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Zeaxanthin-enriched fraction of Dunaliella salina: Acute and chronic assessments

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Abstract

The current work aims to evaluate the oral acute and sub-chronic toxicity of Zeaxanthin-enriched fraction from *Dunaliella salina (D. salina)*. In the acute study, mice were orally administered 0.625, 1.25, 2.5 and 5 g/kg of the Zeaxanthin-enriched fraction as a single dose and observed for two weeks. In the chronic study, Wistar rats were orally administered 250 and 500 mg/kg of the Zeaxanthin-enriched fraction for three months. Complete blood picture (CPC), kidney functions (urea, creatinine, and albumin), liver function enzyme activities (alanine and aspartate amino transferases, alkaline phosphatase, bilirubin, and albumin), and blood glucose level were assessed. Finally, liver, kidney, and heart histopathological investigations were performed. The HPLC analysis showed that hexane/ethanol (2:1, V/V) crude extract of *D. salina* contains zeaxanthin (8.469mg/g crude extract), while the Zeaxanthin-enriched fraction contained Zeaxanthin (14.301 mg/g rich fraction).In acute toxicity, no toxicologically relevant findings were noted after 24 h of the Zeaxanthin-enriched fraction (0.625, 1.25, 2.5 and 5 g/kg) compared with the control group mice. In the chronic study, both sexes of rats treated for three consecutive months with a daily dose of 250 and 500 mg /kg of the Zeaxanthin-enriched fraction did not show any sign of toxicity (no mortality, no patches of yellow color appearance, no hair, no diarrhea loss and no abnormalities on behavior). The Zeaxanthin-enriched fraction also did not change CPC, kidney functions, liver enzyme activities, and blood glucose level, compared to their relative normal values. The histopathological study, showed normal kidney, liver, and heart in the Zeaxanthin-enriched fraction groups. The Zeaxanthin-enriched fraction LD_{50} is greater than 5000 mg/kg and is safe at a dose of 500 mg /kg in a chronic study.

Keywords: Zeaxanthin**;** Acute Toxicity; Chronic Toxicity; Rat; Mice, ***Dunaliella salina***

1. Introduction

Dunaliella salina (D. salina) is a halophilic, unicellular, naked green alga. It has variable cell shape, being oval, pear, spherical, ellipsoidal, or cylindrical shape that change with changing surrounding conditions, frequently becoming spherical in stress conditions. It has a pair of equal -length flagella besides one cup-shaped chloroplast. *D. salina* appears orange to red in color instead of green under different stress conditions due to the accumulation of high amounts of carotenoids in the chloroplast, which protects the chlorophyll and the DNA from damage by the high irradiance (Madkour and Abdel-Daim, 2013). *D. salina* carotenoids area a mixture of the β-carotene isomers all-trans and 9-cis and different isomers of Zeaxanthin Hu et al., 2008; (El-Baz et al., 2019). The high content of carotenoids, mainly β-carotene and Zeaxanthin in *D. salina* provide different health benefits against different cancer cell lines, age-associated cardiac dysfunction, obesity-associated inflammation, and oxidative damage (El-Baz et al., 2020b).

Zeaxanthin (3R, 3′R-*β*, *β*-carotene-3, 3′-diol) is one of the carotenes called xanthophylls produced by *D. salina*; it is more polar than other carotenoids thanks to the existence of hydroxyl groups on the cyclic ring structure (Sajilata et al., 2008). Contrary to α- and β-carotene (provitamin A carotenoids), Zeaxanthin has not the ability to convert to vitamin A. It cannot be biosynthesized in animal tissues, and its existence in different tissues entirely is attributed to the consumption of algal or plant sources (Mares-Perlman et al., 2002). Several studies demonstrated that lutein

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and/or Zeaxanthin, the most important macular pigments, reduce the risk of age-related macular disease and diabetic retinopathy through their potent antioxidant and anti-inflammatory effects(Xue et al., 2015). Preceding scientific reports also showed that Zeaxanthin has various protective effects as it exhibited antioxidant, chemoprotective, cardioprotective, anti-mutagenic, and anti-inflammatory properties (Firdous et al., 2015). Zeaxanthin presented cytotoxic influence against colon adenocarcinoma (HT-29) cells, but was not cytotoxic to normal human colon epithelial (CCD 841 CoTr) cells (Grudzinski et al., 2018). Feeding of zeaxanthin (0.005%, an emulsion with drinking water for 40 weeks) presented cancer-preventive effects against spontaneous liver carcinogenesis in C3H/He male mice (Nishino, 1998). Also, exhibited also a protecting effect with acetic acid-induced ulcerative colitis in rats through its anti-inflammatory and anti-oxidative impacts and reduction of caspase-3 expression (El-Akabawy and El-Sherif, 2019). Besides, the intake of Zeaxanthin improves ulcerative colitis (UC) symptoms in patients associated with UC retardation, besides decreases in fecal blood, mucus, and pus (Głąbska et al., 2016).

Different reports indicated the health benefits of Zeaxanthin, either free or extracted from various sources. But, there are no available reports about the use of Zeaxanthin-enriched fraction of *D. salina*. The present study aims to investigate the acute toxicity using the dose of 5000 mg/kg body weight orally administered to male and female mice and chronic study of the Zeaxanthin-enriched fraction from *D. salina* (250 and 500 mg/kg) orally administered to male and female rats daily for three consecutive months. This is to determine the possibility of using them clinically for the treatment of different diseases as well as for human consumption as dietary supplementation.

1. Materials and methods

1.1. Cultivation of *Dunaliella salina*

Dunaliella salina was isolated from salt deposition basins of The Egyptian Salts and Minerals Company, EMISAL, and grown on Bold media (Stein et al., 1973) containing NaCl with a concentration of 100 g/l.The algal biomass was harvested and inoculated in plastic bottles with a capacity of 17 l containing 15 l of microalgae culture with continuous aeration. After growing for 10 days the culture was transferred to a fully automated and computer-controlled photobioreactor with a capacity of 4000 l. Carbon dioxide was injected 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 harvested by centrifugation at 2000 rpm and then sun-dried at40-45ºC. The dried biomass of *D. salina* was ground thoroughly for cell wall disruption.

1.2. Preparations of algal extract and Zeaxanthin-enriched fraction

The fine powder of *D. salina* (100 g) was soaked with 1000 ml of hexane/ethanol (2:1, v/v) in a 2000 ml conical flask and kept on an orbital shaker (Stuart, England) at 160 rpm at room temperature for 24 h. Then, the extract was centrifuged (Sigma 3-18ks Centrifuge, Germany) at 5000 rpm for 20 min at 25°C to separate cell debris from the supernatant. The extraction step was repeated twice and the pooled supernatants were concentrated using a vacuum rotary evaporator (Heidolph Unimax 2010, Germany) at 40°C to dryness giving the crude extract. The crude extract was subjected to a silica gel column chromatography using silica gel 60-120 µm (Sigma-Aldrich Co., USA). Hexane/ethyl acetate was used as a mobile phase with increasing polarity (0, 10, 20, 30, 50, 70, and100% ethyl acetate). This afforded 40 fractions that collected in 25 ml per each fraction. The collected fractions were subjected to TLC (20 × 20 cm aluminum sheets coated with silica gel 60 F_{254}, Merck, Germany) to detect the presence of Phyto-compounds that were visualized by ultraviolet (UV) fluorescent colors at 254/366 nm UV lamps. The afforded 40 fractions were combined into 5 main fractions (1-5) based on TLC results. Each main fraction was concentrated to dryness using a rotary evaporator. The five main fractions (1-5) were subjected to HPLC analysis versus Zeaxanthin (Sigma-Aldrich Co., USA) that used as standard to determine the Zeaxanthin-enriched fraction. Zeaxanthin-enriched fraction (Fraction 4) was confirmed by HPLC. All the extraction and column chromatography fractionation steps were performed in dim light.

1.3. HPLC analysis of zeaxanthin

D. salina crude extract and Zeaxanthin-enriched fraction were subjected to an Agilent 1260 infinity series HPLC-DAD system (Agilent Technologies, Waldbronn, Germany) equipped with binary gradient Agilent 1260 prep pump (G1361A). An auto-sampler Agilent 1260 prep ALS (G2260A) and Agilent diode array detector 1260 DAD VL (G1315D) was employed for the detection of the separated β-carotene and Zeaxanthin. Agilent 5 Prep- C18 Scalar column (5 µm, 150 mm × 4.6 mm) was utilized for separation. The following solvents were used at a flow rate of 1.25 ml/ min: (A) acetone and (B) methanol: H_2O (9:1 v/v) containing 0.05% BHT. The separation of Zeaxanthin was achieved by a gradient between solvents A and B for 40 min as follows: B was run at 80 to 20% for 25 min, 20% for 10 min, and 20 to 80% for 5 min (Sarada

et al., 2006). The peaks were integrated at 450 nm to identify and quantify Zeaxanthin in the samples. Zeaxanthin (Sigma-Aldrich Co., USA) was used as standard. Zeaxanthin in the sample that was identified and quantified by comparing retention time and the peak area of the unknown peak with the Zeaxanthin standard peak.

1.4. Animals

Male and female mice with an average weight of 20-30 g as well as the male and female Wistar albino rats weighing 120- 130 g were obtained from animal house lab, National Research Centre, Dokki, Cairo, and were used in this study. Animals were housed under normal laboratory conditions for one week before the initiation of biological experiments (adaptation period), housed in a well-ventilated box (22 ± 20 °C) on a twelve hours light and dark cycle. Animals were fed with a natural basal diet. Diets and water were supplied *ad libitum* and had free access to water. Also, they were cared for according to the guidelines for animal experiments which were approved by the Ethical Committee of Medical Research at the National Research Centre, Cairo, Egypt.

1.5. Acute toxicity

Selected 40 mice of the uniform weight of both sexes are taken and divided into 8 groups. Each group contains 5 mice. The Zeaxanthin-enriched fraction gave orally to mice in graded doses 0.625, 1.25, 2.5 and 5 g/kg. The control group received the same volumes of distilled water. The percentage mortality for the Zeaxanthin-enriched fraction was recorded 24 h later (Desoukey et al., 2016; El-Naggar et al., 2018). Observation of mice for 14 days, for any changes in the skin, respiratory, circulatory, autonomic, central nervous systems, somatomotor activity, and behavior pattern. Particular observation for tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma was done.

1.6. Chronic toxicity

Wistar albino rats were divided into sex groups as follows:

Groups 1 and 2: Control male and female rats (15 rats each) as described aforementioned.

Groups 3 and 4: Male and female rats were administered orally 250 mg/kg (1/20 of acute toxicity) of the Zeaxanthin-enriched fraction daily for three consecutive months (15 rats each). The animals were observed daily for signs and behavioral changes.

Groups 5 and 6: Male and female rats were administered orally 500 mg/kg (1/10 of acute toxicity) of the Zeaxanthin-enriched fraction daily for three consecutive months (15 rats each). The animals were observed daily for signs and behavioral changes.

All animals were sacrificed after three months. Fasting blood samples were collected by puncture of sub – tongual vein and left for clotting then, centrifuged at 3000 rpm for 15 minutes to separate serum.

1.7. Estimation of absolute organ weight

Absolute organ weight of all the animals, after 3 months, was recorded, kidneys, heart, liver, were removed, observed macroscopically for lesions and weighed on a weighing balance for determining absolute organ weight.

1.8. Clinical chemistry analysis

Biochemical parameters were determined in serum using Biodiagnostic kits (Bio diagnostics Co., Egypt). Creatinine and total urea were determined according to the methods of Bartles et al. (1972) and Fawcett and Soctt (1960).Liver function enzyme activities, alanine and aspartate aminotransferases (AST and ALT) were determined in rat serum according to the method of Reitman and Frankel (1957) as well as alkaline phosphatase (ALP) was determined according to Belfield and Goldberg (1971) . Bilirubin was determined according to the method of Walter and Gerade (1970). Glucose level was measured using colorimetric kits (Teitz, 1970). In addition, lipid profile was performed.

1.9. Hematological parameters

Blood samples were immediately analyzed for the estimation of numbers of erythrocytes, hemoglobin (Hb), and hematocrit (PCVt) according to Thrall (2004). White blood cell (WBC) was counted.

1.10. Histopathological study

Histopathological examination was carried out on tissue slices of hepatic, cardiac and renal tissues; which were fixed in 10% formaldehyde and embedded in paraffin wax blocks. Sections of 5 μm thick were stained with hematoxylin and eosin (H&E) then examined under a light microscope for determination of pathological changes.

1.11. Statistics

All the values are presented as means ± standard error of the means (SE). Comparisons between different groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey HSD test for multiple comparisons. Graph pad Prism software, version 5 (Inc., San Diego, USA) was used to carry out these statistical tests. The difference was considered significant when $p < 0.05$.

2. Results

2.1. Zeaxanthin contents in the crude extract and Zeaxanthin-enriched fraction of *D. salina*

The extraction of fine powder of *D. salina* with hexane/ethanol (2:1, v/v) revealed crude extract

with extraction yield (3.39%). The HPLC analysis (Figure 1) showed that hexane/ethanol crude extract of *D. salina* contains Zeaxanthin (8.469 mg/g crude extract). The fractionation of *D. salina* crude extract with silica gel column chromatography resulted in an enriched fraction (Zeaxanthin-enriched fraction) that contains high content of the Zeaxanthin when compared with the crude extract. The HPLC analysis (Figure 1) showed that the Zeaxanthin-enriched fraction contains Zeaxanthin (14.301 mg/g rich fraction). The Zeaxanthin-enriched fraction was used to conduct the acute and sub-chronic toxicity.

2.2. Acute toxicity

After 24 h, the results showed that no mortality was observed after Zeaxanthin, up to 5 g/kg. A single oral administration of Zeaxanthin, after 15 days, revealed no significant change in skin and fur, circulatory, respiratory, autonomic, behavioral patterns, and central nervous systems.

2.3. Chronic toxicity

Food and water consumption

The current results revealed no significant difference in the consumption of food and water after three months (Table 1).

Assessment of body weight and absolute organ body weight

The body weight of albino rats was recorded at the end of the experiment (Table 2). It was noticed that the weight of the animals did not change as compared to normal animals. In addition, the absolute organ weight of the treated rats compared with that of the control group animals. No significant differences in liver, kidney and heart organ weight were recorded between the treated rats and those of the control group (Table 1).

Effect on CBC profile

Both sexes of rats treated for three consecutive months with a daily dose of 250 and 500 mg /kg of Zeaxanthin-enriched fraction did not show any sign of toxicity (no mortality, no patches of yellow color appearance, no hair, no diarrhea loss, and no abnormalities on behavior). CBC profile demonstrated that no change in the CBC profile in all Zeaxanthin-enriched fraction treated groups compared to their normal values (Table 2).

Effect on kidney, liver and lipid profiles

Sub-chronic the Zeaxanthin-enriched fraction administration of 250 and 500 mg /kg did not change blood glucose level, kidney and liver functions, in rats (Table 3).

The biochemical parameters of blood, such as serum cholesterol, triglycerides, HDL and LDL of the treated rats and of the untreated rats were alike, suggesting that oral administration of the Zeaxanthin-enriched fraction (Table 4).

2.4. Effect on histopathological study

Heart histopathological investigation declared normal myocardial muscles; normal striation and nucleation in all groups; control male and female as well as treated male and female rats (Figure 2) supplemented for three consecutive months with the Zeaxanthin-enriched fraction of *D. salina* microalgae.

Kidney histopathological investigation showed normal renal histology; normal renal glomeruli and the normal renal tubules in all rat groups (Figure 3).

Liver histopathological investigation showed normal hepatic parenchyma; normal hepatocytes, blood sinusoids, and central veins, in all rat groups (Figure 4).

3. Discussion

D. salina is one of the most important algal strains that accumulate high amounts of carotenoids, especially β-carotene and Zeaxanthin. The extraction by hexane/ethanol (2:1, v/v) in this study showed a positive effect on the recovery of Zeaxanthin from *D. salina*. The content of Zeaxanthin of *D. salina* in the present study is higher than Zeaxanthin content (0.2mg/g dry weight) of Wild-type *D.salina* Teod, while it is lower than that content (6 mg/g dry weight) of new mutant (zea1) of *D. salina* (Jin et al., 2003). The Zeaxanthin content of hexane/ethanol extract of *D. salina* in the present study is lower than Zeaxanthin (11.32mg/g crude extract) content of acetone extract of *Chlorella saccharophila* (Singh et al., 2013); this supports the positive effect of solvent system polarity in the recovery of different categories of natural products and carotenoids (Hejazi et al., 2002;El-Baz et al., 2020a).

In the current acute study, we evaluate the acute and chronic toxicity of Zeaxanthin-enriched fraction from *D. salina*, using a single oral dose of Zeaxanthin (5000 mg /kg), all mice survived with no change in skin and fur, no patches of yellow color appearance, no hair, no diarrhea loss and no abnormalities on behavior. Therefore, the Zeaxanthin LD_{50} values were more significant than 5000 mg/kg in mice, reflecting its safety. In previous acute studies, Zeaxanthin up to 4000 mg/kg produced no mortality in rats and 8000 mg/kg in mice toxicity (Baechtold, 1977). Also, Zeaxanthin in both sexes of albino guinea pigs showed no sensitization or signs of skin irritation (Klecak and Geleick, 1977).

Table (1): Food and water consumption, body weight and absolute organ body weight of male and female rats post chronic administration of Zeaxanthin-enriched fraction.

	Normal male	Normal female	Zeaxanthin (250mg/kg) male	Zeaxanthin	Normal male	Normal female
Body weight (g)	154.02±0.91	155.28±0.86	158.60±0.58	153.64±1.02	161.50±0.86	157.78±2.91
Liver weight (g)	3.63±0.00	3.58±0.04	3.54±0.01	3.67±0.02	3.57±0.03	3.63±0.03
Kidney weight (g)	0.45±0.01	0.47±0.00	0.42±0.00	0.42±0.01	0.45±0.02	0.45±0.01
Heart weight (g)	0.27±0.00	0.28±0.01	0.27±0.00	0.28±0.01	0.28±0.00	0.27±0.00
Water intake (mL)	10.95±0.16	10.49±0.38	10.13±0.28	10.25±0.09	10.98±0.09	10.24±0.11
Food intake (g)	5.58±0.08	5.45±0.04	5.31±0.26	5.36±0.20	5.68±0.02	5.61±0.04

Data are Means ± SE of 15 rats in control groups and 15 rats in the treated groups, where *P < 0.05 represents a significant difference as compared to the control.

Table (2): Blood profile picture of male and female rats post chronic administration of Zeaxanthin-enriched fraction

	Normal male	Normal female	Zeaxanthin (250mg/kg) male	Zeaxanthin (250mg/kg) female	Zeaxanthin (500mg/kg) male	Zeaxanthin (500mg/kg) female
Hb (g/L)	14.35±0.14	14.20±0.61	14.98±0.59	15.18±0.80	14.65±0.79	15.33±0.76
RBCs(million cells/cmm)	7.48±0.21	7.59±0.47	8.54±0.28	8.36±0.49	7.68±0.54	8.94±0.35
haematocrit (%)	44.45±0.95	44.35±1.85	45.93±1.87	44.80±2.49	43.80±2.07	43.23±3.19
MCV (fl)	55.50±0.29	54.75±1.03	53.75±0.63	53.75±0.75	54.50±0.87	53.75±1.44
MCH (pg)	17.50±0.29	18.75±0.48	17.50±0.29	18.25±0.25	17.25±0.25	18.50±0.50
MCHC (g/dl)	32.50±0.29	34.50±0.50	33.75±0.25	34.00±0.00	35.25±1.31	34.50±0.50
RDW-CV (%)	16.40±0.40	15.52±0.70	14.85±0.17	14.63±0.33	15.53±0.58	15.73±1.15
Platelets (10^3/cmm)	438.50±12.84	433.75±10.86	455.50±10.04	437.50±6.18	489.50±25.55	442.50±31.82
MPV (fl)	7.15±0.09	8.00±0.23	8.13±0.17	7.95±0.36	7.65±0.33	8.20±0.20
WBCs (10^3/cmm)	10.20±0.06	9.48±1.58	9.85±1.22	9.68±0.51	11.68±1.04	10.83±0.92
Neutrophils (10^3/cm)	2.57±0.18	2.18±0.01	2.35±0.02	2.68±0.04	2.38±0.17	2.27±0.05
Lymphocyte (10^3/cm)	7.30±0.14	5.54±0.13	6.62±0.17	5.46±0.19	6.98±0.24	5.75±0.23
Monocyte (10^3/cm)	1.79±0.05	1.07±0.27	1.23±0.03	0.98±0.01	1.21±0.23	1.08±0.06

Data are Means ± SE of 15 rats in control groups and 15 rats in the treated groups, where *P < 0.05 represents a significant difference as compared to the control.

Table (3): Creatinine, blood urea nitrogen, liver function enzyme activities, bilirubin and albumin levels in serum of male and female rats post chronic administration of Zeaxanthin-enriched fraction.

	Normal male	Normal female	Zeaxanthin (250mg/kg) male	Zeaxanthin (250mg/kg) female	Zeaxanthin (500mg/kg) male	Zeaxanthin (500mg/kg) female
Creatinine (mg/dl)	0.94±0.19	0.78±0.09	0.91±0.06	0.73±0.07	0.93±0.06	0.80±0.07
BUN (mg/dl)	20.67±2.62	20.67±2.09	21.67±1.65	23.33±1.55	19.67±0.62	21.92±1.49
ALT (IU/ml)	37.00±1.87	38.50±2.02	38.00±3.19	39.25±4.73	38.25±2.06	43.50±3.71
AST (IU/ml)	206.50±2.99	212.33±12.11	206.00±1.15	215.00±9.35	209.33±2.09	217.00±7.68
ALK (IU/ml)	97.50±9.67	105.00±1.73	97.50±9.84	101.00±4.51	82.25±2.78	113.75±1.44
Bilirubin (Mg/dl)	0.80±0.04	0.76±0.03	0.79±0.06	0.75±0.05	0.83±0.04	0.78±0.05
Albumin (g/dl)	2.63±0.17	2.86±0.02	2.54±0.04	2.88±0.03	2.69±0.13	2.89±0.03
Glucose Mg%	**237.00±5.51**	**222.75±20.43**	**234.50±9.46**	**220.00±2.89**	**237.50±2.47**	**232.00±17.86**

Data are Means ± SE of 15 rats in control groups and 15 rats in the treated groups, where *P < 0.05 represents a significant difference as compared to the control.

Table (4): Lipid profile of male and female rats post chronic administration of Zeaxanthin-enriched fraction

	Normal male	Normal female	Zeaxanthin (250mg/kg) male	Zeaxa nthin (250mg/kg) female	Zeaxanthin (500mg/kg) male	Zeaxa nthin (500mg/kg) female
Cholesterol ((mg/dL	51.67±3.82	54.00±5.57	54.33±7.01	52.00±4.77	54.67±2.75	53.00±3.50
Triglyceride ((mg/dL	91.00±4.27	95.33±5.51	95.67±3.40	96.00±3.28	95.33±3.21	97.33±3.62
HDL ((mg/dL	29.67±0.76	29.00±0.87	27.67±0.58	30.00±0.87	31.00±1.00	31.33±1.04
LDL ((mg/dL	23.67±1.26	24.67±1.15	22.33±0.29	24.67±1.44	23.67±1.04	24.33±1.61

Data are Means ± SE of 15 rats in control groups and 15 rats in the treated groups, where *P < 0.05 represents a significant difference as compared to the control.

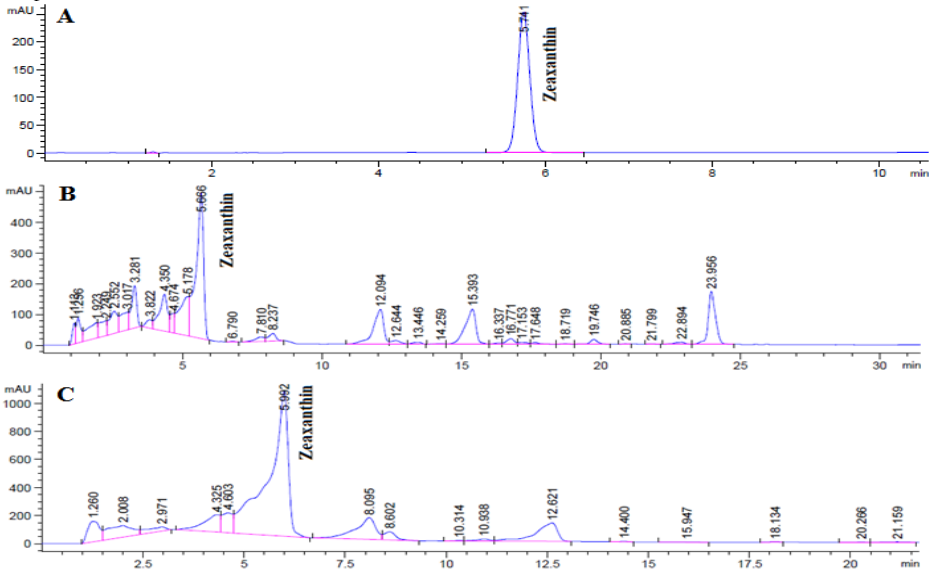

Figure 1. HPLC spectrum at 450 nm of zeaxanthin standard (A) zeaxanthin of Dunaliella salina crude extract (B) and zeaxanthin of Zeaxanthin-enriched fraction (C).

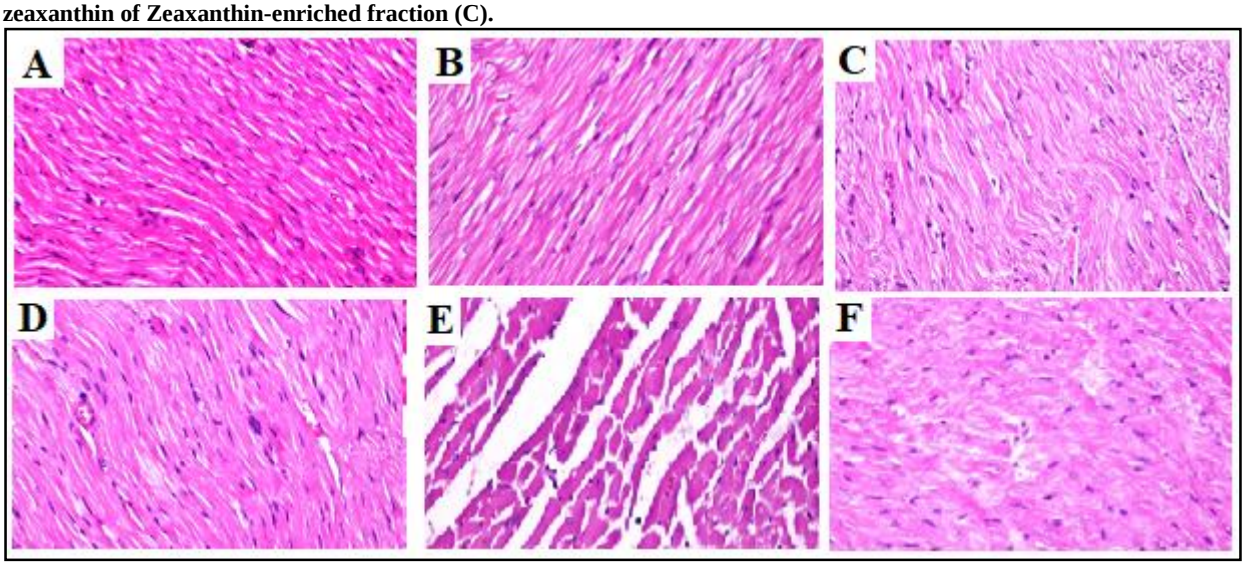

Figure 2. Heart histopathological alterations in A: male (control); B: female (control); C: male (treated); and D: female (treated) rat supplemented for three consecutive months with 250 mg/kg zeaxanthin-enriched fraction of *D. salina* microalgae. E: male (treated); and F: female (treated) rat supplemented for three consecutive months with 500 mg/kg zeaxanthin-enriched fraction of D. salina microalgae **(H&E X200).**

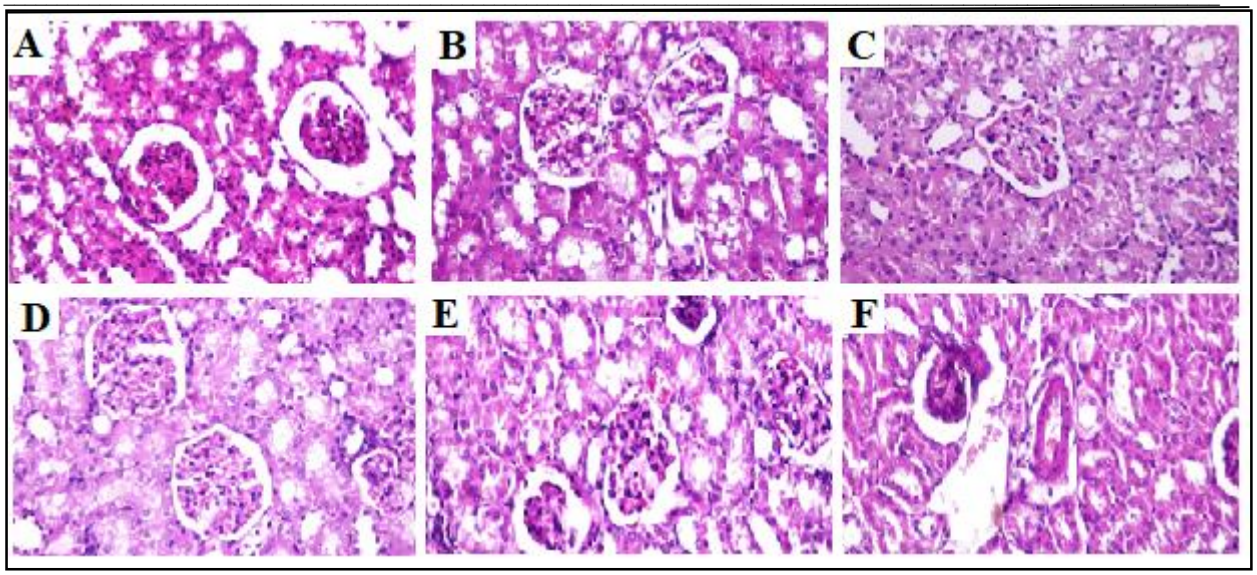

Figure 3. Kidneys histopathological alterations in A: male (control); B: female (control); C: male (treated); and D: female (treated) rat supplemented for three consecutive months with 250mg/kg zeaxanthin-enriched fraction of *D. salina* microalgae. E: male (treated); and F: female (treated) rat supplemented for three consecutive months with 500 mg/kg zeaxanthin-enriched fraction of D. salina microalgae **(H&E X200).**

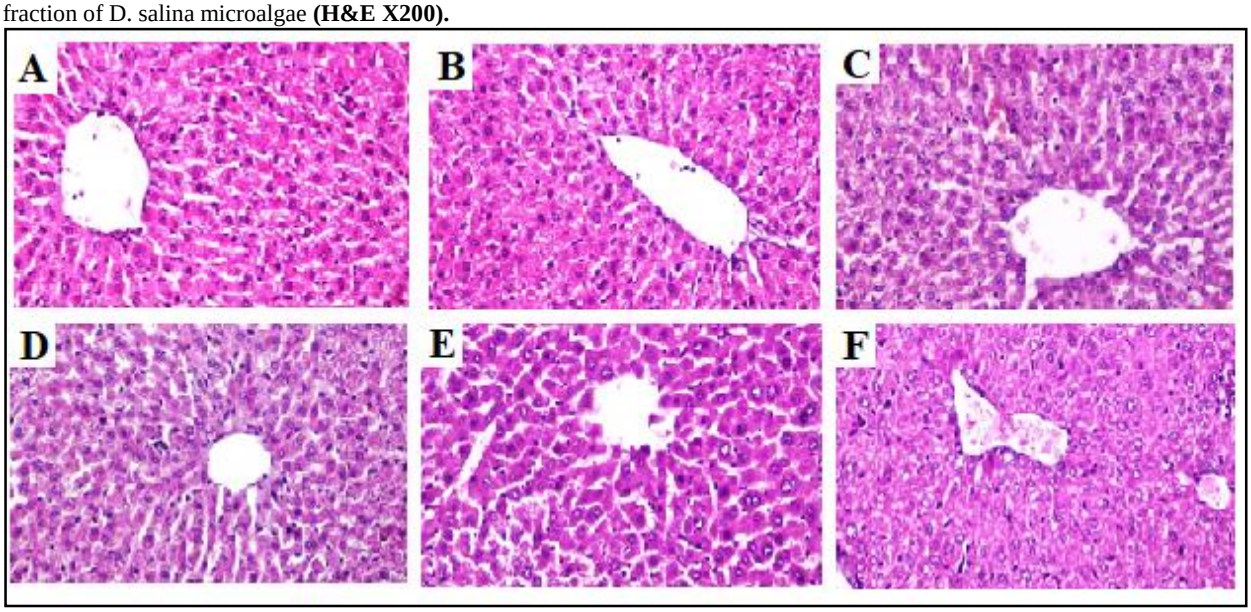

Figure 4. Liver histopathological alterations in A: male (control); B: female (control); C: male (treated); and D: female (treated) rat supplemented for three consecutive months with 250mg/kg zeaxanthin-enriched fraction of *D. salina* microalgae. E: male (treated); and F: female (treated) rat supplemented for three consecutive months with 500 mg/kg zeaxanthin-enriched fraction of D. salina microalgae **(H&E X200).**

Zeaxanthin is a safe natural carotenoid-based on previous studies, so it is valuable to be extracted from algae and to be evaluated for the first time for its safety from algae. In the current sub chronic study with Zeaxanthin-enriched fraction from *D. salina* (250 or 500 mg /kg) for 3 months in both sexes of rats, no change in food and water consumption, no change in body and organ weight, no related hematology and clinical chemistry, or no histopathological effects attributable to the Zeaxanthin treatment. The Zeaxanthin fraction exhibited No Adverse Effect Level (NOAEL) in rats. The safety evaluation of dietary substance Zeaxanthin is consumed at higher intakes for its benefits required more estimation to be available for human needs and protection from diseases. Several human intervention studies are in progress with respect to investigating the function for zeaxanthin. The current work indicates good systemic tolerance of Zeaxanthin, at dosages 250- and 500 mg/day for up to three months. A previous publication reporting toxicology studies for a zeaxanthin concentrate from marigold flowers (*Tagetes erecta L.*) at dose levels of 0, 4, 40, and 400 mg/kg bw/day (gavage) for 13 weeks In Wistar rats. It has no toxicologically significant treatment-related changes(Ravikrishnan et al., 2011). Chronic studies of

synthetic Zeaxanthin formulations (1000 mg/kg) in mice and rats, and 400 mg/kg in dogs, showed no histopathological changes or adverse effects. Zeaxanthin (1000 or 400 mg/kg; in rats or rabbits), neither had fetal toxicity, not teratogenicity. *In vitro* and *in vivo* tests, formulated Zeaxanthin exhibited no mutagenic effect (Edwards, 2016). Previously, Zeaxanthin beadles (9.3%) were administered orally to mice 13-week. Zeaxanthin (250, 500, and 1000 mg/kg) or its beadles (9.3%) in mice showed no hematology or clinical chemistry, No adipose tissue discoloration, or no histopathological findings were observed in zeaxanthin or the beadle (Ettlin et al., 1980). In another toxicity study in rabbits, Zeaxanthin was taken orally in rapeseed oil from day 7 to 19 of gestation, at doses of 100, 200, and 400 mg/kg. On day 30 of gestation, rabbits were Caesarian sectioned, Zeaxanthin showed no embryotoxic or teratogenic at doses up to 400 mg/kg/day in rabbits that associated with no deaths or signs of maternal toxicity, no indication of any embryotoxic or teratogenic effect (Kistler, 1983).

The current histopathological study of Zeaxanthin-enriched fraction from *D. salina* (250 mg /kg) for 3 months in both sex of rats revealed normal histological structure of hepatic lobule, renal parenchyma, and cardiac myocytes in both sexes of rats. These results are supported by the previous study which investigates that Chronic oral Zeaxanthin (0.2 and 20 mg/kg; after a 52-week) did not produce adverse effects in Cynomolgus monkeys (Edwards, 2016). Moreover, in rats, meso-Zeaxanthin, Zeaxanthin rare isomer, gavage administration (200 mg/kg; daily) showed on mortality, clinical signs of toxicity, changes or histopathology suggesting that no adverse effect of Meso-Zeaxanthin (200 mg/kg) in rats for 13 consecutive weeks (Chang, 2006). Also, the toxicological effects of feeding 90-day of Meso-Zeaxanthin (300 mg/kg) were not observed, in both sexes of rats. So, there is no genotoxicity or acute toxicity with Meso-Zeaxanthin (Xu et al., 2013).

Conclusion

The findings of the acute toxicity studies of the Zeaxanthin-enriched fraction exhibit its safety for oral administration up to 5000 mg/kg. The results for different physical parameters, such as food and water consumption, body and organs weight, hematological and biochemical parameters as well as histopathological study reflect the safety of the Zeaxanthin-enriched fraction up to 500 mg/kg. Therefore, it could be concluded that it can be incorporated in oral dosage forms to protect against several diseases.

Declaration of interest

The authors declare no conflict of interest.

Author contribution

Farouk K. El-Baz: Conceptualization, Resources, Funding acquisition, Review & editing. Hanan F. Aly: Methodology. Sami I. Ali: Methodology, Writing - original draft. Abeer A.A. Salama: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - review & editing.

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