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ISOLATION AND CHARACTERIZATION OF CHEMICAL CONSTITUENTS OF *STOECHOSPERMUM MARGINATUM* (DICTYOTALES, PHAEOPHYTA) AND THEIR ANTIMICROBIAL ACTIVITY.

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ABSTRACT

From chloroform and methanolic extracts of *Stoechosermum marginatum* (C. Ag) Kütz. five saturated and four unsaturated fatty acids, three sterols, four diterpenes and D-mannitol were isolated. The fatty acids, analysed as methyl esters, were myristate, pentadecylate, palmitate, margarate and nonadecylate among saturated and tetradecatrienoate, pentadecenoate, hiragonate and oleate among unsaturated ones. The sterols were identified as cholesterol [1], 24-methylene cholesterol [2] and 24-methyl cholesterol [3]. The diterpenoids were determined as 19-acetoxy-5 (R), 15, 18 (R and S)-tetrahydroxyspata-13, 16 (E)-diene, 5 (R), 15, 18 (R and S), 19-tetrahydrowspata-13, 16 (E)-diene [5], 5 (R), 18-dihydroxyspata-13, 16 (E)-dime [6] and 5 (R), 16-dihydroxyspata-13, 17-diene [7]. All these diterpenes showed strong antibacterial activity against three gram +ve and six gram we bacteria. The crude extract was found active against three fungi in 10 mg/ml concentration, whereas the diterpene [5] exhibited activity against only one fungus.

Introduction

The brown seaweed, *Stoechospermum marginatum* (C. Agardh) Kützing abundantly occurs as benthos on sand covered rocks and sandy flats in lower to sub-littoral zone at the coast of Pakistan (Shamed, 1990a). Solimabi *et al.* (1980) characterized a diterpenoid, stoechospermol from this alga collected from Goa (India). Gerwick *at al*. (1981) isolated ten diterpenoids belonging to the class of spatane series and De Silva *et al.* (1982) investigated antibacterial activity of extract from specimens collected from Jaffna (Sri Lanka). However, no *phycochemical* study was carried out on this seaweed occurring along the coast of northern Arabian Sea and no investigation this species collected from the coast of Karachi (Pakistan) to isolate its natural products and to study the antimicrobial activity of some of these chemical constituents.

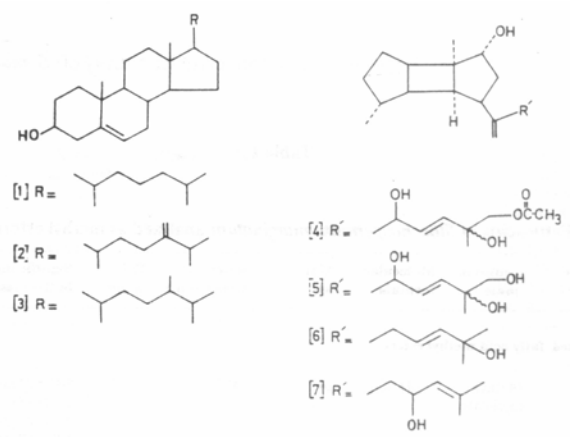
Material and Methods

About 1 kg (dry weight) of Stoechospermum marginatum was collected as drift material from the shallow water sandy flats of Buleji coast, near Karachi in November 1986. It was dried in shade and extracted first with $CHCl_3$ and then with $CHCl_3$-MeOH (1:1, v/v). The $CHCl_3$ extract on evaporation of organic phase under vacuum furnished 11 g of dark brownish residue, which was saponified by refluxing at 100°C for 3 hours with 10% KOH in aqueous ethanol. It was concentrated under vacuum and then ether was added. The aqueous alkaline layer was acidified with HCl and then extracted with Et_2O. The combined ether fraction was dried over anhydrous Na_2SO_4 and on evaporation of ether a residue (12.32 mg) was obtained. The fatty acid methyl esters so obtained were analysed by GC-MS.

The D-mannitol was isolated from methanolic extract through sephadex LH20 in a very pure crystalline form. The purity was checked on TLC developed in EtOAc:Acetic Acid:H2O:MeOH (65:1:15:1.5) and the sugar spraying reagent (orcinol + $FeCl_3$ +H_2SO_4 + EtOH) was used to detect the spot. It was confirmed by comparing it with an authentic sample (Bano *et al,* 1987).

The $CHCl_3$-MeOH extract was evaporated under reduced pressure and the residue thus obtained was subjected on column of silica gel. The elution was carried out with hexane, hexane : Et_2O, Et_2O, $CHCl_3$: MeOH and then pure MeOH. The fraction eluted with hexane : Et2O (95:5) afforded crystalline compound characterized as hexadecanoic acid, while the fraction eluted with hexane : Et_2O (75:25) furnished a mixture of sterols, which were further purified on HPLC using silica gel column (hexane: Et_2O). From the HPLC one major and two minor sterols were isolated and characterized as cholesterol [1], 24-methylene cholesterol [2] and 24-methyl cholesterol [3] respectively.

Further elution with pure ether furnished an oily mixture of compounds, which was further purified on preparative layer chromatography developed in $CHCl_3$:MeOH (9:1). This compound was identified as 19-acetoxy-5 It, 15,18 (R and S)-trihydroxyspata-13, 16 (E)-diene [4]. The fraction eluted with CHCl:MeOH (95:5) yielded a mixture of gummy compounds, which were purified on HPLC using different proportions of *iso*-octane and EtOAc. From the HPLC three compounds were isolated and identified as 5 (R), 15, 18 (R and S), 19-tetrahydroxyspata-13, 16 (E)-diene [5], 5 (R), 18-dihydroxyspata-13, 16 (E)-diene [6] and 5 (R), 16-dihydroxyspata-13, 17-diene [7]. The antibacterial activity of the compounds [4-7] were checked on thin layer plate developed in $CHCl_3$:MeOH (9:1). The crude extract and compound [5] were also checked for antifungal activity. The antimicrobial activity was studied as described earlier (Usmanghani and Shameel, 1986).



The GC-MS was performed on GC-Hewlett Packard with 11/73 DEC computer data system and a 1.2 m x 4 mm packed glass capillary column coated with gas chrome Q (100-120 mesh, OV 101 1%). The column temperature was programmed between 70-250°C with a rate of increase of 80°C per minute. The carrier gas (helium) flow rate was 32 ml/min and injector temperature was 250°C. The ^{1}H-NMR data were recorded on Broker AM-300 at 300 MHz in $CDCl_3$ and FAB mass spectrum were obtained on MAT 312. The high performance liquid chromatography was performed on a Shimadzu LC-6A HPLC apparatus, which consists of dual pump system, Wyctor, detector (R1), Veerdu and integrator, by using a 16 mm x 50 cm preparative silica gel column. The details of the instrumentation may be found in (Hayee-Memon *et al.*, 1991).

Results and Discussion

The $CHCl_3$ extract of *Stoechospermum marginatum* afforded five saturated and four unsaturated fatly acids as methyl esters. They have been analysed through mass and their fragmentation pattern (Table 1). It revealed the occurrence of methyl myristate, pentadecylate, palmitate, margarate and nonadecylate as saturated acids, whereas methyl tetradecatrienoate, pentadecenoate, hiragonate and oleate *were* unsaturated ones. Myristic, palmitic and oleic acids were present in overwhelming quantity as compared to others. Palmitic and oleic acids have also been found in largest quantity among saturated and unsaturated fatty acids respectively in other brown seaweeds of Karachi (Qasim, 1986; Shamed, 1990 b). Tetradecatrienoic and hiragonic acids are triunsaturated fatty acids detected in S. marginatum both in appreciable quantity. The former has also been reported from *Colpomenia sinuosa* (Shaikh *et al,* 1991a) and *Padina tetrastromatica* (Shaikh *et al,* 1991b) and the latter from *Iyengaria stellata* (Shamed, 1990 b). Triunsaturated fatty acids are not very common in brown algae. The $CHCl_3$ extract also provided pure crystalls of D-mannitol. This compound is of usual occurrence in brown algae (e.g. Bano *et al.,* 1987).

Table 1

Fatty acids of *Stoechospermum marginatum* analysed as methyl esters.

Systematic name	Common name	Molecular formula	Mol. wt.	Retention time (min.)	Rel. % age	Significant ions in the mass spectra
A. Saturated fatty acid methyl esters:						
Methyl-*n*-tetradecanoate	Methyl myristate	$C_{15}H_{30}O_2$	242	14' 56"	17.93	**GC-MS m/z 242 (M^+, $C_{15}H_{30}O_2$), 221 (M^+-31), 199 (M^+-43), 185, 178, 157, 143, 129, 111, 87, 74.**
Methyl-*n*-pentadecanoate	Methyl pentadecylate	$C_{16}H_{32}O_2$	256	16' 20"	5.23	**GC-MS m/z 256 (M^+, $C_{16}H_{32}O_2$), 213 (M^+-43), 199, 185, 171, 157, 143, 129, 115, 101, 83, 73.**
Methyl-*n*-hexadecanoate	Methyl palmitate	$C_{17}H_{34}O_2$	270	17' 30"	18.25	**GC-MS m/z 270 (M^+, $C_{17}H_{34}O_2$), 239 (M^+-31), 227 (M^+-43), 213, 199, 185, 171, 157, 143, 129, 115, 101, 87, 74.**
Methyl-*n*-heptadecanoate	Methyl margarate	$C_{18}H_{36}O_2$	284	22' 25"	13.49	**GC-MS m/z 284 (M^+, $C_{18}H_{36}O_2$), 227 (M^+-57), 213, 199, 185, 171, 157, 143, 129, 115, 101, 87, 73.**
Methyl-*n*-nonadecanoate	Methyl nonadecylate	$C_{20}H_{40}O_2$	312	12' 45"	5.55	**GC-MC m/z 312 (M^+, $C_{20}H_{40}O_2$), 269 (M^+-43), 225, 241, 227, 213, 199, 185, 171, 157, 143, 129, 115, 101, 87, 71.**
B. Unsaturated fatty acid methyl esters:						
Methyl-2,4,5-tetradecatrienoate	Methyl tetradecatrienoate	$C_{15}H_{24}O_2$	236	10' 58"	3.80	**GC-MS m/z 236 (M^+, $C_{15}H_{24}O_2$), 193 (M^+-43), 179, 165, 137, 95, 81, 67.**
Methyl-pentadecenoate	Methyl pentadecenoate	$C_{16}H_{30}O_2$	254	23' 10"	7.93	**GC-MS m/z 254 (M^+, $C_{16}H_{30}O_2$), 222 (M^+-32), 208, 194, 180, 166, 152, 87, 74.**

(Continued.....)

Methyl-6, 10,14-hexa-decatrieno-ate	Methyl hiragonate	$C_{17}H_{28}O_2$	264	23' 50"	9.20	GC-MS m/z 264 (M^+, $C_{17}H_{28}O_2$), 232 (M^+ -32), 190 (M^+ -74), 180, 166, 152, 87 74.
Methyh-9-octadece-noate	Methyl oleate	$C_{19}H_{36}O_2$	296	19' 36"	18.57	GC-MS m/z 296 (M^+, $C_{19}H_{36}O_2$), 264 (M^+ -32), 222 (M^+-74), 208, 194, 180, 166, 152, 87, 74.

Table 2.

Sterols of *Stoechospermum marginatum*.

Systematic name	Common name	Molecular formula	Mol. wt.	Mass and fragmen-tation pattern	Spectral data
Cholest-5-en-3β-ol	Choles-terol	$C_{27}H_{46}O$ [1]	386	m/z 386 ($M^{+\cdot}$, $C_{27}H_{46}O$), 371 (M^+ -CH_3), 353 (M^+ -CH_3 -H_2O), 273 (M^+ -C_9H_7 - side chain), 255, 252 (M^+ -side chain-$C_{16}H_{17}$), 231 (M^+ -side chain-ring D cleavage), 229 (M^+ - side chain-C_{16}-C_{17}-OH), 213 (M -side chain-H_2O- ring D cleavage), 121, 107.	- - -
24-methy-lene cho-lest-5-en-3β-ol	24-meth-ylene cholest-erol	$C_{28}H_{46}O$ [2]	398	m/z 398 (M^+, $C_{28}H_{46}O$), 365 (M^+ -CH_3 -H_2O), 314 (M^+ -C_6H_{12}), 271 (M^+ - C_9H_{17} -2H), 255 (M^+ - C_9H_{17} -H_2O), 213 ($C_{12}H_{23}$-H_2O), 185 (M^+ - $C_{14}H_{27}$-H_2O), 171, 145, 121, 95, 81.	^{1}H-NMR ppm 0.67 (3H, s, 18-Me), 0.84 (3H, s, 19-Me), 1.24 (3H, d, J = 7 Hz, 21 Me), 1.60 (6H, m, 26, 27-Me), 3.15 (2H, s, 28-H), 3.50 (1H, m, 3β-H), 5.33 (1H, m, 5-H).
24-methyl-cholest-5-en-3β-ol	24-meth-yl cho-lesterol	$C_{28}H_{49}O$ [3]	400	m/z 400 (M^+, $C_{28}H_{49}O$), 385 (M -CH_3), 382 (M^+ - H_2O), 367 (M^+ -CH_3 - H_2O), 349, 300, 273 M^+-side chain), 271, 255, 231, 213, 199, 145, 121, 81.	^{1}H-NMR 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, 3β-H), 3.48 (1H, m, 3β-H), 5.33 (1H, m, 5-H).

The $CHC1_3$-MeOH extract yielded three sterols, out of which cholesterol was found to be the major and 24-methylene and 24-methyl cholesterol as minor components (Table 2). The mass spectrum of cholesterol exhibited molecules ion peak at m/z 386 with strong peaks at m/z 371 and 255 characteristic of cholesterol [1]. The compound [2] exhibited molecular ion peak at m/z 398 corresponding with the molecular formula C2sH4oO. The peaks appeared at m/z 314, 217, 213 and 171 corresponding with the fragmentation pattern of sterols. The peak at m/z 314 is characteristic for the presence of double bond at C-24 (28) formed due to RDA fragmentation as described by Zielinski *et al* (1981) for 24-methylene cholesterol [2]. The molelcular ion peak at m/z 400 corresponds with the molecular formula C29H49O for compound [3] together with a strong peak at m/z 271 (M^+-side chain-2H), 255, 231 and 213 demonstrating the presence of usual $\Delta^{5,3}$-hydroxy sterol nucleus. The structure thus assigned for [3] is 24-methyl cholesterol. The structures have been confirmed by 'H-NMR spectroscopy. These sterols have also been found in Iyengaria stellata (Usmanghani *et al.,* 1987), *Padina tetras*tr*omatica* (Shaikh *et al,* 1991b) and *Spatoglossum variabile* (Shaikh *et al.,* 1990). Actually cholesterol is the major sterol of Rhodophyta. However, fucosterol or its C-20 and other isomers, the major sterols of Phaeophyta, could not be detected in this seaweed.

The $CHCl_3$ -MeOH extract of the material further provided four diterpenes, all possessing the novel 'spatane' tricyclic diterpenoid ring system. The selected ^{1}H-NMR assignments for these diterpenes have been given in Table 3. The ^{3}H-NMR and mass spectra of the compounds [4, 5, 6 and 7] were found identical with 19-acetoxy-5 (R), 15, 18 (R and S)-trihydroxyspata-13, 16 (E)-diene [4], 5 (R), 15, 18 (R and S), 19-tetrahydroxyspata-13, 16 (E)-diene [5], 5 (R), 18-dihydroxyspata-13, 16 (E)-diene [6] and 5 (R), 16-dihydroxyspata-13, 17-diene [7] respectively as described by Gerwick *et al.* (1981). Dictyotalean marine algae are prolific producers of interesting secondary metabolites, consisting of C-11 acetate-derived compounds (Shaikh *et al*, 1990), compounds of mixed biosynthesis (Gerwick *et al,* 1979), sesquiterpenoids (Fattorusso *et al,* 1978) and diterpenoids (Solimabi *et al.,* 1980). The diterpenoids from this group are particularly unique, as the novel ring systems produced represent unconventional diterpenoid cyclizations not yet observed from terrestrial sources (Gerwick *et al,* 1981). *S. marginatum* and other Dictyotalean seaweeds are frequently encountered as the major vegetation of sand covered rocks in the shallow water of lower littoral and sub-littral zones of tropical seas, although herbivorous predators are enormous in this area. Therefore a correlation between secondary metabolite synthesis within this order of brown algae and predator avoidance is evident.

The methanolic extract of *Stoechospermum marginatum,* has shown strong antibacterial activity and was found most active among 50 species of marine benthic algae (Usmanghani and Shameel, 1986), the active constituents were found to be a mixture of diterpenoid monoacetates having the spatane skeleton (De Silva *et al*., 1982). Therefore the four diterpenoids isolated from our material were tested against three gram positive and six gram negative bacteria (Table 4). No activity was observed against

Salmonella typhi (7) and *Shigella dysenteriae* (8) by any diterpene. The compounds [4 and 5] showed a strong activity against rest of the bacteria, while compound [6] was strongly active only against *Bacillus subtilis* (1) and weakly active against others. The compound [7] was highly active against *Bacillus subtilis* (1), *Shigella sonnei* (9), *Staphylococcus aureus* (2) and *Streptococcus faecalis* (3). Usmanghani and Shameel (1986) observed no activity of the methanolic extract of S. marginatum against any of the four studied gram -ve bacteria, but the compounds [4, 5, 6 and 7] were quite active against four of the six investigated gram -ve species.

Table 3

Selected ^{1}H-NMR assignments for diterpenes isolated from *Stoechospermum marginatum*.

H's at C no.	Compound [4] m/z 378 ($C_{22}H_{34}O_5$)	Compound [5] m/z 336 ($C_{20}H_{32}O_4$)	Compound [6] m/z 304 ($C_{20}H_{32}O_2$)	Compound [7] m/z 304 ($C_{20}H_{32}O_2$)
C_5	3.63(d,J = 3.99 Hz)	3.56(d,J = 3.54 Hz)	3.73(d,J = 4.2 Hz)	3.72(d,J = 4.2 Hz)
C_6	2.18(ddd,J = 13.1, 13.1,4.2 Hz)	2.42(ddd,12.9, 12.9,4.2 Hz)	2.24(ddd,J = 13.1, 13.1,4.2 Hz)	2.25(ddd,J = 13,13, 4 Hz)
C_7	2.81 (m)	3.20 (m)	2.95 (m)	2.97 (m)
C_{11}	0.84(d,J = 6.33 Hz)	1.11(d,J = 6.48 Hz)	0.89(d,J = 6.27 Hz)	0.85(d,J = 6.39 Hz)
C_{12}	0.90 (s)	1.14 (s)	0.97 (s)	0.88 (s)
C_{14}	5.24 (s)	5.52 (s)	4.81 (s)	4.91 (s)
C_{14}	4.86 (s)	5.04 (s)	4.75 (s)	4.84 (s)
C_{15}	4.35(d,J = 6.18 Hz)	3.82(d,J = 3.93 Hz)	2.69(dd,J = 14.9,4.8 Hz)	- - -
C_{16}	5.63 (m)	5.66 (m) 5.61 (m)		4.53(ddd,J = 6.4, 6.4,6.4 Hz)
C_{17}	5.63 (m)	5.66 (m)	5.61 (m)	5.15(d,J = 6.1 Hz)
C_{19}	4.01(d,J = 11.07 Hz)	3.40 (s)	1.70 (s)	1.66 (s)
C_{20}	1.23 (s)	1.38 (s)	1.67 (s)	1.70 (s)

Table 4

Antibacterial activity of diterpenes isolated from *Stoechospermum marginatum*.

Diterpene Compound	Gram positive bacteria			Gram negative bacteria					
	1	2	3	4	5	6	7	8	9
[4]	+ +	+ +	+	+ +	+ +	+ +	-	-	+ +
[5]	+ +	+ +	+ +	+ +	+ +	+ +	-	-	+ +
[6]	+ +	+	+	+	+	+	-	-	+
[7]	+ +	+ +	+ +	+	+	+	-	-	+ +

1 = *Bacillus subtilis*, 2 = *Staphylococcus aureus*, 3 = *Streptococcus faecalis*, 4 = *Escherichia coli*, 5 = *Klebsiella pneumoniae*, 6 = *Pseudomonas aeruginosa*, 7 = *Salmonella typhi*, 8 = *Shigella dysenteriae*, 9 = *Shigella sonnei* (- = no activity, + = active, + + = highly active).

The extract of *S. marginatum* has also exhibited strong antifungal activity (Usmanghani and Shameel, 1986). Therefore the crude extract of our material as well as compound [5] were tested against six fungi (Table 5). No activity was observed against *Aspergillus flatus* (1), *A. niger* (3) and *Trichophyton mentagrophytes* (5). The crude extract showed the activity against *Aspergillus* fumigatus (2), *Paecilomyces lilacinus* (4) and *Trichophyton rubrum* (6) in the concentration of 10 mg/mi. However, the pure compound [5] was found active only against *T. rubrum* (6), indicating that the other terpenoids present in the seaweed are also active.

Table 5

Antifungal activity of diterpenes isolated from *Stoechospermum marginatum*

Compound tested	1	2	3	4	5	6
Crude extract	-	+	-	+	-	+
Compound [5]	-	-	-	-	-	+

1 = *Aspergillus flavus*, 2 = *Aspergillus fumigatus*, 3 = *Aspergillus niger*, 4 = *Paecilomyces lilacinus*, 5 = *Trichophyton mentagrophytes*, 6 = *Trichophyton rubrum* (- = no effect, + = activity present).

In the previous study *Stoechospermum maculatum* J. Ag. was also found to show strong antibacterial and antifungal activities (Usmanghani and Shamed, 1986), but the taxonomic studies revealed that this species is a synonym of S. marginatum (Shameel, 1990a). However, the present prerequisit bioactive study will provided a better insight to start investigations on the phycochemistry of seaweeds and may possibly augment elucidation of their pharmacology.

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