

Evaluation of hepatoprotective and antioxidant activity of astaxanthin and astaxanthin esters from microalga-*Haematococcus pluvialis*

A. Ranga Rao · R. Sarada · M. D. Shylaja · G. A. Ravishankar

Revised: 10 February 2015 / Accepted: 19 February 2015 / Published online: 5 March 2015
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Abstract Effect of isolated astaxanthin (ASX) and astaxanthin esters (ASXEs) from green microalga-*Haematococcus pluvialis* on hepatotoxicity and antioxidant activity against carbon tetrachloride (CCl_4) induced toxicity in rats was compared with synthetic astaxanthin (SASX). ASX, ASXEs, and SASX, all dissolved in olive oil, fed to rats with 100 and 250 $\mu\text{g/kg}$ b.w for 14 days. They were evaluated for their hepatoprotective and antioxidant activity by measuring appropriate enzymes. Among the treated groups, the SGPT, SGOT and ALP levels were decreased by 2, 2.4, and 1.5 fold in ASXEs treated group at 250 $\mu\text{g/Kg}$ b.w. when compared to toxin group. Further, antioxidant enzymes catalase, glutathione, superoxide dismutase and lipid peroxidase levels were estimated in treated groups, their levels were reduced by 30–50 % in the toxin group, however these levels restored by 136.95 and 238.48 % in ASXEs treated group at 250 $\mu\text{g/kg}$. The lipid peroxidation was restored by 5.2 and 2.8 fold in ASXEs and ASX treated groups at

250 $\mu\text{g/kg}$. The total protein, albumin and bilirubin contents were decreased in toxin group, whereas normalized in ASXEs treated group. These results indicates that ASX and ASXEs have better hepatoprotection and antioxidant activity, therefore can be used in pharmaceutical and nutraceutical applications and also extended to use as food colorant.

Keywords *H. pluvialis* · ASX · ASXEs · CCl_4 · Antioxidants · Hepatoprotection · SGPT, SGOT, ALP

Introduction

The imbalance of reactive oxygen species concentration to the antioxidative defense mechanism is said to be oxidative stress (Uttara et al. 2009), which causes various diseases such as immune inflammatory lesions, nervous system, cardiovascular, and cancer (Lee et al. 2012). Carbon tetrachloride is well-known toxicant, supposed to generate free radicals in various tissues. Free radicals can destroy proteins, cell membranes, and nucleic acids (Kadiiska et al. 2005). Carotenoids have shown potential antioxidant properties which inhibit free radical formation in various diseases (Chatterjee et al. 2012). Microalgae are known to produce various bio-active compounds such as astaxanthin, β -carotene, lutein, phycocyanin, and phycoerythrin (Takaichi 2011; Ranga Rao et al. 2009, 2014). β -carotene bioavailability was lower than that of astaxanthin and xanthophyll's (Yeum and Russell 2002). As the natural carotenoids are associated with oxygenated functional group they are absorbed better than synthetic ones (Yeum and Russell 2002).

H. pluvialis is a green microalga, known to produce carotenoids under stress, which contain 70 % mono-ester, 15–20 % di-ester and 4–5 % free form of ASX (Ranga Rao et al. 2010, 2013a). It is approved as an antioxidant food supplement by the Swedish Health Food Council Advisory Board, and permitted as a food colorant in salmon feed, by food and Drug

A. R. Rao · R. Sarada · G. A. Ravishankar
Plant Cell Biotechnology Department, Central Food Technological Research Institute, (Constituent Laboratory of Council of Scientific and Industrial Research), Mysore 570020, Karnataka, India

M. D. Shylaja
Biochemistry and Nutrition Department, Central Food Technological Research Institute, (Constituent Laboratory of Council of Scientific and Industrial Research), Mysore 570020, Karnataka, India

A. R. Rao (✉)
Institute of Ocean and Earth Sciences, University of Malaya, Kuala Lumpur 50603, Malaysia
e-mail: arangarao99@gmail.com

A. R. Rao
e-mail: arangarao@um.edu.my

G. A. Ravishankar
Dayananda Sagar Institutions, Dr.C.D.Sagar Center for Life Sciences, Shavige Malleshwara Hills, 5th Floor, Kumaraswamy Layout, Bangalore 560078, Karnataka, India

Administration in United States (Yuan et al. 2011) for imparting pigmentation. Astaxanthin is used as pigmentation source in trout, farmed salmon, and poultry (Lorenz and Cysewski 2000). Nutraceutical and medical applications of astaxanthin were reported against diseases such as age-related macular degeneration, inflammation, cancer, *Helicobacter pylori* infection (Fassett and Combes 2011). Natural astaxanthin has claimed its importance in functional foods. *H. pluvialis* has been cultivated at large scale in various systems (Wang et al. 2013). In CFTRI laboratory many studies have been conducted on *H. pluvialis*, including its production, characterization of astaxanthin (ASX), astaxanthin esters (ASXEs) etc. Recently our research group reported that the potency of ASXEs on anti-ulcer and anti-skin cancer activity in rat models (Ranga Rao et al. 2013a, b; Kamath et al. 2008). The present study is about the effect of isolated astaxanthin (ASX) and astaxanthin esters (ASXEs) from *H. pluvialis* on CCl₄ induced hepatotoxicity in rats. The current results showed that ASXEs showed better protection than ASX on hepatic biochemical markers and antioxidant enzymes in CCl₄ induced hepatotoxicity in rats.

Materials and methods

Chemicals and reagents

Solvents HPLC grade- methanol, acetonitrile, dichloromethane were purchased from Rankem Chemicals Ltd (Mumbai, India). Analytical grade-hexane, acetone, methanol, chloroform, and petroleum ether were procured from Sisco Chemicals Laboratory (Mumbai, India). The serum glutamate transaminase (SGPT), serum glutamate oxaloacetate (SGOT), alkaline phosphatase (ALP), bilirubin and albumin kits were obtained from Kumar Diagnostics Ltd., Mysore, Karnataka, India. Synthetic astaxanthin procured from Sigma Chemicals, Co (St. Louis, Mo).

Batch culture of *H. pluvialis* and its carotenoid accumulation

H. pluvialis (34-1a) was procured from Sammlung von Algenkulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen, Germany, and grown in bold basal medium. Carotenoids accumulation under stress conditions were reported (Sarada et al. 2002). The encysted algal biomass said to be rich in astaxanthin (ASX) and astaxanthin esters (ASXEs) was harvested and kept at 4 °C until further use.

Extraction of ASX and ASXEs from *H. pluvialis*

Carotenoid from encysted algal biomass was extracted with acetone, followed by centrifugation at 4000×g (C24; Remi Instruments Ltd, Mumbai, India). ASX and ASXEs were

isolated from the carotenoids as earlier reported by Ranga Rao et al. (2013a). Briefly, carotenoid extract was separated on silica gel thin layer chromatography with the solvent ratio of hexane: acetone (7:3, v/v) and the bands were scraped and re-dissolved in acetone. Further acetone was evaporated in the fractions using rota-vapor. The above extraction methods were repeated for 2–3 times in the dark condition to avoid degradation. These fractions were flushed with nitrogen, stored at 0 °C temperature, used it in further experiments.

Characterization of ASX and ASXEs by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectra (LC-MS)

Both, HPLC (Shimadzu 10AS, Kyoto, Japan) reverse phase 25×4.6 mm, 5 μm, C₁₈ column (Wakosil 11 5C 18RS) and Waters 2996 modular HPLC system (auto-sampler, gradient pump, thermo-regulator and DAD), coupled to a Q-Tof Ultima (UK) mass spectrometer were used for the identification and characterization of ASX and ASXEs. HPLC and LC-MS conditions used as per the details given in our earlier reports (Ranga Rao et al. 2010, 2013a).

Animals

The institutional animal ethics committee (IAEC No. 116/08) was approved for the animal experiments. Albino Wistar rats [Out B - Wistar, IND-Cft (2C), 200–220±2 g] were individually maintained at room temperature (28±2 °C), provided 12 h light/dark cycle in the animal house. Fresh pellet diet obtained from Amrut feeds, Sangli, India and tap water were given to rats on daily basis.

Carbon tetrachloride (CCl₄) treatment

Rats were divided into seven groups, each group consisted of six animals, details are provided in Table 1. Group-I served as normal, group-II served as toxin, group-III-VII were given single dose of CCl₄ 2.0 g/kg b.w, dissolved in equal volume of liquid paraffin to produce hepatotoxicity. Administration of ASX, ASXEs and SASX was started 2 weeks prior to CCl₄ treatment. The animals were sacrificed after 24 h of CCl₄ treatment, collected blood from the heart and liver tissues by the method of Chidambara Murthy et al. (2005a, b).

Analysis of hepatic injury

After 24 h of hepatotoxin administration, rats were anesthetized with diethyl ether. The blood was collected by cardiac puncture, allowed to clot for 1–2 h at room temperature and serum was separated by centrifugation at 2500 rpm for 15 min and used to determine the activities of SGPT (Bergmeyer and Horder 1980), SGOT (Bergmeyer et al. 1976), ALP (Szasz

et al. 1974), albumin (Wooton 1964) and bilirubin (Malloy and Evelyn 1937) in normal, toxin and sample treated groups using commercially available enzyme kits.

Analysis of antioxidant enzymes of liver tissue

Activities of catalase was measured by the method of Aebi (1984) and superoxide dismutase by the method of Flohe and Otting (1984). Glutathione peroxidase and glutathione were measured as per the protocols described earlier (Ranga Rao et al. 2013a, b).

Protein estimation, and lipid peroxidation assay

Protein was determined by the procedure of Lowry et al. (1951). Lipid peroxidation activity was determined by the procedure of Buege and Aust (1978), a pink chromogen, a diadduct, formed by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) which can be detected spectrophotometrically at 532 nm.

Histopathological studies

The liver samples were kept for 24 h in 10 % buffered formalin. The tissue sections were made in paraffin blocks, stained with hematoxylin and eosin dye, and were observed in light microscope (Leitz, Germany) at 60x magnification.

Statistical analysis

The values shown as averages of mean $\pm$ standard deviation of six replicates. The values were evaluated by one-way ANOVA followed by Duncan's multiple-range test (*post-hoc*) using Microsoft Excel XP (Microsoft Corp., Redmond, WA) software.

Results and discussion

ASX and ASXEs in batch culture of *H. pluvialis*

Alga was grown in bold basal medium for 3 weeks and the biomass contained 2.35 % (w/w) total carotenoid and 0.20 % (w/w) chlorophyll. ASX and ASXEs were found to be ~2 and 78 % in total carotenoid on dry weight basis. ASX and ASXEs were identified by absorption spectra at 470–476 nm. HPLC profile of isolated ASX from *H. pluvialis* is shown in Fig. 1. As ASX and ASXEs showed better resolution on thin layer chromatography those portions were scraped and pure components were isolated by preparative thin layer chromatography after reconfirming their mobility with the same chromatographic system. Results were characterized by mass spectra. ASXEs- $C_{16:0}$, $C_{17:2}$, $C_{17:1}$, $C_{17:0}$, $C_{18:4}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$,

Table 1 Experimental groups for carbon tetrachloride treatment

Group-I	Without any treatment (normal)
Group-II	CCl_4 +olive oil (toxin)
Group-III	CCl_4 +ASX100 ^a
Group-IV	CCl_4 +ASX250 ^a
Group-V	CCl_4 +ASXEs100 ^a
Group-VI	CCl_4 +ASXEs250 ^a
Group-VII	CCl_4 +SASX100 ^a

^a $\mu\text{g/kg}$ b.w Body weight, CCl_4 , Carbon tetrachloride, ASX Astaxanthin, ASXEs Astaxanthin esters from *H. pluvialis*, SASX Synthetic astaxanthin

$C_{16:0}/C_{16:0}$, $C_{16:0}/C_{18:2}$, $C_{18:1}/C_{18:3}$, $C_{18:1}/C_{18:2}$, $C_{18:1}/C_{18:1}$ were identified by mass spectra. ASXEs fragmentation pattern was interpreted due to breaking of fatty acid and water molecules. Further these molecules were evaluated for their effects on hepatoprotection and antioxidant activity in rats.

Influence of ASX and ASXEs on serum marker enzymes in normal and experimental rats

The levels of serum SGPT, SGOT, and ALP were significantly elevated in CCl_4 treated group of hepatic damage. Treatment of rats along with ASX, ASXEs and SASX at 100 and 250 $\mu\text{g/kg}$ b.w noticeably prevented the CCl_4 induced elevation of SGPT, SGOT and ALP (Fig. 2). The levels of serum enzymes were increased by 1.5–2 folds in CCl_4 as compared to control group. The enzyme activity was increased in CCl_4 toxin administered group and the enzyme activity of SGPT, SGOT and

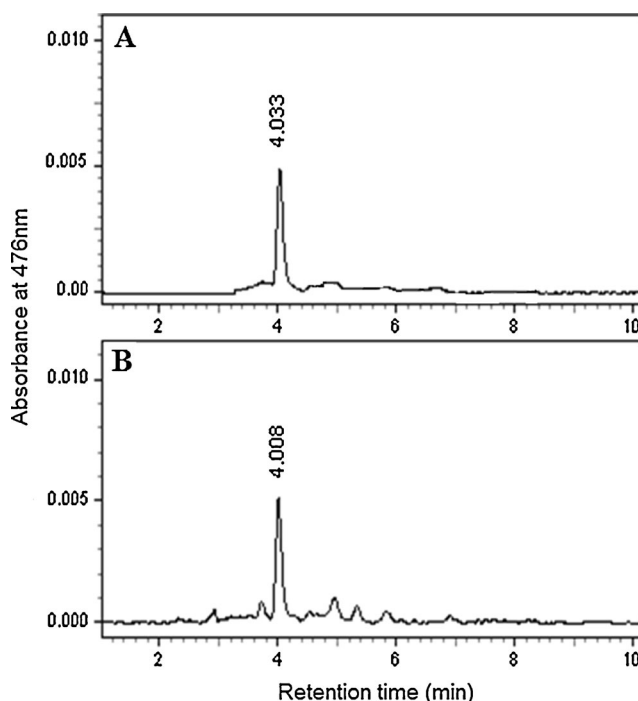


Fig. 1 High performance liquid chromatography (HPLC) profile of standard astaxanthin (a) and isolated astaxanthin (b) from *H. pluvialis*

ALP were 180.39, 196.34 and 125.41 Units/mL respectively. Levels of these enzymes were significantly less in the tested group animals. SGPT activity was 134.85, 112.23 Units/mL in ASX, 129.31, 86.45 Units/mL in ASXEs and 102.28 Units/mL respectively in SASX after their treatment at 100 and 250 $\mu\text{g/kg}$ b.w; whereas for SGOT it was 137.45, 117.45 Units/L in ASX, 117.45, 79.67 Units/L in ASXEs and 85.23 Units/mL in SASX respectively (Fig. 2a and b). The hepatoprotective activity of ASX, ASXEs and SASX on ALP are shown in Fig. 2c. ALP activity was 85.41 Units/mL in normal group, whereas in treated groups it registered 113.45 Units/mL (ASX), 95.78 Units/mL (ASXEs), 89.75 Units/mL (SASX) Units/mL in treatments at 100 $\mu\text{g/kg}$ b.w; and 97.45 (ASX), 80.45 (ASXEs) Units/mL in treatments at 250 $\mu\text{g/kg}$ b.w.

Influence of ASX and ASXEs on protein, albumin, bilirubin contents in normal and experimental rats

The total protein, albumin and bilirubin levels were measured in normal, in experimental rats treated with toxin, ASX, ASXEs and SASX after the administration of CCl_4 (Fig. 3). The protein and albumin levels decreased in toxin group, whereas these levels increased in ASX, ASXEs and SASX treated groups (Fig. 3a and b). The increased serum

bilirubin levels by treatment with toxin, were significantly lowered by treating with ASX, ASXEs and SASX in a dose depend manner (Fig. 3c).

Influence of ASX and ASXEs on antioxidant enzymes, glutathione levels in normal and experimental rats

The liver antioxidant enzymes activity noticeably decreased in the toxin (CCl_4) treated groups and elevated in the ASX, SASX and ASXEs treated groups when compared to normal animals (Table 2). The group treated with ASXEs at 250 $\mu\text{g/kg}$ was more active i.e. protective when compared to toxin and SASX treated group. ASXEs showed hepato-protection which was measured in terms of level of hepatic enzymes namely, catalase, glutathione peroxidase, superoxide dismutase and anti-lipid peroxidation. Rats treated with at 2.0 g/kg b.w. toxin showed the decrease in levels of catalase, glutathione peroxidase and superoxide dismutase by 31.65, 35.17 and 51.70 %, whereas, lipid peroxidation activity increased by 2.4 folds as compared to normal group. However, antioxidant enzymes superoxide dismutase, catalase and peroxidase enzyme activities were preserved in the pretreatment of rats with 250 $\mu\text{g/kg}$ of ASX, SASX and ASXEs treated groups. Catalase restoration was 136.95 % and 238.48 % higher compared to toxin groups respectively at 250 $\mu\text{g/kg}$

Fig. 2 Influence of ASX, ASXEs and SASX administration on serum parameters in carbon tetrachloride treated rats. ASX, ASXEs and SASX treated for 14 days, followed by CCl_4 treatment, and sacrifice 24 h later. Each value represents the average of mean $\pm$ SD ($n=6$), $^{**}P<0.001$ compared to toxin treated group. * $\mu\text{g/kg}$ b.w; body weight; SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxaloacetate transaminase; ALP, alkaline phosphatases; toxin, carbon tetrachloride; ASX, astaxanthin, ASXEs, astaxanthin esters; SASX, synthetic astaxanthin

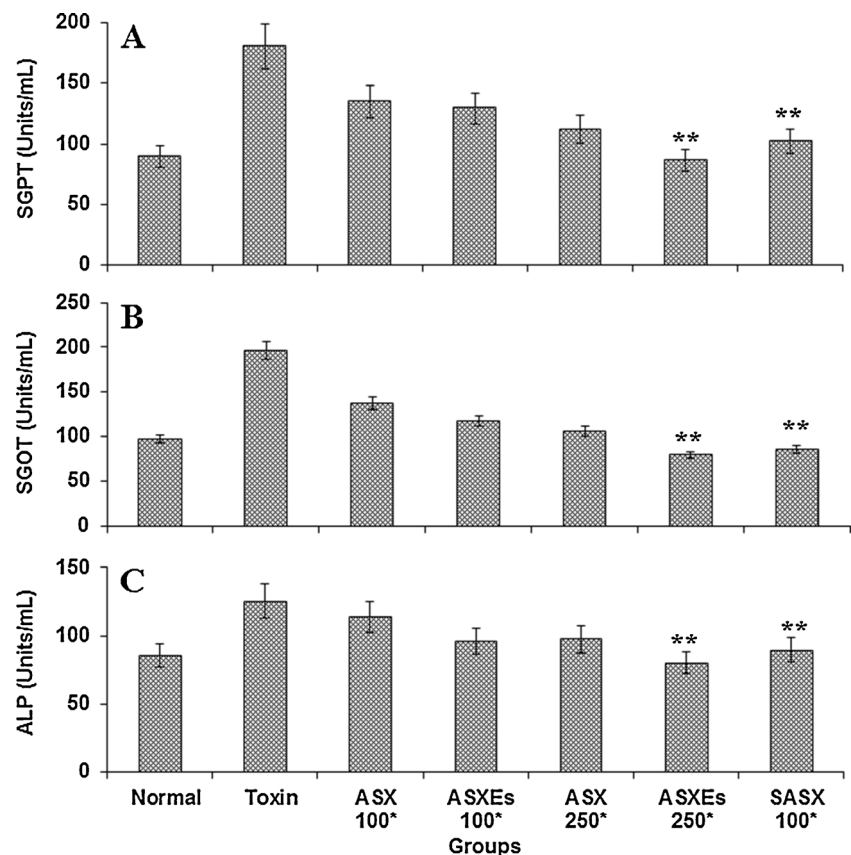
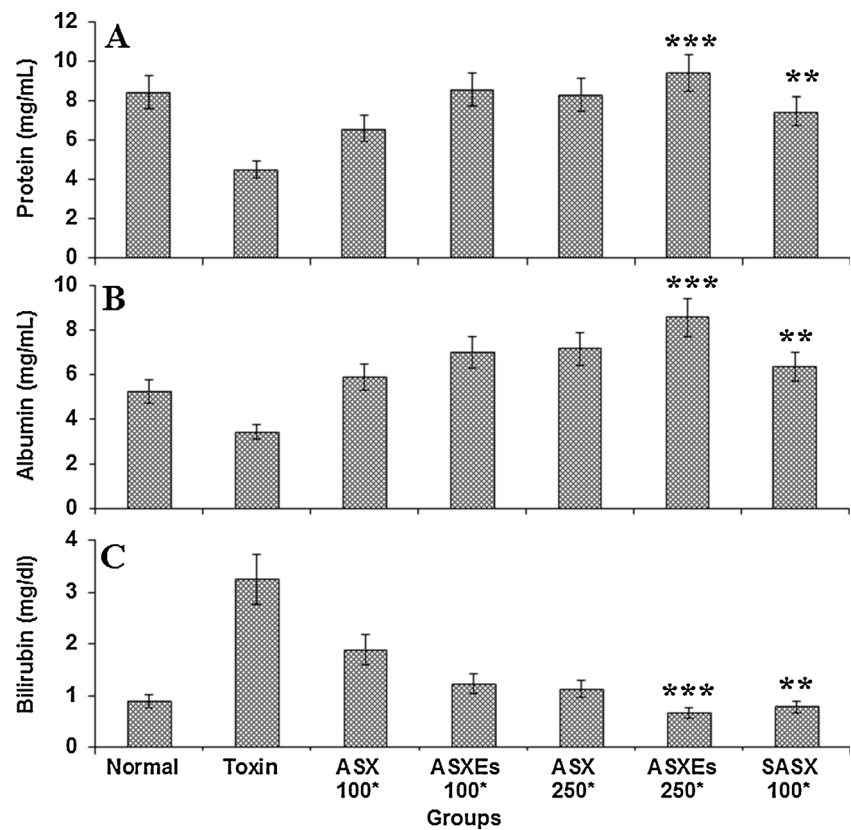


Fig. 3 Influence of ASX, ASXEs and SASX administration on protein, albumin and bilirubin in carbon tetrachloride treated rats. ASX, ASXEs and SASX treated for 14 days, followed by CCl₄ treatment, and sacrifice 24 h later. * $\mu\text{g/kg}$ b.w, each value represents the mean $\pm$ SD ($n=6$), ** $P<0.01$, *** $P<0.001$ compared to toxin treated group. * $\mu\text{g/kg}$ b.w, body weight; toxin, carbon tetrachloride; ASX, astaxanthin, ASXEs, astaxanthin esters; SASX, synthetic astxanthin



of ASX and ASXEs whereas treated with 100 $\mu\text{g/kg}$ SASX treated group was 179.61 %. The peroxidase and superoxide dismutase enzymes also showed similar trend (Table 2). This shows the protection provided by feeding ASX and ASXEs and its ability to maintain these enzyme levels even after toxin treatment. Similarly, the level of GSH was low in toxin treated groups whereas in ASX, ASXEs and SASX treated groups their levels were significantly increased (Table 2). The lipid peroxidation was retained by 5.2 and 2.8 folds in 250 $\mu\text{g/kg}$ b.w of ASX and ASXEs treated groups and 1.9 fold in SASX treated group at 100 $\mu\text{g/kg}$ b.w.

Influence of ASX and ASXEs on liver histopathology in normal and experimental rats

Liver histopathology of normal, toxin, ASX and ASXEs treated groups is shown in Fig. 4. According to histopathology studies, hepatocytes with normal architecture, portal triad, portal veins, hepatic artery and vein were found to be visible in normal group animals, whereas in toxin treated group they were significantly changed. The liver has retained the normal hepatic architecture with minor hemorrhage at 250 $\mu\text{g/kg}$ b.w in ASXEs treated group (Fig. 4) followed by ASX and SASX treated groups.

Table 2 Influence of ASX, ASXEs and SASX on liver antioxidant enzymes and glutathione in carbon tetrachloride treated rats

Groups	Catalase (U/mg protein)	Glutathione peroxidase (U/mg protein)	Superoxide dismutase (U/mg protein)	% Anti-lipid peroxidase	Glutathione (nmol/mg protein)
Normal	457.23 $\pm$ 6.98	10.12 $\pm$ 1.42	20.81 $\pm$ 1.75	22.45 $\pm$ 2.61	11.23 $\pm$ 1.15
Toxin	312.51 $\pm$ 10.37	6.56 $\pm$ 1.74	10.05 $\pm$ 2.38	53.78 $\pm$ 3.80	4.25 $\pm$ 0.93
ASX100 ^a	428 $\pm$ 12. 61**	11.98 $\pm$ 4.63**	12.13 $\pm$ 2.66**	18.91 $\pm$ 2.13**	8.16 $\pm$ 1.84**
ASX250*	543.01 $\pm$ 9.61**	15.98 $\pm$ 2.30**	15.02 $\pm$ 1.73**	26.46 $\pm$ 1.09**	10.47 $\pm$ 1.06**
ASXEs100*	623. 84 $\pm$ 8.25***	21.79 $\pm$ 3.06***	26.13 $\pm$ 3.67***	21.81 $\pm$ 2.74***	15.03 $\pm$ 1.09***
ASXEs250*	745.28 $\pm$ 6.05***	23.65 $\pm$ 1.65***	32.98 $\pm$ 4.01***	10.33 $\pm$ 1.12***	18.69 $\pm$ 2.78**
SASX100*	561.31 $\pm$ 11.82**	11.24 $\pm$ 2.38**	16.81 $\pm$ 3.22**	27. 58 $\pm$ 2.91**	13.52 $\pm$ 2.13**

* $\mu\text{g/kg}$ b.w Body weight, toxin Carbon tetrachloride (CCl₄), ASX Astaxanthin, ASXEs Astaxanthin esters, SASX Synthetic astxanthin. Each value represents the average of mean $\pm$ SD ($n=6$), ** $P<0.01$, *** $P<0.001$ compared to toxin treated group

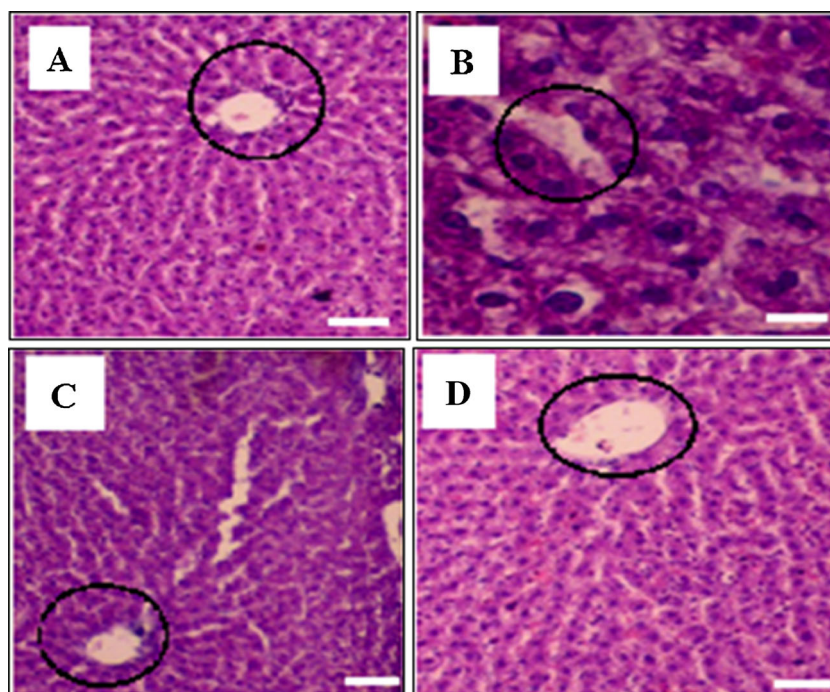
Liver is highly affected by toxic agents as being vital organ to metabolise xenobiotics. The free radicals deactivate liver detoxification enzymes during the catalytic cycle. Carbon tetrachloride has been widely studied as a liver toxicant, and its metabolites formed as trichloromethyl peroxy radical and trichloromethyl radical which are involved in the pathogenesis of liver and kidney damage. The increased lipid peroxidation in liver was triggered by the enormously generated free radicals in the toxic group. The massive free radical generation help the cytotoxicity effect to propagate intracellularly, increasing the interaction of these radicals with phospholipids structure and inducing a peroxidation process that destroys structure of organ (Jaeschke et al. 2012). The interaction of free radical with polyunsaturated fatty acids of the membrane lipids initiate lipid peroxidation, leads to oxidative stress, further forms malondialdehyde (MDA), which is end product of this process (Negre-Salvayre et al. 2008).

This study has shown that the isolated ASX and ASXEs of *H. pluvialis* offered better protection to the toxin induced rats and also maintained their enzyme levels in both serum and liver tissue (Fig. 2 and Table 2). Antioxidant enzymes and glutathione levels were significantly increased in liver tissue by the treatment of ASX and ASXEs, effectively demonstrating their protective effect (Table 2). Biochemical parameters protein, albumin contents declined in toxin treated groups while bilirubin levels were increased. However, these values were significantly protected in the both ASX and ASXEs treated groups (Fig. 3a, b and c). These results indicate that ASX and ASXEs from *H. pluvialis* have a elevated anti-hepatotoxic effect than SASX, these current observations have been shown the potential biological activity of ASXEs. Earlier

studies from our laboratory conducted on the bioavailability, antioxidant effect, gastro-protective effect and anti-cancer activity of ASX and ASXEs from *H. pluvialis* in the rat models, results showed that the antioxidant enzymes levels were significantly increased by ASX and ASXEs treatment (Ranga Rao et al. 2013a, b; Kamath et al. 2008). Risk of various disorders can be reduced by taking carotenoids like astaxanthin, lutein and zeaxanthin (Sanda et al. 2008). Scientific reports indicate that astaxanthin has shown beneficial effects on cardiovascular disease, blood pressure in vitro and in-vivo model, this may be due to its higher antioxidant activity (Fassett and Combes 2011). Recently our research group has shown that skin tumors and tyrosinase enzyme activity significantly decreased, whereas antioxidant enzyme levels increased by ASX and ASXEs treatment in skin carcinogenesis rats (Ranga Rao et al. 2013a). Similar results were observed by astaxanthin treatment against 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin and methylnitrosourea induced toxicity in rats by stimulating the cellular antioxidant enzymes, hindering lipid peroxidation, and protein oxidation (Sanda et al. 2008; Turkez et al. 2012).

Carotenoids pro-antioxidant ability was well defined, in membrane protection ability mostly by preventing lipid peroxidation and restoring various antioxidant enzymes like superoxide dismutase, catalase, and peroxidases (González-Burgos and Gómez-Serranillos 2012). However, protection abilities in ASX and ASXEs treated groups were less exposed. In humans, dietary astaxanthin boosted immune response, reduced DNA oxidative damage and inflammation (Park et al. 2010). Astaxanthin showed various pharmacological activities, including anti-inflammatory, anti-diabetic activities as well as

Fig. 4 Histopathological observation of liver of different treated groups (60x). Section through the liver of normal rats showing central vein and hepatocytes (a), section through the liver of CCl₄-treated rats showing central vein and hepatocytes (b) and section through the liver of ASX, ASXEs (250 µg/kg b.w) treated rats (c and d) showing the central vein (round marking) and hepatocytes



antioxidant properties (Ranga Rao et al. 2010, 2013a, b; Maoka et al. 2012). Lipid peroxidation in the serum and liver of astaxanthin-fed rats treated with CCl₄ was significantly inhibited relative to rats fed a control diet (Kang et al. 2001). Micro algal carotenoids β -carotene from *Dunaliella*, *Spirulina* biomass were enhanced antioxidant enzyme activity and hepatoprotective property on carbon tetra chloride induced toxicity in rats (Chidambara murthy et al. 2005a, b; Vanitha et al. 2007). Lutein producing microalga-*Botryococcus braunii* enhanced bioavailability and antioxidant activity in experimental rats by inhibition of lipid peroxidation (Ranga Rao et al. 2006). Recently Sindhu et al. (2010) reported the carotenoid lutein isolated from marigold flowers (*Tagetes erecta* L.) protected the liver damage in paracetamol, carbon tetrachloride, ethanol induced hepatotoxic rats by increasing liver antioxidant enzyme activity. In another study, astaxanthin showed anti-hypertensive activity in rats (Hussein et al. (2006) which have been used as a model to study the mechanism, pathophysiology, and management of hypertension.

Conclusion

In summary, the current results revealed that ASX and ASXEs prevented hepatic damage induced by carbon tetra chloride with the enhancement of antioxidant enzyme activities. ASX and ASXEs treatments showed potent anti-hepatoprotective and anti-oxidant activity in rats when compared to SASX treated ones. As *H. pluvialis* accumulate ASX and ASXEs which has a higher degree of hepatoprotective and antioxidant activity, they can be used in various formulations and functional foods, which have high demand in the market. Therefore, ASX and ASXEs can be considered for various health applications in food, feed, pharmaceutical and nutraceutical formulations.

Acknowledgments The award of Senior Research Fellowship to ARR by the Indian Council of Medical Research (ICMR), New Delhi, is gratefully acknowledged.

Conflict of interest The authors declare that they have no conflict of interest.

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