Pol. J. Environ. Stud. Vol. 20, No. 5 (2011), 1153-1159

Original Research

Phenolic Compounds Exuded from Two Submerged Freshwater Macrophytes and Their Allelopathic Effects on *Microcystis aeruginosa*

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Received: 16 April 2010
Accepted: 20 September 2010

Abstract

Culture solutions of the submerged freshwater macrophytes *Hydrilla verticillata* and *Vallisneria spiralis*, separately incubated at 10 g of fresh weight (FW) per liter for three days, were extracted by solid phase extraction (SPE) followed by liquid liquid extraction (LLE), and the acquired ethyl acetate fractions were analyzed by gas chromatograph-mass spectrometry (GC-MS) to identify potential allelochemicals exuded from these plant species. Freeze-dried plant tissues were separately methanol-extracted with a similar LLE-GC-MS procedure to compare allelochemical production and exudation. Four phenolic compounds were identified for both species: vanillic acid (VA), protocatechuic acid (PA), ferulic acid (FA), and caffeic acid (CA). *H.verticillata* produced 179 times the amount of phenolic compounds of *V. spiralis.* The phenolic contents in the culture solution were lower than 10% of those in the plant tissues for both submerged species in six of the eight cases. When evaluating the joint effects of the compounds using the Toxicity Index (TI) model, it was observed that the four phenolic compounds exerted additive and synergistic inhibition effects on the growth of *Microcystis aeruginosa* depending on the mixing ratios. These results indicate that *H. verticillata* and *V. spiralis* could release some phenolic allelochemicals to inhibit the growth of *M. aeruginosa*, and the joint action of multiple allelochemicals may be an important allelopathic pattern of submerged macrophytes to inhibit the growth of noxious cyanobacteria in natural aquatic ecosystems.

Keywords: *Hydrilla verticillata*, *Vallisneria spiralis*, exudates, phenolic allelochemicals, joint action

Introduction

Some submerged macrophytes negatively affect the growth of algae and cyanobacteria by releasing secondary metabolites, called allelochemicals, into water [1-5], and allelopathy has been proposed as one of the main mechanisms by which macrophytes control phytoplankton biomass and taxonomic composition in aquatic ecosystems [6].

Hydrilla verticillata and *Vallisneria spiralis* are two common Hydrocharitaceae submerged macrophytes in many shallow lakes of the middle-lower reaches of the Yangtze River, China [7, 8]. Growth of the noxious cyanobacteria *Microcystis aeruginosa* could be inhibited in the presence of *V. spiralis* or the culture filtrates of *H. verticillata* [9, 10], which indicates that the two plants could release allelochemicals into water environment to exert

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allelopathic effects on *Microcystis aeruginosa*. But what compounds are responsible for the algal growth inhibition is not clear.

Phenolic compounds are one major class of identified allelochemicals present in many submerged macrophytes [11-14]. The contents of total phenolic compounds (TPCs) in the exudates of *H. verticillata* and *V spiralis*, and their strong inhibitory effects on the growth of *M. aeruginosa,* have been studied in our previous work [15]. This study tried to identify and quantify individual phenolic compounds exuded from *H. verticillata* and *V. spiralis*, and evaluate the allelopathic effects of the identified compounds on the growth of *M. aeruginosa*.

Materials and Methods

Reagents

N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) used for silanization was purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard reference compounds were vallinic acid (4-hydroxy-3-methoxybenzoic acid, VA), protocatechuic acid (3,4-dihydroxybenzoic acid, PA), ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, FA), and caffeic acid (3,4-dihydroxycinnamic acid, CA) (Fig. 1). The compounds were purchased from Sigma-Aldrich, except protocatechuic acid from Alfa Aesar (Ward Hill, Massachusetts, USA). All solvents and reagents used in this study were of HPLC grade purity.

Plant Materials and Cultivation

H. verticillata and *V. spiralis* were collected from Moon Lake (30º33'N, 114º15'E) in Wuhan, China, and then pre-cultured in the laboratory [15]. When the experiment began, the plants were washed carefully with tap water and later distilled water. One part of the plant materials was weighed and prepared for extraction from plant tissues. The other parts of the plants were cultivated at a density of 10 g FW/L in MIII medium [16] for three days under the same conditions as the pre-cultured (a light intensity of 3000 lux and 12:12 light: dark cycle at 25ºC), and then the allelochemicals in the culture solution were extracted. MIII medium without plants was cultivated under the same conditions as the control.

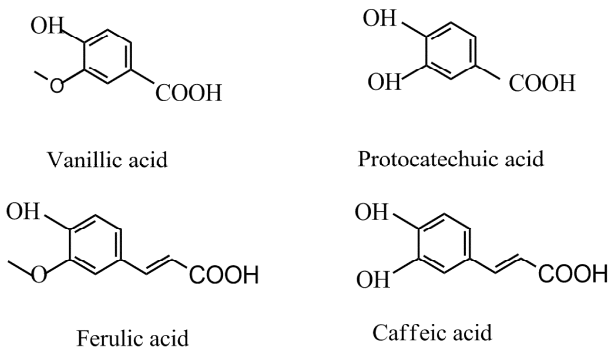

Fig. 1. Chemical structures of phenolic compounds identified in the plant tissues and exudates of *H. verticillata* and *V. spiralis*.

Allelochemical Extraction from Plant Tissues

Extraction from plant tissues was to find the phenolic allelochemicals produced by the two plants. The extraction procedure was based on the methods reported by Gross et al. [17]. The washed plant materials were shock frozen at minus 80ºC, freeze-dried, ground, and weighed. One gram of plant dry powder was soaked in 50 ml of 50% methanol water solution and shaken for 12 h at 100 rpm. Then the suspended samples were centrifuged (15,000 rpm, 20ºC, 30 min), and the supernatant was filtered through GF/F glass microfibre filters (pore size: 0.7 μm, Whatman, Maidstone, UK). The residue was extracted once again as above. The filtrates were combined, evaporated for removing the methanol with a rotary evaporator (40ºC, 70 rpm), and the aqueous phase was subsequently extracted by liquid liquid extraction (LLE) [14] for more thorough elimination of interferents. The ethyl acetate fraction containing phenolic compounds was dried by anhydrous sodium sulfate concentrated with a rotary evaporator (40ºC, 70 rpm) and silytated [18] before gas chromatograph-mass spectrometry (GC-MS) analysis.

Allelochemical Extraction from Plant Exudates

To detect whether the phenolic allelochemicals were released into water environment by the two plants, solid phase extraction (SPE) and subsequent LLE were performed to extract phenolic allelochemicals exuded into the culture solution. Before SPE, the culture solution of plants was filtered through GF/F filters to remove particles and acidified to pH 2 to enhance the binding of phenolic compounds. Oasis HLB cartridges (500 mg, 6 cc, Waters, Milford, Massachusetts, USA) were used to adsorb exuded phenolic compounds. Each cartridge was preconditioned with 15 ml methanol and equilibrated with 15 ml Milli-Q ultra-pure water. Two litres of acidified culture solution were passed over one cartridge at a rate of 3 ml/min, totally 10 L on 5 cartridges for one plant species. Then, the adsorbed cartridges were washed with ultra-pure water, dried under reduced pressure, and finally eluted with 18 ml methanol per one cartridge. The methanol eluates were combined separately for the two plants, evaporated to dryness, and re-dissolved in ultra-pure water. The same LLE procedure and sequent treatment as plant extracts were used for further separation and purification. The control culture solution was extracted likewise.

Preparation of the Standard Solutions

Stock solutions of VA, PA, FA, and CA were prepared in methanol, and combined to make a standard mixture of 50 mg/L for each. An aliquot of this stock mixture was silytated as the methods mentioned above and diluted to make six concentration standard solutions (ranging from 800 to 4,000 μg/L) for quantitative analysis.

Table 1. Ions for quantitative analysis of silylation derivatives of phenolic compounds.

Compound	Molecular mass	Quantitative ion (m/z*)	Confirmation ions (m/z)
Vanillic acid	312	297	312 267
Protocatechuic acid	370	370	193 355
Ferulic acid	338	338	323 308
Caffeic acid	396	396	219 381

*m/z is the abbreviation of mass-to-charge ratio.

Analytical Protocol of GC-MS

An Agilent 6890N GC with an Agilent 5973I MS operated in electron impact mode was used for sample analysis. The capillary column was an HP-5 ms (30 m×0.25 mm×0.25 μm). The initial oven temperature was 50ºC, held for 1 min, then raised to 280ºC at 10ºC/min with a final isotherm of 5 min. The injection port and interface temperature were both 280ºC. One microliter of sample was injected in the splitless mode. Helium carrier gas was maintained at a constant flow rate of 1 ml/min. The ionization source temperature and the MS quadrupole temperature were set at 230ºC and 150ºC, respectively. To identify the phenolic compounds, the MS was operated in full-scan mode, mass fragments of the components were firstly compared to the mass fragmentation data contained in the NIST 02 and then compared to that of the authentic standards for final confirmation. For quantitative analysis, the MS was operated in selected ion monitoring (SIM) mode. The ions monitored for each compound are listed in Table 1. The pretreated standard solutions with different known concentrations were determined to plot the standard curves (Table 2), based on which contents of the four phenolic compounds extracted from plant tissues and plant exudates were calculated.

Bioassay

One axenic strain of *M. aeruginosa* (FACHB 942), obtained from the Culture Collection of the Freshwater Algae, Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), was selected for the bioassay. The cultures were grown in autoclaved BG11 liquid medium [19] under a 12:12 h light: dark cycle with a light intensity of 2500 lux at 25ºC and were manually shaken twice a day during the incubation. The cells in the exponential phase were used for the growth inhibition tests.

Table 2. Standard curves of phenolic compounds for quantitative analysis.

Compound	Equation	Correlation coefficients (γ^2)
VA	Y=13.08X-9795.4	0.992
PA	Y=11.198X-8513.1	0.994
FA	Y=5.1365X-4093.8	0.994
CA	Y=6.0349X-4703.5	0.994

The growth inhibition tests were based on ISO standard 8692 method [20], modified as follows. Each compound (Fig. 1) was dissolved in DMSO and added into 20ml sterilized BG11 medium, to which circa 1×10^6 cells/ml of *M. aeruginosa* were inoculated. The final concentrations of phenolic compounds in the test solution were 0, 1, 2.5, 5, 10, and 20 mg/L for PA and CA, respectively, 0, 5, 20, 40, 80, and 160 mg/L for VA, 0, 5, 20, 80, 160, and 320 mg/L for FA. All the treatments and the control were prepared in triplicate. The 50% inhibition concentration of the test compounds (EC_{50}) was determined after 72 h incubation. The concentration of DMSO in the test solution was lower than 0.1% (v/v), which had no effect on the growth of *M. aeruginosa* in the pre-experiment.

To investigate the joint effects of the four phenolic compounds, three mixtures with different proportions were prepared. The proportion in the mixture I (Mix I) was calculated based on the EC_{50} values of the individual compound obtained in this study. The proportions in mixture II (Mix II) and mixture III (Mix III) were calculated according to the concentrations of the four phenolic compounds in the culture solution of *H. verticillata* and *V. spiralis* determined in the study, respectively. Five concentrations were prepared for each mixture to cover the responses of *M. aeruginosa,* from no effect to total inhibition. The bioassay procedure of the mixtures was the same as that of the individual compounds. According to the EC_{50} values of the mixtures and the individual allelochemicals, Toxicity Index (TI) model, a quantitative method for analyzing the joint effects of multiple mixtures [21] was used to evaluate the joint effects of the four phenolic compounds. TI values were calculated by the following equation:

$$TI=\sum Cmix_i/EC_{50i}$$

...where TI is the toxicity index, $Cmix_i$ is the concentration of component *i* at the EC_{50} value of the mixture ($mg\cdot L^{-1}$), and EC_{50i} is the EC_{50} value of component *i* measured individually ($mg\cdot L^{-1}$). When the TI value is <0.5, it indicates synergistic action, 0.5<TI<2.0 indicates additive action, and TI>2.0 shows anatagonism.

Table 3. Quantities of the phenolic compounds in the plant tissues and the culuture solutions.

		VA	PA	FA	CA
Plant tissues (μg/10g FW)	*V. spiralis*	40.4	11.1	11.9	11.3
	H. verticillata	181.0	288.3	1,134.0	11,762.4
Culture solutions (μg/L)	*V. spiralis*	1.8	1.3	0.9	1.1
	H. verticillata	1.2	12.1	9.1	29.7
Percentage* (%)	*V. spiralis*	4.4	11.7	7.7	10.0
	H. verticillata	0.7	4.2	0.8	0.3

*percentage of the individual phenolic compounds in the plant culture solutions to those in the plant tissues.

Statistical Analysis

EC_{50} and TI values represent means (n=3) with associated error bars (±standard deviation). Statistical analysis of the data were performed using SPSS 13.0. The EC_{50} values among different compounds and TI values among different mixtures were compared using one-way ANOVA at the 0.05 level. Tests of correlation between contents of phenolic compounds in plant extracts and exudates for each plant were performed using Spearman's rank correlation.

Results

Identification of Compounds

Total ion chromatogram (TIC), obtained by GC/MS analysis of the extracted exudates from *H. verticillata* and *V. spiralis*, is shown in Fig. 2. By Comparing retention time and mass spectral pattern with that of authentic standards, peaks at 16.98, 17.61, 20.07, and 20.66 min were identified as the silytated derivate of VA, PA, FA, and CA, respectively, all of which were found in the ethyl acetate fraction of the two plant extracts whereas never detected in the control.

Quantification of Identified Compounds

The total amounts of the four phenolic compounds in the tissues of *H. verticillata* were 13.4 mg/10g FW, in which CA accounted for 88.0% (Table 3). The content of FA in the plant tissues was 1.1 mg/10g FW, and the levels of VA and PA were 0.2 and 0.3 mg/10g FW, respectively. However, the total content of the four phenolic compounds in *V. spiralis* (74.7 μg/10g FW) was much lower, and vanillic acid amounted to approximately 4 times higher than the three other compounds. The total contents of the four phenolic compounds produced by *H. verticillata* were 179 times as high as that produced by *V. spiralis*.

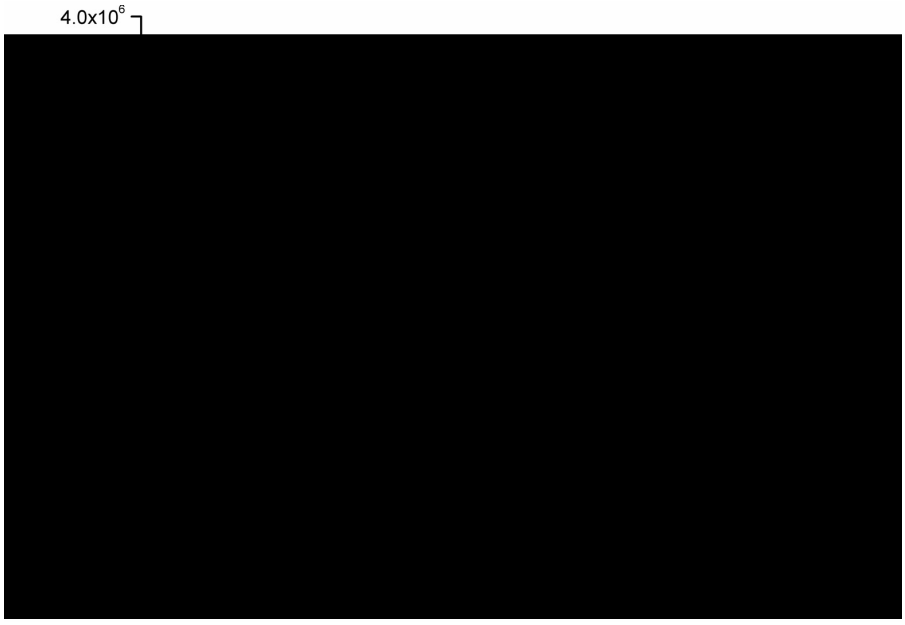

Fig. 2. Total ion chromatogram of standard mixtures and enriched exudates from *H. verticillata* and *V. spiralis* compared with the control culture solution. Compounds are numbered as follows: 1 – vanillic acid (VA), 2 – protocatechuic acid (PA), 3 – ferulic acid (FA), 4 – caffeic acid (CA).

The concentration of the phenolic compounds exuded into the culture solution ranged between 0.9 μg/L (FA for *V. spiralis*) and 29.7 μg/L (CA for *H. verticillata*), and in most cases the concentration of the individual compound was not beyond 10.0 μg/L (Table 3). The total amount of the four phenolic compounds exuded from *H. verticillata* (52.1 μg/L) was approximately 10 times as high as that from *V. spiralis* (5.1 μg/L). The content of each compound except VA exuded from *H. verticillata* was higher than that in *V. spiralis* culture solution.

The percentage of the individual phenolic compounds in the plant culture solution to those in the plant tissues before release varied among plant species and different compounds (Table 3). There was poor correlation between the contents in the culture solution and the contents in the plant tissues ($p>0.05$). In most cases, their contents in the culture solution were lower than 10% of those in the plant tissues.

Inhibitory Effects of Phenolic Compounds on *M. aeruginosa*

Four identified phenolic compounds inhibited the growth of *M. aeruginosa* and their inhibitory effects varied ($p<0.05$). The EC_{50} value of CA was the lowest, whereas that of FA was the highest (Fig. 3). The four compounds also showed joint inhibition on the growth of *M. aeruginosa* (Fig. 4), and the joint action varied with mixing ratios ($p<0.05$). TI values ranged between 0.5 and 2.0 in Mix I and Mix II, which indicated additive action. TI value was lower than 0.5 in Mix III, which meant synergistic action.

Discussion

This study is the first confirmation of the presence of VA, PA, FA, and CA in the culture solution of *H. verticillata* and *V. spiralis*. The evidence of their existence in the two plant tissues provided by previous studies [12, 13] and the present study indicates that *H. verticillata* and *V. spiralis* not only contain these four phenolic compounds in the plant tissues, but also could release them into the surrounding water envrionment. Furthermore, they possessed inhibitory effects on the growth of *M. aeruginosa*. The four compounds are confirmed to be the phenolic allelochemicals of *H. verticillata* and *V. spiralis* according to the prerequisites of allelopathy proposed by Willis [22].

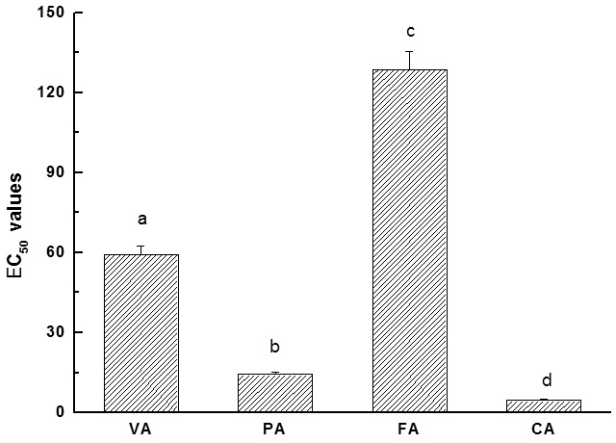

Fig. 3. The 72 h EC_{50} values of individual phenolic compounds against the growth of *M. aeruginosa* (means±standard deviation, n=3). Significant differences between the treatments are indicated with different letters (Tukey test, $p<0.05$).

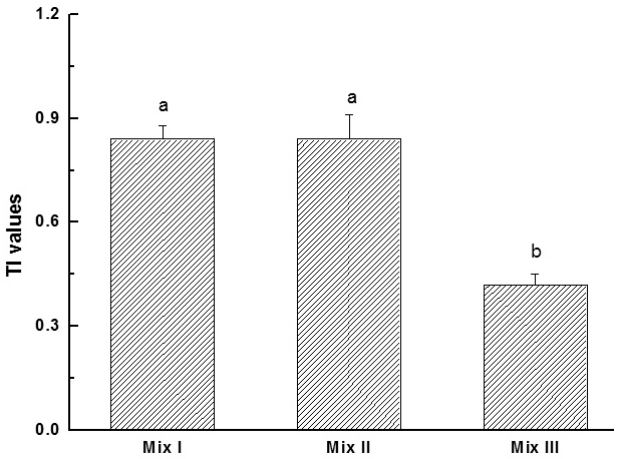

Fig. 4. TI values calculated according to the cell density of *M. aeruginosa* in the mixtures of phenolic compounds (means± standard deviation, n=3). Composition and proportion of mixtures: Mix I, CA:PA:VA:FA, 1:3:13:28; Mix II, CA:PA:VA:FA, 1:0.5:0.05:0.5; Mix II, CA:PA:VA:FA, 1:1:1.5:1. Significant differences between the treatments are indicated with different letters (Tukey test, $p<0.05$).

The amounts of the phenolic allelochemicals exuded by the two plants were not proportional to those in the plant tissues, and a large part of compounds were not released into the surrounding environment but retained in plant tissues. The plant culture density of *H. verticillata* and *V. spiralis* in the present study is 10 g FW/L, which approaches to a moderate level in the natural environment [23-25]. The phenolic contents released by *E. mutallii* increased along with the plant culture density increasing (unpublished data). However, the release is limited. Even if the culture density is beyond 100 g FW/L, the release amount of the phenolic allelochemicals of submerged macrophytes is lower than 100 μg/L [12, 26]. TPCs exuded from *M. spicatum* during two weeks contribute only 1.0% to that in the plant tissues [27]. The similar phenomenon was also observed for wheat when investigating the phenolic allelochemicals in wheat root, shoot, and exudate samples [28]. Some phenolic compounds such as PA and CA are easily oxidized by dissolved oxygen in water environment [26], which may partially reduce the determined phenolic contents after three days of cultivation. Microorganisms are another possible cause for the big differences between production and exudation as they always coexist with the donor plants. Even in the laboratory experiment, it is impossible to exclude the interference of symbiotic and epiphyted microorganisms absolutely [29]. The quick degradation of the exuded phenolic com-

pounds by microorganisms is supposed to be a potential reason why the determined release amount is always very low. To date most quantitative analysis of the exuded allelochemicals are done in the laboratory and the plants are cultivated under the optimum growth conditions [28, 30]. However, plants in natural ecosystems grow in different complex conditions, some of which may induce the release of allelochemicals [29]. For example, TPCs exuded from *M. spicatum* cultivated at high light increases by 60-140% compared to low light cultures [27].

It is impossible a single allelochemical with a low release amount to reach the effective dosage to reduce the cyanobacterial population. However, one plant can release many secondary metabolites simultaneously instead of single compounds into the water envrionment, and they are likely to jointly inhibit the target organisms [26, 30-31]. Ellagic acid, pyrogallic acid, gallic acid, and (+) – catechin, released simultaneously by *M. spicatum*, exert synergistic inhibition action on the growth of *M. aeruginosa* when the mixing proportion is calculated according to the determined contents in the culture solution [26]. In this study, the four phenolic compounds exuded from *H. verticillata* and *V. spiralis* exert additive and synergistic inhibition action against the growth of *M. aeruginosa* depending upon mixing ratios. It is probably still to underestimate the allelopathic effects of the phenolic allelochemicals exuded from *H. verticillata* and *V. spiralis*, since a single determination after three days of cultivation could not reflect the actual situation in natural ecosystems, in which allelochemicals could be released continuously by the donor plants and exert sublethal effects on target organisms during long-term exposure. It is reported that plant exudates with initial addition typically elicit weaker allelopathic effects than with continuous addition [32].

But there are some interspecific differences between the plant species belonging to the same family (Hydrocharitaceae). According to the TPC contents, *H. verticillata* produces significantly higher amounts of phenolic compounds than *V. spiralis* [15]. They also differ by the individual phenolic contents in the present study, which leads to the lower exuded percentage of the phenolic allelochemicals for *H. verticillata* than that for *V. spiralis*. The release differences might influence the allelopathic effects of the two plants. When the mixing ratio is based on the release contents by *H. verticillata* (Mix II), the four phenolic allelochemicals show weaker joint inhibition effects in comparison with *V. spiralis* (Mix III). The ethyl acetate fraction isolated from the aqueous extracts of *H. verticillata* also exhibits weaker algal growth inhibition than that of *V. spiralis* [33].

In the history of allelopathy, studies on allelochemical exudation are much less than studies on allelochemical production. Many antialgal compounds have been extracted from plant tissues [34], which just proves that the donor plants could produce these potential allelochemicals [22]. To test whether these compounds could exert allelopathic effects on the target organisms in natural ecosystems, it is essential to test whether these compounds could exude into the surrounding environment firstly. Hence, more studies on allelochemical exudation need to be done in the future.

Conclusions

VA, PA, FA, and CA were isolated from both plant extracts and exudates of *H. verticillata* and *V. spiralis*, which indicates that the two plants could release the four phenolic compounds into water environment. The contents of the compounds exuded from plants were much lower than their EC_{50} values for *M. aeruginosa*. However, the four phenolic compounds exerted additive and synergistic effects on the growth inhibition of *M. aeruginosa,* depending on mixing ratios. It indicates that joint action of multiple allelochemicals may be an important allelopathic growth inhibition pattern of submerged macrophytes against noxious cyanobacteria.

Acknowledgements

We are grateful to the National Natural Science Foundation of China (30870221, 20877093, 50808172), and the China Postdoctoral Science Foundation Funded Project (20080431013). We are also thankful to Profs. Y. Y. Zhang and B. Y. Liu, Drs. S. P. Cheng, F. He, D Xu, and S. Y. Zhang, and other members of the research group for their kind assistance during our work.

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