et al. 2017). Most such molecules include primary and secondary metabolites that possess biological activity (i.e., antioxidant, anti-inflammatory, antimicrobial,

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antitumor, anti-infective, immunosuppressive, antibacterial), for human and veterinary uses (Abed et al. 2009; Guedes et al. 2011a; Lau et al. 2015; Demay et al. 2019). Pigments, lipids, vitamins, proteins, phenolic compounds, carbohydrates, enzymes, and toxins are included in this long list of compounds that hold a relevant market interest. Several industries are particularly focused on metabolites bearing bioactive properties, to be explored in pharmacological, nutraceutical, and cosmetical fields. For instance, pigments such as phycobiliproteins (PBPs) and carotenoids (i.e., β-carotene, lutein), as well as phenolic compounds can play the role of functional ingredients and thus be employed as health supplements or cosmetic formulas. In the case of the pigments, they can be used as natural food colorants or dyes owing to their colored features. The market estimated for these metabolites has been increasing and is projected to reach US$ 643.13 million in 2026 for carotenoids, in particular β-carotene (Ahuja and Rawat 2020), and for PBPs, specifically phycocyanin, and estimated to double by 2028, with a value of US$ 490 million (Future Market Insights 2018).

Despite their intrinsic potential, only a few species of microalgae and cyanobacteria are currently being produced for commercial purposes. There are thousands of species in nature and a significant portion of those are microalgae and cyanobacteria, but only ca. 30,000 species have been described so far (Guiry 2012). Furthermore, most still lack data able to support (or discard) their biotechnological value. This is the case of *Synechocystis salina*, a marine photosynthetic cyanobacterium (Korelusova et al. 2009). The genus *Synechocystis* is important in the biotechnological field (i.e., *Synechocystis* PCC 6803) because of its simple morphology, ease of transfer in culture, and part of many molecular and genetic tools, and therefore, it is an important model for experimental work (Yu et al. 2013; Santos-Merino et al. 2019).

Studies on *S. salina* have been essentially focused on its ability to remove the organic load of wastewaters (Dominic et al. 2009) for bioremediation purposes, when in consortia with bacteria (Gonçalves et al. 2014, 2016). In parallel, its biomass has been upgraded for production of lipids (Gonçalves et al. 2016) and polyhydroxyalkanoates (PHB). The latter are natural polyesters with potential application in the industry of biodegradable bioplastics as a substitute of petrochemical-based plastics (Meixner et al. 2016). However, assessment of the bioactive potential of such added-value compounds as pigments (i.e., carotenoids, PBPs), lipids, or phenolic compounds have been scarce so far concerning this microorganism. Nevertheless, a number of studies have already unfolded the hidden potential of *Synechocystis* spp., particularly in relation to antimicrobial, cytotoxic, or antioxidant bioactivities (Martins et al. 2008; Plaza et al. 2009; El Semary and El Naby 2010; Sakthivel and Kathiresan 2012; Guedes et al. 2013a). In addition, *Synechocystis* has been reported to produce bartolosides—secondary metabolites accounting for up to 0.6% of its total dry weight (DW), but without any bioactivity assigned to date (Leão et al. 2015; Afonso et al. 2016). *Synechocystis salina* can represent an important source of bartolosides and other putatively added-value compounds. Furthermore, generation of bioactive extracts of *Synechocystis* represents a great opportunity to improve compound recovery techniques, namely, using antioxidant capacity as target, and to explore (re)use of its biomass in combination with GRAS (generally recognized as safe) solvent-mediated extraction. From an industrial perspective, mitigation of processing costs and greener extraction processes are crucial for development of human and animal applications.

In view of the above, this work took as its main goal to chemically characterize strain *S. salina* LEGE 06,155, in terms of pigments [chlorophyll *a* (chl *a*), total carotenoids, PBPs], total lipids, and phenolic compounds, aiming at eventual exploitation for bioactive compounds, namely, with antioxidant capacity. At the same time, single and successive extractions were performed in GRAS solvents with distinct polarities (hexane, ethanol, acetone, and phosphate-buffered saline), in order to ascertain their performance toward extraction of relevant classes of compounds with bioactive properties, while (re)using biomass as per a biorefinery concept.

Materials and methods

Microorganism and biomass production

Synechocystis salina LEGE 06,155 was obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC). *Synechocystis salina* biomass production was performed in 5-L glass flat bottom round flasks (4.8 L of working volume), using Z8 culture medium (Kotai 1972), supplemented with 25 g L^{-1} NaCl and with pH adjusted to 7.20 ± 0.05. The cultures were maintained in batch mode at 25 °C, under a light intensity of 100 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ supplied by fluorescent lamps (Biolux, Osram), and a light/dark cycle of 16 h:8 h (L:D). Agitation was provided by continuously bubbling air at the bottom of the cultures at an airflow of 0.75 $L_{air}\ L_{culture}^{-1}\ min^{-1}$. For each batch, a pre-inoculum was cultivated for 10 days (to ensure it had reached the exponential phase), with an initial optical density of 0.1 ($OD = \lambda_{680}\ nm - \lambda_{750}\ nm$). The biomass production was monitored over time in order to determine the suitable day for cell harvest (i.e., stationary phase). Culture samples were collected regularly and dry weight (DW) was determined. Aliquots of 5 mL of the *S. salina* culture were

filtered through a preconditioned Whatman GF/C (0.45 µm) glass microfiber filter paper and further dried at 100 °C until constant weight. Biomass was harvested by centrifugation (1400 × *g*, 20 min) and frozen at − 20 °C, then freeze-dried, and finally kept in a vacuum desiccator before further analysis.

Extraction procedure

Extracts were obtained by a series of single and successive extractions, performed with solvents of different polarities: hexane (H), acetone (A), ethanol (E), and phosphate-buffered saline (PBS) (20 mM, pH 7.4). All extractions were performed in triplicate, using 85 mg of freeze-dried biomass, 220 mg of glass beads, and 5 mL of solvent using a Precellys homogenizer (Bertin Technologies, France), with 6 min cycle at 8000 rpm (30 s homogenization with 40 s of stopping intervals).

Single extractions were performed by adding 5 mL of each solvent (hexane, acetone, ethanol, or PBS) in separate (series A), and successive extractions were carried out in different series, by using 5 mL of different solvents as H-E-PBS (series B), H-A-PBS (series C) and H-A-E-PBS (series D) and collecting the extract after adding each solvent. All PBS extractions (successive) were performed by vortex stirring for 10 s and centrifuged at 2744 × *g* for 3 min. The supernatants were recovered, evaporated in a rotary vacuum evaporator, and resuspended in DMSO and PBS (1:4 *v/v*) to a final concentration of 10 mg mL^{-1} for further analysis. All extracts were prepared under dimmed light to prevent any oxidation or degradation.

Three replicates of each extract were prepared independently and chemically analyzed in triplicate for each method, unless stated otherwise.

Antioxidant capacity assessment

All samples from both single and successive extractions of *S. salina* were assayed for their antioxidant capacity. Four different radical scavenging assays were employed, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $O_2^{\bullet-}$, and $NO^{\bullet-}$. Samples were prepared in DMSO/PBS (1:4 *v/v*) from mass extracts to a final concentration of 10 mg mL^{-1} and set in a dilution series of 0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 mg mL^{-1}. For each assay, the percentage inhibition was determined as follows:

$$\%_{inhibition} = \frac{\left(Abs_{extract} - Abs_{blanckextract}\right) - Abs_{control}}{Abs_{control}} \times 100\%$$

where $Abs_{extract}$ is absorbance of the extract with the reagent solution; $Abs_{blanck\ extract}$ is absorbance of the extract with the solvent in which the reagent solution is prepared; and $Abs_{control}$ is absorbance of the DMSO/PBS (1:4 *v/v*), the solvent mixture where extracts are dissolved, with the reagent solution.

Using software GraphPrism Plotting, the dose–response curves were plotted and used to determine the corresponding IC_{25} and IC_{50} values.

$ABTS^{\bullet+}$ assay

The radical-scavenging capacity of biological triplicates of *S. salina* extracts were assessed via $ABTS^{\bullet+}$ assay as described elsewhere (Guedes et al. 2013b; Granados-Guzman et al. 2017). This is a spectrophotometric method valid for both lipophilic and hydrophilic antioxidant compounds (Re et al. 1999). Briefly, ABTS radical cation was produced via reaction of potassium persulfate (Sigma) 0.66 mg mL^{-1} and ABTS (Sigma) 38.4 mg mL^{-1}, so as to assure a working absorbance within 0.680–0.720. For the assay, 63 µL of extract samples were reacted with 180 µL of ABTS solution, and the control used 63 µL DMSO/PBS (1:4 *v/v*) and 180 µL of ABTS. The antioxidant capacity of samples was measured in triplicate at λ = 734 nm in the plaque reader after 6-min incubation.

$DPPH^{\bullet}$ assay

The $DPPH^{\bullet}$ is a free radical scavenging method, previously described by Brand-Williams et al. (1995) and adapted to microplate reader by Granados-Guzman et al. (2017), which is particularly appropriate for lipophilic compounds (Niki 2010). DPPH (Sigma) solution was prepared by dissolving 2.85 mg of DPPH in 50 mL methanol (100%), so that absorbance at λ = 515 nm lay between 0.8 and 0.9. For the assay, 63 µL of extract samples were reacted with 180 µL of DPPH solution. The control was made with 63 µL DMSO/PBS (1:4 *v/v*) with 180 µL methanol. Samples were kept in the dark, incubated at room temperature and absorbance at λ = 515 nm immediately read in triplicate after 30-min reaction.

$NO^{\bullet-}$ assay

The $NO^{\bullet-}$ assay was employed to evaluate the antioxidant capacity of *S. salina* extracts, according to the procedure described by Bajpai et al. (2017) with some modifications. Briefly, 50 µL of 5 mM sodium nitroprusside (SNP) was added to 50 µL of the sample extracts prepared with phosphate-buffered saline (20 mM, pH = 7.4). The reaction mixture was incubated at room temperature under continuous light (visible polychromatic light source, with an average light intensity of 23 ± 2 $\mu mol_{photons}\ m^{-2}\ s^{-1}$), over 1-h incubation. Then 50 µL Griess reagent (1% sulfanilamide and 0.1% naphthylethylene-diamine dihydrochloride in 2% (*v/v*) phosphoric acid) was added to the reaction mixture and

absorbance was read in triplicate after 10 min in a plaque reader at λ = 582 nm.

$O_2^{\bullet-}$ assay

In the $O_2^{\bullet-}$ assay, the superoxide radicals were generated by a NADH/PMS system, according to Gülçin et al. (2005). Briefly, the reaction mixture resulted from of addition of 50 µL of extract samples (concentrations ranging from 0.16 to 10 mg mL^{-1}), 50 µL of NADH (166 µM), 150 µL of NBT (43 µM), and 50 µL of PMS (2.7 µM), followed by incubation at room temperature for 10 min. Absorbance was read in triplicate in a plaque reader at 560 nm.

Cytotoxicity assessment (MTT assay)

The extracts were evaluated in terms of cytotoxicity/cell viability, by resorting to the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The rationale of this method is based on reduction of MTT by NAD(P)H-dependent cellular oxidoreductase enzymes, mainly present in the mitochondria, to formazan salts. This reduction leads to formation of insoluble violet/blue formazan salts by the living cells, which after dissolution with DMSO, can be measured at 550 nm (Berridge and Tan 1993; Capasso et al. 2003). This assay was performed on the human endothelial cell line hCMEC, obtained from American Type Culture Collection (ATCC). In brief, cells were seeded in a 96 well plate to a final concentration of 10×10^4 cells mL^{-1} with Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (*v/v*) fetal bovine serum (FBS) and 1% (*v/v*) penicillin–streptomycin (penicillin 100 IU L^{-1}, streptomycin 100 µL mL^{-1}) and incubated for 34 h at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was replaced afterward and cells were incubated with the extracts of *S. salina* in two different concentrations (100 and 200 $\mu g_{extract}$ mL^{-1}) for 24 and 48 h, into a final volume of 100 µL. Cell medium with 1 and 20% of DMSO was added to the plaque as negative control (no cell death) and positive control (cell death by cytotoxicity), respectively. To ascertain cell viability, 20 µL of MTT solution (1 mg mL^{-1}) was added to each well and further incubated for 3.5 h at 37 °C. The culture supernatant was then removed, 100 µL of DMSO was added to each well to dissolve the formazan salts, and absorbance was read at 550 nm. Results are expressed in $mg_{extract}$ mL^{-1} at four independent assays ($n = 4$) and performed in quadruplicate for each sample.

Biochemical characterization of extracts

Determination of pigments content

Chlorophylls and carotenoids determination Identification and quantification of pigments in the organic extracts (hexane, acetone, and ethanol), in particular chlorophylls (chls) and carotenoids (including β-carotene, echinenone, zeaxanthin), were performed in a Waters-Alliance high-performance liquid chromatograph (HPLC) following the method described elsewhere (Guedes et al. 2011b). The HPLC was equipped with a LichoCart 250–4 C18 reversed-phase column (250 × 4 mm, 5-µm bondapack) (Merck, Germany), with a FLR and a PDA detector set to spectrum scanning in the range of λ = 250–750 nm.

Each extract sample, prepared in duplicate as described above, was evaporated in a vacuum rotary evaporator, and the pellet resuspended in a volume of internal standard solution [*trans*-β-apo-8′-carotenal (Sigma, ≥ 96.0% (UV))] to a final concentration of 10 mg mL^{-1}. The internal standard solution was prepared to a final concentration of 170 µL mL^{-1} in a solvent mixture of acetone and ethyl acetate at 9:1 (*v/v*). The samples were eluted in a gradient using ethyl acetate (100%) and acetonitrile:water (9:1) as eluents, over 55 min, with a flow rate of 1 mL min^{-1} and a constant temperature of 25 ± 2 °C.

The pigments were identified by comparison of retention times with those of standards chl *a* (Sigma, ≥ 96.0% (UV)), fucoxanthin (Sigma, ≥ 95.0% (UV)), β-carotene (Extrasynthese, ≥ 98.0% (UV)), and zeaxanthin (Extrasynthese, ≥ 98.0% (UV)), as well as *trans*-β-apo-8′-carotenal (internal standard). Quantification was performed via calibration curves with said standards. Chl *a* and total carotenoids were expressed in mg of freeze-dried biomass (per g_{DW}^{-1}) while the carotenoid profile results were expressed in µg g_{DW}^{-1}.

Phycobiliproteins determination The determination of PBP content, namely, phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE), was assessed in PBS extracts and measured via absorbance at λ = 562, 615, and 652 nm, based on the equations of Bennett and Bogorad (2004) as follows:

$$\text{PC (mg mL} - 1) = (A615) - (0.474\,A652)$$

$$\text{APC (mg mL} - 1) = [(A652) - (9.16\,A652)]/5.09$$

$$\text{PE (mg mL} - 1) = [(A562) - 2.41(\text{PC}) - 0.849\,(\text{APC})\,(9.16\,A652)]/9.62$$

The PBP pigment content was expressed in mg g_{DW}^{-1}.

Total lipid quantification

Total lipid quantification was performed by spectrophotometric analysis, according with Mishra et al. (2014), with some modifications. Briefly, 50 µL of extract samples (at 10 mg mL^{-1}) was added to a glass tube, together with 500 µL of concentrated sulfuric acid (> 95% *v/v*). The samples were incubated for 10 min in an oven at 100 °C and cooled in ice afterward. Then, 1.25 mL of phosphovanillin reagent was added to the samples that were further incubated for 20 min, until achieving room temperature, when a reading was taken at λ = 530 nm in a microplate reader (Multiscan Go, ThermoFisher Scientific, USA). The quantification was performed by standard curve with canola oil, covering the concentration range 0–2 mg mL^{-1} and dissolved in DMSO/PBS (1:4 *v/v*). The total lipid content was calculated via the linear regression equation: Absorbance = 0.9513 × total lipid content ($mg_{COE}\ g^{-1}{}_{DW}$) + 0.0134 ($R^2 = 0.99$), and results were expressed in mg canola oil equivalents (COE) per gram of dry weight (DW)—$mg_{COE}\ g_{DW}{}^{-1}$. All experiments were carried out in triplicate.

Total phenolic compounds quantification

Total phenolic compounds were determined through the Folin-Ciocalteu colorimetric-based method (Folin and Ciocalteu 1927), with modifications. Briefly, aliquots of 25 µL of extract samples (10 mg mL^{-1}) were prepared in a 96-well plate with 125 µL of deionized water and 25 µL of Folin-Ciocalteu reagent and kept in the dark for 5 min. Samples were then incubated with 75 µL of sodium carbonate (6.60 wt%), at room temperature over 60 min. After incubation, absorbance of the reaction mixture was measured at λ = 760 nm using a microplate spectrophotometer (Multiscan Go, ThermoFisher). Gallic acid (Acros Organic), ranging in concentration from 0 to 200 µg mL^{-1} DMSO:PBS (1:4 *v/v*), was used to prepare the standard curve. The total content of phenolic compounds in the extracts was calculated using linear regression equation Absorbance = 0.0052 × phenolic content ($\mu g_{GAE}\ mg\ {}_{DW}{}^{-1}$) + 0.0539 ($R^2 = 0.99$), and results were expressed in µg gallic acid equivalent (GAE) per gram of dried biomass—$\mu g_{GAE}\ g_{DW}{}^{-1}$. All experiments were performed in triplicate.

Statistical analysis

The experimental data was analyzed using software GraphPad Prism, version 8 for Windows (GraphPad Software, USA). Data analyzed were submitted to Shapiro–Wilk test to confirm the normal distribution of the residuals. Then a one-way ANOVA with Tukey's multiple comparison tests was used to assess variance between yields, antioxidant capacity, total content in chls, carotenoids, PBPs, lipids, and phenolic compounds. Student's *t*-tests were performed to ascertain significant differences among the results of the cytotoxicity tests.

Results

Biomass production

To select the best day to harvest the biomass, production of biomass by *S. salina* was monitored over time. The results in Fig. 1 showed that, under the conditions tested, *S. salina* enters the late exponential phase at day 21—so this was the production time selected.

Extraction yields

The extraction yields (% mass weight) of the single and successive extractions applied to *S. salina* were determined to each solvent of each series. The results are depicted in Table 1 showing that the yields can vary depending on the solvent used or combined solvents used. Concerning organic extracts for single extractions, ethanol (series A) stood out with the highest yield of extraction (with 27.91 ± 1.12%), 7.5-fold higher than the acetonic (series A), presenting the lowest yield of extraction. In successive extractions, use of ethanol improved yield (series B and D), yet the extraction in series B was significant better than in series D. Regarding the aqueous extracts, no difference between single and successive extractions ($p > 0.05$) was found.

Antioxidant capacity

The antioxidant capacity of *S. salina*, considering all single and successive extracts, was evaluated by different radical

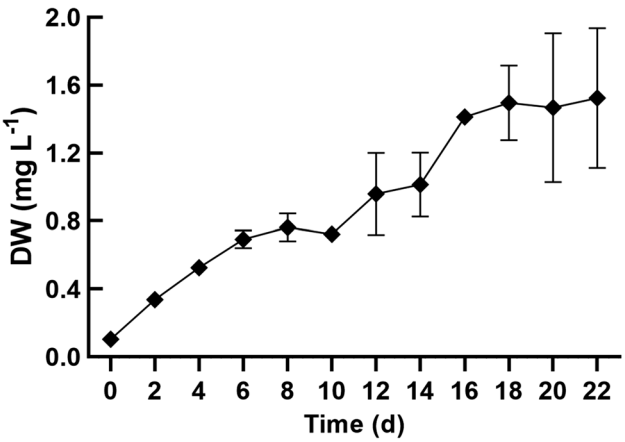

Fig. 1 Growth curve of *S. salina* LEGE 06,155, expressed as dry weight (mg L^{-1}) (-◆-) per day (d). The results are mean ± SD ($n = 3$)

Table 1 Yield of extractions *Synechocystis salina* LEGE 06,155 biomass of the various solvent and series (series A, B, C, and D) used. The results are reported as mean ± SD ($n = 3$) and expressed as percentage of weight of freeze-dried biomass. For each column, different characters mean $p < 0.05$ (one-way ANOVA, Tukey's multiple comparisons test)

Series of extraction	Extracts	Extraction yield (%)	Total extraction yield (%)
Series A (single)	Hexane	3.79 ± 0.19^{a}	3.79 ± 0.19^{a}
	Ethanol	27.91 ± 1.12^{b}	27.91 ± 1.12^{b}
	Acetone	3.72 ± 0.38^{a}	3.72 ± 0.38^{a}
	PBS	$2.64 \pm 0.14^{a,c}$	$2.64 \pm 0.14^{a,c}$
Series B	Hexane	3.79 ± 0.19^{a}	
	Ethanol	26.25 ± 0.21^{b}	33.56 ± 0.64^{c}
	PBS	3.65 ± 0.21^{a}	
Series C	Hexane	3.79 ± 0.19^{a}	
	Acetone	4.34 ± 0.14^{a}	12.26 ± 0.19^{d}
	PBS	4.12 ± 0.13^{a}	
Series D	Hexane	3.79 ± 0.19^{a}	
	Acetone	4.34 ± 0.14^{a}	
	Ethanol	22.66 ± 0.14^{e}	29.75 ± 1.63^{b}
	PBS	3.30 ± 0.98^{a}	

scavenging methods: $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $O_2^{\bullet-}$, and $NO^{\bullet-}$. All extracts showed antioxidant potential in one or more assays. Considering total antioxidant potential, results of the $ABTS^{\bullet+}$ assay (Table 2) showed that the IC_{50} of *S. salina* extracts ranged between 200 ± 45 $\mu g_{extract}$ mL^{-1} (PBS, series A) and 1171.62 ± 138.95 $\mu g_{extract}$ mL^{-1} (acetone, series C). Overall, the aqueous extracts (single and successive) exhibited the highest radical scavenging capacity and the single (series A) was the most active. Considering the organic extracts, the hexane and ethanolic ones (both single and successive of in series B) attained a high antioxidant capacity.

In the evaluation of *S. salina* extracts against radical $DPPH^{\bullet}$ (Table 2), only the IC_{50} of the acetonic extract (series A) could be estimated, i.e., 670.78 ± 67.01 $\mu g_{extract}$ mL^{-1}. However, regarding the values of IC_{25}, it was observed that other extracts possessed potential antioxidant capacity, namely, the acetonic (series C and D) and ethanolic (series A) ones. The IC_{50} and IC_{25} of aqueous extracts of both single and successive extractions could not be determined.

Regarding $NO^{\bullet-}$ assay (Table 2), the IC_{50} values ranged between 440.76 ± 44.88 $\mu g_{extract}$ mL^{-1} (PBS, series B) and 727.94 ± 64.51 $\mu g_{extract}$ mL^{-1} (acetonic, series A); the best results were obtained with PBS. On the other hand, in the $O_2^{\bullet-}$ assay (Table 2), the ethanolic extract (series B) was the only one able to reach the IC_{50} (1073.09 ± 17.38 $\mu g_{extract}$ mL^{-1}).

Extract cytotoxicity

The cytotoxicity of *S. salina* extracts was evaluated using two different concentrations (100 and 200 $\mu g_{extract}$ mL^{-1}), for exposure times of 24 and 48 h. The results are depicted in Fig. 2A and B, for the organic and aqueous extracts, respectively. Within the organic extracts, ethanolic extract (series D) and acetonic one (series A) exhibited cytotoxicity. In the case of ethanolic extract, the cytotoxicity was retained for the two concentrations tested (decrease in cell viability by 20.2 to 28.8%, when compared to the control, $p < 0.05$). A significant cytotoxicity was observed in the acetonic extract (series A), at 200 $\mu g_{extract}$ mL^{-1} for 48 h. It caused a decrease in cell viability of ca. 87%, when compared to the control ($p < 0.05$), yet cytotoxicity was not detected at 100 $\mu g_{extract}$ mL^{-1} concentration (Fig. 2A). For that reason, the latter extract was tested for the intermediate concentration of 150 μg mL^{-1}, but no cytotoxicity was detected (data not shown). Despite not being the focus of this study, it should be highlighted that some of the organic extracts (by 48 h) seemed to stimulate cell proliferation (except for hexane, series A and ethanolic, series D), ranging from 21 to 34% (in relation to the positive control)—relevant from a perspective of cell proliferation concerning formation of new vasculature.

With regard to the PBS aqueous extracts (Fig. 2B), series C and D presented cytotoxicity after 24 and 48 h of exposure at 100 $\mu g_{extract}$ mL^{-1} and showed a significant decrease in cell viability by ca. 18–21%, when compared to the control (DMEM media) ($p < 0.05$). Cells exposed to 200 μg mL^{-1} showed mild signs of cytotoxicity, after 48-h exposure, in particular for series B, C, and D as compared with the control ($p < 0.05$). Therefore, all aqueous extracts showed some signs of mild cytotoxicity, except that extract of PBS (single extraction, series A) which did not present significant decrease in cell viability ($p > 0.05$) for either the tested concentrations.

Extract physicochemical characterization

General biochemical profile

The overall biochemical profile *S. salina*, in each series and single extracts, was determined and results are shown in Fig. 3.

Table 2 Antioxidant capacity of single (series A) and successive extracts (series B, C, and D), of biomass of *Synechocystis salina* LEGE 06,155, represented by IC_{25} and IC_{50} ($\mu g_{extract}$ mL^{-1}) for various colorimetric methods, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $NO^{\bullet-}$, and $O_2^{\bullet-}$. The results are reported as mean ± SD ($n = 3$) and expressed as $\mu g_{extract}$ mL^{-1}. For each column, different characters means $p < 0.05$ (one-way ANOVA; Tukey's multiple comparisons test)

Series of extraction	$ABTS^{\bullet+}$		$DPPH^{\bullet}$		$NO^{\bullet-}$		$O_2^{\bullet-}$	
	IC_{25}	IC_{50}	IC_{25}	IC_{50}	IC_{25}	IC_{50}	IC_{25}	IC_{50}
Series A (single)	194.81 ± 22.23^{a}	443.84 ± 20.18^{a}	1184.75 ± 15.35^{a}	n.d	1754.34 ± 88.26^{a}	n.d	n.d	n.d
	144.05 ± 9.14^{a}	402.58 ± 13.98^{a}	443.33 ± 47.26^{b}	n.d	1181.52 ± 68.17^{b}	n.d	412.35 ± 1.30^{a}	n.d
	355.64 ± 1.02^{b}	980.11 ± 23.91^{b}	69.94 ± 8.84^{c}	670.77 ± 67.01	162. 57 ± 64.51^{c}	727.94 ± 64.51^{a}	926.67 ± 62.00^{b}	n.d
	47.78 ± 4.91^{c}	220.45 ± 23.07^{c}	n.d	n.d	137.30 ± 5.36^{c}	n.d	n.d	n.d
Series B* (successive)	143.71 ± 19.55^{a}	446.32 ± 50.84^{a}	1067.08 ± 85.08^{a}	n.d	760.00 ± 40.81^{d}	n.d	312.88 ± 30.32^{a}	1073.09 ± 17.38
	94.24 ± 2.09^{d}	300.96 ± 17.14^{a,c}	n.d	n.d	124.47 ± 30.72^{c}	440.76 ± 44.88^{b}	n.d	n.d
Series C* (successive)	297.83 ± 11.45^{e}	1171.62 ± 138.95^{d}	435.07 ± 15.63^{b}	n.d	221.50 ± 16.79^{e}	698.68 ± 23.62^{a}	200.63 ± 18.58^{a,c}	n.d
	69.15 ± 1.31^{c,d}	283.69 ± 2.05^{a,c}	n.d	n.d	70.87 ± 20.67^{c,e}	495.48 ± 0.00^{b}	n.d	n.d
Series D* (successive)	297.83 ± 11.45^{e}	1171.62 ± 138.95^{c}	435.07 ± 15.63^{b}	n.d	221.50 ± 16.79^{e}	698.68 ± 23.62^{a}	200.63 ± 18.58^{a,c}	n.d
	148.48 ± 0.25^{a}	648.01 ± 6.02^{e}	937.68 ± 38.83^{a}	n.d	1032.32 ± 85.04^{b}	n.d	n.d	n.d
	101.66 ± 5.83^{d}	349.66 ± 0.28^{a,c}	n.d	n.d	230.84 ± 47.28^{c}	534.25 ± 0.34^{b}	n.d	n.d

*The extracts of each series were analyzed separately

At a first glance, series of successive extraction B, C, and D could afford most metabolites, e.g., chls, carotenoids, PBPs, phenolic compounds, and lipids. In addition, the PBPs are the compounds that contribute the most to the total compounds extracted, with a pronounced percentage from 78 to 81%. Within the organic extracts, in particular hexane, 20-fold highest amount of lipids was produced, when compared to acetonic extract (series A). The hexanolic also presented a considerable percentage of carotenoids and phenolic compounds, up to threefold the acetonic extract (series A) and 25-fold the ethanolic extract that presented the lowest value observed for single extractions (series A). In addition, chls were the preponderant compounds in the acetonic single extract (series A), where they accounted for a total of 89.2% of such pigments, almost eightfold the hexane extract that the lowest percentage. Concerning the aqueous extracts, PC was the pigment that stood out in both single and successive extractions, contributing with up to 60% of the total quantified metabolites—except for the PBS single extract, in which APC stood out with the highest percentage (42.28%).

Pigment content and profile

The pigment contents and profile were determined for single and successive extracts of biomass of *S. salina*. Series B was observed to achieve the overall highest pigment content (25.58 ± 1.13 mg g_{DW}^{-1}) (Table 3). Conversely, the successive extraction (series B) was the most effective in extracting chls, specifically the ethanolic fraction as well as the single ethanolic extraction. The single extraction with acetone (series A) was noted to be more effective in extracting carotenoids. However, the highest ratio of carotenoids/chls was possible to observe in the hexane extract.

Analyzing the carotenoids profile of each organic extract of *S. salina* (Table 4), a total of 8 carotenoids were identified, including carotenes lycopene, α- and β-carotene, β-zeacarotene, and β-carotene-5,6-epoxide and xanthophylls zeaxanthin, lutein, and echinenone. To note that, all presented extracts revealed the presence of up to two non-identified chl *a* derivatives; for the hexane and acetonic extract (single, series A), pheophytin was identified (data not shown). The content and carotenoid profile were dependent on the single or successive extraction.

Regarding the single extractions, acetone produced the extract with all identified carotenoids; in particular, it was the only extract revealing the presence of lycopene (11.30 ± 0.82 μg g_{DW}^{-1}). Conversely, ethanol (single) could not extract both lycopene and zeaxanthin, but stood out by extracting up to fivefold more carotenes (α-carotene, β-carotene, and β-carotene-5,6-epoxide), besides xanthophyll echinenone.

Hexane permitted extraction of some carotenes, in particular β-zeacarotene. In addition, a more refined profile and

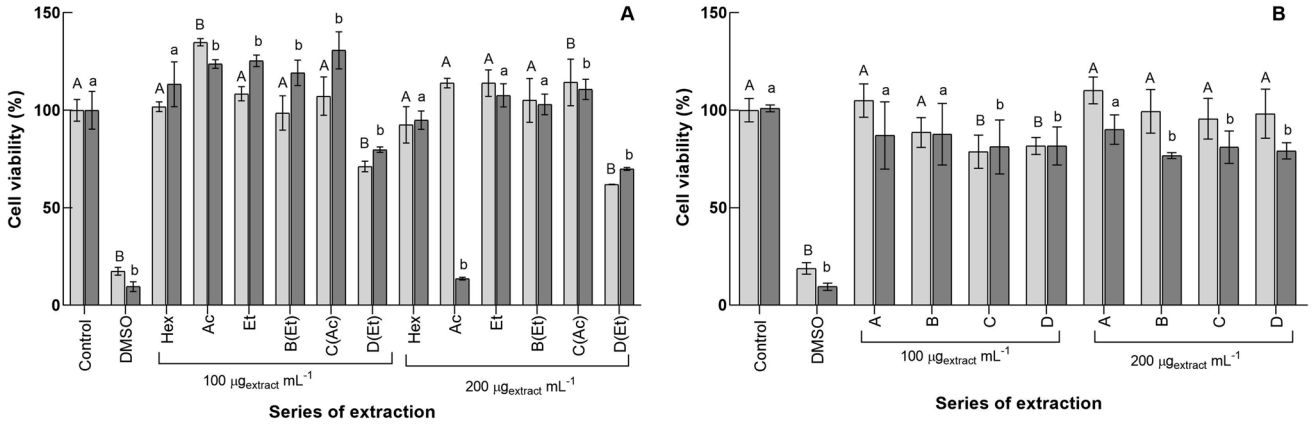

Fig. 2 Cytotoxicity effects of successive *Synechocystis salina* LEGE 06,155 extracts of series A, B, C, and D for the organic (**A**) and aqueous (**B**) extracts against hCMED cells. Organic extracts are represented as follows: B(Et)—series B, ethanolic; C(Ac)—series C, acetonic; and D(Et)—series D, ethanolic. In aqueous extracts, each capital letter refers to each series of single or successive extraction. The positive control (Control) was tested with DMSO (1% *v/v*) in the organic extracts and DMEM media for the aqueous extracts; the negative control (DMSO) was tested with DMSO (20% *v/v*). The extracts were exposed by 24 h (□) and 48 h (▮), at 100 µg$_{extract}$ mL^{-1} and at 200 µg$_{extract}$ mL^{-1}. Results are reported as mean ± SD, and results are expressed in % cell viability. The data were analyzed in comparison with the positive control (no cell death A,a) and the negative control (cell death, B,b). The uppercases represent the treatment at 24 h and the lowercases represent the treatment at 48 h. Different characters mean $p < 0.05$ (Student's *t*-tests)

content were obtained in the successive extractions, particularly regarding xanthophylls. The ethanolic extract (series D) seemed to be more selective for xanthophyll lutein as it presented the highest content (216.14 ± 13.57 µg g_{DW}^{-1}), corresponding to an almost 34-fold increase when compared to the lowest value for acetone (series C). Ethanol (series B) was appropriate to extract general xanthophyll pigments, as carotenes were not found in the profile.

In what pertains to PBP profile and content, both single and successive extracts contained PC, APC, and PE (Table 3). The PC was the pigment with the highest concentration in all successive extraction series, standing out in series C, twice as high as the single extraction with PBS (series A). On the other hand, it was observed that single extraction with PBS enhanced extraction of APC and PE, up to 2.9- and sixfold increase, respectively, when compared to the aqueous successive extractions.

Overall, single and successive extractions (series A and C, respectively) were the best series to extract PBPs, with no significant differences among them ($p > 0.05$); however, the proportion of PC, APC, and PE varied among them.

Total lipid content

Total lipid content of *S. salina* was estimated for all organic extracts in the various series, as depicted in

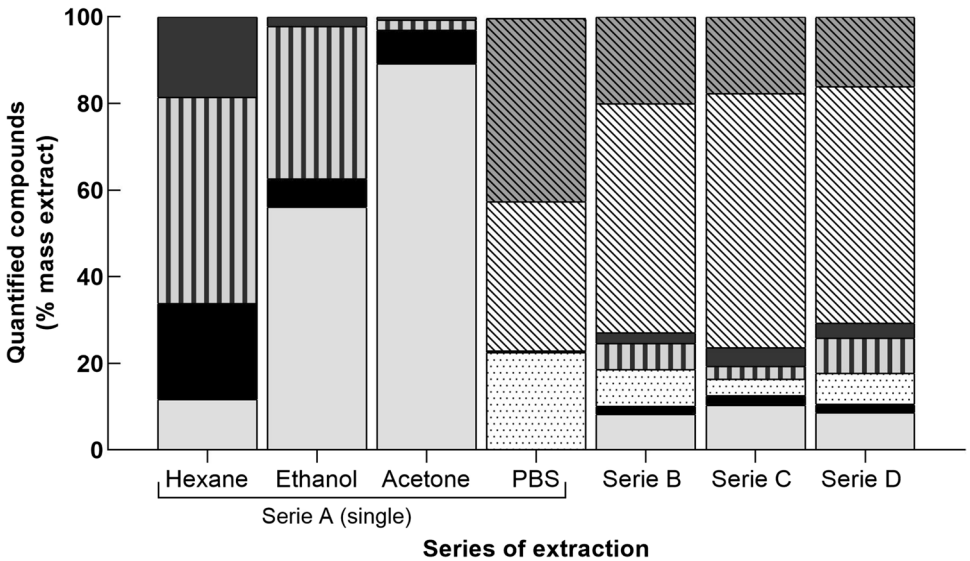

Fig. 3 Biochemical profile of the quantified compounds in the single (series A) and successive extractions (series B, C, and D) of the biomass of *Synechocystis salina* LEGE 06,155 in terms of chlorophylls (□), carotenoids (■), lipids (▥), phenolic compounds (▩), phycoerythrin (⬚), allophycocyanin (▧), and phycocyanin (▨). Results are express in % of mass of extract of the quantified compounds

Table 3 Pigment composition of *Synechocystis salina* LEGE 06,155 extracts, in terms of total chlorophyll *a* (and derivatives), total carotenoids, and total phycobiliproteins, including phycocyanin (PC), allophycocyanin (APC), and PE (phycoerythrin), and overall pigment content in the various series of extraction performed (series A, B, C, and D). The results are reported as mean ± SD ($n = 3$) and expressed in mg g_{DW}^{-1}. For each column, different characters mean $p < 0.05$ (one-way ANOVA, Tukey's multiple comparisons test). Different lowercase characters show significant statistical differences ($p < 0.05$) between extracts (in each column)

Series of extracts	Total chl *a* (and derivatives) (mg g_{DW}^{-1})	Total carotenoids (mg g_{DW}^{-1})	Car/Chls ratio	Phycobiliproteins (mg g_{DW}^{-1})			Total PBP (mg g_{DW}^{-1})	Total pigments (mg g_{DW}^{-1})
				PC	APC	PE		
Series A (single)								
Hexane	0.12 ± 0.00^{a}	0.24 ± 0.03^{a}	1.98^{a}	–	–	–	–	0.36 ± 0.03^{a}
Ethanol	8.32 ± 0.19^{b}	$\mathbf{1.00 \pm 0.04^{b}}$	0.12^{b}	–	–	–	–	9.32 ± 0.19^{b}
Acetone	4.05 ± 0.35^{c}	0.34 ± 0.02^{a}	0.08^{c}	–	–	–	–	4.39 ± 0.35^{c}
PBS	–	–		7.08 ± 0.57^{a}	$\mathbf{8.70 \pm 0.03^{a}}$	$\mathbf{4.31 \pm 0.55^{a}}$	$\mathbf{19.74 \pm 0.92^{a}}$	19.74 ± 0.92^{d}
Series B (successive)								
Hexane	0.12 ± 0.00^{a}	0.24 ± 0.03^{a}	1.98^{a}	–	–	–	–	
Ethanol	10.47 ± 0.78^{b}	0.37 ± 0.00^{a}	0.04^{c}	–	–	–	–	$\mathbf{25.58 \pm 1.13^{e}}$
*ΣH + E	**10.59 ± 0.78**	0.61 ± 0.03	0.06^{c}					
PBS	–	–		9.48 ± 0.41^{a}	3.70 ± 0.28^{b}	1.57 ± 0.18^{b}	14.75 ± 0.80^{b}	
Series C (successive)								
Hexane	0.12 ± 0.00^{a}	0.24 ± 0.03^{a}	1.98^{a}	–	–	–	–	
Acetone	1.87 ± 0.02^{e}	0.08 ± 0.00^{c}	0.04^{c}	–	–	–	–	20.02 ± 1.62^{d}
*ΣH + A + E	1.99 ± 0.02	0.32 ± 0.03	0.16^{b}	–	–	–		
PBS	–	–		$\mathbf{14.16 \pm 1.32^{b}}$	3.21 ± 0.24^{b}	0.71 ± 0.05^{b}	18.08 ± 0.13^{a}	
Series D (successive)								
Hexane	0.12 ± 0.00^{a}	0.24 ± 0.03^{a}	1.98^{a}	–	–	–	–	
Acetone	1.87 ± 0.02^{e}	0.08 ± 0.00^{c}	0.04^{c}	–	–	–	–	
Ethanol	0.39 ± 0.03^{a}	0.22 ± 0.01^{a}	$0,61^{d}$	–	–	–	–	17.18 ± 1.15^{d}
*ΣH + A + E	2.38 ± 0.06	0.54 ± 0.03	0.23^{e}	–	–	–	–	
PBS	–	–		9.98 ± 0.86^{a}	$2.95 \pm 0.20^{b,c}$	1.33 ± 0.13^{b}	14.26 ± 0.75^{b}	

*Σ, sum of each individual extract of the successive extractions

Table 4 Carotenoid profile of *Synechocystis salina* LEGE 06,155 extracts and content in the different single (series A) and successive organic extractions (series B, C and D). The results are reported as mean ± SD ($n = 3$) and are expressed in μg g_{DW}^{-1}. For each row, different characters mean $p < 0.05$ (one-way ANOVA, Tukey's multiple comparisons test)

Identified pigments	Concentration of pigment (μg g_{DW}^{-1})					
	Hexane (single/successive)	Acetone (single)	Ethanol (single)	Ethanol (successive B)	Acetone (successive C/D)	Ethanol (successive D)
Carotenes						
α-Carotene*	21.92 ± 1.91^{a}	23.84 ± 0.74^{a}	106.96 ± 11.88^{b}	n.d	n.d	n.d
β-Carotene	39.20 ± 4.14^{a}	58.72 ± 4.18^{b}	110.97 ± 6.37^{c}	n.d	n.d	n.d
β-Carotene-5,6-epoxide*	n.d	4.82 ± 0.17^{a}	110.81 ± 10.18^{b}	n.d	n.d	n.d
β-zeacarotene*	21.80 ± 1.20^{a}	4.45 ± 0.08^{b}	n.d	n.d	n.d	n.d
Lycopene*	n.d	11.30 ± 0.82	n.d	n.d	n.d	n.d
Xanthophylls						
Lutein	19.74 ± 0.87^{a}	49.73 ± 2.00^{b}	142.60 ± 6.66^{c}	162.41 ± 3.42^{c}	6.43 ± 0.61^{a}	216.14 ± 13.57^{d}
Echinenone	131.06 ± 14.50^{a}	149.36 ± 8.70^{a}	484.71 ± 30.52^{b}	173.07 ± 2.83^{a}	45.11 ± 0.00^{c}	n.d
Zeaxanthin	10.15 ± 0.32^{a}	15.97 ± 0.86	$47.82 \pm 1.19^{b,c}$	35.61 ± 2.00^{b}	27.81 ± 2.65^{b}	n.d

*Concentration expressed in β-carotene equivalents

Fig. 4. The ethanolic fraction was observed as the best extract to retrieve the high concentration of total lipid compounds from the *S. salina* biomass. The single extraction with ethanol was 53.5-fold that of the acetonic extract (series A) which had the lowest content in lipid compounds. The successive extractions did not seem to enhance extraction yield of these compounds, yet series D showed a similar concentration of lipids as the single extraction of ethanol (series A) ($p > 0.05$).

Total phenolic compound content

Synechocystis salina biomass extracts were evaluated for their content in total phenolic compounds (TPC), with results presented in Fig. 5. Overall, single extractions were not so effective in the extraction of TPC, despite the single extraction with ethanol having shown considerably concentrations of TPC, with 0.44 ± 0.04 mg_{GAE} g_{DW}^{-1} (11-fold increase relative to the lowest value, achieved with the single acetonic extraction). On the other hand, the successive extractions were proven to improve the content of such compounds, up to 23-fold more. The use of a less polar solvent as hexane (0.25 ± 0.03 mg_{GAE} g_{DW}^{-1}) helped in the extraction of non-polar compounds and seemed to enhance extraction yield of TPC with polar nature (i.e., series C). Among successive extractions, series B and D attained the best results with no significant differences ($p > 0.05$) between each other, whereas the ethanolic fraction stood out with the highest content (0.58 ± 0.02 mg_{GAE} g_{DW}^{-1}). Series C led to a low cumulative TPC concentration, but was able to extract a substantial quantity of aqueous TPC (0.26 ± 0.00 mg_{GAE} g_{DW}^{-1}), across all series.

Correlation between antioxidant capacity and chemical characterized compounds

The correlation between the antioxidant capacity (IC_{25} and IC_{50} values) and the total content of the compounds here studied and described as having antioxidant capacity in the literature (i.e., Amaro et al. 2015; Pagels et al. 2019) was determined for both organic and aqueous extracts. Only statistically relevant correlations between the antioxidant capacity and the studied compounds are depicted in Table 5. For the organic extracts, the antioxidant capacity presented in $ABTS^{\bullet+}$ had a negative correlation with the content of pheophytin, the carotenoid zeaxanthin, and the content in lipids and phenolic compounds—the negative correlation describes a high antioxidant capacity with the lower IC_{25}/IC_{50} and the increment of content in such compounds. β-Carotene was the only compound (negatively) correlated with the antioxidant capacity for the $NO^{\bullet-}$ assay while for the $O^{\bullet-}$ assay, total carotenoids and phenolic compounds were the compounds most representative. No correlation could be established for $DPPH^{\bullet}$ assay. Regarding the aqueous extracts, the only representative correlation was presented with $ABTS^{\bullet+}$ assay and the APC.

Discussion

The cyanobacterium *S. salina* was investigated as source of added-value compounds, e.g., metabolites with antioxidant properties, to eventually address a number of biotechnological purposes (in the food, feed, nutraceutical, cosmetical, or dye industries). Furthermore, this study resorted to GRAS

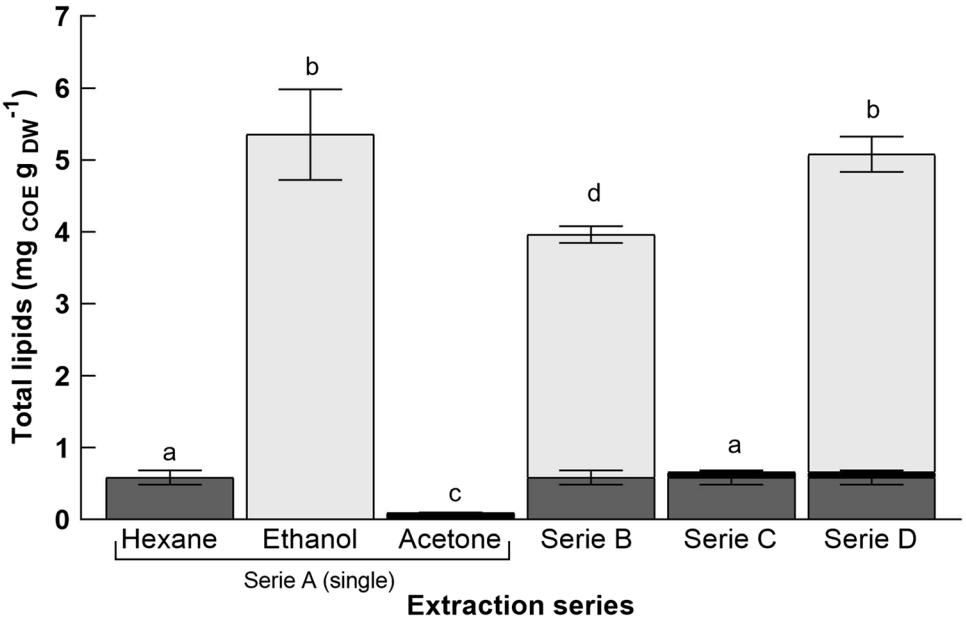

Fig. 4 Total lipid content obtained in the various organic extracts of the single (series A) and successive extractions (series B, C, and D) with the corresponding solvents used hexane (■), ethanol (□), and acetone (■) of biomass of *Synechocystis salina* LEGE 06,155. Results are reported as mean ± SD ($n = 3$) and expressed in mg of canola oil equivalent per g dry weight (mg_{COE} g_{DW}^{-1}). Different characters mean $p < 0.05$ (one-way ANOVA, Tukey's multiple test)

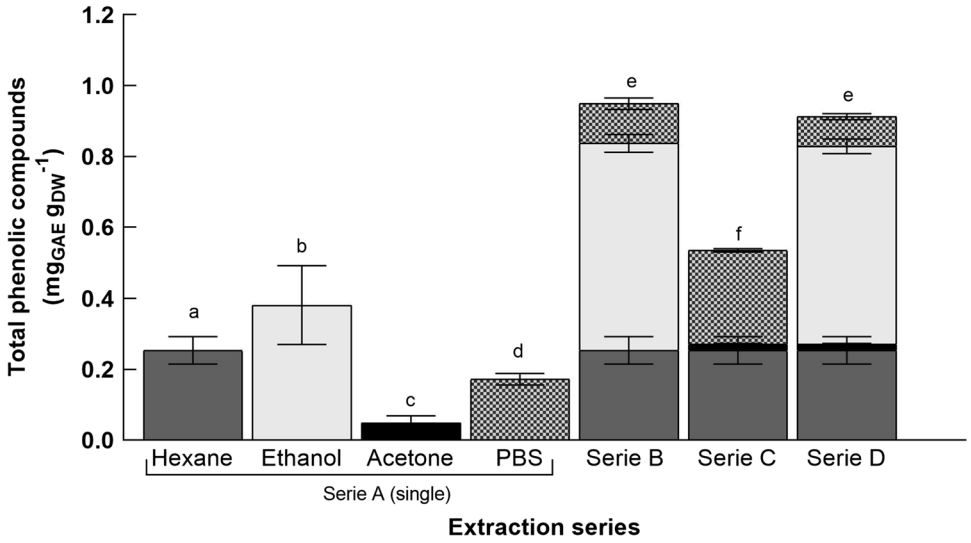

Fig. 5 Total phenolic content in the various organic extracts of the single (series A) and successive extractions (series B, C, and D), with the corresponding solvents used hexane (▩), ethanol (□), acetone (■), and PBS (▩) of the biomass of *Synechocystis salina* LEGE 06,155. Results are reported as mean ± SD ($n = 3$) and expressed in mg gallic acid equivalents per g dry weight (mg$_{GAE}$ g$_{DW}^{-1}$). Different characters mean $p < 0.05$ (one-way ANOVA, Tukey's multiple test)

Table 5 Correlation between the antioxidant capacity (IC_{50}) of $ABTS^{\bullet+}$, $NO^{\bullet-}$, and $O^{\bullet-}$ assays and the content of the chemical characterized compounds in both organic and aqueous extracts, by using Pearson product-moment correlation, with the respective Pearson correlation coefficient (r) and the significant statistical relationship (p value)

$X_{variable}$	$Y_{variable}$	r (x,y)	p value
Organic extracts ($n = 16$)			
$ABTS^{\bullet+}$ (IC_{25})	Pheophytin	−0.85	0.0317
$ABTS^{\bullet+}$ (IC_{50})	Zeaxanthin	−0.58	0.0499
	Lipids	−0.54	0.0462
	Phenolic compounds	−0.60	0.0239
$NO^{\bullet-}$ (IC_{25})	β-Carotene	−1.00	0.0493
$O^{\bullet-}$ (IC_{50})	Total carotenoids	−1.00	0.0384
	Phenolic compounds	−1.00	0.0374
Aqueous extracts ($n = 9$)			
$ABTS^{\bullet+}$ (IC_{50})	APC	−0.95	0.0133

solvents with combined successive extractions, in attempts to make the extraction process greener and safer, besides reusing biomass to obtain the most relevant classes of bioactive compounds.

Regarding yields, the use of more than one solvent seemed to maximize use of biomass, as compared to a single extraction approach. Specifically, the combination of series B led to the highest extraction yields. For the organic extracts, ethanol stood out for its higher yields than any other solvent used (even in single extraction)—which demonstrates that this solvent has an extensive ability to extract metabolites possessing a wide range of polarities. On the other hand, the lowest yields were obtained with acetone (series A), which agrees with the results of Goiris et al. (2012). In the aqueous extracts, the yield of extraction was low, without relevant differences among single or successive extractions ($p > 0.05$), as opposed to the high yields obtained with different types of cyanobacteria and microalgae as reported by Jerez-Martel et al. (2017). However, the type of extraction and solvents used affect the extraction yields, which can also influence the type of compounds extracted (Maadane et al. 2015; Pagels et al. 2020) as apparent in the overall biochemical profile obtained.

Regarding biological activities, all tested extracts, both organic and aqueous, were shown to possess antioxidant potential. The aqueous extracts, in particular the one of series A (single), unfolded the highest antioxidant potential, with the lowest IC_{50} recorded (for $ABTS^{\bullet+}$). The aqueous extracts of series B and C also stood out in the $NO^{\bullet-}$ assay. This noteworthy antioxidant capacity is probably related to the presence of PBPs, in particular with the APC. Such compounds were described as having considerable natural antioxidant features and superior ability to scavenge free radicals (Bhat and Madyastha 2001; Saini et al. 2018; Pagels et al. 2019). For instance, PC of *Spirulina platensis* was shown to protect against oxidative stress and against DNA damage by scavenging free radicals and $ONOO^{\bullet-}$ (peroxynitrite) (Bhat and Madyastha, 2001).

Regarding the organic extracts, interesting antioxidant properties were observed in the acetonic extracts, series A and C, for the assay $NO^{\bullet}$. Such results may be due the presence of carotenes (i.e., β-carotene and derivatives). The acetonic extract, in particular of series A, presented a relatively high concentration of carotenoids—and was the only one with lycopene in its carotenoid profile. Despite this particular pigment did not present representative correlation with the antioxidant capacity of the organic extracts (data not shown), in literature this pigment is described as having high antioxidant capacity, exceeding that of β-carotene or α-tocopherol (Kelkel et al., 2011). Cyanobacterial extracts are composed of complex matrixes with a wide range of

carotenoids and other bioactive compounds such as polyphenols, proteins, or vitamins that can have a synergistic (or antagonistic) behavior (Maadane et al. 2015) and could contribute to the observed antioxidant capacities here studied. This is a possible explanation for the antioxidant capacity observed in the DPPH$^{\bullet}$ assay that could not correlate with any of the compounds here characterized, but shown low IC_{25} and IC_{50} particularly for the acetonic extracts (series A and C). Moreover, the presence of some chl derivatives has also been described as having potent antioxidant capacity (i.e., pheophytin) (Perez-Galvez et al. 2017), which can indeed contribute to a superior antioxidant potential, in particular for the ABTS$^{\bullet+}$ assay.

Considering the ethanolic extracts, the high content in carotenoids, in particular xanthophylls as zeaxanthin, echinenone, and lutein, but also carotenes such as β-carotene and α-carotene, is claimed to relate to its high antioxidant capacity, particularly in the case of ABTS$^{\bullet+}$ (series A and B) and $O_2^{\bullet-}$ (series B). Most such pigments have been described to have potent capacity to scavenge free radicals (Safafar et al. 2015) and can act synergistically (Maadane et al. 2015; Pagels et al. 2020). In addition, the high content total phenolic compounds in these extracts can also support these results, since those compounds possess also antioxidant properties (Jerez-Martel et al. 2017).

Overall, the extracts possess different antioxidant compounds, which work through distinct mechanisms in scavenging the different free radicals used in this study. This is in agreement with Goh et al. (2010), who demonstrated different antioxidant capacities for both non-polar and polar solvent in the case of the marine diatom *Navicula clavata* and the green microalgae *Chlorella marina* and *Dunaliella salina*. The study of cytotoxicity of extracts is essential to anticipate whether these extracts will exert any toxicity against biological systems, while giving insights onto their potential regarding further biotechnological applications, in particular encompassing human and animal uses. We used hCMED cells in our study, a representative model of endothelial cells found across all human body. Such cells are vital in maintenance of several organs (e.g., skin, heart, kidneys, liver) and promoter peripheral vascularization. Hence, they exhibit a relevant biological platform to assess cytotoxicity (Rajendran et al. 2013).

Almost all *S. salina* organic extracts did not shown any signs of cytotoxicity, except the acetonic (series A) and ethanolic (series D) ones. In the latter case, previous extraction with acetone seemed to unveil certain compounds toxic to the cells. In the first case, increasing concentrations up to 200 $\mu g_{extract}\ mL^{-1}$, after 48-h exposure, led to a marked cell death, which seems to indicate a dose-responsive effect: the higher the concentration of the extract, the higher decrease in cell viability with time. These conclusions can be corroborated by the intermediate concentration tested of 150 $\mu g_{extract}\ mL^{-1}$ of this extract (data no shown), where no signs cytotoxicity were observed. On the contrary, this particular extract promoted cell proliferation at 100 $\mu g_{extract}\ mL^{-1}$, after 48-h exposure, as well as other organic extracts including the acetonic (series C) and ethanolic ones (series A and B). Therefore, the positive potential effects on endothelium cells and the potential applications of (some) the organic *Synechocystis* extracts are hereby suggested. The presence of carotenoids and phenolic compounds in those extracts can partially contribute to induce cellular proliferation, thus functioning as growth elicitors. For instance, β-carotene has been reported to play a significant role in early steps on angiogenic activity (Dembińska-Kieć et al. 2006). Therefore, such extracts could be potentially used in the cosmetic field (e.g., skin regenerative properties) and the pharmaceutical field (apparent angiogenic properties). Nevertheless, detailed studies on cell viability, using different cell models and concentrations, are still necessary to confirm such proliferative properties.

The cytotoxicity being present in aqueous extracts was quite unexpected because these are aqueous extracts rich in non-toxic and natural phycobiliproteins and phenolic compounds. A possible explanation is the presence of salts of the buffer phosphate in the mass of extract, which can potentially exert a negative effect upon cell viability. PBS has been reported to show cytotoxicity in other cellular lines (Schuerer et al. 2017). The lyophilized PBS mass was dissolved in neutral pH water, which could lead to pH change of alkaline cell media, observed by an orange/yellowish coloration (data not shown). This means that the nature of the extracts could affect the pH of the media where cells lie on—and, consequently, affect cell viability.

The importance of the combined extraction of metabolites of interest by resorting to successive extraction with GRAS solvents was proven to enhance the contents of several groups of compounds (i.e., pigments and phenolic compounds). For pigments, series B (in particular ethanolic extract) enhanced recovery of chls and carotenoids. Ethanol is one of the most common solvents used to extract photosynthetic pigments that span wide ranges of polarities—which also helps explain our results. In addition, it is suggested that different solvents and combinations thereof (via successive extraction) influence carotenoids profile and content, as observed before (Amaro et al. 2015; Lopes et al. 2020; Schüler et al. 2020). For instance, Lopes et al. (2020) have studied pigments of various terrestrial and aquatic cyanobacteria, in both acetonic and ethanolic (70%) extracts (single extractions). Their results for both solvents (and for the same species) have shown considerably different carotenoid profiles and content. In the present study, ethanolic extracts (single, series A) stood out with the highest quantity of carotenoids extracted, but the successive extraction denoted a more restricted profile which supports the idea

that successive extraction can be more selective to some families of compounds. For instance, extraction with ethanol (series B) was more selective to extract high concentrations of xanthophylls, whereas ethanol (series D) showed more appropriate to extract lutein in higher quantities. This can be advantageous in a biotechnological context, when pigment(s) are required in purer form. *Synechocystis salina* seems to possess carotenoids with a more polar nature, consistent with the high amounts of xanthophylls (lutein and echinenone) found in the ethanolic extracts. The presence of β-carotene, zeaxanthin, and echinenone in *Synechocystis* carotenoid profile is similar to the most common carotenoids present in cyanobacteria (Novoveská et al. 2019). The presence or absence of certain carotenoids (and also different contents) is highly species-dependent and strongly related with the metabolic routes and pathways developed by cyanobacteria, in order to protect and adapt to the environmental conditions where they grow.

Regarding lipid recovery, both ethanol in single extraction (series A) and series D have shown the best results. Phospholipids are the major constituents of lipid membranes (i.e., animals, non-photosynthetic-bacteria, and fungi), yet the lipid composition of cyanobacteria is different and resembles that of chloroplasts of green algae and plants, as they hold a strict evolutionary relation (Raven and Allen 2003). Cyanobacterial lipid membranes are essentially composed by three types of non-phosphorous glycolipids—monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), besides phospholipid phosphatidylglycerol (PG) (Wada and Murata 1998). The galactolipids MGDG and DGDG can be found to higher proportion in the cyanobacterial lipid pool, as they have been reported to account for about 25 to 50% of the total cyanobacterial membrane (Kobayashi 2016). *Synechocystis salina* is probably rich in such galactolipids and this is why ethanol, having an intermediate polarity (close to that of water), was the best solvent to recover such lipids. It should be highlighted that total lipid content of *S. salina*, considering the ethanolic single extract, accounts for up to 6.44% of its DW. In fact, Guedes et al. (2011c) have shown that unicellular cyanobacteria produce low quantities of lipids/fatty acids when compared to microalgae (e.g., *Scenedesmus obliquus*). However, the total lipid content depends not only on the species, but also on the imposed conditions to the culture—light quality and intensity, culture medium and nitrogen source, pH, and temperature (Wada and Murata 1998; Guedes et al. 2011c; Markou and Nerantzis 2013). For instance, *Synechocystis* sp. PCC 6803 wild type accumulated up to 16.8% (DW) under a continuous irradiance of 40 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, at 28 °C (Eungrasamee et al. 2019). The growth stage and culture age can also interfere with total lipid accumulation (Guedes et al. 2011a, b, c).

With regards to TPC, *S. salina* biomass presented different classes of phenolic compounds, both water-soluble and lipophilic, even though to low concentrations, and results suggested series B to best extract such compounds. Phenolic compounds are secondary metabolites with a wide range of chemical structures, which are produced by plants, algae, and cyanobacteria (Freile-Pelegrín and Robledo, 2013). They form a group of interesting bioactive components with radical scavenger properties, preventing and fighting radical oxidative stress, and thus having antioxidant capacity; this is precisely why they are attractive for the pharmaceutical and nutraceutical industries (Jerez-Martel et al., 2017). So far, there are few reports on total phenolic content in cyanobacteria and microalgae (Li et al. 2007; Aydaş et al. 2013; Maadane et al. 2015; Jerez-Martel et al. 2017; Haoujar et al. 2019; Pagels et al. 2020). In some cases, their concentration in cyanobacteria can be up to 78-fold higher compared to the best value found in this study. Care should be taken, however, when comparing such results because the different species of microorganisms studied and their inherent genetics, as well as their growth conditions (i.e., temperature, stress exposure, light, nutrient deprivation) and the analytical method selected to assess extraction (Li et al., 2007; Maadane et al., 2015; Safafar et al., 2015), greatly influence the result obtained.

In brief, our results provide some clues onto the potential biotechnological applications of *S. salina* biomass. Extracts of *S. salina* presented good general antioxidant capacity, with ability to scavenge a wide range of free radicals, as they are rich in several pigments and phenolic compounds, and (some of them) possess the extra advantage of not exhibiting cytotoxicity against human cells. The ethanolic extracts have stood out for their remarkable potential arising from a high content in chlorophyll and carotenoids, besides high yields of extractable substances. Moreover, the profile of ethanolic extracts (series A) revealed the presence of echinenone, β-carotene, and lutein to the highest concentrations. Such extracts have also shown high content in TPC and total lipids, with the advantage of not being cytotoxic; such antioxidant capacity could be applied in the industry of food/feed, dyes, cosmetics, or pharmaceuticals. The aqueous extracts presented the highest antioxidant capacity, in particular those with the single extraction (series A), and the highest global content in terms of PBPs, but also in terms of APC and PE—whereas the aqueous extract of series C exhibited the highest content in terms of PC. Those are quite promising, because of the potential value of such pigments in the industry of dyes and functional food. Despite the cytotoxicity found, at least in the aqueous extracts, further studies are needed to confirm that salts in the PBS extracts are indeed interfering with cell viability. For instance, other cell models (i.e., fibroblasts) may be used to test

cytotoxicity, also useful to give hints on the application in the cosmetic and cosmeceutical fields. The single and combined extraction also proved a good approach to obtain rich extracts in metabolites with added-value and bioactive potential to be applied in different fields of biotechnology, including food, feed, nutraceuticals, or pharmaceuticals.

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Author contribution JA, HMA, and ACG conceived and designed research. JA conducted experiments. JA, GL, and TT performed analytical measurements. JA wrote the manuscript draft. FXM and ACG were responsible for supervision (including revision of manuscript) and funding acquirement. All authors read and approved the manuscript.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest The authors declare no competing interests.

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