

**Poly-unsaturated fatty acid, biodiesel property and anticancer activity analysis of *Monoraphidium griffithii***

**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 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. 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

25 human lung cancer cell line (A549) and the cell viability was 77.87% even at the high concentration of  
26 100 µg/ml against the Vero cell line.

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

30 **Keywords:** Biodiesel, microalgae, anti-cancer activity, cytotoxicity, FAME

## 31 1. INTRODUCTION

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

40 The rich biochemical and physiological properties of microalgae are considered to be valuable  
41 sources of commercial importance. They are able to produce bioactive compounds like antioxidants,  
42 β- carotene, lutein, carotenoids, natural dyes, polyunsaturated fatty acids (PUFAs), lipids, and  
43 pigments [4]. These compounds are being extensively used in the food and fuel sectors as raw  
44 materials. Other sectors of microalgal applications include animal nutrition, human nutrition,  
45 cosmetics, and pharmaceuticals [5]. They are also being used as a tool in environmental  
46 biotechnology for bioremediation, environmental monitoring of toxicants, carbon mitigation,  
47 wastewater treatment, and bioassay [1]. The commercial production of microalgal biomass in bulk  
48 quantities is feasible owing to their fast growth and flexible culture condition. Being photosynthetic  
49 they require minimal nutrients and medium to accumulate and secrete metabolites. Therefore, when a  
50 microalgal strain with scientific importance is identified, it can be commercialized effectively through  
51 mass cultivation in open areas or in photobioreactors to produce large quantities of the targeted  
52 metabolite [6].

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

## 61 **2. MATERIALS AND METHODS**

### 62 **2.1 Sample collection, isolation and culture**

63            The water sample was collected in a sterile container from the Puzhal freshwater lake,  
64 Chennai, Tamil Nadu. The water was filtered and used for the microalgae isolation process. The  
65 filtered sample was serially diluted and inoculated on petri-plate containing Bold's Basal Medium  
66 (BBM) with 1% Agar. The petri plates were placed under continuous white light illumination of 1000  
67 lux with 16:8 light and dark hours until visible colonies appear [8]. The individual cultures were  
68 maintained in BBM in Erlenmeyer flask under the same illumination condition for the growth of  
69 microalgae.

### 70 **2.2 Characterization of isolated microalgae**

71            The morphological characterization of the microalgae was performed through observation  
72 under the light microscope. The shape, cellular arrangements, and nature of the spine were  
73 considered to characterize the microalgae morphologically [9]. These characteristics were compared  
74 with the standard monographs to perform genus-level identification [10]. The DNA was isolated by the  
75 CTAB method and the concentration and purity were analyzed by nano spectrophotometer [11]. The  
76 molecular characterization was done with internal transcribed spacer 2 (ITS 2) primer with sequence  
77 5'- GAGCATGTCTGCCTCAGC -3' for the forward primer and 5'- GGTAGCCTTGCTGAGC -3' for  
78 the reverse primer. The thermal cycle programmed for amplification was: initial denaturation at 95°C  
79 for 5 mins, denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1  
80 min for 35 cycles with a final extension at 72°C for 10 min. The amplified product was checked on a

81 1.8% agarose gel [12]. The PCR product was sequenced, blast analyzed and submitted to Genbank  
82 for accession number.

### 83 **2.3 Biochemical characterization**

#### 84 **2.3.1 Protein estimation**

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

#### 90 **2.3.2 Carbohydrate estimation**

91 Carbohydrate extraction was performed from 2 ml microalgal culture with an OD of 0.6,  
92 centrifuged at 10000 rpm to pellet the cells and extracted with 0.1 N NaOH with a modification of cell  
93 disruption by a bead beater for 15 mins [14]. Then the supernatant was used to estimate the  
94 concentration of carbohydrates by the phenol sulphuric acid method. The absorbance of the sample  
95 was read at 490 nm using a spectrophotometer and compared with the known standard of glucose  
96 [15].

#### 97 **2.3.3 Pigment extraction**

98 Chlorophyll a, b, and carotenoids were extracted from the microalgal culture by using the  
99 solvent method with minor modifications [16]. The pigment extraction was performed with 80%  
100 acetone on a microalgal pellet from a 2 ml culture of OD 0.6 at 630 nm. The modification includes the  
101 sonication of the culture with the solvent for 15 mins under dark condition with a 10 s pulse for every  
102 30 s. Later the culture was left in the dark overnight to enhance the pigment extraction. The  
103 absorbance was read at 646, 663, and 470 nm on a spectrophotometer and substituted on the  
104 standard Welburn equation (1), (2), and (3) [17].

105 
$$C_a = 12.21A_{663} - 2.81A_{646} \quad \dots\dots\dots (1)$$

106 
$$C_b = 20.13A_{646} - 5.03A_{663} \quad \dots\dots\dots (2)$$

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

109

110 **2.3.4 Lipid extraction**

111 Lipid extraction was performed with chloroform and methanol in the ratio of 2:1 with slight  
112 modification by including sonication to disrupt the cells for 10 mins with 10 s pulse for every 30 s [18].

113 The water was added to the solvent mixture and centrifuged at 5000 rpm for 10 mins. The chloroform  
114 layer containing lipids was collected and dried in a pre-weighed vial. The concentration (%) of lipid  
115 was determined gravimetrically by the following formula (4).

116 Lipid concentration (%) = Weight of the lipid / Weight of the sample x 100 .....(4)

117 **2.4 Fluorescence microscopy**

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

120 **2.5 Fatty acid analysis**

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

130 **2.7 Characterization of biodiesel property**

131 The biodiesel property of the FAME content was analyzed by BiodieselAnalyzer© Ver. 2.2  
132 software. The cetane number, kinetic viscosity, density, iodine value, pour point, cloud point, oxidation

133 stability, long-chain saturation factor, cold filter plugging point, higher heating value, allylic position  
134 equivalent, bis-allylic position equivalent, degree of unsaturation, saturated, monounsaturated and  
135 polyunsaturated fatty acids were analyzed and compared with the American Standard for Testing  
136 Materials (ASTM) limits [20].

137

## 138 **2.6 Anticancer activity**

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

$$144 \text{ Cell viability (\%)} = \frac{\text{OD of the sample} - \text{OD of the blank}}{\text{OD of the control} - \text{OD of the blank}} \times 100 \dots\dots(5)$$

145

## 146 **3. RESULTS AND DISCUSSION**

### 147 **3.1 Sample collection, isolation and culture**

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

### 157 **3.2 Characterisation of isolated microalgae**

158 The characterization was made according to its morphological observation it had individual  
 159 cells, spindle-shaped and fusiform in nature. With these observations, the isolated microalgae were  
 160 identified to belong to the genus *Monoraphidium* belonging to the family *Selenastraceae* [24]. The  
 161 photomicrograph of the isolated strain is given in (Fig 1. A). The morphology of the microalgae tends  
 162 to vary depending on the age of the culture and its habitat [25]. About 63 microalgal species were  
 163 identified from Muttukadu estuary and the microalgae were identified with the help of standard books  
 164 and monographs [26]. The concentration and 260/280A ratio of the DNA isolated were 154.8 ng/ $\mu$ l  
 165 and 1.94 respectively, the ITS-2 region was amplified and the product was about 300bp. The  
 166 sequence upon blast analysis with the NCBI database depicted 84% query coverage and 89.55%  
 167 percentage identity to *Monoraphidium griffithii*. The sequence was submitted to Genbank and  
 168 obtained the accession number (OP677755.1). The culture was maintained under the same photo  
 169 condition to facilitate growth and further culturing. Similarly, in a study 6 closely related microalgae  
 170 belonging to *Scenedesmus*, *Desmodesmus*, *Chlorococcum*, and *Chlorella* genera were characterized  
 171 by ITS primer and distinguished further with compensatory base changes (CBC) [12].

### 172 3.3 Biochemical characterization

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

175 Table 1. Biochemical composition of *Monoraphidium griffithii*

Biochemical composition	Concentration
Protein	32.97% $\pm$ 1.648*
Carbohydrate	15.36% $\pm$ 2.857*
Chlorophyll a	13.45 $\pm$ 1.768 ( $\mu$ g/ml)
Chlorophyll b	4.94 $\pm$ 0.48 1( $\mu$ g/ml)
Total Chlorophyll	18.39 $\pm$ 0.635 ( $\mu$ g/ml)
Total Chlorophyll ( $\mu$ g/mg biomass)	26.27 $\pm$ 1.48
Carotenoid	5.62 $\pm$ 0.552 ( $\mu$ g/ml)
Carotenoid ( $\mu$ g/mg biomass)	8.03 $\pm$ 1.92

Lipid	15.89%±1.407*
-------	---------------

177

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

### 179 **3.3.1 Protein estimation**

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

### 191 **3.3.2 Carbohydrate estimation**

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

### 202 **3.3.3 Pigment analysis**

203 Microalgal pigments like chlorophyll and carotenoids are light-harvesting chemicals that play a  
204 role in photosynthesis [33]. These pigments tend to possess anti-inflammatory and anti-oxidant  
205 properties thereby finding their application in the pharmaceutical and cosmetic industry [34]. The total  
206 chlorophyll and carotenoid content of *Monoraphidium griffithii* in the present study were  $26.27 \pm 1.48$   
207 and  $8.03 \pm 1.92$   $\mu\text{g}/\text{mg}$  of the biomass. It was higher than other reported study before culture  
208 modification, were the chlorophyll content of the isolated strain *Monoraphidium sp.* was increased  
209 from  $3.36$   $\text{mg}/\text{g}$  to  $89.53$   $\text{mg}/\text{g}$  after cultivation in dairy wastewater under the mixotrophic condition.  
210 The carotenoid content also tends to increase with changes in culture conditions [32]. A study  
211 reported the content of chlorophyll a and b of *Monoraphidium sp.* to be  $11.264 \pm 0.065$   $\mu\text{g}/\text{ml}$  and  
212  $2.082 \pm 0.067$   $\mu\text{g}/\text{ml}$ , for *Scenedesmus sp.* chlorophyll a and b were  $0.834 \pm 0.004$   $\mu\text{g}/\text{ml}$  and  
213  $0.334 \pm 0.003$   $\mu\text{g}/\text{ml}$ , *Ankistrodesmus sp.* had  $4.038 \pm 0.06$  and  $0.890 \pm 0.046$   $\mu\text{g}/\text{ml}$  and *Selenastrum sp.*  
214 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  
215 denote the change in chlorophyll content with respect to the species and culture conditions. The  
216 increase in chlorophyll content increases photosynthesis thereby increasing the synthesis of energy-  
217 storage compounds and other secondary metabolites. Chlorophyll can be used as a nutraceutical  
218 drug and also as a natural food coloring agent [36]. The isolated species contains a moderate level of  
219 carotenoid which can be used as an antioxidant to suppress oxidative stress.

#### 220 **3.3.4 Lipid content**

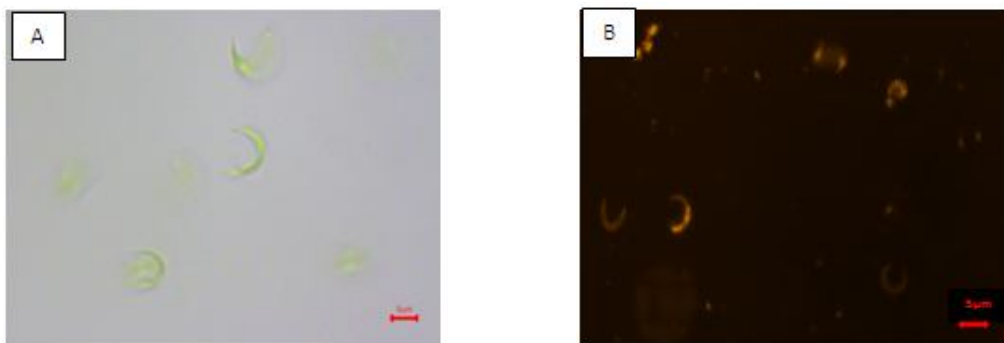
221 Lipids are the major storage components of microalgae. Lipids are used as the raw material  
222 for biodiesel production where the triacylglycerol (neutral lipid) is transesterified to form fatty acid  
223 methyl esters which is the chemical form of biodiesel. The lipid content of *Monoraphidium griffithii* of  
224 the present study was 15.89%. In a previous report the lipid content of *Monoraphidium sp.* was  
225 recorded as  $11.57\% \pm 0.6$  of DCW at the highest biomass productivity condition which was lower than  
226 that found in the present study [37]. In another study on *Monoraphidium pusillum* the lipid content was  
227 found to be between 10-15% in the control and they were able to increase the lipid quantity up to 25%  
228 upon cultivation under nitrogen depletion conditions [38]. This emphasizes the potential to increase  
229 lipid content by changing the culture condition. By increasing the lipid content, the microalgal strain  
230 *Monoraphidium griffithii* can be used as a source for biodiesel production.

#### 231 **3.4 Fluorescence microscopy**

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

234 Fig 1. (A) Photomicrograph of *Monoraphidium griffithii* under light microscope (1000x magnification)

235 (B) Photomicrograph of *Monoraphidium griffithii* under fluorescent microscope excitation at 530 nm



236

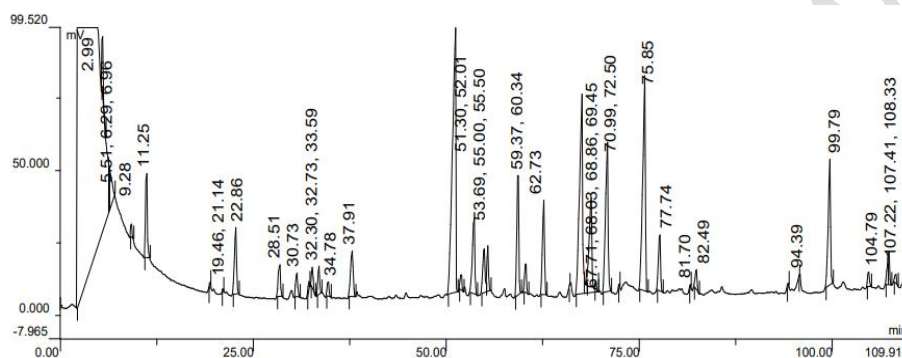
237

### 238 3.5 Fatty acid analysis

239 The GC-FID analysis of the transesterified lipids revealed the presence of different fatty acids  
240 listed in (Table. 2). The chromatogram of the FAME GC-FID analysis is given in (Fig. 2). It contained  
241 33.48% saturated fatty acid, 39.6% monounsaturated fatty acid, and 26.9% polyunsaturated fatty  
242 acid, of which palmitic acid was 18.08%, cis-10 heptadecanoic acid was 17.37%, gamma-linolenic  
243 acid was 11.68%, oleic acid was 10.36%, and linoleic acid was 7.3% alpha-linolenic acid was 2.05%,  
244 and docosahexaenoic acid was 1.27% was predominant in the isolate, other acids were present in  
245 trace amounts. In a study on a *Monoraphidium* sp. the percentage of saturated, monounsaturated,  
246 and polyunsaturated fatty acids were 23.43, 39.03 and 37.54 respectively at 35 days of cultivation  
247 with 0.36 g/L nitrate concentration. The alpha-linolenic acid content was about 2.84%, 1.57%, and  
248 5.42% at a different phase of growth. It was reported that the fatty acid content changed at different  
249 times during the growth phase [37]. The fatty acid profile of *Monoraphidium contortum* was reported to  
250 have saturated, monounsaturated and polyunsaturated fatty acids content of 27.8%, 36.8%, and 20%  
251 respectively after optimization of phosphate and nitrate concentration in the medium and the alpha-  
252 linolenic acid content was about 2.5% in the control and it increased to 7.1% after optimization [39]. In  
253 a study, the fatty acid of *Schizochytrium* microalgae powder was analyzed and found to contain  
254 17.63% docosahexaenoic acid [40]. The strain analyzed in the present study contained found to have

255 33.48% saturated, 39.6% monounsaturated, and 26.9% polyunsaturated fatty acid without any culture  
 256 optimization or substrate addition. These studies denote that the fatty acid profile varies with species  
 257 and substrates used for culture. The *Monoraphidium griffithii* in the present study contains  
 258 polyunsaturated fatty acids such as alpha-linolenic and docosahexaenoic acid, it can be used for  
 259 omega-3 fatty acids enrichment of food and feed research studies.

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



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

265

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

266

### 267 3.6 Biodiesel properties

268 The biodiesel properties of FAME of *Monoraphidium griffithii* analyzed by the  
269 BiodieselAnalyzer© Ver. 2.2 are listed in (Table 3). It revealed that the properties like iodine value,  
270 cetane number, and kinematic viscosity were within the limits of ASTM standards. Since the strain  
271 contained a high content of monounsaturated fatty acids it has better cold flow properties and pour  
272 point making them a feasible biodiesel candidate for usage in cold temperatures [41]. Cetane number  
273 is associated with the ignition quality of the biodiesel. A higher cetane number represents a higher  
274 ignition capacity [42]. The cetane value of 59.14 in the present study denotes the better ignitability of  
275 biodiesel. According to the FAME content, it was also found that the biodiesel from *Monoraphidium*  
276 *griffithii* had better oxidative stability of 7.18 h which denotes the stability of the fuel when exposed to  
277 air [43]. A previous study on the biodiesel property of FAME from *Monoraphidium* sp reported, the  
278 pour point to be 2<sup>0</sup>C, cloud point to be 3<sup>0</sup>C, and the kinematic viscosity (mm<sup>2</sup> /s) to be 48. The  
279 kinematic viscosity seemed to be higher than the standard limits which may hinder the flow and  
280 injection spray property of the fuel [44]. In a similar study, the viscosity at 40<sup>0</sup>C, specific gravity, and  
281 cetane number of the FAME derived from *Monoraphidium* sp were in accordance with the ASTM

282 standard but the iodine value and higher heating value deviated from the standards [45]. A  
 283 comparison of the biodiesel property of two microalgae *Auxenochlorella protothecoides* and *Chlorella*  
 284 *sorokiniana* cultured under different concentrations of glucose and acetate was performed through the  
 285 BiodieselAnalyzer© software and changes in biodiesel properties due to the influence of culture  
 286 conditions were, the percentage of polyunsaturated fatty acid of *A. protothecoides* and *C. sorokiniana*  
 287 increased to 19.66% and 24.73% respectively after cultivation under acetate at a concentration of 30  
 288 g/l, whereas under glucose supplementation at 30 g/l the saturated fatty acid content increased to  
 289 38.35% and 45.75% in *A. protothecoides* and *C. sorokiniana* respectively [46]. Therefore, the  
 290 software gives a rough idea about the biodiesel property. Based on the software analysis, the strain  
 291 used in the present study has biodiesel properties as per ASTM standards.

292

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

295

<b>Biodiesel properties</b>	<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 ( $\text{mm}^2/\text{s}$ ) ( $\nu$ )	2.56	1.9-6.0
Density ( $\text{g}/\text{cm}^3$ ) ( $\rho$ )	0.71	-

296

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

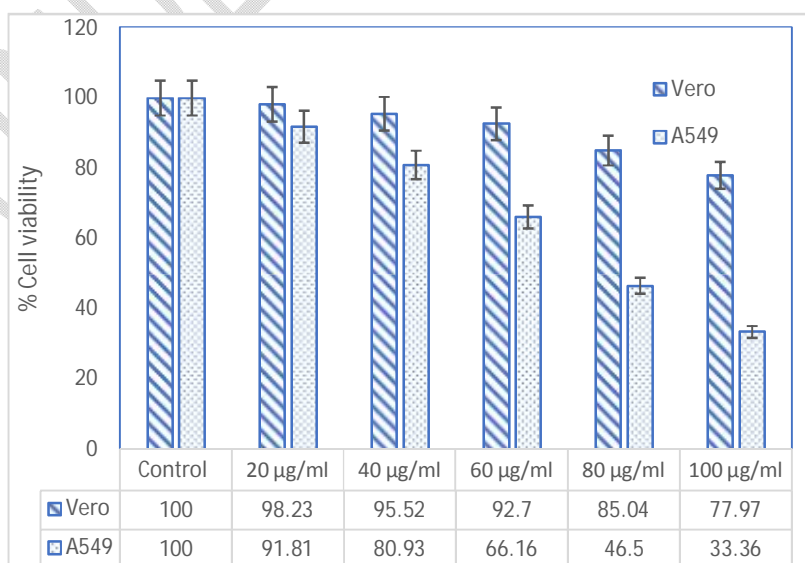
### 298 3.7 Anticancer and cytotoxicity studies

299 The anticancer activity and cytotoxicity of isolated freshwater microalgae *Monoraphidium*  
 300 *griffithii* on the human lung cancer cell line (A549) and Vero cell line is shown in (Fig. 3). The cell  
 301 viability after 24 h decreased upon increasing the sample concentration from 20 to 100  $\mu\text{g}/\text{ml}$ . The  
 302 cell viability decreased to less than 50% (46.5%) at the concentration of 80  $\mu\text{g}/\text{ml}$  against the human  
 303 lung cancer cell line (A549). The cytotoxicity of the microalgae was evaluated on the vero cell line and  
 304 the viability of cells was over 80% up to the concentration of 80  $\mu\text{g}/\text{ml}$  and the viability percentage  
 305 decreased to 77.97% at 100  $\mu\text{g}/\text{ml}$  concentration (Fig. 3).

306 Fig 3. Cell viability percentage of different concentrations of *Monoraphidium griffithii* against human  
 307 lung cancer cell line

308 (A549)  
 309 vero cell

and normal  
 line.



314

315

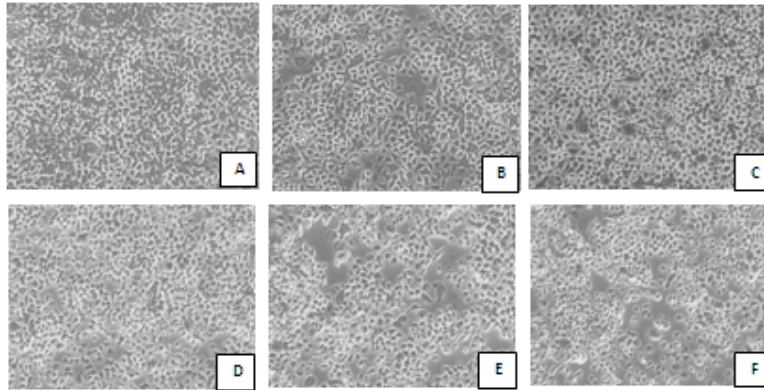
316

317

318

319           These results show that the microalgae *Monoraphidium griffithii* contains compounds that can  
320 be used as an anticancer agent, and on the normal cell line the cell viability was higher, that is  
321 77.97% at the highest concentration of 100 µg/ml, this shows that it is not toxic to cause the death of  
322 normal cells. The morphology of the treated and control cells of the A549 and vero cell line is shown  
323 in (Fig. 4 a & b). The apoptotic cells tend to lose their cellular appendages, desmosomes and become  
324 circular while losing their viability upon treatment with microalgae cells. A similar study on  
325 *Nannochloropsis oculata* methanolic extract on a human breast cancer cell line (MDA-MB-231)  
326 showed a decrease in cell proliferation upon increasing concentration. The cell viability was less than  
327 50% at a concentration of 400 µg/ml after 72 h of induction [47]. In a previous study the anticancer  
328 activity for 8 cyanobacterial species and one microalgae species *Chlorella vulgaris*, were analyzed  
329 and a single concentration of the aqueous extract 100 µg/ml was used across the samples and the  
330 cell viability was checked against Ehrlich Ascites Carcinoma cell (EACC) and Human hepatocellular  
331 cancer cell line (HepG2). The results stated that two cyanobacterial species *Nostoc muscorum* and  
332 *Oscillatoria* sp. showed increased viability than other species against EACC and HepG2 cell lines.  
333 The microalgae *Chlorella vulgaris* showed below 40% cell viability against EACC and 55% cell  
334 viability against HepG2 at 100 µg/ml concentration. In this study, at a concentration of 100 µg/ml, the  
335 viability of cancer cell line decreased to 35.36% which was lower than the reported studies,  
336 suggesting the presence of compounds with enhanced anticancer activity [48]. Anticancer studies  
337 already reported for other species of microalgae were in support of the present report and the ability  
338 of the microalgae *Monoraphidium griffithii* to reduce the viability of cancer cells gives insights into the  
339 presence of certain biologically active moieties that has potential anti-cancer property which can be  
340 studied further with respect to clinical relevance.

341 **Fig. 4** (a) Morphological changes of human lung cancer cell line (A549) at different concentrations of  
342 *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  
343 concentration

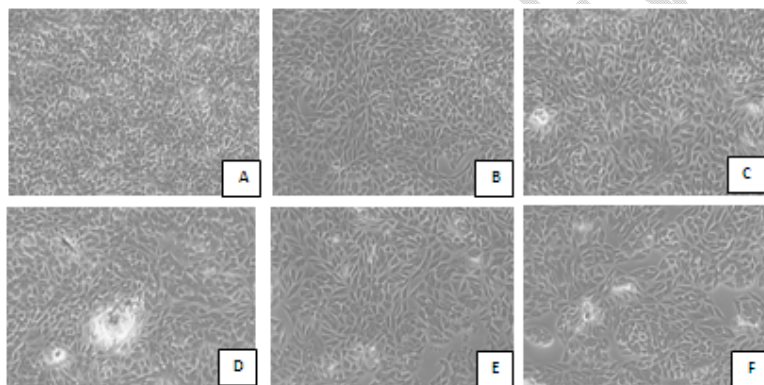


344

345

346 **Fig. 4** (b) Morphological changes of vero cell line at different concentrations of *Monoraphidium griffithii*

347 A-control, B-20 µg/ml, C-40 µg/ml, D-60 µg/ml, E-80 µg/ml, and F-100 µg/ml concentration



348

349

#### 350 4. CONCLUSION

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

358

359 **REFERENCES**

- 360 1. Rizwan M, Mujtaba G, Memon SA, Lee K, Rashid N. Exploring the potential of microalgae for new  
361 biotechnology applications and beyond: a review. *Renew Sustain Energy Rev.* 2018;92:394-04.
- 362 2. Singh A, Nigam PS, Murphy JD. Mechanism and challenges in commercialisation of algal biofuels.  
363 *Bioresour Technol* 2011;102:26–34.
- 364 3. Lee Y. Algal Nutrition Heterotrophic Carbon Nutrition. In: Richmond A, editor. *Handbook of*  
365 *Microalgal Culture: Biotechnology and Applied Phycology.* Oxford; Blackwell Publishing; 2004.
- 366 4. Moreno-Garcia L, Adjallé K, Barnabé S, Raghavan G. Microalgae biomass production for a  
367 biorefinery system: recent advances and the way towards sustainability. *Renew Sustain Energy Rev*  
368 2017;76:493–06.
- 369 5. Suganya T, Varman M, Masjuki H, Renganathan S. Macroalgae and microalgae as a potential  
370 source for commercial applications along with biofuels production: a biorefinery approach. *Renew*  
371 *Sustain Energy Rev* 2016;55:909–41.
- 372 6. Sánchez JF, Fernández JM, Acien FG, Rueda A, Pérez-Parra J, Molina E. Influence of culture  
373 conditions on the productivity and lutein content of the new strain *Scenedesmus almeriensis*. *Process*  
374 *Biochem.* 2008;43:398–05
- 375 7. Chacón-Lee TL, González-Maríño GE. Microalgae for “healthy” foods—possibilities and  
376 challenges. *Crit Rev Food Sci Food Saf.* 2010;9:655–75.
- 377 8. Tale M, Ghosh S, Kapadnis B, Kale S. Isolation and characterization of microalgae for biodiesel  
378 production from Nisargruna biogas plant effluent. *Bioresour Technol.* 2014;169: 328-35.
- 379 9. Alam MM, Mumtaz AS, Russell M, Grogger M, Veverka D, Hallenbeck PC. Isolation and  
380 characterization of microalgae from diverse Pakistani habitats: Exploring third-generation biofuel  
381 potential. *Energies.* 2019;12(14): 2660.
- 382 10. Prescott GW. How to know the freshwater algae. In: *How to know the freshwater algae.* Dubuque,  
383 Iowa: Wm C. Brown Company Publishers; 1964.

- 384 11. Doyle J. DNA protocols for plants. In Molecular techniques in taxonomy. Springer, Berlin,  
385 Heidelberg.1991
- 386 12. Kaur S, Sarkar M, Srivastava RB, Gogoi HK, Kalita MC. Fatty acid profiling and molecular  
387 characterization of some freshwater microalgae from India with potential for biodiesel production. New  
388 Biotechnol. 2012;29(3):332-44.
- 389 13. Richmond A. editor. Handbook of microalgal culture: biotechnology and applied phycology. John  
390 Wiley & Sons. 2008.
- 391 14. Chen Y, Vaidyanathan S. Simultaneous assay of pigments, carbohydrates, proteins and lipids in  
392 microalgae. Anal Chim Acta, 2013;776:31-40.
- 393 15. Varshney P, Beardall J, Bhattacharya S, Wangikar PP. Isolation and biochemical characterisation  
394 of two thermophilic green algal species-*Asterarcys quadricellulare* and *Chlorella sorokiniana*, which  
395 are tolerant to high levels of carbon dioxide and nitric oxide. Algal Res. 2018;30: 28-37.
- 396 16. Yuan H, Zhang X, Jiang Z, Wang X, Wang Y, Cao L, et al. Effect of light spectra on microalgal  
397 biofilm: Cell growth, photosynthetic property, and main organic composition. Renew Energy,  
398 2020;157: 83-89.
- 399 17. Wellburn AR. The spectral determination of chlorophylls a and b, as well as total carotenoids,  
400 using various solvents with spectrophotometers of different resolution. Journal of plant physiology.  
401 1994;144(3):307-13.
- 402 18. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total  
403 lipids from animal tissues. J biol Chem, 1957;226(1): 497-09.
- 404 19. Wahlen BD, Willis RM, Seefeldt LC. Biodiesel production by simultaneous extraction and  
405 conversion of total lipids from microalgae, cyanobacteria, and wild mixed-cultures. Bioresour Technol.  
406 2011;102(3): 2724-30.
- 407 20. Talebi AF, Tabatabaei M, Chisti Y. BiodieselAnalyzer: a user-friendly software for predicting the  
408 properties of prospective biodiesel. Biofuel Res. J, 2014;1(2):55-57.

- 409 21. Chen X, Song L, Wang H, Liu S, Yu H, Wang X, **et al.** Partial characterization, the immune  
410 modulation and anticancer activities of sulfated polysaccharides from filamentous microalgae  
411 *Tribonema* sp. *Molecules*. 2019;24(2):322.
- 412 22. Musharraf SG, Ahmed MA, Zehra N, Kabir N, Choudhary MI, Rahman AU. Biodiesel production  
413 from microalgal isolates of southern Pakistan and quantification of FAMEs by GC-MS/MS analysis.  
414 *Chem. Cent. J.* 2012;6(1):1-0.
- 415 23. Lloyd C, Tan KH, Lim KL, Valu VG, Fun SM, Chye TR, **et al.** Identification of microalgae cultured  
416 in Bold's Basal medium from freshwater samples, from a high-rise city. *Sci. Rep.* 2021;11(1):1-6.
- 417 24. Yu X, Zhao P, He C, Li J, Tang X, Zhou J, **et al.** Isolation of a novel strain of *Monoraphidium* sp.  
418 and characterization of its potential application as biodiesel feedstock. *Bioresour Technol.* 2012;121:  
419 256-62.
- 420 25. Metzger P, Largeau C. *Botryococcus braunii*: a rich source for hydrocarbons and related ether  
421 lipids. *Appl Microbiol Biotechnol* 2005;66:486e96
- 422 26. Sandeep KP, KumaraguruVasangam KP, Kumararaja P, Syama Dayal J, Sreekanth GB,  
423 Ambasankar K, **et al.** Microalgal diversity of a tropical estuary in south India with special reference to  
424 isolation of potential species for aquaculture. *J. Coast. Conserv.* 2019 23(1):253-67.
- 425 27. Becker EW. Micro-algae as a source of protein. *Biotechnol Adv.* 2007;25(2), 207-10.
- 426 28. Amorim ML, Soares J, Coimbra JSJR, Leite MDO, Albino LFT, Martins MA. Microalgae proteins:  
427 Production, separation, isolation, quantification, and application in food and feed. *Crit Rev Food Sci*  
428 *Nutr.* 2021;61(12):1976-02.
- 429 29. Santhakumaran P, Kookal SK, Ray JG. Biomass yield and biochemical profile of fourteen species  
430 of fast-growing green algae from eutrophic bloomed freshwaters of Kerala, South India. *Biomass and*  
431 *bioenergy*, 2018;119:155-65.
- 432 30. Wang Y, Tibbetts SM, McGinn PJ. Microalgae as sources of high-quality protein for human food  
433 and protein supplements. *Foods*, 2021;10(12): 3002.

- 434 31. de Carvalho Silvello MA, Gonçalves IS, Azambuja SPH, Costa SS, Silva PGP, Santos LO **et al.**  
435 Microalgae-based carbohydrates: A green innovative source of bioenergy. *Bioresour*  
436 *Technol.* 2022;344:126304.
- 437 32. Kuravi SD, Mohan SV. Mixotrophic cultivation of *Monoraphidium* sp. In dairy wastewater using  
438 Flat-Panel photobioreactor and photosynthetic performance. *Bioresour Technol.* 2022;348:126671.
- 439 33. Hu J, Nagarajan D, Zhang Q, Chang JS, Lee DJ. Heterotrophic cultivation of microalgae for  
440 pigment production: A review. *Biotechnol Adv.* 2018;36(1):54-67.
- 441 34. Morocho-Jácome AL, Ruscinc N, Martinez RM, de Carvalho JC, Santos de Almeida T, **et al.** (Bio)  
442 Technological aspects of microalgae pigments for cosmetics. *Appl Microbiol Biotechnol.*  
443 2020;104(22):9513-22.
- 444 35. Nayma Z, Khatoun H, Rahman MR, Mukta FA, Sultana R, Nuruzzaman M. Comparative study of  
445 growth, pigments and proximate composition of selected indigenous freshwater microalgae isolated  
446 from Bangladesh. *Journal of Innovation in Applied Research*, 2022;05:02.
- 447 36. Hosikian A, Lim S, Halim R, Danquah MK. Chlorophyll extraction from microalgae: a review on the  
448 process engineering aspects. *Int J Chem Eng.* 2010;2010.
- 449 37. Dhup S, Dhawan V. Effect of nitrogen concentration on lipid productivity and fatty acid  
450 composition of *Monoraphidium* sp. *Bioresour technol.* 2014;152, 572-75.
- 451 38. Bácsi I, Tóthfalusi F, Márton K, B-Béres V, Gonda S. The Effects of Photobioreactor Type on  
452 Biomass and Lipid Production of the Green Microalga *Monoraphidium pusillum* in Laboratory  
453 Scale. *Appl Sci.* 2022;12(4):2196.
- 454 39. Ajayan KV, Manaswini PS, Harilal CC. Effect of nitrate and phosphate levels on biochemical  
455 contents and fatty acid methyl esters profile of *Monoraphidium contortum* (thuret). *Eco*  
456 *Chron.* 2018;13(2):51-59.
- 457 40. **Zhu H, Wang X, Zhang W, Zhang Y, Zhang S, Pang X, et al. Dietary Schizochytrium Microalgae**  
458 **Affect the Fatty Acid Profile of Goat Milk: Quantification of Docosahexaenoic Acid (DHA) and Its**  
459 **Distribution at Sn-2 Position. *Foods.* 2022;11(14):2087.**
- 460

- 461 41. Knothe G. "Designer" biodiesel: optimizing fatty ester composition to improve fuel properties.  
462 Energy & Fuels, 2008;22(2):1358-64.
- 463 42. Atabani AE, Silitonga AS, Badruddin IA, Mahlia TM, Masjuki H, Mekhilef S. A comprehensive  
464 review on biodiesel as an alternative energy resource and its characteristics. Renew Sustain Energy  
465 Rev. 2012;16(4):2070-93.
- 466 43. Atadashi IM, Aroua MK, Aziz AA. High quality biodiesel and its diesel engine application: a  
467 review. Renew Sustain Energy Rev. 2010;14(7):1999-08.
- 468 44. Namitha B, Sathish A, Kumar PS, Nithya K, Sundar S. Microalgal biodiesel synthesized from  
469 Monoraphidium sp., and Chlorella sorokiniana: feasibility and emission parameter  
470 studies. Fuel, 2021;301:121063.
- 471 45. Pikoli, M. R., Sari, A. F., Solihat, N. A., & Permana, A. H. (2019). Characteristics of tropical  
472 freshwater microalgae *Micractinium conductrix*, *Monoraphidium* sp. and *Choricystis parasitica*, and  
473 their potency as biodiesel feedstock. *Heliyon*, 5(12): e02922.
- 474 46. Patel A, Krikigianni E, Rova U, Christakopoulos P, Matsakas L. Bioprocessing of volatile fatty  
475 acids by oleaginous freshwater microalgae and their potential for biofuel and protein  
476 production. Chem Eng J. 2022;438,135529.
- 477 47. Wali AF, Al Dhaheri Y, Ramakrishna Pillai J, Mushtaq A, Rao PG, Rabbani SA, et al. Lc-ms  
478 phytochemical screening, in vitro antioxidant, antimicrobial and anticancer activity of microalgae  
479 *Nannochloropsis oculata* extract. Separations. 2020;8;7(4):54.
- 480 48. Shanab SM, Mostafa SS, Shalaby EA, Mahmoud GI. Aqueous extracts of microalgae exhibit  
481 antioxidant and anticancer activities. Asian Pac J Trop Biomed. 2012;2(8), 608-615.