**MANSOURA JOURNAL OF BIOLOGY**
**Official Journal of Faculty of Science, Mansoura University, Egypt**
**E-mail: scimag@mans.edu.eg** **ISSN: 2974-492X**

# Isolation, cultivation of freshwater chlorophyta and screening of the synthesized bioactive compounds and phytohormone

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**Received:23/8/2024**
**Accepted: 28/9/2024**

**Abstract:** Microalgae are aquatic biochemically diverse assemblage of microorganisms found in both fresh and marine systems, are capable of photosynthesis, and grow as individual cells, chains, or colonies. The scientific research community is concentrating on isolating and extracting primary and secondary metabolites produced by these organisms. The current study aimed to isolate freshwater microalgal strains, determine their growth rates and screen their bioactive compounds to assess their suitability for different applications especially biofertilizer. By applying this methodology, freshwater microalga *Chlorella vulgaris, Tetradesmus dimorphus*, and *Kirchneriella aparta* were isolated and cultivated on Bold Basal Media (BBM). Isolated microalgae exhibited different growth rates, recording 0.45, 0.53, and 0.26, respectively. They have also produced some significant bioactive molecules such as lipids, forming 4.87, 4.71, and 6.59 % from their dry weight, respectively, while carbohydrates content per dry weight percentages equal 14.75, 45.80, and 25.46 %, respectively, and proteins 43.73, 31.26, and 26.52 %, respectively. Additionally, pigments have been identified, such as chlorophyll a, which has been recorded at 13.94, 23.42, and 14.96 mg/g D.w., respectively; chlorophyll b, at 17.43 ±0.21, 18.60 ±3.80, and 20.18 ±3.16 mg/g D.w.; and carotenoid, at 12.18, 12.32, and 7.40 mg/g D.w. Antioxidant compounds are also produced, including flavonoids (12.77, 19.99, and 26.88 mg/g F.w., respectively) and phenols (8.76, 18.79, and 16.38 mg/g F.w., respectively). Furthermore, biostimulants, including kinetin, ziaten, gibrillic acid (GA3), indole acetic acid (IAA), and abscisic acid (ABA) have been estimated, with values varying depending on the species of microalgae. C. vulgaris yielded 0.24, 3.28, 308.40, 13.88, and 3.94 µg/100 mL, whereas T. dimorphus recorded 0.936, 4.36, 812.2, 4.14, and 3.32 µg/100 mL, and K. aparta recorded 0.487, 4.74, 182.6, 9.54, and 10.49 µg/100 mL, respectively and this creates opportunities for its application as a biostimulant.. These findings conclude that these microalgae may be potential candidates for many uses in different sectors and open new horizons in the pharmaceutical and cosmetic industries as ingredients in functional foods and biofertilizers.

**keywords**: Chlorella vulgaris; Tetradesmus dimorphus; Kirchneriella aparta; biochemical composition; antioxidant activity; biostimulants.

## 1. Introduction

Microalgae are a prevalent group of rapidly growing single-celled or simple multicellular microphototrophs [1]. They are photosynthetic prokaryotic or eukaryotic microorganisms that include species belonging to several phyla [2, 3]. They live in salty or freshwater habitats and can be found in a variety of severe environments [4].

Chlorophyta is one of the most remarkable phyla [5], it contains many families, e.g., *Chlorellaceae, Scenedesmaceae*, and *Selenastraceae. Chlorellaceae* organisms have significant scientific and practical importance; they have many physical traits, such as small size, comparable morphological structures, and significant phenotypic flexibility, making it challenging to identify between them [6]. *Chlorella vulgaris* is one of the most remarkable microalgae in the Chlorellaceae family. It is a small, spherical cell that ranges in diameter from 2 to 10 µm and shares many structural characteristics with plants [7]. The family *Scenedesmaceae*, which is a part of the class Chlorophyceae, consists of 43 genera, including *Tetradesmus, Pectinodesmus, Hariotina*, and *Scenedesmus* [8]. *Tetradesmus dimorphus* cells are spindle-shaped and typically form a coenobium that resembles a bundle [9]. The family *Selenastraceae* also belongs to the class Chlorophyceae; it has elongated cells that can be either straight or twisted in different ways e.g *Kirchneriella aparta* [10].

Microalgae have recently received a lot of interest [11], as they are a valuable source of a variety of organic compounds, including proteins, carbohydrates, fibers, vitamins, polyunsaturated fatty acids, inorganic substances, trace elements, and pigments. They have been used various industries, which explains why their production mass is rising on a global scale [12]. Being such a plentiful source of important nutrients, microalgae have become significant source of food, *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Dunaliella salina* are examples of green algae (Chlorophycea) that are extensively marketed and utilized, mostly as ingredients in dietary supplements for people and animals [13]. In addition, *Arthrospira platensis*, one of the most nutrient-dense foods known to man, is becoming increasingly well-known as a food supplement [14]. Furthermore, microalgae are now also sold as fish food [15].

The microalgal pigment content, which varies depending on the species, is frequently utilized in cosmetics [13]. Where microalgal proteins, pigments, vitamins, and other ingredients are beneficial to skin care formulations, which is why microalgae extracts are primarily used in face and skin care products (such as anti-aging creams, refreshing care products, emollient, and as an anti-irritant in peelers), in addition to sun protection and hair care products [16]. *Chlorella* and *Arthrospira* are typical species utilized in cosmetics [12]. In addition to microalgal pigments usage as cosmetic ingredients, they also have commercial use as a natural food colouring [17]. Besides carotenoids, which are common pigments in algae [18] and well-known for their significant role in enhancing antioxidant activity in microalgal biomass [19], microalgae also contain chemical substances that have antioxidant properties called phenols [20], which are known as a common class of secondary metabolites employed in a variety of physiological activities in plants [21], it is also found that microalgae and cyanobacteria contain various types of flavonoids, which are the most prevalent among phenolic chemicals [20, 22]. Due to microalgae's greater photosynthetic efficiency, faster growth rate, and capacity to thrive in non-arable areas with fewer contamination risks, microalgae offer several advantages over terrestrial land plants [23].

Isolation is an essential stage in getting a pure culture for the development of bioprocesses. It is the initial phase of choosing potential microalgae for various uses [24]. For the cultivation of microalgae choosing a suitable tank or container is very crucial, and it ranges from simple bioreactors such as Erlenmeyer flasks, bottles, or jars to high-tech photobioreactors such as thin-film flat-plate photobioreactors and raceways for mass cultivation [25]. To provide nutrients for microalgal growth, culture media are commonly made up of prepared media e.g. f/2 medium [26], Bold's basal medium (BBM), and Blue-Green (BG11) in addition to various wastewaters that are typically enhanced with nitrogen and phosphorus compounds [25, 27]. Furthermore, microalgae are a source of natural phytohormones, particularly auxins, cytokinins, and gibberellic acid [28], which are crucial components in plant biostimulants that promote plant growth and productivity [29]. Also, Wang and Komatsu [30] and Rahman, Li [31] suggested that applying phytohormones topically has been shown to increase plants' ability to withstand heavy metals and flooding stresses. Additionally, phytohormones assist with plants' defenses against biotic stress [32]. Consequently, the utilization of algae as biofertilizers has recently received attention due to its effectiveness and provision of environmental and human safety [33].

This current study aimed to isolate fresh chlorophyta species to determine their growth rates and screen their bioactive compounds, such as carbohydrates, lipids, proteins, chlorophylls, and carotenoids, as well as antioxidant assays, e.g., determination of phenolic and flavonoid contents and DPPH radical in addition to phytohormone estimation.

## 2. Materials and methods

### Isolation and culturing of a microalga

Freshwater samples were collected from the Nile River, Mansoura City, Egypt (31° 1' N, 31° 3' E).

*Chlorella vulgaris*, *Tetradesmus dimorphus*, and *Kirchneriella aparta* were isolated and identified molecularly according to [34]. Utilizing BBM, isolation and purification were carried out using streak plating methods according to [35] in the Phycology Laboratory, Faculty of Science, Mansoura University, Egypt.

### Estimation of the algal growth curve

One colony was picked from each pure single-strain petri dish, transferred to 5 mL of BBM, and allowed to grow until its growth became visible. It was then transferred to 100 mL of BBM and allowed to grow once more at 26 ºC and 27 uE /m/s, while being observed daily by optical density measurements at 440 nm until it reached a steady state. This culture was then used as an inoculum to estimate the growth curve. Each alga was cultivated on BBM medium by a ratio equal to 1:10 (20 mL algal inoculum in 200 mL BBM) in triplicates. The growth was monitored daily by measurement of optical density at 440 nm and cell count to calculate the algal growth curve, The following equations were used to compute the growth rates of the algal cultures by Stein-Taylor [36]:

Growth rate; $\mu = \frac{\ell n\,(N / No)}{dt}$

Where No is the initial cell density (cell/mL) and N is the cell density at a given time t.

The doubling per day rate (D/d) was calculated as follows:

Doubling per day; $D/d = \frac{\mu}{\ell n 2}$

### Evaluation of the biochemical composition of the isolated microalgae

Total protein content was estimated based on Lowery method with a slight modification according to Slocombe [37], total carbohydrate content was determined using phenol sulfuric acid method according to Moheimani [38], and total lipid content was estimated by sulfo-phosho vanillin method based on Byreddy [39].

### Phytohormones Analysis of Isolated Microalgae

Fresh frozen samples of the isolated microalgal biomass were gently delivered to the Faculty of Agriculture, Ain Shams University, Egypt, for phytohormone analysis (auxins, IAA, gibberellins, GA3, cytokinin, CK, and abscisic acid, ABA), following extraction by Shindy and Smith [40].

### Determination of chlorophylls and total carotenoid contents

Pigment content was assessed at the end of the investigation according to [41], and the pigment concentration was estimated using the following equations:

Chlorophyll a (Ch. a) = (11.75* O.D662) – (2.350* O.D645)

Chlorophyll b (Ch. b) = (18.61* O.D645) – (3.960 * O.D662)

Carotenoids = ((1000* O.D470) – (2.270* Ch. a) – (81.4* Ch. b))/227

### Antioxidant assay assessment

### Microalgal extracts preparation

Dry biomass (0.1 g) of the tested microalgae was extracted using 15 mL of methanol with continuous shaking for a week at 30 °C. The extract was filtered using Whatman filter paper (No. 1), and the filtrates were collected and kept at 4 °C for further determination of phenolic, flavonoids contents and DPPH radical.

### Determination of Total phenolic content

Total phenolic content (TPC) was determined spectrophotometrically using Folin– Ciocalteu according to McDonald, Prenzler [42]. Different gallic acid concentrations were used to produce the standard curve. The experiment was carried out in triplicate, and the outcome was presented as the total phenolic content in mg of gallic acid per mL of algal extract.

### Determination of Total flavonoid content

Total flavonoid content was determined spectrophotometrically according to Zhishen, Mengcheng [43]. The results were represented as mean mg of quercetin equivalents per mL of algal extract, based on standards made with known quercetin concentrations.

### Determination of antioxidant activity 2,2-diphenyl-1 picrylhydrazyl (DPPH) radical

According to Amarowicz [44], DPPH radical scavenging activity was performed to determine the total antioxidant capacity. The following equation was used to calculate the percentage of DPPH inhibition:

$$\text{Inhibition (\%)} = [(A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{control}}) * 100]$$

Where $A_{\text{Control}}$ represented the absorbance of the DPPH solution without extract at 517 nm, and $A_{\text{Sample}}$ was the absorbance of isolated microalgae extracts.

### Statistical Analysis

All experiments used a randomized complete block design with three experimental replications. One-way ANOVA was used to analyze the data to find the differences between the means of the three studied algae; according to Duncan's multiple range tests by SPSS (version 22.0, 2013, IBM Corp., Armonk, NY, USA) were declared significant at $P \leq 0.05$. The results were presented as means ± standard error (SE). Figures are plotted by Microsoft Excel 365.

## 3. Results

### Microalgal growth and biomass productivity

The growth of isolated microalgae varied considerably among the different species. Maximum optical density was recorded by *Tetradesmus dimorpohus* (1.267 after 6 days of cultivation), followed by *Chlorella vulgaris* (1.226 after 7 days of cultivation), *Kirchneriella aperta* was the least of them in optical density, recording 0.468 (**Figure 1**). However, *C. vulgaris* was greatly exceeded than *T. dimopohus* in cell count, which recorded $726 \times 10^4$ cell/mL in the sixth day, while *C. vulgaris* recorded $2313 \times 10^4$ cell/mL on the same day. Also, *K. aperta* was the least in cell count, recording $248 \times 10^4$ cell/mL (**Figure 2**). Microalgal species exhibited different specific growth rates and growth doublings, *T. dimorphus* recorded the highest specific growth rate and growth doubling with values of 0.53 and 0.76, respectively, followed by *C. vulgaris* (0.45 and 0.65, respectively), while the lowest were recorded for *K. aperta* (0.26 and 0.38, respectively) as scored in **Table (1)**. Moreover, **Figure 3** illustrates the isolates' growth in the mean of biomass production, *T. dimopohus* and *C. vulgaris* maintained the maximum biomass production (0.242 and 0.181 g/L, respectively), while *K. aperta* was estimated as 0.071 g/L.

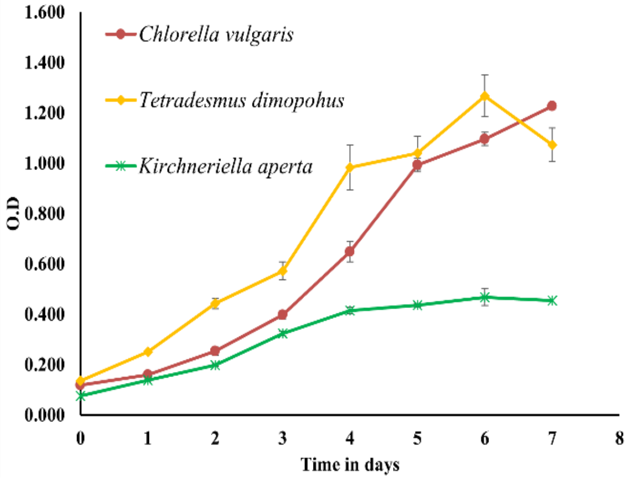


**Figure 1.** Growth curve expressed by optical density for *Chlorella vulgaris*, *Tetradesmus dimorphus*, and *Kirchneriella aperta* cultivated on BBM over time (in days). Data represented as mean ± SE and n=3.

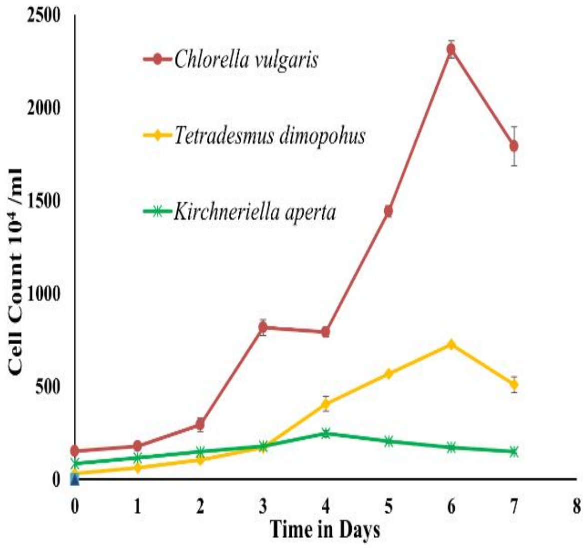


**Figure 2.** Growth curves of *C. vulgaris, T. dimorphus*, and *K.aperta* grown on BBM for 7 days under laboratory-controlled conditions, Data represented as mean ± SE and n=3.

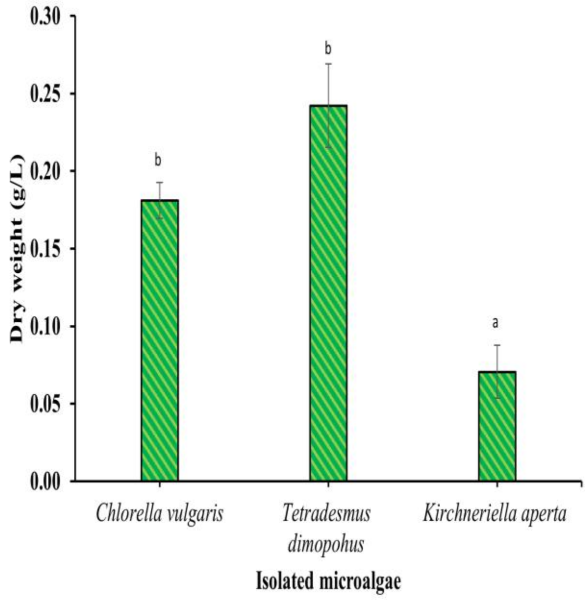


**Figure 3**. Dry weight of *C. vulgaris, T. dimorphus,* and *K. aperta* cultivated on BBM. The results are recorded as Mean of triplicates ± Standard Error (SE). Different superscript letters refer to significant variation with Duncan's test at P ≤ 0.05, where a < b.

**Table 1**: Specific growth rate (μ) and growth doubling per day (D/d) of *C. vulgaris, T. dimorphus*, and *K. aparta* grown on BBM.

| Microalgal species | Growth rate (μ) | Doubling time (D/d) |
|---|---|---|
| ***C. vulgaris*** | $0.45 \pm 0.0165^{b}$ | $0.65 \pm 0.027^{b}$ |
| ***T. dimorphus*** | $0.53 \pm 0.002^{c}$ | $0.76 \pm 0.002^{c}$ |
| ***K. aperta*** | $0.26 \pm 0.017^{a}$ | $0.38 \pm 0.025^{a}$ |

Different superscript letters refer to significant differences (P ≤ 0.05) (Duncan's multiple range test), where a < c.

### 3.2 Pigments content

Figure (4) illustrates that. *T. dimopohus* had the highest chlorophyll a and carotenoid concentrations while coming in second in chlorophyll b concentration, measuring 30.35, 9.38, and 12.87 mg/g D.W., respectively. *Kirchneriella aparta* came in second in both chlorophyll a and chlorophyll b and the lowest in carotenoid, recording 26.44, 16.76, and 2.85 mg/g D.W., respectively., while *C. vulgaris* became second in chlorophyll a, the highest in chlorophyll b, and the lowest in carotenoid, with measurements of 18.52, 17.19, and 8.25 mg/g D.W., respectively.

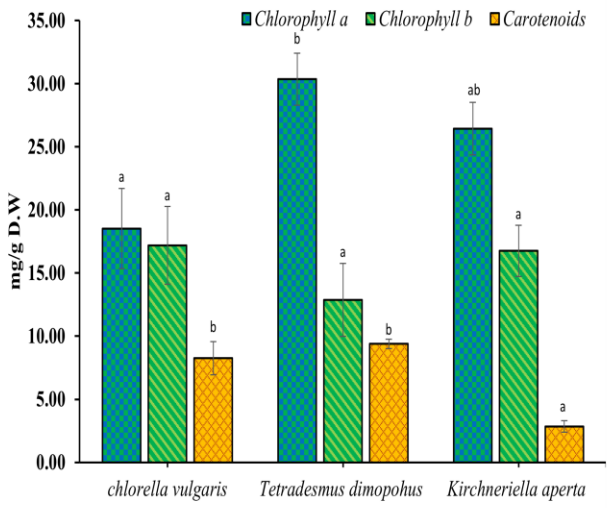


**Figure 4.** Pigments content of *C. vulgaris, T. dimorphus*, and *K.aparta*. Data represented as the mean of triplicates ± SE. Different letters indicate significant differences at (P ≤ 0.05), where a < b.

### 3.3 Biochemical composition of the isolated microalgae

*Kirchneriella aparta* had the highest percentage of total lipid (6.49 %), whereas *T. dimorphus* had the lowest percentage (4.61 %). *Chlorella vulgaris* maintained the highest total protein content (42.73 %) compared with *T. dimorphus* and *K. aperta* (30.26 and 25.52 % respectively). On the other hand, *T. dimorphus* maintained the highest percentage of total carbohydrate content (44.80%) compared to *C. vulgais* and *K. aperta* (13.75 and 21 %, respectively), as illustrated in **Figure (5).**

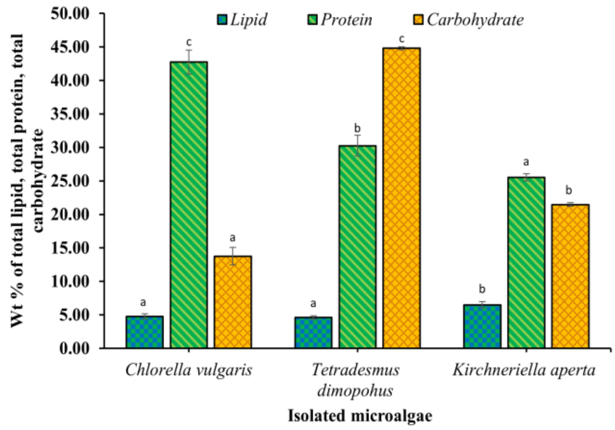


**Figure 5.** Weight percent of the three isolated microalgae's lipid, protein, and carbohydrate content. The results are recorded as mean of triplicates ± SE. Different superscript letters

refer to significant variation with Duncan's test at $P \leq 0.05$, where a < c.

### 3.4 Phytohormones content

Phytohormone content was determined at the end of the growth stationary phase for each isolated microalga. The phytohormone content differed based on species; *C. vulgaris* recorded 13.88 µg/100 mL for IAA, 0.243, 3.28 µg/100 mL for cytokinin (kinetin, zeatin), respectively, 308.4 µg/100 mL for gibberellins ($GA_3$), and 3.94 µg/100 mL for abscisic acid (ABA), while *T. dimopohus* recorded 0.936 and 4.36 µg/100 mL for cytokinin (kinetin, zeatin), respectively, 812.2 µg/100 mL for GA3, 4.14 µg/100 mL for IAA, and 3.32 µg/100 mL for ABA, and *K. aperta* recorded 0.487 and 4.74 µg/100 mL for cytokinin (kinetin, zeatin), respectively, 182.6 µg/100 mL for GA3, 9.54 µg/100 mL for IAA, and 10.49 µg/100 mL for ABA (**Table 2**).

**Table 2.** Phytohormones content (kinetin, zeatin, $GA_3$, IAA, and ABA) of the three isolated microalgae.

| **Microalgae** | **Phytohormones (µg/100 mL)** | | | | |
|---|---|---|---|---|---|
| | **kinetin** | **zeatin** | **GA3** | **IAA** | **ABA** |
| **C. vulgaris** | 0.243 | 3.28 | 308.4 | 13.88 | 3.94 |
| **T. dimopohus** | 0.936 | 4.36 | 812.2 | 4.14 | 3.32 |
| **K. aperta** | 0.487 | 4.74 | 182.6 | 9.54 | 10.49 |

### 3.5 Antioxidants assay

#### 3.5.1 Total phenolics (TP) and total flavonoids (TF) contents

Following the results shown in **Table (3)**, *K. aperta* had higher TP and total flavonoid levels, recording 25.88 and 15.58 mg/g F.W, respectively, followed by *T. dimopohus*, with values of 19.00 and 17.99 ± 2.45 mg/g F.W, while *C. vulgaris* came last, recording 11.77 and 7.76 mg/g F.W, respectively.

**Table 3.** Total phenolic contents (TP), and total flavonoid content (TF) of the three isolated microalgae extracts

| **Microalgal Species** | **Total phenolics content (mg (GAE)/ g D.W)** | **Total flavonoids content (mg (QE)/g D.W)** |
|---|---|---|
| ***C.vulgaris*** | $7.76^{a}$ ±0.83 | $11.77^{a}$ ±1.72 |
| ***T.dimorphus*** | $17.99^{b}$ ±2.45 | $19.00^{ab}$ ±5.39 |
| ***K.aperta*** | $15.58^{b}$ ±0.44 | $25.88^{b}$ ±2.85 |

The results are recorded as mean of triplicates ± SE. Different superscript letters refer to significant variation with Duncan's test at $P \leq 0.05$, where a < b.

#### 3.5.2. DPPH radical inhibition assay of isolated microalgae

The DPPH assay was used to determine the antioxidant potential of the isolated microalgae extracts, as observed in **Figure (6)**. The highest antioxidant capacity of the algal extracts was measured in *T. dimopohus*, which recorded 24.50 %, followed by *C. vulgaris*, with a value of 34.15 %, while *K. aperta* recorded the lowest at 35.65 %.

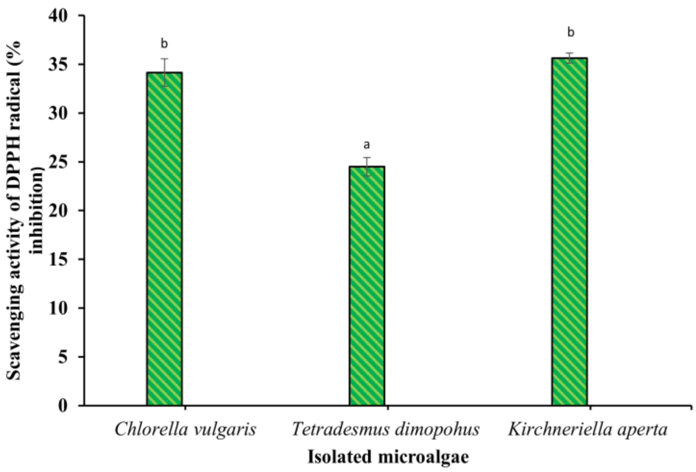


**Figure 6**. Scavenging activity of DPPH radical of the three isolated microalgae extracts. The results are recorded as mean of triplicates ± SE. Different superscript letters refer to significant variation with Duncan's test at $P \leq 0.05$, where a < b.

## 4. Discussion

Microalgae, known as photosynthetic aquatic creatures, have enormous potential as biological factories that may produce various natural products such as proteins, lipids, polysaccharides, carotenoids, phenols, and flavonoids. These physiologically active substances have antioxidant, antiviral, anticancer, and bactericidal properties [45]. In this investigation, three species were isolated from the freshwater: *C. vulgaris*, which was also isolated previously by Gomaa, Refaat [46]; *T. dimopohus*, also isolated by Anh, Van Minh [47]; and *K. aperta*, isolated by Lombardi, Vieira [48]. The growth rate differed substantially across different species; this is also abundantly obvious from Yang and Gao [49] study, which investigated how $CO_2$ concentration affected the rates of growth of *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, *Tetradesmus obliquus*, and other chlorophyta species.

Microalgae have gained a lot of interest as a natural source for pigment manufacturing. Due to their simplicity of cultivation, quick growth rates, and diversity, they naturally create a wide spectrum of colors. They can produce various pigments with different color shades and biological properties. These include phycobiliproteins, carotenoids, xanthophylls, and chlorophylls [50]. These pigments are essential to various industries, including food, medicine, and cosmetics [51]. Microalgae biomass can yield 0.5 to 1.5 % of its dry weight in chlorophylls [52]. According to Jain, Behera [53], the microalgal consortium produced 4.62 μg/mL of Chl a, 4.78 μg/mL of Chl b, and 1.76 μg/mL of carotenoids. According to our results, *C. vulgaris* exceeded *T. dimopohus* in terms of cell count, even though *T. dimopohus* had a higher optical density. Pigment content may assist in explaining this contradiction. *T. dimopohus*, as demonstrated in pigment content results, had the highest concentration of either chlorophyll a or chlorophyll b, which are responsible for absorption at wavelength 440 nm that used to measure *Chlorophyta* growth.

According to our findings, isolated microalgae are a valuable source of components for functional foods since they accumulate lipids, protein, and carbohydrates, as demonstrated by several studies [54, 55]. Furthermore, microalgae are essential sources of antioxidants as they can produce both phenols and flavonoids, which are thought to be the primary sources of plant antioxidant activity [56], in addition to carotenoids which not only harvest light energy but also behave as photoprotective agents against free radicals and harsh environmental conditions [55]. Moreover, the DPPH method used to test antioxidants and their effectiveness, the antioxidant activity estimated in isolated microalgae ranged between 24.50 and 35.65 %; these scavenging values are higher than those reported in similar studies [57, 58].

It has been documented that microalgae generate exogenous and endogenous phytohormones. The most commonly found phytohormones in microalgae were gibberellins, auxins, cytokinins, and abscisic acid [59, 60]. These hormones are chemical compounds created in minimal amounts by plants that trigger or regulate physiological processes in plants, such as growth and development. It was found that factors like light source and intensity, salt stress during growth, and carbon source may affect phytohormone production by microalgae [60]. In this investigation, the phytohormones present in *C. vulgaris*, *T. dimopohus*, and *K. aperta* biomass, particularly auxins, cytokinins, and gibberellins, which can contribute to several plant growth and development aspects when added to the soil or applied by foliar spray [33, 61]. Also, Cruz, da Rosa [62] determined phytohormones in several microalgal species and reported that IAA concentrations ranged from 1.94 to 56.45 nmol/g, whereas those of trans zeatine ranged from 0.06 to 35.52 pmol/g.

## 5. Conclusions

Microalgae are a source of functional chemicals with additional value, which may ultimately be a healthier choice. The results highlighted the ability of *C. vulgaris, T. dimopohus, and K. aperta* to synthesize carbohydrates, proteins, lipids, chlorophylls, carotenoid pigments, and antioxidant compounds, as well as their phytohormone content, so it is crucial to use microalgae as a new natural source of numerous significant bioactive substances to promote global sustainable development since it presents prospects for sustainable development in several sectors. Thus, further studies need to be done on potential microalgal strains and strategies of nutrition and cultivation for enhancing algal growth and yielding more valuable bioactive chemicals to enhance their uses in many applications, in particular, biofertilizers, to improve the surroundings and human safety.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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