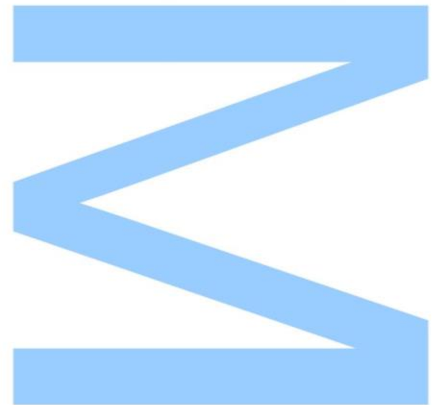
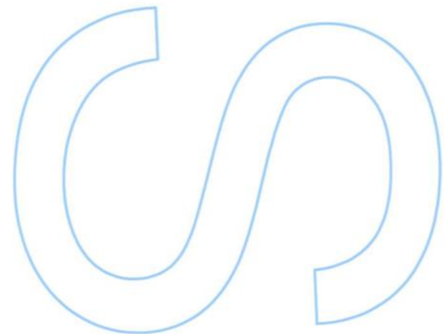
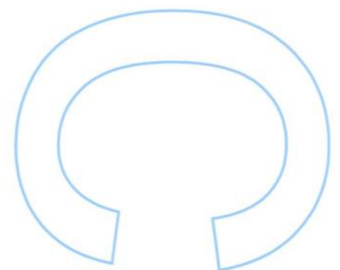
Exploring the Biotechnological Potential of Cyanobacteria in the Treatment of Psoriasis

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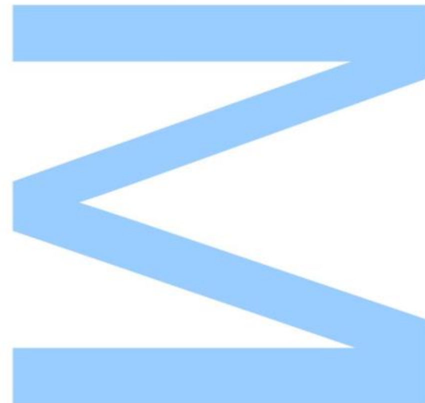

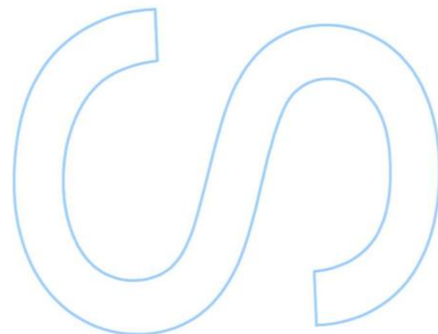

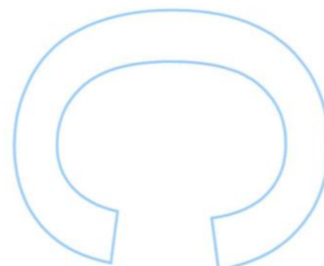

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Abstract

Cyanobacteria, also known as blue-green algae, represent one of the earliest forms of life to inhabit our planet, with deep and essential roles in the carbon and nitrogen biochemical cycles. In the last decades, the research devoted to cyanobacteria has gained increased attention, due to the biotechnological potential of their secondary metabolites in a wide range of areas, such as pharmacology, energy sector, agriculture, cosmetics, wastewater treatment and food/feed industries.

In this study, six cyanobacteria strains (*Alkalinema aff. pantanalense* LEGE15481, *Cyanobium gracile* LEGE12431, *Nodosilinea (Leptolyngbya) antarctica* LEGE13457, *Synechocystis salina* LEGE00037, *Cuspidothrix issatschenkoi* LEGE03282, and *Leptolyngbya*-like sp. LEGE13412) from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGEcc - http://lege.ciimar.up.pt/) of CIIMAR, were selected in order to explore their biotechnological potential in the treatment and management of psoriasis. To achieve this goal, different extracts were prepared, chemically characterized for their pigment profile, by HPLC-PDA, and total phenolic content, through the Folin-Ciocalteu colorimetric assay. The antioxidant potential of the extracts was assessed *in vitro*, by determining their capacity to scavenge superoxide anion radical ($O_2^{\bullet -}$). Considering the problematic of inflammation in psoriasis, as well as the presence of psoriatic plaques, the anti-inflammatory potential of the extracts, together with their capacity to reduce keratinocytes hyperproliferation, was assessed *in vitro* using the macrophages cell line RAW 264.7 and the keratinocytes cell line HaCaT as models.

The highest carotenoids content was found in *Nodosilinea (Leptolyngbya) antarctica* LEGE13457 (63.701 µg/mg of dry extract), with β-carotene as major compound (27.695 µg/mg of dry extract), and the highest content in phenols was found in *Cyanobium gracile* LEGE12431 (22.01 $GAE.mg^{-1}$ of dry extract). Regarding the antioxidant potential, acetone extracts were those presenting the best capacity to scavenge $O_2^{\bullet -}$, with *Nodosilinea (Leptolyngbya) antarctica* LEGE13457 and *Cuspidothrix issatschenkoi* LEGE 03282 presenting the lowest IC_{50} values (0.319 and 0.286 $µg.mL^{-1}$, respectively), and a correlation of this activity with the carotenoids profile and the total phenolic content was noted. Regarding the anti-inflammatory potential, in a general way, acetone extracts were more promising than those obtained with ethanol. All the ethanol extracts increased the nitric oxide (NO) production in RAW 264.7 cells. The acetone extracts of *Nodosilinea (Leptolyngbya)*

antarctica LEGE13457, *Alkalinema aff. pantanalense* LEGE15481 and *Leptolyngbya*-like sp. LEGE13412 presented a mild anti-inflammatory potential, reducing the NO production in about 25%, face to control, and without affecting macrophages viability. Considering the central role of keratinocytes in the pathogenesis of psoriasis, the effect of cyanobacteria extracts towards keratinocytes proliferation was evaluated *in vitro*, after 24 and 48h of exposition to the extracts. With the exception of *Alkalinema aff. pantanalense* LEGE15481, all the tested extracts significantly reduced keratinocytes viability. Going against the goal of the present project, *Leptolyngbya*-like sp. LEGE 13412 and *Nodosilinea (Leptolyngbya) antarctica* LEGE13457 acetone extracts seem to be very promising for further exploitations in the framework of psoriasis, once these two species presented anti-inflammatory potential, reduced keratinocytes hyperproliferation, and were able to scavenge free radicals implicated in the multifactorial framework of the disease.

Keywords: Cyanobacteria, Psoriasis, Carotenoids, Oxidative Stress, Inflammation, Keratinocytes.

Resumo

As cianobactérias, também conhecidas como algas verde-azuis, representam uma das primeiras formas de vida a habitar nosso planeta, desempenhando funções essenciais nos ciclos bioquímicos de carbono e azoto. Nas últimas décadas, a investigação dedicada às cianobactérias ganhou maior atenção, devido ao potencial biotecnológico dos seus metabolitos secundários numa ampla variedade de áreas, como farmacologia, sector energético, agricultura, cosmética, tratamento de águas residuais e indústria alimentar.

Neste estudo, seis estirpes de cianobactérias (*Alkalinema aff. Pantanalense* LEGE15481, *Cyanobium gracile* LEGE12431, *Nodosilinea (Leptolyngbya) antarctica* LEGE13457, *Synechocystis salina* LEGE00037, *Cuspidothrix issatschenkoi* LEGE 03282 e *Leptolyngbya-like* sp. LEGE13412, provenientes da Blue Biotechnology and Ecotoxicology Culture Collection (LEGEcc - http://lege.ciimar.up.pt/) do CIIMAR, foram selecionadas para explorar o seu potencial biotecnológico no tratamento da psoríase. Para atingir esse objetivo, diferentes extratos foram preparados, quimicamente caracterizados no que respeita ao seu perfil de pigmentos, por HPLC-PDA, e conteúdo fenólico total, através do ensaio colorimétrico Folin-Ciocalteu. O potencial antioxidante dos extratos foi avaliado *in vitro*, através da avaliação da sua capacidade de sequestro do radical anião superóxido ($O_2^{\bullet-}$). Considerando a problemática da inflamação na psoríase, bem como a presença de placas psoriáticas, o potencial anti-inflamatório dos extratos, juntamente com sua capacidade de reduzir a hiperproliferação dos queratinócitos, foi avaliado *in vitro*, utilizando linhas celulares de macrófagos RAW 264.7 e queratinócitos HaCaT como modelo.

O maior conteúdo de carotenóides foi encontrado na espécie *Nodosilinea (Leptolyngbya) antarctica* LEGE13457 (63,701 µg/mg of dry extract), sendo o β-caroteno o principal composto (27,695 µg/mg of dry extract), e o maior conteúdo em fenóis foi encontrado na espécie *Cyanobium gracile* LEGE12431 (22,01 GAE.mg^{-1} de extrato seco). Em relação ao potencial antioxidante, os extratos acetónicos foram os que apresentaram melhor capacidade de sequestro do $O_2^{\bullet-}$, tendo as espécies *Nodosilinea (Leptolyngbya) antarctica* LEGE13457 e *Cuspidothrix issatschenkoi* LEGE 03282 apresentado os menores valores de IC_{50} (0,319 e 0,286 µg.mL^{-1}, respectivamente), e tendo sido notada uma correlação dessa atividade com o perfil de carotenóides e o conteúdo fenólico total. Em relação ao potencial antiinflamatório, de uma forma geral, os extratos acetónicos foram mais promissores do que os obtidos com etanol. Todos os extratos etanólicos aumentaram a produção de óxido

nítrico (NO) pelas células RAW 264.7. Os extratos acetónicos da espécie *Nodosilinea (Leptolyngbya) antarctica* LEGE13457, *Alkalinema aff. pantanalense* LEGE15481 e *Leptolyngbya-like* sp. LEGE13412 apresentaram um potencial anti-inflamatório moderado, reduzindo a produção de NO em cerca de 25%, face ao controlo, e sem afetar a viabilidade dos macrófagos. Considerando o papel central dos queratinócitos na patogénese da psoríase, o efeito dos extratos de cianobactérias na proliferação de queratinócitos foi avaliado *in vitro*, após 24 e 48h de exposição aos extratos. Com exceção de *Alkalinema aff. pantanalense* LEGE15481, todos os extratos testados afetaram a viabilidade dos queratinócitos. Indo de encontro ao objetivo do presente estudo, os extratos acetónicos das espécies *Leptolyngbya-like* sp. LEGE 13412 e *Nodosilinea (Leptolyngbya) antarctica* LEGE13457 revelaram-se bastante promissores para futura exploração no âmbito da psoríase, uma vez que estas duas espécies apresentaram potencial anti-inflamatório, capacidade de reduzir a hiperproliferação de queratinócitos, e foram ainda capazes de sequestrar radicais livres com papel importante no quadro multifatorial da doença.

Palavras Chave: Cianobactérias, Psoríase, Carotenoides, Stress Oxidativo, Inflamação, Queratinócitos.

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List of Abbreviations

$^1O_2$ – Singlet oxygen

AOAC - Association of Official Analytical Chemists or AOAC INTERNATIONAL

AP-1 – Activator Protein 1

APX - Ascorbate peroxidase

BBE – Blue Biotechnology and Ecotoxicology

CAT - Catalase

cGMP - Cyclic Guanosine Monophosphate

CLEC7A – C-Type Lectin Domain Family 7 Member A

COX-1 – Cyclooxygenase 1

COX-2 – Cyclooxygenase 2

CRTISO - Carotenoid isomerase

cyt b_6f – Cytochrome b_6f complex

DHA – Dehydroascorbate

DHAR - Dehydroascorbate reductase

DMAPP - Dimethylallyl pyrophosphate

DMSO – Dimethyl sulfoxide

eNOS – Endothelial Nitric Oxide Synthase

EPS – Exopolysaccharide

GAE – Gallic Acid Equivalents

GGPP - Geranylgeranyl pyrophosphate

GGPS - Geranylgeranyl pyrophosphate synthase

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GPX - Glutathione peroxidase

GPX - Guaiacol peroxidase

GR - Glutathione reductase

GSH - Glutathione reduced form

GSSH - Glutathione oxidized form

H_2O_2 – Hydrogen peroxide

HMG-CoA reductase - 3-hydroxy-3-methylglutaryl coenzyme A reductase

HPLC - High Performance Liquid Chromatography

IC – Inhibitory Concentration

IFN-γ – Interferon gamma

IL – Interleukin

iNOS – Inducible Nitric Oxide Synthase

IPI - Isopentenyl diphosphate isomerase

IPP - Isopentenyl pyrophosphate

LCBY - Lycopene β-cyclase

LCYE - Lycopene ε-cyclase

LEGE CC - Blue Biotechnology and Ecotoxicology Culture Collection

LOD – Limit of detection

LOQ – Limit of quantification

LPS – Lipopolysaccharide

MDA - monodehydroascorbate

MDHAR - Monodehydroascorbate reductase

MMP9 - Matrix metalloproteinase 9

MS – Mass Spectroscopy

MTT – 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide

Na^+/K^+- ATPase – Sodium/Potassium Adeniosine triphosphatase

nAChR - Nicotinic Acetylcholine Receptors

NADH - β-nicotinamide adenine dinucleotide reduced form

NADPH – Nucleotide Adenine

NBT - Nitrotetrazolium blue chloride

NF-κB – Nuclear factor kappa-light-chain-enhancer

NK - Natural killer cells

NKT - Natural killer T cells

nNOS – Neuronal Nitric Oxide Synthase

NO – Nitric Oxide

NOS – Nitric Oxide Synthase

$O_2^{\cdot -}$ - Superoxide radical anion

O_2^{2-} - Peroxide ion

OH^- - Hydroxyl radical

OX – Terminal Oxidase

PASI - Psoriasis Area and Severity Index

PC - Plastocyanin

PDA – Photo Diode Array

PDS - Phytoene desaturase

PMS - Phenazine methosulphate

PQ - Plastoquinone

PSI – Photosystem I

PSII – Photosystem II

PSY - Phytoene synthase

PUVA – Psoralen plus ultraviolet light A

ROS – Reactive Oxygen Species

SDH – Succinate dehydrogenase

sGC - Soluble guanylate cyclase

SOD - Superoxide dismutase

SYK – Spleen Tyrosinase Kinase

Th – Helper T cell

TNF *a* - Tumour necrosis factor alpha

TPC – Total Phenolic Content

Treg – Regulatory Tcell

UV – Ultraviolet

VEGF - Vascular endothelial growth factor

VFDF – Very Fast Death Factor

ZDS - ζ-carotene desaturase

Z-ISO - ζ-carotene isomerase

1. Introduction

1.1. Cyanobacteria

Cyanobacteria represent a group of gram negative photoautotrophic prokaryotic organisms capable of same-compartment oxygenic photosynthesis and respiration, of which fossil records date back to roughly 3500 million years ago (Kamal et al., 2014). Geological evidences include the presence of microbially laminated structures (stromatolites); additionally, cyanobacterial microscopic fossils and isotopic carbon data consistent with Rubisco-mediated CO_2 -fixation (Whitton and Potts, 2012), proves the primordial cyanobacteria's pliability and surviving success as an organism. Symbiotic associations of nitrogen fixing cyanobacteria (*Anabaena* or *Nostoc*) can be established with more complex biota, as the floating fern *Azolla* (Vincent, 2009). The complex symbiotic associations between the cyanobacteria *Nostoc* sp. with *Zamia integrifolia* (a species of gymnosperm cycads plants) and *Gunnera* sp. (an example of symbiosis with angiosperm plants), further underlines the great versatility of these organisms regarding survival strategies (Bryant, 1994).

Upon their recognition in the 19th century, cyanobacteria have been included in the division of algae, being commonly denominated as blue-green algae or cyanophytes, due to their plant-alike photosynthetic properties. In 1960, taxonomic classification was greatly influenced by the developments in cellular organization research and, consequently, differentiating eukaryotes and prokaryotes, reformulating the former classification in algae groups, once cyanobacteria are essentially prokaryotes. Thusly, the prokaryotic membrane and cell organization became the unifying factor in the diverse Bacteria domain, where cyanobacteria are now included (Stanier and Bazine, 1977; Sharma et al., 2014) .

1.1.1. Morphology and Diversity

Cyanobacteria are morphologically like gram negative bacteria; they have a prokaryotic nature, with the characteristic cell membrane and peptidoglycan cell wall, normally lacking any type of complex membrane-bound organelles or a membrane enveloped organized nucleus (Stanier and Bazine, 1977). Cyanobacteria are often free living, but display the

capacity to form aggregates in colonies or filaments (**Fig. 1**), regularly surrounded by a mucilaginous sheath, responsible for keeping their shape and structure (Singh et al., 2005).

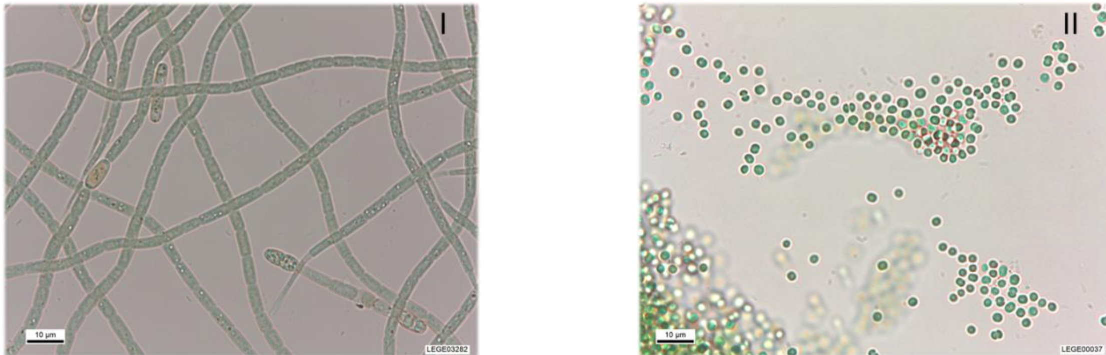


Fig. 1 – Different cyanobacteria morphologies. Representation of filamentous (**I** - *Cuspidothrix issatschenkoi* **LEGE03282)** and unicellular (**II** - *Synechocystis salina* **LEGE00037)** morphologies. (Photographs kindly provided by the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC, http://lege.ciimar.up.pt/), CIIMAR)

In order to better characterize and further study the diversity of these organisms, a classification system was required, therefore, Table 1 shows a fairly accepted model based on morphological and reproductive standpoints (Singh et al., 2005).

Table 1 – Classification of cyanobacteria according to morphological and reproductive characteristics.*

Cyanobacteria Classification		
Morphology	**Reproduction**	**Order**
Unicellular or Colonial	Binary Fission	Chroococcales
Unicellular or Colonial	Multiple Budding Fission	Chamaesiphonales
Filamentous	Trichome Fragmentation	Nostocales
Filamentous Heterocystous	Trichome Fragmentation	Notocaceae Rivulariaceae Scytonemataceae
Branched Filamentous	Trichome Fragmentation	Stigonematales

*(adapted from Singh et al., 2005).

The ability to form multicellular structures gives cyanobacteria the skill to compartmentalize and specialize molecular functions. For instance, the differentiation of vegetative cells leads to the formation of atmospheric nitrogen fixing structures, denominated heterocysts or, in order to cope with unfavorable conditions, to the formation of a tough reproductive structure termed akinete (Flores and Herrero, 2010). Similarly, their reproductive strategies include binary fission, budding fission, single plane trichome fragmentation (false branching) and multiple plane trichome fragmentation (true branching) (Table 1), which are deeply rooted to a taxonomically accurate study regarding the correct identification and characterization of cyanobacteria (Tatsumi et al., 2008). With a set of as much "polyphasic" data gathered, the classification and identification of cyanobacteria came to a 5 subsection system, hence: Subsection I and II comprise unicellular types where the former reproduces by binary fission and the latter can undergo multiple divisions, by formation of a large number of structures termed baeocytes, which eventually grow into full-fledged daughter cells; Subsections III, IV and V consist of filamentous cyanobacteria. Subsection III includes filamentous cyanobacteria consisting of one cell type, whereas Subsections IV and V consist of cyanobacteria that exhibit the ability to fixate nitrogen, and display a certain degree of differentiation, unique trait to prokaryotes, with heterocysts and akinetes. What differentiates subsections IV and V is the already mentioned false and true branching of trichomes, derived from one and multiple planes of division, respectively, providing cyanobacteria one of the most advanced types of morphological structures in the prokaryotic domain (Sharma et al., 2014).

1.1.2. Photosynthesis and Respiration

Cyanobacteria are thought to be unique in the sense that, photosynthesis and respiration are combined and present in the same compartment, the thylakoid, through the combination of transmembrane proteins. The thylakoid is the main photosynthetic structure in cyanobacteria. Here, the internal membrane system, that separates the cytoplasm from the thylakoid lumen, contains intersected photosynthetic and respiratory electron transport chains, represented in Figure 2 (Vermaas, 2001). The electron transport chain is composed by four enzyme complexes, each one with its own function, namely: photosystem II (PSII), cytochrome b_6f (cytbf), photosystem I (PSI), and H^+-translocating ATP synthase (F_0F_1, cF_0F_1) (Pfeil et al., 2014). In cyanobacteria, an antennae structure denominated phycobilisome is responsible for harvesting light energy and migrate it towards both

chlorophyll (closed tetrapyrrole) bearing photosystem complexes (I and II) in the photosynthetic transport chain. They vary in shape and configuration, though a common core-rod structure is displayed.

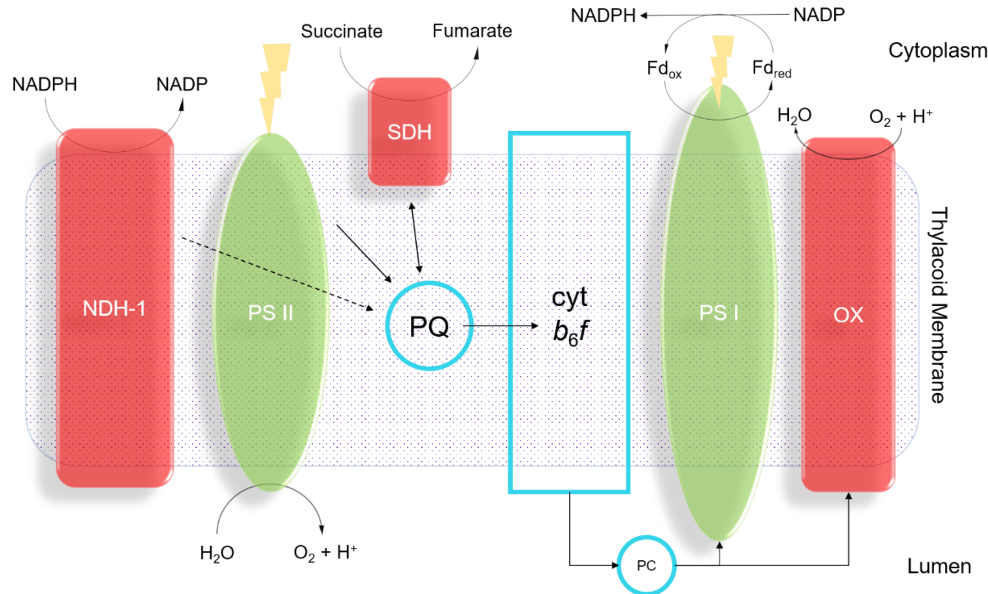


Fig. 2 – Schematic representation of the intersected thylakoid membrane respiratory and photosynthetic electron transport chains. Red protein complexes ■ (NDH-1 – Type 1 NADPH (nicotinamide adenine dinucleotide phosphate) dehydrogenase; SDH – Succinate dehydrogenase; OX – Terminal Oxidase) are exclusively used for the respiratory electron transport chain. Green protein complexes ■ (PS II – Photosystem II; PS I – Photosystem I) are solely used in the photosynthetic electron transport chain; the blue outlined protein complexes □ (PQ Plastoquinone; cyt b_6f – cytochrome b_6f complex; PC - Plastocyanin) are used by both transport chains. Yellow ligh bolts ▼ represent the light stimulation of each photosystem. Adapted from (Vermaas, 2001)

The major molecules that constitute phycobilisomes are pigmented biliproteins (open-chain tetrapyrroles) that subdivide into three energy levels: high energy (phycoerythrins (red) or phycoerythrocyanins (magenta) pigments, with A_{max} at 560 nm), intermediate energy (phycocyanins (cyan-blue pigments), with A_{max} at 620 nm), and low energy (allophycocyanins (blue pigments), with A_{max} at 650 nm) (MacColl, 1998; Whitton, 2012). Regarding light harvest, carotenoids also play an important role. These molecules are normally present and highly conserved throughout evolution in most photosynthetic organisms, including higher plants and algae. They function as key structural components of light harvesting centers, as accessory light harvesting pigments, as substrate for abscisic acid and as one of the foremost components of photoprotection, by dissipating excess energy and scavenging reactive species (DellaPenna, 1999).

Respiration aims to produce energy through the conversion of carbon containing molecules (usually sugars) into carbon dioxide (CO_2) and water (H_2O), achieving the reverse reaction

to photosynthesis, which produces sugars merging carbon dioxide (CO_2) and water (H_2O), using energy from light (Vermaas, 2001).

1.2. Cyanobacteria Bioactive Compounds

Some cyanobacteria, as well as microalgae, appear on historical records of human use, e.g., by Kanembu woman in Chad (Lake Kossorom), the Aztecs in Mexico (Lake Texcoco), also in China, Japan, Burma, Thailand, Vietnam, India and South America, and include the filamentous strains *Spirulina* (correct name *Arthrospira*), *Nostoc* (*N. commune, N. flagelliforme, N. punctiforme*), and the unicellular strains: *Alphanotheca sacrum, Spirogyra and Oedogonium*. All include the use of cyanobacteria biomass in many ways, from the development of characteristic dishes along the generations, to their inclusion in supplements, taking advantage from the active components of these organisms, with interest for human´s health and countries economy (Gantar and Svirčev, 2008). For instance, cyanobacteria are known to produce an important group of biopolymers with significant ecological importance in the microbial community, and economic interest in food, pharmaceutical, bioremediation and flocculation industries, the exopolysaccharides (EPSs). These compounds are complex molecules composed of six or more different monosaccharides out of a pool of at least twelve sugars. Most EPS's fall into one of three categories: *i)* endogenous polysaccharides – found in α-granules, and comprised of a branched glycogen-like polymer; *ii)* cell envelope polysaccharides – the cell wall polysaccharides and external layers of the glycocalyx; and *iii)* extracellular polysaccharides. The glycocalyx can be subdivided into: *a)* a well-structured polysaccharide sheath; *b)* a polysaccharide capsule that extends outside the sheath, although less structured; and *c)* mucilage polysaccharides, which are very loosely associated with the organism and most identifiable as true exopolysaccharide (Whitton, 2012).

The potential for cyanobacteria-based industries is well described throughout their applications in energy (hydrogen production and oil-degrading bacteria), aquaculture, wastewater treatment, food and fertilizers industries. As the research grows, many potential applications are being discovered: for instance, the combination of metabolic engineering with the advances in photobioreactor technology, promise to offer solutions for CO_2 sequestration, biofuels, production of added-value products and inoculants for integrated nutrient and pest management in agriculture (Gupta et al., 2013). Besides this, in recent years, blue biotechnology has gained increased attention, due to the importance of marine

resources on the path of discovering novel bioactive compounds able to fulfil the demanding pharmaceutical industry. Biotechnologically, cyanobacteria are very attractive organisms, being seen as a rich and still underexplored source of bioactive metabolites with a wide range of biological activities with interest for humans' health. Among these compounds, those of greater pharmacological interest include alkaloids, peptides and terpenes (Abed et al., 2009).

Among the bioactive compounds referred above, carotenoids are worth of further exploitation, due to their well-known biotechnological applications, most related to their ability to display positive impacts on oxidative stress portrayed in immune response modulation, signalling transduction between cells, and anti-inflammatory response mechanisms (Vílchez et al., 2011). Nowadays, carotenoids are used in food and nutrition as vitamin A precursors, with results proposing a mitigation of oxidative damage, that constitutes a major risk in chronic diseases. In immune systems regulation, these compounds enhance both specific and nonspecific immune functions, with proposed modus operands that include: *i)* quenching of reactive species; *ii)* quenching of immunosuppressing peroxides; *iii)* helping to maintain membrane fluidity and transmembrane proteins structure, essential for immune functions and *iv)* acting in the release of immunomodulatory lipid molecules, such as prostaglandins and leukotrienes. Regarding antimicrobial agents screening, carotenoids have reported anti-*Helicobacter pylori,* anti-*Staphylococcus aureus*, anti-*Escherichia coli* and anti-*Pseudomonas aeruginosa* activity, which represents a new breeding ground for new antimicrobial compounds against ever present and multidrug resistant bacteria (Kirti et al., 2014).

1.3. Carotenoids

Carotenoids are a group of molecules well known for their colors, ranging from yellow to orange and red. Normally produced and displayed in photosynthetic organisms as light harvesting molecules, carotenoids pass the energy to chlorophylls by a singlet-singlet excitation transfer, and also represent a substantial role in photoprotection by their reactive oxygen species (ROS) quenching capacity, thus preventing oxidative damage (Cogdell, 1985). Heterotrophic organisms don't usually exhibit *de novo* carotenoids synthetization, therefore depending on a regular dietary intake to obtain carotenoids (Stahl and Sies, 2005). Thusly, humans use these bioactive phytochemicals for their already mentioned antioxidant capacity, and for their long-term effects, due to the widespread presence of oxidative stress

in various chronic diseases such as cancer, cardiovascular disease, diabetes, psoriasis and osteoporosis (Rao and Rao, 2013).

1.3.1. Chemical Characterization and Biosynthesis

Carotenoids are tetraterpenoids composed by a carbon chain with multiple conjugated double bonds, with a whole linear structure or with terminal rings (Stahl and Sies, 2005). The biosynthesis of these compounds starts with the accumulation of isopentenyl pyrophosphate C5 (IPP) moieties in the plastids, followed by a condensation of four IPPs to form a C20 geranylgeranyl pyrophosphate (GGPP) molecule, mediated by the isopentenyl diphosphate isomerase (IPI) and geranylgeranyl pyrophosphate synthase (GGPS) enzymes. Here, a link between two GGPP molecules, catalyzed by phytoene synthase (PSY), originates the first C40 carotenoid, phytoene (Figure 3) (Tanaka et al., 2008).

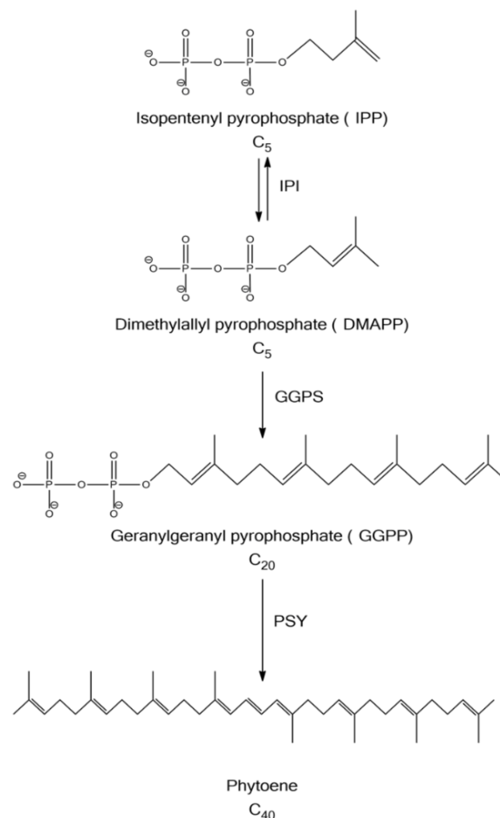


Fig. 3 – **Schematic representation of the first steps of carotenoids biosynthesis.** The accumulation of IPP (Isopentenyl pyrophosphate) is considered to be the first step into the carotenoid pathway. IPI (isopentenyl diphosphate isomerase) changes the position of the double carbon bond yielding DMAPP (dimethylallyl pyrophosphate), the five-carbon building block to synthetize a twenty carbon GGPP (Geranylgeranyl pyrophosphate) molecule, by the action of GGPS (Geranylgeranylpyrophosphate synthase). Finally, the enzyme PSY (Phytoene synthase) produces a 40-carbon chain with three conjugated double bonds denominated Phytoene. Adapted from (Fraser and Bramley, 2004).

The next step, portrayed in Figure 4, focusses on the conjugated double bonds, in a process mediated by an array of four enzymes: phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO). The first three enzymes yield phytofluene, ζ-carotene, neurosporene and lycopene, displaying 5, 7, 9 and 11 conjugated double bonds, respectively. Here, the number of conjugated double bonds results in the colorless phytofluene and phytoene, pale-yellow ζ-carotene, orange neurosporene and red lycopene. Carotenoid isomerase (CRTISO) has the function to convert *cis*-configuration intermediates to *trans*-configuration, resulting in the formation of *all-trans*-lycopene (Tanaka et al., 2008; Cazzonelli and Pogson, 2010). This *cis/trans* configurations gives carotenoids the possibility to present mono- and poly-*cis* isomers in addition to the *all-trans* form. Generally, the *trans* configuration is the most thermodynamically stable and nature predominant, despite the reported presence of various *cis* isomers in blood and tissues (Stahl and Sies, 2005).

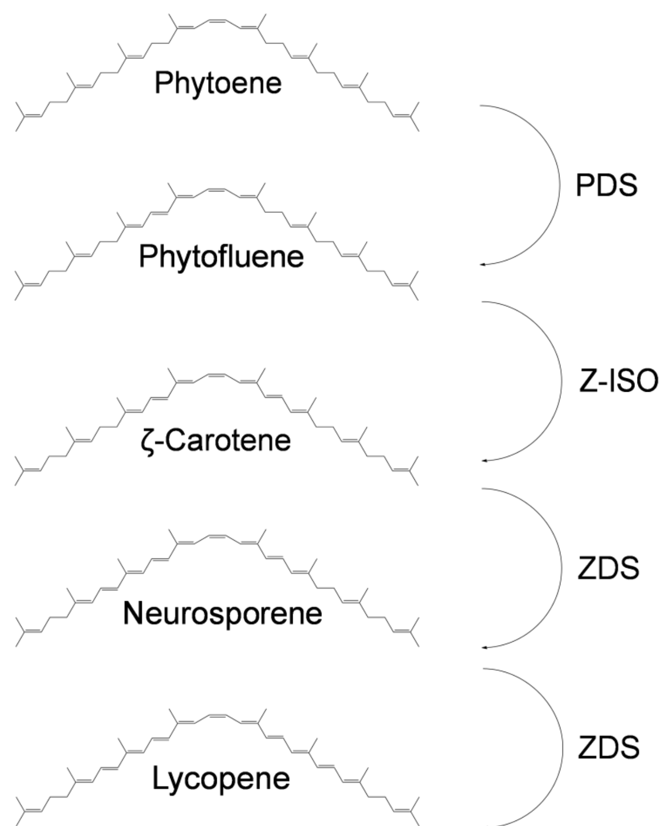


Fig. 4 – **Schematic representation of the second steps of carotenoids biosynthesis.** In this step, the main focus are the conjugated double bounds. Starting with 3 double bonds, phytoene gives origin to phytofluene with 5, by action of PDS (phytoene desaturase), followed by ζ-carotene, with 7, by the action of Z-ISO (ζ-carotene isomerase) and neurosporene, with 9, by action of ZDS (ζ-carotene desaturase). Adapted from (Namitha and Negi, 2010).

Lastly, lycopene goes through a cyclization process involving two enzymes (lycopene *β*-cyclase, LCYB, and lycopene *ε*-cyclase, LCYE) that constitute a branching point in carotenoid synthesis. Here, depending on the type of ring or combination of rings formed on each of the extremities of the molecule, two different pathways emerge, originating different carotenes (tetraterpenoids only comprised of carbon and hydrogen atoms) and xanthophylls (oxygenated tetraterpenoids derivatives due to, e.g., hydroxylation and epoxidation) (Figure 5) (Namitha and Negi, 2010).

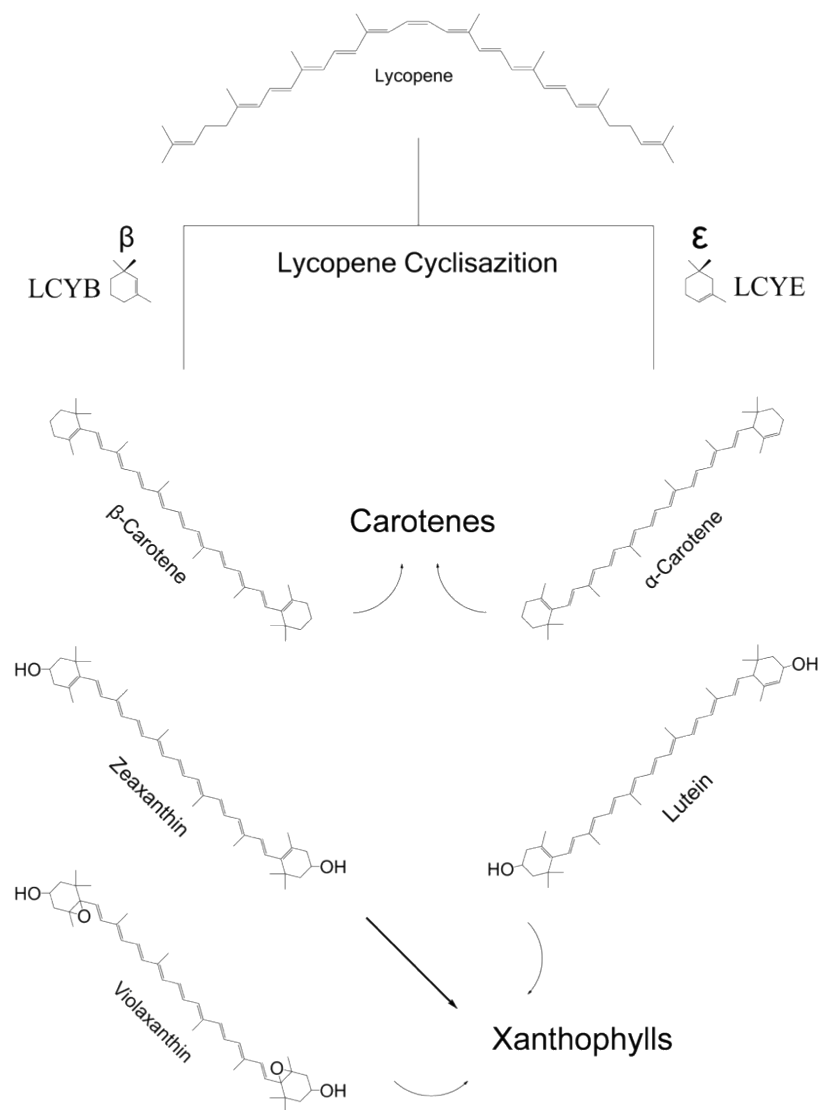


Fig. 5 – **Schematic representation of the third steps of carotenoids biosynthesis.** The CRTISO (carotenoid isomerase) enzyme forms *all-trans* lycopene, that will suffer a cyclization process. The two major enzymes involved in this process are the LCYB (Lycopene β-cyclase) and LCYE (Lycopene ε-cyclase). These enzymes add a β-ring or an ε-ring to the ends of the molecule, creating the branching point in carotenoid biosynthesis. LCYB has the ability to add one or two rings in the same lycopene molecule, leading to the formation of β-carotene; in contrast, LCYE only displays the capacity to add one ring to each lycopene molecule, generating an hybrid β, ε known as α-carotene. The first carotenes may suffer conformation alterations, as well as oxygenations, originating xanthophylls. Adapted from (Fraser and Bramley, 2004).

The qualitative and quantitative analysis of carotenoids in different matrices has gained increasing importance, largely due to their health benefit properties. Carotenoids profiling appears as necessary to understand their importance in humans' metabolism and health.
Even though a variety of methods have been employed in carotenoids profiling over the years, the most commonly used method for identification and quantification of carotenoids remains the High Performance Liquid Chromatography (HPLC) combined with UV–vis detection. Reversed-phase HPLC using C18 columns, with isocratic or gradient modes, constitute a good choice for carotenoids profiling, once this combination enables a significant increase in the interaction between analyte and non-polar stationary phase, leading to enhanced resolution of carotenoids. In recent years optimized processes allowed fast and sensitive methods for carotenoid characterization, including isomers distinction within a short period of time, which represents a valuable tool for profiling these bioactive compounds within a wide range of extracts (Gupta et al., 2015).

1.4. Cyanobacteria and Skin Conditions

Skin health and cyanobacteria came in contact not only by their already mentioned prolific applications in biotechnology but also by a branch of skin therapy called balneotherapy. Balneotherapy consists in the treatment of inflammatory skin diseases based on bathing in mineral water baths or pools with very specific geological formations (e.g. Kangal hot spring in Turkey; Comano spa in Italy; Salies de Béarn in France; and the Blue Lagoon in Iceland) presenting three main characteristics: spring origin, bacteriologically purity and therapeutic potential (Huld Eysteinsdóttir et al., 2017). Much of the chemical composition of these spring waters comes from their geological origin, where temperature and minerals play key roles. However, considering the presence of photosynthetic organisms thriving in extreme oxidizing inducing conditions, a link may be inferred between their presence, the bioavailability of antioxidant metabolites in water, and the amelioration of various diseases (inflammation, psoriasis and rheumatoid arthritis) with a straight link with oxidative stress (Trabelsi et al., 2016). In fact, this theory had been supported in the past, in a study conducted in a thermal lagoon in Iceland, where individuals with psoriasis have significantly improved their condition after bathing. Besides other characteristics of the water, it is believed that there is a link between the presence of the cyanobacteria *Leptolyngbya eredi var. thermalis* and the amelioration of the symptoms, once this species is not known to occur anywhere else in the world (Ólafsson, 1996).

1.4.1. Psoriasis

Psoriasis is a chronic inflammatory disorder with a reported prevalence between 2-3% in the world population (Grozdev et al., 2014). The different types and forms of skin lesions are often characterized by common erythematous papules with white thick scales, in most cases displayed on the extensor aspects of the elbows and knees, scalp, lumbosacral region and umbilicus area, as represented in Figure 6 (Griffiths and Barker, 2007; Nograles et al., 2010).

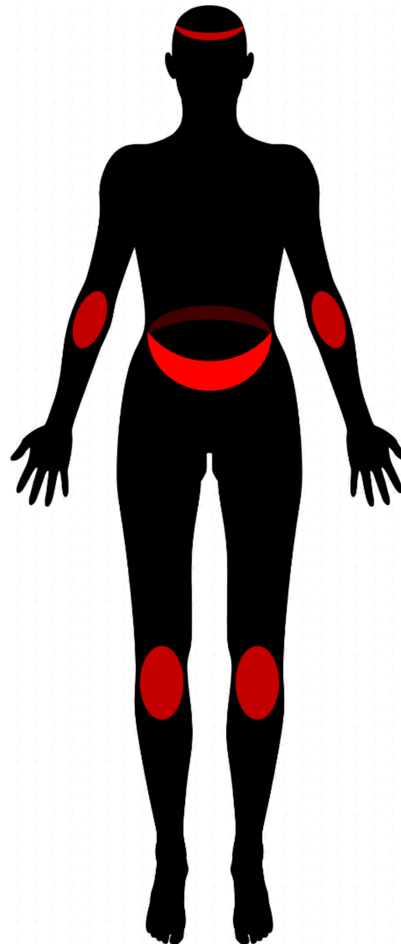


Fig. 6 – Schematic representation of the body areas most commonly affected by psoriasis: scalp, umbilicus area, lumbosacral region, and extensor aspects of both elbows and knees.

Histologically, psoriasis is characterized by hyperplasia, dilatation, proliferation of dermal blood vessels and accumulation of inflammatory cells, in particular neutrophils and T lymphocytes in the dermis (Mak et al., 2009). The vasodilation is mediated through an interaction between a vascular endothelial growth factor (VEGF, an angiogenic factor) and the angiopoietin/Tie signalling pathway, through tumour necrosis factor alpha (TNF *α*) which is, by itself, a key pro-inflammatory cytokine (Mak et al., 2009). Nowadays, six distinct

phenotypes of psoriasis are described: *i) Chronic plaque psoriasis*: affecting 80-90% of patients and being characterized by red, scaly and well demarcated discoid lesions, varying in size and thickness; *ii) Guttate psoriasis*: from the Latin "*gutta*" = drop, is the second most common phenotype, affecting 2% of the patients, and being presented as an acute eruption of teardrop-like skin lesions, affecting mostly the trunk and proximal extremities; *iii) Pustular Psoriasis:* characterized by the extensive development of sterile pustules on the skin's surface, including two subtypes: generalised pustular psoriasis and localised pustular psoriasis; *iv) Erythrodermic psoriasis*: a severe type of psoriasis, rarely appearing as a first occurrence of the disease, and described as presenting generalized erythema and fine scaling involving more than 90% of body surface; *v) Eczematous psoriasis*: eczema and psoriasis may be difficult to differentiate, however, eczema can be viewed as part of a clinical spectrum of psoriasis, or can affect already established psoriatic plaques, ending up in the term eczematous psoriasis; and *vi) Photosensitive psoriasis*: most patients experience a benefit when exposed to sunlight, nevertheless, 5-20% of patients have photosensitive psoriasis, where the exposition to sunlight may develop a photo-disturbed psoriasiform rash. The patients with fair skin of phenotype I or II seem to be the major risk population group (Eysteinsdóttir et al., 2017). The major problem arises in the unique and dynamic interactive pathways between immune cells and skin cells (Baliwag et al., 2015).

Under psoriatic conditions, keratinocytes, the skin key cells, suffer perturbed differentiation and proliferation, demonstrating the following characteristics: *i*) hyperplasia of the epidermis (acanthosis) with loss of granular layer; *ii*) regular elongation of the rete ridges (papillomatosis), *iii*) thickening of the corneal layer (hyperkeratosis), and *iv*) incomplete keratinocyte differentiation with retention of nuclei in the stratum corneum (parakeratosis) (Conrad and Flatz, 2013). Also, increased vascularity in the dermis is often linked with keratinocytes, which are now recognized as key drivers of abnormal dermal vascular proliferation and angiogenesis, with factors like vascular endothelial growth factor (VEGF) that inadvertently interacts with angiopoietin/Tie and is modulated by TNF*α* (Griffiths and Barker, 2007). Together with the above mentioned, the inflammatory framework linked with immune cells, namely T-lymphocytes and neutrophils, creates a network of cytokines (IFN-*γ*, TNF-*α*, interleukins IL-6, IL-8, IL-12, IL-17, IL-23, IL-22) that nowadays constitutes the core focus of investigation towards understanding the pathogenesis of psoriasis (Arican et al., 2005). New therapies may target specific waypoints in the network and hence, arrest the uncontrolled pathway in a more direct and tailored way. In this way, some promising results

have already been reported (Reich et al., 2001). Currently, the severity of psoriasis is measured with a tool denominated PASI (Psoriasis Area and Severity Index) score. The current treatment goal consists in the reduction of PASI ≥ 75% after 10 to 16 weeks (Huld Eysteinsdóttir et al., 2017). The recommended treatment for mild psoriasis begins with a topical therapy comprising corticosteroids, calcipotriol, tazarotene, tar, anthralin and keratolytics. When another type of therapy is needed, phototherapy becomes a choice, where ultraviolet light (UV) is used (broadband 290-320 nm/narrowband 311 nm, UVB), psoralen plus ultraviolet light A (PUVA) and climatotherapy, or systemic treatments (retinoids-acitretin, methotrexate, cyclosporine and fumaric esters) in refractory cases (Huld Eysteinsdóttir et al., 2017). The choice of a treatment consists in the analysis of the effectiveness/side effects, a fine balance representing a suitable therapy for each case. For the three therapies mentioned (topical, photo/climatotherapy and systemic therapies), the most effective is the systemic therapy, however, with more prominent side effects, and the least effective is the topical therapy, being the photo therapy the most commonly used in the majority of the cases (Huld Eysteinsdóttir et al., 2017). Nowadays, research is focused in the screening of alternative therapies, hopping to mitigate unwanted side effects, with effective results, and avoiding multiple therapy, thus constituting a benefit to the patient and to the healthcare services, also targeting the cost/effectiveness threshold that many of the newer compounds represent (Dubois Declercq and Pouliot, 2013).

1.4.1.1. Inflammation

Inflammation can be described as a succession of reactions occurring in a living tissue when it is injured (provided that the injury is not of such degree to destroy its structure and vitality), with the purpose of inactivate or destroy invading organisms, remove irritants and set the stage for tissues repair (Punchard et al., 2004). The signs and symptoms range from vascular permeability, vasodilation, vasoconstriction, and chemotaxis to leukocytes adhesion, pain, fever, and tissue/endothelial damage (Salvemini et al., 2003). As stress builds up in the affected tissue, ROS start to accumulate: e.g. superoxide radical anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), peroxide ion (O_2^{2-}) and singlet oxygen ($^1O_2$) (Apel and Hirt, 2004). These molecules represent, by themselves, messengers in the inflammatory network, their neutralization being important to halt or delay the progression of the inflammatory process (Reuter et al., 2010).

Keratinocytes play a central role in the histological profile and definition of this inflammatory disease. They are able to recruit and activate T cells [divided into T helper (Th1; CD4 +) and T cytotoxic (Tc1; CD8 +) that can be even more subdivided into Th17 cells and regulatory T cells (Treg)] which play a central role in the inflammatory network (Mak et al., 2009). Other cell types also important in this process include innate [keratinocytes, dendritic antigen-presenting cells, neutrophils, macrophages, natural killer (NK) and natural killer T (NKT) cells] and adaptative (CD4+ and CD8+ T lymphocytes) ones (Mak et al., 2009). The expansion and migration of T-cells and neutrophils to the epidermis precedes the beginning of psoriasis as these cells, activated by keratinocytes and antigen presenting dendritic cells (APDC), produce the cytokines IFN-γ, IL-2, and IL-12 that marks the start of the inflammatory pathway where posteriorly, the Th17/Th22 axis becomes the central point of equilibrium, unbalanced in psoriasis (Conrad and Flatz, 2013). Recently, one of the major focus is on the inflammatory mediators IL-17A, IL-22, TNF-α and IL-23, which are products of the imbalance of cytokines and interleukins secreting cells (Rashmi et al., 2009).

In some recent studies cyanobacteria were further highlighted as potential sources for promising anti-inflammatory compounds. Silambarasan et. al. reported the anti-inflammatory activity of *Trichodesmium erythraeum* evaluated through and *in vivo* carrageenan-induced paw oedema in winstar albino rats, reporting up to 57% oedema inhibition for animals treated with 300 $mg.kg^{-1}$ of *Trichodesmium erythraeum* aqueous extract (Silambarasan et al., 2011). In another study Garbacki et. al. reported a significant anti-inflammatory potential of capsular polysaccharides from cyanobacteria belonging to the genus *Phormidium* and *Nostoc,* against an *in vivo* croton oil-induced oedema, in a mice ear skin model. Results showed a 56% decrease in oedema weight (Garbacki et al., 2000). Considering *in vitro* models, Faltermann et. al. and Macagno et. al. reported the anti-inflammatory effects of a serine protease inhibitor from a microcystin-deficient *Planktothrix* strain, and an LPS antagonist from *Oscillatoria Planktothrix* FP1 in dendritic cells. Both demonstrated an effective response in anti-inflammatory biological activities with diverse modes of action (Macagno et al., 2006; Faltermann et al., 2016), thusly demonstrating a promising role for cyanobacteria in the screening for new anti-inflammatory drugs and therapies with relevant potential applications in human inflammatory diseases.

The Nitric Oxide

Nitric oxide (NO) is an inorganic compound, first discovered through endothelial cells´ ability to synthetize it from the conversion of $_L$-arginine into $_L$-citrulline, as a transcellular signal, as represented in Figure 7 (Knowles and Moncada, 1994). NO is synthetized by an enzyme called NOS (Nitric oxide synthase). This enzyme has three isoenzymes (nNOS, eNOS and iNOS) where the calcium-dependent nNOS and eNOS represent neuronal and endothelium NO synthases that produce NO as a neurotransmitter and vasodilator, respectively. INOS, the calcium independent inducible Nitric Oxide Synthase, produces high amounts of NO mainly in response to inflammatory stimuli (Reuter et al., 2010).

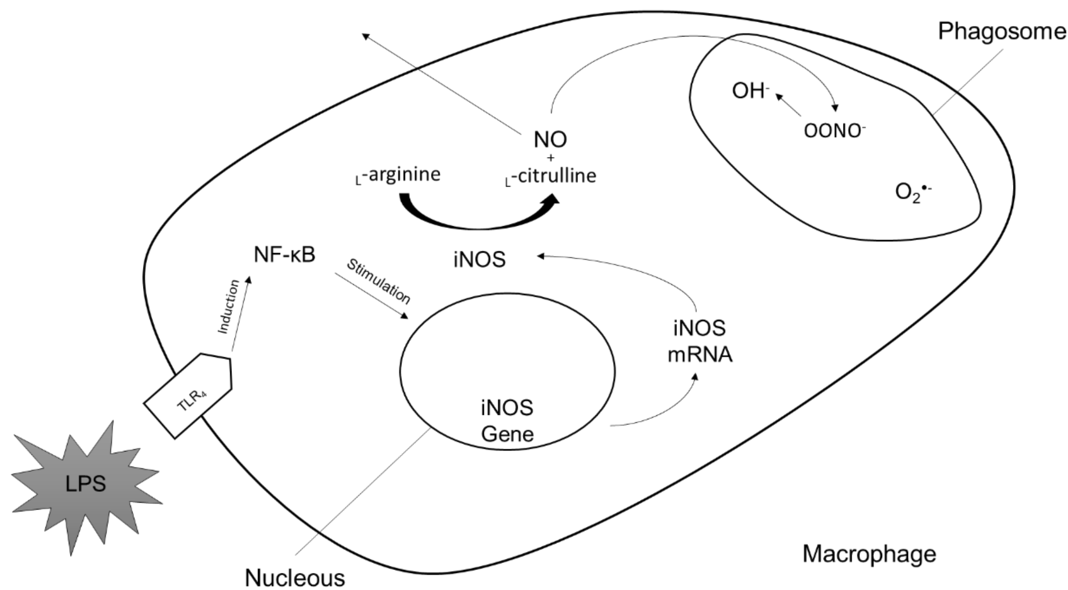


Fig. 7 – Schematic representation of the nitric oxide production in a macrophage model, on the onset of LPS (lipopolysaccharide)-induced inflammation, and its correlation with phagosomic reactive oxygen and nitrogen species. Adapted from (Brubaker et al., 2011).

The inducible isoform acts very differently when compared with the constitutive isoforms: eNOS and nNOS produce NO within seconds, with direct and short activities, while iNOS produces very large amounts of NO in a sustained manner (Salvemini et al., 2003). Large amounts of NO produced by iNOS can become problematic: when large amounts of NO are reached, the consequent increased production of reactive nitrogen and oxygen species leads to a brand-new field of biological effects, including imbalances in iNOS regulation which may emphasise the physiology and pathology of inflammation (Zamora et al., 2000) (Salvemini et al., 2003).

The two major NO signalling pathways are the cGMP-dependent (NO-sGC-cGMP pathway) and the cGMP-independent (NO oxidative pathway) (Vanini et al., 2015). The former represents an interconnected pathway involving soluble guanylate cyclase (sGC) and cyclic GMP (cGMP) whose increase leads to smooth muscle relaxation, vasorelaxation and decrease in platelet aggregation. The latter occurs mainly through S-nitrosylation of the cysteine residues, which affects S-nitrosylation regulated transcription factors such as NF-κB, thus affecting the progression of cancer. On the other hand, S-nitrosylation of caspase-3, caspase-9 and c-Jun N-terminal kinase inhibits apoptosis (Vanini et al., 2015). As such, NO became a central point in inflammation as it represents a sturdy and relevant molecule for anti-inflammatory activity assessment. J. Kim et. al. described an anti-inflammatory activity from *Lyngbya majuscule* isolated compounds (Malyngamide F acetate and Malyngamide S) based on the inhibition of NO production in LPS stimulated RAW 264.7 (Kim and Kim, 2013). Also, Bruno et. al. also reported the anti-inflammatory activity of monogalactosyldiacylglycerol isolated from *Phormidium* sp. in selective *in vivo* models, finding deep correlations between the modus operands of the molecule and its anti-inflammatory activity, by measuring levels of NOS, together with COX-1 and COX-2, well known for their key participation in the multifaceted inflammatory pathways (Bruno et al., 2005). The authors further highlighted the importance of NO in the modulation of inflammation, additionally validating the use of NO as a suitable indicator of the anti-inflammatory potential of extract and isolated compounds.

1.4.1.2. Oxidative Stress and Reactive Oxygen Species

The core basis of what is known as oxidative damage is defined as the imbalance between oxidants and antioxidants, favoring the prior, and potentially leading to tissues damage (Betteridge, 2000). The imbalance between oxidant and antioxidant agents, favoring the accumulation of the first, gives rise to a set of biomolecular damage caused by reactive species upon the constituents of living organisms. The type of damage includes damage to proteins (e.g. damage to the ion channels Na^+/K^+- ATPase, by fragmentation of the peptide chain, alteration of electrical charge of proteins, cross-linking of proteins, and oxidation of specific amino acids); damage to DNA (e.g. degradation of bases, single or double stranded DNA breaks, purine, pyrimidine or sugar-bound modifications, mutations, deletions, and cross-linking with proteins); damage to lipids (e.g. lipid peroxidation with consequent harmful byproducts, and disruption of the membrane lipid bilayer arrangement); and significant

alterations in signal transduction by activation of redox sensitive inflammatory transcription factors (e.g. NF-κB, AP-1, nuclear factor of activated T cells and hypoxia-inducible factor 1) (Halliwell, 1986; Birben et al., 2012).

The foremost reactive species involved in oxidative stress are commonly known as ROS. This term is used to describe short-lived diffusible entities based on oxygen. As an unavoidable outcome of aerobic metabolism, ROS can be classified as free radicals ($O_2^{\bullet-}$, $^{\bullet}OH$,) or nonradical molecules (H_2O_2, $^1O_2$, O_2^{2-}), formed by a leakage of electrons onto oxygen O_2 from the electron transport chain activities of chloroplasts, mitochondria, plasma membrane proteins, peroxisome, apoplast, and endoplasmic reticulum (Sharma et al., 2012). Cell responses to the oxidant/antioxidant balance include increased proliferation, prevention of cell division, senescence, necrosis, apoptosis and even cell death. At the same time demonstrating a double side with benefic effects that include the destruction of invading pathogens (oxidative burst) and, under specific circumstances, modulate the inflammatory response (Halliwell, 1986). As is, the term anti-oxidant is applied to any substance that, when present at low concentrations, compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate, maintaining redox homeostasis (Halliwell, 1991).

The first line of defense against oxidative stress is the prevention of the formation of ROS. The second is interception of the unavoidable formation of ROS by means of scavenging, through enzymatic and nonenzymatic mechanisms. Nonenzymatic mechanisms include the redox buffers ascorbate and glutathione (GSH-reduced form/GSSH-oxidized form), tocopherol (MDA - monodehydroascorbate/ DHA - dehydroascorbate), flavonoids, alkaloids, phenolic compounds, and carotenoids. Enzymatics comprise superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), CAT (catalase), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Apel and Hirt, 2004; Sharma et al., 2012). And finally, the third and last line of defense covers the repair of the inflicted damage consequent of a not completely effective interception step resulting in the accumulation of reactive species (Helmut, 1993). Face to the above, the search for new bioactive compounds able to avoid the establishment of oxidative stress, or capable of scavenging the already formed free radicals, constitutes an asset in the search for new drugs.

Some studies have already addressed the potential of cyanobacteria extracts. S. Patel et. al. described the antioxidant activity of C-phycocyanin isolated from *Lyngbya, Phormidium*

and *Spirulina*, reporting peroxyl radical and •OH scavenging properties correlated with this cyanobacteria molecule (Patel et al., 2006). In other studies, D. Kelman et. al. and O. Babić et. al. tackle the antioxidant capacity of different strains of cyanobacteria, including *Cyanothece, Anabaena, Trichodesmium, Prochlorothrix, Phormidium, Nostoc, Calothrix, Oscillatoria and Synechococcus,* by measuring their antioxidant capacity utilizing FRAP (Ferric Reducing Ability of Plasma) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays. D. Kelman et. al. reported a maximum antioxidant activity for *Trichodesmium,* with mean FRAP values of 66.9 and 41.7 µM per mg, in the non-polar and polar organic extracts, respectively. O. Babić et. al. found an IC_{50} of 30.72 µg.mL^{-1} for a crude ethanol extract of *Calothrix* M2, regarding DPPH radical scavenging, and 22.48 mg of ascorbic acid equivalents (AAE) per mg for a crude *Phormidium* M1 ethanol extract (Kelman et al., 2009; Babić et al., 2016). Hereby, it is demonstrated the antioxidant activity of cyanobacteria extracts as well as cyanobacteria isolated compounds. Additionally, in this studies, strong correlation between phenolic compounds and pigments with the reported antioxidant potential was inferred. This is mainly due to the quenching capacity of these molecules which, due to their molecular nature and specific functions, are responsible for dealing with oxidative stress in aerobic life forms, thus helping to maintain a fine balance in cell redox homeostasis.

2. Objectives

In order to better understand the biotechnological potential of cyanobacteria in the topical treatment of psoriasis, this work focused on the exploitation of different cyanobacteria extracts of the LEGE Culture Collection (lege.ciimar.up.pt) of CIIMAR, based on their chemical characterization and determination of antioxidant and anti-inflammatory potential, as well as their capacity to reduce keratinocytes hyperproliferation, characteristic of psoriatic skin.

The main goal of this project is to broaden the range of currently existing topical therapies for the treatment of psoriasis, aiming to find polyvalent alternatives, and with less side effects, through the exploitation of cyanobacteria.

To achieve the main goal of this project, the following tasks were established:

i. Cyanobacteria biomass production: culture, scale-up and harvest of different strains

ii. Preparation of different cyanobacteria extracts

iii. Chemical characterization of the extracts:
    - Total phenolic content – colorimetric assay;
    - Carotenoids profile by HPLC-PDA;

iv. Biological Activities of the extracts:
    - Radical scavenging capacity *in vitro*, in a cell-free system;
    - Anti-inflammatory potential *in vitro* using the macrophage cell line RAW 264.7;
    - Antiproliferative capacity *in vitro* using the keratinocytes cell line HaCAT;

3. Materials and Methods

3.1. Cyanobacteria Strains

Six cyanobacteria strains isolated from samples across the world, ranging from Portugal to Chile, Antarctica and Brazil, and maintained in the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC, http://lege.ciimar.up.pt/) at the Interdisciplinary Center of Marine and Environmental Research (CIIMAR/CIMAR), were selected to be the studied in this project (Figure 8, Table 2). The strains selected embrace two focal points: the first considers bioactivity studies previously reported (Costa et al., 2016; Morone Bavini et al., 2018), and favoring the least studied strains, and the second focuses the genetic biodiversity of cyanobacteria. The morphology of the selected strains (*Alkalinema aff. pantanalense* **LEGE15481**, *Cyanobium gracile* **LEGE12431**, *Nodosilinea (Leptolyngbya) antarctica* **LEGE13457,** *Synechocystis salina* **LEGE00037,** *Cuspidothrix issatschenkoi* **LEGE03282,** and *Leptolyngbya-like* sp. **LEGE13412**) is displayed in Figure 8.

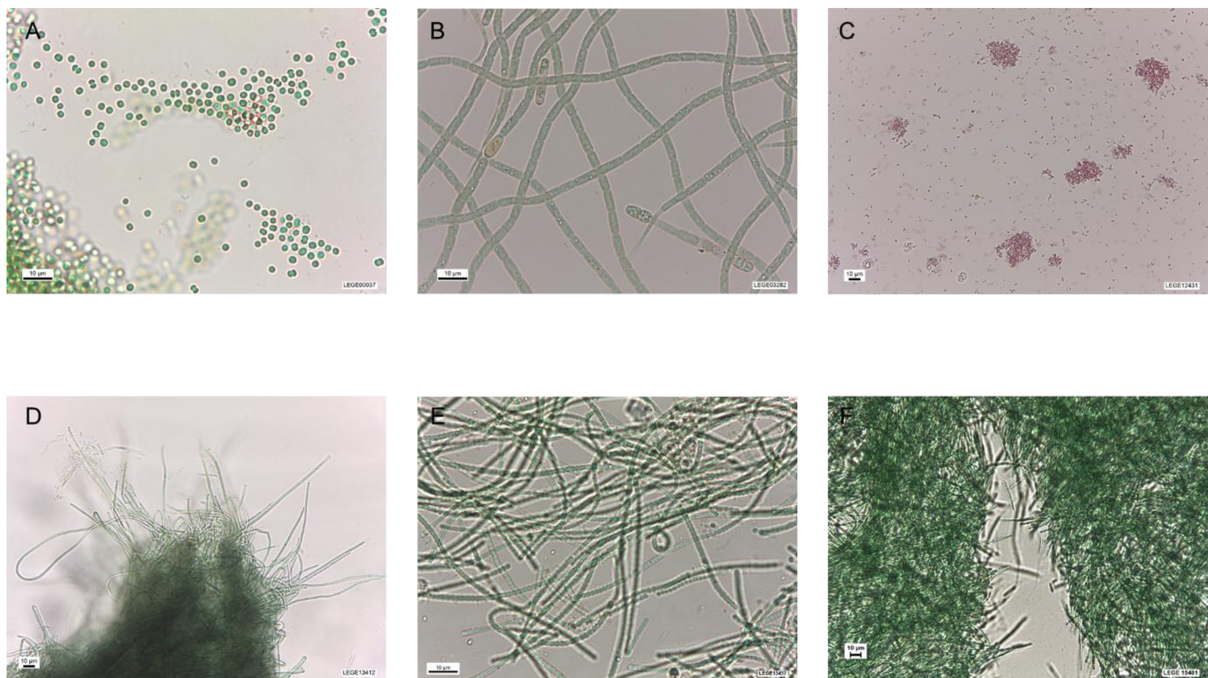


Fig. 8 – Morphology of the selected cyanobacteria strains. A - *Synechocystis salina* **LEGE00037**; **B** - *Cuspidothrix issatschenkoi* **LEGE03282; C -** *Cyanobium gracile* **LEGE12431**; **D** - *Leptolyngbya-like* sp. **LEGE13412**; **E** - *Nodosilinea (Leptolyngbya) antarctica* **LEGE13457**; **F** - *Alkalinema aff. pantanalense* **LEGE15481**. (Photographs kindly provided by the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC, http://lege.ciimar.up.pt/), CIIMAR).

Table 2 - Origin of the selected cyanobacteria strains*

Genus/Strain	LEGECC Code	Origin/Environment
Alkalinema aff. pantanalense	LEGE15481	Amazon River, Macapá – Brazil Freshwater (Smooth Biofilm)
Cyanobium gracile	LEGE12431	Caburgua Lake, La Araucania Region – Chile Freshwater (Aggregate Forming)
Nodosilinea (Leptolyngbya) antarctica	LEGE13457	McMurdo Dry Valleys, Victoria Valley – Antarctica Terrestrial (Smooth Biofilm)
Synechocystis salina	LEGE00037	Moledo Beach, Caminha – Portugal Marine (Aggregate Forming)
Cuspidothrix issatschenkoi	LEGE03282	Maranhão Dam Reservoir, Montargil – Portugal Freshwater (Homogenous Growth)
Leptolyngbya-like sp.	LEGE13412	Porto Metropolitan Area – Portugal Marine (Mucilaginous)

*(Courtesy of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC, http://lege.ciimar.up.pt/), CIIMAR)

3.2. Cyanobacteria Biomass: Culture and Harvest

The selected cyanobacteria strains were requested to the LEGE CC, and the biomass was obtained through a 10-fold scale-up culture process (40 mL, then 400 mL and finally 4 L). Terrestrial and freshwater strains (Table 2) were cultured in liquid Z8 medium (Kotai, 1972; Zickler, 1997) (composed of four solutions, of which: A – $NaNO_3$, $Ca(NO3)_2 \cdot 4H_2O$, and $MgSO_4 \cdot 7H2O$; B – K_2HPO_4, and Na_2CO_3; Fe-EDTA – $FeCl_3$, and EDTA-Na; Micronutrients solution, that includes tungsten (W), molybdenum (Mo), zinc (Zn), cadmium (Cd), coper (Cu), nickel (Ni), chromium (Cr), vanadium (V), aluminum (Al), manganese (Mn) and boron (B)) supplemented with vitamin B12, and for the marine strains, 25 $g.L^{-1}$ of NaCl was added. Growing conditions, at 25°C, comprised a photoperiod of 16 h/8 h light and dark cycles, respectively, with a light intensity of 10 mmol photons $s^{-1}.m^2$. After the strains achieve the proper growth, the biomass was harvested by centrifugation (7000 gs, 15 min; Thermo Scientific Bios 16, Germany). Marine strains were washed with distilled water to remove salts. Fresh harvested biomass was then freeze dried (Telstar LyoQuest) and kept at -20ºC until further analysis.

3.3. Extraction Process

Two kinds of extracts were prepared from cyanobacteria dry biomass: ethanol (70% *v/v*) and acetone. Briefly, 500 mg of dry biomass were suspended in 10 mL of the respective solvent, and sonicated (Vibra-Cell, USA) at a frequency of 70/80 Hz, for 3 minutes with 1-minute intervals in ice, in order to avoid sample heating. Afterwards, the mixture was centrifuged at 15000 x g for 10 minutes at 4°C, (Gyrozen 2236R, South Korea), to allow the sedimentation of all cell debris. The process was repeated five times for each solvent, and the supernatant resulting from the extraction was collected in a round bottomed flask and evaporated under reduced pressure (Buchi R-210 Rotavapor).

After evaporation, the extract was resuspended in a small volume of the original solvent (acetone or ethanol 70%) and transferred to weighted glass vials. The remaining solvent was further evaporated, and the vial was kept in a desiccator overnight to ensure the complete drying of the extract. The resulting mass was determined, and the extraction yield was calculated. The vials containing the dry extracts were stored at -20°C until further chemical and biological analysis. Both biomass and extracts were protected from light with aluminum foil during all the process.

3.4. Phytochemical Analysis

3.4.1. Total Phenolic Content

The total phenolic content (TPC) of the cyanobacteria extracts was determined through the Folin-Ciocalteu assay, as previously described (Barroso et al., 2016; Morone Bavini et al., 2018). Briefly, a volume of 25 µL of each cyanobacteria extract was carefully mixed with 500 µL of deionized water, 100 µL of Na_2CO3 solution (75 gL^{-1}) and 25 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) (or 25 µL of H_2O for blanks). The reaction was conducted for 60 minutes, in the dark, at room temperature. The absorbance of the resulting colored product was determined at 725 nm, using a Synergy HT Multi-detection microplate reader (Biotek, Germany), running GEN5™ software.

Gallic acid was used as reference phenolic compound, and the total phenolic content of the extracts was expressed in µg of Gallic acid equivalents (GAE)/mg dry extract, according to the calibration plot: $y = 2.101x + 0.03651$, $r^2 = 0.9998$ (Figure 9) [limit of detection (LOD) =

0.0021450 $mgGAE.mL^{-1}$, limit of quantification (LOQ) = 0.0064413 $mgGAE.mL^{-1}$]. Results were expressed as Mean±SD of three independent assays, performed in duplicate.

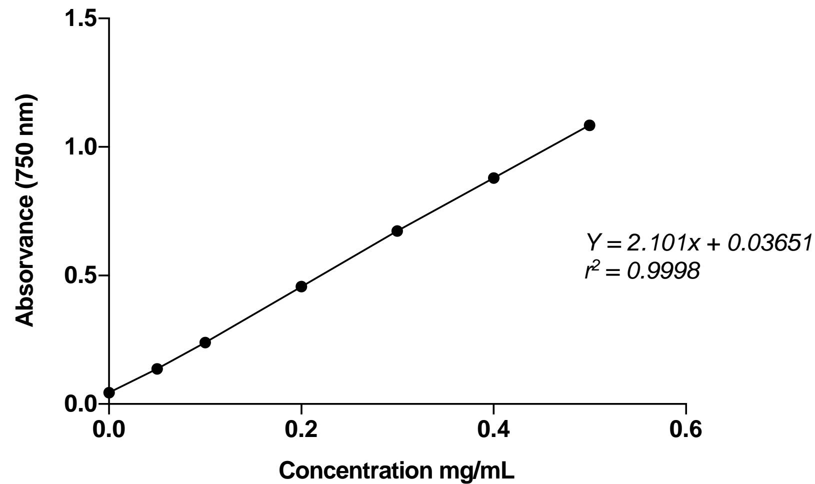


Figure 9: Calibration curve of Gallic acid.

3.4.2. Carotenoids and Chlorophylls profile

The determination of the carotenoids and chlorophylls profile was performed by HPLC with PDA detection, following a method described before (Amaro et al., 2015; Amaro et al., 2018), with minor modifications. The dried ethanol and acetone extracts were resuspended in ethanol (70%, *v/v*) or acetone, to a final concentration of 10 mg/mL or 5mg/mL, respectively, prior to HPLC analysis. Twenty microliters of each extract were analyzed using a Purospher® STAR RP-18 Endcapped (5 µm, 250 x 4 mm) chromatographic column, kept at a constant temperature of 25 ºC during the analysis, with a column heater (Waters, USA). The mobile phase consisted of two solvents, ethyl acetate (A) and acetonitrile:water 9:1 (*v/v*) (B), which were used to install the following gradient: 0% A at 0 min, 60% A at 31 minutes, 60% A at 36 minutes, 100% A at 38 minutes, 100% A at 43 minutes, and 0% A from 50 to 55 minutes. All the solvents used in the chromatographic analysis were filtered using a GH Polypro (GHP) Membrane Disk Filter, with 0.2 µm porosity (Pall, NY, USA) and degasified prior to analysis. The flow rate was 1 mL/min.

The resulting chromatograms were processed using Empower chromatography software (Waters, USA), with spectra data collected in range between 250 to 750 nm. The compounds identification was carried out by comparing the Ultra Violet-Visible spectra alongside the retention times with those of authentic standards. Posterior quantification was attained face to authentic standards, with the chromatograms recorded at 450 nm. Zeaxanthin, lutein,

echinenone, β-carotene and chlorophyll-*a* were quantified with their respective authentic standards. The unidentified carotenoids were quantified as zeaxanthin, β-carotene derivatives and α-carotene derivative as β-carotene, and the unidentified chlorophylls and chlorophyll-*a* derivatives as chlorophyll-*a*.

Table 3 – Calibration Curves of Carotenoid standards and Chlorophyll-a.

Standard	**Calibration Curve**	r^2	**LOD** ($mg.mL^{-1}$) [a]	**LOQ**[2] ($mg.mL^{-1}$)[b]
Lutein	y = 141092914x +5527	0.9998	0.0002867	0.0008688
Chlorophyll-*a*	y = 7471178x + 2673	0.9998	0.0014721	0.0044608
Zeaxanthin	y = 1406075946x - 138160	0.9987	0.0003949	0.0011965
β-Carotene	y = 290231487x + 172758	0.9997	0.0019354	0.0058649
Echinenone	y = 61438587x + 12035	0.9995	0.0005080	0.001538
Neoxanthin	y = 219321360x + 57794	0.9999	0.0004293	0.0013009
Canthaxanthin	y = 5662994x - 2341	0.9994	0.0030490	0.0092395

[a] limit of detection;
[b] limit of quantification;

3.5. Antioxidant Potential

Antioxidant potential of the cyanobacteria ethanol and acetone extracts was assessed by determining their capacity to scavenge ROS. In this assay the $O_2^{\bullet-}$ was selected for the antioxidant potential determination, and measured followed a methodology previously reported (Lopes et al., 2019). A range of dilutions was prepared for each extract. In a 96 wells plate, 50 µL of each dilution was mixed with 50 µL of a solution of β-nicotinamide adenine dinucleotide reduced form (166µM) (NADH) and 150 µL of nitrotetrazolium blue chloride (43 µM) (NBT) (kept protected from light). The reaction (Figure 10) was started by the addition of 50 µL of phenazine methosulphate (2.7 µM) (PMS) and the reduction rate of NBT was monitored at 562 nm for 2 minutes, using a Synergy HT Multi detection microplate reader operated by GEN5™ (Biotek, Germany) operating in kinetic function. Every solution was dissolved in phosphate buffer (19 mM, pH 7.4). At least three independent assays were performed in duplicate. Scavenging activity was expressed as the percentage of inhibition of $O_2^{\bullet-}$, relative to the control, according to the formula:

$$O_2^{\bullet -}\ Scavenging\ (\%) = 100 - [(A_S * 100) \div A_C]$$

Where A_S corresponds to the reaction rate of the sample and A_C to the reaction rate of the control.

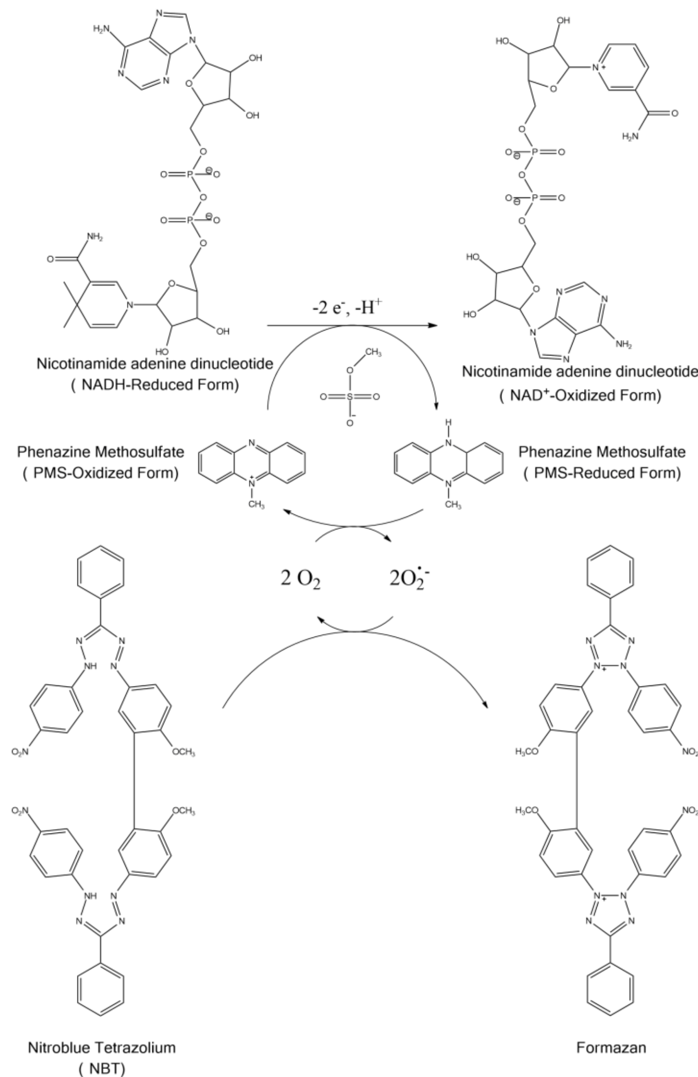


Figure 10 – **Schematic representation of the NADH/PMS/NBT system.** NADH - β-nicotinamide adenine dinucleotide reduced form; PMS - phenazine methosulphate; NBT - nitrotetrazolium blue chloride. Adapted from (Lopes, 2014).

The IC values and respective inhibition curves were calculated with Graphpad Prism® (version 7.0e) software, and the results were expressed as Mean ± SD (μg mL^{-1}) of at least three independent assays performed in duplicate.

3.6. Cell Assays

The murine macrophage cell line RAW 264.7, obtained from the American Type Culture Collection (ATCC), was selected as model for the determination of the anti-inflammatory potential of the cyanobacteria extracts under study.

3.6.1. Cell Culture and Treatments

RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Roti®-CELL) with glutamine, without pyruvate, supplemented with 10% (*v/v*) of inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Penicillin 100 U L^{-1}, Streptomycin 100 μL mL^{-1}) in a humidified atmosphere containing 5% CO_2 at 37°C. Whenever reaching about 80% confluence, RAW 264.7 cells were transferred to new flasks, by scraping. The culture medium was renewed every two days. Similar conditions were used for the maintenance of the keratinocytes cell line HaCAT, which were grown in the same culture medium, without FBS inactivation, and transferred by trypsinization.

Prior to extracts exposition, cells were seeded in 96 wells plates, at a density of 3.5×10^4 cells/well for RAW 264.7 and 2.5×10^3 cell/well for HaCAT, and incubated for 24h. Cyanobacteria extracts were prepared in DMEM, sterilized by filtration using a 0.22 μm pore membrane, and stored at -20°C until cells exposure. Serial dilutions (100, 50, 25, 12.5 and 6.25 μg dry extract/mL) of cyanobacteria ethanol and acetone extracts were prepared in DMEM with 0.25% DMSO, established as the maximum DMSO concentration non-interfering with the assays.

3.6.2. Anti-inflammatory Potential

Upon a harmful stimulus, macrophages overexpress iNOS, leading to an increased release of NO to the extracellular space. One of the formed products, nitrite, is stable and thusly, measurable, being a quite good indicator of the inflammatory state. After a pre-treatment of 2h with the extract's serial dilutions (or vehicle), RAW 264.7 cells were stimulated with *Salmonella enterica* lipopolysaccharide (LPS), at 1 μg mL^{-1}, and further incubated for 22h, at 37°C, in a humidified controlled atmosphere containing 5% CO_2. The effect of the extracts on NO production by RAW 264.7 cells, in the absence of LPS stimulation, was also evaluated, in order to cross out the direct effect of the extracts. NO released to the culture medium was measured through the Griess reaction (Figure 11) as before (Barbosa et al., 2017). Briefly, 75 μL of Griess reagent (sulfanilamide 10 mg mL^{-1} and ethylenediamine 1 mg mL^{-1}, prepared in H_3PO_4 2%) (Sigma-Aldrich, USA), were mixed with 75 μL of cells supernatant and incubated in the dark for 10 minutes. The absorbance of the reaction product was determined at 562 nm. At least four independent assays were performed in duplicate. Dexametasone was used as positive control.

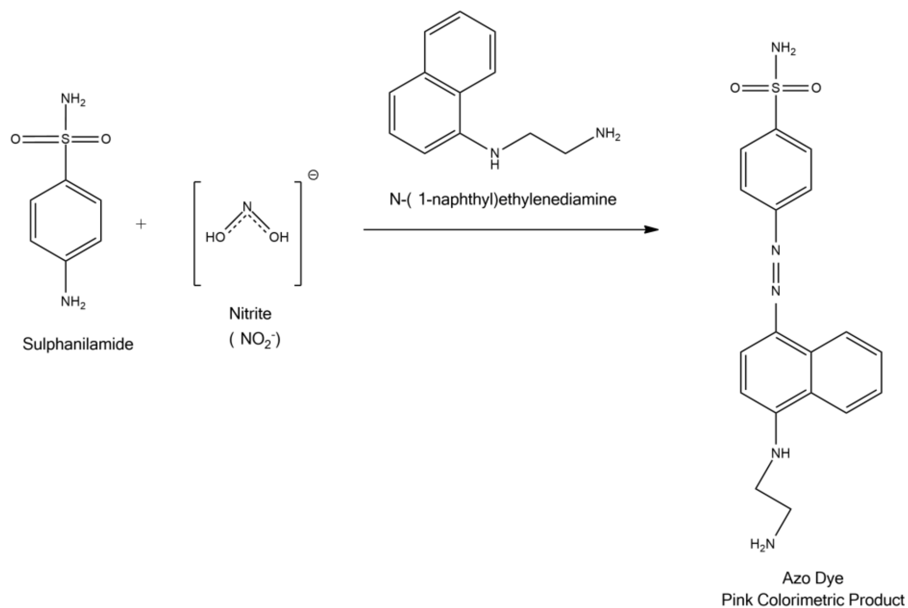


Figure 11 – Schematic representation of the Griess reaction (Promega, 2009).

3.6.2.1. Cytotoxicity on RAW 264.7 cells

In parallel with the determination of the extracellular levels of NO, the cytotoxicity of the extracts was monitored through the 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 12), following a methodology previously described (Barbosa et al., 2017). The assay consisted in the reduction of the yellow MTT to insoluble purple formazan crystals, mainly by the action of mitochondrial dehydrogenases of metabolically active cells. After the incubation period of 24h, 100 µL of MTT (0.5 mg/mL), freshly prepared in DMEM at 37°C, were added to each well and incubated at 37°C for 45 minutes. After that, the supernatant was removed, and the resulting formazan crystals were dissolved with 100 µL of DMSO. The absorbance of the colored product was determined at 515 nm, using a Synergy HT Multi-Detection microplate reader (Biotek, Bad Friedrichshall, Germany) operated by GEN5™ software. Cytotoxicity was expressed as the percentage of MTT reduction, face to the solvent control (0.25% DMSO). At least four independent assays were carried out in duplicate.

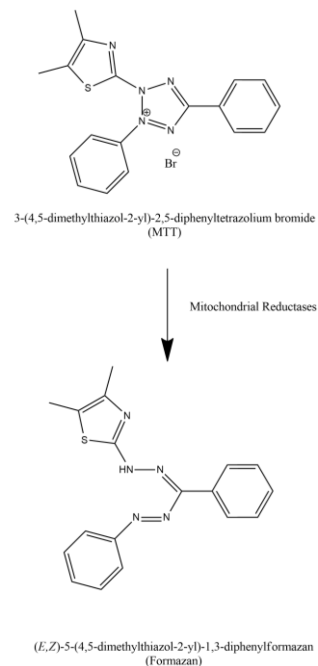


Figure 12 – **MTT reduction to formazan**. (adapted from (Kuete et al., 2017))

3.6.3. Anti-proliferative potential

Psoriatic skin is characterized by the presence of plaques originated through an abnormal proliferation and differentiation of keratinocytes. The capacity of cyanobacteria ethanol and acetone extracts to slow down keratinocytes hyperproliferation was determined by evaluating the viability of the keratinocytes cell line HaCAT after a 24h and 48h of exposition to the extracts, following a methodology previously described (Morone Bavini et al., 2018). Briefly, after 24h and 48h of incubation with the serial dilutions of the extracts, 20 μL of MTT solution were added to the cell's supernatant, and the cells were further incubated at 37ºC for 3.5h. After the incubation period, the culture medium was removed and the MTT assay was carried out as previously described. At least four independent assays were performed in duplicate. DMSO (20%) was used as positive control.

3.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7.0e for Mac OS X (GraphPad Software, La Jolla, California, USA). One-way ANOVA followed by Tuckey' HSD multiple comparisons test, or Two Tailed unpaired t-test were performed.

4. Results and Discussion

4.1. Phytochemical Analysis

4.1.1. Carotenoids and Chlorophylls Profilling

The carotenoid and chlorophylls profiles of the selected cyanobacteria strains were established through HPLC-PDA analysis and are present in Table 4 and Figure 13. Overall, 58 compounds, including 50 carotenoids (1-36, 38, 40, 41, 43, 45-49, 54-58) and 8 chlorophylls (37, 39, 42, 44, 50-53) were detected. Compounds with different retention time but with the same spectra of the standards were labeled as derivatives (10, 15, 17, 18, 21, 25, 27, 36-41, 44-53, 55, 57, 58).

Total carotenoids ranged from 0.059 $\mu g.mg^{-1}$ in *Synechocystis salina* LEGE00037 ethanol extract to 63.701 $\mu g.mg^{-1}$ in *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 acetone extract. Similarly, total chlorophylls fluctuated between 0.067 $\mu g.mg^{-1}$ and 417.576 $\mu g.mg^{-1}$ in *Leptolyngbya-like* sp. LEGE13412 ethanol extract and *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 acetone extract, respectively.

Regarding the carotenes all-*trans* β-carotene (54), α-carotene derivative (55) and 13-cis-β-carotene (56) were present in most of the strains and extracts, all-*trans* β-carotene (54) being the predominant carotene. Also, the highest amount of all-*trans* β-carotene (54) was found in *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 acetone extract (27.695 $\mu g.mg^{-1}$), contrasting with the lowest observed in the acetone extract of *Synechocystis salina* LEGE00037 (0.006 $\mu g.mg^{-1}$) (Table 4).

Concerning xanthophylls, lutein (23) was one of the least present xanthophylls among the samples, only being tentatively identified in *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 and *Leptolyngbya-like* sp. LEGE13412. The contrary was observed for its derivatives, which were much more abundant among the samples. Together with lutein (23), canthaxanthin (29) was one of the less abundant compounds. It was only quantified in the acetone extracts of *Cuspidothrix issatschenkoi* LEGE03282 (9.30 $\mu g.mg^{-1}$) (Table 4, Figure 13), and *Synechocystis salina* LEGE00037 (0.017 $\mu g.mg^{-1}$) (Table 4). Zeaxanthin (24) was present in the majority of the extracts. With the exception of *Cuspidothrix issatschenkoi* LEGE03282 (Figure 13), at least one extract of each strain showed the presence of this xantophyll, the higher values being found in the acetone extracts of *Cyanobium gracile* LEGE12431 (6.299 $\mu g.mg^{-1}$), followed by *Alkalinema aff. pantanalense* LEGE1581 (2.35 $\mu g.mg^{-1}$) (Table 4, Figure 13).

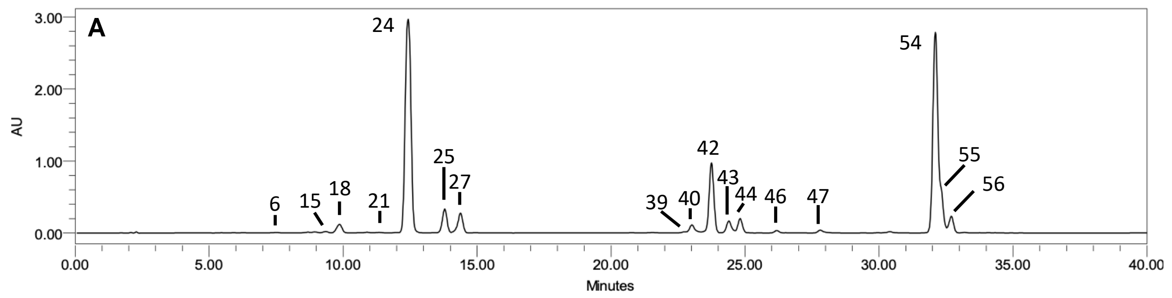
Table 4 – Carotenoids and Chlorophylls content in different extracts of cyanobacteria strains (µg/mg of dry extract), determined by HPLC-PDA[1,2].

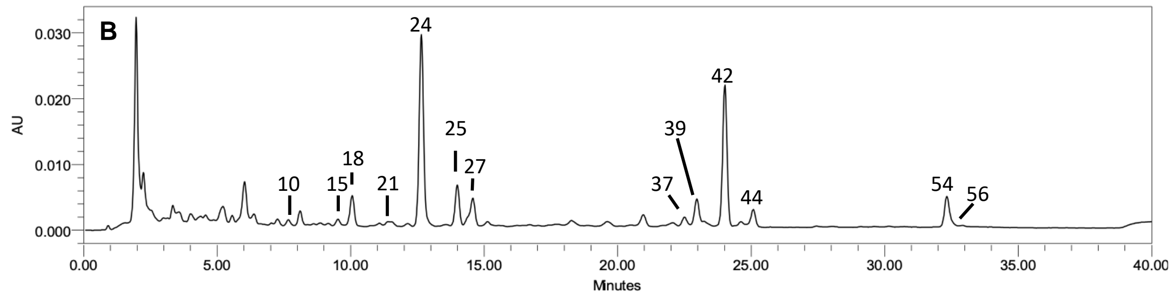
Peak	Compound	RT (min)	*Alkalinema aff. pantanalense* **LEGE15481**		*Cyanobium gracile* **LEGE12431**		*Nodosilinea (Leptolyngbya) antarctica* **LEGE13457**		*Cuspidothrix issatschenkoi* **LEGE03282**		*Leptolyngbya-like* sp. **LEGE13412**		*Synechocystis salina* **LEGE00037**	
			EtOH	**Acetone**	**EtOH**	**Acetone**	**EtOH**	**Acetone**	**EtOH**	**Acetone**	**EtOH**	**Acetone**	**EtOH**	**Acetone**
1	Unidentified Carotenoid	3.13	nd	nd	nd	nd	0.039 ± 0.006	0.093 ± 0.008	nd	nd	nd	nd	nd	nd
2	Unidentified Carotenoid	3.39	nd	nd	nd	nd	0.051 ± 0.008		nd	nd	nd	nd	nd	nd
3	Unidentified Carotenoid	4.31	nd	nd	nd	nd	0.016 ± 0.004	nd	nd	nd	nd	nd	nd	nd
4	Unidentified Carotenoid	4.32	0.033 ± 0.002	0.187 ± 0.003	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5	Unidentified Carotenoid	4.79	nd	nd	nd	nd	0.053 ± 0.003	nd	nd	nd	nd	nd	nd	nd
6	Unidentified Carotenoid	5.15	nd	nd	nd	nd	0.034 ± 0.003	0.451 ± 0.007	0.014 ± <0.001	0.069 ± 0.002	0.011 ± <0.001	0.110 ± 0.008	nd	nd
7	Unidentified Carotenoid	5.68	nd	0.100 ± <0.001	nd	nd	0.080 ± <0.001	nd	nd	nd	0.010 ± <0.001	nd	nd	nd
8	Unidentified Carotenoid	6.28	nd	0.055 ± <0.001	nd	nd	0.011 ± <0.001	nd	0.010 ± <0.001	nd	nd	nd	nd	nd
9	Unidentified Carotenoid	7.01	0.013 ± <0.001	0.050 ± <0.001	nd	nd	0.251 ± 0.004	1.298 ± 0.017	0.011 ± <0.001	0.058 ± <0.001	0.014 ± <0.001	0.410 ± 0.033	0.012 ± <0.001	$4.609E^{-5}$ ± <0.001
10	Beta carotene oxygenated derivative	7.64	nd	nd	nq	nq	1.259 ± 0.011	6.940 ± 0.118	nd	nd	nq	2.826 ± 0.251	nq	nq
11	Unidentified Carotenoid	7.75	nd	nd	0.011 ± <0.001	nq	nd	nd	0.010 ± <0.001	0.055 ± <0.001	nd	nd	nd	nd
12	Unidentified Carotenoid	8.21	0.015 ± <0.001	0.099 ± 0.001	nd	nd	0.055 ± 0001	0.269±<0.001	nd	nd	0.010 ± <0.001	0.082 ± 0.004	nd	nd
13	Unidentified Carotenoid	8.55	0.012 ± <0.001	nd	nd	nd	0.033 ± 0.001	0.127 ± 0.001	nd	nd	nd	nd	nd	$3.410E^{-5}$ ± <0.001
14	Unidentified Carotenoid	8.94	0.012±<0.001	nd	nd	nd	0.039 ± <0.001	0.120 ± 0.003	nd	nd	0.011 ± <0.001	0.061 ± 0.002	nd	
15	Lutein Derivative	9.17	0.014 ± <0.001	0.202 ± 0.014	0.003 ± <0.001	0.384 ± 0.011	0.354 ± 0.019	1.107 ± 0.002	nd	nd	0.004 ± <0.001	0.842 ± 0.126	0.003 ± <0.001	$8.427E^{-5}$ ± <0.001
16	Unidentified Carotenoid	9.68	0.010 ± <0.001	nd	nd	nd	0.054 ± 0.002	0.225 ± 0006	nd	nd	0.011 ± <0.001	0.093 ± 0.010	0.056 ± <0.001	$6.062E^{-4}$ ± <0.001
17	Lutein Derivative	9.87	0.019 ± <0.001	0.394 ± 0.015	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
18	Beta carotene oxygenated derivative	10.02	nd	nd	nq	1.102 ± 0.026	0.865 ± 0.009	5.589 ± 0.069	nd	nd	n.q.	2.255 ± 0.183	0.010 ± 0.001	$8.206E^{-4}$ ± <0.001
19	Unidentified Carotenoid	11.06	nd	nd	nd	nd	0.020 ± < 0.001	0.085 ± < 0.001	nd	nd	nd	nd	0.014 ± <0.001	$4.208E^{-5}$ ± <0.001
20	Unidentified Carotenoid	11.46	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.022 ± <0.001	$9.980E^{-5}$ ± <0.001
21	Lutein Derivative	11.50	nd	nq	nq	0.216 ± 0.007	0.193 ± 0005	0.826 ± 0.012	nd	nd	1.315E-4 ± 0.001	0.616 ± 0.053	nd	nd
22	Unidentified Carotenoid	12.17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.021 ± <0.001	$1.531E^{-4}$ ± <0.001
23	Lutein	12.19	nd	nd	nd	nd	0.152 ± 0005	0.582 ± 0.010	nd	nd	nd	0.315 ± 0.04	nd	nd
24	Zeaxanthin	12.64	0.146 ± 0.005	2.348 ± 0.001	0.033 ± <0.001	6.299 ± 0.025	0.088 ± <0.001	0.625 ± 0.008	nd	nd	0.012 ± <0.001	0.332 ± 0.032	0.052 ± <0.001	$7.090E^{-4}$ ± <0.001
25	Lutein Derivative	14.00	0.129 ± <0.001	2.497 ± 0.268	0.044 ± 0.001	5.916 ± 0.055	0.099 ± <0.001	0.485 ± 0.007	nd	nd	n.q.	0.444± 0.068	0.079 ± <0.001	$8.253E^{-4}$ ± <0.001
26	Unidentified Carotenoid	14.40	0.012 ± <0.001	nd	nd	nd	0.004 ± <0.001	0.170 ± 0.213	nd	0.080 ± <0.001	nd	0.066 ± 0.008	0.016 ± <0.001	$1.384E^{-4}$ ± <0.001
27	Lutein Derivative	14.58	0.078 ± 0.001	1.601 ± 0.081	0.039 ± <0.001	5.327 ± 0.121	nd	nd	nd	nd	0.002 ± 0.002	0.282 ± 0.040	0.001 ± <0.001	$3.845E^{-4}$ ± <0.001
28	Unidentified Carotenoid	14.94	nd	0.064 ± <0.001	nd	0.028 ± <0.001	nd	nd	nd	0.073 ± <0.001	nd	0.032 ± <0.001	nd	$5.055E^{-5}$ ± <0.001
29	Canthaxanthin	15.83	nd	nd	nd	nd	nd	nd	0.230 ± 0002	9.300 ± 0.220	nd	nd	nd	0.017 ± < 0.001
30	Unidentified Carotenoid	16.81	nd	nd	nd	nd	0.013 ± <0.001	0.052 ± <0.001	nd	nd	nd	nd	nd	nd
31	Unidentified Carotenoid	18.32	nd	nd	nd	nd	nd	nd	nd	0.062 ± 0.001	nd	nd	nd	nd
32	Unidentified Carotenoid	18.53	nd	nd	nd	nd	nd	0.061 ± <0.001	nd	nd	nd	nd	nd	nd
33	Echinenone Derivative	18.72	0.249 ± 0.004	5.797 ± 0.119	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

34	Unidentified Carotenoid	20.18	nd	nd	nd	nd	nd	nd	nd	0.079 ± <0.001	nd	nd	nd	nd
35	Unidentified Carotenoid	20.52	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.011 ± <0.001	$7.822E^{-5}$ ± <0.001
36	β-Carotene Oygenated Derivative	21.14	nd	nd	nd	nd	nd	nd	nd	n.q.	nd	nd	nd	nd
37	Chlorophyll-*a* Derivative	22.48	0.526 ± 0.005	nd	0.147 ± 0.010	nd	0.097 ± 0.002	0.672 ± 0.020	nd	nd	nd	1.084 ± 0.007	nd	$7.697E^{-4}$ ± <0.001
38	β-Carotene Oxygenated Derivative	23.11	nd	nq	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
39	Chlorophyll-*a* Derivative	22.94	7.147 ± 0.044	nd	0.502 ± 0.003	4.466 ± 0.083	nd	nd	0.475 ± 0.0103	11.295 ± 0.374	0.036 ± 0.017	nd	1.527 ± 0.016	0.008± <0.001
40	β-Carotene Oxygenated Derivative	23.67	nd	0.043 ± 0.004	nq	1.044 ± 0.021	nd	1.251 ± 0.022	nd	n.q.	nd	0.331 ± 0.042	nd	$1.857E^{-4}$ ± <0.001
41	β-Carotene Oxygenated Derivative	23.79	nd	0.104 ± 0.002	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
42	Chlorophyll-*a*	24.00	9.101 ± 0.030	nd	3.257 ± 0.037	325.047±5.764	11.729 ± 0.034	348.97 ± 4.190	1.854 ± 0.005	196.163 ± 4.469	0.031 ± 0.010	61.726 ± 5.346	8.910 ± 0.033	0.060 ± <0.001
43	Echinenone	24.61	nd	1.584 ± 0.037	nd	7.313 ± 0.152	0.300 ± 0.002	6.481 ± 0.127	0.060 ± <0.001	nd	nd	17.035 ± 1.432	0.779 ± 0.005	0.030 ± <0.001
44	Chlorophyll-*a* Derivative	25.06	0.907 ± 0.011	nd	0.420 ± 0.006	67.075 ± 1.441	1.010 ± 0.032	65.127 ± 0.957	0.464 ± 0.005	nd	nd	15.273 ± 1.314	0.840 ± 0.007	$1.600E^{-2}$ ± <0.001
45	Unidentified Carotenoid	25.59	nd	nd	nd	nd	nd	0.126 ± 0.002	nd	nd	nd	nd	nd	nd
46	Unidentified Carotenoid	26.19	nd	0.106 ± 0.001	nd	0.081 ± 0.001	nd	0.092 ± 0.001	nd	0.112 ± 0.001	nd	0.041 ± <0.001	nd	$3.627E^{-5}$ ± <0.001
47	Unidentified Carotenoid	27.92	nd	0.121 ± 0.003	nd	0.116 ± 0.004	0.015 ± <0.001	0.079 ± 0.003	nd	0.224 ± 0.005	nd	0.114 ± 0.007	nd	$3.827E^{-5}$ ± <0.001
48	Unidentified Carotenoid	28.67	nd	0.207 ± <0.001	nd	nd	nd	0.045 ± <0.001	nd		nd	nd	nd	nd
49	Unidentified Carotenoid	29.07	nd	0.078 ± 0.001	nd	nd	nd	nd	nd	0.077 ± <0.001	nd	nd	nd	nd
50	Unidentified Chlorophyll	29.51	0.391 ± 0.013	5.162 ± 0.081	nd	nd	nd	nd	nq	2.619 ± 0.034	nd	nd	nd	nd
51	Unidentified Chlorophyll	30.03	0.342 ± 0.005	2.100 ± 0.033	nd	nd	nd	nd	0.004 ± 0.007	1.794 ± 0.013	nd	nd	nd	nd
52	Unidentified Chlorophyll	30.61	1.016 ± 0.012	54.138±0.949	nd	nd	nd	2.809 ± 0.053	0.279 ± <0.001	26.106 ± 0.584	nd	8.120 ± 0.435	0.046 ± 0.002	0.007±<0.001
53	Unidentified Chlorophyll	31.19	nd	11.802±0.227	nd	nd	nd	nd	0.007 ± 0.006.	10.198 ± 0.171	nd	2.370 ± 0.042	nq	0.002±<0.001
54	All-*trans* β-Carotene	32.32	nq	15.196±0.779	nq	23.963±0.181	0.859 ± 0.021	27.695±0.337	nq	12.384 ± 0.249	nq	5.762 ± 0.389	nq	0.006±<0.001
55	α-Carotene derivative	32.89	nd	1.624 ± 0.289	nd	4.164 ± 0.257	0.060± 0.010	5.940 ± 0.030	nq	10.135 ± 0.349	nd	0.684 ± 0.141	nq	$4.14E^{-4}$ ± <0.001
56	13-*cis*-β-Carotene	33.37	nd	1.076 ± 0.0310	nq	1.874 ± 0.054	nq	2.884 ± 0.044	nd	1.708 ± 0.066	nd	0.371 ± 0.032	nq	$3.721E^{-4}$ ± <0.001
57	β-Carotene Derivative	34.74	nq	nd	nd	nd	nd	nd	nd	nq	nd	nd	nd	nd
58	β-Carotene Derivative	36.34	nd	n.q.	nd	nd	nd	nd	nd	nq	nd	nd	nd	nd
Total Carotenoids			0.742 ± 0.004^{e}	33.532 ± 0.271^{c}	0.131 ± <0.001^{e}	57.834 ± 0.277^{b}	5.028 ± 0.083^{d}	63.701 ± 0.592^{a}	0.335 ± 0.001^{e}	34.191 ± 0.631^{c}	0.084 ± 0.005^{e}	33.104 ± 2.054^{c}	1.075 ± 0.005^{e}	0.059 ± <0.001^{e}
Total Chlorophylls			19.430 ± 0.043^{f}	73.202 ± 0.912^{e}	4.327 ± 0.041^{h}	396.588 ± 5.154^{b}	12.835 ± 0.045^{g,h}	417.576 ± 3.691^{a}	3.083 ± 0.023^{h}	248.175 ± 3.992^{c}	0.067 ± 0.020^{h}	88.572 ± 5.041^{d}	11.323 ± 0.028^{g,h}	0.095 ± 0.001^{h}
Total Pigments			20.172 ± 0.046^{e}	106.733 ± 1.183^{d}	4.458 ± 0.041^{e,f}	454.422 ± 5.432^{b}	17.864 ± 0.128^{e,f}	481.277 ± 4.283^{a}	3.418 ± 0.022^{e,f}	282.366 ± 4.623^{c}	0.151 ± 0.024^{f}	121.675 ± 7.095^{d}	12.399 ± 0.033^{e,f}	0.153 ± 0.001^{f}

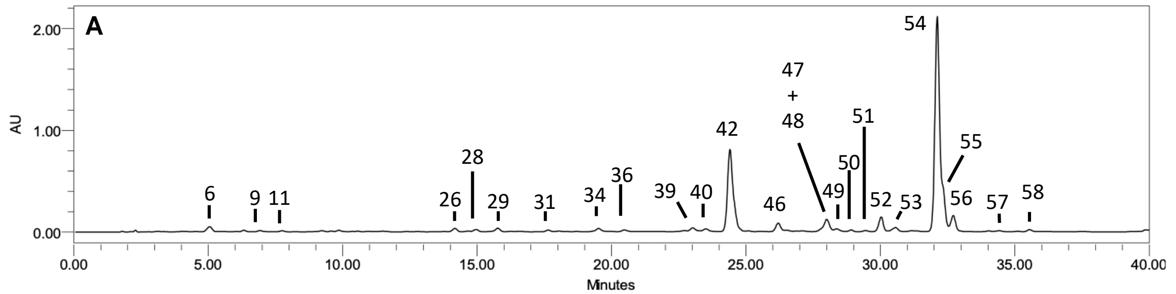
[1] Values are expressed as Mean±SD of three determinations;
[2] nd, not detected; nq, not quantified;
Different superscript letters in the same row denote statistical differences at $p<0.05$ (ANOVA, Tukey HSD)

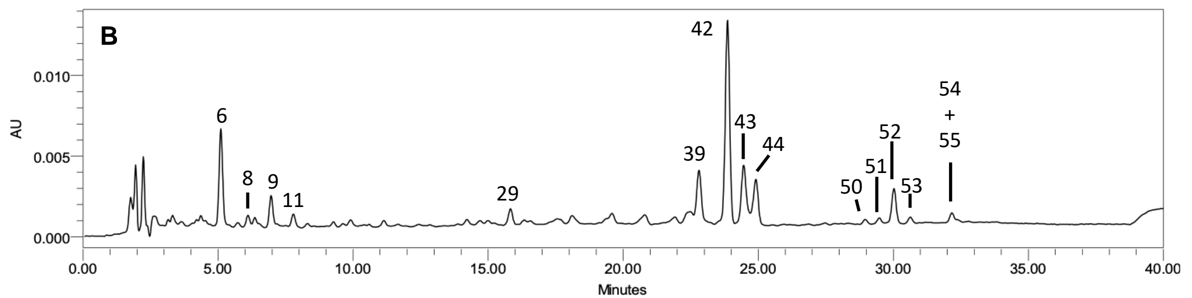
Cyanobium gracile LEGE12431





Cuspidothrix issatschenkoi LEGE03282





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Alkalinema aff. Pantanalense LEGE15481

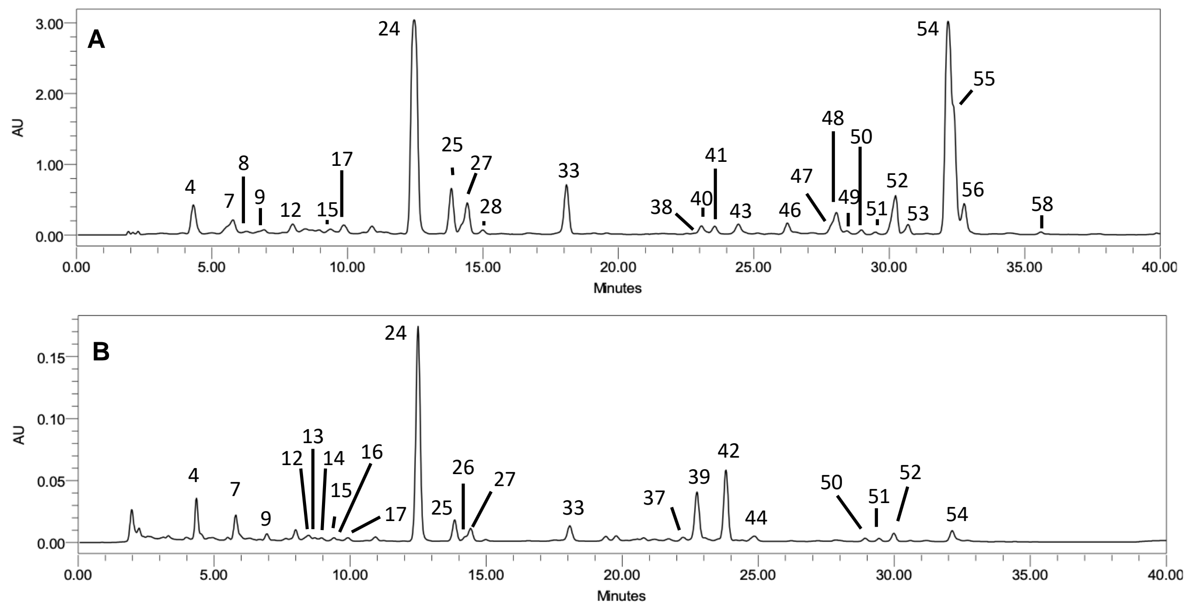

Fig. 13 – Carotenoid and chlorophylls profile of cyanobacteria extracts. HPLC-PDA recorded at 450 nm. **A** - acetone extract and **B** - ethanol extract, of the cyanobacteria strains *Cyanobium gracile* LEGE12431*, Cuspidothrix issatschenkoi* LEGE03282*,* and *Alkalinema aff. pantanalense* LEGE1581. Unidentified carotenoids (4, 6, 7, 8, 9, 11, 12, 13, 14, 16, 26, 28, 31, 34, 46, 47, 48, 49), β-carotene oxygenated derivatives (10, 18, 36, 38, 40, 41), Lutein derivatives (15, 17, 21, 25, 27), Echinenone derivatives (33), Chlorophyll-*a* derivatives (37, 39, 44), Unidentified Chlorophylls (50, 51, 52, 53), Zeaxanthin (24), Canthaxanthin (29), Chlorophyll *a* (42), Echinenone (43), all-trans β-Carotene (54), α-Carotene derivative (55), 13-cis-β-Carotene (56), β-Carotene derivatives (57, 58).

Lastly, echinenone (43) was the most prevalent xanthophyll as it was detected in all strains, with concentrations ranging from 0.030 $\mu g.mg^{-1}$ (in the acetone extract of *Synechocystis salina* LEGE00037) to 17.035 $\mu g.mg^{-1}$ (in the acetone extract of *Leptolyngbya-like* sp. LEGE13412) (Table 4).

Concerning carotenes, β-carotene is considered one of the most impactful carotenoid in human health, its functions including: vitamin A formation, inhibition of lipoxygenases, inhibition of HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase), cell to cell communication through connexin 43 stimuli, participation in the antioxidant/pro-oxidant axis, and influence in immunological responses and hormones fertility regulation (Burri, 1997). Most of the cyanobacteria extracts contained this compound and/or its isomers and derivatives (Table 4, Figure 13). As already mentioned, carotenes come before the oxygenation steps for xanthophyll synthesis and are widespread in nature. The presence of different β-carotene oxygenated derivatives throughout the extracts demonstrates the ability

of cyanobacteria to synthesize a wide range of carotenoids, thus enhancing the potential of these organisms for being explored for biotechnological purposes.

Regarding xanthophylls, the product of carotenes oxygenation, lutein (23), zeaxanthin (24), canthaxanthin (29) and echinenone (43) were tentatively identified (Table 4, Figure 13). The importance of lutein, zeaxanthin and echinenone in human health has already been described, zeaxanthin being reported in the central macular region of the eye's retina and lutein being distributed throughout the retina (Ribaya-Mercado and Blumberg, 2004). Additionally, together with echinenone and canthaxanthin, these molecules display the ability to scavenge ROS, being important antioxidants, also being reported for their tumor suppressing activity (Mathews-Roth, 1982; Fallis, 2013).

The strains *Synechocystis salina* LEGE00037, *Cyanobium gracile* LEGE12431 (Figure 13), and *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 are prone to comparison with other works. Morone et al. (Morone Bavini et al., 2018) described a similar carotenoid profile by HPLC-PDA for strains of the same genera as those analyzed herein. In the present study, *Synechocystis salina* LEGE00037 presented a value corresponding to total carotenoids of 1.075 $\mu g.mg^{-1}$ of dry extract, which corresponds to 145.735 $\mu g.g^{-1}$ of dry biomass (data not shown), and whose order of magnitude is in accordance with the results obtained by (Morone Bavini et al., 2018) (383.89 $\mu g.g^{-1}$ and 634.71 $\mu g.g^{-1}$, for total carotenoids and chlorophylls of *Synechocystis salina* LEGE06099, and 162.43 $\mu g.g^{-1}$ and 4762.90 $\mu g.g^{-1}$ for total carotenoids and chlorophylls of *Synechocystis salina* LEGE06155). Paliwal and co-workers (Paliwal et al., 2015) followed a similar methodology for pigments profiling, obtaining a value of 7.05 $mg.g^{-1}$ for total carotenoids and 12.90 $mg.g^{-1}$ for total chlorophylls, for the strain *Synechocystis* sp. CCNM 2501, which was higher than those obtained by us. These differences can be justified by the different extraction methodology, once Paliwal et al. extracted the compounds from fresh biomass. Regarding chlorophylls, *Synechocystis salina* LEGE00037 presented a total of 1622.578 $\mu g.g^{-1}$, which was lower than the values reported before. This can be justified by genetic differences between species, and also by different culturing conditions, namely photoperiod. Regarding *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457, we observed a higher amount in total carotenoids and chlorophylls content (Table 4), when compared with a strain of the same genus, *Nodosilinea nodulosa* LEGE06102, previously analyzed by Morone and co-workers (Morone Bavini et al., 2018). *Nodosilinea nodulosa* LEGE06102 presented 358.30 $\mu g.g^{-1}$ and 1425 $\mu g.g^{-1}$, dry biomass, of total carotenoids and chlorophylls, while, in the present work, we obtained 657.394 $\mu g.g^{-}$

^{1} and 4309.385 µg.g^{-1} (converted values; data not shown). Although sharing the same genus, species-specific differences regarding pigments profile were clear. Nevertheless, it is worth mentioning that *Nodosilinea nodulosa* LEGE06102 was sampled from a marine biome from the Portuguese coast and is commonly found attached to submerged stones in tidepools, whereas *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 is a terrestrial strain isolated from a frozen deserted Antarctic valley, which underlines a strongly divergent environmental stimuli. The Antarctica extreme conditions (low temperatures, high speed winds, and higher exposure to ionizing radiation) may significantly influence species metabolism, consequently affecting carotenoids and chlorophylls profile of *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457, possibly explaining the differences evidenced above. Lastly, *Cyanobium gracile* LEGE12431 presented an overall higher amount of carotenoids and chlorophylls (Table 4), when compared to the *Cyanobium* sp. LEGE06113 and *Cyanobium* sp. LEGE 07175 previously described (Morone Bavini et al., 2018). *Cyanobium sp.* LEGE06113 displayed 188.84 µg.g^{-1}, and 17963.97 µg.g^{-1}, and *Cyanobium sp*. LEGE 07175 with 169.38 µg.g^{-1}, and 1050.51 µg.g^{-1}, dry biomass, of total carotenoids and chlorophylls, respectively. Nevertheless, *Cyanobium* sp. LEGE06113 displayed a higher amount for total chlorophylls than those obtained herein. Additionally, *Cyanobium gracile* LEGE12431, displayed the highest amount of zeaxanthin and all-*trans* β-carotene among the strains evaluated in our study (Table 4).

In a general way, the pigments profile presented herein is similar to those previously reported by other authors, only differing in the presence of an α-carotene derivative (55) and a β-carotene isomer (13-*cis*-β-carotene - 56), tentatively identified by us (Table 4, Figure 13). For the species *Cuspidothrix issatschenkoi* LEGE03282, *Leptolyngbya-like* sp. LEGE13412 and *Alkalinema aff. pantanalense* LEGE1581, as far as we can ascertain, this is the first time that a carotenoids and chlorophylls profile is established.

4.1.2. Total Phenolic Content

Phenolic compounds are a large class of secondary metabolites that comprise different subgroups of a great variety of structures, as for example phenolic acids, xanthones, stilbenes, chromones, naftoquinones, anthraquinones, anthrones, flavonoids, lignans and lignins (Velderrain-Rodríguez et al., 2014), that are called so by the presence of at least one hydroxylated aromatic ring, the phenol group (Khoddami et al., 2013). These compounds are widely distributed in photosynthetic organisms, having been mainly described in cereal

grains, vegetables, legumes, fruits, nuts, berries and related processed foods (Velderrain-Rodríguez et al., 2014). Due to their molecular structures, phenolic compounds have demonstrated to be potent antioxidants, acting as reducing agents, singlet oxygen quenchers and chelators of metal ions. Other important biological activities have been reported for these compounds, namely anticarcinogenic, antiviral, antimicrobial, antithrombotic, anti-inflammatory and antimutagenic (Mustafa et al., 2010).

The total phenolic content (TPC) of the different extracts of the six cyanobacteria strains explored in study, was determined by the colorimetric method of Folin-Ciocalteu, and the results were expressed in µg of gallic acid equivalents (GAEs)/mg of dry extract (Table 5; Figure 14).

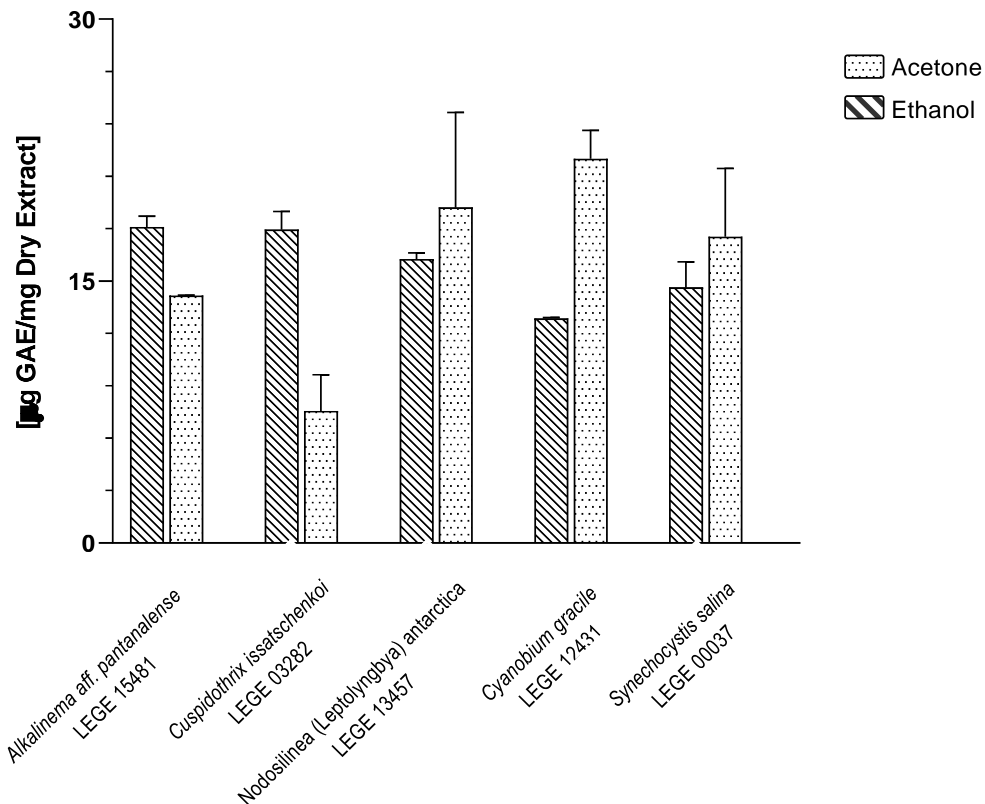

Figure 14 – Quantification of total phenols of cyanobacteria extracts. Results for the Folin Ciocalteu assay for acetone and ethanol extracts of the selected cyanobacteria strains, expressed in µg of GAEs/mg of dry extract. Results are the Mean±SD of three independent assays performed in duplicate.

TPC ranged between 7.58 and 22.01 µg GAE.mg of dry extract $^{-1}$. With the exception of the strain *Leptolyngbya-like* sp. LEGE13412, where the content in phenols was below the limit

of detection of the method, these compounds were quantified in all the remaining samples. There was not observed a clear distinction in the content of phenols according to the extraction solvent, but according to the species under study. *Nodosilinea (leptolyngbya) Antarctica* LEGE 13457, *Cyanobium gracile* LEGE 12431 and *Synechocystis salina* LEGE 00037 presented a higher phenolic content in their acetone extracts, while *Alkalinema aff. pantanalense* LEGE 15481 and *Cuspidothrix issatschenkoi* LEGE 03282 displayed the highest content in total phenols for ethanol extracts (Figure 14, Table 5). The acetone extract of the strain *Cyanobium gracile* LEGE 12431 was the one presenting the highest content in phenols (22.01 µg GAE.mg of dry extract $^{-1}$), and the acetone extract of *Cuspidothrix issatschenkoi* LEGE 03282 the lowest (7.58 µg GAE.mg of dry extract $^{-1}$).

Table 5 – Total phenolic content of cyanobacteria extracts (µg GAE.mg of dry extract^{-1}).

Strains	Extracts	
	Acetone	Ethanol
Alkalinema aff. pantanalense LEGE15481	14.17 ± 0.01^{b}	18.11 ± 0.43^{a}
Cyanobium gracile LEGE12431	22.01 ± 1.1^{a}	12.86 ± 0.04^{b}
Synechocystis salina LEGE00037	17.54 ± 2.76	14.65 ± 1.04
Nodosilinea (leptolyngbya) antarctica LEGE13457	19.23 ± 3.83	16.26 ± 0.25
Cuspidothrix issatschenkoi LEGE03282	7.58 ± 1.46^{b}	17.97 ± 0.71^{a}
Leptolyngbya-like sp. LEGE13412	n.d.	n.d.

Different superscript letters in the same row denote statistical differences at $p<0.05$ (Unpaired *t*-test)
Mean±SD of three independent assays

Previous studies concerning the determination of total phenols have been mainly devoted to strains belonging to the *Leptolyngbya and Synechocystis* genus. Badr and his team (Badr et al., 2019) reported up to 10.41 µg GAE.g^{-1} dry biomass for an extract of *Leptolyngbya*, while Ijaz and Hasnain (Ijaz and Hasnain, 2016), using a chromatographic method, reported a TPC of 829.7 µg.g^{-1} dry extract for a methanol extract of the same species. In another study, Babaoglu et al. (Babaoglu Aydaş et al., 2013) quantified the total phenols in methanol extracts of *Leptolyngbya* and reported a value of 26.6 µg gallic.mg^{-1} of dry extract for *Leptolyngbya* sp. BASO704 and 58.5 µg gallic . mg^{-1} of dry extract for *Leptolyngbya* sp. BASO700. Also, the same research group (Babaoglu Aydaş et al., 2013), explored the TPC in methanol extracts of different strains belonging to the genus *Synechocystis*, and found values of 43.4, 66.0 and 78.1 µg gallic.mg^{-1}. The values obtained for TPC of the cyanobacteria species considered herein were generally lower when compared with the previous studies. *Leptolyngbya*-like sp. LEGE13412 didn't display any quantifiable amount

of phenolic compounds and, therefore, differences in strains, growth conditions and extraction processes might explain the differences observed. As for *Synechocystis salina* LEGE00037, both acetone and ethanol extracts displayed a similar content in phenolic compounds with 17.54 and 14.65 µg GAE.mg dry extract $^{-1}$ even so falling short when comparing with the studies mentioned above.

4.2. Biological Activities

4.2.1. Antioxidant potential

The antioxidant potential of the ethanol and acetone extracts of the selected cyanobacteria strains was determined by the previously described $O_2^{\bullet-}$ scavenging assay. The $O_2^{\bullet-}$ represents one of many ROS produced as a byproduct of energy production in oxygenic respiration performing organisms. Hence, its accumulation or overproduction can lead to a disruption in cell redox homeostasis, being directly or indirectly correlated with a great amount of illnesses. The role of antioxidants in human health is of such importance that, the screening of molecules able to regulate the pro-oxidant/antioxidant axis are a constant asset in studies devoted to the exploitation of the biotechnological applications of different matrices (Shahidi et al., 1992; Valko et al., 2007).

The ethanol and acetone extracts of the cyanobacteria strains under study were assessed for their capacity to scavenge $O_2^{\bullet-}$, through an *in vitro* cell free assay. The dose response curves and IC values for ethanol (Table 6, Figure 15) and acetone (Table 7, Figure 16) extracts are presented below. Concerning ethanol extracts, only *Cuspidothrix issatschenkoi* LEGE 03282 and *Alkalinema aff. pantanalense* LEGE 15481 reached IC_{50}, the former being more effective (0.728 vs 1.322 mg.mL^{-1}) (p<0.05) (Table 6). It is noted that these two species were the ones presenting the highest content in phenols (Table 4), confirming the importance of these compounds in free radical scavenging.

Regarding *Cyanobium gracile* LEGE12431 and *Nodosilinea (Leptolynbbya) antarctica* LEGE13457, these species presented similar IC_{25} values for the ethanol extracts (Table 6), however, lower than that of *Cuspidothrix issatschenkoi* LEGE 03282, once more proving the importance of phenolic compounds in radical scavenging.

The strain *Synechocystis salina* LEGE 00037 displayed an interesting radical scavenging profile. Contrary to the other strains, which presented a dose-dependent radical scavenging capacity, *S. salina* LEGE 00037 had a dual behavior; the ethanol extract seemed to be

antioxidant for lower concentrations, acting in an opposite manner for higher ones (Figure 15).

Table 6 – Inhibitory concentration (IC) values (mg of dry extract.mL^{-1}) assessed for the superoxide anion radical scavenging assay of the ethanol extracts[1,2].

Strains	IC_{25}	IC_{50}
Alkalinema aff. pantanalense LEGE 15481	0.657 ± 0.041[b]	1.322 ± 0.201[b]
Cyanobium gracile LEGE 12431	0.458 ± 0.120[a,b]	-
Nodosilinea (Leptolyngbya) antarctica LEGE 13457	0.730 ± 0.153[b]	-
Synechocystis salina LEGE 00037	0.024*	-
Cuspidothrix issatschenkoi LEGE 03282	0.177 ± 0.039[a]	0.728 ± 0.065[a]

[1]Different superscript letters in each column denote statistical differences at p<0.05 (Ordinary one-way ANOVA)
[2]Mean±SD of at least two independent assays.
* IC_{25} only reached once.

A previous study (Morone Bavini et al., 2018) already reported the antioxidant potential of cyanobacteria ethanol extracts, through the DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) and $O_2^{\bullet-}$ scavenging assays, and included the screening of some strains of the same genera as the ones studied herein: *Synechocystis salina* LEGE06099, *Nodosilinea nodulosa* LEGE06102, *Cyanobium* sp. LEGE06113, *Synechocystis salina* LEGE06155, and *Cyanobium* sp. LEGE07175. The authors found that only *Synechocystis salina* LEGE06099 and *Synechocystis salina* LEGE06155 presented capacity to scavenge $O_2^{\bullet-}$, the first only reaching IC_{10} (1183.33 µg.mL^{-1}) and the latter achieving IC_{50} (1275.86 µg.mL^{-1}). All the other strains revealed to be ineffective to scavenge this radical. In this study, the strain *Synechocystis salina* LEGE 00037 showed an interesting antioxidant profile, represented in Figure 15, suggesting a mild antioxidant capacity at low concentrations, and the opposite behavior at higher ones. To the best of our knowledge, with the exception of *Synechocystis salina*, the antioxidant potential of the remaining species was reported herein for the first time.

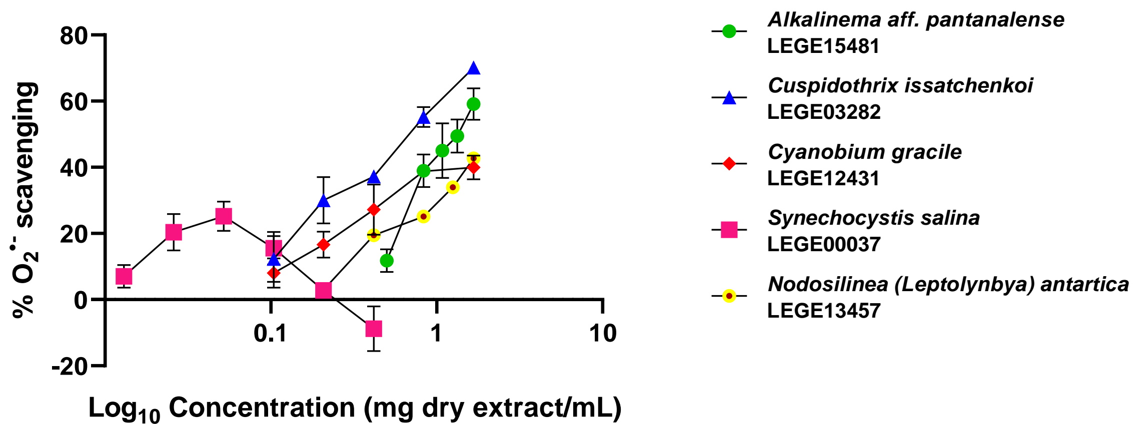

Figure 15 – Radical scavenging profile of cyanobacteria ethanol extracts. Results are expressed as % of superoxide anion radical ($O_2^{\bullet-}$) scavenging; Mean±SD of at least three independent assays performed in duplicate.

Regarding the acetone extracts, only 4 of the studied strains presented the ability to scavenge $O_2^{\bullet-}$. As previously observed for the ethanol extracts, a dose dependent radical scavenging activity was observed (Figure 16).

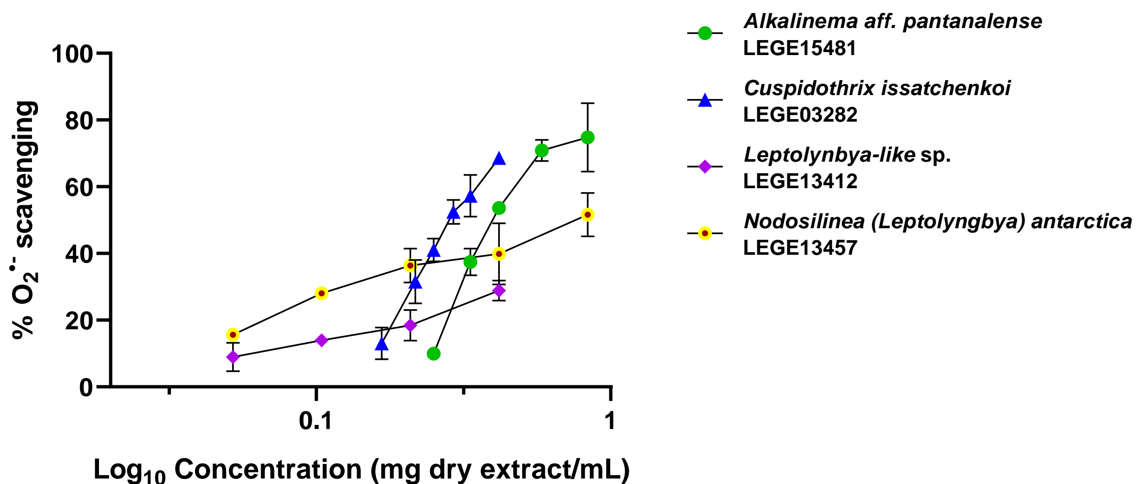

Figure 16 – Radical scavenging profile of cyanobacteria acetone extracts. Results are expressed as % of superoxide anion radical ($O_2^{\bullet-}$) scavenging; Mean±SD of at least three independent assays performed in duplicate.

The most effective strains were *Cuspidothrix issatschenkoi* LEGE 03282, with an IC_{50} of 0.286 mg.mL^{-1} and *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 (p<0.05), followed by *Alkalinema aff. pantanalense* LEGE 15481 (Table 7). The lowest IC_{25} was observed with *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 (0.095 mg.mL^{-1}) (p<0.05), followed by

Cuspidothrix issatschenkoi LEGE 03282, *Leptolyngbya-like* sp. LEGE 13412, and *Alkalinema aff. pantanalense* LEGE 15481 (with IC_{25} values of 0.198, 0.269 and 0.295 $mg.mL^{-1}$, respectively) (Table 7).

Table 7 - Inhibitory concentration (IC) values (mg of dry extract.mL^{-1}) assessed for the superoxide anion radical scavenging assay of the acetone extracts[1,2].

Stains	IC_{25}	IC_{50}
Alkalinema aff. pantanalense LEGE 15481	0.295 ± 0.005^c	0.382 ± 0.009^b
Leptolyngbya-like sp. LEGE 13412	0.269 ± 0.024^c	-
Nodosilinea (Leptolyngbya) antarctica LEGE13457	0.095 ± 0.002^a	0.319 ± 0.009^a
Cuspidothrix issatschenkoi LEGE 03282	0.198 ± 0.008^b	0.286 ± 0.012^a

[1]Different superscript letters in each column denote statistical differences at $p<0.05$ (ANOVA, Tukey HSD)
[2]Mean±SD of at least two independent assays

Anna and co-workers (Anna, 2016) evaluated the antioxidant potential of the cyanobacteria strains *Cyanobium* sp. LEGE 06113, *Synechocystis salina* LEGE 06099, *Cyanobium* sp. LEGE 07175, and *Nodosilinea nodulosa* LEGE 06102, for the radical scavenging activity of their dichloromethane:methanol (2:1, *v/v*) extracts, against $DPPH^{\bullet}$ and $O_2^{\bullet -}$, and presented the results as the maximum percentage of free radical scavenging. The strain *Cyanobium* sp. LEGE 06113 showed a maximum scavenging of 19.53% at 100 µg extract.mL^{-1} for $DPPH^{\bullet}$, with no positive results for $O_2^{\bullet -}$. The highest scavenging potential of *Synechocystis salina* LEGE 06099 was 1.95% at 10 µg extract.mL^{-1} for $DPPH^{\bullet}$, contrasting with the result obtained for $O_2^{\bullet -}$, which reached 49.57% of scavenging at 10 µg extract.mL^{-1}. *Cyanobium* sp. LEGE 07175 exhibited a maximum of 20.43% for $DPPH^{\bullet}$ scavenging at 100 µg extract.mL^{-1}, and 47.39% for $O_2^{\bullet -}$. scavenging at 10 µg extract.mL^{-1}. And finally *Nodosilinea nodulosa* LEGE 06102 displayed 12.22% of $DPPH^{\bullet}$ scavenging at 100 µg extract.mL^{-1} and 28.89% for $O_2^{\bullet -}$ at 10 µg extract.mL^{-1}. In our study the strain *Cyanobium gracile* (LEGE 12431) displayed a maximum $O_2^{\bullet -}$ scavenging of 42.46% at 1.67 mg dry extract.mL^{-1} in the ethanol extract (Table 6), which ranks above *Cyanobium* sp. LEGE 06113 and below *Cyanobium* sp. LEGE 07175 evaluated by these authors. As for *Synechocystis salina* LEGE 00037, a maximum of 30.30% was observed in the ethanol extract at 50 µg dry extract.mL^{-1} (Table 6), which was lower than the result obtained before for *Synechocystis salina* LEGE 06099. These differences between the same species, kept in optimal laboratory conditions, but sampled from different locations across the globe, can be justified by differences in

genetic code, probably due to subjection to different abiotic factors, that lead to different metabolites production. Lastly, in the acetone extract of *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 was perceived a maximum $O_2^{\bullet-}$ scavenging of up to 56% at 1.67 mg dry extract.mL^{-1}, a considerable difference face to results obtained by Anna et al., proving the huge variance that can occur when comparing different species of the same genus..

Overall the best performing strains in antioxidant potential were *Cuspidothrix issatschenkoi* LEGE 03282 and *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457, followed by and *Alkalinema aff. pantanalense* LEGE 15481 (p<0.05), all exhibiting IC_{25} and IC_{50} in one or both extracts. A strong link between this biological activity and the chemical profile of the extracts was noted. In a general way, extracts richer in phenols presented a higher radical scavenging activity, accompanied by carotenoids, which are known for their antioxidant potential. For instance, as it can be seen in Table 4, acetone extracts present a significantly higher carotenoids content than ethanol extracts (p<0.05), also displaying significantly lower IC values (p<0.05) regarding $O_2^{\bullet-}$ scavenging (Table 6; Figure 15). *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 was distinguished by a far greater amount of carotenoids facing the others. While *Cuspidothrix issatschenkoi* LEGE 03282 and *Alkalinema aff. pantanalense* LEGE 15481 presented equivalent amounts of carotenoids as well as phenols, though still displaying significant statistical difference (p<0.05) between IC_{50}. A more specific analysis of carotenoid content (Table 4) can evidence the exclusive presence of canthaxanthin (29) in *Cuspidothrix issatschenkoi* LEGE 03282 acetone extract. Canthaxanthin representing a xanthophyll with well-known antioxidant and immunomodulatory activities (Chan et al., 2009; Esatbeyoglu and Rimbach, 2017), highlighting a possible explanation for the difference observed.

Hereby, several correlations between carotenoids and phenols content and the antioxidant capacity of cyanobacteria extracts were noted, emphasizing their well-established and reported antioxidant properties (Shahidi et al., 1992; Helmut, 1993; Namitha and Negi, 2010; Goiris et al., 2012).

4.2.2. Anti-Inflammatory Potential

The anti-inflammatory potential of the different extracts was assessed through the determination of their capacity to reduce the NO production by RAW 264.7 cells, when stimulated with LPS. As it was already mentioned, NO represents a key molecule in the modulation of inflammatory responses, vasodilation and as neurotransmitter. Its determination through the referred methodology is a good way to screen the anti-inflammatory potential of extracts and isolated compounds.

The results obtained are displayed in Figure 17. As it can be seen through the graphs, two different behaviors were perceived. In a general way, the exposition of RAW 264.7 cells to the cyanobacteria ethanol extracts significantly stimulated the production of NO (Figure 17). This behavior was observed when comparing the NO present in the culture medium of cells treated with the extract, with the NO basal levels of the untreated control. All the ethanol extracts, as well as *Cyanobium gracile* LEGE 12431 and *Cuspidothrix issatschenkoi* LEGE 03282 acetone extracts, increased the NO production in such an extension, that surpassed that of the control when stimulated with LPS. Despite the overstimulation of the production of NO by the extracts, they did not affect macrophages viability, with the exception of *Cuspidothrix issatschenkoi* LEGE 03282 ethanol extract, where an overall decrease of about 30 to 40% in cell viability (Figure 17, A). Of the six acetone extracts, 4 showed a mild anti-inflammatory potential for the highest concentrations tested: *Synechocystis salina* LEGE 00037, *Alkalinema aff. pantanalense* LEGE 15481, *Leptolyngbya-like* sp. LEGE 13412 and *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 demonstrated the ability to reduce extracellular NO by 18, 17, 25, and 27%, respectively, without compromising cellular viability (Figure 17).

In a previous survey, Gomes and co-workers (Gomes Ferreira, 2016) carried out a sizable screening of fractions of different polarities obtained from crude cyanobacteria extracts for the anti-inflammatory potential, using the same cell model applied herein. The strains *Synechocystis* sp. LEGE07211, *Synechocystis* sp. LEGE06079, *Leptolyngbya* sp. LEGE 07075, *Leptolyngbya* sp. LEGE 07084 and *Cyanobium* sp. LEGE07175 studied by these authors can be compared with those analyzed herein once, even differing in species, they share the same genus. The results obtained by Gomes et al. showed a 30% decrease in extracellular NO in a fraction of *Synechocystis* sp. LEGE07211, which was higher than the value we obtained for *S. salina* LEGE00037 acetone extract (Figure 17, B).

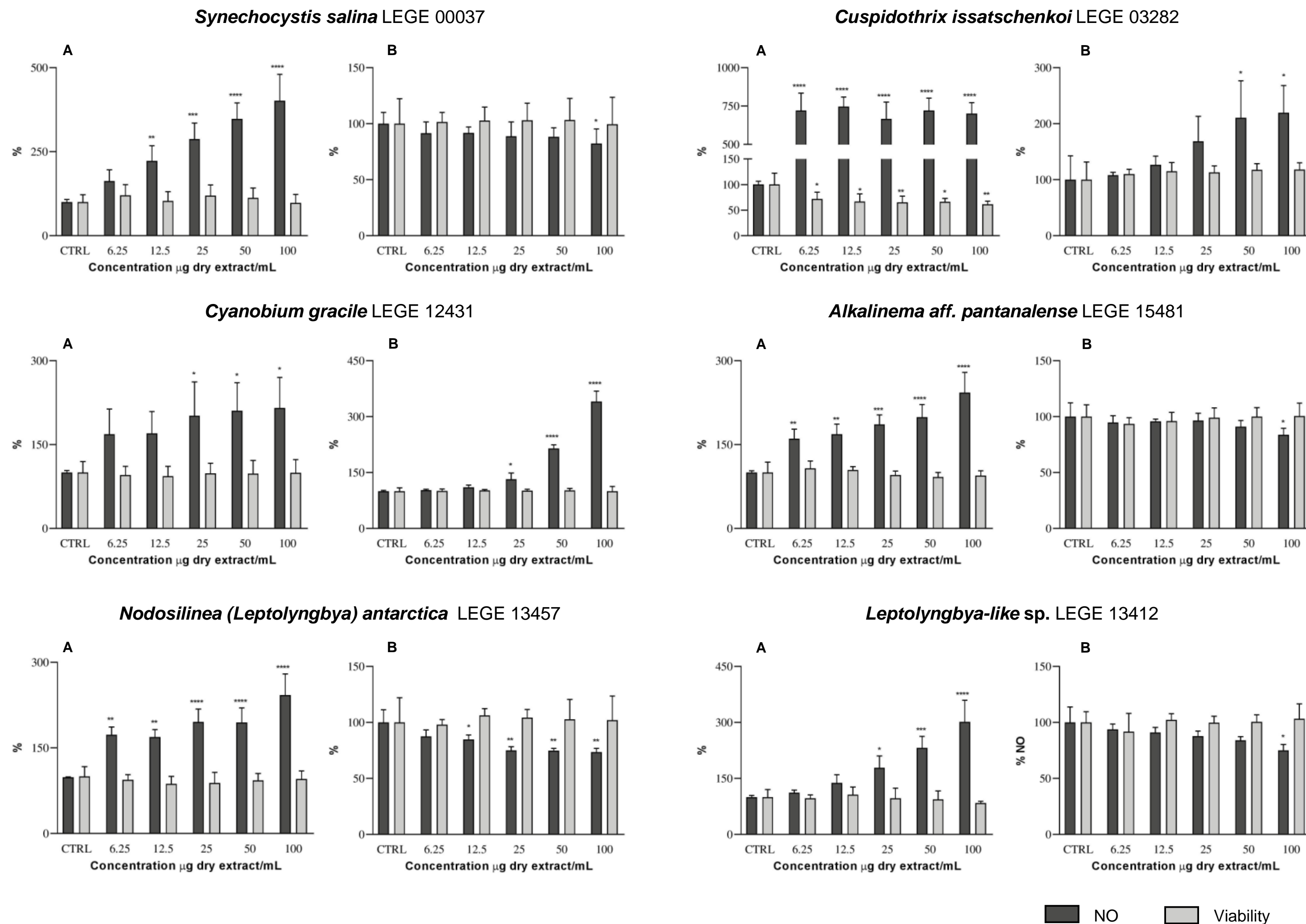

Figure 17 – **Nitric oxide production by RAW 264.7 cells in the presence of cyanobacteria extracts**. **A** – Ethanol extract; **B** – Acetone extract. Results are expressed as % of NO relative to control: with LPS stimulation (*Synechocystis salina* LEGE 00037*, Alkalinema aff. pantanalense* LEGE 15481*, Leptolyngbya-like sp.* LEGE 13412 *and Nodosilinea (Leptolyngbya) antarctica* LEGE 13457) and without stimulation (Ethanol extracts, *Cyanobium gracile* LEGE 12431 and *Cuspidothrix issatschenkoi* LEGE 03282 acetone extracts) Results are expressed as mean ± SD of at least four independent assays, performed in duplicate. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (ANOVA, Tukey HSD)

This variation is clearly due to differences in the composition of the extracts tested, once Gomes et al. tested a fraction from a crude extract, allowing the separation of compounds according to different solvent polarities, hence achieving a narrower screening.

As discussed before, inflammation is characterized by the involvement of an extensive and diverse array of triggers, symptoms, pathways, cells and organs, which all work together in order to overcome the inflammatory state, protecting against injury and infection, and setting the stage for healing. Until now, only few studies have addressed the anti-inflammatory potential of cyanobacteria extracts. Nonetheless, their potential use for tackling inflammation has been subject of some studies.

Anti-inflammatory drugs are met with a high demand, due to the importance of the inflammatory process in multiple human diseases, including psoriasis, Alzheimer's disease, arthritis and more recently cancer (Mantovani et al., 2008; Villoslada et al., 2008). Anti-inflammatory potential research based in the exploitation of cyanobacteria secondary metabolites has thus been increasingly considered. These organisms have not developed an adaptative immune response, and rely mainly on their innate immune system as defense against pathogens, thus demonstrating a selective better production of anti-inflammatory natural products, as a probable important part of their evolutionary strategy (Villa et al., 2010). Indeed, several studies underline this potential, as for example, Malloy et. al. (Malloy et al., 2011) described the anti-inflammatory potential of an oxidized lipopeptide molecule named Malyngamide 2, isolated from cf. *Lyngbya sordida* sample (PNG-06/02/02-3) collected near Dutchess Island in Papua New Guinea. Like our study, the bioactivity assay used consisted on the determination of the NO in the culture medium of LPS-stimulated RAW 264.7 cells. The authors found that malyngamide 2 displayed robust anti-inflammatory properties, inhibiting induced NO production with an IC_{50} of 8.0 μM (Malloy et al., 2011). In another study Ting Chen and co-workers (Chen et al., 2004) valuated the immunomodulating properties of the cyanobacteria produced microcystin-LR, commonly known for causing severe health issues to livestock and human beings. The anti-inflammatory potential was tested utilizing LPS stimulated BALB/c mice peritoneal macrophages, followed by the quantification of NO production in cells culture supernatant, using the Griess reagent method, and additionally complemented with the assessment of mRNA levels of iNOS, as well as known inflammatory mediators (IL-1β, TNF-α, GM-CSF, and INF-γ), through reverse-transcriptional polymerase chain reaction (RT-PCR). The authors found a significant dose-dependent decrease in NO production, and in mRNA levels

of iNOS, IL-1β and TNF-α, when treated with microcystin-LR; mRNA levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and INF-γ were also downregulated, although this time in a dose-independent way. Moreover, *in vivo* models have also emphasized the anti-inflammatory potential of cyanobacteria-derived compounds. Bruno and collaborators (Bruno et al., 2005) reported the anti-inflammatory effect of monogalactosyldiacylglyverol (MGDG), digalactosyldiacylglycerol (DGDG), sulphoquinovosyldiacylglycerol (SQDG) and phosphatidylacylglycerol, isolated from the strain *Phromidium* sp. ETS-05, in a croton-oil induced ear edema, and in carrageenan-induced paw edema, in mouse models, reporting a general dose-dependent response, with lower toxicity than the reference drug indomethacin (Bruno et al., 2005). These works, based in different cells and animal models, demonstrate the potential of different cyanobacteria strains for the screening of new promising anti-inflammatory compounds.

Through the analysis of our results, it seems clear that the anti-inflammatory potential of the extracts has some linkage with their carotenoids content once, acetone extracts, much richer in carotenoids, present more potential to reduce NO production by RAW 264.7 cells. As previously reported in different studies, carotenoids may have an important role in several steps of inflammation. As such, Bendich and Shapiro reported for the first time that dietary canthaxanthin increased mitogen-induced lymphocyte proliferation in a rat model (Bendich and Shapiro, 1986). Okai et. al. tested the effect of canthaxanthin on cultured spleen cells and thymocytes, both types of cells being central in the immune system, reporting a significantly enhancement in the release of IL-1 α and TNF-α (Okai and Higashi-OKai, 1997). Also, Prabhala et. al. observed an enhanced expression of activation markers in human peripheral blood mononuclear cells when treated with canthaxanthin *in vitro* (Prabhala et al., 1989). In another study, Soontornchaiboon and co-workers report the anti-inflammatory effects of the xanthophyll violaxanthin, isolated from the microalga *Chlorella ellipsoidea,* in the same macrophage cell model applied in our study. The authors further assessed the mechanism of the compound through real-time polymerase chain reaction (rtPCR), western blotting and electrophoretic-mobility shift assay (EMSA), reporting a significant inhibition of NO and prostaglandin E_2 production, also suggesting an anti-inflammatory modus operands around the inhibition of NF-κB inflammatory pathway (Soontornchaiboon et al., 2012). β-Carotene, lycopene, lutein and phycobiliproteins are also reported to display anti-inflammatory activities, as described by G. Silambarasan et. al., where aqueous extracts of *Trichodesmium erythraem* displayed a strong anti-inflammatory activity against the

carrageenan-induced paw edema in Winstar albino rats. A 57.5% inhibition of inflammation volume was observed for a dosage of 500 $mg.kg^{-1}$ in 30 min of exposure, comparable with the commercial drug indomethacin. The anti-inflammatory effect was further characterized; phycocyanin seemed to be responsible for the inhibition of the formation of leukotriene, which is known to be an inflammatory metabolite of arachidonic acid (Silambarasan et al., 2011), thusly highlighting a deeper relationship between carotenoids and inflammation.
The anti-inflammatory potential of the cyanobacteria extracts can additionally be linked with their ability to scavenge reactive species (Dedon and Tannenbaum, 2004).
As such, and as already discussed, the antioxidant potential of the extracts can also influence the inflammatory network, through their capacity to scavenge reactive species. Here, a correlation between the presence of antioxidant compounds and anti-inflammatory potential can be inferred. As such and detailed above, the best strains in diminishing extracellular NO were *Synechocystis salina* LEGE 00037, *Alkalinema aff. pantanalense* LEGE 15481, *Leptolyngbya-like* sp. LEGE 13412 and *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457. *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457, together with *Alkalinema aff. pantanalense* LEGE 15481, were distinguished with high contents of both carotenoids and chlorophylls (Table 4, Figure 13), and with high TPC, being among the richer strains (Table 5, Figure 14). Additionally, both strains stood out in the antioxidant $O_2^{\bullet-}$ scavenging assay, being one of the few to reach IC_{50} (Table 7 Figure 16). Regarding *Synechocystis salina* LEGE 00037 and *Leptolyngbya-like* sp. LEGE 13412, these strains neither presented high amounts of carotenoids and chlorophylls (Table 4, Figure 13) nor phenols (Table 5, Figure 14), when compared with the previous, hence deserving a deeper scrutiny. *Leptolyngbya-like* sp. LEGE 13412 did not show measurable content in phenols, and the results for the antioxidant potential only revealed an IC_{25} for the acetone extract (Table 7 Figure 14). So, an elementary carotenoid analysis can justify the anti-inflammatory potential found herein (Table 4 Figure 13). This strain presents exclusively high amounts of echinenone, which comes as a possible explanation for the anti-inflammatory potential displayed (Fraser and Bramley, 2004; Ciccone et al., 2013).
Regarding *Synechocystis salina* LEGE 00037, a correlation between anti-inflammatory potential and carotenoids observed; this strain displayed the lowest total pigments content but, at the same time, ranked third best in total phenols. Thus, its anti-inflammatory potential may be connected with specific phenolic compounds not profiled herein.

Cyanobium gracile LEGE 12431 and *Cuspidothrix issatschenkoi* LEGE 03282 displayed no anti-inflammatory potential, contrary, greatly increasing extracellular NO, even surpassing control LPS stimulation. *Cuspidothrix issatschenkoi* LEGE 03282, in particular, promoted the most intense stimulation on NO production. This may be due to a relatively new report of the presence of the anatoxin-a gene in this strain (Jiang et al., 2015). Anatoxin-a is a naturally-ocurring homotropane nicotinic agonists and is considered a very fast death factor (VFDF) as it acts by binding to nAChR (nicotinic Acetylcholine Receptors) receptors that are found throughout muscle and central nervous system (Wonnacott and Gallagher, 2006). This may also justify the decrease in macrophage viability observed for the ethanol extract of this strain (Figure 17). Overall, going against the main goal of the present project, beyond being good radical scavengers, the acetone extracts of *Leptolyngbya-like* sp. LEGE 13412, *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457 and *Alkalinema aff. pantanalense* LEGE 15481, seem promising for a possible biotechnological application in psoriatic disease, once they presented anti-inflammatory potential, and also free radical scavenging capacity.

4.2.3. Effect on keratinocytes hyperproliferation

Keratinocytes play a central role in the pathogenesis of psoriasis, normally suffering from hyperproliferation and abnormal differentiation, therefore, many of the anti-psoriatic therapies have now focused on the arrest of this hyperproliferation of keratinocytes, aiming for a newfound homeostasis in cell growth and, consequently, a normal epidermis recovery (Tse et al., 2006). With this in mind, the effect of cyanobacteria extracts on keratinocytes proliferation was assessed by measuring their effect on cell viability, after periods of 24 and 48h of exposition to the extracts, through the MTT assay (Figure 18).

Apart from *Alkalinema aff. pantanalense* LEGE 15481 and the ethanol extract of *Nodosilinea (Leptolyngbya) antarctica* LEGE 13457, all the tested extracts were able to significantly affect keratinocytes viability. The most pronounced results were observed with *Cuspidothrix issatschenkoi* LEGE 03282 ethanol extract and with *Leptolyngbya-like* sp. LEGE 13412 acetone extract, which presented a decrease in cell viability up to 50%, for the highest concentration tested (100 µg dry extract.mL^{-1}), after 48 hours of exposure (Figure 18).

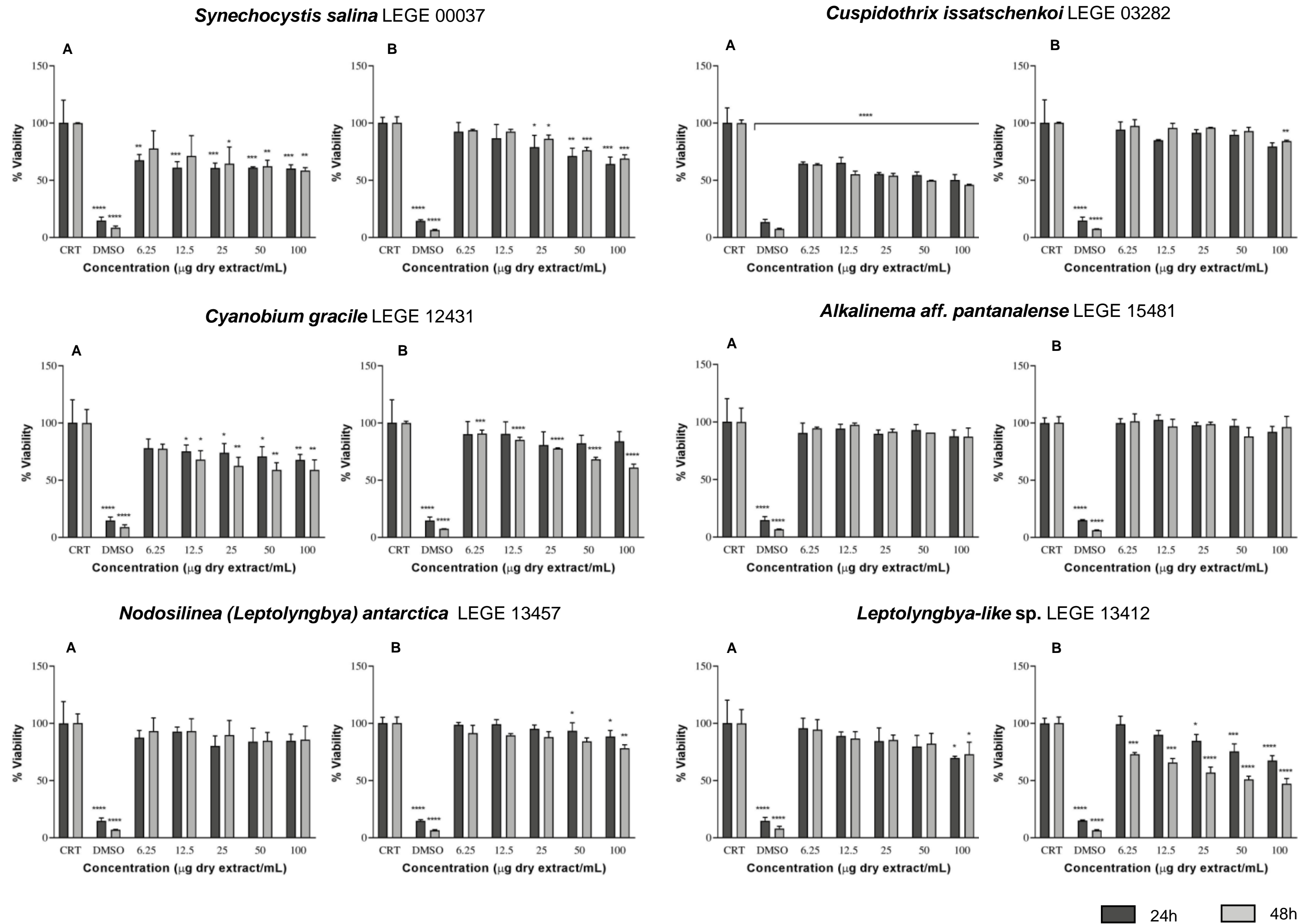

Figure 18 – **Keratinocytes (HaCAT) viability after 24h and 48h of incubation with cyanobacteria extracts. A** – Ethanol extract; **B** – Acetone extract. Results are expressed as Mean±SD of at least four independent assays, performed in duplicate. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (ANOVA, Tukey HS)

The keratinocyte specific profile in psoriasis brings forward objective-oriented assays, and few studies tackled cyanobacteria-derived keratolytic activity. Even so Morone et. al. reported a decrease in keratinocytes viability when exposed to ethanol extracts of *Phormidium* sp. LEGE05292 and *Oscillatoriales* LEGE07167 (Morone Bavini et al., 2018). In the framework of psoriasis, a study by Gudmundsdottir et. al. addressed a much more in-depth anti-inflammatory assay utilizing dendritic cells, T cells and keratinocytes, all key participants in psoriasis and inflammatory diseases (Gudmundsdottir et al., 2019). The authors reported an inhibition of SYK gene (encoding the spleen tyrosine kinase enzyme) and CLEC7A (gene encoding the C-type lectin domain family 7 member A or Dectin-1) expression in the cells treated with exopolysaccharides extracted from *Cyanobacterium aponium* (sampled from the Blue Lagoon in Iceland), both genes deeply rooted with innate immune response. The results revealed a decrease of chemotaxis for T cells in keratinocytes, an inactivation of T cells and regulatory dendritic cells immunosuppression, further characterizing the anti-psoriatic effect of cyanobacteria derived metabolites in a wider assay, with cells characteristically altered in psoriatic pathogenesis (Gudmundsdottir et al., 2019). Furthermore, Tse et. al. portrayed the anti-proliferative effects of traditional Chinese herbs in the treatment of psoriasis, utilizing an HaCat keratinocyte cell model similar to those performed herein, hence validating the cell assay in the overall screening for anti-psoriatic compounds in a topical therapy framework (Tse et al., 2006). T

Despite having reduced keratinocytes viability, the ethanol extracts of the cyanobacteria strains evaluated herein do not meet the goal of the present study, once they showed a pro-inflammatory potential in the assay performed with RAW 264.7 macrophages (Figure 17), which is not desirable in the psoriatic framework. Contrary, some acetone extracts displayed anti-inflammatory potential, by reducing the NO in the culture medium of LPS-stimulated RAW 264.7 cells, also demonstrating the ability to scavenge free radicals and significantly slow down keratinocytes proliferation, which is desirable in the framework of psoriasis. Additionally, a certain differential toxicity was noted, once the majority of the extracts had the ability to reduce keratinocytes viability, without affecting those of macrophages (Figures 17 and 18). The species *Leptolyngbya*-like sp. LEGE 13412 and *Nodosilinea (Leptolyngbya) antarctica* LEGE13457 can thus be pointed as very promising in biotechnology, with a view to the development of topic formulations devoted to slow down inflammation and reduce psoriatic plaque in individuals suffering from psoriasis.

5. Conclusions and Future Perspectives

Cyanobacteria are one of the most prolific and primordial photosynthetic organisms on Earth. Therefore, the screening of cyanobacteria secondary metabolites portrays a robust tool for new drug discovery, aiming to fill the existing therapeutic gaps, and to provide natural alternatives to the existing drugs.

With the present study, the carotenoids profile of six cyanobacteria strains from the LEGEcc were reported for the first time. Both acetone and ethanol extracts were explored in different biologic systems, in specific bioassays selected according to the framework of the psoriatic disease. Two of the selected species stand out, *Leptolyngbya*-like sp. LEGE 13412 and *Nodosilinea (Leptolyngbya) antarctica* LEGE13457, once they met together the major focal points occurring in psoriatic disease, and covered in this project: antioxidant and anti-inflammatory potential, and capacity to reduce keratinocytes hyperproliferation, with differential toxicity for the cell lines analyzed. These species are worth of further exploitation, namely concerning the study of their mechanism of action and the search for the compounds responsible for the bioactivity, once they can constitute a future hope for the patients with this inflammatory disease.

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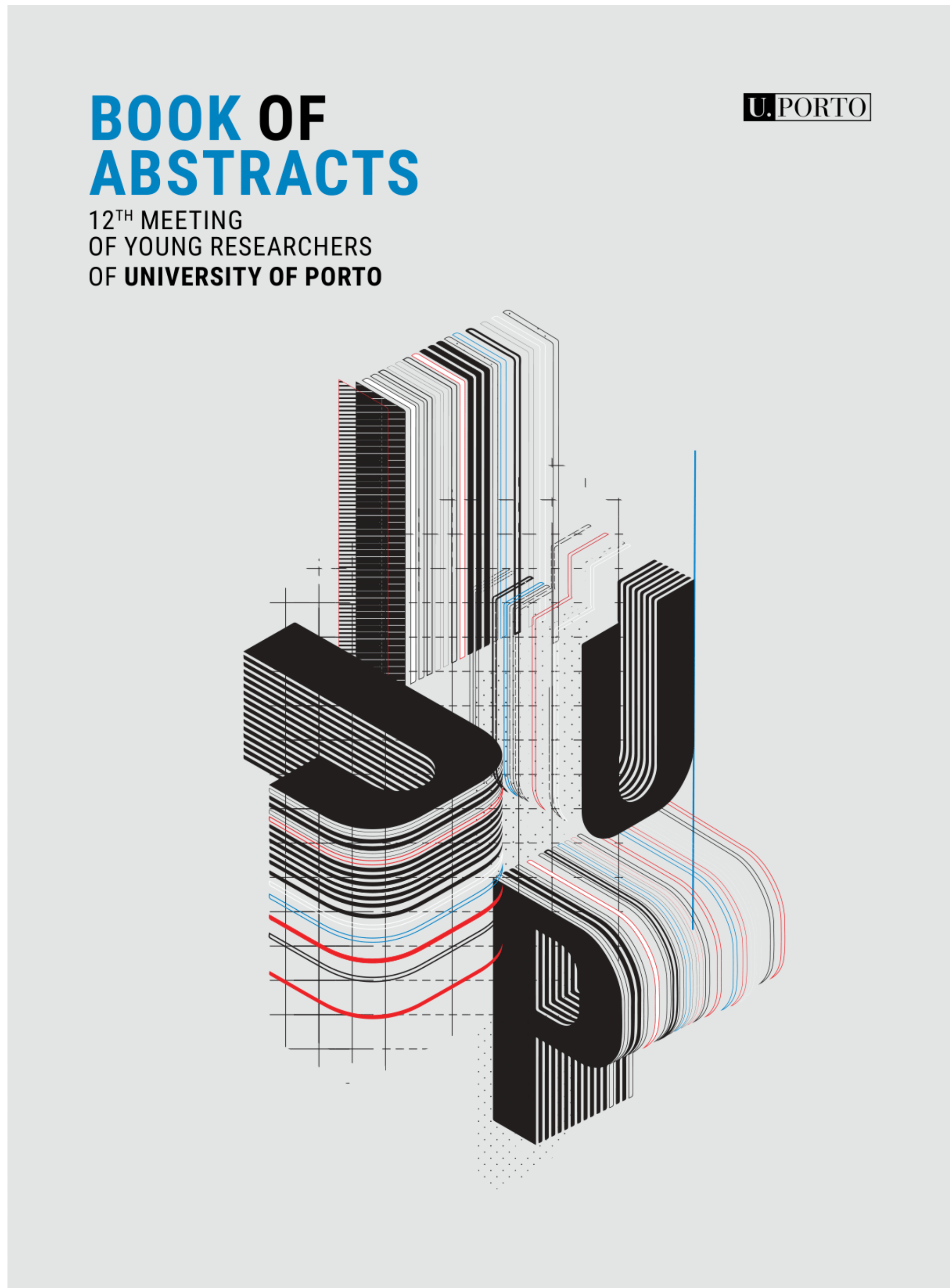
Attachments

Poster Presentations



- 15081 | Cyanobacteria from the LEGE Culture Collection: a road for innovation in human health?

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Ubiquitously distributed through the most different terrestrial and marine environments, cyanobacteria constitute an inspiring source for the search for new bioactive compounds. Of them, carotenoids represent a promising class of secondary metabolites, with recognized beneficial properties for humans´ health.
Different cyanobacteria strains of the LEGE culture collection (lege.ciimar.up.pt), namely those belonging to the genus *Cyanobium* (LEGE12431) and *Nodosilinea* (LEGE13457), were explored regarding their pigments profile and biological activities. The strains under study were cultured and scaled-up until 4L culture. After collection, the biomass was lyophilized and used for the preparation of acetonic (100%) and ethanolic (70%) extracts. The extracts were chemically analysed for their pigments profile, by High Performance Liquid Chromatography (HPLC) with Photo Diode Array (PDA) at 450 nm. The carotenoids qualitative and quantitative profile was established, with xantophylls being dominant over carotenes. The antioxidant activity of the extracts, determined through their capacity to scavenge superoxide radical anion *in vitro*, was positively correlated with their content in carotenoids. In order to find out the potential of these organisms for the treatment of chronic skin inflammatory conditions, the extracts will be screened for their toxicity and further explored for their capacity to reduce inflammation, using the mammal cell model RAW 264.7. Altogether, our results will enrich the knowledge of underexplored cyanobacteria strains, both regarding their metabolome and biological activities.

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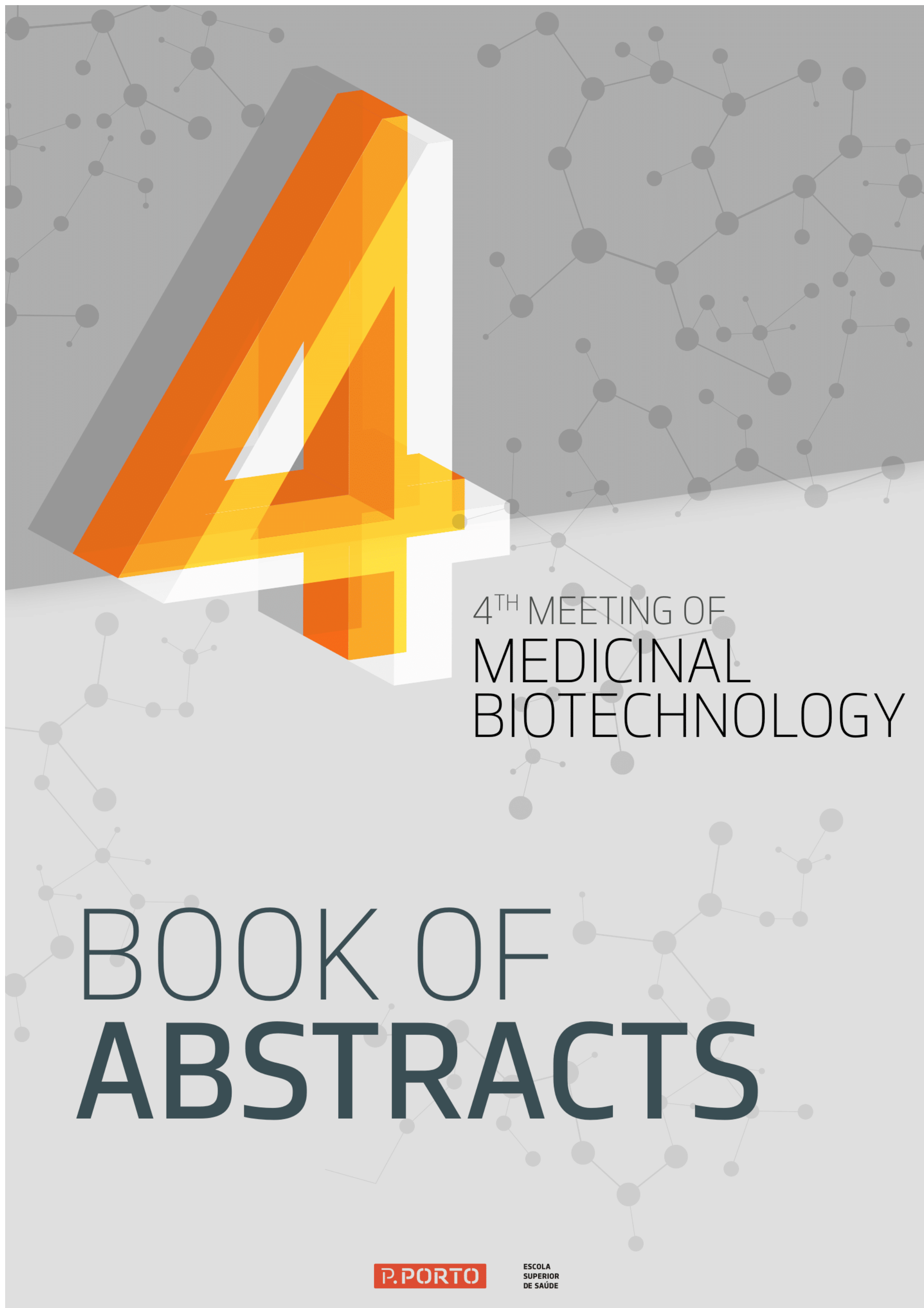
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The biotechnological potential of cyanobacteria bioactive extracts

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Cyanobacteria, formerly known as blue-green algae, represent a vast group of organisms, still underexplored for their potential biotechnological applications in the many branches of biological sciences. The relatively ease of handling regarding cyanobacteria biomass production, and the non-need of arable land occupation, makes these organisms very attractive in the diverse scientific fields. Known for their richness in secondary metabolites, different from those found in higher plants, cyanobacteria are emerging as a possible source of bioactive compounds with promising beneficial effects in humans´ health.
In order to screen the potential biotechnological applications of cyanobacteria, diverse strains from the LEGE culture collection (lege.ciimar.up.pt) were selected for pigment profiling and biological activity assessment. With the aim of screening a wide range of cyanobacteria, strains belonging to the genera *Cyanobium* (LEGE 12431), *Nodosilinea* (LEGE 13457), *Cuspidothrix* (LEGE 03282), and *Alkalinema* (LEGE 15481) were cultured and scaled-up to 4L culture. After harvesting, biomass was lyophilized and extracted with different solvents or solvent mixtures (acetone 100% and ethanol 70%). The extracts were analysed by High Performance Liquid Chromatography (HPLC) with Photo Diode Array (PDA) detection, in order to establish their carotenoids profile, and with the Folin-Ciocalteu assay for total phenols quantification. The antioxidant capacity of the extracts was evaluated against superoxide anion radical, through an *in vitro* cell-free assay, and the biological activity appears to be correlated with the total phenols content and carotenoids profile. In order to determine the potential of cyanobacteria bioactive extracts to be used in the treatment for chronic skin inflammatory diseases, the extracts will be screened for their anti-inflammatory capacity using the macrophage cell line RAW 264.7. This work aims to enrich the current knowledge in underexplored cyanobacteria strains, both regarding their metabolome, and the potential biotechnological application of their bioactive extracts in the treatment of inflammatory process-based diseases.

Keywords: Cyanobacteria, Carotenoids, Inflammation, Oxidative Stress

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