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Original Research

Chemical Composition in Aqueous Extracts of *Potamogeton malaianus* and *Potamogeton maackianus* and their Allelopathic Effects on *Microcystis aeruginosa*

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Abstract

The ethyl acetate fractions of aqueous extracts from two submerged macrophytes *Potamogeton malaianus* and *Potamogeton maackianus* were analyzed by gas chromatograph-mass spectrometry (GC-MS). The allelopathic activities and joint effects of the main components in ethyl acetate fractions on *Microcystis aeruginosa* were also determined. The results indicated that primary compositions in the ethyl acetate fractions were fatty acids, phenolic acids and hydroxy fatty acids that possessed antialgal activities. The joint effect assay for palmitic acid, benzoic acid and lactic acid showed that the additional effects were observed in the mixed organic acid, namely, the inhibitory effects of mixture groups were stronger than that of each compound alone on the growth of *M. aeruginosa*.

Keywords: allelopathic activity, GC-MS, *Potamogeton malaianus*, *Potamogeton maackianus*, *Microcystis aeruginosa*

Introduction

Eutrophication of water due to pollution is one of the most serious environmental problems. The occurrence of toxic cyanobacterial blooms in eutrophic lakes, reservoirs and recreational waters has become a worldwide problem [1]. Harmful algal bloom deterioration of water quality causes serious environmental and economic problems because most of these lakes are used as drinking water sources or supplying industry and agriculture [2]. Therefore, it is necessary to explore an effective and secure new method to control the growth of harmful algae.

Chemical interactions between aquatic plants and microalgae play an important role in control of the algal growth in aquatic ecosystems due to excrete second metabolites by the macrophytes [3]. Organic acids as major allelochemicals in some aquatic plants were reported. The extracts from *Myriophyllum spicatum* contain 12 kinds of phenols and polyphenols (gallic acid and ellagic acid) are reported to be growth inhibition properties for *Anacystis nidulans* (*Cyanobacterium*) and *Selenastrum capricornutum* (green alga) [4]. *Myriophyllum verticillatum* contains 3 polyphenols with inhibitory activity to the growth of

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Synechococcus leopoliensis (*Cyanobacterium*) [5]. Nakai et. al. [6] reported that the *M. spicatum* released 4 polyphenols [ellagic, gallic and pyrogallic acids and (+)-catechin] into the culture solution which inhibited the growth of *M. aeruginosa*. Gross et. al. [7] showed that *M. spicatum* released the allelopathic compounds hydrolyzable tannin (eugeniin), and its derivatives, ellagic and gallic acids.

Potamogeton malaianus and *Potamogeton maackianus* are submersed aquatic macrophytes and commonly found in ponds, lakes and streams in East Asian countries [8]. *P. maackianus* is one of the dominant species in many lakes of the middle-lower reaches of the Yangtze River, China [9]. Wu et al. [10] assessed the allelopathic effects of the submerged macrophyte *P. malaianus* on *Scenedesmus obliquus* by using a two-phase approach under controlled laboratory conditions. He et al. [11] reported on the allelopathic effects of *P. malaianus* and *Najas minor* on growth, photosynthesis and antioxidant systems of *S. obliquus* by coexistence experiments. These results indicate that some macrophytes can be used to control algal growth. But reports about organic acids as allelochemicals in aqueous extracts from *P. malaianus* and *P. maackianus* were rare in literature.

This paper describes the isolation and characterization of organic acids of aqueous extracts from *P. malaianus* and *P. maackianus,* as well as their allelopathic activities on the growth of *M. aeruginosa*. In addition, the joint effects of these allelochemicals on the growth of *M. aeruginosa* are also investigated.

Materials and Methods

Chemicals

Ethyl acetate and n-hexane (HPLC grade) were purchased from the Tedia Company, INC, USA. Dimethyl sulfoxide (DMSO, AR), palmitic acid (AR), benzoic acid (AR), lactic acid (AR) and anhydrous sodium sulfate (AR) were purchased from Chemical Reagent, Shanghai, China.

Preparation of Aqueous Extracts and Fractionation

P. malaianus and *P. maackianus* were collected from a pond in the Wuhan Botanical Garden, Chinese Academy of Science, Wuhan, China. Plant materials were washed free of debris by tap water and later by deionized water, then were dried and powdered. 20 g of the powdered sample was soaked in 300 mL of distilled water for 48 h at room temperature, then filtered with GF/C glass fibre filters (47 mm, 1.2 μm, purchased from Whatman Maidstone, UK) with reducing pressure using a vacuum pump, subsequently collected the filtrate for further fractionations. Fig. 1 shows all the steps used in the study.

The aqueous extracts were fractionated according to Xian et al. [12]. Briefly, the filtrate was adjusted to pH 12 with 2 M NaOH, the alkaline extract was centrifuged at 6,000 rpm for 10 min. The supernatant was transferred to a separating funnel and washed three times with 200 mL hexane. The aqueous fraction was acidified to pH 5 with 2 M HCl and then extracted three times with 100 mL ethyl acetate. The ethyl acetate extracts were first dried with anhydrous sodium sulfate and then evaporated to dryness by rotary evaporator at 39ºC. The ethyl acetate extracts were stored at 4ºC until used for GC-MS analysis and biological assay.

Bioassay

The assay was performed using *M. aeruginosa,* which was the most common [13] to be found in eutrophied lakes. Axenic *M. aeruginosa* were obtained from the Culture Collection of Algae at the Institute of Hydrobiology, Chinese Academy of Sciences. *M. aeruginosa* was cultured in sterilized BG11 medium (pH 7.4) at 25ºC, light intensity of 2,500 lux and 12:12 h light:dark cycle. The algae were cultured for 4 days to reach the exponential phase with the density of 10^5-10^6 cells/mL, which were used for the assay of growth inhibition. The compositions of the algae culture medium were 1,500 mg $NaNO_3$, 40 mg $K_2HPO_4{\cdot}3H_2O$, 75 mg $MgSO_4{\cdot}7H_2O$, 36 mg $CaCl_2{\cdot}2H_2O$, 6 mg citric acid, 6 mg ferric ammonium citrate, 1 mg EDTA(dinatrium-salt), 20 mg Na_2CO_3, 2.86 mg H_3BO_3, 1.81 mg $MnCl_2{\cdot}H_2O$, 0.222 mg $ZnSO_4{\cdot}7H_2O$, 0.079 mg $CuSO_4{\cdot}5H_2O$, 0.390 mg $Na_2MoO_4{\cdot}2H_2O$, and 0.049 mg $Co(NO_3)_2{\cdot}6H_2O$ dissolved in distilled water to a total volume of 1,000 mL. The concentrated ethyl acetate fractions were dissolved in 30 μL DMSO and diluted to 50 mL using sterilized algal culture medium. Assay of algal growth inhibition was performed as per the U.S. EPA method [14]. Briefly, 30 mL algae in

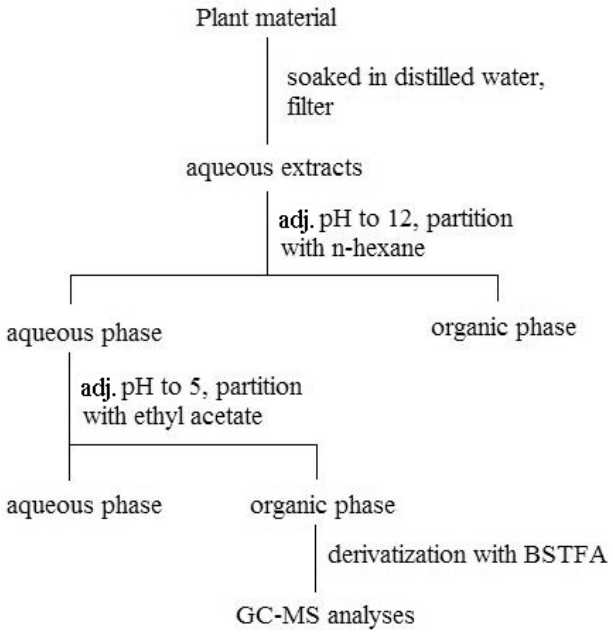

Fig. 1. Flow diagram for extraction of organic acids from *Potamogeton malaianus* and *Potamogeton maackianus.*

exponential phase were placed in sterile 100 mL Erlenmeyer flasks and appropriate amount of ethyl acetate fractions solution was added, mixed well and incubated for 72 h, then the algal growth was monitored using a microscope and hemocytometer to count cell numbers. In control groups, the ethyl acetate fractions were replaced by the algal culture medium.

Inhibitory effects of three organic acids (palmitic acid, benzoic acid and lactic acid) were estimated by five concentrations (5, 20, 80, 160 and 320 mg/L). All procedures of algal growth inhibitory test for three organic acids were as equal as those for the ethyl acetate extracts. The concentration of ethyl acetate fractions resulting in 50% reduction of algal growth rate relative to control (E_rC_{50}) was determined after incubation for 72 h. The percent reduction in growth rate with each ethyl acetate fraction concentration was compared to control value by plotting against the logarithm of the concentration E_rC_{50} values were calculated by linear interpolation [15].

The ratio of the concentration for each compound, designed in the joint toxicity experiment, was equal to the ratio for E_rC_{50} of each compound [16]. All procedures of algae toxicity test for mixtures were as equal as those for the ethyl acetate fractions. In this study, the coefficient of joint effect (K) was used to evaluate the joint toxicity of palmitic acid, benzoic acid and lactic acid. The joint effect was assumed as additional action, and predictive E_rC_{50} (PE_rC_{50}) was handled according to the following equations [16]:

$$\frac{1}{PE_rC_{50}} = \frac{a}{AE_rC_{50}} + \frac{b}{BE_rC_{50}} + \ldots \quad (1)$$

...where: *a* and *b* were the proportion of concentration in mixture (A and B) for A and B, respectively;

AE_rC_{50} and BE_rC_{50} were the E_rC_{50} values of A and B, respectively;

PE_rC_{50} was predictive E_rC_{50} of each mixture.

Then the coefficient of joint effect (K) was calculated according to the following equations [16]:

$$K = \frac{\text{Predictive } E_rC_{50}}{\text{Observed } E_rC_{50}} \quad (2)$$

At last, joint toxicity was evaluated by K as follows [16]:

When $K < 0.57$, it indicates antagonism; $0.57 < K < 1.75$ for addition; $K > 1.75$ for synergism.

Excel 2003 and SPSS 13.0 software were used for statistical analysis.

Identification of Allelochemicals

The dried ethyl acetate fractions were derivatized by adding 0.1 mL *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The mixture was heated at 80ºC for 2 h and derivatized samples were submitted to GC-MS (Agilent 5973, USA) analysis. Mass fragments of the components were compared to the mass fragmentation data contained in the NIST 02.

Results

From Table 1 it can be seen that more than 20 compounds were detected in the aqueous extracts of submerged macrophytes (Table 1) using GC-MS. Organic acids were main components of ethyl acetate fractions of aqueous extracts. While the amount and components of organic acids were different in two macrophytes, fatty acids, phenolic acids and hydroxy fatty acids were primary compositions.

In *P. malaianus*, more than 1% of total content were phenol, benzoic acid, 3-hydroxydecanoic acid, lauric acid, myristic acid, 3-hydroxytetradecanoic acid, palmitic acid, and octadecenoic acid. In *P. maackianus* more than 1% of total content were acetic acid, 2-hydroxylpropanoic acid, benzoic acid, succinic acid, 4-hydroxybenzoic acid,

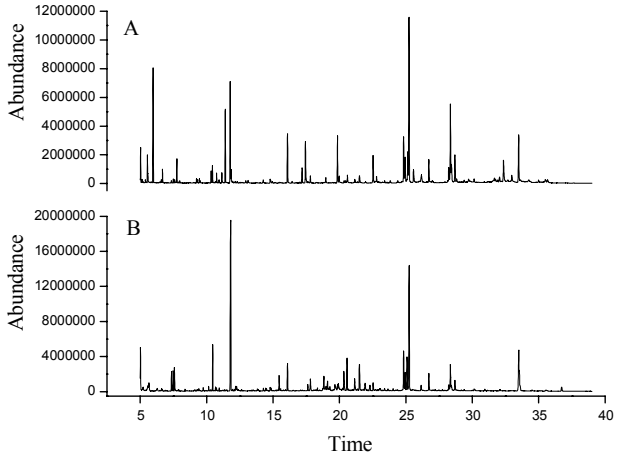

Fig. 2. Total ion chromatogram of ethyl acetate fraction of aqueous extracts from (A) *Potamogeton maackianus* and (B) *Potamogeton malaianus.*

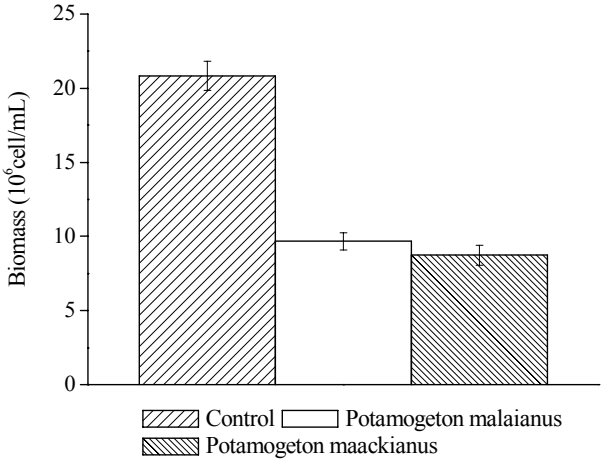

Fig. 3. The inhibitory effects of ethyl acetate fractions from *P. malaianus* and *P. maackianus.* Exposure time was 72 h. All error bars correspond to the standard deviation.

Table 1. Chemical compositions of aqueous extracts of submerged macrophytes.

Rent time (min)	Compounds	*P. malaianus*	*P. maackianus*
5.50	Acetic acid	n.d.	
7.58	Phenol		n.d.
7.74	Lactic acid	n.d.	
9.24	3-Hydroxybutanoic acid	n.d.	
10.16	Phenethyl alcohol		n.d.
10.35	2-Hydroxylhexanoic acid	n.d.	
10.38	3- Methyl -2-hydroxylvaleric acid	n.d.	
10.43	Benzoic acid		
10.66	Octanoic acid		n.d.
11.15	Phenylacetic acid	n.d.	
11.40	Succinic acid	n.d.	
14.28	2-Hydroxy-2-(4-methoxyphenyl)acetic acid		n.d.
16.09	4-Hydroxyphenethyl alcohol		
17.19	4-Hydroxybenzoic acid	n.d.	
17.46	4-Hydroxyphenylacetic acid	n.d.	
17.62	Lauric acid		n.d.
17.81	3-Hydroxydecanoic acid		
19.85	4-Hydroxyphenylpropionic acid		
19.98	Vanillic acid	n.d.	
20.61	Azelaic acid	n.d.	
21.52	Myristic acid		
22.53	3-(4-hydroxy-3-methoxyphenyl)propanoic acid		
24.84	unknown		
24.94	unknown		
25.09	3-Hydroxytetradecanoic Acid		n.d.
25.26	Palmitic acid		
26.74	Margaric acid		
28.24	unknown		
28.36	Octadecenoic acid		
28.43	unknown		
28.70	Octadecanoic acid		

"n.d." undetectable

4-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid, palmitic acid and octadecenoic acid. In *P. malaianus* and *P. maackianus* the highest content of organic acids was palmitic acid.

The allelopathic effects of ethyl acetate fractions from submerged macrophytes and three organic acids were examined by bioassay of *M. aeruginosa*. The results showed that the extracts from the plants inhibited obviously to the growth of *M. aeruginosa* with the inhibition rate of 53.5% and 58.1% for *P. malaianus* and *P. maackianus*, respectively, when the concentration of extracts were 60 mg/L (Fig. 3). The 50 % growth inhibition concentration of three organic acids (palmitic acid, benzoic acid and lactic acid) were found to be 184.6 ± 5.1, 69.3 ± 4.0 and 163.8 ± 4.2 mg /L, respectively.

Allelochemicals in the aquatic environment usually consist of several compounds coexisting instead of existing as individual chemicals. Consequently the joint effect of three organic acids was investigated and the composition of four mixture groups and the proportion of three organic acids in each group were listed in Fig. 4.

From Fig. 4 it was observed that the additional effect of tested chemicals on the growth of *M. aeruginosa* was presented in each group of the compound.

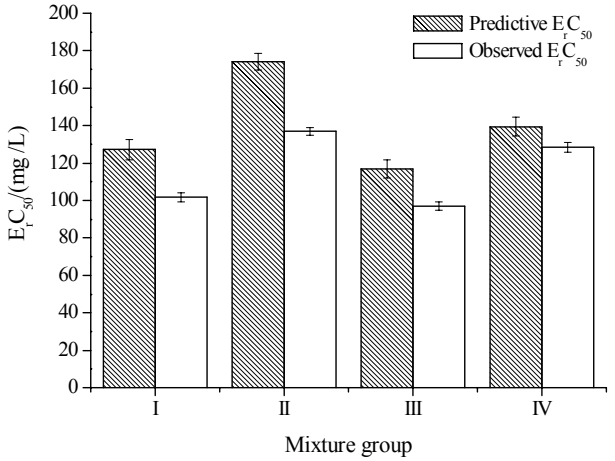

Fig. 4. Joint effect of mixed organic acid on growth of *M. aeruginosa*. Composition and proportion of mixtures:
I, palmitic acid : benzoic acid, 2.7 : 1;
II, palmitic acid : lactic acid, 1.1 : 1;
III, lactic acid : benzoic acid, 2.4 : 1;
IV, palmitic acid : lactic acid : benzoic acid, 2.7 : 2.4 : 1.
The coefficient of joint effect (K) of each mixture group was 1.3, 1.3, 1.2 and 1.1 respectively. All error bars correspond to the standard deviation.

Discussion

Wu et al. [17] reported that the allelopathic effects of weak polar compounds from *P. maackianus* on toxic cyanobacteria (*M. aeruginosa*) and Xian et al. [12] reported the main components of organic acids in *Ceratophyllum demersum, Vallisneria spiralis* and *Hydrilla verticillata*. In this study, strong polar organic acids of aqueous extracts from *P. malaianus* and *P. maackianus* were isolated and characterized. The results indicated that more than 20 fatty acids, phenolic acids and hydroxy fatty acids were detected in the aqueous extracts of the plants. Compared to the results reported by Xian et al. [12], the kinds of the detected organic acids were mostly different. Furthermore, the highest content compound detected in this study was palmitic acid, but they did not find it. There was a difference in the amount and components of organic acids which might be due to the differences of plant species studied.

Strong polar organic acids of aqueous extracts from plants were water-soluble substances that could be easily released into surroundings and contribute more to allelopathic activities. Allelopathic properties of long-chain fatty acids on algae were reported by Proctor [18] and confirmed by Kroes [19]. Tang et al. [20] reported that short-chain fatty acids in decomposing wheat straw in water were able to inhibit the seedling growth of wheat. Xian et al. [12] reported several fatty acids (succinic acid, azelaic acid, aconitic acid, lactic acid, citric acid) with allelopathic activity on *M. aeruginosa*. In addition, the antialgal effects of phenolic acids were reported by some [21-23]. For example, benzoic acid was reported to inhibit hydraulic conductivity and nutrient uptake by plant roots, thus resulting in growth inhibition [21]. In a previous work we had reported the 50% growth inhibitory concentration of 4-hydroxybenzoic acid and benzoic acid for both strains of *M. aeruginosa* (the toxic strain FACHB 942 and the nontoxic strain FACHB 469) [23]. In this study, several phenolic acids, such as benzoic acid and 4-hydroxybenzoic acid, were detected in the aqueous extracts of *P. malaianus* and *P. maackianus*.

The results of antialgal bioassay showed that organic acids in the aqueous extracts of *P. malaianus* and *P. maackianus* had potential allelopathic activities on algal growth. The result indicated that inhibitory effects of these mixture groups were stronger than that of individual compounds on the growth of *M. aeruginosa*. The additional effect was presented between the tested allelochemicals ($0.57 < K < 1.75$). In a previous work it was reported that the joint effects of benzoic acid, 4-hydroxybenzoic acid and 3,4,5-trihydroxybenzoic acid on the growth of *M. aeruginosa* (toxic FACHB 942), and the mixture of phenolic allelochemicals showed the obvious synergistic effects [23]. Because some allelochemicals are simultaneously present in the aquatic environment, the synergistic effects of several allelochemicals are supposed to be an important subject that might reveal the mechanism of markedly reducing the algal population in a natural aquatic ecosystem in the presence of submerged marcrophytes.

Although the synergistic action of organic acids was not found in this study, it is significant to investigate further whether a stronger synergistic effect is present between different kinds of identified allelochemical aquatic environments.

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