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CO₂ enrichment: Enhancing antioxidant, antibacterial and anticancer activities in *Arthrospira platensis*

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22 **Abstract**

23 The effect of CO₂ enrichment on the content of minerals, crude proteins, carbohydrate, and
24 total polyphenols and flavonoids as well as antioxidant, cytotoxic and antibacterial activity of
25 *Arthrospira platensis* were studied. *A. platensis* grown with CO₂ enriched medium had less
26 crude protein but more carbohydrate and phytochemical compounds. Increased calcium and
27 iron contents were also obtained. CO₂ seemed to increase polyphenols, flavonoids and
28 tannins, which in turn increased antioxidant activities using the DPPH assay (IC₅₀ = 1.64 µg
29 mL⁻¹). Moreover, CO₂ enrichment increased antibacterial activity, namely against *E. coli* and
30 *S. enterica*. Cytotoxicity was increased with CO₂, especially with the OVCAR (IC₅₀=17.5 µg
31 mL⁻¹) and MCF-7 (IC₅₀ = 14.5 µg mL⁻¹) cell lines. GC-MS analysis showed that in CO₂ free
32 medium 12 bioactive compounds were found. The most abundant compounds were
33 hexadecanoic acid, methyl ester and n-hexadecanoic acid. However in CO₂ enriched medium
34 results showed the presence of only 7 compounds. Therefore, the use of CO₂ in the production
35 of algae may have a positive environmental benefit to reduce the carbon pollution from the
36 atmosphere and provide a better *Spirulina* production and quality.

37

38 **Key words:** *Arthrospira platensis*, *Spirulina*, CO₂ enrichments, phytochemicals,
39 antibacterial activity, OVCAR, MCF-7.

40 **Introduction**

41 Global atmospheric carbon dioxide (CO₂) concentration has significantly increased since the
42 industrial revolution (270 to 400 ppm), elevating by about 2 ppm/yr during the past few
43 decades (Dlugokencky et al., 2018; Moreira & Pires, 2016). Global CO₂ emissions are about
44 30 Gt/yr, which creates an imbalance in the natural carbon cycle leading to its accumulation
45 in atmosphere (IPPC, 2001). This elevated global atmospheric CO₂ concentration may not
46 only cause climate warming, but also cause profound impacts on the net primary productivity
47 of agricultural and natural ecosystems (Zheng et al., 2018).

48 The reduction of greenhouse gases has become a priority issue (Yoshioka et al., 2013).
49 Photosynthesis by microalgae using atmospheric CO₂ is one process that can reduce CO₂ in
50 the atmosphere (Jeon et al., 2005; Kim & Lee, 2018; Tarko et al., 2012). Cyanobacterium are
51 a blue-green algae found in aquatic and terrestrial habitats. Microalgae produce different
52 compounds, many with bioactivity (Abd El-Baky et al., 2009; Kumari et al., 2014; Wang et
53 al., 2003). Microalgal production in raceway ponds seems to be the best method for large
54 scale production. *A. platensis* is a small spiral-coil shaped blue green algae that grows
55 naturally in mineral-rich alkaline waters, and is increasingly farmed around the world due to
56 its many benefits. Many studies have described *A. platensis* higher productivity with CO₂
57 added to growing medium (Gordillo et al., 1999; Ravelonandro et al., 2011; Singh & Singh,
58 2014). However, none has qualitatively evaluated the bioactive compounds with a CO₂
59 enriched culture media.

60 Generally, outdoor open raceways have been used in the production of microalgae and
61 cyanobacteria at the commercial level (Castro et al., 2015; Costa et al., 2003; Richmond et al.,
62 1999; White & Ryan, 2015).

63 Several reports dealing with the effects of increasing atmospheric CO₂ concentration on the
64 growth parameters and biochemical composition have been done with plants (Reitz et al.,

65 1997; Wang et al., 2003; Yusuke et al., 2007) and algae (Gordillo et al., 1999; Kumari et al.,
66 2014; Singh & Singh, 2014). Algae have an important role in the carbon cycle and their
67 response to elevated CO₂ are of potential benefit (Xu et al., 2010). However, information
68 about the effects of elevated CO₂ on the health-promoting phytochemicals in food algae are
69 limited. This study aims to assess the impact of CO₂ enrichment on phytochemical levels in *A.*
70 *platensis* extracts: (i) polyphenols, flavonoids and tannins content, and (ii) antioxidant (TAC,
71 DPPH and FRAP assays), anti-bacterial and cytotoxic activities.

72

73 **Materiel and Methods**

74 **Algae strain, growth medium and culture conditions**

75 *The A. platensis* strain was obtained from Eden Life (a Tunisian company specializing in the
76 production of microalgae) located in the south of the country (Kettana, Gabes). The *A.*
77 *platensis* algae were cultivated using the standard Zarrouk medium (containing
78 macronutrients: NaHCO₃: 18.0, NaNO₃: 2.5, K₂HPO₄: 0.5, K₂SO₄: 1.0, NaCl: 1.0, CaCl₂:
79 0.04, Na₂EDTA: 0.08, MgSO₄.7H₂O: 0.20, FeSO₄.7H₂O: 0.01 and trace elements: H₃BO₃:
80 2.86, (NH₄)₆ Mo₇O₄: 0.02, MnCl₂.4H₂O: 1.80, CuSO₄.5H₂O: 0.08, ZnSO₄.7H₂O: 0.22 (g/L))
81 (Zarrouk 1966). *A. platensis* cultivation was done in 10 m³ raceway ponds. A raceway is an
82 oblong and superficial recirculating pond with semi-circular ends. A paddlewheel
83 continuously circulates (30 cm/s) and mixes and aerates the algal broth in the channel. Ponds
84 (50 cm depth) were placed in a controlled glass house protected from UV light, using plastic
85 film. In the open raceway ponds, the volume of the medium was maintained unchanged by the
86 replacement of the water lost by evaporation. The cultures were illuminated using halogen
87 spots (Decostar Titan 51, 12V, 35 W; Osram GmbH, Munich, Germany) which provided 52–
88 55 μmol (photon) m⁻² s⁻¹ and light/dark cycles of 16/8 h at 25 °C and aerated continuously
89 with liquid CO₂ at ~50 bar with a flow rate of 0.3 L min⁻¹. The high purity CO₂ (99.9%) was

90 provided by the Air Liquide Co. (Sfax, Tunisia). The bubbling of the CO₂ in the raceway pond
91 is done using an air flow meter diffuser (Air Liquide, HBS 315/8 c7 1206) with a pipe in the
92 form of a T for greater diffusion. The culture was filtered through a fine mesh (about 30 µm)
93 which collects the *Spirulina* biomass. The fresh biomass was then dried using a self made
94 solar panel hot air drier and vacuum stored at room temperature (23±3 °C), before extractions
95 were carried out for a maximum of 10 wk.

96

97 **Proximate Analysis**

98 Analyses of *A. platensis* for ash was done using combustion at 550 °C overnight (Woyewoda
99 et al., 1986). Moisture content was assayed as follows: about 3 L of the algal culture were
100 collected and filtered through a pre-weighed 30 µm mesh filter (Diatex, Laval, France).
101 Samples were weighed before and after drying for 15 h at 105 °C. Water content is calculated
102 by the difference between the fresh weight (fw) and the dry weight (dw). Total sugars were
103 determined using the phenol-sulfuric acid method (Dubois et al., 1956).
104 Kjeldahl apparatus and method (using a DK8 digester and UDK 129 distillation unit, Velp-
105 Scientifica, Usmate, Italy) was used to determine the total nitrogen content in *A. platensis*
106 samples (AOAC, 1996). After total nitrogen content determination, crude protein contents
107 were calculated:

$$108 \quad \mathbf{N\% = V*0.0014*(100/M)}$$

$$109 \quad \mathbf{P\% = N\%*6.25}$$

110 (Where V = sulfuric acid volume and M = dw)

111

112 **Mineral composition determination**

113 Contents of Ca, Cd, Fe, K, Mg, Na, Pb, and Zn were assayed according to the method of
114 Indrayan et al. (2005). Mineral compositions of the sampled were made as follows: 20 to 25

115 mg of dried samples were ashed using 50 mL 1% hydrochloric acid then filtered using a
116 Whatman ashless filter paper (GE Healthcare, GmbH, Freiburg, Germany). Atomic
117 absorption spectrometry (Perkin Elmer Model AAS 4100, Boston, MA, USA) was used for
118 determination of mineral contents. All the analysis were evaluated in triplicate.

119

120 **Soxhlet lipid extraction**

121 Lipid content was analyzed according to the method of Ramluckan et al. (2014). Briefly, dried
122 biomass (0.5 g) was extracted in chloroform for 5 h using a solid-liquid extraction system
123 (Soxhlet extractor CG-1374 GPE Scientific, Ltd., Bedford, UK). The extract was then
124 concentrated on a rotary evaporator and weighed.

125

126 **Preparation of methanol extracts**

127 Dried biomass of *A. platensis* was extracted using methanol with a constant solid/liquid ratio
128 of 1:10 (w/v). The solid–liquid extraction was done for 2 h, where the suspension was
129 continuously mixed using a magnetic agitator (AREX Velp-Scientifica, Italy). The extraction
130 was carried out in the dark at room temperature and then the liquid was separated by
131 centrifugation (2,200 g) for 10 min at 20°C using a HERMLE Z 513 K centrifuge (Hermle
132 Labortechnik, Wehingen, Germany). Then, a rotary vacuum evaporator R-200 (Büchi
133 Labortechnik, Flawil, Switzerland) was used to evaporate the extract. The supernatant
134 samples were dissolved in methanol (10 mg mL⁻¹).

135

136 **Total polyphenols content (TPC)**

137 TPC was determined spectrophotometrically using the Folin-Ciocalteu method (Singleton et
138 al., 1999). Briefly, 0.1 mL of sample extract was mixed with 0.5 mL of 10-fold diluted Folin-
139 Ciocalteu's phenol reagent and allowed to react for 5 min. Then 0.4 mL 7% Na₂CO₃ solution

140 was added and the final volume was brought to 2 mL with distilled water. After 1 h at room
141 temperature, the absorbance at 760 nm was measured using a UV-VIS Spectrophotometer
142 (2005, JP Selecta S.A., Barcelona, Spain). A calibration curve was prepared using gallic acid
143 (GA) as a positive control. Results were expressed as mg GA equivalents(E)/g of the
144 *Spirulina* dw.

145

146 **Total flavonoids content (TFC)**

147 TFC was done using a colorimetric method according to Dewanto et al. (2002). An aliquot
148 (500 μ L) of algae extract or standard solution of catechin (C, 0 to 500 μ g mL⁻¹) was added to
149 1.5 mL distilled water, 150 μ L 5% sodium nitrite solution and mixed for 6 min before
150 addition of 150 μ L 10% aluminum chloride. Volume was adjusted with distilled water to 2.5
151 mL and incubated at room temperature for 6 min. Reaction was completed by adding 500 μ L
152 4% sodium hydroxide. The final absorbance was determined at 510 nm against a blank. **The**
153 **TFC** was reported as mg CE/g dw against the calibration curve of catechin, from 0 to 500
154 μ g mL⁻¹.

155

156 **Tannins content**

157 Condensed tannins were assayed using the method previously described by Sun et al. (1998).
158 *A. platensis* extract (50 μ L) was added to, 3 mL 4% vanillin (w/v in methanol) and 1.5 mL
159 concentrated HCl. Then the absorbance was measured at 500 nm after incubation in the dark
160 (15 min), at room temperature. The content of total condensed tannins in *Spirulina* extract is
161 expressed as mg CE/g dw.

162

163 ***In vitro* antioxidant assays**

164 ***Total antioxidant activity (TAC)***

165 The TAC was determined using the phosphate molybdate method (Prieto et al., 1999).
166 Briefly, 0.1 mL of extract was combined with 1 mL of solution composed of sulfuric acid (0.6
167 M), sodium phosphate (28 mM) and ammonium molybdate (4mM). The mixture was then
168 incubated at 95 °C for 90 min. The absorbance was measured at 695 nm, after cooling to
169 room temperature. Results were expressed as mg GAE/g dw.

170

171 ***DPPH radical scavenging assay***

172 The stable free radical scavenging activity was evaluated using the DPPH assay (Shekhar and
173 Anju, 2014). A methanol extract of *A. platensis* was mixed, at equal volume, with 2,2-
174 diphenyl-1-picrylhydrazyl (DPPH, 100 mM), then incubated at room temperature, for 15 min.
175 The absorbance was determined at 517 nm. Butylated hydroxytoluene (BHT) was used as a
176 positive control. These measurements were done in triplicate and the percentage of inhibition
177 of DPPH oxidation (P_i) was calculated using the following formula:

$$178 \quad P_i = ((A_b - A_s) / A_b) * 100$$

179 Where A_b is the absorbance of the control and A_s is the absorbance of the extract. The 50%
180 inhibitory concentration (IC_{50}) was expressed as the quantity of the extracts to react with a
181 half of DPPH radicals. The IC_{50} values were calculated using linear regression analysis and
182 used to indicate antioxidant capacity.

183

184 ***Ferric Reducing Antioxidant Power assay (FRAP)***

185 The FRAP assay was done according to the process of Benzie and Strain, (1996). The FRAP
186 reagent was prepared from 0.3 M acetate buffer (pH 3.6), 10 mmol 2,4,6-tripyridyl-s-triazine
187 (TPTZ) solution in 40 mmol hydrochloric acid and 20 mmol iron (III) chloride solution with
188 the ratio 10:1:1 (v/v/v). Briefly, 50 μ L of sample of phytochemicals (three replicates) were
189 added to 1.5 mL of the FRAP reagent. Four min after, the absorbance was determined at 593

190 nm. The standard curve was constructed using FeSO₄ solution (from 50 to 200 µg mL⁻¹), and
191 the results were expressed as µg mL⁻¹ Fe (II).

192

193 **Gas Chromatography – Mass Spectrometry (GC-MS)**

194 The chemical identification of volatile compounds from the organic extracts was determined
195 using the method of Kohoude et al. (2017). The gas chromatography-mass spectrometry was
196 an Agilent Technologies GC systems with a GC-7890A/MS-5975C model (Agilent
197 Technologies, Santa Clara, CA, USA) equipped with a fused silica capillary DB-5MS column
198 (5% phenyl-methylpolysiloxane, 30 x 0.25 mm, film thickness 0.25 µm, Agilent
199 Technologies). Chromatographic conditions were; 50 °C hold for 1 min, up to 250 °C at the
200 rate of 10 °C min⁻¹ and then held 1 min at 250 °C. After that, a second gradient was used
201 going to 300 °C at 50 °C min⁻¹ and finally a 300 °C hold for 3 min. For analysis reasons, the
202 samples were dissolved in their principal solvents. Ten µL of each extract was injected (into a
203 200 °C heated injector) with the ratio of 1:10 in the split mode. The carrier gas used was
204 helium with a flow rate of 1 mL min⁻¹. The mass spectrometer was adjusted for an emission
205 current of 10 µA and electron multiplier voltage 1400 - 1500 V. Trap temperature was 250 °C
206 and that of the transfer line was 270 °C. Mass scanning was in the interval of 40 to 650 amu.

207 The identification of the compounds was done using mass spectra comparison with those
208 obtained in NIST08 (National Institute of Standards and Technology, <https://www.nist.gov/>),
209 using AMDIS software (Automated Mass Spectral Deconvolution and Identification System,
210 <https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis>).

211 AMDIS is a software used for GC-MS data interpretation from NIST. The goal was to find
212 the maximum similarity, in terms of spectra, between the compounds detected in extracts and
213 those suggested by the NIST database. Finally, the retention index facilitated certain
214 assignments.

215

216 **Cytotoxicity evaluation**

217 Cytotoxicity of extracts against human breast (MCF-7), ovarian (OVCAR) and colon (HCT-
218 116) cancer cell lines, ordered from the American Type Culture Collection (ATCC Co.,
219 Manassas, VA, USA), was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl
220 tetrazolium bromide (MTT) assay of El Euch et al. (2015). The HCT-116 cell line was grown
221 in Gibco RPMI 1640 medium (Thermo Fisher Scientific, Paisley, UK), while MCF-7 and
222 OVCAR cell lines were grown in Dulbecco's modified eagle medium (DMEM) (Thermo
223 Fisher Scientific) supplemented with 10% faetal calf serum (Gibco, Langley, OK, USA), air
224 and 5% CO₂. **Briefly**, 100 µL of cells were distributed in 96-well plates at a concentration of
225 10⁴ cells/well and incubated at 37 °C for 24 h. **Then**, 100 µL of cells in exponential growth
226 phase were incubated in a fully humidified atmosphere at 37 °C for 48 h with the addition of
227 100 µL of the culture medium, supplemented with 2 mM L-glutamine and 50 µg mL⁻¹
228 gentamycin, containing extracts at a concentration of 50 mg L⁻¹. The medium was removed
229 and cells were treated with MTT solution, i.e., 50 µL, 1 mg mL⁻¹ in phosphate buffered saline
230 (PBS) and incubated at 37 °C for 40 min. MTT solution was then discarded and dimethyl
231 sulfoxide (DMSO) (50 µL) was added to dissolve the blue crystals. A microplate reader
232 (Multiskan Go, F1-01620, Thermo Fisher Scientific, Vantaa, Finland) was used to measure
233 the absorbance at 605 nm. Tamoxifen (100 µM) was used as a positive control.

234

235 **Antibacterial activity**

236 The *A. platensis* extracts were tested individually 9 human-pathogenic microbial strains: 6
237 Gram positive bacteria (*Enterococcus faecalis*; *Listeria monocytogenes*; *Bacillus subtilis*;
238 *Bacillus cereus*; *Bacillus thuringiensis*; *Micrococcus luteus*) and 3 Gram-negative ones

239 (*Salmonella enterica*; *Escherichia coli*; *Klebsiella pneumoniae*). The bacteria used were
240 selected for their involvement in the human skin, oral and intestinal tract.
241 The antimicrobial activities of *Spirulina* extracts were assayed using the well diffusion
242 method according to NCCLS M2-A5 (NCCLS, 1993). Inoculum containing 10^6 colony-
243 forming units (cfu) mL^{-1} of each bacterial culture to be tested was spread on Mueller-Hinton
244 (MH) agar plates with a sterile swab moistened with the bacterial suspension. Subsequently,
245 wells of 6 mm diameter were punched, using a sterile agar cutter, into the MH agar medium.
246 The antimicrobial activity of the *Spirulina* extract was checked by introducing 50 μL of 100
247 $\mu\text{g mL}^{-1}$ concentrations into triplicate wells. An additional wells in each plate was filled with
248 the solvent DMSO, which served as the negative controls while ampicillin (5 $\mu\text{g mL}^{-1}$) was
249 used as positive control. The culture plates were allowed to diffuse for 2 h at room
250 temperature. The plates were then incubated, for 24 h, in the upright position at 37 °C. After
251 that, the antimicrobial activities of the algae extract and the standard antibiotic were
252 determined. Zones of the inhibition around each of the extract and the antibiotic were
253 measured to the nearest mm using a digital caliper (Shanghai Taihai Congliang Ju Co., Ltd.,
254 Shanghai, China). Three replicates were carried out for each test microorganism.

255

256 **Statistical Analysis**

257 Data were expressed as the mean value \pm SD of 3 replicate samples. The statistical analyzes
258 were done using the one way analysis of variance (ANOVA) procedure with the Statistical
259 Package for the Social Sciences (SPSS) version 20.0 software (IBM Corp, released 2011,
260 Armonk, NY, USA). When $p < 0.05$, differences were considered as statistically significant
261 according to Fisher's LSD test.

262 **Results**

263 **Proximate analysis**

264 The proximate composition parameters of *A. platensis* with controlled (-CO₂) or carbon
265 dioxide enriched medium (+CO₂) is reported in Table 1. Results showed low moisture content
266 with both culture media. Increased CO₂ in the culture medium seemed to decrease protein
267 content and to increase carbohydrate, while moisture, ash and fats contents remained
268 unchanged with CO₂ treatment (Table 1).

269

270 **Mineral element composition**

271 Results in Table 2 show that *A. platensis* powder contains high amounts of K, Mg and Na
272 (more than 1.1 g/100 g dw) followed with Ca than iron. Enhanced CO₂ in the culture medium
273 affected the mineral composition differently: No significant trend was detected for K, Mg, Zn
274 and Na, while a significant increase was seen for Fe (26%) and Ca (21%). Pb and Cd were not
275 detected with both media.

276

277 **Phytochemical**

278 Contents of polyphenols compounds expressed as GAE and flavonoids and tannins contents
279 expressed as CE were measured in *A. platensis* methanolic extracts (Fig. 1). Elevated CO₂ in
280 the culture medium (+CO₂) significantly increased the polyphenols, flavonoids and tannins.
281 Amongst the components detected in the methanol extract of *A. platensis*, polyphenols
282 showed the most important increase in CO₂ enriched medium reaching 54%, while flavonoids
283 reached 36% and tannins only 28%.

284

285 **Antioxidant activity**

286 The antioxidant activity of *A. platensis* methanolic extracts was evaluated. Three
287 complementary experiments were used to estimate the antioxidant activity of *A. platensis*:
288 TAC, DPPH free radical-scavenging activity and FRAP. It is important to use different
289 measures of antioxidant capacity, because many factors can influence this capacity. As
290 summarized in Figure 2, TAC ranged from 25 to 35 mg GAE g⁻¹ dw, respectively with CO₂
291 free (-CO₂) and CO₂ fed (+CO₂) medium The half-maximal inhibitory concentration (IC₅₀) is
292 an estimation of the efficiency of a substance in reducing a specific biological and/or
293 biochemical function. It represents the amount of a compound or bioactive molecule that is
294 required for 50% inhibition *in vitro*. *A. platensis* methanol extracts had the capacity to reduce
295 DPPH. CO₂ enrichment induced a stimulation of antioxidant activities, the IC₅₀ were
296 increased by 25 and 65% for DPPH and FRAP, respectively.

297

298 **Antibacterial activity**

299 The antibacterial activities of *A. platensis* methanol extracts was determined against 6 Gram-
300 positive bacteria and 3 Gram-negative ones and results are shown in Table 3. *A. platensis*
301 methanolic extract showed the highest mean ZOI against the Gram-positive *L. monocytogenes*
302 and *B. thuringiensis* while it showed lower ZOI against the other Gram-positive studied
303 bacteria. For Gram-negative bacteria, the maximum ZOI was observed against *K.*
304 *pneumoniae*. *E. coli* showed the minimum ZOI against all the studied bacteria. No ZOI was
305 found in the DMSO blind control whereas in the positive control ampicillin (5 µg) ZOI
306 ranged from 15 to 20 mm against all the studied bacteria. Increased CO₂ concentration level
307 with culture conditions (+CO₂) directly affected responses of *A. platensis* methanol extract
308 with both *E. coli* and *S. enterica* bacteria by increasing ZOI. The other bacteria showed no
309 significant trend with elevated CO₂ in the culture medium.

310

311 **Cytotoxic activity**

312 Anticancer activity of *A. platensis* methanol extracts with different concentrations were
313 determined using the cell lines viability assay. Table 4 summarizes cytotoxic effects of *A.*
314 *platensis* methanol extracts on the three cell lines viability and IC₅₀ values. With controlled
315 conditions (-CO₂), no inhibition was found in the three cancer cell lines. For algae treated
316 with CO₂ *A. platensis* methanol extracts were found to inhibit proliferation of human colon
317 (HCT-116), breast (MCF-7) and ovarian (OVCAR) cancers. The overall results indicated that
318 *A. platensis* methanolic extracts cultivated with elevated CO₂ concentration showed the
319 strongest cytotoxic activities towards cancer cells HCT-116.

320

321 **GC-MS analysis**

322 The *A. platensis* methanolic extract using GC-MS analysis showed the presence of 12 volatile
323 compounds. Table 5 shows the compounds with their retention time (RT) and molecular
324 formula. In the CO₂ free medium, the methanol extract of *A. platensis* showed 12 compounds.
325 The most abundant compound was the hexadecanoic acid, methyl ester. The n-hexadecanoic
326 acid; 8,11-octadecadienoic acid, methyl ester and phytol were also found at intermediate
327 quantities as compared to the other compounds. However in CO₂ enriched medium results
328 shown in Table 5 showed only 7 compounds. Glycerin; 4:3,6-dianhydro- α -d-glucopyranose;
329 heptadecane; 4-hydroxy- β -ionone and 3',8,8'-trimethoxy-3-piperidyl-2,2'-binaphthalene-
330 1,1',4,4'-tetrone were not detected. The production of the n-hexadecanoic acid was reduced by
331 80% with the CO₂ enrichment. The quantities of the other compounds are greater than or
332 equal for the CO₂ enriched system. The majority of compounds extracted were fatty acids or
333 esters of fatty acids.

334

335 **Discussion**

336 CO₂ enrichment did not alter the mineral composition in *A. platensis* (Table 2). *A. platensis*
337 could be considered as good sources of minerals and available carbohydrate with low levels
338 of fats. Previous studies reported different trends of minerals and proximate composition
339 (Babadzhanov et al., 2004; Branger et al., 2003; Oluyemi et al., 2006). This could be due to
340 different growth conditions, locality and culture medium composition.

341 Enhanced CO₂ in the culture medium increased carbohydrate content (Table 1). A stimulation
342 of growth, photosynthesis rate and biochemical composition with elevated CO₂ are shown in
343 most reviews (Bazzaz, 1990; Mattson et al., 2005). Exposure of plants to elevated CO₂
344 usually leads to amplified rates of net photosynthesis which often enhances total non-
345 structural carbohydrates concentration (Booker, 2000). Enhanced polyphenols, flavonoids and
346 tannins in *A. platensis* with CO₂ enrichment was found (Table 3). According to the carbon
347 nutrient balance theory, that with higher atmospheric CO₂, the increases of the carbon to
348 nitrogen ratio induces the synthesis of secondary metabolites by carbon allocation to the
349 secondary metabolism (Tisserat & Vaughn, 2001). CO₂ enrichment conditions significantly
350 enhanced polyphenols content (20 mg g⁻¹) flavonoids (10 mg g⁻¹) and tannins (5.4 mg g⁻¹) in
351 *A. platensis*, and these high contents were associated with elevated antioxidant activity (Fig.
352 2). Elevated CO₂ may allow for the removal of free radicals, which are associated with
353 increased antioxidant capacity (Wang et al., 2003). The polyphenols contained different
354 bioactivities, including anti-inflammatory, antiviral, antioxidant, antithrombotic, vasodilatory,
355 and anticarcinogenic properties (Singh & Patra, 2018). Wu et al. (2005) estimated that the
356 total polyphenols and other metabolites are linked to antioxidant properties.

357 The role of dietary antioxidants and their potential health and disease benefits have been
358 studied (Huang, 2018). Components with antioxidant activity are found in only a few species
359 of algae (Shanab et al., 2012). Phenolic compounds acquire antioxidant activity in biological
360 systems (Lin et al., 2016; Pourreza, 2013). Although the accumulation of polyphenols in

361 plants is well known, the antioxidant properties of algae are poorly known. These results
362 suggested that the methanol extracts of *A. platensis* may be a promising new source of natural
363 compounds with antioxidant ability. The antioxidant activity of *A. platensis* extracts appeared
364 to be related to their high polyphenols content, since the DPPH scavenging activity of the
365 extracts is correlated with the increase of their polyphenols content. The scavenging activity
366 of phenols extracts of *Spirulina maxima* toward DPPH radicals suggested that the antioxidant
367 activity was carried out by mechanisms that needed electron and hydrogen donors (Abd El-
368 Bakyet al., 2009; El Jemli et al., 2016). The polyphenols of *Spirulina* extracts can obstruct the
369 chain reactions of the lipid peroxidation process by providing hydrogen atom to the free
370 radicals. The avoidance of the beginning step of the chain reaction by scavenging free radicals
371 is considered to be an important mode action (Ramdani et al., 2017; Ruberto et al., 2001).

372 Increased CO₂ in the culture medium ameliorated cytotoxic effects against human ovary
373 cancer cells (OVCAR) and breast cancer cell lines (MCF-7) while no cytotoxic effects were
374 detected against colon carcinoma cells (HCT-116) (Table 4).

375 Some studies reported that tumors were prevented with algae extracts (Abu Zaid et al., 2015;
376 Farooqi et al., 2012; Martínez et al., 2018), because of their antioxidant properties (Abu Zaid
377 et al., 2015). Mohd-Syahril et al. (2012) described the importance of chemo in treating cancer.
378 Hence, new anticancer agents should be sought from various resources. An important number
379 of anticancer compounds are natural products or their derivatives, mostly produced by
380 photosynthetic microalgae (Khan et al., 2018).

381 Enriching plants with high CO₂ levels has been shown to increase the bioactive compounds
382 and their biological activities (Idso et al., 2000; Karimi et al., 2016). Variation of secondary
383 metabolites and phytochemicals, for example, total polyphenols, flavonoids, fatty acids and
384 volatile compounds, may result in high anti-cancer activity of algae and or plants.

385 Both extracts of *A. platensis* (-CO₂ and +CO₂) showed different pattern of antibacterial
386 activities. The difference in the response between Gram-negative and Gram-positive bacteria
387 could be due to the difference in their cell wall structure. CO₂ enriched medium seems to
388 enhance the antibacterial effect of *A. platensis* methanolic extracts on *S. enterica*, *E. coli*, and
389 *K. pneumoniae* (Table 3). It appears that extracts with important anti-microbial activities
390 against Gram-negative bacteria do not necessarily exert a high activity against other Gram-
391 negative bacteria compared to Gram-positive bacteria (Elisha et al., 2017).

392 The mechanisms of antitumoral, antiviral and antimicrobial effects of *A. platensis* are due to
393 its composition of endonuclease and fatty acids (specially high amount of γ -linolenic acid),
394 respectively, as well as to its phycocyanin pigment, a brilliant blue polypeptide (Hoseini et al.,
395 2013).

396 The production of volatile compounds was higher without CO₂ than with CO₂ enrichment.
397 This trend can be linked to the production of high molecular weight compounds with CO₂
398 enrichment, and therefore fewer volatiles. These points to the possibility of forming other
399 antioxidant, antibacterial and cytotoxic compounds in a CO₂ enriched medium, such as
400 polysaccharides (Chaiklahan et al., 2013) and/or proteins (Wang & Zhang, 2017).

401

402 **Conclusion**

403 Elevated CO₂ is known to have a positive effect on photosynthetic activity, growth, biomass
404 accumulation and yield. Higher photosynthetic activity can result in carbon accumulation,
405 which can affect a wide range of metabolic functions including primary and secondary
406 metabolisms in algae. CO₂ enrichment of the culture medium of *A. platensis* enhanced the
407 accumulation and distribution of bio-active compounds such as polyphenols and flavonoids,
408 fatty acids or esters, volatile compounds as well as their antioxidant, antibacterial and
409 anticancer activities. The results of antioxidant activity of *A. platensis* indicated a good

410 potential, especially when grown with elevated carbon dioxide. A screening fractionation is
411 planned to identify the most active phenolic compounds in *Spirulina* extracts. The search is in
412 progress to find new safe and effective antioxidants for use in foods and pharmaceutical
413 preparations to replace synthetics. Therefore, the production of algae under CO₂ enrichment
414 could be used as one of the solutions of carbon sequestration to achieve permanent removal of
415 the carbon from the atmosphere. Further extensive experiments will be required to confirm
416 this approach.

417

418 **Conflict of interest**

419 The authors confirm that they have no conflicts of interest with respect to the study described
420 in this manuscript.

421

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429

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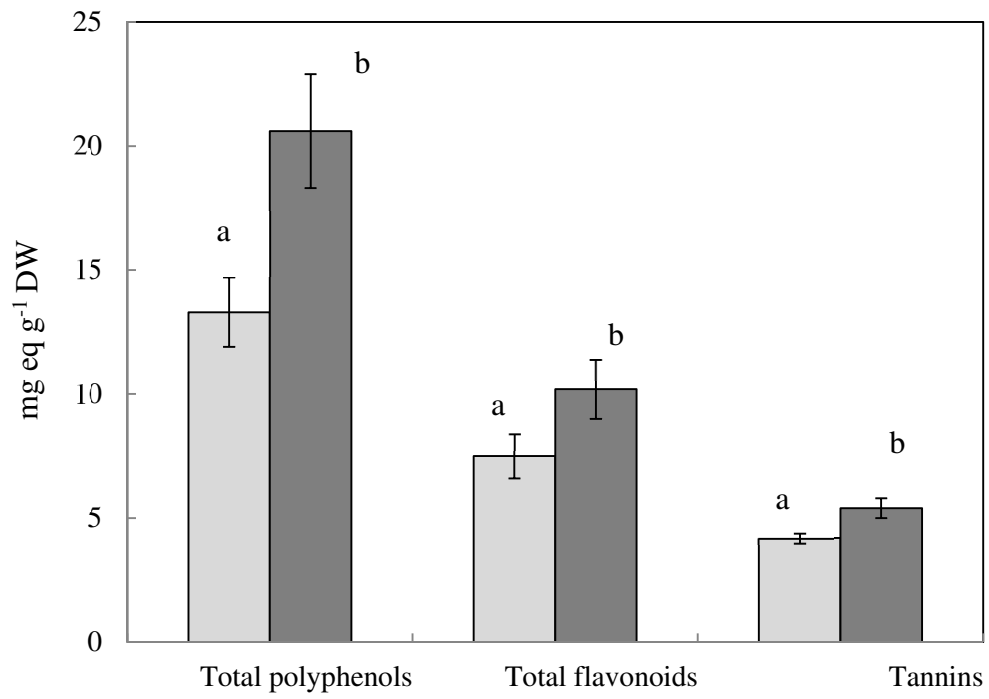
617 **Figure legend:**

618

619 **Figure 1:** Phytochemical composition extract of *A. platensis* grown in control (without CO₂
620 added) or CO₂ enriched medium. Total polyphenols is expressed as gallic acid equivalents
621 (mg GAE g⁻¹dw).The total flavonoids and condensed tannin concentration are expressed as
622 catechin equivalents (mg CE g⁻¹dw). Results are mean of triplicate determinations ± S.D. The
623 same letters are not significantly different.

624

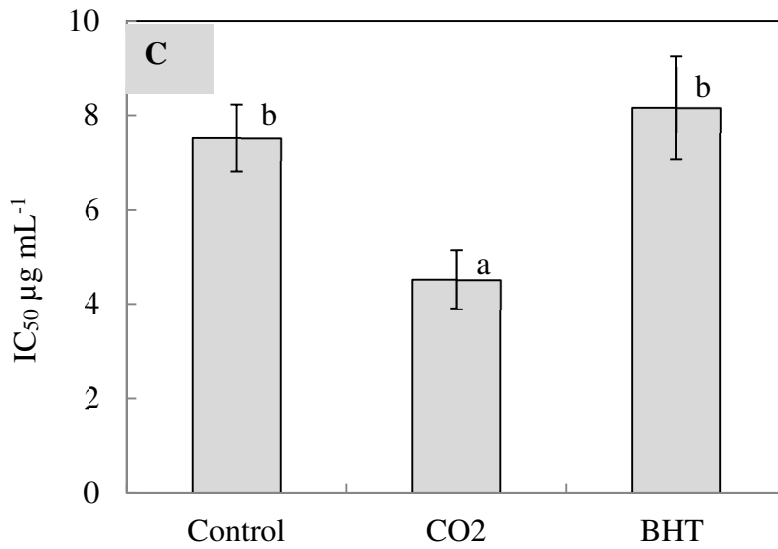
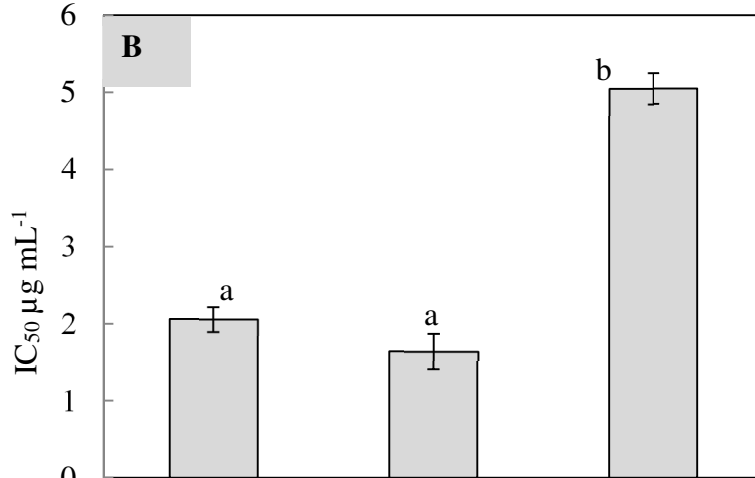
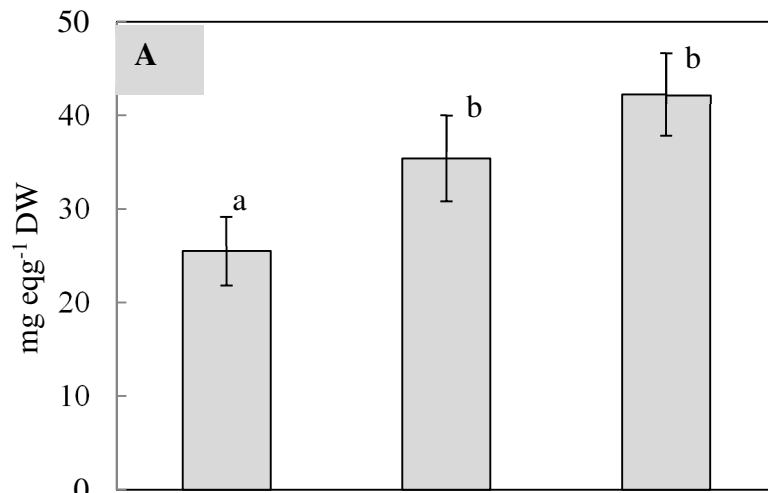
625 **Figure 2:** Antioxidant activities of methanol extract of *A. platensis* grown in control (without
626 CO₂ added) or CO₂ enriched medium, compared to butylated hydroxy toluene (BHT), as a
627 positive control. **A:** Total antioxidant activity (mg GAE g⁻¹dw); **B:** DPPH radical scavenging
628 activity assay (IC₅₀: μg mL⁻¹); **C:** Ferric reducing antioxidant power (FRAP) (IC₅₀: μg mL⁻¹).
629 Results are mean of triplicate determinations ± S. D. The same letters are not significantly
630 different.



1

2 **Figure 1.**

3



7 **Figure 2**

1 **Table 1.** Proximate composition of *Arthrospira platensis* grown in control (-CO₂) or CO₂
2 enriched (+CO₂) medium. Moisture, ash, protein, fats and total sugars are expressed in %.
3 Values are shown as mean±standard deviation (n=3). Values with the different superscript
4 letters (a and b), in the same row, are significantly different at p < 0.05.

5

%	-CO ₂	+CO ₂
Moisture	3.8±0.8a	3.9±1.5a
Ash	16±2a	20±5a
Crude protein (N*6.25)	67±6a	52±4b
Fats	6.0±1.4a	5.3±0.9a
Total sugars	19±3b	36±6a

6

7 **Table 2.** Mineral composition in g (Mg, K and Na) or mg (Ca, Fe, Zn)/100 g dw of
 8 *Arthrospira platensis* grown in control (-CO₂) or CO₂ enriched (+CO₂) medium values are
 9 shown as mean ± standard deviation (n=3). Values with the different superscript letters (a and
 10 b), in the same row, are significantly different at p < 0.05.

	-CO ₂	+CO ₂
Mg	1.24±0.01a	1.25±0.02a
K	1.14±0.01a	1.03±0.02a
Na	1.12±0.02 a	1.02±0.02 a
Ca	160±10b	190±10a
Fe	27±5b	34±4a
Zn	0.50±0.01 a	0.50±0.02 a
Cd	*	*
Pb	*	*

11 (*: not detected).

12

13 **Table 3.** Antibacterial activity of *Arthrospira platensis* grown in control (-CO₂) or CO₂
 14 enriched (+CO₂) medium. Values are shown as mean ± standard deviation (n=3). Values with
 15 different superscript letters (a, b and c), in the same line, are significantly different at p < 0.05.

	Ampicillin	-CO ₂	+CO ₂
Gram-negative bacteria			
<i>Salmonella enterica</i>	17.1±0.4a	14.2±0.3b	18.2±0.5a
<i>Escherichia coli</i> 25922	15.3±0.4b	13.4±0.3c	16.3±0.2a
<i>Klebsiella pneumoniae</i> WHO24	18.4±0.3a	16.2±0.3b	16.3±0.2b
Gram-positive bacteria			
<i>Enterococcus faecalis</i>	19.4±0.1a	15.5±0.2b	14.3±0.2b
<i>Listeria monocytogenes</i>	21±1a	19.3±0.2a	19.4±0.4a
<i>Bacillus subtilis</i>	19.5±0.1a	15.2±0.3b	14.2±0.2b
<i>Bacillus cereus</i>	18.2±0.2a	15.4±0.3b	16.5±0.2b
<i>Bacillus thuringiensis</i>	20.6±0.3a	19.6±0.1a	18.3±0.2b
<i>Micrococcus luteus</i>	20.5±0.1a	14.4±0.3b	13.2±0.2b

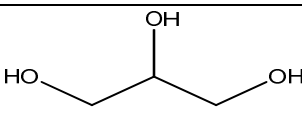
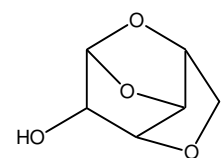
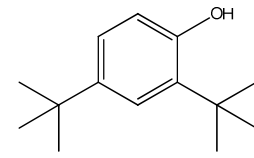

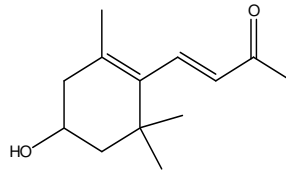
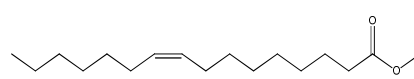
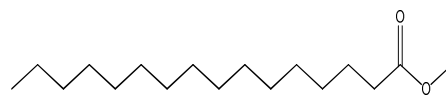
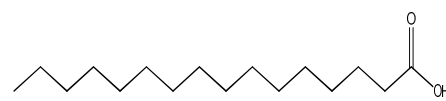
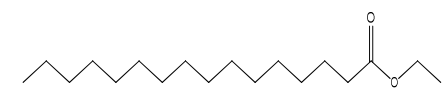
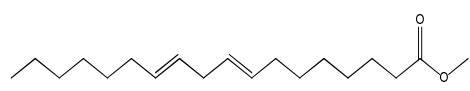
16

17 **Table 4.** Cytotoxicity of methanol extracts of *Arthrospira platensis* on HCT-116, MCF-7 and
18 OVCAR cell lines. IC₅₀ value (μg mL⁻¹). Values are shown as mean ± standard deviation
19 (n=3).

Cell lines	-CO₂	+CO₂	Tamoxifen
OVCAR	>50	17±1	0.19±0.03
MCF-7	>50	14.5±0.5	0.15±0.02
HCT-116	>50	>50	0.14±0.02

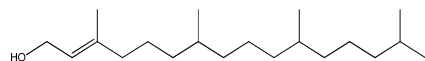
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21 **Table 5:** Phytocomponents identified using GC-MS in the methanol extracts of *Arthrospira platensis*.

Retention time	Molecular compound name	Formula	Peak area (x10 ³ arbitrary units)	
			without CO ₂	with CO ₂
6.35	Glycerin		5	
9.64	1,4:3,6-Dianhydro- α-d-glucopyranose		3	
13.63	Phenol, 2,4- bis(1,1- dimethylethyl)-		3	3
15.77	Heptadecane		9	
15.81	4-Hydroxy-β- ionone		2	
17.96	9-Hexadecenoic acid, methyl ester, (Z)-		8	7
18.16	Hexadecanoicacid, methyl ester		110	100
18.51	n- Hexadecanoicacid		55	11
18.82	Hexadecanoicacid, ethyl ester		4	4
19.80	8,11- Octadecadienoic acid, methyl ester		30	30

19.98

Phytol



25

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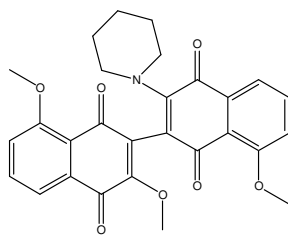
3',8,8'-Trimethoxy-

24.07

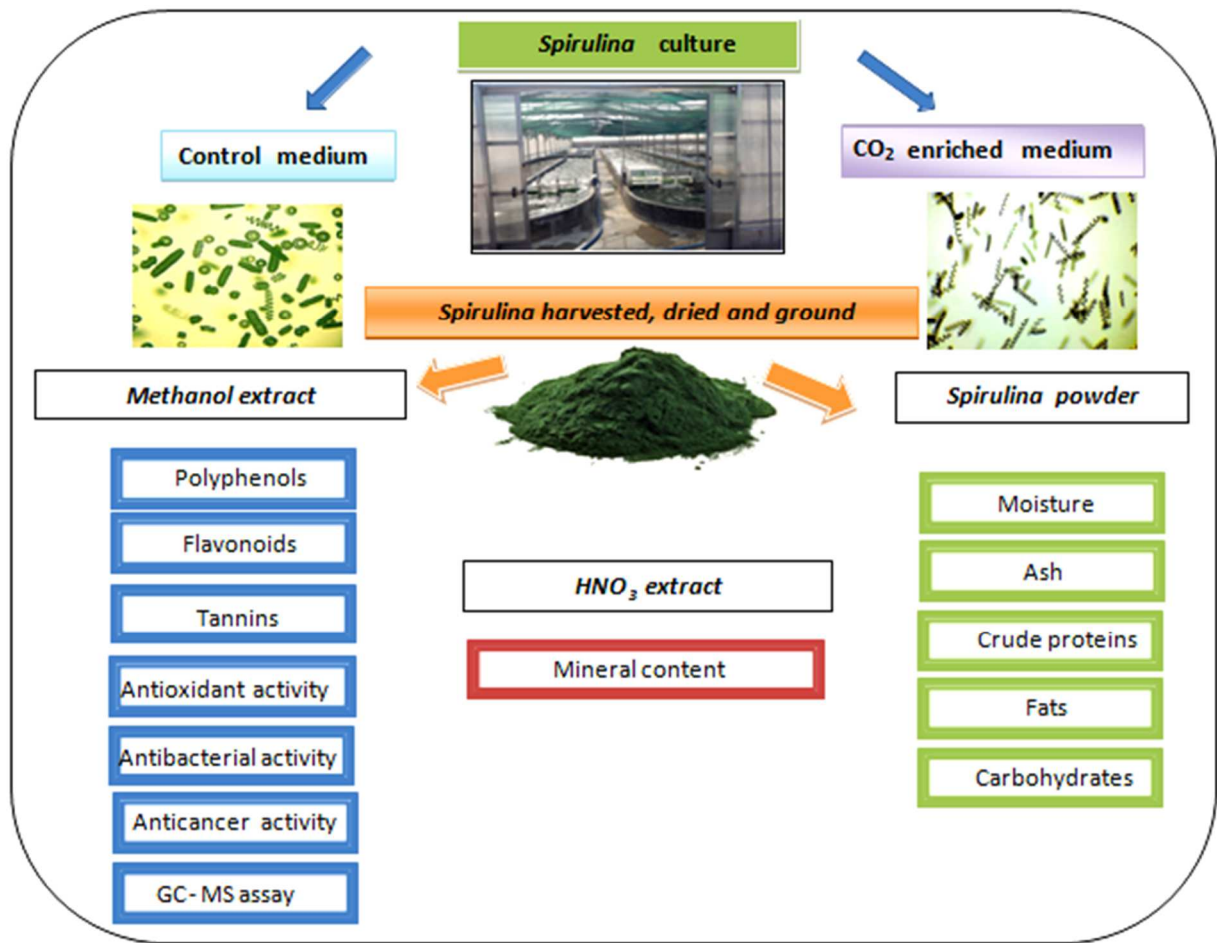
3-piperidyl-2,2'-

binaphthalene-

1,1',4,4'-tetrone



11



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