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Production of Carotenoids from Marine Microalgae and its Evaluation as Safe Food Colorant and Lowering Cholesterol Agents

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Abstract: *Dunaliella salina,* a green marine algae, accumulated high amounts of carotenoids (12.6%, d.w), including β-carotene (60.4%, of total carotenoids), astaxanthine (17.7%), zeananthin (13.4%), lutein (4.6%) and cryptoxanthin (3.9%), when cultivated under salted stress condition combined with low nitrogen level. The algal carotenoids was evaluated as a natural food colorant using male albino rats. Four groups of male rats were fed on standard diet (SD), diets containing 0.5 and 1.0 g /Kg of *Dunaliella* carotenoids and *trans* β-carotene for 6 weeks. The tested diets did not induce any obvious injurious effect or morality of the animals. The values on absolute and relative weights of vital organs, body weight gains, food intake, food efficiency and protein efficiency ratio did not reveal any significant differences among all tested rat groups. The levels of glucose in plasma and hepatic glycogen were not significantly changed compared with the rat control group. Also, the enzyme activities of plasma aspartate (AST) and alanine aminotransferase (ALT), alkaline phosphatase (ALP) and levels of total proteins, urea and creatinine did not show any significant differences compared with rat control group. Whilst, rats fed on carotenoid supplemented diets possessed significant lower levels in plasma and hepatic total lipids, triglycerides, total cholesterol and phosophlipids. Overall consumption of carotenoids, as a natural colorant from *Dunaliella salina* did not result in adverse effects in experimental animals, but might possess hypolipidemic and hypocholesterolemic properties. Therefore, *Dunaliella salina* algae can be further extended to exploit its possible application for various health benefits as nutraceuticals and food additives.

Key words: Microalgae · food additives · natural colorant · *Dunaliella salina*

INTRODUCTION

The halotolerant unicellular green algae *Dunaliella salina* has been widely recognized to accumulate high amounts of carotenoids (up to 13 % w/w) when grown under growth limiting conditions such as nitrogen starvation or high salt concentration and exposed to high light intensity [1-4]. The interest in *D. salina* algae has been renewed in recent years due to the increasing demand for natural pigments of vegetable origin to be used as a substitute for the synthetic counterpart. Carotenoids of algae are being used as a source of colorant or as scavenger and /or quencher of reactive oxygen species (ROS) [5,6] and an enhancer for antibody production [7]. It has been reported that more than 100 tons of carotenoid pigment might be required for inclusion in animal feed [8]. Food additives as antioxidant and colorants are very important ingredients in most convenient foods [9]. The synthetic permitted food additives has been decreased for their suspected action as promoters of carcinogensis and caused liver and renal toxicity [10]. Therefore, there is a great interest in substituting the synthetic food additives with natural pigments as carotenoids.

Carotenoids have been reported to have multiple biological activities such as anticarcinogen [3, 4], antimutagenic [11], antioxidant [12], anti-inflamnatory, antiprolifertive, [12] and antiatherogenic properties [14] and chemopreventive agent against cancer disease in various organs like lung, stomach, colon, breast and prostate [15]. Also, carotenoids can play important role in immune response [7], neoplastic transformation and control of growth and intracellular communication [16]. The biological activities of carotenoids as β-carotene are related in general to their ability to form vitamin A in the body [17]. However, other carotenoids such as

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astaxinthin, lycopene and cryptoxanthin do not form vitamin A. In this context, there are two major hypotheses proposed to explain the biological effects of carotenoids: oxidation and non-oxidative mechanisms [18].

Chronic diseases including cancer and cardiovascular disease are the main causes of death in the world. About 50% of all cancer has been attributed to diet [19]. Oxidative stress induced by ROS is one of the main foci of recent research related to pathogenesis of many diseases. ROS, including oxygen free radicals are causative factors in the etiology of degenerative disorders including some hepatopathies and other serious organ damage due to modify the cellular biomolecules such as lipids, proteins and DNA [20-22]. Carotenoids may protect cells from oxidative stress by quenching singlet oxygen with physical mechanism [11,22].

The search for safe potential source of food additives and supplement is therefore highly desirable. One of our main interests is to assess the suitability of some bioactive compounds obtained from cultivated algae such as carotenoids (red color) of *D. salina* exerted good antioxidant and anticarcinogen activities. Consequently, the main objective of the present work was to study the toxicological safety natural pigment extract from *D. salina.*

MATERIALS AND METHODS

Algal source: Marine microalga *Dunaliella salina* was obtained from the culture collection of Botany Department, Texas University, Austin, Texas, U.S.A.

Growth conditions: The mass culture of *Dunaliella salina* was cultured in 3 aquarium with 40 L of culture medium containing 8% NaCl and 70 ppm nitrogen (as optimum conditions) and 25% NaCl and 5 ppm nitrogen (stress condition), pH was adjusted to 8.5 during autumn season in National Research Centre (NRC). The cultures were gassed with 1.5% volume CO_2 in air and were grown at 20-23°C. The cultures were illuminated with continuous cool white fluorescent lamps (Philips, 40 w) at light intensity level of approximately 400 w m^{-2} [23].

Growth measurements and harvesting: The growth of *Dunaliella salina* thoughtout growth period was measured by dry weight and optical density (O.D.) methods as described by Payer [24]. At stationary phase, algal cells were harvested by centrifugation at 6000 x *g* (4°C) for 15 min and frozen at -20°C.

Extraction of carotenoid pigments: *Dunaliella* carotenoid pigments were extracted from cells with tetrahydrofuran (THF) (1: 10 w/v), in the presence of 30 mg^{-1} a mixture consists of butylated hydroxytoluene (BHT) and magnesium carbonate (1:10, w/w). After 24 h, an aliquot of the pigments extract was filtered and concentrated to about 10 ml, under vacuum, then, the solvent was completely removed under a stream of nitrogen. The extracted pigments were saponified with 25 ml of 10% methanolic potassium hydroxide for 2 h at room temperature and extracted with dichloromethane. The solvent layer was then separated by separator funnel, washed several times with distilled water, dried over Na_2SO_4 and completely removed by nitrogen gas.

Determination of algal total carotenoids: The total carotenoids were spectrophotometriclly estimated at 450 nm by the method of Semenenko and Abdullaev [25]. β-carotene served as a standard compound, was used for preparing the calibration curve.

Identification of carotenoids: Carotenoids were identified by Thermo-separation-liquid chromatography system consisting of a spectra system (UV 2000) detector (at 438 nm) and Spectra System P 2000 pump, on a 250x4.6mm (i.d.) column packed with Chromosil C18 material, 5 μm particle sizes and eluted with acetonitrile: methanol (80:10 v/v), at a flow rate of 1 ml min^{-1}. Some available standard carotenoids: β-carotene, zeaxanthin, lutein, astaxanthin and cryptoxanthin (Sigma Chemical Co.) were also run under the same HPLC conditions [26].

Nutritional evaluation

Animal: The animals used in this study were male Albino rats (Rattus CFT-Wistar strain, n= 28), with an average initial body weight of 80 ± 4.5 g (mean ± SD), obtained from the Research Institute of Ophthalmology (Giza, Egypt). Rats were randomly divided into four groups of 7 rats each. Each group was housed in a stainless-steel wire-bottomed cage in a room with controlled temperature and light.

Diet: According to one factorial experimental design four diets were used, differing in the level of carotenoid supplement. Groups of rats were fed experimental diets as follows: group 1, fed on a standard diet formulated as reported by Venkataraman *et al.* [27] (Negative control, SD); group 2, fed on a standard diet supplemented with synthetic β-carotene (0.5 g/Kg diet) (positive control, SDβC); group 3 was fed on a standard diet supplemented

with *D. salina* carotenoids pigment (0.5 g/Kg diet) (SD*DC*0.5); group 4 fed on a balanced diet supplemented with *D. salina* carotenoids (1.0 g/Kg diet) (SD*DC*1). The experimental feeding of rats on different diets composition was continued for 6 weeks.

Biological analysis: Food intake and body weight gain were monitored throughout the experimental period, food efficiency and protein efficiency ratio were determined at the end of the experiment.

Rat organ weights: At the end of the 6-week experimental period, rats were decapitated, vital organs: liver, kidney, lungs and heart were removed and weighed. The relative organ weights were calculated as follows: weight of organ /total body weight x 100.

Determination of some biochemical parameters in plasma: After the animals were decapitated and the blood was collected, plasma was obtained upon centrifugation at 1500 x g kept frozen until analysis. Clinical chemistry on plasma including: blood glucose, creatinine and bilirubin, plasma total proteins, alkaline phosphatase (ALP, E.C. 3.1.3.1), alanine aminotransferase (ALT, E.C. 2.6.1.2), aspartate aminotransferase (AST, E.C. 2.6.1.1), cholesterol, phospholipids, triglycerides and total lipids were done immediately by using Boehringer Mannheim GmbH Diagnostic kits (Germany).

Determination of liver lipids and glycogen: A portion from liver tissue was crushed to small particles in a glass homogenizer and extracted with 2 ml chloroform and centrifuged at 1500 x *g* to obtain the supernatant. Total lipids and lipid constituents were determined using diagnostic kits. Hepatic glycogen was extracted with potassium hydroxide solution (30 %) and hydrolyzed with sulphuric acid (5M) and then the produced glucose was determined using anthrone reagent as reported by Hassid and Ablraham [28].

Each chemical determination was performed in triplicate and the mean values are presented in the text.

Statistical analysis: Values generated for each treatment group were analyzed for statistical differences by the student's *t*-test.

RESULTS AND DISCUSSION

D. salina was allowed to grow and to accumulate large amounts of carotenoids under optimal conditions (8 % NaCl and 70 mg l^{-1} N) and salted stress (25 % NaCl and 5 mg l^{-1} N) and exposed to light intensity. The maximum dry weight (g L^{-1}) of *D. salina* cells grown under these conditions was found to be 1.43 g L^{-1} and 0.961 g /L respectively, at the beginning of stationary phase (22 days) and the total carotenoid content was found to be 1.56% (w/w) and 12.6 %, respectively (Table 1). It also noticed that the yield of carotenoids was 121.1 mg l^{-1} and 22.31mg l^{-1}, respectively.

The carotenoid compounds of *D. salina* cells grown under both conditions were qualitatively and quantitatively determined by HPLC. As shown in HPLC chromatograms (Fig. 1), *D. salina* carotenoids containing β-carotene as the major compound along with other carotenoids, i.e., -zeaxanthin, cryptoxanthin, lutein and astaxanthin (Table 2). The amount and relative percentage

Table 1: Effect of stress conditions on carotenoids of *Dunaliella salina* at the end of experimental period (22 days)

Algal growth conditions	Dry weight g/l (d.w)	Ratio S/O	Total carotenoid %	Ratio S/O	Yield of carotenoids (mg l^{-1} d.w)	Ratio S/O
Cell cultivated in 70 mg l^{-1} nitrogen +8% NaCl	1.43	1.0	1.56	1.0	22.31	1.0
Cell cultivated in 5 mg l^{-1} nitrogen +25% NaCl	0.961	0.672	12.6	8.1	121.1	5.4

Ratio S/O: Cells under stress conditions(S) / Cells under optimum conditions (O)

Each value repersents the mean of three replicates and based on dry weight

Table 2: Carotenoids profile of *Dunaliella salina* grown under stress conditions

Carotenoids	Cell cultivated in 70 mg l^{-1} N. +8% NaCl	Relative%	Cell cultivated in 5 mg l^{-1} N. +25% NaCl	Relative%	Ratio
β-carotene (μg l^{-1})	5.6	39.4	70.2	60.4	12.5
Zeaxanthin (μg l^{-1})	1.23	8.7	15.6	13.4	5.3
Cryptoxanthin (μg l^{-1})	0.95	6.7	4.5	3.9	4.7
Lutein (μg l^{-1})	2.92	20.6	5.3	4.6	4.3
Astaxanthin (μg l^{-1})	3.5	24.6	20.6	17.7	5.8

Each value repersents the mean of three replicates and based on dry weight All values are significant at (P< 0.5)

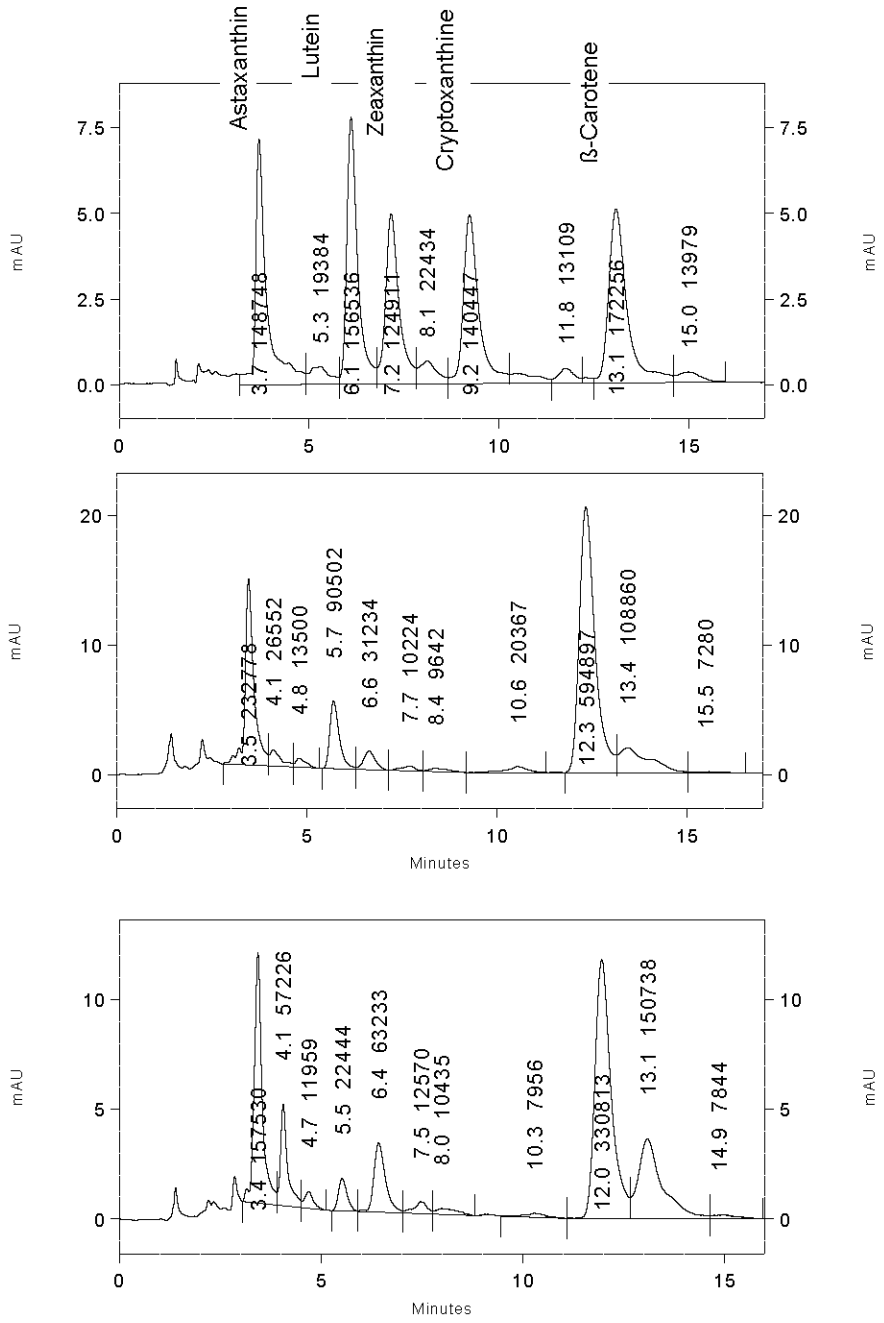

Fig. 1: HPLC profile of *Dunaliella salina* carotenoids (a): HPLC chromatograme of carotenoids standard (b): HPLC chromatograme of *Dunaliell carotenoids* (cells cultived in 70 ppm N.+ 8%NaCl) (c): HPLC chromatograme of *Dunaliell carotenoids* (Cell cultivated in 5 ppm N. + 25 % NaCl)

(in parenthesis) of each carotenoids in algae cell under stress conditions were 70.2 μg l^{-1}, (60.4%), 15.6 μg l^{-1} (13.4%), 4.5 μg l^{-1} (3.9%), 5.3 μg l^{-1} (4.6%) and 20.6 μg l^{-1} (17.7%), respectively. The carotenoids values of cells grown under optimum conditions were 5.6 μg l^{-1} (39.4%), 1.23 μg l^{-1} (8.7%), 0.95 μg l^{-1} (6.7%), 2.92 μg l^{-1} (20.6%) and 3.5 μg l^{-1} (24.6%), respectively. These data revealed that the *D. salina* grown in hyper salted medium combined with low N level can accumulate massive amounts of total carotenoids reaching12.6% of algal dry

Table 3: Effect of *Dunaliella salina* carotenoids on rats body weight gain, food intake, food efficiency and protein efficiency ratio

Supplemented diet	Body weight gain (g)	Food intake g/ 42 days	Food efficiency	Protein efficiency ratio
SD	102.5±6.6[a]	12.63±1.2[a]	19.32±1.05[a]	1.28±0.04[a]
SD+0.5 g/Kg ßC	99.6±7.1[a]	12.46±0.96[a]	19.04±1.08[a]	1.27±0.07[a]
SD +D SC 0.5g/Kg	101.5±5.4[a]	12.75±1.23[a]	18.9±1.27[a]	1.26±0.04[a]
SD + DSC 1.0 g/Kg	115.6±6.5[b]	12.89±1.54[a]	21.35±1.94[b]	1.421±0.05[b]

Each value repersents the mean of 7 rats±S.D. * All values are significant at (P< 0.05)

SD: Standard diet (Negative control)

SD + 0.5βC g/Kg: Standard diet supplemented by 0.5 g/Kg β-carotene (Positive control)

SD + DSC 0.5 g/Kg: Standard diet supplemented by *D. salina* carotenoids 0.5 g/Kg

SD + DSC 1.0 g/Kg: Standard diet supplemented by *D. salina* carotenoids 1.0 g/Kg

Table 4: Relative organ weight (% Body weight) of rats fed on diet supplemented with *Dunaliella salina* carotenoids

Supplemented diet	Liver	Kidney	Lungs	Heart
SD	4.09[a]	0.93[a]	0.55[a]	0.52[a]
SD + 0.5 g/Kg βC	4.02[a]	0.91[a]	0.53[a]	0.52[a]
SD +D SC 0.5g/Kg	3.99[a]	0.92[a]	0.54[a]	0.53[a]
SD + DSC 1.0 g/Kg	3.95[a]	0.93[a]	0.52[a]	0.53[a]

Each value repersents the mean of 7 rats±S.D. * Allvalues are significant at (P< 0.05)

SD: Standard diet (Negative control)

SD+0.5βC g/Kg: Standard diet supplemented by 0.5 g/Kg β-carotene (Positive control)

SD+DSC 0.5 g/Kg: Standard diet supplemented by *D. salina* carotenoids 0.5 g/Kg

SD+DSC 1.0 g/Kg: Standard diet supplemented by *D. salina* carotenoids 1.0 g/Kg

weight and β-carotene was the major constituents (70.2%) of total carotenoid content. On the other hand, under optimal salt and nitrogen levels algal cells did not accumulate large amount of carotenoids and β-carotene still the most abundant constituent of total carotenoids. Therefore, the stress condition enhanced carotenogenesis process in *D. salina* cells. It appears that β-carotene biosynthesis was a direct function of the salt stress and nitrogen-deficiency during growth. The levels of carotenoids accumulated by salt stress combined with nitrogen in *D. salina* cells in this study were high compared with those reported in the literature with the same inductive factor. In this respect, the maximal carotenoid yield (68.8 mg l^{-1}) was obtained from cells grown under stress conditions was in agreement with Ben-Amotz and Avron, [1], Abd El-Baky *et al.* [2,4].

A set of nutritional experiments was carried to elucidate the influence of total carotenoid pigment extracted from *D. salina* cells grown in indoor under optimal and salt stress combined with N-deficiency on rat growth and blood components. A standard diet (SD) without carotenoids was given to rats and the results of this experiment were taken as a guide to evaluate the safety limits of algal pigments. Another experiment was performed where synthetic all-trans β- carotene is given to rats. Consequently, safety measures can be evaluated between the effects of SD, SD + βC0.5 g/Kg and also, between both SD+*D*C0.5 g/Kg and SD + *D*C1.0 g/Kg diets. No rat death was found throughout the feeding experiments of tested diets under study. Animal aspect and behavior were normal in all groups during the test period. Also, animals did not show any toxic symptoms.

The results of body weight, food intake and food efficiency ratio, and protein efficiency in each rat group are shown in Table 3. There was no significant difference (P<0.05) in body weight gains of rats fed SD, SD+βC0.5 g/Kg and SD+*D*C0.5 g/Kg diets, but the body weight gain of SD + *D*C1.0 g/Kg rat group was significantly higher than that in the other groups. No significant differences between tested and both control animals were detected in food daily intake (range 12.46 – 12.89 g/day). The values of food efficiency ratio and protein efficiency were ratio of SD (19.32 and 1.28), SD + βC 0.5 g/Kg (19.04 and 1.27) and SD + *D*C0.5 g/Kg (18.9 and 1.26) groups and did not show any significant changes. Whilst, the values in SD + *D*C1.0 g/Kg (21.35 and 1.42) group was significantly higher than that in other groups.

At necropsy, no effect was observed on absolute fresh or relative organ weights (liver, heart and kidney) among the four tested groups (Table 4). These values revealed that the vital organs did not changed with the administration of carotenoids pigment of *D. salina*. Also,

Table 5: Effect of diet supplemented with *Dunaliella salina* carotenoids on some rat blood constituents

Parameters	Negative control	Positive control	D.s. carotenoids (0.5 g/Kg B.W)	D.s. carotenoids (1.0 g/Kg B.W)
AP (IU/l)	320±15.1[α]	319±13.9[α]	325±14.2[α]	316±13.3[a]
ALT (IU/l)	36.5±1.6[α]	41.2±1.2[α]	28.5±1.9[α]	42.6±2.4[α]
AST (IU/l)	30.2±2.1[α]	29.8±1.9[α]	68.9±2.3[α]	31.6±1.7[α]
Total proteins (g/dl)	6.11±0.23[α]	5.99±0.65[α]	6.25±0.45[α]	6.22±0.57[α]
Creatinine (mg/dl)	0.93±0.02[α]	1.02±0.05[α]	1.01±0.06[α]	0.99±0.03[α]
Bilirubin (mg/dl)	0.12±0.003[α]	0.09±0.002[α]	0.11±0.004[α]	0.09±0.002[α]
Glucose (mg/dl)	96.33±3.8[α]	100.11±4.9[α]	99.01±4.1[α]	98.2±4.3[α]

Each value repersents the mean of 7 rats±S.D. * All values are non significant at (P< 0.05)

Table 6: Influence of diet supplemented with *Dunaliella salina* carotenoids on rats plasma lipids

Supplemented diet	Total lipids (mg/dl)	Ratio[a] %	Ratio[b] %	Total chloesterol (mg/dl)	Ratio[a] %	Ratio[b] %	Phospholipids (mg/dl)	Ratio[a] %	Ratio[b] %	Triglycerides (mg/dl)	Ratio[a] %	Ratio[b] %
SD	592±7.2	0		79.6±1.2	0		20.14±0.64			70.8±3.5	0	
SD + 0.5 g/Kg βC	530±5.6	10.47	0	71.2±0.99*	10.8	0	17.31±0.66*	14.05		64.9±4.6*	8.33	-
SD +D SC 0.5g/Kg	502±4.2*	15.2	5.2	60.6±1.3*	23.86	29.6	15.25±0.41*	24.28	11.9	51.03±2.9*	27.92	21.37
SD + DSC 1.0 g/Kg	480±6.1*	18.91	9.43	52.7±0.89*	33.79	40.03	14.26±0.63*	29.19	19.12	46.50±2.3*	34.32	28.35

Each value repersents the mean of 7 rats±S.D.
* All values are significant at (P< 0.05)
Ratio[a]: Reduction % compared to negative control
Ratio[b]: Reduction % compared to positive control
SD: Standard diet (Negative control)
SD+0.5βC g/Kg: Standard diet supplemented by 0.5 g/Kg β-carotene (Positive control)
SD+DSC 0.5 g/Kg: Standard diet supplemented by *D. salina* carotenoids 0.5 g/Kg
SD+DSC 1.0 g/Kg: Standard diet supplemented by *D. salina* carotenoids 1.0 g/Kg

their organs had no enlargement or non-morphological differences in particular, in kidney and liver and its tissues.

Table 5 represents the results of plasma of ALP, ALT and AST enzyme activities in rats fed on different experimental diets. The activities of these enzymes in all groups did not show any significant difference from one to another. However, the values in the SD + *DC*1.0 g/Kg group are 316, 42.6 and 31.6 IU/l, respectively compared that of SD group (320, 36.5 and 30.2 IU/l, respectively). The plasma protein contents in rats fed on tested diets are shown in Table 5. The algae carotenoids at 0.5 and 1.0 g/Kg level induced slight increase in the protein content (6.25 g/dl and 6.22 g/dl, respectively), compared with SD group (6.11 g/dl). The slight increase indicates that there was a positive correlation between ALT and AST activities. The slight increase of protein content in SD + *DC*0.5 g/Kg and SD + *DC*1.0 g/Kg groups might be attributed to the high turn over to synthesis of protein molecules.

The changes in contents of plasma creatinine, bilirubin, glucose and hepatic glycogen of rats fed on different experimental diets are shown in Tables 5. The results show that there was a little increase in plasma glucose content of the tested groups (98.2 -99.01 mg/dl) and this increase was not significant compared with SD group (96.33 mg/dl). Also, the concentrations of plasma creatinine, bilirubin and hepatic glycogen in tested groups showed non significant differences with the same values of SD group. However, biochemical data indicated that *D. salina* carotenoids at both levels 0.5 and 1.0 g/Kg diet did not exhibit any significant changes in AST, ALT, ALP activities and the levels of glucose, total proteins, urea and creatinine. These findings revealed that *D. salina* carotenoids did not induce any hepatic or kidney injury.

The concentrations of total lipids (TL), total cholesterol (TC), phospholipids (PL) and triglycerides (TG) in plasma and liver of the tested groups are shown in Tables 6 and 7. The results showed that there were significant decreases in plasma total lipids of SDβC (530 mg/dl), SD + *DC*0.5 g/Kg (502 mg/dl) and SD + *DC*1.0 g/Kg (480 mg/dl) groups compared with SD group (592 mg/dl). These changes were tended to be associated with similar changes in the concentrations of TC, PL and TG constituents. The levels of these lipid constituents were reduced in SD + *DC*0.5 g/Kg and SD + *DC*1.0 g/Kg rat groups to 60.6 and 52.7mg/dl, 15.25 and 14.26 mg/dl and 51.03 and 46.5 mg/dl, respectively, compared with that of SD rats group (79.6, 20.14 and 70.8 mg/dl respectively). Surprisingly, feeding on *D. salina* carotenoids diets decreased the plasma and hepatic total lipids and their constituents compared with that feeding on the

Table 7: Effect of deit supplemented with *Dunaliella salina* carotenoids on rats hepatic lipids constituents and glycogen levels

Supplemented diet	Total lipids mg/g	Ratio[a]	Ratio[b]	Total Chloesterol mg/g	Ratio[a]	Ratio[b]	Phospholipids mg/g	Ratio[a]	Ratio[b]	Triglycerides mg/g	Ratio[a]	Ratio[b]	Glycogen g/100gun
SD	41.11±0.93	0		2.1±0.11	0		0.54±0.03	0		16.3±0.32	0		5.18±0.41[a]
SD+0.5 g/Kg βC	33.22±0.65*	19.19	0	1.8±0.09*	14.28	0	0.48±0.04*	11.11	0	13.2±0.41*	19.02	0	5.11 ±0.32[a]
SD+D SC 0.5g/Kg	29.39±0.74*	28.5	11.52	1.7±0.12*	19.04	5.55	0.43±0.02*	20.37	10.42	11.6±0.32*	28.83	12.12	5.01±0.42[a]
SD+DSC 1.0 g/Kg	27.21±0.63*	33.81	18.09	1.5±0.13*	28.57	16.66	0.39±0.03*	27.77	18.75	9.5±0.36*	41.71	28.03	4.99±0.22[a]

Each value repersents the mean of 7 rats±S.D.
*All values are significant at (P< 0.05)
Ratio[a]: Reduction % compared to negative control
Ratio[b]: Reduction % compared to positive control
SD: Standard diet (Negative control)
SD+0.5βC g/Kg: Standard diet supplemented by 0.5 g/Kg β-carotene (Positive control)
SD+DSC 0.5 g/Kg: Standard diet supplemented by *D. salina* carotenoids 0.5 g/Kg
SD+DSC 1.0 g/Kg: Standard diet supplemented by *D. salina* carotenoids 1.0 g/Kg

supplemented β-carotene diets (500 mg/Kg). The levels of TL, TC, PL and TG in liver were significantly decreased to 29.39, 1.7, 0.43 and 11.6 mg/dl in SD + *D*C0.5 g/Kg rat group and 27.21, 1.5, 0.39 and 9.5 mg/dl in SD + *D*C1.0 g/Kg rat group, respectively compared with 41.11, 2.1, 0.54 and 16.3 mg/dl, respectively in liver of SD rat group. According to our knowledge, the hypolipidemic and hypocholesterdemic effects of *D. salina* carotenoids have not previously been investigated. However, several studies were conducted on cholesterol-regulatory properties of many microalgae, *(Spirulina* and *Chlorella)* [29]. For example, in rat, mice and human studies, *Spirulina* showed positive effect with respect to serum cholesterol reduction and the elevation of HDL-cholesterol level and HDL to LDL ratio [30-32]. Also, feeding *Spirulina* supplemented at 5, 10 and 15% of the diets caused a significant inhibition of total HDL-cholesterol, TG and PL in hypolipidemic rats [9,33].

A possible mechanism for lowering plasma cholesterol in animals fed on diet containing *D. salina* carotenoids is that caused a reduction of lipid peroxidation of endoplasmic reticulum which is the site of lipoprotein synthesis. A second possible mechanism for lowering plasma cholesterol concentration in animals fed on diet containing *D. salina* carotenoids is as follows: carotenoids inhibit the cholesterol synthesis through the inhibition of β-hydroxy-β-methylglutaryl CoA (HMG-CoA) synthesis. This enzyme involves in cholesterol biosynthesis, which is expected to parallel to the activity of HMG-CoA reductase the rate limiting enzyme in cholesterol biosynthesis. However, the work of Futhraman *et al.* [34] showed that hypocholesterolemic effect of lycopene and β- carotene was related to suppression of cholesterol synthesis by inhibiting HMG-CoA reductase and augmentation of LDL receptor activity in macrophage. Also, Murillo [35] found that rats fed 1% dietary astaxanthin for 30 days increased HDL cholesterol (good cholesterol) of 57 mg /dl compared to the control diet with 42.4 mg/dl. Conversely, the LDL (bad cholesterol) decreased from control diet of 12.5 mg/dl to 9.6 mg/dl when supplemented with astaxanthin. Hence, *D. salina* carotenoids containing β-carotene (as the major constituent), lycopen, astaxanthin and cryptoxanthin had significant hypocholesterolemic effect. These constituents had been reported to reduce cholesterol biosynthesis, increased HDL level and decreased LDL level. The decrease in the oxidation of these lipid-carriers reduce the risk of atherosclerosis and coronary heart disease. Also, *D. salina* carotenoids had hypotriglyceridemic and phosphlipidemic effects, it might be through increasing the activity of triglyceride lipase and phospholipase, they are key lipolytic enzymes in the metabolism of TG and phospholipids.

Several animal studies have been performed on the safety of carotenoid supplements. For instance, a 13 week study of synthetic lycopene in rats caused no significant adverse affects at levels up to 3 g /Kg body weight /day [36]. McClain and Bausch [37] reported that synthetic lycopene had a large margin effect of safety and antioxidants are needed to prevent lipid degradation and the occurrence of mutagenic materials. A similar duration test with fungal-carotenoids mixed the diet reported the non observed effect at 1% level [38]. A study of developmental toxicity found no material or development effect in rabbits and rat using synthetic lycopene at levels 2 and 3 g /Kg/d, respectively [39]. *D. salina* carotenoids had no gentoxicity, but inhibited spontaneous and γ-ray-induced micronucleus formation and mit-induced chromosomal aberrations in human lymphocytes *in vitro* [40]. Long term administration of β-carotene derived from *D. bardawil* in young male volunteers (60 mg/d) did not induce any side effects or toxicities during the administration period [41]. Also, the algae extract of *Dunaliella* and *Spirulina* has

been shown to prevent tumor development in hamster buccal pouch. This extract was found to contain ≥15 different carotenoids [14].

Finally, the present data on carotenoids extract, the natural red colorant from edible algae *Dunaliella* did not induce any adverse effects in albino rats. Also, at terminal autopsy, vital organs of treated rat groups did not show any biochemical and morphological alteration as compared to control animals. On the other hand, *Dunaliella* extract has been show to have important health benefits as cholesterol, triglycerides and total lipids lowering effect and other additional physiological benefits for human health. These data may be an indicative of safety of *Dunaliella* carotenoid pigment for human consumption.

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