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Table: 1, Figs : 7

Isolation and characterization of antialgal allelochemicals from *Arundo donax* L.

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ABSTRACT

We isolated and characterised the allelochemicals from *Arundo donax* L. (giant reed), these were very inhibitory to toxic and bloom-forming cyanobacterium *Microcystis aeruginosa*. The methanol extract of dry biomass of giant reed shoot quickly inhibited the algal growth. Through solvent extraction, the methanol extract was fractioned into neutral and acidic fractions, both these fractions also inhibited the growth of *M. aeruginosa*, but the neutral fraction was more inhibitoy than acidic fraction. The neutral fraction was analyzed by gas chromatography/mass spectrometry (GC/MS) and was found to contain several potential allelochemicals, including indoles, ketones, esters, alcohols, etc. Among them, 3-(dimethylaminomethyl)indole (i.e. gramine, an alkaloid) was found in the neutral fraction. Gramine inhibited the *M. aeruginosa* at 0.47 $mg \cdot L^{-1}$ medium effective concentration ($EC_{50,3d}$) and proved one of the strongest antialgal allelochemicals from aquatic plants.

Keywords: Algal-bloom control, allelochemical, fractions, giant reed, growth, gramine, GC/MS, methanol extract, *Microcystis aeruginosa*.

INTRODUCTION

In recent years, use of allelochemicals from the aquatic plants to control algal-bloom is hot research topic. Thus many kinds of aquatic plants have been screened and some allelochemicals inhibitory to bloom-forming algae have been isolated and identified (4,7,14,26,29,36,37,39). The inhibitory activity of selective and biodegradable β-asarone isolated from the genus *Acorus* on algal growth was equivalent to copper sulphate (13). Two new lignin compounds are very inhibitory to *Selenastrum capricornutum* at 0.1 $\mu mol \cdot L^{-1}$ concentration (11). Nonanoic acid, cis-6-octadecenoic and cis-9-octadecenoic acids also proved very inhibitory to *Microcystis aeruginosa* at effective concentrations (EC_{50}) of 0.5, 1.6 and 3.3 $mg \cdot L^{-1}$, respectively (31). The allelochemical 2-methylacetoacetate (EMA) inhibited algal growth with EC_{50} of 0.65 $mg \cdot L^{-1}$ (25). Although several kinds of inhibitory allelochemicals have been identified, but most inhibitory to toxic and bloom-forming algae such as *M. aeruginosa* are still few and need to be further explored.

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Arundo donax L. (family *Poaceae*) is major aquatic plant, growing quickly in lakes, streams, drains and other wet places (21). It is native of eastern Asia, especially China (33) and has been intentionally introduced worldwide (32). It produce huge biomass, whose disposal is of increasing concern. Its aquatic extract is very inhibitory and selectively inhibits the *M. aeruginosa* with $EC_{50,6d}$ at 4.66 $g \cdot L^{-1}$, which was much stronger than extracts of other aquatic plants (17). Therefore, it is considered a promising plant resource for exploring and developing safe, novel and effective algicides (17).

The methanol extract of giant reed strongly inhibits the *M. aeruginosa* (18). This study aimed to isolate and characterise the inhibitory allelochemicals from the methanol extract of giant reed, as an alternative method for eco-friendly control of algal blooms.

MATERIALS AND METHODS

The test plants of giant reed were collected from shrubs growing in Jinan City, Shandong Province, China during September–October, 2005. The aboveground parts of the plant were rinsed with axenic distilled water and dried at 60 ˚C for 72 h. *Microcystis aeruginosa* is most undesirable cyanobacterium in China (24,25). *M. aeruginosa* used for antialgal bioassay was supplied by Freshwater Algae Culture of Hydrobiology Collection (FACHB), China (25).

Extract Preparation

Dried material of giant reed was ground into 0.3-mm plant powder. Plant powder (1.5 kg) of giant reed and methanol (15 L) were placed in a steel vat covered with a steel lid. The extract was prepared under ultrasonic conditions (25 kHz, 250 W) at room temperature in dark for 120 min. Then the extract was filtered through a 0.45-µm filter to remove solid particles and then through a 0.22-µm filter to remove microorganisms. The methanol in the extract was removed using a rotary evaporator (100 rpm, 65 ºC) and the extract was weighed and stored at 4 ˚C for later use.

Isolation and Identification of potential allelochemicals

The methanol extract (2 g) was dissolved in 200 mL of ethyl ether and then extracted with 180 mL of NaOH (2 M). The mixture stood for 30 min to separate into two layers—the upper organic layer A and the lower aqueous layer B. The pH of layer A was adjusted to the neutrality. Then layer A was dehydrated with anhydrous sodium sulfate (Na_2SO_4) and evaporated to obtain the neutral fraction C. The pH of layer B was adjusted to 1. Then layer B was extracted with 300 mL of ethyl acetate. The mixture stood for 30 min to separate into two layers—the upper organic layer D and the lower aqueous layer E. The pH of layer D was adjusted to the neutrality. Then layer D was dehydrated with Na_2SO_4 and evaporated to obtain acidic fraction F. Fractions C and F were used for later analysis.

The constituents of fraction C was analyzed by gas chromatography-mass spectrometry (GC-MS) (DSQ) equipped with a VF-5MS column (30 m×0.25 mm×0.25 µm). Helium was used as the carrier gas, the temperatures for injection port and transmission line were 250 ºC, and the temperature programming was set with initial oven temperature at 100 ºC and held for 3 min and the final temperature of the oven was 300 ºC with rate at 10 ºC·min^{-1}. A 2-μL sample was injected with splitless mode. Mass spectra were recorded over 33-650 amu range with electron impact ionization energy 70 eV. Preliminary identification of constituents was based on computer matching components of mass spectral data against the standard NIST library (2002 version) spectra. Relative proportions of the constituents were computed with GC-MS peak areas. The potential allelochemical was determined based on comprehensive analysis of the results from external standard under the same experimental condition and algal bioassay. The external standard compound was obtained from Sigma-Aldrich Company.

Bioassays

M. aeruginosa was maintained in BG11 medium (25). Before the experiment, the algae were pre-cultured [light of 40-60 μmol photons·m^{-2}·s^{-1} (light: dark=14 h: 10 h), at 24-25 ˚C]. In the late exponential phase of growth, algal cultures were diluted with medium and counted with a haemacytometer to ascertain initial algal densities.

Methanol extract, fraction C, fraction F and potential allelochemical (i.e. the compound confirmed by authentic sample) were pre-dissolved in dimethyl sulphoxide (DMSO) to concentration of 40 g·L^{-1}. Bioassay experiments were performed in a 20-mL mixed culture system with a 50-mL conical flask. The mixed system composed of 1 mL algal culture (late exponential phase of growth), test material of giant reed (extract, fraction C, fraction F or potential allelochemical) and culture medium. All treatments were prepared in three replicates. Cultures were shaken by hand thrice daily. Algal growth was monitored using haemacytometers. Extract effects on algae were tested for 7 d and Fraction effects were tested for 11 d. Note: Pre-experiments proved that when the proportion of DMSO added was less than 1 % (v/v), the growth of *M. aeruginosa* was not affected, so the final proportion of DMSO added did not exceed 1% (v/v) in the later experiments.

The concentrations of methanol extract were 0, 0.05, 0.1, 0.25, 0.5 g·L^{-1}; concentration of fraction C (or fraction F) were 0, 0.01, 0.05 and 0.15 g·L^{-1} and concentrations of potential allelochemicals were 0, 0.5, 1, 2, 4 and 8 mg·L^{-1}, respectively.

VI. Data analysis

The changes in algal growth were estimated by % Inhibition (PI), which was calculated as under:

$$PI(\%)=(1-N_e/N_t)\times 100,$$

Where, N_e and N_t are the numbers of algal cells in treatment groups (added with extract, fractions or potential allelochemical) and the controls (Cell·mL^{-1}), respectively. Positive PIs show inhibitory effects on algal growth.

When algal growth was significantly inhibited, the 50 % effective concentration (EC_{50}) was estimated with logistic fitting. Results were analyzed with OriginPro 7.5 program. The mean values and standard deviations (SD) were calculated from three replicates per treatment.

RESULTS AND DISCUSSION

Effects of the Methanol extract

The methanol extract of giant reed inhibited the growth of *M. aeruginosa* (Fig. 1). One d after incubation, the PIs of the extract on *M. aeruginosa* was 50 % (Fig. 2). 0.5 g·L^{-1} extract was more inhibitory (73.8 %) at 7 d after incubation. Obviously, the methanol extract contained allelochemicals to inhibit the growth of *M. aeruginosa*. The methanol extract showed quick inhibitory effects on algal growth just after 1 d and this will be very useful to control algal-bloom. Hence its inhibitory fractions and potential allelochemicals need to be isolated and characterised.

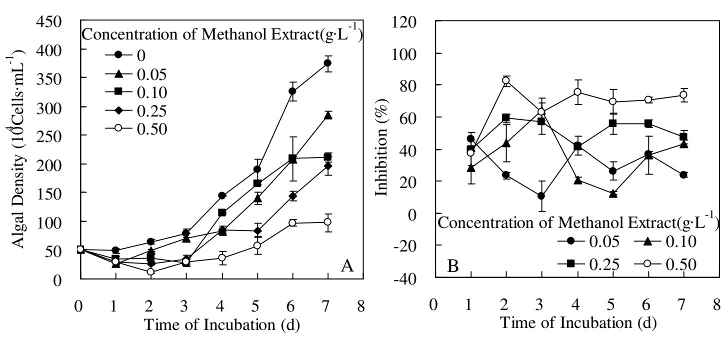

Figure 1. Effects of the methanol extract on the growth of *M. aeruginosa*. A: Growth curve; B: PI curve. All error bars indicate standard deviation (SD) of triplicate tests.

Effects of giant reed fractions

Through liquid-liquid extraction, neutral fraction C and acidic fraction F were obtained from the methanol extract. These two fractions drastically inhibited the growth of *M. aeruginosa* (Figs. 2-4). After 7d of incubation, the mean PI of treatment groups with 0.15 g·L^{-1} of fraction C was 82.0 %; the inhibitory effect of 0.15 g·L^{-1} of fraction F was 90.3 %, i.e. most inhibitory. However, after 11 d of incubation, the concentration of 0.01 g·L^{-1}, acidic fraction F stimulated the algal growth. The change in inhibitory activity of fraction F with extended incubation time might be attributed to its complex constituents. The occurrence of algal re-growth at low concentration of fraction F might be due to the consumption or transformation of active acidic allelochemicals. The stimulatory effects had been also observed in other plant extracts and allelochemicals (6,16,23,30). Thus, growth stimulation occurred at low concentration but high concentrations caused growth inhibition.

As the incubation time was extended, the inhibitory effects of fraction C increased gradually and became stronger than fraction F. After 11 d of incubation, average PI of neutral fraction C at 0.01 g·L^{-1} reached 51.9 %. At concentration above 0.01 g·L^{-1}, the growth of *M. aeruginosa* was completely inhibited. The average PIs of fraction C on *M. aeruginosa* were 92.4 % at 0.05 g·L^{-1} and 99.7 % at 0.15 g·L^{-1}, respectively. Therefore,

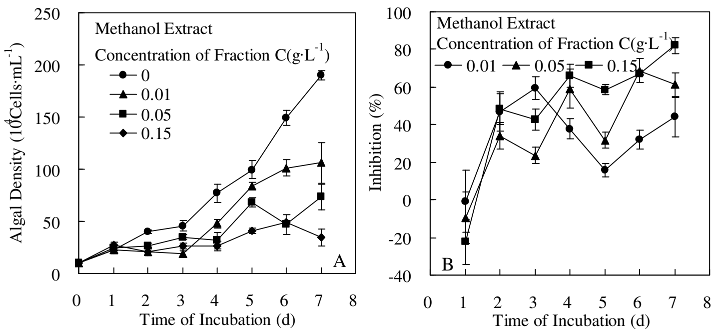

Figure 2. Effects of neutral/alkaline fraction C from methanol extract on the growth of *M. aeruginosa* (0-7 d). A: Growth curve; B: PI curve. All error bars indicate SD of triplicate tests.

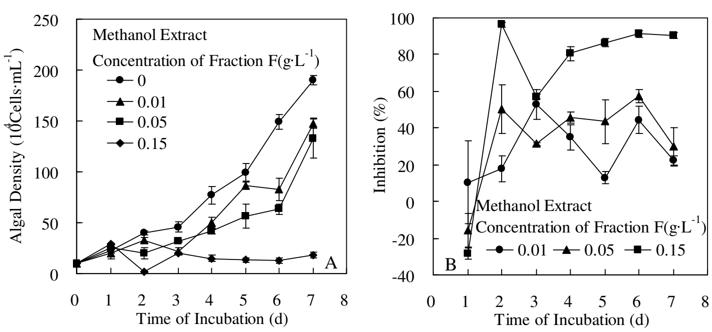

Figure 3. Effects of fraction F from methanol extract on the growth of *M. aeruginosa* (0-7 d). A: Growth curve; B: PI curve. All error bars indicate SD of triplicate tests.

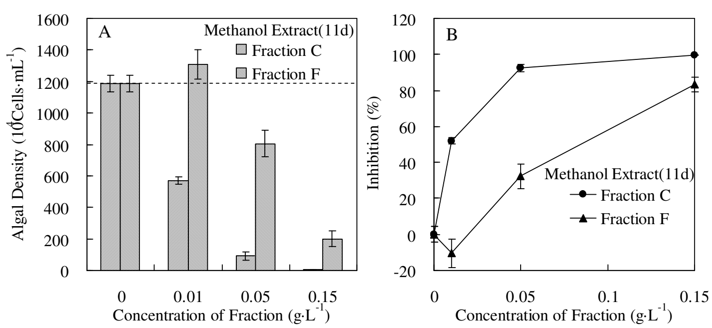

Figure 4. Effects of neutral/alkaline fraction C and acidic fraction F from methanol extract on the growth of *M. aeruginosa* (11 d). A: Growth curve; B: PI curve. All error bars indicate SD of triplicate tests.

it was shown that neutral fraction C was more worthy of being used for later isolation and identification of inhibitory allelochemicals.

Characterization of inhibitory allelochemicals

The analysis with GC-MS identified eleven constituents from the neutral fraction C (Table 1). Indole-like compounds were the main components, (82.16 % of fraction C) obtained from the methanol extract of giant reed. Other three known compounds made up 11.85 % of fraction C.

Table 1. Chemical constituents of fraction C from the methanol extract of giant reed

No.	Compound	Molecular Mass ($g{\cdot}mol^{-1}$)	Retention Time (min)	Relative Amount[a] (%)
1	1H-indole	117	8.01	14.11
2	3-methyl-indole	131	8.93	43.00
3	2,4,6-trimethyl-benzonitrile	145	9.83	20.09
4	3-(dimethylaminomethyl)indole (i.e. gramine)	174	11.75	4.96
5	6,10,14-trimethyl-2-pentadecanone	268	13.71	1.74
6	Unknown compound	207	13.82	2.69
7	1,2-benzenecarboxylic acid, butyl octyl ester	334	14.93	0.72
8	3,7,11,15-tetramethyl-2-hexadecen-1-ol	296	16.40	2.03
9	Unknown compound	341	16.73	5.76
10	4,8,12,16-tetramethylheptadecan-4-olide	324	18.51	1.49
11	Unknown compound	405	22.37	3.40

[a] Relative amount is expressed as peak area ratio.

In other 88.15 % of fraction C including indole-like compounds, several compounds were regarded as potential allelochemicals. Skatole (i.e. 3-methyl-indole) suppresses bacteria and fungi (2), so 3-methyl-indole and even 2,4,6-trimethyl-benzonitrile may have antialgal activities. The indole alkaloid, 3-(dimethylaminomethyl)indole (i.e. gramine) (4.96 %) is major allelochemical to inhibit the growth of some plants and bacteria (5, 27) produced by many terrestrial plants, [barley (*Hordeum vulgare*) (15) and reed canarygrass (*Phalaris arundinacea* L.) (3)]. However, its antialgal activity was only reported on *Anabaena* sp. (PCC7119) (10).

The compound 3,7,11,15-tetramethyl-2-hexadecen-1-ol (2.03 %) called phytol is a chlorophyll-degrading product. The compound 6,10,14-trimethyl-2-pentadecanone (1.74 %) called phytone is a phytol-degrading biomarker in chlorophyll (8) and is important intermediate in preparation of α-tocopherol, with insecticidal activity (28). The compound 4,8,12,16-tetramethylheptadecan-4-olide (a kind of lactide) (1.49 %) is an auto-oxidation product of α-tocopherol (34). Phytol and phytone are degraded products of chlorophyll and may have antialgal activities. Like β-carotene, α-tocopherol is also important non-enzymatic antioxidant. The compound 4,8,12,16-tetramethylheptadecan- 4-olide was produced from α-tocopherol through free radical oxidation process, hence it might also have antialgal activity.

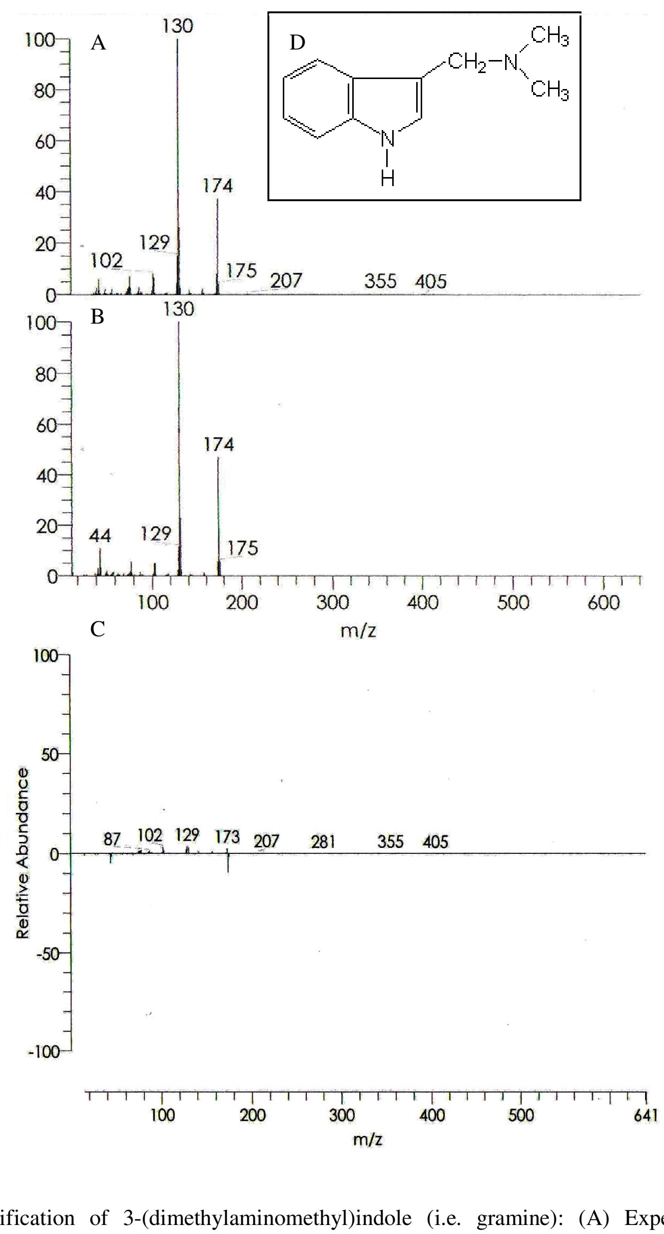

Figure 5. Identification of 3-(dimethylaminomethyl)indole (i.e. gramine): (A) Experimentally observed mass spectra obtained over the retention time 11.69-11.87 min; (B) Library mass spectrum of gramine; (C) Raw data—library entry; and (D) Structure of gramine.

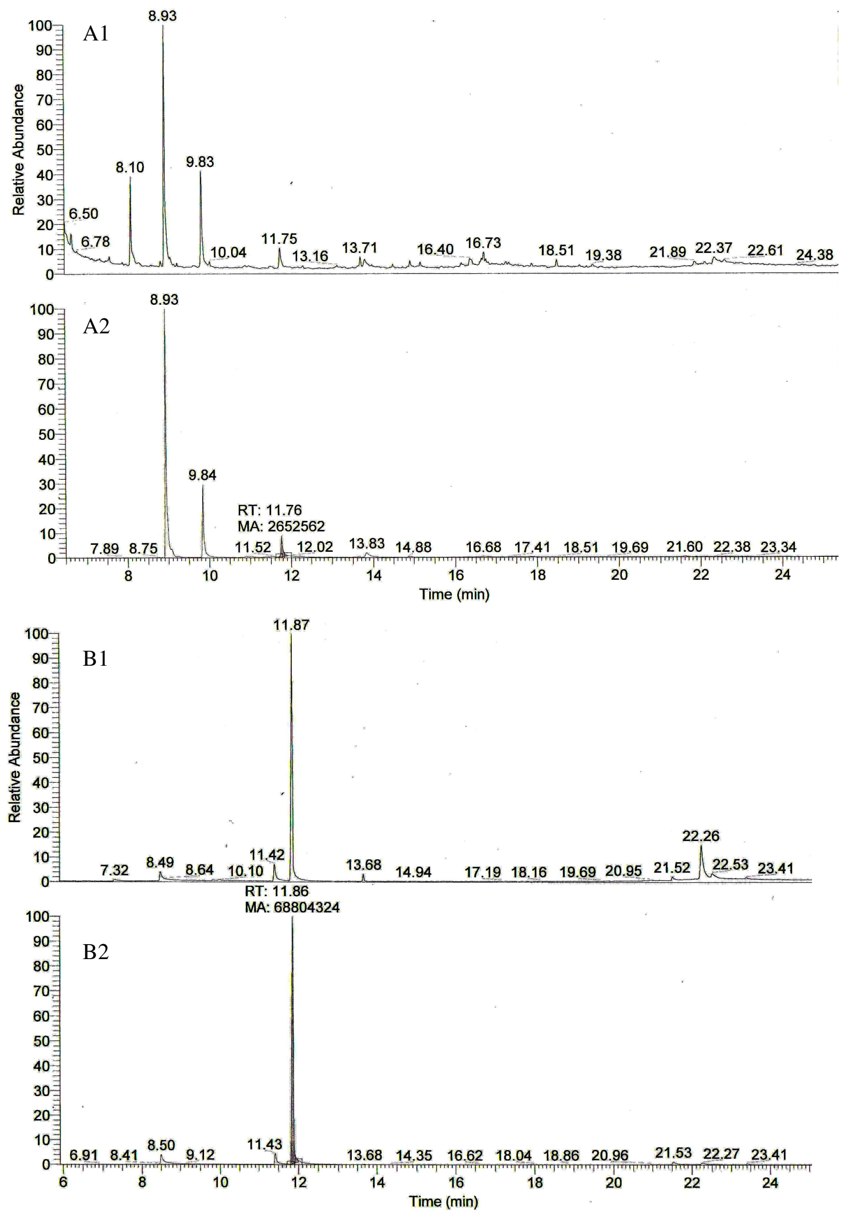

Figure 6. Chromatograms of fraction C from methanol extract of giant reed: (A1, A2) Not spiked, A2 was the extracted ion chromatography of A1; (B1, B2) Spiked with standard sample (gramine), B2 was the extracted ion chromatography of B1.

Gramine: Based on comprehensive comparison and analysis, gramine was confirmed to exist in fraction C (Fig. 5 and Fig. 6). To verify its inhibitory effects, the assay of antialgal activity was done; gramine strongly inhibited the *M. aeruginosa* (Fig. 7). When initial algal density of *M. aeruginosa* was 5×10^4 Cells·mL^{-1}, the $EC_{50,3d}$ of gramine was 0.47 mg·L^{-1} (19). Compared to other antialgal allelochemicals (12,20,31,35,38), gramine is one of the strongest antialgal allelochemicals. Its inhibitory mechanism on *M. aeruginosa* has been reported (19). Gramine content was 0.1 % of fraction C and 0.03 ‰ of dried biomass of giant. Under the optimum conditions of growth, its content was 0.05 % (22).

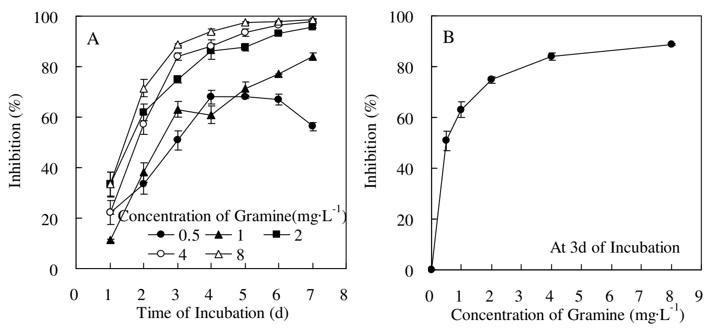

Figure 7. Effects of gramine on the growth of *M. aeruginosa*. A: PI curve (1-7 d); B: PI curve at 3 d of incubation. All error bars indicate SD of triplicate tests.

Allelopathic effects of aquatic plants on algae had been studied for several decades and many kinds of allelochemicals have been isolated and identified viz., long-chain fatty acids, simple phenolic acids, esters, alcohols, ethers, ketones, terpenes and phenanthrenes, indoles, sulfides (or elemental sulfur), lignins, phenylnaphthylamines, phenylpropanoids, simple unsaturated lactones, benzoic acids and their derivatives (20). Some allelochemicals including the gramine, isolated from aquatic plants with *in-vitro* antialgal activities are insoluble or slightly soluble in water. Hence, these will not diffuse normally in water. Therefore, how such allelochemicals (insoluble or slightly soluble in water) play their roles in aquatic environment. The results with *Pistia stratiotes* showed that polar fatty acids could play as micelles to help other non-polar compounds enhance their solubility in aqueous medium, which agrees with former hypothesis (1,9). Therefore, gramine and other allelochemicals insoluble or slightly soluble in water may be mediated with some helping molecules in a similar way. Our current results speculate that which compounds of giant reed suppresses the algal growth in natural water bodies? How the antialgal allelochemicals function in water still needs to be explored further?

Our results have shown an alternative method to control algal blooms in an environment friendly manner. To take full advantage of giant-reed resource and develop safe, novel and effective algicides of plant origin for algal-bloom control in future, much effort is needed to isolate and identify a variety of highly effective antialgal compounds from giant reed.

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