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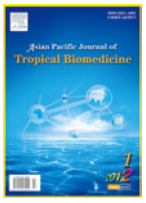

Document heading

Evaluation of antioxidant properties of marine microalga *Chlorella marina* (Butcher, 1952)

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ABSTRACT

Objective: To evaluate the *in vitro* antioxidant capacity of a *Chlorella marina* (Butcher, 1952). **Methods:** Samples were tested for the total phenolic content, antioxidant activity, deoxy ribose radical scavenging activity and reducing power. **Results:** The methanolic extract was found to have high levels of phenolic (0.647±0.052 mg GAE/g), when compared to diethyl ether and hexane. The diethyl ether extract exhibited higher antioxidant potential as (0.816±0.366 mg AscAE/g), higher percentage of deoxy ribose radical scavenging activity (0.399±0.004) and reducing power (2.814±0.014). **Conclusions:** The phenolic compounds were not a major contributor to the antioxidant capacities of these microalgae. This was very different from many other plant species like fruits, vegetables and medicinal plants. The microalgae could contain different antioxidant compounds from other plants.

1. Introduction

In recent years, the use of photosynthetic micro-organisms, such as microalgae, in life sciences has received increasing attentions due to their diverse phytometabolic contents with various chemical structures and biological activities[1]. Use of microalgae for human consumption as a source of high value health food, functional foods and for production of biochemical products, such as vitamins, carotenoids, phycocyanin and polyunsaturated fatty acids including the omega-3 fatty acids have been developed[2-4]. Among the various microalgae that have been explored for their suitability for commercial potential: *Dunaliella* species, *Chlorella* species and *Spirulina* species are three major type that have been used successfully to produced high concentrations of valuable compounds such as lipids, protein and pigments[5-7]. They also have nutritionally potential applications as functional foods which are able to provide additional physiological and pharmacological benefits for human health[1,8,9]. Polyunsaturated fatty acids, sulfated polysaccharides, phycosterols, heat-induced proteins, phenolic compounds, and pigments including carotenoids are the naturally origin functional ingredients which have positive effects on the health of man and animals[10]. A large number of studies on the microalgal bioactive compounds have oriented to the anti-inflammatory, antiviral, antimicrobial, antihelmintic, cytotoxic, immunological, and enzyme inhibition properties[11-13]. β-carotene and other carotenoids (astaxanthin & lutein) are integral part of the photosynthetic apparatus in algae and functions as accessory pigments in the harvesting complex and as protective agents against the active oxygen products (AOS) that are formed from photo-oxidation. These oxygen radicals can react with macromolecules and lead to cellular damages[14,15]. The mechanism of biological effect of illumination (including near-UV-B) appear to involve endogenous photosensitization and formation of AOS, such as from singlet oxygen ($^1O_2$), superoxide radical (O^{-2}), hydroxyl radical (-OH) and hydrogen peroxide (H_2O_2)[15,16]. The algae have developed defiance system against photo-oxidative damage by antioxidative mechanisms to detoxify and eliminate these highly reactive oxygen species.

Moreover, because of phototropic life of microalgae and their

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permanent exposure to high oxygen and radical stresses, they have a high capability for production of numerous efficient protective chemicals against oxidative and radical stressors[17]. This scavenger capacity of micro algal contents bring them up as the potential alternative substances against oxidation-associated conditions like chronic diseases, inflammation, aging or skin UV-exposure. However, these antioxidant products are mainly aimed at the health food market for direct human consumption which recognized as safe[5,6]. Since many authors postulated that a high intake of antioxidant compounds might decrease the risk of cancer, aging, inflammation, stroke disease and neurodegenerative disease (Parkinson's & Alzheimer's) in human and experimental animals[7,18,19].

There are a number of reports on the evaluation of antioxidant activity of some species belonged to genera of Botryococcus[20], Chlorella[21], Dunaliella[22], Nostoc[23], Phaeodactylum[24], Polysiphonia[25], Scytosiphon[26], Spirulina[27,28], etc. The aims of the present study was to identify new sources of safe and inexpensive antioxidants from microalgae using *Chlorella marina* (*C. marina*). The strain was collected from RGCA (Rajiv Gandhi Centre for Aquaculture), Sirkali, Tamilnadu, India. The strain were cultured in laboratory conditions by measuring the antioxidant activity, total phenolic content, Deoxy ribose radical scavenging activity and reducing power in various extracts.

2. Materials and methods

2.1. Chemicals and materials

Folin-Ciocalteu's phenol reagent, Sodium carbonate, Gallic acid, Ascorbic acid, $FeCl_3$, TCA, Potassium ferric cyanide, was purchased from Merck (Mumbai, India). All chemicals used in the experiments were of analytical grade. The *C. marina* were cultured, and the cells were obtained by centrifugation.

2.2. Sample preparation

A precisely weighed ~0.2 g amount of ground freeze dried microalgae was extracted with 2 mL of different solvents methanol, diethyl ether and hexane for 30 min at room temperature (20 ℃). The tube was centrifuged at 4 500 g for 10 min and the supernatant was recovered. The extraction was repeated with 2 mL of three different solvents and the two supernatants were combined. The residue was subsequently extracted twice, for 30 min at room temperature and the supernatants were combined. Then, the residues were further extracted twice with water (2 mL each time) for 30 min at 80 ℃, which was considered appropriate according to the literature[29], and the supernatants were combined.

2.3. Determination of total phenolic content

The total phenolic content of the extracts was estimated by the Folin-Ciocalteu method[30]. Two hundred microlitres of diluted sample were added to 1 mL of 1:10 diluted Folin-Ciocalteu reagent. After 4 min, 800 μL of saturated sodium carbonate (75 g/L) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured using the Perkin Elmer Lamba 25 UV-vis Spectrophotometer. The results were expressed as Gallic acid equivalent (GAE)/g dry weight of microalgae, and calculated as mean value ± SD (n = 3).

2.4. Determination of total antioxidant activity

Total antioxidant activity of crude extracts was determined according to the method of Prieto [31]. Briefly, 0.3 mL of sample solution (0.1 mg/mL) was mixed with 3.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95 ℃ for 90 min under water bath. Absorbance of all the sample mixtures was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid. A calibration curve of ascorbic acid was prepared and the total antioxidant activity was standardized against ascorbic acid equivalents per gram of sample on a dry weight basis.

2.5. De-oxyribose radical scavenging activity

Deoxyribose non-site specific hydroxyl radical scavenging activity of crude extracts was determined according to the method[32]. Briefly, 2.0 mL aliquots of sample were added to the test tube containing reaction mixture of 2.0 mL $FeSO_4.7H_2O$ (10 mM), 0.2 mL EDTA (10 mM) and 0.2 mL deoxyribose (10 mM). The volume was made up to 1.8 mL with phosphate buffer (0.1 M, pH 7.4) and to that 0.2 mL H_2O_2 (10 mM) was added. The mixture was incubated at 37 ℃ under dark for 4 h. After incubation, 1 mL of TCA (2.8%) and TBA (1%) were added to the mixture, and then left to stand under boiling water bath for 10 min. After treatment absorbance was measured at 532 nm. If the mixture was turbid, the absorbance was measured after filtration. Scavenging activity (%) was calculated using the equation given by[33].

2.6. Ferric reducing antioxidant Power (FRAP)

Reducing power of crude extracts was determined by the method prescribed by[34]. Briefly, 1.0 mL of extract containing different concentration of sample was mixed with 2.5 ml of Phosphate buffer (0.2 M, pH 6.6) and 2.5 mL Potassium ferricyanide (1%). Reaction mixture was incubated at 50 ℃ for 20 min. After incubation, 2.5 mL of Trichloroacetic acid (10%) was added and centrifuged (650 g) for 10 min. From the upper layer, 2.5 mL solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%). Absorbance of all the sample solutions was measured at 700 nm. Increased absorbance is indicated increased reducing power.

3. Results

The total phenolic content (TPC) of *C. marina* along with the standard gallic acid is shown in Figure 1. Methanol extract exhibited higher activity followed by diethyl ether

and hexane. All the activities were however; relatively lower than that of standard compound. Methanol extract showed high TPC of (0.647±0.052) mg GAE/g. The minimum activity was noted in diethyl ether extract 0.368 0.126 mg GAE/g. The antioxidant activity of *C. marina* along with standard ascorbic acid as shown in Figure 2. Among the three extracts diethyl ether exhibited higher radical scavenging activity when compared to hexane and methanol. All the activities were however; relatively lower than that of standard compound. The diethyl ether extract showed higher antioxidant potential as (0.816±0.366) mg ascorbic acid equivalents/g. The minimum activities were observed in (0.562±0.172) mg ascorbic acid equivalents /g in methanolic extract.

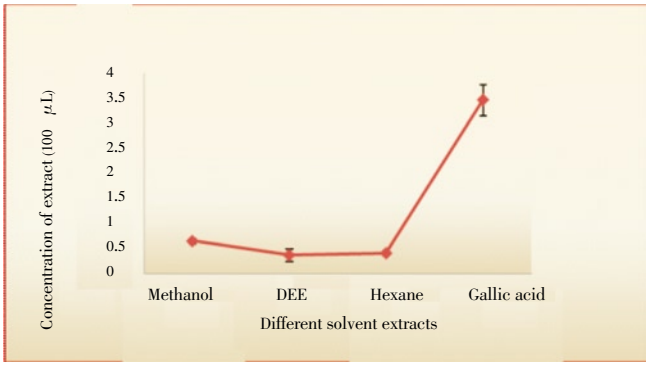

Figure 1. Total phenolic content.

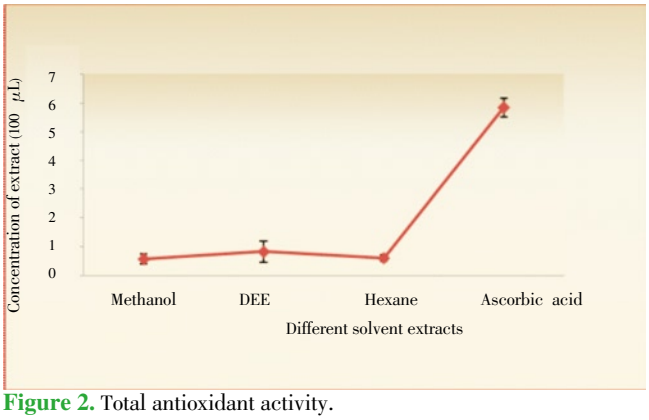

Figure 2. Total antioxidant activity.

The Deoxyribose radical scavenging activity of *C. marina* as shown in Figure 3. Among the three extracts hexane extracts exhibited higher radical scavenging activity when compared to diethyl ether and methanol. The hexane extract showed higher radical scavenging potential as (0.399 ±0.004). The minimum activity was observed in (0.365± 0.001) in methanolic extract. Concentration dependency of antioxidant activity was investigated as a function of reducing power as shown in Figure 4. The reducing capacity of various concentrations of *C. marina* extracts behaved in a dose dependent manner (0.2 to1.0 mg/mL). Similar to the total antioxidant activity (TAA), diethyl ether extract showed better reducing power than methanol and hexane. Among the three extracts, methanolic extract exhibited higher radical scavenging activity when compared to diethyl ether and hexane. Thus, the phenolic compounds were major source to the antioxidative capacities of *C. marina*.

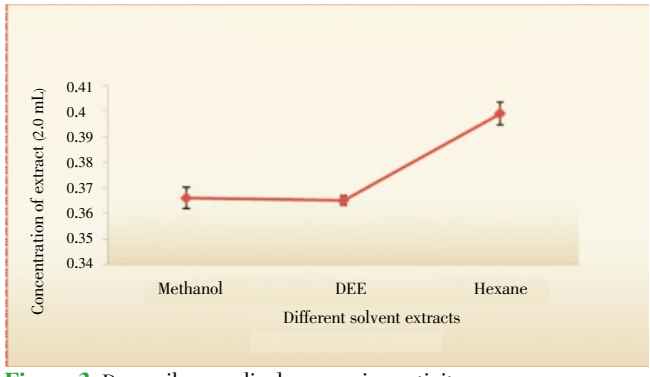

Figure 3. Deoxyribose radical scavenging activity.

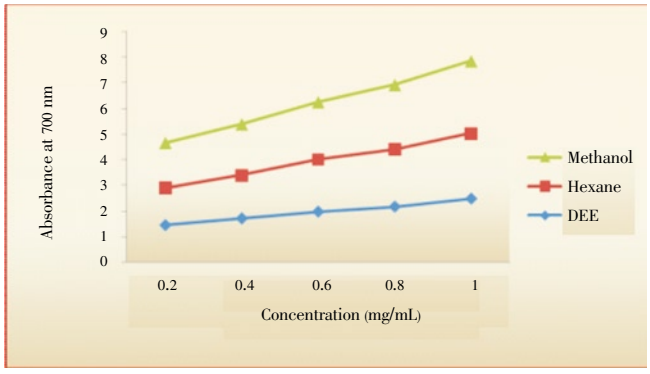

Figure 4. Ferric reducing power.

4. Discussion

Phenolic compounds serve as important antioxidants because of their ability to donate a hydrogen atom or an electron in order to form stable radical intermediates. The total phenolic contents of *C. marina* were determined and expressed as gallic acid equivalent (GAE) in order to make a comparison between different microalgae and identify a natural source for phenolic compounds. The methanolic extract exhibited higher amount of phenols compared to other extracts. In converse to the report of Li [23], the hexane fractions did not possess the highest total phenolic levels. These compounds were mainly detected in water fractions. It was true especially for the extracellular substances. As the exceptions, water fractions of *Fischerella musicola* and *Microchaete tenera* cell masses had more phenolics than their water extracts of the extracellular substance. In DPPH assay, radical scavenging activity was evaluated and probably some other functional component would be effective. It should be noted that the other antioxidant compounds such as carotenoids, polyunsaturated fatty acids and polysaccharides may play an important role[35,36]. There have been few other studies on the relationship between these two parameters for algae and the results were contradictory.

For example, Jimenez-Escrig *et al*[37] described a significant relationship between total antioxidant capacity and total phenolic compounds. However, the report of Li[23] showed the opposite results for three solvent fractions. Therefore, there is, as yet, no definite conclusion on the role of phenolic compounds in antioxidant capacity and further studies are required. The present study, the antioxidant capacity and total phenolic content of *C. marina* were evaluated by FRAP methods. In this assay, the results

demonstrated an important role for natural antioxidants. Phenolic compounds were a major contributor to the antioxidant capacities, but the correlation coefficient between these two parameters were significant only in FRAP assay.

Microalga used in this study, especially *C. marina* may find important and wide applications in the pharmaceutical and food industries because of high antioxidant activities of their substances. Generally, carotenoids have two important roles in photosynthetic organisms[38]. First, they act as accessory light-harvesting pigments, trapping light energy and passing it on to chlorophylls. Second, and more importantly, carotenoids protect the photosynthetic apparatus from light-mediated stress, for example by quenching singlet oxygen ($^1O_2$) generated by photo-oxidation. In the green alga, *C. marina* it has been suggested that photosynthetically produced active oxygen species, O^{2-} (and its products) and $^1O_2$, are involved in triggering β-carotene biosynthesis, and the massive amount of carotenoid accumulated can protect the photosynthetic apparatus against oxidative stress[39].

Carotenoids scavenge several active oxygen species such as $^1O_2$, O^{2-}, H_2O_2, peroxy radicals, and hydroxyl radicals (HO^-) both *in vitro* and *in vivo*[40-44]. In the non photosynthetic bacterium *Deinococcus radiodurans*[45], the fungus *Fusarium aquaeductuum*[46], yeasts *Rhodotorula mucilaginosa*[47,48], carotenoids protect against experimentally induced oxidative damage. The red-pigmented strain of *D. radiodurans* is resistant to HO^-, whereas the colorless strain is significantly sensitive[45]. In *Fusarium aquaeductuum*, H_2O_2 induces carotenoid biosynthesis in the dark, a process that normally occurs only under illumination[46]. In *Rhodotorula mucilaginosa*, β-carotene protects the cells against O^{2-}[47].

Previously it has been reported that antioxidant activity is correlated with polyphenolic contents[49,50]. In previous study, some enzymatic digests did not possess antioxidant activity, although they contained numerous phenolic compounds. Therefore, it can be assumed that polyphenol content is not the only factor that can influence antioxidant activity. In fact, there are other bioactive components such as proteins, polysaccharides and different kinds of pigments which are present in microalgae[51-53]. For example it was established that oligosaccharides, sulfate and glycoprotein components have exhibited antioxidant activities in the red microalga, *Porphyridium* sp.[54].

The reducing capability in 1 mL containing different concentrations was determined (Figure 4). The Ferric reducing power was found to be very high in diethyl ether followed by methanol and hexane fractions as well as the phenolic compounds were not a major contributor to the antioxidant capacities of these microalgae. In fact, microalgae could produce a wide range of antioxidant compounds, including for example, carotenoids, polyunsaturated fatty acids and polysaccharides[35,36].

In a previous study, a significant correlation was demonstrated between the antioxidant activity and phenolic content of four macroalgae[37], although, there have been few other studies on the relationship between these two parameters for algae. Therefore, there is not yet any concrete scientific evidence for how much phenolic compounds contribute to the total antioxidant capacity in algae. Although phenolic compounds can be principal antioxidant compounds in many plant species like vegetables, fruits and medicinal plants[29,55,56], they are less important as antioxidants in microalgae.

In conclusion, the results of this study have highlighted potential antioxidant activity in methanol extracts. As such, the use of *C. marina* as natural antioxidant sources in the food and pharmaceutical industries appears promising and should be investigated further.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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