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Characterization of Microalgal Carotenoids by Mass Spectrometry and Their Bioavailability and Antioxidant Properties Elucidated in Rat Model

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Of the total carotenoids in respective algal samples, β-carotene in *Spirulina platensis* was 69.5%, astaxanthin and its esters in *Haematococcus pluvialis* was 81.38%, and lutein in *Botryococcus braunii* was 74.6%. The carotenoids were characterized by mass spectrometry. A time−course study of carotenoids in rats after administration of microalgal biomass showed peak levels in plasma, liver, and eyes at 2, 4, and 6 h, respectively. β-Carotene accumulation in *Spirulina*-fed rats was maximum in eye tissues at 6 h. Similarly, levels of astaxanthin and lutein in *Haematococcus*- and *Botryococcus*-fed rats were also maximal in eye tissues. Astaxanthin from *H. pluvialis* showed better bioavailability than β-carotene and lutein. The antioxidant enzymes, catalase, superoxide dismutase, peroxidase, and TBARS were significantly high in plasma at 2 h and in liver at 4 h, evidently offering protection from free radicals. This study implies that microalgae can be a good source of carotenoids of high bioavailability and nutraceutical value.

KEYWORDS: *Spirulina*; *Haematococcus*; *Botryococcus*; carotenoids; bioavailability; antioxidant enzymes

INTRODUCTION

There is an increasing interest in the use of antioxidative molecules in health foods since they have the capacity to quench free radicals, thereby protecting cells and tissues from oxidative damage (*1*). They are also useful in preventing deterioration of food products during processing and storage (*2*). Carotenoids also influence cellular signaling and may trigger sensitive regulatory pathways. More than 600 naturally occurring carotenoids have been characterized; among them, β-carotene is recognized as the most prominent antioxidant (*3*).

Spirunlia platensis is a multicellular, filamentous cyanobacterium, or a blue-green alga of cyanophyta. *S. platensis* contains β-carotene, vitamins, and minerals, and it has received attention as a most promising and highly nutritious food source (*4*, *5*). *Spirulina* was proven to exhibit various biological activities such as lowering plasma cholesterol levels and blood pressure and improving the vitamin A levels in blood (*6*, *7*). Biological properties of *Spirulina* are attributed to components including ω-3 or ω-6 fatty acids, β-carotene, α-tocopherol, phycocyanin, and phenolic compounds (*8*). The bioavailability of *Spirulina* carotenoids has been demonstrated in both rats and chicken (*9*, *10*). *Spirulina* has been commercialized in several countries for its use in health foods and for therapeutic purposes, particularly for its valuable constituents, proteins, and vitamins (*11*). It is also a potential therapeutic agent in oxidative stress-induced diseases (*12*, *13*).

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Haematococcus pluvialis is a unicellular green alga belonging to the family chlorophyceae. It is known to accumulate carotenoids under stress conditions (*14*). Astaxanthin is the major carotenoid in *Haematococcus*, and it exists mainly as Astaxanthin esters (AE) (70% monoester, 15−20% diester, and 4−5% free form) (*15*). Astaxanthin has gained nutraceutical and pharmaceutical importance because of its high antioxidant activity (*15*). It is commercially available as an antioxidant food supplement and approved by the Swedish Health Food Council Advisory Board (*16*). Bioavailability of esterified astaxanthin in human volunteers has been investigated (*16*, *17*). In the United States, astaxanthin is permitted for use as a food color additive by the Food and Drug Administration (*18*) via induced pigmentation of cultured salmons. Because of demand for the natural astaxanthin in the fast-growing nutraceutical market, *H. pluvialis* is being cultivated on a large scale as a rich source of natural astaxanthin (*19*).

Botryococcus braunii is a green colonial microalga belonging to the family chlorophyceae. It is known for the production of hydrocarbons, exopolysaccharides, and carotenoids. This alga is grouped into three different races, A, B, and L, depending on the type of hydrocarbons that they synthesize (*20*). The presence of carotenoids is more pronounced in races B and L (*20*). Both of the races produce almost equal amounts of β-carotene, echinenone, canthaxanthin, lutein, violaxanthin, loroxanthin, and neoxanthin. However, lutein is the major carotenoid (22−29%) reported in the linear phase of these races. Canthaxanthin (46%) together with echinenone (20−28%) are the dominating carotenoids in the

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stationary phase (*21*). Grung et al. (*22*) reported the presence of adonixanthin in the "L" race in the stationary phase. Carotenoids such as botryoxanthin-A (*23*), botryoxanthin-B, α-botryoxanthin (*24*), and braunixanthin 1 and 2 (*25*) isolated from the B race may contribute to the color of the algal colonies. The present study was undertaken to evaluate the bioavailability of carotenoids and their antioxidant properties from *S. platensis*, *H. pluvialis*, and *B. braunii* biomasses in an experimental rat model.

MATERIALS AND METHODS

Source of Chemicals. High-performance liquid chromatography (HPLC) grade acetonitrile, methanol, and dichloromethane were purchased from Rankem Chemicals Ltd. (Mumbai, India). Analytical grade acetone, hexane, chloroform, methanol, and petroleum ether were purchased from Sisco Research Laboratories, Pvt, Ltd., (Mumbai, India). Nitroblue tetrazolium (NBT), trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Tris-HCl buffer, standard astaxanthin, xanthophylls (lutein and zeaxanthin), and β-carotene were obtained from Sigma Chemicals Co. (St. Louis, MO).

Microalgae Culture and Quantification of Carotenoids. *S. platensis* was grown in modified Zarrouk's medium (*26*). The cultures were incubated at 25 ± 1 °C under 1.2 ± 0.2 klux light intensity with a 16 h:8 h photoperiod. *H. pluvialis* was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institut, Universitat Gottingen (Gottingen, Germany), and it was maintained on autotrophic medium and agar slants (*27*). *H. pluvialis* grown in modified autotrophic Bold basal medium (*28*) and carotenoid formation was obtained under salinity stress as reported earlier by Sarada et al. (*27*). The encysted red cell biomass rich in carotenoids was harvested, lyophilized, and stored at 4 °C. *B. braunii* (CFTRI-K) was isolated from Indian fresh water bodies of Kodaikanal (latitude 10.31N and longitude 77.32E), India, and identified as race "A" (*29*), and the stock culture was maintained in modified CHU13 medium (*30*). *B. braunii* was grown in liquid medium for the biomass. The cultures were incubated at 25 ± 1 °C under 1.5 ± 0.2 klux light intensity with a 16 h:8 h light:dark cycle. After 4 weeks of growth, the culture was harvested by centrifugation (*20*), and the biomass was lyophilized and used for the extraction of total carotenoids.

A known quantity (1 g) of *S. platensis*, *H. pluvialis*, and *B. braunii* biomasses were homogenized and extracted repeatedly with acetone, chloroform, methanol, and petroleum ether separately. Extracts were pooled, evaporated using a rotavopar (Buchi, Germany), and redissolved in acetone. The absorbance of extracts was measured at 450, 470, 645, and 661.5 nm to estimate the content of total carotenoid and chlorophyll using Lichtenthaler equations (*31*). Astaxanthin was determined at 480 nm using an extinction coefficient of 2500 at 1% level by the method of Davies (*32*).

Bioavailability of Carotenoids. Animal experiments were performed after due clearance from the Institutional Animal Ethics Committee of CFTRI, Mysore (IAEC No. 116/08). Male Wistar rats [OUTB Wistar, IND-Cft 2c] weighing 41 ± 2 g were housed individually in steel cages at room temperature (28 ± 2 °C). A 12 h light/ dark cycle was maintained, and the rats received daily a fresh pellet diet (Amrut feeds, Sangli, India) and had free access to tap water. The leftover diets were weighed and discarded. After 7 days of acclimatization, rats were deprived of food for 12 h and administered a single dose of *S. platensis*, *H. pluvialis*, and *B. braunii* biomass as a source of β-carotene, astaxanthin, and lutein, respectively. Diet samples were processed for the analysis of three carotenoids by HPLC (*33*).

Group of rats (n = 25) were administered by intubations to the stomach a single dose (134, 33, 328 mg/rat) of *S. platensis or H. pluvialis*, or *B. braunii* biomass solubilized in olive oil as source of 200 μM equivalent of β-carotene, astaxanthin, and lutein. Each group was divided into five subgroups (n = 5/subgroup) to measure the time−course response of carotenoids in plasma and tissues for 9 h. A separate group (n = 5) not fed either of the biomasses was considered as 0 h. Rats in 0 h and in each treatment group (n = 5/time point) at 2, 4, 6, and 9 h after gavage were exsanguinated under mild ether anesthesia, blood was collected from the heart into heparinized tubes, and plasma was separated immediately by centrifugation at 1000*g* for 15 min at 4 °C. Liver and eyes were dissected and washed with ice cold saline and immediately stored at −70 °C until analyzed.

HPLC Analyses of Carotenoids. Analyses of individual carotenoids viz. β-carotene, astaxanthin, and lutein from plasma, liver, and eyes of rats were done by HPLC using the method of Baskaran et al. (*34*). In brief, to the plasma (0.8 mL), 3 mL of dichloromethane:methanol (1:2, v/v) containing α-tocopherol (2 mM) was added. After the solution was mixed, hexane (1.5 mL) was added, stirred, and centrifuged at 1000*g* for 15 min, and the resulting upper hexane/dichloromethane phase was collected. This extraction was repeated for the lower phase two more times using 1 mL of dichloromethane and 1.5 mL of hexane. The hexane extracts were pooled and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 μL of mobile phase and used for HPLC analysis of β-carotene, astaxanthin, and lutein.

Liver and eye samples (1 g) were homogenized separately with nine parts of ice-cold isotonic saline with a Potter−Elvehjem homogenizer. β-Carotene, astaxanthin, and lutein were extracted from 0.8 mL of the homogenate and analyzed by HPLC with the same procedure used for plasma.

In the case of the groups fed with the *B. braunii* biomass (lutein source), the liver extract was further saponified by incubating in 2 mL of 10 M potassium hydroxide at 60 °C for 45 min. Subsequently, lutein was extracted and analyzed by HPLC with the same procedure used for plasma. Samples were handled on ice under dim yellow light to minimize isomerization and oxidation of carotenoids by light irradiation.

Liquid Chromatography−Mass Spectrometry (LC-MS) of Carotenoids. The carotenoids were further confirmed in *S. platensis*, *H. pluvialis*, and *B. braunii* biomasses by using the Waters 2996 modular HPLC system (autosampler, gradient pump, thermo-regulator, and diode array detector), coupled to a Q-Tof Ultima (UK) mass spectrometer. In brief, the atmospheric pressure chemical ionization (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.

Antioxidant Enzymes. The catalase assay was carried out by the method of Aebi (*35*). Plasma (0.2 mL) from each group was diluted with 1.9 mL of phosphate buffer (125 mM, pH 7.4). The reaction was initiated by the addition of 1 mL of hydrogen peroxide (H_2O_2) (30 mM). Blank without plasma was prepared with 2.1 mL of phosphate buffer and 1 mL of H_2O_2 (30 mM). The decrease in absorbance due to the decomposition of H_2O_2 was measured at the end of 1 min against the blank at 240 nm. Units of catalase activity were expressed as the amount of enzyme that decomposes 1 μM H_20_2 per minute at 25 °C. The specific activity was expressed in terms of units per mg protein.

The assay of superoxide dismutase (SOD) was measured on the reduction of NBT to water insoluble blue formazan, as described by Fedovich (*36*). To the plasma (0.2 mL), 125 mM sodium carbonate (1 mL), 24 μM NBT (0.4 mL), and 0.1 mM ethylenediaminotetraacetic acid (EDTA) (0.2 mL) were added. The reaction was initiated by adding 1 mM hydroxylamine hydrochloride (0.4 mL). Zero time absorbance was taken at 560 nm followed by recording of the absorbance after 5 min at 25 °C. Units of enzyme required for the reduction of NBT by 50% were determined. The specific activity was expressed in terms of units per mg protein.

The peroxidase assay was carried out as per Nicholas (*37*). To the liver homogenate (0.5 mL), 10 mM potassium iodide (KI) (1 mL) and 40 mM sodium acetate (1 mL) solution were added. The absorbance of KI was read at 353 nm, which indicates the amount of peroxidase. To the reaction mixture, 20 μL of H_2O_2 (15 mM) was added, and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to bring about a one OD per minute. The specific activity was expressed in terms of units per milligram protein.

Lipidperoxidation Assay. TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (*38*). The TBA reactive substances were measured in terms of MDA and expressed as MDA equivalent. To the plasma and liver homogenate (0.5 mL), 1 mL of 0.15 M potassium chloride (KCl) was added, and the peroxidation was initiated by adding 250 μL of 0.2 mM ferric chloride. The reaction mixture was incubated at 37 °C for

Table 1. Components and Carotenoids in Microalgal Biomasses[a]

components/carotenoids	%		
	S. platensis	*H. pluvialis*	*B. braunii*
biomass yield	1.5 ± 0.04	2.2 ± 0.02	2.5 ± 0.05
total carotenoids	2.0 ± 0.08	2.3 ± 0.12	0.35 ± 0.06
total phenolics	0.2 ± 0.01	0.28 ± 0.03	0.17 ± 0.02
astaxanthin (1)	ND	3.8 ± 0.25	0.4 ± 0.02
lutein (2)	14.4 ± 0.58	1.4 ± 0.20	74.6 ± 2.3
zeaxanthin (3)	2.7 ± 0.16	4.2 ± 0.38	1.4 ± 0.18
β-carotene (4)	69.5 ± 2.65	1.7 ± 0.98	1.5 ± 0.10
AE	ND	77.58 ± 3.12	ND
UI	13.4 ± 0.07	11.32 ± 0.16	22.1 ± 0.41

[a] Twenty-one day old cultures analyzed for various parameters. Values are means ± SDs (n = 5); ND refers to not detected. One to four refers to carotenoids, UI refers to unidentified peaks in **Figure 1**, and AE refers to astaxanthin esters.

30 min. The reaction was stopped by adding 2 mL of an ice-cold mixture of 0.25 N HCl containing 15% TCA, 0.30% TBA, and 0.05% BHT and heated at 80 °C for 60 min. The samples were cooled, and results were expressed as MDA equivalent, which was calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. One unit of lipid peroxidation was defined as the amount of enzyme required to convert 1 mole of TBA into thiobarbituric acid reactive substances (TBARS). The specific activity was expressed in terms of units per milligram protein.

Estimation of Protein Content. Protein was determined as per the method of Lowry et al. (*39*)

Statistical Analysis. Data were tested for the homogeneity of variances by the Bartlett test. When homogeneous variances were confirmed, the data were tested by repeated measures analysis of variance (ANOVA), and significant differences in means among groups and at different time points were evaluated by Tukey's test. The percent differences between groups were calculated and considered significant at the level of $p < 0.05$.

RESULTS

Biomass Yield, Total Carotenoid, and Phenolic Content in Microalgae. The yield of biomass, total carotenoid, and phenolic contents in microalgae from 21 day old cultures are shown in **Table 1**. The biomass yield was 1.5, 2.2, and 2.5 g/L in *S. platensis*, *H. pluvialis*, and *B. braunii*. The total carotenoid content was found to be 2, 2.3, and 0.35%, whereas the total phenolic content was 0.2, 0.28, and 0.17% in *S. platensis*, *H. pluvialis*, and *B. braunii*, respectively.

Identification of Carotenoids by HPLC and LC-MS (APCI). The HPLC profiles of carotenoids in the *S. platensis*, *H. pluvialis*, and *B. braunii* are shown in **Figure 1A−C**. Algal biomass contained three classes of pigments such as xanthophylls, chlorophylls, and carotenoids, which were eluted through a C_{18} column and separated within 30 min. The detectable xanthophylls comprised of astaxanthin (peak 1), lutein (peak 2), zeaxanthin (peak 3), and β-carotene (peak 4) and few unidentified peaks. These carotenoids were eluted under isocratic conditions and confirmed by their retention times and the absorption spectra of the respective reference standards. The carotenoid compositions of *S. platensis*, *H. pluvialis*, and *B. braunii* are shown in **Table 1**.

In *S. platensis*, β-carotene (69.5%) was the major one, and others include lutein (14.4%), zeaxanthin (2.7%), and unidentified peaks (13.4%). The *H. pluvialis* biomass contained astaxanthin esters (AE, 77.58%), and others are astaxanthin (3.8%), lutein (1.4%), zeaxanthin (4.2%), β-carotene (1.7%), and unidentified peaks (11.32%). The relative percentage of carotenoids in *B. braunii* was determined for astaxanthin (0.4%), lutein (74.6%), zeaxanthin (1.4%), β-carotene (1.5%), and unidentified (22.1%).

MS (APCI) was applied to confirm the carotenoids from *S. platensis*, *H. pluvialis*, and *B. braunii*. The molecular mass of each carotenoid peak was identified with their respective mass spectra. Neoxanthin, violaxanthin, α-carotene, and β-carotene

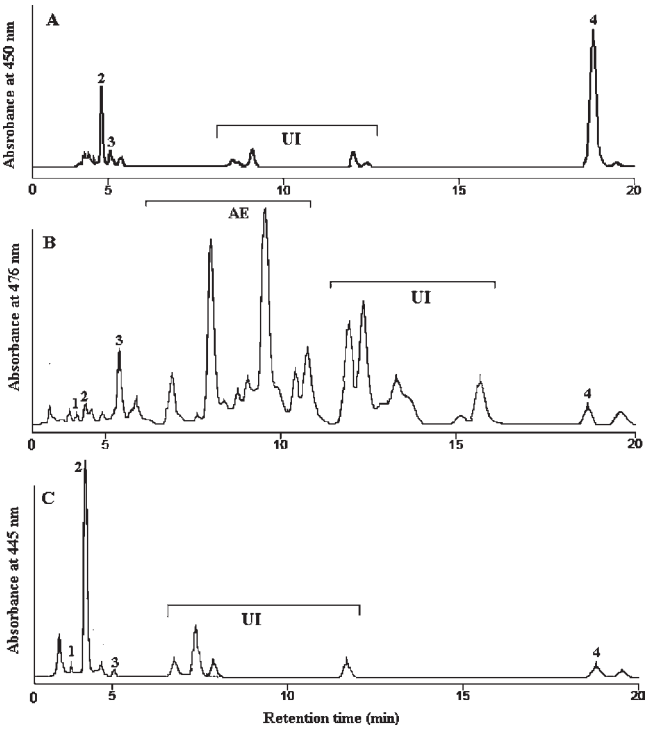

Figure 1. HPLC profile of carotenoids from *S. platensis* (**A**), *H. pluvialis* (**B**), and *B. braunii* (**C**) biomasses. Peaks: 1, astaxanthin; 2, lutein; 3, zeaxanthin; 4, β-carotene; AE, astaxanthin esters; and UI, unidentified peaks.

are isomers having similar molecular masses. On the basis of the fragmentation data, various carotenoids were identified in *S. platensis*, *H. pluvialis*, and *B. braunii* biomasses (**Figure 2A−C**).

Bioavailability of Carotenoids. The time-course response of β-carotene in plasma, liver, and eyes of rats over 0−9 h after gavage of *Spirulina* was examined. β-Carotene was not detected in the plasma, liver, and eye of the 0 h group, but after gavage of the *S. platensis* biomass, its maximum level recorded at 2 h was 70.56 ± 3.14 pmol/mL, at 4 h was 100.35 ± 4.91 pmol/g, and at 6 h was 146.29 ± 6.12 pmol/g in plasma, liver, and eyes respectively (**Figure 3A**). The β-carotene (146.29 ± 6.12 pmol/g) accumulation in the eyes was higher at 6 h than in liver tissues.

Astaxanthin levels in plasma, liver, and eyes of rat over 0−9 h after gavage of the *H. pluvialis* biomass is presented in **Figure 3B**. Astaxanthin was not detected in the 0 h group, but after gavage, however, it was detected in plasma (128.49 ± 2.58 pmol/mL), liver (131.23 ± 4.58 pmol/g), and eye (281.45 ± 5.12 pmol/g). The eye astaxanthin level was markedly higher at 6 h and lower at 9 h than those observed in liver. The mean astaxanthin values of eyes were higher by 3-fold as compared to liver tissues.

Similar to earlier experiments for β-carotene and astaxanthin, lutein levels were determined in samples of plasma, liver, and eyes of rats fed with *B. braunii*. Lutein was not detected in the zero time group; however, it was detectable in 2 h (43.27 ± 2.57 pmol/mL), 4 h (54.63 ± 3.41 pmol/g), and 6 h (259.23 ± 4.81 pmol/g) in plasma, liver, and eyes, respectively (**Figure 3C**). Eye tissues accumulated lutein more than in liver.

Antioxidant Property of Carotenoids. Antioxidant enzymes catalase, SOD, and peroxidase were measured in plasma and liver samples mainly to elucidate the activity of these enzymes upon feeding of algal biomass. Feeding of *S. platensis*, *H. pluvialis*, and *B. braunii* biomasses as a source of β-carotene, astaxanthin, and lutein, respectively, over 0−9 h to rats influenced plasma antioxidant enzymes such as catalase, SOD, and peroxidase (**Table 2**). Results showed maximum plasma catalase,

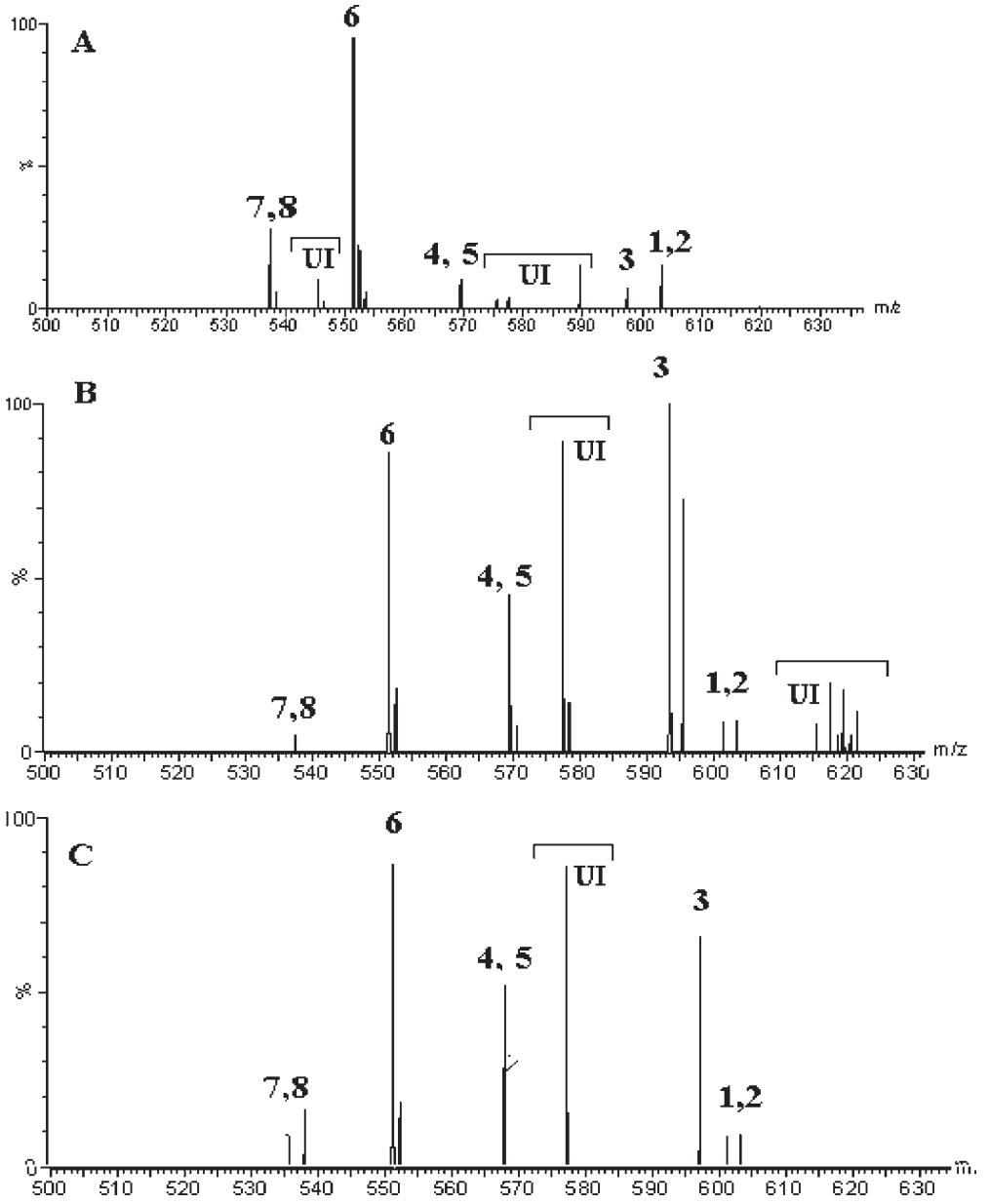

Figure 2. LC-MS (APCI) profile of *S. platensis* (**A**), *H. pluvialis* (**B**), and *B. braunii* (**C**). Peaks: 1, neoxanthin; 2, violaxanthin; 3, astaxanthin; 4, lutein; 5, zeaxanthin; 6, β-cryptoxanthin; 7 and 8, α- and β-carotene; and UI, unidentified peaks.

SOD, and peroxidase activity (116.48, 70.27, and 100.34%) at 2 h after intubation of the *S. platensis* biomass, when compared to the 0 h group. Similarly, the maximum activities of catalase, SOD, and peroxidase (134.06, 60.10, and 104.76%) were observed at 2 h in *H. pluvialis* treatment as a source of astaxanthin, when compared to the 0 h group. Catalase and peroxidase activities were 2.2- and 1.7-fold higher, when compared to SOD. As in the other two groups, the maximum catalase, SOD, and peroxidase activities (101.85, 46.49, and 88.29%) were found at 2 h after intubation of *B. braunii* as a source of lutein, when compared to the 0 h group.

Upon intubation of *S. platensis*, *H. pluvialis*, and *B. braunii* as a source of β-carotene, astaxanthin, and lutein to rats, the hepatic antioxidant enzymes, catalase, SOD, and peroxidase (**Table 3**) were analyzed. The results showed that the maximum catalase (52.06%), SOD (66.4%), and peroxidase (114.2%) activities were noticed at 4 h. As like *S. platensis*, the maximum activities of catalase, SOD, and peroxidase, 52.30, 66.24, and 118.81%, respectively, were also found at 4 h in *H. pluvialis* treatment. Antioxidant enzyme levels were also higher in the *B. braunii* treatment.

Changes of lipid peroxidation in plasma and liver of algal biomass-treated rats are shown in **Table 4**. The lipid peroxidation was found to be 0.71 nmol/mg protein at 2 h in *S. platensis* biomass-treated rats. Similarly, the *H. pluvialis* biomass showed the maximum lipid peroxidation of 0.57 nmol/mg protein at 2 h. As in the other two groups, the *B. braunii* biomass also exhibited 0.79 nmol/mg protein lipid peroxidation at 2 h. In the case of liver, the lipid peroxidation was found to be higher in *Spirulina* fed rats as compared to the other two algae treatments.

DISCUSSION

The present study was conducted to elucidate the bioavailability of β-carotene, astaxanthin, and lutein from microalgae *S. platensis*, *H. pluvialis*, and *B. braunii* biomasses by monitoring the postprandial plasma, liver, and eye response in rats over 9 h after their administration. The results showed that the bioavailability of astaxanthin and its esters from *H. pluvialis* was higher followed by β-carotene and lutein from *S. platensis* and *B. braunii*, respectively. The concentration of all of the carotenoids after a

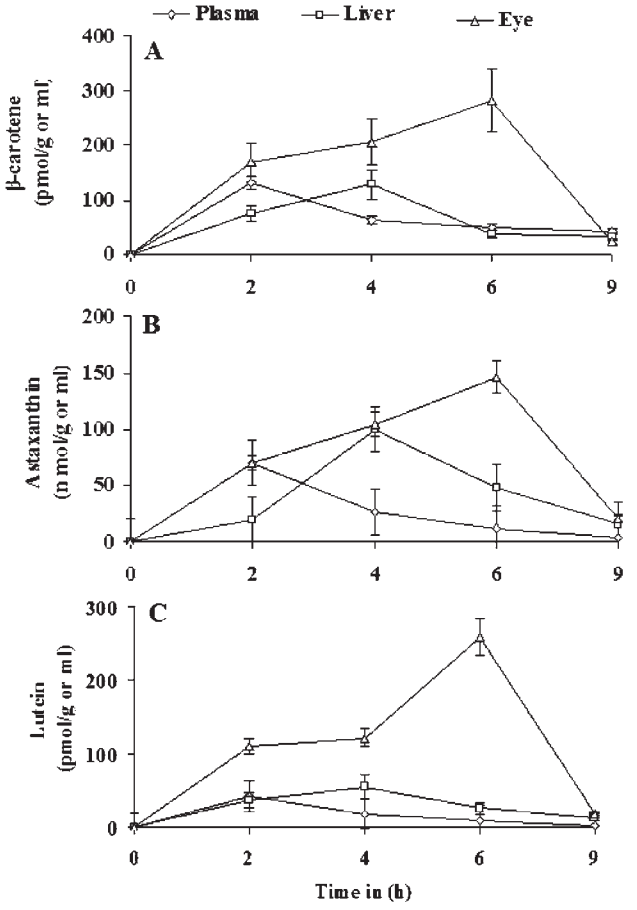

Figure 3. Carotenoids in plasma, liver, and eye in *S. platensis*- (**A**), *H. pluvialis*- (**B**), and *B. braunii*- (**C**) fed rats. Values represent the mean ± SD of five analyses. Values are significant at $p < 0.05$ as compared to the 0 h group.

single dose of microalgal biomass reached a maximum level at 2 h in plasma in all of the groups, although their concentration levels varied among carotenoids significantly (**Figure 3A−C**). This study indicates that the plasma astaxanthin level in rats from *H. pluvialis* biomass was 1.8- and 3.0-fold higher than β-carotene and lutein, whereas the level of β-carotene was 1.6-fold higher than lutein. The peak carotenoid concentration in the eye of experimental rats reached maximum levels at 6 h in all of the groups; however, the concentration varied significantly (**Figure 3A−C**). Evidently, the astaxanthin level in eyes from the *H. pluvialis* biomass was 1.9- and 2.1-fold higher than β-carotene and lutein. Also, the level of β-carotene was 1.1-fold higher than that of lutein. The presence of astaxanthin and its esters in *H. pluvialis* might be an added advantage to influence the higher bioavailability of astaxanthin in rats. The only study on humans to date confirmed the bioavailability of astaxanthin supplied in a single high dosage of 100 mg and its transport in the plasma by lipoproteins (*17*). Lutein and zeaxanthin are reported to be concentrated in the macula of the eye (*40*). The bioavailability of *trans*-β-carotene is reported to be three times more than that of *cis*-β-carotene (*41*). Natural β-carotene has been shown to have a higher bioavailability as compared to synthetic ones (*42*).

The influence of the algal carotenoids on the activities of catalase, peroxidase, and SOD was profound. The animals fed with microalgal biomass resulted in an increase in activity of the liver and plasma antioxidant enzymes (**Tables 2** and **3**), and they possibly act as potent free radical scavengers. Particularly, a high level of carotenoids may act as a strong antioxidant by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction (*43*). Our earlier studies have shown inhibition of lipid peroxidation by *B. braunii* extracts in vitro in brain, kidney, and liver tissues

Table 2. Activity of Catalase, SOD, and Peroxidase in the Plasma of Rats after a Single Dose of *S. platensis*, *H. pluvialis*, and *B. braunii* Biomass as a Source of β-Carotene, Astaxanthin, and Lutein[a]

	U/mg protein		
time (h)	*S. platensis*	*H. pluvialis*	*B. bruanii*
	catalase		
0	106.85 ± 3.59 a	106.85 ± 3.59 a	106.85 ± 3.59 a
2	231.31 ± 8.93 b	250.10 ± 12.03 b	215.68 ± 5.67 b
4	221.67 ± 5.95 b	243.58 ± 8.03 b	198.99 ± 8.07 c
6	176.06 ± 3.42 c	199.23 ± 5.47 c	185.03 ± 11.01 c
9	140.16 ± 2.73 d	178.55 ± 15.42 c	168.17 ± 12.17 d
	SOD		
0	7.57 ± 1.21 c	7.57 ± 1.21 c	7.57 ± 1.21 c
2	12.89 ± 2.65 a	12.12 ± 3.25 a	11.09 ± 1.97 a
4	8.48 ± 1.65 b	11.14 ± 6.32 a	10.35 ± 2.9 a
6	9.46 ± 0.35 b	10.01 ± 0.54 a	10.5 ± 2.2 a
9	11.59 ± 2.72 a	9.09 ± 2.51 b	9.86 ± 0.74 b
	peroxidase		
0	7.35 ± 1.55 c	7.35 ± 1.55 c	7.35 ± 1.55 c
2	14.95 ± 1.76 a	15.05 ± 0.92 a	13.84 ± 1.0 a
4	14.28 ± 3.43 a	12.86 ± 0.61 b	12.27 ± 3.4 b
6	12.53 ± 0.95 b	10.51 ± 0.61 b	10.53 ± 0.91 b
9	10.34 ± 1.99 b	9.95 ± 1.14 b	9.79 ± 0.30 b

[a] Rats were fed with a single dose of biomass containing 200 μM equivalent of carotenoids from the respective biomass and killed at 0, 2, 4, 6, and 9 h after the dose. Values are means ± SD ($n = 5$). Values not sharing a similar letter within the same column in a group over 0−9 h are significantly different ($P < 0.05$) as determined by ANOVA.

Table 3. Antioxidant Enzymes (Catalase, SOD, and Peroxidase) in the Liver of Rats after a Single Dose of *S. platensis*, *H. pluvialis*, and *B. braunii* Biomass as a Source of β-Carotene, Astaxanthin, and Lutein[a]

	U/mg protein		
time (h)	*S. platensis*	*H. pluvialis*	*B. bruanii*
	catalase		
0	251.52 ± 2.10 c	251.52 ± 2.10 c	251.52 ± 2.10 c
2	358.46 ± 4.40 a	336.67 ± 4.51 b	328.11 ± 6.36 b
4	382.48 ± 6.73 a	383.08 ± 1.81 a	371.29 ± 5.68 a
6	370.52 ± 5.26 a	374.45 ± 4.34 a	356.83 ± 2.15 a
9	331.76 ± 7.29 b	366.44 ± 4.34 a	344.72 ± 6.34 a
	SOD		
0	10.34 ± 0.54 c	10.34 ± 0.54 c	10.34 ± 0.54 c
2	13.41 ± 4.75 b	13.80 ± 4.47 b	12.91 ± 2.58 a
4	17.21 ± 1.19 a	17.19 ± 3.87 a	16.30 ± 4.54 a
6	15.03 ± 2.91 a	16.29 ± 1.21 a	15.64 ± 1.81 a
9	14.46 ± 2.96 b	15.65 ± 0.85 a	13.92 ± 0.78 b
	peroxidase		
0	7.44 ± 2.64 c	7.44 ± 2.64 c	7.44 ± 2.64 c
2	9.22 ± 4.39 c	10.11 ± 1.47 c	8.42 ± 2.83 c
4	15.94 ± 2.84 a	16.28 ± 2.25 a	13.34 ± 4.34 a
6	14.94 ± 6.33 a	14.63 ± 3.99 a	12.6 ± 5.95 a
9	11.38 ± 5.2 b	11.32 ± 2.37 b	9.43 ± 1.54 c

[a] Experimental details are the same as in **Table 2**.

Table 4. Antilipid Peroxidation after a Single Dose of *S. platensis*, *H. pluvialis*, and *B. braunii* Biomass as a Source of Astaxanthin, β-Carotene, and Lutein[a]

	TBARS (nmol/mg protein)		
time (h)	*S. platensis*	*H. pluvialis*	*B. braunii*
	plasma		
0	1.34 ± 0.05 b	1.34 ± 0.05 b	1.34 ± 0.05 b
2	0.71 ± 0.04 d	0.57 ± 0.06 d	0.79 ± 0.02 d
4	0.83 ± 0.09 c	0.59 ± 0.08 d	0.81 ± 0.06 d
6	0.87 ± 0.06 c	0.78 ± 0.02 c	0.90 ± 0.03 d
9	0.91 ± 0.09 c	0.82 ± 0.05 c	1.01 ± 0.08 c
	liver		
0	1.87 ± 0.08 a	1.87 ± 0.08 a	1.87 ± 0.08 a
2	0.91 ± 0.05 c	0.84 ± 0.06 c	1.06 ± 0.07 c
4	0.85 ± 0.01 c	0.72 ± 0.05 c	0.90 ± 0.02 d
6	1.12 ± 0.04 b	0.97 ± 0.03 b	1.21 ± 0.03 b
9	1.27 ± 0.06 b	1.02 ± 0.04 b	1.34 ± 0.02 b

[a] Experimental details are the same as in **Table 2**.

of rats (*44*). Antioxidant principles from red seaweed *Kappaphycus* and *Dunaliella salina* that produce β-carotene were also reported (*45*, *46*).

In conclusion, it was evident that an algal biomass is a potential source of carotenoids exhibiting bioavailability in experimental animals. The results also showed increased levels of antioxidant enzymes and prevention of lipid peroxidation through scavenging free radicals and hydroxyl radicals in living cells when the micoalgae biomass was fed to rats. This implies that the *S. platensis*, *H. pluvialis*, and *B. braunii* biomass can be used as source of carotenoids and also as nutritional or nutraceutical supplements.

ABBREVIATIONS USED

Butylated hydroxyanisole; butylated hydroxytoluene; EDTA, ethylenediaminotetraacetic acid; MDA, malondialdehyde; H_2O_2, hydrogen peroxide, TBARS, thiobarbituric acid reactive substances; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; APCI, atmospheric pressure chemical ionization.

LITERATURE CITED

(1) Cao, G.; Prior, R. L. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin. Chem.* **1998**, *44*, 1309–1315.

(2) Shyamala, B. N.; Gupta, S.; Jyothilakshmi, A.; Prakash, J. Leafy vegetable extracts—Antioxidant activity and effect on storage stability of heated oils. *Innovative Food Sci. Emerging Technol.* **2005**, *6*, 239–245.

(3) Olson, J. A.; Krinsky, N. I. Introduction: The colorful fascinating world of the carotenoids: Important physiologic modulators. *FASEB J.* **1995**, *9*, 1547–1550.

(4) Ciferri, O.; Tiboni, O. The biochemistry and industrial potential of *Spirulina*. *Annu. Rev. Microbiol.* **1985**, *39*, 503–552.

(5) Richmond, A. *Spirulina*. In *Micro-algal Biotechnology*; Borowitzka, M. A., Borowitzka, L., Eds.; Cambridge U.P.: Cambridge, 1988; pp 85−121.

(6) Ross, E.; Dominy, W. The nutritional value of dehydrated, blue-green algae (*Spirulina platensis*) for poultry. *Poult. Sci.* **1990**, *69*, 794–800.

(7) Annapurna, V.; Shah, N.; Bhaskaran, P.; Bamji, M. S.; Reddy, V. Bioavailability of *Spirulina* carotenes in preschool children. *J. Clin. Biochem. Nutr.* **1991**, *12*, 145–151.

(8) Chamorro, G.; Salazar, M.; Araujo, K. G; Dos Santos, C. P.; Ceballos, G.; Castillo, L. F. Update on the pharmacology of *Spirulina* (Arthrospira), an unconventional food. *Arch. Latinoam. Nutr.* **2002**, *52*, 232–240.

(9) Kapoor, R.; Mehta, U. Effect of supplementation of blue green alga (*Spirulina*) on outcome of pregnancy in rats. *Plant Foods Hum. Nutr.* **1993**, *43*, 29–35.

(10) Mitchell, G. V.; Grundel, E.; Jenkins, M.; Blakely, S. R. Effects of graded dietary levels of *Spirulina* maxima on vitamin A and E in male rats. *J. Nutr.* **1990**, *120*, 1235–1240.

(11) Hirahashi, T.; Matsumoto, M.; Hazeki, K.; Saeki, Y.; Ui, M.; Seya, T. Activation of the human innate immune system by *Spirulina*: Augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. *Int. Immunopharmacol.* **2002**, *2*, 423–434.

(12) Rimbau, V.; Camins, A.; Pubill, D.; Sureda, F. X.; Romay, C.; González, R.; Jiménez, A.; Escubedo, E.; Camarasa, J.; Pallàs, M. C-phycocyanin protects cerebellar granule cells from low potassium/serum deprivation-induced apoptosis. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2001**, *364*, 96–104.

(13) Bhat, V. B.; Madyastha, K. M. Scavenging of peroxynitrite by phycocyanin and phycocyanobilin from *Spirulina platensis*: Protection against oxidative damage to DNA. *Biochem. Biophys. Res. Commun.* **2001**, *5*, 262–266.

(14) Sarada, R.; Sila, B.; Suvendu, B.; Ravishankar, G. A. A response surface approach for the production of natural pigment astaxanthin from green alga, *Haematococcus pluvialis*: effect of sodium acetate, culture age and sodium chloride. *Food Biotechnol.* **2002**, *16*, 107–120.

(15) Johnson, E. A.; An, G. H. Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.* **1991**, *11*, 297–326.

(16) Odeberg, J. M.; Lignell, A.; Pettersson, A.; Hoglund, P. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid-based formulations. *Eur. J. Pharm. Sci.* **2003**, *19*, 299–304.

(17) Osterlie, M.; Bjerkeng, B.; Liaaen-Jensen, S. Plasma appearance and distribution of astaxanthin E/Z isomers in plasma lipoproteins of men after single dose administration of astaxanthin. *J. Nutr. Biochem.* **2000**, *11*, 482–492.

(18) Turujman, S. A.; Wamer, W. G.; Wei, R. R.; Albert, R. H. Rapid liquid chromatographic method to distinguish wild salmon from aquacultured salmon fed synthetic astaxanthin. *J. Am. Oil Chem. Soc.* **1997**, *80*, 622–632.

(19) Olaizola, M. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25000-liter outdoor photobioreactors. *J. Appl. Phycol.* **2000**, *12*, 499–506.

(20) Dayananda, C.; Sarada, R.; Bhattacharya, S.; Ravishankar, G. A. Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii*. *Process Biochem.* **2005**, *40*, 3125–3131.

(21) Grung, M.; Metzger, P.; Liaaen Jensen, S. Primary and secondary carotenoids in two races of green *Botryococcus braunii*. *Biochem. Syst. Ecol.* **1989**, *17*, 263–269.

(22) Grung, M.; Metzger, P.; Liaaen Jensen, S. Algal carotenoids 53; Secondary carotenoids of algae 4; Secondary carotenoids in the green alga *Botryococcus braunii*, race L, new strain. *Biochem. Syst. Ecol.* **1994**, *22*, 25–29.

(23) Okada, S.; Mastuda, M.; Yamaguchi, K. Botryoxanthin-A a new member of the new class of carotenoids from green micro alga *Botryococcus braunii*, Berkeley. *Tetrahedron Lett.* **1996**, *37*, 1065–1068.

(24) Okada, S.; Tonegawa, I.; Mastuda, M.; Murakami, M.; Yamaguchi, K. Botryoxanthin-B and α-botryoxanthin-A from green micro alga *Botryococcus braunii*. *Phytochemistry* **1998**, *47*, 1111–1115.

(25) Okada, S.; Tonegawa, I.; Mastuda, M.; Murakami, M.; Yamaguchi, K. Braunixanthins 1 and 2, new carotenoids from green alga *Botryococcus braunii*. *Tetrahedron* **1997**, *53*, 11307–11316.

(26) Zarrouk, C. Contribution a letude d'une cyanophycee. Influence de divers factours physiques. et chimiques sur la croissance et la phytosynthese do *S. platensis* maxima. Ph.D Thesis, University of Paris, 1966.

(27) Sarada, R.; Usha, T.; Ravishankar, G. A. Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions. *Process Biochem.* **2002**, *37*, 623–627.

(28) Usha, T.; Sarada, R.; Ravishankar, G. A. Production of astaxanthin in *Haematococcus pluvialis* cultured in various media. *Bioresour. Technol.* **1999**, *68*, 197–199.

(29) Dayananda, C.; Sarada, R.; Kumar, V.; Ravishankar, G. A. Isolation and characterization of hydrocarbon producing green alga *Botryococcus braunii* from Indian freshwater bodies. *Electron. J. Biotechnol.* **2007**, *10*.
(30) Largeau, C.; Casadevall, E.; Berkaloff, C.; Dhamliencourt, P. Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii*. *Phytochemisty* **1980**, *19*, 1043–1051.
(31) Lichtenthaler, H. K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In *Methods in Enzymology*; Packer, L., Douce, R., Eds.; Academic Press: London, 1987; pp 350−382.
(32) Davies, B. H. Carotenoids. In *Chemistry and Biochemistry of Plant Pigments*; Goodwin, T. W., Ed.; Academic Press: London, 1976; pp 38−166.
(33) Lakshminarayana, R.; Raju, M.; Krishnakantha, T. P.; Baskaran, V. Lutein and zeaxanthin in leafy greens and their bioavailability: Olive oil influences the absorption of dietary lutein and its accumulation in adult rats. *J. Agric. Food Chem.* **2007**, *55*, 6395–6400.
(34) Baskaran, V.; Sugawara, T.; Nagao, A. Phospholipids affect the intestinal absorption of carotenoids in mice. *Lipids* **2003**, *38*, 705–711.
(35) Aebi, H. Catalase *in vitro*. In *Methods in Enzymology*; Packer, L., Ed.; Academic Press: New York, 1984; *105*, pp 121−125.
(36) Fedovich, B. C. Superoxide dismutase improved assay and an assay applicable to acrylamide gel. *Anal. Biochem.* **1976**, *10*, 276–287.
(37) Nicholas, M. A. A spectrophotometric assay for iodide oxidation by thyroid peroxidase. *Anal. Biochem.* **1962**, *4*, 311–345.
(38) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **1978**, *52*, 302–310.
(39) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, J. L. Protein measurement with Folin-phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
(40) Landrum, J. T.; Bone, R. A.; Moore, L. L.; Gomez, C. M. Analysis of zeaxanthin distribution within individual human retinas. *Methods Enzymol.* **1999**, *299*, 457–467.
(41) Castenmiller, J. M.; West, C. E. Bioavailability and bioconversion of carotenoids. *Annu. Rev. Nutr.* **1998**, *18*, 19–38.
(42) Ben Amortz, A.; Levy, Y. Bioavailability of natural isomers mixture compared with synthetic all-trans β- carotene in human serum. *Am. J. Clin. Nutr.* **1996**, *63*, 729–734.
(43) Gordon, M. F. The mechanism of antioxidant action *in vitro*. In *Food Antioxidants*; Hudson, B. J. F., London, U. K., Eds.; Elsevier Applied Science: New York, 1990; pp 1−18.
(44) Ranga Rao, A.; Sarada, R.; Baskaran, V.; Ravishankar, G. A. Antioxidant activity of *Botryococcus braunii* extract elucidated in *in vitro* models. *J. Agric. Food Chem.* **2006**, *54*, 4593–4599.
(45) Fayaz, M.; Namitha, K. K.; Murthy, K. N. C.; Mahadeva Swamy, M.; Sarada, R.; Khanam, S.; Subba Rao, P. V.; Ravishankar, G. A. Chemical composition, iron bioavailability and antioxidant activity of *Kappaphycus alvarezzi* (Doty). *J. Agric. Food Chem.* **2005**, *53*, 792–797.
(46) Chidambara Murthy, K. N.; Vanitha, A.; Rajesha, J.; Mahadeva Swamy, M.; Sowmya, P. R.; Ravishankar, G. A. *In vivo* antioxidant activity of carotenoids from *Dunaliella salina*—A green microalga. *Life Sci.* **2005**, *76*, 1381–1390.

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