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Antioxidant and anticancer potential of successive extracts and column fractions of three microalgae were measured for optimum conditions

Fatma-El Zahraa M. Fadl[1], Sanaa M. Shanab[1], Emad A. Shalaby[2]

[1]Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza, Egypt
[2]Department of Biochemistry, Faculty of Agriculture, Cairo University, Giza, Egypt

Three green microalgal species (*Chlamydomonas reinhardtii, Dictyochloropsis splendida*, and *Muriella sp*.) were cultured under four stress culture conditions (nitrogen concentrations, light intensity, photoperiod, and salinity). The present study aims to evaluate the algal growth parameters, and antioxidant and anticancer efficiencies of the stressed biomasses. Extraction was performed by hexane, ethyl acetate, and methanol and produced 36 extracts (E1-E36). Antioxidant activity of the 36 crude extracts was performed by radical DPPH & non-radical $KMnO_4$. E14 of *D. splendida* cultivated on 3g/L nitrogen was the promising crude extract that exhibited the greatest antioxidant activity by both methods. Fractionation of E14 was done by silica gel column chromatography producing 11 fractions (F1—F11). Greater antioxidant activity of F5 and F8 occurred (74.33 ± 0.58, 37.03 ± 0.25% and 74.66 ± 0.53, 37.86 ± 0.32% respectively against DPPH and KMnO4) compared to antioxidant standards (Ascorbic acid, BHT). High-Performance Liquid Chromatography (HPLC) analyses of E14, F5, and F8 were evaluated and 4, 5, and 6 Phenolic compounds were produced respectively. Anticancer activity of E14, F5, and F8 as well as the mixture of F5+F8 was determined against three cancer cell lines (HepG2, MCF7 & PC3) against MTT assay. The obtained results showed that fractions and a mixture of fractions exhibited greater activity and lower IC50 compared to their crude extract E14 (IC50 ranged from 19.78 to 20.31 against HepG2, 18.77 to 38.28 against MCF-7 and from 19.39 to 21.84 µg/ml against PC3).

Keywords: Microalgae, Abiotic Stresses, Growth Rate, Chemical Constituents, Biological Activities

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Fatma-El Zahraa M. Fadl,
Department of Botany and Microbiology,
Faculty of Science, Cairo University,
Giza, Egypt
Email: fatma.fadl.amin@gmail.com
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INTRODUCTION

Macro- and Microalgae are photosynthetic, pigment-containing organisms. They have different sizes ranging from a few microns to several meters. They inhabit mainly freshwater, marine, and terrestrial ecosystems where water (or even humidity), and light are available. Algae live freely planktonic or benthic in the aquatic system fixed on living or non-living substrates. During the exponential growth phase, algae produce primary metabolites (proteins, lipids, and carbohydrates) which are involved in different algal growth processes. At the end of the growth phase and beginning of the stationary phase, they produce secondary metabolites (phenolic substances, flavonoids, alkaloids, etc.) which are important for algal protection and survival (Ibrahim et al., 2023).

Under adverse environmental or cultural conditions, stressed microalgae affected morphologically, physiologically, and biochemically, can withstand physical or chemical stresses by altering their metabolism towards the production of secondary phytochemicals including antioxidant substances and/or antioxidant enzymes to scavenge the significant increase in free radicals produced under stress conditions (Xing et al., 2022; Kolackova et al., 2023; Pereira et al., 2023; Gharib et al., 2024).

The secondary metabolites or active phytochemicals produced under stresses exhibit different biological activities and are of great value in the medical, pharmaceutical, cosmetics as well as food industries (Flefel et al., 2014; Zhou et al., 2022; Elakabawy et al., 2023).

This study aims to assess the growth parameters and biological activities, antioxidant & anticancer of three green microalgae (*Chlamydomonas reinhardtii*, *Dictyochloropsis splendida and Muriella sp.*) cultivated under different physical and chemical culture conditions (light intensity, photoperiods, salinity and nitrogen concentrations).

MATERIALS AND METHODS

Materials

Microorganisms

Microalgal Strains: Three identified freshwater green microalgal species: *Chlamydomonas reinhadtii, Dictochloropsis splendida, and Muriella sp.* (Identified by Prof. Sanaa Shanab according to Bourelly 1972, Prescott 1978 and 1982) were cultured and maintained on BG11, the light intensity of 40 µmol/m^2/s, photoperiod of 16/8 L/D cycle at 25°C and constant aeration was provided by air passed through the bacterial filter of 0.22µ m for 30 days. These algal species were used and mentioned previously (in the studies performed by Ali, 2020; El-Fayoumy, 2020; El-Fayoumy et al. 2020 & 2021 Shanab et al., 2021 & 2022 and Elakbawy et al., 2023). Light in the incubator was provided by cool white*, fluorescent lamps*. Light

intensity was measured by Lux meter [model Lx-101 Digital Luxmeter (made in Taiwan)] and the obtained lux was transferred to µmol /m^2/ s by the equation:

50 Lux = 1µ E =1 µmol /m^2/ s according to (Stain, 1973)

Cell Lines: Three cancer cell lines used in this investigation are human hepatocellular carcinoma (HepG2), breast cancer (MCF-7), and prostate adenocarcinoma (PC3). They were obtained and propagated in the science way for Scientific Research and Consultation Laboratory, Cairo, Egypt, and acquired from the American type of culture collection (ATCC, Manassas, VA, USA).

Chemicals and Solvents: All chemicals, solvents, and phenolic acids standards (for HPLC analysis) were purchased from Merck Co. and Sigma (Aldrich St. Louis, MO, USA).

Methods

Effect of Biotic Stress Conditions on the Growth of *C. reinhardtii, D. splendida,* and *Muriella* sp.

In all experiments, the green microalgal species: (*C. reinhardtii, D. splendida, and Muriella sp*.) were grown on BG11 medium (Stanier et al., 1971) in 1L glass flasks at 25±1°C with constant aeration by air (filtered through 0.22 µm bacterial filter) for 30 days. The effect of nitrogen (0.75, 1.5, and 3 g/L, using $NaNO_3$ as nitrogen source), salinity (1, 2, and 4 g/L) concentrations, light intensity (20, 40, and 60, µmol/m^2/s) photoperiod (8:16, 12:12, and 16:8h, light/dark cycle) on the growth of the studied microalgal species were determined separately. The growth was monitored every 5 days by optical density (O.D. at 680 nm) and Dry weight (DW, g/L).

Analytical Methods

Determination of Cell Growth

Using Optical Density: The growth of algal cells cultured under each stress factor (in triplicates) was determined spectrophotometrically by measuring O.D. at 680 nm at regular intervals of 5 days during the experimental period (Ali, 2020; El-Fayoumy et al., 2020 & 2021).

Dry Weight: The cell dry weight (D.W.) was measured gravimetrically every 5 days throughout cultivation where 20 mL of culture samples were filtered through pre-weighed filter paper (0.45 µm) and washed with deionized water. The filtered cells were dried at 60°C in the oven until constant weight, cooled in a desiccator, and then weighed.

The D.W. was expressed as g/L. The maximum specific growth rate, μ_{max} (d^{-1}) and doubling time (td) (d), were calculated as reported by Levasseur et al., (1993):

$$\mu_{max} = \frac{1}{t} * ln\frac{x_f}{x_0} \dots\dots\dots\dots (1)$$

$td = \ln 2/\mu_{max}$ .... (2)

Where X_f and X_0 are the concentrations of biomass (g/L) at the end and beginning of a batch run respectively, and t is the duration of the run (day). The biomass productivity (BP) (mg $L^{-1}d^{-1}$) and biomass yield (BY) were calculated as mentioned by Vidyashankar et al., (2015):

$$BP = \frac{(X_f - X_0)}{(T_2 - T_1)} \dots\dots\dots\dots (3)$$

$$BY = (X_f - X_0) \dots\dots\dots\dots (4)$$

Where X_f and X_0 are the concentrations of biomass (g/L) at the end and beginning of a batch run, respectively; and T1 and T2 (day).

The biomass productivity (BP) (mg $L^{-1}d^{-1}$) and biomass yield (BY) were calculated as follows by Vidyashankar et al., (2015):

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$$BY = (X_f - X_0) \dots\dots\dots\dots (4)$$

Where X_f and X_0 are the concentrations of biomass (g/L) at the end and beginning of a batch run, respectively; and T1 and T2 (day) represent the incubation period of an experiment at the initial time (day 0) and the final day of incubation, respectively.

Successive Extract Preparation: Air-dried microalgal biomass (10g) was successively extracted with 50 ml of organic solvents of varying polarity (hexane, ethyl acetate, and methanol). Algal biomass was grinded with the solvent in mortar to crash the cells and facilitate the extract. Extractions were filtrated by filter papers and kept in dark bottles in the refrigerator till use. Solvents were evaporated using a rotary evaporator at 40-45°C. Thirty- six crude extracts (E1-E36) were mixed separately with Tween (20%) to obtain the suitable concentrations, then a test for detection of the antioxidant activity was performed for screening and obtaining the most promising algal crude extract.

Factions	Extraction Solvents Ratio (%)	Abbreviation of Extraction Solvents
F1	100% Petroleum ether	100% PE
F2	80% Petroleum ether:20% Ethyl acetate	80% PE:20% EA
F3	60% Petroleum ether:40% Ethyl acetate	60% PE:40% EA
F4	40% Petroleum ether:60% Ethyl acetate	40% PE:60% EA
F5	20% Petroleum ether:80% Ethyl acetate	20% PE:80% EA
F6	100% Ethyl acetate	100% EA
F7	80% Ethyl acetate:20% Methanol	80% EA:20% MeOH
F8	60% Ethyl acetate:40% Methanol	60% EA:40% MeOH
F9	40% Ethyl acetate:60% Methanol	40% EA:60% MeOH
F10	20% Ethyl acetate:80% Methanol	20% EA:80% MeOH
F11	100% Methanol	100% MeOH

Fractionation of Promising Algal Extract: Fractionation of the greatest antioxidant activity crude extract (E14) was performed by silica gel column chromatography and eluted by a mixture of petroleum ether, ethyl acetate, and methanol as follows: (Eleven fractions, F1-F11, are produced).

Biological Activities of Successive Extracts of C. *reinhardtii*, *D. splendida*, and *Muriella sp.* Biomass Cultured on Optimal Conditions for Biomass Production

Antioxidant Activity: Two methods were used to determine the antioxidant activity of obtained extracts:

DPPH Radical Scavenging Activity: The scavenging effects of extracted samples were determined by the method of Yen & Chen, (1995), where DPPH solution (in methanol) was mixed with extracted sample suspension in Tween 20. The absorbance of all the sample solutions and antioxidant standards (Ascorbic acid (AscA) and Butylated hydroxytoluene (BHT) were measured at 517 nm. The percentage (%) of scavenging activity was calculated as the following:

$$\text{Antioxidant activity } (\%) = \left(\frac{A_c - A_t}{A_c}\right) * 100$$

Where A_t and A_c are the respective absorbance of tested samples and DPPH

$KMnO_4$ as Non-Radical Assay: The scavenging effects of crude extracts were performed according to Gaber et al., (2021). A mixture of $KMnO_4$ solution (in methanol) was added to an equal amount of each extract. The absorbance of all the sample solutions (as natural antioxidant standard) was measured at 514 nm. The percentage (%) of scavenging activity was determined and the equation was described previously.

Anticancer Activity

Cell Lines and Culture Conditions

MTT Cytotoxicity Assay: The cytotoxicity of the promising crude extract E14 and its fractions F5, F 8, F5+ F8 were evaluated against the three cancer cell lines: HepG2, MCF-7, PC3 using MTT assay according to El-Fayoumy et al., 2020. The cytotoxic dose that killed 50% of cancer cells (IC_{50}) was determined and cell viability % was calculated by the following formula.

$$\text{Cell viability } (\%) = \left(1 - \frac{\text{OD of treated cells}}{\text{OD of control cells}}\right) * 100$$

Identification of bioactive compounds separated from promising algal fractions by high-performance liquid chromatography analysis

The bioactive compounds in the promising crude extract and its fractions were determined by HPLC (Agilent 1260, USA) injected by a mixture of standard phenolic acids (such as Quercetin, Naringenin, Caffeic acid, Gallic acid, Cinnamic acid, Kaempeferol, etc.). In detail: The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 0.9 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (82% A); 15-16 min (82% A); and 16-20 (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5 µl for each of the sample solutions. The column temperature was maintained at 40°C.

Statistical Analysis

All experiments are conducted in triplicate and data are expressed as mean **+_** standard deviation. One–way ANOVA was used to determine the significant difference in dependent variables (Snedecor and Cochran 1982).

RESULTS AND DISCUSSION

Effect of Different Abiotic Culture Conditions on Growth Parameters of *C. inhardtii., D. splendida,* and *Muriella* sp.

Nitrogen

Nitrogen is the most important macronutrient for algal growth and metabolism. It is incorporated in the synthesis of different nitrogen-containing compounds such as amino acids, proteins, RNA, DNA,

alkaloids, etc., which have a very pronounced and highly important role in primary and secondary metabolism and algal survival. Abiotic nitrogen stress on the three studied green microalgal species was performed using various concentrations (0.75, 1.5, and 3 g/L). The obtained results (Table 1) revealed that, in the absence of nitrogen (N-starvation), all growth parameters were inhibited compared to those under different nitrogen concentrations, which were gradually stimulated, and the doubling time was decreased with the elevation of nitrogen concentrations, as seen in Table 1. Both *C. reinhardtii* and *D. splendida* respond positively to the elevation of nitrogen concentrations (0–3 g/L), while in the case of *Muriella sp*., the highest concentration (3 g/L) was shown to be inhibitory to all growth parameters.

Also, they went parallel with the results published of Ishika et al., 2017, where the increase in phosphorus and nitrogen concentrations was accomplished by an obvious enhancement of growth parameters but induced a decline in lipid accumulation in the studied green algal species. In addition, N-starvation induced a remarkable enhancement in lipid and carbohydrate contents, together with an obvious decline in growth rate, photosynthetic efficiency, and protein content, as reported by Rehman and Anal, (2019). Moreover, lipids were increased by 1.7 folds in *Chlorococcum sp*. TISTR 858 and the maximum lipid content were produced in *Phaeodactylum tricornatum* under nitrogen deficiency as mentioned by Yodsuwan et al., 2017. Increasing nitrogen concentration from 0.0 to 3.0 g/L led to a remarkable increase in biomass and specific growth rate, in the studied algal species. Our result showed an agreement with those obtained by Shalaby 2017, especially in the case of the enhancement of growth parameters with increased nitrogen concentration.

Moreover, Elakabawy et al., 2023 investigated the biological activities and plant growth regulators produced by some green microalgal, including two of our studied species (*Chlorella vulgaris, Muriella sp., and Dictyochloropsis splendida*) and Cyanobacteria species (*Anabaena oryza, Nostoc linkia, and Nostoc muscorum*) cultured in different wastewater concentrations. Growth rate (containing 1.5 g/L N) was estimated as optical density and dry weight) of *C. vulgaris* cultured on control BG11 medium, recorded high productivity and maximum growth rate at different cultivation periods. The combination of BG11 & Treated Sewage Water (TSW) at different percentages (50, 75, & 100%) led to marked variations in growth rate at variable growth periods which correlated with the ratio of TSW percentage. The maximum growth rate was reached when algal cultivation occurred with combined media (BG11, BG11o + TSW) than on the control one.

In the same context, Shanab et al., (2022) investigated the effect of culture media composition, and nutrient stress on algal biomass of the green microalga *Dictyochloropsis splendida*. The obtained results showed the highest growth parameters when the alga was cultured on BG11 medium enriched with high conc. of N, P, & Mg but low conc. of carbonate. Also, growth parameters and lipid content of *C. vulgaris* and *D. splendida* were significantly affected by nitrogen conc. Where the highest biomass productivity and yield of both algae were obtained on cultivation with 3g/L nitrate ($NaNO_3$) and decreased under nitrogen limitation as recorded by Ali, 2020. These results agreed with those of Feng et al., 2021 on Chlorella zofingiensis, with Rios et al., 2015 on *Desmodesmus sp*., on *Nanochloropsis oculata* (Surendhiran and Mani, 2014), and on *Chlorococcum sp.* TISTR 8583 by Rehman and Anal, 2019.

El-Fayoumy, 2020 and El-Fayoumy et al. 2020 & 2021 studied during PhD work growth (as O.D., & D.Wt.), phytochemical composition, and biological activities of different microalgal (*Chlorella vulgaris, Muriellasp., and Dictyochloropsis splendida*) and cyanobacterial species (*Nostoc linckia, Anabaena oryza, and Nostoc muscorum*) cultured under Nitrogen, Sulfur, Zinc, and Cupper stress conditions.

The obtained results revealed that a higher growth rate occurred of the green algal species than the cyanobacterial ones although all the culture conditions and nutritive media are the same. Moreover, *Chlorella vulgaris* and *Nostoc linckia* were cultured under stress conditions of Nitrogen (0.0, 1.5, 3, 6, & 12 g/L), Sulfur (0.0, 0.07, 0.15, 0.3, & 0.6 g/L) Zinc & Cupper as well as mixture of N+S and Cu+Zn. Concerning Nitrogen stress, the results showed significant enhancement of growth with elevation of N. Conc. during the incubation period, especially at conc. 1.5, & 3 g/L nitrogen. Maximum growth rate was recorded at 3g/l nitrate while the higher concentrations (6, & 12 g/L) induced significant retardation of growth for *N. linckia*.

The obtained results agreed with those published by Roopnarain et al., 2015, who indicated that algal biomass productivity is directly proportional to the nitrogen conc. Also, the results went parallel with the results of Panche et al., 2014 who illustrated that nitrogen stress significantly decreased

photosynthetic efficiency and protein content. Also, Li et al., (2019) demonstrated an apparent reduction in biomass of *Porphyridium purpureum* at low nitrogen concentrations. Zarrinmehr et al., 2020 investigated the effect of different Nitrogen conc. (0.0, 36, 72, 144, and 288 mg/L) on the growth rate and biochemical composition of *Isochrysis galbana*. Cell growth, pigment, and protein content were decreased with diminishing nitrogen concentrations. While, on total nitrogen deprivation, carbohydrates showed the highest production. Also, nitrogen content affects the percentage of fatty acids (SFAs and PUFAs).

Salinity

Salinity stress adversely affects the growth and development of plants and algal cells. Salt-adapted or salt-tolerant cells undergo modified biochemical composition, and reduced chlorophyll and protein contents, while an increase in carbohydrates was achieved. Moreover, salinity stress alters the algal metabolic pathway to induce biologically active compounds. Also, salt stress causes an imbalance in cellular ions, leading to ion toxicity, and osmotic stress induces growth inhibition and significant production of compatible solutes which stabilize the macromolecules and organelles (Petrusa & Winicov, 1997).

Table 2 recorded the effect of NaCl concentrations (1, 2, and 4 g/L) on growth parameters and doubling time of the three green microalgae under investigation (C. *reinhardtii, D. splendida, and Muriella sp*.). The obtained results showed that, in the absence of salt stress (0.0 g/L NaCl), all growth parameters showed optimum growth and minimum doubling times. On the gradual elevation of salt concentrations, all growth parameters of the three tested algal species were inhibited and translated into great retardation at the highest concentration used (4 g/L NaCl), which was accompanied by the longest doubling times.

Shalaby et al., 2010 studied the salt stress affecting *Spirulina platensis*, its growth, and its biological activities (antioxidant and antivirus). Their results recorded a marked decrease in dry weight, chlorophyll content, and certain xanthophylls. While B-carotene production was enhanced, especially at higher salt concentrations. Additionally, Ali, (2020) reported that higher growth parameters were recorded when *C. vulgaris* & *D. splendida* were cultured in BG 11 medium devoid of NaCl. High salinity conc. (salinity stress) induced inhibition of cell growth, altered cellular structure, and increased lipid yield as reported by Zhu et al., 2016. Also, retardation of algal growth and increase in lipid content was obtained in *Dunaliella sp.* under salt stress conditions (Cao et al., 2014).

Also, polar extracts of *S. platensis* exhibited antiviral and antioxidant activities compared to the non-polar ones (pet. ether). *Dunaliella salina* and *D. bardawil* were able to grow in culture media with more than 30% NaCl and accumulate remarkable amounts of carotenoids (8–14% of total D.wt.), as reported by Ye et al., 2008, Pan et al., 2024 investigated the effect of different salinity concentrations on growth, physiological and biochemical algal components of *Euchlorocystis marina*. Results showed that high cell density or growth was obtained at a salinity of 15 ppt. while higher salinity (60 ppt.) recorded both the lowest photosynthetic pigment content and highest lipid and polysaccharide accumulations.

Light Intensity

Light is an important physical factor that affects metabolite production in plants and algae, where a positive correlation was found between increasing light intensity and phenolic contents. Moderate light intensity induced higher anthocyanin accumulation in food plants (such as blueberry, raspberry, and black rice). However, continuous darkness for 10 days showed the lowest pigment content, but when it was replaced by continuous irradiance, the highest pigment production occurred (Larsson et al., 1986 and Zhong et al., 1993). Light intensity can affect and control the biomass and lipid production in algae (Sajjadi et al., 2018).

Stress conditions have been widely used to increase production or induce the synthesis of secondary metabolites *in vitro* plant cell culture or algal culture (DiCosmo and Misawa, 1985). Macroalgae (seaweeds), inhabiting the intertidal zone, were subjected to prolonged exposure to high light intensities and elevated temperatures, which can damage the photosynthetic system. These macroalgae have developed mechanisms to prevent and avoid lethal physiological damage (photooxidation of photosystems) and maintain physiological integrity during emersion, as reported by Quaas et al., 2015.

Also, major defence systems have evolved to protect them against free radicals and other ROS species. Production of antioxidant enzymes is the primary evolved defensive enzyme against excessive ROS damage (Asada, 1999). The highly decreased

underwater light intensity in seas may have led to physiological stress in submerged algae, causing retardation in their growth rates and lower production of carbon-based secondary metabolites such as phenols and sugar, as reported by Shalaby, 2017. The obtained results illustrated in Table 3 revealed that growth parameters were highly stimulated by the increase in light intensity from 20 to 40 µmol/m^2/s. However, exposure to more light intensity (60 µmol/m^2/s) caused a marked inhibition of all growth parameters with an increase in doubling time, especially in the case of both *C. reinhardtii and Muriella sp*.

On the contrary, *D. splendida* behaved differently, and not only did it tolerate the high light intensity (60 µmol/m^2/s), but its growth parameters showed a pronounced and significant increase with a continual reduction in doubling time with concentrations. The greatest biomass and lipid yield of *Chlorella vulgaris* and *D. splendida* were obtained at a light intensity of 40 µmol/m^2/s, as reported by Shanab et al., 2022. These results matched well with our results concerning the three studied green algal species. Moreover, Khoeyi et al., 2012 found that, under low light intensity of 37.5 µmol/m^2/s*, Chlorella vulgaris* exhibited slow growth and low biomass, while the increase in light quantity from 37.5 to 62.5 µmol/m^2/s was associated with an obvious significant increase in growth and biomass, while exposure to more light intensity of 100 µmol/m^2/s induced an inhibition in algal biomass.

Growth parameters and lipid content in *Chlorella vulgaris* & *Dictyochloropsis splendida* were altered at the different light intensity used (20, 40, 60, 100, & 140 µE/m^2/s). Greatest biomass in both algal species were recorded under 40 µE/m^2/s, while maximum lipid content was obtained under 140 µE/m^2/s (Ali, 2020 & Shanab et al., 2022). Low light intensity induced reduction in dry weight while higher ones caused damage to the photosynthetic apparatus as reported by (Singh and Saxena, 2015 & Khoey et al., 2012). Also, (Rusngsomboon, 2012) studied the effect of various light intensities (0.3-538 µE/m^2/s) on the biomass and lipid content of *Botryococcus braunii* KMITL2.

High light intensity induced photoinhibition, while low intensity was insufficient for growth may be due to the self-shading effect as mentioned by Quaas et al. 2015. Esteves et al., 2024 studied the effect of various light intensities (291, 527, & 1107 µmol/m^2/s) on the growth and biochemical composition of *Chlorella vulgaris*. Maximum biomass productivity and higher growth rate under high light intensity. While the extremely used light intensity of 1107µmol/m^2/s enhanced the lipid and carbohydrates accumulations. Haris et al., 2022 isolated and identified 3 algal species (*Chlorella vulgaris, Tetraselmis chuii, and Isochrysis galbana*) morphologically and molecularly by 18S rRNA. The optimum salinity level of the culture medium for *T. chuii* was 40 parts per thousand (ppt.) which induced the highest cell density, protein, and carbohydrate contents as well as high lipid. Concerning *Chlorella vulgaris*, 10 ppt. salinity was the best for inducing the highest cell density, and lipid content, while carbohydrates and protein contents were highly produced at 0.0 ppt. salinity. Variation of salinity affects the growth and biochemical composition of the three indigenously isolated and identified algal species.

Photoperiod (Light/Dark Cycle)

Photoperiod means the duration of the light period (in hours) in the algal culture. Algal species respond differently to photoperiods (Patel et al., 2019). Light/Dark (L/D) cycles play an important role and affect the synthesis of organic compounds (as lipids and carbohydrates) and nutrient metabolism, which in turn are involved in microalgal growth and biomass productivity according to the length of the lighted period of the cycle (Krzeminska et al., 2014 & Takache et al., 2015). Shifting photoperiod (L/D cycles) timescales may induce several interactions associated with cell metabolism, such as photon absorption rate, and photosynthetic apparatuses which therefore affect the overall energy dynamics of microalgae (Wahidin et al., 2013).

The biochemical composition of algae is altered by changing the light-dark cycle. Enhancing the frequencies of light and dark periods can significantly increase the efficiency of the photosynthetic process, biomass production, and vice versa (Singh & Singh, 2015). The growth rate and lipid content of *Nannachloropsis sp*. were enhanced when shifting the L/D cycle from 12/12 to 18/6, as reported by Rai et al., 2015.

Table 1. Kinetics of growth of *C. reinhardtii, D. splendida,* and *Muriella* sp. cultured on different concentrations of nitrogen.

Microalgal species	$NaNO_3$ (g L^{-1})	Biomass productivity (*BP*) (mg $L^{-1}d^{-1}$)	Specific growth rate (μ_{max}) (d^{-1})	Biomass yield (*BY*) (g L^{-1})	Doubling time (td) (d)
C. reinhardtii	0	15.64 ± 0.51^{d}	0.13 ± 0.01^{d}	0.31 ± 0.01^{d}	5.09 ± 0.26^{d}
	0.75	37.81 ± 1.36^{c}	0.21 ± 0.01^{c}	0.76 ± 0.03^{c}	3.34 ± 0.06^{c}
	1.5	56.00 ± 2.90^{b}	0.24 ± 0.01^{b}	1.12 ± 0.06^{b}	2.92 ± 0.06^{b}
	3	67.98 ± 3.46^{a}	0.26 ± 0.01^{a}	1.35 ± 0.07^{a}	2.69 ± 0.12^{a}
D. splendida	0	15.31 ± 0.42^{d}	0.10 ± 0.00^{d}	0.38 ± 0.01^{d}	6.67 ± 0.05^{d}
	0.75	22.78 ± 1.08^{c}	0.11 ± 0.01^{c}	0.57 ± 0.03^{c}	5.84 ± 0.08^{c}
	1.5	40.52 ± 5.99^{b}	0.15 ± 0.01^{b}	1.01 ± 0.15^{b}	4.51 ± 0.11^{b}
	3	53.85 ± 1.58^{a}	0.17 ± 0.01^{a}	1.34 ± 0.04^{a}	4.03 ± 0.19^{a}
Muriella sp.	0	21.20 ± 0.72^{d}	0.11 ± 0.01^{b}	0.64 ± 0.02^{d}	6.58 ± 0.12^{c}
	0.75	28.09 ± 0.35^{c}	0.12 ± 0.00^{b}	0.84 ± 0.01^{c}	5.82 ± 0.04^{b}
	1.5	52.09 ± 0.92^{a}	0.14 ± 0.01^{a}	1.56 ± 0.03^{a}	4.83 ± 0.09^{a}
	3	46.31 ± 1.01^{b}	0.14 ± 0.01^{a}	1.42 ± 0.03^{b}	4.98 ± 0.12^{a}

All cultures were incubated under illumination of 40 µmol m^{-2} s^{-1} and photoperiod (16L: 8D) at 25± 1 °C with constant bubbling of air (filtered through 0.22 µm filter). Different superscript letters within the same column for each microalgal species indicate significant differences at p<0.05. The data are presented as mean ± standard deviation of three replicates.

Table 2. Kinetics of growth of *C. reinhardtii, D. splendida,* and *Muriella* sp. cultured on different concentrations of NaCl.

Microalgal species	NaCl (g L^{-1})	Biomass productivity (*BP*) (mg $L^{-1}d^{-1}$)	Specific growth rate (μ_{max}) (d^{-1})	Biomass yield (*BY*) (g L^{-1})	Doubling time (td) (d)
C. reinhardtii	0	56.00 ± 2.90^{a}	0.24 ± 0.01^{a}	1.12 ± 0.06^{a}	2.93 ± 0.06^{a}
	1	40.67 ± 3.26^{b}	0.22 ± 0.01^{b}	0.81 ± 0.07^{b}	3.12 ± 0.12^{b}
	2	25.50 ± 1.10^{c}	0.19 ± 0.01^{c}	0.51 ± 0.02^{c}	3.67 ± 0.10^{c}
	4	22.50 ± 0.83^{d}	0.18 ± 0.01^{d}	0.45 ± 0.02^{d}	3.71 ± 0.08^{d}
D. splendida	0	36.52 ± 2.49^{a}	0.15 ± 0.03^{a}	0.91 ± 0.06^{a}	4.51 ± 0.11^{a}
	1	31.44 ± 1.11^{b}	0.14 ± 0.01^{b}	0.78 ± 0.03^{b}	4.66 ± 0.17^{a}
	2	21.57 ± 0.64^{c}	0.12 ± 0.01^{c}	0.53 ± 0.02^{c}	5.35 ± 0.27^{b}
	4	21.31 ± 0.47^{c}	0.13 ± 0.01^{c}	0.53 ± 0.01^{c}	5.32 ± 0.25^{b}
Muriella sp.	0	52.09 ± 0.92^{a}	0.14 ± 0.01^{a}	1.53 ± 0.03^{a}	4.83 ± 0.09^{a}
	1	35.87 ± 3.68^{b}	0.14 ± 0.01^{a}	1.08 ± 0.11^{b}	5.09 ± 0.18^{b}
	2	21.98 ± 0.64^{c}	0.12 ± 0.00^{b}	0.65 ± 0.02^{c}	5.79 ± 0.12^{c}
	4	19.31 ± 0.58^{d}	0.12 ± 0.01^{b}	0.58 ± 0.02^{c}	5.86 ± 0.63^{c}

All cultures were incubated under illumination of 40 µmol m^{-2} s^{-1} and photoperiod (16L: 8D) at 25± 1 °C with constant bubbling of air (filtered through 0.22 µm filter). Different superscript letters within the same column for each microalgal species indicate significant differences at p<0.05. The data are presented as mean ± standard deviation of three replicates.

Table 3. Kinetics of growth of *C. reinhardtii, D. splendida,* and *Muriella* sp. cultured under different light intensities.

Microalgal species	light intensity (µmol m^{-2} s^{-1})	Biomass productivity (*BP*) (mg $L^{-1}d^{-1}$)	Specific growth rate (μ_{max}) (d^{-1})	Biomass yield (*BY*) (g L^{-1})	Doubling time (td) (d)
C. reinhardtii	20	34.33 ± 1.42^{b}	0.18 ± 0.01^{b}	0.68 ± 0.03^{b}	3.79 ± 0.16^{b}
	40	56.00 ± 2.90^{a}	0.23 ± 0.01^{a}	1.12 ± 0.06^{a}	2.92 ± 0.06^{a}
	60	25.00 ± 1.63^{c}	0.16 ± 0.02^{c}	0.50 ± 0.03^{c}	4.13 ± 0.42^{c}
D. splendida	20	32.25 ± 1.77^{b}	0.15 ± 0.01^{b}	0.81 ± 0.04^{b}	4.50 ± 0.15^{b}
	40	36.52 ± 2.49^{b}	0.15 ± 0.01^{b}	0.91 ± 0.06^{b}	4.51 ± 0.01^{b}
	60	51.05 ± 2.44^{a}	0.17 ± 0.01^{a}	1.27 ± 0.06^{a}	4.04 ± 0.11^{a}
Muriella sp.	20	37.53 ± 1.62^{b}	0.12 ± 0.01^{c}	1.12 ± 0.05^{b}	5.40 ± 0.14^{b}
	40	52.09 ± 0.92^{a}	0.14 ± 0.01^{a}	1.56 ± 0.03^{a}	4.83 ± 0.09^{a}
	60	28.75 ± 0.58^{c}	0.11 ± 0.00^{b}	0.86 ± 0.02^{c}	5.85 ± 0.05^{b}

All cultures were incubated under different light intensities and photoperiod (16L: 8D) at 25± 1 °C with constant bubbling of air (filtered through 0.22 µm filter). Different superscript letters within the same column for each microalgal species indicate significant differences at p<0.05. The data are presented as mean ± standard deviation of three replicates.

To stimulate the microalgal biomass productivity, the light intensity must be increased. The light/dark cycle and light wavelength should be adjusted to obtain an optimal balance between photo protection and photosynthesis (Tang et al., 2011). The green microalgal species *C. reinhardtii, D.* splendida*, and Muriella sp*. were cultivated on BG 11 medium at 25°C using light and dark cycles of 8:16, 12:12, and 16:8 of isoquantic light intensities during the light duration of the L/D cycle (the same light quantity was received in the 8, 12 or 16 hour light periods).

The results illustrated in Table 4 show that, under the shortest L/D cycle of 8–16, all the growth parameters tested showed the lowest results compared to those obtained by the longer photoperiod cycles (12:12 and 16:8). This means that the elongation of the light period led to a marked enhancement of growth parameters, while at the same time causing a decrease in doubling time. The increase in all parameters is largely proportional to the length of the lighted hours of the L/D cycle, in the order 16:8> 12:12> 8:16. The obtained results in this investigation showed an agreement with the results reported by (Shah et al., 2014), who mentioned that the greatest biomass and lipid production of *Chlorela sp*. occurred when exposed to 24 hours of light (24:0 L/D cycle).

Also, Krzeminska et al., 2014 published comparable results, to ours, on *Chlorella minutissma* and other algal species. Moreover, Kumar et al., 2019 and Singh & Singh 2015 published their results on *Scenedesmus dimorphus, Pavolva lutheri, and Scendesmus obliquus,* which went parallel with our results. In addition, maximum growth and lipid content produced in *Picochlorum maculatum* occurred at 18:6 L/D cycle and 150 µmol/m^2/s. Chokshi et al., 2017, Gauthier 2020, and Ali 2020 reported that the increased light period from 8 to 24 with the same isoquantic light intensity showed an important increase in growth parameters and lipid content (by 2-2.5 times) of both green microalgae used in his study, *Chlorella vulgaris and D. splendida,* and the lowest growth rate, biomass, and lipid content were recorded at the photoperiod 8:16 L/D cycle.

Antioxidant Activity (%) of Successive Extracts of Different Algal Biomass Using DPPH and $KMnO_4$ Methods

Extraction of the algal biomass produced under different stress conditions (Nitrogen conc., light intensity, photoperiod, and salinity) for the three green algal species used in this study. Extraction was performed successively using the organic solvents: hexane, ethyl acetate, and methanol for the promising alga and conditions. Table 5 records the algal species, the stress conditions, the organic solvents used for extraction, and the extraction codes (E) for the treatments. In the case of *C. reinhardtii*, the promising N-concentration was 3 g/L, which induced maximum growth parameters and had the codes E1 (for hexane), E2 (for ethyl acetate), and E3 (for methanol extract). Under salinity stress, maximum growth of *C. reinherdtii* occurred at 0.0 g/L NaCl concentration and has the extraction codes E4, E5, and E6 with hexane, ethyl acetate, and methanol, respectively. Light intensity (20, 40, and 60 µE/m^2/s) showed that 40 µE/m^2/s was the optimum light intensity, which induced the greatest growth parameters compared to those of 20 and 60 µE/m^2/s. So, the extraction codes for the light intensity stress were E7, E8, and E9 for the solvents used in extractions, respectively.

In the case of photoperiod, the 16:8 L/D cycle was the optimum for growth parameters and had the extraction codes E10, E11, and E12. This means that for each algal species, four promising culture conditions and extractions were performed by three organic solvents of different polarities, producing 12 extracts. So, for *C. reinhardtii* extraction codes from E1 to E12, for *D. splendida* extraction codes from E13 to E24, and *Muriella sp.* extraction codes from E25 to E36, as illustrated in Table 5. Antioxidant activity was carried out using DPPH and $KMnO_4$ for the selected concentration of each stress factor (Nitrogen. conc., Salinity, Light intensity, and Photoperiod) for each of the studied algal species.

Nitrogen

Figure 1 recorded the antioxidant activity of the nitrogen concentration extracts for *C. reinhardtii, D. splendida, and Muriella sp*. using both the DPPH and $KMnO_4$ methods at 100 µg/mL of the N. conc*. C. reinhardtii* showed that the extract of algal biomass grown under N. conc. 3 g/L was the most promising for the growth parameters tested. The radical DPPH showed significantly similar antioxidant activity in the case of hexane (64.66±1.52%) and ethyl acetate (64.33±1.15%) extracts, while methanol extract showed significantly lower activity (46.00±1.00%) compared to those produced by the non-polar and partially polar solvents. Concerning *D. splendida*, the optimum N. concentration of 3 g/L was extracted by

Table 4. Kinetics of growth of *C. reinhardtii, D. splendida,* and *Muriella* sp. cultured under different photoperiods.

Microalgal species	Photoperiod (Light: Dark) (L:D)	Biomass productivity (*BP*) (mg $L^{-1}d^{-1}$)	Specific growth rate (μ_{max}) (d^{-1})	Biomass yield (*BY*) (g L^{-1})	Doubling time (td) (d)
C. reinhardtii	8:16	38.25 ± 2.36^{b}	0.22 ± 0.01^{b}	0.81 ± 0.05^{c}	3.03 ± 0.12^{c}
	12:12	54.25 ± 5.45^{a}	0.24 ± 0.01^{a}	0.97 ± 0.11^{b}	2.93 ± 0.09^{a}
	16:8	54.25 ± 2.90^{a}	0.24 ± 0.01^{a}	1.12 ± 0.06^{a}	2.92 ± 0.06^{a}
D. splendida	8:16	24.38 ± 2.02^{b}	0.12 ± 0.01^{b}	0.61 ± 0.05^{b}	5.42 ± 0.10^{b}
	12:12	27.45 ± 0.88^{b}	0.13 ± 0.01^{b}	0.69 ± 0.02^{b}	5.15 ± 0.08^{b}
	16:8	36.52 ± 2.49^{a}	0.15 ± 0.01^{a}	0.91 ± 0.06^{a}	4.50 ± 0.10^{a}
Muriella sp.	8:16	23.98 ± 1.68^{c}	0.11 ± 0.00^{c}	0.75 ± 0.05^{c}	5.91 ± 0.09^{c}
	12:12	40.87 ± 1.64^{b}	0.13 ± 0.01^{b}	1.28 ± 0.05^{b}	5.21 ± 0.18^{b}
	16:8	52.09 ± 0.92^{a}	0.14 ± 0.00^{a}	1.58 ± 0.03^{a}	4.82 ± 0.09^{a}

All cultures were incubated under different photo periods at 25± 1 °C with constant bubbling of air (filtered through 0.22 µm filter). Different superscript letters within the same column for each microalgal species indicate significant differences at $p<0.05$. The data are presented as mean ± standard deviation of three replicates.

Table 5. Promising cultural conditions of C. *reinhardtii*, *D. splendida,* and *Muriella sp.* biomass cultivated under different abiotic stress conditions for biomass production (with extract codes for their successive extracts).

Microalgal species	Conditions	Extracts	Extract code
C. reinhardtii	3 g L^{-1} $NaNO_3$	Hexane	E1
		Ethyl acetate	E2
		Methanol	E3
	0 g L^{-1} NaCl	Hexane	E4
		Ethyl acetate	E5
		Methanol	E6
	40 µmol m^{-2} s^{-1}	Hexane	E7
		Ethyl acetate	E8
		Methanol	E9
	16 h:8 h (L:D) Light: Dark cycle	Hexane	E10
		Ethyl acetate	E11
		Methanol	E12
D. splendida	3 g L^{-1} $NaNO_3$	Hexane	E13
		Ethyl acetate	E14
		Methanol	E15
	0 g L^{-1} NaCl	Hexane	E16
		Ethyl acetate	E17
		Methanol	E18
	60 µmol m^{-2} s^{-1}	Hexane	E19
		Ethyl acetate	E20
		Methanol	E21
	16 h:8 h (L:D) Light: Dark cycle	Hexane	E22
		Ethyl acetate	E23
		Methanol	E24
Muriella sp.	1.5 g L^{-1} $NaNO_3$	Hexane	E25
		Ethyl acetate	E26
		Methanol	E27
	0 g L^{-1} NaCl	Hexane	E28
		Ethyl acetate	E29
		Methanol	E30
	40 µmol m^{-2} s^{-1}	Hexane	E31
		Ethyl acetate	E32
		Methanol	E33
	16 h:8 h (L:D) Light: Dark cycle	Hexane	E34
		Ethyl acetate	E35
		Methanol	E36

the three organic solvents and tested for their antioxidant activity by DPPH and $KMnO_4$.

Moreover, the obtained results showed that the ethyl acetate extract of *D. splendida* exhibited the greatest antioxidant activity with DPPH and $KMnO_4$ (85.00±1.00 and 39.43 ±0.51%, respectively) compared to those of methanol (69.00 ±1.00%) and hexane (61.33 ± 0.57%). In *Muriella sp*., the optimum N concentration was 1.5 g/L, and the antioxidant activity reached 60.33 ± 1.52% in ethyl acetate extract, followed in descending order by hexane extract (53.00 ± 1.00%), and the lowest activity was recorded by methanol extract (48.66 ± 0.57%) for DPPH. While $KMnO_4$ showed a similar trend but with lower activity. As shown in Figure 1, results noticed that the most promising extract was that of *D. splendida* grown on 3 g/L nitrogen concentration and extracted by 100% ethyl acetate (exhibited 85.00 ± 1.00%, 39.43 ± 0.51% antioxidant activity with DPPH and $KMnO_4$, respectively).

Salinity

The obtained results in Figure 2 show the promising NaCl concentration in each algal species extracted by the three organic solvents, and the antioxidant efficiency of each was tested using the radical DPPH and the non-radical $KMnO_4$ assays. For all three green algal species used in this study, 0% NaCl was the optimum salinity. Antioxidant activity by DPPH and $KMnO_4$ for *C. reinherdtii* was significantly different, ranging from 70.00 ± 2.00 and 24.83 ± 0.28%, respectively, for hexane extract. The activity decreased with elevation of solvent polarity to 66.67 ± 1.15, 20.03 ± 0.15% in ethyl acetate, and 42.67 ± 2.30, 12.16 ± 0.76% in methanol extract for both antioxidant methods. Concerning *D. splendida*, the activity showed an increment with polarity, it ranged 43.00+1.00, 14.80 ± 0.55% in hexane for DPPH & $KMnO_4$ and in ethyl acetate 50.67 ± 0.57, 17.90 ± 0.31% while in methanol the activity reached 54.67 ± 0.57, 16.20 ± 0.72% respectively. For *Muriella* sp the antioxidant activity decreased with an elevation of polarity. Hexane extraction showed 56.66 ± 0.56 and 19.33 ± 0.64%, while for ethyl acetate, the activity decreased to 53.67 ± 0.57 and 18.10+0.36%, and for methanol, the activity continued its decrease to 43.33 ± 2.30 and 13.13 ± 0.32%.

Light Intensity

Concerning the effect of light intensity 20, 40, & 60 µmol/m2/s, the promising light intensity for both *C. reinherdtii* and *Muriella sp*. was 40 µmol/m2/s, while *D. splendida* preferred the higher light intensity of 60 µmol/m2/s. The antioxidant activity of DPPH and $KMnO_4$ was carried out for the algal species cultured at their optimal light intensity and extracted by hexane, ethyl acetate, and methanol. The obtained data recorded in Figure 3, the antioxidant activity by DPPH, $KMnO_4$ showed significantly different activity for each extract. Hexane extract showed activity ranging from 44.00-1.00 and 27.34-0.40% for DPPH and $KMnO_4$, respectively. While ethyl acetate showed 52.66 ± 2.15, 16.13 ± 0.51%, and methanol extract exhibited 50.67 ± 2.08, 22.13 ± 0.15% antioxidant activity for *C. reinherdti* with DPPH and $KMnO_4$ (Figure 3A).

In the case of *D. splendida*, which prefers a higher light intensity of 60 µE/m2/s, and its promising culture was extracted by hexane (55.32 ± 0.58, 14.10 ± 0.36%), ethyl acetate showed 40.00 ± 1.00, 22.96 ± 0.060% antioxidant activity for both reagents, and methanol extract showed relatively higher activity of 60.33 ± 1.55, 13.06 ± 0.20% by DPPH and $KMnO_4$ (Figure 3B). Concerning *Muriella sp.,* its extracts showed relatively lower antioxidant activity than those of *D. splendida and C. reinherdtii*. Hexane extract showed 39.00 ± 1.00, 15.00 ± 1.00%, and with ethyl acetate extract, the activity increased to 42.32 ± 0.56 and 14.13 ± 0.32%, while methanol showed a relative increase in antioxidant activity to 50.67 ± 0.15 and 18.10 ± 0.17% with DPPH and $KMnO_4$ reagents, respectively (Figure 3C).

Photoperiod

Figure 4 recorded the promising photoperiod regime for each algal species and the extraction of their cultures by hexane, ethyl acetate, and methanol. The optimal photoperiod for the three green algal species was 16:8 (L/D cycle), but their antioxidant activity showed significantly different data with the three organic solvents used. The hexane extract of *C. reinhardtii* showed 57.33 ± 2.52% with DPPH and 12.16 ± 0.20% with $KMnO_4$. The antioxidant activity of ethyl acetate decreased to 48.32 ± 5.13% and 28.00 ± 0.40 for DPPH and $KMnO_4$, respectively. In the case of methanol, the activity reached 63.67 ± 1.51% and 14.90±0.26 for the radical and non-radical methods. Concerning *D. splendida*, hexane extract exhibited 69.67 ± 0.50% activity with DPPH and 24.90 ± 0.36% with $KMnO_4$, while for ethyl acetate and methanol, the activity decreased to 59.33 ± 0.58% and 21.16 ± 0.15% respectively, for DPPH and $KMNO_4$.

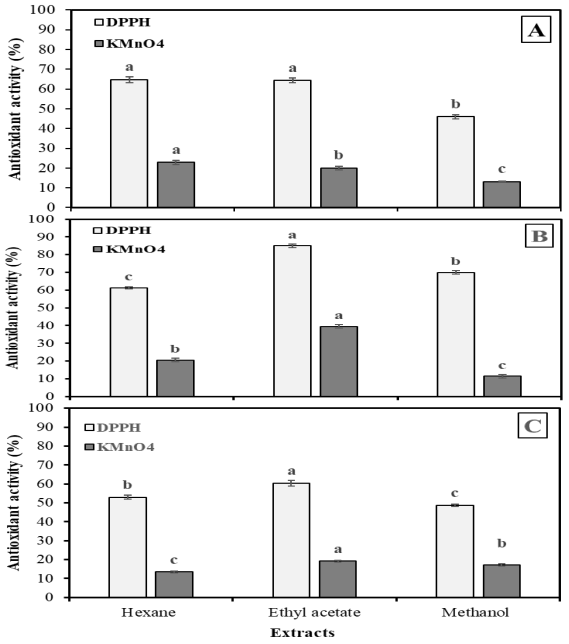


Figure 1. Antioxidant activity (%) of successive extracts of (A) *C. reinhardtii,* (B) *D. splendida,* and (C) *Muriella* sp. a cultured on optimal nitrogen concentrations (3, 3, 1.5 g L^{-1} $NaNO_3$, respectively) based on DPPH and $KMnO_4$ methods at 100 µg mL^{-1}. Different small letters on the same bars indicate significant differences (p<0.05). Error bars represent ± standard deviation of three replicates.

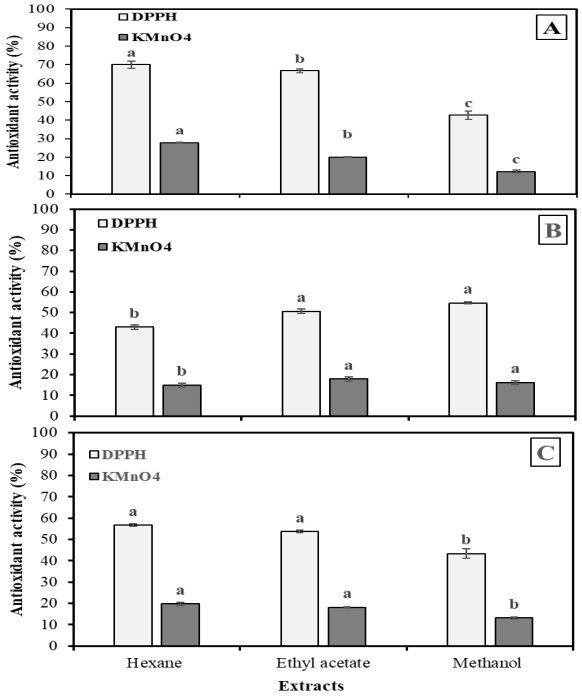


Figure 2. Antioxidant activity (%) of successive extracts of (A) *C. reinhardtii,* (B) *D. splendida,* and (C) *Muriella* sp. biomass cultured on optimal NaCl concentration (0 g L^{-1} NaCl) based on DPPH and $KMnO_4$ methods at 100 µg mL^{-1}. Different small letters on the same bars indicate significant differences (p<0.05). Error bars represent ± standard deviation of three replicates.

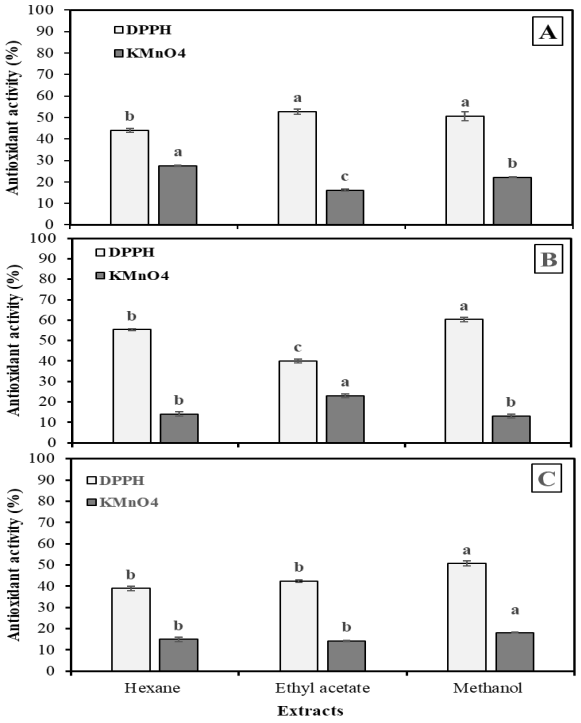


Figure 3. Antioxidant activity (%) of successive extracts of (A) *C. reinhardtii,* (B) *D. splendida* and (C) *Muriella* sp. biomass cultured on optimal light intensities (40, 60, 40 µmol m^{-2} $s^{-1,}$ respectively) based on DPPH and $KMnO_4$ methods at 100 µg mL^{-1}. Different small letters on the same bars indicate significant differences (p<0.05). Error bars represent ± standard deviation of three replicates.

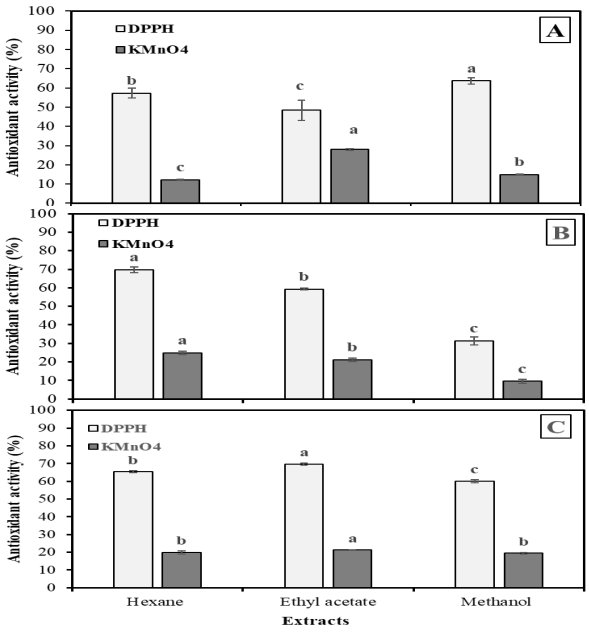


Figure 4. Antioxidant activity (%) of successive extracts of (A) *C. reinhardtii,* (B) *D. splendida,* and (C) *Muriella* sp. cultured on optimal photoperiods (16L:8D) based on DPPH and $KMnO_4$ methods at 100 µg mL^{-1}. Different small letters on the same bars indicate significant differences (p<0.05). Error bars represent ± standard deviation of three replicates.

In the case of methanol extract, the activity continued to decrease to reach 31.31 ± 2.08 and 9.56 ± 0.51 percent for both methods.

Concerning *Muriella sp*., hexane extract showed 65%, ethyl acetate 69%, and methanol 60% for DPPH, while for $KMnO_4$, it showed 19, 21, and 19% for the three extracts, respectively. The antioxidant activity measured in this study by the radical DPPH and the non-radical $KMnO_4$ showed variable results concerning not only the type of algal species used but also the organic solvents and the investigated stress culture conditions (nitrogen concentration, salinity concentration, light intensity, and photoperiod or L/D cycles). Changing the composition of nutrient growth media can induce the production of ROS within the cell. So, correspondingly, an antioxidant response has rapidly occurred to alleviate the imbalance and scavenge the free radicals (Gaber et al., 2021). Also, Shalaby et al., (2023) found that exposing microalgae to abiotic stress factor(s) during cultivation can enhance the accumulation of valuable metabolites within their cells, including carotenoids, antioxidants, and vitamins. These variations in the obtained results in the case of DPPH and $KMnO_4$ may be because the radical DPPH is an unstable compound and reacts potentially faster with different antioxidants that act as hydrogen donors. While the non-radical $KMnO_4$ is more stable and needs more time to react with antioxidant substances by oxidizing double bonds in the reducing agent, as mentioned by Cohen, (1997 & Polewski et al., (2002). The antioxidant activity of the active compounds in different algal extracts may be correlated with the presence of hydroxyl groups and unsaturated bonds in these phytochemicals, which show a high scavenging ability for free radicals, consequently preventing the oxidation processes from occurring, as published by Mustafa 2024). To select the promising extract from the three algal species under investigation, the type of stress condition (Nitrogen conc., light intensity, Salinity, and Photoperiod), and the organic solvent used (hexane, ethyl acetate, and methanol), which exhibited the greatest antioxidant activity (by DPPH and $KMnO_4$), we used statistical method illustrated in the next paragraph (3.3).

Tukey Pairwise Comparisons Based on One-way ANOVA of Antioxidant Activity (%) Promising Extracts Using the DPPH Method

Using a Tukey pairwise comparison based on a one-way ANOVA of antioxidant activity, all types of extracts E1–E36 and their antioxidant activity by DPPH and $KMnO_4$ were indicated. From these results, we found that E 14 (N. conc. 3 g/L) extracted by 100% ethyl acetate showed the greatest antioxidant activity of 85.33% by DPPH reagent, similarly E 14 recorded the greatest antioxidant activity of 84.33% by the non-radical reagent $KMnO_4$. So, the promising crude extract was E 14, which showed pronounced antioxidant activity and belonged to *D. splendida* grown under N. conc. of 3 g/L and extracted with 100% ethyl acetate.

Antioxidant Activity (%) of Fractions of *D. splendida* Ethyl Acetate Extract against DPPH and $KMnO_4$

Fractionation was carried out by column chromatgraphy packed by Silica gel for column, and eluted by petroleum ether, ethyl acetate, and methanol in the ratios indicated in Table 6. Eleven fractions (F1-F11) were obtained from the fractionation of the promising crude extract E14. Antioxidant activity was carried out for the 11 fractions by both the radical (DPPH) and the non-radical ($KMnO_4$) to select the most promising fraction(s) that exhibited the greatest antioxidant activity by both reagents. From the obtained results in Table 6, the fractions from F1 to F11 showed antioxidant activity ranging from 20.66 to 74.66% with the DPPH reagent and from 8.88 to 37.86% with $KMnO_4$. The fractions No. F5 and F8 from all eleven fractions exhibited the greatest antioxidant activity by both DPPH (74.33 & 74.66%) and $KMnO_4$ (37.03 & 37.86%) for F5 and F8, respectively, compared to the natural antioxidant standard ascorbic acid (90.56% and 53.73%) for DPPH and $KMnO_4$. F5 was the fraction of the crude ethyl acetate extract (E14) eluted by 20% pet. ether: 80% ethyl acetate, while F8 was eluted by 60% ethyl acetate: 40% methanol (Table 6).

Anticancer Activity of Concentration of *D. splendida* Ethyl Acetate Extract and its Fractions

The crude ethyl acetate extract of *D. splendida* cultured on 3 g of nitrogen (E14) as well as its fractions (F5 and F8) and mixture of F5 and F8 were investigated for their anticancer activity (cell viability, toxicity, and IC_{50}) against three important cancer cell lines (HepG2, MCF 7, and PC3) and their IC_{50}. The obtained results are recorded in Figure 5, for HepG2, MCF-7, and PC3, respectively. Breast cancer cell lines (MCF-7) and prostate cancer cell lines (PC3) were most commonly affecting women & men in Egypt, while HepG2 affects both populations.

Using extract and fraction concentrations (3.120/ 6.25/ 12.5/ 25/ 50 & 100 µg/ml) against the different

cancer cell lines tested, determine the cell viability, toxicity, and IC_{50} for each extract or fraction concentration and the minimum inhibitory concentration that affects 50% of the cancer cell line, known as IC_{50}. Generally, in all cancer cell lines (HepG2, MCF-7, and PC3), at the lowest extract concentration (3.120 µg/mL), cancer cell viability was nearly unaffected, viability was mostly 99%, and toxicity was near zero (0.24%). With a gradual increase in extract or fraction concentrations, cell viability decreased, and toxicity gradually increased to reach its maximum at the highest extract (or fraction) concentration (100 µg/mL). These were caused by the crude ethyl acetate extract (E14) and fractions 5, 8, or a mixture of F5 and F8.

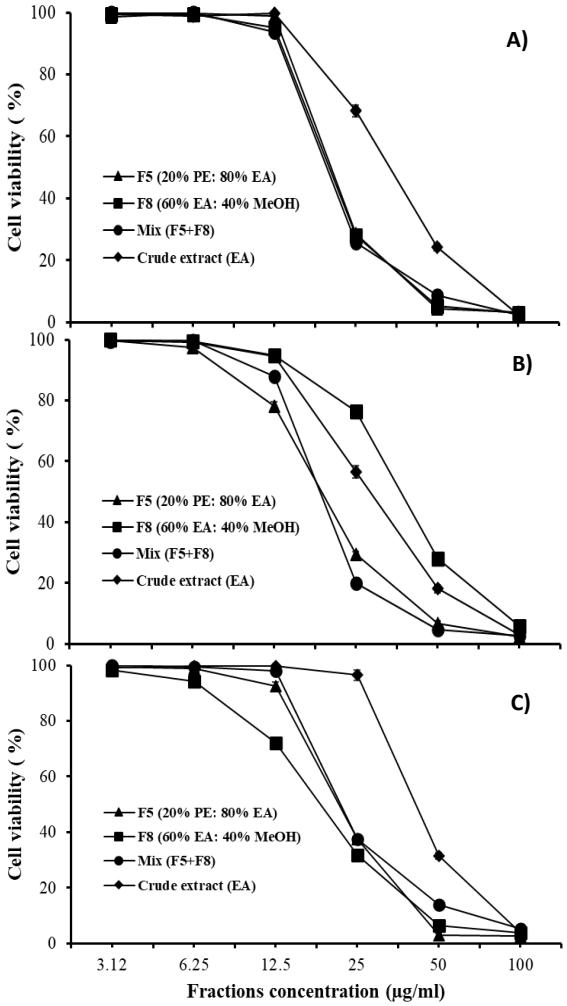


Figure 5. Anticancer activity of different concentrations of *D. splendida* ethyl acetate extract and its fractions on (A) HepG2, (B) Mcf7, (C) Pc3 cancer cell lines.

IC_{50} for HepG2 (Figure 5A) shows that the fractions 5 and 8 showed to be more effective than their crude extract (E14), from which they were fractionated (by IC_{50} of 20.31 and 20.13 µg/ml), and their mixture F5 and F8 was more effective against HepG2, showing less conc. (by IC_{50} of 19.78 µg/ml), which are all more efficient than the crude ethyl acetate (E14) (IC_{50} = 36.26 µg/ml). This means that fractionation led the resulting fractions to be more efficient against the HepG2, and this may be due to certain antagonism or peroxidation occurring between the constituents of the promising crude extract (E14).

Figure 5B shows the effect of extract E14 and its fractions F5 and F8 as well as a mixture of F5 and F8 on MCF-7. The same trend was obvious concerning the extract conc. (or fraction), cell viability and cell toxicity, which were inversely and proportionally correlated with concentration, as in the case of HepG 2. The IC_{50} of the fraction F5 (19.59 µg/ml) as well as that of F5 + F8 (18.77 µg/ml) were about half of the conc. of E14 and F8 (32.29 & 38.28 µg/ml). The IC50s of F5 and F5+F8 have significantly similar efficiency against MCF-7, with IC_{50}s of 19.59 and 18.77 µg/mL, respectively. Concerning prostate cancer cell line in men (PC3), the IC_{50} of crude extract (E14) showed 41.41 µg/ml, while on fractionation, the obtained fractions F5 and F8 showed more pronounced and efficient effect on PC3 with half the concentration in E14 to acquire the same result.

F5 and F8 show lower concentrations of 21.79 and 19.39 µg/mL as their IC_{50}, and even their mixture (21.84 µg/mL) was not significantly different from both fractions separately. This means that fractionation of the promising crude extract (E14) renders its fractions F5 and F8 more effective against PC3, as seen in Figure 5. The obtained results recorded the anticancer activity and toxicity of E14, F5, F8, and F5+F8 on HepG2, MCF-7, and PC3 cancer cell lines.

The promising crude extract E14 (dissolved in 100% ethyl acetate) exhibited 85 and 84% antioxidant activity by DPPH and $KMnO_4$ among the 36 extracts of the studied algal species using the three organic solvents used. Fractionation of E14 by silica gel column chromatography and an elution system of petroleum ether, ethyl acetate, and methanol in certain ratios produced eleven fractions (F1–F11).

Table 6. Antioxidant activity (%) of fractions of *D. splendida* ethyl acetate extract against DPPH and $KMnO_4$ at 100 µg mL^{-1}

Microalgal species	Factions	Extraction solvents ratio (%)	Abbreviation of extraction solvents	Antioxidant activity (%)	
				DPPH method	$KMnO_4$ method
D. splendida	F1	100% Petroleum ether	100 % PE	43.66 ± 1.15^{g}	22.10 ± 0.45^{f}
	F2	80% Petroleum ether: 20% Ethyl acetate	80% PE: 20 % EA	20.67 ± 0.57^{h}	8.88 ± 0.28^{j}
	F3	60% Petroleum ether: 40% Ethyl acetate	60% PE: 40% EA	20.66 ± 1.16^{h}	12.23 ± 0.25^{i}
	F4	40% Petroleum ether: 60% Ethyl acetate	40% PE: 60% EA	51.68 ± 1.53^{f}	21.13 ± 0.15^{f}
	F5	20% Petroleum ether: 80% Ethyl acetate	20% PE: 80% EA	74.33 ± 0.58^{b}	37.03 ± 0.25^{c}
	F6	100% Ethyl acetate	100% EA	63.67 ± 0.57^{d}	27.93 ± 0.11^{d}
	F7	80% Ethyl acetate: 20% Methanol	80% EA: 20 % MeOH	61.65 ± 1.50^{d}	16.86 ± 0.41h
	F8	60% Ethyl acetate: 40% Methanol	60% EA: 40% MeOH	74.66 ± 0.53^{b}	37.86 ± 0.32^{c}
	F9	40% Ethyl acetate: 60% Methanol	40% EA: 60% MeOH	58.32 ± 0.48^{e}	24.93 ± 0.20^{e}
	F10	20% Ethyl acetate: 80% Methanol	20% EA: 80% MeOH	68.33 ± 0.38^{c}	21.63 ± 0.56^{f}
	F11	100% Methanol	100% MeOH	56.56 ± 0. 41^{e}	18.06 ± 0.20^{g}
Standards antioxidant	AscA		AscA	90.56 ± 0.55^{a}	**53.73 ± 0.92^{a}**
	BHT		BHT	89.32 ± 0.92^{a}	**86.33 ± 0.75^{a}**

AscA: Ascorbic acid (natural antioxidant), BHT: Butylated hydroxytoluene (synthetic antioxidant), Different superscript letters within the same column indicate significant differences at $p<0.05$. The data are presented as mean ± standard deviation of three replicates.

Table 7a. List of phytochemical constituents (as µg g^{-1}) in *D. splendida* ethyl acetate crude extract (E14) analyzed by HPLC.

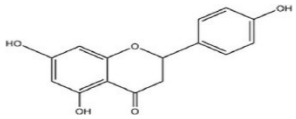
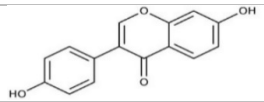
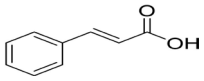
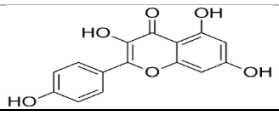
No.	Retention time (min)	Chemical name	Chemical structure	Conc. (µg mL^{-1})	Biological activities	References
1	10.793	Naringenin		2.51	Antioxidant Antitumor Antiviral Antibacterial Anti-inflammatory	(Yining Zhao et al., 2021)
2	11.807	Daidzein		20.89	Antioxidant, Anti-inflammatory Anticancer	(Prakash D. et al.2017)
3	14.115	Cinnamic acid		2	Antioxidant Antimicrobial Anticancer Anti-inflammatory	(Int.J.Mol.Sci. et al., 2022)
4	15.227	Kaempferol		3.24	Antioxidant Anti-inflammatory and antimicrobial	(Sneh Punia et al., 2022)

Table 7b. List of phytochemical constituents (as µg g^{-1}) in fraction 5 (F5) of *D. splendida* ethyl acetate crude extract (E14) analyzed by HPLC.

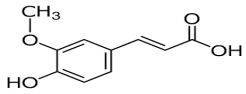
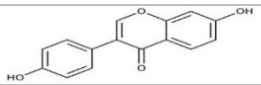
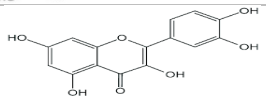
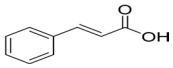
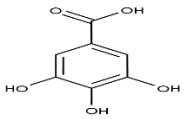
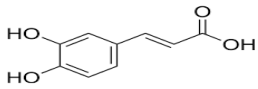
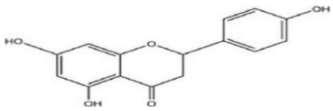
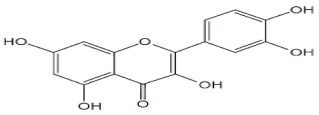
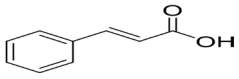
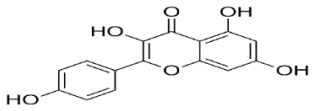
No.	Retention time (min)	Chemical name	Chemical structure	Conc. (µg mL^{-1})	Biological activities	References
1	10.270	Ferulic acid		2.97	Anti-inflammatory Antibacterial Anticancer and Antithrombotic	(Kamila Zdunska et al., 2018)
2	10.793	Naringenin		3.87	Antioxidant Antitumor, Antibacterial Anti-inflammatory	(Yining Zhao et al., 2021)
3	11.807	Daidzein		22.21	Antioxidant Anti-inflammatory Anticancer	(Prakash et al., 2017)
4	12.766	Quercetin		2.44	Anti-inflammatory Antihypertensive Anti-obesity Anti-atherosclerotic.	(Abdelhalim et al., 2018)
5	14.115	Cinnamic acid		14.121	Antioxidant Antimicrobial Anticancer Anti-inflammatory	(Int.J. Mol.Sci et al., 2022)

Table 7c. List of phytochemical constituents (as µg g^{-1}) in fraction 8 (F8) of *D. splendida* ethyl acetate crude extract (E14) analyzed by HPLC.

No.	Retention time (min)	Chemical name	Chemical structure	Conc. (µg mL^{-1})	Biological activities	References
1	3.281	Gallic acid		38.58	Antioxidant, Anti-inflammatory Antineoplastic	(Polewski et al., 2002)
2	6.288	Caffeic acid		2.05	Antioxidant, Anti-inflammatory Anticarcinogenic	(Kaio Murilo et al., 2019)
3	10.793	Naringenin		3.87	Antioxidant Antitumor Antiviral Antibacterial Anti-inflammatory	(Yining Zhao et al., 2021)
4	12.951	Quercetin		14.23	Anti-inflammatory Antihypertensive Anti-atherosclerotic	(Abdelhalim et al., 2018)
5	14.107	Cinnamic acid		0.38	Antioxidant, Antimicrobial Anticancer Anti-inflammatory	(Int.J. Mol.Sci et al., 2020)
6	15.319	Kaempferol		11.46	Antioxidant Anti-inflammatory Antimicrobial	(Sneh Punia et al., 2022)

Selection of the promising fractions by antioxidant assay (radical by DPPH and non-radical by $KMnO_4$) revealed that F5 and F8 (74–75% of antioxidant activity) were the most promising fractions among the eleven fractions produced from E14. Both fractions were tested against three cancer cell lines those women and men suffered from in Egypt (MCF-7, PC3, and HepG2), compared with their crude extract E14 as well as a mixture of both F5 and F8 and determining the IC_{50} in all cases. So, it seems necessary to determine the phytochemicals and their relative concentrations in E14, F5, and F8 to know their active constituents. This was achieved by HPLC recorded in Table 7, in which many phenolic acids were injected as standards.

Ethyl acetate promising crude extract (E14) was shown to contain 4 phenolic compounds, F5 contains 5 compounds, and F8 contains 6 compounds (as seen in Tables 7a–c) with various relative concentrations. The maximum relative concentration in E14 (Table 7a) was Daidzein (20.89 µg/ml); in F5 (Table 7b), also Daidzein recorded 22.21 µg/ml together with Cinnamic acid (14.12 µg/ml). In the case of F8 (Table 7c), gallic acid showed 38.58, quercetin 14.23, and kaempferol 11.46 µg/ml. In addition, in all cases, phenolic compounds are present with small relative contents (3 compounds in each of E14, F5, and F8), as shown in Table 7.

The anticancer activity and the lower IC_{50} are not only due to the increased number of phytochemicals in F5 and F8 or the mixture of F5 and F8, but also to the increased relative concentrations of two or three phenolic compounds in F5 (Daidzein 22.21 and Cinnamic Acid 14.12 µg/ml) and in F8 (Kaempferol 11.46, Quercetin 14.23, and Gallic Acid 38.58 µg/ml), as well as to a larger extent on mixing the two fractions F5+F8. The lower the concentration needed to achieve the IC_{50}, the greater the activity against the cancer cell line tested, which is related to the number and concentration of the active chemical constituents in each tested extract or fraction and the synergism between them (Tables 7a–c). The obtained results in this investigation agreed with those of Jalil et al., 2023; Bourais et al., 2023; Michalaki et al., 2023; Alasalvar et al., 2023; Andishmand et al., 2023 and Yang et al., 2023) concerning the antioxidant and anticancer efficiencies of the phenolic compounds in the investigated extract and fractions.

CONCLUSION

Physical and/or chemical optimum & stress factors (Nitrogen conc., light intensity, salinity, and photoperiod) affect microalgal growth, alter its metabolism, and produce valuable phytochemicals to withstand the adverse stress effects. The investigated green microalgal species respond differently to the studied stresses. Extraction of algal biomasses by hexane, ethyl acetate, and methanol produced 36 crude extracts (E1-E36). Antioxidant activity by DPPH and KMnO4 revealed that E14 was the promising crude extract that showed the greatest antioxidant efficiency. Fractionation of E14 by silica gel column chromatography produced 11 fractions (F1-F11). F5 and F8 exhibited the greatest antioxidant activity by both methods, and they were investigated against HepG2, MCF7, and PC3. Cell viability was inversely proportional to concentration, while the opposite was true for cell cytotoxicity. F5, F8, and F5+F8 showed pronounced anticancer activity and lower IC_{50} values compared to those of their crude extract E14 (where antagonism and peroxidation may occur between its constituents having many OH groups). Antioxidant and anticancer activities may be due to the presence of synergism between many phenolic acids in the active fractions (HPLC analysis).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

THE CONTRIBUTION OF ALL AUTHORS

FE.P. did the practical work and arranged data in Tables and Figures, wrote the draft of the manuscript as well as the reviewer corrections, and was the corresponding author. S.M.M.S and E.A.S shared the supervision of the thesis from which this article was developed, shared the idea of this article, the practical work, revising and evaluating the manuscript at all stages.

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