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Microalgae extracts: Potential anti-*Trypanosoma cruzi* agents?

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ABSTRACT

Introduction: Chagas disease, caused by the protozoan parasite*Trypanosoma cruzi*, has no effective treatment available. On the other hand, microalgae are aquatic organisms that constitute an interesting reservoir of biologically active metabolites. Moreover, some species of green and red algae present anti-protozoan activity. Our aim was to study the antiparasitic effects of aqueous, methanolic and ethanolic extracts from different microalgae.

Methods and Results: : Our results show that the methanolic extracts of *S. obliquus* and *T. suecica* as well as the ethanolic extracts of *C. reinhardtii* and *T. suecica* present trypanocidal activity on the infective extracellular trypomastigotes and intracellular amastigotes. In addition, the ethanolic extract of *C. reinhardtii* potentiates the activity of the conventional antichagasic drug nifurtimox. In order to identify some potential compounds with trypanocidal activity, we performed a phytochemical screening analyzing the presence of phenolic compounds, pigments and terpenoids.

Conclusion: The different microalgae extracts, particularly the ethanolic extract of*C. reinhardtii,* are promising potential candidates for the development of future natural antichagasic drugs.

1. Introduction

Chagas disease (CD) or American Trypanosomiasis is a potentially life-threatening zoonosis caused by the flagellate protozoan parasite *Trypanosoma cruzi*. It has been estimated that approximately eight million people in Latin America are infected with this parasite and 100 million people are living in endemic areas (25 % of the total population in Latin America) and are therefore at risk of infection [1].

T. cruzi presents an indirect life cycle, affecting mammals as its definitive hosts were the parasite develops into two forms: the circulating nonreplicative trypomastigotes and the intracellular replicative amastigotes. [1,2].

The disease evolves in two phases. The acute phase, defined by patent parasitemia, lasts 2–3 months. It is often asymptomatic or involves unspecific flulike symptoms, although 2–12 % of infected individuals die from acute myocardiopathy associated (or not) with a meningoencephalic compromise. Then, the individual enters the chronic phase and remains infected throughout life. Although most patients remain asymptomatic for several months to decades, 30–40 % of them develop cardiac (most commonly) and/or digestive tract (less frequent) pathologies that may lead to premature death or incapacitation [3,4]. The efficacy of benznidazole (Bz) and nifurtimox (Nfx), the two trypanocidal drugs currently employed in the treatment of CD, are far from optimal for the treatment of chronically infected patients. Moreover, antiparasitic treatment is contraindicated during pregnancy, because the risks of using the available drugs on the fetus are unknown, and the risk of adverse reactions is high in adults [5]; therefore, finding new effective and safe drugs remains a current challenge [6].

Diverse aquatic organisms represent a sparsely explored reservoir of biologically active metabolites. In particular, microalgae constitute a vast group of unicellular eukaryotic photosynthetic organisms [7] that is considered part of the earliest forms of life on the earth [8]. Microalgae have been used by indigenous population for centuries. The first use of microalgae by humans dates back 2000 years to the Chinese, who used them to survive during famine [9]. Aztecs also used microalgae species as a food source as was described by Spanish chroniclers [10].The diversity of microalgae (i.e., prokaryotic cyanobacteria and eukaryotic microalgae) is vast, but this diversity has not yet been fully

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exposed. There are more than 50,000 different types of microalgal species present in oceans and fresh water [11]. They are especially valuable due to their high content of natural compounds, including complex organic primary and secondary metabolites, such as phytopigments (xanthophylls and carotenoids), polyunsaturated fatty acids (PUFAs), phenolic substances, docosahexaenoic acid (DHA), vitamins, carbohydrates, tannins, terpenoids and peptides, with different biological activities, including antioxidant, antihypertensive, antimicrobial, carcinogenic and anti-inflammatory properties, among others [12,13,7,11,14].

Interestingly, it has been demonstrated that some species of green and red algae present anti-protozoan activity [15,16]. In particular, lipodepsipeptides isolated from the filamentous cyanobacterium *Oscillatoria nigro-viridis* [17] as well as crude extracts of four green marine algae (*Cladophora rupestris*, *Codium fragile sp., Tomentosoides*, *Ulva intestinalis* and *Ulva lactuca*) [15] present trypanocidal activity.

Here we studied the effect of aqueous, methanolic and ethanolic extracts from 4 species of microalgae and the cyanobacterium *Arthrospira platensis* on the infective trypomastigote and amastigote forms of *Trypanosoma cruzi*. In particular, we assayed extracts from *A. platensis* commonly known as the food supplement spirulina [18], the fresh water microalgae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus,* both employed in the biofuel industry [19,20] as well as the marine microalgae *Tetraselmis suecica* and *Nannochloropsis oculate*, both of which are widely used because of their pigments with nutraceutical and anti-oxidant activity [21,22]. In addition, we performed phytochemical tests in order to identify some potential compounds with trypanocidal activity. Thus, we analyzed the presence of phenolic compounds, pigments and terpenoids. Our results suggest that the methanolic extract of *S. obliquus* and the ethanolic extract of *C. reinhardtii*, are promising potential candidates for the development of future natural anti-chagasic drugs.

2. Materials and methods

2.1. Microalgae species, culture conditions and characteristics of growth

The microalgae species *Chlamydomonas reinhardtii* (strain CC®-1010™), *Scenedesmus obliquus* (strain CCMP-2399) were obtained from the culture collection of the Laboratory of Genetic and Molecular Immunology (GIM, PUCV). *T. suecica* (strain CCAP904) was obtained from the National Center for Marine Algae and Microbiota (NCMA) at the Bigelow Laboratory, USA. *Nannochloropsis oculata* was obtained from the Marine Research Center Quintay (CIMARQ), Valparaíso, Chile, and *Arthrospira platensis* was donated by Aeon Biogroup©. *T. suecica* and *N. oculata* were cultivated in F/2 medium [23], *C. reinhardtii* was maintained in Tris acetate phosphate (TAP) medium [24], *S. obliquus* was maintained in BG-11 medium [25] and *A. platensis* in Zarrouk´s medium [26]. Unialgal cultures from each species were obtained from colonies that were maintained in axenic agar plates with the corresponding medium. Colonies were seeded in liquid medium and cultured until they reached the exponential phase at 21 ± 0,5 °C under controlled lighting (40 W) and were then pumped with an aeration pump (20 L/min). Afterward, aliquots were successively transferred to increasing volumes of the medium and cultured with aeration conditions at the temperature and illumination conditions described previously until a final volume of 20 L was reached [27]. All laboratory cultures were generated without stress induction.

2.2. Microalgae extract sample preparation

Microalgae were collected by centrifugation at 4480 × g for 20 min at 4 °C. Then, the pellets were washed in phosphate-buffered saline (PBS) and centrifuged under the same conditions. The biomass was frozen at −80 °C and lyophilized using a Christ Alpha model 1−2LD (Germany) to obtain dried samples. The extracts were obtained by dissolving in three different solvents: absolute ethanol, absolute methanol or distillated water (0.01 g/mL), stirring for 1 min and maintaining at 4 °C for 24 h in darkness. Then, the extracts were centrifuged at 3000 x g for 5 min at room temperature, and the solvents were evaporated in a rotary evaporator SpeedVac rotary evaporator Concentrator Savant SVC-100H for 24 h. The extracts were suspended in H_2Odd, lyophilized again as described previously, and stored at room temperature in the dark until used. The yield of the extracts was calculated as R = (mg extract/mg dry biomass) x 100.

2.3. Cell culture

Green Monkey (*Cercopithecus aethiops*) renal fibroblast-like cells (Vero cells (ATCC®CCL-81)) were grown in RPMI medium supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicillin–streptomycin) [28]. The cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 24−48 h. Cells were counted in a Neubauer chamber previous to the experiments.

2.4. Parasite cultures and harvesting

Stock cultures of *T. cruzi* epimastigotes (Ypsilon strain) were maintained in axenic conditions at 28 °C in monophasic Diamond's culture medium supplemented with 75 μM hemin, 5 % fetal bovine serum (FBS), 100 μg/mL sodium penicillin and 100 μ/mL streptomycin [2]. Infective extracellular trypomastigotes were obtained from infected Vero cells. Semiconfluent Vero cells were incubated with epimastigotes of *T. cruzi* harvested in the late stationary phase. Trypomastigotes also present in the culture invade Vero cells and replicate intracellularly as amastigotes. After 72 h, amastigotes transform back into trypomastigotes which lyse the host cells. Trypomastigotes were recovered in the supernatant by low-speed centrifugation (500 x g) counted in a Neubauer chamber and used for viability assays [2,29,30].

2.5. Parasite and cell viability measurements

Trypomastigotes (10^7 parasite/mL) or Vero cells (500 cells/mL) were incubated with the different extracts or Nfx re-suspend in dimethyl sulfoxide in culture medium (0.025 % v/v, final concentration), for 24 h. IC50 values were obtained by dose-response analysis were parasites or Vero cells were incubated with different concentration of Nfx (5, 10, 15, 30, 60, 100 μM) or the microalgae extracts (10, 25, 50, 60, 75, 100, 200, 500 and 1000 μg/mL). For combination experiments, parasites or Vero cells were exposed for 24 h to the ethanolic extracts of *C. reinhardtii* and *T. suecica*, the methanolic extracts of *T. suecica* and *S. obliquus*, Nfx and their combinations. For the study about the outcome of the microalgal extract-Nfx combinations, we performed a dose-matrix approach, comparing the effect of the combination of 6 concentrations of the ethanolic extracts of *C. reinhardtii* and *T. suecica*, as well as the methanolic extracts of *T. suecica* and *S. obliquus* (10, 25, 50, 60, 75 and 100 μg/mL) and 6 concentrations of Nfx (5, 10, 15, 30, 60, 100 μM). We analyzed the data using the free software Combenefit [31], and we chose the Bliss independence approach for the data analyses, assuming that both drugs have different action mechanisms.

The effect of the microalgae extracts on trypomastigote and Vero cell viability were evaluated by the tetrazolium salt (MTT) reduction assay. Briefly, 10 mL of 5 mg/mL MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye and 0.22 mg/mL phenazine methosulphate (used as an electron carrier) were added to each well containing 10^6 parasites or 5 × 10^4 cells in 100 mL of RPMI 1640 medium, without phenol red. After incubating the parasites or cells for 4 h at 37 °C, the generated formazan crystals were dissolved in 100 mL of 10 % (w/v) SDS in 0.01 M HCl. The plates were kept overnight at 37 °C, and the optical density (OD) was determined using a microplate reader (LabSystems Multiskan MS, Finland) at 570 nm. Under these

conditions, the OD is directly proportional to the viable number of cells in each well [30].

2.6. Effect on intracellular parasites

Vero cells were detached by trypsinization, sedimented, and re-suspended in media containing 10 % FBS. Then, 2×10^5 cells were seeded into six-well plates. The cells were allowed to adhere to the bottom of the wells for 3 h and were then challenged with the parasites at a Vero cell:parasite ratio of 1:1 for 24 h, after which the supernatant was removed and the cells were exposed to the different microalgae extracts at the IC_{50} concentration or Nfx for other 24 h.

2.7. Morphological analysis

Vero cells were fixed in cold 90 % methanol and washed with PBS and incubated with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Then, the sections were mounted in Vectashield (ScyTek ACA) and observed on an epifluorescence microscope (Motic BA310; Hong Kong, China). Amastigotes were recognized by their morphology, including nuclear size and the presence of a kinetoplast, and analyzed with the MATLAB® software [29]. At least 500 cells were analyzed per condition.

2.8. Determination of parasite DNA by real time PCR

Genomic DNA was extracted from Vero cells with a Wizard Genomic DNA Purification Kit (Promega®, USA) according to the manufacturer's instructions. The resulting DNA was quantified with a μDrop Plate DNA quantification system in a Varioskan Flash Multimode Reader (Thermo Scientific, USA). For amplification of human and parasite DNA, two specific primer pairs were used. A 100 bp human GAPDH sequence was amplified using the primers hGDH-F (5'-TGATGCGTGTACAAGCGTTTT-3') and hGDH-R (5'-ACATGGTATTCACCACCCCACTAT-3'), which were designed using Primer Express software (version 3.0; Applied Biosystems®). For *T. cruzi* DNA detection, a 182 bp sequence of satellite DNA was amplified using the primers TCZ-F (5'-GCTCTTGCCCACAMG GGTGC-3') and TCZ-R (5'-CAAGCAGCGGATAGTTCAGG-3') [32,33]. Each reaction mix contained 200 nM of each primer (forward and reverse), 1 ng of DNA, 12.5 μL of SensiMix® SYBR Green Master Mix (Bioline®, USA) and H_20 for a total volume of 25 μL. The amplification was performed in an ABI Prism 7300 sequence detector (Applied Biosystems®, USA). The cycling program was as follows: an initial incubation at 20 °C for 2 min, a denaturation step at 95 °C for 10 min and 40 amplification cycles of 95 °C (15 s), 60 °C (15 s) and 72 °C (30 s). The final step was a dissociation stage that ranged from 60 to 95 °C (105 s). The relative quantification analysis of the results was expressed as an RQ value determined using the comparative control (ΔΔCt) method [34,35].

2.9. Determination of total phenolic content

TPC was estimated according the Folin-Ciocalteu method described by Ainssworth [36], with slight modifications. One mg of each microalgae extract was added with 1.5 mL of Folin-Ciocalteu reagent (1 N). After 4 min, 1.5 mL of saturated sodium carbonate (75 g/L) was added, and then distilled water until a total of 25 mL. Following 2 h of incubation at room temperature, the absorbance was measured at 765 nm using a spectrophotometer. For the quantification a calibration curve of gallic acid (10 − 250 μg / mL) was used. The results were expressed in mg equivalent of gallic acid (GAE) / g of dry microalgae extract.

2.10. Determination of total flavonoid content

TFC was determined using the spectrophotometric method according [37], with slight modifications. One mg of each microalgae extract was added with 1.5 mL of methanol and 0.1 mL of $AlCl_3$ (10 % w/v). Then, 0.1 mL of potassium acetate (1 M) was added, and then distilled water until a total of 5 mL. After 30 min of incubation at room temperature, the absorbance was measured at 415 nm using a spectrophotometer. Quercetin (10 − 100 μg/mL) was used for the calibration curve. Results were expressed as mg Quercetin Equivalent (QE)/g of dry extract of microalgae.

2.11. Determination of pigment content

Total carotenoids and chlorophylls contents were estimated according Lichtenthaler [38] with slight modifications. One mg of microalgae extract was resuspended in the solvent, vortexed for 1 min and homogenized with 2 cycles (30 s, rate of 6 movements/s) in a FastPrep-24™ MP Biomedicals equipment. The maximum absorption for carotenoids and chlorophylls *a* and *b* was determined spectrophotometrically (Jenway 6800 UV/VIS spectrophotometer) at wavelengths of 470, 652, and 665 nm, and 470, 648, and 664 nm for methanol and ethanol suspensions, respectively. For all pigments, turbidity registered at 750 nm was subtracted. The relative concentration (*C*) of pigments was calculated according the following Eq.s (1 to 6):

Methanol:

$$C_{Chlorophyll\ a} = 16.72 \times Abs_{665.2} - 9.16 \times Abs_{652.4} \quad (1)$$

$$C_{Chlorophyll\ b} = 34.09 \times Abs_{652.4} - 15.28 \times Abs_{665.2} \quad (2)$$

$$C_{Carotenoids} = [(1000 \times Abs_{470} - 1.63 \times C_{Chlorophyll\ a} - 104.96 C_{Chlorophyll\ b})] / 221 \quad (3)$$

Ethanol (95 %):

$$C_{Chlorophyll\ a} = 13.36 \times Abs_{664.1} - 5.19 \times Abs_{648.6} \quad (4)$$

$$C_{Chlorophyll\ b} = 27.43 \times Abs_{648.6} - 8.12 \times Abs_{664.1} \quad (5)$$

$$C_{Carotenoids} = [(1000 \times Abs_{470} - 2.13 \times C_{Chlorophyll\ a} - 97.64 C_{Chlorophyll\ b})] / 209 \quad (6)$$

Pigment concentration (mg of pigment/g of dry extract) was calculated with Eq.s 7 and 8:

$$Chlorophylls = C_{Chlorophyll} \times (volume\ of\ extract / mg\ of\ extract) \times z \quad (7)$$

$$Carotenoids = C_{Carotenoids} \times (volume\ of\ extract / mg\ of\ extract) \times z \quad (8)$$

2.12. Determination of terpenoids

Terpenoids were tested by the Salkowski reaction. 1 mL of each extract/fraction was mixed in 400 μL of chloroform, and concentrated H_2S0_4 (250 μL) was carefully added. The change in colour to brown indicates the presence of terpenoids [39].

2.13. Statistical analysis

For the combination experiments, the data were analyzed using the Combenefit free software [31]. All other statistical analyses were performed using GraphPad Prism (6.0) software. For all experiments, the statistical significance was established at $p < 0.05$. Normal distribution of data was assessed using D'Agostino-Pearsons analysis. One-way or Two-way ANOVA (with Tukey´s or Bonferroni´s post-test) or *t*-test analysis were performed when required.

3. Results

3.1. In vitro anti-Trypanosoma cruzi activity

We assayed the effect of aqueous, methanolic and ethanolic extracts

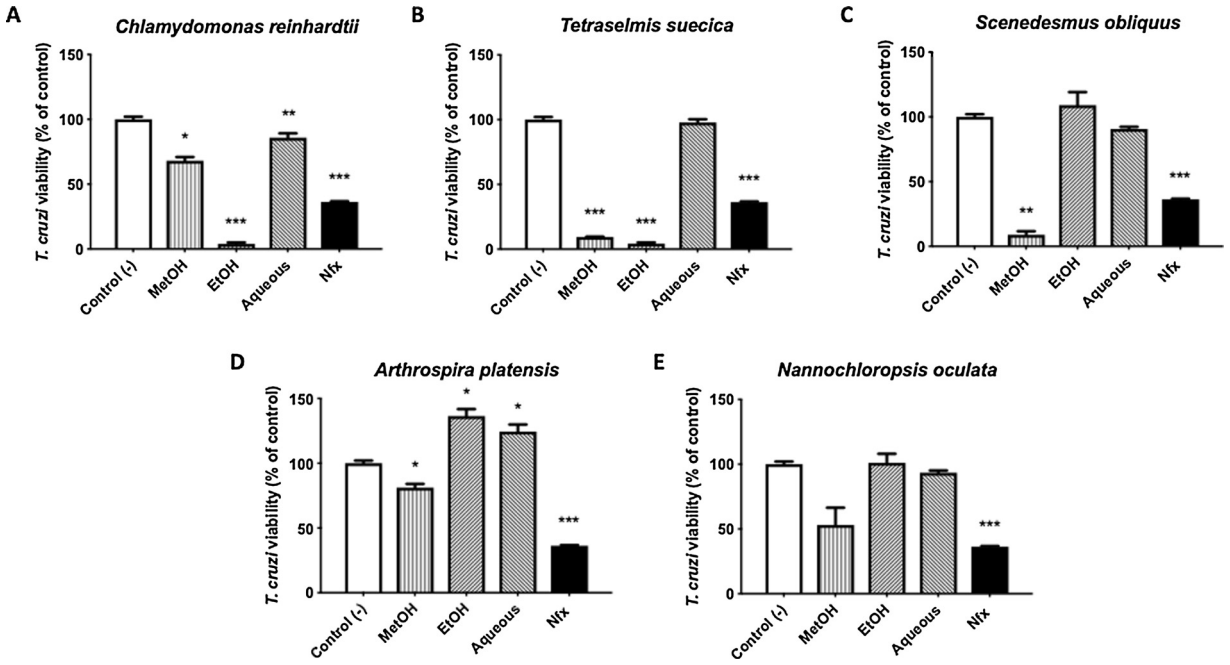


Fig. 1. Effects of the aqueous, methanolic and ethanolic extracts from the different microalgae on *T. cruzi* trypomastigote viability: *T. cruzi* trypomastigotes (10^7 parasites/mL) were incubated in presence and absence of aqueous, methanolic and ethanolic extracts of *C. reinhardtii* (**A**), *T. suecica* (**B**), *S. obliquus* (**C**), *A. platensis* (**D**) and *N. oculata* (*E*) (200 μg/mL) or of Nfx (10 μM) during 24 h and the viability was measured by the MTT method. Only, the ethanolic extracts of *C. reinhardtii* (**A**) and *T. suecica* (**B**) and the methanolic extracts of *T. suecica* (**B**) and *S. obliquus* (**C**) presented higher trypanocidal activity than Nfx. Data were analyzed by ANOVA followed by Dunnett's posttest (*p ≤ 0.01; **p ≤ 0.01; ***p ≤ 0.001).

(200 μg/mL each) of *C. reinhardtii* (Fig. 1A), *T. suecica* (Fig. 1B), *S. obliquus* (Fig. 1C), *A. platensis* (Fig. 1D) and *N. oculate* (Fig. 1E) on the infective trypomastigote form of *T. cruzi*. The parasites were incubated in the presence and absence of each extract or Nfx (10 μM), as reference trypanocidal drug, for 24 h and the parasite viability was measured by the MTT method. Only, the ethanolic extracts of *C. reinhardtii* (Fig. 1A) and *T. suecica* (Fig. 1B) and the methanolic extracts of *T. suecica* (Fig. 1B) and *S. obliquus* (Fig. 1C) presented higher trypanocidal activities than the ~IC_{50} of Nfx at 200 μg/mL. Thus, the parasite viability decreased in the presence of the ethanolic extracts of *C. reinhardtii* and *T. suecica* to 3.91 ± 0.892 % ($p \leq 0.001$) and 4.273 ± 0.724 % ($p \leq 0.001$), respectively. The methanolic extracts of *T. suecica* and *S. obliquus* decreases the parasite viability to 9.423 ± 0.257 % ($p \leq 0.001$) and 8.896 ± 2.328 % ($p \leq 0.001$). Therefore, these four extracts were used for the following experiments.

We further determined the IC_{50} values for the parasitic and mammalian cells and determined the selectivity indexes, which are depicted in Table 1. All IC_{50} values for the trypanocidal activities were between 60 and 70 μg/mL and the IC_{50} values for the mammalian cells ranged between 195 and 680 μg/mL. Thus, the selectivity indexes were 3.3 for the ethanolic extract of *C. reinhardtii,* 4.8 for the ethanolic extract of *T. suecica,* 2.9 for the methanolic extract of *T. suecica* and 11.2 for the methanolic extract of *S. obliquus.*

In addition, we assayed the effect of the four extracts on mammalian cells infected with intracellular amastigotes. Vero cells infected previously with the parasite were incubated in presence and absence of the selected extracts or Nfx at their respective IC_{50} values (Fig. 2).

The four assayed extracts significantly ($p \leq 0.0001$) decreased the percentage of infected cells in a way similar to that of Nfx (Fig. 2A). However, none of the extracts significantly decreased the number of amastigotes per cell (Fig. 2 B). Nevertheless, the ethanolic extract of *C. reinhardtii,* the methanolic extract of *S. obliquus* and Nfx showed a tendency to decrease the average amastigote number per cell. In Fig. 2C representative images of infected Vero cells and the effect of the ethanolic extract of *C. reinhardtii* are shown. We further corroborated the decrease in mammalian cell infection by analyzing parasite DNA load by quantitative PCR. All of the assayed extracts significantly ($p \leq 0.0001$) decreased the parasite DNA load in Vero cells (Fig. 3).

3.2. Anti-Trypanosoma cruzi activity of the selected microalgal extracts in combination with Nfx

To study the outcome of the microalgal extract-Nfx combination (Fig. 4), we performed a dose-matrix approach, comparing the effect of the combination of 6 concentrations of the ethanolic extracts of *C. reinhardtii* and *T. suecica*, as well as the methanolic extracts of *T. suecica* and *S. obliquus* (10, 25, 50, 60, 75 and 100 μg/mL) and 6 concentrations of Nfx (5, 10, 15, 30, 60, 100 μM). We analyzed the data using the free software Combenefit, and we chose the Bliss independence approach for the data analyses, assuming that both drugs have different action

Table 1
Determination of IC_{50} values and selectivity index.

		IC_{50} (μg/mL)		
		VERO® cells	*T. cruzi*	Selectivity index
Ethanolic Extracts	*Chlamydomonas reinhardtii*	220.3 ± 6.7	67.0 ± 4.12	3.3
	Tetraselmis suecica	325.6 ± 9.3	68.4 ± 4.68	4.8
Methanolic Extracts	*Scenedesmus obliquus*	196.1 ± 6.6	67.7 ± 5.17	2.9
	Tetraselmis suecica	680 ± 5.6	60.9 ± 14.30	11.2

Effect of the ethanolic extracts of *C. reinhardtii* and *T. suecica* as well as the methanolic extracts of *T. suecica* and *S. obliquus* on *T. cruzi* trypomastigotes and VERO cells.

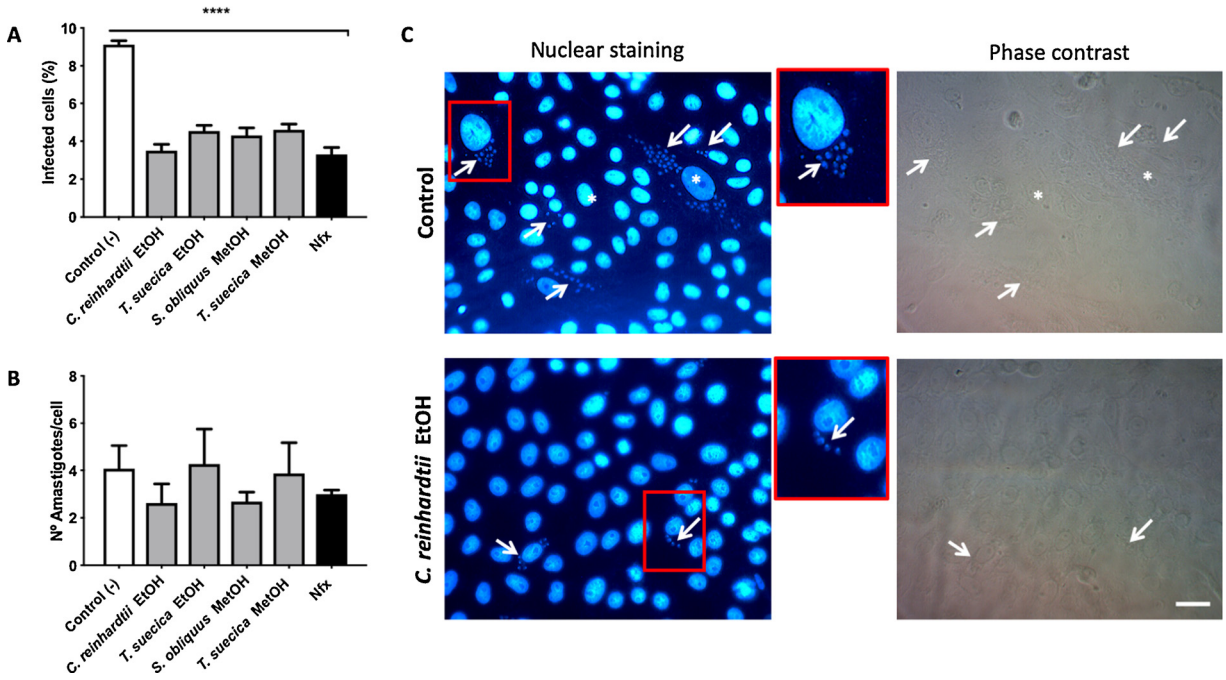


Fig. 2. Effects of the ethanolic extracts of *C. reinhardtii* and *T. suecica* and the methanolic extracts of *T. suecica* and *S. obliquus* on *T. cruzi* amastigotes: Vero cells were exposed to *T. cruzi* trypomastigotes at a 1:1 ratio during 24 h, after which the supernatant was removed and cells were exposed to the different microalgae extracts at their corresponding IC_{50} concentration or Nfx for an additional 24 h. Panel **A** shows the percentage of infected Vero cells and panel **B** shows the number of amastigotes per cell after treatment. Data were analyzed by ANOVA followed by Dunnett's posttest (****$p \leq 0.0001$). Panel **C** shows representative images of infected Vero cells (control) and cells infected but treated with the ethanolic extract from *C. reinhardtii.* Intracellular amastigotes were recognized by their characteristic morphology such as nuclear size and the presence of a kinetoplast (white arrows), Vero cell nuclei are identified by white asterisk. Insets (images with a red border) show an amplified region of infected cell. Cells were then processed for nuclear DAPI staining (panels on the left) and corresponding phase contrast images are shown on the panels on the right. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

mechanisms. For the Nfx-*C. reinhardtii* combination experiments (Fig. 4A), the absolute EC_{50} values (the concentration that decreases the viability of parasites by 50 %) were 14.0 μM for Nfx (panel I) and 69 μg/mL for the ethanolic extract (panel II). The dose-matrix combination (panel III) shows the combination modeling between Nfx and the *C. reinhardtii* extract by using the abovementioned Bliss Independence model. Synergy heatmaps were obtained by comparing the real-data registered by each combination point (panels III and IV), which show a synergistic behavior at low concentrations of Nfx and high concentrations of the *C. reinhardtii* ethanolic extract. In contrast, the combination experiments between Nfx and the ethanolic extract of *T. suecica* showed an antagonistic behavior between both (Fig. 4B). Thus, the EC_{50} for Nfx (I) in this experiment was 10.4 μM and for the *T. suecica* (II) extract, the EC_{50} was 117 μg/mL. The synergy heatmaps (III and IV) show an antagonistic behavior between low concentrations of Nfx and middle to high concentrations of the *T. suecica* extract. The combination experiments between Nfx and each of the methanolic extracts of *T. suecica* and *S. obliquus* did not show either synergic or antagonistic behavior between them as shown in Fig. 4 C and D.

3.3. Phytochemical Screening for potential trypanocidal components in the microalgae extracts

In order to dilucidate some of the potential trypanocidal components in the selected microalgae extracts, we measured the total phenolic content (TPC) expressed as mg gallic acid equivalent (GAE/g) and total flavonoid content (TFC, expressed as mg quercetin equivalent (QE/g) (Table 2). The four microalgae extracts with trypanocidal capacity present phenolic and flavonoid compounds, however the methanolic extract of *T. suecica* showed a significant higher content of TPC (6.74 ± 0.24, $p \leq 0.05$) and TFC (12. 16 ± 0.52, $p \leq 0.05$) compared to the ethanolic one (4.30 ± 0.75 and 8.51 ± 0.94, respectively). *S. obliquus* presented the lowest amount of TPC (3.26 ± 0.22, $p \leq 0.05$) compared to the extracts from *T. suecica* and the ethanolic extract of *C. reinhardtii* (3.55 ± 0.51). The TFC of the *S. obliquus* (11.33 ± 0.77) and *C. reinhardtii* (9.22 ± 0.84) extracts was not significantly different to the observed in the *T. suecica* extracts. In addition, we quantified the contents of chlorophyll *a*, chlorophyll *b* and carotenoids in the different microalgae extracts by spectrophotometric methods (Table 2). These pigments were identified in both extracts from *T. suecica* and in the methanolic extract from *S. obliquus*, in the ethanolic extract from *C. rheinhardtii* only chlorophyll a could be detected. The amount of both chlorophyll a (11.74 ± 0.62) and b (10.34 ± 0.36) pigments was significantly higher ($p \leq 0.05$) in the methanolic extract from *T. suecica* than in the other extracts. On the other hand, the methanolic extract from *S. obliquus* presented the lowest amount of chlorophyll b (1.93 ± 0.11) and carotenoid (1.13 ± 0.02). Additionally, we tested the presence of terpenoids through Salkowski`s test, all of the trypanocidal microalgae extracts were positive for them (Table 2).

In addition, we performed qualitative analysis for polyphenols, lipids and pigments by thin layer chromatography (TLC). Thus, we detected by TLC in the ethanolic and methanolic extract from *T. suecica*, respectively, 6 and 4 spots positive for polyphenols. In the methanolic extract from S. obliquus and the ethanolic extract from *C. rheinhardtii* we detected, respectively 3 and 8 spots positive for polyphenols (Fig. 5).

The TLC analysis for lipids showed 7 positive spots in both ethanolic and methanolic extract from *T. suecica* as well as 1 and 5 in the extracts from *S. obliquus* and *C. rheinhardtii* (Fig. 6). The presence of pigments was also analyzed by TLC, the same positive spots were detected in ethanolic and methanolic extracts from *T. suecica*. Only 2 and 4 positive spots were detected, respectively, in the methanolic extracts from *S. obliquus* and ethanolic extract from *C. rheinhardtii* (Fig. 7).

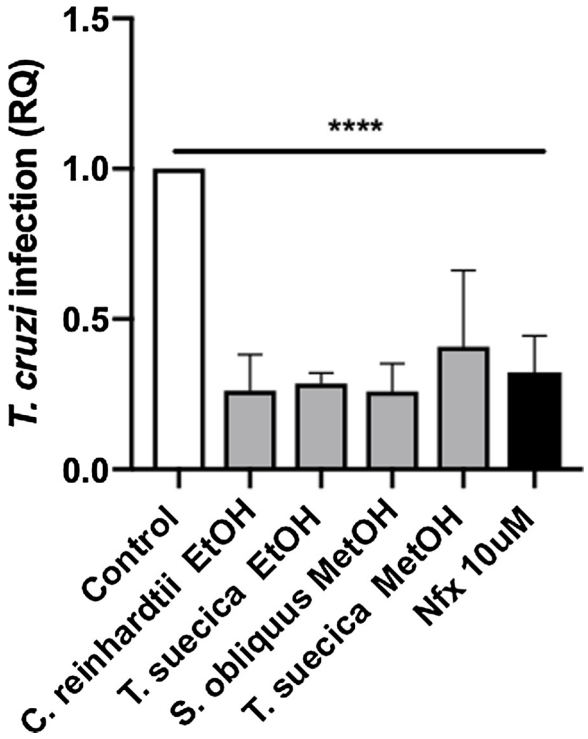


Fig. 3. Effect of the ethanolic extracts of *C. reinhardtii* and *T. suecica* and the methanolic extracts of *T. suecica* and *S. obliquus* on parasite DNA load in Vero cells: Vero cells were exposed to *T. cruzi* trypomastigotes at a 1:1 ratio for 24 h, after which the supernatant was removed and cells were exposed to the different microalgae extracts at the corresponding IC_{50} concentration or Nfx for an additional 24 h. Data are a comparison of parasite DNA in 1 ng of total DNA isolated from infected cells. Real-time quantification by qPCR was performed using the ΔΔCt method. Data represent the means ± SD and were analyzed by ANOVA followed by Dunnett's posttest (****$p \leq 0.0001$).

4. Discussion

According to the World Health Organization (WHO), one of the research priorities for Chagas disease is to develop a treatment that is able to eliminate the parasite from infected individuals in order to decrease the probability of developing the symptoms and to impair parasite transmission [40,41]. However, efficient and safe treatment is far from being available. Both of the currently used drugs, Nfx and Bz, require prolonged treatment (up to 90 days), present low efficacy in the chronic phase of disease, can cause severe adverse effects and are contraindicated during pregnancy due to genotoxic effects [1,40,42]. Moreover, their supply is insufficient [40].

In this context, it is particularly important to search for trypanocidal agents of natural origin. Among the anti-chagasic compounds investigated for treating tropical diseases, the most reported are from plant origins. No antiprotozoal activity has been reported in microalgae, although in these organisms a wide range of molecules of interest to human health and the pharmaceutical industry have been described. The microalgal extracts were found to be more active than total British and Irish red algae extract against *T. cruzi* [15]; enhancing the use of microalgae as emerging trypanocidal agents.

However, there are no preclinical studies of the use of algae extracts for the treatment of Chagas disease. To date, there are several *in vivo* preclinical trials of plant-derived products for the treatment of *T. cruzi*, with very diverse animal models and very variable experimental conditions, which make it difficult to determine their effectiveness [43]. Regarding *in vitro* experiments, it has been reported, that aqueous extracts from the medicinal plants *Enanatia chlorantha* and *Quassia africana*, were active against *T. cruzi* with IC_{50} values of 187 and 188 μg/mL, and selective indexes of 3,0 and 3,3, respectively [44]. These IC_{50} values are lower than those obtained for our extracts, although selectivity indexes are similar; nevertheless, these authors do not explain what parasite form was used. On the other hand, Molina-Garza et al. [45] described growth inhibition (between 88 and 100 %) of the epimastigote form treated for 96 h with 150 μg/mL of ethanolic extracts from the medicinal plants *Eryngium heterophyllum*, *Haematoxylum brasiletto*, *Marrubium vulgare* and *Schinus molle*, with IC_{50} values of 1124; 792; 2266 and 16,31, respectively [45]. In our study, the IC_{50} values were between 609 and 684 for the trypomastigote form treated for 96 h, values that are below the cut-off points for IC_{50} values accepted for natural products (IC_{50} values below 100 μg/mL or 25 μM) [46]

Most recently, Obbo et al. [47] reported the strongest inhibition of *T. cruzi* from the dichloromethane (DCM) extract of the medicinal plant *Baccharoides adoensis* (IC_{50} 2.1 μg/mL), the DCM and the ether extracts of *Khaya anthotheca* (IC_{50} 13.81 μg/mL and 14.51 μg/mL, respectively), the DCM extract of *Momordica foetida* (IC_{50} 17.90 μg/mL), the hexane extract of *K. anthotheca* (IC_{50} 18.57 μg/mL) and the ether extract of *S. pinnata* (IC_{50} 18.20 μg/mL), as compared with the activity of Bz (IC_{50} 0.328 μg/mL) [47].

In this study, extracts with trypanocidal activity were identified from the microalgae *C. reinhardtii*, *S. obliquus* and *T. suecica*, which eliminate approximately 90 % of the tripomastigote infective form of *T. cruzi* (< 200 μg/mL) and were more effective than the drug Nfx (10 μM). These extracts presented high levels of selectivity toward the parasite, particularly, the methanolic and ethanolic extracts of *T. suecica* presented selectivity indexes of 11.2 and 4.8, respectively. Other studies have shown similar selectivity indexes for plant extracts [48,49]

Most drugs that are tested for trypanocidal activity affect only the extracellular blood form of the parasite, being effective in the acute phase of the disease, but not in the chronic phase, so it is necessary to evaluate the drugs in the both trypomastigote and amastigote forms [50]. The ethanolic extract of *C. reinhardtii* and the methanolic extract of *S. obliquus* also demonstrate cytotoxic activity against the amastigote intracellular form, they decreased the parasite DNA load in infected cells by 62.07 % ± 0.33 and 74.1 % ± 0.09, respectively, with reduction levels comparable to those observed with Nfx (63.83 % ± 0.09).

In addition, these extracts reduced the percentage of infected cells, so the ethanolic extracts of *C. reinhardtii* and *T. suecica* decreased from 9.11 % ± 0.21 ($p \leq 0.0001$) to 3.54 % ± 0.34 and 4.54 % ± 0.33, respectively, and the methanolic extracts of *T. suecica* and *S. obliquus* decreased the infection percentage to 4.30 % ± 0.42 and 4.69 % ± 0.31, respectively, of which these results are similar to the decrease induced by Nfx (3.35 % ± 0.37).

Moreover, these extracts also decreased the number of intracellular parasites from an average of 4.38 ± 0.78 amastigotes per cell, to 3.0 ± 0.66 and 2,80 ± 0,36 in cells treated with the ethanolic extract of *C. reinhardtii* and methanolic extract of *S. obliquus*, respectively. This is particularly relevant, considering that the low effects of conventional anti-chagasic drugs is due, at least in part, to the low efficacy against the intracellular forms of the parasite.

On the other hand, therapies based on drug combinations have been proposed as promising alternatives for *T. cruzi* treatment, considering that some chemical or natural compounds that are currently under study have low efficiency, which is why the combined therapy takes on real importance.in order to increase the capacity for action, to decrease the doses used, and to reduce the adverse effects that both drugs generate [50–52].

In this context, four selected extracts (ethanolic from *C. reinhardtii* and *T. suecica*, and methanolic from *S. obliquus* and *T. suecica*) were assayed in combination with the conventional drug Nfx; of these combinations, the ethanolic extract of *C. reinhardtii* (50 μg/mL) showed synergistic action that reduced the IC_{50} of the commercial drug from 14 μM to 5 μM. According to the mathematical models used by the Combenefit software, which analyze the dose-response curves of both, Nfx and extract, indicates that the effect is additive against the parasite

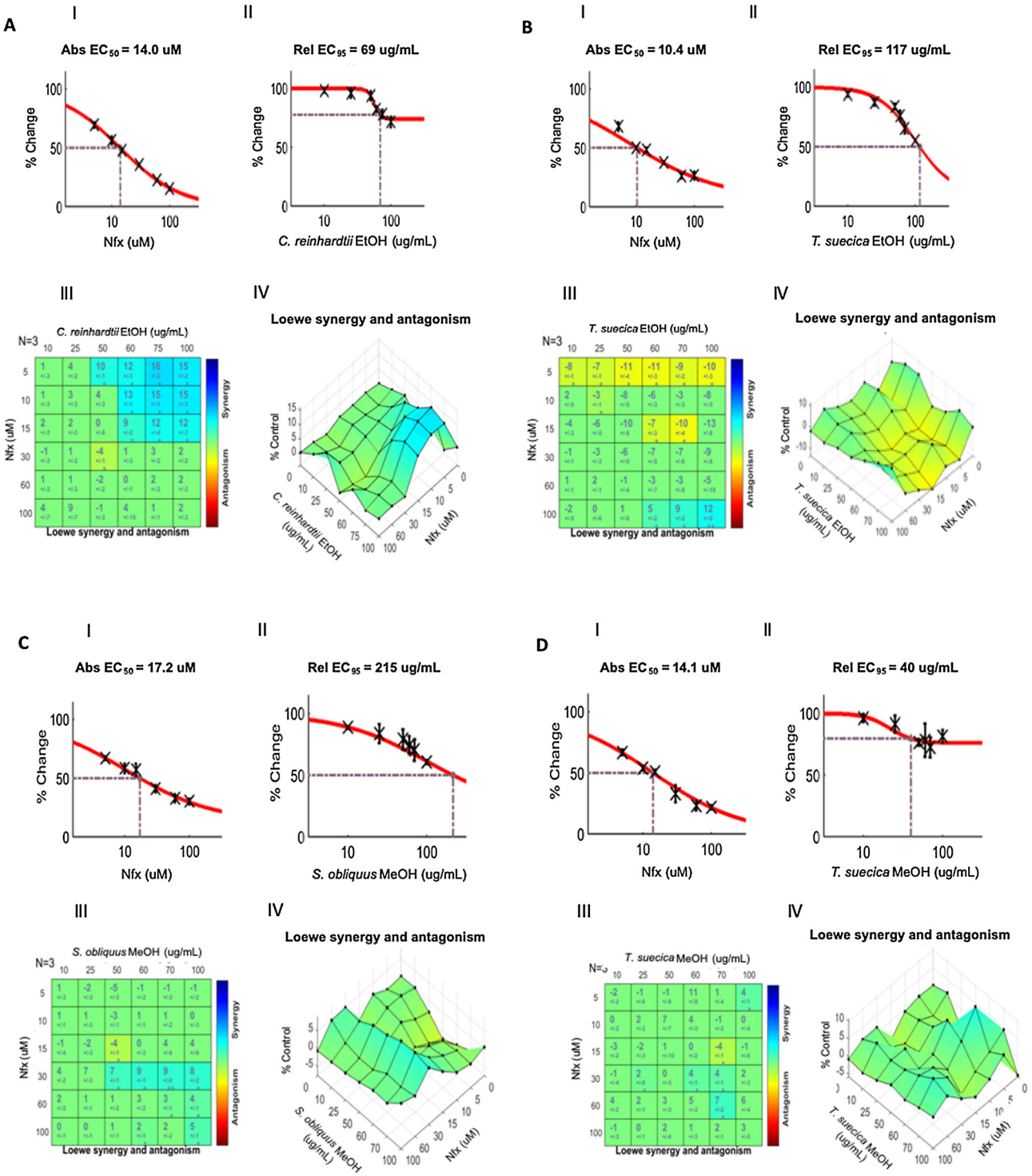


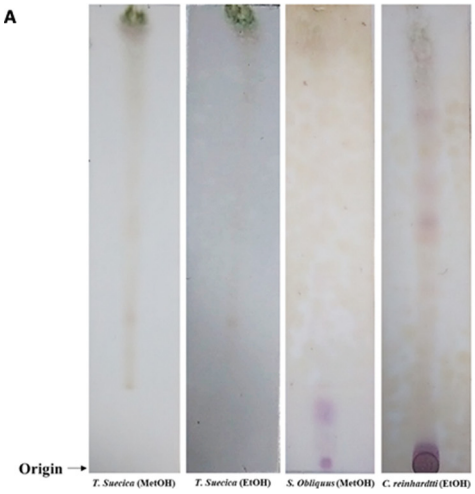
Fig. 4. Effects of the ethanolic extracts of *C. reinhardtii* and *T. suecica,* the methanolic extracts of *T. suecica* and *S. obliquus* and Nfx and their combination on *T. cruzi* trypomastigotes: *T. cruzi* trypomastigotes (10^7 parasites/mL) were incubated in the presence and absence of different concentrations (10, 25, 50, 60, 75 and 100 μg/mL) of ethanolic extracts of *C. reinhardtii* (**A**) and *T. suecica* (**B**) as well as the methanolic extracts of *S. obliquus* (**C**) and *T. suecica* (**D**) or Nfx (5, 10, 15, 30, 60, 100 μM) or their combinations during 24 h. The parasite viability was measured by the MTT method. Panels I and II panels correspond to the dose-response curves for the effect of Nfx or of the different extracts on the cell viability alone, respectively. Panels III shows the real-data obtained when Nfx and the different extracts were combined and was analyzed using the Bliss Independence model. Panel IV shows the difference between the real data and the Bliss model and is expressed as a synergy heatmap. The dose-matrix heatmaps show the synergy score for each combination. Negative values represent an antagonistic combination. The combinations with statistical significance (*$p \leq 0.05$) are colored, and the nonsignificant values are shown in green. The data are expressed as the mean of three independent experiments. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

[31]. Potential drug combination therapies could generate better efficacy, lower development of resistance to microorganisms and lower toxicity, compared to individual drugs [53]. There are studies that have proposed that medicines such as aspirin, dipyridamole or clomipramine showed synergistic activity with trypanocidal drugs, and that compounds such as D,L-buthionine-*S,R*-sulfoximine may decrease the

Table 2
Phenolic compounds, Pigments, terpenoids of each microalgae species for ethanolic and methanolic extracts.

Microalgae	Extract	Phenolic Compounds		Pigments			Terpenoids
		TPC (mg GAE/g)	TFC (mg QE/g)	Chlorophyll*a* (mg/g)	Chlorophyll *b* (mg/g)	Carotenoid (mg/g)	
T. suecica	Ethanolic	4.30 ± 0.75	8.51 ± 0.94	8.47 ± 0.24	5.02 ± 0.46	5.02 ± 0.20	Positive
	Methanolic	6.74 ± 0.24*	12.16 ± 0.52	11.74 ± 0.62*	10.34 ± 0.36*	2.21 ± 0.09*	Positive
S. obliquus	Methanolic	3.26 ± 0.22*	11.33 ± 0.77	6.7 ± 0.06*	1.93 ± 0.11*	1.13 ± 0.02*	Positive
C. reinhardtii	Ethanolic	3.55 ± 0.51	9.22 ± 0.84	8.88 ± 0.05	N.D.	N.D.	Positive

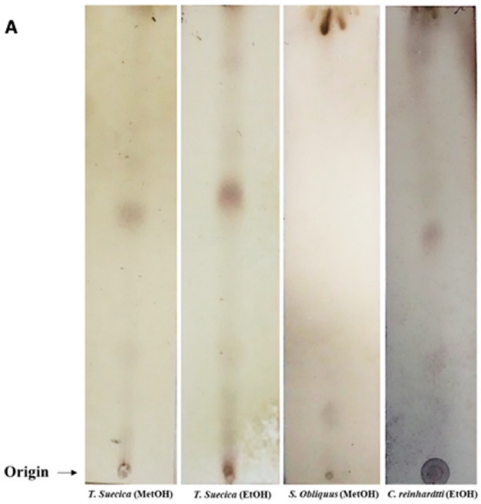
Values marked with asterisk show significant differences for the same solvent ($p < 0.05$).
TPC: Total Phenolic Content; TFC: Total Flavonoid content.
Phenolic compounds, pigments, terpenoids were determined as described in the material and method section.



B

Microalgae	Extract	TLC Polyphenols	
		N° Sp	R_f
T. suecica	Ethanolic	6	0.18/0.31/0.53/0.65/ 0.80/0.95
	Methanolic	4	0.31/0.52/0.68/0.95
S. obliquus	Methanolic	3	0.02/0.12/0.17
C. reinhardtii	Ethanolic	8	0.05/0.5/0.57/0.62/ 0.7/0.750.8/0.85

Fig. 5. TLC analysis of polyphenolic compounds: revealed chromatofolios (A), rate factor of spots positive (B) in methanolic and ethanol extracts of microalgae.



B

Microalgae	Extract	TLC Lipids	
		N° Sp	R_f
T. suecica	Ethanolic	7	0.03/0.1/0.19/0.3 /0.6/0.7/0.88
	Methanolic	7	0.03/0.1/0.19/0.3 0.55/0.7/0.88
S. obliquus	Methanolic	1	0.11
C. reinhardtii	Ethanolic	5	0.26/0.51 0.6/0.8/0.9

Fig. 6. TLC analysis of lipids compounds: revealed chromatofolios (A), rate factor of spots positive (B) in methanolic and ethanol extracts of microalgae.

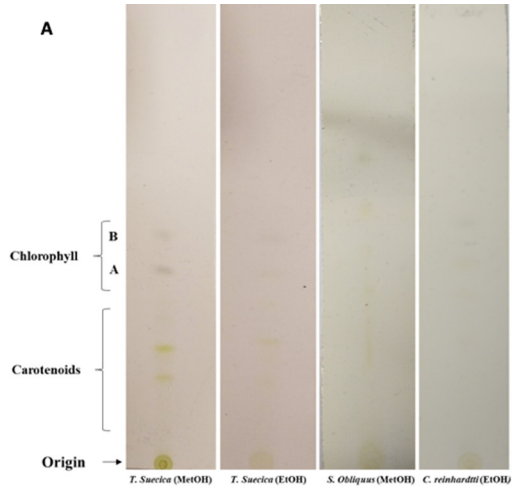
glutathione content, increasing the trypanocidal activity of Nfx or Bz [54–56].

Specific compounds isolated from plants, such as sesquiterpene lactones, have been tested *in vitro* for trypanocidal activity. Example of these include ambrosin, incomptine B and glaucolide, with IC_{50} values lower than Nfx on epimastigote forms [57], and dehydroleucodine and helenalin, which unlike conventional drugs, induce programmed cell death in the epimastigote and trypomastigote forms [2].

In addition, there are few reports about specific compounds isolated from marine microorganism [58]. Thus, lipopeptide viridamide A, obtained from marine cyanobacteria *Oscillatoria nigro-viridis* present activity against *T. cruzi* (IC_{50} 1 μM) [59]. Trypanocidal activity has also been described for the sesquiterpene elatol isolated from the red macroalgae *Laurencia dendroidea*, with IC_{50} values of 45,4, 1,38 and 1,0 against the epimastigote, trypomastigote and amastigote forms, respectively [60].

In order to identify potential trypanocidal compounds present in the microalgae extracts, we performed a phytochemical screening by determining the presence and quantity of phenolic compounds, pigments and terpenoids. Here we studied polyphenols, pigments and terpenes because in previous studies have documented that the microalgae are rich those bioactive compounds [61–63] The different extracts showed

A



B

Microalgae	Extract	TLC Pigments N° Sp	TLC Pigments R_f
T. suecica	Ethanolic	5	0.2/0.25/0.33/0.40/0.49
	Methanolic	5	0.2/0.25/0.33/0.40/0.49
S. obliquus	Methanolic	2	0.44/0.49
C. reinhardtii	Ethanolic	4	0.21/0.32/0.55/0.65

Fig. 7. TLC analysis of pigments compounds: revealed chromatofolios (A), rate factor of spots positive (B) in methanolic and ethanol extracts of microalgae.

the presence of phenolic compounds and particularly of polyphenols (Table 2, Fig. 5). Polyphenols have the capacity to inhibit the growth of different microorganisms, including protozoan ones [64,65]. In addition, it can be seen in the chromatograms (Fig. 5) that the polyphenolic profile of the methanolic and ethanolic extracts of *S. obliquus* and *C. rheinhardtii* are similar; indicating that both species biosynthesize identical polyphenolic compounds and that the polarity of both solvents allows the extraction of these compounds from the plant matrix.

Pigments and its derivatives such as phytol have been studied as antiparasitic compounds [66]; phytol has been described in *Tetraselmis chui* and *S. obliquus* [67]. Terpenoids have also trypanocidal properties, particularly the terpenoids cumanin and cordilin, isolated from *Ambrosia* species, were active against *T. cruzi* epimastigotes (IC_{50} values of 12 μM and 26 μM, respectively), and cumanin and psilostachyin were also active against amastigote forms (IC_{50} values of 8 μM and 21 μM, respectively) [68].

Future studies regarding the effect of the different potential trypanocidal compounds identified in the here studied microalgae extracts, particularly in the ethanolic extract of *C. reinhardtii*, will contribute to the search for new, safer and more effective treatments of this neglected tropical disease.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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