Oceanological and Hydrobiological Studies

International Journal of Oceanography and Hydrobiology

Volume 50, No. 4, December 2021
pages (385-397)

sciendo

ISSN 1730-413X
eISSN 1897-3191

Tunisian inland water microflora as a source of phycobiliproteins and biological activity with beneficial effects on human health

by

Khaled Gharbi[1,*], Afef Fathalli[2], Rym Essid[3], Chiheb Fassatoui[1], Mohamed Salah Romdhane[1], Ferid Limam[3], Amel Ben Rejeb Jenhani[1]

DOI: **10.2478/oandhs-2021-0033**
Category: **Original research paper**
Received: **May 2, 2021**
Accepted: **July 5, 2021**

[1]*Laboratoire Écosystèmes et Ressources Aquatiques et Animale (LR21AGRO1), Institut National Agronomique de Tunisie, Université de Carthage, 43, Avenue Charles Nicolle, 1082, Tunis Mahrajène, Tunisia*

[2]*Institut National des Sciences et Technologies de la mer, Port de pêche 2060, La Goulette, Tunisia*

[3]*Laboratory of Bioactive Substances, Biotechnology Center of Borj Cedria, BP-901, 2050, Hammam-Lif, Tunisia*

* Corresponding author: khaledgharbi10@yahoo.fr

Abstract

Ten monoclonal microalgal cultures were obtained from several Tunisian inland water bodies, and their dichloromethane and methanolic extracts were screened for antibacterial, antileishmanial, and antioxidant properties, as well as phycobiliprotein production capacity. *Cylindrospermopsis raciborskii* has been shown to synthesize high levels of phycocyanin and may be an effective alternative source to other sources used for commercial production of phycocyanin. *Chroococcus* sp. and *Leptolyngbya* sp1. exhibited the strongest radical scavenging activity against DPPH (IC_{50} = 212.15 and 263.91 µg ml^{-1}, respectively), indicating their promising potential for use as new effective and non-toxic antioxidants. Furthermore, *Dunaliella* sp. showed an interesting antileishmanial activity against the pathogens *Leishmania infantum* and *Leishmania major* (IC_{50} = 151 and 284 µg ml^{-1}, respectively), thus representing a good candidate for use against cutaneous and visceral leishmaniasis in Tunisia, a country endemic to these diseases where thousands of new cases are registered every year. These results suggest that the strains of microalgae featured in this work have the potential to serve as natural alternative, safe and sustainable sources of high value-added products that could be used to improve the final biomass value.

Key words: Tunisian inland water, microalgae identification, phytochemical content, biological activity

1. Introduction

Despite tremendous advances in medicine around the world, infectious diseases caused by bacteria, fungi, and different types of protozoa continue to pose a major threat to public health. Their impact is particularly significant in developing countries due to the lack of access to medicines and the emergence and spread of antibiotic resistance, which has increased dramatically over the past decade (Kang et al. 2015). Several antimicrobial chemical agents have been synthesized for certain parasitic and bacterial diseases, however, they are still very limited in number and their use is restricted due to their toxicity, high cost, microbial resistance and low efficacy (Sanchez et al. 2010). According to the Eastern Mediterranean Regional office of the World Health Organization, cutaneous leishmaniasis (CL) caused by the protozoan *Leishmania major* continues to be a major public health problem in Tunisia, considered an endemic country for this disease. It occurs mainly in central and southwestern Tunisia (semi-arid and arid areas) and causes thousands of new cases each year. In some villages, infection rates can reach up to 60% of the population (WHO 2017).

On the other hand, oxidative damage caused by reactive oxygen species (ROS) on lipids, proteins, and nucleic acids can trigger various chronic diseases, such as coronary heart disease, atherosclerosis, cancer, and aging (Ngo et al. 2011). Over the past decades, the food, cosmetics, and nutraceutical industries have been putting enormous efforts into finding natural alternatives to replace synthetic antioxidants, in particular BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole), which several epidemiological studies have shown to be toxic in the medium and long term (Agyei et al. 2015). Such alarming situation has highlighted the urgency of finding new safe alternatives of natural origin, easily accessible, and capable of being produced in a sustainable manner.

In recent years, the use of photosynthetic microorganisms such as microalgae in life sciences has received increasing attention due to their diverse phytometabolic content with varied chemical structures and biological properties. The group of phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin) are among the most valuable accessory pigments that can be extracted from microalgae due to their extremely beneficial effects on health. They are widely recognized as powerful functional ingredients with very interesting applications in various fields such as agri-food, aquaculture, biotechnology, medicine, and pharmacy (Lamers et al. 2010; Jalal et al. 2013).

Currently, the growing demand for functional foods and nutraceuticals has sparked an increasing interest in the use of microalgae as natural sources of high value products, especially phycobiliproteins, which has automatically resulted in their surge in demand in the global market of human health, nutrition, and aquaculture (Jalal et al. 2013; Yang et al. 2013). However, their wide industrial application still requires studies to isolate and characterize more candidate strains from various terrestrial and marine environments in order to select those with interesting levels of accumulation of these valuable molecules.

Therefore, the present work aimed to study the production of phycobiliproteins and the accumulation potential of some local microalgal strains isolated in several Tunisian inland water bodies, as well as to determine their phytochemical composition and to evaluate antioxidant, antibacterial, and antileishmanial properties of their extracts.

2. Materials and methods

2.1. Study area and sample collection

The microalgae strains investigated in this work were collected in eight Tunisian inland water bodies (river, lagoon, dam reservoir, spring) located in the north, center, and south of the country (Table 1).

2.2. Strain isolation and culture medium

All samples were taken to the laboratory where each strain was subjected to capillary isolation in liquid medium, using an inverted microscope (Leica microsystems, Wetzlar, Germany). A single individual was isolated (a single cell, colony, or filament) in 300 µl of the appropriate culture medium, and growth of each isolate was achieved by gradually increasing the culture volume from the initial isolation to the final batch. All strains were cultivated in 2 l laboratory flasks, using a monospecific batch culture system under sterile conditions. Growing was carried out at 25 ± 1°C temperature in a thermostatically controlled room, and using BG11 (Rippka et al. 1979) or CONWAY (Blancheton 1985) media, depending on the origin of each strain (Table 1). Culture media were sterilized by autoclaving at 120°C for 20 min before use. Growth was conducted in a 16:8 h light:dark regime under illumination of cool white fluorescent tubes with a light intensity of approximately 25 µmol m^{-2} s^{-1}. Cells were harvested only in the exponential growth phase by centrifugation (3000 rpm; Thermo IEC CL31R Multispeed centrifuge; Massachusetts – USA),

Table 1

Microalgal strains isolated from Tunisian inland water bodies (river, lagoon, dam reservoir, spring) with location and culture mediums

Strain		Class/Order	Sampling site	Culture medium
Dunaliella sp.	Duna-GV	Chlorophyceae/ Chlamydomonadales	El Grine Sabkha 33°38'55''N; 10°32'33''E S: 56 PSU	CONWAY
Chroococcus sp.	Chroo-CH	Cyanobacteria /Chroococcales	Chanchou River 33°54'3''N; 9°43'22''E S: 4.2 PSU	BG11
Arthrospira platensis	Arthro-KB	Cyanobacteria /Oscillatoriales	Korba lagoon 36°36'43''N; 10°53'13''E S: 16 PSU	BG11
Leptolyngbya sp1.	Osci-NB-01		Nabhena reservoir 36°03'57''N; 9°51'54''E S: 0.5 PSU	BG11
Leptolyngbya sp2.	Lepto-CH		Chanchou River 33°54'3''N; 9°43'22''E S: 4.2 PSU	BG11
Limnotrix sp.	Osci-BM-01		Bir Mcherga reservoir 36°30'32''N; 10°00'36''E S: 1.6 PSU	BG11
Planktothrix agardhii	Plank-SS-01		Sidi Saad reservoir 35°22'51''N; 9°41'20''E S: 1.9 PSU	BG11
Spirulina sp.	Spir-ML		Maltine River 34°24'56''N; 10°19'45''E S: 45 PSU	CONWAY
Anabaenopsis circularis	Pseud-01	Cyanobacteria /Nostocales	Siliana spring 35°54'39''N; 9°20'06''E S: 0.2 PSU	BG11
Cylindrospermopsis raciborskii	Cyl-NB-05		Nabhena reservoir 36°03'57''N; 9°51'54''E S: 0.5 PSU	BG11

S – salinity; PSU – practical salinity unit

then lyophilized using a Christ Alpha 2-4 LD Plus freeze-dryer (Harz, Germany) after a cultivation period between 18 and 25 days. Samples were then stored at –20°C until analysis.

For screening of the investigated biological activity, the lyophilized biomass of each strain was ground using a mortar and pestle for a few minutes and extracted adding 1:10 w/v of solvent mixture (dichloromethane–methanol, 1:1, v/v) with the assistance of an ultrasonic bath (Branson 2800, Branson Ultrasonics Corporation, Danbury, USA) (110 w; 40 kHz) for 15 min at room temperature, and then filtered. The filtrate was subsequently collected and the remaining microalgal material was ground in a mortar, mixed with the same extraction buffer and filtered again until the completion of three successive extraction cycles for maximum extraction yield. The solvents were then evaporated using a Rotavapor (KNF, Freiburg, Germany) and the obtained extracts were stored at –80°C between each analysis.

2.3. Morphological and molecular identification

2.3.1. Morphological identification

Morphology and size of the microalgae strains were observed through a Leica DM LS2 microscope (Leica microsystems, Wetzlar, Germany) with magnification: 400x and 1000x, using a specialized digital camera (HDCE - 50B, Olympus Corporation, Tokyo, Japan) monitored by AxioVision software, version 4.8. Taxonomic identification was based on Geitler (1932), Bourrelly (1966) and Komárek and Anagnostidis (1999, 2005).

2.3.2. Molecular identification

Total genomic DNA was extracted using a DNeasy® Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) following the protocol provided by the manufacturer and stored frozen at –20°C until use.

The DNA extracts were checked by electrophoresis in 1% agarose gel and then quantified with a Thermo Fisher spectrophotometer (Heaios Y, Thermo Scientific; Massachusetts, USA), followed by dilution to 200 ng/µl solutions. Polymerase chain reaction (PCR) amplifications were performed with a DNA Thermal Cycler, model 2720 (Applied Biosystems/ California, USA), using 100 µl of the PCR reaction mixture. For prokaryotic strains, nuclear-encoded 16S rRNA gene segments were amplified with PCR primers 27 Fw and 1494 Rev under the conditions described in Fathalli et al. (2011). For *Dunaliella* sp., MA1 and MA2 primers were used for PCR amplification of 18S rDNA gene segments under the conditions described in Olmos et al. (2000).

All PCR amplifications were performed in 20 µl aliquots, containing 10 pmol of each forward and reverse primers (Invitrogen, Carlsbad, California), 1 × Reaction Buffer (Invitrogen, Carlsbad, California), 250 µM of each deoxynucleoside triphosphate (OMEGA biotec TQAC136), 2.5 mM $MgCl_2$ (Invitrogen, Carlsbad, California), 0.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, California), and 5 to 10 ng of genomic DNA template. All PCR amplicons were analyzed by electrophoresis in 1% agarose gel (Invitrogen, Carlsbad, California), run in 1 × TBE buffer, stained with ethidium bromide, and photographed under UV trans-illumination.

A total of 100 µl of PCR of each amplified product was purified using a PureLink® PCR Purification Kit (Invitrogen, Carlsbad, California), following the protocol provided by the manufacturer, and subsequently sent for direct sequencing. Nucleotide sequences were obtained and submitted to the BLAST (Basic Local Alignment Search Tool) database (http://www.ncbi.nlm.nih.gov/BLAST) for identification.

All sequences were then submitted to the GenBank database (accession numbers are given in Table 2).

2.4. Phytochemical content

2.4.1. Phycobiliproteins

The content of phycobiliproteins (phycocyanin – PC, allophycocyanin – APC, and phycoerythrin – PE) was determined during the exponential growth phase of cyanobacterial strains according to Bennett & Bogorod (1973). For each strain, 10 ml of fresh culture, taken in a culture concentration range of 100 µg of fresh weight per ml, was centrifuged at 3000 rpm for 5 min. The collected cell mass was then washed with buffer solution 1 M Tris HCl (pH 8.1), and one volume of cell mass was subsequently resuspended in five times the volume of the same buffer. In order to extract the pigments, it was necessary to perform cell wall splitting for cyanobacterial strains. For this, continuous freezing at –20°C, thawing at +4°C, and sonication (10 min with cycles of 30 s) were applied to all samples, which allowed destruction of cell walls. The cell fragments were then separated by centrifugation at 12 000 rpm for 10 min, and the supernatant was taken for spectrophotometric determination of phycobiliprotein content by measuring the absorbance at wavelengths of 620, 652 and 562 nm. Absorbance measurements were performed on a UV/Vis spectrophotometer (Heaios Y, Thermo Scientific; Massachusetts, USA), and the amount of PC, APC, and PE in a sample was calculated using the following formula (Bennett & Bogorad 1973; Horvath et al. 2013):

$$\begin{aligned} PC\ [mg\ ml^{-1}] &= (A_{620} - 0.474 \times A_{652}) / 5.34 \\ APC\ [mg\ ml^{-1}] &= (A_{652} - 0.208 \times A_{620}) / 5.09 \\ PE\ [mg\ ml^{-1}] &= (A_{562} - 2.41 \times PC - 0.849 \times APC) / 9.62 \end{aligned} \quad (1)$$

Table 2

Molecular identification of microalgal strains isolated from different Tunisian inland water bodies with % of similarity according to the BLAST database and accession numbers

Strain	Taxon according to BLAST database	% of similarity	Accession numbers	
			16S rRNA	rpoC1
Osci-NB-01	*Leptolyngbya* sp.	99	MG762090	-
Osci-BM-01	*Limnotrix* sp.	100	MG762091	-
Plank-SS-01	*Planktothrix agardhii*	100	MG762092	-
Cyl-NB-05	*Cylindrospermopsis raciborskii*	96	-	HQ389355
Pseud-01	*Anabaenopsis circularis*	99	MG098078	-
Arthro-KB	*Arthrospira platensis*	100	MG098079	-
Lepto-CH	*Leptolyngbya* sp.	99	MG098077	-
Spir-ML	*Spirulina* sp.	99	MG518486	-

where *A* is the absorbance at a specified wavelength.

Each sample was analyzed in triplicate and the buffer was used as a blank.

2.4.2. Total polyphenols

The total content of polyphenols (TPP) of each extract was assessed according to a colorimetric method adapted from Dewanto et al. (2002) using the Folin–Ciocalteu reagent. A volume of 0.125 ml of ethanol extracts was added to 0.5 ml of distilled water and 0.125 ml of Folin–Ciocalteu reagent. After 6 min incubation in the dark, the mixture was added to 1.25 ml of 7% (w/v) sodium carbonate, and the solution was made up to 3 ml with distilled water. The mixture was then incubated for 90 min in the dark and then taken for absorbance measurement at 760 nm, using a UV/Vis spectrophotometer (Optizen 2120 UV plus, Mecasys, Korea). TPP was calculated from the linear equation of a standard curve prepared with gallic acid (concentration range of 50–200 µg ml^{-1}). Results were expressed in mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.4.3. Total flavonoids

Total flavonoid (TF) content was determined colorimetrically according to Heimler et al. (2006). Briefly, 0.075 ml of 7% $NaNO_2$ (w/v) was mixed with 0.25 ml of each extract and 0.15 ml of 10% (w/v) $AlCl_3$. After 5 min incubation at room temperature, 0.5 ml of (1 M) NaOH was added to the mixture and adjusted to 3 ml with distilled water. Absorbance was determined at 510 nm after 15 min incubation at room temperature. The TF content was estimated based on a standard calibration curve (concentration range of 100–750 µg ml^{-1}). Results were expressed in mg catechin equivalent per gram of dry weight (mg CE/g DW).

2.5. Antioxidant activity

Antioxidant activity of microalgal extracts was assessed using 1,1 Diphenyl 2- Picryl Hydrazyl (DPPH) assay for free radical scavenging activity according to Cheel et al. (2007). Samples of microalgae extracts were prepared in methanol at a final concentration ranging from 62.5 µg ml^{-1} to 2 mg ml^{-1}. They were subsequently mixed with 2 ml of (60 µM) DPPH solution prepared in methanol, then incubated in the dark for 30 min at room temperature. Absorbance measurements were taken against a blank at 517 nm using a UV/Vis spectrophotometer and butylated hydroxytoluene (BHT) as a standard. Free radical scavenging activity was determined according to the following equation:

$$\%inhibition = \left(\frac{A_B - A_S}{A_B}\right) \times 100 \quad (2)$$

where A_B = absorbance of blank (containing all reagents except the sample) and A_S = absorbance of a sample.

IC_{50} was expressed as the concentration of extract inhibiting 50% of the DPPH free radical scavenging activity.

2.6. Antibacterial activity

2.6.1. Bacterial strains

Antimicrobial activity of microalgal extracts was tested against reference microorganisms, including Gram-positive bacteria (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* MRSA and *Bacillus cereus* ATCC 14579), and Gram-negative bacteria (*Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853). All bacterial strains were kindly provided by the "Laboratory of Bioactive Substances", Borj Cedria Biotechnology Center, Tunisia.

2.6.2. Antibacterial assay

Antibacterial activity tests were carried out using the agar disc diffusion method according to Celiktas et al. (2007). Bacterial suspensions (10^8 cells ml^{-1}) were inoculated evenly in Mueller-Hinton (MH) agar plates using a cotton swab. Whatman filter paper discs (diameter = 5 mm, porosity = 6 µm) were impregnated with double serial dilutions of the extract, ranging from 31.25 µg ml^{-1} to 1 mg ml^{-1}, then allowed to dry under a flow hood for 3 h before application to the agar surface. Plates were incubated at 37°C for 24 h, and the size of inhibitory zones (including the diameter of the disc) was measured. Clear zones of inhibition around the discs indicate antibacterial activity. The minimum inhibitory concentration (MIC) was determined for extracts with a zone diameter over 10 mm. The MIC was determined on 96-well plates and defined as the lowest concentration of the extract that inhibits the growth of microorganisms. The tetracycline (30 µg/disk) was used as a reference antibiotic.

2.7. Antileishmanial activity

Antileishmanial activity was evaluated in 96-well plates on promastigotes of *Leishmania major* (gLC94) and *Leishmania infantum* (LVMon). In their stationary growth phase, promastigotes were maintained on RPMI-1640 medium (Gibco), containing 100 µg of streptomycin ml^{-1} and 100 U penicillin ml^{-1} (Gibco), supplemented with 10% of fetal bovine serum FBS (Gibco) and incubated at 27°C in a humidified atmosphere with 5% CO_2. Promastigote culture (2×10^5 parasites ml^{-1}) was added to each well. Two-fold serial dilutions of microalgae extracts were added to a final concentration, ranging from 7.81 µg ml^{-1} to 1 mg ml^{-1}, and plates were incubated at 27°C for 72 h (Essid et al. 2015). The parasite viability was assessed after addition of 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) to 10 mg ml^{-1}. After 4 h of incubation in the dark and in the presence of MTT, the medium was removed and formazan crystals were solubilized by adding 100 µl of pure DMSO. The relative amount of formazan blue produced by viable parasites was determined spectrophotometrically using an ELISA plate reader (Synergy HT, Bio-TEK) at 560 nm. Amphotericin B (98% purity, from Sigma-Aldrich, USA) was used as a positive control for all tests. Antipromastigote activity was expressed as the IC_{50} value, defined as the concentration of a compound that inhibits the growth of promastigotes by 50%. Three replicates were performed, and the average was calculated from IC_{50} values obtained for each separate experiment (Essid et al. 2015).

All solvents and reagents used for the extraction procedures, antioxidant activity, and antileishmanial assay were purchased from Sigma-Aldrich (GmbH, Steinheim, Germany). Amphotericin B was purchased from Sigma (98% purity, Sigma–Aldrich, USA).

2.8. Statistical analysis

Assays were conducted in triplicate and data were expressed as the mean ± standard error of three independent measurements. To assess the significance of linear correlations between the antioxidant activity and total phenolic and flavonoid amounts in each microalgal extract, Pearson's correlation analysis was carried out using r-Project software. Differences were considered significant at $p < 0.05$. The inhibitory concentration was calculated from the GraphPad Prism dose– response curve (statistical programmer) obtained by plotting the percent inhibition against concentrations.

3. Results

3.1. Phytochemical content

3.1.1. Phycobiliproteins

Quantitative evaluation of PC, APC, and PE content was carried out on the cyanobacterial strains studied as shown in Fig. 1.

Nostocales cyanobacterium Cyl-NB-05 contained the maximum total phycobiliprotein content (3150 µg ml^{-1} of fresh culture), while the minimal amount was found in Chroo-CH and Pseud-01 (44 µg ml^{-1} each), within the same culture concentration range (~100 µg ml^{-1}). For the top phycobiliprotein producer strain (*Cylindrospermopsis raciborskii*), the level of PC is more than ten times higher than the two other constituents (2873 µg ml^{-1} for PC against 277 µg ml^{-1} for APC and PE, combined). The other strains tested had smaller amounts of PC, ranging from 11 (Pseud-01) to 671 µg ml^{-1} (Plank-SS-01).

In terms of APC production, the maximum yield was found in Osci-NB-01 (362 µg ml^{-1}), which was also the only isolate where the amount of APC largely exceeded its PC level (twice as much). It was closely followed by another Oscillatoriales cyanobacterium, Plank-SS-01 (326 µg ml^{-1}).

In all the strains studied, PE was present in lower amounts compared to the two other constituents, with the exception of Chroo-CH and Pseud-01, where it was the major phycobiliprotein produced.

3.1.2. Total polyphenols and flavonoids

The amounts of total polyphenols and flavonoids (TPP and TF, respectively) contained in the crude extracts of the investigated isolates are shown in Table 3. TPP levels varied between undetectable (*Cylindrospermopsis raciborskii* and *Spirulina* sp.) and 9 mg GAE/g DW for *Chroococcus* sp., while TF amounts ranged between 1.5 (*Cylindrospermopsis raciborskii*) and 74 mg CE/g DW (*Arthrospira platensis*).

3.2. Antioxidant activity

The antioxidant activity of microalgal extracts against DPPH free radicals are shown in Table 3. The higher the DPPH radical scavenging activity of a compound, the lower its IC_{50} value (inversely proportional), since the IC_{50} represents the amount of antioxidants needed to reduce the concentration of a free radical by 50%.

The lowest IC_{50} values were found in *Chroococcus* sp. and *Leptolyngbya* sp1. (212 and 263 µg ml^{-1},

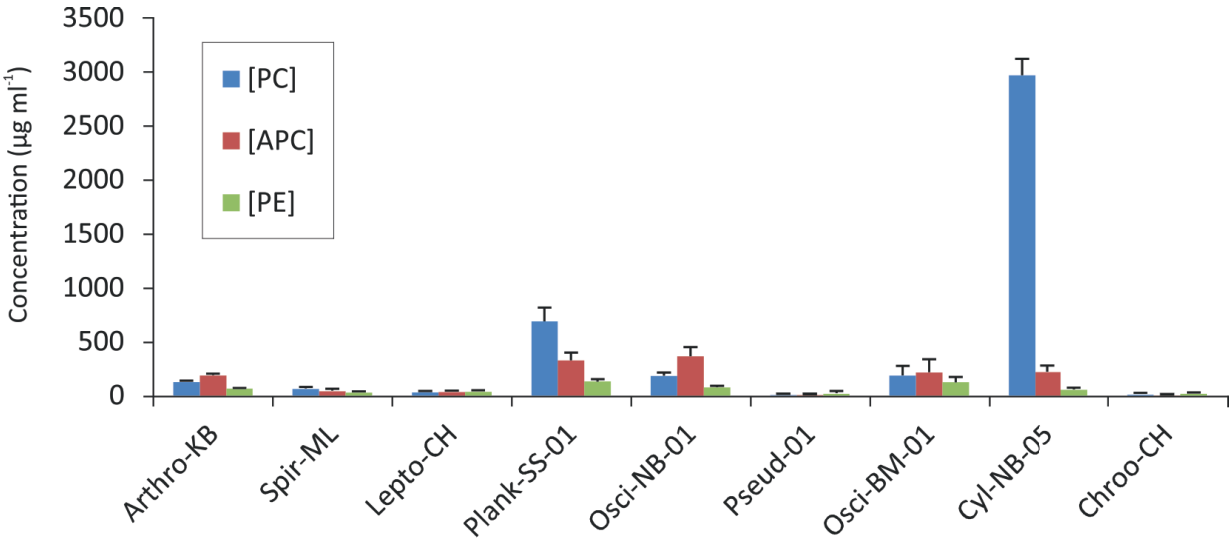

Figure 1

Phycobiliprotein concentration of cyanobacterial strains isolated from different aquatic habitats expressed in µg per ml of fresh culture. Bars indicate the standard deviation of three replicates (PC = Phycocyanin; APC = Allophycocyanin; PE = Phycoerythrin)

respectively), thus indicating their better ability to reduce DPPH radical scavenging activity compared to the other strains investigated. The other strains tested showed higher inhibitory concentrations, ranging from 680 µg ml^{-1} (*Dunaliella* sp.) to 1 mg ml^{-1} (*Arthrospira platensis*). The results also indicated that certain strains exhibited no detectable antioxidant activity at the maximum extract concentration (*Planktothrix agardhii*, *Limnotrix* sp., *Spirulina* sp., and *Cylindrospermopsis raciborskii*).

3.3. Antimicrobial activity

The antibacterial activity of microalgae extracts was studied against human pathogenic bacteria (Table 4). Most strains of microalgae showed no detectable antibacterial activity against all species of bacteria tested, except for *Dunaliella* sp. (Duna-GV), which showed moderate inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA). This antibacterial capacity was materialized by an inhibition

Table 3

Phenolics, flavonoids and antioxidant activity of microalgal extracts

Strain	Code	TPP (mg EqGA/g DW)	TF (mg EqC/g DW)	Antioxidant activity against DPPH (IC_{50}, µg ml^{-1})
Chroococcus sp.	Chroo-CH	8.90 ± 0.25	67.94 ± 1.36	212.15 ± 2.07
Leptolyngbya sp1.	Osci-NB-01	1.81 ± 0.39	58.46 ± 0.73	263.91 ± 14.93
Arthrospira platensis	Arthro-KB	2.85 ± 0.06	74.09 ± 5.28	953.94 ± 28.14
Leptolyngbya sp2.	Lepto-CH	7.75 ± 0.77	68.17 ± 3.96	782.87 ± 4.2
Planktothrix agardhii	Plank-SS-01	4.86 ± 0.3	38.32 ± 5.37	NA
Anabaenopsis circularis	Pseud-01	5.63 ± 0.15	38.05 ± 4.43	912.21 ± 2.21
Cylindrospermopsis raciborskii	Cyl-NB-05	ND	1.48 ± 0.03	NA
Limnotrix sp.	Osci-BM-01	1.77 ± 0.13	36.31 ± 3.12	NA
Spirulina sp.	Spir-ML	ND	5.03 ± 1	NA
Dunaliella sp.	Duna-GV	3.07 ± 0.32	37.94 ± 3.11	681 ± 20,07
BHT	-	-	-	17.34 ± 0.23*

TPP – total polyphenols; TF – total flavonoids; DW – dry weight; GA – gallic acid ; C – catechin ; BHT – butylated hydroxytoluene ; NA – not active at maximum concentration; ND – not detectable.
Results represent means of three replicates with standard deviation (Means ± SD, n = 3)

Table 4

Antibacterial activity of microalgal extracts

Strain	Inhibition zone diameter (mm) [MIC (µg ml^{-1})]					
	B. cereus ATCC 14579	*E. faecalis* ATCC 29212	*S. aureus* ATCC 25923	MRSA	*E. coli* ATCC 35218	*P. aeruginosa* ATCC 27853
Chroo-CH	NA	NA	NA	NA	NA	NA
Osci-NB-01	NA	NA	NA	NA	NA	NA
Arthro-KB	NA	NA	NA	NA	NA	NA
Lepto-CH	NA	NA	NA	NA	NA	NA
Plank-SS-01	NA	NA	NA	NA	NA	NA
Pseud-01	NA	NA	NA	NA	NA	NA
Cyl-NB-05	NA	NA	NA	NA	NA	NA
Osci-BM-01	NA	NA	NA	NA	NA	NA
Spir-ML	NA	NA	NA	NA	NA	NA
Duna-GV	NA	NA	NA	16 ± 0.1 [375]	NA	NA

NA – not active at maximum concentration

zone of 16 mm, corresponding to a minimum inhibitory concentration (MIC) of 375 µg ml^{-1}.

3.4. Antileishmanial activity

The antileishmanial capacity of microalgae extracts was tested against the promastigotes *L. major* (gLC94) and *L. infantum* (LVMon) (Table 5). The unicellular green algae *Dunaliella* sp. (Duna-GV) exhibited the best antileishmanial activity against both leishmaniasis pathogens. The crude extract of Duna-GV reduced the viability of *L. infantum* and, to a lesser extent, that of *L. major* (IC_{50} = 151 and 284 µg ml^{-1}, respectively). It was followed by the Oscillatoriales cyanobacterium *Limnothrix* sp. that showed interesting IC_{50} values against the two pathogens (188 and 377 µg ml^{-1} for *L. infantum* and *L. major*, respectively).

Table 5

Antileishmanial activity of microalgal extracts

Strain	IC_{50} ± SD (µg ml^{-1})	
	L. infantum (LVMon)	*L. major* (gLC94)
Chroo-CH	713.4 ± 0.24	1000 ± 1.96
Osci-NB-01	448.7 ± 0.24	728.5 ± 1.21
Arthro-KB	> 1000	> 1000
Lepto-CH	212.7 ± 1.46	401.9 ± 1.22
Plank-SS-01	1000 ± 1.98	> 1000
Pseud-01	1000 ± 2.38	> 1000
Cyl-NB-05	246.4 ± 1.66	473.8 ± 0.53
Osci-BM-01	188.7 ± 1.35	377.5 ± 0.09
Spir-ML	1000 ± 2.44	> 1000
Duna-GV	151.2 ± 0.37	284.87 ± 1.46
Amphotericin B	0.64 ± 0.24	0.97 ± 0.08

IC_{50} = Inhibitory Concentration 50 (µg ml^{-1}); Amphotericin B = positive control.
SD – standard deviation; each value was represented as mean ± SD (n = 3)

4. Discussion

4.1. Phycobiliproteins

According to the results obtained, the *Cylindrospermopsis* strain Cylind-NB occupies an exceptional position among all the strains studied, being by far the most productive of total phycobiliproteins, with a total amount exceeding 3000 µg ml^{-1}. More interestingly, the PC level in this Nostocales cyanobacterium is more than ten times higher than the two other constituents of phycobiliprotein combined (2873 µg ml^{-1} of PC against 277 µg ml^{-1} for APC and PE, combined). Compared to the results obtained by other authors, the *Cylindrospermopsis* strain tested in the present study appears to be a very rich source of PC. For example, Horvath et al. (2013) reported a PC amount of 5 µg ml^{-1} in a *Cylindrospermopsis raciborskii* strain isolated from surface water samples collected from Lake Balaton (Hungary), using the same extraction method (repeated cycles of freezing and thawing combined with sonication). Aside from the strain effect, which is the main factor explaining the huge difference observed between the two isolates, other factors may also have intervened, notably the high light intensity applied by Horvath et al. (2013), i.e. 40 µmol m^{-2} s^{-1}, compared to the present work (25 µmol m^{-2} s^{-1}). In fact, the negative effect of light intensities above 25 µmol m^{-2} s^{-1} on the cellular amount of PC in cyanobacterial strains has been reported in several studies such as Takano et al. (1995) and De Oliveira et al. (2014). These studies demonstrated that the level of incident light is one of the factors that most affects the cyanobacteria metabolism, and that lower light intensities were more advantageous in terms of total

yields of phycobiliproteins produced by cyanobacteria isolates of the genera *Nostoc* and *Synechococcus*. It has been suggested that this phenomenon is probably due to a strategy of preventing photo-oxidative damage caused by the production of free radicals in cells. Apart from the *Cylindrospermopsis* genius, lower amounts of PC (ranging from 15 to 264 µg ml^{-1}) have also been reported by Basheva et al. (2018) in 18 cyanobacterial strains belonging mainly to the genera *Microcoleus, Nostoc, Phormidesmis, and Leptolyngbya*. These values were obtained under optimal culture conditions, in particular the culture mode (extensive culture), culture medium (liquid Z-medium), pH (8.5), and temperature (24 ± 1°C). On the other hand, a high PC level, quite close to our result, was obtained by Bachchhav et al. (2016) in an Indian strain of *Arthrospira platensis* under a mixotrophic regime and yellow LED illumination. The authors recorded a PC amount of 2500 µg ml^{-1} produced by A. *platensis*, thus proving its significant potential for use in the biotechnological production of this pigment.

To the best of our knowledge, this work is the first to highlight the capacity of the cyanobacterium *Cylindrospermopsis raciborskii* to produce huge PC amounts, making it an effective alternative natural source for the biological production of one of the most valuable microalgal accessory pigments. In fact, many potent bioactivities, with extremely beneficial effects on human health, have been attributed to PC, such as the positive influence on the immune system, especially the defense mechanisms against infectious diseases. For instance, Kawamura et al. (2004) found that cyanobacterial PC enhances the biological defense activity against infectious diseases through sustaining functions of the mucosal immune system, and reduces allergic inflammation by suppressing antigen-specific IgE antibodies. It was concluded that the PC-rich cyanobacterial biomass or its processed products are not only useful dietary supplements, but they also strengthen the defense mechanisms against infectious diseases, food allergies, and other inflammatory diseases.

In addition to the previously described beneficial effects, some epidemiological studies have demonstrated the efficacy of PC as an inhibitor of cancer cell proliferation *in vivo* and *in vitro*. For example, Liu et al. (2000) reported that PC of cyanobacterial origin significantly inhibited the growth of chronic myeloid leukemia/chronic human blast crisis K562 cells in a dose-dependent manner. Tumor cells were arrested in the G1 phase and blocked to progress through the S phase, suggesting that the inhibitory effect of PC against the growth of K562 cells may have occurred through pathways other than apoptosis, and that the altered expression pattern of the c-myc protein may be involved in such inhibition.

Moreover, the neuroprotective role of PC against global cerebral ischemia/reperfusion damage in gerbils has been reported (Penton-Rol et al. 2011). PC was found to exhibit a potent protective effect against neuronal cell death in the hippocampus, and significantly improve locomotor behavior and survival in gerbils after just one week of reperfusion. In another study, Rimbau et al. (2001) found strong evidence for a remarkable protective role of PC against cell death caused by 24 h withdrawal of potassium and serum (K/S) in rat granular cerebellar cell cultures. These findings highlight some promising perspectives on the use of our *Cylindrospermopsis raciborskii* strain as a reliable source of potent and non-toxic natural drugs for the treatment of neuronal damage induced by oxidative stress in neurodegenerative disorders, ischemic strokes, Alzheimer's disease and Parkinson's disease.

4.2. Antioxidant activity

When comparing our results with previous studies, it can be observed that, in general, the levels of antioxidant activity against DPPH radicals obtained in the present work remain relatively less pronounced. IC_{50} values ranging from 67.5 to 119.6 µg ml^{-1} and from 30.72 to 102.47 µg ml^{-1} were reported by Ijaz & Hasnain (2016) and Babic et al. (2016), respectively, for the antioxidant activity of crud extracts of several cyanobacterial strains against DPPH radicals. Similarly, Safafar et al. (2015) recorded 10-fold higher inhibitory activity for *Anabaenopsis* and for *Arthrospira* (IC_{50} = 90 and 100 µg ml^{-1}, respectively) than that recorded for the same strains in our study. On the other hand, the most active isolate against DPPH radicals characterized in the present work (*Chroococcus* sp.) showed much higher antioxidant activity (6 times higher) than that found by Singh et al. (2017) for the same species (IC_{50} = 1260 Vs 212 µg ml^{-1}).

The particularly interesting antioxidant activity recorded in *Chroococcus* sp. and, to a lesser extent, *Leptolyngbya* sp1. may suggest that they are good candidates to be considered as new natural sources of effective and non-toxic antioxidants, with a number of extremely beneficial health effects. In fact, many authors have reported the importance of antioxidant and radical scavenging activity of cyanobacterial origin among the most interesting therapeutic and nutritional functions that are particularly sought after by the food and pharmaceutical industry in recent decades (Hossain et al. 2016). Nevertheless, extracts of these cyanobacterial strains remain remarkably less

active compared to the synthetic standard BHT used as a positive control in the present work (IC_{50} = 17.34 µg ml^{-1}).

This finding appears to be consistent with previous studies, e.g. Blagojevic et al. (2018), where an extremely low IC_{50} value was reported for BHT (about 10 µg ml^{-1}), reflecting much stronger DPPH scavenging efficiency compared to 10 microalgal isolates essayed in their study.

Nevertheless, it should be noted that such extraordinary antioxidant efficiency of BHT among other synthetic commercial antioxidants, especially butylated hydroxyanisole (BHA), tertbutyl hydroquinone (TBHQ), or propyl gallate (PG), is not without a significant cost. In fact, the use of BHT and BHA as food additives to neutralize or delay lipid peroxidation in the food industry has been shown to be associated with several serious health risks. Some epidemiological studies focusing on the medium and long-term health effects of BHT and BHA on animal models have proved that these products are characterized by high toxicity and carcinogenic or mutagenic effects (Lanigan & Yamarik 2002; Agyei et al. 2015).

These adverse health effects attributed to BHT and BHA and other commonly used synthetic antioxidants have put more pressure on nutraceutical, pharmaceutical, and food industries, and have made the need to identify new sources of natural antioxidants as safe alternatives even more appealing and urgent.

Regarding total polyphenols, very few studies have measured the polyphenol content in microalgae compared to other cellular constituents (Goiris et al. 2012). Our results showed TPP levels varying between undetectable values and a maximum of 8.9 mg EqGA/g DW (*Chroococcus* sp.). These levels are within the range reported by Safafar et al. (2015), who assessed the TPP content in different microalgal species belonging to several taxonomic classes (between 4 and 8 mg EqGA/g DW). However, Goiris et al. (2012) and Hossain et al. (2016) reported lower levels, not exceeding 4 mg EqGA/g DW, in extracts from more than 35 industrial microalgal strains. The observed differences can be explained by extrinsic factors such as culture conditions (photoperiod and nutrient concentration) and culture method (autotrophic or mixotrophic) as well as genetic and physiological factors, especially strain intrinsic characteristics, its origin, and even the stage of development at harvest time.

Statistical analysis revealed that there was no significant correlation between antioxidant activity and polyphenol content in the crude extracts, either in terms of PPT (r = 0.142; p = 0.782), or FT (r = 0.214; p = 0.661). The highest DPPH scavenging activity was observed in *Leptolyngbya* sp1., the extract of which contained the lowest concentration of PPT. On the other hand, *Arthrospira platensis* was the richest strain in FT and yet exhibited the lowest DPPH scavenging activity among all the investigated strains. This indicates that probably other compounds contribute strongly to the antioxidant effects observed for the strains tested in the present study. This could be attributed, for instance, to phenolic compounds that cannot be detected by the method adopted here or to other non-phenolic compounds. The later hypothesis seems to be the most consistent with a previous study (Cepoi et al. 2009) where the high antioxidant activity recorded in the biomass of *Arthrospira platensis* and *Nostoc linckia* was suggested to be related to considerable amounts of carotenoids and tocopherols, which were better extracted at higher concentrations of ethanol (55 and 70%). In general, these microorganisms are known for their ability to synthesize various phytochemical compounds such as tannins, terpenoids, UV screening compounds, steroids, and polyunsaturated fatty acids, thus forming a complex mixture of compounds in which antioxidants can act synergistically in a manner that is only partly understood.

4.3. Antimicrobial activity

The antibacterial activity assessment against several human pathogenic bacteria revealed a moderate antibacterial potential of the unicellular green alga *Dunaliella* sp. (Duna-GV) only against methicillin-resistant *Staphylococcus aureus* (MRSA), with a MIC value of 375 µg ml^{-1}, corresponding to an inhibition diameter of 16 mm. This growth inhibitory capacity of such a pathogen, considered the most virulent of the genus *Staphylococcus*, can be qualified as moderate on the basis of the classification criteria provided by Askun et al. (2013). Indeed, the antibacterial activity of plant extracts is considered to be high for MICs below 100 µg ml^{-1}, moderate between 100 and 625 µg ml^{-1}, and inactive for MICs above 800 µg ml^{-1}. The result is consistent with the previous study by Maadane et al. (2017) on several microalgal species, including *Dunaliella* sp. and *Dunaliella salina*, collected along the Moroccan coast, who concluded that the ethanolic extracts of *Dunaliella* strains exhibited moderate inhibitory activity, targeting in particular the pathogens *P. aeruginosa* and *Escherichia coli*.

According to previous research published by Maadane et al. (2015), ethanolic extracts of *Dunaliella* sp. contained significant amounts of carotenoids,

up to 10.8 mg g^{-1} DW, suggesting a possible role of these compounds in the total antimicrobial potential observed in this strain. The last hypothesis seems to be in agreement with Bhagavathy et al. (2011), where different carotenoids extracted from *Chlorococcum humicola* exhibited important antimicrobial activity, affecting both Gram positive and Gram negative bacteria.

4.4. Antileishmanial activity

The antileishmanial activity of microalgal extracts was evaluated against promastigote forms of the parasites *L. infantum* (LVMon) and *L. major* (gLC94). The results showed that the unicellular green algae *Dunaliella* sp. (Duna-GV) exhibited the highest inhibition against the two *Leishmania* pathogens, with IC_{50} values of 151.2 µg ml^{-1} and 284.8 µg ml^{-1} for *L. infantum* and *L. major*, respectively.

At present, marine organisms are increasingly recognized by scientists as an interesting natural source of new bioactive products and a promising alternative in the therapy and control of leishmaniasis. However, very few reports have been published on the antileishmanial capacity of microalgal extracts (Pereira et al. 2015).

To the best of our knowledge, this is the first study to demonstrate the antileishmanial effect of the genus *Dunaliella*. It strongly suggests that this eukaryotic unicellular green microalga may represent a good candidate to be used in the treatment of cutaneous and visceral leishmaniasis caused by the pathogens *L. major* and *L. infantum*. This appears to be of particular interest for Tunisia, a country endemic to CL and VL, where CL caused by *L. major* continues to pose a major health threat and causes thousands of new cases annually, with alarming infection rates reaching up to 60% of the population of certain villages (WHO 2017).

In addition, the results revealed that most of the isolates tested exhibited a particularly targeted antileishmanial activity against *L. infantum* more than *L. major* parasites. This could also be of particular importance in the wider context of the Mediterranean region, where *L. infantum* is the sole causative agent of canine leishmaniasis as well as cutaneous and visceral forms of human leishmaniasis. They are described as endemic in 22 countries of the Mediterranean zone, where they are strongly considered as a serious public and veterinary health problem (Campino et al. 2006). Furthermore, in the Iberian Peninsula in Southwestern Europe, infections with *L. infantum* in humans are mainly associated with immunosuppressive diseases, in particular with co-infection with the AIDS virus, HIV. According to Campino & Maia (2010), no effective human or canine vaccine against this pathogen has been developed, and chemotherapy is the only way to control cases of infection.

5. Conclusion

The present study provides important, clear and reliable information on 10 microalgal strains that colonize several Tunisian inland water bodies with respect to their potential for production of phycobiliproteins, and the evaluation of their antioxidant, antibacterial, and antileishmanial effects. The cyanobacterium *Cylindrospermopsis raciborskii* occupied a unique position among all the investigated isolates, considering its higher production and accumulation capacity of phycocyanin. This is a characteristic that certainly gives this strain a significant potential for use in the biotechnological production of this extremely valuable pigment. The genera *Chroococcus* and *Leptolyngbya* exhibited promising antioxidant activity, making them potential candidates as new natural sources of effective and non-toxic antioxidants. Eukaryotic unicellular green microalgae *Dunaliella* sp. showed the best antileishmanial properties. This result is of particular importance, especially in the context of Tunisia, where cutaneous leishmaniasis caused by *L. major* continues to cause thousands of new cases of infection each year and is a major health problem for the local population.

In view of the results obtained in the present work, we believe that the optimization of culture conditions, especially light and temperature, targeting the most potential strains will have an impact on increasing their potentiality. In addition, the use of genetic engineering as a measure to control the expression level of the genes encoding this activity would be of great benefit.

Acknowledgements

The present work was supported by Laboratoire Ecosystèmes et Ressources Aquatiques et Animales, Institut National Agronomique de Tunisie, University of Carthage with the collaboration of Bioactive Substances Laboratory, Biotechnology Center of Borj-Cedria, Tunisia, which are greatly acknowledged. This research was also financially supported by the Ministry of Higher Education and Scientific Research of the Tunisian government.

References

Agyei, D., Danquah, M.K., Sarethy, I.P. & Pan, S. (2015). Antioxidative peptides derived from food proteins. In: Rani V, Yadav U (eds) Free radicals in human health and disease. Springer, New Delhi. Retrieved November 30, 2020, from SpringerLink https://link.springer.com/chapter/citeas. DOI:10.1007/978-81-322-2035-0_26.

Anonymous, World Health Organisation, Eastern Mediterranean Regional office (WHO EMRO). (2017). Cutaneous leishmaniasis in Tunisia. Retrieved May 16, 2020, from https://www.who.int/leishmaniasis/ burden/ Leishmaniasis_Tunisia/en/.

Askun, T., Tekwu, E.M., Satil, F., Modanlioglu, S. & Aydeniz, H. (2013). Preliminary antimycobacterial study on selected Turkish plants (Lamiaceae) against *Mycobacterium tuberculosis* and search for some phenolic constituents. *BMC Complement Altern. Med.* 13: 365. DOI: 10.1186/1472-6882-13-365.

Babic, O., Kovac, D., Raseta, M., Sibul, F., Svircev, Z. et al. (2016). Evaluation of antioxidant activity and phenolic profile of filamentous terrestrial cyanobacterial strains isolated from forest ecosystem. *J. Appl. Phycol.* 28(4): 2333–2342. DOI:10.1007/s10811-015-0773-4.

Bachchhav, M.B., Kulkarni, M.V., & Ingale, A.G. (2016). Enhanced phycocyanin production from *Spirulina platensis* using Light Emitting Diode. *J. Inst. Eng. India Ser. E.* 98(1): 41–45. DOI: 10.1007/s40034-016-0090-8.

Basheva, D., Moten, D., Stoyanov, P., Belkinova, D., Mladenov, R. et al. (2018). Content of phycoerythrin, phycocyanin, alophycocyanin and phycoerythrocyanin in some cyanobacterial strains: Applications. *Eng. Life Sci.* 18(11): 861–866. DOI: 10.1002/elsc.201800035.

Bennett, A. & Bogorod, L. (1973). Comple-mentary chromatic adaptation in filamentous blue–green alga. *J. Cell. Biol.* 58(2): 419–435. DOI: 10.1083/jcb.58.2.419.

Bhagavathy, S., Sumathi, P. & Jancy Sherene Bell, I. (2011). Green algae *Chlorococcum humicola* - a new source of bioactive compounds with antimicrobial activity. *Asian Pac. J. Trop. Biomed.* 1(1): S1–S7. DOI: 10.1016/S2221-1691(11)60111-1.

Blagojevic, D., Babic, O.B., Raseta, M., Sibul, F.S., Janjusevic, L. et al. (2018). Antioxidant activity and phenolic profile in filamentous cyanobacteria: the impact of nitrogen. *J. Appl. Phycol.* 30(4): 2337–2346. DOI: 10.1007/s10811-018-1476-4.

Blancheton, A. (1985). Production d'Algues unicellulaires. Ifremer report, Ifremer Bibliothèque de PALAVAS, https://archimer.ifremer.fr/doc/00000/1749/.

Bourrelly, P. (1966). Les algues d'eau douce : les algues vertes (Vol. 1). N. Boubée & Cie (Ed.), Paris, France.

Campino, L. & Maia, C. (2010). Epidemiologia das leishmanioses em Portugal (In Portuguese) (English summary). *Acta Méd. Port.* 23(5): 859–864.

Campino, L., Pratlong, F., Abranches, P., Rioux, J.A., Santos-Gomes, G. et al. (2006). Leishmaniasis in Portugal: Enzymatic Polymorphism of *Leishmania infantum* based on Identification of 213 Strains. *Trop. Med. Int. Health.* 11(11): 1708–1714. DOI: 10.1111/j.1365-3156.2006.01728.x.

Celiktas, O.Y., Kocabas, E.E.H, Bedir, E., Sukan, F.V., Ozek, T. et al. (2007). Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations. *Food Chem.* 100(2): 553–559. DOI: 10.1016/j.foodchem.2005.10.011.

Cepoi, L., Rudi, L., Miscu, V., Cojocari, A., Chiriac, T. et al. (2009). Antioxidative activity of ethanol extracts from *Spirulina platensis* and *Nostoc linckia* measured by various methods. *Analele Univ. din Oradea, Fasc. Biol.* 16: 43–48.

Cheel, C.J., Theoduloz, C., Rodriguez, J.A., Caligari, P.D.S. & Schmeda-Hirschmann G. (2007). Free radical scavenging activity and phenolic content in achenes and thalamus from *Fragariachiloensis* ssp. *chiloensis*, *F. vesca* and *F. x ananassa* cv Chandler. *Food Chem.* 102(1): 36–44. DOI: 10.1016/j.foodchem.2006.04.036.

De Oliveira, C.A., Oliveira, W.C., Ribeiro, S.M.R., Stringheta, P.C. & Nascimento, A.G. (2014). Effect of light intensity on the production of pigments in *Nostoc* spp. *Euro. J. of Biol. and Med. Sci. Res.* 2(1): 23–36.

Dewanto, V.W.X., Adom, K.K. & Liu, R.H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agric. Food Chem.* 50(10): 3010–3014. DOI: 10.1021/jf0115589.

Essid, R., Rahali, F.Z., Msaadaa, K., Sghair, I., Hammami, M. et al. (2015). Antileishmanial and cytotoxic potential of essential oils from medicinal plants in Northern Tunisia. *Ind. Crops Prod.* 77: 795–802. DOI: 10.1016/j.indcrop.2015.09.049.

Fathalli, A., Jenhani, A.B., Moreira, C., Welker, M., Romdhane, M. et al. (2011). Molecular and phylogenetic characterization of potentially toxic cyanobacteria in Tunisian freshwaters. *Syst. Appl. Microbiol.* 34(4): 303–310. DOI: 10.1016/j.syapm.2010.12.003.

Geitler, L. (1932). In Rabenhorst's, Koeltz (Ed.), *Cyanophyceae von Europa in Kryptogamen-Flora. Scientific Books*. Koeniggstein W, Germany.

Goiris, K., Muylaert, K., Fraeye, I., Foubert, I., Brabanter, J.D. et al. (2012). Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *J. Appl. Phycol.* 24(6): 1477–1486. DOI: 10.1007/s10811-012-9804-6.

Heimler, D., Vignolini, P., Giulia, D.M., Vincieri, F.F. & Romani, A. (2006). Anti-radical activity and polyphenol composition of local *Brassicaceae* edible varieties, *Food Chem.* 99(3): 464–469. DOI: 10.1016/j.foodchem.2005.07.057.

Hossain, M.F., Ratnayake, R.R., Meerajini, K. & Wasantha Kumara, K.L. (2016). Antioxidant properties in some selected cyanobacteria isolated from fresh water bodies of Sri Lanka. *Food Sci. Nutr.* 4(5): 753–758. DOI: 10.1002/fsn3.340.

Horvath, H., Kovacs, A.W., Riddick, C.A.L. & Présing, M. (2013).

Extraction methods for phycocyanin determination in freshwater filamentous cyanobacteria and their application in a shallow lake. *Eur. J. Phycol.* 48(3): 278–286. DOI: 10.1080/09670262.2013.821525.

Ijaz, S. & Hasnain, S. (2016). Antioxidant potential of indigenous cyanobacterial strains in relation with their phenolic and flavonoid contents. *Nat. Prod. Res.* 30(11): 1297–1300. DOI: 10.1080/14786419.2015.1053088.

Jalal, K.C.A., Shamsuddin, A.A., Rahman, M.F., Nurzatul, N.Z. & Rozihan, M. (2013). Growth and Total Carotenoid, Chlorophyll *a* and Chlorophyll *b* of Tropical Microalgae (*Isochrysis* sp.) in Laboratory Cultured Conditions. *J. Biol. Sci.* 13(1): 10–17. DOI: 10.3923/jbs.2013.10.17.

Kang, H.K., Seo, C.H. & Park, Y. (2015). Marine peptides and their anti-infective activities. *Mar. Drugs* 13(1): 618–654. DOI: 10.3390/md13010618.

Kawamura, N.C., Hirahashi, T., Nagai, T., Yamada, H., Katoh, T. et al. (2004). Phycocyanin enhances secretary IgA antibody response and suppresses allergic IgE antibody response in mice immunized with antigen-entrapped biodegradable microparticles. *J. Nutr. Sci. Vitaminol.* 50(2): 129–136. DOI: 10.3177/jnsv.50.129.

Komárek, J., Anagnostidis, K. (1999). Cyanoprokaryota, Part 1: Chroococcales, Su¨ sswasserflora von Mitteleuropa, Bd 19/1. Spektrum Akademischer Verlag.

Komárek, J., Anagnostidis, K. (2005). Cyanoprokaryota, Part 2: Oscillatoriales, Su¨ sswasserflora von Mitteleuropa, Bd 19/2. Spektrum Akademischer Verlag.

Lamers, P.P., Van de Laak, C.C., Kaasenbrood, P.S., Lorier, J., Janssen, M. et al. (2010). Carotenoid and fatty acid metabolism in light-stressed *Dunaliella salina*. *Biotechnol. Bioeng.* 106(4): 638–648. DOI: 10.1002/bit.22725.

Lanigan, R.S. & Yamarik, T.A. (2002). Final report on the safety assessment of BHT. *Int. J. Toxicol.* 2: 19–94. DOI: 10.1080/10915810290096513.

Liu, Y., Xu, L., Cheng, N., Lin, L. & Zhang, C. (2000). Inhibitory effect of phycocyanin from *Spirulina platensis* on the growth ofhuman leukemia K562 cells. *J. Appl. Phycol.* 12(2): 125–130. DOI: 10.1023/A:1008132210772.

Maadane, A., Merghoub, N., Ainane, T., El Arroussi, H., Benhima, R. et al. (2015). Antioxidant activity of some Moroccan marine microalgae: pufa profiles, carotenoids and phenolic content. *J. Biotechnol.* 215: 13–19. DOI: 10.1016/j.jbiotec.2015.06.400.

Maadane, A., Merghoub, N., Mernissi, N., Ainane, T., Amzazi, S. et al. (2017). Antimicrobial activity of marine microalgae isolated from Moroccan coastlines. *J. Microbiol. Biotechnol. Food Sci.* 6(6): 1257–1260. DOI: 10.15414/jmbfs.2017.6.6.1250-1256.

Ngo, D.H., Wijesekara, I., Vo, T.S., Van, T.Q. & Kim, S.K. (2011). Marine food-derived functional ingredients as potential antioxidants in the food industry: An overview. *J. Appl. Phycol.* 44(2): 523–529. DOI: 10.1016/j.foodres.2010.12.030.

Olmos, J., Paniagua, J. & Contreras, R. (2000). Molecular identification of *Dunaliella* sp. utilizing the 18s rDNA gene. *Lett. Appl. Microbiol.* 30(1): 80–84. DOI: 10.1046/j.1472-765x.2000.00672.x.

Penton-Rol, G., Marín-Prida, J., Pardo-Andreu, G., Martínez-Sánchez, G., Acosta-Medina, E.F. et al. (2011). C-Phycocyanin is neuroprotective against global cerebral ischemia/reperfusion injury in gerbils. *Brain Res. Bull.* 86(1–2): 42–52. DOI: 10.1016/j.brainresbull.2011.05.016.

Pereira, H., Custódio L., Rodrigues, M.J., De Sousa, C.B., Oliveira, M. et al. (2015). Biological Activities and Chemical Composition of Methanolic Extracts of Selected Autochthonous Microalgae Strains from the Red Sea. *Mar. Drugs* 13(6): 3531–3549. DOI: 10.3390/md13063531.

Rimbau, V., Camins, A., Pubill, D., Sureda, F.X., Romay, C. et al. (2001). C-phycocyanin protects cerebellar granule cells from low potassium/serum deprivation-induced apoptosis. *Naunyn Schmiedebergs Arch. Pharmacol.* 364(2): 96–104. DOI: 10.1007/s002100100437.

Rippka, R., Derueles, J., Waterbury, J.B., Herdman, M. & Stanier R.Y. (1979). Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. *J. Gen. Microbiol.* 111(1): 1–61. DOI: 10.1099/00221287-111-1-1.

Safafar, H., Van Wagenen, J., Møller, P. & Jacobsen, C. (2015). Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater. *Mar. Drugs* 13(12): 7339–7356. DOI: 10.3390/md13127069.

Sanchez, L.M., Lopez, D., Vesely, B.A., Della Togna, G., Gerwick, W.H. et al. (2010). Almiramides A-C: discovery and development of a new class of leishmaniasis lead compounds. *J. Med. Chem.* 53(10): 4187–4197. DOI: 10.1021/jm100265s.

Singh, D.P, Prabha, R., Verma, S., Meena, K.K. & Yandigeri, M. (2017). Antioxidant properties and polyphenolic content in terrestrial cyanobacteria. *3 Biotech*. 7(2): 134. DOI: 10.1007/s13205-017-0786-6.

Takano, H., Arai, T., Hirano, M. & Matsunaga, T. (1995). Effects of intensity and quality of light on phycocyanin production by a marine cyanobacterium *Synechococcus* sp. NKBG 042902. *Appl. Microbiol. Biotechnol.* 43: 1014–1018. DOI: 10.1007/BF00166918.

Yang, D.J., Lin, J.T., Chen, Y.C., Liu, S.C., Lu, F.J. et al. (2013). Suppressive effect of carotenoid extract of *Dunaliella salina* alga on production of LPS-stimulated pro-inflammatory mediators in RAW264.7 cells via NF-jB and JNK inactivation. *J. Funct. Foods.* 5(2): 607–615. DOI: 10.1016/j.jff.2013.01.001.