Anti-ageing potential of cyanobacteria: effect on matrix metalloproteinases and oxidative stress

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Abstract

Skin changes are among the first signs of ageing, being exacerbated by environmental factors such as sun exposure and oxidative environment. The most visible signs include the reduction of skin's density and elasticity, the appearance of wrinkles and hyperpigmentation. Matrix metalloproteinases (MMPs) are a group of enzymes responsible for the degradation of most extracellular matrix proteins. The increased activity of MMPs, resulting from metabolism unbalance, environmental stressors and various pathological conditions, can lead to tissues destruction, its inhibition being the focus of many marketed cosmeceutical formulations. Among these enzymes, collagenase, elastase and hyaluronidase are the most important regarding the maintenance of skin structure and function, while tyrosinase is central in the pigmentation processes. Effective inhibition of these enzymes, together with the scavenge of deleterious free radicals, constitutes an effective and multifactorial way to slow-down the skin ageing process, preventing undesirable skin changes.

Well recognized for their enormous potential applications in pharmacology and biotechnology, cyanobacteria appear as an emergent source of bioactive compounds, with the capacity to produce a wide range of bioactive secondary metabolites with diverse chemical structures. Taking advantage of the potential of these microorganisms, this project aims to evaluate the anti-ageing potential of pigments-rich extracts of cyanobacteria of the Blue Biotechnology and Ecotoxicology culture collection (LEGE CC, lege.ciimar.up.pt), in different bioassays involving cell and cell-free systems, unraveling their anti-ageing potential.

The cyanobacteria strains *Leptolyngbya boryana* LEGE 15486*, Cephalothrix lacustris* LEGE 15493*, Leptolyngbya cf. ectocarpi* LEGE 11479*,* and *Nodosilinea nodulosa* LEGE 06104, were selected for this study. Acetone and aqueous extracts were prepared and chemically characterized for their content in phenols, proteins, carotenoids and chlorophylls, and evaluated for their cytotoxicity in different skin cell lines. Due to the nontoxic nature of the extracts, they were explored for their antioxidant potential against the physiological superoxide anion radical ($O_2^{\bullet-}$), and for their capacity to inhibit key enzymes involved in the skin ageing process: tyrosinase, elastase and hyaluronidase. Because UV radiation is one of the main causes of skin damage and ageing, the sun protection potential of the extracts was also determined.

The results obtained showed that the strain *Leptolyngbya boryana* LEGE 15486 contained the highest content in the determined phytochemicals: 3.075 mg GAE g^{-1} for

total phenolic content, 69.218 mg g^{-1} for total proteins, and 0.675 mg g^{-1} for carotenoids. Regarding the $O_2^{\bullet-}$ scavenging activity, *Cephalothrix lacustris* LEGE 15493 and *Leptolyngbya cf. ectocarpi* LEGE 11479 showed the best results, with an IC_{50} of 65.5 µg mL^{-1} (aqueous extract) and 1190.5 µg mL^{-1} (acetone extract), respectively. Concerning the hyaluronidase and elastase inhibition assay, *Leptolyngbya cf. ectocarpi* LEGE 11479 showed some inhibitory activity with IC_{50} values of 863 µg mL^{-1} and 391 µg ml^{-1}, respectively. *Nodosilinea nodulosa* LEGE 06104 presented the best results regarding tyrosinase inhibition, being the only one to achieve an IC_{50} (989.26 µg ml^{-1}).
Regarding the sun protection potential, *Leptolyngbya boryana* LEGE 15486 displayed the best values.

Face to the results, all strains under study have potential for application in cosmetics.

Keywords: Cyanobacteria, skin ageing, matrix metalloproteinases, oxidative stress, carotenoids, phycobiliproteins, UV protection.

Resumo

As alterações cutâneas estão entre os primeiros sinais de envelhecimento, sendo agravadas por fatores ambientais como a exposição solar e o ambiente oxidativo. Os sinais mais visíveis incluem a redução da densidade e elasticidade da pele, o aparecimento de rugas e a hiperpigmentação. As metaloproteinases (MMPs) são um grupo de enzimas responsáveis pela degradação da maioria das proteínas da matriz extracelular. O aumento da atividade das MMPs, resultante do desequilíbrio do metabolismo e de várias condições patológicas, pode levar à destruição dos tecidos, sendo a sua inibição o foco de muitas formulações cosméticas comercializadas. Entre essas enzimas, a colagenase, a elastase e a hialuronidase são as mais importantes para a manutenção da estrutura e função da pele, enquanto que a tirosinase é essencial nos processos de pigmentação. A inibição dessas enzimas, juntamente com o sequestro de radicais livres, constitui uma forma eficaz e multifatorial de retardar o processo de envelhecimento, evitando alterações indesejáveis na pele.

Bem reconhecidas pelo seu enorme potencial de aplicação em farmacologia e biotecnologia, as cianobactérias constituem uma fonte emergente de compostos bioativos, com capacidade de produzir uma ampla gama de metabolitos secundários bioativos com diversas estruturas químicas. Tirando partido do potencial destes microrganismos, este projeto visa avaliar o potencial anti envelhecimento de extratos ricos em pigmentos, obtidos de cianobactérias da coleção de culturas do Blue Biotechnology and Ecotoxicology (LEGE CC, lege.ciimar.up.pt), em diferentes bioensaios celulares e não celulares, revelando o seu potencial de anti envelhecimento. As estirpes de cianobacterias *Leptolyngbya boryana* LEGE 15486*, Cephalothrix lacustris* LEGE 15493*, Leptolyngbya cf. ectocarpi* LEGE 11479*,* e *Nodosilinea nodulosa* LEGE 06104 foram selecionadas para este estudo.

Extratos acetónicos e aquosos foram preparados e caracterizados quimicamente quanto ao seu conteúdo em fenóis, proteínas, carotenoides e clorofilas, e avaliados quanto à sua citotoxicidade em diferentes linhas celulares da pele. Devido à natureza não toxica dos extratos, foi explorado o seu potencial antioxidante contra o radical anião superóxido ($O_2^{\bullet -}$), e a sua capacidade de inibir enzimas essenciais no processo de envelhecimento, como a tirosinase, elastase e hialuronidase. Como a radiação ultravioleta é uma das principais causas de danos e envelhecimento da pele, o potencial de proteção solar dos extratos também foi determinado.

Os resultados obtidos mostraram que em relação ao teor fitoquímico, a estirpe *Leptolyngbya boryana* LEGE 15486 apresentou o os valores mais elevados, com 3.075 mg GAE g^{-1} para o conteúdo total fenólico, 69.218 mg g^{-1} para proteínas totais, e 0.675 mg g^{-1} para os carotenoides. Em relação à atividade de eliminação do $O_2^{\bullet-}$, as estirpes *Cephalothrix lacustris* LEGE 15493 e *Leptolyngbya cf. ectocarpi* LEGE 11479 apresentaram os melhores resultados, com um IC_{50} de 65.5 µg mL^{-1} (extrato aquoso) e 1190.5 µg mL^{-1} (extrato acetónico), respetivamente. Sobre o ensaio da inibição da hialuronidase e elastase, *Leptolyngbya cf. ectocarpi* LEGE 11479 apresentou alguma atividade de inibição com valores de IC_{50} de 863 µg mL^{-1} e 391 µg mL^{-1}, respetivamente. *Nodosilinea nodulosa* LEGE 06104 mostrou ter os melhores resultados quanto à inibição da tirosinase, sendo a única estirpe a atingir um IC_{50} de 989.26 µg ml^{-1}.
Em relação ao potencial de proteção solar, *Leptolyngbya boryana* LEGE 15486 apresentou os melhores valores.

Mediante os resultados, todas as estirpes estudadas têm potencial para aplicação em cosméticos.

Palavras chave: Cianobactérias, envelhecimento da pele, metaloproteinases, stress oxidativo, carotenoides, ficobiliproteínas, proteção UV.

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List of Abbreviations

AA- Amino acid

APC- Allophycocyanin

ATCC-American Type Culture Collection

CIIMAR-Interdisciplinary Center of Marine and Environmental Research

DMAB- 4-(Dimethylamino)benzaldehyde

DMEM Glutamax- Glutamax Dulbecco's Modified Eagle Medium

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

DPPH. 2,2-diphenyl-1-picrylhydrazyl radical

DSCG- Disodium cromoglycate

EC- European commission

ECM- Extracellular matrix

EPS- Exopolysaccharides

GA-Gallic acid

GAE- Gallic acid equivalent

HA- Hyaluronic acid

HAase- Hyaluronidase

HaCAT- Human keratinocyte cell line

hCMEC- Human endothelial cell line

HPLC- High performance liquid chromatography

HLE- Human leukocyte elastase

HQ- Hydroquinone

HYAL- Hyaluronidases

IC_{50}- Concentration required to achieve 50% of inhibition

L-DOPA- L-3,4-dihydroxyphenylalanine

LEGE CC- Blue Biotechnology and Ecotoxicology Culture Collection of CIIMAR

MAA- Mycosporine-like amino acids

M2G- Mycosporine-2-glycine

MMPs- Matrix metalloproteinases

MT-MMPs- Membrane-type MMPs

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

NADH- β-nicotinamide adenine dinucleotide reduced form

NBT- Nitrotetrazolium blue chloride

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

PBPs- Phycobiliproteins

PBS- Phosphate- buffered saline

PC- Phycocyanin

PE- Phycoerythrin

PMS- Phenazine methosulphate

PPE- Porcine pancreatic elastase

PUFAs- Polyunsaturated fatty acids

ROS- Reactive oxygen species

SPF- Sun Protection Factor

TPC- Total phenolic content

TYR- Tyrosine

UVR- Ultraviolet radiation

3T3L1- Mouse fibroblast cell line

1. Introduction

1.1 General Introduction

Representing 16% of the total body weight, skin is the largest organ in the human body. Among its several functions, skin works as a physical barrier and offers protection against diverse harmful stressors, such as chemicals, pathogens and ultraviolet radiation (UVR) [1]. In addition, it is responsible for the synthesis of vitamin D, that is crucial for the development and maintenance of calcium homeostasis, as well as in immune, sensorial and body temperature regulation functions [2,3]. Being one of the most complex organs, skin is composed of three distinct layers, the epidermis, on the surface, the dermis, and the hypodermis [4] (Fig.1). The epidermis is a continually renewing epithelium constituted mainly by keratinocytes and melanocytes, and is the most exposed part of the skin. Its primary function is to protect the skin from potential environmental threats, providing physical, chemical, biochemical, and adaptive immunologic barriers [5].

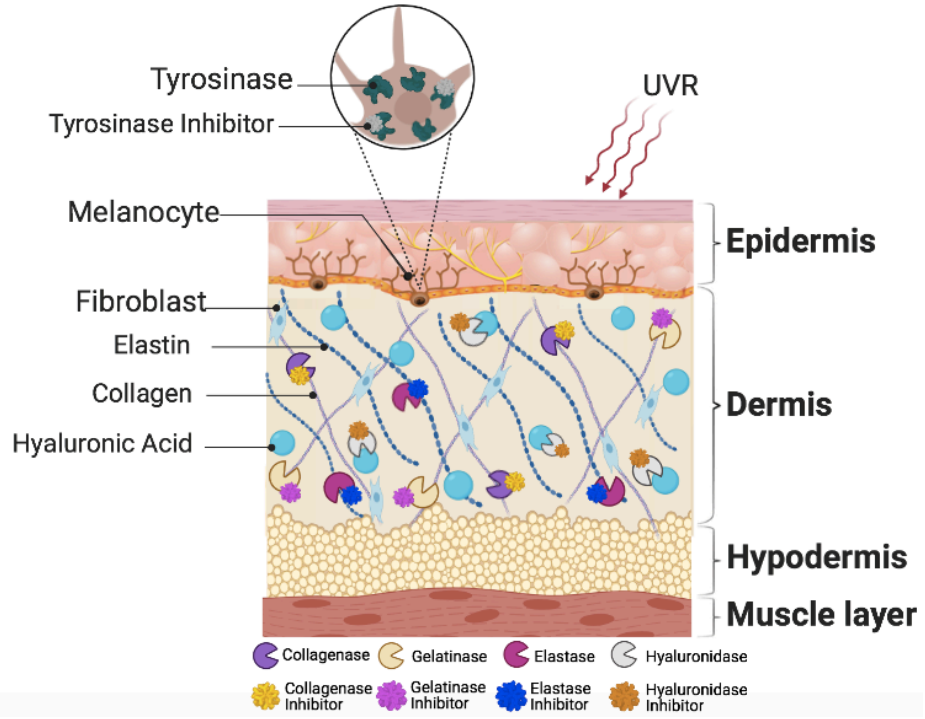


Figure 1. Schematic representation of the skin structure, with emphasis on the main enzymes involved in the aging process and their target molecules. UVR, ultraviolet radiation. *Created with BioRender.com [6].*

Underlying the epidermis, there is a thicker and deeper skin layer called dermis, which is constituted by connective tissue that includes a three-dimensional network of collagen and elastin fibers surrounded by the ground substances, such as hyaluronic acid (HA), and also including cells like fibroblasts and macrophages [4]. Dermis is the most important layer, being responsible for elasticity, flexibility, and aesthetic properties of the skin [4] (Fig.1).

The appearance of the skin, especially of the face, is one of the first characteristics that people notice, its good maintenance having a special individual and social importance, and turning skincare as one of the biggest concerns of modern societies. Skin ageing in humans is a slow and complex process induced by multiple factors, one of them being oxidative stress. Oxidative stress is a result of an increased amount of reactive oxygen species (ROS) formation and/or a reduced capacity for their scavenging by antioxidants. There are two ways of free-radicals production, endogenous and exogenous. In endogenous, it occurs as a natural consequence of gene mutations, cellular senescence, cellular metabolism, decrease in cellular DNA repair capacity, loss of telomeres, chromosomal abnormalities, elevation in O_2 concentration, increased mitochondrial leakage, and hormonal changes, affecting the skin in the same manner as it affects the internal organs [7-9]. Exogenous sources of oxidants are caused by chemicals, toxins, pollutants, radiations such as UV light, and mostly affects sun exposed body areas, for this reason, is also being referred to as photo ageing [10]. Photo ageing is responsible for 80 to 90% of skin ageing [11]. UVR is the most harmful external agent for the skin [1]. These agents have distinct physical properties and can penetrate the skin and exacerbate the enzymatic degradation of collagen and elastin fibers [4]. UVR also induces matrix metalloproteinases (MMPs) activity, which are responsible for degradation of the extracellular matrix (ECM) (Fig. 1). While their upregulation promotes ageing and cancer, they are crucial in epidermal differentiation and prevention of wound scars [12]. Among the causes for the reduction of the antioxidants activity, the most important includes the diminution in antioxidants intake/bioabsorption, and the antioxidant enzymes activity [9].

These alterations commonly affect the epidermal thickness, structure and pigmentation, as well as the morphology and microstructure of deeper layers, resulting in dryness, fragility, enlarged pores, fine lines, and wrinkles [10,13]. Another ageing factor is the lack of skin hydration. Hence, having hydrated skin is an important step to prevent the appearance of wrinkles and keep the deeper layers and the skin barrier protected. Therefore, the skin ages once it becomes dry and the epidermal and the dermal layers become slimmer.

Over the years, there has been an increasing concern with the physical aspect and facial appearance, which made scientific research suffer a significant evolution in this field. The demand for new products that improve skin health, especially from natural sources, has increased. Natural plant sources have been used in cosmetics through the years, but recently, more attention has been paid to extracts from macroalgae and eukaryotic microalgae [14]. Different companies around the world have been investing in research and development of new products with marine origin, able to satisfy consumer's needs, prolong health and beauty, and prevent the ageing process. Thereby, marine bioactive compounds have emerged as new sources of cosmetic ingredients. Ingredients with bioactive functionalities play an important role in skin health and protection. They have different mechanisms that minimize multiple damages occurring during skin ageing, such as wrinkles [4], pigmentation, collagen degradation, loss of elasticity [15] and loss of moisture [16]. Within marine organisms that produce bioactive compounds, cyanobacteria are gaining importance due to their capacity to produce diverse secondary metabolites, with unique structures and mechanisms of action.

1.2 Cosmetics and skin ageing

Cosmetics and skincare products have played diverse and important roles in human history [17]. Ancient cultures were as preoccupied with the aesthetic appearance as we are today. The oldest records came from Egyptians, as they were particularly concerned with physical appearance, and one of their biggest fears was developing facial wrinkles. Due to the dry and hot weather, they used oils and creams for skin protection [18].

Over the years some products like salt, honey, or acids were used to treat and clean the skin. But it was when more complex societies rise that the demand for cosmetic products increased. The modern term “cosmetic”, meaning to “beautify the body”, came from the ancient Roman public baths, where they did their hygiene [19]. Currently, cosmetic products are defined as “*any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odors*” by the European Commission (EC) regulation No 1223/2009 [20].

Nowadays, with the increase in life expectancy and the lifestyle improvement, body, skin and hair appearance is still one of the biggest concerns. Consumers have become more

conscious regarding the usage of cosmetics in their daily life to counteract the visible hallmarks of skin ageing and to step up their style quotient and overall personality. Many people use cosmetic products and their ingredients to enhance one's inherent beauty and physical features and restore the tissue's youthful appearance [8].

The cosmetics or beauty products industry, globally, is one of the sectors that faced the least impact from the economic ups and downs. According to Haddara *et al*., [21], the global cosmetics market is predicted to garner $805.61 billion by 2023. This is because of continuing and growing usage of products by women, and increasingly by men across the world. As we age, the skin naturally weakens, making it difficult to prevent ageing signs, and thus increasing concern on their prevention. Therefore, rapidly demographic ageing has led to robust demand for anti-ageing products in order to prevent wrinkles, age spots, uneven skin tone, hyperpigmentation, and dry skin, creating room for new innovations in cosmetics, thus boosting the industry growth. So, the ageing population is one of the major driver for this market [22]. This growing demand for cosmetic products has in turn led to the growth of the cosmetics market across the world. The global cosmetics market is divided based on the category of cosmetics and includes skin and sun care products, hair care products, deodorants, makeup and color cosmetics, and fragrances. Among these products, skin and sun care hold a considerable amount of percentage share in the global cosmetic products market. Skincare is one of the most technically advanced, complex, and diverse categories of cosmetics [22].

In recent years, the concern for the environment has grown in the cosmetic field. Consumers are now more concerned about sustainability and the impacts of cosmetic industry in the planet. Hence they are starting to look for products with a green life cycle, which implies the packaging, manufacturing, distribution, post-consumer use, and sourcing [23].

Concerning sourcing, the cosmetic industry is being forced to find new ingredients or new sources that are less aggressive to the environment. However, the quality and efficacy must be the same at least. With the improvement of science and research, it was possible to find and confirm the many properties of cyanobacteria. The sustainability of these microorganisms was shown through their low maintenance requirements, their capacity to self-renew, easy genetic manipulation, minimal cultivation space, low environmental impact, carbon neutrality (they use carbon dioxide), basic nutrition requirements and, compared to plants, they have a higher amount of photosynthesis and biomass production, which makes them a sustainable choice for skin care products [14,24-27]. The residual biomass can be used as fertilizer or in animal feed, and it can

also be used to make bio-polyesters, sometimes known as “Green Plastics”, which fits the circular economy concept [14,28,29].

Furthermore, according to different studies, they are potentially useful sources of natural bioactive compounds for the cosmetic industry [25]. They can be used in face lotions, anti-ageing creams, shampoos, body soaps, makeup, and sun protection products [20]. As a result, marine organisms, particularly microorganisms, are considered as a novel and promising source of bioactive molecules, capable of counteracting the processes that cause skin damage and ageing.

1.3 Cyanobacteria bioactive compounds

With approximately 2.6–3.5 billion years, cyanobacteria are by far the most widely occurring photosynthetic organisms [29]. They are morphologically diverse and can be found in almost every habitat, due to their capacity to change their metabolism and adapt [29]. So, it is estimated that only a limited number of species have been studied [30]. This leads to the unknown of high-value metabolites they are able to produce.

It is known that cyanobacteria synthesize proteins, carotenoids, lipids (polyunsaturated fatty acids (PUFAs), hydrocarbons), pigments, polysaccharides (cellulose, alginates, starch), and other compounds, with proven bioactivities in the pharmaceutical, energy, nutrition and cosmetic fields [28,31,32]. Regarding energy application, diverse cyanobacteria are being used to produce bioethanol [29]. Due to their high protein and PUFAs content, they can also be used for human and animal nutrition [28]. In the pharmaceutical field, it is noteworthy the production of grassystatin A–B for lung cancer, kempopeptin A for colon cancer, and dolastatin 15 for breast cancer [33]. Other studies show that they also have antitumor, anticoagulant, anti-inflammatory, and protease inhibitory activities [33]. Regarding cosmetics, their bioactive compounds, mostly in extracts, have been reported to be used in shampoos and body soaps [16,20], face lotions, anti-ageing creams, makeup and sun blockers [14,16,25]. Concerning sunscreens, some of these microorganisms produce UV-absorbing compounds, such as mycosporine-like amino acids (MAAs) and scytonemin, as well as carotenoids, phycobiliproteins and polyphenols, with an important role in preventing oxidative stress through their capacity to scavenge deleterious free radicals [34]. They also produce exopolysaccharides (EPS), with important moisturizing properties [14], metalloproteinases inhibitors [12], and compounds able to inhibit tyrosinase, and thus avoid skin hyperpigmentation [14].

1.3.1 Antioxidants

Cyanobacteria are usually exposed to oxidative stress, which enables them to produce multiple bioactive compounds able to defend them against ROS and free radicals [25]. This capacity has been already demonstrated in several studies. For instance, a methanolic extract of *Arthrospira platensis* has demonstrated high radical-scavenging activity [35]. Another example was presented using *Anabaena sp.* extract, which possesses efficient antioxidant potential, measured by DPPH radical scavenging activity [36]. In a study conducted by Lopes *et al*., [32], five ethanol and acetone extracts from different cyanobacteria strains were characterized for their antioxidant potential against $O_2^{\bullet-}$, and the results were very promising.

Antioxidants like carotenoids, proteins, such PBSs (phycobiliproteins) and phenols can also prevent skin damage by stabilizing free radicals when transferring electrons to an oxidizing agent [25].

Carotenoids are natural isoprenoid fat-soluble pigments that can be orange, yellow, or red [25]. They have gained a lot of attention and interest for being associated with a diminished risk for several degenerative disorders, and antioxidant activity, which is related to the C=C chemical double bonds that are present in their molecules. They can also act as light filters by reducing light exposure [37]. Some cyanobacteria like *Wollea vaginicola*, *Leptolyngbya foveolarum*, and *Synechocystis salina* LEGE 06099 have been highlighted for their significant content in carotenoids, what enhances their interest in the pharmacological and cosmetic fields [14,25].

Regarding proteins, some reports show that their associated bioactivities could be valuable for pharmaceuticals, cosmeceuticals, and nutraceuticals. Some of them are anti-ageing, anti-irritant, antihypertensive, antioxidant, anticoagulant, antiproliferative, and have immune-stimulant activity [38]. An example of the use of proteins for cosmetics is the use of phycobiliproteins (PBSs), which are naturally present in cyanobacteria. One of cyanobacteria defensive mechanism is their capacity to absorb light energy without producing ROS, which is possible due to changes in the content and ratio of phycobilisomes [39]. Phycobiliproteins are water-soluble proteins that are associated with phycobilins (photosynthetic pigments). They are divided into three groups according to their structure and light absorption: phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC) (Table 1).

They are reported to be good antioxidant agents due to their structural resemblance to bilirubin (which eliminates oxygen derivatives). Furthermore, they are related to anticancer and anti-inflammatory activities [25,40]. PC is the most common phycobiliprotein in cyanobacteria, having interesting antioxidant and radical scavenging properties, as well as capacity to inhibit cell proliferation [25,40].

Table 1. Phycobiliproteins and their light absorption range.

Phycobiliproteins	Absorption (nm)
Phycocyanin	610-625
Phycoerythrin	490-570
Allophycocyanin	650-660

Spirulina platensis, *Arthospira maxima* and *Halomicronema* sp. are some cyanobacteria that can be used in pharmaceutical and cosmeceutical industries due to their amount of phycobiliproteins [25,40].

Phenolic compounds are secondary metabolites, originated from primary metabolites, mostly for smell, color, protection against UV radiation, and predators [41]. They are known as radical scavengers, which can also inhibit iron-mediated oxyradical formation to prevent oxidative stress [42]. Moreover, they have one or more hydroxyl groups attached to an aromatic ring, benzene. In terms of classification, they cluster a large and diverse group of chemical compounds, such as flavonoids, tannins, phenolic acids, stilbenes, and lignans [43,44]. In relation to flavonoids they belong to a class of low-molecular weight phenolic compounds and have a polyphenolic structure. There are several subgroups depending on their structure, such as, flavonols (quercetin, rutin, kaemferol), isoflavones (genistein, daidzein, glycitein), flavones (luteonin, apigenin, tangeritin), anthocyanins (cyaniding, malvidin, peonidin), and chalcones [45]. Tannins were originally known as substances used for converting animal skin into leather, but recently have attracted scientific interest due to their antiviral, antitumor and antibacterial activity. They have varying molecular sizes and complexities and are classified into two categories: hydrolysable and condensed tannins [46,47]. Although they are well known in plant kingdom for their crucial role in growth and reproduction, phenolic acids are only now being investigated for their antioxidant properties These compounds are widely diverse and are characterized by hydroxylated aromatic rings [48]. Stilbenes are another class of polyphenols that are already being used for their antioxidant properties, and have been studied for drug development, as exemplified by resveratrol [49].

Lignans are fiber related polyphenols which are present in fiber rich foods. Some studies revealed its importance by associating the consumption of lignans with the reduction of the risk of a wide range of conditions [50].

Regarding their potential for cosmeceutical applications, some studies confirmed that several cyanobacteria were effective as scavengers of free radicals due to their relevant quantities of phenolic compounds. In Jerez-Martel *et al.*, [42] two phenolic acids, gallic acid and chlorogenic acid were found in *Nostoc commune,* which presents a significantly high antioxidant capacity. Blagojevic and co-workers [51] studied ethanolic extracts from ten cyanobacteria of the genera *Nostoc*, *Anabaena* and *Arthrospira*. The results revealed gallic acid, chlorogenic acid, quinic acid, catechin, epicatechin, kaempferol, rutin and apiin among the detected phenols.

Furthermore other studies revealed that these compounds can act as skin-whitening agents and are associated with a broad spectrum of health-promoting effects [52]. They are also becoming promising due to their blue UVR screening properties [53].

Overall, most antioxidants are synthetic, therefore is extremely important to explore new sources of antioxidants.

1.3.2 Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent enzymes, and their main function is to remodel and degrade the ECM [12]. The ECM is a gel-like material made of collagen and elastic fibers dispersed in a ground substance made of glycosaminoglycans, proteoglycans, and connective tissue glycoproteins. It is essential to hold cells together and provide a pathway for nutrients and oxygen [54]. There are several different types of cells capable of producing MMPs in the skin, such as keratinocytes, fibroblasts, macrophages, endothelial cells, mast cells, eosinophils, and neutrophils [4].

Elastin and collagen are important proteins responsible for resistance and elasticity of the skin, being the main components of the connective tissue [55]. Therefore, any alterations in collagen and elastin induced by MMPs are mostly responsible for damage and wrinkle formation [56]. The functional subgroups of MMPs include collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), amid others [57]. Among the most relevant regarding skin ageing are: collagenases, gelatinases, elastase, and hyaluronidases (Fig.1).

1.3.2.1 Collagenases

These enzymes (MMPs-1, -8, -13, and -18) are responsible for the initiation of collagen fragmentation in human skin and control of collagen turnover [58]. They cleave all types of interstitial collagens (I, II, and III) at a single site. Collagen fragments lose stability at body temperature after the cleavage, and their structure is disrupted, contributing to indirect tissue damage [12,59,60].

Hence, there is a need to conserve the dermal matrix by inhibiting the activity of these matrix metalloproteinases, and others. In this regard, marine natural products constitute a new hope for the search of new and innovative bioactive compounds, able to maintain healthy skin and slow-down the skin ageing process [61].

An example is the mycosporine-2-glycine (M2G) (Fig.2), isolated from the cyanobacterium *Aphanothece halophytica*, that has bacterial collagenase inhibitory properties. The ability of M2G to chelate calcium ions and its effectiveness in blocking the synthesis of glycation-dependent protein-protein cross-linking, a process linked to the development of dull skin, were identified as possible mechanisms of enzyme inhibition. Thus, M2G has potential applications for the prevention of skin ageing, being an alluring candidate for new cosmetics [62].

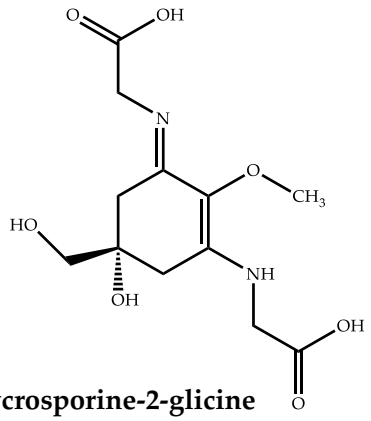


Figure 2. Structure of Mycosporine-2-glycine, a compound with collagenase inhibitory properties.

Another study was conducted and the authors found that *Arthrospira maxima* was able to produce anti-collagenase peptides. In comparison to the synthetic inhibitor (57.13%), the peptide fraction displayed a superior anti collagenase activity (92.5%). These peptides have a sequence that resembles the cleavage point in native collagen

preventing ECM breakdown. The inhibitory activity of collagenase by these peptides was attributed to a competition with the enzyme active site [63].

1.3.2.2 Gelatinases

Gelatinases (MMPs-2 and -9) degrade basement membrane and denatured structural collagens [12,59]. They are essential in degrading collagen fragments after their initial degradation by collagenases [64]. There are also some reports, although in lower amount, on cyanobacteria compounds that inhibit these enzymes.

For instance, the effect of the phycobiliprotein C-phycocyanin from the cyanobacterium *Spirulina platensis* on human gelatinases MMP-2 and MMP-9 was investigated by Kunte and co-workers [65]. It was found that the extract reduced significantly the activity of MMP-2 by 55.13% and MMP-9 by 57.9%, they also reduced the mRNA expression of both enzymes in the hepatocellular cancer cell line HepG2. Another cyanobacteria (*Mastigocladus laminosus*) was subject study and preliminary experiments showed that the polysaccharide fraction could be correlated to the inhibition of MMPs-2 and -9 expression [66].

1.3.2.3 Elastase

Elastin is the second most abundant constituent of the connective tissue in the dermis, and it's degradation results in loss of elasticity and an aged appearance of the skin [67]. Elastase (MMPs-12) is a serine protease that can degrade elastin [4]. MMP-12 is the most effective MMP against elastin and is produced by macrophages and fibroblasts in response to UV radiation [67]. Regarding elastase inhibition activity, it was recently found that the cyclic depsipeptides tutuilamides A−C, from *Schizothrix* sp. and *Coleofasciculus* sp., are potent inhibitors of porcine pancreatic elastase (PPE) [68]. It was also shown that the depsipeptide lyngbyastatin-4, -5, -6, and -7, the cyclodepsipeptide somamide B, the tiglicamides A-C, and largamides A-C produce by *Lyngbya* spp., selectively inhibited PPE *in vitro* (Fig. 3) [69-72].

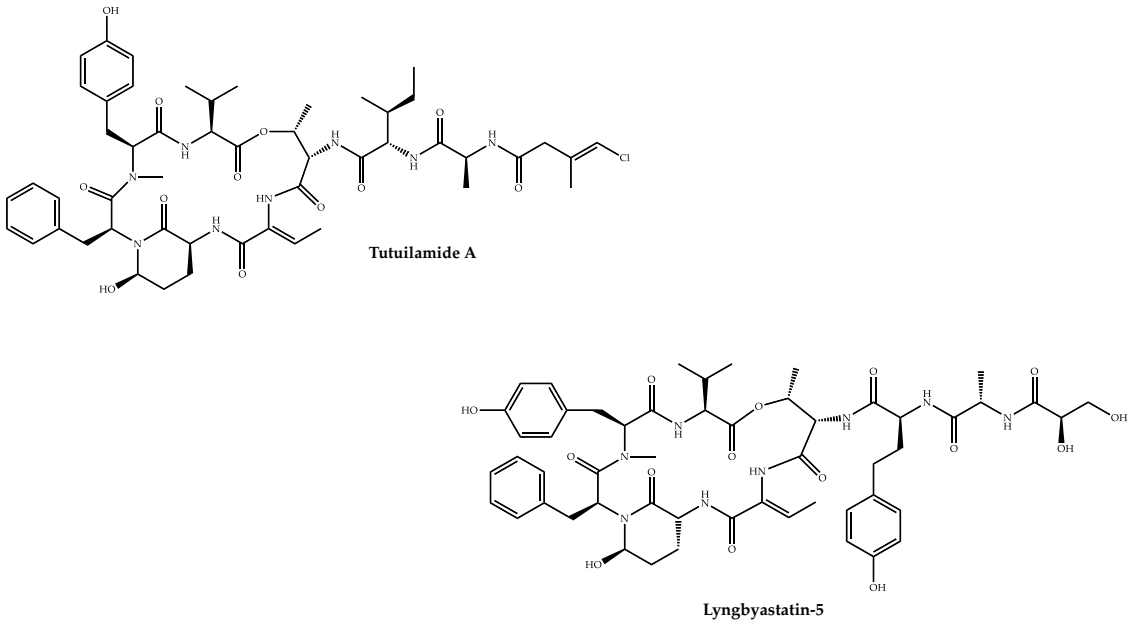


Figure 3. Structure of Tutuilamide A and Lyngbyastatin-5, two compounds with elastase inhibitory properties.

Another cyanobacteria that produces elastase inhibitors is *Planktothris rubescens.* It was conceivable to predict a structure-activity relationship after examining the molecular structure of these compounds, since it was discovered that the molecules flexible side chains displayed marginal selectivity for human leukocyte elastase (HLE). HLE has become more important as a result of its involvement in a variety of disease processes, therefore discovering inhibitors for this enzyme is a strategic therapeutic goal.

1.3.2.4 Hyaluronidases

Skin ageing is also associated with loss of skin moisture, due to the evaporation of the superficial water. Evaporated water is replaced with water from the lower layers in the skin, leading to shrinkage, and in a worst scenario to cell death [73,74]. The relation between skin moisture and wrinkles, revealed that skin hydration reduces the depth of wrinkle furrows significantly [75]. The key molecule involved in skin moisture is hyaluronic acid (HA), found in young skin at the periphery of collagen and elastin fibers [76]. Its main function is to retain water and to lubricate movable parts of the body [77].

Hyaluronidases (HYAL) are enzymes that dissolve HA polymers by cleaving high molecular weight HA into smaller fragments [4,78]. Aged skin, which is less plump than youthful skin, is characterized by decreased levels of HA. This reduction may be involved

in the changes occurring during the ageing process, including wrinkling, altered elasticity, and reduced turgidity [79].

Regarding hyaluronidase (HAase) inhibitory activity, there are still few studies with cyanobacteria, but it was shown by Yamaguchi and his team [80], that a polysaccharide from *Nostochopsis lobatus* MAC0804NAN produced a large amount of polysaccharide with a high inhibitory effect , being about 14.5 times stronger than the natural inhibitor disodium cromoglicate, once more emphasizing the importance of the exploitation of cyanobacteria-derived compounds in this field.

In addition to pure compounds, in a study including *Spirulina platensis,* Fujitani *et al*. [81] discovered that extracts, specifically ethanol-insoluble fractions, could decrease HAase activity. It was found that these types of extracts contained macromolecules such polysaccharides, which may be implicated in hyaluronidase inhibition.

Due to the increased extraction yield and reduced processing costs, using effective extracts as an active ingredient in cosmetics can be an advantage over isolated compounds.

1.3.3 Hyperpigmentation

Skin-whitening, or an aesthetically pleasing skin pigmentary appearance, has been a primary focus of many cosmetic industries. Skin gets darkened at some parts because of UV radiation, ageing, and pregnancy. Although hyperpigmentation is not harmful in any way, sometimes it can cause serious problems, such as melanoma. Thus, several treatment modalities are being investigated for their efficacy in treating skin hyperpigmentation [20,82].

Melanogenesis is a process that involves several chemical and enzymatic reactions to produce melanin, a major component of skin color, and occurs in melanocytes [11]. Hyperpigmentation can occur depending on either the increase in the number of melanocytes (containing melanin) or in the activity of melanogenic enzymes (tyrosinase) [83,84] (Fig.1). The UVR exposure causes an accumulation of an abnormal amount of melanin, which increases ROS production [11]. ROS are produced in the epidermis of the skin and affect the activity of melanocytes, that increase the conversion of tyrosine (TYR) into melanin by oxidation [20].

Tyrosinase is an enzyme that catalyzes melanin synthesis in melanocytes. Therefore, skin pigmentation can be prevented by tyrosinase inhibitors [20,85]. Some of the well-known inhibitors are hydroquinone (HQ), kojic acid and arbutin, however, they have

harmful effects. Curto and his team [86], demonstrated that HQ has mutagenic and cytotoxicity activity against mammalian V79 cells, also causing DNA damaged, and the National Toxicology Program has confirmed some evidence of carcinogenic activity [87,88]. Regarding kojic acid, skin irritation, and allergic dermatitis were developed after using skincare products containing it [89]. Additionally, they have high toxicity, low stability, poor skin penetration, and insufficient activity [90].

So, it became extremely important to find other ways to overcome hyperpigmentation or to find new tyrosinase inhibitors without such side effects. An example is the crude extract of *Arthrospira platensis* that, through its main components, can be a potential candidate for the development of effective and safe skin whitening cosmetics. It was found that some phenolic compounds produced by this species could act as effective inhibitors of tyrosinase [90]. Regarding *in vitro* trials involving humans, the biotechnological company CODIF Research & Nature, produced the extract PHORMISKIN Bioprotech G®, from the cyanobacteria *Phormidium persicinum,* that was able to reduce melanin synthesis. It was reported that, during the experimental period of 28 consecutive days, the skin tone became more uniform and brighter [91].

The use of vitamins C and E is another approach to prevent melanosome formation in the skin [92]. With this in mind, it can be assumed that *Spirulina* sp. constitutes a great candidate for cosmetic purposes due to their significant content in these vitamins [93,94]. In Zolghardi and co-workers [95] is also reported that phenols, polyphenols (flavonoids, isoflavones, flavanones), phenolic acids, stillbenes, and lignans could inhibit tyrosinase activity.

1.3.4 Sun Protection

Even though a growing number of companies have incorporated sun blockers in their products, it is still difficult to convince the public regarding the risks of sun exposure [79]. As said before, sunlight is one of the primary causes of skin ageing (about 80%), being wrinkles and pigmentation the most salient manifestations. An increase in the development of benign and malignant neoplasms derived from UV exposition has also been noted [96]. A study revealed that about one million people are diagnosed with skin cancer every year, and 10000 die from it [97].

The main harmful consequences of sun radiation are caused by UV radiation, which can be divided into three electromagnetic regions: UV-C, from 200 to 290 nm; UV-B, from 290 to 320 nm, and UV-A, from 320 to 400 nm [97]. UV-C radiation is the only one that

is filtered by the atmosphere before entering the earth. UV-B is not fully filtered by the ozone layer and affects primarily the epidermis and dermis, inducing mutations in the DNA, making it the most harmful. UV-A can reach deeper layers and is indirectly mutagenic by ROS generating [53,96,97]. Recently, the biological effects of blue light (400 to 450 nm) was linked to erythema, pigmentation, and free radicals production.

Regarding these facts, sunscreens are really important and necessary for skin protection, mostly in the prevention of diseases and premature ageing. The UV filters used in sunscreens are divided according to their capacity to absorb (synthetic organic) or reflect UVR (physical or inorganic) [53]. Due to some negative effects on humans (allergies) and in the environment (coral bleaching), the area of sunscreens development is very active.

Research on natural products such as natural photoprotectors is gaining considerable attention, considering that they are less toxic and biodegradable, making them more beneficial to humans and environment. In this regard, cyanobacteria are once again great candidates, since they can live in extreme conditions and thus produce a variety of bioactive compounds with diverse activities. Some studies have already reported their effectiveness as sun protector agents, such as those of Hossain and his team [98], who found that *Cephalothrix komarekiana* had a SPF value of 2.37.

Despite the studies presented above, the number of cyanobacteria strains explored in the field of cosmetics is very scarce, considering the potentialities of this resource. Therefore, it is necessary to increase the research on these organisms, especially their extracts. In addition to being easier to obtain rather than isolated compounds, bioactive extracts are economically more attractive, since they present several interesting bioactivities and have higher extraction yields.

Moreover, even though the studied strains show potential cosmetic application, there is still a lack in the tests required to prove their safe and effective use in cosmetic products. In this dissertation, new strains will be studied to broaden current knowledge and to deepen the possibilities of using these compounds in the world of cosmetics.

2. Objectives

Although cyanobacteria are recognized for their enormous biological potential, their interest for cosmetics has only recently arisen, and a very limited number of strains are actually marketed for this purpose. In this regard, this project intends to increase the scientific knowledge of understudied cyanobacteria strains, scientifically and economically valuing this resource in the field of cosmetics.

To achieve this, different extracts of understudied cyanobacteria of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC) will be sequentially produced and explored for their anti-ageing potential, through:

i) Culture and scale-up of 4 cyanobacteria strains selected from the LEGE CC;

ii) Preparation of cyanobacteria bioactive extracts using a sequential extraction scheme;

iii) Assessment of the cytotoxicity of the extracts in different skin cell lines (keratinocytes, fibroblasts and endothelial cells);

iv) Chemical characterization in terms of pigments, phenols and proteins;

v) Evaluation of the antioxidant potential of the extracts against deleterious free-radicals involved in the ageing process;

vi) Evaluation of the capacity of the extracts to inhibit key enzymes involved in the skin ageing process (hyaluronidase, elastase and tyrosinase);

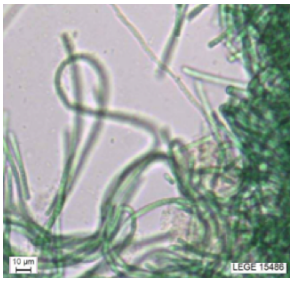
vii) Evaluation of the sun protection factor of the extracts;

Using different cell and cell-free bioassays, the proposed tasks will contribute to highlight the scientific and economic potential of this sustainable resource.

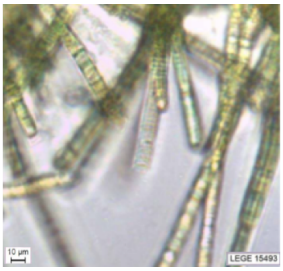
3. Materials and Methods

3.1 Cyanobacteria strains

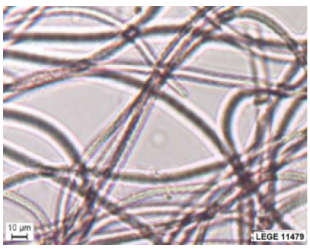
In this project, four filamentous cyanobacterial strains, *Leptolyngbya boryana* LEGE 15486*, Cephalothrix lacustris* LEGE 15493*, Leptolyngbya cf. ectocarpi* LEGE 11479*,* and *Nodosilinea nodulosa* LEGE 06104 were used (Fig. 4). They were isolated from Portuguese marine, and Brazilian freshwater ecosystems, and maintained in the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC) at the Interdisciplinary Center of Marine and Environmental Research (CIIMAR).



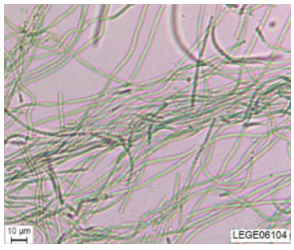
Strain: *Leptolyngbya boryana* **LEGE 15486**
Origin: Brazil, freshwater



Strain: *Cephalothrix lacustris* **LEGE15493**
Origin: Brazil, freshwater



Strain: *Leptolyngbya cf. ectocarpi* **LEGE11479**
Origin: Portugal, marine



Strain: *Nodosilinea nodulosa* **LEGE06104**
Origin: Portugal, marine

Figure 4. Selected cyanobacteria strains and origin. *(Photographs kindly provided by LEGE-CC)*

These strains were selected based on the potential to produce bioactive compounds previously described for the genera, and with interest for cosmetic purposes, such as antioxidant and anti-inflammatory. For instance, the genera *Leptolyngbya* has been shown to produce antioxidant and anticancer compounds, as well as *Nodosilinea*. Regarding the strain from the genera *Cephalothrix*, it was reported that cyanobacteria from Brazil have the ability to biosynthesize cyanopeptolins, which are candidates for anticancer drugs [99-101]. Moreover, the selected strains have not been explored for cosmetic purposes before.

3.2 Culture for biomass production

For biomass production purposes, a scale-up culture scheme was set. Each strain was inoculated in liquid Z8 medium [102], supplemented with 10 μg L^{-1} vitamin B12 and 25 g L^{-1} NaCl for the marine strains. Cultures were maintained at 25 °C, with a light intensity of 10 μmol photons m^{-2} s^{-1} and with a photoperiod of 14h light:10h dark. The strains were cultured in an initial volume of 40 mL. After periodically checking the strains growth, 2 steps of scale-up were follow. The first from 40 to 400 mL and the second from 400 mL to 4 L, until the 4L of culture was ready for collection. The strains were collected by filtration and the marine strains were washed with distilled water to remove the excess NaCl. Then, the biomass was frozen and freeze-dried (Telstar LyoQuest) under reduced pressure (0.1 mbar with condenser at -47ºC). The biomass was stored at -20ºC prior to extracts preparation.

3.3 Cyanobacterial extracts preparation

Two different extracts were sequentially prepared from each strain: acetone and aqueous. First, the acetone extract was prepared, using 2 g of dry biomass suspended in 70 mL of 100% acetone. For the release of the cell content it was necessary to break the cell walls through an ultrasonic bath (Fisherbrand®-FB15053) for 10min, in controlled temperature, in order to avoid degradation of the bioactive compounds. The mixture was then transferred to 50 mL falcons and centrifuged at 10000 Gs for 5 min at 4ºC (Thermo Scientific™ HERAUS Megafuge™ 16R). The resulted supernatant was filtered and then evaporated under reduced pressure by using a rotavapor (BUCHI R-210 Rotary Evaporator) at 200 mBar and -8ºC. This extraction process was performed 4 times for

each strain. The resulted pellet was left to dry in the fume hood, and then extracted with 70 mL of distilled water. The same methodology mentioned above was used, the mixture was placed in an ultrasonic bath for 10 min, then centrifuged at 5000 Gs for 5 min at 4ºC, and finally filtered through cotton. This process was repeated 3 times for each strain. The result solution was frozen and lyophilized. After complete drying, the acetone and aqueous extracts were weighed, and the extraction yield calculated. The resulting extracts were kept at -20 ºC until further analysis.

3.4 Cytotoxicity analysis in skin cell lines

3.4.1 Cell culture

The cytotoxicity of the extracts was evaluated in the human keratinocyte cell line HaCAT, mouse fibroblasts 3T3L1, and human endothelial cells hCMEC. Keratinocytes are essential components of the epidermis, composing about 95% of it. Regarding the dermis, fibroblasts are the most important cells, being responsible for the production of the dermal matrix components, essential for the maintenance of the skin shape and structure. Endothelial cells were also chosen, since the skin is surrounded by many blood vessels [103,104]. Endothelial cell line was provided by Dr. P. O. Couraud (INSERM), while the other cell lines were obtained from the American Type Culture Collection (ATCC). Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM GlutaMAX™, Gibco), supplemented with 10% (*v/v*) fetal bovine serum (Gibco), 1% of penicillin-streptomycin (Pen-Strep 100 IU mL^{-1} and 10mg mL^{-1}, respectively) (Gibco) and 0.1% Amphotericin B (Gibco). Cells maintenance and assays were performed at 37ºC, in a 5% CO_2 humidified atmosphere, and the culture medium was renewed every two days. After reaching 80-90% of cell growth, cells were washed with phosphate-buffered saline (PBS, Gibco), detached with TrypLEX express enzyme (1x) (Gibco), passed for maintenance and seeded for the planned assays.

3.4.2 Cytotoxicity assay - MTT assay

Endothelial cells, fibroblasts and keratinocytes were seeded in 96 well plates, at a density of 1.0 $x10^5$ cells mL^{-1}, 3.3 $x10^4$ cells mL^{-1} and 2.5 $x10^4$ cells mL^{-1}, respectively. After 24 hours of cell adhesion, the culture medium was removed and the cells were exposed for 24 and 48 hours to fresh medium containing the different cyanobacteria extracts in five

serial concentrations of 200, 100, 50, 25 and 12.5 µg mL^{-1}. For the acetone extracts, stock solutions were prepared in dimethyl sulfoxide (DMSO) (Gibco), and diluted with DMEM prior to cells exposure, so that the maximum DMSO concentration did not exceed 1%. Aqueous extracts were prepared in PBS and also diluted with DMEM prior to cells exposition. Negative control was PBS and the control of cells death was also DMSO 20%. After the incubation time, the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) cytotoxicity assay was performed. Briefly, 20µL of MTT solution (1mg mL^{-1}) were added to each well and incubated at 37ºC for 3h. Following the incubation time, the medium was carefully removed, and the purple colored formazan salts were dissolved in DMSO (100 µL). Absorbance was read at 550 nm in a Synergy HT Multi-detection microplate reader (Biorek, Bad Friedrichshall) operated by GEN5™ software. The assay was run in quadruplicate and averaged. For reproducibility, each assay was independently repeated at least three times. Cytotoxicity was expressed as percentage of cell viability, considering 100% viability in the solvent control.

3.5 Chemical profiling of cyanobacteria extracts

3.5.1 Determination of total phenolic content (TPC)

To determine the TPC of the cyanobacteria extracts, a colorimetric assay was used, based on the Folin-Ciocalteu reagent, according to Barroso *et al.*, [105] with slight modifications. The acetone extracts were solubilized in DMSO, and the aqueous extracts in water. To summarize, in an Eppendorf, 25µL of each extract (10mg mL^{-1}) was completely mixed with 25µL of Folin-Ciocalteu reagent (Sigma-Aldrich), 200µL of Na_2CO_3 solution (75g L^{-1}), and 500µL of deionized water. For the blanck, the Folin-Ciocalteu reagent was replaced by deionized water. The absorbance of the colored product formed was measured at 725nm, using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5™ software. Standard curves for TPC quantification were obtained using seven concentrations of gallic acid (GA) (0.025 to 0.5 mg mL^{-1}), prepared in the same solvent as the extracts to be tested. The respective linear regression equations are displayed in Figure 5. Three independent determinations were carried out in duplicate. The concentration of total phenolics was expressed as µg GA equivalents (GAE) mg^{-1} dry extract and in mg GAE g^{-1} dry biomass.

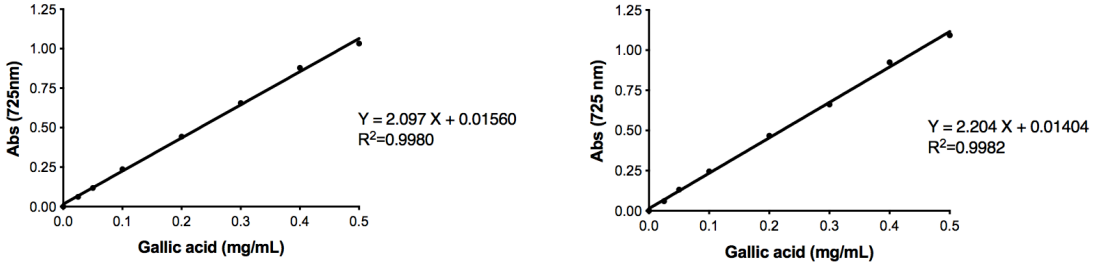


Figure 5. Standard calibration curves for Gallic acid in acetone (right) and water (left).

3.5.2 Determination of total proteins

Total proteins concentration was determined using the BCA Protein Assay kit (#23227, Thermo-Scientific). Aqueous extracts were prepared in water, while acetone extracts were prepared in DMSO. Briefly, in a 96 well plate, 25µL of each extract (1mg mL^{-1}) was mixed with 200µL of working reagent. The absorbance was measured at 562nm, using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5^{TM} software. A standard curve ($y=-126.87x^3+547.73x^2+483.85x-10.017$; $R^2=0,999$ and $y=162.87x^3-248.51x^2+932.13x-11.715$; $R^2=0,999$) was obtained for each extract, using nine concentrations of albumin (BSA) (25 to 2000 µg mL^{-1}) to quantify the proteins. Three independent experiments were carried out in triplicate. The total proteins were expressed as µg BSA equivalents (BSA) mg^{-1} dry extract and in mg BSA g^{-1} dry biomass.

3.5.3 Pigments determination

The pigments present in both aqueous and acetone extracts, including carotenoids, chlorophyll *a* and phycobiliproteins, were quantified spectrophotometrically.

Chlorophyll-*a* and derivatives were quantified using a calibration curve obtained with the commercial standard (Sigma-Aldrich), prepared using the same solvent as those used to solubilize cyanobacteria extracts: $y=8.0791x - 0.0022$ ($R^2=0.996$) water for aqueous extracts and $y=23.292x + 0.0032$ ($R^2=0.999$) DMSO for the acetone extracts.

Total carotenoids were expressed in beta-carotene and quantified using a calibration curve prepared with the commercial standard (Sigma-Aldrich). Similarly to the above, the linear regression equations were calculated with the respective solvents: water ($y=22.611x - 0.0032$; $R^2=0.999$) for aqueous extracts and DMSO ($17.133x + 0.0099$; R2=0.990) for acetone extracts. The concentration of total carotenoids was expressed

as µg beta-carotene equivalents (BCE) mg^{-1} dry extract and mg BCE g^{-1} dry biomass. Calibrations curves for both standards were obtained using five different concentrations (0.001 to 0.025 mg mL^{-1}).

The spectrophotometric determinations were performed in a 96-well plate, at different wavelengths (450nm for beta-carotene, 663nm for chlorophyll *a* and derivatives, and 562, 615 and 645nm for phycobiliproteins), using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5™ software.

For phycobiliproteins, phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE), the formulas used by Pagels *et al*., [106] were employed:

$$Phycocyanin(PC) = \frac{A_{615nm} - 0{,}474 \times A_{652nm}}{5.34}$$

$$Allophycocyanin(APC) = \frac{A_{652nm} - 0{,}208 \times A_{615nm}}{5.09}$$

$$Phycoerythrin(PE) = \frac{A_{562nm} - 2{,}41 \times PC - 0.849 \times APC}{9.62}$$

The experiment was carried out in triplicate. The results were expressed in mg of the respective phycobiliprotein by grams of dry extract ($mg_p\, g_E^{-1}$).

3.6 Antioxidant potential

3.6.1 Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity

The free radical scavenging assay of the $O_2^{\bullet-}$ was performed to evaluate the antioxidant potential of the cyanobacteria extracts, according to Barbosa and co-workers [107], with minor modifications. The aqueous extracts were prepared in water, while acetone extracts were prepared in DMSO. Five serial dilutions were prepared for each extract, and tested in order to evaluate the extract behaviour and to determine the effective concentration to achieve 50% of scavenging (IC_{50}), 25% (IC_{25}) and 10% (IC_{10}), whenever possible. All reagents were dissolved in phosphate buffer (19 µM, pH 7.4). A volume of 50 µL of each dilution was mixed with 50 µL of 166 µM β-nicotinamide adenine dinucleotide reduced form (NADH) solution and 150 µL of 43 µM nitrotetrazolium blue chloride (NBT) in a 96 wells plate. After the addition of 50 µL of 2.7 µM phenazine methosulphate (PMS), the radical scavenging activity of the samples was monitored with a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by

GEN5™ software, in kinetic function, at room temperature, for 2 minutes, at 562 nm. Three independent assays were performed in triplicate. GA was used as positive control (1 mg mL^{-1}). The results were expressed as percentage of radical scavenging in comparison to the untreated control, according to the formula:

$$\%\ O_2^{\bullet-}\ (scavenging) = 100 - \left[\frac{m_s * 100}{m_c}\right]$$

Where m_c is the velocity of reaction (absorbance units per second) of the control, and m_s is the velocity of reaction (absorbance units per second) of the sample.

The results for the calculated IC values were expressed as mean $\pm$ SD (µg mL^{-1}) of at least three independent assays performed in duplicate. The IC values and the corresponding dose-response curves were calculated with Graphpad Prism ® software (version 9).

3.7 Enzymes inhibition

3.7.1 Hyaluronidase inhibition assay

The hyaluronidase inhibition assay was slight modified from those proposed by Ferreres *et al*., [108]. Briefly, in a reaction tube, 25 µL of each extract (9 mg mL^{-1}), 175 µL hyaluronic acid (HA) (0.7 mg mL^{-1}), and 25 µL of hyaluronidase (HAase) (900U mL^{-1} in NaCl 0.9%) were mixed. Aqueous extracts were prepared in water, and the acetone extracts were prepared in DMSO. After 30 minutes of incubation at 37ºC, the reaction was stopped by adding 25 µL of di-sodium tetraborate (0.8M in water), and then heating for 3 min in a boiling water bath. The reaction tubes were cooled at room temperature and then 375 µL of DMAB [4-(Dimethylamino)benzaldehyde] solution was added. After 20 minutes of incubation ate 37ºC the absorbance of the colored product formed was measured in a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5™, at 560nm. For the negative control, 25 µL of the solvent was added instead of the extract, and the enzyme was substituted for NaCl 0,9%. DSCG (disodium cromoglycate) was used as positive control.

Three independent assays were performed in triplicate. The results were expressed as percentage of enzyme inhibition in comparison to the untreated control, according to the formula:

$$Inhibition(\%) = 100 - \left[\frac{A_s - A_w}{A_c} \times 100\right]$$

Where A_c is the absorbance of the control, the A_w is the absorbance of the untreated

sample, and A_S is the absorbance of the sample.

3.7.2 Elastase inhibition assay

Porcine pancreatic elastase inhibition assay was performed according to Mota and co-workers [109] with slight modifications. For the aqueous extracts water was used, and for acetone extracts, DMSO. Briefly, in a 96-wells plate it was mixed 50 µL of each extract (1 mg mL^{-1}), 90 µL of HEPES buffer (0.1 M), 10 µL of *N*-succinyl-Ala-Ala-Ala p-nitroanilide substrate (100 µM), 70 µL of acetate buffer (200 mM), and 30 µL of elastase (1 U mL^{-1}). After that, the plate was incubated at 37ºC for 10 min, and the absorbance of the reaction product was measured in a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5™, at 405nm. For negative control (0% inhibition), the reaction was conducted with DMSO or H_2O instead of the extract, and ascorbic acid was used as positive control. Three independent assays were performed in triplicate. The results were expressed as percentage of enzyme inhibition in comparison to the untreated control, according to the formula:

$$Inhibition(\%) = 100 - \left[\frac{A_s - A_w}{A_c} \times 100\right]$$

Where A_c is the absorbance of the control, the A_w is the absorbance of the untreated sample, and A_S is the absorbance of the sample.

3.7.3 Tyrosinase inhibition assay

The tyrosinase inhibitory activity assay was performed according to Adhikari *et al*.,[110] with slight modifications. Briefly, in a 96-well plate, 20 µL of each extract (10 mg mL^{-1}) were mixed with 100 µL of tyrosinase (30 U mL^{-1} in phosphate buffer). Aqueous extracts were prepared in water, while acetone extracts were prepared in DMSO. The mixture was incubated at 30ºC during 10 min. Then 80 µL of L-DOPA (L-3,4-dihydroxyphenylalanine) solution (2.5 mM in phosphate buffer) were added, and the absorption (T0) was immediately read with a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5™ software, at 475nm. After 8 minutes the absorbance was measure again (T8). For negative control (0% inhibition),

the reaction was conducted with DMSO or H_2O instead of extract. Kojic acid (20 mg mL^{-1}) was used as positive control. The percentage of inhibition was calculated according to the formula:

$$Inhibition\ (\%) = 100 - \left(\frac{A_{sample}}{A_{control}} \times 100\right)$$

Where A_{sample} is the absorbance of the samples (T8-T0), and $A_{control}$ is the absorbance of the control (T8-T0). Three independent assays were performed in triplicate.

3.8 Sun Protection Factor (SPF)

The *in vitro* sun protector factor was performed according to Rohr and co-workers [111] with slight modifications. Aqueous extracts were prepared in water, while acetone extracts were prepared in acetone. Briefly, the absorbance of 2 mL of each extract (200, 500, and 1000 µg mL^{-1}), was measured in a spectrophotometer (from 290 to 320 nm, 5 in 5 nm). The SPF was calculated using an estimation formula developed by Mansur [112]:

$$SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I\ (\lambda) \times Abs\ (\lambda)$$

Where $EE(\lambda)$ is the erythemal effect spectrum, $I\ (\lambda)$ is the solar intensity spectrum, $Abs(\lambda)$ is the absorbance of extract sample, and CF is the correction factor (=28), that was determined using a standard sunscreen by Lancaster® with a known SFP value of 30.

3.9 Statistical analysis

Statistical analysis was performed using GraphPad PRISM software (GraphPad® software, CA, USA) (Version 9 for MacOS). Data were analyzed for normality and homogeneity of variances by Kolmogorov-Smirnov and Leven's tests, and then submitted to one-way ANOVA followed by Tuckey' HSD multiple comparisons test, or Two Talied unpaired t-test.

4. Results and Discussion

In the present study, four cyanobacteria strains were obtained and cultured. When growth was deemed favorable for the highest biomass concentration, the harvest process took place for the posterior extraction process. After the sequential extraction, the cyanobacteria extracts were analyzed for their cytotoxicity using different skin cell lines: endothelial, fibroblasts, and keratinocytes cells. Once the toxicity in these cell lines was determined for cosmetic ingredients, the extracts followed the further chemical and biological analysis.

4.1 Cyanobacteria culture and sequential extraction

The process of growing the strains up to the required amount took about four months. During that period, there was a renew of culture medium that followed the scale-up process to boost biomass growth. There was also a special care to keep the same environmental conditions, such as sufficient amount of light, water temperature, pH, oxygen, and nutrient levels. The morphological characteristics and changes of cyanobacteria were further assembled through the growing process.

After the exponential growth phase the strains were collected and the extraction process started. Each extraction takes about a day, but as it is sequential, with the same amount of biomass it is possible to obtain completely different extracts, with different compounds and bioactivities. This makes the procedure more sustainable, environmental friendly and economically attractive.

Regarding the extraction yield (Table 2), it is possible to verify that the acetone extracts have much lower values than the aqueous ones, what may occur because of the different types of compounds that are extracted with each solvent.

It should be noted that *Leptolyngbya boryana* LEGE 15486 has higher values and in the aqueous extract is quite noticeable. Once again, these results can have the same explanation as mentioned above.

Table 2. Cyanobacteria extraction yield (%).

Strains	Acetone	Aqueous
Nodosilinea nodulosa LEGE 06104	0.6	9.0
Leptolyngbya cf. ectocarpi LEGE 11479	1.6	14.5
Cephalothrix lacustris LEGE 15493	2.0	9.5
Leptolyngbya boryana LEGE 15486	2.9	22.0

4.2 Cytotoxicity

Cytotoxicity tests are indispensable during cosmetics production, since they predict health risks connected with the use of the extracts as bioactive ingredients. In this sense, the cytotoxicity of the extracts was evaluated in three different cell lines. Due to logistical constraints, the fibroblasts (3T3L1) used in this study were from mouse, while endothelial cells (hCMEC) were from brain barrier. Keratinocytes (HaCat), on the other hand, were from human skin.

Endothelial cells form a barrier between the vessels walls and blood; they were chosen accounting their presence in dermis which, contrary to epidermis, is an irrigated layer of the skin [113]. The cytotoxicity results (Fig.6) show that none of the strains was toxic (<70% viability) for the cell lines under study, and under the tested concentrations. In general, it is also noticeable that the concentration of 200 µg dry extract mL^{-1} (24h) presents lower values. It is also possible to verify that the aqueous extracts have slightly greater viability than acetone extracts.

The data showed that cells exposed to the acetone extract of *Cephalothrix lacustris* LEGE 15493, at 200 µg dry extract mL^{-1} (24h), presented the lowest viability, but not less than 70% (Fig. 6, e). The non-toxicity of the extracts can be confirmed after 48h of exposition, when cells viability increased to about 90%.

Regarding the dermis, fibroblasts are the most important cells, being responsible for the production of the dermal matrix components, essential for the maintenance of skin shape and structure, such as collagen and hyaluronic acid [14,104].

Once again, the results showed that both aqueous and acetone extracts were non-toxic under the tested concentrations (Fig. 7). In fact, in the aqueous extracts, there seems to be a slight increase in viability compared to the control, while at 48h, the percentage of viability reduces. In *Leptolyngbya boryana* LEGE 15486 (24h) the increase was up to

115% cell viability (Fig. 7, d). *Cephalothrix lacustris* LEGE 15493, and *Leptolyngbya cf. ectocarpi* LEGE 11479 acetone extracts also showed a significant increase after 48h in 200 µg dry extract mL^{-1}, and 12.5 and 50 µg dry extract mL^{-1}, after 24h, respectively. The fact that there is a narrow increase within 24 hours can be beneficial to the skin, as it can lead to an upturn in the dermal matrix cells and thus contribute to skin repair. However, further studies are needed to prove this induction of cell proliferation and to verify that it only happens in this type of cell line, not presenting risks for other cells.

Regarding the concentrations, only the acetone extract of *Nodosilinea nodulosa* LEGE 06104 presented a decrease in cells viability with increasing concentration.

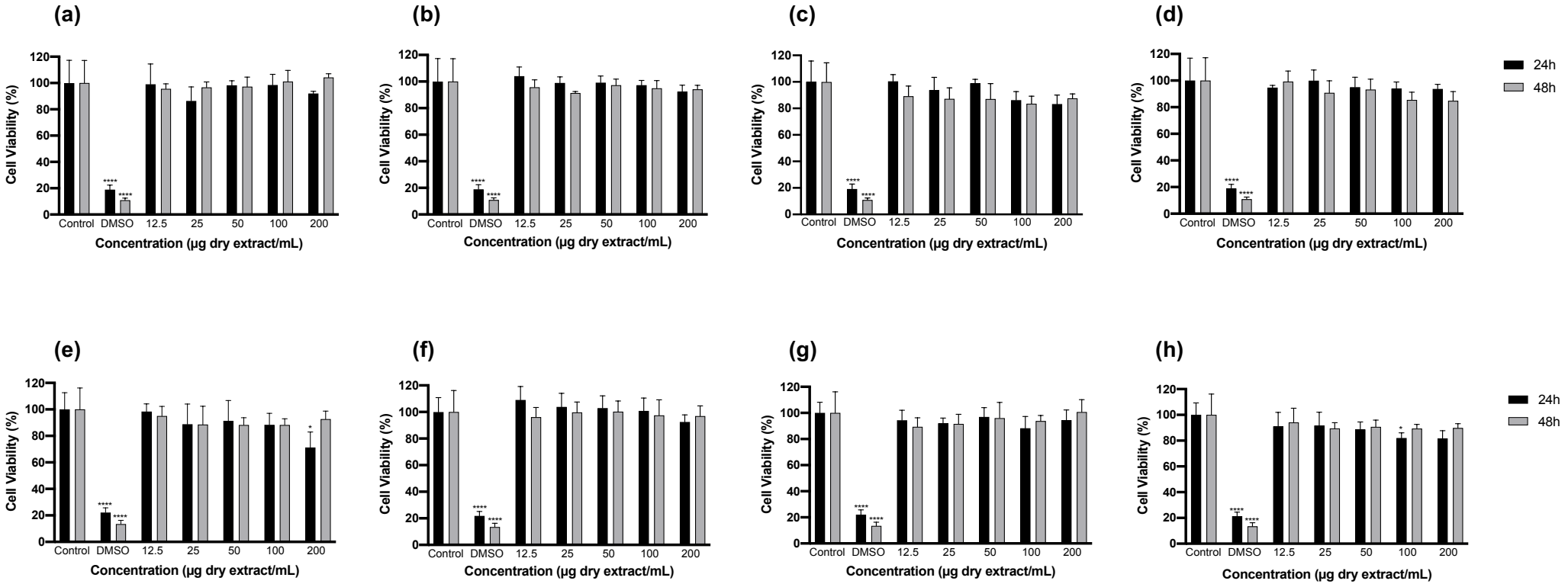


Figure 6. Endothelial cells (hCMEC) viability after 24 and 48 h of incubation with cyanobacteria aqueous **(a-d)** and acetone **(e-h)** extracts. **(a) (e)** *Cephalothrix lacustris* LEGE 15493**, (b) (f)** *Nodosilinea nodulosa* LEGE 06104**, (c) (g)** *Leptolyngbya cf. ectocarpi* LEGE 11479 and **(d) (h)** *Leptolyngbya boryana* LEGE 15486. Results are expressed as % of MTT reduction vs. the untreated control. DMSO (20%) represents the positive control. Results are expressed as the mean ± SD of at least three independent assays, performed in quadruplicate. Statistical differences at **p*<0.05, ** *p*<0.01, *** *p*<0.001, **** *p*<0.0001 (One way ANOVA).

Keratinocytes are an essential component of the epidermis, composing about 95% of it, and are present in all four layers [103]. They play an important role in providing structure and defense to the skin [114]. As it is possible to observe in Figure 8, the extracts show no toxicity for the keratinocytes cell line, having viability higher than 80%.
All values presented are lower than the control, except for *Leptolyngbya cf. ectocarpi* LEGE 11479, in the acetone extract, only at 200 µg dry extract mL^{-1} (24h).
Concerning the viability between 24 and 48 hours, it is not possible to obtain a correlation.
Regarding the control with DMSO, it is expected that the viability values are higher than those previously reported for the other cell lines, once keratinocytes are more resistant cells.

As it was possible to verify by the results presented, none of the extracts was considered toxic to the studied cell lines, thus all of them proceed on to the following assays.

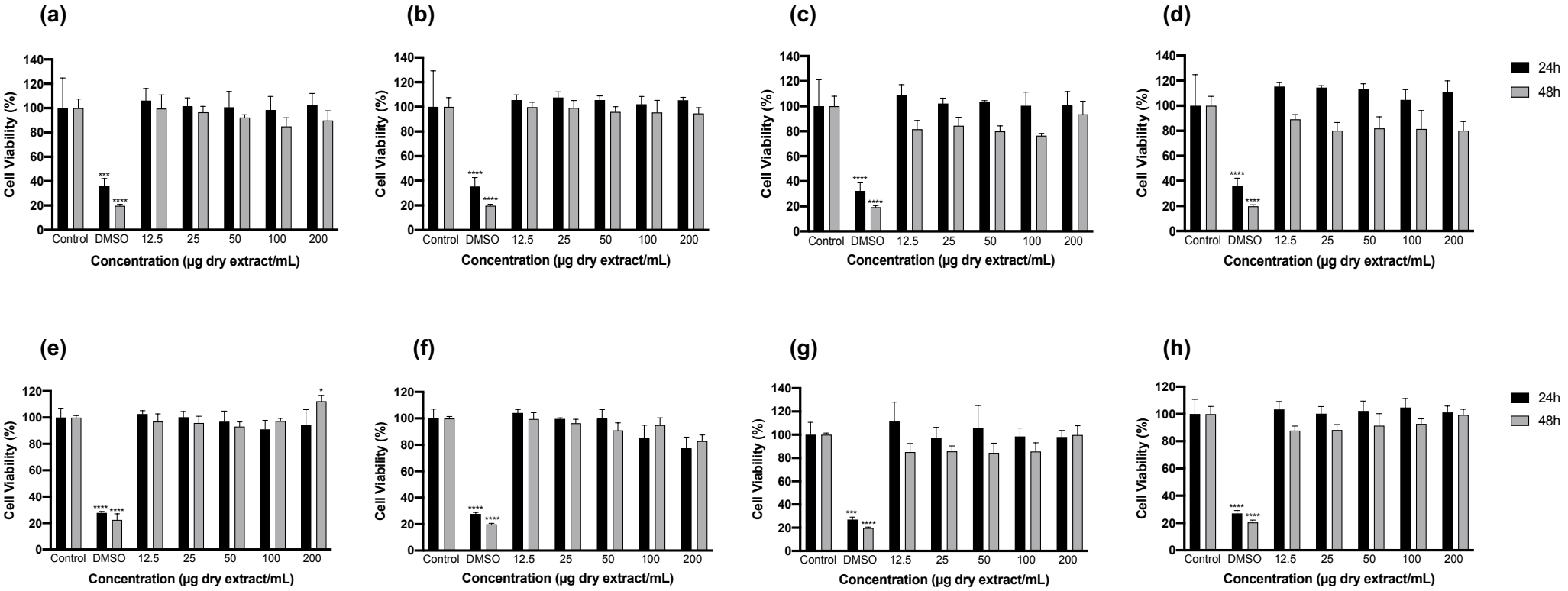


Figure 7. Fibroblasts cells (3T3L1) viability after 24 and 48 h of incubation with cyanobacteria aqueous **(a-d)** and acetone **(e-h)** extracts. **(a) (e)** *Cephalothrix lacustris* LEGE 15493**, (b) (f)** *Nodosilinea nodulosa* LEGE 06104**, (c) (g)** *Leptolyngbya cf. ectocarpi* LEGE 11479 and **(d) (h)** *Leptolyngbya boryana* LEGE 15486. Results are expressed as % of MTT reduction vs. the untreated control. DMSO (20%) represents the positive control. Results are expressed as the mean ± SD of at least three independent assays, performed in quadruplicate. Statistical differences at *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (One way ANOVA).

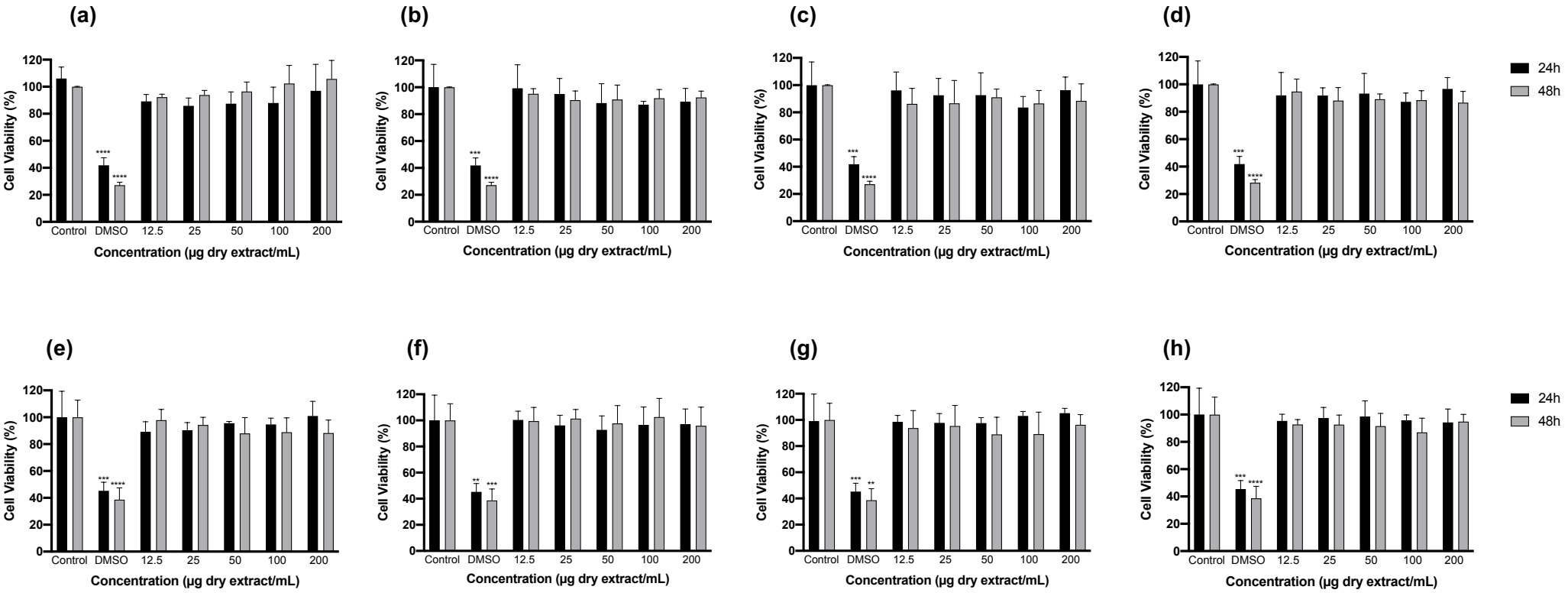


Figure 8. Keratinocytes cells (HaCAT) viability after 24 and 48 h of incubation with cyanobacteria aqueous **(a-d)** and acetone **(e-h)** extracts. **(a) (e)** *Cephalothrix lacustris* LEGE 15493**, (b) (f)** *Nodosilinea nodulosa* LEGE 06104**, (c) (g)** *Leptolyngbya cf. ectocarpi* LEGE 11479 and **(d) (h)** *Leptolyngbya boryana* LEGE 15486. Results are expressed as % of MTT reduction vs. the untreated control. DMSO (20%) represents the positive control. Results are expressed as the mean ± SD of at least three independent assays, performed in quadruplicate. Statistical differences at *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (One way ANOVA)

4.3 Chemical profiling

In most cases, the antioxidant capacity of the extracts is not only based on a single compound but in a mixture of different antioxidant compounds acting synergistically [52]. Therefore, a chemical characterization was performed, and the quantitative profile of phenols, proteins, carotenoids, and pigments was established. However, a study with a comprehensive qualitative analysis, for instance using HPLC- PDA and authentic standards for comparison would be an added value.

As mentioned before, these compounds are crucial to confer biological activities to the extracts, and thus, their determination can predict the potential of a natural source to be explored for cosmetic applications.

It is also important to mention that different physiological conditions can influence the amount of compounds and their different activities.

As the extraction is sequential, the value for dry biomass includes the values obtained in both extractions.

4.3.1 Phenols

The TPC of both extracts was measured through the Folin-Ciocalteu colorimetric assay. Even considering the inherent limitations of the method, as the formation of colored products with some compounds with no phenolic nature (e.g. with aromatic rings in their chemical structures), this is a standard assay widely used for a quick determination of total phenols, allowing the comparison of different samples and consequently predicting their antioxidant potential.

Table 3 contemplates the data (expressed in GAEs) for the total phenolic content of the eight extracts explored in the present study.

The highest phenolic content was found in *Leptolyngbya boryana* LEGE 15486, with 3.08 mg GAE g^{-1} of dry biomass, followed by *Cephalothrix lacustris* LEGE 15493, with 1.31 mg GAE g^{-1} of dry biomass, and *Leptolyngbya cf. ectocarpi* LEGE 11479, with 1.23 mg GAE g^{-1}. The lowest value was found for *Nodosilinea nodulosa* LEGE 06104, with 0.59 mg GAE g^{-1} dry biomass.

Table 3. Quantification of Total Phenolic Content (TPC) of cyanobacteria extracts [1,2,3].

Strains	μg GAE mg^{-1} dry extract		mg GAE g^{-1} dry biomass
	Aqueous	Acetone	
Nodosilinea nodulosa LEGE 06104	6.52 ± 0.38	11.23 ± 1.55	0.59 ± 0.03
Leptolyngbya cf. ectocarpi LEGE 11479	8.49 ± 1.18	17.59 ± 2.29	1.23 ± 0.17
Cephalothrix lacustris LEGE 15493	13.75 ± 0.28	nd	1.31 ± 0.03
Leptolyngbya boryana LEGE 15486	13.98 ± 0.90	7.10 ± 1.73	3.08 ± 0.20

[1]Expressed in gallic acid equivalents (GAE).
[2]Mean ±SD of three independent experiments.
[3]nd, not detected.

Overall, the freshwater strains presented higher values than those from marine environment. When comparing the two strains of the same genera it is possible to verify that *Leptolyngbya boryana* LEGE 15486 (freshwater) presented higher phenolic content. Despite being from the same genus and subjected to similar culture conditions, they belong to different species, are from different habitats, and are exposed to various types of stress, that molds diverse genetic codes.

It is also noteworthy that the phenolic values (dry extract) found for the acetone extracts are higher in the marine strains, while the freshwater strains presented better results with the aqueous extraction.

When making comparisons with other strains, we should consider the extraction method and the solvent used. Phenols have a polar and a nonpolar part in their molecules and possess a solubility preference to solvents like alcohols and acetone (intermediate polarity) rather than water (more polar). However, the solubility of phenols in different solvents cannot be based on their polarities, since other parameters like temperature and pH can have a great influence on their solubility [115].

Morone and co-workers [31] used the same methodology and found that *Nodosilinea nodulosa* LEGE 06102 had 1.23 mg GAE g^{-1}, however, the authors used ethanol as extraction solvent which certainly lead to the differences observed. Moreover, even being similar, the species were collected from different locations and have a different genetic code, what may influence compounds production. For all the seven strains tested by the authors, the highest value was found in *Synechocystis salina* LEGE 06099 (2.45 mg GAE g^{-1}). In another study, with *Nodosilinea antarctica* LEGE 13457 the TPC content was 19.23 μg GAE mg^{-1} (acetone extract) and no phenols were detected in *Leptolyngbya*-like sp. LEGE 13412, which demonstrates the high variability among similar species. They also reported data for other strains, being *Cyanobium gracile* LEGE

12431 the one presenting the highest content in phenols, with 22.01 μg GAE mg^{-1} dry acetone extract [32].

Trabelsi and team members [99] reported that the thermophilic cyanobacterium *Leptolyngbya* sp. had 139 mg GAE g^{-1}, this is probably due to the high-temperature levels of the habitat, and the solvent (methanol) used, which has high polarity. It is referred that, to avoid oxidative stress induced by the high temperatures, cyanobacteria produce higher amounts of phenols, flavonoids and others. Another research group [116] also reported a TPC of 6.24 mg GAE g^{-1} for *Leptolyngbya* sp. KC45, which provides from a location with ambient temperatures of approximately 40–45°C.

To the best of our knowledge, there are no previous reports regarding TPC in *Cephalothrix sp.*, therefore a comparison cannot be made.

Comparing with one of the most known and used cyanobacteria, *Spirulina* sp. (1.78 mg GAE g^{-1}), the strain *Leptolyngbya boryana* LEGE 15486 presented very promising results [117].

The values obtained for TPC of the cyanobacteria species considered herein were generally lower when compared with the previous studies regarding the same genera. Nevertheless, they still have antioxidant potential, which is one of the most desired activity in cosmetic products, thus being worth of further exploitations.

4.3.2 Proteins

Proteins are macromolecules consisting essentially of polymers of structural units called amino acids (AAs). No cell works properly without proteins, and their structures range from primary to quaternary [118]. When a linear chain of AAs folds into a particular three-dimensional conformation, it constitutes the most basic level or primary structure. The secondary structure is achieved due to self-folding forces (hydrogen bonds, salt, and disulfide bridges) or hydrophobic/ hydrophilic interactions. Those same forces can cause further arrangements and arise to a tertiary protein structure. When a protein incorporates more than one polypeptide chain, it is named a quaternary structure [118,119].

As previously mentioned, collagen, elastin, and keratin are skin proteins. To recapitulate, collagen is the most abundant, making about 80% of our skin. Together with elastase, it is responsible for giving structure, and its lower levels can cause wrinkles and fine lines. Keratin, gives the rigidity present in the skin surface, hair, and nails, also offering protection [120].

Regarding other important proteins in the body, we have hemoglobin, the protein that transports O_2 in the blood, plasma proteins, which includes albumin and lipoproteins, and muscle proteins, including actin, myosin, and troponin [118].

Nevertheless, we investigate the presence of total proteins and then a more specific family of colored pigment proteins called phycobiliproteins, which have already shown their relevance in this area.

4.3.2.1 Total Proteins

Proteins are generally undervalued, however they compose a large fraction of the cyanobacteria biomass [121]. It is reported that proteins have antioxidant and immune-stimulant properties and also confer moisture retention in the skin, which is essential to prevent skin ageing [53].

With this in mind, the cyanobacteria extracts explored in the present study were characterized for their proteins content. The results (Table 4) revealed that *Leptolyngbya boryana* LEGE 15486 has the higher content of total proteins (69.22 mg g^{-1}), followed by *Cephalothrix lacustris* LEGE 15493, *Leptolyngbya cf. ectocarpi* LEGE 11479, and *Nodosilinea nodulosa* LEGE 06104, with 49.52, 26.93, and 15.23 mg g^{-1}, respectively. This differences may happen because some cyanobacteria have higher amounts of structural proteins.

Table 4. Total proteins content in the cyanobacteria strains[1].

Strains	μg BSA mg^{-1} dry extract		mg BSA g^{-1} dry biomass
	Aqueous	Acetone	
Nodosilinea nodulosa LEGE 06104	169.18 ± 2.21	134.76 ± 2.41	15.23 ± 0.20
Leptolyngbya cf. ectocarpi LEGE 11479	185.69 ± 0.78	136.63 ± 3.60	26.93 ± 0.11
Cephalothrix lacustris LEGE 15493	521.18 ± 0.60	218.23 ± 3.48	49.52 ± 0.06
Leptolyngbya boryana LEGE 15486	314.60 ± 5.90	177.27 ± 5.89	69.22 ± 1.30

[1]Values are expressed as mean ± SD of three determinations.

It is possible to verify that the aqueous extraction resulted in higher values than the acetone one. Also, the marine strains presented lower values.

We were not able to compare the results with strains of the same genera, due to insufficient information and research.

In the work developed by Vega *et al*., [53], five cyanobacteria were studied, and their proteins content was measured. When comparing with our strains, the values are higher, however more biomass was used in the extraction and the solvents were different. Nevertheless, because of their bioactivities, cyanobacteria proteins and derivatives might be an interesting choice for cosmetics.

4.3.2.2 Phycobiliproteins

Data concerning phycobiliproteins content is summarized in tables 5 to 7.
In this work, the sequential extracts were obtained using ultrasonication, which may explain the release of PBPs in the first extraction with acetone, even though being water soluble molecules. Furthermore, when selecting a disruption method, it is important to assure that the method is harsh enough to release the cell content, without damaging it. Regarding phycocyanin, *Leptolyngbya boryana* LEGE 15486 is the one with the highest value (15.97 mg g^{-1}), followed by *Cephalothrix lacustris* LEGE 15493 (7.72 mg g^{-1}), *Nodosilinea nodulosa* LEGE 06104 (2.58 mg g^{-1}), and *Leptolyngbya cf. ectocarpi* LEGE 11479 (0.84 mg g^{-1}). In relation to LEGE 15486, LEGE 15493, and LEGE 06104, PC was dominant. These results are in accordance with the appearance in color of the biomass and the aqueous extracts. LEGE 11479 has a pink/purple coloration in the biomass and the extract, and the other ones are green in the biomass and blue in the extracts. LEGE 15486 has the most intense blue color and the LEGE 06104 the weakest.

Table 5. Phycocyanin content in the cyanobacteria strains[1].

Strains	μg mg^{-1} dry extract		mg g^{-1} dry biomass
	Aqueous	Acetone	
Nodosilinea nodulosa LEGE 06104	28.65 ± 3.21	101.31 ± 0.42	2.58 ± 0.29
Leptolyngbya cf. ectocarpi LEGE 11479	5.76 ± 0.14	103.48 ± 0.14	0.84 ± 0.02
Cephalothrix lacustris LEGE 15493	81.27 ± 5.79	81.54 ± 2.03	7.72 ± 0.55
Leptolyngbya boryana LEGE 15486	72.58 ± 14.29	118.42 ± 1.81	15.97 ± 3.14

[1]Values are expressed as mean ± SD of three determinations.

The content of APC (Table 6) was higher in LEGE 15486, with 7.25 mg g^{-1}, followed by LEGE 15493, LEGE 11479 and LEGE 06104. Once again, the same can be said about the color of the extracts, since the APC has a bluish-green color.

About PE (Table 7), the higher content was found in LEGE 15486, succeeded by LEGE 11479, LEGE 15493, and LEGE 06104.

Although the differences between the values are not much, it would be expected for LEGE 11479 to have higher values, due to its pink/purple color. This can be explained by the fact that the extraction yield was superior. As is possible to observe in table 2, LEGE 15486 was the one with the highest yield. And the values are calculated with the yield data.

When comparing strains in terms of phycobiliproteins, it is extremely important to pay attention to the culture conditions, once differences in light, nitrogen, temperature, pH, carbon, and salinity, can drastically influence their production [40].

Table 6. Allophycocyanin content in the cyanobacteria strains[1].

Strains	μg mg^{-1} dry extract		mg g^{-1} dry biomass
	Aqueous	Acetone	
Nodosilinea nodulosa LEGE 06104	11.74 ± 0.11	308.17 ± 8.86	1.06 ± 0.01
Leptolyngbya cf. ectocarpi LEGE 11479	8.93 ± 0.38	301.50 ± 4.98	1.30 ± 0.06
Cephalothrix lacustris LEGE 15493	33.03 ± 1.45	632.14 ± 22.98	3.15 ± 0.14
Leptolyngbya boryana LEGE 15486	32.78 ± 5.78	416.00 ± 6.66	7.25 ± 1.27

[1]Values are expressed as mean ± SD of three determinations.

In Pumas and co-workers [116], the thermotolerant cyanobacteria *Leptolyngbya* sp. KC45 showed a content of PE of almost 100 mg g^{-1}, followed by about 40 and 43 mg g^{-1} for APC and PC, respectively. In addition to the differences in the species and cultivation, it was possible to correlate the color of the strain (red-brown) with the amount of PE.

To our knowledge, there is no more information that allow us to make comparisons with the other species of the same genera.

Table 7. Phycoerythrin content in the cyanobacteria strains[1].

Strains	μg mg^{-1} dry extract		mg g^{-1} dry biomass
	Aqueous	Acetone	
Nodosilinea nodulosa LEGE 06104	4.9 ± 0.40	68.52 ± 1.53	0.44 ± 0.04
Leptolyngbya cf. ectocarpi LEGE 11479	7.87 ± 0.2	67.53 ± 2.68	1.14 ± 0.03
Cephalothrix lacustris LEGE 15493	11.19 ± 0.14	74.51 ± 2.99	1.06 ± 0.01
Leptolyngbya boryana LEGE 15486	5.50 ± 0.71	110.33 ± 0.13	1.21 ± 0.16

[1]Values are expressed as mean ± SD of three determinations.

Comparing with other genera, specifically with the widely known *Spirulina* sp., it presented values of total PBPs of about 19 mg g^{-1} [117]; our strain LEGE 15486 had a total of 24.43 mg g^{-1}, which enhances its economic interest. In the same study, the authors reported values of 127.01 mg g^{-1} of total PBPs of *Lyngbya* sp. In another study they presented data regarding 18 strains, where the higher amount was found in *Anabaena* NCCU-9 , with 91.54 mg g^{-1} [122].

4.3.3 Carotenoids and Chlorophyll *a*

As shown in table 8, the total carotenoid concentration ranged from 0.30 to 0.68 mg g^{-1} dry biomass. Among the cyanobacteria strains under study, the highest content was found in *Leptolyngbya boryana* LEGE 15486 (0.68 mg g^{-1}), followed by *Leptolyngbya cf. ectocarpi* LEGE 11479 and *Cephalothrix lacustris* LEGE 15493 (0.44, 0.43 mg g^{-1}, respectively). Once again the lowest value was detected in *Nodosilinea nodulosa* LEGE 06104.

Table 8. Carotenoid content in the cyanobacteria strains[1].

Strains	μg mg^{-1} dry extract		mg g^{-1} dry biomass
	Aqueous	Acetone	
Nodosilinea nodulosa LEGE 06104	3.35 ± 0.35	125.34 ± 5.0	0.30 ± 0.03
Leptolyngbya cf. ectocarpi LEGE 11479	3.01 ± 0.23	89.07 ± 5.0	0.44 ± 0.03
Cephalothrix lacustris LEGE 15493	4.54 ± 0.61	137.50 ± 2.8	0.43 ± 0.06
Leptolyngbya boryana LEGE 15486	3.04 ± 0.93	159.39 ± 5.0	0.68 ± 0.21

[1]Values are expressed as mean ± SD of three determinations.

The results show higher values in the acetone extraction, and for the freshwater strains. The content in carotenoids was generally lower compared to chlorophyll *a* (Table 8 and 9), with *Leptolyngbya boryana* LEGE 15486 having repeatedly the higher value (3.80 mg g^{-1}) in chlorophyll *a*. It was succeed by *Cephalothrix lacustris* LEGE 15493 (1.87 mg g^{-1}), *Nodosilinea nodulosa* LEGE 06104 (0.50 mg g^{-1}), and *Leptolyngbya cf. ectocarpi* LEGE 11479 (0.49 mg g^{-1}).

Table 9. Chlorophyll *a* and derivatives content in the cyanobacteria strains[1].

Strains	μg mg^{-1} dry extract		mg g^{-1} dry biomass
	Aqueous	Acetone	
Nodosilinea nodulosa LEGE 06104	5.51 ± 0.31	117.89 ± 4.86	0.50 ± 0.03
Leptolyngbya cf. ectocarpi LEGE 11479	3.38 ± 0.31	114.25 ± 2.73	0.49 ± 0.04
Cephalothrix lacustris LEGE 15493	19.58 ± 1.04	264.94 ± 11.84	1.87 ± 0.10
Leptolyngbya boryana LEGE 15486	17.25 ± 3.91	190.02 ± 6.38	3.80 ± 0.86

[1]Values are expressed as mean ± SD of three determinations.

Lopes and her team [32] obtained 0.657 mg g^{-1}, dry biomass, of total carotenoids for *Nodosilinea antarctica* LEGE 13457 acetone extract (converted values). For chlorophyll *a* content, it was reported 348.97 μg mg^{-1} dry extract; in this work the content was lower, being 117.894 μg mg^{-1}. The authors also provided data for *Leptolyngbya*-like sp. LEGE 13412 in μg mg^{-1} dry extract, with 33.104 and 61.726, for carotenoids, and chlorophyll *a*, respectively. When comparing with our results the values are significantly lower. This difference can be explained due to the fact that the strains presented in this work are aquatic (Portugal and Brazil), and the others are from Antarctica, were the conditions are severe and possibly more likely for the production of these compounds. It has been reported that species from that environment are exposed to high radiation, temperature fluctuations, desiccation periods, and hydrodynamics [53]. In that conditions, cyanobacteria produce excessive ROS, which can lead to DNA mutations, lipid peroxidation, or proteins denaturalization, affecting important biological processes such as photosynthesis.

Another study revealed that *Nodosilinea nodulosa* LEGE 06102 presented 0.371 mg g^{-1} dry biomass (ethanol extract) of total carotenoids [31]. These results are similar and/or lower than ours, once the authors did not detect chlorophyll *a*. They also studied other six strains and the results didn't vary much, what may be explained by the very similar culture conditions and methodology and those used herein.

With regard to the content of chlorophyll *a*, it is possible to verify that the freshwater strains have higher values, alike TPC.

As it can be seen through table 9, the acetone extracts were more effective in the extraction of this type of pigment. It is also significant that the acetone extracts are green, so this results are in agreement with the laboratory observations.

Although having the same genera, differences regarding pigments profile were clear in *Leptolyngbya* sp. Therefore, different cultivation conditions, nutrients supply, among other things may have led to the various results.

The final antioxidant capacity might be higher or lower, and many times independently of the content of the bioactive compounds, what may happen by additive, synergistic or antagonistic effects [52]. Thus, it would be important to further perform a qualitative profile of the extracts, and evaluate the mechanisms underlying the antioxidant activity of the bioactive compounds.

From the overall results, the strain *Leptolyngbya boryana* LEGE 15486 presents the highest values. The fact that the extraction yield is also the highest is a plus point. Therefore, this cyanobacteria has great potential in the cosmeceutical and pharmaceutical fields. Moreover, successive extractions seem to be a good strategy to maximize the biomass, and reduce waste.

4.4 Antioxidant Potential

Superoxide anion radical scavenging activity

The $O_2^{\bullet-}$ is an extremely important ROS. It causes oxidative damage to the human body and, finding mechanisms that inhibit its action is essential, not only in the field of cosmetics, but also for the amelioration and prevention of a wide array of diseased. The $O_2^{\bullet-}$ scavenging activity data is summarized in Table 10 and Figure 9.

Regarding the aqueous extracts, where more promising results were obtained, we have *Cephalothrix lacustris* LEGE 15493 with the lowest IC_{50} (65.5 μg mL^{-1}) followed by *Leptolyngbya boryana* LEGE 15486 (99.5 μg mL^{-1}), and *Nodosilinea nodulosa* LEGE 06104 (101.3 μg mL^{-1}). For *Leptolyngbya cf. ectocarpi* LEGE 11479, only the IC_{25} was reached (233 μg mL^{-1}) for the higher concentration tested (Figure 9).

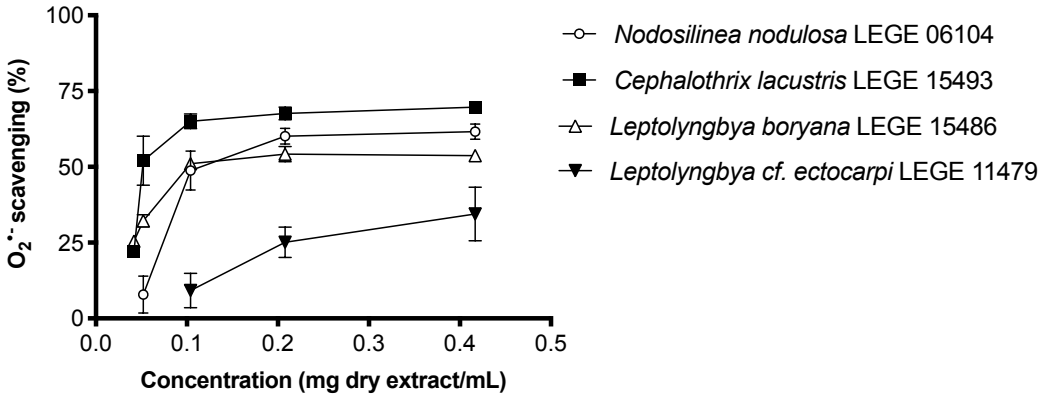


Figure 9. Superoxide radical scavenging activity of aqueous extracts of *Nodosilinea nodulosa* LEGE 06104, *Cephalothrix lacustris* LEGE 15493, *Leptolyngbya boryana* LEGE 15486, and *Leptolyngbya cf. ectocarpi* LEGE 11479. Values are expressed as mean ± SD, n=3.

In relation to the acetone extracts, only LEGE 11479 revealed some potential, with IC_{50}, and IC_{25} of 1190.5 and 580 μg mL^{-1} respectively. Once again the freshwater strains presented the best results.

The question of the correlation between antioxidant compounds such as, phenols, carotenoids, chlorophylls, and antioxidant activity, like the $O_2^{\bullet-}$ scavenging activity, is a controversial issue. Some studies proved a close correlation, on the other hand, others did not find a significant link between those compounds and other antioxidant activities. In this case, it was possible to obtain a negative correlation for the aqueous extracts once, in a general way, the strains with higher amount of phenolic compounds, carotenoids, chlorophylls, and proteins had better results (lower IC values) in the $O_2^{\bullet-}$ scavenging activity. In Lopes [32] and Morone [31], it was also reported that a higher content of chlorophylls could contributed to $O_2^{\bullet-}$ scavenging.

Regarding acetone extracts, it was only possible to correlate the amount of total phenols with the $O_2^{\bullet-}$ scavenging activity, since the *Leptolyngbya cf. ectocarpi* LEGE 11479 had the best values for both assays.

An inquisitive result appears in LEGE 15486 acetone extract; despite having the highest amount of carotenoids, it only reached IC_{25}, indicating that different contributions of carotenoids depend on their chemical features. Also, the antioxidant capacity of carotenoids decrease in the presence of hydroxyl and keto groups, and increase with upturn numbers of conjugated double bonds [32]. Therefore, the reactivity between carotenoids and $O_2^{\bullet-}$ differs, showing that the antioxidant activity depends upon various antioxidant compounds. As a matter of fact, the qualitative analysis of the carotenoids

profile would be helpful to infer about the contribution of any individual compound.

Table 10. Inhibitory concentration (IC) values (μg mL^{-1}) obtained for the $O_2^{\bullet-}$ scavenging activity of cyanobacteria extracts[1,2].

Strains	Acetone		Aqueous
	IC_{25}	IC_{50}	IC_{50}
Nodosilinea nodulosa LEGE 06104	1121.50 ± 89.80	nd	101.33 ± 22.94
Leptolyngbya cf. ectocarpi LEGE 11479	580.00 ± 29.69	1190.50 ± 108.19	nd
Cephalothrix lacustris LEGE 15493	1080.00 ± 254.93	nd	65.50 ± 7.78
Leptolyngbya boryana LEGE 15486	1190.33 ± 110.82	nd	99.50 ± 0.71

[1]Values are expressed as mean ± SD of three determinations. [2] nd: Not determined.

A comparison of antioxidant capacities among different cyanobacteria is challenging due to the different methods applied. In Morone and co-workers [31], the lowest IC_{50} was 822.70 μg mL^{-1} for *Phormidium* sp. LEGE 05292, ethanol extraction, and *Nodosilinea nodulosa* LEGE 06102 did not display $O_2^{\bullet-}$ scavenging activity. Lopes *et al.,* [32] reported for *Nodosilinea (Leptolynbbya) antarctica* LEGE13457 and *Cuspidothrix issatschenkoi* LEGE 03282 an IC_{25} of 319 and 286 μg mL^{-1} for acetone extracts. *Leptolynbbya-like* sp. LEGE 13412 didn't present any activity. They also reported a higher effectiveness in the acetone extracts rather than the ethanol ones. In Amaro and co-workers [123], the IC_{50} values for *Gloeothece* sp. and *Scenedesmus obliquus* (M2-1) were 1394 and 826 μg mL^{-1} respectively.

Comparing with the same type of solvent, in general, our results are less promising. On the other hand, the aqueous extracts revealed an enormous potential.

4.5 Enzymes inhibition

4.5.1 Hyaluronidase

Regarding HAase, only three strains presented the capacity to inhibit this enzyme, acting in a dose-dependent manner (Figure 10). *Leptolyngbya cf. ectocarpi* LEGE 11479 (IC_{50} =863 μg mL^{-1} and IC_{25} =489 μg mL^{-1}) in aqueous extracts, *Cephalothrix lacustris* LEGE

15493 and (IC_{25} =832 μg mL^{-1}) and *Nodosilinea nodulosa* LEGE 06104 (IC_{25} =995 μg mL^{-1}) in acetone extracts. In this case, the marine strains presented more promising results.

When comparing with the reference drug Di-sodium cromoglicate (DSCG), IC_{50} =1105 μg mL^{-1}, it is possible to conclude that the extracts under study are active in a similar concentration range.

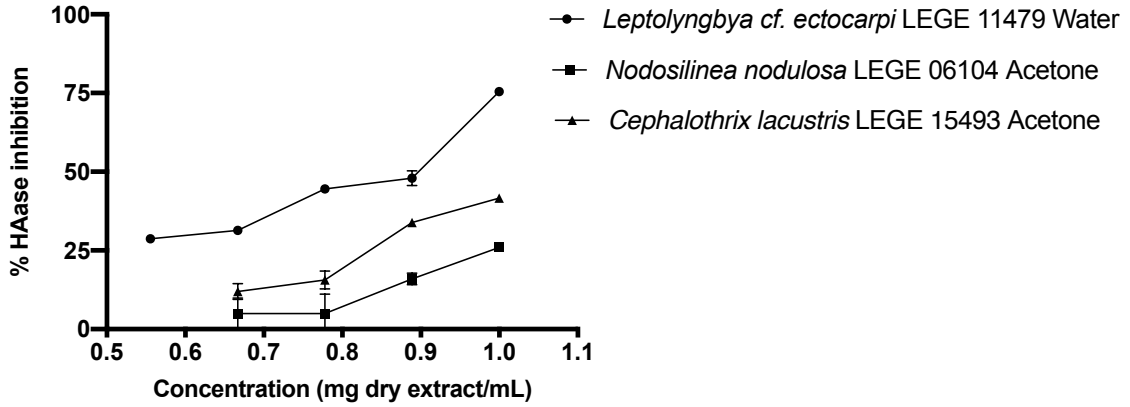


Figure 10. Inhibitory activity of cyanobacteria extracts of *Nodosilinea nodulosa* LEGE 06104, *Cephalothrix lacustris* LEGE 15493, and *Leptolyngbya cf. ectocarpi* LEGE 11479 against hyaluronidase (HAase). Values are expressed as mean ± SD, n=3.

Although in lower amount, there are some reports on the potential of cyanobacteria compounds and extracts to act upon hyaluronidase. In Morone and co-workers [31], two cyanobacteria (*Tychonema* sp. LEGE 07196 and *Cyanobium* sp. LEGE 07175) showed strong inhibitory activity, with IC_{50} =182.74 and 208.36 μg mL^{-1} for ethanol extracts.

For an ethanol-insoluble fraction of *Spirulina platensis* it was reported an IC_{50} of 150 µg ml^{-1} [81].

Another study, conducted by Yamaguchi & Koketsu [80], showed that *Nostochopsis lobatus* MAC0804NAN can produce a large amount of polysaccharides with a high inhibitory effect (IC_{50}= 7.18 µg ml^{-1}). It was also reported that an *Arthrospira*-derived peptide may be involved in hyaluronidase inhibition [63].

Once again these results support the utilization of cyanobacteria compounds as anti-ageing ingredients for cosmeceutical industry.

4.5.2 Elastase

Considering elastase inhibitory activity, it was only in the acetone extracts that some interesting activity was found (Fig. 11). Once again, only *Leptolyngbya cf. ectocarpi* LEGE 11479 reached IC_{50}, with a value of 391 µg ml^{-1} (IC_{25} was 74 µg ml^{-1}). *Nodosilinea nodulosa* LEGE 06104, *Cephalothrix lacustris* LEGE 15493, and *Leptolyngbya boryana* LEGE 15486 only reached IC_{25}, with values of 126, 86, and 99 µg ml^{-1}. Also in this case, the marine strains obtained better results.

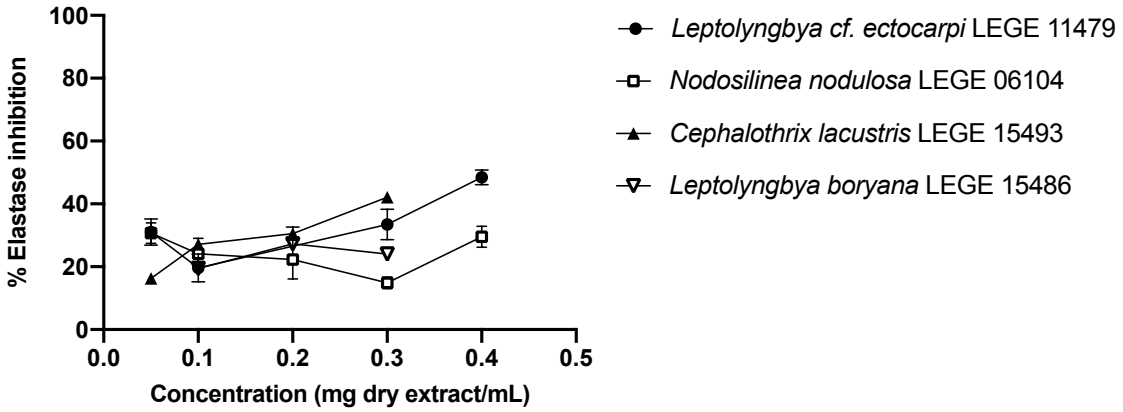


Figure 11. Inhibitory activity of cyanobacteria acetone extracts of *Leptolyngbya cf. ectocarpi* LEGE 11479, *Nodosilinea nodulosa* LEGE 06104, *Cephalothrix lacustris* LEGE 15493, and *Leptolyngbya boryana* LEGE 15486 against elastase. Values are expressed as mean ± SD, n=3.

It was not possible to make comparisons with strains of the same genera due to the lack of data. Regarding other strains, it was discovered that *Nostoc minutum* produced microviridins-type peptides and nostopeptins, with IC_{50} = 1.3 and 11.0 µg ml^{-1} [124,125]. Microviridins B and C obtained from *Microcystis aeruginosa* inhibited elastase effectively, with IC_{50} values of 0.044 and 0.084 µg ml^{-1} [126]. Most of the available data regarding elastase inhibition is about isolated compounds, so comparisons with the extracts from our strains are difficult to make.

The results regarding elastase inhibition of our strains are inferior to those presented. However, they still have some potential, as they have shown activity in all different assays.

4.5.3 Tyrosinase

Even though the majority of the available studies are trifling and explores mushroom tyrosinase as enzymatic model, which makes it difficult to translate the results to human environment, this enzyme as high similarity and homology compared to human tyrosinase [95]. So, in this study, the same model was used, as the enzyme is easier to purchase, the protocols are optimized, and so was possible to compare results.
Once again it was only in the acetone extracts that some activity was found, the results are expressed in Figure 12 and Table 11.

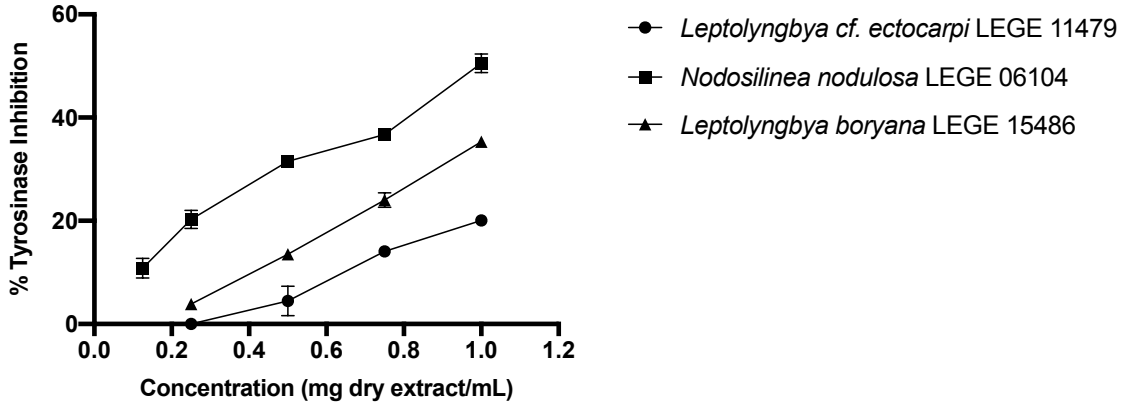


Figure 12. Inhibitory activity of cyanobacteria acetone extracts of *Leptolyngbya cf. ectocarpi* LEGE 11479, *Nodosilinea nodulosa* LEGE 06104, and *Leptolyngbya boryana* LEGE 15486 against tyrosinase. Values are expressed as mean ± SD, n=3.

Nodosilinea nodulosa LEGE 06104 obtained the utmost results, being the only to achieve the IC_{50} with 989.26 ± 4.3 μg mL^{-1}. Alongside is *Leptolyngbya boryana* LEGE 15486 with IC_{25} =784. 78 ± 4.33 μg mL^{-1} and *Leptolyngbya cf. ectocarpi* LEGE 11479 only reached IC_{20}. More promising results were found in the marine strains.
In the work of Morone and co-workers [31], from the seven ethanol extracts tested, none of the strains revealed inhibition of this enzyme. An interesting aspect is that one of the studied strains was *Nodosilinea nodulosa* LEGE 06102 that, in our case, was the one with the best results, once again emphasizing the importance of genetic codes differences among strains of the same species.
It was not possible to make comparisons with the other strains of the same genera due to the lack of information.

Table 11. Inhibitory concentration (IC) values (μg mL^{-1}) obtained for tyrosinase activity of cyanobacteria extracts[1,2].

Strains	IC_{50}	IC_{25}	IC_{20}	IC_{15}
Nodosilinea nodulosa LEGE 06104	989.26 ± 4.3	323.37 ± 41.1	nd	nd
Leptolyngbya cf. ectocarpi LEGE 11479	nd	nd	980.99 ± 4.7	806.03 ± 13.9
Leptolyngbya boryana LEGE 15486	nd	784. 78 ± 4.33	nd	518. 48 ± 40.2

[1]Values are expressed as mean ± SD of three determinations.
[2] nd: Not determined.

Concerning other strains, in the work conducted by Yabuta and team members [127], it was reported that the hot water extract of *Nostochopsis* sp. significantly inhibited the tyrosinase activity (IC_{50}= 250 μg mL^{-1}). This is a very interesting result because is a much better value than the one we obtained, and with aqueous extract. They attribute the result to low molecular compounds released from pycobiliproteins by heat treatment. One compound can be a potent peroxy radical scavenger, named biline moiety.
Another study evaluated the inhibitory activity of *Arthrospira platensis* ethanol (IC_{50}= 14000 μg mL^{-1}) and water (IC_{50}= 72000 μg mL^{-1}) extracts [90]. They explained this values due to substances like ferulic and caffeic acids in ethanol extract.

4.6 Sun Protector Factor

The photoprotection capacity was measured only for the UVR-B, since it is the most harmful radiation. Three different concentrations of the extracts were tested (Table 12).
Regarding acetone extracts, the highest value was found for *Leptolyngbya boryana* LEGE 15486 in the lower concentration, followed by *Leptolyngbya cf. ectocarpi* LEGE 11479 and *Cephalothrix lacustris* LEGE 15493, also in the lower concentration, and lastly *Nodosilinea nodulosa* LEGE 06104 in the highest concentration.
It is curious that with the increasing concentrations, the SFP value does not increase, with the exception of LEGE 06104.
In aqueous extracts the highest values were all obtained at the highest concentrations, in the following order: LEGE 15486, LEGE 15493, LEGE 11479, and LEGE 06104.
Hossain and his team [98] reported that the SPF value for *Cephalothrix komarekiana* extract was 2.37. Another group found that the SPF of a methanol extract of *Aphanizomenon flos-aquae* was 4 [128]. However, the information about the

concentration of the extract used by the authors was not available, so comparisons were not possible to make.

Table 12. Sun protector factor of cyanobacteria extracts[1].

Strains	Acetone			Aqueous		
	200 μg mL^{-1}	500 μg mL^{-1}	1000 μg mL^{-1}	200 μg mL^{-1}	500 μg mL^{-1}	1000 μg mL^{-1}
Nodosilinea nodulosa LEGE 06104	3.50 ± 0.07	5.20 ± 0.19	6.30 ± 0.09	1.30 ± 0.02	3.61 ± 0.03	7.27 ± 0.05
Leptolyngbya cf. ectocarpi LEGE 11479	10.74 ± 0.41	8.43 ± 0.32	7.44 ± 0.15	1.97 ± 0.02	5.56 ± 0.00	11.54 ± 0.00
Cephalothrix lacustris LEGE 15493	7.79 ± 0.51	5.85 ± 0.22	5.06 ± 0.16	2.87 ± 0.02	7.38 ± 0.43	14.86 ± 0.42
Leptolyngbya boryana LEGE 15486	19.22 ± 1.50	15.06 ± 0.11	12.00 ± 0.78	3.05 ± 0.16	5.74 ± 0.02	17.16 ± 0.02

[1]Values are expressed as mean ± SD of three determinations

The results show that these strains could be a good option as biological photoprotectors. More studies are needed to evaluate if they can be included in lotions to act as sunscreens, or act as a booster to other sunscreens.

5. Conclusions

The growing research in natural sources, more specifically from marine origin, has provided a countless number of new molecules with promising bioactivities worth of further exploitation in the field of skin aging. As it is known, from previous studies, cyanobacteria produce a wide range of bioactive substances including phenolic compounds, proteins, and pigments with antioxidant capacity. Since there is very scarce information about cyanobacteria compounds for skin ageing, this work contributed immensely for this purpose. Furthermore, the presented findings indicate that some cyanobacteria strains can be utilized for providing high-value compounds for cosmeceutical and pharmaceutical industry. Due to their antioxidant, anti-enzymatic and photoprotection capacity.

The non-cytotoxic acetone and aqueous extracts of the different species displayed promising results for different areas, but further biochemical studies are needed to identify the chemical structures of the bioactive compounds. In general all strains have potential, more specifically *Leptolyngbya boryana* LEGE 15486 for antioxidant and sun protection activity, and *Leptolyngbya cf. ectocarpi* LEGE 11479 for enzymatic inhibition activity.

It was also verified that depending on the solvent used and extraction techniques the results can vary. Moreover, the strategy of using sequential extraction increases the biotechnological value of the strain.

However further exploitation is needed since there are many other strains to discover and study that can be used in cosmeceutical area.

6. Future Perspectives

The growing research in natural sources, more specifically from marine origin, has provided a countless number of new molecules with promising bioactivities worth of further exploitation in the field of skin aging. Besides the inherent advantages of using cyanobacteria as metabolic producers, it has been demonstrated herein their huge potential to target specific enzymes involved in the aging process, most of the times with an activity significantly higher than that of the reference drugs currently in use. Allied to this, the concern in delaying the effects of aging has been the fuel for the investment in the search for new, innovative, effective and eco-friendly compounds, aiming the discovery of the perfect anti-aging formulation.

In this regard, it seems unquestionable that further toxicological and biotechnological studies will be the next steps to evaluate the safety and effectiveness of these molecules in anti-aging formulations. New assays, such as HPLC- PDA, collagenase inhibition, and in the long run testes with human volunteers will be made to increase knowledge, that will much probably revolutionize the cosmetic market.

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