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Antioxidant and Anticancer Potential of Astaxanthin from Micro Green Alga *Haematococcus pluvialis* during Diethyl Nitrosamine (DEN) Induced Experimental Hepatocellular Carcinogens in Wistar Rats

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Abstract

Haematococcus pluvialis is a potent organism for the production of Astaxanthin (ASX), is a high value ketocarotenoid. The natural and nutritional red carotenoid pigment is used as dietary supplements. Liver cancer is the fifth most common cancer in worldwide but because of very poor prognosis; it is the third most common cause of death among other cancers. There are currently limited therapeutic regimens available for effective treatment of this cancer. The present study was designed to evaluate the beneficial effects of dietary pigment astaxanthin, against Diethyl Nitrosamine (DEN) -induced oxidative stress and liver carcinogenesis' in Wistar rats. ASX was orally administered to experimental rats at 20 mg/kg body weight prior to cancer induction. In the present study, we investigated the anticancer effects of Astaxanthin (ASX) during DEN-induced Hepatocellular Carcinoma (HCC) in male Wistar rats. DEN treatments resulted in increased levels of Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP), Acid Phosphatase (ACP), and Lactate Dehydrogenase (LDH) in blood serum, and decrease the antioxidant enzymic parameters such as Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (Gpx), Glutathione Reductase (GR) and non-enzymic parameters Vitamin A, C, and E in liver homogenates. Pretreatment with ASX significantly attenuated these alterations and increase in antioxidant parameters and decreased the levels of AST, ALT, ALP, ACP and LDH in blood serum. Nevertheless, the experimental and control rats of liver morphological, histopathological examination and immunohistochemical expression of COX-2 and PCNA analysis are authenticated the protective effects of astaxanthin treated rats. These findings suggest that ASX is a potent antioxidant and anticancer agent against DEN induced HCC.

Keywords: *Haematococcus pluvialis*; Astaxanthin (ASX); Hepatocellular carcinoma; Antioxidants; COX-2; PCNA

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Introduction

Antioxidants are quenchers of free radicals that are responsible for inducing oxidative stress generated *via* Reactive Oxygen Species (ROS) in the body; thus they prevent several ROS induced degenerative diseases such as cancer, ulcer, diabetes, cardiovascular diseases etc [1]. HCC is a major healthcare problem worldwide because it is the 5th most common cancer with more than one million fatalities occurring annually worldwide [2]. Liver is one of the most important organs in energy metabolism. Most apolipoproteins, endogenous lipids, and lipoproteins are synthesized in the liver, which depends on the integrity of cellular functions of this organ. Under normal physiological conditions liver ensures homeostasis of lipid and lipoprotein metabolism. HCC impairs this process leading to alterations in the lipid and lipoprotein patterns [3]. It is less common in most parts of the developed western world but appears to be increasing substantially in incidence and its incidence is very high in Asian countries [4]. These highly variable geographical distributions of HCC indicate that environmental factors are of major causative importance [5]. The reason for relatively high

incidence in Asian countries is due to the widespread contamination of foods with mycotoxins, moderately high prevalence of Hepatitis B virus, Hepatitis C virus-related chronic liver disease, which are considered as the most important risk factors for the development of HCC. The mortality rate remains high from HCC despite treatment with recent results showing 1-year, 3- years and 5-years survival rates of 66.1%, 39.7%, and 32.5%, respectively [4].

DEN a hepatocarcinogen, is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication and is normally used as a carcinogen to induce liver cancer in animal models [6]. The presence of nitroso compounds and their precursors in human environment together with the possibility of their endogenous formation in human body have led to suggestions of their potential involvement in human cancers [7]. The main cause for concern is that DEN is found in a variety of foods like cheese; soybean; smoked, salted, and dried fish; cured meat; and alcoholic beverages [8]. It is also found in tobacco smoke at a concentration ranging from 1 ng/cigarette to 28 ng/cigarette and in baby bottle nipples at a level of 10 ppb [9].

The micro alga, *Haematococcus pluvialis* (chlorophyte) is one of the richest sources of astaxanthin accumulating up to 2% to 3% of dry weight and constitutes ~85% to 88% of total carotenoid [10]. As attributed to carotenoid and closely associated to *β*-carotene, lutein, and zeaxanthin, astaxanthin shares with them many of the general metabolic and physiological activities. On the other hand, ASX has unique chemical properties based on its molecular structure. The presence of the Hydroxyl (OH) and keto (C=O) moieties on each ionone ring explains some of its unique features, namely, a higher antioxidant activity. ASX (3,3'-dihydroxy- β,β-carotene-4,4'-dione) has been documented to provide important metabolic functions in animals, including conversion to Vitamin A [11], enhancement of immune response [12], and protection against diseases such as cancers by scavenging oxygen radicals [13]. The antioxidant activity of ASX has been reported to approximately 10 times stronger than that of other carotenoids tested [14]. ASX also shows strong activity as an inhibitor of oxygen radical-mediated LPO [15].

In recent years, a number of studies on astaxanthin have *in vitro* and *in vivo* demonstrated its antioxidant effect, for example, the quenching effect on singlet oxygen, a strong scavenging effect on superoxide, hydrogen peroxide, and hydroxyl radicals and an inhibitory effect on lipid peroxidation [16,17]. In addition to these, several other biologic activities of ASX, including anti-cancer, anti-inflammatory, antidiabetic, immunomodulatory activities and a neuroprotective effect, also have been reported [18]. From the above reports Astaxanthin is known to be a powerful antioxidant, although these facts suggested that ASX might be a potent candidate for a natural hepatoprotective agent, further basic evidence to demonstrate the hepatoprotective effect of ASX is needed.

Cyclooxygenases also known as prostaglandin H2 syntheses are the rate-limiting enzymes involved in the conversion of arachidonic acid into Prostaglandins (PGs) [19]. Two isoforms of COX exist, with diverse tissue distributions. COX-1 is constitutively expressed in many tissues and cell types, whereas COX-2 is an inducible isoform that participates in pro-inflammatory responses in response to certain stimuli such as mitogens, cytokines and growth factors [20-22]. Upregulation of COX-2 has also been implicated in cancer development and growth. Recent reports on COX-2 expression in cancers show that this enzyme stimulates angiogenesis and is associated with tumor growth, invasion, and metastasis [20,23].

Proliferating Cell Nuclear Antigen (PCNA) is a 36-kDa protein functions as a cofactor of DNA-polymerase and an important marker for evaluating the proliferation of several cancers including HCC [24-26]. PCNA is synthesized during the late G1-early S phase of the cell cycle, immediately preceding the onset of DNA synthesis, is most abundant during the S phase, and declines during the G2/M phase [27,28]. X-ray crystallography has shown that PCNA molecules form a trimeric ring around DNA [29]. The detection of PCNA using immunohistochemical methods is a common way to study the proliferating activity of transformed cells. Reducing cellular proliferation was one of the hallmarks of controlling the carcinogenic process and higher expression of PCNA protein was closely related to increased proliferation because it plays an essential role in nucleic acid metabolism as a component of the replication [30]. The present study was conduct to delineate the role of ASX on the expression of COX-2 and PCNA during DEN-induced hepatocarcinogenesis in Wistar rats. To our understanding, no reports have been documented on the same, by morphometric evaluation of the liver along with histological observation and immunohistochemical analysis.

Materials and Methods

Algal culture

Haematococcus pluvialis Flotow was obtained from the culture collection of Algae, Centre for Advanced Studies in Botany, University of Madras, Chennai, India. The culture was maintained in Bold Basal Medium (BBM) pH – 6.8, temp 23°C-25°C and 12/12 h light and dark conditions (Figure 1).

Chemicals and reagents

Diethyl Nitrosamine (DEN) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Separation of carotenoids by Thin Layer Chromatography (TLC)

The *H. pluvialis* extract were analyzed by using TLC aluminum sheets (10 cm × 10 cm) pre-coated with silica gel 60 (Merck Ltd, New Delhi). *H. pluvialis* extract was spotted on TLC sheet and developed using solvent system (acetone: n hexane, 3:7) [31]. The developed allowed to dry at room temperature and carotenoids were identified by comparing with plates were identified by comparing with authentic astaxanthin.

Estimation of carotenoids

The absorbance of the extracts was read at 476 nm using a Milton Roy UV-Spectrophotometer. Total carotenoids contents were calculated using [32]. Astaxanthin was analyzed by the method of Davies [33]. Extractability of astaxanthin was calculated for all samples as per the procedure of Kobayashi et al. [34] (Figure 2).

Analysis of carotenoids by HPLC

Carotenoid extracts were subjected to HPLC (JASCO HPLC 1500 Series) analysis using Lichrospher C18 100 RP (25.0 cm × 4.6 mm × 5 μm)) column, PU 1580 Pump, MD 1515 PDA detector. The following solvents were used at a flow rate of 1.25 mL min^{-1}: (A) acetone and (B) methanol: H_2O (9:1 v/v). The separation of carotenoids 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. The separated carotenoids and astaxanthin esters were identified using a photodiode array detector [35]. The peaks were integrated at 476 nm to quantify free astaxanthin and astaxanthin esters Figure 3, 4.

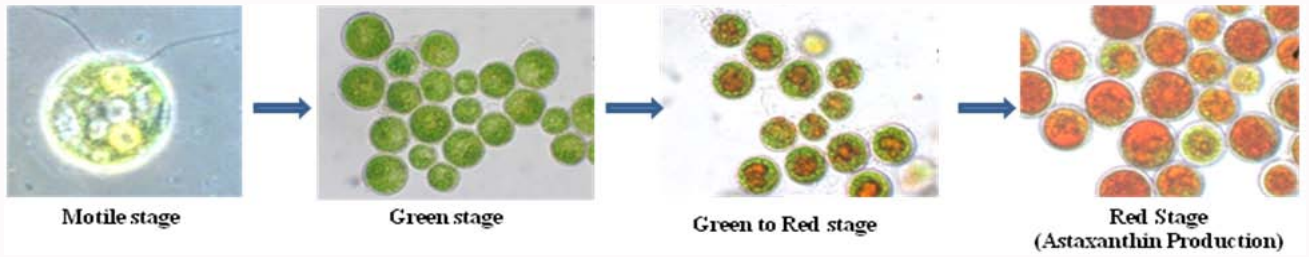

Figure 1: *Haematococcus pluvialis* life cycle.

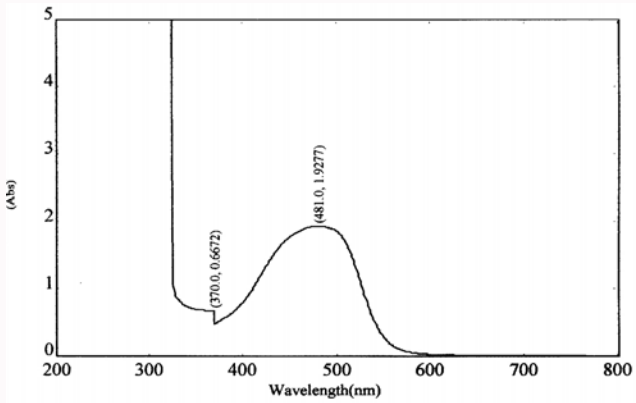

Figure 2: Adsorption spectrum of Astaxanthin from *Haematococcus pluvialis*.

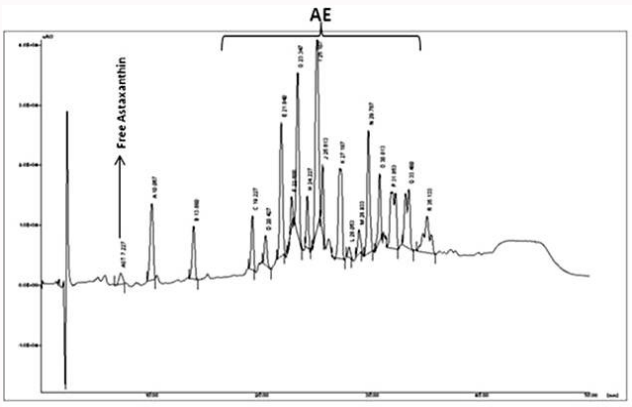

Figure 3: HPLC analysis of Astaxanthin from *Haematococcus pluvialis*.

Animals

Male, Wistar strain of albino rats weighing about 185 g to 220 g were procured from Tamilnadu Veterinary & Animal Science University (TANUVAS), Madhavaram, Chennai, India. The animals were housed in cages under proper environmental conditions and were fed with a commercial pelleted diet (M/s Hindustan Foods Ltd., Bangalore, India). The animals had free access to water. All the experiments were designed and conducted according to the ethical norms approved by Institutional Animal Ethics Committee guidelines, India (IAEC No. 03/017/09).

Experimental design

Initially an effective dosage fixation studies was conducted with Astaxanthin dissolved in saline, at five different doses (5, 10, 15, 20 and 25 mg/kg body weight) to determine the optimum dosage. It was observed that Astaxanthin (ASX) at the dose of 20 mg/kg body weight

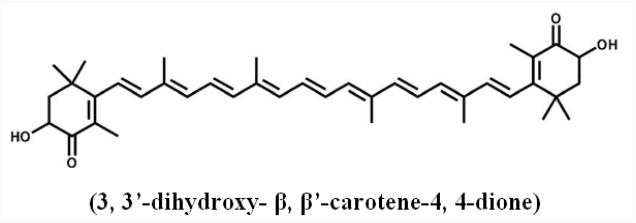

Figure 4: Chemical Structure of Astaxanthin (C_{42} H_{54} O_4).

significantly (P<0.05) altered the activities of marker enzymes such as acid phosphatase, alkaline phosphatase, aspartate transaminase, alanine transaminase, LDH in liver tissues and the activities of enzymic and non-enzymic antioxidant parameters in the liver to near normal values in DEN-induced rats during the experimental study. Hence, the dose of 20 mg/kg was chosen for the further study.

The experimental animals were divided into five groups, each group comprising six animals shown in Table 1.

After the experimental period the rats were anesthetized followed by cervical decapitation.

Biochemical studies

After cervical decapitation, the blood samples were collected from the experimental animals and liver tissue was removed and washed in ice-cold saline. The total protein in the serum was estimated by the method of Lowry, et al. [37].

Determination of liver marker enzymes

The activities of liver marker enzymes Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were assayed by the method of [38], Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) by the method of [39], Lactate Dehydrogenase (LDH) by the method of [40], were estimated in the blood serum of experimental animals.

Determination of oxidant and antioxidant activity

Lipid peroxidation was determined in the liver tissue and plasma by measuring the formation of Thiobarbituric Acid Reactive Substances (TBARS) according to the method of [41]. Hydroperoxides were estimated both in plasma and in tissue homogenate by the method described by [3].

The activities of enzymic antioxidants Superoxide Dismutase (SOD) [42], Catalase (CAT) [43], Glutathione Peroxidase (GPx) [44] and Glutathione Reductase (GR) [45] were assayed in the liver tissue homogenates. The non-enzymic antioxidant Vitamin A was determined by the method of Bayfield and Cole. [46]; Vitamin C [47] and Vitamin E [48] were assayed in liver tissue homogenates.

Table 1: The experimental animals were divided into five groups, each group comprising six animals.

Group I	Normal control rats fed with standard diet for 16 weeks.
Group II	Rats were induced with hepatocellular carcinoma by providing 0.01% DEN through drinking water for 15 weeks [36].
Group III	Rats pre-treated with 20 mg/kg body weight 1 week before the administration of 0.01% DEN and continued along with carcinogen till the end of the experiment (i.e. 16 weeks).
Group IV	Rats post-treated with 20 mg/kg body weight for 5 weeks, i.e. after the administration of DEN for 10 weeks, Astaxanthin was supplemented with diet for 5 weeks along with the carcinogen and continued till the end of experiment.
Group V	Rats were treated with Astaxanthin alone by oral gavage daily at a dose of 20 mg/kg body weight (based on effective dosage fixation studies) for 16 weeks

Table 2: Effect of astaxanthin on body weights of rats subjected to DEN.

Experimental groups	Initial body weight (g)	Final body weight (g)	Body weight gain (%)
Control	198.25 ± 0.37	246.84 ± 4.3	57.43 ± 0.29
DEN	205.10 ± 0.28[a]	240.08 ± 3.7[a]	35.85 ± 0.21[a]
DEN+Astaxanthin (Pre-initiation)	200.47 ± 0.42[b]	287.88 ± 4.9[b]	80.35 ± 0.31[b]
DEN+Astaxanthin (Post-initiation)	196.55 ± 0.41[c]	274.45 ± 14.03[c]	68.85 ± 0.3[c]
Astaxanthin (alone)	210.28 ± 0.38	304.1 ± 4.49	94.45 ± 0.31

DEN: Diethylnitrosamine

Values are given as mean ± SD for groups of six rats each. Values are given statistically significant at P<0.05. Values not sharing a common superscript letter (a–d) differ significantly

Histopathological studies

Liver tissue samples were fixed in 10% buffered formalin for 24 h. The processed tissues were embedded in paraffin blocks and sections made were stained with hematoxylin and eosin dye [49]. The sections were analyzed by observing under light microscope (Leitz, Germany) at 10x magnification.

Immunohistochemistry

Immunohistochemical staining was carried out following the method of Ramakrishnan et al. [50]. The tissue sections were deparaffinized in two changes of xylene at 60°C for 20 min each and hydrated through a graded series of alcohol, the slides were incubated in a citrate buffer (pH 6.0) for three cycles of 5 min each in a microwave oven for antigen retrieval. The sections were then allowed to cool to room temperature and then rinsed with 1x Tris-Buffered Saline (TBS), and treated with 0.3% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity. Nonspecific binding was blocked with 3% Bovine Serum Albumin (BSA) at room temperature for 1 h. The sections were then incubated with primary antibodies (COX-2 & PCNA goat polyclonal & rabbit polyclonal antibody) at a dilution of 1:500, overnight at 4°C. After incubation, the liver tissue section were rinsed with 1X TBS containing 0.05% Tween 20 twice and incubated with secondary antibody, (anti-goat & goat anti-rabbit HRP conjugate) at a dilution of 1:2000, for 1 h at 4°C. The peroxidase activity was visualized by treating the slides with 3,3'-diaminobenzidine tetrahydrochloride (SRL, Mumbai, India), the slides were counterstained with Meyer's hematoxylin. Negative controls were incubated with TBS instead of primary antibodies. Quantitative analysis was made in a blinded manner under bright field in Carl Zeiss Axiostar plus microscope. Each section was examined at high magnification (40x) and number of COX-2 and PCNA positive cells per 100 tumor cells was reported.

Data analysis

All the data were evaluated with SPSS/10 software. Hypothesis testing methods included one way Analysis of Variance (ANOVA) followed by Least Significant Difference (LSD) test. p<0.05 was considered to indicate statistical significance. All these results were expressed as mean SD for six animals in each group.

Results

The effect of DEN and ASX on change in body weight and percentage of body weight gain is shown in Table 2. Induction of DEN to rats (Group II) resulted in loss of body weight, which also exhibited decreased percentage of body weight gain when compared with the (Group I) control rats. Administration of ASX to DEN-induced rats (Groups III & IV) showed significant increase in body weight when compared with DEN-induced rats with more pronounced percentage of body weight gain observed in ASX pre-treated rats (Group III). Body weight changes in ASX (Group V) treated rats were monitored and compared with the control to determine the toxic effects, if any, of ASX during the treatment period. No noticeable changes were observed between the groups that prove the non-toxic nature of ASX.

Astaxanthin decreased the levels of marker enzymes in serum of DEN-induced animals

Figures 5-7 shows the activity of marker enzymes AST, ALT,

Table 3: Levels of Thiobarbituric Acid Reactive Substances (TBARS) and Hydroperoxides in Plasma and liver of control and experimental group of rats.

Experimental groups	Liver		Plasma	
	TBARS (mmol/100 g tissue)	Hydroperoxides (mmol/100 g tissue)	TBARS (nmol/ml)	Hydroperoxides (10-5 mmol/dl)
Control	35.67 ± 2.85[a]	11.52 ± 0.94[a]	4.36 ± 0.26[a]	11.73 ± 0.72[a]
DEN	63.8 ± 6.21[b]	23.36 ± 2.28[b]	8.50 ± 0.65[b]	19.8 ± 1.54[b]
DEN+Astaxanthin (Pre-initiation)	42.39 ± 3.68[c]	14.67 ± 1.29[c]	4.92 ± 0.32[c]	13.25 ± 0.90[c]
DEN+Astaxanthin (Post-initiation)	46.35 ± 3.7[d]	18.59 ± 1.5[d]	5.65 ± 0.33[d]	15.24 ± 1.21[d]
Astaxanthin (alone)	36.21 ± 2.93[a]	12.74 ± 1.04[a]	3.82 ± 0.23[a]	11.02 ± 0.69[a]

DEN: Diethylnitrosamine; TBARS: Thiobarbituric Acid Reactive Substances

Values are given as mean ± SD for groups of six rats each. Values are given statistically significant at P<0.05. Values not sharing a common superscript letter (a–d) differ significantly

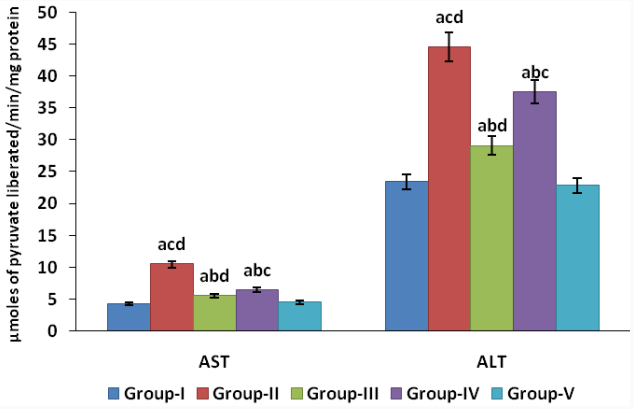

Figure 5: The effect of Astaxanthin on the levels of serum aminotransferase activity (AST, ALT) during DEN-induced hepatocarcinogenesis. Results are expressed as mean ± SD, (n=6), P<0.05, compared with [a]Group I, [b]Group II, cGroup III, and [d]Group IV. Units are expressed as µmoles of private liberated per minute per mg protein for both AST and ALT.

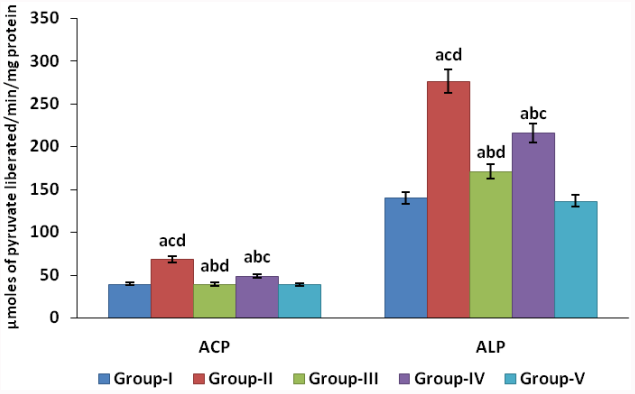

Figure 6: The effect of astaxanthin on the levels of phosphatases activity (ACP, ALP) in control and experimental animals. Results are expressed as mean ± SD, (n=6), P>0.05, compared with [a]Group I, [b]Group II, [c]Group [III], and [d]Group IV. Units are expressed as µmoles of phenol liberated per minute per mg protein for both ACP and ALP.

ACP, ALP and LDH in the serum of control and experimental groups of animals. DEN-induced Group II animals exhibited a significant elevation in the activity of these marker enzymes when compared with Group I normal control animals. ASX treated animals from Groups III and IV showed a significant decrease in the levels of these enzymes when compared with Group II animals. It can be seen that DEN administered animals (Group II) showed marked increase in the levels of liver marker enzymes when compared with control animals. However, on ASX treatment on Groups III and IV, there was a reversal in these values toward normal control.

In vivo antioxidant activity of Astaxanthin

Table 3 shows the levels of TBARS and hydroperoxides in the liver and plasma of control and experimental groups of rats. Significantly elevated levels of TBARS and hydroperoxides were observed in DEN-induced rats (Group II) as compared with the control (Group I) and ASX treated animals (Group V). DEN-induced with ASX treated (Group III& IV) rats significantly lowered the liver and plasma levels of TBARS and hydroperoxides as compared with DEN-induced rats (Group II), with more pronounced effect observed in ASX pre-initiation group (Group III).

The activities of enzymic antioxidants in liver homogenates

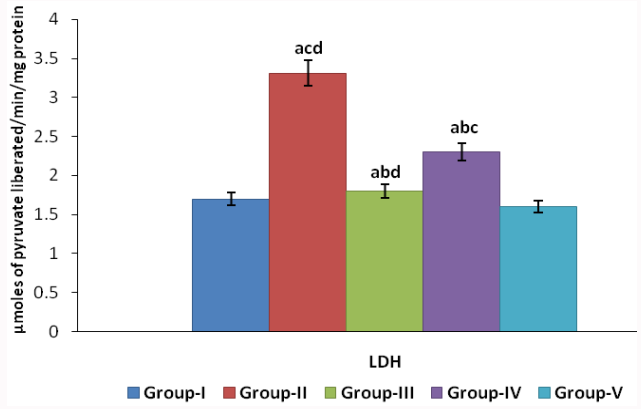

Figure 7: Effect of Astaxanthin on the specific activity of serum LDH during hepatocarcinogenesis. Results are expressed as mean ± SD, (n=6), P<0.05, compared with [a]Group I, [b]Group II, [c]Group III, and [d]Group IV. Units are expressed as µmoles of private liberated per minute per mg protein.

of control and experimental group of animals are shown in Table 4. A significant descends in the activities of SOD, CAT, GPx and GR was evident in DEN-induced rats (Group II) when compared with the control (Group I) and ASX treated rats (Group V). ASX administration to DEN-induced rats significantly increased the activities of these enzymes as compared with DEN-induced Group II; with ASX pre-initiation group (Group III) exhibited more prominent activities of these enzymic antioxidants as compared with post-initiation group (Group IV).

The levels of Vitamin A, C and E in liver tissue homogenates of control and experimental group of rats are shown in Table 5. A significant decrease in the levels of Vitamin A, C and E was evident in DEN induced rats (Group II) when compared with control (Group I) and ASX treated rats (Group V). ASX administration to DEN-induced rats significantly increased the levels of these antioxidants as compared with DEN-induced Group II, with ASX pre-initiation (Group III) exhibited more pronounced effect when compared with post-initiation (Group IV).

Histopathological analysis

Animals treated with ASX (20 mg/kg b. wt.) against diethylnitrosamine were selected for the microscopical assessment (histopathological changes) on the basis of the observed biochemical and cytogenetic results. Figure 8 show the hematoxylin eosin stained liver section of the control and experimental rats. Control animals showed normal architecture of the liver. DEN alone administered animals had shown diffuse hepatocytic damage areas of necrosis, congestion, cells with pyknotic nuclei (Figure 8). Significantly reduced morphological signs of cell damage and inflammation of liver were evident in ASX pre-initiation group (Figure 2e) when compared with post-initiation group (Figure 2f). Not much deviation in the architecture of the liver section of ASX alone served animals was noted in comparison to the control animals.

Astaxanthin down regulated the protein level expressions of COX-2 and PCNA in rat liver carcinogenesis

Immunohistochemical analysis of COX-2 and PCNA in liver of control and experimental group of animals. Figure 9 shows the levels of COX-2 in the liver of control and experimental animals. Tumor-induced Group II animals showed a significant increase in the number of the COX-2 expression when compared with Group

Table 4: Effect of Astaxanthin on the concentration of enzymatic-antioxidant parameters.

Experimental groups	SOD	CAT	GPx	GR
Control	9.25 ± 0.37	53.84 ± 4.3	7.43 ± 0.29	141.4 ± 11.31
DEN	5.15 ± 0.28[a]	34.08 ± 3.7[a]	3.85 ± 0.21[a]	112.52 ± 13.5[a]
DEN+Astaxanthin (Pre-initiation)	8.47 ± 0.42[b]	49.88 ± 4.9[b]	6.35 ± 0.31[b]	129.3 ± 12.93[b]
DEN+Astaxanthin (Post-initiation)	7.55 ± 0.41[c]	42.45 ± 14.03[c]	5.85 ± 0.3[c]	111.32 ± 10.57[c]
Astaxanthin (alone)	9.28 ± 0.38	54.1 ± 4.49	7.45 ± 0.31	142.25 ± 11.66

DEN: Diethylnitrosamine

Effect of Astaxanthin on the concentration of low molecular weight antioxidants are mean ± S.D. for six animals for each group. The symbols 'a-c' represent significance at P<0.05, where a = comparison with Group I and b = comparison with Group II

Table 5: Effect of Astaxanthin on the concentration of non – enzymic antioxidant parameters.

Experimental groups	Vitamin A	Vitamin C	Vitamin E
Control	5.04 ± 0.20	3.74 ± 0.14	1.85 ± 0.07
DEN	2.95 ± 0.15[a]	1.37 ± 0.07[a]	0.98 ± 0.05[a]
DEN+Astaxanthin (Pre-initiation)	4.26 ± 0.21[b]	3.15 ± 0.151[b]	1.78 ± 0.08[b]
DEN+Astaxanthin (Post-initiation)	4.01 ± 0.2[c]	2.98 ± 1.5[c]	1.15 ± 0.05[c]
Astaxanthin (alone)	5.25 ± 0.22	3.98 ± 0.15	1.87 ± 0.07

DEN: Diethylnitrosamine

Effect of Astaxanthin on the concentration of low molecular weight antioxidants are mean ± S.D. for six animals for each group. The symbols 'a' and 'b' represent significance at P<0.05, where a = comparison with Group I and b = comparison with Group II

I normal control animals. ASX treated Groups III and IV showed a significant decrease in the level of COX-2 when compared with tumor bearing animals of Group II.

Figure 10 shows immunohistochemical staining of PCNA in the liver of control and experimental group of animals. DEN-induced Group II showed a significant increase in the number of PCNA positive cells when compared with Group I normal control animals, while ASX treated Group III and IV significantly decreased the number of PCNA positive cells when compared with DEN-induced animals.

Discussion

The current study addressed the identification of a potent antioxidant ASX from *H. pluvialis* and determined its antioxidant and anticancer potencies at *in vivo* models. Liver is one of the most important organs in energy metabolism. Most apolipoproteins, endogenous lipids, and lipoproteins are synthesized in the liver, which depends on the integrity of cellular functions of this organ. Under normal physiological conditions liver ensures homeostasis of lipid and lipoprotein metabolism. HCC impairs this process leading to alterations in the lipid and lipoprotein patterns [3].

Hepato specific enzymes were activated when hepatocellular damage give rise to abnormalities of liver function and these enzymes are remarkably increased in HCC [36]. One of the most sensitive and dramatic indicators of hepatocyte injury is the release of intracellular enzymes, such as transaminases, phosphates, and LDH in the circulation after DEN administration. The measurement of phosphatase activity is useful as an indicator of liver function [51]. In the liver, it is closely connected with lipid membrane in the canalicular zone, so that any interference with the bile flow, whether extra-hepatic or intra-hepatic leads to increased serum levels of ACP and ALP activities. Aminotransferases (AST and ALT) are reliable marker enzymes of liver and they are the first enzymes to be used in diagnostic enzymology when liver damage has occurred [52]. Because of their intracellular location in the cytosol, toxicity affecting the liver with subsequent breakdown in membrane architecture of the cells leads to their spillage into serum, and their concentration rises in the latter. LDH release into the serum reflects a nonspecific alteration in the plasma membrane integrity and permeability or may be due to its overproduction by tumor cells. LDH is a fairly sensitive marker of solid neoplasm [53] and many studies revealed increased LDH activity in various types of tumor [36,54,55]. In the present study the levels of marker enzymes were increase in serum was observed, when administration of DEN and treatment with ASX showed a significant reduction in the levels of marker enzymes in serum.

Several studies have demonstrated the antioxidant potential of carotenoids that can significantly reduce free radicals and the oxidative load to help the body maintain a healthy state. Studies have shown that *in vivo* supplementation with exogenous astaxanthin is capable of suppressing the formation of byproducts (e.g., TBARS) of membrane LPO induced by superoxide anion. Studies in the past have attributed that the LPO attenuating effect of ASX to its molecular structure [56]. The above reports were substantiated by our study as administration of ASX to DEN-induced rats significantly reduced the levels of TBARS and hydroperoxides proving its potential antioxidant ability. The enzymatic antioxidants such as SOD, CAT, GPx and GR limit the effects of oxidant molecules on tissues and are activated against oxidative cell injury [57]. These enzymes work synergistically to eliminate active oxygen species and small deviations in physiological concentrations may have a dramatic effect on the resistance of cellular macro molecules and DNA to oxidative damage [58]. The decreased activities of these enzymic antioxidants observed in DEN-induced rats might be due to increased oxidative stress as enhanced levels of LPO along with the reduced activities of enzymic antioxidants has been observed during DEN-induced malignant alterations [56]. In this study, ASX administration prevented DEN-induced changes in enzymatic antioxidant enzymes and this could be attributed to its high scavenger potential, which would protect the tissues against free radicals generated by DEN, sparing antioxidant defenses [59,60].

The non-enzymatic antioxidant parameters Vitamins A, C, and E prevent its oxidation. Vitamin A is involved in epithelial cell

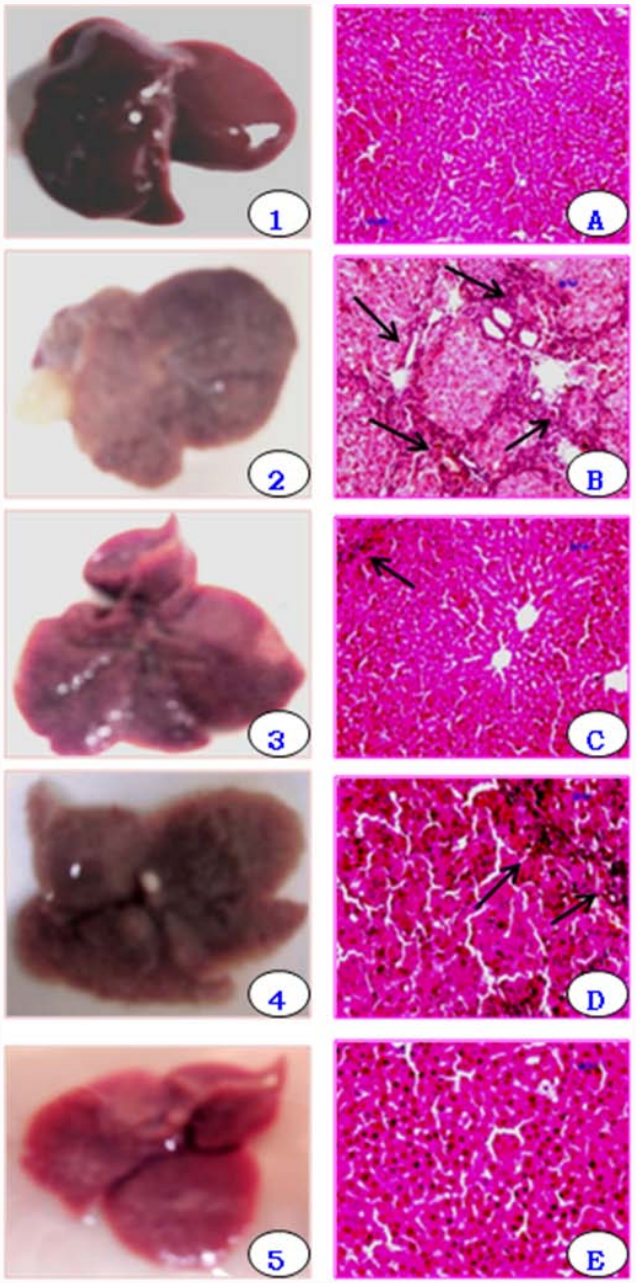

Figure 8: (I) Macroscopic appearance of the livers from control and experimental group of animals (1-5); (II) Histopathological alterations in liver of control and experimental groups of rats. (A) Control, (B) Diethylnitrosamine (DEN)-induced, (C) DEN + astaxanthin (pre-initiation), (D) DEN + astaxanthin (post-initiation). (E) Astaxanthin alone.

differentiation and is known to inhibit carcinogenesis, by preventing biological oxidative damage. Vitamin A at pharmacological doses is reported to alleviate oxidative stress mediated membrane LPO and membranes enriched with vitamin A are protected against oxidative stress *in vivo* and exhibit resistance to LPO induced *in vitro* [61]. Vitamin C scavenges ROS generated during the metabolism of carcinogen and thus possibly protects the genetic material at the initiation and promotion stages of carcinogenesis [62]. Vitamin E scavenges free radicals to prevent LPO of polyunsaturated fatty acids, which can act as promoters of carcinogenesis [63]. Therefore, the decreased concentrations of Vitamins A, C and E in DEN administered animals may be due to increased utilization of these antioxidants to

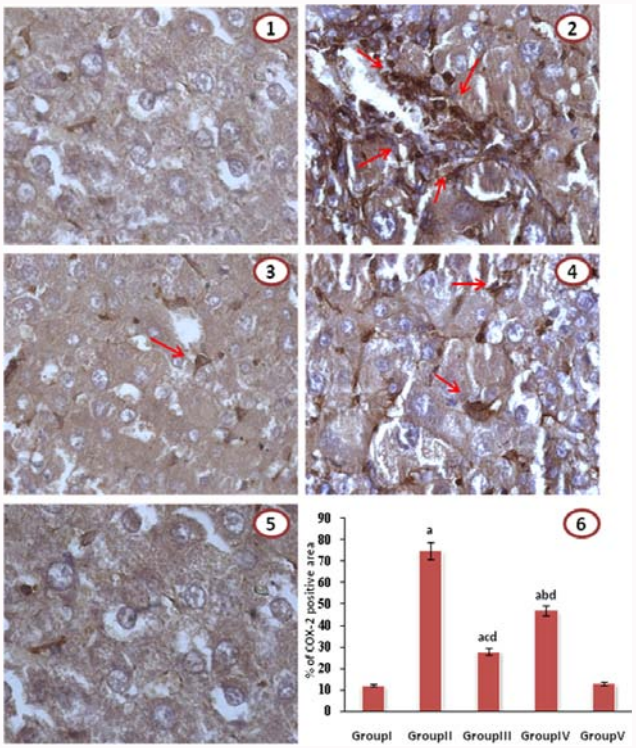

Figure 9: Immunohistochemistry.
Plate-I: Representative Immunohistochemical staining of COX-2 in the liver of control and experimental animals at 40x of original magnification. Plates 1–5 represent the liver sections of Groups I–V of experimental animals, respectively. Plate 6 represents quantification of immunostained section of COX-2. Each section was examined at high magnification and the ratio of positive area to hepatic tissue area was calculated. The result was regarded as the mean of five different fields on each section. Results are expressed as mean ± SD. (n=6) P<0.05 compared with [a]Group I, [b]Group II, [c]Group III, [d]Group IV; → shows immunostained areas.

counter LPO. Administration of ASX preserved the normal levels of these non-enzymic antioxidants in DEN-induced rats, which might be due to its antioxidant property as ASX has been reported to suppress production of free radicals and subsequent oxidative stress [64]. The histopathological observations clearly showed that ASX administration at pre-initiation stage greatly influences liver carcinogenesis by altering the efficacy at which DEN can initiate histological changes. Well differentiated signs of tissue injury and tumors were observed in liver tissue sections due to DEN induction that was restored to normalcy in ASX pre-treated rats. The ability of ASX to restore the histological changes induced by DEN indicates the anti-carcinogenic potential of this carotenoid. A previous report suggested that elevated LPO induced certain histological alteration in liver tissues of DEN-induced rats. ASX ameliorated the histopathological alterations in DEN-induced rats probably by extenuating the levels of LPO. Thus, the histological findings clearly support the biochemical data and suggest that ASX may play a promising anticancer role with respect to liver carcinogenesis.

ASX treatment significantly attenuated these alterations and decreased the levels of COX-2 and PCNA. Cell proliferation is thought to play an important role in several steps of the carcinogenic process. PCNA functions as a cofactor of DNA-polymerase and an important marker for evaluating the proliferation of several cancers including HCC [24,25]. The detection of PCNA using immunohistochemical methods is a common way to study the proliferating activity of

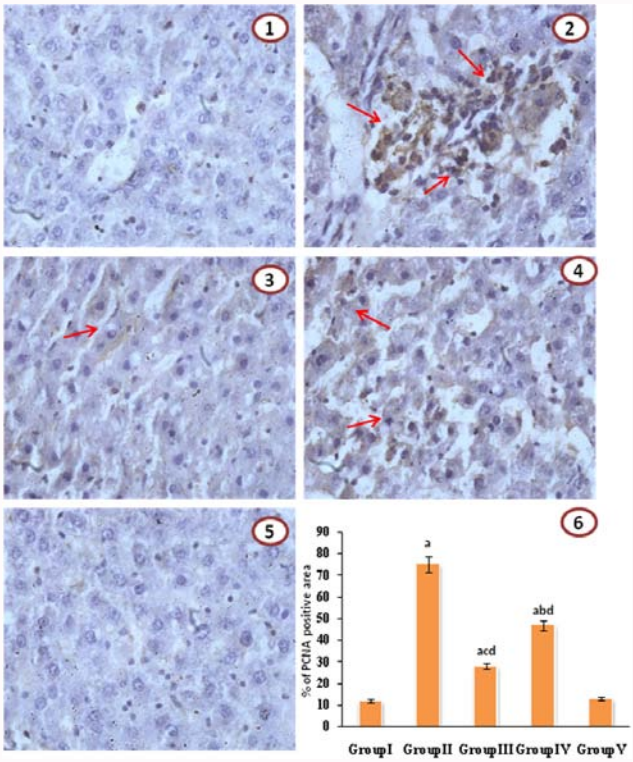

Figure 10: Immunohistochemistry.
Plate-II: Representative immunohistochemical staining of PCNA in the liver of control and experimental animals at 40x of original magnification. Plates 1–5 represent the liver sections of Groups I–V of experimental animals, respectively. Plate 6 represents quantification of immunostained section of PCNA. Each section was examined at high magnification and the ratio of positive area to hepatic tissue area was calculated. The result was regarded as the mean of five different fields on each section. Results are expressed as mean ± SD. (n=6) P<0.05 compared with [a]Group I, [b]Group II, [c]Group III, [d]Group IV; → shows immunostained areas.

transformed cells. Elevated expression of PCNA in the liver of DEN-administered animals indicates the hyper proliferative activity of tumor cells. Reducing cellular proliferation was one of the hallmarks of controlling the carcinogenic process and higher expression of PCNA protein was closely related to increased proliferation because it plays an essential role in nucleic acid metabolism as a component of the replication [30]. ASX administration resulted in decreased expression of this proliferative marker, which clearly revealed its antiproliferative activity in HCC.

COX2 is a key enzyme in arachidonic acid metabolism, is consistently absent in normal tissue, and is increased in the tumor. Increased expression of COX-2 in HCC was observed by several investigators [21,22,26,65]. In the present study, increased expression of COX-2 was observed in the liver of Group II hepatoma-bearing animals. It is possible that the increased levels of COX-2 serve to lower the intracellular levels of free arachidonic acid, thereby preventing carcinogenesis. ASX treatment significantly inhibited the expression of COX-2, thereby maintaining increased levels of free arachidonic acid in the cells, which might have resulted in the suppression of carcinogenesis.

In conclusion, we found that the ASX has exhibited potent antioxidant, immunomodulating and enzyme inducing properties, all of which suggest a potential role for this carotenoid in the prevention of cancer. Moreover, its unique structural properties and its lack of pro-oxidant activity make it a prime aspirant for further investigation in this area of human health. More research is needed on the absorption and metabolism of this promising anticancer agent in humans, and on its interactions with other carotenoids and Vitamins in the human system.

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