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Identification of Carotenoids from Green Alga *Haematococcus pluvialis* by HPLC and LC–MS (APCI) and Their Antioxidant Properties

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***Haematococcus pluvialis*, a green alga, accumulates astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) upto 2–3% on a dry weight basis. In the present study, identification of carotenoids from *Haematococcus* cyst cell extract by HPLC and LC–MS (APCI) and their antioxidant properties were evaluated in *in vitro* model systems. The extract exhibited 89% and 78% antioxidant activities in the β-carotene linoleate model and the hydroxyl radical scavenging model, at 9 ppm of total carotenoid, respectively. The extract also showed 80%, 85%, and 79% antioxidant activities against lipid peroxidation in the kidney, brain, and liver of rats. Low-density lipoprotein oxidation induced by Cu^{2+} ions was also protected (45%, 64%, and 75%) by the extract in a dose-dependent manner with different carotenoid levels. Thiobarbituric acid reactive substances concentration in the blood, liver, and kidney of rats were also significantly ($p<0.005$) decreased in *H. pluvialis*-treated rats. The potent antioxidant activity is attributable to various carotenoids present in the extract.**

Keywords: *H. pluvialis*, carotenoids, antioxidant properties, HPLC, LC–MS (APCI)

Free radicals (hydroxyl and peroxy radicals) and highly reactive forms of oxygen are produced in the body during normal metabolic reactions and processes. Oxidative damage has been linked to aging, atherogenesis, ischemia-reperfusion injury, infant retinopathy, age-related macular degeneration, and carcinogenesis. There is increasing interest in the use and evaluation of antioxidant activity in food and pharmaceutical preparations. This interest is mainly due to the role of reactive oxygen species (ROS) in the aging process and pathogenesis of many diseases in which ROS are mainly involved. Many studies have shown that these ROS, including oxygen free radicals, are causative factors in the etiology of degenerative disorders including some hepatopathies and other serious organ damage [23]. They can also prevent the chain reaction of production of free radicals initiated by the degradation of polyunsaturated fatty acids, which can dramatically accelerate the degradation of lipid membranes. According to *in vitro* and *in vivo* studies, several classical antioxidants have been shown to protect various cells like hepatocytes and nephrocytes against lipid peroxidation [9] or inflammation, thereby preventing the occurrence of hepatic necrosis, kidney damage, and other radical-associated activities [33].

H. pluvialis is a unicellular green alga belonging to the family Chlorophyceae. It is known to accumulate carotenoids under stress conditions. It is one of the potent biological sources for astaxanthin production. Astaxanthin is the major carotenoid in *Haematococcus* and it exists mainly as astaxanthin esters (monoester, 70%; diester, 15–20%; and free form, 4–5%) [10]. Astaxanthin has gained nutraceutical and pharmaceutical importance owing to its high antioxidant activity [10]. It is commercially available as an antioxidant food supplement and approved by the Swedish Health Food Council Advisory Board [19]. It is produced from the microalga *Haematococcus pluvialis*, and the recommended daily dose is 4 mg [19]. In the United States, astaxanthin is permitted for use as a food color additive in salmonid feed by the Food and Drug Administration [30]. Owing to its strong antioxidant activity, 10 times higher than β-carotene and more than 500 times effective than α-tocopherol, astaxanthin has been proposed as the super vitamin E [17]. Kobayashi and Sakamoto [12] have reported higher antioxidant activities for astaxanthin esters than β-carotene in methylene blue-sensitized photooxidization of the linoleic acid model. They also reported that the antioxidant activities of β-carotene and free astaxanthin were dependent on the polarity of the solvent, whereas astaxanthin esters were independent of the polarity. Gradelet *et al.* [8] have demonstrated the preventive

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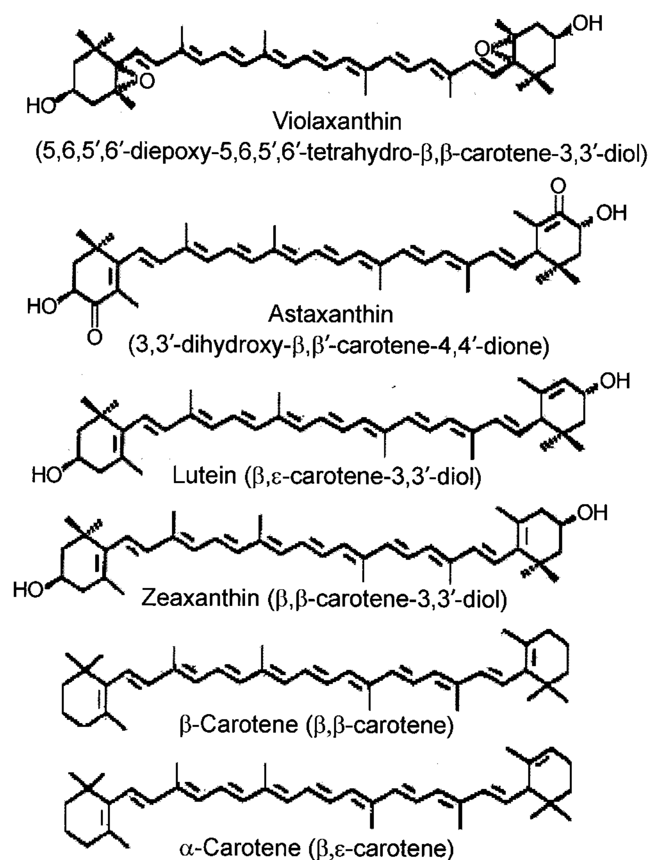

Fig. 1. Chemical structure of major carotenoids present in *H. pluvialis*.

effects of astaxanthin against aflatoxin B_1 carcinogenicity. It has important metabolic functions in animals and man, such as enhancement of the immune response, and protection against degenerative diseases by scavenging of oxygen free radicals [17]. Moreover, astaxanthin has been found to provide many essential biological functions, including protection against lipid-membrane peroxidation of essential polyunsaturated fatty acids and proteins, DNA damage, and ultraviolet light effects [26]. Currently, there is demand for the natural astaxanthin in the fast growing nutraceutical market. *H. pluvialis*, being a rich source of natural astaxanthin, is now cultivated on a large scale [20]. Ceron *et al.* [3] reported that astaxanthin esters (diester and monoester) have shown higher antioxidant activities than free astaxanthin and other carotenoids in *Haematococcus pluvialis*. Therefore, the present study was focused on evaluation of the antioxidant activity of *H. pluvialis* extract using different *in vitro* models. With astaxanthin and its esters being the major proportion (85–88%) of total carotenoids, the total carotenoid extract was taken for the experiments. The chemical structure's of carotenoids (violaxanthin, astaxanthin, lutein, zeaxanthin, α-carotene, and β-carotene) found in *H. pluvialis* are shown in Fig. 1.

Materials and Methods

Chemicals

All the chemicals and solvents used were of analytical/HPLC grade and were obtained from Ranbaxy Fine Chemicals Ltd., (Mumbai, India). Acetone, acetonitrile, methanol, dichloromethane, Tween-40, Folin–Ciocaltaeu reagent, ethylenediamine tetraacetic acid (EDTA), ascorbic acid, linoleic acid, ferrous sulfate, and ferrous chloride were also obtained from Ranbaxy Fine Chemicals Ltd. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), dimethylsulfoxide (DMSO), trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), Nash reagent, Tris-HCl buffer, potassium ferric cyanide, egg lecithin, standard astaxanthin, violaxanthin, lutein, zeaxanthin, and α,β-carotene were obtained from Sigma Chemicals, Co. (St. Louis, MO, U.S.A.).

Animals

Male albino rats (35±2 g) of Out B-Wistar, IND-Cft (2C), 4 weeks old, were used in this study. The rats were housed under normal laboratory conditions (27±2°C, 12/12 h light dark/cycle) with free access to standard rat feed (Amrut Feeds, Sangli, India) and water. Blood collection from animal experiments were performed after due clearance from the institutional animal ethics committee.

Cultivation of *H. pluvialis*

H. pluvialis (19-1a) was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen, Germany, and was maintained on autotrophic medium agar slants [25].

Growth and Carotenoid Formation in *H. pluvialis* Culture

H. pluvialis culture was grown in modified autotrophic bold basal medium (BBM) [31], and carotenoid formation was obtained under salinity stress [25]. The encysted red cells rich in carotenoids were harvested, freeze-dried, and stored at 4°C.

Analytical Methods

Estimation of chlorophyll, total carotenoids, and astaxanthin. A known quantity of encysted cells of *H. pluvialis* was homogenized with a mortar and pestle in a dark room and extracted repeatedly with acetone. Total carotenoid and chlorophyll contents were analyzed as per the procedure of Lichtenthaler [16] by measuring the absorbance at 470 nm for carotenoids, and 645 and 661.5 nm for chlorophyll (Shimadzu UV–Vis Spectrophotometer UV-160-A). The total carotenoid content was expressed in terms of percent dry weight. The astaxanthin content was determined at 480 nm by using an extinction coefficient of 2,500 at the 1% level by the method of Davies [5].

HPLC analysis of *H. pluvialis* extract. Carotenoids of the *H. pluvialis* extract were analyzed using an HPLC (Shimadzu 10AS, Kyoto, Japan) reverse phase 25 cm×4.6 mm, 5 μm, C_{18} column (Wakosil 11 5C 18RS) with an isocratic solvent system consisting of dichloromethane:acetonitrile:methanol (20:70:10, v/v/v) at a flow rate of 1.0 ml/min. All the carotenoids were monitored at 476 nm with a UV–visible detector (Shimadzu, Kyoto, Japan). The peak identification and λ_{max} values of these components were confirmed by their retention times and characteristic spectra of standard chromatograms recorded with a Shimadzu model LC-10AVP series equipped with a SPD-10AVP photodiode array detector. They were quantified from their peak areas in relation to the respective reference standards.

Liquid chromatography–mass spectroscopy (LC–MS) in atmospheric pressure chemical ionization (APCI) of *H. pluvialis* extract. The carotenoids were identified in *H. pluvialis* by utilizing the Waters 2996 modular HPLC system (autosampler, gradient pump, thermoregulator, and DAD), coupled to a Q-Tof Ultima (U.K.) mass spectrometer. In brief, the APCI source was heated at 130°C and the probe was kept at 500°C. The corona (5 kV), HV lens (0.5 kV), and cone (30 V) voltages were optimized. Nitrogen was used as the sheath and drying gas at 100 and 300 l/h, respectively. The spectrometer was calibrated in the postitive mode and $[M+H]^+$ ions were recorded. Mass spectra of carotenoids were acquired with an *m/z* 400–600 scan range at 450 nm by a diode array detector and confirmed with respective standards.

Antioxidant Assays

Preparation of *H. pluvialis* extract for antioxidant assays. A known quantity (2 g) of freeze-dried *H. pluvialis* biomass was extracted with acetone repeatedly, and the pooled (total volume 150 ml) acetone extract was concentrated by rotary evaporation (Buchi, Germany) and redissolved in ethanol and used for different *in vitro* assays. The acetone extract was analyzed for carotenoid content, chlorophyll content, total phenolics, and reducing power.

Determination of total phenolic compounds. The concentration of total phenolic compounds in the extracts was determined according to the procedure of Ranga Rao *et al.* [24] and expressed as caffeic acid equivalents. In brief, samples and standards were prepared in acidified (3 g/l, HCl) methanol:water (60:40, v/v) and 100 µl of each was added separately to 2 ml of 2% Na_2CO_3. After 5 min, 100 µl of 50% Folin–Ciocalteu reagent was added and the mixture was allowed to stand at room temperature for 30 min. The absorbance was measured at 750 nm using a spectrophotometer (Shimadzu 160A). The blank consisted of all reagents and solvents without sample or standard. The standard caffeic acid was prepared at concentrations of 10–100 µg/ml. The phenolic concentration in the algal extract was determined by comparison with the standards.

Antioxidant assay using β-carotene linoleate model system (β-CLAMS). The antioxidant activities of *H. pluvialis* extract was evaluated by the procedure of Fayaz *et al.* [7]. In brief, β-carotene (0.2 mg), 20 mg of linoleic acid, and 200 mg of Tween-40 (polyoxyethylene sorbital monopalmitate) were mixed in 0.5 ml of chloroform. The chloroform was removed at 40°C under vacuum using a rotary evaporator (Buchi, Germany). The resulting mixture was diluted with triple distilled water (10 ml), mixed well for 1–2 min, and made up to 50 ml with oxygenated water. Aliquots (4 ml) of this emulsion were transferred to different test tubes containing test samples (5, 7, and 9 ppm of total carotenoids). Butylated hydroxy anisole (BHA) was used for comparative purposes. A control, 4 ml of the above emulsion without β-carotene, was prepared. The tubes were placed in a water bath maintained at 50°C. Absorbance of all the samples at 470 nm was taken at zero time (t=0). Measurement of absorbance was continued until the color of β-carotene disappeared in the control group (t=180 min) at 15-min intervals. A mixture prepared as above without β-carotene served as the blank. All determinations were carried out in triplicates. A dose-response relationship of antioxidant activity for *H. pluvialis* extract was determined at different concentrations. The antioxidant activity (AA) of the *H. pluvialis* extract was evaluated in terms of bleaching of the β-carotene using the following formula.

$$AA=100[1-(A_0-A_t)/(A^0_0-A^0_t)]$$

where A^0 and A^0_0 are the absorbance values measured at zero time incubation for test sample and control, respectively, and A_t and A^0_t are the values after incubation for 180 min.

Assay for hydroxyl radical scavenging activity. The hydroxyl radical scavenging activity of *H. pluvialis* extract was determined according to the procedure of Fayaz *et al.* [7]. In brief, different concentrations (5, 7, and 9 ppm total carotenoids) of *H. pluvialis* extracts were taken and the solvent was evaporated to dryness under a stream of nitrogen. The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), 167 µM iron:EDTA mixture (1:2, w/w), 0.1 mM EDTA, 2 mM ascorbic acid, and 33 mM Me_2SO in a final volume of 3.0 ml. The reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by the addition of 1 ml of TCA (17.5%) and 2 ml of Nash reagent and left at room temperature for 15 min. The intensity of yellow color was measured spectrophotometrically (Shimadzu 160A) at 412 nm against a reagent blank. The percentage hydroxy radical scavenging activity of sample was calculated as % inhibition relative to the control.

Assay for lipid peroxidation using brain and kidney homogenates. Brain and kidney of normal albino male rats were homogenized with a Polytron in 10 ml of ice-cold Tris-HCl buffer (20 mM, pH 7.4) by the procedure of Chidambaramurthy *et al.* [4]. The homogenate was centrifuged at 24,795 ×*g* for 15 min. The supernatants (1 ml) were incubated with different levels of *H. pluvialis* extracts (5, 7, and 9 ppm total carotenoids) in the presence of 10 µM $FeSO_4$ and 0.1 mM ascorbic acid at 37°C for 1 h. The reaction was terminated by addition of 1.0 ml of TCA (28%) and 1.5 ml of TBA (1%). The solution was heated at 100°C for 15 min, cooled to room temperature, centrifuged at 4,696 ×*g* for 15 min, and the color of the MDA–TBA complex in the supernatant was read at 532 nm using a spectrophotometer. BHA was used as a positive control. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio $(\%)=(A-A_1)/A\times100$, where A is the absorbance of the control, and A_1 is the absorbance of the test sample.

Assay for inhibitory effect on lipid peroxidation. Assay of the inhibitory effect of *H. pluvialis* extract on lipid peroxidation was carried out as given in Ranga Rao *et al.* [24]. Normal rats were anesthetized with diethyl ether and sacrificed by exsanguination. The perfused liver was isolated and homogenized with 9 parts of isotonic phosphate buffer saline using a Potter–Elvehjem homogenizer at 4°C. The homogenate was centrifuged at 2,800 ×*g* for 15 min and supernatant was used for the *in vitro* lipid peroxidation assay. In brief, to different concentrations of sample extracts (5, 7, and 9 ppm total carotenoids), 1 ml of 0.15 M potassium chloride and 0.5 ml of rat liver homogenate were added. Peroxidation was initiated by adding 100 µl of 0.2 mM ferric chloride. After incubation at 37°C for 30 min, the reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 15% TCA, 0.38% TBA, and 0.5% BHT. The reaction mixture was heated at 80°C for 60 min, cooled, centrifuged at 2,800 ×*g*, and the supernatant was read at 532 nm. A control without added sample extract was also run simultaneously. The percentage of lipid peroxidation inhibitory activity (% LP) was calculated as lipid peroxidation=1-(sample OD/blank OD)×100.

Assay for antioxidant activity on liposome model system. The lipid peroxidation inhibitory activity of the *H. pluvialis* extracts in a liposome model system was determined according to the method of Duh and Yen [6]. Egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in an ultrasonic homogenizer (Sonoplus HD 2200). *H. pluvialis* extracts at different concentrations (5, 7, and 9 ppm total

carotenoids) were added to 1 ml of liposome mixture and to the control (without test samples). Lipid peroxidation was induced by adding 10 µl of $FeCl_3$ (400 mM) and 10 µl of L-ascorbic acid (200 mM). After incubation at 37°C for 1 h, the reaction was stopped adding 2 ml of 0.25 N HCl containing 150 mg/ml TCA and 3.75 mg/ml TBA. The reaction mixture was subsequently boiled for 15 min, cooled to room temperature, centrifuged at 2,800 ×*g* for 15 min, and the absorbance of the supernatant was read at 532 nm by spectrophotometer.

Antioxidant activity on human low-density lipoprotein (LDL) oxidation. Plasma was separated from blood drawn from human volunteers under the supervision of medical doctor, CFTRI and stored at 4°C until use. Isolation of LDL from the plasma and test of antioxidant activity of the algal extract were done according to the procedure of Ranga Rao *et al.* [24]. To the *H. pluvialis* extract (5, 7, and 9 ppm total carotenoids), 40 µl of copper sulfate (2 mM) was added and made upto 1.5 ml with phosphate buffer (50 mM, pH 7.4). A tube without extract and copper sulfate served as the negative control and another tube without copper sulfate served as the positive control. All the tubes were incubated at 37°C for 45 min. To the aliquots of 0.5 ml drawn at 2 h, 4 h, and 6 h intervals from each tube, 0.25 ml of TBA (1% in 50 mM NaOH) and 0.25 ml of TCA (2.8%) were added. The tubes were incubated again at 95°C for 45 min, cooled to room temperature, and centrifuged at 4,600 ×*g* for 15 min. A pink chromogen was extracted and read at 532 nm by a spectrophotometer.

Reducing power of *H. pluvialis*. The reducing power of *H. pluvialis* extract was determined as per the procedure given in Ranga Rao *et al.* [24]. The extract (1 mg/ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferric cyanide [$K_3Fe(CN)_6$, 1%] and then incubated at 50°C for 20 min. To this mixture, 2.5 ml of TCA (10%) was added and centrifuged at 4,600 ×*g* for 20 min. The upper layer (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%), and read at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of protein. Protein was estimated in the animal tissues and algal samples by using the method of Lowry *et al.* [18].

Statistical analysis. Results were expressed as the mean±SD of three replicates. The difference between groups was statistically analyzed by using one-way ANOVA.

Results

Carotenoid and Chlorophyll Content in *H. pluvialis* Extract

The acetone extract of *H. pluvialis* contained 2.2% (w/w) total carotenoid, 0.22% (w/w) chlorophyll, and 0.18% phenolics. The astaxanthin content was found to be 1.94% of biomass on dry weight basis (*i.e.*, it constituted 88% of total carotenoids). The absorption spectra of the extract from *H. pluvialis* cells showed a major peak at 470–474 nm (carotenoid) and a minor peak at 661–663 nm (chlorophyll). The acetone extract used for different antioxidant assays contained 5, 7, and 9 ppm levels of total carotenoids.

Carotenoid Composition in *H. pluvialis* by HPLC

In the order of elution through a C_{18} column, the carotenoids are xanthophylls, chlorophylls, and hydrocarbon carotenoids, and these pigments were separated within 21 min (Fig. 2). The relative percentage of xanthophylls such as violaxanthin (1.5%), free astaxanthin (4.5%), lutein (3.8%), zeaxanthin (2.1%), α-carotene (1.7%), β-carotene (3.4%), and astaxanthin esters (83.7%) were determined. These carotenoids were eluted under isocratic condition and confirmed by their retention time and absorption spectra of respective reference standards, as shown in Fig. 3. Among the carotenoids eluted, astaxanthin and its esters formed the major proportion of carotenoids followed by violaxanthin, free astaxanthin, lutein, zeaxanthin, α-carotene, and β-carotene.

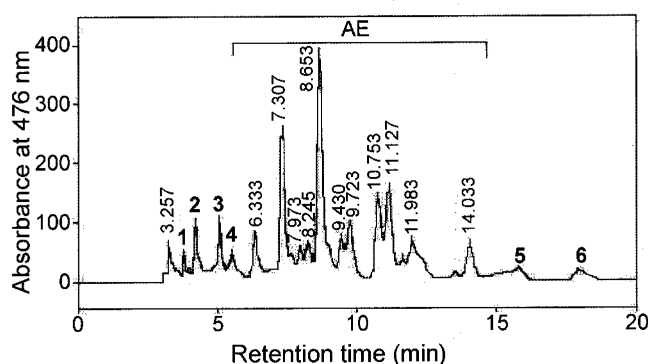

Fig. 2. HPLC profile of carotenoids from *H. pluvialis* extract: **(1)** violaxanthin, **(2)** astaxanthin, **(3)** lutein, **(4)** zeaxanthin, **(AE)** astaxanthin esters, **(5)** α-carotene, and **(6)** β-carotene.
Refer to Methods section for HPLC conditions.

Identification of Carotenoids from *H. pluvialis* by LC–MS (APCI)

LC–MS (APCI) has been applied for analysis of different carotenoids of *H. pluvialis*. LC–MS (APCI) was used for determination of the molecular mass of each peak. Because the ionization mode was positive, most of the *m/z* data are $[M+H]^+$ and the mass data of compounds identified are given in Table 1. Based on the mass fragmentation interpretation, violaxanthin, neoxanthin, astaxanthin, lutein, zeaxanthin, α-carotene, β-carotene, echinenone, 7,8,7',8'-tetradehydro-astaxanthin, and antheraxanthin were identified in *H. pluvialis* extract. The carotenoids and mass spectral fragmentation data are shown in Fig. 4.

Antioxidant Assay Using β-Carotene Linoleate Model System (β-CLAMS)

The mechanism of bleaching of β-carotene is a free-radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid [1, 4, 7]. β-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules causing these to lose their double bonds by oxidation; the compound loses its chromophore and characteristic orange color, which can be monitored

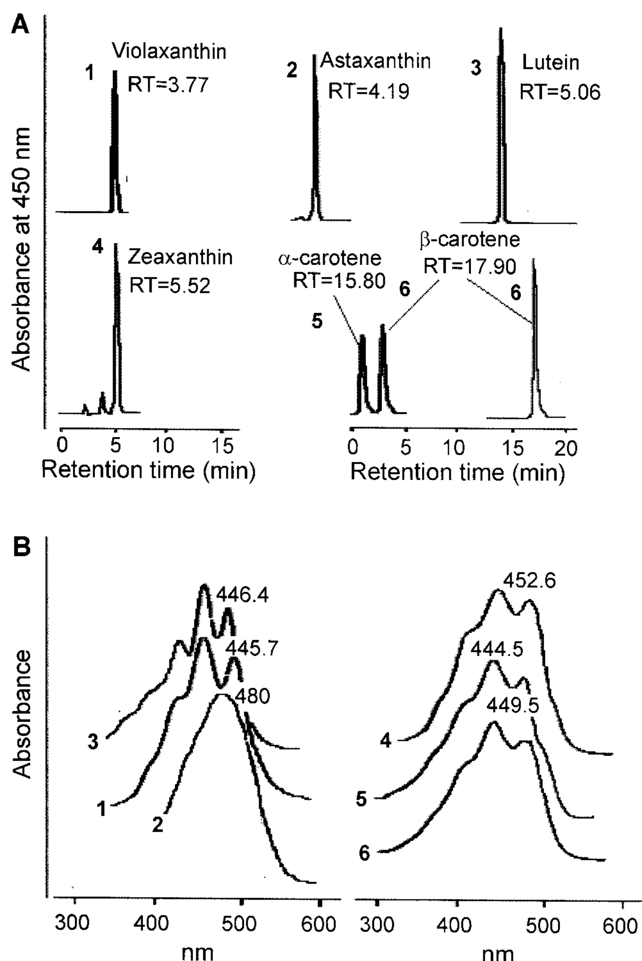

Fig. 3. HPLC profiles of standard carotenoids (**A**) and absorption spectra (**B**) of carotenoids eluted from *H. pluvalis*: (**1**) violaxanthin, (**2**) astaxanthin, (**3**) lutein, (**4**) zeaxanthin, (**5**) α-carotene, and (**6**) β-carotene.

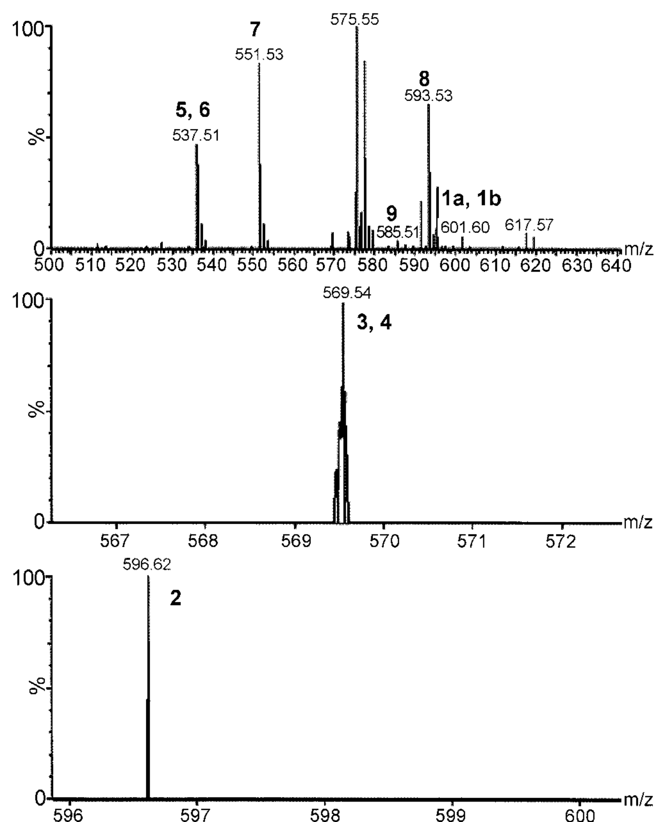

Fig. 4. LC–MS (APCI) profile of carotenoids from *H. pluvialis* extract: (**1a**) violaxanthin, (**1b**) neoxanthin, (**2**) astaxanthin, (**3**) lutein, (**4**) zeaxanthin, (**5**) α-carotene, (**6**) β-carotene, (**7**) echinenone, (**8**) 7,8,7',8'-tetradehydroastaxanthin, and (**9**) antheraxanthin.

spectrophotometrically. The antioxidant activity of *H. pluvialis* extract as measured by the bleaching of β-carotene is shown in Fig. 5A. *H. pluvialis* exhibited 67%, 78%, and 89% of antioxidant property (ED_{50}=4.5 ppm) in the β-carotene linoleate model system at 5, 7, and 9 ppm levels of carotenoid, which is 85–95% of BHA.

Hydroxyl Radical Scavenging Activity

The hydroxyl radical is supposed to be one of the fast initiators of the lipid peroxidation process, obstructing hydrogen atoms from unsaturated fatty acids. The hydroxyl radical scavenging activity of *H. pluvialis* was estimated

Table 1. Identification of carotenoids from *H. pluvialis* extract elucidated by $APCI^+$ ion mode showing their molecular mass and molecular formula.

Peak No	Identification of carotenoids	Molecular formula	Approximate molecular mass	Fragmentation mass	Identity
1a	Violaxanthin	$C_{40}H_{56}O_4$	600.41	601.60	$[M+H]^+$
1b	Neoxanthin	$C_{40}H_{56}O_4$	600.41	601.60	$[M+H]^+$
2	Astaxanthin	$C_{40}H_{52}O_4$	596.38	596.62	$[M+H]^+$
3	Lutein	$C_{40}H_{56}O_2$	568.42	569.54	$[M+H]^+$
4	Zeaxanthin	$C_{40}H_{56}O_2$	568.42	569.54	$[M+H]^+$
5	α-Carotene	$C_{40}H_{56}$	536.43	537.51	$[M+H]^+$
6	β-Carotene	$C_{40}H_{56}$	536.43	537.51	$[M+H]^+$
7	Echinenone	$C_{40}H_{54}O$	550.41	551.53	$[M+H]^+$
8	7,8,7',8'-Tetradehydroastaxanthin	$C_{40}H_{48}O_4$	592.35	593.53	$[M+H]^+$
9	Antheraxanthin	$C_{40}H_{56}O_3$	584.42	585.51	$[M+H]^+$

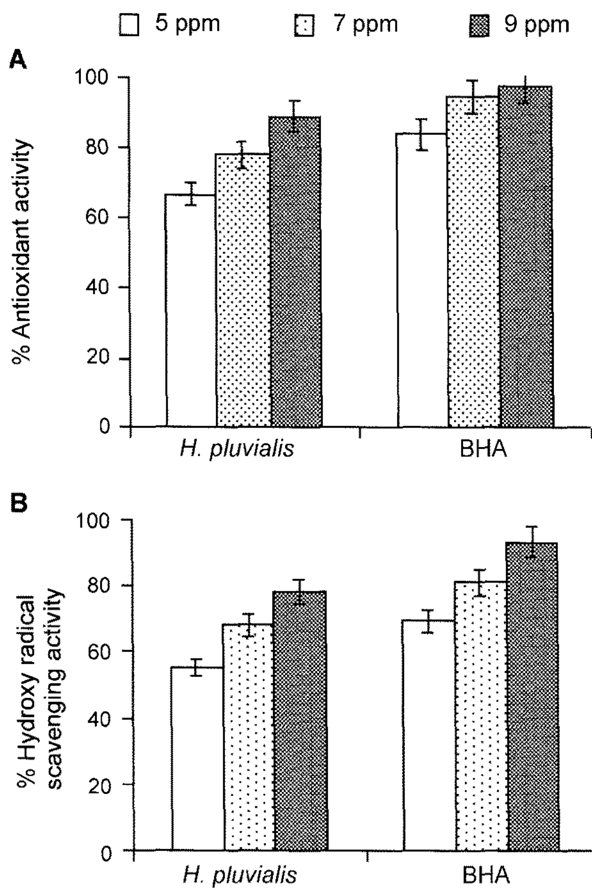

Fig. 5. Antioxidant activity of *H. pluvialis* extract from the (**A**) β-CLAMS method, and (**B**) hydroxy radical scavenging.
Data represent an average of 3 replicates. Bars indicate mean±SD.

by generating the hydroxyl radical using the Fe^{3+}/ascorbic acid system. The hydroxyl radicals formed by the oxidation reacts with DMSO to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. The hydroxyl radical scavenging activity of the algal extract is shown in Fig. 5B. *H. pluvialis* exhibited 55%, 68%, and 78% of hydroxyl radical scavenging activities at 5, 7, and 9 ppm levels of carotenoid (ED_{50}= 3.7 ppm), which is 85–90% of BHA. The *H. pluvialis* extract showed an inhibitory effect on lipid peroxidation.

Changes of Lipid Peroxide Level in Rat Tissues

As observed in model systems, *H. pluvialis* extract also exhibited an inhibitory effect on lipid peroxidation in brain (85%), kidney (80%), and liver (79%) at the 9 ppm level of carotenoid (ED_{50}=4.5 ppm) concentration (Fig. 6A, 6B, and 6C) and it was found to be dose-dependent. The data show that the inhibitory activity of algal extract on lipid peroxidation in the brain was similar to that in the kidney and liver. This can be attributed to the same mechanism that may be involved in these tissues.

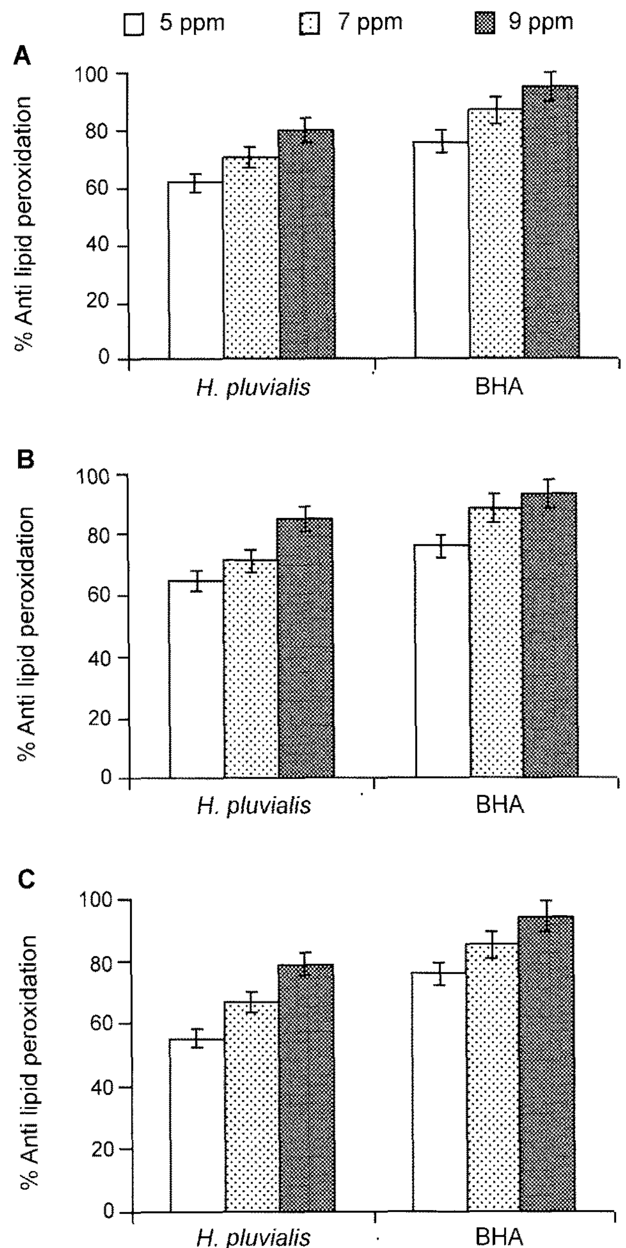

Fig. 6. Antilipid peroxidation of *H. pluvialis* extract on (**A**) kidney, (**B**) brain, and (**C**) liver.
Data represent an average of 3 replicates. Bars indicate mean±SD.

Inhibitory Effect on Lipid Peroxidation in Liposomes

Lipid peroxidation is a free-radical-mediated propagation of oxidative damage to polyunsaturated fatty acids involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants. To evaluate the antioxidant activity of the algal extract, a liposome model system was used. Malondialdehyde is the major product of lipid peroxidation that reacts with TBA to form a pink chromogen (diadduct), which can be detected spectrophotometrically at 532 nm. The antioxidative

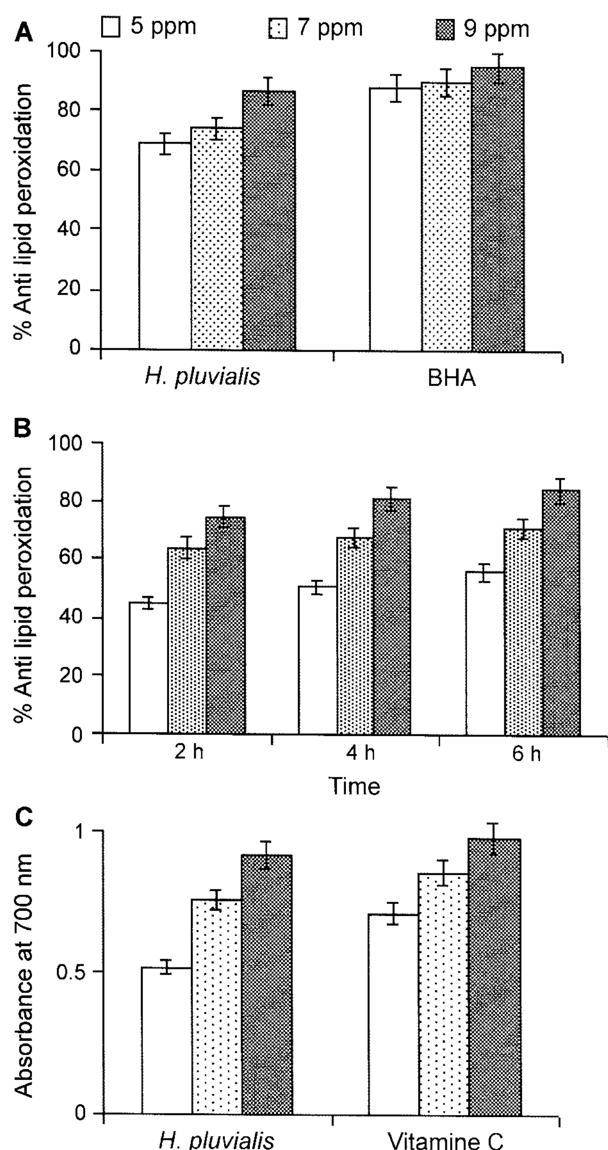

Fig. 7. **(A)** Antilipid peroxidation of *H. pluvialis* extract on liposome, **(B)** time course effect of *H. pluvialis* extract on human low-density lipoproteins, and **(C)** reducing power of *H. pluvialis* extract.

Data represent an average of 3 replicates. Bars indicate mean±SD.

property of the algal extract in the liposome system, induced by $FeCl_3$ plus ascorbic acid, is shown in Fig. 7A. As in BHA, the antioxidant property of the alga extract in the liposome system was significantly higher and it was found to be dose-dependent. The extract exhibited 69%, 74%, and 87% inhibition of peroxidation of lecithin at the 5, 7, and 9 ppm levels of carotenoid (ED_{50}=3.6 ppm). The result shows that the extracts used have a strong antioxidant action in the liposome model system. The carotenoids present in green algae *Dunaliella salina* [4], *Botryococcus braunii* [24], and seaweed *Kappaphycus alvarazzi* [7] may prevent the destructive effect of lipid peroxides *in vitro* by lowering their levels. Moreover, they have reported that the antioxidant property of the algae and the seaweed is due to the higher levels of carotenoids and phenolic compounds present in them. Similarly, the *H. pluvialis* extracts used in this study may play an important role in protecting the cells from lipid peroxides.

Inhibitory Effect on LDL Oxidation

Oxidative modification is known to play an important role in the pathogenesis of atherosclerosis and coronary heart diseases, and the dietary antioxidants that protect LDL from oxidation may therefore reduce atherogenesis [22, 29]. The antioxidant activity of *H. pluvialis* extracts against human LDL oxidation is shown in Fig. 7B. The polyunsaturated fatty acids of human LDL were oxidized and the malondialdehyde formed was estimated by using the TBA method. The average induction time for copper-mediated LDL oxidation was around 20 min without addition of algal extracts. The algal extract protected LDL from oxidation, as measured by the prolongation of induction time of the formation of conjugated dienes. The algal extract exhibited 45%, 64%, and 75% protection at the 5, 7, and 9 ppm levels of carotenoid (ED_{50}=5 ppm), respectively, at the end of 2 h after induction of oxidation. Protection was 51%, 68%, and 82% and 56%, 73%, and 84% at the 5, 7, and 9 ppm levels of carotenoids used at the end of 4 h and 6 h, respectively. The result indicates a dose-dependent inhibition effect of *H. pluvialis* against LDL oxidation.

Reducing Power

The reducing power of *H. pluvialis* extract was found to be 0.52, 0.765, and 0.89 at the 5, 7, and 9 ppm levels of carotenoids compared with standard vitamin C (Fig. 7C). The presence of reducing power indicates that the *H. pluvialis* extract has electron donors and can react with free radicals to convert them to more stable products and terminate the radical chain reaction. The antioxidant action of reductones is based on the breaking of the free radical chain by the donation of a hydrogen atom. The reducing power of *H. pluvialis* extract indicates that the marked antioxidant activity of algae is believed to be due to the presence of carotenoids. In particular, high level of astaxanthin may act as a strong antioxidant as reductones by donating the electrons and reacting with free radicals to convert them to a more stable product and terminate the free radical chain reaction.

DISCUSSION

The role of antioxidants in health and disease has been realized beyond doubt and the search for different sources of antioxidants, especially natural ones, has acquired newer

dimensions. The algae, both micro and macro, are also under exploration for bioactive molecules. Astaxanthin, astaxanthin esters, and total carotenoid from *H. pluvialis* were evaluated for anti-ulcer properties in ethanol-induced gastric ulcer in rats by Kamath *et al.* [11]. The antioxidant activity of algal extracts was reported to be dependent on the chemical components of the extracts that mainly consisted of carotenoids, polyphenols, tocopherols, and vitamin C [1, 4, 7, 24]. These substances can act as potent antioxidants in protecting lipid peroxidation, and by free radical scavenging and hydroxy radical scavenging activities by different modes of action [1]. Palozza and Krinsky [21] reported astaxanthin as a highly potential antioxidant in protecting membranous phospholipids and other lipids against peroxidation. Velioglu *et al.* [32] reported a strong relationship between the total phenolic content and antioxidant activity in selected fruits, vegetables, and grain products. Active oxygen species regulate carotenoids biosynthesis in some microorganisms such as yeast *Phaffia rhodozyma* [27], and green algae *H. pluvialis* [13] and *Dunaliella bardawil* [28]. The protective action of β-carotene against oxidation, oxidation-mediated diseases in animals, and the carotenoid isomers in algae have shown to inhibit the LDL-oxidation in diabetes mellitus patients [15]. Natural β-carotene was shown to have higher bioavailability compared with synthetic ones [2]. Lutein constituted the major carotenoid in *B. braunii* extract with a bioavailability higher than β-carotene, and is considered to be an active agent in the prevention of chronic diseases such as cataracts, age-related macular degeneration, and atherosclerosis, besides its use as feed additive in poultry farming as well as food dye [14]. The present result shows that *H. pluvialis* extract is capable of preventing lipid peroxidation through scavenging free radicals and hydroxy radicals in living cells. Therefore, *H. pluvialis* extract can be used for various applications such as health supplements, pharmaceuticals, and nutraceuticals.

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