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Antioxidant activity of *Micractinium* sp. (Chlorophyta) extracts against H_2O_2 induced oxidative stress in human breast adenocarcinoma cells

Onur Bulut[1,2], Işkın Engin Köse[2], Çağla Sönmez[3]✉ & Hüseyin Avni Öktem[2]

In response to the growing demand for high-value bioactive compounds, microalgae cultivation has gained a significant acceleration in recent years. Among these compounds, antioxidants have emerged as essential constituents in the food, pharmaceutical, and cosmetics industries. This study focuses on *Micractinium* sp. ME05, a green microalgal strain previously isolated from hot springs flora in our laboratory. *Micractinium* sp. cells were extracted using six different solvents, and their antioxidant capacity, as well as total phenolic, flavonoid, and carotenoid contents were evaluated. The methanolic extracts demonstrated the highest antioxidant capacity, measuring 7.72 and 93.80 µmol trolox equivalents g^{-1} dry weight (DW) according to the DPPH and FRAP assays, respectively. To further characterize the biochemical profile, reverse phase high-performance chromatography (RP-HPLC) was employed to quantify twelve different phenolics, including rutin, gallic acid, benzoic acid, cinnamic acid, and β-carotene, in the microalgal extracts. Notably, the acetone extracts of *Micractinium* sp. grown mixotrophically contained a high amount of gallic acid (469.21 ± 159.74 µg g^{-1} DW), while 4-hydroxy benzoic acid (403.93 ± 20.98 µg g^{-1} DW) was the main phenolic compound in the methanolic extracts under heterotrophic cultivation. Moreover, extracts from *Micractinium* sp. exhibited remarkable cytoprotective activity by effectively inhibiting hydrogen peroxide-induced oxidative stress and cell death in human breast adenocarcinoma (MCF-7) cells. In conclusion, with its diverse biochemical composition and adaptability to different growth regimens, *Micractinium* sp. emerges as a robust candidate for mass cultivation in nutraceutical and food applications.

Keywords Microalgae, Antioxidants, Gallic acid, 4-Hydroxy benzoic acid, Oxidative stress, Cytoprotective effect

The concept of oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) production and the body's ability to counteract their harmful effects, underscores the critical role of antioxidants in maintaining health. Reactive oxygen species can induce damage to cellular components, including proteins, lipids, and DNA, with prolonged exposure correlating strongly with various diseases. Antioxidants, encompassing vitamins, phenolic compounds, and carotenoids, constitute a vital component of the human diet, primarily sourced from fruits and vegetables[1]. Carotenoids such as astaxanthin, lycopene, lutein and β-carotene, are a class of natural pigments abundant in yellow, orange and dark green leafy plants. β-carotene, in particular, functions as an antioxidant by scavenging various free radicals. Polyphenols, another crucial group of antioxidants, include phenolic acids, flavonoids, tannins, lignans, and stilbenes, and are predominantly found in vegetables, fruits, cereals, herbs, and spices[2,3].

Antioxidants act through diverse mechanisms, such as inhibiting enzymes like glutathione S-transferase, chelating trace metals involved in ROS production, and up-regulating antioxidant defense pathways. The documented anti-carcinogenic and anti-proliferative activities of phenolic compounds on various tumor cell lines

[1]Department of Bioengineering, Faculty of Engineering and Architecture, Konya Food and Agriculture University, 42080 Konya, Türkiye. [2]Department of Biological Sciences, Middle East Technical University, Universiteler Mah., Dumlupinar Blv., No. 1, 06800 Ankara, Türkiye. [3]Department of Medical Biology, Faculty of Medicine, Atilim University, 06830 Ankara, Türkiye. ✉email: cagla.altun@gmail.com

highlight their potential therapeutic benefits[4–7]. Furthermore, the positive cognitive outcomes in patients with Down syndrome associated with early developmental stage consumption of phenolic-rich dietary supplements add to the versatile spectrum of their effects, including antimicrobial, anti-inflammatory, and anti-biofilm activities[8–11]. Studies also reveal the efficacy of using dietary antioxidants in combination with chemo and radiation therapy in cancer patients to suppress the toxicity-related side effects of such treatments[12].

In response to contemporary lifestyle challenges, where poor dietary habits and environmental factors contribute to oxidative stress, there is a growing global demand for antioxidants. In addition to their well-documented health benefits, antioxidants serve a crucial role in the preservation of packaged foods by inhibiting oxidation processes, thereby extending the shelf life of products. The food industry traditionally leaned towards synthetic antioxidants for this purpose; however, mounting concerns about their potential adverse health effects have prompted a shift in consumer preferences. There is now a strong inclination towards the use of natural antioxidants, driving researchers to explore new biological sources that are rich in these natural compounds[13,14].

Microalgae emerge as promising natural sources of antioxidants due to their adaptability to adverse environmental conditions and their capacity to accumulate essential secondary metabolites, including phenolic compounds and carotenoids, with robust antioxidant capabilities. Notably, the antioxidant activity varies among microalgae species and cultivation conditions[15,16].

Microalgal extracts have demonstrated cytotoxic effects on various human cancer cells including liver, colon, breast, lung, and brain, while some of them also exhibiting the ability to inhibit oxidative stress without causing cytotoxicity[17–21]. β-carotene from *Dunaliella salina*, polyunsaturated fatty acids from *Nannochloropsis salina*, sterols in *N. oculate* extracts and violaxanthin, a carotenoid compound, isolated from *D. tertiolecta* are examples of microalgal compounds with documented anti-cancer properties[22–25].

Micractinium is a genus of green microalgae in the family *Chlorellaceae* (Chlorophyta). Different species of *Micractinium* are adapted to diverse geographical locations and exhibit high phenotypic plasticity[26]. Various strains of *Micractinium* showed promising biotechnological potential as feed supplement, biofuel, and in wastewater treatment[27,28]. Thermophilic/thermotolerant strains of *Micractinium* are advantageous in lowering the operational costs of cultivation in large scale bioreactors[29]. *Micractinium* sp. METUNERGY05 (ME05), used in this study, was previously isolated in our laboratory from hot springs of Haymana, Turkey[30]. It is a thermotolerant strain which is suitable for biodiesel production and can be cultivated both mixotrophically and heterotrophically using by-products of a sugar factory as sole carbon source, which reduces the operational costs[31,32].

The aim of this study was to assess the antioxidant properties, along with the phenolic, flavonoid, and carotenoid contents of *Micractinium* sp. extracts cultivated under both mixotrophic and heterotrophic conditions. To achieve this, we employed six different solvents for the extraction of microalgal biomass. Utilizing RP-HPLC analysis, we explored the diverse profile of phenolics present in the extracts, providing valuable insights into the intricate biochemical composition of *Micractinium* sp. Subsequently, we evaluated the cytoprotective properties of the methanolic extracts of *Micractinium* sp. Specifically, we investigated their effectiveness against hydrogen peroxide (H_2O_2)-induced oxidative stress, shedding light on the potential bioactivity of these extracts in mitigating cellular damage. Our findings highlight *Micractinium* sp. as a promising natural source of antioxidants for nutraceutical applications and the food industry. The versatility of *Micractinium* sp. in adapting to different cultivation conditions further enhances its appeal as a bioresource with broad applications.

Materials and methods

Chemicals and reagents

All chemicals and solvents used in this study were purchased from Sigma–Aldrich, AppliChem GmbH and Merck Company, and were analytical or HPLC grade. Molasses used during heterotrophic cultivation was obtained from Konya Sugar Factory, Türkiye.

Microalgal culture and extract preparation

Growth and culture conditions of Micractinium sp.

The green microalga *Micractinium* sp. cells were cultured in Tris–Acetate-Phosphate (TAP) growth medium[33]. Detailed morphological, biochemical, and molecular characterization of *Micractinium* sp. ME05 strain was previously reported by Onay et al.[30].

For mixotrophic cultivation, *Micractinium* sp. was inoculated in 1 L TAP medium and grown at 25 °C under photoperiod (16:8 h of light: dark) at 54 µmol m^{-2} s^{-1} light intensity with constant shaking until cells reached the stationary phase and were harvested. Heterotrophic cultivation was carried out by inoculating $2–3 \times 10^5$ cells mL^{-1} *Micractinium* sp. into 1 L Bold's Basal Medium (BBM) supplemented with 19 g of molasses hydrolysate in 2-L Erlenmeyer flasks. The composition of BBM and molasses, preparation of molasses hydrolysate and the optimum molasses hydrolysate amount for the highest biomass concentration were previously described by Engin et al.[31]. *Micractinium* sp. cells were grown under complete darkness at 30 °C with air supply at 0.5 L min^{-1} through an aquarium pump. The growth went by for 5 days until harvesting.

Preparation of microalgal extracts

Micractinium sp. cells cultivated either mixotrophically or heterotrophically were centrifuged at 3600×*g* for 20 min. The cell pellet was lyophilized using a freeze-dryer and ground to a fine powder. Six different solvents, namely methanol, ethanol, acetone, hexane, ethyl acetate and water were used as extractants. 200 mg of lyophilized microalgae was extracted with 5 ml of solvent at room temperature by sonication in an ultrasonic water bath for 20 min followed by stirring on an orbital shaker for 1 h. The extract was centrifuged at 3800×*g* for 10 min and the aqueous phase was collected in separate bottles. The residual pellets were re-suspended in the same solvents and re-extracted as previously described in Bulut et al.[34]. The extracts were combined and filtered

through a 0.45 µm pore size polytetrafluoroethylene (PTFE) syringe filter and dried using a rotary evaporator. The dried residues were weighed to calculate the extraction yields. Methanol was used as the vehicle solution for solubilization of the dried residues at a concentration of 20 mg mL^{-1}. The solubilized extracts were stored at – 20 °C until further analysis.

Antioxidant measurements

Measurement of the total phenolic content

The Folin & Ciocalteu spectrophotometric method was used to measure the total phenolic content (TPC) of the microalgal extracts. The protocol previously reported by Bulut et al. was followed[34]. 100 µL sample was mixed with 400 µL of Folin-Ciocalteu reagent (1:10 diluted in ultrapure water). The mixture was vortexed thoroughly and allowed to stand at room temperature for 5 min. 500 µL of 7.5% (w/v) $NaNO_2$ solution was added to the mixture and the tubes were incubated for 1.5 h in the dark at room temperature. Following the incubation, 200 µL of sample was transferred to a clear 96-well microplate and the absorbance of each well was measured at 760 nm using a UV–Vis microplate reader. A standard curve prepared by serial dilution of gallic acid solutions ranging from 10 to 400 mg L^{-1} was used for calibration. TPC of the extracts was calculated as gallic acid equivalents using the regression equation of the standard curve. TPC was expressed as mg gallic acid equivalents per gram dry weight of sample (mg GAE g^{-1} DW). The analyses were performed as biological triplicates.

Measurement of the total flavonoid content

The total flavonoid content (TFC) of the microalgal extracts was measured by the aluminum chloride method[34,35]. One milliliter of extract was diluted 1:5 with ultrapure water; mixed with 0.3 mL of 5% (w/v) $NaNO_2$ and incubated at ambient temperature for 5 min. The sample was mixed with 0.3 mL of 10% (w/v) $AlCl_3$ in ethanol after incubation at ambient temperature for 5 min. This step was followed by another incubation at ambient temperature for 6 min. Then, 2 mL of 1 M NaOH was added to the mixture and the total volume was adjusted to 10 mL with ultrapure water. After a brief vortex, 200 µL of the sample was transferred to a clear 96-well microplate and the absorbance of each well was measured at 510 nm using a UV–Vis microplate reader. A standard curve was prepared with serial dilution of quercetin solutions ranging from 10 to 400 mg L^{-1}. Total flavonoid content of the microalgal extracts was calculated using the regression equation of this standard curve. The results were expressed as mg quercetin equivalents per gram dry weight of sample (mg QE g^{-1} DW).

Measurement of the total carotenoid content

Total carotenoid content of the microalgal extracts was calculated following the method by Lichtenthaler and Buschmann[36]. Absorbance of the methanolic extracts was recorded at 470, 652 and 665 nm using a UV–Vis spectrophotometer and total carotenoid content was calculated according to the Lichtenthaler equations as follows:

$$c_a\left(\mu g\ mL^{-1}\right) = 16.72A_{665} - 9.16A_{652}$$

$$c_b\left(\mu g\ mL^{-1}\right) = 34.09A_{652} - 15.28A_{665}$$

$$c_{(x+c)}\left(\mu g\ mL^{-1}\right) = (1000A_{470} - 1.63c_a - 104.96c_b)/221$$

where c_a and c_b are concentrations of chlorophyll a and b, respectively, and $c_{(x+c)}$ is the concentration of the total carotenoids. The results were expressed as mg carotenoid per gram dry weight of sample (mg carotenoid g^{-1} DW).

DPPH assay

The DPPH radical scavenging activity of the microalgal extracts was measured according to Cheng et al.[37]. Briefly, 100 µL of microalgal extracts at concentrations ranging from 50 to 2000 µg mL^{-1} was mixed with 100 µL of 0.2 mM DPPH solution in a clear 96-well plate. The mixture was incubated in the dark at ambient temperature for 30 min, and the absorbance was recorded at 515 nm using a microplate reader with the trolox solution as the positive control and the DPPH solution as blank[38]. The percentage of scavenged DPPH• radical was calculated according to the following equation:

$$\text{DPPH scavenging activity (\%)} = [1 - (As - Asc)/Ac] \times 100$$

where *As* is absorbance of the sample (100 µL of sample with 100 µL of DPPH• radical solution), *Asc* is absorbance of the sample control (100 µL of sample with 100 µL of methanol) and *Ac* is absorbance of the control (100 µL of methanol with 100 µL of DPPH• radical solution). A standard curve was prepared with serially diluted trolox solutions in the range of 2.5 to 80 µmol L^{-1} concentrations. Total antioxidant capacity of the microalgal extracts was calculated as trolox equivalents using the regression equation of the standard curve. The results were expressed as micromol of equivalent trolox per gram of dried weight (µmol TE g^{-1} DW) and (%) DPPH radical scavenging activity of the extract.

FRAP assay

The antioxidant capacity of the extracts was also evaluated by the FRAP assay through monitoring the reduction of Fe^{3+}-TPTZ to blue-colored Fe^{2+}-TPTZ[38]. The working FRAP solution was freshly prepared by mixing ten volumes of acetate buffer, one volume of TPTZ solution and one volume of ferric chloride hexahydrate solution and warmed at 37 °C in a water bath prior to use. 25 µL of the microalgal extract at concentrations between 50 and 2000 µg mL^{-1} was mixed with 175 µL of pre-warmed FRAP solution in a clear 96-well microplate. The microplate was allowed to stand at room temperature for 30 min in the dark. The absorbance of each sample was measured at 593 nm using a microplate reader. Trolox solutions ranging from 5 to 20 µmol L^{-1} were used for preparation

of a standard curve. Total antioxidant capacity of the microalgal extracts was calculated as trolox equivalents using the regression equation of the standard graph. FRAP values were expressed as μmol trolox equivalents per gram dry weight of sample (μmol TE g^{-1} DW)[34].

Reverse phase high performance liquid chromatography (RP-HPLC) analysis

Twelve selected phenolic compounds; namely, gallic acid, benzoic acid, 4-hydroxy benzoic acid, vanillic acid, syringic acid, cinnamic acid, coumaric acid, caffeic acid, chlorogenic acid, rosmarinic acid, quercetin and rutin were identified in the microalgal extracts by reverse phase HPLC (Waters Alliance 2695, Waters Corporation, USA) coupled to a UV/Vis detector (Waters 2489 detector) as described in[34]. The microalgal extract at a concentration of 1000 ppm was passed through a 0.45 μm PTFE syringe filter prior to injection. The chromatographic separation was performed in a C18 analytical column (ACE 5, AC Technologies, Scotland). Elution was carried out with a gradient pump mode involving three mobile phases; mobile phase A: 2% (v/v) acetic acid, mobile phase B: acetonitrile and 0.5% (v/v) acetic acid (1:1 v/v) and mobile phase C: acetonitrile. The gradient was set as following: 0–8 min: 95% A and 5% B; 8–10 min: 80% A and 20% B; 10–17 min: 78% A and 22% B; 17–19 min: 75% A and 25% B; 19–30 min: 73% A and 27% B; 30–35 min: 60% A and 40% B; 35–40 min: 55% A and 45% B; 40–45 min: 35% A and 65% B; 56–50 min: 10% B and 90% C; 50–52 min: 100% C; and 52–60 min: 95% A and 5% B. The flow rate was 1.2 mL min^{-1}, the injection volume was 20 μL and the column temperature was maintained at ambient temperature. Simultaneous monitoring was done via a UV/Vis detector with reference wavelength of 280 nm. Retention times and peak areas of both authentic standards and microalgal extracts were monitored automatically by Empower 3 Chromatography Data Software (Waters Corporation, USA). The concentration of individual phenolic compounds was quantified by comparison of the chromatographic peaks of the microalgal extracts to those of authentic standards.

To identify the β-carotene content, a RP-HPLC system equipped with a Shimadzu LC-20AD pump (Shimazdu, Kyoto, Japan) and Shimazdu SPD-20A UV/Vis detector was used. An Inertsil ODS-2 C18 analytical column was used for the chromatographic runs (GL Sciences, Tokyo, Japan). The gradient pump mode consisting of two mobile phases (mobile phase A: 90% acetonitrile in water, mobile phase B: ethyl acetate) was used for elution at a flow rate of 1.0 mL min^{-1}. The peaks were detected at 450 nm. β-carotene concentration in the microalgal extracts was identified by comparison of the peak areas of the samples to the authentic standards.

Cell culture and assays

Inhibition of the H_2O_2-induced reactive oxygen species (ROS) generation in MCF-7 cells

The effect of the methanolic microalgal extracts on inhibition of the intracellular H_2O_2-induced ROS generation in MCF-7 cells was evaluated by a fluorescence assay using the cell-permeant probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as described by Zhuang et al. with minor modifications[39]. MCF-7 cells were pretreated with 50, 100, 200 or 400 μg mL^{-1} of the methanolic extracts, separately for 48 h in a 96-well black cell culture plate. Cells treated with the culture medium containing VS and ascorbic acid (8 μg mL^{-1}) were used as the negative and the positive controls, respectively. After 48 h incubation with the methanolic extracts, the cells were exposed to 0.5 mM of H_2O_2 for 6 h to induce intracellular oxidative stress via ROS generation. The cells were washed twice with PBS and incubated with serum-free medium containing DCFH-DA (20 μM) for 30 min in the dark at 37 °C. Cells were immediately washed with PBS. The formation of the fluorescent 2',7'-dichlorofluorescein (DCF) due to oxidation of the non-fluorescent DCFH-DA by intracellular oxidative stress was detected by a fluorescence microplate reader with an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

Apoptosis assay

The cytoprotective effect of the methanolic extracts of mixotrophically grown *Micractinium* sp. on H_2O_2-induced apoptosis of MCF-7 cells was determined by an Annexin V-FITC and propidium iodide (PI) double-staining apoptosis assay kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. MCF-7 cells were firstly treated with the microalgal extracts and then, with H_2O_2 as described in Sect. 2.11. The cells were collected by trypsinization, washed with PBS twice, and resuspended in 200 μL of binding buffer containing 5 μL of Annexin V-FITC. After incubation of the cells for 15 min at room temperature in the dark, 10 μL of PI was added to cells and incubated for 10 min in an ice bath in the dark. Finally, the cells were analyzed using a flow cytometer.

Statistical analysis

All experiments in this study were carried out in biological triplicates. Results were expressed as mean ± standard error. The analysis of the mean values was performed using the analysis of variance (ANOVA) test and Tukey's post-hoc comparison test. A p-value < 0.001, < 0.01, and < 0.05 was considered as highly significant (***), very significant (**) and significant (*), respectively in statistical terms. Pearson's correlation of determination (R^2) was used to compute correlations among antioxidant assays, TPC and TFC under different growth conditions. The statistical analysis was conducted using R version 3.4.2.

Results

Extraction yields of *Micractinium* sp. in different solvents

In this study, six different solvents with varying polarity, namely, methanol, ethanol, acetone, hexane, ethyl acetate and water were used to extract antioxidants from either mixotrophically or heterotrophically grown *Micractinium* sp. The extraction yields are given in Table 1. The highest extraction yield of 30.40 ± 0.94% was obtained in methanol followed by 28.16 ± 1.08% in water ($p = 0.91$) for mixotrophic growth. For heterotrophic growth, the highest yield was equal in methanol (38.23 ± 3.90%) and water (38.33 ± 0.34%) ($p = 1.0$). The lowest extraction yield was in ethyl acetate (11.54 ± 1.47%) and acetone (8.29 ± 1.41%) for mixotrophic and heterotrophic cultivation,

Solvent	Extraction yield (%)	
	Mixotrophic growth	Heterotrophic growth
Methanol	30.40 ± 0.94 b	38.23 ± 3.90 b
Ethanol	14.85 ± 1.88 a	17.78 ± 4.78 a
Acetone	16.46 ± 0.95 a	8.29 ± 1.41 a
Hexane	12.70 ± 2.62 a	15.45 ± 2.00 a
Ethyl acetate	11.54 ± 1.47 a	13.92 ± 1.31 a
Water	28.16 ± 1.08 b	38.33 ± 0.34 b

Table 1. Extraction yields of *Micractinium* sp. extracts prepared using different solvents and growth conditions. Results are expressed as mean ± standard error of three measurements ($n = 3$). Means with different letters in the same column are statistically significant ($p < 0.05$).

respectively. The difference in extraction yields of methanol and water with respect to acetone, ethyl acetate, ethanol and hexane was highly significant ($p < 0.001$) for both mixotrophic and heterotrophic growth conditions.

Antioxidant capacity of *Micractinium* sp. measured by DPPH and FRAP assays

Antioxidant capacity of *Micractinium* sp. extracts in six different solvents under two different growth regimens was measured by DPPH and FRAP assays. The results are given in Table 2. DPPH assay results are expressed both as % DPPH radical scavenging activity of microalgal extracts at 1 mg mL^{-1} concentration and as micromoles trolox equivalent (TE) per gram dry weight (g^{-1} DW) of microalgae. The former expression does not take into account the extraction yield of the samples in each solvent. For mixotrophic growth, the highest antioxidant capacity was measured in the methanolic extracts as 7.72 ± 0.95 and 93.80 ± 6.28 µmol TE g^{-1} DW followed by ethanol extracts as 6.41 ± 1.33 and 79.83 ± 7.56 µmol TE g^{-1} DW by DPPH and FRAP assays, respectively. Similarly, the highest antioxidant capacity in heterotrophically grown samples was recorded in the methanolic extracts as 6.82 ± 1.31 and 64.91 ± 4.28 µmol TE g^{-1} DW by DPPH and FRAP assays, respectively. The antioxidant capacities of mixotrophically grown microalgae were higher compared to heterotrophic samples. Particularly, the difference in antioxidant capacities measured by FRAP assay between mixotrophic and heterotrophic growth in methanol, ethanol and acetone extracts was statistically significant. The correlation between DPPH and FRAP assays was found to be highly significant ($p < 0.001$) for both conditions, and the coefficient of determination (R^2) values for these assays were calculated as 0.57 and 0.65 for mixotrophic and heterotrophic growth, respectively. The low correlation between two methods can be explained by the methodological differences in detection and measurement of the antioxidants[34].

Total phenolic, flavonoid and carotenoid contents of *Micractinium* sp. extracts

Total phenolic contents (TPC) of *Micractinium* sp. grown under two different growth conditions and extracted in six different solvents are given in Table 3. The highest TPC was found in methanolic extracts as 18.11 ± 2.17 mg GAE g^{-1} DW and 11.47 ± 1.41 mg GAE g^{-1} DW for mixotrophic and heterotrophic growth, respectively. The difference between TPCs of two growth conditions in methanolic extracts is very significant ($p = 0.009$). These results are consistent with a previous report in which, both *Chlorella vulgaris* and *Scenedesmus obliquus* had higher polyphenols in the mixotrophic culture compared to the heterotrophic culture[40]. The lowest TPCs of both mixotrophic and heterotrophic cultivation were measured in hexane extracts as 3.87 ± 0.83 mg GAE g^{-1} DW and 2.40 ± 0.19 mg GAE g^{-1} DW, respectively. The difference in results was not statistically significant.

The highest total flavonoid content (TFC) of mixotrophic *Micractinium* sp. was detected in the methanolic extracts (5.72 ± 0.26 mg QE g^{-1} DW) followed by the ethanol extracts (5.21 ± 1.70 mg QE g^{-1} DW) (Table 3). TFC of heterotrophically grown cell extracts was the highest in methanol with a concentration of 3.22 ± 0.27 mg QE

Solvent	Mixotrophic growth			Heterotrophic growth		
	(%) DPPH Radical scavenging effect[1]	DPPH (µmol TE g^{-1} DW)	FRAP (µmol TE g^{-1} DW)	(%) DPPH Radical scavenging effect[1]	DPPH (µmol TE g^{-1} DW)	FRAP (µmol TE g^{-1} DW)
Methanol	39.61 ± 4.37 bc	7.72 ± 0.95 a	93.80 ± 6.28 c	28.10 ± 2.36 ab	6.82 ± 1.31 b	64.91 ± 4.28 d
Ethanol	64.15 ± 5.24 b	6.41 ± 1.33 ac	79.83 ± 7.56 ac	35.75 ± 3.94 ac	3.82 ± 0.62 ab	50.43 ± 10.45 bd
Acetone	46.48 ± 1.39 ab	4.97 ± 0.45 ab	68.88 ± 2.96 a	44.35 ± 9.44 a	2.32 ± 0.30 a	35.70 ± 1.85 ab
Hexane	30.55 ± 19.09 ac	2.05 ± 0.28 b	15.70 ± 2.25 b	14.24 ± 5.28 bc	1.12 ± 0.47 a	11.73 ± 2.14 c
Ethyl acetate	54.71 ± 1.42 ab	4.10 ± 0.45 ab	29.06 ± 6.32 b	31.30 ± 3.42 ab	2.79 ± 0.54 a	14.55 ± 3.33 ac
Water	17.65 ± 2.91 c	2.93 ± 0.64 bc	7.69 ± 0.43 b	9.00 ± 2.01 b	1.68 ± 0.53 a	1.40 ± 0.50 c

Table 2. Antioxidant capacity of *Micractinium* sp. extracts in different solvent extracts determined by DPPH and FRAP assays. Results are mean ± standard error of three measurements ($n = 3$). [1] Radical scavenging effects of algal extracts at 1 mg mL^{-1} concentration. Means with different letters in the same column are statistically significant ($p < 0.05$).

Solvent	Mixotrophic growth			Heterotrophic growth		
	Total phenolic content (mg GAE g^{-1} DW)	Total flavonoid content (mg QE g^{-1} DW)	Carotenoid content (mg g^{-1} DW)	Total phenolic content (mg GAE g^{-1} DW)	Total flavonoid content (mg QE g^{-1} DW)	Carotenoid content (mg g^{-1} DW)
Methanol	18.11 ± 2.17 b	5.72 ± 0.26 a	2.27 ± 0.18 b	11.47 ± 1.41 c	3.22 ± 0.27 b	1.65 ± 0.01 b
Ethanol	7.40 ± 1.46 a	5.21 ± 1.70 a	NT	6.67 ± 1.07 b	1.89 ± 0.35 a	NT
Acetone	7.37 ± 0.83 a	4.21 ± 0.68 ab	3.02 ± 0.11 a	4.77 ± 0.35 ab	1.40 ± 0.16 a	0.32 ± 0.05 a
Hexane	3.87 ± 0.83 a	1.07 ± 0.17 b	NT	2.40 ± 0.19 a	0.86 ± 0.07 a	NT
Ethyl acetate	8.02 ± 0.52 a	2.39 ± 0.08 ab	3.17 ± 0.21 a	4.76 ± 0.09 ab	2.09 ± 0.45 ab	0.32 ± 0.06 a
Water	6.65 ± 1.32 a	1.15 ± 0.07 b	NT	7.07 ± 0.66 b	1.47 ± 0.02 a	NT

Table 3. Total phenolic, flavonoid and carotenoids of *Micractinium* sp. extracts prepared using different solvents and growth conditions. Results are mean ± standard error of three measurements ($n = 3$). NT: Not Tested. Means with different letters in the same column are statistically significant ($p < 0.05$).

g^{-1} DW. The difference in TFC of the methanolic extracts between mixotrophic and heterotrophic cultivation was not statistically significant. However, there was a significant reduction in TFC of ethanol extracts from heterotrophic samples (1.89 ± 0.35 mg QE g^{-1} DW) compared to ethanol extracts of the mixotrophic culture (5.21 ± 1.70 mg QE g^{-1} DW).

The total carotenoid content (TCC) of *Micractinium* sp. was calculated in methanol, acetone and ethyl acetate extracts as 2.27 ± 0.18 mg g^{-1} DW, 3.02 ± 0.11 mg g^{-1} DW and 3.17 ± 0.21 mg g^{-1} DW, respectively under mixotrophic cultivation (Table 3). TCC of heterotrophically grown microalgae was recorded as 1.65 ± 0.01 mg g^{-1} DW in methanol, 0.32 ± 0.05 mg g^{-1} DW in acetone and 0.32 ± 0.06 mg g^{-1} DW in the ethyl acetate extracts. The difference in carotenoid content between mixotrophic and heterotrophic growth was statistically significant for ethyl acetate extracts ($p < 0.05$).

Correlation of the antioxidant capacity with the phenolic, flavonoid, and carotenoid contents

The correlation of determination (R^2) values between the antioxidant capacity and the total phenolics, flavonoids and carotenoids of *Micractinium* sp. in different solvent extracts were calculated both for mixotrophic and heterotrophic growth conditions. The R^2 value between the DPPH assay and TPC in ethanol extracts of mixotrophically cultivated microalgae was 0.99 ($p = 0.01$). This result is consistent with the strong correlation between TPC of *Chlorella vulgaris* and *Scenedesmus obliquus* and the DPPH assay reported in Shetty and Sibi[40]. In the same study, it was shown that the contribution of phenolics to the antioxidant potential was irrespective of the cultivation mode[40]. In the present study, other R^2 values of 0.90 or higher were obtained between DPPH or FRAP assay and TPC, TFC and TCC in various solvent extracts of *Micractinium* sp. both under mixotrophic and heterotrophic growth conditions; however, none of them were found to be statistically significant ($p > 0.05$) (Supplementary Tables 1 and 2). The statistically significant positive correlation between DPPH assay and TPC in the ethanol extracts suggests that polyphenols that are highly soluble in ethanol greatly contribute to the antioxidant activity of microalgae. Ethanol is also advantageous as a solvent as it is safe for human consumption[5].

Identification of selected phenolic compounds in *Micractinium* sp. extracts by RP-HPLC

Twelve different phenolic compounds that fall in three categories, namely, flavonols; rutin and quercetin, benzoic acid derivatives; 4-hydroxy benzoic acid, benzoic acid, gallic acid, syringic acid and vanillic acid and cinnamic acid and derivatives; caffeic acid, rosmarinic acid, coumaric acid and chlorogenic acid were quantified by RP-HPLC in methanol, acetone and ethyl acetate extracts of the mixotrophically and heterotrophically grown *Micractinium* sp. (Table 4). Gallic acid (469.21 ± 159.74 µg g^{-1} DW) in the acetone extracts of mixotrophic microalgae was the highest phenolic compound detected. Under heterotrophic growth, 4-hydroxy benzoic acid (403.93 ± 20.98 µg g^{-1} DW) in the methanolic extracts was the most abundant phenolic compound. Strikingly, the amount of the same compound in the methanolic extracts of mixotrophic *Micractinium* sp. was only 1.98 ± 0.91 µg g^{-1} DW. Acetone is a powerful solvent of flavonols and consistently rutin concentration in acetone extracts (212.09 ± 122.46 µg g^{-1} DW in mixotrophic samples) was significantly higher than the other solvents. Overall, there were considerable differences in the amounts of phenolic compounds between mixotrophic and heterotrophic microalgal extracts.

The amount of the carotenoid, β-carotene was quantified in the methanolic extracts of the mixotrophically or the heterotrophically cultivated *Micractinium* sp. by RP-HPLC (Table 5). β-carotene content under mixotrophic growth (52.28 ± 1.45 µg g^{-1} DW) was slightly higher than the β-carotene amount under heterotrophic growth (45.99 ± 3.46 µg g^{-1} DW). Although different detection and calculation methods have been used, β-carotene accounts for approximately 2% of the total carotenoids in methanolic extracts of *Micractinium* sp.

Inhibitory effect of *Micractinium* sp. extracts on intracellular ROS generation

Micractinium sp. methanolic extract with the highest antioxidant activity under mixotrophic cultivation was tested for its ability to inhibit intracellular oxidative stress induced by H_2O_2 in MCF-7 cells by the DCFH-DA assay. This method is based on the oxidation of non-fluorescent DCFH-DA into fluorescent DCF by intracellular ROS. Therefore, the intensity of intracellular fluorescent signal is proportional to the amount of intracellular

Phenolic compound	Amount[1] (μg g^{-1} DW)					
	Mixotrophic growth			Heterotrophic growth		
	Methanol	Acetone	Ethyl acetate	Methanol	Acetone	Ethyl acetate
Benzoic acid derivatives						
Gallic acid	129.08 ± 2.65 a	469.21 ± 159.74 a	ND	125.06 ± 11.68 a	12.93 ± 3.93 a	ND
Benzoic acid	13.62 ± 2.63 a	37.84 ± 2.20 a	8.77 ± 2.55 a	107.20 ± 16.85 b	28.09 ± 14.36 a	18.50 ± 3.56 a
4-Hydroxy Benzoic acid	1.98 ± 0.91 a	0.95 ± 0.12 a	1.10 ± 0.63 a	403.93 ± 20.98 b	40.48 ± 4.10 a	4.75 ± 0.60 a
Vanillic acid	13.37 ± 7.72 a	5.37 ± 1.54 a	ND	47.66 ± 2.44 b	11.91 ± 1.19 a	4.46 ± 0.18 a
Syringic acid	27.99 ± 6.87 ab	9.32 ± 0.58 a	5.01 ± 0.61 a	56.42 ± 13.04 b	5.70 ± 0.53 a	6.77 ± 0.88 a
Cinnamic acid derivatives						
Cinnamic acid	10.34 ± 6.86 b	18.06 ± 0.77 b	ND	196.44 ± 6.70 c	51.88 ± 2.26 a	38.24 ± 3.61 ab
Coumaric acid	19.36 ± 15.25 a	4.41 ± 1.46 a	9.58 ± 1.29 a	10.85 ± 1.49 a	0.55 ± 0.43 a	5.94 ± 0.02 a
Caffeic acid	16.46 ± 9.50 a	4.13 ± 0.99 a	ND	3.87 ± 0.86 a	4.51 ± 0.30 a	ND
Chlorogenic acid	11.27 ± 5.61 a	2.30 ± 0.10 a	1.55 ± 0.89 a	2.78 ± 1.05 a	0.79 ± 0.55 a	1.86 ± 0.08 a
Rosmarinic acid	34.84 ± 2.89 a	1.83 ± 0.51 a	1.98 ± 1.15 a	18.45 ± 10.60 a	ND	4.22 ± 0.59 a
Flavonols						
Quercetin	65.63 ± 0.49 a	2.70 ± 0.94 b	30.02 ± 13.28 ab	37.09 ± 8.67 ab	28.20 ± 5.81 ab	14.24 ± 1.66 b
Rutin	53.91 ± 0.58 a	212.09 ± 122.46 a	ND	39.89 ± 0.10 a	80.33 ± 38.33 a	ND

Table 4. The phenolic compounds in different solvent extracts of *Micractinium* sp. identified by RP-HPLC. [1] Results are mean ± standard error of two measurements. ND: Not Detected. Means with different letters in the same row are statistically significant ($p < 0.05$).

Growth	β-Carotene amount [1] (μg g^{-1} DW)
Mixotrophic	52.28 ± 1.45 a
Heterotrophic	45.99 ± 3.46 a

Table 5. β-carotene amounts in methanolic extracts of *Micractinium* sp. identified by RP-HPLC. [1]Results are mean ± standard error of two measurements. Means with different letters in the same column are statistically significant ($p < 0.05$).

ROS[41]. H_2O_2 was used to promote oxidative stress as it is a relatively stable ROS generator and can result in the accumulation of ROS within the cell at elevated concentrations leading to cell damage and death[39,41].

Pre-incubation of MCF-7 cells with the microalgal extracts for 48 h significantly changed the levels of intracellular ROS (Fig. 1A). The inhibitory effect of the microalgal extracts on the intracellular ROS levels occurred in a concentration-dependent manner. Pre-treatment with the methanolic extract reduced intracellular ROS in MCF-7 cells by 23.80% and 72.60% at the lowest (50 μg mL^{-1}) and the highest concentration (400 μg mL^{-1}), respectively. Concentration-dependent inhibitory effect of the microalgal extracts was highly significant compared to the control cells treated with the vehicle solution (methanol) only ($p < 0.001$).

Cytoprotective activity of *Micractinium* sp. extracts against H_2O_2-induced cell apoptosis

The methanolic extract, which exhibited the highest antioxidant activity in the mixotrophically cultivated *Micractinium* sp. was evaluated for its ability to protect MCF-7 cells against H_2O_2-induced apoptosis. Upon treatment with 1 mM H_2O_2, the percentage of viable, necrotic, and apoptotic MCF-7 cells were calculated as 54.83 ± 3.87, 34.56 ± 2.92, and 10.60 ± 0.95%, respectively (Fig. 1B). The percentage of viable MCF-7 cells pre-treated with the methanolic extract of *Micractinium* sp. increased to 74.00 ± 2.32% ($p < 0.001$). Pre-treatment with the methanolic microalgal extract led to a two-fold decrease in the number of necrotic cells (17.36 ± 1.78%) and caused a slight reduction in the apoptosis rate (8.60 ± 1.45%).

Discussion

The pursuit of finding a single solvent capable of solubilizing all target compounds during extraction is challenging. Phenolic compounds, characterized by diverse chemical structures, often form attachments to sugars or proteins in vivo, impacting their solubility across various solvents[42,43]. Consequently, we assessed the extraction yields of antioxidants from *Micractinium* sp. under both mixotrophic and heterotrophic conditions using six different solvents with varying polarities. The selection of different solvents aimed to encompass a wide range of polarities, thereby enhancing our understanding of solvent-specific extraction efficiencies and the nature of the compounds being extracted. In our study, methanol and water emerged as the most efficient solvents, yielding the highest extraction percentages under both growth conditions. This finding aligns with previous studies demonstrating the efficacy of methanol and water in extracting bioactive compounds from microalgae[44,45]. These

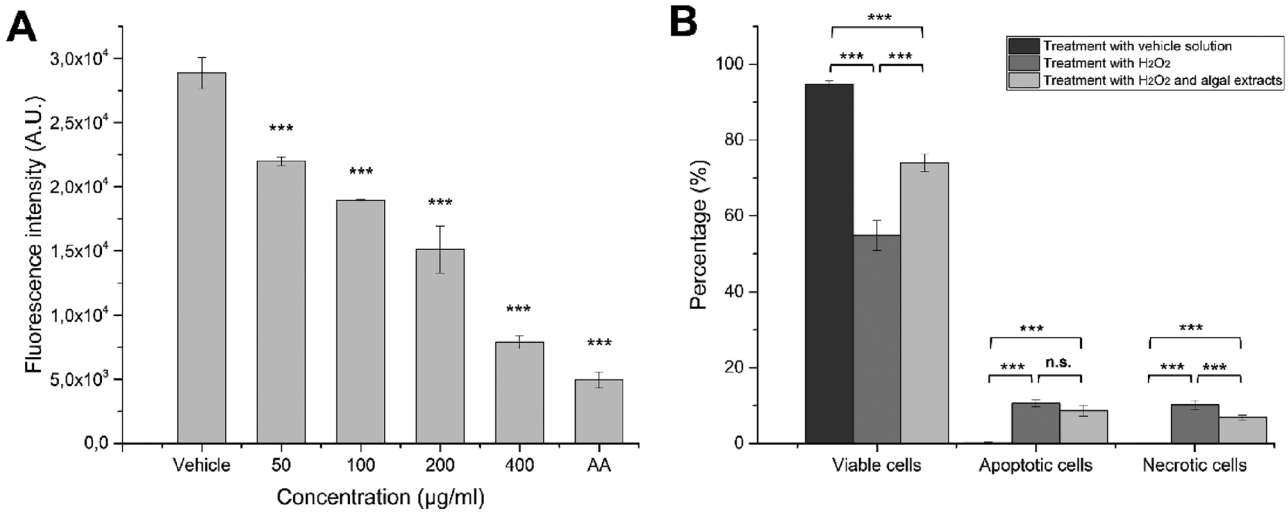

Figure 1. (**A**) Inhibitory effects of methanolic extracts of *Micractinium* sp. and ascorbic acid (AA) on intracellular ROS generation in H_2O_2-induced MCF-7 cells. (**B**) Cytoprotective effects of the methanolic extract of *Micractinium* sp. on H_2O_2-induced MCF-7 cell apoptosis and necrosis. (***) indicates the significance at $p < 0.001$.

results underscore the importance of solvent selection in optimizing extraction efficiency and yield. Furthermore, the significant disparity in extraction yields between methanol and water compared to other solvents highlights the critical influence of solvent polarity on the extraction of bioactive compounds.

The antioxidant capacities of various macroalgae and microalgae have been extensively studied, with notable variations observed across different species and strains[43,46,47]. Notably, our study is the first to evaluate the antioxidant capacity of a *Micractinium* species. Methanolic extracts exhibited the highest antioxidant capacity under both growth conditions, as evidenced by both DPPH and FRAP assays. This observation aligns with prior research highlighting the superior antioxidant potential of methanolic extracts from various microalgae species[42]. The strong correlation between the DPPH and FRAP assays suggests their complementary nature in assessing antioxidant capacity, despite methodological differences[48]. The observed moderate correlation coefficients indicate that while both assays provide valuable insights into the antioxidant potential of microalgal extracts, they may capture different aspects of antioxidant activity.

Comparisons of antioxidant capacities across studies are challenging due to variations in laboratory conditions and methodologies. However, two thermo-tolerant strains, *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03, isolated from the same thermal waters as *Micractinium* sp., were recently evaluated for their antioxidant capacity[21,34]. Our results demonstrated that ethyl acetate and water extracts of *Micractinium* sp. exhibited higher antioxidant capacity than *Scenedesmus* sp. ME02 but lower than *H. tetrachotoma* ME03. The observed differences in antioxidant capacity among the thermo-tolerant strains can be attributed to genetic variability, which influences the production and composition of antioxidant compounds. Each strain has unique metabolic pathways that determine the types and amounts of antioxidants synthesized, affected by enzyme activities and metabolic fluxes. Additionally, strain-specific adaptations to their thermal environments may result in the production of unique antioxidants that confer thermal stress protection. The presence and concentration of secondary metabolites, which act as antioxidants and are often species-specific, further contribute to the variations in antioxidant capacity[15,49].

Microalgae cultivation methods, including autotrophy, heterotrophy, and mixotrophy, significantly influence their biochemical content. In this study, we evaluated the antioxidant activity of *Micractinium* sp. cells grown under mixotrophic and heterotrophic conditions. The notable contrast in antioxidant capacities between these samples underscores the impact of cultivation mode on the biochemical composition and subsequent antioxidant properties of *Micractinium* sp. Heterotrophic cultivation, particularly when utilizing molasses and vinasse as carbon sources, proved advantageous for achieving higher biomass and lower costs. *Micractinium* sp. demonstrated adaptability to various growth and temperature regimens, further influencing its biochemical content[30,50].

Surprisingly, limited information exists on how heterotrophic growth affects the antioxidant capacity of microalgae compared to other cultivation conditions. In a previous study, the antioxidant activities of *Chlorella vulgaris* and *Scenedesmus obliquus* across autotrophic, mixotrophic, and heterotrophic conditions were compared[40]. Consistent with our findings, methanolic extracts of both microalgae exhibited reduced antioxidant potential during heterotrophic growth compared to autotrophic and mixotrophic conditions. This decline in antioxidant activity under heterotrophic conditions aligns with the understanding that environmental factors, including light and ultraviolet exposure, along with internal processes such as photosynthesis, generate ROS. To counteract oxidative damage from ROS, microalgae produce antioxidants as a defense mechanism. Importantly, microalgae cultivated under mixotrophic conditions are exposed to light and rely on both photosynthesis and an additional carbon source in the culture medium for energy. Consequently, it is plausible to expect a higher antioxidant capacity in mixotrophic cultures compared to heterotrophic growth conditions due to the increased

antioxidant activity prompted by the combined effects of photosynthesis and light exposure. This discovery emphasizes the complex relationship between microalgae cultivation methods, environmental influences, and their antioxidant responses, providing insight into the subtle changes in their biochemical processes across different growth environments[32,49,51].

Studies on other microalgae, such as *Tetraselmis suecica* and *Hindakia tetrachotoma* ME03, have highlighted their antioxidant activity and potential applications in the cosmetic and biotechnological industries[17,21]. The present investigation into *Micractinium* sp. ME05 extracts expands the limited knowledge of the in vitro antioxidant activity of green microalgae, further supporting their potential for biotechnological applications.

The evaluation of total phenolic contents (TPC) in *Micractinium* sp., cultivated under varied growth conditions and extracted using different solvents, provided insights into the phenolic composition of the microalgal biomass. Consistent with prior research, *Micractinium* sp. extracted with polar solvents exhibited higher concentrations of phenolic compounds compared to nonpolar hexane extracts, regardless of the cultivation method[34,46]. Methanolic extracts showed the highest TPC, aligning with previous studies suggesting the effectiveness of methanol in extracting phenolic compounds from microalgae[52]. Interestingly, hexane extracts displayed the lowest TPC across both growth conditions, indicating the limited capacity of this solvent to extract phenolic compounds from *Micractinium* sp. Additionally, our study identified a higher phenolic content in *Micractinium* sp. compared to *Scenedesmus* sp. ME02 in ethyl acetate and water extracts, revealing differences in the composition of these two freshwater strains isolated from the same thermal flora[34]. These findings underscore the critical role of solvent selection in optimizing phenolic extraction efficiency.

The health-promoting properties of flavonoids, commonly found in fruits and vegetables, are extensively documented in scientific literature, yet research on flavonoid content in microalgae remains relatively scarce[53]. However, insights from previous studies have illuminated the presence of a diverse array of flavonoids across different classes of microalgae, albeit in smaller quantities compared to terrestrial plants[54]. Methanolic extracts emerged as the most efficient solvent for extracting flavonoids from *Micractinium* sp., consistent with its effectiveness in extracting other bioactive compounds. Importantly, our findings reveal that *Micractinium* sp. exhibits a higher total flavonoid content compared to several other microalgae species, as demonstrated in previous studies[34,52]. This observation underscores the potential of *Micractinium* sp. as a promising natural source of flavonoids, suggesting its suitability as a potential substitute for synthetic antioxidants in the industry. The relatively higher flavonoid content in *Micractinium* sp. extracts, particularly when cultivated under mixotrophic conditions, highlights the importance of cultivation strategies in modulating the biochemical composition and potential applications of microalgae-derived products.

The total carotenoid content (TCC) in *Micractinium* sp. exhibits significant variation across different cultivation modes, with higher concentrations observed in mixotrophic cultures compared to heterotrophic cultures. This study represents the first attempt to compare the TCC of microalgae under different cultivation modes, exploring the dynamics of carotenoid accumulation in response to varied growth conditions. In a previous study, two *Micractinium* sp. strains, designated as CCNM 1006 and CCNM 1041, were evaluated for their total carotenoid contents. Both strains displayed slightly higher quantities of total carotenoids compared to *Micractinium* sp. As part of a broader study encompassing 57 distinct microalgae strains, *Micractinium* sp. fell within the medium range concerning its TCC[28]. The pivotal role of carotenoids in microalgae involves safeguarding chlorophylls from the detrimental effects of light exposure by scavenging ROS[55]. Our findings indicate a higher accumulation of carotenoids in mixotrophic microalgae compared to cultures grown heterotrophically. This underscores the vital role of carotenoids in responding to light exposure, a phenomenon crucial for mitigating oxidative stress through ROS scavenging. Importantly, our study marks the first attempt to compare the TCC of microalgae grown under distinct cultivation modes, offering valuable insights into the dynamics of carotenoid accumulation in response to varied growth conditions.

Despite the well-established role of phenolic compounds in plant antioxidant capacity, their contribution to microalgal antioxidant potential remains debated[21,34,40,56]. The diverse nature of microalgal antioxidants collectively contributes to their overall antioxidant capacity. Therefore, we investigated the profile of phenolics, and β-carotene present in the extracts. This study stands out as the first to quantify individual phenolic compounds in a *Micractinium* species and compare their relative quantities under distinct cultivation modes. Twelve phenolic compounds, categorized into flavonols, benzoic acid derivatives, and cinnamic acid derivatives, were quantified in methanol, acetone, and ethyl acetate extracts from both mixotrophically and heterotrophically grown microalgae.

In this comparative exploration, *Chlorella pyrenoidosa*, another freshwater green microalga, exhibited considerably lower concentrations of gallic acid compared to *Micractinium* sp., showcasing the distinct composition of these two species[47]. 4-hydroxy benzoic acid content (20 μg g^{-1} sample) was higher in *C. pyrenoidosa* compared to mixotrophic *Micractinium* sp. (approximately 2 μg g^{-1} DW in methanol extract) but much lower than in heterotrophically grown culture (approximately 400 μg g^{-1} DW in methanolic extract). The significant increase in 4-hydroxy benzoic acid content under heterotrophic growth in *Micractinium* sp. raises questions about the underlying mechanisms governing these variations, especially considering its known antimicrobial properties used in various industries (food, pharmaceutical, and cosmetics).

Interestingly, despite *Micractinium* sp. displaying a higher total flavonoid content than *Scenedesmus* sp. ME02, specific flavonoids like quercetin and rutin were found to be significantly lower in *Micractinium* sp. This suggests the possible presence of other, unexplored flavonoids in *Micractinium* sp., hinting at the complexity and diversity of its biochemical profile[34]. Additionally, the comparison of cinnamic acid derivatives, chlorogenic, and caffeic acid concentrations between *Scenedesmus* sp. and *Micractinium* sp. adds another layer to the variations in flavonoid composition within different microalgal species collected from the same geothermal flora[54].

In emphasizing the importance of specific compounds within microalgae, it is crucial to consider the diverse range of metabolites and their potential applications. Major carotenoid groups, including carotenes (such as β-carotene and lycopene) and xanthophylls (like lutein, astaxanthin, and fucoxanthin), each serve distinct roles.

The prevalence of β-carotene in green microalgae like *Dunaliella salina* and *Spirulina maxima* underscores their nutritional significance, while *Haematococcus pluvialis* stands out as a key source of astaxanthin—a commercially valuable product renowned for its various health benefits[2,55]. In our study, we also quantified β-carotene in methanolic extracts of both mixotrophically and heterotrophically cultivated *Micractinium* sp., noting slightly higher β-carotene content in mixotrophic cultures compared to heterotrophic ones. Despite this minor difference, β-carotene accounted for approximately 2% of the total carotenoids in methanolic extracts of *Micractinium* sp., highlighting its substantial presence. These findings underscore the influence of cultivation conditions on carotenoid biosynthesis, with light exposure likely boosting β-carotene production in mixotrophic cultures due to its role in photoprotection and light harvesting.

The methanolic extract of *Micractinium* sp., which demonstrated the highest antioxidant activity under mixotrophic cultivation, underwent further evaluation for its potential to mitigate intracellular oxidative stress and apoptosis induced by H_2O_2 in MCF-7 cells. Utilizing the DCFH-DA assay, a well-established method for measuring intracellular ROS levels, our study revealed a significant reduction in ROS in a concentration-dependent manner following pre-incubation with the microalgal extract[41]. This substantial decrease in ROS levels is particularly noteworthy as it highlights the potent antioxidant capacity of extracts in protecting cells from oxidative damage induced by H_2O_2, a stable ROS generator known to cause significant cellular damage at elevated concentrations[39]. The concentration-dependent response observed in this study aligns with the notion that higher concentrations of antioxidants can more effectively neutralize ROS, thereby providing greater protection against oxidative stress.

Apoptosis, or programmed cell death, represents a fundamental cellular process crucial for maintaining tissue homeostasis and eliminating damaged or aberrant cells. Dysregulation of apoptotic pathways is closely associated with various pathological conditions, including cancer[57]. In our study, we explored the potential of *Micractinium* sp. extracts in modulating apoptotic responses induced by H_2O_2, a potent oxidizing agent known to trigger apoptotic cascades in cancer cells[39]. Our findings reveal a significant reduction in apoptotic rates in MCF-7 breast adenocarcinoma cells pre-treated with *Micractinium* sp. methanolic extracts, suggesting a cytoprotective effect against H_2O_2-induced apoptosis. This finding suggests that the methanolic extract of *Micractinium* sp. not only scavenges ROS effectively but also enhances cell survival under oxidative stress conditions. The significant improvement in cell viability and prevention of necrotic and apoptotic cell death pathways underscore the therapeutic potential of *Micractinium* sp. extracts in combating oxidative stress-related cellular damage.

These findings align with prior research indicating that microalgal extracts possess robust antioxidant properties and effectively alleviate oxidative stress in diverse cell lines[17,21,58]. In a previous study, Bechelli et al. investigated the cytotoxic effects of algae, including *Dunaliella salina* extracts, on normal hematopoietic and leukemia cells by Annexin staining, demonstrating a significant reduction in cell viability induced by *D. salina* ethanolic extracts[59]. Similarly, Karakaş et al. demonstrated that the cytotoxic effects of extracts from *Chlorella protothecoides* and *Nannochloropsis oculate* on human brain glioblastoma and colon colorectal carcinoma cell lines[20]. To the best of our knowledge, the current study marks the first demonstration of in vitro cytoprotective activity in cell extracts from a *Micractinium* species. Furthermore, while other studies have explored the cytotoxic effects of various algae extracts on different cell lines, this study uniquely demonstrates the in vitro cytoprotective activity of *Micractinium* species, opening avenues for further investigations into specific bioactive compounds.

The ability of *Micractinium* sp. extracts to modulate cell death pathways and enhance cellular viability in the face of oxidative stress holds significant implications for biomedical applications. While our study provides valuable insights into the cytoprotective effects of *Micractinium* sp. extracts against H_2O_2-induced oxidative stress in breast adenocarcinoma cells, several avenues for future research warrant exploration. Further elucidation of the underlying molecular mechanisms governing the cytoprotective activity of *Micractinium* sp. extracts, including their impact on apoptotic signaling pathways and cellular redox balance, is essential for fully harnessing their therapeutic potential.

In addition to whole cell extracts, specific bioactive compounds derived from microalgae have been examined for their antioxidant activity on cell lines. For instance, β-carotene extracted from *D. salina* strongly reduced cell viability of prostate cancer cells[23]. Another carotenoid, violaxanthin isolated from *D. tertiolecta* showed anti-cancer activity on MCF-7 cells[22]. Polyunsaturated fatty acids extracted from *Nannochloropsis salina* also exhibited in vitro anti-proliferative effect on MCF-7 cells[25]. While these studies highlight the potential of individual compounds, the use of crude extracts is also important. Crude extracts contain a complex mixture of various bioactive compounds that can work synergistically, potentially enhancing their overall antioxidant and cytotoxic effects. This synergism can lead to a more effective mitigation of oxidative stress and inhibition of cancer cell proliferation compared to isolated compounds. Therefore, exploring the bioactivity of crude extracts provides a holistic understanding of their therapeutic potential and can uncover interactions that may be missed when studying single compounds. *Micractinium* sp. contains a rich profile of fatty acids, which may collectively contribute to its antioxidant activity.

The versatile characteristics of *Micractinium* sp., including its adaptability to both mixotrophic and heterotrophic conditions, wide temperature range (16–50 °C), and diverse biochemical composition, position it as an ideal candidate for mass cultivation with promising applications in the nutraceutical and food industries. Our study represents the first attempt to quantify specific phenolic compounds in a *Micractinium* species and compare their concentrations under different cultivation methods. Significantly, the antioxidant-rich extracts of *Micractinium* sp. exhibited a notable inhibitory effect on ROS production and apoptosis induced by H_2O_2 in MCF-7 cells. This discovery provides valuable insights into the relatively unexplored field of in vitro antioxidant activity of green microalgae for potential biotechnological applications. Future investigations focusing on the identification and characterization of specific bioactive compounds derived from *Micractinium* sp. can further enhance our understanding of its antioxidant activity, both in vitro and in vivo, thus contributing to the advancement of microalgal biotechnology.

Conclusion

In this study, the evaluation of antioxidant activity in mixotrophically and heterotrophically grown *Micractinium* sp. cells using six different solvents for extraction has yielded significant insights. Among these solvents, methanol emerged as particularly effective, with *Micractinium* sp. methanolic extracts demonstrating the highest antioxidant activity. The notable reduction in oxidative stress and the observed cytoprotective effects on MCF-7 cells underscore the therapeutic potential of *Micractinium* sp., particularly in addressing oxidative stress-related disorders.

A comprehensive comparative analysis revealed intriguing distinctions between mixotrophically and heterotrophically grown microalgal extracts. Overall, mixotrophic samples exhibited a superior antioxidant capacity, accompanied by higher levels of total phenolics, flavonoids, and carotenoids. This suggests that the cultivation method has a significant impact on the biochemical composition of *Micractinium* sp., influencing its potential health-promoting attributes.

Specifically, mixotrophic samples displayed elevated concentrations of gallic acid and rutin, compounds associated with various health benefits. In contrast, heterotrophic samples showcased substantial accumulations of 4-hydroxy benzoic acid and cinnamic acid, indicating a distinct biochemical profile under these growth conditions.

This study breaks new ground by quantifying the amounts of these phenolic compounds in a *Micractinium* species for the first time. Moreover, it pioneers the documentation of the antioxidant and cytoprotective activities of *Micractinium* sp., expanding the understanding of its potential applications in microalgal biotechnology.

Future investigations could focus on the targeted extraction of specific bioactive compounds from *Micractinium* sp. This approach would allow for a more detailed exploration of the in vitro and in vivo antioxidant activities, both in isolation and in conjunction with whole cell extracts. Such focused studies will undoubtedly contribute to unraveling the therapeutic potential and specific health benefits associated with *Micractinium* sp. ME05.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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References

1. Forman, H. J. & Zhang, H. Targeting oxidative stress in disease: Promise and limitations of antioxidant therapy. *Nat. Rev. Drug Discov.* **20**(9), 689–709 (2021).
2. Maoka, T. Carotenoids as natural functional pigments. *J. Nat. Med.* **74**(1), 1–16 (2020).
3. Zhang, H. & Tsao, R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Curr. Opin. Food Sci.* **8**, 33–42 (2016).
4. Brewer, M. S. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. *Compr. Rev. Food Sci. Food Saf.* **10**(4), 221–247 (2011).
5. Dai, J. & Mumper, R. J. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules* **15**(10), 7313–7352 (2010).
6. Abbaszadeh, H., Keikhaei, B. & Mottaghi, S. A review of molecular mechanisms involved in anticancer and antiangiogenic effects of natural polyphenolic compounds. *Phytother. Res.* **33**(8), 2002–2014 (2019).
7. Yuan, M. *et al.* The role of bioactive compounds in natural products extracted from plants in cancer treatment and their mechanisms related to anticancer effects. *Oxid. Med. Cell. Longev.* **66**, 22 (2022).
8. Vacca, R. A. *et al.* Plant polyphenols as natural drugs for the management of down syndrome and related disorders. *Neurosci. Biobehav. Rev.* **71**, 865–877 (2016).
9. Lobiuc, A. *et al.* Future antimicrobials: Natural and functionalized phenolics. *Molecules* **28**(3), 1114 (2023).
10. Quradha, M. M. *et al.* Comparative assessment of phenolic composition profile and biological activities of green extract and conventional extracts of *Salvia sclarea*. *Sci. Rep.* **14**(1), 1885 (2024).
11. Lee, A. H. *et al.* Fucoxanthin from microalgae *Phaeodactylum tricornutum* inhibits pro-inflammatory cytokines by regulating both NF-κB and NLRP3 inflammasome activation. *Sci. Rep.* **11**(1), 543 (2021).
12. Ferdous, U. T. & Yusof, Z. N. B. Medicinal prospects of antioxidants from algal sources in cancer therapy. *Front. Pharmacol.* **12**, 593116 (2021).
13. Poljsak, B., Kovač, V. & Milisav, I. Antioxidants, food processing and health. *Antioxidants* **10**(3), 433 (2021).
14. Franco, R., Navarro, G. & Martínez-Pinilla, E. Antioxidants versus food antioxidant additives and food preservatives. *Antioxidants* **8**(11), 542 (2019).
15. Coulombier, N., Jauffrais, T. & Lebouvier, N. Antioxidant compounds from microalgae: A review. *Mar. Drugs* **19**(10), 549 (2021).
16. Almendinger, M. *et al.* Characterization of selected microalgae and cyanobacteria as sources of compounds with antioxidant capacity. *Algal Res.* **53**, 102168 (2021).
17. Sansone, C. *et al.* The green microalga *Tetraselmis suecica* reduces oxidative stress and induces repairing mechanisms in human cells. *Sci. Rep.* **7**(1), 1–12 (2017).
18. El-Baz, F. K., Hussein, R. A., Mahmoud, K. & Abdo, S. M. Cytotoxic activity of carotenoid rich fractions from *Haematococcus pluvialis* and *Dunaliella salina* microalgae and the identification of the phytoconstituents using LC-DAD/ESI-MS. *Phytother. Res.* **32**(2), 298–304 (2018).
19. Abd El-Hack, M. E. *et al.* Microalgae in modern cancer therapy: *Current knowledge*. *Biomed. Pharmacother.* **111**, 42–50 (2019).
20. Karakaş, C. Y., Tekarslan Şahin, H., Inan, B., Özçimen, D. & Erginer, Y. Ö. *In vitro* cytotoxic activity of microalgal extracts loaded nano–micro particles produced via electrospraying and microemulsion methods. *Biotechnol. Prog.* **35**(6), e2876 (2019).
21. Bulut, O., Sönmez, Ç. & Öktem, H. A. *Hindakia tetrachotoma* ME03 (Chlorophyta) has high phenolic content, antioxidant capacity, and attenuates H_2O_2-induced oxidative stress and apoptosis in human cells. *Phycologia* **66**, 1–10 (2023).
22. Pasquet, V. *et al.* Antiproliferative activity of violaxanthin isolated from bioguided fractionation of *Dunaliella tertiolecta* extracts. *Mar. Drugs* **9**(5), 819–831 (2011).

23. Jayappriyan, K. R., Rajkumar, R., Venkatakrishnan, V., Nagaraj, S. & Rengasamy, R. In vitro anticancer activity of natural β-carotene from *Dunaliella salina* EU5891199 in PC-3 cells. *Biomed. Prev. Nutr.* **3**(2), 99–105 (2013).
24. Sanjeewa, K. K. A. *et al.* Anti-Inflammatory and anti-cancer activities of sterol rich fraction of cultured marine microalga *Nannochloropsis oculata*. *Algae* **31**(3), 277–287 (2016).
25. Sayegh, F. *et al.* Production of polyunsaturated single cell oils possessing antimicrobial and anticancer properties. *Ann. Microbiol.* **66**(3), 937–948 (2016).
26. Krivina, E. *et al. Micractinium lacustre* and *M. thermotolerans* spp. nov. (Trebouxiophyceae, Chlorophyta): Taxonomy, temperature-dependent growth, photosynthetic characteristics and fatty acid composition. *Algal Res.* **71**, 103042 (2023).
27. Abou-Shanab, R. A. *et al.* Cultivation of a new microalga, *Micractinium reisseri*, in municipal wastewater for nutrient removal, biomass, lipid, and fatty acid production. *Biotechnol. Bioprocess Eng.* **19**, 510–518 (2014).
28. Paliwal, C. *et al.* Microalgal carotenoids: Potential nutraceutical compounds with chemotaxonomic importance. *Algal Res.* **15**, 24–31 (2016).
29. Malavasi, V., Soru, S. & Cao, G. Extremophile microalgae: The potential for biotechnological application. *J. Phycol.* **56**(3), 559–573 (2020).
30. Onay, M., Sonmez, C., Oktem, H. A. & Yucel, A. M. Thermo-resistant green microalgae for effective biodiesel production: Isolation and characterization of unialgal species from geothermal flora of Central Anatolia. *Bioresour. Technol.* **169**, 62–71 (2014).
31. Engin, I. K., Cekmecelioglu, D., Yücel, A. M. & Oktem, H. A. Heterotrophic growth and oil production from *Micractinium* sp. ME05 using molasses. *J. Appl. Phycol.* **30**(6), 3483–3492 (2018).
32. Engin, I. K., Cekmecelioglu, D., Yücel, A. M. & Oktem, H. A. Evaluation of heterotrophic and mixotrophic cultivation of novel *Micractinium* sp. ME05 on vinasse and its scale up for biodiesel production. *Bioresour. Technol.* **251**, 128–134 (2018).
33. Sonmez, C., Elcin, E., Akın, D., Oktem, H. A. & Yucel, M. Evaluation of novel thermo-resistant *Micractinium* and *Scenedesmus* sp. for efficient biomass and lipid production under different temperature and nutrient regimes. *Bioresour. Technol.* **211**, 422–428 (2016).
34. Bulut, O. *et al.* Phenolic compounds, carotenoids, and antioxidant capacities of a thermo-tolerant *Scenedesmus* sp. (Chlorophyta) extracted with different solvents. *J. Appl. Phycol.* **31**(3), 1675–1683 (2019).
35. Herald, T. J., Gadgil, P. & Tilley, M. High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. *J. Sci. Food Agric.* **92**(11), 2326–2331 (2012).
36. Lichtenthaler, H. K. & Buschmann, C. Chlorophylls and carotenoids: Measurement and characterization by UV–Vis spectroscopy. *Curr. Protoc. Food Anal. Chem.* **1**(1), F4–F3 (2001).
37. Cheng, Z., Moore, J. & Yu, L. High-throughput relative DPPH radical scavenging capacity assay. *J. Agric. Food Chem.* **54**(20), 7429–7436 (2006).
38. Firuzi, O., Lacanna, A., Petrucci, R., Marrosu, G. & Saso, L. Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. *Biochimica et Biophysica Acta BBA-General Subjects* **1721**(1–3), 174–184 (2005).
39. Zhuang, Y., Ma, Q., Guo, Y. & Sun, L. Protective effects of rambutan (*Nephelium lappaceum*) peel phenolics on H_2O_2-induced oxidative damages in HepG2 cells and d-galactose-induced aging mice. *Food Chem. Toxicol.* **108**, 554–562 (2017).
40. Shetty, V. & Sibi, G. Relationship between total phenolics content and antioxidant activities of microalgae under autotrophic, heterotrophic and mixotrophic growth. *J. Food Resour. Sci.* **4**(1), 1–9 (2015).
41. Oparka, M. *et al.* Quantifying ROS levels using CM-H2DCFDA and HyPer. *Methods* **109**, 3–11 (2016).
42. Monteiro, M. *et al.* Effect of extraction method and solvent system on the phenolic content and antioxidant activity of selected macro-and microalgae extracts. *J. Appl. Phycol.* **32**, 349–362 (2020).
43. Farvin, K. S. & Jacobsen, C. Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chem.* **138**(2–3), 1670–1681 (2013).
44. Wang, T., Jonsdottir, R. & Ólafsdóttir, G. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chem.* **116**(1), 240–248 (2009).
45. Jerez-Martel, I. *et al.* Phenolic profile and antioxidant activity of crude extracts from microalgae and cyanobacteria strains. *J. Food Qual.* **2017**, 1–8. https://doi.org/10.1155/2017/2924508 (2017).
46. Goiris, K. *et al.* Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *J. Appl. Phycol.* **24**(6), 1477–1486 (2012).
47. Machu, L. *et al.* Phenolic content and antioxidant capacity in algal food products. *Molecules* **20**(1), 1118–1133 (2015).
48. Munteanu, I. G. & Apetrei, C. Analytical methods used in determining antioxidant activity: A review. *Int. J. Mol. Sci.* **22**(7), 3380 (2021).
49. Gauthier, M. R., Senhorinho, G. N. A. & Scott, J. A. Microalgae under environmental stress as a source of antioxidants. *Algal Res.* **52**, 102104 (2020).
50. Engin, I. K., Cekmecelioglu, D., Yücel, A. M. & Oktem, H. A. Enhancement of heterotrophic biomass production by Micractinium sp. ME05. *Waste Biomass Valoriz.* **9**(5), 811–820 (2018).
51. Abreu, A. P., Morais, R. C., Teixeira, J. A. & Nunes, J. A comparison between microalgal autotrophic growth and metabolite accumulation with heterotrophic, mixotrophic and photoheterotrophic cultivation modes. *Renew. Sustain. Energy Rev.* **159**, 112247 (2022).
52. Safafar, H., Van Wagenen, J., Møller, P. & Jacobsen, C. Carotenoids, phenolic compounds and tocopherols contribute to the antioxidative properties of some microalgae species grown on industrial wastewater. *Mar. Drugs* **13**(12), 7339–7356 (2015).
53. Kozłowska, A. & Szostak-Węgierek, D. Flavonoids—Food sources, health benefits, and mechanisms involved. In *Bioactive Molecules in Food. Reference Series in Phytochemistry* (eds Mérillon, J. M. & Ramawat, K.) (Springer, 2019). https://doi.org/10.1007/978-3-319-78030-6_54.
54. Goiris, K. *et al.* Detection of flavonoids in microalgae from different evolutionary lineages. *J. Phycol.* **50**(3), 483–492. https://doi.org/10.1111/jpy.12180 (2014).
55. Sathasivam, R. & Ki, J. S. A review of the biological activities of microalgal carotenoids and their potential use in healthcare and cosmetic industries. *Mar. Drugs* **16**(1), 26 (2018).
56. Li, H. B. *et al.* Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food Chem.* **102**(3), 771–776 (2007).
57. Vitale, I. *et al.* Apoptotic cell death in disease—Current understanding of the NCCD 2023. *Cell Death Differ.* **30**(5), 1097–1154 (2023).
58. Vahdati, S. N., Lashkari, A., Navasatli, S. A., Ardestani, S. K. & Safavi, M. Butylated hydroxyl-toluene, 2,4-Di-tert-butylphenol, and phytol of Chlorella sp. protect the PC12 cell line against H_2O_2-induced neurotoxicity. *Biomed. Pharmacother.* **145**, 112–415 (2022).
59. Bechelli, J., Coppage, M., Rosell, K. & Liesveld, J. Cytotoxicity of algae extracts on normal and malignant cells. *Leukemia Res. Treat.* **6**, 66 (2011).

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Author contributions

O.B, Ç.S and H.A.Ö. designed research, O.B. and I.E.K performed the experiments, O.B. and Ç.S. analyzed the data, O.B. and Ç.S. wrote the manuscript, All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Ç.S.

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