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PHYCOCHEMICAL INVESTIGATIONS ON CENTROCERAS CLAVULATUM *(CERAMIALES, RHODOPHYTA)*

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ABSTRACT

The methanolic extract of a marine red alga *Centroceras clavulatum* (C Ag.) Mont., collected from the coast of Karachi, was studied for its fatty and sterol compaitions. The analysis of fatty acid methyl earn by GC-MS revealed the presence of methyl laurate, tridecylate, myristate pentadecylate palmitate, margarate, nonadecylate arachidate behenate, tricomsanoate and pentacosanoate among saturated and methyl palmitoleate and heptadecylenate among unsaturated acids. The sterol composition divulged the presence of 22-dehydrocholesterol, cholesterol. 24-methyl-cholesterol and β-sitosterol, identified through mass spectrometry and ^{1}H-NMR spectroscopy.

Introduction

Phycochemistry is a new term introduced by Shameel (1990). Very little attention has been paid on the phycochemistry of *Centroceras Kutzing.* A few studies have been carried out on fatty acid composition (Johns *et al,* 1979), sterol content (Matsuhiro and Urzua, 1984), carbohydrate distribution (Nagashima, 1976) and amino-acids and proteins (Impellizzeri *et al.,* 1975) of various species of this genus.

Centroceras clavulatum (C. Agardh) Montage is a filiform, irregularly dichotomous, articulated and thread like dark red alga, having fronds matted together forming very low cushions, upto 2 mm high and attached firmly with the substratum (Anand, 1943). It grows abundantly on sand covered rocks and sandy bottom pools of lower littoral at Karachi, Lasbela and other coastal areas of Pakistan (Shameel, 1987; Shameel *et al.,* 1989) and has not yet been investigated phycochemically. In this paper an attempt has been made to present the results of a detailed study on the fatty acid and sterol compositions of this seaweed.

Materials and Methods

The specimens of *Centroceras clavulatum* were collected from sand covered lower littoral rocks and muddy flats of Buleji, near Karachi during March 1987. They were

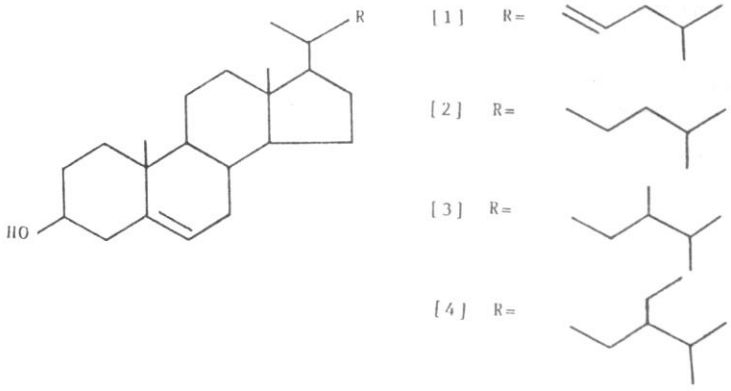
thoroughly cleaned to remove the attached sand and mud particles, in which they were found embeded, and dried in shade.

Extraction and esterification of fatty acids

The dried material (250 g) was percolated in methanol for about three weeks and this procedure was repeated three times. The combined extracts were concentrated under vacuum to give a dark pasty residue. The methanolic extract was saponified in 10% KOH (25 ml refluxed for three hours) and the concentrated extract, so obtained, was fractionated between ethyl acetate and water. The aqueous alkaline layer was thereafter acidified with HCl and extracted with ethyl acetate. The EtOAc combined layer on evaporation under reduced pressure gave a residue weighing 0.4 g. The etherification of the residue was carried out with diazomethane. The aliquots were injected directly into the gas chromatograph attached to mass spectrometer (GC-MS). The details of GC-MS have previously been reported (Hayee-Memon *et al.*, 1991).

Extraction and purification of sterols

A preliminary extraction of air dried material was carried out under reflux with methanol at 100 °C. The extract (2.5 g) was fractionated with ethyl acetate and *al*, 1991).water. The EtOAc fraction, so obtained, afforded a dark reddish brown residue weighing 1.5 g. The EtOAc extract was column chromatographed with silica gel in hexane, hexane-ether, chloroform and chloroform-methanol in increasing order of polarity. The different sterols so obtained with eluting solvents were 22-dehydro-cholesterol [1] and cholesterol [2] with hexane-ether (80:20, v/v) and 24-methyl cholesterol [3] and ß-sitosterol [4] with hexane-ether (75:25, v/v). Further purification of a mixture of these sterols was affected with preparative thin layer chromatography (TLC) and repeated crystallization. After ascertaining the purity of the compounds they were subjected to mass spectrometry and ^{1}H-NMR spectroscopy. The details of instrumentation have previously been described (Hayee-Memon *et al.*, 1991).



Results and Discussion

The GC-MS data of the methylated fatty acids revealed the presence of eleven saturated acids (Table 1). The significant ions in the mass spectra of these acids are given below:

Table 1

Fatty acid composition of *Centroceras clavulatum* analysed as methyl esters.

Common name	Systematic name	Molecular formula	Mol. wt.	Relative Ret. time	Relative percentage
Saturated fatty acid methyl esters					
Methyl laurate	Methyl-*n*-dedocanoate	$C_{13}H_{26}O_2$	214	1.50	7.06
Methyl tridecylate	Methyl-*n*-tridecanoate	$C_{14}H_{28}O_2$	228	1.68	7.22
Methyl myristate	Methyl-*n*-tetradecanoate	$C_{15}H_{30}O_2$	242	1.76	14.76
Methyl pentadecanoate	Methyl-*n*-pentadecanoate	$C_{16}H_{32}O_2$	256	1.95	19.90
Methyl palmitate	Methyl-*n*-hexadecanoate	$C_{17}H_{34}O_2$	270	1.97	15.89
Methyl margarate	Methyl-*n*-heptadecanoate	$C_{18}H_{36}O_2$	284	2.15	12.35
Methyl nonadecylate	Methyl-*n*-nonadecanoate	$C_{20}H_{40}O_2$	312	2.55	6.09
Methyl arachidate	Methyl-*n*-eicosanoate	$C_{21}H_{42}O_2$	326	2.61	4.65
Methyl behenate	Methyl-*n*-docosanoate	$C_{23}H_{46}O_2$	354	2.85	2.72
Methyl tricosanoate	Methyl-*n*-tricosanoate	$C_{24}H_{48}O_2$	368	3.09	2.08
Methyl pentacosanoate	Methyl-*n*-pentacosanoate	$C_{26}H_{52}O_2$	396	3.98	1.92
Unsaturaid fatty acid methyl esters					
Methyl palmitoleate	Methyl-*n*-hexadecenoate	$C_{17}H_{32}O_2$	268	0.98	2.24
Methyl heptadecylenate	Methyl-*n*-heptadecenoate	$C_{18}H_{34}O_2$	282	1.92	3.04

Methyl laurate: GC-MS, m/z 214 (M^+, $C_{13}H_{26}O_2$, 50%), 171 (M^+ -43, 93%), 157 (M^+ -57, 97%),143 (91%),129 (94%), 115 (89%), 101 (87%), 87 (96%), 73 (100%).

Methyl tridecylate: GC-MS, m/z 228 (M^+ , $C_{14}H_{28}O_2$, 42%), 185 (M^+ -43, 30%), 171 (M^+ -57, 13%), 157 (12%), 143 (38%), 129 (51%), 115 (20%), 101 (86%), 87 (93%), 73 (100%).

Methyl myristate: GC-MS, m/z 242 (M^+, $C_{15}H_{30}O_2$, 1%), 199 (M^+ 43, 17%),185 (M^+ -57, 30%), 171 (13%), 157 (11%), 143 (38%), 129 (50%), 115 (19%), 101 (87%), 87 (94%), 73 (100%).

Methyl pentadecylate: GC-MS, m/z 256 (M^+ , $C_{16}H_{32}O_2$, 7%), 213 (M^+43,12%), 199 ($M^{'}$ -57, 17%), 185 (20%), 171 (8%), 157 (7%), 143 (11%), 129 (36%), 115 (16%), 101 (12%), 87 (22%), 73 (100%).

Methyl palmitate: GC-MS, m/z 270 (M^+ , $C_{17}H_{34}O_2$, 67%), 239 (M^+ -31, 28%), 227 (M^+ -43, 44%), 213 ($M^{'}$ -57, 15%), 199 (24%), 185 (36%), 171 (35%), 157 (21%), 143 (87%),129 (91%), 115 (70%), 101 (86%), 87 (93%), 73 (100%).

Methyl margarate: GC-MS, m/z 284 (M^+ , $C_{18}H_{36}O_2$, 16%), 241 (M^+ -43, 9%), 227 (M^+ -57, 69%), 213 (76%), 199 (65%), 185 (77%), 171 (84%), 157 (92%), 143 (90%), 129 (87%), 115 (91%), 101 (93%), 87 (94%), 73 (100%).

Methyl nonadecylate: GC-MS, m/z 312 (M^+ , $C_{2o}H_{4o}O_2$, 3%), 269 (M^+ 43, 2%), 255 (M^+ -57, 3%), 241 (7%), 227 (8%), 213 (17%), 199 (10%), 185 (16%), 171 (12%), 157 (13%), 143 (38%), 129 (43%), 115 (22%), 101 (24%), 87 (90%), 73 (100%).

Methyl arachidate: GC-MS, m/z 326 (M^+, $C_{21}H_{42}O_2$, 13%), 295 (M^+ -31, 4%), 283 (M^+ -43, 6%), 269 (M^+ -57, 4%), 255 (3%), 241 (7%), 227 (8%), 213 (15%), 199 (10%), 185 (15%), 167 (25%), 153 (27%), 139 (45%), 125 (54%), 111(35%), 97 (71%), 87 (80%), 73 (100%).

Methyl beheoate: GC-MS, m/z 354 (M^+ , $C_{23}H_{46}O_2$, 27%), 311 (M^+ -43, 3%), 297 (M - 57, 2%), 283 (8%), 269 (9%), 255 (18%), 241 (7%), 227 (10%), 213 (27%), 199 (13%), 185 (14%), 171 (14%), 157 (15%), 143 (17%), 129 (24%), 115 (22%), 101 (36%), 87 (69%), 73 (100%).

Methyl tricosanoate: GC-MS, m/z 368 (M^+ , C24H4g02, 42%), 325 (M^+ -43, 12%), 311 (M` -57, 3%), 297 (3%), 283 (7%), 269 (9%), 255 (18%), 241 (8%), 227 (9%), 213 (27%), 199 (13%), 185 (13%), 171 (12%), 157 (15%), 143 (17%), 129 (25%), 115 (11%), 101 (15%), 87 (39%), 73 (100%).

Methyl pentacosanoate: GC-MS, m/z 396 (M^+, $C_{26}H_{52}O_2$, 6%), 353 (M^+ -43, 83%), 339 (M^+ -57, 5%), 325 (3%), 311 (3%), 297 (3%), 283 (7%), 269 (9%), 255 (18%), 241 (8%), 227 (9%), 213 (27%), 199 (13%), 185 (13%), 171 (12%), 157 (15%),143 (17%), 129 (25%),115 (11%), 101 (35%), 87 (69%),73 (100%).

Among these fatty esters (Table 1) Me pentadecylate was found in highest proportion (20%), followed by Me palmitate (16%) and Me myristate (15%). Usually palmitate add is found in largest quantity in the red seaweeds of Karachi (Qasim, 1986; Shamed, 1990), while in certain red algae margaric add is present in highest amount (Afaq-Husain et al, 1991; Hayee-Memon *et al*, 1991). In this respect C clavulatum differs from other red seaweeds. It possessed Me behenate, tricosanoate and pentacosanoate in very small proportion (<4%). This is also quite unusual as other investigated red algae of Karachi do not contain saturated fatty acids higher than C_{18} (Qasim, 1986). Arachidonic acid, which is fairly widely distributed in higher plants and usually absent in the red seaweeds of Karachi (Shamed, 1990), was present in appreciable quantity (5%) in this alga. However, red algae from other seas bear a high concentration of C_{20} acids (Lie Ken Jie, 1989), this appears to be an adaptation necessary for survival in the marine environment.

The GC-MS data of the methylated fatty acid fraction divulged the presence of only two unsaturated fatty acids. The significant ions in their mass spectra are as follows:

Methyl palmitoleate: GC-MS, m/z 268 (M^+ , $C_{17}H_{12}O_2$, 1%), 225 (M^+ -43, 2%), 194 (M^+ -74, 4%), 180 (5%), 166 (8%), 152 (4%), 138 (11%), 124 (31%), 110 (42%), 96 (27%), 82 (35%), 68 (21%), 54 (100%).

Methyl heptadecylate GC-MS, m/z 282 (M^+, $C_{18}H_{34}O_2$, 1%), 239 (M^+-43, 3%), 208 (M^+ -74, 2%), 194 (3%), 180 (2%), 166 (4%), 152 (4%), 138 (5%), 124 (7%), 110 (15%), 96 (32%), 82 (48%), 68 (59%), 54 (100%).

Palmitoleic acid was found to be present in several red algae of Karachi and appears to be of usual occurrence, but heptadecylenic acid has so far been reported only from *Halymenia porphyrodies* (Shameel 1990) and is, therefore, of restricted occurrence. It is also very interesting that oleic acid, which is commonly present in all the investigated red seaweeds of Karachi (Qasim, 1986), could not be detected in this alga. Oleic add is of universal occurrence among seaweeds and has been detected along with β-iodopalmitic add in *C. clavulatum* collected from Atlantic Ocean (Takahashi, 1976), but both these acids were absent in the specimens of Arabian Sea. Our specimens exhibit remarkable difference in the fatty add composition than those from Australian waters (Johns *et al,* 1979). This indicates that sometimes the local oceanic conditions have a great bearing on the general metabolism of seaweeds.

The preparative TLC and repeated crystallization afforded four sterols (Table 2) and the following spectral data were recorded through ^{1}H-NMR spectroscopy. The mass spectral assignments of the isolated sterols and their fragmentation pattern are also given:

Table 2

Sterol composition of *Centroceras clavulatum*.

Common name	Systematic name	Molecular formula	Mol. wt.	Relative percentage
22-Dehydrocholesterol	22-Dehydrocholest-5-en-3β-ol	$C_{27}H_{44}O$ [1]	384	22.64
Cholesterol	Cholest-5-en-3β-ol	$C_{27}H_{46}O$ [2]	386	57.08
24-Methyl cholesterol	24-Methylcholest-5-en-3β-ol	$C_{28}H_{48}O$ [3]	400	11.17
β-Sitosterol	24R-Ethylcholest-5-en-3β-ol	$C_{29}H_{50}O$ [4]	414	8.49

22-Dehydrocholesterol [1]: GC-MS, m/z 384 (M^+ $C_{27}H_{44}O$), 313 (M^+ -Cash), 271 (M - C_8H_{15} -2H), 255 (M^+ -C_8H_{15}-H_2O), 217,185, 157, 127, 95, 69.

^{1}H-NMR (400 MHz, $CDCl_3$), ppm: 0.67 (3H, s, 18-Me), 0.83 (3H, d, J= 6.0 Hz, 26-Me), 0.85 (3H, d, J = 5.6 H4 27-Me), 1.00 (3H, s, 19-Me), 1.22 (311, d, J = 7.0 Hz, 21-Me), 3.51(3-11), 4.22 (1H, I, J = 6.0 Hz, 6-H).

Cholesterol [2]: GC-MS, m/z 386 (M^+, C2711460), 371(M^+ -CH_3), 353 (M^+ -CH3 - H20), 273 (M^+-side chain), 255 (M + -side chain-H_2O), 231, 229, 213,121, 107.

^{1}H-NMR (400 MHz, $CDCl_3$ ppm: 0.65 (3H, s, 18-Me), 0.87 (3H, s, 19-Me), 1.24 (3H, d, J = 7.0 Hz, 21-Me), 1.57 (6H, m, 26, 27-Me), 3.52 (3-H), 5.72 (1H, m, 6-H).

24-Methyl cholesterol [31: GC-MS, m/z 400 (M^+ $C_{28}H_{48}O$), 385 (M^+ -CH_3) 382 (M^+ - H_2O), 367 (M -CH_3-H_2O), 314, 297, 269, 217, 227, 149, 69.

^{1}H-NMR (400 MHz, $CDCl_3$), ppm: 0.66 (3H, s, 18-Me), 0.89 (3H, s, 19-Me), 1.24 (1H, d, J = 7.0 Hz, 21-Me), 1.60 (6H, m, 26, 27-H), 3.48 (1H, m, 3-H), 5.33 (1H, m, 6-H).

ß-Sitosterol [4]: GC-MS, m/z 414 (M^+, $C_{29}H_{50}O$), 399 (M^+ -CH_3), 396 (M^+ -H_2O), 381 (M^+-CH_3 -H_2O), 342, 301, 273, 255.

^{1}H-NMR (300 MHz, $CDCl_3$), ppm: 0.76 (3H, s, C-18H), 0.83-0.99 (12H, m, 21, 26, 27, 29-H), 1.05 (3H, s, 19-H), 3.20 (1H, m, 3-H), 534 (1H, t, J = 6 Hz, 6-H).

Cholesterol was predominant (57%) in *C. clawlalum* and was followed by 22-dehydrocholesterol (23%, Table 2). This is quite usual, as cholesterol or its biosynthetic precursor desmosterol is the main sterol component in the members of six orders of

colonial Rhodophyta (Goodwin, 1974), while 22-dehydrocholesterol is the major component in certain other red algae (Chardon-Loriaux *et al*, 1976). The major sterols of red algae are C_{27} containing compounds. The 24-methyl cholesterol and ß-sitosterol were present in small amount (8-11%) in *C. clavulatum*, they have also been detected in small quantities in various red algae (Kabore *et al,* 1983; Afaq-Husain *et al,* 1991). However, desmosterol and 24-methylene cholesterol, which were found in *C. pacificum* and other species (Urzua *et al.,* 1982; Matsuhiro and Urzua, 1984), could not be detected in C. *clavulatum.* This signifies differences among various species of *Centroceras.* Uptil now no other species has been reported from the coast of Pakistan.

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