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PHYCOCHEMISTRY AND BIOACTIVITY OF TWO STONEWORT ALGAE (CHAROPHYTA) OF SINDH

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Abstract

Two stonewort green algae, *Chara contraria* A. Braun *ex* Kützing and *Nitella flexilis* (Linnaeus) C. A. Agardh were collected from Kinjhar and Haleji lakes, Distt. Thatta, Sindh during October to December 1999. Their methanol extracts revealed the presence of a variety of saturated and unsaturated fatty acids (FAs) by GC-MS and β-sitosterol by EI-MS & ^{1}H-NMR. In *C. contraria,* 9 saturated, 5 mono- and 2 tri-unsaturated FAs were detected, and *N. flexilis* contained 5 saturated, 7 mono-, 2 di-, 2 tri- and 3 poly-unsaturated FAs; while 4 FAs remained unidentified. The C13:1, C14:0, C16:0, C16:3, C17:2, C17:3, C29:0 and C29:3 acids were most commonly present in appreciable quantities (8.053-20.423 %) in them. Their methanol extracts showed little antibacterial activity but a strong antifungal activity against 6 of the 10 tested fungal pathogens. They displayed no bioactivity in brine shrimp bioassay and insecticidal tests. *Chara contraria* exhibited appreciable phytotoxicity (6.66 %) and antitumour activity (31 %) but *N. flexilis* did not, they showed specific differences.

Introduction

A large number of green seaweeds growing at the seashore of Karachi and the adjacent coastal areas of Sindh have been investigated phycochemically (Usmanghani *et al*., 1985; Qasim, 1986; Shameel, 1987, 1993; Aliya *et al*., 1995; Aliya & Shameel 1999, 2003), and their bioactivities were studied (Usmanghani *et al*., 1984; Naqvi *et al*., 1992; Aliya *et al*., 1994; Atta-ur-Rahman *et al*., 1997; Rizvi & Shameel, 2003). Only a few studies were conducted on stonewort green algae of this area (Khaliq-uz-Zaman *et al*., 1998, 2001; Shameel *et al*., 1999). This paucity of knowledge initiated a research program to compare the observations made on green seaweeds with their freshwater counterparts of Sindh (Ghazala *et al*., 2003, 2004a-d). The present investigation is a continuation of this program in which taxonomy, phycochemistry and bioactivity of two stonewort algae *Chara contraria* A. Brun *ex* Kützing and *Nitella flexilis* (Linnaeus) C. A. Agardh have been studied.

Materials and Methods

Collection of material: Specimens were collected during October to December 1999 from the bottom of a shallow water pond at Hyderabad and from sandy bottom of Kinjhar and Haleji lakes of District Thatta (Sindh) at a depth of 10-40 cm.

Detection of fatty acids: The algae under investigation weighting 1 kg fresh weight (*C. contraria*) and 4811.2 g dry wt. (*N. flexilis*) were percolated with *n*-hexane: chloroform (1:1, v/v) in a respirator for two weeks. The extract so obtained was reduced under vacuum and partitioned between EtOAc and water (1:1, v/v), which on evaporation

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yielded 15 g (*C. contraria*) and 10 g (*N. flexilis*) of residue. It was saponified, refluxed, esterified, methylated and finally subjected to GC-MS, as described in Ghazala *et al*., (2004a).

Isolation of sterol & terpene: Thalli were thoroughly washed with tap water to remove debris and animal castings. Dried and chopped material weighing 650.5 g was soaked in methanol for almost three weeks. The MeOH fraction on evaporation under vacuum gave 38.4 g thick crude algal material, and the slurry was loaded on to silica gel column for chromatography eluting with *n*-hexane: chloroform and finally with methanol in increasing order of polarity by 2 % (v/v) respectively, as given by Ghazala *et al*., (2003).

Bioactivity tests: The methodologies for bactericidal activity, antifungal bioassay, food poisoning and phytotoxic activities, brine shrimp bioassay and insecticidal activity were the same as have been described previously (Ghazala *et al*., 2003, 2004a).

Antitumour activity: Potato tubers obtained from local markets were sterilized by immersion in sodium hypochlorite for 20 minutes. After removing their ends potatoes were soaked for 10 minutes or more in Clorox. A core of the tissue was extracted from each tuber with 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 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 ^{o}C for 12-20 days after which the tumours produced were counted with the 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

***Chara contraria* A. Braun *ex* Kützing:** Thallus coarse, usually heavily encrusted, 20-30 cm high; stem up to 730 µm in diameter, stem cortex regularly diplostichous; stipulates in double whorls, paired with each leave; leaves 6-8 in a whorl, incurved or spreading; terminal cell of the stem and leaves ecorticated; sex organs monoecious, both organs produced at the same node, antheridia 311-470 µm in diameter, oogonia 800 µm long, spiral cells showing 13-15 convolutions (Fig. 1).

***Nitella flexilis* (Linnaeus) C.A. Agardh:** Thallus 30-40 cm high; stem and leaves uncorticated, encrusted with lime; stem up to 0.8 mm thick; internodes 4.5-9.0 cm long; leaves up to 6 in a whorl, forked; last segment unicellular; stipule-circle lacking; antheridia borne above oogonia; crown developed on ooganium by tips of 5 two-celled coiled tubes (Fig. 2).

Detection of fatty acids: The GC-MS of the methylated fatty acids (FAs) revealed the presence of 9 saturated and 7 unsaturated FA-methyl esters in *C. contraria* and 9 saturated and 14 unsaturated FA-methyl esters in *N. flexilis*, while 4 FAs remained unidentified (Tables 1 & 2).

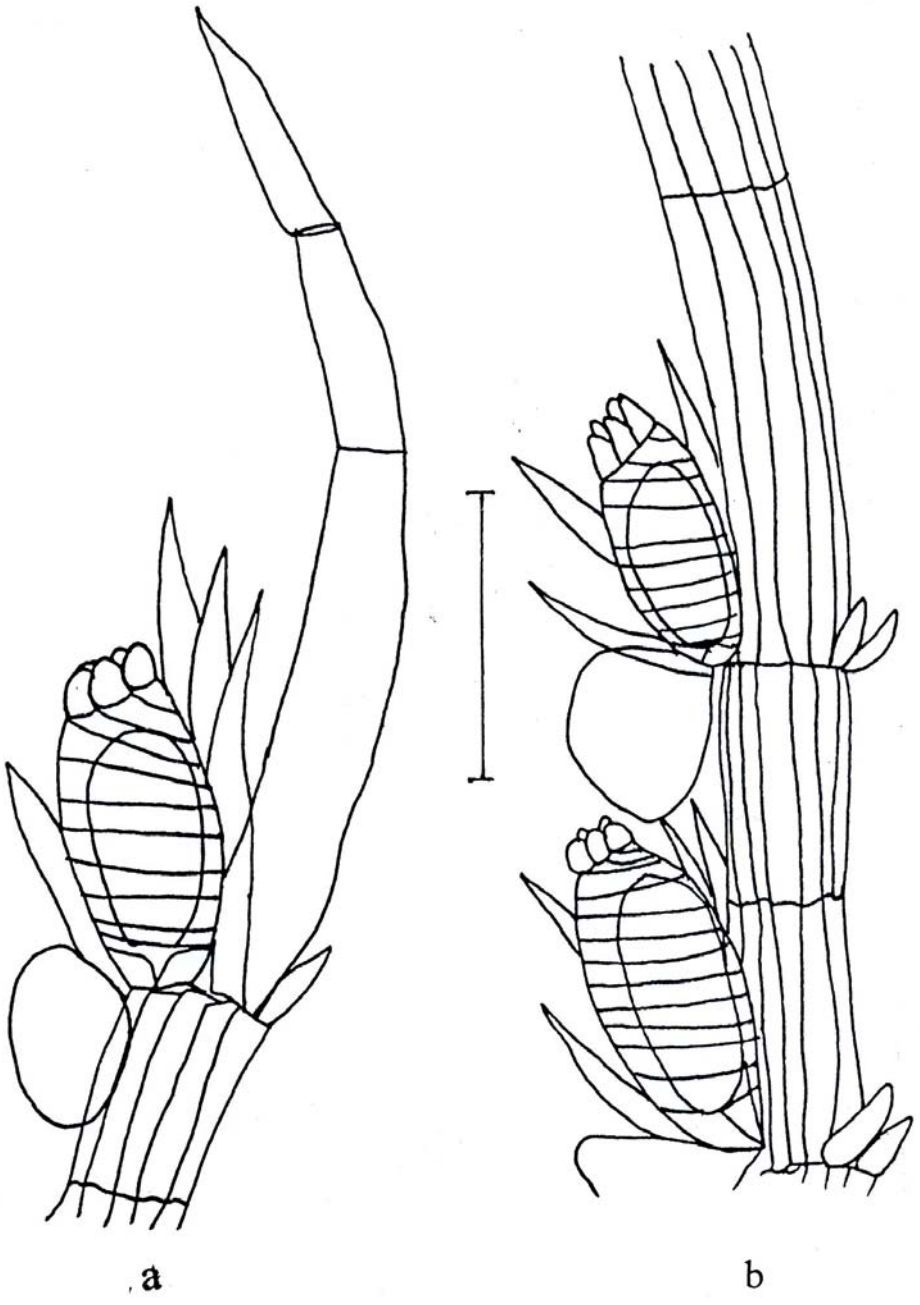

Fig. 1. *Chara contraria*: **a.** branchlet with globule and nucule, **b.** main thallus (scale: 400 μm).

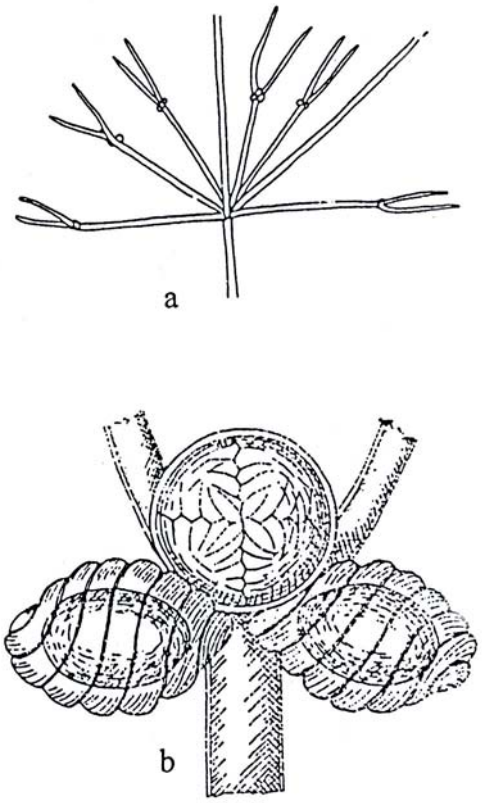

Fig. 2. *Nitella flexilis* (Linnaeus) C.A. Agardh: **a.** part of thallus showing whorl of forked leaves, **b.** reproductive organs (x 50).

Extraction of sterol and terpene: Pure sterol compound was eluted in a mixture of *n*-hexane: chloroform (90:10) from column and purified on preparative thick layer silica gel glass plates in solvent system *n*-hexane: chloroform (80:20). Purity was then checked on TLC card in the above solvent system and by spraying with Ce $(SO_4)_2$ a pinkish red spot was found. After ascertaining the purity of the compound the isolated sterol was subjected to spectral analysis (EIMS and ^{1}H-NMR). In this way β-sitosterol was identified, but *trans*-phytol could not be detected (Table 3).

Bioactivities: Methanol extract of *C. contraria* showed no bioactivity against any one of the 10 tested bacterial species but displayed a strong antifungal activity against 6 of the 10 tested fungal organisms (Table 5). It exhibited phytotoxicity against *Lemna minor* of low quality at the three concentrations and its cytotoxic activity against brine shrimp was observed to be non-significant. It revealed an appreciable antitumour activity, which enhanced from 10 to 31 % with increasing concentration of the extract. The extract of *N. flexilis* exhibited antibacterial activity against 3 of the 10 tested bacterial species and antifungal activity against 6 of the 10 tested fungal organisms (Table 3). Its cytotoxic and insecticidal activities were found to be non significant.

Table 1. Fatty acids detected as methyl esters in three fractions (A-C) of *Chara contraria* and four fractions (A-D) of *Nitella flexilis.*

Acid type	Systematic name	Common name	Molecular formula	Mol. wt.	Rel. % age
		Chara contraria			
	Fraction A				**99.754**
C14:0	*n*-Tetradecanoate	Myristate	$C_{15}H_{30}O_2$	242	2.448
C15:0	*n*-Pentadecanoate	Pentadecylate	$C_{16}H_{32}O_2$	256	1.53
C16:0	*n*-Hexadecanoate	Palmitate	$C_{17}H_{34}O_2$	270	6.12
C17:3	5,8,11-Heptadecatrienoate	-	$C_{18}H_{30}O_2$	278	38.862
C18:1	8-Octadecenoate	Oleate	$C_{19}H_{36}O_2$	296	5.506
C18:0	9-Methyl-heptadecanoate	-	$C_{19}H_{38}O_2$	298	2.448
C19:1	10-Nonadecenoate	Nonadecylenate	$C_{20}H_{38}O_2$	310	1.836
C20:1	9-Eicosenoate	Gadoleate	$C_{21}H_{40}O_2$	324	7.65
C22:0	Docosanoate	Behenate	$C_{23}H_{46}O_2$	354	3.06
C29:3	Nonacosatrienoate	-	$C_{30}H_{54}O_2$	446	7.956
-	Unidentified	-	-	330	7.65
-	Unidentified	-	-	410	9.18
-	Unidentified	-	$C_{29}H_{52}O_2$	432	5.508
	Fraction B				**99.918**
C20:1	9-Eicosenoate	Gadoleate	$C_{21}H_{40}O_2$	324	4.697
C22:1	11-Docosenoate	Cetoleate	$C_{23}H_{44}O_2$	352	14.518
C23:1	Tricosenoate	-	$C_{24}H_{46}O_2$	366	3.843
C24:0	*n*-Tetracosanoate	Lignocerate	$C_{25}H_{50}O_2$	382	17.934
C29:3	Nonacosatrinoate	-	$C_{30}H_{54}O_2$	446	53.375
C29:0	*n*-Nonacosanoate	-	$C_{30}H_{60}O_2$	452	5.551
	Fraction C				**99.735**
C17:0	*n*-Heptadecanoate	Margarate	$C_{18}H_{36}O_2$	284	9.81
C18:0	9-Methyl-heptadecanoate	-	$C_{19}H_{38}O_2$	298	17.004
C29:1	10-Nonadecenoate	Nonadecylenate	$C_{20}H_{38}O_2$	310	7.194
C21:0	*n*-Heneicosanoate	-	$C_{22}H_{44}O_2$	340	23.871
C22:1	11-Docosenoate	Cetoleate	$C_{23}H_{44}O_2$	452	41.856
		Nitella flexilis			
	Fraction A				**99.99**
C11:0	Undecanoate	Undecylate	$C_{12}H_{24}O_2$	200	0.6711
C14:1	9-Tetradecenoate	Mysistoleate	$C_{15}H_{28}O_2$	240	2.013
C15:3	3,7,11-Trimethyl-2,6,10-dodecatrienoate	-	$C_{16}H_{26}O_2$	250	10.066
C15:0	*n*-Pentadecanoate	Pentadecylate	$C_{16}H_{32}O_2$	256	6.039

Table 1 (Cont'd.)

Acid type	Systematic name	Common name	Molecular formula	Mol. wt.	Rel. % age
C16:3	Hexadecatrienoate	-	$C_{17}H_{28}O_2$	264	32.212
C17:2	9,12-Heptadecadienoate	-	$C_{18}H_{32}O_2$	280	42.279
C18:5	Octadecapentaenoate	-	$C_{19}H_{28}O_2$	288	3.355
C18:2	5,8-Octadecadienoate	-	$C_{19}H_{34}O_2$	294	3.355
	Fraction B				**99.99**
C14:1	9-Tetradecenoate	Mysistoleate	$C_{15}H_{28}O_2$	240	21.333
C14:0	*n*-Tetradecanoate	Mysistate	$C_{15}H_{30}O_2$	242	25.997
C16:0	*n*-Hexadecanoate	Palmitate	$C_{17}H_{34}O_2$	270	41.329
C18:1	8-Octadecenoate	Oleate	$C_{19}H_{36}O_2$	296	1.999
C18:0	*n*-Octadecanoate	Stearate	$C_{19}H_{38}O_2$	298	1.333
-	Unidentified	-	-	274	7.999
	Fraction C				**99.99**
C8:1	7-Octynoate	-	$C_9H_{14}O_2$	154	1.923
C13:1	9-Tridecenoate	Decylacrylate	$C_{14}H_{26}O_2$	226	59.613
C14:1	13-Tetradecynoate	-	$C_{15}H_{26}O_2$	238	1.923
C15:4	Pentadecatetraenoate	-	$C_{16}H_{24}O_2$	248	1.923
C16:1	9-Hexadecenoate	Palmitoleate	$C_{17}H_{32}O_2$	268	17.307
C18:5	Octadecapentaenoate	-	$C_{19}H_{28}O_2$	288	3.846
C18:2	5,8-Octadecadienoate	-	$C_{19}H_{34}O_2$	294	2.884
C19:1	10-Nonadecenoate	Nonadecylenate	$C_{20}H_{38}O_2$	310	1.923
-	Unidentified	-	-	190	4.807
-	Unidentified	-	-	318	3.846
	Fraction D				**99.99**
C13:1	9-Tridecenoate	Decylacrylate	$C_{14}H_{26}O_2$	226	14.691
C14:0	*n*-Tetradecanoate	Myristate	$C_{15}H_{30}O_2$	242	29.855
C15:3	3,7,11-Trimethyl-2,6,10-dodecatrienoate	-	$C_{16}H_{26}O_2$	250	12.795
C15:0	*n*-Pentadecanoate	Pentadecylate	$C_{16}H_{32}O_2$	256	3.791
C16:0	*n*-Hexadecanoate	Palmitate	$C_{17}H_{34}O_2$	270	23.695
C18:5	Octadecapentaenoate	-	$C_{19}H_{28}O_2$	288	4.739
C18:2	5,8-Octadecadienoate	-	$C_{19}H_{34}O_2$	294	3.791
C18:0	*n*-Octadecanoate	Stearate	$C_{19}H_{38}O_2$	298	3.317
C22:4	6, 9,12,15-Docosatetraenoate	-	$C_{23}H_{38}O_2$	346	3.317

Mol. wt. = Molecular weight, Rel. % age = Relative percentage.

Table 2. Total fatty acids analysed as methyl esters present in the methanol extracts of *Chara contraria* and *Nitella flexilis*.

Acid type	Systematic name	Molecular formula	Mol. wt.	Rel. % age	
				C.C.	N.F.
I. Saturated fatty acids				**43.829**	**34.005**
C11:0	*n*-Undecanoate	C12H2402	200	-	0.167
C14:0	*n*-Tetradecanoate	$C_{15}H_{30}O_2$	242	0.815	13.963
C15:0	*n*-Pentadecanoate	$C_{16}H_{32}O_2$	256	0.509	2.457
C16:0	*n*-Hexadecanoate	$C_{17}H_{34}O_2$	270	2.037	16.256
C17:0	*n*-Heptadecanoate	$C_{18}H_{36}O_2$	284	3.266	-
C18:0	9-Methyl-heptadecanoate	$C_{19}H_{38}O_2$	298	6.477	1.162
C21:0	*n*-Heneicosanoate	$C_{22}H_{44}O_2$	340	7.949	-
C22:0	*n*-Docosanoate	$C_{23}H_{46}O_2$	354	1.018	-
C24:0	*n*-Tetracosanoate	$C_{25}H_{50}O_2$	382	5.972	-
C29:0	*n*-Nonacosanoate	$C_{30}H_{60}O_2$	452	15.786	-
II. Monounsaturated fatty acids				**15.063**	**30.677**
a. Monoenoic fatty acids				**15.063**	**29.711**
C13:1	9-Tridecenoate	$C_{14}H_{26}O_2$	226	-	18.57
C14:1	9-Tetradecenoate	$C_{15}H_{28}O_2$	240	-	5.836
C16:1	9-Hexadecenoate	$C_{17}H_{32}O_2$	268	-	4.326
C18:1	8-Octadecenoate	$C_{19}H_{36}O_2$	296	1.833	0.499
C19:1	10-Nonadecenoate	$C_{20}H_{38}O_2$	310	3.006	0.480
C20:1	9-Eicosenoate	$C_{21}H_{40}O_2$	324	4.111	-
C22:1	11-Docosenoate	$C_{23}H_{44}O_2$	352	4.834	-
C23:1	Tricosenoate	$C_{24}H_{46}O_2$	366	1.279	-
b. Monoynoic fatty acids				-	**0.96**
C8:1	7-Octynoate	$C_9H_{14}O_2$	1.54	-	0.480
C14:1	13-Tetradecynoate	$C_{15}H_{26}O_2$	238	-	0.480
III. Diunsaturated fatty acids				-	**13.076**
C17:2	9,12-Heptadecadienoate	$C_{18}H_{32}O_2$	280	-	10.569
C18:2	5,8-Octadecadienoate	$C_{19}H_{34}O_2$	294	-	2.507
IV. Triunsaturated fatty acids				**33.364**	**13.768**
C15:3	3,7,11-Trimethyl-2,6, 10-dodecatrienoate	$C_{16}H_{26}O_2$	250	-	5.715
C16:3	Hexadecatrienoate	$C_{17}H_{28}O_2$	264	-	8.053
C17:3	5,8,11-Heptadecatrienoate	$C_{18}H_{30}O_2$	278	12.941	-
C29:3	Nonacosatrienoate	$C_{30}H_{54}O_2$	446	20.423	-
V. Polyunsaturated fatty acids				-	**4.294**
C15:4	Pentadecatetraenoate	$C_{16}H_{24}O_2$	248	-	0.480
C18:5	Octadecapentaenoate	$C_{19}H_{28}O_2$	288	-	2.985
C22:4	6,9,12,15-Docosatetraenoate	$C_{23}H_{38}O_2$	346	-	0.829
IV. Unidentified fatty acids				**7.437**	**4.161**
-	-	-	190	-	1.201
-	-	-	274	-	1.999
-	-	-	318	-	0.961
-	-	-	-	7.437	-

Mol. wt. = Molecular weight, Rel. % age = Relative percentage, C.C. = *Chara contraria*, N.F. = *Nitella flexilis.*

Table 3. Comparison of phycochemistry and bioactivity of stonewort algae.

Compounds/ Organisms	***Chara contraria***	***Nitella flexilis***
I. Natural products	**%**	**%**
B-Sitosterol	-	+
Trans-phytol	-	-
II. Antibacterial activity	**mm**	**mm**
Bacillus cereus	-	6
Corynebacterium diphtheriae	-	†6.5
Escherichia coli	-	-
Klebsiella pneumoniae	-	-
Proteus mirabilis	-	-
Pseudomonas aeruginosa	-	-
Salmonella typhi	-	-
Shigella boydii	-	†8
Staphylococcus aureus	-	-
Streptococcus pyogenes	-	-
III. Antifungal activity	**%**	**%**
Alternaria alternata	-	-
Curvularia lunata	2.9	3.0
Drechslera australiensis	-	-
Fusarium solani	-	-
Fusarium sporotrichoids	3.2	3.5
Fusarium proliferatum	-	-
Macrophomina phaseolina	3.5	3.7
Rhizoctonia solani	3.5	3.6
Sclerotium rolfsii	3.6	3.4
Trichoderma harzianum	3.3	3.0
IV. Phytotoxicity	**%**	**%**
500 µg/mL concentration	6.66	X
V. Brine shrimp bioassay		
1000 µg/mL LD_{50}	-	-
VI. Insecticidal activity		
Tribolium castaneum	x	-
VII. Antitumour activity	**%**	**%**
100 µg	31	x

+ = Present, - = Absent /no activity, x = Not tested, †= Decrease in bacterial population/ unit area.

Discussion

The methanol extract of *C. contraria* revealed the presence of 9 saturated and 7 unsaturated FAs, while 1 FA could not be identified (Table 2). Nonacosatrienoic acid (C29:3) was found to be in largest amount (20.423 %) as compared to all the other FAs, so much so that it appeared to be the most dominant acid, while pentadecylic acid (C15:0) was present in the smallest amount (0.51 %). Other prevalent acids were nonacosanoic (C29:0, 15.786 %) and 5, 8, 11-heptadecatrienoic (C17:3, 12.94 %) acids. Unsaturated FAs were found to be in slightly larger quantity (48.427 %) than saturated FAs (43.829 %), while a proportion of 7.437 % remained unidentified. In a previous study a total number of 23 FAs were detected in *C. corallina* var. *wallichii* including a wide variety of saturated (36.5 %) and unsaturated (63.5 %) FAs and hexadecadienoic acid occurred in greatest amount (23.7 %), while pentadecylic (10.61 %) and palmitic (17.69 %) acids were the major SFAs (Khaliq-uz-Zaman *et al.,* 1998). In this way the two species of *Chara* differed from each other.

β-Sitosterol was isolated from *C. contraria*. It has also been obtained from the MeOH extract of *C. wallichii* in a dominant proportion (76.8 %) apart from three other sterols *i.e.* cholesterol, clerosterol and stigmasterol which occurred in small quantities (Shameel *et al.,* 1999). Sitosterol and fucosterol were detected as principal sterols in *N. opaca* (Heilbron, 1942); while clionasterol, an isomer of sitosterol and 28-isofucosterol were found to be the major sterols of *C. vulgaris* and *N. flexilis* (Patterson, 1972). In *C. australis* and *C. buckelii* also sitosterol was observed to be present along with several other sterols (Patterson *et al.,* 1991). It appears that β-sitosterol is the major sterol of *Chara*.

The extract of *C. contraria* exhibited a variable amount of bioactivity against a variety of tests (Table 3). A strong antibacterial, antifungal and phytotoxic activities were also noted in the MeOH, EtOAc-soluble part of MeOH extract and 4 isolated sterols from *C. wallichii* against a variety of test organisms (Khaliq-uz-Zaman *et al.,* 2001). Functional derivaties of 2-(methylthio) propane-1,3-dithiol, extracted from *C. globularis*, were found to possess insecticidal properties (Jacobsen & Pedersen, 1983). Subsequently *C. aspera* was observed to accumulate benzo[a]pyrene, a carcinogenic compound in its thallus (Isha *et al.,* 1987).

The methanol extract of *N. flexilis* revealed the presence of 5 saturated and 14 unsaturated FAs (Table 2). The former were present in smaller amount (34.005 %) than the latter (61.82 %). Similar observations were made on *C. corallina* var. *wallichii* (Khaliq-uz-Zaman *et al.,* 1998) and several other freshwater green algae of Sindh (Ghazala *et al*., 2004a-d). Tridecenoic acid (C13:1) was found to be in largest quantity (18.576 %) and undecanoic acid (C11:0) occurred in the smallest amount (0.167 %). The unsaturated acids included 7 mono-, 2 di-, 2 tri- and 3 poly-unsaturated FAs. The monounsaturated acids also included 2 monoyenoic acids (C8:1 and C14:1) having one triple bond. The polyunsaturated acids were having 4-5 double bonds. Such acids were not detected in the previous studies on *Chara* (Khaliq-uz-Zaman *et al.,* 1998).

From sterol and terpene fractions of the methanol extract of *N. flexilis* no natural product could be detected. A variety of sterols have been isolated previously from *C. corallina* var. *wallichii* (Khaliq-uz-Zaman *et al.,* 1998). The crude extract of *N. flexilis* showed a strong antifungal activity and a slight antibacterial activity. It did not display any significant cytotoxic and insecticidal activity (Table 3). It was observed that the

activity of thiol containing enzymes of *Nitella* is influenced by *p*-benzoquinone and pyrocatechol (Ivanova & Stom, 1980). *Nitella* spp., have the enzymatic machinary to decompose pyrocatechol, hydroquinone, phenol, *p*-cresol, guaiacol and other benzene derivatives (Stom *et al.,* 1979). The MeOH extract, its EtOAc-soluble part and four sterols isolated from *C. wallichii* showed bioactivity against 10 species of bacteria and 10 of fungi and its phytotoxicity against *Lemna minor* (Khaliq-uz-Zaman *et al.,* 2001).

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