Larvicidal activity of extracts from three *Plumbago* spp against *Anopheles gambiae*

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Three Plumbago *spp have been tested for mosquito larvicidal activity. The crude extracts exhibiting the highest larvicidal activity against* Anopheles gambiae *were hexane (LC_{50} = 6.4 µg/mL) and chloroform (LC_{50} = 6.7 µg/mL) extracts from* Plumbago zeylanica *Linn, chloroform (LC_{50} = 6.7 ug/mL) extract from* Plumbago stenophylla *Bull and ethyl acetate (LC_{50} = 4.1 µg/mL) extract from* Plumbago dawei *Rolfe. These LC_{50} values were within 95% confidence limits. 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin) 1 (LC_{50} = 1.9 µg/mL) and β-sitosterol 2 were characterised from ethyl acetate extract of root bark of* P. dawei, *a native medicinal plant growing in Kenya, based on spectral analysis and comparisons with data in literature.*

Key words: *Plumbago zeylanica* - *Plumbago stenophylla* - *Plumbago dawei* - larvicidal activity - 5-hydroxy-2-methyl-1,4-naphthoquinone - *Anopheles gambiae*

The repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations. It has also resulted in the development of resistance (Brown 1986), undesirable effects on non-target organisms and fostered environmental and human health concern (Cheng et al. 2003). These side effects have initiated a search for alternative control measures. Natural products of plant origin with insecticidal properties have been assayed in the recent past for the control of a variety of insect pests and vectors. These include essential oils (Harve & Kamath 2004) and azadiractins (Obomanu et al. 2006). Phytochemicals derived from plant sources can act as larvicides, insect growth regulators, repellents and ovipositor attractants, as observed by many researchers (Venketachalam & Jebasan 2001, Thomas et al. 2004).

Plumbaginaceae is a plant family consisting mostly of shrubs that is found in scarcely distributed grassland steppes of arid and coastal regions. They are slow growing, highly branched evergreen shrubs (Kokwaro 1993). Extracts of several Plumbaginacea family members have been investigated for anti-malarial, anti-bacterial and anti-protozoal properties (Saraswathy et al. 2003, Parekh & Chanda 2006, Dorni et al. 2007). Here we report the larvicidal activity of organic extracts of three *Plumbago* spp commonly found in the Coastal and Eastern Regions of Kenya. The species investigated were *Plumbago zeylanica* Linn, *Plumbago dawei* Rolfe and *Plumbago stenophylla* Bull. *P. zeylanica* is traditionally used topically for skin disorders and orally for hookworms. Additionally, in some communities, this species is used as a traditional medicine against malaria. The decoction of the leaf of *P. dawei* is consumed as a purgative and *P. stenophylla* root extracts are used for the treatment of wounds (Kokwaro 1993). In addition to the characterisation of larvicidal activity, phytochemical investigation of the most active extracts was carried out.

MATERIALS AND METHODS

Plant material - The roots of *P. zeylanica*, *P. dawei* and *P. stenophylla* were collected from Kibwezi, Mwatate and Arabuko, respectively, in June 2005, and identified by SG Mathenge of the Nairobi University Botany Department. Voucher specimen 2005/10, 2005/11 and 2005/12, respectively, were deposited at the University Herbarium, Botany Department, University of Nairobi.

Extraction - The air dried root bark of the plant (1 kg) was crushed and extracted sequentially with hexane, chloroform, ethyl acetate and methanol at RT for three days. The extract was filtered and the solvent removed at low temperature (35-40^{0}C) and reduced pressure on a rotary evaporator.

Fractionation and purification - All solvents used were of analytical grade (E. Merck, D-6100 Darmstadt, F. R. Germany). Melting point was determined on micro-capillary melting point apparatus, Yanaco model MP 500D and is uncorrected. Analytical thin layer chromatography (TLC) was performed using pre-coated silica gel 60 F_{254} plates (5 x 10 cm x 0.20 mm) with fluorescence indicator. For preparative TLC (PTLC), 20 x 20 cm x 0.25 mm plates were used. The spots were first visualised using a multi-band UV-254/366 nm lamp (UV GL-58). The TLC plates were then sprayed with 2% concentrated H_2SO_4 in metha-

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nol and kept in the oven at 105^{0}C for 10 min. ^{1}H NMR was recorded at 200 MHz and ^{13}C NMR (DEPT) recorded at 50 MHz on Varian Gemini 200 MHz instrument at RT; chemical shifts are reported in ppm upfield of tetramethylsilane and multiplicities are reported as singlet (s), doublet (d), triplet (t) and quartet (q). MS analyses were carried out in Finigan Mat SSQ700 single quadrupole instrument and Autospec time of flight spectrometer EI-MS and ES-MS.

The root bark of *P. dawei* (410 g) was extracted (RT) with ethyl acetate (1.5 L) for three days. The extract (3 g) was fractionated by column chromatography on silica gel 230-400 mesh eluting with C_6H_{14}: EtOAc (9:1, 8:2, 7:3 and 100% EtOAc) to afford 56 fractions (Fr_{1-56}). The first solvent system yielded fractions Fr_{1-11}(76 mg, 0,0253%). The second system gave fractions Fr_{12-34} (401 mg, 13,37%) while the third gave fractions Fr $_{35-56}$ (106 mg, 0,0353%). Fraction Fr_{12-34} gave compound 1, which was obtained as bright yellow needle-like crystals with melting point 77.8-77.4ºC. PTLC purification (using 8: 2 C_6H_{14}: EtOAc) of fraction Fr_{35-56} gave 2 as a colourless crystalline solid with melting point 130-132ºC.

Larvicidal bioassays - Larvicidal tests were carried out on late 3rd and newly emerged 4th instar *Anopheles gambiae* larvae. The larvae were reared under standard conditions in the mosquito insectary of the International Centre of Insect Physiology and Entomology, Nairobi, Kenya. *A. gambiae* larvae were reared on fish food and maintained at 27 ± 1^{o}C, > 70% relative humidity, with a photoperiod of 16 h. Twenty-five larvae were used (5 insects per 30 mL vial) for each experiment in five replicates. The larvae were considered dead if they were immobile, unable to reach the water surface and lacked head to tail flexion in response to tapping the beaker with the end of a pencil. The larvae were scored for percent mortality after 24, 48 and 72 h. Deltamethrin was used as a positive control.

Larvicidal activity of extracts/compounds was determined on the basis of the percentage mortality of the larvae introduced into the test vials. Mortality was monitored after 24, 48 and 72 h until emergence of adults in the test at different concentration ranges. Percentage mortality (PM) was determined using the equation:

$$\% PM = [\text{mean death count/initial larvae population}] \times 100$$

Relative potency values LC_{50} were determined by probit analysis.

RESULTS AND DISCUSSION

The yields of the crude extracts from the three species are presented in Table I. The MeOH extracts gave the highest yields from each species.

Larvicidal bioassays on crude extracts and isolated compounds - Larvicidal activities of crude organic extracts from *Plumbago* spp are summarised in Tables II-IV. They are presented as percentage mortalities (% mortality).

From the results, all crude extracts (except that of methanol) (Table II) achieved maximum mortality of 100% at 100 µg/mL; 84-98% at 10 µg/mL; 26-98% at 5 µg/mL and 6-88% at 1 µg/mL (24-72 h). Mortality increased when the larvae were exposed for a longer period. *P. dawei* extracts had the highest activity, followed by *P. zeylanica* (Table IV) and finally *P. stenophylla* (Table III). The results reveal that the extracts of different plants varied significantly in their larvicidal potential.

The larvicidal activity of the organic extracts was found to be species-dependent. For *P. dawei*, EtOAc extract showed the highest larvicidal activity (Table V). For *P. zeylanica,* the extract of C_6H_{14} was the most active, while for *P. stenophylla* the $CHCl_3$ extract was the most active.

From the data in Table V, the ethyl acetate extract from the root bark of *P. dawei* displayed LC_{90} and LC_{50} values of 10.6 and 4.10 µg/mL, respectively, making it the most active of all extracts tested. This extract was

TABLE I

Yields of extracts of *Plumbago* spp root bark

	Yield %			
Solvent	C_6H_{14}	$CHCl_3$	EtOAc	MeOH
Species				
Plumbago dawei	0.4	0.15	0.5	8.2
Plumbago zeylanica	0.5	0.2	0.7	4.15
Plumbago stenophylla	0.4	1.0	2.6	9.13

TABLE II

Percentage mortality of *Plumbago dawei* extracts against *Anopheles gambiae* larvae

		Mortality (X ± se)[a] %			
Extract	Time (h)	1 µg	5 µg	10 µg	100 µg
C_6H_{14}	24	24 ± 1.41	70 ± 4.78	86 ± 2.45	100
$CHCl_3$	24	6 ± 0.89	26 ± 10.77	84 ± 5.09	100
EtOAc	24	46 ± 1.79	86 ± 5.10	98 ± 2.00	100
MeOH	24	na	na	na	na
C_6H_{14}	48	38 ± 1.41	78 ± 2.00	92 ± 2.00	-
$CHCl_3$	48	22 ± 0.89	54 ± 7.78	98 ± 4.47	-
EtOAc	48	68 ± 1.41	92 ± 3.74	100	-
MeOH	48	na	na	na	na
C_6H_{14}	72	62 ± 1.41	88 ± 2.00	100	-
$CHCl_3$	72	54 ± 1.79	64 ± 7.78	98 ± 4.47	-
EtOAc	72	88 ± 1.10	98 ± 2.5	-	-
MeOH	72	na	na	na	na

a: each percentage mortality is the mean (X) and standard deviation (se) as the average from five repetitions performed on 25 larvae per group of *Anopheles gambiae*. Relative potency for deltamethrin was 0.06 %. na: no activity.

TABLE III

Percentage mortality of *Plumbago stenophylla* extracts against *Anopheles gambiae* larvae

		Mortality (X ± se)[a] %			
Extract	Time (h)	1 µg	5 µg	10 µg	100 µg
C_6H_{14}	24	20 ± 1.41	46 ± 1.41	84 ± 1.41	100
$CHCl_3$	24	28 ± 1.41	58 ± 7.35	96 ± 2.45	100
EtOAc	24	22 ± 1.41	50 ± 10.0	90 ± 3.16	100
MeOH	24	na	na	na	na
C_6H_{14}	48	32 ± 2.61	60 ± 2.00	96 ± 2.46	-
$CHCl_3$	48	50 ± 2.00	70 ± 4.47	100	-
EtOAc	48	46 ± 2.00	62 ± 9.49	100	-
MeOH	48	na	na	na	na
C_6H_{14}	72	58 ± 2.28	78 ± 2.00	100	-
$CHCl_3$	72	76 ± 1.79	86 ± 5.10	-	-
EtOAc	72	62 ± 1.41	72 ± 8.60	-	-
MeOH	72	na	na	na	na

a: each percentage mortality is the mean (X) and standard deviation (se) as the average from five repetitions performed on 25 larvae per group of *Anopheles gambiae*. Relative potency for deltamethrin was 0.06 %. na: no activity.

TABLE IV

Percentage mortality of *Plumbago zeylanica* extracts against *Anopheles gambiae* larvae

		Mortality (X ± se)[a] %			
Extract	Time (h)	1 µg	5 µg	10 µg	100 µg
C_6H_{14}	24	34 ± 1.41	76 ± 4.00	90 ± 2.00	100
$CHCl_3$	24	18 ± 1.26	40 ± 5.48	88 ± 3.74	100
EtOAc	24	20 ± 1.41	70 ± 6.32	86 ± 4.00	100
MeOH	24	na	na	na	na
C_6H_{14}	48	56 ± 2.19	86 ± 2.45	92 ± 4.47	-
$CHCl_3$	48	38 ± 2.06	54 ± 5.10	90 ± 10.0	-
EtOAc	48	42 ± 0.89	96 ± 2.45	100	-
MeOH	48	na	na	na	na
C_6H_{14}	72	80 ± 1.41	90 ± 0.00	94 ± 5.48	-
$CHCl_3$	72	64 ± 2.00	94 ± 2.45	94 ± 5.48	-
EtOAc	72	86 ± 1.41	100	-	-
MeOH	72	na	na	na	na

a: each percentage mortality is the mean (X) and standard deviation (se) as the average from five repetitions performed on 25 larvae per group of *Anopheles gambiae*. Relative potency for deltamethrin was 0.06 %. na: no activity.

selected for further analysis and was subjected to chromatographic fractionation. The fractions obtained were then tested for their larvicidal activity to establish the chemicals responsible in these species.

Three major fractions were obtained when the EtOAc extract of *P. dawei* was subjected to column chromatography. Table VI shows the lethal concentration values of these fractions. $Fr_{1\text{-}11}$ (LC_{50} = 110.0 µg/mL) and $F_{12\text{-}34}$ (LC_{50} = 1.9 µg/mL) exhibited the strongest larvicidal activities, while $Fr_{35\text{-}56}$ with (LC_{50} = 16.1 µg/mL) was the least active.

The larvicidal activities obtained in this study are in agreement with reported data. For example, Sosan et al. (2001) reported the larvicidal activities of essential oils of *Ocimum gratissium, Cymbopogon citrus* and *Ageratum conyzoides* against *Aedes aegypti,* achieving 100% mortality at concentrations of 120, 200 and 300 ppm, respectively. Das et al. (2007) reported that the LC_{90} values of methanol and ethanol extracts from *Aristolochia saccata* roots, *Annona squamosa* leaves and *Gymnopetelu cochinchinensis* fruits/pericarp against *Aedes albopictus* and *Culex quinquefasciatus* larvae ranged from 31.80-155 ppm. These results show that the larvicidal activities of plant extracts vary according to the plant species, part of the plant used, application method and geographical location where the plants were grown. However, ours is the first report of the larvicidal activities of these *Plumbago* spp.

The naphthoquinone, plumbagin, 1 was obtained from fractions $F_{12\text{-}34}$, while β-sitosterol 2 was obtained from $Fr_{35\text{-}56}$. These two compounds were subjected to larvicidal assays. The concentration of 1 used ranged from 1-5 µg/mL over a period of 24 h. The results confirm that the larvicidal activity of *P. dawei* is due to 1. Plumbagin has been isolated in a range of *Plumbago* species (Croft et al. 1989, Panichayupakaranant & Tewtrakul 2002, Ribeiro de Paiva et al. 2003) and also in the leaves, roots and stem bark of different plant species (Borges-Argáez et al. 2007, Dzoyem et al. 2007, Ganapaty et al. 2006). This compound has been reported to exhibit anti-protozoal, anti-fungal, cytotoxic and anti-bacterial effects (Cai et al. 2000, Wang et al. 2001, Inbaraj & Chignell 2003). In contrast, α-sistosterol, 2 did not show any larval mortality at the tested concentration ranges.

TABLE V

Lethal concentrations (LC) of most active extracts of *Plumbago* spp

		LC (95% confidence limits)			
Species	Extract	LC_{25}	LC_{50}	LC_{75}	LC_{90}
Plumbago dawei	EtOAc	2.5	4.1	6.7	10.6
Plumbago zeylanica	C_6H_{14}	3.1	6.4	13.4	26.2
Plumbago stenophylla	$CHCl_3$	4.3	6.7	10.5	15.6

relative potency for deltamethrin was 0.06%.

TABLE VI

Lethal concentrations (LC) values of *Plumbago dawei* EtOAc fractions

Fractions	LC (µg/mL) (95% confidence limits)			
	LC_{25}	LC_{50}	LC_{75}	LC_{90}
Fr_{1-11}	36.0	110.0	335.5 0	926.3
Fr_{12-34} (1)	1.7	1.9	2.1	2.3
Fr_{35-56} (2)	13.5	16.1	19.2	22.3

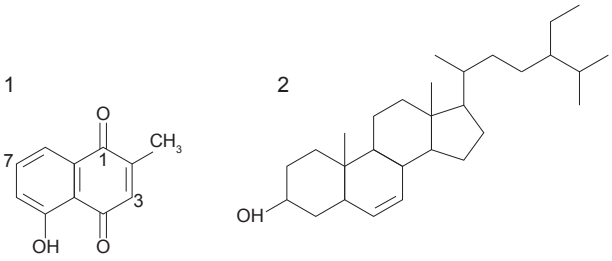
relative potency for deltamethrin was 0.06%.

In conclusion, it is evident that all three *Plumbago* species tested and the isolated naphthoquinone 1 possess remarkable larvicidal activity. Larvicides of great value could be developed from these plants as an alternative means to control mosquitoes and thus help combat malaria.

Identification of isolated and purified compounds

5-Hydroxy-2-methyl-1,4-naphthoquinone(Plumbagin) - This was obtained from fraction Fr_{12-34} and identified from 1D and 2D NMR data, mass spectral data and by comparing these spectroscopic data with those reported in literature. Plumbagin (Figure) was obtained as bright yellow needles (401 mg), mp 77.8-77.4°C [literature value 76.8-77.4°C (Dinda et al. 1998, Hazra et al. 2002)]; ^{1}H-NMR ($CDCl_3$, 200 MHz,), δ 2.20 (3H, s, CH_3), 6.81 (1H, s, H-3), 7.24 (1H, d, J = 7.8 Hz, H-8), 7.61 (2H, t, J = 7.8, 2.4 Hz, H-6 and H-7), 11.98 (1H, s, OH). ^{13}C-NMR ($CDCl_3$, 50 Hz), δ: 16.8 (C-9, q), 115.3 (C-4a, s), 119.6 (C-8, d), 124.4 (C-6, d), 132.3 (C-8a, s), 135.7 (C-7, d), 136.4 (C-3, d), 149.9 (C-2, s), 161.4 (C-5, s), 185.1 (C-1, s), 190.5 (C-4, s). EIMS: *m/z* 188 (M^+, 100%, $C_{11}H_8O_3$), 173 (M-CH_3, 2.9), 160 (3.5), 131 (71.4), 120 (4.7), 92 (72.9), 77 (3.4) and 63 (85).

17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (β-Sitosterol) - This compound (Figure) was identified by comparing the spectral data obtained with those reported in the literature (Dey & Harbone 1991). The stereochemistry at the C-24 asymmetric centre was investigated by a careful comparison with the ^{1}H NMR spectra of both epimers at C-24 in similar steroids (Gamze et al. 2002). The steroid was obtained as colourless crystals (13 mg), mp 130-132ºC; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.67 (3H, s, H-18), 1.0 (3H, s, H-19), 0.81 (3H, dd, H-27), 0.84 (3H, t, H-29), 0.82 (3H, d, H-26), 0.93 (3H, d, H-21), 3.50 (1H, m, H-3), 5.34 (1H, d, 6-H); the rest of the protons were found in a complex multiplet signal between α 1.13-2.34. ^{13}C NMR ($CDCl_3$, 50 Hz) δ 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 31.6 (C-8), 50.1 (C-9), 36.5 (C-10), 23.0 (C-11), 39.7 (C-12), 42.3, (C-13), 56.6 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.1 (C-20), 19.0 (C-21), 33.9 (C-22), 26.0 (C-23), 45.7 (C-24), 29.7 (C-25), 18.8 (C-26), 19.8 (C-27), 21.1 (C-28), 12.0 (C-29); EIMS: *m/z*, 414 ($[M]^+$, 4.2, $C_{29}H_{50}O$), 396 ($[M-H_2O]^+$, 1.8), 381 ($[M-H_2O-CH_3]^+$, 1.2), 303 ($[M-C_7H_{11}O]^+$, 1.6), 301 ($[M-C_8H_{17}]^+$, 1.0), 273 ($[M-C_{10}H_{20}]^+$, 1.4), 231 ($[M-C_{13}H_{25}]^+$, 1.0), 213 ($[231-H_2O]^+$, 1.7), 255 ($M-H_2O-C_{10}H_{21}]^+$, 14), 43 ($(C_3H_7)^+$, 100).



Compounds isolated from EtOAc extract of *Plumbago dawei*: 1: Plumbagin; 2: β-Sitosterol.

Though compounds 1 and 2 have been reported elsewhere in literature (Dey & Harbone 1991**,** Hazra et al. 2002), this is the first time they are being reported from the *Plumbago* species growing in Kenya.

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