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ANTIBACTERIAL STUDY AND FATTY ACID ANALYSIS OF LIPIDS OF THE SPONGE *Myrmekioderma granulata*

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The composition of the lipophilic extract of the sponge Myrmekioderma granulata *(Esper) collected from 13 m depth of the Bay of Bengal of the Orissa coast was investigated. Fatty acids as well as volatiles and sterols were identified. 4,8,12-Trimethyltridecanoic acid was identified for the first time along with the important PUFAs such as linoleic acid (n-6, C18:2), dihomo-γ-linolenic acid (n-6, C20:3), 5,8,11,14-eicosatetraenoic acid (n-3, C20:4), and 5,8,11,14,17-eicosapentaenoic acid (EPA) (n-3, C20:5) from this species. The branched polyunsaturated fatty acids like br-C26:2, 25-methyl-5,9-heptacosadienoic acid and 24-methyl-5,9-heptacosadienoic acid were also identified by GC-MS. The lipid extract exhibited limited activity against different pathogens.*

Key words: sponge, *Myrmekioderma granulata* (Esper), fatty acid, sterols, volatiles, antibacterial.

The literature shows studies of bioactive components from some species of the genus *Myrmekioderma*. The diterpenes cyanthiwigins A-AA isolated from *Myrmekioderma styx* showed activity against hepatitis, HIV, and tuberculosis [1]. A high-molecular-weight inhibitor specific to the β-1,3-glucanases of marine molluscs has been isolated from the tropical sponge *Myrmekioderma granulata* [2]. It was also found to have antibacterial activity at a concentration of 10 μg/mL against *Escherichia coli* [3], but much less work has been done on the lipid content of different species of the genus *Myrmekioderma*. Myrmekiodermin glycolipid ether was isolated from the marine sponge *Myrmekioderma* sp. [4]. The new lipid myrmekiosides, which have antitumor properties, were isolated from *Myrmekioderma* sp. [5]. Novel iso/anteiso nonacosadienoic acids were also identified from the phospholipid of *Myrmekioderma styx* [6].

This work aims at extending our knowledge on sponge lipids, sterols, and volatiles, especially for the species *Myrmekioderma granulata*, and on its antibacterial activity against different pathogens.

GC-MS analysis (Table 1) of total lipids of *M. granulata* showed the presence of 27 fatty acids. Saturated linear FAs constitute 15.68% of the total FA content, and the acids C16:0, C17:0, and C18:0 were dominant. A significant proportion of the branched saturated fatty acids (28.41%) was present in *M. granulata*, br-C16:0, br-C14:0, and br-C24:0 being the major ones among them. It is considered that saturated iso and anteiso C15-C20 acids have a bacterial origin [7]. The ratio of iso and anteiso FAs was much higher in sponges, a significant part of whose biomass was compounded by bacterial symbionts. Thus, in sponge *M. granulata* the content of iso and anteiso fatty acids may be due to symbiotic bacteria. Only one isoprenoid fatty acid, i.e., 4, 8, 12-trimethyltridecanoic acid, which is very often found in marine sponges, was identified from this species.

The content of 18:1(Δ^9) was dominant (10.82%) among all the linear monoenoic FAs found in *M. granulata*.

Important PUFAs such as linoleic acid (*n*-6, C18:2), dihomo-γ-linolenic acid (*n*-6, C20:3), eicosatetraenoic acid (*n*-3, C20:4), and eicosapentaenoic acid (EPA) (*n*-3, C20:5) were found. Both linear and branched demospongic acids were identified in *M. granulata.* The composition of *M. granulata* FAs of normal structure as well as polyenoic acids is sufficiently characteristic of the marine sponges [8].

This is the first analysis of the fatty acid profile of the total lipid composition of *M. granulata*. Among four species of the genus *Myrmekioderma*, only the fatty acid profile of the phospholipid of the species *M. styx* was studied [6]. We have compared the fatty acid profile of *M. granulata* with the fatty acid profile of the phospholipids of *M. styx*. It is interesting to note that most of the fatty acids of *M. granulata* were also found in the phospholipids of *M. styx*.

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TABLE 1. GC-MS Analysis of FAME of Total Lipid of *Myrmekioderma granulata*

Compound	RT	Total, %	Compound	RT	Total, %
14:0	5.354	1.30	18:2 (9,12)	12.255	1.31
13:0 br	5.593	1.45	19:0	12.667	1.60
14:0 br	5.940	4.21	20:3, *n*-3	16.492	2.41
14:0 br	6.121	1.36	20:4	17.174	0.95
15:0	6.473	0.92	20:5 (5,8,11,14,17)	17.395	1.12
15:0 br	7.235	1.2	23:0	18.663	0.57
16:0	7.947	4.90	24:0 br	22.189	11.21
cis-16:1 (9)	8.354	0.87	25:0 br	25.858	2.14
ai-16:0	8.508	4.97	26:2 (5,9)	27.262	2.95
16:0 br	8.821	1.87	26:2 br (5,9)	29.119	10.75
17:0	9.197	2.54	27:2 (5,9)	29.762	3.43
18:0	11.312	3.85	27:2 br (5,9,25-methyl)	32.892	3.29
18:1 (9)	11.881	10.82	27:2 br (5,9-24-methyl)	35.128	4.84
18:1 (10)	11.648	0.85			

RT: retention time.

TABLE 2. Composition of the Volatile Compounds and Sterols in *M. granulata*

Compound	% of composition	Compound	% of composition
Acids		Hydrocarbons	
2-Nonynoic acid	4.49	Tridecane, 2-methyl	13.46
Hydrocarbons		Tetracosane	10.67
Cyclopropane, [3-chloropropyl] methylene	0.49	Tricosane	8.20
Butane, 1-(2, 2-dichloro-3-ethylcyclopropyl)	0.23	Pentacosane	4.75
1-Pentene, 5-chloro-4-(chloromethyl)	0.39	Sterols	
1-Chlorodecane	7.22	Cholesta-3,5-diene	0.55
1-Nonene, 4,6,8-trimethyl	10.10	Cholesterol	17.81
Decane, 2-methyl	12.06	Ergosta-5,22-dien-3-ol	8.02
Heptadecane, 2-methyl	14.05	Ergosta-5-en-3-ol	4.84
Octadecane, 2-methyl	13.84	β-Sitosterol	68.76

Saturated fatty acids, monobranched saturated fatty acids, and polybranched saturated fatty acids were common in both species, with varying compositions, and their contents were relatively greater in *M. styx* than in *M. granulata*.

MUFAs such as 16:1(Δ^9) and 18:1(Δ^9) were common in both species, while the percentage of 18:1(Δ^9) was abnormally greater in *M. granulata* (10.82%) than in *M. styx* (0.6%). 16:1(Δ^{11}) was present in *M. styx*, while 16:1(Δ^{10}) was present in *M. granulata*. Demospongic acids such as 26:2 ($\Delta^{5,9}$) and 27:2 ($\Delta^{5,9}$) and branched demospongic acids such as 25-methyl C27:2 and 24-methyl C27:2 were common in both species and their percentage compositions were greater in *M. granulata* than in *M. styx*.

A striking difference between the two species is that the important PUFAs such as 20:3($\Delta^{5,8,11}$), 20:5($\Delta^{5,8,11,14,17}$), and 18:2($\Delta^{9,12}$) were present only in *M. granulata* and absent in *M. styx*.

Volatile compounds often possess valuable biological activities. They serve as allelochemicals defending the organism from bacteria, fungi, and viruses. Analogous to other investigated sponges [9], there are a limited number of volatile compounds in *M. granulata* (Table 2), which contains mainly branched saturated hydrocarbons. The significant concentrations of these compounds are an indication of the participation of bacteria (coming from the diet or associated with the tissues) in their formation [9]. Contrary to most of the other marine organisms, the investigated sponge contains no esters of fatty acids.

Sterols were analyzed by gas chromatography (qualitative and quantitative analyses) and by GC-MS. The results obtained are summarized in Table 2. It is evident that, in contrast to most of the marine sponges investigated till now, the sterol composition of this sample is very simple, in accordance with the earlier results of Sica et al. [10]. β-Sitosterol and cholesterol are the main sterols in the sample investigated by us, accompanied by low concentrations of sterols typical of marine sponges.

Some of the minor sterols are characteristic for phytoplankton [11]. Thus, participation of some phytoplankton species cannot be excluded.

The lipids exhibited trace activity against *Staphylococcus aureus* (human pathogen) and *Staphylococcus aureus* (fish pathogen). Antimicrobial activity is exhibited by many lipids of sponges, including fatty acids [12, 13]. *cis*-9-Octadecenoic and *cis*-9,12-octadecadienoic acids [13, 14] have maximum antimicrobial activity. Hence, the inhibitory activity of the fatty acid components of the lipid extract of the sponge might be partly due to the presence of some polyunsaturated fatty acids.

EXPERIMENTAL

General Experimental Procedures. FAME analyses were performed on a Shimadzu QP-5000 GC-MS equipped with FID, and a 25 m × 0.25 mm, 0.25 μm film thickness, WCOT column coated with 5% diphenylsiloxane, supplied by J & W (DB-5). Helium was used as the carrier gas at a flow rate of 1.2 mL/min and a column pressure of 42 KPa. The column temperature was programmed from 120–300°C at 2°C/min, 300°C for 10 min, with total run time of 100 min using 70 eV ionization voltage (EI). Peak identification was carried out by comparison of the mass spectra with those available in the NIST and Wiley libraries.

Sponge Material. Sponge specimen *Myrmekioderma granulata* (Esper) (Class: Demospongiae Sollas, Order: Halichondrida Vosmaer, Family: Axinellidae Ridley and Dendy) was collected from 13 m depth of the Bay of Bengal of Orissa coast during February-March 2006 from newly found ridge lineation [15].

Extraction. The sponges were thoroughly washed, cut, and air-dried. Ten grams of each species was homogenized and successively extracted three times with chloroform–methanol (2:1, v/v) to isolate the lipids. Crude lipid extracts were purified by "Folch wash" [16] to remove nonlipid contaminants. The chloroform phase was separated from the combined extract, dried over anhydrous sodium sulfate, and concentrated under nitrogen atmosphere.

Preparation of Fatty Acid Methyl Esters. The fatty acids so obtained were converted to the corresponding methyl esters. The fatty acids (10 mg) were 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°C for 2 hour. After cooling, the methyl esters were extracted with petroleum ether and simultaneously neutralized and dried over sodium sulfate – sodium biocarbonate mixture. The solvent was evaporated to dryness at reduced pressure at 40°C in a water bath. These fatty acid methyl esters (FAME) were then analyzed by GC-MS for identification.

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 [17]. Volatiles were extracted from the distillate with diethyl ether (yield: 3 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% diphenylsiloxane, supplied by J & W (DB-5). Helium was used as the carrier gas at a flow rate of 1.2 mL/min and a column pressure of 42 KPa. The column temperature was programmed from 40°C to 280°C at a rate of 4°C/min using 70 eV ionization voltage (EI).

Isolation and Analysis of Sterols. Part of the lipophilic extract (100 mg) was chromatographed on a silica gel column with mixtures of hexane and acetone in ascending polarity. The fractions containing sterols according to TLC were combined and purified by preparative TLC with hexane–acetone 9:1 as mobile phase. The sterols obtained (3 mg) were analyzed by GC-MS. The temperature programme was 80–200°C at 2°C/min, 5-min hold, 200–300°C at 10°C/min and a 20 min hold. The injector temperature was 300°C and the detector temperature was 300°C. The carrier gas was helium at a flow rate 1.2 mL/min.

Antibacterial Activity Testing of Lipid Extracts of *Myrmekioderma granulata* (Esper). The antibacterial assay of lipid extracts of *M. granulata* (200 μg/6 mm disc) was carried out against five fish pathogens (*Edwardsiella tarda*, *Staphylococcus aureus*, *Micrococcus* sp., *Pseudomonas aeruginosa*, and *Escherichia coli*) and five human pathogens (*Staphylococcus aureus* and *Salmonella typhii*) including three MDR (Multi drug resistant) strains (*Staphylococcus pyogenes*, *Acinetobacter* sp., and *Salmonella typhii*) by the disc-assay method [18].

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