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Larvicidal and Pupicidal Activities of Alizarin Isolated from Roots of *Rubia cordifolia* Against *Culex quinquefasciatus* Say and *Aedes aegypti* (L.) (Diptera: Culicidae)

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Abstract
The mosquitocidal activities of different fractions and a compound alizarin from the methanol extract of *Rubia cordifolia* roots were evaluated on larvae and pupae of *Culex quinquefasciatus* Say and *Aedes aegypti* (L.) (Diptera: Culicidae). Larvae and pupae were exposed to concentrations of 2.5, 5.0, 7.5 and 10 ppm for fractions and 0.5, 1.0, 1.5 and 2.0 ppm for compound. After 24 h, the mortality was assessed and the LC_{50} and LC_{90} values were estimated for larvae and pupae. Among the 23 fractions screened, fraction 2 from the methanol extract of *R. cordifolia* showed good mosquitocidal activity against *C. quinquefasciatus* and *A. aegypti*. LC_{50} and LC_{90} values of fraction 2 were 3.53 and 7.26 ppm for *C. quinquefasciatus* and 3.86 and 8.28 ppm for *A. aegypti* larvae, and 3.76 and 7.50 ppm for *C. quinquefasciatus* and 3.92 and 8.05 ppm for *A. aegypti* pupae, respectively. Further, the isolated compound alizarin presented good larvicidal and pupicidal activities. LC_{50} and LC_{90} values of alizarin for larvae were 0.81 and 3.86 ppm against *C. quinquefasciatus* and 1.31 and 6.04 ppm for *A. aegypti* larvae, respectively. Similarly, the LC_{50} and LC_{90} values of alizarin for pupae were 1.97 and 4.79 ppm for *C. quinquefasciatus* and 2.05 and 5.59 ppm for *A. aegypti* pupae, respectively. The structure of the isolated compound was identified on the basis of spectroscopic analysis and compared with reported spectral data. The results indicated that alizarin could be used as a potential larvicide and pupicide.

Introduction

Mosquitoes are small insects in the order Diptera. Many species of mosquitoes serve as important vectors of several diseases. *Culex quinquefasciatus* Say is an important vector of lymphatic filariasis in tropical and subtropical regions, as it vectors *Wuchereria bancrofti* (Holder 1999). According to World Health Organization report (1984), about 90 million people worldwide are infected with *W. bancrofti* and ten times more people are at the risk of being infected. Alone in India, 25 million people harbour microfilaria (mf) and 19 million people suffer from filarial disease manifestations (NICD 1990, Reegan *et al* 2015). *Aedes aegypti* (L.) is the primary vector involved in the transmission of dengue, chikungunya and Zika viruses (Harrington *et al* 2005, Kannathasan *et al* 2011, Yakob & Walker 2016). Many Asian countries including India are endemic for dengue fever. Many sporadic dengue cases have been reported from various parts of India (Akram & Ahmed 2005) and a major outbreak was recorded during 2012. During this outbreak, a total

of 5376 dengue cases and 39 deaths were detected in the state Tamil Nadu, southern India (Kannan 2012, Reegan *et al* 2014). The climatological condition also favoured rapid increase of *A. aegypti* populations.

For the past several decades, synthetic insecticides have been used against the aquatic stages of vector mosquitoes. The usage of synthetic insecticides—pyrethroids, organophosphates, organochlorines and carbamates—is increasing year after year, and continuous application of these insecticides pose a major threat to environment and human health (Shaalan *et al* 2005, Sutthanont *et al* 2010, Madhu *et al* 2010, Bayen 2012). Phytocompounds isolated from plants are target specific and safe to all associated organisms. Hence, plant-derived products would be a good alternative to synthetic insecticides.

Decoction from *R. cordifolia* roots is prescribed to cure jaundice, paralytic affections and urinary dysfunctions (Devi Priya & Siril 2014). Roots of *R. cordifolia* have also been used as astringent, thermogenic, febrifuge, antidysenteric, antihelmintic, galactopurifier, ophthalmic and rejuvenant and used to treat cough, bladder and kidney stones, and joint inflammation (Sivarajan & Balachandran 1994). In our preliminary study, methanol extract of *R. cordifolia* roots showed higher mosquitocidal activity than hexane and chloroform extracts against *C. quinquefasciatus* and *A. aegypti*. We report here the isolation and identification of an active molecule from root extracts of *R. cordifolia* against larvae and pupae of *C. quinquefasciatus* and *A. aegypti*.

Material and Methods

Insect rearing

Culex quinquefasciatus and *Aedes aegypti* larvae were reared in tap water at 27 ± 2°C, 75–85% RH with13:11 L/D photoperiod. Larvae were fed with dog biscuits and Brewer's yeast in the ratio of 3:2. Pupae were transferred from the rearing trays to plastic cups (250 mL) containing tap water and placed in breeding cages (60 × 60 × 60 cm dimension) for adult emergence. Adults were fed with wet raisins and 10%

Table 1 Lethal concentrations (in ppm) of different fractions of *Rubia cordifolia* methanol extract against larvae of *Culex quinquefasciatus*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Intercept ± SE	Slope ± SE	χ^2
			LL	UL		LL	UL			
Culex quinquefasciatus	Fraction 1	5.60	5.0	6.25	16.05	12.99	22.05	2.9 ± 0.25	2.8 ± 0.31	3.3*
	Fraction 2	3.53	3.16	3.87	7.26	6.52	8.32	2.7 ± 0.25	4.0 ± 0.3	5.6*
	Fraction 3	4.32	3.83	4.80	11.27	9.62	14.08	3.03 ± 0.24	3.08 ± 0.31	2.6*
	Fraction 4	17.72	11.84	50.72	167.27	55.85	3673.75	3.35 ± 0.25	1.31 ± 0.32	1.3*
	Fraction 5	15.86	11.48	32.22	99.02	43.33	702.53	3.06 ± 0.27	1.61 ± 0.33	2.3*
	Fraction 6	8.15	6.78	10.80	47.90	27.06	154.82	3.48 ± 0.23	1.66 ± 0.30	2.0*
	Fraction 7	11.93	9.12	20.72	87.18	39.15	576.83	3.40 ± 0.24	1.48 ± 0.31	2.3*
	Fraction 8	7.06	6.19	8.26	25.53	18.35	44.56	3.05 ± 0.25	2.29 ± 0.31	5.9*
	Fraction 9	10.38	8.46	14.77	53.19	29.84	171.42	3.16 ± 0.25	1.80 ± 0.31	4.1*
	Fraction 10	8.93	7.50	11.68	42.30	25.70	110.09	3.19 ± 0.25	1.89 ± 0.31	4.6*
	Fraction 11	11.28	8.88	17.76	69.70	34.77	320.82	3.29 ± 0.25	1.62 ± 0.31	1.2*
	Fracton 12	8.84	7.24	12.30	55.54	29.69	210.78	3.47 ± 0.24	1.60 ± 0.30	4.4*
	Fraction 13	9.92	8.24	13.39	45.08	27.07	120.45	3.05 ± 0.26	1.94 ± 0.32	1.6*
	Fraction 14	10.02	8.29	13.70	47.05	27.77	131.72	3.08 ± 0.26	1.90 ± 0.32	0.9*
	Fraction 15	10.93	8.79	16.11	57.87	31.45	204.14	3.16 ± 0.25	1.77 ± 0.32	0.5*
	Fraction 16	6.97	6.04	8.30	28.77	19.70	56.10	3.24 ± 0.24	2.08 ± 0.30	2.8*
	Fraction 17	12.58	10.01	19.07	56.48	31.60	182.89	2.83 ± 0.28	1.96 ± 0.34	1.6*
	Fraction 18	10.91	8.89	15.55	51.53	29.54	155.69	3.02 ± 0.26	1.90 ± 0.32	0.3*
	Fraction 19	3.59	3.19	3.96	7.93	7.05	9.24	2.92 ± 0.24	3.72 ± 3.72	5.5*
	Fraction 20	7.17	6.22	8.56	29.01	19.90	56.26	3.19 ± 0.24	2.11 ± 0.30	5.9*
	Fraction 21	4.00	3.50	4.48	10.91	9.28	13.74	3.22 ± 0.23	2.94 ± 0.31	0.2*
	Fraction 22	10.87	8.60	16.83	68.14	34.17	309.60	3.33 ± 0.25	1.60 ± 0.31	0.2*
	Fraction 23	5.11	4.41	5.84	19.14	14.42	30.50	3.41 ± 0.23	2.23 ± 0.29	2.6*

LC_{50} - lethal concentration that kills 50% of the exposed larvae, LC_{90} - lethal concentration that kills 90% of the exposed larvae.

LL lower limit (95% confidence limit), *UL* upper limit (95% confidence limit).

*$p \leq 0.05$, level of significance of chi-square values.

Table 2 Lethal concentrations (in ppm) of different fractions of *Rubia cordifolia* methanol extract against larvae of *Aedes aegypti*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Intercept ± SE	Slope ± SE	χ^2
			LL	UL		LL	UL			
Aedes aegypti	Fraction 1	12.65	9.66	21.83	81.85	38.34	461.44	3.25 ± 0.25	1.58 ± 0.31	0.2*
	Fraction 2	3.86	3.47	4.23	8.28	7.38	9.61	2.72 ± 0.25	3.86 ± 0.35	0.7*
	Fraction 3	9.34	7.90	12.06	38.81	24.65	89.87	2.98 ± 0.26	2.07 ± 0.32	0.5*
	Fraction 4	9.28	7.87	11.94	38.05	24.35	86.48	2.97 ± 0.26	2.09 ± 0.32	3.09*
	Fraction 5	7.88	6.83	9.56	30.75	20.94	60.43	3.05 ± 0.25	2.16 ± 0.31	1.9*
	Fraction 6	7.86	6.96	9.15	24.07	17.97	38.73	2.63 ± 0.27	2.63 ± 0.33	2.8*
	Fraction 7	10.99	9.07	15.19	45.62	27.63	119.06	2.84 ± 0.27	2.07 ± 0.33	0.3*
	Fraction 8	12.01	9.39	19.28	69.92	35.17	311.54	3.1 ± 0.2	1.6 ± 0.3	0.7*
	Fraction 9	12.75	10.06	19.80	60.29	32.79	211.49	2.90 ± 0.28	1.89 ± 0.34	0.5*
	Fraction 10	13.40	10.21	23.17	78.30	37.81	396.38	3.11 ± 0.26	1.67 ± 0.32	0.3*
	Fraction 11	13.51	10.58	21.45	60.94	33.16	214.02	2.78 ± 0.29	1.95 ± 0.35	1.3*
	Fracton 12	12.89	10.07	20.63	65.40	34.29	255.48	2.98 ± 0.27	1.81 ± 0.33	0.05*
	Fraction 13	8.60	7.42	10.63	32.68	22.01	65.72	2.93 ± 0.26	2.21 ± 0.32	2.3*
	Fraction 14	7.74	6.85	9.05	24.57	18.17	40.29	2.72 ± 0.26	2.55 ± 0.33	4.9*
	Fraction 15	10.40	8.57	14.36	47.49	28.06	132.36	3.02 ± 0.26	1.94 ± 0.32	0.1*
	Fraction 16	11.28	9.24	15.87	47.81	28.46	130.56	2.84 ± 0.27	2.04 ± 0.34	0.5*
	Fraction 17	11.75	9.49	17.14	52.64	30.17	159.12	2.89 ± 0.27	1.96 ± 0.33	0.2*
	Fraction 18	7.77	6.81	9.20	26.88	19.24	47.23	2.88 ± 0.26	2.37 ± 0.32	1.7*
	Fraction 19	5.56	2.41	11.07	15.71	8.99	3613.04	2.88 ± 0.43	2.84 ± 0.55	6.0*
	Fraction 20	7.13	6.31	8.25	23.42	17.40	38.04	2.88 ± 0.25	2.48 ± 0.32	2.4*
	Fraction 21	5.67	5.10	6.30	15.47	12.69	20.75	2.78 ± 0.25	2.94 ± 0.32	3.8*
	Fraction 22	9.84	8.30	12.85	39.17	24.97	89.87	2.87 ± 0.27	2.13 ± 0.33	1.6*
	Fraction 23	6.30	5.65	7.07	18.0	14.34	25.51	2.75 ± 0.25	2.81 ± 0.32	3.4*

*$p \leq 0.05$, level of significance of chi-square values.

sucrose solution soaked in cotton. Adult females were deprived of sucrose for 6 h and then provided with a mouse placed in a breeding cage overnight for blood feeding. The ovitrap, containing water at the bottom and filter paper on the sides of a 500-mL plastic container was placed in the breeding cage, and the eggs were collected after 3 days. Third instars and pupae were used for the experiment.

Plant material

Roots of *R. cordifolia* (Fig 1) were collected from Kalakkad Mundanthurai Tiger Reserve forest (KMTR) in Southern Western Ghats of Tirunelveli District, India. The plant material was authenticated by Dr. S. Mutheeswaran, Taxonomist at Entomology Research Institute, Loyola College, Chennai. A voucher specimen (ERI-LA-MOS-304) was deposited in the herbarium of the institute.

Extraction

The roots were shade dried and coarsely powdered using an electric blender. The powdered root (1 kg) was extracted twice with methanol by cold percolation (48 h). The extract was filtered through Whatman No. 1 filter paper and concentrated in a rotary evaporator and finally dried under vacuum.

Chromatographic separation

The methanol extract (91 g) was subjected to column chromatography on a silica gel (100–200 mesh) column packed in hexane. The column was eluted with solvents of increasing polarity in the order hexane, ethyl acetate and methanol and their mixtures. Similar fractions were combined based on their TLC profiles. Finally, 23 fractions were obtained. Each fraction was subjected to mosquitocidal activity at the concentrations of 2.5, 5.0, 7.5 and 10 ppm. Fraction 2 eluted with hexane:ethyl acetate (90:10) showed significant mosquitocidal activity. Based on the bioassay results, fraction 2 was selected for further identification of the bioactive compound.

Bioassays

Larvicidal and pupicidal activities were evaluated using the method prescribed by World Health Organization (2005)

Table 3 Lethal concentrations (in ppm) of different fractions of *Rubia cordifolia* methanol extract against pupae of *Culex quinquefasciatus*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Intercept ± SE	Slope ± SE	χ^2
			LL	UL		LL	UL			
Culex quinquefasciatus	Fraction 1	4.78	4.24	5.31	13.23	11.02	17.25	3.02 ± 0.24	2.89 ± 0.31	4.9*
	Fraction 2	3.76	3.40	4.10	7.50	6.76	8.56	2.53 ± 0.26	4.27 ± 0.37	5.7*
	Fraction 3	11.85	9.03	20.84	90.59	39.82	651.11	3.44 ± 0.24	1.45 ± 0.30	0.9*
	Fraction 4	11.76	9.23	18.61	68.33	34.65	297.01	3.20 ± 0.25	1.67 ± 0.32	0.5*
	Fraction 5	8.66	7.39	10.92	36.15	23.39	80.14	3.06 ± 0.25	2.06 ± 0.31	3.1*
	Fraction 6	8.86	7.98	10.16	21.13	16.68	30.73	1.78 ± 0.35	3.39 ± 0.41	3.0*
	Fraction 7	9.28	7.87	11.94	38.05	24.35	86.48	2.97 ± 0.26	2.09 ± 0.32	3.0*
	Fraction 8	11.59	9.42	16.62	50.18	29.34	143.86	2.85 ± 0.27	2.01 ± 0.34	1.0*
	Fraction 9	10.08	8.41	13.51	43.10	26.46	108.62	2.96 ± 0.26	2.03 ± 0.32	0.6*
	Fraction 10	8.57	7.35	10.65	33.96	22.51	71.02	3.0 ± 0.25	2.14 ± 0.32	0.8*
	Fraction 11	10.66	8.92	14.24	40.80	25.82	95.40	2.73 ± 0.28	2.19 ± 0.34	1.6*
	Fracton 12	8.05	6.71	10.58	46.52	26.58	145.56	3.47 ± 0.23	1.68 ± 0.30	1.8*
	Fraction 13	8.14	7.06	9.91	30.93	21.12	60.33	2.98 ± 0.25	2.21 ± 0.32	3.8*
	Fraction 14	8.23	7.32	9.58	23.72	17.91	37.34	2.44 ± 0.29	2.78 ± 0.35	3.5*
	Fraction15	8.00	6.84	9.96	35.41	22.81	79.63	3.20 ± 0.24	1.98 ± 0.31	4.5*
	Fraction 16	11.79	9.48	17.40	54.61	30.80	172.59	2.93 ± 0.27	1.92 ± 0.33	1.2*
	Fraction 17	6.89	5.99	8.16	27.65	19.20	52.24	3.21 ± 0.24	2.12 ± 0.30	2.5*
	Fraction 18	6.12	5.28	7.19	26.59	18.35	51.30	3.41 ± 0.23	2.01 ± 0.29	1.5*
	Fraction 19	4.64	3.40	5.82	47.32	23.94	259.31	4.15 ± 0.21	1.27 ± 0.28	2.8*
	Fraction 20	7.69	6.62	9.39	32.50	21.55	68.15	3.18 ± 0.24	2.04 ± 0.31	2.3*
	Fraction 21	6.46	5.65	7.52	24.65	17.69	43.34	3.21 ± 0.24	2.20 ± 0.30	1.1*
	Fraction 22	6.28	5.47	7.32	24.87	17.69	44.60	3.28 ± 0.24	2.14 ± 0.30	3.0*
	Fraction 23	6.81	5.98	7.92	24.45	17.75	41.79	3.07 ± 0.24	2.30 ± 0.31	5.1*

LC_{50} - lethal concentration that kills 50% of the exposed pupae, LC_{90} - lethal concentration that kills 90% of the exposed pupae.

LL lower limit (95% confidence limit), *UL* upper limit (95% confidence limit).

*$p \leq 0.05$, level of significance of chi-square values.

with slight modifications. Fractions were tested at 2.5, 5.0, 7.5 and 10 ppm using acetone. Each treatment, including control, was replicated five times. Twenty third instars and pupae (In WHO protocol: 25 third instars used) of *C. quinquefasciatus* and *A. aegypti* were used for each replicate. Azadirachtin and temephos were used as positive controls, and acetone was used as a negative control. The dead larvae and pupae were registered after 24-h exposure period. The percent mortality was calculated and subjected to corrections according to Abbott (1925) using:

$$1-\frac{n \text{ in T after treatment}}{n \text{ in C after treatment}} \times 100$$

where *n* is the number of larvae, *T* is the treated and *C* is the control. The corrected percentage mortality value for each concentration was considered to estimate LC_{50} and LC_{90} values using US EPA probit analysis software (version 1.5).

Identification of the active compound

The active fraction 2 was crystallised from hexane-ether mixture to get the active compound. The structure of the compound was elucidated on the basis of spectroscopic data. UV–vis spectrum was collected on a Shimadzu UV–Vis spectrophotometer in methanol. IR spectrum was obtained on a Perkin-Elmer FT-IR grating spectrophotometer in KBr disc. ^{1}H and ^{13}C NMR were produced on a Bruker Instrument at 400 and 100 MHz in DMSO d6, respectively.

Larvicidal and pupicidal activity of the compound

The larvicidal and pupicidal activities of the isolated compound were performed as earlier mentioned. Test concentrations were 0.5, 1.0, 1.5 and 2.0 ppm.

Table 4 Lethal concentrations (in ppm) of different fractions of *Rubia cordifolia* methanol extract against pupae of *Aedes aegypti*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Intercept ± SE	Slope ± SE	χ^2
			LL	UL		LL	UL			
Aedes aegypti	Fraction 1	10.16	8.56	13.33	39.18	25.07	89.02	2.79 ± 0.27	2.18 ± 0.33	0.7*
	Fraction 2	3.92	3.54	4.28	8.05	7.23	9.25	2.56 ± 0.25	4.09 ± 0.36	2.8*
	Fraction 3	7.32	6.58	8.30	20.19	15.84	29.42	2.48 ± 0.28	2.90 ± 0.34	4.9*
	Fraction 4	12.67	9.98	19.80	61.81	33.21	224.52	2.94 ± 0.27	1.86 ± 0.33	0.2*
	Fraction 5	14.69	10.99	26.72	82.15	39.15	433.97	2.99 ± 0.27	1.71 ± 0.34	0.2*
	Fraction 6	10.78	8.83	15.13	49.20	28.75	141.07	2.99 ± 0.26	1.94 ± 0.32	0.7*
	Fraction 7	9.42	7.96	12.21	39.25	24.84	91.69	2.98 ± 0.26	2.06 ± 0.32	1.4*
	Fraction 8	10.27	8.59	13.71	41.87	26.09	101.60	2.87 ± 0.27	2.10 ± 0.33	0.9*
	Fraction 9	10.16	8.44	13.72	44.40	26.94	115.43	2.98 ± 0.26	2.00 ± 0.32	0.6*
	Fraction 10	8.84	7.57	11.11	35.13	23.08	75.01	2.97 ± 0.26	2.13 ± 0.32	1.6*
	Fraction 11	8.04	7.09	9.48	25.73	18.81	43.20	2.70 ± 0.27	2.53 ± 0.33	1.9*
	Fracton 12	9.47	8.00	12.27	39.06	24.79	90.57	2.96 ± 0.26	2.08 ± 0.32	1.6*
	Fraction 13	9.73	8.32	12.32	34.51	23.13	70.31	2.69 ± 0.28	2.33 ± 0.34	1.2*
	Fraction 14	6.90	6.17	7.83	20.31	15.77	30.19	2.70 ± 0.26	2.73 ± 0.32	2.7*
	Fraction 15	9.50	8.15	11.94	33.722	22.74	67.65	2.72 ± 0.28	2.33 ± 0.34	0.9*
	Fraction 16	7.30	6.47	8.44	23.27	17.39	37.34	2.80 ± 0.26	2.54 ± 0.32	2.3*
	Fraction 17	11.64	9.29	17.52	58.97	32.05	207.26	3.06 ± 0.26	1.81 ± 0.32	0.3*
	Fraction 18	7.05	6.29	8.06	21.31	16.34	32.49	2.73 ± 0.26	2.66 ± 0.32	2.7*
	Fraction 19	4.90	4.35	5.44	13.58	11.28	17.80	3.00 ± 0.24	2.89 ± 0.31	4.5*
	Fraction 20	5.40	4.83	6.00	14.91	12.26	19.91	2.87 ± 0.25	2.90 ± 0.31	5.1*
	Fraction 21	5.46	4.88	6.08	15.30	12.51	20.63	2.88 ± 0.25	2.86 ± 0.31	5.8*
	Fraction 22	4.93	1.16	9.15	12.73	7.61	7189.93	2.84 ± 0.49	3.11 ± 0.63	7.8*
	Fraction 23	6.81	6.12	7.66	18.92	15.01	27.01	2.59 ± 0.27	2.88 ± 2.88	5.47*

*$p \leq 0.05$, level of significance of chi-square values.

Results

Bioassay results of crude chromatographic fractions

Among the 23 fractions screened, fraction 2 was found to be the most effective, with LC_{50} and LC_{90} values of 3.53, 7.26 ppm and 3.86, 8.28 ppm for larvae of *C. quinquefasciatus* and *A. aegypti*, respectively. Bioactivity of this fraction was followed by fraction 19, which yielded LC_{50} and LC_{90} values of 3.59, 7.93 ppm and 5.56, 15.71 ppm for larvae of *C. quinquefasciatus* and *A. aegypti*, respectively (Tables 1 and 2). The LC_{50} and LC_{90} values of fraction 2 for pupicidal activity were 3.76, 7.50 ppm and 3.92, 8.05 ppm for pupae of *C. quinquefasciatus* and *A. aegypti*, respectively (Tables 3 and 4). All other fractions tested showed only moderate activity against the larvae and pupae of both mosquito species.

Fig 1 *Rubia cordifolia* whole plant (**a**) and its root (**b**).

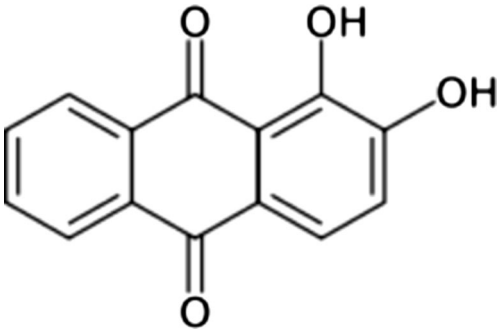

Fig 2 Structure of alizarin.

Identification of the active compound

The active molecule present in fraction 2 was identified as alizarin (1) [1,2-dihydroxyanthraquinone-9,10 anthraquinone] (Fig 2). It was obtained as reddish orange crystals from hexane–ether mixture: mp 148°C. UV: λ_{max} MeOH 249, 270, 435 nm (Fig 3). IR: ν_{max} MeOH KBr 3422, 3369 (hydroxyl) 3074 (aromatic) 1662, 1631 (quinine carbonyl) 1588, 1457 (aromatic) 1340, 1294, 1191, 1037, 1017, 895, 840, 756, 711 (aromatic) (Fig 4). ^{1}H NMR (δ DMSO d6, 400 MHz): 7.23 (1H,d,J = 8.4Hz,H3), 7.66 (^{1}H,d,J = 8.4Hz,H-4), 7.93 (2H,m,H6 and H7), 8.16 (^{1}H,m,H8), 8.20 (1H,m,H5) (Fig 5). ^{13}C NMR (δ DMSO d6, 100 MHz): 150.17 (C-1), 152.17 (C-2), 120.23 (C-3 and C4), 123.18 (C4a), 125.89 (C5 and C8), 132.96 (C-8a), 134.52 (C-6) 133.46 (C-7), 188.19 (C-9) 115.65 (C-9a) 179.96 (C-10), 132.24 (C-10a) (Fig 6). The physical and spectroscopic data were comparable with those reported in the literature (Ahmed *et al* 2014, Mahendra *et al* 2014). (See supplementary Material for Figs 3 to 6).

Larval and pupal mortality caused by alizarin

Exposure of larvae and pupae of *C. quinquefasciatus* and *A. aegypti* to alizarin increased mortality in a concentration-dependent manner. LC_{50} and LC_{90} values of alizarin for *C. quinquefasciatus* and *A. aegypti* larvae were 0.81, 3.56 ppm and 1.31, 6.04 ppm, respectively (Tables 5 and 6). Similarly, LC_{50} and LC_{90} values of alizarin for *C. quinquefasciatus* and *A. aegypti* pupae were 1.97, 4.79 ppm and 2.05, 5.59 ppm, respectively (Tables 7 and 8). There was no mortality in controls, and all larvae and pupae were active and exhibited normal movement. Convulsions were observed at 2 ppm of alizarin and dead larvae and pupae settled down as already reported (Reegan *et al* 2013).

Discussion

Mosquitoes are one of the most dangerous insects since they vector several pathogens to humans. *Culex quinquefasciatus* and *A. aegypti* are well established in tropical and subtropical regions, and they have also developed resistance to chemical insecticides (Tikar *et al* 2009, Llinás *et al* 2010, Mulyatno *et al* 2012, Chen *et al* 2013, Grisales *et al* 2013). Hence, plant extracts and isolated compounds would be good alternatives to control vector mosquitoes.

Table 5 Lethal concentrations of alizarin (in ppm) against larvae of *Culex quinquefasciatus*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Slope ± SE	Intercept ± SE	χ^2
			LL	UL		LL	UL			
Culex quinquefasciatus	Alizarin	0.81	0.59	1.01	3.56	2.41	8.443	2.1 ± 0.3	5.1 ± 0.1	4.1*
	Azadirachtin	0.28	0.12	0.37	0.55	0.46	0.66	4.3 ± 1.1	7.4 ± 0.3	0.1*
	Temephos	0.65	0.56	0.73	1.62	1.42	1.93	3.2 ± 0.3	5.5 ± 0.1	1.7*

*$p \leq 0.05$, level of significance of chi-square values.

Table 6 Lethal concentrations of alizarin (in ppm) against larvae of *Aedes aegypti*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Slope ± SE	Intercept ± SE	χ^2
			LL	UL		LL	UL			
Aedes aegypti	Alizarin	1.31	1.05	1.72	6.04	3.5	21.86	3.4 ± 0.4	4.7 ± 0.1	3.3*
	Azadirachtin	0.34	0.22	0.43	1.04	0.90	1.27	2.6 ± 0.3	6.2 ± 0.1	3.7*
	Temephos	0.92	0.11	1.66	1.82	1.17	646.1	4.3 ± 0.9	5.1 ± 0.1	2.4*

Positive control values-Reegan *et al* 2014

*$p \leq 0.05$, level of significance of chi-square values.

Table 7 Lethal concentrations of alizarin (in ppm) against pupae of *Culex quinquefasciatus*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Slope ± SE	Intercept ± SE	χ^2
			LL	UL		LL	UL			
Culex quinquefasciatus	Alizarin	1.97	1.74	2.21	4.79	4.14	5.79	2.4 ± 0.2	4.0 ± 0.1	5.4*
	Azadirachtin	0.47	0.41	0.52	0.80	0.72	0.92	5.6 ± 0.7	6.8 ± 0.2	0.3*
	Temephos	0.75	0.68	0.82	1.52	1.37	1.74	4.1 ± 0.3	5.5 ± 0.1	4.8*

*$p \leq 0.05$, level of significance of chi-square values.

Table 8 Lethal concentrations of alizarin (in ppm) against pupae of *Aedes aegypti*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Slope ± SE	Intercept ± SE	χ^2
			LL	UL		LL	UL			
Aedes aegypti	Alizarin	2.05	1.71	2.84	5.59	3.69	13.77	2.9 ± 0.5	4.1 ± 0.1	3.3*
	Azadirachtin	0.58	0.51	0.65	1.25	1.12	1.44	3.8 ± 0.3	5.9 ± 0.1	3.6*
	Temephos	1.00	0.21	1.89	2.21	1.38	962.19	3.7 ± 0.7	4.9 ± 0.1	4.1*

Positive control values-Reegan *et al* 2014

*$p \leq 0.05$, level of significance of chi-square values.

In the present study, fraction 2 from the methanol extract of roots of *R. cordifolia* eluted with hexane:ethyl acetate (90:10) recorded good mosquitocidal activity against *A. aegypti* followed by fraction 19. LC_{50} and LC_{90} values for fraction 2 for third instars of *C. quinquefasciatus* and *A. aegypti* corroborated earlier findings of Muthu *et al* (2012) of the potential of plant extracts—*Clerodendrum phlomidis*—as sources of active molecules with insecticide activity against *C. quinquefasciatus* and *A. aegypti*.

The present study revealed that alizarin belongs to the anthraquinone group. Alizarin exhibited good larvicidal and pupicidal activities against both mosquito species. The activity was higher against *C. quinquefasciatus* than in *A. aegypti*. The estimated LC_{50} values for larvae and pupae of both mosquitoes are close to those reported by Han *et al* (2013) for lansiumamide B against early fourth instars of *A. albopictus*.

In conclusion, the fraction 2 and the isolated compound alizarin from methanol extract of *R. cordifolia* roots produced good activity against larvae and pupae of *C. quinquefasciatus* and *A. aegypti*. These results suggested that the isolated compound alizarin could be used in mosquito control programmes.

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