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Research Article

DISCOVERY OF PHOTOCYTOTOXIC CHLOROPHYLL DERIVATIVES FROM ANTARCTIC, ARCTIC, AND TROPICAL *CHLORELLA*

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ABSTRACT

Objective: The objective of the study is to investigate tropical and polar *Chlorella* strains as natural sources of photosensitizers and determine how environmental adaptation to extreme habitats influences photocytotoxic metabolite production for photodynamic therapy (PDT).

Methods: Four *Chlorella* strains, tropical (TRP), Antarctic (ANS and ANT), and Arctic (ARC) were cultured under controlled laboratory conditions for 10 and 15 days. Methanolic and butanolic extracts were screened for photocytotoxicity against HL60 leukemia cells using an MTT assay under broad-spectrum light irradiation. Active extracts were dereplicated by ultra-performance liquid chromatography-photodiode array-mass spectrometry (UPLC-PDA-MS) and high-resolution mass spectrometry (HRMS)/MS to identify known and novel photosensitizers.

Results: Of the 16 extracts tested, only day-15 methanolic extracts showed strong light-dependent cytotoxicity. TRP and ANT reduced HL60 viability by more than two-fold upon irradiation compared to dark controls. Metabolomic profiling identified nine known chlorophyll-derived photosensitizers and revealed three previously unreported chlorophyll-based compounds (m/z 623, 531, 663) with distinct Soret and Q-band absorptions. The tropical strain TRP, adapted to high irradiance and thermal stress, yielded the highest diversity and abundance of photosensitizers. In contrast, polar strains produced lower levels, possibly reflecting culture conditions that did not replicate their native extreme light regimens.

Conclusion: This study highlights *Chlorella* as a sustainable reservoir of photosensitizers and shows that adaptation to extreme environments shapes their biosynthetic potential. The discovery of three novel chlorophyll derivatives expands the repertoire of natural photosensitizers and underscores the promise of microalgae as biofactories for next-generation PDT agents.

Keywords: *Chlorella*, Photodynamic therapy, Photocytotoxicity, Chlorophyll derivatives, Cyclic tetrapyrroles.

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INTRODUCTION

Photodynamic therapy (PDT) has gained increasing attention as a minimally invasive modality for cancer and infectious disease treatment [1,2]. By activating a photosensitizer (PS) with light of a specific wavelength, PDT generates reactive oxygen species that induce targeted cell death, vascular damage, and antitumor immune responses. Compared with conventional therapies, it offers superior selectivity, reduced systemic toxicity, and repeatability without cumulative side effects. Despite this promise, the broader application of PDT is constrained by fundamental challenges, including shallow light penetration, dependence on oxygen availability, and limited diversity of clinically effective photosensitizers [1,3].

The discovery of novel PS molecules with enhanced photophysical properties is therefore critical for advancing PDT. Natural pigments, particularly chlorophyll derivatives, are attractive candidates due to their strong light absorption and singlet oxygen generation [4-6]. Microalgae of the genus *Chlorella* represent a sustainable and underexplored source of such compounds [7]. Notably, strains adapted to extreme environments, such as polar regions, produce elevated levels of pigments and stress-protective metabolites, suggesting untapped potential for phototoxic applications [8,9].

Here, we report the screening of four *Chlorella* strains originating from tropical (TRP, Malaysia) [10,11], Antarctic (ANS and ANT) [12], and Arctic (ARC) environments to identify phototoxic compounds [12,13]. By leveraging physiological adaptations to contrasting climates, this study explores evolutionary stress responses as a bioprospecting strategy for next-generation photosensitizers. *Chlorella* strains originating from extreme environments have evolved enhanced biosynthetic capacity for photocytotoxic chlorophyll derivatives as an adaptive response to their growing environment, particularly those that are exposed to intense irradiance. The findings provide new insights into the role of microalgae in sustainable drug discovery and the future development of PDT agents.

METHODS

Chemicals and reagents

Analytical and high-performance liquid chromatography (HPLC)-grade solvents were obtained from Merck KGaA (Germany) and Fisher Scientific (USA). Dimethyl sulfoxide (molecular biology grade, Sigma-Aldrich, USA) was used to dissolve algal extracts for photocytotoxicity assays. RPMI 1640 media (with and without phenol red) and fetal bovine serum (FBS) were purchased from Gibco and Sigma-Aldrich, respectively. Pheophorbide-a, used as a reference photosensitizer, was obtained from Frontier Scientific (USA).

Microalgal strains and culture conditions

Four *Chlorella* strains representing distinct climatic origins were obtained for this study: TRP, a tropical isolate from a freshwater pond at Universiti Malaya, Malaysia [10]; ANS, derived from Antarctic snow; ANT, recovered from Antarctic soil, both from the

Windmill Islands region [12]; and ARC, collected from rock runnel in Ny-Ålesund, Arctic [12,13]. All strains were cultured in Bold's Basal Medium (BBM) and maintained under continuous illumination with cool white, fluorescent light at 42 µmol m^{-2} s^{-1}. To simulate native environmental conditions, TRP was incubated at ambient room temperature, while the polar strains (ANS, ANT, and ARC) were maintained at 4°C.

Experimental cultivation and biomass harvesting

Each strain was inoculated into 250 mL Erlenmeyer flasks containing 100 mL BBM and grown for 10 and 15 days in duplicate. Cultures were harvested by centrifugation (4000 rpm, 15 min) to separate cell pellets and supernatant.

Preparation of extracts

Cell pellets were freeze-dried, weighed, and extracted with methanol (5 mL per sample) using sonication (5 min). Supernatants were subjected to liquid–liquid extraction with n-butanol (3 × 100 mL), and pooled butanolic fractions were concentrated.

Fractionation and purification

Methanolic extracts of TRP were fractionated by solid-phase extraction (SPE) on RP-18 cartridges with sequential elution using 70:30 MeOH: H_2O, 80:20 MeOH: H_2O, 100% MeOH (two fractions), and 100% acetone. Photocytotoxic fractions (Fractions 3–5) were analyzed by HPLC, and compounds with unknown m/z values (623, 531, 663) were isolated for structural characterization.

Ultra-performance liquid chromatography-photodiode array-mass spectrometry (UPLC-PDA-MS) and high-resolution mass spectrometry (HRMS) analysis

Dereplication of the extracts and fractions was performed using UPLC-PDA-MS. Samples (20 mg/L) were analyzed on a reversed-phase UPLC system. The mobile phases consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient program was as follows: 60% B from 0–0.1 min; 60–85% B from 0.1–2 min; 85–100% B from 2–5.5 min; an isocratic hold at 100% B from 5.5–12.5 min; and re-equilibration to 60% B from 12.5–15 min. PDA detection was monitored at 200, 400, and 650 nm.

HRMS and tandem MS/MS were performed on an Acquity™ UPLC-PDA coupled with a Synapt HDMS Q-TOF system (Waters, USA) in positive ESI mode. The source capillary voltage and source temperature were set to 2.70 kV and 100°C, respectively. The collision energy was set to 6.0 V. The desolvation gas flow was 700 L/h. Tuning of the MS was carried out using leucine-enkephalin (500 pg/mL; calculated [M + 1]: 556.2771). Calibration was performed using sodium formate (0.5 M), and accuracy was verified with caffeine (195.0879, 1.5 ppm). Chromatographic separation used a BEH C18 column (2.1 × 50 mm, 1.7 µm). The molecular mass and ultraviolet-visible (UV-Vis) spectral data were obtained and compared with those in the in-house photosensitizer library [14].

Cell culture and photocytotoxicity assay

HL-60 cells (ATCC, USA) were maintained in RPMI 1640 medium supplemented with 10% FBS. Cells (15,000/well) were seeded in 96-well plates using phenol-red-free RPMI medium. The extracts were tested at 20 µg/mL (DMSO ≤0.01%). After a 2-h incubation with test compounds, cells were irradiated using a calibrated broad-spectrum LED array (Solis-LED 90, Thorlabs Inc., USA) delivering a fluence of 4.1 J/cm^2 at a fluence rate of 6.8 mW/cm^2. The emission profile spanned 400–700 nm, with peak outputs at 435 nm and 660 nm, corresponding to the Soret and Q-band absorption maxima of chlorophyll-derived photosensitizers. The distance between the light source and the cell monolayer was maintained at 15 cm. Dark controls were shielded from light using opaque barriers. Post-irradiation, cells were incubated for 24 h before viability assessment using the MTT assay (5 mg/mL, 4 h, 37°C). Formazan crystals were solubilized with DMSO, and absorbance was measured at 570 nm using a microplate reader [15]. Pheophorbide-a served as a positive control, and dark toxicity was evaluated in parallel to distinguish light-dependent cytotoxicity.

Statistical analysis

All assays were conducted in quadruplicate. Results are expressed as mean±SD. Bar chart and statistical analysis were done using GraphPad Prism 9.

RESULTS

Photocytotoxic activity of *Chlorella* extracts

A total of 16 extracts were obtained from four *Chlorella* strains, comprising eight methanolic (cell-derived) and eight butanolic (supernatant-derived) extracts harvested on days 10 and 15. Photocytotoxic activity was assessed using an MTT-based short-term survival assay (Fig. 1).

Four methanolic extracts harvested on day 15 displayed moderate to strong photocytotoxic activity at 20 µg/mL, whereas no strong photocytotoxic effect was observed in day 10 methanolic extracts. Notably, methanolic extracts of *Chlorella* TRP (tropical) and ANT (Antarctic) induced more than a two-fold reduction in cell viability under light irradiation compared to dark controls. By contrast, none of the butanolic extracts exhibited significant photocytotoxicity, with the exception of the day 10 extract from ANT, which reduced cell viability by >80% in both irradiated and non-irradiated conditions, suggesting general cytotoxicity rather than light activation.

Dereplication and identification of photosensitizers

UPLC-PDA-MS dereplication revealed the consistent presence of cyclic tetrapyrrolic compounds across the photocytotoxic extracts, although at varying intensities (Supplementary Figs S1-S4). Nine known photosensitizers were identified based on retention times, UV–Vis spectra, and MS/MS data (Table 1). These included chlorophyll-a and chlorophyll-b derivatives such as pheophorbide-a. Carotenoids were also detected but were excluded from further consideration given their established photoprotective, rather than photocytotoxic functions (Fig. 2).

The analysis also detected the presence of three minor compounds which could potentially be new chlorophyll-based photosensitizers (Table 1). Compound A with a detected *m/z* $[M+H]^+$ of 623 showed strong UV-Vis absorption at 436 and 650 nm which is characteristic of a chlorophyll-b-type structure. Compound B with a detected *m/z* $[M+H]^+$ of 531 showed strong UV-Vis absorption at 407 and 658 nm, which is characteristic of a chlorophyll-a-type structure. Compound C with a detected *m/z* $[M+H]^+$ of 663 showed strong UV-Vis absorption at 410 and 658 nm, which is characteristic of a chlorophyll-a-type structure. The deduction is made based on the difference of UV-Vis data recorded as well as a difference in MS/MS spectra as compared to the standard compounds. Compound A was identified in both ANT (Antarctic) and ARC (Arctic) extracts, suggesting a polar strain association. Compound B was present in TRP (tropical) and ANS (Antarctic) strains, indicating broader distribution across climatic origins. Compound C was exclusively detected in ANT, highlighting its potential as a strain-specific metabolite.

Strain-dependent differences in photosensitizer production

Among the four strains, the tropical *Chlorella* TRP methanolic extract exhibited the strongest photocytotoxic activity, correlating with both the highest diversity (nine compounds) and relative abundance of chlorophyll-derived photosensitizers. Antarctic (ANS and ANT) and Arctic (ARC) strains contained fewer and lower amounts of photosensitizers.

DISCUSSION

This study shows that *Chlorella* strains produce chlorophyll-derived photosensitizers with strain- and time-dependent differences in activity. Extracts harvested at day 15 were consistently more active

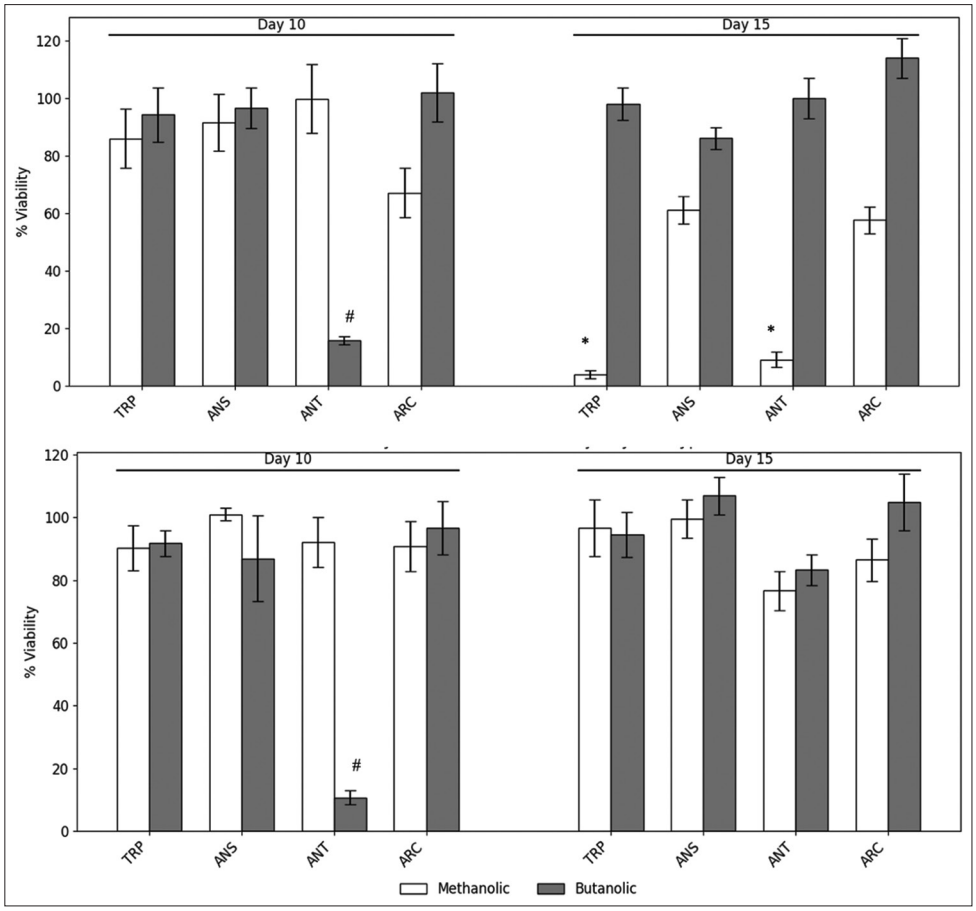

Fig. 1: HL60 viability treated with methanolic and butanolic extracts harvested on day 10 and day 15. Top: Unirradiated treatment; Bottom: Irradiated treatment. The results were the mean of 4 biological replicates. Error bars representing standard deviation. #Cell viability was significantly reduced compared to control, with no difference between irradiated and non-irradiated conditions, indicating general (non-photoactivated) toxicity. *p<0.05 compared to untreated control

Table 1: Photosensitizers identified and mass value distribution between samples

Photosensitizers	Mass (*m/z*)	Sample*			
		TRP	ANS	ANT	ARC
Purpurin-18	565	√	—	—	—
Purpurin-18 methyl ester	579	√	—	—	—
Pheophorbide-a	593	√	√	√	√
13- Pheophorbide-a methyl ester	607	√	—	—	—
13- hydroxypheophorbide-a	609	√	√	√	√
13- hydroxypheophorbide-a methyl ester	623	√	—	—	√
15- hydroxypurpurin-7-lactone methyl ester	625	√	—	—	—
15- hydroxypurpurin-7-lactone methyl diester	639	√	—	—	—
15-methoxypurpurin-7-lactone methyl diester	653	√	—	—	—
Compound A	623	—	—	√	√
Compound B	531	√	√	—	—
Compound C	663	—	—	√	—

*√: Detected, —: Not detected

than those at day 10, suggesting that chlorophyll degradation into active tetrapyrrolic derivatives occurs more prominently in the later growth phase. Chlorophyll breakdown products such as pheophorbides are well-established photosensitizers due to their strong absorption in the therapeutic window (600–700 nm) and high singlet oxygen quantum yields [16,17].

Strain origin strongly influenced photosensitizer yield. The tropical *Chlorella* TRP outperformed polar strains both in activity and compound diversity. This difference may reflect ecological adaptations: polar strains were cultured at low irradiance (42 μmol m^{-2}/s), far below natural polar summer conditions, which can exceed 1,500 μmol m^{-2}/s [18-20]. Light availability is a major regulator of chlorophyll biosynthesis [21,22], and insufficient irradiance likely limited pigment accumulation and subsequent photosensitizer formation in polar strains. Adjusting cultivation to mimic native high-light environments may enhance pigment production and phototoxicity in psychrotolerant species.

Cyclic tetrapyrroles display distinctive UV-Vis patterns within the 400–440 nm regions, which is known as the Soret band, followed by 3–5 Q bands with one of the strongest absorbing Q bands, namely Q_y, in the 600–700 nm regions. The presence of side chains with different functional groups (electron-releasing or -withdrawing) has been observed to shift the absorption wavelengths of the Soret and Q bands in the UV-Vis spectroscopy. For example, the Soret band of chlorophyll-b is red-shifted by about 30 nm compared to that of chlorophyll-a because of the presence of -CHO group at C-7 position. This distinctive UV-Vis absorption feature is one way to distinguish a chlorophyll-a derivative from a chlorophyll-b structure. Here, all the photosensitizers identified from the extracts are derivatives of chlorophyll-a and -b. These compounds are likely products yielded from the breakdown of chlorophylls through a stepwise enzymatic pathway that removes the magnesium central atom as well as the phytyl group at the C-17 position, to result in the formation of cyclic tetrapyrroles that are de-metallated and without the C-17 aliphatic phytyl group, as depicted in Fig. 2. As

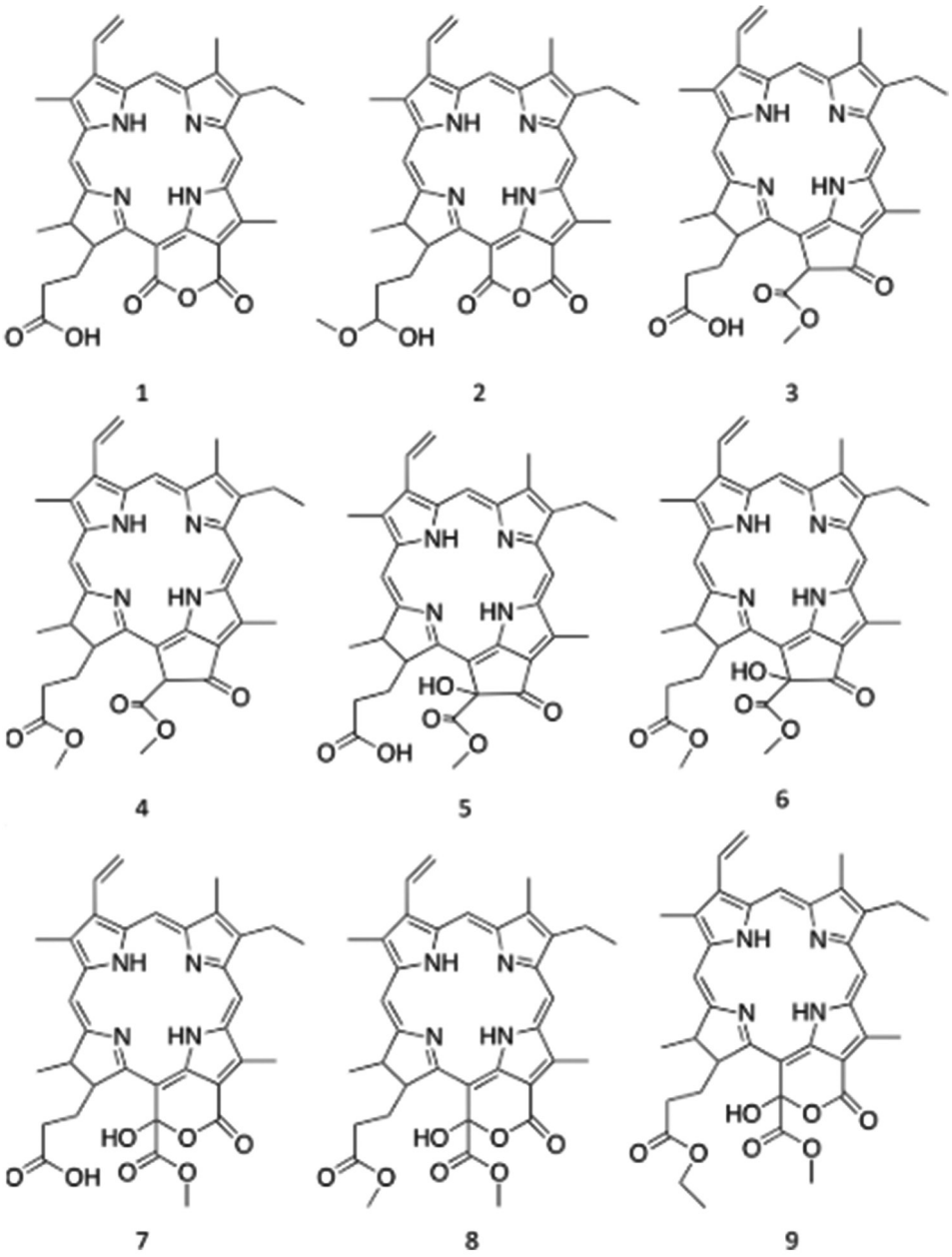

Fig. 2: Structure of known photosensitizers identified from phototoxic *Chlorella* extracts. (1) Purpurin-18; (2) Purpurin-18 methyl ester; (3) Pheophorbide-a; (4) 13-Pheophorbide-a methyl ester; (5) 13-hydroxypheophorbide-a; (6) 13-hydroxypheophorbide-a methyl ester; (7) 15-hydroxypurpurin-7-lactone methyl ester; (8) 15-hydroxypurpurin-7-lactone methyl diester; (9) 15-methoxypurpurin-7-lactone methyl diester

only *Chlorella* extracts harvested on day-15 showed photocytotoxicity but not the extracts harvested on day-10, the degradation of chlorophyll to form the active photosensitizer derivatives probably accelerated only after day-10.

The discovery of three minor, putatively novel chlorophyll derivatives (Compounds A-C), adds to the chemical diversity of algal tetrapyrroles. Structural modifications, such as electron-donating or -withdrawing substituents, are known to induce bathochromic or hypsochromic shifts in absorption spectra [23]. Carotenoids detected in the extracts were excluded from photocytotoxic consideration, consistent with their established role as photoprotectants rather than photosensitizers [24]. This supports the specificity of chlorophyll-derived compounds as the drivers of photocytotoxicity in this system.

Overall, these findings highlight *Chlorella*, particularly TRP, as a promising microalgal source of natural photosensitizers for PDT. Previous research has mainly emphasized its applications in bioremediation and biofuels [25,26], but here, we demonstrate biomedical relevance. This expands the scope of algal bioprospecting, positioning microalgae as sustainable reservoirs of novel phototherapeutic agents.

CONCLUSION

This study provides the first comparative evaluation of tropical and polar *Chlorella* strains as sources of natural photosensitizers for PDT. Methanolic extracts, particularly from tropical *Chlorella* TRP, demonstrated strong photocytotoxic activity associated with a diverse profile of chlorophyll-derived tetrapyrroles. Three previously unreported minor compounds with chlorophyll-like spectral features

were also detected, suggesting potential new photosensitizers. Together, these findings highlight *Chlorella* as a sustainable platform for the discovery of next-generation PDT agents and underscore the importance of strain origin and cultivation conditions in optimizing photosensitizer production.

Future work should focus on optimizing cultivation conditions, particularly light intensity and nutrient regimens, to enhance photosensitizer yields in polar *Chlorella* strains. Structural elucidation and functional validation of the three newly detected chlorophyll derivatives are the critical next steps to confirm their novelty and therapeutic potential. Expanding this screening approach to additional extremophilic microalgae may uncover further phototoxic metabolites with unique structural diversity. Finally, translating these findings into pre-clinical models will be essential to establish efficacy, selectivity, and safety, thereby bridging the gap from algal bioprospecting to clinical PDT applications.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the University of Malaya Algae Culture Collection (UMACC) for providing the *Chlorella* extracts used in this study. The Chlorella strains used in this study were originally obtained from the University of Malaya Algae Culture Collection (UMACC), with strain codes as follows: TRP (UMACC 001), ANS (UMACC 234), ANT (UMACC 237), and ARC (UMACC 263). We also extend our sincere thanks to Cancer Research Malaysia (formerly Cancer Research Initiatives Foundation, CARIF), where the majority of the experimental work and analyses were carried out during N.K.W.'s attachment. Our appreciation goes to the dedicated staff members of CARIF, whose contributions and support were invaluable to the success of this project. This publication is supported by Quest International University under the QIU Academic Publication Funding.

AUTHORS' CONTRIBUTIONS

N.K.W.: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing-original draft. K.H.W.: Data analysis, Writing-original draft, Visualization. N.Y.P.: Writing–review & editing, Critical revision. B.S.T: Writing–review & editing. J.N.: Writing – review & editing.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPORTING INFORMATION

UPLC-PDA-MS chromatograms of TRP, ANS, ANT, and ARC are available as supporting information.

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SUPPLEMENTARY MATERIAL

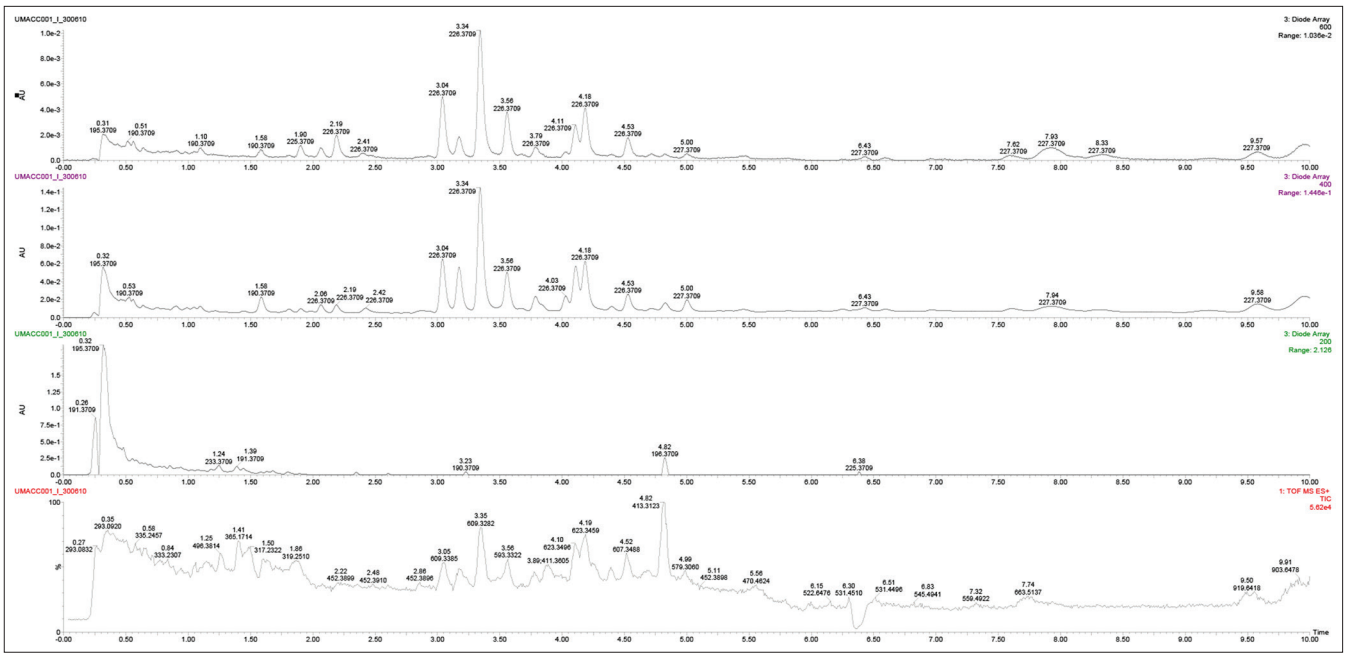

Fig. S1: UPLC chromatograms of the TRP methanol extract at 200, 400, and 600 nm (top to bottom), obtained using an Acquity BEH C18 column (1.7 µm, 2.1 × 50 mm). The mobile phase was acetonitrile–water with 0.1% formic acid, applied using a 60–100% acetonitrile gradient. The total ion chromatogram was recorded in positive ESI mode.

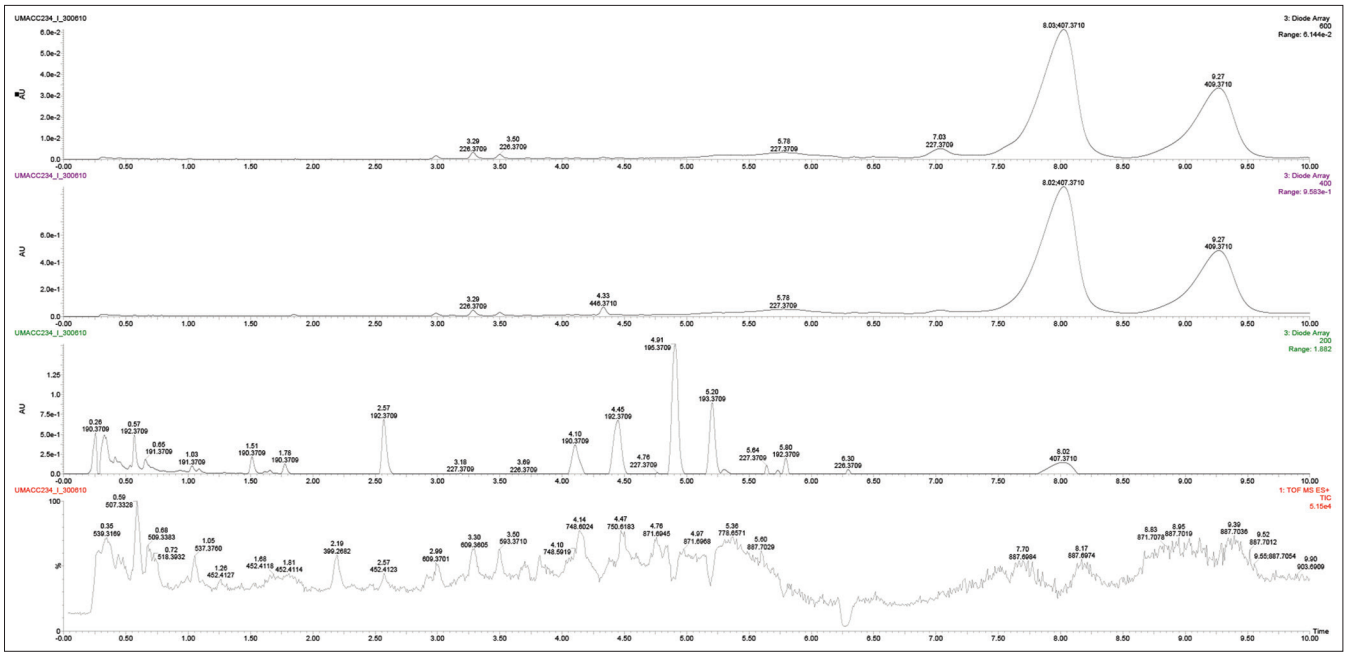

Fig. S2: UPLC chromatograms of the ANS methanol extract at 200, 400, and 600 nm (top to bottom), obtained using an Acquity BEH C18 column (1.7 µm, 2.1 × 50 mm). The mobile phase was acetonitrile–water with 0.1% formic acid, run with a 60–100% acetonitrile gradient. The total ion chromatogram was acquired in positive ESI mode.

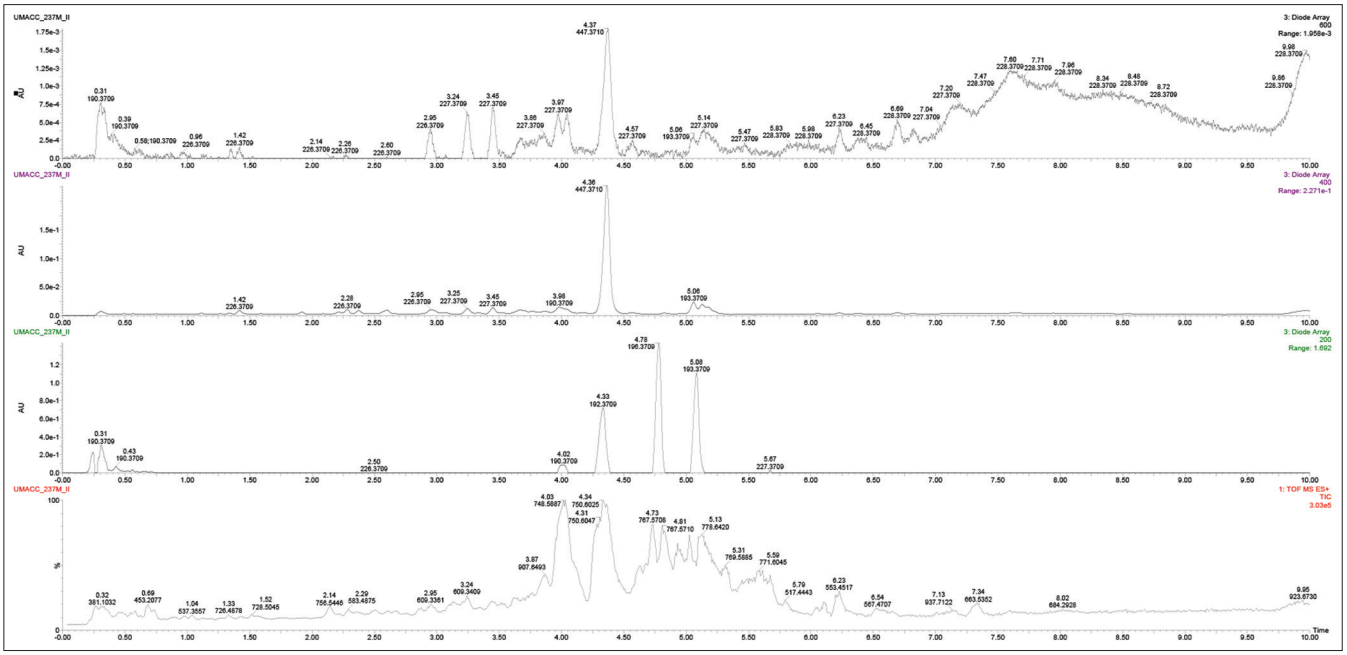

Fig. S3: UPLC chromatograms of the ANT methanol extract at 200, 400, and 600 nm (top to bottom), obtained using an Acquity BEH C18 column (1.7 µm, 2.1 × 50 mm). The mobile phase was acetonitrile–water with 0.1% formic acid, applied with a 60–100% acetonitrile gradient. The total ion chromatogram was recorded in positive ESI mode.

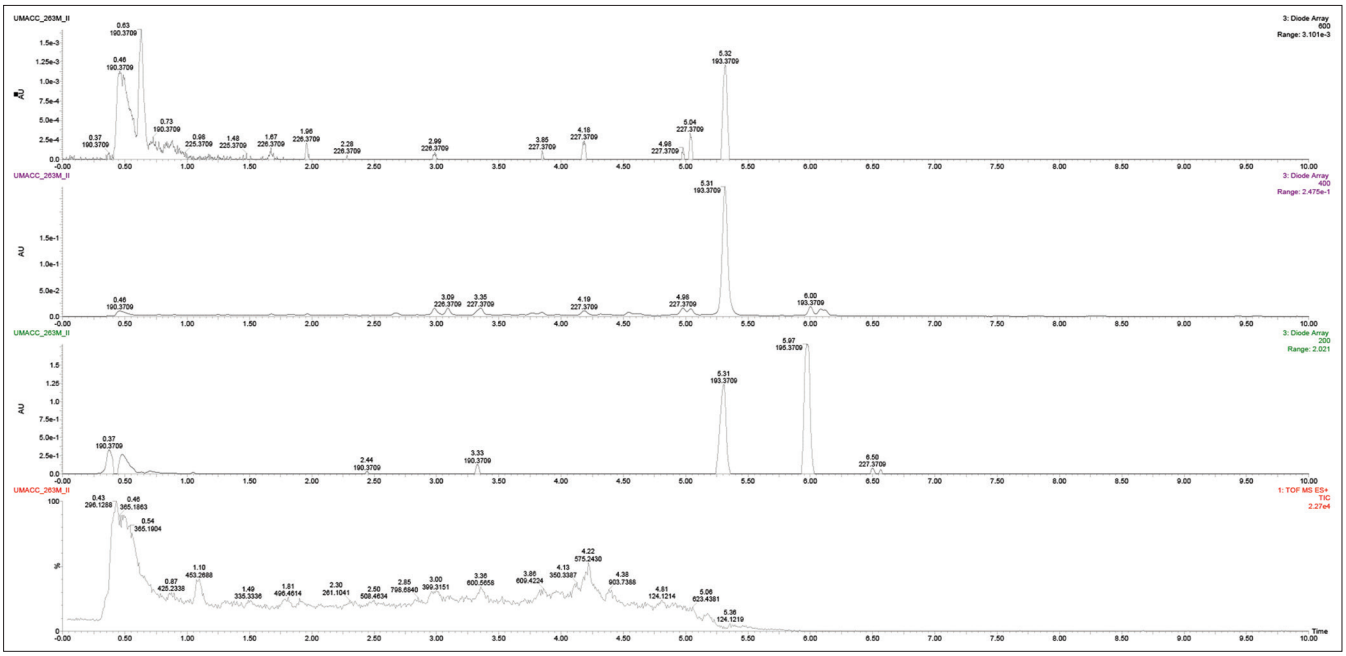

Fig. S4: UPLC chromatograms of the ARC methanol extract at 200, 400, and 600 nm (top to bottom), obtained using an Acquity BEH C18 column (1.7 µm, 2.1 × 50 mm). The mobile phase was acetonitrile–water with 0.1% formic acid, applied with a 60–100% acetonitrile gradient. The total ion chromatogram was recorded in positive ESI mode.