Original Article

Chemical Composition, Antioxidant, and Cytotoxic Potential of *Nannochloropsis* Species Extracts

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Abstract

Context: Screening of natural biomolecules from microalgae. **Background:** The microalgae were recognized for their biological and pharmacological importance of active natural products with high antioxidant and antiproliferative profile. In the preliminary screening, three species *Nannochloropsis* sp. (NC) (green algae), *Amphora* sp. (diatom), and *Nostoc* sp. (blue-green algae) were tested and *Nannochloropsis* was selected based on their scavenging properties. **Objective:** The objective of the study is to explore the biological information of microalgal species where the clinical investigation is still quite limited. **Materials and Methods:** The phytochemical screening of selected NC. primarily comprises saponins, terpenoids, flavonoids, and phenols which were confirmed by high-performance thin-layer chromatography, Fourier transform infrared, and gas chromatography–mass spectra analysis. **Results:** The ethyl acetate extract *Nannochloropsis* hexane (EAENH) fraction showed 40.61 mg GAE/g, 68.77 mg QE/g, 5.73 mg/g, and 57.38 mg CHL/g for total phenolic, flavonoid, carotenoid, and sterol content, respectively. Moreover, antioxidant activities were evaluated for the extract showing high flavonoid and phenolic contents after partial purification with hexane. The half inhibitory concentration (IC_{50}) values for EAENH was found to be 13.9, 21.22, and 14.58 µg/mL for 1,1-diphenyl-2-picrylhydrazyl radical, hydrogen peroxide, and reducing power assays, respectively. The antiproliferative activity of EAENH on human non-small lung cancer cell line (A549) IC_{50} value was 175 µg/mL using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **Conclusion:** The present study confirmed that the bioactive components present in the EAENH were accountable for excellent antioxidant and cytotoxic properties.

Keywords: Antioxidant, cytotoxic, gas chromatography–mass spectra, high-performance thin-layer chromatography, *Nannochloropsis*

Introduction

Microalgae are photosynthetic eukaryotes which comprise prime elements of freshwater and marine phytoplankton. They primarily act as a food source for other marine organisms and an excellent source of lipids, pigments, carotenoids, omega-3-fatty acids, and surplus biochemical.[1] In living schemes beneath stress conditions, the excessive generation of hydroxyl (OH) and alternative extremely reactive oxygen species (ROS) generates oxidative injury through the several biomolecules with ROS as well as DNA.[2] Very few studies were undergone to explore the quantification and documentation of antioxidant compounds of microalgae even though more antioxidant profile in microalgae have been affected[3] including the impact of phenolic in microalgae resistance systems opposing ROS accumulation.[4]

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Carotenoids are the principal antioxidant compounds from microalgae. They can be divided into two groups: carotenes and xanthophylls. Acetylenic and allenic carotenoids, such as fucoxanthin, neoxanthin, and violaxanthin correspondingly,[5] are vastly epitomized in red and green algae, and thirty various carotenoids as a minimum had been recognized in this class.[6] It has been stated that carotenoids have diverse biological properties such as antioxidant, anti-inflammatory, antiproliferative, antiatherogenic, and chemotherapeutic agent to treat several types of cancer such as stomach, lung, liver, breast, colon, and prostate.

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Cell disruption is generally essential for recovering intracellular products from microalgae. The cell walls can intensely modify any extraction method by lowering the cell biodegradability.[7] The cell disruption methods such as mechanical and chemical treatments including high-pressure homogenizers, supercritical fluid, pressurized liquid, ultrasounds, microwaves, autoclaving, and addition of sodium hydroxide, hydrochloric acid, or alkaline lysis have been used effectively.[8] The above techniques have some hitches correlated to the thermal denaturation of by-products that might be due to their raised extraction temperature.[9-11] Hence, selecting of a suitable extraction method counts on numerous factors such as biomass with its extract, end use, and thermolability.[12,13]

The solvents are extensively used to extract algal metabolites from algal biomass.[14] For instance, solvent residues, the presence of the cell wall and physiological properties such as location of the bioactive content stored in the cell could prevent direct contact between the solvent and cell membrane that hinder the extraction.[15] At times, solvent extraction of dry biomass has evidenced successful recovery of intracellular metabolites than wet biomass.[16] Homogenization disrupts the cell wall when cells are forced through a small opening at high pressures allowing the extraction of biomolecules.[17] Supercritical fluid extraction using carbon dioxide (CO_2) as an extraction fluid illustrates an effective substitute to conventional techniques in relation to purity and yield.[9,12] The pressurized liquid extraction has validated to be a sound alternative to improve the extraction yield of lipids and carotenoids.[18]

Chlorella ovalis, *Nannochloropsis oculata,* and dinoflagellate *Amphidinium carterae* showed antiproliferative and anti-inflammatory properties.[19] *Nannochloropsis* sp. (NC) exhibited strong antioxidant activates in a similar study performed earlier.[20] Very few therapeutic biochemical acquired from algae have been successfully marketed and many are underneath clinical trials.[21] Hence, the assessment of such properties endures a motivating and worthwhile task, mainly for discovering innovative sources of natural antioxidants.

NC of the Eustigmatophyceae class and Chlorophyceae green algal group around 2–5 μm width is spherical and unicellular[22] which stores carotenoids in stressful states. *Amphora,* considered as the principal species, is a key group of marine and freshwater diatoms which are under class Bacillariophyceae, order Thalassiophysales, and family Catenulaceae. *Nostoc* belongs to family Nostocaceae, and order Nostocales are present in diverse environments that form colonies comprising filaments of moniliform cells coming under cyanobacteria.[23]

It is necessary to conduct a comprehensive screening of the therapeutic activities for NC since only few studies have been done. The principal stimulation to initiate this work attributes to inadequate data on biochemicals and their properties in algal species. The foremost objective of this paper is to select the microalgae after primary 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) screening, followed by extraction with solvents, identification and quantification of bioactive compounds, further assessed by performing phytochemical and biological screening including *in vitro* antioxidant and cytotoxic assays. The fractions showing high polyphenolic contents are directed to thin-layer chromatography (TLC) analysis to identify the active biochemicals followed by high-performance TLC (HPTLC), Fourier transform infrared spectra (FTIR), and gas chromatography–mass spectra (GCMS) analysis.

Materials and Methods

Reagents and chemicals

Methanol, ethanol, dichloromethane, hexane, diethyl ether, and acetone were purchased from Virat Lab, Hyderabad, India. DPPH, potassium ferricyanide ($K_3Fe[CN]_6$), l-ascorbic acid, and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich, India. Quercetin, cholesterol, and gallic acid were procured from Molychem Pvt. Ltd. Mumbai, India. For assays, hydrogen peroxide, ferrous chloride ($FeCl_2$), ferric chloride ($FeCl_3$), ferrous sulfate, aluminum chloride, sodium bicarbonate, ferric chloride, and silica gel G were purchased from Hi-Media Laboratories and Molychem Pvt. Ltd. Mumbai, India. Folin–Ciocalteu phenol reagent, Liebermann–Burchard reagent, ninhydrin reagent, anisaldehyde spray, vanillin, hydrochloric acid, sulfuric acid, methanol, n-hexane, acetonitrile, chloroform, and benzene were procured from SD Fine Chemicals Limited, Mumbai. All the other chemicals and solvents used were of analytical grade.

Sample collection

The sources of three microalgae NC (green algae), *Amphora* sp. (diatom), and *Nostoc* sp. (blue-green algae) were collected from the Marine Department, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. Microalgae were raised in seawater with a salinity of 28/30 ppt. The green and blue-green algae were nurtured in Conway media, which were supplemented with the main mineral solution, a silicate solution, a trace metal solution, and a vitamin solution as nutrients. The diatoms were grown well in TMRL media, where the main mineral solution was incorporated. The media was maintained in pH 8 with proper aeration and illumination at 23°C ± 2°C. The compositions of the media were given in Table 1. For every 5 days, the microalgae were collected and rinsed with either distilled water or ammonium sulfate to carry out the preliminary screening. Then, they were dried under shade, finely powdered, frozen, and stored at 20°C.[24]

Preliminary screening for selection of microalgae

Initially, preliminary antioxidant screening was quantified at each stage of growth (lag, exponential, stationary, and decline phases) for NC, *Amphora* species, and *Nostoc* species using DPPH assay. Once the algae have been chosen, cultures were grown for 13 days (the stationary phase), and the cells were harvested and dried to estimate the biomass.

Solvent extraction and partial purification

Freeze-dried microalgae (5 g) were extracted with 500 mL of solvents ethyl acetate, ethanol, and acetone for 20 min at 40°C with rotational velocity 6000 rpm using an Ultra-Turrax T-25 Homogenizer. The resulting slurry was then cooling centrifuged at 3000 rpm for 15 min and filtered. The filter cake was re-extracted for 20 times until it became colorless. The filtrates were combined and concentrated using a rotary vacuum evaporator at 30°C–45°C. All procedures were done in the absence of light, and extraction conditions were given in Table 2. Later, extracts were lyophilized and the quantity of substances extracted was expressed as percentage by weight. The freeze-dried powder was considered as the crude extract of NC.[25] To the crude extract, equal quantity of hexane were added to the separator funnel, kept aside undisturbed, and the upper phase rich in biochemical had been collected after phase separation.[26] For separating active compounds, the resulting hexane phase separations ethyl acetate extract *Nannochloropsis* (EAEN), ethanol extract *Nannochloropsis* (EEN), and acetone extract *Nannochloropsis* (AEN) were partially purified further using an open silica column chromatography and eluted with mixture of hexane: EA, EA: methanol, and toluene: EA step-gradient elution with successive ratios, and nearly five fractions were collected.[27] Then, the fractions were subjected to TLC, detected active fraction for EAEN hexane (EAENH), EEN hexane (EENH), and AENH were evaporated resulting in a concentrated thick residue.

Table 1: Medias used for cultivating microalgae

Ingredients	Quantity
Conway' or Walne's medium	
Nutrient solution a per liter of DW	
FeCl3.6H2O	1.3 g
MnCl2.4H2O	0.36 g
H3BO3	33.6 g
EDTA (disodium salt)	45.0 g
NaH2PO4.2H2O	20.0 g
NaNO3	100.0 g
TMS stock	1.0 ml
TMS B per 100 ml DW	
ZnCl2	2.1 g
CoCl2.6H2O	2.0 g
(NH4) 6Mo7O24.4H	0.9 g
CuSO4.5H2O	2.0 g
This solution is normally cloudy. Acidify with a few drops of concentrated HCl to give a clear solution	
Vitamin solution C per 100 ml	
Cyanocobalamin	10.0 mg
Thiamine	10.0 mg
Biotin	200.0 µg
Medium per liter	
Nutrient solution (A)	1.0 ml
Trace metal solution (B)	0.5 ml
Vitamin solution (C)	0.1 ml
Sterilized seawater	1.0 liter
TMRL medium (Tung Kang Marine Research Laboratory)	
Potassium nitrate	10 g/100 ml of DW
Sodium orthophosphate	1 g/100ml of DW
Ferric chloride	0.3 g/100 ml of DW
Sodium silicate	0.1 g/100 ml of DW

HCl: Hydrochloric acid, TMS: Trace metal solution, DW: Distilled water, EDTA: Ethylenediaminetetraacetic acid

Phytochemical and biochemical screening

The powdered extracts were utilized for phytochemical tests with little modifications.[28] The quantity of chlorophyll extracted was calculated based on the equations of MacKinney.[29] Total carotenoid and chlorophyll contents were examined as per the Lichtenthaler HK protocol, 1987, by measuring the absorbance at 470 nm for carotenoids and 645 nm (chlorophyll b) and 661.5 nm (chlorophyll a).[30] The EAENH, EENH, and AENH were evaluated for biochemical composition such as phenols, flavonoids, and sterols using gallic acid, quercetin, and cholesterol as standard correspondingly.[31]

Thin-layer chromatography and high-performance thin-layer chromatography

The Merck aluminum plate precoated with silica gel $60F_{254}$ of 0.2-mm thickness TLC plate was prepared with solvent toluene–ethyl acetate–formic acid (8:2:0.2, v/v/v), and fractions were spotted on the bottom of the plate and run in the solvent. The plate was detected through CAMAG TLC visualizer under ultraviolet at 254 and 366 nm,[32] immersed in vanillin–sulfuric acid reagent, and kept in oven at 105°C until the color of the spots was appeared and documented. For HPTLC fingerprint profile, the TLC plate developed above was scanned at a wavelength of 254 and 366 nm using CAMAG TLC Scanner 3 using D2 lamp.[33]

Fourier transform infrared spectra and gas chromatography–mass spectra

FTIR spectra were collected for EAENH at a resolution of 4 cm^{-1} in transmission mode range between 4000 and 400 cm^{-1} using Shimadzu IR spectrophotometer, model 840, Japan. GCMS analysis of EAENH was run using

Table 2: Extraction conditions for *Nannochloropsis* spp.

Microalgae	Solvents used	Number of extraction cycles	Weight of the extract (g)	Percentage yield extraction
Dried *Nannochloropsis* spp. microalgal powder (5 g)	Ethanol (25 ml and 20 min for each cycle)	20	0.6	23.33
	Ethyl acetate (25 ml and 20 min for each cycle)	20	1.9	38.88
	Acetone (25 ml and 20 min for each cycle)	20	0.5	17.77

Shimadzu/QP2020GC instrument coupled with MS-5975 inert MSD and triple-axis mass selective ion detector. The documentation of phytochemical components was attained using the National Institute of Standards and Technology MS library database.[34]

Antioxidant assays

1,1-diphenyl-2-picrylhydrazyl radical assay

In this assay, 50 µL of a freshly prepared ethanol-DPPH solution (0.3 mM) was mixed with 200 µL of EAENH, EENH, and AENH and allowed to react at 37°C in the dark. After 1 h, the absorbance was noted at 517 nm. The mixture of DPPH solution (50 µL) and ethanol (200 µL) was used as negative control. Ascorbic acid was used as positive control, at the same concentrations of the fractions (10–100 µg/mL). A blank solution was the mixture of 250 µL of ethanol and sample extracts.[35-38] The results were converted into percentage antioxidant activity (AA) using the following equation:

$$\% \text{ DPPH scavenging activity} = \frac{(\text{Control absorbance} - \text{Sample absorbance}) \times 100}{\text{Control absorbance}}$$

Hydrogen peroxide assay

Hydrogen peroxide scavenging activity was estimated by incubating the reaction mixture with different concentrations (10–100 µg/ml) of EAENH, EENH, and AENH (1 ml), 2.5 ml of phosphate buffer (pH 7.4; 100 mM), and 400 µl of H_2O_2 (5 mM) for 20 min.[35,39] The reaction mixture was observed at 610 nm after incubation. The blank was considered as mixture without sample and ascorbic acid as control for every experiment.

Ferric oxide reducing power assay

Several concentrations (10–100 µg/ml) of EAENH, EENH, and AENH (1 ml) were dissolved into 0.2-M phosphate buffer (pH 6.6) with 1% of potassium ferricyanide (2.5 ml) and robustly mixed. The reaction was ceased by adding 1 ml of 10% TCA after incubation at 50°C for 20 min. The reaction mixture was centrifuged at 3000 rpm for 15 min, and ferric chloride was dissipated into the supernatant. The solution was measured at 700 nm against ascorbic acid as a control and reducing power was estimated.[40-42]

Cell viability assay

Cell viability test was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Vijayan *et al*., 2018.[43,44] Human non-small lung cancer cell line (A-549) was cultured with DMEM media supplemented with 10% fetal bovine serum and antibiotic mixture (penicillin, ampicillin, and streptomycin 100 Units/mL) maintained at 37°C, 95% humidity, and 5% CO_2. The cells were seeded into a 96-well plate at the density of 2×10^4 per well. After 24 h of incubation at 37°C, the cells were treated with concentrations of EAENH fractions in the range 20-200 µg/mL (microgram per milliliter) by serial dilution with dimethyl sulfoxide and vincristine drug as standard (0.1g/mL). The A-549 cells were trypsinized and counted using hemocytometer. Then, 100 µl of A-549 cells was added to the poly-L-lysine-coated 96-well plate and incubated at 37°C in a humidified 5% CO_2 incubator. After 24-h incubation, old medium was replenished with fresh medium, and 50 µl of EAENH fraction was added and incubated for 48 h at 37°C in a humidified 5% CO_2 incubator. Then, 30 µl of 0.5% w/v MTT was added and incubated for 4 h at 37°C. Add 50 µl of acidic-isopropanol after incubation for 30 min at 37°C to dissolve the formazan formed. Then, absorbance was observed at 554 using a microplate reader. Measurements were performed in triplicates, and the concentration that can induce 50% of cytotoxicity was determined graphically using GraphPad Prism software.

The percentage of cell viability was calculated based on the following formula:

$$\% \text{ Cell Viability} = (\text{Abs [sample]}/\text{Abs (control)}) \times 100.$$

All values were represented as the mean ± standard deviation of triplicates ($n = 3$) of each experiment. The data were analyzed using analysis of variance (ANOVA). The findings with $P \leq 0.05$ were measured to be statistically significant. The data were statistically calculated by Microsoft Excel 2007 and linear regression analysis using GraphPad Prism (Windows version 6.01, GraphPad Software, La Jolla, California, USA).

Results

Preliminary screening

Natural antioxidants have been exploited as the potential remedial agent against many diseases including cancer, inflammatory diseases, and aging. The radical scavenging activities (RSAs) of various cell concentrations at different growth phases showed a concentration dependency [Figure 1]. The NC biomass dry weight was noticed to be 5 g. The percentage yield extractions of ethyl acetate (1.9 g), ethanol (0.6 g), and acetone (0.5 g) were determined to be 38.88%, 23.33%, and 17.77% w/w correspondingly where ethyl acetate gave the highest.

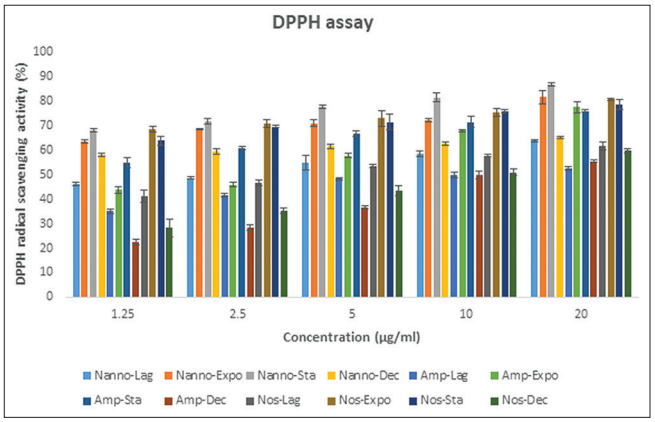

Figure 1: Antioxidant preliminary screening for *Nannochloropsis* sp., *Amphora* sp., and *Nostoc* sp. quantified at different growth phases

Phytochemical screening

In the present study, phytochemical screening for EAENH, EEN hexane (EENH), and AEN hexane (AENH) was performed [Table 3].

Total phenolic, flavonoid, carotenoid, chlorophyll, and sterol content of *Nannochloropsis*

The EAENH contained 215.7-µg/mL chlorophyll a and 345.4-µg/ml chlorophyll b, and total carotenoid content (TCC) ranges between 3.12 and 6.13 mg/g. The highest TCC was exhibited by acetone extract compared to EENH and AENH. Total sterol content (TSC) of the algal extract and its fractions were measured using Liebermann–Burchard reaction. The calibration curve for cholesterol was measured. The microalgae *Schizochytrium aggregatum* extracts rich in lipids have been recognized with antioxidant property.[45] From Table 4, the EAENH was shown to exhibit significantly higher ($P < 0.05$) fatty acids, flavonoids, terpenoids, carotenoids, and polyphenolic contents than the other.

Thin-layer chromatography and high-performance thin-layer chromatography analysis

The partially purified EAENH fraction was tested for bioactive compounds using TLC. The colored spots obtained and the R_f values were compared with the standards published in the previous articles to find out phytochemical constituents.[46] The typical R_f values were given in Table 5. Toluene: ethyl acetate: formic acid (8:2:0.2) was used as the solvent system. The R_f values of the spots were presented in Figure 2.

HPTLC profile revealed clear documentation of the foremost phytochemical components, i.e., terpenoids, flavonoids, and

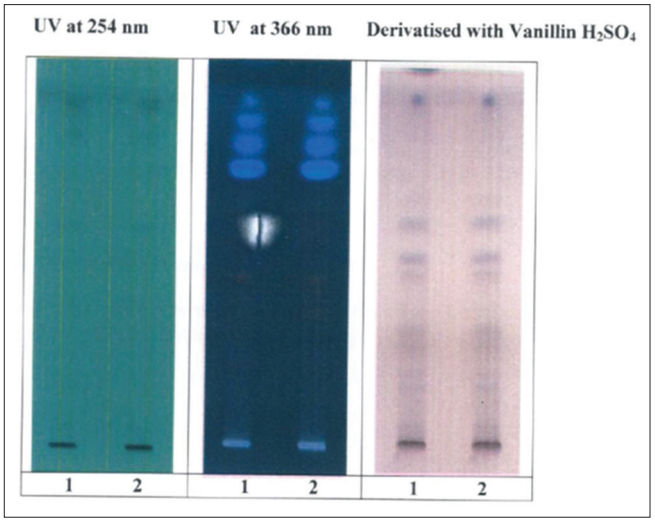

Figure 2: Thin-layer chromatography photodocumentation of EAH fraction ultraviolet at 254 nm and ultraviolet at 366 nm and derivatized with vanillin H2SO4

Table 3: Phytochemical screening of *Nannochloropsis* bioactive fractions

Phytochemical constituents	Tests	EAENH	EENH	AENH
Tannins	Gelatin test	-	-	-
Phenols	Ferric chloride test	++	++	+
Carotenoids	Chloroform and sulfuric acid test	++++	++++	++++
Alkaloids	Mayer's test	+	+	+
	Wagner's test	+	+	+
Flavonoids	Alkaline reagent test	++	++	++
	Ammonium test			
Proteins	Biuret test	+	+	+
Saponins	Foam test	+++++	+++++	++++
Terpenoids/steroids	Liebermann-Burchard reaction	++++	++++	+++
Carbohydrates	Molisch's test	+++	+++	++
	Benedict's test			
Reducing sugars	Fehling's test	++	++	++
Quinones	Borntrager's test	-	-	-

+++: Appreciable amount (positive within 5 min), ++: Moderate amount (positive after 5 min but within 10 min), +: Trace amount (positive after 10 min but within 15 min), -: Negligible, EAENH: Ethyl acetate extract *Nannochloropsis* hexane, EENH: Ethanol extract *Nannochloropsis* hexane, AENH: Acetone extract *Nannochloropsis* hexane

Table 4: Total biochemical contents of *Nannochloropsis* spp.

Biochemical content	Ethyl acetate extract (EAENH)	Ethanol extract (EENH)	Acetone extract (AENH)
TPC (mg GAE/g)	40.61±1.8	26.42±1.5	21.55±1.7
TFC (mg QE/g)	68.77±2.56	48.31±4.86	42.88±5.23
TCC (mg/g)	5.73±0.09	3.12±0.03	6.13±0.13
TSC (mg CHL/g)	57.38±0.3	35.22±0.7	32.65±0.5

Values were reported as mean±SD of three parallel measurements. TPC: Total phenolic content (mg GAE/g extract), TFC: Total flavonoid content (µg QE/g extract), TCC: Total carotenoid content as per Lichtenthaler equations (mg/g extract), TSC: Total sterol content (mg cholesterol/g extract), SD: Standard deviation, EAENH: Ethyl acetate extract *Nannochloropsis* hexane, EENH: Ethanol extract *Nannochloropsis* hexane, AENH: Acetone extract *Nannochloropsis* hexane

saponins present in the EAENH fraction of NC which might correlate to some polar and nonpolar compounds, confirmed by specific R_f values for each standard (sesquiterpene: 0.89, quercetin: 0.53, and stearic acid: 0.39). Toluene: ethyl acetate: formic acid (8:2:0.2) was learned to be the best solvent system. Terpenoids were detected at daylight, 254 nm and 366 nm before derivatization.[47] Eight different terpenoids were separated by seeing in the R_f range of 0.06 to 0.94 [Figure 3a]. The highest and lowest peak areas 23553.7 AU and 227.9 AU were observed at the R_f of 0.89 and 0.18, respectively. Nine compounds were identified to be flavonoids at the R_f in the range of 0.19–0.96 [Figure 3b]. The highest peak area was 2720.0 AU and that of the lower was 227.5 AU observed at R_f of 0.92 and 0.18, respectively.[48] Thirteen different types of saponins were observed. The R_f values for the saponins were in the range of 0.05–0.91.[49] The highest peak area was 9045.6 AU and that of the lowest one was 107.7 AU which were observed at R_f of 0.91 and 0.05, respectively [Figure 3c].

Table 5: Rf values of the ethyl acetate extract *Nannochloropsis* hexane fraction

UV at 254 nm		UV at 366 nm		Derivatized with vanillin-sulfuric acid	
R_f	Color	R_f	Color	R_f	Color
0.06	Green	0.37, 0.47	Red	0.17, 0.21	Gray
0.26, 0.60	Green	0.68	Green	0.28	Gray
0.86, 0.94	Green	0.76, 0.83	Fluorescent blue	0.32, 0.48	Gray
		0.89, 0.95	Fluorescent blue	0.52, 0.62	Purple
				0.95	Blue

UV: Ultraviolet

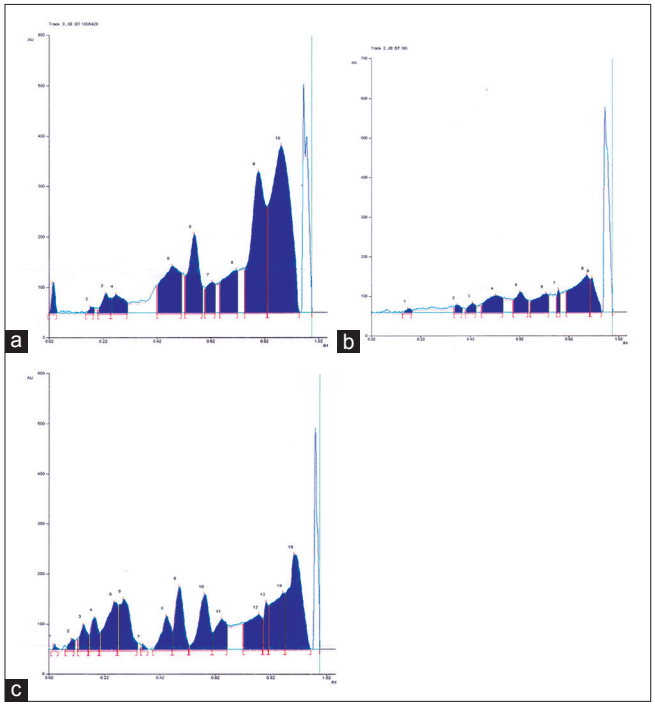

Figure 3: High-performance thin-layer chromatography fingerprint profile of NCEH fractions for terpenoids (a), flavonoids (b), and saponins (c)

Fourier transform infrared spectra analysis

The FTIR spectrum of NC powder [Figure 4a] and partially purified EAENH fraction [Figure 4b] was presented. The absorption band at 3662 cm^{-1} indicates O–H stretching of flavonoid or phenol, and a weak band at 3409 cm^{-1} shows N–H stretching regarding amide A band of protein. An intense band at 2973 $cm^{-1}$was due to $–NH_3^+$ stretching and N–H stretching of amide band of protein, which indicates that the bands were related to carbohydrates. A weak absorption band at 2904 and 2938 cm^{-1} was due to the $–NH_3^+$ stretching and N–H stretching of amide band, respectively, that implies the protein existence. A sharp dominant peak observed at 1230 and 1647 cm^{-1} was due to the C = O stretching of fatty acid esters which signify the prevalence of lipids and a weak absorption band observed at 1452 cm^{-1} due to the CH_2 and CH_3 bending of methyl and C–O stretching of a carboxylic group. The intense band at 1406 cm^{-1} shows amino acids C = O stretching and C = C stretching of phenols and flavonols. The dominant peak at 1256 cm^{-1} shows *P* = O symmetric stretching of phosphodiester bond of nucleic acids and phospholipids. An intense peak was found at 1051 cm^{-1} due to the O–H stretching of flavonol or phenol. The band observed at 880 cm^{-1} shows carbohydrates C–O stretching and 751 cm^{-1} shows C–H bending vibration of an alkyl group. The dominant intensified and weak IR peaks of EAENH imply the presence of the fatty acid esters, lipids, proteins, phenols, and carbohydrates.

Gas chromatography–mass spectra analysis

The predominant constituents of the EAENH fraction were octadecanoic acid (10.9%) pursued by

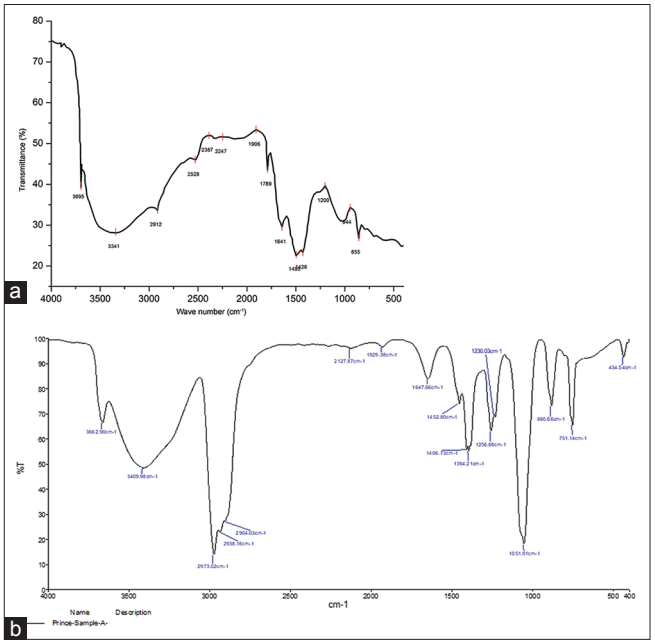

Figure 4: Fourier transform infrared spectra spectrum of the *Nannochloropsis* powder (a) and NCEH fraction (b)

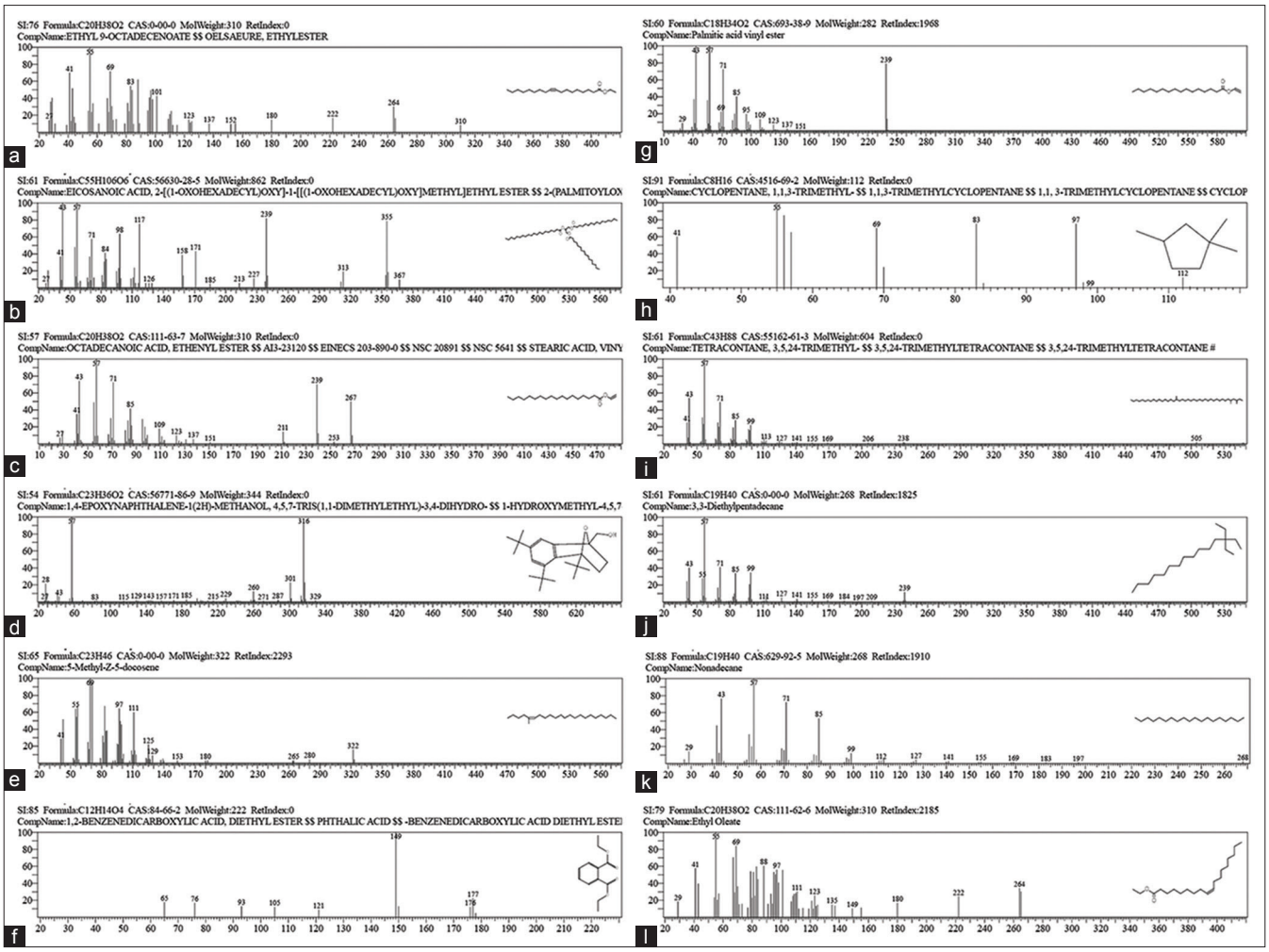

Figure 5: (a) "Ethyl 9-Octadecenoate" at retention time 21.77 and Hit 2. (b) "Eicosanoic acid, 2-[(1-oxohexadecyl)oxy]-1-[[(1-oxohexadecyl)oxy]methyl] ethyl ester" at retention time 23.3 and Hit 5. (c) "Octadecanoic acid, ethenyl ester" at retention time 24.17 and Hit 1. (d) "1,4-epoxynaphthalene-1 (2H)-methanol, 4,5,7-tris (1,1-dimethylethyl)-3,4–dihydro-" at retention time 21.08 and Hit 2. (e) "5-Methyl-Z-5-docosene" at retention time 20.73 and Hit 4. (f) "1,2-Benzenedicarboxylic acid, diethyl ester" at retention time 13.21 and Hit 1. (g) "Palmitic acid vinyl ester" at retention time 24.38 and Hit 3. (h) "1,1,3-Trimethyl cyclopentane" at retention time 7.63 and Hit 2. (i) "3,5,24-Trimethyl tetracontane" at retention time 11.96 and Hit 3. (j) "3,3-Diethylpentadecane" at retention time 9.49 and Hit 5. (k) "Nonadecane" at retention time 5.97 and Hit 1. (l) "Ethyl Oleate" at retention time 19.17 and Hit 1

hexadecanoic acid (8.32%), octadecanoic acid, ethenyl ester (6.87%), 1,4-epoxynaphthalene-1 (2H)-methanol, 4,5,7-tris (1,1-dimethylethyl)-3,4–dihydro-(7.1%), 5-methyl-Z-5-docosene (3.24%), 1,2-benzenedicarboxylic acid (5.61%), and 1,2-cyclopentanediol (4.81%). In the detected 50 compounds, almost 30 were obtainable in traces [Figure 5].

Antioxidant activities

The higher scavenging activity might be credited to the escalated concentration of fatty acids, terpenoids, flavonoids, and polyphenols. From the results, the EAENH fraction showed an excellent AA followed by EENH and AENH fractions. The half inhibitory concentration (IC_{50}) values for EAENH fraction were found to be 13.9, 21.22, and 14.58 µg/mL; for EENH fraction were 31.84, 36.97, and 27.88 µg/mL; and for AENH fraction were 48.01, 57.59 and 33.58 µg/mL for DPPH, hydrogen peroxide assay (HPA), and ferric oxide reducing power assay assays, respectively, which indicate EA as the suitable solvent for extracting biochemicals from NC [Figure 6].

Cell viability assay

The phytochemicals in EAENH fraction confer cytotoxicity as they induce apoptosis by generating RSAs. The standard values were statistically significant compared to control cells ($P < 0.001$). The cell viability of A-549 cells decreased with increase in the concentration of the fraction and highest in 200 µgmL^{-1} with an IC_{50} value of 175 µg/mL which implies NC fraction as a moderate anticancer agent [Figure 7].

The ANOVA of antioxidants assays revealed statistically significant effects owing to the concentration of the fractionated solvent and interactions of the concentration of fractionated

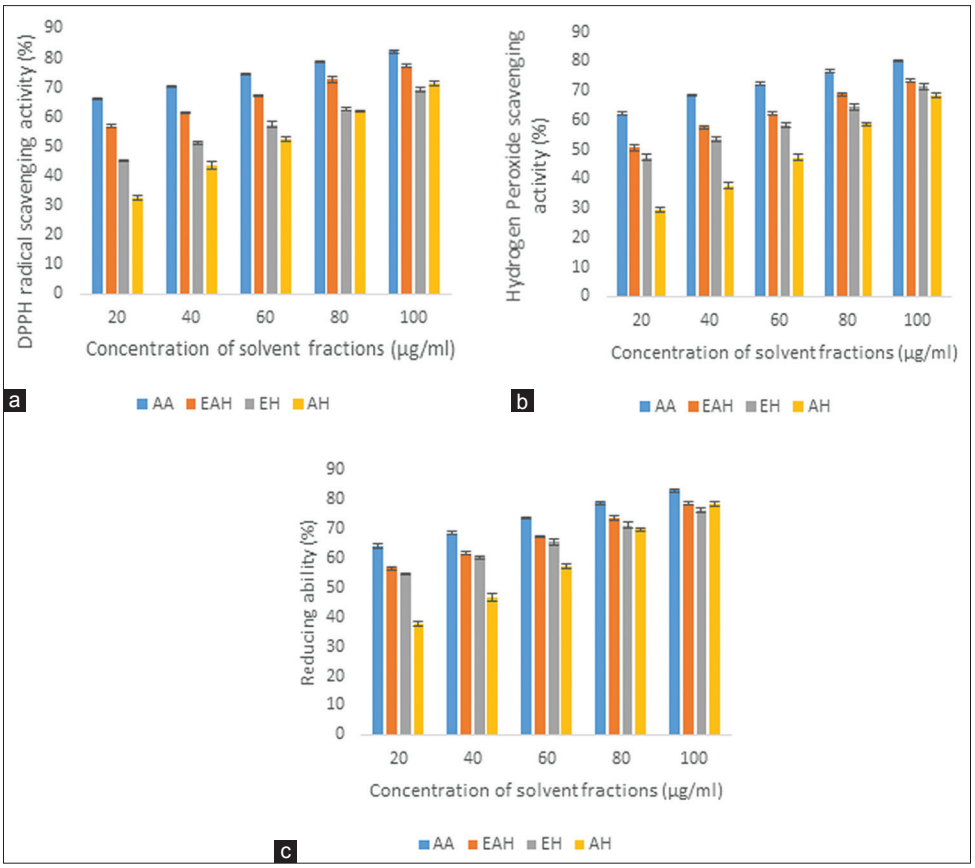

Figure 6: Radical scavenging activity of *Nannochloropsis* sp. EAH fractions at different concentrations. (a) 1,1-diphenyl-2-picrylhydrazyl radical activity, (b) hydrogen peroxide scavenging activity, and (c) reducing power. Each value represents mean ą standard error of mean ($n = 3$)

solvents. The EENH and AENH showed significantly lower AA ($P < 0.005$) compared to EAENH fractions which were concentration dependent.

Discussion

From the preliminary antioxidant assessment, the stationary phases of NC and *Amphora* sp. displayed potent RSAs compared to the *Nostoc* sp. Hence, the stationary phase of NC species was selected for further extraction processes, partial purification, and biochemical studies.

The solvent selection was significant to uphold the extraction since it establishes the degree of affinity to the chemical composition of the constituents to be extracted. The extraction proficiency was highly reliant on polarity of the organic solvent or solvent mixture used. In this study, the extraction process effect on yield and biological screening was assessed with solvents of differing polarities such as acetone (0.355), ethyl acetate (0.460), and ethanol (0.654).[50] The greener solvents used were food grade, less toxic, easily available, and extract phytochemicals effectually.[51] The extraction yield from high to low were as follows: ethyl acetate > ethanol > acetone.

In general, the yield improves with increase in the polarity of solvents, wherein this case ethyl acetate gave higher yield which might attribute to the type of algae, biochemical accumulation in algae, solvent polarity, storage and extraction conditions.[52,53] Moreover, the differences in solvent polarity used determine the type, composition, and AA of phytochemicals.[54] Ethyl acetate effectively extracts alkaloid, glycosides, terpenoid, sterol, and flavonoid. Ethanol can successfully separate polar compounds such as sugar, amino acid, glycoside compounds, phenolic compounds with low and medium molecular weights, flavonoid, terpenoid, anthocyanin, saponin, tannin, phenone, flavone, and polyphenol.[55] Acetone was used to extract photosynthetic pigments with a wide range of polarity.[51]

Phytochemical screening showed a positive test for flavonoids, proteins, carbohydrates, terpenoids/steroids, phenols, and saponins which were evidenced by HPTLC analysis. The total phenolic content (TPC), total flavonoid content (TFC), TCC, and TSC of the EAENH were efficiently good.

The quantity of TPC was higher in polar than nonpolar solvents. The deviations in TPC from solvent extracts were ascribed to the polarities of various compounds present in the algae. The TPC, TFC, and TSC were in the order as follows: EAENH > EENH > AENH. Overall, the extractability of a specific compound was a function of the ratio of solute to solvent. In this study, the recovery of TPC seemed to be reliant on the solvent typology, its polarity index, and solubility of

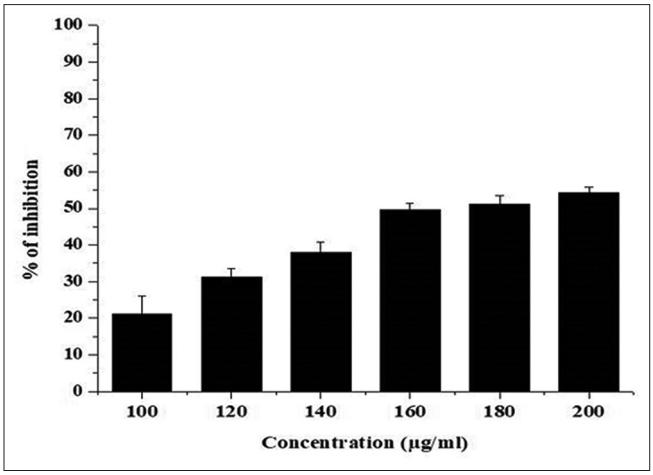

Figure 7: Cell viability assay (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) of ethyl acetate extract *Nannochloropsis* hexane. Each value represents mean ą standard error of mean (n = 3)

TPC in the extraction solvent. The solubility of polyphenols or flavonoids depends on the molecular size, hydroxyl groups, and length of the hydrocarbon.[56]

The results indicate that the maximum TFC was shown by EAENH which might be attributed to the purification and concentration of polyphenolics through the fractionation process, which was possibly responsible for its significant antioxidant property. Position OH and double bonds in flavonoid were determined to give escalated AA.[57]

The TCC was in the order as follows: AENH > EAENH > EENH. The presence of conjugated double bonds in oxygenated diterpenoids, i.e., xanthophylls rich in EAENH attribute to its effectual AA. The TSC was predominant in EAENH compared to the other fractions accountable for its escalated antioxidant property. The present TLC and HPTLC studies validated the presence of saponins, flavonoids, terpenoids, chlorophylls, diterpenes, and phenols at different R_f levels in the EA microalgal extracts of the study species, NC.[46]

The absorption spectra at 1647 cm^{-1} characteristics of C = O groups in lipid esters and the range from 2938 to 1647 cm^{-1} characteristics of CH_2 and CH_3 groups in lipid acyl chains were weakened when compared to microalgae powder spectra after the extraction processes using EA and hexane. The GCMS of EAENH fraction composed mainly of isoprenoids, saturated fatty acids, oxygenated tetraterpenes, a sesquiterpene, diterpenes, oxygenated diterpenes, unsaturated hydrocarbons, aliphatic alkanes, aliphatic esters and heterocyclic compounds, aliphatic alcohols, aromatic compounds, aliphatic amide, and fatty acid ester.

The decline in the DPPH radical concentration was imputed to the scavenging proficiency of the active fractions of NC. The scavenging effect increased with an increase in the fraction concentrations, and ascorbic acid was the standard used. The conjugated double bonds in the xanthophylls, i.e., terpenoids mark it to be an effective antioxidant.[58]

The dose-dependent HPA of EAENH fraction showed excellent scavenging. Goh *et al.* and Sanjeewa *et al.* suggest that various extracted solvents of *Chaetoceros* sp. and NC comprise different powerful antioxidant compounds capable to scavenge various forms of free radicals. The EAENH fraction showed definitive scavenging control and increased as the concentration increased up to 100 µg/mL ($P < 0.05$).

The antioxidant potential ranking order was as follows: EAENH > EENH > AENH. The antioxidant assays indicate that all extracts were acknowledged of donating an electron or hydrogen to the radicals which were regulated by polyphenols. The reports were concurring with earlier studies where algal extracts illustrated affirmative biological activities in relation to antioxidant and antimicrobial activities which were contributed by the distinct biochemicals in NC.[59]

Antiproliferative effect of the EAENH was investigated using human non-small lung cancer cell line (A-549). The cell toxicity was found to be dose dependent. Recent research stated the antioxidant and anticancer activities of ethanol extract of freshwater microalga *Chloromonas* sp. (ETCH) could serve as potential therapeutic candidate against human cancers such as HeLa, A375, and Hs578T.[60] Similar study revealed the phytochemicals of *Chlorella vulgaris* could be responsible for exhibiting anticancer activities against MCF-7 cancer cell lines with IC_{50} value of 31.2 µg/ml.[61]

Conclusion

This study reported that EAENH fractions showed potential antioxidant and moderate cytotoxic activity. The phytochemical assessment established the dominance of saponins, terpenoids, flavonoids, and phenolic acids supporting its valuable antioxidant and antiproliferative properties which were further authenticated by HPTLC, FTIR, and GCMS analysis. Future researches are essential to efficaciously isolate and purify the biochemicals from the hexane fractions signifying their potential to explore expanded investigations for enhancing their production by implementing advanced molecular biotechnological techniques. It was evident that secondary metabolites enriched in *Nannochloropsis* act as a prime basis for further pharmacological studies. This green alga also serves as a promising candidate which could be endurably utilized as an immense treasure for the invention of novel therapeutic agents against oxidative stress and cancer.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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