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Phytochemistry and Bioactivity of Some Freshwater Green Algae from Pakistan

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Abstract

Eleven green algae belonging to the phyla Volvocophyta Chlorophyta, and Charophyta were collected from various freshwater habitats of Sindh (Pakistan) during February 1995 and November 1998 and investigated. Their methanol extracts revealed the presence of D-norandrostane-16-carboxylic acid, β-sitosterol, and *trans*-phytol. The unsaturated fatty acids were found in larger proportion (54–94%) than the saturated fatty acids (6–40%). The C15:0 and C16:0 were the most commonly occurring fatty acids, followed by C18:1, C19:1, C15:3, and C17:3 acids. These algae resembled green seaweeds of Pakistan to a great extent in their fatty acid composition. Their methanol extract showed poor antibacterial but a strong antifungal activity. They displayed a significant phytotoxic activity but non-significant cytotoxic, insecticidal, and antitumor activities. Algae belonging to the three phyla exhibited differences in their fatty acid, sterol and terpene compositions as well as in their bioactivities. Species belonging to the same genera revealed specific differences among themselves.

Keywords: Algae, antibacterial, antifungal, antitumor, cytotoxicity, fatty acids, phytotoxicity, sterol, terpene.

Introduction

Algae are a very important component of the aquatic ecosystem. They inhabit the pelagic as well as benthic environments of the hydrosphere in a variety of forms. This variation is more pronounced in green algae, which taxonomically belongs to different phyla, such as Prochlorophyta, Volvocophyta, Euglenophyta, Chlorophyta, and Charophyta (Shameel, 2001). Apart from taxonomy, green algae of Pakistan have been investigated from various viewpoints. From time-to-time, a large number of green seaweeds growing at the seashore of Karachi and the adjacent coastal areas of Pakistan have been investigated phycochemically (Usmanghani et al., 1985, 1986; Qasim, 1986; Shameel, 1987, 1990, 1993; Aliya et al., 1991, 1993, 1995; Ahmad et al., 1992, 1993; Aliya & Shameel, 1993, 1999), their bioactivities were studied (Usmanghani et al., 1984; Usmanghani & Shameel, 1986; Naqvi et al., 1992; Amjad & Shameel, 1993; Aliya et al., 1994; Atta-ur-Rahman et al., 1997; Rizvi et al., 2000), and quite interesting results were obtained. There are also a few studies made on the brackish water green algae (Khaliq-uz-Zaman et al., 1998, 2001), but hardly any such studies have been conducted on any freshwater green alga of this area. This information remained lacking about the phytochemistry and bioactivity of the freshwater counterparts of green seaweeds to make a sound comparison. This paucity of knowledge initiated the current investigation.

Materials and Methods

Collection of material

Eleven green algae were collected from various habitats of Sindh, Pakistan, during February 1995 and November 1998 (Table 1). They were thoroughly washed to remove extraneous materials and dried in shade. Voucher specimens were preserved in 5% formaline solution and deposited in Seaweed Biology & Phycochemistry Lab., MAHQ Biological Research Centre, University of Karachi. Identification of the material was made by the second author.

Extraction and identification of fatty acids

The dried algal material was soaked in methanol for 2 weeks at room temperature. The mixture was filtered

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Table 1. Details for the investigated algal material.

No. Alga	Locality	Place	Date
Volvocophyta			
Volvocophyceae			
Tetrasporales			
Tetrasporaceae			
1. *Tetraspora cylindrica* (Wahlenberg) Agardh	Thermal effluents	Jamshoro	Jan.–Mar. 1996
2. *T. gelatinosa* (Vaucher) Desvaux	Industrial wastewater	Hyderabad	Dec. 1995
Chlorococcales			
Chlorococaceae			
3. *Chlorococcum humicolum* (Nägeli) Rabenhorst	Riverine ponds	Hyderabad	Sep. 1998
Hydrodictyaceae			
4. *Hydrodictyon reticulatum* (Linnaeus) Lagerheim	Rice fields	Bhit Shah, Hala	Nov. 1995
Chlorophyta			
Ulvophyceae			
Chaetophorales			
Chaetophoraceae			
5. *Chaetophora elegans* (Roth) Agardh	Rice fields	Tando Muhammad Khan	Sep.–Nov. 1998
Zygnemophyceae			
Zygnemales			
Zygnemaceae			
6. *Spirogyra hyalina* Cleve	Riverine pond	Hyderabad	May–July 1998
7. *Zygenma czurdae* Randhawa	Back-water ponds	Khari bus stand	Dec. 1995
8. *Zygnema stellinum* (Vaucher) C.A. Agardh	Sim Nala Bridge	Tando Adam	Feb. 1995
9. *Zygnema tenue* Kützing	Riverine ponds	Hyderabad	Feb. 1995
Siphonocladophyceae			
Cladophorales			
Cladophoraceae			
10. *Rhizoclonium hieroglyphicum* (C.A. Agardh) Kützing	Saline ponds	Hyderabad	Oct. 1995
Charophyta			
Charophyceae			
Charales			
Characeae			
11. *Chara contraria* A. Braun ex Kützing	Riverine ponds	Hyderabad	Oct.–Dec. 1996

to remove an undissolved portion, and the filtrate was evaporated in a rotary evaporator under reduced pressure. This was repeated three-times and eluted in a dark colored, thick and syrupy residue (Fig. 1). The residue was dissolved in a small quantity of methanol and poured on silica gel (about 10 g) to prepare a slurry. The solvent was allowed to evaporate in the open atmosphere. A medium-sized column was packed with fresh silica using *n*-hexane as solvent, which was allowed to run for 1 day. After packing the column, the slurry was loaded and a layer of silica gel was placed above it, which prevented the disturbance of slurry.

Some fractions were directly treated with diazomethane and others dissolved in EtOAc and distilled water. The lower aqueous layer, containing a water-soluble part, and the upper organic layer (EtOAc layer), containing organic compounds, were separated by a separating funnel. The EtOAc layer was dried overnight and fractions were kept in round bottles at 250°C for methylation (Fig. 2). The aliquots were directly injected into gas chromatography-mass spectrumitor (GC-MS) for analysis. The instrumentation consisted of Hewlett Packard GC with a 11/73 DEC computer data system and a 1.2 m × 4 mm packed glass-capillary column 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, and the 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. Two compounds were eluted: β-sitosterol in 8.5 hexane:1.5 $CHCl_3$ and diterpenoid (*trans*-phytol) in $CHCl_3$:MeOH (9.5:0.5, v/v). Ceric sulfate spray was used for visualization. Structures were determined by HR-MS, ^1H NMR and ^{13}C NMR spectroscopy. The following spectral data

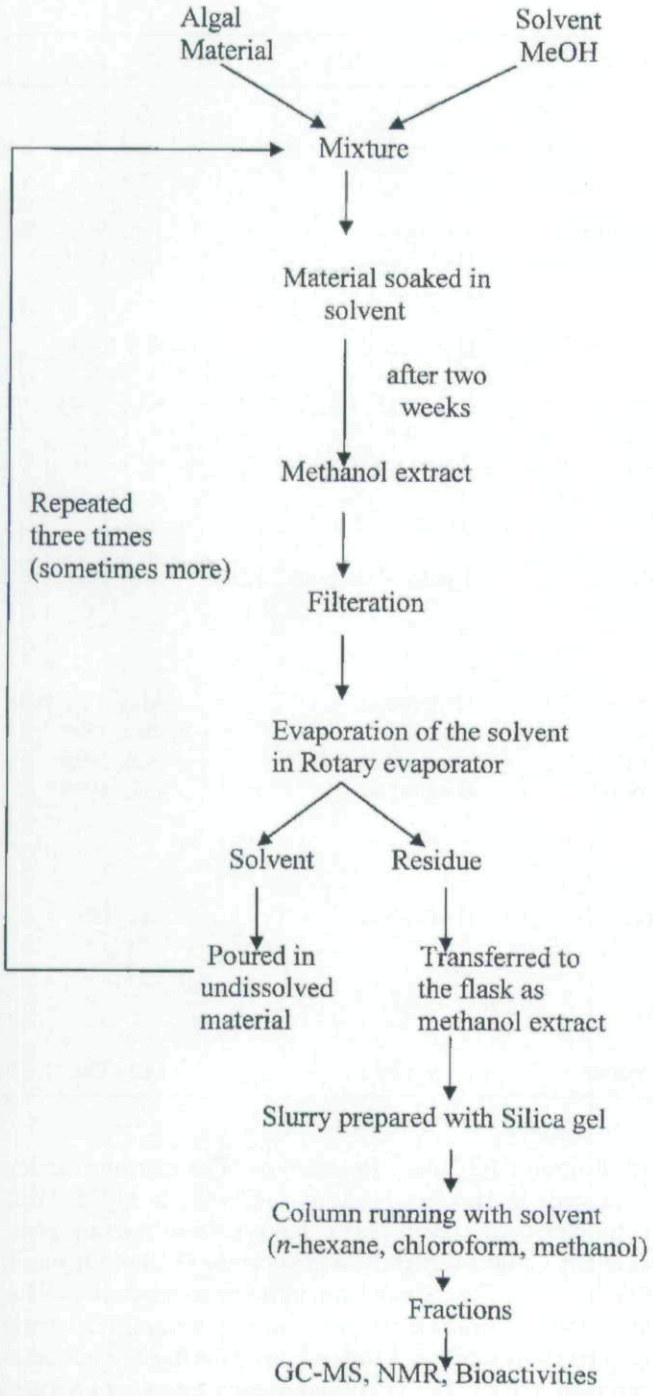

Figure 1. Extraction of methanol extract.

were obtained, which led to the identification of the compounds:

β-*Sitosterol*

EIMS: m/z 414 (M^+, $C_{29}H_{50}O_2$), 399 (M^+-CH_3), 396 (M^+-H_2O), 381 ($M^+-CH_3-H_2O$), 342, 301, 273, 255. ^1H NMR ($CDCl_3$, 300 MHz): σ 0.76 (3H, *s*, C-18 H), 0.83–0.99 (12 H, *m*, H-21, H-26, H-27, and H-29), 1.05

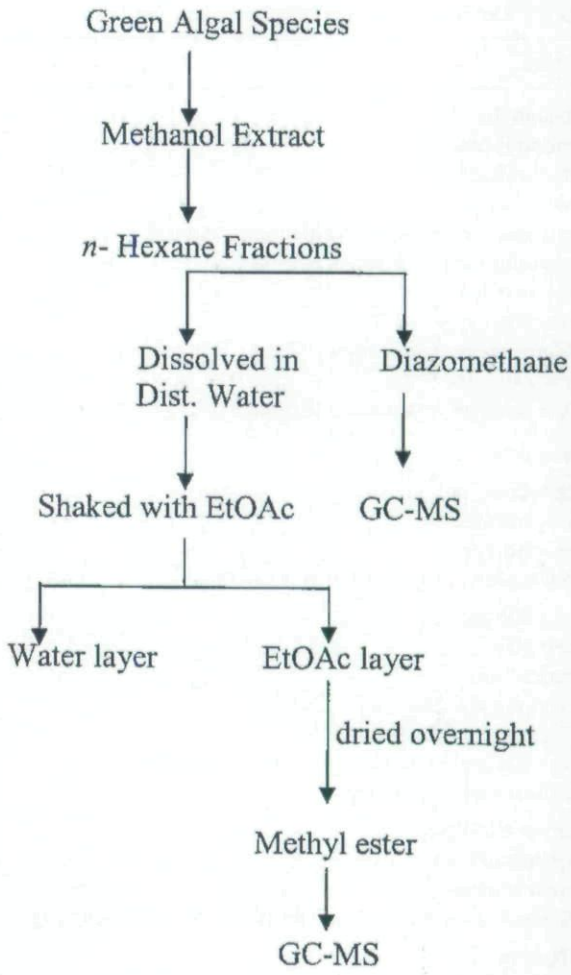

Figure 2. Detection of fatty acids.

(3H, *s*, H-19), 3.20 (1H, *m*, H-3), 5.34 (1H, *t*, $J = 6.0$ Hz, H-6) ppm.

trans-*Phytol*

IR ($CHCl_3$): 3340 (OH), 1625 (olefinic) cm^{-1}. FDMS: m/z 296 (M^+, 100%). EIMS (rel int %): m/z 296 (M^+, $C_{20}H_{40}O$, 2%), 279 (M^+-OH, 2%), 278 (M^+-H_2O, 3%), 252 (M^+-CH_2OH-CH, 1%), 197 ($M^+-C_6H_{11}O_3$, 13%), 113 (5%), 98 (2%), 85 (28%), 71 (100%). ^1H NMR ($CDCl_3$, 300 MHz): δ 0.83 (3H, *d*, $J = 6.5$ Hz), 0.84 (*d*, $J = 6.5$ HZ), 8.5 (6H, $J = 6.6$ Hz), 1.66 (3H, *s*), 1.98 (2H, *t*, $J = 7.6$ Hz, H-2), 4.14 (2H, *d*, $J = 6.9$ Hz), and 5.40 (1H, *t*, $J = 6.9$ Hz) ppm.

Bactericidal activity

Wells were placed in the medium with the help of sterile metallic borers with their centers at least 24-mm apart. Bacterial inoculums about 2 to 8 h old containing approximately 10^4–10^6 colony forming units (CFU)/ml were spread on the surface of nutrient agar with the help

of sterile cotton swabs, which were rotated firmly against the upper inside well of the tube to express excess fluid. The entire agar surface of the plate was streaked three-times with the swab while turning the plate at 60° between each streaking. A recommended concentration of the sample (2 mg/ml) in dimethylsulfoxide (DMSO) was then added to the respective wells. Other wells were 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 the diameter of zones showing complete inhibition (in mm) and growth inhibition was calculated with reference to a positive control (Amtul, 1997).

Antifungal bioassay

The antifungal activity was initially investigated in agar wells and potato dextrose agar (PDA) medium. Broth of boiled potatoes, sugar, and agar (20 g each) were dissolved in 1 l of distilled water and autoclaved. Each plate contained 20 ml of medium with diluted crude extract of the sample. Each Petri plate having 5 holes containing different concentrations of extracts and one disk of fungus was used, and 3–5 such Petri dishes were prepared for each experimental sample. Initially, 3 + 1 sterilized test tubes were taken and 0.9 ml methanol was added in each test tube (with sterilized pipette). About 0.1 ml of extract was transferred in test tube no. I [1/10 dilution], from which 0.1 ml was transferred to the test tube II [1/100 dilution], and 0.1 ml from test tube II 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 each hole in an anticlockwise direction. An inoculum dish (5-mm diameter) 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 toward each hole and compared with the control hole (Najeeb, 1997).

Antifungal activity was also studied by a food poisoning method. In this method, Czapek's Doxagar (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), $FeSO_4 \cdot H_2O$ (0.1 g), sucrose (30.0 g), and agar (20 g) were dissolved in 1 l distilled water and autoclaved at 121°C temperature for 2 h. About 20 ml of this medium were poured into each sterile Petri dish and different fungal species were added for inhibition studies (Najeeb, 1997).

Phytotoxic activity

The E-medium for *Lemma aequinoctialis* Welw. was prepared by mixing various inorganic constituents (0.68 g KH_2PO_4, 1.515 g KNO_3, 1.180 g $Ca(NO_2)_2{\cdot}4H_2O$, 0.492 g $MgSO_4 \cdot 7H_2O$, 0.00286 g H_3BO_3, 0.00362 g $MnCl_2 \cdot H_2O$, 0.00540 g $FeCl_3 \cdot 6H_2O$, 0.00022 g $ZnSO_4 \cdot 5H_2O$, 0.00022 g $CuSO_4 \cdot 5H_2O$, 0.00012 g $Na_2MO_4 \cdot 2H_2O$, and 0.01120 g EDTA) in 1 l distilled water. The pH was adjusted between 5.5–6.0 by adding KOH pellets. It was then autoclaved at 121°C for 15 min. About 12 mg of extract were dissolved in 1.5 ml of solvent (methanol/ethanol) serving as stock solution. Thirty sterilized vials were inoculated with 100, 10, and 5 µl of 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. Each vial was placed in a Petri dish filled with about 2 cm of water; the container was sealed with novix and a glass plate. The plates were examined twice during incubation. Dishes with vials were placed in a growth cabinet for 7 days. The numbers of fronds per vial were counted and recorded on the seventh day. Percent growth regulation was calculated with reference to the control (Farhana, 1997).

Brine shrimp bioassay

The assay was conducted by taking the hatching tray (a rectangular dish, 22 × 32 cm) half-filled with brine solution (sea salt 38 g/l of d/w), and then 500 mg of eggs of brine shrimp (*Artemia salina*) were sprinkled and the lid was placed. The tray was allowed to incubate for hatching at 27°C for 2 days. The brine shrimp larvae were collected using a Pasteur pipette under a light source. The sample (20 mg) was dissolved in 2 ml of methanol and then 500, 50, and 5 µl of this solution were transferred to vials and diluted with the solvents to give 1000, 100, and 10 µl/ml concentrations, respectively. The solvent was evaporated overnight. The volume was raised to 5 ml with a 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 were analyzed with Finney computer program to determine LD_{50} values at 95% confidence intervals (Mansoor, 1997).

Insecticidal activity

The insects (*Tribolium castaneum*, common grain pest) were exposed to the test compound by direct contact toxicity method using filter paper impregnated with test sample (1571.33 µg/cm^3). Afterwards, 10 adult insects of different types and of the same age were transferred to Petri dishes. A check batch of negative control was treated with solvent for determination of solvent effect. Another batch supplemented with reference insecticides,

that is, Mortein Coopex (Reckilt Benckiser Pak. Ltd.) was used. All of them were kept without food for 24 h. Mortality counts were carried out after a 24 h exposure period (Farhana, 2000).

Antitumor activity

Potato tubers were obtained from local markets and kept under refrigeration until used. Tuber surfaces were sterilized by immersion in sodium hypochlorite solution (50%) for 20 min. The ends were removed and the potatoes were soaked for 10 min or more in Clorox. A core of the tissue was extracted from each tuber with a sterilized cork borer (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 disks with a surface-sterilized scalpel and knife. The disks were then transferred to a 1.5% agar plate (1.5 g of Merck 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, and after incubation the tumors were counted with naked eye. Significant activity was indicated when two or more independent assays gave consistent 20% or greater inhibition (Parveen, 1997).

Results and Discussion

Eleven commonly occurring species of freshwater green algae, as shown in Table 1, were collected from water channels, riverine ponds, canals, and saline water ponds present in the districts of Hyderabad, Tando Muhammad Khan, Dadu, and from the neighboring areas of Jamshoro (Pakistan) and have been investigated phytochemically and investigated for bioactivity. Taxonomically, all of them were found to be known species; they belonged to 3 Phyla, 5 classes, 6 orders, 7 families, and 8 genera according to the recent classification (Shameel, 2001). Although they have already been previously described taxonomically, all the investigated species were studied for the first time phytochemically as well as from the viewpoint of bioactivity during this research program.

Fatty acids

Although analysis of fatty acids (FAs) does not require great bulk of the collected material, it is extremely difficult to collect freshwater microalgae in appreciable quantity. Therefore, out of 11 investigated species, the FAs were thoroughly detected only in 8 algae. Due to scarcity of material, only 4–5 FAs were found in *Tetraspora gelatinosa* and *Zygnema czurdae*, while no FA could be detected in *Spirogyra hyalina*, although they were all treated by the same method. Altogether, 42 different FAs have been detected (Table 2) including 14 saturated fatty acids (SFAs), 27 unsaturated fatty acids (UFAs), and 1 steroidal FA. The steroidal FA was identified as D-norandrostane-16-carboxylic acid (Fig. 3; [**1**]) on the basis of following spectral data:

D-Norandrostane-16-carboxylate (**1**): GC-MS: *m/z* (rel int %) 304 (M^+, $C_{20}H_{33}O_2$, 3%), 288 (M^+-16, 4), 288 (M^+-40, 7), 250 (6), 240 (12), 226 (100), 220 (30), 211 (47), 205 (30), 189 (14), 178 (9), 165 (10), 152 (6), 134 (6), 112 (12), 97 (9), 89 (6), 83 (10), 68 (8), 53 (4).

The steroidal FA is a derivative of an animal hormone, the testerone (17β-hydroxyandrost-4-en-3-one, [**2**]). The UFAs consisted of 13 mono-unsaturated fatty acids (MUFAs), 5 di-unsaturated fatty acids (DUFAs), 5 tri-unsaturated fatty acids (TUFAs), and 4 poly-unsaturated fatty acids (PUFAs). This indicates that UFAs of the investigated species exhibited greater diversity than that of SFAs. In a study carried out on 12 green seaweeds of the Karachi coast, the UFAs were also found in greater number than SFAs (Shameel, 1993). This shows uniformity in the marine and freshwater green algae of Pakistan.

Practically in all the investigated species, UFAs were found in a larger proportion (54–94%) than the SFAs (6–40%). Only in *Rhizoclonium hieroglyphicum* the SFAs were detected in a larger amount (66.29%) than the UFAs (16.52%), while in *Tetraspora cylindrica* (44.868% and 44.9%) and *Zygnema czurdae* (50.5% and 49.5%), both the categories of FAs were almost in equal quantity. There are ambiguities in certain species due to some unidentified FAs, which were present in an appreciable amount (4.8–17.2%). They remained unidentified because their GC-MS spectra could not be obtained due to some technical difficulties. Similarly, in a variety of marine benthic algae growing along the coast of Karachi, UFAs were detected in a greater quantity than the SFAs (Aliya & Shameel, 1993, 1998, 1999). In this regard, marine and freshwater green algae of Pakistan behaved similarly.

Pentadecylic (C15:0) and palmitic (C16:0) acids were the most commonly occurring FAs, detected in eight out of the nine investigated species (due to scarcity of material, three species could not be analyzed properly). They were followed by oleic (C18:1) and nonadecylenic (C19:1) acids, which could be detected in seven species, and pentadecatrienoic (C15:3) and hexadecatrienoic (C16:3) acids were observed in six investigated species. The studies conducted on marine algae from Karachi also showed the common occurrence of palmitic and oleic acids in their thalli (Qasim, 1986, Shameel, 1990, 1993; Shameel & Khan, 1991).

Palmitic and oleic acids were detected in the overwhelming quantity in most of the previously investigated green seaweeds not only from the coast of Pakistan

Table 2. Relative percentages of the fatty acids detected in 11 species of freshwater algae of Pakistan.

	Approximate relative percentages in algae										
Fatty acids	1	2	3	4	5	6	7	8	9	10	11
Saturated											
C13:0	–	–	–	–	–	–	–	–	–	5.8	–
C14:0	3.133	–	2.00	–	–	–	–	–	8.24	0.53	0.81
C15:0	9.596	–	22.86	12.35	23.6	–	–	3.044	21.63	8.855	0.51
C16:0	4.24	–	1.25	3.13	–	–	5.94	16.947	2.57	32.01	2.07
C17:0	0.867	–	–	3.06	12.6	–	–	–	15.45	4.335	3.16
C18:0	2.55	–	–	2.96	–	–	–	7.55	–	–	6.30
C19:0	3.359	–	–	1.65	–	–	–	–	–	–	–
C20:0	–	–	1.00	3.60	–	–	–	8.00	2.57	–	–
C21:0	17.02	–	–	–	–	–	–	–	–	7.155	7.70
C22:0	–	–	–	–	3.00	–	–	–	–	4.245	1.03
C23:0	3.757	–	–	–	–	–	–	–	–	–	–
C24:0	–	–	0.75	–	–	–	–	4.22	–	0.66	5.82
C27:0	0.346	–	–	–	–	–	–	–	–	2.655	–
C29:0	–	–	–	–	–	–	–	–	–	–	4.46
Total	44.868	–	27.86	26.75	39.3	–	5.94	39.761	50.46	66.29	31.86
Mono-unsaturated											
C10:1	–	–	–	–	–	–	–	–	–	8.74	–
C12:1	0.37	–	–	3.96	7.22	–	–	–	3.09	–	–
C13:1	–	–	–	5.31	3.6	–	–	–	–	–	–
C14:1	0.93	–	–	2.44	–	–	–	40.579	2.06	–	–
C15:1	–	–	–	3.21	–	–	68.58	–	–	–	–
C17:1	3.68	–	16.63	–	–	–	13.5	–	32.44	1.325	–
C18:1	0.150	–	5.83	–	–	–	6.48	1.226	3.60	0.795	1.84
C19:1	1.85	–	8.90	3.87	7.8	–	–	8.75	3.09	–	2.93
C20:1	2.60	–	1.00	–	–	–	–	–	–	–	4.08
C21:1	13.352	–	–	–	–	–	–	3.77	–	3.005	–
C22:1	1.849	–	–	1.02	–	–	–	–	–	–	17.95
C23:1	–	–	–	–	–	–	–	–	–	–	1.24
C24:1	–	–	–	1.02	–	–	–	–	–	–	–
Total	24.781	–	32.36	20.62	18.62	–	88.56	58.325	44.28	13.87	28.04
Di-unsaturated											
C5:2	0.115	–	–	–	–	–	–	–	–	–	–
C14:2	–	–	–	7.39	8.96	–	–	–	–	–	–
C16:2	2.023	–	–	7.09	–	–	–	–	–	–	–
C17:2	7.167	–	2.5	2.50	–	–	–	–	–	0.795	–
C18:2	1.156	–	–	2.87	–	–	–	–	–	0.66	–
Total	10.461	–	2.5	19.85	8.96	–	–	–	–	1.455	–
Tri-unsaturated											
C11:3	0.115	–	–	–	–	–	5.40	–	–	–	–
C15:3	9.028	17.612	29.38	14.42	8.32	–	–	–	–	0.53	–
C17:3	1.62	23.8	6.25	1.32	–	–	–	–	5.15	–	12.99
C19:3	–	–	–	–	–	–	–	–	–	–	–
C29:3	–	–	–	0.90	19.4	–	–	–	–	–	19.98
Total	9.143	41.412	35.88	16.64	27.72	–	5.40	–	5.15	0.53	32.97
Poly-unsaturated											
C14:5	–	–	–	–	–	–	–	–	–	1.235	–
C15:4	–	30.084	–	–	–	–	–	–	–	–	–
C20:6	–	–	1.25	–	–	–	–	–	–	–	–
C22:6	–	–	–	–	–	–	–	–	–	5.6	–
Total	–	30.084	1.25	–	–	–	–	–	–	6.835	–

(*Continued*)

Table 2. Continued.

Fatty acids	Approximate relative percentages in algae										
	1	2	3	4	5	6	7	8	9	10	11
Steroidal FA C19:4	–	28.084	–	–	–	–	–	–	–	–	–
Unidentified	10.25	–	–	16.01	4.8	–	–	5.824	–	17.185	7.46
Total acids	24	04	13	20	09	–	05	09	11	18	16

–, not detected.

1, *Tetraspora cylindrica*; 2, *T. gelatinosa*; 3, *Chlorococcum humicolum*; 4, *Hydrodictyon reticulatum*; 5, *Chaetophora elegans*; 6, *Spirogyra hyalina*; 7, *Zygnema czurdae*; 8, *Z. stellinum*; 9, *Z. tenue*; 10, *Rhizoclonium hieroglyphicum*; 11, *Chara contraria.*

(Qasim, 1986; Shameel, 1987, 1990, 1993; Aliya et al., 1995) but also those growing at other coasts in the world oceans (Karawya et al., 1982; Parekh et al., 1984; Sridharan et al., 1993; Manoharan & Subramanian, 1995; Eienkov et al., 1996; Kafanova et al., 1996). In the current species, both of them were found in small quantities (8.52% and 2.85% on the average), while myristic (12.81%), arachidic (10.63%), heptadecylenic (13.52%), and pentadecatrienoic (13.72% on the average) acids were present in much larger proportion. In this respect, freshwater green algae appeared to behave differently than green seaweeds of Pakistan.

Tetraspora cylindrica exhibited the largest diversity as it contained 24 different FAs, and the next diverse species was *Hydrodictyon reticulatum* with 20 different FAs. *Zygnema stellinum* appeared to be the least diverse species with 9 FAs. However, only four FAs were detected in *T. gelatinosa*, five FAs in *Z. czurdae*, and no FA in *Spirogyra hyalina*. This is only due to scarcity of the available material, therefore these figures may not be completely accurate. All the investigated species exhibited great variation in their FA composition. Even

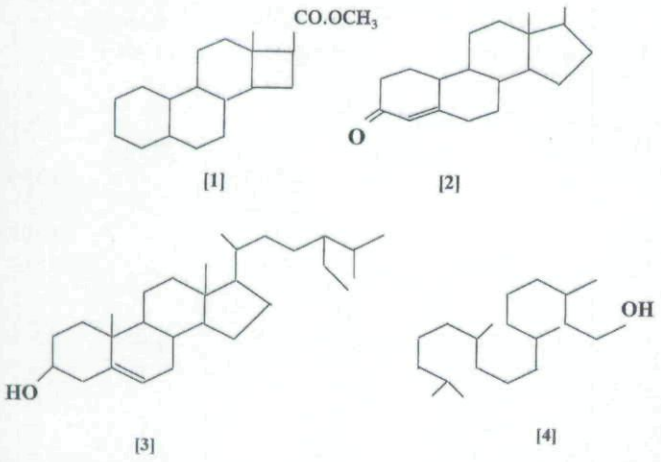

Figure 3. Structure of the isolated natural products: [**1**] = D-norandrostane-16-carboxylate, [**2**] = 17β-hydroxyandrost-4-en-3-one (testosterone), [**3**] = β-sitosterol, [**4**] = *trans*-phytol.

the two species of *Tetraspora* and three species of *Zygnema* differed from one another to a great extent. This indicates that different species of the same genus may behave variably in their FA composition. Such specific differences have also been observed among green seaweeds of Karachi coast (Aliya & Shameel, 1993, 1998).

The SFAs ranged from C13 to C29, the MUFAs from C10 to C24, the DUFAs from C5 to C18, TUFAs from C11 to C29, while PUFAs exhibited the shortest range from C14 to C22. The MUFAs showed the largest range of FAs. The PUFAs were found only in *T. gelatinosa*, *Chlorococcum humicolum*, and *Rhizoclonium hieroglyphicum*, and they were mainly C14, C15, C20, and C22 acids, while in other studies on freshwater green algae it was observed that their FA pattern is characterized as lacking in C20 acids but containing large amounts of C16- and C18-PUFAs (Menzel & Wild, 1989). Studies on other freshwater green algae showed the presence of palmitic, linoleic and linolenic acids (El-Sayed, 1983; Stefanov et al., 1996). The current results agree with these observations.

When FA composition of a different group of green algae (belonging to different phyla) was compared, it appeared that the species of the phylum Volvocophyta showed the presence of the largest number of FAs with large amounts of C15:0, C15:3, C15:4, C17:1, and C21:0 acids. They not only possessed PUFAs but also steroidal FA, therefore all the six categories of FAs were found in them. Members of the phylum Chlorophyta exhibited the smallest number of FAs with a few DUFAs and TUFAs and no PUFA or steroidal FA. They contained large amounts of C14:1, C15:1, C16:0, C17:0, C17:1, and C19:3 acids. Species of the phylum Charophyta revealed an intermediate number of FAs with no DUFA, PUFA, and steroidal FA. The acids present in large proportion were C17:3, C22:1, and C29:3. Similarly, the proportion of UFAs was largest in the members of the Volvocophyta and smallest in the species of the Chlorophyta, while that of Charophyta occupies an intermediate position. Further data are required to evaluate the validity of these observations.

Sterols and terpenes

Extraction of sterols and terpenes requires a huge collection of material, and freshwater algae having small thalli and not growing abundantly could not be collected in vast quantity. Therefore, a detailed analysis of sterol and terpene fractions was not possible. Only one sterol, that is, β-sitosterol (having cholesta-skeleton: 24r-ethylcholest-5-en-3β-ol) and only one terpene, that is, *trans*-phytol (an acyclic diterpene alcohol: 3,7,11,15-tetramethyl-hexadec-2-en-1-ol) could be detected in them (Table 3); for their structures see Fig. 1 (**3** and **4**). The two investigated species of *Tetraspora* exhibited a similarity by possessing both these natural products, while the three investigated species of *Zygnema* showed differences among themselves, that is, *Z. tenue* possessed them but *Z. czurdae* and *Z. stellinum* lacked them. All the four investigated species of the phylum Volvocophyta showed the presence of both of them. Out of six investigated species of the phylum Chlorophyta, three exhibited their presence, three lacked them, and one investigated member of the phylum Charophyta possessed only β-sitosterol and lacked *trans*-phytol. However, the data sets are limited so no general conclusions may be drawn about their distribution in various phyla.

The brassinosteroids previously isolated from *Hydrodictyon reticulatum* (Yokata et al., 1987) and cholesterol previously found in *Spirogyra crassa* (Stefanov et al., 1996) could not be detected in the current specimens, most probably due to small quantity of the available material. The alga *H. reticulatum* is believed to synthesize spinasterol having the same configuration as the higher plant sterols, while other members of the order Chlorococcales were found to possess a variety of Δ^5, Δ^7, and $\Delta^{5,7}$-sterols (Goodwin, 1974). A large number of sterols (Aliya & Shameel, 1993, 1998, 1999) and acyclic diterpenes (Aliya et al., 1994) have been isolated from green seaweeds of Pakistan and other coastal areas (Aknin et al., 1992). Whether such a variety of sterols and terpenes may also be displayed by freshwater green algae cannot be stated with certainty. However, in another study from the current lab, four different sterols have been isolated from *Chara wallichii* (Khaliq-uz-Zaman et al., 2001). This and other studies on Charales (Heilbron, 1942; Patterson, 1972; Patterson et al., 1991) indicate the presence of a variety of natural products in freshwater green algae.

Antibacterial activity

Tetraspora gelatinosa was available only in a very small amount, therefore it could not be investigated. Ten algal species were tested against five Gram-positive and six Gram-negative species of bacteria but the results were not very promising (Table 4). *Acromonas hydrophila* was tested against the methanol extracts of only 2 algal species out of 10; and *Rhizoclonium hieroglypiycum* was tested against 4 Gram-positive and 4 Gram-negative bacteria out of 11 species. *Shigella boydii* was found to be affected most against algal extracts, four of the nine investigated extracts displayed a positive activity against it by showing decrease in the bacterial population per unit area. It appeared to be the most sensitive bacterium. This was followed by *Klebsiella pneumoniae* that was affected by the methanol extracts of four of the nine tested algal species. *Corynebacterium diptheriae* was affected by the extract of only 1 species out of 10 tested algae. The rest of the bacterial species remained unaffected by any one of the tested extracts. *Tetraspora cylindrical* appeared to be the most active alga by showing activity against three bacterial species. All the tested algae of the phylum Volvocophyta exhibited bioactivity, most of the members of the phylum Chlorophyta were inactive while only two of them revealed bioactivity, and the only member of the phylum Charophyta remained inactive.

The MeOH extract and its EtOAc-soluble part of *Chara corallina* were active against a variety of Gram-positive and Gram-negative bacteria (Khaliq-uz-Zaman et al., 1998). The aqueous extract of *Chara globularis* is reported to show antibiotic activity against a natural population of bacteria from pond water (Anthoni et al., 1987). The sterols extracted from *Chara wallichii* exhibited bioactivity against several species of bacteria (Khaliq-uz-Zaman et al., 2001). This indicated that freshwater green algae may also display antibacterial activity, and all of them are not so inactive as shown in the current results.

Table 3. Sterol and terpene isolated from 11 species of freshwater algae of Pakistan.

			Algal species										
Sterol and terpene	Molecular formula	Mol wt	1	2	3	4	5	6	7	8	9	10	11
β-Sitosterol	$C_{29}H_{50}O$	414	+	+	+	+	−	+	−	−	+	+	+
Trans-phytol	$C_{20}H_{40}O$	296	+	+	+	+	−	+	−	−	+	+	−

+, present; −, absent.

1, *Tetraspora cylindrica*; 2, *T. gelatinosa*; 3, *Chlorococcum humicolum*; 4, *Hydrodictyon reticulatum*; 5, *Chaetophora elegans*; 6, *Spirogyra hyalina*; 7, *Zygnema czurdae*; 8, *Z. stellinum*; 9, *Z. tenue*; 10, *Rhizoclonium hieroglyphicum*; 11, *Chara contraria*.

Table 4. Antibacterial activity (in mm) exhibited by the methanol extracts of 10 species of freshwater algae of Pakistan.

	Algal species									
Bacterial species	1	2	3	4	5	6	7	8	9	10
Gram-positive										
Alteromonas hydrophila	–	×	×	×	×	×	×	×	–	×
Bacillus cereus	–	–	–	–	–	–	–	–	×	–
Corynebacterium diphtheriae	6*	–	–	–	–	–	–	–	–	–
Staphylococcus aureus	–	–	–	–	–	–	–	–	–	–
Streptococcus pyogenes	–	–	–	–	–	–	–	–	–	–
Gram-negative										
Escherichia coli	–	–	–	–	–	–	–	–	–	–
Klebsiella pneumoniae	7.5*	7.5*	9*	9*	–	–	–	–	×	–
Proteus mirabilis	–	–	–	–	–	–	–	–	–	–
Pseudomonas aeruginosa	–	–	–	–	–	–	–	–	–	–
Salmonella typhi	–	–	–	–	–	–	–	–	–	–
Shigella boydii	8*	9*	8*	–	8*	–	–	–	×	–

–, no activity; x, not tested, *, decrease in bacterial population/unit area only.
1, *Tetraspora cylindrica*; 2, *Chlorococcum humicolum*; 3, *Hydrodictyon reticulatum*; 4, *Chaetophora elegans*; 5, *Spirogyra hyalina*; 6, *Zygnema czurdae*; 7, *Z. stellinum*; 8, *Z. tenue*; 9, *Rhizoclonium hieroglyphicum*; 10, *Chara contraria.*

Antifungal activity

The methanol extracts of all 11 algal species were tested against 3 facultative parasites, 6 plant parasites, and 1 saprophyte by agar well diffusion method (Table 5). Only one facultative parasite was affected by algal extracts, while two of them did not show any effect. Four plant parasites were affected by the crude extracts of algae, and two of them remained unaffected. Growth of the single treated saprophyte was inhibited by algal extracts. Plant parasitic fungi appeared to be highly susceptible against compounds extracted from freshwater algae. It was very interesting to note that all the tested algal extracts exhibited similar effects against each of the fungal species, hence no conclusion will be drawn at this point about which algal species is most active and which fungus is most sensitive against such activity.

When antifungal activity was tested by a food poisoning method, it uniformly gave similar results against all the tested fungal organisms (Table 6). *Curvularia lunata* was tested only against *Tetraspora cylindrica*, which actually replaced *Fusarium proliferatum*. The rest of the organisms gave similar results, without much variation. The MeOH extract, its EtOAc-soluble parts, and four sterols isolated from *Chara corallina* displayed strong

Table 5. Antifungal activity shown by the methanol extracts of 11 species of freshwater algae by agar well diffusion method.

	Growth inhibition in cm										
Fungal species	1	2	3	4	5	6	7	8	9	10	11
Facultative parasites											
Alternaria alternata	–	–	–	–	–	–	–	–	–	–	–
Curvularia lunata	2.8	3.4	3.1	2.9	3.5	2.9	3.0	2.3	3.5	3.4	2.9
Drechslera australiensis	–	–	–	–	–	–	–	–	–	–	–
Plant parasites											
Fusarium solani	–	–	–	–	–	–	–	–	–	–	–
Fusarium sporotrichoids	4.1	3.7	3.3	2.5	3.3	3.2	2.9	2.2	3.7	3.0	3.2
Fusarium proliferatum	–	–	–	–	–	–	–	–	–	–	–
Macrophomina phaseolina	4.0	3.5	3.5	3.4	3.5	3.5	3.5	3.5	3.5	3.9	3.5
Rhizoctonita solani	4.0	3.4	3.2	3.2	3.0	3.5	3.1	3.7	3.5	3.5	3.5
Sclerotium rolfsii	3.8	3.6	3.6	3.8	3.5	3.6	3.5	1.8	3.3	3.3	3.6
Saprophyte											
Trichoderma harzianum	3.7	3.1	3.6	3.2	3.3	3.3	3.5	3.4	2.8	4.0	3.3

–, no activity.
1, *Tetraspora cylindrica*; 2, *T. gelatinosa*; 3, *Chlorococcum humicolum*; 4, *Hydrodictyon reticulatum*; 5, *Chaetophora elegans*; 6, *Spirogyra hyalina*; 7, *Zygnema czurdae*; 8, *Z. stellinum*; 9, *Z. tenue*; 10, *Rhizoclonium hieroglyphicum*; 11, *Chara contraria.*

Table 6. Antifungal activity displayed by the methanol extracts of seven species of freshwater algae by food poisoning method.

Fungal organisms	Growth inhibition in cm						
	1	2	3	4	5	6	7
Curvularia lunata	3.6	×	×	×	×	×	×
Fusarium proliferatum	×	6.0	7.1	6.2	8.0	8.0	7.0
Fusarium solani	6.0	5.7	7.6	6.9	8.0	7.9	7.0
Macrophomina phaseolina	6.8	7.5	8.3	8.5	8.0	7.5	8.4
Rhizoctonia solani	4.36	1.4	6.9	8.5	7.5	4.3	7.0
Sclerotium rolfsii	7.5	6.1	7.1	8.2	6.1	6.0	8.9
Trichoderma harzianum	7.9	7.9	8.1	8.5	8.2	7.8	7.9

×, not tested.
1, *Tetraspora cylindrica*; 2, *Chlorococcum humicolum*; 3, *Hydrodictyon reticulatum*; 4, *Chaetophora elegans*; 5, *Zygnema czurdae*; 6, *Z. stellinum*; 7, *Rhizoclonium hieroglyphicum*.

antifungal activity against a variety of fungal species (Khaliq-uz-Zaman et al., 1998). In these studies, no activity was seen against *Drechslera rostrata* and *D. australiensis*, indicating that the genus *Drechslera* is resistant against the bioactive constituents of freshwater green algae.

Other bioactivities

The methanol extracts obtained from 10 species of freshwater algae were tested against *Lemna* spp. for phytotoxic activity and brine shrimp larvae for cytotoxic activity (Table 7, A). Extracts obtained from eight species were tested against *L. aequinoctialis* and two of those were tried against *L. minor*. The results of the former were 14% to 100% while those of the latter were only 7% to 13%. Most of the investigated algal species showed more than 71% phytotoxic activity against *L. aequinoctialis*; only *Tetraspora cylindrica* showed lesser activity (14.28%). *Chlorococcum humicolum* could not be tested for phytotoxic activity. The methanol and ethanol extracts as well as isolated sterols of *Chara wallichii* also exhibited significant phytotoxic activity against *L. aequinoctialis* (Khaliq-uz-Zaman et al., 2001).

All the investigated extracts of algal species displayed non-significant results of cytotoxic activity through the brine shrimp bioassay (Table 7, B). The cytokinins,

Table 7. Phytotoxic and cytotoxic activities exhibited by the methanol extracts of 11 species of freshwater algae of Pakistan.

	Growth inhibition in %										
	1	2	3	4	5	6	7	8	9	10	11
A. Phytotoxic activity against *Lemna* spp.											
Lemna aequinoctialis	14.28	100	×	100	80.95	100	100	100	71.42	×	×
L. minor	×	×	×	×	×	×	×	×	×	13.3	6.66
B. Brine shrimp bioassay (LD_{50} in µg/ml)	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000

× = not tested.
1, *Tetraspora cylindrica*; 2, *T. gelatinosa*; 3, *Chlorococcum humicolum*; 4, *Hydrodictyon reticulatum*; 5, *Chaetophora elegans*; 6, *Spirogyra hyalina*; 7, *Zygnema czurdae*; 8, *Z. stellinum*; 9, *Z. tenue*; 10, *Rhizoclonium hieroglyphicum*; 11, *Chara contraria*.

Table 8. Insecticidal activity shown by the methanol extracts of eight species of freshwater algae against *Tribolium castaneum*.

Dose in µl/cm²	Algal species							
	1	2	3	4	5	6	7	8
1571.70 µl/cm²	–	–	–	–	–	–	–	–

–, no activity.
1, *Tetraspora cylindrica*; 2, *T. gelatinosa*; 3, *Chlorococcum humicolum*; 4, *Hydrodictyon reticulatum*; 5, *Spirogyra hyalina*; 6, *Zygnema czurdae*; 7, *Z. stellinum*; 8, *Z. tenue*.

Table 9. Antitumor activity of the methanol extract of *Chara contraria.*

µg/µl	% Inhibition
10	10
50	15
100	31

Inhibition >20% shows significant result. Drug used as positive control: *vinbastune.*

known to be ubiquitous amoung higher plants, have been isolated from *Chara globularis* (Zhang et al., 1989), abscisic acid (ABA) has been detected in *C. foetida* (Tietz et al., 1989), and sterols present in *C. corallina* were found to exhibit cytotoxic activity (Khaliq-uz-Zaman et al., 1998). These observations indicate that freshwater green algae may also possess some cytotoxic substances.

None of the eight algal extracts tested for their bioactivity against the insect (pest) *Tribolium castaneum* showed any activity (Table 8). However, *Chara globularis* is reported to contain compounds with insecticidal properties (Jacoben & Pedersen, 1983).

Antitumor activity was studied only in the extract of *Chara contraria* (Table 9). Due to shortage of material, the antitumor activity could not be investigated in extracts of other freshwater green algae. However, it was found that the activity enhanced from 10% to 31% with increasing concentration of the extract.

References

Ahmad VU, Aliya R, Perveen S, Shameel M (1992): A sterol glycoside from marine green alga *Codium iyengarii. Phytochemistry 31*: 1429–1431.

Ahmad VU, Aliya R, Perveen S, Shameel M (1993): Sterols from marine green alga *Codium decorticatum. Phytochemistry 33*: 1189–1192.

Aknin M, Moellet-Nzaou R, Kornprobst JM, Gaydou EM, Samba A, Miralles J (1992): Sterol composition of twelve Chlorophyceae from the Senegalese coast and their chemotaxonomic significance. *Phytochemistry 31*: 4167–4169.

Aliya R, Shameel M (1993): Phycochemical examination of three species of *Codium* (Bryopsidophyceae). *Bot Mar 36*: 371–376.

Aliya R, Shameel M (1998): Phycochemical investigations on air-dried material of five species of *Caulerpa* (Bryopsidophyceae). *Bot Mar 41*: 125–132.

Aliya R, Shameel M (1999): Phytochemical evaluation of four coenocytic green seaweeds from the coast of Karachi. *Pak J Mar Biol 5*: 65–76.

Aliya R, Shameel M, Usmanghani K, Ahmad VU (1991): Analysis of fatty acids from *Codium iyengarii* (Bryopsidophyceae). *Pak J Pharm Sci 4*: 103–111.

Aliya R, Shameel M, Usmanghani K, Ahmad VU (1993): Fatty acid composition of five species of *Caulerpa* (Bryopsidophyceae) from north Arabian Sea. *Mar Res 2*: 25–34.

Aliya R, Shameel M, Perveen S, Ali MS, Usmanghani K, Ahmad VU (1994): Acyclic diterpene alcohols isolated from four algae of Bryopsidophyceae and their toxicity. *Pak J Mar Sci 3*: 15–24.

Aliya R, Shameel M, Usmanghani K, Sabiha S, Ahmad VU (1995): Fatty acid composition of two siphonaceous green algae from the coast of Karachi. *Pak J Pharm Sci 8*: 47–54.

Amjad MT, Shameel M (1993): Comparative haemagglutinic activity in the species of *Caulerpa* and *Ulva* (Chlorophyta) of Karachi coast. *Pak J Mar Sci 2*: 113–117.

Amtul Z (1997): Antibacterial techniques in bioassay guided fractions. In: Amtul Z, ed., *Workshop on Use of Bioassay Techniques for New Drug Development*. HEJ Res Inst Chem, Karachi Univ., pp. 5–16.

Anthoni U, Nielsen PH, Smith-Hansen L, Wium-Andersen S, Christophersen C (1987): Charamin, a quarternary ammonium ion antibiotic from the green alga *Chara globularis. J Org Chem 52*: 694–695.

Atta-ur-Rahman, Khan AM, Shabbir M, Abid M, Chaudhary MI, Nasreen A, Maqbool MA, Shameel M, Sualeh R (1997): Nematocidal study of marine organisms. *Pak J Nematol 15*: 95–100.

Eienokov I, Stefanov K, Alexandrova M, Dimitrove-Konaklieva S, Papov S (1996): Lipid composition of some Bulgarian *Cladophora* species. *Bot Mar 39*: 79–82.

El-Sayed MM (1983): Fatty acid composition of some algal lipids from the Red Sea. *J Fac Mar Sci 3*: 141–148.

Farhana K (1997): The *Lemna*-bioassay for inhibitors and promoters of plant growth. In: Amtul Z, ed., *Workshop on Use of Bioassay Techniques for New Drug Development*. HEJ Res Inst Chem, Karachi Univ., pp. 5–17.

Farhana K (2000): Screening of insecticides by impregnation method on storage pests. In: *Mannual of Bioassay Techniques*. Worksh Tech Prod Develop Med Plants, HEJ Res Inst Chem, Pakistan & Inter-Isl Network Trop Med, Malaysia., pp. 15–17.

Goodwin TW (1974): Sterols. In: Stewart WDP, ed., *Algal Physiology and Biochemistry*. London: Blackwell, pp. 266–280.

Helles SR, Milne GWA (1978): *EPA/NIH Mass Spectral Data Base*. 4 Vols., Washington, DC. NIBS, U.S. Government Printing Office, 3975 pp.

Heilbron IM (1942): Some aspects of algal chemistry. *J Chem Soc 79*: 78–89.

Jacobsen N, Pedersen LEK (1983): Synthesis and insecticidal properties of derivatives of propane-1,3-dithiol (analogs of the insecticidal derivatives of dithiolane and trithiane from the alga *Chara globularis* Thuillier). *Pestic Sci 14*: 90–97.

Kafanova TV, Busarova NG, Isai SV, Zuyagintseva TYA (1996): Fatty acids and prostaglandins of thermophilic cyanobacteria. *Khim Prir Soedin 6*: 869–874.

Karawya MS, Fiad SA, Naim N, Hamdy AA, El-Refai AH, Sallam LA (1982): Chemical composition of some Egyptian marine algae. II. Fatty acid composition. *Egypt J Pharm Sci 21*: 349–356.

Khaliq-uz-Zaman SM, Shameel S, Shameel M, Leghari SM, Ahamad VU (1998): Bioactive compounds in *Chara corallina* var. *wallichi* (A. Br.) R. D. Wood (Charophyta). *Pak J Bot 30*: 19–31.

Khaliq-uz-Zaman SM, Simin K, Shameel M (2001): Antimicrobial activity and phytotoxicity of sterols from *Chara wellichi* A. Br. (Charophyta). *Pak J Sci Ind Res 44*: 301–304.

Manoharan C, Subramanian G (1995): Effect of effluents on lipid content of *Oscillatoria pseudogerminata* var *unigranulata* Biswas. *Geobios* (Jodhpur, India) *22*: 141–142.

Mansoor S (1997): Brine-shrimp bioassay to screen *in vivo* lethality of bioactive compounds. In: Amtul Z, ed., *Workshop on Use of Bioassay Techniques for New Drug Development*. HEJ Res Inst Chem, Karachi Univ, p. 3–4.

Menzel K, Wild A (1989): Fatty acid composition in the lipids of some marine Chlorococcales and Eustigmatales. *Z Naturforsch C Biosci 44*: 743–748.

Muto SK, Niimura, Oohara M, Oguchi Y, Matsunaga K, Hirose K, Kakuchi J, Sugita N, Furusho T, et al. (1988): Polysaccharides from marine algae and antiviral drugs containing the same as active ingredient. *European Patent Application EP* 295, *956*: 13 pp.

Najeeb F (1997): Use of antifungal bioassay for the screening of antimicrobial agents. In: Amtul Z, ed., *Workshop on Use of Bioassay Techniques for New Drug Development*. HEJ Res Inst Chem, Karachi Univ., pp. 17–18.

Naqvi SBS, Sheikh D, Usmanghani K, Shameel M, Sheikh R (1992): Screening of marine algae of Karachi for haemagglutinin activity. *Pak J Pharm Sci 5*: 129–138.

Parekh KS, Parekh HH, Rao PS (1984): Fatty acid content of some Indian marine algae. *Indian J Mar Sci 13*: 45–46.

Patterson GW (1972): Sterols of *Nitella flexilis* and *Chara vulgaris*. *Phytochemistry 11*: 3481–3483.

Patterson GW, McKenna KC, Lusby WR, Bisson MA (1991): Sterols of the Charophyceae. *J Nat Prod 54*: 1141–1143.

Qasim R (1986): Studies on fatty acid composition of eighteen species of seaweeds from the Karachi coast. *J Chem Soc Pak 8*: 233–230.

Rizvi MA, Farooqui S, Shameel M (2000): Bioactivity and elemental compositon of certain seawoods from Karachi coast. *Pak J Mar Biol 6*: 207–218.

Shameel M (1987): Studies on the fatty acids from seawoods of Karachi. In: Alahi I, Hussain F, eds., *Modern Trends of Plant Science Research in Pakistan. Proceed Nat Conf Plant Sci 3*: 183–186.

Shameel M (1990): Phycochemical studies on fatty acids from certain seaweeds. *Bot Mar 33*: 429–432.

Shameel M (1993): Phycochemical studies on fatty acids composition of twelve littoral green seaweeds of Karachi coast. In: Tirmizi NM, Kazmi QB, eds., *Proceedings of the National Seminar on Study and Management in Coastal Zones in Parkistan*. Karachi: Pak Nat Commis UNESCO, pp. 17–25.

Shameel M (2001): An approach to the classification of algae in the new millennium. *Pak J Mar Biol 7*: 233–250.

Shameel M, Khan R (1991): Fatty acid composition of nine green seaweeds. *Bot Mar 34*: 501–504.

Sridharan R, Babu JM, Tridevi GK, Mathur HH (1993): Fatty acid composition of some marine algae from Indian waters. *Bioact Comp Mar Org Indo-US Symp*, pp. 201–204.

Stefanov K, Dimitrov K, Dimitrova KS, Kirisheva I, Popv S (1996): Lipid and sterol composition of the freshwater alga *Spirogyra crassa*. *Arch Hydrobiol 135*: 523–527.

Tietz A, Ruttkowski U, Kochler R, Kasparik W (1989): Further investigations on the occurrence and the effects of abscisic acid in algae. *Biochem Physiol Pflanz 184*: 259–266.

Usmanghani K, Shameel M (1986): Studies on the antimicrobial activity of certain seaweeds from Karachi coast. In: Ahmed R, San Pietro A, eds., *Prospects for Biosaline Research*. Proc US-Pak Biosal Res Worksh, Karachi, pp. 519–526.

Usmanghani K, Shameel M, Sualeh M, Khan K, Mahmood ZA (1984): Antibacterial and antifungal activities of marine algae from Karachi seashore of Pakistan. *Fitoterapia 55*: 73–77.

Usmanghani K, Shameel M, Alam M (1985): Fatty acids of the seaweed *Ulva fasciata* (Chlorophyta). *Sci Pharm 53*: 247–251.

Usmanghani K, Shameel M, Alam M (1986): Studies on fatty acids of a green alga *Ulva fasciata* from Karachi coast. In: Ahmad R, San Pietro A, eds., *Prospects for Biosaline Research*. Proc US-Pak Biosal Res Worksh, Karachi, pp. 527–531.

Yasuno M, Sugaya Y, Iurakuma T (1985): Effects of insecticides on the benthic community in a model stream. *Environ Poll Ser A, 38*: 31–43.

Yokota T, Kim SK, Fukui Y, Takahashi N, Takeuchi Y, Takematsu T (1987): Brassinosteroids and sterols from a green alga, *Hydrodictyon reticulatum*: configuration at C-24. *Phytochemistry 26*: 503–506.

Zhang W, Yamane H, Takahashi N, Chapman DJ, Phinney HO (1989): Identification of a cytokinin in the green alga *Chara globularis*. *Phytochemistry 28*: 337–338.