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Allelopathic effects of submerged macrophyte *Chara vulgaris* on toxic *Microcystis aeruginosa*

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ABSTRACT

Microcystis aeruginosa is major bloom-forming cyanobacteria in eutrophic freshwater and the toxic microcystins secreted by it have proved hazardous to aquatic environment. In this study, we investigated the allelopathic effects of *Chara vulgaris* on growth and development of toxic *M. aeruginosa*. The allelochemicals of *C. vulgaris* were purified and identified and their allelopathic effects were studied in cyanobacterial assays. We found that reciprocal allelopathy exists between the *M. aeruginosa* and *C. vulgaris*. The *C. vulgaris* allelochemicals included 3 fatty acids [(Z,Z)-9,12 -octadecadienoic (ODEA, 18:2), tetradecanoic (TDA, 14:0) and hexadecanoic acids (HAD,16:0)], which inhibited the growth of toxic *M. aeruginosa* and the ODEA proved most potent. The combined activity of these three fatty acids exerted synergistic inhibitory effects on the growth of toxic *M. aeruginosa*. We have found that *C. vulgaris* and its allelochemicals may control the *M. aeruginosa* bloom and it may be useful to recover the eutrophic waters.

Keywords: Allelopathy**,** allelochemicals, *Chara vulgaris*, *Microcystis aeruginosa*

INTRODUCTION

In recent years, water bloom has become very serious problem in eutrophic freshwater body. *Microcystis aeruginosa* is most common bloom-forming cyanobacteria in eutrophic freshwater and the toxic microcystins secreted by it have become hazardous to aquatic environment. Recently the control of toxic cyanobacteria growth has drawn increasing attention, hence, some methods have been developed (i). use of yellow loess, clay, copper sulfate and (ii). biological agents, e.g. bacteria (10), viruses (2) and zooplankton (18) to control cyanobacteria blooms. Although these methods are effective in some short-term experiments, but their negative effects on environment have made them impractical (21,7,8). The aquatic macrophytes play major role in stabilizing the clear water state in shallow mesotrophic and eutrophic lakes. They improves the water condition (22) through many mechanisms including the excretion of substances inhibitory to phytoplankton growth (4,5,15). The charophyte meadow greatly improves the water transparency than other macrophytes (16), owing to rapid colonization (19,14) and fast

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emergence after the lake restoration through bio-manipulation (12,13). Some compounds (dithiolane and trithiane) isolated from *Chara globularis* inhibits the photosynthesis of epiphytic diatom *Nitzschia palea* Kützing (24,25).

To further explore the allelopathic effects of charophyte and to understand the mechanism of cyanobacteria inhibition, we studied (i). allelopathic effects of *Chara vulgaris* on toxic *Microcystis aeruginosa*, (ii). separated and identified the unknown allelochemicals from *C. vulgaris* and (iii) determined their effects on toxic *Microcystis aeruginosa* (to find the efficient cyanobacteria controlling agents, harmless to the environment). To develop new potential strategies for water treatment by using *C. vulgaris* and its allelochemicals.

MATERIALS AND METHODS

C. vulgaris was collected from a clear pond from our University Campus, and Toxic *M. aeruginosa* FACHB-942 was provided by Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China. Amberlite^{TM} XAD1180, (Z,Z)-9,12- octadeca-dienoic acid tetradecanoic and hexadecanoic acids were purchased from the Chemical suppliers in Beijing.

Chara vulgaris Allelopathic effects on *M. aeruginosa*

The young sprouts of *C. vulgaris* were chosen and washed in flowing water to remove debris and then rinsed in sterile water thrice. The cleaned plants were immersed in 70% ethanol for 30 s and 10% sodium- hypochloride solution for 10 s and then rinsed with sterile water thrice (6) and dried with sterilized filter paper. Sterilized *C. vulgaris* was cultured in 2000 mL beaker with 1200 mL Hoagland medium (0.1×). The beakers were covered by sterilized filter paper and then placed in an incubator for acclimatized culture using light intensity of 6000 lx at 25±1°C and photoperiod 14L:10D for 14 days. The *M. aeruginosa* was also inoculated into 500 mL sterilized flasks filled with 200 mL Hoagland medium (0.1×) and then placed in the same incubator and at the same conditions for acclimatization and culture, all the flasks were shaken four times at set time everyday (28, 29).

Based on pre-experiments, different concentrations (10^3, 10^5, 10^7cells /mL) of toxic *M. aeruginosa* FACHB-942 (exponential growth phase) were respectively co-cultured with above mentioned acclimatized cultured *C. vulgaris* (5 g fresh weight) in 500 mL Erlenmeyer flask containing 300 mL Hoagland medium (0.1×). The control groups were prepared with separate culture of *M. aeruginosa* (10^3, 10^5, 10^7cells/mL) and *C. vulgaris* (5g fresh weight). All the Erlenmeyer flasks were stuffed up with sterile silica gel-stoppered and cultured under the conditions as described above for 12 days. These experiments were repeated twice and replicated thrice. During the experiments, 1 mL cultural solution was collected from each flasks every other day to determine the *M. aeruginosa* growth by counting the numbers. The inhibitory ratio (IR) was calculated as under:

$$IR = 1 - (N_t / N_o) \times 100 \quad (11)$$

Where, N_t : Number of *M. aeruginosa* in treatment and N_o : Number of *M. aeruginosa* in control.

To evaluate the reciprocal responses between *M. aeruginosa* and *C. vulgaris*, at the end of experiments *C. vulgaris* was separated, rinsed with distilled water and its biomass was recorded and the growth inhibition (%) over control was also calculated as per above formula. Where N_t : Biomass of *C. vulgaris* in treatment and N_o : Biomass of *C. vulgaris* in control.

***Chara vulgaris* extraction:** The *C. vulgaris*, after cleaning and drying at room temperature, was ground into powder. Organic solvents with different polarities (ethyl ether, acetone, methanol and ethyl acetate) were used for extraction. All these solvents were purified before using. Soxhlet extractors were used for *C. vulgaris* powder extraction (9) and extraction was done for 12 h and the four extracts obtained were evaporated in vacuum at 50°C. The final powdered products were stored at 4°C for further use.

Extracts bioassays: The toxic *M. aeruginosa* FACHB-942 was inoculated into 250 mL sterilized flasks filled with 100 mL BG-11 medium. These flasks were placed in an incubator (light intensity of 4000 lx at 25±1°C and photoperiod 12L:12D) for 7 days and all flasks were shaken daily four times at set time (28,29).

The extracts from *C. vulgaris* were dissolved in 0.05 % dimethyl sulfoxide (DMSO, AR). At this concentration, DMSO would not be expected to influence the growth of *M. aeruginosa*, (26). *M. aeruginosa* (2 mL, in exponential growth phase) was inoculated into sterilized 50 mL flasks filled with 25 mL BG-11 medium, followed by adding dissolved extracts at the final concentration of 0.1mg/mL. The final density of *M. aeruginosa* was approximately 10^5-10^6 cells/mL. The control flasks contained all the aforementioned components except *C. vulgaris* extract. Each experiment was conducted thrice under the same cultural conditions. The mixture in flasks was incubated at 25±1°C for 5 days and all the flasks were shaken daily four times. At the end of incubation, the *M. aeruginosa* cells were counted and the inhibitory ratio of extracts on *M. aeruginosa* was calculated.

Extracts purification: Extract with the most potent inhibitory effect on *M. aeruginosa* was selected as mentioned above. The extract was purified by elution using ethyl ether, acetone, ethyl acetate, 80% and 100% methanol, respectively, through Amberlite^{TM} XAD_{1180} macroporous resin. The macroporous resin was packed into a glass column with a diameter to length ratio of 1:10. A separation funnel was fixed at the top opening of the burette with an elution rate of 1 mL/min. Fractions from the elute were collected and then the eluting reagents were vapourized by vacuum at 50°C. To further determine the anti-cyanobacterial activity of each fraction of the extract, the fraction was dissolved with 0.05% DMSO and assayed. The inhibitory effects of each fraction were evaluated by bioassay using *M. aeruginosa*. The fraction with the best inhibitory effect was analyzed by using a gas chromatograph equipped with a mass selective detector (GC-MSD, Agilent Science and Technology Co. Ltd., America).

Allelochemicals identification: GC-MSD analysis was carried out on HP 6890 GC, FID coupled with inert-5975 MSD, which was equipped with a HP-1 column (30 m × 0.32 mm × 0.25 μm). Injector temperature was 280°C; transfer line temperature was 280°C; oven temperature was kept at 70°C for 2 min, programmed to increase to 120°C at a rate of 8°C /min, kept constant at 120°C for 1 min, then programmed to increase to 280°C at a rate of 10°C /min, and then kept isothermal for 10 min. The carrier gas used was helium (99.99%) with the injected amount of 0.1 μL (split ratio 1:100); ionization energy was 70 eV; scan time, 1s; mass range, 20–550 amu.

Allelochemicals assays

The allelopathy of each identified compound on *M. aeruginosa* was evaluated in cyanobacterial assay. The allelopathic compounds were commercially obtained and were dissolved in 0.05% DMSO. A series of concentrations of the relevant compounds were used in the assay to generate a dose-response on the inhibition of *M. aeruginosa*. In control only 0.05% DMSO was used without any allelopathic compound. The samples were cultivated under the aforementioned conditions for 6 days. The numbers of *M. aeruginosa* were monitored daily to assess the inhibitory effects of observed compounds on cyanobacteria. The half inhibiting concentration ($EC_{50,}$ i.e., the concentration of each allelochemical from *C. vulgaris* that inhibits normal growth of alga by 50%, mg/L) was obtained using a regression equation of allelochemical concentration and the inhibitory ratio.

Collective activity of allelochemicals: The collective activity of allelochemicals on inhibition of *M. aeruginosa* was studied using previously described methods (17). Briefly, experimentally obtained inhibitory effects from a mixture of the identified allelochemicals was compared with those predicted, based on the sum of inhibitory effects from each individual allelochemical. The predicted inhibitory effect (PIE) of a mixture of the allelochemicals was calculated by Colby's equation (1),

i.e., $\text{PIE}[\%]= (\underline{A}\times\underline{B}\times\underline{C}\times\ldots\times\underline{N})/(100)^{n-1}$

Where A, B, C, . . .N : Normalized maximum growth with allelochemical $\underline{A}$, $\underline{B}$, $\underline{C}$,…$\underline{N}$ at the added amount a, b, c,…n ug/L, respectively, n: Total number of allelochemicals considered.

PIE : The predicted normalized maximum growth of *M. aeruginosa* when inhibited by a mixture of allelochemicals, hence, the inhibition (%) was Total percentage (100%) minus the PIE (%).

Statistical analysis: SPSS 13.0 programmes were used for the statistical analysis. A one-way analysis of variance (ANOVA) was applied in comparing the *M. aeruginosa* inhibitory ratios by *C. vulgaris* and the allelochemicals under different conditions, and the differences in *M. aeruginosa* densities and *C. vulgaris* biomasses among the control and treatment were assessed using paired *t*-test.

RESULTS

Allelopathic interactions between *Chara vulgaris* and toxic *M. aeruginosa*

Different densities of toxic *M. aeruginosa* had differently responded to *Chara vulgaris* (Fig. 1). In first 6-days, the *Chara vulgaris* had no inhibitory effects on *M. aeruginosa* at lower density (10^3 cells/mL). The inhibitory effects increased at the moderate density (10^5 cells/mL). *C. vulgaris* magnitude of inhibition on *M. aeruginosa* decreased at higher density (10^7 cells/mL). At day 12, the Inhibition (%) over control (IRs) were 12.07, 89.16 and 29.21% for 10^3, 10^5, 10^7 cells/mL groups, respectively (Fig. 2). 10^5 cells/mL group showed the strongest allelopathic inhibitory effect, the IRs of the three groups analysed by paired *t*-test, the IR of the moderate density (10^5 cells/mL) group had significant differences from those of the other two groups ($p<0.05$).

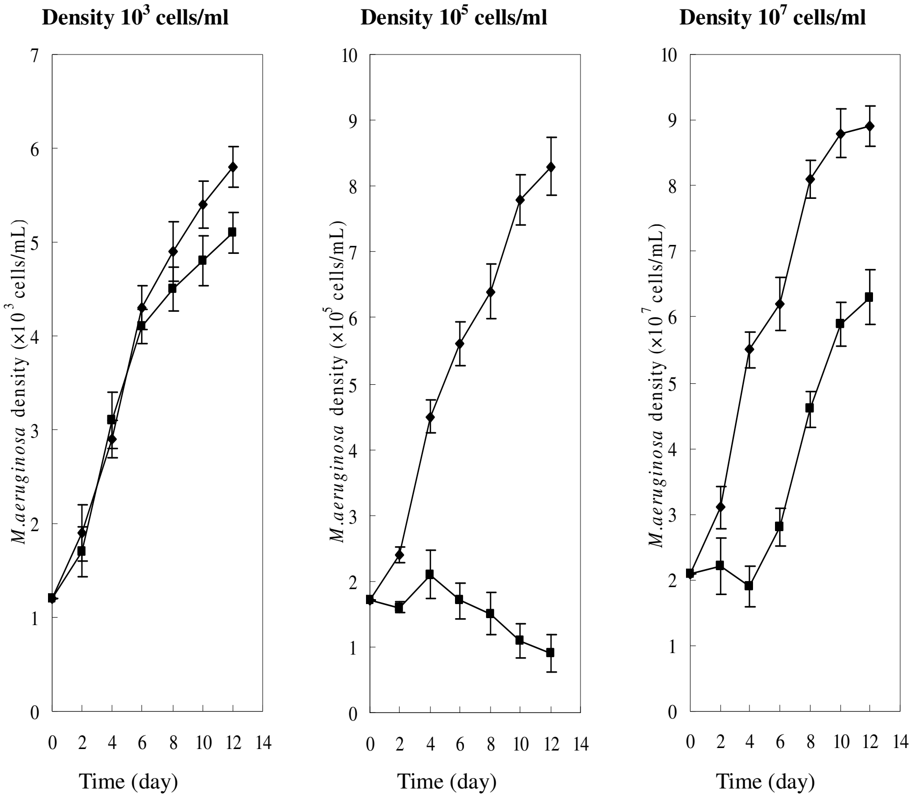

Figure 1. Allelopathic inhibitory effects of *C. vulgaris* on difference densities of *M. aeruginosa* (□ Controls ■ Treatments)

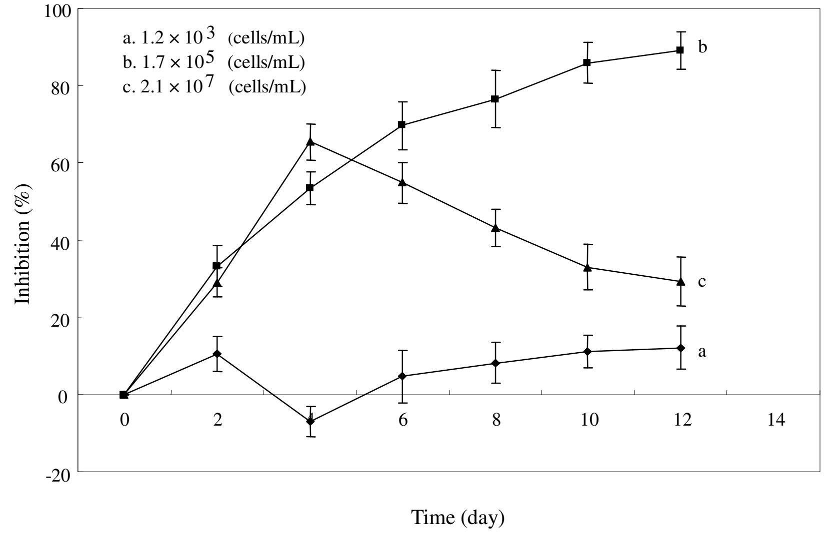

Figure 2. The Inhibition (%) over control (IRs) of *M. aeruginosa* treated by *C. vulgaris*

While, the biomass of *C. vulgaris* in all groups changed differently on day 12. The Inhibition (%) over the control were 3.12, 5.27 and 40.63% for 10^3, 10^5, 10^7 cells/mL groups, respectively (Fig. 3), only 10^7 cells/mL group the biomass of *C. vulgaris* decreased obviously, and the IR compared with the control had significant difference ($p<0.05$).

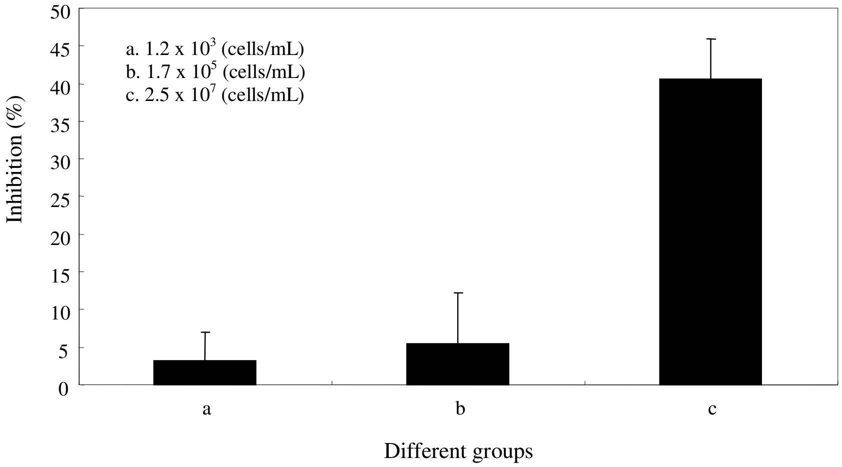

Figure 3. The Inhibitions (%) over control (IRs) of *C. vulgaris* in various densities of *M. aeruginosa* groups

A certain amount of target organism stimulated the synthesis and release of allelochemicals. Allelopathy occurs only when the target organism that reached threshold stimulus. Besides, the allelopathic interactions intensity is correlated to appropriate contents. That is why the IRs of 10^3 and 10^7 cells/mL groups to *M. aeruginosa* were much lower than 10^5 cells/mL group, because the density of *M. aeruginosa* in 10^3 cells/mL group was not high enough to stimulate the *C. vulgaris* to release the allelochemicals; whereas, the *M. aeruginosa* density in 10^7 cells/mL group was so high that the cyanobacteria itself can produce and release lots of allelochemicals such as microcystins etc and contrarily inhibited the growth of *C. vulgaris*. We found that *C. vulgaris* effectively inhibited the growth of *M. aeruginosa* in 10^5 cells/mL group, this suggested that *M. aeruginosa* can be controlled by *C. vulgaris* in their appropriate contents.

Allelopathy of *Chara vulgaris* extracts

All four extracts of *C. vulgaris* inhibited the *M. aeruginosa* growth by 23~87% than control (Table 1). However, the methanol extract was most potent (87.53% inhibition over the control). Therefore, methanol extract was used for further isolation of allelochemicals.

Table 1. Inhibitory effects of different extracts of *Chara vulgaris* on the growth of *M. aeruginosa*

Inhibiting substances conc (0.1 mg / mL)	*M. aeruginosa* density ($\times10^5$ cells /mL)	Inhibition (%) over control (IRs)
Control	18.41 ± 2.61	
Ethyl ether extract	11.79 ± 3.53	35.95 ± 0.91*
Acetone extract	11.33 ± 2.85	38.44 ± 0.68*
Methanol extract	5.74 ± 1.72	68.81 ± 0.23* *
Ethyl acetate extract	12.42 ± 3.64	32.53 ± 0.67*

*p<0.05; * *p<0.01 vs. control.

The methanol extract was eluted with ethyl ether, actone, ethyl acetate, and methanol (80 and 100%) and five fractions were obtained. These fractions were assayed for their inhibitory effects on the cyanobacterial growth. Among these five fractions, the elutions using 80 and 100% methanol were more inhibitory to *M. aeruginosa* and caused 52.33 and 83.72% reductions, respectively (Table 2). The remaining 3 elutions with ethyl ether, actone, ethyl acetate were less potent. Based on these results, we used the fraction from 100% methanol eluate to analyze the possible compounds by GC-MSD.

Table 2. Allelopathic effects of various fractions of *Chara vulgaris* methanol extract on the growth of *M. aeruginosa*

Fractions conc (0.05 mg / mL)	*M. aeruginosa* density ($\times10^5$ cells /mL)	Inhibition (%) over control (IRs)
Control	19.37 ± 1.83	
Ethyl ether eluate	14.86 ± 2.71	23.28 ± 2.65*
Acetone eluate	13.49 ± 2.15	30.35 ± 1.92*
Ethyl acetate eluate	15.48 ± 2.53	20.82 ± 2.16*
80% Methanol eluate	8.64 ± 1.76	55.39 ± 1.27* *
100% Methanol eluate	5.83 ± 2.64	77.58 ± 3.62* *

*p<0.05; * *p<0.01 vs. control.

Allelochemicals Identification

GC-MSD analysis showed that multiple peaks appeared on the total ion chromatogram for the eluate of 100% methanol. By comparing the mass spectral peak patterns with those stored in mass spectral library of U.S. National Institute of Standards and Technology (NIST 02.L), three compounds viz., tetradecanoic acid (TDA, 14:0), hexadecanoic acid (HAD, 16:0) and (Z,Z)-9,12- octadecadienoic acid (ODEA, 18:2) were identified from the elute of 100% methanol.

Anti-cyanobacterial effect of identified fatty acids

To assess the effect of above compounds on *M. aeruginosa* growth, the cyanobacterial assay was conducted using each identified fatty acid at different concentrations (ODEA 0.01, 0.05, 0.10 mg/L; TDA 4.00, 8.00, 12.00 mg/L; HAD 5.00, 10.00, 15.00 mg/L, respectively). All three fatty acids inhibited the growth of *M. aeruginosa* in a concentration- and time-dependent manner (Fig. 4). ODEA caused maximum growth inhibition (EC_{50} of 0.042 ± 0.012 mg /L) of *M. aeruginosa*. TDA and HDA were less in inhibitory to the growth of *M. aeruginosa* and their EC_{50} were, 12.799 ± 0.471 and 17.167 ± 0.794 mg /L, respectively. Thus ODEA, TDA and HAD might be the allelochemicals that contributes to the inhibitory effect of *C. vulgaris* on *M. aeruginosa.*

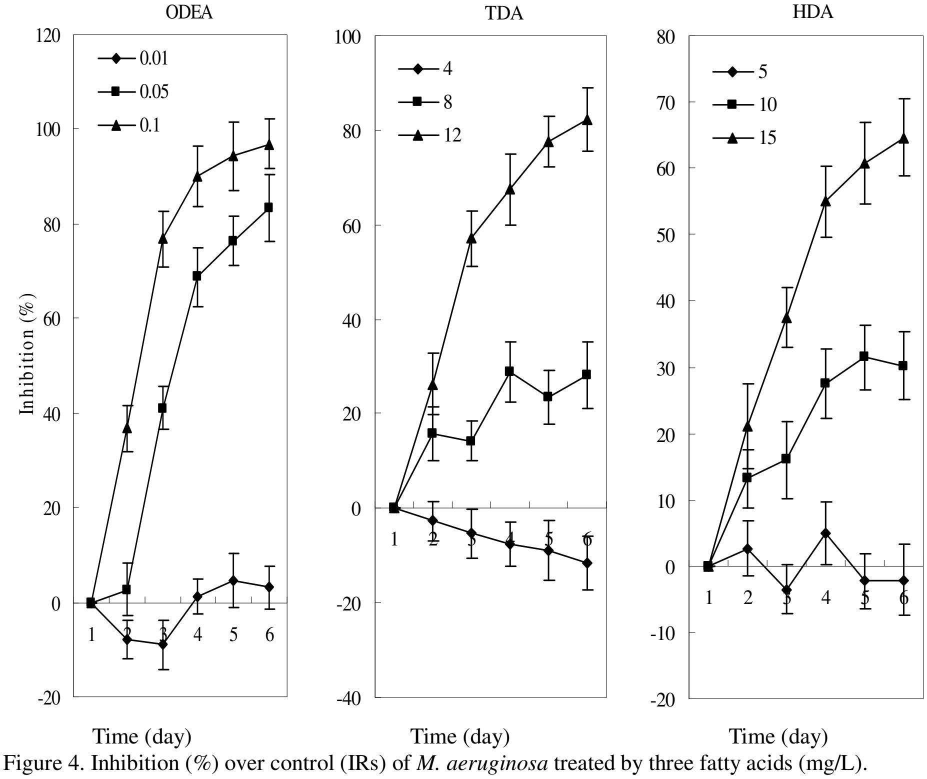

Figure 4. Inhibition (%) over control (IRs) of *M. aeruginosa* treated by three fatty acids (mg/L).

Collective activity of identified fatty acids

To study the collective activity of ODEA, TDA and HAD, these were mixed and used in the cyanobacterial assay. The final concentrations of each fatty acid in mixture was determined based on its individual inhibitory effect (as shown in Figure 4), i.e. 0.03, 10.67 and 16.55 mg/L for ODEA, TDA and HAD, respectively. A synergistic action on *M. aeruginosa* inhibition occurred when ODEA, TDA and HAD were used together (Fig. 5). The mixtures drastically inhibited the growth of *M. aeruginosa* by 95.1% on sixth day of incubation, the experimental inhibitions (%) compared to predicted inhibitions (%) were significantly different ($p<0.05$). Considering that relatively low concentrations of these fatty acids were used, this combination might be potentially safe to other water living organisms, while still effective in controlling cyanobacterial growth, thus would be practically useful to treat the water-blooms.

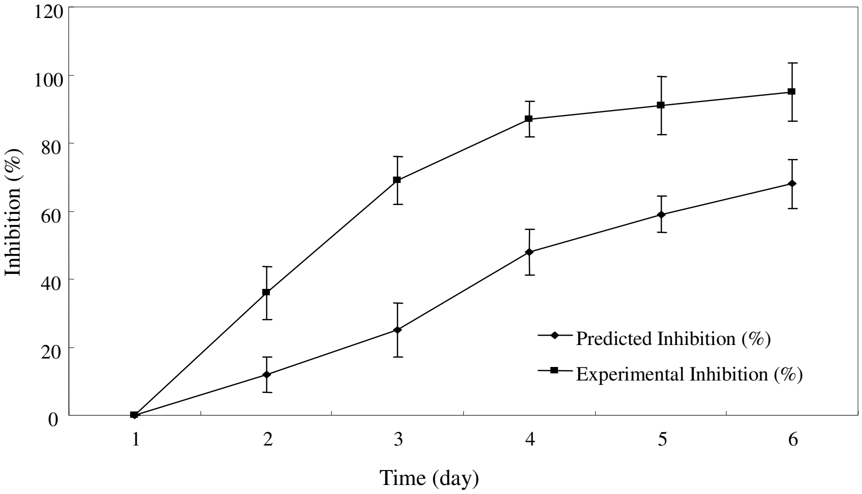

Figure 5. Inhibition (%) over control (IRs) of *M. aeruginosa* treated by mixtures of three fatty acids

DISCUSSION

Allelopathy plays major role in the population dynamics and species distribution within an ecosystem (20). Low phytoplankton densities in *Chara* dominated water systems have led to the hypothesis of allelopathy (3). Our results demonstrated that allelopathic interactions occured between the *M. aeruginosa* and *C. vulgaris* in coexistent assays, and when in their appropriate contents the *C. vulgaris* dominates through inhibiting the growth of *M. aeruginosa*. This view agrees with other researchers', e.g. allelopathic interactions between the two different organisms are density dependent and affects their mutual growth even in the presence of an adequate nutrient supply (23).

In our study, we didn't measure the nutrients consumed, because the nutrition in the medium was sufficient for the organisms during the study period (6,27). There was no light competition due to different growth patterns of *C. vulgaris* being submerged and *M. aeruginosa* floating, respectively. Besides, we used full side lights in the incubator. Compared to recorded irradiance levels (≈30–130 μmol m^{-2} s^{-1}) for cyanobacterial or aquatic plants culture (6), we kept irradiance at 6000 lx i.e. about 120 μmol m^{-2} s^{-1}) to maintain adequate growth of *C. vulgaris* and *M. aeruginosa*.

We isolated and identified 3 allelochemicals (ODEA, TDA and HAD) from *C. vulgaris* and all these significantly inhibited the growth of *M. aeruginosa*. The inhibitory effects of each individual fatty acid was concentration-dependent and they showed synergistic inhibitory effects when used together. Among the three fatty acids, ODEA was unsaturated fatty acid; TDA and HAD were saturated fatty acids and TDA had short carbon chain. ODEA proved most potent to inhibit the growth of *M. aeruginosa,* indicating that the unsaturated fatty acid might be more powerful than saturated fatty acid. Among the two saturated fatty acids TDA and HAD, TDA was more inhibitory to the *M. aeruginosa* growth than that of HAD. It suggested that the fatty acid with a shorter carbon chain caused more growth inhibition in *M. aeruginosa*. The remaining question is, how these fatty acids inhibits the cyanobacteria and how the microcystins secreted by cyanobacteria inhibits the *C. vulgaris* growth.

This is the first study to show (i). the allelopathic interactions between the *M. aeruginosa* and *C. vulgaris,* (ii). dominative allelopathic inhibition of *C. vulgaris* on *M. aeruginosa* and (iii). to identify and bio-test the allelochemicals of *C. vulgaris* and their inhibitory effects on *M. aeruginosa*. The study may help in developing new strategies to control cyanobacteria using the *C. vulgaris* and its allelochemicals prior to cyanobacteria blooms.

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