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Safety of Astaxanthin-Rich Fraction of *Haematococcus pluvialis* Microalgae

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Abstract

Astaxanthin, is a valuable component of a natural origin, is promising as a food supplement worldwide. The principle commercial productive source of astaxanthin is the green microalgae Haematococcus pluvialis (H. pluvialis). The present study was aimed to investigate the acute and chronic dietary supplementation of an Astaxanthin-Rich Fraction (ARF) of *H. pluvialis*.The extraction of *H. pluvialis* fine powder with dichloromethane: methanol (3:1, V/V) revealed 6.1% containing all-trans-astaxanthin (10.85 mg/g, crud extract) as quantified by HPLC. While, the ARF fractionated by column chromatography contains all-trans-astaxanthin (16.21 mg/g of astaxanthin). The LD_{50} of the biomass was greater than 5000mg/kg body weight for 24 h. In chronic toxicity study, rats and mice (15/sex/group) were supplemented daily for three continuous months with 500 mg /kg /bodyweight /day with ARF. The ARF supplementation was associated with yellow color staining of fur and feces. Various blood and chemical parameters were included in this study as profile of blood picture (CBC), hepatic enzymes, total bilirubin (TB), renal tests, albumin, and glucose levels. Examination of liver, kidney and heart architectures was also performed. The exposing of the two sexes of mice and rats to 500 mg /kg/day of ARF did not exhibit any toxicity symptoms and no mortality, no effect on the levels of blood chemistry and biomarkers as well as pathological examination. So, it could be concluded that, ARF is safe at the dose 50mg/kg in chronic toxicity, suggesting its promising benefits as food additives beside its usefulness as a therapeutic candidate against different prospective illness.

Keywords: Astaxanthin; H. pluvialis; toxicity; liver enzymes; hematological parameters; histopathology.

1. Introduction

Astaxanthin is considered as ketocarotenoid pigment of a lipid-soluble with a specific orangey-red color. It is a xanthophyll pigment transformed in distinctive photosynthetic and organisms of marine origin in addition to a few plants and microorganisms [1]. *Haematococcus pluvialis* (*H. pluvialis*), a microalga of green single-celled , may be great known as those grade regular wellspring about astaxanthin for employments of commercial uses around several producing organisms of astaxanthin, , much appreciated to its intense capability to secondary synthesizing astaxanthin that might achieve up to 4% (w/w, dry weight basis) particularly under distinctive stress states [2]. The chemical structure of astaxanthin is characteristic by having two similar twin deviated carbon atoms at the positions of 3 and 3′ the ring of β-ionone, with a group of hydroxyl with thirteen alternating single -double bonds at the both ends of polyene carbon backbone [3].Three characteristics isomers of astaxanthin namely (3R, 3′R), (3S, 3′S), and (3R, 3′S) and could be found naturally as esterified fatty acid astaxanthin or as astaxanthin in free forms. High amounts of fatty acid astaxanthin are produced from H. pluvialis while, little amounts of free form astaxanthin are detected (3S, 3′S). However, non-esterified form of astaxanthin (3R, 3′R) are produced from Xanthophyllomyces dendrorhous [4, 5].Astaxanthin is not produced in the cells of animals with no provitamin A effect and has strong reinforcement activity, almost ten times more than different carotenoids, including β- carotene, zeaxanthin, canthaxanthin, Furthermore lutein, and about 100 times more stupendous over α-tocopherol[6]. Astaxanthin shows strong scavenging activity of free radicals in vitro and a powerful prevention effect against oxidative injury by stimulating indigenous antioxidant enzymes defense system as superoxide dismutase, catalase, peroxidase etc. in vivo models [6-8]. Different studies either in

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vivo or in vitro have declared that astaxanthin has a great different biological effects , such as an antitumor [9] , anti-Helicobacter pylori , normalized blood pressure[10,11] and shows a cardioprotective effects[12,13]. However, the great different useful astaxanthin effects have been determined to some extent, its injurious effect have not been studied. The present study aimed to study the chronic supplementation with 500 mg/kg body weight /day for three continuous months.

2. Materials and methods

2.1. Cultivation of *H. pluvialis*

H. pluvialis was isolated from the freshwater community of River Nile and grown on BG11 media [5,14].

2.2.Quantification and identification of H. pluvialis astaxanthin

2.3. Preparations of algal extract and astaxanthin enrich fractions

The fine powder of *H. pluvialis* (100 g) was soaked in 1000 ml of dichloromethane: methanol (3:1, 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) and hexane/ethyl acetate as mobile phase with increasing polarity (0, 10, 30, and100% ethyl acetate) then ethyl acetate: methanol (1:1) that afforded 10 fractions that collected in 50 ml per each fraction. The 10 fractions were subjected to TLC (20 × 20 cm aluminum sheets coated with silica gel 60 F254, Merck, Germany) to detect the presence of phyto-compounds that were visualized by ultraviolet (UV) fluorescent colors at 254/366 nm UV lamps. Astaxanthin enriches fraction was combined based on TLC result and confirmed by HPLC. All the extraction and column chromatography fractionation steps were performed in dim light.

2.4. HPLC analysis of astaxanthin

H. pluvialis crude extract and astaxanthin enrich 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 autosampler Agilent 1260 prep ALS (G2260A), and Agilent diode array detector 1260 DAD VL (G1315D) was employed for the detection ofastaxanthin. Agilent 5Prep-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: H2O (9:1 v/v) containing 0.05% BHT. The separation of astaxanthin 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 [15]. The peaks were integrated at 450 nm to quantify astaxanthin. All-trans-astaxanthin (Sigma-Aldrich Co., USA) was used as standard. Astaxanthin was identified and quantified by comparing retention time and the peak area of the unknown peak with the all-trans-astaxanthin standard (Fig.1).

2.5. Biological Study

2.5.1. *Animals*

Male and female Swiss 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 (NRC), Dokki, and Giza were used in this study. Animals were acclimatized for one week before the starting of the experiment (adaptation period).The animals were housed with a well-ventilation (20 °C) with a cycle of twelve hours. Normal basal diets and water were supplied adlibitum. Animals were cared according to the Ethical Committee guide lines of NRC, Giza, Egypt for animal experiments.

2.5.2. *Acute toxicity*

Treatment in an experiment designed to determine the oral LD50, a *H. pluvialis* astaxanthin biomass suspension in water solution was administered to Waster rats (4/sex/group) as a single oral dose of 500-5000 mg /kg body weight *via* gavage. Animals were observed for 24 h for signs of morbidity or mortality. The control group was treated with water vehicle. The animals were observed during 24 h investigation [16-18].

2.5.3. *Experimental design*

One hundred and twenty male and female mice and rats were used in this study; thirty mice and rats for each gender were supplemented orally with 500 mg /kg body weight of *H. pluviali s*astaxanthinmicroalgae and were divided into eight groups as follows:Groups 1 and 2: Control male and female mice (15 mice each)were daily orally administered 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 of *H. pluvialis* astaxanthin for three 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 *H. pluvialis* astaxanthin daily for three consecutive months (15 mice each). The animals were observed daily for their behaviors food and water intakes.After the three 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 [19-21]. Biochemical parameters were determined in serum using Biodiagnostic kits (Bio diagnostics Co., Egypt).

2.5.4. *Biochemical parameters*

Liver function enzyme activities, alanine and aspartate aminotransferases (AST and ALT), as well as alkaline phosphatase (ALP), were estimated in mice and rat sera [22, 23] respectively. The levels of total urea and creatinine were determined according to the methods of Bartles et al. [24] and Fawcett and Scott [25]. The level of bilirubin was also determined [26] .Glucose level was measured using colorimetric kits according to the method of Trinder [27].

2.5.5. *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 hours. 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 55–60°C). Liver, Kidney & heart sections of 4 µm thickness were prepared and stained with haematoxylin and eosin. Paraffin sections were stained in Harris's haematoxylin for 5 minutes. Sections were washed in runningwater for bluing and then stained in 1% watery eosin for 2 minutes, washed in water, dehydrated, cleared, and mounted using Canada balsam.Eight microscopic fields per section were examined using alight microscope (Olympus BX50, Japan) under2 high-power magnification (200).

2.6. 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 unshared letters are significant at $P \leq 0.05$.

3. Results

The extraction of fine powder of *H. pluvialis* in dichloromethane: methanol (3:1, V/V) revealed crud extract with an extraction yield (6.1%) containing all-trans-astaxanthin (10.85 mg/g, crud extract) as quantified by HPLC. While the astaxanthin enriches fraction fractionated by silica column chromatography contains all-trans-astaxanthin (16.21 mg/g of astaxanthin enriches fraction) .

Acute toxicity study reveal, the oral LD50 of ARF was up to 5,000 mg/kg body weight. Series concentration from 500 -5000 mg mg/kg body weight were used from ARF for 24 h and showed no mortality, no toxic effects and no behavioral abnormalities, suggesting that the ARF is experimentally non-toxic and no treatment –dependent harmed effect.

In chronic toxicity, exposed of both sexes of rats and mice to daily 500 mg /kg body weight of ARF of *H. pluvialis* for three continuous months didn't give any harmfulness indication in the two sexes of mice and rats (no mortality, no going bald, no loose bowels, and so on). Moreover, irregularities on conduct, food and water admissions, and wellbeing status among the treated creatures didn't notice. The dietary supplementation of the ARF to experimentally animals in long term chronic study was associated with staining of fur and feces with red-orange color.

The presented Tables (1&4) declared insignificant changes in blood picture profile in ARF -treated male and female mice and rats comparing with related control groups. Also, insignificant difference in hepatic enzymes and glucose levels after long term supplementation of 500mg/kg ARF of *H. pluvialis* to both sexes of mice and rats was detected (Tables 2 and 5), comparing with corresponding control. Further , urea, creatinine and albumin levels showed also insignificant difference after ARF supplementation to both sexes of mice and rodents compared to control groups (Tables 3and 6). Pathological examination showed that, normal cardiac myocytes with normal histological striation and nucleation of treated cardiac tissue. Normal renal glomeruli and tubules of kidney rats and mice was also noticed post chronic administration of 50mg/kg body weight of ARF of *H. pluvialis*. Liver tissue of ARF treated rats showed also, normal hepatic parenchyma , hepatocytes, blood sinusoids, portal area as well as normal lobule in the two sexes of mice and rodents (Figures 2and 3), compared to their corresponding control groups.

Table 1: Profile of blood picture of mice (male and female) after supplementation with 500 mg /kg /body weight of carotenoids rich fraction - extracted from microalgae *H. pluvialis.*

Biomarkers	Control Male	Control Female	Treated Male	Treated Female
HB (g/dL)	15.00 ±1.00[a]	14.00± 0.60 [a]	14.80 .12 ± 0. 67[a]	14. 00 ± 0.66[a]
RBCs(Xmillion cells/ cmm)	5.00 ± 0.77[a]	4.60± 0.98 [a]	4.64 ± 0.90[a]	4.53 ± 0.66[a]
PCV (%)	42.00± 2.10[a]	41.90 ± 2.66[a]	40.76 ± 3.11[a]	40.45 ± 5.10[a]
Haematocrit (HCT)%	45.10±3.00[a]	42.10±2.22[a]	43.10±2.50	42.90±2.10[a]
MCV(fl)	89.80±5.28[a]	87.10±6.00[a]	88.00±4.20[a]	87.20±9.20[a]
MCH(pg)	29.67±1.98[a]	28.65±2.09[a]	82.65±3.10	27.98±5.96
MCHC(g/dL)	33.89±4.90[a]	34.00±2.00 [a]	33.00±2.28[a]	32.55±3.00[a]
RDW-CV(%)	14.50±0.88[a]	14.20±0.75[a]	13.50±3.33[a]	13.20±2.00[a]
MPV(fl)	7.90±0.09[a]	8.40±0.65[a]	8.20±1.88[a]	8.00±0.32[a]
WBCs(× 10^3 /cmm)	6.60 ± 3.43[a]	7.00 ± 1.00 [a]	8. 10 ± 4.53[a]	7.70 ± 1.00[a]
Neutrophils %	60.69 ± 4.12[a]	56.00 ± 2.00[a]	65.00 ± 11.10[a]	60.00± 7.00[a]
Eosinophil %	0.20 ± 0.04[a]	0.17 ± 0.02[a]	0.78 ± 0.02[a]	0.90 ± 0.04[a]
Lymphocyte%	34.00 ± 5.00[a]	35.00 ± 3.10[a]	36.00 ± 4.00[a]	37.99 ±5.90[a]
Monocyte%	2.00± 0.12[a]	1.60 ± 0.05[a]	2.98± 0.50[a]	2.21± 0.50[a]
Platelets (x 10^3/cmm)	300.00 ± 12.00[a]	332.00 ± 60.00[a]	350.00 ±13.00[a]	400.00 ± 33.00[a]

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

Table 2: Hepatic enzymes levels in mice (male and female) after supplementation with 500 mg /kg /body weight of astaxanthin rich fraction - extracted from microalgae *H. pluvialis*.

Groups	ALT (U/l)	AST (U/l)	Bilirubin(mg/dl)	ALP(U/l)	Glucose (mg/dl)
Control Male Mice	40. 99 ± 3.1100[a]	134.00 ± 9.20[a]	0.87 ± 0.19[a]	105.00 ± 8.00[a]	100.00 ± 6.00[a]
Control Female Mice	42.00 ± 2.90[a]	130.10 ± 9.21[a]	0.82± 0.66[a]	112.00± 9.90[a]	114.00± 3.20[a]
500mg/kg astaxanthin Rich Fraction- Male mice	43.00 ± 2.55[a]	126.50 ± 9.00[a]	0.78± 0.10[a]	122.00 ± 9.65[a]	117.00± 3.10[a]
500mg/kg astaxanthin Rich Fraction-Female mice	45.00 ± 3.69[a]	119.60± 5.71[a]	0.80±0.22[a]	126.00± 6.11[a]	122.00± 7.60[a]

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

Table 3: Levels of urea, creatinine and albumin in mice (male and female) after supplementation with 500 mg /kg /body weight of astaxanthin rich fraction - extracted from microalgae *H. pluvialis*.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Albumin (mg/dl)
Control Male Mice	48.60 ± 4.22a	0.52 ± 0.01a	2.98 ± 0.60a
Control Female Mice	44.00 ± 3.00a	0. 49 ± 0.02a	2.95± 0.31a
500mg/Kg astaxanthin Rich Fraction- Male mice	45.00 ± 5.00a	0.60 ± 0.07a	2.78 ± 0.30a
500mg/Kg astaxanthin Rich Fraction-Female mice	43.00 ± 4.00a	0.64± 0.01a	2.76±0.56a

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

Table 4: Profile of blood picture in rats (male and female) after supplementation with 500 mg /kg/body weight of 50 mg /kg astaxanthin-rich fraction extracted from microalgae *H. pluvialis*.

Biomarkers	Control Male	Control Female	Treated Male	Treated Female
HB (g/dL)	15.00 ±1.00[a]	14.00± 0.60 a	14.80 .12 ± 0. 67[a]	14. 00 ± 0.66[a]
RBCs(Xmillion cells/ cmm)	5.00 ± 0.77[a]	4.60± 0.98 a	4.64 ± 0.90 [a]	4.53 ± 0.66 [a]
PCV (%)	42.00± 2.10[a]	41.90 ± 2.66 a	40.76 ± 3.11[a]	40.45 ± 5.10[a]
Haematocrit (HCT)%	45.10±3.00[a]	42.10±2.22[a]	43.10±2.50	42.90±2.10[a]
MCV(fl)	89.80±5.28[a]	87.10±6.00[a]	88.00±4.20[a]	87.20±9.20[a]
MCH(pg)	29.67±1.98[a]	28.65±2.09[a]	82.65±3.10	27.98±5.96
MCHC(g/dL)	33.89±4.90[a]	34.00±2.00 [a]	33.00±2.28[a]	32.55±3.00[a]
RDW-CV(%)	14.50±0.88[a]	14.20±0.75[a]	13.50±3.33[a]	13.20±2.00[a]
MPV(fl)	7.90±0.09[a]	8.40±0.65[a]	8.20±1.88[a]	8.00±0.32[a]
WBCs(× 10^3 /cmm)	6.60 ± 3.43[a]	7.00 ± 1.00 [a]	8. 10 ± 4.53[a]	7.70 ± 1.00 [a]
Neutrophils %	60.69 ± 4.12a	56.00 ± 2.00a	65.00 ± 11.10[a]	60.00± 7.00[a]
Eosinophil %	0.20 ± 0.04 [a]	0.17 ± 0.02[a]	0.78 ± 0.02[a]	0.90 ± 0.04[a]
Lymphocyte%	34.00 ± 5.00[a]	35.00 ± 3.10 a	36.00 ± 4.00[a]	37.99 ±5.90[a]
Monocyte%	2.00± 0.12 [a]	1.60 ± 0.05 [a]	2.98± 0.50 [a]	2.21± 0.50[a]
Platelets (x 10^3/cmm)	300.00 ± 12.00[a]	332.00 ± 60.00 [a]	350.00 ±13.00 [a]	400.00 ± 33.00 [a]

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

Table 5: Hepatic enzymes levels in rats (male and female) after supplementation with 500 mg /kg /body weight of astaxanthin rich fraction - extracted from microalgae *H. pluvialis*.

Groups	ALT (U/l)	AST (U/l)	Bilirubin(mg/dl)	ALP(U/l)	Glucose (mg/dl)
Control Male rats	31. 61 ± 12.00[a]	90.00 ± 9.12[a]	0.62 ± 0.11[a]	80.00 ± 4.60[a]	83.00 ± 3.00[a]
Control Female rats	27.00± 3.00[a]	85.95 ± 6.90[a]	0.68± 0.11[a]	75.00± 6.10[a]	117.00± 10.00[a]
500mg/Kg Carotenoids Rich Fraction- Male rats	29.00 ± 2.03[a]	87.00 ± 8.00[a]	0.67 ± 0.10[a]	78.00 ± 6.70[a]	100.00± 6.20[a]
500mg/Kg Carotenoids Rich Fraction-Female rats	28.13 ± 2.51[a]	94.60± 8.10[a]	0.60±0.01[a]	82.00 ± 7.00[a]	116.00± 10.10[a]

Data are Means ± SD of 15 rats in treated group. Statistical analysis is carried out using Co-state and SPSS computer programs (version 8), where 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 astaxanthin rich fraction - extracted from microalgae *H. pluvialis*.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Albumin (mg/dl)
Control Male rats	27.66 ± 2.00[a]	0.200 ± 0.05[a]	3.08 ± 0.90[a]
Control Female rats	25.00 ± 1.60[a]	0.160 ± 0.11[a]	3.12± 0.19[a]
500mg/Kg Carotenoids Rich Fraction- Male rats	28.00 ± 2.00 [a]	0.174 ± 0.03[a]	3.60 ± 0.51[a]
500mg/Kg Carotenoids Rich Fraction-Female rats	27.00 ± 2.00[a]	0.167± 0.04[a]	3.54±0..49[a]

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

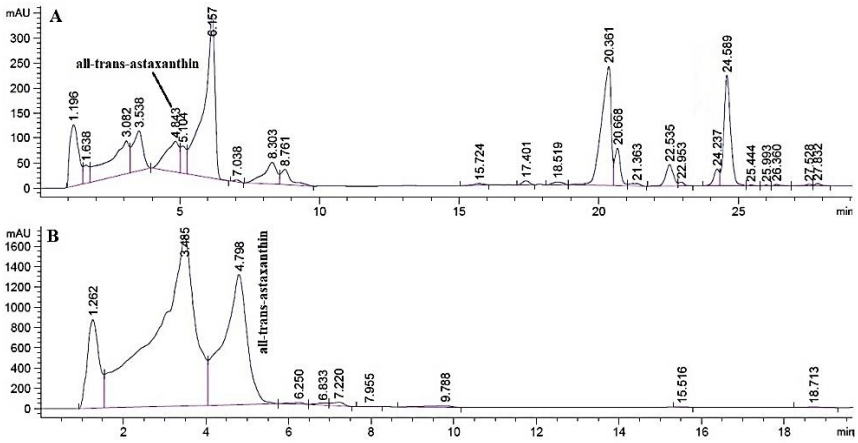


Figure 1. HPLC chromatogram at 450 nm of all-trans-astaxanthin in the crude extract (A), and astaxanthin enriches fraction (B) of *H. pluvialis*.

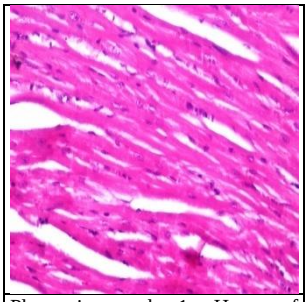
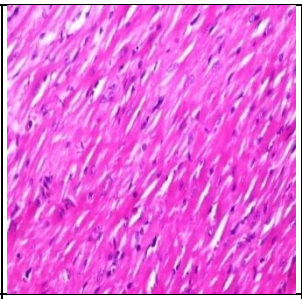
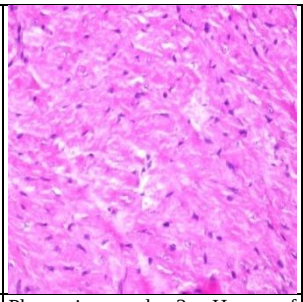
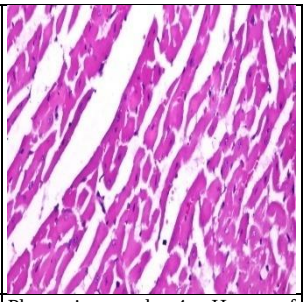
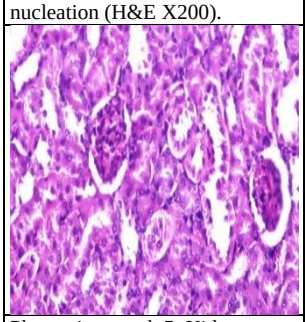
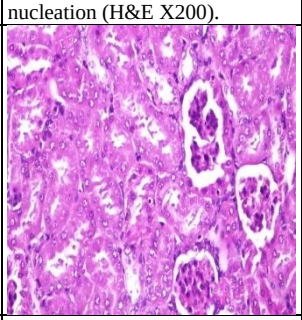
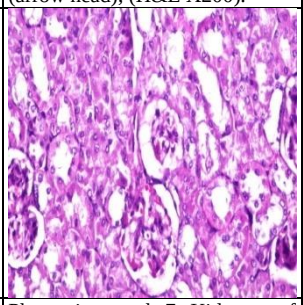
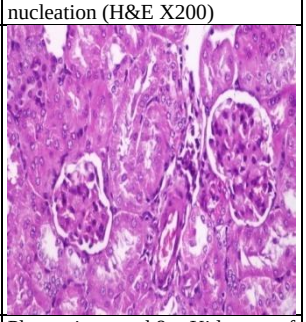
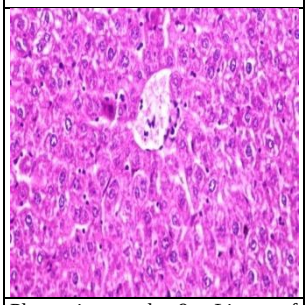
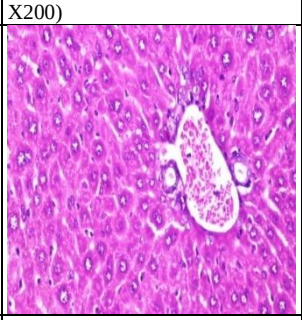
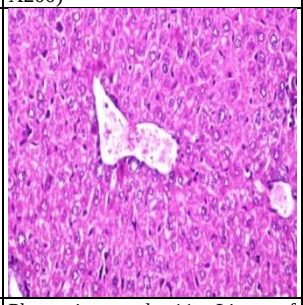
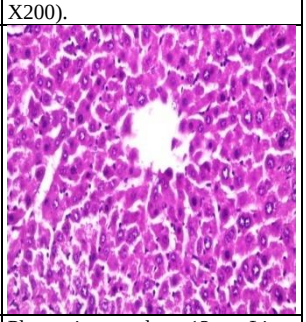
			
Photomicrograph 1: Heart of control female mice showing normal myocardial muscles; note the normal striation and nucleation (H&E X200).	Photomicrograph 2: Heart of control male mice showing normal myocardial muscles; note the normal striation and nucleation (H&E X200).	Photomicrograph 3: Heart of treated female mice showing muscle hyalinosis with homogenous muscle bundles (arrow head), (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 showing female 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)	Photomicrograph8: 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 mice of showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and portal area, (H&E X200)	Photomicrograph 11: Liver of treated female mice showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and portal area, (H&E X200).	Photomicrograph 12: Liver showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).

Fig.2. Histopathological alterations in heart ,liver and kidney of female and male mice supplemented for three consecutive months with 500mg/Kg body weight of astaxanthin-rich fraction of microalgae *Haematococcus pluvialis* .

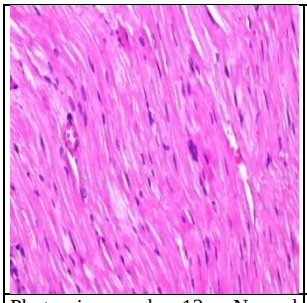
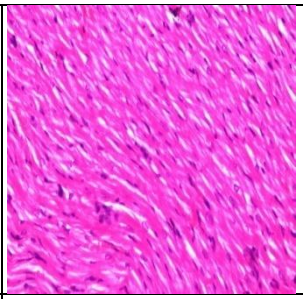
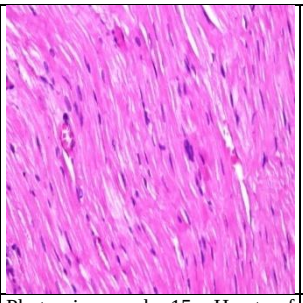
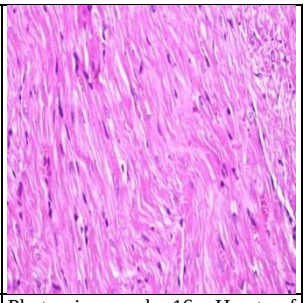
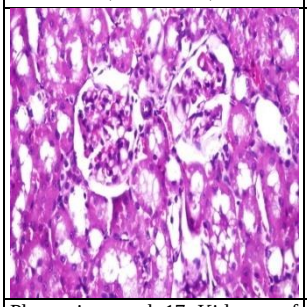
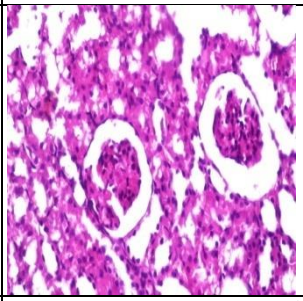
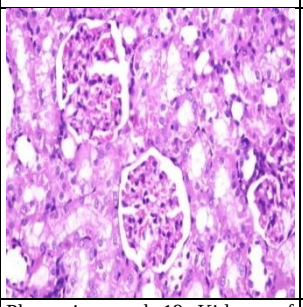
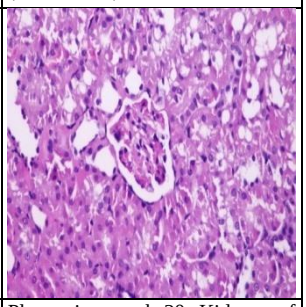
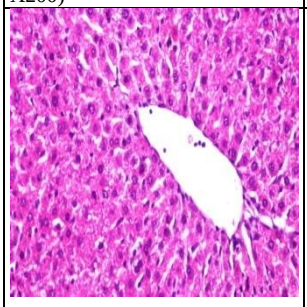
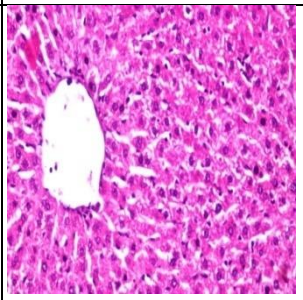
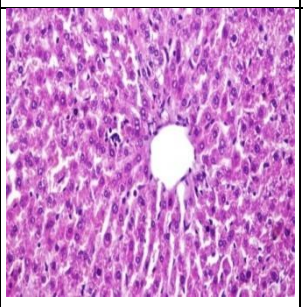
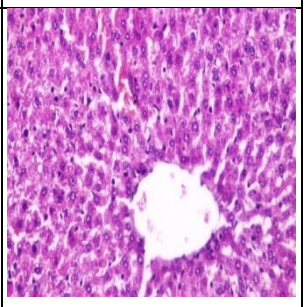
			
Photomicrograph 13: Normal heart of female control rats showing myocardial muscles; note the normal striation and nucleation (H&E X200)	Photomicrograph 14: Normalheart of male control rats showing myocardial muscles; note the normal striation and nucleation (H&E X200)	Photomicrograph 15: Heart of treated female rats showing normal 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, normal renal glomeruli and normal renal tubules, (H&E X200).	Photomicrograph 20: Kidneys of treated male rats showing normal renal histology, 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 Liver showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).	Photomicrograph 24: Liver of treated male rats liver showing normal hepatic parenchyma; note the normal hepatocytes, blood sinusoids, and central veins, (H&E X200).

Fig.3. Histopathological alterations in heart ,kidney and liver of female and male rats supplemented for three consecutive months with 500mg/kg body weight of astaxanthin-rich fraction of microalgae *Haematococcus pluvialis.*

4. Discussion

H. pluvialis accumulates the largest yield (about 97%) of its astaxanthin mainly as a mixture of mono- and di-esters in the cell's cytoplasm, while the lowest portion of the yield is existent as free astaxanthin [5, 28]. The carotenoid extract of H. pluvialis in the present study contains adequate amounts of free astaxanthin based on our HPLC analysis, these results agree with former reports [5, 7].

Acute toxicity study of astaxanthin enrich fraction of microalgae *H. pluvialis* revealed no toxicological features and no mortality post the supplementation of mice and rodents with serial concentrations (500-up to5000 mg/kg body weight) of ARF for 24 hours. Additionally, in this research we evaluate chronic toxicity of ARF. In this study , 500 mg/kg b.wt/day of ARF of *H. pluvialis* were used to investigate the effect of long –term ARF supplementation of in the two sexes of mice and rodents and for three continues months . The presented data showed no significant differences in blood chemistry parameters ; Hb , PCV, WBC, RBC, platelets, and other blood cells ;neutrophil , eosinophil , monocytes and lymphocytes /animals /sex/ group compared with their related control groups . Also, hepatic enzymes; AST, ALT, ALP, showed no noticeable changes. Beside, bilirubin and glucose levels showed no marked significant differences /animals /sex/group compared to their relative control groups. Additionally, urea, creatinine and albumin levels did not exhibit significant difference in the two sexes of mice and rodents compared to their control groups. Pathological examination for hepatic, cardiac and renal tissues revealed no remarkable alterations and no injurious effects after supplementation with ARF (500mg /kg body weight /day) for three successive months in the tissue architectures of animals /sex/group compared to their related control groups.

Previously investigations covered the impact of astaxanthin supplementation. Ono et al. [29] declared that F344 rats exposed to daily highest doses of Haematococcus extract (5%) which is comparable to 0.25% astaxanthin; 125 mg/kg/day for continuous ninety days did not influence body weight gain, feed utilization, morphology of blood cells, and chemistry of plasma, weight of different organs or pathological examination. Takahashi et al. [30] also found that, orally daily admission of 500 mg kg/day ARF of *H. pluvialis* for ninety continous days to Sprague–Dawley rats did not show treatment-related unfavorable impacts. Stewart et al. [31], declaring that, male and female rodents exposed to 500 mg astaxanthin/kg/day of an astaxanthin-enrich fraction of *H. pluvialis* as dietary intake supplement for continuous ninety days showed no significant biological harmed impact on a different parameters of health. Based on Stewart et al. [31] study, the no-observed-adverse-effect-level (NOAEL) of the astaxanthin biomass in diet was found to be 20%. The comparable dose of the astaxanthin biomass for the rats of male and female was 14,161 and 17,076 mg/kg body weight/day, respectively. So, the astaxanthin resulting dose was 465 and 557 mg/kg/day, respectively.

In a parallel line with the present study Spiller and Dewell [32], found that , adult subjects obtained 6 mg /kg / day declared normalization in blood pressure , blood chemistry parameters analyzed as blood cells count and reported no clinical importance after continuous eight weeks of H. pluvialis extract intakes .

Further , and in a good concomitant with the present study , Satoh et al.[33] ascertained , no variation was found when adult human supplemented with up to 20 mg of oily product of astaxanthin of *H. pluvialis* , Puresta®, (comparable to dialcohol of astaxanthin),continuously daily for one month. Puresta® safety has been already examined in a clinical report where 17 adult subjects were obtained Puresta® comparable to 8 mg dialcohol of astaxanthin two times /day for continuous 12 weeks [34]. The authors added that, no significant variations and no subject reports of subject reports of unfriendly encounters during treatment, in addition, beneficial outcomes of this product were seen on metabolic disorder and intellectual capacity. Our present findings uphold the safety of astaxanthin (500 mg/kg body weight /day, with no negative consequences for blood chemistry or hematology.as well as biomarkers.

The extraction of fine powder of *H. pluvialis* with dichloromethane: methanol (3:1, V/V) revealed crud extract with an extraction yield (6.1%) containing all-trans-astaxanthin (10.85 mg/g, crud extract) as quantified by HPLC. While, the astaxanthin enriches fraction fractionated by silica column chromatography contains all-trans-astaxanthin (16.21 mg/g of astaxanthin enriches fraction).

The present results declare no mortality was observed in acute toxicity study of ARF and the LD50 is up to 5000 mg / kg body weight for 24 hr. Also, chronic toxicity revealed the safety of ARF after daily orally supplementation with 500 mg/kg for three continuous months. Different blood chemistry and biochemical parameters showed no significant variations and no noticeable changes in pathological investigation of liver , heart and kidney tissues in therapeutic groups of the two sexes of mice and rats compared to their related control levels.

5. Conclusions

The extraction of fine powder of *H. pluvialis* with dichloromethane: methanol (3:1, V/V) revealed crud extract with an extraction yield (6.1%) containing all-

trans-astaxanthin (10.85 mg/g, crud extract) as quantified by HPLC. While, the astaxanthin enriches fraction fractionated by silica column chromatography contains all-trans-astaxanthin (16.21 mg/g of astaxanthin enriches fraction).The present results declare no mortality was observed in acute toxicity study of ARF and the LD50 is up to 5000 mg / kg body weight for 24 hr. Also, chronic toxicity revealed the safety of ARF after daily orally supplementation with 500 mg/kg for three continuous months. Different blood chemistry and biochemical parameters showed no significant variations and no noticeable changes in pathological l investigation of liver , heart and kidney tissues in therapeutic groups of the two sexes of mice and rats compared to their related control levels.

Conflicts of interest

"There are no conflicts to declare".

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