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PHYCOCHEMISTRY AND BIOACTIVITY OF CERTAIN FRESHWATER GREEN ALGAE OF SINDH

B. GHAZALA[1], MUSTAFA SHAMEEL[1], M. IQBAL CHOUDHARY[2], SALEEM SHAHZAD[1] AND SULTAN MAHMOOD LEGHARI[3]

[1]*Department of Botany, University of Karachi, Karachi-75270*
[2]*HEJ Research institute of Chemistry, University of Karachi, Karachi-75270*
[3]*Deprtment of Freshwater Biology & Fisheries, University of Sindh, Jamshoro-76080*

Abstract

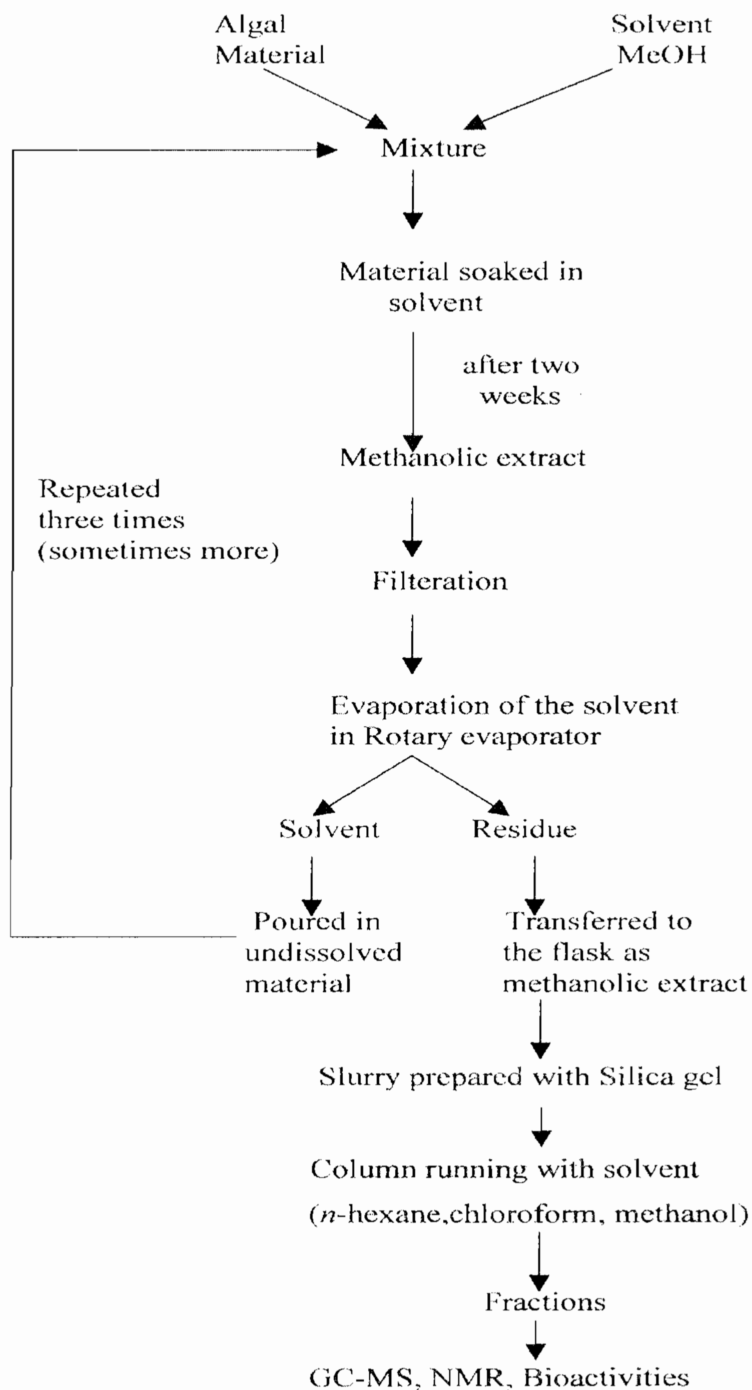
Methanol extracts of five species of freshwater green algae, *Chara cotraria* A. Braun *ex* Kützing, *Chlorococcum humicolum* (Nägeli) Rabenhorst, *Hydrodictyon indicum* Iyengar, *Rhizoclonium hieroglyphicum* (C. A. Agardh) Kützing and *Zygnema stellinum* (Vaucher) C. A. Agardh, collected during September and December 1995 from water channels at Bhitshah, Tandoadam, Hyderabad, and Jamshoro in the province of Sindh (Pakistan), were phycochemically studied by GC-MS & NMR spectroscopy and investigated against seven different tests of bioactivity. Thirty-three different fatty acids ranging from C10 to C29 were detected, 13 being saturated and 20 unsaturated ones. Among them 13 were monounsaturated, 4 diunsaturated and 3 triunsaturated acids, polyunsaturated acids could not be detected. Palmitic acid was found to occur in highest amount (on the average 9.5 %), but oleic acid was present in the small proportion (0.79 – 5.83 %). A sterol (β-sitosterol) and an acyclic diterpenoid (*trans*-phytol) have also been isolated and chemically elucidated. Strong bioactivities were observed regarding bactericidal and antifungal activities by all the investigated species, *H. indicum* and *Z. stellinum* exthibited positive activity against six different tests of bioactivity, *C. contraria* appeared to be the least active.

Introduction

Freshwater green algae usually belong to the divisions Volvocophyta, Chlorophyta and Charophyta (Shameel, 2001). As primary producers they play a very important role in the aquatic ecosystem. There is a luxurient growth of algal flora in the water bodies scattered all over Sindh (Pakistan). Several studies have been made on the habitat and general occurrence (Pervaiz & Ahmed, 1981; Shameel & Butt, 1984; Aisha & Zahid, 1991), taxonomy (Aizaz & Farooqui, 1972; Farzana & Nizamuddin, 1979; Ahmed *et al.*, 1983; Leghari & Arbani, 1983), palaeontolgy (Leghari & Nizamani, 1984; Sahito *et al.*, 1986, 1988), ecology (Nazneen, 1974; Leghari & Arbani, 1984), physiology and biochemistry (Hasni, 1991) of freshwater algae growing in these water masses, but no detailed investigation has so far been carried out on their phycochemistry, toxicity and bioactivity.

Materials and Methods

Chara contraria, Chlorocoum humicolum, Hydrodictyon indicum, Rhizoclonium hieroglyphicum and *Zygnema stellinum* were collected during September and December 1995 from water channels at Bhitshah, Tandojam, Hyderabad and Jamshoro in the province of Sindh (Pakistan). They were thoroughly washed with tap water to remove sand particles and animal castings, dried under sunshade, chopped in small pieces and soaked in methanol for 1 or 2 weeks. Procedure for the extraction of methanolic extracts of these algae has been presented in Scheme 1.



Scheme 1. Extraction of methanolic extract.

Detection of fatty acids: The extract was dissolved in a little quantity of methanol and poured on silica gel (about 10 g). The solvent was allowed to evaporate in the open atmosphere. Medium sized column was packed with fresh silica using *n*-hexane as solvent, which was allowed to run for one day. After packing the column, slurry was loaded and a layer of silica gel was placed above it, which prevented the disturbance of slurry. The mixture of products were dissolved in EtOAc and distilled water. Lower layer contained water soluble part and upper layer (EtOAc layer) contained organic compounds; the two layers were rendered apart by separating funnel. The EtOAc layer was dried overnight and fractions were treated with diazomethane. The reaction mixture was kept overnight at room temperature and then evaporated under reduced pressure. The aliquotes were directly injected into GC-MS for analysis. It was performed on a Hewlett Packard GC with a 11/73 DEC computer data system and a 1.2 m x 4 mm packed glass-capillary coloumn coated with gas chrome Q (100-120 mesh, OV 101 1%). The column temperature was programmed for 70 to 250 °C with a rate of increase of 8 °C per min. The carrier gas (He) flow rate was 32 mL/min., injector temperature was 250 °C. The fatty acid methyl esters so obtained were identified by matching their spectra with those in the NBS mass spectral library (Helles & Milne, 1978).

Isolation of sterols and terpenes: The fractions were subjected to column chromatography on silica gel using a mixture of solvent systems; where two compounds were eluted *i.e.* β-sitosterol in 8.5 hexane: 1.5 $CHCl_3$ and diterpenoid (*trans*-phytol) in hexane: $CHCl_3$. Ceric sulphate spray was used for visualization. They were chemically elucidated by HR-MS, ^{1}H- and ^{13}C-NMR spectroscopy.

Phytotoxic activity: Medium for culturing *Lemna aequinoctialis* Welw. was prepared by mixing various inorganic constituents in 1L distilled water. The pH was adjusted between 5.5 - 6.0 by adding KOH pellets. It was then autoclaved at 121°C for 15 minutes. About 12 mg of extract was dissolved in 1.5 mL of solvent (Methanol/Ethanol) serving as stock solution. Thirty sterilized vials were inoculated with 100 μL, 10 μL and 5 μL of solution pipetted from the stock solution for 500, 50 and 25 ppm. Solvent was allowed to evaporate overnight under sterilized conditions. About 2 mL of E-medium was added and then a single plant of *L. aequinoctialis*, each containing a rosette of three fronds, was placed in each vial. Experimental vials were supplemented with algal extract, and reference plant growth inhibitors and promoters were used serving as negative and positive controls respectively. For positive control Paraquat (ICI Pak. Ltd.) was used. Each vial was placed in Petri dish filled with about 2 cm of water; the container was sealed with novix and a glass plate. Plates were examined twice during incubation. Dish with vial was placed in growth cabinet for seven days. The numbers of fronds per vial were counted and recorded on the 7th day. Interpretation of result was analyzed as growth regulation in percentage, calculations were made with reference to the control. The details may be found in Farhana (1997).

Bactericidal activity: The wells were dug in the medium with the help of sterile metallic borers with their centers at least 24 mm apart. About 2-8 h old bacterial inoculum containing approx. $10^4 - 10^6$ colony forming units (CFU)/mL were spread on the surface of nutrient agar with the help of a sterile cotton swab, which is rotated firmly against the upper inside well of the tube to express excess fluid. Entire agar surface of the plate was

streaked with the swab three times turning the plate at 60 °C between each streaking. Recommended concentration of the sample (2 mg/mL) of dimethyl sulphoxide (DMSO) was then added to respective wells. Other wells supplemented with DMSO and reference antibacterial drugs served as negative and positive controls respectively. The plates were incubated immediately at 37 °C for 14-19 h or more. Activity was determined by measuring diameter of zones showing complete inhibition (in mm) and growth inhibition was calculated with reference to positive control. Theory and details of the procedure may be found in Amtul (1997).

Brine shrimp bioassay: It was conducted by taking the half filled hatching tray (a rectangular dish (22 x 32 cm) with brine solution (sea salt 38 g/L of d/w), then 500 mg eggs of brine shrimp (*Artemia salina*) were sprinkled and the lid was placed. It was allowed to incubate for hatching at 27 °C for 2 days. The brine shrimp larvae were collected through a light source and Pasteur pipette. The sample (20 mg) was dissolved in 2 mL of respective solvent and then 500 µL, 50 µL and 5 µL of this solution was transferred to vials corresponding to 1000, 100 and 10 µg/mL respectively and was evaporated overnight. After two days of hatching, larvae were placed in each vial using a Pasteur pipette. The volume was raised to 5 mL with syringe, by adding seawater and was incubated at 27 °C for 24 h under illumination. After 24 h the number of survivors were counted and recorded. The data was analyzed with Finney computer program to determine LD_{50} values at 95 % confidence intervals. The details of the procedure are given by Mansoor (1997).

Antifungal activity: It was investigated with agar well and PDA medium. Broth of boiled potatoes, sugar and agar (20 g each) were dissolved in 1L distilled water and autoclaved. Each plate contained 20 mL medium with diluted crude extract of the sample. Each Petri plate having 5 holes containing different concentrated extracts and one disc of fungus was used and 3-5 such Petri dishes were prepared for each experimental sample. Initially 3+1 sterilized test tubes were taken, 0.9 mL methanol was added in each test tube (with sterilized pipette). About 0.1 mL extract was transferred in test tube I [1/10 dil], from which 0.1 mL was transferred to the test tube II [1/100 dilution]. From this test tube 0.1 mL was added to the test tube III [1/1000 dilution]. A poured plate was taken and 5 holes of 0.5 mm diameter were made along the periphery. One drop of each treatment was added in a hole in anticlockwise direction. An inoculum dish (5 mm diam.) of a test fungus was placed in the center of each of the three replicate plates. It was incubated at room temperature and the growth of the test fungus was recorded towards each hole and compared with the control hole.

Food poisoning activity: In this activity Czapek's Dox agar (CZDA) medium was used in which $NaNO_3$ (3.0 g), K_2HPO_4 (1.0 g), $MgSO_4$ (0.5 g), KCl (0.5 g), $FeSO_4.7H_2O$ (0.1 g), sucrose (30.0 g), agar (20 g) was dissolved in 1 L distilled water and autoclaved at 121 °C temperature for 2 h. About 20 mL of this medium was poured into each sterile Petri dish and different fungal species were added for inhibition.

Antitumour activity: Galsky's plate disc assay was modified by N.R. Ferrigni & J.L. McLaughlin for the solution assay of plant extracts and to test the effectiveness of the modified assay as an antitumor prescreen for crude plant extracts. Potato tubers were

obtained from local markets and kept under refrigeration until used. Tuber surfaces were sterilized by immersion in sodium hypochlorite for 20 minutes. The ends were removed and the potatoes were soaked for 10 minutes more in Clorox. A core of the tissue was extracted from each tuber with sterilized cork borer of 1.5 cm radius. The 2 cm pieces were removed from each end and discarded, the remainder of the cylinder was cut into 0.5 cm discs with a surface-sterilized scalpel and knife. The discs were then transferred to 1.5 % agar plate (1.5 g of Merk agar dissolved in 100 mL of distilled water, autoclaved and 20 mL of it poured into each sterile Petri plate). The extracts were dissolved in 2 mL of DMSO. Plates were incubated at 27 °C for 12-20 days after incubation and the tumors were counted with naked eye. Significant activity was indicated when two or more independent assays gave consistent 20 % or greater inhibition. Details of the procedure may be found in Parveen (1997).

Results and Discussion

In the methanolic extracts of algae 39 different fatty acids (FA) were detected, 13 of them were saturated (SFA) and 20 unsaturated (UFA) acids (Table 1). Among UFAs 13 were monounsaturated (MUFA), 4 diunsaturated (DUFA) and 3 triunsaturated (TUFA) acids; polyunsaturated fatty acids (PUFA) with more than three double bonds could not be detected. It appeared that the major proportion was of SFAs and MUFAs, which were equal in number. Totally the UFAs were much more than SFAs (Table 2). This is contrary to the observations made on the marine green algae growing at the coastal waters of Karachi (Shameel, 1990, Shameel & Khan, 1991), they were found to contain SFAs in much greater quantity than UFAs. This may probably be due to their differences in adaptations in the marine as compared to the freshwater environment.

Hydrodictyon indicum contained the highest number of FAs (19), while *Zygnema stellinum* revealed the presence of smallest number (9 only) of them. It is interesting to note that *Rhizoclonium hieroglyphicum* and *Chara contraria* contained equal number (16) of FAs. Fatty acids of much higher numbers were detected in the littoral green seaweeds of Karachi coast (Shameel, 1993). This may also be an adaptation to the marine environment. Pentadecylic and palmitic acids were the only FAs found in all the five species. These are also commonly occurring FAs in the marine benthic algae studied from the coast of Karachi (Shameel, 1990). Thirteen FAs *i.e.* caproleic, lauroleic, tridecanoic, decylacrylic, undecadienoic, pentadecenoic, hexadecadienoic, nonadecanoic , gadoleic, tricosenoic, tetracosenoic, heptacosanoic and nonacosanoic acids were found only in any one of the five investigated species, four of them were saturated and nine unsaturated FAs. These FAs were found to occur rarely in the coenocytic green seaweeds of the northern Arabian Sea (Aliya *et al.*, 1995).

The detected FAs ranged from C10 to C29. Palmitic acid was found to occur in highest amount in all the five investigated species (on the average 9.512 %) as compared to the rest of the acids. The same observations were made in a variety of marine algae growing at the coast of Pakistan (Usmanghani & Shameel, 1994). The marine green algae also showed the presence of oleic acid in an appreciable quantity (Shameel, 1993; Aliya *et al.*, 1995), but the investigated five species of freshwater green algae differed from them; oleic acid was present in them in a small amount (0.79-5.83 %). This may be again due to the difference in their environments.

Table 1. Details of the fatty acids detected in the freshwater green algae of Sindh.

Type of acids	Systematic name	Common name	Molecular formula	Molecular weight
C10:1	9-Decenoic acid	Carproleic acid	$C_{10}H_{18}O_2$	184
C12:1	9-Dodecenoic acid	Lauroleic acid	$C_{12}H_{22}O_2$	212
C13:0	*n*-Tridecanoic acid	-	$C_{12}H_{26}O_2$	228
C13:1	Tridecenoic acid	Decylacry-lic acid	$C_{13}H_{24}O_2$	226
C14:0	*n*-Tetradecanoic acid	Myristic acid	$C_{14}H_{28}O_2$	242
C14:1	9-Tetradecenoic acid	Myristoleic acid	$C_{14}H_{26}O_2$	240
C14:2	7-Ethyl-3-methyl-2,6-undecadien-oic acid	-	$C_{14}H_{24}O_2$	238
C15:0	*n*-Pentadecanoic acid	Pentadecyli-c acid	$C_{15}H_{30}O_2$	256
C15:1	Pentadecenoic acid	Pentadecy-lenic acid	$C_{15}H_{28}O_2$	254
C15:3	3,7,11-Trimethyl-2,6,10-dodecatr-ienoic acid	-	$C_{15}H_{24}O_2$	250
C16:0	*n*-Hexadecanoic acid	Palmitic acid	$C_{16}H_{32}O_2$	270
C16:2	Hexadecadienoic acid	-	$C_{16}H_{28}O_2$	266
C17:0	*n*-Heptadecanoic acid	Margaric acid	$C_{17}H_{34}O_2$	284
C17:1	9-Heptadecenoic acid	Heptadecy-lenic acid	$C_{17}H_{32}O_2$	282
C17:2	9,12-Heptadec-adienoic acid	-	$C_{17}H_{30}O_2$	280
C17:3	5,8,11-Heptadeca-trienoic acid	-	$C_{17}H_{28}O_2$	278
C18:0	*n*-Octadecanoic acid	Stearic acid	$C_{18}H_{36}O_2$	298
C18:1	8-Octadecenoic acid	Oleic acid	$C_{18}H_{34}O_2$	296
C18:2	5,8-Octadecadienoic acid	Linoleic acid	$C_{18}H_{32}O_2$	294
C19:0	*n*-Nonadecanoic acid	Nonadecylic acid	$C_{19}H_{38}O_2$	312
C19:1	10 Nonadecenoic acid	Nonadecy-lenic acid	$C_{19}H_{36}O_2$	310
C20:0	*n*-Eicosanoic acid	Arachidic acid	$C_{20}H_{40}O_2$	326
C20:1	9-Eicosenoic acid	Gadoleic acid	$C_{20}H_{38}O_2$	324
C21:0	*n*-Heneicosanoic acid	-	$C_{21}H_{42}O_2$	340
C21:1	Heneicosenoic acid	-	$C_{21}H_{40}O_2$	338
C22:1	13-Docosenoic acid	Cetroleic acid	$C_{22}H_{42}O_2$	352
C23:1	Tricosenoic acid	-	$C_{23}H_{44}O_2$	366
C24:0	*n*-Tetracosanoic acid	Lignoceric acid	$C_{24}H_{48}O_2$	382
C24:1	15-Tetracosenoic acid	-	$C_{24}H_{46}O_2$	380
C27:0	*n*-Heptacosanoic acid	-	$C_{27}H_{54}O_2$	424
C29:0	*n*-Nonacosanoic acid	-	$C_{29}H_{58}O_2$	452
C29:3	Nonacosatrienoic acid	-	$C_{29}H_{52}O_2$	446

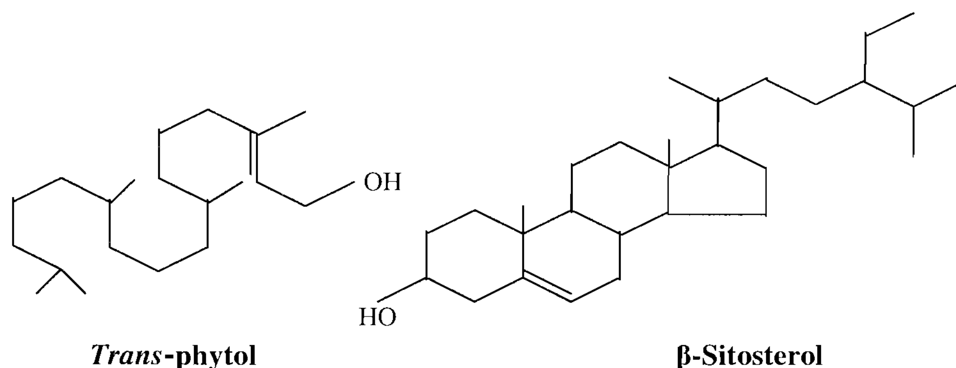
Table 2. Relative percentages of the acids present in the freshwater green algae of Sindh.

Fatty acids	*Chlorococcum humicolum*	*Hydrodictyon indicum*	*Zygnema stellinum*	*Rhizoclonium hieroglyphicum*	*Chara contraria*
C10:1	-	-	-	8.74	-
C12:1	-	3.96	-	-	-
C13:0	-	-	-	5.85	-
C13:1	-	5.13	-	-	-
C14:0	2.00	-	-	0.53	0.81
C14:1	-	2.44	39.76	-	-
C15:0	-	7.39	-	-	-
C15:0	22.85	12.35	3.00	8.85	0.51
C15:1	-	3.21	-	-	-
C15:3	29.39	14.42	-	0.53	-
C16:0	1.25	3.13	16.89	32.01	2.07
C16:2	-	7.09	-	-	-
C17:0	-	3.06	-	4.33	3.16
C17:1	16.63	-	-	1.32	-
C17:2	-	2.5	-	0.79	-
C17:3	6.25	-	-	-	12.99
C18:0	-	2.96	7.55	-	6.3
C18:1	5.83	-	1.2	0.79	1.84
C18:2	2.5	2.87	-	0.66	-
C19:0	-	1.65	-	-	-
C19:1	8.9	3.87	9	-	2.93
C20:0	1.00	3.6	8.00	-	-
C20:1	-	-	-	-	4.08
C21:0	-	-	-	7.15	7.7
C21:1	1.00	-	3.77	3.00	-
C22:0	-	-	-	4.24	1.03
C22:1	-	1.02	-	-	17.95
C23:1	-	-	-	-	1.24
C24:0	0.75	-	4.22	0.66	5.82
C24:1		1.02	-	-	-
C27:0		-	-	2.65	-
C29:0		-	-	-	4.46
C29:3		0.9	-	-	19.98
T.No.	**12**	**19**	**9**	**16**	**16**

T.No. = Total numbers of the fatty acids present.

Apart from FAs two other natural products have been isolated from *Rhizoclonium hieroglyphicum* and *Chara contraria*, one being a sterol *i.e.* β-sitosterol and the other an

acyclic diterpenoid *i.e. trans*-phytol. The β-sitosterol is a commonly occurring sterol of coenocytic green seaweeds (Aliya & Shameel, 1993, 1998). *Trans*-phytol is a side-chin component of chlorophylls a and b, it occurs as a tail of haem-nucleus. Both these chlorophyll types are found in dominant quantities among all the photosynthesizing green algae (Aliya *et al.*, 1994). From NMR-spectra their structures were elucidated as follows:



***Trans*-phytol** **β-Sitosterol**

Table 3. Bioactivity of the methanol extracts obtained from freshwater green algae of Sindh.

	Freshwater green algae				
	Chlorococcum humicolum	*Hydrodictyon indicum*	*Zygnema stellinum*	*Rhizoclonium hieroglyphicum*	*Chara contraria*
Phytotoxic bioassay	-	+	+	+	+
Bactericidal activity	+	+	+	+	+
Brine shrimp bioactivity	+	+	+	+	-
Insecticidal bioactivity	+	+	+	-	-
Food poisoning activity	+	+	+	+	-
Antitumour activity	-	-	-	-	+
Antifungal activity	+	+	+	+	+

All the five investigated species of freshwater green algae exhibited a strong bioactivity when treated with seven different tests (Table 3). Among these tests bactericidal and antifungal activities were displayed by the methanolic extracts of all the five species, while other tests have shown a positive activity by some species and no activity by others. *Chlorococcum humicolum* did not exhibit any phytotoxic activity against *Lemna aequinoctialis*, while rests of the four species have shown a positive activity. It appears to contain no toxic substances, that is why both of them may grow side by side in water channels. A variety of marine benthic algae were found to exhibit bioactivity and phytotxicity of varied nature (Rizvi *et al.*, 2000). This may be due to their protective mechanism against herbivory.

Hydrodictyon indicum and *Zygnema stellinum*, being most active, exhibited a positive activity against six different tests of bioactivity. They are commonly growing algae in the freshwater channels, pools and ditches. Probably due to this reason they eliminate other species and dominate in the population. *Chara contraria* showed a positive activity against only four tests and appeared to be the least active. In a previous study a variety of sterols isolated from *Chara wallichii* were found to exhibit strong antimicrobial activity and phytotoxicity (Khaliq-uz-Zaman *et al.*, 2001).

Although the results are interesting but inadequate to draw some definite conclusions. A variety of more freshwater algae are to be investigated to obtain a clear picture, therefore, these studies are in continuation as it is a Ph. D. program.

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