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Short Communication

Isolating and Identifying Organic Acids from *Portulaca oleracea* and Determining Their Anti-cyanobacterial Activity

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Abstract

We explored the possibility of using allelochemicals from terrestrial plants to solve the problem of cyanobacterial blooms in eutrophicated shallow lakes. The highly effective inhibitory allelochemicals of purslane (*Portulaca oleracea* L.) and purslane seeds were extracted and purified through a series of procedures, and the chemical components of the purified extracts were analyzed by gas chromatography-mass spectrometry (GC-MS). The extracts contained 12 fatty acids and three phenolic acids, of which nine compounds were found to be inhibitors of harmful cyanobacteria. The inhibitory ratios of the purified extracts of purslane and purslane seeds on *M. aeruginosa* were 97.4 and 81.6%, respectively, when the cyanobacterial were exposed under 15.0 mg/L of extracts. The purified extracts exhibited significant anti-cyanobacterial activities, the organic acids that may contribute to the allelopathic effects of the purslane and purslane seeds on *M. aeruginosa*.

Keywords: organic acid, *Portulaca oleracea*, *Microcystis aeruginosa*, cyanobacterial inhibition activity, GC-MS

Introduction

Microcystis aeruginosa is a common freshwater cyanobacterium in freshwater lakes and reservoirs worldwide. Microcystins produced by *M. aeruginosa* have been implicated in wildlife, livestock, and pet fatalities as well as human poisonings worldwide [1-7]. The control of *Microcystis* blooms is an urgent issue with regard to improving water and ecosystem quality and public health.

In recent efforts to control toxic bloom-forming *Microcystis*, algicides from natural biomaterials (allelochemicals) have received attention as alternatives to chemical agents [8]. Traditional approaches to studying allelochemicals have focused on the isolation of allelopathic compounds from aquatic macrophytes [9-12]. A few studies have focused on the potential algaecide isolation from terrestrial plants, such as barley straw, *Salvia miltiorrhiza* Bung, and *Radix Astragali* [13-16].

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Purslane (*Portulaca oleracea* L.) is one of the most used medicinal plants, being a common, herbaceous succulent annual plant. It can be found all over the world in the temperate and tropical regions from Europe to Africa, Asia, America, and Australia [17]. It has been used as a food and medicinal plant for thousands of years in China as well as many other nations [18]. Compounds have been separated and identified from purslane – for example, five alkaloids and six fatty acids [17, 19]. Four fatty acids (palmitic, linoleic, oleic, and stearic acid) have been shown to markedly inhibit the growth of *M. aeruginosa*, *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, *Selenastrum capricornutum*, *Monoraphidim contortum,* and *Chlorella vulgaris* [20-23].

Based on the literature, the extracts of purslane may contain plentiful organic acids that could inhibit the growth of harmful cyanobacteria. Therefore, the goals of this work were to isolate and identify organic acids from extracts of purslane seeds and purslane plants except seeds (hereinafter referred to as purslane), and to study their allelopathic effects on *M. aeruginosa*.

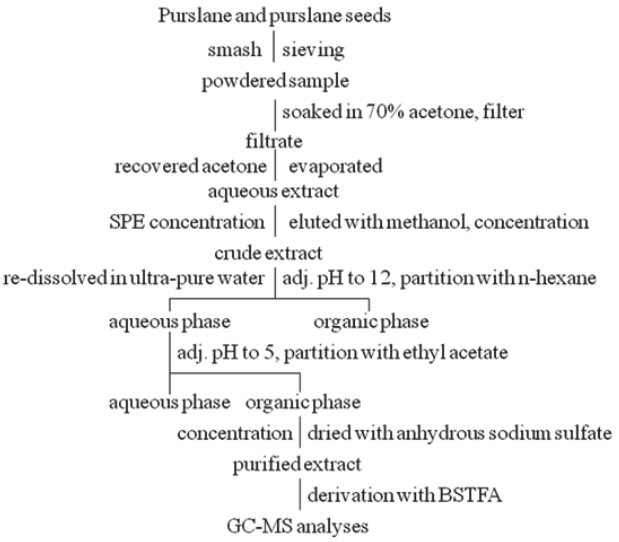

Fig. 1. Flow diagram for extraction of organic acids from *Portulaca oleracea* and *Portulaca oleracea* seeds.

Materials and Methods

Preparation of Plant Material Extracts

Purslane and purslane seeds were naturally dried on trays away from sunlight at room temperature. The dry weights of the purslane and purslane seeds were measured, and then they were powdered to obtain 20-mesh particles. A powder sample (5 g) was extracted with 100 mL of 70% acetone at room temperature (25ºC) for 24 h. The extract was filtered through GF/C glass fiber filters (47 mm, 1.2 µm, Whatman Maidstone, UK) for particle removal. After removing the acetone by reduced pressure distillation at 35ºC, an aqueous solution of organic acids was obtained. The polyphenol content was determined according to Cai et al. [24]. Solid phase extraction (SPE) was employed to extract the organic acids from the aqueous solution. Before SPE, Oasis HLB cartridges (500 mg, 6cc, Waters, Milford, Massachusetts, USA) were preconditioned with 15 mL of methanol and equilibrated with 15 mL of Milli-Q ultra-pure water. The aqueous solution was passed over the cartridge at a rate of 3 mL/min. The adsorbed cartridges were washed with ultra-pure water, dried under reduced pressure, and finally eluted with 18 mL of methanol per cartridge. The eluted methanol was evaporated by a rotary evaporator at 40ºC. The crude organic acids were re-dissolved in ultra-pure water. To separate and purify organic acids, the aqueous solution was adjusted to pH 12 using 2 M NaOH, and 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 of hexane. The aqueous fraction was acidified to pH 5 using 2 M HCl and then extracted three times with 100 mL of ethyl acetate. The ethyl acetate extracts were first dried with anhydrous sodium sulfate and then evaporated to dryness by a rotary evaporator at 39ºC. The purified organic acids were stored at 4ºC until use in GC-MS analysis and biological assay.

Bioassay

Axenic *M. aeruginosa* were obtained from the Culture Collection of Algae at the Institute of Hydrobiology, Chinese Academy of Sciences. The cyanobacterial were cultured in sterilized BG11 [25] medium (pH 7.4) at 25ºC with a light intensity of 2,500 lux and 12:12 h light:dark cycle. The cyanobacterial were cultured for four days to reach the exponential phase with a density of 10^5-10^6 cells/mL and then used for the growth inhi-bition assay. The growth medium used in all cultures was BG11.

The concentration-response relationships between the allelochemicals and the tested organisms were studied in 50 mL flasks containing 25 mL of test solution (in BG11), to which 10^6 cells mL^{-1} of *M. aeruginosa* were inoculated. The tested organisms were exposed, in triplicate, to one concentration level and a control, respectively. The final concentrations of compounds in the test solution were 20 mgL^{-1} and 15 mgL^{-1}, respectively, for the crude and purified allelochemicals obtained from purslane and purslane seeds. In control groups, the ethyl acetate fractions were replaced by the algal culture medium.

The inhibition ratio of every component based on the cell density of the tested organisms and control was determined after exposure for 72 h. The algal growth was monitored using a microscope and hemocytometer to count cell numbers. The stock solutions of extracts were prepared with DMSO, the concentration of which was less than 0.2% (v/v) in the test solution. The test results indicated that the concentrations of DMSO added had no effect on the growth of the tested organisms [26].

Table 1. Compounds isolated from purslane and purslane seeds.

Rent time (min)	Compounds	Purslane	Purslane seed
15.32	butanedioic acid	-	+
17.46	phenylpropionic acid	-	+
21.69	p-hydroxybenzoic acid	+	+
22.11	lauric acid	+	-
24.24	vanillic acid	+	-
24.93	myristic acid	+	+
26.73	pentadecanoic acid	+	+
28.43	palmitoleic acid	+	-
28.73	palmitic acid	+	-
29.81	heptadecanoic acid	+	-
31.27	linoleic acid	+	+
31.34	oleic acid	+	-
31.64	stearic acid	+	+
34.34	arachidic acid	-	+
36.85	behenic acid	-	+

"+"detectable, "–"undetectable

Identification of Allelochemical

The purified organic acids were analyzed by gas chromatography-mass spectrometry (GC-MS) (Agilent computerized system consisting of a 6890 gas chromatograph coupled to an Agilent 5973N quadrupole mass spectrometer) using a HP-5MS capillary column (30 m × 0.25 mm × 0.25 mm id). The oven temperature was programmed as follows: 60ºC (initial temperature, maintained for 1 min), heated to 260ºC at a rate of 4ºC/min, and maintained for 20 min. Helium was used as the carrier gas with a flow rate of 1 mL/min. The mass fragments of the components were compared to the NIST 02 mass fragmentation data.

Results

The results showed that polyphenol contents of the purslane and purslane seeds were 0.96 % and 0.26%, respectively. The total polyphenol content of the purslane was higher than that of the purslane seeds.

The components of the extracts from purslane and purslane seeds are shown in Table 1. Fifteen organic acid compounds were detected in the extracts of the two plant materials. The compositions of the organic acid compounds were different in the two plant materials. There were 11 organic acids in the extract of purslane and 9 in that of purslane seeds.

When *M. aeruginosa* was exposed to the respective cr ude extracts of 20.0 mg/L and purified extracts of 15.0 mg/L from purslane and purslane seed, the inhibitory ratios were all observed in the range of 81.6-98.2% (Table 2). Hence, the crude and purified extracts exhibited significant anti-cyanobacterial activities, and the allelopathic effects of the purified extracts were higher than those of the crude extracts.

Discussion

In the current work, the extracts contained 12 fatty acids and three phenolic acids, of which 12 compounds were found to be inhibitors of harmful cyanobacteria, and the purified extracts from the purslane and purslane seeds exhibited significant anti- cyanobacterial activities. *Portulaca oleracea* contains organic acids, which are widely distributed in aquatic and terrestrial environments [11, 27-31], indicating the possibility that organic acids in the aquatic environment may significantly affect cyanobacterial growth. Table 3 shows that nine organic

Table 3. Median effective concentrations (EC_{50}, mg/L) for the growth inhibition of *M. aeruginosa* of nine tested organic acids.

Organic acids	Median effective concentrations (EC_{50}, mg/L)	References
lauric acid	4.56	22
myristic acid	12.80±0.47	21
palmitic acid	17.17±0.79	21
linoleic acid	0.04±0.01	21
oleic acid	1.6±0.4	11
stearic acid	19.85	22
butanedioic acid	117±3	32
p-hydroxybenzoic acid	32.5±2.00	32
vanillic acid	57.17	33

The given values are mean ± S.D. of three repeated tests.

Table 2. Inhibitory effects of crude and purified extracts from purslane and purslane seeds on growth of *M. aeruginosa.*

	Purslane (crude) (%)	Purslane seed (crude) (%)	Purslane (purified) (%)	Purslane seed (purified) (%)
Inhibitory ratio	98.20±1.64	95.76±1.10	97.43±0.63	81.63±2.59

acids (lauric, myristic, palmitic, linoleic, oleic, stearic, butanedioic, p-hydroxybenzoic, and vanillic) inhibited the growth of *M. aeruginosa.*

Nakai et al.[11] found that (i) length of carbon chain, (ii) number of unsaturated linkages, and (iii) positions of any double bonds may affect the anti-cyanobacterial activities of fatty acids. Free fatty acids exerted cytotoxic effects on the plasma membranes of some species of phytoplankton, resulting in an alteration in membrane permeability [20]. Lu et al. [34] found that daily low-dose exposure to the allelochemical pyrogallol inhibited the growth of *M. aeruginosa* and affected the morphological characteristics and metabolic state. Damage to the plasma membranes allowed K^+ to leak out from the cellular interior. The lethal effects of fatty acids may be caused by the lytic effect on the stressed cells via disruption of the cell plasma membranes. As a secondary effect, fatty acids seem to cause the dissociation of phycobilins from the thylakoid membranes. In addition, Zhang et al. [33] found that vanillic acid triggered the generation of superoxide anion radicals ($O_2^{\bullet-}$). The $O_2^{\bullet-}$ might induce a lipid peroxidation that might change cell membrane penetrability, thereby leading to the eventual death of *M. aeruginosa*.

Conclusions

In the current work, organic acids were isolated and identified from purslane and purslane seeds. The purified extracts contained 12 fatty acids and three phenolic acids, of which nine compounds were found to be inhibitors of harmful cyanobacteria. Hence the purified extracts exhibited significant anti-cyanobacterial activities that may contribute to the allelopathic effects of the purslane and purslane seeds on *M. aeruginosa*.

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