MATERIALS & METHODS
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2.0. Material used for experiments

2.0.1. Algal genotype used in the present study

The different genotypes used in the study include,

i. *Dunaliella bardawil* was isolated from the Sambar salt lake of Rajasthan, India.

ii. *Dunaliella bardawil* strains V-101, V-102, AP-504, Ne-17 and T-34 were gift from Dr. Rengaswamy, DOS in Botany, Annamalai University, Chennai.

2.0.2. Glasswares

All glasswares used for the experiments viz., conical flasks, culture tubes, culture bottles, measuring cylinders, volumetric flasks etc, were from Borosil or Vensil Ltd, Mumbai, India.

2.0.3. Chemicals used for experiments

All the chemicals used were of analytical grade obtained from Hi-media Laboratories, Qualigens Fine Chemicals and SISCO Research Laboratory Chemicals, Mumbai, India. Chemicals used for large-scale cultivation were of commercial grade purchased from M/s Mysore pure chemicals, Mysore, India. Standard β-carotene, lycopene, lutein, retinol, primers and cell culture media chemicals were procured from Sigma Chemicals Ltd, St Louis, Missouri, USA. Solvents used were of analytical and HPLC grade, obtained from Qualigens Fine Chemicals, Mumbai; Rankem Pvt Ltd, Mumbai and E. Merck Ltd, Mumbai, India. Different diagnostic kits used for biochemical analysis were obtained from Span Diagnostics Ltd, Bangalore, India. Diet used for experimental animals were from Amrut Laboratory Feed Product, Bangalore, India.
2.1. Maintenance of *Dunaliella bardawil* cultures and in vivo culture conditions

2.1.1. Maintenance of the germplasm

The axenic cultures of *D. bardawil* were maintained in AS-100 media (Vonshak, 1986) with modification. Chemical composition of the medium (AS-100) is presented in Table-7. The Tris buffer (1gL⁻¹) used in AS-100 medium was replaced with NaHCO₃ (4.0gL⁻¹). The pH was adjusted to 7.5 using a pH meter (Cyber Scan 510, Oakton, USA) prior to autoclaving at 121°C, for 20min. The solid medium was prepared by gelling with 1.5% (w/v) tissue culture grade agar (Hi-media, Mumbai, India), in test tubes and in Petriplates, each containing 15mL of the medium. The tubes and flasks were closed with cotton plugs. The slant, Petriplates and flasks were inoculated with *D. bardawil* cultures, under aseptic conditions in laminar airflow cabinet (Airflow control systems, Bangalore, India). Algal pure cultures were maintained in solid medium as well as in liquid medium in Erlenmeyer flasks.

**Table 7. Chemical composition of AS-100 media**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>(g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄</td>
<td>2.44</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.3</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.05</td>
</tr>
<tr>
<td>KCl</td>
<td>0.6</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>1.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>50.0</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>1.0</td>
</tr>
<tr>
<td>Trace metal solution</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>3.426 gL⁻¹</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>1.215 mgL⁻¹</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.432 mgL⁻¹</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>31.5 mgL⁻¹</td>
</tr>
<tr>
<td>Conc·H₂SO₄</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂·4H₂O</td>
<td>31.19 mgL⁻¹</td>
</tr>
<tr>
<td>Chelated Iron solution</td>
<td></td>
</tr>
<tr>
<td>(10g of Na₂EDTA dissolved in 500 mL of hot water + 0.81g of FeCl₃·6H₂O in 500mL of 0.1N HCl, mix and make up to 1L)</td>
<td>3 mL of stock</td>
</tr>
</tbody>
</table>

2.1.2. Growth of *Dunaliella* in AS 100 medium

The cultures were maintained at 20-22°C in Erlenmeyer flasks under 12:12 hrs light/dark cycle of light (1.5-2 Klux), provided by universal-white lamps. The cultures were shaken manually once a day. Light intensities were measured using a lux meter
(TES 1332, Digital lux meter, Thaïwan). The growth of *D. bardawil* was monitored by the following parameters.

### 2.1. 3. Parameters for measurement of growth

#### i. Cell count

Growth of *D. bardawil* was measured in terms of cell number and counted using haemocytometer after fixing the cells by adding a drop of dilute hydrochloric acid (0.1N HCl). Cell count was expressed as number of cells/mL. In order to know the growth, optical density (OD) was also measured at 590 nm.

#### ii. Fresh weight (FW) and Dry weight (DW)

The fresh weight of algae was determined after centrifugation of the culture at 5000 rpm for 10 min and removing the excess of moisture using blotting paper. In order to determine the dry weight, algal cells after centrifugation were washed with distilled water three to four times to remove salt and other media chemicals and dried in a hot air oven (Sanyo, Electric Biomedical Co., Ltd, Japan) at 60 ± 2°C till a constant weight was attained on a glass Petridish. Biomass was expressed as gram per liter of culture.

Specific growth rate was calculated using the formula,

\[
\text{Specific growth rate} = \frac{\text{Final biomass} - \text{Initial biomass}}{\text{Initial biomass}} \times \frac{\text{Number of days}}{2}
\]

#### iii. Estimation of pigments

##### a. Chlorophyll content

*D. bardawil* cells were taken into a graduated tube and centrifuged at 5000 rpm for 15 minutes. A known amount of pellet was homogenized with acetone using pestle and mortar. The supernatant was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and absorbance was measured spectrophotometrically (Shimadzu-160A, Japan) at 645 nm and 661.5 nm against acetone blank. Concentration of chlorophyll a and b were calculated by the equation of Lichtenthaler (1987) and expressed as mg g⁻¹ biomass.

\[
\text{Chl a} = 11.24 \times \text{OD}_{661.5} - 2.04 \times \text{OD}_{645.0}
\]

\[
\text{Chl b} = 20.13 \times \text{OD}_{645.0} - 4.19 \times \text{OD}_{661.5}
\]
b. Carotenoid content

Carotenoids were extracted with acetone as mentioned above and analyzed using High Performance Liquid Chromatography (HPLC).

2.1.4. Identification and estimation of carotenoids

2.1.4.1. Identification of carotenoids by Thin Layer Chromatography

*D. bardawil* biomass was taken in mortar along with glass powder and acetone, subjected for mechanical grinding for 2-3 minutes, until the biomass becomes colorless. Whole process was carried out under yellow light in order to minimize the loss due to photo degradation. The whole solution was centrifuged at 5000 rpm for 5 minutes and the supernatant was collected, concentrated and redissolved in acetone. This extract was spotted on silica gel TLC plates (Merck Ltd, 20 × 20 cm, 0.5mm thickness) and the carotenoids were fractionated using acetone: hexane: acetic acid (7:3:0.1). The individual carotenoids and the total extract were identified using HPLC as explained below.

2.1.4.2. Identification of carotenoids by High Performance Liquid Chromatography (HPLC)

The total extract and individual carotene resolved through TLC was identified using high-performance liquid chromatography on a Bondapak C18 column (5µ × 250 mm) with methanol: acetonitrile: chloroform (47:47:6) as mobile phase at a flow rate of 1mL min⁻¹. Parameters were controlled by a Shimadzu LC10 AS liquid chromatograph equipped with a dual pump and a photodiode array detector (Model SPD-10A) set at 450 nm. The recorder Shimadzu C-R7A chromatopac was set at a chart speed of 2.5 cm min⁻¹. Samples were (10µl) injected with Rheodyne 7125 injector. Peak identification was achieved by comparing with their respective standards (Sigma, USA) and confirmed by spiking the standards with individual samples.
2.1.5. Growth and carotenoids production in *D. bardawil* under different culture conditions

In order to achieve high growth and induce accumulation of carotenoids, algal cells were cultured under different culture conditions like light, salt and different metal ions, after a growth period of 7±2 days.

2.1.5.1. Influence of light on the growth and carotenoid production

To study the effect of light intensity on growth and carotenogenesis efficiency, cultures were grown under different light regimes as given below.

(a) Culturing algae in controlled conditions with a light intensity of 1.5-2 Klux provided by cool white fluorescent lamps and a temperature of 20-22°C with a 12:12 hrs light/dark cycle (Indoor laboratory culture condition).

(b) Culturing algae in outdoor ponds equipped with a shade net for achieving a light intensity of 15-20 Klux at a temperature of 22-28°C.

(c) Culturing algae under direct sunlight in outdoor ponds subjected to a light intensity of 30-35 Klux at a temperature of 30-35°C.

The cultures were daily monitored for cell count, chlorophyll and carotenoid contents as explained earlier (Section 2.1.3 and 2.1.4).

2.1.5.2. Influence of sodium chloride on the growth and carotenoid production

To study the effect of sodium chloride content on growth and carotenogenesis in *D. bardawil* cells, the cultures were grown in different concentration of NaCl ranging from 0.5 to 4.0 M. The cultures were monitored for cell count, chlorophyll and β-carotene content.

2.1.5.3. Influence of carbon dioxide on growth of *D. bardawil*

To study influence of carbon dioxide on the growth of *D. bardawil*, the cultures were grown in two-tier flask as explained by Tripati et al (2001). A two-tier culture vessel consisting of two 250 mL small neck Erlenmeyer flask was employed. The lower compartment of the flask contained 100mL of 3M buffer mixture (KHCO₃/K₂CO₃ at 73; 27), to generate 2% CO₂ partial pressure was used. The upper chamber was inoculated with media containing known inoculum of *D. bardawil* cells. The cultures were monitored for cell count, chlorophyll and β-carotene content.
2.1.5.4. Influence of metal ions on the growth and carotenogenesis in *D. bardawil*

Micronutrients/metal ions play a crucial rule in growth of any organism. In order to know the effect of various divalent metal ions on the growth of *D. bardawil*, growth was monitored with different metal ions like Fe (0.2-2.0 mM), Zn (0.2-1.0 µM) and Mn (2.0-10 µM). Effect of these micronutrients on growth and carotenogenesis was studied.

2.1.6. Scale up studies in outdoor conditions

Once the culture was established in the laboratory conditions of 1.5-2Klux light intensity and a temperature of 20-22°C, scale up studies was carried out. Gradually the indoor grown cultures of *D. bardawil* was scaled up to 500mL and 1L flasks, 5L and 20L carboys and finally transferred to rectangular open glass tanks (40cm height X 40cm long X 20cm wide) with 20-25L culture capacity. During transferring of cultures from indoor to outdoor, initially carboys were closed with cotton plugs and the closures were removed gradually during 2nd and 3rd cycle to expose the cultures completely to an open system. In continuation of scale up studies, *D. bardawil* culture was grown in circular cement tank of 500L capacity with culture volume of 200 ± 50L. Culture tank was maintained in natural environmental conditions of temperature and light. The high light caused bleaching and death of the algal cells, hence direct sunlight on the culture was avoided during vegetative growth phase by covering with green house shade net. The cultures were daily mixed in order to prevent settling of cells at the bottom of the tank.

The cultures from the circular tanks were transferred to raceway tanks of 5.0m² of 500L culture capacity for vegetative growth. Initially 250L media was inoculated with 40 ± 5L of green culture of14-15 days old, having cell count of ~ 40.0 × 10⁴ cells mL⁻¹ under 50 % cut-off light (15-20 Klux) provided by green house shade net. The cultures were mixed occasionally using paddle wheel at 10-12 rpm, for ten minutes, twice or thrice a day depending on the light intensity. After a growth period of 20 days the cultures were kept for carotenogenesis by direct exposure to sunlight under outdoor condition of 30-35 Klux light intensity. The contaminations observed under outdoor conditions such as *Chlorella*, Protozoa and Diatoms were controlled by the addition of sodium chloride (2.0% w/v) to the medium.
2.1.7. Harvesting of algal cells

*D. bardawil* cells are 4-10 μm wide, 6-15 μm long and flagellate organisms with high motility and hence it is difficult to harvest this algae unlike other algal forms. The harvesting was achieved either by batch or continuous centrifugation.

2.1.7.1. Batch Centrifugation

To harvest the algal biomass in a batch mode, bowl centrifuge was used at a speed of 5000rpm (M/s West Folia, Germany). Thirty liter of culture was manually fed at a time to the centrifuge. The rotor speed was 5000 rpm with flow of the culture adjusted to 5L hr⁻¹. Biomass was collected in a cone shaped rotor and the media collected after the centrifuge was recycled for further cultivation.

2.1.7.2. Online centrifugation

Online centrifugation was used to harvest large amount of culture. A batch of 500 L culture was fed to online centrifuge (M/s Sharples, UK) at the rate of 8-10 L hr⁻¹, at an average speed of 7500 ± 1500 rpm. Biomass settled on the inner side of cylindrical tube was collected and the media was recycled.

2.1.8. Drying of *D. bardawil* biomass

Different drying methods were employed to study the effectiveness of drying processes, so that the method should not affect the concentration of pigment, in *Dunaliella*, the carotene content. The different drying methods employed were oven drying, sun, shade, freeze and spray drying.

i. Sun drying: The wet biomass of *Dunaliella* was spread as a thin layer of ~0.5 cm thickness on an aluminum tray. Samples were kept under direct sunlight (~27 ± 3 °C) for 5-6 hr. Sample was analyzed for moisture content and also for the carotene content. After complete drying total loss of carotenoids were recorded and percentage loss of pigments were calculated.

ii. Shade drying: This was carried out in a similar manner as described above except the trays were kept in shade avoiding direct sunlight (20-25°C).
iii. Oven drying: The wet biomass of *Dunaliella* was spread as a thin layer of ~0.5 cm thickness on an aluminum tray inside hot air oven (Sanyo, Electric Biomedical Co., Ltd, Japan). Algal biomass was dried at 45 ± 2°C till a constant weight was achieved. The dried biomass was analyzed for moisture and carotene content.

iv. Freeze-drying: This was carried out in a freeze drier (Model-10XB, Lyophylization Systems Inc. USA) for 7 hours by spreading the sample in a tray. The samples were analyzed for moisture and pigments content after freeze drying.

v. Spray drying: Algal biomass was subjected for spray drying using spray drier (Bowen Eng Inc., New Jersey USA). Feed rate of sample containing 15-20% solids was at 100 mL min⁻¹ (6 L Hr⁻¹). Inside temperature was maintained at 160± 5°C and outlet was maintained at 80±5°C. Sample was fed using the blower at an air pressure of 2.2-2.5 kg in⁻².

2.1.9. Storage of dry biomass

Equal weight (5g) aliquots of dried biomass were placed in brown glass vials, filled with nitrogen gas and stored at –80, 0, 25 and 37°C for 3 months under complete dark conditions. Aliquots (50 mg) of powder were drawn every two weeks for analysis.

2.1.9.1. Color changes during storage

The color change of carotenoid powder during storage was studied by using a Hunterlab color measurement system (Labscan XE, USA), which was used to determine the Hunter L, a and b values. ‘L’ was used to denote the brightness of the powder, while ‘a’ and ‘b’ denotes the intensity of red and yellow color respectively.

2.1.9.2. Analysis of carotenoids in stored samples

Aliquots of samples were analyzed for the carotenoid content by HPLC as explained earlier (section 2.1.4).
2.1.10. Nutritional composition of *Dunaliella bardawil* biomass

### 2.1.10.1 Moisture content

Moisture content was estimated by the method described by Raghuramulu et al (1983). A known amount of algal biomass was dried in hot air oven at 70 ± 2°C to get a constant weight. The dried biomass was cooled in a desiccator and weighed. The loss in weight was recorded and expressed as percentage of moisture content.

### 2.1.10.2. Ash content

Ash content was estimated according to the method described by Raghuramulu et al (1983). A known weight of the algal material was initially charred on a silica crucible and placed in a muffle furnace at 400-450 °C for 6 hours till the charred material becomes white. The dish was allowed to cool to room temperature in a desiccator till a constant weight is obtained. The difference in initial and final weight was taken as total ash content.

### 2.1.10.3. Estimation of mineral content

The mineral content was estimated by the method described by Raghuramulu et al (1983). The ash obtained as above was dissolved with a small amount of water (0.5 to 1.0mL) and mixed with 5mL concentrated Hydrochloric acid to prepare the solution. The mixture was evaporated to dryness, redissolved in 5mL HCl, dried and repeated again. Finally dissolved in 4mL of HCl and filtered through whatmann filter (#40) paper and made to known volume. The resulting ash solution was aspirated into the flame generated atomic absorption spectroscopy (AAS; Perkin-Elmer, USA, Model-3100) and the determination of metals and minerals were executed with the following operation conditions (Table 8) with acetylene air flame (acetylene pressure-75psi and air pressure 8psi) on the flame generating system.

**Table 8. The conditions of atomic absorption spectroscopy**

<table>
<thead>
<tr>
<th>Metal analyzed</th>
<th>Hollow cathode lamp</th>
<th>Wavelength (mA)</th>
<th>Lamp Current (mA)</th>
<th>Slit width (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>Ca</td>
<td>422.7</td>
<td>10</td>
<td>0.7</td>
</tr>
<tr>
<td>Fe</td>
<td>Fe</td>
<td>248.3</td>
<td>20</td>
<td>0.2</td>
</tr>
<tr>
<td>Cu</td>
<td>Cu</td>
<td>324.8</td>
<td>15</td>
<td>0.7</td>
</tr>
<tr>
<td>Mg</td>
<td>Mg</td>
<td>285.2</td>
<td>06</td>
<td>0.7</td>
</tr>
</tbody>
</table>
2.1.10.4. Crude fiber
Crude fiber was determined by the method described by Mahadevaswamy (1996). A known quantity of algal biomass was taken in a conical flask to which, 50 mL of 1.25 \% sulphuric acid was added and boiled for 30 minutes. The mixture was filtered and the residue was washed with distilled water till free from acid. The residue was quantitatively transferred into the original flask and digested for 30 minutes with 50 mL of 1.25 \% sodium hydroxide solution. The digested residue was filtered and washed with distilled water and quantitatively transferred to a previously weighed silica crucible. The crucible was dried at 110^{\circ}C for 3 hours and weighed again. The difference in weight before and after drying was expressed as crude fiber content.

2.1.10.5. Estimation of protein
Protein content of the biomass was calculated using total nitrogen content, which was estimated by micro-Kjeldhal method (Raghuramulu et al, 1983). A known amount of algal sample was digested in concentrated sulphuric acid with a catalyst (copper sulphate, potassium sulphate and selenium dioxide) for 6-8 hours, until the solution becomes clear. The digested solution was cooled and made up to a known volume. An aliquot was distilled by adding excess of 40\% (w/v) sodium hydroxide. The liberated ammonia was absorbed in 2 \% (w/v) boric acid and titrated against 0.014N hydrochloric acid. The nitrogen content was expressed as crude protein by multiplying with factor 6.25.

2.1.10.6. Estimation of lipids
2.1.10.6.1. Extraction and purification of lipid
Lipids were extracted, purified and quantified according to the method of Mahadevappa and Raina (1978). Fifty grams of cells was sonicated with 10 mL water, and lipids were extracted with chloroform:methanol (2:1) solvent mixture at room temperature for 3-4 h. The water phase was removed by allowing the two phases to separate using a separating funnel and the chloroform: methanol layer was collected.

<table>
<thead>
<tr>
<th>K</th>
<th>K</th>
<th>404.4</th>
<th>12</th>
<th>0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>Na</td>
<td>589.0</td>
<td>08</td>
<td>0.2</td>
</tr>
<tr>
<td>Zn</td>
<td>Zn</td>
<td>213.9</td>
<td>10</td>
<td>0.7</td>
</tr>
</tbody>
</table>
The organic layer was flash evaporated to dryness and the difference in weight before and after drying was expressed as total lipid. The lipid fraction was dissolved in 1 ml chloroform and stored at -20°C for further analysis.

2.1.10.6.2. Separation of lipids
Neutral and polar lipids were resolved from the total extract by thin-layer chromatography (TLC) as described by Mahadevappa and Raina (1978). Initially neutral lipids were separated using petroleum ether: diethyl ether: acetic acid (80:20:1) in which polar lipids would remain at the origin. The resolved neutral lipids (excluding the origin) were scrapped off the plate, re-extracted in chloroform: methanol (2:1) and re-chromatogramed individually in the same solvent system. The polar lipid present at the origin was likewise scrapped off, extracted in chloroform: methanol (2:1) and the lipids thus resolved were chromatographed in chloroform: methanol (4:1) to resolve polar lipids. Individual fractions were scrapped off the plate and quantified using gas chromatography.

2.1.10.6.3. Quantification of lipid fractions
The TLC plates were lightly sprayed with 50% H₂SO₄ and charred to locate the various lipid components. The relative concentrations of these components were determined by an automatic TLC scanner (Model 2, mounted on a Flourimeter, Model III; Turner Associates, CA, USA). Identification of the lipids on the plates was carried out by comparing with authentic standards and with the use of specific spray reagents (Siakator and Rouser, 1965). Sugar (Dubious et al. 1956), protein (Lowry et al. 1951), phosphorous (Lowry et al. 1954) and sterol (Lowry 1968) in the lipid were estimated by standard procedures.

2.1.10.6.4. Identification of lipids by Gas Chromatography (GC)
Fatty acid methyl esters were prepared using the method of methanolic boron trifluoride and were analyzed by gas chromatography. A GC model Shimadzu-15A, (Shimadzu Corporation, Kyoto, Japan) equipped with a 15% capillary diethylene glycol succinate (DEGS) (Shimadzu Corporation, Kyoto, Japan) column (30 m X 0.25 mm i.d.) and a flame ionization detector, was used. Nitrogen flow was 1 mL min⁻¹, and the temperature programming was 120°C (1 min) with a 10°C min⁻¹ increase to
180\(^{\circ}\)C (4 min), and then 4\(^{\circ}\)C min\(^{-1}\) to reach 280\(^{\circ}\)C (2 min). The injector and detector temperatures were set at 250\(^{\circ}\)C. Peaks were identified by comparison with authentic fatty acids methyl ester standards (Sigma).

### 2.1.10.7. Estimation of carbohydrate

Carbohydrate was estimated as described by Raghuramulu et al (1983). Known amount of algal biomass (1g) was hydrolyzed by keeping it in waterbath for 3 hrs with 5 mL of 2.5N HCl. The mixture was cooled and neutralized with Na\(_2\)CO\(_3\) until effervescence ceases. The mixture was made to 100mL and centrifuged. Aliquots of the supernatant (100, 200, 300µL...) were taken in different tubes, and made to 1mL. To this 1mL phenol, followed by 5mL H\(_2\)SO\(_4\) was added to each tube. The blank was run with 1 mL of distilled water. After cooling (10 minute at room temperature), the brown color developed was measured at 490 nm against reagent blank. Glucose (5-25 µg) was used as standard. The amount of carbohydrate was calculated using standard graph.
SECTION II
ANALYSIS OF GENES INVOLVED IN CAROTENOID BIOSYNTHESIS PATHWAY DURING LIGHT INDUCED CAROTENOGENESIS

2.2.1. Growth and cultivation of *D. bardawil*
In this section the effect of light intensity on growth and carotenogenesis, the regulation of genes involved during carotenogenesis were studied. *D. bardawil* cultures were grown under the different light regimes as given below.
(a) Culturing algae in controlled conditions with a light intensity of 1.5-2 Klux provided by cool white fluorescent lamps and a temperature of 20-22°C with a 12:12 hrs light/dark cycle (Indoor laboratory culture condition).
(b) Culturing algae under sunlight in outdoor ponds equipped with a screen for achieving a light intensity of 15-20 Klux at a temperature of 22-28°C.
(c) Culturing algae under direct sunlight in outdoor ponds subjected to a light intensity of 30-35 Klux at a temperature of 25-30°C.
The cultures were daily monitored for cell count, chlorophyll and carotenoid contents as explained earlier (Section 2.1.3 and 2.1.4).

2.2.2. Isolation of genomic DNA from *D. bardawil*
Genomic DNA was isolated from algal cells using the Gen Elute Plant Genomic DNA isolation kit (Sigma, USA).

2.2.3. RNA Extraction and transcript analysis
Total RNA was isolated using an RNA isolation kit (Ambion, USA) from aliquots of frozen cells harvested at different stages of carotenoid accumulation subjected to different light intensities. To avoid possible RNase contamination, all plastic wares were treated with 0.1% diethyl pyrocarbonate and the working area, electrophoresis tank, and other required materials were treated with RNase Zap (Sigma, USA). The quality and concentration of RNA were checked on denaturing agarose gel and quantified by measuring absorbance at 260 nm in a UV spectrophotometer (Sambrook et al, 1989). All RNA samples were subjected to DNase (DNasefree, Ambion, USA) treatment to avoid possible amplifications from contaminant genomic DNA.
The primers for all the candidate genes were designed using Primer3 software (Rozen and Skaletsky, 2000) are listed in Table 9. A control PCR was run on extracted RNA samples to check for the absence of genomic DNA. First strand cDNAs were synthesized using first strand synthesis kit (Ambion USA) from 400 ng of total RNA in 10 μL of final volume, using M-MuLV reverse transcriptase and oligo-dT (18-mer) primer following the instructions of the manufacturer. One μL of cDNA was used in semi quantitative RT-PCR reaction using specific primers and specific annealing temperatures (Table 9).

2.2.4. Polymerase chain reaction (PCR)

PCR was performed using primers designed for particular genes (Table 9). The PCR mixture (25 μL) contained 50 ng of cDNA prepared from different treated *D. bardawil* as the template, 1X PCR buffer, 2.5 mM of dNTPs and 1 unit of *Taq* DNA polymerase (MBI Fermentas), 25 pmoles of each primer (Genosys, Sigma USA). PCR for phytoene synthase (*PSY*), phytoene desaturase (*PDS*) and lycopene cyclase (*LCY*) gene was performed at initial denaturation at 94°C for 5 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at different annealing temperatures for candidate genes and 1 min extension at 72°C with a final extension of 72°C for 10 min. PCR for β-carotene hydroxylase (*CH*) was carried out as described above with annealing at 55°C for 1 min. The thermal cycler used was Primus 25 PCR system (MWG, AG Biotech, Germany). An aliquot of 12.5 μL from each PCR reaction was fractionated on a 1.5% (w/v) agarose gel in Tris acetate EDTA (TAE) buffer.

**Table 9. Primers and the PCR conditions used for different genes**

<table>
<thead>
<tr>
<th>Target gene amplified</th>
<th>Primer Name</th>
<th>Primer sequence 5’ – 3’</th>
<th>RT PCR Product size (bp)</th>
<th>Source</th>
<th>Ann.Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dunaliella</em> sp. phytoene synthase mRNA</td>
<td>PSYF</td>
<td>ATGTCTATGATGGATGCCAGGAG</td>
<td>442</td>
<td>DQ463305</td>
<td>50°C</td>
</tr>
<tr>
<td></td>
<td>PSYR</td>
<td>CTTCAGGCGGTGATACATGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dunaliella bardawil</em> mRNA for phytoene desaturase.</td>
<td>PDSF</td>
<td>TCTTTGGTGGTACACACTACA</td>
<td>664</td>
<td>Y14807</td>
<td>48°C</td>
</tr>
<tr>
<td></td>
<td>PDSR</td>
<td>TTGCTACCATGTCGTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.5. Agarose gel electrophoresis

i. Preparation of 50X TAE buffer (1000mL)

242.0 mgL⁻¹ Tris base, 57.1 mgL⁻¹ glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0) was dissolved and made to 1L.

ii. Preparation of DNA loading dye (6X)

0.25% Xylene cyanol, 0.25% Bromophenol blue and 30% Glycerol was mixed well and stored at 4°C

The gel casting boat was sealed with adhesive tape and the comb was placed on it for making the wells. Agarose (Electrophoretic grade SRL, India) was used at 1.5% (w/v) level and melted in 1X TAE. Agarose was allowed to cool to about 50°C and poured into the sealed gel casting boat. The gel was allowed to set for 30 min. The comb and adhesive tapes were removed and the gel was placed in the electrophoresis tank (Bangalore genei, India). Electrophoresis was carried out at 80V using a power pack (Consort Power Pack- E861, Belgium). The tank was filled with 1X TAE buffer to the electrode chamber to cover the gel to a depth of about 1 mm. The samples were loaded by mixing 10 μL aliquot with 2 μL of loading dye. The samples were run at 50 volts till the loading dye reaches ¾th of the gel. The gel was removed from the tank and placed in ethidium bromide (5μg mL⁻¹) solution for 15 min, subsequently destained with distilled water to remove unbound dye.
Ethidium bromide stained gels were observed under UV light and photographed with a Digital Imaging System (HeroLab, Germany). The transcript abundance of PSY, PDS, LCY and CH were quantified using the intensity histogram.
2.3.1. Acute oral toxicity study in rats

The study was designed to investigate the toxicological effects of oral administration of *D. bardawil* biomass after a single oral dose. The toxicological symptoms were monitored for 15 days. This will provide information on the health hazards likely to arise from a short-term exposure by the oral route.

2.3.1.1. Maintenance of rats for experimentation

Five male and five female rats of 6-8 weeks old, weighing 80-100g rats were used in the study. The rats were acclimatized for 7 days and the test substance; *D. bardawil* biomass (5000mg Kg⁻¹ b.w) was freshly prepared in distilled water (vehicle) and administered orally. The control group animals were treated with only distilled water. The animals were caged in a group of 5 according to sex; in polypropylene cages fitted with wire mesh tops and paddy husk bedding. The room was well ventilated with a temperature of 24± 2°C, with 60% relative humidity and 12hr light/dark cycle. The standard rat pellet feed and water was given *ad libitum*. All experimental animals were observed for 15 days after *D. bardawil* oral administration. Observation was made 3 times on the day of dosing and twice daily thereafter for the remaining 14 days. Body weights were recorded initially and weekly.

2.3.1.2. Biochemical observations

The following clinical laboratory determinations were made in all the animals at the end of the experiment. The organs were weighed and collected at the termination of the experiment. Haematological observations like Red blood cell (RBC) count, White blood cell (WBC) count, Hemoglobin (Hb) count, Platelet count, Neutrophils (N), Lymphocytes (L), Monocyte (M), Eosinophils (E) and Basophils (B) count were determined using baker hematology system.

Serum biochemistry parameters like total protein, serum aspartate aminotransferase (AST), serum alanine amino transferase (ALT) and serum alkaline phosphatase (ALP) were measured using Span diagnostic kits, India.
2.3. 2. Sub chronic (90 days repeated) oral toxicity study in rats
The toxicological effects of 90 days repeated oral administration of *D. bardawil* biomass in albino rats was assessed. This will provide information on the possible health hazards likely to arise from repeated exposure over a limited period of time.

2.3. 2.1. Maintenance of rats for experimentation
Thirty healthy rats (15 male and 15 female, in a group of five each) were acclimatized for 7 days. The first group was kept as control given only the vehicle i.e. distilled water. Second group was given 100 mg kg\(^{-1}\) b.w. *D. bardawil* biomass and third group was administered with 1000 mg kg\(^{-1}\) b.w. *D. bardawil* biomass for 90 days. The rats were observed daily for behavior, appearance, toxicological signs and symptoms, if any. Body weight and feed consumption was recorded at weekly intervals.

2.3.2.2. Biochemical observations
All the biochemical estimations were carried out as explained earlier (section 2.3.1.2)
SECTION IV

BIOAVAILABILITY AND BIOCONVERSION OF CAROTENOIDS FROM
D. BARDAWIL - IN VITRO, INVIVO AND CELL LINE STUDIES

2.4.1. In vitro bioavailability of carotenoids from D. bardawil biomass by simulated (in vitro) digestion method

The standard procedure given by Garrett et al, (1999b) was used for the in vitro digestion. Briefly the homogenized meal was subjected to acidification (pH 2) with 1M HCl and 2 mL of porcine pepsin (40 mg mL\(^{-1}\) in 0.1M HCl). The homogenate was transferred to a clean amber bottle and incubated at 37\(^{0}\)C in a shaking water bath (95 rpm) for 1 hour. Next, the pH of the partially digested sample was raised to 5.3 by the addition of sodium bicarbonate (0.9M), followed by the addition of a mixture of bile extract and pancreatin (9mL containing 2mg mL\(^{-1}\) pancreatin and 12mg mL\(^{-1}\) bile extract in 100mM sodium bicarbonate solution). The pH of the sample was increased to 7.5 by the addition of 1N sodium hydroxide and 10mL aliquots of the incompletely digested meal were transferred to three amber glass bottles sealed and incubated at 37\(^{0}\)C for 2 hours to complete the intestinal phase of the in vitro digestion. The digesta was centrifuged and the aqueous fraction was extracted three times with hexane. All the fractions were pooled and evaporated to dryness in a rotary evaporator (Buchi Rotavapour/R-205, Flawil, Switzerland). The residue was dissolved in 500\(\mu\)L of mobile phase solvent, filtered through a 0.25 \(\mu\)m membrane filter and analyzed by HPLC for carotenoids with the conditions as explained earlier (section 2.1.4).

2.4.2. In vivo bioconversion of carotenoids from D. bardawil

2.4.2.1. Bioconversion of D. bardawil carotenoids to vitamin A by intestinal perfusion method

The study technique of Wang et al, (1993) with a modification was employed to study the in vivo bioconversion of carotenoids from D. bardawil. The experiment was carried out by perfusing \(\beta\)-carotene extract from D. bardawil biomass into the intestine of rat and monitoring the level of retinol conversion for a varied interval of 15, 30, 60 and 120 min.
2.4.2.2. Maintenance of rats for experimentation

The male Wister strain rats (weighing about 100-120g each) were used for this study. These animals were housed under the following conditions: temperature- 25±2°C, relative humidity 55±5%, and a 12 hr light/dark cycle. The animals were housed in polypropylene cages (3 rats/cage) on soft sawdust bedding, fed with commercial basal diet (Amrut feeds, Bangalore, India) and water *ad libitum*. After 7 days of acclimatization, rats were deprived of diet for 12 hours before using for perfusion study.

2.4.2.3. Experimental design

The animals were anaesthetized using diethyl ether anaesthesia. Through the midline abdominal incision, the proximal end of intestine was flushed with a normal saline and then with the known volume of carotenoid extract from *D. bardawil* biomass. To prevent the perfusate from washing back into the stomach or continuing to large intestine, both distal and proximal ends were tied immediately. The water soluble carotenoid extract was prepared as below. A known concentration of carotenoid extract in acetone was dried under nitrogen flush. To the dried extract 20 µL of Tween-20 was added and vortexed vigorously. This is made to known volume by addition of phosphate buffer saline to get a final concentration. This carotene preparation was perfused and the whole intestine was removed from the group of rats in a definite interval of 15, 30, 60 and 120 mins.

2.4.2.4. Analysis of carotene and vitamin A

The perfusate from the intestine was decanted to a graduated tube, and intestine was excised from the rat. The extraction of carotenoids and vitamin A was carried out according to Schmitz et al, (1991). Briefly, intestine was homogenized with ethanol: water (1:1) with BHT, then saponified using 5mL of 10% NaOH in ethanol for 30 min at 60°C. To the saponified mixture 10 mL of water was added and extracted with hexane. The hexane fraction was dried and redissolved in acetone. HPLC analysis was carried out at 450 nm for carotenoids and at 320 nm for vitamin A with the conditions as explained earlier (section 2.1.4). The percentage increase in retinol content was monitored during different time interval.
2.4.3. Studies on bioavailability of carotenoids from *D. bardawil* biomass by feeding trials

2.4. 3.1. Maintenance of rats for experimentation

The male Wistar strain rats (weighing about 100-120g each) were used for this study. The animals were housed under the following conditions: temperature 25 ± 2°C, relative humidity 55 ± 5%, and a 12 hr light/dark cycle. The animals were housed in polypropylene cages (4 rats/cage) on soft sawdust bedding. Throughout the experiment, rats were given commercial basal diet (Amrut feeds, Bangalore, India) and water *ad libitum*. After 7 days of acclimatization, rats were deprived of diet for 12 hours before administering β-carotene (single dose study). Otherwise rats were received same diet throughout the experimental period (multiple dose study). All the experiments were carried out under the regulation of Institute Animal Ethical Committee.

2.4. 3.2. Test compound and levels of dose administration

*D. bardawil* biomass {100µg β-carotene equivalent (~3.5mg D. bardawil biomass) per kg body weight} was ground with minimal amount of water to form uniform slurry. Standard β-carotene (100µg kg⁻¹ body weight) was prepared in a water-soluble form and used for the experiment.

2.4. 3.3. Single dose studies

The groups of rats (n=36) were administered orally with single dose of *D. bardawil* biomass and with synthetic β-carotene after overnight fasting. Each group was further divided in to 8 subgroups (n= 4) and a control group to measure the retinol and β-carotene concentration of serum and liver at different intervals. Control group was fed with the same amount of water. Rats from control and in each treatment group at 0, 2, 4, 6 and 8 hr after gavage were sacrificed with diethyl ether anesthesia. Blood was collected directly from the heart and kept at 4°C. Serum was separated by centrifugation at 2500 rpm for 20 min, and stored at -70°C for further analysis. The livers were removed and washed with ice-cold saline and stored at -70°C until it is processed for carotene and vitamin A estimation. All the animals were fasted till the last blood drawing.
The quantity of retinol converted was measured in terms of area under curve (Parvin et al, 2000). The area under curve is the serum concentration Vs time of a known drug/product. It is also known as the total amount of drug/product absorbed by the body at definite time period. It is very useful to study the relative efficacy of different drug/product at a given time. The area under curve was calculated using the software ‘origin’.

2.4. 3.4. Multiple dose study
For multiple dose study, three groups of rats (n=4) were individually caged in metabolic cages. Two groups were administered daily with doses of either synthetic β-carotene or D.bardawil biomass for a period of 7 days. Clinical signs and general appearances were checked daily and body weights were measured once in a week. Urine and faecal matter from each rat was collected into amber bottles everyday and preserved at -70°C until analysis. Urine was collected daily in amber colored bottles containing 2mL of toluene (to prevent bacterial growth) from day 2 onwards. Urine volume was determined by measuring cylinder and corrected for the added toluene. Faecal matter was collected separately from the individual rats for 3 days and weight was noted down. Rats were weighed initially and at the end of the experiment. Before the day of necropsy, the animals were deprived of food overnight and sacrificed by anaesthetizing with diethyl ether. Blood was collected directly from the heart and kept at 4°C. Serum was separated by centrifugation at 2500 rpm for 20 min, and stored at -70°C for further analysis. The livers were removed and washed with ice-cold saline and stored at -70°C until the analysis.

2.4. 3.5. Estimation of carotenoids and vitamin A in intestine and liver
The extraction of carotenoids and vitamin A was carried out according to Schmitz et al, (1991) as explained earlier (section 2.4.2.4). The HPLC estimation was carried out at 450 nm for carotenoids and at 320 nm for vitamin A.

2.4.3.6. Analysis of carotenoids and vitamin A in urine and faecal matter
Aliquots of urine and faecal samples from each rat were taken for analysis either fresh or frozen at –70°C. Urine samples were filtered through Whatmann No.1filter paper and faecal matter (1g) was homogenized with 2 mL water and extraction was carried out as explained earlier (2.4.2.4).
2.4.3.7. Estimation of serum triglycerides
Triglyceride in the serum was estimated using kits from Span diagnostics, India.

2.4.4. Biological activity of *D. bardawil* carotenoids on primary cell lines

2.4.4.1. Maintenance of experimental rats
The maintenance of rats was given in earlier section 2.4.2.2.

2.4.4.2. Isolation of rat intestinal epithelial cells
Overnight, fasted rats were sacrificed by ether anesthesia. The small intestine was
removed and flushed gently with normal saline containing 1.0mM dithiothreitol.
Intestinal epithelial cells were prepared according to Upreti et al (2005). In brief, the
caecal end of the intestine was ligated and solution A containing 1.5mM KCl, 96mM
NaCl, 27mM sodium citrate, 8mM KH2PO4 and 5.6 mM Na2HPO4 (pH 7.3) was filled
after clamping the other end with artery forceps. The intestine was then immersed in
Solution A and incubated at 37°C for 15 min in a constant temperature shaker bath.
After incubation the intestine was emptied and fluid discarded. The intestine was then
filled with solution B containing 1.5mM EDTA and 0.5mM dithiothreitol in Phosphate
buffer saline (pH 7.2) and immersed in solution A for incubation. After 4 min
incubation, the contents were emptied into a plastic centrifuge tube to recover the first
epithelial cell population. The process of filling the intestine with solution B and
collecting the washings were repeated twice. All the cell population were pooled and
centrifuged at 900rpm for 5 mins and washed twice with 4mM EDTA solution
containing 15mM β-mercaptoethanol to remove phosphate buffer. The resulting cell
mass is known as primary intestinal epithelial cells. These cells were suspended in
Dulbecco's Modification of Eagle's (DME) medium (pH 7.2 containing 100 IU mL⁻¹
penicillin and streptomycin and 10% fetal bovine serum). These cells were grown in a
tissue culture plates on an incubator under 5% CO₂ atmosphere at 37°C with 95%
relative humidity.

2.4.4.3. Trypan blue dye exclusion assay to study the cell viability of intestinal
epithelial cells
An aliquot of cells (100µL) were dispersed in phosphate buffer saline (pH 7.0) and
mixed with trypan blue (100µL containing 0.2mg mL⁻¹) for 1 min (Altman et al,
1993). The stained (non-viable cells) and non-stained cells (viable cells) were observed under the microscope and counted separately using haemocytometer. The percentage of viable cells was calculated using the formula,

\[
\% \text{ of viable cells} = \frac{\text{Total no. of cells} - \text{Total no. of dead cells}}{\text{total no. of cells}} \times 100
\]

2.4.4.4. Treatment of cells to study the uptake of carotene and conversion to vitamin A in cell lines

*D. bardawil* carotenoid extract and synthetic β-carotene was prepared in phosphate buffer saline containing 5 and 10 µM of carotenoid content as explained in section 2.4.2.3. Approximately equal numbers of (5 X 10⁷ cells/mL) intestinal epithelial cells were suspended in media with and without carotene extract and incubated at 37°C with 5% CO₂ and 95% relative humidity. Aliquots of samples were withdrawn at different time periods (30, 60, 120 and 180 min) and the cells were pelleted by centrifugation at 8000rpm for 10 mins. The carotenoids and vitamin A in the cell pellet were extracted with acetone and analyzed by HPLC conditions as explained in section (2.1.4) at 450 nm for carotenoids and at 320 nm for vitamin A.
SECTION V

BIOLOGICAL ACTIVITY OF D. BARDAWIL BIOMASS ON CCl₄ INDUCED TOXICITY: BENEFICIAL ATTRIBUTES OF D. BARDAWIL AND ITS POTENTIAL TO MODULATE EXPERIMENTALLY INDUCED DISEASE CONDITIONS IN DIFFERENT ORGANS

2.5. Modulatory effect of D. bardawil on CCl₄ induced toxicity

The possible beneficial attributes of D. bardawil biomass and its potential to modulate experimentally induced disease conditions on blood parameters, liver and kidney were studied in this section.

2.5.1. Experimental design

Albino rats of Wister strain (120–150g body weight) bred in the Animal House of Central Food Technological Research Institute were used for the study. Animals were grouped into six groups each consisting of 6 rats (3 males and 3 females, maintained separately). The carotenoid rich biomass of D. bardawil was given at two different doses, i.e., 2.5 and 5.0 g kg⁻¹ body weight (approximately equivalent to 50 and 100 mg of β-carotene kg⁻¹ body weight) and synthetic β-carotene (50 mg kg⁻¹ body weight) as a single dose for a period of 14 days.

The six groups are as follows,

Group-1 Normal (receiving normal basal diet without toxin treatment),
Group-2 Control (receiving normal basal diet with toxin treatment),
Group-3 D. bardawil biomass was fed at 5g kg⁻¹ body weight,
Group-4 D. bardawil biomass was fed at 2.5g kg⁻¹ body weight along with CCl₄,
Group-5 D.bardawil biomass fed at 5g kg⁻¹ body weight with CCl₄ and
Group-6 was treated with synthetic β-carotene at 50mg kg⁻¹ body weight along with CCl₄.

The dosage of CCl₄ was decided based on earlier reports (Chidambara Murthy et al, 2002). The animals of all the groups except group 1 and 3 were given single dose of CCl₄ (2mL kg⁻¹ b. w.) on 15th day dissolved in olive oil (1:1). Animals of group 1 and 3 were given same dose of olive oil as a vehicle.
2.5.2. Maintenance of rats for experimentation
The animals were housed in a room with a barrier system, and maintained under the following conditions: temperature 24±1°C, relative humidity 55±5%, and a 12 hr light/dark cycle. The animals were housed in polypropylene cages (3 rats/cage) on soft sawdust bedding. Throughout the experiment, rats were given commercial basal diet and water *ad libitum*. All the experiments were carried out under the regulation of Institute Animal Ethical Committee.

2.5.3. Test compound and administration dose levels
*D. bardawil* biomass was ground with minimal amount of water to form uniform slurry. Experimental groups were fed with either *D. bardawil* biomass or synthetic β-carotene once a day for 14 days by forced feeding using an oral gavage method. On 15th day the animals were administered with single dose of CCl₄ (2mL kg⁻¹ b.w) in olive oil, and sacrificed after 6 hrs of the dosage.

2.5.4. Biochemical screening
Clinical signs and general appearances were checked daily and body weights were measured once in a week. Before the day of necropsy, the animals were deprived of food overnight and sacrificed by anaesthetizing the animals with ether. Blood was collected from the animals and the serum obtained was analyzed. The organs for the analysis were transferred to ice-cold containers for various biochemical estimations. A piece of liver was stored in 10% formalin solution for histopathological examination.

2.5.5. Serum biochemistry
Serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) were measured by the DNPH method (King 1965). Serum alkaline phosphatase (ALP) activity was assayed by the method of King and Armstrong (1988) using the commercially available kits (M/s SPAN diagnostic reagent kits, Mumbai, India). Serum Creatinine and Bilirubin content was estimated using diagnostic kits from SPAN diagnostics Ltd, India.
2.5.6. Liver parameter evaluation
The liver parameters were analyzed by estimating the lipid peroxidation and histopathological examination.

2.5.6.1. Lipid peroxidation
Liver tissues were homogenized in 0.1 mol L⁻¹ Tris-buffer (pH 7.4) and centrifuged. The particle-free homogenate was used for various biochemical analyses. Extent of lipid peroxidation was measured by quantifying the malondialdehyde formed in terms of thiobarbituric acid reactive substances (TBARS) and expressed in terms of nmol mg⁻¹ protein (Buege and Aust, 1978).

2.5.6.2. Histopathological studies
A portion of tissue was fixed in freshly prepared Bovin’s fluid (Saturated solution of picric acid 80%, formaldehyde (commercial) 15 % and glacial acetic acid 5 %). The tissues were processed according to the method of Lillie (1965). Six µm thick paraffin sections were prepared and stained with hematoxylin and eosin for histopathological examination.

2.5.7. Kidney parameter evaluation
Kidney parameters studied include the lipid peroxidation in renal tissues and creatinine estimation in serum.

2.5.7.1. Lipid peroxidation
Kidney tissues were homogenized in 0.1 mol L⁻¹ Tris-buffer (pH 7.4) and centrifuged. The particle-free homogenate was used for the biochemical analysis. Extent of lipid peroxidation was measured by quantifying the malondialdehyde formed in terms of thiobarbituric acid reactive substances (TBARS) and expressed in terms of nmol mg⁻¹ protein (Buege and Aust, 1978).

2.5.7.2. Estimation of serum creatinine
Serum Creatinine, indicator of kidney damage was estimated using diagnostic kit from SPAN diagnostics Ltd, India
2.5.8. Estimation of protein
Protein content was determined using the method of Lowry et al, (1951).

2.6. Statistical analysis
All the in vitro experiments were carried out three times in triplicates. Results were expressed as mean ± SD in case of in vitro experiments, and mean ± SE in case of in vivo analysis. Analysis of variance (ANOVA) was used for comparison of mean values and the values are considered as statistically significant at p < 0.05 or 0.01.