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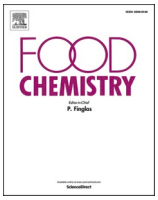

Sustainable approach towards isolation of photosynthetic pigments from *Spirulina* and the assessment of their prooxidant and antioxidant properties

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ABSTRACT

Carotenoids, chlorophyll and phycocyanin are three types of photosynthetic pigments found in *Spirulina* that differ in colour, composition, stability, solubility, and commercial importance. Such diversity of structures creates a challenge to extract these pigments simultaneously from the same batch of raw material in an efficient and sustainable manner. This study demonstrates that water can be successfully used as a single solvent together with combined (non)mechanical cell membrane disruption techniques (ultrasonication, centrifugation, freezing/thawing cycle) to extract these photosynthetic pigments from the same batch. This water-based approach delivers a significant improvement in isolating green pigments, which are often overlooked during extraction due to a preference for blue and yellow pigments. Chlorophyll was quantitatively converted to its stable derivatives to carry out a comparative analysis of antioxidant properties (DPPH, TEAC, FRAP), singlet oxygen production and intracellular activities (MTT, ROS assays) using Caco-2 cells.

1. Introduction

Natural or naturally derived bioactive compounds support many vital applications in pharmaceutical, food, cosmetics, feed, and biofuel industries (Pagels, Pereira, Vicente, & Guedes, 2021). Among commercially viable marine algae, cyanobacteria represent a renewable source of bioactives as it is cultivated on a large scale due to the ability to survive in harsh environmental conditions (Morocho-Jácome et al., 2020). *Spirulina* is a commercial name given to *Arthrospira platensis* and *Arthrospira maxima* species of cyanobacteria. For centuries, *Spirulina* has been used in many parts of the world as a food source; the United Nations classified it as "one of the best foods of the future" and an important natural food source (Moorhead, Capelli, & Cysewski, 1993) Although *Spirulina* is an excellent source of polysaccharides, lipids, and proteins, it is also an important source of edible natural colouring agents, which showed health benefits and are often a part of nutraceuticals, cosmeceuticals, and drugs (Jaeschke, Teixeira, Marczak, & Mercali, 2021). Although *Spirulina* is considered safe, raw extracts can be contaminated with cyanotoxins such as nodularins and microcystins, which are synthesized by a range of cyanobacteria, posing a hazard to human health (Schreidah, Ratnayake, Senarath, & Karunarathne, 2020). Moreover, extracts may contain heavy metals, so the production of pure photosynthetic pigments has become even more important. However, the economics of any pure bioactive is driven by the production costs which can be as high as 60 % of the total expenditure (Pagels et al., 2021). The commercial value of *Spirulina* is primarily directed by phycocyanin (the most common phycobiliprotein), 80 % of which is utilized as a blue pigment by the food and cosmetic industry (Pagels et al., 2021). But *Spirulina* is also a source of other important photosynthetic pigments such as yellow xanthophylls and green chlorophylls; even more, the unique photosynthetic system of cyanobacteria produces primarily chlorophyll *a*. However, the developed practical strategies are mainly tailored to isolate blue phycocyanin overlooking chlorophylls (Minchev, Petkova, & Milkova-Tomova, 2020). The limited number of extraction strategies is due the chlorophyll's stability towards heat $\geq$ 60 °C, light, and pH at 7.5. Methanol was the first solvent used to extract

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chlorophyll *a* from *Spirulina* but due to its toxicity, it has been replaced by other solvents such as ethanol and acetone (Andersen, 2005). Recent studies reported a successful attempt to extract chlorophyll using supercritical carbon dioxide (Marzorati, Parolini, Colombo, Idà, Schievano, & Verotta, 2020) and ionic liquid (Martins et al., 2021).

A green biorefinery concept is a very attractive alternative that can potentially deliver a wide range of complex molecules as opposed to traditional extractions of value-added chemicals. But the pigment fraction i.e. the total phytochemical content, tends to be very small and is the most complex one to separate due to different solubilities and stabilities of the diverse structures (Pérez-Gálvez, Viera, & Roca, 2020). Already developed practical strategies fail to deliver pigments such as phycobiliproteins, carotenoids and chlorophylls from the same source, simultaneously. For example, when isolating blue colorants, green and yellow fractions are usually discarded. This is especially true, when the concentration of target colorants is relatively low requiring less sustainable and labour-intense approaches (Marzorati et al., 2020).

The current study aims to develop a smart extraction method that avoids loss of target molecules and minimizes waste to reduce production costs. To further decrease a detrimental impact (both economic and environmental), this study improves the existing extraction - purification stages, which can help to advance the overall purification process. This work demonstrates the potential of the green pigments by studying their antioxidant and prooxidant properties, in comparison to phycocyanin.

2. Materials and methods

2.1. Chemicals and reagents

Spirulina powder (*Arthrospira platensis*) was purchased from Nukraft brand. All chemicals and solvents were obtained from Fisher Scientific (Loughborough, UK) and Sigma Aldrich (Dorset, UK). Caco-2 cells were purchased from EACC, European Collection of Authenticated Cell Collection. Dulbecco's modified Eagle Medium (DMEM), Foetal Bovine Serum (FBS), antibiotic (10,000 U/mL penicillin and 10 mg streptomycin/mL) and minimum essential medium (MEM) non-essential amino acids were all purchased from Gibco Life Technologies (NewYork, USA). Thiazolyl Blue Tetrazolium Bromide (MTT), Neutral Red, hydrogen peroxide solution, 30 % (w/w) in water, Sulforhodamine B (SRB), trichloroacetic acid (TCA), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH2-DA), acetic acid and Trizma base were all obtained from Sigma–Aldrich.

2.2. Ultrasound assisted water extraction of photosynthetic pigments

Approximately 100 g of *Spirulina* powder was dispersed in 400 mL of distilled water and allowed to freeze for 24 h in the freezer (−20 °C). Then the mixture was thawed at + 50 °C and sonicated by sonication bath at 40 kHz for 3 h. The mixture reached + 50 °C after 2 h of sonication time. The supernatant was filtered through a Buechner funnel with sealed-in coarse porosity filter (90–130 μm), and the solid residue remaining on the filter was washed three times with 20 mL of distilled water. The combined water filtrate was centrifuged at 1207 × 'g force' for 15 min yielding 10.8 g of dark green solid, which was dried in air for 72 h in the dark and was stored at −20 °C under nitrogen atmosphere. Blue aqueous solution (200 mL) containing phycocyanin was stored at + 4 °C in the dark, prior to use. (Fig. 1)

2.3. Ultrasound assisted acetone extraction of photosynthetic pigments

Approximately 100 g of *Spirulina* powder was mixed with 400 mL of acetone and left in the freezer (−20 °C) for 24 h. Then the mixture was thawed at + 50 °C and sonicated by sonication bath at 40 kHz for 3 h. The mixture reached + 50 °C after 1 h of sonication time. The supernatant was filtered through a Buechner funnel, and the solid residue remaining on the filter was washed three times with 20 mL of acetone. The combined green acetone extract was evaporated under reduced pressure, yielding 7.4 g of dark green precipitate this sample was stored at −20 °C under nitrogen atmosphere. (Fig. 1)

2.4. Relative efficiency of the extraction method: Extraction yield

The extraction yield (%) from powdered *Spirulina* algae was calculated as follows: [(the mass of extracted solid, g) / (the mass initial dry material, g)] × 100 % (Ferreira-Santos, Miranda, Belo, Spigno, Teixeira, & Rocha, 2021).

2.5. Isolation and purification of phycocyanin

Approximately, 200 mL of crude blue aqueous extract (from *sec*. 2.2) was treated by gradually adding 10 g of solid ammonium sulphate to reach the saturation. The mixture was stirred for 1 h and kept overnight at + 4 °C in the dark. The mixture was centrifuged at 1207 × 'g force' for 15 min, then 18 g of a blue precipitate was obtained. The blue precipitate was dissolved in sodium phosphate solution (0.005 M, pH = 7) at a ratio 1:5 (w/v) and kept at + 4 °C in the dark for 24 hrs than the mixture was centrifuged at 1207 × 'g force' for 15 min and filtered yielding 15 g of phycocyanin (Marzorati et al., 2020). The purity and grade were calculated using HPLC and UV–vis absorption methods.

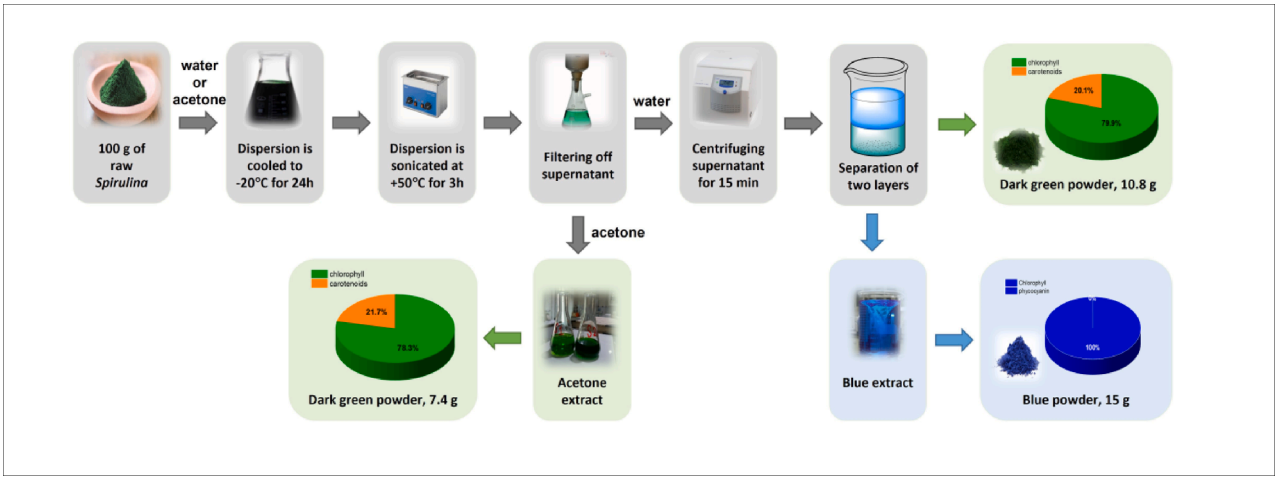

Fig. 1. Separation strategy for photosynthetic pigments from *Spirulina* by water vs. acetone.

2.6. Isolation and purification of chlorophyll derivatives

Thin layer chromatography (TLC) technique was used to predetermine the separation of pigments in two different solvent systems: (a) hexane–acetone 70:30 (v/v) and (b) dichloromethane–tetrahydrofuran 95:5 (v/v). Briefly, the crude green extract was analyzed using TLC Silica gel 60 F254 aluminium coated plates (Merck). The solvent system of hexane–acetone 70:30 (v/v) showed the best separation **(SI Fig. S1)** and was therefore used in flash chromatography to purify the pigments. Here, 6 g of crude green extract was submitted to a normal phase flash chromatography procedure by applying this extract on top of a glass column (5 × 150 cm) packed with 190 g silica gel 60 mesh, eluted using a hexane–acetone 90:10 (v/v) system **(SI Fig. S2)**. The polarity of the system was gradually raised until the ratio of hexane–acetone 60:40 (v/v) was reached. Four coloured fractions were collected: orange, green, grey and yellow (ca. 200 mL each). These fractions were evaporated under reduced pressure using a standard rotary evaporator at pressure 10 mbar, yielding β-carotene (0.9 g), pheophorbide *a* (1.4 g), pheophytin *a* (2.6 g), and lutein (0.5 g).

2.7. Conversion of chlorophyll a from green extract into methyl pheophorbide a

Approximately, 1 g of crude green solid extract (from ***sec.* 2.2**) was treated following modified method (Smith, Goff, & Simpson, 1985) In brief, 110 mL of methanol containing 5 % of sulfuric acid was added to 1 g of the dark green solid. The mixture was de-oxygenated by bubbling nitrogen through the solution and left to stir under nitrogen atmosphere for 12 h in the dark at room temperature. The residue was diluted with dichloromethane; this solution was rinsed with water (50 mL), then with 40 mL of saturated solution of aqueous sodium bicarbonate (10 %) and dried over sodium sulphate. The solvent was removed under reduced pressure leaving a green precipitate which was recrystallized from dichloromethane/methanol yielding 0.4 g of green methyl pheophorbide *a*.

2.8. Quantification of the individual pigments in extracts

For the quantitative determination of pigments, stock solutions were prepared from the green extracts at a concentration of 1 mg/mL in 80 % acetone. Briefly, measurements were performed using a quartz cuvette containing 30 μL of stock solution for green extracts with 3 mL of 80 % acetone and 200 μL blue (phycocyanin) extract with 3 mL of distilled water. The absorbances of the green extracts were measured at 663, 645 and 470 nm against a blank (80 % acetone). For the phycocyanin extract, the absorbance was measured at 652 and 620 nm against a blank (distilled water).

The concentration of chlorophylls, total carotenoids (Wellburn, 1994) and phycocyanin (Bennett & Bogorad, 1973) was determined by following equations:

$$C_{chlorophyll\ a}\left(\frac{\mu g}{mL}\right) = 12.25 \times A_{663} - 2.79 \times A_{645} \quad (1)$$

$$C_{chlorophyll\ b}\left(\frac{\mu g}{mL}\right) = 21.5 \times A_{645} - 5.10 \times A_{663} \quad (2)$$

$$C_{carotenoids}\left(\frac{\mu g}{mL}\right) = \frac{1000 \times A_{470} - 1.82C_{(chlorophyll\ a)} - 85.02C_{(chlorophyll\ b)}}{198} \quad (3)$$

$$C_{phycocyanin}\left(\frac{mg}{ml}\right) = \frac{A_{620} - 0.474 \times A_{652}}{5.34} \quad (4)$$

Where *A* is absorbance (a.u.) at the corresponding wavelength for the sample. The concentration of each pigment is expressed as mg of pigment per g of powdered *Spirulina* (mg/g).

2.9. Assessment of antioxidant activities

The antioxidant capacity of pure pigments was evaluated using 2,2,-diphenyl-1-picryl (DPPH), ferric-reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC). Stock solutions (10 mg/mL) of each purified compound were prepared in ethanol / DMSO mixture (60:40, v/v) and further diluted according to the assay requirements (0, 25, 50, 100, 150, 200 and 250 μg/mL concentrations). Trolox (10 mg/mL) was used as standard to cover a range of 0–1000 μg/mL, diluted in ethanol / DMSO mixture (60:40, v/v). Briefly, the antioxidant capacities of pigments were determined by DPPH according to the method described by (Hsu, Chao, Hu, & Yang, 2013) with modifications. Fresh ethanol solution of DPPH reagent (120 μM) was prepared; 10 μL of samples were mixed with 300 μL of DPPH reagent and incubated for 30 min at room temperature. Subsequently, absorbance was measured at 515 nm. Trolox equivalent antioxidant capacity (TEAC) was conducted based on the method described by (Ganwarige Sumali N. Fernando, Wood, Papaioannou, Marshall, Sergeeva, & Boesch, 2021); 10 μL of samples were mixed with 300 μL of $ABTS^{+}$ solution and incubated for 10 min at room temperature in the dark. Then, the absorbance was measured at 734 nm. The method described in (Ganwarige Sumali N Fernando, Sergeeva, Frutos, Marshall, & Boesch, 2022) was used to perform FRAP assay; 10 μL of samples were mixed with 300 μL of FRAP reagent and incubated for 15 min at 37 °C. Subsequently, the absorbance of the colored product was taken at 593 nm. All assays were performed in 96-well plates and absorbance measurements were recorded by a microplate reader (TECAN, Mannedorf, Switzerland). In addition, the results are expressed as mean ± standard deviation as μg Trolox Equivalent Antioxidant Capacity per mL of pigments (μg TE/mL).

2.10. Cell culture and cytotoxicity assays

Caco-2 cells were routinely cultivated in DMEM medium with 10 % FBS, 1 % antibiotics, 1 % non-essential amino acids under standard conditions (5 % CO_2, 37 °C). The cytotoxicity of the four pigment samples (pheophytin *a*, phycocyanin, pheophorbide *a*, methyl pheophorbide *a*) was determined by MTT assay and neutral red assay as previously described (Wang et al., 2022). Briefly, Caco-2 cells were seeded at 1.0×10^5 cells per well in 24-well plates. Upon reaching 90 % confluence, the medium was removed, and cells were incubated in DMEM containing different concentrations of pigments (0, 1, 2.5, 5, 10, 25, 50, 100 μg/mL). The samples were dissolved in DMSO/ethanol (80:20, v/v) or in the case of phycocyanin, in distilled water, with max solvent concentration of 0.2 % (1 %, 2 %). After 24 h, the medium was replaced by DMEM containing MTT (1 mM). Following 2 h incubation at 37 ℃, the cells were washed with DPBS and DMSO added to each well followed by 10–15 min on a shaker. Then, 3 × 200 μL from each well were transferred on a 96 well pate and absorbance was recorded at 570 nm using microplate reader (Tecan Spark 10 M), against DMSO as the control. For the neutral red assay, cells were incubated for 2 h with neutral red dye (40 μg/mL) in FBS-free DMEM medium. Subsequently, bleaching solution (50 % ethanol, 49 % deionized H_2O, 1 % acetic acid) was used to dissolve the dye. The absorbance was recorded at 540 nm. Cell viability in both assays was calculated as percentage of treated cells in relation to negative control.

2.11. *Intracellular reactive* oxygen species (ROS) assay

For the ROS assay, Caco-2 cells were seeded in 96-well plates at a density of 7.0×10^4 cells/well in a volume of 100 μL (Lee, Park, & Jeong, 2021; Wang et al., 2022). After 24 h the medium was removed and replaced by 100 μL medium containing 30 μM of DCFH2-DA. Following a 40 min incubation at 37℃, the cells were washed and incubated with pigment samples at different concentrations (0,1, 5, 10, 25, 50, 100 μg/mL), in phenol red-free and FBS-free DMEM. Curcumin (10 μM) was used as positive control. After 1 h, H_2O_2 was added at a final

concentration of 200 μM. To determine non-stimulated ROS levels, cells were in parallel incubated without hydrogen peroxide. The fluorescence intensity was read after 1 h with the TECAN reader in scan mode, at excitation of 485 nm and emission of 535 nm. Subsequently, protein content was determined by adding 50 μL of SRB-TCA (SRB in 10 % TCA solution) to each well, incubating the plate for 15 min at room temperature, then removing the solution and washing with 200 μL of 1 % acetic acid. Finally, the solution was removed, and 100 μL of trizma base (10 mM) was added for 5 min. The fluorescence intensity was read at excitation of 540 nm and emission of 590 nm. The levels of ROS were calculated as ROS generation of sample treated cells in percentage of negative control and normalized according to protein content.

2.12. Assessment of prooxidant activity via singlet oxygen ($^1O_2$) generation assay

Photostability Analysis. The photostability of the individual compounds (pheophorbide *a*, pheophytin *a*, methyl pheophorbide *a*, phycocyanin, and 1,3-diphenylisobenzofuran) was determined according to (Martins et al., 2021) with some modifications. In brief a quartz cuvette containing 10 μL of the stock solution of an individual compound (pheophorbide *a*, pheophytin *a*, methyl pheophorbide *a*, phycocyanin, or 1,3-diphenylisobenzofuran) was irradiated in DMF (3 mL) during the 120 *sec* irradiation period with visible light (Xenon lamp – 50 W) using each of the following long-pass cut-off filters (420, 455, 475, 495 nm). The absorption region was recorded and the photostability was expressed as the ratio (%) of the absorbance of a characteristic band of the compound at a given time of irradiation (A_t) and its absorbance prior to the start of the irradiation process (A_0).

Singlet oxygen generation and quantum yield. A modified photochemical method was used to determine the ability of pigments (phycocyanin, pheophorbide *a*, pheophytin *a* and methyl pheophorbide *a*) to produce singlet oxygen using DPBF (1,3-diphenylisobenzofuran) as a singlet oxygen trapping agent and methylene blue as a reference compound.(Rogers, Sergeeva, Paszko, Vaz, & Senge, 2015; Tan et al., 2017) Briefly, the stock solutions of DPBF (1 mg/mL, 1.5 mM) and the pigments (1.5 mM) and phycocyanin (1 mg/mL) were prepared in DMF. The first sample contained 40 μL of DPBF stock solution in 3 mL of DMF. The remaining samples (including reference sample) contained 10 μL of the stock solution of individual pigment and 40 μL of DPBF stock solution in 3 mL of DMF. The photooxidation rate was assessed by measuring DPBF absorbance at 410 nm every 6 *sec* for 2 min while illuminating the solution with visible light (Xenon lamp – 50 W) using a long-pass cut-off filter (495 nm).

Singlet oxygen ($^1O_2$) quantum yield (ΦΔ). (ΦΔ) of the isolated pigments was calculated in a relative manner using methylene blue (MB) as a reference. A linear plot of $\ln(A_0/A_t)$ vs. the irradiation time (t, *sec*) was obtained, where A_0 and A_t are the absorbances of DPBF at 410 nm before irradiation and at every 6 *sec* during illumination (120 *sec*), respectively. Singlet oxygen ($^1O_2$) quantum yield (ΦΔ) was calculated using the following equation:

$$\Phi = \Phi_R\left(\frac{k_S}{k_R}\right)\left(\frac{I_{aR}}{I_{aS}}\right)$$

where *R* and *S* are the reference (MB) and the sample, respectively; *k* is the slope of the linear plot of $\ln(A_0/A_t)$ vs. time; I_a is the total amount of light absorbed. The samples and reference were measured under the same conditions.

2.13. Data analysis

All experiments of antioxidant and prooxidant activities were carried out in triplicates. All cell culture experiments were performed in duplicates and repeated on three passages of cells. All results were expressed as mean value ± standard deviation (SD). The graphs were plotted with Origin Pro 2020 for Windows. Topspin software was used to analyse the NMR data and Chem Draw Prime 2019 for Windows was used to represent chemical structures.

3. Results and discussion

3.1. Development of sustainable extraction approach

This study explores a water-based method to simultaneously isolate three types of photosynthetic pigments: carotenoids, chlorophylls and phycocyanin from the same batch of *Spirulina*. Two key steps in all extraction methodologies are the selection of an appropriate/ compatible solvent and method for cell wall disruption. (Minchev et al., 2020) Because the efficiency of the solvent extraction strongly depends on the solubility and stability of these molecules in the selected medium, the current separation strategy is based on the relationship between the chemical composition of pigments and their water solubility. Carotenoids are lipophilic pigments and are generally soluble in aprotic organic solvents such as acetone, whereas phycocyanin is hydrophilic. In contrast, chlorophylls are amphiphilic due to their hydrophilic and lipophilic character. Thus, they can by dissolved in either polar or non-polar solvents to a varying degree (Pérez-Gálvez et al., 2020).

The disruption of the cell wall is a key step affecting the yield and purity of the extract, it can also facilitate the process of releasing pigments based on the type of bonding that holds them in place within the plant cell. For example, phycocyanin is covalently bound and chlorophyll *a* is non-covalently bound to light-harvesting proteins. There are two principal pathways for cell wall disruption: (i) non-mechanical pathway e.g., thermal, chemical, and enzymatic treatments, and (ii) mechanical pathway e.g., ultrasonication, electric field, and microwaves (Pagels et al., 2021). This study employed mechanical separation techniques such as ultrasonication and centrifugation with a non-mechanical freezing/thawing. These steps can efficiently enhance the overall separation process as they exploit the properties of the material such as its size, shape and density as well as its average viscosity and solubility in the chosen medium (Anlauf, 2007).

This study also compares this methodology to the traditional acetone extractions as the solvent choice determines the sustainability of the process e.g., green (water, supercritical carbon dioxide) vs. traditional organic solvents (Marzorati et al., 2020). Among organic solvents, acetone provides the widest coverage in terms of the pigment's solubility; therefore, it is preferred in quantitative analysis of pigments. Moreover, several studies showed acetone to be better extraction solvent for chlorophylls as other common solvents such as ethanol may increase the activity of chlorophyllase, which is the enzyme that increases its activity with cell wall disruption leading to the degradation of chlorophylls during or after extraction (Andersen, 2005).

Our separation strategy using water and a comparative acetone extraction is outlined in Fig. 1. A batch prepared by suspending 100 g of *Spirulina* in 400 mL of water was cooled down (−20 °C) for 24 h and then sonicated allowing efficient breakdown of cell wall and release of the pigments. The removal of cell debris at this stage provides a fast and efficient separation of the supernatant, which is saturated with yellow, green, and blue pigments. This step is crucial for the successful separation of the pigments at the centrifugation stage, which can separate chlorophylls and carotenoids from phycocyanin due to the difference in their solubility in water as well as their molecular size. The top aqueous layer contained 100 % of phycocyanin, while a bottom dark green viscous fraction consisted of chlorophyll/ its derivatives (79.9 %) and carotenoids (20.1 %). This key difference in the current approach to remove cell debris at earlier stage enables efficient separation by centrifugation, while the published methods (Tavanandi, Mittal, Chandrasekhar, & Raghavarao, 2018) wasted chlorophyll content when discarding the supernatant and cell debris at the same stage. To demonstrate this point, a water extraction was carried out under previously published conditions skipping the first filtration step prior

centrifugation. Here, the supernatant contained mostly phycocyanin with the chlorophyll fraction being negligible.

Additionally, a traditional acetone extraction combined with sonication and centrifugation was tested; this method yielded only the extract containing carotenoids (21.7 %) and chlorophylls (78.3 %). But the total pigment content isolated from 100 g of *Spirulina*, is only 7.4 g which is significantly lower than the total pigment content isolated by our water method. The overall yield of green and yellow pigments from water extraction is 46 % higher than that achieved by the acetone-based method, and 69 % higher for chlorophyll pigments.

The concentration range for the individual pigments in water and acetone green extracts showed that chlorophyll *a* was the dominant pigment. The content of chlorophyll *a* was 68.94 ± 0.04 mg g^{-1} for the water and 34.20 ± 0.04 mg g^{-1} for the acetone extract. The concentrations of carotenoids were 17.30 ± 0.03 mg g^{-1} for water and 8.35 ± 0.04 mg g^{-1} for the acetone method. Published studies to extract pigments from *Spirulina* by green solvents reported 5.7 mg g^{-1} for chlorophyll *a* in $scCO_2$ extraction (Marzorati et al., 2020), while the use of ionic liquids delivered 4.36 ± 0.78 mg g^{-1} of chlorophyll *a* (Martins et al., 2021). Closer to our results is the study based on water extraction under rotary shaking conditions for cell wall disruption, reporting yields of 25.43 ± 0.02 mg g^{-1} for chlorophyll *a* and 4.25 ± 0.10 mg g^{-1} for carotenoids. (Gabr, El-Sayed, & Hikal, 2020) This difference in the efficiency of extractions underlines the importance of cell wall disruption method, where freezing and thawing increased extraction efficacy, as ice crystals can break the cell membrane during freezing.(Andersen, 2005) The subsequent ultrasonication allows an effcient permeation of the plant cell by the solvent molecules, which also explains why acetone extaction is less efficient. The concentration of phycocyanin in the water extract was 7.55 ± 1.14 mg g^{-1}, which is similar to other studies; the yield can be further improved through increasing the number of freezing/thawing cycles and the duration of ultrasonication (Jaeschke et al., 2021).

3.2. Purification and chemical functionalization of raw green extract

A limited use of chlorophyll itself is mainly driven by its ease of degradation, when exposed to light, moisture, or harsh environment. (Činčárová, Hájek, Dobřichovský, Lukeš, & Hrouzek, 2021). The degradation is an inevitable process already starting in the raw material itself. Analytical studies (Činčárová et al., 2021) showed *Spirulina* contains first-generation degradation products such as pheophytin *a* and pheophorbide *a* that are formed *via* the removal of the central magnesium ion followed by the hydrolysis of phytyl chain from chlorophyll *a* (Fig. 2). Chlorophyll derived pigments are known for their dose-dependent antioxidant properties, but under certain conditions, they can also be phototoxic making them promising photosensitizers. Thus, isolation and purification steps to convert chlorophyll into more stable products were incorporated as part of overall strategy.

An initial phytochemical screening of green extract using thin layer

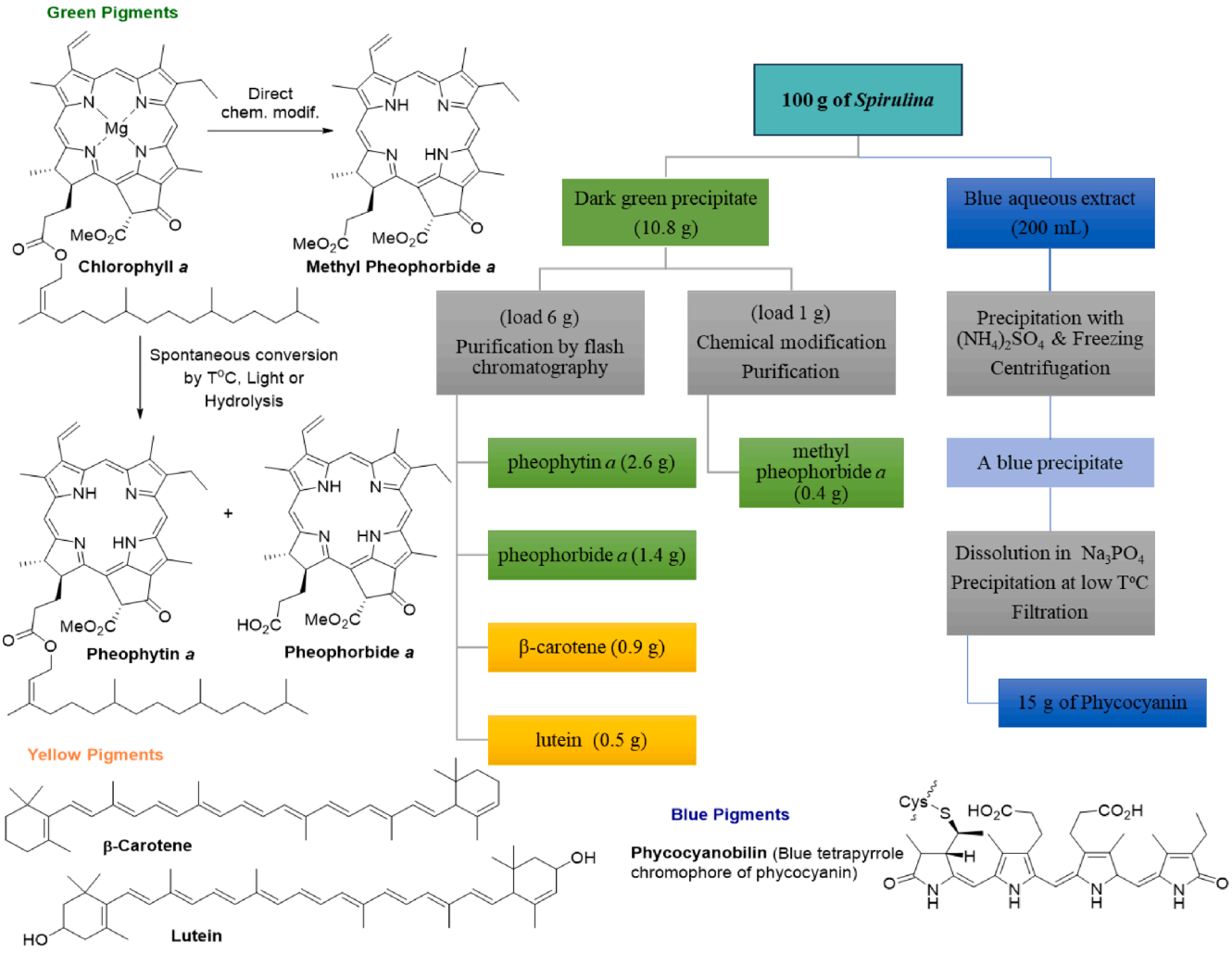

Fig. 2. Isolation and purification of photosynthetic pigments from *Spirulina*. Chemical structures of the key isolated compounds: β-carotene, lutein, pheophytin *a*, pheophorbide *a*, methyl pheophorbide *a*, phycocyanin. Chemical transformations involving chlorophyll *a*.

chromatography analysis confirmed the presence of xanthophylls (yellow), chlorophyll *a* (green) and β-carotene (orange) along with pheophytin *a* and pheophorbide *a*. Optimal solvent mixtures were determined using the retention factor, which indicates the separation and elution efficiency of a particular compound in this solvent. The hexane–acetone 90:10 (v/v) solvent system showed to be practical to separate pigments with RF between 0.2 and 0.9 (**SI Fig. S1**) and was adopted in the isolation of the compounds by flash chromatography. The method involved loading 190 g of silica (60–120 mesh) with 6 g of the crude dried green extract. The following pigments were isolated: β-carotene – 0.9 g, pheophorbide *a* – 1.4 g, pheophytin *a* – 2.6 g, and lutein – 0.5 g. (Fig. 2).

To demonstrate versatility of using untreated extracts, all chlorophyll derived molecules were converted into one single lipophilic methyl pheophorbide *a*. This can be achieved by treating 1 g of a raw green solid extract with acid in methanol followed by re-crystallization yielding 0.4 g of the compound with the purity of 94.5 %. The protocol involves the removal of magnesium ion through acid hydrolysis and esterification with methanol to obtain methyl pheophorbide *a* (Fig. 2). This method can be used to prepare a variety of lipophilic derivatives using different alcohols for the esterification step.

The purity of the isolated pigments was confirmed by HPLC-DAD analysis using a reverse column and the mobile phase containing trifluoroacetic acid (50–95 %) and acetonitrile monitored at 430 nm (Soret band) and 620 nm (Q-band). A simultaneous detection at both wavelengths is very important as it ensures the samples are not contaminated by carotenoids (430 nm) or phycocyanin (620 nm). The retention times at 430 nm and 620 nm were 8.60 min for pheophytin *a*, 8.73 min for pheophorbide *a*, and 8.65 min for methyl pheophorbide *a* (**SI Fig. S5**). The purity of the isolated pigments was calculated using the DAD chromatogram peak area at 430 nm. A high purity (>95 %) was confirmed for pheophorbide *a* and methyl pheophorbide *a*, while pheophytin *a* showed 92 % under these conditions due to racemization facilitated by TFA (Davani, Terenzi, Tumiatti, De Simone, Andrisano, & Montanari, 2022).

3.3. Further identification of chlorophyll derivatives

Methyl pheophorbide *a*, pheophorbide *a* and pheophytin *a*, are structurally very similar; their identity was confirmed by NMR (**SI Table S1, SI Fig. S3a-c**), UV–Vis (**SI Fig. S3d**) and accurate MS (**SI Fig. S4**). The UV–vis absorption spectra are similar featuring an intense Soret band at ca. λ_{max} 410 nm and the series of so-called Q bands between 505 nm and 665 nm with the intensity several times lower than that of the Soret band. Despite the structural complexity, ^{1}H NMR spectra of these pigments feature several characteristic regions: (1) the 7–10 ppm range contains three signals (singlets) for *β*- and *meso*-Hs; (2) the 3.3–4.9 ppm range contains four singlets of methyl groups at the pyrrole ring; (3) 6–8 ppm range contains signals of the vinyl group (as ABX system) present in chlorophyll *a* and its derivatives; (4) the 0.8–5 ppm range contains protons of the phytyl chain, when de-pytylation occurs, these signals disappear (Katz & Brown, 1983).

The mass spectrometry (MS) analysis confirmed the structures of the chlorophyll derivatives. Pheophytin *a* gave a monoisotopic *m/z* 887.5697 value corresponding to [M + H_2O] (Freitas et al., 2019), pheophorbide *a* and methyl pheophorbide *a* showed monoisotopic $[M + H]^+$ signals of *m/z* 593.2773 (Miranda et al., 2017) and 607.2752 (Smith et al., 1985), respectively.

3.4. Purification of blue extract and identification of phycocyanin

Most of the studies in the literature on phycocyanin extractions focused on maximizing the extract yield overlooking other phytochemicals (Jaeschke et al., 2021). In this study, an extract containing phycocyanin was obtained by separating the upper blue aqueous layer from the viscous green fraction (**Fig. 1**). This blue extract was treated with $(NH_4)_2SO_4$, frozen and centrifuged to yield a blue precipitate (**Fig. 2**), which was re-dispersed in K_3PO_4 and filtered yielding 15 g of blue powder.

To confirm structural features of phycocyanin, its spectral properties, chemical purity, and the protein content were assessed. Phycocyanin belongs to the phycobiliprotein (PBP) family, which can be broadly divided into three classes based on their spectral properties: phycoerythrin (λ_{max} 565 nm), phycocyanin (λ_{max} 620 nm), and allophycocyanin (λ_{max} 650 nm). In this study, the UV–vis absorption spectrum of the purified phycocyanin shows a prominent peak at λ_{max} 620, which corresponds to phycocyanin. (**SI Fig. S6b**)

To further characterize the purified samples, infrared (IR) spectroscopy was employed. (**SI Table S2, SI Fig. S6a**). IR analysis confirmed the presence of the key functional group regions: (1) centered at 3466 cm^{-1} denotes the stretching vibrations of N—H and O—H, (2) centered at 1638 cm^{-1} refers to the stretching vibrations of C═O (amide), (3) centered at 1460 cm^{-1} indicates C═C and O—C—O stretches, (4) centered at 1101 cm^{-1} refers to C—O stretching; this data is consistent with the literature (Izadi & Fazilati, 2018).

The purity of phycocyanin was determined by HPLC/DAD. A single peak with the retention time of 1.88 min was recorded in the reverse phase (DAD 620 nm, 100 % purity) confirming that phycocyanin did not contain chlorophyll and its derivatives. (**SI Fig. S6c**) Furthermore, the structural study of phycocyanin (Dagnino-Leone et al., 2022) shows that it is a chromophore bound to two distinct types of polypeptides with a large molecular weight β-unit (MW:14–21 kDa) and a low α-unit (MW:12–19 kDa), generally present in equal amounts. Thus, the purity of phycocyanin relative to the polypeptide content is an essential factor that determines its application. (Jaeschke et al., 2021) The presence of the protein content in phycocyanin extracts can be assessed by calculating the ratio between the absorption of phycocyanin at 620 nm and the absorption of all proteins at 280 nm (A_{620}/A_{280}). Based on the classification the A_{620}/A_{280} ratio greater than 4.0 is analytical grade, A_{620}/A_{280} between 0.7 and 3.9 is reagent grade, while the ratio below 0.7 is considered food grade. (Marzorati et al., 2020) In this study, the purity of phycocyanin was at food grade level (0.6). When lyophilizing *Spirulina*, the purity of phycocyanin usually does not exceed 0.6; however, dialysis can be performed on the extracted samples to increase purity to 2.2 (Minchev et al., 2020).

3.5. Antioxidant and prooxidant evaluation

Unique structural features allow chlorophyll, and its derivatives exhibit both antioxidant and pro-oxidant properties. Antioxidant potential of chlorophyll derivatives and phycocyanin was highlighted by several studies (Pérez-Gálvez et al., 2020). However due to challenging purification, very few have analysed methyl pheophorbide *a*, pheophorbide *a*, pheophytin *a* in either their pure form or/ and isolated by the same method as impurities can greatly influence the bioactivity. Also, some studies report on pro-oxidant activities (singlet oxygen production) of phycocyanin. In this study, methyl pheophorbide *a*, pheophorbide *a*, and pheophytin *a* were isolated in their pure form by flash chromatography allowing more accurate bioactivity analysis. In the same process, phycocyanin of food grade purity and free from chlorophyll and its derivatives was isolated. This allows a meaningful comparative analysis of the dual activity of these biologically relevant molecules by testing their potential as antioxidants *in vitro* in the dark. As well as the ability of these pigments to produce single oxygen under illumination conditions.

To note that there is a plethora of studies assessing antioxidant and radical scavenging activity of β-carotene and lutein, therefore, their analysis was not covered in this study.

3.5.1. General antioxidant capacity: DPPH, TEAC and FRAP assays

It is accepted that a single assay cannot provide a full picture of antioxidant activity due to different mechanisms and limitations of

individual assays (Prior, Wu, & Schaich, 2005). Moreover, analysis of impurified raw extracts is problematic due to the presence of multiple classes of antioxidants e.g., small molecules and proteins. Additionally, pigments can interfere with colorimetric analysis as they can give their own signals in the working region of the assay affecting the results. Thus, ferric-reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), and free radical scavenging activity (DPPH) assays were employed to provide a comprehensive rating of antioxidant capacity. The FRAP assay measures antioxidant capacity directly by reducing ferric (III) ions to ferrous (II) ions *via* a single electron transfer mechanism (SET), while DPPH and TEAC assess antioxidant capacity by scavenging free radicals through both SET and hydrogen atom transfer (HAT). (Prior et al., 2005)

All purified chlorophyll derivatives showed a dose-dependent response in DPPH, TEAC and FRAP assays. (Fig. 3) When compared at the highest concentration of 250 μg/mL, methyl pheophorbide *a* displayed the best radical scavenging activity followed by pheophorbide *a* and pheophytin *a* (Fig. 3). This trend is confirmed by other studies using TEAC assay. (Pérez-Gálvez et al., 2020) The results of FRAP and TEAC assays are very similar. As $ABTS^{\cdot+}$ radical in TEAC assay has a redox potential (+0.68 V) close to the one in FRAP (Fe^{2+}/Fe^{3+}) with latter being purely SET process, it is possible to conclude that chlorophyll derivatives predominately act through electron transfer rather than hydrogen transfer (HET). This fact can help to explain lower antioxidant activity in the DPPH assay, as DPPH is a stable radical that can act as radical as well as oxidant that can be quenched *via* HET and SET mechanisms. Additionally, a strong interference between chlorophyll derivatives and DPPH radicals at 515 nm, especially at higher concentrations, can obscure the results. (**SI Fig. S8**) The difference in the radical scavenging ability in the chlorophyll derivatives is driven by the side chain: phytyl – in pheophytin *a*, methoxy (–OMe) – in methyl pheophorbide *a*, and hydroxy (–OH) – in pheophorbide *a*. (Fig. 2) The methoxy group is electron donating, which increases the stability of the resonance system in the macrocyclic tetrapyrroles that may lead to an increased radical scavenging activity, while a lesser stability is associated with an electron withdrawing group such as –OH. This can result in a decrease in radical scavenging activity (Hoshina, Tomita, & Shioi, 1998). Moreover, the presence of a phytyl chain can provide a steric hinderance delaying the interaction, and consequently, slowing down quenching of radicals.

Several studies have claimed that phycocyanin has a high radical scavenging activity *in vitro*. (Renugadevi, Nachiyar, Sowmiya, & Sunkar, 2018) Although phycocyanin demonstrated a dose-dependent response in TEAC, FRAP and DPPH assays, its activity was significantly lower than that of green pigments in this concentration range. A low response in DPPH assay can be explained by steric bulkiness of phycocyanin. It is

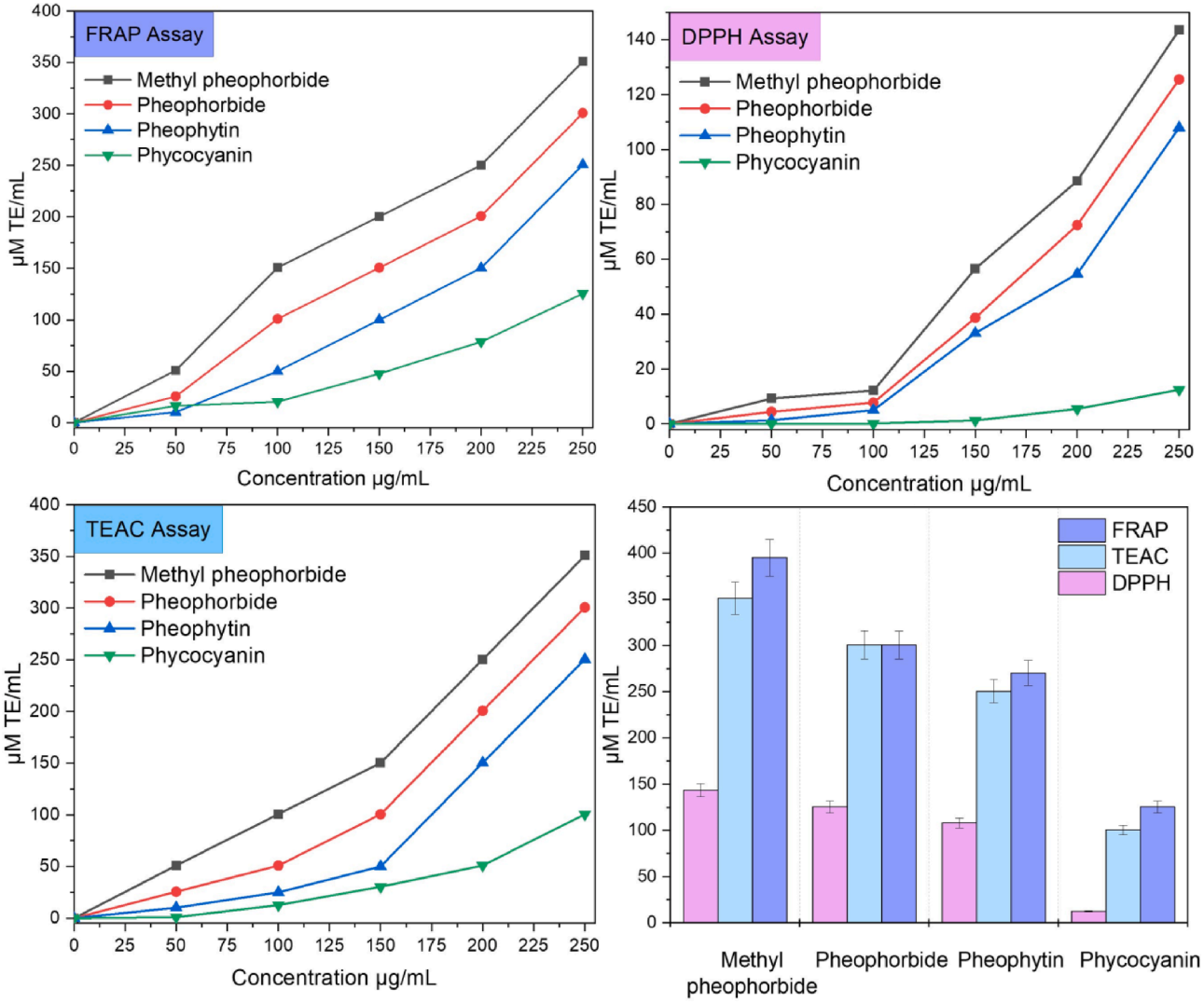

Fig. 3. Dose-dependent antioxidant activity of purified pigments assessed by FRAP, DPPH and TEAC assays. Comparative analysis of individual pigments at the highest concentration (250 μg/mL).

known that antioxidant activity measured by DPPH is higher for small molecules, where unhindered and rapid access to DPPH radical is favored. Lower antioxidant activity indicates that the mechanism of action by which phycocyanin quenches these radicals is limited. Similar to green pigments, the FRAP assay delivers best results indicating its potential to act as an electron donor, hence demonstrating that phycocyanin acts preferentially through SET electron transfer mechanism (Prior et al., 2005).

The activity of purified pigments decreases in the following order: methyl pheophorbide *a* > pheophorbide *a* > pheophytin *a* > phycocyanin. This difference in radical scavenging activity is due to the chemical structure of the chromophore in the pigments. Radical scavenging activity of antioxidants is often associated with an increased number of hydrogen donors such as hydroxy (–OH) and amino (–NHR) groups. Although chlorophyll derivatives and phycocyanin have some structural similarities such as tetrapyrrole chromophore with hydroxy and NHR groups, the hydrogen transfer ability of these groups in phycocyanin is rather reduced due to being a part of carboxylic and pyrrole units, respectively (Fig. 2). However, chlorophyll derivatives are macrocyclic tetrapyrroles, which are aromatic compounds (18π-electrons), while the phycocyanin chromophore is an open chain tetrapyrrole. Noteworthy, chlorophyll derivatives are chlorins, which are porphyrins with one reduced βC-βC single bond, which can be easily oxidized to βC = βC double bond. This facile oxidation improves the stability of the macrocycle and hence, is a favored process, which can also explain their readiness to act through SET mechanism in the antioxidant assays. And finally, phycocyanin is a protein-based pigment with a limited accessibility to the chromophore unit; the protein content can impact on the activity i.e. analytical grade has a higher activity than food grade. This may indeed be a factor in the lower antioxidant capacity when compared to the chlorophyll derivatives.

3.5.2. *Effect of algal pigments on viability of Caco-2 cells*

In this study, MTT and Neutral Red (NR) assays were employed to determine the impact of individual algal pigments on cell viability. All pigments exerted dose-dependent cytotoxicity towards Caco-2 cells except phycocyanin, which did not reduce cell viability, even at the highest concentration of 100 μg/mL. Pheophorbide *a* and pheophytin *a* showed a noticeable reduction in cell viability when the concentrations reached 25 μg/mL ($p < 0.05$) (Fig. 4a). Methyl pheophorbide *a* only demonstrated a decreased cell viability at 100 μg/mL. In contrast, NR assay results for all samples indicated no significant cytotoxicity in the target concentration range (0—–100 μg/mL) (Fig. 4b). This different outcome in the cytotoxicity for these assays is likely due to the different mechanisms of action. The MTT assay is based on the enzymatic conversion of MTT to its insoluble formazan in the mitochondria, while the NR assay depends on the uptake and lysosomal accumulation of the supravital dye (Fotakis & Timbrell, 2006). Moreover, it is known that pheophorbide *a* can accumulate in mitochondria leading to a targeted phototoxicity when activated by light (Tang, Liu, Zhang, Fong, & Fung, 2009). Thus, a high concentration of green pigments could have some effect on the mitochondrial respiratory activity leading to a decreased viability of Caco-2 cells. Previous studies on the cytotoxicity of green pigments using MTT assay reported differing observations. For example, pheophorbide *a* (0.5–1.0 μM) and pheophytin *a* (5–30 μM) fractions from the algae *Saccharina japonica* did not impact cell viability of LPS-stimulated RAW 264.7 macrophage cells when analysed by MTT assay (Islam et al., 2013). Similarly, using MTT assay, C-phycocyanin from

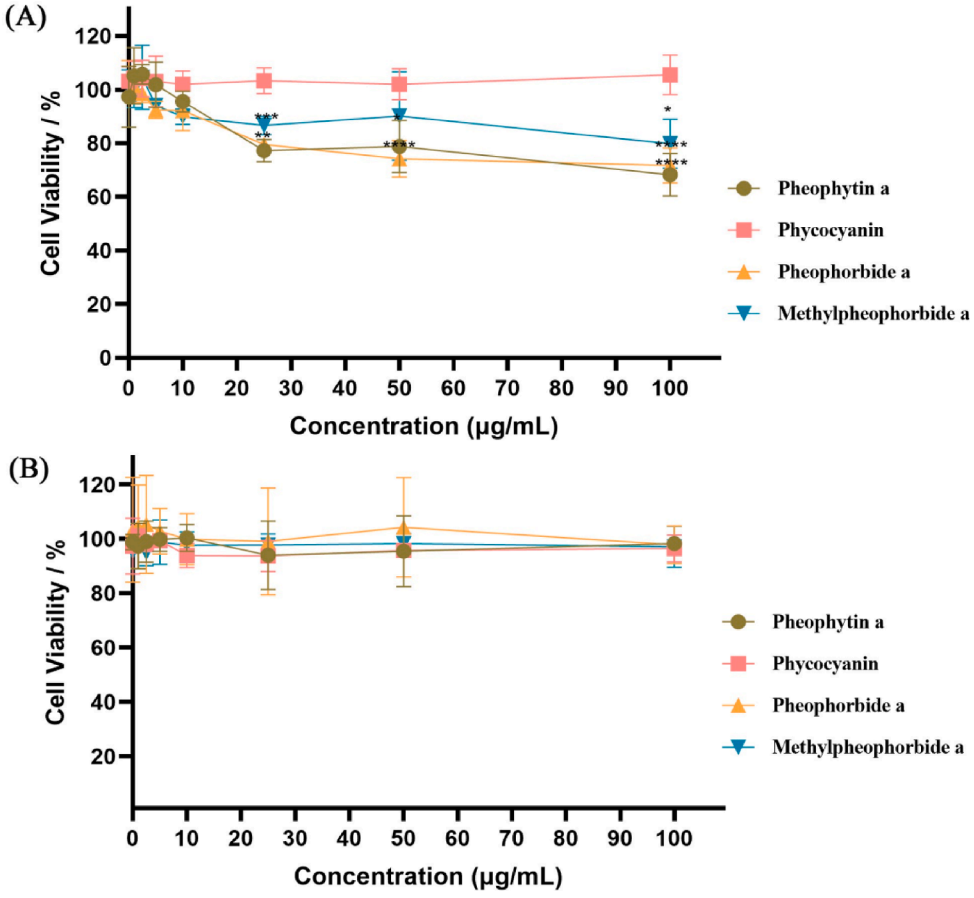

Fig. 4. Effect of purified algal pigments on viability of Caco-2 cells determined by MTT (A) and Neutral Red assay (B). The results are presented as mean value ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

blue green algae did not show toxicity towards RAW 264.7 cells, even at concentrations up to 250 μg/mL) (Cherng, Cheng, Tarn, & Chou, 2007). Differing sensitivity of individual cell lines towards algal pigments could in part explain variable findings in the literature regarding their cytotoxicity.

3.5.3. Effect of algal pigments on intracellular generation of ROS

The intracellular antioxidant capacity of algal samples against H_2O_2-induced ROS generation in Caco-2 cells is shown in Fig. 5. Under H_2O_2-induced conditions, pheophytin *a*, pheophorbide *a* and methyl pheophorbide *a* demonstrated a dose-dependent reduction in ROS levels, starting from 25 μg/mL, 25 μg/mL, and 5 μg/mL, respectively. In contrast, phycocyanin exerted significant intracellular antioxidant capacity at 1 μg/mL, which did not display a clear dose-dependent pattern across the range of concentrations tested (Fig. 5b). Indeed, the protective effects of marine algae on cancer, neurodegenerative, inflammatory and other ROS-related diseases have been linked to the presence of bioactives such as the photosynthetic pigments (Pradhan, Nayak, Patra, Jit, Ragusa, & Jena, 2020). Previous research has shown that phycocyanin had antiviral effects by reducing the level of cellular ROS (Jadaun et al., 2022). Pheophorbide *a* was reported to significantly reduce UVB-induced ROS accumulation by 36.5 % and 46.2 % at concentrations of 0.1 and 1 μM in CCD-986sk cells (Lee et al., 2021). Our comparative analysis showed that methyl pheophorbide *a* seemed to reduce ROS generation more effectively than pheophorbide *a*, which might be due to the structural difference discussed under general antioxidant activity. Overall, the intracellular results are in line with the findings observed for DPPH, TEAC and FRAP assays.

As well, the baseline (cells without being exposed to H_2O_2, blue line) for pheophytin *a*, pheophorbide *a,* and methyl pheophorbide *a* slightly increased at concentration of 50 μg/mL, 100 μg/mL and 100 μg/mL, respectively. Phycocyanin showed no effect on ROS generation in cells without H_2O_2 induction. An increase of the baseline in the samples containing green pigments could be due to color interference between the samples and assay, as pheophytin *a*, pheophorbide *a,* and methyl pheophorbide *a* were observed to have their own signals under the same measurement conditions of ROS detection (**SI** Fig. S9).

3.5.4. Prooxidant activities

It is well known that porphyrins are excellent Type II photosensitizers (PS) with good capabilities to generate singlet oxygen ($^1O_2$) upon irradiation with the visible light. This feature is widely explored in photomedical applications such as photodynamic therapy (PDT). Although chlorophyll derivatives exhibit $^1O_2$ production, the comparative analysis between the derivatives is lacking. This study compared the ability of pheophorbide *a*, pheophytin *a*, methyl pheophorbide *a* and phycocyanin to produce singlet oxygen using methylene blue as a reference. This $^1O_2$ method is based on measuring a decrease in the absorption at λ_{max} 410 nm of a trapping agent (1,3-diphenylisobenzofuran, DPBF) in DMF in the presence of PS and light. The decrease in absorption occurs due to the photooxidation of DPBF by $^1O_2$ generated by PS, which leads to DBB product of ring-opening reaction(Rogers et al., 2015) and is shown in **Fig. 6**.

Initial experiments were aimed to establish the photostability of pigments and DPBF to visible light using long-pass cut-off filters: 420 nm, 455 nm, 475 nm, and 495 nm during the 120 s irradiation period with Xenon lamp (50 W). On its own, DPBF shows photodegradation of up to 50 % by visible light when 420 nm filter is used. The degree of degradation falls to 20 % and 5 % for 455 nm and 475 nm filter, respectively. Finally, 100 % of photostability is achieved when using the 495 nm long-pass cut-off filter (**SI** Fig. S10). In contrast, all pigments under investigation exhibit a remarkable photostability regardless of the filters used. Effectiveness of pigments to generate singlet oxygen was probed using 495 nm filter and the results are shown in (**Fig. 6**). Pheophorbide *a*, pheophytin *a,* and methyl pheophorbide *a* are effective in $^1O_2$ generation, while phycocyanin shows only marginal activity, when compared to chlorophyll derivatives. The results also indicate that the efficacy of methyl pheophorbide *a* to generate singlet oxygen is

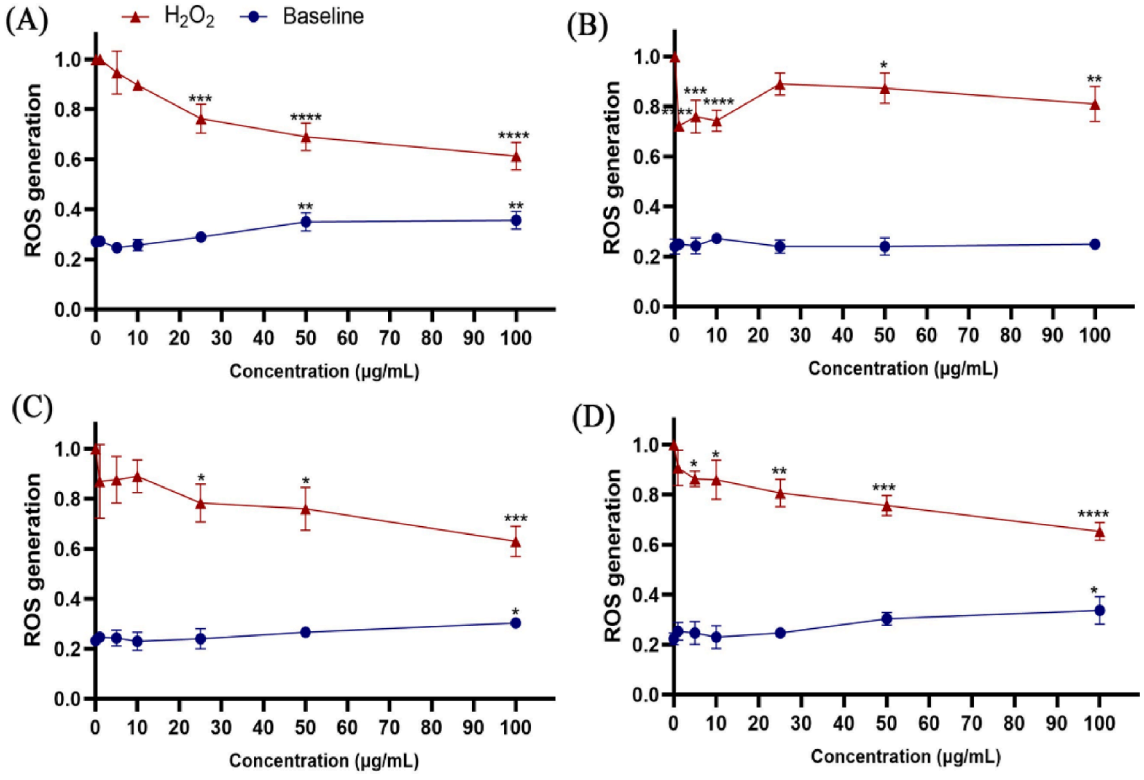

Fig. 5. Effect of pheophytin *a* (A), phycocyanin (B), pheophorbide *a* (C), methyl pheophorbide *a* (D) against H_2O_2-stimulated ROS generation in Caco-2 cells. The results are presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

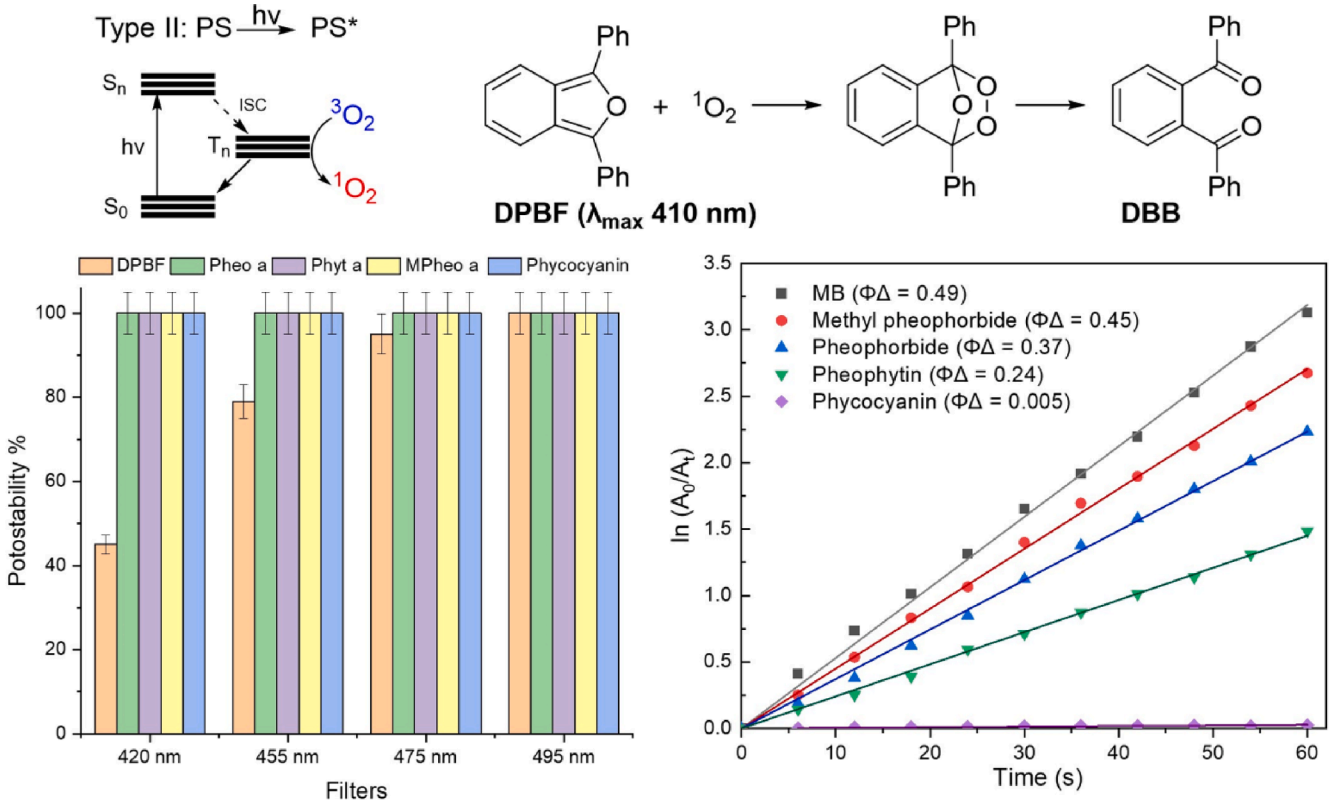

Fig. 6. The photostability ratio of DPBF and pigments at 410 nm when illuminated by visible light (Xenon lamp – 50 W) using four cut-off filters at 120 *sec*. Photooxidation reaction between DPBF and ^{1}O2 generated by PS. The linear fitting curve comparison of pigments and methylene blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

slightly higher than that of pheophorbide *a*, and pheophytin *a*.

The $^1O_2$ quantum yield (ΦΔ) for the pigments was obtained from the DPBF reaction using methylene blue (MB) as a standard. The linear plots of y (ln A) = f(t) was obtained by plotting $\ln(A_0/A_t)$ values of the DPBF absorbance at 410 nm against the reaction time (t) under irradiation. (Fig. 6) The $^1O_2$ quantum yield of MB measured in DMF is 0.49, which marks efficient and powerful photosensitizer. Remarkably similar to MB, methyl pheophorbide *a* gave (ΦΔ) of 0.45, which is the highest value among chlorophyll derivatives. This was followed by pheophorbide *a* (ΦΔ 0.37) and pheophytin *a* (ΦΔ 0.24). In contrast, the singlet oxygen quantum yield of phycocyanin is only about 0.005 under these conditions.

It was speculated that high FRAP values may correlate with the tendency to become pro-oxidants under certain conditions, which had been demonstrated for some flavones and flavanones. In this study, this trend shows that the high values in FRAP indeed correspond to high $^1O_2$ quantum yield.

4. Conclusion

To summarize, a sustainable approach was developed to extract photosynthetic pigments from *Spirulina* by freezing, thawing, centrifugation and ultrasound assisted extraction using water as a sole solvent. This approach succeeded in producing three main types of photosynthetic pigments: carotenoids, chlorophylls and phycocyanin from the same batch of *Spirulina*. The steps to convert chlorophyll into stable products such as pheophorbide *a* and pheophytin *a* were incorporated. Additionally, the treatment of chlorophyll extract with acid followed by methylation led to the production of methyl pheophorbide *a*. Overall, the current results demonstrate that the combination of environmentally friendly solvent with a variety of cell membrane disruption techniques can successfully recover photosynthetic pigments in a highly efficient manner and thereby contribute to a more efficient use of bioresources.

A comparative analysis of green and blue pigments revealed that green pigments have significantly higher activity as antioxidants and prooxidants compared to phycocyanin. The superior antioxidant activity of green pigments can be attributed to their unique structure and ability to undergo SET, also the presence of electron rich functional groups enhances this ability. Equally, the prooxidant properties to generate singlet oxygen are excellent and on par with methylene blue, which is well-known PS. In contrast, phycocyanin showed exceptionally low activity to generate singlet oxygen. The demonstration and comparison of these activities and the availability of the method may change the outlook for a wider utilization of these green pigments.

CRediT authorship contribution statement

Shorog Alotaiby: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Xiao Zhao:** Methodology, Writing – review & editing. **Christine Boesch:** Methodology, Validation, Investigation, Writing – review & editing, Supervision. **Natalia N. Sergeeva:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137653.

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