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Bioactivity-guided isolation of mosquitocidal constituents from the rhizomes of *Plumbago capensis* Thunb

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ABSTRACT

A bioassay-guided fractionation and chemical examination of chloroform extract of *Plumbago capensis* roots resulted in isolation and characterization of two new napthaquinone derivatives (**4**, **8**) along with six known compounds (**1**–**3**, **5**–**7**). Their structures were determined on the basis of extensive spectroscopic (IR, MS, 1D and 2D NMR) data analysis and by comparison with the literature data. All the compounds were tested for their mosquito larvicidal activity against fourth instar larvae of *Aedes aegypti*, and compared with that of rotenone. Among the tested compounds, isoshinanolone (**3**) and plumbagin (**1**) showed excellent toxicity with LC_{50} values of 1.26 and 5.43 μg/mL. New compound (**8**) displayed moderate toxicity against the tested mosquito species.

The growing demand for natural products has intensified, as they are biodegradable, eco-friendly, and safe to the environment. It is well-known that plant-derived natural products are extensively used as biologically active compounds, particularly in the area of infectious diseases, which represent a serious problem to health, being one of the main causes of morbidity and mortality worldwide.[1] Mosquitoes are being the most important vectors for the transmission of malaria, filariasis, and viral diseases.[2] The yellow fever mosquito, *Aedes aegypti* is widely distributed in the tropical and subtropical zones, and responsible for the transmission of dengue virus, causative agent of dengue fever and dengue hemorrhagic fever/dengue shock syndrome.[3] Recently, in India, the incidence of dengue fever has increased significantly. It has been estimated that over 1,80,000 cases have occurred in India since December 2005.[4] The continuous use of synthetic insecticides and insect growth regulators for the eradication of *A. aegypti* has been effective but nevertheless has led to the outbreak of insect species showing pesticide resistance. Apart from providing treatment to the infected, an effective and sustainable vector control strategy needs to be adopted. Numerous methods are used for the vector control like insecticide spraying, personal protection measures, larval and environmental control. The limitations of current insecticidal agents and rapid development of drug resistance have highlighted the need for the discovery of new insecticidal agents.[5] In this respect, use of plant based insecticides[6,7] can be considered to be an important alternative strategy for the control of *A. aegypti* larvae, since they constitute a rich source of bioactive compounds that are biodegradable to nontoxic products and potentially suitable for use in integrated pest management programs.

Plumbaginaceae is an economically important family, consisting mostly of shrubs distributed throughout Asia and Africa. The genus *plumbago* comprises 10–20 species of flowering plants, and known for its antimicrobial,[8] cytotoxic,[9] antimalarial[10] and antiprotozoal properties.[11] Unlike the more popular *Plumbago zeylanica*, *Plumbago capensis* is a lesser known species in ethnopharmacognosy. The herbaceous perennial plant, *P. capensis* has been traditionally used as one of the principle component in Ayurvedic preparation, Siddharna Yoga.[12] The main constituents of *P. capensis* are the napthaquinones.[13] In an attempt to add value to agricultural products, recently we have examined the connectivity between antifeedant activity and Michael adducts of plumbagin.[14]

As part of our continuing efforts directed towards the discovery of the structurally interesting and biologically active compounds from the Indian medicinal plants,[15] it was noticed that the $CHCl_3$ extract of *P. capensis* roots showed moderate mosquitocidal activity against fourth instar larvae of *A. aegypti*. Further, fractionation of this extract led to the isolation and identification of two new napthaquinone derivatives with moderate mosquitocidal activity along with other known compounds possessing potent larvicidal potentials. In this Letter, we report isolation, identification, and

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structure elucidation of two new napthaquinone derivatives, their mosquito controlling ability and discuss the structure–activity relationship for this family of natural mosquitocidal agents isolated from this plant. Structures of the new compounds were established using IR, MS, 1D and 2D NMR (HSQC, HMBC, COSY and NOESY) spectroscopic techniques (Fig. 1).

The roots of *P. capensis* Thunb (10 kg) were shade dried, powdered and extracted with chloroform in a soxhlet apparatus for 72 h. The resulting chloroform extract was evaporated to dryness under reduced pressure, affording syrupy residue (12 g). Then this chloroform extract was subjected to column chromatography on a silica gel column (60–120 mesh, 150 × 15 cm) and eluted with a step wise gradient of hexane/EtOAc (99:1, 98:2, 92:8, 90:10, 88:12 by volume) to afford a total of 102 fractions of 50 mL each. Column fractions were analyzed by TLC (Silica Gel 60 F254, hexane/EtOAc, 85:15), and fractions with similar TLC patterns were combined to give five major fractions (F_1, F_2, F_3, F_4, and F_5). The bioactive fraction F_1 (8 g), showed a strong larvicidal activity (100% mortality) against *A. aegypti* at 25 mg/L, which was purified by silica gel column chromatography (100–200 mesh, hexane/EtOAc, 99:1) to give orange needles of compound **1** (7.5 g). The active fraction F_2 eluting with hexane/EtOAc (92:8) was rechromatographed by silica gel with hexane/EtOAc (92:8) to give compound **2** (226 mg) and further eluted with hexane/EtOAc (90:10) to give compound **3** (826 mg).The fraction F_3 was then dissolved in MeOH and purified by repeated semi preparative HPLC using a 60 × 16 mm, 15 micron, 100 Å Eurospher RP 18 column, eluting with a MeCN/H_2O mixture (7:3, v/v) at a flow rate of 4 mL/min, with detection by UV absorption at 260 nm, to afford compound **4** (3 mg) with a retention time of 4.48 min and compound **5** (200 mg) with a retention time of 4.88 min. Fraction F_4 was rechromatographed on a silica gel column (100–200 mesh) with an isocratic elution using solvent system (hexane/chloroform/acetone, 7:2.8:0.2, by volume) to give an orange solids, which were identified as compound **6** (22 mg) and compound **7** (30 mg). Fraction F_5 was chromatographed on a silica gel column (60–120 mesh, 50 × 5 cm) and, eluted with a step wise gradient of chloroform/methanol (99:1, by volume) to give compounds **8** (10 mg).

Compound **4** was isolated as pale yellow solid with $[\alpha]_D^{25}$ +5.20 (*c* 1, $CHCl_3$) and mp 165 °C. The IR spectrum indicated the presence of OH (3426 cm^{-1}), carbonyl (1673 cm^{-1}) and aromatic functional groups. The molecular formula was determined as $C_{11}H_{12}O_3$ by HRESIMS, which provided a pseudomolecular ion peak at *m*/*z* 193.0869 [M^++H], in conjunction with its ^{13}C NMR. The ^1H NMR spectrum of **4** (in $CDCl_3$) displayed signals of nonequivalent methylene protons at δ 2.44 (1H, ddd, *J* = 7.2 Hz, 10 Hz, 17.3 Hz, H-3ax) and δ 2.31 (1H, m, H-3 eq) and three adjacent aromatic hydrogens at δ 7.11 (1H, d, *J* = 7.5 Hz), 7.51 (1H, *t*, *J* = 7.5 Hz) and 6.91 (1H, d, *J* = 8.5 Hz) assigned to 1,2,3-fused benzene ring. The presence of the signals at δ 1.18 (3H, d, *J* = 6.8 Hz, H-2, -CH_3), δ 4.51 (1H, dd, *J* = 5.3, 7.0 Hz, H-4) and δ 12.35 (1H, s, OH) associated data described above was indicative of napthaquinone derivative.[16] ^{13}C NMR spectrum of **1** (Table 1), together with the information from a DEPT spectrum, showed the presence of 11 carbon signals assigned to one methyl (secondary), one methylene (dihydro napthaquinone moiety), five methines (three olefinic, one oxymethine and one methine) and four nonhydrogen-bearing carbons (one carbonyl and three olefinic). Comparison of the NMR data with those reported for isoshinanolone was indicative of a similar structure but with different substitutions (C-2 and C-3 positions) and stereochemistry due to the differences in the chemical shifts of the C-2/C-3 signals.[16]

A comprehensive analysis of the 2D NMR data (HMBC, COSY and HSQC) of **4** facilitated the proton, carbon assignments and establishment of the final structure. Interpretation of ^1H–^1H COSY experiment revealed the sequential correlations of H-5 through H-6 to H-7. Further, it was confirmed by its HMBC correlations from H-5/C-6, C-10; H-6/C-5, C-7; H-7/C-6, C-8 (Fig. 2). The position of the methyl group at C-2 was supported by HMBC correlations from H_3-2 (CH_3)/C-2, C-3, C-1 (δ 203.89). The HMBC spectrum (Fig. 2) also revealed cross peaks between the methine proton (H-2) at δ 2.95 to the carbonyl carbon (δ 203.89), and two methylene protons (H-3ax, H-3eq) showed correlation to C-4 (δ 73.91). In the ^{13}C NMR spectrum, signal at δ 203.89 was assigned to carbonyl group at C-1, corresponding with the HMBC cross-peaks between OH-8 (δ 12.35)/C-1 (δ 203.89).

The relative configuration of **4** was established by analysis of ^1H coupling patterns and the NOESY spectrum (Fig. 3). Thus, using H-4 as anchoring point, the methyl group at C-2 was positioned equatorial (α-position) based on the NOESY correlation network, which showed the strong correlation of H-4/H-3eq; H_3-2/H-3eq. Further, occurrence of NOE cross peaks between H-3ax and H-2

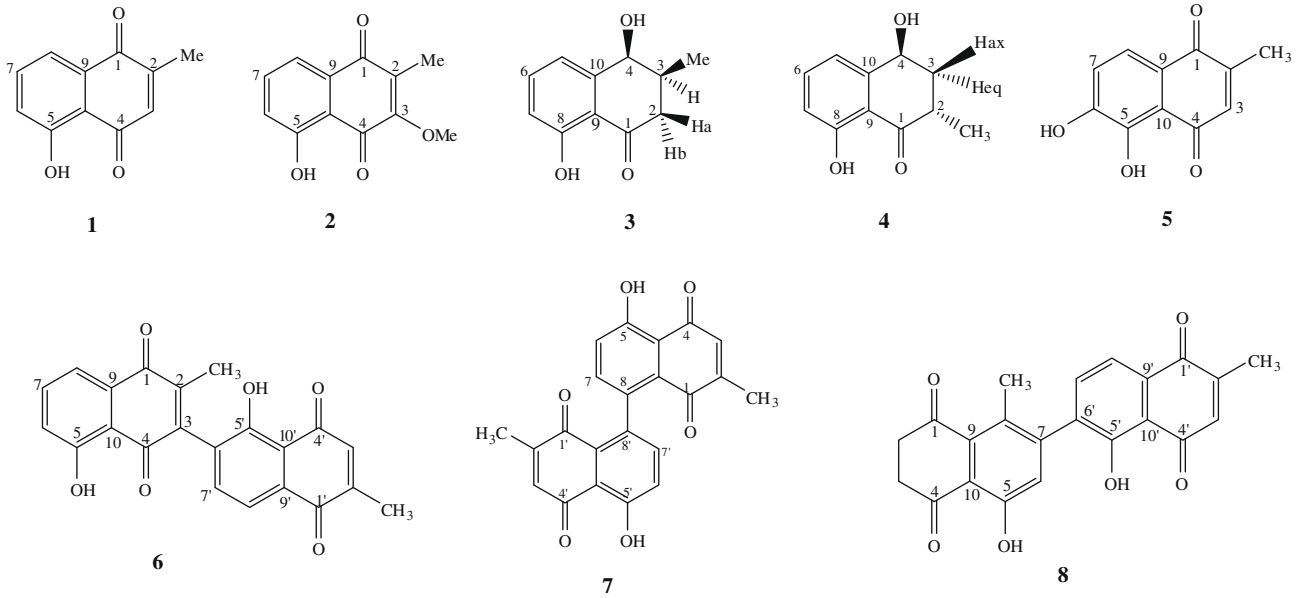

Figure 1. Isolated compounds from hexane extract of *Plumbago capensis*.

Table 1
^{1}H NMR and ^{13}C NMR data of compounds **4** and **8**

Position	Compound **4**		Compound **8**	
	δ_c	δ_H multiplicity	δ_c	δ_H multiplicity
1	203.89	—	184.90	—
2	37.70	2.95(1H, ddd, J = 4.2,13.7,17.3 Hz)	37.74	2.92 (2H, t, J = 6.0 Hz)
3	43.62	2.44 (1H, ddd, J = 7.2, 17.3 Hz), 2.31 (1H, m)	38.66	2.65 (2H, t, J = 6.0 Hz)
4	73.91	4.51 (1H, dd, J = 5.3, 7.0 Hz)	204.74	—
5	117.36	7.11 (1H, d, J = 7.5 Hz)	154.76	12.36 (OH, s)
6	136.69	7.5 (1H, t, J = 7.5 Hz)	122.65	7.20 (1H, s)
7	117.50	2.56-2.64 (1H, m)	—	—
8	162.72	12.35 (OH, s)	143.16	—
9	115.07	—	131.86	—
10	146.36	—	115.21	—
2-CH_3	—	1.18 (3H, d, J = 6.8 Hz)	—	—
1′	—	—	182.43	—
2′	—	—	149.97	—
3′	—	—	135.66	6.83 (1H, q, J = 1.5 Hz)
4′	—	—	190.77	—-
5′	—	—	159.22	12.30 (OH, s)
6′	—	—	129.79	—
7′	—	—	138.56	7.53(1H, d, J = 8.0 Hz)
8′	—	—	119.24	7.73 (1H, d, J = 8.0 Hz)
9′	—	—	132.20	—
10′	—	—	115.49	—
8-CH_3	—	—	14.43	2.07 (3H, s)
2′-CH_3	—	—	16.52	2.22 (3H, d, J = 1.5 Hz)

Assignments were based on 2D NMR including DQF-COSY, HSQC, HMBC and NOESY. Well-resolved couplings are expressed with coupling patterns and coupling constants in hertz in parentheses. For overlapped signals, only chemical shift values are given.

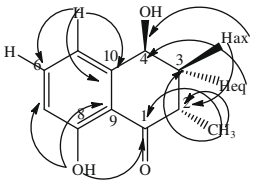

Figure 2. Key HMBC correlations of compound **4**.

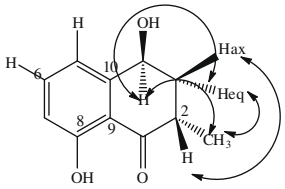

Figure 3. NOESY correlations of compound **4**.

and absence of the NOE correlations between H-4 and H-2 supported the α-position of the methyl group. It is important to note that due to insufficient sample of compound **4**, we are unable to establish the absolute stereochemistry by CD spectrum. However, based on the NOE observations and comparison of the positive optical rotation ($[\alpha]_D^{25}$ +5.20) of **4** with the optical rotation of related structures possessing the same chiral skeleton[17] with the same with achiral substituents led to the configuration of **4** as 4*S*,2*S*. Thus, compound **4** was characterized as (2*S*,4*S*)-4,8-dihydroxy-2-methyl-3,4-dihydronapthalen-1-(2*H*)-one for which the name isoplumbagolone is proposed.

Compound **8** was isolated as orange needles with a melting point of 114–116 °C. The HR-EI mass spectrum showed a molecular ion at *m*/*z* 377.1054 [M$^+$+H], suggesting a molecular formula $C_{22}H_{16}O_6$. The UV spectrum showed absorption maxima at λ_{max} (MeOH) 266 nm, 242 nm, 205 nm and 196 nm which are typical of binapthaquinone skeleton. The ^{1}H NMR spectrum of **8** (Table 1) displayed well separated two methyl groups, one giving rise to a singlet at δ 2.07 and the other to a narrow doublet (J = 1.5 Hz) at 2.22 ppm, coupled to a vinylic proton which appears as a quartet at δ 6.83 (1H, J = 1.5 Hz, H-3′). It also showed the signals at δ 7.73 (1H, d, J = 8.0 Hz, H-8′), δ 7.53 (1H, d, J = 8.0 Hz, H-7′), typical of *ortho* coupled aromatic protons. In addition, two triplets at δ 2.92 (2H, t, J = 6.0 Hz, H-2) and δ 2.65 (2H, t, J = 6.0 Hz, H-3) suggested the presence of two adjacent methylene groups and two sharp singlets at δ 12.30 (1H, s, OH-5′), δ 12.36 (1H, s, OH-5) were assigned to chelated hydroxyl groups (exchangeable with D_2O). The ^{13}C NMR spectrum displayed the presence of 22 carbon atoms (Table 1), and were further classified by DEPT experiments into categories of two methylenes, four methines, two methyls and fourteen quaternary carbons including four carbonyls (δ 184.90, δ 204.74, δ 182.43 and δ 190.77). The ^{1}H and ^{13}C data in association with the molecular composition highly suggested the structure of **8** to be a dimeric napthaquinone, with the structure similar to that of chitranone.[18]

Interpretation of 2D NMR (COSY, HMQC, and HMBC) data enabled the proton, and carbon assignments (Table 1). A close comparison of NMR data revealed one of the napthaquinone units in **8** from C-1′ to C-10′ to be in agreement with that of plumbagin as indicated by the presence of aromatic protons (δ 7.53, 7.73, each doublets), methyl group (δ 2.07), vinylic proton (δ 6.83,q) and chelated hydroxyl group and two carbonyls. Further, HMBC correlations between Me-2′ and C-2′ (δ 149.97), C-3′ (135.66), and C-1′ (δ 182.43), between H-8′ and C-9′ (δ 132.20), C-1′(δ 182.43) and C-8′ (δ 119.24), and between OH-5′ (δ 12.30) and C-10′ (δ 115.49) and C-5′ (δ 159.22) supported the presence of plumbagin unit (Fig. 4). With respect to the second napthaquinone part of **8**, the NMR data of were compatible to those of dihydronapthaquinone, as indicated by the presence of two triplets at δ 2.92 (2H, t, J = 6.0 Hz, H-2) and δ 2.65 (2H, t, J = 6.0 Hz, H-3), and the ^{13}C NMR resonances at δ 184.90 (C-1), δ 37.74 (C-2), 38.66 (C-3), 204.74 (C-4), for dihydronapthaquinone.

After assigning the constitutional units of new dimer **8**, we turned our attention to establish their connectivity. Thus, the two units depicted above were assembled according to HMBC and COSY correlations. In the NMR spectrum of **8**, a vinylic proton which appears as a quartet at 6.83 (J = 1.5 Hz) and two triplets at δ 2.92 (2H, t, J = 6.0 Hz) and δ 2.65 (2H, t, J = 6.0 Hz) assigned two adjacent methylene groups of reduced quinine moiety (assigned by COSY) were seen. Based on these evidence, the connection of napthaquinoe units was supposed to be realized through aromatic ring inter-reaction. The ^{1}H–1H COSY relations between H-7′/H-8′ indicated that dimeric linkage in the plumbagin moiety could be through C-6′. Finally, interpretation of the HMBC correlations of H-6/C-7, C-6′, Me-8/C-7 and H-7′/C-6′, C-7 allowed the assignment

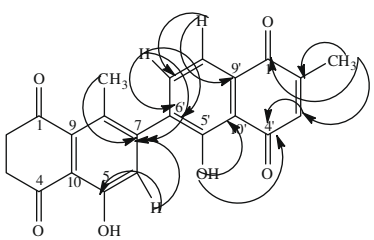

Figure 4. Key HMBC correlations of compound **8**.

of napthaquinone units linking from C-6′ to C-7. Based on these data, compound **8** was characterized as 2,3-dihydro-5,5′-dihydroxy-8-methyl-(7,6′-binaphthalene)-1,1′,4,4′-tetraone and trivially named as chitranane.

In addition to the above two new compounds, six known compounds were also isolated from chloroform extract. By comparison of their physical and spectroscopic data with literature, they were characterized as plumbagin (**1**),[19] 3-*O*-methyl droserone (**2**),[20] isoshinanolone (**3**),[16] 6-hydroxy plumbagin (**5**),[21] chitranone (**6**),[18] and maritinone (**7**),[22,18b] respectively (Fig. 1).

In the present study, mosquitocidal activities were investigated for chloroform extract of *P. capensis* roots and isolates against fourth instar larvae of *A. aegypti*.[23–25] Fractionation of the active chloroform extract led to the isolation of two new compounds (**4**, **8**) and six known compounds (**1**–**3**, **5**–**7**) with better mosquitocidal potentials against *A. aegypti*. As demonstrated in Table 2, all the isolates displayed varying degrees of mosquitocidal potentials (LC_{50} range from 1.26 to 40.66 μg/mL). Among the test compounds, isoshinanolone (**3**), plumbagin (**1**), 6-hydroxy plumbagin (**5**) exhibited potent larvicidal activity against *A. aegypti*. Isoshinanolone (**3**) is highly active in controlling *A. aegypti* larvae with IC_{50} value of 1.26 μg/mL. Plumbagin occupied at second place in controlling the *A. aegypti* larvae with IC_{50} value of 5.43 μg/mL and this observation is in line with the earlier reports about the mosquito larvicidal potential of plumbagin.[26] Though it is difficult to discuss the structure activity relationship criteria responsible for the mosquitocidal activities in this set of compounds, presence of reduced quinine ring (ring B), hydroxyl group at 4th position and methyl group at 3rd position (compound **3**) appears to be important in imparting the mosquitocidal activity when compared with **1**, **2**, **5**. Furthermore, dimeric napthaquinones (**6**, **7** and **8**) were less potent than the corresponding monomeric napthaquinones (**1**, **2** and **3**). It is noteworthy to mention that absence of the methyl group at 3rd position led to drastic decrease in the activity (compound **4**). It is interesting that the plumbagin showed 100% toxicity at 6.5 μg/mL, while its structural analogue, hydroxyl plumbagin (**5**) showed same percentage of larval mortality at 20 μg/mL. This may be due to the interference of hydroxyl group with active site in the hydroxy plumbagin (**5**), which reduces its larvicidal activity. Chitranone (**6**) and isoplumbagolone (**4**) did not produce any mortality in the larval populations even at higher concentrations tested (60 μg/mL). Overall, these results imply that the mode of perception as well as the substitution pattern on the napthaquinone skeleton differed considerably against the mosquito species examined in this study. However, some of these compounds were highly promising as toxicants as well as mosquito larvicides against *A. aegypti*.

In conclusion, the *P. capensis* root derived materials are promising for managing field populations of *A. aegypti*. These activities in conjunction with the previously reported activities against the several other pests, suggest that plumbagin (**1**), isoshinanolone (**3**) has the potential to be developed as an effective and alternative pest managing natural agents. Further studies are required on the mode of action, dosage dependent and their effects on the environment for their use as a natural larvicides.

Table 2
Mosquito larvicidal activity of constituents derived from *P. capensis* against fourth instar larvae of *A. aegypti*

Compounds	Activity (μg/mL) (95% FL)[a]	
	LC_{50} (LCL–UCL)	LC_{90} (LCL–UCL)
1	5.43 (5.11–5.70)	6.56 (6.14–7.73)
2	31.47 (22.17–36.29)	55.72 (46.89–93.69)
3	1.26 (0.90–01.73)	4.10 (2.66–12.007)
4	NA	NA
5	13.64 (12.20–14.90)	19.28 (17.09–25.27)
6	NA	NA
7	40.66 (31.07–44.02)	53.87 (48.79–65.93)
8	31.21 (27.68–34.33)	42.72 (38.07–55.09)
Rotenone	6.20 (4.70–7.80)	25.00 (19.01–38.12)

[a] FL: fiducial limits LCL: lower confident limits, UCL: upper confident limits, NA: not active at highest concentration tested (60 μg/mL).

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