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## TETRANORTRITERPENOIDS AND STEROIDAL GLYCOSIDES FROM THE SEEDS OF *AZADIRACHTA INDICA* A. JUSS

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The paper describes the isolation of two new tetranortriterpenoids limbocinin (**1**) and limbocidin (**2**) and two new glycosides (**3**) and (**4**) of stigmasterol which have been isolated from the neutral fraction of the seeds of *Azadirachta indica* A. Juss. Their structures have been elucidated through spectroscopic methods.

*Key words: Azadirachta indica* A. Juss, Meliaceae, Neem seed, Limonoids, Stigmasterol glycosides.

### INTRODUCTION

The neem tree (*Azadirachta indica* A. Juss: Meliaceae) is indigenous to Asia, Africa and other tropical parts of the world and almost every part of the tree has long been used for the treatment of a variety of human ailments [1, 2]. In continuation of studies in the constituents of various parts of neem [3-5], two new tetranortriterpenoids limbocinin (**1**) and limbocidin (**2**) have been isolated from the neutral fraction of the ethanolic extract of Neem seed, along with two new glycosides (**3**) and (**4**) of stigmasterol. The structures of these constituents have been established through spectral studies.

### EXPERIMENTAL

IR (in $CHCl_3$) and UV (in MeOH) spectra were measured on JASCO-A-302 and Hitachi-U-3200 spectrophotometer respectively. Mass spectra was recorded on finnigan MAT 112 spectrometer, exact masses have been measured through peak matching. FAB mass (positive) spectra were run on JMS HX-110 double focussing mass spectrometer, operating at an accelerating voltage of 10 kv, using methanol as a solvent and glycerol as a matrix on the target. The samples were ionized by bombardment with Xenon (gas) atoms. The $^1H$ NMR spectra were recorded in $CDCl_3$ on Bruker Aspect AM-400 spectrometer, operating at 400 MHz. The chemical shifts are recorded in ppm ($\delta$) and coupling constants (J) are in Hz. The purity of compounds was checked on tlc. Silica gel E´. Merck 9385 was used for the flash column chromatography (Eyela EF-10).

*Plant material.* The plant material examined in the present studies was collected from Karachi region and a voucher specimen (No. NM-1) has been deposited in the herbarium of the Botany Department of Karachi University.

*Extraction of plant material.* The ethanolic extract of Neem seed (8 kg) was divided into acidic and neutral fractions. The neutral fraction was partitioned into petroleum ether soluble and insoluble portions and the latter was successively treated with ether and ethyl acetate. Each of these was concentrated and treated with an excess of petroleum ether, affording an ether-petroleum ether soluble (A) and insoluble (B) portions and ethyl acetate-petroleum ether soluble (B) and insoluble (C) fractions. Fractions B and C were combined on the basis of tlc, freed of the solvent and the residue was successively treated with 50% and 80% aqueous methanol. The 50% and 80% aqueous methanol soluble portions were combined freed of the solvent and treated with ether to give ether soluble and insoluble fractions. The latter was taken in a small quantity of ethyl acetate and poured in an excess of petroleum ether affording a light yellow powder (0.506 gm) which was filtered and subjected to flash column chromatography (petroleum ether, petroleum ether-ethyl acetate in order of increasing polarity). The petroleum ether-ethyl acetate (1:1) eluate was rechromatographed on flash column (petroleum ether, petroleum ether-ethyl acetate in order of increasing polarity). The petroleum ether-ethyl acetate (6:4) eluate on thin layer chromatography (silica gel SIF 254 precoated aluminium card chloroform, methanol (9.8:0.2) furnished limbocinin (5.5 mg) limbocidin (4.85 mg), stigmasterol glycoside **3** (4.12 mg) and **4** (4mg) in the order of polarity.

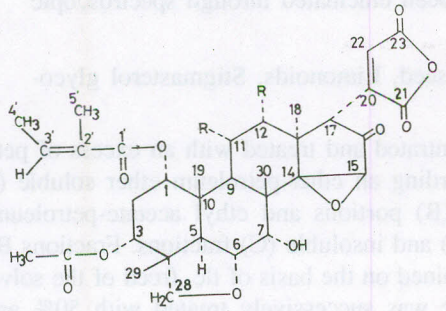
*Limbocinin.* (**1**) FABMS (rel. intensities %) *m/z* 629 [$M^+$+1] (10), 610 (4), 568 (3), 538 (3), 531 (4), 528 (4), 513 (5), 510 (5), 502 (10), 471 (4), 468 (4), 450 (5), 431 (5) and 97 (100). EIMS (rel. intensities %) *m/z* 430 (1), 412 (17), 397 (1.5), 393 (7), 339 (2), 251 (6), 206 (7), 109 (30) and 83 (100).

*Limbocidin.* (**2**) FABMS (rel. intensities %) *m/z* 661 [$M^+$+1] (7), 628 (10), 607 (4), 564 (4), 464 (8), 405 (4) and 97 (10) EIMS (rel. intensities %) 628 (2), 610 (4), 551 (13), 528 (7), 510.2284 ($C_{29}H_{34}O_8$, $M^+$–3x$H_2O$-side chain-H) (9), 412 (22), 109 (24), 97 (45) and 83 (100). UV $\lambda_{max}$ 203 nm and IR $\upsilon_{max}$ 3200-3600 (–OH), 1700-1780 (ester, anhydride carbonyl and cyclopentanone) and 1600 $cm^{-1}$ (C=C).

*Stigmasterolglycoside.* (**3**) FABMS (rel. intensities %) *m/z* 717 [$M^+$+1] (3), 677 (4), 412 (2) and 315 (3). EIMS (rel. intensities %) *m/z* 412. 3749 (calc. for $C_{29}H_{48}O$,

412.3705), (1.5) 368.3452 ($C_{27}H_{44}$) (0.5), 229.1958 ($C_{17}H_{15}$) (2), 159 (3.5) and 145 (3.5). UV $\lambda_{max}$ 203 nm and IR $\upsilon_{max}$ 3250-3600 (-OH), 1700-1760 (C=O) and 1340-1380 $cm^{-1}$ ($CH_3$).

*Stigmasterolglycoside.* (**4**) FABMS (rel. intensities %) 719 [M⁺+1] (2). EIMS (rel. intensities %) 412.3733 (calc. for $C_{29}H_{48}O$, 412.3705) (3), 395.3641 ($C_{29}H_{47}$) (2), 369.3481 ($C_{27}H_{45}$), (2), 147 (23), 145.0104 ($C_5H_5O_5$) (22) and 83.0497 ($C_5H_7O$) (100). UV $\lambda_{max}$ 203 nm and IR $\upsilon_{max}$ 3200-3600 (OH), 1700-1760 (C=O) and 1325-1380 $cm^{-1}$ ($CH_3$).



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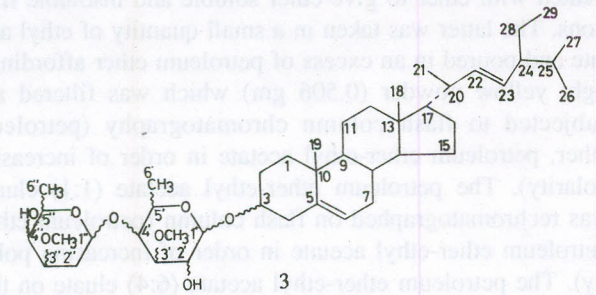
2. R = OH



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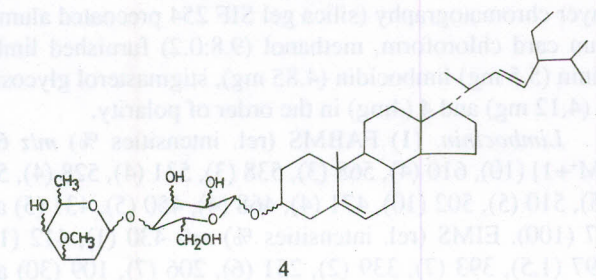

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## RESULTS AND DISCUSSION

The FAB mass spectrum of limbocinin (**1**) showed the molecular ion peak at m/z 629 [M⁺+1]. Its UV spectrum showed absorption at 205 nm and IR spectrum displayed peaks at 3200-3600 (OH), 1700-1770 (ester, anhydride carbonyl and cyclopentanone), 1600 (C=C) and 1340-1380 $cm^{-1}$ ($CH_3$). A quartet of quartet at δ 6.96 ($J_{3',4'}$ = 8.40, $J_{3',5'}$ = 1.30 Hz, H-3′), a quartet of doblet at δ 1.80 ($J_{4',3'}$ = 8.40, $J_{4',5}$ = 1.30 Hz, H-4′) and a broad doublet at δ 1.92 ($J_{5',3'}$ = 1.30 Hz, H-5′) in the $^1$H NMR spectrum (Table 1) showed the presence of a tigoloyloxy group whereas a three-proton singlet at δ 1.98 exhibited an acetoxy function. Four one-proton doublets at δ 2.81 ($J_{5,6}$ = 13.00 Hz), 4.18 ($J_{7,6}$ = 2.90 Hz), 3.70 ($J_{gem}$ = 7.36 Hz) and 3.61 ($J_{gem}$ = 7.36 Hz) attributable to H-5, H-7, H-28a and H-28b respectively, along with a double doblet at δ 4.01 (H-6) showed an ether

Table 1. $^1$H NMR spectral data ($\delta_H$ ppm and J Hz) of **1** and **2**

| Assignment | **1** | **2** |
|---|---|---|
| H-1 | 4.98(t) | 4.97(t) |
| | $J_{1,2}$ 2.44 | $J_{1,2}$ 3.28 |
| H-2 | 2,29(m) | 2.32(m) |
| H-3 | 4.88(t) | 4.87(t) |
| | $J_{3,2}$ 2.68 | $J_{3,2}$ 3.28 |
| H-5 | 2.81(d) | 2.70(d) |
| | $J_{5,6}$ 13.00 | $J_{5,6}$ 12.48 |
| H-6 | 4.01(dd) | 3.96(dd) |
| | $J_{6,5}$ 13.00 | $J_{65}$ 12.48 |
| | $J_{6,7}$ 2.90 | $J_{6,7}$ 3.44 |
| H-7 | 4.18(d) | 4.23(d) |
| | $J_{6,7}$ 2.90 | $J_{7,6}$ 3.44 |
| H-9 | 2.76(m) | 3.54(d) |
| | | $J_{9,11\beta}$ 9.64 |
| H-11α | 1.51-1.80(m) | – |
| H-11β | 1.51-1.80(m) | 3.64(m) |
| H-12α | 1.51-1.80(m) | – |
| H-12β | 1.88(ddd) | 3.47(d) |
| | $J_{gem}$ 17.40 | $J_{12\beta,11\beta}$ 5.24 |
| | $J_{12\beta,11\alpha}$ 1.50 | |
| | $J_{12\beta,11\beta}$ 1.50 | |
| H-15 | 3.33(s) | 3.44(s) |
| H-18 | 0.87(s) | 0.93(s) |
| H-19 | 0.96(s) | 1.20(s) |
| H-22 | 6.90(s) | 6.74(s) |
| H-28a | 3.70(d) | 3.70(d) |
| | $J_{gem}$ 7.36 | $J_{gem}$ 7.20 |
| H-28b | 3.61(d) | 3.59(d) |
| | $J_{gem}$ 7.36 | $J_{gem}$ 7.20 |
| H-29 | 1.22(s) | 1.28(s) |
| H-30 | 1.35(s) | 1.34(s) |
| H-30' | 6.96(qq) | 6.96(qq) |
| | $J_{3',4'}$ 8.40 | $J_{3',4'}$ 7.60 |
| | $J_{3',5'}$ 1.30 | $J_{3',5'}$ 1.26 |
| H-4' | 1.80(qd) | 1.83(qd) |
| | $J_{4',3'}$ 8.40 | $J_{4',3'}$ 7.60 |
| | $J_{4',5'}$ 1.30 | $J_{4',5'}$ 1.26 |
| H-5' | 1.92(brd) | 1.89(brd) |
| | $J_{5',3'}$ 1.30 | $J_{5',3'}$ 1.26 |
| O-C-$CH_3$ | 1.98(s) | 1.99(s)) |

linkage between C-28 and C-6 and an oxygen substituent at C-7 as observed in vilasinin [6] and salannin [7]. A sharp one-proton singlet at δ 3.33 showed an epoxy ring between C-14 and C-15 and carbonyl function at C-16 as in epoxyazadiradione [8]. The signals for the β-substituted furan ring, a common feature of meliacins were absent in the $^1$H NMR spectrum and instead, the presence of a cyclic anhydride was indicated by a singlet at δ 6.90 (H-22) which is comparable with the value reported for a similar moiety [9]. These features together with four methyl singlets in the $^1$H NMR spectrum, suggested that **1** is a tetranortriterpenoid. Absence of the carbomethoxy and vinylic methyl signals indicated that **1** has an intact ring C of vilasinin [6] instead of C-seco ring of salannin [7]. This was supported by a doublet of double doublet of H-12β at δ 1.88 (J = 17.40,, 1.50, 1.50 Hz) and multiplets of H-12α, H-11α and H-11β (δ 1.51-1.80) and H-9 (δ 2.76). Furthermore, as the characteristic signal of H-17 was missing in the $^1$H NMR spectrum and also the molecular weight required 16 a.m.u. to be accounted for, a hydroxyl group was placed at C-17. The ester groups were placed at C-1 and C-3 as both H-1 (δ 4.98, t, $J_{1,2}$ = 2.44 Hz) and H-3 (δ 4.88, t, $J_{3,2}$ = 2.68 Hz) showed couplings with the same methylene protons (H-2). Their coupling constants further showed that both the substituents have axial (α) orientation. Finally a comparison of chemical shifts of H-1 and H-3 with those of salannin led to place the tigoloyloxy function at C-1 and acetoxy group at C-3. In the light of these observations structure **1** has been assigned to limbocinin.

The FABMS spectrum of limbocidin (**2**) showed the molecular ion peak at *m/z* 661 [M$^+$+1]. Other spectral features of **2** were very similar to those of **1** and besides the signals noted above for **1**, the $^1$H NMR spectrum of **2** showed only two additional sets of signals. Thus, it exhibited a one-proton doublet at δ 3.47 (J = 5.24 Hz) and a one-proton multiplet at δ 3.64. The chemical shifts of these protons suggested two oxygen substituents and a difference of 32 a.m.u. in **2** (*m/z* 660) from those of **1** (*m/z* 628), demonstrated their nature as hydroxyl functions. The only positions to locate these were at C-11 and C-12, which was also supported by the signal of H-9 resonating as a doublet at δ 3.54 (J = 9.64 Hz). Further, the coupling constant of H-9 and H-12 demonstrated that both the hydroxyl groups have equatorial (α) dispositions. Thus limbocidin is 11, 12-dihydroxy limbocinin and it was confirmed through a fragment at *m/z* 510.2284 ($C_{29}H_{34}O_8$) resulting from the loss of three molecules of water and the C-17 side chain. It is the first report of isolation of limonoids with a cyclic anhydride side chain at C-17 and also the first instance of isolation of 17-hydroxy tetranortriterpenoids with ring D of epoxyazadiradione. The stereochemistry of furan ring in **1** and **2** could not be determined as they were obtained as minor constituents.

The EIMS of **3** indicated a peak at *m/z* 412, the peak matching of which gave its composition as $C_{29}H_{48}O$. As it gave positive sterolic test, it was assumed that **3** is stigmasterol, however, their co-chromatography revealed that **3** is highly polar as compared to stigmasterol or any other free sterols and therefore the possibility of its glycosidic nature was considered. This was evident from its FAB mass which gave the molecular ion peak at *m/z* 716 exhibiting that *m/z* 412 noted in the EIMS is actually the molecular weight of the aglycone portion, and the 304 a.m.u. left had to be taken for the sugar portion. Two anomeric protons at δ 4.28 (d, J = 10.30 Hz) and 4.22 (dd, J = 9.50, 3.00 Hz), two methoxy groups (δ 3.33 and 3.41) and two three-protons doublets at δ 1.28 (J = 6.50 Hz) and δ 1.30 (J = 6.64 Hz) exhibited the possibility of two sugar moieties in the molecule. The significant ions at *m/z* 145 and 159 indicated that these are hexose methyl ethers (for instance D-diginose and or D-thevetose). A one-proton multiplet at δ 3.63 for H-3 and a three proton multiplet between δ 5.20-5.40 for H-6, H-22, and H-23 apart from methyl signals at δ 0.67(s), 0.82 (s), 1.00 (d, J = 7.12 Hz), 0.84 (d, J = 7.00 Hz), 0.79 (d, J = 7.5 Hz) and 0.80 (t, J = 7.50 Hz) attributable to H-18, H-19, H-21, H-26 and H-29, are comparable with those reported for stigmasterol [10] and therefore led to decide that **3** is a glycoside of stigmasterol.

Compound **4** with a molecular ion peak at *m/z* 719 [M$^+$+1] has very similar spectral data to those of **3** with only a difference of 2 a.m.u. The ion at *m/z* 412 ($C_{29}H_{48}O$ vide peak matching) and signals in the $^1$H NMR spectrum showed that they differ only in the nature of sugar molecules. The significant ions at *m/z* 145 and 162 and only one methoxyl signal (δ 3.61) in the $^1$H NMR spectrum suggested that one of the sugars is a methoxy hexose (e.g. D-diginose) whereas the other is glucose. For an exact identification of the sugar molecules in **3** and **4** and their linkages, further studies are needed after the compounds are isolated in the larger quantities in the future working.

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