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FATTY ACID PROFILE AND STEROL COMPOSITION OF THE MARINE SPONGE *Azorica pfeifferae*

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Marine sponges are the most primitive multicellular animals, which contain many bioactive and structurally different metabolites, including lipids [1, 2]. Regular research into the lipids of sponges began in the 1970s [3] and continue till today because of their great diversity of fatty acids, sterols, etc. Studies on fatty acids and sterols as lipid components of sponges and other organisms have been extensively taken up by a number of investigators [4, 5].

Azorica pfeifferae (Carter), is a marine sponge with a cuplike hard structure, which is widely distributed in the Bay of Bengal of the Orissa coast [6]. There are only a few investigations on this species. The alcohol extract of this species has shown antifouling activity [7]. The bacteria isolated from this sponge produces industrial enzymes [8], but no investigation on the chemistry of this species has been done so far. This paper presents a study of fatty acid profile and sterol composition of the total lipid of *A. pfeifferae* for the first time.

FAME Analysis. The fatty acid profile was characterized by linear saturated fatty acids, branched saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids (Table 1). In the sponges, the content of saturated fatty acid can reach 80% of the total FA , as, e.g., in the sponges *Agelas dispar* and *Ciona aprica* [9, 10]. In the present investigation, the percentage of straight chain fatty acids was 30.02% of the total fatty acids. All the saturated fatty acids of linear structure from C11:0 to C25:0 were revealed except C12:0, C13:0, C19:0, and C23:0. Generally, among the saturated fatty acids, C16:0 and C18:0 dominated in most of the sponges, but, interestingly, in the present investigation, the main saturated fatty acid is C25:0 (11.57%), which is a very rare and important observation. There are very few species in which the main saturated fatty acid is a long-chain fatty acid, such as in *C. aprica*, in which the main saturated fatty acid was C24:0 (16.7%) [9]. The total percentage of branched chain fatty acids was found to be 46.63%, which is an important observation. The species *Geodia gibberosa* also contains 40–50% of branched fatty acids [11]. Generally, branched saturated fatty acids with total carbon atoms from C14:0 to C29:0 have been found in sponges among which C15:0–C22:0 acids were the most widespread, which is very much similar to the results obtained from *A. pfeifferae*. Among the branched fatty acids, the amount of 11-methyloctadecanoic acid (18:0) was significantly high, i.e., 25.92% of the total FA. The presence of saturated iso- and ante-iso acids is known to have a bacterial origin [12, 13]. So, the presence of symbiotic bacteria cannot be excluded. In sponges, polymethyl branched saturated FAs are the usual isoprenoid FAs, but it is interesting that no polybranched fatty acid is identified from this species. Generally, in various sponge species, the percentage of monoenes, varies from 2% to 50% of the total FA content. The species under investigation contains only 4.06% of monoenes, which includes 13-C18:1 and 11-C19:1. C16 monoenic acid prevailed in most of the sponge species. This sponge species is unique in that it is completely devoid of C16 monoenic FA. Again, 9-C18:1 and 11-C18:1 are the principal isomers of C18 monoenes in sponges [14], but in the present investigation, 13-C18:1 is identified, which is a notable observation. Another interesting monoene, 11-C19:1, was identified (1.85%) from this species, which is rare [15]. In sponges, the polyenic FAs are represented exclusively by dienes, the ratio of which can reach 6–12% of the total FA [10]. In the present investigation, the polyunsaturated FA of this species is represented by four dienes with a total content of 19.31%. An important PUFA, linoleic acid 9,12-C18:2, which is generally found to be 0.2–6.6% of the total FA in many marine sponges [16, 17], was identified (2.94%) along with three demospongic acids, which include 5,9-C23:2, 5,9-C25:2, and 5,9-C26:2. Among the observed demospongic acids, the content of 5,9-C25:2 was significant (9.64%). 5,9-C23:2, which is rarely observed, was also identified from this species [18].

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TABLE 1. GC-MS Analysis of FAME of Total Lipid of *A. pfeifferae*

Name of FAMEs	Retention time	% of FAME	Name of FAMEs	Retention time	% of FAME
11:0	3.04	1.22	18:0	10.34	6.07
14:0	6.37	0.45	11-19:1	10.47	1.85
13-Methyl-14:0	7.04	2.98	11-Methyl-18:0	10.75	25.92
12-Methyl-14:0	7.12	1.62	17-Methyl-18:0	10.91	1.30
15:0	7.40	0.49	20:0	12.07	0.87
14-Methyl-15:0	8.05	1.03	21:0	12.60	0.73
16:0	8.43	4.89	22:0	13.40	0.67
15-Methyl-16:0	8.87	6.31	15-Methyl-22:0	13.99	3.27
14-Methyl-16:0	9.06	3.37	5,9-23:2	14.30	3.75
17:0	9.13	1.52	24:0	14.51	1.54
16-Methyl-17:0	9.44	0.83	5,9-25:2	15.33	9.64
9,12-18:2	9.87	2.94	25:0	15.50	11.57
13-18:1	10.11	2.21	5,9-26:2	16.29	2.98

TABLE 2. Sterol Composition of *A. pfeifferae*

Compound	Retention time	Content, %	Mass fragmentation
Ergosta-5,22-dien-3β-ol	32.030	0.59	398, 385, 366, 351, 324, 314, 299, 273, 282, 255, 227, 213
Cholestane-3β-ol methyl ether	32.150	0.29	402, 387, 370, 355, 345, 328, 300, 271, 262, 248, 230, 215, 201
Cholesteryl methyl ether (5-cholesten-3β-ol methyl ether)	32.358	3.58	400, 385, 368, 353, 326, 301, 275, 255, 247, 228, 213, 199
Stigmasta-7,22-dien-3β-ol	33.083	4.76	412, 398, 380, 365, 353, 338, 314, 299, 285, 267, 255, 228, 213, 199
Ergosta-7-en-3β-ol	33.908	2.19	400, 385, 382, 368, 353, 313, 299,287, 274, 255, 229, 213, 199, 187
β-Sitosterol	34.217	2.49	414, 399, 382, 367, 340, 315, 300,289, 255, 228, 213, 199
Stigmasta-5,22-dien-3β-ol methyl ether	34.525	1.87	426, 414, 415, 394, 379, 367, 351,328, 314, 300, 285, 267, 255, 213, 199
Stigmast-5-en-3β-ol methyl ether	35.808	2.85	428, 413, 412, 396, 366, 354, 329, 303, 287, 275, 255, 213, 199
Stigmast-7-en-3β-ol	36.217	16.43	414, 382, 367, 357, 327, 301, 287, 274, 255, 245, 229, 213, 201, 187
Stigmasterol methyl ether	36.733	0.90	426, 411, 394, 379, 361, 328, 313, 296, 281, 271, 253, 239, 229, 211, 199
Cholest-7-en-3β-ol, acetate	38.433	64.05	428, 413, 396, 381, 371, 355, 329, 315, 302, 287, 273, 255, 245, 229, 213, 201

Sterol Analysis. A total of 11 individual mono- and di-unsaturated sterols were identified from the liphophilic extract of *A. pfeifferae.* The retention time and mass fragmentation pattern are presented in Table 2. The sterols were identified by comparing their mass fragmentation pattern with the NIST/Wiley library and were confirmed by the literature. The most abundant sterol was cholest-7-ene-3β-acetate (64.05%). A number of the sterols were found as sterol ethers, along with stigmasterol and β-sitosterol. Stigmasterol exhibited profound inhibitory effect on HIV reverse phase transcriptase and tumor promotion in two-stage carcinogenesis in mice [19–20]. Sitosterol also inhibited carcinogenesis and tumor promotion *in vivo* [20]. The anti-inflammatory activity of the mixture of sitosterol and stigmasterol after topical application is also reported in the literature [21]. Sterol ethers with a C-7 double bond are used for the treatment of some prostate problems, and these are very rarely found in plants [21]. Interestingly, a number of sterols with the C-7 double bond were identified from this species. Five sterol ethers (8.59% of total sterol content) were identified from this species, which are well characterized by the loss of the ether moiety in the mass fragmentation pattern. A sterol ether, 24(*R*)-methyl-5α-cholest-7-enyl-3β-methoxymethyl ether, isolated from the marine sponge *Scleritoderma* sp. cf. *paccardi* was found to show cytotoxic activity [22]. So, the presence of a number of sterol ethers in *A. pfeifferae* may be of practical importance.

A specimen of *Azorica pfeifferae* (Carter) (Class: Demospongiae, Order: Epipolasida Sollas, Family: Scleritodermidae Sollas) was collected by SCUBA from 30 m depth in the Bay of Bengal during Februry–March 2007 from the coral ridge of lineation. The collected sponge material was immediately put into 70% methanol during transport to the laboratory for preservation and identification.

The sponge sample was thoroughly washed with distilled water and air-dried under shade. Ten grams of sponge material was homogenized, air-dried under shade, and successively extracted three times with chloroform–methanol (2:1, v/v) to isolate lipids. The crude lipid extracts were purified by "Folch wash" to remove non-lipid contaminants. The chloroform phase was separated from the combined extract, dried over anhydrous sodium sulfate, and concentrated under nitrogen atmosphere.

Part of the lipophilic extract (100 mg) was taken in 5 mL of ethanolic KOH and then refluxed on a water bath at 70–80°C for 4 hours. After cooling, the free fatty acids were isolated and dried over anhydrous sodium sulfate. The free fatty acids were dissolved in 4 mL of 5% HCl in methanol and 0.5 mL of benzene, and then the mixture was refluxed on a water bath at 70–80°C for 2 hours. After cooling, the methyl esters were extracted with petroleum ether, and simultaneously neutralized and dried over anhydrous sodium sulfate. The solvent was evaporated to dryness at reduced pressure at 40°C in a water bath. These fatty acid methyl esters (FAMEs) were analyzed by GC-MS for identification.

FAME analyses were performed on an Agilent GC-MS system equipped with FID on an HP-1 column (25 m × 0.25 mm, 0.25 μm film thickness). 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 for fatty acid methyl esters from 120–280°C at 2°C/min, 280°C for 10 min, with a total run time of 90 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 and confirmed by comparison of retention times as well as mass fragmentation of authentic standards C4–C24 (Supelco standard FAME mixtures).

Isolation and Analysis of Sterols. Part of the lipophilic extract (100 mg) was taken in 5 mL of ethanolic KOH and then refluxed on a water bath at 70–80°C for 4 hours. The solution was diluted with distilled water and extracted with diethyl ether. The ether extract was dried and freed of the solvent by evaporation at 40°C to get an unsaponifiable portion, which is chromatographed on a silica gel column with a mixture of hexane and acetone with increasing polarity. The fractions containing sterols, according to TLC, were combined and purified by preparative TLC with hexane–acetone 9:1 as the mobile phase. The obtained sterols (3 mg) were analyzed by GC-MS. The temperature program was 150–200°C at 6°C/min and 5 min hold, and 200–280°C at 4°C/min and 20 min hold. The injector temperature was 300°C and the detector temperature was 300°C. The carrier gas was helium, and the sterols were identified.

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