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## Phytochemical and Biological Studies on Proteins Isolated from Different Microalgal Species

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**Abstract**

Microalgae are rich in high protein content that enhances their nutritional and therapeutic benefits in addition to their ability for high mass production. Therefore, the present study was designed to reveal the biological efficiency of the natural proteins isolated form *Dunaliella salina*, *Scenedesmus obliquus* and *Spirulina platensis*. During the current study, it was found that the highest protein content was noticed in *S. platensis* (48.44%) followed by *D. salina* (31.75%) then *S. obliquus* (28.56%). The protein content decreased upon applying some stress conditions on *S. platensis* and *S. obliquus*. The electrophoretic detection of the proteins isolated from the microalgal species showed that the vegetative and stressed *S. obliquus* were physiologically similar to *S. platensis* by 42.11 and 57.14%, respectively. *D. salina* was similar to *S. platensis* by 46.15%. As compared to the vegetative *S. obliquus*, the stressed *S. obliquus* and *D. salina* were similar to 30.77 and 33.33%, respectively. The *D. salina* was similar to the stressed *S. obliquus* by 57.14%. The protein pattern was electrophoretically represented by 10 denatured bands in *S. platensis*, 9 bands in the vegetative *S. obliquus*, 4 bands in the stressed *S. obliquus* and 3 bands in *D. salina*. There was one common band identified in all microalgal species at relative mobility (Rf) 0.95 (Molecular weight (Mwt) 5.27 KDa). There were 5 characteristic (unique) bands identified in the vegetative *S. obliquus* at Rfs 0.07, 0.17, 0.50, 0.58 and 0.86 (Mwts 214.68, 137.74, 42.37, 30.18 and 12.44 KDa, respectively). The amino acids that were analyzed in the isolated protein hydrolysate showed that 15 amino acids were noticed in *S. platensis* and *S. obliquus*. While in *D. salina*, 17 amino acids were identified. Glutamic acid was the most predominant amino acid and exhibited the highest percentages in *D. salina*, *S. obliquus* and *S. platensis* (4.96%, 3.3% and 7.58%, respectively). Additionally, the *in-vitro* studies on the isolated proteins showed that the *D. salina* protein exhibited the highest antioxidant activity at a dose of 100 µg/ml (308.78%) followed by the *S. platensis* and *S. obliquus* proteins (140.81% and 124.49%, respectively). Furthermore, *D. salina* and *S. obliquus* showed moderate anticholinesterase activity (34% and 36%, respectively). Therefore, microalgal proteins might be used as therapeutic antioxidants and might be used in combination with treatments of Alzhiemer's disease (AD).

***Keywords***: *Dunaliella salina*, *Scenedesmus obliquus*, *Spirulina platensis*, Proteins, Antioxidants, Electrophoresis.

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## 1. Introduction

The recent study emphasized that algae have attracted global attention due to its potential to produce lots of valuable natural products **[1,2]**. Microalgae are rich sources of the biologically active (primary and secondary) metabolites that have been isolated and being developed as an effective alternative to antibiotics to be gained importance especially to combat disease problem **[3,4]**. It is well known that microalgae are rich in protein contents. Some of them contain protein contents higher than animal or plant sources, e.g. *Spirulina paltensis* (*S. platensis*) is considered as protein source more than dried skimmed milk, chicken, fish, beef and peanuts **[5]**. Moreover, they are commercially important due to its ability for high mass production with low cost in addition to its nutritional, therapeutic and pharmacological benefits **[6]**. Therefore, the recent studies directed to search in different microalgal species to be evaluated phytochemically and biologically.

The protein content was studied widely followed by analyzing the major amino acids in *S. platensis*, *Scenedesmus obliquus* (*S. Obliquus*), *Dunaliella salina* (*D. salina*). It was found that the major amino acids were represented by glutamic acid, aspartic acid and leucine in *S. platensis* and *D. salina.* While in *S. obliquus*, the major amino acids were represented by glutamic acid, alanine and aspartic acid **[5]**.

It is well known that *Spirulina* is a commercial alga containing various antioxidants (especially phycocyanin) that exhibit protective efficiency against diabetic nephropathy **[6]**. It was reported that phycobiliprotein including allo-phycocyanin, phycocyanin and phycoerythrin represented about 20% of the total protein content in *S. platensis* **[5]**. The C-Phycocyanin derived from *S. platensis* is considered as a novel hypocholesterolemic protein **[7]**. It is a complete protein containing all essential amino acids **[8]**.

**Nuhu [9]** showed that *Spirulina* displayed anticancer and antimicrobial via the production of phycocyanin, phycocyanobilin and allophycocyanin. Also, **Greque de Morais *et al.* [10]** reported that phycocyanin, found in *Spirulina* had an anti-inflammatory activity. Additionally, **Vadiraja *et al.* [11]** found that C-phycocyanin isolated from *Spirulina* reduced $CCl_4$ induced liver injury in rats and may act as a scavenger against attack of free radicals.

In 2018, **Darvish *et al.* [12]** isolated proteins from *D. salina* by hydrolysis and they found that peptide fractions (<3 kDa and 63 kDa) significantly reduced viability of colon cancer cells and exhibited antimicrobial effect against *Escherichia coli*, *Staphylococcus aureus* and *Helicobacter pylori*. Concerning *S. obliquus*, an increase in body weights was observed in children fed with diets incorporated with *Scenedesmus* sp. Also, mycosporine-like amino acids and sporopollenin were extracted and used as UV screening compounds for skin protection. These amino acids are important for skin hydration, elasticity and photoprotection. Therefore, they are included in cosmetics **[13]**. Moreover, the protein hydrolysates isolated from *S. obliquus* have a potential antioxidant effect **[14]**.

The current work was designed to study the proteins isolated from three microalgal species that selected to be under investigation and followed by evaluating their biological activities as cytotoxic, antiviral, antioxidant and anticholinesterase.

## 2. Materials and Methods

### *2.1. Algae material*

*S. platensis* that belongs to Cyanophyta was cultivated using Zarrouk medium **[15]**. *S. obliquus* and *D. salina* that belong to Chlorophyta were cultivated using BG-II medium **[16]** and artificial sea water **[17]**, respectively. All algae species were cultivated and collected from the Algal Biotechnology Unit, Biological and Agricultural Research Division, National Research Centre, Dokki, Giza, Egypt. For each algal species, the stress was achieved in the growth basically by increasing salinity to 2.0% Sodium chloride, 45 mM sodium acetate (as organic carbon) and 125 ppm ferrous sulfate as iron **[18]**. Vegetative and stress-growth were performed within a 200-L vertical sheet photo bioreactor **[19]**. Growth conditions were varied based in growth site (in and out-door). Also, harvesting, purification and drying of these algal species were performed **[20,21]**.

### *2.2. Separation of algal protein by salting method*

The dried powdered algae (400g) were defatted with petroleum ether (60-80%) and chloroform. Half the defatted algae were stirred in sodium chloride solution (10%) for 1hr several times (100ml) and then filtered. An equal volume of trichloroacetic acid (TCA) solution (10%) was added to the filtrate. The protein was precipitated as white flocculent amorphous precipitate, collected by centrifugation, washed with TCA solution (5%) then with ether and absolute ethanol and dried in vacuum desiccators. The crude dried protein was dialyzed by parchment membrane. The non-dialyzable fraction was collected and lyophilized by freeze drying according to the method described by **Aly *et al.* [22]**.

### *2.3. Estimation of total protein*

The total protein was estimated by Micro-Kjeldahl method **[23]**. In brief, a sample of a dried algal powder (0.2-0.3g) was weighed into a digestion flask containing sulphuric acid (10ml) and 0.5g digestion mixture $K_2SO_4$-$CuSO_4$ (1:4). The sample was then incinerated and the digested sample was quantitatively transferred into the Markham micro-Kjeldahl with the least amount of ammonia-free distilled water and then 20ml of NaOH solution (50%) was added. A strong current of steam was passed and the ammonia was distilled into a 10 ml of boric acid solution (2.5%). The ammonia was then titrated against HCl using mixed indicator (0.4 ml methylene blue and 0.1 ml methyl red) until a faint red end point was obtained. Percent of the total protein was calculated using the equation:

Total protein (%):

Titration Reading X HCl Concentration X 0.014 X 6.25 X 100/weight of sample

### *2.4. Electrophoretic pattern*

#### *2.4.1. Preparation of the samples*

The samples were rapidly freezed with liquid nitrogen and ground then homogenized in water-soluble extraction buffer (0.2 mg/ml). The homogenates were centrifuged at 10,000 rpm for 10 min. The clear supernatant containing water-soluble proteins was transferred to new tubes. The protein concentration was estimated in all samples using bovine serum albumin as standard based on method suggested by **Bradford [24]**.

#### *2.4.2. Electrophoretic Protein*

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at the concentration 12% according to protocol documented by **Laemmli [25]** using Mini-gel electrophoresis (BioRad, USA). The denatured gel was stained by commassie Brilliant Blue G-250 for visulaizing the protein bands.

#### *2.4.3. Data Analysis*

The denatured protein bands in polyacrylamide gel were analyzed using Quantity One software (Version 4.6.2). The relative mobility (Rf), band % (B%) and quantity (Quant.) of the electrophoretically separated proteins were determined in addition to the molecular weights (Mwts) which was estimated in comparison to marker of standard molecular weights with regularly spaced bands ranging from 5 to 245 KDa. The similarity index (SI%) and genetic distance (GD%) were calculated according to the equation suggested by **Nei and Li [26]**.

### *2.5. Amino acids analysis*

The separated proteins were hydrolysed with HCl (6N) and then the amino acids were analyzed by LC 3000 amino acid analyzer (Eppendorf-Biotronik, Germany) according to the method described by **Salman *et al.* [27]**. The obtained data were subjected to statistical analysis using general linear models (GLM) procedure of Statistical analysis system (SAS) and significance was declared at $P<0.05$.

### *2.6. Biological studies*

#### *2.6.1. Cytotoxic activity*

The cytotoxic activities of the isolated proteins were evaluated on human hepatocarcinoma (HEPG-2), adenocarcinoma (MCF-7) and colon carcinoma (HCT116) cell lines. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan **[28]**. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program (Version 14). Difference was considered significant where $P<0.05$. Also, a probit analysis was carried for determination of $IC_{50}$ and $IC_{90}$ using SPSS program.

#### *2.6.2. In vitro antiviral activity*

##### *2.6.2.1. Preparation of culture cells*

Human hepatocyte (Huh 7.5), MA104, BGM and Vero cell lines were used for growth of HCV genotype 4a [ED-43/SG-Feo (VYG) replicon], rotavirus Wa, coxsakievirus B4 and HSV1, respectively. They were obtained from the Holding Company for Biological Products & Vaccines VACSERA, Egypt and cultured using specific growth media Dulbecco´s Modified Eagle Medium (DMEM) then kept in $CO_2$ incubator. The cells were seeded in 96-well tissue culture plates (Greiner Bio-One, Germany) and incubated at 37 °C in a humidified atmosphere of $CO_2$ (5% v/v). After incubation for 24 hrs, the medium was discarded from confluent cells monolayers and replenished with 100 µL of bi-fold dilutions of different tested samples prepared in DMEM (GIBCO BRL). For cell controls, 100 µL of DMEM without samples was added.

##### *2.6.2.2. The non toxic dose on human Huh 7.5, MA104, Vero and BGM cell lines*

Each sample of isolated proteins (50 mg) was dissolved in bi-fold distilled water and decontaminated by adding 24 µL of mixture (100X) of antibiotic-antimycotic [penicillin G sodium (10 000 IU), streptomycin sulfate (10000 µg) and amphotericin B (250 µg)]. The non toxic dose of the samples was evaluated by inoculating tenfold serial dilution of each decontaminated sample in Huh 7.5, MA104, and Vero and BGM cells. The cell

morphology and cell viability were examined by using the inverted light microscopy and trypan blue dye exclusion method, respectively **[29]**.

*2.6.2.3. The antiviral effect on HCV genotype 4a, rotavirus Wa, Coxsackievirus B4 and HSV1 strains*

The HCV RNA was quantified in replicon cells after treatment with the samples as initial titers according to the method described by **Saeed *et al.* [30]** while rotavirus Wa strain, Coxsackievirus B4 and Herpes simplex virus type 1 were determined in culture cells according to the method suggested by **Schmidtke *et al.* [31]**.

*2.6.3. Antioxidant study*

The scavenging activity of the proteins isolated from *D. salina*, *S. obliquus* and *S. platensis* against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals was quantified according to the method of **McCue *et al.* [32]**. Briefly, 1 mL of the DPPH solution (0.1mM) was added to 1 mL of each protein sample and ascorbic acid (reference drug) at three concentrations (1, 10 and 100 µg/mL). After incubation for 30 min., the discoloration was measured at 517 nm. Each experiment was carried out at least in triplicate. The scavenging ability of DPPH• was calculated using the equation:

$$\textbf{Scavenging effect (\%):}\ A0 - A1\ \textbf{X}\ 100$$

Where *A0* is the absorbance of DPPH• solution (without the tested sample) and *A1* is the absorbance of the tested proteins with DPPH• solution. Statistical analysis was carried out using two ways ANOVA coupled with CO-state computer program.

*2.6.4. Acetyl cholinesterase (AChE) enzyme activity*

The AChE activity was measured by using spectrophotometer based on Ellman's method **[33]**. This method depends on ability of the enzyme to hydrolyse substrate of acetylthiocholine to product of thiocholine which reacts with Ellman's reagent to produce colored product measured by spectrophotometer at 412 nm. In brief, 250 µL of samples and standard drug at three concentrations of 1, 10 and 100 µg/ mL placed in test tubes containing 1710 µL Tris–HCl buffer (50 mM, pH 8.0), 10 µL AChE (6.67 $UmL^{-1}$) and 20 µL 5,5'-dithio-bis[2-nitrobenzoic acid] (DTNB) (10 mM). Positive control namely galanthamine was prepared in serial concentration as same as test sample by dissolving in Tris–HCl buffer. The mixture was incubated for 15 min at 37 ºC. Then, 10 µL of acetylthiocholine iodide (200 mM) was added to the mixture and the absorbance was measured at 412 nm every 10 Sec. for 3 mins with blank containing buffer instead of enzyme solution. The enzyme inhibition (%) was calculated from the rate of absorbance change with time (V= Abs/Δt). The data were analyzed statistically using two ways ANOVA coupled with CO-state computer program.

## 3. Results and Discussion

*Content of total protein*

Both of *S. obliquus* and *S. platensis* were cultivated under the vegetative and stressed conditions. As revealed in **Table 1**, it was noticed that the highest protein content exist in the vegetative *S. platensis* followed by *D. salina* then vegetative *S. obliquus*. Although the protein content decreased in the stressed forms of both *S. obliquus* and *S. platensis*, it is higher in the stressed *S. obliquus* than the stressed *S. platensis*. This was in accordance with **Markou [34]** who emphasized that stress conditions applied to *S. Platensis* decreased the protein content. Also, synthesis of the phycobilins (protein-binded pigments) decreases in *S. Platensis* under stress conditions.

*Electrophoretic protein pattern*

Proteins play an effective role inside the cells and they are susceptible to be oxidized depending on relative content of oxidation-sensitive amino acid residues **[35,36]**. Electrophoretic technique used as a tool for separating and identifying the different proteins in addition to analyzing stoichiometry of their specific subunits **[37]**. The SI is related to the qualitative mutation that occurs due to disappearance of normal bands with existence of one or more abnormal ones. The SI is inversely correlated to genetic variation. The low SI values between the compared groups indicate differences in number and arrangement of electrophoretically separated bands **[2,38]**. Otherwise, the alterations that were detected by altering quantities of normally identified bands belong to the quantitative mutations and the SI is only correlated to the qualitative alterations **[39]**.

As presented in **Fig. 1** and compiled in **Table 2**, there are physiological correlations among the three studied microalgal species. It was noticed that the vegetative and stressed *S. obliquus* were physiologically similar to *S. platensis* by 42.11 and 57.14% (GD 57.89 and 42.86%, respectively). *D. salina* was similar to *S. platensis* by 46.15% (GD 53.85%). As compared to the vegetative *S. obliquus*, the stressed *S. obliquus* and *D. salina* were similar to 30.77 and 33.33% (GD 69.23 and 66.67%, respectively). The *D. salina* was physiologically similar to the stressed *S. obliquus* by 57.14% (GD 42.86%). Data depicted in **Table 3** showed that there was one common band identified at Rf 0.95 and Mwt 5.27 KDa. In *S. platensis*, the protein pattern was electrophoretically represented by 10 denatured protein bands identified at Rfs 0.11, 0.22, 0.33, 0.47, 0.60, 0.73, 0.82, 0.84, 0.95 and 0.97 (Mwts 174.08,

115.40, 71.80, 48.58, 27.66, 19.92, 15.90, 13.91, 5.27 and 4.27 KDa ; B% 6.80, 6.82, 6.64, 7.38, 6.95, 9.29, 12.69, 13.75, 15.00 and 14.68% and Qty 4.76, 5.34, 5.60, 4.56, 7.73, 6.12, 3.14, 3.40, 7.10 and 3.33, respectively). In vegetative *S. obliquus*, the protein pattern was represented by 9 bands identified at Rfs 0.07, 0.17, 0.33, 0.50, 0.58, 0.72, 0.81, 0.86 and 0.95 (Mwts 214.68, 137.74, 71.54, 42.37, 30.18, 20.40, 17.00, 12.44 and 5.46 KDa ; B% 8.38, 8.31, 8.18, 8.67, 8.83, 10.86, 14.02, 15.04 and 17.71% and Qty 4.31, 4.12, 4.35, 6.00, 2.04, 1.35, 9.94, 8.80 and 7.22, respectively). There were 5 characteristic (unique) bands identified at Rfs 0.07, 0.17, 0.50, 0.58 and 0.86 (Mwts 214.68, 137.74, 42.37, 30.18 and 12.44 KDa ; B% 8.38, 8.31, 8.67, 8.83 and 15.04% and Qty 4.31, 4.12, 6.00, 2.04 and 8.80, respectively). While in the stressed *S. obliquus*, no characteristic bands were noticed. The protein pattern was represented by 4 bands identified at Rfs 0.11, 0.23, 0.80 and 0.94 (Mwts 176.42, 112.29, 17.27 and 5.75 KDa ; B% 18.34, 18.21, 30.73 and 32.72% and Quant. 2.89, 3.98, 9.67 and 12.87, respectively). As regard to *D. salina*, it was noticed that there were only three protein bands identified at Rfs 0.11, 0.72 and 0.94 (Mwts 171.77, 20.48 and 5.75 KDa ; B% 25.83, 34.22 and 39.95% and Qty 9.90, 8.42 and 4.91, respectively). No characteristic bands were noticed but the last protein band is considered as common band (Rf 0.94, Mwts 5.75 KDa ; B% 39.95 and Qty 4.91).

*Amino acids analysis*

Types and concentrations of the amino acid analyzed in the proteins separated from vegetative and stressed microalgal species were compiled in **Table 4** and illustrated in **Fig. 2**. Fifteen amino acids were noticed in both of *S. obliquus* and *S. platensis* while 17 amino acids in *D. salina*. **Glutamic acid** is the most predominant amino acid in all studied microalgal species. Their percentages were about 4.96, 3.3 and 7.58% in *D. salina, S. obliquus* and *S. platensis*, respectively. Alanine & isoleusine were more predominant amino acids in *S. platensis*. Alanine & leucine were more predominant amino acids in *S. obliquus*. As regard to *D. salina*, aspartic acid & leucine were more predominant amino acids.

It was noticed that *S. platensis* was found to be rich in protein (48.44%) and glutamic acid was found to be the most predominant amino acid in the three microalgal species. This was in accordance with **Barka and Blecker [5]** and supported by **Yucetepe *et al.* [40]** who emphasized that *S. platensis* was found to have significant antioxidant activity due to the high digestability properties of the proteins isolated from *S. platensis*. The antioxidant activity of protein hydrolysate isolated from *S. platensis* might refer to high glutamic acid percentage (7.58%) and also being rich in essential, non-essential and hydrophobic amino acids. This was in agreement with **Afify *et al.* [41]** who postulated that the antioxidant activity of protein hydrolysates depends on amino acids and peptides composition. Therefore, the low molecular weight peptides (4.27, 5.27, 13.91, 15.90, 19.92, 27.66 and 48.58 KDa) that were identified during the present study in the protein hydrolysate isolated from *S. platensis* were responsible for increasing its antioxidant activity. The latter authors added that a mixture of free amino acids, di, tri and oligo-peptides were responsible for modifying the functional characteristics of proteins and increasing number of the polar groups and solubility of the hydrolysate.

*Cytotoxic activity*

It was found that the proteins separated from the three microalgal species exhibited non significant cytotoxic activity against HEPG-2, MCF-7 and HCT-116 cell lines as compared to standard doxorubicin **(Table 5 and Fig. 3)**.

*Antiviral activities*

The proteins isolated from the three microalgal species exhibited non-significant antiviral activity (20%) against HCV4a, rotavirus Wa, coxsakievirus B4 and HSV1 viruses at the non-toxic doses 0.7, 0.8 and 0.9 mg/ml for *D. salina, S. obliquus* and *S. platensis*, respectively.

*DPPH• free radical scavenging activity*

As presented in **Table 6**, the proteins isolated from the three microalgal species exhibited scavenging activity against DPPH• free radical in dose dependent manner. The proteins isolated from *D. salina* showed antioxidant activity higher than standard ascorbic acid. It exhibit highly significant antioxidant activity 300.16 and 308.78 at the doses 10 and 100 µg/ml, respectively. The antioxidant activity of ascorbic acid was about 80.00 and 94.82% at the doses 10 and 100 µg/ml, respectively. Moreover, the proteins isolated from *S. obliquus* and *S. platensis* at a dose of 100 µg/ml showed significant antioxidant activity that was about 124.49 and 140.81%, respectively in comparison with standard ascorbic acid.

During the current experiment, it was found that the proteins isolated from *D. salina* exhibit highly significant and promising antioxidant activity. This was in accordance with **Afify *et al.* [14]** who stated that the antioxidant activity of the isolated proteins might be attributed to the presence of cystine amino acid. Moreover, all amino acids can generally interact with free radicals, but the most effective are those containing nucleophilic sulfur-containing side chains (Cys and Met) or aromatic side chains (Trp, Tyr and Phe) which can easily donate hydrogen atoms. In the present study, glutamic acid, cysteine and glycine

were noticed in *D. salina*. This was in agreement with **Mcbean [42]** who postulated that these amino acids were the immediate amino acid precursors of reduced glutathione (GSH), hydrogen sulfide and taurine which had significant antioxidant and/or neuroprotective properties. The free sulfhydryl group on cysteine can adopt a number of oxidation states, ranging from reversible disulfides (–S–S–) and sulfenic acids (–SOOH) to the more oxidized irreversible sulfinic (–SOO2H) and sulfonic acids (–SOO3H) which may arise as a result of chronic and/or severe oxidative stress. GSH had dual action in antioxidant protection and maintenance of the antioxidant capacity of neurons.

As supported in the current study by the electrophoretic assay in the proteins isolated from *D. salina*, it was noticed that the proteins were rich in low molecular weight peptides (5.75 and 20.48 KDa) which increased their antioxidant activity as stated by **Afify *et al.* [41]** who reported that low molecular weight protein hydrolysates possess scavenging activities against reactive oxygen species (ROS) than high molecular weight ones since small peptides can more easily access the oxidative mechanism to donate protons to free radicals, and stabilize ROS through a direct electron transfer. Additionally, *S. obliquus* exhibited significant antioxidant activity and this was in agreement with **Ishaq *et al.* [13]** who reported that *Scenedesmus* sp. possess antioxidant properties that could be applied in the nutraceutical industry. **Afify *et al.* [14]** postulated that the better antioxidant activity of protein hydrolysates in *S. obliquus* might be due to the peptide structure, hydrophobicity, type and size of the amino acids accessing the donation of protons to free radicals and hence stabilizing ROS. Also, protein hydrolysates and peptides could easily enter into target organs through membrane lipid bilayers by the aid of their hydrophobicity due to the presence of hydrophobic amino acids (alanine, leucine and phenylalanine) in *S. obliquus*. The low molecular weight peptides (5.46, 12.44, 17.00, 20.40, 30.18 and42.37 KDa) that were identified by electrophoretic assay in protein hydrolysate isolated from *S. obliquus* are able to increase the antioxidant activity as supported previously by **Afify *et al.* [41]**.

*Acetylcholinesterase inhibition activity*

Data presented in **Table 7** showed that the proteins isolated from the three microalgal species exhibited marked inhibitory effect on cholinesterase activity at low dose (1 µg/ml). While at the highest dose (100 µg/ml), the proteins isolated from *D. salina* and *S. obliquus* exhibit marked anticholinesterase activity as compared to standard galanthamine.

The oxidative stress plays vital role in development of neurodegenerative disorders including Alzheimer's disease (AD). As stated by **Mcbean [42]**, the antioxidant properties of the studied microalgal species were positively related to their anticholinesterase activities. In the present study, *S. obliquus* showed inhibitory effect on cholinesterase activity for the first time. Concerning *D. salina*, its good anticholinesterase activity might be attributed to presence of cystine amino acid which is reduced to cysteine that is an important substrate for GSH synthesis in the brain to perform a variety of neuroprotective functions **[43]**. Findings of the current study were supported by **Aly *et al.* [44]** who postulated that *D. salina* exhibited neuro-modulating effect against AD in rats in comparison with Donepezil reference drug. Therefore, the current study recommended demand for the studied microalgal products to be used as functional foods and therapeutic agents.

**Table 1.** Total protein content in the different microalgal species under vegetative and stressed conditions

| | *D. salina* | *S. obliquus* | | *S. platensis* | |
|---|---|---|---|---|---|
| | | **Veg.** | **Stress** | **Veg.** | **Stress** |
| N% | 5.08 | 4.57 | 3.45 | 7.75 | 2.02 |
| Protein Content | **31.75** | **28.56** | 21.56 | **48.44*** | 12.63 |

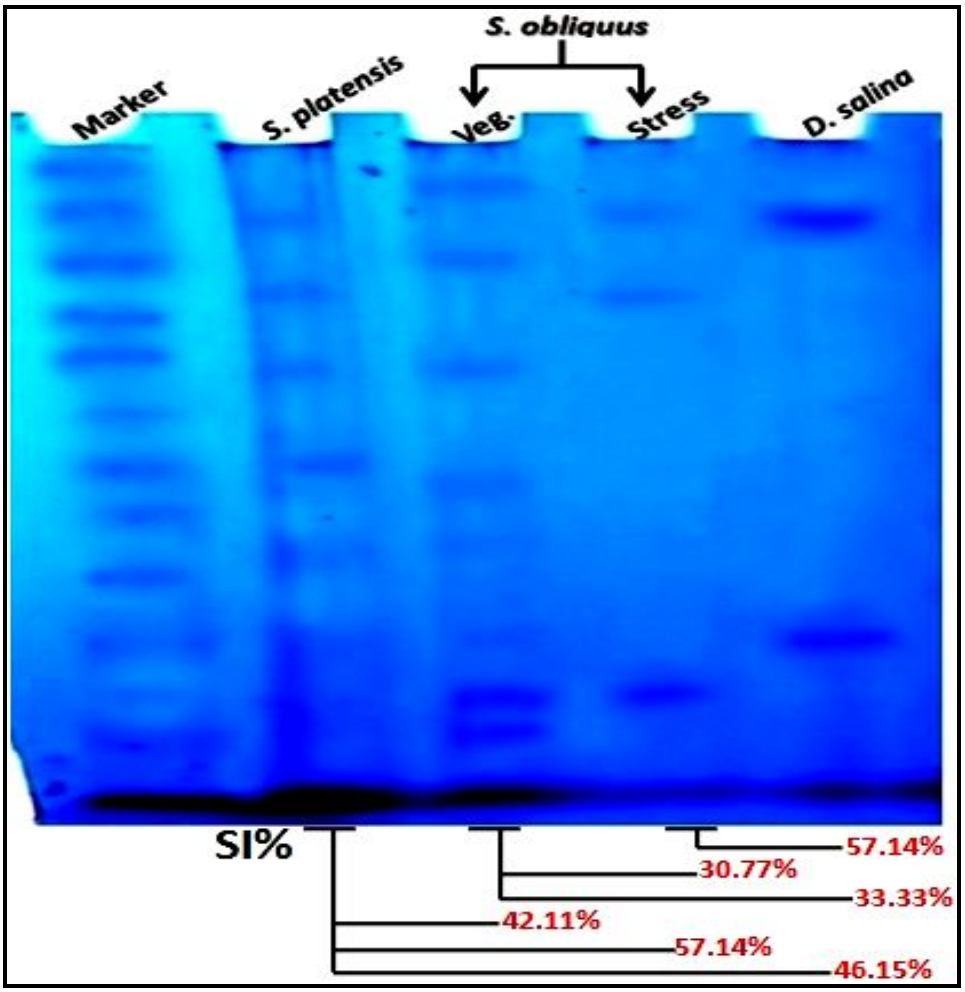


**Fig. 1.** Electrophoretic protein pattern showing the physiological differences in denatured proteins among the three studied microalgal species.

**Table 2.** Data of Similarity Index (SI%) and Genetic Distance (GD%) showing the differences in electrophoretic protein pattern among the three studied microalgal species.

| | | | *S. platensis* | *S. obliquus* | | *D. salina* |
|---|---|---|---|---|---|---|
| | | | | Vegetative | Stress | |
| | | | SI% | | | |
| *S. platensis* | | GD% | - | 42.11 | 57.14 | 46.15 |
| *S. obliquus* | Vegetative | | 57.89 | - | 30.77 | 33.33 |
| | Stress | | 42.86 | 69.23 | - | 57.14 |
| *D. salina* | | | 53.85 | 66.67 | 42.86 | - |

**Table 3.** Data of the electrophoretic protein pattern showing the physiological differences in denatured proteins among the three studied microalgal species.

| ***S. platensis*** | | | | | ***S. obliquus*** | | | | | | | | | | ***D. salina*** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **Vegetative** | | | | | **Stress** | | | | | | | | | |
| **Rf** | **Mwt** | **Int.** | **B%** | **Qty** | **Rf** | **Mwt** | **Int.** | **B%** | **Qty** | **Rf** | **Mwt** | **Int.** | **B%** | **Qty** | **Rf** | **Mwt** | **Int.** | **B%** | **Qty** |
| - | - | - | - | - | 0.07 | 214.68 | 116.12 | 8.38 | 4.31 | - | - | - | - | - | - | - | - | - | - |
| 0.11 | 174.08 | 113.35 | 6.80 | 4.76 | - | - | - | - | - | 0.11 | 176.42 | 117.57 | 18.34 | 2.89 | 0.11 | 171.80 | 154.58 | 25.83 | 9.90 |
| - | - | - | - | - | 0.17 | 137.74 | 115.11 | 8.31 | 4.12 | - | - | - | - | - | - | - | - | - | - |
| 0.22 | 115.40 | 113.80 | 6.82 | 5.34 | - | - | - | - | - | 0.23 | 112.29 | 116.73 | 18.21 | 3.98 | - | - | - | - | - |
| 0.33 | 71.80 | 110.70 | 6.64 | 5.60 | 0.33 | 71.54 | 113.38 | 8.18 | 4.35 | - | - | - | - | - | - | - | - | - | - |
| 0.47 | 48.58 | 123.11 | 7.38 | 4.56 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | - | - | - | - | 0.50 | 42.37 | 120.13 | 8.67 | 6.00 | - | - | - | - | - | - | - | - | - | - |
| - | - | - | - | - | 0.58 | 30.18 | 122.33 | 8.83 | 2.04 | - | - | - | - | - | - | - | - | - | - |
| 0.60 | 27.66 | 115.93 | 6.95 | 7.73 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 0.73 | 19.92 | 155.02 | 9.29 | 6.12 | 0.72 | 20.40 | 150.47 | 10.86 | 1.35 | - | - | - | - | - | 0.72 | 20.48 | 204.84 | 34.22 | 8.42 |
| 0.82 | 15.90 | 211.70 | 12.69 | 3.14 | 0.81 | 17.00 | 194.25 | 14.02 | 9.94 | 0.80 | 17.27 | 196.96 | 30.73 | 9.67 | - | - | - | - | - |
| 0.84 | 13.91 | 229.29 | 13.75 | 3.40 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| - | - | - | - | - | 0.86 | 12.44 | 208.32 | 15.04 | 8.80 | - | - | - | - | - | - | - | - | - | - |
| 0.95 | 5.27 | 250.13 | 15.00 | 7.10 | 0.95 | 5.46 | 245.31 | 17.71 | 7.22 | 0.94 | 5.75 | 209.76 | 32.72 | 12.87 | 0.94 | 5.75 | 239.13 | 39.95 | 4.91 |
| 0.97 | 4.27 | 244.82 | 14.68 | 3.33 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

**Rf:** Relative Mobility, **Mwt:** Molecular Weight, **Int.:** Band Intensity, **B%:** Band Percent, **Qty:** Band Quantity.

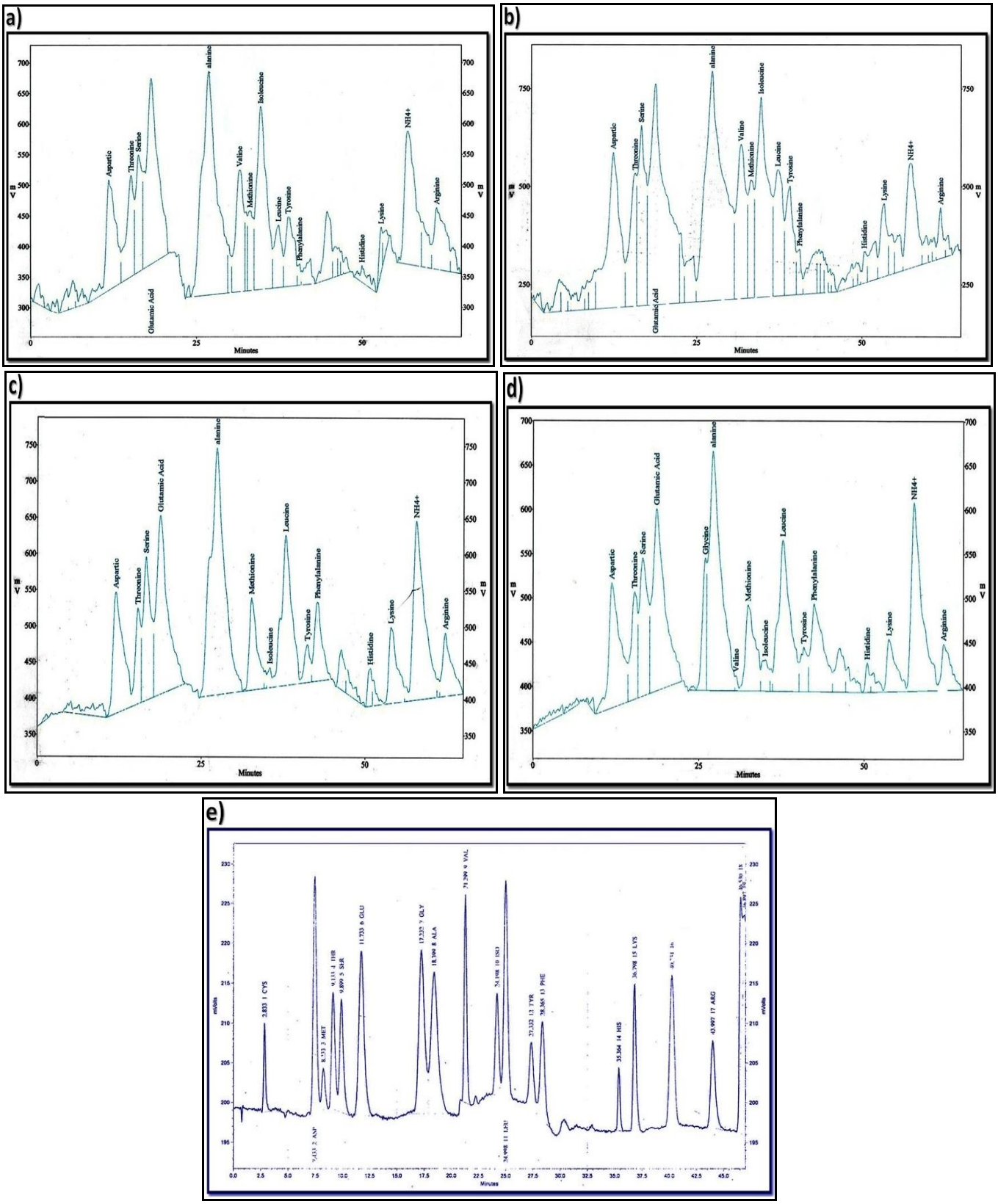


**Fig. 2.** Data of the amino acids analysis in protein hydrolysate of *S. platensis* (**a)** vegetative & **b)** stressed), *S. obliquus* (**c)** vegetative & **d)** stressed) and *D. salina* (**e)** vegetative).

**Table 4.** Data of amino acids analyzed in the proteins separated from the different vegetative and stressed microalgal species

| Amino acids | | *S. platensis* | | | *S. obliquus* | | | *D. salina* | |
|---|---|---|---|---|---|---|---|---|---|
| **Type** | **Name** | **RRt** | **Conc.%** | | **RRt** | **Conc.%** | | **RRt** | **Conc.%** |
| | | | **Vegetative** | **Stress** | | **Vegetative** | **Stress** | | |
| **Essential amino acids** | **Histidine** | 2.74 | 0.35 | 1.10 | 2.70 | 1.09 | 0.38 | 3.01 | 0.78 |
| | **Isoleucine** | 1.90 | 5.60 | 10.38 | 1.89 | 0.11 | 0.42 | 2.06 | 1.99 |
| | **Leucine** | 2.05 | 1.49 | 5.29 | 2.01 | 2.35 | 3.5 | 2.13 | 3.96 |
| | **Lysine** | 2.90 | 0.51 | 2.61 | 2.87 | 1.09 | 1.01 | 3.14 | 2.3 |
| | **Methionine** | 1.82 | 1.81 | 4.05 | 1.74 | 1.38 | 2.10 | 0.70 | 0.75 |
| | **Phenylalanine** | 2.23 | 0.28 | 1.28 | 2.27 | 1.22 | 2.78 | 2.42 | 2.52 |
| | **Threonine** | 0.83 | 1.76 | 3.26 | 0.82 | 0.57 | 0.99 | 0.78 | 1.90 |
| | **Tryptophan** | - | - | - | - | - | - | - | - |
| | **Valine** | 1.73 | 1.94 | 4.27 | 1.46 | - | 0.05 | 1.82 | 2.72 |
| **Non-Essential amino acids** | **Alanine** | 1.48 | 5.92 | 11.24 | 1.46 | 2.94 | 3.15 | 1.57 | 3.51 |
| | **Arginine** | 3.36 | 4.38 | 2.13 | 3.31 | 1.69 | 1.68 | 3.75 | 2.50 |
| | **Aspartic acid** | 0.65 | 2.87 | 8.52 | 0.64 | 1.61 | 2.46 | 0.63 | 4.01 |
| | **Cystine** | 1.00 | - | - | - | - | - | 0.24 | 0.86 |
| | **Glutamic acid** | - | **7.58** | **18.29** | **1.00** | **3.30** | **4.80** | **1.00** | **4.96** |
| | **Glycine** | - | - | - | - | - | 0.32 | 1.47 | 2.52 |
| | **Proline** | 0.92 | - | 6.29 | 1.003 | 1.02 | 1.20 | 1.01 | 1.37 |
| | **Serine** | 2.15 | 1.46 | 4.02 | 0.89 | 1.02 | 1.37 | 0.84 | 1.59 |
| | **Tyrosine** | 3.12 | 2.8 | 5.98 | 2.19 | 0.49 | 0.89 | 2.33 | 1.75 |
| **$NH4^+$** | | 3.08 | 2.74 | 3.41 | 3.08 | 2.05 | 2.65 | - | Un-detected |
| **Total no. of detected amino acids** | | | 15.00 | 16.00 | | 15.00 | 17.00 | | 17.00 |
| **Total no. of essential amino acids** | | | 8.00 | 8.00 | | 7.00 | 8.00 | | 8.00 |
| **Total no. of detected non essential amino** | | | 6.00 | 7.00 | | 7.00 | 8.00 | | 9.00 |

**Table 5.** Cytotoxic activity of the proteins isolated from the three microalgal species

| Cell line | Dose (µg/ml) | 6.25 | 12.50 | 25.00 | 50.00 | 100.00 |
|---|---|---|---|---|---|---|
| | **Isolated proteins** | **Inhibition (%)** | | | | |
| **HEPG-2** | ***D. Salina*** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | ***S. obliquus*** | 0.00 | 0.00 | 3.50±2.33$^{a}$ | 11.03±1.56$^{b}$ | 34.5±2.34$^{c}$ |
| | ***S. platensis*** | 0.00 | 0.00 | 0.00 | 11.8±1.22$^{b}$ | 36.1±3.14$^{c}$ |
| | **Doxorubicin** | 13.7±1.40$^{b}$ | 32.4±2.23$^{c}$ | 60.2±3.56$^{d}$ | 97.2±4.78$^{e}$ | 100.00±1.37$^{e}$ |
| **MCF-7** | ***D. Salina*** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | ***S. obliquus*** | 0.00 | 0.00 | 0.00 | 5.9±0.20$^{a}$ | 24.3±1.30$^{b}$ |
| | ***S. platensis*** | 0.00 | 0.00 | 0.00 | 5.6±0.17$^{a}$ | 16.7±2.93$^{a}$ |
| | **Doxorubicin** | 16.7±1.11$^{b}$ | 32.7±3.76$^{c}$ | 57.60±2.49$^{d}$ | 84.3±4.10$^{g}$ | 100.00±1.63$^{f}$ |
| **HCT-116** | ***D. Salina*** | 0.00 | 0.00 | 0.00 | 10.7±1.21$^{b}$ | 26.70±2.68$^{b}$ |
| | ***S. obliquus*** | 0.00 | 0.00 | 0.00 | 0.00 | 12.6±1.66$^{b}$ |
| | ***S. platensis*** | 0.00 | 0.00 | 0.00 | 0.00 | 10.80±1.56$^{b}$ |
| | **Doxorubicin** | 10.2±1.21$^{b}$ | 18.4±1.11$^{b}$ | 35.4±3.06$^{c}$ | 65.2±4.00$^{d}$ | 100±1.07$^{f}$ |

Data were represented by mean ±SD of three replicate. The similar letters indicated non-significant difference and the different letters indicated significant difference at $P \leq 0.05$.

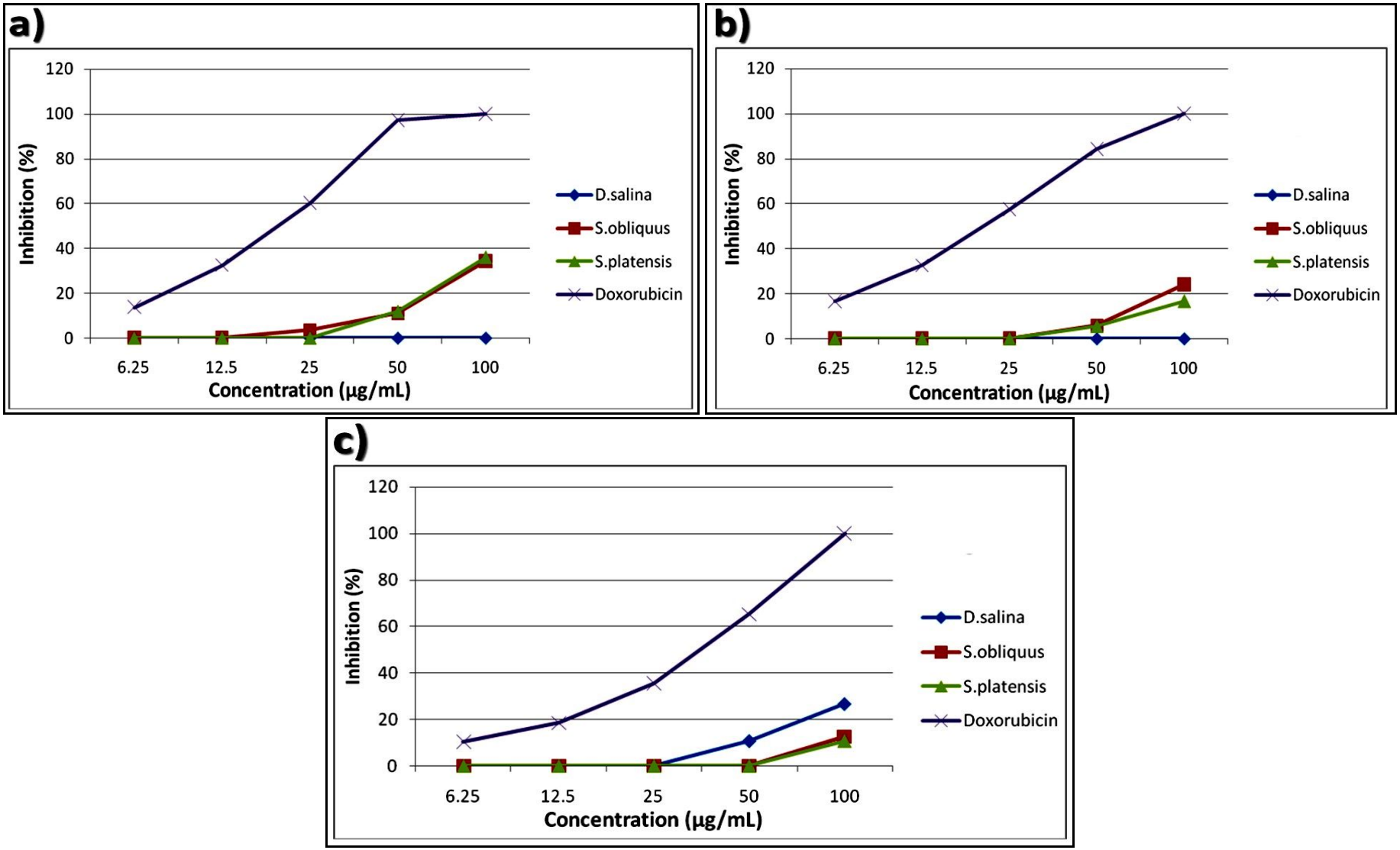


**Fig. 3.** Cytotoxic activity of the isolated proteins against **a)** HEPG-2, **b)** MCF-7 and **c)** HCT-116 human cell line *in vitro*.

**Table 6.** The DPPH· Scavenging activity of the proteins isolated from the different microalgal species.

| Isolated proteins | Inhibition (%) ± SD | | |
|---|---|---|---|
| | **1 µg/ml** | **10 µg/ml** | **100 µg/ml** |
| **Control** | 0.98±0.05[a] | 1.01±0.02[a] | 0.98±0.005[a] |
| ***D. Salina*** | **40.82±1.33[b]** | **300.16±14.65[c]** | **308.78±19.23[c]** |
| ***S. obliquus*** | 8.16±0.89[d] | 30.62±1.76[e] | **124.49±5.33[f]** |
| ***S. platensis*** | 14.29±0.87[g] | 32.65±2.21[h] | **140.81±7.78[f]** |
| **Ascorbic acid** | 29.32±1.23 [h] | 80.00±10.22[o] | 94.82±1.10[j] |

Data were represented by mean ±SD of three replicate. The similar letters indicated non-significant difference and the different letters indicated significant difference at *P≤0.05*.

**Table 7.** Inhibitory effect against Cholinesterase activity of the proteins isolated from the different microalgal species.

| Isolated proteins | Inhibition (%) ± SD | | |
|---|---|---|---|
| | **1 µg/ml** | **10 µg/ml** | **100 µg/ml** |
| **Control** | 2.02±0.12[a] | 1.81±0.34[a] | 1.98±0.02[a] |
| ***D. Salina*** | 32.00 ±1.83b | 30.00±1.35b | 34.00±2.98b |
| ***S. obliquus*** | 25.00±0.56c | 27.00±0.90c | 36.00±0.88b |
| ***S. platensis*** | 32.00±2.61[b] | 18.00±0.11[d] | 6.00±0.25[e] |
| **Galanthamine** | 56.00±3.55[f] | 60.60 ±2.77[f] | 78.00±3.09[u] |

Data were represented by mean ±SD of three replicate. The similar letters indicated non-significant difference and the different letters indicated significant difference at *P≤0.05*.

## 4. Conclusion

The highest protein content was noticed in *S. platensis* (48.44%) followed by *D. salina* (31.75%) then *S. obliquus* (28.56%). Proteins isolated from vegetative and stressed *S. obliquus* were physiologically similar to that isolated from *S. platensis* by 42.11 and 57.14% (GD 57.89 and 42.86%, respectively). *D. salina* was similar to *S. platensis* by 46.15% (GD 53.85%). As compared to the vegetative *S. obliquus*, the proteins isolated from stressed *S. obliquus* and *D. salina* were similar to 30.77 and 33.33% (GD 69.23 and 66.67%, respectively). The proteins from *D. salina* were similar to the stressed *S. obliquus* by 57.14% (GD 42.86%). In the *D. salina*, the protein pattern was represented electrophoretically by 3 bands identified at Rfs 0.11, 0.72 and 0.94. In *S. obliquus*, 9 de-natured bands were identified at Rfs 0.07, 0.17, 0.33, 0.50, 0.58, 0.72, 0.81, 0.86 and 0.95. While in *S. platensis*, the protein pattern was represented by 10 de-natured protein bands identified at Rfs 0.11, 0.22, 0.33, 0.47, 0.60, 0.73, 0.82, 0.84, 0.95 and 0.97. It was found that 15 amino acids were identified in the proteins isolated from *S. platensis* and *S. obliquus.* While in *D. salina,* 17 amino acids were identified. Glutamic acid was the most predominant amino acid and it exhibited the highest percentages in *D. salina*, *S. obliquus* and *S. platensis* (4.96%, 3.3% and 7.58%, respectively). Additionally, the proteins isolated from *D. salina* exhibited the highest antioxidant activity at a dose of 100 µg/ml (308.78%) followed by the *S. platensis* and *S. obliquus* proteins (140.81% and 124.49%, respectively). Furthermore, *D. salina* and *S. obliquus* showed moderate inhibitory effect at a dose of 100 µg/ml on cholinesterase activity (34% and 36%, respectively). Therefore, microalgal proteins might be used as therapeutic antioxidants and might be used in combination with AD treatments.

## 5. Conflict of Interests

The authors responsible for the manuscript theoretically and practically declare that there are no financial or non-financial conflicts of interests.

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