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Egyptian *Arthrospira* phytosterols: production, identification, antioxidant and antiproliferative activities

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Abstract

Cultivation of microalgae as a source of phytosterol and other lipid compounds has gained more attention for commercial applications in pharmaceutical, cosmetic and food function industries. In this research, native *Spirulina maxima* SM from Egypt was grown in individual cultures containing various concentrations of nitrogen (N), phosphorus (P) and sulfur (S) elements in order to elucidate the effect of its elements on lipid and phytosterols production and to evaluate its antioxidant and antiproliferative activities. The results revealed that the SM was able to grow in different concentrations of testing elements S (from 0.3 to 2.4 mM), P (from 0.3 to 2.4 mM) and N (0.3 to 3.2 mM) with significant differences. A high potential for production of MS biomass, total lipid and phytosterol contents were obtained in individual cultures containing 0.6 mM N, 0.6 mM P and 0.80 mM, respectively. Therefore, these concentrations (combination of S+P+N element) were selected for cultivation of SM at large scale in a column photobioreactor (PBR 300 L) to induce sufficient SM biomass so that, we can obtain an adequate amount of total lipid and phytosterol contents. Phytosterols (PS) of native SM grown in the 300 L PBR were partially purified from unsaponified extracts of SM total lipid followed by its purification by crystallization process. The identification and quantification of PS profile were performed by GC-FID analysis. The results revealed high levels of campsterol, D^7-Avena sterol, β-sitosterol, stigmasterol and other compounds. These PS compounds showed marked *in vitro* superoxide, DPPH and ·OH radical scavenging activity, which was comparable with the results obtained with standard antioxidants BHA or α-tocopherol. Moreover, SM phytosterols exhibited anti-proliferative activity against three human cancer cell lines (MCF-7, Hep-G2 and HCT-116) with IC_{50} values less than 11.62 μg/mL as assessed by *in vitro* MTT colorimetric method. Thus, SM phytosterol may be considered as a potential natural source of promising ingredient in the future for a range of health applications for human, cosmetic industries and in functional food.

Keywords: antioxidant; antiproliferative; microalgae *Spirulina maxima*; phosphorus; phytosterols; sulfur and nitrogen limitation

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Introduction

The phytosterol compositions of the microalgae are structurally related to cholesterol, but differ from cholesterol in the structure of the side chain. Currently, phytosterols (C28 and C29 sterols) are playing an important role in nutraceutic, pharmaceutical and functional foods industries. Also, they are precursors of vitamin D2 and other bio-active molecules (Andrade *et al.*, 2018). Phytosterols (PSs) showed some therapeutic applications to treat hypercholesterolemia and have lowering effect on total and low-density-lipoprotein (LDL)-cholesterol in human and may have value in treating complications of diabetes and hypertension (Luo *et al.*, 2015; Abdul *et al.*, 2016). In addition, PSs may possess anti-inflammatory, anti-atherogenicity, anti-cancer and anti-oxidative activities and may provide protective effect against nervous system disorders such as autoimmune encephalomyelitis or Alzheimer's disease (Ahmed *et al.*, 2015; Koh *et al.*, 2018). The world market for PSs was estimated at 1000 tons per year, and the market is expanding, due to increase its application in cosmetic and pharmaceutical industries. In 2012, the phytosterols markets in European were about 196 million US$ and global market was about 300 million US$ and it is expected to increase by 7-9% annually (Borowitzka, 2013). Nowadays, the consumers are actively seeking products containing health-promoting ingredients such as phytosterols, phycocynin and polyunsaturated fatty acids, to improve the state of health or well-being and reduce the risk of disease (Abd El Baky *et al.*, 2014, 2016). Thus, research is focusing on identifying new natural sources of phytosterols and microalgae which have been demonstrated as a suitable alternative source of these functional compounds (Borowitzka, 2013; Andrade, 2018). However, some phytosterols (ß-sitosterol, campesterol and stigmasterol) are subject to the FDA health claim and it could be used in the food market, including breakfast cereals, cereal bars and milk (Luo *et al.*, 2015; Srigley and Haile, 2015).

Microalgae are the main source of phytosterols for zooplankton and fish and these compounds become increased in all arthropods which food quality greatly depends on the sterol composition of algal species in the zooplankton diet (Martin-Creuzburg and Von Elert, 2004). However, the biochemical composition of microalgae under normal conditions is characterized by a wide percentage of proteins (up to 60%), carbohydrates (up to 40%), lipids (up to 15%) and carotenoids (Abd El Baky and El Baroty, 2012, 2013). The biochemical flexibility (capacity of protein, lipids and pigments (include: chlorophyll, carotenoids and phycobilliproteins) accumulation is depending on the microalgae species and on the environmental and nutritional conditions (Abd El Baky *et al.*, 2009; El Baroty *et al.*, 2011). The main environmental factors influencing biosynthesis pathways of bio-molecules are light intensity, pH and salinity, while the nutritional factors have included the availability and source of nitrogen, carbon and iron (Abd El Baky and El Baroty, 2012, 2016; Shanthi *et al.*, 2018). However, microalgae have many advantages to be utilized for production of bio-molecules that possess high growth rate, the possibility of culturing them in non-arable lands and the use of less- and lower-quality water. In algae, research has focused on the diverse sterol composition of different species but there are few studies that deal with the influence of culture conditions on the sterol content or composition of microalgae. Gordillo *et al.* (1998) suggests that the phytosterol content of algae changes under different environmental conditions, such as nutrient sources or light intensity. As yet, however, simultaneous effects of nutrient availability on quantity and quality of sterol accumulation in *S. maxima* grown at large scale in photobioreactor have not been considered. Therefore, in this investigation, we examined the influence of adding various concentrations of phosphorus, sulfur and nitrogen elements on *S. maxima* growth medium on total lipid contents and phytosterol yield. The best concentration produces TL and PS was selected for cultivation of SM at large scale in photobioreactor (combination of S+P+N element, PBR 300 L) to induce and obtain highest values of total lipid and phytosterol PS contents. The PS composition, its antioxidant and antiproliferative activities were determined.

Materials and Methods

Algal source

Blue green algae, native *Spirulina (Arthropira) maximas* (SM) use in this study, were isolated from Egypt, and maintenance in our current culture collection, algae unit in Plant Biochemistry Department (it cited in EMCCN), National Research Centre, Giza, Egypt. The details of the SM strain and the protocol for the maintenance of pure culture in Zarrouk's medium (Zarrouk, 1966) as described previously (Abd El Baky *et al.*, 2006).

Reagents

All reagents and chemicals used in the experiments were of analytical grade and purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and were stored according to the vendor's instructions.

Cultivation of algae

Effect of phosphorus concentration on lipid accumulation and *Spirulina platensis* biomass

The SM was cultivated in Zarrouk's medium (Zarrouk, 1966). Phosphorus (P) was supplied as KH_2PO_4 with the serious concentrations of viz., 0.3, 0.6, 1.2 mM and 2.4 mM phosphorus into a different flask (2 L) containing 1.7 L Zarrouk medium. Aeration was accomplished using air pumps to achieve an air flow rate of 20 L/h. The cultures were gassed with 0.03% volume CO_2 in an air and temperature was maintained at 25 °C ±3 °C. The pH of all media was adjusted to 8.5. The cultures were illuminated with continuous 10 cool white fluorescent lamps (Philips 40 W) provided an illumination of 2500 lux. In all cultivated flasks, conductivity, salinity, pH and temperature were daily measured within 10 days of growth by Hanna (HI 09812-5) conductivity. The purity of cultures was periodically checked by microscopic observation following taxonomy guidelines. All solutions and glassware were autoclaved at 121 °C for 15 min prior to use.

Effect of sulfur concentration on lipid accumulation and SM biomass

The sulfur concentrations were adjusted to 0.20, 0.40, 0.80, 1.60 and 3.20 mM by supplementation with as potassium sulfate K_2SO_4 into a different flask (2 L) containing 1.7 L Zarrouk medium. All flasks were incubated at the same conditions as described previously.

Effect of nitrogen concentration on lipid accumulation and SM biomass

Effect of nitrogen, four concentrations of KNO_3 (0.3, 0.6, 1.2, and 3.2 mM) were maintained in flask (2 L) containing 1.7 L Zarrouk medium. All flasks were incubated at the same conditions as described previously.

Cultivation of S. maxima at large scale

The SM was cultured in Zarrouk's medium containing a combination of nitrogen, sulfur and phosphorus limitation (0.6 mM N + 0.6 mM P + 0.80 mM S) in 300 L column photoreactor.

The cultivation of SM culture in photobioreactor was achieved at the same conditions as described previously.

Growth measurements and harvesting

The growth rate of SM was monitoring every three days through the entire cultivation period by determining the cell dry weight (dw, gravimetrically) and by optical density at OD 560 nm. A good linear relationship between the biomass DW concentration and the OD 560 nm was recorded. The cells were harvested at the stationary phase (10 days), by centrifugation at 10,000 x g (4 °C) for 15 min and the cell masses were stored at -20 °C until analysis. All analytical determinations were performed in triplicate and the mean values were recorded.

Determination of lipid content

Lipid extraction

The harvested cells (10 g) were washed several times with deionized water, lyophilized for lipid extraction (Bligh and Dyer, 1959). The total lipids were extracted with chloroform-methanol (2:1, v/v), and then separated into chloroform (bottom layer) and aqueous methanol layers by the addition of methanol and water to give a final solvent ratio of about chloroform: methanol: water of 1:1:1. The organic (chloroform) layer was cleaned using a saturated NaCl water solution and the chloroform phase was recovered using a separator funnel. The chloroform phase was dried with sodium sulfate overnight, and evaporated to dryness under reducing pressure. Purified lipids were measured gravimetrically and stored at -20 °C under nitrogen gases to prevent of lipid auto-oxidation or used directly for subsequent analysis.

Extraction of phytosterols from MS oil

The total lipids were saponified by refluxing with 5% (w/v) KOH methanol/water (4:1, v/v) solution for 3 hrs. After addition of 2 volumes of distilled water, unsaponified in the combined solution was then extracted four times with 25 mL of di ethyl ether. The ether phases were then combined, dried with sodium sulfate overnight, filtered and evaporated to dryness. The residue weighted after drying to constant weight.

Separation of Spirulina maxima phytosterols

Total sterols from unsaponified material were semi purified by crystallization process as described by Chuanphongpanich *et al.* (2006).

Crystallization and filtration

A portion of the unsaponified material (USM, 0.2 g) was dissolved in the minimal volume of hot ethylene dichloride and then subsequent freezing to precipitate sterol fractions. The resulting crystals of steroid fraction were collected by vacuum filtration. When all the crystals have been transferred, they were washed with a small amount of the cold ethylene dichloride and the solvent was completely removed in a vacuum. The sterol yield was 0.16 g (80% of the USM).

Recrystallization

The crystals (0.1 g) were dissolved in hexane (20 ml), then a volume of water (10 ml) was added to react with the sterols (one water molecule per two sterol molecules) to form a semi hydrate slurry, which is insoluble in hexane, and will form a precipitate. The resulting slurry, of which seemed to be phytosterols fraction were obtained and it is probable that there are several sterols present in this SM. The fraction was filtered to separate the crystals from the solution. The crystals of steroids fraction were filtered and washed with cold hexane and the solvent was completely removed under N gases. The sterols yellow crystals yield was 0.072 g.

Identification of Spirulina maxima phytosterols

The steroid of SM was analyzed with an HP 5890 gas chromatograph equipped with FID detector and DB-5 capillary column (30 m, 0.25 mm (5%-phenyl) - 95%-methyl-polysiloxane, 0.25 um film thickness, 28 0°C temperature injector and 300 °C temperature transfer line. The oven temperature was programmed as follows: initial temperature 100 °C for 2 min, increase 10 °C/min up to 300 °C and then hold (isothermal) at 300 °C for 20 min. Helium was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The identification of sterols was based on the comparison of their retention times relative to authentic sterols cholesterol (5a-cholesten-3β-ol), β-sitosterol (5-cholesten-24β-ethyl-3β-ol), campesterol (5-cholesten-24-β-methyl-3b-ol), ergosterol (24 β-methyl-cholesta-5,7,22-trien-3ß-ol) and stigmasterol (5,22-cholestadien-24- ethyl-3β-ol) represented the main phytosterols) standards (Aldrich-Sigma, purity > 98% by GC). The relative percentage (%) of each component was evaluated by comparing its average peak area to the total areas. Moreover, the identification of SM phytosterols was confirmed based on steroids the elution order in the literature data

steroids as following order: cholesterol < brassicasterol < campesterol < campestanol < stigmasterol < sitosterol < sitostanol < Δ5-avenasterol (Winkler-Moser, 2018).

Antioxidant activity

DPPH radical scavenging assay

The SM phytosterolss was assayed with DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, previously dissolved in methanol. Methanolic solution of DPPH radicals was freshly prepared at a concentration of 0.02%, this solution was stable for more than 2 h. For assay, in test tube 40 µl was added to 1 ml conained series concentration of 1.0 to 32.0 ug/ml of MS–PS and was vortexed for 20 s and incubated in the dark at ambient temperature 30 °C ± 2 °C. Against a blank of pure methanol without DPPH, the decrease in absorption at 515 nm was measured in 1-cm quartz cells after 30 min of mixing using UV/ visible spectrophotometer (Thermo, USA). The inhibition percent was calculated from the following equation:

Inhibition % = $(A_0 - A1) / A_0) \times 100$ (Equation I)

Where: A_0 is the absorbance of control and A1 absorbance of the test. The obtained dose response curves were used to calculate the IC_{50} value, which is the effective concentration at which the antioxidant activity is 50%.

ABTS radical scavenging assay

ABTS radical scavenging activity was determined according to the Re *et al.* (1999) method. The ABTS solution was prepared and stored in the dark at room temperature for 16 h. The working ABTS solution was prepared by adding 1 mL of the solution was diluted with 40 mL deionized to give an absorbance equal to 0.70 ± 0.02 at 734 nm. To 100 µL ABTS solution, 400 µL test samples SM-PS at serial concentrations (2 to 64 µl/ml) were added.

After 10 min, the absorbance of the solution was read at 734 nm. The scavenging capability of ABTS was calculated using the following equation: (Equation I).

Hydroxyl radicals $HO^{\cdot}$ radical-scavenging activity

Hydroxyl radicals were generated by a Fenton reaction ($Fe3^{+}$- ascorbate-EDTA-H_2O_2 system), and the scavenging capacity towards $HO^{\cdot}$ radicals was determined spectrophotometrically at 532 nm by using deoxyribose method (Halliwell *et al.*, 1987). The negative control without any antioxidant or test sample (SM–PS) was considered 100% deoxyribose oxidation. The % hydroxyl radical scavenging activity of test sample was determined accordingly in comparison with negative control. BHT and alpha-tecopherol was taken as the positive control. The inhibition % was expressed, according to the following equation: (%) = $[A_0 - (A1 - A2)]/A_0 \times 100$, where: A_0 is the absorbance of the control without a sample, A1 is the absorbance in the presence of the sample and deoxyribose and A2 is the absorbance of the sample without deoxyribose.

Superoxide radical scavenging assay

The superoxide $O^{\cdot -}$ radicals generated from the photo reduction of riboflavin was detected by Nitro blue tetrazolium (NBT) reagent (contained EDTA (0.1 M), 0.0015% NaCN, riboflavin (0.12 mM), NBT (1.5 mM)). In test tube, the NPT mixture and various concentrations of tested sample (SM-PS) and phosphate buffer (67 mM, pH 7.8) were added in a total volume of 3 ml. The tubes were uniformly illuminated for 15 min and optical density was measured at 530 nm before and after the illumination (McCord and Fridovich, 1969). The percentage inhibition was calculated by using Equation (I). The BHT and alpha-tocopherol was used as a positive control. All experimental procedures were performed in triplicate and their mean values (standard deviation) were given.

Antiproliferative activity of SM phytosterols

Cell culture

The antiproliferative effect of SM was investigated on three human cancer cell lines MCF-7 (breast adenocarcinoma cells), Hep-G2 (hepatocellular carcinoma cells) and HCT-116 (colon carcinoma cells) were provided by the Cambrex BioScience (Copenhagen, Denmark). Antiproliferative of the prepared steroid of SM and etoposide (positive control, 5 -25 ug/ml) samples on the cancer cells was determined using MTT (-3(4, 5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) *in vitro* method. The cancer cells were cultured in DMEM (Dulbeco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/l), penicillin G sodium (100 U/ml), streptomycin sulfate (100 units/ml), amphotericin B (250 ng/ml) and maintained at 37 °C with 5% CO_2 in a humidified atmosphere of 95% air. For sub-culturing, monolayer cells were harvested after trypsin/EDTA treatment at 37 °C. Cells were used when confluence had reached 75%.

Cytotoxic assay

Cells ($0.5X10^5$ cells/ well) were seeded in 96-wells microplates, and treated with 20 µl of SM solution (DMSO, dimethyl dimethylsulphoxide) at concentrations ranging from 1.0 - 100 µl/l; subsequently the cells were incubated for 48 h at 37 °C. After incubation, cell suspension was mixed with 2 µl of MTT (5mg /ml of MTT in 0.9% NaCl) solution/ well and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 µl of acidified isopropanol (0.04 N HCl in absolute isopropanol) / well and plate was shacked at room temperature. Viable cells were determined by the absorbance at 570 nm with a microplate reader (BioRad, Richmond, CA). Measurements were performed in three independed assays, using five replicates for each assay and the concentration required for a 50% percent inhibition of viability (IC_{50}) was determined.

Statistical analysis

All measurements were carried out on samples drawn in triplicate and the data presented are mean of three replicates (±SD)standard deviations. Statistical analyses were performed using one-way analysis of variance ANOVA, and the significance of the difference between the means was determined by Duncan's multiple range procedure. A p-value ($P < 0.05$) less than 0.05 were regarded significantly different.

Results and Discussion

Recently, several research initiatives have proven that *Spirulina* sp**.** microalgae biomass appear to be the one of the promising sources for the technological production of value-added phycocyanine and other fine chemicals (Colla *et al.*, 2007). To enhance the feasibility of microalgae as a source of lipid rich in ώ-3 and steroids, many reports have searched for microalgae species which not only have high biomass productivity, but also have high lipid content, when cultivated under define conditions, such as high iron concentration, nitrogen and phosphate limitation and salt stress (Abd El Baky *et al.*, 2016, 2009).

Effect of phosphorus (P) concentrations on SM growth and total lipid content

The impact of various phosphorus concentrations (P, 0.3, 0.6, 1.2 mM and 2.4 mM) on the biomass (DW), total lipid productivity and total lipid contents of SM cells grown in Zarrouk's medium for 15 days are shown in Table 1. The values of these parameters were significantly different ($P>0.5\%$) among all cultures, with various degrees. The maximum biomass concentration (DW) of 2.76 ±0.21 DW g L^{-1} was obtained at 2.4 mM P, followed by 1.87 ± 0.12 g L^{-1} at 1.2 mM P. At low P (0.3 mM) concentration, the lower DW was obtained with a value of 0.754 ± 0.08 DW g L^{-1}. Trend of biomass yield in SM cells was almost linear with increasing P in growth medium. However, Celekli *et al.* (2009) observed that phosphate concentration more

than 0.5 g /l causes a decrease ($p < 0.01$) in biomass production in *S. platensis* cultuers. Chiu *et al.* (2009) found that optimal growth potential of *Chlorella* sp. and *N. oculata* was recorded at 2 mM P concentration. Whereas, in *D. terticlecta* culture the higher growth rates were obtained at 1.6 m MP (Tang *et al*., 2010).

As shown in (Table 1), the total lipid content (19.13 - 39.22 %, w/ DW) in SP cells showed increasing trends with the decreased of P level in growth medium. The TL contents in culture cultivated at 2.4, 1.2, 0.6- and 0.3-mM phosphorus were found to be 19.13%, 27.98 % and 39.22%, 35.14%, respectively. Thus, highest accumulation of TL (39.22%, w/w) and lipid yields (0.659 g/L) were obtained at 0.6 mom, this could attribute to high microalgae growth and contribute to more biomass as nitrogen source. These data were in accordance with the results of (Tang *et al*., 2011) who reported that the accumulation of TL was enhanced as resulted to changing the phosphorus concentration to be 0.6 mM.

Effect of sulfur concentration on SM growth and total lipids

As shown in Table 2, the DW biomass of cultures supplemented with the 160 mg L^{-1} sulfur concentration (SCs, 3.09 ± 0.32 g mL^{-1}) was higher than that obtained in cultures supplemented with 20, 40 and 80 mg L^{-1} SCs (1.75 ± 0.14, 2.32 ± 0.17 and 2.67 ± 0.23 g L^{-1}). Total lipid contents (TL) and lipid yield (LY) (in parentheses) of SM cells showed an increased trend with increasing sulfur concentration in a nutrient medium (Table 2). The maximum TL was 34.29% (0.916 g/L) was obtained at 80 mg L^{-1} sulfur, followed by 30.74% (0.713 g/L) at 40 mg L^{-1} sulfur (0.472 g/L) at 20 mg L^{-1} sulfur , then 18.43% (0.569 g/L) at 160 mg L^{-1} sulfur. In general, the levels of TL and lipid yield (LY) in algal culture supplemented with S concentrations range between 20 - 160 mg L^{-1} significantly ($P<0.05$) increased with sulfur limitation. Liu *et al.* (2008) reported that a significant increase in lipid content (20-50%, dw) occurred in many microalgae after being subjected to phosphate and sulfur depletion, which the metabolic pathways could be modified toward lipid biosynthesis.

Table 1. Effect of phosphorus concentrations on lipid content and biomass of *Spirulina maxima*

Phosphorus conc. (mM)	Biomass (dry weight g/L)		Lipid content (%)	lipids yield (g/L)
0.3	0.75	±0.08[a]	35.14±1.41[c]	0.265±0.057b
0.6	1.68	±0.11b	39.22±1.98[d]	0.659±0.076[d]
1.2	1.87	±0.12c	27.98±0.697[b]	0.523±0.087a
2.4	2.76	±0.21d	19.13±0.576[a]	0.528±0.077c

Each value represents the mean of three replicates and based on dry weight
All values are significant at ($P< 0.5$).

Table 2. Effect of sulfur concentrations on lipid content and biomass of *Spirulina maxima*

Sulfur conc. (mM)	Biomass (dry weight g/L)		Lipid content (%)	Lipids yield (g/L)
0.20	1.75	±0.14[a]	26.98±1.04[b]	0.472±0.068[a]
0.40	2.32	±0.17[b]	30.74±1.34[c]	0.713±0.085[c]
0.80	2.67	±0.23[c]	34.29±1.22[d]	0.916±0.078[d]
1.60	3.09	±0.32[d]	18.43±0.97[a]	0.569±0.061[b]

Each value represents the mean of three replicates and based on dry weight
All values are significant at ($P< 0.5$).

Effect of nitrogen concentration on SM growth and total lipids

Nitrogen starvation approaches were as a main factor affecting for accumulation of high lipid content in several microalgae species (Abd El Baky and El Baroty, 2013, 2016). As shown in Table 3, the DW biomass of cultures supplemented with the 2.4 mM nitrogen concentration (2.982 g mL-1) was higher than that obtained in cultures supplemented with 0.3, 0.6- and 1.2-mM nitrogen concentration (1.54, 1.89 and 2.59 g L-

1). Total lipid contents (TL) and lipid yield (LY) (in parentheses) of SM cells showed an increased trend with increasing N concentration in a nutrient medium (Table 3). The maximum TL were of 44.65% (0.647 g/L) was obtained in 0.3 mM N culture, followed by 43.22% (0.816 g/L) in 0.6 mM (0.793 g/L) at 1.2 mM N culture, then 12.45% (0.371 g/L) in 2.4 mM N culture. In general, the levels of TL and LY in microalgae culture cultivated in 0.3-2.4 mM N-medium significantly ($P<0.05$) increase with N depletion. However, a possible reason for this could be that N ions are essential for the cell growth and membrane biosynthesis; therefore, the metabolic pathway could be shift from the protein biosynthesis to lipid biosynthesis (Liu *et al*., 2008; Abd El Baky and El Baroty, 2017).

Table 3. Effect of nitrogen starvation on lipid content and biomass of *Spirulina maxima*

Nitrogen conc. (mM)	Biomass (dry weight g/L)	Lipid content (%)	Lipids yield (g/L)
0.3	1.54[a]	44.65[c]	0.647[b]
0.6	1.89[b]	43.22[c]	0.816[d]
1.2	2.59[c]	30.65[b]	0.793[c]
3.2	2.98[d]	12.45[a]	0.371[a]

Each value represents the mean of three replicates and based on dry weight
All values are significant at ($P< 0.5$).

Influence of combination of phosphorus, sulfur and nitrogen limitation on the total lipid content and phytosterol contents of SM grown in 300 L photobioreactor (a large scale)

To our knowledge, this is the first report of Egyptian native microalgae, identified as S. maxima cultivated under artificial light and in medium containing three limited concentration of P+S+N, in 300L closed column photobioreactor (PBR, at large scale) to produce sufficient biomass to obtain an adequate amount of phytosterols compounds to perform biological assays. Data in Table 4 indicate that the P+S+N limitation in the nutrient growth medium led to an increase in the lipid content. However, total lipids and phytosterols content in SM were 47.33 and 5.54% (dw), respectively. Therefore, under combination of three (P+S+N) elements condition, the S. maxima produced higher amount of phytosterols (5.54% of lipid) content than that did in individual element cultures. However, in over all, our results representing that an increase in P, S and N concentrations (over than 0.6, 0.6, 0.8 mM) in growth medium resulted in a significant decrease in total sterols yield. However, no report on the effect of nitrogen and phosphorus levels in growth of on sterol accumulation in Spirulina species. Ahmed *et al.* (2015) reported that no significant change in sterol content could be found at high nitrate or phosphate levels or even at nitrate- and/or phosphate-deprived conditions. On the other hand, interaction effects was found between the light and nutrient concentrations (e.g. phosphorus and/or silicate) on phytosterol content in Scenedesmus and Cyclotella species, that the higher phytosterol content was obtained in cultures grow in low light intensity coupled with low P content than that made in high P treatment (Piepho, 2010).

Our results corroborate with that reports on a positive interaction between KNO_3 concentrations with an increase of biomasses in several Spirulina species (Colla *et al*., 2007). Generally, Spirulina maxima cells can be manipulated the lipid content which yields a maximum value of about 47.33% in SM cells grown at phosphorous, sulfur and nitrogen limitation. Also, the results revealed that SM oil containing significant amounts of phytosterols compounds and may help for health promotion and disease prevention.

Table 4. Effect of nitrogen, phosphorus and sulfur starvation on lipid and phytosterols content and biomass of *Spirulina maxima* grown in photobioreactor (300 L)

Nitrogen, phosphorus and Sulfur limitation	Biomass (dry weight g/L)	Lipid content (%)	lipids yield (g/L)	Phytosterols (%)
0.6 mM N + 0.6 mM P + 0.80 mM S	2.76±0.21	47.33±1.32	1.31±0.14	5.54±0.42

Each value represents the mean of three replicates and based on dry weight

Identification of the phytosterol content in SM grown in photobioreactor

Phytosterols (PS) of SM grown in 300 L PBR were partially purified from unsaponified extract of SM total lipid and followed purified by recrystallization process. The GC/FID method employed in the present study achieved a good separation of β-sitosterol, stigmasterol, campesterol and D7-Avena sterol which are among the important phytosterols. β-Sitosterol, D7-Avena sterol and other phytosterols in SM grown under the combined element limitation (Table 5). The yields (mg/g dw) and relative percentage (%, of the sterol fraction) of the stagmasterol with value of 1.01 mg/g dw cells and 6.44%, respectively followed by campesterol 3.16 mg/g dw cells and 21.11%, respectively, then b-Sitosterol (7.72 mg/g dw and 49.23%), D7-Avena sterol (1.62 mg/g dw and 12.12%) and ergosterol (0.91 mg/g dw and 5.23%) were also presence a trace phytosterols compounds. Interestingly, a significant variation in quantity of phytosterols content in SM cell was appeared. However, phytoesterol structurally similar to cholesterol and an integral component of algae cell membranes, which playing an important role in the cell stability.

Table 5. Identification of *Spirulina maxima* phytostreol

Steroids	Relative content mg/g (of steroids extract)	Relative (%[a])
Campesterol	3.16	22.42
Stigmasterol	1.23	7.56
Cholesterol	1.01	6.44
Beta-Sitosterol	0.91	5.23
D^7-Avena sterol	7.2	49.23
Ergosterol	1.62	12.12

[a]Sterol content is expressed in % of total sterol fraction

Our results revealed that the b-Sitosterol and D^7-Avena sterol was the major phytosterol in SM grown at algae scales in tubler BPR. These results were comprabole with that reported in several previous studies. In early report on phytosterols in *Spirulina maxima* (blue-green algae,), were by Nadal (1971) who isolated cholesterol and sitosterol as the major phytosteols compounds**.** Paoletti *et al.* (1976) found that in three *Spirulina* species, 24 ethylcholest-5-ene-3b-ol (sitosterol or clionasterol) was the predominant sterol and with a relatively significant amount of cholesterol (3.5 - 14%), on the contrary, in green algae. 24-Ethylcholesterol (sitosterol or clionasterol, 79.5%) and cholesterol (8.5%) was identified as a predominated phytosteroids compound in *Spirulina maxima* (Rzama *et al.*, 1994). However, despite the differences in the relative amounts in sterol composition among blue green algae, *Scenedesmus* and *Chlorella* species, and it is often found that two or three particular sterols dominate ((sitosterol and ergosterol) (Paoletti *et al.*, 1976; Rzama, 1994).

The sterol content in several blue green algae was ranged between 0.003 and 0.025% of dry algal biomass (Razama, 1994). Francavilla *et al.* (2010) reported that the yields of total sterols were 1.3% and 0.89% of dw in *D. tertiolecta* and *D. salina*, respectively, when grow at 0.6 M NaCl salinity. However, microalgae biotechnology has development due to novel production technologies which allow obtaining high biomass productivities (Chisti, 2007) and major yields of favorite bioactive compounds (Molina Grima *et al.*, 2003).

On the other hand, the production of phytosterols could be considered as an added-value for *Spirulina* sp. biomass, which could be cultivated at large scales for production of crude lipids (rich in w3 linolenic acid), proteins, vitamins and also other high added-value byproducts, including phycocynin, carotenoid and antioxidant compounds (Abd El Baky and El Baroty, 2010, 2015) as well as they can reduce atmospheric CO_2 (CO_2 bio-mitigation) applying as model of biorefinery (Bai *et al.*, 2011). Hence, production and extraction of high-value phytosterols accompanied the lipid products can potentially offset the high cultivation and production costs and may make *Spirullina* biomass economic for application is feasible.

Antioxidant activity

Oxidative stress plays a significant pathological role in induction of many human diseases (Nithiya *et al.*, 2011). Thus, antioxidants are extensively studied for their capacity to protect organism and cell from oxidative damage to biomolecules like DNA, lipids and proteins that play role chronic diseases such as emphysema, cirrhosis, cancer, coronary heart diseases and brain dysfunction (Abd El Baky *et al.*, 2009).

Antioxidant activity of SM steroids by scavenging radical assays

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in food and biological systems. The reduction of $^{\cdot}OH$, ABTS, DPPH and $O^{\cdot -}$ is indicative of the capacity of the SM steroids sample to scavenge free radicals, independently of any enzymatic activity. Table 6, illustrates the DPPH, ABTS, $^{\cdot}OH$ and superoxide radical scavenging ability of steroids and antioxidant standards (BHT and alpha-tecopherol). Steroids fraction of SM showed excellent DPPH, ABTS, OH and superoxide radicals scavenging activity with dose dependent and IC_{50} values were 25.73 ± 1.42, 15.24 ± 1.44, 21.11 ± 1.18 and 19.13 ± 1.34 μg/ml, respectively. Thesevalues for BHA and alpha-tecopherol (in parentheses) were 16.11 ± 1.33 (14.11 ± 2.22), 11.67 ± 0.87 (11.18 ± 0.56), 12.44 ± 0.88 (14.21 ± 0.42) and 14.11 ± 0.91 μg/ml (16.34 ± 0.54 μg/ml), respectively. However, the efficiency of antioxidant capacity was found to be dose depending on manner and on scavenging of radical mechanisms. Based on IC_{50} values, for scavenging of ABTS, antioxidant efficiency of SM phytosterols was close to that recorded for BHA and alpha-tecopherol standard antioxidants Their result also showed that SP steroids possessed higher scavenging activity in ABTS (act as electron donors) assay than the DPPH (act as hydrogen donating) assay at all different doses used. As shown in Table 5, the four radical systems used for antioxidant evaluation of SM phytosterols showed differences in antioxidant activity could be due to the different mechanisms in radical scavenging. Yu *et al.* (2002) reported that two or more radical systems are required to assess the radical-scavenging activity of antioxidant. However, scavenging (O_2•-, OH•, LOO•) of free radicals is a potential health interest, that play an important role in the progression of many diseases and pathological disorders, such as atherosclerosis, cataracts and Alzheimer's and Parkinson's diseases, diabetes (type 2) and some types of cancer (Wojcik *et al.*, 2010 and Bachiega *et al.*, 2016). The phytosterols b-sitosterol, stigmasterol, campesterol showed an antioxidant action against oxidation of methyl linoleate in aqueous solution. Its effect due to suppressed the auto-oxidation chain reaction of lipid materials and thereby inhibiting the propagation and promoting termination reactions (Yoshida and Nika, 2003). Vivacons and Moreno (2005) also suggest that phytosterols (b-sitosterol) are responsible for the preventive effects on the development of diseases due to its antioxidant effect. On the other hand, Gordon *et al.* (1983) reported that the phytosterols, particularly 5-avenasterol act as antioxidants and as antipolymerization agents in frying oils.

Table 6. *In vitro s*uperoxide, DPPH and OH radical scavenging activity of *Spirulina maxima* phytosterols, BHA and α-tocopherol

Assay	IC_{50} (μg ml^{-1})			LSD (P< 0.05)
	BHA	ß-Tocopherol	*SM phyto*sterol	
DPPH· scavenging radical)	16.11 ±1.33	14.11 ±2.22	25.73 ±1.42	1.86
ABTS scavenging radical	11.67 ±0.87	11.18 ±0.56	15.24 ±1.44	1.52
OH· scavenging radical	12.44 ±0.88	14.21 ±0.42	21.11 ±1.18	1.78
Superoxide scavenging radical	14.11 ±0.91	16.34 ±0.54	19.13 ±1.34	1.95

Table 7. Antiproliferation activity of *Spirulina maxima agtensis* steroid against three model's human cancer cell lines and Paclitaxel standard anticancer drug

[a]Human cancer cell lines	IC_{50} μg /ml	
	SM phytosterols	[b]Paclitaxel
MCF-7	5.49	0.45
HepG2)	11.42	0.52
HCT-116	6.68	0.35

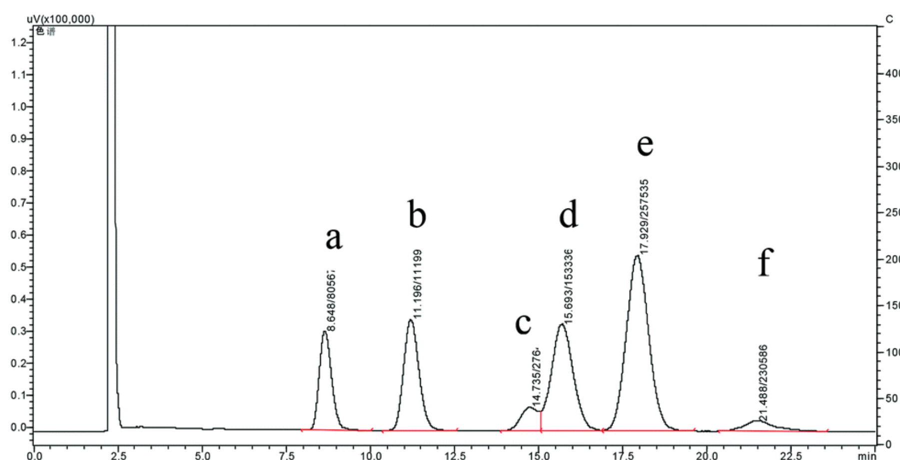

Figure 1. GC/FID Identification of *Spirulina maxima* phytosterol

In vitro antiproliferative activity

The phytosterols of SM cells grown at large scale in photobioreactor (300 L, P+S+N) were tested in vitro for its potential human cancer cell growth inhibitory effect on three (MCF-7, Hep G2 and HCT-116) cancer cell line using MTT assay, that assay is widely used to quantify cell viability and proliferation (Table 7). The incubation of three cell lines with increasing concentration of SM steroids caused a gradual inhibition of cell growth as concluded from its low IC_{50} value of 5.49, 11.42 and 6.68 μg/ml, respectively. The IC_{50} value of Paclitaxel anticancer drug against tested cell lines was 0.45, 0.52 and 0.35 μg/ml, respectively. The anticancer action of SM phytosterols (containing ß-sitosterol, campesterol and stigmasterol) may be compatible with the potential inhibitory effect of phytosterols compounds against breast, colon and prostate human cancer cell lines (Cilla *et al.*, 2015). Baskar *et al.* (2010) and Jayaprakasha *et al.* (2010) found that ß-sitosterol (from sour

orange) induced a significant dose-dependent growth inhibition of colon 320 cells and HT-29 cells by inducing apoptosis action throughout scavenging of reactive oxygen species. Also, ß-sitosterol significantly inhibited the growth and induced apoptosis in SGC-7901 human stomach cancer cells (Zhao *et al.*, 2009). In addition, phytosterols isolated from evening primrose oil and its main components ß-sitosterol and campesterol showed a reduction in cell proliferation, apoptosis induction and arrest the cell cycle of colon cancer HT-29 (Montserrat-de la Paz *et al.*, 2015). Stigmasterol isolated from *Navicula incerta* marine algae induced apoptosis in human hepatocarcinoma Hep G2 cells (Kim *et al.*, 2014). Moreover, it has been reported that the antioxidant activity of phytosterols may explain, at least in part, their anticancer effect in mice (Liu *et al.*, 2010), and can be attributed to protect the cells from oxidative stress damage by inhibiting mitochondrial ROS formation (Llaverias *et al.*, 2013). In general, the phytosterols may be inhibiting the cancer cell growth due to its function in proliferation, signal transduction and modulating the activity of membrane-bound enzymes of cancer cells, slowing of cell cycle progression and the inhibition of tumor metastasis (Bradford and Awad, 2007; Woyengo *et al.*, 2009).

Conclusions

Cultivation of SM under N, P and S nutrient insufficient condition, the total lipid and phytosterol contents were enhanced compared with that at high concentrations. At large scale in 300L photobireactor, culture of SM in combination of N, P and S limitation media, the highest total lipid and phytosterol was achieved comparable with that in individual cultures. The phytosterols of partially purified phytosterol-rich fraction isolated from unsaponified extract of total lipid of SM cells followed by recrastilization, showed antioxidant and *in vitro* inhibitory effect on the growth of three human (MCF-7, HepG2 and HCT-116) cancer cell line. Thus, these results are quite related to the phytosterol (PS) contents of the *Spirulina maxima* fractions and might provide a promising role in future medicines as anti-oxidant and anti-cancer.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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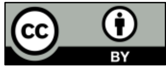

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