

## Characterization of Nutraceutical Compounds in *Blue Green Alga Spirulina maxima*

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**Abstract:** This work was conducted to evaluate the influence cultures condition (nitrogen concentration in growth medium) on characterization of some nutraceutical compounds in algal *Spirulina maxima* and antioxidant and antibacterial activities as well as chemical composition of organic extract obtained from different cultures were examined. The amounts of total carotenoids, chlorophylls-derived and phenolic compounds were associated inversely with concentration of nitrogen in growth media. The antibacterial test results showed that all *S. maxima* extracts exhibited a great potential antibacterial activity against 6 bacterial strains with inhibition zones ranged 7-18 mm and MICs ranged 30-40  $\mu\text{g mL}^{-1}$ . Also, all *S. maxima* extracts possessed a potent antioxidant properties as compared to commercial antioxidants, when assessed by three different methods included rapid TLC screening,  $\beta$ -carotene/linoleic acid and 1,1-diphenyl-2-picrylhydrazyl free radical scavenging. The chromatographic analyses of *Spirulina* organic extracts with TLC and HPLC showed that carotenoids, chlorophyll-derived and phenol compounds were present as main constituents and quantity changed significantly dependant on culture condition. Thus, it could be suggested that the *Spirulina* is useful bio-system for production bioactive compounds possess an antioxidants and antimicrobial principles and as natural pigments.

**Key words:** *Spirulina maxima* • Organic extract • Antioxidant activity • Antimicrobial activity

### INTRODUCTION

Recently, many studies have focused on physiological properties and the presence of high value chemical such as antiviral or antioxidant compounds in blue green alga *Spirulina* [1-3]. The occurrence of many compounds possess antioxidant activity in biological systems in higher plant-derived is well documented, but in microalgae are less well documented [4]. *Spirulina platensis* or its extracts could show therapeutic properties, which include the ability to prevent and inhibit cancers, to decrease blood cholesterol levels, stimulate the immunological system, to reduce the nephrotoxicity of pharmaceuticals and toxic metals and provide protection against the harmful effect of radiation [5, 6]. Meanwhile, *Spirulina* deserves special attention both as a source of protein-rich material of nutritional or industrial use (blue pigments) and for its health-enhancing properties [7]. These functional properties have been attributed to different compounds such as, phycocyanins, carotenoids, organic acids, sulfated polysaccharide and polyunsaturated fatty acids [2, 3, 8,9].

Chronic diseases including cancer and cardiovascular diseases are the main causes of death in the world, which the oxidative stress induced by reactive oxygen species (ROS) is one of the foci related to this disease. ROS are highly reactive oxidant molecules that are generated endogenously through regular metabolic activity, lifestyle activity and diet and they are reacting with cellular components, causing oxidative damage to such critical cellular biomolecules such as lipid, protein and DNA. Thus, it is strong evidence that this damage may play a significant role in causation of several chronic diseases [10]. In order to protect the body against the consequences of oxidative stress, one way by which a substance can interfere with these processes is by acting as antioxidant or free radical scavenger. Thus, antioxidant plays an important role in the protection of cells against oxidative damage caused by ROS [1, 11].

Now-a-days, food industries demand new food ingredients obtained from natural source, in order to developed novel functional foods or nutraceuticals. Algal species are an important alternative material to extract natural antioxidative compounds able to delay or prevent

the oxidative damage caused by ROS molecules [12, 12]. Among algal species, *Spirulina* has been reported to prevent oxidative damage by scavenging free radicals and active oxygen and hence can indirectly reduce cancer formation in human body. It has been reported that, increased consumption of foods, which are rich with free radical scavenging activity, leads up to a doubling of protection against many common types of cancer formations [14].

In this report, the influence of nitrogen concentration in growth media production of some nutraceutical compounds by *Spirulina maxima* was investigated. Also, organic extracts of different algal cultures were assessed for their antioxidant and antimicrobial activity as well as chemical constituent of extracts was identified.

## MATERIALS AND METHODS

**Algal Source:** The blue green algae, *Spirulina 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<sup>-1</sup> sodium nitrate as nitrogen source [15], this medium also being used in this work but the concentration of the sodium nitrate was modified.

**Growth Conditions:** Large-scale cultivation on Zarrouk's medium containing five nitrogen concentrations (2.5, 1.78, 1.25, 0.624 and zero g/L, as NaNO<sub>3</sub>) at pH 10.5 was done in five aquariums (30 L, each). The cultures were gassed with air containing 0.3% CO<sub>2</sub> (v/v) and continuously illuminated with ten cool white fluorescent lamps (40 W each, Philips). The culture temperature was maintained at 28±2°C.

**Growth Measurements:** The monitor of algal growth was measured spectrophotometrically as described by Payer [16]. Briefly, 10 ml of cultures were taken every 3 days and measured at 670 nm density. The calculated biomass (the average of three experiments) was used to obtain maximum specific growth rates ( $\mu_{max}$ ) from the log phase of the growth curves by exponential regression. Productivities were calculated from the equation  $P = (X_i - X_0)/t_i$ , where  $P$  = productivity (mg L<sup>-1</sup> day<sup>-1</sup>),  $X_0$  = initial biomass density (mg L<sup>-1</sup>),  $X_i$  = biomass density at time  $i$  (mg L<sup>-1</sup>) and  $t_i$  = time interval (h) between  $X_0$  and  $X_i$ . [4].

**Determination of Dry Weight:** Ten ml from different cultures were filtered under vacuum through filter

Table 1: Growth parameter and extraction yield of *Spirulina maxima* grown at sodium nitrate concentrations

Na NO <sub>3</sub> (g L <sup>-1</sup> )	$\mu_{max}$ (day <sup>-1</sup> )	$P_{432}$ (mg L <sup>-1</sup> day <sup>-1</sup> )	Extraction yield (%)
2.500	0.094±0.003	36.5±0.12	11.0
1.875	0.089±0.005	34.33±0.21	12.1
1.250	0.081±0.004	32.35±0.32	12.3
0.625	0.074±0.004	30.15±0.71	13.2
0.000	0.069±0.003	28.91±0.53	13.9

All values show mean±standard deviation

$\mu_{max}$  = maximum specific growth rate

$P_{432}$  = productivity at 432 h = 18 days

membrane (0.45  $\mu$ m) and washed, several times with distilled water to remove soluble salts. Then the algae cells were dried at 80°C for 30 min and weighed.

**Preparation of Organic Extracts (COEs):** The fresh filtrated *S. maxima* samples (ca. 5 g) were homogenized with 50 mL dichloromethane/methanol (1:1, v/v), then the homogenate was filtered and the filtrate was evaporated under vacuum to dryness under vacuum at 40°C. The extraction yields were (based on dry weight, D.W) 11.0, 12.0, 12.3, 13.0 and 13.9 % from cells grown in medium containing five sodium nitrate at 2.5, 1.785, 1.25, 0.625 and zero g/L, respectively, (Table 1). All extraction was done in duplicate. The dry extracts were kept at 4°C under N<sub>2</sub> when not in use.

**Determination of Total Phenol Content:** The concentration of phenolics in *Sp. maxima* samples were estimated by the Folin-Ciocalteu procedure of Singleton *et al.* [17] and expressing results in gallic acid equivalent, a naturally occurring phenol.

**Determination of Chlorophyll:** One gram of *S. maxima* was homogenized in 20 ml acetone (80%) and allowed to stand overnight in dark at 4°C for complete extract followed by centrifugation at 10,000 xg for 5 min. The contents of total chlorophyll (T-Chl), chlorophyll a (Chl-a) and chlorophyll b (Chl-b) in the supernatant were determined spectrophotometrically according to Lichtenthaler [18] method.

**Determination of Carotenoids:** The total carotenoids in *Sp. maxima* samples were determined spectrophotometrically at 450 nm according to AOAC standard methods [19].  $\beta$ -carotene served as a standard compound was used for preparing the calibration curve.

**Determination of Algal Tocopherols:** Tocopherols were determined by HPLC equipped with Spectra System UV2000 detector at 290 nm and separated on a 250×4.6 mm (i.d) column packed with Vydac and eluted with 90:10 acetonitrile: methanol (v/v) at a flow rate of 1 mL min<sup>-1</sup>. Standard of α-tocopherol was run under the same conditions [20].

**Extraction and Determination of Phycocyanin:** The concentration of total blue pigment phycocyanin was determined by spectrophotometrically at 280, 615 and 652 nm; respectively as reported by Silverira *et al.* [21]. Phycocyanin concentration (PC) and extraction purity (EP) were calculated by following equation: (PC) = OD<sub>615</sub> - 0.474 (OD<sub>652</sub>)/5.34 mg mL<sup>-1</sup> and (EP) = OD<sub>615</sub>/OD<sub>280</sub> respectively.

#### Chromatographic Analysis

**TLC Analysis:** Two μL of different organic extract solution (20 mg mL<sup>-1</sup>) was subjected to thin layer chromatography (TLC) on 10 ×20 cm glass plates, coated with 0.2 mm silica of gel 60 F254 (Merck, Germany) and allowed to dry for a few minutes, then developed with hexane/acetone (75:25, v:v). The separated compounds were located and identified by visualizing TLC plates either with solution composed of 1% FeCl<sub>3</sub>-1% K(CN)<sub>6</sub> and under UV and without reagents to identified chemical constituents.

**HPLC Analysis:** The chemical constituents in *S. maxima* extracts were identified by the HPLC method already reported [22], using Dionex Summit IV HPLC system consisted of a Dionex P680 dual gradient pump, a ASI-100 auto-sampler equipped with a 20- μL loop and PDA-100 photodiode array detector. A reverse phase column C18 (250×4.6 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 0.9 mL min<sup>-1</sup> according to a step gradient, lasting 35 min, which started from 25% B, changing at 50% in 1 min, rising up to 100% B at minute 10. 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 comparing with data appearing in the literature.

#### Determination of Antioxidant Capacity

**Rapid Screening of Antioxidant Capacity with TLC-autography Methods:** The TLC-autography technique was used for detecting antioxidant activity of COE-*Sp. m*, in which, the separated compounds on TLC plates were sprayed with a oxidizing solution of β-carotene/linoleic acid mixture (contained 9 mg β-carotene and 27 μL linoleic acid in 90 mL chloroform/ethanol, 2:1, v/v) and/or with 0.5 mmol/L 2,2-diphenyl-1-picrylhydrazyl (methanolic DPPH) radical to detect antioxidant active compounds as reported by Pratt and Miller, [23] and Jaime *et al.* [24], respectively. The protecting against the bleaching β-carotene gave orange spots and scavenging DPPH radical gave yellow colored spots [24] were taken as positive results.

#### β-carotene/linoleic Acid Bleaching in Aqueous Media:

The procedure is based on a previously reported method [25] with slight modifications. Five mg of β-carotene dissolved in 2 mL chloroform were added into flask containing 27 μL linoleic acid and 500 mg Tween-20, the solvent was evaporated under vacuum. Then, 250 mL ultra-pure water was added and emulsification was achieved by agitation in ultrasonic bath for 15 min. A 200 μL of each *S. maxima* extracts (containing 50-200 μg mL<sup>-1</sup> of 0.5 % Tween-20 solution) was added into flask containing 50 mL of reaction mixture, which were subjected to thermal auto-oxidation at 50±2°C for 7 h. The absorbance of 4 mL from reaction mixture at 20 min intervals was measured at 470 nm against blank, which were prepared by added 2 mL of chloroform to reaction mixture instead of β-carotene. All samples were assayed in triplicate. Antioxidant capacities of algal extracts were compared with those of BHT, BHA and β-tocopherol (200 ppm) and control. Inhibition of bleaching β-carotene (I %) was calculated as following:

$$I (\%) = (A_{\text{initial}} - A_{\text{sample}} / A_{\text{initial}}) \times 100,$$

where A initial is the absorbance at zero time and A samples is the absorbance after 2 h.

**DPPH• Free Radical-scavenging Assay:** The DPPH assay was carried out following the method of Tagashira and Ohtake [26]. The four different concentrations of the respective *S. maxima* extracts were added to 25 mL of 0.004 % DPPH radical. The reaction mixture were shaken vigorously and then kept in dark at 30±1°C. At 15 min

intervals up to 180 min, the absorbance of the resulting solution was measured at 517 nm, against blank. The differences in absorbance between a test sample and a control (methanolic DPPH) was considered as activity. The methanol was used to adjust zero and BHT, BHA and  $\beta$ -tocopherol (at 200 ppm) were used as reference standard. The radical scavenging activity of *S. maxima* extracts in the reaction mixture was calculated from a calibration curve at 517 nm. All tests were run in triplicate and averaged,

**Antimicrobial Activity:** Microorganisms For the antibacterial evaluation, strains from the Fisher Scientific Co. (Texas, USA) were used (*Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Micrococcus luteus*, *Klebsiella pneumoniae*, *Serratia marcescens*).

#### Antibacterial Testing

**Preparation of Inoculums:** The bacteria strains were inoculated on nutrient broth (Difco) and incubated for 24 h at  $30 \pm 0.1^\circ\text{C}$ . Adequate amounts of autoclaved Difco Agar medium were dispensed into sterile plates and allowed to solidify under aseptic conditions. The 0.1 mL of the test organisms was inoculated with a sterile swab on the surface of appropriate solid medium in plates and then was incubated at  $27^\circ\text{C}$  for 1 h.

**Antibacterial Assay:** The antibacterial activity was evaluated with the paper disk diffusion method [27]. Briefly, 6 mm paper discs were impregnated with 4-16  $\mu\text{L}$  of three different concentrations (1, 2 and 4  $\text{mg disk}^{-1}$ ) of the respective *S. maxima* organic extracts (25 mg of extracts  $0.1\text{ mL}^{-1}$  of Dimethylsulfoxide, DMSO) and allow to dried. The agar plates inoculated with the test organisms were incubated for 1 h. Then tested paper disks were applied on the plates and incubated at  $30 \pm 0.1^\circ\text{C}$  for 24-48 h. After incubation, all plates were observed for zones of growth inhibition and the diameters of these zones were measured in millimeters. The antimicrobial agent chloramphenicol (Sigma Chemical) was included in the assays as positive control (10 and 20  $\mu\text{g disk}^{-1}$ ). Inhibitory activity of DMSO was also tested. All tests were performed under sterile conditions in duplicate and repeated three times.

**Minimal Inhibitory Concentration (MIC):** The minimal inhibitory concentration tests were carried out according the methods of the European Pharmacopoeia [28]. MIC was defined as the lowest algal extracts concentration

showing no visible bacterial growth after incubation time for 24 h at  $37^\circ\text{C}$ .

**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.

## RESULTS AND DISCUSSION

Table 1 shows the specific growth rate ( $\mu_{\text{max}}$ ) and productivity  $P_{432}$  at 18 days for *S. maxima* grown at different  $\text{NaNO}_3$  concentration. The result revealed that concentration of  $\text{NaNO}_3$  ranged from 0.069 to 0.094 had significant influences on  $\mu_{\text{max}}$  and productivity, where among lower  $\text{NaNO}_3$  level ( $0.625\text{--}0.0\text{ g L}^{-1}$ ) did not show any significant affect of their growth parameters. This mean that concentration of nitrogen in media could be reduced to  $0.625\text{ g L}^{-1}$  media with out loss of productively this lead to decrease production cost in large-scale cultivation.

Some microalgae species is well known due to its ability to accumulated high amounts of carotenoids, phycocyanin and  $\alpha$ -tocopherol under different condition of stress. In several study, the affect *S. maxima* algae growth seems to be associated negatively with enhancement of carotenoids biosynthesis [6, 20]. Table 2, shows the influenced on N concentration the growth and the concentration of total carotenoids (TCOR),  $\alpha$ -tocopherol (TOC), total phenols compounds (TPC), total chlorophyll (T-Chl) and phycocyanin (Phy) of nutrient-deprived cultures of algae *Spirulina maxima* are compared. The nitrogen starvation led to accumulation of high amount of carotenoids and tocopherol, which was found in compatible with significant decreased of the biomass weight, growth rate and Phy, TPC and Chl contents. Thus, the concentration of sodium nitrate in Zarrouk's medium exhibited a significant effect on the production of all phytochemical constituents. For instance, the highest concentration of carotenoid contents was obtained in culture grown either at lowest ( $0.625\text{ g L}^{-1}$ ) or in free  $\text{NaNO}_3$  medium (zero  $\text{g L}^{-1}$ ), with values of 16.53 and  $20.0\text{ mg g}^{-1}\text{ (d w)}$ , respectively. In contrast, lowest TCR content ( $7.32\text{ mg g}^{-1}\text{ d w}$ ) was found in culture grown at highest  $\text{NaNO}_3$  ( $2.5\text{ g/L}^{-1}$ ). This finding has been shown by previous workers [13] that the production of carotenoids occurs in microalgae cells grown under specific conditions, including high light intensity and high concentration of salt coupled with

Table 2: Influence of sodium nitrate concentration on total chlorophylls, total carotenoids, total phenol compounds,  $\alpha$ -tocopherol and total phycocyanin contents in *Spirulina maxima*

Na NO <sub>3</sub> (g L <sup>-1</sup> ) concentrations	Total Chl. (mg g <sup>-1</sup> )	Total carotenoids (mg g <sup>-1</sup> )	Total phenol compounds (mg g <sup>-1</sup> )	$\alpha$ -Tocopherol $\mu$ g Kg <sup>-1</sup>	Total phycocyanin (mg g <sup>-1</sup> )
2.500	16.23 $\pm$ 1.24 <sup>a</sup>	7.32 $\pm$ 0.46 <sup>a</sup>	4.98 $\pm$ 0.25 <sup>a</sup>	0.266 $\pm$ 0.04 <sup>a</sup>	106.4 $\pm$ 2.15 <sup>a</sup>
1.875	14.35 $\pm$ 1.21 <sup>b</sup>	8.25 $\pm$ 0.35 <sup>b</sup>	4.23 $\pm$ 0.21 <sup>a</sup>	0.44 $\pm$ 0.01 <sup>b</sup>	92.9 $\pm$ 1.35 <sup>b</sup>
1.250	12.63 $\pm$ 1.11 <sup>c</sup>	12.35 $\pm$ 0.94 <sup>c</sup>	3.22 $\pm$ 0.22 <sup>b</sup>	0.501 $\pm$ 0.03 <sup>b</sup>	70.1 $\pm$ 0.14 <sup>c</sup>
0.625	8.12 $\pm$ 0.94 <sup>d</sup>	16.53 $\pm$ 0.98 <sup>d</sup>	3.55 $\pm$ 0.27 <sup>b</sup>	0.978 $\pm$ 0.7 <sup>c</sup>	55.7 $\pm$ 1.67 <sup>d</sup>
0.000	7.69 $\pm$ 0.54 <sup>e</sup>	20.00 $\pm$ 1.11 <sup>e</sup>	3.08 $\pm$ 0.25 <sup>b</sup>	1.32 $\pm$ 0.05 <sup>c</sup>	45.3 $\pm$ 1.23 <sup>d</sup>
LSD at level (P<0.01)	1.22	1.13	1.21	0.65	7.58

All values show mean of three replicates,  $\pm$  standard deviationValues are significant at (P< 0.01),  $\pm$ SDTable 3: HPLC profile of photosynthetic pigments in *Spirulina maxima* organic extracts as affected by different sodium nitrate concentrations

		Relative area peak (%)				
RF	Compounds	<i>S. mx.</i> COE grown in 2.5 g L <sup>-1</sup> NaNO <sub>3</sub>	<i>S. mx.</i> COE grown in 1.875 g L <sup>-1</sup> NaNO <sub>3</sub>	<i>S. mx.</i> COE grown in 1.250 g L <sup>-1</sup> NaNO <sub>3</sub>	<i>S. mx.</i> COE grown in 0.625 g L <sup>-1</sup> NaNO <sub>3</sub>	<i>S. mx.</i> COE grown zero NaNO <sub>3</sub>
3.46	Violaxanthin	27.02	0.31	1.26	0.64	2.02
4.50	Unknown	2.04	4.49	2.85	1.10	5.06
4.92	Unknown			0.14		9.81
5.20	Antheraxanthin	16.77	0.30	39.48	2.37	
5.70	Unknown		0.48			0.43
6.27	Unknown		1.12	1.08	3.32	2.57
6.46	Unknown					0.51
7.27	Astaxanthin	0.06	11.69	0.13	2.29	1.51
7.98	Lutein	0.03	1.08	0.30	1.10	2.93
8.62	Unknown				0.93	0.54
8.81	Zeaxanthin	0.04	1.16	0.16	1.02	
9.57	Cryptoxanthin	0.26	3.26	0.28	1.34	1.93
10.57	Chlorophyll a	10.19	2.56	6.34	14.83	12.87
11.22	Chlorophyll b	0.41	39.22	13.30	0.16	9.19
12.67	$\alpha$ -carotene	5.13	6.96	5.29	2.16	8.36
13.68	Carotene- isomers	0.55	0.50	0.60	0.94	35.97
15.10	9-cis- $\beta$ -carotene	35.75	22.90	24.16	63.77	0.14
15.63	Unknown	0.24	0.30	0.31	0.40	0.47
16.88	Carotenoids compounds	0.18	0.20	0.27	0.35	0.58
17.66	Unknown	0.27	0.37	0.36	0.54	0.22
18.55	Unknown	0.13	0.15	0.12	0.23	95.11
Total identified compounds		98.52	97.05	96.41	97.28	4.89
Unknown		1.48	2.95	3.59	2.72	

Rf refers to retention time

nutrient imbalance. It seems that the division of algae cells grown under N starvation are blocked, while photosynthesis continues, leading to storage specific compounds such as carotenoids and triglycerides. That accumulation of these compounds, do not require nitrogen for their biosynthesis and synthesizing enzymes also may be less susceptible to disorganization than in the system responsible for synthesis other compounds [12, 29].

*S. maxima* grown at lower N concentration (0.652 and zero NaNO<sub>3</sub> g L<sup>-1</sup>), characterized with lower cellular contents of TPC and T-Chl, with values was 3.55 and 3.08 mg g<sup>-1</sup> and 8.12 and 7.69 mg g<sup>-1</sup>, respectively. In contrast, the concentration of these were increased significantly in cells grown in media containing higher sodium nitrate level (2.5 g L<sup>-1</sup>) with values were 4.98 and 16.23 mg g<sup>-1</sup>, respectively. Thus, higher quantities of TPC and T-Chl are synthesized in *S. maxima* grown in media containing higher sodium nitrate level. For phycocyanin,

blue pigment at lower sodium nitrate levels (0.625 and 0 g L<sup>-1</sup>) *S. maxima* had lower concentration of phycocyanine with values 55.7 and 45.3 mg g<sup>-1</sup> as compared with that value 106.4 mg g<sup>-1</sup> in cell grown under higher sodium nitrate level (2.5 g L<sup>-1</sup>). This finding can explain with that the sodium nitrate is required for synthesis of the amino acid, which makes up proteins and other cellular components such as chlorophylls and phycocyanin [4, 9]. However, with regard to T-Chl, TPS and Phy, higher concentration of NaNO<sub>3</sub> resulted in an increase in these compounds, similar to that was obtained by Abd El Baky [9] and Colla *et al.* [4].

**Identification of the Chemical Composition of *Spirulina* Organic Extracts by Chromatographic Methods:** By using TLC and HPLC technique, chlorophyll-derived and many carotenoids compounds were identified in organic extracts of *S. maxima* grown in different culture medium (Table 3 and Fig. 1).

Tests were carried out in triplicate

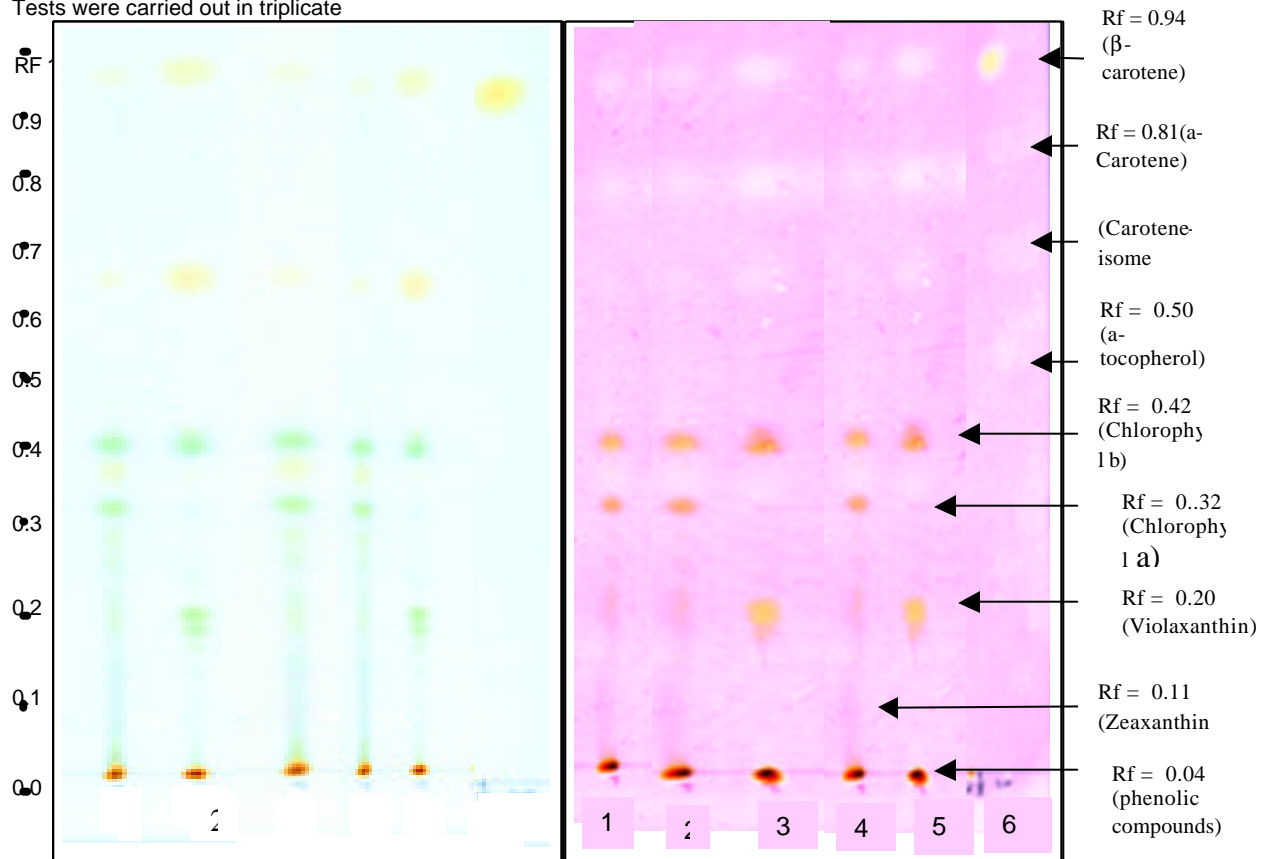


Fig. 1: (a) TLC chromatogram of *Spirulina maxima* organic extracts, (b) TLC chromatogram of *Spirulina maxima* organic extracts stained with DPPH $\cdot$ , stained with 0.004% DPPH $\cdot$  methanolic solution

1: *Sp. mx.* COE grown in 2.5 g L $^{-1}$  NaNO $_3$   
 2: *Sp. mx.* COE grown in 1.875 g L $^{-1}$  NaNO $_3$   
 3: *Sp. mx.* COE grown in 1.250 g L $^{-1}$  NaNO $_3$   
 4: *Sp. mx.* COE grown in 0.625 g L $^{-1}$  NaNO $_3$   
 5: *Sp. mx.* COE grown in 0.0 g L $^{-1}$  NaNO $_3$   
 6:  $\beta$ -carotene and  $\alpha$ -tocopherol reference

Table 4: Antibacterial activities (inhibition zone in diameter (mm) around the disks <sup>a</sup> and MIC<sup>b</sup>) of *Spirulina maxima* organic extract organic extract

			<i>S. mx.CO.E</i> grown					<i>Sp. mx.CO.E</i> grown				<i>Sp. mx.CO.E</i> grown				<i>Sp. mx.CO.E</i> grown				<i>Sp. mx.CO.E</i> grown			
Reference antibiotic			in 2.5 g L <sup>-1</sup> NaNO <sub>3</sub>					1.875 Na NO <sub>3</sub> (g L <sup>-1</sup> )				1.25 Na NO <sub>3</sub> (g L <sup>-1</sup> )				0.625 Na NO <sub>3</sub> (g L <sup>-1</sup> )				zero Na NO <sub>3</sub> (g L <sup>-1</sup> )			
10 20			2 4 8					2 4 8				2 4 8				2 4 8 MIC				2 4 8			
Microbactra strains	MIC		MIC					MIC				MIC				MIC				MIC			
	(μg/disk)	μg mL <sup>-1</sup>	(mg/disk)					(mg/disk)				(mg/disk)				(mg/disk)				(mg/disk)			
<i>B. cereus</i>	15	20	20	7	7	13	50	5	8	12	45	5	9	12	40	4	8	12	30	4	8	12	30
<i>B. subtilis</i>	16	21	20	5	7	13	50	4	7	13	45	4	8	12	40	4	8	12	30	5	7	11	30
<i>Kl. pneumoniae</i>	17	22	20	4	8	13	50	4	7	12	45	4	8	12	40	4	7	12	30	5	7	11	30
<i>M. luteus</i>	18	23	20	4	8	13	50	4	7	12	45	4	8	12	40	4	8	11	30	5	7	10	30
<i>S.marcescens</i>	13	18	20	4	8	13	50	4	7	11	45	4	8	12	40	4	8	11	30	5	7	11	30
<i>Staph. aureus</i>	14	19	20	4	9	14	50	4	7	11	45	4	8	12	40	3	7	11	30	5	7	11	30

<sup>a</sup>: Values represent the mean of three replicates and rebated three times<sup>b</sup>: MIC: Minimum inhibition concentration, values given as μg mL<sup>-1</sup> for samples and for chloramphenicol

The TLC chromatogram of organic extracts of *S. maxima* provided evidence for the presence of several coloring pigments bands. Of which, blue, red, orange, yellow green and others, corresponded to photosynthetic pigments includes: Phycocyanine, carotenoids and chlorophyll-derived were identified in all *S. maxima* cultures extracts. The main pigments zeaxanthin, violaxanthin, Chl-*a*, chl-*b* and  $\alpha$ - and  $\beta$ -carotene were found at different  $hR_f$  ( $hR_f = R_f \times 100$ ) values of 50, 18, 31, 41, 81 and 94, respectively. These coloring bands on TLC plates were found at in agreement with colors of main pigments described in *Spirulina* [6, 30]. In additions, phenolics compounds were located on TLC plates at the start line or at less than  $hR_f = 10$ , that bands gave a blue colored after spraying with either  $FeCl_3$ - $FeK_3(CN)_6$  and Folin-Ciocalteu reagents. However, the relative density of bands separated on TLC plates, showed that *Spirulina* coloring compounds were varied as resulted to N level in Zarrouk's medium.

The all organic extracts of *Spirulina* grown at different N concentration, were separated and identified by using HPLC-DAD, Table 4). Considerable variation was found among all *Spirulina* extracts. The HPLC profile of these extracts showed that a large number of pigments were present in significant relative percentage (% of total area). At low N levels,  $\beta$ - and  $\beta$ -carotene, carotene-isomers and Chl-*b* were the most abundant constituents in the *Spirulina*, as compared with that did in cellsgrown optimal N level. The higher proportions of carotenoids in *S. maxima* was obtained at lower N concentrations (1.25, 0.625 and zero g/l of NaNO<sub>3</sub>), than those obtained at optimal N level (2.5 g l<sup>-1</sup> of NaNO<sub>3</sub>). These results are in agreement to previous studies with *S. plantensis*, which indicate an inverse relationship between increase proportion carotenoids compounds and N concentration in growth medium [20].

**Antibacterial Activity:** There is a growing interest in isolating antimicrobial substances from cyanobacteria. Some species such as *Anabaena*, *Oscillatoria*, *Spirulina* and *Synechocystis* exhibited antimicrobial activity against some pathogenic organisms [3, 31]. In this study, the antibacterial activity of *S. maxima* organic extracts assayed against of six bacteria was qualitatively and quantitatively assessed by evaluating the inhibition zones, zone diameter and MIC values. Generally, the all *S. maxima* extracts were found to be effective against all tested bacteria and these antibacterial activity was found in a dose depended manner, this being in agreement with that found by Ozdemir *et al.* [3]. As shown in Table 4, the most susceptible bacterium was *K. pneumoniae* and *S. marcescens* to organic-extracts of *Spirulina maxima* with highest inhibition zone values ranged 4-13 mm at concentration was 2-8 mg/disk. It is interesting to note that all *S. maxima* organic extracts manifested similar degrees of susceptibility towards to both gram positive and gram negative bacteria. Thus, the antibacterial activity could be due to presence of some active component containing in all organic extracts in particular the lipophilic and phenol compounds. Organic extracts of *Spirulina maxima* grown at high N level (2.5 g/l of NaNO<sub>3</sub>) showed lower inhibition zone values ranged from 4 to 13 mm, compared to that values ranged from 4 and 12 mm to organic extracts obtained for *Spirulina* grown at low NaNO<sub>3</sub> level (0.652 and zero g l<sup>-1</sup>). On the other hand, all *Spirulina* extracts showed good potential of antibacterial activities against all of 6 bacteria with MICs ranged from 30-50 μg mL<sup>-1</sup>. Among all extracts of *S. maxima*, extract of cells grown at higher N levels (0.625 and zero NaNO<sub>3</sub> g L<sup>-1</sup>) were the most potent against all bacterium with MIC values of 30 μg mL<sup>-1</sup>, as compared to that value 40 μg mL<sup>-1</sup> for extracts of algal grown in free N media. The maximal inhibition zones and

MIC values for bacterium strains sensitive to chloramphenicol standard antibiotic, were in ranged of 13-23 mm and 20 µg mL<sup>-1</sup>, respectively (Table 4). Higher antibacterial activity of *Spirulina* could be explained by presence of some active component containing in the organic extracts in particular the lipophilic compound.

In this study, the data seemed to indicate that the antimicrobial activity was related to the amounts of lipophilic and lipid soluble phenol compounds containing in *Spirulina* organic extracts. These are in agreement with those published by Mundt *et al.* [31] and Ozdemir *et al.* [3], in that antimicrobial activity of microalgae against some pathogenic organisms could be due to its containing fatty acids and hydroxyl unsaturated fatty acids, glycolipid and phenol compounds. Hence, the non-polar extracts of some algae exhibited greater antimicrobial activity than polar one and their activity can be attributed to the presence of fatty acids and lipid-soluble phenolic-terpenoid compounds [32]. In previously paper we found that *Spirulina maxima* had a large amounts of total lipids rich in omega-6 polyunsaturated fatty acids (ω6-PUFAs), in particular linoleic (C18:2 ω6) and gamma linolenic (γGLA, C18:3) acids when grown under low N levels [6]. Hence, the organic solvent used in this work, is able to extractable all of these components. Therefore, these findings were generally confirmed higher antimicrobial activity found in *Spirulina* extracts. Furthermore, the differences in antibacterial activity among all *Sp. maxima* extracts may be correlated with total quantity of lipophilic and phenol-lipid soluble compounds in these extracts. Prindle and Wright [33] mentioned that the antibacterial effects of phenolic compounds are in concentration dependent. At low concentration, phenols affect enzyme activity, especially of those enzymes associated with energy production; while at greater concentrations, they cause protein denaturation. In addition, effect of phenol and/or fatty acids on microbial growth could be the result of the ability of these compounds to alter microbial cell permeability, permitting the loss of macro-molecules from the interior and could be also interact with membrane proteins causing a deformation in there structure and functionality as well as affecting in cellular activity or disturb genetic [31].

#### Antioxidant Capacity of Crude Organic Extracts *Sp. maxima*

**Preliminary TLC Assay:** The TLC screening assay of the antioxidant activity of organic extracts from *S. maxima* grown at different N level is choice as the rapid method for detect activity of natural compounds [34].

Table 5: Scavenging activity of *Spirulina maxima* organic extracts on DPPH•

Samples	Inhibition (%) <sup>a</sup>	IC <sub>50</sub> <sup>b</sup> (µg mL <sup>-1</sup> )
<i>Sp. mx.COE</i> grown in 2.5 g L <sup>-1</sup> NaNO <sub>3</sub>	60.33±1.12	30.00
<i>Sp. mx.COE</i> grown in 1.875 g L <sup>-1</sup> NaNO <sub>3</sub>	63.65±0.92	28.00
<i>Sp. mx.COE</i> grown in 1.250 g L <sup>-1</sup> NaNO <sub>3</sub>	71.22±1.11	26.00
<i>Sp. mx.COE</i> grown in 0.625 g L <sup>-1</sup> NaNO <sub>3</sub>	81.14±1.25	23.00
<i>Sp. mx.COE</i> grown in 0.00 g L <sup>-1</sup> NaNO <sub>3</sub>	75.36±1.45	22.00
TBA	93.00±1.68	13.80
BHT	95.58±1.87	14.60
α-Tocopherol	91.54±1.95	16.00
LSD at level (P<0.01)	2.36	2.14

a: Percentage of antioxidant inhibition was calculated from following equation:

$$\%a = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

Where A<sub>blank</sub> = absorbance of methanolic DPPH

A<sub>sample</sub> = absorbance of DPPH radical + samples

IC<sub>50</sub><sup>b</sup>: Concentration (µg mL<sup>-1</sup>) for a 50% inhibition was calculated from the plot of inhibition (%) against *Spirulina maxima* extracts concentration

#### Bleaching B-carotene

**Linoleic Acid-TLC (BC-TLC) Assay:** The separated organic extracts of *S. maxima* on TLC plats were sprayed with β-carotene/linoleic acid mixture, 2 h after, which the back ground was bleached, bands in a yellow color considered as antioxidant activate. As show in chromatogram, bands at *hR<sub>f</sub>* = 95, 81, 65, 41, 31 and >10 corresponded to lipid-soluble compound include: carotenoids and phenol compounds were appeared to have antioxidant activity.

**DPPH -TLC (DPPH-TLC) Assay:** The fast DPPH-TLC assay was used to detect the antioxidant activity of *Sp. maxima* organic extracts. In this assay, bands with antioxidant activity were able to reduce the stable radical DPPH to the pale yellow colored diphenyl-pirylhydrazyl on a violet background, within few min (<3 min). Bands at *R<sub>f</sub>* = 0.95, 0.81, 0.65, 0.41, 0.31 and 0.10 exhibited a potent DPPH radical scavenging. All organic extracts of *S. maxima* was found to have the approximately similar results as antioxidant. Therefore, antioxidant capacity coincides with carotenoids and phenols components containing in organic extracts of *S. maxima* grown under different levels of nitrogen (Fig. 1).

**Antioxidant Activity of *S. maxima* Organic Extracts in Aqueous Model System:** This set was conducted to derstanding of antioxidant activity of algal extract in biological system, because the lipid compounds such as unsaturated fatty acids are usually present in an aqueous medium in such system.

The antioxidant activity of *S. maxima* organic extracts in aqueous emulsion was evaluated by measured the rate of β-carotene bleaching method (Table 5 and Fig. 2). As



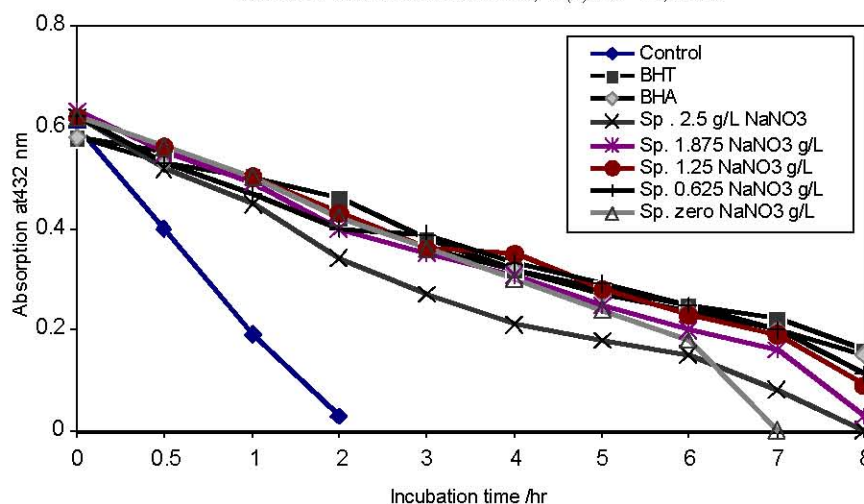


Fig. 3: Effect of *Spirulina maxima* organic extracts obtained from cells grown natural sea water (NSW) and artificial sea water (ASW) on bleaching under sodium nitrate concentrations on  $\beta$ -carotene coupled with autoxidized linoleic acid

compared with the control (without-antioxidant), all *Spirulina* organic extracts and BHT and BHA were able to inhibit the  $\beta$ -carotene degradation due to induction of free peroxy radicals ( $\text{LOO}^\bullet$ ) generated from linoleic acid auto-oxidation. Thus, these extracts exhibited antioxidant properties. In order to compare the antioxidative capacity of the *S. maxima* extracts values of the relative inhibition percentage (I %) of  $\beta$ -carotene oxidation was calculated. These values for extracts obtained from *S. maxima* grown in media containing 2.5, 1.875, 1.25, 0.625 and zero  $\text{NaNO}_3 \text{ g L}^{-1}$ , BHT and BHA (at  $100 \mu\text{g mL}^{-1}$ ) were 80.76, 85.57, 88.23, 85.85, 87.43, 92.18 and 89.58 %, respectively. By comparing among all values of inhibition %, the *S. maxima* organic extracts showed a significant antioxidant activity as that was found for commonly synthetic antioxidant BHT and/or BHA.

**Free Radical Scavenging Activity (FRSA):** Free radical scavenging activity (FRSA) of differ organic extracts of *S. maxima* and reference substance: BHA, BHT and  $\alpha$ -tocopherol for reduce the stable free radical DPPH are shown in Table 4. The strongest effect was observed for all organic extracts derived from *Sp* grown in media containing 2.5, 1.875, 1.25, 0.625 and zero  $\text{NaNO}_3 \text{ g L}^{-1}$ , with an  $\text{IC}_{50}$  value of 30.0, 28.0, 26.0, 23.0 and  $22.0 \mu\text{g mL}^{-1}$ , respectively. These values could be compared with the synthetic antioxidant: TOC, BHT and BHA, that possesses high inhibitory activity, with  $\text{IC}_{50}$  values 14.6, 13.8 and 16.0, respectively. In fact, the inhibitory activity of *S. extracts* was found to be closed to those of commercial antioxidant agent. Thus, crude organic

extracts of *S. maxima* showed strongest radical scavenging activity. This finding suggests that the presence of electron and/or hydrogen donating constituent in algal organic extracts such as phycocyanin, carotenoid and phenol compounds. However, the positive correlations between phenol, phytosterol and carotenoids contents in *Spirulina* and its antioxidant activity is well documented by Abd El-Baky [9]; Khan *et al.* [1] and Athukorela *et al.* [13]. Generally, the antioxidant results of inhibition of  $\beta$ -carotene bleaching and DPPH radical scavenging activity, it may be possible to find effective antioxidants compounds in *Spirulina maxima*. The total phenolics, phycocyanin, total carotenoids and chlorophyll-derived containing in the organic extracts might explain their high antioxidant activity. For instance, algal phenol compounds are effective antioxidant that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the inhibition or propagation of oxidizing chain reactions. The phenolics ( $\text{ArOH}$ ) easily transfer a hydrogen atom to lipid peroxy radical ( $\text{LOO}^\bullet$ ) and form the aryloxy ( $\text{ArO}^\bullet$ ), which being incapable of acting as a chain carrier, couple with another radical thus quenching the chain radical process ( $\text{ArO}^\bullet + \text{LOO}^\bullet \rightarrow \text{non radical products}$ ) [35]. In other words, antioxidant activity of phenol compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizer free radical, quenching singlet and triplet oxygen, or decomposing peroxides [36]. Similar antioxidant properties were reported for carotenoid compounds that is capable to scavenging of free radicals and quench singlet oxygen primarily by physical

mechanism [37]. In addition, phycocyanine, one of the major pigments in *Spirulina*, possesses significant antioxidant and radical scavenging properties as well as can inhibit lipid peroxidation mediated by ROS and could be act as chelating agent [1].

Finally, organic-extracts of *Spirulina maxima* grown at different N concentration possess strongest antibacterial and antioxidant properties when compared to commercial antibiotic (chloramphenicol) and synthetic antioxidants ( $\beta$ -Tocopherol, BHA and BHT). Thus, it can be suggested that the *Spirulina* organic-extracts may exert a better function in inhibit growth of pathogenic bacteria and in free radical scavenging and may be a promising alternative to synthetic substances as natural compound with high antibacterial and antioxidant activities and as food colorant as well as use as nutraceutical products. Their activities can be improved by changing the culture condition and the production of these agents may be promoted

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