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Isolation and identification of antialgal compounds from *Potamogeton maackianus* by activity-guided fractionation

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ABSTRACT

Potamogeton maackianus is major submerged species in China. Its antialgal allelopathic activities have been demonstrated, but its antialgal compounds are not fully known. To investigate the antialgal chemicals of *P. maackianus*, EtOH extracts of this macrophyte were further isolated by activity-guided fractionations. The antialgal bioassays of fractions were tested on algal specie, *Selenastrum capricornutum*. Through column chromatography (silica gel chromatography, ODS C18 P-HPLC, Sephadex LH-20) and preparative thin layer chromatography (P-TLC), six antialgal compounds (PK1-6) were isolated and their structures were determined by mass spectrum (EI-MS) and nuclear magnetic resonance (NMR) spectroscopy (^{1}H, ^{13}C, 13C-DEPT, H-H COSY, HSQC and HMBC). PK1-PK6 were identified as stigmasterol (PK1), 4-hydroxybenzoic acid (PK2), 6-hydroxy-α-ionone (PK3), (E)-4-(4-hydroxy-3,3,8-trimethyl-9- -oxa-bicycle[6.1.0]nonan-4-yl) but-3-en-2-one (PK4), 1*H*-indole-3-carbaldehyde (PK5) and lignan (+)-pinoresinol (PK6), respectively. All six compounds were isolated for the first time from *P. maackianus*, while, PK3 was isolated from natural plants for first time and PK4 is a new compound. The results of bioassay showed that algal growth were obviously inhibited by the six isolated compounds at 20 mg L^{-1}, and antialgal activities followed the order: PK3, PK4 > PK6 > PK5 > PK2 > PK1. The PK3 and PK4 completely inhibited the growth of *S. capricornutum* but stigmasterol was less inhibitory (30.4%) to PK1.

Key words: Activity-guided fractionation, Allelochemicals, Antialgal compounds, Macrophyte, *Potamogeton maackianus, Selenastrum capricornutum*

INTRODUCTION

Aquatic macrophytes and algae are antagonistic to each other in aquatic ecosystems (10,11,19,24). The discovery of their allelopathy potential, hinted that macrophytes might be used as competitor for algae (9) and their antialgal allelochemicals may be used for algal control. Allelopathic algal growth inhibition by submerged plants is one of the mechanisms to stabilize the macrophyte-dominated clear water state (30) and was proposed as a measure to control the undesired algal growth in aquatic ecosystems (25). Thus algal growth could be controlled by using the antialgal allelopathic compounds extracted from the macrophytes.

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The algal population decreases in the presence of *Potamogeton* species in aquatic systems (13). The allelopathy and antialgal allelochemicals of such species have drawn more attention in past several decades. The *Potamogeton malaianus* inhibits the *Scenedesmus obliquus* (39), while *P. pectinatus* inhibits the *Selenastrum capricornutum* and *Microcystis aeruginosa* (3,43). To know the compounds acting in antialgal activities and the structures of these active compounds, isolation and identification are used to search the allelochemicals present in *Potamogeton* species in phytoplankton (1,4,18,36), among them many compounds are algicidal (1,4,37). *P. maackianus* is most inhibitory to growth of *S. capricornutum* and *M. aeruginosa* (40,44), however, its isolated antialgal compounds are still unknown.

This study aimed to isolate the allelochemicals from *P. maackianus* extracts by activity-guided fractionation and chromatography. The isolated active compounds were identified using mass (MS) spectrometry and Nuclear magnetic resonance (NMR) spectrometry and their allelopathic effects on algae were discussed.

MATERIALS AND METHODS

I. General instrumentation

P. maackianus was collected in August, 2006 from the Liangzihu Lake, Hubei Province, China. Plant materials were washed free of debris, frozen-dried, powdered and stored in air-tight containers at -80°C in dark until use.

Column chromatography was performed on Merck Kieselgel 60 (230-400 mesh), Sephadex LH-20 (Pharmacia) and ODS C18 (YMC, 50 μm) under a medium pressure. Analytical thin layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} with 0.2 mm film thickness. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates with 0.5 mm film thickness. Nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Bruker AM-300 spectrometer in 0.5 mL solutions of $CDCl_3$ or CD_3OD at 25°C. All chemical shifts are reported as values relative to TMS. Proton-detected heteronuclear correlations were measured using heteronuclear multiple-quantum correlation (HMQC) (optimized for $^{1}J_{HC}$=145 Hz) and heteronuclear multiple-bond correlation (HMBC) (optimized for $^{1}J_{HC}$=7 Hz). EI mass spectra were obtained at 70 eV on a Finigan TRACE 2000 apparatus.

II. Extraction and isolation of antialgal compounds

Plant materials (2.0 kg) were refluxed in 95% EtOH in ratio of 1:30 (w/v). After 72 h, the solvent was removed from the extract in vacuum to give 51 g extracts. The bioactive extracts was suspended in 2 L 80% MeOH solutions and then partitioned with hexane and EtOAc in sequence as per the method of Gunatilaka *et al.* (12). Antialgal effects of fractions were tested in bioassay and the EtOAc fraction (16 g) with stronger allelopathic potential was then chromatographed on a Si gel column made up in n-hexane and eluted with increasing percentages of EtOAc. Fifteen fractions were obtained based on their TLC profile. Of these, only fractions 1, 5, 7 and 11 obtained by elution with hexane-EtOAc (19:1, 17:3, 15:5, and 13:7) respectively were bioactive.

Fraction 1 (350 mg) was a liquid mixture of very low polarity and was not further separated. Fraction 5 (900 mg) was purified on Sephadex LH-20 [hexane–$CHCl_3$–MeOH

(3:1:1)] to give PK1 (150 mg). Fraction 7 (500 mg) was chromatographed on Sephadex LH-20 eluting with $CHCl_3$–MeOH (1:1) to give pure PK2 (40 mg). Fraction 11 was chromatographed on silica gel eluting with hexane–EtOAc mixtures. Hexane–EtOAc (1:1) gave fraction 11-6 (1 g), which shown the strongest antialgal activity. Fraction 11-6 was further separated by RP-18 P-HPLC [MeOH–H_2O (4:6)] to obtain the most active fraction 11-6-1, which was purified on Sephadex LH-20 [$CHCl_3$–MeOH (1:1)] to give bioactive mixtures A and B. A was purified by preparative TLC with $CHCl_3$–MeOH (19:1) to give PK3 (2 mg) and PK4 (4 mg), B with $CHCl_3$–EtOAc (16:4) to give PK5 (5 mg) and PK6 (20 mg).

III. Bioassay

The antialgal test was done on green alga *S. capricornutum,* which was pre-cultured in SE medium (8), as per the 96-well microplate technique described by Environment Canada (8), which was widely used to test the algal growth inhibition of compounds isolated from macrophytes (2,37). The fractions or pure compounds were initially dissolved in dimethyl sulfoxide (DMSO) and then diluted in the algal culturing medium containing $1*10^5$ algal cells mL^{-1}. 96-well microplates filled with the prepared medium and algae were placed in an incubator at 25°C under continuous irradiance (3000 lux) for 72 h. Each test concentration of fractions was replicated 15 times and the control groups were prepared only with DMSO. The OD_{450nm} of resuspended algal cells was measured by microplate photometer (SLT Spectra). The experiments were repeated twice and the growth inhibition (%) at specific test substance concentration was calculated compared to control group. The effects of DMSO on the growth of *S. capricornutum* were studied before the bioassay of crude extracts and pure compounds, the results showed that there are no differences between the control groups and the treatments (0.33% v/v DMSO, Fig. 1, $p>0.1$).The validity of test was controlled with a reference toxicant, potassium dichromate ($K_2Cr_2O_7$), and a 72 h IC_{50} of 0.67 mg L^{-1} (0.49–0.81) was obtained with five replications of four concentrations between 0.18 and 1.07 mg L^{-1}. The 72 h IC_{50} value given by the Algaltoxkit FTM producer was 0.38 mg L^{-1}. Highest DMSO level in the test wells did not exceed 0.33% (v/v).

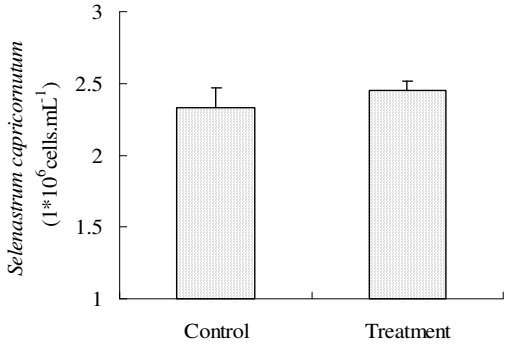


Figure 1. The effects of DMSO at 0.33% v/v on the growth of *Selenastrum capricornutum*. The columns, showed no difference ($p > 0.1$) with the control. The bars of column are the standard deviation of triplicates ($n = 3$).

Statistical analysis: The Homogeneity of Variances of data in different fractions or compounds treatments was tested using Levene's Test. Statistical differences between the control and treatments were tested using one way-ANOVA with SPSS software (Version 13.0, SPSS Inc. Chicago, IL, USA) at 95% confidence level.

RESULTS AND DISCUSSION

Algal growth inhibition of active fractions and compounds isolated from *P. maackianus*

Three extracts were obtained from the EtOH extracts of *P. maackianus* through liquid-liquid extraction (LLE). The hexane extract and EtOAc extract were more inhibitory (77.2% and 63.0%), respectively to algal growth than MeOH extract (Table 1). Because of less quantity of hexane extract, the hexane extract couldn't be further fractioned. The EtOAc extract was fractioned by silica gel column chromatography and 15 fractions (1–15) were obtained. Among them, fraction 1, 5, 7 and 11 showed inhibitory activities on growth of algae (Table 1). Because of the lowest polarity, fraction 1 could not be separated effectively in common column chromatography. Fraction 11 had more weight and higher activity than other fractions in antialgal test. The further separation of fractions 5, 7 and 11 were processed as per the antialgal test and six compounds were isolated.

Table 1. Inhibition (%) of *Potamogeton maackianus* extracts on *Selenastrum capricornutum*

Fractions and compounds	Growth inhibition (%) [a]
EtOH extract (50 mg/L)	55.7±4.5*
n-hexane extract (50 mg/L)	77.2±5.3*
EtOAc extract (50 mg/L)	63.0±6.9*
Methanol extract (50 mg/L)	-3.5±6.1 [c]
1 (50 mg/L)	30.8±2.1*
5 (50 mg/L)	49.6±5.0*
7 (50 mg/L)	52.4±5.2*
11 (50 mg/L)	56.8±9.6*
11-6 (20 mg/L)	47.9±4.5*
11-6-1(20 mg/L)	67.3±4.3*
A (20 mg/L)	87.6±7.8*
B (20 mg/L)	69.7±3.8*
PK1 (20 mg/L)	32.1±2.1*
PK2 (20 mg/L)	51.2±2.3*
PK3 (20 mg/L)	100 [b]*
PK4 (20 mg/L)	100 [b]*
PK5 (20 mg/L)	87±5.9*
PK6 (20 mg/L)	89±5.0*

a Values are means ± standard deviation; b Test group entirely inhibits the algal growth; c Negative is where the treatment boosted the algal growth compared with the control; * $p<0.05$

Structure identification of antialgal compounds isolated from *P. maackianus*

PK1, PK2 and PK6 were identified as stigmasterol, 4-hydroxybenzoic acid and lignan (+)-pinoresinol, respectively, by comparing of their spectroscopic properties data

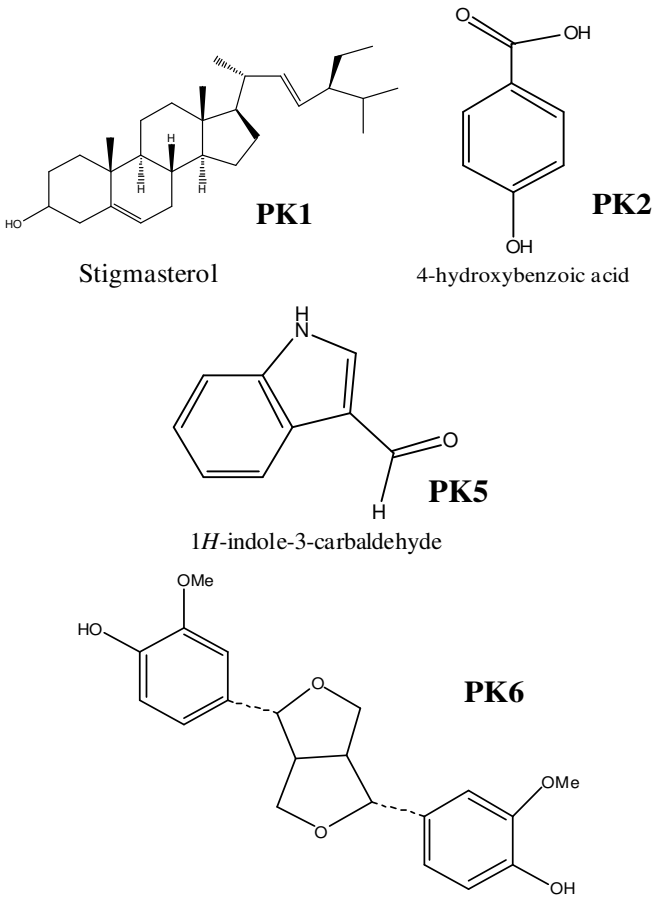


Figure 2. The chemical structures of compound PK1-2 and PK 5-6.

(MS and NMR) with published data of others (6,20,35). PK5 was determined as 1*H*-indole-3-carbaldehyde, owing to matching of their MS and NMR spectra. The chemical structures of the PK1-2 and 5-6 identified were shown in Fig. 2.

PK3 had a molecular formula $C_{13}H_{20}O_2$ in accordance with the molecular ion at m/z 208 in the EI-MS spectrum and 13 carbon signals in the ^{13}C-NMR (Table 2). Spectral data of PK3 (Table 2) were in good agreement with structure of 6-hydroxy-α-ionone (14). PK4 showed 15 carbon signals in the ^{13}C NMR spectrum (Table 3), which, along with the molecular ion at m/z 258 in the EI-MS spectrum, justified a molecular formula $C_{15}H_{24}O_3$. According to the NMR spectrum (^{1}H, ^{13}C, ^{13}C-DEPT, H-H COSY, HSQC and HMBC), the chemical structure of PK4 was determined as (E)-4-(4- hydroxy-3,3,8- trimethyl-9-oxa-bicycle[6.1.0]nonan-4-yl) but-3-en-2-one. The chemical structure and structure key HMBC correlations of PK3 and PK4 were shown in Fig. 3.

Table 2. ^{1}H and ^{13}C chemical shifts of Compound PK3

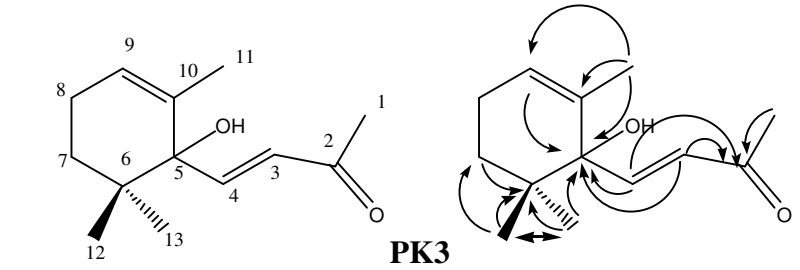
Position	δ (^{1}H-NMR)		δ (^{13}C-NMR)		HMBC (H to C)
	δ (ppm)	Peak (J)	δ (ppm)	Peak	
1 (CH3)	2.30	s	28.6	q	C-2
2 (C=O)			198	s	
3 (=CH)	6.45	d (16Hz)	130.6	d	C-2, 5
4 (=CH)	6.82	d (16Hz)	145.2	d	C-2, 5
5 (C-OH)			79.5	s	
6 (C)			41.7	s	
7 (CH2)	2.50 1H	d (17.1Hz)	49.8	t	C-6
	2.33 1H	d(17.1Hz)			
8 (CH2)	1.24	brs	29.9	t	
9 (=CH)	5.95	brs	128.0	d	C-5
10(=C)			160.0	s	
11(CH3)	1.88	s	18.9	q	C-5, 9, 10
12(CH3)	1.02	s	24.6	q	C-5, 6, 7, 13
13(CH3)	1.10	s	23.2	q	C-5, 6, 7, 12

Table 3. ^{1}H and ^{13}C chemical shifts of Compound PK4

Position	δ (^{1}H-NMR)		δ (^{13}C-NMR)		HMBC (H to C)
	δ (ppm)	Peak (J)	δ (ppm)	Peak	
1 (CH_3)	2.28	s	28.5	q	C-2, 3
2 (C=O)			197.6	s	
3 (=CH)	6.29	d (15.3Hz)	132.8	d	C-2
4 (=CH)	7.04	d (15.3Hz)	145.2	d	C-2, 5
5 (C-OH)			69.7	s	
6 (CH_2)	1.24	m	29.9	t	
7 (CH_2)	1.24	m	53.6	t	
8 (CH_2)	1.64 1H	m	40.8	t	C-9,10
	2.36 1H	m			
9 (C)			67.5	s	
10(CH)	3.90	m	64.2	d	
11(CH_2)	1.24 1H	m	46.9	t	
	1.63 1H	m			
12(C)			35.5	s	
13(CH_3)	1.19	s	20.1	q	C-8, 9
14(CH_3)	0.98	s	25.2	q	C-5, 11, 12, 15
15(CH_3)	1.19	s	29.6	q	C-5, 11, 12, 14

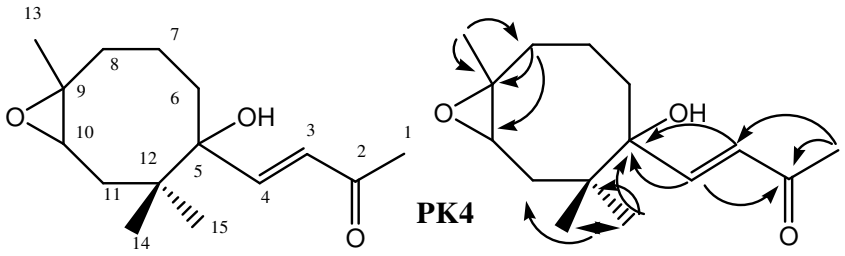
The algal growth was test, when treated with six compounds at 20 mg L^{-1} concentration and showed following antialgal activities PK3 > PK4 > PK6 > PK5 > PK2 > PK1 (Table 1). The PK3 and PK4 completely inhibited the growth of *S. capricornutum*, while the isolated stigmasterol caused the lowest inhibition (30.4%).

Isolation and identification of allelochemicals is most important in allelopathy research (31). Allelochemicals mainly include sesquiterpene lactones, organic acids, phenols, quinines, coumarins, flavonoids, anthraquinone and tannins (16,26,27,31,32). Till now many compounds have shown antialgal allelopathic activities. To search the natural



PK3

(*E*)-4-(1-hydroxy-2,6,6-trimethylcyclohex-2-enyl)but-3-en-2-one



PK4

(*E*)-4-(4-hydroxy-3,3,8-trimethyl-9-oxa-bicyclo[6.1.0]nonan-4-yl)but-3-en-2-one

Figure 3. The chemical structures and key HMBC correlations of Compound PK 3-4.

antialgal compounds, many *Potamogeton* species had been studied (4,5,37). Some compounds [such as ent-labdane diterpenes (5,37), furano-diterpenes (4), lactone diterpenes (2)] isolated from the Potamogetonaceae show high antialgal activities. Although the allelopathic inhibitory activities of *P. maackianus* on algae were observed (43), but their antialgal compounds isolated from *P.maackianus* were still unknown. In this paper, six active compounds were isolated from *P. maackianus* by Activity-Guided Fractionation and their structures were determined by EI-MS and NMR spectrum. All the six compounds were isolated for the first time from *P. maackianus* and furthermore, PK4 was a new compound.

Some phytosterols, such as stigmast-4-en-3,6-dione (45) inhibits the algae growth, but other sterols have no antialgal activity (6). DellaGreca *et al.* (6) found that pure sterol did not inhibit algal growth, but sterol mixtures or sterol extracts had some antialgal activities in processes of isolation and purification, this might be contributed by some minor components. In this study, the isolated stigmasterol had antialgal activity, and this could be due to some minor unidentified components or their synergistic action.

The phenolic acids have allelopathic antialgal activity (11,23,24) and are main allelochemicals released from the barley straw to inhibit the algal blooms (28,38). Phenolic acid could inhibit the growth of algae by influencing the photosynthetic pigment (23), alkaline phosphatase activity (7,11), electron transfer chain (7,21), gene expression and antioxidant enzymes (33). Phenolic acids are one of the main allelochemicals in *Myriophyllum spicatum* (15). The well-known allelochemical 4-Hydroxybenzoic acid, isolated from many plants has antialgal activity (34,42). The 4-hydroxybenzoic acid is present in macrophytes and can be secreted into surrounding water to inhibit algae growth (11).

The structure of PK3 (6-hydroxy-α-ionone) was similar to 6-hydroxy-3-oxo -α-ionone, which has antialgal activity (41). Although 6-hydroxy-α-ionone could be synthesized with its isomeric compound (14), but was isolated from the natural plant for the first time. PK4 was identified as new compound containing an eight-membered ring, its structure fragment was almost similar to PK3. Many compounds with ketone functional group, have antialgal activities, such as ethyl-2-methylacetoacetate (EMA) from *Phragmites communis* (22). PK3 and PK4 were most inhibitory to algae due to their α, β-unsaturated ketones structure.

PK5 was identified as 3-indole-formaldehyde (1*H*-indole-3- carbaldehyde) an alkaloid. Alkaloids have antialgal activities (17) and some antialgal alkaloids were isolated from the *Potamogeton* species (29). PK6 was lignin (+)-pinoresinol, which was previously isolated from *Zantedeschia aethiopica* (6) and is antialgal.

CONCLUSIONS

In the investigation of antialgal chemicals from *P. maackianus*, 6 compounds were isolated by activity-guided fractionations. The compounds were identified and characterized by their spectroscopic features (EI-MS and NMR). All the 6 compounds were isolated for the first time from *P. maackianus.* Among them, PK3 was isolated first time from a plant and PK4 was a new compound. Further studies are needed to determine the concentrations of these compounds in plant issues and to explore their antialgal mechanism of action.

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