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

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Cancer cases results in 13% of all deaths worldwide. Harmful side effects to patients under to conventional treatments have led to beneficial alternative therapies search. Microalgae synthesize compounds with known *in vitro* and *in vivo* biological activity against different tumor cell lines. The native microalgae from Nuevo Leon State, Mexico, represent an unexploited source with antitumor potential. Our objective was evaluate the *in vitro* cytotoxic effect of Nuevo Leon State native *Chlorella sorokiniana* (Chlorellales: Chlorellaceae) and *Scenedesmus* sp. (Chlorococcales: Scenedesmaceae). Native microalgae crude organic extracts cytotoxicity against murine L5178Y-R lymphoma cells and their effect on cell lymphoproliferation were evaluated using the MTT colorimetric method. The cell death mechanism was analyzed *in vitro* by acridine orange and ethidium bromide staining and by DNA degradation in 2% agarose gel electrophoresis. Results data indicated significant ($p<0.05$) 61.89% ± 3.26% and 74.77% ± 1.84% tumor cell toxicity by *C. sorokiniana* and *Scenedesmus* sp. methanol extracts, respectively. The extracts were shown to induce tumor cell death by the mechanism of apoptosis. This study contributes to Mexican microalgae biodiversity knowledge and provides a potential antitumor agents source.

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Short title: Antitumor activity of two microalgae

Abstract.

Cancer cases results in 13% of all deaths worldwide. Harmful side effects to patients under to conventional treatments have led to beneficial alternative therapies search. Microalgae synthesize compounds with known *in vitro* and *in vivo* biological activity against different tumor cell lines. The native microalgae from Nuevo Leon State, Mexico, represent an unexploited source with antitumor potential. Our objective was evaluate the *in vitro* cytotoxic effect of Nuevo Leon State native *Chlorella sorokiniana* (Chlorellales: Chlorellaceae) and *Scenedesmus* sp. (Chlorococcales: Scenedesmaceae).

Native microalgae crude organic extracts cytotoxicity against murine L5178Y-R lymphoma cells and their effect on cell lymphoproliferation were evaluated using the MTT colorimetric method. The cell death mechanism was analyzed *in vitro* by acridine orange and ethidium bromide staining and by DNA degradation in 2% agarose gel electrophoresis.

Results data indicated significant ($p<0.05$) 61.89% ± 3.26% and 74.77% ± 1.84% tumor cell toxicity by *C. sorokiniana* and *Scenedesmus* sp. methanol extracts, respectively. The extracts were shown to induce tumor cell death by the mechanism of apoptosis. This study contributes to Mexican microalgae biodiversity knowledge and provides a potential antitumor agents source.

Keywords: apoptosis; cytotoxicity; lymphoproliferation; microalgae; mouse lymphoma; tumor cell toxicity.

INTRODUCTION

Microalgae are unicellular, simple, primitive, and photosynthetic organisms, which produce bioactive compounds with anti-infectious, anti-inflammatory, antiviral, antibacterial, and antitumor activities (Shanab et al, 2012; El Baky et al., 2014). Common failure of conventional therapy against cancer indicates a critical need for beneficial alternative therapeutic agents (Rengarajan et al. 2013). Anti-tumor activity of microalgal compounds can be explained by their ability to cross the lipophilic membranes and interact with proteins involved in apoptosis. In addition, several microalgal compounds induce DNA-dependent DNA polymerases inhibition, cyclins expression alteration, or major transduction pathways interference. Microalgal compounds anticancer property has also been related to immune response stimulation (Baudelet et al. 2013). The objective of the present study was to evaluate the potential of the two native microalgae, *C. sorokiniana* and *Scenedesmus* sp. extracts, isolated from Nuevo Leon, Mexico, against murine L5178Y-R lymphoma cells. To our knowledge, this is the first report of antitumor activity of microalgae isolated from this particular region.

MATERIALS AND METHODS

Microalgae strains and culture.

In the present study, two microalgae isolated from Nuevo Leon sate, Mexico, were tested. *C. sorokiniana* was isolated from San Juan River in the municipality of Cadereyta (26°21′55″N 98°51′15″O), whereas *Scenedesmus* was obtained from Pesquería River in the municipality of Apodaca (25°47′06″N 100°03′04″O). *C. sorokiniana* molecular identification

using the P2F (5′-GGC TCA TTA AAT CAG TTA TAG-3′) and P2R (5′-CCT TGT TAC GA(C/T) TTC TCC TTC-3′) primers (Lee & Hur, 2009), to assess the 18S gene amplification, was previously reported by Cantú-Bernal (2017). L-glutamine and penicillin-streptomycin solutions were purchased from Life Technologies (Grand Island, NY). RPMI 1640 medium, fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), *N*, *N*-dimethylformamide (DMF), phosphate buffered saline (PBS), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Vincristine was obtained from Vintec (Columbia, S.A. de C.V., Ciudad de Mexico). Extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37°C in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7. Water samples were taken on 50 ml sterile Falcon tubes and kept at 5°C ± 2°C on ice. Then, 5 ml were transferred to 250 ml Erlenmeyer flasks, containing 100 ml of LC culture medium, as reported by López-Chuken et al. (2010). Flasks were incubated at room temperature (25°C± 3°C) in a continuous shaker at 120 x g and under light radiation using 100 Watt white fluorescent light bulb as a continuous artificial light source (1000 lux approximately). Flasks were incubated for 14 d until green growth was observed, after which, 100 µl were transferred to Petri dishes containing the same culture medium, but solidified with 1.5% of bacteriological agar. Inoculated dishes were incubated at 30°C ± 2°C by using a 100 watt white fluorescent light bulb as a continuous artificial light until isolated green colonies were observed. Single colonies were collected using a bacteriological loop and placed in Erlenmeyer flasks containing 100 ml of algal liquid culture medium. Flasks were then incubated under the same conditions described above. This process allowed us selecting a single microalgae genus by picking up a single colony; however, given that microalgae tend to grow in consortia with bacteria and yeasts, microalgal cultures were treated with an antibiotic and

antimycotic solution containing 500 UI/ml penicillin, 500 µg/ml streptomycin, 50 µg/ml gentamicin, and 1.25 µg/ml fungizone. For this, 5 ml of liquid culture medium with antibiotics were placed in 15 ml conical tubes, after which 0.25 ml of the algal culture were added and tubes were incubated for 48 h, under same shaking and lighting conditions described above. After the incubation period, 500 µl of the cultures were transferred into 50 mL of sterilized LC algal liquid culture medium without antibiotics producing axenic cultures of *C. sorokiniana* and *Scenedesmus* sp. isolates. Each axenic culture was grown for 7 d in 1-L Erlenmeyer flasks containing 500 ml of LC media until exponential growth phase was reached (based on growth curve, data not shown). Next, each complete culture was transferred to individual bioreactor tanks containing 14.5 L of LC microalgae medium. Photobioreactor tanks were designed by López-Chuken work team, and consisted of circular acrylic tanks of 30 cm of diameter and height; aeration was supplemented by air pumps with an adapted 0.2 µm filter at 1-L/min flow rate, radiated by continuous artificial LED white lights at 1500 lux of intensity, and agitation by rotary plastic pallets at 50 rpm (supplementary material Fig. S1). Biomass production in bioreactors was monitored every 2 d (Tuesdays, Thursdays, and Saturdays) by taking a 10 ml sample with a sterile pipette and filtering through a previously weighed 0.7 µm-pore size microfiber paper. Then the paper was dried at 70°C inside an oven and weighed again; this monitoring process was repeated until the biomass production showed no increase. Once the maximum biomass production was reached, bioreactor tanks were stored at 4°C ± 2°C, until most microalgae biomass precipitated; then, the supernatant was decanted. The collected wet biomass was the centrifuged at 9000 rpm for 10 min (THERMO Scientific, ST 16 R, Waltham MA) and frozen dried (Labconco, Kansas City, MO).

Tumor cytotoxicity and apoptosis assays.

C. sorokiniana and *Scenedesmus* sp. dried biomass was extracted separately with 300 ml methanol using a Soxhlet system during 48 h; after this, methanol was removed by rotary evaporation and then by vacuum desiccation until dryness was obtained. Extracts were dissolved in RPMI medium at a concentration of 1-mg/ml and kept frozen. This solvent was chosen based on previous results where methanol extracts showed the highest cytotoxic activity against L5178Y-R cell line, whereas chloroform and hexane extracts showed no cytotoxic effect (data not shown). The tumor cell line L5178Y-R (mouse DBA/2 lymphoma) was purchased from the American Type Culture Collection (LY-R, ATCC® CRL-1722™, Rockville, MD), maintained in culture flasks with RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5% penicillin-streptomycin solution (referred as complete RPMI medium) at 37°C, in a humidified atmosphere of 5% CO_2 in air. Cellular density was kept between 10^5 and 10^6cells/ml. To determine the cytotoxic effect of *C. sorokiniana* and *Scenedesmus* sp. methanol extracts against L5178Y-R tumor cells, cell cultures were collected and washed three times in RPMI medium, then suspended and adjusted to $5x10^4$ cells/ml with complete RPMI medium. One hundred microliters of the cell suspensions were then added to flat-bottomed 96-well plates (Becton Dickinson, Cockeysville, MD), containing 100 µl of complete RPMI, methanol microalgae extracts at various concentrations, vincristine (250 µg/ml) as positive control, and RPMI medium as negative control; all treatments were tested in triplicate. Microplates were incubated for 48 h at 37°C with 5% CO_2, then 15 µl of MTT were added (0.5 µg/ml, final concentration), and cultures were incubated for 3 additional hours. After this, supernatant was removed and 80 µl of DMSO were added to all wells. Optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (DTX 880 Multimode detector, Becton

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

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

Apoptosis induction by *C. sorokiniana* and *Scenedesmus* sp. methanol extracts against L5178Y-R cell line was evaluated *in vitro* by acridine orange and ethidium bromide staining. For this, $1x10^6$ L5178Y-R tumor cells were placed in 24-well plates in the presence of 500 µg/ml methanol extracts, and incubated for 24 h. Then, 500 µl of RPMI, plus 1-µl of acridine orange and 100 µg/mL ethidium bromide (1:1 ratio) were added to the wells. Next, cultured cells were incubated for 5 min, washed with 1-ml PBS, and suspended in 100 µl of RPMI medium; after incubation period, 10 µl of cell suspension were placed between a slide and a coverslip for fluorescence microscope visualization. Acridine orange stains viable cells and dead cells (green cells), whereas ethidium bromide only stains those cells that have lost the integrity of their membrane (orange cells). Therefore, viable cells appear in a uniform green tone, cells found in apoptosis appear in a spotty green or granular in the center due to the condensation of chromatin and fragmentation of the nucleus, whereas cells in necrosis appear in a uniform orange hue (Coligan et al. 1995).

In addition, apoptosis induction was evaluated by DNA degradation (Orozco-Flores et al. 2017). Cells were incubated for 48 h with *C. sorokiniana* and *Scenedesmus* sp. methanol extracts at 500 µg/ml, testing their respective negative (culture medium) and positive (20 µg/ml actinomycin D) controls. After the incubation period, cells were collected and centrifuged at 2000 rpm for 10 min, then washed with PBS and extracted using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, MA). In order to visualize the extracted DNA, the sample was separated

by 2% agarose gel electrophoresis, using SB buffer for the electrophoretic shift at 70V for 20 min and 110V for 1 h. After this, gel was stained with 5 ng/ml ethidium bromide and photographs were documented under High Performance Ultraviolet Transilluminator (UVP, LLC. Upland, CA) light. DNA like-ladder fragmentation indicates apoptotic activity, whereas DNA scan represents cell death by necrosis.

Animals.

Six- to eight-week old Balb/c female mice were purchased from Harlan Mexico S.A. de C.V. (Mexico, D.F.). They were kept in a pathogen- and stress-free environment at 24°C, under a light-dark cycle (light phase, 06:00-18:00 h) in a One Cage 2100™ System (Lab Products, Inc., Seaford, DE), and given water and food *ad libitum*. Animals were euthanized by asphyxiation in a 100% CO_2 chamber. Experiments involving the use of animals were reviewed and approved by our institutional animal care and use committee before being initiated, and were performed in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in March 1999.

Murine thymus lymphocyte viability assay.

Thymus was immediately removed after mouse death. Single-cell suspensions were prepared by disrupting the organ in RPMI 1640 medium. Cell suspensions were washed three times in this medium, and suspended and adjusted at $1x10^7$ cells/ml in complete RPMI medium. Thymus lymphocyte viability was determined by a colorimetric technique using MTT (7). Thymus suspensions (100 µg of 1 X 10^7 cells/ml) were added to flat-bottomed 96-well plates (Becton Dickinson) containing triplicate cultures (100 µg/well) of complete RPMI medium

(unstimulated control), or 100 µl of *C. sorokiniana* and *Scenedesmus* sp. methanol extracts at various concentrations, for 48 h at 37°C in 95% air-5% CO_2 atmosphere. After incubation for 44 h, MTT was added, and cultures were additionally incubated for 4 h. Cell cultures were then incubated for 16 h with extraction buffer (100 µl/well), and optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (Becton Dickinson) at 570 nm (7).

All experiments were repeated at least three times with similar results. The results were expressed as means ± SEM of triplicate determinations from a representative experiment. Statistical significance was assessed by one-way analysis of variance and by the Student's *t* test.

RESULTS

Microscopic evaluation revealed the presence of *C. sorokiniana* and *Scenedesmus* sp. (Table 1), whose isolated colony cultures were then produced under axenic conditions. To our knowledge, this is the first report of Nuevo Leon, Mexico native microalgae, identified as *C. sorokiniana* and *Scenedesmus* sp., showing cytotoxicity against a murine lymphoma tumor cell line. Once the biomass was dried, collected, and weighed separately for each bioreactor, the yield by *C. sorokiniana* was of 0.24 g/L (± 0.01), whereas for *Scenedesmus* sp. was of 0.30 (± 0.01) g/L (Fig. 1).

Microalgae methanol extracts tested *in vitro* against tumor cell resulted in concentration-dependent activity against the murine tumor cell line L5178Y-R. *C. sorokiniana* extract caused significant ($p < 0.05$) 17% and 61% tumor cell toxicity at concentrations of 250 and 500 µg/ml, respectively, whereas that of *Scenedesmus* sp. induced 15%, 25%, and 75% cytotoxicity at

concentrations of 125, 250, and 500 µg/ml, respectively (Fig. 2). *C. sorokiniana* extract resulted in 74.4% tumor cell toxicity, 66% apoptosis, and 9% necrosis, whereas *Scenedesmus* sp. extract caused 54% tumor cell toxicity, 51% apoptosis, and 3% necrosis (Fig. 3).

Cytotoxicity of *C. sorokiniana* and *Scenedesmus* sp. methanol extracts did not negatively change normal mouse thymus lymphocytes resulting in up to 26% and 19% (not significant) lymphocyte toxicity (Fig. 4).

DISCUSSION

To our knowledge, this is the first report of Nuevo Leon, Mexico native microalgae, identified as *Chlorella* sp. and *Scenedesmus* sp., showing cytotoxicity against a murine lymphoma tumor cell line. Previous reports have shown microalgae potential for wastewater treatment and biodiesel production (Reyna-Martínez et al. 2014; Beltrán-Rocha et al. 2017). In the present study, microalga isolates were grown under artificial light, closed photobioreactors, and previously established physicochemical conditions. *C. sorokiniana* and *Scenedesmus* sp. production in the photobioreactors was stopped after 29 d, because no additional biomass production was observed. Biomass production was lower compared with other reports using batch culture and phototrophic conditions (Brennan and Owende 2010; Chen et al. 2011). However, sufficient biomass was produced to obtain an adequate amount of aqueous and methanol extracts to perform biological assays.

In vitro tumor cell toxicity assays resulted in concentration-dependent activity against the murine tumor cell line L5178Y-R (up to 61.9% and 74.8% cytotoxicity at 500 mg/mL *Chlorella* sp. and *Scenedesmus* sp. extracts, respectively). These results are comparable to other reports

showing about 50% *in vitro* cytotoxicity by microalga extracts against cervical cancer (Yusof et al. 2010, Kyadari et al. 2013).

Microalgae-induced tumor cytotoxicity was observed to be mediated by apoptosis, as determined by the acridine orange and ethidium bromide staining, as well as DNA fragmentation (ladder pattern) (Nagata 2000). In fact, microalga isolates methanol extracts resulted in similar effects against the cell line compared with actinomycin D, compound that resulted in cellular apoptosis (Quintanilla-Licea et al. 2012). After testing crude extracts of the cyanobacteria *Nostoc* sp., tested against human pancreatic tumor cells PaTu 8902, Voráčová et al. (2017) found that apoptosis was mostly mediated by caspases 3 and 7.

Tumor cancer cells may develop as a result of *in situ* formation of nitrosamines from secondary amines and nitrite in an acidic environment of the stomach. There are chemical agents known as chemopreventers, which help to reverse, suppress or prevent these nitrosamines. In fact, ascorbic acid or phenolic compounds are chemopreventers, since they prevent or reduced nitrosamines formation (Jahan *et al.* 2017). It has been shown that microalgae have the ability to synthesize a number of bioactive compounds, including bioactive peptides, fucans, galactans, alginates, phenolic compounds, phycocyanins, phycobiliproteins, eicosapentanoic and arachidonic acids, carotenoids, tocopherols, sterols, and terpenoids (Lordan et al. 2011). Some of these compounds may be responsible for the cytotoxicity induced by *C. sorokiniana* and *Scenedesmus* sp. methanol extracts, against the murine lymphoma cell line L5178Y-R. Lymphocyte cytotoxicity results were comparable with other reports showing about 50% *in vitro* cytotoxicity by microalga extracts against cervical cancer cells (Yusof et al., 2010; Kyadari et al., 2013).

The bioguided fractionation of these extracts is ongoing, and further studies of the isolated pure compounds will be performed.

CONCLUSION

The native microalgae *C. sorokiniana* and *Scenedesmus* sp. isolates from Nuevo Leon, Mexico water bodies were produced under a semi-pilot level using closed photobioreactors, with artificial illumination and aeration. The produced microalgae methanol extracts were cytotoxic against the murine tumor cell line L5178Y-R *in vitro,* by the mechanism of apoptosis, without affecting normal murine lymphocytes.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Figures legends

Fig. 1. Biomass production time-course by *Chlorella sorokiniana* and *Scenedesmus* sp. isolates. Data represent means ± SEM.

Fig. 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.

Fig. 3. Effects of *Chlorella 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 *Chlorella sorokiniana* and *Scenedesmus* sp. methanol extracts, and actinomycin D (20 µg/ml).

Fig. 4. 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^{o}C, and cell viability was determined as detailed in the text.

Supplementary material

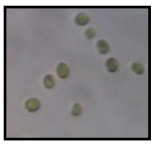
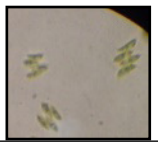
Figure S1. Bioreactor tanks designed by López-Chuken *et al*. (2010) consisting of circular acrylic tanks (30 cm of diameter and height) where aeration was supplemented by air pumps with an adapted 0.2 μm filter at 1-L min^{-1} flow rate; radiated by continuous artificial illumination (LED white lights at 1500 lux of intensity), and under agitation by rotary plastic pallets at 50 x g.

Table 1(on next page)

Table 1.

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.	

1

Figure 1(on next page)

Biomass production

DOCTOR : Fig. 1. Biomass production time-course by *Chlorella sorokiniana* and *Scenedesmus* sp. isolates. Data represent means ± SEM.

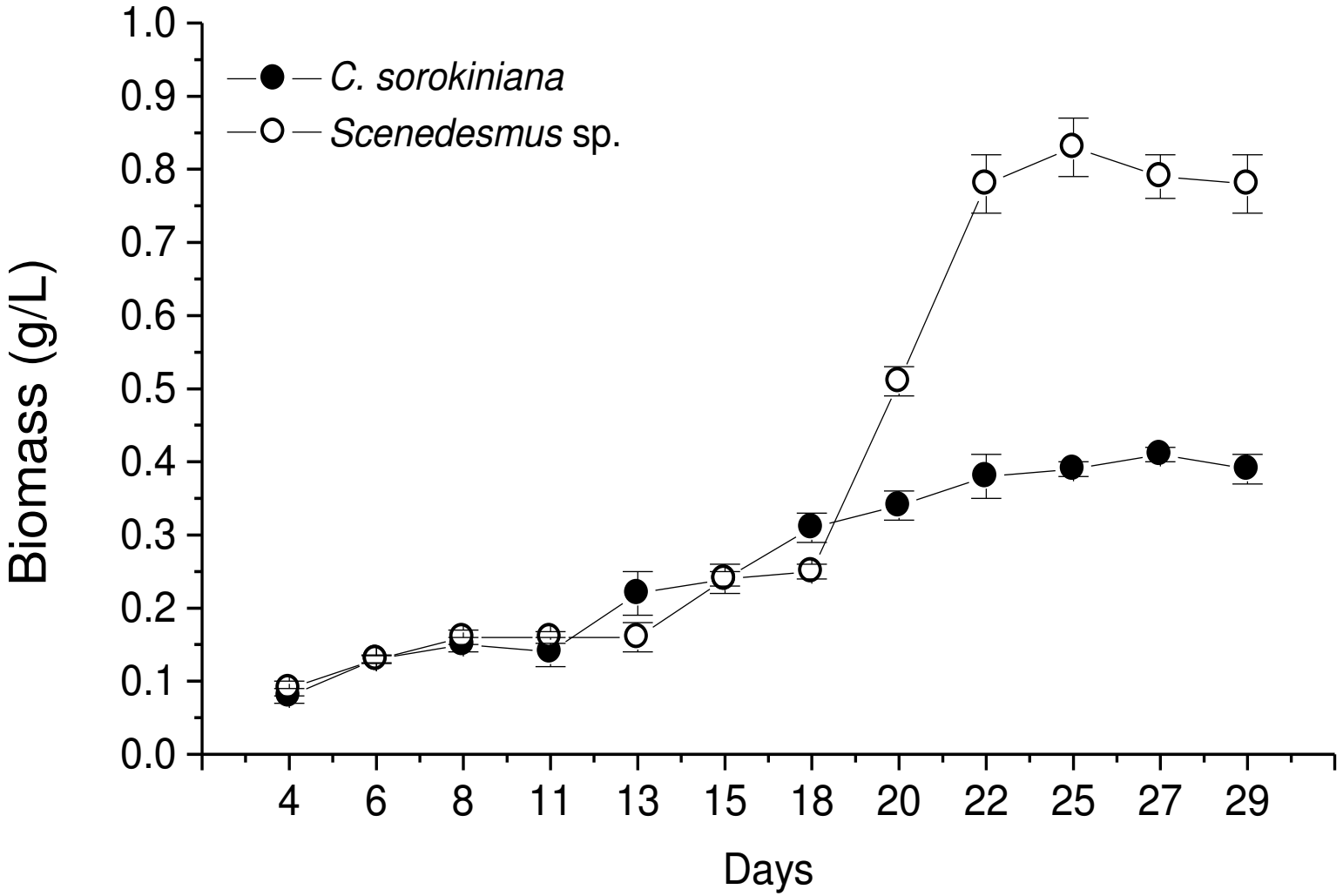

C. sorokiniana
Scenedesmus sp.
Biomass (g/L)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
4
6
8
11
13
15
18
20
22
25
27
29
Days

Figure 2(on next page)

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

DOCTOR : Fig. 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.

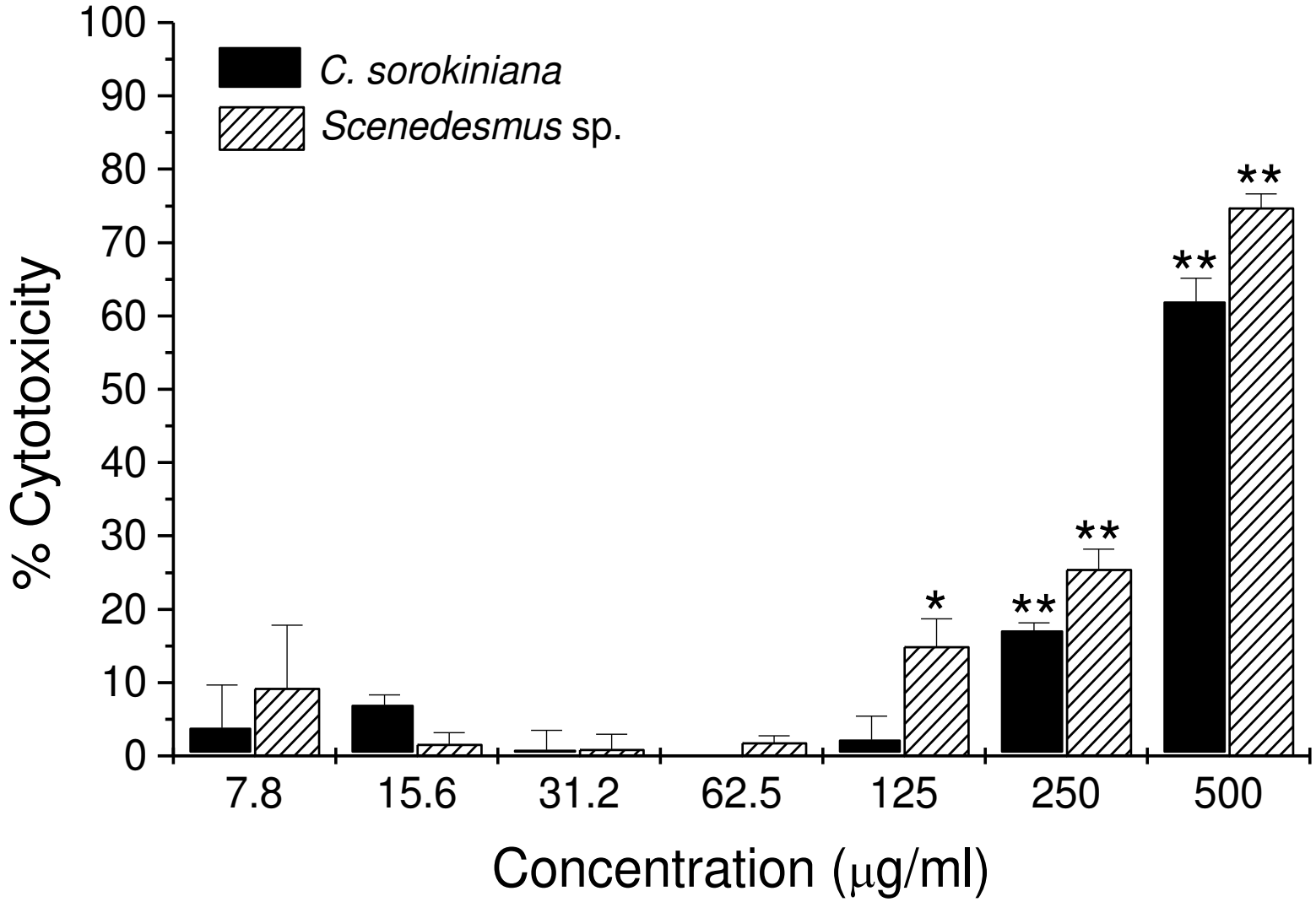

C. sorokiniana
Scenedesmus sp.
% Cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
7.8
15.6
31.2
62.5
125
250
500
Concentration (μg/ml)
*
**
**
**
**

Figure 3(on next page)

Percentage of viable, apoptotic, and necrotic L5178Y-R cells

DOCTOR : Fig. 3. Effects of *Chlorella 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 *Chlorella sorokiniana* and *Scenedesmus* sp. methanol extracts, and actinomycin D (20 µg/ml).

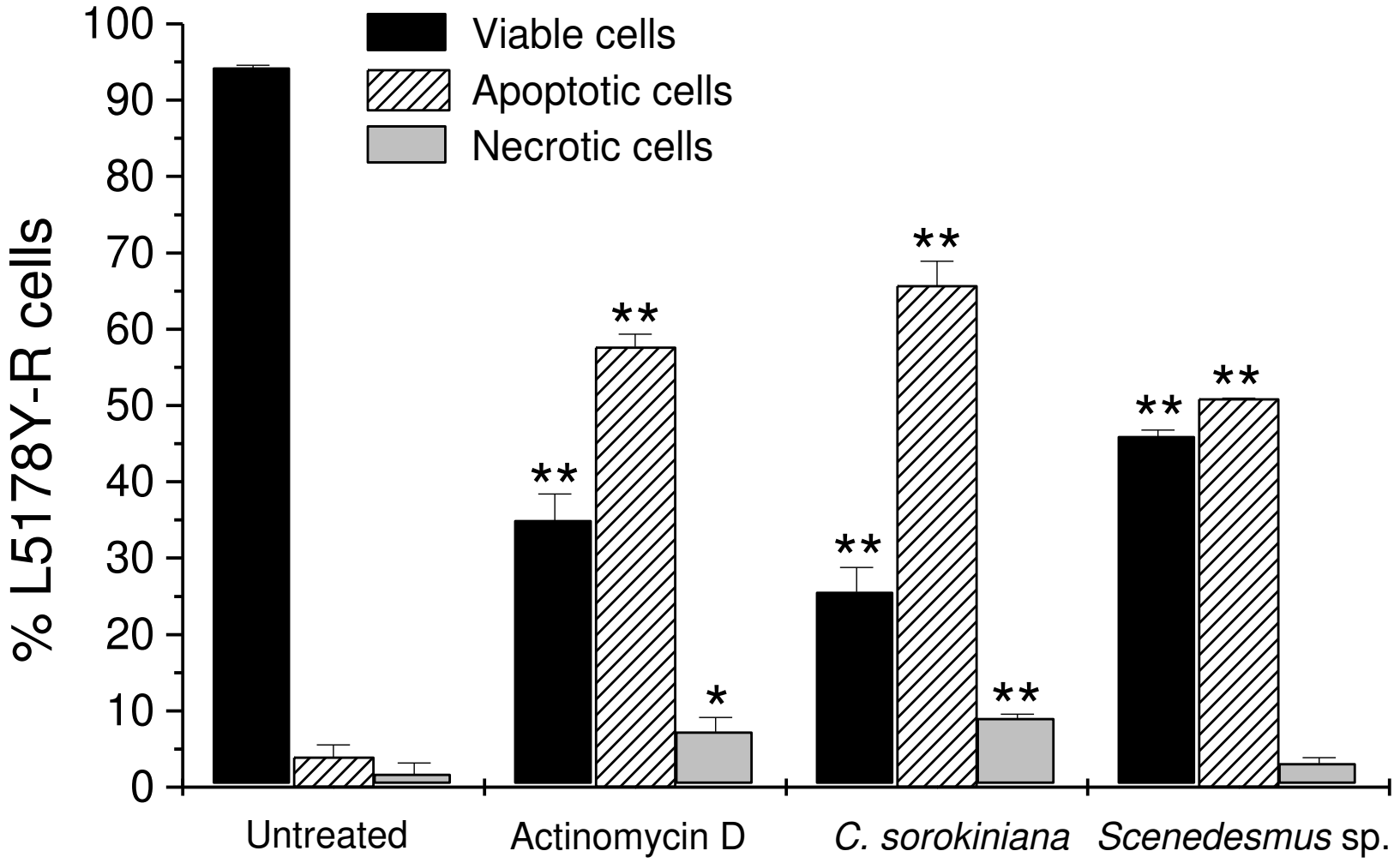
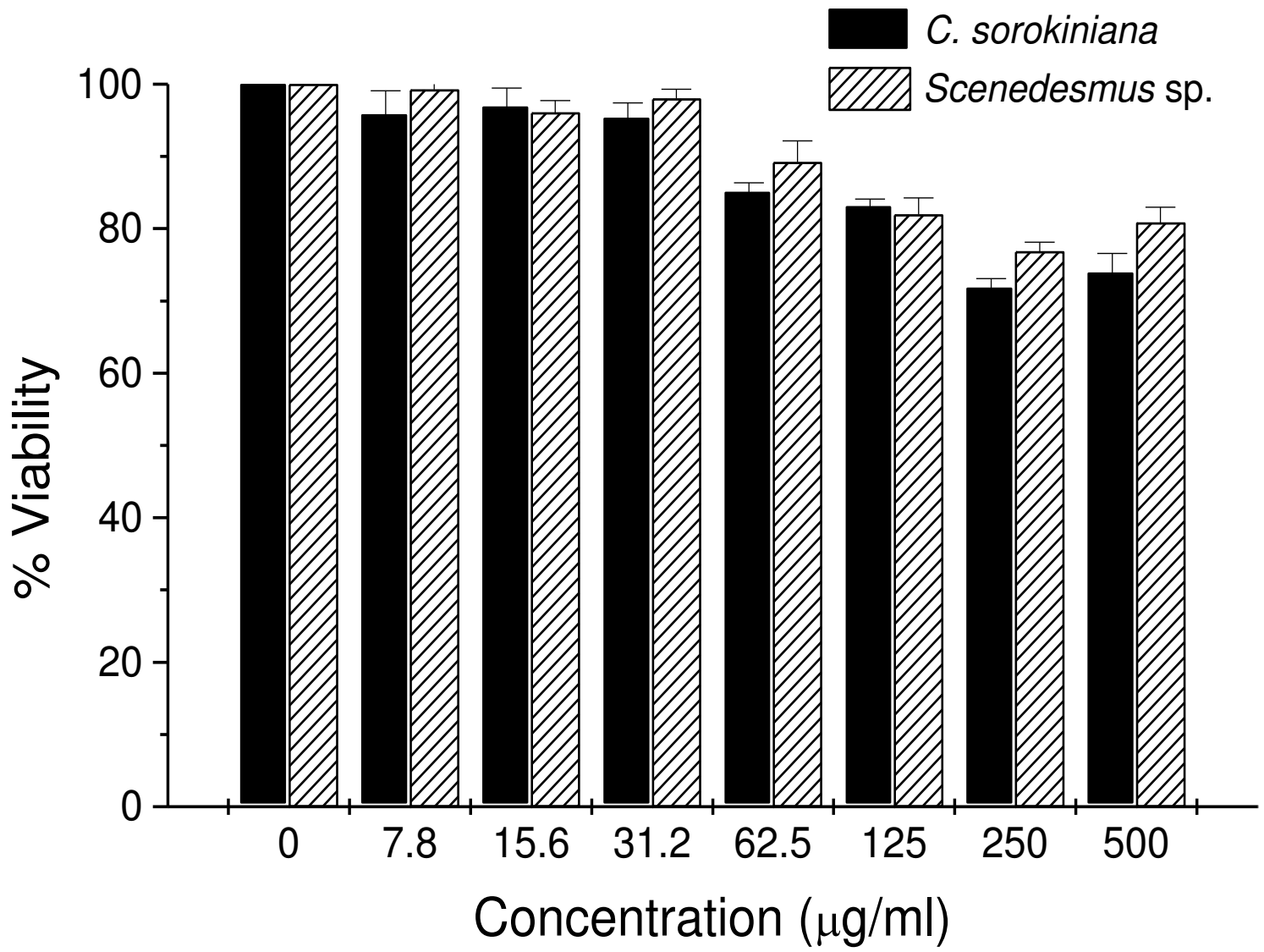

Viable cells
Apoptotic cells
Necrotic cells
% L5178Y-R cells
100
90
80
70
60
50
40
30
20
10
0
**
**
*
**
**
**
**
Untreated
Actinomycin D
C. sorokiniana
Scenedesmus sp.

Figure 4(on next page)

Thymus lymphocyte viability

DOCTOR : Fig. 4. 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.


C. sorokiniana
Scenedesmus sp.
% Viability
100
80
60
40
20
0
0
7.8
15.6
31.2
62.5
125
250
500
Concentration (μg/ml)