

SCREENING FOR AQUATIC MACROPHYTE SPECIES TO INHIBIT MICROCYSTIS AERUGINOSA: AN APPROACH USING ETHANOLIC EXTRACTS

Seleção de espécies de macrófitas aquáticas inibidoras de *Microcystis aeruginosa*: uma abordagem utilizando extratos etanólicos

Augusto Lima da Silveira^{1,2}, Lucia Regina Rocha Martins¹, Thomaz Aurélio Pagioro¹

¹ Departamento de Química e Biologia, Universidade Tecnológica Federal do Paraná (UTFPR) – Curitiba, PR, Brazil; ² Programa de Pós-Graduação em Ecologia e Conservação, Universidade Federal do Paraná (UFPR) – Curitiba, PR, Brazil.

Silveira, A. L. da*
augusto.ls13@gmail.com
<https://orcid.org/0000-0002-8484-3712>

Martins, L. R. R.
lcmartins.utfpr@gmail.com
<https://orcid.org/0000-0002-9018-7176>

Pagioro, T. A.
thomazap@gmail.com
<https://orcid.org/0000-0002-2169-6989>

* Corresponding author:
(Departamento de Química e Biologia) UTFPR Curitiba, sede Ecoville. R. Dep. Heitor Alencar Furtado, 5000 – Cidade Industrial De Curitiba, Curitiba – PR, 81280-340.

Abstract: Cyanobacterial blooms are a threat to freshwater resources globally, driven by anthropogenic factors like climate change and eutrophication. *Microcystis aeruginosa*, a dominant cyanobacterial species, produces microcystins, hepatotoxins that can affect environmental health. Conventional treatments do not completely remove microcystins, demanding the development of complementary strategies for contamination control. Therefore, allelochemical compounds from aquatic macrophytes may represent a more sustainable approach for controlling *M. aeruginosa* growth and microcystin production in aquatic ecosystems. This study aimed to assess the inhibitory effects of ethanolic extracts from aquatic macrophytes on *M. aeruginosa*. Furthermore, a preliminary investigation was conducted to determine the presence of phytochemical compounds with allelopathic activity in the extracts. Seven aquatic macrophytes species were used to obtain allelochemical extracts: *Eichhornia azurea*, *Eleocharis acutangula*, *Ludwigia peruviana*, *Myriophyllum aquaticum*, *Pontederia cordata*, *Sagittaria montevidensis*, and *Typha domingensis*. In static bioassays *M. aeruginosa* cultures were exposed to 0.1, 50 and 500 mg.Extract.L⁻¹ for 10 days. Inhibition rate (IR), cell density, microcystins production and cell viability were assessed. All the ethanolic extracts presented inhibitory effects in *M. aeruginosa*. The ethanolic extract of *M. aquaticum* exhibited superior performance compared to other extracts. It demonstrated an adequate extraction yield (20.29%), the highest inhibition rate (99.1%), low microcystin production, and exhibited cyanocidal effects. The presence of allelochemical compounds, such as phenolic compounds and saponins, probably contributed to the observed inhibition effects in the bioassays. These results highlight the potential of ethanolic extracts from aquatic macrophytes in controlling *M. aeruginosa*, requiring further toxicological and phytochemical investigations to enhance efficiency and ensure safe use. Furthermore, the findings highlight the potential of aquatic macrophyte extracts as a complementary and sustainable approach for controlling cyanobacterial blooms.

Keywords: Aquatic plants, Bioassay, Cyanobacteria, Eutrophication, Microcystins.

Resumo: As florações de cianobactérias representam uma ameaça aos recursos hídricos em todo o mundo, impulsionadas por fatores antropogênicos, como as mudanças climáticas e a eutrofização. *Microcystis aeruginosa* é uma espécie dominante de cianobactéria que produz microcistinas, hepatotoxinas que afetam a saúde ambiental. Os tratamentos de água convencionais não removem completamente as microcistinas, o que exige o desenvolvimento de métodos complementares para o controle da contaminação. Nesse contexto, o uso de compostos aleloquímicos provenientes de macrófitas aquáticas pode ser uma abordagem mais sustentável para o controle do crescimento de *M. aeruginosa* e da produção de microcistinas nos ecossistemas aquáticos. O presente estudo teve o objetivo de avaliar os efeitos inibitórios de extratos aleloquímicos de macrófitas aquáticas sobre *M. aeruginosa*. Além disso, foi realizada uma investigação fitoquímica preliminar para identificar compostos com atividade alelopática nos extratos. Sete espécies de macrófitas aquáticas foram utilizadas para a obtenção dos extratos etanólicos: *Eichhornia azurea*, *Eleocharis acutangula*, *Ludwigia peruviana*, *Myriophyllum aquaticum*, *Pontederia cordata*, *Sagittaria montevidensis* e *Typha domingensis*. Em bioensaios estáticos, culturas de *M. aeruginosa* foram expostas a 0,1; 50 e 500 mg.Extrato. L⁻¹ por 10 dias. Foram avaliados a taxa de inibição (IR), densidade celular, produção de microcistinas e viabilidade celular. Todos os extratos etanólicos apresentaram efeitos inibitórios sobre a *M. aeruginosa*. O extrato etanólico de *M. aquaticum* demonstrou maior desempenho em comparação aos outros extratos, com rendimento de extração adequado (20,29%), maior taxa de inibição (99,1%), restrição na produção de microcistinas e efeitos cianocidas. A presença de compostos aleloquímicos, como compostos fenólicos e saponinas, provavelmente contribuiu para os efeitos inibitórios observados nos bioensaios. Esses resultados destacam o potencial dos extratos etanólicos de macrófitas aquáticas no controle de *M. aeruginosa*, demandando investigações toxicológicas e fitoquímicas adicionais para aumentar eficiência e garantir o uso seguro. Além disso, indicam o potencial de extratos de macrófitas aquáticas como uma abordagem complementar e sustentável no controle de florações de cianobactérias.

Palavras-chave: Bioensaio, Cianobactérias, Eutrofização, Microcistinas, Plantas aquáticas.

INTRODUCTION

Cyanobacterial blooms represent a significant threat to the quality and availability of freshwater resources globally. Anthropogenic factors, including climate change, intensified eutrophication, and alterations in land use, have contributed to the frequency and duration of these events (Van de Waal et al., 2024). The concern regarding intense cyanobacterial growth arises not only from the production of taste and odor compounds in the water but also from the production of cyanotoxins, which pose risks to human health and the environment (Wang et al., 2024). During blooms, other impacts such as oxygen consumption related to the decomposition can directly affect aquatic life (Harris et al., 2024).

The cyanobacterial species *Microcystis aeruginosa* demonstrates a high capacity for environmental dominance, and its intense growth is frequently recorded in common blooms in water resources (Zhang et al., 2022; Zhou et al., 2021). This species can produce microcystins, which are classified as hepatotoxins due to their mechanism of action (T. Li et al., 2023). The group of microcystins comprises 329 known variants of molecules with the general structure D-Ala¹-X²-D-MeAsp³-Z⁴-Adda⁵-D-Glu⁶-Mdha⁷. Variations in the molecule mainly result from substitutions by different amino acids at positions X and Z. To date, the Microcystin-LR variant containing leucine and arginine is the most common and the one with the highest toxicity (Janssen et al., 2023; Jones et al., 2021).

Microcystins can bioaccumulate throughout the food chain due to properties of the molecule such as low degradability. The presence of this cyanotoxin increases the likelihood of liver cancer development and can lead to the animals and humans death (Chen et al., 2024). Due to the risks involved, the World Health Organization (WHO) sets the safe exposure limit for microcystins at 1 µg.L⁻¹, and in the case of exposure periods up to one week, up to 12 µg.L⁻¹ (WHO, 2021). Therefore, control measures are imperative to mitigate exposure risks, given that concentrations of microcystins reaching up to 12000 µg.L⁻¹ have been documented in tropical lentic water bodies across Latin America during periods of intense blooms (Aguilera et al., 2023).

To maintain water resources within safe limits regarding microcystins, some treatment technologies have been implemented. Conventional treatment technologies involve coagulation and flocculation processes followed by sedimentation and filtration. However, these methods have limitations as they can remove cyanobacterial cells (which internally store most of the microcystins) but demonstrate low efficiency in removing microcystins already released into the water (Barancheshme et al., 2024). Therefore, depending on the concentration of microcystins in the water, it is necessary to adopt methods such as activated carbon filtration, UV radiation, and oxidative processes, which are more effective in removing this cyanotoxin when it is dissolved (Mokoena, 2024).

These water treatment methods still have limitations such as high implementation and maintenance costs, as well as incomplete removal of microcystins. In this context, the development of alternative and more sustainable methods for treating water affected by cyanobacterial blooms acquires fundamental importance (Akyol et al., 2021; Munoz et al., 2021). Thus, the use of aquatic macrophytes in water treatment processes emerges as a Nature-Based Solution (NBS) with potential for controlling cyanobacterial blooms (Martinez i Quer et al., 2024).

Aquatic macrophytes are macroscopic plant species that inhabit aquatic environments either seasonally or permanently. They play a crucial ecological role in regulating productivity processes, contributing to biomass and oxygen production in aquatic ecosystems (Bomfim et al., 2025). This diverse group comprises different life forms, including submerged, floating, and emergent species. The richness and diversity of macrophyte species can serve as indicators of environmental quality due to their preferences for specific conditions such as light availability, humidity, temperature, and pH (Szoszkiewicz et al., 2025).

Aquatic macrophytes can remove organic pollutants through mechanisms, such as adsorption, absorption, and transformation. Consequently, some approaches employ these plant species in phytoremediation processes (Marques et al., 2024). Another approach to phytoremediation involves the allelopathic processes, given their natural occurrence and potential applicability.

In a competitive context, through allelopathic mechanisms, primary producers can produce secondary metabolites to stimulate or inhibit the growth of potential competitors (Wang & Liu, 2023). The application of allelopathic mechanisms is one promising approach for bloom control, as aquatic macrophytes produce compounds with inhibitory effects on cell duplication and cyanotoxin production in cyanobacteria (Wang & Liu, 2023). Aquatic macrophytes produce allelochemicals throughout their life cycle, substances used in competition processes for resources in the environment. These substances play a role in interfering with the growth of other species, potentially competing for light, space, and nutrients. Allelochemicals have effects, especially on primary producers, the main competitors, and act both in inhibiting and stimulating growth, depending on the competitive strategy (Schandry & Becker, 2020). Substances with allelopathic potential include mainly phenolic compounds, fatty acids, alkaloids, terpenoids, flavonoids, etc., produced according to environmental conditions and the species of aquatic macrophytes (Li et al., 2021).

Allelochemicals that can influence the growth of the species *M. aeruginosa* primarily act by inhibiting electron flow and reducing the content of photosynthetic pigments in the cells. However, these effects are often observed under conditions of high allelochemical concentration, which are rarely encountered in the environment due to factors such as the photodegradation of these substances. Consequently, the concentration of allelochemicals under environmental conditions tends to be below that required for bloom control (Luo et al., 2024). Therefore, to enhance inhibitory capacity, some studies employ extracts from aquatic macrophytes to increase the effectiveness of allelochemical action (Maredová et al., 2021).

Allelochemical compounds exhibit lower persistence in the environment compared to conventional algicides and cyanocides due to their high biodegradability. Additionally, these compounds often demonstrate selectivity in their mechanism of action, resulting in low toxicity to non-target species. These characteristics confer environmentally safe and sustainable potential in the application of the method (Tazart et al., 2021).

Given the potential of allelochemical compounds in controlling environmental impacts resulting from cyanobacterial blooms, we conducted a preliminary investigation of aquatic macrophyte species that could be used in water treatment processes. Therefore, the study aimed to assess the inhibitory effects of allelochemicals from aquatic macrophytes on *Microcystis aeruginosa*. Furthermore, a preliminary investigation was conducted to determine the presence of phytochemical compounds with allelopathic activity in the extracts.

The search for sustainable methods to control cyanobacterial blooms can contribute to overcoming limitations of conventional treatments in removing microcystins. Phytoremediation using aquatic macrophytes, particularly through allelopathic mechanisms, has demonstrated potential for mitigating these impacts. However, the effectiveness of these mechanisms under environmental conditions is limited. Therefore, this study aims to evaluate the allelochemicals extracted from different aquatic macrophyte species in controlling *Microcystis aeruginosa* cell proliferation and microcystin production, contributing to reducing the demand for conventional treatment systems. For that, we used three concentrations of ethanolic extracts from seven macrophyte species and analyzed their substances with allelopathic potential on *Microcystis aeruginosa*. We expected that the extracts would exhibit inhibitory effects on cyanobacterial growth and toxin production, and that such effects could be associated with the presence of allelochemical classes identified through preliminary phytochemical screening.

MATERIAL AND METHODS

Culture conditions and acclimation of M. aeruginosa

The experiments were conducted using *Microcystis aeruginosa* from an inoculum of the BB005 strain, which was obtained from the Department of Botany at the Federal University of São Carlos. This strain of cyanobacteria was isolated from the Barra Bonita reservoir in São Paulo, where toxic blooms occur.

Laboratory cultures were established in ASM-1 culture medium (Gorham et al., 1964), and maintained under controlled conditions. A 12-hour photoperiod, light intensity of $34 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ and a temperature of $25 \pm 2^\circ\text{C}$ were chosen based on previous studies that demonstrated optimal cell growth under similar conditions (Jiang et al., 2008; Soares et al., 2004). *M. aeruginosa* strain was acclimated to laboratory conditions for two months. During this period, cultures were subcultured every 10 days at a 10% inoculum rate, enabling the achievement of exponential growth phase with a cellular concentration ranging from 10^6 cells.mL⁻¹.

Aquatic macrophytes collection and ethanolic extracts preparation

Aquatic macrophytes were collected from a lentic water body in the Curitiba Metropolitan Region, located in Parana state, Brazil ($25^\circ 48'S$, $49^\circ 13'$) during the spring season in South America. A total of seven macrophytes species were sampled, namely: *Eichhornia azurea* (Sw.) Kunth (emergent), *Eleocharis acutangula* (Roxb.) Steud. (emergent), *Ludwigia peruviana* (L.) Hara (emergent), *Myriophyllum aquaticum* (Vell.) Verdc. (submerged / emergent), *Pontederia cordata* L. var. *cordata* (emergent), *Sagittaria montevidensis* Cham. & Schltdl (emergent) and *Typha domingensis* Pers. (emergent). Selection of aquatic macrophyte species was based on their abundance during the collection period.

The entire plants samples were collected (aerial plant tissue and roots), rapidly rinsed with tap water to eliminate impurities, and subsequently dried at 33°C for 15 days. After drying, the material was ground into a powder and sieved, at which point the dry weight was determined. The extraction process for each collected species was conducted following the method proposed by (Victório et al., 2010).

Ethanol 80% was employed in a 2:1 ratio (ethanol/dried plant, v/v) for three cycles of 45 minutes in an ultrasonic bath. In each cycle, the mixture was filtered through a Whatman® filter (110 mm) using a vacuum pump system. The liquid extract was collected, and the plant material was re-extracted with an equal volume of alcohol. Following three cycles, the liquid extracts were combined and subjected to evaporation using a rotary evaporator (Fisatom® 801)

until complete ethanol removal. After evaporation, residual moisture was removed through freezing and lyophilization (Liotop® L101). Solid extracts were stored at -20°C until bioassays preparation, to minimize active plant compounds degradation.

The extraction efficiency was calculated based on the mass of dry extract obtained at the end of the process compared to the dry weight of the ground and sieved plant material.

Inhibition and cell viability bioassays

The evaluation of the inhibitory effects of ethanolic extracts from aquatic macrophytes was conducted through 10 days static bioassays. The cultivation conditions during the bioassays remained the same as the acclimation period. Each ethanolic extract obtained was individually tested for inhibitory effects on *M. aeruginosa*.

The bioassays were performed in triplicate using randomly positioned 250 mL Erlenmeyer flasks at the cultivation site. Randomization was implemented to mitigate any potential bias. The aim was to ensure that the positioning of the flasks did not influence the results.

Each bioassay was conducted using 150 mL aliquots from the same culture of *M. aeruginosa* containing 10^6 cells.mL⁻¹, consistent with concentrations observed in bloom events (Toxic Cyanobacteria in Water, 2021). These aliquots were then added to previously sterilized 250 mL Erlenmeyer flasks.

Prior to bioassays conducting, known concentrations of dried ethanolic extract were pre-solubilized in dimethyl sulfoxide (DMSO) to ensure uniform exposure of the cultures across all bioassays (proportion 3:1 considering solvent : extract). The ethanolic extract and DMSO mixture were adjusted to 10 mL in a volumetric flask with ASM-1 culture medium. Thus, it was possible to obtain a stock solution with a known extract concentration. Considering the bioassays were conducted in 150 mL of culture, three experimental conditions were prepared in cultures containing 0.1, 50, and 500 mg.L⁻¹ of ethanolic extract from aquatic macrophytes. Considering that the study aims to search for species with inhibitory potential against *M. aeruginosa*, these concentrations were

chosen to allow evaluation at three screening concentrations in different ranges (referred in this study as low, intermediate, and high concentrations, respectively). The exposure time and concentration parameters were established based on preliminary assays and the evaluation of the *M. aeruginosa* growth curve.

Furthermore, two controls were included for each bioassay: the first containing only the 150 mL aliquot of *M. aeruginosa* culture, and the second containing the culture along with the highest volume of DMSO used in the extract solubilization process. These conditions were necessary to assess the growth conditions of the culture and to verify any potential influences of the solvent on cell duplication.

Samples were collected at 0, 2, 5, 7 and 10 days to evaluate cell density using a Neubauer chamber (depth of 0,100 mm) in an optical microscope (Olympus BX51). The inhibition rate (IR) was determined using Equation 1, as proposed by Cheng et al. (2008):

$$IR = [1 - (C/C_0^{-1})]. 100 \quad (1)$$

Where:

C = cell density after 10 days;

C₀ = cell density in control after 10 days.

Cell viability tests were conducted at the end of bioassays. This step aimed to evaluate whether the extracts presented cyanocidal or cyanostatic effects on cyanobacteria. Cell viability tests provide insights into whether the extracts not only affected the growth of *M. aeruginosa* but also blocked or temporarily inhibited the cellular replication process. Thus, on the tenth day, aliquots were collected from the test flasks with the highest IR and transferred to test tubes containing fresh sterile ASM-1 culture medium. The inoculation rate used was the same as that employed in the inhibition experiments. No extracts were added at this stage and the new cultures were monitored in 10 days by cell counting.

Microcystin-LR analysis

In addition to assessing their effects on cell duplication, the inhibitory effects of ethanolic extracts from aquatic

macrophytes were evaluated regarding the production of Microcystin-LR (MC-LR). For the analysis of microcystin-LR, samples were collected at the end of the inhibition bioassays (10 days). To carry out the MC-LR tests, an analytical blank was prepared using ASM-1 culture medium with DMSO in the same proportion as in the tests.

Analysis of MC-LR was conducted using commercially available semi-quantitative tests (ET 022 EnviroLogix®) with absorbance readings performed on a spectrophotometer (Varian Cary 50) at a wavelength of 450 nm. The readings were carried out using an acrylic cuvette with a 10 mm optical path, two polished faces and a useful volume of 1 ml for the sample. Although a semi-quantitative test was used, the calibration curve exhibited sufficient linearity for sample analysis ($r^2=0.9585$; $p<0.0210$). The detection limit was 0.25 µg.L⁻¹, and the linear equation of the model was $y = 1.178 - 0.331x$, determined from the absorbance of the analytical standards following the processing defined by the semi-quantitative test method.

Preliminary phytochemical analysis of the ethanolic extracts

Phytochemical assays were conducted on the extracts to assess the presence of substances with allelopathic potential in *M. aeruginosa*. This step served as a screening investigation to identify allelochemical compounds, including steroids, triterpenes, saponins, tannins, and flavonoids, as well as to quantify phenolic compounds. For this purpose, 200 mg of extracts were utilized in the phytochemical investigations. To remove moisture, the extracts were placed in a vacuum desiccator for 2 hours.

The qualitative analysis of allelochemicals entailed chemical reactions resulting in observable changes, such as the formation of precipitates, color alterations, and foam formation. These changes served as indicators for the presence or absence of compounds belonging to the classes of steroids, triterpenes, saponins, tannins, and flavonoids. For steroids and triterpenes, the Liebermann-Burchard test was employed; for saponins, the foam formation and persistence test were conducted; for tannins and phenols, a 2% ferric chloride reaction was employed, and for flavonoids,

the approach involved observing pH-induced changes Phani et al. (2013) adapted by Matias et al. (2019). Total phenolic compounds were quantified using a quantitative method and expressed as mg GAE.g⁻¹ (gallic acid equivalent). For each extract, 10 mg was diluted in 10 mL of methanol. Subsequently, 500 µL of Folin-Ciocalteu reagent was added to 100 µL of the diluted sample, followed by the addition of 6 mL of distilled water. The mixture was vortexed for 60 s, then 2 mL of Na₂CO₃ (15% solution) was added and vortexed for 30 s. After 2 hours, the absorbance was measured at a wavelength of 750 nm using a spectrophotometer (Varian, Cary 50) with a quartz cuvette (10 mm optical path). The concentration of total phenolic compounds was determined by reference to a gallic acid calibration curve (Swain & Hillis, 1959 adapted by Marques et al. 2012). The calibration curve exhibited sufficient linearity for phenolic compounds analysis ($r^2=0.9961$; $p<0.0010$) and the linear equation of the model was $y = 1.178 - 0.331x$, determined from the absorbance of the analytical standards.

Statistical Analysis

The changes in cellular density over time for each bioassay were statistically evaluated. The Levene and Kolmogorov-Smirnov tests were employed to assess the homogeneity and normality of variables, respectively. Subsequently, repeated measures two-way ANOVA was applied to normally distributed data, considering cell density, extract concentration, and time as factors. When significant differences were detected, Tukey's test was performed for post hoc comparisons. Statistical analyses and graphical representations were performed using Prism 6 (version 6.0.1) software, with significance set at $p < 0.05$.

RESULTS

The extraction efficiency varied according to the species of aquatic macrophyte used, as observed in Table 1. The species with the highest efficiency in the extraction process was *S. montevidensis* (22.63%), while the one with the lowest yield was *L. peruviana* (7.55%).

The results indicated that the experimental condition of 0.1 mg.Extract.L⁻¹ exhibited the ability to inhibit *M. aeruginosa* cell duplication in three species of aquatic macrophytes (Table 1). At a concentration of 50 mg.Extract.L⁻¹, inhibition occurred from six species, and at 500 mg.Extract.L⁻¹, all tested species influenced cell density. Regarding the inhibition rate (IR), it is evident that the highest effectiveness for the tested species occurs under the conditions of 50 and 500 mg.Extract.L⁻¹, with rates exceeding 60%. The species *M. aquaticum* exhibited the highest IR compared to others at both higher extract concentrations.

Table 1
Extraction efficiency for the ethanolic extracts and inhibition rate (IR) of *Microcystis aeruginosa*, after 10 days, according to the experimental condition

Macrophyte species	Extraction process		IR (%)		
	Dry weight (g)	Efficiency (%)	Ethanolic extract concentration		
			0.1 mg.L ⁻¹	50 mg.L ⁻¹	500 mg.L ⁻¹
<i>Eichhornia azurea</i>	40	14.50	25.4 ± 6.0	27.6 ± 2.8	88.7 ± 1.6
<i>Eleocharis acutangula</i>	25	12.60	n.o.	19.6 ± 8.6	96.0 ± 0.3
<i>Ludwigia peruviana</i>	40	7.55	17.2 ± 4.6	19.6 ± 9.0	98.1 ± 0.1
<i>Myriophyllum aquaticum</i>	50	20.29	n.o.	60.1 ± 1.2	99.1 ± 0.3
<i>Pontederia cordata</i>	20	12.40	12.5 ± 1.2	12.0 ± 3.9	89.8 ± 0.9
<i>Sagittaria montevidensis</i>	30	22.63	n.o.	n.o.	22.5 ± 5.3
<i>Typha domingensis</i>	30	10.90	n.o.	23.0 ± 3.2	79.6 ± 1.5

IR values are presented as mean ± standard deviation. Non-observed inhibitions are indicated as n.o.

The presence of solvent (DMSO) did not interfere with cell duplication, as no differences in *M. aeruginosa* cell density were observed between the two controls of the bioassays ($P>0.7390$). Thus, the results will be presented with reference to the control without DMSO.

The distribution of variables met the normality assumptions, enabling the application of two-way repeated measures ANOVA. The ethanolic extracts from aquatic macrophytes use affected cellular density in the bioassays (Table S1). Figure 1 graphically illustrates the differences observed between the control condition and the treatments, as well as compares cellular densities within the same

experimental condition for the extracts from different species tested.

The concentration of 500 mg.Extract.L⁻¹ was most effective in reducing the cell duplication of *M. aeruginosa* among the tested conditions. The influence of this condition occurred as early as the second day of exposure, except for the bioassay with the extract of *T. domingensis* ($F_{16,40} = 34.6$, $P = 0.1705$) where effects were only observed on the fifth day. In the 50 mg.Extract.L⁻¹ condition, effects on cell density are predominantly observed after the fifth day of exposure, except for the extracts of *M. aquaticum* ($F_{16,40} = 423.4$, $P = 0.0001$) and *S. montevidensis* ($F_{16,40} = 9.9$, $P = 0.0080$) which showed reduced cell density as early as the second day of exposure. Even at the lowest extract concentration, 0.1 mg.Extract.L⁻¹, effects on cell density were observed. For the extracts obtained from *M. aquaticum* ($F_{16,40} = 423.4$, $P < 0.0001$) and *S. montevidensis* ($F_{16,40} = 9.9$, $P = 0.0098$), cell density was affected on the fifth day, a shorter time compared to the other extracts.

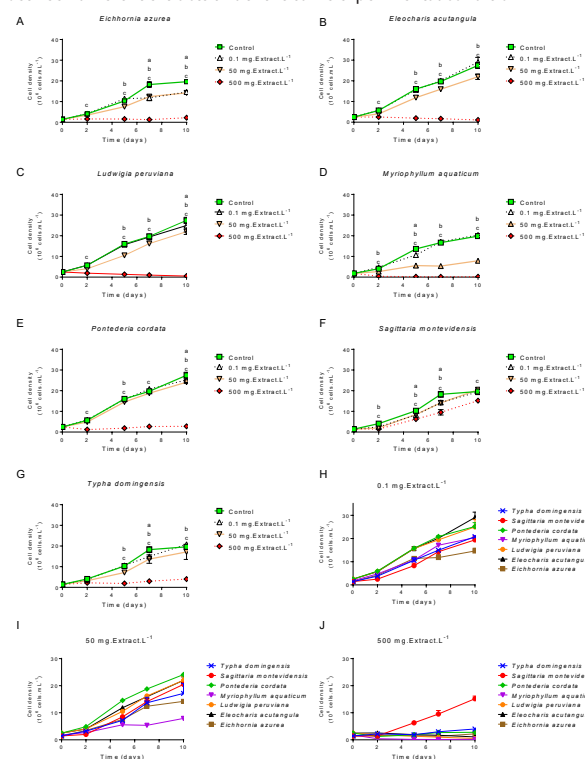
Since the concentration of 500 mg.Extract.L⁻¹ showed the highest IR in *M. aeruginosa* for all species, this experimental condition was used to assess post-exposure cell viability. After ten days of exposure to the extracts, some cultures regrowth (Table 2). Thus, treatments using extracts from *E. azurea*, *P. cordata*, *S. montevidensis*, and *T. domingensis* increased cell density at the end of the viability test (10 days). Conversely, treatments using extracts from *E. acutangula*, *L. peruviana*, and *M. aquaticum* did not show viable cells at the end of the viability test.

The concentration of MC-LR in the treatments were lower than that observed for the control condition and were above the detection limit for the treatments with extracts from the species *E. acutangula* and *P. cordata* (Table 2).

The preliminary phytochemical analysis revealed the diversity of composition among the ethanolic extracts of aquatic macrophytes. The presence of compounds with allelopathic potential was observed, as shown in Table 3. Regarding phenolic compounds, the ethanolic extract of the species *M. aquaticum* exhibited the highest concentration, while the extract of the species *S. montevidensis* showed the lowest level of phenolic compounds.

Figure 1

Cell density of *Microcystis aeruginosa* throughout the exposure bioassays to ethanolic extracts of aquatic macrophytes and the comparison of cell density between different extracts under the same experimental condition



Note: (A) *Eichhornia azurea*; (B) *Eleocharis acutangula*; (C) *Ludwigia peruviana*; (D) *Myriophyllum aquaticum*; (E) *Pontederia cordata*; (F) *Sagittaria montevidensis*; (G) *Typha domingensis*; (H) Comparison between 0.1 mg.Extract.L⁻¹ treatment; (I) Comparison between 50 mg.Extract.L⁻¹ treatment; (J) Comparison between 500 mg.Extract.L⁻¹ treatment. Values represented as mean $\pm$ standard deviation (vertical bars). Differences between treatment and control condition are indicated by different lowercase letters: (a) difference between control condition and treatment of 0.1 mg.Extract.L⁻¹, (b) difference between control condition and treatment of 50 mg.Extract.L⁻¹, and (c) difference between control condition and treatment of 500 mg.Extract.L⁻¹ ($p < 0.05$). Statistical analysis was performed using repeated measures two-way ANOVA and Tukey's test ($N=3$).

Table 2

Microcystin-LR (MC-LR) production and post-exposure cell density to the condition of 500 mg.Extract.L⁻¹ (10 days), and cell density after viability testing in sterile ASM-1 medium without further addition of extract (10 days)

Treatment	MC-LR ($\mu\text{g.L}^{-1}$)	Cell density after extract exposure ($10^6 \text{ cells.mL}^{-1}$)	Cell density after viability test ($10^6 \text{ cells.mL}^{-1}$)
Control	1.184	2.18 ± 0.03	10.20 ± 0.91
<i>Eichhornia azurea</i>	n.d.	0.281 ± 0.024	5.41 ± 0.34
<i>Eleocharis acutangula</i>	0.610	0.0235 ± 0.0037	n.d.
<i>Ludwigia peruviana</i>	n.d.	0.0693 ± 0.0033	n.d.
<i>Myriophyllum aquaticum</i>	n.d.	0.0171 ± 0.0037	n.d.
<i>Pontederia cordata</i>	0.267	0.317 ± 0.054	8.35 ± 0.38
<i>Sagittaria montevidensis</i>	n.d.	2.02 ± 0.04	13.61 ± 0.33
<i>Typha domingensis</i>	n.d.	1.21 ± 0.05	11.63 ± 0.11

Note: Values are presented as mean $\pm$ standard deviation. Non-detected concentrations are indicated as n.d.

Table 3
Qualitative and quantitative phytochemical analysis of the aquatic macrophyte ethanolic extracts

Ethanolic extracts	Steroids and Triterpenes		S	P	HPT	CT	FFX	Total phenolics (mg.L ⁻¹)
	FS	FPT						
Eichhornia azurea	+					+	+	93.3 ± 8.1
Eleocharis acutangula	+		+			+	+	209.3 ± 12.3
Ludwigia peruviana	+		+		+		+	311.1 ± 17.1
Myriophyllum aquaticum	+		+		+		+	405.2 ± 15.2
Pontederia cordata	+					+	+	90.4 ± 11.2
Sagittaria montevidensis		+				+	+	77.0 ± 7.9
Typha domingensis	+			+			+	126.7 ± 11.3

Note: FS: Free steroids; FPT: Free pentacyclic triterpenoids; S: Saponins; P: Phenols; HPT: Hydrolysable pyrogallallic tannins; CT: Catechetic tannins; FFX: Flavones, flavonols, and xanthenes. (+) indicates phytochemical compound presence. Total phenolics presented as mean ± standard deviation.

DISCUSSION

The hypotheses proposed in this study were confirmed. The extracts inhibited *Microcystis aeruginosa* growth, with variations in effectiveness among macrophyte species. Additionally, some extracts suppressed microcystin production and exhibited cyanocidal effects, supporting the influence of phytochemical classes on the observed outcomes. All extracts in the present study inhibited cell duplication at the highest concentration (500 mg.Extract.L⁻¹). Notably, *M. aquaticum*, *L. peruviana*, and *E. acutangula* extracts exhibited the highest inhibition rates at this concentration and influenced cell duplication at the intermediate concentration (50 mg Extract.L⁻¹). Additionally, extracts from these three species demonstrated a cyanocidal effect, as *M. aeruginosa* cells did not restarted duplication even after extract exposure stopped and nutrient supply was restored. Furthermore, *M. aquaticum* and *L. peruviana* extracts not only exhibited the highest inhibition rates but also suppressed microcystin production in cultures.

Most studies on cyanobacteria inhibition through allelopathic mechanisms evaluate the aquatic macrophyte *M. aquaticum*. This species has demonstrated potential for cyanobacteria control due to the allelochemicals it produces.

Wang et al. (2017) assessed the ability of the species *M. aquaticum* to affect the cyanobacterium *M. aeruginosa*. Growth was strongly inhibited and was associated with increased lipoperoxidation in *M. aeruginosa* membrane, leading to cell lysis. Cheng et al. (2008) observed that water used in the cultivation of *M. aquaticum* negatively affected the photosynthetic capacity in *M. aeruginosa* by reducing the concentration of chlorophyll-a, phycocyanin, and allophycocyanin. Regarding the production of allelochemicals by *M. aquaticum*, Kitamura et al. (2023) noted that the inhibition of *M. aeruginosa* occurred more intensely for extracts obtained during the autumn. In another approach using co-culture experiments with *M. aquaticum*, the inhibition of *M. aeruginosa* was 100% in cell duplication, > 98% in the concentration of photosynthetic pigments, and 79% in the production of microcystins (Kitamura et al., 2022). In this way, the inhibition observed in present study suggests that the allelochemicals produced by *M. aquaticum* were present in the extract at concentrations sufficient to interfere with the metabolic processes of *M. aeruginosa*.

Our results demonstrate that *M. aquaticum* extract has the potential to mitigate environmental impacts caused by cyanobacterial blooms, due to the inhibitory effects observed. The extract obtained from the *M. aquaticum* species showed the best results in application. This extract had the second highest extraction yield (20.29%), the highest inhibition rate (between 60.1 and 99.1%) and restricted the production of MC-LR. Additionally, a cyanocidal effect was observed, as even after interrupting exposure to the extract, *M. aeruginosa* was unable to increase cell density even with a new nutrient supply.

Conversely, considering the evaluated parameters, the extract from the species *S. montevidensis* exhibited the lowest inhibition rate (22.5%). This result becomes evident when assessing that the cell density, although lower compared to the control condition, increased throughout the exposure period at all three tested extract concentrations. Despite slightly inhibiting cell duplication, after interrupting exposure to the extract and providing a new nutrient source, cell density increased. These results indicate an inhibition low effectiveness of the allelochemicals extracted from the aquatic macrophyte.

The aggregation state of *M. aeruginosa* cells may have influenced the low inhibition observed for the extract from the species *S. montevidensis*. The strain BB005 of the present study tended to form colonies under the culture conditions. Zhao et al. (2013) evaluated the aquatic macrophyte belonging to the same genus, *Sagittaria sagittifolia*, in co-culture conditions regarding inhibitory effects. In the study, *S. sagittifolia* inhibited 96.5% of the growth of *M. aeruginosa* cultures within seven days when the bioassay was conducted for cultures with unicellular growth. For *M. aeruginosa* cultures with colonial growth, inhibition was 40%, achieved only after 15 days of exposure.

When comparing species of aquatic macrophytes regarding their inhibitory potential on *M. aeruginosa*, El-Sheekh et al. (2017) found the potential of the ethanolic extract of *Eichhornia crassipes*. At a concentration of 80 mg.L⁻¹, the extract of this species showed inhibitory activity at least five times higher than the extracts of *Ceratophyllum demersum*, *C. submersum*, *Polygonum tomentosum*, and *Saccharum spontaneum*. Thus, the results obtained in the present study support the potential use of species of the genus *Eichhornia* sp. for the inhibition of *M. aeruginosa*.

M. aeruginosa was also inhibited using an extract of *Ludwigia adscendens* at a concentration of 10 mg.L⁻¹. In this case, the effectiveness of the extract in inhibition decreased as the concentration of nutrients available to the cyanobacterium increased. Therefore, the application of extracts containing allelochemicals in natural environments should also be evaluated for conditions where nutrient restriction is not feasible (Sim et al., 2024). In the present study, the extract of *L. peruviana* exhibited the second highest inhibition rate at 500 mg.Extract.L⁻¹, restricted the production of MC-LR, and showed a cyanocidal effect. These results provide perspective on the use of aquatic macrophyte species of the *Ludwigia* sp. genus in controlling *M. aeruginosa* blooms.

The species *P. cordata* in co-culture conditions has already been shown to be effective in controlling *M. aeruginosa*, with a 95.5% inhibition within seven days of exposure (Zhao et al., 2013). Additionally, Y. Li et al. (2023) observed that in the presence of *P. cordata*, the levels of chlorophyll-a and the activity of the enzyme superoxide dismutase (SOD) decreased in *M. aeruginosa*. Under these conditions, inhibition results mainly from deficits in photosynthetic activity and the

fragility of the antioxidant system, as reduced SOD activity makes the cells more susceptible to oxidative damage. The results obtained in the present study reinforce this inhibitory capacity of *P. cordata*. The ethanolic extract of this species at a concentration of 500 mg.L⁻¹ resulted in an inhibition rate of 89.8%, as well as leading to lower synthesis of MC-LR compared to the control condition.

The allelopathic activity of the species *T. domingensis* has previously been evaluated concerning the cyanobacterium *Lyngbya majuscula*. The allelochemical compounds produced were responsible for altering oxygen production, affecting cellular metabolism (Prindle & Martin, 1996). Thus, the results obtained in the present study bring perspectives for the use of this aquatic macrophyte in control actions for the species *M. aeruginosa* as well, due to the observed inhibitory effects.

Few studies have linked the genus *Eleocharis* sp. to allelopathic activity against *M. aeruginosa* and cyanobacteria in general. Nakai et al. (1999) found that the species *Eleocharis acicularis* exhibited slight inhibitory activity against *M. aeruginosa* in co-culture conditions. In contrast, the present study observed high inhibitory activity with the extract of *E. acutangula* concerning cell density and cyanocidal effect. However, the production of MC-LR increased over the exposure period.

Analysis of cyanotoxin production reveals that the extract of *E. acutangula*, while reducing cell concentration, resulted in higher microcystin-LR production compared to other extracts. This phenomenon occurs when *M. aeruginosa* cultures are exposed to pyrogallol and hydroquinone polyphenols, phytochemicals produced by plants. Interestingly, parameters such as cell concentration and MC-LR do not exhibit a significant statistical correlation. The photosynthetic apparatus of the cells is damaged, resulting in inhibition by mechanisms not yet fully understood, and the surviving cells produce higher levels of cyanotoxin (Dziga et al., 2007, 2009).

Lower MC-LR production observed when cultures were exposed to extracts of *E. azurea*, *L. peruviana*, *M. aquaticum*, *P. cordata*, and *T. domingensis* can be attributed to the presence of phenolic compounds. These compounds may form complexes with certain cellular proteins, thereby limiting enzymatic action (Quideau, 2013). This mechanism likely

modifies MC-LR production by the cells, as biosynthesis occurs through specific genes responsible for coding the microcystin synthetase enzyme (Bittencourt-Oliveira et al., 2011).

Cellular viability following exposure to the extracts is related to factors such as degradability and the physicochemical characteristics of the allelochemicals. When cell cultures resume cell duplication, it indicates that favorable conditions have returned, either due to the depletion of allelochemicals or even degradation. Another aspect to consider is that more hydrophilic compounds have a greater capacity to generate cyanocidal effects (Gil et al., 2021). Therefore, the occurrence of cyanostatic effects for the majority of the tested extracts can be explained by the fact that some allelochemicals have low persistence, which allowed the regrowth of the cultures (Sim et al., 2024).

The presence of allelochemicals from aquatic macrophytes can interfere with the photosynthetic capacity, nutrient uptake, and other physiological processes in algae and cyanobacteria. Allelochemicals reported to have these effects include tannins, flavonoids, phenolic acids, alkaloids, terpenoids, and some volatile organic compounds (Wimalasena et al., 2023). In the present study, the observed cyanocidal effects upon exposure to extracts of *E. acutangula*, *L. peruviana*, and *M. aquaticum* may result from the presence of these compounds. These three extracts exhibited the highest concentrations of phenolic compounds and the presence of tannins, as evidenced by preliminary phytochemical analysis. Additionally, these extracts were the only ones that tested positive for saponins. Saponins can affect the cell membranes of *M. aeruginosa* by disrupting the antioxidant system and increasing susceptibility to lipid peroxidation, causing damage that hinders cell duplication (Luo et al., 2013). Therefore, the cyanostatic effects observed upon exposure to extracts of *E. azurea*, *P. cordata*, *S. montevidensis*, and *T. domingensis* reflect the diversity in the allelochemicals spectrum of action, as well as the variability of effects among different species of aquatic macrophytes.

CONCLUSION

Our results demonstrate prospects in using ethanolic extracts of aquatic macrophytes in *M. aeruginosa* toxic

blooms remediation, as a Nature-Based Solution (NBS) for water treatment. We highlight that all tested extracts showed inhibitory effects, and in some cases, even at a concentration of 0.1 mg.Extract.L⁻¹. Overall, the highest concentration exhibited the greatest effectiveness, as it resulted in the highest inhibition rates. Regarding exposure time, the effects were predominantly observed on the fifth day. Under the tested conditions, the extract of *M. aquaticum* showed the best performance, exhibiting the highest inhibition of cell duplication, reduced MC-LR production, and cyanocidal effects. In the same perspective, the extract of *L. peruviana* stands out for its efficiency in inhibitory effects on *M. aeruginosa*. Preliminary phytochemical investigation demonstrated the diversity of allelochemical compounds produced by aquatic macrophytes. Thus, future approaches require detailed toxicological and phytochemical studies to better identify compounds with activity against cyanobacteria and, at the same time, predict possible deleterious effects on non-target species. The results are also important in highlighting the potential of ethanol extraction, contributing to a more sustainable and economically viable application, considering the low toxicity of this solvent. Future studies should explore strategies to improve the efficiency of extract production and application. Additionally, ecotoxicological assessments are essential to ensure the safety of its use. Finally, the data obtained contribute to increasing knowledge about potential species, as four of the seven tested aquatic macrophyte species have no records in the literature regarding inhibitory effects on cyanobacteria.

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SUPPLEMENTARY MATERIAL 1 – S1

Results from the comparison between control conditions and treatments using two-way repeated measures ANOVA and Tukey's test in the Eichhornia azurea ethanolic extract bioassay

ANOVA table	DF	Sum of squares	Mean square	F	P
Model	16	536.5	33.5	87.85	< 0.0001
Error	40	15.3	0.4		
Total	56	551.8			
P values for the comparison between treatment and control conditions					
Treatment	Time (days)				
	0	2	5	7	10
0.1 mg.Extract.L ⁻¹	-	0.9996	0.2436	<0.0001*	<0.0001*
50 mg.Extract.L ⁻¹	-	0.7487	<0.0001*	<0.0001*	<0.0001*
500 mg.Extract.L ⁻¹	-	0.0002*	<0.0001*	<0.0001*	<0.0001*

(*) indicate P < 0.05

Results from the comparison between control conditions and treatments using two-way repeated measures ANOVA and Tukey's test in the Eleocharis acutangula ethanolic extract bioassay

ANOVA table	DF	Sum of squares	Mean square	F	P
Model	16	1088	67.98	158.8	< 0.0001
Error	40	17.1	0.4		
Total	56	1105.1			
P values for the comparison between treatment and control conditions					
Treatment	Time (days)				
	0	2	5	7	10
0.1 mg.Extract.L ⁻¹	-	>0.9999	0.9505	0.9585	0.0722
50 mg.Extract.L ⁻¹	-	0.0563	<0.0001*	<0.0001*	<0.0001*
500 mg.Extract.L ⁻¹	-	<0.0001*	<0.0001*	<0.0001*	<0.0001*

(*) indicate P < 0.05

Results from the comparison between control conditions and treatments using two-way repeated measures ANOVA and Tukey's test in the Ludwigia peruviana ethanolic extract bioassay

ANOVA table	DF	Sum of squares	Mean square	F	P
Model	16	1047	65.4	116.9	< 0.0001
Error	40	22.4	0.6		
Total	56	1069.4			
P values for the comparison between treatment and control conditions					
Treatment	Time (days)				
	0	2	5	7	10
0.1 mg.Extract.L ⁻¹	-	>0.9999	0.9874	0.9932	0.0091*
50 mg.Extract.L ⁻¹	-	0.1573	<0.0001*	<0.0001*	<0.0001*
500 mg.Extract.L ⁻¹	-	<0.0001*	<0.0001*	<0.0001*	<0.0001*

(*) indicate P < 0.05

Results from the comparison between control conditions and treatments using two-way repeated measures ANOVA and Tukey's test in the Miryophyllum aquaticum ethanolic extract bioassay

ANOVA table	DF	Sum of squares	Mean square	F	P
Model	16	785.3	49.1	423.4	< 0.0001
Error	40	4.6	0.1		
Total	56	789.9			
P values for the comparison between treatment and control conditions					
Treatment	Time (days)				
	0	2	5	7	10
0.1 mg.Extract.L ⁻¹	-	0.1337	<0.0001*	0.5459	0.3665
50 mg.Extract.L ⁻¹	-	0.0001*	<0.0001*	<0.0001*	<0.0001*
500 mg.Extract.L ⁻¹	-	<0.0001*	<0.0001*	<0.0001*	<0.0001*

(*) indicate P < 0.05

Results from the comparison between control conditions and treatments using two-way repeated measures ANOVA and Tukey's test in the *Pontederia chordata* ethanolic extract bioassay

ANOVA table	DF	Sum of squares	Mean square	F	P
Model	16	866.8	54.18	214.6	< 0.0001
Error	40	10.1	0.3		
Total	56	876.9			
P values for the comparison between treatment and control conditions					
Treatment	Time (days)				
	0	2	5	7	10
0.1 mg.Extract.L ⁻¹	-	0.9852	0.9875	0.1366	0.0002*
50 mg.Extract.L ⁻¹	-	0.4112	0.0235*	0.3131	<0.0001*
500 mg.Extract.L ⁻¹	-	<0.0001*	<0.0001*	<0.0001*	<0.0001*

(*) indicate P < 0.05

Results from the comparison between control conditions and treatments using two-way repeated measures ANOVA and Tukey's test in the *Sagittaria montevidensis* ethanolic extract bioassay

ANOVA table	DF	Sum of squares	Mean square	F	P
Model	16	90.9	5.7	9.9	< 0.0001
Error	40	22.9	0.6		
Total	56	113.80			
P values for the comparison between treatment and control conditions					
Treatment	Time (days)				
	0	2	5	7	10
0.1 mg.Extract.L ⁻¹	-	0.0733	0.0098*	<0.0001*	0.9975
50 mg.Extract.L ⁻¹	-	0.0080*	0.0289*	<0.0001*	0.5809
500 mg.Extract.L ⁻¹	-	0.0005*	<0.0001*	<0.0001*	<0.0001*

(*) indicate P < 0.05

Results from the comparison between control conditions and treatments using two-way repeated measures ANOVA and Tukey's test in the *Typha domingensis* ethanolic extract bioassay

ANOVA table	DF	Sum of squares	Mean square	F	P
Model	16	480.5	30.0	34.6	< 0.0001
Error	40	34.7	0.9		
Total	56	515.2			
P values for the comparison between treatment and control conditions					
Treatment	Time (days)				
	0	2	5	7	10
0.1 mg.Extract.L ⁻¹	-	0.9959	0.9973	0.0022*	0.6161
50 mg.Extract.L ⁻¹	-	0.8347	0.0042*	<0.0001*	0.0386*
500 mg.Extract.L ⁻¹	-	0.1705	<0.0001*	<0.0001*	<0.0001*

(*) indicate P < 0.05