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STUDIES ON THE PHYCOCHEMISTRY AND BIOLOGICAL ACTIVITY OF *SPIROGYRA RHIZOIDES* (CHLOROPHYCOTA)

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Abstract

The unbranched filaments of a grass green macroalga, *Spirogyra rhizoides* Randhawa were collected from freshwater habitats at Nai Baran in the Sindh Province of Pakistan and extracted in methanol. The extract revealed the presence of 22 different fatty acids, including 11 saturated and 11 unsaturated acids by GLC and GC-MS. The former acids were slightly larger in proportion (52.48%) than latter ones (47.49%). Margaric (10.33%), parinaric (11.34%) and oleic (12.86%) acids were found in highest proportion, while palmitic acid occurred in small proportion (4.84%). Furthermore, two sterols (isodecortinol & dinosterol), one monoterpene (9-hydroxygeraniol), one sesquiterpene (isoafracinol), one triterpene (30-*nor*-cyclopterospermone) and a polysaccharide (xylasmacin) were also obtained from the extract by (EI, FAB, FD & HR)-MS and (^{1}H & ^{13}C)-NMR spectroscopic techniques. The extract showed strong antimicrobial activity against 14 bacterial and 20 fungal species, including 7 human-, 5 plant- pathogens and 8 saprophytes.

Introduction

Spirogyra rhizoides Randhawa is a filamentous green macroalga of the family Zygnemaceae order Zygnemales, class Zygnemophyceae, phylum Chlorophycota; (Shameel, 2001, 2008). It commonly occurs in the freshwaters of Pakistan. Irrespective of several studies made on the taxonomy of various species of *Spirogyra* Link growing in Pakistan (Masud-ul-Hasan, 1978; Masud-ul-Hasan & Yunus, 1989; Shahida *et al*., 2005; Gul *et al*., 2007, Husna *et al*., 2007, Sarim *et al*., 2007; Zarina *et al*., 2007; Shahnaz *et al*., 2008; Ghazala *et al*., 2009; Masud-ul-Hasan *et al*., 2010 etc.), only a few investigations were made on their phycochemistry (Ghazala *et al*., 2005; Valeem & Shameel, 2006; Aftab & Shameel, 2009). The present study was undertaken to investigate the phycochemistry and biological activity of *S. rhizoids*.

Materials and Methods

Spirogyra rhizoides commonly occurs in ponds, pools and small streams at Nai Baran, near Thatta Road Bridge., it was collected being attached with rhizoids to the stones embedded in the mud in the slow running water at Nai Baran (with rain water) on the National Highway, Hyderabad to Thatta. The methods used for the extraction of algal specimens and the saponification, esterification and identification of the fatty acids, as well as the purification and chemical elucidation of the isolated natural products by GLC, GC-MS, (EI, FAB, FD & HR) –MS and (^{1}H & ^{13}C)-NMR spectroscopic techniques from the extract were the same as described recently (Khalid *et al*., 2010). The procedures for different tests conducted for the biological activity have also been described there in detail.

Results

Detection of fatty acids: Three fractions obtained from column chromatography of the extract of *S. rhizoides* were analysed for fatty acids, where fraction A was eluted from column in *n*-hexane (100), fraction B in *n*-hexane: chloroform (95:05), fraction C in *n*-hexane: chloroform (90:10). All of them were methylated by diazomethane and analysed initially by GLC and finally by GC-MS. Identification of the individual fatty acids was carried out by matching their mass spectra with the NBS mass spectral library (Helles & Milne, 1978). As a result of that 22 different fatty acids were detected, including 11 saturated and 11 unsaturated acids (Table 1).

Extraction of sterols: Two sterols were identified from the fractions eluted from the silica gel column, where compound 1 was eluted in mixture form in *n*-hexane: chloroform (60:40) from column and purified on preparative thick layer silica gel glass plates in solvent system of *n*-hexane: chloroform (1:1). Its purity was checked on TLC card in the solvent system of *n*-hexane: chloroform (60:40) and after spraying with Ce $(SO_4)_2$ a pure purple spot was obtained. After using various spectroscopic methods it was identified as isodecortinol. The compound 2 was purified and eluted from column in *n*-hexane: chloroform (70:30). It was further purified on preparative silica gel glass plates in solvent system of *n*-hexane: chloroform (60:40). Its purity was then checked on TLC card in the solvent system of *n*-hexane: chloroform (60:40), and a red spot was found after spraying with Ce $(SO_4)_2$. After using various types of spectroscopy it was identified as dinosterol.

Some physical properties of the identified sterols are shown in the Table 1. The following spectral data were obtained, on the basis of which these compounds were identified:

Isodecortinol (Fig. 1[1]): IR ($CHCl_3$) ν_{max}: 3460, 3050, 1650, 890 cm^{-1}. **FD-MS** (rel. int.): *m/z* 428 $[M]^+$ (100). **EI-MS** (rel. int.): *m/z* 428 (8), 410 (70), 395 (5), 392 (4), 344 (2), 326 (3), 285 (4), 269 (4), 227 (3), 211 (4), 175 (10), 161 (22), 135 (24), 107 (30), 95 (44), 81 (58), 69 (55), 55 (100). **HR-MS** (rel. int. %): *m/z* 428.3662, $C_{29}H_{48}O_2$ $[M]^+$, 410.3581 $C_{29}H_{46}O$ $[M\text{-}H_2O]^+$, 395.3396, $C_{28}H_{43}O$ $[M\text{-}H_2O\text{-}Me]^+$, 392.9759, $C_{29}H_{44}$ $[M\text{-}H_2O]^+$, 344.2682, $C_{23}H_{36}O_2$ $[M\text{-}C_6H_{12}]^+$, 288.2041, $C_{19}H_{28}O_2$ [M-side chain 2H]$^+$, 133, 119, 107, 105, 93, 81, 69, 55. **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ 5.28 (1H, t, J=2.12 Hz, H-6), 4.71 (1H, br.s, H-26), 3.83 (1H, dt, J=7.8, 1.8 Hz, H-7), 3.54 (1H, m, H-3), 1.55 (3H, s, H-27), 1.04 (3H, s, H-19), 0.91 (3H, d, J=6.6 Hz, H-21), 0.87 (3H, t, J=6.7 Hz, H-29), 0.67 (3H, s, H-18) ppm. **^{13}C-NMR** ($CDCl_3$, 75 MHz, δ ppm): see Table 3.

Table 1. Fatty acids detected in the methanol extract of *Spirogyra rhizoides.*

Systematic name	Common name	Mol. formula	Mol. wt.	Rel. % age
Saturated acids:				52.48
n-Heptanoic	Heptylic	$C_7H_{14}O_2$	130	2.41
n-Tridecanoic	Tridecylic	$C_{13}H_{26}O_2$	214	2.56
n-Tetradecanoic	Myristic	$C_{14}H_{28}O_2$	228	4.87
n-Pentadecanoic	Pentadecylic	$C_{15}H_{30}O_2$	242	6.56
n-Hexadecanoic	Palmitic	$C_{16}H_{32}O_2$	256	4.84
n-Heptadecanoic	Margaric	$C_{17}H_{34}O_2$	270	10.33
n-Octadecanoic	Stearic	$C_{18}H_{36}O_2$	284	4.14
n-Docosanoic	Behenic	$C_{22}H_{44}O_2$	340	5.32
n-Tetracosanoic	Lignoceric	$C_{24}H_{48}O_2$	368	1.50
n-Pentacosanoic	Pentacosoic	$C_{25}H_{50}O_2$	382	4.13
Tridecatrienoic	—	$C_{13}H_{20}O_2$	208	4.81
n-Hexacosanoic	Cerotic	$C_{26}H_{52}O_2$	396	5.82
Unsaturated acids:				**47.49**
9-Tetradecenoic	Myristoleic	$C_{14}H_{26}O_2$	226	2.62
6,10,14-Hexadeca- trienoic	Hiragonic	$C_{16}H_{26}O_2$	250	1.05
Heptadecatrienoic	—	$C_{17}H_{28}O_2$	264	3.45
Heptadecenoic	Heptadecylenic	$C_{17}H_{32}O_2$	268	1.60
9,11,13,15-Octadeca- tetraenoic	Parinaric	$C_{18}H_{28}O_2$	276	11.34
9,12,15-Octadeca- trienoic	Linolenic	$C_{18}H_{30}O_2$	278	1.07
9,12,-Octadecadienoic	Linoleic	$C_{18}H_{32}O_2$	280	3.60
9-Octadecenoic	Oleic	$C_{18}H_{34}O_2$	282	12.86
Nonadecenoic	Nonadecylenic	$C_{19}H_{36}O_2$	296	3.85
9-Eicosenoic	Gadoleic	$C_{20}H_{38}O_2$	310	1.24

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

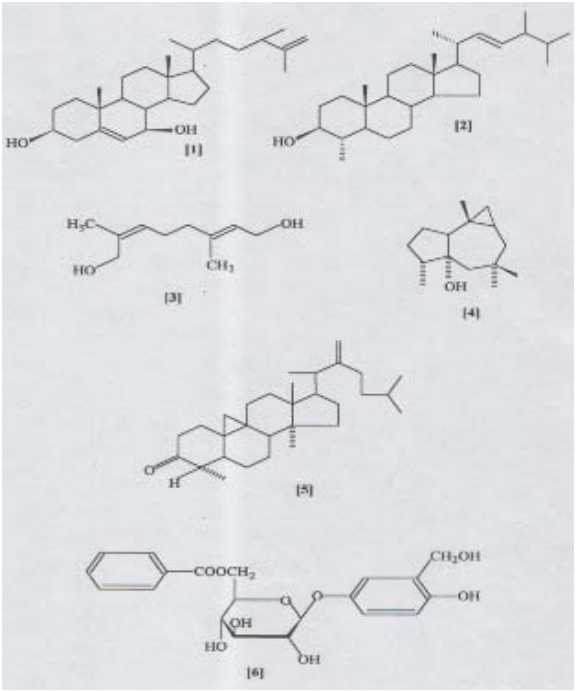

Fig. 1. Natural products isolated from *Spirogyra rhizoides*: **[1]** = Isodecortinol, **[2]** = Dinosterol, **[3]** = 9-Hydroxygeraniol, **[4]** = Isoafricinol, **[5]** = 30- *Nor*-cyclopterospermone, **[6]** = Xylasmacin.

Dinosterol (Fig. 1[2]): HR-MS (rel. int. %): *m/z* $C_{30}H_{50}O$ (calcd. 428.4041; found 428.4054), 316 (88), 287 (100), 271 (64). **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ 0.70 (C_3H, s), 0.80 (3H, d, J=7 Hz0, 0.84 (3H, s), 0.85 (3H, d, J=7 Hz), 0.94 (6H, d, J=6.5 Hz, isopropyl), 0.95 (3H, d, J=6 Hz), 4.87 (1H, g, J=1.2, 10 Hz), 3.10 (1H, m) ppm. Decoupling study showed the presence of a partial structure –CHCH=C (CH_3)– which seemed to be located in the side chain leaving a few possibilities.

Isolation of a monoterpene: A monoterpene was identified from the fractions eluted from the silica gel column. It was eluted in pure form in solvent system *n*-hexane: chloroform (80:20). Its purity was checked on TLC card in solvent system *n*-hexane: chloroform (70:30), and by spraying with Ce $(SO_4)_2$ and on heating it produced a single red spot. After using different spectroscopic techniques it was identified as 9-hydroxygeraniol. Some of its physical properties are given in the Table 2. The following spectral data were obtained, on the basis of which it was identified:

9-Hydroxygeraniol (Fig. 1[3]): Colourless gum; **EI-MS** (rel. int. %): *m/z* 170.132 (15, $M)^+$ (calcd. for $C_{10}H_{18}O_2$: 170, 132), 152 (23, $M\text{-}H_2O)^+$, 139 (44, $M\text{-}CH_2OH)^+$, 121 (46), 119 (43), 109 (62), 95 (40), 93 (46), 91 (40), 85 (48), 83 (52), 81 (49), 70 (51), 69 (100), 67 (55), 56 (40), 55 (63). **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ C_1=4.15 (br.d), C_2=5.63 (br.t), C_4=2.06, C_5=2.03 (br.t), C_6=5.05 (br.t), C_8=4.02 (br.s), C_9=1.65 (br.s), C_{10}=1.55 (br.s) ppm.

Table 2. Natural products obtained from methanol extract *of Spirogyra rhizoides.*

Str. No.	Common name	Mol. formula	Mol. wt.	Mel. Pt	$[\alpha]_d$ ($CHCl_3$)
	Sterols:				
1.	Isodecortinol	$C_{29}H_{48}O_2$	428		
2.	Dinosterol	$C_{30}H_{50}O$	428	220-222°	5°
	Terpenes:				
3.	9-Hydroxygeraniol	$C_{10}H_{18}O_2$	170		
4.	Isoafracinol	$C_{15}H_{26}O$	222	-	13.3°
5.	30-*Nor*-cycloptero-spermone	$C_{30}H_{48}O$	424		
	Carbohydrate:				
	Xylasmacin	$C_{20}H_{22}O_9$	406	149-151°	-30°

Str. No. = Structure number in Fig. 14, **Mol. Wt.** = molecular weight, **Mel. Pt.** = melting point

Table 3. ^{13}C-NMR chemical shifts of the compounds obtained from methanol extract of *Spirogyra rhizoides.*

Carbon No.	Isodecortinol	Isoafracinol	Carbon No	Isodecortinol isodecortinol isodecortinol
1.	36.8	53.9	16	29.4
2.	31.5	18.7	17	55.3
3.	71.4	22.7	18	11.7
4.	41.6	20.9	19	19.1
5.	143.4	41.0	20	35.4
6.	125.3	34.0	21	18.6
7.	73.3	46.4	22	33.6
8.	40.8	85.7	23	29.6
9.	48.2	85.7	24	33.6
10.	36.2	31.3	25	17.7
11.	21.0	23.5	26	147.6
12.	39.52	2.1	27	111.3
13.	42.83	1.1	28	26.3
14.	55.93	1.6	29	11.9
15.	26.41	2.3		

The compound was heated with Ac_2O at 80° C for 2.5 h to give its diacetate which is colorless oil. **IR** (CCl_4) ν_{max}: 1750, 1250 (Oac), 160 (C=C). **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ C_1=4.62 (br.d), C_2=5.62 (br.t), C_4=2.13 (br.t), C_5=2.08 (br.t), C_6=5.08, C_8=4.53 (br.s), C_9=1.67 (br.s), C_{10}=1.58 (br.s) ppm.

Separation of a sesquiterpene: A sesquiterpene was purified and eluted from column in *n*-hexane:chloroform (70:30). It was further purified on preparative silica gel glass plates in solvent system of *n*-hexane: chloroform (60:40). Its purity was then checked on TLC card in the solvent system of *n*-hexane:chloroform (60:40), and a purplish spot was observed after spraying with $Ce(SO_4)_2$. After using various types of spectroscopy it was identified as isoafricanol. Some of its physico-chemical properties are shown in the Table 2. The following spectral data were obtained, on the basis of which it was identified:

Isoafracinol (Fig. 1[4]): EI-MS (rel. int. %): *m/z* 222.197272 (39.2) $[M]^+$ $C_{15}H_{26}O$, 207 (29.7), 204 (48.0), 189 (33.6), 165 (39.5), 125 (37.9), 109 (61.4), 98 (100), 83 (69.8). **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ 1.26 (1H, dd, J=12.5 Hz, H-3α), 1.09 (3H, s, H-13), 0.96 (3H, s, H-14), 0.90 (3H, s, H-12), 0.88 (3H, d, J=6.7 Hz, H-15), 0.66 (1H, ddd, H-4), 0.38 (1H, dd, J=4.1, 8.3 Hz, H-3β), 0.12 (1H, dd, J=4.5 Hz, H-3α) ppm. **^{13}C-NMR** ($CDCl_3$, 75 MHz, δ ppm): see Table 3.

Detection of a triterpene: A triterpene was purified and eluted from column in *n*-hexane:chloroform (30:70). It was further purified on preparative silica gel glass plates in solvent system of *n*-hexane:chloroform (20:80). Its purity was then checked on TLC card in the solvent system *n*-hexane:chloroform (10:90) and a purplish spot was observed after spraying with $Ce(SO_4)_2$. After using various types of spectroscopy it was identified as 30-*nor*-cyclopterospermone. Some of its physical properties are given in the Table 2. The following spectral data were obtained, on the basis of which it was identified:

30-*Nor*-cyclopterospermone (Fig. 1[5]): EI-MS: Characteristic mass spectral fragments: M^+=424 (62), Ion.a=299 (53), Ion.b=300 (27), Ion.c=175 (50). **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ 0.33 (1H), 0.58 (1H), 0.88, 0.96, 1.02 (18H, 6Me), 4.60 (2H, 7) ppm.

Extraction of a polysaccharide: The residue from pooled fractions eluted with chloroform:methanol (95:5), was crystallized and recrystallized from methanol to afford fine white needle like crystals. Its purity was checked on TLC card in solvent system chloroform:methanol:water (4:6:0.5), by spraying with $Ce(SO_4)_2$, and on heating it produced a single dark purple spot. After using different spectroscopic techniques it was identified as xylasmacin. Some of its physico-chemical properties are shown in the Table 2. The following spectral data were obtained, on the basis of which it was identified:

Xylasmacin (Fig. 14[6]): UV (MeOH) λ_{max}: 227.5 (log ε 4.31), 275 (3.50), & 282.5 (3.56); (0.1N NaOH) 227.5 (log ε 4.39), 273 (3.75), 280 (3.75) & 305 (3.77). **IR** (KBr) ν_{max}: 3600 (s) (br), 2910 (w), 2890 (w), 1730 (s0, 1610 (s), 1590 (m), 1500 (s), 1455 (s), 1280 (s), 1120 (s), 1075 (s), 860 (m) & 710 (s) cm^{-1}. **EI-MS** (rel. int. %): *m/z* 406 (M^+, 1), 267 (3), 249 (3), 140 (59), 123 (29), 122 (100), 105 (52), & 77 (17). **^{1}H-NMR** ($CDCl_3$/DMSO-d, 300 MHz): δ 4.33 (2H, s, $ArCH_2O$), 5.02 (1H, d, J=8.4 Hz, anomeric H), 6.52 (1H, dd, J=8.7 & 3 Hz, H-4), 6.80 (1H, d, J=3 Hz, H-5), 6.93 (1H, d, J=8.7 Hz, H-3) & 7.44-8.17 (5H, m). **HR-MS** (rel. int. %): *m/z* obsd. 406.3951, calcd. for $C_{20}H_{22}O_9$ 406.3929.

Biological activities: The crude methanol extract indicated a strong antibacterial activity against all of the 14 tested bacterial organisms (Table 4) and showed strong antifungal activity against all 20 tested fungal species including 7 human pathogens, 5 plant pathogens and 8 saprophytes (Table 5). Therefore, methanol extract of *Spirogyra rhizoides* revealed very promising results of antimicrobial activities.

Table 4. Antibacterial activity shown by the methanol extract of *Spirogyra rhizoides*.

Bacterial culture	Zone of inhibition (mm)	Reference drugs	Zone of inhibition (mm)
Bacillus cereus	8	Amoxicillin $(H_2O)_3$	19
		Ampicilln $(H_2O)_3$	19
Corynebacterium diphtheriae	18	Amoxicillin $(H_2O)_3$	-
		Ampicillin $(H_2O)_3$	16
Escherichia coli	12	Ainoxicillin $(H_2O)_3$	12
		Ampicillin $(H_2O)_3$	14
Klebsiella pneumoniae	12	Amoxicillin $(H_2O)_3$	-
		Ampicillin $(H_2O)_3$	9
Listeria monocytogenes	19	Amoxicillin $(H_2O)_3$	12
		Ampicillin $(H_2O)_3$	12
Proteus mirabilis	23	Amoxicillin $(H_2O)_3$	20
		Ampicillin $(H_2O)_3$	20
Proteus valgaris	9	Amoxicillin $(H_2O)_3$	10
		Ampicillin $(H_2O)_3$	10
Pseudomonas aeruginosa	17	Amoxicillin $(H_2O)_3$	-
		Ampicillin $(H_2O)_3$	12
Salmonella typhi	21	Amoxicillin $(H_2O)_3$	20
		Ampicillin $(H_2O)_3$	21
Shigella boydii	25	Amoxicillin $(H_2O)_3$	21
		Ampicillin $(H_2O)_3$	22
Staphylococcus aureus	19	Amoxicillin $(H_2O)_3$	22
		Ampicillin $(H_2O)_3$	22
Streptococcus faecalis	28	Amoxicillin $(H_2O)_3$	17
		Ampicillin $(H_2O)_3$	20
Streptococcus pyogenes	9	Amoxicillin $(H_2O)_3$	11
		Ampicillin $(H_2O)_3$	11
Vibrio choleriae	14	Amoxicillin $(H_2O)_3$	11
		Ampicillin $(H_2O)_3$	11

- = Not tested

Discussion

The methanol extract of Spirogyra *rhizoides* yielded 22 fatty acids (FAs), out of which 11 were SFAs and 11 UFAs (Table 1). Among them, SFAs were present in a slightly large amount (52.48%) than UFAs (47.49%). Among them C17:0, C 18:1 and C 18:4 acids were present in the dominating amount (10.33-12.86%). Similar results were also shown by the species of *Spirogyra* collected from estuarine environment at Miani Hor (Aftab & Shameel, 2009) indicating that species may occur in the freshwater or estuarine environment but behave similarly. Although oleic acid was present in appreciable proportion (12.86%) but it contained very small quantity (4.84%) of palmitic acid. Usually the green seaweeds from the coast of Karachi as well as freshwater green algae of Sindh contained these acids in highest perpotion (Qasim, 1986; Shameel, 1990, 1993; Aliya & Shameel, 1999; Ghazala & Shameel, 2005). These acids were also found in overwhelming quantity in different species of *Spirogyra* collected from freshwater habitats of Sofia, Bulgaria (Ivanova *et al*., 2002).

Table 5. Antifungal activity exhibited by the methanol extract of *Spirogyra rhizoids*.

Fungal culture	Colony sample	Diam. (mm) control	Inhibition %	MIC µg/mL miconazole	Ketoconazole
Human pathogens:					
Allescheria boydii	12	82	90.24	0.05	0.1-4
Candida albicans	45	95	90.52	0.1-2.0	0.1-8.0
Epidermophyton floccosum	23	108	89.81	0.5-1.0	0.1-8.0
Microsporum canis	07	53	75.47	0.5-10	0.05-12.8
Trichophyton longifusus	10	35	45.71	2.54	5.20
Trichophyton mentagrophytes	09	102	42.15	2.59	5-19
Trichophyton semii	09	95	92.63	2.59	5.19
Plant pathogens:					
Fusarium oxysporm	14	89	88.76	-	-
Macrophomina phaseolina	17	98	85.71	-	-
Pythium aphanidermatum	17	58	72.41	-	-
Pythium oedochilum	21	47	38.29	-	-
Rhizoctonia solani	19	66	72.72	-	-
Saprophytes:					
Aspergillus flevus	19	98	76.53	-	-
Drechslera rostrata	17	60	70.00	0.3	0.3
Gliocladium virens	11	102	88.23	-	-
Nigrospora oryzae	20	65	81.53	0.3	0.3
Paecilomyces lilacinus	19	86	80.23	-	-
Stachybotrys atra	12	84	67.85	0.3	0.3
Trichoderma hamatum	18	60	73.33	-	-
Trichoderma harzianum	23	104	82.69	-	-

MIC = Minimum inhibitory concentration of standard drugs, - = Not tested

Spirogyra is a commonly occurring filamentous alga in the freshwater habitats of Sindh, Pakistan. Its investigated species indicated the presence of two sterols, one monoterpene, one sesquiterpene, one triterpene and one carbohydrate in its methanol extract. A variety of sterols and monosaccharides like rhamnose, arabinose, xylose and galactoose *etc.* have bean detected in several species of *Spirogyra* collected from lakes and ponds near Sofia, Bulgaria (Mitova *et al*., 1999). This indicates that the species of *Spirogyra* are very rich in the contents of a variety of natural products.

In the ponds and lakes, the species of *Spirogyra* provide a good feeding material for the herbivorous fishes and simultaneously clean the water reservoirs by their antimicrobial activities. That is why the species, studied here, exhibited quite promising results in its bioactivity tests. Similar results have also been obtained about the bioactivity of the species of *Spirogyra* collected from the estuarine environment of Miani Hor (Aftab & Shameel, 2009). Previously, different species of *Spirogyra* were found to show promising results in their tests of antibacterial activity (Yamaguchi & Yamazaki, 1999), general bioassay (Li *et al*., 2002) and allelopathic activity (Mohammed, 2002) *etc*. This justifies their luxuriant growth in the freshwater and estuarine environments.

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