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Pigments profile and antioxidant potential of extremophile cyanobacteria isolated from the Mexican Volcanic Lake Chichonal

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ABSTRACT

Cyanobacteria are ancient and prolific microorganisms for which the current knowledge remains limited. The present work constitutes the first report on the presence of the cyanobacteria *Tolypothrix* sp. LEGE 221228, *Nostoc* sp. LEGE 221229, *Scytonema* sp. LEGE 221230 and *Drouetiella* sp. LEGE 221231 in the volcanic crater lake in Chichonal (Chiapas-Mexico), and in the characterization of their pigments profile and antioxidant potential, through the application of biorefinery approaches. The identification of cyanobacteria species was achieved through advanced morphology-based methods for cyanobacterial taxonomy. The HPLC-PDA analysis of the cyanobacteria acetone extracts revealed β-carotene, echinenone and chlorophyll-*a* as the main pigments. *Drouetiella* sp. LEGE 221231 presented the highest β-carotene concentration (63.67 μg/mL), total carotenoids (87.92 μg/mL) and chlorophylls (20.88 μg/mL), closely followed by *Tolypothrix* sp. LEGE 221228 (with 51.37, 75.14 and 14.88 μg/mL, respectively), this being the first report on the carotenoid and chlorophylls profile of these strains. Aqueous extracts revealed the highest content of phycoerythrin in *Tolypothrix* sp. LEGE 221228 (50.61 mg/g) and of phycocyanin in *Nostoc* sp. LEGE 221229 (57.66 mg/g), standing out in superoxide anion radical scavenging, while the acetonic ones were more effective in scavenging nitric oxide radical. For both, *Tolypothrix* sp. LEGE 221228 was the most effective, presenting the lowest IC_{50} (53.75 and 57.68 μg/mL, respectively). Overall, the present study enriches the knowledge on biodiversity of cyanobacteria from under-explored environments, enhancing their potential biotechnological applications as producers of added value secondary metabolites with interest in food, pharmaceutical and cosmetic industries.

1. Introduction

Cyanobacteria are one of the principal oxygen producers of the planet, however, there is still a lack of knowledge on the species that can be found in certain areas of the world. Extreme environments are one of them, often being dominated by large colonies of cyanobacteria [1,2]. As a diverse group of photosynthetic prokaryotes, these organisms are adapted to extreme temperatures and pH, and tolerate high salt concentrations, being also found in salt flats and hypersaline lakes. Not only, cyanobacteria are resilient to high levels of ultraviolet (UV) radiation, and can adapt to high pressure, low oxygen, and high concentrations of minerals. They are often found in nitrogen-poor soils and have the ability to fix atmospheric nitrogen, making them important contributors to nitrogen cycling in these ecosystems [3]. These prolific microorganisms can also be used to monitor aquatic systems, due to the toxins produced by some species. For instance, along Mexico territory,

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water from lakes or rivers is commonly used to irrigate the soils, for agriculture practices and also for animals consumption [4]. However, there's a lack of studies regarding the monitoring of the presence of cyanobacteria toxins, as well as the identification of the species responsible for their production, which can constitute an environmental and health issue [5].

Beyond aquatic systems monitoring, cyanobacteria hold a significant economic potential, particularly in the field of biotechnology, with clinical, pharmaceutical, cosmetic, and textile applications, due to their ability to produce a wide variety of secondary metabolites, in which pigments are included [6,7]. Notably, the pigments produced by cyanobacteria have unique characteristics, making them an appealing alternative to synthetic compounds, namely their non-toxic nature, biocompatibility, cost-effective extraction methods, high relative abundance, and ability to be sustainably produced [8]. Among the pigments most commonly produced by cyanobacteria are chlorophylls, carotenoids, and the phycobiliproteins (PBPs) including phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC). Chlorophylls-*a* and *b* predominate in cyanobacteria, being at the basis of the formation of the diverse range of greens [9]. Along with chlorophylls, carotenoids are among the most abundant pigments in nature, serving various functions like extending the range of light absorption of photosynthetic organisms such as algae and cyanobacteria, to protect them against photo-oxidative stress, by quenching excited molecules and reactive oxygen (ROS) and nitrogen species (RNS) [10]. These tetraterpenoids are categorized into different groups based on the presence or absence of oxygen in their molecules. The non-oxygenated derivatives are referred to as carotenes, and the oxygenated ones as xanthophylls, with hydrophilic properties due to the presence of hydroxyl and keto groups at the end of the rings [11]. The commercial value of carotenoids is given by their antioxidant properties, vibrant colors, and health-promoting attributes, among others. Among the wide variety of carotenoids produced by cyanobacteria, β-carotene and its isoforms take particular significance, due to their pro-vitamin A activity and strong antioxidant capacity [12]. Regarding PBPs, they consist of colored tetrapyrrole billiproteins absorbing visible radiation whereas chlorophyll exhibits low absorption. These pigments are organized in specific arrangements called phycobilisomes [7], being categorized by their absorption spectra into PE, PC and APC, with absorption maxima of 565 nm, 620 nm, and 650 nm, respectively. PC has applications in various domains, such as food additives, healthy food, cosmetics and pharmaceuticals [13], due to its well-known anti-inflammatory, anti-cancer and anti-oxidant properties.

Molecules with antioxidant properties constitute one of the first lines of defense against biological damage, their exploitation from natural sources with sustainable production being subject of a growing interest by the scientific community. Oxidative stress can be defined as an imbalance between pro-oxidants and antioxidants, accompanied by the dysregulation of redox circuits and resultant macromolecular damage [14]. Its establishment is highly correlated with and excessive production and deficient depuration of reactive species, produced as intermediates or by products of cellular metabolism, catalyzed by enzymes located within various organelles, such as plasma membrane, cytosol, mitochondria, peroxisomes, and endoplasmic reticulum [15]. Among them, superoxide anion ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), and nitric oxide ($^{\bullet}NO$) radicals, as well as hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO), can be highlighted as the main contributors to the establishment of oxidative state, leading to cell and tissue damage, and playing an important role in the onset and worsening of several metabolic disorders and chronic diseases [16–18].

The present work constitutes the first report on the presence of the cyanobacteria *Tolypothrix* sp. LEGE 221228, *Nostoc* sp. LEGE 221229, *Scytonema* sp. LEGE 221230 and *Drouetiella* sp. LEGE 221231 in the volcanic crater lake in Chichonal (Chiapas-Mexico), and in the characterization of their pigments profile and antioxidant potential against relevant physiological free radicals, in a biorefinery approach where a single biomass is subjected to sequential extraction steps.

2. Materials and methods

2.1. Sampling, cyanobacteria isolation and culture conditions

Biological samples were collected from the crater lake at El Chichón volcano, which is situated in the Chiapanecan Volcanic Arc, in south-eastern Mexico and north-western Chiapas. Its coordinates are 17.36° N, 93.23° W, and it has a maximum elevation of 1100 m above sea level (MASL). The lake is 160 m deep, and the region receives an average annual rainfall of 4000 mm [19]. Biofilms were scraped from rocks and sandy soil near the lake around the volcano area, in freshwater, marine, brackish and terrestrial habitats, on April 2022, and the samples were assigned with the codes MEX19C, MEX18A, MEX13B and MEX14A. The samples were placed in falcon tubes with Z8 media [20] and brought to the Blue Biotechnology Ecotoxicology and Health (BBEH) group of CIIMAR for strains isolation. In laboratory, the 4 environmental samples were transferred to 40 mL plastic flasks with Z8 medium, under aseptic conditions, to enrich the cultures and allow the diversity of microorganisms to appear, and maintained at 25 °C with a light/dark cycle of 14/10 h and a light intensity of 10–30 μmol photons m^{-2} s^{-1}. After a period of 3 to 4 weeks, the first microphotographs were taken, using a Leica DMLB light microscope coupled to a Leica ICC50 HD digital camera (Leica Microsystems, Germany), and the diversity of cyanobacteria present in the environmental samples was recorded. Two main strategies were adopted to isolate the cyanobacterial strains: streak plate method [21] using solid Z8 medium with agar and cycloheximide to avoid the microalgae present, and the separation of individual trichomes by micromanipulation [22]. After 15–20 days, biomass was harvested for DNA extraction.

2.2. Morphological characterization and identification

Morphological analyses of the cyanobacterial isolates were performed during the exponential phase of growth (2 to 3-week-old cultures), using a Leica DMLB light microscope coupled to a Leica ICC50 HD digital camera (Leica Microsystems, Germany). Morphometric measurements were then performed using the image analysis software Leica Application Suite version 4.2.0 (Leica Microsystems), at 40× and 100× magnification. Microphotographs of each isolate were recorded and analyzed by the LEICA LAS AF version 1.8.0 image analysis software. The morphological identification was performed according to the classical taxonomy of Komárek, J., & Anagnostidis, K. [23] and Komarek, J. [24]. The shape and size of vegetative cells, the presence and position of heterocysts and akinetes, the color and chromatic adaptation capability (CCA) of the isolates, the number of trichomes per filament, the presence or absence of sheaths, the constrictions at the cross-wall and the necridic cells were registered. Measurements were carried out for three-week old cultures and each measurable character of an isolate was measured 20 times, in different positions of the slide preparation.

2.3. DNA extraction, amplification (PCR) and sequencing

Four cyanobacteria strains were characterized for the first time. After 15–20 days of cultivation, Genomic DNA was extracted using the Genomic DNA Mini Kit (Invitrogen, USA), according to the manufacturer's instructions for Gram-negative bacteria. The identification of the strains was performed by 16S rRNA sequencing, PCR amplification was performed using the oligonucleotide primers set 27F [25], CYA-781R [26], CYA-359F [26], 1494R [25] and 23S30R [26]. PCR reactions were performed in a final volume of 20 μL containing 1× Green GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 125.0 mM of each deoxynucleotide triphosphate, 1.0 μM of each primer, 0.5 U of GoTaq Flexi DNA Polymerase (Promega, USA), 10 mg/mL of bovine serum albumin (BSA), and

10–30 ng of template DNA, on a Veriti Dx Thermal Cycler (Invitrogen, USA). The PCR conditions were the following: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. The PCR reactions were performed in duplicate. PCR products were separated by 1.5 % agarose gel stained with SYBR® safe (Invitrogen, USA) and DNA fragments with the expected size were excised and purified using NZYGelpure (NzyTech, Genes and Enzymes, Lisbon, Portugal), according to the manufacturer's instructions. Since the sequences were obtained by direct sequencing of purified amplicons, internal primers CYA359F, CYA781R and 1494R were used to improve the quality of sequences. The sequencing was performed at GATC Biotech (Germany) and the nucleotide sequences obtained were manually inspected for quality and assembled using the Geneious 11.1.5 software (Biomatters Ltd., New Zealand). The obtained consensus sequences were submitted to the GenBank database of the National Center for Biotechnology Information (NCBI) (GenBank (http://www.ncbi.nlm.nih.gov) for alignment employing the Basic Local Alignment Search Tool (BLASTn) algorithm of nucleotides in the 16 S rRNA sequences database to determine their phylogenetic affiliations.

2.4. Phylogenetic analysis

A total of 222 sequences were used in the final analysis, including: two strains of *Gloeobacter violaceus* PCC 7421 and *Gloeobacter kilaueensis* JS1 as an outgroup, 220 sequences of cyanobacteria, including type, reference and related strains belonging to the orders Nostocales, Oculatellales, Pseudanabaenales, Gomontiellales and Chroococcidiopsidales, retrieved from GenBank (National Center for Biotechnology Information, NCBI, Bethesda, MD, USA), and four sequences of isolates obtained in this work. Multiple sequence alignment was carried out using ClustalW in MEGA7 [27,28] and sequences were manually proofread and edited. The best fit model was assessed using jModelTest 2.1.10 [29] according to the Bayesian information criterion and Akaike information criterion scores. Maximum likelihood analysis was carried out using substitution model GTR + G + I with 1000 bootstrap resampling replicates using the MEGA7 software [28]. The final phylogenetic tree was visualized and edited on Interactive Tree of Life [30].

2.5. Cultivation of biomass

The strains were cultured in Z8 medium [20] at 25 °C, with a light intensity of 10–30 μmol photons m^{-2} s^{-1} and a photoperiod of 14 h of light and 10 h of darkness, and followed a scale-up process of 10-fold increase, from 40 mL to 4 L. Two stages of scale-up were conducted. The first stage increased the volume from 40 to 400 mL, and the second stage from 400 mL to 4 L, the strains being maintained with constant aeration until collection. The strains were harvested by filtration. The resulting biomass was frozen and freeze-dried (Telstar LyoQuest) under reduced pressure (0.1 mbar with the condenser at −47 °C) and stored at −20 °C prior to extracts preparation.

2.6. Extracts preparation

Two distinct types of extracts were sequentially obtained using solvents of different polarities: acetone and water. Initially, the acetonic extract was prepared by suspending 1 g of dried biomass in 25 mL of acetone. To help release the cellular content, cell walls were disrupted using an ultrasonic bath (Fisherbrand®-FB15053) during 5 min, maintaining the temperature controlled (under 30 °C) to prevent degradation of bioactive compounds. The mixture was then filtered by cotton, and the extraction procedure was repeated 4 times. The filtrate resulting from the extractions was combined and evaporated under reduced pressure using a rotavapor (BUCHI R-210 Rotary Evaporator). Subsequently, the extract was transferred to weighted vials, which were stored at −20 °C for subsequent chemical and biological analysis.

The leftover biomass resulting from the acetonic extraction was left to completely dry overnight (in a hotte), before aqueous extracts preparation. The aqueous extract was prepared by suspending the dried biomass in 25 mL of distilled water. The same methodology was applied, with controlled temperature, to prevent bioactive compounds degradation. The mixture was filtered, and the process was repeated 4 times. The extract was transferred to falcons and centrifuged at 10000 G for 5 min at 4 °C (Thermo ScientificTM HERAUS MegafugeTM 16R). The resulting solution was frozen and lyophilized. After completely drying, the acetone and aqueous extracts were weighed, and the extraction yield was calculated. The resulting extracts were stored at −20 °C until further analysis.

2.7. Chemical profile

2.7.1. Carotenoid and chlorophylls

Acetone extracts were dissolved in HPLC-grade methanol to a final concentration of 5 mg/mL, and filtered through a 0.22 μm pore membrane. Carotenoid analysis was conducted using a Waters Alliance 2695 high-performance liquid chromatography (HPLC) system with a photodiode array (PDA) detector (USA) for resolution, detection, and identification of compounds of interest. The stationary phase was a YMC Carotenoid C30 (250 × 4.6 mm; 5 μm) column, kept at constant temperature (25 °C) with a column heater (Waters Corporation, Milford, CT, USA). The mobile phase consisted of two solvents: methanol (A) and tert-butyl methyl ether (B) (VWR Prolabo), starting with 95 % A and initiating a gradient to achieve 10 % B at 5 min, 18 % B at 20 min, 30 % B at 28 min, 50 % B from 31 to 37 min, 80 % B from 38 to 47 min, and 5 % B from 48 to 50 min. The flow rate was 0.90 mL/min, and the injection volume was 5 μL. Data were processed using Empower chromatography software (Waters, USA) [31].

Spectral data from all peaks were collected in the range of 250 to 750 nm. Compounds were identified by comparing their retention times and UV–Vis spectra with those of authentic standards. Carotenoids quantification was achieved by measuring the absorbance recorded in the chromatograms relative to external standards at 450 nm. Canthaxanthin, chlorophyll-*a*, echinenone, lutein, and β-carotene were quantified using authentic standards. Unidentified carotenoids were quantified as β-carotene and chlorophyll derivatives as chlorophyll-*a*. Calibration curves were constructed with five different concentrations of standards, chosen to represent the range of compounds concentrations in the samples.

2.7.2. Phycobiliproteins

The PBPs present in the aqueous extracts were determined spectrophotometrically. Aqueous extracts were resuspended in water to a final concentration of 0.5 mg/mL. PBPs were determined by measuring the absorbances at different wavelengths, in a 1 cm pathlength cell, and using the corresponding formulas, as previously described [32]:

$$\text{Phycoerythrin (PE)} = \frac{\text{A562nm} - 2.41 \times \text{PC} - 0.849 \times \text{APC}}{9.62}$$

$$\text{Phycocyanin (PC)} = \frac{\text{A615nm} - 0.474 \times \text{A652nm}}{5.34}$$

$$\text{Allophycocyanin (APC)} = \frac{\text{A652nm} - 0.208 \times \text{A615nm}}{5.09}$$

The experiment was carried out in duplicate, and the results were expressed in μg of the respective phycobiliprotein per mg of dry extract.

2.8. Evaluation of the antioxidant potential

2.8.1. Superoxide anion radical scavenging

The ability of cyanobacteria extracts to scavenge the $O_2^{\bullet-}$ was

performed according to Barbosa and co-workers [33]. The aqueous extracts were dissolved in water, while the acetone extracts were prepared in DMSO. The $O_2^{\bullet-}$ scavenging ability of the different extracts was determined using five serial dilutions, prepared for each cyanobacteria extract (from 0.078 mg/mL to 1.25 mg/mL). Briefly, an aliquot of 50 μL of each dilution was transferred to a 96 wells plate and mixed with 50 μL of 166 μM nicotinamide adenine dinucleotide (reduced form NADH) solution and 150 μL of 43 μM nitrotetrazolium blue chloride (NBT). After adding 50 μL of phenazine methosulphate (PMS) 2.7 μM, the radical scavenging potential of the samples was determined by monitoring their effect in the reduction rate of NBT, using a Synergy HT Multidetector Microplate Reader operated by GEN5TM (Biotek, BadFriedrichshall, Germany) in kinetic function, at room temperature, for 2 min, at 562 nm. All reagents were dissolved in phosphate buffer (19 mM, pH 7.4). Results were expressed as the percentage of $O_2^{\bullet-}$ scavenging face to the untreated control. Gallic acid was used as positive control. The results for the calculated IC values were expressed as mean ± SD (μg/mL) of at least three independent assays performed in duplicate. The IC values and the corresponding dose-response curves were calculated with GraphPad Prism® software (version 9.2 for Windows).

2.8.2. Nitric oxide radical scavenging

The $^{\bullet}NO$ scavenging activity was performed following the protocol described by Lopes et al., [34], with minor modifications. Five serial dilutions were prepared for each cyanobacteria extract (from 0.078 mg/mL to 0.25 mg/mL). An aliquot of 75 μL of each serial dilution was transferred to a 96-well plate and mixed with 75 μL of SNP (sodium pentacyanonitrosylferrate) 2 % for aqueous extracts and 2.5 % for acetonic extracts, dissolved in phosphate buffer (KH_2PO_4). The mixture was incubated for 60 min at room temperature and under light effect. Then, 75 μL of H_3PO_4 2 % was added to the blanks, while 75 μL of Griess reagent (dissolved in H_3PO_4 buffer 2 %) was added to the remaining wells. The plate was incubated for 5 min at room temperature, in the dark, and the absorbance was read using a Synergy HT Multidetection Microplate Reader operated by GEN5TM ((Biotek, BadFriedrichshall, Germany), at 562 nm. Gallic acid was used as a positive control. Results were expressed as % of $^{\bullet}NO$ scavenging in comparison to the untreated control. The results for the calculated IC values were expressed as mean ± SD (μg/mL) of at least three independent assays performed in duplicate. The IC values and the corresponding dose–response curves were calculated with GraphPad Prism® software (version 9.2 for Windows).

2.9. Assessment of the anti-hyperpigmentation potential

The tyrosinase inhibition assay was performed according to Adhikari et al. [35], with slight modifications, to evaluate the anti-hyperpigmentation potential of the cyanobacteria extracts. Five serial dilutions, from 0.063 to 10 mg/mL, were prepared for each extract. Briefly, in a 96-well plate, 10 μL of each extract were mixed with 90 μL of buffer, and 50 μL of enzyme (50 UI/mL in phosphate buffer). Acetone extracts and aqueous extracts were resuspended in DMSO and water, respectively. The mixture was incubated at room temperature during 5 min. After that, 50 μL of L-DOPA (L-3,4-dihydroxyphenylalanine) solution (2.5 mM in phosphate buffer) was added, and the absorption was recorded in kinetic mode in a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall, Germany) operated by GEN5TM software, at 475 nm. After 10 min, the reaction rate was recorded. Negative control was performed in the absence of extract, and kojic acid was used as a positive control. Three independent assays were performed in duplicate. The results were expressed as the percentage of enzyme inhibition in comparison to the untreated control. The results for the calculated IC values were expressed as mean ± SD (μg/mL) of at least three independent assays performed in duplicate. The IC values and the corresponding dose-response curves were calculated with GraphPad Prism® software (version 9.2 for Windows).

2.10. Statistical analysis

Statistical analysis was performed using IBM® SPSS® STATISTICS software, version 27.01.0, IBM Corporation, New York, NY, USA (2015). Data were analyzed for normality and homogeneity of variances using Kolmogorov–Smirnov and Leven's tests, and submitted to one-way ANOVA, using Tukey's HSD (honest significant difference) as a post hoc test. A Pearson correlation was conducted to compare normalized expression data between the chemical profile and the biological activities of cyanobacteria extracts.

3. Results and discussion

The formation of a crater lake in April 1982 marked the beginning of a long-term study of lake properties. Initially, three small lakes were observed, which eventually merged into one by November of the same year [36]. Various changes in the Lakes' physical and geochemical properties have been observed over time. The pH levels of the water have fluctuated significantly since its formation, with a range from an extremely acidic value of 0.56 in January 1983 to 2.57 in January 2003, 2.32 in January 2009, and 2.54 in April 2014, as reported by Armienta and co-workers [37]. Similarly, the water temperature has varied from 52 to 58 °C in January 1983 to 31–35 °C in January 2003, 29.6–42.9 °C in January 2009, and 39–44.2 °C in April 2014, with the lowest temperature range (20.1–28.8 °C) recorded in October 2010. The water volume of the lake has also undergone noticeable changes, ranging from 3×10^4 m^3 to 130.6×10^4 m^3 [38–40]. The geochemical composition of the lakes' water also exhibited significant variations in the concentration of key ions over time, especially SO_4^{2-} and Cl^-. These changes were attributed to the influence of near-neutral geyser-like springs into the lake water, the input of hydrothermal waters and H_2S-rich gases, and the effects of tectonic and meteorological factors [37,41,42]. Additionally, the physicochemical characteristics of the fluids emanating from El Chichón are contingent upon the prevailing hydrogeological conditions within the system, which are susceptible to alterations induced by volcanic phenomena. These unique characteristics increase the interest in discovering and exploring life forms capable of colonizing this environment.

3.1. Morphological and phylogenetic analysis

From the four samples collected around the underexplored location of the Chichonal volcano area (Fig. 1), and assigned with the codes MEX19C, MEX18A, MEX13B and MEX14A, we could isolate four cyanobacterial strains, which are detailed in Table 1.

The morphological analysis allowed the strain MEX19C to be identified as *Drouetiella* Mai (Oculatellales), and revealed that the strains MEX18A, MEX13B and MEX14A were similar to *Scytonema* Bornet & Flahault, *Tolypothrix* Bornet & Flahault and *Nostoc* Bornet et Flahault, respectively (Fig. 2 and Table 1).

The 16S rRNA gene ML phylogeny (Fig. 3) confirmed the identification of MEX019C as *Drouetiella*, in the Oculatellales cluster. The strain MEX013B is positioned among *Tolypothrix* strains [43], it is possible to assign the strain to this genus. The other strains are positioned in the order Nostocales, but in unidentified clades. The strain MEX14A is close related to *"Nostoc"* strains, which are phylogenetically distant to the type species *Nostoc commune* Bornet & Flahault. Finally, the strain MEX018B is within a clade composed by "*Scytonema*" [43] strains, which are distant to the type *S. hofmanni*, and neither can be assigned to this genus. Considering that, these two latter strains must be described as new genera in further taxonomical studies and in this paper, we will consider them as unidentified Nostocales.

Cyanobacteria taxonomy classification is in constant evolution, beginning with the Komarek et al. [24] with the classification system directed to higher levels of orders and family's traditional taxa, combined with the polyphasic approach. Recently, in 2023, an updated and

Fig. 1. Map showing the sampling site where the environmental samples were collected.

Table 1
List of the cyanobacteria strains selected for this study, their morphological features and information about the sampling location.

User Code	LEGE Code	Morphological features	Strain identification	Measures (WxL or diameter)[a]		Habitat | Sample description	Environment
				Vegetative Cell	Heterocysts and Akinetes		
Mex 13B	LEGE 221228	Trichomes cylindrical and slightly attenuated towards the ends; No ramifications and no visible sheaths; Olive green filaments;	*Tolypothrix* sp.	(4.2 ± 0.6 × 5.0 ± 0.6)	Absent	Ferrous river next to camp; Sand wall with black spots	Subaerial
Mex 14A	LEGE 221229	Trichomes distinctly constricted at cross-walls; cylindrical vegetative cells; no ramification and no visible heterocyst; colorless sheaths with mucilage; blue-green filaments;	*Nostoc* sp.	(4.9 ± 0.5 × 6.3 ± 0.9)	Absent	Ferrous river next to camp; Green mat on sand	Subaerial
Mex 18A	LEGE 221230	Trichomes constricted at cross-walls; ramifications after trichome disintegration; rectangular cells; rounded apical cell; Sheaths firm; With barrel-shaped heterocysts; Blue-green filaments;	*Scytonema* sp.	(10.8 ± 0.8 × 7.1 ± 1.5)	Present (11.3 ± 1.2 × 8.8 ± 0.7)	Road outside camping; Black mat on bricks	Subaerial
Mex 19C	LEGE 221231	Trichomes slightly constricted at the cross-walls; squared cells with different sizes; cylindrical apical cells; Sheath clear and thin; Blue-green filaments;	*Drouetiella* sp.	(2.4 ± 0.2 × 1.9 ± 0.3)	Absent	Road outside camping; Black mat on tree	Subaerial

[a] W: width; L: length; Measures in μm.

more robust taxonomic system with new orders, families and intermixed groups merging some of the existing order was published [44].

After the amplification of the 16S rRNA gene with the specific primers listed on Table 1, on the materials and methods section, we obtain fragments between 1000/1300 bp, which is expected for the 16S rRNA gene amplification in cyanobacteria. The comparison with the database available helped mostly for genus-level identification and provided enough of a hint for the further identification of cyanobacteria based on modern morphology-based system for cyanobacterial taxonomy. The four isolated strains from Mexico were classified according to this new taxonomic revision, shown in the phylogenetic tree, and deposited in the BBEH Culture Collection – LEGE-CC of CIIMAR with the respective codes (Table 2, Fig. 3).

3.2. Pigments characterization

Beyond the morphological and phylogenetic analysis of the extremophile cyanobacteria strains isolated from the Chichonal volcano

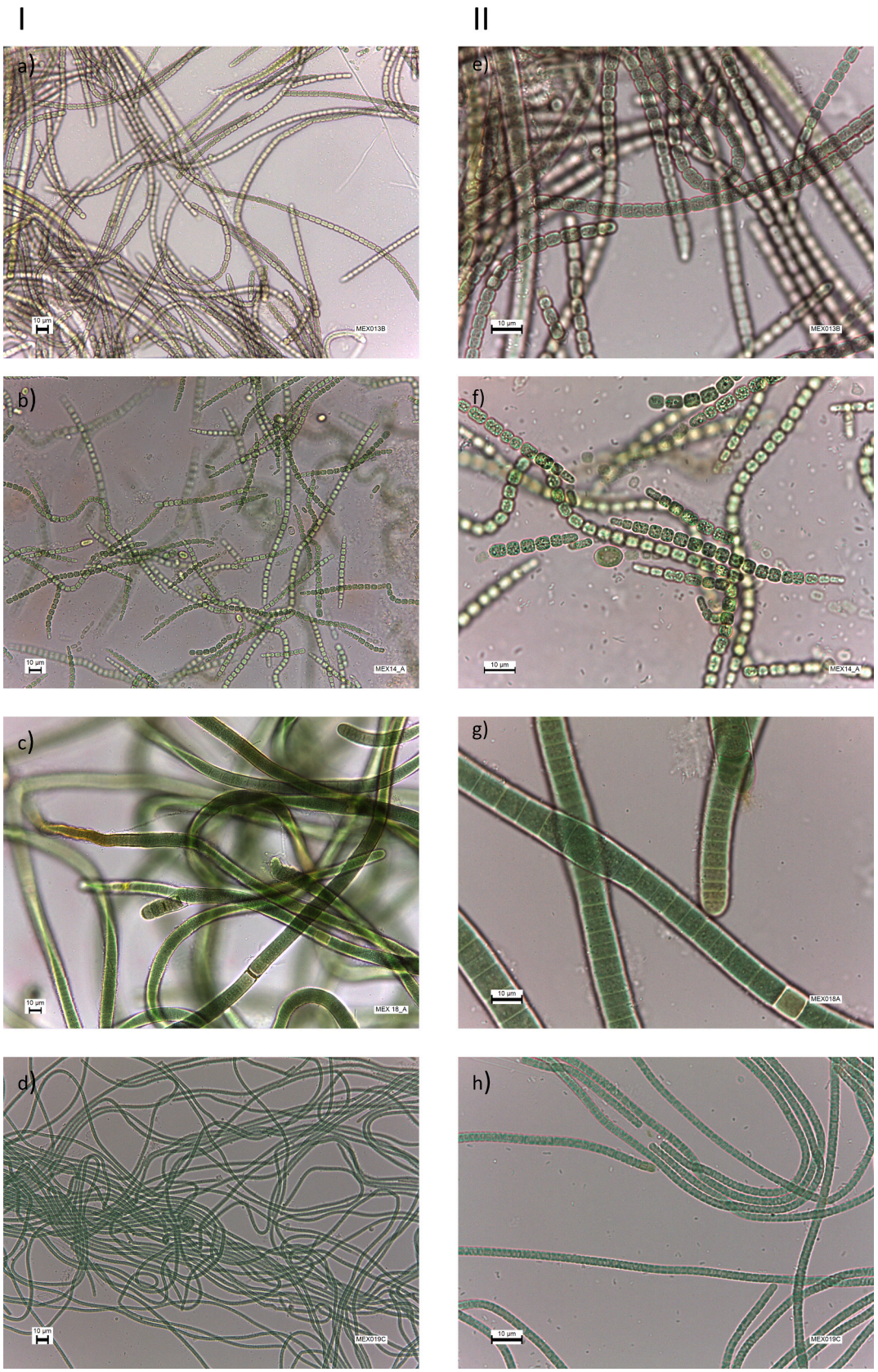

Fig. 2. Microphotographs of the isolated cyanobacterial strains: column I (x 40) a) MEX13B; b) MEX14A; c)MEX18A; d) MEX19C; and column II (x100) in the same order of strains.

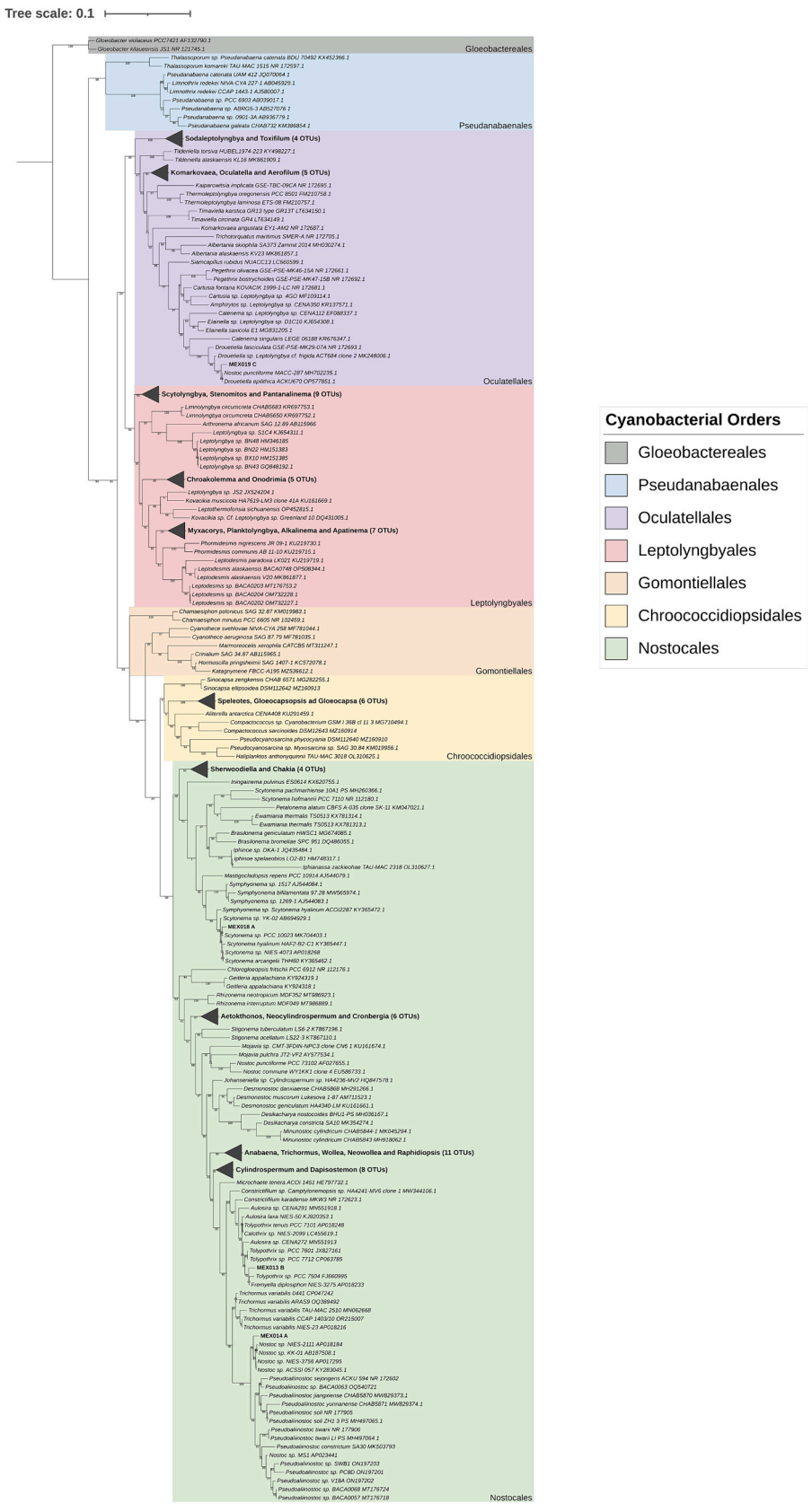

Fig. 3. Maximum likelihood phylogenetic tree based on 222 partial 16S rRNA gene sequences of cyanobacteria. *Gloeobacter violaceus* PCC 7421 and *Gloeobacter kilaueensis* JS1 were used as outgroup. The strains used in this work are indicated in bold. The different color segments represent the different cyanobacterial orders.

Table 2
List of the cyanobacteria strains molecular identification and their BLASTn outputs.

User Code	LEGE Code	Fragment Size (bp)	Date of BLAST	Query Coverage (%)	Identity (%)	BLASTn outputs (Accession Number)
Mex 13B	LEGE 221228	1312	16/10/2023	99	99.24	*Fremyella diplosiphon* NIES-3275 (AP018233.1)
				99	99.24	*Tolypothrix* sp. PCC 7712 (CP063785.1)
				99	99.21	*Tolypothrix* sp. PCC 7504 (FJ661002.1)
Mex 14 A	LEGE 221229	1171	28/03/2023	100	99.06	*Nostoc* sp. NIES-2111 DNA (AP018184.1)
				100	99.06	*Nostoc* sp. NIES-3756 DNA (AP017295.1)
				100	99.06	*Nostoc* sp. KK-01 (AB187508.1)
Mex 18A	LEGE 221230	1041	28/03/2023	100	99.62	*Scytonema* sp. PCC 10023 (MK704403.1)
				100	99.42	*Scytonema hyalinum* HAF2-B2-C1 (KY365447.1)
				100	99.42	*Scytonema* sp. NIES-4073 (AP018268.1)
Mex 19C	LEGE 221231	1252	01/08/2023	100	99.04	*Nostoc punctiforme* MACC-287 (MH702235.1)
				100	99.04	*Drouetiella epilithica* strain ACKU670 (NR_186875.1)
				100	99.04	*Drouetiella epilithica* ACKU670 (OP577851.1)

area in Mexico, this study focused on the chemical characterization and exploitation of the biological activities of their extracts, with a view to their biotechnological valorization. To assess their biological properties and extract their pigments, a sequential extraction method was employed using acetone and water. Our results demonstrated the high effectiveness of this technique, enabling us to obtain extracts with distinct polarities and distinctive chemical and biological attributes. This research emphasized the remarkable qualities of this sequential and cost-effective extraction approach. The extraction yields are displayed in Table 3.

The extraction yield was significantly higher with water when compared to acetone. This difference can be directly attributed to the distinct affinities of various compounds for each solvent, influenced by their molecular weight and polarity, as further elaborated in this study. Notably, among the strains examined, *Nostoc* sp. LEGE 221229 exhibited the highest extraction yield for both solvents, this finding holding economic significance, especially in the context of potential industrial applications.

The acetone and water extracts were chemically characterized, encompassing phycobiliproteins (PBPs), carotenoids, and chlorophylls. This analysis aimed to juxtapose the chemical profiles resulting from the diverse extraction solvents and to establish a connection between the chemical composition and the biological activities.

3.2.1. Carotenoid and chlorophylls

HPLC-PDA analysis of the acetone extracts from various cyanobacterial strains enabled the detection of several compounds, including three xanthophylls: lutein (4), canthaxanthin (7), and echinenone (13); two carotenes: β-carotene (16) and a β-carotene derivative (17), as well as chlorophyll-*a* (5) and a chlorophyll-*a* derivative (6), with a good chromatographic resolution (Fig. 4). Additionally, seven compounds exhibiting the same spectra of the identified carotenoids were detected in certain strains. However, because they displayed different retention times when compared with the standards, they were categorized as unidentified carotenoids (1, 2, 3, 8, 9, 10, 11, 12, 14, 15). Table 4 presents the qualitative and quantitative pigments profile of the cyanobacteria strains under study, and their chromatographic profiles are displayed in Fig. 4.

In terms of chlorophylls, their total content ranged from 10.55 to 20.88 μg/mg across the strains. Chlorophyll-*a* (5) was consistently present in all strains. The highest chlorophyll content was observed in *Drouetiella* sp. LEGE 221231 (20.88 μg/mg), followed by *Tolypothrix* sp. LEGE 221228 (15.46 μg/mg), *Nostoc* sp. LEGE 221229 (12.93 μg/mg), and *Scytonema* sp. LEGE 221230 (10.55 μg/mg) (Table 4). Regarding carotenoid concentrations, the total carotenoids ranged from 48.8 to 87.916 μg/mg of dry extract. The highest carotenoid content was also found in *Drouetiella* sp. LEGE 221231 (87.92 μg/mg), followed by *Tolypothrix* sp. LEGE 221228 (75.14 μg/mg), *Scytonema* sp. LEGE 221230 (57.82 μg/mg), and *Nostoc* sp. LEGE 221229 (48.8 μg/mg).

Among carotenoids, lutein (4) was detected in only two strains, namely *Scytonema* sp. LEGE 221230 and *Drouetiella* sp. LEGE 221231, being significantly higher in the last one ($p < 0.05$). Cataxanthin (7) was identified in all strains except *Drouetiella* sp. LEGE 221231, with the highest content found in *Scytonema* sp. LEGE 221230 (0.95 μg/mg) ($p < 0.05$). Echinenone (13) was found in all strains, with the highest content observed in *Scytonema* sp. LEGE 221230 (11.43 μg/mg), followed by *Tolypothrix* sp. LEGE 221228, *Nostoc* sp. LEGE 221229, and *Drouetiella* sp. LEGE 221231, with 6.36, 4.13 and 3.18 μg/mg, respectively. Beta-carotene (16) was the major carotenoid in all the studied strains, with the highest content found in *Drouetiella* sp. LEGE 221231 (63.67 μg/mg) ($p < 0.05$), followed by *Tolypothrix* sp. LEGE 221228 (51.38 μg/mg), *Scytonema* sp. LEGE 221230 (30.09 μg/mg), and *Nostoc* sp. LEGE 221229 (27.69 μg/mg) (Table 4, Fig. 4).

Jaiswal and co-workers [45] explored the pigments variation of a *Tolypothrix* sp. strain during different incubation periods, and reported maximum values of 9.12 and 0.79 μg/mg of dry weight, for total chlorophylls and carotenoids, respectively. The total amount of chlorophylls reported was superior to that of carotenoids, which differs from the results obtained herein. The absence of a qualitative carotenoid profile, and the pigments characterization in the fresh culture, hampers a direct comparison with the present work. In the present study, *Tolypothrix* sp. LEGE 221228 presented a significantly higher content in carotenoids when compared to chlorophylls (Table 4), what may be explained by the different origin of this strain that, being exposed to extreme environmental conditions, may produce higher amounts of carotenoids as a protection mechanism against oxidative stress. In fact, it has been demonstrated that extremophilic cyanobacteria produce a number of exclusive and distinctive metabolites in order to survive in adverse conditions. In response to stress factors and environmental changes, cyanobacteria can adapt the carotenoid production to enhance photoprotection and energy dissipation. This is facilitated by the Orange Carotenoid Protein (OCP), a water-soluble protein that contains a single molecule of hydroxyechinenone. The OCP plays a critical role in protecting against oxidative damage and dissipating energy in cyanobacteria. In fact, the slr1963 gene, constitutively expressed and encoding OCP, has its transcription temporary increased in response to high light or salt stress [46]. To our knowledge, this is the first report on the carotenoid profile of *Tolypothrix* sp.

In line with our research, Palmer et al. [47] also conducted studies on the *Nostoc* sp. strain. The authors utilized ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MSE) to establish the chemical profile, and identified echinenone as the major compound, followed by caloxanthin and

Table 3
Cyanobacteria extraction yield ($mg_{dry\ extract}/g_{dry\ biomass}$).

Strain	Acetone extract	Water extract
Tolypothrix sp. LEGE 221228	24.7	152.5
Nostoc sp. LEGE 221229	35.5	226.4
Scytonema sp. LEGE 221230	15.7	99.5
Drouetiella sp. LEGE 221231	25.5	100.2

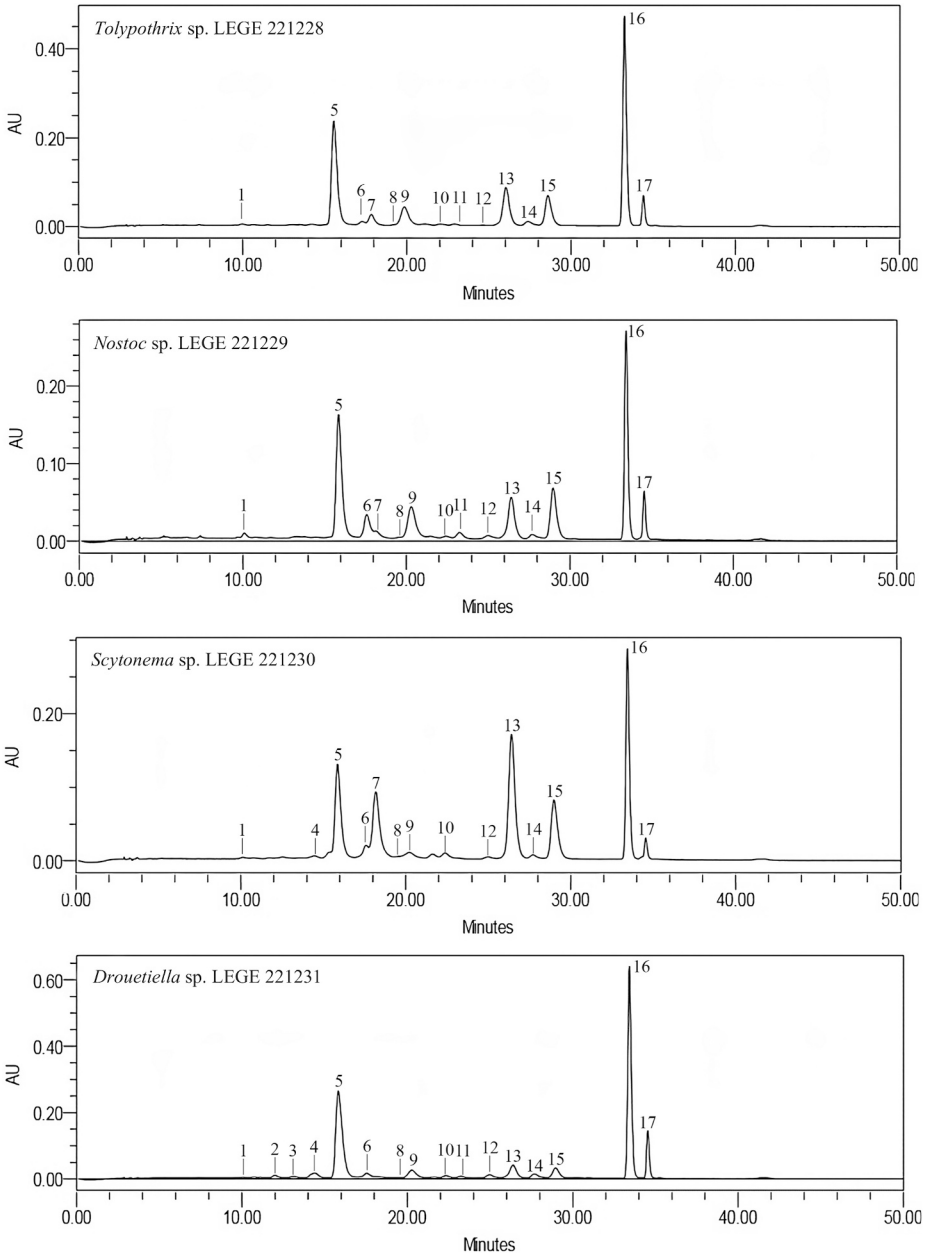

Fig. 4. Carotenoid and chlorophylls profile of acetone extracts of cyanobacteria strains: *Tolypothrix* sp. LEGE 221228, *Nostoc* sp. LEGE 221229, *Scytonema* sp. LEGE 221230 and *Drouetiella* sp. LEGE 221231. HPLC-PDA was recorded at 450 nm. Unidentified carotenoids (1,2,3,8,9,10,11,12,14,15), lutein (4), chlorophyll-*a* (5), chlorophyll-*a* derivative (6), canthaxanthin (7), echinenone (13), β-carotene (16) and β-carotene derivate (17).

β-cryptoxanthin. Echinenone was also characterized in the present study as the major xanthophyll, but β-carotene appeared as the most representative carotenoid, contrary to Palmer and co-workers, that only reported trace amounts of β-carotene isomers. On the other hand, Hashtroudi et al. [48] reported β-carotene as the major compound of a species from the genus *Nostoc* isolated from a paddy field in Iran. The major qualitative differences stand on the presence of zeaxanthin, reported by the previous authors and not identified herein, and the presence of canthaxanthin found by us and reported later by Yang et al. [49]. This xanthophyll is characterized for protecting chlorophyll and carotenoids from degradation under photoinhibitory conditions, that may occur under extreme environmental conditions such as water stress, heat or extreme light exposure [50], which is in accordance with the origin of the samples isolated herein.

Lakatus et al. investigated *Syctonema* sp. strains collected in Puerto Ayacucho [51] and reported significant amounts of chlorophyll-*a*, canthaxanthin, echinenone, and β-carotene, which is consistent with the results obtained later by Asencio et al. [52] and by us. The authors also concluded that different β-carotene derivatives appeared to be strongly influenced by irradiance. Although scytonemin is the characteristic compound of *Scytonema* sp., responsible to cope up against strong solar radiation, it has been reported that carotenoids and chlorophyll-*a* are also present during high irradiance, enabling the organisms to thrive in extreme environmental conditions [53].

To our knowledge, this is the first report on the carotenoid and chlorophylls profile of *Drouetiella* sp. Of the analyzed strains, *Drouetiella* sp. was the one presenting the highest amount of carotenoids and chlorophylls, with β-carotene being the major compound. The major difference between this species and the remaining ones stands on the absence of canthaxanthin, and in the higher amount of lutein, chlorophyll-*a* and β-carotene. Overall, the qualitative profile was similar between all the extremophile cyanobacteria analyzed.

Table 4
Carotenoid and chlorophylls content (μg/$mg_{dry\ extract}$) of the cyanobacteria strains, as determined by HPLC-PDA[1,2].

Peak	Compound	RT (min)	*Tolypothrix* sp. LEGE 221228	*Nostoc* sp. LEGE 221229	*Scytonema* sp. LEGE 221230	*Drouetiella* sp. LEGE 221231
1	Chlorophyll-*a* derivative	10.05	0.09 ± 0.03 [a]	0.37 ± 0.01 [b]	0.35 ± 0.07 [b]	0.03 ± 0.01 [a]
2	Unidentified carotenoid	11.09	nd	nd	nd	0.70 ± 0.03
3	Unidentified carotenoid	13.00	nd	nd	nd	0.36 ± 0.04
4	Lutein	14.03	nd	nd	0.71 ± 0.09 [a]	1.65 ± 0.11 [b]
5	Chlorophyll-*a*	15.38	14.88 ± 0.31 [b]	10.49 ± 0.04 [a]	9.03 ± 0.21. [a]	17.75 ± 0.42 [c]
6	Chlorophyll-*a* derivative	17.41	0.48 ± 0.07 [a]	2.08 ± 0.04 [c]	1.17 ± 0.05 [b]	2.05 ± 0.12 [c]
7	Canthaxanthin	17.77	0.28 ± 0.02 [b]	0.14 ± 0.01 [a]	0.95 ± 0.02 [c]	nd
8	Unidentified carotenoid	20.15	4.06 ± 0.16 [c]	4.13 ± 0.01 [c]	1.68 ± 0.15 [a]	2.44 ± 0.08 [b]
9	Unidentified carotenoid	21.48	0.28 ± 0.02 [b]	0.27 ± 0.01 [b]	0.54 ± 0.01 [c]	0.18 ± 0.01 [a]
10	Unidentified carotenoid	22.27	0.26 ± 0.03 [a]	0.24 ± 0.002 [a]	1.03 ± 0.01 [c]	0.52 ± 0.02 [b]
11	Unidentified carotenoid	23.08	0.31 ± 0.002 [a]	0.78 ± 0.08 [b]	nd	0.39 ± 0.01 [a]
12	Unidentified carotenoid	24.92	0.18 ± 0.1 [a]	0.60 ± 0.05 [b]	0.53 ± 0.01 [b]	1.00 ± 0.02 [c]
13	Echinenone	26.34	6.36 ± 0.11 [c]	4.13 ± 0001 [b]	11.43 ± 0.11 [d]	3.18 ± 0.05 [a]
14	Unidentified carotenoid	27.63	0.84 ± 0.1 [a, b]	0.55 ± 0.01 [a]	0.60 ± 0.05 [a]	1.04 ± 0.01 [b]
15	Unidentified carotenoid	28.90	4.81 ± 0.17 [b]	4.56 ± 0.01 [b]	6.02 ± 0.04 [c]	2.19 ± 0.08 [a]
16	β-Carotene	33.40	51.37 ± 0.53 [b]	27.69 ± 0.20 [a]	30.09 ± 0.48 [a]	63.67 ± 1.23 [c]
17	β-Carotene derivative	34.37	6.35 ± 0.15 [b]	5.73 ± 0.02 [b]	4.22 ± 0.04 [a]	11.65 ± 0.31 [d]
Total carotenoids			75.14 ± 1.36 [c]	48.42 ± 0.40 [a]	57.20 ± 1.01 [b]	87.92 ± 1.92 [d]
Total chlorophylls			15.45 ± 0.41 [c]	12.93 ± 0.09 [b]	10.55 ± 0.33 [a]	20.88 ± 0.60 [d]

[1] Values are expressed as mean ± SD of two determinations.
[2] Different superscript letters in the same row denote statistical differences at $p < 0.05$ (ANOVA, Tukey's HSD). Nd: Not detected; RT: retention time.

3.2.2. Phycobiliproteins

Cyanobacteria, previously recognized as blue-green algae, belong to a bacterial phylum capable of harnessing energy through oxygenic photosynthesis. These microorganisms employ phycobiliproteins to capture light energy, distinguishing them from higher plants. These proteins form phycobilisomes into thylakoid membranes, serving as the principal light-harvesting complex of Photosystem-II, and transmit light energy to the photosynthetic reaction center in cyanobacteria. The central constituents of the phycobilisome are the bilin-containing proteins PE, PC and APC. C-phycocyanin (C-PC) is the predominant pigment within the phycobilisome complex, being universally present in all cyanobacterial species [54]. PBPs have a wide array of applications, serving as natural colorants in the food industry, dietary supplements, cosmetics, and even as fluorescent markers in immunoassays. Beyond their industrial uses, PBPs have been recognized for their medicinal potential, backed by documented anti-inflammatory, antiviral, anti-cancer, immunostimulatory, and antioxidant properties [55]. Table 5 provides a comprehensive overview of the PBPs content of the strains under study.

Considering these outcomes, it is noteworthy that *Tolypothrix* sp. LEGE 221228 exhibits a higher abundance of PE ($p < 0.05$) compared with the other strains. Similarly, *Nostoc* sp. LEGE 221229 demonstrates an elevated PC content, correlating with the vibrant red/pink and blue visual appearance of the aqueous extracts of these strains.

Tolypothrix tenuis, a species belonging to the same genus as that reported herein, has been previously explored for its content in phycobiliproteins, presenting 39.01, 20.04 and 42.04 μg/mg of dry weight, of PC, APC and PE, respectively [45], which is in agreement with the values reported herein. However, the studies devoted to the exploitation of the pigments of the genus *Tolypothrix* are scarce. Regarding *Nostoc* sp., some discrepancies were observed. For instance, the study of Soule and co-workers [56] reported a major amount of PC in *Nostoc punctiforme* (which is in accordance with the results obtained herein), followed by PE and APE in similar amounts. The authors also demonstrated that the pigments content varied with radiation. Shinde et al. analyzed *Nostoc ellipsosporum*, *Nostoc entophytum* and *Nostoc punctiforme*, and found that PC was dominant, followed by APC and lastly by PE, in significantly lower amounts [57]. This analysis allows us to realize that the content in phycobiliproteins, as well as in other pigments, is highly dependent on species-specific factors. *Scytonema* sp. Is another example of this; Pandey and co-workers [58] reported higher levels of PE (31.5 μg/mg), which is in inverse proportion to our finding (6.62 μg/mg). Similarly, lower levels of PC and APC were found by us when compared with their work (22.2 μg/mg and 24.2 μg/mg, respectively). Our results are consistent with those of Asencio et al. [52], where PE is not always present depending on the *Scytonema* cultures distinguished by cluster analysis. Regarding *Drouetiella* sp., to our knowledge, this is the first report on the PBPs profile of this strain. Overall, taking the present results and the mentioned studies into consideration, it seems that the PBPs profile cannot be taken as a chemotaxonomic marker of this strains.

Table 5
Phycobiliproteins content (μg/mg of dry extract) in cyanobacteria aqueous extracts[1,2].

Strain	Phycoerythrin	Phycocyanin	Allophycocyanin
Tolypothrix sp. LEGE 221228	50.61 ± 0.95 [d]	39.46 ± 0.81 [c]	16.54 ± 0.32 [b]
Nostoc sp. LEGE 221229	13.50 ± 0.57 [c]	57.66 ± 0.54 [d]	7.25 ± 0.38 [a]
Scytonema sp. LEGE 221230	6.62 ± 0.05 [a]	12.95 ± 0.14 [a]	8.33 ± 0.22 [a]
Drouetiella sp. LEGE 221231	9.57 ± 0.007 [b]	15.22 ± 0.13 [b]	15.63 ± 0.278 [b]

[1] Values are expressed as the mean ± SD of three independent experiments.
[2] Different superscript letters in the same column correspond to statistical differences at $p < 0.05$.

3.3. Assessment of antioxidant capacities

When endogenous detoxification mechanisms fail or prove inadequate, the potential for heightened oxidative damage and its consequential harmful impacts increases substantially [59]. Consequently, discovering mechanisms that can hinder the detrimental effects of free radicals becomes a matter of paramount importance. This significance is not only confined to the realm of cosmetics but also extends to the enhancement and prevention of an extensive spectrum of diseases. The IC values corresponding to $O_2^{\bullet -}$ scavenging by cyanobacteria aqueous extracts are displayed in Table 6, while their dose-dependent behavior is represented in Fig. 5.

Tolypothrix sp. LEGE 221228 was the most effective of the studied strains, reaching the lowest IC_{50} (53.75 μg/mL), followed by *Drouetiella* sp. LEGE 221231 and *Scytonema* sp. LEGE 221230. *Nostoc* sp. LEGE 221229 was the less effective, presenting the highest values (Table 6). It is worth mentioning that a significant negative correlation has been found between $O_2^{\bullet -}$ scavenging and PC (−0.813, $p < 0.05$), suggesting the contribution of this phycobiliprotein to radical scavenging.

Concerning the acetonic extracts, none of the strains exhibited a

Table 6
Inhibitory concentration (IC) values (μg/mL) of cyanobacteria aqueous extracts against superoxide anion radical ($O_2^{\bullet-}$)[1,2].

Strains	IC_{25}	IC_{50}
Tolypothrix sp. LEGE 221228	20.29 ± 0.01 [a]	53.75 ± 0.02 [a]
Nostoc sp. LEGE 221229	51.11 ± 9.36 [b]	166.13 ± 27.32 [b]
Scytonema sp. LEGE 221230	54.85 ± 8.42 [b]	94.66 ± 1.56 [a]
Drouetiella sp. LEGE 221231	44.1 ± 5.61 [a,b]	89.67 ± 1.86 [a]

[1] Values are expressed as the mean ± SD of three independent experiments.
[2] Different superscript letters in the same column correspond to statistical differences at $p < 0.05$.

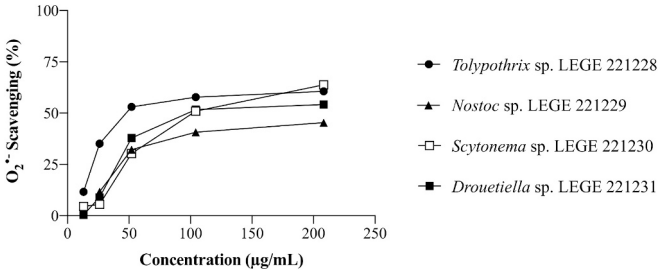

Fig. 5. Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity of cyanobacteria aqueous extracts. Values are expressed as the mean ± SD of at least three independent experiments, performed in duplicate.

significant capability to scavenge $O_2^{\bullet-}$, when compared with the aqueous extract. The highest activity observed reached 22 % in the case of *Nostoc* sp. LEGE 221229, followed by *Tolypothrix* LEGE 221228 sp. and *Drouetiella* sp. LEGE 221231 with 9 %, and *Scytonema* sp. LEGE 221230 with 6 %, for the highest concentration tested (300 μg/mL).

Nowruzi et al. [60] have recently explored the $O_2^{\bullet-}$ scavenging ability of C-phycoerythrin purified from a *Nostoc* sp. strain, and found a concentration-dependent activity, in line with the results obtained by us with the phycobiliproteins-rich extracts. The authors found an IC_{50} value of 57 μg/mL, which is close to the IC_{50} value obtained by us for *Tolypothrix* sp. LEGE 221228. Although a significant negative correlation has been observed between the radical scavenging and the content of PC, the contribution of the other PBP for $O_2^{\bullet-}$ scavenging is unquestionable. Moreover, a synergistic activity is probable and advantageous, the use of extracts avoiding the expensive and time consuming methodologies needed for compounds purification and isolation. In fact, Sonani and co-workers [61] have explored the ability of purified phycobiliproteins to scavenge different free radicals, and found IC_{50} values of 185.3, 328.2 and 314.7 μg/mL for PE, PC and APC, respectively, which demonstrates the probable synergic effect of the different PBPs within the extracts analyzed herein.

Together with $O_2^{\bullet-}$, $^{\bullet}NO$ plays an important role in oxidative stress. This pivotal physiological regulator, endogenously produced by nitric oxide synthases (NOS), has a remarkable diffusion capacity and reactivity, and participates in diverse metabolic processes, including cardiac oxygen metabolism modulation, insulin secretion, glucose metabolism, cardiovascular and renal regulation, and neural processes [62]. But NO's multifunctional signaling role extends across human tissues. In skin, $^{\bullet}NO$ production is increase by UVA and UVB exposure which also stimulates tyrosinase activity and melanin synthesis [63]. This significance extends beyond cosmetics, encompassing diverse conditions like hyperpigmentation disorders such as melasma. IC values obtained for $^{\bullet}NO$ scavenging by aqueous and acetone extracts of the strains under study are displayed in Table 7, while their dose-dependent behavior is represented in Fig. 6.

All the extracts analyzed presented a dose-dependent behavior, with acetonic extracts being significantly more effective than aqueous ones regarding $^{\bullet}NO$ scavenging. For the aqueous extracts, *Tolypothrix* sp. LEGE 221228 was the most effective, being the only able to reach IC_{50}.

Table 7
Inhibitory concentration (IC) values (μg/mL) of cyanobacteria aqueous and acetone extracts against nitric oxide radical ($^{\bullet}NO$)[1,2].

Strains	Acetone Extracts		Aqueous Extracts	
	IC_{25}	IC_{50}	IC_{25}	IC_{50}
Tolypothrix sp. LEGE 221228	29.73 ± 14.47	57.68 ± 11.41 [a]	689.91 ± 100.69	1220.00 ± 39.60
Nostoc sp. LEGE 221229	30.8 ± 0.01	81.69 ± 0.01 [b]	–	–
Scytonema sp. LEGE 221230	22.37 ± 6.64	64.52 ± 11.52 [a]	–	–
Drouetiella sp. LEGE 221231	30.02 ± 1.17	101.23 ± 9.57 [c]	803.37 ± 92.39	–

[1] Values are expressed as the mean ± SD of three independent experiments.
[2] Different superscript letters in the same column correspond to statistical differences at $p < 0.05$.

Drouetiella sp. LEGE 221231 extract only reached IC_{25}, and a neglectable % of $^{\bullet}NO$ scavenging was displayed by the remaining aqueous extracts, for the highest concentration tested (300 μg/mL) (Table 7).

On the other hand, acetone extracts demonstrated a significantly better efficacy to scavenge $^{\bullet}NO$, with *Tolypothrix* sp. LEGE 221228 being the most effective strain, with an IC_{50} value of 57.7 μg/mL, closely followed by *Scytonema* sp. LEGE 221230 (Table 7). A significant negative correlation has been observed between $^{\bullet}NO$ scavenging and β-carotene (−0.764, $p < 0.05$), suggesting that this compound contributes to the radical scavenging activity of acetone extracts. On the other hand, a positive correlation was noted for PC (0.970, $p < 0.01$), suggesting that this phycobiliprotein may hamper the scavenging of nitrogen free radicals.

Few reports have addressed the free radicals scavenging ability of cyanobacteria extracts. Arun et al. [64] evaluated the capacity of a methanol extract of *Nostoc muscorum* to scavenge $^{\bullet}NO$, and obtained a value of 23.58 %. The fact that the authors did not present the result in terms of dry weight of extract, prevents a direct comparison with the present results. Amaro et al. [65] reported that microalgae extracts of higher polarity (ethanol) were more prone to $O_2^{\bullet-}$ scavenging, while those of lower polarity (acetone) were more effective in scavenging $^{\bullet}NO$, which is in accordance with the results obtained by us.

3.4. Assessment of anti-hyperpigmentation potential

Tyrosinase holds a prominent role as the rate-limiting catalyst in the melanin synthesis. This enzymatic function has led to its applications in both the cosmetic industry and pharmaceuticals. Melanin stands as the principal determinant of skin color, assuming a pivotal role in various physiological processes, and serving as an exceptional photoprotective agent, by absorbing detrimental UV rays and subsequently dissipating their energy as innocuous heat. However, the excessive accumulation of melanin can contribute to cosmetic concerns such as freckles, age spots, and melasma, potentially giving rise to more severe dermatological issues. Consequently, the regulation of melanogenesis emerges as a crucial strategy in the management of complications linked to irregular skin pigmentation [66].

Of the strains explored herein, only the aqueous extract of *Nostoc* sp. LEGE 221229 inhibited tyrosinase, with IC_{25} and IC_{50} values of 70.2 ± 0.01 μg/mL and 929.85 ± 0.03 μg/mL, respectively. This observation provides a glimpse into the limited but existent ability of tyrosinase inhibition by this specific strain. Even though the studies regarding tyrosinase inhibition by cyanobacteria extracts are scarce, this bioactivity has been recently explored by Morone et al. [31] and Favas et al. [67]. Contrary to the results obtained by us, Morone and co-workers [31] reported tyrosinase inhibition by cyanobacteria acetone extracts, with IC_{50} values ranging from 465.9 to 849.5 μg/mL. As well, Favas and co-workers only found activity for the acetone extracts, with only one strain reaching the IC_{50} with a value near 1 mg/mL. To the best of our

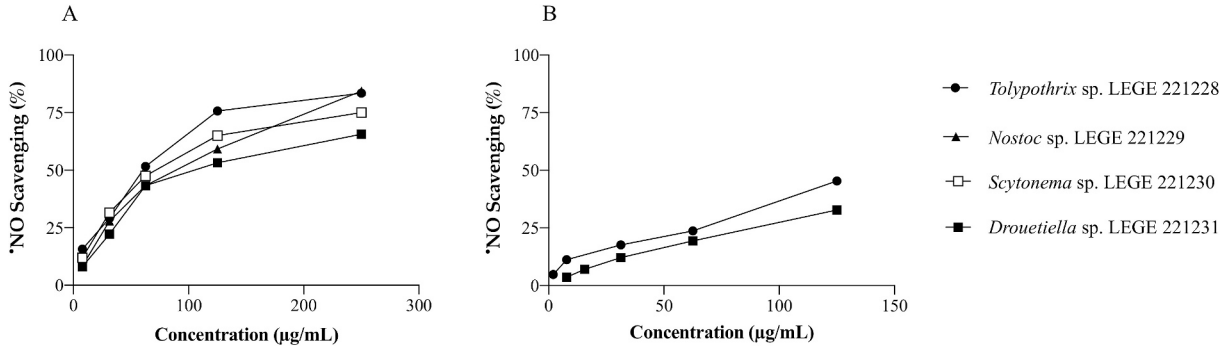

Fig. 6. Nitric oxide anion (•NO) scavenging activity of cyanobacteria acetone (A) and aqueous (B) extracts. Values are expressed as the mean ± SD of at least three independent experiments, performed in duplicate.

knowledge, there have been no previous reports documenting tyrosinase inhibitory activity for the strains examined in the present study. Nevertheless, considering the significant concentration of PC in the *Nostoc* sp. LEGE 221229, it is plausible to correlate a portion of these findings to this specific phycobiliprotein. This association is supported by the work of Wu et al. [68], who demonstrated the capacity of C-PC to exert inhibitory effects on both melanin synthesis and tyrosinase expression, thereby modulating melanogenesis.

Indeed, the current scientific literature reflects a significant gap regarding the detailed analysis of the correlation between the chemical composition and the biological activities of extracts from various strains of cyanobacteria. This lack of detailed studies underscores the need for more thorough research that can elucidate the specific mechanisms by which the chemical components of these organisms influence their biological properties. Future investigations in this area would not only contribute to our understanding of biotechnology but also pave the way for the development of innovative therapeutic applications.

4. Conclusions

The present work gives a valuable contribute to the knowledge of cyanobacteria biodiversity of underexplored habitats, being the first of his kind reporting the above cyanobacteria strains in the volcanic lake Chichonal, in Mexico. In addition, it emphasizes the potential of cyanobacteria biomass in the discovery of bioactive extracts with different biotechnological applications. The sequential extraction process allowed the extraction of secondary metabolites with different chemical characteristics and bioactivities, maximizing the extraction yield in a concept of biorefinery. Of the isolated strains, *Drouetiella* sp. LEGE 221231 stood out for its pigments content, while *Tolypothrix* sp. LEGE 221228 revealed the most promising biological activity for both carotenoids and phycobiliproteins, their further exploitation as antioxidants and colorants in the food and cosmetic industries being worthy of further exploitation.

CRediT authorship contribution statement

Raquel Silva: Writing – original draft, Methodology, Investigation, Formal analysis. **Talita Gonçalves:** Writing – original draft, Methodology, Investigation, Formal analysis. **Janaína Morone:** Writing – review & editing, Methodology. **Gabriela Alves Moreira:** Writing – review & editing, Methodology. **João Morais:** Writing – review & editing, Methodology, Formal analysis. **Guilherme Scotta Hentschke:** Writing – review & editing, Validation, Formal analysis. **Peggy Elizabeth Álvarez-Gutiérrez:** Writing – review & editing, Resources. **Ramón Alberto Batista-García:** Writing – review & editing, Resources. **Vitor Vasconcelos:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Graciliana Lopes:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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