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Table: 1, Figs : 3

Allelopathic effects of phenolic compounds present in submerged macrophytes on *Microcystis aeruginosa*

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ABSTRACT

In laboratory studies, the allelopathic effects of 3 (Hydrocharitaceae family) submerged macrophytes (*Elodea nuttallii* (Planch) St. John, *Hydrilla verticillata* (L.f.) Royle and *Vallisneria spiralis* L.) were investigated on two strains of *Microcystis aeruginosa*. Both aqueous methanol extracts and exudates of three macrophytes inhibited the growth of both strains of *Microcystis aeruginosa*. After 3-days culture, *E. nuttallii, H. verticillata* and *V. spiralis* excreted 0.8, 0.3 and 1.0% of total phenolic compounds (TPC), respectively, into the surrounding water. After removing phenolic compounds by polyvinylpolypyrrolidone (PVPP), the plant exudates showed very weak activity. The inhibitory rates of exudates of *E. nuttallii*, *H. verticillata* and *V. spiralis,* against non-toxic *M.aeruginosa* were decreased by 35.7, 43.4 and 59.1% respectively. Thus 3 submerged macrophytes released the phenolic compounds into the surrounding water, to inhibit the growth of *M. aeruginosa.* This information may help us in understanding the mechanism of allelopathy in aquatic ecosystems and to control the algal bloom in eutrophic water bodies.

Key words: Allelopathy, *Elodea nuttallii*, *Hydrilla verticillata*, *Microcystis aeruginosa,* phenolic compounds, *Vallisneria spiralis*.

INTRODUCTION

The submerged macrophytes negatively affects the development and composition of phytoplankton through releasing the allelochemicals into aquatic ecosystems (5,12). This may be one of the mechanisms to control the noxious algal bloom and stabilize the macrophyte-dominated clear-water state in shallow eutrophic lakes (17). Recent research has focussed to extract and identify the compounds directly from the tissues of submerged macrphytes with organic solvents or water, to control the agal growth. From tissues of *Myriophyllum spicatum*, 12 phenolic compounds were isolated and some of these inhibit the algal growth (14). Wium-Andersen *et al.* (20) found a sulphur compound from *Ceratophyllum demersum* with allelopathic properties. Greca *et al.* (2) extracted the anti-algal furano-diterpenes from *Potamogeton natans* L. Among all classes of identified allelochemicals, phenolic compounds are major ones, present in many submerged macrophytes. Besides, water soluble phenolic compounds (due to their hydroxyl groups), are exuded into the surrounding water and inhibits the algal growth. *M. spicatum*, releases 4 algae-inhibiting polyphenols into the culture solution (17), however, more research on the release pattern of allelochemicals from submerged macrophytes is needed.

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Elodea nuttallii (Planch) St. John, *Hydrilla verticillata* (L.f.) Royle and *Vallisneria spiralis* L. are 3 common spp. (Hydrocharitaceae family) in many shallow lakes of Yangtze river, China. *E. nuttallii*, is native of North America and was introduced from Japan in 1990s and the others two are native species. It is known that some Hydrocharitaceae plants are allelopathic to algae (1,5,21,24) and phenolic compounds are also present in *Hydrilla* (7,15,22).

This study aimed to investigate (i) the allelopathic activity of above 3 Hydrocharitaceae family spp. and (ii) to test whether phenolic compounds present in these plant spps contribute to the allelopathic effects against two strains of cyanobacterium (*Microcystis aeruginosa*). Therefore, the content of total phenolic compounds (TPC) was determined in plant tissue and exudates. The inhibitory effects of plant extracts and exudates before and after removing phenolic compounds were also evaluated.

MATERIALS AND METHODS

Macrophytes Cultivation: *E. nuttallii*, *H. verticillata* and *V. spiralis* were collected from Moon Lake (30°33´N, 114°15´E), Wuhan, China and then cultured with sediment from same sites to supply nutrients in laboratory (light intensity of 3000 lux and 12:12 h light:dark cycle at 25°C). Fifteen days before the start of experiment, the fresh plants were carefully rinsed with tap water to remove debris and microorganisms and then precultured in MIII medium (10) under the same incubated conditions to grow new shoots. The culture medium was replaced every 5 days.

Plants Extraction: At the start of experiment, the fresh plants were selected and rinsed with distilled water and then cultured in MIII medium at density of 10g FW/l for 3 days, under the same conditions mentioned above. After 3-days cultivation, the plants were freeze-dried, weighed (DW), homogenized and then extracted for 2 h with 50% aqueous methanol (v/v). The extracts were centrifuged (15000 rpm, 20° C, 30 min) and the supernatant was filtered through GF/F filters (Whatman, Maidstone, UK). The residue was extracted once again as above. The filtrates were combined, evaporated to dryness with a rotary evaporator (40°C, 70rpm) and redissolved in 50% methanol.

Culture solution was acidified to pH 4, using acetic acid to enhance the binding of phenolic compounds (6) and then filtered through washed GF/F filters to remove particles. Filtrates were passed through Oasis HLB solid phase extraction (SPE) catridges (500mg, Waters), each pre-conditioned with 7 ml methanol (Chromatographic grade) and equilibrated with 7 ml Milli-Q ultrapure water. Adsorbed compounds in the columns were eluted twice with 10 ml methanol and the elution was combined, evaporated to dryness with a rotary evaporator (40° C, 70 rpm), and redissolved in 50% methanol.

Determination of TPC (Total phenolic compounds): TPC of plant extracts and exudates was determined by the Folin-Ciocalteau colorimetric method (9,18). Briefly, 2 ml samples were mixed with 1 ml Folin-Ciocalteau Reagent, mixed well and incubated at room temperature for 5 min and then 1 ml 10% Na_2CO_3 was added to the mixture and shaked. Absorbance was measured at 765 nm after 2 h incubation at 25° C on water bath and the results were expressed as gallic acid equivalents.

Removal of Phenolic Compounds: Polyvinylpolypyrrolidone (PVPP) was used to remove the phenolic substances from the samples (11). The aqueous aliquots of extracts and exudates (adjusted to pH 4 with acetic acid) were mixed with insoluble PVPP (Sigma P-6755, to final concentration of 5% [w/v]) respectively to precipitate phenolic compounds. After incubation over night at 8±2 °C, the suspension was centrifuged, the supernatant was evaporated to dryness and redissolved in 50% methanol (1).

Bioassays: The two axenic strains of *M. aeruginosa* used in bioassay were obtained from the Culture Collection of the Freshwater Algae, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China (Table 1). The strain which produces microcystin during axenic culture is classified as toxic and the other which do not produce microcystin is non-toxic (23). The cultures were grown in sterilized BG11 nutrient solution (16) under fluorescent light (2500 lux, 12:12 h light:dark cycle) at 25±1 °C and were manually shaken twice a day during incubation. Cyanobacterial cells in exponential phase were prepared for bioassay of crude extracts and enriched exudates of 3 plants, before and after PVPP treatment.

Table 1. Test strains of *M. aeruginosa*

Strain code	Sampling stations	Shape	Property (23)
FACHB-942	Dianchi Lake, China	Unicell	Toxic, bloom-forming species
FACHB-469	France	Unicell	Nontoxic species

The growth inhibition test was done as per ISO standard 8692 (8) with some modifications. A small volume of each sample (equivalent to 0.1 g plant DW for plant extracts and 0.2 g plant DW for enriched exudates) was added to a 250-ml Erlenmeyer flask containing 100 ml sterilized BG11 medium and the cyanobacterial cells were immediately inoculated ($1.0\pm0.5\times10^6$ cells ml^{-1}). Under the identical conditions mentioned above, the inoculum was incubated for 72 h and then the growth rate was monitored by counting the cell numbers by a optical microscope (Olympus BH2, Japan) using a hemocytometer. In control, the extracts or exudates were replaced by equivalent solvent. Each treatment was repeated 3 times. The inhibition rate was calculated over the control.

Statistical analysis: Data represent means (n=3) with associated error bars (±SE). SPSS 13.0 was used for statistical analyses of the data. The content of TPC among different plants was compared using one-way ANOVA. The comparison of inhibition rate before and after removing phenolic compounds was made with paired-samples T Test (α=0.05).The inhibition effects among different plants and cyanobacterium species were analyzed with Multivariate ANOVA at a significance level of $P<0.05$. All data were tested for normal distribution (Kolmogorov-Smirnov test) and variance homogeneity (Levene's test). Multiple comparisons between the 3 plant spp. were performed with a Tukey test.

RESULTS AND DISCUSSION

TPC in Plants and Culture solution

When investigating the allelopathic effects of aquatic plants, we should not only consider the allelochemicals plants produce, but also their release into surrounding water to affect the target organisms, excluding herbivore, competition and other physical or biotic factors (19). Based on these principles, the content of TPC in plants and exudates were analyzed. The phenolic compounds were present in all 3 Hydrocharitaceae family plants and their surrounding culture solution (Fig. 1). TPC in *H. verticillata* was highest (15.65±0.21 mg g^{-1} DW), but was lowest (33.02±1.20 µg L^{-1}) in its culture solution. However, *E. nuttallii* and *V. spiralis* showed no significant differences ($p = 0.671$) as per their TPC, which on average was 12.91±0.32 mg g^{-1} DW in plants ($p = 0.068$) and 73.73±0.58 µg L^{-1} in culture solution. There were some interspecific differences between the *H. verticillata* and other plants ($p < 0.001$).

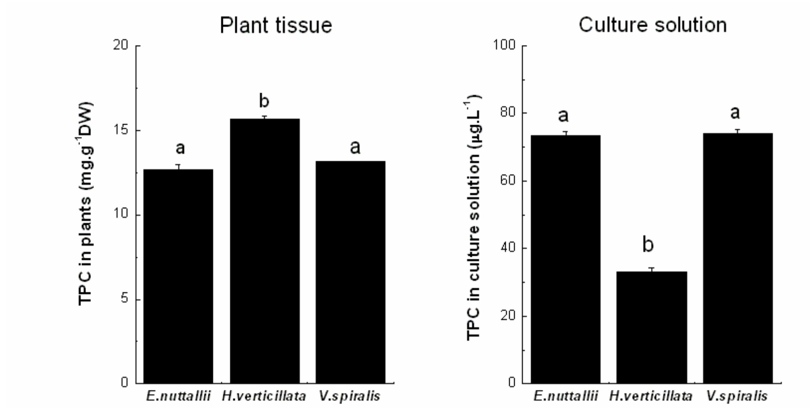

Figure 1. The content of TPC in plants and culture solution of 3 submerged macrophytes. Significant differences between the treatments are indicated with different letters (Tukey test,a=0.05)

The total exuded phenolic compounds during 3-days were 0.8% and 1.0% of the extracted phenolic compounds, respectively for *E. nuttallii* and *V. spiralis*. The release rate for *H. verticillata* was the lowest (only 0.3%). The data revealed that only a small amount of secondary metabolites produced by plants could be released into surrounding water, as per literature. Gross (3) found that the exuded phenolic compounds during two weeks contributed only about 1.0% to TPC in *M. spicatum* plant tissue.

Growth Inhibition of Plant Extracts

The crude extracts of 3 submerged macrophytes significantly inhibited the growth of two strains of *M. aeruginosa*. The inhibition rate reached 80% to the toxic strain (Fig. 2.A) and 60% to non-toxic one (Fig. 2.B) when 1 mg DW ml^{-1} biomass equivalent was applied. After removing the phenolic compounds from these extracts by PVPP precipitation, the changes in inhibitory activity depended upon the *M. aeruginosa* strains.

The inhibition rate in toxic strain, was similar to that before the PVPP treatment ($p>0.05$). However after PVPP treatment, the *E. nuttallii* and *H. verticillata* crude extracts drastically reduced the growth of non-toxic *M. aeruginosa* (Fig. 2), because PVPP also decreased the inhibitory effects of crude extracts.

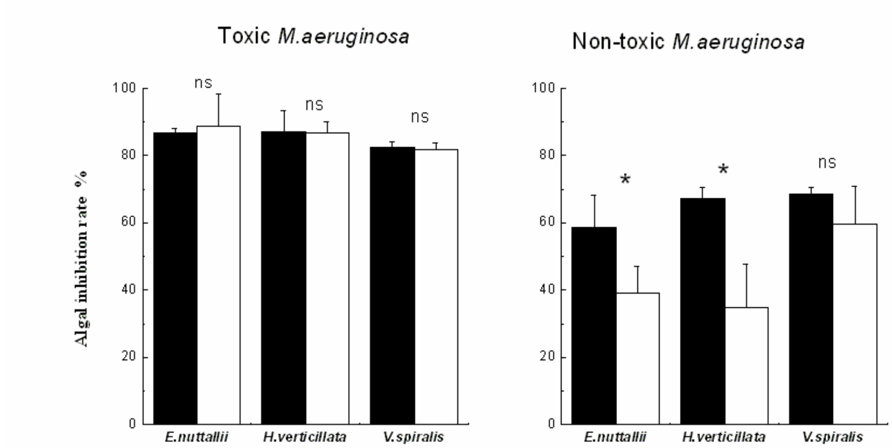

Figure 2. Comparison of the growth inhibition rate of extracts against toxic and non-toxic strain of *M.aeruginosa* 72 h after incubation before (black bars) and after removing phenolic compounds (white bars) n=3, ±1 SE. *, Significant differences occurred (a=0.05) after removing phenolic compounds. ns, Nonsignificant difference.

The responses of two strains of *M. aeruginosa* to plant extracts were significantly different. The toxic strain FACHB942 was more sensitive than non-toxic FACHB 469. Mulderij *et al*. (13), reported that toxic cyanobacterium NIVA-CYA 140 was more sensitive to allelochemicals than non-toxic cyanobacterium NIVA-CYA 43, when evaluating the allelopathic potential of exudates from the aquatic macrophyte, *Stratiotes aloides*, on the growth of toxic and non-toxic *M. aeruginosa*.

Growth Inhibition of Exudates

After SPE enrichment, the exudates (equivalent to 2 mg DW plants ml^{-1}) from the 3 plants inhibited the growth of toxic strain by > 50%, but inhibition rate varied significantly to non-toxic strain (Fig. 3). *E. nuttallii* showed the strongest allelopathic inhibitory effects (65.2% inhibition) to non-toxic *M. aeruginosa* followed by *H. verticillata* (44.6%) and *V. spiralis* (39.6%).

After removing the phenolic compounds from the exudates by PVPP precipitation, the inhibitory activity to cyanobacteria was decreased significantly ($p < 0.05$) (Fig. 3). The PVPP treatment decreased the magnitude of growth inhibition to non-toxic *M. aeruginosa* by 35.7, 43.4 and 59.1% for *E. nuttallii*, *H. verticillata* and *V. spiralis,* respectively.

Comparing with phenolic compounds extracted from plant tissue, the exuded phenolic compounds played a great role to inhibit the growth of *M. aeruginosa*. As

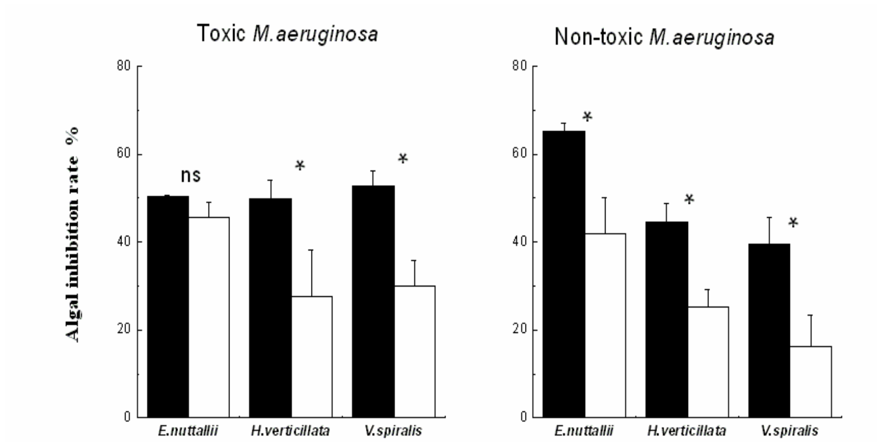

Figure 3. Comparison of the growth inhibition rate of exudates against toxic and non-toxic strain of *M.aeruginosa* 72 h after incubation before (black bars) and after removing phenolic compounds (white bars) n=3,±1 SE. *,Significant differences occurred (a=0.05) after removing phenolic compounds. ns, Nonsignificant difference.

mentioned earlier, because of their high water solubility, phenolic compounds are easily released into the surrounding water by aquatic plants than other classes of potential allelochemicals, which makes the hypothesis reasonable that phenolic compounds can be partly responsible for the reduction of algal growth in natural aquatic ecosystems. Till now, this hypothesis has been confirmed on *M. spicatum*, phenolic compounds, i.e. especially the released polyphenols are very inhibitory to algae (4,17). For the test plants of this study, there was some indirect proof. Erhard *et al.* (1) found that main active fractions obtained by further separation of methanolic extracts of *Elodea* were hydrophilic and slightly lipophilic compounds, which were supposed to be responsible for algal growth reduction. Xian *et al.* (24) isolated several phenolic acids from aqueous extracts of *V. spiralis* and *H. verticillata*, which showed the potential allelopathic activities on algal growth.

Although *E. nuttallii*, *H. verticillata* and *V. spiralis* are from same family but, there were still some interspecific differences. Firstly, the TPC of *H. verticillata* was significantly different than other two species. It produced higher amounts of phenolic compounds but released very little into the culture solution, which was supposed to be related to the characteristics of individual compounds. Rao *et al.* (15) reported that *Hydrilla* was rich in gibberellin-like substances and dihydroxyphenolic acids but these were absent in plant leachate.

The bioassay results showed that *E. nuttallii* was significantly different from other two plants in algal growth inhibition of plant exudates ($p<0.01$), whereas, the differences in plant extracts were not significant ($p>0.05$). Considering the prerequisites of allelopathy (19), the inhibitory effects of exudates represent the negative allelopathy of plants. *E. nuttallii* was exotic compared to other two native species, which seemed to be a good explanation for the differences in allelopathy. However, more evidence from molecules and genes to species interactions in laboratory conditions and in situ is needed to demonstrate this hypothesis clearly.

CONCLUSIONS

The 3-submerged macrophytes (Hydrocharitaceae family): *E. nuttallii*, *H. verticillata* and *V. spiralis*, allelopathically inhibited the toxic and non-toxic *M. aeruginosa spp*. Besides they released the phenolic compounds in their surrounding water and thereby inhibited the growth of *M. aeruginosa*. Further research is needed to identify the phenolic allelochemicals in the exudates and their mechanism of allelopathic inhibition in freshwater ecosystems.

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