Salt Stress Enhancement of Antioxidant and Antiviral Efficiency of *Spirulina platensis*

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Abstract: Cultivation of *Spirulina platensis* under salt stress conditions (0.02 (control), 0.04 and 0.08 M NaCl) led to a remarkable alteration of algal metabolism as well as an enhancement or induction of biologically active compounds concerning algal growth, salt stress caused a decrease in dry weight, chlorophyll a content as well as certain xanthophylls (neoxanthin and violaxanthin) while β-carotene production was stimulated especially at higher salt concentrations. Biochemical analysis of salt stressed alga revealed that lipid content was slightly increased together with certain saturated and unsaturated fatty acids espcially the polyunsaturated ones (Gamma linolenic acid). Electrophoretic analysis of soluble protein pointed out that certain high molecular weight protein bands were not detected comparing with the protein marker. Five new protein bands of molecular weights 190, 158, 113, 77 and 28 kDa were recorded, in addition to an increase in the intensity of 6 already existing bands. Phosphate buffer and water extracts of the alga exhibited antiviral activities against both Hepatitis-A-virus-type-MBB (HAV-MBB strain, RNA virus) and Herpes simplex-virus-type-1 (HSV-1, DNA virus). Water extracts was found to be more effective than phosphate buffer extracts in inducing antiviral activities (98%) espicially against HSV-1 virus. The same water extract of the salt stressed alga demonstrated higher anticoagulating activity compared with those of heparin and the positive control measured by clotting time assay. Antioxidant activity of the algal successive extracts against 2, 2 diphenyl-1-picrylhydrazyl and 2,2'- azino-bis (ethylbenzthiazoline-6- sulfonic acid) radical methods revealed moderate antioxidant activity of the non-polar algal extracts (petroleum ether) which were doubled with increasing extract concentration (55.0, 60.4 and 62.3, 66.8 µg/ml at 0.02, 0.04 M NaCl respectively) the lowest activity was recorded by the partialy polar (ethyl acetate) algal extract of both concentrations at all salinity levels. While the polar extracts (ethanol and water) showed higher antioxidant activities which were doubled with increasing extract concentration. Ethanolic algal extract (of 100 µg/ ml at 0.08 M NaCl) exhibited the highest antioxidant activity compared with those of the synthetic antioxidant butylated hydroxy anisol as standard (85.0, 89.9 and 86.0, 91.8% respectively). [Journal of American Science. 2010;6(10):38-51]. (ISSN: 1545-1003).

Key words: Antioxidant-Antivirus -Biochemical studies- -*Spirulina platensis*- Salt stress

1. Introduction

Many cyanobacteria and microalgae were considered as a natural source of various biologically and pharmacologically active compounds with structurally complex molecules which are difficult or impossible to be produced by chemical synthesis (Smith and Doan, 1999).

Genus *Spirulina* has gained an importance and international demand for its high phytonutrients value and pigments which have applications in healthy foods, animal feed, therapeutics and diagnostics (Becker, 1994; Vonshak and Tomaselli, 2000). *Spirulina* has been used as food and nutritional supplements since long time (Dillon *et al*., 1995). It is generally a rich source of protein, vitamins, essential amino acids, minerals, essential fatty acids such as γ-linolenic acid and sulfolipid (Mendes *et al*., 2003). Moreover in addition to ω-3 and ω-6-poly unsaturated fatty acids,it has also phycocyanin and other phytochemicals (Chamorro *et al*., 2002).

Some *Spirulina* species exhibit antibacterial (Ozdemir *et al*., 2004), antiplatelet (Hsiao *et al*., 2005), antihepatoxic (Mohan *et al*., 2006) and antiviral activities (Hernandez-Corona *et al*., 2002). *Spirulina* as many other cyanobacteria species have the potential to produce a large number of antimicrobial substances, so they are considered as suitable candidates for exploitation as biocontrol agents of plant pathogenic bacteria and fungi (Kulik, 1995).

Salinity represents one of the most important factors exerting stress injury on the growth and metabolism of plants.

Salt stress causes an imbalance of the cellular ions resulting in ion toxicity and osmotic stress, leading to retardation of growth either directly by salt or indirectly by oxidative stress induced by reactive oxygen species (ROS).

Salinity can cause significant accumulation of compatible solutes which acts as enzyme producers, stabilizing the structure of macromolecules and organelles (Dahlich *et al*.,

1983). Salinity stress may alter the metabolic pathways of stressed organism(s) leading to either enhancement or induction of biologically active compounds.

The present work aimed to investigate the different biological activities of *Spirulina platensis* and the relations with its biochemical composition, pigments and different constituents which may vary with salt stress culture conditions.

2. Material and Methods

Chemicals and reagents:

Pure hexane, chloroform, ethanol, ether, acetone, methanol and acetic acid were purchased from E.Merch Co. (Germany), and distilled before use. Butylated hydroxy anisole, Tween 20, 2, 2 diphenyl-1-picrylhydrazyl (DPPH) and 2,2'- azino-bis (ethylbenzthiazoline-6- sulfonic acid (ABTS), standard fatty acids and hydrocarbons were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Algal species and culture conditions

Spirulina platensis was obtained from the lab of phycology In Botany Department, Faculty of Science, Cairo University, Egypt. The alga was cultivated on liquid Zarrouk medium (Zarrouk 1966). Different sodium chloride concentrations were used (0.02 (control), 0.04 and 0.08 M NaCl). *Spirulina platensis* was cultured (Botany Department, Faculty of Science, Cairo University, Egypt) in 3 L flasks containing 2l Zarrouk medium (pH 9) containing different salt concentration, 200 ml algal inoculums supplemented with aeration tubes. Cultures were incubated at 25 ºC ±1ºC, light intensity of 40 µE / m²/s (Cool white fluorescent lamps), photoperiod of 16/8 light, dark cycles, for 20 days after which algal cells were harvested by centrifugation at 10,000 rpm / 5 min, frozen by liquid nitrogen and stored at -20 ºC till use.

Determination of growth rate by dry weight method

Algal samples from the different salt concentrations (10 ml) were filtered under vacuum through 0.45 µm filter membrane and washed several times with distilled water. Then, algal cells were dried at 100 ºC for 30min and weighed (Abd El-Baky *et al.*, 2003).

Phytochemical analysis

Extraction and determination of algal pigments

Determination of chlorophyll was carried out according to Holden (1965) method, where the fresh sample (0.5g) was grinded in a mortar with acetone in presence of calcium carbonate then filtered. The absorbance of extracts was measured at 663 and 645 nm in 1cm quartz cell against 80% aqueous acetone as blank.

Extraction and determination of water soluble pigments (phycobiliprotein):

The water soluble phycobiliproteins pigments including allophycocyanin (APC), phycocyanin (PC) and C-phycoerytherine (C-PC) were extracted from algal cells (1g) with 10 ml phosphate buffer (0.05 M, pH 6.8) according to the method described by Bryant*l*,. 1979. The absorbance (A) of the extracts was recorded at the following wave lengths: 650nm; 620nm and 565nm.

Identification of *Spirulina* lipophilic pigments by thin layer chromatography (TLC):

Photosynthetic pigments were separated from 10 µl of total lipid extract by silica gel thin layer (60 F_{254}, E.Merck, Germany). The following five solvents systems were used: 1- Hexane : acetone, (7: 3 v/v); 2- Toluene : acetone, (6: 4 v/v); 3- Hexane : ether : formic acid, (80: 20 2 v/v/v); 4- Acetone 5% in chloroform and 5- Pet-ether (40-60ºC) : acetone, (7 : 3 v/v). Identification of individual pigment were determined by R_f value on TLC plate which was compared with that of authentic sample and with values reported in literature (Schneider 1966).

Electrophoretic fractionation of soluble proteins

Polyacrylamide gel electrophoreses in the presence of Sodium Dodecyl Sulphate (SDS-PAGE) was used for determining the molecular weight of protein fractions (Water soluble protein) according to method of Laemmli **(**1970**).** Standard molecular weight proteins marker was obtained from Sigma, this marker content proteins at varied molecular weight: 119 (β-galactosidase), 98 (Bovin serum albumin), 52 (Ovalbumin), 36 (Carbonic anhydrase) and 30 (Soybean trypsin inhibitor) kDa.

Extraction and determination of total lipids:

Lipids were extracted by the modified method described by Xu *et al.* (1998).

Separation and identification of fatty acids

Fatty acids methyl esters (FAME) were analyzed by gas liquid chromatography (GLC) according to Farag *et al.* (1986) under specific conditions of column. The separated fatty acids were identified by comparing their retention times with those of standard fatty acid methyl ester (purity 99% by GLC, sigma Co.). Also, Co-chromatography and GC/MS methods were used for verification of the peaks identity and position of double bond in fatty acid molecules.

Separation and identification of unsaponified matter

The unsaponifiable compounds were identified by GLC using an instrument equipped with a flame ionization detector (FID). The unsaponifiable compounds (hydrocarbon and sterols) were identified by comparing their retention times with those of standard hydrocarbons from C8 to C36 and some authentic sterols (Cholesterol, stigmasterol and β-sitosterol).

Biological Activities

Antiviral activity of algal extracts

Preparation of samples for antiviral bioassay

Extracts were dissolved as 100mg each in 1ml of 0.1 M phosphate buffer (pH 7). The final concentration was 100 µg/µl (Stock solution)

Viruses used

Herpes simplex virus type 1 (HSV-1) and Hepatitis A virus (cell culture adapted strain MBB). The two viruses were obtained from virology laboratory, NRC. Viruses were propagated and titrated on Vero cell (HSV-1) and HepG2 for HAV-MBB strain.

Cytotoxicity assay

Double fold dilution of each sample was prepared in deionized water (1:2 to 1:256) and diluted samples were inoculated in 96 well. Twice culture plate containg confluent monolayer of Vero cell and another plate containing HepG2. Cells were incubated at 37 ºC overnight and examined microscopically for cytopathic effect (CPE). The lower dilutions, which showed no morphological changes on cell cultures, were selected for antivirus bioassay (Abad *et al*., 2000).

Plaque infectivity reduction assay

The method described by Silva *et al.* (1997) was used where, a 6-well plate was cultivated with Vero cell (HSV) and another plate containing HepG2 (HAV) culture (10^5cell/ml) and incubated for 2 days at 37^0 C. virus was diluted to give 10^7 PFU/ml as final concentrations and mixed with the algal extract at the previous concentration and incubated overnight at 4^0 C. Growth medium was removed from the multiwell plate and virus-compound mixture was inoculated (100µl/well). After contact time, inocula were aspirated, agarose were overlaid, and plates were left to solidify and incubated until the development of virus plaques. Cell sheets were fixed in 10% formalin and stained with crystal violet stain. Virus plaques were counted and the percentage of reduction was calculated.

Mode of action

The inhibition mechanism of virus by crude algal extracts was studied using two methods: Viral replication according to Amoros *et al*. (1994) and Viral adsorption according to Zhang *et al.* (1995).

Determination of anticoagulation activity:

The anticoagulating activity of water algal extracts were investigated using the method of USA pharmacopia (1985) as follow: Each tubes, 0.8 ml of extract solution (1 %), 0.8 ml of standard heparin sodium solution (0.5 U.S.P unit/0.8 ml), or 0.8 ml saline solution was added. Then, 1 ml plasma and 0.2 ml of calcium chloride solution (1%) were added in each tube. The tubes were stopped immediately, and inverted three times in such a way to mixed the contents that the entire inner surface of the tube became wet. The time required for clotting was determined.

Determination of Antioxidant activity:

a- DPPH method

The 2, 2 diphenyl-1-picrylhydrazyl (DPPH) test was carried out as described by Burits and Bucar (2000). One ml of *Spirulina* extract (Hexane, chloroform, ethyl acetate, ethanol and water extracts) at different concentration was mixed with 1ml DPPH reagent (0.002% (w/v) /methanol water solution). After an incubation period, the absorbance was measured at 517nm. BHA was used as positive control and extracts concentration providing 50% inhibition (IC50) was calculated from the graph plotting inhibition percentages against extract concentration.

%Antioxidant activity =Ac-At / Ac x 100 where: At was the absorbance of the algal extract samples and Ac the absorbance of methanolic DPPH solution.

b- ABTS method

This assay was based on the ability of different substances to scavenge 2,2'- azino-bis (ethylbenzthiazoline-6- sulfonic acid (ABTS.+) radical cation in comparison to the standard BHA (50 and 100 µg/ml). According to the method of Re *et al*. (1999), the antioxidant activity of the tested samples was calculated by determined the decrease in absorbance at different concentrations (50 and 100 µg/ml) by using the following equation: Antioxidant activity= ((Ac-At)/ Ac) x 100, where: At and Ac are the respective absorbances of tested samples and $ABTS^{\cdot+}$.

Statistical analysis

Data were subjected to an analysis of variance, and the means were compared using the "Least Significant Difference (LSD)" test at the 0.05

and 0.01 levels, as recommended by Snedecor and Cochran (1982).

3. Results and Discussion

Concerning the biochemical analysis of the salt stressed *S. platensis*, the algal growth was slightly affected by low salt concentration (0.02M NaCl), increasing salinity (0.04 and 0.08 M) led to a marked and progressive inhibition of growth translated as a decrease in algal dry weight (Fig. 1). Our results were in accordance with the results recorded by Fodorpataki and Bartha (2004) where salt stressed (0.5 M) *Scenedesmus opoliensis* led to an increase in rate of cell divisions, decrease in biomass, chlorophyll content.

Sudhir *et al.* (2005), reported that 0.8 M NaCl caused a remarkable decrease in photosystem II (PS II) mediated oxygen evolution activity of *S. platensis*. Our results were in agreement with those of Abdel-Rahman *et al.* (2005), who reported that higher salt concentrations (150-250 mM NaCl) reduced growth, carbohydrate and protein contents of both *Chlorella vulgaris and Chlorococcus humicola.* Also the obtained results of salt stressed *S. platensis* went parallel with those of Shanab (2007), Shanab and Galal (2007) where the salt tolerant *Chlorella sp* grown under different NaCl conc. (100-400 mM) their dry weight as well as pigment content were decreased at all NaCl conc. While the salt sensitive *Scendesmus sp* recorded a remarkable decrease in dry weight and pigment content at the lowest salt conc. used (100 mM). In concomitant with the obtained results in this investigation.

The inhibition of growth under salt stress conditions was certainly due to alteration of algal metabolism which might be directed towards the production of substances which have a role in algal salt tolerance or defense mechanism. Chlorophyll content of *S. platensis* (Table 1) was affected by salt stress conditions where chlorophyll a, neoxanthin, violaxanthin were recorded at low salinity and faintly detected at higher once (using TLC) while the reverse was shown by β-Carotene. The water soluble phycobiliprotein (Table 2) composed of phycocyanin (CPC), phycoerythrin (PE) and allophycocyanin (APC). Increasing salt conc. enhanced the production of both phycocyanin and phycoerythrin while allophycocyanin (APC) production was inhibited leading to a marked decrease in total phycobiliprotein content. Our results went parallel with those of many investigators (Cifferi 1983, Piorreck *et al*., 1984, Becker 1994 and Rogel-Yogui *et al*., 2004) where different salt and nitrogen concentrations induced changes of chlorophyll and phycobiliprotein pigment contents of *Spirulina* species. It seems that under severe salt stress, the algal defense mechanisms do not allowed to spend too much energy for the synthesis of many new chlorophyll molecules and binding proteins which may explain the decrease in chlorophyll and allophycocyanin contents in our results (Fodorpataki and Bartha .,2004). Moreover, these were confirmed by the absence of 47 kDa chlorophyll protein and 94 kDa protein linking phycobilisomes to thylakoid from *Spirulina platensis* SDS-electropheritic analysis (Garnia *et al*., 1994; Fodorpataki and Bartha, 2004).

Salt stress conditions not only affected algal growth, pigment content but also protein and lipid production of the stressed alga. Analysis of soluble proteins (by SDS electrophorsis) of *S. platensis* cultivated under different salt concentrations and recorded in Table (3) and Fig. (2), revealed that, no protein bands of high molecular weight(190-117), were recorded at the highest NaCl conc. used (0.08 M). While two new highly intensive protein bands of molecular wts, 113, 77 were recorded only at higher NaCl conc. Also certain bands were present at low and moderate salt conc. (0.02 and 0.04 M) but absent (not detected) at higher ones (0.08 M). Moreover six protein bands were detected at low and/or moderate salt conc. but their intensities were highly increased at higher salt stress conditions (of M.wts 106, 90, 82, 67, 35 and 30). Absence of either new protein bands or an increase in the intensity of 42 and 37 KDa bands confirmed the obtained results concerning the decrease in total phycobiliprotein pigments under salt stress conditions. The obtained results concerning protein analysis of salt stressed *S. platensis* was comparable to those of S. *maxima* cultivated under nitrogen stress condition (Shalaby 2004). Both *Spirulina* species have two specific new protein bands of molecular weight 113 and 76 in addition to a highly intensive band at M.wt 103. Higher numbers of new protein bands were recorded in *S. maxima* at different nitrogen conc. and not equivalent to similar bands (of the same M.wt) produced by *S. platensis* under salinity stress conditions. These differences may be due to variable metabolic processes in both species and to the availability of nitrogen (essential for protein synthesis) in study of ***S. maxima*** and present only as normal medium constituent in experiments of *S. platensis.*

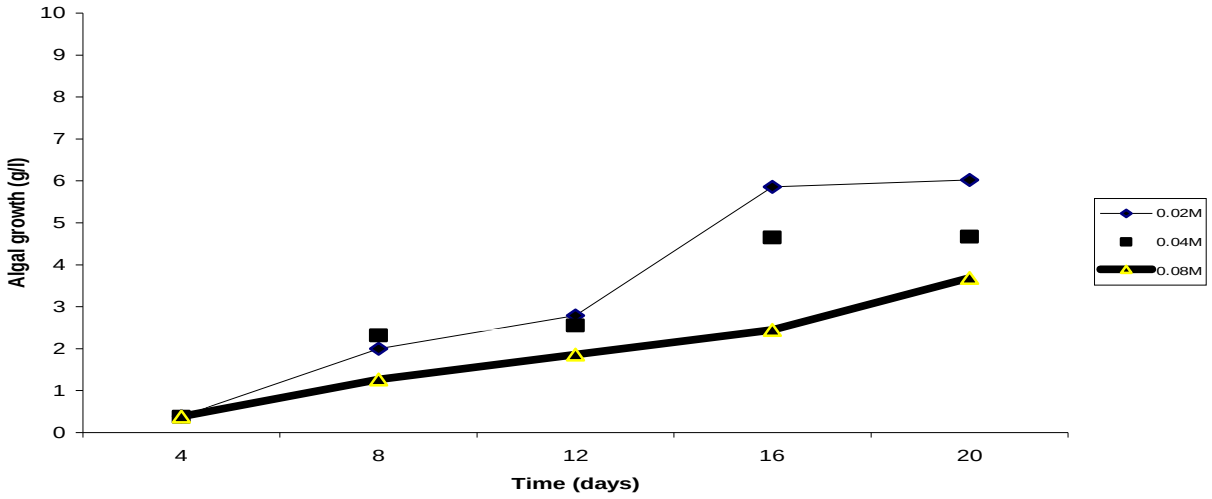

Fig.1: Growth rate of *Spirulina platensis* cultivated under different salt concentration (0.02, 0.04 and 0.08 M NaCl) during 20 days incubation period, represented as dry weight (g/l).

Table (1): Separation of *Spirulina platensis* Lipid soluble pigments produced under salt stress conditions by TLC using different organic solvent systems:

Pigments	hR_f	NaCl Concentration (M)		
		0.02 (control)	0.04	0.08
Myxoxanthophyll	5.0	+	+	+
Neoxanthin	10	+	-	-
Violaxanthin	22	+	-	-
Lutein	41	+	+	+
Chlorophyll a	67	+	-	-
β-carotene	88	+	+	+

* +: present -: absent; hRf: Rf x 100

Table (2): Relative percent of phycobiliprotein pigments (CPC, APC and PE) of *Spirulina platensis* cultivated under salt stress conditions (0.02, 0.04 and 0.08 M NaCl).

Concentration of NaCl (M)	Phycobiliprotein pigments (g/100 g F.wt)			
	Phycocyanin (CPC) %	Allophycocyanin (APC)%	Phycoerytherin% (PE)%	Total phycobilin %
0.02 (Control)	**1.76**	5.70^{a}	0.45^{b}	7.91^{a}
0.04	**1.80**	4.50^{b}	0.65^{a}	6.95^{b}
0.08	**1.91**	2.05^{c}	0.74^{a}	4.70^{c}
LSD	NS	0.1006	0.1006	0.1006

Each value is presented as mean of triplet treatments, means within each row with different letters (a-c) differ significantly at P # 0.05 according to Duncan's multiple range test,

Table (3): SDS-Electrophoretic analysis of soluble proteins produced by the salt stressed *S.platensis* cultivated under different NaCl Concentration (0.02, 0.04 and 0.08 M).

Protein band	Molecular weight (kDa)	hR_f	Concentration of NaCl (M)		
			0.02 (Control)	0.04	0.08
High molecular weight (HMW, %)					
1*n.p	190	0.7	-	5.99	-
2	180	2.1	4.1	-	-
3	167	8.1	1.4	2.72	-
4	160	10.2	3.45	3.09	-
5*n.p	158	10.8	-	0.73	-
6	150	13.3	1.32	1.55	-
7	147	14.3	2.4	-	-
8	145	15.7	3.0	2.65	-
9	137	18.8	2.94	2.15	-
10	133	20.9	1.2	2.60	-
11	124	24.3	4.56	3.79	-
12	117	27.8	5.6	4.31	-
13*n.p	113	28.8	-	-	8.62
14	106	31.8	2.23	2.32	5.53
15	103	33.3	1.32	-	12.14
16	101	34.3	2.2	-	-
Medium molecular weight (MMW, %)					
17	97	35.9	2.25	3.23	-
18	93	37.3	1.36	3.28	-
19	90	39.1	2.30	1.60	14.05
20	89	39.9	2.9	3.94	-
21	82	43.1	4.19	4.16	11.98
22*n.p	77	46.8	-	-	8.96
23	73	48.7	4.48	4.32	-
24	67	52.3	1.06	3.34	13.47
25	64	53.1	0.36	4.94	-
26	58	57.8	5.64	5.65	-
27	50	60.5	4.5	3.41	-
Low molecular weight (LMW, %)					
29	47	65.4	5.3	3.53	-
30	40	71.3	2.88	4.36	-
31	38	77	3.77	8.00	8.08
32	35	81	2.64	5.34	6.53
33	30	82.3	10.23	3.68	10.64
34*n.p	28	86.7	-	1.84	-
35	25	90.2	5.78	3.48	-

*: not present; n.p: new protein; hR_f**:** $R_f \times 100$

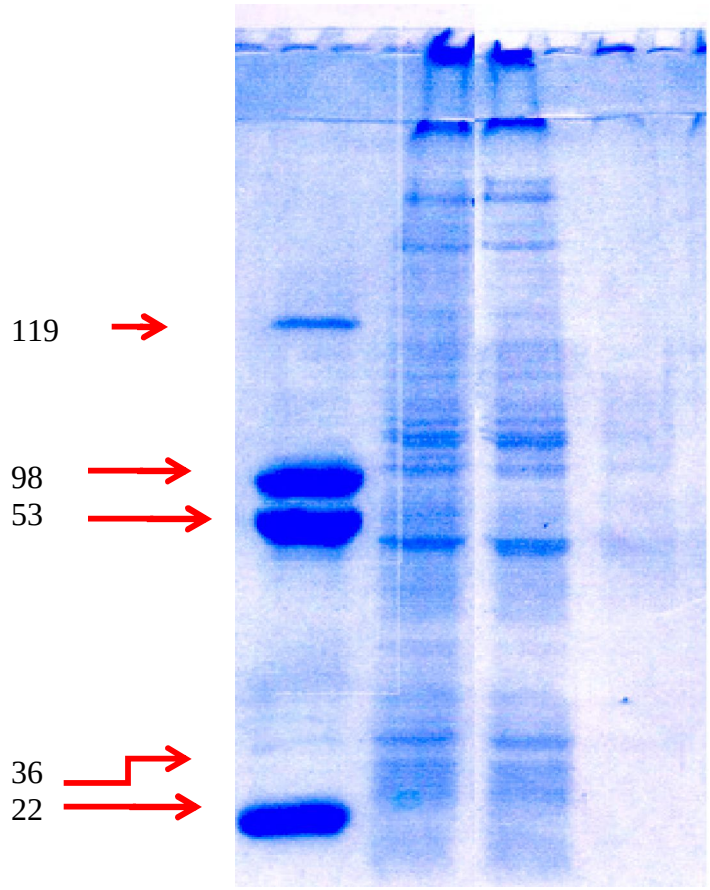

Fig.2: SDS-Electrophoretic analysis of soluble proteins produced by the salt stressed *S .platensis* cultivated under different NaCl Concentration (0.02, 0.04 and 0.08 M).

Concerning lipids of cyanobacteria including *Spirulina*, they are esters of glycerol and fatty acids; they may be either saturated or unsaturated and may have polyunsaturated fatty acids. Total lipid content of salt stressed S. platensis was slightly increased at higher salt conc. (8.0, 8.0 and 9.0% at 0.02, 0.04 and 0.08 M NaCl respectively). Total lipids contained fatty acids and hydrocarbons which were affected by culture conditions as salinity, N-starvation, light intensity (Tedesco and Duerr 1989, Abd El-Baky *et al*., 2004). Generally, exposure of microalgae to any deleterious environmental change responds in many different ways, one of which is the modification of lipid composition in order to maintain the critical degree of membrane fluidity (Romano *et al*., 2000). The relative percentage of fatty acids in stressed *S. platensis* lipids illustrated in Table (4) recorded remarkable changes which were induced by various salt concentrations. The % of total saturated fatty acids produced were 85.6, 37.2 and 67.24% in 0.02, 0.04 and 0.08 M NaCl respectively, while the unsaturated fatty acids were 14.9, 62.8 and 32.6% including 7.2, 46.3 and 14.2 % polyunsaturated fatty acids. These results clearly showed that lipids at lower salt conc. have majority % of saturated fatty acids and as salinity increased (from 0.02 to 0.04 M) unsaturation also increased including large percentage of polyunsaturated fatty acids (C16:2, C16:3, C18: 2 and C18:3). At higher salt conc. (0.08 M) % unsaturated fatty acids decreased due to the desaturation, oxidative peroxidation and consequently changes in membrane fluidity, permeability and cellular metabolic functions (Bandopadhyay *et al*., 1999, Singh *et al*., 2002).

Table (4): Relative percentage (%) of fatty acids and hydrocarbons in *S.platensis* lipid extract produced under different NaCl concentrations (0.02, 0.04 and 0.08 M).

Fatty acids	Sodium chloride conc. (M)			Hydrocarbons	Sodium chloride conc. (M)		
	0.02	**0.04**	**0.08**		**0.02**	**0.04**	**0.08**
C8:0	0.4	0.2	0.24	**C12**	-	-	-
C10:0	-	-	-	**C15**	-	-	-
C12:0	0.4	1.3	4.2	**C18**	-	19.7	0.5
C14:0	10.5	-	10.5	**C20**	11.3	-	-
C16:0	29.8	18.0	25.2	**C21**	0.8	53.7	2.5
C16:1 (ω 7)	2.9	5.5	3.6	**C22**	2.8	1.9	28.3
C16:2 (ω 2)	2.7	2.3	5.9	**C26**	19.0	2.9	2.9
C16:3 (ω 3)	2.9	22.5	6.6	**C28**	13.6	15.2	12.3
C17:0	-	-	-	**C29**	22.3	3.7	43.9
C18:0	44.5	17.7	27.1	**C30**	11.1	1.7	9.5
C18:1 (ω 9)	2.8	11.0	15.8	**C32**	18.0	1.2	-
C18:2 (φ6)	2.0	7.4	-	**C34**	1.0	-	-
γC18:3(ω6)	1.6	14.1	0.7	**C36**	-	-	-
Total saturated acids	85.6	37.2	67.24				
Total monounsaturated fatty acids	5.7	16.5	19.4				
Total polyunsaturated fatty acids	9.2	46.3	13.2				
Total omega 6 fatty acids	3.6	21.5	0.7				
Total omega 3 fatty acids	2.9	22.5	6.6				
Total lipids%	8.0±0.4	8.0±0.2	9.0±0.6				
TU/Ts	0.17	1.6	0.48				

*Each value represents the average of three replicate run.
TU/TS: Total unsaturated/Total Saturated ; *- : not present

Relative percentage of hydrocarbons was also affected by salinity stress where C12, C15 and C36 were not detected at all NaCl conc, while C18 was only produced with moderate percentage at 0.04 M then markedly decreased at higher salinity level. C20 was only recorded at lower salt conc. on the other hand C21, C22, C26, C28, C29 and C30 were present at all salinity levels but with different relative percentages, C21 was highly increased at moderate salinity conc. (53.7%) then dropped to 2.5% at high salt conc., while C22 markedly increased at higher NaCl conc. (28.3%). Also C29 its relative % at low NaCl conc. (22.3%) was doubled at higher salt stress condition to reach 43.9%. C32 was markedly decreased with increasing NaCl concentration from 0.02 to 0.04 (18, 1.2%) then completely disappeared at higher salinity level. The obtained biochemical analysis of *S. platensis* encouraged the investigation of various biological activities.

Concerning the antiviral activity, Table (5) showed that algal water extract (50 µg/ml) of low salt concentration exhibited (0.02 M) relatively higher (60.0%) antihepatitis A virus-type MBB more than phosphate buffer extract (9.0%) of the same concentration and the activity of the latter extract increased (56.0-58.0%) at moderate salt concentration (0.04 M) using 20 and 50 µg/ml extract concentration respectively. On the other hand, the antiviral activity against herpes simplex virus –type 1 showed a comparable activity by both water and phosphate buffer extracts of both concentrations at all salinity levels with maximum antiviral activity (98.0%) at 50 ug/ml extract concentration.

Table (5): Antiviral activity (%) of phosphate buffer and water extracts of the salt stressed *S. platensis* (at conc.20 and 50 µg/ml) using Hepatitis A virus type MBB (HAV-MBB) and Herpes Simplex virus type 1 (HSV-1).

NaCl (M)	HAV-MBB virus (RNA virus)				HSV-1 virus (DNA virus)			
	20 µg/ml		50 µg/ml		20 µg/ml		50 µg/ml	
	Phosphate buffer	Water extract	Phosphate buffer	Water extract	Phosphate buffer	Water extract	Phosphate buffer	Water extract
0.02 (Control)	9.0[c]	40.0[a]	9.0[c]	60.0[a]	90.0	96.0[a]	93.0[b]	98.0[a]
0.04	56.0[a]	32.0[b]	58.0[a]	34.0[b]	88.0	90.0[b]	90.0[c]	94.0[b]
0.08	32.0[b]	25.0[c]	37.0[b]	25.0[c]	90.0	96.0[a]	98.0[a]	98.0[a]
LSD	2.0318	1.1006	2.465	2.465	NS	1.123	2.250	2.250

Each value is presented as mean of triplet treatments, means within each row with different letters (a-c) differ significantly at P # 0.05 according to Duncan's multiple range test,

The antiviral activity against HSV-1 (DNA virus) was markedly pronounced (98.0%) than that against HAV-MBB (60.0%) which is an RNA virus. These activities which were shown to be controlled by both type and concentration of algal extract (water or phosphate buffer, at 20 and 50 µg/ml) may be induced by the sulphated polysaccharide and tannins in *S. platensis* extracts (Witvrouw and De clereq 1997). These antivirus substances may interfere at one or more of viral stages, either at the stage of virus attachment or penetration to the host cell, or at the virus replication or the virus maturity and release stages. The obtained results concerning these activities against HAV-MBB and HSV-1 viruses were in agreement with the data obtained by Hayashi *et al*. (1996) who found that water extract of *S.platensis* inhibited the replication in vitro of herpes simplex virus type 1 in Hela cell within the concentration range 80-50 ug/ml. our results were also in accordance with those reported by Witvrouw and De clercq 1997, who emphasized that sulphated polysaccharides were found to be potent and selective inhibitors of HIV-1 replication in cell culture. Moreover, Ayehunine *et al.* (1998) reported that an aqueous extract of *S. platensis* inhibited HIV-1 replication in Human T-cell lines and langerhans cells and their antiviral activity was found in polysaccharides fraction. Our results were confirmed by and coincided with the results reported by Shalaby (2004), where phosphate buffer and water extracts of *S. maxima* cultivated under different N-Conc. Exhibited weak antiviral activity against HAV-MBB and highly pronounced activity against HSV-1 viruses. He added that the sulphated polysaccharides produced from fractionation of water extract (called Ca-*Spirulina*) caused the inhibition of virus penetration into the host cells.

Experiments carried out with dextran sulphate revealed that the antiviral activity increased with increasing molecular weight and degree of sulfation of the sulphated polysaccharides. Many microalgal polysaccharides significantly inhibit the infection of Vero cell by HSV-1, HSV-2 and VZV viruses, and these compounds did not show any cytotoxic effect even at greater dose concentration (Huleih *et al*., 2001).

The activity of polar extract of *Spirulina* platensis (at concentration 20 and 50 µg/ml) against HSV-1 and the clinical strain were evaluated by the plaque reduction assay. the algal extract did not induce any effect on virus replication The effect of algal extract on virus adsorption In the second set of experiment, the inhibitory effect of extract of *Spirulina* sp (at concentration 20 and 50 µg/ml) on virus adsorption to host cell was measured by monitoring the attachment of infectious HSV virions on to host cells in the presence of their extract. The results indicated that algal extracts completely inhibited (99.99%) the cell-associated infectivity. as compared with that in control levels. These results were agreement with these obtained by Boyed *et al.* (1997) who found that the antiviral activity of cyanovirin- N (CV-N) isolated from *Nostoc sp* against HIV-2 is due, at least in part, to unique, high-affinity interaction of CV-N with the viral surface envelope glycoprotein gp120.

The mode of action of *S. platensis* extracts (at concentration 20 and 50 µg/ml) against both viruses were evaluated by the plaque reduction assay. The results reported that the microalgae extract did

not induce any effect on virus replication algal extracts completely inhibited (100%) the cell-associated infectivity as compared with that in control levels.

Regarding the anticoagulation activity of the hot water extract of salt stressed *S. platensis*, the obtained results (Fig.3) showed that great anticoagulating efficiency (expressed by clotting time assay) compared with that of the standard anticoagulatant heparin (sulfate glucouronic acid) to be 13, 17 min respectively. our results were in agreement with those recorded by Shalaby (2004) on *S.maxima* where the clotting times were inversely proportional to nitrate concentration in the growth media (11, 12, 13 at 410, 205, 102.5 ppm N compared with 16 min in case of heparin). This activity was reported to have a close relation with the water extract containing sulfated polysaccharides and phenolic compounds and depend upon the molecular size, type of sugar and sulphate content and position of the active components (Shanmugam and Mody 2000). Therefore, in future algal sulphated polysaccharides water extracts can be used as anticoagulant/antithrombitic agent, in medical purposes, replacing the known heparin which was extracted from internal organs of higher animals and exhibited haemorrhagic like side effects.

Extracts of *S. platensis* by organic solvents of different polarities and concentrations showed that the polar solvents (ethanol and water) extracts at higher concentration (100 ug/ml) exhibited higher antioxidant activity (85.0, 89.9% by DPPH and ABTS respectively) comparable to the standard antioxidant, BHA (86.5 and 91.8 %). This is followed in the second order by the non-polar (pet. ether) extract at high concentration (100 µg/ml) of both low and moderate salinity levels (55.0, 62.3% and 60.4, 66.8% by DPPH and ABTS respectively) while the partially polar ethyl acetate extracts demonstrated the lowest antioxidant activities at all salt concentrations (ranged from 0.0 to 23.6%) as recorded in Tables 6 and7.

The obtained results revealed that polar antioxidant substances might be present in the polar *Spirulina* extract to which attributed the antioxidant activity. These polar substances were found in extracts of different red, brown and green seaweeds (Matsukawa *et al*., 1997, Anggediredja *et al*.1997, Lim *et al*. 2002, Santoso *et al*. 2004, Zhang *et al*. 2007, Shanab 2007 and Shalaby 2008) as well as in microalgae and cyanobacterial species (Abd El-Baky, 2003, Reddy *et al*. 2003, Benedetti *et al*. 2004, Shalaby 2004)., these substances mainly include phycocyanin pigment, sulphated polysaccharides and phenolic compounds which are largely present in most macro, micro and cyanobacterial species which exhibited pronounced antioxidant activity. Non-polar antioxidant substances were recorded also in microalgae as *Chlorella vulgaris*, *Dunaliella salina* (Nirupama, 2004, Murthy *et al*. 2005 and Zhang-Bao *et al*. 2004) in cyanobacteria (Shalaby 2004) in addition to the different seaweed species (Krinsky 1989, Hyun *et al*., 2003, Xi *et al*., 2003, Sook *et al*., 2004, Yuan *et al*., 2005, Shanab 2007 and Shalaby 2008). These non-polar substances include carotenoids (β-carotene, Astaxanthin, and Zeaxanthin), chlorophylls and fatty acids which were largely enhanced by salinity stress and reported to have higher antioxidant activities (Endo *et al*., 1985; Murthy *et al*., 2005).

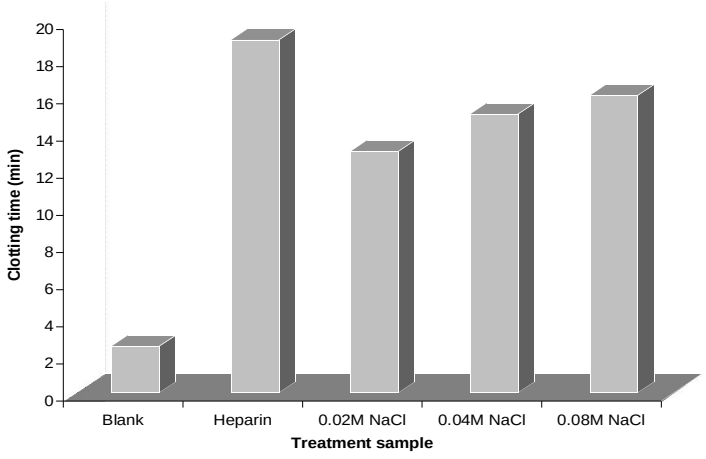

Fig.3: Anticoagulation activity (clotting time) of the hot water extracts of the salt stressed *S. platensis*.

Table (6): Antioxidant activity (%) of salt stressed *S. platensis using* different solvent extracts (pet. ether, ethyl acetate, ethanol and water) of different concentration (50 and 100 µg/ml), butylated hydroxyl anisole (BHA) was used as standard antioxidant against DPPH radical.

NaCl (M)	Solvent extract	Concentration (µg/ml)	
		50	100
0.02 (Control)	Pet.ether (40-60)	29.0±2.3^{m}	55.0±3.2^{f}
	Ethyl acetate	0.0±0.0^{u}	9.0±0.5^{s}
	Ethanol	31.5±1.5^{k}	75.0±3.8^{b}
	Water	30.0±2.4^{l}	65.0±2.6^{d}
0.04	Pet.ether (40-60)	29.0±0.8^{m}	62.3±2.8^{e}
	Ethyl acetate	0.0±0.0^{u}	11.0±0.9^{r}
	Ethanol	20.5±2.5^{o}	44.0±4.5^{h}
	Water	20.0±1.6^{p}	45.0±3.5^{g}
0.08	Pet.ether (40-60)	11.0±0.1^{r}	25.0±0.89^{n}
	Ethyl acetate	5.0±0.6^{t}	17.0±1.0^{q}
	Ethanol	32.0±2.8^{j}	85.0±4.8^{a}
	Water	35.6±4.3^{i}	65.8±3.8^{c}
BHA		60.6±2.6^{e}	86.5±4.5^{a}
LSD		0.9179	

Each value is presented as mean of triplet treatments, means within each row with different letters (a-s) differ significantly at P # 0.05 according to Duncan's multiple range test,

Table (7): Antioxidant activity (%) of salt stressed *S. platensis using* different solvent extracts (pet. ether, ethyl acetate, ethanol and water) of different concentration (50 and 100 µg /ml), butylated hydroxyl anisole (BHA) was used as standard antioxidant against ABTS radical.

NaCl (M)	Solvent extract	Concentration (µg/ml)	
		50	100
0.02 (control)	Pet.ether (40-60)	28.4±1.5^{jk}	60.4±4.1^{e}
	Ethyl acetate	5.6±0.5^{p}	9.4±1.0^{o}
	Ethanol	34.8±1.0^{i}	81.7±3.5^{b}
	Water	32.5±2.6^{i}	64.8±2.4^{d}
0.04	Pet.ether (40-60)	29.9±3.4^{ij}	66.8±3.6^{cd}
	Ethyl acetate	6.8±0.5^{op}	16.5±2.3^{m}
	Ethanol	22.4±0.3^{l}	45.8±1.8^{g}
	Water	26.5±1.2^{k}	50.8±5.0^{f}
0.08	Pet.ether (40-60)	12.3±0.08^{n}	26.8±1.3^{k}
	Ethyl acetate	8.9±2.0^{o}	23.6±2.4^{l}
	Ethanol	40.8±1.2^{h}	89.9±5.6^{a}
	Water	40.5±3.0^{h}	68.9±3.1^{c}
BHA		67.4±2.3^{d}	91.8±4.5^{a}
LSD		2.825	

Each value is presented as mean of triplet treatments, means within each row with different letters (a-p) differ significantly at P # 0.05 according to Duncan's multiple range test.

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