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Inhibitory effects of *Cyperus alternifolius* on growth of *Microcystis aeruginosa* and identification of algicidal substances

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ABSTRACT

We tested the inhibitory effects of *Cyperus alternifolius* aquatic weed on growth of *Microcystis aeruginosa* and also identified some allelochemicals released by this aquatic plant. We found that the accumulation of allelochemicals in planting water solutions inhibited the growth of *M. aeruginosa*, especially after long cultivation time, which caused the accumulation of allelochemicals. The inhibitory rates of planting water solutions 5, 10 and 15 days after culture on *M. aeruginosa* were 29.3 %, 44.5 % and 59.0 %, respectively. Eleven compounds 8-fatty acids and 3-phenolics, with potential allelopathic activity, were identified in the extracts of planting water. These findings provide a basis for the use of artificial floating-bed plants to control the toxic cyanobacteria.

Key words: Accumulation, algicidal compound, allelochemicals, cyanobacteria, *Cyperus alternifolius*, *Microcystis aeruginosa*, fatty acids, phenolics.

INTRODUCTION

Microcystis blooms in eutrophic water bodies is a global problem, as it decreases the dissolved oxygen content and produces odour compounds and toxins (9,20). Monitoring and controlling the growth of undesirable algal blooms is of urgent concern in aquatic microbiology (1,22). The discovery of allelopathy in plants and microorganisms brought up the possibility of controlling the microalgal growth using the aquatic plants (5). Biological control based on the allelopathic effects between macrophyte and *Microcystis* blooms are of particular interest due to their low cost, practicability and long-term ecological safety.

Many studies have shown the feasibility of inhibiting the microalgae growth with aquatic plants. After investigating the allelopathic effects produced by nine species of macrophytes, Nakai *et al*. (13) found that *Myriophyllum spicatum* produced strong inhibitory effects on two species of harmful cyanobacteria (*Microcystis aeruginosa* and *Phormidium tenue*). In addition, other researchers showed that submerged plants *Elodea nuttalli*, *Hydrilla verticillata* and *Vallisneria spiralis* adversely influenced the algal growth (4). Also emerged aquatic plants [*Stratiotes aloides* (11) and *Thalia dealbata* (26) and floating *Pistia stratiotes* (23)], significantly inhibited the growth of *M. aeruginosa*. The latter study also showed that root exudates from *P. stratiotes* were very inhibitory to *M. aeruginosa* growth than leaf leachates, leaf volatilization and residue decomposition.

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With soil-less culture technology it is possible to use aquatic plants as artificial floating-beds to inhibit the microalgae growth. In a floating-bed system, the roots of aquatic plants are in close contact with microalgal cells, resulting in stronger allelopathic effects. Some plants that have been used in artificial floating-beds, such as *Typha angustifolia*, *Scirpus tabernaemontani* and *Phragmites australis*, have shown anti-cyanobacterial allelopathic effects (15). *Cyperus alternifolius* (family Cyperaceae) is a perennial plant with evergreen foliage and has often been used for artificial floating-beds. It has high efficiency in assimilating the excessive nutrients (8) and other micro-pollutants such as oxybenzone (2). Moreover, *C. alternifolius* inhibited the growth of *M. aeruginosa* through oxidative damage in semi-continuous co-culture system (28). In co-culture the growth of *M. aeruginosa* was inhibited by the allelochemicals released into the water (21,23). In that case, polyphenols and fatty acids caused the allelopathic effects (12,14).

Studying allelopathic effects of macrophytes on phytoplankton must be done avoiding the interference of light and nutrients as growth limiting resources. However, in a co-culture system, manipulating light and nutrient conditions may also influence the production of inhibitory compounds of algal growth. Therefore, to better understand the allelopathy in aquatic plants, it is crucial to study the effects of allelochemicals with the individual isolated compounds. Many studies investigated the extracts of aquatic plant tissues and identified allelochemicals such as phenolic acids, fatty acids and terpenoids (24). However, the compounds isolated from the extracts of aquatic plant tissues are not necessarily released into the planting water. Therefore, assessment of allelochemicals that are really released in to planting water is necessary. However, studies about anti-cyanobacterial allelochemicals found in the planting water of *C. alternifolius* are scarce.

This study aimed to evaluate the allelopathic effects of *C. alternifolius* planting water on growth of *M. aeruginosa*. The effects of collection time of planting water and growth stages of C. *alternifolius* on allelopathic activity were also studied. The allelochemcials identified in *C. alternifolius* planting water, to provide new insights into the allelopathic mechanisms of *C. alternifolius* against *Microcystis* overgrowth in natural water bodies.

MATERIAL AND METHODS

I. Plants and Cyanobacterium

The study was conducted from April, 2017 to January, 2018. *C. alternifolius* plants were collected from Zhuanghang Experimental Base, Shanghai Academy of Agricultural Sciences (longitude: 121.39, latitude: 30.89, mean height above sea level: 4 m, annual rainfall: 1200 mm, maximum and minimum temperature: 38 ℃ and -3 ℃). Prior to experiments, *C. alternifolius* plants were acclimatized to natural river water for 10 days. *M. aeruginosa* (Kützing) Lemmermann (NIES-44), obtained from the National Institute for Environmental Studies (NIES collection, Tsukuba, Japan) was used for the cyanobacterial assays. The strain was isolated from Lake Kasumigaura Ibaraki, Japan in August 1974 and was maintained in axenic conditions. The cell size (minimum-maximum dimensions) was 6-8 μm. *M. aeruginosa* was cultivated under light of 20-30 μmol photons m^{-2} s^{-1} (light:dark cycle, 10 h: 14 h) in a modified C (CB) medium (12) at 25 °C.

II. Inhibitory effects of *C. alternifolius*

To study the inhibitory effects of *C. alternifolius* plants at different times of cultivation, the plant roots were carefully washed and transplanted into plastic bucket

containing 8 L distilled water. On day 5, 10 and 15 day, 500 mL samples of planting water from inside the plastic buckets were collected and filtered with glass microfiber filters (GF/F 47 mm, 0.52 μm) (Whatman GE) and stored at 4 °C for further experiments. Every time planting water was sampled from the plastic bucket, *C. alternifolius* plants were taken out and weighed on an electronic scale (ME4002E, Mettler Toledo, Switzerland).

To study the inhibitory effects of *C. alternifolius* in different stages, 500 mL samples of planting water were collected from three different stages. The first growth stage, was a small plant (211.7 g) and the next two growth stages of the same plant were at 15 and 30 days. In this experiment, *C. alternifolia* were cultured in same conditions as above. The fresh weights of whole plant and root were measured on each sampling. Before the further allelopathy experiment, the planting water samples from the second and third stages were diluted to the same fresh weight proportion as the first stage (26.5 g L^{-1}).

To perform the allelopathy experiments, planting water samples from the two experiments described above were processed in the same way. Nutrient levels were adjusted to those in CB medium. 10 mL of planting water samples were sterilized by filtration using Millex -GP needle filters (33 mm dia, 0. 22 μm pore-size, Millipore, USA). These 10 mL samples were placed into a 50 mL Erlenmeyer flask, to which 100 μL of algae suspension were immediately inoculated. A final concentration of approximately 10^5 cells mL^{-1} from exponential growth phase was used for these allelopathy experiments. The experiments lasted 15 days. The *M. aeruginosa* cell abundances were monitored every three days using a microscope hemocytometer (XB-K-25, Anxin Optical Instrument Manufacturing Co., Ltd, China). Control groups were prepared with sterilized CB medium and algae solution. Each experiment was performed in triplicates.

III. Collection of root exudate concentrates

The root exudate concentrates (EC) in the planting water were collected by a solid extraction procedure. First, 4 mL of methanol were used to activate the solid extraction column (Oasis HLB, Waters) that was cleaned by distilled water at pH 1.5 adjusted by hydrochloric acid. The filtered planting water collected in the first experiment of section II was also adjusted to pH 1.5 using hydrochloric acid. Then, 150 mL of sample were passed through the cartridge at flow rate of 3.0 mL min^{-1}. The adsorbates in the extraction cartridge were eluted with 20 mL methanol. Finally, the volume of elution was reduced to 5 mL under reduced pressure with rotary evaporator. The EC was stored at 4 °C before use in the following analysis.

IV. Inhibitory effects of three identified allelochemicals

To evaluate inhibitory effects of the allelochemicals from *C. alternifolius* that were identified, three of these compounds were chosen. We tested Dodecanoic acid (DA), tetradecanoic acid (TA), 2,6-bis(dimethylethyl)-4-methyl phenol (BHT) and a mixture of all these components. The components in the individual solutions were prepared at concentrations of 0.1, 1.0, 2.5 and 5.0 mg L^{-1}. The mixture solution contained the same concentration of each compound as in the individual compound solution. These solutions were tested in 96 h bioassay with *M. aeruginosa*. All the compounds were purchased from Sinopharm Chemical Reagent Co., Ltd, China. The compounds were dissolved in methanol and filtered through an autoclaved membrane filter (0. 22 μm, Millipore, USA), after which 1 mL of this filtrate was added to 100 mL of sterile CB medium. *M. aeruginosa* was immediately inoculated (about 10^5 cell mL^{-1}). The control groups were prepared by adding 1 mL filtered methanol into algae medium.

V. Analytical methods

Dissolved organic carbon (DOC) concentrations on each tested plant culture solution were determined by a total organic carbon analyzer (TOC5000, Shimadzu).

The allelochemical component of the secretion concentrate was analyzed by high-resolution gas chromatography mass spectrometry (GC-MS, Hewlett Packard 6890 series/ 5973 series) equipped with a HP-5 column (30.0 m□250 μm, 0.25 μm). The GC condition was as follows: injection temperature was at 250 °C, oven temperature was at 100 °C for 1 min and programmed to increase to 180 °C at a rate of 8 °C/min, then a further increase to 280 °C at a rate of 4 °C/min, which was kept constant for 10 min. The 2 mL secretion concentrate evaporated at 40 °C under a gentle stream of nitrogen. The resultant residue was treated with 200 μl N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) for 3 h at 50 °C for trimethylsilyl (TMS) derivatization. After removing the excess BSTFA under a nitrogen stream for 10 min, the sample was dissolved in a small volume of ethyl acetate (40 μL) and analyzed by GC/MS in the electron ionization mode. The compounds were identified from the mass spectra patterns after matching with the mass spectral library version 2 at the U.S. National Institute of Standards and Technology.

VI. Statistical analysis

The inhibitory effects of *C. alternifolius* on the *M. aeruginosa* growth were estimated by inhibitory rate (IR), calculated as under:

$$IR\ (\%) = (1-N/N_0)*100$$

Where N_0 and N are the algal cell numbers in the control group and the treatment group, respectively. Results were expressed as mean values ± standard deviation (SD). Significant differences among the experimental results were determined by one-way analysis of variance (ANOVA) followed by the least significance difference (LSD). The semi-effective concentration (EC_{50}) was calculated using probit regression. All the statistical analysis was performed by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

In control group with planting water collected at different times, *M. aeruginosa* grew from 10^5 to 10^7 cells mL^{-1} during the 15-day period. In treatment groups, the growth of *M. aeruginosa* was inhibited 6 days after incubation, although the differences were not always significant. The cell densities between the control and treatment groups were significantly different [Fig. 1(a)] 12-days after the incubation with planting water collected on 5th day ($F = 7.90$, $p < 0.05$) and 10th day ($F = 14.67$, $p < 0.05$). With planting water from the 15th day, the cell abundances of *M. aeruginosa* in treatment group differed from control 12 days after incubation ($F = 89.49$, $p < 0.01$). After 15 days of incubation with the planting water extracted from the 5, 10 and 15 days, the IRs of *M. aeruginosa* were 15.1 %, 47.1 % and 66.7 %, respectively. The fresh weight (FW) of whole *C. alternifolius* plant increased from 102.2 to 196.4 g L^{-1} from 5 to 15 days [Fig. 1(b)] and the DOC of the collected planting water changed from 1.58 mg L^{-1} on 5th day to 2.34 mg L^{-1} on 15th day (Fig. 1).

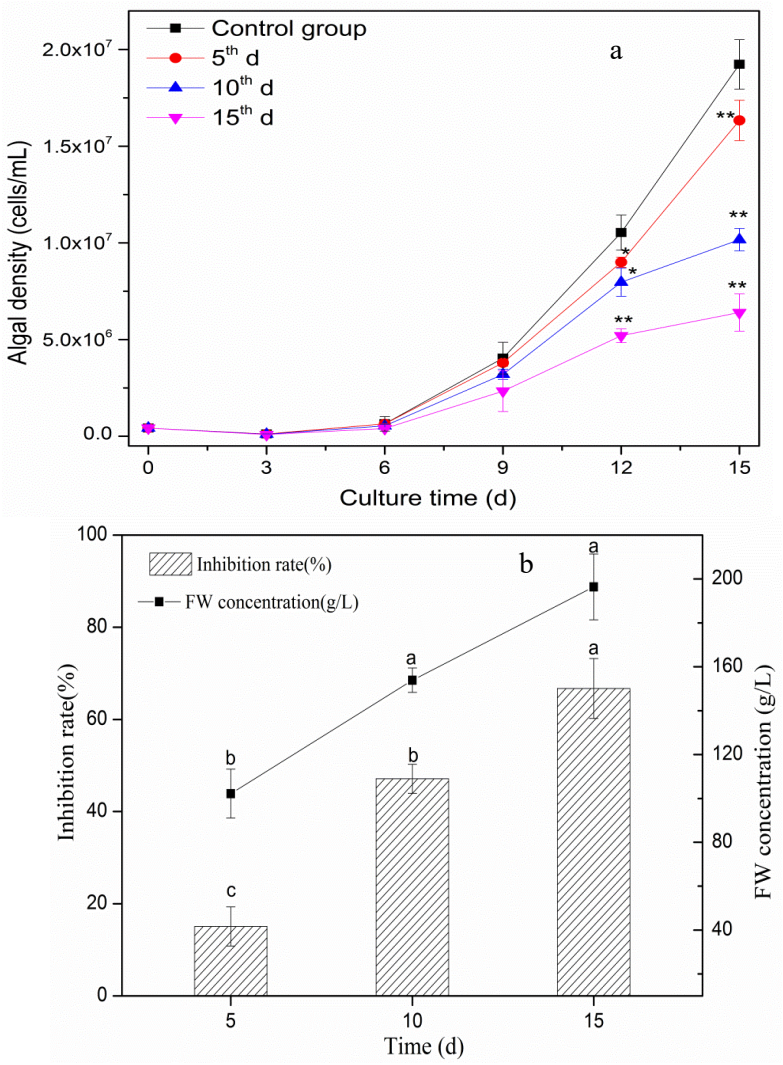


Figure 1. (a) Cell density of *M. aeruginosa* during the 15-day exposure planting water of *C. alternifolius* collected on different days. (b) Inhibition rate of planting water collected at different time periods after the 15-day co-culturing with *M. aeruginosa* and the change of fresh weight (FW) concentration of *C. alternifolius* at various collection time periods. Lower case letters a, b and c indicate statistically significant differences (one-way ANOVA) among the different inhibition rates and FW concentrations of *C. alternifolius* at various time periods. Data are presented as the mean ± standard deviation ($n = 3$). The significant difference versus control group is (*$p < 0.05$, ** $p < 0.01$).

Growth inhibition by allelochemicals released from macrophytes is the direct evidence of allelopathic activity against phytoplankton (12,14). The use of floating-bed plants allelochemicals could represent a novel and safe method to control the algal blooms.

The present research suggested that the planting water of one kind of floating-bed plant, *C. alternifolius*, could significantly inhibit the growth of *M. aeruginosa*. These inhibitory effects were related to the time of collection of the planting water and the plant biomass. The accumulation of allelochemicals in planting water probably contributed to increase of DOC with plant culture time, due to the continuous release of these compounds.

In studying the allelopathic effects on microalgae, the effects of growth phase need to be considered (11). Even though cells in exponential growth phase were used, the cell abundances did not change in the initial culture stages, even in control groups [Fig. 1(a)]. This may be due to their adaptation to new growth conditions. After this initial phase, by day 6, the growth in all groups increased considerably. Meanwhile, the allelochemicals started to reduce the growth of *M. aeruginosa* in treatment groups.

In the experiment using planting water from different growth stages of *C. alternifolius*, the inhibition rates were normalized according to the fresh weight for each collecting time. At day 15, the FW of *C. alternifolius* were 26.5, 68.8 and 204.7 g/L for each of three growth stages. The minimum plant FW was the standard for dilution of planting water in other growth stages. The IRs of planting water collected were 33.2 %, 34.4 % and 35.5 %, respectively for each stage, none of which were significantly different (Fig. 2). The inhibitory effects of different growth stages of *C. alternifoila* were not significant against *M. aeruginosa*. These results were consistent with previous researches (6, 7, 23) which showed that it is the plant biomass rather than growth phase that determines the effects on algal inhibition (Fig. 2). The compounds present in planting water of *C. alternifolius* were identified by GC-MS. A total ion chromatogram of EC of the *C. alternifolius* planting water is shown in Fig. 3. Eight fatty acids (Butanedioic acid, 2,3-dihydroxy propanoic acid, 2-hydroxy heptanoic acid, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, 9-cis-octadecenoic acid, and octadecanoic acid) and three phenolic compounds (2,6-bis(dimethylethyl)-4-methyl phenol, 4-hydroxybenzoic acid, and 1,2-benzenedicarboxylic acid) were identified after matching the mass spectra patterns. (Fig. 4). Their retention time and predicted formula structure are shown in Table 1. The allelochemicals from *C. alternifolia* planting water have rarely been identified, but certain species of *Cyperaceae* - i.e., *C. rotundus* (16, 18), *C. longus* (10), *C. esculentus* (17), *C. kyllingia* and C. *brevifolius* (19) are known to contain allelochemicals (sesquiterpenes, dicarboxylic, phenolic and fatty acids). Reported EC_{50} values were 117 mg L^{-1} for butanedioic acid (25), 0.42 mmol L^{-1} (58.01 mg L^{-1}) for 4-hydroxybenzoic acid (27), 4.56 mg L^{-1} for dodecanoic acid, 15.50 mg L^{-1} for tetradecanoic acid, 18.23 mg L^{-1} for hexadecanoic acid, 19.85 mg L^{-1} for octadecanoic acid and 1.33 mg L^{-1} for 9-cis-octadecenoic acid (4). The allelopathic effects of 4-identified allelochemicals (2,3-dihydroxy propanoic acid, 2-hydroxy heptanoic acid, 2,6-bis(dimethylethyl)-4-methyl phenol, and 1,2-benzenedicarboxylic acid) on *M. aeruginosa* are reported first time. (Fig. 3, Fig. 4 and Table 1).

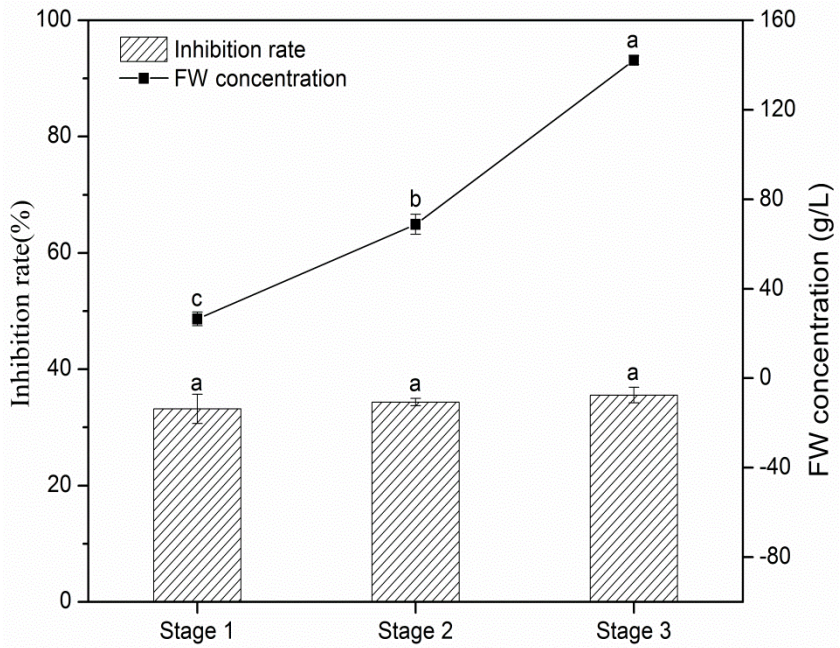


Figure 2. Inhibition rate on *M. aeruginosa* of planting water with the same fresh weights (FW) of *C. alternifolius* in three growth stages after 15-day cultivation. Lower case letters a, b and c indicate statistically significant differences (one-way ANOVA) among the different inhibition rates and FW of *C. alternifolius* in different growth stages. Data are the mean ± standard deviation (n = 3).

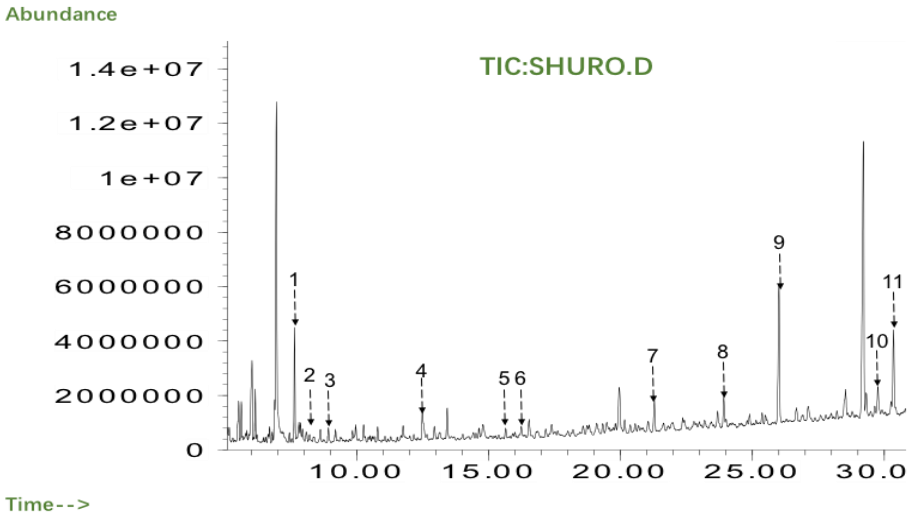


Figure 3. A total ion chromatogram of the solid extract of the *C. alternifolius* culture solution (1-11 is the compound numbers in Table 1).

a

b

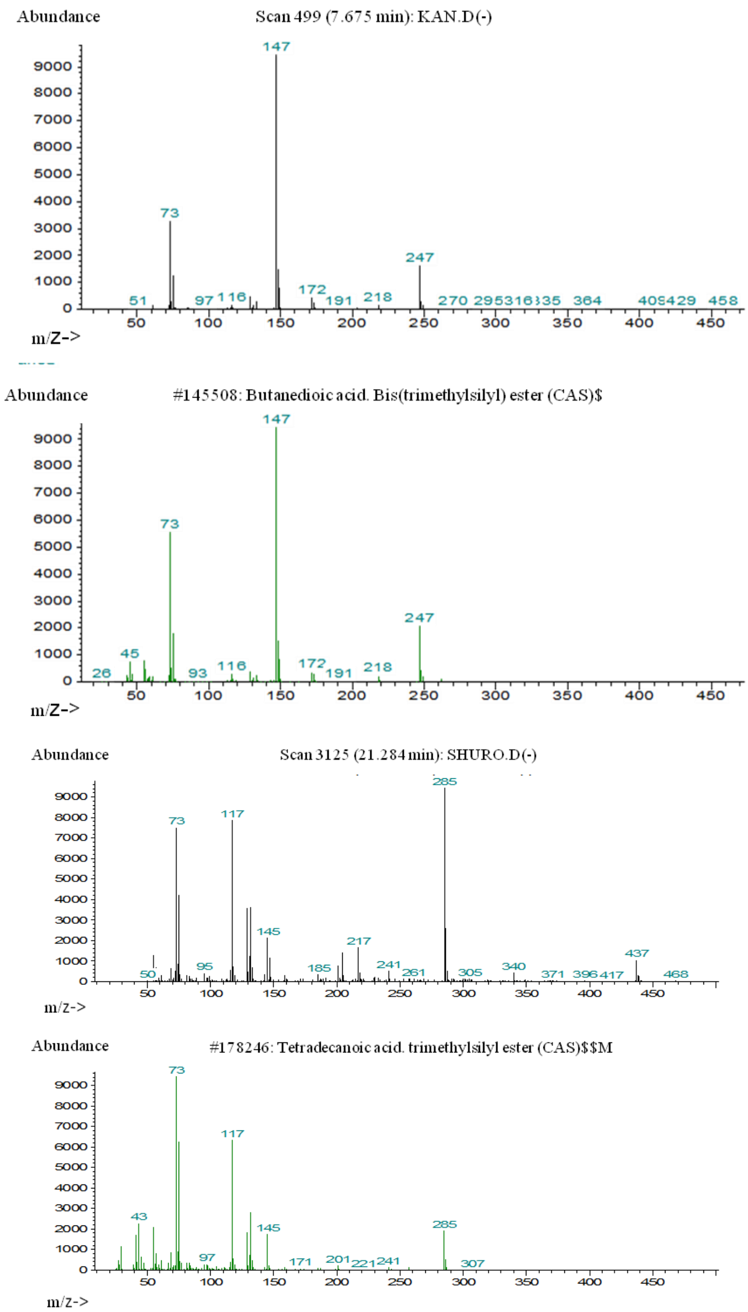
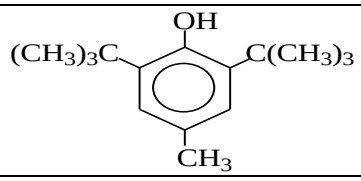
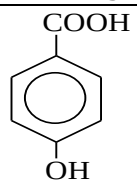
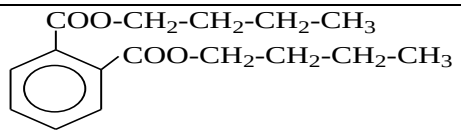


Figure 4. A comparison of (a) a fragment pattern of the peak at 7.675 min with that of butanedioic acid-TMS ester and (b) a fragment pattern of the peak at 21.284 min with that of tetradecanoic acid-TMS ester.

Table 1. Eleven Identified Allelochemicals in the *C. alternifolius* Planting Water

Compound number	Retention time (min)	Compound name	Predicted (structural) formula
1	7.675	Butanedioic acid-TMS	$HOOC\text{-}CH_2\text{-}CH_2\text{-}COOH$
2	8.209	2,3-dihydroxy propanoic acid-TMS	$C(OH)H_2\text{-}C(OH)H\text{-}COOH$
3	8.925	2-hydroxy heptanoic acid-TMS	$CH_3\text{-}(CH_2)_4\text{-}C(OH)H\text{-}COOH$
4	12.465	2,6-bis(dimethylethyl)-4-methyl phenol	
5	15.646	4-hydroxybenzoic acid	
6	16.231	Dodecanoic acid-TMS	$CH_3\text{-}(CH_2)_{10}\text{-}COOH$
7	21.284	Tetradecanoic acid-TMS	$CH_3\text{-}(CH_2)_{12}\text{-}COOH$
8	23.916	1,2-benzenedicarboxylic acid-TMS	
9	26.000	Hexadecanoic acid-TMS	$CH_3\text{-}(CH_2)_{14}\text{-}COOH$
10	29.757	9-cis-octadecenoic acid-TMS	$CH_3\text{-}(CH_2)_7\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOH$
11	30.347	Octadecanoic acid-TMS	$CH_3\text{-}(CH_2)_{16}\text{-}COOH$

The IRs of tested dodecanoic acid (DA), tetradecanoic acid (TA), 2,6-bis(dimethylethyl)-4-methyl phenol (BHT) alone and their mixture at different concentration are shown in Fig. 5. At each concentration level, DA had the lowest inhibitory effect on *M. aeruginosa* growth and the mixture was more inhibitory than individual components. These IRs exhibited a dose-dependent effect (one-way ANOVA, $F = 513.9$, $p < 0.001$ for TA, $F = 1000.7$, $p < 0.001$ for BHT, $F = 1157.6$, $p < 0.001$ for DA, $F = 215.9$, $p < 0.001$ for the mixture). The EC_{50} values calculated in range of 0.1-5.0 mg L^{-1} during 96 h (95% confidence interval) for TA, BHT, DA and mixture were: 0.69 [0.33, 0.98], 0.91 [0.53, 1.24], 2.80 [2.50, 3.13] and 0.44 [0.09, 0.71] mg L^{-1}, respectively. In this experiment,

the EC_{50} of dodecanoic acid (2.80 mg L^{-1}) and tetradecanoic acid (0.69 mg L^{-1}) were lower than in previous reports, perhaps because of different culture conditions or strains of target species (Fig. 5).

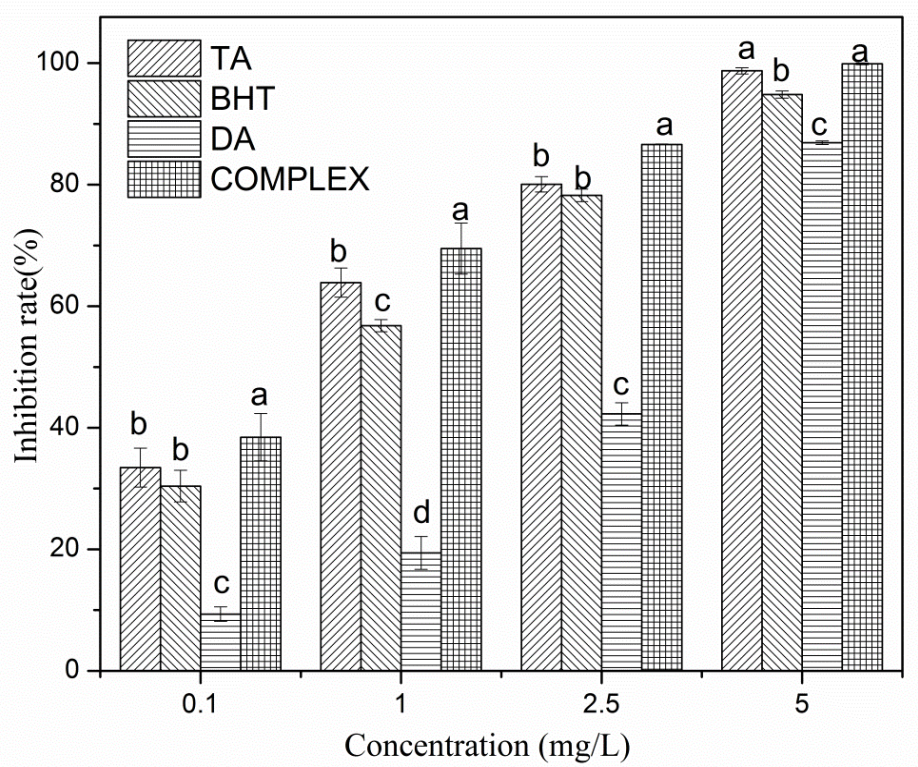


Figure 5. Inhibitory effects on *M. aeruginosa* of three components and their mixture (COMPLEX) of identified allelochemicals from *C. alternifolius*. Lower case letters a, b, c and d indicate statistically significant differences (one-way ANOVA) among the different inhibition rate of allelochemicals (F = 577.62). Data are presented as mean ± standard deviation (n = 3).

Although the identified components may have contributed to the inhibitory effects of *C. alternifolius* on *M. aeruginosa*, the test of allelopathic effects from individual standard commercial samples is not enough to understand the whole potential allelopathic effects of this plant. First, the concentrations (normally at μg L^{-1} level) of allelochemicals secreted by plants into natural water bodies are lower than the EC_{50} values of individual allelochemicals studied in laboratory (3). Second, some non-volatile compounds or non-detectable organic compounds in the planting water, which also played role in anti-cyanobacterial processes, may be overlooked. Third, many allelochemicals co-existing in the solution could have synergetic inhibitory effects.

CONCLUSIONS

The allelochemicals accumulation in the planting water from the floating-bed plant, *C. alternifolius*, depended on the accumulating time and plant biomass. The growth stages did not affect the allelochemicals release. To find the allelochemicals responsible for the *C. alternifolius*, allelopathy the potential anti-cyanobacterial species and their density in co-culture solution should be determined.

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