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Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae

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Abstract

In order to identify new sources of safe and inexpensive antioxidants, the antioxidant capacity and total phenolic content of different fractions of 23 microalgae were evaluated, using Trolox equivalent antioxidant capacity assay and the Folin–Ciocalteu method, respectively. The microalgae were extracted using hexane, ethyl acetate and water by a three-step sequential extraction procedure. Most of these microalgae were evaluated for the first time for their antioxidant activities. It was found that the microalgae *Synechococcus* sp. FACHB 283, *Chlamydomonas nivalis* and *Nostoc ellipsosporum* CCAP 1453/17 possessed the highest antioxidant capacities and thus could be potential rich sources of natural antioxidants. In addition, the correlation coefficients between the antioxidant capacities and the phenolic contents were very small in hexane ($R^2 = 0.0075$), ethyl acetate ($R^2 = 0.5851$) and water ($R^2 = 0.3599$) fractions. Thus, 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.
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Keywords: Microalgae; Antioxidant capacity; Phenolic content; Trolox equivalent antioxidant capacity assay

1. Introduction

The oxidative damage caused by reactive oxygen species on lipids, proteins and nucleic acids may trigger various chronic diseases, such as coronary heart disease, atherosclerosis, cancer and ageing (Finkel & Holbrook, 2000; Madhavi, Deshpande, & Salunkhe, 1996). Epidemiological studies have demonstrated an inverse association between intake of fruits and vegetables and mortality from age-related diseases, such as coronary heart disease and cancer, which may be attributed to their antioxidant activity (Eberhardt, Lee, & Liu, 2000; Gey, 1990; Willett, 1991). On the other hand, some synthetic antioxidants, such as BHT and BHA, need to be replaced with natural antioxidants, as they were found to be toxic and carcinogenic in animal models (Ito et al., 1986; Safer & Al-Nughamish, 1999). Thus, it is important to identify new sources of safe and inexpensive antioxidants of natural origin.

Algal biomass and algae-derived compounds have a very wide range of potential applications, from animal feed and aquaculture to human nutrition and health products. Some algae are considered as rich sources of natural antioxidants (Chkhikvishvili & Ramazanov, 2000; Huang & Wang, 2004). Although macroalgae have received much attention as potential natural antioxidants (Duan, Zhang, Li, & Wang, 2006; Kuda, Tsunekawa, Hishi, & Araki, 2005; Zhang et al., 2003), there has been very limited information on antioxidant activity of microalgae (Herrero, Martin-Alvarez, Senorans, Cifuentes, & Ibanez, 2005;

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Murthy et al., 2005; Tannin-Spitz, Bergman, van-Moppes, Grossman, & Arad, 2005). Microalgae may serve as a continuous and reliable source of natural products, including antioxidants, because they can be cultivated in bioreactors on a large scale (Chen, 1996). Furthermore, the qualities of the microalgal cells can be controlled, so that they contain no herbicides and pesticides, or any other toxic substances, by using clean nutrient media for growing the microalgae (Li & Chen, 2001; Li, Jiang, & Chen, 2002). The value of microalgae as a source of natural antioxidants is further enhanced by the relative ease of purification of target compounds (Li, Chen, Zhang, Yang, & Xu, 2001).

Microalgae represent an almost untapped resource of natural antioxidants, due to their enormous biodiversity, much more diverse than higher plants. However, not all groups of microalgae can be used as natural sources of antioxidants, due to their widely varied contents of target products, growth rate or yields, ease of cultivation, and/or other factors. Reports on the antioxidant activity of microalgae are limited, especially concerning the relationship between their phenolic content and antioxidant capacity. Therefore, it was desirable to identify some rich sources of antioxidants from a large group of microalgae and to evaluate the relationship between these two parameters.

The aims of this study were to identify new sources of safe and inexpensive antioxidants from 23 microalgae, using Trolox equivalent antioxidant capacity (TEAC) assay, to determine their total phenolic contents and to investigate the relationship between antioxidant capacity and phenolic content.

2. Materials and methods

2.1. Chemicals and materials

Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, sodium carbonate, Folin–Ciocalteu's reagent and gallic acid were purchased from Sigma–Aldrich (St. Louis, MO). Ethanol was obtained from Merck (Darmstadt, Germany). All chemicals used in the experiments were of analytical grade.

The 23 selected microalgae were cultured in our laboratory, 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 hexane for 30 min at room temperature (20 °C). The tube was centrifuged at 4500*g* for 10 min and the supernatant was recovered. The extraction was repeated with 2 ml of hexane and the two supernatants were combined. The residue was subsequently extracted twice with ethyl acetate (2 ml each time) 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 °C, which was considered appropriate according to the literature (Cai, Luo, Sun, & Corke, 2004), and the supernatants were combined. The hexane and ethyl acetate extracts were purged to dryness using nitrogen, and together with the water extracts were stored at 0 °C before use. The water extract was directly used in the antioxidant assay, while hexane and ethyl acetate extracts were diluted appropriately with ethanol and immediately used in the antioxidant assay.

2.3. Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant capacity of the extract was evaluated using a Spectronic Genesys 5 spectrophotometer, by the improved $ABTS^{\cdot+}$ method, as described by Re et al. (1999) with slight modification. Briefly, $ABTS^{\cdot+}$ radical cation was generated by a reaction of 7 mM ABTS with 2.45 mM potassium persulfate. The reaction mixture was allowed to stand in the dark for 16 h at room temperature and used within two days. The $ABTS^{\cdot+}$ solution was diluted with ethanol, to give an absorbance of 0.700 ± 0.050 at 734 nm. All samples were diluted appropriately to give absorbance values 20–80% of that of the blank. Fifty microlitres of diluted sample were mixed with 1.9 ml of diluted $ABTS^{\cdot+}$ solution. The mixture was allowed to stand for 6 min at room temperature and the absorbance was immediately recorded at 734 nm. Trolox solution (final concentration 0–15 μM) was used as a reference standard. The results were expressed as μmol Trolox/g dry weight of microalgae, and calculated as mean value ± standard deviation (SD)($n = 3$).

2.4. Determination of total phenolics

Total phenolic content was estimated by the Folin–Ciocalteu method (Singleton & Rossi, 1965). 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. Gallic acid (0–500 mg/l) was used for the standard calibration curve. The results were expressed as gallic acid equivalent (GAE)/g dry weight of microalgae, and calculated as mean value ± SD ($n = 3$).

3. Results and discussion

3.1. Antioxidant capacities of the microalgae

In this study, the antioxidants of microalgae were fractionated to hexane extractable, ethyl acetate extractable and water-soluble by a three-stage sequential extraction procedure. The antioxidant capacities of the fractions were evaluated using the improved $ABTS^{\cdot+}$ radical decolorisation assay, one of the most commonly employed methods for measuring antioxidant capacity, which measures the ability of a compound to scavenge $ABTS^{\cdot+}$ radical. It is recommended for use in plant extracts because the long wavelength absorption maximum at 734 nm eliminates

Table 1
Antioxidant capacities (μmol Trolox/g) of different fractions of 23 microalgae

Algae species	Hexane fraction	Ethyl acetate fraction	Water fraction	Total
Anabaena flos-aquae FACHB 245	9.27 ± 0.27	5.83 ± 0.24	2.16 ± 0.22	17.26 ± 0.73
Chlamydomonas nivalis	11.41 ± 0.13	10.79 ± 0.28	1.92 ± 0.07	24.13 ± 0.47
Chlorella protothecoides #7	3.49 ± 0.13	5.73 ± 0.22	0.01 ± 0.00	9.22 ± 0.35
Chlorella pyrenoidosa #1	8.48 ± 0.42	5.80 ± 0.11	3.05 ± 0.13	17.32 ± 0.66
Chlorella pyrenoidosa #2	2.14 ± 0.06	3.92 ± 0.17	3.91 ± 0.09	9.97 ± 0.33
Chlorella pyrenoidosa #3	2.14 ± 0.04	6.08 ± 0.21	7.49 ± 0.20	15.71 ± 0.44
Chlorella vulgaris #4	2.11 ± 0.12	3.50 ± 0.24	3.59 ± 0.34	9.20 ± 0.70
Chlorella vulgaris #5	5.53 ± 0.05	6.54 ± 0.26	3.43 ± 0.20	15.50 ± 0.51
Chlorella vulgaris #8	1.43 ± 0.18	2.49 ± 0.08	1.75 ± 0.14	5.67 ± 0.39
Chlorella zofingiensis	1.83 ± 0.21	2.31 ± 0.02	1.19 ± 0.01	5.33 ± 0.24
Crypthecodinium cohnii 30556	0.01 ± 0.00	0.37 ± 0.04	5.91 ± 0.17	6.27 ± 0.20
Crypthecodinium cohnii 316	0.37 ± 0.01	0.01 ± 0.00	1.80 ± 0.05	2.17 ± 0.06
Crypthecodinium cohnii 50051	0.41 ± 0.03	0.25 ± 0.06	2.38 ± 0.14	3.03 ± 0.23
Crypthecodinium cohnii RJH	0.36 ± 0.05	0.31 ± 0.06	5.41 ± 0.19	6.08 ± 0.30
Nitzschia laevis	2.21 ± 0.32	5.30 ± 0.20	6.12 ± 0.25	13.62 ± 0.76
Nostoc ellipsosporum CCAP 1453/11	2.39 ± 0.26	6.52 ± 0.54	3.10 ± 0.35	12.00 ± 1.15
Nostoc ellipsosporum CCAP 1453/16	0.08 ± 0.00	0.40 ± 0.10	1.89 ± 0.26	2.37 ± 0.36
Nostoc ellipsosporum CCAP 1453/17	3.84 ± 0.09	8.03 ± 0.89	9.23 ± 0.86	21.09 ± 1.83
Nostoc ellipsosporum CCAP 1453/19	1.80 ± 0.24	4.31 ± 0.25	7.70 ± 0.24	13.82 ± 0.73
Schizochytrium sp. #5	0.12 ± 0.03	0.16 ± 0.18	4.57 ± 0.11	4.85 ± 0.32
Schizochytrium mangrovei BF3	0.01 ± 0.00	0.01 ± 0.00	1.33 ± 0.12	1.33 ± 0.12
Synechococcus sp. FACHB 283	7.17 ± 0.32	16.00 ± 0.61	6.39 ± 0.30	29.56 ± 1.24
Thraustochytrium sp. 26185	0.01 ± 0.00	0.26 ± 0.03	2.00 ± 0.10	2.26 ± 0.13

colour interference in plant extracts (Awika, Rooney, Wu, Prior, & Cisneros-Zevallos, 2003).

Table 1 shows the antioxidant capacities of the 23 selected microalgae. The microalgae had very diverse radical scavenging capacity (1.33–29.56 μmol Trolox/g). This was expected because the samples were selected from different families. However, it was surprising that among the same family, and even among the same species, different species or strains could exhibit very different radical scavenging ability. For example, *Chlorella zofingiensis* possessed much lower total antioxidant capacity than *Chlorella pyrenoidosa*. Among different strains of *Chlorella pyrenoidosa*, strains 1 and 3 had much higher total antioxidant capacity than strain 2. Similar variation in antioxidant capacity was also observed among strains of *Crypthecodinium cohnii* and *Nostoc ellipsosporum*. This justifies the importance of rapid testing to identify potent species or strains from these plentiful resources.

The antioxidant compounds of microalgae could be of very different polarity, and as a result were extracted into different polarity solvents (Table 1). For the hexane fractions, the antioxidant capacities ranged from 0.01 to 11.41 μmol Trolox/g. *Chlamydomonas nivalis* was found to have the highest antioxidant capacity (11.41 μmol Trolox/g), followed by *Anabaena flos-aquae* FACHB 245 (9.27 μmol Trolox/g) and *Chlorella pyrenoidosa* #1 (8.48 μmol Trolox/g). For the ethyl acetate fractions, the antioxidant capacities ranged from 0.01 to 16.00 μmol Trolox/g. *Synechococcus* sp. FACHB 283 had the highest antioxidant capacity (16.00 μmol Trolox/g), followed by *Chlamydomonas nivalis* (10.79 μmol Trolox/g) and *Nostoc ellipsosporum* CCAP 1453/17 (8.03 μmol Trolox/g). For the water fractions, the antioxidant capacities ranged from 0.01 to 9.23 μmol Trolox/g. *Nostoc ellipsosporum* CCAP 1453/17 possessed the highest antioxidant capacity (9.23 μmol Trolox/g), followed by *Nostoc ellipsosporum* CCAP 1453/19 (7.70 μmol Trolox/g) and *Chlorella pyrenoidosa* #3 (7.49 μmol Trolox/g). When the sum of TEAC for the three fractions of microalgae was taken into consideration, the total antioxidant capacities ranged from 1.33 to 29.56 μmol Trolox/g. *Synechococcus* sp. FACHB 283, *Chlamydomonas nivalis* and *Nostoc ellipsosporum* CCAP 1453/17 had the highest antioxidant capacities (>20 μmol Trolox/g); *Chlorella pyrenoidosa* #1, *Anabaena flos-aquae* FACHB 245, *Chlorella pyrenoidosa* #3 and *Chlorella vulgaris* #5 had significant antioxidant capacities (>15 μmol Trolox/g); *Nostoc ellipsosporum* CCAP 1453/19, *Nitzschia laevis* and *Nostoc ellipsosporum* CCAP 1453/11 had relatively high antioxidant capacities (>10 μmol Trolox/g).

The antioxidants from *Synechococcus* sp. FACHB 283 were mainly in the ethyl acetate fraction. The antioxidants of *Chlamydomonas nivalis* were extractable using hexane and ethyl acetate. The antioxidants from *Nostoc ellipsosporum* CCAP 1453/17 were mainly in the ethyl acetate and water fractions. To our knowledge, there was no prior report as to the antioxidant activities of the extracts from these three microalgae and this study provided valuable preliminary data, through demonstration of their high antioxidant capacities. They were potentially rich sources of natural antioxidants.

3.2. Total phenolic contents of the microalgae

Phenolic compounds such as flavonoids, phenolic acids, and tannins are considered to be major contributors to the antioxidant capacity of plants. These antioxidants also pos-

sess diverse biological activities, such as anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activities. These activities may be related to their antioxidant activity (Chung, Wong, Huang, & Lin, 1998). Thus, the total phenolic content of the different fractions of 23 microalgae was also evaluated, using the Folin–Ciocalteu method. The variation of phenolic content was quite large (Table 2). The phenolic content of hexane fractions varied from 2.12 to 39.87 mg GAE/g. *Nostoc ellipsosporum* CCAP 1453/17 (39.87 mg GAE/g), *Chlorella protothecoides* #7 (14.35 mg GAE/g) and *Chlorella pyrenoidosa* #3 (13.90 mg GAE/g) were found to have the highest phenolic contents. Other microalgaes with significant phenolic content (>10 mg GAE/g) were *Schizochytrium* sp. #5, *Crypthecodinium cohnii* 50051, *Chlorella pyrenoidosa* #2, *Chlorella vulgaris* #4, *Chlorella vulgaris* #8 and *Crypthecodinium cohnii* 316. The phenolic content of ethyl acetate fractions ranged from 0.01 to 9.80 mg GAE/g, and most of the ethyl acetate fractions showed low contents of phenolic compounds (<5 mg GAE/g). The ethyl acetate fraction of *Nostoc ellipsosporum* CCAP 1453/17 had the highest phenolic content (9.80 mg GAE/g), followed by *Chlamydomonas nivalis* (8.12 mg GAE/g) and *Synechococcus* sp. FACHB 283 (5.64 mg GAE/g). The phenolic content of water fractions varied from 0.95 to 10.68 mg GAE/g; *Nostoc ellipsosporum* CCAP 1453/17 had the highest phenolic content (10.68 mg GAE/g), followed by *Nostoc ellipsosporum* CCAP 1453/19 (4.38 mg GAE/g) and *Nitzschia laevis* (3.88 mg GAE/g). Based on the sum of three fractions, the total phenolic content ranged from 3.59 to 60.35 mg GAE/g. *Nostoc ellipsosporum* CCAP 1453/17 (60.35 mg GAE/g), *Chlorella protothecoides* #7 (19.03 mg GAE/g) and *Chlorella pyrenoidosa* #3 (17.24 mg GAE/g) possessed the highest phenolic contents. *Schizochytrium* sp. #5, *Chlorella pyrenoidosa* #2, *Chlamydomonas nivalis* and *Chlorella vulgaris* #4 had relatively high total phenolic contents (>15 mg GAE/g). A previous study demonstrated the presence of macroalgae *Cystoseira compressia* and *Sargassum furcatum* (Chkhikvishvili & Ramazanov, 2000). For microalgae, there has been very limited information on their phenolic contents.

3.3. Correlation between antioxidant capacity and phenolic content

The correlation coefficient (R^2) between the antioxidant capacity and the phenolic content of the 23 microalgae was determined (Fig. 1). The correlation coefficient between the antioxidant capacities and the phenolic contents was found to be very small in hexane ($R^2 = 0.0075$), ethyl acetate ($R^2 = 0.5851$) and water ($R^2 = 0.3599$) fractions as well as the total (0.1156). For Fig. 1 A, C and D, even if the point on the far right end (i.e. *Nostoc ellipsosporum* CCAP 1453/17) was ignored, the correlation coefficients were still very small (0.0658, 0.1688 and 0.0375, respectively). Thus, 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 (Chen, 1996; Chen, Li, Wong, Ji, & Jiang, 2005).

In a previous study, a significant correlation was demonstrated between the antioxidant activity and phenolic content of four macroalgae (Jimenez-Escrig, Jimenez-Jimenez, Pulido, & Saura-Calixto, 2001), although, there have been few other studies on the relationship between these two parameters for algae. Therefore, there is not yet any con-

Table 2
Phenolic contents (mg GAE/g) of 23 microalgae

Algae species	Hexane fraction	Ethyl acetate fraction	Water fraction	Total
Anabaena flos-aquae FACHB 245	5.48 ± 0.08	2.99 ± 0.03	2.52 ± 0.01	10.99 ± 0.12
Chlamydomonas nivalis	4.55 ± 0.13	8.12 ± 0.11	2.40 ± 0.02	15.07 ± 0.26
Chlorella protothecoides #7	14.35 ± 0.07	2.01 ± 0.01	2.67 ± 0.06	19.03 ± 0.14
Chlorella pyrenoidosa #1	4.00 ± 0.03	4.59 ± 0.14	1.87 ± 0.03	10.46 ± 0.20
Chlorella pyrenoidosa #2	11.90 ± 0.40	2.04 ± 0.01	1.17 ± 0.06	15.11 ± 0.47
Chlorella pyrenoidosa #3	13.90 ± 0.13	2.18 ± 0.01	1.16 ± 0.01	17.24 ± 0.15
Chlorella vulgaris #4	11.57 ± 0.07	2.22 ± 0.02	1.22 ± 0.05	15.01 ± 0.14
Chlorella vulgaris #5	3.12 ± 0.08	3.69 ± 0.03	1.77 ± 0.01	8.58 ± 0.12
Chlorella vulgaris #8	11.43 ± 0.07	1.60 ± 0.01	0.97 ± 0.01	14.00 ± 0.08
Chlorella zofingiensis	3.47 ± 0.01	3.31 ± 0.01	1.09 ± 0.01	7.87 ± 0.03
Crypthecodinium cohnii 30556	2.70 ± 0.05	1.05 ± 0.02	2.55 ± 0.01	6.30 ± 0.08
Crypthecodinium cohnii 316	10.57 ± 0.05	0.85 ± 0.01	0.95 ± 0.03	12.37 ± 0.08
Crypthecodinium cohnii 50051	12.68 ± 0.17	1.07 ± 0.01	1.11 ± 0.02	14.86 ± 0.19
Crypthecodinium cohnii RJH	9.07 ± 0.15	1.12 ± 0.01	1.40 ± 0.08	11.59 ± 0.24
Nitzschia laevis	2.37 ± 0.77	2.37 ± 0.04	3.88 ± 0.01	8.62 ± 0.82
Nostoc ellipsosporum CCAP 1453/11	5.14 ± 0.82	3.12 ± 0.01	2.65 ± 0.04	10.91 ± 0.87
Nostoc ellipsosporum CCAP 1453/16	3.43 ± 0.35	1.57 ± 0.04	2.20 ± 0.03	7.20 ± 0.42
Nostoc ellipsosporum CCAP 1453/17	39.87 ± 1.92	9.80 ± 0.05	10.68 ± 0.30	60.35 ± 2.27
Nostoc ellipsosporum CCAP 1453/19	2.68 ± 0.08	2.70 ± 0.08	4.38 ± 0.03	9.76 ± 0.19
Schizochytrium sp. #5	13.61 ± 0.01	0.96 ± 0.01	1.37 ± 0.03	15.94 ± 0.05
Schizochytrium mangrovei BF3	2.22 ± 0.03	0.01 ± 0.00	1.37 ± 0.01	3.59 ± 0.04
Synechococcus sp. FACHB 283	2.12 ± 0.01	5.64 ± 0.07	2.80 ± 0.03	10.56 ± 0.11
Thraustochytrium sp. 26185	4.00 ± 0.04	1.22 ± 0.02	1.48 ± 0.01	6.70 ± 0.07

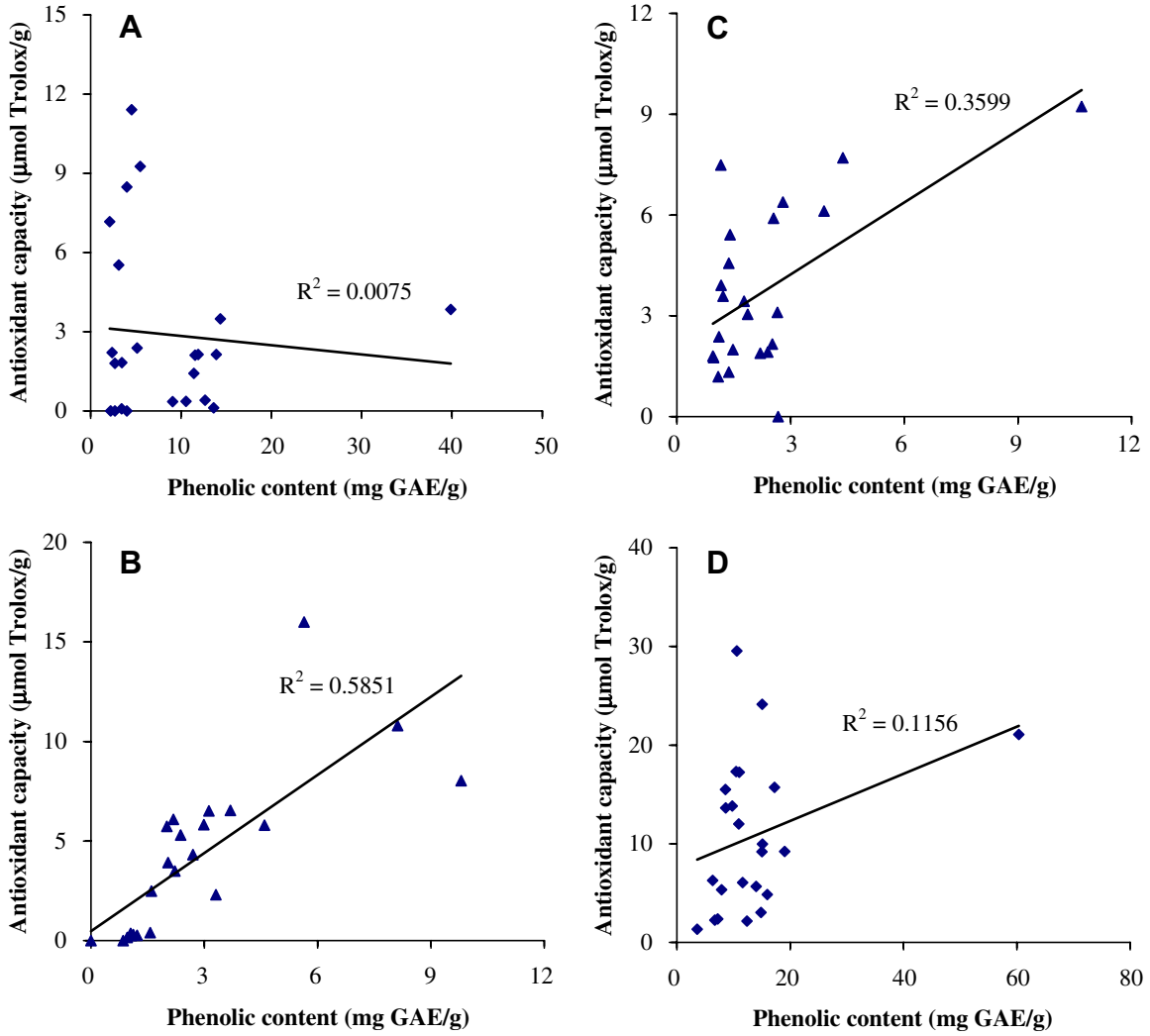

Fig. 1. Correlation between the antioxidant capacity and phenolic content of hexane (A), ethyl acetate (B) and water (C) fractions, as well as the total (D), for the extracts of the microalgae. GAE: gallic acid equivalents.

crete scientific evidence for how much phenolic compounds contribute to the total antioxidant capacity in algae. As far as we know, no microalgae have been investigated for the relationship between antioxidant activity and phenolic content. In this study, a relatively large number of microalgae samples were systematically evaluated for the first time, to establish the relationship between these two parameters. Although phenolic compounds can be principal antioxidant compounds in many plant species like vegetables, fruits and medicinal plants (Cai et al., 2004; Soong & Barlow, 2004; Wong, Li, Cheng, & Chen, 2006), they are less important as antioxidants in microalgae, as evidenced by the poor correlation between antioxidant capacity and phenolic content.

In conclusion, the antioxidant capacity and phenolic content of the 23 microalgae were evaluated. *Synechococcus* sp. FACHB 283, *Chlamydomonas nivalis* and *Nostoc ellipsosporum* CCAP 1453/17 were found to have the highest antioxidant capacities and thus could be potential rich sources of natural antioxidants. The correlation coefficient between the antioxidant capacities and the phenolic contents was very small, and phenolic compounds were not a major contributor to the antioxidant capacities of these microalgae. This was very different from many other plant species like vegetables, fruits and medicinal plants.

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