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Isolation and Cytotoxic Activity of Acyclic Triterpene Callicarpenol from *Callicarpa macrophylla*

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The dried fruit tissue of *Callicarpa macrophylla*, one new acyclic triterpene, 2,6,14,18,22-pentamethyl-*n*-tetracos-9-en-17α-ol-27-oic acid (**1**) was isolated along with known compounds β-sitosterol, oleanolic acid and β-sitosterol-3-O-β-D-glucoside.The structure of the new compound was elucidated by 500 MHz NMR using 1D and 2D spectral methods, *viz.*, ^{1}H, ^{13}C, ^{1}H-^{1}H COSY and HETCOR aided by EIMS, CIMS, FABMS and IR, UV and chemical methods. The compound **1** was found to have cytotoxic effects against P388 murine leukemia cells, the β-sitosterol and β-sitosterol-3-O-β-D-glucoside had no cytotoxicity.

Key Words: Callicarpenol, Acyclic triterpene, *Callicarpa macrophylla*, Cytotoxic activity.

INTRODUCTION

Callicarpa macrophylla Vahl. (Verbenaceae), a shrub growing abundantly in the Upper Gangetic plain, is reported to be useful in rheumatic disorders. The leaves, applied hot, are reported to give relief in rheumatic pains. A paste of the seed is employed in treating oral ulcers, in leprosy and as diuretic; the seeds and roots are used as stomachic[1]. Due to the medicinal importance of *C. macrophylla* in the Indian system of medicine[2], chemical investigations of different parts like seeds, leaves and bark of the plant have been carried out by several groups[3–16]. However, the fruits have not been investigated chemically; for instance, its fruits are used by the Tharus of Kheri District, Uttar Pradesh, India for blisters and boils on tongue[17].

The constitutents of *C. macrophylla* fruit tissues have been examined and isolated one new acyclic triterpene callicarpenol **1** along with the known compounds β-sitosterol, oleanolic acid, β-sitosterol-3-O-β-D-glucoside and other compounds, mostly sugar molecules. This paper deals with the isolation and structural elucidation of the new acyclic triterpene and its cytotoxicity.

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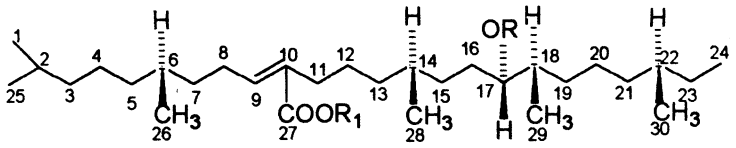

Fig. 1. Chemical structures of **1**, **1a** and **1**: **1**: R = H; R_1 = H; **1a**: R = Ac, R_1 = H; **1b**: R = H, R_1 = Me.

EXPERIMENTAL

Melting points were determined on Electrochemical Eng. melting point apparatus and optical rotation was measured on an AA-10 model polarimeter. IR spectra were recorded on a Thermo Mattson 60-AR spectrophotometer. UV spectra were recorded using a UV-Vis spectrometer TU-180_{PC}. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were obtained on a Brucker Avance (DRX-500) using $CDCl_3$, CD_3OD, C_5D_5N as solvents. EI-Mass spectra were recorded on a JEOL JMS-SX 102 A and CIMS on a Jeol JMS-AX 505 WA. Column chromatography was performed over silica gel 70-230 (Merck) or ODS silica gel [Lichroprep RP-18 (40–63 μm)] (Merck). HPLC was performed on a C-18 column by using isocratic solvent system (MeOH : H_2O, 9 : 1) and a flow rate of 1 mL/min. TLC analyses were performed on precoated silica gel glass plates 60 F_{254} (Merck) and visualized under UV light and by spraying with (vanillin 1 g : sulfuric acid 5 mL : ethanol 94 mL) solution followed by heating (100–110°C).

Bioassay for cytotoxic activity: The cytotoxic assays were performed by using the 3-(4,5-dimethy(thiazol-2-yl)2,5-diphenyl) tetrazolium bromide (MTT) assay method[18]. The murine P388 leukemia cells were cultured in RPMI 1640 medium (Nissui) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and kanamycin (5.3 mL/L) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. 100 μL of cell suspension was added to each well (3×10^3 cells/well) of a 96-microwell plate (Iwaki, flat bottom, treated polystyrene) and incubated for 24 h. Test compounds were dissolved in DMSO in various concentrations (100, 30, 10, 3, 1, 0.3, 0.1 μg/mL) and 10 μL of the test solution or DMSO (control) was added to each well. The plate was kept in an incubator for 48 h. After termination of cell culture by adding 20 μL MTT (5% in PBS) to each well, the plate was further incubated for 4 h. To each well was added 100 μL of 10% SDS-0.01 N HCl. The plate was read on a microplate reader (MPR A4i, Tosoh) at 550 nm. A dose-response curve was plotted for each compound and the concentrations giving 50% inhibition of cell growth (IC_{50}) were recorded.

Plant material: Fruit tissue (250 g) of *C. macrophylla* was collected from the Himalayas of Kumaun region, Nanital city (India) in August 2001 when white in colour and dried at low temperature in an oven and authenticated by Prof. Y.P.S. Pangtey, plant taxonomist, Department of Botany, Kumaun University, India. A voucher specimen (No. KUPH 70-71) was deposited at the herbarium of Kumaun University, Nainital, India.

Extraction and isolation: The dried powered fruit tissue (250 g) of *C. macrophylla* after defatting with petroleum ether (40–60°C) was extracted with 95% EtOH (3 × 1 L) at room temperature. The combined extract was evaporated to dryness (30 g). The residue was separated on a silica gel column (70–230 mesh, 700 g, 5.0 × 75 cm) and eluted with a gradient of hexane (hex), hex/EtOAc (9 : 1, 8 : 2, 7 : 3, 1 : 1), EtOAc, EtOAc/MeOH (9.5 : 0.5, 9 : 1, 7 : 3) and MeOH (each fraction 500 mL): fractions 1–2 in hex, 3–5 in hex/EtOAc (9 : 1), 6–11 in hex/EtOAc (8 : 2), 12–15 in hex/EtOAc (7 : 3), 16–20 in hex/EtOAc (1 : 1), 21–22 in EtOAc, 23–28 in EtOAc/MeOH (9.5 : 0.5), 29–30 in EtOAc/MeOH (9 : 1), 31–34 in EtOAc/MeOH (7 : 3) and 35–40 in MeOH. The initial fractions 1–5 were insufficient to purify a compound, while fraction 6 (2.4 g) was chromatographed on a silica gel (70–230 mesh 100 g) by normal full form using CH_2Cl_2/MeOH (99.8 : 0.2, 99.6 : 0.4, 99.4 : 0.6, 99.2 : 0.8, 99 : 1, each fraction 200 mL) as eluants to yield five fractions. Fractions 1–3 were combined on the basis of TLC to give pure compound β-sitosterol (100 mg; R_f = 0.38; CH_2Cl_2/MeOH, 97 : 3) and also confirmed with authentic sample from Sigma through Co-TLC. Fractions 16 and 17 were crystallized in a tube and the solid compound insoluble in hexane and after filtration through sintered funnel was obtained in the impure state (yield 50 mg); the same was chromatographed on an ODS silica gel (Lichroprep RP-18, Merck) by normal CC using sequential mixtures of H_2O/MeOH as eluants (elution order 80 : 20, 60 : 40, 40 : 60, 20 : 80, MeOH (100%) and each fraction 150 mL) to yield five fractions. Fractions 4 (30 mg) in H_2O/MeOH (20 : 80) still showed impurities in reverse phase TLC and was further purified by C_{18} HPLC column by using MeOH/H_2O (9 : 1) as solvent system to collect the pure compound **1** (R_f = 0.41; $CHCl_3$/MeOH, 9.5 : 0.5). The HPLC chromatograms of compound **1** before and after separation are shown in Figs. 3 and 4. Fractions 21 (2.1 g) was chromatographed on silica gel by normal CC using $CHCl_3$/MeOH to give only oleanolic acid (5 mg) and fractions 23 (2.1 g) after CC over silica gel using $CHCl_3$/MeOH yielded the pure compound β-sitosterol-3-O-β-D-glucoside (80 mg). Oleanolic acid and β-sitosterol-3-O-β-D-glucoside were identified through Co-TLC with the previously isolated sterol glycosides from several plants. The other polar fractions contain only sugar molecules on the basis NMR.

2,6,14,18,22-pentamethyl-*n*-tetracos-9-en-17α-ol-27-oic acid (1): R_f 0.41 ($CHCl_3$: MeOH, 9.5 : 0.5); colourless powder; m.p. 219–221°C; $[\alpha]_D^{24}$ + 12.40° (MeOH); UV λ_{max} (MeOH) 216 nm; IR (KBr, cm^{-1}) ν_{max}: 3435 (OH), 3385, 2927, 2867, 1693, 1635, 1458, 1383, 1279, 1235, 1150, 1035, 990; FABMS (positive mode) m/z 467 $[M + H]^+$; FABMS (negative mode) m/z 465 $[M - H]^-$; CIMS (%) m/z 467 (MH^+, 8); EIMS m/z (rel. int., %) 466 $[M]^+$ ($C_{30}H_{58}O_3$) (4.8), 253 (100), 237 (4.1), 213 (11.3), 211 (28.7), 207 (42.3), 192 (13.8), 177 (13.6), 162 (4.2), 157 (4.9), 149 (4.1), 147 (7.9), 135 (6.5), 133 (30.6), 122 (3.6), 121 (7.8), 119 (13.5), 107 (7.9), 96 (9.1), 94 (8.1), 80 (8.3), 69 (9.5), 55 (11.9); ^1H NMR (500 MHz; MeOD) and ^{13}C NMR (125 MHz; MeOD) δ (Table-1).

Acetylation of (1): Compound **1** (2 mg) was refluxed with acetic anhydride (1 mL) and pyridine (0.5 mL) at 80°C over a water bath for 4 h. After usual work up, **1** yielded solid compound **1a**; m.p. 143–145°C; ^1H-NMR (500 MHz; $CDCl_3$) δ (Table-1).

TABLE-1
^1H AND ^{13}C NMR DATA OF COMPOUND **1**[a] AND ITS DERIVATIVES **1a**[b] (ACETYL) AND **1b**[b] (METHOXY DERIVATIVE)

Position	^1H		^{13}C	^1H (acetyl derivative)		^1H (methoxy derivative)	
	α	β		α	β	α	β
1.	0.97 d (3.1)	–	17.79	0.99 d (3.1)	–	0.95 d (3.1)	–
2.	2.21 m	–	40.59	2.11 m	–	2.17 m	–
3.	1.37 m	1.07 m	28.92	1.35 m	1.17 m	1.33 m	1.08 m
4.	1.39 m	1.29 m	24.52	1.38 m	1.31 m	1.49 m	1.30 m
5.	1.37 m	1.02 m	24.23	1.32 m	1.17 m	1.28 m	1.14 m
6.	1.62 m	–	54.55	1.64 m	–	1.63 m	–
7.	2.03 m	1.07 m	38.28	2.08 m	1.17 m	2.01 m	1.22 m
8.	2.19 d (11.3)	1.93 dd (3.5, 5.2)	39.97	2.18 d (11.3)	1.95 d (3.5)	2.18 d (11.3)	1.92 m
9.	5.22 m	–	127.03	5.28 d (11.3)	–	5.26 m	–
10.	–	–	139.82	–	–	–	–
11.	2.03 dd (3.7, 9.8)	1.92 m	40.16	2.23 m	2.02 m	2.33 m	1.90 m
12.	1.33 m	1.57 m	34.50	1.40 m	1.58 m	1.49 m	1.56 m
13.	1.49 m	1.51 m	28.92	1.46 m	1.52 m	1.34 m	1.52 m
14.	1.65 m	–	56.91	1.67 m	–	1.68 m	–
15.	1.35 m	1.02 m	25.49	1.35 m	1.14 m	1.32 m	1.14 m
16.	1.33 m	1.39 m	40.95	1.37 m	1.40 m	1.34 m	1.49 m
17.	–	3.14 ddd (4.0, 7.0, 4.2)	79.87	–	4.51 ddd (4.0, 7.0, 4.3)	–	3.13 m
18.	1.93 m	–	40.57	1.91 m	–	1.91 m	–
19.	1.07 m	1.29 m	28.92	1.23 m	1.26 m	1.22 m	1.28 m
20.	1.35 m	1.02 m	28.92	1.37 m	1.08 m	1.33 m	1.08 m
21.	1.37 m	1.37 m	25.49	1.52 m	1.55 m	1.52 m	1.56 m
22.	1.68 m	–	43.41	1.69 m	–	1.68 m	–
23.	1.35 m	1.29 m	28.06	1.35 m	1.31 m	1.34 m	1.30 m
24.	1.00 t (12.2)	–	19.63	0.99 t (11.2)	–	0.92 t (11.2)	–
25.	0.87 d (6.4)	–	17.98	0.93 d (6.1)	–	0.94 d (6.1)	–
26.	0.85 br s	–	21.72	0.86 d (6.4)	–	0.88 d (6.4)	–
27.	–	–	181.92	–	–	–	–
28.	0.89 d (6.4)	–	29.38	0.91 d (6.3)	–	0.90 d (6.3)	–
29.	0.78 d (7.1)	–	16.17	0.78 d (7.1)	–	0.80 d (7.0)	–
30.	1.11 br s	–	16.52	1.07 br s	–	0.98 br s	–
Others	–	–	–	2.05 br s (OAc)	–	3.16 br s (OMe)	–

[a] In CD_3OD. [b] In $CDCl_3$. J values (in Hz) are in parenthesis

Methylation of (1): Compound **1** (2 mg) was taken in a flask and ethereal solution of diazomethane was added at room temperature. After usual wok up, **1** yielded **1b**; ^{1}H-NMR (500 MHz; $CDCl_3$) δ (Table-1).

RESULTS AND DISCUSSION

The ethanol extract of *C. macrophylla* fruits was separated by column chromatography over silica gel, Lichroprep RP-18 (ODS silica gel) and after HPLC (high pressure liquid chromatography) separation over C_{18} column yielded one new acyclic triterpene compound **1** named as callicarpenol, which gave effervescence with sodium bicarbonate solution and decolorized bromine water indicating the presence of a carboxylic group and unsaturation in the molecule. Its IR spectrum displayed characteristic absorption bands for hydroxyl (3435 cm^{-1}) and carboxyl (3385, 1693 cm^{-1}) groups and unsaturation (1635 cm^{-1}). Its mass spectrum displayed a molecular ion peak at m/z 466 in the EIMS, CIMS and FABMS (positive and negative mode) consistent to a molecular formula for acyclic triterpene, $C_{30}H_{58}O_3$. It indicated two double bond equivalents; each one of them was adjusted in the olefinic linkage and the carboxylic group. The prominent ion peaks appearing at m/z 211 $[C_{10}–C_{11}$ fission$]^+$, 237 $[M\text{-}211\text{-}H_2O]^+$, 253 $[C_{13}–C_{14}$ fission$]^+$, 207 $[253\text{-}HCOOH]^+$, 192 $[207\text{-}Me]^+$, 149 $[192\text{-}C_3H_7]^+$, 135 $[149\text{-}CH_2]^+$, 121 $[135\text{-}CH_2]^+$, 107 $[121\text{-}CH_2]^+$, 177 $[192\text{-}Me]^+$, 162 $[177\text{-}Me]^+$, 147 $[162\text{-}Me]^+$, 122 $[207\text{-}C_6H_{13}]^+$, 94 $[122\text{-}C_2H_4]^+$, 80 $[94\text{-}CH_2]^+$ and 157 $[C_{16}\text{-}C_{17}$ fission$]^+$ suggested the existence of vinylic linkage at Δ^9, carboxylic function at C-27 and hydroxyl group at C-17. The mass fragmentation patterns also are shown in Fig. 2.

The ^{1}H-NMR spectrum of **1** displayed a one-proton deshielded multiplet at δ 5.22 assigned to vinylic H-9. A one-proton doublet of double doublet at δ 3.14 (J = 4.0, 4.2, 7.0 Hz) was attributed to α-oriented H-17 carbinol proton. A three-proton triplet at δ 1.0 (J = 12.2 Hz) was ascribed to C-24 primary methyl protons. Four doublets at δ 0.97 (J = 3.11 Hz), 0.87 (J = 6.4 Hz), 0.89 (J = 6.4 Hz) and 0.78 (J = 7.1 Hz) and two broad signals at δ 0.85 and 1.11, all integrating for three protons each, were assigned to C-1, C-25, C-28, C-29, C-26 and C-30 secondary methyl protons, respectively. Two one-proton double doublets at δ 1.93 (J = 3.5, 5.2 Hz) and δ 2.03 (J = 3.7, 9.8 Hz); a one-proton doublet at δ 2.19 (J = 11.2 Hz) and a one-proton multiplet at δ 1.92 were associated to C-8 and C-11 methylene protons adjacent to the vinylic linkage. The remaining methylene and methine protons resonated in the range δ 2.21-1.02 (Table-1). The presence of all methyl signals between δ 0.78–1.11 indicated their location at the saturated carbons. The absolute configuration of **1**, the 8β-proton signal at δ 1.93 with coupling constants of 3.5 and 5.2 Hz due to interaction of 8, 9-*cis* indicated *cis*-orientation of 9H. The β-configuration of C-26, C-28, C-29 and C-30 methyl functionalities was derived on the basis of biogenetic analogy of triterpenes. Besides the well known squalene, the acyclic triterpenes reported are hemidesmusoic acid from the roots of *Hemidesmus indicus*[19], cressatriterpenic acid from the aerial parts of *Cressa cretica*[20], anaphlisqualenol and anaphranasoic

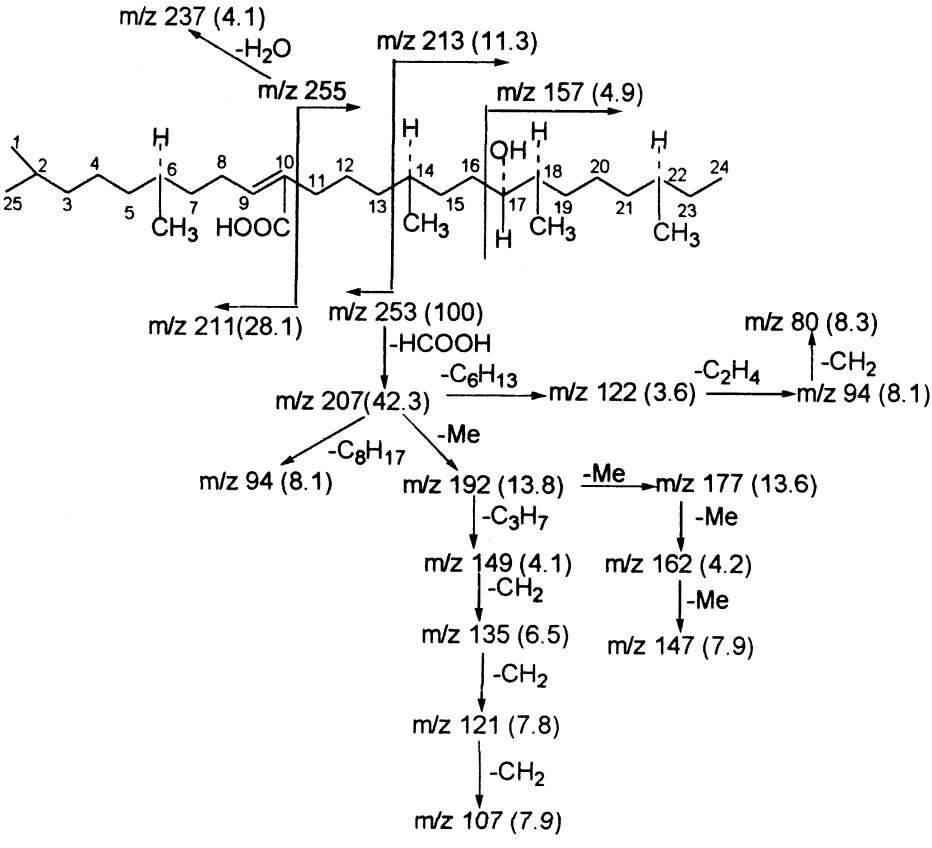

Fig. 2. Mass fragmentation pattern of **1**

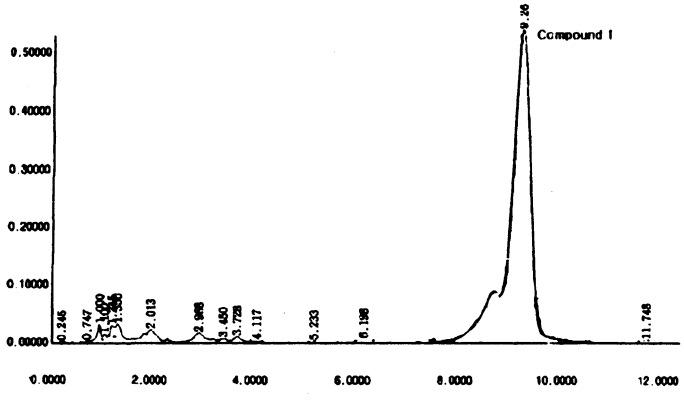

Fig. 3. HPLC chromatogram of compound **1** before HPLC separation

acid from *Anaphalis araneosa*[21] and scoparoic acid from the roots of *Artemisia scoparia*[22].

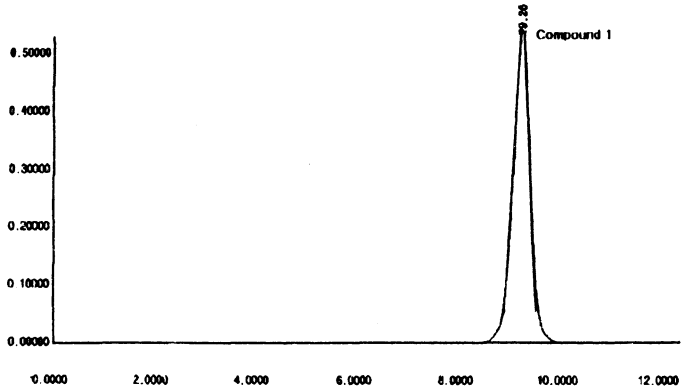

Fig. 4. HPLC chromatogram of compound **1** after HPLC separation

The ^{13}C-NMR spectrum of **1** exhibited important signals for C-27 carboxylic (δ 181.92), vinylic C-9 (δ 127.03) and C-10 (δ 139.82) and carbinol C-17 (δ 79.87) carbons. The methyl carbon signals appeared at δ 17.79 (C-1), 19.63 (C-24), 17.98 (C-25), 21.72 (C-26), 29.38 (C-28), 16.17 (C-29) and 16.52 (C-30). The remaining methine and methylene carbons resonated between δ 24.23–56.91 (Table-1). The ^{1}H-^{1}H COSY spectrum showed a correlation between H-17, H-18 and H-16. The ^{1}H-^{13}C HETCOR spectrum displayed correlation of C-27 with C-9; C-17 with C-15, C-16, C-18, C-19 and C-29; and C-9 with C-6, C-7 and C-8. Acetylation of **1** yielded a monoacetyl product **1a** supporting the existence of one acetylable hydroxyl group in the molecule. The ^{1}H-NMR of **1a** showed signals at δ 2.05 (3H, s) for one alcoholic acetyl group. Treatment of **1** with ethereal solution of diazomethane formed a monomethoxy derivative **1b**. The ^{1}H-NMR of **1b** showed signals at δ 3.16 (3H, s) for one carboxylic methoxy group. On the basis of these evidences the structure of **1** has been established as 2,6,14,18,22-pentamethyl-*n*-tetracos-9-en-17α-ol-27-oic acid.

Cytotoxic activity: The diterpenoids and triterpenoids have been reported to show cytotoxic activity against leukemia cells[18, 23]. The compound **1**, *β-sitosterol and* β-sitosterol-3-O-β-D-glucoside showed cytotoxic activity against P388 murine leukemia cells with IC_{50} values of 9, 50, 100 μg/mL respectively. The results demonstrated that compound **1** had lower cytotoxicity and other known compounds almost no activity.

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