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Original Research

Composition of Ethyl Acetate Extracts from Three Plant Materials (Shaddock Peel, Pomegranate Peel, Pomegranate Seed) and Their Algicidal Activities

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Abstract

Many studies have involved the isolation and identification of allelochemicals from aquatic plants, but the algicidal properties of terrestrial plants have received less attention. This study aims to identify allelochemicals of ethyl acetate extracts from three plant materials (shaddock peel, pomegranate peel, pomegranate seed) and to investigate their inhibitory effects on *Microcystis aeruginosa*. The ethyl acetate extracts of the three plant materials were identified by GC-MS. Finally, 19 kinds of compounds (including organic acids, ester, ketone, sterol, etc.) were obtained and eight kinds of organic acids and N-phenyl-2-Naphthalenamine were proved to be allelochemicals. The inhibitory effects of the ethyl acetate extracts were also explored by *M. aeruginosa* bioassay. This showed that the inhibition percentages of ethyl acetate extracts of the three plant materials on the growth of *M. aeruginosa* were 43.9%, 47.5%, and 40.3%, respectively, when the algae were treated at a dosage of 20 mg/L extracts.

Keywords: allelopathic effect, shaddock peel, pomegranate peel, pomegranate seed, *Microcystis aeruginosa*

Introduction

Eutrophication is a worldwide problem in aquatic ecosystems and cyanobacterial blooms can cause severe water quality deterioration due to toxin production, hypoxia, off-flavor problems that lead to illness in animals and humans [1-3]. Therefore, the removal of harmful cyanobacterial blooms is a crucial step for the maintenance of safe water supplies and for the safety of aquatic products [4].

Recent years have seen many studies relating to the isolation and identification of allelochemicals from aquatic plants [5-8], while the algicidal properties of terrestrial plants still get less attention [9]. Up to now, the extracts of many terrestrial plants also show inhibitory effects against cyanobacteria, such as barley straw [10-13], Chinese traditional medicines [9, 14, 15], and so on.

Pomegranate (*Punicagranatum*), belonging to the family Punicaceae [16], is one of the oldest edible fruits. It has been cultivated extensively in Mediterranean countries, Iran, India, and to some extent in the U.S. (California), China, Japan, and Russia [17]. Pomegranate peels and seeds

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as a byproduct of pomegranate processing are about 26-30%, 20% (w/w) of the whole fruit weight, respectively [18, 19], and are a good source of secondary products such as tannins, organic acid, polyphenol, and alkaloids [20-22]. Shaddock, like other citrus fruits, has a small edible portion and large amounts of waste materials such as peels and seeds, and peels contain considerable phenolic compounds [23]. At present, the polyphenols have shown significant inhibition to the growth of harmful algae. Therefore, these previous results indicate that the shaddock peel, pomegranate peel, and pomegranate seed could be used as an algicide.

The objectives of this study are to identify the allelochemicals from the ethyl acetate extracts of shaddock peel, pomegranate peel, and pomegranate seed, and to test their algicidal activities.

Materials and Methods

Preparation of Plant Materials Extracts

Shaddock peel, pomegranate peels, and seeds were naturally dried on trays away from sunlight at room temperature. The dry weight of peels and seeds were measured and powdered to get 20 mesh size. Five g of the shaddock peel, pomegranate peel, and seed powder were extracted with 100 mL of ethyl acetate (HPLC) at room temperature (25ºC) for 24 h. The extract was filtered through GF/C glass fibre filters (47 mm, 1.2 μm, purchased from Whatman Maidstone, UK) for removal of particles. The extracts were first dried with anhydrous sodium sulfate (AR) 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.

Identification of Allelochemical

The dried ethyl acetate extracts were analyzed by GC-MS (Agilent computerized system consistiing of a 6890 gas chromatograph coupled to a 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 at 60ºC (initial temperature maintained 1 min) to reach 260ºC at a rate of 4ºC/min, kept constant 20 min. Helium was used as carrier gas with a flow rate of 1 mL/min. Mass fragments of the components were compared to the mass fragmentation data contained in the NIST 02.

Bioassay

Axenic *Microcystis aeruginosa* were obtained from the culture collection of algae at the Institute of Hydrobiology, Chinese Academy of Sciences. The algae were cultured in sterilized BG11 medium (pH 7.4) [24] at 25ºC with light intensity of 2500 lux, 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 dose-response relationships between the allelochemicals and the tested organisms were studied in 50 mL flasks containing 25 mL test solution, to which 10^6 cells $m \cdot L^{-1}$ 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 $mg \cdot L^{-1}$ for ethyl acetate extracts from pomegranate peel, pomegranate seed, and shaddock peel, respectively. The inhibition percentages of each ethyl acetate extract based on cell density of the tested organisms and control were determined after exposure for 72 h. The stock solutions of ethyl acetate extracts were prepared with dimethyl sulfoxide (DMSO, AR), which in 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.

Results

Total ion chromatogram was obtained by gas chromatograph-mass spectrometry (GC-MS) analysis of the ethyl acetate extracts from shaddock peel, pomegranate peel, and pomegranate seed. The chemical composition of the ethyl acetate extracts from three plant materials were listed in Table 1. Nineteen compounds were analyzed and identified by GC-MS, among which fatty acids, phenolic acids, and sterol were primary compositions. The amount and components of three ethyl acetate extracts from shaddock peel, pomegranate peel, and pomegranate seed were different (Table 1).

In order to investigate the allelopathic potential of shaddock peel, pomegranate peel and pomegranate seed, the allelopathic effects of ethyl acetate extracts from three plant materials were determined by bioassay of *M. aeruginosa*. The inhibition percentages of ethyl acetate extracts on *M. aeruginosa* were 47.5%, 40.3% and 43.9% when the algae were treated by 20 mg/L extracts of pomegranate peel, pomegranate seed, and shaddock peel, respectively. Hence, these three plant materials exhibited significant anti-cyanobacterial activities (Table 2).

Discussion

Allelochemical (lauric, myristic, hexadecanoic, linoleic, oleic, stearic, *cis*-6-octadecenoic, gallic acid, and N-phenyl-2-naphthalenamine) identified in this study were found to be significantly active against some harmful algae (Table 3). Especially phenolic acid (gallic acid) and unsaturated fatty acid (linoleic, oleic, and *cis*-6-octadecenoic acid) showed stronger algicidal activities. The 50% inhibitory concentrations of each compound based on the cell density or chlorophyll *a* of the tested algae (EC_{50}) were determined after algae were exposed for one to seven days. This indicates that these identified compounds might potentially be effective biological algicides and serve as allelochemicals to control nuisance algal growth (Table 3).

Table 1. The analytical results of ethyl acetate extracts from three plant materials by GC-MS.

Rent time (min)	Compounds	Pomegranate peel	Pomegranate seed	Shaddock peel
5.86	2-ethyl-1,3-dimethyl-benzene	-	-	+
6.91	Glycerol	+	-	-
9.30	Dodecanoic acid	-	-	+
10.05	Unknown	+	+	-
10.26	Unknown	+	+	+
10.34	Unknown	+	+	+
10.37	Tetradecanoic acid	-	-	+
11.00	Gallic acid	+	-	+
11.37	Hexadecanoic acid	+	+	+
11.75	Heptadecanoic acid	-	-	+
11.89	*cis*-6-octadecenoic acid	+	+	+
12.36	Linoleic acid	-	+	+
12.39	Oleic acid	+	+	-
12.53	Stearic acid	+	+	-
12.66	Unknown	-	+	+
12.76	Unknown	-	+	+
12.77	N-phenyl-2-naphthalenamine	+	-	-
13.08	Unknown	+	+	+
15.00	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	+	-	-
15.86	Unknown	-	+	+
19.64	1-iodo-octadecane	+	-	-
21.46	Stigmastan-3,5-diene	+	-	+
22.25	Unknown	+	-	-
25.30	Stigmasterol	-	-	+
25.86	22,23-dihydro-stigmasterol	+	-	-
26.94	*β*-sitosterol	+	+	+
32.06	Friedelan-3-one	-	-	+

"+" detectable, "-" undetectable

Allelochemicals produced by plant exert inhibitory effects on a variety of algae [6, 25-28] may play important roles in the interaction between plants and phytoplankton species in aquatic ecosystems [8]. The fatty acids and polyphenol show allelopathic antialgal activities [6, 27, 29-31] and are the main allelochemicals released from the barley straw and *Myriophyllum spicatum* to inhibit the algal blooms. For example, the well-known allelochemical gallic acid, isolated from many plants, shows antialgal activity

Table 2. Inhibitory effects of ethyl acetate extracts from three plant materials on growth of *Microcystis aeruginosa.*

	Pomegranate peel	Pomegranate seed	Shaddock peel
Inhibition ratio	47.5 %	40.3%	43.9 %

[29, 32]. Gallic acid is present in macrophytes and can be secreted into surrounding waters to inhibit algae growth [27].

Fatty acids are widely distributed in aquatic and terrestrial environments [33], which could inhibit the growth of harmful algae. To discuss the difference of algae growth inhibition by different fatty acids, it is essential to identify the key structures that induce such effects. Nakai et al. [6] found that:

(i) length of carbon chain
(ii) number of unsaturated linkages
(iii) positions of any double bonds may affect the anti-cyanobacterial activities of fatty acids.

Fatty acids and polyphenols were the main allelochemicals, the inhibition mechanism had been reported in some of the literature [34-41]. Researchers believe that allelochemicals are toxic to phytoplankton, and the toxic effects are multiple. For example, Wu et al. [34] found that fatty acids primarily affect the plasma membranes, leading to a change in membrane permeability and dissociation of phycobilins from the thylakoids. Severe damage to the plasma membranes would give rise to a disruption of the stressed cells. In addition, allelochemicals can damage cell membranes [35], lyse target cells [36], and influence enzyme activity [37-39], the electron transfer chain [40], and gene expression [41].

Conclusions

To summarize, the present study isolated and identified allelochemicals of ethyl acetate extracts from three plant materials (shaddock peel, pomegranate peel, pomegranate seed). Allelochemicals (lauric, myristic, hexadecanoic, linoleic, oleic, stearic, *cis*-6-octadecenoic, gallic acid, and N-phenyl-2-naphthalenamine) identified in this study were found to be significantly active against some harmful algae. Especially phenolic acid (gallic acid) and unsaturated fatty acid (linoleic, oleic and *cis*-6-octadecenoic acid) showed stronger algicidal activities. The results suggest that these ethyl acetate extracts from three plant materials (shaddock peel, pomegranate peel, pomegranate seed) may serve as environmentally friendly agents for controlling the growth of *M. aeruginosa*.

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Table 3. EC_{50} (mg/L) of 9 allelochemicals on several algae.

Compounds	*Microcystis aeruginosa*	*Chlorella pyrenoidosa*	*Scenedesmus obliquus*	*Selenastrum capricornutum*	*Monoraphidiu mcontortum*	*Chlorella vulgaris*
Lauric acid	4.563[a]	6.265[a]	5.813[a]	10.36[b]	>400[c]	>400[c]
Myristic acid	12.799±0.471[d]	16.003[a]	15.977[a]	18.65[b]	>400[c]	>400[c]
Hexadecanoic acid	17.167±0.794[d]	19.876[a]	20.146[a]	35.46[b]	9.2±0.6[c]	59.1±1.5[c]
Linoleic acid	0.042±0.012[d]	0.089[a]	0.059[a]	17.27[b]	8.0±0.1[c]	9.4±0.2[c]
Oleic acid	1.332[a]	1.475[a]	1.406[a]	8.08[b]	12.1±0.9[c]	12.4±0.3[c]
Stearic acid	19.849[a]	20.849[a]	20.754[a]	40.27[b]	177±15[c]	200±25[c]
cis-6-octadecenoic acid	3.3±0.4[f]					
Gallic acid	1.0[e]					
N-phenyl-2-naphthalenamine	5[g]					

[a] quoted from [42]; [b] quoted from [43]; [c] quoted from [34]; [d] quoted from [44]; [e] quoted from [27]; [f] quoted from [6]; [g] quoted from [45]

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