Research Articles

Isolation and Identification of Antialgal Compounds from the Leaves of *Vallisneria spiralis* L. by Activity-Guided Fractionation

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Abstract

Background, Aims and Scope. *Vallisneria spiralis* Linn., a common submerged macrophyte, is widely available in quiet waters of lakes, ponds, marshes and streams in Southeast Asia. *V. spiralis* plays a significant role not only in decreasing eutrophication of water body for its productivity, but also in inhibiting the growth of blue-green algae? The aim of the paper involves the isolation and identification of allelochemicals from extracts of *V. spiralis* by activity-guided fractionation and column chromatography.

Methods. Leaves of *V. spiralis* was washed free of debris, air-dried and refluxed in 95% EtOH. The extract was isolated using column chromatography and fractionation with antialgal activity. Potential allelochemicals were analyzed by high-resolution gas chromatography-mass spectrometry (HRGC-MS).

Results. Two fractions with strong antialgal activity were isolated using column chromatography and activity-guided fractionation from the extract of *V. spiralis*. 2-Ethyl-3-methylmaleimide, dihydroactinidiolide and 4-oxo-β-Ionone were identified in the first fraction, and 3-hydroxy-5,6-epoxy-β-ionone, loliolide, 6-hydroxy-3-oxo-α-ionone and an unknown compound in the second fraction. They had strong inhibitory effects on *Microcystis aeruginosa* Kütz.

Discussion. 2-Ethyl-3-methylmaleimide is a byproduct of photooxidation of chlorophyll, and five other compounds identified were derivatives of β-carotene. HRGC-MS and derivatization technology were used to identify and confirm their molecular structures. The formula of the unknown compound was $C_{16}H_{19}NO_4$. Metabolites of plant pigments had strong inhibitory activities on growth of algae.

Conclusions. Six compounds had been identified in *V. spiralis*, among them, 2-ethyl-3-methylmaleimide was the main allelochemical, and derivatives of ionone were also potential allelochemicals.

Recommendations and Perspectives. The results of our research could help us to study further mechanisms of inhibitory effect on algae and develop new potential antialgal substances.

Keywords: 2-ethyl-3-methylmaleimide; activity-guided fractionation; allelochemicals; antialgal compounds; blue-green algae; cyanobacteria; derivatives of ionone; eutrophication; high-resolution gas chromatography-mass spectrometry; macrophyte; *Vallisneria spiralis* Linn.

Introduction

Vallisneria spiralis Linn. (family Hydrocharitaceae; common name: 'flat grass', 'noodle grass' and 'channel grass') is a submerged macrophyte that is widely distributed in shallow-water lakes in Asia and growing well in good transparence, deep-thick silt and slow-flow water areas [1]. *V. spiralis* plays a significant role in decreasing eutrophication of water body for its productivity and disposing industrial wastewater for its adaptability and well potential pollutant absorption [2–3], and it would maintain a good transparency of about 4 m at a high coverage (>90%) even in a eutrophic level [4]. The dense growth of *V. gigantea* can inhibit cyanobacterial growth in shallow reservoirs [5].

Allelopathy of interspecies was widely discovered in terrestrial plants, and allelopathic species were chosen to increase their output and quality. In aquatic ecosystems, varieties of submerged macrophytes were found to effectively inhibit the growth of blue-green algae. Culture solutions of Chara [6–8], *Myriophyllum spicatum* [9–10], *Ceratophyllum demersum* [11–14], *Stratiotes aloides* [8,15] and *V. spiralis* [16] showed allelopathic activities to deleterious algae including cyanobacteria. Most of them demonstrated only the phenomenon of allelopathy between macrophytes and phytoplankton, but dealt with few allelopathic compounds [10].

A plant may interfere with the growth of its neighbors directly through resource competition or chemical inhibition (allelopathy), or indirectly if it harbors or attracts organisms, such as herbivores, that affect its neighbors. These various mechanisms of interference may interact and are thus difficult to distinguish experimentally [17]. In aquatic ecosystems, studying allelopathic effects of macrophytes on phytoplankton must exclude light and nutrient limitation as growth inhibiting factors for phytoplankton. However, isolation and identification of allelochemicals are very important to investigate allelopathy of macrophytes on phytoplankton. Gross [18] reviewed allelopathic substances from aquatic macrophytes that inhibited phytoplankton growth. Phenolic acid and polyphenols in *M. spicatum* [19–22] and sulfur or lipophilic, labile sulfur compounds in *C. demersum* [23] were identified as major algicides. Leu [22] had studied the mechanism of polyphenol allelochemicals on algae and demonstrated that an inhibition of the PS II in photosynthe-

sis of cyanobacteria would be significant to cause allelopathy. Through the studies of dissolved organic carbon, N and P nutrients, *V. americana* exerted a strong but indirect effect on bacteria by modifying nutrient conditions and/or suppressing phytoplankton [24–25]. Until now, however, allelochemicals in *V. spiralis* were still unknown.

This research involved an isolation of allelochemicals from extracts of *V. spiralis* by activity-guided fractionation and column chromatography (CC). The six compounds were identified using high-resolution gas chromatography-mass spectrometry and trimethylsilyl derivatization technology, and their allelopathic effects in environment were discussed.

1 Experimental Section

Macrophytes. *Vallisneria spiralis* Linn. was collected from the East Taihu Lake of China in September 2003, free of roots. Plant material was washed free of debris, air-dried, shattered powder, and stored in air-tight containers at –4°C in the dark until use.

1.1 Isolation and identification of antialgal compounds

Plant material (800 g) was refluxed in 5 l 95% EtOH. After 12 h, the solvent was removed from the extract in vacuum. The extract was suspended in distilled water, and then partitioned sequentially with 0.5 l petroleum ether, EtOAc, $CHCl_3$, and n-BuOH. Allelopathic effects of the fractions were screened with the bioassay noted below, and the $CHCl_3$ fraction with the strongest allelopathic potential was then subjected to silica gel CC with 150 ml of EtOAc-MeOH, respectively (1:0, 9:1, 3:1, 1:1, 1:3, 1:9, 0:1). Seven fractions (**A-G**) without solvent were obtained. The first fraction was further separated by CC with 150 ml n-hexane-EtOAc, respectively (3:1, 1:1 and 0:1), and three fractions (**A1**, **A2**, **A3**) without solvent were obtained. **A1** was a liquid mixture of low polarity (350 mg), and **A3** was a solid mixture of strong polarity (182 mg).

The constituents of **A1** and **A3** were analyzed by high-resolution gas chromatography-mass spectrometry (HP6890-Micromass GCT) equipped with a HP-5 column (30 m × 0.25 mm × 0.25 µm). GC condition: injection temperature 280°C, oven temperature was kept at 120°C for 1 min, programed to increase to 250°C at a rate of 10°C/min, kept constant for 10 min. MS condition: multiple mass analysis, tolerance = 5.0 mDa, DBE (Double Bond Equivalents), –0.5~20. Trimethylsilyl derivatives with *N,O*-bis(trimethylsilyl)trifluoracetamide (BSTFA) were also analyzed. Condition of preparing and analyzing derivatives: 0.2 mg extracts and 0.1 ml BSTFA was added and kept for 30 min in 50–60°C for complete derivatization, the temperature of GC injection was set at 280°C, the oven temperature was programed at 80°C, isothermal 1 min, followed by ramping to 280°C at a rate of 10°C/min, and then isothermal for 10 min, MS ionization was set in the electron impact mode (EI) at 70 eV. Constituents were identified by peak matching against standards in the Wiley Library. The relative amounts of constituents were calculated by integrating all peaks with areas greater than 1%.

1.2 Bioassay

Algal experiments were performed with *Microcystis aeruginosa* Kütz, the most undesirable blue-green algae responsible for lake eutrophication in southern Asia. The algae, *M. aeruginosa*, were obtained from the Environmental Biological Laboratory, Nanjing University. Before the experiment, the algae were cultivated in axenic MA liquid medium [26] at 25±1°C and at a light intensity of 70–100 µmol $m^{-2}s^{-1}$ until the concentration of algae reached about 10^5 cells/ml. The composition of the MA liquid medium was 50 mg $Ca(NO_3)_2{\cdot}4H_2O$, 100 mg KNO_3, 33 mg NaCl, 50 mg $NaNO_3$, 30 mg $MgSO_4$, 5 mg Na_2EDTA, 0.5 mg $FeCl_3{\cdot}6H_2O$, 1 mg $ZnSO_4$, 7 mg $CoSO_4{\cdot}7H_2O$, 0.6 mg $(NH_4)_2\,MoO_4{\cdot}2H_2O$, 20 mg H_3BO_3,100 mg KH_2PO_4, 500 mg Bicine in 1000 ml of distilled water.

Algal growth inhibition was performed as an ISO 8692 method [27] with some modification using *M. aeruginosa* as a test alga to replace *Scenedesmus subspicatus* and *Selenastrum capricornutum*. 10 mg mixtures were dissolved into 50 µL dimethyl sulfoxide (DMSO) in sterile foam-stoppered 250 ml Erlenmeyer flasks and diluted with 150 ml algae medium, and then algae were inoculated into them. The algae were cultivated for 3–5 d at 25°C, and their growth was monitored with a microscope and a hemocytometer by counting the cell number at the stationary phase in 0 h, 72 h and 120 h, light dark ratio 14:10. Control groups were prepared without the sample. Each experiment included triplicate treatments and the experiments were repeated twice. The percentage growth inhibition at specific test substance concentration was calculated compared to the control group.

2 Results and Discussion

Four extracts were obtained from leaves of *V. spiralis*. Antialgal activities showed that the inhibitory effect of chloroform extract whose percentage inhibition of growth rate was up to 91%, was the strongest. However, inhibitory effects of n-butanol extract was the weakest, only 15% (**Table 1**). The

Table 1: Percentage inhibition from leaf extract of *V. spiralis* on *M. aeruginosa* (the concentration is about 66 mg l^{-1})

Fraction	Growth inhibition (%) [a]
Petroleum ether	35.5±5.0
EtOAc	65.2±7.2
$CHCl_3$	91.6±10.5
n-BuOH	15.3±2.3
A	100 [b]
B	–1.4±1.2 [c]
C	–11.3±3.0
D	–18.7±5.1
E	–20.0±6.5
F	32.0±10.8
G	–11.0±8.0
A1	100
A2	42.3±5.5
A3	100

[a] Values are means ± standard deviation
[b] 100, test group entirely inhibits the algal growth
[c] Negative is where the treatment boosted the algal growth compared with the control

Table 2: Allelochemicals identified from **A1** and **A3**

Compound (A1)	RT (min)	RA (%)	Formula	Fragment ion (m/z)
1	4.38	77.1	$C_7H_9NO_2$	139 (M^+, 100*), 124 (35), 121 (7), 110 (10), 96 (16), 81 (11), 67 (37), 53 (15)
2	8.09	18.9	$C_{11}H_{16}O_2$	180 (M^+, 17), 165 (6), 152 (15), 137 (49), 124 (13), 111 (100), 109 (48), 67 (17)
3	9.58	4.0	$C_{13}H_{18}O_2$	206 (M^+, 60), 191 (17), 163 (100), 149 (34), 135 (24), 121 (46), 91 (22), 43 (59)
Compound (A3)	**RT (min)**	**RA (%)**	**Formula**	**Fragment ion (m/z)**
4	9.81	38.3	$C_{13}H_{20}O_3$	224 (M^+, 0.4), 182 (11), 125 (9), 124 (11), 123 (100), 95 (9), 79 (8), 43 (34)
5	10.88	10.1	$C_{11}H_{16}O_3$	196 (M^+, 0.3), 178 (100), 163 (49), 135 (35), 111 (33), 109 (16), 95 (17), 67 (17)
6	11.27	44.5	$C_{13}H_{18}O_3$	222 (M^+, 0.3), 166 (15), 125 (9), 124 (100), 123 (11), 95 (11), 67 (7), 43 (23)
Unknown	11.69	7.1	Mw 289	289 (M+, 0.3), 173 (19), 131 (21), 128 (25), 115 (67), 87 (60), 73 (28), 69 (100)

RT: Retention time; RA: Relative amount; data in parentheses were relative abundance ratio

extract of chloroform was fractioned by silica gel column chromatography and seven fractions (**A–G**) were obtained. Fraction **A** showed the strongest inhibitory activity on growth of algae, fraction **F** was in the next place, and other fractions boosted the algal growth inversely (see Table 1). The further separation of **A** was processed by CC and three fractions were obtained as follows: **A1**, **A2** and **A3**, and antialgal activities showed **A1** > **A3** > **A2** (see Table 1). The growth of alga was fully inhibited at the concentration of 66 mg/l for **A1**and **A3**, and the concentration was about equal to IC_{50} value of **A2**.

The analysis with HRGC-MS had identified three compounds in **A1**, 2-ethyl-3-methylmaleimide (compound **1**), dihydroactinidiolide (compound **2**) and 4-oxo-β-ionone (compound **3**), the relative amounts were 77.1%, 18.9% and 4%, respectively (**Table 2**). In fraction **A3**, three compounds, 3-hydroxy-5,6-epoxy-β-ionone (compound **4**), loliolide (compound **5**) and 6-hydroxy-3-oxo-α-Ionone (compound **6**) were identified, and one unknown compound was also separated. Relative amounts of the four compounds were 38.3%, 10.1%, 44.5% and 7.1%, respectively (see Table 2). The chemical structures of the six compounds identified were shown in **Fig. 1**, and their elemental compositions were obtained in the data of HRGC-MS (**Table 3**).

In **A1**, compound **1** with the highest content and strong irritative odor (about 336 mg/kg) is a simple alkaloid, which originated from photooxidation of chlorophyll [28] and also had been detected from ground water [29], caffeinated beverages [30] and essential oil of *V. spiralis*. Alkaloid is an important kind of secondary metabolite contained in varieties of plants including freshwater macrophytes. *V. spiralis* can produce compound **1** and excrete it into the outer environment. Ostrofsky and Zettler [31] found the presence of alkaloids in fifteen species of aquatic vascular plants, including *V. americana* and their potential deterrents to herbivores. Compound **2**, dihydroactinidiolide, which was an important flavor component from tobacco, tea, coffee juice [32], *Centaurea* [33] and macrophytes (34), was a very active allelochemical and could strongly inhibit the growth of many aquatic macrophytes, especially algae [35]. Dihydroactinidiolide could be released from an aquatic plant, *Eleocharis coloradoenis*, which had distinct allelopathy on other coexisting macrophytes [36–37]. Compound **3** is a derivative of ionone. It is easily obtained from commercially available β-ionone by a two-step protocol involving selective epoxidation of the free double bond followed by a base catalyzed isomerization [38]. Ayers et al. [39] were the first to identify alpha-ionone, β-ionone and β-ionone-5,6-epoxide as off-flavor components of dehydrated carrots. The ionones have a floral character and are typically used in the formu-

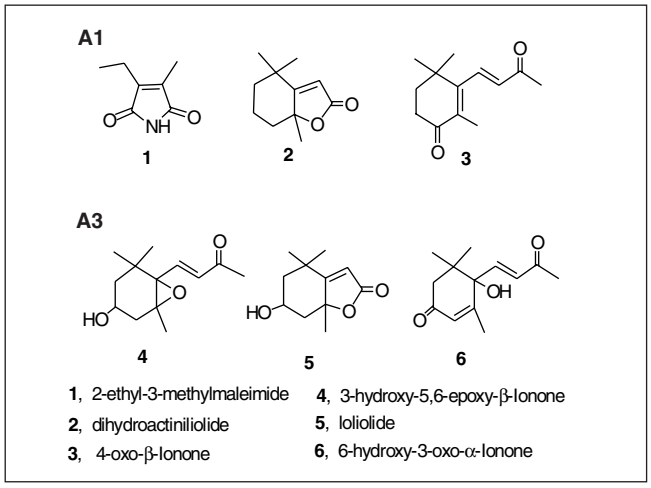

Fig. 1: Allelochemicals isolated from *V. spiralis*

Table 3: Empirical formula, observed and calculated mass/charge ratios, double bond equivalents (DBE), and mass errors of the molecular ions observed in the product ion mass spectrum of the six compounds

Compounds	Predicted formula	Observed mass (Da)	Calculated mass (Da)	DBE	Error (mDa)	Error (ppm)
1	$C_7H_9NO_2$	139.0620	139.0633	4.0	–1.3	9.6
2	$C_{11}H_{16}O_2$	180.1149	180.1150	4.0	–0.1	–0.7
3	$C_{13}H_{18}O_2$	206.1339	206.1307	5.0	3.2	15.6
4	$C_{13}H_{20}O_3$	224.1410	224.1412	4.0	–0.2	–1.1
5	$C_{11}H_{16}O_3$	196.1086	196.1099	4.0	–1.3	–6.6
6	$C_{13}H_{18}O_3$	222.1220	222.1256	5.0	–3.6	–16.2

lation of raspberry flavorings. In plants, the biological roles of carotenoid-derived metabolites, except for abscisic acid, are not very well known. It has been shown that 3-hydroxy-β-ionone isolated from dwarf bean shoots could inhibit the growth of lettuce seedlings [40].

In **A3**, all of the three compounds identified were derivatives of carotenoid. Compound **5**, loliolide, is a potent cytotoxic agent and prepared by citral [41]. Compound **4** and **6** are also derivatives of ionone. Carotenoid-derived metabolites such as α-ionone, β-ionone, and their related derivatives are important components of floral scents [42]. Compound **4** and **5** had been separated and characterized as phytotoxic constituents from the aqueous extracts of *Bunias orientalis* leaves [43]. Compound **6**, which was also separated from *Heliabthus annuus*, inhibited 45% of the growth of *Lepidium sativum* at the concentration of 10^{-4}~10^{-6} mol/l [44]. Similarly, blumenin, a glycosylated cyclohexone produced from root carotenoids, inhibited fungal colonization and arbuscule formation during the initial stages of mycorrhiza development in barley and wheat [45].

Compound **4**, **5** and **6** have hydroxy groups in their structures. Their relative abundance ratio of molecular ion was lower than 1%. In order to confirm their molecular weight, trimethylsilyl derivatives of **A3** had been made. After derivatization molecular ion peak of compound **4**, **5** and the unknown increased 72 mass number than their real molecular weights except compound **6** (**Table 4**), relative abundance ratio increased more than ten times. So we could conclude that this unknown compound contained a dissociative hydroxy group. HRGC-MS spectrum showed that the formula of the TMSi of unknown compound was $C_{19}H_{27}NO_4Si$ (found 361.1724, calc 361.1709), and formula of the unknown was deduced at $C_{16}H_{19}NO_4$. In terms of compound **6**, the hydroxy group is in the sixth carbon of the molecular structure, and the carbon is a quarternary structure. Because of space hindrance, the hydroxy group was too difficult to react with derivatization reagent.

The six isolated and identified compounds are derivatives of β-carotene, except compound **1**. Photo-oxidation of carotenoids in living plants (i.e. flowers) is often responsible for their odor. β-Carotene, when cleaved, gives rise to various compounds. Among them, β-ionone, 5,6-epoxy-β-ionone and dihydroactinidiolide are often associated with fruity, floral and woody notes to varying degrees. Pathways of compounds **2**, **3**, **4**, **5** and **6** production were proposed (**Fig. 2**). Enzymatic catalysis was very important, especially lipoxygenase in the processes of their biosynthesis. The pathway that dihydroactinidiolide was produced through β-ionone had been proposed by Bosser [46]. However, the chemical pathway may involve the formation of 5,8-epoxy- β-carotene from β-carotene and the further cleavage into dihydroactinidiolide [47]. As the derivatives of chlorophyll or carotene, all of compounds **1–6** are secondary metabolites with biological activities from above references, although their inhibitory effects on algae were seldom involved. And these compounds were considered as allelochemical standard chemicals and were used to detect their allelopathic activities and calculate their 50% effective inhibition concentration (EC_{50}).

Table 4: Molecular ion and relative abundance ratio of compounds in **A3**

Compound	MI (m/z)	TMSi-MI (m/z)
4	224 (0.4)*	296 (2)
5	196 (0.3)	268 (18)
6	222 (0.3)	222 (0.5)
Unknown	289 (0.3)	361 (7)

MI: Molecular Ion; TMSi-MI: Trimethylsilyl Molecular Ion;
Data in parentheses were relative abundance ratio

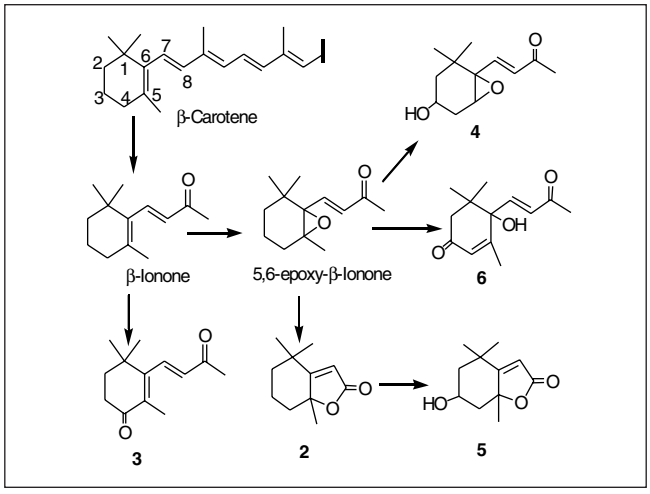

Fig. 2: The pathway of compounds **2**, **3**, **4**, **5** and **6** from β-carotene

The authors [48] studied inhibitory activity of aquatic extracts of submerged macrophytes on *M. aeruginosa* and found that allelopathic activity was affected by the content of phenolic acids which was lower in *V. spiralis*. Within an aquatic ecosystem, allelopathic substances exuded from macrophytes can inhibit blue-green alga. However, cyanobacteria can also influence the content of nitrogen and phosphorus in the surface of sediments [49], toxins exuded from deleterious algae also inhibit the growth of macrophytes, and microcystin produced by *M. aeruginosa* was harmful to the plant life of *V. spiralis*. When the concentration of microcystin was at 10 mg/l, the root growth and leaf formation were inhibited significantly and the root hairs disappeared [50]. Microcystin, as an allelochemical, could influence not only the growth of aquatic macrophytes but also the function of water bodies and its removal, thus deserving more attention [51]. Allelopathy between *V. spiralis* and *M. aeruginosa* is a sophisticated interaction process. The chemical interactions between macrophytes and alga were mutual and influenced by environmental factors such as nutrients, temperature, pH and their densities [52], etc.

3 Conclusions and Recommendations

Seven compounds which have strong allelopathic activities on *M. aeruginosa* were isolated, and six of them had been characterized by HRGC-MS. The structure of the seventh compound (unknown compound) was further being analyzed using tandem mass spectrometry. In the leaves of *V. spiralis* 2-ethyl-3-methylmaleimide was firstly found to be an important potential allelopathic substance, and the decomposition products of β-carotene were also seen to demonstrate strong inhibitory effects on cyanobacteria.

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