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Full Length Research Paper

Production of phenolic compounds from *Spirulina maxima* microalgae and its protective effects

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The purpose of this study was to illustrate the enhancing process of phenolics synthesis in *Spirulina maxima* grown in Zarrouk's medium supplemented with different concentration of $NaNO_3$ and/or combined with phenylalanine (L-PA). Also, the protective efficacy of *Spirulina* polyphenolic (*S*PP) extracts against CCl_4-induced microsomal lipid peroxidation and scavenging of hydroxyl radical formation were performed. The results revealed that the levels of $NaNO_3$ and L-PA in growth medium had positive effects on the production of biomass (34-64 mg/day), total phenolics (4.51-16.96 mg/g d.w) and flavonoids (1.32-5.12 mg/g d.w) contents. The highest levels of these compounds were obtained in Zarrouk's medium containing 3.77 g/L $NaNO_3$ and 100 mg/L L-PA. The HPLC-DAD profile of all phenolic extracts of *Spirulina* showed the presence of large numbers of phenolic acids and flavonoids, in variable levels. Gallic, chlorogenic, cinnamic, pinostrobin and *p*-OH-benzoic were found as the most abundant constituents among different extracts. Polyphenolic of *Spirulina* exhibited antioxidant effects on CCl_4-induced lipid peroxidation (inhibition %) in liver homogenate and on DPPH radical scavenging activity (with IC_{50} values ranged from 23.22 to 35.62 µg/ml), in dose-depended manner. Their protective potential was comparable to that of standard phenolic antioxidants (BHT, BHA and α-tocopherol, with IC_{50} values ranged from 13.22 to 23.62 µg/ml). Thus, *S. maxima* can be regarded as potent natural sources of nutraceutical and functional ingredients.

Key words: *Spirulina maxima,* antioxidant activity, carbon tetrachloride, hepatoprotective effects.

INTRODUCTION

Nowadays, one of the main considerable interest areas of research in food science and technology is the so-called functional foods because they can provide physiological nutritional and energetic benefits such as, antihypertensive and antioxidant properties (Diplock et al., 1999; Plaza et al., 2008). Antioxidant components are micro-constituents present in the diet that can delay or inhibit lipid oxidation, by inhibiting the initiation or propagation steps of oxidizing chain reactions, and involved in scavenging free radicals (Chadwick et al., 2003). Plants have reported to contain a wide variety of antioxidant components, such as phenolic and carotenoids compounds (Katalinic et al., 2004). Several epidemiological studies indicated a negative correlation between the intake of dietary phenols and coronary heart disease (Knekt et al., 1996), cancer and osteoporosis (Sun et al., 2002), stroke (Keli et al., 1996) and other degenerative diseases (Randhir et al., 2002). Furthermore, phenols have been reported to exhibit pharmacological properties such as anticarcinogenic, antiviral, antimicrobial, anti-inflammatory or antitumoral (Kono et al., 1995; Shetty, 1997; Abd El-Bakey et al., 2008).

Recently, much attention has been focused on the microalgae as sources of novel, biologically active compounds such as phycobiline, phenols, terpenoids, steroids and polysaccharide (Qi et al., 2006; Li et al., 2007; Abd El Baky et al., 2008). However, the occurrence of phenolic compounds in blue green algae is less documented than that in higher plants (Santoyo et al., 2006; Colla et al., 2007). Algal phenolic compounds were reported to be a potential candidate to combat free radicals, which are harmful to our body and food systems (Adamson et al., 1999; Estrada et al., 2001). *In vitro* studies demonstrated that the *Spirulina* and *Nestoc* spe-

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cies have several therapeutic properties, due to their ability to scavenge superoxide and hydroxyl radicals and inhibit lipid peroxidation (Khan et al., 2005; Li et al., 2007).

The influence of growth conditions on the chemical composition of *Spirulina* has been studied by many researchers with the purpose of optimizing the production of economically and nutritionally interesting compounds, especially pigments, antioxidants vitamins and phycocyanin (Tanticharoen et al., 1994; Abd El-Baky, et al., 2006 and 2007). However, the manipulating growth conditions for biomass production and productivity are usually used in the commercial production of potentially useful compounds such as ω-3 fatty acids, carotenoids and phenolics (Abd El-Baky et al., 2007).

In the present study, we hypothesized that nutrient application in *Spirulina maxima* medium containing nitrogen and L-phenylalanine (L-PA, as a precursor for phenylpropanoid synthesis) at different concentrations may result in accumulation of phenolic compounds (PC). The parameters measured to characterize their effect were the change in algal growth and characterization of phenolics compounds. Also, the bioactivity as natural nutraceuticals and functional ingredients of their phenolic substances was evaluated.

MATERIALS AND METHODS

Algal source

The blue green algae, *S. maxima*, was obtained from the Culture Collection of Texas University, Austin, Texas, USA. Strain was maintained in Zarrouk's medium, a standard synthetic medium containing 2.5 g L^{-1} sodium nitrate as nitrogen source (Zarrouk, 1966). However, this medium was used in this work containing variable concentrations of sodium nitrate.

Growth conditions

Large-scale cultivation on Zarrouk's medium containing four nitrogen concentrations (2.5, 3.38 and 3.75 g/L, as $NaNO_3$) at pH 10.5 was done in five aquariums (30 L, each). The cultures were gassed with air containing 0.3% CO_2 (v/v), and continuously illuminated with ten cool white fluorescent lamps (40 W each, Philips). The culture temperature was maintained at 28± 2ºC.

Growth measurements

The algal growth (biomass gL^{-1}) was spectophotometrically determined as described by Payer (1971). The calculated biomass (the average of three experiments) was used to obtain maximum specific growth rates (μ_{max}) from the log phase of the growth curves by exponential regression. Productivities was calculated from the equation P= (Xi -X0) / t_i, where *P* = productivity (mg $L^{-1}day^{-1}$), *X*0 = initial biomass density (mg L^{-1}), *Xi* = biomass density at ti (mg L^{-1}) and ti = time interval (h) between X0 and Xi (Colla et al., 2007).

Determination of dry weight

Ten ml from different cultures were filtered under vacuum through filter membrane (0.45 µm) and several time washed with distilled water to remove soluble salts. Then the algae cells was dried at 80ºC for 30 min and weighed.

Preparation and extraction of crude phenols of algae

The algae were harvested by continuous flow centrifugation (3 L/h) at 2000 x g for 30 min at 4°C and then the resulting whole-cell pellet was weighed. A 4 g of pellet was re-suspended in ethanol (20 mL) and sonicated to disrupt cells, then homogenized for 3 min at room temperature. The homogenate was centrifuged at 2000 x g for 15 min at 4°C, the resulting supernatant was centrifuged again (2000 x g for 10 min). The resulting supernatant was filtered through Millipore filters (0.45 µm pore size) and the filtrate was evaporated to dryness to yield of crude algal ethanolic extracts which contained the phenolic compounds.

Determination of total phenolics content (TPC)

The total phenolic content of tested samples was spectrophotometercally determined by the Folin-Ciocalteu method using gallic acids (10-200 µg/mL) as a standard (Singleton et al., 1999). Five replicates were used for each biomass sample.

Determination of flavonoids content (TFC)

The total flavonoid content was spectrophotometercally determined by the aluminum chloride method based on the formation of complex flavonoid- aluminum (Djeridane et al., 2006). One milliliter of flavonoids extract was mixed with 1 mL of $AlCl_3$ methanolic solution (2%, w/v). After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm. The contents of TFC were estimated from the standard calibration curve of 10-100 µg/ mL quercetin.

HPLC analysis

The chemical constituents of the *S. maxima* extracts were identified by HPLC method reported by Mendiola et al. (2005). Dionex Summit IV HPLC system consisted of a Dionex P680 dual gradient pump, an ASI-100 auto-sampler equipped with a 20- µL loop and PDA-100 photodiode array detector were used. A reversed phase column C18 (250 x 4.6 mm, 5 µm partials) was used. The mobile phase was a mixture of solvent A (methanol/ammonium acetate 0.1 N; 7:3, v/v) and solvent B (pure methanol) at rate of 0.9 ml min^{-1} as a step gradient, lasting 35 min, which started from 25% B, changing at 50% in 1 min, rising up to 100% B at 10 min. Then, the mobile phase composition was kept constant until the end of the analysis. Total acquisition time was 35 min. The temperature was set at 25°C. The identification of the peaks was performed, when possible, using standards. When no standards were available, tentative identification was done based on UV-Vis spectra characteristics and compared with that data appearing in the literature.

Antioxidant assays

The antioxidant activity of the phenolics *S. maxima* extracts was determined by two methods namely DPPH• free-radical scavenging and inhibition of lipid peroxidation of hepatic microsomes induced by CCl_4.

DPPH• free- radical scavenging assay

The method described by Tagashira and Ohtake (1998) was used

Table 1. Growth parameter of *Spirulina maxima* grown under sodium nitrate and phenylalanine concentrations.

Treatments	μmax (day^{-1})	Δ t (h)	P_{432} (mg L^{-1} day^{-1})	Extraction yield
2.5 g L^{-1} $NaNO_3$ (g l^{-1})	0.099 ± 0.003	24-432	37.50 ± 0.11	10.14
2.5 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	0.130 ± 0.003	24-432	42.60 ± 0.14	11.65
2.5 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	0.151 ± 0.003	24-432	46.70 ± 0.13	13.63
3.125 g L^{-1} $NaNO_3$ (g L^{-1})	0.111 ± 0.004	24-432	45.50 ± 0.14	11.25
3.125 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	0.183 ± 0.005	24-432	51.80 ± 0.12	13.58
3.125 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	0.201 ± 0.003	24-432	57.70 ± 0.15	14.51
3.777 g L^{-1} $NaNO_3$ (g L^{-1})	0.156 ± 0.003	24-432	51.20 ± 0.13	12.64
3.777 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	0.192 ± 0.004	24-432	56.80 ± 0.12	14.98
3.777 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	0.251 ± 0.004	24-432	65.40 ± 0.13	16.35

Except for Δt, all values show mean ± standard deviation.
μmax = Maximum specific growth rate; μt = start–end of the exponential growth phase; P_{432} = productivity at 432 h.

in order to assess the DPPH radical scavenging of phenol extracts of *S. maxima* (PES). To 25 ml of 0.004% of DPPH reagent, different concentration of PES (ranged 5- 100 μg/mL) was added. The reaction mixture was vortexed and incubated in a dark at 30 ± 1ºC. The absorbance was monitored at 517 nm for 90 min, at 15 min intervals, against a blank (pure methanol). The BHT, BHA and α-tocopherol (100 μg/mL) were used as reference standards. The radical scavenging activity of PES in the mixture was calculated from a calibration curve. All tests were run in triplicates and average values were presented in the text. Extracts concentration providing 50% inhibition (IC_{50}) was calculated from graph plotted of inhibition percentage against extract concentration.

Lipid peroxidation of hepatic microsomes induced by CCl_4

The hepatic microsomes were prepared from livers of male Wister rats (body weight 120 ± 5 g). The microsomal fraction was isolated by differential centrifugation as described by Kornbrast and Mavis (1980). Five rats were decapitated and allowed exsanguinations. Then, the liver was quickly excised and washed several times with ice cold 0.15 M KCl, then homogenized in the same saline solution. The homogenate was filtered and centrifuged successively at 3000 x g (at 4℃) for 10 min; the supernatants were separated and then, centrifuged at 20,000 x g (10 min). The resulted supernatants were centrifuged at 105,000 x g for 1 h at 4℃, and then the microsomal pellets were re-suspended in ice cold Tris–buffer (pH 7.4), kept at –30℃ until used.

Lipid oxidation of hepatic microsomal was induced by treatment of a 200 μL of rat hepatic microsomal protein (10 mg) with 10 μL of carbon tetrachloride (CCl_4, as oxidizing reagent) in phosphate buffer (total volume 3 mL). Then, one mL of TPC of different extracts (at concentration of 50 and 100 μg/ mL, final volume) was added. The reaction mixture was incubated at 37±1℃ for 45 min. The butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) were used as antioxidant standards (at 50 and 100 μg/mL). At intervals time up to 45 min, the TBARs products in the reaction mixture was determined.

The concentration of TBARs (as a product of lipid peroxidation) was measured according to the method developed by Haraguchi et al. (1997). The malondialdehyde (MDA) contents in 1 mL of the reaction mixture were quantizied by reaction with 2.5 mL of TBA-TCA reagent (consisted of 1 ml thiobarbituric acid (0.38%) and 1.5 mL of 15% trichloroacetic acid). Then, the mixture was incubated at 90°C for 30 min. After centrifugation (at 1000 x g for 5 min), the TBARS content in the supernatant was spectrophotometercally determined at 532 nm using the molar extinction coefficient of chromophore (1.56×10^{-5} $M^{-1}cm^{-1}$). The TBARs concentration in liver microsomes was normalized with protein concentration.

Statistical analyses:

Data obtained from measurements for each variable were subjected to analysis of variance using the COSTAT computer package (Cohort Software, CA, USA). The mean values were compared with LSD test.

RESULTS AND DISCUSSION

As shown in Table 1, the concentration of $NaNO_3$ (2.5-3.77 g/L) in Zarrouk's medium exhibited a significant affect on the values of productivity at 432 h (ranged from 37.5 to 51.2 mg/L) and μ_{max} (0.10 to 0.16 day^{-1}) of *S. maxima* cultures. Furthermore, their values ranged from 42.6- 65.4 mg/L and 0.13-0.25 day^{-1}, respectively in culture cultivated in the medium containing $NaNO_3$ (2.5-3.77 g/L) combined with L-PA (50-100 mg/L^{-1}). Thus, L-PA has an important influence on the productivity and μ_{max} of *S. maxima* culture. This is to be expected for cultured containing L-PA because this amino acid may play an impotent role as growth factors, which lead to an enhanced synthesized of protein and other cellular components and/or an increased rate of photosynthesis. Therefore, the biomasses of algal cells increased as compared with that in control. As mentioned by Colla et al. (2007), a significant positive interaction between $NaNO_3$ concentration and increase of biomasses was found in *S. platensis.* Also, the increase of biomasses in this experiment is in accordance with those obtained in three species of *Sprulina; Sprulina platensis, Sprulina lonar and Sprulina laxissma* (Susinjan and Shivaprakash, 2005).

Influenced of sodium nitrate level on total phenolics and flavonoids contents

Table 2 shows that the phenolic and flavonoid contents of

Table 2. Influence of sodium nitrate and phenylalanine concentrations on phenolics and flavonoids content of *Spirulina maxima.*

$NaNO_3$ / phenylalanine	Total phenol (mg g^{-1} DW)	Phenolic (%)	Ratio[a]	Total flavonoids (mg g^{-1} DW)	Flavonoids (%)	Ratio[a]
2.5 g L^{-1} $NaNO_3$ (g l^{-1})	4.51 ± 0.23	0.45	1.00	1.32 ± 0.03	0.13	1.0
2.5 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	5.68 ± 0.33	0.57	1.26	1.54 ± 0.08	0.15	1.2
2.5 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	7.36 ± 0.36	0.74	1.63	1.94 ± 0.06	0.19	1.5
3.125 g L^{-1} $NaNO_3$ (g L^{-1})	5.19 ± 0.35	0.52	1.15	1.45 ± 0.07	0.14	1.1
3.125 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	8.64 ± 0.27	0.86	1.91	2.25 ± 0.13	0.22	1.7
3.125 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	10.95 ± 0.47	1.09	2.42	3.31 ± 0.21	0.33	2.5
3.777 g L^{-1} $NaNO_3$	6.54 ± 0.54	0.65	1.45	1.93 ± 0.05	0.19	1.5
3.777 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	12.94 ± 0.93	1.29	2.86	4.65 ± 0.14	0.46	3.5

All values show mean of three replicates, ± standard deviation.
Values are significant at ($P < 0.01$).

S. maxima significantly affected with concentration of $NaNO_3$ in Zarrouk's medium. At higher $NaNO_3$ concentration (3.12 and 3.77 g/L), *Spirulina* had higher values of cellular total phenolics (5.12 and 6.54 mg g^{-1}) and flavanoids contents (1.45 and 1.93 mg g^{-1}) compared to those values 4.51 and 1.32 mg g^{-1} in culture grown under optimal level (2.5 g^{-1} $NaNO_3$). Thus, a significant positive correlation between $NaNO_3$ concentration and the accumulation of higher amounts of phenolic contents in *S. maxima* was observed. These results revealed that nitrogen is required for synthesis of the aromatic amino acids (AAA), which may be playing an important role as precursors for biosynthesis of phenolic compounds. Therefore, the second set of experimental was conducted to evaluate the affect of adding L-phenylalanine (L-PA) as AAA to growth media of *S. maxima* on production of phenolic compounds.

Influence of L-PA combined with sodium nitrate levels on total phenolic and flavonoid contents

Total phenolic contents (TPC)

As shown in Table 2, the levels of cellular contents of the phenolic (TPC) and flavenoids (TFC) were strongly affected by concentration of both nitrogen and L-PA presence in the culture media. The synthesis of their compounds being almost folded in cultures cultivated at higher $NaNO_3$ and L-PA levels. The highest amount of TPC (16.96 mg/g d.w) and TFC (1.69 mg/g d.w) was obtained in culture grown in medium contained higher levels of L-PA (100 mg L^{-1}) coupled with highest $NaNO_3$ (3.77 g/L) concentration. While, those values were 5.68 and 0.57 mg/g d.w in culture grown at optimal $NaNO_3$ (2.5 g/L) concentration combined with low L-PA level (50 mg /L). Thus, there seems to be a linear relationship between the levels of TPC and TFC and its concentration of $NaNO_3$ and L-PA in Zarrouk's medium. Therefore, a combination between $NaNO_3$ and L-PA in growth medium showed a significant affect on production high amounts of TPC and TFC. The amounts of TPC and TPC (in parentheses) of *S. maxima* grown in medium contained 2.5, 3.12 and 3.77 g/L $NaNO_3$ combined with L-PA at 50 and 100 mg L^{-1} were found to be about 1.26 (1.63), 1.91 (2.42) and 2.86 (3.75)-fold and (1.63),1 (2.) and 2. (3.5)-fold, respectively, those obtained in *Spirulina* grown in standard Zarrouk's medium (in absent of L-PA). Consequently, an interesting direct correlation between higher accumulation of cellular phenolic compounds and the supplementation of growth medium with L-PA was observed. This result clearly indicates that the L-PA has a stimulatory role on an accumulation of phenolics in *Spirulina.* It is known that L-PA plays an important role in biosynthesis of phenolics, which L-PA acts as a precursor for phenyl-propanoid pathway that synthesizes phenolic compounds. This pathway is responsible for the synthesis of a wide variety of phenolics metabolites such as salicylates, cumarins and flavanoids (Dixon and Paiva, 1995). According to previous studies, the biosynthetic pathways that lead to the formation of phenyl-propanoid compounds are increased with entry of L-PA. Their pathway is also important for biosynthesis of cinnamic acid, which leads to increase the phenol synthesize in plants (Herrmann, 1998; Shetty and Randhir, 2005). However, in green algae the amino acid phenylalanine could be converted by an ammonia lyase to *trans*-cinnamic acid, which is then converted to cumaric acid that in turn is converted to caffeic acid, these compounds being converted through several chemical reactions to phenols and flavonoids (Taiz and Zeigler, 1998; Rechner et al., 2001).

Identification of phenol compounds

The HPLC-DAD profile of phenolics extracts of *Spirulina* grown in Zarrouk's medium contained different concentration of $NaNO_3$ combined with L-PA supplementation showed the presence of a large number of phenolic acids

Table 3. Influence of sodium nitrate and phenylalanine concentrations on phenolics composition of *Spirulina maxima*.

$NaNO_3$	2.5 g L^{-1}			3.125 g L^{-1}			3.777 g L^{-1}		
Phenylalanine	**0.0**	**50 mg L^{-1}**	**100 mg L^{-1}**	**0.0**	**50 mg L^{-1}**	**100 mg L^{-1}**	**0.0**	**50 mg L^{-1}**	**100 mg L^{-1}**
Gallic acid	19.82	16.0	12.15	17.91	13.3	6.94	15.5	8.23	4.78
P-OH benzoic acid	14.18	12.31	10.27	12.18	8.27	7.62	11.7	6.12	3.32
Chlorogenic acid	13.21	10.21	7.21	9.21	3.24	3.54	13.7	6.23	1.14
Vanillin acid	7.12	5.25	3.51	5.31	4.33	3.85	1.21	0.57	0.23
Caffeic acid	6.36	4.31	2.22	4.95	3.61	2.97	7.92	2.44	1.78
Syringic acid	…….	5.21	4.25	……	2.85	1.75	……..	1.54	0.84
Salicylic acid	4.12	2.37	1.82	3.00	2.85	1.49	1.22	0.34	0.19
O-Coumaric acid	4.13	3.21	2.47	2.66	1.92	1.21	2.79	1.21	0.11
Fuerulic acid	3.12	3.02	3.11	2.96	4.51	5.67	3.14	5.31	7.22
Cinnamic acid	1.05	4.57	7.54	3.85	10.99	15.77	8.25	15.65	18.14
Quercetin	1.14	4.25	6.32	1.85	2.45	3.54	2.35	3.21	4.23
Genstein	1.22	0.52	……	……	……	……	……	……	……
Unknown	1.15	2.11	……	……	……	……	……	……	……
Unknown	1.35	2.98	…….	…….	…….	0.73	2.39	1.69	……
Unknown	1.92	2.51	2.11	1.48	2.13	1.05	2.39	1.98	…….
Kaermphereol	1.33	2.81	3.51	3.34	4.51	5.21	3.51	6.52	7.38
Euganol	1.09	1.89	3.53	5.11	5.39	3.85	1.13	4.31	7.25
Unknown	1.25	1.81	2.33	3.55	4.81	1.35	1.8	2.45	2.71
Chrysin	1.02	……	……	……	……	……	……	……	…….
Galangin	0.54	0.65	0.89	1.14	1.87	2.11	1.57	2.65	3.25
Unknown	1.53	1.83	2.13	2.06	3.35	0.53	……	……	……
Unknown	1.52	1.98	2.28	1.95	0.97	1.17	3.64	1.35	2.17
Unknown	2.29	3.11	4.51	3.62	2.65	1.35	1.10	1.98	2.65
Pinostrobin	7.28	10.25	15.31	11.1	15.54	29.73	15.8	25.98	33.61
Unknown	…….	1.17	0.82	1.31	……..	1.28	2.83	…….	…….
Total identified	**97.74**	**99.12**	**98.29**	**98.54**	**99.51**	**97.08**	**96.1**	**97.78**	**99.77**

and few number of flavonoids (due to the lacking of flavonoids standard) with considerable variation in relative area % (Table 3). Of which, the gallic, chlorogenic, cinnamic, *p*-OH-benzoic, quimic, caffeic, vanillic and ferulic acids were the most abundant constituents (<10 % - >1%, of total chromatographic area). All these constituents were pronouncedly decreased with increasing concentration of L-PA and $NaNO_3$ in growth medium. For instant, the values of caffeic, vanillic and ferulic acids in cultures grown at higher $NaNO_3$ (3.77 g L^{-1}) level combined with low (50 mg/L) and high (100 mg/L) L-PA (in parenthesis) were 2.44 (1.78), 0.57 (0.23) and 5.31% (7.22 %), respectively. In contrast, their components were found at higher values of 6.36, 7.12 and 3.12% in S*pirulina* grown on a medium containing low $NaNO_3$ (2.5 g/L) level only. With regard to flavonoids constituents, quaristin (1.14- 6.32%) is present as the main constituents in *Spirulina* grown under different culture condition. In the current study, phenolic acids constituent in *Spirulina* culture decreased by increasing the levels of L-PA and $NaNO_3$ in growth medium. This phenomenon is associated with increase flavonoid constituents. This means that the levels of L-PA and $NaNO_3$ in growth medium had a negative correlation with synthesis of phenolic acids and positively correlated with flavonoids constituents. According to Miranda et al. (1998), the main phenolic compounds found in *Spirulina* are *s*alicylic, *trans*-cinnamic, synaptic, chlorogenic and caffeic acids. However, the metabolic pathways for the formation of phenolics compounds in *Spirulina* and their importance are still unknown (Colla et al., 2007).

Antioxidant activity

The potential health benefits of algal bio-active compounds such as phenolics are widely investigated. In humans, they are shown to be strong antioxidants, which might prevent oxidative damage to biomolecular like DNA, lipids and proteins that play role chronic diseases such as cancer and brine dysfunction (Droge, 2002). Thus, the antioxidant activity of the total phenolic of the different *Spirulina* culture was evaluated by two models system (Tables 4 and 5).

Table 4. Scavenging activity of *Spirulina maxima* organic extracts on DPPH• radical.

$NaNO_3$ (g L^{-1})/phenylalanine (mg L^{-1})	IC_{50}[b] (µg mL^{-1})
BHA	14.8
BHT	16.6
α-Tocopherol	18.8
2.5 g L^{-1} $NaNO_3$	35.62
2.5 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	31.27
2.5 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	29.35
3.125 g L^{-1} $NaNO_3$ (g L^{-1})	33.51
3.125 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	30.25
3.125 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	27.31
3.777 g L^{-1} $NaNO_3$	30.54
3.777 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	25.14
3.777 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	23.22
LSD at level (P< 0.01)	1.36

IC_{50}[b]: Concentration (µg/ml) for a 50% inhibition was calculated from the plot of inhibition (%) against *Spirulina maxima* extracts concentration. Tests were carried out in triplicate.

Scavenging effect on DPPH radicals

Free radical scavenging is generally the accepted mechanism for antioxidants inhibiting lipid peroxidation, in a relatively short time. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability (Abd El-Baky et al., 2007).

As shown in Table 4, phenolic extracts of *S. maxima* grown in different medium (*Sp*.TPC) possessed a good ability of scavenging DPPH radical activity (RSA) compared to positive synthetic antioxidants. The RSA of these algal extracts may vary depending on the actual phenolic contents. This was expected because the samples were rich in phenolic acid compounds that exhibited the highest RSA (Table 2). The IC_{50} values for *Spirulina* phenolic extracts ranged from 23.22 to 35.62 µg/ml, while IC_{50} values for α--TOC, BHT and BHA were 18.8, 16.6 and 14.80 µg/ml, respectively. Thus, *Sp*.TPC induces significant DPPH radical scavenging activity and their activity was found to be closed to those of commercial antioxidant. However, the DPPH scavenging activity of *S. maxima* extracts is in positive correlated with increase of phenolic content in those extracts. Thus, the antioxidant nature of *S. maxima* extracts is apparently related to their high phenoilc contents.

The scavenging activity of *Spirulina* phenol extracts toward DPPH radicals suggests that the extracts had antioxidant activity through electron and hydrogen donors' mechanisms. In other words, *Spirulina* extracts containing phenolic compounds are able to donate hydrogen atom to the free radical thus stopping the propagation chain reaction during lipid oxidation process. It is known that the prevention of the chain initiation step by scavenging various reactive species such as free radicals is considered to be important mode action (Halliwell and Gutteridge, 1989; Ruberto et al., 2001).

Protection of the *S. maxima* phenol extracts against hepatic microsomal lipid-peroxidation-induced by CCl_4

In order to determine the antioxidant activity of *S. maxima* (Santoyo et al., 2006) in terms of the mechanisms of its hepatic-protective effect through inhibiting the oxidative stress in rat hepatic homogenate, the level of TBA-MDA complex as oxidative marker was determined (Table 5 and Figure 1). As regard to TBARS formation, the amounts of MDA contents were significantly elevated from 0.53 to 14.59 µmol/mL, after 45 min of incubation of *in vitro* hepatotoxicity model (CCl_4-induced lipid peroxidation of rats liver microsomes). Thus, the enhanced lipid peroxidation in rats liver microsomes can be processed in non-enzymatic and enzymatic ways. As shown in Table 5, the enhanced CCl_4-lipid peroxidation in a model system was significantly inhibited by catalysis with *S. maxima* phenolic extracts and commercial antioxidant standard with various degrees. Moreover, it is clear that *S. maxima* phenolic extracts behave as inhibitors of CCl_4-induced lipid peroxidation independent of concentration. The catalyzed hepatic microsomes with *S. maxima* phenolic extracts at 50 and 100 (in parentheses) µg/mL caused an inhibition of MAD formation ranging from 25.33 to 73.09% (from 42.42 to 80.5%), compared to control. At same concentrations, the reference compound BHT inhibited MAD formation by 40.23 and 69.61%, respec-tively. Thus, *S. maxima* phenolic extracts exhibited more prominent effects in inhibition of CCl_4 -induced lipid peroxidation compared to that BHT. However, the inhibition of MAD formation of tested extracts is positively correlated with increase of phenolic contents in those extracts. The algal phenol extracts caused a significant decrease in the level of MDA contents with increasing concentration of phenol contents (50-100 µg/mL), that led to a progressive inhibition of the CCl_4-induced MDA formation with a concomitant increase of hepatic microsomal resistance to oxidation. These results clearly suggested that inhibition of CCl_4-induced lipid peroxidetion is related to the abilities of the phenolic and flavonoid compounds presence in *Spirulina* extracts to inhibit hepatic oxidative enzymes (cytochrome *P*450 system). Droge (2002) reported that certain flavonoids and phenols have protective effect on liver damage due to its antioxidant properties.

However, the experimental results in DPPH assay demonstrate that *S. maxima* phenolic extracts exercises free radical scavenging activity. Therefore, inhibiting TBA formation substantiates the antioxidant capacity of *S.*

Table 5. Effect of algal phenolic extracts on inhibition of rats liver microsome lipid peroxidation induced by CCl_4 system.

Treatments	Conc. (µg/mL)	Incubation period (min)						Mean of Inh. (%)
		15 TBARs		30 TBARs		45 TBARs		
		µmol/mL	Inh. (%)	µmol/mL	Inh. (%)	µmol/mL	Inh. (%)	
CCl_4 (control)		7.53	0.00	11.29	0.00	14.59	0.00	0.00
BHT	50	5.23	30.54	6.35	43.75	7.82	46.40	40.23
	100	2.82	62.54	3.25	72.68	3.85	73.61	69.61
2.5 g L^{-1} $NaNO_3$	50	6.23	17.26	8.35	26.04	9.82	32.69	25.33
	100	5.33	29.22	7.32	35.16	8.25	43.45	35.94
2.5 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	50	5.44	27.76	7.45	34.01	8.26	43.38	35.05
	100	4.39	41.69	5.22	53.76	6.52	55.31	50.25
2.5 $NaNO_3$ + 100 mg L^{-1} phenylalanine	50	4.82	35.98	6.23	44.82	7.35	49.62	43.47
	100	3.11	58.68	4.33	61.64	5.21	64.29	61.53
3.125 g L^{-1} $NaNO_3$	50	5.85	22.31	7.83	30.64	8.32	42.97	31.97
	100	4.98	34.06	6.34	43.84	7.31	49.89	42.59
3.125 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	50	5.11	32.14	6.35	34.75	8.27	43.33	36.74
	100	3.21	57.37	4.15	63.24	4.84	66.82	62.47
3.125 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	50	4.15	44.88	5.28	53.23	6.34	56.54	51.55
	100	2.22	70.52	2.55	77.41	3.11	78.68	75.53
3.777 g L^{-1} $NaNO_3$	50	5.45	27.62	6.82	39.59	7.33	49.76	38.99
	100	4.35	42.23	5.21	53.85	5.53	62.09	52.72
3.777 g L^{-1} $NaNO_3$ + 50 mg L^{-1} phenylalanine	50	2.42	67.86	4.31	61.82	5.94	59.29	62.99
	100	1.21	83.93	2.52	77.68	2.71	81.14	80.92
3.777 g L^{-1} $NaNO_3$ + 100 mg L^{-1} phenylalanine	50	1.81	75.96	3.22	71.48	4.11	71.83	73.09
	100	1.01	86.58	1.21	89.28	1.23	91.57	89.14

TBARs, Thiobarbturic acid reactive substance; TBARs formation was expressed as µM; Inh, Inhibition (%) was expressed as the reduction of TBARs formation from sample to control; Mean of Inh (%); Mean of Inhibition (%) at different time intervals (15, 30 and 45 min).
All values are means of 3 replicates.

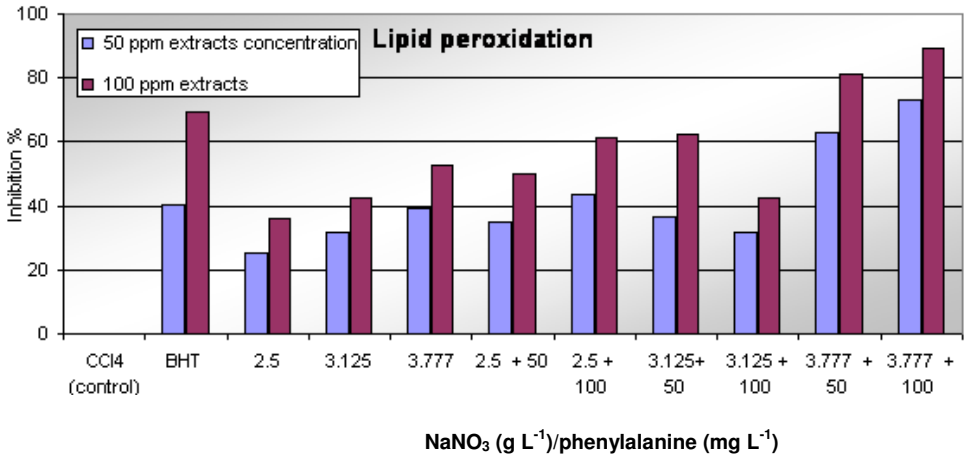

Figure 1. Effect of algal phenolic extracts on inhibition of rats liver microsome lipid peroxidation induced by CCl_4 system.

maxima in protecting hepatic microsomal at physiological pH from oxidative degradation. Therefore, *Spirulina* phenolic extract is capable of acting as anti-hepatotoxicity agent. A possible mechanism for the protection of CCl_4-induced liver scavenger may be by intercepting those radicals involved in CCl_4 metabolism by hepatic micro-

somal enzymes.

It is well established that CCl_4 induces hepato-toxicity by cytochrome *P*450 mediated reactions to produce CCl_4–derived radicals such as trichloromethyl ($\bullet CCl_3$) and trichloromethyl peroxyl ($\bullet OOCCl_3$) (Santoyo et al., 2006). These activated radicals bind covalently to the macromolecules (lipids and proteins) and induce peroxidative degradation of membrane lipids rich in polyunsaturated fatty acids and finally to cell death (Johnston and Kroening, 1998). Moreover, this oxidative process is one of the principal causes of hepatotoxicity of CCl_4 (Cotran, et al., 1994; Opoku et al., 2007). Therefore, the antioxidant activity and/or the inhibition of free radical generation are important in terms of protecting the liver from CCl_4–induced damage (Opoku et al., 2007). According to Cervato et al. (2000) and Athukorala et al. (2006), phenolic metabolites are effective in the inhibition of all phases of the peroxidative process: first neutralizing free radicals, then blocking the peroxidation catalysis with oxidizing agent and finally through interruption of lipid–radical chain reactions.

It is interesting to note that algal phenolic extracts could afford protection against CCl_4-induced lipid peroxidation *in vivo*. Therefore, it could be suggested that the algal extracts may exert a better function in free radical scavenging and inhibition of cytochrome *P*450 mediated reactions responsible for metabolism of xenobiotics and thereby may affect their toxicity and carcinogenicity. Also, algal phenol compounds may be a promising alternative to synthetic substances as natural compound with high antioxidant activity. In addition, *S. maxima* may be exploited as potent natural sources of nutraceutical and functional ingredients. These properties can be improved by changing the culture conditions and the production of these agents could be promising.

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