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TWO NEW CONSTITUENTS FROM THE BARK OF *HOLARRHENA PUBESCENS*

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Abstract -Two new compounds, norkurchamide (3,20-dioxo-11α-hydroxycona-1,4-diene) (**1**) and pubatriol (3-oxo-11α,19,22-trihydroxycona-1,4-diene) (**2**) have been isolated from the bark of *Holarrhena pubescens*. Their structures have been established through spectroscopic studies.

Holarrhena pubescens [synonym: *H. antidysenterica Apocynaceae*] inhabits the forest areas of the sub-continent. It has been extensively studied mainly because the bark commonly known as "Kurchi" is highly reputed in traditional medicine as a remedy for amoebic dysentery and other intestinal ailments.[1,2] The plant has also been reported to possess anthelmintic, appetiser, astringent and antidiarroeal properties.[3] A hot decoction of bark is used as a gargle in toothache.[4] Chemical studies on *H. pubescens* were undertaken as far back as 1858 when the alkaloid conessine was first isolated by Hains[5] from the bark of the plant. Since then this plant has been extensively studied for its alkaloids[6] due to their pharmacological significance. Studies undertaken by this group on the bark of the plant led to the isolation of several new compounds.[7-11] In continuation of these investigations two new compounds of conanine series have now been obtained. The isolation and structure elucidation of these constituents are being reported in this paper. The structural studies are based on 1D and 2D NMR experiments (COSY 45, HMQC and HMBC) and other spectral evidences.

Norkurchamide (**1**) showed M^+ peak at *m/z* 327.1894 corresponding to the molecular formula $C_{20}H_{25}NO_3$ showing nine double bond equivalents. Its IR (1660 and1620 cm^{-1}), UV (237 nm) absorptions and the NMR spctral signals (Broad Band, DEPT, HMQC; Table 1) at δ 7. 81 (d, *J* =10.3 Hz, H-1), 6.15 (dd, *J* =10.3, 1.8 Hz, H-2), 6.09 (t, *J* =1.8 Hz, H-4), 158.7 (C-1), 125.3 (C-2), 186.5 (C-3), 124.7 (C-4) and 167.2 (C-5) indicated the 1,4- dien-3-one system in ring A which was supported by prominent MS fragments[6] at *m/z* 107.0472 (C_7H_7O) and 121.0676 (C_8H_9O) in the HRMS[14] (*vide* structure). A signal at δ

at δ 3.71 observed as a dt (*J* = 9.1, 9.1, 5.3 Hz) was attributed to the axial proton geminal to a hydroxyl group. This was placed at C-11 with α-disposition on the basis of the NMR spectral data (Table 1). The ^{1}H-NMR spectrum of **1** showed only one tertiary methyl group at δ 1.25 (H-19). The absence of the second angular methyl group of steroids and a peak at *m/z* 57.0210 (C_2H_3NO) indicated the presence of conanine type of skeleton.[12] This fragment also justified the remaining oxygen function which was placed in ring E as a carbonyl of lactam function (IR: 1710 cm^{-1}; ^{13}C-NMR: δ 174.4).

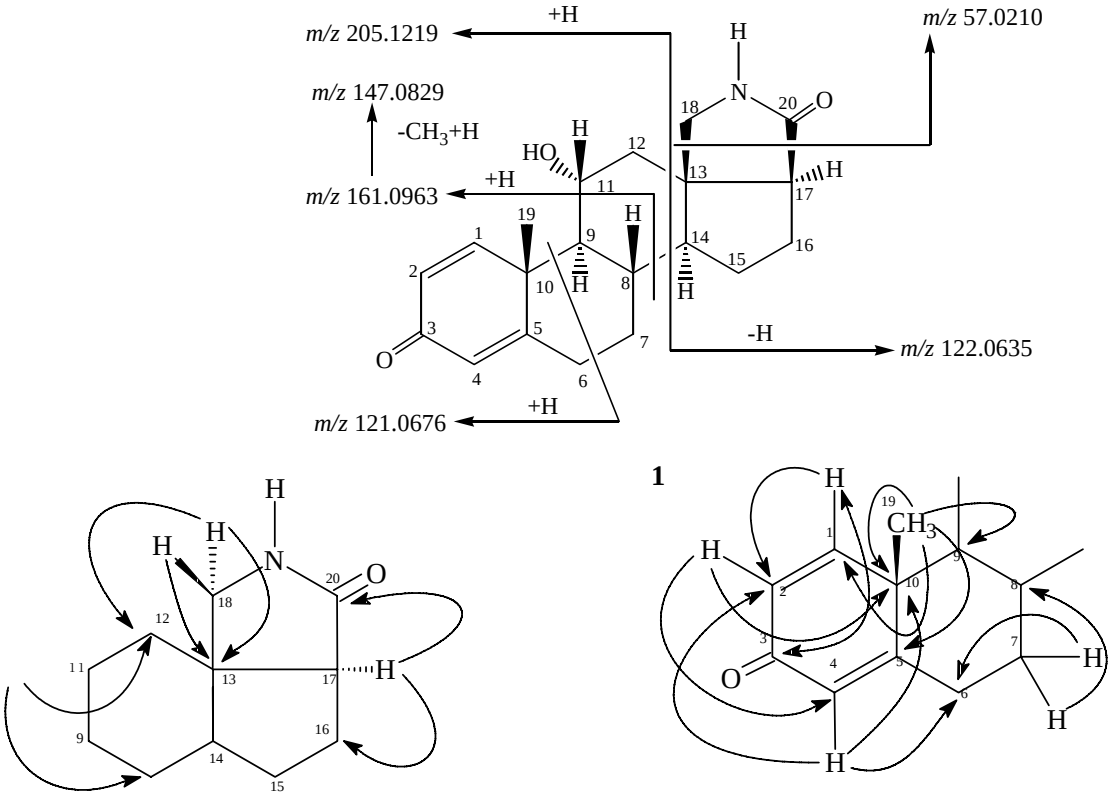
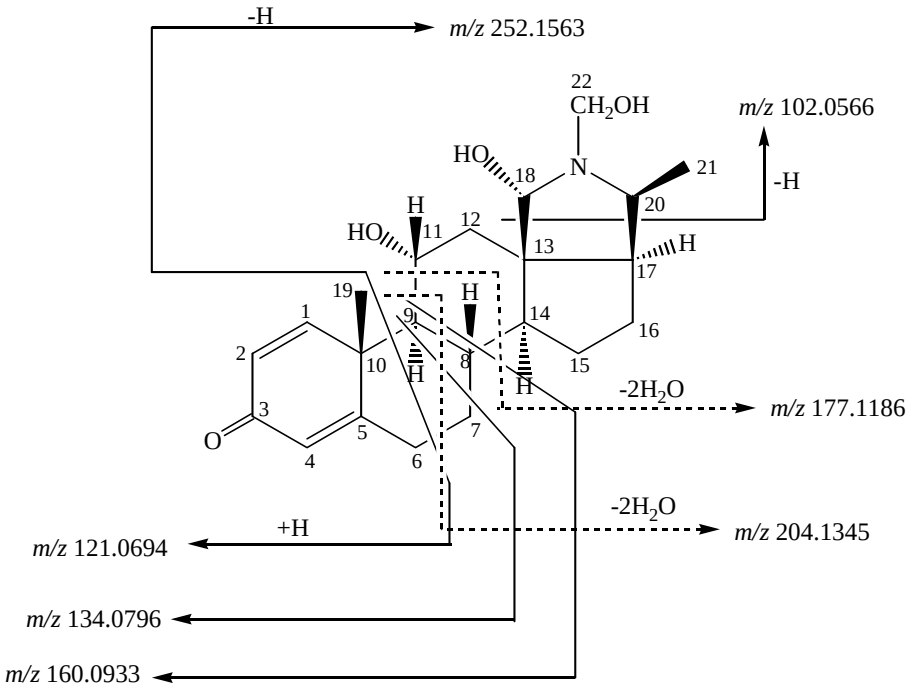


Figure 1 (Arrows show HMBC correlations for norkurchamide)

Two AB doublets at δ 3.85 and 3.51 with a geminal coupling constant of 8.8 Hz were assignable to H-18a and H-18b and the absence of 21-CH_3 signal and presence of a one proton triplet at δ 2.30 with coupling constant 7.8 Hz due to H-17 allowed to locate this amido carbonyl group at C-20 which was fully supported by the HMBC correlations shown in the partial structure (Figure 1). On the basis of these observations **1** has been assigned the structure as 3,20-dioxo-11α-hydroxycona-1,4-diene. The connectivities of various protons and carbons could be established from HMQC spectrum. The stereochemistry of various centres was deduced through comparison of ^{13}C NMR chemical shifts of **1** with those reported for compounds with similar partial structures[6] as well as interactions observed in NOESY plot. Thus H-11 (β) showed connectivity with H-8, H-18 and H-19 whereas H-17 (α) had contours related to H-14 which was connected with H-9.



2

The molecular formula ($C_{22}H_{31}NO_4$) of pubatriol (**2)** was obtained through accurate MS measurement (found 373.2225) of the molecular ion which showed eight double bond equivalents. Its IR (1660 and 1620 cm^{-1}) and UV (245 nm) absorptions and the signals in the NMR spectra (Table 2) at δ 7.87 (1H, d, *J* =10.3 Hz, H-1), 6.15 (1H, dd, *J* =10.3, 1.8 Hz, H-2), 6.08 (1H, t, *J* =1.8 Hz, H-4), 154.4 (C-1), 127.4 (C-2), 186.4 (C-3), 124.6 (C-4), 167.4 (C-5) were suggestive of the 1,4-dien-3-one system in ring A, which was supported by a significant mass ion at *m/z* 121.0694[14] (*vide* structure) in the HRMS. The IR (3650 cm^{-1}) and NMR spectral data (Table 2) further showed the presence of OH groups. Thus the ^{1}H-NMR spectrum revealed a one-proton doublet of triplet at δ 3.86 (*J* =11.0, 11.0, 4.7 Hz) which was attributable to the axial hydrogen at C-11, geminal to an OH group (*loc. cit.*). This proton had a cross peak at δ 71.8 in the HMQC plot. The mass fragments at *m/z* 252.1563 and 102.0566 suggested the location of the remaining two hydroxyl groups in the heterocyclic ring of the molecule. The appearance of H-21 at δ 1.10 (d, *J*= 6.7 Hz) showed that the two hydroxyl groups must be placed at C-18 and at *N*-methyl carbon. A broad singlet at δ 4.47 for H-18 and two doublets at δ 4.21, and 4.17 with geminal coupling constant of 11.0 Hz for the non-equivalent hydroxymethylene protons H-22a and H-22b confirmed the location of these groups, which got supportive evidence by the signals in the ^{13}C-NMR at δ 94.7 and 86.7 assignable to these carbons respectively. On the basis of these observations **2** has been assigned the structure as 3-oxo-11α,18, *N*-methyl-trihydroxy-cona-1,4-diene. The connectivities of various protons and carbons could be established from HMQC spectrum. The comparable carbon chemical shifts of **2** with those of **1** particularly of rings A and B, and part of rings C, D and E suggested the same stereochemistry of C-8-C10, C-14 and C-17 in both the compounds. Further the NOESY plot showed interactions of H-9 with H-

14 and H-14 with H-17 suggesting that these lie in the same plane (α). The α orientation of the hydroxyl group at C-18 could be decided on the basis of NOESY and inspection of Drieding model. In the NOESY plot an interaction of H-18 with both H-8 and H-21 as well as with H-22 was noted and the model suggested that it was possible with H-18*β* which comes closer to H-21 in this orientation. An interaction between H-18 and H-11 (*β*) was also noted along with other expected relations.

EXPERIMENTAL

General Methods

Mps are uncorrected. MS were recorded on a Finnigan MAT 112 and 312 double-focusing mass spectrometer connected to a PDP 11/34 computer system; NMR spectra ($CDCl_3$ 400 MHz for ^1H and 75 MHz for ^{13}C) were recorded on a Bruker AM 400 and AM 300 FT NMR respectively. The chemical shifts are reported in δ (ppm) and the coupling constants are in Hz. The ^{13}C-NMR spectral assignments (Tables 1 and 2) have been made partly through a comparison of the chemical shifts with the published data for similar compounds[6] and partly through the appearance of signals in DEPT, HMQC (Tables 1 and 2) and HMBC (Fig. 1 and 2) spectra. Precoated thin layer cards (DC-karten SiF) were used for TLC. The petroleum ether used was of the boiling range 60-70^oC. Flash column chromatography was performed on model Eyela EF-10, si gel used was from Merck 9385 and Al_2O_3 was from Merck 90.

Plant Material

The bark of *H. pubescens (H. antidysenterica)* was supplied by the courtesy of Hamdard Foundation Pakistan Ltd. It was identified by Miss Ashreen Jahan, botanist, Hamdard Foundation Pakistan Ltd.

Extraction and Isolation

Uncrushed bark (10 kg) was macerated with 10% methanolic NaOH (10 L) for 48 h at 28^oC, and repeatedly percolated with MeOH for 48 h (five times) at the same temperature following the reported procedure[15] in order to hydrolyze the tannates. Each extract was neutralized with 30 % aqueous HOAc. The pH of the syrupy concentrate (2 L) obtained on removal of the solvent from the combined extracts under reduced pressure was reduced to acidic by adding 10 % aqueous HOAc at 28^oC and the solution was extracted with EtOAc. The aqueous phase was basified with 20% NH_4OH and again shaken out with EtOAc. The moist EtOAc phase was treated with a vigorous stream of CO_2. The precipitate containing the carbonate bases was filtered and the filtrate was dried over anhydrous Na_2SO_4 and freed of the solvent under reduced pressure. The residue (20 g) was divided into petroleum ether soluble and petroleum ether insoluble fractions. The petroleum ether soluble fraction yielded conessine (9 g) according to the reported

isolation procedure.[1] The petroleum ether insoluble portion (11 g) when dissolved in 10% aqueous AcOH and treated with $(NH_4)_2SO_4$, furnished colorless precipitate of sulfates which was filtered. The sulfate mother liquour was made alkaline with 10% aqueous NaOH and extracted out with EtOAc. The EtOAc phase was washed with H_2O to neutral pH, dried (Na_2SO_4) and evaporated under reduced pressure to give a colorless residue (9.5 g). This residue was subjected to flash column chromatography (Al_2O_3, Merck 90; petroleum ether, petroleum ether-EtOAc, in order of increasing polarity.). Nine fractions were ultimately obtained on combining the eluates on the basis of TLC. The major fraction eluted with $CHCl_3$ and was re-chromatographed on flash column (si gel; petroleum ether, petroleum ether-EtOAc, in order of increasing polarity). The petroleum ether-EtOAc (1.5:8.5) eluate furnished fraction A (41 mg) and petroleum ether-EtOAc (1:9) eluate yielded fraction B (42 mg). The crude fractions A and B afforded **1** (26 mg) and **2** (14 mg) respectively on purification over thin layer plates of si gel with solvent systems $CHCl_3$-MeOH (8.7:1.3 and 8:2 respectively).

Table 1. ^{1}H- and ^{13}C-NMR spectral data for norkurchamide (**1**).[a]

C	δC	H	δH	Multiplicity	*J* value (Hz)
1	158.7	1	7.81	d	10.3
2	125.3	2	6.15	dd	10.3,1.8
3	186.5	-	-	-	-
4	124.7	4	6.09	t	1.8
5	167.2	-	-	-	-
6	33.0	6a	2.22	m	-
		6b	1.18	m	-
7	29.6	7a	1.40	m	-
		7b	1.34	m	-
8	35.9	8	1.42	m	-
9	59.1	9	1.21	m	-
10	43.9	-	-	-	-
11	70.1	11β	3.71	ddd	9.1,9.1, 5.3
12	49.1	12α	2.52	dd	11.9, 4.7
		12β	1.54	m	-
13	54.0	-	-	-	-
14	56.4	14	1.30	m	-
15	26.2	15a	1.70	m	-
		15b	1.62	m	-
16	33.6	16a	2.48	m	-
		16b	2.34	m	-
17	53.7	17	2.30	t	7.8
18	72.9	18a	3.85	d	8.8
		18b	3.51	d	8.8
19	18.5	19	1.25	s	-
20	174.4	-	-	-	-

[a]The assignments are based on COSY-45°, *J*-resolved, Broad band ^{1}H-decoupled, DEPT, HMQC and HMBC spectra.

3,20-Dioxo-11α–hydroxycona-1,4-diene (**1**) was obtained as orange rods (MeOH); mp 130-132^{o}C; UV λ_{max} (MeOH) 246.5 and 206.0 nm; IR ν_{max} ($CHCl_3$), 3400, 2950, 1710, 1660, 1620, 1600, 1110 cm^{-1}; EIMS (70 eV) *m/z* $(M)^+$ 327.1894 ($C_{20}H_{25}NO_3$ requires M^+ 327.1834) (21), 205.1219 (34), 161.0963 (18), 147.0829 (14), 122.0635 (100), 121.0676 (21), 107.0472 (30.5), 57.0210 (21); ^{1}H-NMR and ^{13}C-NMR: Table 1. HMBC: Figure 1.

3-Oxo-11α, 19, 22-trihydroxy-cona-1,4-diene (**2**) was obtained as orange rods (MeOH); mp 110-112^{o}C; UV λ_{max} (MeOH) 245.9 and 210.0 nm; IR ν_{max} ($CHCl_3$), 3650, 2950, 1660, 1620, 1600, 1110 cm^{-1}; EIMS (70 eV) *m/z* $(M)^+$ 373.2225 ($C_{22}H_{31}NO_4$ requires M^+ 373.2252) (41), 252.1563 (8), 204.1345 (16), 177.1186 (11), 160.0933 (12), 134.0796 (14), 121.0694 (30), 102.0566 (100); ^{1}H-NMR and ^{13}C-NMR: Table 2.

Table **2**. ^{1}H- and ^{13}C-NMR spectral data for pubatriol (**2**).[a]

C	δC	H	δH	Multiplicity	*J* value (Hz)
1	158.4	1	7.87	d	10.3
2	127.4	2	6.15	dd	10.3,1.8
3	186.4	-	-	-	-
4	124.6	4	6.08	t	1.8
5	167.4	-	-	-	-
6	33.1	6a	2.10	m	-
		6b	1.28	m	-
7	29.7	7a	1.38	m	-
		7b	1.21	m	-
8	34.4	8	1.38	m	-
9	59.4	9	1.54	m	-
10	44.8	-	-	-	-
11	71.8	11β	3.86	ddd	11.0, 11.0, 4.7
12	49.2	12a	2.50	m	-
		12b	1.64	m	-
13	47.2	-	-	-	-
14	51.0	14	1.36	m	-
15	27.3	15a	1.74	m	-
		15b	1.54	m	-
16	33.6	16a	2.44	m	-
		16b	2.38	m	-
17	51.2	17	1.18	m	-
18	94.7	18	4.47	br. s	-
19	14.1	19	1.24	s	-
20	63.5	20	3.70	m	-
21	18.6	21	1.10	d	6.7
22	86.7	22a	4.21	d	11.0
		22b	4.17	d	11.0

[a]The assignments are based on COSY-45^{o}, *J*-resolved, Broad band ^{1}H-decoupled, DEPT, HMQC and HMBC spectra.

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