APPENDICES
THEVETIA PERUVIANA KERNEL OIL: A POTENTIAL BACTERICIDAL AGENT

V.K. SAXENA*, S.K. JAIN
Natural Products Laboratory, Department of Chemistry, University of Sagar, Sagar (M.P) 470 003 India

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SUMMARY. The seed kernels of T. peruviana yielded 58% of a pale yellow oil, having the following composition: palmitic acid, 15.6; stearic acid, 10.5; oleic acid, 60.9; linolenic acid, 5.2; linoleic acid, 7.8; behenic acid, 0.2; and erucic acid, 0.1. The oil was found to possess strong bactericidal activity against Bacillus subtilis and Staphylococcus aureus and least against Vibrocholerae.

Thevetia peruviana (Pers.), a member of the family Apocynaceae, is widely cultivated in the gardens throughout India. The kernel oil has been used externally for curing skin diseases. ¹ We have chemically investigated the oil by GLC and have tested its antibacterial activity.

The mature fruits of T. peruviana were collected from trees grown at Sagar and seeds were separated and shelled. The kernels were crushed and then Soxhlet extracted with petrol (60-80°C). Excess of hexane was added to the extract and the hexane-insoluble matter was removed by filtration. The filtrate, after evaporation of the solvent, gave 58% of a pale yellow oil (Table 1). After saponification (KOH and alcohol), the mixed fatty acids were esterified with BF₃-methanol (14%) ² and GLC analysed (Varian Aerograph 180°C, 6 ft × 1/8

<table>
<thead>
<tr>
<th>Constant</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific gravity at 25°C</td>
<td>0.904</td>
</tr>
<tr>
<td>Refractive index at 25°C</td>
<td>1.4603</td>
</tr>
<tr>
<td>Acid value</td>
<td>4.8</td>
</tr>
<tr>
<td>Saponification value</td>
<td>198.84</td>
</tr>
<tr>
<td>Iodine value (W/m²)</td>
<td>87.15</td>
</tr>
<tr>
<td>Unsaponifiable matter</td>
<td>1.5%</td>
</tr>
</tbody>
</table>

Table 1 - Physico-chemical constants of the oil.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Retention time (tₚ) (Min)</th>
<th>Composition (% by wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>6.2</td>
<td>15.6</td>
</tr>
<tr>
<td>18:0</td>
<td>11.4</td>
<td>10.5</td>
</tr>
<tr>
<td>18:1</td>
<td>12.6</td>
<td>60.9</td>
</tr>
<tr>
<td>18:2</td>
<td>14.9</td>
<td>5.2</td>
</tr>
<tr>
<td>18:3</td>
<td>19.7</td>
<td>7.4</td>
</tr>
<tr>
<td>22:0</td>
<td>40.5</td>
<td>0.2</td>
</tr>
<tr>
<td>22:1</td>
<td>44.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 2 - Fatty acid composition of Thevetia peruviana kernel oil.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Diameter of zone inhibition in mm*</th>
<th>Control**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pure oil 1:100 1:250 1:500 1:1000</td>
<td></td>
</tr>
<tr>
<td><em>S. albus</em></td>
<td>16 13 12 10 08 15</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>20 19 18 17 07 17</td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>20 18 16 15 09 18</td>
<td></td>
</tr>
<tr>
<td><em>B. pumilus</em></td>
<td>18 16 15 14 08 16</td>
<td></td>
</tr>
<tr>
<td><em>B. anthracis</em></td>
<td>15 14 13 12 06 12</td>
<td></td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>12 11 09 08 05 10</td>
<td></td>
</tr>
</tbody>
</table>

* including the diameter of filter paper disc.
** 1000 ppm griseofulvin.

Table 3 - Bactericidal activity of *Thucvetia peruviana* kernel oil.

in stainless steel column packed with 15% diethylene glycol succinate on Chromosorb W 60-80 mesh). Fatty acid composition (% by wt.) is shown in Table 2.

**Bactericidal activity of the kernel oil**

Solutions of five different dilutions (1:100, 1:250, 1:500, and 1:1000) of kernel oil in ethylene glycol were prepared and tested against *S. albus*, *S. aureus*, *B. subtilis*, *B. pumilus*, *B. anthracis* and *V. cholerae*. Griseofulvin (1000 ppm) was used as control. Bactonutrient agar alongwith Saboraud's dextrose agar was employed as medium. Sterilized paper discs (10 mm diam.) of Whatman No. 1 filter paper were thoroughly soaked in pure oil and in different dilutions and placed over the seeded agar plates.

The bactericidal activity was measured as zones of inhibition around the discs after incubating them at 33°C and the observation is recorded in Table 3.

In view of its strong bactericidal activity, the oil may potentially be used for manufacture of bactericidal soaps.

REFERENCES

AMINO-ACID COMPOSITION OF NERIUM OLEANDER STEM

V. K. SAXENA, A. K. JAIN AND S. K. JAIN

Natural Products Lab., Chemistry Department, Sagar University, Sagar (M.P.) 470 003.

(Received 5 September, 1988)

Stems of *Nerium oleander* (N. O. Apocynaceae) were studied for amino-acid make up.

**Experimental and Discussion**

50 mg dried, defatted powder of stems were hydrolysed with 6 N HCl and 6 N NaOH respectively in sealed tubes at 100-110° till the hydrolysate was negative to biuret test. The hydrolysates were concentrated under reduced pressure and then taken in 10% isopropanol and analysed by paper chromatography\(^4\). Quantitative determination of individual amino acids was made by photometric method\(^4\).

Presence of 16 amino-acids was observed, the composition expressed as mg glycine per 16 mg nitrogen being alanine (0.99 mg), arginine (1.12 mg), aspartic acid (1.98 mg), cystine (1.09 mg), glutamic acid (2.27 mg), glycine (2.01 mg), histidine (0.89 mg), leucine and isoleucine (2.13 mg), lysine (0.78 mg), phenylalanine (1.02 mg), proline (1.32 mg), serine (1.05 mg), threonine (0.12 mg), tryptophan (0.14 mg), tyrosine (1.20 mg) and valine (0.95 mg). The predominating amino-acids are glutamic acid, leucine and isoleucine, and glycine. However, for use of such stems as source for the amino-acids should take due care of the cardio-toxic glycosides present in these.

**References**

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Paper No. 272/R/2—"Non-saponifiable....Analysis"
Entitled:

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Sagar-470 003 (M.P.)
NON-SAPONIFIABLE CONSTITUENTS OF NERIUM OLEANDER

KERENEL OIL - GC-MS ANALYSIS

V.K. SAXENA, S.K. JAIN and G.C. SAMAIYA
Natural Products Laboratory, Department of Chemistry,
University of Sagar, SAGAR (M.P.) 470 003 INDIA

ABSTRACT

Nerium oleander Linn (N.O. Apocynaceae) is an glabrous shrub of India\(^1\), and is reported to possess cardiotonic properties\(^2\) Phytoc-chemical investigations on the plant was undertaken to study non- saponifiable constituents of kernel oil.

The Kernel oil obtained in 2\(1\%\) yield was worked up by column chromatography, gas liquid chromatography and mass spectroscopy. The oil has been found to consists \(\beta\). Sitosterol, 49.02\%; \(\alpha\)-spinasterol, 27.11\%; Stigmasterol, 19.01\% andcampsterol, 5.85\%.

PROCEDURE AND RESULTS

The mature fruits of Nerium oleander were collected from trees grown at Sagar and their pericorop was removed. The air-dried, powdered kernels 100 g were extracted with petroleum ether (60–80°C) in a soxhlet extractor for 48 hours. Evaporation of the extract under reduced pressure afforded a 2\(1\%\) of pale yellow oil. It was purified by activated animal charcoal. The oil 5 g was saponified with 10% methanolic KOH and the yellow coloured non-saponifiable matter 2.2 g was recovered in the usual way.

The non-saponifiable matter was taken up in solvent ether and chromatographed over silica gel. Elution with hexane followed by increasing amount of ether in hexane (5, 10, 15, 20\%) furnished sterols in the last elute. The crude mixture was recrystallised
from methanol ether (4:1) to provide a crystalline solid, M.P. 130-32°, it gave positive Libermann-Buchard test confirming the steroidal nature of the compound, it was homogenous on TLC.

**Gas-Liquid chromatography**

GLC analysis of sterols were carried out on a Pye-Unicam 104 using 3% OV-17 column and FID detector, operating conditions being column temperature 260°, injection port and FID temperature 280° carrier gas N₂ at flow rate 50 ml h⁻¹. GLC analysis gave four peaks from β-sitosterol (Rₜ 1.52); α-spinasterol (1.72); stigmasterol (1.37) and campesterol (1.28). GLC analysis confirmed that it was a mixture of β-sitosterol, 48.02%; α-spinasterol (27.11%); stigmasterol, 19.01% and campesterol, 5.85%.

Further confirmation of this steroidal composition was carried out by EIMS of steroidal fraction, which showed ions peaks at M/Z 414, 412, 400 suggesting it to be mixture of β-sitosterol M₁ 414, stigmasterol M₂ 412 and campesterol M₃ 400 in addition to α-spinasterol due to presence of ion peak at 271 (100%) which appears mainly due to α-spinasterol.

**REFERENCES**

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Prof. Dr. E. Reinhard
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D-7400 Tübingen 1

Prof. V.K. Saxena
Dept. of Chemistry
Dr. H.S. Gour University
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Scientist-in-Charge

Chemistry Section.

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A Novel Cardiotonic Glycoside from the Roots of

\textit{Nerium oleander}

V.K. Saxena\textsuperscript{1,2} and S.K. Jain\textsuperscript{1}

1. Natural Products Laboratory, Department of Chemistry, Dr. Harisingh Gour University, SAGAR - 470 003 (M.P.) INDIA.

2. Address for correspondence.
Abstract: A novel cardiotonic glycoside, 16-dianhydrogitoxigenin-3-O-β-D-glucopyranosyl (1→4)-O-β-D-xylopyranoside has been isolated from the MeOH-soluble fraction of roots of N. oleander and has been identified by chemical and spectral studies.
Introduction

*N. oleander* Linn. (1-2) (Apocynaceae) is a widely available evergreen glabrous, ornamental shrub in mediterranean region. It's various vegetative parts have been reported to possess cardiotonic activity. The cardiotonic property of *N. oleander* has been attributed partly to the presence of cardenolides: oleandrine, digitoxigenin, and uzarigenin. In our research programme on cardiotonic drugs we have undertaken on this plant and report the presence of a new glycosidic cardenolide. It was identified as 14,16-dianhydrogitoxigenin-3-O-β-D-glucopyranosyl (1→4) O-β-D-xylopyranoside.

Results and Discussion

Compound 1, C_{34}H_{48}O_{12}, obtained as a colourless crystalline substance responded to positive Kedde's, Keller-Kiliani, and Legal's test (3-5) confirming its cardenolide nature. Peak in the IR spectrum of 1 at 3300 cm^{-1} indicated the presence of free OH group(s). Preparation of a hexa-acetyl derivative 2, C_{46}H_{60}O_{18}, suggested 6 acetylatable OH groups. Acid hydrolysis of 1 with 7% ethanolic H_{2}SO_{4}, gave a cardiogenin 3, C_{25}H_{30}O_{3}, glucose and xylose 1 mol. each respectively. The IR spectrum of 3 showed peak at 3450 cm^{-1}, indicating the presence of free OH group. Compound 3 on acetylation with Ac_{2}O/Pyridine afforded a mono-acetate 4, C_{25}H_{32}O_{4}, suggesting one acetylatable OH group in it, and 3 on Jones oxidation afforded another compound 5, which gave positive Zimmermann test for 3 keto group (6).

The cardenolide nature of 3 was also supported by MS fragmentation pattern showing the loss of H_{2}O (m/z 336) followed by an methyl
(m/z 321) from the molecule. The base peak (m/z 203) resulted from the loss of ring D and side chain (7). Moreover, peak m/z 111 was characteristic of butenolide ring in cardenolide (8).

The UV spectrum of 3, λ\textsubscript{max} (MeOH) 280 nm exhibited presence of conjugated carbonyl group, while the presence of a lactone ring was supported by an IR peak at 1740 cm\textsuperscript{-1}. Appearance of a triple doublet at 6 5.08 (2H) and broad singlet at 6 5.86 (1H) in \textsuperscript{1}H-NMR was a diagnostic feature of lactone ring (9). These assignments were confirmed in the \textsuperscript{13}C-NMR spectrum by resonance at 6 176.87 [1C, s, C(23)], 173.57 [1C, s, C(20)], 119.55 [1C, d, C(22)], 72.10 [1C, t, C(21)]. The A/B ring juncture was determined to be 5β since C-18 methyl carbon is found at 6 9.60 [1C, q, C(18)] (10).

Location of lactone ring at C-17 position in the ring D was established by KMnO\textsubscript{4} oxidation of methyl derivative of 1, which furnished a compound 7. Molecular formula C\textsubscript{21}H\textsubscript{30}O\textsubscript{3}, which was found to be 3β-methoxy-5β-ethyl-14,16-enic acid confirmed by mmp, Co-TLC and Co-PC with authentic sample.

Another peak in the IR spectrum of 3 at 1640 cm\textsuperscript{-1} showed the presence of unsaturation, which was further supported by the fact that aglycone on catalytic hydrogenation formed hexa-anhydo derivative 8, C\textsubscript{23}H\textsubscript{36}O\textsubscript{3}, mp 153-54° thereby confirming the presence of three double bond in it.

The position of the one of the double bond in 3 was confirmed at C-20(22), because 3 responded to (+) Legal test (5) for α:β unsatura-
tion in the lactone ring which was also supported by $\lambda_{\text{max}}$ (MeOH) 219 nm.

Compound 3 gave positive TNM test (11) suggesting that the remaining two olefinic bonds were present in the nucleus. The position of these olefinic bonds were fixed on the basis of decoupling experiments, the two protons resonating at $\delta$ 5.08 (2H, ddd, J=2,2,1 Hz) were assigned to 21-H, while the absorption at $\delta$ 5.87 (1H, broad singlet) was assigned to 22H. The two remaining absorption at $\delta$ 6.09 (1H, dd, J=2, 1 Hz) and $\delta$ 6.76 (1H, d, J=2 Hz) were assigned to vinylic protons at C-16 and C-15 respectively. The coupling between C-16 and C-15 protons was confirmed by irradiation at $\delta$ 6.75 which simplified the absorption at $\delta$ 6.09 to a fine doublet (J=1 Hz), and irradiation at $\delta$ 6.09, which collapsed the doublet at $\delta$ 6.75 to a singlet and simplified the absorption at $\delta$ 5.08, indicating the long-range coupling between the C-21 protons and H-16 proton.

The position of attachment of sugar residue to cardiogenin was shown to be at C-3 as the glycosidic cardenolide did not respond to Zimmermann test, while the oxidised 14,16-dianhydrogitoxigenin did characteristic for C$_3$-OH group.

The $^1$H- and $^{13}$C-NMR spectra both revealed the presence of one oxymethine proton, resonating as a double doublet at $\delta$ 3.08, was typical of H-3 with coupling constants indicative of an axial configuration thereby requiring the normal equatorial (3P) oxygen.

The $^1$H-NMR spectrum of 2 at 90 MHz not only confirmed the derived structure but also established the configuration of the glyco-
sidic linkages. The two double doublets (J=9 and 3Hz) of one proton each at δ 4.46 and δ 4.35 could be easily assigned to two anomic protons of the two sugars. The large coupling constant (J=9 Hz) of these two anomic protons were typical of an axil configuration suggesting that the sugar moieties were in 4C1 D conformation joined to the aglycone through a β-D-glycosidic linkage (12).

The cardiac glycoside when hydrolysed by enzyme diastase (13) yielded D-xylose and D-glucose, giving first appearance of D-glucose on partial hydrolysis. Consumption of 3.02 moles of periodate to produce 1.01 mole of formic acid per mole of the glycoside and hydrolysis of the permethylated glycoside (14) yielded 2,3-di-O-methyl-D-xylose (confirmed by Co-PC and Co-TLC) and 2,3,4,6-tetra-O-methyl-D-glucose (confirmed by Co-PC and Co-TLC) and indicated the sugar moieties as β-D-glucopyranosyl (1→4)-O-β-D-xylopyranoside.

On the basis of above evidence I was identified as 14,16-dianhydrogitoxigenin-3-O-β-D-glucopyranosyl (1→4)-O-β-D-xylopyranoside, which was further supported by 13C-NMR (20 MHz, DMSO-d6, δ ppm) data of I which showed (for sugar) doublets at 102.79, 101.89 (C1', C1") 72.48, 75.98, 77.06, 72.89, 76.46, 70.28, 74.82 (C2'-C4', C2"-C5") triplets at 63.66, 61.21 (C5', C6'), (for aglycone) singlets at 176.87 (C23), 173.57 (C20), 158.05 (C17), 146.31 (C14), 54.24 (C13), 36.29 (C10), doublets at 135.89 (C16), 119.55 (C22), 108.38 (C15), 70.74 (C3), 45.18 (C9), 41.84 (C8), 36.85 (C5), triplets at 72.10 (C21), 37.12 (C12), 33.51 (C4), 30.74 (C1), 27.96 (C2), 26.66 (C6), 24.04 (C7), 21.44 (C11), quartets at 23.94 (C19), 9.60 (C18).
Experimental

Plant material: The plant *Nerium oleander* was supplied by M/S. L.R. Brothers, Saharanpur and authenticated by the Botany Department of this University. An herbarium specimen (No. V-XX) has been deposited at herbarium room No. 36 of the Chemistry Department. Dried and powdered roots were extracted exhaustively with hot 90% EtOH. The extract was concentrated under reduced pressure to a viscous mass. It was segregated into petroleum-ether (60-80°C), C₆H₆, CHCl₃, EtOAc, Me₂CO, and MeOH-soluble fractions.

Isolation and identification: The concentrated MeOH-soluble fraction showed a single spot on TLC [Si gel 60 plates solvent system CHCl₃:Me₂CO:MeOH:H₂O (60:20:14:2 v/v) sprayed by Kedde's reagent (2% 3,5-dinitrobenzoic acid followed by KOH in MeOH)]. The fraction was purified over a Si gel column (60-120 mesh) and eluted with CHCl₃:MeOH (7:4 v/v), providing compound 1: colourless solid (0.032%), mp 258-260°C, C₃₄H₄₈O₁₂ (calcd C 62.954, H 7.612 found C 62.968, H 7.623); EIMS [M]+ 648, UV λmax (MeOH), 220 and 281 nm; EIMS m/z (%): [M]+ 648 (24.3), 486 (29.8), 354 (40.8), 339 (28.0), 336 (24.9), 321 (13.8), 280 (31.0), 271 (15.9), 269 (21.5), 253 (23.3), 234 (6.5), 216 (6.8), 203 (100), 166 (28.8), 163 (29.3), 162 (22.0), 148 (23.1), 139 (8.4), 133 (20.9), 125 (26.3), 121 (4.3), 117 (11.9), 111 (4.0), 107 (8.9); IR νmax (KBr), 3300, 2960, 2870, 1742, 1712, 1640, 1630, 1432, 1390, 1380, 1345, 1305, 1210, 1195, 1160, 1030, 895 cm⁻¹.

Acetylation of 1 to 2: Treatment of 1, with Ac₂O and pyridine (5:1 v/v) at 110°C for 4 h yielded a hexaacetate as white needles, mp 227-229°C, C₄₆H₆₀O₁₈ (calcd C 61.329, H 6.713 found C 61.338, H 6.725),
EIMS [M]+ 900; ¹H-NMR (90 MHz, DMSO-d₆, δ ppm) 0.92 (3H, s, Me-18), 0.98 (3H, s, Me-19), 1.24-2.00 (polymethylene-CH₂- and -CH=), 2.03 (3H, s, OAc-4"), 2.06 (2 x 3 H, 2 x s, OAc-2" and OAc-3"), 2.09 (3H, s, OAc-2'), 2.12 (3H, s, OAc-3'), 2.15 (3H, s, OAc-6"), 3.08 (1H, dd, J=11, 4 Hz, H-3), 3.34-4.30 (11H, m, sugar protons), 4.35 (1H, dd, J=9, 3 Hz, anomeric sugar proton H-1'), 4.46 (1H, dd, J=9, 3 Hz, anomeric sugar proton H-1"), 5.06 (2H, ddd, J=2, 2, 1 Hz, H-21), 5.87 (1H, bs, H-22), 6.08 (1H, dd, J=2, 1 Hz, H-16) 6.75 (1H, d, J=2 Hz, H-15).

Acid hydrolysis of 1: Compound 1 was hydrolysed (7% ethanolic H₂SO₄) by refluxing for about 8 h, after removal of EtOH it yielded aglycone 3. The hydrolysate was neutralized with BaCO₃, BaSO₄ was filtered off, and filtrate was concentrated under vaccum. It was examined on PC [n-BuOH:HOAc:H₂O (4:1:5)] and showed the presence of D-glucose and D-xylose. The quantitative estimation of sugar in the hydrolysate showed the presence of 1 mol. of glucose and 1 mol. of xylose (15).

Identification of aglycone 3: Colourless needles, mp 176-177°, C₂₃H₃₀O₃ (calcd C 77.928, H 8.531 found C 77.942, H 8.548), EIMS (M)+ 354, TLC homogenous [Rf : 0.81, CHCl₃:MeOH:H₂O (80:18:2 v/v)]; UV λmax (MeOH) 219 and 280 nm; IR νmax (KBr) 3450, 2962, 2968, 1740, 1712, 1642, 1632, 1450, 1390, 1378, 1340, 1308, 1208, 1190, 1150, 1032, 890 cm⁻¹; EIMS m/z (%) [M]+ 354 (40.3), 339 (28.2), 336 (25.1), 321 (14.2), 271 (15.6), 269 (21.0), 253 (23.0), 234 (6.4), 216 (6.8), 203 (100), 166 (28.6), 162 (22.0), 148 (23.0), 139 (84), 133 (21.0), 125 (26.3), 121 (4.2), 111 (4.0), 107 (8.8). It formed a monoacetate 4: mp 168-169°, C₂₅H₃₂O₄ (calcd C 75.724, H 8.135 found C 75.748, H 8.149),
EIMS [M]+ 396; 1H-NMR (90 MHz, CDCl3, δ ppm) 0.94 (3H, s, Me-18), 0.98 (3H, s, Me-19), 1.22-2.00 (polyethylene -CH2- and -CH=), 2.06 (3H, s, OAc-3), 3.10 (1H, dd, J=11, 4 Hz, H-3), 5.08 (2H, ddd, J=2.1 Hz, H-21), 5.86 (1H, bs, H-22), 6.09 (1H, dd, J=2.1 Hz, H-16), 6.76 (1H, d, J=2 Hz, H-15).

Jones oxidation of 3 to 5: A solution of 1 (100 mg) in ether (50 ml) was added K2Cr2O7 (200 mg) in 0.1 ml of concentrated H2SO4 and 3 ml of H2O. The reaction mixture was stirred overnight. The product was purified by silica gel chromatography using hexane:EtOAc. Recrystallisation from EtOAc:hexane yielded 5, mp 191-193⁰, C23H28O3 (calcd C 78.455, H 8.007 found C 78.463, H 8.011), EIMS [M]+ 352.

Methylation of 3 to 6: To the solution of 1 (100 mg) in dry acetone (20 ml) was added dry K2CO3 (200 mg) and Me2SO4 (2.0 ml). The reaction mixture was stirred at 50⁰ for 5 hr, then diluted with H2O and extracted with ether. The ethereal extract was dried over anhydrous Na2SO4 and concentrated to dryness, and the product crystallized from MeOH to give 6 as white needles, mp 128-130⁰, C24H32O3 (calcd C 78.225, H 8.753 found C 78.238, H 8.764), EIMS [M]+ 368.

Catalytic hydrogenation of 3 to 8: A solution of 3 (100 mg) in 20 ml dry MeOH was reduced with H2 in presence of 5%Pd/C at room temperature from 4 hr. It was filtered and solvent removed under reduced pressure to give a residue which crystallized from MeOH, mp 153-154⁰, C23H36O3 (calcd C 76.625, H 10.065 found C 76.635, H 10.073), EIMS [M]+ 360.
Attachment of aglycone with sugar: Compound 1 was treated with Me₂SO₄ in aq. NaOH giving methylated glycoside. It was hydrolysed with 4N H₂SO₄. After usual workup, the methylated sugars were identified by Co-PC and Co-TLC as 2,3-di-O-methyl-D-xylose and 2,3,4,6-tetra-O-methyl-D-glucose.

Periodate oxidation: Compound 1 was dissolved in MeOH and treated with NaIO₄ for 48 hr. The liberated HCOOH and consumed periodate were estimated by the Jones method (16).

Enzymatic hydrolysis: Compound 1 in MeOH was mixed with an equal volume of almond emulsion solution and left at room temperature for 24 hr. The examination of hydrolysate on PC showed the presence of D-glucose and D-xylose.

Acknowledgement

We warmly thank to the Director, Central Drug Research Institute, Lucknow for spectral analysis. Thanks are also due to Mr. S.D. Deousker, for typing the manuscript.
1. $R = H$;  2. $R = Ac$.

3. $R = H$
4. $R = Ac$
5. $R = Me$
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>$^1$H-NMR</td>
<td>proton magnetic resonance</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
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<td>CDCl$_3$</td>
<td>deuterated chloroform</td>
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<td>bs</td>
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References


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1. The manuscript is to be resubmitted in the proper form (vide Information for Authors, copy enclosed) for which the original manuscript is returned herewith.
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Title of paper –

\[ L(3b)_{14,16,18} \text{ diamyladco} \text{ oxo} \]

Yours faithfully,

K Chandrasekhar

Dr. V.K. Saxena
Deptt. of Chem.
Dr. H.S. Gour University
Sagar 470 003 (M.P.)
Organic Chemistry


V.K. SAXENA* and S.K. Jain
Natural Products Laboratory, Department of Chemistry,
Dr.H.S.Gour University, SAGAR - 470 003 (M.P.) INDIA

Abstract

A novel cardenolide 3β-hydroxy-19-oxo-5β-carda-14,16,20(22)-trienolide (1) has been isolated from MeOH-soluble fraction of the stems of Nerium oleander and has been identified by chemical and spectral analysis.

Introduction

Nerium oleander Linn. (Apocynaceae) is a widely available evergreen, glabrous, ornamental shrub in the mediterranean region and in India.1,2 Its various vegetative parts have been reported to possess cardiotonic activity. Earlier workers have reported digitalinum verum, oleandrin, nerifolin, adynerin, neriantin, odorobiosides G and K, odorotriosides G and K and cornerine from the different vegetative parts of the plant. From our research program on cardiotonic drugs we now report a new cardenolide. It was identified as, 3β-hydroxy-19-oxo-5β-carda-14,16,20(22)-trienolide.
Results and Discussion

Compound 1, C_{23}H_{28}O_{4}, obtained as a colourless crystalline substance responded to positive Kedde's\textsuperscript{3}, Keller-Kilian\textsuperscript{4} and Legal's test\textsuperscript{5} confirming its cardenolide nature, and also supported by a ms fragmentation pattern showing the loss of H_{2}O (m/z 350), followed by an aldehyde (m/z 321) from the molecule. The base peak (m/z 217) resulted from the loss of ring D and side chain\textsuperscript{6}. Moreover, peak m/z 111 is characteristic of the butenolide ring in cardenolides\textsuperscript{7}.

A peak in ir at 3450 cm\textsuperscript{-1} indicated the presence of a free OH group. Preparation of a mono-acetyl derivative (2), C_{25}H_{30}O_{5}, suggested one acetylable OH group. Compound 1 on Jones oxidation afforded a compound 4, which gave positive Zimmermann test for 3 keto group\textsuperscript{8,9}.

The uv spectrum of 1, \textlambda_{max}(MeOH) 280 nm exhibited the presence of conjugated carbonyl group, while the presence of a lactone ring was supported by an ir peak at 1740 cm\textsuperscript{-1}. Appearance of triple doublets at 5.06 (2H, J=2.1,1 Hz) and broad singlet at 5.87 (1H) in \textsuperscript{1}H nmr was a diagnostic feature of a lactone ring.\textsuperscript{10}

A peak in the ir spectrum of 1 at 1646 cm\textsuperscript{-1} showed the presence of unsaturation, which was supported by the fact that compound 1 on catalytic hydrogenation gave hexahydro derivative(9, thereby confirming the presence of three double bonds in it.
The position of one double bond was established at C-20(22), since compound I produced deep red colour in pyridine with alkaline sodium nitroprusside\textsuperscript{5} and this position was further supported by uv absorption at 223 nm characteristic of \( \alpha: \beta \) unsaturated lactone ring.

Compound I gave positive TNM\textsuperscript{11} test suggesting that the remaining olefinic bonds were present in the steroidal nucleus. Their position was fixed at C-14(15) and C-16(17), because \(^1\text{H} \) nmr absorption at \( \delta 6.08 \) (1H, dd, J=2, 1 Hz) and \( \delta 6.75 \) (1H, d, J=2 Hz) were assigned to vinylic protons at C-16 and C-15 respectively.

The position of lactone ring was established at C-17, since methyl derivative of I on KMnO\textsubscript{4} oxidation in acetone yielded 3\( \beta \)-methoxy-19-oxo-5\( \beta \)-eti-14,16-enic acid (6) which was confirmed by m.m.p., Co-tlc and superimposable ir spectrum.

On the basis of the above evidence I was identified as, 3\( \beta \)-hydroxy-19-oxo-5\( \beta \)-carda-14,16,20(22)-trienolide and was further supported by \(^{13}\text{C} \) nmr (20 MHz, DMSO-\textsubscript{d}\textsubscript{6}, \( \delta \) ppm) data of I which showed signals at 30.72 (C-1), 27.93 (C-2), 66.76 (C-3), 33.52 (C-4), 36.89 (C-5), 26.64 (C-6), 24.08 (C-7), 36.72 (C-8), 41.19 (C-9), 36.26 (C-10), 21.47 (C-11), 37.74 (C-12), 54.28 (C-13), 146.34 (C-14), 108.32 (C-15), 135.87 (C-16), 158.08 (C-17), 17.80 (C-18), 195.73 (C-19), 173.58 (C-20), 72.14 (C-21), 119.58 (C-22) and 176.84 (C-23).
Experimental

Plant Material: The plant *N. oleander* was supplied by M/s L.R. Brother, Saharanpur and authenticated by the Botany Department of this university. An herbarium specimen (No V-XIX) has been deposited at herbarium room No. 36 of the Chemistry Department. Dried and powdered stems were extracted exhaustively with hot 90% EtOH. The extract was concentrated under reduced pressure to a viscous mass. It was segregated into petroleum-ether (60-80⁰) -, C₆H₆-, CHCl₃-, EtOAc-, Me₂CO- and MeOH- soluble fractions.

Isolation and identification: The concentrated MeOH-soluble fraction showed a single spot on tlc [Si gel 60 plates solvent system CHCl₃-MeOH-H₂O (80:18:2 v/v) sprayed by Kedde’s reagent]. The fraction was purified over a Si gel column (60-120 mesh) and eluted with CHCl₃-MeOH (3:4 v/v), providing compound 1: colourless crystalline solid (0.028%), m.p. 179-81⁰ (Found: C, 74.998; H, 7.685. C₂₃H₂₈O₄ calcd. for: C, 74.972; H, 7.659%); uv λmax (MeOH) 223 and 280 nm; elms m/z (%) [M]⁺ 368 (45.0), 350 (23.2), 339 (21.7), 321 (28.3), 285 (41.1), 283 (29.4), 267 (80.6), 247 (69.8), 229 (18.1), 217 (100), 179 (57.0), 176 (47.2), 161 (9.1), 153 (8.6), 139 (6.8), 135 (22.9), 133 (10.9), 121 (7.3), 111 (49.0); ir λmax (KBr) 3450, 2960, 2880, 2830, 1740, 1732, 1710, 1646, 1631, 1450, 1377, 1340, 1305, 1205, 1195, 1155, 1030, 890 and 830 cm⁻¹.
Acetylation of 1 to 2: A solution of 1 (200 mg) in pyridine (20 ml) and Ac₂O (20 ml) was stirred overnight at room temp. The solution was evaporated in vacuo and residue was crystallized from MeOH to give colourless needles (160 mg), m.p. 171-73° [Found: C, 73.17; H 7.38; C₂₅H₃₀O₅ calcd for: C, 73.146; H, 7.366%]; eims [M]+ 410; ¹H nmr (90 MHz, DMSO-d₆, δ ppm) 0.98 (3H, s, Me), 1.24-2.00 (polymethylene-CH₂- and -CH=), 2.06 (3H, s, -OAc), 4.09 (1H, m, H-3α), 5.06 (2H, ddd, J=2,1,1 Hz, H-21), 5.87 (1H, bs, H-22), 6.08 (1H, dd, J=2,1 Hz, H-16), 6.75 (1H, d, J=2 Hz, H-15), 10.02 (1H, s, -CHO).

Methylation of 1 to 3: To the solution of 1 (200 mg) in dry acetone (100 ml) was added dry K₂CO₃ (500 mg) and Me₂SO₄ (1.0 ml). The reaction mixture was stirred at 50° for 5 hr, then diluted with H₂O and extracted with ether. The Et₂O was evaporated to dryness and the product crystallized from MeOH to give 3 (150 mg) as a white needles, m.p. 118-19°, eims [M]+ 382 (Found: C, 73.928; H, 7.942. C₂₄H₃₀O₄ calcd for: C, 73.903, H, 7.932%).

Jones oxidation of 1 to 4: A solution of 1 (100 mg) in ether (100 ml) was added K₂Cr₂O₇ (500 mg) in 0.1 ml of concentrated H₂SO₄ and 5 ml of H₂O. The reaction mixture was stirred overnight. The product was purified by Si gel chromatography using hexane-EtOAc. Recrystallisation from EtOAc-hexane yielded 4, m.p. 150-51°, eims [M]+ 366 (Found: C, 75.129, H, 7.164. C₂₃H₂₆O₄ calcd for: C,75.116; H,7.152%).
Catalytic hydrogenation to 1 to 5: A solution of 1 (100 mg) in 20 ml dry MeOH was reduced with hydrogen in presence of 5% Pd/C at room temperature for 4 hr. It was filtered and solvent removed under reduced pressure to give a residue which crystallised from MeOH, m.p. 162-63°, eims [M]+ 374 (Found : C, 73.778; H, 9.246. C_{23}H_{34}O_4 calcd for : C, 73.765; H, 9.232%).

Acknowledgements

The authors are thankful to Prof.S.P. Banerjee, Head, Department of Chemistry, for facilities and to the Director, Central Drug Research Institute, Lucknow for spectral analysis.
2. R = Ac

AC₂O/PY

1. R = H

Pd/C

H₂

DMS + dry K₂CO₃
in dry acetone

3. R = Me

KMnO₄
Oxidn

MeO