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Original Research

Composition and Anti-Cyanobacterial Activity of Essential Oils from Six Different Submerged Macrophytes

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Abstract

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. The inhibitory allelopathy of plants on harmful algae, which has drawn extensive attention recently, is low-cost and ecologically safe, and some good results have been reported. In this study, volatile organic compounds as major allelochemicals have been isolated from some aquatic plants and identified. The chemical compositions of essential oil isolated from *Potamogeton cristatus*, *Potamogeton maackianus*, *Potamogeton lucens*, *Vallisneria spinulosa*, *Ceratophyllum demersum*, and *Hydrilla verticillata* were analyzed by GC/MS. Thirty components were identified in the oils, mainly including fatty acids, ester, sterol, and ketone, etc. Inhibitory effects of essential oils on *Microcystis aeruginosa* were also investigated. The inhibition ratio of essential oils on *M. aeruginosa* was 30.2-41.7% when the treatment concentration of extracts was at a level of 50.0 mg/L. Hence, the essential oils isolated exhibited a significant anti-cyanobacterial activity.

Keywords: anti-cyanobacterial activity, essential oil, *Microcystis aeruginosa*, GC/MS, submerged macrophytes

Introduction

Allelochemicals from macrophytes or other organisms that inhibit microalgal growth have gained great interest due to their environmental potential as algaecides in controlling water blooms or red tide [1]. In aquatic ecosystems, varieties of submerged macrophytes were found to effectively inhibit the growth of blue-green algae [2]. For example, *Myriophyllum spicatum* [3-5], *Vallisneria spiralis* [2], *Potamogeton crispus* [6], *Ceratophyllum demersum*, and *Najas marina* [7] showed allelopathic effects on nuisance algae. Xian et al. [2] found that six allelochemicals (2-ethyl-3-methylmaleimide, dihydroactinidiolide, 4-oxo-β-ionone, 3-hydroxy-5, 6-epoxy-β-ionone, loliolide, and 6-hydroxy-3-oxo-α-ionone) and an unknown compound from *V. spiralis* had strong inhibitory effects on *Microcystis aeruginosa* Kütz. *M. spicatum* could release ellagic acid, gallic acid, pyrogallic acid, (+)-catechin, hydrolyzable tannin (eugeniin) and β-1,2,3-tri-O-galloyl-4,6-(S)-hexahydroxydiphenoyl-D-glucose (tellimagrandin II) into the culture solution, which inhibits the growth of *M. aeruginosa*

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[3, 8, 9]. Tellimagrandin II is an effective inhibitor of microalgal exoenzymes, and tellimagrandin II has at least two modes of action: inhibition of exoenzymes and inhibition of PSII [9]. Gao et al. [10] researched the algae inhibition activity of phenolic compounds exuded by culture solutions of two submerged freshwater macrophytes (*V. spiralis* and *Hydrilla verticillata*) incubated at 10g of fresh weight for three days. Results indicated that two plants could release some phenolic allelochemicals into culture solutions to inhibit the growth of *M. aeruginosa*. Gross et al. [7] showed that for *Najas*, 50% methanol and for *Ceratophyllum* 50% acetone yielded the strongest inhibition in the agar-diffusion assay with various filamentous or chroococcal cyanobacteria as target species. Further fractionation by liquid-liquid extraction (LLE) and solid phase extraction (SPE) procedures showed that both aquatic plants appeared to have more than one active fraction, one being hydrophilic and one moderately lipophilic. The water-soluble allelochemicals may inhibit phytoplankton growth, whereas the lipophilic allelochemicals may act through direct cell-cell contact, e.g., against epiphytes. These results clearly indicate that some macrophytes can be used to control nuisance cyanobacterial growth.

In this study we described the results of GC/MS analyses of essential oils from *Potamogeton cristatus*, *Potamogeton maackianus*, *Potamogeton lucens*, *Vallisneria spinulosa*, *Ceratophyllum demersum*, *Hydrilla verticillata,* and their anti-cyanobacterial activities.

Materials and Methods

Chemicals

Anhydrous sodium sulfate (AR), alcohol (AR), diethyl ether (AR), and dimethyl sulfoxide (DMSO, AR) were purchased from Chemical Reagent Company, Shanghai, China. Methanol (HPLC) was purchased from Tedia Company, Inc., USA.

Macrophytes and Extraction of Chemicals

P. cristatus, *P. maackianus*, *P. lucens*, *V. spinulosa*, *C. demersum,* and *H. verticillata* were collected from Wuhan Botanical Garden, Chinese Academy of Sciences. Plant materials were washed free of debris with regular water and later by deionized water, they were then dried and powdered. An appropriate amount of the powdered sample was soaked in alcohol for 72 hours at room temperature, then filtered with GF/C glass fiber filters (47 mm, 1.2 μm, purchased from Whatman Maidstone, UK). Pressure was reduced using a vacuum pump, and the filtrates subsequently collected. The alcohol filtrates were evaporated to be close to dryness by rotary evaporator at 39ºC, then an appropriate amount of ultrapure water was added to the alcohol filtrates, and finally fractionated with diethyl ether three times. The diethyl ether filtrates were first dehydrated with sodium sulfate anhydrate and then evaporated to dryness by the rotary evaporator at 39ºC. The diethyl ether extracts were stored at 4ºC until they were used for GC-MS analysis and biological assay. Fig. 1 shows all the steps used in the study.

Bioassay

Axenic *M. aeruginosa* obtained from the Culture Collection of Algae at the Institute of Hydrobiology, Chinese Academy of Sciences, was used for cyanobacterial assays. The algae were cultured in sterilized BG11 medium (pH 7.4) under a light intensity of 2500 lux at 25ºC, on a 12:12 hour light:dark cycle. The algae were cultured for four days to reach the exponential phase with the density of 10^5-10^6 cells/mL, and were used for the assay of growth inhibition. The growth medium of all cultures was BG11 [11].

The concentration-response relationships between the allelochemicals and the tested organisms were studied in 50 mL flasks containing 20 mL test solution, to which 10^6 cells·mL^{-1} of *M. aeruginosa* were inoculated. The tested

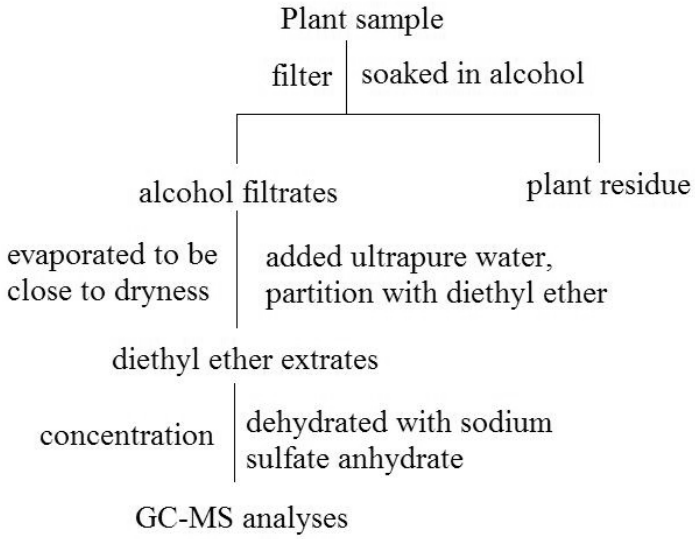

Fig. 1. Flow diagram for the extraction of essential oils from *Potamogeton cristatus*, *Potamogeton maackianus*, *Potamogeton lucens*, *Vallisneria spinulosa*, *Ceratophyllum demersum*, and *Hydrilla verticillata*.

organisms were exposed, in triplicate, to one concentration level and a control, respectively. The final concentrations of compounds in the test solution were 50 mgL^{-1} for essential oils from *P. cristatus*, *P. maackianus*, *P. lucens*, *V. spinulosa*, *C. demersum*, and *H. verticillata*. The 50% inhibition concentrations of all essential oils based on cell density of the tested organisms (EC_{50}) were determined after exposure for 72 hours. The stock solutions of essential oils were prepared with DMSO, which in the test solution was lower than 0.2% (v/v). The test results indicated that the concentrations of DMSO added had no effect on the growth of the tested organisms.

Identification of Essential Oils

The dried essential oils were analyzed by gas chromatograph-mass spectrometry (GC-MS) (Agilent computerized system consisting of a 6890 gas chromatograph coupled with an Agilent 5973N quadrupole mass spectrometer) using an HP-5MS capillary column (30 m×0.25 mm×0.25 mm ID). The oven temperature was set at 60ºC (initial temperature was maintained for 1 min) and reached 260ºC at a rate of 4ºC/min, and kept constant for 20 min. Helium was used as carrier gas with a flow rate of 1 mL/min. Mass spectral patterns of the peaks were identified by comparing them with patterns stored in the US National Institute of Standards and Technology (NIST) mass spectral library ver. 2.0.

Results

Characterization of volatile components from *P. cristatus*, *P. maackianus*, *P. lucens*, *V. spinulosa*, *C. demersum*, and *H. verticillata* were analyzed and identified by gas chromatograph-mass spectrometry (GC-MS). From Table 1 it was found that more than 30 compounds were detected in the essential oils of six submerged macrophytes (Table 1). The composition of the volatile components were different in the six plants. Major volatile components identified from the six macrophytes were fatty acids, ester, ketone, sterol, alkane, amide, naphthalene, and phenanthrene. There were differences in amounts and components of essential oils, which might be due to the differences of plant species. Xian et al. [12] also reported the composition of the volatile substance from *C. demersum*, compared to the results of our studies. There were differences in amounts and components of volatile oils from *C. demersum*, which might be due to the different extraction methods.

Table 2 showed the anti-cyanobacterial activities of essential oils from *P. cristatus*, *P. maackianus*, *P. lucens*, *V. spinulosa*, *C. demersum*, and *H. verticillata* on *M. aeruginosa*. These results indicated that the essential oils isolated from the six submerged macrophytes inhibited the growth of *M. aeruginosa* with inhibition rates being 35.1, 36.2, 32.9, 41.7, 30.2, and 36.6%, respectively, when the treatment concentration of extracts was at a level of 50.0 mg/L.

Discussion

Harmful cyanobacterial blooms have increased globally in frequency and intensity and research on their controlling topic are developing simultaneously [13]. Recently, much effort has been paid to allelochemicals, which are potentially important sources of selective and biodegradable algicides [14]. Some allelochemicals have been isolated and their algicidal activities have been demonstrated, such as alkaloids (gramine, berberine) from *Arundo donax* L. and *Coptis chinensis* [15, 16], phenolic acids, hydroxy fatty acids, fatty acids from *Potamogeton malaianus*, *Potamogeton maackianus*, *Typha latifolia*, and *Arundo donax* [17, 18].

Submerged macrophytes can stabilize clear-water states in shallow eutrophic lakes by releasing allelopathic compounds that reduce epiphyton and phytoplankton biomass [19]. Zhang et al. [20] isolated and identified 3 allelochemicals (linoleic acid, tetradecanoic, and hexadecanoic acids) from submerged macrophytes (*Chara vulgaris*), which inhibited the growth of toxic *M. aeruginosa* and the linoleic acid proved most potent. The combined activity of these three fatty acids exerted synergistic inhibitory effects on the growth of toxic *M. aeruginosa*. The previous reports indicated that four fatty acids (dodecanoic acid, tetradecanoic acid, hexadecanoic acid, linoleic acid) could control the growth of toxic *M. aeurginosa*, *Chlorella pyrenoidosa* Chick, and *Scenedesmus obliques* Kütz [21]. Linoleic acid was found to be the most inhibitory for *M. aeruginosa*. In addition, linoleic acid also was clearly able to inhibit the growth of *Phormidium tenue* [22], and *Selenastrum capricornutum* [23]. Fatty acids are widely distributed in aquatic and terrestrial environments, indicating the possibility that fatty acids in aquatic environments may affect cyanobacterial growth [4]. In our previous works, the EC_{50} of dihydroactinidiolide and β-ionone on *M. aeruginosa* were reported at 30.1±1.7 and 25.3±2.1 mg/L, respectively [24]. These compounds were also found in this study.

Three phthalate compounds (dimethyl phthalate, diethyl phthalate, 1,2-benzenedicarboxylic acid mono(2-ethylhexyl)ester) were identified in the oils. Certain allelochemicals of phthalate had been studied for controlling microalgae growth. For example, diethyl phthalate could inhibit the growth of *Dunaliella salina* [25], and dibutyl phthalate was able to inhibit the growth of *Gymnodinium breve* [26]. Bie et al. [26] reported the mechanism of inhibitory action of dibutyl phthalate (DBP) on red tide algae *G. breve*. The effects of DBP on malonaldehyde (MDA), subcellular structure and superoxide dismutase (SOD) isoforms were investigated. The results showed that MDA accumulated in the algae cell under DBP exposure, and for the 3 $mg{\cdot}L^{-1}$ DBP-treated algae culture a peak value of 0.34 $\mu mol{\cdot}(10^9\ cells)^{-1}$ occurred at 72 h, which was about 2.3 times that of the control. TEM pictures showed the disruption of DBP on the subcellular structure of *G. breve*. A morphological phenomenon appeared whereby the algae cell was commonly found with small tubules or apical parts around the cell membrane, and almost all normal cell

Table 1. Composition of essential oils from six submerged macrophytes.

Rent time (min)	Compounds	*P. cristatus*	*P. maackianus*	*P. lucens*	*V. spinulosa*	*C. demersum*	*H. verticillata*
7.25	1-methyl-naphthalene	+	+	+	+	+	+
8.28	dimethyl phthalate	+	-	+	+	+	+
8.31	unknown	+	+	+	+	+	+
8.49	β-ionone	+	+	-	-	+	+
8.64	butylated hydroxytoluene	+	+	+	+	+	+
8.82	dodecanoic acid	-	-	-	-	-	+
8.87	dihydroactinidiolide	+	+	-	-	+	+
9.12	diethyl phthalate	+	+	+	+	+	+
9.48	ethyl citrate	+	+	+	+	+	+
9.57	diisobutyl adipate	+	+	-	+	+	+
9.65	heptadecane	+	+	+	-	+	+
9.99	tetradecanoic acid	+	+	+	+	+	+
10.16	tetradecanoic acid ethyl ester	+	-	-	-	+	-
10.20	octadecane	+	+	+	-	+	+
10.33	phenanthrene	-	-	-	+	+	+
10.45	6,10,14-trimethyl-2-pentadecanone	+	+	-	-	+	+
10.56	pentadecanoic acid	+	-	-	+	+	+
10.67	unknown	+	+	-	+	+	+
10.72	nonadecane	+	+	-	-	-	+
11.03	Z-11-hexadecenoic acid	-	-	+	+	-	+
11.09	hexadecanoic acid	-	+	-	-	+	+
11.15	unknown	+	+	+	+	+	+
11.20	hexadecanoic acid ethyl ester	+	+	+	+	+	+
11.79	phytol	+	+	+	+	+	+
11.94	linoleic acid	+	+	+	+	+	+
12.01	linoleic acid ethyl ester	+	+	-	-	+	+
12.16	unknown	-	-	-	-	+	-
12.93	(Z)-9-octadecenamide	+	+	-	-	-	+
13.88	1,2-benzenedicarboxylic acid mono(2-ethylhexyl) ester	+	+	+	+	+	+
14.10	unknown	-	-	+	-	-	-
14.75	11-decyl-heneicosane	-		-	-	-	-
18.32	unknown	-	-	+	-	-	+
19.95	vitamin E	-	-	-	-	+	-
21.96	campesterol	-	-	-	-	+	-
22.59	stigmasterol	-	+	+	-	-	+
23.86	g-sitosterol	+	-	-	-	+	+

"-"undetectable, "+"detectable

Table 2. Inhibitory effects of essential oils from the six submerged macrophytes on growth of *M. aeruginosa*.

	P. cristatus	*P. maackianus*	*P. lucens*	*V. spinulosa*	*C. demersum*	*H. verticillata*
Inhibition ratio	35.1%	36.2%	32.9%	41.7%	30.2%	36.6%

Table 3. EC_{50} (mg/L)of four fatty acids on toxic *M. aeurginosa*, *C. pyrenoidosa* Chick, and *S. obliques* Kütz (the fifth day) [21].

Compounds	*Microcystis aeruginosa*	*Chlorella pyrenoidosa*	*Scenedesmus obliquus*
Dodecanoic acid	4.563	6.265	5.813
Tetradecanoic acid	15.498	16.003	15.977
Hexadecanoic acid	18.234	19.876	20.146
Linoleic acid	0.047	0.089	0.059

organelles were indistinguishable in the end. Chen [27] studied the release of phthalate esters and bioavailability during decomposition of tested submerged plants (*H. verticilla*) in the laboratory. He thought that the possible degradation pathway of DBP and di(2-ethylhexyl) phthalate (DEHP) were conferred by kinds of degradation products. That is to say, DBP and DEHP were first hydrolyzed into corresponding MBP, MEHP, and alcohols under the effect of esterase, and then to o-phthalic acid and benzoic acid gradually. At last, they were degraded into CO_2 and H_2O.

These fatty acids, phthalate, dihydroactinidiolide, and β-ionone were found to be significantly active against cyanobacterial at low concentrations and are promising chemical agents for harmful algae control.

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