Metabolomics and Biological activities of *Chlorella vulgaris* grown under modified growth medium (BG_{11}) composition

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ABSTRACT

The aim of this work was to determine the biochemical compounds and evaluate the biological activities of Chlorella vulgaris cultivated under a biotic stress condition (various Zinc and Cupper conc.). The growth rate was recorded as well as determination of active compounds, pigments and defense enzymes, in addition to the biological activities as antioxidant, antimicrobial and anticancer. The obtained results revealed that, higher copper concentrations [0.632 mg /L (Cu)] showed an inhibitory effect to growth while 1.76 mg /L (Zn) enhanced growth which reached its maximum at 25th day of cultivation. Furthermore, combination of 0.88mg/L (Zn) and 0.316mg /L (Cu) induced an increase in growth rate, catalase, tannins, lipid peroxidation and glutathione-S-transferase and a decrease in flavonoids, phenolic content, protein and antioxidant activity. Also, the results of antioxidant activity showed that, elevation of Zn conc. induced an augmentation of antioxidant activity either by DPPH(2, 2 diphenyl-1-picrylhydrazyl) or ABTS (2, 2'- azino-bis ethylbenzthiazoline-6-sulfonic acid), with maximum activity at 0.88 mg/L Zinc conc. (89.91%) even exceeded those of control (85.62%). While more elevated Zn conc. (1.76 mg/L) induced lower activity when compared with synthetic antioxidant standard (Butylated hydroxyl toluene, BHT). Concerning antimicrobial activity, Gram +ve bacteria, Staphylococcusaureus recorded moderate activity in sulfur-contained extract. Cytotoxicity of three cancer cell lines was inversely proportional to extracts conc. used, where the higher conc. (500µg/ml) showed the lowest cell viability of the tested cell lines which ranged from 22.06 to 69.89%. 0.316 mg/L (Cu) of conc. 500 µg/ml recorded the lowest

cell viability of (32.166 %) in breast cell line , (22.06%) in colon cell line, and 27.18 06%) in cervical cell line.

Keywords:*Chlorella vulgaris*, Chemical compositions, Anticancer, Antioxidant Antimicrobial, Defense enzymes

INTRODUCTION

Microalgae include unicellular, simple, primitive, and photosynthetic organisms and are considered a potentially valuable and new source of different biologically active molecules for applications in various fields such as food industry, agriculture, pharmaceutical, nutraceutical, and cosmetic sectors (Shalaby, 2011; Shanab et al., 2012; Shalaby and Dubey, 2018). *Chlorella sp.* is a microalga that potential by used for food supplement, pharmaceuticals, animal feed, aquaculture and cosmetics (Widayat et al., 2018). They can be easily cultured and harvested, have short generation times and enable an environmentally-friendly approach to drug discovery by overcoming problems associated with the over-utilization of marine resources and the use of destructive collection practices (Lauritano et al., 2016).

The bioactive compounds from natural sources has beneficial effects on health (Herrero et al., 2013) and used for the treatment of different human diseases (Newman and Cragg, 2012).

Several studies on the impact of various stresses including heavy metal exposure on microalgal growth have been done (Pinto et al., 2003; Zouari et al., 2016). Algae often minimize free radical damage by inducing an antioxidant defensive system (Li et al., 2006; Olivares et al., 2016), such as non-enzymatic (e.g., glutathione (GSH), tocopherols, ascorbate (ASC), and carotenoids), and enzymatic (e.g., superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX)), which are known to be involved in protecting plants against high toxic levels of heavy metals (Pinto et al., 2003; Sáeza et al., 2015; Machado and Soares, 2016; Moenne et al., 2016). A large number of algal extracts and / or extracellular products have proven antimicrobial, antitumor, antioxidant and antiviral activities (Ghasemi et al., 2004; Ozemir et al., 2004).

This study aimed to identify the secondary metabolites produced by *Chlorella vulgaris* cultivated undernutrient stress concentration especially copper and zinc as micronutrients by an increase or decrease of the element content in the culture medium (BG_{11}). Nutrient stress was performed by single nutritive component (Cu, Zn) and in combination of two elements (Cu+Zn) as well as the biological activities of these metabolites (antioxidant, antimicrobial and anticancer activities) were targeted in this study.

Statement of Novelty

- First time for cultivation of *chlorella vulgaris* under these conc. of microelements.
- Enhancement of the production of bioactive compounds from *C.vulgaris* as antioxidant, anticancer.
- Production of *C.vulgaris* biomass in modified growth media more than BG_{11} (control media).

MATERIALS AND METHODS

Chemicals and drugs

Pure hexane, chloroform, ethanol, ether, acetone, methanol and methylene chloride were purchased from E. Merck Co. (Darmstadt, Germany). Sulfarhodamine, 2, 2 diphenyl-1-picrylhydrazyl (DPPH), 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid ($ABTS.^{+}$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid and other materials were of the highest available commercial grade.

Algal cultivation

The green microalga *Chlorella vulgaris* used in this study was kindly isolated and identified by Dr.Sanaa Shanab, Professor of Phycology in the Department of Botany and Microbiology, Faculty of Science, Cairo University, according to Bourelly 1972, Prescott 1978, 1982, then molecularly identified by sigma company (6^{th} October, Egypt).

The alga was cultivated on BG_{11} medium (Stanier et al., 1971) and incubated at controlled culture conditions of temperature (25±2°C) light intensity ($40\mu E/m^2/S$), light duration (16-8 L/D cycles) with continuous aeration (60 bubbles/min).

Preparation of axenic culture (free from bacteria)

Purification of *Chlorella vulgaris* culture from bacteria is necessary for this study concerning secondary metabolites production without interference of bacteria mixed with the alga under investigation. Mixture of antibiotics; penicilliumG, dihydrostreptomycin sulfate and gentamycin sulfate at different concentrations according to the method described by Andersen (2005). The axenic culture of the alga was used in all experimental work.

Bacterial and fungal cultures

For determination of antimicrobial activity of different algal extracts, it was tested in vitro against G+ve and G-ve bacterial strains using agar well diffusion method.

The Gram positive bacterial strains, *Staphylococcus aureus* (ATCC: 6538) and *Streptococcus mutans*, (ATCC: 25175), the gram negative strains; *Escherichia coli* (ATCC: 9637) and *Klebsiella pneumonia* (ATCC: 10031) were cultured using nutrient agar medium and incubated at the favorable culture condition. Fungal strains used in this study were *Aspergillus niger* (ATCC: 32856) and *candida albicans* (ATCC: 10231).

Cell line cultures (for anticancer activity).

Human breast adenocarcinoma (MCF-7), human colorectal carcinoma (HCT 116) and human cervical cancer (Hela) were propagated in RPMI-1640 medium L-Glutamine (lonzaverviers SPRL, Belgium cat#12-604F), this medium was supplemented with 10%fetal bovine serum (FBS) (seralab, UK cat#EU-000-H) and 1% antibiotic, Antimycotic). The cells were incubated in 5% Co_2 humidified at 37°c for growth.

Antioxidant enzymes kits

5-a-Catalase enzyme (Bio diagnostic). It is an antioxidant enzyme used against H_2O_2 which cause intracellular damage.The kit include: (1) phosphate buffer PH7 (100 mM/L), (2) H_2O_2 substrate as standard (500 mM/L), 3-chromogen inhibitor and (4) enzyme peroxidase (>2,000/L), 4-Aminoantipyrine preservative (2 mM/L).

5-b-Glutathione-S-transferase (GST) [Bio diagnostic]. They are multifunctional enzymes which play a key role in cellular detoxification. The enzymes protect cells against toxicants by conjugating them to glutathione and neutralizing their electrophilic sites. GST kit measures total GST activity by recording the absorbance at 340 nm'

5-c-Lipid peroxidation (Malondialdehyde) [Bio diagnostic]. Thiobarbituric acid (TBA) react with malondialdehyde (MDA) in acidic medium at 95°c for 30 min. to form thiobarbituric acid reactive product determined spectrophotometrically by absorbance at 534 nm (of the pink product).The kit include: (1) standard (10 nmol/ml) (2) Thiobarbituric acid detergent stabilizer (25 mmol/L).

Modification of the chemical composition of the culture medium

Studying the effect of varying nutritive elements provided by the culture medium (BG_{11}), an increase or decrease of certain element concentration was performed (as single element stress). copper and zinc concentrations used were; zero, 0.158, 0.316 and 0.632 mg/L for copper and zero, 0.44, 0.88 and 1.76 mg/L for zinc respectively. Combination of the two major nutrients used was copper and zinc at concentration 0.316 mg/L for copper and 0.88 mg/L for zinc (as double element stress).

Determination of algal growth rates

Growth of *chlorella vulgaris* culture (free from bacteria) was determined by measuring the optical density at 680 nm at 5 day interval though the incubation period of 30 days at the controlled culture conditions.

Determination of algal dry weight

Algal dry biomass was determined at 5 day interval of the incubation period (using 20 ml algal suspension), filtration, washing and drying at 105°c for 24 h according to the method described by Talukdar (2012).

Determination of chlorophylls and carotenoids

Pigments were determined according to Lichtenthaler and Wellburn (1985) using 96% methanol and recording the absorbance at the wavelengths 666, 653 and 470 nm and using the following equations:

$$\text{Chlorophyll a (mg/g)} = 15.65 * A_{666} - 7.340 * A_{653}$$
$$\text{Chlorophyll b (mg/g)} = 27.05 * A_{653} - 11.2 * A_{666}$$
$$\text{Total carotenoids (mg/g)} = 1000 * A_{470} - 2.860 * C_a - 129 * C_b / 245$$

Where A_{666}, A_{653} and A_{470} nm are the absorbance at the indicated wavelengths.

Extraction of algal biomass

The dried algal biomass were extracted three times with organic solvent mixture of methanol and methylene chloride (1:1) for 40 minutes followed by centrifugation, filtration and evaporation of solvents using rotary evaporator at 40-50 °C. The obtained crude extract was expressed as percentage of the dried biomass weight used (mg extract/g dry biomass weight).

Determination of the total phenolic contents

Phenolic contents in the crude extract was determined according to the method of Taga et al. (1984) and expressed as Gallic acid equivalent/gram of the alga (GAE/g).

Determination of Flavonoid contents

Flavonoid content in the algal extract was determined by the spectrophotometric method recorded by Quettier et al. (2000) and expressed in terms of Rutin equivalent (mg of Ru/g of extract)

Determination of Tannins

Using Vanillin hydrochloride method of Broadhurst and Jones (1978). The amount of Tannic acid in the sample was recorded from the standard curve and expressed as tannic equivalents.

Estimation of total protein

Extraction of protein was performed according the modified method of Rausch (1981) and total protein concentration was determined using the method of Lowry et al. (1951) and absorbance was recorded at 720 nm. Total protein was determined using the calibration curve of BSA (0.2-40 µg/ml).

Determination of antioxidant enzymes and Lipid peroxidation Catalase estimation. Algal homogenate+phosphate buffer+H_2O_2 at 25°c, incubate for 1 min. then chromogen –inhibitor and enzyme peroxidase and preservative after incubation for 10 min. at 37°C, record absorbance of sample against sample blank and standard against standard blank using methods of Aebi (1984) and Fossati et al. (1980) catalase activity U/g=$A_{standard}$-$A_{sample}/A_{standard}$*1/gm biomass used.

Glutathionne-S-transferase. Algal homogenate and phosphate buffer at PH 7.4 + glutathione reduced (GSH). Incubation at 37°C for 5 min. then add CDNB and incubate at 37°C for 5 min. and finally mix with TCA, centrifuge at 3,000 rpm for 5 min. record the absorbance of sample (A_{sample}) against blank at 340 nm, calculate the activity as U/g biomass=A_{sample} *2.812/g biomass sample according to the method reported by Habig et al. (1974).

Lipid peroxidation (MDA determination method). Algal homogenate+phosphate buffer (PH 7.5), centrifuge at 4,000 rpm for 15 min. supernatant+chromogen, heat in boiling water bath for 30 min. coal and record the absorbance of algal sample (A_{sample}) against blank and standard against distilled water at 354 nm. MDA in algal sample (nmol/g algal biomass) = $A_{sample}/A_{standard}$*10/g tissue used. According the method described by Satoh (1978) and Ohkawa et al. (1979).

Biological activities of algal extract. Determination of biological activities of the crude extract produced from *chlorella vulgaris* cultured in normal BG_{11} growth medium (control) and altered (stressed) BG_{11} medium with modified contents of Copper and Zinc, single or in combination, the following activities were performed:

Antioxidant activity

-*DPPH radical scavenging activity*. The scavenging effects of crude Methanol: Methylene chlorid (1:1), extract was determined by the method of Yen and Chen (1995), where, 2.0 ml of 0.16 mM DPPH solution (in methanol) was added to a test tube containing 2.0 ml aliquot of sample. The mixture was vortexed for 1 min and kept at room temperature for 30 min. in the dark. The absorbance of all the sample solutions and BHT as synthetic standard were measured at 517 nm. The percentage (%) of scavenging activity was calculated as the following:

% Antioxidant activity = (Control-Sample x 100) / Control

Where control in DPPH solution (0.16 mM).

-*ABTS radical cation scavenging assay*. This assay was based on the ability of different substances to scavenge (2, 2'- azino-bis ethylbenzthiazoline-6-sulfonic acid ($ABTS^{\cdot+}$)) radical cation in comparison to a standard (BHT). The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 4-16 hrs. until the reaction was completed and the absorbance was stable. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for measurements

according to Pellegrini et al., 1999). The photometric Assay was conducted on 0.9 ml of $ABTS^{+}$. and 0.1 ml of tested samples and mixed for 45 s, and measurements were taken at 734 nm after 1 min. The antioxidant activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the following equation: $E= ((A_c-A_t)/A_c) \times 100$, where: At and Ac are the respective absorbance of tested samples and $ABTS^{\cdot+}$

Increased regeneration of ROS occurred under stress conditions, the defense system in the form of low molecular weight substances (as ascorbic acid, β-carotene, glutathaione) and/or antioxidant enzymes (superoxide dismutase/catalase/peroxidase/glutathione-S-transferas) were produced in order to scavenge the ROS and avoid their harmful action on biomolecules and biomembranes.

Antimicrobial assay

To investigate the produced secondary metabolites produced under normal and stressed culture conditions of the selected alga for this study *(Chlorella vulgaris)*, agar well diffusion method (Scott, 1989) was used. The algal crude extracts dissolved in DMSO were tested at a concentration of 15 mg/ml against both G+ve and G-ve bacterial strains and fungal strains (*Aspergillusniger* and *candida albicans*) which were previously cultured on their specific culture medium and incubated in their favorable conditions. (The activity was expressed as diameter of inhibition zones in mm), Ampicillin and Gentamicin were used as standards for G+ve and G-ve bacteria respectively and DMSO as -ve control, while Nystain was used as standard drug for fungi.

Anticancer activity

Cytotoxic effect of crude extracts was tested against different cancer cell lines (by MTT assay (El-Far et al., 2009; Dharmendra and Maurya, 2011; Ranganathan et al., 2015)) using 96-well plate in triplicates and the crude extracts dissolved in DMSO were tested against Breast, Hela and colon canceroma cell lines, Data were calculated as percentage of cell viability % cell viability = Mean absorbance in test wells/mean absorbance in control wells*100

Statistical analysis

Data were subjected to an analysis of variance, and the means were compared using the 'Least Significant Difference (LSD)' test at 0.05 levels, as recommended by Snedecor and Cochran (1982). Data are presented as mean ± SD.

RESULTS

Growth rate

The growth rate of *Chlorella vulgaris* cultivated under various zinc concentrations (of BG_{11} medium) was recorded in Table 1 the results revealed that, in zero (zinc) conc. an increase in growth rate during the incubation period until the 25^{th} day of cultivation after which growth starts to decline. In control

media (0.22 mg/L Zinc) a gradual increase of growth was recorded with maximum (0.995±0.01 g/L) at the 20th day then growth decreased. In 1X Zinc conc, (0.44 mg/L) an increased growth gradually recorded till the 25th day of cultivation (0.432±0.102 g/L). In 2X Zinc conc. (0.88 mg/L) a pronounced enhancement of growth was shown more than those of 0.44 mg/L Zn with maximum at 25th day of cultivation (0.528±0.107 g/L). In 4X Zinc conc. (1.76 mg/L) the results showed maximum growth rate was always recorded during all of the period of cultivation compared to all Zn conc. (0.44 and 0.88 mg/L Zn) even more than the control was showed (at 25th day) recording 1.005±0.14 g/L by the end of cultivation period (30 days) growth decline was recorded in all cases but still 4X Zn showed comparable maximum growth (0.916±0.112 g/L) as indicated in Table 1.

The data in Table 2 showed that an obvious increase in growth rate during the incubation period from 0-25th day of cultivation of *C.vulgaris* at all copper conc. with lower frequency increase in 0.632mg/L Cu conc. than those of 0.158, 0.316 mg/L Cu and even those of control and zero Cu conc. Maximum growth was noticed in 2X Cu (1.44±0.018 g/L) in the 20th and at the 25th day of cultivation (1.609±0.02) followed in descending order by the growth rate at 1X Cu conc. (1.200±0.0014 g/L). By the end of cultivation time (30 days) and with the start of stationary phase of growth, a decline of growth was observed, but still 0.316 mg/L Cu conc. showed higher growth rate compared to all other conc. (1.470±0.011 g/L).

Table 3 recorded the combined effect of the two micronutrients (Cu and Zn) in 2X conc. during 30 days of cultivation. The combination of Cu + Zn showed an inhibitory effect of growth compared to those of control especially at the first 10 days of cultivation after which a steady increase of growth was noticed since the 20th and 25th day of cultivation compared to control. The combination Zn+Cu, induced comparable growth rate from 20-30 days of cultivations as shown in Table 3.

Table 1. Growth rate of *chlorella vulgaris* (O.D and dry wt. (g/L)) cultivated under different Zinc concentrations.

Days	(0) Zn		Cont. (0.22mg/L)		1X Zn (0.44 mg/L)		2x Zn (0.88 mg/L)		4X Zn (1.76 mg/L)	
	Wt.	**O.D**	**WT.**	**O.D**	**WT.**	**O.D**	**WT.**	**O.D**	**WT.**	**O.D**
0	0.02± 0.004^{g}	0.002± 0.0005^{g}	0.026± 0.004^{f}	0.002± 0.0006^{f}	0.03± 0.004^{f}	0.003± 0.00153^{g}	0.03± 0.004^{f}	0.003± 0.00153^{g}	0.02± 0.003^{g}	0.003± 0.0015^{g}
5	0.08± 0.002^{f}	0.049± 0.001^{f}	0.22± 0.003^{e}	0.089± 0.004^{e}	0.036± 0.04^{e}	0.02± 0.001^{f}	0.08± 0.004^{e}	0.05± 0.002^{f}	0.06± 0.006^{f}	0.04± 0.002^{f}
10	0.11± 0.004^{e}	0.07± 0.002^{e}	0.38± 0.035^{d}	0.27± 0.006^{d}	0.105± 0.005^{d}	0.07± 0.002^{e}	0.169± 0.007^{d}	0.11± 0.002^{e}	0.21± 0.007^{e}	0.13± 0.002^{e}
15	0.17± 0.002^{d}	0.11± 0.0031^{d}	0.50± 0.008^{c}	0.36± 0.009^{c}	0.27± 0.007^{c}	0.17± 0.0021^{d}	0.35± 0.009^{c}	0.22± 0.0026^{d}	0.54± 0.009^{d}	0.34± 0.004^{d}
20	0.246± 0.003^{c}	0.16± 0.005^{c}	0.995± 0.01^{a}	0.63± 0.01^{a}	0.33± 0.01^{b}	0.21± 0.004^{b}	0.48± 0.11^{b}	0.31± 0.002^{c}	0.66± 0.0105^{c}	0.42± 0.003^{C}
25	0.505± 0.008^{a}	0.32± 0.001^{a}	0.60± 0.008^{b}	0.55± 0.008^{b}	0.43± 0.10^{a}	0.27± 0.00153^{a}	0.53± 0.11^{a}	0.398± 0.003^{a}	1.01± 0.140^{a}	0.64± 0.005^{a}
30	0.48± 0.005^{b}	0.30± 0.002^{b}			0.27± 0.006^{c}	0.172± 0.004^{c}	0.48± 0.10^{b}	0.34± 0.00265^{b}	0.92± 0.112^{b}	0.58± 0.002^{a}

Note: Data are presented as means ± SD (n=3) in each column and for each part means with different letters are significantly different (P<0.05, One Way Anova, Fisher's LSD comparison).

Table 2. Growth rate of *chlorella vulgaris* (O.D and dry wt. (g/L) cultivated under different Copper concentrations.

Days	(0) CU		Cont. (0.079mg/L)		1X CU (0.158 mg/L)		2x CU (0.316 mg/L)		4X CU (0.632 mg/L)	
	Wt.	O.D	WT.	O.D	WT.	O.D	WT.	O.D	WT.	O.D
0	0.03± 0.003^{g}	0.002± 0.0005^{g}	0.026± 0.0037^{f}	0.002± 0.0005^{f}	0.03± 0.004^{g}	0.003±0 .0016^{f}	0.03± 0.003^{f}	0.003± 0.0016^{g}	0.030± 0.003^{g}	0.003± 0.0015^{g}
5	0.099± 0.005^{f}	0.063± 0.002^{f}	0.216± 0.0030^{e}	0.09± 0.004^{e}	0.10± 0.003^{f}	0.063±0 .0011^{e}	0.120± 0.003^{e}	0.076± 0.00152^{f}	0.083± 0.0012^{f}	0.053± 0.001^{f}
10	0.21± 0.007^{e}	0.13± 0.002^{e}	0.38± 0.04^{d}	0.28± 0.01^{d}	0.22± 0.006^{e}	0.14± 0.003^{d}	0.42± 0.005^{d}	0.27± 0.002^{e}	0.17± 0.003^{e}	0.102± 0.002^{e}
15	0.51± 0.009^{d}	0.32± 0.004^{d}	0.50± 0.008^{c}	0.36± 0.009^{c}	0.54± 0.008^{d}	0.441±0 .0036^{c}	1.018± 0.012^{c}	0.65± 0.0021^{d}	0.25± 0.006^{d}	0.16± 0.0015^{d}
20	0.72± 0.010^{c}	0.45± 0.005c^{2}	0.995± 0.010^{a}	0.63± 0.01^{a}	0.997± 0.010^{b}	0.689±0 .004^{b}	1.44± 0.018^{b}	0.918± 0.004^{c}	0.46± 0.010^{c}	0.2907± 0.0031^{c}
25	0.93± 0.01^{a}	0.592± 0.0056^{a}	0.600± 0.008^{b}	0.550± 0.008^{b}	1.20± 0.014^{a}	0.78± 0.004^{a}	1.61± 0.02^{a}	0.99± 0.00666^{a}	0.95± 0.04^{a}	0.60± 0.005^{a}
30	0.78± 0.01^{b}	0.497± 0.0037^{b}			0.84± 0.01^{c}	0.63± 0.003^{b}	1.47± 0.01^{b}	0.94± 0.012^{b}	0.81± 0.020^{b}	0.51± 0.003^{b}

Note: Data are presented as means ± SD (n=3) in each column and for each part means with different letters are significantly different (P<0.05, One Way Anova, Fisher's LSD comparison).

Table 3. Growth rate of *chlorella vulgaris* (O.D and dry wt. (g/L) cultivated on BG_{11} medium with modified Cu and Zn concentrations and combined effect of two elements.

Days	Cont.		(0.316 mg/L CU+ 0.88 mg/LZn)	
	WT.	O.D	WT.	O.D
0	0.026±0.0037^{f}	0.002±0.0006^{f}	0.03±0.0020^{f}	0.003±0.0016^{f}
5	0.216±0.0030	0.089±0.004^{e}	0.086±0.003^{e}	0.055±0.003^{e}
10	0.383±0.035^{d}	0.277±0.0060^{d}	0.315±0.005^{d}	0.201±0.002^{d}
15	0.500±0.008^{c}	0.360±0.0085^{c}	0.442±0.007^{c}	0.280±0.0035^{c}
20	0.995±0.010^{a}	0.630±0.010^{a}	1.121±0.012^{b}	0.710±0.007^{b}
25	0.600±0.008^{b}	0.550±0.0076^{b}	1.310±0.020^{a}	0.830±0.0015^{a}
30			1.100±0.013^{b}	0.770±0.002^{b}

Note: Data are presented as means ± SD (n=3) in each column and for each part means with different letters are significantly different (P<0.05, One Way Anova, Fisher's LSD comparison).

Pigments

The obtained results recorded in Table 4 showed that, in case of (Zn) conc. the elevation of Zinc conc. induced a gradual increase in carotenoids and total pigments which reached its maximum at 1.76 mg/L Zn (2.243±0.082, 6.07±0.438 mg/g). Concerning (Cu) conc. also, the increase in Cu conc. induced an obvious enhancement of pigment content which reached the maximum carotenoids of 3.77±0.2 mg/g and total pigments of 16.349±0.350 mg/g at 0.316 mg/g (Cu) conc. Combination of 0.88 mg/L (Zn) and 0.316 mg/L (Cu) led to an inhibition of pigment production comparable to those produced singly in Zn and Cu conc. (2.825±0.066, 10.81±0.191 mg/g of carotenoids and total pigments respectively).

Table 4. Pigments contents (as mg/g) produced by *C.vulgaris* cultivated under Copper and Zinc conc. separately or in combination.

Treatments	Chl a	Chl b	Carotenoids	Total pigments
zinc conc. (mg/L)				
Control	3.540±0.154^{c}	0.158±0.0068^{e}	1.666±0.100^{c}	5.364±0.0500^{c}
0	2.37±0.130^{d}	1.014±0.0136^{c}	0.9±0.005^{d}	4.300±0.148^{d}
0.44	1.74±0.027^{e}	1.132±0.0887^{b}	0.5±0.0045^{e}	3.387±0.111^{e}
0.88	4.66±0.318^{b}	1.192±0.0291^{a}	2.079±0.0043^{b}	7.937±0.340^{b}
1.76	5.214±0.408^{a}	0.852±0.0320^{d}	2.243±0.0824^{a}	8.310±0.365^{a}
copper conc. (mg/L)				
Control	3.540±0.154^{e}	0.158±0.0068^{f}	1.666±0.100^{d}	5.364±0.0500^{e}
0	3.48±0.419^{e}	0.402±0.0068^{d}	2.100±0.1000^{e}	5.307±0.1528^{e}
0.158	5.160±0.121^{c}	0.676±0.0545^{c}	2.533±0.1523^{b}	8.369±0.300^{c}
0.316	8.891±0.089^{a}	3.688±0.072^{a}	3.77±0.200^{a}	16.349±0.350^{a}
0.632	3.713±0.088^{d}	0.391±0.0698^{e}	1.340±0.0053^{f}	5.447±0.4014^{e}
0.88mg/L Zn+ 0.316mg/L Cu	7.721± 0.083^{b}	0.8377±0.0971^{b}	2.825±0.0661^{c}	10.808±0.191^{b}

Note: Data are presented as means ± SD (n=3) in each column and for each part means with different letters are significantly different (P<0.05, One Way Anova, Fisher's LSD comparison).

Flavonoids and Phenolic compounds content

The obtained data in Table 5 for Cultivation of *C.vulgaris* on BG_{11} medium contained different Zn and Cu concentrations separately or in combination revealed that, at 0.44 mg/L Zn the alga produced a significant large content of flavonoids (23.89±1.336 mg/g) which is much higher than that produced by the control alga (21.495±2.20 mg/g) while lower (zero Zn conc.) or higher Zn conc. (0.88 and 1.76 mg/L) produced lower flavonoid contents compared to both those of control and 0.44 mg/L Zn as shown in Table 5.

Concerning copper, the conc. 0.316 mg/L Cu induced the production of the greatest flavonoid content (which reached 25.062±1.503 mg/g) exceeded that of the control (21.495± 2.2 mg/g) as well as those produced by lower (zero Cu) and higher Cu concentrations (0.158, 0.632 mg/g).

Combination of 0.88 mg/L Zn and 0.316 mg/L Cu induced much lower flavonoid content (7.529±0.74 mg/g) which equivalent to about half of that produced at 0.88 mg/L Zn (15.35 mg/g) and third that produced at 0.316 mg/L Cu (25.06 mg/g).

Also, The obtained data illustrated in Table 5 revealed that, Zn conc., 0.88 mg/L Zn produced the maximum phenolic content (60.166±1.25 mg GA/g) which represent more than double fold those of the control (25.92±0.425 mg GA/g) and it was followed in descending order by the quantity of phenolic content produced at 0.44 mg/L Zn (53.76±0.68 mg GA/g) while zero Zn and 1.76 mg/L Zn induced lower and comparable phenolic contents (20.00 and 19.34 mg GA/g respectively).

In case of Cu concentrations, increasing Cu concentrations (0, 0.158, 0.316 mg/L) induced an increased phenolic production with the maximum content of 35.20±1.058 mg GA/g at 0.316 mg/L Cu, which was about 1.5 folds that of the control (24.8±0.42 mg GA/g).

Combination of 0.88 mg/L Zn and 0.316 mg/L Cu led to extremely lower phenolic production (22.62±0.713 mg GA/g) synthesized by each of single micronutrients (60.166 in case of 0.88 mg/L Zn and 35.20 in case of 0.316 mg/L Cu).

The obtained results in Table 5 showed that, in case of Zn conc., Zn starvation produced the highest tannin content (1.7187±0.054 mg/g).

While in (Cu) conc., the more elevated Cu conc. (0.632 mg/L) produced the highest tannin content (1.412±0.0826 mg/g). Combination of 0.88 mg/L (Zn) and 0.316 mg/L (Cu) induced an increased amount of tannin (1.5426±0.1124 mg/g) which exceeded those produced separately by both Zn and Cu at the same concentrations.

Table 5. Flavonoids, phenolic and Tannins contents (expressed as mg /g dry wt.) produced by *C. vulgaris* grown under Copper and Zinc conc. separately or in combination.

Treatments	Flavonoids contents as (mg of Rutin/g dry wt.)	Phenolic contents as (mg Gallic acid equivalent/g dry wt.)	Tannins conc. as (mg Tannic acid equivalent/g dry wt.)
Zinc conc. (mg/L)			
Control	21.495±2.20^{b}	25.92±0.425^{c}	1.052±0.0989^{b}
0	13.28±1.027^{d}	20.00±0.579^{d}	1.719±0.0545^{a}
0.44	23.892±1.336^{a}	53.76±0.680^{b}	1.004±0.0836^{b}
0.88	15.348±0.664^{c}	60.166±1.25^{a}	0.613±0.0620^{c}
1.76	7.095±0.364^{e}	19.34±1.23^{d}	0.49±0.0437^{c}
Copper conc. (mg/L)			
Control	21.50±2.20^{b}	24.80±0.424^{c}	1.05±0.0989^{c}
0	14.47±1.064^{d}	25.92±0.425^{bc}	0.61±0.0288^{d}
0.158	16.00±1.35^{c}	27.16±0.854^{b}	0.508±0.0284^{d}
0.316	25.06±1.503^{a}	35.20±1.058^{a}	1.317±0.0653^{b}
0.632	1.35±0.370^{f}	15.02±0.159^{e}	1.41±0.0826^{ab}
0.88mg/L Zn+ 0.316mg/L Cu	7.53±0.732^{e}	22.62±0.713^{d}	1.543±0.1124^{a}

Note: Data are presented as means ± SD (n=3) in each column and for each part means with different letters are significantly different (P<0.05, One Way Anova, Fisher's LSD comparison).

Antioxidant activity

ROS are products of a normal cellular metabolism and play vital roles in the stimulation of signaling pathways in plant and animal cells in response to changes in intra- and extracellular environmental conditions. Proteins, nucleic acids and lipids were also significant targets for oxidative attack, and modification of these molecules can increase the risk of mutagenesis. Therefore, antioxidants are good scavengers for ROS and free radicals. In other word it defends and protect cells from their bad action. The antioxidants prevent damages in proteins, DNA (protect from mutation) and lipid peroxidation (protect plasma membrane) in living cells (normal cells).

In the current study, Concerning Zinc conc. (0, 0.44, 0.88, 1.76 mg/L) the obtained results in Table 6 revealed that, elevation of Zn conc. induced an augmentation of antioxidant activity either by DPPH (30 and 60 min.) or ABTS, with maximum activity at 0.88 mg/L Zinc conc. (89.91%) even exceeded those

of control (85.62%). Higher Zn conc. (1.76 mg/L) induced lower activity when compared with synthetic antioxidant standard (BHT).

Concerning Cu concentrations (0, 0.158, 0.316, 0.632 mg/L), the conc. 0.158 mg/L and to less extend 0.316 mg/L Cu, induced higher antioxidant activity recorded by both DPPH and ABTS which exceeded those of the control (Table 6). Combination of 0.88 mg/L Zn + 0.316 mg/L Cu induced a relatively lower antioxidant activity by both DPPH and ABTS assays (73.066, 79.9 and 55.93% respectively) which were much lower not only by those produced by both control and Standard antioxidant (BHT) but also by separate elements at the same conc. as shown in Table 6.

Table 6. Antioxidant activity (%) of *chlorella vulgaris* extract cultivated in different Zinc, Copper combination of Cu and Zn. concentration by DPPH and ABTS.

Treatments	DPPH at 30 min	DPPH at 60 min	ABTS
Zinc conc. (mg/L)			
Control	85.62±0.629^{b}	84.09±0.944616^{b}	61.18±0.742^{d}
0	64.155±1.003^{d}	71.24±0.773^{d}	52±0.980^{e}
0.44	81.683±1.533^{c}	81.283±0.831^{c}	72.1±0.655^{c}
0.88	89.916±0.728^{a}	89.13±0.615^{a}	75.23±0.585^{b}
1.76	82.133±0.907^{c}	82.32±0.470^{c}	49.25±0.901^{f}
Copper conc. (mg/L)			
0	74±0.952^{c}	74.6±0.360^{f}	54.8±0.721^{e}
0.158	87.30±0.60^{a}	84.836±0.660^{c}	78.03±0.850^{b}
0.316	86.293±0.525^{ab}	86.166±0.665^{b}	72.84±0.752^{c}
0.632	74.14±0.593^{c}	77.896±0.661^{e}	40.616±0.625^{f}
0.88mg/L Zn+ 0.316mg/L Cu	73.066±1.006^{c}	79.95±0.676^{d}	55.93±0.901^{e}
BHT	85.633±0.550^{a}	89.5±0.55 ^{a}	90.82±0.687^{a}

Note: Data are presented as means ± SD (n=3) in each column and for each part means with different letters are significantly different (P<0.05, One Way Anova, Fisher's LSD comparison).

Antimicrobial activity

Antimicrobial activity (expressed as diameter of inhibition zone in mm) of *C.vulgaris* (extract) cultivated in stressed culture of BG_{11} medium contained 0.158 and 0.316 mg/L (Cu), 0.44 mg/L and 0.88 mg/L (Zn) against standard antibacterial G-ve, G+ve (Gentamycin, Ampicillin respectively) and standard antifungal drugs (Nystatin) were given in Table 7, the obtained results revealed

that, in gram –ve bacteria, only 0.88 mg/L (Zn) produced a moderate antibacterial activity (13.3±0.5 mm) against *E. coli* while *Klebsiella pneumonia* gave no activity with any of the test extracts.

In Gram+ve bacteria, *Staphylococcus aureus* produced variable antibacterial activities with the test extracts ranged between 15.3±0.6 to 20.6±0.6 mm. 0.158, 0.316 mg/L (Cu) extracts produced highinhibition zones of 15.3±0.6 and 17.6±0.6 mm. respectively. The highest antibacterial activity was produced by (Zn) containing extracts (0.44 and 0.88 mg/L) recording an activity of 20.6±0.6 and 19.0±1.00 mm which were near to that of standard Ampicillin. Relatively lower activities were obtained with the same extracts against *Streptococcus mutans* giving an activity ranged 10.3±0.5-14.6±0.5 mm.

Concerning fungal sp. *Aspergillus niger* have no activity while the yeast *Candida albicans* produced moderate activity (10.3-11.6 mm) with extracts from algae cultivated under Zn stress while Cu conc. have no activity.

Table 7. Antimicrobial activity (as diameter of inhibition zone in mm) of *C. vulgaris* (extract) cultivated in BG_{11} medium contained stressed conc. of Zn and Cu.

Sample microorganism	0.158 mg/L (Cu)	0.316 mg/L (Cu)	0.44 mg/L (Zn)	0.88 mg/L (Zn)	Standard antibiotic
Gram negative bacteria					**Gentamicin**
Escherichia coli (ATCC:9637)	NA	NA	NA	13.3±0.5	27±0.5
Klebsiella pneumonia (ATCC:10031)	NA	NA	NA	NA	25±0.5
Gram positive bacteria					**Ampicilin**
Staphylococcus aureus (ATCC:6538)	15.3±0.6	17.6±0.6	20.6±0.6	19.0±1.0	22±0.1
Streptococcus mutans (ATCC:25175)	NA	10.3±0.5	12.6±0.5	14.6±0.5	30±0.5
Fungi					**Nystatin**
Candida albicans (ATCC:10231)	NA	NA	11.6±0.5	11.3±0.5	21±0.5
Asperagillus Nigar (ATCC:32856)	NA	NA	NA	NA	20±0.5

Antitumor activity

MTT assay was used to assess the cytotoxicity of the crude extract of *C.vulgaris* against three different solid tumor cell lines. Different cell lines were used according to their origin and morphology as well as sensitivity and receptor sites behavior. The cytotoxicity parameter, IC_{50} was calculated. The obtained results of the crude extract (Figure 1) showed moderate potency against HeLa and HCT 116 cell lines for algae cultived under 0.316 mg/L Cu with IC_{50} of 94.2 and 145 µg/mL respectively, followed by algae cultived under 0.158 mg/L Cu with IC_{50} 187 and 197 µg/mL respectively. However, MCF-7 cell lines showed relatively higher resistance against the crude extract with IC_{50} ranged from 303 to 757 µg/mL for all tested extracts (Figure 1).

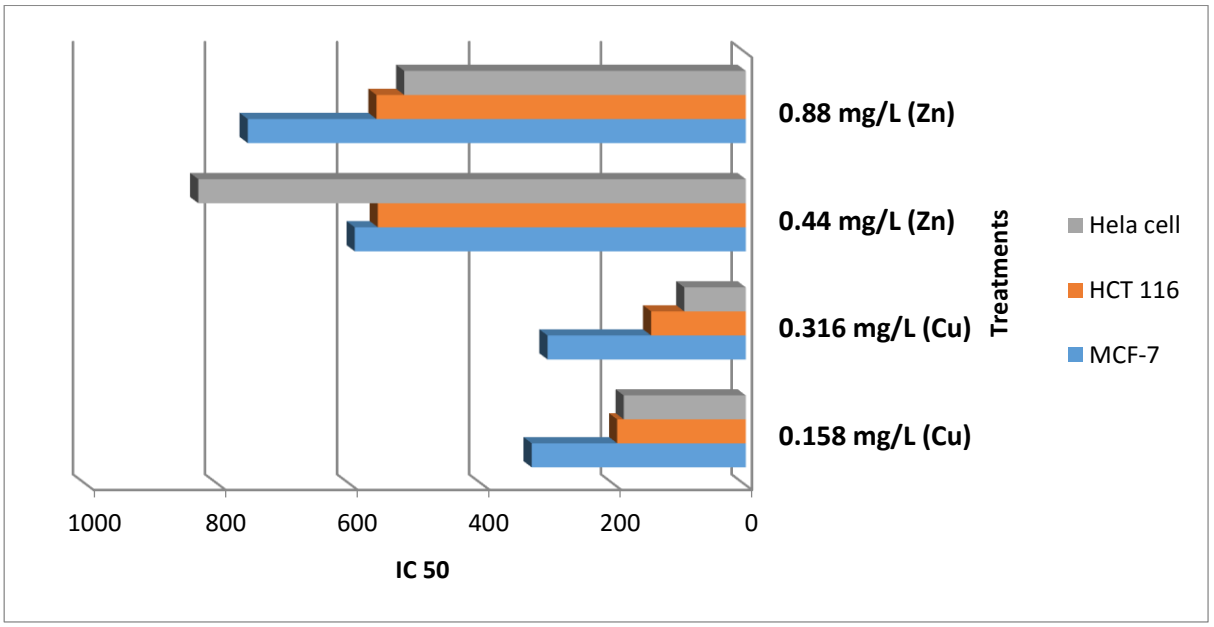

Figure 1. Cytotoxity (IC_{50}) of tested extracts from *chlorella vulgaris* cultivated under diff. conc. from Zn and Cu on various cancer cell lines.

DISCUSSION

The growth rate results of *Chlorella vulgaris* cultivated under various zinc concentrations (of BG_{11} medium) may be due to that Zinc can easily be rationalized since it is a micronutrient required for microalgal metabolism (Bascik-Remisiewicz et al., 2009) as it is a part of prosthetic moieties of some of its relevant enzymes. In our study, the toxicity effect of zinc was progressively decreased when the exposure time was increased which was judged by the increased growth rate of the cultures. This may be due to the accumulation of zinc onto algal cells in higher concentrations which significantly affect/reduced the available zinc ion concentration and its toxicity to algal surviving cells in the final period of experiment as agreed by Lim et al. (2006).

However, the declined growth of *C.vulgaris* at high copper conc. may be due to the resistant of *C.vulgaris* to tested Cu concentrations (from 0.158 mg/L to 0.632 mg/L Cu) and copper, seems to regulate the expression of functional activity as conc. factor for different algal enzymes as reported by Harris (1992). Also, these results were in agreement with results obtained by Mamboya et al. (1999) who reported that, copper is acutely toxic to *Padinaboergesenii* at concentrations higher than 500 mg Cu/L. Low concentration of copper is toxic to *P. boergesenii* after a prolonged exposure of 21 days.

The data in Table 3 showed that the combination of Cu + Zn showed an inhibitory effect of growth compared to those of control this may be due to the accumulation of zinc or Cu onto algal cells in higher concentrations which significantly affect/reduced the available zinc and cu ions concentration and its toxicity to algal surviving cells in the final period of experiment as agreed by Lim et al. (2006).

The elevation of Zinc and copper conc. induced a gradual increase in pigments contents (carotenoids and total pigments) in *C.vulgaris*, these results may be due to the gene expression from the effect of Zn and Cu stress on algal cell and led to increase of algal pigments as deference compounds against this abiotic stress such as carotenoids, these results were in agreement with those obtained by Juan et al. (2005) Who reported that As for the moderate concentrations of Cu (up to 0.1 mM), they caused an increase in carotenoids, which protect against stress, since these pigments are responsible for the suppression of singlet oxygen ($^1O_2$), reducing lipid peroxidation and consequent oxidative damage.

From the data in Table 5, cultivation of algal cell under Zn conc. produced a significant large content of flavonoids (23.89±1.336 mg/g), However, Concerning copper, the conc. 0.316 mg/L Cu induced the production of the greatest flavonoid content. Furthermore, Combination of 0.88 mg/L Zn and 0.316 mg/L Cu induced much lower flavonoid content (7.529±0.74 mg/g), these results may be due to the gene expression from the effect of Zn and Cu stress on algal cell and led to increase of antioxidant compounds such as phenolic compounds, flavonoids, carotenoids. These results were in agreement with the results obtained by Sakihama et al. (2002), who found that Phenolic compounds responded to stress. However, at 0.1 mM the values presented a significant increase when compared to the control. Thus, these compounds may be acting as antioxidants as well as playing the role of chelating agents (Michalak, 2006). The observed increase in the concentration of phenolic compounds is confirmed by the data shown for anthocyanins and carotenoids, which are also compounds formed by aromatic rings. All these molecules along with proline (neutralization of OH- and $^1O_2$), which presented the same behavior, probably have protective role in plant physiology in response to Cu stress in different cellular compartments, since anthocyanins are found in the vacuole, carotenoids in the chloroplast as well as proline in the cytosol.

The obtained results in Table 5 showed that, in case of Zn conc., Zn starvation produced the highest tannin content (1.7187±0.054 mg/g).

While in (Cu) conc., the more elevated Cu conc. (0.632 mg/L) produced the highest tannin content (1.412±0.0826 mg/g). Combination of 0.88 mg/L (Zn) and 0.316 mg/L (Cu) induced an increased amount of tannin (1.5426±0.1124 mg/g) which exceeded those produced separately by both Zn and Cu at the same concentrations.

The antioxidant activity of active ingredients of the promising extracts from *C.vulgaris* cultivated under 0.44 and 0.88 mg/L Zn and 0.158 and 0.316 mg/L Cu may be correlated with the presence of hydroxyl group and unsaturated bonds in the chemical structure of its compounds which show high ability for scavenging free radicals and prevent the oxidation processes, as shown in Table 9, the promising extract are rich with bioactive compounds as antioxidant compounds such as Milbemycin b, cyclononasiloxane, hexadecanoic acid, 10-octadecanoic acid, Tetrakis, cyclotrisiloxane. These observations were in agreement with the previously published results (Kadri et al., 2011; Abd El-Aty et al., 2014; Khurgain et al., 2017; Sayik et al., 2017). Also, the obtained results correlated with data in Table 5, there are strong correlation between the antioxidant activity of promising extracts (Table 6) and concentration of flavonoids and phenolic compounds in Table 5, the algae cultivated under 0.44 and 0.88 mg/L Zn produced 23.89 and 15.34 mg/g flavonoids and 53.76 and 60.16 mg/g phenolic content. However, algae cultivated under 0.158 and 0.316 mg/L Cu produced 16.0 and 25.06 mg/g flavonoids and 27.16 and 35.20 mg/g phenolic content.

The obtained data recorded in Table 8 revealed that Concerning Zn, increased Zn conc., induced a gradual increase in lipid peroxidation while 1.76 mg/L (Zn) produced the highest lipid peroxidation (198.85±2.00 nmol/g). Moreover in case of 0.316 mg/L Cu conc. induced the greatest lipid peroxidation content (277.77±2.45 nmol/g) was induced. Combination of 0.88 mg /L (Zn) and 0.316 mg/L (Cu) led to the production of greater lipid peroxidation (217.74±2.50 nmol/g) than those produced by separate (Zn), 137.84±1.32 but less than that of (Cu) at the same concentrations (277.77±2.45). Concerning Glutathaione-S-transferase, (U/g tissue), the data recorded in Table 8 illustrated that, (Zn) conc., 1.76 mg/L produced 309.412±2.59 U/g while in case of (Cu) conc., 0.632 mg/L induced 345.93±3.06 U/g of the enzyme. Combination of 0.88 mg/L Zn and 0.316 mg/L Cu induced greater enzyme content (291±3.13 U/g). Concerning catalase activity (%), (Zn) conc., 1.76 mg/L Zn induced the greatest enzyme content (%) which reached 52.09±1.57%. Concerning (Cu) conc. the highest Cu conc. 0.632 mg/L induced the maximum catalase activity (57.97±1.93%). Combination of 0.88 mg/L (Zn) + 0.316 mg/L (Cu) produced an increased a catalase activity (47.3±1.70%) which was much more than those produced by 0.88 mg/L Zn but less than those produced by 0.316 mg/ L Cu separately. These results may be due to the mechanism of effect of heavymetal as inducer of oxidative stress in algal cell of *Chlorella* microalga activated a variety of antioxidative enzymes such as

SOD, GST, CAT and APX to diminish the ROS. These biological responses can be interpreted as a tolerant mechanism as reported by Ajayan and Selvaraju (2012).

Table 8. Lipid peroxidation (MAD n mol/g), Protein (mg/g), Glutathaione-S-transferase (U/g tissue), Catalase activity (%) produced by *C.vulgaris* cultivated under Copper and Zinc conc. separately or in combination.

Treatments	Lipid peroxidation (MAD n mol/g)	Protein conc. as mg/g	GST (U/g)	Catalase activity (%)
Zinc conc.(mg/L)				
Control	66.51±0.67^{e}	125.15±3.77^{b}	69.87±1.90^{d}	33.53±0.96^{d}
0	170.21±1.40^{b}	80.52±2.26^{d}	190.00±2.23^{b}	40.09±1.65^{b}
0.44	130.08±1.52^{d}	74.40 ±3.92^{e}	175.82±1.98^{c}	37.97±1.02^{c}
0.88	137.84±1.32^{c}	113.88±3.18^{c}	191.23±2.03^{b}	41.90±1.30^{b}
1.76	198.85±2.00^{a}	172.30±3.16^{a}	309.412±2.59^{a}	52.09±1.57^{a}
copper conc. (mg/L)				
Control	66.51±0.67^{e}	125.15±3.77^{d}	69.87±1.90^{f}	33.53±0.96^{e}
0	90.34±0.89^{d}	122.66 ±2.58^{e}	117.61±1.66^{e}	32.79±0.94^{e}
0.158	248.03±2.06^{b}	171.89.84^{c}	170.58±1.90^{d}	45.215±1.06^{d}
0.316	277.77±2.45^{a}	472.28±2.02^{a}	224.32±1.95^{c}	55.128±1.23^{b}
0.632	250.64±2.01^{b}	103.57±1.24^{f}	345.93±3.06^{a}	57.97±1.93^{a}
0.88 mg/L Zn	217.74±2.50^{c}	172.30±3.16^{a}	291.50±3.13^{b}	47.3±1.70^{c}

Note: Data are presented as means ± SD (n=3) in each column and for each part means with different letters are significantly different (P<0.05, One Way Anova, Fisher's LSD comparison).

The antimicrobial activity of *C.vulgaris* cultivated under Zn and Cu conc. may be due to the types and content of secondary metabolites which synthesized during algal cultivated under Cu, Zn stress, from these metabolites which recorded antimicrobial activites: Milemycin b, cycloheptasiloxane, cyclooctasiloxane, hexadecanoicbacid, 2-hexadecanoic-1-Ol, cyclodecasiloxane, 4-Eicosaenoic acid, Tetrakis, 13-Docosenoic acid, Cyclotrisiloxane, Octasiloxane as shown in Table 9. these compounds have high ability to inhibit microbial growth by its effect on microbe replication, microbe enzymes, cell membrane etc. as reported by Rollas and Kücükgüzel, 2007; Kalaisezhiyen and Sasikumar, 2012; Krishnaveni et al., 2014; Khurgain et al., 2017, Mebude and Adeniyi, 2017; Sayik et al., 2017.

From the obtained data in Figure 1 , it is clear that the cytotoxicity pattern of the crude extract of *C.vulgaris* on both HCT 116 and HeLa cell lines is similar while differs on MCF-7. These results indicate that the effect of crude extract on HCT 116 and Hela cells is concentration dependant through the concentrations tested (1-1000 µg/mL). This effect can be explained as receptor independent for these type of cells (Westerink and Schoonen, 2007).

Also, the cytotoxic profile of the crude extract may be due to its content from bioactive compounds as mention in Table 9, the data revealed that the algal cultivated under 0.316 Cu mg/L was rich with different anticancer compounds such as Milbemycin b, cycloheptasioxane, hexadecanoic acid, cyclotrisiloxane, 1,2-benzenedicarboxylic acid. Furthermore, alga cultivated under 0.158 mg/L (Cu) was rich with the following anticancer compounds: Toosendanin, Milbemycin b, cycloheptasioxane, hexadecanoic acid and cyclotrisiloxane as recorded by Rollas and Kücükgüzel 2007; Kalaisezhiyen and Sasikumar, 2012; Krishnaveni et al., 2014; Khurgain et al., 2017; Mebude and Adeniyi, 2017 and Sayik et al., 2017 (Table 9).

CONCLUSION

From our data, it can be concluded that the pronounced results may encourage a country-wide project for making a pharmaco-economic value of microalgae in Egypt. In this concern, our results support the use of *Chlorella vulgaris* for the production of active compounds especially for pharmaceutical remedies. In addition, they have scavenging antioxidant properties against the reactive oxygen species. The present study reveals the high potency of crude extracts as antioxidants and potential anticancer in comparison to synthetic standards. This crude extract represents favorable economic and industrial value for the production of commercial products. the biological activities of *C.vulgaris* increased for more than 2 times when cultivated under stress of Cu and Zn ions and it can used for the production of bioactive compounds with high percentage.

Table 9. GC/mass for most 4 promising *chorella vulgarisextracts* (as antioxidant) cultivated under cultivated under various Zn and Cu conc.

No.	Compound name	Molecular weight	Samples (Area %)				Biological activity	References
			0.158 mg/L CU	0.316 mg/L CU	0.44 mg/L Zn	0.88 mg/L Zn		
1	2-(3',4'-bis(trimethylsilyloxy)phenyl)-2-oxoethyl-n,n-bis(trimethylsilyl)-amine	455	0.79					
2	TOOSENDANIN	574	0.66				Anti-cancer, Anti-botulismicageny	Zhang et. al., 2018
3	Milbemycin b, 13-chloro-5-demethoxy-28-deoxy-6,28-epoxy-5-(hydroxyimino)-25-(1-methylethyl)-, (6R,13R,25R)-	603	0.98	1.65	2.20	0.94	Antioxidant, Antimicrobial, Antiviral, Anti-inflammatory, Ant diabetic activity	Khurgain et. al., 2017
4	3-Pyridinecarboxylic acid, 2,7,10-tris(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-1,1,3,6,9-pentamethyl-4-oxo-4a,7a-epoxy-5H-cyclopenta[a]cyclopropa[f]cycloundecen-11-yl ester, [1aR-(1aR*,2R*,3S*,4aR*,6S*,7S*,7aS*,8E,10R*,11R*,11aS*)]-	597	0.08					

Table 9. Cont.

No.	Compound name	Molecular weight	Samples (Area %)				Biological activity	References
			0.158 mg/L CU	0.316 mg/L CU	0.44 mg/L Zn	0.88 mg/L Zn		
5	Cycloheptasiloxane, tetradecamethyl-	518	2.82	2.48	2.40		antibacterial, antifungal, antifouling, immunomodulatory and antitumor activities	Patil and Jadhav, 2014
6	Cyclooctasiloxane, hexadecamethyl-	592	5.66	5.80	4.54	0.85	Antimicrobial activity	Mebude and Adeniyi, 2017
7	s-(tert-butyl) [2-(5-hydroxy-2-pentynyl)-3-oxocyclopentyl]ethanethioate	296	0.10					
8	Cyclononasiloxane, octadecamethyl	666	6.13	11.59	8.37		Antioxidant activity	Kadri et. al., 2011
9	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-	206	0.15	0.15			Antioxidant, Antiviral, Antidiabetic analgesic activity.	Prakash and Suneetha, 2014
10	DOTRIACONTANE	450	0.09				Antimicrobial activty	Cherchi et. al., 2001
11	Dimethoxyglyceroldocosyl ether	460	0.16				Antimicrobial activity	Kenawy et. al., 1998; Mohamed et. al., 2015
12	Ethanol, 2-(9-octadecenyloxy)-, (Z)-	312	0.16					

Table 9. Cont.

No.	Compound name	Molecular weight	Samples (Area %)				Biological activity	References
			0.158 mg/L CU	0.316 mg/L CU	0.44 mg/L Zn	0.88 mg/L Zn		
13	FLAVONE 4'-OH,5-OH,7-DI-O-GLUCOSIDE	594	0.08	0.12			Antioxidant activity	Rice-Evans et. al., 1996
14	17-Pentatriacontene	490	0.14	0.27				
15	Hexadecanoic acid, methyl ester	270	1.24	0.90	5.13	2.34	Antioxidant, antimicrobial, hypocholesterolemic, nematicidal, pesticidal, hemolytic, antiandrogenic, hemolytic, 5-alpha reductase inhibitor cancer enzyme inhibitors in pharmaceutical, cosmetics, and food industries	Sayik et. al., 2017; Kalaisezhiyen and Sasikumar, 2012
16	Cis-2-phenyl-1, 3-dioxolane-4-methyl octadec-9, 12, 15-trienoate	440	0.15	0.26			Antibacterial, Antifungal activity	Sosa et. al., 2016
17	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16 ,16,18,18 ,20,20- ICOSAMETHYLCYCLODECASI L OXANE	740	5.27					

Table 9. Cont.

No.	Compound name	Molecular weight	Samples (Area %)				Biological activity	References
			0.158 mg/L CU	0.316 mg/L CU	0.44 mg/L Zn	0.88 mg/L Zn		
18	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	342	0.55				Antimicrobial activity	Krishnaveni et. al., 2014
19	4H-1-benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-á-dglucopyranosyl-5,7-dihydroxy-	610	0.37	0.41			Antioxidant, antimicrobial, cancer enzyme inhibitors in pharmaceutical, cosmetics, and food industries	Sayik et. al., 2017
20	2-HEXADECEN-1-OL, 3,7,11,15-TETRAMETHYL-, [R-[R*,R*-(E)]]-	296	0.61	0.25	1.09		anticancer, anti-inflammatory and antimicrobial ,antioxidant activities	Kalaisezhiyen and Sasikumar, 2012; Mohamed et. al., 2015
21	10-Octadecenoic acid, methyl ester	296	1.82	1.33			Antioxidant, antimicrobial, cancer enzyme inhibitors in pharmaceutical, cosmetics, and food industries	Sayik et. al., 2017, Kalaisezhiyen and Susikumar, 2012; Sosa et. al., 2016
22	Cyclodecasiloxane, eicosamethyl-	740	48.53	53.78	10.67		Antimicrobial activity	Jasim et. al., 2015

Table 9. Cont.

No.	Compound name	Molecular weight	Samples (Area %)				Biological activity	References
			0.158 mg/L CU	0.316 mg/L CU	0.44 mg/L Zn	0.88 mg/L Zn		
23	11-Eicosenoic acid, methyl ester	324	4.50	3.13	8.66	19.65	Antimicrobial activity	Rahbar et. al., 2012
24	TETRAKIS (dimethylsilylcarbodiimide)	392	0.39	0.12	0.30	0.71	Antioxidant, Antibacterial activity	Abd El-Aty et. al., 2014
25	13-Docosenoic acid, methyl ester, (Z)-	352	1.19	0.84	2.22		Antimicrobial activity	Krishnaveni et. al., 2014
26	THIENO[3,4-C]PYRIDINE, 1,3,4,7-TETRAPHENYL	439	0.29		1.49	6.56	Anticonvulsant, antidepressant, analgesic, anti-inflammatory,antiplatelet, antimalarial,antimicrobial,antimycobacterial,antitumoral,antiviral and antischistosomiasis activities	Rollas and Kücükgüzet, 2007
27	spirosolan-3-ol, 28-acetyl-, acetate (ester), (3á,5à,22á,25s)-	499	0.43	0.27	2.70			
28	Cyclotrisiloxane, hexaphenyl-	594	16.64	15.91	36.25	48.91	Antioxidant, Antimicrobial, Antiviral, Anti-inflammatory, Ant diabetic activity	Khurgain et. al., 2017
29	6,9,12,15-Docosatetraenoic acid, methyl ester	346		0.13			Phytopharmaceutical importance	Srivastava et. al., 2015

Table 9. Cont.

No.	Compound name	Molecular weight	Samples (Area %)				Biological activity	References
			0.158 mg/L CU	0.316 mg/L CU	0.44 mg/L Zn	0.88 mg/L Zn		
30	1,2-Benzenedicarboxylic acid, butyl octyl ester	334		0.23			Antioxidant, antimicrobial, anticancer	Sayik et. al., 2017
31	Octasiloxane	578			3.70		Antimicrobial, antioxidant, anticancer	Venkatesh et. al., 2014
32	9-Octadecanoic acid (Z)-, methyl ester	296			7.4	3.68	Antimicrobial, antioxidant, anticancer	Sayik et. al., 2017
33	Trilinolein	878				2.13	Anti-ischemic and antioxidant activity	Srivastava et. al., 2015
34	Cholesta-8, 24-diene-3-ol, 4, 14-dimethyl-acetate	454				0.57	Antiviral activity	Mohamed et. al., 2015

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