



Astaxanthin- Enriched Fraction from the Microalga *Haematococcus pluvialis* Mitigates Kidney Injury via Modulation of Nrf2/Keap1 and MAPK/ERK Pathways in Rats

Farouk K. El-Baz¹ · Sami I. Ali¹ · Abeer Salama² · Rania Elgohary³

Received: 5 September 2022 / Accepted: 17 January 2023 / Published online: 16 February 2023
© The Author(s) 2023

Abstract

Acute kidney injury is a complication of using gentamicin in high doses and is associated with severe morbidity and mortality. Astaxanthin, the red xanthophyll carotenoid pigment, has an antioxidant power higher than 100 times that of vitamin E and 10 times more than that of β -carotene, making it one of the strongest bioactive carotenoids. *Haematococcus pluvialis*, Haematococcaceae, microalga produces high amounts of astaxanthin in the form of astaxanthin esters besides adequate amounts of free astaxanthin. We attempted to explore the potential therapeutic effects of astaxanthin- and astaxanthin ester-enriched fractions from *H. pluvialis* against acute kidney injury induced by gentamicin and their mechanisms of action in rats. Adult male Wister albino rats were injected with gentamicin (100 mg/kg, *i.p.*) for seven consecutive days to induce acute kidney injury, and these rats were treated daily with astaxanthin ester-enriched fractions (30 mg/kg; *p.o.*) for 14 days. LC/ESI-MS analysis revealed the presence of different isomers of free astaxanthin, astaxanthin monoesters, and astaxanthin diesters in the astaxanthin- and astaxanthin ester-enriched fractions. Astaxanthin and its esters ameliorated kidney dysfunction, modulated nuclear factor erythroid 2 related factor 2/Kelch-like erythroid associated protein 1/hypoxia-inducible factor- α , and suppressed mitogen-activated protein kinases/extracellular signal-regulated kinases/renin-angiotensin system expression. Astaxanthin and its ester improved early acute kidney injury via modulation of the Nrf2/Keap1 antioxidant defense pathway and MAPK/ERK signaling cascade.

Keywords Chlorophyta · Carotenoids · Xanthophylls · Kidney dysfunction · Proinflammatory markers · Oxidative stress

Introduction

Acute kidney injury is the case of systemic vasoconstriction, intravascular hypovolemia, and organ dysfunction, which result from oxidative stress. In acute kidney injury, free radicals are involved in cellular function disruptions and renal dysfunction followed by apoptosis (Quiros et al. 2011). Gentamicin, an aminoglycoside antibiotic, is used

in gram-negative bacteria infection treatment, but high doses of gentamicin produce serious side effects such as nephrotoxicity through its accumulation in the renal proximal tubular cells leading to renal dysfunction and cell injury (Quiros et al. 2011). The administration of gentamicin enhances the generation of reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, and superoxide anions in the renal tubule cells, which causes nephrotoxicity. Oxidative stress also provokes the release of proinflammatory mediators such as mitogen-activated protein kinases (MAPK), which causes kidney injury (Subramanian et al. 2015). Nuclear factor erythroid-2-related factor-2 (Nrf2) redox-sensitive transcription factor plays a vital role in cellular defense against oxidative stress (El-Baz et al. 2022). It activates the response of the antioxidant elements and the antioxidant enzymes such as glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) in the cell. It protects against gentamicin-induced oxidative stress in rat kidneys (Subramanian et al. 2015).

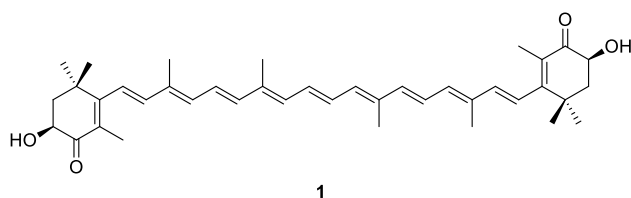
✉ Sami I. Ali
sb.ali@nrc.sci.eg

¹ Plant Biochemistry Department, National Research Centre, 33 El Buhouth St.(Former El-Tahrir St.), Dokki, Cairo 12622, Egypt

² Pharmacology Department, National Research Centre, 33 El Buhouth St.(Former El-Tahrir St.), Dokki, Cairo 12622, Egypt

³ Narcotics, Ergogenics and Poisons Department, National Research Centre, 33 El Buhouth St.(Former El-Tahrir St.), Dokki, Cairo 12622, Egypt

Astaxanthin (**1**; 3,3'-dihydroxy- β,β' -carotene-4,4'-dione), the red xanthophyll carotenoid pigment, has an antioxidant power higher than 100 times that of vitamin E and 10 times more than that of β -carotene, making it one of the strongest bioactive carotenoids (El-Baz et al. 2021). The microalga *Haematococcus pluvialis*, a freshwater species of Chlorophyta from the family Haematococcaceae, produces astaxanthin as astaxanthin esters. *Haematococcus pluvialis* can accumulate excessive amounts of astaxanthin, as well as its monoester (C16:2, C16:1, and C16:0) and diesters (C18:4/C18:3 and C18:1/C18:3) of saturated and unsaturated fatty acids (Todorovic et al. 2021). Therefore, this microalga has become one of the main sources of astaxanthin for health food supplements and pharmaceutical applications. Approved doses varied in different countries and ranged between 2 and 24 mg (Brendler and Williamson 2019). *H. pluvialis* produces the 3*S*,3'*S'* isomer (**1**) of astaxanthin, which is the most efficient for human implementations because of a superior bioavailability, compared to the other isomers of 3*R*,3'*S'* (meso form) and 3*R*,3'*R'* (Stachowiak and Szulc 2021).



Astaxanthin is the only carotenoid that passes through the blood–brain barrier (Fanaee-Danesh et al. 2019). It exhibited protective effects against tissue damage and oxidative stress in the *in vitro* and the *in vivo* experiments, and cured liver fibrosis (El-Baz et al. 2020 and 2021). It also protects against inflammation and apoptosis of tubular epithelial cells in ischemia/reperfusion-induced renal injury (Qiu et al. 2015). Despite these benefits, the effect of astaxanthin and its esters in gentamicin-induced acute kidney injury has not been investigated. So, we extracted astaxanthin- and astaxanthin ester-enriched fractions from *H. pluvialis* and evaluated their therapeutic effect against gentamicin-induced acute kidney injury in rats. We hypothesize that the potent antioxidant properties of astaxanthin and its esters can mitigate gentamicin-induced acute kidney injury in rats through the modulation of the Nrf2/ Keap1 and MAPK/ERK pathways.

Materials and Methods

Microalgae Cultivation

Haematococcus pluvialis, Haematococcaceae, was isolated from a freshwater community of the River Nile in Cairo, Egypt, and identified by Prof. Gamila H. Ali, professor of

water pollution, Environmental Research & Climate Changes Institute, National Research Centre, Cairo, Egypt, and then was grown on BG11 media (Abdo et al. 2013). Cultivation was conducted in plastic bottles with a capacity of 17 l containing 15 l of microalgal culture with continuous aeration. The culture temperature was 22 ± 3 °C. Fluorescent light was used to supply a constant light intensity of 100 $\mu\text{mol}/\text{m}^2/\text{sec}$ for the culture. After growing for 10 days the culture was transferred to a fully automated and computer-controlled vertical 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 $896 \times g$ for 15 min using a basket centrifuge. Samples were washed twice with water, dried in an oven at 50 °C, ground into a homogenous powder, and stored in a refrigerator for further chemical and biological investigation.

Astaxanthin Quantification

Extraction and Fractionation

The fine powder of *H. pluvialis* (100 g) was soaked with 1000 ml of hexane:ethyl acetate (1:1, v/v) in a 2000-ml conical flask and kept on an orbital shaker (Stuart, England) at $0.23 \times g$ at room temperature for 24 h. Then, the extract was centrifuged (Sigma 3-18ks Centrifuge, Germany) at $4248 \times g$ 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 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, and 100% ethyl acetate) then ethyl acetate:methanol (1:1) that afforded 10 fractions that collected in 50 ml per fraction. The 10 fractions were subjected to TLC (20 $\times$ 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. Fractions were combined into three fractions based on TLC results and concentrated to dryness using a rotary evaporator. Astaxanthin- and astaxanthin ester-enriched fractions were recognized by HPLC.

HPLC–DAD Analysis

Haematococcus pluvialis crude extract and astaxanthin- and astaxanthin ester-enriched fractions were subjected to analysis with 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). An Agilent 5 Prep C-18 Scalar column (5 μm , 150 mm $\times$ 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 carotenoids and 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 (Sarada et al. 2006). 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 comparison with retention time and the peak area of the unknown peak with the all-*trans*-astaxanthin standard.

UHPLC-ESI-MS Analysis

UHPLC-ESI-MS positive ion acquisition mode was carried out on a XEVOTQD triple quadrupole instrument, Waters Corporation, Milford, MA01757 USA, mass spectrometer. Mass Lynx™ software version 4.1 (Waters, Milford, MA, USA) was used to control the instruments, and for data acquisition and processing. UHPLC chromatographic separations were performed on a reversed-phase column ACQUITYUPLC-BEH C-18 (1.7 μm , 2.1 $\times$ 50 mm) (Waters, Milford, MA), and a gradient system with the mobile phase consisting of solvent A: acetonitrile: methanol (7:3, v/v) and solvent B: H₂O 100%. The gradient program used is shown in Table S1. ESI-mass conditions were *m/z* 50–3200 mass range: polarity, positive mode; capillary voltage set at 3 kV; cone, 30 V; extractor 2 V; RF 0.5 V; source temperature, 150 °C; probe temperature, 450 °C; cone gas flow, 10 l/h; dis-solvation gas flow, 440 l/h; and collision gas flow, 0.15 ml/min, following the methods of Rivera et al. (2011). The column was thermo-stated at 32 °C and the sample temperature was set at 25 °C. Before use, all solutions were filtered through 0.2 μm Membrane Nylon White Plain (Millipore, Bedford, MA, USA). Chromatographic peaks were identified by comparing positive ion ESI-MS spectra against known standards or *m/z* of compounds previously reported in the literature.

Animals

Adult male Wister albino rats weighing 120–150 g were purchased from the animal house colony of the National Research Centre (Dokki, Cairo, Egypt) and were kept at room temperature 25 °C $\pm$ 2 and with a light program of 12-h on/off. During the whole experiment, standard food and water were submitted to the animals under conventional

laboratory conditions. The rats were allowed to adapt to these conditions for 2 weeks before beginning the experimental protocol. All experimental procedures were conducted according to the ethical principles and guidelines of the use, care, and handling of experimental animals adopted by the Medical Research Ethics Committee at the National Research Centre, Egypt, and approved under Reg. No. 19/116, which is based on the Principles of Laboratory Animal Care (NIH No. 85:23 revised 1985). All experimental procedures were conducted in compliance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Experimental Design

Rats were grouped into 4 groups, each one consisting of eight rats: group I: normal control group received saline; group II: rats were injected with gentamicin (100 mg/kg, intraperitoneally (*i.p.*) (Erdem et al. 2000) for 7 consecutive days and served as the acute kidney injury group; group III and IV: rats received daily astaxanthin- or astaxanthin ester-enriched fractions (30 mg/kg/day, *p.o.*) (Deng et al. 2017) during 14 days after induction of acute kidney injury with gentamicin (80-mg ampules were purchased from Alexandria Chemical Co., Egypt).

Blood and Tissue samples

At the end of the experimental period, blood samples were withdrawn from the retro-orbital vein of each animal, and centrifuged at 250 $\times g$ for 10 min to obtain serum samples that were stored at –20 °C. The animals were sacrificed by decapitation; one kidney from each rat was immediately dissected out, washed with ice-cooled physiological saline, and homogenized in phosphate-buffered saline (PBS) (pH 7.4) as 20% (w/v) for the biochemical measurements. The other kidney was kept for histopathological assessment.

Biochemical Analysis

The obtained serum was used to estimate creatinine and urea levels using biondiagnostic kits (Egypt). Mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), renin-angiotensin system (RAS), and hypoxia-inducible factor- α (HIF- α), nuclear factor erythroid 2-related factor 2 (Nrf2), kelch-like ECH-associated protein 1 (Keap1) were determined using ELISA kits procured from Sunlong Biotech Co., Ltd, China. Kidney contents of MAPK, ERK, RAS, HIF- α , Nrf2, and Keap1 were determined by ELISA kits. The manufacturer's instructions were followed for calculating the results. Standards and samples were instilled into wells with immobilized antibodies specific for each

parameter, and then, horseradish peroxidase–conjugated streptavidin was instilled into the wells and incubated for 60 min at 37 °C, which were aspirated and washed 5 times with kit washing solution. Chromogen A and B solutions were added to the wells and incubated for 15 min at 37 °C; the developed color is proportional to the bound amounts of MAPK, ERK, RAS, HIF- α , Nrf2, and Keap1. After 10 min, color intensity was measured at 450 nm (Asfour et al. 2020).

Histological Examination

The dissected kidneys of distinct groups were fixed in 10% formalin. Fixation for 1 or 2 days was followed by dehydration in ascending grades of alcohol (70%, 90%, and three changes in absolute alcohol), clearance with xylene, impregnation in three successive changes in soft paraffin at 50 °C, and finally embedded in paraffin wax to obtain solid blocks containing the tissue. Serial transverse sections of 7 μ m thick were cut. Paraffin sections were mounted on glass slides covered by albumin glycerin and then stained with hematoxylin and eosin. Hematoxylin and eosin sections were evaluated qualitatively under light microscopy.

Statistical Analysis

All the values are presented as means $\pm$ standard error of the means (SE). Data of this study were evaluated by one-way analysis of variance followed by Tukey's multiple comparisons tests. GraphPad Prism software, version 5 (GraphPad Software Inc.; San Diego, USA) was used to conduct these statistical tests. The difference was considered significant when $p < 0.05$.

Results and Discussion

LC/ESI–MS Analysis

The extraction of fine powder of *H. pluvialis* with hexane:ethyl acetate (1:1, v/v) provided a crude extract with a yield (2.83%, dry weight) containing astaxanthin (11.91 mg/g, extract) as measured by HPLC (Fig. S1A). Based on HPLC analysis, the silica gel column chromatography fractionation gave different fractions from *H. pluvialis* extract such as astaxanthin ester–enriched fraction and astaxanthin–enriched fraction (Fig. S1B and S1C). The astaxanthin–enriched fraction contains 17.8 mg/g, while the astaxanthin ester–enriched fraction contains mono- and di-esters of astaxanthin.

The LC/ESI–MS analysis of all-*trans*-astaxanthin used as standard showed a remarkable m/z value of 597.32 $[M+H]^+$ and 619.50 $[M+Na]^+$, as previously described (Todorovic et al. 2021). We observed a remarkable m/z value of 611.40; it

is assumed to be $[M-H_2O+MeOH]^+$ of astaxanthin. The adducts with solvents methanol (MeOH) and water (H_2O) were observed as well at m/z values of 660.32 $[M+2MeOH+H]^+$ and 669.38 $[M+MeOH+H_2O+Na]^+$ with low intensity compared with high intensity of $[M+Na]^+$ (Fig. S2A and S3A). LC/ESI–MS positive mode and the MS² data applied to identify free astaxanthin isomers and astaxanthin mono- and di-esters in *H. pluvialis* fractions are presented in Table S2 for the astaxanthin ester–enriched fraction and in Table S3 for the astaxanthin–enriched fraction. The mass spectrum (Fig. S2B) of the astaxanthin ester–enriched fraction showed 5 isomers of free astaxanthin, 13 molecules of astaxanthin monoester (ME C14:2, C16:1, C17:3, C18:3, C21:1, C21:2, C23:2), and 9 molecules of astaxanthin diester (DE C10:0/C11:1, C10:0/C14:1, C10:1/C14:2, C13:0/C14:2, C14:0/C14:0, C12:0/C14:2, C17:1/C16:2, C17:1/C18:1). Molecules were identified based on the molecular weight (Table S2). For the astaxanthin–enriched fraction, the LC/ESI–MS spectrum (Fig. S2C) revealed 21 isomers of free astaxanthin, 7 molecules of astaxanthin monoester (ME C14:2, C17:3, and C21:2), and one molecule of astaxanthin diester (DE C14:0/C14:0). Molecules were identified based on the molecular weight (Table S3). The MS pattern of most astaxanthin isomers showed $[M+Na]^+$ with low intensity and $[M+MeOH+H_2O+Na]^+$ with high intensity, while the astaxanthin radical parent ion $[M]^+$ at m/z 593 and other solvent adducts such as $[M+3H_2O+Na]^+$ at m/z 673 and $[M+MeOH+H]^+$ at m/z 629 were observed in few astaxanthin isomers such as astaxanthin isomer 19 (Fig. S3B) and astaxanthin isomer 24 (Fig. S3C). All astaxanthin isomers showed a remarkable m/z value of 611 ($M-H_2O+MeOH$) with variable intensities, the same as that of the standard.

The addition of formic acid to the used mobile phase in the present study to improve carotenoid separation showed a remarkable effect on the ionization of astaxanthin as it leads to the observation of different ionization adducts as presented in Fig. S3. The astaxanthin isomers in the present study showed a remarkable fragmentation pattern, the same as previous reports of different carotenoids (Todorovic et al. 2021), through the elimination of 92 Da that corresponds to polyene toluene (C_7H_8), elimination of 106 Da that corresponds to polyene xylene (C_8H_{10}), and elimination of 18 Da of H_2O . The fragment ion at m/z 413.13 ($MH-92-92$, 100%), made from losing two units of (C_7H_8), was a characteristic pattern for astaxanthin isomers 4, 5, 7, 21, 23, 24, and 26, while fragment ion at m/z 385.96 ($M-106-106$, 100%), made from losing two units of (C_8H_{10}), was a characteristic pattern for astaxanthin isomer 8. In addition, the loss of H_2O in fragment ions at m/z 315.05 ($M-106-106-4H_2O$, 100%) and 345.12 ($M-106-92-3H_2O$, 100%) was a characteristic pattern for astaxanthin isomer 9 and 11, respectively (Tables S2 and S3). The astaxanthin monoesters (ME) were assigned as $[ME+H]^+$ and showed a remarkable fragmentation pattern containing a common fragment ion at m/z

611 (ME – FA + MeOH), and few ME showed the fragment ion at m/z 579 (ME – FA) as presented in Tables S2 and S3. The ESI–MS spectrum of astaxanthin monoester (ME C18:3) is presented in Fig. S4A; the m/z 857.87(10) represents $[M+H+ME\ C18:3]^+$; the MS² product ions showed m/z 785.44 (ME – 4H₂O, 9), m/z 727.57 (ME – 92 – 4H₂O, 20), m/z 611.21 (ME – FA + MeOH, 18), m/z 579.46 (ME – FA, 40), and m/z 301.06 (ME – 92 – 92 – 92, 100). The astaxanthin diesters (DE) were assigned also as $[DE+H]^+$ and showed a remarkable fragment ion of one fatty acid elimination $[DE-FA]^+$ beside the fragment ion of both fatty acids elimination $[DE-FA-FA]^+$ as presented in Tables S2 and S3. The ESI–MS spectrum of astaxanthin diester (DE C17:1/C16:2) is presented in Fig. S4B; the m/z 1081.23 (56) represents $[M+H+DE\ C17:1/C16:2]^+$; the MS² product ions showed m/z 832.97 (DE – FA_{C16:2}, 2), m/z 813.94 (DE – FA_{C17:1}), m/z 568.05 (DE – FA_{C16:2} – FA_{C17:1}), and m/z 552.39 (DE – FA_{C16:2} – FA_{C17:1} – H₂O, 100%). We found that the loss of fatty acid and water is the common fragmentation pattern of astaxanthin esters in *H. pluvialis*. The mass difference between the protonated molecule and fragment ions in the mass spectrum can be utilized only to assign acyl chains, not to determine the position of double bonds, so it is difficult to determine different isomers of astaxanthin and astaxanthin esters in *H. pluvialis*. The current findings agree with other findings that have identified different isomers of astaxanthin mono- and diesters (Miao et al. 2006; Holtin et al. 2009; Todorovic et al. 2021).

Creatinine and Urea Levels

Acute kidney injury induced by injection of gentamicin after 7 days was evidenced by a significant increase in serum creatinine and urea levels by 67 and 75%, respectively, as compared to the normal control group. Astaxanthin and astaxanthin ester administration provided marked protection manifested as significantly decreased serum levels of creatinine by 24 and 32% and urea by 38 and 42%, respectively, as compared to the gentamicin group (Table 1).

Table 1 Effect of astaxanthin (1) and astaxanthin ester-enriched fractions on serum levels of creatinine and urea

	Normal control	Gentamicin (100 mg/kg)	Astaxanthin (30 mg/kg)	Astaxanthin esters (30 mg/kg)
Creatinine (mg/dl)	0.96 ± 0.03	1.6 ± 0.06 ^a	1.22 ± 0.08 ^b	1.09 ± 0.06 ^b
% of gentamicin			76%	68%
Urea (mg/dl)	43.6 ± 1.9	76.2 ± 11.9 ^a	47 ± 0.84 ^b	43.9 ± 2.9 ^b
% of gentamicin			62%	58%

Data are presented as mean ± SE for each group ($n=8$) and % of the gentamicin group. Statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple-comparison tests

^aStatistically significant from the normal control group

^bStatistically significant from the gentamicin group at $p < 0.05$

Nrf2 and Keap 1 Kidney Contents

Injection of gentamicin showed a significant reduction in Nrf2 and a significant increase in Keap 1 tissue levels by 34 and 161%, respectively, as compared with the normal control values. On the other hand, administration of astaxanthin and astaxanthin esters showed a significant increase in tissue levels of Nrf2 by 32 and 39% and a decrease in Keap1 by 25 and 38%, respectively, as compared with the gentamicin group (Fig. 1).

HIF-α Kidney Content

HIF-α content decreased by 70% after gentamicin injection as compared with the normal control value. In contrast, astaxanthin and astaxanthin esters exerted an antioxidant effect by increasing HIF-α content significantly by 60 and 149%, respectively, as compared with the gentamicin group (Fig. 1c).

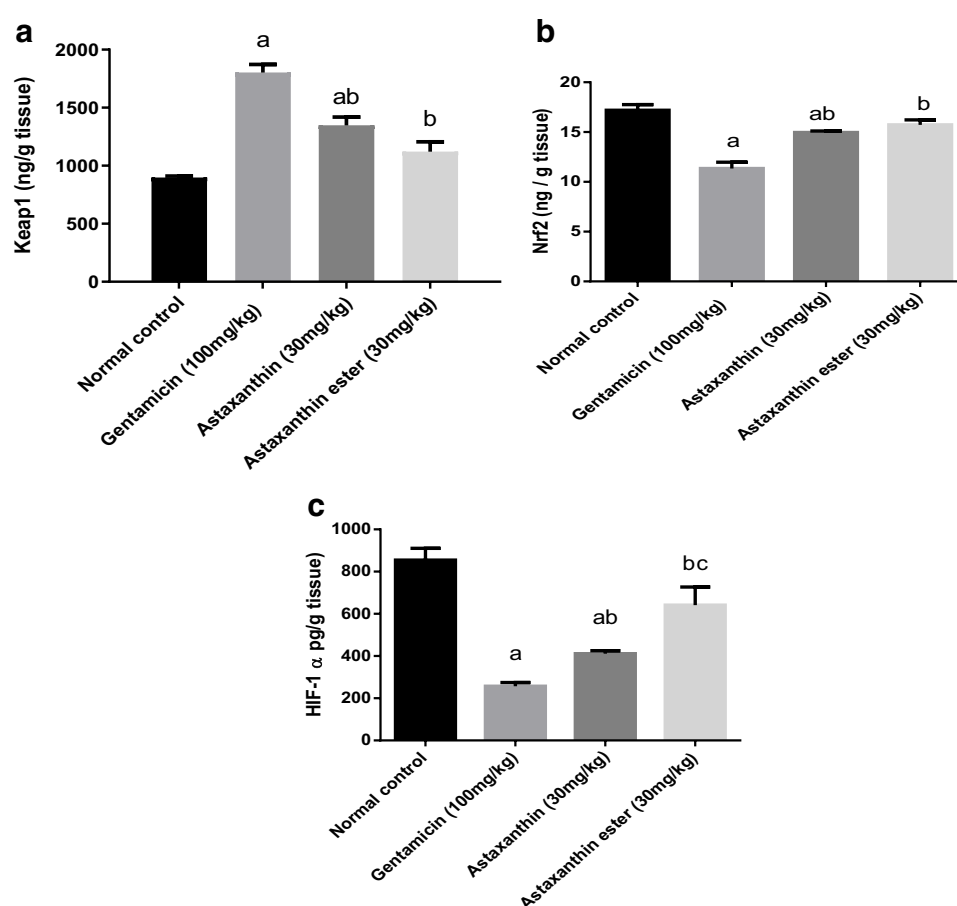
MAPK and ERK Kidney Contents

Gentamicin injection induced a significant increase in MAPK and ERK tissue levels by 3.4-fold and 2.6-fold, respectively, as compared with the normal control values as a sign of kidney inflammation. On the other hand, administration of astaxanthin and astaxanthin esters showed a significant decrease in the level of MAPK by 55 and 77% and ERK by 46 and 65%, respectively, as compared with the gentamicin group (Fig. 2a,b).

RAS Kidney Content

Gentamicin injection induced a significant increase in RAS tissue levels by twofold as compared with the normal control value as a sign of kidney inflammation. On the other hand, treatment with astaxanthin and astaxanthin esters decreased the level of RAS by 20% and 60% as compared with the gentamicin group (Fig. 2c).

Fig. 1 Effect of astaxanthin (1)–enriched fraction and astaxanthin ester–enriched fraction on **a** Nrf2, **b** Keap1, and **c** HIF-1 α kidney contents. Data are presented as mean $\pm$ SE for each group ($n=8$). Statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple-comparison tests. a, statistically significant from the normal control group. b, statistically significant from the gentamicin group at $p<0.05$



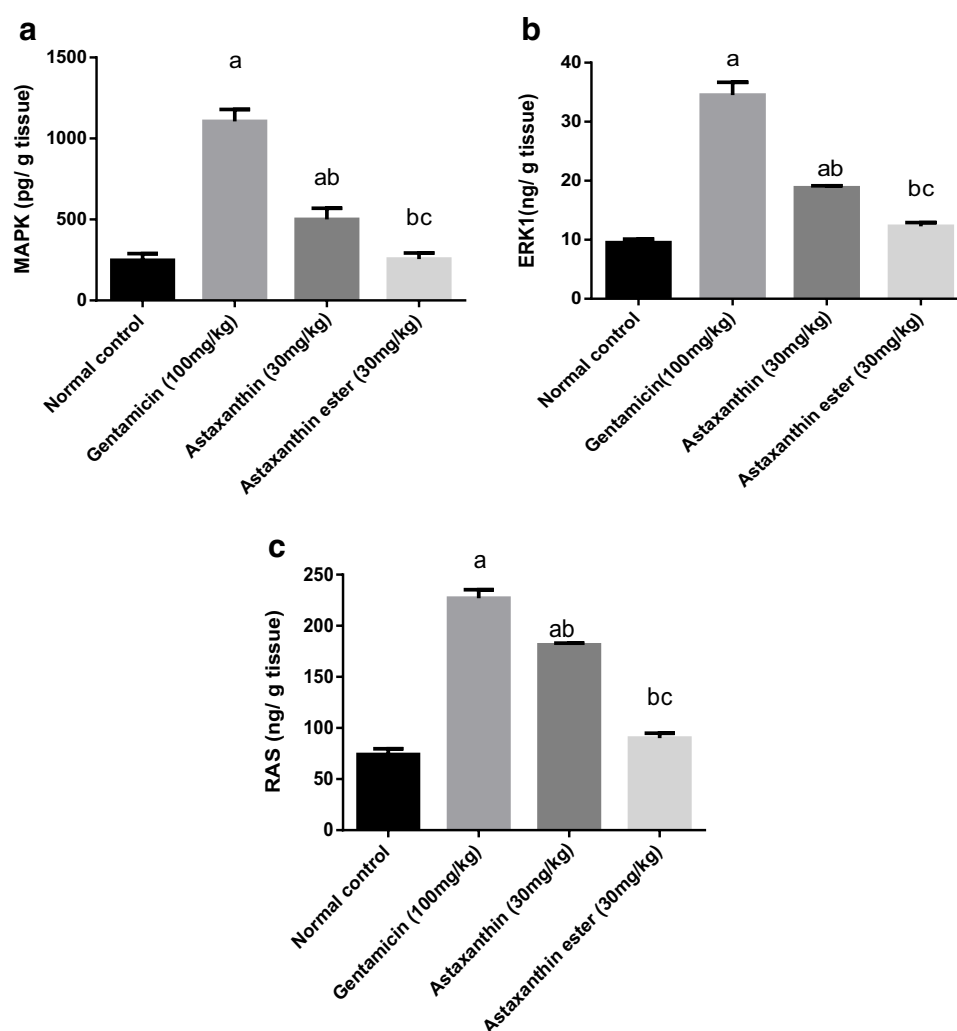
Histopathological Results

In the control group, the kidney section showed no histopathological alterations and showed ordinary histological architecture of average glomeruli with average Bowman's spaces, average proximal tubules with preserved brush borders, and average distal tubules (Fig. 3a). The gentamicin treatment revealed remarkable histopathological alterations in the kidney section, including markedly atrophied glomeruli with widened Bowman's spaces, proximal tubules with markedly apoptotic epithelial lining, and mild interstitial inflammatory infiltrate as shown in Fig. 3c, d). The control group also showed normal histopathological structure in the renal medulla, with average collecting tubules with average epithelial lining and average interstitium (Fig. 3e). The gentamicin group showed remarkable histopathological changes in the renal medulla, including collecting tubules with mildly apoptotic epithelial lining, intra-tubular hyaline casts, and mildly congested peri-tubular capillaries (Fig. 3f). The treatment with astaxanthin and astaxanthin esters also improved the gentamicin histopathological changes in the renal medulla and showed average collecting tubules with average epithelial lining and average interstitium (Fig. 3g, h).

In the gentamicin animal model, its accumulation in the renal tubules induces oxidative stress in the kidney, which causes nephrotoxicity. In the current study, the histopathological results showed that gentamicin-treated rats showed markedly atrophied glomeruli and apoptotic epithelial lining of proximal tubules with inflammatory infiltrate. These results are in accord with earlier reports, which reported that gentamicin causes kidney histopathological injuries such as proximal tubule epithelial edema, desquamation, and tubular fibrosis and necrosis (Xu et al. 2008). Our results showed that the administration of astaxanthin and astaxanthin esters mitigated all kidney and renal medulla histopathological alterations caused by gentamicin in rats. Administration of gentamicin increased serum creatinine and urea levels in our study, which is in consensus with the results of Xu et al. (2008), who reported that gentamicin elevated kidney function levels. Astaxanthin and astaxanthin ester administration in this study alleviated kidney dysfunction by decreasing the serum levels of creatinine and urea. Administration of astaxanthin attenuated elevated kidney function, oxidative stress, and apoptosis in acute kidney injury (Guo et al. 2015).

Nrf2 is a leading transcription factor that controls the endogenous antioxidant defense system via controlling

Fig. 2 Effect of astaxanthin (1)–enriched fraction and astaxanthin ester–enriched fraction on **a** MAPK, **b** ERK, and **c** RAS kidney contents. Data are presented as mean $\pm$ SE for each group ($n=8$). Statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple-comparison tests. a, statistically significant from the normal control group. b, statistically significant from the gentamicin group. c, statistically significant from the astaxanthin group at $p<0.05$



different antioxidant-responsive elements (ARE) genes. Gentamicin treatment suppresses renal Nrf2 translocation and activation and prevents ARE gene expression. This result may be related to the impact of gentamicin on Keap1 expression or dissociation (Ali et al. 2021). Our findings showed that astaxanthin and astaxanthin esters increased the levels of Nrf2 and decreased the levels of Keap1 in the kidney tissues. Thus, they can alleviate the kidney damage induced by the oxidative stress of gentamicin in rats by regulating the Nrf2/Keap1 pathway. Astaxanthin regulated oxidative stress and kidney injury–related Nrf2/Keap1 pathway induced by ochratoxin in mice (Ruíz-Salinas et al. 2020). The positive role of astaxanthin and astaxanthin esters in regulating the Nrf2/Keap1 pathway in the present study is due to their potent antioxidant properties. Due to its special structure, astaxanthin exhibits higher antioxidant effects than other antioxidants because of its high ability to bind to the cell membrane from the inside to the outside. The astaxanthin end ring can capture free radicals on the surface and inside the cell membrane, while the double-single bond

chain can capture free radicals only inside the cell membrane (Pashkow et al. 2008).

Upregulation of a regulatory defense factor against hypoxia-inducible factor- α (HIF- α) plays a vital role in acute or chronic kidney disease against podocyte injury with suppression of oxidative stress (Weidemann et al. 2008). HIF has an antioxidant effect also in diabetic nephropathy and renal ischemia–reperfusion injury and has a renal tubular cell antiapoptotic effect in cisplatin-induced nephrotoxicity (Weidemann et al. 2008). Gentamicin-induced nephrotoxicity elevated renal HIF-1 α mRNA levels (Xu et al. 2008). Therefore, HIF- α may be involved in gentamicin-induced renal injury. This study showed that astaxanthin and astaxanthin esters have a therapeutic effect against gentamicin-induced renal tubule cell injuries through modulation of HIF- α activity. In oxidative stress, the activation of different pro-inflammatory mediators including nuclear factor-kappa beta (NF)- κ B, TGF- β 1, and MAPKs leads to the progression of kidney damage. MAPK engages in cellular growth, differentiation, proliferation, and apoptosis regulation. MAPK promotes the

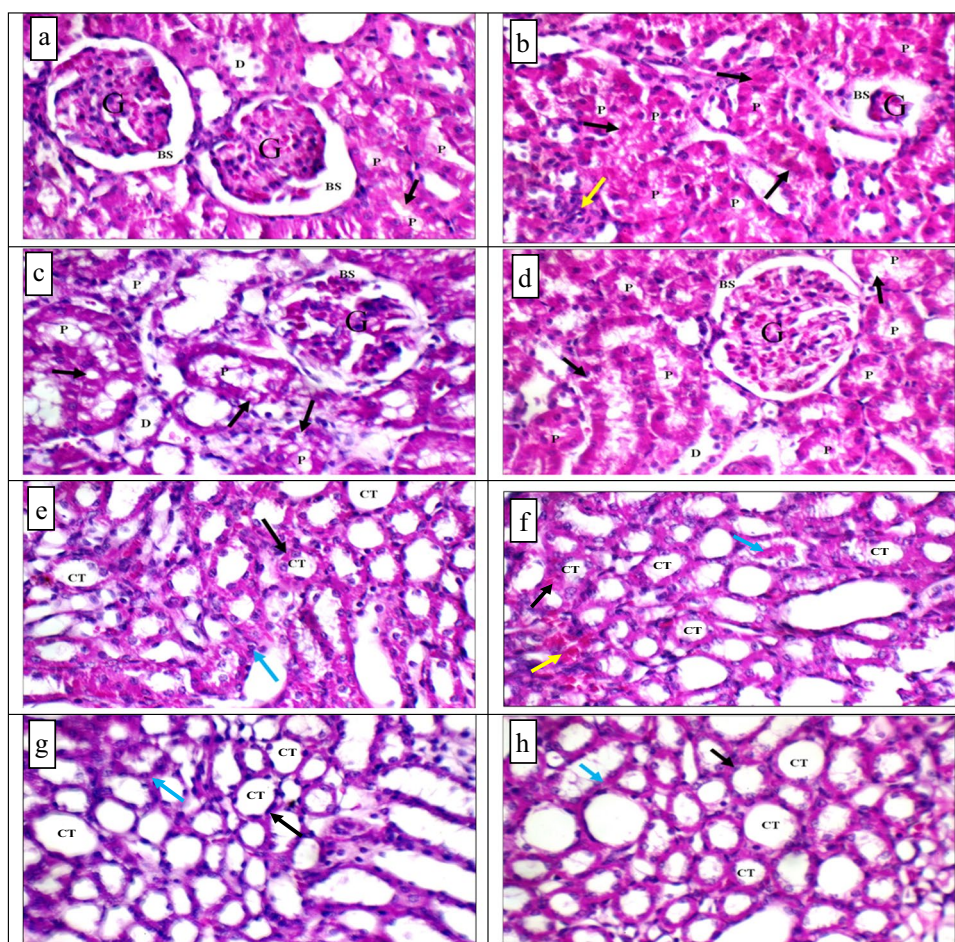


Fig. 3 Kidney section of **a** normal rats showing the normal architecture of average glomeruli (G) with average Bowman's spaces (BS), average proximal tubules (P) with preserved brush borders (black arrow), and average distal tubules (D). **b** Gentamicin-treated rats showing markedly atrophied glomeruli (G) with widened Bowman's spaces (BS), proximal tubules (P) with markedly apoptotic epithelial lining (black arrow), and mild interstitial inflammatory infiltrate (yellow arrow). **c** Kidney section of astaxanthin-enriched-fraction-treated rats showing mildly congested glomeruli (G) with average Bowman's spaces (BS), proximal tubules (P) with markedly edematous epithelial lining (black arrow), and average distal tubules (D). **d** Kidney section of astaxanthin ester-enriched fraction-treated rats showing mildly congested glomeruli (G) with average Bowman's spaces (BS), proximal

tubules (P) with mildly edematous epithelial lining (black arrow), and average distal tubules (D) (H&E×400). Kidney section in the renal medulla of **e** normal rats showing the normal architecture of average collecting tubules (CT) with average epithelial lining (black arrow) and average interstitium (blue arrow). **f** Gentamicin-treated rats showing collecting tubules (CT) with mildly apoptotic epithelial lining (black arrow), intra-tubular hyaline casts (blue arrow), and mildly congested peri-tubular capillaries (yellow arrow). **g** Astaxanthin-enriched fraction-treated rats showing average collecting tubules (CT) with average epithelial lining (black arrow) and average interstitium (blue arrow). **h** Astaxanthin ester-enriched fraction-treated rats showing average collecting tubules (CT) with average epithelial lining (black arrow) and average interstitium (blue arrow) (H&E×400).

activation of NF- κ B that is present in every cell cytoplasm; upon stimulation, it enhances TNF- α and IL-6 expressions aggravating the cellular damage (Qiao et al. 2012). In this study, gentamicin elevated kidney contents of MAPK and ERK. These results explain the vital role of proximal tubular epithelial cells in producing cytokines and cell adhesion molecules, provoking infiltrating cell recruitment, and activation at the inflammatory sites. Our findings showed that astaxanthin and astaxanthin esters alleviated MAPK/ERK signaling pathways. Thus, they may be considered a potential therapy for the renal disease caused by gentamicin through

the inactivation of MAPK/ERK pathways; consequently, they can inhibit the inflammatory process and apoptosis in the kidney cells, as shown in the histopathological results. In a previous study, astaxanthin reduced acetaldehyde-induced apoptosis and inhibited MAPK signaling pathway activation in diabetic cardiomyopathy (Zhang and Xu 2018).

The kidney is a unique organ containing a renin-angiotensin system (RAS) component as angiotensin II. Oxidative stress also affects the proinflammatory effect of angiotensin II produced in the distal and proximal tubules, the collecting ducts, and renal endothelial cells. RAS disturbance is one of the chief

pathways implicated in both glomerular and tubulointerstitial injuries resulting in renal injury through direct effects of angiotensin II type 1 receptor and also due to inflammatory cytokine activation (Al-Kuraishy et al. 2019). In the current study, astaxanthin and astaxanthin esters ameliorated the elevated levels of RAS induced by gentamicin in the kidney. Consequently, they can inhibit inflammation caused by RAS disturbance. In another study, astaxanthin modulated RAS and decreased TGF- β 1 levels and calcium oxalate crystal deposition in the kidney (Alex et al. 2014). The therapeutic role of the astaxanthin and astaxanthin ester fractions in the present study might be due to the high contents of different isomers of free astaxanthin and astaxanthin mono- and di-esters; isomers of astaxanthin may be of 9-*cis*, 13-*cis*, and 15-*cis* isomers not only all-*trans* which exert high therapeutic effects against gentamicin-induced kidney damage in rats. The isomers of 9-*cis* and 13-*cis*-astaxanthin exhibited a higher antioxidant effect than all-*trans*-astaxanthin against oxidative stress in the human intestinal epithelial Caco-2 cells (Yang et al. 2017).

Conclusion

The LC–ESI–MS analysis revealed different isomers of free astaxanthin and astaxanthin mono- and di-esters in the astaxanthin-enriched fraction and astaxanthin ester-enriched fraction of *H. pluvialis*. Due to its special structure, astaxanthin exhibits the highest antioxidant capacity, which may attract much attention to its use in the pharmaceutical and food industries. Astaxanthin-enriched fraction and astaxanthin ester-enriched fraction of *H. pluvialis* exert reno-therapeutic effects on renal tubular epithelial cells against gentamicin-induced acute kidney injury. They showed high therapeutic effects against gentamicin-induced kidney damage in rats by activating the antioxidant defense pathway of Nrf2 and HIF- α as well as suppressed Keap1/MAPK/ERK/RAS pathways.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s43450-023-00361-2>.

Acknowledgements The authors would like to express their sincere thanks to the National Strategy for Biotechnology and Genetic Engineering (Ministry of Scientific Research—Academy of Scientific Research and Technology) and Science and Technology Center (STC) for providing funds and support. The authors would also like to thank the National Research Centre (NRC) for providing all possible facilities and help.

Author Contribution FKE: conceptualization, resources, funding acquisition, review, and editing; SIA: conceptualization, methodology, validation, formal analysis, investigation, and manuscript writing, review, editing, and publication steps; AAS: conceptualization, methodology, validation, formal analysis, investigation, and manuscript writing, review, and editing; RE: methodology, formal analysis, and manuscript writing. All of the authors have read and approved the final manuscript.

Funding Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). Open access for publication was provided by the Science, Technology, and Innovation Funding Authority (STDF).

Data Availability All data generated or analyzed during this study are included in the manuscript and in the supplementary file.

Declarations

Ethics approval All experimental procedures were conducted according to the ethical principles and guidelines of the use, care, and handling of experimental animals adopted by the Medical Research Ethics Committee at the National Research Centre, Egypt, and approved under Reg. No. 19/116, which is based on the Principles of Laboratory Animal Care (NIH No. 85:23 revised 1985). All experimental procedures were conducted in compliance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Abdo SM, Ahmed E, El-Enin SA, El-Din RS, El Diwani G, Ali G (2013) Growth rate and fatty acids profile of 19 microalgal strains isolated from river Nile for biodiesel production. *J Algal Biomass Utln* 4:51–59
- Al-Kuraishy HM, Al-Gareeb AI, Al-Naimi MS (2019) Renoprotective effect of irbesartan in a rat model of gentamicin-induced nephrotoxicity: role of oxidative stress. *J Lab Physicians* 11:200–205. https://doi.org/10.4103/JLP.JLP_136_18
- Alex M, Sauganth Paul M, Abhilash M, Mathews VV, Anilkumar T, Nair RH (2014) Astaxanthin modulates osteopontin and transforming growth factor β 1 expression levels in a rat model of nephrolithiasis: a comparison with citrate administration. *BJU Int* 114:458–466. <https://doi.org/10.1111/bju.12537>
- Ali FE, Sayed AM, El-Bahrawy AH, Omar ZM, Hassanein EH (2021) Targeting KEAP1/Nrf2, AKT, and PPAR- γ signals as a potential protective mechanism of diosmin against gentamicin-induced nephrotoxicity. *Life Sci* 275:119349. <https://doi.org/10.1016/j.lfs.2021.119349>
- Asfour MH, Kassem AA, Salama A, Abd El-Alim SH (2020) Hydrophobic ion pair loaded self-emulsifying drug delivery system (SEDDS): a novel oral drug delivery approach of cromolyn sodium for management of bronchial asthma. *Int J Pharm* 585:119494. <https://doi.org/10.1016/j.ijpharm.2020.119494>
- Brendler T, Williamson EM (2019) Astaxanthin: how much is too much? A safety review. *Phytother Res* 33:3090–3111. <https://doi.org/10.1002/ptr.6514>

- Deng Z-Y, Shan W-G, Wang S-F, Hu M-M, Chen Y (2017) Effects of astaxanthin on blood coagulation, fibrinolysis and platelet aggregation in hyperlipidemic rats. *Pharm Biol* 55:663–672. <https://doi.org/10.1080/13880209.2016.1261905>
- El-Baz FK, Ali SI, Basha M, Kassem AA, Shamma RN, Elgohary R, Salama A (2020) Design and evaluation of bio-enhanced oral tablets of *Dunaliella salina* microalgae for treatment of liver fibrosis. *J Drug Deliv Sci Technol* 59:101845. <https://doi.org/10.1016/j.jddst.2020.101845>
- El-Baz FK, Salama A, Ali SI, Elgohary R (2021) *Haematococcus pluvialis* carotenoids enrich fractions ameliorate liver fibrosis induced by thioacetamide in rats: modulation of metalloproteinase and its inhibitor. *Biomed Res Int* 2021:6631415. <https://doi.org/10.1155/2021/6631415>
- El-Baz FK, Salama A, Ali SI, Elgohary R (2022) Lutein isolated from *Scenedesmus obliquus* microalga boosts immunity against cyclophosphamide-induced brain injury in rats. *Sci Rep* 12:22601. <https://doi.org/10.1038/s41598-022-25252-9>
- Erdem A, Gündogan NÜ, Usübütün A, Kılınc K, Erdem ŞR, Kara A, Bozkurt A (2000) The protective effect of taurine against gentamicin-induced acute tubular necrosis in rats. *Nephrol Dial Transplant* 15:1175–1182. <https://doi.org/10.1093/ndt/15.8.1175>
- Fanaee-Danesh E, Gali CC, Tadic J, Zandl-Lang M, Kober AC, Agujetas VR, de Dios C, Tam-Amersdorfer C, Stracke A, Albrecher NM (2019) Astaxanthin exerts protective effects similar to bexarotene in Alzheimer's disease by modulating amyloid-beta and cholesterol homeostasis in blood-brain barrier endothelial cells. *Biochim Biophys Acta - Mol Basis Dis* 1865:2224–2245. <https://doi.org/10.1016/j.bbadis.2019.04.019>
- Guo S-X, Zhou H-L, Huang C-L, You C-G, Fang Q, Wu P, Wang X-G, Han C-M (2015) Astaxanthin attenuates early acute kidney injury following severe burns in rats by ameliorating oxidative stress and mitochondrial-related apoptosis. *Mar Drugs* 13:2105–2123. <https://doi.org/10.3390/md13042105>
- Holtin K, Kuehnle M, Rehbein J, Schuler P, Nicholson G, Albert K (2009) Determination of astaxanthin and astaxanthin esters in the microalgae *Haematococcus pluvialis* by LC-(APCI) MS and characterization of predominant carotenoid isomers by NMR spectroscopy. *Anal Bioanal Chem* 395:1613. <https://doi.org/10.1007/s00216-009-2837-2>
- Miao F, Lu D, Li Y, Zeng M (2006) Characterization of astaxanthin esters in *Haematococcus pluvialis* by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Anal Biochem* 352:176–181. <https://doi.org/10.1016/j.ab.2006.03.006>
- Pashkow FJ, Watumull DG, Campbell CL (2008) Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. *Am J Card* 101:S58–S68. <https://doi.org/10.1016/j.amjcard.2008.02.010>
- Qiao H, Zhang X, Zhu C, Dong L, Wang L, Zhang X, Xing Y, Wang C, Ji Y, Cao X (2012) Luteolin downregulates TLR4, TLR5, NF-κB and p-p38MAPK expression, upregulates the p-ERK expression, and protects rat brains against focal ischemia. *Brain Res* 1448:71–81. <https://doi.org/10.1016/j.brainres.2012.02.003>
- Qiu X, Fu K, Zhao X, Zhang Y, Yuan Y, Zhang S, Gu X, Guo H (2015) Protective effects of astaxanthin against ischemia/reperfusion induced renal injury in mice. *J Transl Med* 13:28. <https://doi.org/10.1186/s12967-015-0388-1>
- Quiros Y, Vicente-Vicente L, Morales AI, López-Novoa JM, López-Hernández FJ (2011) An integrative overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin. *Toxicol Sci* 119:245–256. <https://doi.org/10.1093/toxsci/kfq267>
- Rivera S, Vilaró F, Canela R (2011) Determination of carotenoids by liquid chromatography/mass spectrometry: effect of several dopants. *Anal Bioanal Chem* 400:1339–1346. <https://doi.org/10.1007/s00216-011-4825-6>
- Ruiz-Salinas AK, Vázquez-Roque RA, Díaz A, Pulido G, Treviño S, Floran B, Flores G (2020) The treatment of Goji berry (*Lycium barbarum*) improves the neuroplasticity of the prefrontal cortex and hippocampus in aged rats. *J Nutr Biochem* 83:108416. <https://doi.org/10.1016/j.jnutbio.2020.108416>
- Sarada R, Vidhyavathi R, Usha D, Ravishankar G (2006) An efficient method for extraction of astaxanthin from green alga *Haematococcus pluvialis*. *J Agric Food Chem* 54:7585–7588. <https://doi.org/10.1021/jf060737t>
- Stachowiak B, Szulc P (2021) Astaxanthin for the food industry. *Molecules* 26:2666. <https://doi.org/10.3390/molecules26092666>
- Subramanian P, Anandan R, Jayapalan JJ, Hashim OH (2015) Hesperidin protects gentamicin-induced nephrotoxicity via Nrf2/HO-1 signaling and inhibits inflammation mediated by NF-κB in rats. *J Funct Foods* 13:89–99. <https://doi.org/10.1016/j.jff.2014.12.035>
- Todorovic B, Grujic VJ, Krajnc AU, Kranvogel R, Ambrožić-Dolinšek J (2021) Identification and content of astaxanthin and its esters from microalgae *Haematococcus pluvialis* by HPLC-DAD and LC-QTOF-MS after extraction with various solvents. *Plants* 10:2413. <https://doi.org/10.3390/plants10112413>
- Weidemann A, Bernhardt WM, Klanke B, Daniel C, Buchholz B, Câmpean V, Amann K, Warnecke C, Wiesener MS, Eckardt K-U (2008) HIF activation protects from acute kidney injury. *J Am Soc Nephrol* 19:486–494. <https://doi.org/10.1681/ASN.2007040419>
- Xu EY, Perlina A, Vu H, Troth SP, Brennan RJ, Aslamkhan AG, Xu Q (2008) Integrated pathway analysis of rat urine metabolic profiles and kidney transcriptomic profiles to elucidate the systems toxicology of model nephrotoxicants. *Chem Res Toxicol* 21:1548–1561. <https://doi.org/10.1021/tx800061w>
- Yang C, Zhang H, Liu R, Zhu H, Zhang L, Tsao R (2017) Bioaccessibility, cellular uptake, and transport of astaxanthin isomers and their antioxidative effects in human intestinal epithelial Caco-2 cells. *J Agric Food Chem* 65:10223–10232. <https://doi.org/10.1021/acs.jafc.7b04254>
- Zhang B, Xu D (2018) Protective effects of astaxanthin on diabetic cardiomyopathy in rats. *CYTA-J FOOD* 16:909–915. <https://doi.org/10.1080/19476337.2018.1503617>