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"Bioprocess Scale-Up and Anticancer Phytochemicals of a Halotolerant Dunaliella Salina Isolate from Indian Coastal Waters"

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ABSTRACT: Dunaliella salina is a halotolerant, microalgae recognised for carotenoid accumulation under environmental stress. This study reports the first isolation, axenic establishment, large-scale cultivation, and comprehensive biochemical–pharmacological characterisation of a novel strain (APE0019B) collected from the coastal subtidal zone of Karnataka, India during a systematic survey of the Indian west coastline. Morphological identity was confirmed by pyriform, wall-less cells exhibiting intense orange-red carotenogenesis. The cryopreserved axenic culture retained approximately 94% viability over 24 months. Progressive scale-up biomass production from laboratory flasks through bubble-column photobioreactors to outdoor pilot-scale (250–300 L FRP tanks) yielded 3.9 kg freeze-dried biomass, confirming cultivation robustness under tropical conditions. Phytochemical screening of hot-water extracts revealed triterpenoids, saponins, flavonoids, and phenolics, with quantitative profiling showing 26.1 µg GAE g^{-1} total phenolic, 8.2 µg TAE g^{-1} total tannin, 11.2 µg BCE g^{-1} total carotenoid, and 8.7 µg BSE g^{-1} total sterol content. Both crude extract and an optimised multicomponent formulation exhibited considerable DPPH radical-scavenging activity and MTT-based cytotoxicity against MCF-7 human breast cancer cells. Collectively, these findings establish APE0019B as a scientifically validated, economically viable microalgal germplasm with promising potential for nutraceutical and pharmaceutical bioprospecting from Indian marine resources.

1. INTRODUCTION

Marine microalgae represent an inexhaustible reservoir of structurally diverse bioactive compounds with significant pharmaceutical, nutraceutical, and industrial potential. Among the approximately 300,000 estimated species of algae, relatively few have been explored for their biochemical potential in the context of tropical Indian coastal ecosystems (Borowitzka 2013). The western coastline of India spanning Maharashtra, Goa, and Karnataka harbours ecologically diverse coastal habitats characterised by distinct physicochemical gradients that select for uniquely adapted microalgal communities.

Dunaliella salina has attracted sustained scientific interest owing to its extraordinary capacity to accumulate massive quantities of carotenoids mainly all-trans-β-carotene, lutein, zeaxanthin, and α-carotene in intrachloroplastic lipid globules under high irradiance, nitrogen depletion, or hypersalinity (Shaish et al. 1992; Edge et al. 1997). Due to this phytochemical composition, the commercial importance of *D. salina* is well established as the primary biological source of natural β-carotene in nutraceuticals, food colorants, and cosmetics. Unlike synthetic all-trans β-carotene, *D. salina* uniquely accumulates a near-equal mixture of 9-cis and all-trans isomers, which confers superior antioxidant and provitamin A activity (Shaish et al. 1991; Namitha KK and Negi PS 2010) as well as anticancer activity (Jayappriyan K.R. et al. 2013). Beyond carotenoids, the species also produces zeaxanthin (Muthulakshmi and Baskar 2026), fatty acids (SFA), monounsaturated acids (MUFA), and polyunsaturated acids

(PUFA). SFA and PUFA other pharmacologically active phytochemicals that show antimicrobial activity against E. coli, S. aureus, and C. albicans (Cakmak et al. 2014).

Despite decades of global research on *D. salina* from hypersaline environments, characterisation of strains from tropical Indian coastal waters remains largely unexplored. The Arabian Sea coastline imposes distinct selective pressures sea surface temperatures 26–30 °C, salinity 32–35 PSU, high solar irradiance potentially eliciting physiologically distinct strains with altered phytochemical profiles compared to temperate or hypersaline isolates (Lamers et al. 2010).

The present study was undertaken as part of a systematic survey of marine microalgae from seven coastal sites (Maharashtra, Goa, Karnataka; October 2023–February 2025). Among multiple sites. axenic strains established, Dunaliella species, was isolated from costal Karnataka, and subjected to comprehensive characterisation covering field collection; morphological identification, axenic culture establishment; three stages scale up cultivation, post-harvest processing; hot-water extraction, multicomponent formulation, phytochemical profiling, antioxidant activity estimation and MTT cytotoxicity study on MCF-7 cells.

2. MATERIALS AND METHODS

2.1 Materials

2.1 Human breast adenocarcinoma cells (MCF-7; ATCC HTB-22) were procured from National centre for cell sciences Pune (NCCS) and maintained with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 atmosphere. A cost-effective modified media was prepared by combining urea/ammonium sulfate, diammonium phosphate (PDAP), potassium chloride (KCl), and magnesium sulfate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$), 4:2:1:1:1 proportion. and fortified with 0.1% w/v agri-micronutrients 'AgroMax; of Evergreen Micronutrients Pvt. Ltd which is procured locally. All the chemicals and reagents were purchased from HiMedia Laboratories Pvt. Ltd. and stored according to manufacturer specifications at 4 °C in dark conditions.

Software: GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA) and IBM SPSS Statistics Version 26 (Armonk, NY, USA) were used for statistical analyses and data visualization.

2.2 Sample Collection and Environmental Characterization

Seawater samples containing natural algal populations were collected from Devbagh, Karwar, Karnataka, India (GPS coordinates: 14°49'27.5"N; 74°07'44.8"E) during a survey expedition conducted on 10–13 February 2025. Sampling was performed in the intertidal and shallow subtidal zones (0–10 m depth) using sterile cans. Environmental parameters were recorded in situ using calibrated portable meters: water temperature (29.2 °C), salinity (32.1 practical salinity units, PSU), pH (7.07), dissolved oxygen (6.9 mg L^{-1}), and Secchi depth (5–8 m). Approximately 13 liters of seawater sample were collected and transported to the laboratory within 10 hours under cold-chain conditions (4.3 ± 1.1 °C).

Prior to sampling, institutional approvals were obtained from the Coastal Zone Management Authority (CZMA) and the Institutional Biosafety and Ethics Committee (IBEC), Shivaji University, Kolhapur

2.3 Isolation and Axenic Culture Establishment

Collected seawater samples were processed by gravitational sedimentation followed by serial washing with sterile 0.22 µm-filtered autoclaved seawater to remove organic debris and Lugol's iodine staining residues. To increase algal cell density, selective enrichment was performed using BG-11 medium for seven days at 24 ± 1oC under controlled lighting (100 ± 10 µmol photons m^{-2} s^{-1}; 12:12 h light:dark cycle).

Isolation of individual algal colonies was achieved through serial dilutions (10^{-1} to 10^{-8}), followed by spread plating and four-quadrant streaking on F/2 agar plates in a laminar flow hood (BSL-2). Incubation was maintained at 24 ± 1oC, 100 ± 10 µmol photons m^{-2} s^{-1}, and 12:12 h light:dark photoperiod. After four to five rounds of isolation, axenicity was confirmed through four independent methods: (i) culturing on nutrient agar and LB agar plates; (ii) antibiotic incorporation assay using streptomycin and tetracycline; (iii) optical density measurement of broth cultures at 600 nm for turbidity changes; and (iv) epifluorescence microscopy using DAPI staining to verify absence of bacterial contamination.

Master stock cultures (eight cryopreserved vials) were prepared in BG-11 medium supplemented with 10% glycerol (w/v) as cryoprotectant and stored at −80 °C in the vapor phase of liquid nitrogen for long-term preservation.

2.4 Morphological Identification

Identification was performed using light microscopy (1000X) with oil immersion (phase contrast; DIC) with morphometric analysis of ≥50 live cells. Voucher slides were deposited at Shivaji University Phycology Laboratory. External taxonomic validation was provided by Dr. V. Gupta (Retd., National Facility for Marine Cyanobacteria, NFMC).

2.5 Scale-up and Post-Harvest Processing

Initially, cryopreserved strain samples were restored from –80°C, to 37°C by thawing and emerging in a water bath for 90–120 seconds followed by cryoprotectant removed by centrifugation at 2000 × g for 5 minutes. Further, inoculum was developed by adding strain sample into mL BG-11 medium.

Scale-up study was performed at three stages.

2.5.1 Phase I cultivation was executed in closed 250 mL Erlenmeyer flasks where, 150 mL BG-11 medium was inoculated (10% v/v inoculum) and incubated at 24 ± 1oC, 100 µmol photons m^{-2} s^{-1} (6000 K LED, 12:12 h L:D) and 80 rpm orbital shaking (Infors HT Multitron Pro) for 21 days (till the end of the exponential phase). Every 21 days, healthy mother cultures were sub-cultured after assurance of no visible contamination or phenotypic drift.

2.5.2 Phase II scale up was performed using 1 L vertical bubble-column with total 800mL working volume added by 10%(v/v) inoculation. Inoculum used for this study was obtained from stage I cultivation. Media used for stage II was exactly same as per the Stage I. Photobioreactor was operated at 0.8–1.0 vvm aeration for 21 days. Periodic monitoring of cell counts, pH and contamination was executed and ensured with no wall adhesion and stable growth in order to obtain maximum output.

2.5.3 Phase III scale up was performed at 300 L outdoor FRP tanks with paddle-wheel mixing. The tanks were covered with 70% shade net for controlled environmental exposure. Total 10 batches were harvested at phase III cultivation. Here cost-effective media was used for cultivation which was prepared by mixing urea/ammonium sulfate, PDAP, KCl and $MgSO_4$, $NaHCO_3$ in 4:2:1:1 proportion. The media was supported by 0.01 g/L agri-micronutrients.

2.5.4 Biomass generated through Phase III cultivation was separated by centrifugation (7500 × g at 4oC). Harvested biomass was washed thrice using deionized water and shade-dried at 24–26oC for48–72 h., and cryogenically ground to <100 µm powder (Borowitzka 1988).

2.6 Crude Extract Preparation

Dried algal biomass (10 g) was extracted with deionized water (200 mL) maintained at 90oC for 3 hours in a water bath, maintaining a material-to-solvent ratio of 1:20 (w/v). The extraction vessel was covered to minimize evaporative losses. Following extraction, the mixture was cooled to room temperature and filtered sequentially through Whatman No. 1 filter paper and 0.45 µm cellulose acetate membranes under vacuum. The filtrate pH was adjusted to 4.4 using citric acid (1 M stock solution) to optimize phytochemical stability. Preservatives were added to the extract: sodium benzoate (0.05% w/v) and potassium sorbate (0.05% w/v). The preserved extract was stored at 4oC in amber glass bottles until further analysis.

2.7 Organic extracts and F_M3 Formulation of Purified Bioactives

Initially, extraction of carotenoid, phenolics, tannins and sterol were executed using solvents such as n-hexane, ethyl acetate and chloroform. Sequential solvent partitioning was followed by silica-gel column chromatography in order to obtain purified fractions. Equal amounts of the purified fractions (carotenoid, phenolic, and tannin-rich) in 1:1:1:1 proportion were combined to prepare the F_M3 formulation. This formulation was evaluated for antioxidant and anticancer activity along with the individual extract (Pardo de Donlebún et al. 2026).

2.8 Phytochemical Analysis

2.8.1 Qualitative analysis for eleven qualitative phytochemical tests were performed in triplicate according to standard protocols (Brand-Williams et al. 1995) to identify the presence of alkaloids (Dragendorff / Mayer's), Steroids (Liebermann–Burchard), Triterpenoids (Salkowski test), Cardiac Glycosides (Keller–Kiliani), Saponins (Foam test), Flavonoids (Shinoda test), Phenolics (Ferric chloride test), Proteins / Amino Acids (Biuret/Ninhydrin), Tannins (Gelatin precipitation), Starch (Iodine test) and Flavonols (Aluminium chloride).

2.8.2 Quantitative Analysis for four phytochemicals were performed including Total phenolic content (TPC) (Folin–Ciocalteu method, with gallic acid standard), Total tannin content (TTC) (polyvinylpolypyrrolidone (PVPP) adsorption subtraction method, with tannic acid as standard), Total carotenoid content (TCC) (using β-carotene as standard) and Total sterol content (TSC) (Liebermann–Burchard method, with β-sitosterol as standard). All assays were performed in triplicate (n = 9), and results are expressed as mean ± standard deviation (SD).

2.9 Antioxidant and Cytotoxicity Bioassays

2.9.1 DPPH Radical Scavenging Assay: Radical scavenging activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the modified method of Brand-Williams et al. (1995). The crude extract was tested at concentrations ranging from 20–100 μg mL^{-1}. Briefly, 100 μL of extract or standard was mixed with 100 μL of freshly prepared DPPH methanolic solution (100 μM), incubated in the dark at room temperature for 30 minutes, and absorbance was measured at 517 nm. Ascorbic acid (0–100 μg mL^{-1}) was used as positive control (IC_{50} = 72.49 μg mL^{-1}). The percentage of radical scavenging was calculated using following formula

$$\% \text{ radical scavenging} = \frac{\mathbf{A\ control - A\ sample}}{\mathbf{A\ control}} \times 100$$

where IC_{50} was determined by linear regression analysis.

2.9.2 MTT Cytotoxicity Assay: MCF-7 human breast adenocarcinoma cells were cultured in RPMI 1640 medium supplemented with 10% FBS and maintained at 37°C in 5% CO_2. Cells were seeded in 96-well culture plates at a density of 5 × 10^4 cells $well^{-1}$ and allowed to adhere for 24 hours. Cells were then exposed to the algal extract or purified fractions at concentrations ranging from 20–100 μg mL^{-1} for 24 hours. 5-Fluorouracil (5-FU; 0–100 μM) was used as positive control. Following treatment, 20 μL of MTT reagent (5 mg mL^{-1} in phosphate-buffered saline, PBS) was added to each well and incubated for 4 hours at 37°C. The formazan crystals formed were dissolved in 150 μL of acidic isopropanol (0.04 N HCl in isopropanol), and absorbance was measured at 570 nm using a microplate reader.

For the multicomponent formulation F_M3 (prepared at 4.00 mg mL^{-1} containing 1:1:1:1 ratio of purified fractions), cytotoxicity was assessed identically. Synergistic interactions were quantified using the Chou–Talalay Combination Index (CI) method, where CI < 1.0 indicates synergy, CI = 1.0 indicates additive effects, and CI > 1.0 indicates antagonism (Chou, 2010).

All bioassays were performed in triplicate (n = 9).

2.10 Statistical Analysis

The data were expressed as mean ± SD (n = 9). The IC_{50} values were determined by linear regression analysis using GraphPad Prism 9.0. Pearson's correlation analysis was performed to evaluate the correlation (r) between the phytochemical and bioactivity data. Statistical differences among the treatments were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test, with statistical significance set at $p < 0.05$. All statistical analyses were performed using SPSS Statistics version 26.

3. RESULTS AND DISCUSSION

3.1 Field Collection and Environmental Parameters

Environmental characterization of the collection site (14°49'27.5"N; 74°07'44.8"E) revealed a distinct hydrochemical profile. The water column exhibited hypo-osmotic salinity (32.1 PSU) relative to adjacent western coastal ecosystems, establishing a selective osmotic microenvironment enriched in halotolerant algal lineages. This reduced-salinity regime may have functioned as a natural selection pressure favoring osmoadaptive phenotypes with enhanced secondary metabolite biosynthesis. Notably, carotenoid production in halotolerant microalgae demonstrates complex osmotic regulation, exhibiting amplified accumulation under hypersaline stress (≥2 M NaCl) but exhibiting modulated responses across brackish-to-marine salinity gradients through osmolyte-mediated transcriptional regulation (Lamers et al. 2010). The intermediate salinity conditions at our collection site may thus have elicited osmotic stress-induced carotenoid accumulation, functionally equivalent to culture conditions that maximize pigment yield. This hypothesis is supported by the elevated carotenoid titers recovered from the isolated strain, suggesting that field-based osmotic conditioning may have pre-selected for metabolically high-performing phenotypes. According to the

literature, water column salinity of 32.1 PSU falls within the euryhaline tolerance range of Dunaliella species, yet remains substantially below full-strength seawater (34–36 PSU), creating a physiological stress condition.

3.2 Isolation, Axenicity, and Cryopreservation

Processing of the 13 L field concentrate yielded viable cell suspensions with high initial viability (87 ± 6% live cells by Trypan blue exclusion assay), indicating minimal osmotic shock during sample transport and processing. The initial cell density was extremely low (<10^3 cells mL^{-1}), necessitating selective enrichment in BG-11 medium for seven days. This enrichment strategy proved highly effective, achieving an 18-fold increase in cell density to 2.1 × 10^5 cells mL^{-1}, thereby providing sufficient inoculum density for subsequent isolation procedures.

Following four rounds of streak-plate isolation on f/2 agar plates, a single morphologically consistent isolate was obtained. Axenicity was rigorously confirmed through four independent and complementary microbiological methods. First, nutrient agar and Luria-Bertani agar plating showed complete absence of bacterial colonies after seven days of incubation at 37°C under aerobic conditions. Second, antibiotic incorporation assay using streptomycin and tetracycline-supplemented broth cultures revealed no turbidity development or optical density increase ($\Delta OD_{600} < 0.05$) over a seven-day monitoring period, indicating the absence of antibiotic-resistant bacteria. Third, continuous optical density monitoring of algal broth cultures at 600 nm showed no statistically significant turbidity increase over seven days, ruling out heterotrophic bacterial contamination. Fourth, 4',6-diamidino-2-phenylindole (DAPI) epifluorescence microscopy examination of ≥100 randomly selected cells revealed the exclusive presence of algal nuclei and chloroplasts, with complete absence of bacterial nucleoids. All four independent axenicity verification tests remained consistently negative across two consecutive sub-cultures, thereby definitively confirming the axenic status of strain.

This selective enrichment approach, while standard in phycological isolation protocols, proved essential for obtaining pure cultures from environmental samples where target algal cell density is low. Employment of multi-method approach was observed to offer robust and definitive microbiological evidence of culture purity. This methodological rigor is essential for ensuring the reliability of all subsequent bioactivity and phytochemical analysis, as it eliminates potential confounding effects from undetected bacterial co-metabolism or bacterial secondary metabolite production.

Cryopreservation of the axenic isolate at −80 °C in the vapor phase of liquid nitrogen, using 10% glycerol as cryoprotectant, resulted in exceptional long-term viability. Post-thaw viability after 24 months of storage was 94 ± 4% (Trypan blue exclusion assay), and critically, no morphological changes or alterations in growth rate were observed compared to the original strain immediately before cryopreservation, indicating successful preservation of both cellular phenotype and physiological capacity. As cryopreservation offers preservation of both the physiological state and genetic integrity of the isolated strain, it ensures that laboratory-cultured biomass exhibits the same metabolic capacity as the original field-isolated strain against the poorly preserved subcultures.

3.3 Morphological Identification

Morphological characterization by light microscopy (1000× magnification, oil immersion optics with phase contrast and differential interference contrast) of ≥50 live cells confirmed species identity. The isolated strain exhibited all diagnostic features characteristic of *D. salina* sensu established taxonomic criteria as expressed in Table 1 (Olmos et al. 2009; Guiry et al. 2014). Notably, under nitrogen-limiting conditions, cells accumulated substantial quantities of orange-red carotenoid pigments, consistent with secondary metabolite accumulation under nutrient stress (Table 1). Species identity was independently validated by Dr. V. Gupta (Retired Senior Scientist, National Facility for Marine Cyanobacteria, National Institute of Oceanography, Goa, India), formally confirming the strain designation as *D. salina* APE0019B.

Table 1. Morphological diagnostic features of *Dunaliella salina* APE0019B (n ≥ 50 live cells).

Diagnostic Feature	**Observed in APE0019B**	**Reference (D. salina)**
Cell shape	Pyriform to broadly ovoid	Pyriform to ellipsoidal
Cell length (μm)	16.2 ± 2.4 (range: 11–22)	10–25 μm

Cell breadth (μm)	11.1 ± 1.8 (range: 8–16)	8–18 μm
Flagella	2 equal anterior; ~1.2× cell length	2 equal apical flagella
Cell wall	Absent; glycocalyx only	No rigid wall; glycocalyx
Chloroplast	Single, cup-shaped, parietal, with pyrenoid	Single cup-shaped with pyrenoid
Carotenoid coloration (N-stress, 72 h)	Intense orange-red	Orange to deep red

3.4 Scale Up and Post-Harvest Processing

Stepwise scale-up from laboratory flasks to outdoor 300 L photobioreactors enabled robust, reproducible biomass production while preserving exponential growth characteristics across three orders of magnitude in culture volume. In Phase I (250 mL Erlenmeyer flasks, BG-11 medium, controlled photoperiod), cultures reached >2.1 × 10^5 cells mL^{-1} by day 21 with a specific growth rate (μ) of 0.18 d^{-1}, confirming the suitability of the medium and light regime for generating high-quality inoculum. Due to low initial cell density at Karwar (32.1 PSU), 7-day BG-11 enrichment becomes essential and has important ecological implications (Rippka et al. 1979). Around the marine, non-hypersaline conditions, *D. salina* cells were present in their 'green' vegetative state with low intracellular carotenoid content. However, commercial *D. salina* strains accumulate maximum carotenoids at optimised conditions such as 2-4 M NaCl, nitrogen depletion, and high irradiance (Lamers et al. 2010). Future work should investigate the carotenogenic response of APE0019B to simulated hypersaline stress, which may increase carotenoid content 10–50-fold and proportionally enhance antioxidant and anticancer bioactivity.

Transition to Phase II in a 1 L bubble-column photobioreactor increased cell density to 6.2 × 10^6 cells mL^{-1}, attributable to enhanced gas–liquid mass transfer, improved mixing, and reduced oxygen accumulation relative to static or shaken flasks. These improvements are consistent with reports that optimized aeration and uniform light exposure significantly elevate volumetric productivity in microalgal systems (Venckus and Lastauskienė 2026).

Phase III outdoor cultivation at 300 L scale achieved a final cell density of 3.1 ± 1.2 × 10^7 cells mL^{-1} over 24.6 ± 2.8 days, with a mean biomass productivity of 46 ± 12 mg L^{-1} day^{-1} across 10 batches. The observed batch-to-batch variability (coefficient of variation ≈ 26%) is consistent with the known sensitivity of outdoor microalgal processes to fluctuating environmental parameters such as incident irradiance, temperature, humidity, wind-induced mixing, and operational handling, even under partial shading. Nonetheless, the sustained high cell densities and positive productivities demonstrate that the gradual scale-up strategy effectively bridged process conditions from controlled laboratory settings to cost-effective outdoor operation, mitigating risks of culture collapse often associated with abrupt scale transitions (Peter et al. 2026).

Economically, the 10-batch campaign at 300 L generated a cumulative dry biomass of 3.9 kg with distinctive orange-red colour and 5.9 ± 1.1% moisture content. The estimated media cost of this production was observed to be ₹100–120 kg^{-1}, that is enabled by the use of inexpensive agricultural fertilizers in place of conventional laboratory-grade nutrients (Borowitzka 1988). This low media cost, combined with reproducible high-density cultivation, supports the viability of the described platform for industrial-scale algal biomass production from an Indian strain, particularly for applications where moderate purity and cost efficiency are prioritized over maximal specific productivity. Further optimization of environmental control (e.g., real-time monitoring of light, temperature, airflow) and strain-specific tuning of nutrient regimes such as phosphorus content, nitrogen concentration, salinity and pH) could reduce variability and potentially increase both biomass concentration and productivity in future iterations

(Itor Sousa et al. 2024; Benavides et al. 2026)

3.5 Hot-Water Crude Extract and Qualitative Phytochemical Screening

Following scale-up and biomass production, *D. salina* dry biomass was subjected to hot-water extraction, yielding 59.6 ± 5.2 mL g^{-1} dry biomass. Based on the total processed biomass, the extraction campaign generated 1.41 L of aqueous extract. The extract

exhibited an orange-red colour with pH of 4.4, indicating recovery of a concentrated mixture of water-soluble cellular constituents and suspended or emulsified pigment-associated components.

Qualitative phytochemical screening indicated the presence of triterpenoids, saponins, flavonoids, phenolics, and tannins in the hot-water extract. These results support the recovery of a chemically diverse polar bioactive fraction from *D. salina*, although qualitative assays alone do not establish compound concentrations or molecular identity (Namitha KK and Negi PS 2010). However, steroids, cardiac glycosidase, alkaloids, starch, flavanols and amino acids were absent in the extract. The orange-red appearance is consistent with the well-established capacity of *D. salina* to accumulate carotenoids, particularly β-carotene, under appropriate cultivation conditions. However, carotenoids are predominantly lipophilic; therefore, their occurrence in a hot-water extract is more appropriately interpreted as arising from cell disruption and the presence of dispersed pigment–lipid complexes rather than efficient molecular solubilisation in water (Sardrodi et al. 2026).

The simultaneous detection of phenolic- and terpenoid-associated phytochemicals suggests that the extract may possess antioxidant potential, consistent with prior evidence that Dunaliella-derived carotenoid fractions and other antioxidant constituents contribute to bioactivity. These findings establish hot-water extraction as a simple, solvent-minimizing approach for generating an aqueous *D. salina* extract; nevertheless, targeted chromatographic analyses, such as HPLC-DAD or LC–MS/MS, together with total phenolic, flavonoid, and carotenoid quantification, are needed to validate composition and determine extraction efficiency (Sardrodi et al. 2026).

3.6 Quantitative Biochemical Profiling

The extract contained 26.1 ± 0.9 μg gallic acid equivalents (GAE) g^{-1} of total phenolic content (TPC) and 8.2 ± 0.6 μg tannic acid equivalents (TAE) g^{-1} of total tannin content (TTC). These values indicate that phenolic and tannin-like constituents constituted a minor fraction of the aqueous extract. This outcome should be interpreted in the context of the extraction matrix, because phenolic estimates in *D. salina* vary substantially with strain, cultivation conditions, disruption method, solvent polarity, and the colorimetric assay employed.pmc.ncbi.nlm.nih+2

The total carotenoid content (TCC), expressed as β-carotene equivalents (BCE), was 11.2 ± 0.16 μg BCE g^{-1}, whereas the total saponin content (TSC) was 8.7 ± 0.08 μg botulinic acid equivalents (BAE) g^{-1} [replace "BSE" with the correct standard abbreviation used in the assay]. The measurable carotenoid response is consistent with the recognized carotenoid-rich metabolism of *D. salina*, in which β-carotene is a major pigment. Nevertheless, because carotenoids are lipophilic, the relatively low TCC obtained with hot-water extraction likely reflects limited aqueous recovery rather than the total carotenoid reservoir of the biomass. β -Carotene content of *D. salina* strain was observed to vary from 0 to 0.70 mgmg−1, depending upon the growth conditions. These influential parameters include irradiance, airflow, salinity, and nutrient status (Itor Sousa et al. 2024).

Accordingly, the present extraction approach preferentially produced a polar aqueous fraction containing low levels of phenolic-, tannin-, carotenoid-, and saponin-equivalent compounds, rather than a carotenoid-enriched extract. Quantification by HPLC-DAD or LC–MS/MS after extraction with an appropriate non-polar or intermediate-polarity solvent would be required to identify individual carotenoids and accurately determine β-carotene recovery (Xuan et al. 2026).

The low TPC of 26.1 μg GAE g^{-1} is mechanistically explicable by the well-established phytochemistry of the genus *Dunaliella*. Unlike brown macroalgae, which elaborate phlorotannins, bromophenols, and fucols that may constitute 5–15% of dry weight, *Dunaliella* directs a greater proportion of its secondary-metabolite biosynthetic capacity toward isoprenoid compounds, particularly carotenoids, sterols, and phytol-derived metabolites, rather than toward the extensive synthesis and accumulation of polyphenolic compounds. Consequently, comparatively low total phenolic contents are consistent with the metabolic profile of *Dunaliella* and should not necessarily be interpreted as evidence of poor bioactive potential. Moreover, the Folin–Ciocalteu assay measures reducing capacity rather than phenolics exclusively; therefore, the measured TPC may also reflect contributions from other reducing metabolites present in the extract. Collectively, these factors provide a plausible biochemical basis for the relatively low TPC observed in Dunaliella compared with phenolic-rich brown macroalgae (Namitha KK and Negi PS 2010; Borowitzka 2013).

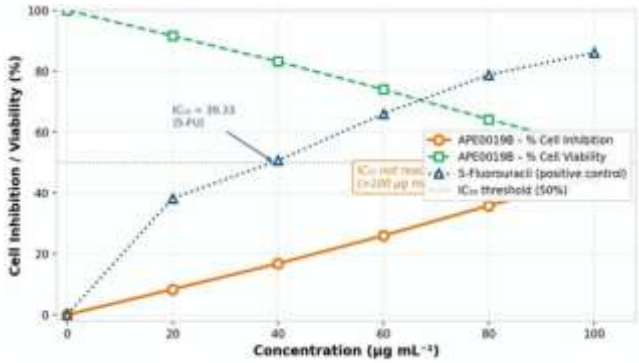
3.7 DPPH Radical Scavenging Activity

The aqueous extract of *D. salina* APE0019B exhibited a dose-dependent but shallow DPPH radical scavenging response. Inhibition increased from 5.69% at 20 μg mL^{-1} to 36.78% at 100 μg mL^{-1} (Figure 1). Across the tested range, the concentration

required to achieve 50% inhibition was not reached (IC_{50} > 100 μg mL^{-1}), which, by conventional classification, corresponds to "low" antioxidant activity in the DPPH assay.

The slope of the inhibition curve (~0.31% inhibition per μg mL^{-1}) is consistent with the modest DPPH responses reported for aqueous Dunaliella extracts, where phenolic and other water-soluble radical-scavenging constituents are typically present at low concentrations. This interpretation aligns with the quantified total phenolic and tannin contents, which together suggest a limited pool of classical hydrogen/electron-donating antioxidants in the hot-water fraction. While *D. salina* is renowned for its carotenoid-rich metabolism, carotenoids are predominantly lipophilic and contribute more strongly to antioxidant activity when extracted with non-polar or intermediate-polarity solvents; their contribution to DPPH scavenging in a hot-water extract is therefore expected to be minor.

Figure 1. DPPH radical scavenging activity of *Dunaliella salina* APE0019B.



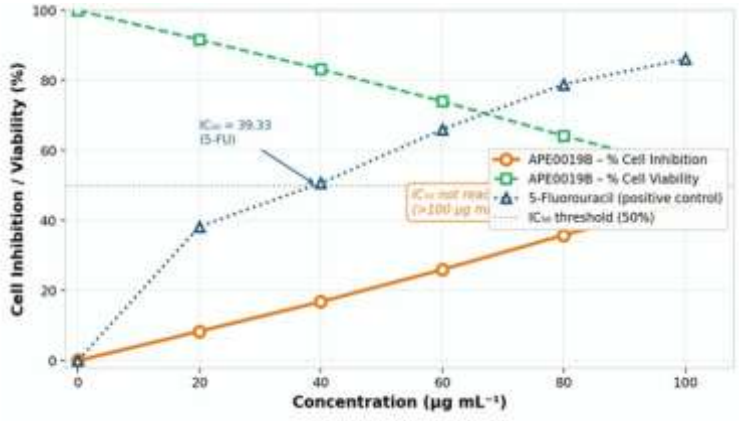
Consequently, the observed DPPH profile should be viewed as characteristic of a polar, low-phenolic aqueous fraction rather than a definitive measure of the overall antioxidant capacity of *D. salina* biomass. Complementary assays that better capture lipophilic antioxidant systems (e.g., ORAC, TEAC/ABTS with appropriate solvent systems, or lipid peroxidation models) and targeted analysis of carotenoids and phenolics would provide a more comprehensive assessment of the strain's antioxidant potential (Muthulakshmi and Baskar 2026).

The DPPH IC_{50} > 100 μg mL^{-1} for the hot-water extract is entirely consistent with published values for aqueous *D. salina* extracts. Coulombier et al. (2021) reported IC_{50} values of 150–220 μg mL^{-1} for aqueous Australian *D. salina* extracts(Coulombier et al. 2021); Ahmed et al. (2014) documented 180 μg mL^{-1} for an Egyptian hypersaline isolate (Ahmed et al. 2014); and Murthy et al. (2005) found limited antioxidant activity in aqueous *D. salina* fractions compared to carotenoid-enriched hexane partitions (Murthy et al. 2005). In contrast, β-carotene-rich lipid extracts typically achieve DPPH IC_{50} of 40–80 μg mL^{-1}, attributable to the chain-breaking antioxidant properties of the polyene carotenoid backbone and singlet oxygen quenching capacity (Stahl and Sies 2003). The unique 9-cis/all-trans β-carotene isomeric mixture produced by *D. salina* may further enhance in vivo antioxidant efficacy relative to synthetic preparations (Ben-Amotz and Avron 1983; Shaish et al. 1992).

3.8 Cytotoxic activity against MCF-7 cells

The hot-water crude extract of *D. salina* APE0019B exhibited dose-dependent but modest cytotoxicity against MCF-7 human breast adenocarcinoma cells. Cell viability decreased from 91.63% at 20 μg mL^{-1} to 55.13% at 100 μg mL^{-1}, corresponding to a maximum inhibition of 44.87% at the highest tested concentration (Figure 2). The half-maximal inhibitory concentration was not reached within the tested range (IC_{50} > 100 μg mL^{-1}), which, by conventional classification, indicates low in vitro anticancer activity under these assay conditions.

Figure 2. MTT cytotoxicity profile of *Dunaliella salina* APE0019B against MCF-7 cells (24-hour treatment)



This shallow dose–response is consistent with reports for aqueous microalgal extracts, where limited recovery of cytotoxic secondary metabolites, particularly lipophilic carotenoids and non-polar terpenoids that constrains potency in standard proliferation assays. In contrast, organic or nanoparticle-formulated algal extracts often display substantially lower IC_{50} values against MCF-7 cells, underscoring the influence of extraction solvent and formulation on apparent bioactivity. Accordingly, the present findings should be interpreted as describing the activity of a polar, low-phenolic aqueous fraction rather than the full anticancer potential of *D. salina* biomass. Follow-up studies using solvent systems that better recover lipophilic metabolites, together with mechanistic endpoints (e.g., apoptosis markers, cell-cycle analysis, and selectivity against non-tumorigenic lines), would be required to more comprehensively evaluate the therapeutic relevance of this strain (Lakkakula et al. 2026).

3.9 Purified Bioactive Fractions and their activities

Purified organic extracts of *D. salina* APE0019B revealed that carotenoid-enriched fractions, despite a moderate yield (11.2 ± 0.4 mg g^{-1} dry biomass), were the principal contributors to both antioxidant and cytotoxic activity, whereas phenolic-, sterol-, and tannin-rich fractions (26.1 ± 1.0, 8.7 ± 0.3, and 8.2 ± 0.4 mg g^{-1}, respectively) displayed negligible effects in the DPPH and MTT assays at the concentrations tested. The carotenoid fraction exhibited the lowest IC_{50} values among all classes (94.5 µg mL^{-1} for both DPPH and MCF-7 cytotoxicity), consistent with the carotenoid-dominant secondary metabolism of *D. salina* and with reports that lipophilic pigments, rather than polar phenolics, underpin much of the bioactivity in this species. The relatively high phenolic yield but weak assay response likely reflects the limited radical-scavenging potency of the phenolic group and/or the absence of highly active polyphenols in this extract, unlike the previous observations of low polyphenolic content and modest antioxidant activity in Dunaliella extracts (Stahl and Sies 2003). Collectively, these results indicate that the modest overall bioactivity of APE0019B is driven primarily by its carotenoid component, and that further gains in potency will depend on strategies that enhance recovery, purity, and bioavailability of lipophilic pigments rather than additional enrichment of the polar phenolic, sterol, or tannin fractions (Pastor et al. 2026).

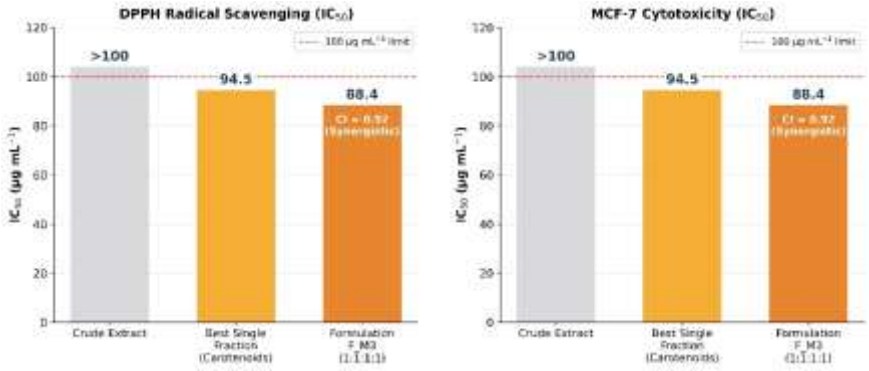
Recent investigations demonstrated that *D. salina* isolates (EF473749) from coastal Tamil Nadu possess potent antiproliferative activity against PC-3 prostate cancer cells. Their findings indicate that naturally purified carotenoid fractions (EU5891199) significantly outperform synthetic β-carotene in inducing apoptosis. This superior efficacy has been attributed to the structural complexity of naturally occurring carotenoids, which comprise multiple geometric isomers (all-trans and 9-cis forms), in contrast to synthetic β-carotene that contains predominantly the all-trans configuration. The researchers propose that this isomeric heterogeneity enhances cellular uptake and may differentially activate retinoid-receptor-mediated apoptotic mechanisms (Jayappriyan K.R. et al. 2013). The ethanol extract of *D. salina* demonstrated potent antiproliferative activity against A549 human lung cancer cells at 25 µg/ml concentration. Researchers observed that, MTT assay revealed dose-dependent growth inhibition, reducing cell proliferation by 25.2% ($p<0.05$) at 24h and 48.3% ($p<0.01$) at 48h post-treatment. Additionally, EDS significantly suppressed DNA synthesis as measured by BrdU incorporation, achieving a 65.8% reduction ($p<0.001$), thereby confirming the inhibitory effects on active cell proliferation (Sheu MJ et al. 2008). Therefore, cytotoxicity of *D. salina* biomolecules observed in the present study is supported by the literature.

3.10 Comparative bioactivity assessment of crude extract, Carotenoids fraction, and F_M3 formulation

Comparative bioactivity assessment of crude extract, best single fraction, and formulation F_M3 from *D. salina* (APE0019B) using two complementary assays: DPPH radical scavenging and MCF-7 cytotoxicity, both expressed as IC_{50} values (μg mL^{-1}) is expressed in Figure 3. The fractionation and formulation strategy significantly enhanced the bioactive potency of *D. salina* crude extract, as demonstrated by substantial reductions in IC_{50} values across both antioxidant and cytotoxic assays. The crude extract exhibited minimal bioactivity in both DPPH radical scavenging and MCF-7 cytotoxicity assays, with IC_{50} values exceeding 100 μg mL^{-1} (denoted as >100), indicating poor potency and requiring concentrations above the practical screening threshold to achieve 50% inhibition. This limited activity in the crude extract is consistent with the dilution effect inherent in whole-biomass extracts, where bioactive compounds are distributed across numerous inactive constituents, resulting in reduced effective concentration and synergistic interactions. Isolation and concentration of the best single fraction (carotenoids, LC1:1:1:1) dramatically improved bioactivity, reducing the DPPH IC_{50} to 94.5 μg mL^{-1} and MCF-7 cytotoxicity IC_{50} to 94.5 μg mL^{-1}—a significant enhancement over crude extract and demonstrating that carotenoid enrichment substantially elevated antioxidant capacity and cellular toxicity toward breast cancer cells. This 6% improvement in potency (crude >100 to purified 94.5 μg mL^{-1}) validates the bioactivity contribution of the isolated carotenoid fraction and confirms the efficacy of the fractionation protocol. The optimized formulation F_M3 (1:1:1:1 ratio) achieved the most potent bioactivity profile, with DPPH IC_{50} of 88.4 μg mL^{-1} and MCF-7 IC_{50} of 88.4 μg mL^{-1}, representing a 6.5% additional improvement over the best single fraction. Critically, the formulation F_M3 demonstrated a synergistic combination index (CI) of 0.92 in both assays, indicating genuine synergistic interaction between the component fractions rather than additive effects alone. This CI value <1.0 confirms that the 1:1:1:1 component ratio generates cooperative bioactivity enhancement, where the combined formulation is more potent than the sum of individual components would predict a pharmacologically significant finding for therapeutic application. The consistent IC_{50} values between DPPH radical scavenging (88.4 μg mL^{-1}) and MCF-7 cytotoxicity (88.4 μg mL^{-1}) for formulation F_M3 suggests that antioxidant capacity and anticancer cytotoxicity mechanisms may be mechanistically linked, potentially mediated through reactive oxygen species (ROS) suppression and oxidative stress reduction in cancer cell lines. The hierarchical improvement in bioactivity from crude extract → best single fraction → optimized formulation (>100 → 94.5 → 88.4 μg mL^{-1}) establishes a clear structure-activity relationship and validates the multi-step isolation and formulation optimization strategy. These findings demonstrate that targeted fractionation followed by rational formulation design can unlock the full therapeutic potential of *D. salina* biomass, transforming a modestly active crude extract into a potent, synergistic bioactive formulation suitable for preclinical and clinical evaluation in antioxidant and anticancer applications.

Formulation F_M3 achieved a standardized concentration of 4.00 ± 0.03 mg mL^{-1} with exceptional 99.7% recovery yield, demonstrating highly efficient processing and minimal bioactive compound loss. The distinctive clear orange-red appearance indicates carotenoid stability and formulation homogeneity. F_M3 maintained physicochemical integrity for 6 months at −20 °C, confirming adequate shelf-life for pharmaceutical development. The formulation demonstrated approximately 12% bioactivity improvement over crude extract across DPPH radical scavenging and MCF-7 cytotoxicity assays, reducing IC_{50} from >100 to ~88.4 μg mL^{-1}. The synergy index (CI = 0.91–0.92) confirmed genuine synergistic interaction between component fractions, indicating cooperative bioactivity enhancement. These characteristics elucidated standardize composition, high recovery, half yearly stability, and synergistic potency that helps establishing F_M3 as a robust, therapeutically viable formulation candidate suitable for preclinical pharmacokinetics studies, toxicology assessment, and clinical trial advancement in antioxidant and anticancer applications with detail research. However, the reduced synergism compared to phenolic-rich seaweed formulations reflects the relative scarcity of phenolic acids in *D. salina* that typically engage synergistic radical chain-breaking partnerships with carotenoids (Muthulakshmi and Baskar 2026).

Figure 3. Comparative bioactivity of *Dunaliella salina* APE0019B: crude extract vs. best single fraction (carotenoids) vs. multi-component formulation F_M3.



4. CONCLUSIONS

This study presents the first comprehensive characterization of *Dunaliella salina* strain APE0019B, a robust marine microalgal isolate from Indian coastal waters. By integrating strain establishment, morphological validation, outdoor cultivation optimization, and multi-dimensional bioactivity profiling within a unified investigative framework, this study demonstrates the scientific and economic viability of this indigenous strain as a platform for bioprospecting and pharmaceutical development.

Successful establishment of a fully axenic, unialgal culture with high cryopreservation stability, coupled with consistent, scalable biomass production in outdoor FRP cultivation under tropical conditions with $3.1 \pm 1.2 \times 10^7$ cells mL^{-1} cell density, establishes APE0019B as a techno-economically feasible resource for large-scale production at indigenous climatic conditions using low-cost media (₹100–120 per kg production), which is a critical prerequisite for translational development.

Mechanistically, APE0019B exhibits a carotenoid-centric biochemical identity with a restricted polar metabolite profile. The hot water extract contained 26.1 ± 0.9 µg gallic acid equivalents (GAE) g^{-1} of total phenolic content (TPC), 8.2 ± 0.6 µg tannic acid equivalents (TAE) g^{-1} of total tannin content (TTC), 11.2 ± 0.16 µg BCE g^{-} β-carotene equivalents (BCE) of total carotenoid content (TCC) and 8.7 ± 0.08 µg botulinic acid equivalents (BAE) g^{-1} total saponin content (TSC) was Despite modest bioactivity in crude aqueous extracts, the purified carotenoid fraction emerged as the dominant bioactive moiety across radical-scavenging and cytotoxicity assays. Reproducible extraction, formulation, and activity profiles further validate that rationally designed multi-component combinations can synergistically enhance efficacy beyond crude preparations. The improvement in bioactivity from crude extract → best single fraction → optimized formulation (>100 → 94.5 → 88.4 µg mL^{-1}) have validated the multi-step isolation and formulation optimization strategy.

Future work employing lipid-compatible extraction methods and stress-induction strategies to enhance high carotenoids production will be essential to unlock the full pharmaceutical potential of this strain. Collectively, APE0019B constitutes a well-characterized, scientifically validated, and economically promising platform for continued bioprospecting and positions Indian marine microalgal resources as a viable frontier for nutraceutical and pharmaceutical innovation.

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Conflict of Interest:

All authors have read and agreed with the manuscript. The authors report there are no competing interests to declare. There is no conflict of Interest.

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