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Lipid Composition and Antibacterial Screening of Lipophilic Extract of a Marine Sponge *Haliclona* sp. Collected from the Bay of Bengal (Orissa Coast), India

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The fatty acid composition as well as volatiles and antibacterial screening of lipophilic extract of the sponge *Haliclona* sp. were analyzed. The fatty acids are characterized by linear saturated, short chain unsaturated, long chain unsaturated and long chain polyunsaturated fatty acids. Linear saturated fatty acids constituted 52 % of the total fatty acid content. An important polyunsaturated fatty acid (PUFA) *i.e.*, linoleic acid was found to be 3.38 % of the total fatty acid content. The content of long chain fatty acids $\Delta^{5,9}$, was significant in *Haliclona* sp. (23.64 %). The antibacterial screening of the lipid extract of *Haliclona* sp. against different pathogens showed marked results.

Key Words: Marine sponge, *Haliclona* sp., Fatty acid, Volatiles, Antibacterial.

INTRODUCTION

Haliclona sp. belongs to the class Demospongiae. Demosponges possess unusual membranes lipids reflecting specific enzyme systems which control biosynthetic properties such as carbon chain elongation and introduction of distinctive double bonds. In particular, many investigations revealed the presence of unique long-chain fatty acids (LCFA, > 24) in demosponges, the so called demospongic acid. These compounds provide excellent targets for chemotaxonomic analyses at the class level[1,2].

Several species of the genus *Haliclona* from tropical and Antarctica region are reported to contain biologically active compounds[3,4]. Many *Haliclona* species are known to be especially rich in sterols[5]. Ang *et al.*[6], isolated manzamine-A, a promising new antimalarial agent. Novel alkaloids are isolated from the sponge *Haliclona cribricutis*[7]. Salicylihamide A was isolated from a *Haliclona* sp. as a selective inhibitor of V-ATPase and has been shown to be 60-fold more cytotoxic to certain cancer cells than to their normal noncancerous counterparts[8]. An antifouling hexapeptide was isolated from *Haliclona* sp.[9]. Two long chain C_{33} polyacetylenic compounds halicynones A and B isolated from the marine sponge *Haliclona* sp., exhibited strong antitumor activity[10]. A pentacyclic sulfated hydroquinone, phuklona sulfate with anti HIV activity was isolated from *Haliclona* sp.[11]. New cerebrosides with long-chain fatty acid moieties have been isolated from an extract of marine sponge *Haliclona* sp.[12]. Lembehyne A, a novel fatty acid (C_{36} linear diacetylene alcohol) derived from the *Haliclona* sp. induces neuronal differentiation in neuroblastoma cell[13]. Novel di-, tri- and tetraenoic fatty acids with *bis*-methylene-interrupted double bond systems were isolated from the sponge *Haliclona cinerea*[14]. The fatty acids (methyl esters) of *Haliclona* sp. collected from Sula-ridge, Norway from 290 m depth and from Gujarat coast were studied[15,16], but the fatty acid profile of the total lipid of the *Haliclona* sp. is not studied.

As described above, a limited literature is available on the total lipid composition of the *Haliclona* sp. The present paper describes the study of fatty acid profile[17], volatiles of the lipid of *Haliclona* sp. and the antibacterial screening of the lipid extract against different pathogens.

EXPERIMENTAL

Sponge material: Specimens of *Haliclona* sp. (class Demospongiae, order Haplosclerida, family Haliclonidae) collected by SCUBA during February 2006 from 13m depth of Bay of Bengal of the Orissa coast, were stored in ethanol and transported to the laboratory. The sample was identified up to genus level by Dr. P.A. Thomas, Ex-Emeritus Scientist (ICAR), Trivandrum, Kerala, India.

Extraction: Ten grams of sponge species was homogenized, air-dried under shade and successively extracted[18] three times with chloroform-methanol (2:1, v/v) to isolate lipids (0.5 g). Crude lipid extracts were purified by "Folch wash" to

remove non-lipid contaminants[19]. The chloroform phase was separated from the combined extract, dried over anhydrous sodium sulphate and concentrated under nitrogen atmosphere. The yield of lipid was *ca.* 5 % of the dry weight of the sponge.

Preparation of the fatty acid methyl esters: Part of lipophilic extract (100 mg) was dissolved in 4 mL of 5 % hydrochloric acid in methanol and 0.5 mL benzene and then the mixture was refluxed in a silicone bath at 80-100 ºC for 2 h. After cooling, the methyl esters were extracted with petroleum ether, simultaneously neutralized and dried over sodium sulphate -sodium bicarbonate mixture. The solvent was evaporated to dryness under reduced pressure at 40 ºC in a rotary evaporator (Heidolph, Laborota 4000). These fatty acid methyl esters (FAME) were then analyzed by GC and GCMS for identification.

Fatty acid methyl ester (FAME) analysis: Fatty acid methyl ester analyses were performed on a Shimadzu QP-5000 GC equipped with FID and a 25 m × 0.25 mm, 0.25 µm film thickness, WCOT column coated with 5 % diphenyl siloxane, supplied by J and W (DB-5). Helium was used as the carrier gas at a flow rate of 1.2 mL/min, at a column pressure of 42 KPa. The column temperature was programmed for fatty acid methyl esters (FAMEs) from 120-300 ºC at 2 ºC/min, 300 ºC for 10 min, with total run time of 100 min using 70 eV ionization voltage (EI). Peak identification was carried out by comparison of their chromatographic retention time with those of authentic standards C4-C24 (Supelco standard FAME mixtures).

GCMS analyses were performed on Shimadzu QP-5000 equipped with a mass selective detector. Operation conditions were-carrier gas, He; ionization voltage, 70 eV and the column condition of use were the same as above. Individual components were identified by comparison of the mass spectra with those available in the NIST and WILEY libraries [Shimadzu-Wiley RegistryTM, 8th Edition Mass Spectral Library and Shimadzu-the NIST 08 mass spectral Library (NIST/EPA/NIH)-New 2008 Version].

Isolation and analysis of the volatile compounds: Part of the lipophilic extract (100 mg) was subjected to a 4 h distillation-extraction in a Lickens-Nickerson apparatus[20]. Volatiles were extracted from the distillate with diethyl ether (yield: 4 mg) and investigated by Shimadzu QP-5000 GC-MS with a 25 m × 0.25 mm, 0.25 µm film thickness, WCOT column coated with 5 % diphenyl siloxane, supplied by J and W (DB-5). Helium was used as the carrier gas at a flow rate of 1.2 mL/min, at a column pressure of 42 KPa. The column temperature was programmed from 40-280 ºC at a rate of 4 ºC/min using 70 eV ionization voltage (EI).

Antibacterial assay of lipid extracts of *Haliclona* sp.: The antibacterial assay of lipid extracts of *Haliclona* sp. collected from 13 m depth (200 µg/6 mm disc) was carried out against five fish pathogens (*Edwardsiella tarda*, *Staphylococcus aureus*, *Micrococcus* sp., *Pseudomonas aeruginosa* and *Escherichia coli*), two human pathogens (*Staphylococcus aureus* and *Salmonella typhii*) including three MDR (multidrug resistant) strains (*Staphylococcus pyogenes*, *Acinetobacter* sp. and *Salmonella typhii*) by disc-assay method[21].

Briefly, each extract (200 µg) in appropriate solvent was applied to a sterile paper disc (6 mm in diameter, Whatman No. 1). After solvent evaporation, the discs were placed on nutrient Agar (Himedia, India) test plates inoculated with the overnight culture of the test pathogen (10^6 CFU/mL) in Brain Heart Infusion (BHI) broth. The plates were incubated for 48 h at 37 ºC. Discs loaded with respective solvent used for dissolution were taken as control after evaporation of the solvent. The zone of inhibition around the disc (average of three experiments) was measured. Determination of minimum inhibitory concentration (MIC) of the lipid extract was carried out by the same method as above.

The test bacterial fish pathogen cultures were obtained from Central Institute of Fresh Water Aquaculture, ICAR, Bhubaneswar. Human pathogens (MDR) were obtained from National Institute of Oceanography, Goa, India.

RESULTS AND DISCUSSION

Analysis of fatty acid methyl ester: The fatty acid composition of sponge *Haliclona* sp. is given in Table-1. They are characterized by linear saturated, short chain unsaturated, long chain unsaturated and long chain poly unsaturated acids.

TABLE-1
GCMS ANALYSIS OF FAME OF TOTAL LIPID OF *Haliclona* sp.

Retention time	Compound	Total (%)
5.365	Tetradecanoic acid (14:0)	2.41
5.836	Tetradecenoic acid (14:1)	2.23
6.136	br-Tetradecanoic acid (br-14:0)	3.78
6.485	Pentadecanoic acid (15:0)	2.39
7.235	br-Pentadecanoic acid (br-15:0)	1.31
7.259	br-Pentadecanoic acid (br-15:0)	1.20
7.970	Hexadecanoic acid (16:0)	19.39
9.664	Heptadecanoic acid (17:0)	4.43
11.335	Octadecanoic acid (18:0)	10.14
11.682	br-Octadecanoic acid (br-18:0)	2.40
13.510	Octadecenoic acid (18:1)	1.07
13.655	9,12-Octadecadienoic acid (18:2)	3.38
18.212	Docosanoic acid (22:0)	0.96
21.585	Tetracosanoic acid (24:0)	2.22
22.243	5,9-Tetracosadienoic acid (24:2)	18.64
23.311	Pentacosanoic acid (25:0)	1.34
24.350	br-Tetracosanoic acid (br-24:0)	3.13
29.028	Heptacosanoic acid (27.0)	8.72
29.748	5,9-Heptacosadienoic acid (27:2)	5.00

br: Branched.

The content of linear saturated acids (C-14 to C-27) was 52 % of the total fatty acid content, where linear fatty acids like 19:0, 20:0, 21:0 and 23:0 were absent. Particularly a high concentration of hexadecanoic acid (16:0, 19.39 %) and octadecanoic acid (18:0, 10.14 %) were revealed in the present investigated *Haliclona* sp. The *Haliclona* sp. collected from Gujarat coast, India also contained good amount of 16:0 (18.5 %) and 18:0 (18.3 %)[16] and these two acids (16:0 and 18:0) were also identified in good amount from a *Haliclona* sp. collected from Sula-ridge, Norway from 290 m depth[15] which are similar with present results and it is chemotaxonomically significant. The above findings suggest that *Haliclona* sp. is a good source of important fatty acids like palmitic acid (16:0) and stearic acid (18:0). The identification of linear fatty acids C14-C21 indicates the presence of symbiotic bacteria[22]. A saturated long chain fatty acid 27:0 was found in a good quantity (8.72 %).

Five branched fatty acids were found, among which br-14:0 (3.78 %) and br-24:0 (3.13 %) were dominant. Br-24:0 is a rare acid which is also found in Caribbean sponge *Cribrochalina vasculum*[23].

Linoleic acid, an important poly unsaturated fatty acid which was identified in many marine sponges generally constituted 0.2-6.6 % of the total fatty acid content[24,25] and the content of this acid is found to be 3.38 % in present investigated *Haliclona* sp.

From literature it was found that in *Haliclona* sp., the polyenic fatty acid is represented exclusively by dienes and the content of LCFA $\Delta^{5,9}$ (long chain fatty acid with double bond at $\Delta^{5,9}$ position) was significant with 27:2 was the major one[15]. In present study, the content of long chain fatty acids $\Delta^{5,9}$ was also significant in *Haliclona* sp. (23.64 %) with 24:2 is the major one (18.64 %) and 27:2 constituted 5.0 % of the total fatty acid content which is an important observation and chemotaxonomically significant.

Volatile compounds: Volatile components of the sponge were isolated by distillation-extraction and investigated by GC-MS. The results obtained are presented in Table-2.

TABLE-2
COMPOSITION OF THE VOLATILE COMPOUNDS IN *Haliclona* sp.

Volatile compounds	Composition (%)
2-Cyclopentene-1-undecanoic acid	6.79
Cyclopentaneundecanoic acid, methyl ester	8.99
5-Cyclopropylcarbonyloxy pentadecane	42.55
1-Chloro-1-hexene(E)	3.76
1,2-Dichlorohexane	15.12
2-Cyclopropylcarbonyloxy tridecane	12.70
1-Chlorodecane	4.37

The literature on sponge volatiles is rather scarce. Roussis *et al*.[26] examined the content of volatile metabolites on two sponges of the genus *Plakortis* (Demospongiae, Homosclerophorida) finding that oxygenated hydrocarbons were the major volatiles in sponges in *P. lita*. In contrast, non-oxygenated hydrocarbons were the major volatiles in *P. angulospiculatus*. Similar results were also obtained in the present study, 5-cyclopropylcarbonyloxy pentadecane was predominant (42.55 % from the total volatile compounds). The percentage of 2-cyclopropylcarbonyloxy tridecane was 12.70 %. The other branched hydrocarbons were 1-chloro-1-hexene (E) (3.76 %), 1,2-dichlorohexane (15.12 %) and 1-chlorodecane (4.37 %). Two free fatty acids 2-cyclopentene-1-undecanoic acid and cyclopentaneundecanoic acid methyl ester were also present in good amount (6.79 and 8.99 %, respectively). These compounds possess some biological activities, which could improve the resistance of the sponge towards pathogens and predators.

Antibacterial assay: The antibacterial screening of the lipid extract of *Haliclona* sp. had exhibited broad spectrum antibacterial activity (Table-3) against all the pathogens at low concentration which was comparable to standard antibiotics tested (Table-4). The MIC value of the test compounds was quite promising as only 100 and 50 µg of the respective lipid extract were required to inhibit the growth of the most of the tested pathogens (Table-5).

The bioactivity of *Haliclona* sp. is already reported in the literature. The methanolic extract and aqueous methanol extract of *Haliclona* sp. exhibited, antiviral, antibacterial and antifungal activities[16,27-29]. The ethanol extract of *Haliclona* sp. collected from Mactan Island, Philippines had shown strong antimicrobial activity against *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*[30].

Antimicrobial activity is exhibited by many lipids of sponges including fatty acids[31-33]. In the present investigation, *Haliclona* sp. contains important fatty acids like oleic acid

TABLE-3
IN VITRO ANTIBACTERIAL ACTIVITY OF LIPID EXTRACT OF *Haliclona* sp. (ZONE OF INHIBITION IN MM INCLUDING 6 mm DISC)

Marine Sponge	Lipid extract (amount per disc)	Diameter of zone of inhibition (mm)									
		S. aureus[1]	*E. tarda*[2]	*S. aureus*[2]	*S. typhii*[1]	*S. pyogenes* (MDR)	*Acinetobacter* sp. (MDR)	*S. typhii* (MDR)	*Micrococcus* sp.[2]	*P. aeroginosa*[2]	*E. coli*[2]
Haliclona sp.	200 µg	6	8	8	7	8	8	9	8	6	Tr

1 = Human pathogen, 2 = Fish pathogen, MDR = Multi drug resistant, Tr = trace inhibition (although observable, the zone of inhibition could not be measured), '–' = Not done.

TABLE-5
MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES OF ACTIVE LIPID EXTRACT (ZONE OF INHIBITION IN MM INCLUDING 6 mm DISC)

Marine Sponge	Lipid extract (amount per disc) (µg)	Diameter of zone of inhibition (mm)									
		S. aureus[1]	*E. tarda*[2]	*S. aureus*[2]	*S. typhii*[1]	*S. pyogenes* (MDR)	*Acinetobacter* sp. (MDR)	*S. typhii* (MDR)	*Micrococcus* sp.[2]	*P. aeroginosa*[2]	*E. coli*[2]
Haliclona sp.	100	Tr	7	-ve	7	7.5	Tr	7	7	7	6
	50	Tr	6.5	-ve	7	6	-ve	6	Tr	Tr	6

1 = Human pathogen, 2 = Fish pathogen, MDR = Multi drug resistant, Tr = trace inhibition (although observable, the zone of inhibition could not be measured), '–' = Not done.

TABLE-4
ACTIVITY OF STANDARD
ANTIBIOTICS AGAINST PATHOGENS

Pathogens	Standard antibiotics		Polymyxin-B (300 U)
	Gentamycin (10 µg)	Streptomycin (10 µg)	
Edwardsiella tarda	S	I	R
Pseudomonas aeroginosa	S	I	R
Escherichia coli	S	S	S
Staphylococcus aureus	S	R	R
Micrococcus sp.	S	S	Not done
Salmonella typhii	S	S	Not done

S = Sensitive (≥ 12 mm), I = Intermediate (9-11 mm), R = Resistant (no zone).

(C18:1, *cis*, 1.07 %), linoleic acid (C18:2, 3.38 %) and a number of branched fatty acids. Hence, the inhibitory activity of the sponge might be partly due to the presence of important essential poly unsaturated fatty acids.

Conclusion

The main focused on sponge lipid mainly for search of new fatty acid structure, evaluating new sources of major poly unsaturated fatty acid of biological interest and also developing tropic and/or chemotaxonomic biomarkers in ecosystem. An important essential polyunsaturated fatty acid *i.e.*, linoleic acid was identified in the lipid composition of the *Haliclona* sp. The content of LCFA $\Delta^{5,9}$ (long chain fatty acid with double bond at 5,9 position), was significant in *Haliclona* sp. and the antibacterial screening of the lipid also showed broad spectrum activity against different human and fish pathogens. So, the lipid composition of *Haliclona* sp. is worth studied.

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