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Selective Inhibition on Growth and Photosynthesis of Harmful Cyanobacteria (*Microcystis aeruginosa*) by Water Soluble Substances of *Dendranthema indicum* Flowers

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Abstract: Harmful cyanobacterial blooms become serious environmental issues in various waterbodies, including aquaculture ponds, which inherently need a high biomass and healthy composition of phytoplankton to sustain their high productivity. Indoor bioassays were conducted to investigate the effects of an aqueous extract of *Dendranthema indicum* flowers on cyanobacteria and green algae species. The thermal stability and polarity of the water-soluble active substances of *D. indicum* flowers were also assessed based on the growth and photosynthesis responses of *Microcystis aeruginosa*. There was obvious growth promotion of green algae, including *Chlorella vulgaris*, *Kirchneriella* sp. and *Haematococcus pluvialis* and strong growth inhibition of toxigenic and non-toxicogenic *M. aeruginosa* by aqueous extracts of *D. indicum* flowers at concentrations ranging from 0.5 to 2.0 g·DW·L⁻¹. The cell concentrations of *M. aeruginosa* and *C. vulgaris* were 46.5% and 242.2% of those in the corresponding controls after a 10-day exposure to aqueous extracts of *D. indicum* flowers at 1.0 g·DW·L⁻¹. There must be some water-soluble active allelochemicals released from *D. indicum* flowers that are responsible for the selective inhibition of *M. aeruginosa* rather than green algae. The inhibition ratio of the growth and photosynthesis of *M. aeruginosa* by aqueous extracts of *D. indicum* flowers at 2.0 g·DW·L⁻¹ prepared with water at 25 °C and 100 °C were above 90% and 80% compared to the cell density and performance index on absorption basis (PIABS) value of *M. aeruginosa* in the control on day 6, without significant differences. It indicates that the active substances of *D. indicum* flowers were thermally stable. The methanol fraction eluted from solid phase extraction (SPE)-enriched aqueous extracts of *D. indicum* flowers showed the strongest inhibition of the growth and photosynthesis of *M. aeruginosa* compared to the other four fractions. It indicates that the most polar substances of *D. indicum* flowers were responsible for the selective inhibition of *M. aeruginosa*. More experiments are required to identify the responsible active substances and reveal the underlying mechanisms of aqueous extracts of *D. indicum* flowers that selectively inhibit cyanobacteria and regulate the phytoplankton community structure.

Keywords: *Dendranthema indicum* flowers; green algae; cyanobacteria; chemical characteristics; photosynthetic activity

1. Introduction

Harmful cyanobacterial blooms have increasingly become a global environmental and public issue due to their damage to water ecosystems and threat to human health [1]. The frequency,

magnitude, and duration of harmful cyanobacterial blooms have been found to have increased globally [2]. *Microcystis aeruginosa* is the most widely distributed species causing harmful cyanobacterial blooms in freshwater worldwide [3]. It is also the most frequently found cyanobacterial taxon in aquaculture water [4]. Some *M. aeruginosa* strains naturally produce microcystins, e.g., microcystin-YR, microcystin-LR, and microcystin-RR, or off-flavor compounds, e.g., geosmin and 2-methylisoborneol [5], which have substantial adverse impacts on drinking water and aquatic food security and consequently present health risks to humans [6].

Multiple countermeasures, including nutrient management [7,8], hydrodynamic regulation [9,10], chemical treatments [11,12], and biological control [13,14], have been developed and used to control cyanobacterial blooms. However, it is still a challenge to control harmful cyanobacterial blooms effectively and protect harmless aquatic organisms, especially for aquaculture ponds. There are 2.53×10^6 ha of pond-based freshwater aquaculture in China [15], which accounts for more than half of the total aquaculture area in China and contributes to the global aquatic food supply [16]. The most urgent need is for green technology to selectively inhibit cyanobacteria but promote the growth of beneficial algae to ensure pond fishery productivity.

It has been reported that some cyanobacteria species are more susceptible than green algae to active substances derived from aquatic plants, such as *Myriophyllum spicatum* [17,18], and terrestrial plants, such as *Salix atrocinerea* [19]. This provides a possibility to explore plant-derived chemical resources to regulate and improve phytoplankton community structure in aquaculture ponds. Compared to aquatic plants, terrestrial plants contain more abundant active compounds, which have been widely used in the fields of traditional Chinese medicine and biopesticides [20,21]. They have been also used as feed additives and immunostimulants to improve aquaculture environments and aquatic product quality [22]. Compositae is the largest family of angiosperms, it is widely distributed throughout the world and is famous for its bioactivity and various active secondary metabolites, including terpenoids, polyacetylenes, phenolics, and organic acids [23]. Some of these compounds have been isolated and identified to be antimicrobial agents [24], anti-inflammatory agents [25], and antimalarial drugs [26]. The inhibition effects of Compositae plants on *M. aeruginosa* were found for a few species, such as *Artemisia lavandulifolia* [27], however, whether they can selectively control harmful cyanobacteria rather than beneficial algae, so as to regulate freshwater phytoplankton composition, is not clear.

Dendranthema indicum is one available Compositae species that is widely distributed in most areas of China. Its dried buds and flowers have been used as a popular tea, as an antipyretic to clear the eye and the mind, and as a remedy for common symptoms including headache, dizziness, and red eye [28]. It has been suggested to be a valuable source of antimicrobial ingredients in the food industry [29]. To assess its potential to control harmful cyanobacteria, our preliminary experiments indicated that *D. indicum* flowers could effectively inhibit the growth of one *M. aeruginosa* strain. As for its selectivity, we proposed a hypothesis that *D. indicum* flowers may selectively inhibit *M. aeruginosa* rather than green algae. To test this hypothesis, the concentration-dependent effects of water extracts of *D. indicum* flowers were investigated using the green algae *Chlorella vulgaris* and the cyanobacteria *M. aeruginosa*. Its species-specific inhibition effects were further tested using two other green algae species and two *M. aeruginosa* strains. To explore the responsible substances, the basic chemical properties, including the thermal stability and polarity of the selective inhibition substances from *D. indicum* flowers, were also investigated in two separate experiments, where chlorophyll fluorescence rise kinetics were observed to reveal their effects on the status and performance of photosynthetic system II (PSII) in *M. aeruginosa* cells.

2. Materials and Methods

2.1. Culture Condition

Two harmful cyanobacterial strains, i.e., one toxigenic (FACHB 905) and one non-toxigenic *M. aeruginosa* (FACHB 526), and three beneficial green alga strains, i.e., *C. vulgaris* (FACHB 8),

Kirchneriella sp. (FACHB 1433) and *Haematococcus pluvialis* (FACHB 1164), were purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology. All the cultures were grown in 250 mL conical flasks containing 100 mL of autoclaved BG 11 medium under $25 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of light intensity with a 12:12 h light: dark cycle at 25 °C. The cultures were manually shaken twice each day and cells in exponential growth phase were prepared for the experiments.

2.2. Experimental Design for Selective Effects of Aqueous Extract from *D. indicum* Flowers

The dry flowers of *D. indicum* used in the study were collected from Dabie Mountain, Anhui Province, China. They were added into glass beakers, immersed in distilled water, and then they stood at room temperature for 8 h to prepare the aqueous extracts of *D. indicum* flowers. The aqueous extracts were filtered by sterile gauze and a 0.22 μm filter membrane in turn, and then they were ready for the experiments.

To analyze the concentration-dependent effects of aqueous extracts of *D. indicum* flowers on phytoplankton species, the cells of *C. vulgaris* and toxigenic *M. aeruginosa* were used for bioassays. The final concentration of the aqueous extracts of *D. indicum* flowers in the bioassays were equivalent to adding 0.5, 1.0, 2.0, 4.0 and 8.0 g·DW *D. indicum* flowers per liter, respectively. The initial cell density for both strains was between $5.0\text{--}5.5 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$. The control for each strain without the addition of plant aqueous extracts was set simultaneously. Three replicates were prepared for both the treatments and controls. The bioassays lasted 10 days. Sub-samples were collected at 9 a.m. on days 0, 2, 4, 6, 8, and 10 for cell counts.

To further testify the species-specific inhibition by aqueous extracts of *D. indicum* flowers, the responses of *Kirchneriella* sp. and *H. pluvialis*, toxigenic and non-toxigenic *M. aeruginosa*, to aqueous extracts of 1.0 g·DW·L⁻¹ *D. indicum* flowers, were compared. The initial OD₆₈₀ for each strain was 0.03, and the corresponding cell density was $6.7 \times 10^4 \text{ cells}\cdot\text{mL}^{-1}$ for *Kirchneriella* sp., $9.8 \times 10^3 \text{ cells}\cdot\text{mL}^{-1}$ for *H. pluvialis*, $5.3 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$, and $5.2 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$ for non-toxigenic and toxigenic *M. aeruginosa*. The control for each strain without the addition of plant aqueous extracts was set simultaneously. Three replicates were prepared for both the treatments and controls. The bioassays lasted 3 days. Sub-samples were collected at the beginning and the end for cell counts and photosynthesis pigments analysis.

2.3. Characterization of Its Selective Inhibition Substances

To test the thermal stability of selective inhibition substances from *D. indicum* flowers, pure water at 25 °C and 100 °C was used to prepare aqueous extracts of *D. indicum* flowers. Pure water in conical flasks was placed in an incubator at 25 °C for 12 h to obtain the extraction water at 25 °C. Pure water in conical flasks was boiled at 100 °C to obtain the extraction water at 100 °C. Two kinds of aqueous extracts from *D. indicum* flowers were prepared as per the protocols mentioned in Section 2.2, and cooled to room temperature. The toxigenic *M. aeruginosa* cells were used to compare the inhibition effects of the two kinds of aqueous extracts of 2.0 g·DW·L⁻¹ *D. indicum* flowers in a 9-day bioassay. The control without plant aqueous extracts was set simultaneously. Three replicates were prepared for both the treatments and controls. The initial cell density of *M. aeruginosa* was $4.6 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$. Sub-samples were collected on day 0, day 3, day 6, and day 9 to measure the cell density, chl *a* concentration, and chlorophyll fluorescence parameters of *M. aeruginosa*.

To test the polarity of selective inhibition substances from *D. indicum* flowers, the aqueous extracts from *D. indicum* flowers were prepared as per the protocols mentioned in Section 2.2. The prepared aqueous extracts were enriched using solid phase extraction (SPE) with activated Oasis HLB cartridges (500 mg, 6cc. Waters, Milford, MA, USA). The five enriched cartridges were eluted with hexane, dichloromethane, ethyl acetate, acetone, and methanol, respectively. The eluted solvents were dried with nitrogen, and the eluted fractions were re-dissolved in DMSO for bioassays with toxigenic *M. aeruginosa* in a 9-day bioassay. The initial cell density of *M. aeruginosa* was $4.5 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$, and the final concentration of each eluting fraction in the bioassay was equivalent to 2.0 g·DW·L⁻¹

D. indicum flowers. The control without eluting fractions was set simultaneously. Three replicates were prepared for both the treatments and controls. The final volume of the cosolvent DMSO in the control and the treatments accounted for 0.1%, which showed no significant inhibition of *M. aeruginosa* in our preliminary experiment. Sub-samples were collected on day 0, day 3, day 6, and day 9 to measure the cell density, chl *a* concentration, and chlorophyll fluorescence parameters of *M. aeruginosa*.

2.4. Determination of Growth Parameters

Five-milliliter sub-samples were collected every time and fixed with Lugol's solution. A cell count was conducted for each sample with a Sedgewick Rafter counter under light microscopy (BA210, MOTIC, Xiamen, China) at $\times 400$ magnification. The daily growth rate of each strain was calculated according to the following equation

$$\text{Daily growth rate (day}^{-1}\text{)} = \ln (N_{t2}/N_{t1})/(t_2 - t_1) \quad (1)$$

where N_{t2} and N_{t1} are the counted cell density of one strain in the control or treatments on the days of t_2 and t_1 , respectively.

2.5. Measurement of Photosynthesis Pigments

A 5-mL subsample of each culture was centrifuged at $7100\times g$ for 10 min and the pellets were harvested and extracted with 95% ethanol for 24 h in the dark. The extracts were centrifuged again at $7100\times g$ for a further 10 min. The absorbance of the supernatant was measured at 665, 649, and 470 nm. The concentrations of chl *a* and carotenoids in the extracts were calculated as described previously [30].

2.6. Measurement of Chlorophyll Fluorescence Parameters

Chlorophyll fluorescence transient O-J-I-P and JIP tests were used to assess the effects of aqueous extracts of *D. indicum* flowers on the photosynthetic activity of *M. aeruginosa*. A 5-mL subsample of *M. aeruginosa* was collected in a tube, dark-adapted for 20 min, and the chlorophyll fluorescence rise kinetics (OJIP curves) were measured with an AquaPen-C AP-C 100 fluorometer (Photon Systems Instruments, Drasov, Czech Republic) according to the manufacturer's instructions. The fluorescence intensity at 2 ms (J-step), 30 ms (I-step), and the maximum fluorescence (P-step) were denoted as F_j , F_i , and F_p , respectively. Selected JIP test parameters quantifying PSII behavior were automatically calculated from the above time-resolved fluorescence and the formulae listed in Table 1 [31].

Table 1. The formulae and illustrations of the selected JIP test parameters in the study.

Parameters	Formulae and Terms	Illustrations
V_j Mo	$V_j = (F_j - F_o)/(F_m - F_o)$ $Mo = 4(F_{300\mu s} - F_o)/(F_m - F_o)$	Relative variable fluorescence intensity at the J-step Approximated initial slope of the fluorescence transient
Quantum efficiencies or flux ratios		
F_v/F_m	$F_v/F_m = TR_o/ABS = 1 - F_o/F_m = \phi Po$	Maximum quantum yield for primary photochemistry
Ψ_o	$\Psi_o = ETo/TR_o = (F_m - F_j)/(F_m - F_o)$	Probability that a trapped exciton moves an electron into the electron transport chain beyond QA (at $t = 0$)
ϕEo	$\phi Eo = ETo/ABS = (F_m - F_j)/F_m$	Quantum yield for electron transport (at $t = 0$)
Specific activities or specific fluxes		
ABS/RC	$ABS/RC = Mo \cdot (1/V_j) \cdot (1/\phi Po)$	Absorption flux per reaction center
TR_o/RC	$TR_o/RC = Mo \cdot (1/V_j)$	Trapped energy flux per reaction center (at $t = 0$)
ETo/RC	$ETo/RC = Mo \cdot (1/V_j) \cdot \Psi_o$	Electron transport flux per reaction center (at $t = 0$)
DI_o/RC	$DI_o/RC = (ABS/RC) - (TR_o/RC)$	Dissipated energy flux per reaction center (at $t = 0$)
Performance indexes		
PI_{ABS}	$PI_{ABS} = (RC/ABS) \cdot [\phi Po / (1 - \phi Po)] \cdot [\Psi_o / (1 - \Psi_o)]$	Performance index on absorption basis

2.7. Data Analysis

Data were analyzed with Microsoft Excel 2010 and SPSS 23 (IBM, Armonk, NY, USA). The inhibition ratio of plant aqueous extracts or the eluting fractions on the targeted organisms was calculated according to the following equation:

$$\text{Inhibition ratio (\%)} = 100 \times (P_c - P_t)/P_c \quad (2)$$

where P_t is the value of each parameter for the specific tested strains in the treatments and P_c is that in the control. ANOVA and a subsequent Tukey's test were used to analyze the differences between treatments. Statements of significance refer to a less than 5% level of statistical confidence.

3. Results

3.1. Concentration-Dependent Effects of Aqueous Extracts from *D. indicum* Flowers

The cell concentrations of *C. vulgaris* increased from 5.5×10^5 cells·mL⁻¹ to 7.9×10^6 cells·mL⁻¹ in the control over 10 days. Aqueous extracts of *D. indicum* flowers at 0.5–2.0 g·DW·L⁻¹ significantly promoted the growth of *C. vulgaris* ($p < 0.05$). When the concentrations of *D. indicum* flowers in the treatments were 0.5 g·DW·L⁻¹, 1.0 g·DW·L⁻¹, and 2.0 g·DW·L⁻¹, the growth of *C. vulgaris* was 235.5%, 384.2%, and 303.4% of that in the control on day 4, respectively, and 184.5%, 242.2%, and 262.7% on day 10, respectively. When the concentrations of *D. indicum* flowers were 4.0 g·DW·L⁻¹ and 8.0 g·DW·L⁻¹, the growth of *C. vulgaris* was 17.0% and 12.9% relative to the control on day 4, and 29.3% and 2.0% on day 10 (Figure 1A). The growth of *C. vulgaris* was significantly inhibited by aqueous extracts of *D. indicum* flowers at a concentration higher than 4.0 g·DW·L⁻¹ ($p < 0.05$).

The cell concentrations of *M. aeruginosa* increased from 5.0×10^5 cells·mL⁻¹ to 2.1×10^7 cells·mL⁻¹ in the control over 10 days. A concentration-dependent inhibition of aqueous extracts of *D. indicum* flowers at 0.5–8.0 g·DW·L⁻¹ on the growth of *M. aeruginosa* was recorded (Figure 1B). In the treatments with aqueous extracts of *D. indicum* flowers at 0.5 g·DW·L⁻¹, the growth of *M. aeruginosa* from day 4 to day 10 ranged from 89.0% to 57.2% of that in the control. When the concentration of *D. indicum* flowers was 1.0 g·DW·L⁻¹, the growth of *M. aeruginosa* from day 4 to day 10 ranged from 49.0% to 46.5% of that in the control. When the concentration of *D. indicum* flowers was 2.0 g·DW·L⁻¹ or higher, cell concentrations of *M. aeruginosa* were even lower than those on day 0, and lower than 10% of those in the control on each sampling day, indicating thorough inhibition by aqueous extracts of *D. indicum* flowers.

Figure 1. The cell density changes of *C. vulgaris* (A) and *M. aeruginosa* (B) exposed to varying concentrations of aqueous extracts of *D. indicum* flowers (g·DW·L⁻¹) in mono-culture bioassays. Different letters indicate significant differences among varying groups at $p < 0.05$ level.

3.2. Species-Specific Effects of Aqueous Extracts from *D. indicum* Flowers

During the 3-day bioassays, the responses of two green algae strains and two cyanobacterial strains to aqueous extracts from *D. indicum* flowers differed significantly ($p < 0.05$). The daily growth rate, chl *a*, and carotenoid contents of *Kirchneriella* sp. and *H. pluvialis* were promoted significantly compared to the control, whereas those of toxigenic and non-toxicogenic *M. aeruginosa* were inhibited significantly by the aqueous extracts from *D. indicum* flowers (Figure 2A–C). The promotion ratio of *Kirchneriella* sp. and *H. pluvialis* by the plant extracts was 126.4% and 58.6% based on the daily growth rate, 40.4% and 72.9% based on chl *a* content, and 35.5% and 84.5% based on carotenoid contents. The inhibition ratio of toxigenic and non-toxicogenic *M. aeruginosa* by the plant extracts was 33.3% and 31.6% based on daily growth rate, 20.7% and 13.8% based on chl *a* content, and 23.5% and 16.7% based on carotenoid contents (Figure 2D).

Figure 2. Effects of water extracts from *D. indicum* flowers at 1.0 g·DW·L⁻¹ on daily growth rate (A), chlorophyll *a* content (B), and carotenoid content (C) of *Kirchneriella* sp. (Kir.), *H. pluvialis* (Hae.), and non-toxicogenic (N-Mic.) and toxicogenic *M. aeruginosa* (T-Mic.), and the inhibition ratios according to the three parameters (D) during 3-day bioassays. * indicates significant differences between the control and the treatment at $p < 0.05$ level.

3.3. Effects of Water Extraction Temperature on Cyanoabacterial Inhibition by Aqueous Extracts of *D. indicum* Flowers

There was no significant difference in the responses of *M. aeruginosa* to water-soluble substances extracted from *D. indicum* flowers in normal temperature water (25 °C) or boiling water (100 °C) during a 9-day bioassay ($p > 0.05$, Figure 3). The cell density and chl *a* concentration of *M. aeruginosa* in the control increased 27-fold and 14-fold from day 0 to day 9, respectively. However, the cell density and chl *a* concentration of *M. aeruginosa* treated with both kinds of plant aqueous extracts increased only twofold (Figure 3A,B). The corresponding inhibition ratios increased over time for both treatments. The inhibition ratios of the plant extracts prepared with water at 25 °C and 100 °C reached up to 93.1% and 93.6% compared to the cell density in the control, and 86.1% and 89.1% relative to the chl *a* concentration of *M. aeruginosa* in the control on day 9 (Figure 3C,D).

Figure 3. Cell density (A) and chl *a* content (B) of *M. aeruginosa* in response to water-soluble substances extracted from *D. indicum* flowers at $2.0 \text{ g} \cdot \text{DW} \cdot \text{L}^{-1}$ with normal temperature water (25°C) and boiling water (100°C), and the corresponding inhibition ratio in terms of cell density (C) and chl *a* content (D) in a 9-day bioassay. Different letters indicate significant differences among different groups at $p < 0.05$ level.

The fast chlorophyll fluorescence induction curves of *M. aeruginosa* treated with aqueous extracts of *D. indicum* flowers using water at 25°C and 100°C showed no significant difference ($p > 0.05$), but were significantly lower than that in the control ($p < 0.05$, Figure 4). The fluorescence intensity at 2 ms (F_0) and 30 ms (F_t) and the maximum fluorescence intensity (F_p) of *M. aeruginosa* cells in the control increased 5.1, 5.3, and 5.8-fold from day 3 to day 9. However, the extent of the increase of the corresponding values was lower than 2.0-fold in both treatments. The gap between the control and the treatments increased over time. Compared to the control, the F_p values decreased by 79.65% and 84.22% in the treatments with normal temperature water (25°C) and boiling water (100°C) on day 9, respectively.

The parameters extracted from OJIP curves using JIP test analysis, including F_v/F_m , ψ_o , ϕE_o , and PIABS of *M. aeruginosa* cells in both treatments, showed no obvious differences ($p > 0.05$), but were significantly lower than those in the control ($p < 0.05$) after 9 days. The ETo/RC of *M. aeruginosa* cells in both treatments was also significantly lower than that in the control ($p < 0.05$). The values of ABS/RC and DIO/RC of *M. aeruginosa* in both treatments were higher than those in the control, and the differences between the control and the treatments increased over time. However, there was no significant difference in TRo/RC between the control and the treatments (Table 2).

Figure 4. The fast chlorophyll fluorescence induction curves of *M. aeruginosa* in response to water-soluble substances extracted from *D. indicum* flowers at $2.0 \text{ g} \cdot \text{DW} \cdot \text{L}^{-1}$ with normal temperature water (25°C) and boiling water (100°C) during a 9-day bioassay. (A) day 0; (B) day 3; (C) day 6; (D) day 9.

Table 2. The JIP test parameters of *M. aeruginosa* in response to water-soluble substances extracted from *D. indicum* flowers at $2.0 \text{ g} \cdot \text{DW} \cdot \text{L}^{-1}$ with normal temperature water (25°C) and boiling water (100°C) in a 9-day bioassay. Different letters indicate significant differences among the control and the treatments on the same day at $p < 0.05$ level.

Parameters	Time	Treatments		
		Control	25°C	100°C
Fv/Fm	day 3	0.44 ± 0.01^a	0.36 ± 0.01^b	0.37 ± 0.01^b
	day 6	0.55 ± 0.01^a	0.36 ± 0.08^b	0.41 ± 0.04^b
	day 9	0.54 ± 0.01^a	0.41 ± 0.03^b	0.41 ± 0.04^b
ψ_o	day 3	0.61 ± 0.01^a	0.55 ± 0.03^b	0.55 ± 0.02^b
	day 6	0.67 ± 0.01^a	0.49 ± 0.03^b	0.47 ± 0.02^b
	day 9	0.66 ± 0.02^a	0.58 ± 0.01^{ab}	0.53 ± 0.07^b
ϕ_{Eo}	day 3	0.27 ± 0.01^a	0.19 ± 0.01^b	0.21 ± 0.01^b
	day 6	0.37 ± 0.01^a	0.18 ± 0.03^b	0.19 ± 0.02^b
	day 9	0.35 ± 0.02^a	0.24 ± 0.02^b	0.21 ± 0.02^b
ABS/RC	day 3	2.30 ± 0.17^a	2.86 ± 0.32^a	2.65 ± 0.15^a
	day 6	1.72 ± 0.18^a	2.72 ± 0.52^b	2.47 ± 0.25^{ab}
	day 9	1.69 ± 0.06^a	2.26 ± 0.30^b	2.10 ± 0.19^{ab}
TRo/RC	day 3	1.02 ± 0.09^a	1.02 ± 0.10^a	0.99 ± 0.05^a
	day 6	0.94 ± 0.09^a	0.95 ± 0.07^a	1.00 ± 0.03^a
	day 9	0.91 ± 0.02^a	0.92 ± 0.07^a	0.85 ± 0.11^a
ETo/RC	day 3	0.62 ± 0.05^a	0.55 ± 0.06^a	0.55 ± 0.04^a
	day 6	0.63 ± 0.06^a	0.47 ± 0.02^b	0.47 ± 0.01^b
	day 9	0.59 ± 0.01^a	0.53 ± 0.03^b	0.45 ± 0.00^c
DIo/RC	day 3	1.28 ± 0.08^a	1.85 ± 0.22^b	1.66 ± 0.12^{ab}
	day 6	0.78 ± 0.09^a	1.77 ± 0.56^b	1.47 ± 0.24^b
	day 9	0.78 ± 0.04^a	1.34 ± 0.23^b	1.25 ± 0.14^b
PIABS	day 3	0.54 ± 0.05^a	0.23 ± 0.04^b	0.28 ± 0.03^b
	day 6	1.43 ± 0.22^a	0.21 ± 0.09^b	0.25 ± 0.06^b
	day 9	1.32 ± 0.20^a	0.44 ± 0.13^b	0.38 ± 0.10^b

3.4. Effects of Eluting Solvents on Cyanoabacterial Inhibition by SPE-Enriched Aqueous Extracts of *D. indicum* Flowers

SPE-enriched aqueous extracts of *D. indicum* flowers were eluted with five solvents with increasing polarities, i.e., hexane, dichloromethane, ethyl acetate, acetone, and methanol, in sequence. The obtained five eluting fractions showed polarity-dependent inhibition effects on *M. aeruginosa* (Figure 5). The cell density of *M. aeruginosa* in the control increased 33-fold over 9 days, 43-fold when treated with the hexane fraction, eightfold with the dichloromethane fraction, fivefold with the ethyl acetate fraction, threefold with the acetone fraction, and twofold with the methanol fraction (Figure 5A). The chl *a* concentration of *M. aeruginosa* in the control increased 10-fold for 9 days, 12-fold for the hexane fraction, eightfold for the dichloromethane fraction, sevenfold for the ethyl acetate fraction, threefold for the acetone fraction, and twofold for the methanol fraction (Figure 5B). The hexane fraction showed significant promotion effects on *M. aeruginosa*, and the maximum promotion ratio was 28.9% and 38.6% in terms of cell density and chl *a* content, respectively, on day 9. However, the inhibition ratio of the other four eluting fractions was above 50% from day 3 and increased over time. The inhibition ratio of the methanol fraction on the cell density and chl *a* content of *M. aeruginosa* was the highest, 94.9% and 85.6%, on day 9 (Figure 5C,D).

Figure 5. Cell density (A) and chl *a* content (B) of *M. aeruginosa* treated with the eluting fractions of SPE-enriched aqueous extracts from *D. indicum* flowers at 2.0 g DW L^{-1} and the corresponding inhibition ratio based on cell density (C) and chl *a* content (D) in a 9-day bioassay. Different letters indicate significant differences among different groups at $p < 0.05$ level.

The polarity-dependent inhibition effects of aqueous extracts from *D. indicum* flowers on the fast chlorophyll fluorescence induction curves of *M. aeruginosa* were also observed (Figure 6). The F_j , F_i , and F_p values of the typical OJIP curves of *M. aeruginosa* in the control increased 5.2, 5.2, and 4.8-fold from day 3 to day 9. The addition of the hexane fraction promoted the chlorophyll fluorescence intensity of *M. aeruginosa* compared to the control. However, the other four fractions showed strong

inhibition effects, and the inhibition degree enhanced with the polarity of the solvents. The differences increased with time. The F_p value increased by 5.04% for the hexane fraction, and decreased by 60.51% for the dichloromethane fraction, 71.97% for the ethyl acetate fraction, 85.62% for the acetone fraction, and 92.32% for the methanol fraction by day 9.

Figure 6. The fast chlorophyll fluorescence curves of *M. aeruginosa* treated with the eluting fractions of SPE-enriched aqueous extracts from *D. indicum* flowers at $2.0 \text{ g} \cdot \text{DW} \cdot \text{L}^{-1}$. (A) Day 0; (B) day 3; (C) day 6; (D) day 9.

The parameters extracted from the OJIP curves of *M. aeruginosa* cells were affected by eluting fractions of *D. indicum* flowers (Table 3). There were no significant differences in F_v/F_m values between the control and the hexane fraction ($p > 0.05$). The F_v/F_m values of *M. aeruginosa* cells treated with the other four eluting fractions showed obvious polarity-dependent decreases on day 6 and day 9 and were significantly lower than that in the control ($p < 0.05$). The F_v/F_m value of *M. aeruginosa* cells treated with the methanol fraction was the lowest, as it decreased by 28.50% on day 3, 29.11% on day 6, and 32.01% on day 9. The ψ_o value of *M. aeruginosa* for the control and hexane fraction was significantly higher than that for the other four fractions ($p < 0.05$). It increased slightly for all the treatments from day 3 to day 9. The ϕE_o value of *M. aeruginosa* cells in the control and hexane fraction showed no significant differences ($p > 0.05$) but were significantly higher than those in the other four fractions ($p < 0.05$). It increased slightly for the four eluting fractions with higher polarity from day 3 to day 9, and the polarity-dependent inhibition was obvious on day 6 and day 9. The ABS/RC and DIO/RC values did not show strict polarity-dependent tendencies, but they were the highest in the methanol fraction. The values of TRo/RC and ETo/RC of *M. aeruginosa* for the dichloromethane fraction and ethyl acetate fraction were the lowest, but they were not significantly different from those for the acetone and methanol fractions in most cases ($p > 0.05$). The PIABS values of *M. aeruginosa* showed decreasing trends, along with an increase in solvent polarity, with the lowest for the methanol fraction on day 6 and day 9. The inhibition ratio of PIABS values in *M. aeruginosa* cells by the methanol fraction was 76.84% on day 3, 81.24% on day 6, and 72.02% on day 9 compared to that in the control.

Table 3. The JIP test parameters of *M. aeruginosa* treated with the eluting fractions of SPE-enriched aqueous extracts from *D. indicum* flowers at 2.0 g·DW·L^{−1} in a 9-day bioassay. Different letters indicate significant differences among the control and the treatments on the same day at $p < 0.05$ level.

Parameters	Time	Treatments					
		Control	Hexane	Dichloromethane	Ethyl Acetate	Acetone	Methanol
Fv/Fm	day 3	0.53 ± 0.01 ^a	0.52 ± 0.01 ^a	0.38 ± 0.02 ^b	0.37 ± 0.01 ^b	0.40 ± 0.01 ^b	0.38 ± 0.01 ^b
	day 6	0.53 ± 0.01 ^a	0.51 ± 0.01 ^a	0.44 ± 0.00 ^b	0.42 ± 0.01 ^b	0.38 ± 0.01 ^c	0.37 ± 0.01 ^c
	day 9	0.53 ± 0.02 ^a	0.51 ± 0.01 ^a	0.50 ± 0.00 ^{ab}	0.48 ± 0.01 ^b	0.44 ± 0.01 ^c	0.36 ± 0.01 ^d
ψ_o	day 3	0.79 ± 0.01 ^a	0.77 ± 0.03 ^a	0.61 ± 0.01 ^c	0.66 ± 0.01 ^b	0.70 ± 0.01 ^b	0.68 ± 0.02 ^b
	day 6	0.80 ± 0.01 ^a	0.81 ± 0.00 ^a	0.69 ± 0.01 ^{cd}	0.74 ± 0.01 ^b	0.71 ± 0.01 ^{bc}	0.66 ± 0.03 ^d
	day 9	0.79 ± 0.03 ^a	0.81 ± 0.01 ^a	0.71 ± 0.01 ^b	0.75 ± 0.02 ^b	0.74 ± 0.00 ^b	0.74 ± 0.01 ^b
ϕEo	day 3	0.42 ± 0.01 ^a	0.40 ± 0.02 ^a	0.23 ± 0.02 ^b	0.24 ± 0.01 ^b	0.28 ± 0.01 ^b	0.25 ± 0.00 ^b
	day 6	0.42 ± 0.01 ^a	0.41 ± 0.01 ^a	0.31 ± 0.01 ^b	0.31 ± 0.01 ^b	0.27 ± 0.01 ^c	0.25 ± 0.01 ^d
	day 9	0.37 ± 0.03 ^a	0.36 ± 0.01 ^a	0.36 ± 0.01 ^a	0.36 ± 0.01 ^a	0.32 ± 0.01 ^b	0.27 ± 0.00 ^c
ABS/RC	day 3	2.43 ± 0.23 ^a	2.51 ± 0.25 ^a	3.03 ± 0.44 ^a	3.69 ± 0.74 ^a	2.88 ± 0.20 ^a	3.16 ± 0.21 ^a
	day 6	2.36 ± 0.07 ^b	2.51 ± 0.09 ^b	2.40 ± 0.06 ^b	2.96 ± 0.04 ^{ab}	2.88 ± 0.34 ^{ab}	3.44 ± 0.54 ^a
	day 9	2.43 ± 0.06 ^b	2.93 ± 0.12 ^b	2.16 ± 0.12 ^c	2.26 ± 0.07 ^c	2.71 ± 0.20 ^b	3.31 ± 0.08 ^a
TRO/RC	day 3	1.28 ± 0.11 ^a	1.30 ± 0.13 ^a	1.16 ± 0.11 ^a	1.37 ± 0.28 ^a	1.16 ± 0.08 ^a	1.19 ± 0.10 ^a
	day 6	1.24 ± 0.03 ^a	1.29 ± 0.04 ^a	1.06 ± 0.02 ^b	1.24 ± 0.05 ^a	1.10 ± 0.17 ^b	1.28 ± 0.22 ^a
	day 9	1.24 ± 0.06 ^a	1.30 ± 0.02 ^a	1.08 ± 0.06 ^b	1.09 ± 0.03 ^b	1.19 ± 0.06 ^{ab}	1.19 ± 0.03 ^{ab}
ETo/RC	day 3	1.01 ± 0.09 ^a	1.00 ± 0.13 ^a	0.71 ± 0.06 ^b	0.90 ± 0.18 ^a	0.81 ± 0.06 ^{ab}	0.80 ± 0.05 ^{ab}
	day 6	0.99 ± 0.0 ^a	1.04 ± 0.03 ^a	0.73 ± 0.03 ^b	0.92 ± 0.04 ^{ab}	0.78 ± 0.11 ^b	0.84 ± 0.11 ^a
	day 9	0.98 ± 0.08 ^a	1.05 ± 0.01 ^a	0.77 ± 0.04 ^b	0.82 ± 0.04 ^b	0.88 ± 0.04 ^{ab}	0.88 ± 0.02 ^{ab}
DIo/RC	day 3	1.15 ± 0.12 ^a	1.21 ± 0.12 ^{ab}	1.87 ± 0.33 ^b	2.33 ± 0.46 ^b	1.71 ± 0.12 ^b	1.97 ± 0.11 ^b
	day 6	1.12 ± 0.06 ^a	1.22 ± 0.07 ^a	1.34 ± 0.04 ^{ab}	1.72 ± 0.03 ^b	1.79 ± 0.17 ^{bc}	2.15 ± 0.32 ^c
	day 9	1.42 ± 0.05 ^b	1.63 ± 0.09 ^b	1.08 ± 0.07 ^c	1.16 ± 0.04 ^c	1.52 ± 0.14 ^b	2.12 ± 0.06 ^a
PIABS	day 3	1.73 ± 0.23 ^a	1.43 ± 0.24 ^a	0.33 ± 0.09 ^b	0.32 ± 0.08 ^b	0.55 ± 0.06 ^b	0.40 ± 0.04 ^b
	day 6	1.86 ± 0.14 ^a	1.75 ± 0.12 ^a	0.74 ± 0.04 ^b	0.70 ± 0.05 ^b	0.54 ± 0.06 ^b	0.35 ± 0.10 ^c
	day 9	1.73 ± 0.29 ^a	1.13 ± 0.13 ^a	1.13 ± 0.09 ^a	1.08 ± 0.00 ^a	0.82 ± 0.10 ^{ab}	0.48 ± 0.01 ^b

4. Discussion

4.1. Selective Growth Inhibition of Cyanobacteria and the Promotion of Green Algae by Aqueous Extracts of *D. indicum* Flowers

A high phytoplankton abundance with a healthy community structure is necessary to improve fishery productivity and the water environment of aquaculture ponds [32,33]. Mechanical removal, water change, engineering measures, and investment in chemical algicides are normally used to control harmful cyanobacteria blooms occurring in the middle and late stages of pond culture [15,34,35]. Nutrient enrichment and the addition of beneficial algae are traditionally used to improve the phytoplankton abundance and structure during the early stage of aquaculture [36,37]. The present study provides a new way to promote green algae and inhibit *M. aeruginosa* simultaneously by aqueous extracts of *D. indicum* flowers. The anti-inflammatory and antimicrobial activities of *D. indicum* extract have been reported [38,39], and our findings reveal its potential to selectively control harmful cyanobacteria and regulate phytoplankton composition to improve fishery productivity in aquaculture ponds.

The concentration-dependent inhibition effects of the aqueous extracts of *D. indicum* flowers on *M. aeruginosa* were found in our bioassays. It is consistent with previous studies showing the concentration-dependent allelopathic effects of aquatic plants on cyanobacteria [40]. Their promotion effects on the green algae *C. vulgaris* were obvious with a slightly enhanced trend when the flower concentration increased from 0.5 to 2.0 g·DW·L^{−1}, whereas their concentration-dependent inhibition effects on *C. vulgaris* were obvious when the flower concentration ranged from 4.0 to 8.0 g·DW·L^{−1}. These results indicated that the added dose played a critical role in the selective inhibition of harmful cyanobacteria by the aqueous extracts of *D. indicum* flowers. The concentration range of the plant extracts to exhibit the selective inhibition of harmful cyanobacteria and the promotion of beneficial

eukaryotic algae needs to be ascertained at larger and longer timescales to ensure their successful application in the restoration of healthy phytoplankton communities in aquaculture ponds.

4.2. Chemical Characteristics of the Selective Inhibition Substances of *D. indicum* Flowers

The significant inhibition of *M. aeruginosa* and the promotion of green algae by aqueous extracts of *D. indicum* flowers were observed under identical culture conditions, excluding nutrients and light limitation or the impacts of any coexisting organisms. It indicated that there must be some water-soluble active substances derived from *D. indicum* flowers contributing to the selective inhibition effects on *M. aeruginosa*. There was no significant difference in inhibition effects on *M. aeruginosa* by aqueous extracts prepared with water at 25 °C and 100 °C. It indicated that the selective inhibition substances of *D. indicum* flowers were thermally stable. The methanol fraction eluted from SPE-enriched aqueous extracts of *D. indicum* flowers showed the strongest inhibition on *M. aeruginosa* compared to the other four fractions. It indicated that the most polar substances of *D. indicum* flowers were responsible for the selective inhibition of *M. aeruginosa*. These results together provided clues to reveal the basic chemical characteristics of selective inhibition substances of *D. indicum* flowers for *M. aeruginosa*. It was very helpful for us to identify the specific substances and their application in the ecological control of harmful cyanobacteria and the regulation of phytoplankton composition. Duddleoside, luteolin, chlorogenic acid, caffeic acid, and 3,5-dicaffeoylquinic acid were typical active substances of *D. indicum* flowers [41]. Flavonoids, including linatin, luteolin, quercetin, and apigenin, were detected in *D. indicum* flowers from various locations in China [42]. Luteolin showed the strongest inhibition effects on the growth and photosynthesis activity of *M. aeruginosa* from among the selected three flavonoids [43]. Whether these co-existing substances contribute to the selective inhibition of cyanobacteria needs further research.

4.3. Photosynthetic Activity of *M. aeruginosa* Treated with Water-Soluble Substances of *D. indicum* Flowers

The chlorophyll fluorescence transients and subsequent JIP test have been widely used to reflect the status and behavior of PSII in phototrophs, including plants and phytoplankton [31,44]. The obvious decrease in the fluorescence transients of *M. aeruginosa* treated with aqueous extracts of *D. indicum* flowers indicated that the successive reduction in the electron acceptor pools of PSII, including Q_A , Q_B , and PQ pool, was seriously affected by water soluble substances extracted from *D. indicum* flowers [31]. The parameter F_v/F_m , the maximum quantum yield of primary photochemistry, has been widely used to reflect the potential photosynthetic capacity of PSII in phototrophs [31,43]. The inhibition ratios of the F_v/F_m values of *M. aeruginosa* by aqueous extracts of *D. indicum* flowers and the methanol eluting fractions were higher than 20% in most cases. The parameter PIABS reflects the performance index on an absorption basis, which is the combination of three independent parameters, including RC/ABS, F_v/F_m , and ψ_o [31]. Its values decreased by more than 50% in the treatments with aqueous extracts of *D. indicum* flowers and the methanol eluting fractions. The extent of the change in PIABS was more consistent with the extent of the change in cell density and chl *a* of *M. aeruginosa* than F_v/F_m , which was also found in previous literature [44]. The significant decrease in ψ_o , ϕE_o , and ETo/RC indicated that the performance of electron transfer from Q_A^- to Q_B was seriously inhibited by polar active substances of *D. indicum* flowers, and the acceptor side of the electron transport of PSII was a target site. The parameter ABS/RC reflects absorption flux per reaction center [45]. The increase in ABS/RC for *M. aeruginosa* treated with aqueous extracts of *D. indicum* flowers and the methanol fraction indicated the changes in the RC number. Since the trapped energy flux per reaction center (TRo/RC) did not change significantly, the decrease in the electron transport flux per reaction center (ETo/RC) corresponded to the increase in the dissipated energy flux per reaction center (DIo/RC) [31].

4.4. The Application of Water-Soluble Substances of *D. indicum* Flowers in Aquaculture Ponds

The present study provided the possibility to use water-soluble substances of *D. indicum* flowers to selectively control harmful *M. aeruginosa* proliferation and regulate phytoplankton community structure in aquaculture ponds. However, verifying this possibility fully and revealing the

underlying mechanisms are necessary before its final application. The cyanobacteria proportion in total phytoplankton density treated with aqueous extracts of *D. indicum* flowers was obviously reduced in our outdoor mesocosms, but the dissolved carbon, nitrogen, and phosphorus concentrations were increased compared to the control (unpublished data). It indicated that a more ecological approach to regulate phytoplankton composition using *D. indicum* flowers was to input the specific responsible substances in aqueous extracts of *D. indicum* flowers in aquaculture ponds. Hence, identifying specific active substances was a crucial step to promote its application.

The well-known benefits to human health and antimicrobial activity also provided a possibility of *D. indicum* flowers to prevent and cure fish disease and promote the quality of aquatic products [28,29], which needs to be verified experimentally using specific substances isolated from *D. indicum* flowers. Medicinal plant derivatives have been widely used as immunostimulants [46]. Once the responsible substances isolated from *D. indicum* flowers were proved to be eco-friendly cyanocides, enough substance sources for wide application are necessary. In addition to extraction from *D. indicum* flowers, more plant sources need to be screened, and synthetic analogs are also feasible. Some plant-derived active compounds for medical, agronomic, and environmental applications have been successfully synthesized and commercially obtained [12,31].

5. Conclusions

The present study demonstrated the strong growth inhibition of toxigenic and non-toxicogenic *M. aeruginosa* and the obvious growth promotion of green algae, including *C. vulgaris*, *Kirchneriella* sp., and *H. pluvialis*, by aqueous extracts of *D. indicum* flowers. The responsible substances of *D. indicum* flowers were thermally stable with high polarity. The acceptor side of the electron transport of PSII was a target site of *M. aeruginosa* cells in response to water-soluble substances of *D. indicum* flowers. More experiments are required to identify the responsible active substances and reveal the underlying mechanisms of aqueous extracts of *D. indicum* flowers to selectively inhibit cyanobacteria and regulate the phytoplankton community structure.

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