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Growth inhibition and oxidative stress of cyanobacteria induced by sanguinarine and 6-methoxydihydro-chelerythrine isolated from *Macleaya microcarpa*

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ABSTRACT

To investigate the algicidal allelochemicals from *Macleaya microcarpa* we used the activity-guided isolation. Five solvents (petroleum ether, chloroform, acetone, ethanol and water) were employed for the extraction of *M. microcarpa*. Among them, only the ethanol extract was active (7d-EC_{50} = 95.52 mg L^{-1}) and hence, was subjected to further isolation and purification using various chromatographic techniques. Two benzophenanthridine alkaloids with potent algicidal activity, sanguinarine and 6-methoxy-dihydrochelerythrine were isolated. The algicidal effects of these compounds were species-specific, both sanguinarine and 6-methoxy-dihydrochelerythrine were active against *Microcystis aeruginosa* with 3 d-EC_{50} values of 0.47 and 3.43 mg L^{-1}, and the 7 d-EC_{50} values of 0.36 and 2.87 mg L^{-1}, respectively. However, sanguinarine showed low inhibition on *Chlorella pyrenoidosa* and *Scenedesmus obliquus* with 3 d-EC_{50} values of 5.32 and 9.70 mg L^{-1}, respectively. 6-Methoxy-dihydrochelerythrine had little effect on *C. pyrenoidosa* and *S. obliquus*. The active compounds could cause oxidative damage in *M. aeruginosa* based on the observed malondialdehyde (MDA) content decrease in superoxide dismutase (SOD) activity and total antioxidant capacity (T-AOC). These results indicated that *M. microcarpa* extract and the isolated allelochemicals may serve as new agents for controlling the growth of toxic *M. aeruginosa* in eutrophic water bodies.

Keywords: Acetone, algicidal activity, allelochemical, chloroform, ethanol, fractions, *Macleaya microcarpa*, 6-methoxy-dihydrochelerythrine, *Microcystis aeruginosa*, oxidative stress, sanguinarine, petroleum ether, water

INTRODUCTION

In recent years, nutrient inputs, climate changes and the construction of water barriers have attributed to the development of harmful algal blooms (HABs) that adversely affects the water supplies, fisheries, transportation and even tourism by causing huge economic losses (23). *Microcystis* is most common bloom-forming genera widely distributed in eutrophic and hypereutrophic lakes and rivers, where they develop dense, potentially toxic blooms (18). *Microcystis aeruginosa* produces large number of diverse, biochemically active secondary metabolites that cause animal or human health hazards and even death (7). Therefore, control and elimination of cyanobacterial blooms have become big problem.

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Studies to develop effective strategies to control cyanobacterial-bloom have been done for several years. Many herbicides (e.g., diquat, simazine, and diuron) (20,36) as well as antibiotics (34) have shown strong inhibitory or killing effects on the growth of the bloom-forming algae. However, most of these chemical agents were limited due to serious drawbacks (short-term effect, secondary contamination, and toxicity to other non-target aquatic organisms). Even a particular compound, selectively affects only cyanobacteria, but the increase in potential health risks in drinking water supplies limits its application (11,20). Biological control methods [virus (5) or bacteria (35)] have adverse ecological consequences. Hence, new environment-friendly methods to control HABs is needed.

Hence, there has been growing interest for the growth inhibition of cyanobacteria by allelochemicals released by plants and many plants secrete allelopathic substances phytotoxic to cyanobacteria (13,29). Many bioactive substances have been isolated and purified successfully. Phenolic acids and polyphenols are most studied (28,42) and other substances have also been isolated for algicidal activities, including fatty acids from *Chara vulgaris* (41), asarone from *Acorns tatarinowii* (30) and gramine from *Arundo donax* L. (16). In our previous study, 5-medicinal plants (*Salvia miltiorrhiza*, *Acorus tatarinowii*, *Polygonum cuspidatum*, *Phellodendron amurense*, *Crataegus pinnatifida*) with algicidal activity against *M. aeruginosa* were identified (40). These studies indicates the possibility of control of harmful algae by using allelochemicals from plants.

M. microcarpa (family Papaveraceae) is distributed widely in North America, Europe and China, hence, has attracted much attention for containing high concentrations of bioactive benzophenanthridine and isoquinoline alkaloids. In traditional Chinese medicine, the aerial part of *Macleaya* herb is used for its analgesic and anti-inflammatory properties in humans. *M. microcarpa* is also successfully used in animal husbandry (19), veterinary medicine and agriculture (8,9). However, there has not been full evaluation of antialgal substances from *M. microcarpa*, which might provide a safe and effective method for environmental water management. This study on *M. microcarpa* algae control using this Chinese medicinal plant was conducted in due to popularity of this medicinal plant, its low toxicity and the principle of allelopathy.

This study aimed to (i) Determine the algicidal components of *M. microcarpa* using various chromatographic techniques by activity-guided isolation, (ii) Evaluate the algicidal effects of active compounds from *M. microcarpa* against *M. aeruginosa*, *Chlorella pyrenoidosa*, and *Scenedesmus obliquus*, and (iii) Investigate the oxidative stress (lipid peroxidation level, superoxide dismutase activity and total antioxidant capacity) of *M. aeruginosa* induced by active compounds.

MATERIALS AND METHODS

I. Plants materials

Aerial parts of *M. microcarpa* were collected in Shaan'xi province, China, in September, 2011. The plant was authenticated by Prof. X.L. He (Northwest A&F University, Shaanxi, China) and a voucher specimen (no.: SZ-1047) has been deposited in the College of Life Science of the University. The aerial parts were oven-dried at 40 ℃, crushed and screened through a 40-mesh stainless steel sieve. The powdered sample was freeze-dried at - 45℃ to ensure a complete removal of water.

II. Algal cultures

The algae tested in this study were: *M. aeruginosa* (FACHB-905), *C. pyrenoidosa* (FACHB-9) and *S. obliquus* (FACHB-416), which were provided by the FACHB collection (Freshwater Algae Culture of Hydrobiology Collection, Chinese Academy of Sciences). Before the experiment was done, batch cultures of tested algae were grown in sterilized BG11 medium at 25℃ under a 90 μmol photons m^{-2} s^{-1} of illumination intensity with photoperiod of 14:10 (light: dark). New cultures were started approximately every 7-days and until the concentration of algae reached approximately 10^6 cells mL^{-1}.

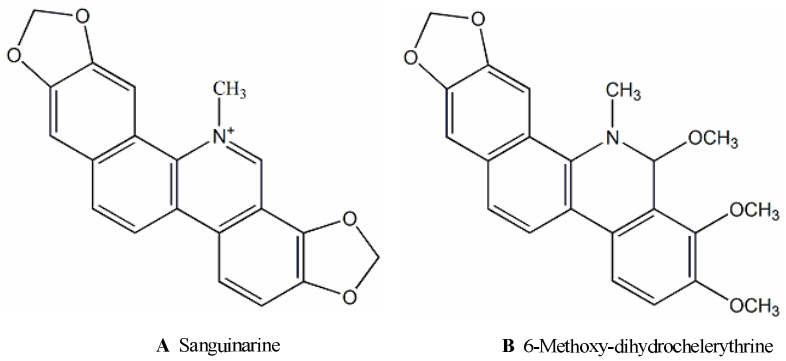
III. Selection of extraction solvent

Five powdered samples (20.0 g each), were extracted separately under reflux with 200 mL each of petroleum ether, chloroform, acetone, ethanol, and water for 2 h, and this process was repeated 3 times. The filtrates were then concentrated under vacuum on reduced pressure to give 5-residues. The 5-residues were then dissolved in dimethyl sulfoxide (DMSO) or distilled water (for water extract) to prepare the stock solutions of 200 g L^{-1} which were used for the algicidal efficacy tests. The control groups without plant extract were kept under the same conditions like test groups. To discard the possible effects of DMSO on algae, other controls containing similar percentage of DMSO were included.

IV. Isolation of algicidal compounds

Based on the previous trials, ethanol was used to extract *M. microcarpa*, to obtain the most effective constituents. The powdered dry sample (2.0 kg) was extracted with ethanol (10 L×3 times) overnight and then filtered through Whatman no. 1 filter paper (Whatman, Maidstone, England). The ethanol filtrates were combined and evaporated to dryness under reduced pressure at 50°C yielding 215.7 g of chloroform extract. Part of the ethanol extract (100.0 g) was eluted with a petroleum ether-ethyl acetate gradient (50:1 to 0:1, v/v) and then with a gradient of ethyl acetate-methanol (1:0 to 0:1, v/v) through silica gel (silica gel: 100-200 mesh) column chromatography to give six fractions (A-F) affording 454 fractions (500 mL each). TLC analysis was performed on silica gel using the same solvent system as the mobile phase. Compounds were visualized under UV light (254 nm and 365 nm) or by spraying the plates with ethanol-sulphuric acid reagent, and these fractions were then pooled in five new fractions as follows: Fr.A (1-58, 11.2 g), Fr.B (59-119, 13.5 g), Fr.C (120-196, 20.3 g), Fr.D (197-280, 11.7 g), Fr.E (281- 454, 17.8 g). Among them, the Fr.C and Fr.D showed higher activity than the other fractions.

Fraction C (15.0 g) was subjected to a silica gel column (350.0 g, 4×80 cm, 300-400 mesh), eluted with a gradient of petroleum ether/ethyl acetate/methanol (50:1:0 to 1:1:5, v/v/v) yielding 126 fractions (300 mL each), and combined to 2 subfractions: Sfr.C1 (2.5 g) and Sfr.C2 (7.8 g) based on TLC analysis. After applied to a Sephadex LH-20 (Amersham Biosciences) column with chloroform/methanol (1:1) as eluent, repetition of recrystallization on Sfr.C2 led to the isolation of the active compound sanguinarine (175.0 mg). Similarly, fraction D (8.0 g) was chromatographed on silica gel by eluting with a petroleum ether/ethyl acetate/methanol gradient (20:1:0 to 0:2:1, v/v/v) and finally eluting with methanol to give 3 subfractions. Recrystallization on Sfr. D2 gave active compound 6-methoxy-dihydrochelerythrine (68.0 mg). The structures of 2 compounds were found by NMR data by comparing with literature (30,32) and their structures are presented in Fig. 1.



A Sanguinarine **B** 6-Methoxy-dihydrochelerythrine

Figure 1. Chemical structures of sanguinarine ($C_{20}H_{14}NO_4$) (A) and 6-methoxy-dihydrochelerythrine ($C_{22}H_{21}NO_5$) (B).

V. Bioassays

For the bioassays, the pure compounds and the crude extracts were dissolved in DMSO into a series of concentration gradients and stored at -20℃ until further required. A 0.3% (v/v) DMSO solution was used to increase the water solubility of the crude extracts.

The algicidal efficacies of fractions and the pure compounds were studied using the U.S. Environmental Protection Agency (U.S. EPA) standard method (38) with some modifications. The active fractions were tested against *M. aeruginosa* and the active compounds were tested against *M. aeruginosa*, *C. pyrenoidosa*, and *S. obliquus* in conical flasks, to which 10^6 cells mL^{-1} of algae were inoculated. The designed concentration gradients of extracts (50, 100, 200, 400, and 800 mg L^{-1}), fractions (100, 200 and 400 mg L^{-1}), and pure compounds (0.125, 0.25, 0.5, 1, and 2 mg L^{-1} for sanguinarine; 1.25, 2.5, 5, 10, and 20 mg L^{-1} for 6-methoxy-dihydrochelerythrine) were added and negative control groups containing no plant extract were set up under the same conditions as the test groups. Algal growth was monitored with a microscope and a hemocytometer by counting the cell number daily for 7 days. Each experiment included triplicate treatments and the experiments were repeated twice. The growth inhibition (%) at specific test substance concentration was calculated compared to control group. The inhibitory ratio (IR) of the extracts on the algal growth was estimated by inhibition over control as under:

$$IR(\%) = [1 - (N / N_0)] \times 100$$

Where, N_0 and N are the cell numbers in control and treatment cultures, respectively.

VI. Oxidative stress in *M. aeruginosa* caused by allelochemicals

A study of oxidative stress effects in *M. aeruginosa* caused by allelopathically active compounds (purity: 98.2% for sanguinarine and 97.3% for 6-methoxy-dihydrochelerythrine) was carried out. Active fractions were added into algal medium at designed concentrations and the algae were then inoculated to give the cell density of approximately 10^6 cells mL^{-1}. The algae were incubated under the same conditions described above for 3 d. The malondialdehyde (MDA) content, superoxide dismutase

(SOD) activity, and total antioxidant capacity (T-AOC) were analyzed at 12, 24, and 48 h. Each experiment included triplicate treatments and was conducted in duplicate.

Lipid peroxidation was determined by measuring the amount of total malondialdehyde (MDA) (24). For each 2-mL aliquot, 2.5 mL of TCA 0.1% was added as an extracting agent and followed by centrifugation at 10,000 rpm for 15 min at 4℃. A mixture was made with 1 mL of supernatant and 4 mL of 0.5% TBA (in 20% TCA) and incubated at 95℃ for 30 min. The reaction was stopped under an ice bath and again centrifuged at 10,000 rpm for 15 min. The absorbance of the supernatant at 532 nm was recorded and corrected for unspecific turbidity by subtracting the value at 600 nm. The concentration of total MDA was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

The crude enzymes were extracted in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.05% (v/v) TritonX-100, 2% (w/v) polyvinyl pyrrolidone and 1 mM ascorbic acid. SOD activity of *M. aeruginosa* was determined by measuring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm as suggested by Beauchamp and Fridovich (4). One unit of SOD was defined as the amount causing 50% inhibition of the photochemical reduction of NBT.

Total antioxidant capacity (T-AOC) was analyzed by the Ferric-reducing antioxidant power (FRAP) assay according to the procedure of Benzie and Strain (3) with slight modification. Briefly, the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol tripyridyltriazine solution in 40 mmol HCl and 20 mmol Fe^{3+} chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily. 100 μL of sample were added to 2 mL of the FRAP reagent, and the absorbance of the reaction mixture were then recorded at 593 nm after warmed to 37 ℃ in a water bath. One unit of T-AOC was defined as causing a 0.01 increase of absorbance of the reaction mixture.

All enzymatic activities were calculated per mg of protein. Total soluble protein content was determined by coomassie blue-dye binding assay (2) using bovine serum albumin as a standard protein.

Statistical analysis: When algal growth was inhibited, the median effective concentration (EC_{50}) was calculated using probit analysis. The data were analyzed by one-way ANOVA and expressed as the arithmetic mean ± standard deviation (SD).

RESULTS

Selection of extraction solvent

The ethanol extract proved most active against the tested alga *M. aeruginosa* with the inhibitory ratio (IR) of 98.6 % (at 800 mg L^{-1}) and EC_{50} of 95.52 mg L^{-1} after 7 d of treatment. The chloroform extract was the next most effective, with IR of 76.5% and EC_{50} of 234.46 mg L^{-1}, followed by the acetone extract with algicidal efficacies of 62.6% and EC_{50} of 302.25 mg L^{-1}, respectively. The petroleum ether and water extracts were least active. The solvent control DMSO had only weak algicidal activity (Table 1, Fig. 2). The ethanol extract showed quick inhibitory effects on algal growth after just 1 d (Figure 2). Thus, its inhibitory fractions and potential allelochemicals need to be isolated.

Table 1. Algicidal effects of *M. microcarpa* extracts on *M. aeruginosa* 7-days after exposure

Extract solvent	IR%	EC_{50} (mg L^{-1})
Petroleum ether	22.6 ± 3.7	>800
Chloroform	76.5 ± 4.5	234.46 ± 19.4
Acetone	62.6 ± 3.9	302.25 ± 23.7
Ethanol	98.6 ± 3.2	95.52 ± 10.9
Water	43.3 ± 2.7	>800
DMSO (0.3%)	5.1 ± 1.2	-

All values were expressed as mean ± standard deviation of three replicates.

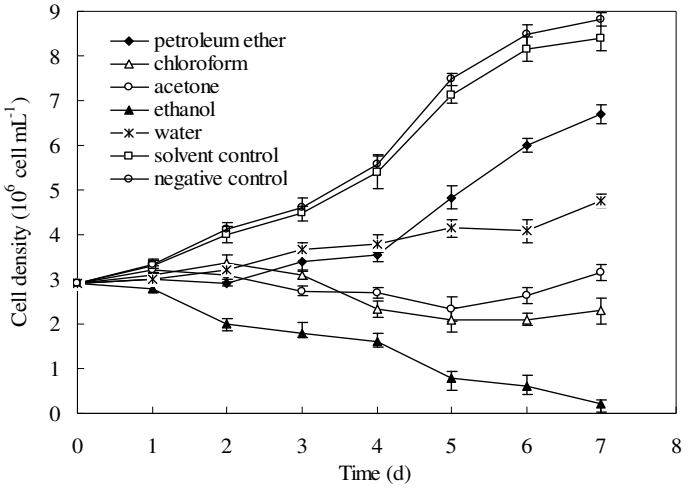


Figure 2. Effect of extracts (at 800 mg L^{-1}) of *M. microcarpa* on growth of *M. aeruginosa*. All error bars indicate standard deviations of three replications.

Algicidal effects of active fractions

To determine the active compounds in ethanol extract, it was fractioned by silica gel column chromatography and five fractions (A - E) were obtained. Of the 5-fractions, Fr. C and Fr. D possessed algicidal activity, with strong inhibition (92.4% and 89.1% at 400 mg L^{-1}, respectively) in *M. aeruginosa* (Figure 3A). Hence, these two fractions were further purified. Rechromatography of Fr. C afforded two major subfractions Sfr. C1 and Sfr. C2. Sfr. C2 had algicidal efficacy of 95.3% at 400 mg L^{-1} and has the potential for further separation. The active Fr. D was subjected to open column chromatography and finally yielded three subfractions : Sfr. D1, Sfr. D2, and Sfr.D3. The Sfr.D2 possessed the highest algicidal efficacy of 92.0 % at 400 mg L^{-1} concentration (Fig. 3B). Fractions and subfractions with the IR < 50% were not studied further.

Algicidal effects of pure compounds

The two isolated compounds (sanguinarine and 6-methoxy-dihydrochelerythrine) inhibited the growth of *M. aeruginosa* (Fig. 4). However, sanguinarine showed higher algicidal efficacy than 6-methoxy-dihydrochelerythrine. The inhibitory effects of

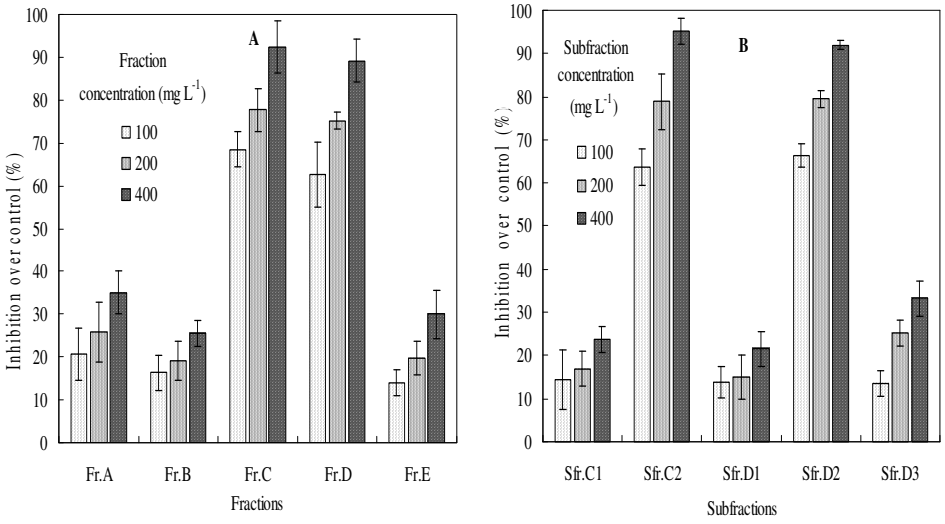


Figure 3. Inhibitory effects of concentrations of fractions (A) and subfractions (B) from ethanol extract of *M. microcarpa* on *M. aeruginosa* 7- days after exposure. All error bars indicate standard deviation of the three replications.

these compounds were concentration dependent and the algal growth was moderately stimulated at low concentrations, but was remarkably inhibited at higher concentrations.

Sanguinarine exhibited strong inhibitory effect on *M. aeruginosa* with 3-d and 7-d EC_{50} values of 0.47 and 0.36 mg L^{-1}, respectively, whereas it was less inhibitory to *C. pyrenoidosa* and *S. obliquus* with 3-d and 7-d EC_{50} of 5.32, 9.70, 4.74, and 4.24 mg L^{-1}, respectively (Table 2). In 6-methoxy-dihydrochelerythrine, the 3-d and 7-d EC_{50} values against *M. aeruginosa* were 3.43 and 2.87 mg L^{-1}, respectively. 6-Methoxy-dihydrochelerythrine was only slightly inhibitory to *C. pyrenoidosa and S. obliquus* and the 3-d EC_{50} values were over 20 mg L^{-1}, and 7-d EC_{50} were 9.75 and 11.02 mg L^{-1}, respectively.

Table 2. Algicidal effects of sanguinarine and 6-methoxy-dihydrochelerythrine isolated from *M. microcarpa* on *M. aeruginosa*, *C. pyrenoidosa* and *S. obliquus* on EC_{50} (mg L^{-1}) 3-days and 7-days after exposure

Algae spp.	Sanguinarine		6-Methoxy-dihydrochelerythrine	
	3d- EC_{50} (mg L^{-1})	7d-EC_{50} (mg L^{-1})	3d- EC_{50} (mg L^{-1})	7d-EC_{50} (mg L^{-1})
M. aeruginosa	0.47 ± 0.21	0.36 ± 0.27	3.43 ± 0.61	2.87 ± 0.56
C. pyrenoidosa	5.32 ± 0.62	4.74 ± 0.54	>20.0	9.75 ± 1.77
S. obliquus	9.70 ± 1.89	4.24 ± 0.48	>20.0	11.02 ± 2.76

All values were expressed as mean ± standard deviation of three replicates.

Oxidative stress in *M. aeruginosa*

The MDA production in *M. aeruginosa* was decreased slightly ($P < 0.05$) 12 h after exposure to 0.5 and 1 mg L^{-1} of sanguinarine (Fig. 5A). With time extension, the

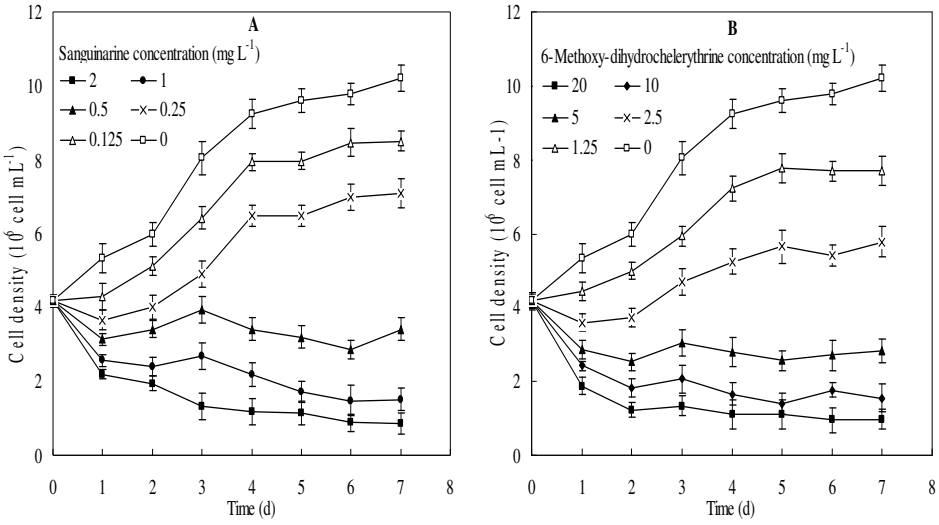


Figure 4. Effect of concentrations of sanguinarine (A) and 6-methoxy-dihydrochelerythrine (B) on growth curves of *M. aeruginosa*. All error bars indicate standard deviation of the three replications.

MDA content increased greatly at 24 and 48 h ($P < 0.01$). The application of sanguinarine at 2 mg L^{-1} after 48 h increased the MDA content 2.3-folds higher than control ($P < 0.01$). The applied 6-methoxy-dihydrochelerythrine, increased the MDA content by no difference ($P < 0.05$) at 12 h to a remarkable increase ($P < 0.01$) 24 and 48 h after treatments, relative to control (Fig. 5B). In both sanguinarine and 6-methoxy-dihydrochelerythrine treatments, the MDA production was positively correlated with these compounds' applied concentrations and increased with exposure time.

Application of sanguinarine (Fig. 5C) increased the SOD activity in *M. aeruginosa* Cells 12 h after exposure. However, when the exposure time was extended to 24 h, the SOD activity decreased ($P < 0.01$). After 48 h exposure to 0.5, 1 of sanguinarine, the activity decreased 26.2, 36.1, and 57.9 % than control, respectively. Contrarily the increase in concentration of 6-methoxy-dihydrochelerythrine (Fig. 5D), decreased the SOD activity. After 24 h exposure, 2.5 and 5 mg L^{-1} 6-methoxy-dihydrochelerythrine significantly decreased the SOD activity to decrease ($P < 0.05$) and after 48 h, the decrease became more pronounced ($P < 0.01$).

When the algal cells were exposed to concentration series of sanguinarine (Fig. 5E), the antioxidant activity did not change 12 h after exposure. After 24 h, exposure to 0.5 and 2 mg L^{-1} of sanguinarine the T-AOC increased ($P < 0.05$). However, 48 h after treatment with 0.5, 1, and 2 mg L^{-1} sanguinarine the T-AOC decreased by 23.8, 42.8, and 38.9%, respectively, than control ($P < 0.01$). While 6-Methoxy-dihydrochelerythrine increased the T-AOC with increasing concentration after 12 and 24 h (Figure 5F). However, the effect was less with exposure time of 48 h ($P < 0.01$).

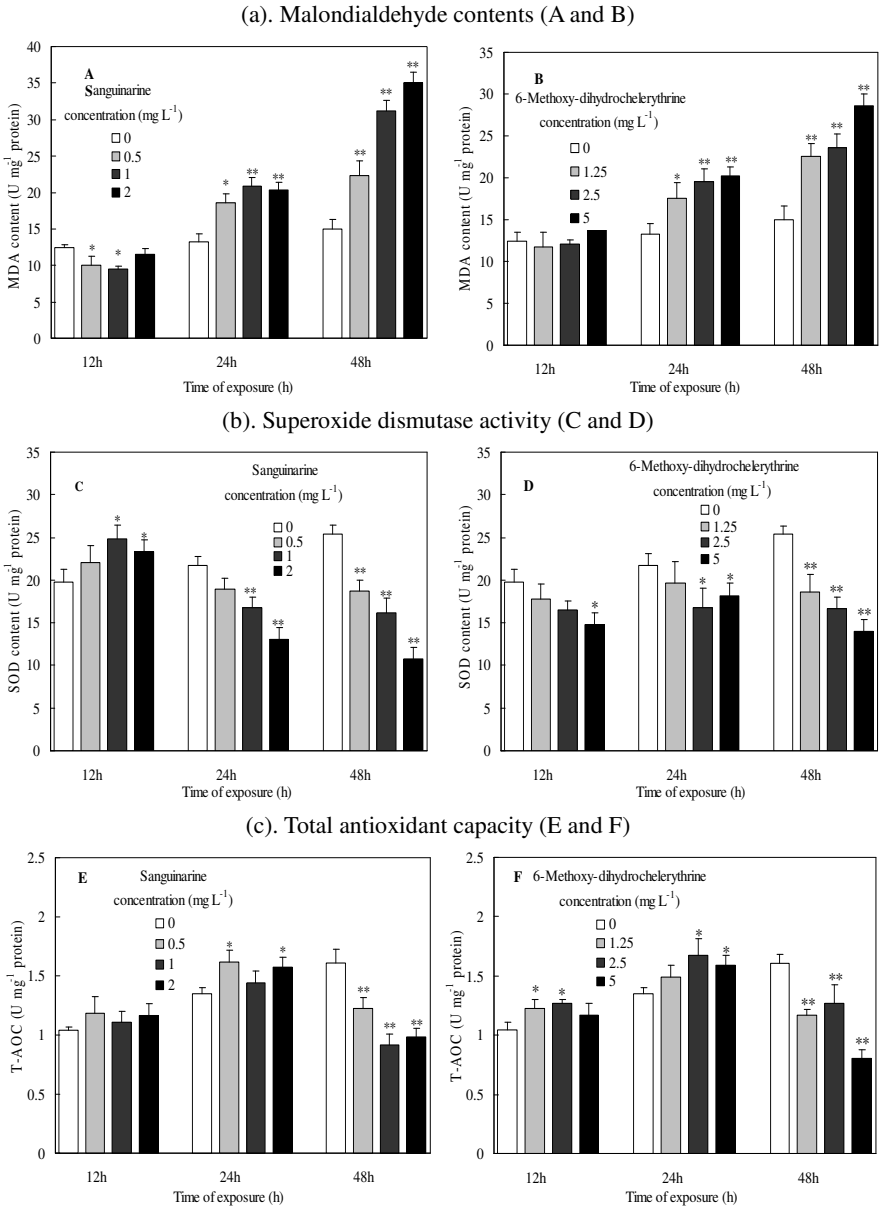


Figure 5. Effects of active compounds on Malondialdehyde contents (A and B), Superoxide dismutase activity (C and D), and Total antioxidant capacity (E and F) of *M. aeruginosa* at various durations of exposure. All error bars indicate standard deviation of the three replications.. Statistical differences from the control group are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$).

DISCUSSION

Water resource management is challenged worldwide to reduce the vulnerability of threats of harmful cyanobacterial blooms and the occurrences of HABs which damage other species in aquatic environment, especially commercially cultivated animals. The studies to search effective algicides for water-bloom controls have been done for several years, but recently, much effort has been made to find alternative agents from different organisms (14,17). Allelochemicals from medicinal plants are attractive sources for their huge biodiversity and safety. Although many medicinal plants possess antiviral and antibacterial activities (26,37), little information is available on medicinal plants processing algicidal activity.

In this study, the ethanolic extract proved effective than other four extracts (Table 1), thereby indicating that the active constituents were mainly concentrated in the ethanol extract. The other extracts or fractions with lower algicidal activity were not further isolated even though they might contain compounds with high activity, but present in low concentrations. The two active compounds (sanguinarine and 6-methoxy-dihydrochelerythrine) showed much higher activities than original ethanolic extract, this indicated that these two compounds may be responsible for the algicidal properties of ethanol extract.

M. microcarpa has been widely used in traditional medicine in China for hundreds of years. Currently, the extracts from this medicinal plant are components of veterinary or human phyto-preparations and of oral-hygiene agents such as dentifrices and chewing gums. *M. microcarpa* was on the European Food Safety Authority (EFSA) list of plants exploited free use as a component in feed additives in animal production (10). The powdered rhizomes or herbs of *M. microcarpa* or *Chelidonium majus* with a standardized amount of sanguinarine (commercial name Sangrovit®) has been recommended as feed additives in pork industry and in aquaculture (19,33). Thus it should be especially suitable for use in aquaculture farms, lakes or drinking water reservoir as an algicide with low toxicity and being economical. Thus, it is worthy of further research as botanical algicide.

Both the ethanol extracts of *M. microcarpa* and its active components showed quick deterimental allelopathic effects on the bloom-forming algae. Following the activity-guided fractionation, the ethanol extract was further isolated and purified yielding two active compounds: sanguinarine and 6-methoxy-dihydrochelerythrine. Both these compounds have algicidal efficacies against *M. aeruginosa* with the 3 d- EC_{50} value of 0.47 mg L^{-1} for sanguinarine and 3.43 mg L^{-1} for 6-methoxy-dihydrochelerythrine (Table 2). Thus, it may be suitable in an emergency to control algal blooms.

Generally, algal inhibitors can have two types of toxicity towards cyanobacteria: (i). Cyanocidal effects (lethal) and (ii). Cyanostatic effects (inhibits new growth). The toxins may be released from algal blooms, when their cell wall is destroyed especially those produced by *Microcystis* species (25,39). Lee *et al.* (21) studied the effect of selenium on the growth of cyanobacterium and found that 100 mg L^{-1} SeO_2 with EDTA and 50 mg L^{-1} SeO_2 without EDTA had cyanocidal effects on *Anacystis nidulans*. In this study, when the doses of sanguinarine and 6-methoxy-dihydrochelerythrine were < 0.5 mg L^{-1} and 5 mg L^{-1}, the cyanostatic effects were observed (Fig. 4). There was no growth in culture and the *M. aeruginosa* population was inhibited after exposure.

The responses of algal cells to effects of allelochemicals vary greatly among species. Both sanguinarine and 6-methoxy-dihydrochelerythrine have strong algicidal activity against *M. aeruginosa* but weak inhibition of *C. pyrenoidosa* and *S. obliquus*. Similar phenomena have been observed in other studies (22,27), The allelochemicals in *Myriophyllum spicatum* caused drastic inhibition of *M. aeruginosa* but weak inhibition in *Anabaena flos-aquae* and *Phormidium tenue* (27), EMA isolated from *Phragmites communis* showed strong growth inhibition of *C. pyrenoidosa* and *M. aeruginosa* but no inhibition of *Chlorella vulgaris* (22). The selective property of these compounds is useful to develop the novel algicides to control harmful cyanobacteria blooms.

Both sanguinarine and 6-methoxy-dihydrochelerythrine belong to the benzophenanthridine alkaloids (QBA), which are found in plants in the families Papaveraceae, Fumariaceae, and Rutaceae. QBA possess various bioactivities [antibacterial (12), anticarcinogenic (1) and antispasmodic property (15)]. Sanguinarine can induce cytotoxicity in liver cells through oxidation of protein thiols, thereby resulting in oxidative stress on the cells and disturbance of mitochondrial function (6). In the present study, antioxidant responses and lipid damages in *M. aeruginosa* after exposure to QBA were found. Lipid peroxidation was evaluated by measuring the formation of malondialdehyde (MDA) which is the main decomposition product of peroxides derived from polyunsaturated fatty acids. The increase in MDA levels (Fig. 5A, 5B) indicates that the cells of *M. aeruginosa* were under serious oxidative stress. SOD is an important enzyme in the antioxidant enzymatic defense system and converts the superoxide radical to H_2O_2. Sanguinarine exposure may induce the production of reactive oxygen and trigger the enzymatic antioxidant defense system as indicated by the increase in SOD activity at 12 h (Figure 5C). The rise of SOD activity in *M. aeruginosa* was consistent with the decrease in lipid peroxidation (lower MDA values) and suggests that the microalgae were able to efficiently scavenge the hydrogen peroxide and avoid excessive oxidative damage in the early stage of exposure. The decrease in SOD and T-AOC (Fig. 5E, 5F) after 48 h suggests that the antioxidant defence system might be the site of action of QBA on *M. aeruginosa*, thus it cannot alleviate the burden of reactive oxygen at the anaphase of exposure. However, the mode of action of QBA on *M. aeruginosa* still needs to be confirmed.

CONCLUSIONS

The sanguinarine and 6-methoxy-dihydrochelerythrine from *M. microcarpa* selectively inhibited the growth of *M. aeruginosa* and the algicidal effect may be due to oxidative damage and the antioxidant system destruction in the test algae. Therefore, sanguinarine and 6-methoxy-dihydrochelerythrine may be novel algicides control cyanobacteria. Also, further investigations related to the ecological risks associated with field studies need to be considered.

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REFERENCE

1. Ahsan, H., Reagan-Shaw, S., Breur, J. and Ahmad, N. (2007). Sanguinarine induces apoptosis of human pancreatic carcinoma AsPC-1 and BxPC-3 cells via modulations in Bcl-2 family proteins. *Cancer Letters* **249**: 198-208.
2. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.
3. Benzie, I.F.F. and Strain, J.J. (1996). The ferric reducing ability of plasma as a measure of ‘‘antioxidant power’’ the FRAP assay. *Analytical Biochemistry* **239**: 70-76.
4. Beauchamp, C. and Fridovich, I. (1971). Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**: 276-287.
5. Bouvier, T. and Del, Giorgio, P.A. (2007). Key role of selective viral-induced mortality in determining marine bacterial community composition. *Environmental Microbiology* **9**: 287-297.
6. Choy, C.S., Cheah, K.P., Chiou, H.Y., Li, J.S., Liu, Y.H., Yong, S.F., Chiu, W.T., Liao, J.W. and Hu, C.M. (2008). Induction of hepatotoxicity by sanguinarine is associated with oxidation of protein thiols and disturbance of mitochondrial respiration. *Journal of Applied Toxicology* **28**: 945-956.
7. Dörr, F.A., Pinto, E., Soares, R.M. and Azevedo, S.M.F.O. (2010). Microcystins in South American aquatic ecosystems: occurrence, toxicity and toxicological assays. *Toxicon* **56**: 1247-1256.
8. Drsata, J., Ulrichova, J. and Walterova, D. (1996). Sanguinarine and chelerythrine as inhibitors of aromatic amino acid decarboxylase. *Journal of Enzyme Inhibition* **10**: 231-237.
9. Feng, G., Zhang, J., Li, X.W., Feng, J.T. and Zhang, X. (2008). Insecticidal activity of alkaloids from *Macleaya microcarpa* against several species of insect pests. *Journal of Zhejiang University (Agric & Life Sci)* **34**: 187-192.
10. Franz, Ch., Bauer, R., Carle, R., Tedesco, D., Tubaro, A., Zitterl-Eglseer, K. (2005). Study on the assessments of plants/herbs, plant/herb extracts and their naturally or synthetically produced components as additives for use in animal production. CFT/EFSA/FEEDAP/2005/01: 140-149.
11. Gary, J.J. and Philip, T.O. (1994). Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Research* **28**: 871-876.
12. Godowski, K.C. (1989). Antimicrobial action of sanguinarine. *The Journal of Clinical Dentistry* **1**: 96-101
13. Gross, E.M., Erhard, D., Ivanyi, E. (2003). Allelopathic activity of *Ceratophyllum demersum* L. and *Najas marina* ssp. Intermedia (Wolfgang) Casper. *Hydrobiologia* **506**: 583-589.
14. Gross, E.M., Hilt, S., Lombardo, P. and Mulderij, G. (2007). Searching for allelopathic effects of submerged macrophytes on phytoplankton -state of the art and open questions. *Hydrobiologia* **584**: 77-88.
15. Hiller, K.O., Ghorbani, M. and Schilcher, H. (1998). Antispasmodic and relaxant activity of chelidonine, protopine, coptisine and *Chelidonium majus* extracts on isolated guinea-pig ileum. *Planta Medica* **64**: 758-760.
16. Hong, Y., Hu, H.Y., Sakoda, A. and Sagehashi, M. (2010). Isolation and characterization of algicidal allelochemicals from *Arundo donax* L. *Allelopathy Journal* **25**: 357-368.
17. Jüttner, F., Todorova, A.K., Walch, N. and Philipsborn, W. (2001). Nostocyclamide M: a cyanobacterial cyclic peptide with allelopathic activity from *Nostoc* 31. *Phytochemistry* **57**: 613-619.
18. Jüttner, F., Watson, S.B., von Elert, E. and Köster, O. (2010) β-Cyclocitral, a grazer defence signal unique to the cyanobacterium *Microcystis*. *Journal of Chemical Ecology* **36**: 1387-1397.
19. Kosina, P., Walterovaa, D. and Ulrichovaa, J. (2004). Sanguinarine and chelerythrine: assessment of safety on pigs in ninety days feeding experiment. *Food and Chemical Toxicology* **42**: 85-91.
20. Lam, A.K.Y., Prepas, E.E., Spink, D. and Hrudey, S.E. (1995). Chemical control of hepatotoxic phytoplankton blooms -implications for human health. *Water Research* **29**: 1845-1854.
21. Lee, L.H., Lustigman, B., Murray, S. and Koepp, S. (1999). Effects of selenium on the growth of cyanobacterium *Anacystis nidulans. Bulletin of Environmental Contamination and Toxicology* **62**: 591-599.
22. Li, F.M. and Hu, H.Y. (2005). Isolation and characterization of a novel algicidal allelochemical from *Phragmites communis*. *Applied and Environmental Microbiology* **71**: 6545-6553.
23. Liu, W. and Qiu, R. (2007). Water eutrophication in China and the combating strategies. *Journal of Chemical Technology and Biotechnology* **82**:781-786.

24. Mishra, Y., Bhargava, P. and Rai, L. (2005). Differential induction of enzymes and antioxidants of the antioxidative defence system in *Anabaena doliolum* exposed to heat stress. *Journal of Thermal Biology* **30**: 524-531.
25. Momani, F.A., Smith, D.W. and EI-Din, M.G. (2008). Degradation of cyanobacteria toxin by advanced oxidation processes. *Journal of Hazardous Materials* **150**: 238-249.
26. Mukhtar, M., Arshad, M., Ahmad, M., Pomerantz, R. J., Wigdahl, B. and Parveen, Z. (2008). Antiviral potentials of medicinal plants. *Virus Research* **131**: 111-120.
27. Nakai, S., Inoue, Y. and Hosomi, M. (1999). Growth inhibition of blue-green algae by allelopathic effects of macrophytes. *Water Science and Technology* **39**: 47-53.
28. Nakai, S., Inoue, Y. and Hosomi, M. (2000). *Myrophyllum spicatum*-released allelopathic polyphenols inhibiting growth of blue-green algae *Microcystis aeruginosa*. *Water Research* **34**: 3026-3032.
29. Nam, S., Joo, S., Kim, S., Baek, N.I., Choi, H.K. and Park, S. (2008). Induced metabolite changes in *Myriophyllum spicatum* during co-existence experiment with the cyanobacterium *Microcystis aeruginosa*. *Journal of Plant Biology* **51**: 373-378.
30. Perez, G.R.M., Vargas, S.R., Diaz, G.G. and Martinez-Martinez, F.J. (2002). Identification of benzophenanthridine alkaloids from *Bocconia arborea* by gas chromatography–mass spectrometry. *Phytochemical Analysis* **13**: 177-180.
31. Pollio, A., Pinto, G., Ligrone, R. and Aliotta, G. (1993). Effects of the potential allelochemical a-asarone on growth, physiology and ultrastructure of two unicellular green algae. *Journal of Applied Phycology* **5**: 395-403.
32. Qin, H.L., Wang, P. and Li, Z.H. (2004). The establishment of the control substance and 1H nuclear magnetic resonance fingerprint of *Macleaya microcarpa* (Maxim.) Fedde. *Chinese Journal of Analytical Chemistry* **32**: 1165-1170. (Cinese).
33. Rawling, M.D., Merrifield, D.L. and Davies, S.J. (2009). Preliminary assessment of dietary supplementation of Sangrovit® on red tilapia (*Oreochromis niloticus*) growth performance and health. *Aquaculture* **294**: 118-122.
34. Robinson, A.A., Belden, J.B. and Lydy, M.J. (2005). Toxicity of fluoroquinolone antibiotics to aquatic organisms. *Environmental Toxicology and Chemistry* **24**: 423-430.
35. Su, J., Yang, X., Zhou, Y. and Zheng, T. (2011). Marine bacteria antagonistic to the harmful algal bloom species *Alexandrium tamarense* (Dinophyceae). *Biological Control* **56**: 132-138.
36. Swain, N., Rath, B. and Adhikary, S.P. (1994). Growth-response of the cyanobacterium *Microcystis Aeruginosa* to herbicides and pesticides. *Journal of Basic Microbiology* **34**: 197-204.
37. Tohidpour, A., Sattari, M., Omidbaigi, R., Yadegar, A. and Nazemi, J. (2010). Antibacterial effect of essential oils from two medicinal plants against methicillin-resistant *Staphylococcus aureus* (MRSA). *Phytomedicine* **17**: 142-145.
38. U.S. EPA. (1989). Algal, *Selenastrum capricornutum*, growth test method 1003.0. *In Short-term Methods for Estimating the Chronic Toxicity of Effluent and Receiving Waters to Freshwater Organisms*. EPA 600/4-89/001. Cincinnati, Ohio, USA.
39. Wu, Y., Kerr, P.G., Hu, Z. and Yang, L. (2010). Removal of cyanobacterial bloom from a biopond-wetland system and the associated response of zoobenthic diversity. *Bioresource Technology* **101**: 3903-3908.
40. Yi, Y.L., Lei, Y., Yin, Y.B., Zhang, H.Y. and Wang, G.X. (2012). The antialgal activity of 40 medicinal plants against *Microcystis aeruginosa*. *Journal of Applied Phycology* **24**: 847-856
41. Zhang, T.T., He, M., Wu, A.P. and Nie, L.W. (2009). Allelopathic effects of submerged macrophyte *Chara vulgaris* on toxic *Microcystis aeruginosa*. *Allelopathy Journal* **23**: 391-401.
42. Zhang, T.T., Zheng, C.Y., Hu, W., Xu, W.W. and Wang, H.F. (2010). The allelopathy and allelopathic mechanism of phenolic acids on toxic *Microcystis aeruginosa*. *Journal of Applied Phycology* **22**: 71-77.