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Inhibitory Effects and Mechanisms of *Hydrilla verticillata* (Linn.f.) Royle Extracts on Freshwater Algae

T.-T. Zhang · M. He · A.-P. Wu · L.-W. Nie

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Abstract To pursue an effective way to control freshwater algae, four extracts from a submerged macrophyte *Hydrilla verticillata* (Linn.f.) Royle were tested to study its inhibitory effects on *Anabaena flos-aquae* FACHB-245 and *Chlorella pyrenoidosa* Chick FACHB-9. Extract with the highest inhibiting ability was further studied in order to reveal the inhibitory mechanism. The results demonstrated that *H. verticillata* extracts inhibited the growth of *A. flos-aquae* and *C. pyrenoidosa*, and methanol extract had the highest inhibiting ability. The mechanism underlying the algal growth inhibition involves the superoxide anion radical generation that induces the damage of cell wall and release of intracellular components.

Keywords Inhibition · *Hydrilla verticillata* · *Anabaena flos-aquae* · *Chlorella pyrenoidosa*

Lake eutrophication is currently one of the most prominent environment issues. In an eutrophic water body, the input of excess amount of nutrients, such as nitrogen and phosphorous, results in a gradual increase of phytoplankton biomass and formation of water blooms, hence inhibiting or destroying the lake ecosystem and aquatic functions. To effectively utilize water resources and manage the aquatic environment, an important mission for scientific researchers is to take measures to control nuisance algal growth. Submerged macrophytes are of great importance for the biological structure and water quality of shallow lakes (Jeppesen et al. 1998). They are believed to play a key role in sustaining the clear water state by competing with algae for nutrients and light (Ozimek et al. 1990) and directly reducing phytoplankton and periphyton growth by allelopathy.

Invasive in most of its range, *Hydrilla verticillata* is a submerged macrophyte, which has been found that all the clear-water lakes with the abundant submerged vegetation (Wang et al. 2004). We have demonstrated the allelopathic inhibition of *H. verticillata* on *Scenedesmus obliquus* by quasi continuous addition culture solution of *H. verticillata* and co-cultivated with *H. verticillata* (Zhang et al. 2007a). The main purpose of the present study was to further reveal the inhibitory effect of *H. verticillata* extracts on other algae.

Materials and Methods

Hydrilla. verticillata was collected from Jinghu, a clear lake in Wuhu City, Anhui Province, P.R. China, and washed with flowing water to remove surface deposited inorganic and organic materials, and rinsed in sterile water, and dried at room temperature, and then blended into powder and weighed. The algae species (from culture stock) *Anabaena flos-aquae* (cyanobacterium) and *Chlorella pyrenoidosa* (green) were provided by the Institute of Hydrobiology, the Chinese Academy of Sciences.

Prior to the initiation of the experiments, the single algae species (*A. flos-aquae* and *C. pyrenoidosa*) were grown in 250 mL sterilized flasks filled with 100 mL 245 medium (Zhang et al. 2007b) and HB-4 medium (Li et al. 1959; Xi et al. 2004) respectively, and then placed in an incubator, using a light intensity of 4,000 lx at 25 ± 1°C and photoperiod 12 L:12 D for 7 days.

T.-T. Zhang · M. He · A.-P. Wu · L.-W. Nie (✉)
Provincial Laboratory for Conservation and Utilization of Important Biological Resources in Anhui College of Life Sciences, Anhui Normal University, Wuhu 241000, People's Republic of China
e-mail: lwnie@mail.ahnu.edu.cn

Organic solvents with different polarities were used during this extraction phase, such as ethyl ether, acetone, methanol and ethyl acetate; and all of these solvents were purified before using. Soxhlet extractors were used for the *H. verticillata* powder extractions (Jin et al. 2003); and extractions were conducted for 12 h when using these organic solvents, respectively. By using this method, four different extracts were obtained.

Under aseptic condition, 54 sterilized flasks were filled with 20 mL 245 medium and 2 mL *A. flos-aquae* alga species in the exponential growth phase, the other 54 sterilized flasks were filled with 20 mL HB-4 medium and 2 mL *C. pyrenoidosa,* then added with four different *H. verticillata* extracts and copper sulfate, respectively. Extracts and copper sulfate final concentrations were 0.2, 0.6 and 1.0 mg mL^{-1}, respectively; and the final algae concentrations were approximately 10^5–10^6 cells mL^{-1}, respectively. For the control, all the components are the same except without using *H. verticillata* extract. All experiments were conducted in triplicate, the cultural conditions were same as mentioned above.

After 6 days incubation, the condition of algae growth were counted using a hemocytometer and the average was calculated based on triplicates. The relationship between extract concentration and algae concentration was plotted on a curve. Similarly, plots of inhibiting substance concentration against concentration of algae solution were obtained for ethyl ether, acetone, methanol, ethyl acetate and copper sulfate, respectively.

Chlorophyll a measurement is another applicable method in determining alga amount. The colorimetric method to test chlorophyll a was reported earlier (He and Wang 2001; American public health association 1985). Briefly, 20 mL cultivated algae solution from the above experiment with 1.0 mg mL^{-1} extract or copper sulfate group and control group was used to determine the content of chlorophyll a. After 0.2 mL $MgCO_3$ suspension was added to the solution, the sample was centrifuged at 3,500×*g*, for 20 min, and the sediment was grinded and extracted with acetone, then the values of OD_{663}, OD_{645} and OD_{630} were measured. The content of chlorophyll a was calculated using the formula chlorophyll a (mg L^{-1}) = 11.64 (OD_{663}) − 21.16 (OD_{645}) + 0.11 (OD_{630}).

The extract with the highest inhibiting ability was defined through the above-stated procedures. The extract then underwent studies of the inhibitory mechanism.

A reliable method to test damage of cell membranes is to measure the electrical conductivity (EC). Samples used are cultured in 100 mL flask with 100 mL mixture containing alga and the *H. verticillata* extract at varying concentration from 0 to 1.5 mg mL^{-1}. Samples of 5 mL were removed from the flasks every day during the 3 days experimental period. Samples were immediately filtered with a 0.22 μm Millipore filter to remove the algal cells. The supernatant was analyzed using a portable conductivity meter (Cole-Parmer Instrument Company, USA).

Since proteins and nucleotides have strong UV absorption at 280 and 260 nm respectively, and are easily detected using a UV–Vis spectrophotometer (Hewlett-Packard Co.), this method is widely used in determining membrane integrity. The experiments were conducted as described in the EC assay above. The OD of the supernatant was measured at 260 and 280 nm using a UV–Vis spectrophotometer. The OD ratio between the experimental group and the control group was used to evaluate the releasing level of proteins and nucleotides.

Superoxide anion radical ($O_2^{\bullet -}$) was assayed by a new spectrophotometer method (Xiao et al. 1999). The reaction of hydroxylamine and $O_2^{\bullet}$ produces $NO_2^{\bullet -}$, which was developed by adding sulphanilic acid and α-naphthylamine. There was a special absorption at 530 nm when the sample was tested by spectrophotometer, and its color responsiveness quantitatively correlates $O_2^{\bullet -}$ amount. Briefly, 0.5 g algal cells coming from the experiments as described in the EC assay were homogenized with ice-cold PBS buffer (6 mL, 65 mM, pH 7.8), filtered with filter paper, and centrifuged at 3,400 r min for 10 min at 4°C.1 mL supernatant was added with 0.9 mL PBS buffer (65 mM, pH 7.8) and 0.1 mL hydroxylamine hydrochloride (10 mM), followed by incubation at 25°C water bath for 20 min. After incubation, 0.5 mL mixture was added with 0.5 mL sulphanilic acid (17 mM) and 0.5 mL α-naphthylamine (17 mM), incubated for 20 min at 25°C, and extracted with equal volume of butanol. The samples were settled for 10 min at room temperature and the butanol phase was taken for measurement at 530 nm using aspectrophotometer.

Data were recorded as mean ± standard deviation and analysed by SPSS 13.0. One-way ANOVA followed by LSD test was used to test for the differences between individual means. The 0.05 level or less was selected as the point of minimum statistical significance in every comparison.

Results and Discussion

A comparison of four extracts of *H. verticillata* resulting from the use of four different solvents and copper sulfate solution was conducted during this research. By using two testing methods, the algae growth inhibition abilities of each extract were determined. Results are presented in Tables 1 and 2.

From Tables 1 and 2, we can see that all extracts from *H. verticillata* inhibited both *A. flos-aquae* and *C. pyrenoidosa* growth to a certain extent, and with *A. flos-aquae* being more sensitive. Among the four extracts of

Table 1 Regression equations and half-inhibiting concentration (EC_{50}, mg mL^{-1}) of *H. verticillata* extracts on the growth of *A. flos-aquae* and *C. pyrenoidosa*

Algae growth inhibiting substances	*A. flos-aquae*		*C. Pyrenoidosa*	
	Regression equation	EC_{50}	Regression equation	EC_{50}
Ethyl ether extract	$Y = 5.04 \times 10^6 e^{-7.47X}$	0.86	$Y = 5.10 \times 10^6 e^{-6.32X}$	0.98
Acetone extract	$Y = 5.33 \times 10^6 e^{-6.59X}$	0.89	$Y = 5.37 \times 10^6 e^{-6.47X}$	1.03
Methanol extract	$Y = 5.01 \times 10^6 e^{-12.33X}$	0.59	$Y = 5.24 \times 10^6 e^{-12.43X}$	0.67
Ethyl acetate extract	$Y = 4.84 \times 10^6 e^{-5.42X}$	1.28	$Y = 5.02 \times 10^6 e^{-7.67X}$	1.09
Copper sulfate	$Y = 5.24 \times 10^6 e^{-10.17X}$	0.65	$Y = 5.37 \times 10^6 e^{-10.47X}$	0.69

Table 2 Effects of *H.verticillata* extracts on chlorophyll a (mg L^{-1}) removal rate (RR%) of *A. flos-aquae* and *C. pyrenoidosa*

Inhibiting substances (1.0 mg mL^{-1})	*A. flos-aquae*		*C. Pyrenoidosa*	
	Chlorophyll a	RR	Chlorophyll a	RR
Control	276.23 ± 12.35	0.0	225.18 ± 10.76	0.0
Ethyl ether extract	107.03 ± 7.48[a]	61.25	104.46 ± 13.38[a]	53.61
Acetone extract	84.46 ± 14.51[a]	69.42	124.21 ± 18.45[a]	44.84
Methanol extract	31.20 ± 8.32[b]	88.71	44.36 ± 11.33[b]	80.30
Ethyl acetate extract	101.24 ± 14.67[a]	63.35	98.13 ± 12.40[a]	56.42
Copper sulfate	37.34 ± 9.54[b]	86.48	51.27 ± 9.55[b]	77.23

Compared with control group: [a] $p < 0.05$; [b] $p < 0.01$

H. verticillata, the extract obtained by using methanol showed relatively better results, and even better than that of using copper sulfate. So the further study on the methanol extract was conducted in order to reveal the allelopathic mechanism on the algae.

The cytoplasmic cell membrane is the target for many antimicrobial agents (Denyer 1995; Denyer and Stewart 1998). Release of intracellular components is a good indicator of membrane integrity (Chen and Cooper 2002). Small ions such as potassium and phosphate tend to leach out first. The value of EC is a positive correlation with the damage of algal cell membranes. Figure 1 shows that the EC value rose as the concentration of methanol extract increased and the acting time prolonged for the two algal species tested.

The release of macromolecules including proteins, DNA, RNA and other materials often follows the release of small ions (Chen and Cooper 2002). Figures 2 and 3 show the leakage and release of proteins and the nucleotides upon the addition of varying concentrations of *H. verticillata* methanol extract to the two alga species. Starting on day 1, addition of *H. verticillata* methanol extract induced a gradual increase of OD_{260} and OD_{280} values for *C. pyrenoidosa*, and a quicker elevation of OD_{260} and OD_{280} values for *A. flos-aquae*, both in a concentration-dependent manner. The difference between *A. flos-aquae* and *C. pyrenoidosa* might be that the algal structure of *A. flos-aquae* is more sensitive to the foreign allelopathic molecules, and the cell is damaged and the function is handicapped in a shorter time. These data suggest that *H. verticillata* methanol extract led to a damage of algae cells that caused the release of nucleotides and protein.

From these results (Figs 1, 2, 3), we can draw the following conclusions: (1) It is positively correlated between the values of EC, proteins, nucleotides and the damage of

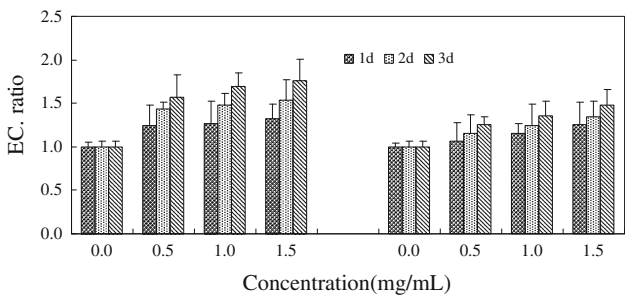

Fig. 1 Effect of methanol extraction from *H. verticillata* on the release of algae (*left*: *A. flos-aquae*; *right*: *C. Pyrenoidosa*) intracellular electrolyte. Data are expressed as the ratio between the EC of the experimental group and the control group (*bar* = SD, n = 3)

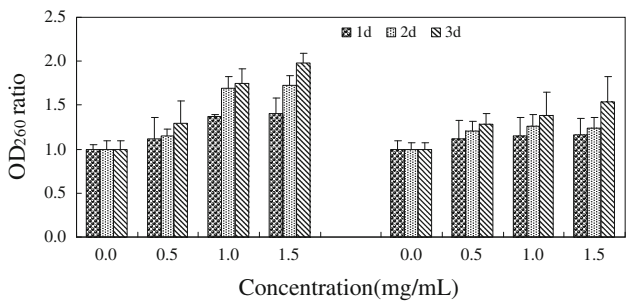

Fig. 2 Effect of methanol extraction from *H. verticillata* on the release of algae (*left*: *A. flos-aquae*; *right*: *C. Pyrenoidosa*) intracellular nucleotides electrolyte. Data are expressed as the ratio between the OD_{260} of the experimental group and the control group (*bar* = SD, n = 3)

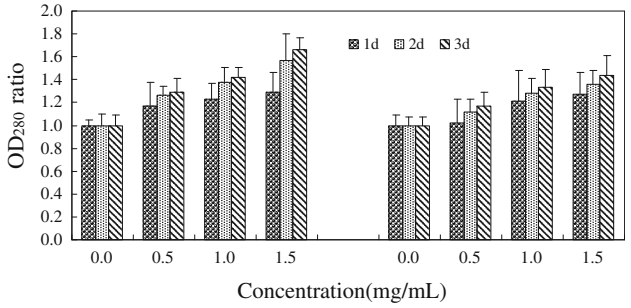

Fig. 3 Effect of methanol extraction from *H. verticillata* on the release of algae (*left*: *A. flos-aquae*; *right*: *C. Pyrenoidosa*) intracellular proteins electrolyte. Data are expressed as the ratio between the OD_{280} of the experimental group and the control group (*bar* = SD, n = 3)

algal cell membranes, especially for *A. flos-aquae*; (2) the substantial release of the proteins and nucleotides from the algal cells suggested that the cellular membrane was irreversibly damaged by *H. verticillata* methanol extract, which resulted in the disintegration of algal cells and the potential termination of algal blooms.

To evaluate whether *H. verticillata* methanol extract interferes with the $O_2^{\bullet-}$ generation, we conducted the $O_2^{\bullet-}$ assay. As can be seen in Fig. 4, the $O_2^{\bullet-}$ OD_{530} ratio between the experiment group and the control group increased significantly starting on day 1, even at the lowest concentration of 0.5 mg mL^{-1} *H. verticillata* methanol extract. The $O_2^{\bullet-}$ OD_{530} ratio reached the maximal point at the concentration 1.5 mg mL^{-1}. These results indicate that *H. verticillata* methanol extract does interfere with the $O_2^{\bullet-}$ radical generation, and that $O_2^{\bullet-}$ is the precursor for active free radicals that have potential for reacting with biological macromolecules and thereby inducing cell damage. It was implicated in several cell-damaged processes, due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Also, $O_2^{\bullet-}$ has been initiated lipid peroxidation directly.

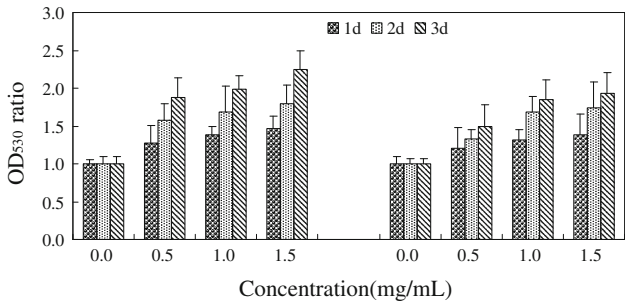

Fig. 4 Effect of methanol extraction from *H. verticillata* on the algae (*left*: *A. flos-aquae*; *right*: *C. Pyrenoidosa*) $O_2^{\bullet-}$ radical generation at the experiments. Data are expressed as the ratio between the OD_{530} of the experimental group and the control group (*bar* = SD, n = 3)

It is well known that $O_2^{\bullet-}$ plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA.

Growth inhibition of the cyanobacterium *Microcystis* caused by other aquatic macrophytes was already reported by other investigators (Nakai et al. 2001, 2005; Körner and Nicklisch 2002). Our present study showed *H. verticillata* might be an effective way to control freshwater algae. We compared the responses of cyanobacteria (*A. flos-aquae)* and non-cyanobacterial (*C. pyrenoidosa*) species and found the cyanobacteria to be more sensitive to extracts from *H. verticillata* than the non-cyanobacterial species and therefore we agree with the hypothesis(Körner and Nicklisch 2002) that cyanobacteria are more sensitive to allelopathic substances than other algae. Our study also indicated that one of the allelopathic mechanisms is due to the $O_2^{\bullet-}$ generation, which induce oxidative damage of the cell membrane and the release of proteins and DNA.

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