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Antioxidant Activity of *Botryococcus braunii* Extract Elucidated in Vitro Models

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Botryococcus braunii is a green colonial microalga that is used mainly for the production of hydrocarbons, exopolysaccharides, and carotenoids. In the present study, the antioxidant properties of acetone extracts of *B. braunii* were evaluated using in vitro model systems such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxy radical scavenging, and lipid peroxidation in human low-density lipoprotein and rat tissues. Acetone extracts of *B. braunii* (equivalent to 10 ppm total carotenoid) exhibited 71 and 67% antioxidant activity in DPPH and hydroxyl radical scavenging model systems, respectively. Similarly, the extract also showed 72, 71, and 70% antioxidant activity in the liver, brain, and kidney of rats. Low-density lipoprotein oxidation induced by Cu^{2+} ions was also protected (22, 38, and 51%) by the algal extract in a dose-dependent manner (4, 6, and 8 ppm levels of total carotenoid). Thiobarbituric acid reactive substances concentration in the blood, liver, and kidney of rats was also significantly decreased in *B. braunii* treated samples compared with those of control. Carotenoids (violaxanthin, astaxanthin, lutein, zeaxanthin, chlorophylls a and b, and α, β-carotene) identified in the *B. braunii* acetone extract may be exhibiting antioxidant activity. Among the carotenoids, lutein represents more than 75% of the total carotenoids. *B. braunii* extract was shown to be effective for protecting biological systems against various oxidative stresses in vitro. This is the first report on the antioxidant properties of *B. braunii*.

KEYWORDS: ***Botryococcus braunii*; antioxidant activity; microalga; lipid peroxidation; carotenoids**

INTRODUCTION

There is an expanding quest surrounding the use of antioxidative molecules because they have the capacity to quench free radicals, thereby protecting cells and tissues from oxidative damage. They are also useful in preventing the deterioration of food products during processing and storage. More than 600 naturally occurring carotenoids have been characterized; among them, β-carotene is recognized as the most prominent antioxidant (*1*). Apart from this, carotenoids also influence cellular signaling and may trigger sensitive regulatory pathways. Only bacteria, fungi, plants, and algae can synthesize carotenoids.

Botryococcus braunii is a green colonial microalga belonging to the family Chlorophyceae and is grouped into three different races, A, B, and L, depending on the type of hydrocarbons they synthesize (*2*). This alga is mainly known for the production of hydrocarbons, exopolysaccharides, and carotenoids. *B. braunii* undergoes a color change because of the accumulation of secondary carotenoids in the matrix. The presence of carotenoids is more pronounced in races B and L (*3*). 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 and 29%) reported in the linear phase of these races. Canthaxanthin (46%) together with echinenone (20−28%) are the dominating carotenoids in the stationary phase (*3*). Grung et al. (*4*) reported the presence of adonixanthin in the "L" race in the stationary phase. Some newly identified carotenoids such as botryoxanthin-A, (*5*), botryoxanthin-B, and α-botryoxanthin (*6*) and braunixanthins 1 and 2 (*7*) isolated from the B race may contribute to the color of the algal colonies. The carotenoid composition and antioxidant properties of *B. braunii* (race "A") have not been studied so far. Hence, the present study was undertaken to evaluate the composition of biomolecules (carotenoids and phenolics) in *B. braunii* extracts and to study its antioxidant properties using in vitro model systems.

MATERIALS AND METHODS

Chemicals. All of the chemicals and solvents were of analytical and high-performance liquid chromatography (HPLC) grade and were obtained from Ranbaxy Fine Chemicals Ltd. (Mumbai, India). Standard lutein, β-carotene, α-carotene, astaxanthin, violaxanthin, and zeaxanthin were obtained from Sigma Chemicals Co. (St. Louis, MO).

Animals. Male albino rats of OUTB-Wistar, IND-cft(2c) of four weeks of age (35 ± 2 g) were used in this study. The rats were housed

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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 human subjects and animal experiments were performed after due clearance from the institutional animal ethics committee.

Algal Culture. *B. braunii* (LB 572) was obtained from the University of Texas. A stock culture was maintained on both liquid and agar slants of modified Chu 13 medium (*8*) by regular subculturing at two-week intervals. Culture flasks were incubated at 26 ± 1 °C with 1.7647 × 10^{-7} erg m^{-2} s^{-1} and a 16/8 h light/dark cycle. After four weeks of growth, the culture was harvested by centrifugation (*2*); the biomass was lyophilized and used for the extraction of carotenoids.

Preparation of *B. braunii* Extracts by Different Solvents. A known quantity of freeze-dried *B. braunii* biomass (100 mg) was ground well using a mortar and pestle, and carotenoids were extracted with different solvents (each 20 mL), namely, acetone, methanol, ethanol, chloroform/methanol (1:1 and 2:1, v/v), petroleum ether, hexane, and ethyl acetate. The extraction was repeated until the sample became colorless (total volume 80 mL). The crude extract (80 mL) was evaporated to dryness in a rotary evaporator (Buchi, Germany) at 30−35 °C and redissolved in a known volume of the respective solvent system. Each extract was tested for antioxidant assay by the hydroxyl and radical scavenging method (*9*). Aliquots of different solvent extracts were taken in terms of carotenoid concentration. After comparing the antioxidant activities of *B. braunii* extract in different solvent systems, acetone extract was found to exhibit higher antioxidant activity. Hence, acetone extract was used for detailed experimental studies.

Estimation of Carotenoids and Chlorophylls. Carotenoids and chlorophylls were extracted from *B. braunii* according to the procedure of Lichtenthaler (*10*). In brief, the freeze-dried biomass of *B. braunii* (10 g) was mixed with ice-cold acetone using a mortar and pestle, and carotenoids and chlorophylls were extracted with acetone (50 mL) repeatedly until the extract became colorless (total volume, 200 mL). The pooled extract absorbance was read at 450, 645, and 661.5 nm. Total chlorophyll and carotenoid contents were quantified by using Lichtenthaler equations (*10*). The acetone extract was concentrated by rotary evaporation (Buchi, Germany) and further dried under nitrogen gas. The residue was redissolved in ethanol (30 mL) and taken for different in vitro antioxidant assays.

HPLC Profile of Carotenoids in *B. braunii* Extract. The carotenoid profile of *B. braunii* acetone extract was analyzed using HPLC (Shimadzu 10AS) on a 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 of the carotenoids were monitored at 450 nm with a UV−visible detector (Shimadzu, 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 modal LC-10AVP series equipped with (SPD-10AVP) photodiode array detector. They were quantified from their peak areas in relation to the respective reference standards.

Determination of Total Phenolic Compounds. The concentration of total phenolic compounds in the extracts was determined according to the method of Taga et al. (*11*) 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 were 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. Absorbance was measured at 750 nm using a spectrophotometer (Shimadzu 160A). The blank consisted of all reagents and solvents without a sample or standard. The standard caffeic acid was prepared at concentrations of 10−100 μg/mL. The phenolic concentration in the alga extract was determined by comparison with the standards.

Assay for Radical Scavenging Activity. The acetone extract of *B. braunii* was assayed for radical scavenging activity by the procedure of Duh and Yen (*12*). *B. braunii* acetone extracts at different levels (total carotenoid contents of 6, 8, and 10 ppm) were taken, and the solvent was evaporated under nitrogen. The reaction mixture consisted of *B. braunii* extract, 1 mL of methanol, and 4 mL of a 0.5 mM methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH). The mixture was vortexed, and after allowing the mixture to stand at room temperature for 20 min, the absorbance of the remaining DPPH was read at 517 nm against the blank. The radical scavenging activity of the extract is expressed as a percentage decrease in the absorbance of DPPH against that of the blank.

Assay for Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity of *B. braunii* was determined according to the method of Klein et al. (*13*). Different concentrations (6, 8, and 10 ppm total carotenoids) of *B. braunii* acetone extracts were taken, and the solvent was evaporated to dryness under nitrogen. The reaction mixture consisted of a 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 trichloroacetic acid (TCA; 17.5%) and 2 mL of Nash reagent and left at room temperature for 15 min. The intensity of the yellow color was measured spectrophotometrically (Shimadzu 160A) at 412 nm against the reagent blank. The percentage of hydroxy radical scavenging activity of the sample was calculated as the percent inhibition relative to the control.

Assays for Lipid Peroxidation using Brain and Kidney Homogenates. For the in vitro studies, the brain and kidney of normal albino male rats were homogenized with a polytron (speed setting 7−8) in 10 mL of ice-cold Tris−HCl buffer (20 mM, pH 7.4) by the method of Liu and Ng (*14*). The homogenate was centrifuged at 14 000 rpm for 15 min. The supernatants (1 mL) were incubated with different levels of *B. braunii* acetone extracts (6, 8, and 10 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 the addition of 1.0 mL of TCA (28%) and 1.5 mL of thiobarbituric acid (TBA; l%). The solution was heated at 100 °C for 15 min, cooled to room temperature, and centrifuged at 2500 rpm for 15 min, and the color of the MDA−TBA complex in the supernatant was read at 532 nm using a spectrophotometer. Butylated hydroxyanisole was used as a positive control. The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = $(A - A_l)/A \times 100\%$, where A is the absorbance of the control and A_l is the absorbance of the test sample.

Assay for the Inhibitory Effect on Lipid Peroxidation. The inhibitory effect on the lipid peroxidation of *B. braunii* acetone extract was analyzed by the method of Halliwell and Gutteridge (*15*). Normal rats were anesthetized with diethyl ether and sacrificed by exsanguination. The perfused liver was isolated and homogenized with nine parts isotonic phosphate buffer saline using a Potter−Elvehjem homogenizer at 4 °C. The homogenate was centrifuged at 1500 rpm for 15 min, and the supernatant was used for the in vitro lipid peroxidation assay. In brief, to different concentrations of sample extractions (6, 8, and 10 ppm total carotenoids), were added 1 mL of 0.15 M potassium chloride and 0.5 mL of rat liver homogenate. 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, and centrifuged at 1500 rpm, and the supernatant was read at 532 nm. A control without the added sample extract was also run simultaneously. The percentage of antilipid peroxidative activity (%ALP) was calculated as %ALP = 1 − (sample OD/blank OD) × 100.

Assay for Antioxidant Activity on Liposome Model System. The lipid peroxidation-inhibitory activity of the *B. braunii* extracts in a liposome model system was determined according to the method of Duh and Yen (*12*). Egg lecithin (3 mg/mL phosphate buffer, pH 7.4) was sonicated in an ultrasonic homogenizer (Son plus HD 2200). *B. braunii* extracts of different concentrations (6, 8, and 10 ppm total carotenoids) were added to 1 mL of a 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 by adding 2 mL of 0.25 N HCl containing 150 mg/mL of TCA and 3.75 mg/mL of TBA. The reaction mixture was subsequently boiled for 15 min, cooled to room temperature, and centrifuged at 1500 rpm for 15

Table 1. Total Chlorophyll, Carotenoid, and Phenolic Contents of *B. braunii* (Dry Weight Basis) by Using Different Solvent Systems[a]

B. braunii extract	total chlorophyll μg/mg	total carotenoid μg/mg	total phenolics μg/mg
acetone	3.59 ± 0.05	2.33 ± 0.12	64.95 ± 0.07
methanol	3.04 ± 0.07	2.31 ± 0.07	128.03 ± 0.48
ethanol	1.31 ± 0.03	0.99 ± 0.00	84.30 ± 0.42
petroleum ether	0.62 ± 0.01	1.10 ± 0.15	44.40 ± 0.38
hexane	0.15 ± 0.07	0.62 ± 0.03	92.10 ± 0.35
chloroform/methanol 1:1	11.61 ± 0.09	4.68 ± 0.05	92.15 ± 0.70
chloroform/methanol 2:1	12.58 ± 0.12	7.56 ± 0.11	44.21 ± 0.38
ethyl acetate	6.42 ± 0.11	3.46 ± 0.03	24.15 ± 0.67

[a] Data represent mean ± SD of three replicates.

min, and the absorbance of the supernatant was read at 532 nm by a spectrophotometer.

Antioxidant Activity on Human Low-Density Lipoprotein (LDL) Oxidation. Plasma was separated from blood drawn from human volunteers and stored at 4 °C until used. The isolation of LDL from the plasma and measurement of the antioxidant activity of the algal extract were done according to the method of Princen et al. (*16*). In brief, to the *B. braunii* extract (6, 8, and 10 ppm total carotenoids) was added 40 μL of copper sulfate (2 mM) and the volume made up to 1.5 mL with phosphate buffer (50 mM, pH 7.4). A tube without extract and copper sulfate served as a negative control, and another tube without copper sulfate served as a positive control. All of the tubes were incubated at 37 °C for 45 min. To the aliquots of 0.5 mL drawn at 2, 4, and 6 h intervals from each tube were added 0.25 mL of TBA (1% in 50 mM NaOH) and 0.25 mL of TCA (2.8%). The tubes were incubated again at 95 °C for 45 min, cooled to room temperature, and centrifuged at 2500 rpm for 15 min. A pink chromogen was extracted and read at 532 nm by a spectrophotometer.

Reducing Power of Algal Extracts. The reducing power of algal extracts was determined according to the method of Oyaizu (*17*). The acetone extract of *B. braunii* (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%] then incubated at 50 °C for 20 min. To this mixture 2.5 mL of TCA (10%) was added, and the mixture was centrifuged at 3000 rpm 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 an increased reducing power.

Determination of Protein. Protein was estimated in animal tissues and the algal sample by using the method of Lowry et al. (*18*).

STATISTICAL ANALYSIS

Results were expressed as the mean ± the standard deviation (SD) of three replicate analyses. The difference between the groups was statistically analyzed by using one-way ANOVA.

RESULTS AND DISCUSSION

Carotenoid and Phenolic Contents in *B. braunii* Extract by Different Solvents. Different solvent extracts obtained from *B. braunii* were analyzed for chlorophylls, total carotenoids, and total phenolics, and the results are presented in **Table 1**. Maximum chlorophyll (11.6 ± 0.09 to 12.6 ± 0.12 μg/mg) and carotenoid (4.7 ± 0.05 to 7.5 ± 0.11 μg/mg) contents were obtained in the extracts of chloroform/methanol (1:1 and 2:1, v/v), while the maximum total phenolics content (128 ± 0.48 μg/mg) was obtained in methanol extract. Of all the solvents used, chlorophylls and carotenoids were found to be less in the petroleum ether and hexane, while the total phenolic content was least in the ethyl acetate extract.

Antioxidant Activity of *B. braunii* Extract by Different Solvent Systems. The different solvent extracts of *B. braunii* were evaluated for both radical scavenging activities and

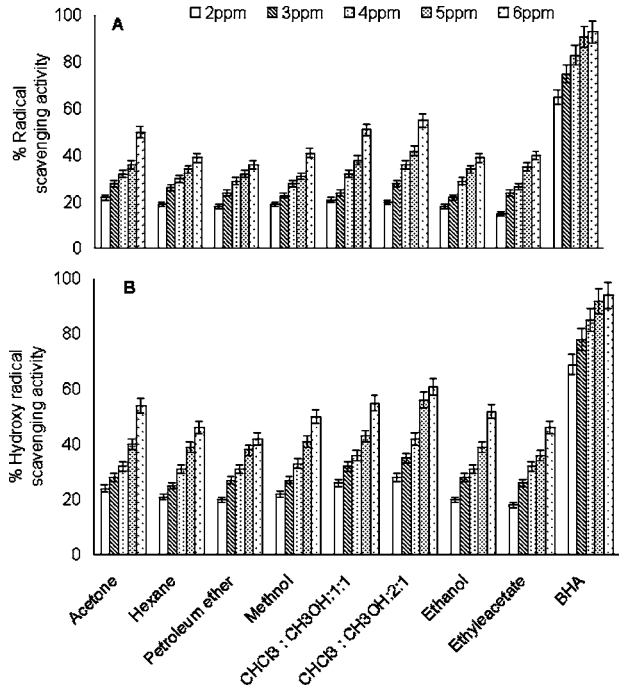

Figure 1. Antioxidant activity of *B. braunii* extract using different solvent systems. (**A**) DPPH method and (**B**) hydroxy radical scavenging activity. Data represent an average of three replicates. Bars indicate mean ± SD.

compared with standard butylated hydroxy anisole (BHA). Different solvent extracts are equated in terms of carotenoid content and assayed at 2−6 ppm of the total carotenoid content. All of the solvent extracts showed concentration-dependent antioxidant activity. It is evident from **Figure 1A,B**, that there is no major difference in the antioxidant activity of *B. braunii* extract by different solvents, although a marginal increase (15−22%) in the antioxidant activity was observed in the acetone and chloroform/methanol extracts compared to those of the remaining extracts. The chloroform/methanol (1:1 and 2:1, v:v) extracts showed a 51% and 55% scavenging activity at the 6 ppm level compared with that of BHA. Acetone fraction extracts at the 6 ppm level showed 50% antioxidant activity in the DPPH model system. Similarly, the acetone and chloroform/methanol (1:1 and 2:1, v:v) extracts showed 54%, 55%, and 61% scavenging activity at the 6 ppm concentration as compared with that of BHA by using the hydroxyl radical scavenging activity model system. The absence of a significant difference among different solvent extracts may be due to the assay of activity in terms of total carotenoid equivalents. Although high antioxidant activity was observed in the chloroform/methanol extract, when compared with other solvent extracts, it was not used for further studies as it contained a significantly high level of chlorophyll. Therefore, because the acetone extract had the next highest antioxidant activity, it was selected for detailed studies. However, acetone was evaporated and redissolved in ethanol for experimental studies.

Total Carotenoid, Chlorophyll, and Phenolic Content in the *B. braunii* Acetone Extract. The acetone extract of *B. braunii*, which is used for detailed antioxidant activity studies, constituted 2.33% on a dry weight basis. The total phenolic, carotenoid, and chlorophyll contents in original algal extract were found to be 0.96%, 0.13%, and 0.056%, while they were 0.96% (w/w), 2.3% (w/w), and 5.4% (w/w) in the acetone extract, respectively. The acetone extract used for different antioxidant assays contained 6, 8, and 10 ppm levels of total carotenoids.

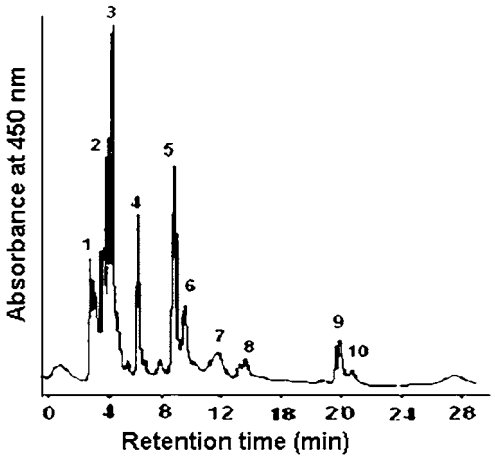

Figure 2. HPLC profile of carotenoids isolated from *B. braunii*. Peaks: (**1**) violaxanthin, (**2**) astaxanthin, (**3**) lutein, (**4**) zeaxanthin, (**5** and **6**) chlorophylls b and a, (**7** and **8**) unknown, (**9**) β-carotene, and (**10**) α-carotene. HPLC conditions are described in the text.

Carotenoid Composition in *B. braunii* Extract by HPLC. The HPLC profile of carotenoids in the *B. braunii* extract is shown in **Figure 2**. The *B. braunii* have three classes of pigments. In the order of elution through a C_{18} column, these are xanthophylls, chlorophylls, and hydrocarbon carotenoids, and these pigments were separated within 21 min. The detectable xanthophylls were comprised of violaxanthin (peak 1), astaxanthin (peak 2), lutein (peak 3), and zeaxanthin (peak 4) and then the chlorophylls a and b (peaks 5 and 6) and α- and β-carotene (peaks 8 and 9). These carotenoids were eluted under isocratic conditions and confirmed by their retention times and the absorption spectra of the respective reference standards, **Figure 3**. Among the carotenoids, lutein was the major carotenoid (75% of the total carotenoids), followed by astaxanthin (18%), β-carotene (6%), α-carotene (0.3%), violaxanthin (0.2%), and zeaxanthin (0.3%). The level of total xanthophylls (oxygenated carotenoids) found in *B. braunii* (A race) is about 94%, while hydrocarbon carotenoids constitute only 6%. The levels of lutein in the cases of the B (22−29%) and L (7−10%) races were reported to be significantly lower than that of race A (*3*, *4*). Further, hydroxylation of hydrocarbon carotenoids is known to be responsible for the formation of 3-hydroxy cyclic carotenoids and epoxy carotenoids. The presence of traces of α-carotene and a lower level of β-carotene in *B. braunii* may therefore be related to the conversion of these compounds to lutein. This may be a reason for the higher content of lutein in this alga.

DPPH Radical Scavenging Activity. DPPH is a stable free radical containing an odd electron in its structure and usually is used for detecting radical scavenging activity in chemical analyses. The degree of decrease in the absorbance of DPPH indicates the free-radical scavenging potentials. The free-radical scavenging potentials of the acetone extract of *B. braunii* at different concentrations were analyzed by the DPPH method, and the results are shown in **Figure 4A**. The *B. braunii* extract exhibited a dose-dependent free-radical scavenging ability at all of the tested concentrations. It exhibited 56, 62, and 68% free-radical activity at the 6, 8, and 10 ppm levels of carotenoids, that is, about 80−90% activity of the respective level of BHA. The activity of the extract is attributed to its hydrogen-donating ability (*19*). The antioxidants are believed to intercept the free-radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not propagate further oxidation of the lipid (*20*).

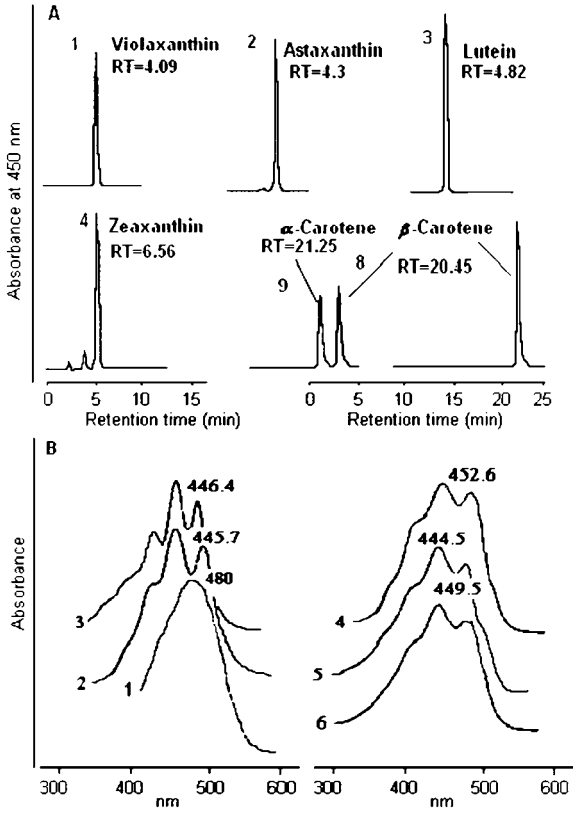

Figure 3. HPLC profiles of standard carotenoids (**A**) and absorption spectra (**B**) of carotenoids eluted from *B. braunii*: (**1**) astaxanthin, (**2**) violaxanthin, (**3**) lutein, (**4**) zeaxanthin, (**5**) α-carotene, and (**6**) β-carotene.

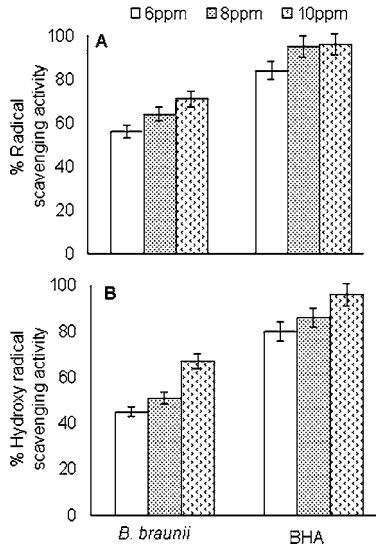

Figure 4. Antioxidant activity of acetone extract of *B. braunii*. (**A**) DPPH method and (**B**) hydroxy radical scavenging activity. Data represent an average of three replicates. Bars indicate mean ± SD.

Hydroxy 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 (*21*). The hydroxyl radical scavenging activity of *B. braunii* was estimated by generating the hydroxyl radical using an Fe^{3+}/ascorbic acid system. The hydroxyl radicals formed by the oxidation react with dimethyl sulfoxide to yield formaldehyde that 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

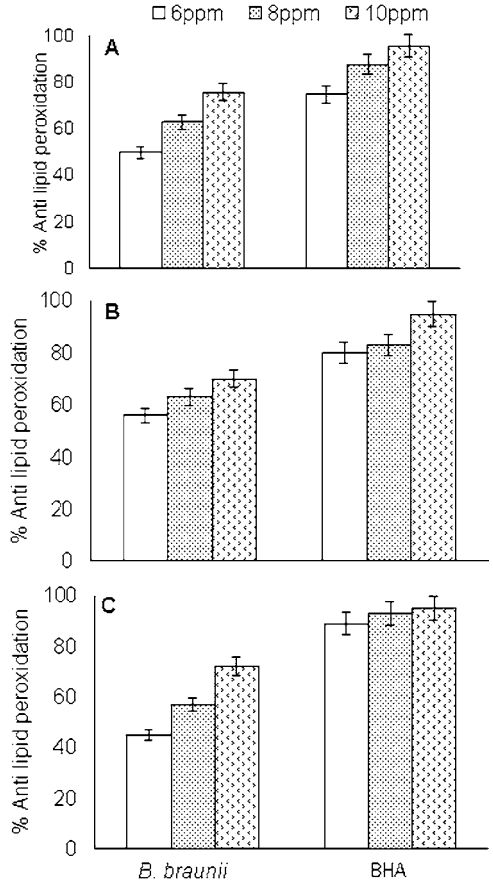

Figure 5. Lipid peroxidation activity of acetone extract of *B. braunii.* (**A**) Brain, (**B**) kidney, and (**C**) liver. Data represent an average of three replicates. Bars indicate mean ± SD.

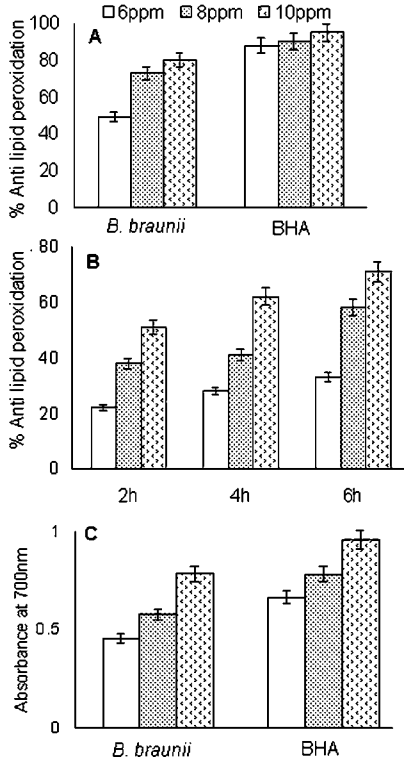

Figure 6. Lipid peroxidation activity of acetone extract of *B. braunii.* (**A**) Liposomes, (**B**) human LDL, and (**C**) reducing power. Data represent an average of three replicates. Bars indicate mean ± SD.

Figure 4B. The *B. braunii* extract exhibited 45−65% hydroxyl radical scavenging activity at 6−10 ppm, which is 50−75% that of BHA. The ability of the *B. braunii* extract showed an inhibitory effect on lipid peroxidation.

Changes of Lipid Peroxide Level in Rat Tissues. As observed in the model systems, the *B. braunii* extract also exhibited an inhibitory effect on lipid peroxidation in the brain (55%), kidney (58%), and liver (47%) at 4 ppm (**Figure 5A**−**C**), and it was found to be dose-dependent. The data show that the inhibitory activity of the algal extract on lipid peroxidation in the brain was similar to that in the kidney and liver. This could possibly be attributed to the same mechanism being involved in these tissues.

Inhibitory Effect on Lipid Peroxidation in Liposomes. Lipid peroxidation is a free-radical-mediated propagation of oxidative damage to polyunsaturated fatty acid 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 (*22*). 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 property of the algal extract in the liposome system, induced by $FeCl_3$ plus ascorbic acid, is shown in **Figure 6A**. As with BHA, the antioxidant property of the alga extract in the liposome system was significantly higher and was found to be dose-dependent. The extract exhibited 45, 65, and 78% inhibition of the peroxidation of lecithin at the 6, 8, and 10 ppm levels of carotenoid, respectively. The result shows that the extracts used have a strong antioxidant action in the liposome modal system. Chidamabara Murthy et al. (*23*) and Fayaz et al. (*9*) reported that carotenoids present in the green marine alga *Dunaliella salina* and the seaweed *Kappaphycus alvarazzi* may prevent the destructive effect of lipid peroxides in vitro by lowering the lipid peroxides. Further, they have reported that the antioxidant properties of the alga and seaweed are due to the higher levels of carotenoids and phenolic compounds present in them. Similarly, the *B. braunii* extracts used in this study may play an important role in protecting the cells of lipid peroxides (*24*).

Inhibitory Effect on LDL Oxidation. Oxidative modification is known to play an important role in the pathogenesis of atherosclerosis and coronary heart diseases (*25*), and the dietary antioxidants that protect LDL from oxidation may therefore reduce atherogenesis (*26*). The antioxidant activity of *B. braunii* extracts against human LDL oxidation is shown in **Figure 6B**. The polyunsaturated fatty acids of human LDL were oxidized, and the malondialdehyde formed has been estimated by using the TBA method. The average induction time for copper-mediated LDL oxidation was around 20 min without the addition of algae extracts. The algal extract protected LDL from oxidation as measured by the prolongation of the induction time of the formation of conjugated dienes. The algal extract exhibited 22, 38, and 51% protection at the 6, 8, and 10 ppm levels of carotenoid at the end of 2 h after the induction of oxidation. Whereas, it was 28, 43, and 62% and 33, 58, and 71% protection at the 6, 8, and 10 ppm levels of carotenoids used at the end of 4 and 6 h. The result indicates a dose-dependent inhibition effect of *B. braunii* against LDL oxidation.

Reducing Power. The reducing power of *B. braunii* extract was found to be 0.48, 0.63, and 0.790 at the 6, 8, and 10 ppm levels of carotenoids compared with standard vitamin C (**Figure 6C**). The presence of reducing power indicates that the *B.*

braunii extract has electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reactions. Yen and Duh (*12*) reported the reducing power of peanut hulls. Gordon (*27*) reported that 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 *B. braunii* extract indicates that the marked antioxidant activity of algae is believed to be due to the presence of carotenoids. Particularly, a high level of lutein (75%) may act as a strong antioxidant as do reductones by donating the electrons and reacting with free radicals to convert them to a more stable product and terminate free radical chain reactions.

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, were also under exploration for bioactive molecules including antioxidants. Fayaz et al. (*9*) reported antioxidant characteristics from the red seaweed *Kappaphycus*, and Aboul-Enein et al. (*28*) reported the antioxidant activity of microalgal extracts on lipid peroxidation. Chidambara Murthy et al. (*23*) reported antioxidant characteristics from *Dunaliella salina*, which produces β-carotene. 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, vitamin C, and so forth. These substances can act as potent antioxidants in protecting lipid peroxidation, free radical scavenging, and hydroxy radical scavenging activities by different modes of actions (*28*).

Several studies have reported on the relationship between phenolic content and antioxidant activity. Velioglu et al. (*29*) reported a strong relationship between the total phenolic content and antioxidant activity in selected fruits, vegetables, and grain products. However, there is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidant (*30*). Active oxygen species regulate carotenoid biosynthesis in some microorganisms such as yeast, *Phaffia rhodozyma* (*31*), green algae, *Haematococcus pluvialis* (*32*), and *Dunaliella bardawil* (*33*). The accumulated carotenoids might function as a protective agent against oxidative stress damage (*31*, *33*). Carotenoids scavenge/quench several active oxygen species such as O_2, H_2O_2, peroxy radicals, and hydroxy radicals (OH) both in vitro and in vivo (*34*−*36*).

The present results substantiate that *B. braunii* extract is capable of preventing lipid peroxidation through scavenging free radicals and hydroxy radicals in living cells. The acetone extract basically consists of carotenoids and polyphenols that are reported to have antioxidant activity. Lutein constituted the major carotenoid in *B. braunii* extract, whose bioavailability is higher than that of 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 and is used as a feed additive in poultry farming as well as a food dye (*37*, *38*). Therefore, the present study indicates the antioxidant potential of *B. braunii* for various health supplements, pharamaceuticals, and nutraceuticals. This is the first report on the evaluation of the carotenoid composition and antioxidant properties of *B. braunii*.

ABBREVIATIONS USED

DPPH, 1,1-diphenyl-2-picrylhydrazyl; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid; TBA, thiobarbituric acid; LDL, low-density lipoprotein; MDA, malondialdehyde; BHA, butylated hydroxy anisole.

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