Antioxidant Properties of Ethanol Extracts from *Chlorella sorokiniana* and *Scenedesmus quadricauda* in the Ca Cam River, Ho Chi Minh City, Vietnam

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Abstract
Microalgae from the Chlorophyta group have garnered attention for their potential as sustainable sources of bioactive compounds applicable in the food, biostimulant and pharmaceutical sectors. This investigation focused on Chlorella sorokiniana and Scenedesmus quadricauda, both isolated from the Ca Cam River in Ho Chi Minh City, Vietnam. The main objectives were to extract and characterize chlorophyll a and b, carotenoids and phycocyanin and to evaluate their total antioxidant capacity. Ultrasound treatment was used to disrupt the microalgal cells followed by extraction with various solvents. The antioxidant activity of the extracts was measured using the DPPH assay. Chlorella sorokiniana showed higher relative humidity (86.61 ± 2.11%) and contained significantly more pigments than Scenedesmus quadricauda (79.67 ± 0.52%) (p = 0.044).

Specifically, Chlorella sorokiniana had a higher chlorophyll a content (1,630 ± 88.63 µg/g) compared to Scenedesmus quadricauda (1,310 ± 108.25 µg/g) (p = 0.032). At low concentrations (110-130 mg/µL), the antioxidant inhibition of these strains ranged from 49-52% (p < 0.001). The highest antioxidant capacity in Chlorella sorokiniana was 52%, equivalent to 1.038 µg of vitamin C, while Scenedesmus quadricauda exhibited approximately 43%, equivalent to 1.08 µg of vitamin C.

Keywords: *Chlorella sorokiniana, Scemedemuus quadricauda,* Chlorophyll a and b, Carotenoids, Phycocyanin, Antioxidants.

Introduction

Antioxidants are compounds that, even in small amounts (and/ or low concentrations), can significantly delay or inhibit the oxidation of other molecules[14]. They are essential for protecting cells from oxidative damage and are abundant in foods, particularly fruits and vegetables, as well as in dietary supplements. Key antioxidants include β-carotene, lutein, lycopene, selenium, vitamins A, C and E, manganese and zeaxanthin[51].

Beyond traditional sources like plants, algae are an exceptional reservoir of bioactive compounds, particularly antioxidants. Different species of algae and their specific compounds exhibit varying degrees of antioxidant capacity.

Algae's high total antioxidant capacity (TAC) is largely due to compounds such as phycocyanins, vitamins, carotenoids, polyphenols, fatty acids and phycobiliproteins. These compounds help algae defend against environmental stressors and when combined, they can outperform even the most potent known antioxidants.[5,49] Microalgae found in diverse aquatic environments from freshwater lakes to oceans, are well-known for their antioxidant production[33]. These organisms convert solar energy into biomass through photosynthesis, generating antioxidants to protect against reactive oxygen species (ROS) formed during metabolic processes[33]. As a result, they accumulate bioactive compounds that defend cells against oxidative stress and damage[46]. Rich in carotenoids, which give them vibrant colors like green, yellow, orange and red, microalgae produce potent antioxidants such as β-carotene, astaxanthin, lutein and zeaxanthin which neutralize free radicals and protect cells from oxidative damage[7,34,50] (Table 1).

Recent research has unveiled the diverse array of bioactive compounds synthesized by microalgae, many of which possess potent antioxidant activities. These include carotenoids, polyphenols, phycobilins, tocopherols and essential fatty acids[20,40]. β-carotene, lutein and astaxanthin, among others, are particularly effective at scavenging free radicals and singlet oxygen species, thereby enhancing cellular protection against oxidative damage[26,33]. Additionally, these compounds have shown promising anticancer properties in both laboratory and animal studies.

Microalgae display extensive diversity, encompassing thousands of identified species categorized into various taxonomic groups based on cellular characteristics, pigmentation and morphology. Major groups include Bacillariophyceae, Cyanophyceae, Chlorophyta, Coccolithophyceae, Cryptophyceae, Euglenophyceae, Eustigmatophyceae, Haptophyta, Miozoa and Porphyridiophyceae. Chlorophyta, notable for chlorophyll *a* and chlorophyll *b*, β-carotene and xanthophylls, was extensively studied.[20,46]

Chlorella sorokiniana, isolated by Sorokin in 1953, is a robust single-celled alga, typically 2 to 4.5 µm in diameter. It exhibits mixotrophic growth, utilizing various carbon and nitrogen sources, suitable for cultivation on waste feedstocks[24].

Table 1
Antioxidants, Their Algal Sources and Potential Functions

Algal species	Types of antioxidants	Functions
Anabaena vaginicola	Carotenoids	Free radical scavenging[15].
Chlorella ellipsoidea, *Chlorella vulgaris*, *Dunaliella salina*	β-carotene; Pigments	Free radical scavenging, health food, natural coloring agents, additives to food, protecting tissues from chemical damage, cancer and other age-related disorders and inhibition of colon cancer[6,29,37].
Chlorella thermophila	Chlorophylls and carotenoids	The extraction efficiency of chlorophyll and carotenoids from wet microalgae biomass, using ethanol and heat drying, is essential for their application as natural food colorings and antioxidants. These compounds also possess properties beneficial for wound healing and exhibit antimutagenic effects[42].
Porphyridium sp. *Spirulina* sp., *Arthrospira platensis*	Carotenoids, chlorophylls, phycocyanin (blue) and phycoerythrin (red)	Natural colors and additives to cosmetics[48] and supercritical-CO_2 ($scCO_2$), with minimized modifications on the biomass[27,32].
Skeletonema marinoi	Pigments, peptides and vitamins	Reactive oxygen species attenuation[45].
Synechococcus sp.	Chlorophylls *a*, *b*, *c* and *d* and total chlorophylls	Assessment of Chlorophylls in natural assemblages of aquatic plants[39].

Studies highlight its resilience in wastewater environments[38], thriving under conditions like carbon dioxide supplementation[25] and elevated temperatures[10]. Its dry weight typically comprises of 40% protein, 30-38% carbohydrate and 18-22% lipid[13,18]. Notably, *C. sorokiniana* biomass contains valuable compounds including antioxidants like carotenoids, up to 0.69% of its dry weight under extreme conditions[28].

Scenedesmus, a genus of freshwater green microalgae, forms colonies 2 to 10 µm in diameter. This autosporic genus often clusters in groups of four or eight cells within a parental mother wall, rich in proteins, carbohydrates and lipids[43]. Among its 72 identified species, *Scenedesmus quadricauda* is notable for bioenergy production[52]. Colonies typically appear as clusters of four to eight cells[31].

Water content or relative humidity (RH%) significantly influences algae applications. Algal-based biostimulants enhance fruit weight[47], hydroponically cultivate lettuce, act as bio-fertilizers and combat algae biofouling on surfaces including fired bricks[36]. Algal water extracts stimulate growth parameters, chlorophyll content, yield and fruit quality of tomato plants under salinity stress[30].

In this study, we evaluated and compared the concentrations of chlorophyll a and chlorophyll b, carotenoids and phycocyanin in wet biomass from the isolated strains *Chlorella sorokiniana* and *Scenedesmus quadricauda* from the Ca Cam River in Ho Chi Minh City, Vietnam. We also investigated and evaluated the antioxidant capacity based on wet microalgae mass between these two strains.

Material and Methods

Experiment design: In October 2023, microalgae samples were collected from the Ca Cam River (10°73'22.1" N, 106°72'85.0" E) in Ho Chi Minh City, Vietnam and analyzed at the Microbiology Laboratory of the University of Science, Ho Chi Minh City. Identified strains included *Chlorella sorokiniana* Shihira and R.W. Krauss 1965 (Class: Trebouxiophyceae) and *Scenedesmus quadricauda* (Turpin) Brébisson 1835 (Class: Chlorophyceae), both from the phylum Chlorophyta. For cultivation, microalgae were pre-cultured and inoculated at 10% (v/v) into Bold's basal medium (BBM) [8], divided into two 100 mL flasks totaling 200 mL per culture. Conditions were maintained at 25 ± 1°C with a pH range of 6.4–6.8 under continuous LED light providing 664–737 lux. Laboratory humidity was kept at 51 ± 1%. Cell growth was monitored daily at 750 nm using SP-UV 1100 UV-VIS Spectrophotometer (Figure 1).

Biomass formation and extract preparation: The microalgae cultures were grown until reaching mid-logarithmic phase, with cell densities reaching approximately 4.1 × 10^6 cells/mL by day 13 for *C. sorokiniana* and 3 - 3.5 × 10^6 cells/mL by day 11 for *S. quadricauda*. Algal cells were harvested during the logarithmic growth phase using a Hettich centrifuge model EBA 20 (Germany). Ethanol extracts were prepared with absolute ethanol (HPLC grade, ACS certified) (Fisher, CAS 64-17-5) following methods by Hemalatha et al[16] and Saranya et al[41]. Fresh microalgae samples (4.0 - 5.0 g) were placed in 35 mL screw-capped centrifuge tubes and extracted with 96% ethanol at room temperature using a Japan-made ultrasonic cleaner (40 kHz frequency, 60 W power, model JP-010 2L)[23].

Successive extractions were carried out with ethanol solutions at 96%, 72%, 48% and 24% concentrations (v/v/v/v), with each extract filtered through Whatmann GF/A filter paper. The final volume of each strain's extract was adjusted to 100 mL and stored at -20°C for subsequent antioxidant activity assays[32].

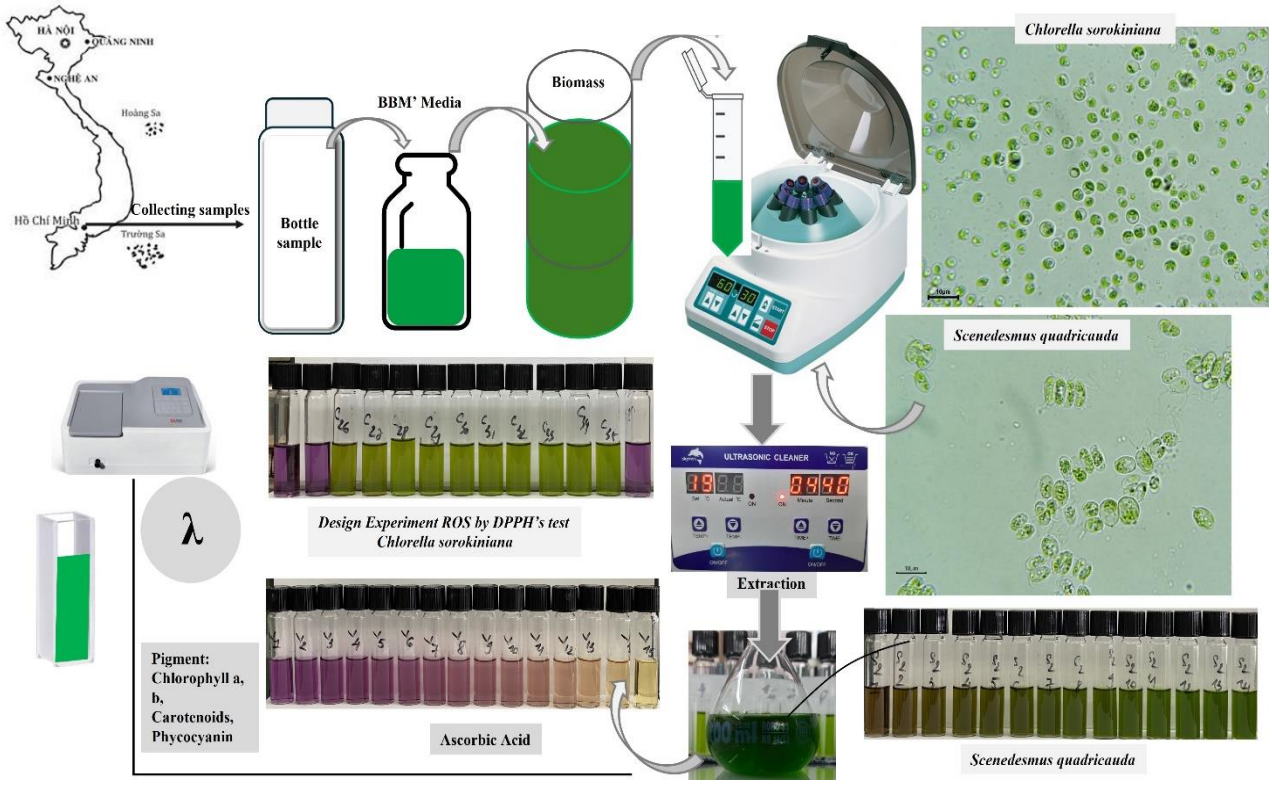

Fig. 1: Schematic step - by - step approach of this study.

Determination of pigment content

Chlorophylls and carotenoids: To quantify chlorophyll a, chlorophyll b and carotenoid contents, around 0.1 up to 0.2 g of fresh microalgae sample was extracted in 10 mL of 96% ethanol using ultrasonic baths operating at 46 kHz for 10 minutes at 75°C, with two repetitions. Each extraction was conducted in triplicate. The resulting extracts were filtered and analyzed spectrophotometrically at wavelengths of 662 nm, 645 nm and 470 nm. Chlorophyll and carotenoid concentrations were determined using equations described by Lichtenthaler and Buschmann[22].

$$C_a, \mu g/mL = 13.95 \times A_{665} - 6.88 \times A_{649} \quad (1)$$

$$C_b, \mu g/mL = 24.9 \times A_{649} - 7.32 \times A_{665} \quad (2)$$

$$C_{x+c}, g/L = (1{,}000 \times A_{470} - 2.05 \times C_a - 114.8 \times C_b)/245 \quad (3)$$

where C_a is chlorophyll a, C_b is chlorophyll b and $C_{(x+c)}$ is total carotene.

Phycocyanin[32]: For phycocyanin extraction, 0.1 - 0.2 g of fresh sample was placed in a 15 mL polypropylene centrifuge tube and mixed with 10 mL of extraction saline buffer. The buffer composition included 8.77 g NaCl, 2.01 g KCl, 11.36 g Na_2HPO_4 and 3.72 g Na_2EDTA per liter of distilled water. After incubating at 4°C for 16 hours, the samples underwent sonication for 10 minutes and subsequent centrifugation at 4°C and 4,000 rpm for 20 minutes. Phycocyanin extracts were then analyzed using the SP-UV 1100 UV-VIS Spectrophotometer at wavelengths of 615 nm and 682 nm. Pigment concentration, yield and purity index were calculated using specific equations:

$$Phycocyanin\ concentration, mg/mL = \frac{A_{615} - 0.475 \times A_{652}}{5.34} \quad (4)$$

$$Purity\ index = \frac{A_{615}}{A_{280}} \quad (5)$$

$$Yield = \frac{PC \times V}{WB} \quad (6)$$

where PC was the concentration of phycocyanin in mg/mL, V was the volume of extract in mL and WB was the biomass of fresh microalgae sample in grams.

Antioxidant activity assessment of ethanol extracts[21,44]: To assess antioxidant activity, varying volumes (50 µL - 4,000 µL) of each ethanol extract were mixed with 500 µL of freshly prepared 0.6 mM DPPH (2,2'-diphenyl-1-picrylhydrazyl) solution in methanol (HPLC grade ≥ 99.8%, CAS: 67-56-1, Fisher Chemical, USA). The mixture was incubated in the dark at room temperature for 30 minutes, followed by measuring absorbance at 517 nm against a blank. A standard curve was generated using L-Ascorbic acid solutions (Sigma, CAS 50-81-7) ranging from 0 to 40 µg/mL. Results were expressed as microgram equivalents of ascorbic acid (AAE)/µL ($y = 2.7316x - 8.922$, $R^2 = 0.9929$) (Figure 2). Percentage inhibition (I %) was calculated using the following equation:

$$I\ \% = \frac{A_0 - A_1}{A_0} \times 100 \quad (7)$$

where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

Determination of Relative Humidity (RH) (%)[2,4]: Fresh microalgae samples underwent relative humidity analysis

using a Gallenkamp hot box oven (Size 2, Model: OVB 305, Sanyo Weiss). The samples were dried at 105℃ ± 3 for 3 hours, with hourly weight recordings. Post-drying, samples were cooled in a desiccator and promptly re-weighed to minimize moisture loss. Water content was calculated based on the mass difference before and after drying, using the following equation:

Percent Total Dry Matter (Total DM):

$$\text{Total DM} = \left\{\frac{(\text{Dry weight of sample and Dish} - \text{Tare weight of Dish})}{(\text{Initial Weight of Sample and Dish} - \text{Tare Weight of Dish})}\right\} \times 100 \quad (8)$$

Percent Total Moiture:

$$\% \text{ Total Moisture} = 100 - \% \text{ Total DM} \quad (9)$$

where DM represents dry matter content.

Data Analysis: All assays for chlorophyll a and chlorophyll b, carotenoids, phycocyanin and antioxidant activities were conducted in triplicate. Results are presented as mean ± standard error of the mean (SEM). Tukey's Honestly Significant Difference (HSD) test was used to identify statistically significant differences between samples. Statistical analyses were performed using R software (version 4.3.1), with significance set at $p < 0.05$.

Results and Discussion

Relative Humidity (RH%): *Chlorella sorokiniana* exhibited a higher relative humidity (RH%) of 86.61 ± 2.11% compared to 79.67 ± 0.52% for *Scenedesmus quadricauda*. Typically, food materials should maintain a moisture content below 80% for optimal preservation. However, water plays a crucial role in the lifecycle of microalgae cells. Extracts from these cells have shown significant benefits as biostimulants in various applications such as enhancing fruit growth[47], supporting hydroponically grown lettuce[11] and acting as bio-fertilizers[36]. In contrast, functional foods derived from microalgae are often produced from dried biomass, making water content a critical factor during production. Consequently, the dry biomass of *S. quadricauda* exceeds that of *C. sorokiniana.*

Chlorophyll content: Chlorophyll, an essential compound found in various products, is highly valued for its natural food coloring, antioxidant properties and antimutagenic effects[22]. It primarily exists in two forms: chlorophyll a and chlorophyll b[17]. Our analysis shows that *C. sorokiniana* contains significantly higher levels of chlorophyll a compared to chlorophyll b, with concentrations of 1,633 ± 88.63 µg/mg and 1,313 ± 108.25 µg/mg, respectively (p = 0.032*).

Furthermore, *C. sorokiniana* exhibits markedly higher chlorophyll a content than *S. quadricauda*. However, there was no statistically significant difference in chlorophyll b concentration between the two species, with averages of 1,045 ± 43.21 µg/mg for *C. sorokiniana* and 1,029.03 ± 63.48 µg/mg for *S. quadricauda* (p = 0.99) (Figure 3).

Ethanol extracts of *Spirulina* sp. predominantly contained chlorophyll a, except when extracted at 20℃ for 2-3 hours at a frequency of 35 kHz[32]. The maximum chlorophyll a content (5.46 mg/g) was observed at 40℃ after 2 hours and a frequency of 45 kHz. Previous studies by Sarkar et al[42] highlighted that wet biomass of *Chlorella thermophila* yielded 2.7 times more chlorophyll and 6.7 times more carotenoids compared to dry biomass. The highest chlorophyll yield (~60 mg/g - dry biomass) was achieved at 58℃ after 6 minutes of homogenization, with an extraction efficiency of ~94%. Additionally, Marzorati et al[27] reported chlorophyll a and b contents from dry *Spirulina* sp. biomass to be 5.7 ± 0.2 mg/g and 3.4 ± 0.3 mg/g respectively.

Chlorophyll is typically extracted from dried biomass using organic solvents or supercritical fluid extraction followed by fractionation to separate chlorophyll pigments and their derivatives[17].

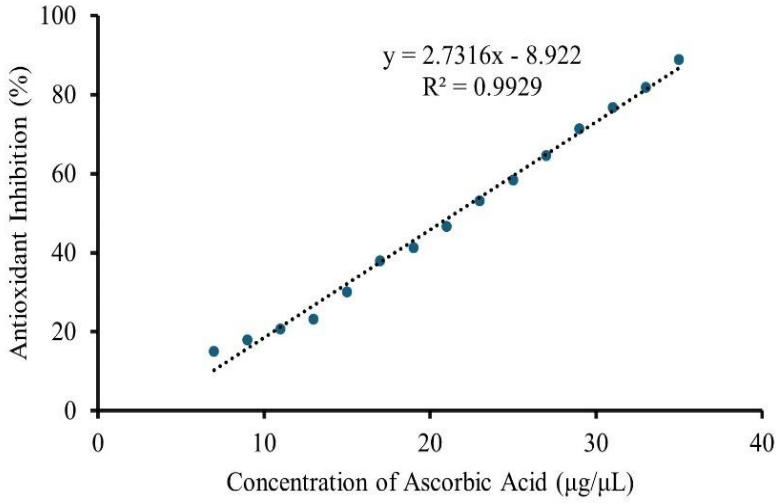

Fig. 2: The standard curve of Ascorbic Acid

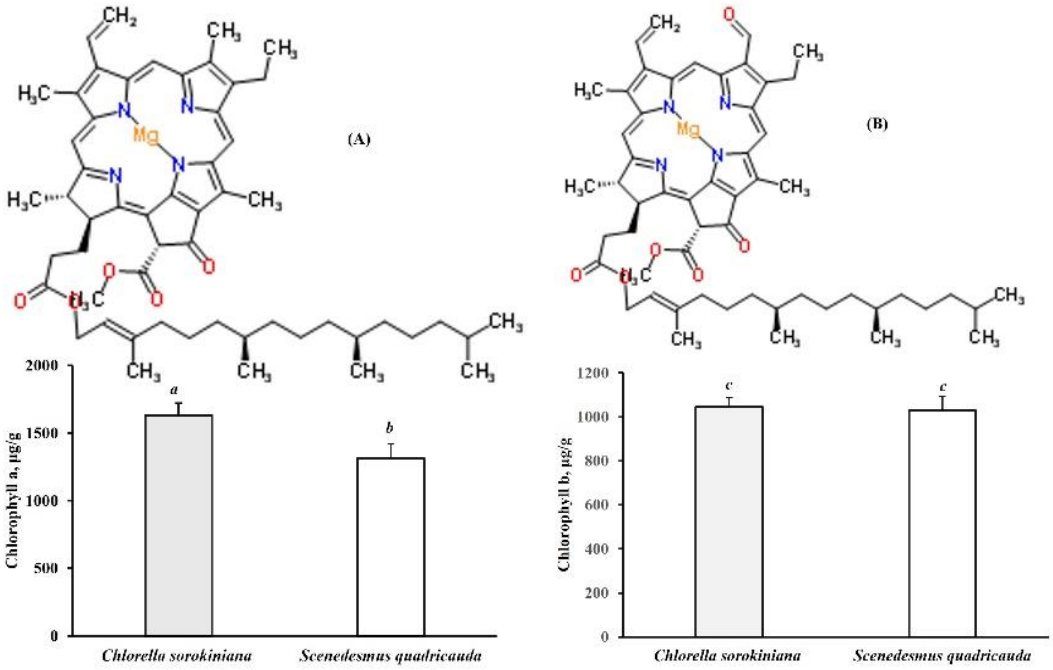

Fig. 3: Relative expressions of chlorophyll *a* and *b* were extracted of *Chlorella sorokiniana* and *Scenedesmus quadricauda*: (A) content of chlorophyll a, (B) content of chlorophyll b. (Different letters within each column indicate significant differences according to TukeyHSD's test at p < 0.05) (Note: Structure of chlorophyll a and b from Zeece[53])

Extensive research has optimized these processes for use in pharmaceuticals, cosmetics and natural food coloring, owing to their antioxidant and antimutagenic properties. Seven distinct chlorophyll compounds were identified in both *C. sorokiniana* and *S. bijuga*, albeit in varying proportions. Chlorophylls constituted 7.6% and 10.2% of the biomass composition in *C. sorokiniana* and *S. bijuga* respectively[12].

Chlorophyll extraction from dried biomass typically employs organic solvents or supercritical fluid extraction methods, followed by fractionation to isolate chlorophyll pigments and their derivatives[17]. Extensive research has optimized these processes for pharmaceutical, cosmetic and natural food coloring applications, leveraging their antioxidant and antimutagenic properties. Seven distinct chlorophyll compounds were identified in both *C. sorokiniana* and *S. bijuga*, albeit in varying proportions. Chlorophylls constituted 7.6% and 10.2% of the biomass composition in *C. sorokiniana* and *S. bijuga* respectively[12].

Carotenoid content: Carotenoids, natural lipophilic pigments, provide red, yellow and orange coloration to various organisms and are widely utilized as dyes, flavorings and nutritional supplements. Our analysis found no significant difference in carotenoid concentrations between *C. sorokiniana* and *S. quadricauda* ($p = 0.99$). Specifically, *C. sorokiniana* exhibited higher carotenoid content compared to *S. quadricauda* (804.71 ± 100.51 µg/g and 732.83 ± 41.70 µg/g respectively) (Figure 4). Wet microalgal biomass yields significantly more pigments compared to dry biomass, influenced by the extraction solvent and method employed[42]. Matsukawa et al[28] noted that carotenoids can constitute up to 0.69% of dry weight under extremophilic conditions.

Fernandes et al[12] identified 17 distinct carotenoids in *C. sorokiniana* and *S. bijuga* using High-performance liquid chromatography coupled with diode array and mass spectrometry detectors (HPLC-PDA-MS/MS). *C. sorokiniana* contained 11 carotenoids (1,408.46 µg/g), while *S. bijuga* harbored 16 (1,195.75 µg/g). Predominantly, all-trans-lutein and all-trans-β-carotene were the major carotenoids identified, with all-trans-lutein being notably more abundant in *C. sorokiniana* (59.01%) and all-trans-β-carotene, more prevalent in *S. bijuga* (13.88%).

Phycocyanin content: Phycocyanins, derived from blue-green algae (Cyanophyta) such as *Spirulina platensis*, *Porphyridium* sp. and *Spirulina* sp. [27,32,48], thrive under alkaline conditions (pH 7.2 to 9.0) and a salinity of 30 g/L, with optimal growth at 27°C. Our analysis found no significant difference in phycocyanin concentrations between *C. sorokiniana* (2.02 ± 0.09 mg/g) and *S. quadricauda* (1.51 ± 0.11 mg/g) ($p = 1.00$) (Figure 5). Kamble et al[19] achieved a phycocyanin yield of 0.26 mg/mL using ultrasound at 40 kHz for 40 minutes. Studies also suggest that a frequency of 50 kHz maximizes phycocyanin extraction, yielding between 0.57 mg/g to 43.75 mg/g with a C-phycocyanin concentration of 0.21 mg/g.

Phycocyanins are essential components of phycobiliprotein complexes in photosynthetic light-harvesting antennae. Known for their vivid colors and intense fluorescence, these pigments are categorized into phycoerythrin (red) and

phycocyanin (blue) groups. They contain various bioactive compounds that exhibit anti-inflammatory properties, inhibit lipid peroxidation and scavenge free radicals, thereby mitigating oxidative stress. [9]

Antioxidants: The optimal antioxidant capacity was assessed in both fresh microalgae strains across concentrations ranging from 2 to 180 mg/µL. At concentrations between 110 and 130 mg/µL, the total antioxidant capacity (TAC) peaked at 49% to 52%. Notably, *C. sorokiniana* exhibited higher antioxidant activity compared to *S. quadricauda* (Figure 6). Statistical analysis indicated a significant positive correlation between microalgae concentration and inhibitory capacity ($p < 0.001$**) (Figures 7 and 8). However, concentrations above 130 mg/µL led to a decline in reactive oxygen species (ROS) proportion (Figure 7).

The maximum antioxidant capacity for *C. sorokiniana*, at 52%, equated to 1.038 µg of ascorbic acid (Vitamin C) while *S. quadricauda* showed approximately 1.08 µg (~43%) under similar conditions. This activity may vary with processing time, as absorbance measurements were taken every 30 minutes over 90 minutes to assess antioxidant potential[26].

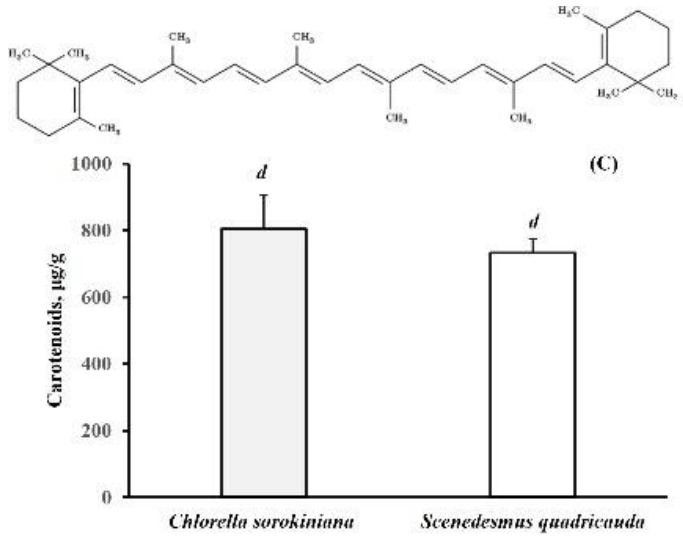

Fig. 4: Relative expression of the total carotenoid contents for ethanolic extracts of *Chlorella sorokiniana* and *Scenedesmus quadricauda.* (Note: Structure of carotenoids from Zeece[53])

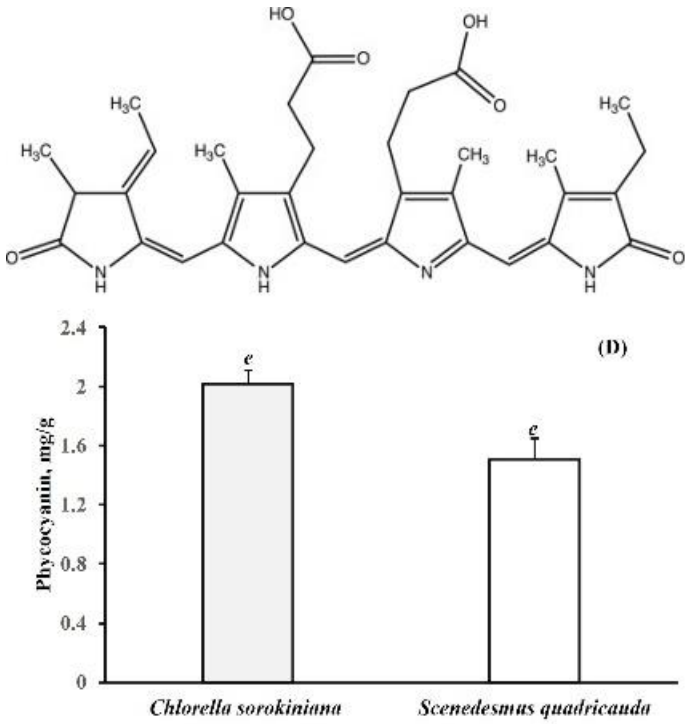

Fig. 5: Relative expression of phycocyanin contents for ethanolic extracts of *Chlorella sorokiniana* and *Scenedesmus quadricauda* (Note: Structure of phycocyanin from Zeece[53]).

Augustina et al[1] reported that *C. vulgaris* exhibited its highest antioxidant capacity at a 0.1% concentration, corresponding to 11.83 mg of vitamin C per 100 g sample. In cosmetic formulations, the antioxidant capacities were 4.95 mg Vit. C per 100 g sample (IC_{50} 719.75 mg/ml) for cream and 4.73 mg vitamin C per 100 g sample (IC_{50} 660 mg/ml) for lotion, with no observed microbial contamination. This suggests potential applications of *C. vulgaris* as an active ingredient in cosmetics[1].

Silva et al[43,44] demonstrated a 95.53% inhibition of DPPH oxidation for carotenoid extracts and 96.09% for phenolic compounds from *S. obliquus*. Other microalgae strains, such as *Dunalliela* sp., *Tetraselmis* sp. and *Nannochloropsis gaditana*, also exhibited substantial antioxidant potential, with DPPH inhibition capacities exceeding 80%[26].

The decline in inhibition percentage over time was attributed to carotenoid oxidation by free radicals generated from DPPH[44]. Phenolic compounds primarily exert antioxidant activity through their redox properties, facilitating the absorption and neutralization of free radicals.

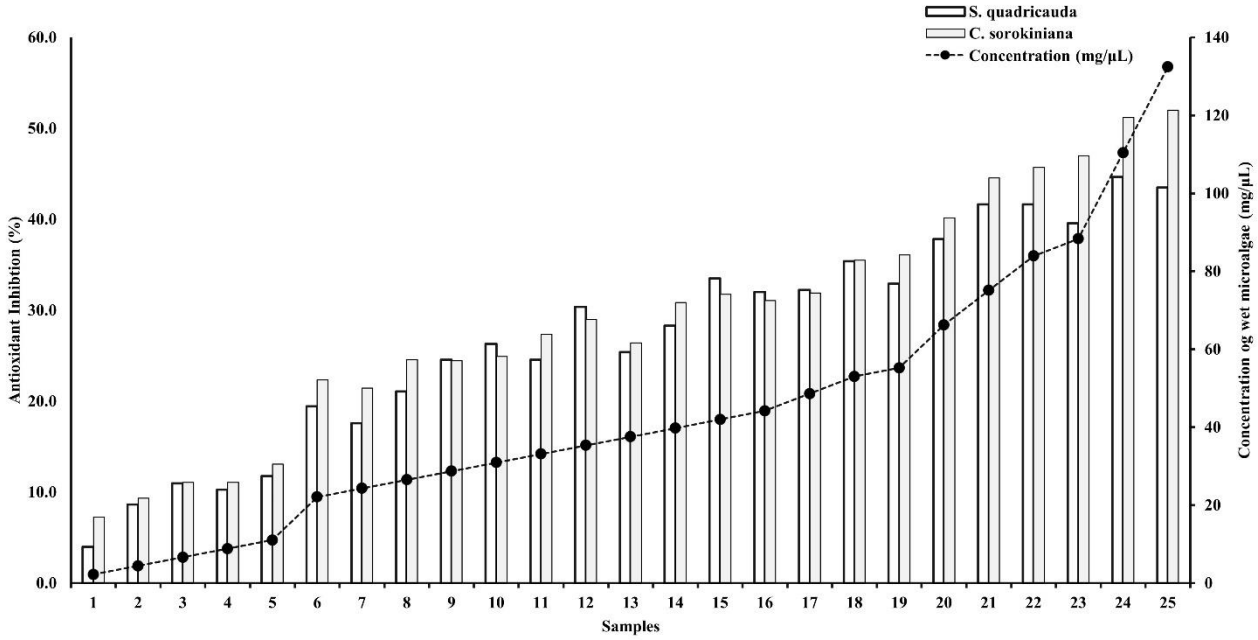

Fig. 6: Percentage of antioxidants between *Chlorella sorokiniana* and *Scenedesmus quadricauda* corresponding to the fresh microalgae.

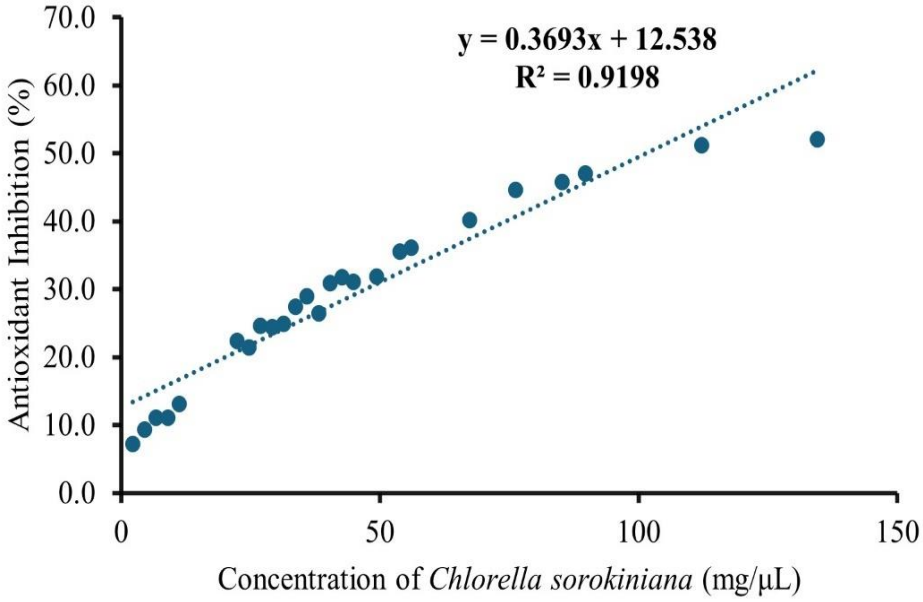

Fig. 7: The standard curve of concentraion of *Chlorella sorokiniana*

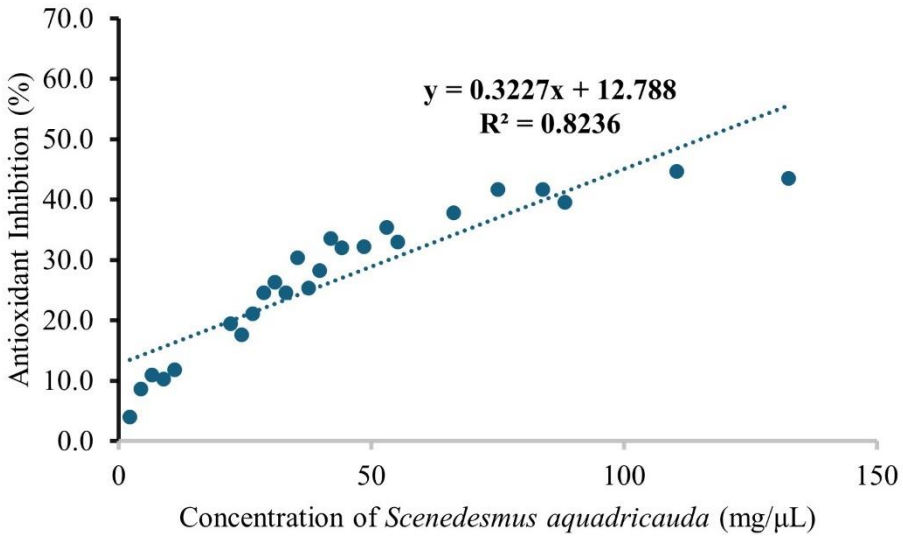

Fig. 8: The standard curve of concentraion of *Scenedesmus quadricauda*

Conclusion

Chlorella sorokiniana exhibited significant concentrations of chlorophylls (a and b), carotenoids and phycocyanin, along with robust antioxidant capacity, achieving inhibition rates ranging from 48% to 52% even at low concentrations. In contrast, *Scenedesmus quadricauda* had a shorter cultivation period for biomass harvesting but did not show significantly higher levels of pigments or antioxidants compared to *C. sorokiniana*. Overall, both microalgae species represent promising natural sources of biologically active compounds with substantial potential for applications.

Acknowledgement

This research was funded by the University of Science, VNU-HCM under the grant number T2023-127**.**

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(Received 31st July 2024, accepted 05th September 2024)