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Isolation and Identification of Extracts of *Eichhornia crassipes* and Their Allelopathic Effects on Algae

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The growth of some plants can be affected by certain chemical substances released from other plants; this phenomenon is called "allelopathy" in the botany. In addition, some higher aquatic plants release phytotoxins to inhibit the growth of algae (Rice 1984), which in fact might provide a way to control the eutrophication of waters. Lake eutrophication is currently one of the most prominent environmental issues. In an eutrophic water body, the input of excess amount of nutrients, such as nitrogen and phosphorous, stimulates the growth of algae and other aquatic plants, accelerates lake aging, hence inhibiting or destroying the lake ecosystem and aquatic functions.

Eichhornia crassipes (a.k.a. water hyacinth) is an aquatic plant exhibiting the affects stated above (Sun 1989 and 1990). Further investigation on the mechanisms of Eichhornia crassipes' allelopathic effects on algae is required.

MATERIALS AND METHODS

Fresh Eichhornia crassipes were collected from the Caohai part of Dianchi Lake in Yunnan province, China. Root samples of this plant were taken and all impurities were eliminated and dried at room temperature and then blended into powder and weighed. The algae species used in this experiment were pure culture of Chlorella sp. and Scendesmus obliquus provided by Institute of Hydrobiology, the Chinese Academy of Sciences. Algae growth inhibiting experiments were conducted during the logarithmic growth phase. Silicon gel powder (mesh sizes 100-200, Tianjin No.3 Chemical solvent factory). The UV/visible spectrophotometer (Model 751, Shanghai Precision & Scientific Instrument Co., LTD, China). Bench top centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, China). GC/MSD (HP5890 II GC/5971A/MSD/ Chemstation, Hewlett Packard, USA).

The research experiment used various solvents to extract Eichhornia crassipes root. As a result, five distinct substances were then isolated and identified using GC/MS. All of these substances exhibited the ability to inhibit algae growth through three measurement methods.

Organic solvents with different polarities were used during this extraction phase,

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such as ethyl ether, acetone and ethyl acetate; and all of these solvents were purified before using (Yan 1991).Soxhlet extractors were used for the Eichhornia crassipes root powder extractions; and extractions were conducted for 10 hr, 14 hr and 12 hr when using ethyl ether, acetone and ethyl acetate, respectively. By using this method, three different extracts were obtained.

Inhibition to algae growth of extracts was examined through algae growth inhibition band experiments (Dai et al.1997), algae counting (Dai et al.1997) and Chlorophyll-a measurement (APHA et al.1989) methods. Algae growth inhibition experiments were performed by using three Eichhornia crassipes root extracts and a copper sulfate solution (10mg mL^{-1}). From these experiments, the extract with the highest inhibition ability was determined.

First, a solution having a volume of 0.1 mL and a concentration of 10 mg mL^{-1} of extracts and copper sulfate solution were placed on several separate pieces of round filter paper (11 mm in diameter). After the solvent had completely evaporated, the filter papers were placed onto solid medium plates, then inoculated with algae species in their logarithmic growth phase; the plates were then irradiated with 4000 – 5000 Lux lights. The irradiation cycle was 16 hr in light followed 8 hr in darkness. The growth of algae in and around the filter paper were observed. Inhibition effect existed only if there was no algae growth within the band around the filter paper. Based on these observations, size of algae growth inhibition band was measured.

Next, solutions of 0, 0.5, 1.0, 1.5, 2.0 mL with a concentration of 10mg mL^{-1} of ethyl ether extracts were placed in five identical flasks; appropriate amounts of ethyl ether were added to each flask and to bring the volume of all the solutions to 2 mL. After the solvents evaporated, 2 mL of algae culture and 8 mL growth medium (aquatic medium # 4) were added to each flask. Then, the flasks were irradiated under 4000-5000 Lux lights for 16 hr followed 8 hr in darkness. After 7d of incubation, algae numbers were counted using a hemocytometer and the average was calculated based on four replicates. The relationship between extract concentration and algae concentration was plotted on a curve. Similarly, plots of inhibiting substance concentration against concentration of algae were obtained for acetone extract, ethyl acetate extract and copper sulfate solution, respectively.

Chlorophyll-a measurement is another applicable method in determining algae amounts. 2mL with a concentration of 10mg mL^{-1} ethyl ether, acetone and ethyl acetate extracts and copper sulfate solutions were prepared and kept in four identical sterilized flasks. After the solvents had completely evaporated, 10 mL algae culture were added to each flask and then incubated along with a corresponding blank sample. The flasks were irradiated by 4000-5000 Lux lights for 16 hr followed by 8 hr in darkness at a temperature of 24℃. After 96 hr, all samples were measured for amounts of Chlorophyll-a and the corresponding removal rates of Chlorophyll-a were calculated.

Extracts with the highest inhibiting ability were defined through the above-stated procedure. The extract then underwent multi-step elution through silicon gel powder. Chlorophyll-a levels were monitored following each elution to continue testing the inhibition ability of the Chlorella sp. A flow chart of the operation is

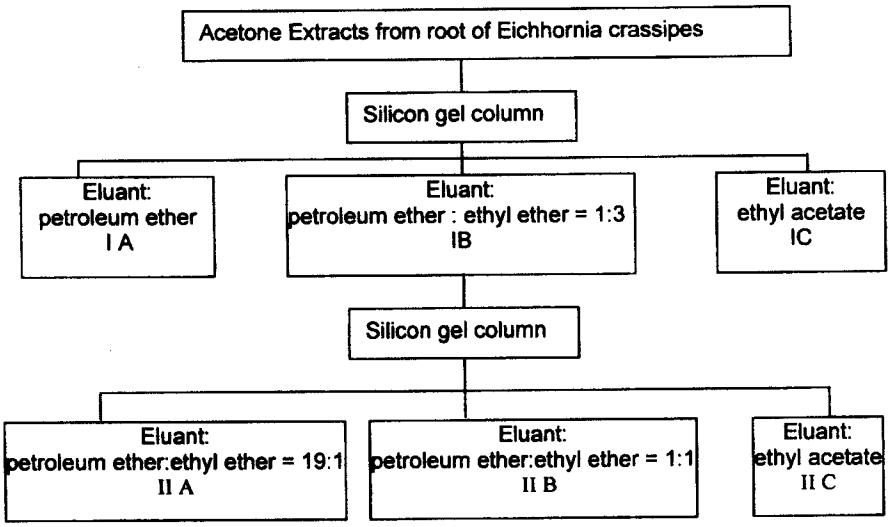

Figure 1. Flow chart of multi-step elution

presented in Figure 1. The silicon gel powder was packed into a glass column with a diameter to length ratio of 1:10. A separation funnel was fixed at the top opening of the burette with an elution rate of 1 mL min^{-1}.

The GC/MS conditions used were as follows: quartz capillary column (30 m × 0.2 mm); GC injector temperature, 280℃; 2 μL sample injection volume; column temperature program was, 40℃ (2min) $\xrightarrow{10℃/min}$ 200℃ $\xrightarrow{20℃/min}$ 280℃; carrier gas, helium (99.99%); MS, EI ion source and 30 – 550amu scanning range.

RESULTS AND DISCUSSION

A comparison of three extracts of Eichhornia crassipes root resulting from the use of three different solvents and copper sulfate solution was conducted during this research. By using three algae growth inhibition testing methods, the algae growth inhibiting abilities of each extract were determined. Results are presented in Tables 1, 2 and 3.

Table 1. Comparison of the allelopathic effect of extracts from Eichhornia crassipes root with copper sulfate solution on algae (by algae growth inhibiting band experiments).

Parameters		Ethyl ether extract	Acetone extract	Ethyl acetate extract	Copper sulfate solution
Chlorella sp.	Diameter/cm	1.19	1.30	0.89	1.28
	Standard deviation/cm	0.019	0.031	0.024	0.062
Scendesmus obliquus	Diameter/cm	1.36	1.25	0.98	1.31
	Standard deviation/cm	0.052	0.059	0.041	0.044

Table 2. Regression equations and half-inhibiting concentrations (by the algae counting method) of the allelopathic effect of extracts on algae.

Algae growth inhibiting substances	Regression equation	Half inhibiting concentration / mg mL^{-1}
Ethyl ether extract	$Y = 4.86 \times 10^6 e^{-6.67X}$	0. 91
Acetone extract	$Y = 4.95 \times 10^6 e^{-11.35X}$	0. 58
Ethyl acetate extract	$Y = 5.00 \times 10^6 e^{-7.85X}$	0. 82
Copper sulfate solution	$Y = 5.34 \times 10^6 e^{-11.12X}$	0. 62

Table 3. Chlorophyll-a removal rate (Chlorophyll-a measurement method) of allelopathic effect of extracts on algae.

Algae growth inhibiting substances	Chlorella sp.		Scendesmus obliquus	
	Chlorophyll-a /mg m^{-3}	Removal rate / %	Chlorophyll-a /mg m^{-3}	Removal rate / %
Blank	28.670	0.0	20.750	0.0
Ethyl ether extract	8.172	71.5	3.188	84.6
Acetone extract	6.279	78.1	3.943	80.9
Ethyl acetate extract	8.011	72.1	5.457	73.7
Copper sulfate solution	5.733	80.0	5.733	72.4

From table 1 to 3, we can see that all extracts from Eichhornia crassipes root inhibit algae growth to a certain extent in a variety of ways. The three testing methods used each have their own advantages and disadvantages; for example, the algae counting method is done quite easily by using a microscope to count numbers of algae, but one drawback is it not applicable when multi-cellular algae (e.g., Scendesmus obliquus) is involved. The algae growth inhibition band method is relatively simple and straightforward, but it cannot indicate levels of algae growth inhibition ability accurately. Regarding the Chlorophyll-a measurement method, it can measure both quantitatively and qualitatively, but this measurement method resulted in the destruction of the sample and could not be used in circumstances requiring continuous testing. Therefore, in this study, we combined all three methods in order to get the most accurate results. Extracts from Eichhornia crassipes root obtained by using acetone showed relatively better results among the three methods employed. In order to determine the effective components contained in these extracts, additional research was conducted to isolate and identify them using the GC/MS method.

Three substances - IA, IB and IC were obtained from our research (see Figure 1). The respective allelopathic effect on algae was determined using Chlorophyll-a measurement after each elution; the results are presented in Table 4. After the first elution, eluant IB showed the highest inhibiting ability. Through further elution, products II A, II B and II C were obtained (see Figure 1). The II A product was a white, solid crystal with one single peak on gas chromatogram and with a

retention time of 8.044 min. Through mass spectrometer determination, its molecular weight was found to be 98. Standard Spectrum databases identified it as β – D dehydrated pyranose.

Table 4. Allelopathic effect of products obtained from Eichhornia crassipes root extract and copper sulfate solutions on algae (by Chlorophyll-a measurement method).

Algae growth inhibiting substance	Chlorophyll-a /mg m^{-3}	Removal rate / %
Blank	29.10	0
Acetone extract	6.28	78.4
I A	21.36	26.6
I B	1.83	93.7
I C	15.37	47.2
II A	14.56	50.0
II B	1.35	95.4
II C	1.38	95.3
Copper sulfate solution	5.73	80.3

II B was a yellow substance with three single peaks on gas chromatogram. The retention time of II B_1 was 23.012 min, II B_2 was 26.506 min, and II B_3 was 31.572 min. After the mass spectrometer and Standard Spectrum database identification, it was concluded that II B actually consisted of three different substances: II B_1, isocyanoethyl acetate ($C_3H_8O_2N$), II B_2, 2-2-dimethyl cyclopentanone ($C_7H_{12}O$) and II B_3, propane amide (C_3H_7NO). IIC was a colorless oil-like substance and its GC spectrum had one single peak with a retention time of 8.052 min. MS determination and Standard Spectrum database identified it as pelargonic acid ($C_9H_{18}O_2$).

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