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Egeria densa organic extracts: an eco-friendly approach to suppress *Microcystis aeruginosa* growth through allelopathy

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ABSTRACT

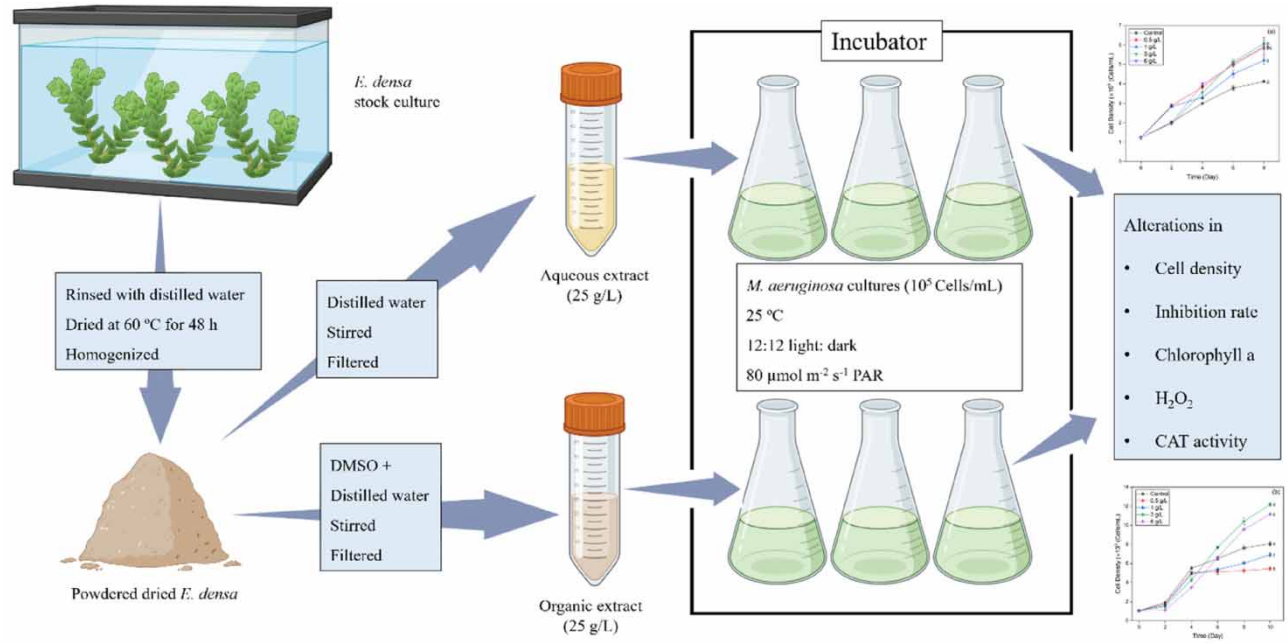
Macrophytes play a significant role in shaping plankton communities by shading, controlling water turbulence, and nutrient availability, while also producing allelochemicals causing varying effects on different organisms. Many researchers have shown that when live macrophytes are present, they inhibit cyanobacteria. However, their widespread use is often limited due to numerous concerns, including invasive characteristics. This study focused on the applicability of *Egeria densa* extracts to suppress *Microcystis aeruginosa*. We employed pure water and dimethyl sulfoxide, to obtain compounds from *E. densa*. The results revealed that *E. densa* aqueous extracts stimulated *M. aeruginosa* growth, whereas organic extracts exhibited suppression. Specifically, at low concentrations of organics extracts (0.5 and 1 g/L), after day 4, the growth inhibition was confirmed by significantly higher ($p < 0.05$) stress levels shown in cells treated with low concentrations. The highest inhibition rate of 32% was observed at 0.5 g/L. However, high concentrations of organic extracts (3 and 6 g/L), showed increased growth compared with control. These results suggest that high concentrations of organic extracts from *E. densa* potentially suppress allelochemical-induced *M. aeruginosa* inhibition due to high nutrient availability. In comparison with an aqueous solvent, the use of organic solvent seems to be more effective in efficiently extracting allelochemicals from *E. densa*.

Key words: allelopathy, cyanobacteria, growth inhibition, macrophytes, plant extracts

HIGHLIGHTS

- Organic solvent extracts of *E. densa* allelochemicals are more effective than aqueous solvent extracts.
- Low-concentration of *E. densa* organic extracts inhibit *M. aeruginosa* growth more effectively than high concentrations and aqueous extracts.
- The amount of nutrients in extracts is a determining factor for the effectiveness of allelochemicals.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The growth of harmful cyanobacteria in freshwater poses numerous problems to the ecosystem, such as hypoxia, aquatic fauna mortality, and the release of toxins into the water, including microcystins, cylindrospermopsin, anatoxins, and saxitoxins (Dolman *et al.* 2012; Paerl & Otten 2013). There are three potential bloom-forming toxin-releasing cyanobacteria genera: *Microcystis*, *Oscillatoria*, and *Anabaena*. These species are more hazardous due to their ability to produce secondary metabolites and endotoxins. *Microcystis* spp., the most common species, known for its ability to form harmful blooms in freshwater bodies worldwide, has been extensively studied by researchers more than other cyanobacteria species (Dolman *et al.* 2012; Preece *et al.* 2017). The hepatotoxic microcystins produced by *Microcystis* spp. are the most common cyanotoxin and are known to cause serious health and environmental problems not only in aquatic organisms but also in human health as well (Preece *et al.* 2017).

Over the years, studies extensively aimed at mitigating cyanobacterial growth and harmful cyanobacteria blooms (HABs); among the various *in situ* control methods, leveraging the natural allelopathic abilities of plants, including macrophytes, to inhibit cyanobacterial growth has proven to be an effective method compared to many other conventional *in situ* control approaches. Conventional methods such as algaecide application, coagulant use, ultrasonic technology, aeration towers, and sediment dredging are practiced as cyanobacteria suppression methods (Paerl & Otten 2013; Sample & Fox 2013). However, all have costly initial application or maintenance and cause negative effects on ecosystem members (Paerl & Otten 2013; Chorus & Welker 2021). Particularly, the use of algaecides, such as copper sulfate, was prohibited due to the potential for these chemicals to accumulate in lake sediments and subsequently be released into the water, potentially harming aquatic organisms (Sukenik & Kaplan 2021). Moreover, ultrasound technology has the potential to affect fish skin due to ultrasound-induced cavitation (Renkel *et al.* 1999). Additionally, ultrasonic waves can lead to cell lysis in cyanobacteria, resulting in the release of intracellular toxins or an increase in toxin production as a defense mechanism against sonication (Zhang *et al.* 2006; Dehghani 2016). Furthermore, dredging activities typically involve significant expenses related to logistics, such as pumping, water drainage, water treatment, sediment transportation, and disposal (Oldenborg & Steinman 2019; Kibuye *et al.* 2021).

The presence of macrophyte beds has a significant impact on the abundance and composition of plankton communities in aquatic environments (Jasser 1995). Due to coexistence within the same environment, these communities are interdependent on shared resources. As a result, the presence of macrophyte vegetation leads to reduced light availability, decreased water

turbulence, lower nutrient levels, and allelopathic interactions which suppresses the growth of plankton, including cyanobacteria (Vanderstukken *et al.* 2011; Senavirathna *et al.* 2021). An important factor to consider is the inhibitory effect exerted by allelochemicals produced by macrophytes, which additionally suppress the growth of cyanobacteria (Hu & Hong 2008; Amorim *et al.* 2019). Macrophytes possessing allelopathic potential play a crucial role in the remediation of water bodies affected by HABs, which is a significant environmental concern in both natural and artificial shallow water systems globally.

Allelopathy is a biological phenomenon characterized by the capability of plants to influence neighboring plants or other organisms through the release of chemical substances into their immediate surroundings, resulting in repressive or stimulative effects (Hu & Hong 2008). The production of allelochemicals represents a natural strategy employed by macrophytes to reduce competition for primary resources and ensure their well-being (Mulderij *et al.* 2007). Several macrophyte species, including *Myriophyllum* spp., *Ceratophyllum demersum*, *Egeria densa*, and *Hydrilla verticillata*, have been identified as strong producers of allelochemicals (Hu & Hong 2008; Vanderstukken *et al.* 2011). Studies by Amorim *et al.* (2019) have shown a significant reduction in *Microcystis aeruginosa* biomass in the presence of *C. demersum*. Similarly, Wang *et al.* (2017) and Nakai *et al.* (2000) have reported the successful inhibition of *M. aeruginosa* growth by the macrophyte genus *Myriophyllum* spp. Although many macrophytes inhibit cyanobacteria growth when present as live plants, the feasibility of utilizing such macrophytes is often limited owing to their invasive nature. Therefore, the utilization of biologically derived substances (BDS) extracted from cyanobacteria-inhibiting macrophytes represents a promising alternative for harnessing the potential of these plants.

Previous studies have shown that the extracts derived from macrophyte species have been found to suppress cyanobacteria growth (Wang *et al.* 2017). The inhibition ability of those allelochemicals present in macrophyte extracts on cyanobacteria is not only species-specific but depends on various other factors such as pretreatment method, utilized solvent, and extraction method on macrophytes. Pretreatments such as mechanical grinding and prolonged stirring are important to induce the release of allelochemicals in the plants (Chen *et al.* 2012). Various aqueous and organic solvents were employed to extract allelochemicals from different parts of macrophytes to inhibit harmful cyanobacteria growth. Distilled or pure water was utilized as a successful aqueous solvent by many researchers (Li *et al.* 2016; Hua *et al.* 2018; Wang *et al.* 2022). Some researchers used a combination of aqueous and organic solvents such as methanol (Gil *et al.* 2021), ethanol (Maredová *et al.* 2021), or dimethyl sulfoxide (DMSO) (Yi *et al.* 2012) to increase the solubility of the allelochemical compounds. On the other hand, some researchers exclusively employed organic solvents, such as hexane, ethyl acetate, and ethanol solutions (Zhang *et al.* 2015), acetone, and dichloromethane (Gao *et al.* 2020) to extract allelochemicals.

Studies investigated the allelopathic inhibitory activities of various macrophyte extracts against *M. aeruginosa*. Research conducted by Zhang *et al.* (2016) observed a sharp decrease in total algal biomass including the dominant *M. aeruginosa* by using the organic extracts derived from macrophyte *Acorus calamus* roots using 95% ethanol as the solvent. According to a study conducted by Li *et al.* (2016), an aqueous extract derived from *Sagittaria trifolia* tubers was able to inhibit *M. aeruginosa* growth by 90% after 6 days. In another study, *M. aeruginosa* density was reduced by 82%, and a significant increase in oxidative stress markers was evidenced in cyanobacterial cells by *Ranunculus aquatilis*-dried tissue extracts (Tazart *et al.* 2021). Submerged macrophytes *Potamogeton pectinatus* and *C. demersum* aqueous extracts with 50 and 100% acetone and ethanol solvents also inhibited *M. aeruginosa* (Ghobrial *et al.* 2015). Furthermore, the extracts from *Chara globularis*, *Ceratophyllum submersum*, *Elodea nuttallii*, *H. verticillata*, *Myriophyllum heterophyllum*, *M. spicatum*, and *Vallisneria americana* demonstrated the ability to inhibit the growth of cyanobacteria. Most importantly, all these species are submerged macrophytes (Maredová *et al.* 2021).

E. densa is another submerged macrophyte, widely recognized for its ability to produce allelochemicals with anti-cyanobacterial properties as a live plant when they coexist in the same environment (Hu & Hong 2008; Vanderstukken *et al.* 2011; Senavirathna *et al.* 2021). In a mesocosm study conducted by Vanderstukken *et al.* (2011), it was demonstrated that *E. densa* is recognized as an effective inhibitor of cyanobacteria. The results of our previous experiment (Wijesinghe *et al.* 2023) also exhibited that the fresh tissue aqueous extracts obtained from *E. densa* co-existed with *M. aeruginosa* performed a considerable suppression than the extracts obtained from *E. densa* monoculture. However, there was limited research on the application of *E. densa* extracts on cyanobacteria control. *E. densa* is an invasive, perennial submerged macrophyte species found in water bodies around the world (Zehnsdorf *et al.* 2015). This submerged macrophyte has become a threat to the natural vegetation in temperate, subtropical and tropical ecosystems because of its invasive nature, the rapid growth which eventually crowds out other natural flora (Dutartre *et al.* 1999). The extraction of natural allelochemicals from invasive macrophyte species is a sustainable method, given their abundance in water bodies. Thus, in this study, we selected *E. densa*

as the subject to investigate a potential approach for utilizing extracts from dried plants to suppress *M. aeruginosa*. The cyanobacterium *M. aeruginosa* was selected for this study due to its ubiquitous and its tendency to form toxic blooms nature in water bodies worldwide (Chen *et al.* 2012). The selection of aqueous or organic solvents to obtain the allelopathic compounds from the plant materials is crucial, as some compounds are readily dissolved in water, while others need organic solvents to increase their water solubility. Therefore, the effects of *E. densa* extracts derived from both aqueous (pure water) or organic (DMSO) solvents on *M. aeruginosa* growth were assessed. Further, the study examined the effect of *E. densa* organic extracts on oxidative damage and antioxidant responses in *M. aeruginosa*.

2. MATERIALS AND METHODS

2.1. Preparation of cultures

2.1.1. *M. aeruginosa*

M. aeruginosa strains (NIES-111) were obtained from the National Institute of Environmental Studies (NIES), Japan. The cultures were pre-cultured in a sterile BG-11 medium (Rippka *et al.* 1979) using autoclaved 300-mL Erlenmeyer flasks. The cultures were maintained in an incubator (LH-55RDS, NK Systems Limited, Tokyo, Japan) at 20 °C under a light intensity of 25–30 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) on a photoperiod of 12:12 h light: dark cycle which was maintained using an automatic setup device (REVEX PT7, Saitama, Japan). The cultures were covered with porous stoppers (SILICOSEN, Shin-Etsu Polymer Co., Ltd, Japan) for better aeration and gently shaken every day. The stock cultures in the exponential growth stage were used for the experiments.

2.1.2. *E. densa*

E. densa certified for agrochemicals and chemicals-free were commercially purchased and cultured in 20-L (25 cm × 24 cm × 40 cm, height × width × length) glass tanks in a temperature-controlled room at 25 ± 2 °C. Macrophytes were cultured in nutrient-removed river sand as the substrate. A commercial aquaponic solution (Hyponex concentrated nutrient solution, Hyponex, Osaka, Japan) was provided as the growth medium. The cultures were exposed to a 12:12 h light: dark cycle with a light intensity of 80–85 μmol m^{-2} s^{-1} PAR. The water levels of the culture tanks were kept constant by replenishing them with deionized water.

2.2. Preparation of *E. densa* extracts

The aqueous and organic extracts of *E. densa* were prepared according to Shi *et al.* (2020) and (Yi *et al.* 2012), respectively with modifications. Healthy *E. densa* plants were selected and washed gently once with tap water and three times with distilled water to remove any kind of detritus and oven dried at 60 °C for 48 h. The dried plant materials were cut into 2 cm small pieces and homogenized in a blender (IFM-C20G, Iwatani Cooperation, Osaka, Japan) and the obtained powder was utilized to prepare the extracts.

For the aqueous solvent, 10 g of dry *E. densa* powder was mixed with 400 mL of ultra-pure water (1:40 *w/v*) to get 25 g/L stock and stirred at 150 rpm for 24 h in dark conditions. The solution was filtered through 0.45 μm and the obtained filtrate was utilized as the *E. densa* aqueous extract and stored at 4 °C. The dry plant powder (10 g) was mixed with 99% DMSO maintaining a 1:2 ratio (sample/solvent) and then topped up with ultra-pure water to obtain 25 g/L stock solution with 5% (*v/v*) final DMSO. The mixture was stirred at 150 rpm continuously for 24 h in dark conditions before passing through 0.45 μm filter units. The obtained filtrates were utilized as the *E. densa* organic extract and stored at 4 °C.

2.3. Experimental design

2.3.1. Effect of *E. densa* extracts on *M. aeruginosa* growth

As shown in Figure 1, *M. aeruginosa* cultures with equal cell densities (10^5 Cells/mL) were separately exposed to *E. densa* aqueous and organic extracts in concentration gradients of 0, 0.5, 1, 3 and 6 g/L. Triplicates were maintained for each extract concentration and cultures were acclimatized for 3 days before the introduction of extracts. All experiments were conducted under controlled laboratory conditions in sterilized 300-mL flasks, each containing 200 mL of *M. aeruginosa* cells in BG-11 medium. The cultures were maintained at 25 ± 1 °C and subjected to a 12:12 light: dark photoperiod using an automatic setup device (REVEX PT7, Saitama, Japan) with a light intensity of 80 μmol m^{-2} s^{-1} PAR. Additionally, control experiments were carried out without any plant extracts to establish baseline growth. During the exposure period to *E. densa* extracts, each cell suspension was gently shaken three times a day. The growth and oxidative stress parameters of *M. aeruginosa* were

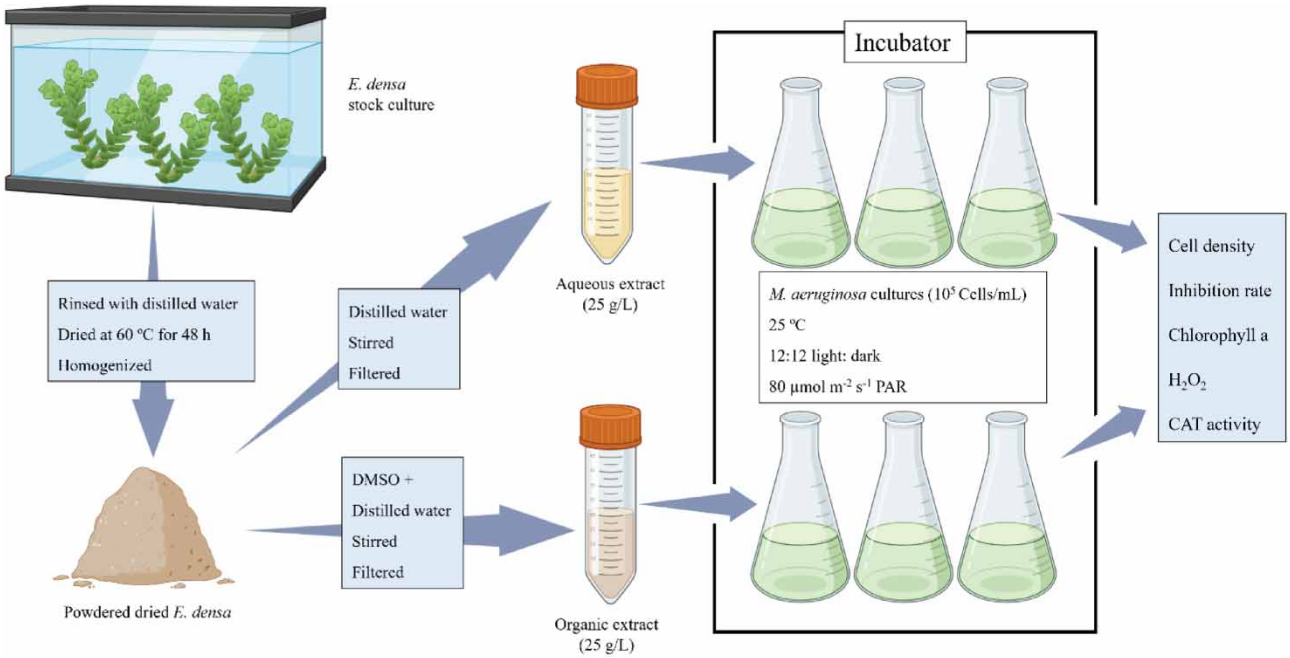


Figure 1 | Experimental procedure from *Egeria densa* culture to *Microcystis aeruginosa* treatment and analysis (Created with BioRender.com).

quantified for a duration of 10 days at 48-h intervals to identify the effect of different *E. densa* extract concentrations on *M. aeruginosa*. Cell counts, inhibition rate (IR), total protein content, and chlorophyll a (Chl-a) concentration were analyzed to evaluate the growth of *M. aeruginosa*. The oxidative stress levels of *M. aeruginosa* cells were determined using hydrogen peroxide (H_2O_2) and catalase (CAT) activity.

2.3.2. Effect of DMSO on *M. aeruginosa* (preliminary experiment)

A preliminary experiment was conducted to assess the effect of DMSO on *M. aeruginosa* before commencing the main experiments. The concentration gradient of prepared *E. densa* extracts ranged from 0.5 to 6 g/L, with DMSO concentration ranging from 0.1 to 2%. Consequently, we exposed *M. aeruginosa* cultures at a cell density of 1×10^5 cells/mL to corresponding DMSO percentages without *E. densa* extracts under similar experimental conditions as described in 2.3.1. This experiment was conducted for a duration of 12 days, during which we monitored cell densities at 72-h intervals.

2.3.3. Effect of low concentrations of *E. densa* organic extracts on *M. aeruginosa*

To investigate the impact of low concentrations of *E. densa* organic extracts on the growth of *M. aeruginosa*, an experiment was conducted using a gradient of extract concentrations at 0, 0.1, 0.2, 0.3, and 0.4 g/L. We exposed *M. aeruginosa* cultures, initially at a cell density of 1×10^5 cells/mL, to the varying concentrations of *E. densa* organic extract under identical experimental conditions as described in 2.3.1. This experiment was conducted for a duration of 10 days, during which we monitored cell densities at 48-h intervals.

2.3.4. Effect of continuous supplying of *E. densa* organic extracts on *M. aeruginosa*

To assess the sustained inhibitory effect of *E. densa* organic extracts, the most *M. aeruginosa* inhibitive concentrations were chosen. The *M. aeruginosa* cultures were subjected to continuous exposure to selected extract concentrations at regular intervals of 24 h for 4 days. The experimental setups were carried out under similar conditions as described in Section 2.3.1.

2.3.5. Effect of phosphate on *M. aeruginosa* growth

To assess the impact of the available phosphate (PO_4^{3-}) in the organic extract on the growth of *M. aeruginosa*, we exposed *M. aeruginosa* cultures to PO_4^{3-} concentrations equivalent to the available PO_4^{3-} levels in *E. densa* extracts at 0.5, 3, and 6 g/L (Figure 6(b)), without adding *E. densa* extracts. Additionally, another group of *M. aeruginosa* cultures was exposed

to equivalent PO_4^{3-} concentrations in groups of 0.5, 3, and 6 g/L, along with 0.5 g/L of *E. densa* extract. A control group of cells was maintained without either PO_4^{3-} or *E. densa* extracts. We monitored cell density every 48 h, and the experiment was conducted for 10 days. The experimental setups were carried out under similar conditions as described in Section 2.3.1. This experiment was conducted for a duration of 10 days, during which we monitored cell densities at 48-h intervals.

Before starting each set of experiments *M. aeruginosa* cultures were acclimatized for 3 days. The experiments were conducted under consistent conditions, with triplicates maintained to ensure reliable and consistent results.

2.4. Analytical methods

2.4.1. Growth parameters of *M. aeruginosa*

2.4.1.1. Cell count. Cell counts were determined by microscopically (BZ-X810, KEYENCE Corporation, Osaka, Japan) counting with a hemocytometer (DHC-N01, C-Chip, NanoEnTec Inc, Guro-gu, Seoul, Korea). The cell count was expressed as cells/mL.

2.4.1.2. Inhibition rate (IR). The percentage of growth inhibition with the presence of different extract concentrations was calculated compared to the control group. The inhibitory effect of the extract on cyanobacteria growth was calculated according to the following Equation (1).

$$\text{IR } \% = \left[1 - \left(\frac{N}{N_o}\right)\right] \times 100 \quad (1)$$

N and N_0 stands for the cell density of the treatment sample and cell density of the control sample, respectively.

2.4.1.3. Total protein. To determine the total protein content of *M. aeruginosa*, the Bradford method (Bradford 1976) was employed. The *M. aeruginosa* cell pellets were mixed with 0.5 M NaOH and heated for 10 min at 70 °C in a water bath. The cooled mixture was then centrifuged at 10,000× *g* for 10 min using a centrifuge (TOMY MX-105), and the resulting supernatant was mixed with Bradford reagent (Wako Chemical, Tokyo, Japan). The mixture was incubated in the dark for 10 min at 25 ± 2 °C, and the absorbance was measured at 595 nm using a spectrophotometer (UV-1280, Shimadzu, Kyoto, Japan). The protein content was estimated using a standard curve and expressed as μg/mL.

2.4.1.4. Chlorophyll-a (Chl-a). The concentration of Chl-a was quantified by homogenizing the *M. aeruginosa* cell pellets in 1 mL of 95% ethanol. The samples vibrated for approximately 1 min and incubated overnight in dark conditions at room temperature (25 ± 2 °C). The samples were then centrifuged at 10,000× g for 10 min at 4 °C, and the supernatant was used to measure the absorbance at 665 and 649 nm wavelengths using a UV-1280 spectrophotometer. Chl-a concentration was calculated using Equation (2) as described by (Hua *et al.* 2018):

$$\text{Chl a} = (13.95 \times A_{665}) - (6.88 \times A_{649}) \quad (2)$$

A_{665} and A_{649} stands as the absorbance values at 665 and 649 nm, respectively, and the Chl-a concentration was expressed as μg/μg of protein.

2.4.2. Oxidative stress parameters of *M. aeruginosa*

2.4.2.1. Cellular hydrogen peroxide (H_2O_2). To quantify the cellular H_2O_2 content, a method proposed by (Jana & Choudhuri 1984) was followed with modifications. Specifically, 1 mL of 0.1 M phosphate buffer (pH 6.5) was added to the *M. aeruginosa* cell pellets, which were then vortexed (Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY, USA) and centrifuged at 10,000× *g* for 10 min at 4 °C to extract the cellular content of H_2O_2. The supernatants were collected and mixed with a reaction mixture containing 0.1% titanium chloride in 20% H_2SO_4 (v/v), and the mixture was incubated for 1 min before centrifugation at 25 ± 2 °C. The supernatant's absorbance was measured at 410 nm using a UV-1280 spectrophotometer. The H_2O_2 concentration per protein was expressed as μmol/μg.

2.4.2.2. Catalase (CAT) activity. Catalase activity in cyanobacteria was quantified according to (Aebi 1984) with modifications. The cell pellets were homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, and then centrifuged at 10,000× *g* for 10 min at 4 °C. CAT activity was measured by combining 10 μL of

supernatant with 10 μL of 750 mM H_2O_2 in 980 μL of 50 mM potassium phosphate buffer (pH 7.0), and the absorbance was measured at 240 nm using a UV-1280 spectrophotometer for 3 min at 0.5-s intervals. CAT activity was calculated using Equation (3).

$$k = \frac{2.3}{\Delta t} \times \log \frac{E_1}{E_2} \tag{3}$$

The rate constant (k) is considered a direct measure of the catalase concentration, and E_1 is the higher first value of the linear extinction, and E_2 is the lower final value of absorption. Also, Δt is the time interval.

2.4.3. *E. densa* extract analysis

2.4.3.1. Dissolved organic carbon (DOC) concentration. The total organic carbon analyzer (TOC-2300, Hiranuma Sangyo Co., Ltd, Ibaraki, Japan) was utilized to measure the concentration of DOC of the aqueous and organic *E. densa* extracts. The catalytic oxidation method was used: TOC = TC (total carbon) – IC (inorganic carbon) to measure the DOC concentrations in the samples filtered through 0.45 μm filters.

2.4.3.2. Determination of UV_{254} absorbance. The UV_{254} absorbance was measured using a UV-1280 spectrophotometer. Extracts were filtered through 0.45 μm filters and distilled water was utilized as the control before taking the absorbance values.

2.4.3.3. Determination of specific ultraviolet absorbance. Specific ultraviolet absorbance (SUVA) is a widely used approach to assess the aromaticity and nature of DOC in a water sample. SUVA is calculated using the ratio of UV_{254} absorbance to DOC concentrations. High SUVA values indicate a greater presence of aromatic compounds in a sample. In this study, SUVA values were used to compare the availability of certain compounds in both aqueous and organic extracts of *E. densa,* which may potentially inhibit cyanobacteria growth. SUVA values of *E. densa* aqueous and organic extracts were calculated using the UV_{254} absorbance and corresponding DOC concentration. Calculation of SUVA was performed using the following Equation (4) (Yan *et al.* 2021).

$$\text{SUVA}\left(\frac{\text{L}}{\text{mg.m}}\right) = \frac{\text{UV}_{254}\ \text{Absorbance}\ (\text{cm}^{-1})}{\text{DOC}(\frac{mg}{L})} \times 100 \tag{4}$$

2.4.3.4. Three-dimensional excitation-emission matrix. Three-dimensional excitation-emission matrix (3D EEM) is another method that can be used to identify the compounds present in the organic extract. The mechanism of fluorescence spectrometry is that organic molecules in the sample instantly ($\sim 10^{-8}$ s) absorb light energy at certain wavelengths and produce emission light with greater wavelengths than the excitation wavelengths (Zhang *et al.* 2019). The excitation and emission wavelengths of each organic compound are unique and fixed (Hu & Yin 2017). Therefore, this method is widely used to identify the groups of various organic compounds in a sample (Hu & Yin 2017; Zhang *et al.* 2019; Yin *et al.* 2022). A 3D EEM of the *E. densa* organic extract was obtained using a fluorescence spectrophotometer (LS 45, Perkin Elmer, Waltham, MA, USA) to identify the presence of organic compounds in *E. densa* extracts. The excitation wavelengths were changed from 200 to 600 nm and the emission wavelength was from 300 to 625 nm at a scanning speed of 1,500 nm/min. Their possible functional classes were determined by comparing the 3D EMMs with those published in the literature (Hu & Yin 2017; Zhang *et al.* 2019). The humification index was calculated according to the regions where the peaks were produced to understand the characteristics of compounds available in the extract.

2.4.3.5. Humification index. The humification index was calculated by dividing the fluorescence intensity in the 435 → 480 nm humic-like region by the intensity in the 300 → 345 nm aromatic protein region of the excitation–emission matrix graph at specific excitation and emission wavelength pairs (Ohno 2002) (Equation (5)).

$$\text{Humification Index} = \frac{\text{Fluorescence intensity at } 435 \rightarrow 480\text{nm}}{\text{Fluorescence intensity at } 300 \rightarrow 345\ \text{nm}} \tag{5}$$

2.4.3.6. Phosphate (PO_4^{3-}). The PO_4^{3-} concentrations of *E. densa* aqueous and organic extracts filtered through 0.45 μm filters were evaluated using PAKTEST Phosphate (Range 2–100 mg/L) and Digital pack test meter (DPM-MTSP, KYORITSU CHEMICAL Corp, Japan).

2.4.3.7. Nitrate (NO_3^-). The NO_3^- concentrations of *E. densa* aqueous and organic extracts filtered through 0.45 μm filters were evaluated using PACKTEST Nitrate (Range 1–45 mg/L) and Digital pack test meter (DPM-MTSP, KYORITSU CHEMICAL Corp, Japan).

2.5. Data analysis

Data were analyzed using SPSS statistical software (Version 20.0, IBM SPSS) with a significance level of 5% ($p \leq 0.05$). Growth and stress parameters under different extract conditions were compared using one-way analysis of variance (ANOVA) with the post-hoc Tukey HSD (honestly significant difference) test. Each experiment was performed in triplicates. All of the graphs were created using OriginLab software 2022 (OriginLab Corporation, Northampton, MA, USA). All graphical images were created with BioRender.com.

3. RESULTS AND DISCUSSION

3.1. Effect of DMSO on *M. aeruginosa* growth (preliminary experiment)

M. aeruginosa cultures exposed to different percentages of DMSO did not exhibit a significant effect on growth, even up to the maximum percentage of 2%, as compared to the control culture without DMSO (Figure 2). A previous study by Kamaya *et al.* (2003) utilized a maximum concentration of 0.3% (*v*/*v*) DMSO, as it was observed to cause no influence on the growth of algae *Selenastrum capricornutum*, a finding supported by Yi *et al.* (2012) for *M. aeruginosa*. Moreover, all treated cell cultures in this study consistently exhibited a progressive increase in cell density during the exposure period, similar to the control cell culture. These results indicate that the varying percentage of DMSO present in the *E. densa* extract concentration gradient used in this study does not affect the growth of *M. aeruginosa*.

3.2. Comparison of *E. densa* aqueous and organic extracts on *M. aeruginosa* growth

As shown in Figure 3(a) and 3(b), the cell densities of the *M. aeruginosa* exposed to aqueous and organic extracts were compared to investigate the inhibitory effects of each extract at different concentrations. The initial cell density was fixed at $1 \pm 0.04 \times 10^5$ cells/mL for all treatments.

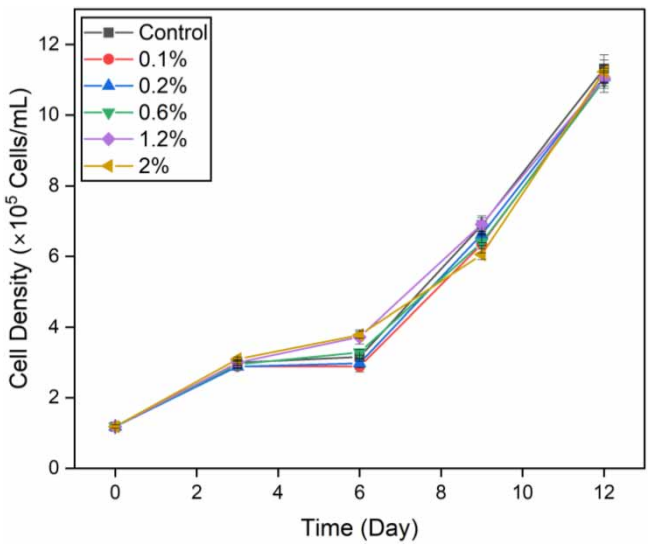


Figure 2 | Variation in cell density of *M. aeruginosa* corresponding to the DMSO percentage gradient during the exposure period. Error bars represent the standard deviation ($n = 3$).

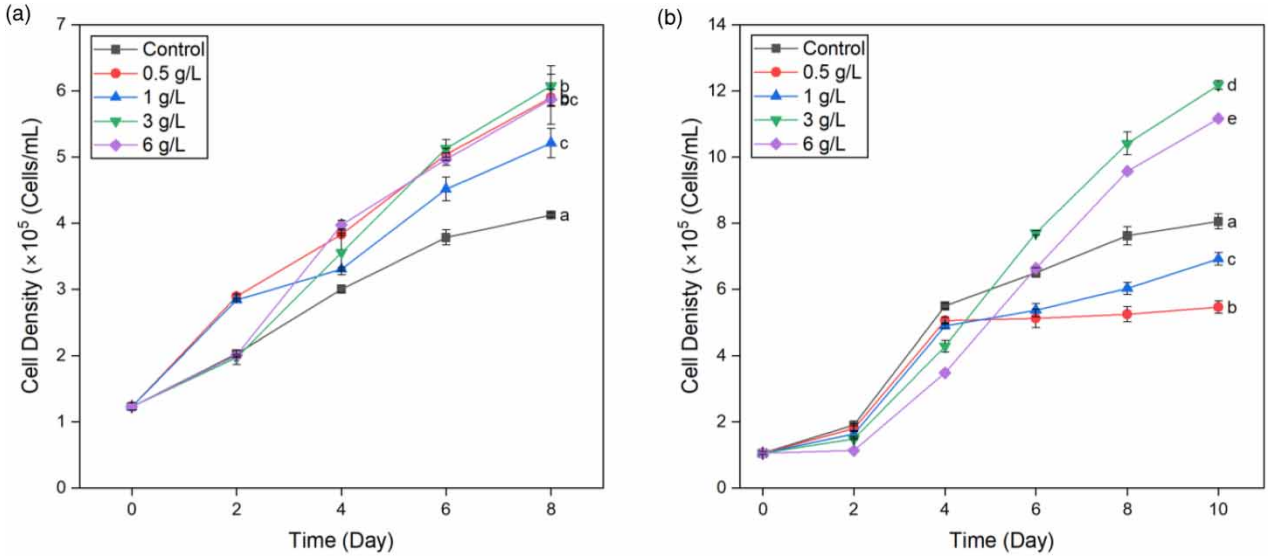


Figure 3 | Cell density of *M. aeruginosa* concerning *E. densa*. (a) Aqueous extract gradient and (b) organic extract gradient throughout the exposure period. Error bars represent the standard deviation ($n = 3$). Different lower-case letters indicate the significant difference in cell densities of *M. aeruginosa* at different extract concentrations at the end of the exposure period.

After a 48-h exposure period, it was observed that the cultures treated with high concentrations of aqueous extracts (3 and 6 g/L) exhibited an approximately similar cell count compared to the control culture (Figure 3(a)). However, as the treatment progressed, the cell count in all cultures treated with aqueous extracts significantly increased compared to both the initial cell density (Day 0) and the cell density of the control culture ($p < 0.05$). In other words, *M. aeruginosa* cultures treated with aqueous extracts did not show any inhibition throughout the entire exposure period.

By contrast, the *M. aeruginosa* cultures treated with *E. densa* organic extracts exhibited a concentration-dependent effect on growth. Initially, all treated samples displayed suppressed growth compared to the control group within the first 4 days (Figure 3(b)). The extract concentrations higher than 3 g/L showed a continuous increase in cell density, even surpassing the control cell suspension. Especially, the lowest cell density of $3.48 \pm 0.16 \times 10^5$ cells/mL was observed at 6 g/L on day 2 before experiencing a sharp subsequent increase. In contrast, the lower concentrations (0.5 and 1 g/L) demonstrated a comparatively higher cell density than the higher extract concentrations within the first 4 days, yet still lower than the control at day 4. However, their growth was subsequently suppressed during the remaining exposure time. Accordingly, the cultures treated with 0.5 g/L displayed the lowest IR of 5% on day 2, which then increased steadily during the exposure period. Interestingly, this group ultimately achieved the highest IR of 32%, surpassing all other treatment groups by the end of the exposure period (Figure 4). The sample treated with 0.5 g/L extract consistently exhibited growth suppression throughout the experiment, showing a cell count of $5.45 \pm 0.18 \times 10^5$ cells/mL on day 10, which was significantly lower than the control ($F(4, 10) = 385.607$, $p < 0.001$). Similarly, the cultures treated with 1 g/L followed a similar trend as cultures treated with 0.5 g/L, with eventual increases in cell density surpassing the 0.5 g/L group by day 6. After 10 days of exposure, the 1 g/L treated cultures displayed a significantly lower cell density than the control ($F(4, 10) = 385.607$, $p = 0.001$) but significantly higher than the 0.5 g/L treated cultures ($F(4, 10) = 385.607$, $p < 0.001$).

3.3. Effects of low concentrations of *E. densa* organic extract on *M. aeruginosa* growth

The exposure of *M. aeruginosa* cultures to low concentrations (0, 0.1, 0.2, 0.3 and 0.4 g/L) of *E. densa* organic extracts exhibited a concentration-dependent effect on growth (Figure 5). The 0.3 and 0.4 g/L concentration treated group showed lower cell densities than the other groups and control. However, the lowest cell density was exhibited by the 0.4 g/L group as $5.7 \pm 0.08 \times 10^5$ cells/ mL at day 10. The 0.4 g/L group also showed the lowest cell density after the first day of exposure as $0.87 \pm 0.06 \times 10^5$ cells/ mL to other groups and control. By the end of the exposure, the 0.4 g/L concentration treated cultures demonstrated a controlled growth than the other cultures and the control. This observation tallies with the results of the previous experiment where 0.5 g/L concentration exhibited continuous growth control toward the end of subsequent

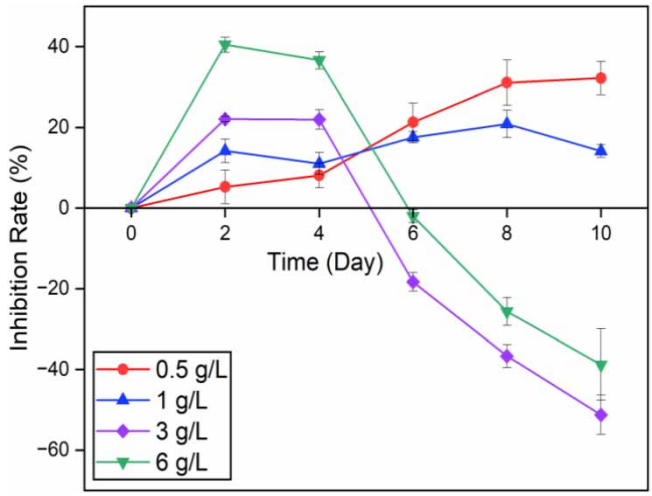


Figure 4 | IR of *M. aeruginosa* cultures exposed to *E. densa* organic extract gradient throughout the experiment period. Error bars represent the standard deviation ($n = 3$).

exposure time. However, 0.4 g/L concentration exhibited an IR of 27% as the highest IR which is lower than the IR of 0.5 g/L (32%) at day 10. Since the final cell density of the 0.5 g/L concentration ($5.45 \pm 0.18 \times 10^5$ cells/mL) was lower than the final cell density of the 0.4 g/L concentration at the end of the exposure, the 0.5 g/L concentration was considered the effective organic extract concentration. Hence, these results demonstrated that organic extracts at low concentrations appear to be effective compared with an aqueous extract or high concentrations of organic extracts. This phenomenon is potentially owing to the presence of available nutrients and organic matter.

Table 1 compares the organic carbon, PO_4^{3-}, and NO_3^- contents of the stocks of both aqueous and organic extracts of *E. densa*.

Figure 6 shows the variation of NO_3^-, PO_4^{3-}, and DOC with increasing extract concentration.

The aqueous extract exhibited higher NO_3^- concentrations than the organic extract, with the 6 g/L aqueous extract recording the highest value of 2.7 ± 0.2 mg/L (Figure 6(a)). Particularly, NO_3^- concentrations were below the detection limit of 1 mg/L in both aqueous and organic extracts for lower concentrations (0.5 and 1 g/L). In contrast, *E. densa* organic extracts

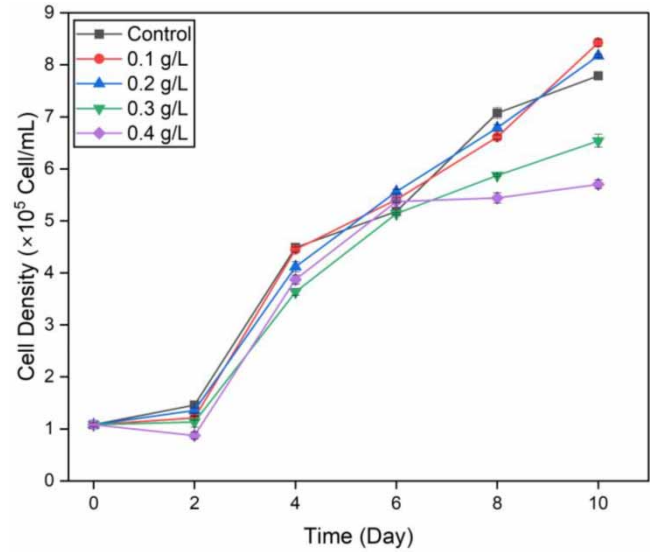


Figure 5 | Cell densities of *M. aeruginosa* cultures exposed to 0, 0.1, 0.2, 0.3, and 0.4 g/L concentrations of *E. densa* organic extracts throughout the exposure period. Error bars represent the standard deviation ($n = 3$).

Table 1 | Organic carbon, PO_4^{3-}, and NO_3^- contents of *E. densa* aqueous and organic stock extract solutions

Parameter	Aqueous extract (mg/L)	Organic extract
Nitrate (NO_3^-)	9.6	4.6
Phosphate (PO_4^{3-})	315.5	420
Dissolved organic carbon (DOC)	2,360	4,780

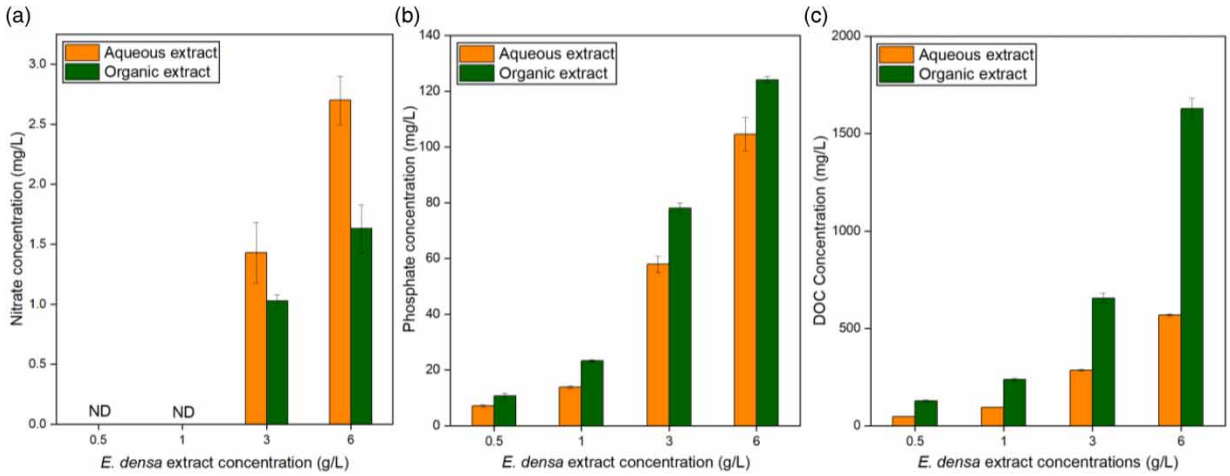


Figure 6 | Variations of NO_3^- (a), PO_4^{3-} (b), and DOC (c) concentrations in *E. densa* aqueous and organic extracts. Error bars represent the standard deviation ($n = 3$). 'ND' refers to 'Not Detected,' and the limit of detection (LOD) for NO_3^- and PO_4^{3-} is set at 1 mg/L.

showed higher PO_4^{3-} concentrations compared to the aqueous extract. Both extracts exhibited a consistent increase in PO_4^{3-} concentration as the extract dosage increased, with the highest concentration of 124 $\pm$ 1.2 mg/L observed in the 6 g/L group of organic extract (Figure 6(b)). The results indicated that the higher concentrations of extracts contained high amounts of favorable nutrients, especially PO_4^{3-} and NO_3^- which may have suppressed the inhibitive effect of allelochemicals by assisting the fast growth of *M. aeruginosa*. Dissolved phosphorus is known to be the key nutrient for algal growth (House 2003). The initial suppression of growth in the cultures treated with high extract concentrations (3 and 6 g/L) can be attributed to the inhibitive effect of high concentrations of allelochemicals. Nevertheless, prolonged exposure to high concentrations of extracts may have triggered the survival of *M. aeruginosa* cells. This response could potentially result from the availability of abundant nutrients in the medium, leading to rapid growth after day 4 (Figure 3(b)).

To evaluate the impact of the PO_4^{3-} concentration within the organic extract on the growth of *M. aeruginosa*, we exposed *M. aeruginosa* cultures to varying PO_4^{3-} concentration. When the cultures were exposed to PO_4^{3-} concentration equivalent to the amount available in 0.5 g/L of the extract, they exhibited more than a two-fold increase in cell density compared to cultures exposed to the 0.5 g/L extract concentration by the end of the exposure period (Figure 7(a)). This finding confirms that the 0.5 g/L extract concentration can inhibit the growth of *M. aeruginosa*, possibly due to the presence of allelochemicals, even the low concentration of PO_4^{3-} concentrations is available. However, the cell cultures exposed to PO_4^{3-} concentrations equivalent to the amounts available in 3, 6 g/L with 0.5 g/L extract did not show any significant difference when compared to cell cultures exposed to PO_4^{3-} only samples. In all these cases, cell density increased by the end of the exposure period. This suggests that high concentrations of PO_4^{3-} suppress the activity of allelochemicals present in the extracts.

Furthermore, DOC concentration in the extracts is an indicator of the availability of organic compounds within them. In the organic extract (excluding the DOC values of DMSO), the DOC concentration was approximately three times higher than that recorded in the corresponding aqueous extracts at the same extract concentration (Figure 6(c)). As a result, the aqueous extract did not suppress the growth of *M. aeruginosa* compared to the control culture at any extract concentration level during the exposure period (Figure 3(a)).

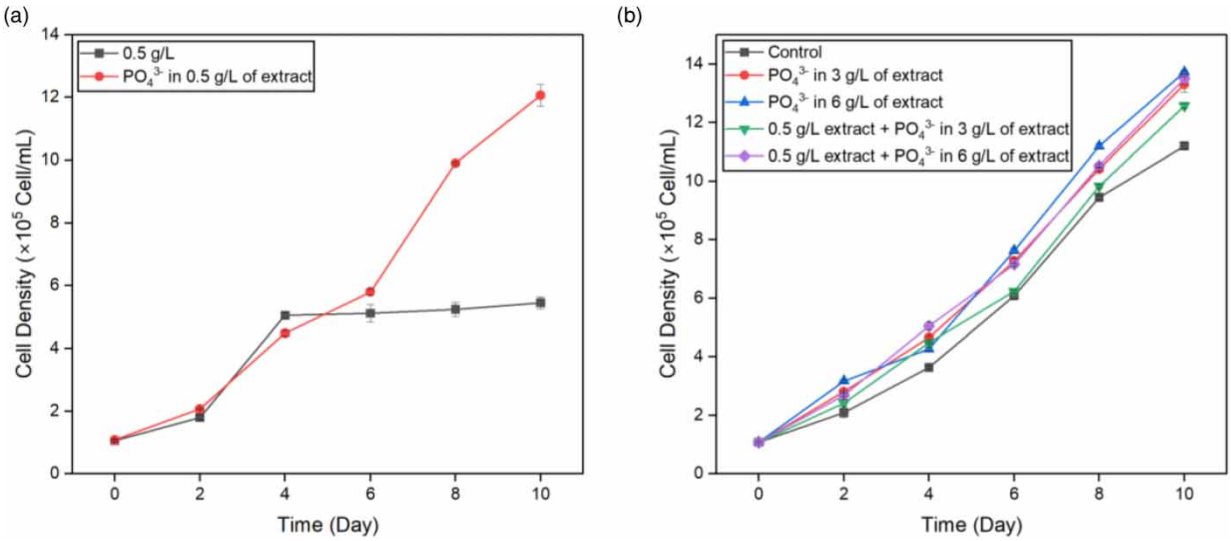


Figure 7 | Cell densities of *M. aeruginosa* cultures exposed to (a) 0.5 g/L extract concentration and PO_4^{3-} in 0.5 g/L and (b) PO_4^{3-} in 3 and 6 g/L, mixture of 0.5 g/L extract with PO_4^{3-} in 3 g/L and PO_4^{3-} in 6 g/L. Error bars represent the standard deviation ($n = 3$).

The inability of the aqueous extract to suppress the *M. aeruginosa* growth indicates a lower availability of anti-cyanobacterial organic chemicals dissolved in the solvent. Consequently, aqueous solvents may not be the most suitable option for extracting allelochemicals from the *E. densa*. On the other hand, following 48 h of exposure to organic extract, high concentrations (3 and 6 g/L) resulted in growth suppression of *M. aeruginosa*, with the 6 g/L concentration exhibiting the lowest cell density ($3.48 \pm 0.16 \times 10^5$ cells/ mL). Furthermore, after 6 days, cultures treated with 0.5 and 1 g/L concentrations exhibit a sustained suppression of *M. aeruginosa* growth, indicating that extracts at low concentrations are more effective. Yi *et al.* (2012) stated that numerous compounds produced by plants have limited water solubility. The results obtained in this study proved that allelochemicals in *E. densa* exhibit better solubility in DMSO. Multiple previous studies have similarly reported successful inhibition of cyanobacteria growth by utilizing various organic plant extracts (Hu & Hong 2008; Yi *et al.* 2012; Zhang *et al.* 2015). The extracts of aquatic macrophytes consist of numerous compounds, including phenolics, tannin, lignin, and aromatic compounds. These substances collectively contribute to the overall composition of available humic substances (Kang *et al.* 2020; Yin *et al.* 2022). According to Zhou *et al.* (2019), phenolic compounds are the most common and vital plant allelochemicals. SUVA is a widely used method for evaluating the aromaticity and composition of DOC in a sample. The corresponding DOC values of both aqueous and organic stocks are presented in Table 1. The elevated SUVA values occur owing to an increase in the proportion of recalcitrant organic compounds such as phenolic compounds and humic substances (Kang & Mitchell 2013). However, SUVA values of aqueous and organic extract stocks observed in the study were 0.46 and 0.22, respectively. A lower SUVA value was reported for organic extract may be due to the high concentration of non-aromatic compounds present in the organic extracts.

The results of the 3D EEM analysis on the aqueous extract of *E. densa* produced four peaks, while the organic extract produced five peaks, as shown in Figure 6(a) and 6(b), respectively. These peaks elucidate the availability of different organic compounds present within extracts. The corresponding excitation and emission wavelengths of each peak are shown in Table 2.

According to the findings of Zhang *et al.* (2019), the peaks B and Q belong to the aromatic protein region which is referred to as aromatic amino acids (e.g., tyrosine and tryptophan). The peaks B, C, Q, and R observed in 300–345 nm emission wavelengths are located within the region associated with protein-like compounds. A higher proportion of protein-like compounds accelerates the growth of *M. aeruginosa*, as most growth-promoting compounds are included in the protein group. The peaks labeled as A, D, P, S, and T, occurring in emission wavelengths of 435–480 nm, are located in a region recognized to be associated with humic-acid-like compounds (Hu & Yin 2017; Yin *et al.* 2022). Specifically, peaks A and P represent the phenolic compounds (Traversa *et al.* 2010). These compounds might have allelopathic properties that potentially contribute to the inhibitory impact of *M. aeruginosa*. When comparing the intensities of phenolic-type peaks, peak P corresponding to the

Table 2 | Excitation and emission wavelength ranges of the peaks in 3D EEM analysis of *E. densa* aqueous and organic extracts

	Peak	Ex/Em (nm)	Compound type	Reference
Aqueous	A	357/445	Humic-acid-like (Phenolic)	Traversa *et al.* (2010)
	B	277/345	Aromatic protein	Yin *et al.* (2022)
	C	217/345	Protein-like compounds	Ouyang *et al.* (2020); Yin *et al.* (2022)
	D	215/445	Humic-acid-like	Gu *et al.* (2017)
Organic	P	364/455	Humic-acid-like (Phenolic)	Traversa *et al.* (2010)
	Q	278/350	Aromatic protein	Yin *et al.* (2022)
	R	232/350	Protein-like compounds	Ouyang *et al.* (2020)
	S	236/460	Humic-acid-like	Gu *et al.* (2017)
	T	442/520	Humic-acid-like	Gu *et al.* (2017)

organic extract showed a higher intensity which represents the concentration than peak A in the aqueous extract EEM spectrum (Figure 8). The calculated humification index of the aqueous extract showed a value of 0.7, indicating higher concentrations of protein-like compounds and lower concentrations of humic-like compounds in *E. densa* aqueous extract. The humification index of organic extract exhibits a value of 1.45, which is attributed to the lower concentration of protein-like compounds and higher humic acid-like compounds in the *E. densa* organic extract. This result also confirms the ability of organic extracts to inhibit *M. aeruginosa* growth.

3.4. Effect of *E. densa* organic extracts on chlorophyll-a concentration, oxidative stress, and antioxidant activities of *M. aeruginosa*

The chlorophyll concentration is a direct indicator to evaluate the disturbance in photosynthesis, trapping capacity in the photosynthetic system and the energy flow in algae (Li *et al.* 2016). As described by Wang *et al.* (2022) the suppressed production of chlorophyll pigments can diminish photosynthesis which can lead to termination of cell growth and eventual cell death. The Chl-a is considered one of the important photosynthetic pigments and thus can be used as an indicator to investigate the photosynthetic capacity in *M. aeruginosa* as well as the biomass indirectly (Li *et al.* 2016; Wang *et al.* 2022). The production of Chl-a in the *M. aeruginosa* cultures was also affected by the exposure to *E. densa* organic extract, with varying concentrations yielding different results (Figure 9). The cultures treated with a concentration of 0.5 g/L exhibited the lowest Chl-a concentration on day 6, measuring $4.8 \pm 0.6 \times 10^{-3}$ μg/μg protein. Throughout the entire exposure period, the cultures

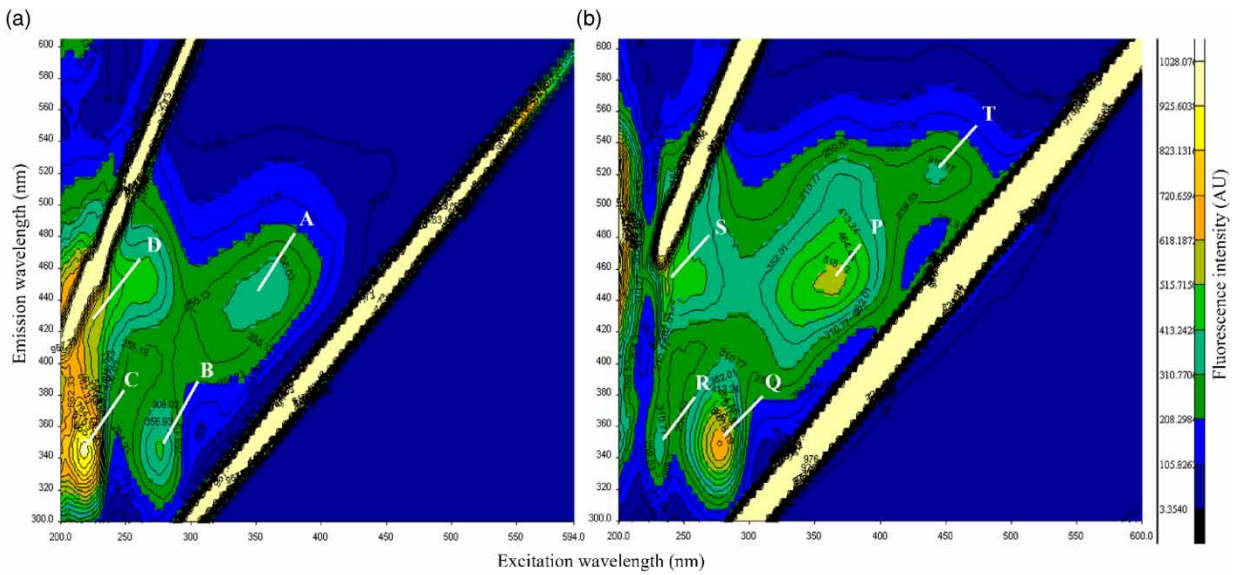


Figure 8 | 3D EEM of *E. densa* aqueous (a) and organic (b) extract. A-D and P-T indicate the different peaks produced by the aqueous and organic extracts, respectively.

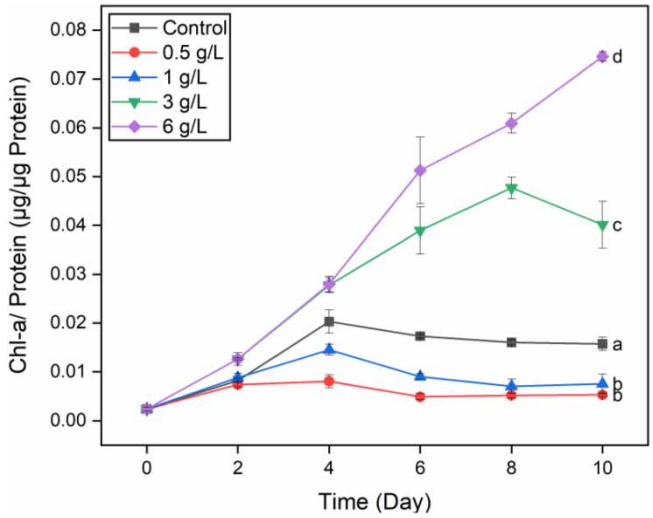


Figure 9 | Chl-a concentration per unit protein of *M. aeruginosa* cultures exposed to *E. densa* organic extract gradient throughout the experiment period. Error bars represent the standard deviation ($n = 3$). Different lower-case letters indicate the significant difference of Chl-a per protein concentration of *M. aeruginosa* at different extract concentrations at the end of the exposure period.

treated with 0.5 g/L consistently showed the lowest Chl-a concentrations compared to the other treatment groups. In contrast, the higher concentrations of *E. densa* organic extract increased Chl-a concentration over time. However, 0.5 and 1 g/L concentration groups exhibited significantly lower Chl-a concentrations than the control group at day 10 ($F(4, 10) = 428.18$, $p < 0.001$) and ($F(4, 10) = 428.18$, $p = 0.001$), respectively.

The Chl-a concentration mirrored the growth variations showed by cell counts as a whole by exhibiting low Chl-a concentrations in cultures treated with low organic extract concentrations. The 0.5 and 1 g/L treatments showed significantly lower Chl-a concentrations than the control ($p < 0.05$) by the end of the exposure time (Figure 9). This indicates that the low concentrations of *E. densa* organic extracts affected the photosynthesis systems of *M. aeruginosa* cells disturbing the Chl-a production of the cells. However, the high organic extract concentrations (3 and 6 g/L) exhibited higher Chl-a concentrations than the control by the end of exposure time. This exhibits the opposite of what Wang *et al.* (2022) witnessed in the inhibitory effects of *Vallisneria* aqueous extract, where high extract concentrations inhibit *M. aeruginosa* growth while low concentrations stimulated it. The high nutrient-absorbing nature of *E. densa* (Vanderstukken *et al.* 2011) may have been attributed to higher nutrient availability in the extract with the extracted allelochemicals. Which created an additional nutrient effect, that surpassed the allelochemical effect of high concentrations on the *M. aeruginosa* cells.

3.4.1. H_2O_2 concentration and CAT activity

The reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) are naturally generated and continuously involved in many reactions in plant metabolism and plant growth as a normal phenomenon (Sharma & Dubey 2005). However, when cells are under various environmental stresses ROS production was stimulated resulting in the accumulation of H_2O_2 and other radicals (Sharma & Dubey 2005; Wang *et al.* 2022). Which leads to oxidative stress and when not regulated by antioxidative activities, eventual cell death (Sharma & Dubey 2005). Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) act as defense mechanisms to protect the cells by eliminating excessive H_2O_2 (Hua *et al.* 2018; Wang *et al.* 2022). Numerous studies have investigated the oxidative damage and the antioxidant defense activities in cyanobacteria caused by various allelochemicals derived from plants (Li *et al.* 2016; Hua *et al.* 2018; Tazart *et al.* 2021; Wang *et al.* 2022). Polyphenols and alkaloids are commonly known compounds found in the various plant tissue extracts that have been studied for their potential inhibitory mechanisms on cyanobacteria. Previous research suggests that the suppression of alkaline phosphatase and disruption of the electron transfer chain within photosystem II (PS II) could potentially serve as inhibitory mechanisms of polyphenols against *Microcystis* species (Dziga *et al.* 2006; Shao *et al.* 2013). The deexcitation of excited energy through a non-photochemical pathway increases due to the interruption of the electron transfer via PSII, consequently leading to an elevation in ROS within the cells (Shao *et al.* 2013).

The H_2O_2 concentration was investigated as a measure of oxidative stress in *M. aeruginosa* cultures treated with *E. densa* organic extracts. On day 4, the cultures treated with a concentration of 0.5 g/L *E. densa* organic extract showed a significantly high H_2O_2 concentration of 1.11 ± 0.04 μmol/μg protein, compared to the control ($F(4, 10) = 32.357$, $p < 0.001$) (Supplementary material, Figure S1). Indicating lowest extract concentration caused the highest oxidative damage in the cultures. However, the cultures treated with both lower concentrations (0.5 and 1 g/L) exhibited a decrease in H_2O_2 concentration over time during the subsequent exposure period. The H_2O_2 concentration of the culture treated with 6 g/L concentration exhibited the highest value (0.77 ± 0.11 μmol/mg) on day 2 which tally with the lowest growth on the same day by 6 g/L culture. The high concentration of allelochemicals in the extract may have induced the H_2O_2 production in the cells, thus oxidative damage, and destroying cells. The high H_2O_2 concentration in cultures treated with low extract concentration could be an effect of allelochemicals catching up when the received low amount of nutrients were consumed by the *M. aeruginosa*. Therefore, it was clear that the exposure time gets longer the cultures treated with low concentrations exhibited a decreasing trend of H_2O_2 concentration while the high extract-treated cultures showed higher H_2O_2 concentrations. Even though the high extract concentration did not reflect an impact on growth in *M. aeruginosa*, the increased H_2O_2 concentration exhibited that *M. aeruginosa* cells were subjected to oxidative stress. This elucidates that the allelochemical effect provided by high extract concentrations has been overpowered by the nutrient effect of the extracts in terms of oxidative damage as well.

The production of antioxidant enzymes is reported to increase in response to the rapid accumulation of H_2O_2, targeting to reduce oxidative damage to cells by breaking down H_2O_2 into H_2O and O_2 (Sharma *et al.* 2012). In this study, we also observed that a significantly high ($F(4, 10) = 55.5$, $p < 0.001$) CAT activity rate of $8.7 \pm 0.8 \times 10^{-4}$ sec^{-1} at day 4 in 0.5 g/L concentration corresponds to the high H_2O_2 concentration in the culture on the same exposure day. The CAT activity also mirrors the pattern of H_2O_2 concentration in cultures treated with high extract concentrations (3 and 6 g/L) by day 10 (Supplementary material, Figure S2), indicating the induction of antioxidant enzyme production to mitigate oxidative stress. However, the CAT activity rate decreased with the exposure time after reaching its peak on day 4. According to Wang *et al.* (2022), this reduction could have occurred as a result of the effect of allelochemicals, which may induce severe stress in the cells and lead to a rapid production of H_2O_2 that suppress the limits of cells' antioxidant defense mechanisms, resulting in a reduction of CAT activity.

3.5. Effects of 0.5 and 1 g/L concentrations of *E. densa* organic extract on continuous exposure to *M. aeruginosa*

The concentrations of 0.5 and 1 g/L were chosen based on the observed inhibitory effect of *M. aeruginosa* when exposed to a concentration gradient of *E. densa* organic extract. These concentrations demonstrated significantly higher ($p < 0.05$) inhibitory effects on the growth of *M. aeruginosa* than the control group. The *M. aeruginosa* cultures at an initial cell density of 1×10^5 cells/mL were continuously exposed to low concentrations (0.5 and 1 g/L) of *E. densa* organic extracts at 24-h intervals. As shown in Figure 10, we observed an initial growth suppression during the first 72 h for both concentrations.

During continuous exposure to both selected concentrations, the cultures of *M. aeruginosa* exhibited lower cell densities compared to the control group for the first 72 h (Figure 10(a)). The cultures exposed to the initial dose of 1 g/L showed the lowest cell density after 24 h, measuring $0.8 \pm 0.02 \times 10^5$ cells/ mL. The cultures treated with a concentration of 1 g/L displayed the highest IR of 29% after 24 h of continuous exposure, which subsequently decreased over time (Figure 10(b)). On the other hand, the cultures treated continuously with a concentration of 0.5 g/L exhibited an IR of 24% at 72 h. After 72 h, the cell count of both concentration groups started to exceed that of the control culture. A similar trend was observed in Chl-a concentration. The lowest Chl-a concentration was shown by the culture, continuously exposed to 0.5 g/L at 24 h as $5.37 \pm 0.3 \times 10^{-3}$ μg/μg protein (Figure 10(c)). This continuous exposure approach was conducted to understand the dominance of nutritional effects over allelochemical inhibitory effects. The results also confirmed that following three doses, *M. aeruginosa* showed a higher growth rate than control cell culture. This observation confirms that a high concentration of nutrients overpowers the influence of allelochemicals. Interestingly, H_2O_2 concentration (Figure 10(d)) and CAT activity rates (Figure 10(e)) remained consistently high throughout the 96 h of exposure to both concentrations (0.5 and 1 g/L). Particularly, the culture exposed to 1 g/L concentration showed the highest oxidative damage, accompanied by the greatest antioxidant concentration. The *M. aeruginosa* cells may have been stressed because of the continuous addition of extracts that consisted of both nutrients and allelochemicals. The elevated CAT activity rate indicates that the increased H_2O_2 concentration is effectively regulated by the antioxidative defense mechanisms of the cells. The increased CAT activity serves to

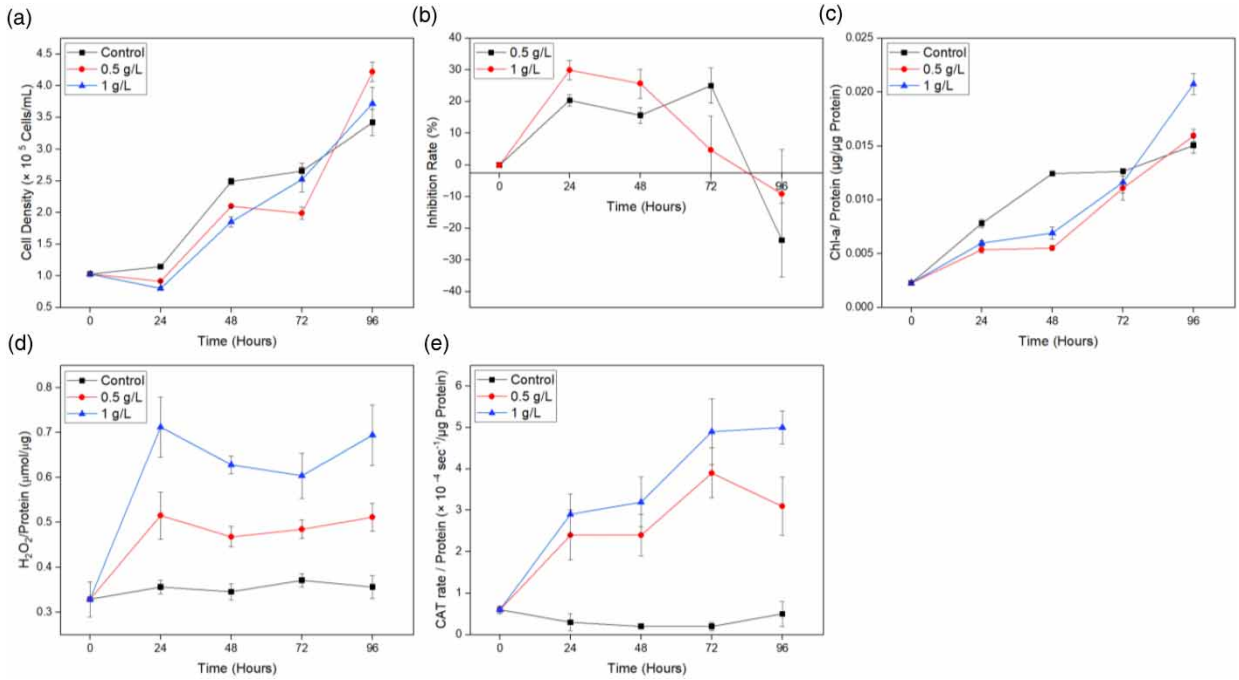


Figure 10 | Cell density (a), IR (b), Chl-a concentration (c), H_2O_2 concentration (d), and CAT activity rate (e) of *M. aeruginosa* cultures exposed to 0.5 and 1 g/L concentrations of *E. densa* organic extracts, along with the control cell culture, throughout the exposure period. Error bars represent the standard deviation ($n = 3$).

neutralize H_2O_2, effectively scavenging free radicals and preventing further oxidative damage within the cells (Wang *et al.* 2022).

4. CONCLUSIONS

The results revealed that low concentrations (0.5 and 1 g/L) of the organic extracts showed inhibitory effects on *M. aeruginosa*, while aqueous extracts stimulate the growth of *M. aeruginosa*. This difference was further supported by the DOC concentrations and distinct fluorescence fingerprints of organic compounds observed in EEM. The coexistence of higher nutrient levels such as PO_4^{3-} in both extracts contributes to enhancing the growth of *M. aeruginosa*. The absence of organic compounds that are responsible for anti-cyanobacterial activities in the aqueous extracts limited its ability to suppress *M. aeruginosa* growth. The DMSO organic extract of *E. densa* demonstrated the potential for bioremediation application. Specifically, the application of low concentrations (0.5 and 1 g/L) of organic extracts effectively suppressed the growth of *M. aeruginosa* and induced significant oxidative damage by the fourth day of exposure. However, higher concentrations (3 and 6 g/L) of organic extracts promoted the growth of *M. aeruginosa* due to high nutrient availability, while simultaneously inducing oxidative damage over time, which was continuously regulated by an antioxidant mechanism. The results confirm that the high availability of nutrients in the *E. densa* organic extracts at high concentrations disrupts the inhibitory effect of allelochemicals. Therefore, compared with an aqueous extract, the organic extract at low concentrations appears to be successful in suppressing *M. aeruginosa* growth. These results provide an approach to the sustainable use of strong anti-cyanobacterial properties in invasive macrophyte species that cannot be introduced as live plants for bioremediation in aquatic ecosystems.

This method is exceptionally well-suited for water bodies where the use of live macrophytes is impractical, such as boat yards, man-made shallow ponds in parks, or ornamental fountains, though not so for the application for large water bodies such as lakes and large reservoirs. However, the ecotoxicity of the extracts with strong inhibitory abilities needs to be investigated prior to the consideration of field applications as a crucial matter since there can be negative influence on non-target beneficial species in the ecosystem, whereas extracts obtained from *Corallina pilulifera* exhibited a nonselective

toxicity of several microalgae species (Jeong *et al.* 2000). An extract obtained from eucalyptus leaves showed a toxicity on zebra fish at higher concentrations (Zhao *et al.* 2019).

AUTHOR CONTRIBUTIONS

A.W. was involved in conceptualization; methodology; investigation, writing – original draft, M.D.H.J.S. was involved in conceptualization; methodology; investigation; resources; writing – review and editing; supervision, T.F. was involved in resource collection and supervision.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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