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Bioactive potential of *Cyanobium* sp. pigment-rich extracts

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Abstract

Cyanobacteria are able to synthesize high-value compounds, such as pigments, known for their bioactivities and several industrial uses. One of the key points for the extraction of pigments is solvent selection. Solvent interferes on pigments concentration, thus affecting the bioactive potential of the extracts. In this work, using frozen-dried biomass of *Cyanobium* sp. LEGE 06113, organic and aqueous pigment-rich extracts were obtained by a classic extraction methodology using four solvents — acetone (A), ethyl acetate (EA), ethanol (E) and water (W). In order to increase the efficiency of extraction from the cyanobacterium biomass, successive extractions were performed by using water after organic extraction (A-W, E-W, EA-W) and acetone after the aqueous extraction (W-A). Extraction yield and profile of carotenoids, phycobiliproteins, and phenolic compounds were quantified. The bioactive potential of *Cyanobium* sp. extracts was assessed in terms of antioxidant capacity ($ABTS^{\bullet+}$, $^{\bullet}NO$, $O_2^{\bullet-}$ scavenging), anti-inflammatory capacity (COX inhibition), and cytotoxicity (HepG2). W-A showed the higher antioxidant capacity and higher content in carotenoids. E-W showed the highest content in phycobiliproteins and great antioxidant capacity. In terms of anti-inflammatory capacity, 100 $\mu g_E\ mL^{-1}$ of E-W extract exhibited capacity to inhibit both COX-1 and COX-2 enzymes. Finally, in what concerns the cytotoxic evaluation, E, W, A-W, E-W, and EA-W revealed to have no cytotoxic effects in concentrations up to 750 $\mu g_E\ mL^{-1}$. Overall, this work constitutes a valid contribution for the valorisation of *Cyanobium* sp. pigment-rich extracts for biotechnological applications.

Keywords Cyanobacteria · Carotenoids · Phycobiliproteins · Antioxidant capacity · Anti-inflammatory capacity · Cytotoxicity

Introduction

Pigments from natural sources, and in particular from cyanobacteria, are emerging as higher market price components. They represent the main source of revenues in industries that use cyanobacterial biomass, when compared to macromolecules obtained from these organisms — e.g. carbohydrates, proteins, and lipids (Ruiz et al. 2016).

Cyanobacterial pigments have been used in several industrial applications, such as pharmaceutical, nutraceutical, cosmetic, food and feed, due to their wide range of bioactive properties. This multipurpose characteristic of cyanobacterial pigments is leading to a raising increase in the production of these organisms, as well as the development of new industrial level techniques of extraction and purification of phycobiliproteins — the main group of pigments in cyanobacteria. Currently, *Arthrospira platensis* (Spirulina) is the main cyanobacterium used to produce pigments, but the existence of other promising species indicates the need of new options for the market (Pandey et al. 2013).

Although the pigment composition varies in cyanobacteria cells within species, pigments are usually divided in three major groups: chlorophylls, carotenoids, and phycobiliproteins (Masojídek et al. 2013).

Concerning carotenoids, eukaryotic microalgae and vascular plants (in the case of lutein) are still the most used organisms. Successful cases of microalgae-based carotenoids bioprocesses include *Dunaliella salina* as source of β-carotene and *Haematococcus pluvialis* as source of astaxanthin. Also, pilot-scale projects for the production of lutein using *Muriellopsis* sp. and *Scenedesmus almeriensis* have also been studied (Del Campo et al. 2007), even though marigold flower remains the industrial source for this compound. In the case of

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phycocyanin, a compound mostly found in cyanobacteria (but also in some other algae), the main source for industrial application is *A. platensis* (Pagels et al. 2019).

In terms of downstream processing, pigments can be sold as pigment-rich biomass, though the bioactive properties are increased within the purification process (Yu et al. 2017; Pagels et al. 2019). Due to the high resistance of the cells, extraction of cyanobacterial pigments requires both mechanical and chemical methodologies. The specific methodology varies within species due to the variety of cyanobacteria morphology (Pan-utai and Iamtham 2018). The chemical extraction is based on the solubility of pigments in specific solvents, in the case of phycobiliproteins to aqueous solvents, and in the case of carotenoids, organic solvents — e.g., acetone and ethanol. However, the number of solvents and extraction processes will depend on the desired purity (Stewart and Farmer 1984; Moreno et al. 1997).

In terms of biotechnological application, cyanobacterial pigments hold great potential to pharmaceutical and nutraceutical formulations; such compounds have proven to be antioxidant and anti-inflammatory agents, also with neuroprotective and hepatoprotective properties (Sekar and Chandramohan 2008). Phycocyanin has been widely described as strong antioxidant, with significant radical scavenging properties against different deleterious free radicals (Benedetti et al. 2004; Sekar and Chandramohan 2008; Pagels et al. 2019); its anti-inflammatory capacity has also been described in several tissues (Sekar and Chandramohan 2008), being known to suppress the expression of inflammatory cofactors related to cancer and other diseases (Reddy et al. 2000; Bhat and Madyastha 2001). Moreover, some carotenoids, such as β-carotene and lutein, can be used as antioxidant to prevention of arthritis, coronary heart diseases, and premature aging (Mattson 2004; Törnwall et al. 2004). However, even with great biotechnological potential, cyanobacteria pigments are still scarce in the market and one of the biggest challenges is how to reduce cost in order to attract market interest.

Cyanobium sp. LEGE 06113 is a marine cyanobacterium that has been described as promising for biotechnological applications, due to the production of hierridin B and C which have antitumor and antimalarial properties (Leão et al. 2013; Freitas et al. 2016; Costa et al. 2019). However, the products described require large amounts of biomass and purification process. In order to overcome additional costs, it is expected to increase value for the biomass of this cyanobacterium in order to be applied in a large-scale perspective. Thus, as a photosynthetic organism, it is possible to take advantage of the pigments produced by this cyanobacterium for several industrial applications (Mandal et al. 2020). For that, a deeper study on the potential of *Cyanobium* sp. pigments was needed. Therefore, this work aims to exploit *Cyanobium* sp. biomass as source of pigments, its bioactive application as well as the optimization of solvent extraction and biomass reuse for successive extractions.

Material and methods

Cyanobacterium biomass

Cyanobium sp. LEGE 06113, obtained from Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC), was grown in batch mode in 5-L glass flat bottom round flasks at 25 °C BG11 medium (Allen 1968; Henrard et al. 2015). Light was provided by fluorescent lamps (Biolux, Osram) with an intensity of 200 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and a light:dark cycle of 16:8 h. Constant air flow was also assured at 0.75 $L_{air}\ L_{culture}^{-1}\ min^{-1}$. The biomass was harvested (ca. 1.0 g L^{-1}) by centrifugation after 25 days when the culture reached the stationary growth phase. The biomass was then freeze-dried and stored in low humidity (desiccator) in darkness, until further analyses.

Pigments-targeted extraction

In order to obtain pigment-rich extracts, single and successive extractions of *Cyanobium* sp. were performed using four solvents — acetone (A), ethyl acetate (EA), ethanol (E) and water (W).

Single extractions

The extractions were performed, in triplicate, using 250 mg of freeze-dried biomass and with a final solvent volume of 15 mL. To improve the extraction rate, cells were crushed using a bead beater Precellys Homogenizer (Bertin, France), using 3 cycles with 5 mL of solvent each (6 series of 8000 rpm for 30 s with 45 s of pause) and 670 mg of 0.1-mm beads to maximize cell disruption. Extracts were centrifuged at 2000×*g* for 10 min, and the supernatant was dried — A, EA, and E by a rotavapor system and W by freeze-drying. All extracts were stored in low humidity (desiccator), in the dark, until further analyses.

Successive extractions

The remaining biomass from the single extractions was used for the attainment of four successive extracts. An aqueous extract was obtained from the biomass previously used in the extractions with A, EA, and E (A-W, EA-W, and E-W), and an acetonic extract was obtained from the remaining biomass after W extraction (W-A). Extracts were centrifuged, and the supernatants were dried — acetonic extract by a rotavapor system and the aqueous extracts by freeze-drying. All extracts were stored in low humidity (desiccator) in the dark until further analyses.

Carotenoids characterization and quantification

The profile and quantification of carotenoids was determined for the organic extracts (A, E, EA and W-A) by high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection (Waters Alliance 2695, USA), following the method described elsewhere (Guedes et al. 2011). The extracts were resuspended in acetone:acetonitrile (9:1) in 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 in a 4 × 250 mm Purospher Star RP-18e (5 μm) column (Merck), the mobile phase in ethyl acetate (solvent A) and acetonitrile:water at 9:1 (v/v) (solvent B). An overall flow rate of 1 mL min^{-1} was set with a gradient installed with solvents A and B along the elution time: 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 data from all peaks were collected in the range 250 to 750 nm. The compounds were identified by comparing the retention times and the UV-visible spectrum with the authentic standards. Standards (HPLC grade) were purchased at Extrasynthese (lutein, zeaxanthin and β-carotene) and at Sigma (echinenone). The calibration curves, correlation coefficient (R^2), limit of detection (LOD = 3 × So/b), and limit of quantification (LOQ = 10 × So/b, where *So* is the standard deviation of signal-to-noise ratio and *b* is the calibration plot slope) for the analyzed carotenoids are shown in the Table 1. The results were expressed in mg of the respective carotenoid by grams of dry extract ($mg_C\ g_E^{-1}$).

Phycobiliproteins quantification

The phycobiliproteins quantification was performed in triplicates and calculated as described by Bennett and Bogorad (1973) using the following formulae: phycocyanin (PC) = $(A_{615} - (0.474 \times A_{652}))/5.34$; allophycocyanin (APC) = $(A_{652} - (0.208 \times A_{615}))/5.09$; phycoerythrin (PE) = $(A_{562} - 2.41 \times PC - 0.849 \times APC)/9.62$. Aqueous extracts (W, A-W, EA-W and E-W) were resuspended to a final concentration of 2 mg mL^{-1}. The results were expressed in mg of the respective phycobiliprotein by grams of dry extract ($mg_P\ g_E^{-1}$).

Total phenolic content quantification

Total soluble phenolic content of the extracts was performed in triplicates and determined by Folin-Ciocalteu method (Folin and Ciocalteu 1927) adapted by Maadane et al. (2015), using gallic acid as a standard. The results were expressed as milligrams of gallic acid equivalents (GAE) per grams of dry extract ($mg_{GAE}\ g_{extract}^{-1}$).

Antioxidant capacity assessment

The antioxidant capacity of each extract was accessed by $ABTS^{\bullet+}$, $O_2^{\bullet-}$ and $^{\bullet}NO$ scavenging assays (Pinho et al. 2011; Guedes et al. 2013). For all the assays, the extracts were resuspended in phosphate buffer (100 μM) with 20% of dimethyl sulfoxide (DMSO) (v/v) to a final extract concentration of 10 mg mL^{-1}. A series of dilutions of each extract were tested in order to determine the concentration able to scavenge 50% and 25% of the radicals (IC_{50} and IC_{25}). For the three assays, the IC values were calculated with GraphPad Prism software (version 7.0) through curve spline interpolation. All the assays were performed in chemical and biological triplicates. Final concentration range tested varied according the reaction volume of each assay (32–389 $\mu g_E\ mL^{-1}$ for $ABTS^{\bullet+}$, 42–333 $\mu g_E\ mL^{-1}$ for $O_2^{\bullet-}$ and 83–667 $\mu g_E\ mL^{-1}$ for $^{\bullet}NO$).

Anti-inflammatory capacity

The anti-inflammatory capacity of W-A and E-W extracts was assessed through an enzyme inhibitory assay — inhibition of COX-2 enzymatic activity, using the COX inhibitor screening assay kit (Cayman Chemical), according to the manufacturer's instructions. Dried extracts were resuspended in DMSO and diluted to a final concentration of 100 $\mu g_E\ mL^{-1}$.

Cytotoxicity evaluation

The human liver cell line HepG2 (American Type Culture Collection) was cultured in Dulbecco's modified Eagle

Table 1 Calibration curves of authentic standards used for carotenoids profiling

Standards	Calibration curve (mg L^{-1})	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Lutein	$y = 133{,}772{,}885x - 22{,}683$	0.999	0.882	2.675
Zeaxanthin	$y = 1{,}399{,}289{,}886x - 119{,}637$	0.998	0.315	0.954
β-carotene	$y = 290{,}230{,}023x + 172{,}819$	0.999	1.935	5.864
Echinenone	$y = 61{,}438{,}587x + 12{,}034$	0.999	0.507	1.538

LOD, limit of detection; *LOQ*, limit of quantification; *y*, peak area; *x*, concentration

medium (DMEM, Gibco) with glutamine, without pyruvate, supplemented with 10% (v/v) of fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin (penicillin 100 IU L^{-1}, Sstreptomycin 100 μL mL^{-1}), and 0.1% (v/v) of amphotericin B, at 37 °C, in a humidified atmosphere containing 5% CO_2. The culture medium was renewed every 2 days, and cell passages were made at about 80% confluence. Prior to extracts exposition, cells were seeded in 96-well plates, at a density of 3.3×10^4 cells mL^{-1} and incubated for 24 h. Extracts serial dilutions (46.9 to 750.0 μg mL^{-1}) were prepared in DMEM with 1% DMSO, established as the maximum DMSO concentration not interfering with the assay. The cytotoxicity of the extracts (W-A and E-W) was evaluated by measuring their effect on cells viability, through the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay is based in the conversion of MTT to formazan crystals by living cells, which can be quantified by optical density measurement at 515 nm, after formazan crystals dilution with DMSO (Lopes et al. 2012). The assay, performed in duplicate, was independently repeated four times. Cytotoxicity was expressed as a percentage of MTT reduction face to untreated control (negative control, with 1% DMSO and DMSO (20%) as positive control).

Statistical analysis

Experimental data were analyzed using GraphPad Prism V. 7.0. A Shapiro-Wilk test of normality was performed before the analysis of variance (ANOVA) to confirm the normal distribution of the residuals. A two-way ANOVA with Tukey's multi-comparison test was used to found differences between extraction yields, antioxidant capacity, carotenoids, phycobiliproteins, and phenolic content and cytotoxicity evaluation.

Results

Extraction yields

A total of eight extracts was obtained and the extracts named after the solvents used to obtain them: four organic extracts — A, EA, E and W-A; and four aqueous extracts — W, A-W, AE-W and E-W. The yield of each extract is depicted in Table 2.

The use of different solvents led to different yields. Among the organic solvents, the higher extraction yield was 26.98 ± 2.49% obtained with E, 3.5-fold higher than the EA extraction, which showed the lowest extraction yield. In what concerns the aqueous extractions, the higher yields were obtained with a single extraction of W and EA-W extractions attaining 47.04 ± 5.12 and 45.74 ± 1.66%, respectively, with no significant differences ($p > 0.05$). The single extraction of W extraction showed a yield 2-fold higher than the E-W successive extraction, which revealed to be the lowest yield among the aqueous extracts.

Table 2 Yield (average ± standard deviation, $n = 3$) of the solvent-assisted extractions (% of biomass) for the *Cyanobium* sp. Solvents: acetone (A), ethyl acetate (EA), ethanol (E) and water (W). Different lowercase letters in the same column show statistical differences ($p < 0.05$)

Organic		Aqueous		Total
Solvent	Yield (%)	Solvent	Yield (%)	Combined yield (%)
A	9.27 ± 1.27 [a]	A-W	36.03 ± 2.81 [b]	45.31 ± 1.84 [a]
EA	7.65 ± 0.10 [a]	EA-W	45.74 ± 1.66 [a]	54.40 ± 1.07 [b]
E	26.98 ± 2.49 [b]	E-W	23.59 ± 0.65 [c]	50.57 ± 2.5 [a, b]
W-A	10.32 ± 0.35 [a]	W	47.04 ± 5.12 [a]	55.94 ± 5.29 [b]

Extracts biochemical profile

The carotenoid profile and quantification were assessed for all the extracts obtained with organic solvents — A, E, EA and W-A, and the results are depicted in Fig. 1A. The carotenoid profile of these extracts was similar but with different content, containing echinenone, zeaxanthin, lutein, and β-carotene as the main compounds, and also unidentified derivatives of those. Among these extracts, A and W-A showed the highest content in total carotenoids, 4.37 ± 0.73 and 3.97 ± 0.94 mg_C g_E^{-1}, respectively, with no significant differences ($p > 0.05$). These two extracts contained c.a. 1.4-fold more carotenoids than the extract with the least carotenoid content, E. The concentration of the identified carotenoids was also higher in the A and W-A extracts with equal amounts of lutein (0.31 ± 0.03 and 0.30 ± 0.03 mg g_E^{-1}), zeaxanthin (1.06 ± 0.10 and 1.07 ± 0.09 mg g_E^{-1}) and β-carotene (1.40 ± 0.16 and 1.46 ± 0.25 mg g_E^{-1}). An exception for echinenone which was highly extracted with A, 1.12 ± 0.11 mg g_E^{-1}, 1.3-fold greater than the W-A.

The phycobiliprotein profile and quantification were assessed for the aqueous extracts — W, A-W, EA-W and E-W, and the results are summarized in Fig. 1B. All extracts showed to have phycocyanin and allophycocyanin in its composition, although in different concentrations, and phycoerythrin was not found in any extract. The E-W was the extract with higher content in total phycobiliproteins, 341.29 ± 4.72 mg_P g_E^{-1}, 2.0-fold more content than the one with lower concentration, EA-W. The content in specific phycobiliproteins was also higher in E-W, having 2.8-fold more phycocyanin and 1.9-fold more allophycocyanin than EA-W.

The total content in phenolic compounds was determined for all extracts, and the results are depicted in Fig. 1C and 1D. For the extracts performed with organic solvents (Fig. 1C), A showed the highest content, 83.1 ± 4.1 (mg_{GAE} g_E^{-1}), being

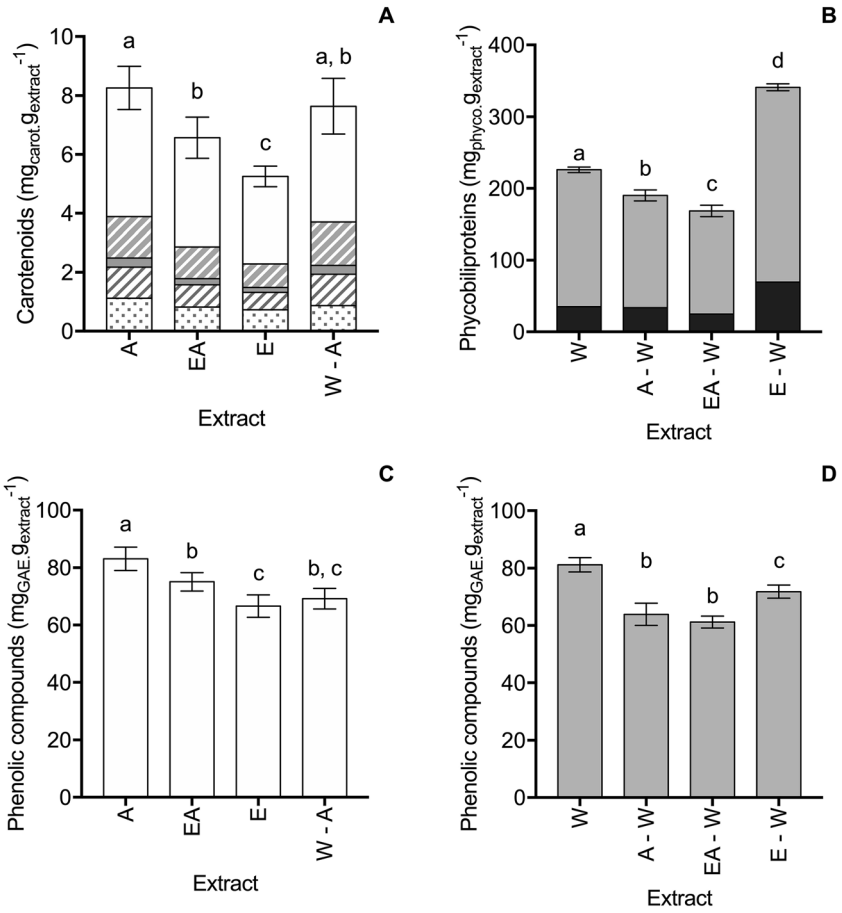

Fig. 1 Biochemical composition (mean ± standard deviation, $n = 3$) of *Cyanobium* sp. extracts, in terms of **A** total carotenoids ($mg_C\ g_E^{-1}$) — bar colors represent specific identified carotenoids: echinenone; zeaxanthin; lutein; β-carotene; and other carotenoids. **B** Total phycobiliproteins ($mg_P\ g_E^{-1}$) — bar colors represent specific identified phycobiliproteins: phycocyanin and allophycocyanin. **C** Total phenolic compounds in organic extracts ($mg_{GAE}\ g_E^{-1}$). **D** Total phenolic compounds ($mg_{GAE}\ g_E^{-1}$) in aqueous extracts. Solvents: acetone (A), ethyl acetate (EA), ethanol (E) and water (W). Different lowercase letters in the same graphic show statistical differences ($p < 0.05$)

1.2-fold higher than E and W-A, which were the ones with lower content in phenols, with no statistical differences between them ($p > 0.05$). For the aqueous extracts (Fig. 1D), W showed the highest content in phenolic compounds with 81.2 ± 2.5 ($mg_{GAE}\ g_E^{-1}$), showing a phenolic content 1.3-fold higher than the other extracts with lower phenolic content, A-W and EA-W, with no statistical differences between them ($p > 0.05$).

Antioxidant capacity of pigment-rich extracts

The antioxidant capacity of all extracts was assessed by three different assays — $ABTS^{\bullet+}$, $O_2^{\bullet-}$, and $^{\bullet}NO$ assay. In order to compare the antioxidant capacity of the extracts, the IC_{50} and IC_{25} were calculated for each assay. The IC values calculated for all extracts in the three assays are depicted in Table 3.

The assays revealed that all extracts have antioxidant capacity, although it varies between extracts and assays. In the $ABTS^{\bullet+}$ assay, among the organic extracts, W-A revealed to have the lower IC_{50} of 69.7 ± 4.1 $\mu g_E\ mL^{-1}$. The EA extract was the only one not reaching IC50 in the range of concentrations tested, being only possible to calculate an IC_{25} of 108.4 ± 4.2 $\mu g_E\ mL^{-1}$. All the aqueous extracts reached IC_{50}, and no significant differences were observed between them ($p > 0.05$), showing an overall IC_{50} average of 165.6 ± 19.7 $\mu g_E\ mL^{-1}$.

In the $^{\bullet}NO$ scavenging assay, it was only possible to calculate the IC_{50} of two organic extracts, EA and W-A. Between these two, W-A had a significant lower value of IC_{50}, 162.0 ± 25.9 $\mu g_E\ mL^{-1}$, being 1.6-fold better than EA. Regarding the other two extracts, E and A, it was not possible to calculate IC_{50} but only IC_{25}. E showed a significantly lower IC_{25} value (94.0 ± 10.4 $\mu g_E\ mL^{-1}$) when compared with A. All the aqueous extracts attained IC_{50} values, and particularly A-W, EA-W, and E-W extracts did not show any significative difference ($p > 0.05$), having an overall average of 159.7 ± 26.7 $\mu g_E\ mL^{-1}$, 1.9-fold better than W, which showed the highest IC_{50} value being significantly different from the others ($p < 0.05$).

In $O_2^{\bullet-}$ scavenging assay, it was not possible to calculate an IC_{50} for any extract, revealing that extracts do not hold a strong scavenging capacity against this radical. Among the organic extracts, it was only possible to calculate the IC_{25} for A at 199.6 ± 20.4 $\mu g_E\ mL^{-1}$. For aqueous extracts, the lowest IC_{25} calculated was 68.8 ± 7.3 $\mu g_E\ mL^{-1}$ for the A-W extract being significantly lower than the EA-W and E-W values, the W extract did not reach IC_{25}.

Among organic extracts, it is outstanding that W-A shows a stronger antioxidant capacity than A in the $ABTS^{\bullet+}$ and $^{\bullet}NO$

Table 3 IC_{50} and IC_{25} of all *Cyanobium* sp. extracts for $ABTS^{\bullet+}$, $^{\bullet}NO$, and $O_2^{\bullet-}$ assays (average ± standard deviation, $n = 3$). Solvents: acetone (A), ethyl acetate (EA), ethanol (E) and water (W). Different lowercase letters for organic extracts and capital letters for aqueous extracts show statistically significant differences ($p < 0.05$) for each method

Solvent	Inhibitory concentration ($\mu g_E\ mL^{-1}$)					
	$ABTS^{\bullet+}$		$^{\bullet}NO$		$O_2^{\bullet-}$	
	IC_{50}	IC_{25}	IC_{50}	IC_{25}	IC_{50}	IC_{25}
Organic						
A	297.0 ± 33.0 ^{a}	119.0 ± 9.9 ^{a}	-	180.7 ± 19.5 ^{a}	-	*199.6 ± 20.4*
EA	-	108.4 ± 4.2 ^{a}	336.7 ± 7.2 ^{a}	116.7 ± 15.9 ^{b}	-	-
E	139.2 ± 6.6 ^{b}	55.7 ± 1.5 ^{b}	-	*94.0 ± 10.4* ^{c}	-	-
W-A	*69.7 ± 4.1* ^{c}	*34.1 ± 1.0* ^{c}	*162.0 ± 25.9* ^{b}	-	-	-
Aqueous						
W	*172.1 ± 22.8* ^{A}	59.9 ± 6.7 $^{A, B}$	298.7 ± 4.9 ^{A}	*121.3 ± 16.9*	-	-
A-W	*176.6 ± 22.8* ^{A}	70.1 ± 1.4 $^{A, B}$	*164.5 ± 27.6* ^{B}	-	-	*68.8 ± 7.3* ^{A}
EA-W	*177.5 ± 29.8* ^{A}	71.9 ± 7.6 ^{A}	*130.7 ± 13.7* ^{B}	-	-	174.0 ± 4.8 ^{B}
E-W	*136.3 ± 16.0* ^{A}	*48.5 ± 8.2* ^{B}	*183.5 ± 16.3* ^{B}	-	-	106.1 ± 30.4 ^{C}

Italicized letters in IC values represent the highest value for the group of extracts

assays even though both extracts showed similar (and the highest) content in carotenoids and that A had more phenolic content than W-A.

Anti-inflammatory capacity

For the analysis of anti-inflammatory capacities, only the most promising extracts were evaluated—one carotenoid rich extract, W-A, and one phycobiliprotein rich extract, E-W. The first one was chosen since it had the best antioxidant capacity results in the $ABTS^{\bullet+}$ and $^{\bullet}NO$ assays (Table 3) and higher content in total carotenoids (Fig. 1A). The E-W extract was chosen due its high content in total phycobiliproteins (Fig. 1B) when compared to the other aqueous extracts, and also a high antioxidant capacity in the $ABTS^{\bullet+}$ and $^{\bullet}NO$ assays (Table 3). Results for the COX inhibitory assay are depicted in Fig. 2.

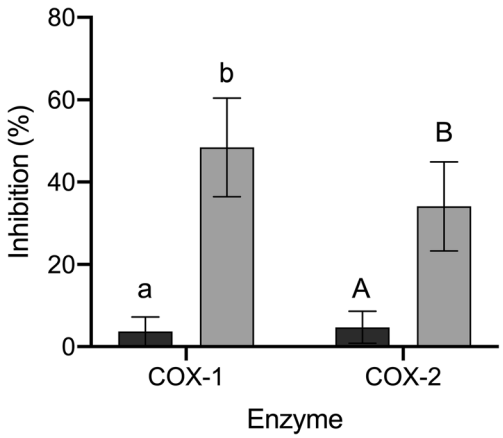

Fig. 2 COX inhibition activity (average ± standard deviation, $n = 3$) of *Cyanobium* sp. pigment-rich extracts — W-A and E-W. Solvents: acetone (A), ethyl acetate (EA), ethanol (E) and water (W). Different lowercase letters for COX-1 and uppercase letters for COX-2 mean significant difference between the extracts ($p < 0.05$)

Results show that E-W seems to inhibit both COX enzymes, while W-A showed a low or absent capacity.

Cytotoxicity of pigments-rich extracts

Considering a nutraceutical approach for the extracts analyzed herein, their cytotoxicity was evaluated in HepG2 cell line, and the results are shown in Fig. 3.

Three of the organic extracts (Fig. 3A, B and D) had cytotoxic effects, A and W-A extracts were cytotoxic in all tested concentration, since the cell viability percentage was significantly different from the control ($p < 0.05$), while EA showed a dose-dependent response, with toxicity only in the highest concentration. E did not have a cytotoxic effect on the HepG2 cell lines in none of the concentrations tested reaching a cell viability with no significative differences from the control ($p > 0.05$). In the case of aqueous extracts (Fig. 3E–H), none of the extracts showed a cytotoxic effect on the HepG2 cell lines for the range of concentration tested ($p > 0.05$).

Discussion

Taking in account the market for natural pigments being based in extracts (Panis and Carreon 2016), the present study evaluated different ways of extraction and therefore the effects on the bioactive potential of the strain. Also, it was possible to give an insight on the biotechnological potential of the extracts by characterizing them in terms of pigments and phenolics content and the bioactive potential in terms of antioxidant, and anti-inflammatory capacity, considering also the toxicity and safety of their use.

Regarding organic extracts and carotenoids, cyanobacteria in general are not the first option as source of such pigments, due to a low content of total carotenoids and yield of organic

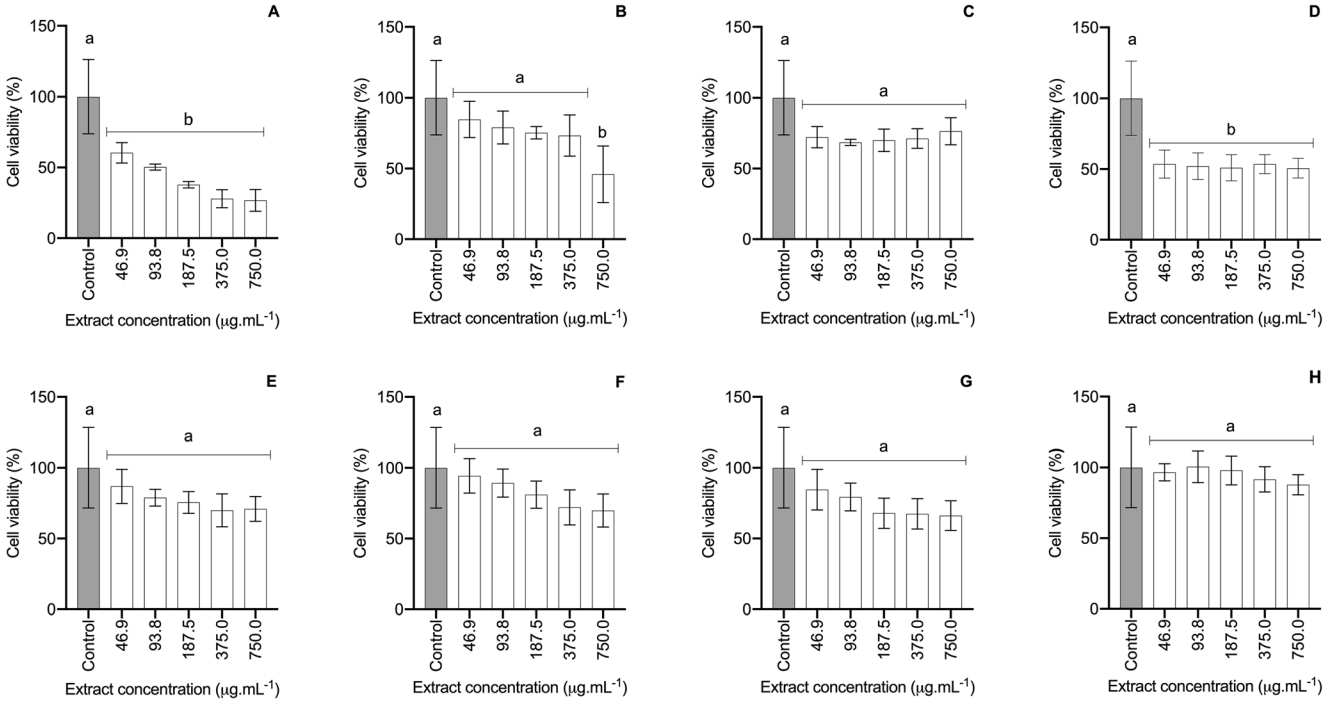

Fig. 3 Cytotoxicity (average ± standard deviation, $n = 3$) of *Cyanobium* sp. extracts to HepG2 cells. **A** A; **B** EA; **C** E; **D** W-A; **E** W; **F** A-W; **G** EA-W; and **H** A-W. Solvents: acetone (A), ethyl acetate (EA), ethanol (E) and water (W). Different lowercase letters in the same graphic means significant differences between the extract concentration and the control ($p < 0.05$)

extracts (Mandal et al. 2020). Here, the use of organic extracts for biotechnological applications is based on the possibility of co-products and an extent use of the biomass. Concerning the yield of organic extracts, the higher values were found using E as solvent. Similar results were reported by Giorgis et al. (2017), where the lowest yields were obtained using A as a solvent and the highest using E. The yield of extraction is dependent of the solvent used and evidences that polar solvents lead to higher yields have already been reported by Jerez-Martel et al. (2017).

In terms of carotenoids, it was verified that echinenone, zeaxanthin, lutein, and β-carotene represent the main carotenoids found in this strain, ca. 50% of total carotenoids. Also, it was observed that the profile did not change by using the tested solvents and only the amount extracted was affected. When it comes to the content, A extracts led to the highest content achieved, either as a single solvent extraction or after a water extraction. Similar results concerning the extraction of carotenoids were reported by Amaro et al., (2015) where the A extracts showed to be the richest in total carotenoids.

Overall, the yield of aqueous extracts was higher than the organic ones, probably due to the high content in aqueous soluble compounds in cyanobacteria cells, like phycobiliproteins — which is the main class of pigments in these organisms (Pagels et al. 2019). Among aqueous extracts, the highest extract yield was found in EA-W and W extracts. Regarding the content of phycobiliprotein, it is notorious that allophycocyanin is the main phycobiliprotein present in this strain and that no phycoerythrin was found. The high content in allophycocyanin also adds biotechnological potential to the extracts since this pigment, isolated from *A. platensis*, demonstrated antiviral activity (Shih et al. 2003).

Moreover, successive extractions seem to represent a good strategy to maximize the use of biomass. As the results demonstrate that the combined yield of the single extraction with the respective successive extraction can reach ca. 56% of the biomass, reducing the waste and increasing the valorization of the bioprocess associated to this cyanobacterium.

The searching for new sources of natural compounds has increased as the society demands a greener approach for health care market, being one of the aspects that increase their value the bioactive potential (Mandal et al. 2020). Cyanobacteria, and in special their pigments, appear as alternative for these needs. *Cyanobium* sp. extracts here exploited, overall, have potential to be used as antioxidant and anti-inflammatory. Moreover, the absent cytotoxicity in high concentrations of most all extracts reveals that these extracts have a potential safe profile and are worth of further exploitation regarding their application in the field of nutraceuticals.

The finding regarding antioxidant capacity suggests that *Cyanobium* sp. pigment-rich extracts can be use as source of antioxidant compounds, in special the W-A extract, and it was clear that the W extraction prior to the acetonic one increased the antioxidant capacity of the extract, i.e., the first W extraction might have concentrated lipophilic compounds such as carotenoids into the biomass after the first

extraction, increasing the efficiency of the A extraction and thereafter, leading to a higher antioxidant capacity of the extract. Results obtained by Trivedi et al. (2016) showed significant increases on the extraction efficiency when using successive extractions compared to singular ones. Furthermore, *Cyanobium* sp. extracts are a complex mixture of compounds and its antioxidant activity cannot be correlated to the content of a single group of compounds such as carotenoids. Synergistic or antagonistic interactions between different molecules should be considered. Therefore, the high content of known antioxidant compounds, like phenolics or carotenoids, in a crude extract like A do not necessarily imply a higher antioxidant activity, when compared to other extracts (Amaro et al. 2015; Babić et al. 2016).

Although the differences in the antioxidant capacity being less accentuated than in the organic extracts, some similar trends can be found among aqueous extracts. Again, the increase on the antioxidant capacity seems to be more related to the use of successive extraction and consequently to possible concentration of hydrophilic compounds such as phycobiliproteins into the remaining biomass. This can be observed in the W extract; although having a high content in phycobiliproteins and phenolic compounds, it was the extract with less $^{\bullet}NO$ scavenging capacity and it did not show $O_2^{\bullet-}$ scavenging capacity. Also, the $^{\bullet}NO$ and $O_2^{\bullet-}$ scavenging capacity increased in all aqueous successive extractions. Moreover, despite the phycobiliprotein content and the phenolic content being the highest and second highest, respectively, E-W extract exhibited a similar antioxidant capacity to the other aqueous extracts.

Comparing the antioxidant capacity with other cyanobacteria and microalgae studies, the IC_{50} values obtained in $ABTS^{\bullet+}$ assay revealed to be more promising than the ones reported for the cyanobacterium *Chroococcus minutus* and green microalgae *Chlorella saccharophila* (Priya and Murugesan 2014) and in the same range of the ones reported for the cyanobacterium *Gloeothece* sp. and green microalgae *Scenedesmus obliquus* (M2–1) (Amaro et al. 2015). In the same way, the IC_{25} values obtained in the $O_2^{\bullet-}$ assay with *Cyanobium* sp. extracts showed to be more promising than those obtained with *Scenedesmus obliquus* (M2–1) extracts (Amaro et al. 2015).

Additionally, when it comes to the anti-inflammatory potential of the extracts, only E-W and W-A extracts were evaluated as the most promising extracts from their content, being the E-W extract the one able to inhibit both enzymes COX 1 and 2. COX enzymes are responsible for producing prostaglandins and to induce responses to inflammatory stimuli, and the inhibition of these enzymes can reduce the symptoms as pain and fever (Ferrer et al. 2019).

Phycobiliproteins extracts have been previously reported for their anti-inflammatory properties (Leung et al. 2013; Pagels et al. 2019), which supports the present results. Thus, the potential for anti-inflammatory application goes beyond the inhibition of COX enzymes, as other mechanisms of inflammation exist. As referred in this study, W-A had the best results for the $^{\bullet}NO$ antioxidant assay. $^{\bullet}NO$ is part of the inflammatory pathway and its scavenging is a primarily indicator for a nutraceutical applicability. Lopes et al. (2020) showed the application of carotenoids-rich acetonic extracts from cyanobacteria as anti-inflammatory components by reducing $^{\bullet}NO$ formation in RAW 264.7 macrophages.

Finally, besides presenting important biological activities with biotechnological potential, for instance, in the industry of nutraceuticals, bioactive extracts should also be screened for their cytotoxicity. All pigment-target extracts from *Cyanobium* sp. were submitted to cytotoxicity analysis, using the human liver cell line HepG2. The liver is a primordial organ regarding drug metabolism and detoxification. It is the first organ perfused by drugs after gut absorption and contains a significantly amount of specialized enzymatic systems, with a crucial importance in xenobiotic metabolism. The cytotoxicity results are important to indicate a possible biotechnological use of the extracts, as E, W, A-W, E-W, and EA-W extracts showed no cytotoxicity in the tested concentrations (up to 750 $\mu g_E\ mL^{-1}$). Thus, the toxicity of some of the organic extracts indicates that in order to use these extracts, further purification is needed.

Conclusion

Cyanobium sp. LEGE 06113 is here proposed as a source of pigments and bioactive extracts. Results showed that phycobiliproteins, especially allophycocyanin, were the main pigments present in this strain, but the profile of carotenoids also seems promising, as echinenone, lutein, zeaxanthin, and β-carotene are present. Moreover, the strategy of using successive extractions can contribute to a better use to the biomass and increase the biotechnological value of the strain. Furthermore, it was observed that *Cyanobium* sp. can be used as source of bioactive compounds, as it was possible to obtain non-cytotoxic extracts with antioxidant and anti-inflammatory capacity, in special E-W extract.

Authors' contributions FP, DS, HMA, and ACG conceived and designed research. FP and DS conducted experiments. FP, DS, GL, and HMA performed analytical measurements. FP and DS wrote the manuscript. ISP, VV, and ACG were responsible for the supervision and funding acquirement. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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