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Phytochemical and Biological Studies of Carotenoids in Some Microalgae (*Dunaliella salina*, *Scenedesmus obliquus* and *Spirulina platensis*)

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Abstract

In the current work, carotenoids of the three microalgae [*Spirulina platensis* (*S. platensis*), *Scenedesmus obliquus* (*S. obliquus*) and *Dunaliellasalina* (*D. salina*)] were extracted, quantified and exposed to phytochemical and biological (cytotoxic, antiviral, antioxidant, and anticholinesterase) activities. Also, some stress conditions were applied to *S. platensis* and *S. obliquus* to raise their carotenoid content. D. *salina* was found to contain the highest percent of carotenoids (0.67%) followed by *S. obliquus* (0.64 %), then *S. platensis* (0.5%). Six carotenoids were detected and identified in *S. platensis* and *S. obliquus*while four carotenoids were detected in *D. salina*. Lutein, β -carotene and diatoxanthin were predominant in *S. platensis* while β -carotene, diatoxanthin and echinenone were predominant in *D. salina*. Furthermore, echinenone, diatoxanthin and anthraxanthin were predominant in *S. obliquus*. *D. salina* carotenoid fraction showed potent cytotoxic activity on hepatocellular carcinoma (HepG2), breast cancer (MCF-7) and colorectal cancer (HCT116) cell lines with an IC50 24.7, 66.4 and 46.7 µg/ml, respectively. Also, *S. platensis* carotenoid fraction showed a significant cytotoxic activity on HepG2, MCF7 and HCT116 cell lines with an IC50 31.1, 69.9 and 62 µg/ml, respectively.*D. salina* and *S. platensis* exhibited a significant antioxidant activity using1, 1- diphenyl-2picryl-hydrazyl (DPPH) with a concentration dependent manner. Also, *D. salina* and *S. obliquus* inhibited the activity of acetylcholinesterase to 60.00 and 30.00% at 10 µg/ml dose, respectively. So, *D. salina* and *S. platensis* carotenoid fractions are recommended to be included in anticancer drug researches while *D. salina* and *S. obliquus* carotenoid fractions should have a consideration in the treatment of neuro- degeneration disease.

Keywords: Spirulinaplatensis; Scenedesmusobliquus; Dunaliellasalina; carotenoids; cytotoxic activity; antioxidant, anticholinesterase

1. Introduction

In the last era, cancer together with some viral diseases had a wide occurrence [1].Cancer, an abnormal overgrowth of cells destroying the surrounding sound tissue, is considered one of the major causes of death around the world and it might start at one body part and made metastasis to other parts. Chemotherapy and radiation, mainly used in the treatment of cancer, had many side effects such as baldness and anorexia [2]. Also, reactive oxygen species (ROS) were responsible for different diseases such as Parkinson, Alzheimer, cardiovascular diseases, cancer and diabetes [3]. Moreover, Alzheimer's disease (AD), a neurodegenerative disorder, which originate from various causes including increased level of the enzyme acetylcholinesterase (AChE) may progress to dementia and CNS dangerous illness [4].

So, the production of new anticancer, antiviral, antioxidant and anticholinesterase drugs with no side effects had an increasing demand. Natural products had the advantage of lower adverse effects [4]. One of the greatest critical divisions of natural products are microalgae which were later used widely to produce a fortified food [5] and are rich in antioxidant compounds especially carotenoids. These carotenoids were also reported to treat different types of cancers [2].

Carotenoids, a naturally occurring pigments in plants and photosynthetic organisms, include β-carotene (a precursor for vitaminA), lutein (prevent age related diseases) and lycopene (active against prostate tumors) [6] and also have a greatest effect in the chronic diseases prevention [7]. Microalgae of marine origin such as *D. salina* produce a highly active antioxidant carotenoids especially β-carotene [3]. It was reported

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that cis-β-carotene occurring in *D. salina* had an overhand antioxidant activity in vivo than the synthesized trans-β-carotene [8]. Also, *D. salina* beta carotenes protect against fibrosarcoma in rats [9]. Moreover, *D. salina* carotenoids were reported to have a hepatoprotective effect [10] and inhibit acute myelogenous leukemia cell lines [11]. Furthermore, Dunaliella was also reported to contain another carotenoid called zeaxanthin, a valuable antioxidant that prevent and treat Age-Related Macular Degeneration (ARMD) that lead to loss of vision [12]. Additionally to β -carotene, it was reported that astaxanthin, lycopene, zeaxanthin, lutein, actaxanthin and cryptoxanthin carotenoids occur in the blue green microalga *Spirulina platensis* and contribute to its antioxidant properties [13,14]. Spirulina extracts containing various carotenoid compounds and tocopherols were also found to have chemopreventive effect [14]. As the microalgae had a biologically valuable carotenoids, they were subjected to some stress conditions that increased their carotenoids content [15]. This study focus on chemical comparison between carotenoids isolated from microalgae *(S. platensis*, *S. obliquus* and *D. salina*) as well as evaluate their cytotoxic, antiviral, antioxidant and anticholinesterase activities.

2. Materials and Methods

2.1. Algae material

Algae strains were obtained from algal biotechnology unit, National Research Centre (prof. Dr. Abo El-Khair Badawy Elsayed).The used strains were *Spirulina platensis* belonging to Cyanophyta grown using Zarrouk medium, *Scenedesmus obliquus* and *Dunaliella salina* belonging to Chlorophyta grown using BG-II medium and artificial sea water, respectively. Stress growth for each alga was achieved basically by increasing salinity concentration to 2.0% Sodium chloride, 45 mM organic carbon as Sodium acetate and 125 ppm iron as ferrous sulfate. Vegetative and stress-growth were performed within a 200-L vertical sheet photo bioreactor **.**Growth conditions were varied based in growth site (in and out-door)**.** Also, harvesting, purification and drying of the microalgae under study were performed [16]**.**

2.2. Quantitative determination of carotenoids

Five ml of algal suspension were centrifuged at 3500 rpm for 5 minutes. Supernatant was disposed of and the leftover pellet is re-suspended with 5% KOH in 30% methanol to recuperate carotene, homogenized and saved for 5 minutes in water shower at 70oC. Re-axis, dispose of supernatant and the leftover was re-removed by 5ml DMSO after the expansion of 5 drops of concentrated acidic corrosive [17]**.** Rotator again at 1000xg for 5 minutes. Carotenes absorbance was estimated at 468 nm and focus was determined (mgl-1) as indicated by Davis [18] as:

Total carotene (mgl^{-1}) = (A-B) × D × F

Where A= Absorbance of the sample at 468 nm; B= Absorbance of the blank at 468 nm; F= 4.6 (factor) and D= dilution factor.

2.3. Carotenoids extraction

Carotenoids were extricated from the dried powders of the microalgae (50 gm) by a combination of CH3)2CO and oil ether (1:1, v/v) at the room temperature until dry. After washed for a few times with water (multiple times), the upper stage was gathered and joined as rough concentrate. The unrefined concentrate was separated, dissipated to dryness in a rotating evaporator and re-suspended in oil ether. Saponification was done in petroleum ether arrangement by adding 40% w/v KOH in methanol to a last grouping of 4% w/v KOH. The combination was kept in obscurity for 12 h at room temperature. The pet. ether extricate was washed a few times with water until all the KOH is taken out and dissipated on an evaporator at 400C and broke up in a little petrol ether[19]**.**

2.4. ESI^+ -MS fragmentation

Instrument:

The Analysis was performed on a TSQ Quantum Access MAX triple stage quadrupole mass spectrometer, Thermo Scientific, New York, USA, furnished with an electrospray ionization (ESI) worked in the positive particle ization mode. Chromatography was carried on Accela U-HPLC framework which was made out of Accela 1250 quaternary siphon and Accelaopenautosampler, New York, USA (worked at 250C). X-calibursoft product rendition 2.2 was utilized to control all boundaries of UPLC, MS and investigation the acquired information.

Chromatographic conditions:

Separation of analytes by chromatographic method (1020, Sigma, New York, USA) was done on Hypersil Gold segment (C18 fortified ultrapure silica based section) 50 mm × 2.0 mm (1.9 µm) from Thermoscientific, New York, USA. Elution was performed isocratically at room temperature utilizing new arranged versatile period of Acetonitrile (A): 0.2% formic corrosive watery arrangement (B) 0.1 % formic corrosive in methanol at a stream pace of 250 µl/min. The boundaries for investigation were completed utilizing positive particle mode as follows: source temperature 150 °C, cone voltage 30 eV, hairlike voltage 3 kV, desolvation temperature 440 °C,

cone gas stream 50 L/h, and desolvation gas stream 900 L/h. Mass spectra (Model 3342S, Sigma, New York, USA) were recognized in the ESI positive particle mode between m/z 100–1000.

2.5. Biological studies

2.5.1. Cytotoxic activity on hepatocarcinoma, adenocarcinoma, and colon carcinoma human cell lines

Cytotoxic effect of microalgal carotenoid extracts was evaluated on hepatocellular carcinoma (HepG2), breast cancer (MCF7), and colorectal carcinoma (HCT116) human cell lines. Cell viability was assessed by the mitochondrial-dependent reduction of yellow MTT (3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan [16].

2.5.2. Biological *in-vitro* antiviral activity against HCV genotype 4a, rotavirus Wa, coxsakie virus B4 and Herpes simplex 1 (HSV1):

2.5.2.1. Culture cells for in vitro antiviral

Human hepatocyte (Huh 7.5), MA104, BGM and Vero cell lines (acquired from the Holding Company for Biological Products and Vaccines VACSERA, Egypt) were utilized for development HCV genotype 4a[ED-43/SG-Feo (VYG) replicon], rotavirus Wa, coxsackie virus B4, and HSV1, separately. They were refined utilizing explicit development media Dulbecco´s Modified Eagle Medium (DMEM) and will be kept in a CO2 hatchery. The cells were cultivated in 96-well tissue culture plates (Greiner Bio-One, Germany) and hatched at 37 °C in a humidified environment of 5% (v/v) CO2. After 24 h brooding, the medium was disposed of from intersecting cells monolayers and recharged with 100 µL of bi-overlap weakening of various examples tried arranged in DMEM (GIBCO BRL). For cell controls, 100 µL of DMEM without tests were added [16].

2.5.2.2. Determination of the nontoxic dose of Huh 7.5, MA104, Vero, and BGM cell lines

Each example of microalgal carotenoid separate (50 mg) was broken up in bi-overlap refined water and sterilized by adding 24 µL of 100× combination of anti-microbial antimycotic [penicillin G sodium (10000 IU), streptomycin sulfate (10000 µg) and amphotericin B (250 µg)]. To assess the nontoxic portion of the examples, ten times sequential weakening of each purified test was vaccinated in Huh 7.5, MA104, Vero and BGM cells. The upset light microscopy and trypan blue color avoidance strategy were utilized for inspecting cell morphology and cell reasonability, individually [16].

2.5.2.3. Determination of antiviral effect on HCV genotype 4a, rotavirus Wa, Coxsackie virus B4, and HSV1 strains

HCV RNA in replicon cells while rotavirus Wa strain, Coxsackie virus B4 and Herpes simplex virus type 1 in cultured cells were quantified as mentioned in **Singab et al.** [16].

2.5.3. The antioxidant study using DPPH free radical scavenging activity

Quantitative measurement of free radical scavenging properties of microalgal carotenoid extracts isolated from *S. platensis, S. obliquus and D. salina* was carried out according to the method reported in **Singab et al.**[16].which stated that 0.1mM solution of 1, 1- diphenyl-2picryl-hydrazyl (DPPH.) was prepared in 100 ml absolute methanol and 1 mL of this solution was added to 1 mL of each microalgal carotenoid extract sample and ascorbic acid (reference drug) at three concentrations (1, 10 and 100 µg/mL). Discoloration was measured at 517 nm after incubation for 30 min. Measurements were taken at least in triplicate. The scavenging ability of DPPH• was calculated using the following equation:

Scavenging effect (%) = $A0-A1A0\times100$

Where A0 is the absorbance of DPPH• solution (without the tested microalgal carotenoid extracts) and A1 is the absorbance of the tested microalgal carotenoid extracts with DPPH• solution.

2.5.4. Assay of acetyl cholinesterase (AChE) enzyme activity by the spectrophotometric method [16]**:**

AChE activity was was estimated by utilizing spectrophotometer dependent on Ellman's technique. The chemical hydrolyzes the substrate acetylthiocholine bringing about the item thiocholine which responded with Ellman's reagent (DTNB) to create 2-nitrobenzoate-5mercaptothiocholine and 5-thio-2-nitrobenzoate which can be recognized at 412 nm. In test tube 1710 µL of 50 mMTris–HCl cushion pH 8.0 and 250 µL of carotenoid tests of tried microalgae and standard medication at three groupings of 1, 10 and 100 µg/mL, 10 µL 6.67 UmL-1 AChE and 20 µL of 10 mM of DTNB (5,5'- dithio-bis[2-nitrobenzoic acid]) in cradle were added. Positive control in particular galanthamine was set up in sequential fixation as same as tried examples by dissolving in 50 mMTris–HCl support pH 8.0. The combination was hatched for 15 min at 37 ºC. At that point, 10 µL of acetylthiocholine iodide (200 mM) in support was added to the combination and the absorbance was estimated at 412 nm each 10 sec for 3 min. For a clear, the support rather than protein arrangement was utilized. The protein hindrance (%) was determined from the pace of absorbance change with time (V= Abs/Δt) according to calculation as follows:

Inhibition (%) = 100 - Change of sample absorbance X 100 change of blank absorbance

The experiment was done in triplicate for each concentration of the tested samples that inhibit the

hydrolysis of the substrate (acetylcholine). The percent of acetylcholinesterase inhibition was calculated as follows: % Inhibition = 100 − [Absorbance of the tested carotenoid extract /Absorbance of the control] × 100.

2.5.5. Statistical analysis

Data of cytotoxic activity were investigated by one way analysis of variance (ANOVA) utilizing the Statistical Package for the Social Sciences (SPSS) program, rendition 14 (IBM programming, NY, USA). The thing that matters was viewed as significant where P<0.05. Furthermore, a probit examination was conveyed for IC50 and IC90 assurance utilizing SPSS 11 program. Then again, statistical examination for antioxidant and anticholinestrase analysis was done utilizing two ways ANOVA combined with CO-state PC program.

2. 3. Results and Discussion

3. 3.1. Phytochemical investigation

4. 3.1.1. Quantitative estimation of carotenoids

D. salina was found to have the highest carotenoids content followed by *S. obliquus* then *S. platensis*as illustrated in table (1). Also, carotenoids content increased in the stress forms of both *S. platensis* and *S. obliquus* more than twice times (2.6 times and 2.4 times, respectively).

3.1.2. Electron spray ionization- Mass spectrum in the positive ion mode (ESI$^+$-MS):

ESI positive mode mass spectra of *S. obliquus* and *S. platensis* showed molecular ions at m/z537, 551, 567, 569, 585 and 601 identified as β-carotene, echinenone, diatoxanthin, lutein, antheraxanthin and violaxanthin as shown in Figures (1 and 2, respectively) while the ESI positive mode mass spectrum of *D. salina* showed molecular ions at m/z537, 551, 567 and 585 identified as β-carotene, echinenone, diatoxanthin and antheraxanthin. Beta-carotene, diatoxanthin and echinenone were found to be predominant in *Dunaliella salina* while echinenone, diatoxanthin and antheraxanthin were predominant in *Scenedesmus obliquus*. Furthermore, lutein, beta-carotene and diatoxanthin were predominant in*spirulina platensis*.

Six compounds were identified by mass fragmentation in *D. salina, S. obliquus* and *S. platensis* as compiled in Table (2) .These compounds were:

Compound 1: was identified in the three microalgae (*D. salina, S. obliquus*and *S. platensis*) as β-carotene with a molecular weight of 536 and a molecular formula $C_{40}H_{56}$. Its mass spectrum displayed a protonated molecular ion $[M+H]^+$ at m/z 537 representing the base peak as shown in Table (2). Detected fragment ion at m/z 446 was corresponding to elimination of toluene $[M+H-92+1]^+$ while fragment ion at m/z 137 $[M+H-400]^+$ was corresponding to cleavage between carbon 7 and 8 of the polyene chain. Also, fragment ion at m/z 431 $[M+H-106]^+$ was corresponding to elimination of xylene. Moreover, fragment ions at m/z 203, 243, 269, and 295 resulted from the cleavage of C11-C12, C13-C14, C15-C16 and C17-C18 carbon-carbon double bonds, respectively accompanied by hydrogen transfer to the ions. These data agree with Breemen et al. [6], Ren and Zhang [19] and Rivera et al. [20].However, some fragments showed m/z +1 as 446 instead of 445 which may be due to artifact as reported by Nicolescu[21],who showed the effect of impurities on the mass spectrum.

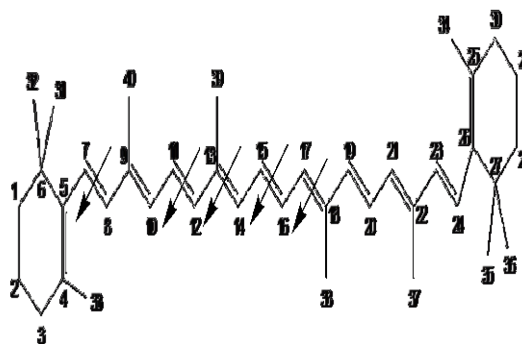


Table 1: Quantitative estimation of the carotenoids content of *D. salina, S. obliquus and S. platensis*

Sample name	Total carotenoids (mgL^{-1})
S. platensis vegetative	0.50
S. platensis stress	1.30
S. obliquus vegetative	0.63
S. obliquus stress	1.50
D. salina vegetative	0.67

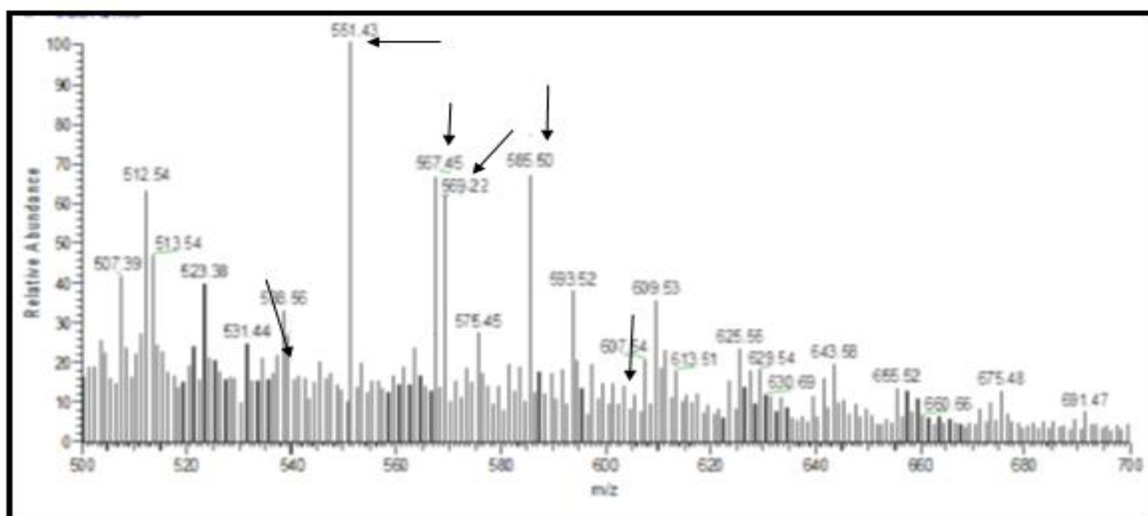


Fig. 1: ESI positive ion mode mass spectrum of carotenoids isolated from *Scenedesmus obliquus*.

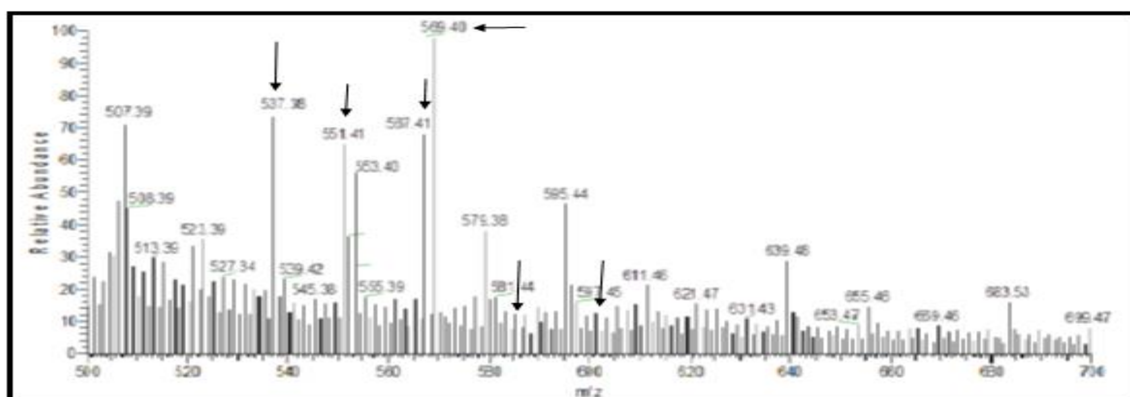


Fig. 2: ESI positive ion mode mass spectrum of carotenoids isolated from *Spirulina platensis*

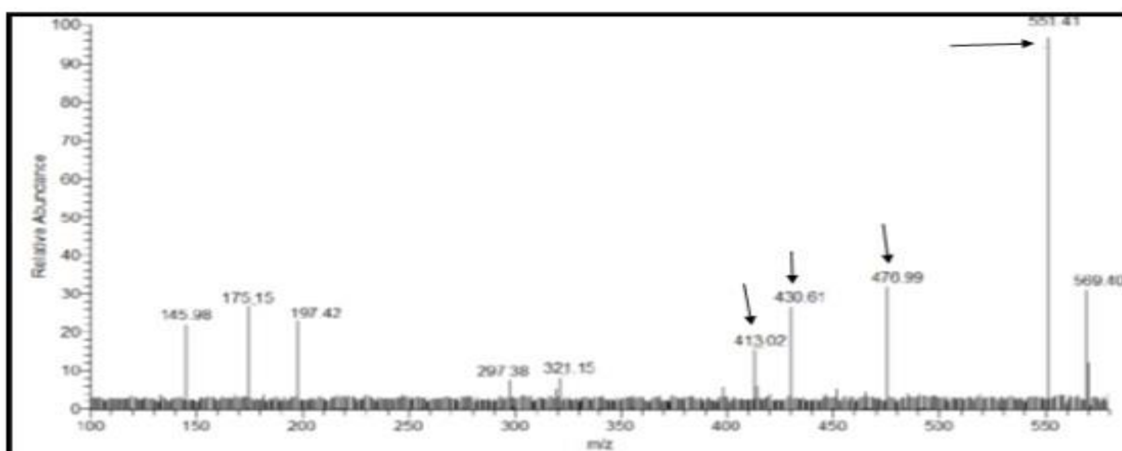
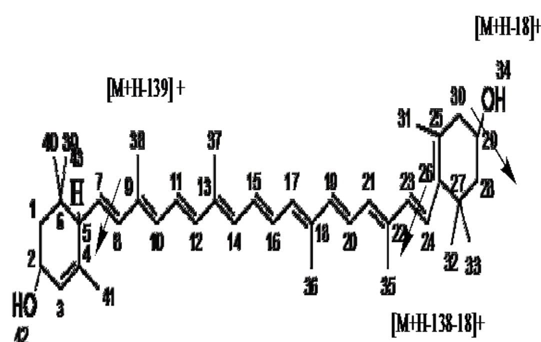


Fig. 3: Mass spectrum of compound 2 (Lutein)

Table 2: Mass fragments of microalgae (*Dunaliella salina* ,*Scenedesmus obliquus* and *Spirulina platensis*):

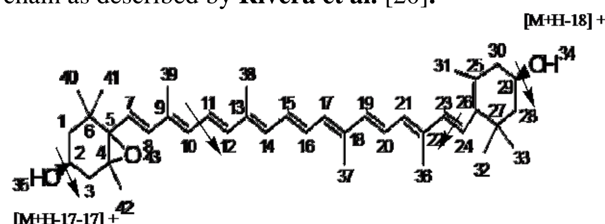
Identified carotenoid	$[M+H]^+$	Relative abundance (%)			Fragment ions of the carotenoids of each microalga with their relative abundance
		D.salina	*S.obliquus*	*S.platensis*	
β-carotene (compound 1)	537	14%	21%	**74%**	537(100%)$[M+H]^+$,446$[M+H-92+1]^+$ (34%),431$[M^+-106]$ (19%) , 203(15%),137$[M^+-400]$ (10%), 243(9%), 295(9%) and 269(8%) **(Rivera *et al.*, 2014, Breemen *et al.*, 2012** and **Ren and Zhang, 2008).**
Lutein (compound 2)	569	-	62%	**100%**	551$[M+H-18]^+$(100%),477$[M+H-92]^+$(32%) ,569(30%),175(27%), 430$[M+H-139]^+$(27%) and 413$[M+H-138-18]^+$ (16%) (**Rivera *et al.*, 2014** and **Ren and Zhang, 2008**).
Antheraxanthin (compound 3)	585	9%	66%	12%	221$[M+H-364]^+$(100%),567$[M+H-18]^+$(57%),585(40%), 551$[M+H-17-17]^+$(40%),134(33%) and119(28%) (**Rivera *et al.*, 2014**).
Violaxanthin (compound 4)	601	-	14%	12%	583$[M+H-18]^+$(76%),565$[M+H-18-18]^+$(48%), 601(45%),503(17%),547(15%), 355(15%), 445(14%),431(13%), 397(12%)and 221(7%) (**Rivera *et al.*, 2014, De Rosso & Mercadante, 2007** and **Ren and Zhang, 2008**).
Echinenone (compound 5)	551	10%	100%	64%	56(34%),203$[M+H-347]^+$(20%),133(20%),69(16%), 536$[M+H-15]^+$ (4%) (**Rivera *et al.*, 2014 and Breemen et al., 2012)**
Diatoxanthin (compound 6)	567	12%	66%	68%	567(81%) ,199(32%), 310(20%),549$[M+H-18]^+$(20%),217(16%) ,443 $[M+H-18-106]^+$ (14%),175(13%), 145(13%) (**Neto et al., 2016**).

Compound 2: was identified in both *S. obliquus*and *S. platensis* as lutein (molecular weight 568 and molecular formula $C_{40}H_{56}O_2$) which was a hydroxycarotenoid. Its fragmentation pattern was illustrated in Figure (3) and Table (2) showing the protonated molecular ion $[M+H]^+$ at m/z 569. Detected typical fragment ion at m/z 551 given by a compound bearing a hydroxyl group is the loss of a molecule of water $[M+H-18]^+$ as described by Rivera et al. [20] and Ren and Zhang [19] and this fragment represented the base peak. Also, fragment ion at m/z 477 was corresponding to elimination of toluene $[M+H-92]^+$ while fragment ion atm/z 430 $[M+H-139]^+$ was corresponding to the loss of the terminal ring containing the unconjugated C3-C4 and C7-C8 double bonds and can be used to distinguish between lutein and its isomer, zeaxanthin. Furthermore, fragment ion at m/z 413 $[M+H-138-18]^+$ was corresponding to elimination of β-ring containing a hydroxyl group (cleavage of C23-C24 double bond).

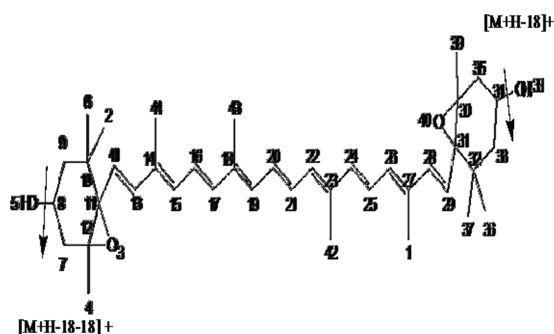


Compound 3: was identified in the three microalgae (*D. salina, S. obliquus*and *S. platensis*) as**antheraxanthin** which was an epoxy carotenoidhaving a molecular weight 584 and a molecular formula $C_{40}H_{56}O_3$. Its mass spectrum displayed a protonated molecular ion $[M+H]^+$ at m/z 585 as shown in Figure (4) and Table (2). Observed fragment ion at m/z 567 was corresponding to loss of

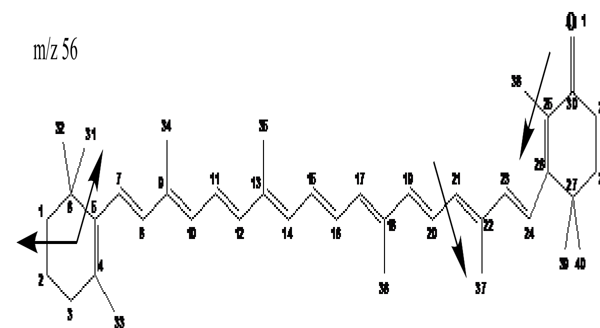
one molecule of water $[M+H-18]^+$ and fragment ion at m/z 551 $[M+H-17-17]^+$ was corresponding to loss of two hydroxyl groups. Furthermore, the mass spectrum of compound 3 showed a fragment ion at m/z 221 produced by cleavage between C10 and C11 which is characteristic to epoxy group fused to hydroxy β ring [20]**.** Also, fragment ion at m/z 134 was characteristic for hydroxycarotenoids and corresponded to the dehydroxylated terminal ring with cleavage between C23 and C24. On the other hand, fragment ion at m/z 119 represent cleavage in the conjugated polyene chain as described by **Rivera et al.** [20]**.**



Compound 4: was identified in both *S.obliquus*and *S. platensis* as violaxanthin having a molecular weight 600 and molecular forrmula $C_{40}H_{56}O_4$. The mass fragmentation pattern of compound 4 Figure (5) and Table (2) showed protonated molecular ion $[M+H]^+$ at m/z 601. Also, the mass spectrum of compound 4 showed molecular ions at m/z 583 $[M+H-18]^+$ and 565 $[M+H-2H_2O]^+$ corresponding to loss of one and two water molecules, respectively. In addition, other fragment ions at m/z 547, 503, 445, 431, 397, 371, 355 and 221 were found in the mass spectrum which also reported by Ren and Zhang [19] and identified as violaxanthin.

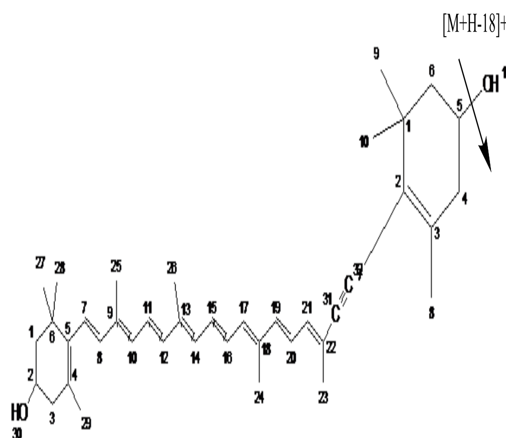


Compound 5: was identified in the three microalgae (*D. salina, S. obliquus*and *S. platensis*) as echinenone which was a ketocarotenoid having a molecular weight 550 and molecular formula $C_{40}H_{54}O$. The fragmentation pattern of compound 5 was illustrated in Table (2) showing protonated molecular ion $[M+H]^+$ at m/z 551. The molecular ion at m/z 203 is a characterof carotenoids containing a keto group as the only substituent on the β-ring and this ion is formed by cleavage between carbons 20 and 21 with the positive charge remaining at the ketone moiety as described by Rivera et al. [20]. Loss of an uncharged radical containing the ketone was characteristic of carotenoids containing a keto group conjugated to the polyene chain. Product ion at m/z 536 may be due to loss of a methyl radical from β-ring. Product ion at m/z 56 indicates the presence of a Ɛ-ring end group as shown in Rivera et al. [20] and explained by Retro-Diels-Alder fragmentation of the ring containing the ketone described by Breemen et al**.** [6]**.** Fragments at m/z 133 and 69 represented cleavage at multiple positions of



conjugation in the polyene chain.

Compound 6: was identified in the three microalgae (*D. salina, S. obliquus*and *S. platensis*) as diatoxanthin which was a hydroxycarotenoid having amolecular weight 566 and molecular formula $C_{40}H_{54}O_2$. Its fragmentation pattern was summarized in Table (2) showing a protonated molecular ion $[M+H]^+$at m/z 567. Also, fragment ion at m/z 549 was corresponding to loss of one molecule of water $[M+H-18]^+$ followed by loss of xylene producing a molecular ion at m/z 443 $[M+H-18-106]^+$[20]**.**Also, Fragments detected in our study at m/z 443, 217, 199, 310, 175 and 145 were reported by Neto et al. [22]**,**which confirmed that the identified compound was **diatoxanthin**.



3.2. ***In-vitro*** **bioactivity studies of the carotenoid content of microalgae (*D. salina, S. obliquus* and *S. platensis*)**

3.2.1. Cytotoxic activity study:

In comparison with Doxorubicin as a reference drug, *S. platensis* and *D. salina s*howed highly significant inhibition against liver cancer (100% at 100 µg/ml dose), significant inhibition against breast (70.5 % and 69.7%, respectively at 100 µg/ml dose) and also significant inhibition against colon cancer (91% and 80.7%, respectively at 100 µg/ml dose) while *S. obliquus* showed good inhibition against liver cancer (60.2% at 100 µg/ml dose) as shown in Table (3) and Figures (6-8).

Table 3. Cytotoxic activity of carotenoids isolated from *Dunaliella salina* ,*Scenedesmus obliquus* and *Spirulina platensis*

Cell line	Tested Carotenoids	% of inhibition						
	Dose (µg/ml)	6.25	12.5	25	50	100	IC_{50}	IC_{90}
HePG2	*D. salina*	10.4±0.05[a]	25.3±1.10[b]	66.2±4.4[c]	90.1±6.12[d]	100±5.11[e]	24.7	42.7
	S. obliquus	0	2.4±0.4[f]	9.7±0.02[a]	24.6±1.11[b]	60.2±5.11[c]	-	-
	S. platensis	14.2±0.66[g]	26.4±1.00[b]	49.2±3.55[h]	75.00±2.00[c]	100±5.00[e]	31.1	57.2
	Doxorubicin	13.7±1.22[g]	32.4±2.00[b]	60.2±3.00[c]	97.2±3.4[e]	100±7.37[e]	21.6	37.8
MCF7	*D. salina*	3.5±0.05[f]	9.8±0.05[a]	23.5±1.10[b]	51.2±1.00[h]	70.5±3.00[c]	66.4	115.2
	S. obliquus	0	0	0	6.5±0.03[i]	21.21±0.10[b]	-	-
	S. platensis	1.3±0.005[j]	6.2±0.08[i]	19.5±1.10[b]	45.7±4.59[h]	69.7±5.40[c]	69.9	116.9
	Doxorubicin	16.7±1.60[g]	32.7±3.00[b]	57.6±4.00[c]	84.3±5.22[d]	100±1.63[e]	26.1	45.02
HCT116	*D. salina*	2.00±0.15[f]	10.00±0.25[a]	30±2.22[b]	72±3.00[c]	91±5.76[d]	46.7	78.8
	S. obliquus	0	0	3.5±0.35[f]	11.9±0.03[a]	27.5±2.40[b]	-	-
	S. platensis	3.8±0.06[f]	9.5±0.04[a]	21.3±2.34[b]	46.5±4.59[h]	80.7±2.55[c]	62	104.5
	Doxorubicin	10.2±0.05[a]	18.4±0.60[g]	35.4±2.22[b]	65.2±3.60[c]	100±1.63[e]	37.6	65.1

Fig. 6: Cytotoxic activity of carotenoids isolated from *Dunaliella salina*, *Scenedesmus obliquus* and *Spirulina platensis* against heptocelluar carcinoma (HepG2) cell line in-vitro.

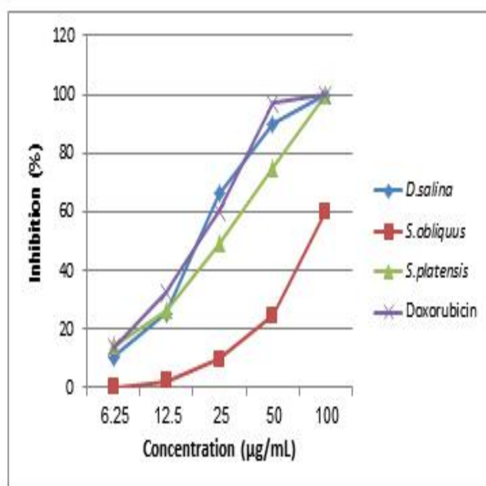


Fig. 7: Cytotoxic activity of carotenoids isolated from *Dunaliella salina* ,*Scenedesmus obliquus* and *Spirulina platensis* against (breast cancer) MCF7 cell line *in-vitro.*

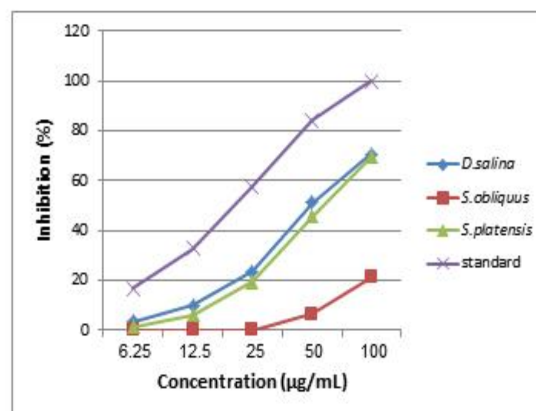
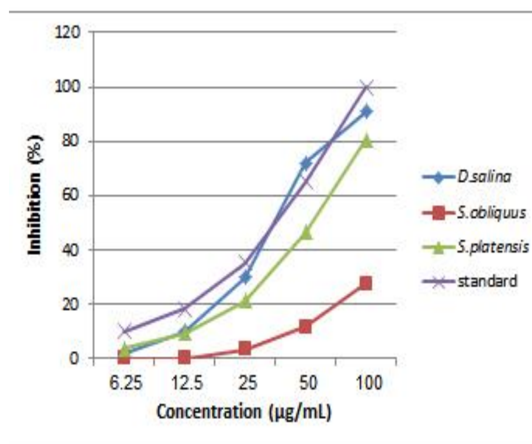


Fig. 8: Cytotoxic activity of carotenoids isolated from*Dunaliellasalina Scenedesmus obliquus* and *Spirulina platensis* against colorectal cancer (HCT116) *in-vitro*.



3.2.2. Antiviral study:

The three microalgae were found to have weak activity against the tested viruses (HCV genotype 4, rotavirus Wa strain, Herpes simplex virus (HSV1) and Coxsackie virus B4).

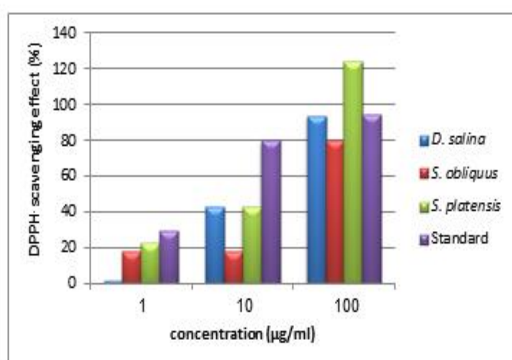
3.2.3. Antioxidant bioassay (DPPH· free radical scavenging activity):

In the present study, we noticed that the antioxidant activities of all the microalgal carotenoid extracts were dose dependent as shown in Table (4) and Figure (9). At 100 µg/ml, *S. platensis* carotenoids showed the highest significant antioxidant activity followed by *D. salina* then *S. obliquus*. At 10 mg/ml dose, carotenoid extracts of *D. salina* and *S. platensis* had significant antioxidant activity. Also, the antioxidant activity of *D. salina* carotenoid extract didn't appear at low dose but appeared obviously at higher doses.

Table 4: Antioxidant activity of carotenoids isolated from *Dunaliella salina Scenedesmus obliquu s*and *Spirulin aplatensis*

carotenoids	% of inhibition		
	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	2.042.04±0.30[b]	42.85±3.93[c]	93.87±2.22[d]
S. obliquus	18.37±0.38[e]	18.00±0.64[e]	80.20±3.54[f]
S. platensis	22.45±0.88[e]	42.85±0.65[c]	124.00±13.87[g]
Ascorbic Acid	29.32±1.23[h]	80.00±10.22[o]	94.82±1.10[i]

Fig. 9: *In-vitro* antioxidant Scavenging activity of carotenoids isolated from *Dunaliella salina ,Scenedesmus obliquus* and *Spirulina platensis*



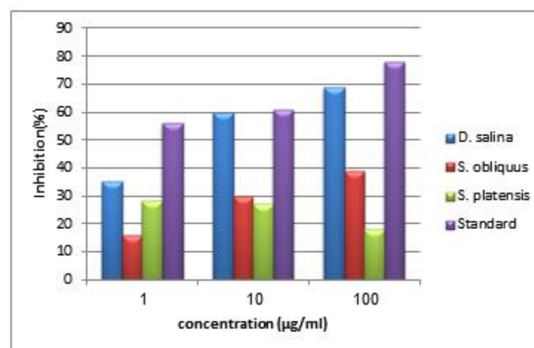
3.2.4. Cholinesterase inhibition activity

As shown in Table (5) and Figure (10), the anticholinesterase activities of Dunaliella and Scenedesmuswere dosedependent while it was inversely proportioned with the dose in Spirulina**.**Also,Dunaliella exhibit the highest significant cholinesterase inhibition activity in comparison with galanthamine standard.

Table 5: Cholinesterase inhibition activity of carotenoids isolated from *Dunaliella salina ,Scenedesmus obliquus* and *Spirulina platensis*

Tested material	% of inhibition		
	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*carotenoids	35.00±1.89[bf]	60.00±3.56[c]	69.00±3.00[d]
S.obliquus carotenoids	16.55±0.23[e]	30.00±0.87[b]	39.00±1.33[f]
S.platensis carotenoids	31.00±0.73[b]	16.00±0.28[e]	6.00±0.76[g]
Galanthamine standard	56.00±3.55[f]	60.60±2.77[f]	78.00±3.09[a]

Fig. 10: Cholinesterase inhibition activity of carotenoids isolated from *Dunaliella salina ,Scenedesmus obliquus* and *Spirulina platensis.*



From the previously mentioned data, six carotenoids (β-carotene, lutein, antheraxanthin, violaxanthin, echinenone and diatoxanthin) were detected in *S. platensis* and *S. obliquus* while lutein and violaxanthin were absent in *D. salina*. β-carotene, diatoxanthin and echinenone were noticed to be predominant in *D. salina* while echinenone, diatoxanthin and anthraxanthin were predominant in *S. obliquus*. Furthermore, lutein, β–carotene and diatoxanthin were predominant in *S. platensis*. In the present study, *D. salina* carotenoids fraction had highly significant *in-vitro* anticancer and significant antioxidant activities which may be due to the predominance of β-carotene and the high percentage of the total carotenoids. Also, the predominance of Lutein and β-carotene in *S. platensis* caused significant anticancer and high significant antioxidant activities. On the other hand, *S. obliquus* had moderate *in-vitro* antioxidant and anticancer activities against hepatocarcinoma which may be due to the less predominance of β-carotene. The predominance of β-carotene gave highly significant anticancer and anticholinesrerase activities to *D. salina* and *S. platensis* carotenoid fractions as described by Osganian [23], Tapiero et al. [24] and Hosokawa et al. [25], as they stated that carotenoids forestall the chain response creation of free radicals started by the corruption of polyunsaturated fats, subsequently forestalling the quickened debasement of lipid films. These properties of carotenoids are utilized to clarify the epidemiological and trial contemplates that show that dietary carotenoids repress the beginning of certain malignancies. Also, the promising anticholinesrerase activity of *D. salina* run in parallel with Aly et al. [5] who found that *D. salina* exhibitted neuro-modulating effect against Alzheimer's disease (AD) in rats in comparison with donepezil reference drug. Moreover, the predominance of β-carotene together with lutein explain the maximum antioxidant activity of *S. platensis* agreeing with **Woodall et al.** [26]**.** who stated that carotenoids, such as β-carotene, astaxanthin, lutein, and lycopene, are acceptable

antioxidant agents in lipid stages, working as free radical or singlet oxygen quenchers. Singlet oxygen (1O2), a free radical is known to harm DNA. Carotenoids act by retaining the energy of the singlet oxygen into the carotenoid chain, which prompts the corruption of the carotenoid particle, yet shields different atoms or tissues from harm. Also, **Aly et al.** [5], declared that carotenoids are well-known for their effect to disrupt, singlet oxygen by physical quenching activity. Further, carotenoids can quench radicals by hydrogen atom transfer or by accepting electrons from radical.

The mechanism of action of β-carotene as antioxidant was by $^{1}O_2$ quencher, inhibits $Na^{+}K^{+}$-ATPase, stimulates catalase , glutathione transferase and radical scavenger as described by Raposo et al. [3]**.** Also, the unique molecular structures of some carotenoids, which have additional oxygenic functional groups (epoxy, hydroxyl, carbonyl and/or carboxyl), together (or not) with special unique allenic features and several conjugated double bonds, provide them with a strong antioxidant activity[3]and this happened with lutein (hydroxyl carotenoid), violaxanthin (epoxycarotenoid), diatoxanthin (hydroxyl carotenoids), anthraxanthin (epoxycarotenoid) and echinenone (ketocarotenoid) detected in our study.

The property of the studied microalgae carotenoids as powerful free radical scavengers and anticholinesterase will be useful in treatment of Alzheimer disease (AD) and cancer since one of the characteristic features that happen in AD is increment in the activity of acetyl cholinesterase (AChE), the enzyme answerable hydrolysis of acetylcholine, from brain cholinergic and non-cholinergic neurons [4]**.** The aerobic metabolism of the brain normally delivers hydrogen peroxide as a bi-product. H2O2 is frequently viewed as a poisonous particle for a wide scope of living frameworks. Hydrogen peroxide has likewise been accounted for to be ensnared in serious obsessive conditions, for example, malignancy, ischemia and neurodegenerative sicknesses [4]**.** In our study, the *in-vitro* anticholinesterase activity of *S. obliquus* was firstly reported. As these carotenoids had more applications, we apply some stress conditions to increase the yield of carotenoids as described by El-sayed[15]**.**

4. Conclusion

S. platensis carotenoid fraction (having lutein, beta carotene and diatoxanthin predominantly) and *D. salina* (having beta-carotene, diatoxanthin and echinenone predominantly) were recommended to be used *in-vitro* against HepG2, MCF7 and HCT116 cancer cell lines and as antioxidants. On the other hand, *S. obliquus* (having echinenone, diatoxanthin and antheraxanthin predominantly) may be used *in-vitro* against hepatocarcinoma. Also, D. *salina* is recommended to be used in researches of Alzheimer's disease.

5. Acknowledgment

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6. Conflicts of Interest

No conflicts, informed consent, or human or animal rights are applicable to this study.

7. Declaration of authors' contributions

Professors Nabaweya A. Ibrahim and Azza A. Matloub provide the critical role in experimental design and handling of experiments. While, professor Abo El-Khair B. El-Sayedprovide the all different algal materials. Also the authors Prof.HananF.Aly and Asmaa S. AbdElsamiae provide critical roles in data evaluation, statistical analysis critical writing of manuscript and results interpretation**.**

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