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Phytoconstituents and In Vitro Anti-oxidant, Anti-viral, Anti-hyperlipidemic and Anticancer Effects of Chlorella vulgaris Microalga in Normal and Stress Conditions

Amal M El-Feky[1], Marwa M Elbatanony[1], Asmaa F Aboul Naser[2], Omnia M Kutkat[3], Abo El-Khair B El-Sayed[4], Manal A Hamed*[2]

[1]*Pharmacognosy Department, National Research Centre, Dokki, Giza, Egypt*

[2]*Department of Therapeutic Chemistry, National Research Centre, Dokki, Giza, Egypt*

[3]*Department of Water Pollution Research, Center of Scientific Excellence for Influenza Virus, National Research Centre, Dokki, Giza, Egypt*

[4]*Algal Biotechnology Unit, Biological and Agricultural Research Division, National Research Centre, Dokki, Giza, Egypt*

[*]***Corresponding author:****Manal A Hamed, Professor, Department of Therapeutic Chemistry, National Research Centre, El-Buhouth St., Dokki, Giza, Egypt, E-mail: Manal_hamed@yahoo.comn*

ABSTRACT

The aim of this study is to investigate the phytochemical constituents of the microalga Chlorella vulgaris using two different cultivation conditions and validate their antioxidant, antiviral, antihyperlipidemic as well as cytotoxic effects against different cell lines; prostate cancer cell line (PC3), hepatocellular carcinoma (HePG2), caucasian breast adenocarcinoma (MCF7) and normal skin fibroblast (BJ1). Alga was heterotrophically grown with a full nitrogen content of the growth medium in both normal and stress growth conditions. Stress was performed by potassium starvation, salting out and ferric chloride in the presence of sodium acetate. Crude protein content for vegetative and stressed C. vulgaris resulted 46% and 30%, respectively. The total carbohydrates of vegetative and stressed types were 25% and 18%, respectively, while the amount of the isolated polysaccharides was 22.6 and 16.5%. Rhamnose (21.27 and 14.89%) and galactose (17.63 and 15.59%) were found to be the main sugars of vegetative and stress alga, respectively; while ribose was the minor identified sugar (3.23 and 1.80%). The total identified fatty acids (7 fatty acids) were 11.25 and 10.29%. Omega 3 (α-linolenic, eicosapentaenoic, and docosahexaenoic acids) and omega 6 fatty acids (arachidonic and linoleic acids) were present in vegetative and stressed alga. Fat- and water-soluble vitamins showed that vitamin E is the major one (181.24 and 49.17 mg.100g^{-1}) following by vitamin B complex (48.34 and 35.05 mg.100g^{-1}). In addition, 6 main pigments and 11 phytosterols were also detected. Total chlorophyll decreased in the stressed C. vulgaris, while content of total carotenoids showed an inverse trend comparing to the vegetative one. Also, the stressed pigment fraction of C. vulgaris exhibited the strongest antioxidant activity against both DPPH and ABTS (32.30 ± 0.51 and 44.44 ± 0.84mg/g), respectively. Contradictory, the vegetative polysaccharides fraction exhibited anti-proliferative effect against prostate cancer cell line and hypolipidemic effect on β-hydroxy-β-methylglutaryl Coenzyme A reductase enzyme; the key enzyme of cholesterol biosynthesis. The vegetative pigment fraction showed the highest inhibition effect on the propagation of influenza virus by 96.10%. In conclusion, the stressed pigments fraction of C. vulgaris recorded in vitro antioxidant effects, while vegetative polysaccharides fraction showed hypolipidemic, anti-proliferative and anti-prostate cancer. Additionally, the vegetative pigments fraction recorded anti-influenza virus effect.

Keywords:*Chlorella vulgaris*; pigments; phytosterols; antioxidant; cytotoxic; hypolipidemic; antiviral

INTRODUCTION

Microalgae are rich sources of biologically active metabolites. Pharmaceutical researches nowadays are focusing on isolation and extraction of primary and secondary metabolites of these organisms and investigating their antimicrobial, antioxidant, anti-inflammatory, antiviral and antitumor activities. The capacity of algae to exhibit such vast range of activities is due to the presence of various secondary metabolites mainly pigments, proteins, carbohydrates, lipids, terpenoids and steroids [1,2].

Algae response to environmental changes is a result of photosynthetic activity adaptation. Since the synthesis of lipids in chlorella takes place mainly in chloroplasts [3], the changes in concentration of pigments (chlorophyll and carotenoids) and lipids content are considered as markers for evaluation of the overall functional status and effectiveness of the algae to response to the impact of cultivation conditions [4].

Chlorella vulgaris belongs to the genus of single-cell green algae; Chlorella (Family; *Chlorellaceae*). It is a spherical member of the phylum Chlorophyta (green algae) which is mainly produced in Taiwan [5], where it is commonly utilized in industry due to its high protein content and valuable essential amino acids composition [6]. In addition, it has high contents of β-1,3-glucan, vitamins, minerals, β-carotene, chlorophyll and Chlorella growth factor (CGF) [7].

Molecular and cellular level studies on algae have indicated their effect as potent cancer inhibitors [8]. Raikar et al. stated that the methanol extract of *Chlorella vulgaris* showed free radical scavenging effect and cytotoxic activity on human breast and liver cancer cell lines.

The aim of the present work was to explore the phytochemical composition and certain biological activities in vegetative and stressed forms of *C. vulgaris* to establish the optimal growth conditions that maximize production of secondary metabolites and testing them as antioxidant, anticancer, antiviral and antihyperlipidemic agents.

MATERIAL AND METHODS

Microalga production

The green microalga *Chlorella vulgaris* (Algal Biotechnology Unit, NRC) was heterotrophicallyindoor grown under BG-11 nutrient growth medium [9]. Urea 0.53 $g.l^{-1}$ substituted 1.5 $g. l^{-1}$ sodium nitrate [10]. Heterotrophic growth was performed using sodium acetate (15 mM for vegetative growth and 45 mM for stress). Illumination and other growth conditions was employed as mentioned by El‑Sayed et al. [11]. Scaling up (outdoor cultivation) was achieved using 1200L open plate photobioreactor. Culture maintaining and harvesting were operated according to Hassan et al. [12].

Growth technique

Fresh and healthy prepared inoculum (ca120 L) was transferred to the bioreactor and diluted three times with tape water and enriched by nutrients. Once growth became dense, a sequences dilution was performed till the desired volume (1200 L) with enrichment of nutrients. 10 days later as growth reached the maximum, a part of algal broth was harvested to obtain the vegetative cells. Then, the volume adjusted again to reach the same growth. Full optimized grown cells were enriched by sea salt (2%); sodium acetate (45 mM) and ferrous sulfate (125 ppm). Growth is achieved till completely yellow-orange biomass was formed. Harvesting and drying were performed**.**

Quantitative assessment of protein and carbohydrates

The total protein content of vegetative and stressed *C. vulgaris* was estimated by micro-kjeldahl method using Markham distillation apparatus as stated by El‑Sayed et al. Total carbohydrates of vegetative and stressed *C. vulgaris* was determined by the phenol-sulfuric acid method using glucose as standard [13].

Extraction of the polysaccharides

Dried powder of vegetative and stressed *C. vulgaris* (150 g) was macerated in 1.0L of distilled water three times till colourless followed by centrifugation (4300 rpm/20 min). The supernatant was filtered and concentrated to 1/5 of the original volume. Subsequently, absolute ethanol was added to the concentrated solution. The ethanol mixture was placed in a freezer overnight, followed by centrifuging (4300 rpm/10 min). The precipitate was washed by acetone, suction-filtered, and then dried [14,15]. GLC analysis of the mucilage hydrolysates was carried out according to Gertz [16] on GLC HP 6890 (Fluka, Switzer-land). Quantitative determination was based on peak area measurement while qualitative identification was carried out by comparison of the retention times of the peaks with those of the authentic sugars [17].

Extraction of natural pigments

The natural pigments were extracted from 150 g dried powder *C. vulgaris* with a mixture of equal volumes of acetone and n-hexane at the room temperature until colorless. The mixture was washed with water for many times, the n-hexane layer was collected and combined as carotenoid containing extract, filtered and evaporated to dryness in a rotary evaporator and kept in refrigerator till analysis [18]. All steps of the extraction process were performed in dark to avoid cis-trans photo-isomerization and photo destruction as carotenoids are sensitive to light and heat [19]. Pigments content were spectrophotometric estimated regarding to chlorophylls a and b, total chlorophylls and carotenoids [19] at 470, 651 and 664 nm, respectively. The amounts of chlorophyll a, chlorophyll b, total chlorophylls, total carotenoids, chlorophylls ratio (a/b) and pigment index (the sum of carotenoids/chlorophyll a) were calculated according to the formulas as stated by Bodnar et al. [20].

Determination of β-carotene and its derivatives

HPLC Agilent Packared (series1200) equipped with auto-sampling injector, ultraviolet (UV) detector at 461 nm. Procedure adopted according to Elsawi et al. [21]. Peaks area was determined by comparing with reference standards using mean values obtained from at least three injections. ESI-MS positive ion acquisition mode was carried out on a XEVO TQD triple quadruple instrument. The peaks and spectra were analysed using the Maslynx 4.1 software and were identified by comparing their retention time (Rt) with the available literature.

LC-ESI-MS

ESI-MS positive ion acquisition mode was carried out on a XEVO TQD triple quadruple instrument, where HPLC-MS system was composed of an autosampler injector (Switzerland), waters corporation (Milford, MA01757, U.S.A) and mass spectrometer. Column: ACQUITY UPLC-BEH

C18 1.7 µm- 2.1 × 50 mm. Mobile phase elution was made with the flow rate of 0.2 mL/min using gradient mobile phase comprising two eluents: eluent A is H_2O acidified with 0.1% formic acid and eluent B is MeOH acidified with 0.1% formic acid. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time (Rt) and mass spectrum with reported data.

Vitamins profile of vegetative and stressed *C. vulgaris*

Fat and water soluble-vitamins were analysed according to method suggested by Hasan et al. [22] using HPLC system (Shimadzu-UFLC Prominence), equipped with an auto sampler (Model-SIL 20AC HT) and UV-visible detector (Model-SPD 20A) as mentioned by Aboulthana et al. [23].

Determination of total phenolics

The total phenolic content was assayed in the pigment extract by the Folin-Ciocalteu method as stated by El-Feky et al. [24]. Content of the phenolics was expressed in terms of gallic acid equivalent (µg gallicacid. g^{-1} of extract).

Volatile composition in vegetative and stressed C. vulgaris

Chemical compositions of the volatile constituents in vegetative and stressed *C. vulgaris* were qualitative and quantitative determined using gas chromatograph coupled with a mass spectrometer (GC/MS Aglient 6890, 70 eV). Based on the chromatogram, the composition of the volatiles can be identified by comparing the retention time of each peak and its area [25].

ASSESSMENT OF ANTIOXIDANT ACTIVITY

DPPH radical scavenging activity

This test was measured as described by Blois [26]. The ability of the extract to scavenge the DPPH free radicals was calculated using the following equation:

DPPH scavenging activity (%) $=\frac{(A0-A1)}{A0} \times 100$; Where, A0 is the absorbance of the control and A1 is the absorbance of the extract.

ABTS radical scavenging activity

ABTS radical-scavenging activity of the extract was determined according to Re et al. [27]. The inhibition percentage of ABTS radical was calculated using the following formula: ABTS scavenging activity (%) = (A0-A1)/A0×100. Where A0 is the absorbance of the control, and A1 is the absorbance of the extract.

***In vitro*hypolipidemic activity**

The fractions were evaluated for their hypolipidaemic activity by estimation of β-hydroxy-β-methylglutaryl coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34. [28]. The reaction mixture consisted of 40-unit HMG-CoA reductase, 0.15 µmol HMG-CoA substrate, 0.1 mL of the tested samples and 0.1 M potassium phosphate buffer (3.5 mM EDTA, 10 mM dithiothreitol, 0.1 g/l bovine serum albumin and 0.30 µmol NADPH). Incubation at 37°C for5 min took place and the decrease in absorbance due to the oxidation of NADPH to NADP was measured at 340 nm after 1-2 min.

$$\text{Enzyme activity µmol/mg protein} = \frac{\Delta A}{E} \times \frac{1}{\text{mg dried extract}}$$

ΔA is the difference between absorbance measurements.
E = extinction coefficient of NADPH ($6.22 \times 10^{-1} \times \mu mol^{-1}\ cm^{-1}$).

Cytotoxicity assay

For influenza assay, samples were diluted with Dulbecco's Modified Eagle's Medium (DMEM). Stock solutions of the test compounds were prepared in 10% DMSO in dd H_2O. The cytotoxic activity of the samples was tested in Madin Darby Canine kidney (MDCK) cells by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method [29]. Briefly, the cells were seeded in 96 well-plates (100 µl/well at a density of 3×10^5 cells/ml) and incubated for 24 hrs at 37°C in 5% CO_2. After 24 hrs, cells were treated with various concentrations of the tested compounds in triplicates. After further 24 hrs, the supernatant was discarded and cell monolayers were washed with sterile phosphate buffer saline (PBS) 3 times and MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37°C for 4 hrs followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCL in 50 ml isopropanol). Absorbance of formazan solutions were measured at λmax 540 nm with 620 nm as a reference wave length using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined.

For, cancer cell lines cytotoxicity, cells were suspended in RPMI 1640 medium [(for HePG2-MCF7 and HCT116-DMEM for PC3)], 1% antibiotic-antimycotic mixture (10,000 U/ml potassium penicillin, 10,000 µg/ml streptomycin sulfate and 25 µg/ml amphotericin B) and 1% L-glutamine at 37ºC under 5% CO_2. Cells were batch cultured for 10 days, then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37ºC for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). A positive control which composed of 100 µg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions. The plot of% cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50 and 90% cytotoxicity.

$$\% \text{ cytotoxicity} = \frac{(\text{absorbance of cells without treatment} - \text{absorbance of cells with treatmement})}{\text{absorbance of cells without treatment}} \times 100$$

Antiviral assay (plaque reduction assay)

Assay was carried out according to the method of Hayden et al. [30] in a six well plate where MDCK cells (10^5 cells/ml) were cultivated for 24 hrs at 37°C. A/CHICKEN/M7217B/1/2013 (H5N1) virus was diluted to give 104PFU/well and mixed with the safe concentration of the tested samples, and incubated for 1 hour at 37°C before being added to the cells. Growth medium was removed from the cell culture plates and the cells were inoculated with (100 µl/well) virus with the tested compounds, after 1-hour contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose and the tested samples were added onto the cell monolayer, plates were left to solidify and incubated at 37°C till formation of viral plaques (3 to 4 days). Formalin (10%) was added for two hours then plates were stained with 0.1% crystal violet in distilled water. Control wells were included where untreated virus was incubated with MDCK cells and finally plaques were counted and percentage reduction in plaques formation in comparison to control wells was recorded as following

$$\% \text{ inhibition} = \frac{\text{viral count (untreated)} - \text{viral count (treated)}}{\text{viral count (untreated)}} \times 100$$

Statistical analysis

Data of the *in vitro*antioxidants estimation were expressed as mean of % of inhibition of triplicate reading in each concentration. Data of the *in vitro* hypolipidemic activity were expressed as mean ± SD of six values in each concentration. Statistical analysis was done by using one-way analysis of variance (ANOVA), CoStat software Computer Program accompanied by *post-hoc* test at least significance difference (LSD) between groups at $p< 0.05$.

RESULTS AND DISCUSSION

Microalgae are considered a viable source of protein, where the total proteins content in mature *C. vulgaris* varies according to growth conditions. In our study and in accordance with Seyfabadi et al. [31] and Bleakley and Hayes [32], the total protein contents were 46.00 and 30.00% in vegetative and stressed *C. vulgaris*, respectively. The amount of the isolated polysaccharides was 22.60 and 16.50% of the total carbohydrates which reached 25.00 and 18.00% of dry weighted vegetative and stressed Chlorella, respectively. Starch and cellulose are the most abundant polysaccharides in C. vulgaris; they serve as energy storage for the cells. In addition, one of the most important polysaccharides detected in *C. vulgaris* is the β1-3 glucan [33], with multiple health and nutritional benefits. The major identified sugars in vegetative and stressed *C. vulgaris* were identified as rhamnose (21.27 and 14.89%) and galactose (17.63 and 15.59%), respectively, while ribose was the minor identified sugar (3.23 and 1.80%) by the same respect. Yaakob et al. [34] stated that the polysaccharides in *C. vulgaris* are useful for human health as being immuno-stimulant and free-radical scavenger (Tables 1 and 2).

Table 1. Major biochemical composition of *Chlorella vulgaris*

Chlorella form	(%)	
	Total protein	Total carbohydrates
Vegetative	46	25
Stress	30	18

Values are expressed as% (w/w) of total protein and carbohydrate contents of vegetative and stressed *C. vulgaris.*

Table 2. GLC analysis of *C. vulgaris* polysaccharides

Authentic sugars	R_t (min.)	Relative percentage(%) of total polysaccharides hydrolyzate	
		Vegetative	Stressed
Arabinose	10.18	10.57	4.97
Xylose	10.23	13.34	7.38
Ribose	10.74	3.23	1.8
Rhamnose	11.85	21.27	14.89
Galactose	16.74	17.63	15.59
Mannose	17.04	6.45	3.94
Glucose	17.25	5.67	2.68
Total identified sugars		78.16%	51.25%

Values are expressed as relative% of total polysaccharides hydrolyzate.

From Table 3, it can be observed that the content of chlorophyll a, b and total chlorophylls were decreased in the stressed *C. vulgaris*, while the content of total carotenoids showed an inverse trend comparing to the vegetative one. These changes in pigments contents are considered to be an adaptation mechanism to stress conditions as reported by Seyfabadi et al. [31]. On the other hand, chlorophyll a/b ratio can characterize the potential photochemical and biosynthetic activity of algae. Thus under stress conditions, chlorophyll a/b ratio decreased when compared with the vegetative one which is a sign of successful formation of chlorella physiological adaptation [35,36]. Under stress environmental condition, a decrease in chlorophyll a content was observed as it is less stable in comparison with chlorophyll b and accordingly the relationship between these two forms of pigment decreased. When this take place, pigment index increases due to chlorophyll a destruction under unfavorable conditions and enhanced formation of the carotenoids that act as a supporting and protective agent in the photosynthesis phenomena. Therefore, the dynamics of photosynthetic pigments content and changes in their ratio indicate Chlorella adaptation in response to the stress conditions.

Table 3. Pigments content in vegetative and stressed *C. vulgaris*

Pigment content	mg/g (mean ± SD)	
	Vegetative	Stressed
Chlorophyll *a*	10.497 ± 0.012	4.784 ± 0.281
Chlorophyll *b*	12.315 ± 0.319	6.210 ± 0.198
Chlorophylls *a/b* ratio	0.852 ± 0.029	0.771 ± 0.204
Total chlorophyll	26.540 ± 0.034	17.756 ± 0.315
Total carotenoids	34.756 ± 0.215	45.597 ± 0.117
Pigment index	3.311 ± 0.176	9.531 ± 0.115
β-carotene	28.840 ± 0.217	39.398 ± 0.427

Table 4. LC/MS analysis of vegetative and stressed *C. vulgaris* pigments

Pigments	Composition%		[M + H]+	Molecular formula	Main fragments (m/z)
	Vegetative	Stressed	(m/z)		
β-Carotene	8.62	18.79	537	$C_{40}H_{56}$	480, 444, 388
Fucoxanthin	2.57	10.94	681	$C_{42}H_{58}O_6$	109, 581, 641
Lutein	9.51	9.87	569	$C_{40}H_{56}O_2$	551, 459, 429
Zeaxanthin	11.62	11.58	569	$C_{40}H_{56}O_2$	551, 533.
Chlorophyll b	15.93	6.14	908	$C_{55}H_{70}MgN_4O_6$	629, 597, 569
Chlorophyll a	10.38	4.27	894	$C_{55}H_{72}MgN_4O_5$	615, 583, 555
Total pigments	58.63	61.14			

Table 5. LC/MS analysis of vegetative and stressed *C. vulgaris* phytosterols

Phytosterol	Composition%		[M + H]+	Molecular formula	Main fragments (m/z)
	Veg.	Str.	(m/z)		
Cholesterol	0.5	1.35	386	$C_{27}H_{46}O$	368, 301, 255,213,135
Ergosterol	0.53	0.85	396	$C_{28}H_{44}O$	363, 337, 271,253
24-Methylenecycloartanol	0.28	0.61	441	$C_{31}H_{52}O$	425, 422, 407, 379, 315, 300, 203.
β-Sitosterol	0.84	0.98	415	C29H50O	396, 314, 271, 255, 213.
Lupenone	0.46	0.56	424	$C_{30}H_{48}O$	424, 409, 381, 368
Lupeol	0.87	0.56	427	$C_{30}H_{50}O$	411, 393, 383, 370
Campesterol	0.43	0.57	401	$C_{28}H_{48}O$	385, 367
Gramisterol	0.08	0.98	413	$C_{29}H_{48}O$	397, 379, 328, 285, 269.

Fucosterol	0.96	0.92	413	$C_{29}H_{48}O$	397, 379, 314, 299, 281, 229
24-Methylene-ergosta-7-en-3β-ol	0.78	0.42	399	$C_{28}H_{46}O$	381, 365, 314, 299
Cycloeucalenol	0.57	0.27	427	$C_{30}H_{50}O$	411, 408, 393, 353, 300.
Total phytosterols	6.3	7.66			

Table 6. LC/MS analysis of vegetative and stressed *C. vulgaris* fatty acids

Fatty acid	**Composition%**		**[M + H]+**	**Molecular formula**	**Main fragments (m/z)**
	Veg.	**Str.**	**(m/z)**		
Lauric acid	0.95	1.52	201	$C_{12}H_{24}O_2$	157, 129, 85,73
Myristic acid	0.83	0.98	229	$C_{14}H_{28}O_2$	199, 185, 171, 143
palmitic acid	0.72	1.34	257	$C_{16}H_{32}O_2$	213, 185, 185, 129
α-Linolenic acid	2.28	1.82	279	$C_{18}H_{30}O_2$	264, 235, 135, 149, 79
Linoleic acid	2.54	1.48	281	$C_{18}H_{32}O_2$	164, 150, 136, 110, 95
Oleic acid	1.58	1. 03	283	$C_{18}H_{34}O_2$	264, 222, 207, 180, 151, 97
Eicosapentaenoic acid	1.27	1.14	303	$C_{20}H_{30}O_2$	299, 287, 272, 252, 208, 175
Docosahexaenoic acid	1.08	0.98	329	$C_{22}H_{32}O_2$	2,86,245
Total fatty acids	11.25	10.29			
α-Tocopherol	6.21	2.76	431	$C_{29}H_{50}O_2$	415, 205, 165
Total identified compounds	82.39	81.85			

LC/MS analysis of the natural pigment extract of vegetative and stressed *C. vulgaris* led to identification of six main pigments (11 phytosterols and 7 fatty acids) (Tables 3-6). We also recognized the presence of chlorophyll b (15.93%) as the major pigment in vegetative and β-carotene (18.79%) as the main one in the stressed *C. vulgaris* with [M + H] + m/z 908 and 537, respectively [37]. The quantitative analysis of the pigments revealed that the major components of carotenoids present in stressed type, while the chlorophylls was predominating in the vegetative one. The content of the identified phytosterols in the stressed type was 7.66% and the vegetative one was 6.30%. This distribution was attributed to their defence role against stress conditions [38].

The total identified fatty acids in vegetative and stressed *C. vulgaris* were 11.25 and 10.29%, respectively. Omega 3 (α-linolenic, eicosapentaenoic, and docosahexaenoic acids) and omega 6 fatty acids (arachidonic and linoleic acids) were present in vegetative and stressed *C. vulgaris* in considerable amounts. According to Adarme-Vega et al. [39], *C. vulgaris* is an excellent alternative nutritive source, especially when added to infant milk formula due to its richness in omega 3 and 6.

Carotenoids are commercially significant since they are widely used as coloring agents in neutraceuticals, pharmaceuticals, cosmetics and foods. Jayappriyan et al. [40] mentioned that β-carotene is considered as a potential agent in prevention of the human prostate cancer cell line and prevent the progress of premalignant conditions to head and neck cancers [41]. Also, Eiichi et al. [42] stated that fucoxanthin is one of the major xanthophylls and possesses unique chemical features and pharmacological effects. It is able to inhibit expression of the N-myconcogene, cell cycle progression in human neuroblastoma cell lines. Moreover, two hydroxylated carotenoids; rhodopin and fucoxanthin have been detected in *C. Vulgaris*. During the growth phase other carotenogenic pathways are activated which allow the possibilities of various oxidative transformations of β-carotene to other hydroxylated/oxidized forms [43].

On the other hand, epidemiological data suggest that the phytosterols are associated with a reduction in colon, breast and prostate cancers, as they play important roles in enabling more robust antitumor responses, including the boosting of immune response and influencing hormonal dependent growth of endocrine tumors. In addition, phytosterols directly inhibits tumor growth, including the slowing of cell cycle progression, the induction of apoptosis and the inhibition of tumor metastasis [44].

The fatty acids constituents such as oleic, linoleic and palmitic acids have a great influence in the maintenance of health and protection from cancer as they possess antiproliferative and cytotoxic activity through inhibition of tumor viability metastasis, transcription factors and cell proliferation in several carcinoma cell types [45,46]. In recent years, fatty acids production in large scale from microalgae have created considerable interest among researchers because of the health benefit of mono and polyunsaturated-fatty acids [47,48].

Chlorella vulgaris has an important vitamin profile as shown in Table 7.Vitamin A was presented in the algae in reasonable amount (13.52 and 9.87 mg/100 g in vegetative and stressed forms respectively), and have antioxidant activity that acts as free radical's scavenger together with improving blood circulation due to its richness in vitamins E (181.24 and 49.17 mg/100 g in vegetative and stressed forms, respectively) [49,50]. In addition, vitamin B complex occupies the second position in the total vitamins content; 48.34 and 35.05 mg/100 g in vegetative and stressed form respectively. It promotes red blood cells growth and maintains healthy skin, hair and muscles [51]. According to Khariy et al. [52], vitamins are sensitive to growth conditions; thus, the concentration in the vegetative form is more than that of stressed one.

Table 7. Vitamins profile in vegetative and stressed *C. vulgaris.*

Vitamin	Content (mg/100 g) mean ± SD	
	Vegetative	Stressed
A (Retinol)	13.52 ± 0.001	9.87 ± 0.151
B1 (Thiamine)	40.36 ± 0.024	31.58 ± 0.064
B2 (Riboflavin)	2.50 ± 0.054	N/A
B6 (Pyridoxine)	5.48 ± 0.018	3.47 ± 0.057
C (Ascorbic acid)	1.43 ± 0.184	0.85 ± 0.194
E (Tocopherol)	181.24 ± 0.084	49.17 ± 0.218
Total identified vitamins	244.53	94.94

The total phenolic content in the pigment extract of vegetative and stressed algae was 21.19 ± 0.49 and 32.89 ± 0.62 µg gallic acid equivalents/g, respectively (Table 8). Soobrattee et al. [56] reported that the phenolic compounds have redox properties, as their free radicals scavenging ability is facilitated by their hydroxyl groups, so that, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity.

Table 8. Total phenolic content of vegetative and stressed *C. vulgaris* in pigment extract.

Extract	**Total phenols (µg GAE/g)**
	Mean ± SD
Vegetative pigments	21.19 ± 0.49
Stressed pigments	32.89 ± 0.62

Different chemical classes of the volatile compounds were identified and quantified using GC/MS analysis (Table 9). Thirty compounds were identified from the vegetative form representing 88.51%, while thirty-one compounds (95.88%) were presented in the stressed form. The fatty acid contents of *C. vulgaris* are influenced by the environmental and cultural conditions that affected its growth [53]. Noticeable changes were reported in the volatile components of vegetative and stressed chlorella, where an increased in lipid content of the stressed *C. vulgaris* was observed. This was attributed to the presence of microalgae that accumulated high lipid levels under stressful conditions and stored them as triglycerides [54]. Table 6 showed that the total saturated and unsaturated fatty acids content in the vegetative *C. vulgaris* were 28.74 and 29.91%, while in stressed form were 35.18 and 22.21% respectively. These results are in convenience with that reported by Chen et al. [55] who illustrated that when *C. vulgarisis* grown under favorable growth conditions, its lipoidal constituents are suitable for nutritional consumption because of its richness in polyunsaturated fatty acids such as linoleic acid, linolenic acid, and eicosapentaenoic acid.

Table 9. GC/MS analysis of vegetative and stressed *C. vulgaris* volatile constituents.

Class	**Compound**	**Molecular weight**	**Molecular Formula**	**%**	
				Vegetative	**Stressed**
Hydrocarbons	Eicosane	282	$C_{20}H_{42}$	0.45	0.48
	Docosane	310	$C_{22}H_{46}$	1.59	1.68
	9-Hexyl Heptadecane	324	$C_{23}H_{48}$	----	0.39
	Pentacosane	352	$C_{25}H_{52}$	1.57	2.61
	Heptacosane	380	$C_{27}H_{56}$	1.27	1.58
	Nonacosane	408	$C_{29}7H_{60}$	2.95	4.98
Total hydrocarbons content				7.83	11.72

Aldehydes and ketones	Pentanedial	100	$C_5H_8O_2$	0.75	1.89
	7,9-Dodecadienal	130	$C_{12}H_{20}O$	1.05	1.09
	2-Tridecadienal	194	$C_{13}H_{22}O$	2.28	2.36
	2-(1'-Ethylhexyl) cyclopentanone	196	$C_{13}H_{24}O$	----	0.57
	16-Octadecenal	266	$C_{18}H_{34}O$	1.87	3.32
Total Aldehydes and ketones content				5.95	8.93
Fatty alcohols	4-Methyl-2-octyn-4-ol	140	$C_9H_{16}O$	----	2.58
	3,7-Dimethylnonanol	172	$C_{11}H_{24}O$	1.98	1.99
Total fatty alcohols content				1.98	4.57
Saturated fatty acids	Dodecanoic acid (Lauric acid)	200	$C_{12}H_{24}O_2$	10.58	10.98
	Tetradeconoic acid (Myristic acid)	228	$C_{14}H_{28}O_2$	7.02	9.21
	n-Hexadecanoic acid (palmitic acid)	256	$C_{16}H_{32}O_2$	5.25	7.58
	Octadecanoic acid (Stearic acid)	284	$C_{18}H_{36}O_2$	5.89	7.41
Total saturated fatty acids content				28.74	35.18
Unsaturated fatty acids	(9Z)-Hexadec-9-enoic acid (Palmitoleic acid)	254	$C_{16}H_{30}O_2$	5.58	4.43
	Heptadecenoicacid	268	$C_{17}H_{32}O_2$	0.91	0.98
	Linolenic acid	278	$C_{18}H_{30}O_2$	8.02	5.78
	9,12-Octadecadienoic acid (Linoleic acid)	280	$C_{18}H_{32}O_2$	6.65	4.97
	Octadec-9-enoic acid (Oleic acid)	282	$C_{18}H_{34}O_2$	5.25	4.45
	Eicosapentaenoic acid	302	$C_{20}H_{30}O_2$	2.45	0.62
	Arachidonic acid (Eicosatetraenoicacid)	304	$C_{20}H_{32}O_2$	1.05	0.98
Total unsaturated fatty acids content				29.91	22.21
Fatty acid esters	14-Methyl pentadecanoate	270	$C_{17}H_{34}O_2$	1.71	---
	Ethyl hexadecanoate (Ethyl palmitate)	284	$C_{18}H_{36}O_2$	3.25	2.98
	Methyl (9E)9-octadecen-12-ynoate	292	$C_{19}H_{32}O_2$	0.98	1.03
	Methyl 12(2-octylcyclopropyl) dodecanoate	366	$C_{24}H_{46}O_2$	0.56	---
Total fatty acid esters content				6.5	4.01
Carotenoids	1,2-Dihydro-ψ,ψ-Caroten-1-ol (Rhodopin)	554	$C_{40}H_{58}O$	2.64	3.04
	Fucoxanthin	658	$C_{42}H_{58}O_6$	1.68	2.75
Total carotenoids content				4.32	5.79
Steroids	Stigmast-5-en-3-ol (Clionasterol)	414	$C_{29}H_{50}O$	1.68	1.74
	Ergosterol	396	$C_{28}H_{44}O$	0.75	0.82
	28-Isofucosterol	412	$C_{29}H_{48}O$	0.85	0.91
Total steroids content				3.28	3.47
Total identified compounds				88.51	95.88

Algae rich in secondary metabolites including phenolics, pigments and carotenoids, have antioxidant activity due to various chemical constituents. The stressed pigments extract of *C. vulgaris* exhibited the strongest antioxidant activity against both DPPH and ABTS (44.446 ± 0.84 and 32.303 ± 0.51 mg/g), respectively (Table 10). This antioxidant effect is due to the presence of phytosterols and phenolic compounds [56]. The same authors added that the antioxidative phytochemicals in natural sources are classified as carotenoids, phenolics, alkaloids as well as nitrogen and organosulfur compounds.

Table 10. Antioxidant activity of polysaccharides and pigment extracts of *C. vulgaris.*

Sample (100 µg/ml)	DPPH	ABTS
Vegetative polysaccharides	9.938 ± 0.32	12.621 ± 0.14
Stressed polysaccharides	12.937 ± 0.21	15.770 ± 0.27
Vegetative pigments	26.870 ± 0.41	38.902 ± 0.43
Stressed pigments	32.303 ± 0.51	44.446 ± 0.84

The examined fractions recorded hypolipidemic effects by variable degrees where, the vegetative polysaccharides fraction recorded the most potent effect. It showed reduction in β-hydroxy-β-methylglutaryl Coenzyme A reductase enzyme (the rate limiting enzyme in cholesterol biosynthesis) by 85.97, 92.42, 95.42, 96.39 and 97.75% at concentration of 0.01, 0.1, 1.00, 10 and 100 mg, respectively (Table 11). Drugs that decrease cholesterol, such as fibrates and bile acid sequestrants, have been used for several decades, but their adverse effects led to the discover of statins; β-hydroxy-β-methylglutaryl coenzyme A reductase (HMG CoA) inhibitors [57,58]. In view of the adverse effects associated with these drugs, many researchers are focused their works on the natural products with lipid-lowering potential and no side effects [59]. Nakamura et al. [60] postulated the role of polysaccharides as a lipid lowering agent.

Table 11. *In vitro* hypolipidemic effects of polysaccharides and pigment extracts of *C. vulgaris* on HMG CoA reductase enzyme.

Fractions Conc. (mg/ml)	Control	Pigment vegetative	Pigment stress	Polysaccharides vegetative	Polysaccharides stress
100 mg	2.67 ± 0.24^{a}	0.31 ± 0.07^{d}	0.66 ± 0.14^{c}	0.06 ± 0.01^{e}	0.98 ± 0.07^{b}
	(---)	(-88.38)	(-75.28)	(-97.75)	(-63.29)
10 mg	6.11 ± 0.61^{a}	2.11 ± 0.54^{d}	3.13 ± 0.55^{c}	0.22 ± 0.03^{e}	4.23 ± 0.43^{b}
	(---)	(-65.46)	(-48.77)	(-96.39)	(-30.76)
1 mg	32.77 ± 2.31^{a}	3.90 ± 0.87^{d}	6.55 ± 0.54^{bc}	1.50 ± 0.42^{e}	6.87 ± 3.25^{b}
	(---)	(-88.09)	(-80.01)	(-95.42)	(-79.03)
0.1 mg	142.20 ± 3.11^{a}	19.24 ± 0.76^{d}	62.44 ± 2.25^{c}	10.33 ± 1.35^{e}	80.55 ± 3.14^{b}
	(---)	(-86.46)	(-56.09)	(-92.73)	(-43.35)
0.01 mg	400.70 ± 9.24^{a}	90.45 ± 1.25^{d}	116.43 ± 4.33^{c}	56.18 ± 2.11^{e}	190.14 ± 3.23^{b}
	(---)	(-77.42)	(-70.94)	(-85.97)	(-52.54)

The primary screening of the anti-proliferative effects of different fractions of *C. vulgaris* on algae against human prostate, hepatocellular and pcaucasian breast adenocarcinoma cell lines as well as the normal skin fibroblast cells were illustrated in Table 12. The lethal concentrations of the samples which caused 50% cells death were recorded in Table 12, where, the vegetative polysaccharides fraction showed anti-prostate cancer effect at concentration 80.80 µg/ml, respectively and had no effect on normal skin fibroblast cells. These observations were in line with the results of Sithranga and Kathiresan [61] and Raikar et al. [62] who recorded algal bioactive components with potent anti-cancer effects. This effect is due to the presence of certain vitamins as antioxidants, presence of phytosterols, fatty acids, steroids and sulphated polysaccharides [63].

Table 12. Percentage mortality and LC50 of polysaccharides and pigment extracts of *C. vulgaris* on different cancer cell lines.

100 ug/ml	BJ1		PC3		HePG2		MCF7	
	%	LC_{50}	%	LC_{50}	%	LC_{50}	%	LC_{50}
Pigment vegetative	100	18.30 ± 0.85	29.7	----	38.6	----	100	31.50 ± 3.02
Pigment stress	64.2	74.70 ± 2.05	28.9	----	13.5	----	77.5	56.80 ± 3.21
Polysaccharides vegetative	14.3	----	56.7	80.80 ± 3.36	23.5	----	15.6	----
polysaccharides stress	74.3	64.70 ± 2.55	75.8	63.30 ± 3.744	46.9	98.40 ± 2.50	12.3	86.70 ± 5.90

The antiviral effect was investigated in Influenza virus (M7217B) 2013 (H5N1). The results were summarized in Tables 13 and 14, where the vegetative pigment fraction at concentration 0.1 µg/µl recorded 96.10% inhibition. Concentrations less than 0.1 µg/µl was illustrated in Tables 14 and 15, where the vegetative pigment fraction of *Chlorella vulgaris* alga showed the highest inhibition effect on the propagation of influenza

virus (A/chicken/Egypt/M7217B/2013) H5N1 at 0.08 µg/ul. This study against Influenza virus (M7217B) 2013 (H5N1) was performed for the first record for *C. vulgaris* and giving promising results. The vegetative pigment fraction proved to be more effective than the stressed one, this may be due to higher concentrations of chlorophyll (a and b) which possessed a diversity of other antiviral activities [64].

Table 13. Antiviral activity (MTT cytotoxicity assay) against Influenza virus (M7217B) 2013 (H5N1).

Sample name	**IC_{50} (µg/µl) mean ± SD**
Vegetative polysaccharide	0.094 ± 0.04
Stressed polysaccharide	0.094 ± 0.18
Vegetative pigment	0.095 ± 0.14
Stressed Pigments	0.120 ± 0.20

Table 14. Antiviral activity (plaque reduction assay) against influenza virus (M7217B) 2013 (H5N1).

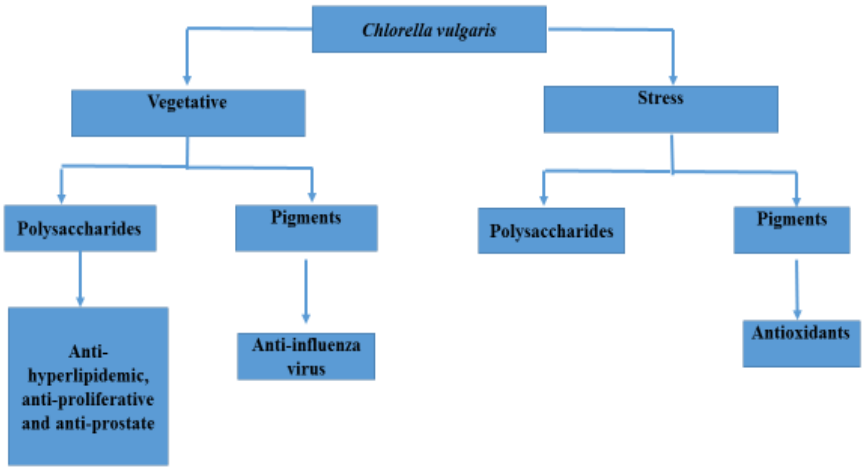
Sample name	**Conc. µg/µl**	**Initial viral count**	**Viral count (PFU/ml)**	**Inhibition%**	**Comments**
Vegetative polysaccharide	0.1	7.7×10^6	$5.6*10^6$	27.20%	
	0.2	7.7×10^6	$5.2*10^6$	32.40%	
	0.4	7.7×10^6	$4.4*10^6$	42.80%	
Stressed polysaccharide	0.1	7.7×10^6	$6.3*10^6$	18.10%	
	0.2	7.7×10^6	$4.3*10^6$	44.10%	
	0.4	7.7×10^6	$4*10^6$	48%	
Vegetative pigment	0.1	7.7×10^6	$3*10^5$	96.10%	
	0.2	7.7×10^6	Non countable	Effect on the sheet of the cells.	
	0.4	7.7×10^6	Non countable	Effect on the sheet of the cells.	
Stressed Pigments	0.1	7.7×10^6	Non countable	No Inhibition	Effect on the sheet of the cells.
	0.2	7.7×10^6	Non countable	No Inhibition	
	0.4	7.7×10^6	Non countable	No Inhibition	

Table 15. Confirmation of plaque reduction assay for vegetative pigment.

Conc. µg/ul	**Inhibition%**
0.02	92.8
0.04	94.2
0.06	96.8
0.08	98.8

In summary, Figure 1 illustrated diagrammatic presentation of the biologically active fractions of *Chlorella vulgaris* as antioxidant, anti-hyperlipidemic, anti-proliferative and anti-cancer agent.

Figure 1: Diagrammatic presentation of the biologically active fractions of *Chlorella vulgaris.*



CONCLUSION

C. vulgaris algae had different phytochemical constituents and recorded certain biological activities. The stressed pigments fraction had *in vitro* antioxidant effects, while the vegetative polysaccharides fraction recorded anti-hyperlipidemic, anti-proliferative and anti-prostate cancer effects. In addition, the vegetative pigments fraction recorded anti-influenza virus activity. Much detailed studies are needed to confirm these effects with special emphasis to the most effective biological molecule.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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