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Ulcer preventive and antioxidative properties of astaxanthin from *Haematococcus pluvialis*

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ABSTRACT

The anti-ulcer properties of astaxanthin fractions such as total carotenoid and astaxanthin esters from *Haematococcus pluvialis* were evaluated in ethanol-induced gastric ulcers in rats. Since oxygen radical release is a pathogenic factor of ethanol-induced gastric damage, astaxanthin — a free radical scavenger, was investigated as a potential ulcer preventive agent. Astaxanthin fractions — total carotenoid and astaxanthin esters were orally administered to experimental rats at 100, 250 and 500 μg/kg b.w. prior to ulcer induction. Alcian blue binding assay indicates that, total carotenoid and astaxanthin esters at 500 μg/kg b.w could protect gastric mucin ~40% and 67% respectively. Pre-treatment with astaxanthin esters, also resulted in significant increase in antioxidant enzyme levels — catalase, superoxide dismutase, and glutathione peroxidase in stomach homogenate. Histopathological examination substantiated the protective effect of astaxanthin in pre-treated rats. The increased antioxidant potencies such as free radical scavenging activity with an IC_{50} of ~8 μg/ml and reducing power abilities (59×10^3 U/g) *in vitro*, reveal that *H. pluvialis* astaxanthin may protect gastric mucosal injury by antioxidative mechanism. In addition, ~23 fold increased lipoxygenase-inhibitory property, in comparison with standard astaxanthin and significant H^+, K^+-ATPase-inhibitory activity of astaxanthin esters, in comparison with known proton pump blocking anti-ulcer drug — omeprazole, may envisage the potential gastroprotective effect by regulating the gastric mucosal injury and gastric acid secretion by the gastric cell during ulcer disease.

1. Introduction

Antioxidants are quenchers of free radicals that are responsible for inducing oxidative stress generated via reactive oxygen species in the body; thus they prevent several reactive oxygen species-induced degenerative diseases such as cancer, ulcer, diabetes, cardiovascular diseases etc. (Ariga, 2004). Now a days, gastric hyperacidity and gastric ulcers are reported to be the most common pathological conditions resulting from uncontrolled acid secretion and pepsin activity (Kaviani et al., 2003). The imbalance between acid secretion within the lumen and mucosal protection by gastric mucin impairs contractibility of the gastric wall and leads to gastric ulcers (Sachs et al., 1995; Das et al., 1997; Rastogi et al., 1998; Galunska et al., 2002; Lai et al., 2003). The continuous use of non-steroidal anti-inflammatory drugs by the global population, stressful lifestyle and inadequate intake of nutritious foods/nutraceuticals are adding to the increased incidence of ulcers worldwide (Miller, 1987; Langman et al., 1991). Commercially available drugs for treatment of this disease, when used on long term basis, are known to cause unpredictable side effects (Debashis et al., 2002) and this warranted identification of safer alternative sources for ulcer management.

Previously we have shown that various water-soluble antioxidants in their free and bound form offer potent anti-ulcer effect (Siddaraju and Dharmesh, 2007). Besides, we could also show that a micro alga, *Haematococcus pluvialis* (chlorophyte) is one of the richest sources of astaxanthin accumulating up to 2–3% of dry weight and constitutes ~85–88% of total carotenoid (Tripathi et al., 1999). Chemically astaxanthin is a ketocarotenoid (3,3′-dihydroxy-β,β-carotene-4,4′-dione) and is the principal pigment of salmonoids and shrimps, various birds and many other organisms (Shahidi et al., 1998). Sensitive fluorimetric assay procedures adopted by Naguib (2000) reveal that astaxanthin has a higher antioxidant activity than lutein, lycopene, ∝/β-carotene, and ∝-tocopherol. Astaxanthin has 100 times and 10 times greater antioxidant activity than vitamin E and β-carotene, respectively, as indicated by investigators from various laboratories (Kurashige et al., 1990; Miki, 1991; Lorenz and Cysewski, 2000). Generally, most astaxanthin-producing organisms, including *H. pluvialis*, synthesize the 3*S*, 3′*S*-isomer. Synthetic astaxanthin resembles the natural astaxanthin molecule produced in living organisms and it consists of a mixture of 1:2:1 of isomers — 3*S*, 3′*S*, 3*R*, 3′*S* and 3*R*, 3′*R* respectively (Higuera-Ciapara et al., 2006). However, it is unique for *H.*

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pluvialis that astaxanthin occurs in three different forms that can be classified as free (~5%), monoesters (~70%) and diesters (~25%).

Utility of astaxanthin as pigmentation source in farmed salmon, trout and poultry is well documented (Lorenz and Cysewski, 2000). The use of astaxanthin as a nutraceutical and a medicinal ingredient against degenerative disease such as cancer, for prevention of age-related macular degeneration, inflammation, *Helicobacter pylori* infection (Wang et al., 2000) and for enhancement of immune responses have been attributed to its antioxidant properties (Jyonouchi et al., 2000; Kang et al., 2001). Mammals lack the ability to synthesize astaxanthin or to convert dietary astaxanthin into vitamin A.

In the current study, we investigate the antioxidant and anti-ulcer potency of esterified astaxanthin (~95%), saponified astaxanthin and total carotenoid from *H. pluvialis*. The results have highlighted the increased potency of esterified astaxanthin fraction.

2. Materials and methods

2.1. Algal strain

Green alga *H. pluvialis* (SAG 19-a) was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Germany. The algal culture was maintained and cultivated as described by Tripathi et al. (1999) in Bold's basal medium under autotrophic conditions. The red encysted cells of *Haematococcus* were harvested, freeze-dried and used for extraction of astaxanthin.

2.2. Preparation of astaxanthin samples

Total carotenoid from *Haematococcus* biomass was extracted as described previously (Sarada et al., 2002) by homogenizing with acetone, followed by centrifugation at 4000 ×*g* (C24; Remi Instruments Ltd, Mumbai, India). This cell free extract was designated as total carotenoid. Total carotenoid was subjected to preparative thin layer chromatography (TLC) using the solvent system acetone: hexane (3:7 v/v) and, separated astaxanthin ester bands were scraped from TLC plates and resuspended in acetone (Fiksdahl et al., 1978). Astaxanthin ester in acetone was saponified with equal volume of diethyl ether and 2% methanolic KOH for 4 h at 0 °C according to the method modified from Yuan and Chen (1999), to obtain free astaxanthin. To this suspension, 10% NaCl was added and repeatedly extracted with diethyl ether. Astaxanthin containing ether layer was collected and concentrated by vacuum to remove residual ether. This concentrated astaxanthin fraction was redissolved in acetone and was designated as saponified astaxanthin. Astaxanthin content in the above fractions was quantified by the method of Davies (1976). All the above extractions were carried out in dark condition. The fraction containing vials were flushed with nitrogen and stored at 0 °C till further use. The astaxanthin esters were further analysed by HPLC in comparing with standard astaxanthin (Sigma Chemical Co., USA) as described by Brinda et al. (2004).

2.3. Assessment of gastric ulcers; protection by total carotenoid and astaxanthin esters

Healthy Albino Wistar rats of both sexes (175±25 g) used for the experiments were maintained under standard conditions of temperature, humidity and light and were provided with standard rodent pellet diet (M/s Sai Durga feeds, Bangalore, India) and tap water *ad libitum*. The study was approved by the institutional ethical committee, which follows the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. 49, 1999), Government of India, New Delhi, India. All animals were classified into 14 groups of 6 animals each and their body weights were recorded on first and final days of pre-treatment. Total carotenoid and astaxanthin esters (dissolved in groundnut oil) were ingested to groups 3–5 and 8–10 at dosages of 100, 250 and 500 μg/kg b.w. respectively. For comparative analysis omeprazole (dissolved in normal saline) at a dose of 20 mg/kg b.w. treated and vehicle (groundnut oil — 1 ml/kg b.w.) control groups were maintained as groups 13 and 14 respectively. Group 2 ingested with normal saline but subjected to ulceration with alcohol treatment was considered as ulcerous group. Groups 6, 7 and 11, 12 served as sample controls for carotenoid and astaxanthin esters at 250 and 500 μg/kg b.w. respectively. Healthy controls were also treated with normal saline. Pre-treatment was done with either normal saline/sample/standard anti-ulcer drug for 21 days. At the end of the 21 day treatment, all the rats were fasted for 18 h with free access to drinking water and coprophagy was avoided by keeping the animals in wire bottom cages. Gastric ulcers were induced in all groups of animals except healthy and sample control groups by orally administering 100% ethanol at dose of 5 ml/kg b.w. (Lee et al., 2006). After 1 h of ethanol administration, all the animals were dissected under ether anesthesia to ensure little or no stress to the animals and the total number of mucosal lesions/ulcers per stomach was counted (Srikanta et al., 2007) and expressed as ulcer index (UI). Lower to higher grading was assigned to milder to severe symptoms, respectively. The following are descriptions of ulcer scores: 0.5 = red colouration, 1.0 = spot ulcers, 2.0 = hemorrhagic streaks more than 3 mm and less than 5 mm, 3.0 = ulcers/hemorrhagic streaks more than 5 mm. The calculated total ulcer score for each experimental group and mean ulcer score of each experimental group were expressed as the ulcer index.

2.4. Determination of gastric mucin

Gastric mucin was determined in control, treated and ulcer-induced groups according to the method described by Corne et al. (1974). The glandular part of each stomach (0.5 g) was placed in 10 ml of 1% Alcian blue solution in 0.16 M sodium acetate (pH 5.8) for 2 h. The dye complex was extracted with 10 ml of 0.5 M magnesium chloride solution, centrifuged at 1500 ×*g* for 10 min and the supernatant was measured spectrophotometrically at 580 nm.

2.5. Histopathological studies

Gastric 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 (Sibilia et al., 2003). The sections were analysed by observing under light microscope (Leitz, Germany) at 10× magnification.

2.6. Determination of changes in the antioxidant enzymes and lipid peroxidation level

The stomach and liver tissues were weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 5% (w/v). The homogenates were centrifuged at 1000 ×*g* at 4 °C for 20 min and the clear supernatants were used for estimation of antioxidant enzyme activities. Superoxide dismutase activity (SOD, EC 1.15.1.1) was determined by measuring the inhibition of the cytochrome *c* reduction using xanthine/xanthine oxidase O_2-generating system at 550 nm (Flohe and Otting, 1984). One unit of superoxide dismutase activity was defined as the amount of enzyme that caused 50% inhibition of cytochrome *c* reduction under the assay conditions. Glutathione peroxidase activity (GSHPx, EC 1.11.1.9) was measured by monitoring the oxidation of NADPH at 340 nm as described by Flohe and Gunzler (1984) in a coupled assay with glutathione reductase. Specific activity was defined as the unit of the enzyme activity per mg of protein. The activity of catalase (CAT, EC 1.11.1.6)

was measured by monitoring the decrease in absorbance of hydrogen peroxide at 240 nm and specific activity was defined as the unit of the enzyme activity per mg of protein (Abei, 1984). Lipid peroxidation was estimated in terms of malondialdehyde, which reacts with thiobarbituric acid to form a red colour complex absorbing at 532 nm (Ohkawa et al., 1979).

2.7. Inhibition of H^+, K^+-ATPase and lipoxygenase activities in vitro

Gastric membrane containing H^+, K^+-ATPase was prepared (Das et al., 1997) from mucosal stomach scrapings of sheep and was homogenized in 20 mM Tris–HCl buffer (pH 7.4). Homogenate was centrifuged for 10 min at 5000 ×*g* and the resulting supernatant was subsequently centrifuged at 5000 ×*g* for 20 min. Thus prepared parietal cell extract was used to determine the H^+, K^+-ATPase inhibition, as standardized in our laboratory previously (Yougender et al., 2007). Briefly the enzyme extract was incubated with different fractions of astaxanthin, in a reaction mixture containing 16 mmol/l Tris buffer (pH 6.5). The reaction was initiated by adding substrate (2 mmol/l ATP, 2 mmol/l $MgCl_2$ and 10 mmol/l KCl) and after 30 min of incubation at 37 °C, the reaction was stopped by the addition of an assay mixture containing 4.5% ammonium molybdate and 60% perchloric acid. Inorganic phosphate (Pi) formed was measured spectrophotometrically at 400 nm. Enzyme activity was calculated as μmol of inorganic phosphate (Pi) released per 1 h per mg protein at various doses of astaxanthin fractions.

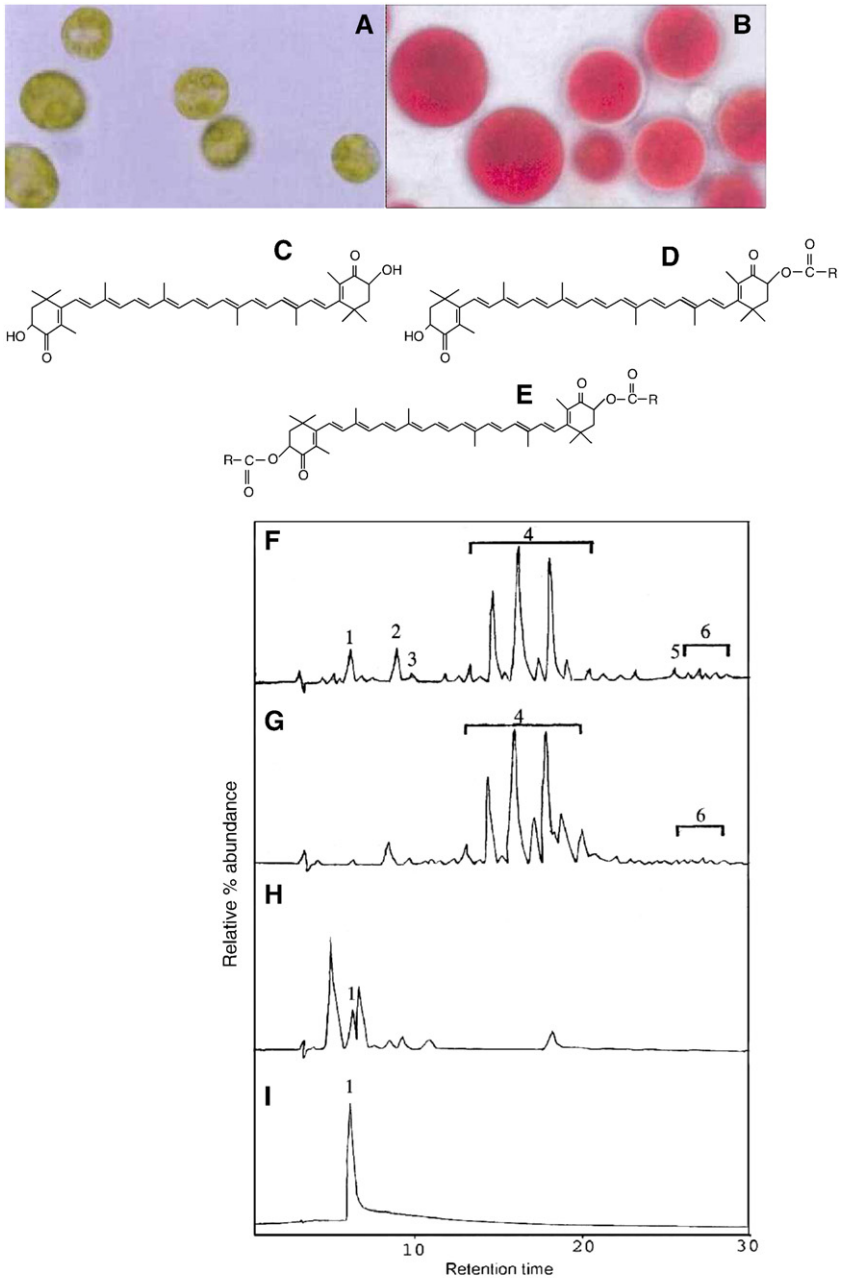

Fig. 1. Different stages of *Haematococcus* cells, structures of astaxanthin esters and their HPLC profile. Growing cells of green alga *Haematococcus* (A) and astaxanthin accumulated *Haematococcus* cells (B) (magnification — 40×). Structure of free/saponified (C), monoester (D) and diester (E) of astaxanthin (*R* = saturated or unsaturated alkyl chains). HPLC profile of total carotenoid (F), esters of astaxanthin (G), saponified astaxanthin (H) and synthetic astaxanthin (I). 1 — free astaxanthin, 2 — lutein, 3 — canthaxanthin, 4 — astaxanthin monoesters, 5 — β-carotene and 6 — astaxanthin diesters.

Lipoxygenase dependent lipid peroxidation was measured spectrophotometrically as the increase in the absorbance of lipid hydroperoxide formation at 234 nm as described by Thippeswamy and Naidu (2005). Briefly, the reaction mixture in a final volume of 1.0 ml contained 100 μM linoleic acid, and 5.0 nM soybean lipoxygenase in 50 mM tris (hydroxymethyl) amino methane (Tris) buffer of pH 7.4. The increase in absorption due to the formation of lipid hydroperoxide was monitored at 234 nm with a Shimadzu UV 160-A spectrophotometer. Soybean lipoxygenase enzyme was incubated with astaxanthin esters for 5 min prior to initiation of reaction with linoleic acid. An extinction coefficient of 25 mM^{-1} cm^{-1} was used for quantification of lipid hydroperoxide.

2.8. Determination of in vitro antioxidant activity

The free radical scavenging activity of astaxanthin was determined on the basis of its ability to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. An aliquot of 100 μl of astaxanthin fractions at various concentrations were added to 3 ml of 0.004% methanol solution of DPPH. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging effect}(\%) = (\text{OD of the control} - \textit{OD of the sample})/(\text{OD of the control}) \times 100$$

OD = optical density at 517 nm.

The reducing power of astaxanthin fraction was determined based on the ability of astaxanthin fractions to reduce potassium ferricyanide, which results in a blue colour formation that was absorbed maximally at 700 nm. Briefly different concentrations of astaxanthin fractions were mixed with a reaction mixture containing 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An equal volume of 10% trichloro acetic acid was added to the mixture and centrifuged at 3000 ×*g* for 10 min. The upper layer of the solution was mixed with distilled water

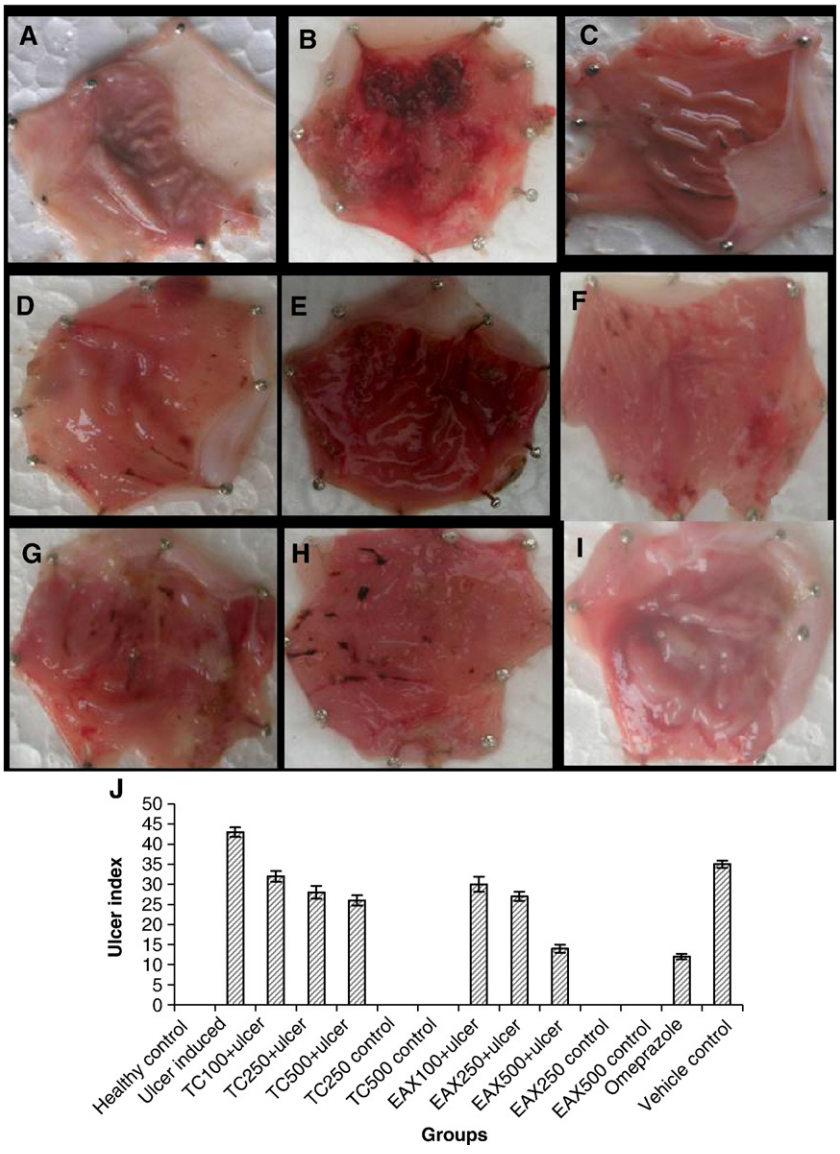

Fig. 2. Macroscopic observation and ulcer index of stomach from ulcer-induced and astaxanthin/omeprazole treated animals. A — Healthy control, B — ulcerated, C — omeprazole treated, D — TC 100 μg/kg b.w., E — TC 250 μg/kg b.w., F — TC 500 μg/kg b.w., G — EAX 100 μg/kg b.w., H — EAX 250 μg/kg b.w., I — EAX 500 μg/kg b.w., J — ulcer index (TC = total carotenoid, EAX = astaxanthin esters).

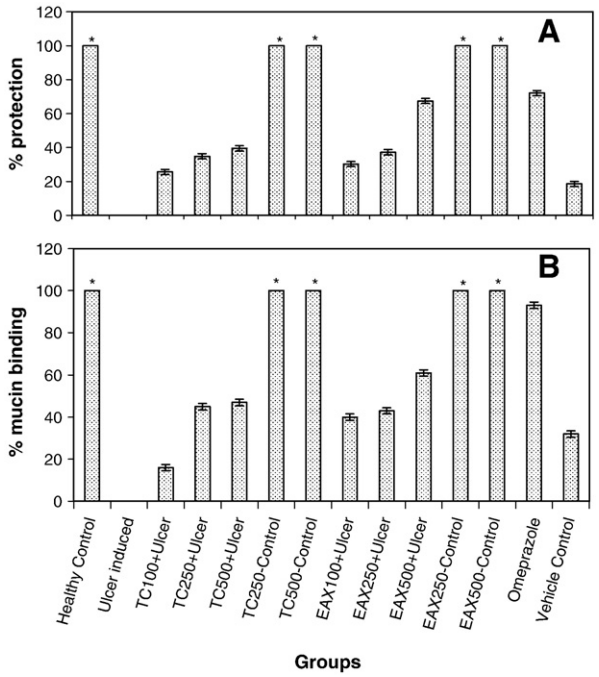

Fig. 3. Protection offered by total carotenoid and astaxanthin esters against ethanol-induced ulcer (A), and mucin binding (B) as measured by Alcian blue staining. * — The healthy control and sample (total carotenoid and astaxanthin ester) control groups were not subjected to ulcer induction, hence considered as 100% protected.

and 0.1% $FeCl_3$ at a ratio of 1:1:2 (v/v/v), and the absorbance was measured at 700 nm (Siddaraju and Dharmesh, 2007).

2.9. Statistical analysis

All the values are expressed as mean ± S.D. After calculation of mean and standard deviation, Duncan test was performed using the software — statistical package for social sciences (SPSS Inc., version 10.0.5) to obtain the significance between the treated groups and the control groups. A value of $P<0.05$ was considered to indicate a significant difference between the groups.

3. Results

3.1. Isolation and characterization of total carotenoid, astaxanthin ester and free saponified astaxanthin from H. pluvialis

Astaxanthin rich *H. pluvialis* encysted cells appeared red (Fig. 1B), as opposed to green cells, which are at the growing stage (Fig. 1A). After extraction, the total carotenoid content was found to be ~2.0% (w/w) on dry weight basis. Thin layer chromatographic (TLC) pattern of total carotenoid showed prominent astaxanthin ester band at relative front (Rf) values of 0.9 and 0.78. These ester bands upon saponification yielded astaxanthin (Fig. 1F–I). The mono- (Fig. 1D) and diesters (Fig. 1E) collectively represented around 95% of astaxanthin in total carotenoid. Fig. 1G depicts the abundance of astaxanthin monoester followed by diester.

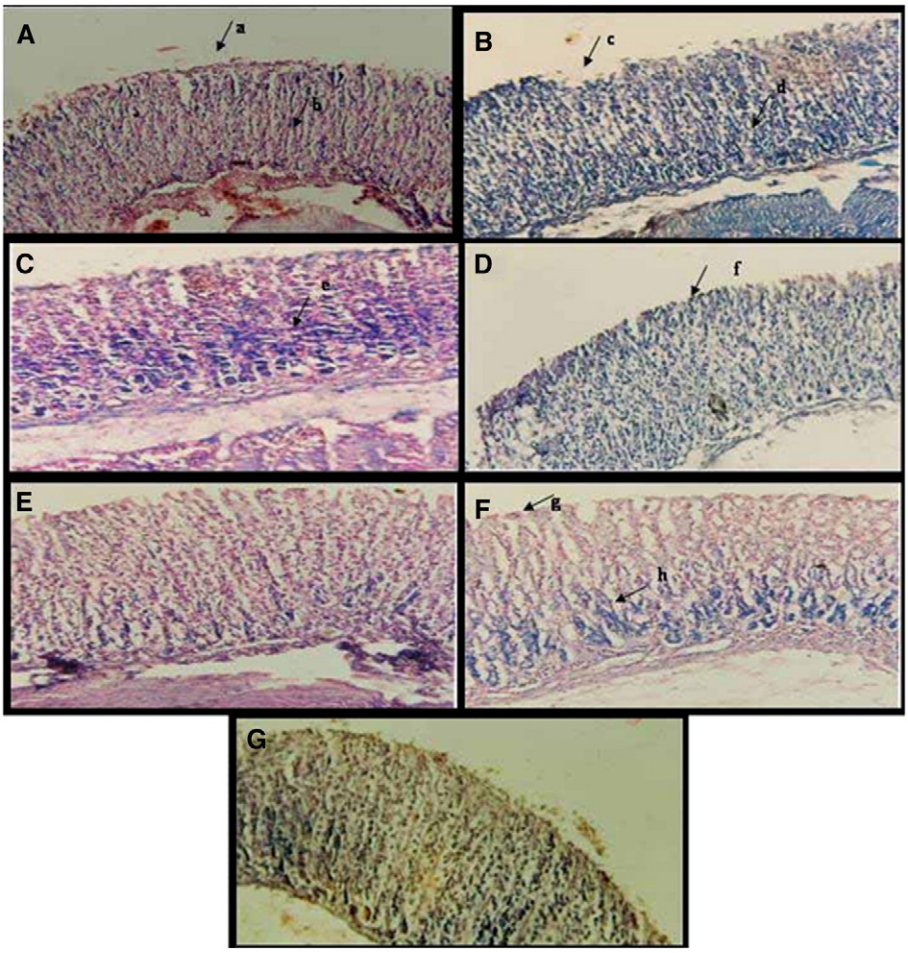

Fig. 4. Histopathological observation of stomach from ulcer-induced and astaxanthin/omeprazole treated animals. A–D indicates hematoxylin and eosin staining sections (magnification 10×). Control (A) shows intact mucosal epithelium (a) with organized glandular structure (b). Ulcer induction (B) showed damaged mucosal epithelium (c) and disrupted glandular structure (d). C & D and E & F showed a recovery in mucosal epithelium (f, g) and reorganized glandular structure (e, h) by total carotenoid and astaxanthin treatment respectively. Omeprazole (G) also showed mucosal protection.

Table 1
Effect of astaxanthin on antioxidant enzymes in stomach homogenate

Groups	SOD (U/mg)	Glutathione peroxidase (U/mg)	Catalase (U/mg)	TBARS (nmol MDA/mg)
1. Healthy control	95.20^{a}±2.86	32.50^{b}±1.30	1.57^{b}±0.03	2.73^{a}±0.07
2. Ulcer induced	25.30^{e}±0.76	10.59^{e}±0.42	0.17^{d}±0.01	8.20^{e}±0.09
3. TC100*	21.64^{d}±0.32	35.86^{b}±1.44	1.34^{c}±0.03	7.04^{d}±0.08
4. TC 250*	23.15^{e}±0.69	43.98^{a}±1.76	2.09^{a}±0.04	7.53^{d}±0.10
5. TC 500*	37.83^{c}±1.13	46.45^{a}±1.89	2.24^{a}±0.04	5.74^{c}±0.08
6. TC250* control	80.47^{b}±0.79	30.90^{c}±1.30	1.26^{c}±0.03	3.63^{b}±0.09
7. TC500* control	89.23^{b}±0.83	37.63^{b}±1.49	1.13^{g}±0.02	3.25^{ab}±0.08
8. EAX 100*	39.23^{c}±1.18	35.67^{b}±1.42	1.73^{b}±0.04	5.75^{c}±0.08
9. EAX 250*	32.67^{d}±0.98	24.69^{d}±0.98	1.62^{b}±0.03	5.20^{c}±0.07
10.EAX 500*	89.76^{b}±0.98	21.60^{d}±0.86	2.67^{a}±0.05	5.74^{c}±0.09
11.EAE250* control	76.45^{b}±0.71	32.82^{b}±1.26	1.20^{c}±0.03	2.93^{a}±0.09
12.EAE500* control	79.32^{b}±0.83	34.60^{b}±1.43	1.35^{c}±0.04	3.09^{a}±0.10
13.Omeprazole 20 mg	24.92^{e}±0.75	29.86^{d}±1.19	1.96^{ab}±0.04	6.84^{d}±0.12
14.Vehicle control	23.05^{e}±0.69	17.08^{e}±0.68	0.18^{d}±0.01	3.45^{b}±0.08

TC — Total carotenoid, EAX — astaxanthin esters, *μg/kg b.w.
Results are expressed as mean±S.D. Range was provided by Duncan multiple test at $P<0.05$. Different letters a to d in the column represent values that are significantly different when ulcer-induced group was compared with healthy control and sample treated groups. a: Less or not significant; b: less significant; c: moderately significant; d: very significant and e: most significant.

3.2. Macroscopic assessment of gastric mucosal protection by H. pluvialis astaxanthin

Ethanol administration induced severe lesions including inflammatory patches, bleeding in mucosa and ulcers with different size and degree in ulcerated rats (Fig. 2) with an ulcer index of 43. No such gastric lesions and bleeding were noticed in healthy control groups (Fig. 2). Pre-treatment with astaxanthin esters showed 3 fold reduction in ulcer index at 500 μg/kg b.w. as opposed to that of ulcer-induced animals (Fig. 2J). Relatively only 40% protection was observed in total carotenoid treated group at similar dosage (Fig. 3A).

Analogous to this, 61% mucin binding in astaxanthin ester treated group at 500 μg/kg b.w. (Fig. 3B) revealed that protection against ulcer may partly be via inhibiting mucosal damage that is generally caused by ethanol.

3.3. Histopathological analysis

Deep erosions with discontinuous mucosal layer were observed in ulcer-induced rats (Fig. 4B). Rats pre-treated with astaxanthin esters at 500 μg/kg b.w. showed normal histology or only very superficial lesions (Fig. 4F). Healthy controls showed intact mucosal epithelium (Fig. 4A). The recovery of mucosal layer was observed in astaxanthin esters (Fig. 4E and F) and total carotenoid (Fig. 4C and D) treated groups when compared to ulcer-induced stomach. Protective ability was comparable with that of the known anti-ulcer drug, omeprazole (Fig. 4G).

3.4. Changes in the antioxidant enzymes and lipid peroxidation level

3 and 10 fold depletions in antioxidant enzymes — superoxide dismutase/glutathione peroxidase and catalase levels respectively were observed in ulcerated rats (Table 1). Pre-treatment of rats with astaxanthin esters at 500 μg/kg b.w. had normalized the superoxide dismutase, catalase and glutathione peroxidase levels.

The antioxidant enzyme activity in serum and liver homogenates is shown in Table 2. A 2 to 3 fold increase in thiobarbituric acid reactive substances in ulcerated animals when compared to healthy animals was significantly normalized with total carotenoid and astaxanthin esters treatment, suggesting the action of total carotenoid and astaxanthin esters against biochemical changes induced by ethanol. However, no significant difference was observed between ulcerated and omeprazole treated groups since the mechanism of action is not by antioxidative but probably via inhibition of H^+, K^+-ATPase.

3.5. Ability of astaxanthin to inhibit H^+, K^+-ATPase and lipoxygenase enzyme in vitro

H^+, K^+-ATPase inhibitors such as omeprazole are anti-ulcerative agents since they block the upregulated activity of H^+, K^+-ATPase. Saponified astaxanthin showed maximum inhibition of H^+, K^+-ATPase activity followed by astaxanthin esters and total carotenoid (Fig. 5).

Table 2
Effect of astaxanthin on antioxidant enzymes in serum and liver homogenate

Groups	SOD (U/mg)	Glutathione peroxidase (U/mg)	Catalase (U/mg)	TBARS (nmol MDA/mg)
Serum				
1. Healthy control	25.30^{a}±0.76	17.72^{a}±0.98	0.70^{b}±0.04	1.34^{b}±0.05
2. Ulcer induced	11.82^{e}±0.35	07.20^{e}±0.40	0.25^{e}±0.01	3.76^{e}±0.15
3. TC100*	19.40^{cd}±0.58	13.20^{d}±0.73	0.43^{c}±0.02	2.97^{d}±0.12
4. TC 250*	21.28^{b}±0.64	12.20^{d}±0.67	0.50^{c}±0.02	3.10^{e}±0.12
5. TC 500*	19.30^{cd}±0.58	18.80^{a}±1.04	0.71^{b}±0.03	0.82^{a}±0.03
6. TC250* control	23.20^{ab}±0.70	12.20^{d}±0.57	0.34^{d}±0.01	1.64^{b}±0.06
7. TC500* control	22.10^{b}±0.60	17.00^{b}±0.98	0.67^{b}±0.03	0.93^{a}±0.03
8. EAX 100*	20.16^{c}±0.60	14.10^{d}±0.78	1.13^{a}±0.05	2.04^{c}±0.08
9. EAX 250*	18.91^{d}±0.57	16.90^{b}±0.93	0.64^{b}±0.03	1.94^{c}±0.08
10.EAX 500*	25.28^{a}±0.76	15.80^{c}±0.87	0.87^{b}±0.04	1.56^{b}±0.06
11.EAE250* control	20.00^{c}±0.64	19.40^{a}±1.20	0.42^{c}±0.02	1.25^{b}±0.03
12.EAE500* control	26.80^{a}±0.64	18.20^{a}±1.10	0.45^{c}±0.02	1.53^{b}±0.04
13.Omeprazole 20 mg	16.50^{d}±0.49	12.60^{d}±0.69	1.37^{a}±0.06	3.46^{e}±0.14
14.Vehicle control	08.90^{e}±0.27	05.10^{e}±0.28	0.31^{d}±0.01	1.60^{b}±0.06
Liver				
1. Healthy control	2.10^{d}±0.07	10.64^{a}±0.53	0.77^{a}±0.05	3.25^{b}±0.15
2. Ulcer induced	1.30^{e}±0.05	2.65^{e}±0.13	0.30^{e}±0.02	5.97^{e}±0.27
3. TC100*	3.27^{b}±0.09	7.15^{c}±0.36	0.59^{b}±0.04	5.34^{d}±0.24
4. TC 250*	3.36^{b}±0.10	6.20^{c}±0.31	0.38^{d}±0.03	5.46^{d}±0.25
5. TC 500*	4.05^{a}±0.11	8.04^{b}±0.40	0.61^{b}±0.04	3.89^{c}±0.18
6. TC250* control	2.21^{d}±0.09	4.69^{d}±0.28	0.58^{b}±0.04	3.00^{b}±0.17
7. TC500* control	3.69^{b}±0.10	8.22^{b}±0.50	0.63^{b}±0.04	3.72^{c}±0.20
8. EAX 100*	2.86^{c}±0.08	3.57^{e}±0.18	0.21^{e}±0.01	5.57^{d}±0.25
9. EAX 250*	2.57^{d}±0.08	4.57^{d}±0.23	0.44^{c}±0.03	5.77^{e}±0.26
10.EAX 500*	2.66^{d}±0.08	5.02^{d}±0.25	0.70^{a}±0.05	3.66^{c}±0.17
11.EAE250* control	2.32^{d}±0.06	9.41^{a}±0.39	0.63^{b}±0.04	3.68^{c}±0.19
12.EAE500* control	4.47^{a}±0.14	9.59^{a}±0.43	0.69^{a}±0.05	3.48^{c}±0.17
13.Omeprazole 20 mg	2.13^{d}±0.07	9.65^{a}±0.48	0.38^{d}±0.03	6.37^{e}±0.29
14.Vehicle control	1.54^{e}±0.06	5.20^{d}±0.26	0.20^{e}±0.01	2.71^{a}±0.12

TC — Total carotenoid, EAX — astaxanthin esters, *μg/kg b.w.
Results are expressed as mean±S.D. Range was provided by Duncan multiple test at $P<0.05$. Different letters a to d in the column represent values that are significantly different when ulcer-induced group is compared with healthy control and sample treated groups. a: Less or not significant; b: less significant; c: moderately significant; d: very significant and e: most significant.

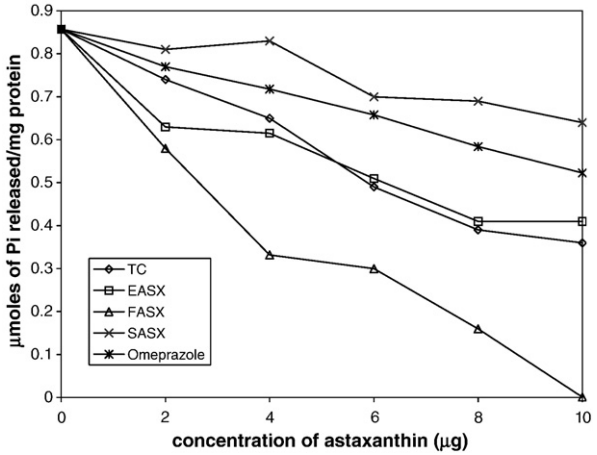

Fig. 5. Inhibition of H^+, K^+-ATPase enzyme activity. TC = total carotenoid, EAX = astaxanthin esters, FASX = saponified astaxanthin, SASX = synthetic astaxanthin.

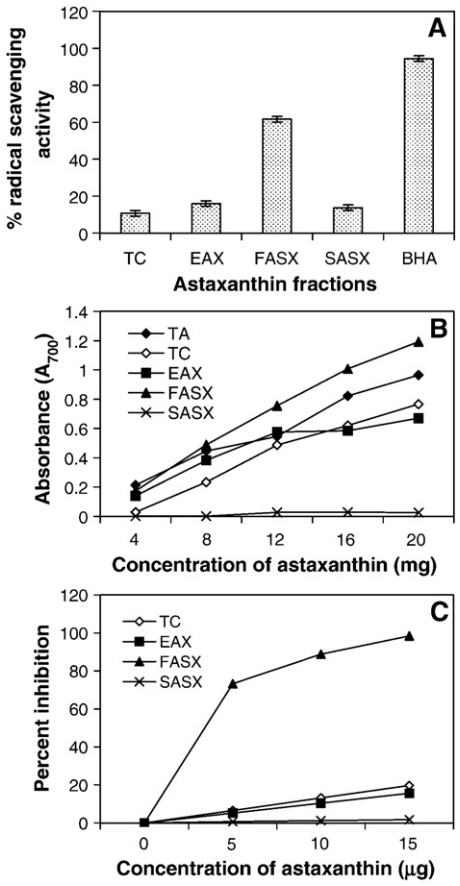

Fig. 6. *In vitro* antioxidant activity of astaxanthin fractions from *Haematococcus*. A. Free radical scavenging activity, B. reducing power, C. lipoxygenase-inhibitory activity of astaxanthin. TC = total carotenoid, EAX = astaxanthin esters, FASX = saponified astaxanthin, SASX = synthetic astaxanthin, TA = tannic acid, BHA = butylated hydroxyl anisole.

Standard astaxanthin did not show any inhibition while astaxanthin esters showed inhibition with an IC_{50} of 18.2 µg/ml which is better than that of the known H^+, K^+-ATPase inhibitors like omeprazole which has an IC_{50} of 25 µg/ml. In addition, saponified astaxanthin could also show 6 and 7 fold higher lipoxygenase-inhibitory activities (Fig. 6C) with an IC_{50} of 3.4 µg/ml, when compared to total carotenoid and astaxanthin esters respectively (Table 3). Increased potency of saponified astaxanthin, when compared to synthetic astaxanthin, may suggest the possibility of altered structure during saponification, which needs to be evaluated.

3.6. *In vitro* antioxidant activity of astaxanthin from *H. pluvialis*

The 1,1-diphenyl-2-picrylhydrazyl radical scavenging activities of total carotenoid, astaxanthin esters and saponified astaxanthin were compared with the activities of synthetic astaxanthin and butylated hydroxyl anisole (BHA; Fig. 6). Saponified astaxanthin showed the maximum free radical scavenging activity (IC_{50} of 8.1 µg/ml) that is 4.5 fold higher in comparison to standard astaxanthin (IC_{50} 36.5 µg/ml). Saponified astaxanthin was also demonstrated to exhibit maximum reducing power (59,600 U/g) followed by total carotenoid (38,350 U/g) and astaxanthin esters (33,550 U/g) (Fig. 6B). Dose dependent increase in activity suggests that, it is proportionally increased to the concentration of astaxanthin in the sample.

4. Discussion

The present study demonstrates for the first time that orally administered total carotenoid and astaxanthin esters exerts a dose-dependent gastroprotective effect on acute, ethanol-induced gastric lesions in rats. Ethanol consumption, leading to health complication in humans reportedly has become a serious problem throughout the world (Tapiero, 2004). The release of oxygen-derived free radicals has drawn attention as a possible pathogenic factor of gastric mucosal injury associated with ethanol (Szabo et al., 1992; Smith et al., 1996). Ethanol has been known to penetrate rapidly into the gastric mucosa and this causes membrane damage, erosion of gastric cells, impairment in H^+ pumping into the gastric lumen and hence gastric ulceration. Investigations of Szabo et al. (1992) and Terano et al. (1986) have revealed that the ethanol-induced gastric damage is mediated by the generation of free radicals.

Having understood that free radical scavenging and antioxidant activities play an important role in the prevention of free radical-related diseases, including aging and ulcers (Packer, 1995), the current study addressed the identification of a potent antioxidant — astaxanthin from *H. pluvialis* and determined its antioxidant and anti-ulcer potencies in both *in vitro* and *in vivo* models.

Besides preventing the extreme reactivity of reactive oxygen species, the control of acid secretion is essential for the treatment of gastric ulcer (Rao et al., 2000). While acid secretion by parietal cells is regulated through several stimulatory receptors, such as histamine H_2 and muscarinic M_3, the final step is mediated by gastric pump, also called the proton pump (Hersey et al., 1995). Thus the effective therapeutic control of acid secretion involves both the blockade of these receptors and the inhibition of the proton pump. In this study saponified astaxanthin from *H. pluvialis* has shown maximum H^+, K^+-ATPase-inhibitory activity that implies its ulcer preventive effect.

Recently, Kim et al. (2005) documented that astaxanthin from yeast — *Xanthophyllomyces* exhibited the ability to inhibit ethanol-induced gastric ulceration and they proposed that the inhibition of gastric ulceration is via activation of antioxidant enzyme — superoxide dismutase, catalase and glutathione peroxidase. *H. pluvialis*, synthesizes the 3*S*, 3′*S*-isomer, whereas yeast *Xanthophyllomyces* produces the opposite isomer having the 3*R*, 3′*R*-configuration (Visser et al., 2003). In the present paper, we address the efficacy of astaxanthin ester in comparison with total carotenoid extract from *Haematococcus* at doses of 100, 250 and 500 µg/kg b.w. against ethanol-induced ulceration. It is well known that astaxanthin is a highly lipophilic compound; therefore the function of astaxanthin as a free radical scavenger and antioxidant is likely assisted by the ease with which it crosses morphophysiological barriers. A study by Tso and Lam (1996) has demonstrated that astaxanthin can cross blood retinal barrier in mammals and can extend its antioxidant benefits beyond that barrier. In addition to the fact that astaxanthin can readily enter into subcellular compartments, where free radicals may be generated, it has no known toxic effects (Guerin et al., 2003).

Presence of astaxanthin esters in *H. pluvialis* has an added advantage that, generally carotenoids, although potential antioxidants, they may lack such properties *in vivo*, because of the pro-oxidant effect. Esterified astaxanthin shows comparatively better stability than free astaxanthin, and hence it may exhibit more health

Table 3
In vitro antioxidant property of astaxanthin fractions

Astaxanthin sample	Free radical scavenging activity IC_{50} (µg/ml)	Reducing power activity (U/g)	H^+, K^+-ATPase inhibition activity IC_{50} (µg/ml)	Lipoxygenase inhibition activity IC_{50} (µg/ml)
Total carotenoid	46.7	38,350	14.4	19.1
Astaxanthin ester	31.2	33,550	18.2	24.4
Saponified astaxanthin	8.1	59,600	6.2	3.4
Synthetic astaxanthin	36.5	1300	36.0	568.1
Butylated hydroxyl anisole	8.5	–	–	–
Omeprazole	–	–	25	–
Tannic acid	–	48,250	–	–

beneficial effects than free astaxanthin. *H. pluvialis* may be a potential natural source for the isolation of esterified astaxanthin and to deploy them for health beneficial effects against several disorders. Further, carotenoid esterification does not pose impediment for bioavailability in humans (Bowen et al., 2002), hence astaxanthin esters can play a role in ulcer prevention.

Inhibition of 15-lipoxygenase enzyme by total carotenoid, saponified astaxanthin and astaxanthin esters of *H. pluvialis* (Fig. 6C) has been demonstrated in the current study. The Saponified astaxanthin showed potent inhibitory activity when compared to both total carotenoid and astaxanthin esters. The process of oxidation of low-density lipoprotein is mediated by 15-lipoxygenase, and is believed to play a key role in mediating inflammatory reactions in ulcerous condition (Steinberg, 1999; Gundersen et al., 2003). Ulcerogens such as alcohol and non-steroidal anti-inflammatory drugs have been known to inhibit leukotrienes and prostaglandins that are important for proliferation of mucin synthesizing-mucosal cells. Inhibitors of lipoxygenases thus would contribute potentially towards the regulation of inflammatory reactions towards the synthesis of gastric mucin and hence mucosal protection during ulcerous condition. Results may imply their beneficial role in the potential management of ulcers.

The present data on *in vivo* anti-ulcer properties of total carotenoid and astaxanthin esters, thus suggest that astaxanthin esters may be a major anti-ulcer component present in the *H. pluvialis* extract. Further evaluation of biochemical changes like catalase, superoxide dismutase, glutathione peroxidase in control, ulcer-induced and treated animal groups revealed that the anti-ulcerogenic potency may be due to a) inhibition of H^+, K^+-ATPase which suppresses the acid secretion, b) upregulation of mucin content partially which protects the gastric mucosal layer against oxidative damage leading to ulceration and c) increase of antioxidant status which would eliminate the oxidative stress condition during ulceration.

Ever since the role of carotenoids is known in the literature for their strong antioxidant and health beneficial properties against oxidative stress induced chronic diseases, several fruits and vegetable sources were explored. *Haematococcus* being a micro alga, there are multi-advantages, like it's utilization as a nutraceutical or food ingredient and it has been approved by the US Food and Drug Administration (21 CFR 190.6) as a dietary ingredient. Presence of higher levels of astaxanthin esters in *Haematococcus* may also reduce the requirement in quantity, so that, an added advantage of supply to a larger population can be anticipated.

In conclusion, this is the first evidence that *Haematococcus* astaxanthin exerts a potent gastroprotective activity against ethanol-induced lesions in rats, upon oral administration. The antioxidative mechanism of *Haematococcus* astaxanthin against ethanol-induced lesions is also supported by its *in vitro* antioxidant potency.

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