



# **Poly-unsaturated Fatty Acid, Biodiesel Property and Anticancer Activity Analysis of *Monoraphidium griffithii***

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## **Authors' contributions**

This work was carried out in collaboration among all authors. Author VV designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MS and KGT managed the analyses of the study. Author GS and SMS managed the literature searches. All authors read and approved the final manuscript.

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## **ABSTRACT**

**Aim:** The aim of the study was to isolate and characterize freshwater microalgae from Puzhal lake, Chennai, Tamil Nadu, and to analyze its biochemical composition, poly-unsaturated fatty acid content, anticancer activity, cytotoxicity, and biodiesel properties.

**Methodology:** Pure isolate obtained from freshwater was characterised biochemically and identified by ITS-2 RNA sequence as *Monoraphidium griffithii*. The microalgal culture was maintained under white light illumination for a photoperiod of 16:8 light/dark hours respectively. The

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saturated and poly-unsaturated fatty acid profiling was performed in a Gas Chromatography-Flame ionization detector and the biodiesel properties were analyzed through BiodieselAnalyzer© Ver. 2.2 software. The anti-cancer and cytotoxicity studies were performed using the human lung cancer cell line (A549) and Vero cell line at 20, 40, 60, 80, and 100 µg biomass/ml concentration.

**Results:** The biochemical composition was determined to be 32.97%±1.648 protein, 15.36%±2.857 carbohydrate, 15.89%±1.407 lipid, 26.27±1.48 µg/mg chlorophyll a+b and 8.03±1.92 µg/mg carotenoid. The fatty acid profiling revealed the presence of 19 different fatty acids of which Palmitic acid was 18.08%, cis-10 heptadecanoic acid was 17.37%, alpha-linolenic acid was 2.05%, and docosahexaenoic acid was 1.27%. The biodiesel properties were within the American standard for testing materials (ASTM) limits. 46.5% cell viability was attained at a microalgal concentration of 80 µg/ml against the human lung cancer cell line (A549) and the cell viability was 77.87% even at the high concentration of 100 µg/ml against the Vero cell line.

**Conclusion:** The isolated microalgae *Monoraphidium griffithii* can be used for the production of biodiesel. The presence of alpha-linolenic and docosahexaenoic acid and anticancer activity makes them a source for human and animal nutrition.

**Keywords:** Biodiesel; microalgae; anti-cancer activity; cytotoxicity; FAME.

## 1. INTRODUCTION

Microalgae is a unicellular organism with versatile applications. There are numerous species of microalgae found predominantly in aquatic and few in terrestrial ecosystems [1]. They are photosynthetic organisms that require sunlight, CO<sub>2</sub>, and dissolved nutrients from their habitats for growth [2]. Microalgae are regarded to absorb more CO<sub>2</sub> from the atmosphere than trees. Being photoautotrophic in nutrition they produce organic compounds such as glucose and energy-giving substances on their own through the biological system. These primary organic compounds are used for the metabolism of other biochemical components like protein, carbohydrates, lipids, pigments, and secondary metabolites [3].

The rich biochemical and physiological properties of microalgae are considered to be valuable sources of commercial importance. They are able to produce bioactive compounds like antioxidants, β-carotene, lutein, carotenoids, natural dyes, polyunsaturated fatty acids (PUFAs), lipids, and pigments [4]. These compounds are being extensively used in the food and fuel sectors as raw materials. Other sectors of microalgal applications include animal nutrition, human nutrition, cosmetics, and pharmaceuticals [5]. They are also being used as a tool in environmental biotechnology for bioremediation, environmental monitoring of toxicants, carbon mitigation, wastewater treatment, and bioassay [1]. The commercial production of microalgal biomass in bulk quantities is feasible owing to their fast growth and flexible culture condition. Being

photosynthetic they require minimal nutrients and medium to accumulate and secrete metabolites. Therefore, when a microalgal strain with scientific importance is identified, it can be commercialized effectively through mass cultivation in open areas or in photobioreactors to produce large quantities of the targeted metabolite [6].

Though extensive research is being carried out on the biochemical composition and biotechnological importance of microalgae only a very few species are commercialized for their applications which mainly include animal nutrition, human nutrition, pharmacological, and cosmetic sectors. Examples are *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina* and *Arthrospira (Spirulina) maxima* [7]. Therefore, this study was targeted to isolate microalgae from a freshwater source, identify, and analyze their biochemical composition namely protein, lipids, carbohydrate, chlorophyll, carotenoid and to analyze the saturated and polyunsaturated fatty acid composition, biodiesel properties, anti-cancer activity and cytotoxicity.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection, Isolation and Culture

The water sample was collected in a sterile container from the Puzhal freshwater lake, Chennai, Tamil Nadu. The water was filtered and used for the microalgae isolation process. The filtered sample was serially diluted and inoculated on petri-plate containing Bold's Basal Medium (BBM) with 1% Agar. The petri plates were placed under continuous white light

illumination of 1000 lux with 16:8 light and dark hours until visible colonies appear [8]. The individual cultures were maintained in BBM in Erlenmyer flask under the same illumination condition for the growth of microalgae.

## 2.2 Characterization of Isolated Microalgae

The morphological characterization of the microalgae was performed through observation under the light microscope. The shape, cellular arrangements, and nature of the spine were considered to characterize the microalgae morphologically [9]. These characteristics were compared with the standard monographs to perform genus-level identification [10]. The DNA was isolated by the CTAB method and the concentration and purity were analyzed by nano spectrophotometer [11]. The molecular characterization was done with internal transcribed spacer 2 (ITS 2) primer with sequence 5'- GAGCATGTCTGCCTCAGC -3' for the forward primer and 5'- GGTAGCCTTGCCTGAGC -3' for the reverse primer. The thermal cycle programmed for amplification was: initial denaturation at 95°C for 5 mins, denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min for 35 cycles with a final extension at 72°C for 10 min. The amplified product was checked on a 1.8% agarose gel [12]. The PCR product was sequenced, blast analyzed and submitted to Genbank for accession number.

## 2.3 Biochemical Characterization

### 2.3.1 Protein estimation

The culture with OD value 0.6 @ 630 nm was centrifuged at 10000 rpm for 10 mins to pellet the cells. The pellet was washed twice with distilled water. The protein was extracted by the trichloroacetic acid (TCA) with a modification of washing the protein pellet with acetone and resuspended with phosphate buffer pH 7.4 [13]. The concentration was determined with Bradford reagent and bovine serum albumin (BSA) as standard. The absorbance was read at 595 nm.

### 2.3.2 Carbohydrate estimation

Carbohydrate extraction was performed from 2 ml microalgal culture with an OD of 0.6 @ 630 nm, centrifuged at 10000 rpm to pellet the cells and extracted with 0.1 N NaOH with a

modification of cell disruption by a bead beater for 15 mins [14]. Then the supernatant was used to estimate the concentration of carbohydrates by the phenol sulphuric acid method. The absorbance of the sample was read at 490 nm using a spectrophotometer and compared with the known standard of glucose [15].

### 2.3.3 Pigment extraction

Chlorophyll a, b, and carotenoids were extracted from the microalgal culture by using the solvent method with minor modifications [16]. The pigment extraction was performed with 80% acetone on a microalgal pellet from a 2 ml culture of OD 0.6 at 630 nm. The modification includes the sonication of the culture with the solvent for 15 mins under dark condition with a 10 s pulse for every 30 s. Later the culture was left in the dark overnight to enhance the pigment extraction. The absorbance was read at 646, 663, and 470 nm on a spectrophotometer and substituted on the standard Welburn equation (1), (2), and (3) [17].

$$C_a = 12.21A_{663} - 2.81A_{646} \quad \dots\dots\dots (1)$$

$$C_b = 20.13A_{646} - 5.03A_{663} \quad \dots\dots\dots (2)$$

$$C_t = \frac{1000A_{470} - 3.27C_a - 104C_b}{198} \quad \dots\dots\dots (3)$$

### 2.3.4 Lipid extraction

Lipid extraction was performed with chloroform and methanol in the ratio of 2:1 with slight modification by including sonication to disrupt the cells for 10 mins with 10 s pulse for every 30 s [18]. The water was added to the solvent mixture and centrifuged at 5000 rpm for 10 mins. The chloroform layer containing lipids was collected and dried in a pre-weighed vial. The concentration (%) of lipid was determined gravimetrically by the following formula (4).

$$\text{Lipid concentration (\%)} = \frac{\text{Weight of the lipid}}{\text{Weight of the sample}} \times 100 \quad \dots\dots\dots (4)$$

## 2.4 Fluorescence Microscopy

The microalgal lipid was stained by a lipophilic dye, Nile red [9] at 1.5 µg/ml concentration. The stained cells were viewed under the fluorescent microscope with excitation at 530 nm.

## 2.5 Fatty Acid Analysis

One hundred mg of biomass was transesterified by adding 4 ml of methanol with 1.8% conc. H<sub>2</sub>SO<sub>4</sub> and heating at 80<sup>o</sup>C for 1 hr and the fatty acid methyl esters (FAME) were separated by adding chloroform and water. The chloroform layer containing FAME was dried and analyzed by Gas Chromatography- Flame Ionisation detector (GC-FID) with Supelco 37 component FAME mix, CRM47885 (Sigma-aldrich) as standard [19]. The FAME sample was dissolved in dichloromethane and 2 µl of the sample was injected to GC (Michro-9100, NETEL) with a fused silica column of dimensions 30m\*25µm\*0.25µm. The oven temperature was set to 100<sup>o</sup>C for 10 min and 1<sup>o</sup>C/min raise up to 240<sup>o</sup>C. The temperature of the flame ionization detector was 250<sup>o</sup>C and the carrier gas used was nitrogen.

## 2.6 Characterization of Biodiesel Property

The biodiesel property of the FAME content was analyzed by BiodieselAnalyzer© Ver. 2.2 software. The cetane number, kinetic viscosity, density, iodine value, pour point, cloud point, oxidation stability, long-chain saturation factor, cold filter plugging point, higher heating value, allylic position equivalent, bis-allylic position equivalent, degree of unsaturation, saturated, monounsaturated and polyunsaturated fatty acids were analyzed and compared with the American Standard for Testing Materials (ASTM) limits [20].

## 2.7 Anticancer Activity

The anticancer activity of the microalgae was analyzed by using human lung cancer cell line (A549) and cytotoxicity was analyzed by Vero cell line [21]. The microalgae were used at five different concentrations of 20, 40, 60, 80, and 100 µg/ml. The culture was incubated with the cell line for 24 h and the cytotoxicity was analyzed by MTT assay by measuring the absorbance at 570nm. The cell viability percentage for each concentration was calculated using the formula (5).

$$\text{Cell viability (\%)} = \frac{\text{OD of the sample} - \text{OD of the blank}}{\text{OD of the control} - \text{OD of the blank}} \times 100 \quad (5)$$

## 3. RESULTS AND DISCUSSION

### 3.1 Sample Collection, Isolation and Culture

About 10-15 colonies were found on the BBM agar plate inoculated with the collected freshwater sample. A single pure strain of microalgae was obtained after several serial dilutions of the obtained colonies after microscopic observation and maintained in BBM. The culture was maintained under white light illumination for a photoperiod of 16:8 light and dark hours. BBM contains the necessary nutrients needed for the growth of microalgae. 20 different microalgal strains were isolated from freshwater resources in Pakistan by serial dilution and streak plating and cultured in BBM for maintenance [22]. In a similar study, 94 culturable pure isolates of microalgae were obtained through plating and serial dilution techniques from freshwater sources of Singapore city and maintained in BBM for further characterization [23].

### 3.2 Characterization of Isolated Microalgae

The characterization was made according to its morphological observation it had individual cells, spindle-shaped and fusiform in nature. With these observations, the isolated microalgae were identified to belong to the genus *Monoraphidium* belonging to the family *Selenastraceae* [24]. The photomicrograph of the isolated strain is given in (Fig 1. A). The morphology of the microalgae tends to vary depending on the age of the culture and its habitat [25]. About 63 microalgal species were identified from Muttukadu estuary and the microalgae were identified with the help of standard books and monographs [26]. The concentration and 260/280A ratio of the DNA isolated were 154.8 ng/µl and 1.94 respectively, the ITS-2 region was amplified and the product was about 300bp. The sequence upon blast analysis with the NCBI database depicted 84% query coverage and 89.55% percentage identity to *Monoraphidium griffithii*. The sequence was submitted to Genbank and obtained the accession number (OP677755.1). The culture was maintained under the same photo condition to facilitate growth and further culturing. Similarly, in a study 6 closely related microalgae belonging to *Scenedesmus*, *Desmodesmus*, *Chlorococcum*, and *Chlorella* genera were characterized by ITS primer and distinguished

further with compensatory base changes (CBC) [12].

### 3.3 Biochemical Characterization

The protein, carbohydrate, pigment, and lipid content of the isolated microalgae is given in Table 1.

#### 3.3.1 Protein estimation

Microalgae containing an increased amount of protein are considered as a source of food and feed material for human and animal consumption [27]. The proteins can be extracted, concentrated, purified and added to any food source for human consumption [28]. The microalgae *Monoraphidium griffithii* isolated in the present study tend to contain about  $32.97\% \pm 1.648$  of protein which makes this a capable strain to include in food and feed formulation. A study on fourteen isolates from Kerala tabulated their biochemical composition. The protein content differed between various species of the *Monoraphidium* genera, namely, *Monoraphidium griffithii* isolated contained  $44.36\% \pm 3.64$  which was higher than the present study whereas, *Monoraphidium contortum* contained  $12.84\% \pm 1.01$  protein which was lower to the present study and *Monoraphidium litorale* contained  $44.35\% \pm 4.04$  of protein of dry cell weight (DCW) [29]. Various other studies suggested that the protein level of the microalgae varies according to the strain, genus, and habitat [30].

#### 3.3.2 Carbohydrate estimation

The carbohydrate content of the isolated microalgae was estimated to analyze its potential to be used as a source for biofuel and bioenergy production. Microalgal polysaccharides are regarded as energy-rich compounds which can be converted to value-added products [31]. It can be utilized to produce a number of intriguing chemicals, including bioethanol, biobutanol, biomethane, and biohydrogen.  $15.36\% \pm 2.857$  DCW of carbohydrate was present in *Monoraphidium griffithii* which was lower to 8-14% present in *Spirulina platensis* and 12-17% present in *Chlorella vulgaris* [29]. Whereas in another study, *Monoraphidium sp.* contained 4.1 mg/g of protein which increased to 88.8 mg/g after cultivation in dairy wastewater [32]. The presence of polysaccharides adds commercial importance to microalgae and changing microalgal culture conditions

and the addition of substrates facilitates a higher yield of carbohydrates favoring commercialization.

#### 3.3.3 Pigment analysis

Microalgal pigments like chlorophyll and carotenoids are light-harvesting chemicals that play a role in photosynthesis [33]. These pigments tend to possess anti-inflammatory and anti-oxidant properties thereby finding their application in the pharmaceutical and cosmetic industry [34]. The total chlorophyll and carotenoid content of *Monoraphidium griffithii* in the present study were  $26.27 \pm 1.48$  and  $8.03 \pm 1.92$   $\mu\text{g}/\text{mg}$  of the biomass. It was higher than other reported study before culture modification, were the chlorophyll content of the isolated strain *Monoraphidium sp.* was increased from 3.36 mg/g to 89.53 mg/g after cultivation in dairy wastewater under the mixotrophic condition. The carotenoid content also tends to increase with changes in culture conditions [32]. A study reported the content of chlorophyll a and b of *Monoraphidium sp.* to be  $11.264 \pm 0.065$   $\mu\text{g}/\text{ml}$  and  $2.082 \pm 0.067$   $\mu\text{g}/\text{ml}$ , for *Scenedesmus sp.* chlorophyll a and b were  $0.834 \pm 0.004$   $\mu\text{g}/\text{ml}$  and  $0.334 \pm 0.003$   $\mu\text{g}/\text{ml}$ , *Ankistrodesmus sp.* had  $4.038 \pm 0.06$  and  $0.890 \pm 0.046$   $\mu\text{g}/\text{ml}$  and *Selenastrum sp.* had  $3.746 \pm 0.032$  and  $0.914 \pm 0.045$   $\mu\text{g}/\text{ml}$  of chlorophyll a and b respectively [35]. These studies denote the change in chlorophyll content with respect to the species and culture conditions. The increase in chlorophyll content increases photosynthesis thereby increasing the synthesis of energy-storage compounds and other secondary metabolites. Chlorophyll can be used as a nutraceutical drug and also as a natural food coloring agent [36]. The isolated species contains a moderate level of carotenoid which can be used as an antioxidant to suppress oxidative stress.

#### 3.3.4 Lipid content

Lipids are the major storage components of microalgae. Lipids are used as the raw material for biodiesel production where the triacylglycerol (neutral lipid) is transesterified to form fatty acid methyl esters which is the chemical form of biodiesel. The lipid content of *Monoraphidium griffithii* of the present study was 15.89%. In a previous report the lipid content of *Monoraphidium sp.* was recorded as  $11.57\% \pm 0.6$  of DCW at the highest biomass productivity condition which was lower than that found in the

present study [37]. In another study on *Monoraphidium pusillum* the lipid content was found to be between 10-15% in the control and they were able to increase the lipid quantity up to 25% upon cultivation under nitrogen depletion conditions [38]. This emphasizes the potential to increase lipid content by changing the culture condition. By increasing the lipid content, the microalgal strain *Monoraphidium griffithii* can be used as a source for biodiesel production.

### 3.4 Fluorescence Microscopy

The Nile red stained (1.5 µg/ml) microalgal cell observed under a fluorescent microscope at excitation wavelength 530 nm is given in (Fig 1, B).

### 3.5 Fatty Acid Analysis

The GC-FID analysis of the transesterified lipids revealed the presence of different fatty acids listed in (Table. 2). The chromatogram of the FAME GC-FID analysis is given in (Fig. 2). It contained 33.48% saturated fatty acid, 39.6% monounsaturated fatty acid, and 26.9% polyunsaturated fatty acid, of which palmitic acid was 18.08%, cis-10 heptadecanoic acid was 17.37%, gamma-linolenic acid was 11.68%, oleic acid was 10.36%, and linoleic acid was 7.3% alpha-linolenic acid was 2.05%, and docosahexaenoic acid was 1.27% was predominant in the isolate, other acids were present in trace amounts. In a study on a *Monoraphidium* sp. the percentage of saturated, monounsaturated, and polyunsaturated fatty acids were 23.43, 39.03 and 37.54 respectively at 35 days of cultivation with 0.36 g/L nitrate concentration. The alpha-linolenic acid content was about 2.84%, 1.57%, and 5.42% at a different phase of growth. It was reported that the fatty acid content changed at different times during the growth phase [37]. The fatty acid profile of *Monoraphidium contortum* was reported to have saturated, monounsaturated and polyunsaturated fatty acids content of 27.8%, 36.8%, and 20% respectively after optimization of phosphate and nitrate concentration in the medium and the alpha-linolenic acid content was about 2.5% in the control and it increased to 7.1% after optimization [39]. In a study, the fatty acid of *Schizochytrium* microalgae powder was analyzed and found to contain 17.63% docosahexaenoic acid [40]. The strain analyzed in the present study contained found to have 33.48% saturated, 39.6% monounsaturated, and 26.9% polyunsaturated

fatty acid without any culture optimization or substrate addition. These studies denote that the fatty acid profile varies with species and substrates used for culture. The *Monoraphidium griffithii* in the present study contains polyunsaturated fatty acids such as alpha-linolenic and docosahexaenoic acid, it can be used for omega-3 fatty acids enrichment of food and feed research studies.

### 3.6 Biodiesel Properties

The biodiesel properties of FAME of *Monoraphidium griffithii* analyzed by the BiodieselAnalyzer© Ver. 2.2 are listed in (Table 3). It revealed that the properties like iodine value, cetane number, and kinematic viscosity were within the limits of ASTM standards. Since the strain contained a high content of monounsaturated fatty acids it has better cold flow properties and pour point making them a feasible biodiesel candidate for usage in cold temperatures [41]. Cetane number is associated with the ignition quality of the biodiesel. A higher cetane number represents a higher ignition capacity [42]. The cetane value of 59.14 in the present study denotes the better ignitability of biodiesel. According to the FAME content, it was also found that the biodiesel from *Monoraphidium griffithii* had better oxidative stability of 7.18 h which denotes the stability of the fuel when exposed to air [43]. A previous study on the biodiesel property of FAME from *Monoraphidium* sp reported, the pour point to be 2°C, cloud point to be 3°C, and the kinematic viscosity (mm<sup>2</sup> /s) to be 48. The kinematic viscosity seemed to be higher than the standard limits which may hinder the flow and injection spray property of the fuel [44]. In a similar study, the viscosity at 40°C, specific gravity, and cetane number of the FAME derived from *Monoraphidium* sp were in accordance with the ASTM standard but the iodine value and higher heating value deviated from the standards [45]. A comparison of the biodiesel property of two microalgae *Auxenochlorella protothecoides* and *Chlorella sorokiniana* cultured under different concentrations of glucose and acetate was performed through the BiodieselAnalyzer© software and changes in biodiesel properties due to the influence of culture conditions were, the percentage of polyunsaturated fatty acid of *A. protothecoides* and *C. sorokiniana* increased to 19.66% and 24.73% respectively after cultivation under acetate at a concentration of 30 g/l, whereas under glucose supplement at 30 g/l the saturated fatty acid content increased to

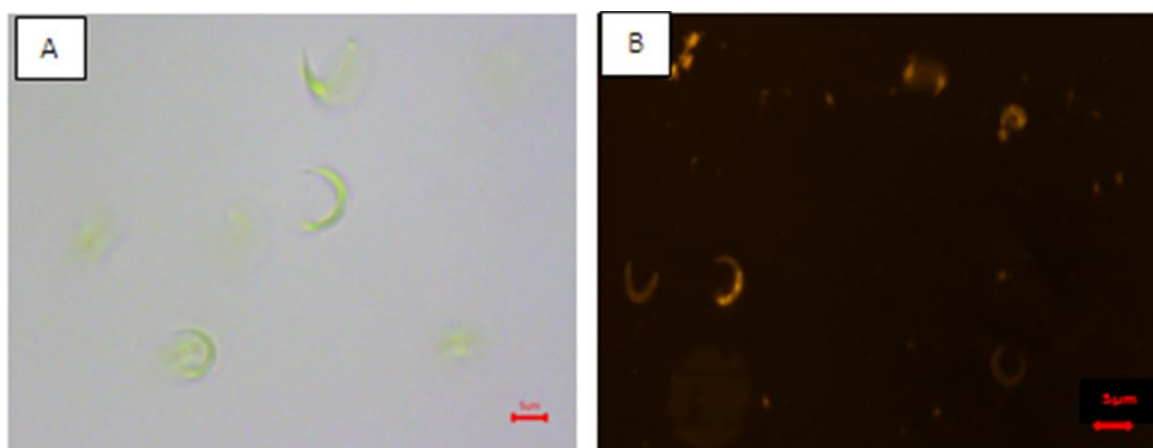
38.35% and 45.75% in *A. protothecoides* and *C. sorokiniana* respectively [46]. Therefore, the software gives a rough idea about the biodiesel

property. Based on the software analysis, the strain used in the present study has biodiesel properties as per ASTM standards.

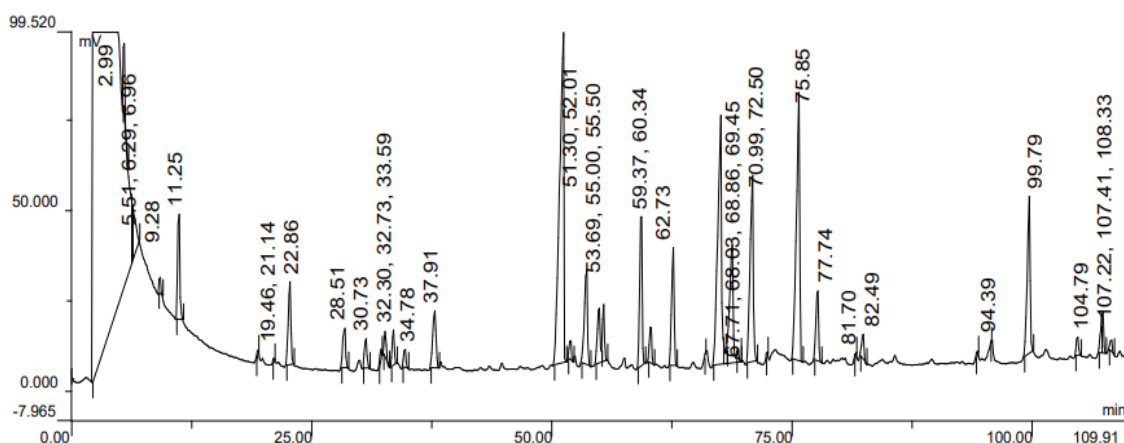
**Table 1. Biochemical composition of *Monoraphidium griffithii***

Biochemical composition	Concentration
Protein	32.97%±1.648*
Carbohydrate	15.36%±2.857*
Chlorophyll a	13.45±1.768 (µg/ml)
Chlorophyll b	4.94±0.481 (µg/ml)
Total Chlorophyll	18.39±0.635 (µg/ml)
Total Chlorophyll (µg/mg biomass)	26.27±1.48
Carotenoid	5.62±0.552 (µg/ml)
Carotenoid (µg/mg biomass)	8.03±1.92
Lipid	15.89%±1.407*

(\* percentage with respect to the weight of biomass)



**Fig. 1. (A) Photomicrograph of *Monoraphidium griffithii* under light microscope (1000x magnification) (B) Photomicrograph of *Monoraphidium griffithii* under fluorescent microscope excitation at 530 nm**



**Fig. 2. Chromatogram of Fatty acid methyl esters in GC-FID**

**Table 2. Fatty acid composition of *Monoraphidium griffithii***

Component Name	Concentration (%)
Butyric acid (C4:0)	1.46 ±0.007
Caprylic acid (C8:0)	2.63 ±0.021
Capric acid (C10:0)	3.01 ±0.042
Undecanoic acid (C11:0)	1.25 ±0.014
Lauric acid (C12:0)	0.83 ±0.028
Tridecanoic acid (C13:0)	2.38 ±0.035
Palmitic acid (C16:0)	18.08 ±0.049
Palmitoleic acid (C16:1)	2.96 ±0.035
Heptadecanoic acid (C17:0)	2.84 ±0.057
Cis 10 Heptadecanoic acid (C17:1)	17.37 ±0.07
Stearic acid (C18:0)	1.00 ±0.014
Elaidic acid (C18:1 cis 9t)	3.51 ±0.028
Oleic acid (C18:1 cis 9c)	10.36 ±0.057
Linolelaidic acid (C18:2)	4.60 ±0.015
Linoleic acid (C18:2 cis 9,12)	7.30 ±0.036
Gamma-Linolenic (C18:3)	11.68 ±0.016
Alpha-Linolenic acid (C18:3 cis 9,12,15)	2.05 ±0.021
Nevronic acid (C24:1)	5.40 ±0.02
Docosahexaenoic acid (C22:6)	1.27 ±0.018

**Table 3. Biodiesel properties based on fatty acid profile of *Monoraphidium griffithii* ON089666.1 upon analysis in Biodieselanalyzer software version 2.2**

Biodiesel properties	<i>Monoraphidium griffithii</i> biodiesel	ASTM Standard fuel parameters
Saturated fatty acids (%) (SFA)	33.48	-
Monounsaturated fatty acids (%) (MUFA)	39.6	-
Polyunsaturated fatty acids (%) (PUFA)	26.9	-
Degree of unsaturation (DU)	93.21	-
Saponification value (mg/g) (SV)	178.97	-
Iodine value (IV)	78.47	130 (Max)
Cetane number (CN)	59.14	47 (min)
Long-chain saturation factor (LCSF)	2.31	-
Cold filter plugging point (°C) (CFPP)	-9.23	-
Cloud point (°C) (CP)	4.52	-
Pour point (°C) (PP)	-1.92	-
Allylic position equivalent (APE)	65.31	-
Bis-allylic position equivalent (BAPE)	39.46	-
Oxidation stability (h) (OS)	7.18	-
Higher heating value (HHV)	31.12	-
Kinematic viscosity (mm <sup>2</sup> /s) (v)	2.56	1.9-6.0
Density (g/ cm <sup>3</sup> ) (p)	0.71	-

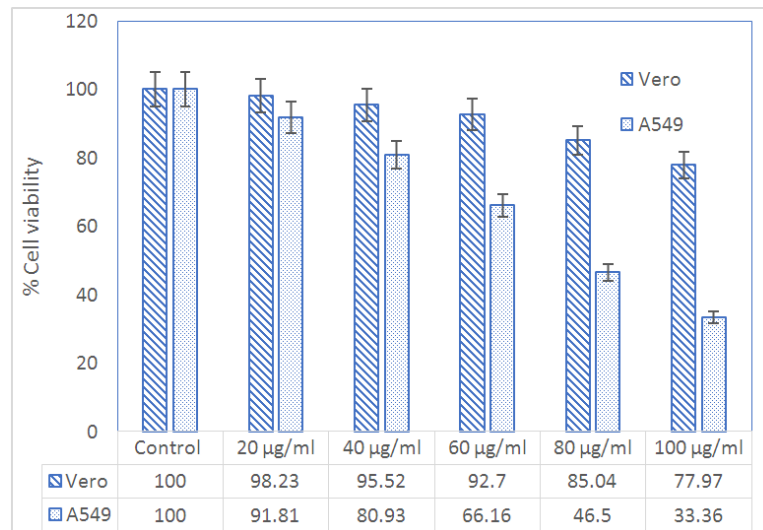
*min- minimum; Max- maximum; - no standard limits classified by ASTM standard*

### 3.7 Anticancer and Cytotoxicity Studies

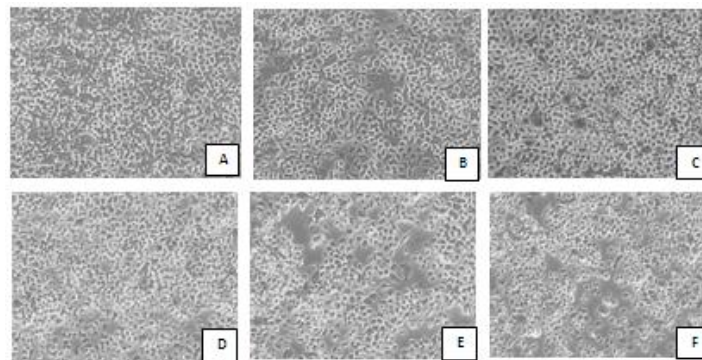
The anticancer activity and cytotoxicity of isolated freshwater microalgae *Monoraphidium griffithii* on the human lung cancer cell line (A549) and Vero cell line is shown in (Fig. 3). The cell viability after 24 h decreased upon increasing the sample concentration from 20 to

100 µg/ml. The cell viability decreased to less than 50% (46.5%) at the concentration of 80 µg/ml against the human lung cancer cell line (A549). The cytotoxicity of the microalgae was evaluated on the vero cell line and the viability of cells was over 80% up to the concentration of 80 µg/ml and the viability percentage decreased to 77.97% at 100 µg/ml concentration (Fig. 3).

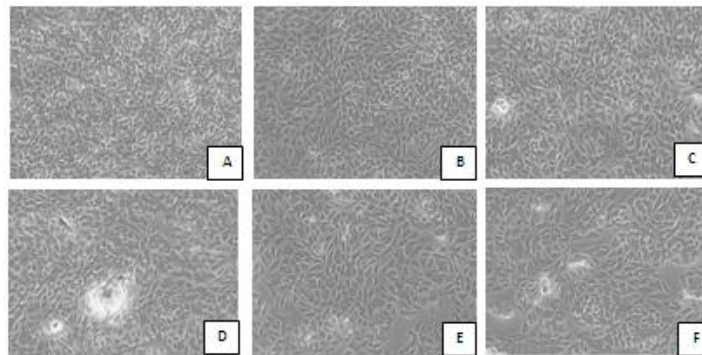




**Fig. 3. Cell viability percentage of different concentrations of *Monoraphidium griffithii* against human lung cancer cell line (A549) and normal vero cell line**



**Fig. 4. (a) Morphological changes of human lung cancer cell line (A549) at different concentrations of *Monoraphidium griffithii* A-control, B-20 µg/ml, C-40 µg/ml, D-60 µg/ml, E-80 µg/ml, and F-100 µg/ml concentration**



**Fig. 4. (b) Morphological changes of vero cell line at different concentrations of *Monoraphidium griffithii* A-control, B-20 µg/ml, C-40 µg/ml, D-60 µg/ml, E-80 µg/ml, and F-100 µg/ml concentration**

These results show that the microalgae *Monoraphidium griffithii* contains compounds that can be used as an anticancer agent, and on the

normal cell line the cell viability was higher, that is 77.97% at the highest concentration of 100 µg/ml, this shows that it is not toxic to cause the

death of normal cells. The morphology of the treated and control cells of the A549 and vero cell line is shown in (Fig. 4a & b). The apoptotic cells tend to lose their cellular appendages, desmosomes and become circular while losing their viability upon treatment with microalgae cells. A similar study on *Nannochloropsis oculata* methanolic extract on a human breast cancer cell line (MDA-MB-231) showed a decrease in cell proliferation upon increasing concentration. The cell viability was less than 50% at a concentration of 400 µg/ml after 72 h of induction [47]. In a previous study the anticancer activity for 8 cyanobacterial species and one microalgae species *Chlorella vulgaris*, were analyzed and a single concentration of the aqueous extract 100 µg/ml was used across the samples and the cell viability was checked against Ehrlich Ascites Carcinoma cell (EACC) and Human hepatocellular cancer cell line (HepG2). The results stated that two cyanobacterial species *Nostoc muscorum* and *Oscillatoria* sp. showed increased viability than other species against EACC and HepG2 cell lines. The microalgae *Chlorella vulgaris* showed below 40% cell viability against EACC and 55% cell viability against HepG2 at 100 µg/ml concentration. In this study, at a concentration of 100 µg/ml, the viability of cancer cell line decreased to 35.36% which was lower than the reported studies, suggesting the presence of compounds with enhanced anticancer activity [48]. Anticancer studies already reported for other species of microalgae were in support of the present report and the ability of the microalgae *Monoraphidium griffithii* to reduce the viability of cancer cells gives insights into the presence of certain biologically active moieties that has potential anti-cancer property which can be studied further with respect to clinical relevance.

#### 4. CONCLUSION

The results of the present study denoted that the isolated freshwater microalgae *Monoraphidium griffithii* is a rich source of carbohydrates, lipids, proteins, alpha-linoleic acid and docosahexaenoic acid. The fatty acid profile by FAME analysis showed that the isolated freshwater microalgae have good biodiesel properties as per ASTM standards and better cold flow properties. The isolated strain also has anticancer activity and it is non-toxic to normal cells. Therefore, the isolated strain was found to have the potential to be used for biodiesel production and also to be used as food and feed additive.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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