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# Assessment of β-carotenoids extracted from *Dunaliella salina* microalgae:Toxicological aspects

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**Abstract**

The acute and chronic toxicity of the β-carotenoid-rich fraction (BCRF) from *Dunaliellasalina* (Chlorophyta) microalgae was evaluated by the supplementation of high doses of BCRF to experimental animals. The HPLC analysis showed that hexane crudeextract of *D. salina* contains 0.813% of β-carotene. Various biomarkers were involved in this study, which includes hematological and biochemical analyses such as complete blood count, liver enzymes, and kidney function. In addition albumin and glucose levels were determined. The histopathological examination was also performed on hepatic, renal, and cardiac architectures to evaluate its safety. An acute toxicity study revealed no toxicological relevance post administration of serial doses (500 mg/kg body weight up to 5000 mg/kg b.wt.).The supplementation of experimental animals (male and female mice and rats) with a dose of 500 mg/kg body weight of βCRF of *D. salina* daily for three consecutive months did not show any toxicity symptoms in both genders of mice and rats and/or mortality. Histopathological observation exhibited no noticeable change in the liver, kidney and heart architectures of both male and female mice and rats. Therefore, BCRF of *D. salina* LD50 is up to 5000 mg/kg, and it is safe at the dose of 500 mg/kg b.wt. in chronic toxicity.

Key words: *Dunaliella salina*; β carotene; biochemical functions; hematological parameters;Histopathological investigation

## 1. Introduction

*Dunaliellasalina* micro algae is a rich source of 9-cis β carotene, which has been distinguished as a significant biomolecule in the therapy of various illnesses. It, is a halotolerantchlorophyte, which is perhaps the most extravagant source of natural carotenoids, and amasses up to 10% of the dry biomass as β-carotene under conditions that are

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roblematic for development, i.e., intensity of light. temperatures, supplement limit, and degree of saltiness [1].Recently, carotenoids of natural origin have medical advantages that stand out, and these advantages were acquired from carotenes and xanthophylls that have free radical scavenging activity [2].Microalgae are considered the most extravagant carotenoid sources of natural origin, particularly *D. salina* green algae [3].Three important items are obtained from algae: β-carotene, glycerol, and proteins. B-carotene, with cell reinforcement properties, is utilized as a provitamin A food additive, coloring agent and used in industries that produced health product [4].

Two types of carotene have been recognized, is based on the arrangement of isomer, Cis or Trans (Z/E), and protein supplement. Carotene in thylakoid membranes comprises essentially of all-trans β-carotene and might be essentially communicated; the 'aggregated' β-carotene, which is found in lipid and proline –rich globules and in the globule of protein (the βC-plastoglobulin) in the thylakoid of the chloroplast show the two cis/trans (Z/E) designs are in the proportion ~1[5]. The accumulation of β-carotene inside the lipid globules in the spaces between thylakoids are found in the chloroplast [6]. Subsequently, it has been suggested that the increment in the unsaturated fat substance under stress conditions can be a part of the way it is ascribed to the expansion in β-carotene [7].
The presence of high 9-cis βC contents in *D. salina* is of the greatest interest in drug therapy. The 9-cis βC has a higher cancer prevention action than all-trans βC and more efficient *in vivo* [8]. The 9-cis βC has been implicated in dystrophies of retinal therapies, persistent psoriasis, coronary heart diseases and as anti-aging drug [9]. Interestingly, a synthetic 9- cis βC has newly been found to repress the degeneration of eye cup photoreceptor from mice bearing retinoid cycle hereditary deformity [10].

In light of the abundant accessibility of β-carotene in Dunaliella, its dramatic impact to lessen the danger of lung, throat, pancreas, stomach, bosom, skin, colon, and ovary malignancy were recorded[11,12]. The avoidance of heart and chronic illness and dangerous tumors, increment of lymphocytes cell division, extension of the immunology reaction, neoplastic changes, and growth control are different attributes of this substance [11, 13].
Carotenes are specifically noteworthy at the commercial level as they are critical for human nourishment .Microalgae have been used for helpful applications for several years , and their extracts or potentially extracellular products revealed antimicrobial activity[14]. An investigation by **Herrero et al.** [15] reported the antibacterial and antifungal activities of different extracts of *D. salina* on various microorganisms that are significant in the different sectors of food industry because of its several characteristics, which is of great economic importance.

No data are accessible in the literaturesconducted to date, incorporated the safety of β-carotenoid-rich fraction (βCRF) extracted from *D. salina* microalgae. Hence, the present study aimed to determine for the first time the safety (chronic toxicity) of βCRF extracted from *D. salina* microalgae. In the current study, 500 mg/kg body weight of BCRF was administered for three successive months in both sexes of mice and rats. Blood parameters and different biomarkers as hepatic enzymes, renal examinations as well as albumin and glucose levels were evaluated.

## 2. Materials and Methods

### 2.1. Cultivation of *D. salina*

*D. salina* was isolated from salt deposition basins of the Egyptian Salts and Minerals Company, EMISAL, and grown on BG11 media [16], containing NaCl with 100 g/L. The biomass of algae was harvested and inoculated in plastic bottles with a capacity of 17 L containing 15 L of microalgae culture with continuous aeration. After 10 days of growing, the culture was moved to a fully computerized and controlled photobioreactor with a limit of 4000 L. Carbon dioxide was infused into the culture as a carbon source. The culture was left to grow until the biomass reached 2–2.5 g/l. Algal biomass was left to develop until the biomass reached 2–2.5 g/L. Then, the algal biomass was collected by centrifugation at 2000 rpm and afterward sun-dried at 40$^{o}$C -45 $^{o}$C. The dried biomass of *D. salina* was ground altogether for cell divider interruption [17].

### 2.2. Extraction of β-carotene from *D. salina* powder

Two kg of *D. salina* powder were extracted with *n*-hexane (10×1L) in portions (250g each) by sonication (Elma Transsonic TS 540 Germany). The collected n-hexane extract was evaporated under vacuum at 40ºC to give 16.5 g of semisolid hexane extract. Part of the resulting orange residue was purified by dissolving in acetone and filtration of the precipitated white solid. The purified acetone extract was evaporated under vacuum at 40ºC and then partitioned between n-hexane and 90% methanol. The original n-hexane extract together with the purified acetone extract and its hexane and methanol fractions were subjected to HPLC analysis for the determination of extract and/or fraction with the highest concentration of β-carotene. There was no significant difference between the β-carotene concentration in all extracts and fractions. Therefore, the original n-hexane extract was chosen for further biological investigations. The combined n-hexane extract of *D. salina* was subjected to HPLC analysis for the determination of its β-carotene.

### 2.3. HPLC Analysis

An Agilent technologies 1100 series HPLC was used, equipped with an Agilent G1322A quaternary pump and degasser, UV detector and Agilent Chem Station software, Santa Clara, California, United States software. Samples were injected into a Lichrosphere 100 RP-18, 5 µm, 250 mm × 4.6 mm column (Merck, Germany) maintained at a temperature of 25 °C, guarded by a 5µm, 10 mm × 4 mm guard column. Stepwise gradient elution was carried out starting with 60% methanol/ acetonitrile with increasing polarity, with 100% methanol after 3 min, then continued with 100% methanol (isocratic elution) for further 22 min, with a flow rate of 1.5 ml/min then it was increased after 4 min - 2.4 ml/min, which was a total run of 25 min; the system was equilibrated with 60% methanol/acetonitrile, at the end of each run. The injection volume was 20 µl and detection was made at 450 nm. The same RP-HPLC method was used for the identification of β-carotene in different extracts and fractions of *D. salina* and for the standardization of the bioactive extracts.

### 2.4. Standardization of β-carotene content in *D. salina*

A standard stock solution of β-carotene was prepared, by dissolving 1.1 mg of reference β-carotene (Type II, synthetic, crystalline, and 95% purity, Sigma, USA) in 5 ml ethyl acetate HPLC grade, in a stoppered measuring flask of 5 ml capacity (220 µg/ml). Serial dilutions were prepared to yield a series of five different concentrations (220, 132, 110, 55 and 27.5µg/ml). Each prepared dilution was injected in triplicates. The standard calibration curve was constructed by plotting the mean peak area (y) *versus* concentration (x). Linearity was assessed using the linear regression analysis, which was calculated by the least square method. The sample solution was prepared by dissolving 50 mg of the hexane extract in 5 ml ethyl acetate HPLC grade, in stoppered measuring flask 5 ml capacity (final concentration 10 mg/ml), filtered through membrane filter PTEF 25/045 µm. Aliquots (20 µl, each) were directly injected in triplicates into the HPLC system, the peak areas were recorded, and the β-carotene content (% w/w) was calculated.

### 2.5. Animals

Male and female Swiss mice with an average weight of 20–30 g as well as the male and female Wistar albino rats weigh of 120–130 g were obtained from Animal House Lab, National Research Centre (NRC), Dokki, Cairo were used in this study. Animals were acclimatized for 1week before the start of the experiment (adaptation period).The animals were housed with good ventilation (20 °C) with a cycle of 12 h. Normal basal diets and water were supplied *adlibitum*. The animals were cared for according to the Ethical Committee guidelines of NRC, Cairo, Egypt, for animal experiments.

### 2.6. Acute toxicity

A total of 40 mice were selected with uniform weight of both sexes and divided into four groups of 10 mice each. βCRF of *D. salina* microalgae orally administered to mice in graded doses of 500-5000mg/kg. The control group received the same volumes of distilled water. The percentage mortality for extracts was recorded 24 h later. The mice were observed for 14 days, for any changes in the skin, respiratory, somatomotor activity, and behavioural patterns. Particular observation was done for tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma [18-20].

### 2.7. Chronic toxicity

#### 2.7.1. Experimental design

One hundred and twenty male and female mice and rats were used in this study; 30 mice and rats for each gender were supplemented orally with 500 mg/kg body weight of βCRF of *D. salina* microalgae and were divided into eight groups as follows:
Groups 1 and 2: Control male and female mice (15 mice each) were daily orally administered with 0.9% normal saline solution for three consecutive months.
Groups 3 and 4: Male and female mice (15 mice each) were supplanted orally daily with 500 mg/kg body weight (1/10 LD50) of BCRF of *D. salina* for 3 consecutive months.

Groups 5 and 6: Control male and female rats (15 rats each) as described aforementioned.
Groups 7 and 8: Male and female rats were administered orally daily with 500 mg/kg body weight of BCRF of *D. salina*daily for 3 consecutive months (15 mice each). The animals were observed daily for behavioural, food, and water intakes.
After the 3 months, all animals were sacrificed. Fasting blood samples were collected by puncture of the sublingual vein and left for clotting then, centrifuged at 3000 rpm for 15 min to separate serum for liver and kidney function tests[21-23].Biochemical parameters were determined in serum using Bio diagnostic kits (Bio diagnostics Co., Egypt).

### 2.8. Biochemical parameters

Liver function enzyme activities, alanine, and aspartate aminotransferases

(ALTandAST) as well as alkaline phosphatase (ALP) were estimated in mice and rat sera [24, 25].
The levels of total urea and creatinine were determined according to the methods of **Bartles et al.** [26] and **Fawcett and Scott** [27].The level of bilirubin was also determined [28]. The glucose level was measured using colorimetric kits according to the method of **Trinder**[29].

### 2.9. Histological Examination

At the end of the experiment, parts of the liver, kidney, and heart were removed carefully and fixed in 10% formalin for 24 h. Samples were washed under tap water, dehydrated in ascending grades of ethanol (50%, 70%, 80%, 90%, and 100%), cleared in xylene, and embedded in paraffin wax (melting point 55°C –60°C). The liver, kidney, and heart sections of 4 $\mu$m thickness were prepared and stained with hematoxylin and eosin. Paraffin sections were stained in Harris's hematoxylin for 5 min. Sections were washed in running water for bluing and then stained in 1% watery eosin for 2 min, washed inwater, dehydrated, cleared, and mounted using Canada balsam. Eight microscopic fields per section were examined using a light microscope (Olympus BX50, Japan) under 2 high-power magnifications (X200)[30].

### 2.10 Statistical analysis

Results were expressed as mean ± SD, where n = 15. Statistical analysis for biochemical parts is carried out using the SPSS computer program (version 8) combined with a co-state computer program, where different letters are significant at P≤0.05.

## 3. Results

The extraction of fine powder of *D. salina* with n-hexane revealed a crude extract with extraction yield (0.825%). The HPLC analysis presented in Figure (1) indicated that n-hexane crude extract of *D. salina* contains β-carotene (0.813%).

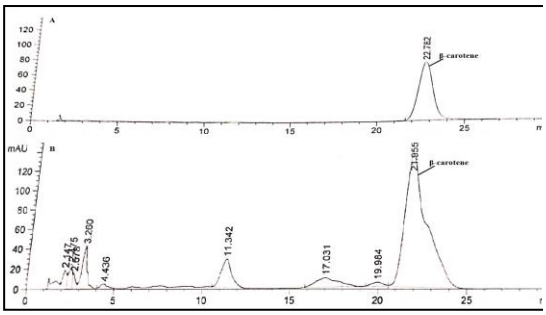
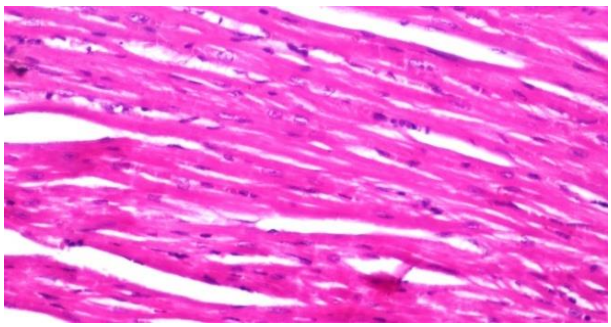



**Figure 1. HPLC chromatogram at 450 nm of β-carotene used as standard (A); and hexane extract of *D. salina*(B).The HPLC analysis indicated that n-hexane crude extract of *D. salina* contains β-carotene (0.813 %).**

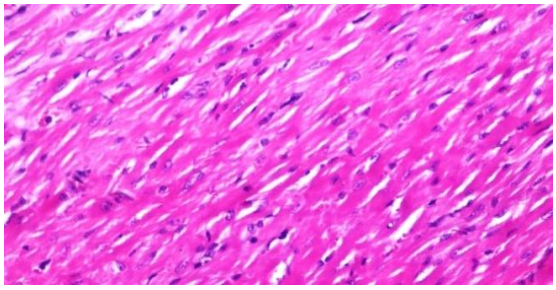
An acute toxicity study revealed no toxicological features and no mortality post the supplementation of rats with serial concentrations (500 up to 5000 mg/kg body weight) of β-carotene-enriched fraction for 24 h.

In chronic toxicity, exposed of both sexes of rodents and mice to daily 500 mg/kg body weight of βCRF for 3 continuous months did not have any harmful indications in both sexes in mice and rodents (no mortality, no going bald, no loose bowels, no patches of yellow shading appearance, and so on… ). Moreover, irregularities in conduct, food, water admissions, and well-being status among the treated creatures were not noticed. The presented Tables (1 and 4) declared insignificant changes in the blood picture profile in BCRF-treated male and female mice and rats when compared with related control groups. Insignificant difference in hepatic enzymes and glucose levels after long-term supplementation of 500 mg/kg βCRF in both sexes of mice and rats was detected (Tables 2 and 5), comparing with corresponding control. Further, urea, creatinine, and albumin levels also showed an insignificant difference after βCRF supplementation in both sexes of mice and rats relative to their control groups (Tables 3 and 6).

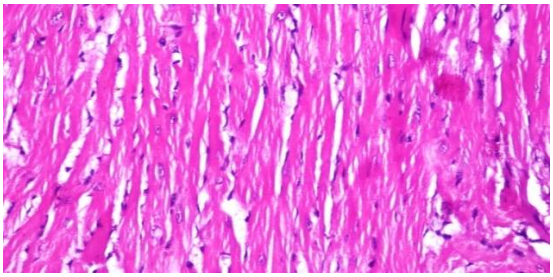
Histopathological examination showed normal cardiac myocytes with normal histological striation and nucleation of treated cardiac tissue. Normal renal glomeruli and tubules of the kidney in rats and mice were also noticed post chronic administration of 500 mg/kg body weight of βCRF of *D. salina*. The liver tissue of βCRF-treated rats also showed normal hepatic parenchyma, hepatocytes, blood sinusoids, portal area, and normal hepatic lobule in both genders of mice and rodents (Figures 2 and 3) as compared to their corresponding control groups



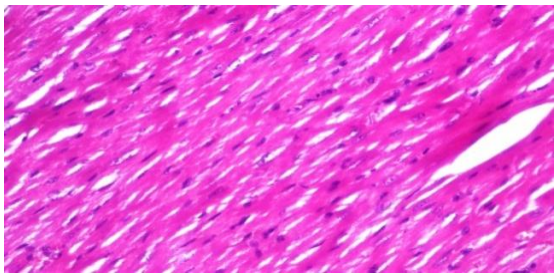
Photomicrograph 1: Heart of control female mice showing normal myocardial muscles; note the normal striation and nucleation(H&E X200).



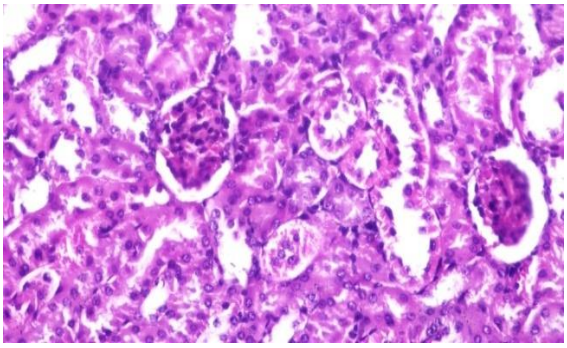
Photomicrograph 2: Heart of control male mice showing normal myocardial muscles; note the normal striation and nucleation (H&E X200).



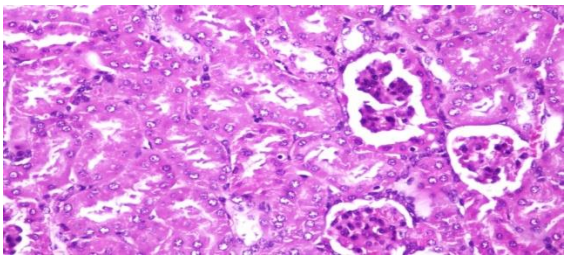
Photomicrograph 3: Heart of treated female mice showing normal myocardial muscles; note the normal striation and nucleation (H&E X200).



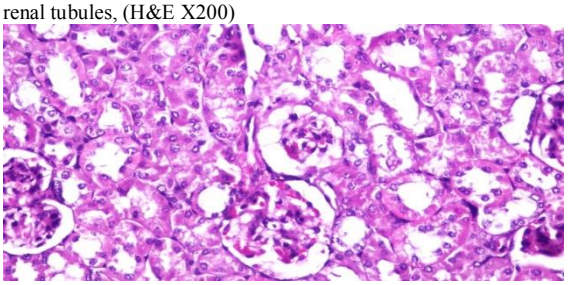
Photomicrograph 4: Heart of treated male mice showing normal myocardial muscles; note the normal striation and nucleation (H&E X200).



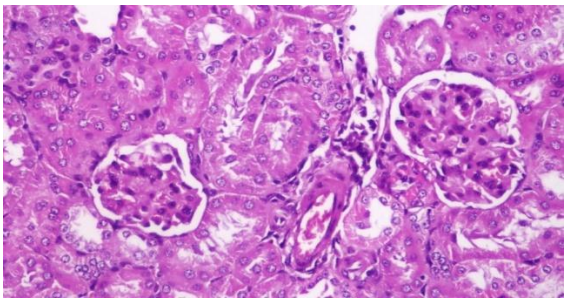
Photomicrograph 5: Kidneys of treated female mice showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, (H&E X200).



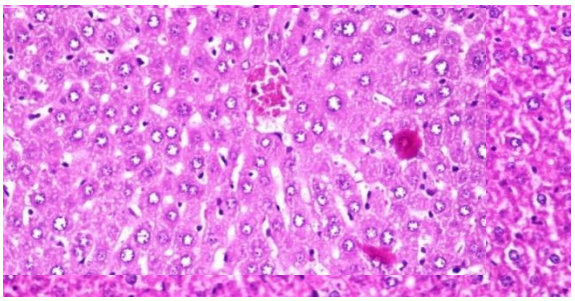
Photomicrograph 6: Kidneys of control male mice showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, (H&E X200)



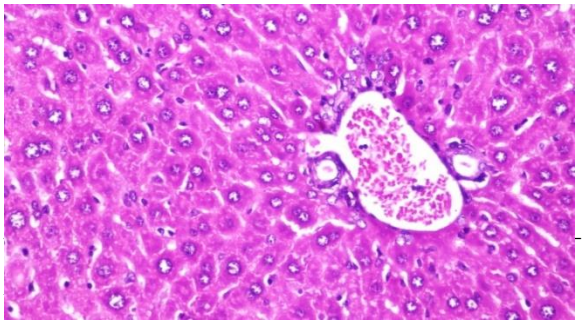
Photomicrograph 7: Kidneys of treated female mice showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, (H&E X200).



Photomicrograph 8: Kidneys of treated male mice showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, (H&E X200).

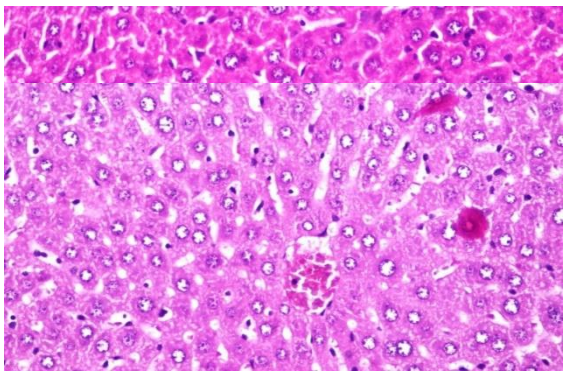


Photomicrograph 9: Liver of control female mice showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).



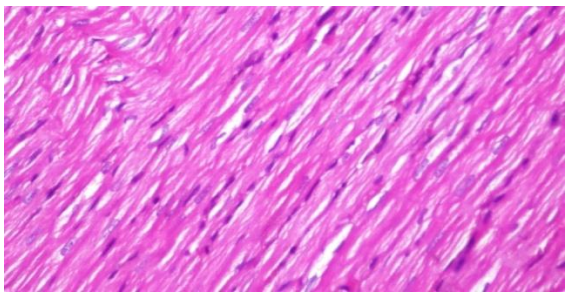
Photomicrograph 10: Liver male control miceof showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and portal area, (H&E X200).

Photomicrograph 11: Liver of female treated mice showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).

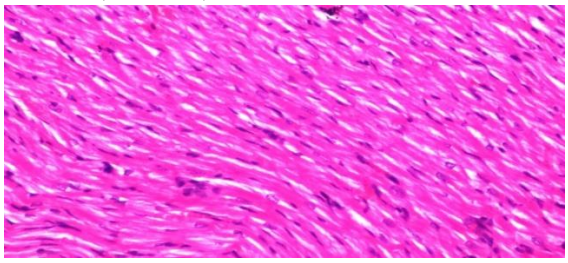


Photomicrograph 12: Liver of treated showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).

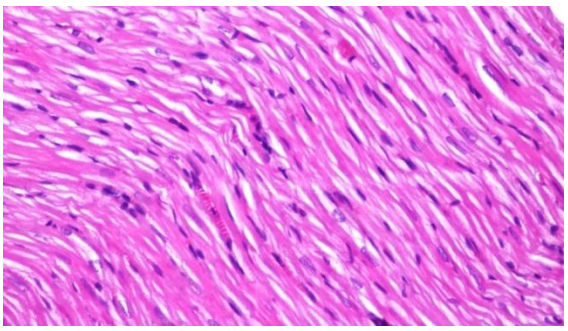
**Figure 2: Histopathological alterations in female and male mice supplemented for three consecutive months with 500 mg/Kg body weight of βCRF of *D. salina*microalgae.**



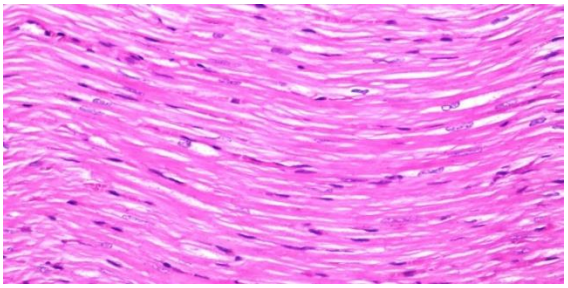
Photomicrograph 13: Normal heart of female control female rats showing myocardial muscles; note the normal striation and nucleation (H&E X200)**.**



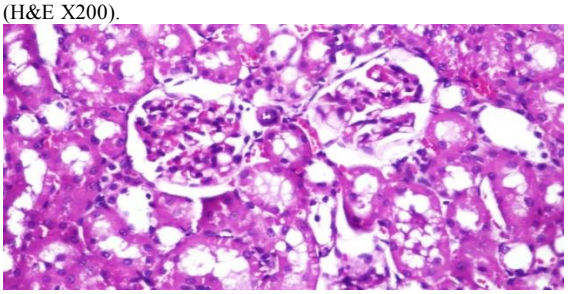
Photomicrograph 14: Normal heart of male control rats showing myocardial muscles; note the normal striation and nucleation (H&E X200).



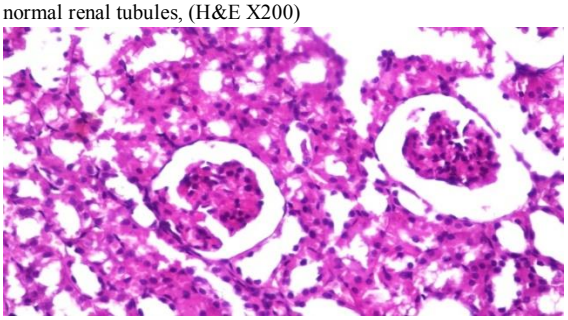
Photomicrograph 15: Heart of treated female rats showing normal myocardial muscles; note the normal striation and nucleation (H&E X200).



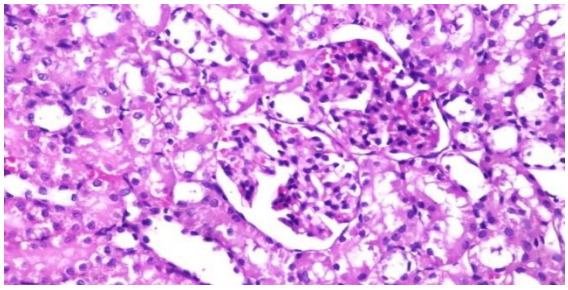
Photomicrograph 16: Heart of treated male rats showing normal myocardial muscles; note the normal striation and nucleation (H&E X200).



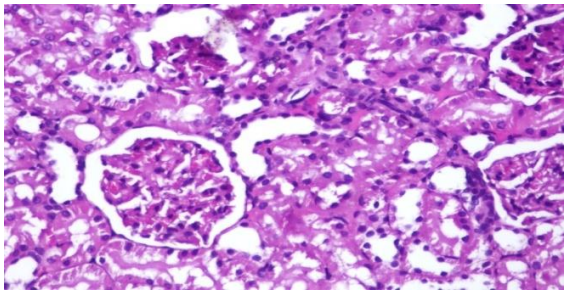
Photomicrograph 17: Kidneys of control female rats showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, (H&E X200)



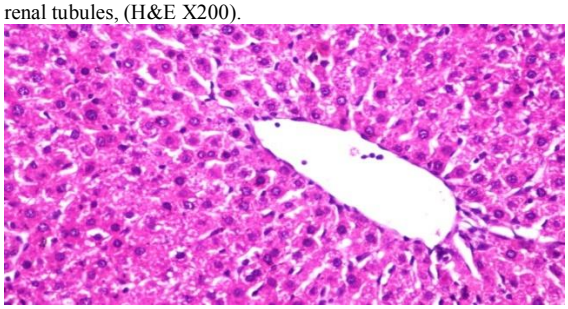
Photomicrograph 18: Kidneys of control male rats showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, (H&E X200)



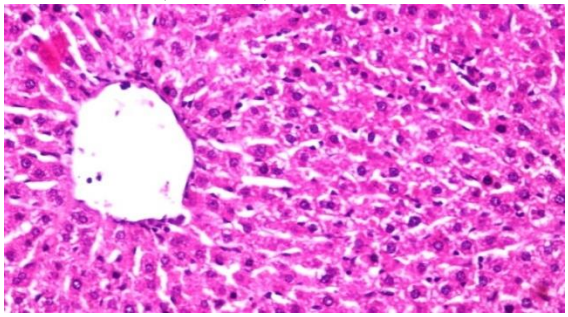
Photomicrograph 19: Kidneys of treated female rats showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, (H&E X200).



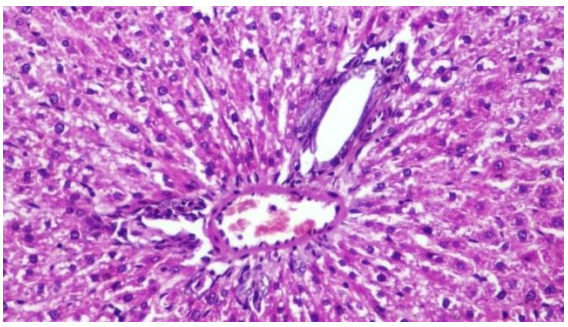
Photomicrograph 20: Kidneys of treated male rats showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, (H&E X200).



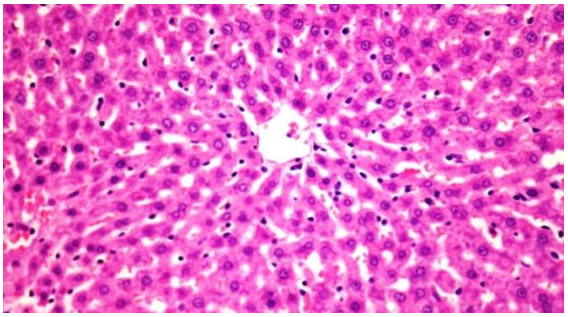
Photomicrograph 21: Liver of control female rats showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).



Photomicrograph 22: Liver of control male rats showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).



Photomicrograph 23: Liver of treated female rats showing normal portal area with normal bile duct, normal hepatic artery and normal portal vein, (H&E X200).



Photomicrograph 24: Liver of treated male rats showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).

**Figure 3.Histopathological alterations in female and male rats supplemented for three consecutive months with 500mg/Kg body weight of βCRF of *D. salina*microalgae**

## 4. Discussion

The β-carotene content (0.813%) of *D. salina* extracted with hexane in the present study, which is higher than the β-carotene content (0.159 mg/g algal powder) of ethanol/water (7:3, v/v) extract of *D. salina*[31] and (5.07 mg/g crude extract) acetone extract of *Chlorella saccharophila*(5.07 mg/g crude extract)[32].β-carotene is the most carotenoids produced in *D. salina* in high amounts. It is a well-known natural antioxidant that protects human cells against free radicals-induced dysfunction particularly singlet oxygen[33].

Thus far, no data are available in the literature for acute and chronic toxicities of BCRF extracted from *D. salina* microalgae. Acute toxicity study of **βCRF**extracted from microalgae *D. salina*revealed no toxicological features and no mortality post the supplementation of rats with serial concentrations (500 up to 5000 mg/kg body weight) of β-carotene-enriched fraction for 24 h .

Additionally, in this research, we evaluate chronic toxicity of β-carotene-enriched fraction. In this study, 500 mg/kg b.wt./day of βCRF of *Dunaliella* were used to investigate the effect of long-term administration of β-carotene-enriched fraction in both genders of mice and rats and for 3 consecutive months. The present data data showed no significant changes in hematological parameters, including Hb, PCV, WBC, RBC, platelets, and other blood cells; neutrophil, eosinophil, monocytes, and lymphocytes/animals/sex/groups as compared to their corresponding control groups. Moreover, liver enzymes such as AST, ALT, and ALP showed no significant changes. Besides, bilirubin and blood glucose levels showed no detectable significant differences in the animals/sex/group as compared to their relative control groups. Additionally, urea, creatinine, and albumin levels showed no significant differences in the two sexes of mice and rodents related to their control groups. Histopathological examination of different organs, namely liver, heart, and kidney, revealed no remarkable alterations and no adverse effects post oral supplementation with 50 mg/kg body weight of **βCRF**/day for 3 continuous months in the tissue architectures of animals/sex/group as compared to control groups.

**Heywood et al.**[34] study the safety of β-carotene, in subchronic and chronic experiments with animals and reported that β-carotene exerted no mutagenic properties in mice and the embryo toxicity studies on rodents and rabbits also reported the same; there was no confirmation of embryo toxicity and no intervention with the function of reproduction in rodents administered orally up to 1000 mg/kg/day doses. However, in contrast to our findings, **Heywood and their colleagues** [35]observed that the treatment of dogs and mice, however, not in rodents with β-carotene, the liver appears with vacuolated cells, eccentric nuclei, and associated with lipid deposition. It was proposed that the vacuolization were cells of fat storage. The differences in histological examination between our study and the one studied previously may be related to the long period of examination, the high oral doses, sources of β-carotene, and the purity.

Table 1. Profile of blood picture of mice (male and female) after supplementation with 500 mg/kg/body weight of βCRF - extracted from microalgae *Dunaliellasalina*

| | Control Male | Control Female | Treated Male | Treated Female |
|---|---|---|---|---|
| HB (g/L) | 11.20 ±0.40a | 11.00± 1.00 a | 12.10 ± 0. 67b | 11. 80 ± 0.28b |
| RBCs(million cells/ uL ) | 6.12 ± 0.76a | 6.10 ± 0.85 a | 6.20 ± 0.81 a | 6.23 ± 0.66 a |
| PCV (%) | 41.78 ± 3.22a | 40.67 ± 3.10 a | 40.76 ± 3.11a | 40.45 ± 5.10a |
| WBCs(× $10^9$ /L) | 9.90 ± 4.50a | 10.00 ± 5.50 a | 10. 00 ± 4.53a | 10.06 ± 3.00 a |
| Neutrophils (×$10^9$ /L) | 20.69 ± 0.78a | 19.90 ± 2.00a | 20.00 ± 1.10a | 19.25± 1.34a |
| Eosinophil (x100 cells /ul) | 1.70 ± 0.34 | 1.67 ± 0.46a | 1.95 ± 0.92a | 1.69 ± 0.74a |
| Lymphocyte($10^9$ /L) | 76.00 ± 3.56a | 77.00 ± 5.00 a | 76.80 ± 5.90a | 77.62 ± 8.22a |
| Monocyte($10^9$ /L) | 1.69± 0.25a | 1.66 ± 0.45 a | 1.67 ± 0.60 a | 1.70± 0.20a |
| Platelets (x $10^9$ /L) | 700.00 ± 30.55a | 690.00 ± 60.00 a | 750.00 ± 60.00 a | 700.00 ± 33.00 a |

Data are means ± SD of 15 mice in the treated group. Statistical analysis is carried out using co-state and SPSS computer programs (version 8), where the unshared letter is significant at P ≤0.05.

**Table 2**

**Hepatic enzyme levels in mice (male and female) after supplementation with 500 mg/kg/body weight of βCRF- extracted from microalgae *Dunaliellasalina***

| Groups | ALT (U/l) | AST (U/l) | Bilirubin(mg/dl) | ALP(U/l) | Glucose (mg/dl) |
|---|---|---|---|---|---|
| Control Male Mice | 37.71 ± 1.00a | 118.65 ± 10.10a | 0.82 ± 0.10a | 85.90 ± 3.66a | 90.00 ± 3.00a |
| Control Female Mice | 38.81 ± 3.00a | 120.15 ± 7.22a | 0.76± 0.21a | 80.00± 8.90a | 100.00± 6.55a |
| 50mg/kg Carotenoids Rich Fraction- Male mice | 40.00 ± 2.33a | 124.22 ± 10.00a | 0.78 ± 0.08a | 75.00 ± 6.70a | 86.90± 5.11a |
| 50mg/kg Carotenoids Rich Fraction-Female mice | 43.45 ± 2.60a | 114.67± 9.11a | 0.70±0.06a | 82.00± 4.07a | 110± 6.65a |

Data are means ± SD of 15 mice in the treated group. Statistical analysis is carried out using co-state and SPSS computer programs (version 8), where the unshared letter is significant at P ≤0.05.

**Table 3**

**Levels of urea, creatinine, and albumin in mice (male and female) after supplementation with 500 mg/kg/body weight of βCRF - extracted from microalgae *Dunaliellasalina***

| Groups | Urea (mg/dl) | Creatinine (mg/dl) | Albumin (mg/dl) |
|---|---|---|---|
| Control Male Mice | 35.66 ± 2.19a | 0.155 ± 0.04a | 2.78 ± 0.60a |
| Control Female Mice | 34.00 ± 3.65a | 0.145 ± 0.12a | 2.85± 0.11a |
| 50mg/kg Carotenoids Rich Fraction- Male mice | 33.00 ± 1.77a | 0.154 ± 0.07a | 2.75 ± 0.11a |
| 50mg/kg Carotenoids Rich Fraction-Female mice | 32.90 ± 3.08a | 0.147± 0.01a | 2.84±0.12a |

Data are means ± SD of 15 mice in the treated group. Statistical analysis is carried out using co-state and SPSS computer programs (version 8), where the unshared letter is significant at P ≤0.05.

**Table 4**

**Profile of Blood picture in rats (male and female) after Supplementation with 500 mg/kg/body weight of βCRF - extracted from microalgae *Dunaliellasalina***

| Groups | ALT (U/l) | AST (U/l) | Bilirubin(mg/dl) | ALP(U/l) | Glucose (mg/dl) |
|---|---|---|---|---|---|
| Control Male Mice | 31. 61 ± 12.00a | 90.00 ± 9.12a | 0.62 ± 0.11a | 80.00 ± 4.60a | 83.00 ± 3.00a |
| Control Female Mice | 27.00± 3.00a | 85.95 ± 6.90a | 0.68± 0.11a | 75.00± 6.10a | 117.00± 10.00a |
| 50mg/kg Carotenoids Rich Fraction- Male mice | 29.00 ± 2.03a | 87.00 ± 8.00a | 0.67 ± 0.10a | 78.00 ± 6.70a | 100.00± 6.20a |
| 50mg/kg Carotenoids Rich Fraction-Female mice | 28.13 ± 2.51a | 94.60± 8.10a | 0.60±0.01a | 82.00 ±7.00a | 116.00± 10.10a |

Data are means ± SD of 15 mice in the treated group. Statistical analysis is carried out using co-state and SPSS computer programs (version 8), where the unshared letter is significant at, where unshared letter is significant at $P \leq 0.05$.

**Table 5. Hepatic enzyme levels in rats (male and female) after supplementation with 500 mg/kg/body weight of βCRF - extracted from microalgae *Dunaliellasalina***

| Biomarkers | Control Male | Control Female | Treated Male | Treated Female |
|---|---|---|---|---|
| HB (g/L) | 13.75 ±1.22a | 13.00± 1.98 a | 14.20 ± 1. 60b | 14. 80 ± 0.28b |
| RBCs(million cells/ uL ) | 7.30 ± 0.76a | 7.20 ± 0.65 a | 8.00 ± 0.98 a | 7. 80 ± 0.88 a |
| PCV (%) | 34.65 ± 2.12a | 34.00 ± 1.90 a | 35.00 ± 1.66a | 33.00 ± 1.04a |
| WBCs($\times 10^9$ /L) | 11.00 ± 2.92a | 10.10 ± 1.88 a | 10. 50 ± 2.13a | 10.06 ± 2.70 a |
| Neutrophils ($\times 10^9$ /L) | 16.69 ± 2.00a | 16.00 ± 1.70a | 15.00 ± 1.65a | 16.25± 2.90a |
| Eosinophil (x100 cells /ul) | 1.69 ± 0.11a | 1.68 ± 0.15a | 1.69 ± 0.11a | 1.66± 0.12a |
| Lymphocyte($10^9$ /L) | 81.00 ± 5.22a | 79.00 ± 6.11 a | 72.00 ± 4.00a | 79.62 ± 7.88a |
| Monocyte($10^9$ /L) | 13.00± 1.25a | 12.90± 1.45 a | 14.60 ± 1.33 a | 14.70± 2.00a |
| Platelets (x $10^9$ /L) | 705.00 ± 20.55a | 652.00 ± 34.00 a | 673.00 ± 33.00 a | 600.00 ± 33.00 a |

Data are means ± SD of 15 mice in the treated group. Statistical analysis is carried out using co-state and SPSS computer programs (version 8), where the unshared letter is significant at $P \leq 0.05$.

**Table 6**

**Levels of urea, creatinine, and albumin in rats (male and female) after supplementation with 500 mg/kg/body weight of βCRF - extracted from microalgae *Dunaliellasalina***

| Groups | Urea (mg/dl) | Creatinine (mg/dl) | Albumin (mg/dl) |
|---|---|---|---|
| Control Male Mice | 27.66 ± 2.00a | 0.200 ± 0.05a | 3.08 ± 0.90a |
| Control Female Mice | 25.00 ± 1.60a | 0.160 ± 0.11a | 3.12± 0.19a |
| 50mg/kg Carotenoids Rich Fraction- Male mice | 28.00 ± 2.00 a | 0.174 ± 0.03a | 3.60 ± 0.51a |
| 50mg/kg Carotenoids Rich Fraction-Female mice | 27.00 ± 2.00a | 0.167± 0.04a | 3.54±0..49a |

Data are means ± SD of 15 mice in the treated group. Statistical analysis is carried out using co-state and SPSS computer programs (version 8), where the unshared letter is significant at $P \leq 0.05$.

In the context of chronic toxicity and in good agreement with our results, **Xue et al.**[35]tested the subacute oral toxicity of carotenoids mixture from citrus peel (Nanfengmiju, *Citrus reticulata* Blanco) and declared that the level of doses 200, 500, and 2000 mg/kg body weight/day given orally to rodents/sex/group for 28 days showed no significant effect of dose on consumption and efficiency of food, gain of body weight, clinical symptoms or ophthalmoscopic indications, and different biochemical investigations. Histopathological examination revealed no observed adverse effect, which suggests that the safety of carotenoids mixture from citrus peel was at least up to 2000 mg/kg body weight/day. However, **Blomhoff**[36] illustrated that huge contrasts in time and dose are expected to imitate hypervitaminosis A. High doses of vitamin A in food and oily solutions are very much endured, whereas emulsified compounds have higher harmfulness. The teratogenicity might be incited by daily doses as low as 40,000 IU of vitamin A (12 mg of retinol) in oil during the first trimester.

## 5. Conclusion

The HPLC analysis showed that hexane crude extract of *D. salina* contains 0.813% of β-carotene. In acute toxicity, no mortality and no toxicological features were seen post 24 h up to 5000 mg/kg body weight. In chronic toxicity, no noticeable significant changes were detected in all blood and biochemical analysis and in pathological investigation of architectures of the heart, kidney, and liver in the two sexes of rodents and mice after long-term supplementation with 500 mg/kg body weight of BCRF extracted from *D. salina* for 3 consecutive months. These results documented that BCRF of *D. salina* LD50 is up to 5000 mg/kg and is safe at the dose of 500 mg/kgb.wt.in chronic toxicity.

## Statement of Informed Consent

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

## Conflicts of Interest

No conflicts of interest.

**Declaration of authors' contributions**

## Declaration of authors' contributions

Professors Farouk K. ElBaz and Hanan F. Aly made important contributions toward experimental design, handling of experiments, and critical writing of manuscript. Also, Professor Farouk K. ElBaz provided algal materials. The authors, Prof.AbeerSalama and Sami I. Ali, made contributions in the extraction and separation of beta carotene by HPLC, data evaluation, statistical analysis, and results interpretation.

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