Antitumor activity of *Chlorella sorokiniana* and *Scenedesmus* sp. microalgae native of Nuevo León State, México

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Cancer cases result in 13% of all deaths worldwide. Unwanted side effects in patients under conventional treatments have led to the search for beneficial alternative therapies. Microalgae synthesize compounds with known *in vitro* and *in vivo* biological activity against different tumor cell lines. Therefore, native microalgae from the State of Nuevo Leon, Mexico may become a potential source of antitumor agents. The aim of the present study was to evaluate the *in vitro* cytotoxic effect of Nuevo Leon regional *Chlorella sorokiniana* (Chlorellales: Chlorellaceae) and *Scenedesmus* sp. (Chlorococcales: Scenedesmaceae). Native microalgae crude organic extracts cytotoxicity against murine L5178Y-R lymphoma cell line and normal lymphocyte proliferation were evaluated using the MTT reduction colorimetric assay. Cell death mechanism was analyzed by acridine orange and ethidium bromide staining, DNA degradation in 2% agarose gel electrophoresis and caspases activity. Results indicated significant ($p<0.05$) 61.89% ± 3.26% and 74.77% ± 1.84% tumor cytotoxicity by *C. sorokiniana* and *Scenedesmus* sp. methanol extracts, respectively, at 500 µg/mL, by the mechanism of apoptosis. This study contributes to Mexican microalgae biodiversity knowledge and their potential as antitumor agent sources.

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2   **Antitumor activity of *Chlorella sorokiniana* and *Scenedesmus* sp. microalgae native of**

3   **Nuevo León State, México**

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19   **Short title**: Antitumor activity of two microalgae

20 **Abstract**.

21 Cancer cases result in 13% of all deaths worldwide. Unwanted side effects in patients under

22 conventional treatments have led to the search for beneficial alternative therapies. Microalgae

23 synthesize compounds with known *in vitro* and *in vivo* biological activity against different tumor

24 cell lines. Therefore, native microalgae from the State of Nuevo Leon, Mexico may become a

25 potential source of antitumor agents. The aim of the present study was to evaluate the *in vitro*

26 cytotoxic effect of Nuevo Leon regional *Chlorella sorokiniana* (Chlorellales: Chlorellaceae) and

27 *Scenedesmus* sp. (Chlorococcales: Scenedesmaceae). Native microalgae crude organic extracts

28 cytotoxicity against murine L5178Y-R lymphoma cell line and normal lymphocyte proliferation

29 were evaluated using the MTT reduction colorimetric assay. Cell death mechanism was analyzed

30 by acridine orange and ethidium bromide staining DNA, degradation in 2% agarose gel

31 electrophoresis and caspases activity.

32 Results indicated significant ($p<0.05$) 61.89% ± 3.26% and 74.77% ± 1.84% tumor cytotoxicity

33 by *C. sorokiniana* and *Scenedesmus* sp. methanol extracts, respectively, at 500 μg/mL, by the

34 mechanism of apoptosis. This study contributes to Mexican microalgae biodiversity knowledge

35 and their potential as antitumor agent sources.

36 **INTRODUCTION**

37   Most commercialized drugs are synthetic derivatives from natural products, or are the

38 result of the systematic screening of terrestrial organisms, such as plants or microorganisms.

39   Analysis of molecules produced by aquatic organisms has shown that microalgae

40 synthesize a large number of bioactive compounds, including pigments, sterols, polyphenols,

41 fatty acids, proteins, vitamins, alkaloids, and sulfated polysaccharides. This group of

42 microorganisms is extremely diverse and represents a number of unexploited natural sources for

43 bioactive agents. Furthermore, the use of polluting elements such as nitrogen, phosphorus, and

44 sulphur for microalgae growth can be considered an advantage, since such elements can be also

45 metabolized by harmful aquatic weeds to proliferate.

46   Microalgae are unicellular, simple, primitive, and photosynthetic organisms, producing

47 bioactive compounds for pharmaceutical and biotechnological applications (Shanab et al. 2012,

48 El Baky et al. 2014, Shalaby 2011**)**, which have shown antiviral, antimicrobial,

49 immunomodulatory, and antitumor properties (Lordan et al. 2011; Teas and Irhimeh 2012).

50   Common failure of conventional therapy against cancer indicates a critical need for

51 beneficial alternative therapeutic agents (Rengarajan et al. 2013). Antitumor activity of

52 microalgal compounds can be explained by their ability to cross the lipophilic membranes and

53 interact with proteins involved in apoptosis. In addition, several microalgal compounds induce

54 DNA-dependent DNA polymerases inhibition, cyclins expression alteration, or major

55 transduction pathways interference. Microalgal compounds have been related to immune

56 response stimulation (Baudelet et al. 2013), as well as cytotoxic against several cancer cell lines

57 (Shanab et al. 2012, Lin et al. 2017). If any compound shows cytotoxic activity against cancer

58 cells, it is important to discriminate if this compound does not represent a threat to normal cells

59 and if its cellular toxicity mechanism is via necrosis or apoptosis. Both apoptosis and necrosis

60   can occur independently, sequentially and/or simultaneously, where the stimuli degree and/or

61   type determines either apoptotic or necrotic death cell (Elmore, 2007). In some cases, cancer

62   chemotherapy treatments result in DNA damage, leading to apoptotic cell death (Elmore, 2007).

63   Apoptosis involves DNA damage and caspases activation. *Chlorella* spp. extracts have resulted

64   in cell death via DNA damage (Yusof et al. 2010) and caspases activation, demonstrating the

65   apoptosis pathway (Lin et al. 2017).

66   The aim of the present study was to evaluate the potential of Nuevo Leon, Mexico native

67   microalgae, *C. sorokiniana* and *Scenedesmus* sp. extracts, isolated from Nuevo Leon, Mexico,

68   against murine L5178Y-R lymphoma cells. To our knowledge, this is the first report of antitumor

69   activity of microalgae isolated from this geographical area.

70   **MATERIALS AND METHODS**

71   **Reagents, culture media, and tumor cells.**

72   L-glutamine and penicillin-streptomycin solutions were purchased from Life Technologies

73   (Grand Island, NY). Concanavalin A (Con A), RPMI 1640 medium, fetal bovine serum (FBS),

74   sodium dodecyl sulfate (SDS), N, N-dimethylformamide (DMF), phosphate buffered saline

75   (PBS), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained

76   from Sigma-Aldrich (St. Louis, MO). Vincristine was obtained from Vintec (Columbia, S.A. de

77   C.V., Ciudad de México). Extraction buffer was prepared by dissolving 20% (wt/vol) SDS at

78   37°C in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7.

79   The tumor cell line L5178Y-R (mouse DBA/2 lymphoma) was purchased from the American

80   Type Culture Collection (LY-R, ATCC® CRL-1722™, Rockville, MD), maintained in culture

81   flasks with RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5%

82   penicillin-streptomycin solution (referred as complete RPMI medium) at 37°C, in a humidified

83   atmosphere of 5% CO_2 in air. Cellular density was kept between 10^5 and 10^6 cells/mL.

84   **Microalgae strains and culture.**

85   *C. sorokiniana* was isolated from San Juan River in the municipality of Cadereyta

86   (26°21′55″N 98°51′15″O), whereas *Scenedesmus* was obtained from Pesquería River in the

87   municipality of Apodaca (25°47′06″N 100°03′04″O) Nuevo Leon, Mexico. *C. sorokiniana*

88   molecular identification using the P2F (5´-GGC TCA TTA AAT CAG TTA TAG-3´) and P2R (5´-

89   CCT TGT TAC GA(C/T) TTC TCC TTC-3´) primers (Lee and Hur 2009), which amplifies for a

90   1700 bp fragment of the 18S gene, as previously reported by Cantú-Bernal (2017). Amplification

91   conditions were an initial denaturation cycle at 95°C for 5 min, 30-35 denaturation cycles at 95°C

92   for 30 sec, alignment at 50-55°C for 30 sec, and an extension process at 72°C for 105 sec,

93   followed by a final extension at 72°C for 7 min. The PCR product was confirmed by

94   electrophoresis on 1.5% agarose gel at 100 Volts for 35 min, were the expected 1700 bp band was

95   observed. Once the PCR product was confirmed, the band was purified, for which the Wizard SV

96   Gel and PCR clean-up system kit (Promega, Invitrogen) was used. For the band sequencing, the

97   product was sent to the synthesis and sequencing unit of the Institute of Biotechnology,

98   Universidad Nacional Autónoma de México. The edition and analysis of the *Chlorella* sp.

99   sequence similarity percentage was carried out using the program Bioedit Sequence Alignment

100   Editor v. 7.1.9 by sequence identity matrix means, after being compared with sequences reported

101   in the GenBank.

102   For microalgae culture, water samples were taken on 50 mL sterile Falcon tubes and kept

103   at 5°C ± 2°C on ice. Then, 5 mL were transferred to 250 mL Erlenmeyer flasks, containing 100

104   mL of LC culture medium, as developed and reported by López-Chuken et al. (2010). Flasks

105   were then incubated at room temperature (25°C± 3°C) in a continuous shaker at 120 x g and

106 under light radiation using 100 Watt white fluorescent light bulb as a continuous artificial light

107 source (1000 lux approximately). Flasks were incubated for 14 d until green growth was

108 observed, after which, 100 µL were transferred to Petri dishes containing the same culture

109 medium, but solidified with 1.5% of bacteriological agar. Inoculated dishes were incubated at

110 30°C ± 2°C by using a 100 watt white fluorescent light bulb as a continuous artificial light until

111 isolated green colonies were observed. Single colonies were collected using a bacteriological

112 loop and placed in Erlenmeyer flasks containing 100 mL of algal LC liquid culture medium.

113 Next, flasks were incubated under the same conditions described above. This process allowed us

114 selecting a single microalgae genus by picking up a single colony; however, given that

115 microalgae tend to grow in consortia with bacteria and yeasts, microalgal cultures were treated

116 with an antibiotic and antimycotic solution containing 500 UI/mL penicillin, 500 µg/mL

117 streptomycin, 50 µg/mL gentamicin, and 1.25 µg/mL fungizone. For this, 5 ml of LC liquid

118 culture medium with antibiotics were placed in 15 mL conical tubes, after which 0.25 mL of the

119 algal culture were added and tubes were incubated for 48 h, under same shaking and lighting

120 conditions described above. After the incubation period, 500 µL of the cultures were transferred

121 into 50 mL of sterilized LC liquid culture medium without antibiotics producing axenic cultures

122 of *C. sorokiniana* and *Scenedesmus* sp. isolates. Each axenic culture was grown for 14 d in 1-L

123 Erlenmeyer flasks containing 500 mL of LC liquid culture medium, until exponential growth

124 phase was reached (based on growth curve, supplemental Fig. S1). Next, each complete culture

125 was transferred to individual bioreactor tanks containing 14.5 L of LC culture medium.

126 Photobioreactor tanks were designed by López-Chuken work team, and consisted of circular

127 acrylic tanks of 30 cm of diameter and height; aeration was supplemented by air pumps with an

128 adapted 0.2 µm filter at 1-L/min flow rate, radiated by continuous artificial LED white lights at

129 1500 lux of intensity, and agitation by rotary plastic pallets at 50 rpm (supplementary material

130 Fig. S2). Biomass production in bioreactors was monitored every 2 d (Tuesdays, Thursdays, and

131   Saturdays) by taking a 10 mL sample with a sterile pipette and filtering through a previously

132   weighed 0.7 μm-pore size microfiber paper. Then the paper was dried at 70°C inside an oven and

133   weighed again; this monitoring process was repeated until the biomass production showed no

134   increase. Once the maximum biomass production was reached, bioreactor tanks were stored at

135   4°C ± 2°C, until most microalgae biomass precipitated, then, the supernatant was decanted

136   (supplementary material Fig. S3). The collected wet biomass was the centrifuged at 9000 rpm for

137   10 min (ST16R model, Thermo Fisher Scientific, Waltham MA) and frozen dried (Labconco,

138   Kansas City, MO).

139   Biomass dried samples of *C. sorokiniana* and *Scenedesmus* sp. were placed in separate Whatman

140   cellulose extraction thimbles (33×80 mm, thickness 1.5 mm) (Sigma-Aldrich) and placed in a

141   Soxhlet extraction apparatus (Reyna-Martinez et al. 2014), which is a continuous system

142   consisting of a flat bottomed round flask, an extraction chamber with a siphon, and a condenser.

143   This method was selected since this extraction is very practical and recommended by most of the

144   methanol-soluble compounds for biological material recovering. A round flask filled with 600

145   mL of methanol was used and the extraction lasted 48 h for each microalgae. Methanol was

146   selected based on previous results where methanol extracts showed the highest cytotoxic activity

147   against L5178Y-R cell line; whereas chloroformic extracts did not show cytotoxic effects and

148   hexane itself showed cytotoxicity against the tumor cell line tested. After the biological material

149   compounds were extracted with methanol, the solutions were filtered using Whatman filter paper,

150   and solvent was evaporated using a rotary evaporator, leaving approximately 10 to 15 mL of

151   liquid material. Remaining solvent was further removed by a vacuum desiccator. Extracts were

152   dissolved in RPMI medium at a concentration of 1 mg/mL and kept frozen until use. From this

153   stock, serial 1:1 dilutions from 500 to 7.8 μg/mL were prepared.

154   The tumor cell line L5178Y-R (mouse DBA/2 lymphoma) was purchased from the American

155   Type Culture Collection (LY-R, ATCC® CRL-1722™, Rockville, MD), maintained in culture

156   flasks with RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5%

157   penicillin-streptomycin solution (referred as complete RPMI medium) at 37°C, in a humidified

158   atmosphere of 5% CO_2 in air. Cellular density was kept between 10^5 and 10^6cells/mL.

159   **Tumor cytotoxicity and apoptosis assays.**

160   To determine the cytotoxic effect of *C. sorokiniana* and *Scenedesmus* sp. methanol extracts

161   against L5178Y-R tumor cells, cell cultures were collected and washed three times in RPMI

162   medium, then suspended and adjusted to 5×10^4 cells/mL with complete RPMI medium. One

163   hundred microliters of the cell suspensions were then added to flat-bottomed 96-well plates

164   (Becton Dickinson, Cockeysville, MD), containing 100 μL of complete RPMI, methanol

165   microalgae extracts at various concentrations, vincristine (250 μg/mL) as positive control, and

166   RPMI medium as negative control; all treatments were tested in triplicate. Microplates were

167   incubated for 48 h at 37°C with 5% CO_2, then 15 μL of MTT were added (0.5 μg/mL, final

168   concentration), and cultures were incubated for 3 additional hours. After this, supernatant was

169   removed and 80 μL of DMSO were added to all wells. Optical densities, resulting from dissolved

170   formazan crystals, were then read in a microplate reader (DTX 880 Multimode detector, Becton

171   Dickinson, Austria) at 570 nm (Gomez-Flores et al. 2009). The percentage of cytotoxicity was

172   calculated as follows:

173   % Cytotoxicity = 100-[(A_{570} in extract-treated cells/ A_{570} in untreated cells) X 100].

174   Apoptosis induction by *C. sorokiniana* and *Scenedesmus* sp. methanol extracts against L5178Y-R

175   cell line was evaluated *in vitro* by acridine orange and ethidium bromide staining. For this, 1×10^6

176   L5178Y-R tumor cells were placed in 24-well plates in the presence of 500 µg/mL methanol

177   extracts, and incubated for 24 h. Then, 500 µL of RPMI, plus 1-µL of acridine orange and 100

178   µg/mL ethidium bromide (1:1 ratio) were added to the wells. Next, cultured cells were incubated

179   for 5 min, washed with 1-mL PBS, and suspended in 100 µL of RPMI medium; after incubation

180   period, 10 µL of cell suspension were placed between a slide and a coverslip for fluorescence

181   microscope visualization (Inverted Tissue Culture Fluorescence Microscope Olympus IX-70,

182   **Representaciones y Distribuciones FAL, S.A. de C.V., Naucalpan, Estado de México)**.

183   Acridine orange stains viable cells and dead cells (green cells), whereas ethidium bromide only

184   stains those cells that have lost the integrity of their membrane (orange cells). Therefore, viable

185   cells appear in a uniform green tone, cells found in apoptosis appear in a spotty green or granular

186   in the center due to the condensation of chromatin and fragmentation of the nucleus, whereas

187   cells in necrosis appear in a uniform orange hue (Coligan et al. 1995).

188   In addition, apoptosis induction was evaluated by DNA degradation (Orozco-Flores et al. 2017).

189   For this, cells were incubated for 48 h with *C. sorokiniana* and *Scenedesmus* sp. methanol

190   extracts at 500 µg/mL, testing their respective negative (culture medium) and positive (20 µg/mL

191   Actinomycin D) controls. After the incubation period, cells were collected and centrifuged at

192   2000 rpm for 10 min, then washed with PBS and extracted using the AxyPrep Multisource

193   Genomic DNA Miniprep Kit (Axygen, MA). In order to visualize the extracted DNA, the sample

194   was separated by 2% agarose gel electrophoresis, using SB buffer for the electrophoretic shift at

195   70V for 20 min and 110V for 1 h. After this, gel was stained with 5 ng/mL ethidium bromide and

196   photographs were documented under High Performance Ultraviolet Transilluminator (UVP, LLC,

197   Upland, CA) light. DNA like-ladder fragmentation indicates apoptotic activity, whereas DNA

198   smear represents cell death by necrosis.

199   In early apoptosis stages caspase enzymes are activated. Caspase participate in the cleavage of

200   protein substrates leading to cell disassembly. Cleavage of protein substrates leads to a

201   fluorescent monoamide formation and finally to a rhodamine 110 conversion. For apoptotic

202   pathway involving caspases, caspase can be monitored by measuring fluorescence intensity using

203   microplate wells (Towhid et al. 2013). For this, L5178Y-R cells ($5X10^5$ cells/well) were seeded

204   in a 48 wells plate, and treated with Actinomycin D (800 ng/mL) as positive control, or *Chlorella*

205   and *Scenedesmus* methanolic extracts at 500 µg/mL. Cultures were then incubated for 24 h at 37º

206   C, after which, activated caspases were detected with the CaspGLOW™ red active caspase

207   staining kit following manufacturer´s instructions. Fluorescence intensity was measured at

208   Ex/EM= 540/570 nm in a Varioskan Lux Multimode Reader (Thermo Fisher Scientific).

209   **Animals.**

210   Six- to eight-week old Balb/c female mice were purchased from Harlan Mexico S.A. de

211   C.V. (Mexico, D.F.). They were kept in a pathogen- and stress-free environment at 24ºC, under a

212   light-dark cycle (light phase, 06:00-18:00 h) in a One Cage 2100™ System (Lab Products, Inc.,

213   Seaford, DE), and given water and food *ad libitum*. Animals were euthanized by asphyxiation in

214   a 100% CO_2 chamber. Experiments involving the use of animals were reviewed and approved by

215   our institutional animal care and use committee before being initiated, and were performed in

216   accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the

217   Society of Toxicology in March 1999.

218   **Murine thymus lymphocyte viability assay.**

219   Thymus was immediately removed after mouse death. Single-cell suspensions were

220   prepared by disrupting the organ in RPMI 1640 medium. Cell suspensions were washed three

221   times in this medium, and suspended and adjusted at $1x10^7$ cells/mL in complete RPMI medium.

222   Thymus lymphocyte viability was determined by a colorimetric technique using MTT (Gomez-

223   Flores et al. 2009). Thymus suspensions (100 µg of 1 X 10^7 cells/mL) were added to flat-

224   bottomed 96-well plates (Becton Dickinson) containing triplicate cultures (100 µg/well) of

225   complete RPMI medium (unstimulated control), or 100 µL of *C. sorokiniana* and *Scenedesmus*

226   sp. methanol extracts at various concentrations, for 48 h at 37°C in 95% air-5% CO_2 atmosphere.

227   After incubation for 44 h, MTT (0.5 mg/mL final concentration) was added, and cultures were

228   additionally incubated for 4 h. Cell cultures were then incubated for 16 h with extraction buffer

229   (100 µL/well), and optical densities, resulting from dissolved formazan crystals, were then read

230   in a microplate reader (Becton Dickinson) at 570 nm (Gomez-Flores et al. 2009).

231   All experiments were repeated at least three times with similar results. The results were

232   expressed as means ± SEM of triplicate determinations from a representative experiment.

233   Statistical significance was assessed by one-way analysis of variance and by the Student's *t* test.

234   **RESULTS**

235   Microscopic evaluation revealed the presence of *C. sorokiniana* and *Scenedesmus* sp.

236   (Table 1), whose isolated colony cultures were then produced under axenic conditions. Culture of

237   both microalgae in photobioreactor tanks showed that the exponential growth phase started after

238   12 d by *C. sorokiniana*, whereas for *Scenedesmus* sp., that started after 19 d (Fig. 1). Once the

239   biomass was dried, collected, and weighed separately for each bioreactor, the yield by *C.*

240   *sorokiniana* was of 0.24 g/L (± 0.01), whereas for *Scenedesmus* sp. was of 0.30 (± 0.01) g/L.

241   Microalgae methanol extracts tested *in vitro* against tumor cell resulted in concentration-

242   dependent activity against the murine tumor cell line L5178Y-R. *C. sorokiniana* extract caused

243   significant ($p < 0.05$) 17% and 61% tumor cell toxicity at concentrations of 250 and 500 µg/mL,

244   respectively, whereas that of *Scenedesmus* sp. induced 15%, 25%, and 75% cytotoxicity at

245   concentrations of 125, 250, and 500 µg/mL, respectively (Fig. 2). Collected data were used to

246   determine the inhibitory concentration mean (IC_{50}) of *C. sorokiniana* and *Scenedesmus* sp.,

247   methanolic extracts. The observed IC_{50} for *C. sorokiniana, Scenedesmus* sp., and vincristine were

248   460.0 ± 21.5, 362.9 ± 13.5, and 76.83 ± 2.55 µg/mL, respectively.

249   *C. sorokiniana* and *Scenedesmus* sp. methanol extracts were shown to cause DNA

250   fragmentation in L5178Y-R cells, with the typical latter pattern, after 24 h of treatment, which

251   was comparable with the results obtained with actinomycin D (Fig. 3A). Caspase activity assay

252   showed that *Scenedesmus* sp. resulted in significantly higher ($P < 0.05$) apoptosis compared with

253   the control (Fig. 3B). The AOPI staining analysis revealed that *C. sorokiniana* extract resulted in

254   74.4% tumor cell toxicity, 66% apoptosis, and 9% necrosis, whereas *Scenedesmus* sp. extract

255   caused 54% tumor cell toxicity, 51% apoptosis, and 3% necrosis (Fig. 3C and 3D).

256   Cytotoxicity of *C. sorokiniana* and *Scenedesmus* sp. methanol extracts did not

257   significantly alter normal murine thymus lymphocyte viability, resulting in up to 26% and 19%

258   not significant lymphocyte toxicity (Fig. 4).

259   **DISCUSSION**

260   To our knowledge, this is the first report of Nuevo Leon, Mexico native microalgae,

261   identified as *C. sorokiniana* and *Scenedesmus* sp., showing cytotoxicity against a murine

262   lymphoma tumor cell line. Previous reports have shown microalgae potential for wastewater

263   treatment and biodiesel production (Reyna-Martínez et al. 2014, Beltrán-Rocha et al. 2017). In

264   the present study, microalga isolates were grown under artificial light, closed photobioreactors,

265   and previously established physicochemical conditions. Biomass production was monitored

266   every 2 d by taking a 10 mL sample, filtered, dried, and weighed during the fermentation time-

267   course. Biomass calculated from time-course data indicated that *Scenedesmus* sp. resulted in

268   higher (twice as much) biomass production compared with that produced by *C. sorokiniana*,

269   since values were higher than 0.8 and up to 0.4, respectively (Fig. 1). Nonetheless, after

270   collecting the final biomass produced by each microalgae, *Scenedesmus* sp. resulted in only 18%

271   more biomass, compared with *C. sorokiniana*, for a total of 13.74 g and 11.21 g dried biomass,

272   respectively. *C. sorokiniana* and *Scenedesmus* sp. production in the photobioreactors was

273   stopped after 29 d, because no additional biomass production was observed. Biomass production

274   was lower compared with other reports using batch culture and phototrophic conditions (Brennan

275   and Owende 2010, Chen et al. 2011). However, sufficient biomass was produced to obtain an

276   adequate amount methanol extract to perform biological assays.

277   *In vitro* tumor cell toxicity assays resulted in concentration-dependent activity against the

278   murine tumor cell line L5178Y-R (up to 61.9% and 74.8% cytotoxicity at 500 µg/mL *C.*

279   *sorokiniana* and *Scenedesmus* sp. extracts, respectively). These results are comparable with other

280   reports showing about 50% *in vitro* cytotoxicity by microalga extracts against cervical cancer

281   (Yusof et al. 2010, Kyadari et al. 2013).

282   Apoptosis is the best known pathway for programmed cell death. Apoptosis and necrosis

283   can occur independently, sequentially or simultaneously. The type and/or the stimuli degree may

284   determine if cells die by apoptosis or necrosis. At low doses, a variety of injurious stimuli such as

285   heat, radiation, hypoxia, and cytotoxic anticancer drugs can induce apoptosis, or lead to necrosis

286   at higher doses (Elmore, 2006). After cells enter the apoptotic process, their DNA degrades,

287   showing a ladder pattern of multiples of approximately 200 base pairs, which can be observed

288   when extracting the DNA and making an agarose gel electrophoresis. Apoptosis involves the

289   activation of caspases enzymes linked to the initiating stimuli. Caspase-3 is required for

290   apoptosis-associated chromatin margination, DNA fragmentation, and nuclear collapse of the cell

291   (Mantena et al. 2006). After testing *C. sorokiniana* and *Scenedesmus* sp. methanolic extracts,

292   using the caspase-3/7 microplate assay, results demonstrated that only the *Scenedesmus* sp.

293   methanolic extract was significantly different compared with the untreated cells (negative

294   control), whereas no differences were observed with either actinomycin D or *C. sorokiniana*

295   methanolic extract. Microalgae-induced tumor cytotoxicity was observed to be mediated by

296   apoptosis, as determined by the acridine orange and ethidium bromide staining, as well as DNA

297   fragmentation (ladder pattern) (Nagata 2000). In fact, microalga isolates methanol extracts

298   resulted in similar effects against the cell line compared with actinomycin D, compound that

299   resulted in cellular apoptosis (Quintanilla-Licea et al. 2012). After testing crude extracts of the

300   cyanobacteria *Nostoc* sp., against human pancreatic tumor cells PaTu 8902, Voráčová et al.

301   (2017) found that apoptosis was mostly mediated by caspases 3 and 7. In summary, DNA

302   fragmentation, acridine orange/ ethidium bromide staining, and caspases results support apoptosis

303   as the cell-death mechanism by the tested microalgae methanolic extracts (Towhid et al. 2013).

304   Tumor cancer cells may develop as a result of *in situ* formation of nitrosamines from

305   secondary amines and nitrite in an acidic environment of the stomach. There are chemical agents

306   known as chemopreventers, which help to reverse, suppress or prevent these nitrosamines

307   formation. In fact, ascorbic acid or phenolic compounds are chemopreventers, since they prevent

308   or reduced nitrosamines formation (Jahan *et al.* 2017). It has been shown that microalgae

309   synthesize a number of bioactive compounds, including bioactive peptides, fucans, galactans,

310   alginates, phenolic compounds, phycocyanins, phycobiliproteins, eicosapentanoic and

311   arachidonic acids, carotenoids, tocopherols, sterols, and terpenoids (Lordan et al. 2011). Some of

312   these compounds may be responsible for the cytotoxicity induced by *C. sorokiniana* and

313   *Scenedesmus* sp. methanol extracts, against the murine lymphoma cell line L5178Y-R.

314   In a recent report, *C. sorokiniana* water extracts were evaluated against two human non-

315   small cell lung cancer (A549 and CL1-5 human lung adenocarcinoma cells) cell lines using a

316   subcutaneous xenograft tumor model. Results demonstrated the tumors growth inhibition after

317   extract oral intake *in vivo*, being mitochondrial-mediated apoptosis (Lin et al. 2017).

318   In the present study, not significant lymphocyte cytotoxicity was observed by *C.*

319   *sorokiniana* and *Scenedesmus* sp. methanol extracts. Nevertheless, results were comparable with

320 other reports, showing lower than 20% lymphocyte cytotoxicity but around 50% cytotoxicity

321 against cervical cancer cells by microalga extracts *in vitro* (Yusof et al. 2010, Kyadari et al.

322 2013).

323   The bio-guided fractionation of these extracts is ongoing, and further studies of the

324 isolated pure compounds will be performed.

325 **CONCLUSION**

326   The native microalgae *C. sorokiniana* and *Scenedesmus* sp. isolates from Nuevo Leon,

327 Mexico water bodies were produced under a semi-pilot level using closed photobioreactors, with

328 artificial illumination and aeration. The produced microalgae methanol extracts were cytotoxic

329 against the murine tumor cell line L5178Y-R *in vitro,* by the mechanism of apoptosis, without

330 affecting normal murine lymphocytes.

331 **REFERENCES**

332 **Baudelet PH, Gagez AL, Bérard JB, Juin C, Bridiau N, Kaas R, Thiéry V, Cadoret JP, Picot**

333   **L, 2013.** Antiproliferative activity of *Cyanophora paradoxa* pigments in melanoma, breast

334   and lung cancer cells. *Marine Drugs* **11**: 4390-4406, DOI 10.3390/md11114390.

335 **Beltrán-Rocha JC, Barceló-Quintal ID, García-Martínez M, Osornio-Berthet L, Saavedra-**

336   **Villarreal N, Villarreal-Chiu J, López-Chuken UJ, 2017**. Polishing of municipal

337   secondary effluent using native microalgae consortia. *Water Science and Technology* **75**:

338   1693-1701 DOI 10.2166/wst.2017.046.

339   **Brennan L, Owende P, 2010.** Biofuels from microalgae-A review of technologies for production

340   processing, and extractions of biofuels and co-products. *Renewable & Sustainable Energy*

341   *Reviews Journal* **14**: 557–577 DOI 10.1016/j.rser.2009.10.009.

342   **Cantú-Bernal SH, 2017.** Estudio de la vida de anaquel de probióticos en presencia de la

343   microalga *Chlorella* sp. en flan. MSci. Thesis dissertation. Universidad Autónoma de

344   Nuevo León, Facultad de Ciencias Biológicas. México,

345   **Chen CY, Yeh KL, Aisyah R, Lee DJ, Chang JS, 2011.** Cultivation, photobioreactor design and

346   harvesting of microalgae for biodiesel production: A critical review. *Bioresource*

347   *Technology* **102**: 71-81 DOI 10.1016/j.biortech.2010.06.159.

348   **Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, 1995.** Related isolation

349   procedures and functional assays. *In: Current Protocols in Immunology.* Ed: Coico R. John

350   Wiley & Sons, Inc., 1: 3.17.1 ISBN: 978-0-471-52276-8.

351   **El Baky HHA, El-Baroty GS, Ibrahim EA, 2014.** Antiproliferation and antioxidant properties

352   of lipid extracts of the microalgae *Scenedesmus obliquus* grown under stress conditions.

353   *Der PharmaChemica* **6**: 24-34.

354   **Elmore S, 2007.** Apoptosis: a review of programmed cell death. *Toxicologic Pathology* **35**: 495-

355   516 DOI 10.1080/01926230701320337

356   **Gomez-Flores R, Verastegui-Rodriguez L, Quintanilla-Licea R, Tamez-Guerra P, Monreal-**

357   **Cuevas E, Tamez-Guerra R, Rodriguez-Padilla C, 2009.** Antitumor properties of

358   *Gymnosperma glutinosum* leaf extracts. *Cancer Investigation* **27**: 149-155 DOI

359   10.1080/07357900802192190.

360   **Jahan A, Ahmad IZ, Fatima N, Ansari VA, Akhtar J, 2017.** Algal bioactive compounds in the

361   cosmeceutical industry: a review. *Phycologia* **56**: 410-422,

362   **Kyadari M, Fatma T, Azad R, Velpandian T, 2013.** Evaluation of antiangiogenic and

363   antiproliferative potential of the organic extract of green algae *Chlorella pyrenoidosa*.

364   *Indian Journal of Pharmacology* **45**: 569-574 DOI 10.4103/0253-7613.121366.

365   **Lee HJ, Hur SB, 2009.** Genetic relationships among multiple strains of the genus *Tetraselmis*

366   based on partial 18S rDNA sequences. *Algae* **24**: 205-212 DOI

367   10.4490/ALGAE.2009.24.4.205.

368   **Lin PY, Tsai CT, Chuang WL, Chao YH, Pan IH, Chen YK, Lin CC,Wang BY, 2017.**

369   *Chlorella sorokiniana* induces mitochondrial-mediated apoptosis in human non-small cell

370   lung cancer cells and inhibits xenograft tumor growth *in vivo*. *BMC Complementary and*

371   *Alternative Medicine* **17**: 88 DOI 10.1186/s12906-017-1611-9

372   **Lordan S, Ross P, Stanton C, 2011.** Marine bioactives as functional food ingredients: potential

373   to reduce the incidence of chronic diseases. *Marine Drugs* **9**: 1056-1100 DOI

374   10.3390/md9061056.

375   **López-Chuken U, Young S, Guzman-Mar L, 2010.** Evaluating a 'biotic ligand model' applied

376   to chloride-enhanced Cd uptake by *Brassica juncea* from nutrient solution at constant Cd^{2+}

377   activity. *Environmental Technology* **31**: 307-318 DOI 10.1080/09593330903470685

378   **Mantena SK, Sharma SD, Katiyar, S.K, 2006.** Berberine, a natural product, induces G1-phase

379   cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma

380   cells. *Molecular Cancer Therapeutics* **5**: 296-308 DOI 10.1158/1535-7163.MCT-05-0448

381   **Nagata S, 2000.** Apoptotic DNA fragmentation. *Experimental Cell Research* **256**: 12-18 DOI

382   10.1006/excr.2000.483

383   **Orozco-Flores AA, Valadez-Lira JA, Oppert B., Gomez-Flores R, Tamez-Guerra R,**

384   **Rodriguez-Padilla C, Tamez-Guerra P, 2017.** Regulation by gut bacteria of immune

385   response, *Bacillus thuringiensis* susceptibility and hemolin expression in *Plodia*

386 *interpunctella*. *Journal of Insect Physiology* **98**: 275-283 DOI

387 10.1016/j.jinsphys.2017.01.020

388 **Quintanilla-Licea R, Morado-Castillo R, Gomez-Flores R, Laatsch H, Verde-Star J,**

389 **Hernandez-Martinez H, Tamez-Guerra P, Tamez-Guerra R, Rodriguez-Padilla C,**

390 **2012.** Bioassay-guided isolation and identification of cytotoxic compounds from

391 *Gymnosperma glutinosum* leaves. *Molecules* **17**: 11229-11241 DOI

392 10.3390/molecules170911229.

393 **Rengarajan T, Rajendran P, Nandakumar N, Balasubramanian MP, Nishigaki I, 2013.**

394 Cancer preventive efficacy of marine carotenoid fucoxanthin: cell cycle arrest and

395 apoptosis. *Nutrients* **5**: 4978-4989 DOI 10.3390/nu5124978

396 **Reyna-Martínez R, Gomez-Flores R, López-Chuken U, González-González R, Fernández-**

397 **Delgadillo S, Balderas-Rentería I, 2014.** Lipid production by pure and mixed cultures of

398 *Chlorella pyrenoidosa* and *Rhodotorula mucilaginosa* isolated in Nuevo Leon, Mexico.

399 *Applied Biochemistry and Biotechnology* **175**: 354-359 DOI 10.1007/s12010-014-1275-6.

400 **Shalaby E, 2011.** Algae as promising organisms for environment and health. *Plant Signaling and*

401 *Behavior*. **9**: 1338-1350. DOI 10.4161/psb.6.9.16779.

402 **Shanab SM, Mostafa SS, Shalaby EA, Mahmoud GI, 2012.** Aqueous extracts of microalgae

403 exhibit antioxidant and anticancer activities. *Asian Pacific Journal of Tropical Medicine* **2**:

404 608-615 DOI 10.1016/S2221-1691(12)60106-3.

405 **Teas J, Irhimeh R, 2012.** Dietary algae and HIV/AIDS: proof of concept clinical data. *Journal*

406 *of Applied Phycology* **24**: 575-582 DOI 10.1007/s10811-011-9766-0.

407 **Towhid ST, Liu GL, Ackermann TF, Beier N, Scholz W, Fuchß T, Lang F, 2013.** Inhibition of

408 colonic tumor growth by the selective SGK inhibitor EMD638683. *Cellular Physiology*

409 *and Biochemistry*, **32**: 838-848.

410   **Voráčová K, Paichlová J, Vicková K, Hrouzek P, 2017.** Screening of cyanobacterial extracts

411   for apoptotic inducers: a combined approach of caspase-3/7 homogeneous assay and time-

412   lapse microscopy. *Journal of Applied Phycology* **29**: 1933–1943 DOI 10.1007/s10811-017-

413   1122-6.

414   **Yusof Y, Saad S, Makpol S, Shaaman N, Ngah W, 2010.** Hot water extract of *Chlorella*

415   *vulgaris* induced DNA damage and apoptosis. *Clinics* **65**: 1371-1377 DOI 10.1590/S1807-

416   59322010001200023.

417 **Figures legends**

418   Fig. 1. Biomass production time-course by *Chlorella sorokiniana* and *Scenedesmus* sp. isolates.

419   Data represent means ± SEM.

420   Fig. 2. L5178Y-R tumor cell toxicity of *Chlorella sorokiniana* and *Scenedesmus* sp. methanol

421   extracts. Microalgae methanol extracts were tested against L5178Y-R cells *in vitro* at

422   concentrations ranging from 7.8 to 500 µg/mL, as detailed in the text. Positive control

423   vincristine caused 85% ± 1.22 cytotoxicity at 250 µg/mL. Data represent means ± SEM.

424   *P<0.05; **P<0.01.

425   Fig. 3. (A) Agarose gel showing the cellular DNA fragmentation by line L5178Y-R after

426   treatment with microalgae methanolic extracts. Lane 1, 100 bp molecular weight marker;

427   lanes 2 and 3, cellular DNA after treatment with *Chlorella sorokiniana* methanolic

428   extracts at 500 and 250 µg/mL, respectively; 4 and 5 cellular DNA after treatment with

429   *Scenedesmus* sp. methanolic extracts at 500 and 250 µg/mL, respectively; lane 6, cellular

430   DNA treated with actinomycin D at 20 µg/mL. (B) Detection of caspase 3/7 enzymes

431   activity in L5178Y-R cells (CaspGLOW™), testing 500,000 cells per well on the same

432   day, untreated or treated with Actinomycin D (800 ng/mL) as positive control, and *C.*

433   *sorokiniana* or *Scenedesmus* sp. methanol extracts **at** 500 µg/mL, incubated by 24 h at

434   37°C and reading fluorescence intensity at Ex/EM = 540/570 nm. (C) Effects of *C.*

435   *sorokiniana* and *Scenedesmus* sp. methanol extracts on percent viable, apoptotic, and

436   necrotic cells. Percentage of viable, apoptotic, and necrotic L5178Y-R cells after 24 h

437   treatment with 500 µg/mL *C. sorokiniana* and *Scenedesmus* sp. methanol extracts, and

438   actinomycin D (20 µg/mL). (D) L5178Y-R cells stained with

439   acridine orange/ethidium bromide used to discriminate viable, apoptotic and necrotic cells

440   after *C. sorokiniana* and *Scenedesmus* sp. methanol extracts treatment.

441   Fig. 4. Effects of *Chlorella sorokiniana* and *Scenedesmus* sp. methanol extracts on viability of

442   normal murine thymus lymphocytes. Thymus lymphocyte viability was determined by a

443   colorimetric technique using MTT (7). Thymus suspensions were incubated with culture

444   medium alone or with *C. sorokiniana* and *Scenedesmus* sp. methanol extracts at various

445   concentrations, for 48 h at 37ºC, and cell viability was determined as detailed in the text.

Locations from Nuevo Leon state, Mexico, where microalgae were isolated.

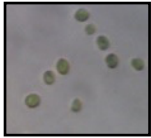
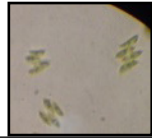
Table 1. Locations from Nuevo Leon state, Mexico, where microalgae were isolated

Location	Microalga	Microscopic shape (100x)
San Juan River, Cadereyta, N.L. 25° 31´17" – 100° 0´34"	*Chlorella sorokiniana*	
Pesquería River, Apodaca, N.L. 25° 46´ 34" – 100°12´35"	*Scenedesmus* sp.	

Figure 1

Biomass production time-course by *Chlorella sorokiniana* and *Scenedesmus* sp. isolates.

Data represent means ± SEM.

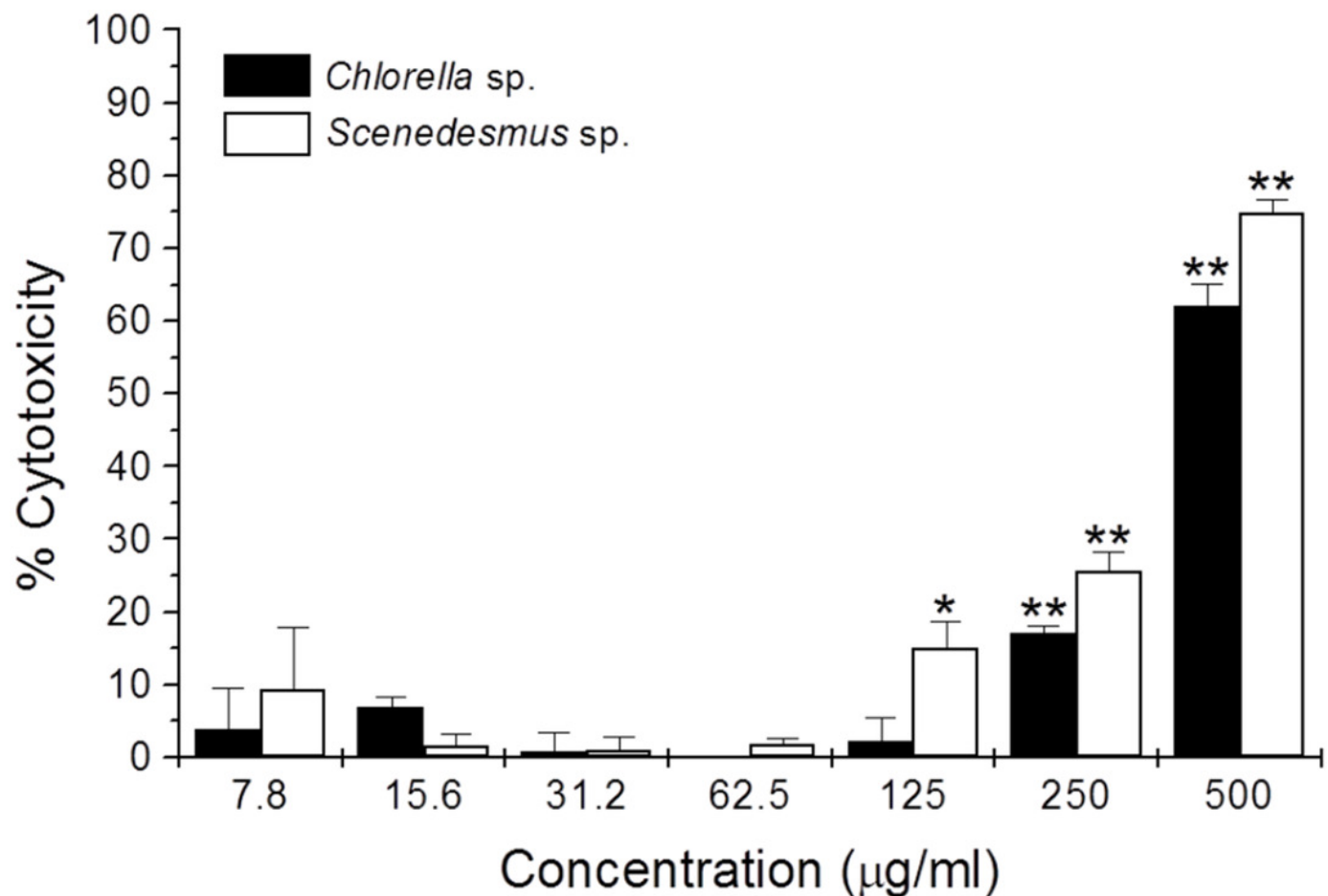



Figure 2

L5178Y-R tumor cell toxicity of *Chlorella sorokiniana* and *Scenedesmus* sp. methanol extracts.

Microalgae methanol extracts at concentrations ranging from 7.8 to 500 µg/ml were tested against L5178Y-R cells *in vitro*, as detailed in the text. Positive control vincristine caused 85% ± 1.22 cytotoxicity at 250 µg/ml. Data represent means ± SEM. *P <0.05; **P <0.01.

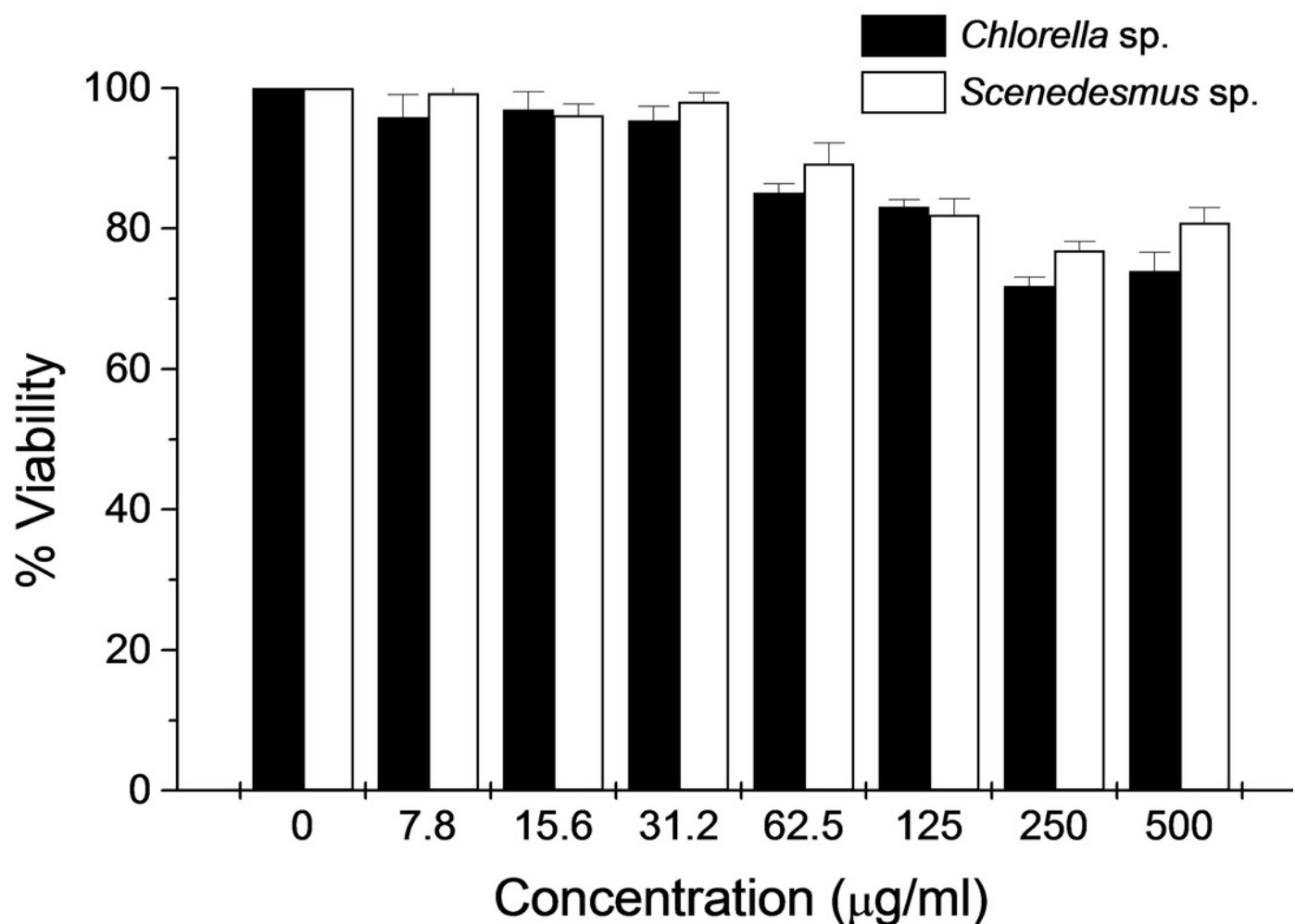



Figure 3

Figure 3

(A) Agarose gel showing the cellular DNA fragmentation by line L5178Y-R after treatment with microalgae methanolic extracts. Lane 1, 100 bp molecular weight marker; lanes 2 and 3, cellular DNA after treatment with *Chlorella sorokiniana* methanolic extracts at 500 and 250 μg/ml, respectively; 4 and 5 cellular DNA after treatment with *Scenedesmus* sp. methanolic extracts at 500 and 250 μg/ml, respectively; lane 6, cellular DNA treated with actinomycin D at 20 μg/ml. (B) Detection of caspase 3/7 enzymes activity in L5178Y-R cells (CaspGLOW™), testing 500,000 cells per well on the same day, untreated or treated with Actinomycin D (800 ng/mL) as positive control, and *C. sorokiniana* or *Scenedesmus* sp. methanol extracts **at** 500 μg/mL, incubated by 24 h at 37°C and reading fluorescence intensity at Ex/EM = 540/570 nm. (C) Effects of *C. sorokiniana* and *Scenedesmus* sp. methanol extracts on percent viable, apoptotic, and necrotic cells. Percentage of viable, apoptotic, and necrotic L5178Y-R cells after 24 h treatment with 500 μg/ml *C. sorokiniana* and *Scenedesmus* sp. methanol extracts, and actinomycin D (20 μg/ml). (D) L5178Y-R cells stained with acridineorange/ethidiumbromide used to discriminate viable, apoptotic and necrotic cells after *C. sorokiniana* and *Scenedesmus* sp. methanol extracts treatment.

A — Lanes 1–6, DNA ladder gel showing 1500bp, 500bp, 200bp markers.

B — Relative fluorescence units (540/570 nm) for Untreated, Actinomycin D, *C. sorokiniana*, *Scenedesmus* sp.

C — % L5178Y-R cells showing Viable cells, Apoptotic cells, Necrotic cells for Untreated, Actinomycin D, *Chlorella* sp., *Scenedesmus* sp.

D — Fluorescence micrographs: Untreated, Actinomycin D, *C. sorokiniana*, *Scenedesmus* sp.

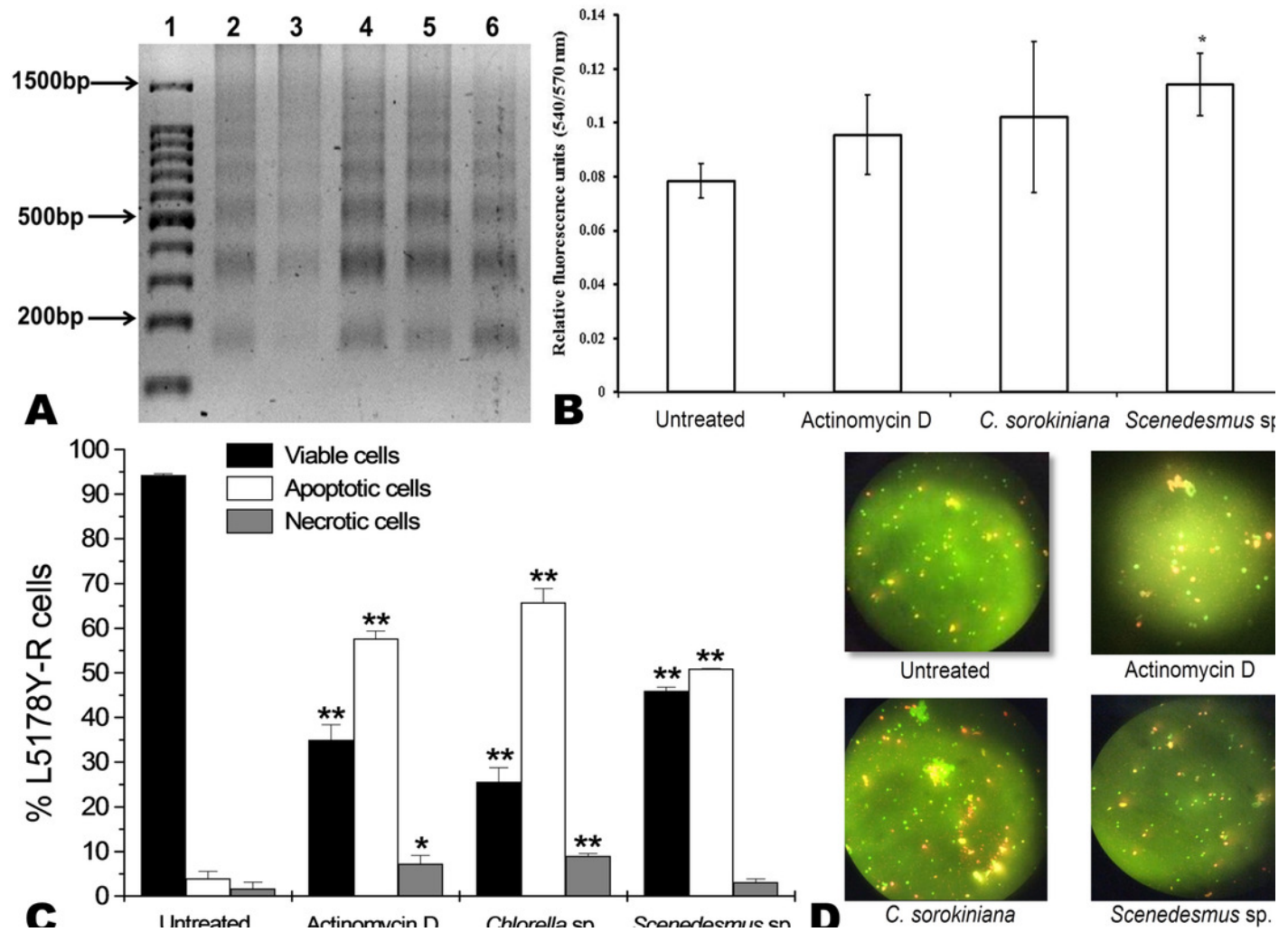



Figure 4

Thymus lymphocyte viability

Effects of *Chlorella sorokiniana* and *Scenedesmus* sp. methanol extracts on viability of normal murine thymus lymphocytes. Thymus lymphocyte viability was determined by a colorimetric technique using MTT (7). Thymus suspensions were incubated with culture medium alone or with *Chlorella sorokiniana* and *Scenedesmus* sp. methanol extracts at various concentrations, for 48 h at 37°C, and cell viability was determined as detailed in the text.