Studies on the Chemical Constituents of *Phyllanthus fraternus*

[1]HABIB-UR-REHMAN, [2]ATTA-UR-RAHMAN
[2]M. I. CHOUDHARY AND [2]A. R. RAZA
[1]Department of Chemistry
University of Azad Jammu and Kashmi
Muzaffarabad-13100, Pakistan
[2]H. E. J. Research Institute of Chemistry/
International Centre for Chemical Sciences
University of Karachi, Karachi–75270, Pakistan

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Summary: Phytochemical studies on *Phyllanthus fraternus* have resulted in the isolation of a new pentacyclic triterpene, olean-18-en-3α-l (**1**). Its structure was determined on the basis of spectral studies. Another known compound, phyllanthin (**2**) was also isolated and its ^{1}H-NMR and ^{13}C-MR chemical shift assignments revised on the basis of extensive NMR studies. Besides, the two known compounds, 1-triacontanol and β-sitosterol, were isolated from the plant and identified on the basis of their spectral studies and literature evidences.

Introduction

The geneus Phyllanthus comprises 700 species. Only ten species, *P. amrus, P. acidus, P. embilica, P. fraternus, P. maderaspatensis, P. parvifolius, P. reticulats, P. rotundifolius, P. urinaria* and *P. virgatus* are found in Pakistan. The plants of the genus find wide application in the folk medicine system for the treatment of jaundice and liver ailments [1]. Several active chemical consti-tuents have been isolated from different species of the genus. *P. accuminatus* has been known to have active chemical constituents, antineoplastic bisa-bolene glycoside, phyllanthoside and phyllanthostatins [2,3]. The two flavonoids designated as FG-1 and FG-2, isolated from *P. fraternus*, exhibit oral hypoglycemic activity in alloxan treated albino rats [4]. The mean reduction of blood sugar was found to be about 20% with FG-1 and 25% with FG-2. The structures of these compounds have not been determined so far. *P. niruri* yielded active substances, securinine, ellagic acid, bursehernin, epiallocatechin, quercetin, rutin and 1-triacontanol [4-9]. Securinine showed CNS stimulant activity with strychnine-like action but with lower toxicity. The $GABA_A$ receptor antagonist activity, causes respiratory stimulation and increases muscle tone and hypertension [5]. Ellagic acid exhibited astringent (intestinal) and haemostatic activity [6]. Bursehernin exhibited antitumor activity [7]. Epigallocatechin showed antisorbutic properties [8]. Quercetin exhibited antioxidant activity [9]. Rutin showed antihaemorihagic activity while 1-triacontanol act as a plant growth hormone [9]. Phyllanthostatin-A isolated from *P. acuminatus* showed cytostatic activity [10].

Phyllanthus fraternus syn. (Euphorbiaceae) is widely distributed in Pakistan, Kashmir, North Western India, Middle East, Africa and West Indies. Literature studies showed that the plant possesses several interesting biological proprieties [11] and contains verity of structures [12-14]. Our recent investigations on the plant have resulted in the isolation of a new pentacyclic triterpene, olean-18-en-3α-1 (1). Its structure has been determined on the basis of spectral studies including 2D NMR experiments.

The plant also yielded another compound, phyllanthin (**2**) reported earlier [15], however, detailed spectral studies including 2D NMR experiments led us to revise the reported ^{1}H-NMR and ^{13}C-NMR shift assignments. Know substances, 1-triacontanol and β-sitosterol have also bee isolated from the plant and identified on the basis of spectral studies and literature evidences [16,17]. These compounds have not been previously reported from this plant. This, therefore, represent the first report of the presence of phyllanthin [15], 1-triacotanol [16] and β-sitosterol [17] in the plant.

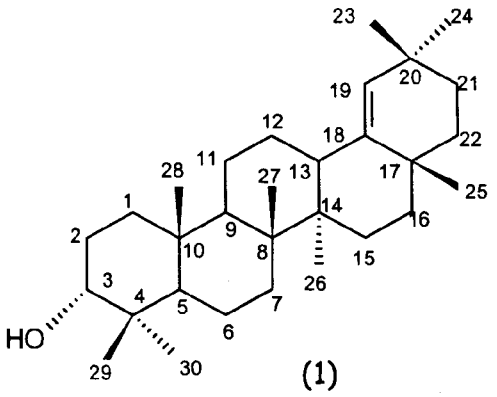
Results and Discussion

Olean-18-en-3α-l (1)

The MeOH extract of the plant was subjected to the column chromatography. The column was eluted with increasing polarities of pet. ether through butanol. Elution with pet. ether/chloroform (17:3)

afforded a semipure compund which was purified on HPLC with pet. ether/chloroform (9:1) to afford a pure compound, olean-18-en-3α-1 (1).

The UV spectrum (MeOH) of the compound 1 showed λ_{max} absorption at 211 nm, indicating the absence of any conjugated system and represents an isolated C=C bond. The IR spectrum ($CHCl_3$) displayed major absorptions at 3410 cm^{-1} and 2825 cm^{-1}, indicating the presence of O-H and C-H functions in the molecule.



(1)

The mass spectrum showed the molecular ion peak at m/z 426, corresponding to the molecular formula $C_{30}H_{50}O$, indicating 6 degrees of unsaturation in the molecule. Other major peaks were found to occur at m/z 411 and 408, indicating the loss of CH_3 and H_2O group from the molecule.

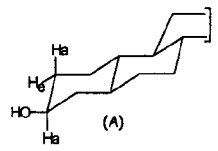
The ^{1}H-NMR spectrum ($CDCl_3$) showed eight methyl singlets, indicating the presence of oleanane skeleton. The 3-H showed HMBC interactions with 2-C and no coupling interactions were observed with olefinic carbon. This indicated that both double bound and OH group are not in the same ring. This was also confirmed by the HOHAHA experiments. No coupling interactions were observed between olefinic C or 1-H with 2-C and 5-C in the spectrum. The 2-H also showed HMBC coupling with 2-C and 5-C in the HOHAHA spectrum.

The ^{13}C-NMR spectrum ($CDCl_3$) showed 30 carbon resonances, 8 methyls, 10 methylenes, 5 methines and 7 quaternary carbons in the molecule. The multiplicity of the carbon signals were determined by the DEPT experiments. The proton and carbon shift assignments were made with the help of ^{13}C-NMR (BB and DEPT) and 2D NMR (HMBC, HMQC and HOHAHA) experiments. The complete ^{1}H-NMR and ^{13}C-NMR shift assignments are presented in Table-1.

Table-1: ^{1}H-NMR and ^{13}C-NMR Chemical Shift Assignments of 1.

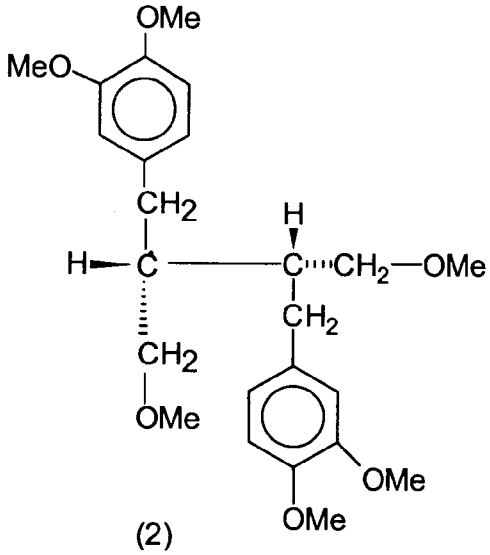
C.No.	C-Chemical Shifts (δ)	Multi-plicity Shifts (δ)	H-Chemcial
01	29.7	CH_2	1.24 (1H, m, Hα), 1.28 (1H, m, Hβ)
02	33.2	CH_2	1.62(1H, d, Hα, J=3.4Hz) 1.65(1H, d, Hβ, J=3.1Hz)
03	76.3	CH	3.38 (1H, t, J=2.9 Hz)
04	37.6	C	--
05	49.4	CH	1.25 (1H, m)
06	18.2	CH_2	1.47 (2H, m)
07	27.0	CH_2	1.81 (2H, d, J=3.8Hz)
08	41.0	C	--
09	50.5	CH	1.42 (1H, d, J=5.4Hz)
10	52.1	C	--
11	25.4	CH_2	1.52 (1H, m, Hα) 1.56 (1H, m, Hβ)
12	30.4	CH_2	1.78 (2H, d, J=2.0Hz)
13	45.3	CH	1.66 (1H, d, J=3.1Hz)
14	28.2	C	--
15	33.0	CH_2	1.50 (1H, m, Hα) 1.53 (1H, m, Hβ)
16	26.6	CH_2	0.97 (2H, d, J=6.6Hz)
17	38.4	C	--
18	139.9	C	--
19	126.6	CH	5.42 (1H, s)
20	33.7	C	--
21	42.8	CH_2	1.41 (2H, d, J=4.3Hz)
22	29.1	CH_2	1.33 (1H, m, Hα, J=3.8Hz) 1.36 (1H, m, Hβ)
23	22.1	CH_3	0.83 (3H, s)
24	28.3	CH_3	0.94 (3H, s)
25	14.0	CH_3	0.74 (3H, s)
26	15.7	CH_3	0.81 (3H, s)
27	17.5	CH_3	0.94 (3H, s)
28	22.0	CH_3	0.76 (3H, s)
29	30.3	CH_3	0.93 (3H, s)
30	18.6	CH_3	1.01 (3H, s)

The stereochemistry at 3-C was established on the basis of the values of the coupling constants. The 3-H showed a triplet at δ 3.38 (J=2.9 Hz). This suggested that 2-H and 3-H are not antiperiplaner (i.e. not at 180°), but make an approximately 60^0 angle with each other. Possibility 'A' is ruled out because no Jaa (7-10 Hz) was observed instead both Jae and Jee (2-3Hz) was observed. Based on the above spectral studies, structure **1** was assigned to olean-18-en-3α-1.



Phyllanthin (2)

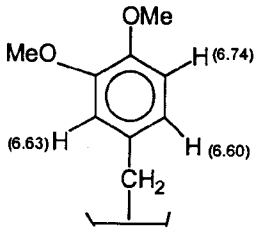
The MeOH extract of the plant was subjected to column chromatography. The column was eluted with pet. ether/$CHCl_3$ (1:3). This afforded white crystals (11.8 mg). The compound was identified as phyllanthin (**2**) on the basis of comparison of its spectral data with that of reported in literature [15]. However, based on our detailed spectral studies, reported ^{1}H-NMR and ^{13}C-NMR shift assignments have been revised.



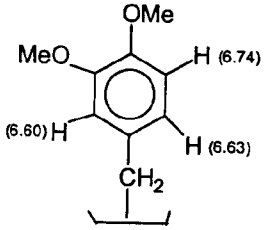
(2)

The ^{1}H-NMR spectrum showed three 3H singlets at δ 3.28, δ3.76 and δ3.80, one 2H singlet at δ3.28, two doublets at δ 6.60 (J=2.0 Hz) and δ 6.74 (J=8.0Hz), one triplet at δ 6.63 (J_1=8.0Hz, J_2=2.0Hz) and a multiplet at δ 2.04. The three singlets indicating 3-OCH_3 groups while two doublets and a triplet showed the presence of 3 aromatic protons in a trisubstituted benzene ring.

The ^{13}C-NMR (BB) showed only 12 carbon resonances which showed that the molecule is a dimer of two 12 carbon monomers. The DEPT spectra showed 3 quaternary carbons (δ 133.8, δ 147.8 and δ 148.9), 4 methines (δ 40.9, δ111.3, δ 112.4 and δ121.3), 2 methylenes (δ 35.0, δ72.8) and 3 methyl carbons (δ 55.8, δ 56.0 and δ 58.8). The comparison of ^{1}H-NMR and ^{13}C-NMR shift assignments of the compound and its physical constants (optical rotation and melting point) with the reported values indicated that the compound in hand was phyllanthin. However, COSY-45°, HMBC and HMQC spectra revealed that the proton and carbon shift assignments reported earlier require revision. The COSY-45° spectrum showed coupling between 5′-H and 6′-H. This showed that the two OMe groups are at *meta* and *para* positions to the alkyl group. These studies led us to revise the ^{1}H-NMR chemical shift assignments as under.

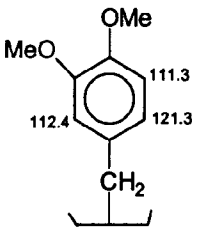


Reported ^{1}H-δ

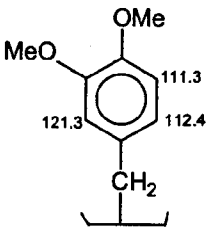


Revised ^{1}H-δ

The carbon signal at δ 111.3 showed HMQC interaction with the proton signal at δ 6.74 (5′–H). The carbon signals at δ 112.4 and δ 121.3 showed coupling interactions with the proton signals at δ 6.60 (2′–H) and δ 6.63 (6′–H) respectively in the HMQC spectrum. The above spectral studies led us to revise the reported ^{13}C-NMR chemical shift assignments of phyllanthin (1) as under. The complete ^{1}H-NMR and ^{13}C-NMR chemical shift assignments are presented in Table–2.



Reported ^{13}C-δ



Revised ^{13}C-δ

Phyllanthin [15] is a dimeric compound with two asymmetric centers. As it a optically active ($[\alpha]_D^{25}$ = + 12.4°), the possibilities of its configura-

tions as RS and SR (both configurations possess a plane of symmetry σ and a center of symmetry S_2), which result optically inactive compound, may be ruled out. Hence, the optically active compound can only possesses RR or SS configuration.

Table-2: ^{1}H-NMR and ^{13}C-NMR Chemical Shift Assignments of **2**.

C.No.	C-Chemical Shifts (δ)	Multi-plicity	H-Chemical Shifts (δ)
01′	133.8	C	- -
02′	112.4/121.3$^+$	CH	6.60/6.63$^+$ (1 H, d, J=2.0Hz)
03′	148.9*	C	- -
04′	147.3*	C	- -
05′	111.3	CH	6.74(1H, d, J=8.0Hz)
06′	121.3/112.4$^+$	CH	6.63/6.60$^+$ (1H, dd, J_1=8.0Hz, J_2=2.0Hz)
Ar-CH_2	35.0	CH_2	2.65 (2H, t, J=6.3Hz)
R-CH	40.9	CH	2.04 (1H, m)
O-CH_2	72.8	CH_2	3.28 (2H, s)
RO-CH_3	56.0	CH_3	3.76 (3H, s)
Ar-OCH_3	55.8	CH_3	3.80 (3H, s)
Ar-OCH_3	55.8	CH_3	3.28 (3H, s)

*Assignments exchangeable. $^+$The ^{1}H and ^{13}C-shifts where revised (2′-C & 6′-C; 2′-H & 6′-H), the reported shift assignments are given oblique.

1-Triacontanol

The methanol extract of the plant was subjected to column chromatography. Elution of the column with pet. ether/$CHCl_3$ (4:1) afforded white crystals (14.8 mg), identified as 1-triacontanol on the basis of comparison of its spectral data with those reported in literature [16]. This represent the first report of the isolation of 1-triacotanol from *P. fraternus*, reported earlier from other species of the plant.

β-Sitosterol

The methanol extract of the plant was subjected to the column chromatography. The column was eluted with pet. ether/$CHCl_3$ (3:1) as the solvent system. This afforded a pure compound (18.1 mg), identified as β-sitosterol on the basis of comparison of its spectral data with those reported in literature [17].

Experimental

Plant Material

The plant material was collected from District Muzaffarabad. The plant was identified by the plant taxonomist at the Department of Botany, University of Azad Jammu and Kashmir, Muzaffarabad. The voucher specimen is deposited in the herbarium of the department.

Experimental Procedure

UV spectra were recorded in MeOH on Shimadzu UV-240 spectrophotometer. IR spectra were recorded in $CHCl_3$ on JASCO IR-1 instrument. The ^{1}H-NMR spectra were recorded in $CHCl_3$ on Bruker AMX-500 NMR spectrometer. The ^{13} C-NMR spectra were recorded in $CDCl_3$ at 125 MHz on the same instrument. Mass spectra were recorded on a Varian MAT 112 mass spectrometer connected to a PDP 11/34 DEC computer system. Optical rotations were measured on JASCO DIP-360 digital polarimeter. Melting points were determined in glass capillary using Gallenkamp melting point apparatus.

Isolation of 1

The dry whole plant (20 kg) was soaked in 30 litres of MeOH for 2 weeks and the extract was concentrated under vacuum. The MeOH extract was subjected to the Silica-gel column chromatography. The column was eluted with increasing polarities of pet. ether/$CHCl_3$ (1:1), $CHCl_3$, $CHCl_3$/MeOH (1:1), MeOH, MeOH/BuOH (1:1) and BuOH to afford fractions, F1 (0.4 g), F2 (4.1 g), F3 (182.0 g), F4 (143.8 g), F5 (24.8 g), F6 (0.8 g) and F7 (0.7 g) respectively. The fraction F2 (4.0 g) was rechromatographed on silica-gel column. The column was eluted with pet. ether/$CHCl_3$ (17:3) to afforded fraction F8 (116.2 mg). This material was again subjected to the Silica-gel column chromatography and eluted with pet. ether/$CHCl_3$ (19:1) to afford the semipure compound (7.5 mg) which was purified on HPLC with pet. ether/$CHCl_3$ (9:1) as the solvent system. This afforded a pure compound (Rf = 0.74, 5.5 mg, 2.8×10^{-5}% yield), m.p. 180^{0}C, $[\alpha]_D^{25}$ = +57 (c=3.5mg/100ml, $CHCl_3$).

Spectral Data of 1

UV (MeOH) λ_{max} nm (log∈): 211 (3.49): IR ($CHCl_3$) ν max (functions): 3410 (O-H), 2835 (C-H); MS m/z (%) 426.3862 ($C_{30}H_{50}O$, calcd. 426.3862, 100 %), 411(8), 408 (5), 393(4), 259(14), 216(12) 190(35), 119 (33), 69 (55); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: Table-1; ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: Table-1.

Isolation of 2

The fraction F2 was subjected to the PTLC experiments with pet. ether/$CHCl_3$ (1:3) as the solvent system. This afforded a crystalline compound (Rf=0.65, 19.8 mg, $9.9x10^{-4}$ % yield), m.p. 93°C (reported 96°), $[\alpha]_D^{25}$= +12.4 (c=1.45mg/ 100ml, $CHCl_3$).

Spectral Data of 2

^{1}H-NMR ($CDCl_3$, 400MHz) δ: Table–2: ^{13}C-NMR ($CDCl_3$, 100MHz) δ: Table- 2.

Isolation of 1-Triacontanol

The fraction F2 was loaded on a Silica gel column. The elution of the column with pet. ether/$CHCl_3$ (4:1) afforded a semi pure compound. The compound was purified on PTLC with pet. ether/$CHCl_3$ (9:1) as the solvent system. This afforded an optically inactive compound (Rf =0.74, 14.8 mg, $8.7x10^{-5}$ % yield), m.p. 83-84°C (reported 86.5°), identified as 1-triacontanol.

Isolation of β-Sitosterol

The fraction F3 was subjected to the Silica gel column chromatography. The column was eluted with pet. ether/$CHCl_3$ (3:1) to afford a semi pure compound. The repeated column chromatography and its elution with pet. ether/$CHCl_3$ (7:8) afforded a pure compound identified as β-sitosterol (Rf= 0.44, 18.1 mg, 9.1 x 10^{-5}% yield), m.p. 130°C (reported 136-137°), $[\alpha]_D^{25}$= -35 (c=2.9 mg/100ml, $CHCl_3$).

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