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Original Article

The Bioactivity and Phycochemistry of Two Species of *Cladophora* (Siphonocladophyceae) from Sindh

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Abstract: The coenocytic filaments of two green algae, *Cladophora glomerata* (Linnaeus) KÜtzing and *C. okamurai* (Ueda) van den Hoek from freshwater habitats of Sindh Province of Pakistan and extracted in methanol. Their crude extracts showed strong antimicrobial activity against 14 bacterial and 20 fungal species including 7 human-, 5 plant- pathogens and 8 saprophytes, but their cytotoxic activity against brine shrimp larvae was non-significant. Methanol extracts of the two species indicated the presence of 38 different fatty acids including 15 saturated and 23 unsaturated acids, while acids of both the categories were in almost equal proportion (46.5-51.9 % saturated, 48.1-53.5 % unsaturated). Furthermore 4 sterols, 4 terpenes, 2 carbohydrates and 1 glycoside were also obtained from these extracts. Both the species differed in the composition of their natural products.

Keywords*:* Algae, *Chlorophycota*, *Cladophora,* phycochemistry, fatty acids, sterols**,** terpenes, carbohydrates

1. INTRODUCTION

Cladophora KÜtzing, *nom. cons.* is a thick-walked, coenocytic, partly siphonaceous and branched green macroalga of the family Cladohporaceae, (order Cladophorales, class siphonocladophyceae, phylum chlorophycota; *fide* [1, 2]). Its species are found in streams and lakes worldwide. They occur both in the freshwater and marine environments of Sindh [3]. Irrespective of several studies made on the taxonomy of its various species [4], only a few investigations were made on their bioactivity and phycochemistry [5, 6]. Therefore, the present study was undertaken to investigate two commonly occurring species of *Cladophora* of this region for this purpose.

2. MATERIALS AND METHODS

Cladophora glomerata (Linnaeus) KÜtzing 1843: 266 is commonly found in slow running water and water-falls being attached with stones, on walls, grasses, aquatic plants and pieces of wood. Specimens were collected at Jamshoro (Sindh, Pakistan) from a pond (waste of Thermal Power and Sandoze Industry). It was found showing luxurious growth at the rocky bottom of the pond. This species usually occurs throughout the year. While the specimens of *Cladophora okamurai* (Ueda 1932) van den Hock 1963 were collected from bottom of the water ponds at Gadap and Kotri, and brought to the laboratory for investigation. The methods used for the extraction of algal material and different tests conducted for the bioactivity of crude extracts were the same as described recently [7]. The procedure adopted for the saponifacation, 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

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techniques from algal extracts have already been mentioned earlier in detail [8].

3. RESULTS AND DISCUSSION

3.1. Biological Activities

The methanol extracts of the two species of *Cladophora* showed a strong antibacterial activity against all 14 tested bacterial organisms and exhibited strong antifungal activity against all 20 tested fungal species including 7 human pathogens, 5 plant pathogens and 8 saprophytes (Table1). Therefore, their extracts presented very promising results of antimicrobial activities, but their cytotoxic activity against brine shrimp larvae was non-significant (Table 1), and both the species behaved similarly in this regards. A verity of cytokinins, zeatins, isopentanyladenine derivatives, dihyfrozeations, benz The extracts of both the species showed very promising results of antimicrobial and cytotoxic ortho- and meta-topolin derivatives have been detected in

Table 1. Antimicrobial activity in the methanol extracts of two investigated species of *Clodophora*.

No.	Organism	*C. glomerata*	*C. okamurai*
Antibacterial Activity:		**mm**	**mm**
1.	*Bacillus cereus*	22	18
2.	*Corynebacterium diphtheriae*	17	15
3.	*Escherichia coli*	09	16
4	*Klebsiella pneumoniae*	06	12
5.	*Listeria monocytogenes*	14	16
6.	*Proteus mirabilis*	22	20
7.	*Proteus valgaris*	13	18
8.	*Pseudomonas aeruginosa*	17	14
9.	*Salmonella typhi*	24	19
10.	*Shigella boydii*	19	21
11.	*Staphylococcus aureus*	22	19
12.	*Streptococcus faecalis*	14	10
13.	*Streptococcus pyogenes*	21	22
14.	*Vibrio choleriae*	15	13
Antifungal Activity:		**%**	**%**
1.	*Allescheria boydii*	84.14	81.70
2.	*Candida albicans*	86.31	84.21
4.	*Microsporum canis*	75.47	75.47
5.	*Trichophyton longifusus*	62.85	65.71
6.	*Trichophyton mentagrophyte s*	88.23	86.27
7.	*Trichophyton semii*	85.26	85.26
8.	*Fusarium oxysporm*	78.65	79.77
9.	*Macrophomina phaseolina*	87.75	81.63
10.	*Pythium aphanidermatum*	72.41	70.68
11.	*Pythium oedochilum*	61.70	74.46
12.	Rhizoclonia soloni	77.27	78.78
13.	*Aspergillus flevus*	81.63	85.71
14.	*Drechslera rostrata*	80.00	76.66
15.	*Gliocladium virens*	81.37	86.27
16.	*Nigrospora oryzae*	78.46	81.53
17.	*Paecilomyces lilacinus*	79.06	86.04
18.	*Stachybotrys atra*	84.52	84.52
19.	*Trichoderma hamatum*	73.33	76.66
20.	*Trichoderma harzianum*	90.38	84.61
Cytotoxic Activity:		**µg/mL**	**µg/mL**
1.	*Artemia salina*	127	110

cladophora[a]. Distribution of growth in marine bactria was observed by the use of aqueous, ethanol and dichloromethane extracts of cladophora [10]. Its extracts were found helpful in the retardation of cardiovascular diseases and preservation of healthy cardiovascular function [11] as well as for the treatment of diabetes and diabetic complication [12]. The polysaccharides extracted from cladophora were observed to prevent dental plaque maturation and enhance nonspecific biological response [13]. Autotoxin a extracted from C. fracta was found to elicit an increase in peroxidase and glutathione 5-transferase activities in aquatic plants like lemna minor indicating a sound phytotoxicity [14]. The two investigated species presented several specific differences in their FA-compositions. In *C. glomerata* the UFAs were present in greater amount (53.46 %) than the SFAs (46.5 %), while *C. okamurai* contained SFAs in slightly grater proportion (51.88 %) than the UFAs (48.07 %). Type of MUFAs found in one species were not present in the other species. Although, polyunsaturated PUFAs up to 6 double bonds DBs were present in *C. glomerata*, no such FA was found in the other species. Similarly oleic acid was found in a small proportion (2.71 %) in *C. okamurai*, but could not be detected in other species. The C 20:0 acid was found in the highest amount (13.21 %) in *C. okamurai*, while the other species contained C 16:0 and C16:3 acids in the largest proportion (20.75-22.12 %). Similar results were shown by the species of *Cladophora* collected from estuarine and marine environments (Orhan *et al.* 2003). Although C16:0 and C18:1 acids were found in high proportions in several green seaweeds of Karachi (Shameel 1993), palmitic acid was not detected in the highest amount and was not common in *C. uncinella* [5]. Some observation have a different metabolic pattern than culture algae, cladophora is a coenocyte its marine as well as freshwater species behave similarly.

3.2. Detection of Fatty Acids (FAs)

Two fractions obtained from column chromatography of the extract of *C. glomerata* were analysed for fatty acids, where fraction A was eluted from column in *n*-hexane (100 %) and fraction B in *n*-hexane:chloroform (95:05). They were methylated by diazomethane and analyzed initially by GLC and finally by GC-MS. Identification of the individual fatty acids was carried out by matching their mass spectra with NBS mass spectral library [15]. Wherefrom 22 different fatty acids were detected, including 10 saturated and 14 unsaturated acids. While among four fractions obtained from column chromatography of the extract of *C. okamurai*, fraction A was eluted from column in pure *n*-hexane (100), fraction B in *n*-hexane:chloroform (95:05), fraction C in *n*-hexane: chloroform (90:10) and fraction D in *n*-hexane:chloroform (85:15). All of them were methylated and analysed as above. Identification of the individual fatty acids was carried out similarly which revealed 24 different fatty acids, including 10 saturated and 14 unsaturated acids (Table 2). Altogether 38 different FAs were detected including 15 saturated and 23 unsaturated acids. The UFAs contained 11 mono, 4 di, 3 triunsaturated and 2 polyunsaturated acids. The UFAs were present in (48.07-53.46%) as compared to the SFAs (46.50-51.88%). This is in agreement with the previous investigations on green algae of Sindh [5,17,19]. The literature citation on green algae, in general, also showed the abundance of UFAs [20]. Palmitic acid (c16:0) was detected in an appreciable proportion (4,32-20.75%), this is also in agreement with the previous studies on green algae of Sindh. However, oleic acid (c18:1) was present only in low proportion (2.71%), while it could not be detected in C. glomerata. It was found as the common UFA in the previous studies [6,22].

The SFAs showed a range between C7 and C24 chin lengths, with the exception of C10-C13 all the acids of this range were detected. Arachidic acid (c20:0) was not found in appreciable proportion (13.21 %) in C.okamurai, it was not detected in the previous studies [21,22]. This acid is fairly widely distributed in green algae [19]. The UFAs exhibited the range between c10 & c29 chain lengths with 1-6 degrees of unsaturation, while c12 and c23-c28 acids were not observed (Table 2). Members of the chlorophyta in general have rather use of the c20:5 and slightly more of the arachidonic acid (c20:4), and linoolenic acid (c18:3) is more abundant [23-25], while these acids were not observed in the present species.

3.3. Extraction of Sterols

Two sterols were identified from the fractions of *C.glomerata* eluted from the silica gel column, where compound 1 was eluted between in mixture form in *n*-hexane: chloroform (80:20) from

Table 2. Fatty acids detected in the methanol extracts of two investigated species of *Cladophora.*

Acid type Compounds		Relative Percentages	
		C. glomerata	*C. okamurai*
Saturated Fatty Acids:		**46.50 %**	**51.88 %**
C 7:0	2,3-Dimethoxy-*n*- propanoic	--	4.53
C 7:0	*n*-Heptanoic	--	3.30
C 8:0	*n*-Octanoic	0.68	2.30
C 9:0	*n*-Nonanoic	--	5.72
C 14:0	*n-Tetradecanoic*	1.99	--
C 15:0	*n-Pentadecanoic*	1.76	--
C 16:0	*n*-Hexadecanoic	20.75	4.32
C 17:0	*n*-Heptadecanoic	1.65	6.03
C 18:0	*n-Octadecanoic*	13.70	--
C 19:0	*n-Nonadecanoic*	3.37	--
C 20:0	*n*-Eicosanoic	1.31	13.21
C 21:0	*n*-Heneicosanoic	-	-3.43
C 22:0	*n*-Docosanoic	0.46	5.02
C 23:0	*n*-Tricosanoic	--	4.02
C 24:0	n-Tetrecosanoic	0.83	--
Unsaturated Fatty Acids:		**53.46%**	**48.07%**
C 10:1	9-Decenoic	0.66	--
C 11:2	3,8-Dimethyl-2, 7-nonadienoic	1.21	6.48
C 11:3	Undecatrienoic	--	1.42
C 13:1	Tridecenoic	2.85	1.63
C 14:1	9-Tetradecenoic	2.44	1.67
C 14:3	Tetradecatrienoic	4.72	--
C 14:4	Tetradecatetraenoic	2.36	--
C 15:1	Pentadecenoic	--	3.74
C 15:2	Pentadecadienoic	1.01	--
C 15:3	3,7,11-Trimethyl-2, 6,10-dodecatrienoic	0.68	3.85
C 16:	9-Hexadecenoic	3.37	--
C 16:2	Hexadecadienoic	2.36	--
C 16:3	6,10,14-Hexadeca trienoic	22.12	--
C 17:1	Heptadecenoic	--	3.73
C 17:3	Heptadecatrienoic	6.42	5.75
C 18:1	9-Octadecenoic	--	2.71
C18:2	9,12-Octadecadienoic	2.04	--
C 19:1	Nonadecenoic	--	1.78
C 20:1	9-Eicosenoic	--	2.97
C 20:6	Eicosahexaenoic	1.22	--
C 21:1	Heneicosenpic	--	2.03
C 22:	11-Docosenoic	--	4.02
C 29:3	Nonacosatrienoic	--	6.56

column. It was purified on preparative thick layer silica gel glass plates in solvent system *n*-hexane:chloroform (70:30). Its purity was checked on TLC card in solvent system *n*-hexane:chloroform (70:30) and by spraying with $Ce(SO_4)_2$, which on heating produced a single pink red spot. After using different spectroscopic techniques it was identified as β-sitosterol (Fig. 1 [2]). The Compound 2 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 *n*-hexane:chloroform (1:1). Its purity was checked on TLC card in solvent system *n*-hexane:chloroform (60:40) and after spraying with $Ce(SO_4)_2$ a pure purple spot was found, it was identified as ergosterol (Fig. 1 [1]). Two sterols were identified from the silica gel column of *C.*

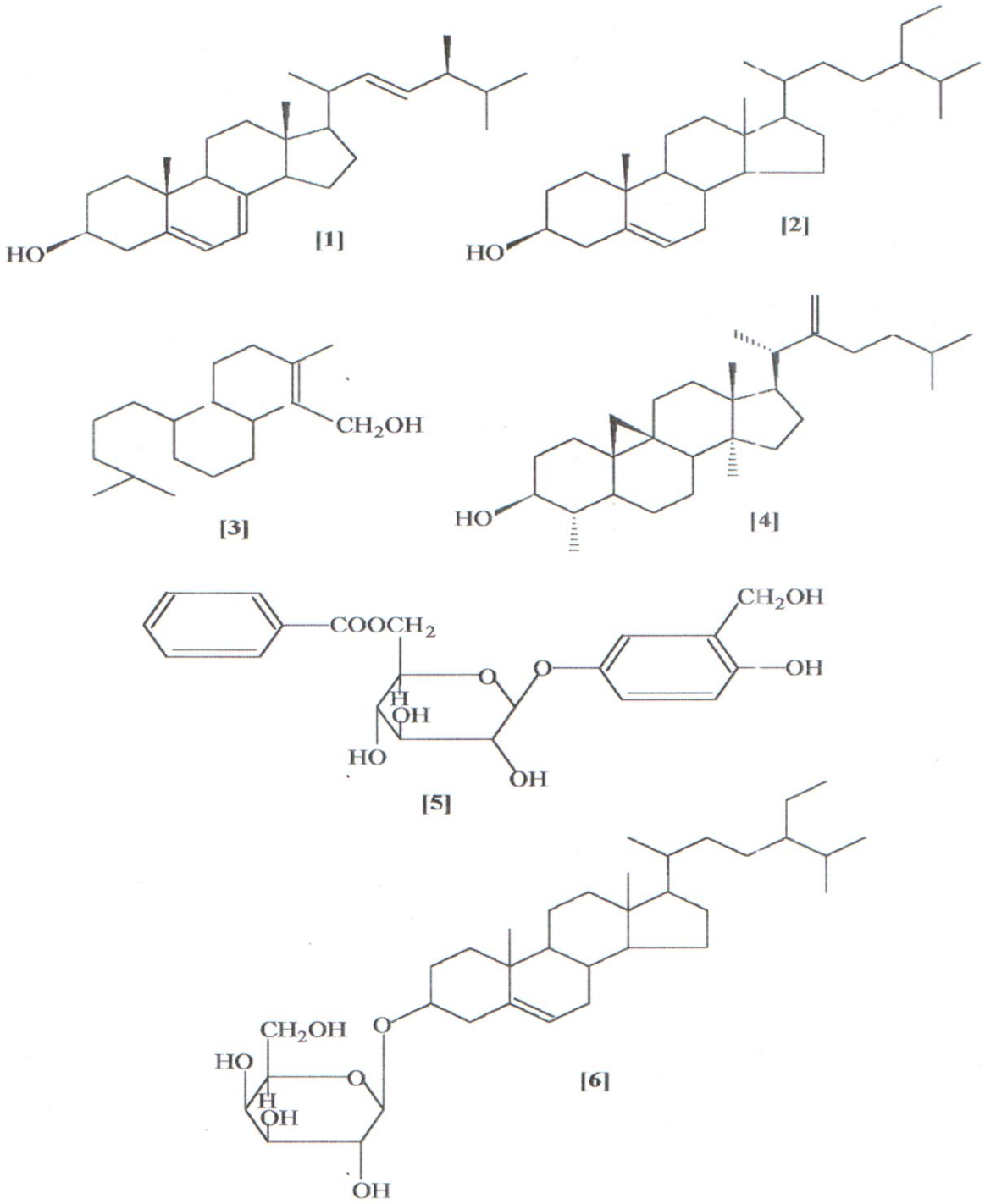

Fig. 1. Natural products obtained from *Cladophora glomerata* : 1] = Ergosterol, [2] = β-Sitosterol, [3] = *Trans*-phytol, [4] = 30-*Nor*-cyclopterospermol, [5] = Xylosmacin, [6] = β-Sitosteryl galactoside.

okamurai, where compound 1 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 checked on TLC card in the above system and a purplish spot was found after spraying with $Ce(SO_4)_2$, it was identified as Decortinol (Fig. 2 [2]). The compound 2 was eluted in mixed form in solvent system *n*-hexane:chloroform (60:40) from column and purified on preparative thick layer silica gel glass plates in solvent system

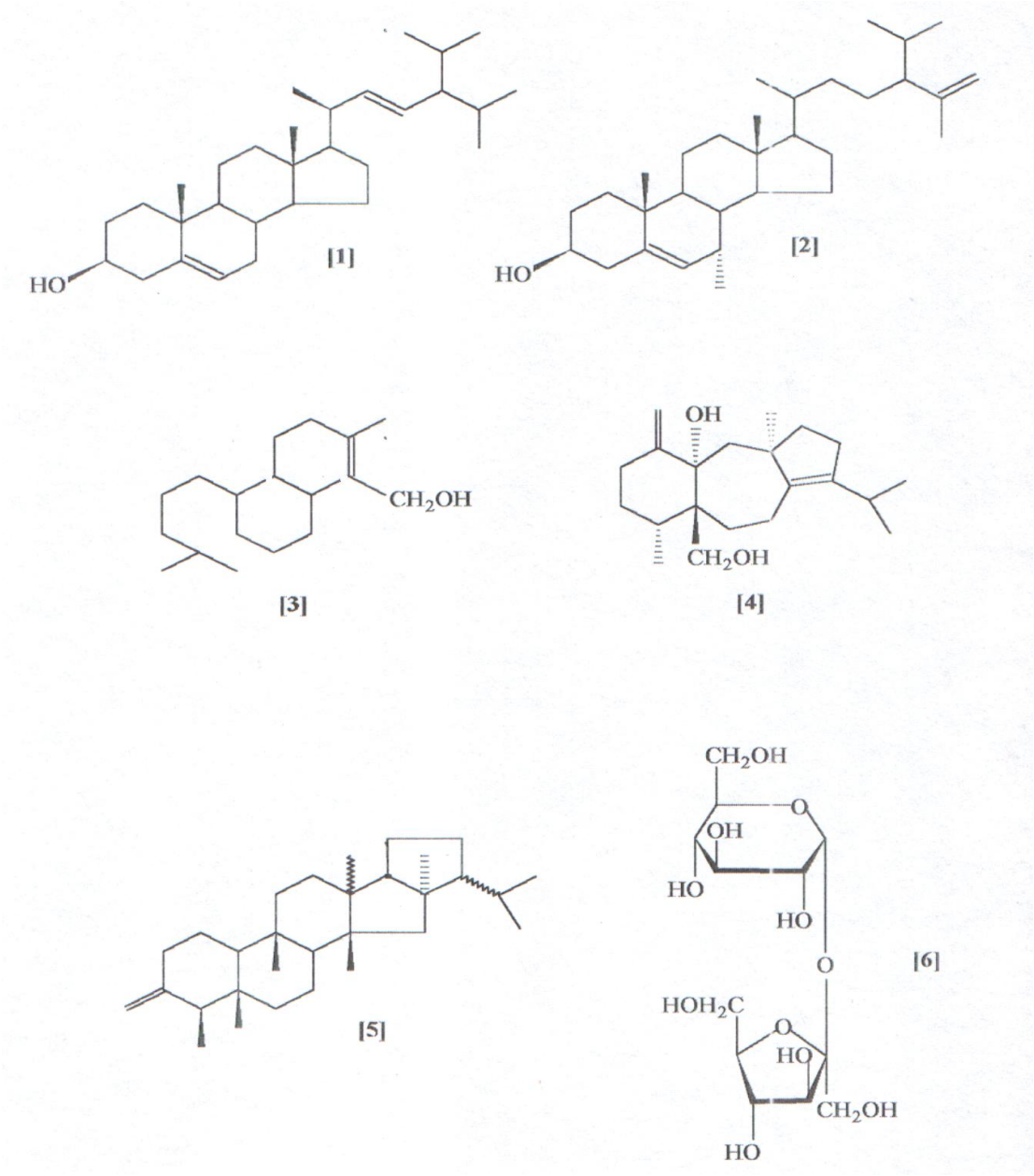

Fig. 2. Natural products isolated from *Cladophora okamurai* : [1] = 22-Dehydro-24-isopropyl cholesterol, [2] = Decortinol, [3] = *Trans*-phytol, [4] = Dictintriol, [5] = Filican-3-one, [6] = Sucrose.

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 pinkish red spot was found, it was identified as 22-dehydro-24-isopropyl cholesterol (Fig. 2 [1]). Some physical properties of the indentified sterols are given in the Table 3.

Four sterols were isolated from methanol extxacts of both the investigated algae, two from one algae and two from the other (Table 3). Both the species different from one another in the type of sterols present. This further indicates that these two species of cladophora are genetically different. Although cholesterol was not extracted in them which is the common sterol of green macroalgae [27], but its 22-dehydro-24-isoprophyl derivative was found in C.okamurai. β-sitosterol was present in c.glomerata, which appears to be a common sterol of freshwater green algae of Sindh as has been isolated from many species during previous studies [18, 19].

Table 3. Natural products extracted from methanol extracts of two investigated species of *Cladophora.*

[1]Str. No.	Common Name	Molecular Formula	[2]Mol. Wt.	[3]Mel. Pt.	$[\alpha]_d$ ($CHCl_3$)
Sterols:					
1[1].	Ergosterol	$C_{28}H_{44}O$	396		
1[2].	β-Sitosterol	$C_{29}H_{50}O$	414	134.5°	-40°
2[1].	22-Dehydro-24-isopropyl cholesterol	$C_{30}H_{50}O$	426		
2[2].	Decortinol	$C_{29}H_{48}O$	428		
Terpenes:					
1[3].	*Trans*-phytol	$C_{20}H_{40}O$	296		
1[4].	30-*Nor*-cyclopter-spermol	$C_{30}H_{50}O$			
2[4].	Dictintriol	$C_{20}H_{32}O_3$	320		-63.39°
2[5].	Filican-3-one	$C_{30}H_{50}O$	426	248-249°	-25.4°
Carbohydrates:					
1[5].	Xylosmacin	$C_{20}H_{22}O_9$	406	149-151°	-30°
2[6].	Sucrose	$C_{12}H_{22}O_{11}$	342	185-187°	+66.5°
Glycoside:					
1[6].	β-Sitosteryl galactoside	$C_{35}H_{61}O_6$	577	275-277°	-63°

[1]Str. No. = Structure Number in Figs. 1 & 2, **[2]Mol. Wt**. = Molecular Weight, **[3]Mel. Pt.** = Melting Point.

3.4. Isolation of Terpenes

A diterpene was purified and eluted from column of *C. glomerata* 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 checked on TLC card in the solvent system of *n*-hexane:chloroform (60:40) and a purplish spot was found after spraying with $Ce(SO_4)_2$, it was identified as 3,7,11,15-tetramethyl-hexane-2-en-1-ol (*trans*-phytol) (Fig. 1 [3]. Two diterpenes were identified from the fractions eluted from the silica gel column of C.okamurai, compound 1 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 checked on TLC card in the solvent system of *n*-hexane:chloroform (60:40) and a purplish spot was observed by spraying with $Ce(SO_4)_2$, it was identified as Dictintriol (Fig. 2 [4]). The compound 2 was eluted in mixture from *n*-hexane:chloroform (70:30) from column and purified on thick layer silica gel glass plates in solvent system *n*-hexane:chloroform (60:40). 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 found, it was also identified as *trans*-phytol (Fig. 2 [3]). A triterpene was purified and eluted from column of *C. g lomerata* in *n*-hexane: chloroform (10:90). It was further purified on preparative silica gel glass plates in solvent system of pure chloroform. Its purity was checked on TLC card in the solvent system of chloroform: methanol (9.5:0.5) and a purplish spot was found after spraying with $Ce(SO_4)_2$, it was identified as 30-*nor*-cyclopterspermol (22-methylene-29-*nor*-cycloartan-3β-ol Fig. 1 [4]). Another triterpene was purified and eluted from column of C.okamurai 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 checked on TLC card in the solvent system of *n*-hexane: chloroform (20:80) and by spraying with $Ce(SO_4)_2$ a pure purple spot was observed, it was identified as filican-3-one (Fig.

2[5]. Some physical properties of the identified terpenes are given in the Table 3.

Four terpenes were extracted from both the investigated species, including two diterpenes and two triterpenes (Table 3). Among tham trans-phytol was the only common compound, while both the species differed from one another in the type of terpenes present in them. This terpene, which is 3,7,11,15-terramethyl hexane-2-en-1-ol, appears to be of common occurrence in the green algae, as it was detected in several species of freshwater algae od Sindh [18, 19]. It is a non-cyclic diterpene.

3.5. Extraction of Glycoside and Carbohydrates

The residue from pooled fractions eluted with chloroform:methanol (90:10), was crystallized and recrystallized from methanol to afford fine white needle like crystals. Purity was then checked on TLC card in solvent system chloroform:methanol:water (4:6:0.5) and then by spraying with $Ce(SO_4)_2$. On heating it produced a single dark purple spot. After using different spectroscopic techniques it was identified as xylosmacin (Fig.1 [5]). The residue from pooled fractions of *C. okamuari* eluted with chloroform:methanol (95:5), was crystallized and recrystallized from methanol to afford fine white needles. Purity was then checked on TLC card in solvent system chloroform:methanol:water (4:6:0.5) and by spraying with $Ce(SO_4)_2$. On heating it produced a single dark purple spot, it was identified as sucrose (Fig. 2 [6]). A glycoside was eluted from column of *C. glomerata* in chloroform:methanol (95:5). It was further purified on preparative silica gel glass plates in solvent system of chloroform:methanol (90:10). Its purity was checked on TLC card in the solvent system of chloroform:methanol (90:10) and after spraying with $Ce(SO_4)_2$ a pinkish red spot was found, it was identified as β-sitosteryl galactoside (Fig 1[6]). Some of the identified carbohydrates and glycosides physical properties are given in the Table 3.

Cladophora appears to be rich in secondary metabolites. It is interesting to note that all of the isolated natural products (except trans-phytol) were present in one species and absent in other. This confirms that the two species under investigation are genetically different from one another, and hence differ in their general metabolism. It was further observed that the same species growing in the estuarine environment indicates that biosynthesis of their natural products is genetically controlled, which is not influenced by aquatic environments. A variety of crystalline cellulose and proteins have been isolated from cladophora [27]. The most majority amoun aminoacods which composed these proteins were glutamine and aspastic acids, alamine, lencine and phenylalamine [28]. A new class of peptides related to rapid replication has been detected in cladophora [29].

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