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Two insecticidal tetranortriterpenoids from *Azadirachta indica*

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Abstract

Two new triterpenoids, 6α-*O*-acetyl-7-deacetylnimocinol [24,25,26,27-tetra-norapotirucalla-(apoeupha)-6α-acetoxy-7α-hydroxy-1,14,20,22-tetraen-21,23-epoxy-3-one] (**1**) and meliacinol [24,25,26,27-tetranorapotirucalla-(apoeupha)-1α-trimethylacryloxy-21,23-6α,28-diepoxy-16-oxo-17-oxa-14,20,22-trien-3α,7α-diol] (**2**) were isolated from the methanolic extract of the fresh leaves of *Azadirachta indica* (neem). Their structures have been elucidated through spectral studies, including 2D-NMR (COSY-45, NOESY, HMQC and HMBC). The bioactivity of these as well as of nimocinol, reported earlier from the same source, is reported. The first compound and nimocinol showed toxicity on fourth instar larvae of mosquitoes (*Aedes aegypti*) with LC_{50} values of 21 and 83 ppm, respectively. The second compound had no effect upto 100 ppm. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Azadirachta indica*; Meliaceae; Fresh leaves; Bioactivity; Tetranortriterpenoids; Meliacinol; 6α-*O*-acetyl-7-deacetyl nimocinol

1. Introduction

Azadirachta indica A. Juss (Meliaceae) is indigenous to the Indo-Pak subcontinent. Its different parts are highly reputed in folklore and traditional systems of medicine for the treatment of a variety of human ailments, particularly against diseases of bacterial and fungal origin (Nadkarni, 1976; Chopra, Nayar & Chopra, 1956). The anti-inflammatory, anti-pyretic, anti-tumor as well as pesticidal activities (Schmutterer, 1995) of its various parts are also well known. In continuation of our studies on its constituents (Siddiqui, Faizi & Siddiqui, 1984a.; Siddiqui, Siddiqui, Ghiasuddin & Faizi, 1990; Siddiqui, Siddiqui, Ghiasuddin & Faizi, 1991; Siddiqui, Ghiasuddin, Faizi & Siddiqui, 1992; Siddiqui, Ghiasuddin & Faizi, 1998), two new tetracyclic triterpenoids, 6α-*O*-acetyl-7-deacetylnimocinol (**1**) and meliacinol (**2**) have been isolated along with the earlier reported terpenoid nimocinol (Siddiqui, Siddiqui, Faizi & Mahmood, 1984b). The insecticidal properties of these have been determined on the fourth instar larvae of mosquitoes (*Aedes aegypti*).

2. Results and discussion

The neutral part of the methanolic extract of the fresh, undried, uncrushed leaves furnished two new tetranortriterpenoids **1** and **2**. Their isolation was achieved through solvent separation, followed by purification using vacuum liquid chromatography and successive preparative TLC on precoated alumina cards (see Section 3).

Compound **1** was assigned the molecular formula $C_{28}H_{36}O_5$ (EI-HRMS *m*/*z* 452.2593, calculated for $C_{28}H_{36}O_5$, 452.2562). The UV spectrum exhibited absorption maximum at 204 nm and IR spectrum

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showed absorption bands at 3450 (OH), 1660–1725 (carbonyls of α-β-unsaturated ketone and ester), 1600 (C=C) and 1375 (geminal methyl) cm^{-1}. The terpenoidal nature of **1** was indicated by the presence of five quaternary methyl singlets at 0.82, 1.17, 1.19, 1.24 and 1.31 in the ^{1}H-NMR spectrum (Table 1). Two AB doublets at δ 7.10 ($J = 10.1$ Hz, H-1) and δ 5.90 ($J = 10.1$ Hz, H-2) in the ^{1}H-NMR spectrum confirmed the-ring A 1-ene-3-one system of nimocinol (Siddiqui et al., 1984b). It was supported by mass fragment **a** [*m*/*z* 137.0962; $C_9H_{13}O$] and **b** [*m*/*z* 149.0987; $C_{10}H_{13}O$]. The ^{1}H-NMR spectrum further showed the presence of α-oriented hydroxy and acetoxy (δ_H 1.95) substituents at C-7 and C-6, respectively, by their geminal proton signals at δ 4.07 (*d*, $J = 2.5$ Hz) and δ 5.48 (*dd*, $J = 12.3$, 2.5 Hz) and a one-proton doublet at δ 2.75 ($J = 12.3$ Hz) for H-5. The ^{1}H-NMR spectrum further showed a one-proton double doublet at δ 5.57 ($J = 3.6$, 1.7 Hz) due to H-15. Its relatively downfield shift was attributable to the hydroxyl group at C-7 instead of the acetoxy group; as observed earlier, the chemical shift of H-15 is strongly influenced by the nature of H-7 substituent and vice versa (Powell, 1966).The corresponding carbon appeared at δ 119.0 (C-15) along with C-14 at δ 158.6. Two one-proton double doublets were observed at δ 7.25 ($J = 1.6$, 0.9 Hz; H-21) and δ 6.27 ($J = 1.6$, 0.9 Hz; H-22) and a one-proton triplet at δ 7.37 ($J = 1.6$ Hz; H-23). The spectral data of rings (A–D) and the furan ring of **1** showed its close structural relationship with nimocinol (Siddiqui et al., 1984b). However, in nimocinol H-6, H-7 and H-15 appeared at δ 4.30, 5.30 and 5.37, respectively, while the signals corresponding to the same protons in **1** were observed at δ 5.48, 4.07 and 5.57. Mass fragments **b** (*m*/*z* 149.0987, $C_{10}H_{13}O$), **c** (*m*/*z* 243.1381, $C_{16}H_{19}O_2$), **d** (*m*/*z* 229.1212, $C_{15}H_{17}O_2$) and **e** (*m*/*z* 161.0999, $C_{11}H_{13}O$) also confirmed the respective positions of acetoxy and hydroxyl groups. These spectral data led to its characterization as 24,25,26,27-tetranor-apotirucalla-(apoeupha)-6α-acetoxy-7α-hydroxy-1,14,20,22-tetraen,-21,23-epoxy-3-one. The ^{1}H- and ^{13}C-NMR assignments are based on 2D experiments (COSY-45, HMBC, HMQC) and comparison of the data with those of compounds having similar partial structures (Siddiqui et al., 1984b; Lavie, Levy & Jain, 1971).

Table 1
^{13}C- and ^{1}H-NMR spectral data (Broad Band, DEPT and HMQC) and long range correlations (HMBC) of **1**

C/H No.	δ_C	δ_H	*J* (Hz)	C/H long range correlations
1	157.3	7.10	*d*, (10.1)	
2	126.1	5.90	*d*, (10.1)	
3	205.9	–	–	
4	40.5	–	–	
5	49.8	2.75	*d* (12.3)	H-7
6	76.0	5.48	*dd* (12.3, 2.5)	H-1, H-7, H-29
7	68.5	4.07	*d* (2.5)	H-30
8	45.4	–	–	
9	37.1	2.30	*dd*, (12.6, 6.5)	
10	43.1			
11	16.3	2.00–2.25	*m*	
		2.20–2.39	*m*	
12	33.6	2.41–245	*m*	
		2.00–2.25	*m*	
13	47.0	–		
14	158.6	–		
15	119.0	5.57	*dd* (J = 3.6, 1.7)	
16	34.3	2.41	*ddd* (J = 14.4, 7.2, 3.3)	
17	51.6	2.46	*dd* (J = 7.2, 3.3)	H-18
18	20.7	0.82	*s*	
19	14.0	1.17	*s*	
20	124.5	–	–	
21	142.5	7.25	*dd* (J = 1.6, 0.9)	H-23
22	110.9	6.27	*dd* (J = 1.6, 0.9)	H-23
23	139.6	7.37	*t* (J = 1.6)	
28	27.0	1.19		
29	19.6	1.24		
30	20.2	1.31		
–O$\underline{C}$OCH$_3$	171.9			
–O–COC̲H$_3$	21.2	1.95		

Meliacinol **2** has the molecular formula $C_{32}H_{42}O_8$ (EI-HRMS *m*/*z* 554.2914, calc. for $C_{32}H_{42}O_8$, 554.2879). Its IR spectrum exhibited bands at 3450 (OH), 1735 (ester carbonyl), 1720 (α,β-unsaturated-δ-lactone) and 1140 and 1080 (ether linkage) cm^{-1}. The NMR spectral data were indicative of the tetranortriterpenoidal character of **2** containing an α,β-unsaturated-δ-lactone ring-D of deoxy-gedunin and a furan side chain as discussed below. These data further disclosed that one of the tertiary methyl groups is functionalized. Thus, the ^{1}H-NMR spectrum (Table 2) showed four tertiary methyl singlets at δ 0.93 (H-18), 1.12 (H-19), 1.23 (H-29) and 1.28 (H-30) and two one-proton doublets at δ 2.61 ($J = 12.0$ Hz) and δ 4.15 ($J = 3.0$ Hz) related to H-5 and H-7, respectively. The chemical shift of H-7 (δ 4.15) is upfield as compared to H-7 (δ 5.24) of deoxy-gedunin (Powell, 1966), indicating a hydroxyl group instead of an acetoxy group at C-7. The ^{1}H-NMR spectrum further showed two one-proton triplets at δ 4.96 ($J = 3.0$ Hz) and δ 3.85 ($J = 3.5$ Hz) attributed to H-1 and H-3, respectively. The triplet at δ 3.85 shifted to δ 5.24 ($J = 3.5$ Hz) on acetylation, whereas the doublet of H-7 remained unaffected, thus confirming the acetylation of the hydroxyl group at C-3 only. It may be noted that though both the hydroxyl groups at C-3 and C-7 are axial, the latter failed to acetylate being more crowed and only the mono-acetyl derivative was obtained under the conditions of the reaction. Two one-proton double doublets at δ 3.97 ($J = 12.0$, 3.0 Hz) and 2.58 ($J = 9.5$, 3.0 Hz) were attributable to H-6 and H-9, respectively. The chemical shift and multiplicity of H-6 suggested an oxygen function at C-6 and the presence of only four methyl signals located on sp^3 carbons in the ^{1}H-NMR spectrum as well as calculation of unsaturations and oxygens in the molecule were indicative of an ether linkage between C-6 and C-28. This was supported by the presence of a pair of doublets at δ 4.10 ($J = 7.5$ Hz) and δ 3.62 ($J = 7.5$ Hz) for H-28a and H-28b in the ^{1}H-NMR spectrum and the corresponding

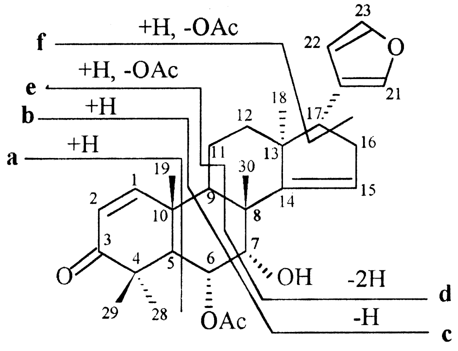
Table 2
^{13}C- and ^{1}H-NMR spectral data (broad band, DEPT and HMQC) and long range correlations (HMBC) of **2**

C/H No.	δ_C	δ_H	*J* (Hz)	C/H long range correlations
1	72.0	4.96	*t*, ($J = 3.0$)	H-19, H-3, H-5
2	29.6	2.00	*m*	H-1, H-29, H-19
3	71.0	3.85	*t*, ($J = 3.5$)	H-1, H-28, H-5
4	44.2	–	–	–
5	39.4	2.61	*d*, ($J = 12.0$)	H-29, H-7
6	72.3	3.97	*dd*, ($J = 12.0$, 3.0)	H-5, H-7, H-28
7	84.0	4.15	*d*, ($J = 3.0$)	H-6, H-30
8	44.2	–	–	–
9	39.0	2.58	*dd*, ($J = 9.5$, 3.0)	H-5, H-3
10	40.6	–	–	–
11	17.3	1.37	*m*	H-19
12	30.5	1.70	*m*	–
13	49.4	–	–	–
14	173.0	–	–	–
15	115.0	5.80	*t*, ($J = 1.5$)	H-7
16	164.0	–		
17	88.9	5.40	*t*, ($J = 1.5$)	H-12
18	19.7	0.93	*s*	–
19	15.2	1.12	*s*	–
20	129.8	–	–	–
21	138.8	7.23	*dd*, ($J = 1.5$, 0.5)	H-22, H-23
22	111.0	6.28	*dd*, ($J = 1.5$, 0.5)	H-21, H-23
23	143.0	7.29	*t*, ($J = 1.5$)	H-22, H-21
28a	77.8	4.10	*d*, ($J = 7.5$)	–
28b		3.62	*d* ($J = 7.5$)	
29	20.4	1.23	*s*	–
30	30.8	1.28	*s*	–
1′	172.0	–	–	–
2′	146.6	–	–	–
3′	158.0	–	–	–
4′	27.7	1.94	*br.s*	H-5′, H-6′
5′	20.9	2.14	*br.s*	H-4′
6′	13.5	1.70	*br.s*	

carbon at δ 77.8 in the ^{13}C-NMR spectrum identified unambiguously on the basis of HMQC. The ^{1}H-NMR spectrum further showed two one-proton signals with very fine splittings of 1.5 Hz at δ 5.80 and 5.40 corresponding to H-15 and H-17, respectively. These signals and their corresponding carbons at δ 115.0 and δ 88.9, respectively, along with the carbons at δ 173.0 (C-14) and δ 164.0 (C-16) were suggestive of ring-D of 14,15 deoxy-gedunin (Lavie et al., 1971). H-15 of **2** appeared downfield as compared to those of deoxy-gedunin and nimocinol which supported the hydroxy substituent at C-7 in **2** instead of acetoxy group (Powell, 1966). Similarly, the double bond at C-14 resulted in a downfield shift of H-7. Two one-proton double doublets observed at δ 7.23 ($J = 1.5$, 0.5 Hz) and 6.28 ($J = 1.5$, 0.5 Hz) and a one-proton triplet at δ 7.29 ($J = 1.5$ Hz) which correlated with carbons at δ 138.8, δ 111.0 and δ 143.0 were assigned to H-21, H-22 and H-23, respectively. These values for the furan ring (protons and carbons) are comparable with those of deoxy-gedunin (Powell, 1966). The ^{1}H-NMR spectral data of rings A and B are comparable with those reported for rings A and B of limbonin and vilasinin-1,3-diacetate (Siddiqui et al., 1990; Kraus & Cramer, 1981) and showed an α-oriented ester unit ($C_6H_9O_2$) at C-1, which was unambiguously identified as trimethyl acrylate from its NMR data δ_H: 1.94, H-4′; 2.14, H-5′, and 1.70, H-6′; δ_C 172.0 (C-1′); 146.6 (C-2′); 158.0 (C-3′); 27.7 (C-4′); 20.9 (C-5′); 13.5 (C-6′)} and loss of a mass fragment **c** ($C_9H_{15}O_3$). All the proton and carbon assignments were supported by interactions observed in the COSY-45 plot, HMQC and HMBC spectra, and are consistent with the reported values of compounds with similar partial structures (Powell, 1966; Lavie et al., 1971; Siddiqui et al., 1990; Kraus, Cramer, Bokel & Sawitzki, 1981; Siddiqui et al., 1984a). The molecular formula displayed 12 double-bond equivalents, three of which were accounted for by the furan ring, four by two α,β-unsaturated carbonyl systems, one by the ether ring between C-6 and C-28, and the remaining four by the four rings (A–D) of the tetracyclic nucleus. The stereochemistry of various centres has been confirmed through NOESY, which showed spatial connectivites of H-1 with H-2; H-2 with H-3; H-7 with H-6 and H-30; H-15 with H-17; H-18 with H-22 and H-22 with H-23.

In light of these spectral data, the structure of **2** has been deduced as 24,25,26,27-tetranorapotirucalla-(apoeupha)-1α-trimethylacryloxy-21,23-6,28-diepoxy-16-oxo-17-oxa-14,20,22-trien-3α, 7α-diol.

Both **1** and **2** and nimocinol (Siddiqui et al., 1984a), were tested for their effects on mosquito (*Aedes aegypti*) fourth instar larvae. **1** and nimocinol showed toxicity with LC_{50} 21 and 83 ppm, respectively, while **2** had no effect upto 100 ppm.



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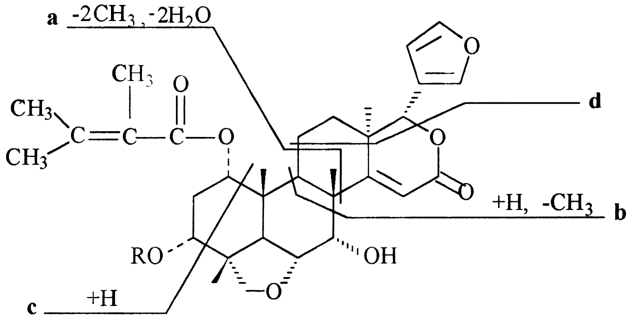

2 R=H
2a R=Ac

3. Experimental

3.1. General

IR ($CHCl_3$) and UV (MeOH) spectra were measured on JASCO-A302 and Hitachi-3200 spectrophotometers, respectively. The ^{1}H-NMR spectra were recorded in $CDCl_3$ on a Bruker Aspect AM 300 operating at 300 MHz, while the ^{13}C-NMR spectra (BB, DEPT, HMQC and HMBC) were recorded in $CDCl_3$ on a Bruker Aspect AM-500 spectrometer operating at 125 MHz. The chemical shifts (δ) are recorded in ppm and coupling constants (J) are in Hz. TLC was performed on precoated alumina (Riedel-de Haen Dc-cards ALF) cards. Plates were visualized under UV light (254 and 366 nm).

3.2. Plant material

Leaves were collected in spring from the Karachi region and identified by Prof. Dr. S.I. Ali, Department of Botany University of Karachi, and a voucher specimen (No. NM-1) has been deposited in the Herbarium of the Department of Botany, University of Karachi.

3.3. Extraction and isolation

The fresh, uncrushed leaves (20 kg) were repeatedly (5×) extracted with MeOH at room temperature. The combined extract, after removal of the solvent under reduced pressure, was partitioned between EtOAc and H_2O. The EtOAc layer was washed, dried (anhydrous Na_2SO_4), treated with charcoal and filtered. The charcoal bed was successively eluted with EtOAc and C_6H_6–MeOH (1:1; v/v). The EtOAc and C_6H_6–MeOH filtrates were combined and the solvent removed at reduced pressure. The residue thereby obtained, was divided into petrol-soluble and petrol-insoluble fractions. The latter fraction was treated with 4% Na_2CO_3 to separate the acidic and neutral fractions. The EtOAc layer containing the neutral fraction was washed with H_2O, dried (anhydrous Na_2SO_4) and evaporated under vacuum. The neutral fraction thereby obtained was divided into petrol-soluble and petrol-insoluble fractions, and the latter was successively treated with different percentages of aqueous MeOH [10%, 20%,..., 100%]. As a result, several fractions were obtained and combined on the basis of their TLC spectrum. The 70 and 80% aq. MeOH fractions were combined together and subjected to VLC (silica gel-60 GF_{60}-254; petrol, petrol–EtOAc in order of increasing polarity up to a ratio of 7:3 and then $CHCl_3$, $CHCl_3$–MeOH, MeOH in order of increasing polarity). The petrol–EtOAc (7.5:2.5) eluate furnished a fraction (1.05 g) which was resolved into two major and four minor spots on precoated alumina cards (petrol–EtOAc, 7:3). The major components '**a**' and '**b**' showed single spots on TLC and '**a**' was identified as nimocinol (Siddiqui et al., 1984b). The ^1H-NMR of '**b**' revealed that it was still a mixture of several constituents with one major band. After a number of trials, it could ultimately be purified on precoated alumina cards (petrol–EtOAc, 7.5:2.5) to afford compound **1** (24 mg). The minor components did not afford any workable quantities.

The petrol–EtOAc (1:1) and $CHCl_3$ eluates were combined together (105 mg) and subjected to alumina coated preparative TLC cards ($CHCl_3$–MeOH, 9.95:0.05) affording a major component (75 mg) showing a single spot on TLC. The ^1H-NMR spectrum indicated that it was still a mixture of several constituents with one major band, which after a number of trials, could ultimately be purified on precoated alumina cards (petrol–EtOAc; 7:3) to afford meliacinol (27 mg).

*3.4. 6-O-acetyl-7-deacetylnimocinol (**1**)*

Slender rods (MeOH) 24 mg, mp 60–62°C; $[\alpha]^{27}_{D}$ + 6.6 ($CHCl_3$, *c*, 0.12); UV λ_{max} MeOH nm: 204; IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1660–1725 (carbonyls of α,β-unsaturated ketone and ester), 1600 (C=C) and 1375 (geminal methyls). HR-EIMS *m/z* (rel.int): 452.2593 [M^+; $C_{28}H_{36}O_5$, calculated for $C_{28}H_{36}O_5$ 452.2562] (21.6), 313.2135 ($C_{21}H_{29}O_2$, fragment **f**) (11.0), 243.1381 ($C_{16}H_{19}O_2$, fragment **c**) (10.8), 229.1212 ($C_{15}H_{17}O_2$, fragment **d**) (17.0), 161.0999 ($C_{11}H_{13}O$, fragment **e**) (27.2), 149.0987 ($C_{10}H_{13}O$, fragment **b**) (32.1), 137.0962 ($C_9H_{13}O$, fragment **a**) (51.4), ^1H- and ^{13}C-NMR (Table 1).

*3.5. Meliacinol (**2**)*

27 mg; fine needles, mp 178–179°C; $[\alpha]^{27}_{D}$ + 7.78° ($CHCl_3$; *c*, 0.18); UV λ_{max} MeOH nm: 205; IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1735, 1720 (ester and lactone carbonyls) and 1140 and 1080 (ether linkage). EIMS *m/z* 554 (M^+); HRMS *m/z* (rel. int); 554.2914 (M^+; calculated for $C_{32}H_{42}O_8$, 554.2879] (32.2), 421.1982 [$C_{26}H_{29}O_5$, M^+-$C_6H_9O_2$,] (2.7), 284.1353 [$C_{18}H_{20}O_3$, fragment **a**] (4.7), 259.1296 [$C_{16}H_{19}O_3$] (12.8), 230.0936 [$C_{14}H_{14}O_3$, fragment **b**] (8.1), 171.0944 [$C_9H_{15}O_3$, fragment **c**] (3.9), 165.0847 [$C_{10}H_{13}O_2$] (4.3) 135.0815 [$C_9H_{11}O$, fragment **d**] (3.9). ^1H- and ^{13}C-NMR (Table 2).

*3.6. Acetylation of **2***

To a solution of **2** (10 mg) in pyridine (1 ml) was added acetic anhydride (1 ml) and kept at room temperature overnight. On usual workup, the mono

Table 3
Toxicity of **1** against the fourth instar larvae of mosquitoes (*Aedes aegypti*) 24 h of treatment showing % mortality at 95% confidence limit

S. No.	Concentration (ppm)	Mean mortalities (%)	SD[a]	SE[b]	Range = mean ± SE × 2.58
1	21.0	50	7.07	3.16	41.84–58.15
2	31.5	62	4.48	2.00	56.84–67.16
3	42.0	72	4.48	2.00	66.84–77.16
4	52.5	84	5.48	2.45	77.68–90.32
5	63.0	92	4.48	2.00	86.84–97.16

[a] SD: standard deviation.

[b] SE: standard error.

Table 4
Toxicity of nimocinol against the fourth instar larvae of mosquitoes (*Aedes aegypti*) 24 h of treatment showing % mortality at 95% confidence limit

S. No.	Concentration (ppm)	Mean mortalities (%)	SD[a]	SE[b]	Range = mean ± SE × 2.58
1	41.40	08	4.48	2.00	2.84–13.16
2	51.75	14	5.48	2.49	7.68–20.31
3	62.10	28	4.48	2.00	22.84–33.16
4	72.45	44	5.48	2.45	37.38–50.32
5	82.80	54	5.48	2.45	47.68–60.32

[a] SD: standard deviation.
[b] SE: standard error.

acetylated product **2a** was obtained showing a single spot on TLC; UV λ_{max} (nm): 205; IR ν_{max} (cm^{-1}): 1720–1735 (ester carbonyls); EIMS: m/z (M^+-8); ^{1}H-NMR δ 5.24 (^{1}H, *t*, $J = 3.5$ Hz; H-3), 2.03 (3H, *s*, $OCOCH_3$).

4. Insecticidal activity

4.1. Insects

Aedes aegypti larvae (P.C.S.I.R. strain) were reared in the laboratory of the Zoology Department, Karachi University under controlled temperature (28 ± 1°C). They were fed with sterilized powder of dried prawns.

4.2. Biological tests (screening procedure)

Ten early-fourth instar mosquito larvae were collected in five ml of the rearing tap water and transferred to 100 ml glass beakers containing 45 ml distilled water. The compounds were tested at 28 ± 1°C at 5 final concentrations (Tables 3 and 4). Controls consisted of water alone. Each concentration and control was run in duplicate and mortality was recorded after 24 h.

4.3. Accurate tests

The WHO modified method of application was followed, in which a batch of 10 insects (fourth instar larvae) was released into a 100 ml beaker containing 50 ml filtered tap water. The concentrations selected in the preliminary screening of each compound were tested at 28 ± 1°C. A group of seven beakers was set up, five for different concentrations and one for each control and check. Each experiment was repeated five times. The experiment was discarded if mortality exceeded 10% in the controls. Mortality was recorded after 24 h and data were corrected using Abbott's formula.

4.4. Calculation of LC_{50}

The lethal concentration LC_{50} was calculated using probit analysis (Raymond, Proto & Ratsira, 1993).

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