HYPOTENSIVE CONSTITUENTS FROM THE BARK OF *HOLARRHENA PUBESCENS (HOLARRHENA ANTIDYSENTERICA)*

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Abstract- Two new alkaloids of conanine series, holamide (*N*-(*N'*-methylacetamido)-3-oxo-1,4-conadiene) (**1**), pubescinine (11α-acetoxy-*N*-demethyl-3-oxo-1,4,17(20)-conatriene) (**2**) and a known alkaloid *norconessine* (3-*N*-methylamino-5-conanene) (**3**), have been isolated from the bark of *Holarrhena pubescens* Buch. Ham (*Holarrhena antidysenterica* Linn.) (Apocynacea). Their structures have been established through spectroscopic studies. Both **1** and **2** showed hypotensive activity in rats at a dose of 3 mg/kg.

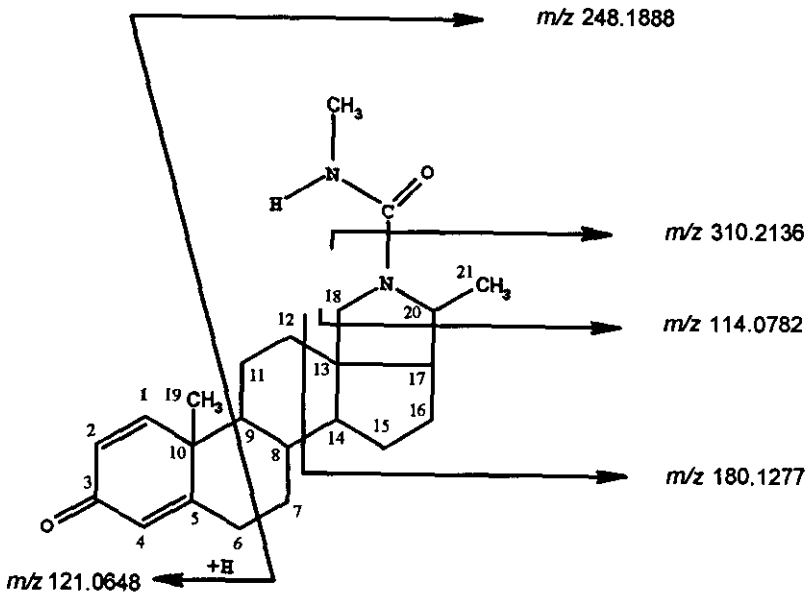
Holarrhena pubescens (Buch. Ham) (Syn: *Holarrhena antidysenterica* L.), belonging to the Apocynaceae family, inhabits the drier forest area of India, Pakistan and other regions of the sub-continent.[1] The bark commonly known as 'kurchi' is highly reputed in traditional medicine as a remedy for amoebic dysentery and other intestinal ailments. Under the name conessi bark, kurchi has found considerable reputation in Europe also as a cure for dysentery.[2] The plant has also been reported to possess anthelmintic, appetising, astringent and anti-diarrhoeal properties.[3] The basic fractions and pure alkaloids have been studied by various groups for different pharmacological properties and were found to affect blood pressure in frogs,[4] cerebrospinal nervous system of mouse, rat, guinea pig and frog[5,6] and to exhibit digitalis like cardiotonic activity on the rabbit heart.[7] The alkaloids also possess antibacterial activity.[8] In view of these properties present studies were undertaken on the isolation of kurchi alkaloids monitoring their effect on the blood pressure of rats. On preliminary screening the methanolic bark extract showed a dose dependent hypotensive effect in anesthetized rats up to a dose range of 10-30 mg/kg. The extract was subjected to a systematic bioassay-guided isolation procedure which resulted in the isolation and structure elucidation of six

alkaloids namely pubescine, norholadiene, pubescimine, pubescinine, holamide and norconessine. The chemistry of pubescine, norholadiene and pubescimine has been reported in an earlier communication.[9] Pubescine and norholadiene did not affect the blood pressure upto a dose of 3 mg/kg while the activity of pubescimine could not be determined due to limited quantity. Holamide (**1**) and pubescinine (**2**), two further new natural products showed hypotensive activity in rats at a dose of 3 mg/kg. Norconnesine (**3**) which had been reported earlier from the same source[10] was inactive at these doses. The structure elucidation of these constituents (**1,2** and **3**) is based on detailed nmr studies (Tables 1-2) and other spectral evidences, which also led to a complete assignment of all the ^{1}H and ^{13}C-nmr data of norconessine for the first time.

RESULTS AND DISCUSSION

Holamide (**1**) has the molecular formula $C_{23}H_{32}N_2O_2$ (HRms) showing nine double bond equivalents. The compound showed two singlets for tertiary methyls at δ 2.71 (N-Me) and 1.29 (Me-19), a doublet at δ 1.45 ($J=6.56$ Hz, Me-21) and two AB doublets at δ 3.17 and 3.00 each for one proton ($J=12.06$ Hz, H-18a and H-18b) in the ^{1}H-nmr spectrum (Table 1). These features suggested that **1** belongs to the conanine series of steroidal alkaloids.[11] It exhibited strong absorptions in the ir spectrum at 1660, 1615 and 1600 cm^{-1} and uv absorption at 240 nm indicating the presence of a conjugated carbonyl moiety.[12] The ^{1}H-nmr spectrum further showed three deshielded olefinic protons at δ 7.00 (1H, d, $J=10.16$ Hz, H-1), 6.21 (1H, dd, $J=10.16$, 1.84 Hz, H-2) and 6.05 (1H, t, $J=1.84$ Hz, H-4). The chemical shifts and coupling constants of the olefinic signals along with a peak at *m/z* 121.0648 in the HRms located a 1,4-dien-3-one moiety in ring A of the steroidal molecule. A strong absorption at 1725 cm^{-1} in the ir spectrum indicated the presence of another carbonyl group which gave a signal at δ 168.07 in the ^{13}C-nmr spectrum (Table 2). Upfield chemical shifts of C-18 (δ 48.13) and C-20 (δ 57.16) suggested the position of this carbonyl group on the ring nitrogen.[13] These structural features still left an *N*-CH_3 moiety to be placed which was taken as a part of the *N*-methylacetamido function at the nitrogen of the conanine skeleton. It was further confirmed by a peak at *m/z* 114.0782 ($C_5H_{10}N_2O$) resulting through the characteristic fragmentation of the 18,20-epimino group.[13] On the basis of these observations structure (**1**) has been assigned to holamide.

Pubescinine (**2**) showed the M^+ peak at *m/z* 367.2135 corresponding to the molecular formula $C_{23}H_{29}NO_3$ showing ten degrees of unsaturation. Its ir (1660, 1620 and 1595 cm^{-1}), uv (242 nm) absorptions and ^{1}H-nmr signals (Table 1) at δ 6.84 (d, $J=10.27$, Hz, H-1), δ 6.17 (dd, $J=$ 10.27, 1.88 Hz, H-2) and δ 6.12 (t, $J=1.88$ Hz, H-4) indicated that it also has the 1,4-dien-3-one system in ring A which was supported by strong peaks[12] at *m/z* 121.0688 (C_8H_9O) and 107.0480 (C_7H_7O) in the HRms (vide structure). However, in contrast to **1, 2** showed only Me-19 at δ 1.28 (Table 1) while the doublet of Me-21 was missing. Instead, a three- proton singlet was observed at δ 2.28 due to a vinylic methyl which indicated a double bond in the 18,20-epimino ring of the conanine series of alkaloids at either C-17 (20) or between C-20 and N. Its position at C-17(20) was confirmed by recording the ^{1}H-nmr in deuterated methanol with a trace of acids when, in contrast to the observation in case of an imine double bond (i.e between C-20 and N),[15] in this case an exchange of the methyl protons (H-21) with deuterium and their disappearance was not noted. A strong absorption of 1720 cm^{-1} in the ir spectrum, a singlet at δ 2.05 in the ^{1}H-nmr spectrum and a fragment at *m/z* 307.1915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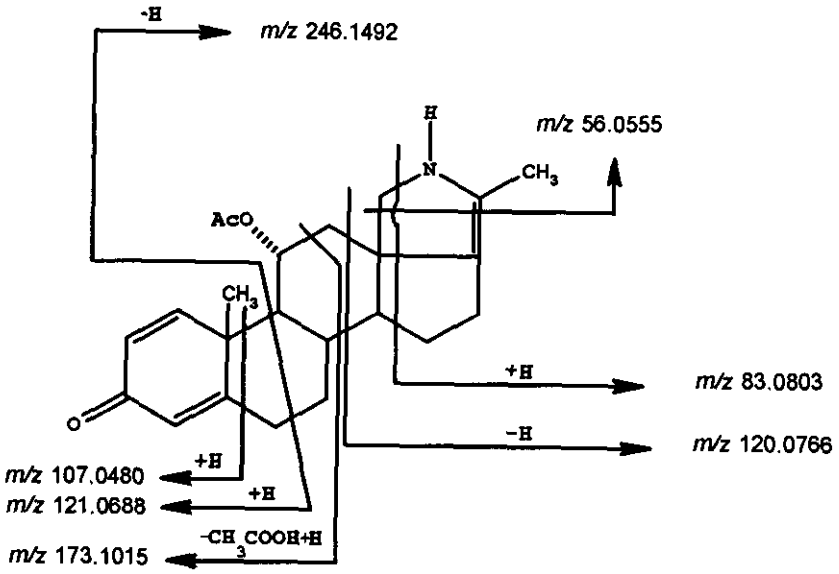
1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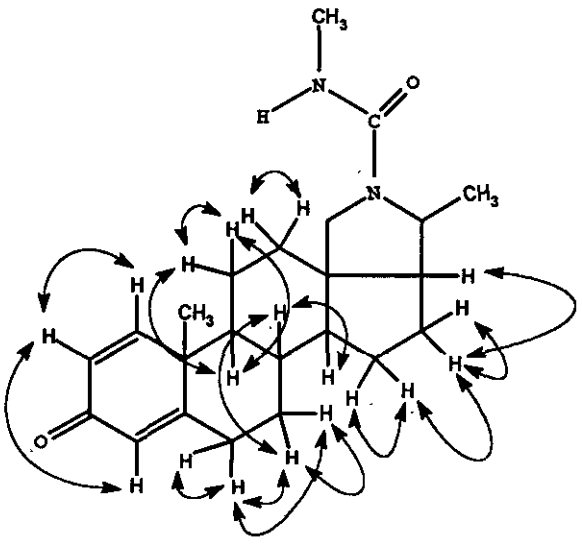
2



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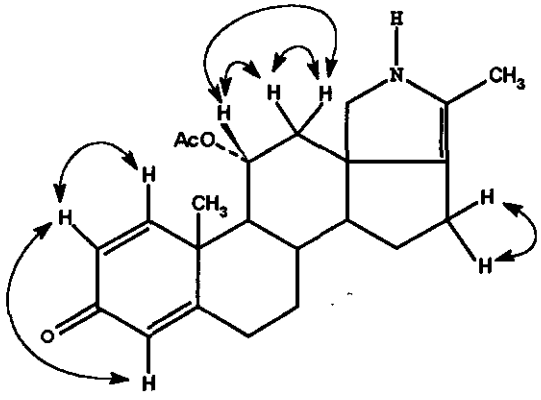

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Figure 1. COSY connectivities for compounds **1** and **2.**

(M^+-CH_3COOH) in the HRms showed the presence of an acetyl group. Its geminal proton appeared at δ 5.16 as a doublet of triplet (J = 10.42 and 5.02 Hz). The multiplicities and coupling constants suggested its axial nature and decided the location of the *O*-acetyl group at either C-7 or C-11 with equatorial disposition. The upfield shift (δ 6.84) of H-1 of **2** as compared to its shift (δ 7.00) in compound (**1**) led to place the acetoxy group at C-11. This assignment was further supported by comparable chemical shifts of H-1 of compound (**2**) with those of other 11-acetoxy compounds.[11] On the basis of these observations structure (**2**) has been assigned to pubescinine which was further corroborated by the ^{1}H (Table 1) and ^{13}C-nmr shifts (Table 2) and various fragments in the HRms (Scheme-2) as well as cosy-45 connectivities (Fig-1).

Compound (**3**) , molecular formula $C_{23}H_{38}N_2$ (M^+ 342.3012) was identified as norconessine, on the basis of detailed ir, uv, ^{1}H-nmr and ^{13}C-nmr data (vide experimental). This compound has been reported previously from the same source.[10]

Intravenous adminstration of the crude extract (CE) of *Holarrhena pubescens* at a dose of 3 mg/kg , produced no effect while at 10-100 mg /kg, it produced a dose-dependent fall in mean arterial blood pressure in anesthetized rats (Table 3). At the dose of 10 mg/kg, % decrease in mean blood pressure was 15.2 ± 1.04 whereas 26.20 ± 2.75 % reduction was observed at the dose of 30 mg/kg. The next higher dose (100 mg/kg) caused further decrease in BP of the magnitude of 35.86± 3.01%.
Activity-directed fractionation of the crude extract (CE) resulted in the isolation of two hypotensive pure compounds (**1** and **2**) along with a compound (**3**) which was inactive at the dose of 10 mg/kg. Due to short supply, these pure compounds were tested at a smaller dose (3 mg/kg) only. At this dose compound (**1**) was slightly less active than compound (**2**) with % reduction in blood pressure of 29.33 ± 3.81% and 34.98 ± 2.0 % respectively. With the crude extract (CE), comparable effects were observed at doses 20-30 times higher than for the pure compounds. This indicates that as compared to the CE, pure compounds are highly concentrated in activity as expected.

EXPERIMENTAL

Melting points are uncorrected. Mass spectra were recorded on a Finnigan MAT 112 and 312 double focussing mass spectrometers connected to a PDP 11/34 computer system; nmr spectra ($CDCl_3$): 400 MHz for ^{1}H and 75 MHz for ^{13}C. The chemical shifts are reported in δ (ppm) and the coupling constants are in Hz. The ^{13}C nmr spectral assignments (Table 2) have been made partly through a comparison of the chemical shifts with the published data for similar compounds[16] and partly through the appearance of signals in DEPT and HMQC spectra (Table 2). Precoated thin layer cards (DC-karten SiF) were used for tlc. Petroleum ether used was of the boiling range 60-70^{o}C.

Wistar normotensive rats (200-250 g) were anesthetized with thiopental sodium (50-80 mg kg^{-1}, i.p). The trachea was exposed and cannulated to facilitate spontaneous respiration. The arterial blood pressure was recorded from the carotid artery *via* the arterial cannula connected to a pressure transducer (model Statham P_{23} AC strain gauge transducer)coupled with Grass model 7D polygraph. Drugs were slowly injected *via* the jugular vein. Mean blood pressure was calculated by the following formula: diastolic blood pressure plus one-third of pulse width.[17]

Table 1. ^{1}H -Nmr data of **1-3**.

	1	**2**	**3**
H	δ_H	δ_H	δ_H
1a	7.00 d, (10.16)	6.84 d, (10.07)	1.90 m
1b	-	-	1.02 m
2a	6.21 dd,(10.16,1.84)	6.17 dd, (10.07,1.88)	1.75 m
2b	-	-	1.55 m
3	-	-	3.32 m
4a	6.05 t, (1.84)	6.12 t, (1.88)	2.04 m
4b	-	-	1.52 m
6a	2.40 m	2.32 m	5.36 dd, (13.63,4.42)
6b	2.20 m	2.10 m	-
7a	2.40 m	1.58 m	2.00 m
7b	2.35 m	1.42 m	1.42 m
8	1.38 m	1.02 m	2.06 m
9	2.15 m	1.50 m	2.14 m
11a	1.92 m	5.16 ddd, (10.42,10.42,5.02)	1.68 m
11b	1.40 m	-	1.18 m
12a	2.02 m	1.88 m	1.62 m
12b	1.98 m	1.12 m	1.48 m
14	1.30 m	2.22 m	1.22 m
15a	1.80 m	1.78 m	1.98 m
15b	1.52 m	1.60 m	1.98 m
16a	2.02 m	2.50 m	2.40 m
16b	1.08 m	2.36 m	1.18 m
17	1.10 m	-	2.78 m
18a	3.17 d, (12.06)	3.30 m	3.04 d, (12.29)
18b	3.00 d, (12.06)	1.40 m	2.93 d, (12.29)
19	1.29 s	1.28 s	0.97 s
20	3.73 m	-	3.70 m
21	1.45 d, (6.56)	2.28 s	1.38 d, (6.54)
$N\text{-}CH_3$ (Amide)	2.71 s	-	-
NH	5.41 s	-	-
$COCH_3$	-	2.05 s	-
$N\text{-}CH_3$ (22)	-	-	2.58 s
$N\text{-}CH_3$ (23)	-	-	2.88 s

Table 2. ^{13}C-Nmr data of **1-3**.

	1	**2**	**3**
C	δ_C	δ_C	δ_C
1	155.08	156.31	37.05
2	127.79	126.23	26.44
3	186.05	185.92	65.81
4	124.15	124.96	35.85
5	168.07	167.82	138.67
6	33.45	33.80	122.54
7	32.47	29.70	37.05
8	37.97	35.43	33.71
9	51.30	56.87	49.08
10	43.23	43.30	36.69
11	22.03	70.84	21.95
12	25.93	41.29	26.44
13	54.12	53.92	51.70
14	53.45	49.80	52.83
15	23.60	24.00	23.81
16	35.54	37.64	32.07
17	52.04	124.21	54.80
18	48.43	59.20	60.97
19	18.65	19.02	19.71
20	57.16	154.20	59.18
21	13.85	21.69	12.36
22 (N-C=O)	168.07	-	-
N-CH_3 (Amide)	29.66	-	-
N-CH_3 (22)	-	-	40.24
N-CH_3 (23)	-	-	41.32
$COCH_3$	-	21.49	-

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

Uncrushed bark (10 kg) was macerated with 10% methanolic NaOH (10 l) for 48 h at 28°C and repeatedly percolated with MeOH for 48 h (five times) at the same temperature. Each extract was neutralized with 30% aq. HOAc. The syrupy concentrate, obtained on removal of the solvent from the combined extracts under reduced pressure produced a dose-dependent fall in mean arterial blood pressure in anesthetized rats. The pH of this concentrate was stumped down by adding 10% aqueous HOAc at 28 °C and it was shaken out with EtOAc and water. The aqueous phase was basified with 20% ammonia and 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 to give a residue (Fraction **I** ; 20 g) which 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.[18] The petroleum ether insoluble portion (11 g) when dissolved in 10% aqueous AcOH and treated with $(NH_4)_2 SO_4$, furnished colourless precipitate of sulfates (Fraction **II**) which was filtered. The sulfate mother liquor was made alkaline with 10 % aqueous NaOH and extracted out with EtOAc which on usual working afforded a colourless residue (Fraction **III**; 9.5 g). **I, II** and **III** were tested for their effect on blood pressure on anesthetized rats. Fraction **I** was found inactivce (upto 30 mg/kg) in lowering blood pressure whereas both **II** and **III** were hypotensive, **III** being more active. Since **III** was most active therefore it was subjected to flash column chromatography (Model Aldrich; Al_2O_3, Merck 90; petroleum ether, petroleum ether-EtOAc, in order of increasing polarity).

Nine fractions (Fractions **IIIa**, **IIIb**, **IIIc**, **IIId**, **IIIe**, **IIIf**, **IIIg**, **IIIh**, **IIIi**) were ultimately obtained on combining the eluates on the basis of tlc. All these were tested on anesthetized rats. Fractions **IIIe, IIIf, IIIh** and **IIIi** were hypotensive (**IIIe** being most active at the dose of 1-30 mg/kg. Fractions **IIIc** and **IIIg** both exhibited biphasic response (hypertensive followed by hypotensive), while **IIIb** and **IIId** were inactive upto the doses of 30 mg/kg. Fraction **IIIe** (850 mg) being most active, was selected for further purification.

It was rechromatographed on a flash column (Eyela EF-10 Si gel, E. Merck 9385, $CHCl_3$, $CHCl_3$-MeOH, in order of increasing polarity). The $CHCl_3$-MeOH (8.8 : 1.2) eluate furnished pubesimine (32 mg). The $CHCl_3$-MeOH (9.5:0.5) eluate furnished four main fractions named as A, B, C and D in order of polarity. A and D were identified as **1** (25 mg) and **3** (30 mg) while B on purification on thick layer chromatography over silica gel with solvent system $CHCl_3$-MeOH (9.5:0.5) afforded **2** (18 mg). Similarly fraction C on thick layer chromatography Silica gel $CHCl_3$-MeOH (9.5:0.5) afforded pubescine and norholadiene. Pubescimine, pubescine and norholadiene were found inactive (up to the dose of 30 mg/kg) in lowering blood pressure and their chemistry has been reported earlier.[9] The activities of **1** and **2** are noted in Table 3 along with that of the crude extract (CE).

Table 3. Hypotensive effect of the crude extract (CE) and pure compounds (**1** and **2**) of *Holarrhena pubescens* on anesthetized rats.

Dose (mg/kg)	% Fall in mean blood pressure		
	CE	**1**	**2**
3	NE	29.33 ± 3.81	34.98 ± 2.01
10	15.20 ± 1.04	ND	ND
30	26.20 ± 2.75	ND	ND
100	35.86 ± 3.01	ND	ND

Values shown are mean ± SEM of three determinations expressed as percent of control values. ND denotes not determined and NE denotes no effect.

N-(N'-methylacetamido)-3-oxo-1,4-conadiene (**1**).- Plates (acetone); mp 248-250°C,$[\alpha]_D$ +126(*c* 0.331, $CHCl_3$). uv λ_{max} (MeOH) 240 and 205 nm; ir ν_{max} ($CHCl_3$) 3450, 1725, 1660, 1615 and 1600 cm^{-1}; Elms *m/z* (%) [M^+] 368.2485 ($C_{23}H_{32}N_2O_2$ requires 368.2463) (2), 310.2136 (71), 248.1888 (4), 180.1277 (4), 121.0648 (99) and 114.0783 (8); ^{1}H-nmr : Table 1; ^{13}C-nmr: Table 2; COSY: Figure 1.

11α-Acetoxy-N-demethyl-3-oxo-1,4,17(20)-conatriene (**2**).- Fine needles (acetone); mp 138-140°C,$[\alpha]_D$ +51 (*c* 0.276, $CHCl_3$). uv λ_{max} (MeOH) 242 and 205 nm; ir ν_{max} ($CHCl_3$) 3400, 1720, 1660, 1620 and 1595 cm^{-1}; Elms (%) *m/z* [M^+] 367.2135 ($C_{23}H_{29}NO_3$ requires 367.2147) (100), 307.1915 (98), 246.1492 (30), 173.1015 (24), 121.0688 (73), 120.0766 (12), 107.0480 (17), 83.0803 (18) and 56.0555 (11); ^{1}H-nmr : Table 1; ^{13}C-nmr : Table 2 ; COSY: Figure 1.

3-N-Methylamino-5-conanene (**3**).- Plates (acetone); mp 227-228°C,$[\alpha]_D$ +6.1 (*c* 0.59, $CHCl_3$). uv λ_{max} (MeOH) 280 and 208 nm; ir ν_{max} ($CHCl_3$) 3400, 2820 and 1600 cm^{-1}; Elms *m/z* (%) [M^+] 342.3012 ($C_{23}H_{38}N_2$ requires 342.3034) (66), 327.2831 (100), 191.1633 (4), 151.1377 (10), 138.1271 (10), 137.1266 (8), 84.0800 (42), 71.0725 (16); ^{1}H-nmr : Table 1;^{13}C-nmr: Table 2.

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