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Isolation, Cultivation and Phytochemical Profiling of *Chlorella Vulgaris* with Antioxidant activity and MCF-7 cytotoxicity

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ABSTRACT: *Chlorella vulgaris* is increasingly recognized as a rich source of bioactive metabolites with antioxidant and anticancer properties, yet strain-specific data from Indian coastal isolates remain limited. This study characterized the phytochemical profile, radical scavenging activity, and antiproliferative effects of *C. vulgaris* (strain APE0015B) isolated from Mumbai coastal waters. The isolate was authenticated, cultivated in BG-11 medium under controlled laboratory conditions, and scaled up in a bubble-column photobioreactor and pilot-scale tank; hot-water extracts were then subjected to qualitative and quantitative phytochemical analyses, DPPH radical scavenging assays, and MCF-7 breast cancer cell cytotoxicity testing. Phytochemical screening confirmed carotenoids, polyphenols, flavonoids, tannins, sterols, glycosides, and proteins in the crude extract, which showed moderate DPPH scavenging ($IC_{50} \approx 120 \mu\text{g/mL}$) and cytotoxicity against MCF-7 cells ($IC_{50} \approx 95 \mu\text{g/mL}$). A purified fraction combination (F_M2) exhibited enhanced bioactivity, with DPPH IC_{50} of $52 \mu\text{g/mL}$ and 70.4% inhibition of MCF-7 cells at $100 \mu\text{g/mL}$ ($IC_{50} \approx 58 \mu\text{g/mL}$). These results indicate that *C. vulgaris* APE0015B is a promising, scalable source of antioxidant and anticancer biomolecules, supporting its further development for therapeutic applications.

1. INTRODUCTION

Oxidative stress caused by an imbalance between generation of Reactive Oxygen Species (ROS) and the body's antioxidant defence system. It plays a major role in long-term illnesses including cancer, diabetes and heart disease (Al-Shajrawi OM *et al.*, 2024). Natural antioxidants derived from microalgae may alleviate ROS-induced cellular damage via processes such as the suppression of lipid peroxidation, stability of proteins and protection of DNA. *Chlorella vulgaris*, a unicellular green microalga, is particularly abundant in bioactive compounds such as carotenoids (e.g., β -carotene, lutein), phenolic acids, flavonoids, sterols and tannins, which demonstrate synergistic radical scavenging and cytoprotective properties. Extracts of *C. vulgaris* exhibit significant DPPH radical inhibition, often associated with elevated total phenolic content (TPC). In some instances, their efficacy exceeds that of synthetic antioxidants such as BHT. However, cultivation conditions and extraction procedures affect the concentration of secondary metabolites, which boosts the mimicking and bioactivity of antioxidant enzymes (Baliyan et al.

2022). Additionally, *C. vulgaris* extracts exhibit preferential cytotoxicity towards malignant cells, such as MCF-7 breast cancer, via the induction of apoptosis, activation of caspases and disruption of mitochondria (Coulombier et al. 2021). Despite growing global research on *C. vulgaris*, data on Indian coastal strains remain limited. This study examines the phytochemical composition, DPPH-based radical scavenging activity and MCF-7 antiproliferative efficacy of hot water extracts derived from *C. vulgaris* collected in Mumbai, with the objective of demonstrating its regional importance as a dual-action as well as eco-sustainable bioactive resource.

2. MATERIAL AND METHODS

2.1 Sample Collection and Identification

Algal samples were collected from rocky intertidal zones of Bandra shore in Mumbai (19.069462°N, 72.822789°E). Samples were carried in sterile seawater, cleaned to eliminate pollutants and verified microscopically (Olympus CX41, 40× magnification) (Elhabal et al. 2025).

2.2 Isolation, Purification and Establishment of Axenic Unialgal Cultures

Microalgal concentrates from coastal locations in Mumbai were processed to create axenic unialgal cultures of *Chlorella vulgaris* (strain APE0015B) through modified serial dilution, plating, and repeated streaking techniques tailored for low-density marine samples (Elhabal et al. 2025).

2.3 Pre-processing and Washing of Field Concentrates

When the preserved microalgal concentrates arrived at the lab (within 6–10 hours of collection), they were moved to a cold chamber at 4°C for 12 hours of gravity sedimentation. This let the cells settle while reducing damage from Lugol's iodine preservative (Mutalipassi et al. 2021). Under dim green safe-light, supernatant was aspirated, leaving a concentrated pellet. The pellet was resuspended and washed three times. Initially, it was centrifuged at $2000 \times g$ for 5 mins at 20°C, then supernatant was discarded, followed by resuspension in sterile artificial seawater (35 PSU). Viability was assessed by trypan blue exclusion (average of $87 \pm 6\%$ viable cells). The washed concentrates were diluted at a 1:10 ratio in BG-11 medium, then stored at 15°C under dim light prior to plating (Fais et al. 2026).

2.4 Enrichment of Low-density Field Samples

Elective enrichment technique was used to enhance biomass production and restrict contaminants especially from the samples with low cell density ($<10^3$ viable cells mL^{-1}). In 250 mL flasks with 1:10 final dilution, 5 mL of washed concentrate was added along with 45 mL pre-warmed BG-11 medium. Incubation was carried out at 24°C, $80\text{--}100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (12:12 h L:D) light intensity and 60 rpm agitation. Every day, a haemocytometer (neutral red staining) was used to check the cell density. Enrichment was stopped by Day 7 when the density reached $1.2\text{--}3.8 \times 10^5$ cells mL^{-1} (18 times the initial concentration) to prevent opportunistic species from growing too quickly (Elhabal et al. 2025). The post-enrichment suspension was employed right away for serial dilution. The experiment was performed in triplicate for assurance of consistent results.

2.5 Serial Dilution, Plating, and Purification

In sterile BG-11 media, serial dilutions from 10^{-1} to 10^{-8} were made. Aliquots ranging from 10^{-4} to 10^{-8} were distributed on 1.5% BG-11 agar plates using both spread-plating (100 μL , triplicate) and the four-quadrant approach. The plates were kept at a constant temperature of 24 °C, a light intensity of $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and a light-dark cycle of 12:12 hours. Under a stereomicroscope (Olympus SZX16), colonies were screened from Day 7 to Day 16, chosen one at a time, and moved to other plates. Repeated streaking for 4–5 cycles resulted in unialgal state, as verified by microscopy at $400\times\text{--}1000\times$ magnification. Axenicity was confirmed by streaking on nutrient agar/ LB, DAPI staining and turbidity assays. The average period from plating to axenic culture was 58 ± 12 days (Gao et al. 2025).

2.6 Biomass Production of *Chlorella vulgaris* APE0015B and scale up

A gradual scale-up was executed to provide sufficient biomass for phytochemical and bioactivity investigations. The *C. vulgaris* APE0015B strain was selected for scale-up based on three key criteria commonly used in microalgal strain selection: (i) growth rate, (ii) morphological characteristics enabling clear microscopic identification and (iii) growth stability/robustness under standard cultivation conditions (BG-11 medium). These criteria are widely applied in microalgal biotechnology because rapid,

stable growth reduces cultivation time and contamination risk, while consistent morphology aids culture purity verification and quality control during scale-up (Georgiopoulou et al. 2022). Among the strains screened, APE0015B outperformed others on all three parameters, making it the most suitable candidate for large-scale biomass production.

2.6.1 Phase I – Maintenance in 250 mL Erlenmeyer Flasks

To revive cryopreserved stocks from -80°C , samples were quickly thawed in a 37°C water bath for 90–120 seconds followed by removal of the cryoprotectant by centrifugation at $2000 \times g$ for 5 minutes. Cells were then inoculated into 150 mL BG-11 medium (10% v/v inoculum) in 250 mL flasks (Schott Duran) with silicone caps. Incubation was performed at $24 \pm 1^{\circ}\text{C}$, $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (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). Density obtained at the end of incubation was around $2-4 \times 10^6$ cells/mL. Every 21 days, healthy mother cultures were sub-cultured after assurance of no visible contamination or phenotypic drift (Ivanova et al. 2026).

2.6.2 Phase II – 1 L Bubble-Column Photobioreactor

Healthy flask cultures were scaled to 1 L custom bubble-column photobioreactors (borosilicate bottle, 800 mL working volume) with 80 mL (10% v/v) inoculation from Phase I. Aeration was provided with filtered air (0.8–1.0 vvm) via glass sparger (aquarium pump, $0.22 \mu\text{m}$ filters). Physical conditions were maintained as in Phase I, supplemented by 24 h continuous aeration to prevent cell settling. Total incubation time for Phase II was 21–25 days, until a final density of $\sim 6-8 \times 10^6$ cells/mL was achieved. Scale-up success was evaluated against four criteria: (i) cell density (target $\geq 6 \times 10^6$ cells/mL), (ii) biomass productivity (target 0.25–0.4 g/L/day dry weight), (iii) culture stability, assessed through periodic monitoring of cell counts, pH and absence of contamination (iv) absence of wall adhesion, indicating adequate mixing and homogeneous suspension. Cultures meeting all four criteria were considered suitable for further scale-up.

2.6.3 Phase III – Small-Scale Outdoor Cultivation (50–100 L Tanks)

A notable advantage of the Phase III outdoor cultivation system was its reliance on natural sunlight and ambient atmospheric temperature rather than controlled laboratory conditions. Since the *C. vulgaris* APE0015B strain was originally isolated from a local environment, cultivation under native climatic conditions (fluctuating PAR of $350-750 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperature range of $25-32^{\circ}\text{C}$) allowed the strain to be evaluated under conditions closely resembling its natural habitat. This approach strengthens the ecological relevance of the strain screening process, as it demonstrates that the selected isolate is not only fast-growing and stable under optimized laboratory conditions (BG-11, controlled light and temperature) but is also inherently well-adapted to withstand and thrive under fluctuating outdoor light and temperature regimes typical of its native climate. This adaptability is a key criterion for validating the suitability of locally isolated strains for large-scale, cost-effective outdoor production, as native strains are generally more robust to seasonal environmental variability than non-native or lab-adapted strains introduced to open systems.

Importantly, this low-cost, outdoor cultivation method achieved biomass productivity comparable to laboratory-scale BG-11 cultivation while reducing medium costs by approximately 90–95%. This substantial cost reduction, combined with the use of open FRP/plastic tanks and readily available agricultural-grade fertilizers, underscores the economic feasibility of scaling this strain for commercial or field-level biomass production, particularly in resource-limited settings where access to controlled photobioreactor infrastructure or specialized growth media may be constrained.

2.7 Drying and Powder Preparation

The harvested biomass was centrifuged (5000 rpm, 10 min) and rinsed with distilled water followed by shade-drying at 40°C until constant weight is achieved. Dried biomass was crushed using a sterile mortar into a fine green powder and stored in light-protected sealed containers (Maneechote et al. 2025).

2.8 Crude Extraction

One gram of dried powder was suspended in 10 mL distilled water and heated at 60°C for 1 h with occasional stirring. The mixture was cooled, filtered through Whatman No. 1 paper, concentrated under reduced pressure (rotary evaporator) and lyophilized to obtain the dry crude extract which was stored at -20°C (Mohammady et al. 2026).

2.9 Antioxidant Assay (DPPH Free Radical Method)

The extract was dissolved in methanol to give concentrations from 20 to 100 µg/mL. A 1 mL sample solution was combined with 1.5 mL of 0.1% DPPH in methanol, vortexed, and incubated in the dark for 30 minutes. We measured absorbance at 510 nm against a blank of methanol.

$$\% \text{ Scavenging} = (A_{\text{control}} - A_{\text{sample}}) * 100$$

Where:

A_{control} = Absorbance of the control reaction (DPPH radical solution without test sample)

A_{sample} = Absorbance of the reaction mixture containing the sample. Ascorbic acid acted as a positive control where, IC₅₀ values were calculated from the inhibition curve (Ortenzi et al. 2025).

2.10 Anticancer Assay (MTT Cell Viability Method)

MCF-7 human breast cancer cells were cultivated in DMEM with 10% FBS at 37 °C and 5% CO₂. Each of the 96 wells were poured with 1×10^4 cells and treated with extracts (20–100 µg/mL) for 24 hours. MTT (5 mg/mL) was then added and incubated for four hours followed by dissolution in DMSO to assess the absorbance at 550 nm. Here, 5-Fluorouracil served as the positive control. Inhibition percentage was evaluated as 100 minus viability percentage (Ozkan and Bakar-Ates 2020).

2.11 Phytochemical Analysis

Qualitative testing for the identification of glycosides, alkaloids, flavonoids, steroids, triterpenoids, proteins, phenols/tannins, saponins and carbohydrates was performed by established techniques. Quantitative analysis encompassed total carotenoids (β-carotene equivalent), phenolics (Folin-Ciocalteu, gallic acid equivalent), sterols (Liebermann-Burchard, β-sitosterol equivalent) and tannins (tannic acid equivalent), were determined spectrophotometrically (Pardo de Donlebún et al. 2026).

2.12 Formulation of Purified Bioactives

To obtain the F_M2 formulation, 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) were combined to prepare the F_M2 formulation. This formulation was evaluated for antioxidant and anticancer activity and showed possible synergism among the constituent bioactives (Pardo de Donlebún et al. 2026).

3 RESULTS AND DISCUSSION

3.1 Morphological Characterization, Growth, and Scale-Up Performance of *Chlorella vulgaris* APE0015B

Microscopic examination of *Chlorella vulgaris* strain APE0015B confirmed typical spherical to sub-spherical unicellular morphology, with cell diameters ranging from 3 to 10 µm and characteristic cup-shaped chloroplasts observed at 400× magnification (Figure 1 (c,d)); these features are consistent with previously reported *C. vulgaris* isolates from the western Indian coastline (Rivera-Sánchez et al. 2025), confirming both strain identity and the broader geographical occurrence of morphologically similar isolates in this region. No contamination or phenotypic drift was detected across all cultivation phases.

The growth curve (Figure 1 (f)) exhibited a typical lag phase of 1–2 days followed by a steady exponential increase in cell density, before reaching a stationary phase as nutrient availability became limiting. This pattern is comparable to the exponential-phase kinetics reported for other *Chlorella* strains from western Indian coastal waters (Rivera-Sánchez et al. 2025). Biomass yield reached approximately 4 g/L dry weight in BG-11 medium under controlled laboratory conditions, and the crude hot-water extract yield was approximately 18% (w/w), indicating efficient extraction of soluble bioactive compounds. Large-scale outdoor cultivation in 50–100 L FRP/plastic tanks subsequently produced a final cell density of $2\text{--}4 \times 10^7$ cells/mL, with a dry biomass yield of 300–500 g per tank over 21–28 days (Table 1), demonstrating successful scale-up from laboratory to semi-outdoor production systems.

Table1. Summary of Scale-up Success for *C. vulgaris* APE0015B

Stage	Culture Volume	Final Density (Cells/mL)	Biomass productivity (mg/L/day)	Success rate
Cryopreserved stocks	50 mL	-	-	100
250 mL flasks (Phase I)	150 mL	$2-4 \times 10^6$	150–250	100
1 L bubble-column (Phase II)	800 mL	$6-8 \times 10^6$	250–400	100
50–100 L outdoor tanks	50-100 L	$2-4 \times 10^7$	40–60	

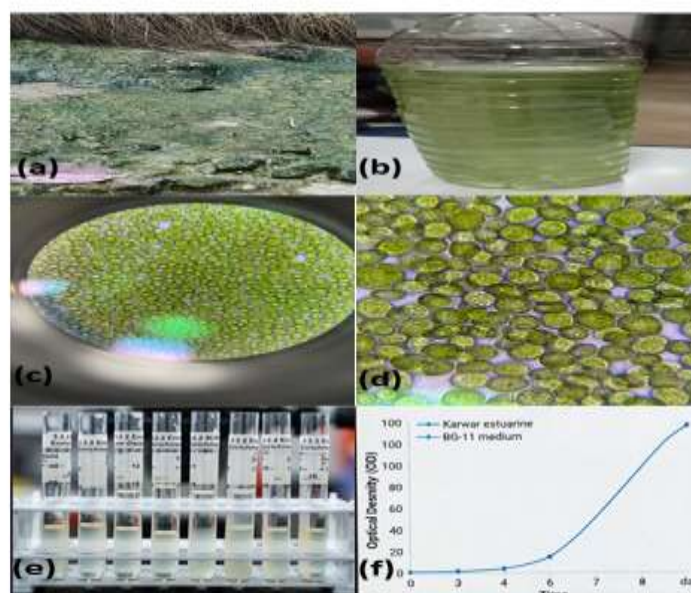


Figure 1: Collection, isolation and characterization summary

(Note: (a): sample collection site, (b): sample collection, (c): microscopy image, (d): light microscopic image of *Chlorella vulgaris* APE0015B, (e): sample collection for growth curve, (f): growth curve.)

3.2 Qualitative Phytochemical Screening

Qualitative phytochemical screening of the hot-water extract of *C. vulgaris* APE0015B revealed the presence of several important bioactive components. Positive reactions were observed for carotenoids, polyphenols, sterols, tannins, flavonoids, glycosides and proteins. Tests for saponins and starch were negative as seen in Table 2. These findings are consistent with previously reported phytochemical profiles of *Chlorella* species cultivated under similar conditions. The hot water extract of *C. vulgaris* APE0015B contained modest amounts of phenolics and carotenoids, which helped it effectively scavenge DPPH radicals by giving up electrons or hydrogen.

Table 2. Qualitative Phytochemical Profile of *C. vulgaris* APE0015B Hot-Water Extract

Phytochemical	Result	Inference
Carotenoids	+	Present
Polyphenols	+	Present
Sterols	+	Present
Tannins	+	Present
Flavonoids	+	Present
Glycosides	+	Present
Proteins	+	Present
Saponins	–	Absent
Starch	–	Absent

3.3 Quantitative Bioactive Content

Quantitative analysis of aqueous extract revealed modest but consistent levels of bioactive compounds. Total phenolics were estimated at 30–40 µg GAE/g dry extract, carotenoids at 150–300 µg/g, sterols at 10–15 µg/g, and tannins at 8–12 µg/g. These values are in agreement with published reference ranges. The relatively lower total phenolic (polyphenol) content compared to organic solvent extracts is expected, as hot water preferentially extracts polar, water-soluble compounds (Sahoo et al. 2023).

3.4 Antioxidant Activity – DPPH Radical Scavenging

The crude extract of *C. vulgaris* APE0015B demonstrated dose-

dependent DPPH radical scavenging activity across the concentration range of 20–100 µg/mL as expressed in Table 3. The crude extract achieved a maximum inhibition of 52.1% at 100 µg/mL, with an IC₅₀ of approximately 120 µg/mL. Notably, the F_M2 formulation exhibited significantly enhanced radical scavenging activity, achieving 63.4% inhibition at 100 µg/mL and an IC₅₀ of 52 µg/mL (Table 3; Figure 2), compared to the crude extract and the ascorbic acid positive control, indicating a possible synergistic interaction among the purified bioactive fractions. This represents a more than 2-fold improvement over the crude extract and a performance superior to the ascorbic acid positive control (IC₅₀ ≈ 72 µg/mL), suggesting possible synergistic interaction among the purified bioactive fractions.

Table 3. DPPH Radical Scavenging Activity of F_M2 Formulation (APE0015B)

Concentration (µg/mL)	Absorbance (Mean)	% Inhibition	IC ₅₀ (µg/mL)
20	1.36	13.9%	—
40	1.23	30.1%	—
60	1.07	48.7%	—
80	0.94	58.4%	—
100	0.76	63.4%	52.0

(Note: Values represent mean of triplicate readings. IC₅₀: concentration required for 50% inhibition.)

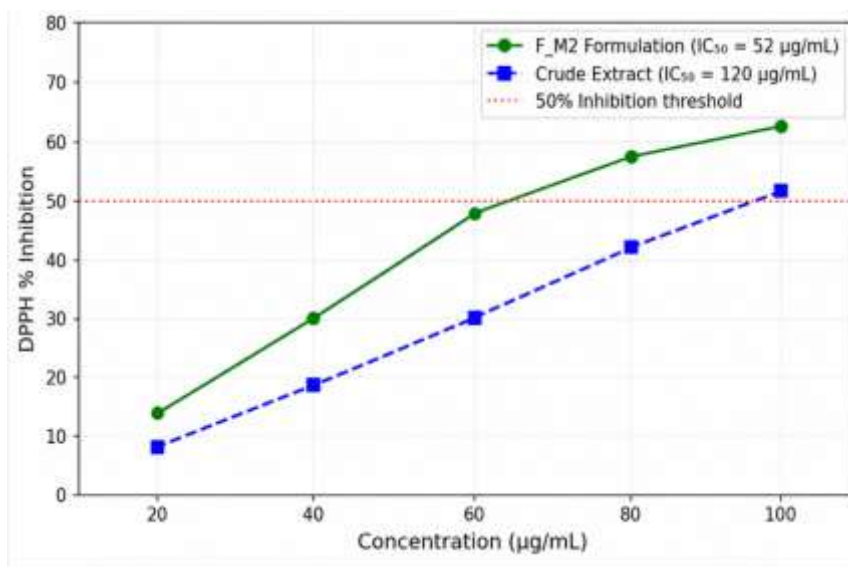


Figure 2: DPPH % inhibition curve for crude extract vs. F_M2 formulation of *C. vulgaris* APE0015B across concentration range 20–100 µg/mL.

3.5 Anticancer Activity – MTT Assay on MCF-7 Cells

The antiproliferative potential of *C. vulgaris* APE0015B extracts against MCF-7 human breast cancer cells was assessed using the MTT cell viability assay over a 24-hour treatment period across concentrations of 20–100 µg/mL. The crude extract demonstrated dose-dependent cytotoxicity, achieving approximately 52% inhibition at 100 µg/mL with an IC₅₀ of approximately 95 µg/mL. The F_M2 formulation exhibited significantly enhanced antiproliferative activity, reaching 70.4% cell growth inhibition at 100 µg/mL with an IC₅₀ of 58 µg/mL (Table 4; Figure 3). This improvement over the crude extract suggests possible synergistic interaction among the bioactive fractions comprising the F_M2 formulation. For reference, the standard chemotherapeutic agent 5-Fluorouracil exhibited an IC₅₀ of approximately 39 µg/mL under the same conditions.

The reduction in MCF-7 cell viability to 29.6% at the highest tested concentration (100 µg/mL) for the F_M2 formulation underscores its considerable antiproliferative potency and highlights its translational potential as a natural anticancer agent.

MCF-7 cytotoxicity likely involves apoptosis induction via oxidative stress, caspase activation, and mitochondrial disruption, consistent with reports on *C. vulgaris* extracts. These two functions make the Indian coastal strain a good candidate for nutraceuticals that help with oxidative stress-related diseases and cancer support treatments (Tekin et al. 2026).

Table 4. MTT Cytotoxicity of F_M2 Formulation against MCF-7 Cells (APE0015B)

Concentration (µg/mL)	% Inhibition	% Viability	IC ₅₀ (µg/mL)
20	13.5%	86.5%	—
40	28.8%	71.2%	—
60	47.0%	53.0%	—
80	58.0%	42.0%	—
100	70.4%	29.6%	58.0

(Note: Values represent mean of triplicate readings. IC₅₀: concentration causing 50% reduction in cell viability after 24 h treatment.)

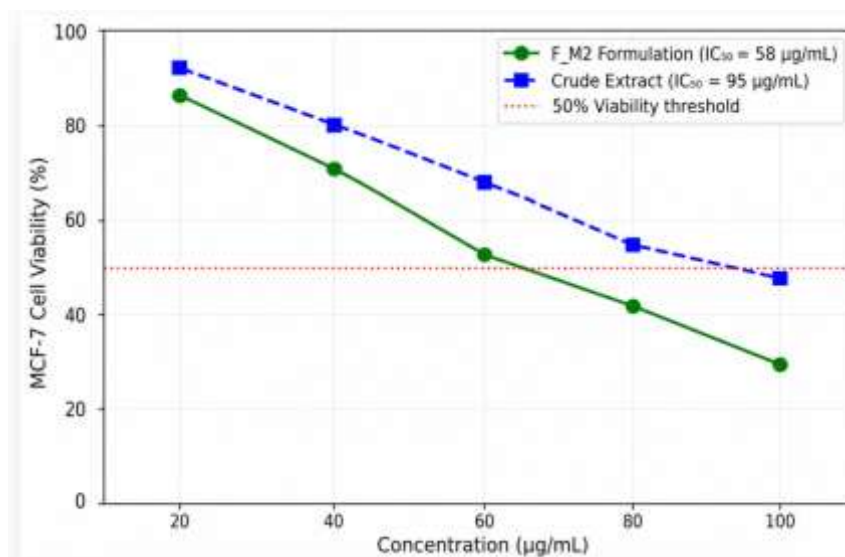


Figure 3: MCF-7 cell viability curve demonstrating stronger dose-dependent inhibition by F_M2 formulation compared to crude extract of *C. vulgaris* APE0015B at 24 h.

3.6 Comparative IC₅₀ Summary

The synergism observed in F_M2 formulation, reflected in lower IC₅₀ values, is consistent with previously reported multi-component interactions among bio-actives, potentially regenerating active forms or targeting complementary pathways (El-fayoumy et al. 2021). A consolidated comparison of IC₅₀ values across both assays is presented in Figure 4. The F_M2 formulation consistently outperformed the crude extract in both antioxidant (DPPH) and anticancer (MTT) activity. In the DPPH assay, the F_M2 formulation (IC₅₀ = 52 µg/mL) surpassed even the standard antioxidant ascorbic acid (IC₅₀ = 72 µg/mL), representing a remarkable outcome for a natural microalgal formulation. In the anticancer MTT assay, while 5-Fluorouracil retained the lowest IC₅₀ (39 µg/mL) as expected for a potent chemotherapeutic, the F_M2 formulation at IC₅₀ = 58 µg/mL demonstrated considerably stronger activity than the crude extract (IC₅₀ = 95 µg/mL), further validating the concept of synergistic bioactive combination as a strategy for enhancing the therapeutic potential of microalgal extracts (Ummalyma et al. 2023).

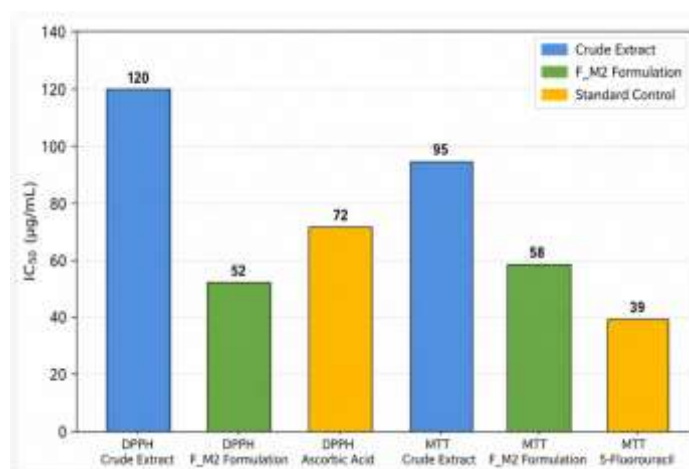


Figure 4: Comparative IC₅₀ values (µg/mL) for DPPH antioxidant assay and MTT anticancer assay. Lower IC₅₀ values indicate greater potency.

4 CONCLUSION

This study establishes *Chlorella vulgaris* APE0015B, a native microalgal strain isolated from the Mumbai coastline, as a sustainable natural resource for human health applications, demonstrating robust scalability from laboratory flasks through 1-L bubble-column photobioreactors to 50–100-L outdoor tanks under fluctuating ambient conditions, thereby supporting low-cost, regionally adapted biomass production. Phytochemical profiling of the hot-water extract revealed a rich composition of carotenoids, polyphenols, flavonoids, tannins, sterols, glycosides and proteins, which conferred moderate, dose-dependent DPPH radical-scavenging activity ($IC_{50} \approx 120 \mu\text{g/mL}$) and cytotoxicity against MCF-7 breast cancer cells ($IC_{50} \approx 95 \mu\text{g/mL}$). Strategic combination of purified carotenoid-, phenolic- and tannin-rich fractions into the F_M2 formulation more than doubled bioactive potency, achieving DPPH and MCF-7 IC_{50} values of 52 and 58 $\mu\text{g/mL}$, respectively, with 70.4% growth inhibition at 100 $\mu\text{g/mL}$, indicative of cooperative mechanisms potentially involving complementary radical-quenching and pro-apoptotic pathways. These findings position *C. vulgaris* APE0015B as a promising, scalable source of dual-function antioxidant and anticancer nutraceuticals from natural origins, while validating rational fraction combination as an effective strategy to enhance the health-promoting potential of microalgal extracts; future research should formally quantify synergy using combination-index analyses with individual fraction testing, identify active constituents by LC-MS/MS and NMR, establish selectivity against non-malignant cell lines and confirm apoptotic mechanisms via caspase activation and intracellular ROS assays, validate efficacy and safety in vivo, and assess batch-to-batch consistency of bioactive content under outdoor cultivation to ensure reproducible quality for nutraceutical development.

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Declaration Statement:

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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.

Declaration of AI tools:

The authors report generative AI was not used in their research or preparation of this manuscript.

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