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# CHEMICAL INVESTIGATION AND ANTIBACTERIAL STUDY OF HEXANE EXTRACT OF LEAVES OF *Finlaysonia obovata*

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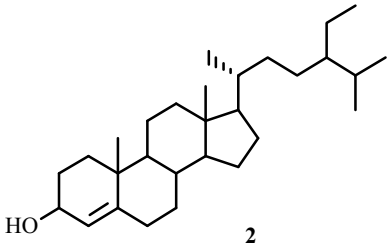
*Finlaysonia obovata*, a latex – exuding mangrove plant (Fam. Periplocaceae), is found in the tidal flats in India, Burma, and Malay, the leaves of which are reported to be eaten as salad in the Moluccas. Mangrove latex-bearing plants were found to show antibacterial and antiviral activity [1]. Earlier we have studied the antibacterial activity, performed the GCMS analysis of extracts, studied the lipids of the leaf, and isolated a rare antibacterial triterpene from *F. obovata* [2, 3]. The present paper deals with the antibacterial screening of column fractions of an active hexane extract, and isolation and spectral characterization of stigmast-4-en-3$\beta$-ol from leaves of *F. obovata*.

The antibacterial assay of the hexane extract of leaves of *F. obovata* was carried out against seven freshwater fish pathogenic bacteria (see Experimental) [3]. The hexane extract was found active against all except *S. aureus* and *E. tarda*. This extract was further taken up for fractionations and isolations of secondary metabolites. The results of anti-pathogenic screening of the active column fraction of the hexane extract [compound **2** is isolated] are presented in Table1. The column fraction (EtOAc:hexane-1:9) showed activity against *S. aureus*, which clearly proves the enrichment of the active components during fractionation.

Column chromatography of the hexane extract over silica gel yielded two compounds **1, 2**, which were identified by color reactions, TLC, IR, and preparation of acetates. Fraction 1 (hexane–EtOAc, 9.7:0.3) eluate, on crystallization from ethyl acetate, afforded a crystalline solid, lupeol acetate (**1**).

Fraction 2 (hexane–EtOAc, 9:1), after purification and after repeated crystallization from methanol, yielded a white crystalline compound **2**, mp 122–123°C. It gave a positive Liebermann-Burchard test for steroids. Also it gave a purple color with acid spray, which indicated that **2** might be a sterol.




**2**

Its molecular weight by mass spectrometry was 414, and elemental analysis gave the formula $C_{29}H_{50}O$. The IR spectrum showed the presence of hydroxyl group (3382 and 1034 $cm^{-1}$) and unsaturation (1658 $cm^{-1}$ and 970 $cm^{-1}$). EI mass spectrum of the compound displayed a molecular ion $[M]^+$ at *m/z* 414 (100%), in agreement with the formula $C_{29}H_{50}O$. Other major fragments were at *m/z* 399, 396, 381, 381, 351, 273 , 255, and 213. The fragmentation pattern suggested that it was a $C_{29}$ sterol with one double bond and a $C_{10}$ saturated side chain.

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TABLE 1. Screening results of Active Column Fraction of Hexane Extract of Leaves of *Finlaysonia obovata* [from Which Compound **2** Is Isolated]. (Zone of Inhibition in mm including 6 mm disc)

| Sample | *Micrococcus* sp. | *A. hydrophila* | *E. coli* | *P. aeruginosa* | *V. alginolyticus* | *S. aureus* | *E. tarda* |
|---|---|---|---|---|---|---|---|
| Column fraction (EtOAc:H-1:9) | 12 mm | Trace | -ve | 12 mm | 13 mm | 12 mm | -ve |

EtOAc = ethyl acetate; H = hexane; (-ve) = no zone.

$^1$H NMR spectrum of compound **2** supported the existence of a trisubstituted double bond [(δ 5.35, a doublet)] as well as a terminal isopropyl group in the side chain [δ 0.83 (3H, d, J = 6.8 Hz, $C_{26/27}$-Me), δ 0.85 (3H, d, J = 6.8 Hz, $C_{27/26}$-Me)]. Two singlets at δ 0.61 and 1.23 were present, corresponding to $C_{18}$ and $C_{19}$ methyl protons, respectively. One triplet and one doublet each integrating for 3H appeared at δ 1.02 and 1.15 due to $C_{29}$ and $C_{21}$ methyl protons, respectively. Signals due to protons attached to carbon-bearing –OH group were observed at δ 3.5 (m). The OH group at C-3 is $\beta$ and equatorially oriented on the basis of biogenetic considerations. The OH group at C-3 was also established from its $^{13}$C NMR spectrum by the signal of the C-3 at 71.79 by comparison with the $^{13}$C NMR values of the compound having an allylic $\beta$-hydroxyl group, *viz.*, 7$\beta$-hydroxysitosterol [4] and bufadienolides [5].

$^{13}$C NMR assignment of compound **2** was based on DEPT experiments in comparison with the data of related compounds, *viz.*, stigmast-5-en-3-ol, stigmast-4-en-3-one, and 7$\beta$-hydroxysitosterol [4]. The fragments at *m/z* 55, 69 also indicated that the hydroxyl was in ring A, at C-3. The MS-data further confirmed the identity of compound **2** as stigmast-4-en-3$\beta$-ol [6].

The mass fragmentation pattern, NMR data, and DEPT spectrum values of the compound with a melting point of 122–123°C (Lit. value 124–125°C) revealed that it was stigmast-4-en-3$\alpha$-ol. It was first prepared by reducing cholestenone with aluminum isopropylate [7]. The formation of the 4-en-3$\beta$-ol derivative by a biosynthetic scheme from campestanol is ubiquitous in the plant kingdom [8].

The results of the present study showed that the column fractions of the hexane extract of the leaf have strong antibacterial activity against fish pathogens. Literature reveals that the hexane and chloroform extract of the plants show antimicrobial activity, antiinflammatory activity, etc. [9 – 12]. The hypoglycemic effect of stigmast-4-en-3$\beta$-ol has already been reported in the literature [6].

Hence, the inhibitory activity of the hexane extract is probably due to the presence of stigmast-4-en-3$\beta$-ol.

**Plant Material.** *F. obovata* is collected from Bhitarkanika mangrove forest of Orissa (during the late winter season) and was identified by K.S. Murthy, I/C SMP unit, Central Research Unit (AY), Bhubaneswar.

The $^1$H NMR spectra were recorded at 400 MHz and $^{13}$C NMR spectra at 100 MHz on a AL-400 MHz FT-NMR, (JEOL, Japan).

**Extraction of the Plant Material.** The leaves of the plant (1 kg) were cut, shade dried, and powdered, and extraction was carried out with different solvents (1:2 vol./vol., thrice) sequentially in increasing order of polarity using hexane, chloroform, ethyl acetate, and alcohol by soaking overnight at ambient temperature. The extracts were freed from solvent under reduced pressure. The residues thus obtained are finally dried under vacuum and used for *in vitro* screening of antibiotic activity.

**Antibiotic Activity Testing of Extract/Column Fractions.** The antibacterial activity of the hexane extract (500 µg/50 µL/6 mm disc) has already been tested against seven freshwater fish pathogenic bacteria, viz., *Micrococcus Sp*. (multidrug resistant strain), *Aeromonas hydrophila, Pseudomonas aeruginosa*, *Vibrio alginolyticus*, *Staphylococcus aureus*, *Escherichia coli*, and *Edwardsiella tarda* by the disc-assay method [13]. The hexane extract was found active against all except *S. aureus* and *E. tarda*. So, this extract was further taken up for fractionation, and the antibacterial screening of column fractions of the hexane extract (200 µg/50 µL /6 mm disc) was carried out.

Briefly, extract (500 µg/50 µL) and column fractions (200 µg /50 µL) of the appropriate solvent were applied to sterile filter paper discs (6 mm diameter). After solvent evaporation the discs were placed on nutrient agar (Himedia, India) test plates inoculated with an overnight culture of the test pathogen ($10^6$ CFUmL$^{-1}$) in brain heart infusion (BHI) broth. The plates were incubated for 48 h at 37°C. Discs loaded with the respective solvent 50 (µL) 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.

The test bacterial pathogen cultures were obtained from stock cultures maintained in the Pathology Laboratory of the Central Institute of Freshwater Aquaculture, ICAR, Bhbaneswar.

**Fractionation of Hexane Extract and Isolation of Pure Compounds.** The active crude hexane extract was chromatographed on a column packed with silica gel (100–200 mesh), and the fractions were monitored by thin layer chromatography (TLC). The hexane-ethyl acetate (9.7:3) eluate on crystallization from a $CHCl_3$–MeOH mixture afforded a crystalline solid, lupeol acetate (**1**). The hexane-ethyl acetate (9:1) eluate on crystallization from methanol afforded a crystalline solid, compound **2**.

**Lupeol acetate (1):** crystallized from $CHCl_3$–MeOH, 7 mg; mp 218–219°C; MF: $C_{32}H_{52}O_2$; EIMS *m/z:* 468 $[M]^+$; $[\alpha]_D^{29}$ +28.4° (0.1g/100 mL, $CHCl_3$); IR (KBr, ν, $cm^{-1}$): 1725, 1245, 872, 898, $^1$H NMR (400 MHz, $CDCl_3$, δ): 0.76, 0.79, 0.84, 0.95–0.98 (6H), 1.04, 1.70. Identical with lupeol acetate by mmp and superimposable IR.

**Compound 2:** crystallized from $CHCl_3$–MeOH. (10 mg); mp (122–123°C) (Lit. value 124–125°C); $[\alpha]_D^{29}$ –35° (*c* 0.1g/100 mL, $CHCl_3$); MF: $C_{29}H_{50}O$. EIMS *m/z*: 414 $[M]^+$; IR ($CHCl_3$, ν, $cm^{-1}$): 3382, 2989.38, 2366.62, 1658, 1034, 970; $^1$H NMR (400 MHz, $CDCl_3$, δ): 0.61 (3H, s), 0.83 (3H, d), 0.85 (3H, d), 1.23 (3H, s), 1.02 (3H, t), 1.15 (3H, d), 1.2 (3H, s), 3.5 (1H, d), 5.35 (1H, d); $^{13}$C NMR (100 MHz, $CDCl_3$, δ): 37.23 (C-1, $CH_2$), 31.65 (C-2, $CH_2$), 71.79 (C-3,CH), 121.72 (C-4,CH), 140.74 (C-5, C), 25.41 (C-6, $CH_2$), 31.89 (C-7, $CH_2$), 30.93 (C-8, CH), 50.09 (C-9, CH), 36.48 (C-10, C), 21.05 (C-11, $CH_2$), 39.74 (C-12, $CH_2$), 42.29 (C-13, C), 56.74 (C-14, CH), 24.29 (C-15, $CH_2$), 28.23 (C-16, $CH_2$), 56.01 (C-17, CH), 11.96 ( C-18, $CH_3$), 19.38 (C-19, $CH_3$), 36.13 (C-20, CH), 18.76 (C-21, $CH_3$), 33.91 (C-22, $CH_2$), 26.01 (C-23, $CH_2$), 45.80 (C-24, CH), 29.11 (C-25, CH), 19.02 (C-26, $CH_3$), 19.82 (C-27, $CH_3$), 23.03 (C-28, $CH_2$), 11.83 (C-29, $CH_3$); EIMS *m/z:* 414 $[M]^+$, 399 $[M\text{-}Me]^+$, 396 $[M–H_2O]$, 381 [396–Me], 351 $[381–2\times Me]^+$, 273 [M-side chain]$^+$, 255 $[273–H_2O]^+$, 213 [255-ring D fusion].

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