Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 110 (2008) 436–445
FOOD CHEMISTRY
www.elsevier.com/locate/foodchem

Iron-chelating ability and antioxidant properties of phycocyanin isolated from a protean extract of *Spirulina platensis*

Paloma Bermejo *, Enrique Piñero, Ángel Mª Villar

Departamento de Farmacología, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain

Received 8 September 2007; received in revised form 7 November 2007; accepted 11 February 2008

Abstract

The *in vitro* scavenger activities of different reactive oxygen species (superoxide radical, hydroxyl radical, hydrogen peroxide, hypochlorous acid and peroxyl radical), the effects on lipid peroxidation and the iron-chelating ability of a *Spirulina platensis* protean extract and the biliprotein, phycocyanin, isolated from this microalga were studied. *S. platensis* protean extract inhibited the generation of hydroxyl radical ($IC_{50} = 537$ μg/ml for the system with EDTA and 1500 μg/ml without EDTA), the production of peroxyl radical ($IC_{50} = 230$ μg/ml), and the lipid peroxidation process ($IC_{50} = 2320$ μg/ml for the enzymatic system and 2180 μg/ml for the non-enzymatic system). Besides, phycocyanin inhibited hydroxyl and peroxyl radicals and the lipid peroxidation process. The iron ions decreased the maximum fluorescence emission spectra of *S. platensis* protean extract and phycocyanin and it was an indicator of the metal-chelating activity. The antioxidant properties of *S. platensis* and phycocyanin may arise from both radical-scavenging and metal chelation. Our results suggest that *S. platensis* could be used as a dietary supplement to prevent some diseases where free radicals are involved.

Keywords: *Spirulina platensis*; Phycocyanin; Free radical scavenger; Lipid peroxidation; Iron-chelating activity

1. Introduction

Spirulina platensis is a cyanobacterium classified as blue-green algae. Nowadays, it is gaining more attention because of its nutritional and various medicinal properties. Its nutritional value derives from its high protein content (about 70%) (Khan, Bhadouria, & Bisen, 2005; Simpore et al., 2006). Several studies have reported that *Spirulina* can prevent or inhibit cancers in animals (Mohan et al., 2006; Roy et al., 2007; Zhang, Lin, Sun, & Deng, 2001). Also, *in vitro* and animal studies suggest that *Spirulina* possesses antiviral effects (Gorobets, Blinkova, & Baturo, 2002; Hernandez-Corona, Nieves, Meckes, Chamorro, & Barron, 2002; Shih, Tsai, Li, Chueh, & Chan, 2003). Besides, *Spirulina* is a powerful stimulant for the immune system, as shown in animal experiments, by increasing the phagocytic and the natural killer activities (Qureshi & Ali, 1996). Moreover, hypocholesterolemic effects have been reported in some animal studies (Chen, Chen, & Tung, 1981; Nagaoka et al., 2005; Samuels, Mani, Iyer, & Nayak, 2002). Finally, *Spirulina maxima*, administered intraperitoneally, has shown to significantly reduce carbon tetrachloride-induced hepatotoxicity (Torres-Duran et al., 1999).

In recent years, there has been an explosive interest in the use of antioxidant nutritional supplements (Gigante et al., 2007; Simpore et al., 2006). Epidemiological evidence suggests that intake of some vitamins, minerals, and other food constituents may help to protect the body against heart disease, cancer and the aging process, and that antioxidants may have a protective effect, either in preventing theses diseases or lessening their severity (Hsia et al., 2007; Luchsinger, Tang, Miller, Green, & Mayeux, 2007; Marcason, 2007; Wu, Ho, Shieh, & Lu, 2005). Several activities of the antioxidants are mediated by inhibition of reactive oxygen species (ROS), which are generated during the oxidative burst. Thus, the usefulness of antioxidants in protecting cellular

* Corresponding author. Tel.: +34 91 3941767; fax: +34 91 3941726.
E-mail address: bescos@farm.ucm.es (P. Bermejo).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.02.021

components against oxidative stress is well established (Mohan et al., 2006).

S. platensis contains phycobilisomes as light-harvesting protein-pigment complexes. Phycobilisomes are mainly composed of polypeptides named phycobiliproteins. The two more important phycobiliproteins which occur in this microalga are phycocyanin and allophycocyanin; both of them have the same chromophore group. Moreover, *Spirulina* contains a whole spectrum of natural mixed carotene and xanthophyll phytopigments which, together with phycocyanin, seem to be related to its antioxidant activity (Bhat & Madyastha, 2000; Miranda, Cintra, Barros, & Mancini Filho, 1998).

In this work, we report the results of the iron-chelating ability and the antioxidant capacity of both a *S. platensis* protean extract and the phycobiliprotein phycocyanin isolated from this microalga. These factors prompted us to investigate the activity of these products on the formation and scavenging activities, *in vitro*, of various ROS: superoxide radical, hydroxyl radical, hydrogen peroxide, peroxyl radical and hypochlorous acid, and the effects on enzymatic and non-enzymatic lipid peroxidation.

2. Materials and methods

2.1. Chemicals

Sodium dihydrogen phosphate 2-hydrate, di-sodium hydrogen phosphate 2-hydrate, phosphoric acid, sodium hypochlorite (NaOCl), sulphuric acid, anhydrous ferric chloride ($FeCl_3$) and hydrochloric acid were purchased from Panreac (Barcelona, Spain). Bio-Gel P gel, and hydroxyapatite were obtained from Bio-Rad. The 2,2′-azobis (2-amidinopropane) hydrochloride (ABAP) was obtained from Polyscience. DEAE Sephadex A-50, sodium chloride, xanthine, xanthine oxidase, cytochrome *C* type III, ethylenediaminetetraacetic acid (EDTA), superoxide dismutase, 2-deoxy-D-ribose, hydrogen peroxide (30%), thiobarbituric acid (TBA), mannitol, guaiacol, horseradish peroxidase, α1-antitrypsin, pancreatic elastase, *N*-succinyl-ala-ala-ala-*p*-nitroanilide, lysozyme, *Micrococcus lysodeikticus*, sucrose, potassium chloride, Folin & Ciocalteu's phenol reagent, ferrous sulphate ($FeSO_4$), adenosine diphosphate (ADP) and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), were purchased from Sigma (St. Louis, MO, USA). Ethanol and ascorbic acid were obtained from Merck (Darmstadt, Germany).

2.2. Protean extract preparation

S. platensis (1 g) (powder provided by GENIX Company, Cuba) was mixed with distilled water (25 ml) for 24 h at 4 °C. The crude phycocyanin obtained was centrifuged (2500 g) and the supernatant was precipitated with 50% ammonium sulphate and centrifuged again (2500 g). The supernatant was discarded and the blue precipitate was dissolved in distilled water, dialyzed (for 24 h) and lyophilized.

2.3. Purification of phycocyanin

The lyophilized aqueous extract was dissolved in 2.5 mM phosphate buffer (pH 7.0) and applied to a Bio-Gel P gel (Bio-Rad) with an exclusion limit of 1500–20,000 Da. Three fractions were obtained, one with a molecular weight higher than 20,000 Da (fraction 1), and two others (fractions 2 and 3) with lower molecular weight. Fraction 1 was then placed onto a hydroxyapatite column (Bio-Rad) and eluted with phosphate buffers of increasing ionic strength, from 2.5 to 100 mM. The fraction with the highest absorbance 620/280 ratio, representative measure of the quantity of phycocyanin, was eluted from 50 to 70 mM at 0.5 ml/min of flow rate (Boussiba & Richmond, 1979). This fraction was further purified by DEAE Sephadex A-50 (Sigma) chromatography in a gradient of 0.15–0.45 M NaCl in 50 mM phosphate buffer (pH 8.0) and a flow rate of 0.5 ml/min (Binder, Wilson, & Zuber, 1972).

2.4. Superoxide radical-scavenger activity

Superoxide radicals were generated by the xanthine/xanthine oxidase system and measured by the cytochrome *C* reduction method (Paya, Halliwell, & Hoult, 1992a). Reaction mixture contained the following: 50 mM phosphate buffer, pH 7.4, 1 mM EDTA, 100 μM xanthine and 100 μM cytochrome *C*, Type III and the assayed samples (*S. platensis* protean extract and phycocyanin) or phosphate buffer (for control reactions). The adding of 0.066 U xanthine oxidase started the reaction and the rate of cytochrome *C* reduction was measured at 550 nm. Superoxide dismutase was used as reference inhibitor.

2.5. Hydroxyl radical-scavenger activity

The hydroxyl radical-scavenging activity was determined according to the deoxyribose method (Aruoma, 1994; Halliwell, Gutteridge, & Aruoma, 1987). The scavenging activity of *S. platensis* protean extract and phycocyanin was measured by the competition between deoxyribose and *S. platensis* protean extract or phycocyanin for the hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (nonsite-specific assay) or Fe^{3+}/ascorbate/H_2O_2 (site-specific assay; in order to check metal chelation activity). Briefly, for the nonsite-specific hydroxyl radical system, the reaction mixture containing different concentrations of the protean extract (5–2000 μg/ml) or phycocyanin (200 μg/ml), 2.8 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, 0.1 mM EDTA and 1 mM H_2O_2 in phosphate buffer (20 mM pH 7.4) were incubated in a water bath at 37 °C for 30 min. For the site-specific hydroxyl radical system, EDTA was replaced by phosphate buffer. The extent of deoxyribose degradation was measured by the thiobarbituric acid (TBA) method (Mihara & Uchiyama,

1978). TBA (300 μl, 0.6% w/v) and phosphoric acid (1 ml) were added to the mixture which was heated at 100 °C for 45 min. After cooling on ice, the absorbance was measured at 535 nm. Mannitol, a classical hydroxyl radical-scavenger, was used as positive control. The hydroxyl radical-scavenging activity was calculated using the following formula:hydroxyl radical-scavenger activity $(\%) = [(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

The IC_{50} (defined as the sample concentration at which 50% of hydroxyl radical was scavenged) was calculated for each sample.

2.6. Hydrogen peroxide-scavenger activity

The production of hydrogen peroxide was followed spectrophotometrically by the guaiacol-peroxidase reaction, which renders a brown colour measurable at 436 nm. Reaction mixture of 1 ml contained 150 mM phosphate buffer, pH 7.4, 0.33 mM guaiacol and 50 μg/ml of horseradish peroxidase and the reaction was initiated by adding hydrogen peroxide. The reaction mixture with *S. platensis* protean extract or phycocyanin was preincubated with concentrations up to 1 mM with 0.1 or 1.0 mM hydrogen peroxide for 30 min at 25 °C, and then the remaining hydrogen peroxide was measured using the peroxidase system (Aruoma, Halliwell, Hoey, & Butler, 1989). *N*-acetylcysteine was used as reference inhibitor.

2.7. Hypochlorous acid assay

Reaction with hypochlorous acid (HOCl) was studied using the elastase assay (Paya et al., 1992a) (Aruoma, 1997). One of the major extracellular targets of HOCl attack is α1-antiproteinase, the main circulating inhibitor of serine proteases, as elastase. Thus, elastase activity may be used to evaluate either α1-antiproteinase activity or hypochlorous acid scavenging capacity. Actually, in the presence of HOCl, the elastase activity was maximum, decreasing when previously we added a compound with hypochlorous acid-scavenging capacity. For the assay, 75 μM HOCl was prepared immediately before use by adjusting a solution of NaOCl to pH 6.2 with diluted sulfuric acid. The concentration of HOCl was further determined, spectrophotometrically at 235 nm, using the molar extinction coefficient of 100 M^{-1} cm^{-1} (Paya et al., 1992a). For the assay, 75 μM HOCl and the samples to be tested (*S. platensis* protean extract or phycocyanin) were incubated in a final volume of 35 μl in phosphate buffer pH 7.4. To the reaction mixture, α1-antiproteinase (final concentration 0.77 mg/ml) was added. The reaction mixture was then incubated at 25 °C for 1 h. Any HOCl remaining was diluted out to the point at which it could not affect elastase, by addition of 2 ml of phosphate-buffered saline (PBS), and followed by 30 μl of 0.1% (w/v) solution of pig pancreatic elastase in the same buffer. After incubation for a further 30 min, the elastase activity remaining was measured by adding 80 μl of a 2.5 mg/ml solution of elastase substrate (*N*-succinyl-ala-ala-ala-*p*-nitroanilide), and monitoring increases in absorbance at 410 nm (Paya et al., 1992a).

2.8. Peroxyl radical-scavenger activity

Peroxyl radicals were generated by thermal decomposition of an aqueous-soluble azocompound, 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP). Protection by *S. platensis* protean extract or phycocyanin against inactivation of lysozyme by the peroxyl radicals was assayed by measuring the loss of turbidity in suspensions of *M. lysodeikticus*. The lysozyme activity was measured by monitoring the loss of turbidity at 450 nm, which resulted from adding lysozyme to a suspension of lyophilized *M. lysodeikticus*. *S. platensis* protean extract, phycocyanin or phosphate buffer (for control reactions) was incubated in 50 mM phosphate buffer, pH 7.4, containing 0.068 mM lysozyme and 10 mM ABAP. The final volume of the reaction mixture was 600 μl. The reaction mixture was incubated for 90 min at 45 °C. Fifty microliters of this mixture were added to 950 μl of a suspension of 0.3 mg/ml *M. lysodeikticus* (dissolved in Dulbecco's buffer). The amount of peroxyl radicals was determined by measuring the linear rate of absorbance change at 450 nm (Paya, Halliwell, & Hoult, 1992b). Mannitol was used as reference compound.

2.9. Microsomal lipid peroxidation

2.9.1. General

All experiments were carried out according to the guidelines for the care and use of experimental animals. Male Wistar rats weighing 250 ± 20 g were starved overnight before the experiments. The rats were killed by cervical dislocation, livers were removed and liver microsomes were prepared as previously described (Cai, Fang, Ma, Yang, & Liu, 2003). The livers were then cut into small pieces and homogenized in ice-cold 0.25 M sucrose to give a 10% (w/v) suspension. Microsomal fractions were isolated by differential ultracentrifugation removal of the nuclear faction at 8000 g for 10 min and removal of mitochondrial fraction at 18,000 g for 10 min. The microsomal fraction was sedimented at 105,000 g for 60 min, and washed two times with 0.15 M KCl at 105,000 g for 30 min. The membranes, suspended in 0.1 M phosphate buffer, pH 7.5, were stored at −80 °C until used. Microsomal protein content was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.9.2. Non-enzymatic system (Fe^{2+}/ascorbate)

Reaction mixture contained 1.25 mg microsomal protein/ml, 50 mM phosphate buffer, pH 7.4, 1.6 mM ascorbic acid and *S. platensis* protean extract or phycocyanin or phosphate buffer (for control reactions). Reaction was

started with the addition of 0.2 mM $FeSO_4$ followed by incubation at 37 °C for 30 min.

2.9.3. Enzymatic system (Fe^{3+}-ADP/NADPH)

Reaction mixture contained 0.25 mg microsomal protein/ml, 10 mM phosphate buffer, pH 7.4, ADP 1.7 mM and Fe^{3+} 100 μM and *S. platensis* protean extract or phycocyanin or phosphate buffer (for control reactions). Peroxidation was started by adding NADPH (400 μM) followed by incubation at 37 °C for 30 min.

Accumulation of lipid peroxidation products was measured according to the Buege and Aust method (Buege & Aust, 1978). One ml of phosphoric acid at 1% and 300 μl of 0.6% thiobarbituric acid (TBA) (w/v) were added and the mixture was heated in a water bath at 100 °C for 45 min. The absorbance of the resulting solution was measured at 535 nm.

2.10. Iron-chelating assay

The fluorescence spectra of *S. platensis* protean extract (0.2 mg/ml) and phycocyanin (0.2 mg/ml) in the presence or absence of different concentrations of $FeSO_4$ and $FeCl_3$ (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) were recorded using a spectrofluorometer (Perkin Elmer LS 50). The increase of the metal concentration causes the decrease in the peak amplitude and is accompanied by a blue shift of fluorescence peak. The mixtures were incubated at 25 °C during 15 min. Later, the fluorescence spectra of each sample were measured in 1 cm^3 quartz cells. The solution was excited at 488 nm and the fluorescence was monitored at 620 nm. These spectra were compared with the corresponding ones without ion iron. Excitation and emission slits were set at 5 nm for all measurements.

2.11. Statistics

Results were expressed as mean of percentage inhibition ± standard error of the mean (SEM). The Newman–Keuls test was used for comparison between means. p-Values lower than 0.05 were considered significant.

3. Results and discussion

3.1. Purification of phycocyanin

In order to purify phycocyanin, *S. platensis* protean extract was separated in a Bio-Gel filtration column, with exclusion limit between 1500 and 20,000 Da, into three fractions. The absorbance 620/280 ratio is a representative measure of the quantity of phycocyanin and this value is used as a criterion of purity of this phycobiliprotein. Fraction 1 showed an absorbance 620/280 ratio significantly higher than the protean extract (0.900 ± 0.0210; 0.520 ± 0.0024, respectively). Fractions 2 and 3 presented a ratio close to zero, which is an indication of the absence of phycocyanin (Boussiba & Richmond, 1979).

Fraction 1 was transferred to a hydroxyapatite column equilibrated with 2.5 mM phosphate buffer, pH 7.0. The fraction with the higher Absorbance 620/280 ratio (fraction 4) was eluted between 50 and 70 mM and the absorbance 620/280 ratio was 2.16 ± 0.180. This value was significantly higher than that of fraction 1. The complete purification of phycocyanin was achieved by ion-exchange chromatography on DEAE Sephadex A-50 from fraction 4. Phycocyanin was eluted between 0.35 and 0.45 M, pH 8.0, and exhibited an absorbance 620/280 ratio of 3.90 ± 0.200. The ratio for phycocyanin at the end of the process was approximately 4, and this was the reported value for pure phycocyanin (Eisele, Bakhru, Liu, MacColl, & Edwards, 2000). Previously, we had demonstrated that an increase in phycocyanin content was related to an increase in the antioxidant activity in different fractions, and therefore phycobiliprotein phycocyanin must be the component mainly responsible for the antioxidant activity (Pinero Estrada, Bermejo Bescos, & Villar del Fresno, 2001).

3.2. Scavenger activity of S. platensis protean extract and phycocyanin on superoxide radical, hydrogen peroxide and hypochlorous acid

In this study, we applied several *in vitro* assays in order to evaluate the antioxidant effect of *S. platensis* protean extract and phycocyanin isolated from this extract. The results showed that *S. platensis* protean extract had no scavenger activity for superoxide radical, hydrogen peroxide or hypochlorous acid at the concentrations assayed (between 5 μg/ml and 2 mg/ml). Like *S. platensis*, phycocyanin did not show scavenger activity for superoxide radical, hydrogen peroxide or hypochlorous acid at the concentration assayed (200 μg/ml).

Table 1
Effect of different concentrations of *Spirulina platensis* protean extract and phycocyanin on hydroxyl radical generation

Spirulina platensis protean extract (μg/ml)	With EDTA % inhibition	Without EDTA % inhibition
5	8.05 ± 1.94*	8.32 ± 3.15*
10	12.5 ± 2.20*	12.4 ± 1.10*
25	17.2 ± 1.03*	15.1 ± 1.22*
50	22.6 ± 2.01*	19.4 ± 2.04*
100	28.7 ± 2.23*	22.2 ± 1.83*
200	38.1 ± 0.90*	28.7 ± 1.07*
500	46.7 ± 2.03*	36.4 ± 1.21*
1000	56.1 ± 1.05*	42.0 ± 2.19*
2000	70.3 ± 1.19*	54.7 ± 1.10*
Phycocyanin (200 μg/ml)	44.4 ± 1.86*	39.5 ± 0.87*
Reference compounds		
Mannitol (200 μg/ml)	13.8 ± 2.03*	
Deferroxamine (200 μg/ml)		24.6 ± 2.15*

* The % inhibition values are significantly different from the control group ($p < 0.05$).

3.3. Scavenger activity of S. platensis protean extract and phycocyanin on hydroxyl radical

Table 1 shows the ability of *S. platensis* protean extract to inhibit hydroxyl radical-mediated deoxyribose degradation in a reaction mixture with (nonsite-specific assay) or without EDTA (site-specific assay). The relative extents of inhibition of deoxyribose degradation will give an indication of hydroxyl radical-scavenger and/or iron-chelating activities.

The deoxyribose assay quantifies the extent to which a substance can inhibit deoxyribose oxidation caused by hydroxyl radical (generated using Fenton chemistry). In reactions containing EDTA, hydroxyl radicals are generated by the Fe–EDTA complex and are subsequently released to free solution. Since EDTA is in stoichiometric excess of ferric ions, there will be very little interaction between ferric ions and deoxyribose. In this case, inhibition of deoxyribose degradation reflects the ability of a substance to directly compete with the reaction between deoxyribose and hydroxyl radical. Clearly, the *S. platensis* protean extract possesses hydroxyl radical-scavenger activity. When EDTA is omitted from the reaction, iron ions are free to bind to the deoxyribose molecule and hydroxyl radical generation becomes site-specific. Under these conditions, any inhibition of deoxyribose degradation upon addition of the *S. platensis* protean extract is likely to be due to iron ion-chelating effects that compete with the binding of the deoxyribose molecule.

Under both conditions, *S. platensis* protean extract inhibited the generation of hydroxyl radical in a concentration-dependent manner. The IC_{50} value (the concentration which produces a 50% reduction of oxidation) was 537 μg/ml for the system with EDTA and 1500 μg/ml without EDTA.

The relationship between the 1/absorbance values and the different concentrations of the protean extract assayed in the hydroxyl radical generation system in the presence of EDTA and absence is depicted in Fig. 1. When there is no chelating agent in the generative system, Fe^{2+} tends to bind to the carbohydrate, forming hydroxyl radical. In that case, in the absence of EDTA, the antioxidant capacity manifested by the *S. platensis* protean extract was due to its ability to react with Fe^{2+} ions.

The slope values corresponding to the assay carried out in the presence of EDTA and the inhibition values as a result of different concentrations of the aqueous extract in this system are higher than the slope values and the inhibition values obtained from the system without EDTA. Thus, this shows that the *S. platensis* protean extract has chelating capacity for Fe^{2+}.

On the other hand, phycocyanin inhibited hydroxyl radical-mediated deoxyribose degradation in a reaction mixture with or without EDTA ($44.4\% \pm 1.86$ and $39.5\% \pm 0.87$, respectively). Our results demonstrate a higher hydroxyl radical-scavenger activity of phycocyanin obtained from *S. platensis* than the activity of phycocyanin extracted from *Arthospira maxima* (Miranda et al., 1998; Romay et al., 1998).

3.4. Peroxyl radical-scavenging activity

The hydrophilic radical initiator, 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP), was used as a source of peroxyl radicals. Different concentrations of *S. platensis* protean extract were tested as possible scavengers of peroxyl radical (Table 2). There is a clear dose-dependent relationship between the inhibition levels of radical generation and the *S. platensis* protean extract concentration, with an $IC_{50} = 230$ μg/ml. Besides, phycocyanin inhibited the production of peroxyl radical, showing behaviour similar to that of ion hydroxyl radical-scavenger activity ($56.1\% \pm 2.22$).

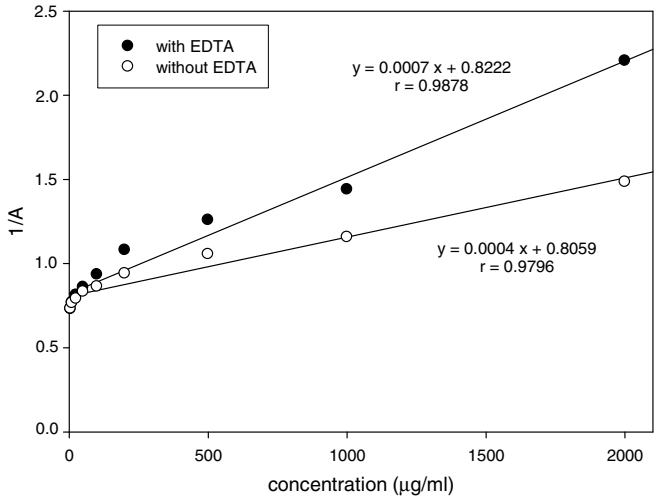

Fig. 1. The plot of 1/absorbance *versus* the *Spirulina platensis* protean extract concentrations (using EDTA and without EDTA in the ·OH generative system).

Table 2
Effect of different concentrations of *Spirulina platensis* protean extract and phycocyanin on peroxyl radical generation

Spirulina platensis (μg/ml)	% inhibition ± SEM
5	8.95 ± 2.32*
10	16.0 ± 2.84*
25	21.1 ± 1.74*
50	27.6 ± 0.89*
100	36.5 ± 3.10*
200	45.4 ± 2.05*
500	56.1 ± 1.88*
1000	67.8 ± 2.31*
2000	83.5 ± 1.02*
Phycocyanin (200 μg/ml)	56.1 ± 2.22*
Reference compound	
Mannitol (200 μg/ml)	12.9 ± 1.75*

* The % inhibition values are significantly different from the control group ($p < 0.05$).

Table 3
Effect of different concentrations of *Spirulina platensis* protean extract and phycocyanin on the inhibition of the microsomal lipid peroxidation

Spirulina platensis (μg/ml)	Non-enzymatic system % inhibition	Enzymatic system % inhibition
5	1.32 ± 0.95	–
10	2.12 ± 1.76	2.05 ± 1.84
25	4.93 ± 1.50*	4.41 ± 0.83*
50	12.5 ± 1.73*	11.7 ± 1.10*
100	14.9 ± 1.95*	15.4 ± 1.91*
200	21.1 ± 1.32*	18.7 ± 2.02*
500	26.3 ± 0.70*	20.5 ± 2.68*
1000	35.6 ± 1.37*	32.6 ± 3.34*
2000	48.1 ± 2.17*	46.8 ± 3.15*
Phycocyanin (200 μg/ml)	27.1 ± 1.39*	29.3 ± 2.85*
Reference compound		
BHT (200 μg/ml)	54.5 ± 3.49*	75.1 ± 2.84*

* The % inhibition values are significantly different from the control group ($p < 0.05$).

3.5. Microsomal lipid peroxidation

The results obtained from the evaluation of the effect of *S. platensis* protean extract on the lipid peroxidation process in rat hepatic microsomes are shown in Table 3. *S. platensis* protean extract had a significant effect on the inhibition of the peroxidation process at the concentrations tested in this study. The IC_{50} value was 2320 μg/ml for the enzymatic system and 2180 μg/ml for the non-enzymatic system.

The results obtained show that the inhibitory activity on lipid peroxidation of *S. platensis* protean extract was lower than was the antioxidant capacity of these samples against hydroxyl and peroxyl radicals.

Phycocyanin inhibited the lipid peroxidation process in rat hepatic microsomes for the enzymatic system (29.3% ± 2.85) and for the non-enzymatic system (27.1% ± 1.39). The results obtained show that the inhibitory activity on lipid peroxidation of phycocyanin was lower than the antioxidant capacity of this compound against hydroxyl and peroxyl radicals. This may be due to the low lipophilic nature of phycocyanin, which will delay their access to the membrane and the initiation of the peroxidation process.

Previously, it was reported that the phycocyanin from *A. maxima* inhibited lipid peroxidation with an $IC_{50} = 12$ mg/ml; using a sixfold lower dose, the phycocyanin of *S. platensis* origin was able to inhibit this reaction by a 27.1% (Romay et al., 1998).

The inhibitory effect observed on microsomal lipid peroxidation is most probably due to a metal-binding capacity of both *S. platensis* protean extract and phycocyanin. One indication of such an action is the ability of *S. platensis* protean extract and phycocyanin to inhibit deoxyribose damage in a site-specific manner (in the absence of EDTA). In the present study it was noticed that *S. platensis* protean extract and phycocyanin inhibited the liver microsomal lipid peroxidation in a concentration-dependent fashion with an IC_{50} value of 11.4 mM (Fig. 3a and 3b). Earlier it was shown that phycocyanin inhibited the iron-ascorbate-induced rat liver microsomal lipid peroxidation with an IC_{50} of about 327 mM (12 mg/ml) (Romay et al., 1998).

We found a clear difference between the inhibition effects of the generation of hydroxyl, peroxyl and lipid peroxidation, caused by the phycocyanin and the *S. platensis* protean extract. At a concentration of 200 μg/ml, we obtained higher antioxidant activity than that of a similar concentration of the extract. This demonstrates that phycocyanin is one of the main components responsible for the antioxidant capacity of the aqueous extract of *S. platensis*.

3.6. Iron-chelating activity

To confirm the iron-binding properties of *S. platensis* protean extract and phycocyanin, fluorescence quenching experiments were carried out and it was found that both *S. platensis* protean extract and phycocyanin interacted with iron ion. The chelating activity of *S. platensis* protean extract instigated a great variation in the fluorescence spectrum. The main components of *S. platensis* protean extract are aromatic rings that could interact with metals, resulting in changes in their fluorescence properties upon iron-binding. We obtained the fluorescence spectrum of the *S. platensis* protean extract, and we compared it with the resulting spectra of the mixture of *S. platensis* protean extract with increasing concentrations of ions Fe^{2+} and Fe^{3+}. Fig. 2a and 2b show the fluorescence spectrum of the aqueous extract of *S. platensis* with different concentrations of $FeSO_4$ and $FeCl_3$, respectively.

The fluorescence spectra of *S. platensis* protean extract had a peak at 620 nm. This peak is due to phycocyanin, the major component of this microalga. The phycocyanin peak decreased significantly under iron stress.

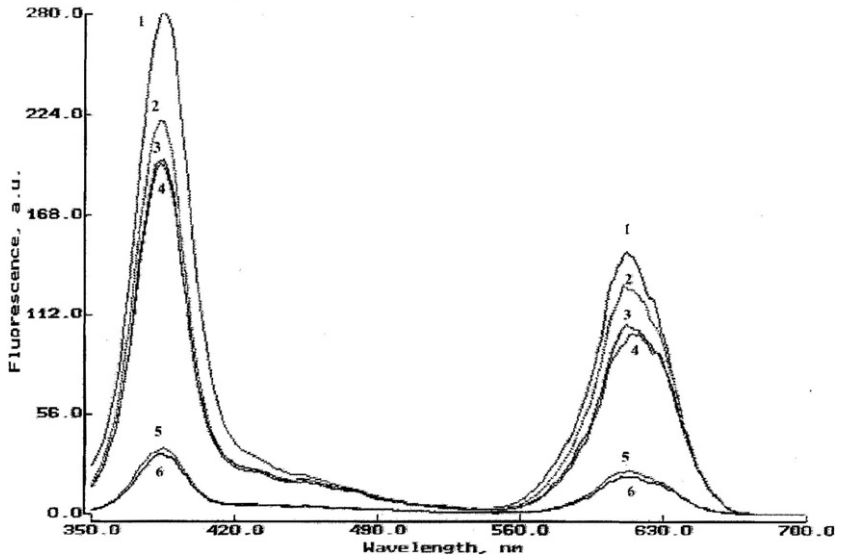

Fig. 2a. Changes in fluorescence spectra of *Spirulina platensis* protean extract after addition of different concentrations of Fe^{2+}. (1) *Spirulina platensis* protean extract alone; (2) *Spirulina platensis* protean extract + Fe^{2+}, 0.1 mg/ml; (3) *Spirulina platensis* protean extract + Fe^{2+}, 0.2 mg/ml; (4) *Spirulina platensis* protean extract + Fe^{2+}, 0.3 mg/ml; (5) *Spirulina platensis* protean extract + Fe^{2+}, 0.4 mg/ml and (6) *Spirulina platensis* protean extract + Fe^{2+}, 0.5 mg/ml. The characteristic peak of phycocyanin can be recorded at 620 nm.

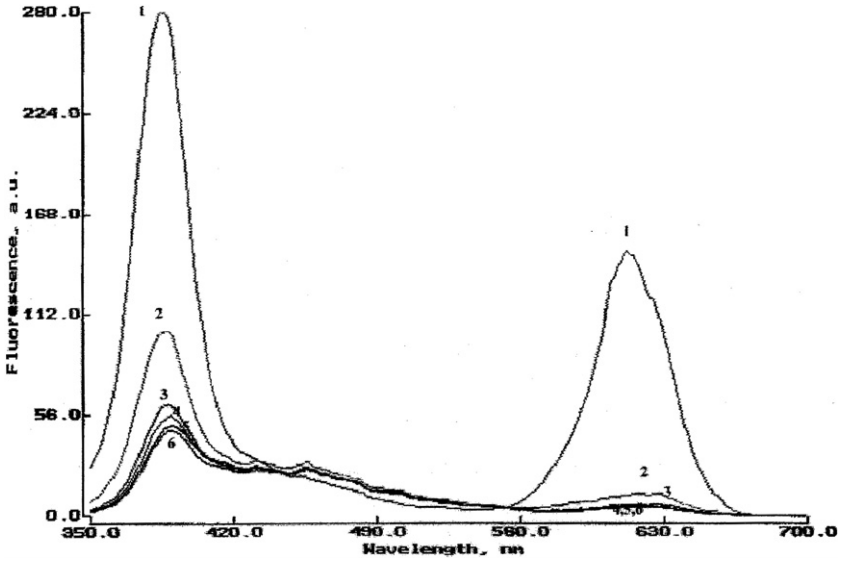

Fig. 2b. Changes in fluorescence spectra of *Spirulina platensis* protean extract after addition of different concentrations of Fe^{3+}. (1) *Spirulina platensis* protean extract alone; (2) *Spirulina platensis* protean extract + Fe^{3+}, 0.1 mg/ml; (3) *Spirulina platensis* protean extract + Fe^{3+}, 0.2 mg/ml; (4) *Spirulina platensis* protean extract + Fe^{3+}, 0.3 mg/ml; (5) *Spirulina platensis* protean extract + Fe^{3+}, 0.4 mg/ml and (6) *Spirulina platensis* protean extract + Fe^{3+}, 0.5 mg/ml. The characteristic peak of phycocyanin can be recorded at 620 nm.

We also measured the fluorescence spectra of phycocyanin isolated from *S. platensis* in the presence of Fe^{2+} and Fe^{3+} ions (Fig. 3a and 3b, respectively). The increase of the metal concentration caused the decrease in the peak amplitude and was accompanied by a blue shift of the fluorescence peak. Fe^{3+} ions quenched the fluorescence more strongly than did Fe^{2+}. With the smaller concentration of $FeCl_3$, a total decrease of the maximum fluorescence emission spectra was obtained. This could be due to the reaction between the aqueous extract and Fe^{3+} which is more probable that the reaction between the aqueous extract and Fe^{2+}.

The results obtained corroborate previous works in which the microalga *S. platensis* has been used as a metal concentrator (Choudhary, Jetley, Abash Khan, Zutshi, & Fatma, 2007; Solisio, Lodi, Torre, Converti, & Del Borghi,

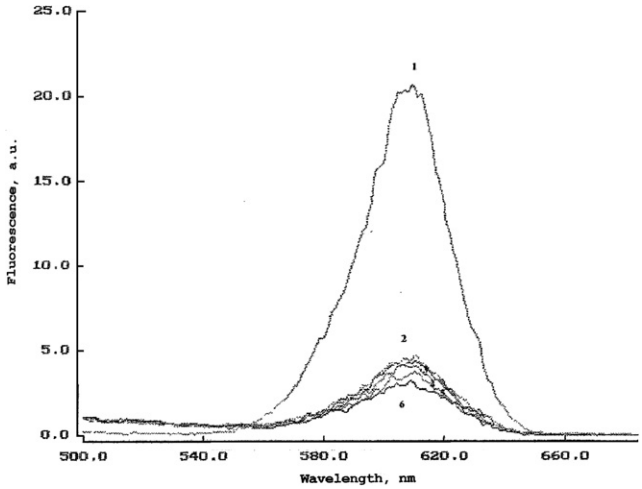

Fig. 3a. Changes in fluorescence spectra of phycocyanin after addition of different concentrations of Fe^{2+}. (1) Phycocyanin alone; (2) phycocyanin + Fe^{2+}, 0.1 mg/ml; (3) phycocyanin + Fe^{2+}, 0.2 mg/ml; (4) phycocyanin + Fe^{2+}, 0.3 mg/ml; (5) phycocyanin + Fe^{2+}, 0.4 mg/ml and (6) phycocyanin + Fe^{2+}, 0.5 mg/ml. The characteristic peak of phycocyanin is shown at 620 nm.

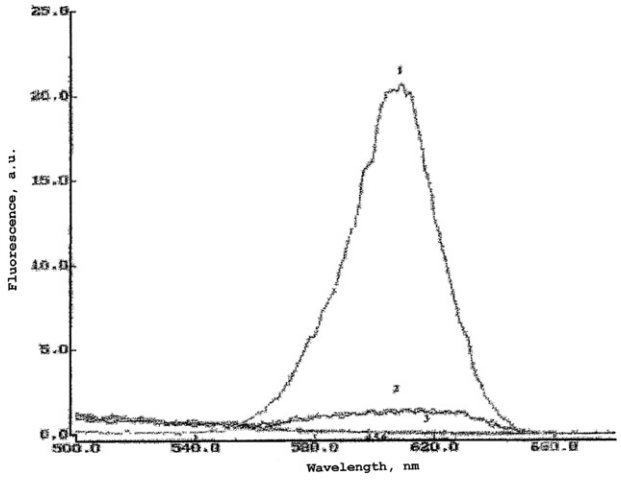

Fig. 3b. Changes in fluorescence spectra of phycocyanin after addition of different concentrations of Fe^{3+}. (1) Phycocyanin alone; (2) phycocyanin + Fe^{3+}, 0.1 mg/ml; (3) phycocyanin + Fe^{3+}, 0.2 mg/ml; (4) phycocyanin + Fe^{3+}, 0.3 mg/ml; (5) phycocyanin + Fe^{3+}, 0.4 mg/ml and (6) phycocyanin + Fe^{3+}, 0.5 mg/ml. The characteristic peak of phycocyanin is shown at 620 nm.

2006; Vannela & Verma, 2006a, 2006b). The adsorption capacity of marine algae would be due to the functional groups present on the cell surface, mainly protein carboxylic groups and functional side chains of amino-acids, such as histidine, cysteine, aspartic acid and glutamic acid (Ashmead, Graff, & Ashmead, 1985). In particular, histidine and the carboxylic group were shown to bind copper (II) effectively, probably because of its bidentate structure (Xue, Stumm, & Sigg, 1988).

4. Conclusions

In this study, it was demonstrated that *S. platensis* protean extract possessed an excellent antioxidant activity, based on different assays. Our results show that *S. platensis* protean extract scavenged hydroxyl and peroxyl radicals and, in addition, showed inhibitory activity against lipid peroxidation. Both radicals (hydroxyl and peroxyl) are among the most important free radicals which can be generated as harmful byproducts and have been implicated in lipid peroxidation and some diseases. The peroxyl radical is generated in normal metabolic reactions by all aerobic organisms and is also the source of the highly biologically reactive, hydroxyl radical. Therefore, scavenging of these free radicals by *S. platensis* can be an effective prevention for a living organism against oxidative stress. An antioxidant can function either by inhibiting the processes activating free radical formation by intercepting the formation of the reactive radical species, and/or inhibiting free radical action by scavenging the radical and preventing the initiation of damaging processes, or by suppressing amplification of the radical damage by further intercepting attack of

secondary-derived radicals on their biological components (propagation) (Rice-Evans & Diplock, 1993). Besides, *S. platensis* has iron-chelating properties affecting iron ions which are known to catalyze many processes leading to the appearance of free radicals.

The antioxidant properties of *S. platensis* and phycocyanin may arise from both radical-scavenging and metal chelation. Most antioxidant capacities of *S. platensis* protean extract are attributable to the biliproteins contained in this microalga, such as phycocyanin. Our study suggests that *S. platensis* could be used to produce a natural dietary antioxidant supplement or added to healthy food products, such as cereals, fruit bars or drinks, to prevent some chronic diseases where free radicals are involved.

References

Aruoma, O. I. (1994). Deoxyribose assay for detecting hydroxyl radicals. *Methods in Enzymology, 233*, 57–66.

Aruoma, O. I. (1997). Scavenging of hypochlorous acid by carvedilol and ebselen in vitro. *Gen Pharmacol, 28*(2), 269–272.

Aruoma, O. I., Halliwell, B., Hoey, B. M., & Butler, J. (1989). The antioxidant action of *N*-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radical Biology and Medicine, 6*(6), 593–597.

Ashmead, H. D., Graff, D. J., & Ashmead, H. H. (1985). *Intestinal absorption of metal ions and chelates*. Springfield, IL: Charles C. Thomas.

Bhat, V. B., & Madyastha, K. M. (2000). C-phycocyanin: A potent peroxyl radical scavenger in vivo and in vitro. *Biochemical and Biophysical Research Communications, 275*(1), 20–25.

Binder, A., Wilson, K., & Zuber, H. (1972). C-phycocyanin from the thermophilic blue-green alga Mastigocladus laminosus, isolation, characterization and subunit composition. *FEBS Letters, 20*(1), 111–116.

Boussiba, S., & Richmond, A. E. (1979). Isolation and characterization of phycocyanins from the blue-green alga *Spirulina platensis*. *Archives of Microbiology, 120*, 155–159.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology, 52*, 302–310.

Cai, Y.-J., Fang, J.-G., Ma, L.-P., Yang, L., & Liu, Z.-L. (2003). Inhibition of free radical-induced peroxidation of rat liver microsomes by resveratrol and its analogues. *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease, 1637*(1), 31–38.

Chen, L. C., Chen, J. S., & Tung, T. C. (1981). Effects of spirulina on serum lipoproteins and its hypocholesterolemic activities. *Taiwan Yi Xue Hui Za Zhi, 80*(9), 934–942.

Choudhary, M., Jetley, U. K., Abash Khan, M., Zutshi, S., & Fatma, T. (2007). Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. *Ecotoxicology and Environment Safety, 66*(2), 204–209.

Eisele, L. E., Bakhru, S. H., Liu, X., MacColl, R., & Edwards, M. R. (2000). Studies on C-phycocyanin from Cyanidium caldarium, a eukaryote at the extremes of habitat. *Biochimica et Biophysica Acta, 1456*(2–3), 99–107.

Gigante, D. P., Buchweitz, M., Helbig, E., Almeida, A. S., Araujo, C. L., Neumann, N. A., et al. (2007). Randomized clinical trial of the impact of a nutritional supplement "multimixture" on the nutritional status of children enrolled at preschools. *Journal de Pediatria, 83*(4), 363–369.

Gorobets, O. B., Blinkova, L. P., & Baturo, A. P. (2002). Action of *Spirulina platensis* on bacterial viruses. *Zeitschrift fur Immunitatsforschung-Immunobiology*(6), 18–21.

Halliwell, B., Gutteridge, J. M., & Aruoma, O. I. (1987). The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry, 165*(1), 215–219.

Hernandez-Corona, A., Nieves, I., Meckes, M., Chamorro, G., & Barron, B. L. (2002). Antiviral activity of *Spirulina maxima* against herpes simplex virus type 2. *Antiviral Research, 56*(3), 279–285.

Hsia, J., Heiss, G., Ren, H., Allison, M., Dolan, N. C., Greenland, P., et al. (2007). Calcium/vitamin D supplementation and cardiovascular events. *Circulation, 115*(7), 846–854.

Khan, Z., Bhadouria, P., & Bisen, P. S. (2005). Nutritional and therapeutic potential of Spirulina. *Current Pharmaceutical Biotechnology, 6*(5), 373–379.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.

Luchsinger, J. A., Tang, M. X., Miller, J., Green, R., & Mayeux, R. (2007). Relation of higher folate intake to lower risk of Alzheimer disease in the elderly. *Archives of Neurology, 64*(1), 86–92.

Marcason, W. (2007). Is supplementation of B vitamins still recommended to reduce the risk of heart disease? *Journal of the American Dietetic Association, 107*(3), 525.

Mihara, M., & Uchiyama, M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Analytical Biochemistry, 86*(1), 271–278.

Miranda, M. S., Cintra, R. G., Barros, S. B., & Mancini Filho, J. (1998). Antioxidant activity of the microalga *Spirulina maxima*. *Brazilian Journal of Medical and Biological Research, 31*(8), 1075–1079.

Mohan, I. K., Khan, M., Shobha, J. C., Naidu, M. U., Prayag, A., Kuppusamy, P., et al. (2006). Protection against cisplatin-induced nephrotoxicity by Spirulina in rats. *Cancer Chemotherapy and Pharmacology, 58*(6), 802–808.

Nagaoka, S., Shimizu, K., Kaneko, H., Shibayama, F., Morikawa, K., Kanamaru, Y., et al. (2005). A novel protein C-phycocyanin plays a crucial role in the hypocholesterolemic action of *Spirulina platensis* concentrate in rats. *Journal of Nutrition, 135*(10), 2425–2430.

Paya, M., Halliwell, B., & Hoult, J. R. (1992a). Interactions of a series of coumarins with reactive oxygen species. Scavenging of superoxide, hypochlorous acid and hydroxyl radicals. *Biochemical Pharmacology, 44*(2), 205–214.

Paya, M., Halliwell, B., & Hoult, J. R. (1992b). Peroxyl radical scavenging by a series of coumarins. *Free Radical Research Communications, 17*(5), 293–298.

Pinero Estrada, J. E., Bermejo Bescos, P., & Villar del Fresno, A. M. (2001). Antioxidant activity of different fractions of *Spirulina platensis* protean extract. *Il Farmaco, 56*(5–7), 497–500.

Qureshi, M. A., & Ali, R. A. (1996). *Spirulina platensis* exposure enhances macrophage phagocytic function in cats. *Immunopharmacology and Immunotoxicology, 18*(3), 457–463.

Rice-Evans, C. A., & Diplock, A. T. (1993). Current status of antioxidant therapy. *Free Radical Biology and Medicine, 15*(1), 77–96.

Romay, C., Armesto, J., Remirez, D., Gonzalez, R., Ledon, N., & Garcia, I. (1998). Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. *Inflammation Research, 47*(1), 36–41.

Roy, K. R., Arunasree, K. M., Reddy, N. P., Dheeraj, B., Reddy, G. V., & Reddanna, P. (2007). Alteration of mitochondrial membrane potential by *Spirulina platensis* C-phycocyanin induces apoptosis in the doxorubicinresistant human hepatocellular-carcinoma cell line HepG2. *Biotechnology and Applied Biochemistry, 47*(Pt 3), 159–167.

Samuels, R., Mani, U. V., Iyer, U. M., & Nayak, U. S. (2002). Hypocholesterolemic effect of spirulina in patients with hyperlipidemic nephrotic syndrome. *Journal of Medical Food, 5*(2), 91–96.

Shih, S. R., Tsai, K. N., Li, Y. S., Chueh, C. C., & Chan, E. C. (2003). Inhibition of enterovirus 71-induced apoptosis by allophycocyanin isolated from a blue-green alga *Spirulina platensis*. *Journal of Medical Virology, 70*(1), 119–125.

Simpore, J., Kabore, F., Zongo, F., Dansou, D., Bere, A., Pignatelli, S., et al. (2006). Nutrition rehabilitation of undernourished children utilizing Spiruline and Misola. *Nutriton Journal, 5*, 3.

Solisio, C., Lodi, A., Torre, P., Converti, A., & Del Borghi, M. (2006). Copper removal by dry and re-hydrated biomass of *Spirulina platensis*. *Bioresource Technology, 97*(14), 1756–1760.

Torres-Duran, P. V., Miranda-Zamora, R., Paredes-Carbajal, M. C., Mascher, D., Ble-Castillo, J., Diaz-Zagoya, J. C., et al. (1999). Studies on the preventive effect of *Spirulina maxima* on fatty liver development induced by carbon tetrachloride, in the rat. *Journal of Ethnopharmacology, 64*(2), 141–147.

Vannela, R., & Verma, S. K. (2006a). Co^{2+}, Cu^{2+}, and Zn^{2+} accumulation by cyanobacterium *Spirulina platensis*. *Biotechnology Progress, 22*(5), 1282–1293.

Vannela, R., & Verma, S. K. (2006b). Cu^{2+} removal and recovery by Spi SORB: Batch stirred and up-flow packed bed columnar reactor systems. *Bioprocess Biosystems Engineering, 29*(1), 7–17.

Wu, L. C., Ho, J. A., Shieh, M. C., & Lu, I. W. (2005). Antioxidant and antiproliferative activities of Spirulina and Chlorella water extracts. *Journal of Agricultural Food Chemistry, 53*(10), 4207–4212.

Xue, H.-B., Stumm, W., & Sigg, L. (1988). The binding of heavy metals to algal surfaces. *Water Research, 22*(7), 917–926.

Zhang, H. Q., Lin, A. P., Sun, Y., & Deng, Y. M. (2001). Chemo- and radioprotective effects of polysaccharide of *Spirulina platensis* on hemopoietic system of mice and dogs. *Acta Pharmacologica Sinica, 22*(12), 1121–1124.