Chapter

Antioxidant Properties of Metabolites from New Extremophiles Microalgal Strain (Southern, Tunisia)

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Abstract

With the demand for bioproducts that can provide benefits for biotechnology sectors like pharmaceuticals, nutraceuticals, and cosmeceuticals, the exploration of microalgal products has turned toward extremophiles. This chapter is intended to provide an insight to most important molecules from halotolerant species, the cyanobacteria *Phormidium versicolor* NCC-466 and *Dunaliella* sp. CTM20028 isolated from Sfax Solar Saltern (Sfax) and Chott El-Djerid (Tozeur), Tunisia. These microalgae have been cultured in standard medium with a salinity of 80 PSU. The *in vitro* antioxidant activities demonstrated that extremolyte from *Dunaliella* and *Phormidium* as, phycocaynin, lipids, and polyphenol compound presents an important antioxidant potential.

Keywords: microalgae, halophile, biomolecule, antioxidant properties

1. Introduction

The primary producers of oxygen in aquatic environments are algae, especially planktonic microalgae. They play an important role in carbon dioxide (CO_2) recycling through photosynthesis [1]. Microalgae have been divided into ten groups, which refer to the color of the cell including: Cyanobacteria, blue-green algae; Chlorophyta, green algae; Rhodophyta, red algae; Glaucophyta; Euglenophyta; Haptophyta; Cryptophyta; photosynthetic Stramenopiles; Dinophyta; and Chlorarachniophyta [2]. Cyanobacteria are much closer to bacteria in terms of structure and their cells lack both nucleus and chloroplasts. Cyanobacteria are also known as a source of pigments, chlorophyll (a), phycocyanin, phycoerythrin, xanthophyll, and ß-carotene. Microalgae are widely distributed in nature and adapted to different environments from fresh to hypersaline water ecosystems. Salt lakes in arid regions (sabkhas) and solar salterns are an examples of high salty environments inhabited by extremely halophilic microorganisms that include halophilic Archaea (halobacteria), halophilic cyanobacteria, and green algae [3–5]. These microorganisms must have specific adaptive strategies for surviving in high salinity conditions to prevent the loss of cellular water under high osmolarity in

hypersaline conditions [6]. Halophiles generally develop two basic mechanisms: (i) halobacteria and microalgae accumulate KCl (potassium chloride) in their cells to maintain high intracellular salt concentrations, osmotically at least equivalent to the external concentrations (the "salt-in" strategy); (ii) other halophiles produce or accumulate low molecular weight compounds (osmolyte or compatible solute) that have osmotic potential.

Microalgae provide many biotechnology applications in various industrial sectors such as food, cosmetics, pharmaceuticals, energy and environmental industries. Hyperhalophilic microalgae and their bioproducts, has gained a great deal of attention in the last decade. They are well known for their production of high value products such as β-carotene, lipids, and omega 3 fatty acids.

There are high demands for novel lead molecules for new classes of pharmaceutical and research biochemicals, and in combination, these drivers have led to an increased interest in microalgae and cyanobacteria as sources of both bioactive natural products.

Cyanobacteria species contain potential products for medicinal [7] and energy applications [8]. Some of this group has secondary metabolites that can potentially be used as therapeutic agents, such as antivirals, immunomodulators, inhibitors, cytostastics and antioxidants [9]. Several natural compounds such as vitamin C, tocopherol, and numerous plant extracts have been commercialized as natural antioxidants to fight against oxidative stress associated with various chronic diseases including atherosclerosis, diabetes mellitus, neurodegenerative disorders, and certain types of cancer [10]. Antioxidants are a crucial defense against free radical-induced damage [11].

Microalgae are abundant in nature and can be used as a renewable source of natural antioxidants [12]. Free radicals including reactive oxygen species (ROS), such as superoxide (O2•−), hydroxyle (OH•) and Hydrogen Peroxide (H_2O_2), and reactive nitrogen species (RNS) are generated during normal cellular metabolism. These free radicals are highly reactive species and play a dual role in humans as both beneficial and toxic compounds depending on their concentration. At low or moderate concentration, these reactive species exert beneficial effects on cellular redox signaling and immune function. At high concentration, however, these radical species produce oxidative stress, a harmful process that can lead to cell death through oxidation of protein, lipid, and DNA [11, 13].

A number of microalgae have been used in the commercial production of pigments with antioxidant properties, for example: astaxanthin from *Haematococcus pluvialis*, ß carotene from *Dunaliella salina*, as well as phycobiliproteins from *Arthrosphira* and *Phorphyridium* [12]. The review here in is about antioxidant capacity of the majors compounds extracted from new strain of hyperhalophilic microalgae (*Dunaliella* sp.) from salt lake Chott El-Djerid and cyanobacteria (*Phormidium versicolor*) from Sfax Solar Saltern (Tunisia).

2. Methods of cultivation and antioxidant assays

2.1 Isolation and principal production of the culture of new highly halophilic microalgae strains

Although most species of green algae (Chlorophyceae) are moderately halophilic, a few of them, including *Dunaliella salina*, are extremely halophilic species [3]. They are responsible for most of the primary production in hypersaline environments [4]. *Dunaliella salina* is the most important species of the genus for

beta-carotene production. Several investigations have demonstrated that *D. salina* produces more than 10% of the dry weight [14]. Lutein, chlorophyll, and other pigments and carotenoids are also produced by the genus of *Dunaliella*, under the same stressful environmental conditions [15]. Lipids for aquaculture, human nutrition, and biodiesel production have also been investigated in *Dunaliella* species [16].

Dunaliella sp. CTM 20028 have been isolated for the first time from Chott El-Djerid (Southern Tunisia) with a mean salinity of 142 PSU [17]. Chott El-Djerid (5. 000 km^2) consists of salty shallow pools and marshes, and it is covered by a large salt pan during the dry season (June to August). The water emerges into the Chott El-Djerid trough a thinclay aquiclude of Quaternary age [18]. This generally allows temporary flooding of the Chott during winter. The climate of the area is arid-saharian with a mean annual rainfall between 80 and 140 mm and mean temperature of 21 °C. The elevation of the Chott surface is controlled by the position of the water table and the associated capillary fringe [19].

After acclimatation and purification, *Dunaliella* sp. was cultured in optimized f/2 Provasoli medium. Culture was carried out in 200 ml flask at 31 °C, 21 rad/s agitation and 54 mmol photon/m^2/s continuous illumination intensity supplied by cool-white fluorescence tubes and in a saturated atmosphere to 0.1 v/v/m CO2.

Cyanobacteria *Phormidium versicolor* NCC466 have been isolated from hypersaline ponds (75 PSU) of Sfax Solar Saltern (Central Tunisia). The solar saltern studied is located in the central-eastern coast of Sfax (Tunisia, 34°39'N and 10°42′E), and consists of a series of shallow interconnected ponds (20–70 cm depth) extending over an area of 1.500 ha. The salinity of water ponds varied from 45 to 450 PSU. The morphometric characteristics of the Saltern were reported elsewhere [20]. This Saltern show high microalgae diversity, 13 diatoms, 26 Dinoflagellates, 5 cyanobacteria and 2 Chlorophyceae [5]. *Phormidium versicolor* was identified according to its internal transcribed spacer sequence based on the rDNA sequence (GenBank accession number NCC 466). It was grown in 250 mL Erlenmeyer flasks in batch containing 100 mL of a modified BG11 medium. The flasks were placed in homeothermic incubator at 25 °C under a light intensity of 100 μM photons m^{-2} s^{-1}, with a 14/10 h light/dark cycle for 11 days.

2.2 Extraction of metabolite and in vitro antioxidant evaluation

Total lipids were extracted at the end of the exponential phase of growth of *Dunaliella*'s cells according to the method of [21]. The phycocyanin pigment was isolated from *P. versicolor* using the method developed by [22]. However, the phenolic and total flavonoids content were determined in ethanolic extract according to [23, 24], respectively.

2.2.1 In vitro free radical scavenging and antioxidant assays

The antioxidant potential of the lipid extract (LE) of *Dunaliella* from Chott El-Djerid in batch culture was assessed on the basis of the 2,2-Diphenylpicrylhydrazyl (DPPH) and superoxide anion radical-scavenging activities. When DPPH radicals encounter a proton donating substrate, such as an antioxidant, the radicals would be scavenged and the absorbance would be reduced [25]. Antioxidant potential of C-PC was evaluated by Superoxide (O2•−) scavenging, Hydroxyl (OH•) and Nitric oxide (NO) scavenging capacity. Moreover, the ability of C-phycocyanin to inhibit the lipid peroxidation was assessed using the method described by [26].

The free radical scavenging capacity of phenolic and flavonoids compounds extracted from *P. versicolor* was assessed through DPPH, NO and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) tests. The antioxidant activities of polyphenol were expressed as IC50, defined as the concentration of the these compounds required causing a 50% decrease in initial DPPH, NO and ABTS concentration.

3. Lipid antioxidant properties of *Dunaliella* sp. from Chott El-Djerid

Lipid compounds such as wax, fat, fat-soluble vitamins, oil, triacylglycerols, phospholipids, co-enzymes (ubiquinone), pigments (carotenoids), and more, could be found in plants or animals. Lipids are formed from long-chain hydrocarbons and sometimes contain other functional groups of oxygen, phosphorus, nitrogen, and sulfur. They are insoluble in water, but soluble in organic solvents such as chloroform, hexane, and ether. As invascular plants, microalgae produce both polar and neutral lipids. There is a wide range of bio-based lipid products that can be harvested from microalgal biomass. Microalgae lipids offer great potential in terms of biotechnology applications (e.g. food, food supplements, energy, cosmetics, and pharmaceuticals). In functional food, the use of microalgal lipids has already been established as an industry. The type and quality of the lipid products depend on microalgae species, culture conditions, and recovery methods.

The present study is the first comprehensive *in vitro* study revealing the protective effect of the lipidic extract (LE) of the *Dunaliella* sp. from Chott El-Djerid [17]. The in vitro antioxidant activity demonstrated that LE presents an important antioxidant potential. The DPPH radical-scavenging activity was investigated at different concentrations from 0.1 to 3 mg/mL of the LE. LE exhibited an interesting radical scavenging activity that was concentration dependent (**Figure 1A**). The IC50 value obtained was about 0.1 ± 0.02 mg/mL which, is only 1.4 times higher than those of control, ascorbic acid and BHT. The antioxidant effect of *Dunaliella* sp. lipid extract was assessed at aconcentration of 1, 2, and 3 mg/mL. The results show that the concentration of 2 and 3 mg/mL of *Dunaliella* sp. Lipid extract indicate a high radical scavenging ability compared with the ascorbic acid and BHT and that of 1 mg/mL of LE presents high activity compared with BHT as positive standard.

The low IC50 indicates the higher free radical-scavenging ability of *Dunaliella* sp.-LE, which contained a high amount of essential fatty acid [17]. In addition, these authors reported that *Dunaliella* sp.-LE exhibited a strong NBT (Nitroblue-terazolium) photoreduction inhibition. Omega-3 EFAs is well documented for the

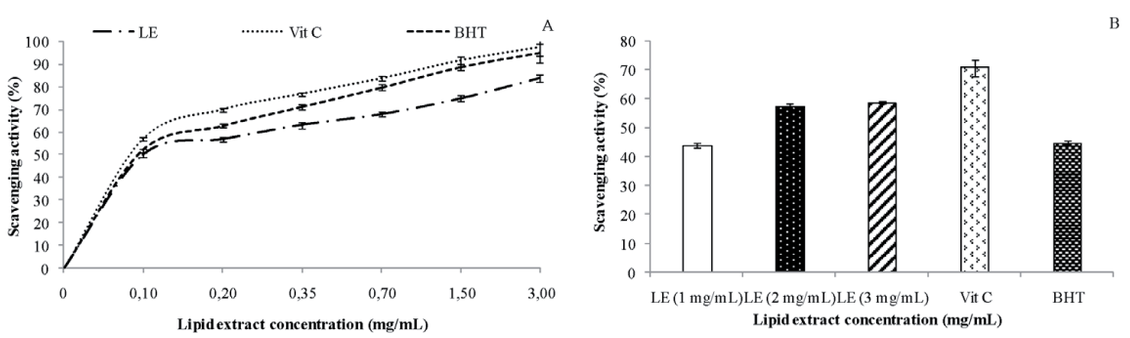

Figure 1.
Antioxidant activities of Dunaliella salina *lipid extract (LE) determined by two methods: DPPH-scavenging activity (A) and superoxide anion scavenging (B) and compared with synthetic antioxidants: Vitamin C (Vit C) and BHT. Data are presented as mean ± SD [17].*

attenuation of oxidant mediated organ damage induced by various xenobiotics and disease states [27]. Moreover [17], stated that LE of *D. salina* from Chott El-Djerid enhance the anticoxidant effect against Ni-induced toxicity by in vitro and in vivo test.

4. Phycocyanin pigments from *Phormidium versicolor* NCC466 from Sfax solar saltern

Phycocyanin (C-PC) isa hetero-oligomer consisting of a grouping of subunits that are organized into complexes called « phycobilisomes » [28]. C-PC possess a number of unique properties that make it useful colorant, including a higher molecular absorbance, fluorescence quantum yields, stable oligomers, and high photosatbility [29]. Phycocyanin has primarily been used as natural dye; however, it is increasingly being used as nutraceuticals or in ither biotechnological applications [29]. However, to the best of our knowledge, the antioxidant capacity of *P. versicolor* phycocyanin fraction (C-PC) has not been proved.

P. versicolor phycocyanin had a strong ability to scavenge free radicals (**Figure 2**). The ability of C-PC to scavenge the O2• − and OH• radicals were measured and compared with that of the positive control (ascorbic acid and BHT) (**Figure 2(a)** and **(b)**). C-PC presented the highest scavenging activity against O2• − and OH• radicals ((87.42 and 88.75% at 1 mg. mL^{-1}), respectively). Phycocyanin fractions isolated from cyanobacteria species were reported to be very efficient free radical

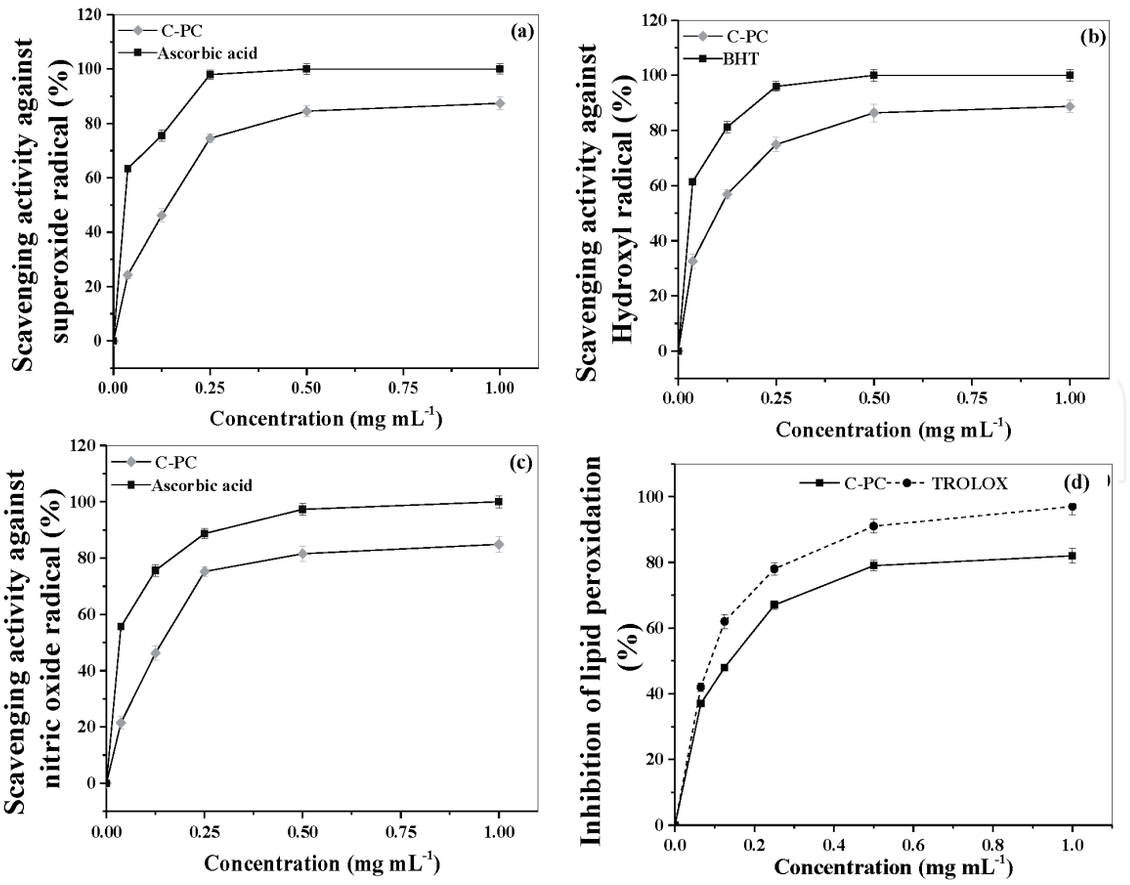

Figure 2.
Antioxidant activity of C-PC extract on (a) superoxide radical, (b) hydroxyl radical, (c) nitric oxide radical and (d) inhibition of lipid peroxidation. BHT, ascorbic acid, TROLOX were used as standard. Values are presented as mean ± SD (n = 3).

scavengers and exhibit the highest antioxidant activity [30]. All phycocyanin extracts showed fairly moderate to high scavenging capacity against free radicals. As for nitric oxide radical (NO•), the C-PC showed a strong NO• scavenging activity reaching up to 84.87% (**Figure 2c**).

Several studies showed that phycocyanin isolated from cyanobacteria species exhibited strong antioxidant properties and can be protected cells against oxidative stress [31, 32]. Moreover, in vitro studies suggest that phycocyanin of *Spirulina* enhance antioxidant enzyme activity and inhibit lipid peroxidation in cells. The effect of *P. versicolor* phycocyanin (C-PC) on ferrous sulfate induced lipid peroxidation *in vitro* was illustrated in **Figure 2d**. Indeed, the inhibition rats of lipid peroxidation of C-PC varied between 37.65 and 82.31%.

The results here in suggested that administration of C-PC in reaction mixture significantly inhibited lipid peroxidation. The present finding revealed that C-PC had a strong effect and had antagonized action against ferrous sulfate induced lipid peroxidation *in vitro*. In this regards, Thangam et al. [33] showed that phycocyanin isolated from *Oscillatoria tenuis* possesses excellent antioxidant activity against DPPH radical, OH• and nitric oxide. Similarly, Ou et al. [31] indicated that *Spirulina maxima* phycocyanin protects human hepatocyte cell line L02 against H_2O_2 induced lipid damage. C-PC from halophilic *P. versicolor* could be used to produce a natural antioxidant complement or added to healthy food products.

5. Antioxidant properties of polyphenolic compounds from *P. versicolor* NCC466

Polyphenols represent a group of chemical compounds emerging from a common intermediate, phenylalanine, or a close forerunner, shikimic acid [34]. Polyphenols are able to protect cells from oxidative stress by various mechanisms; they can chelate transition metal ions, can inhibit lipid peroxidation by trapping the lipid alkoxyl radical, or can directly scavenge molecular species of active oxygen [34]. Flavonoids are a class of phenolic metabolites that have strong chelating and antioxidant properties [34]. Their tendency to inhibit free radical-mediated events is controlled by their chemical structure. This structure–activity relationship has been well established *in vitro* as previously reported [35, 36]. *P. versicolor* exhibited a high amount of phenolics and flavonoids reaching 408 ± 18.8 mg GAE g^{-1} FW and 13,67 ± 0.788 mg QEq g^{-1} FW, respectively (**Table 1**). These amounts are signficantly higher than those recorded in *Dunaliella salina* from Sfax Solar Saltern [37]. These later recorded 0.086 ± 0.002 mg GAE g^{-1} FW and 0.006 ± 0.0001 mg QEq g^{-1} FW respectively for phenolics and flavonoids. Total antioxidant capacity (TAC) of phenolics and flavonoids extracted from *P. versicolor* are high about 0.94 ± 0.02 mg Eq g-1 FW. The IC50 concentrations DPPH, ABTS and NO scanvenging were low (0.007 to 0.031 mg. l^{-1}), suggested a high antioxidant activity of polyphenols and flavonoids extract from *P. versicolor* on the ROS (**Table 1**).

Antioxidant test	***Polyphenols and flavonoids extract***	***Standard***
DPPH (mg. l^{-1})	0.031 ± 0.08	0.077 ± 0.06 (BHT)
ABTS (mg. l^{-1})	0.015 ± 0.01	0.098 ± 0.02 (TROLOX)
NO (mg. l^{-1})	0.007 ± 0.03	0.094 ± 0.01 (Vit C)

Table 1.
Antioxydant capacity (IC50 concentrations) of phenolics and flavonoids metabolites extracted from P. versicolor *NCC466. BHT, Trolox and vitamin C represent the standard.*

6. Conclusion

News hyerhalophilic microlagae strains, *Dunaliella* sp. and *Phormidium versicolor* NCC466 are rich in lipid and phycocyanin even secondary metabolite such polyphenloic compounds. Scavenging activity tests indicated that these extremoplytes have an excellent capacity as natural antioxidant.

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