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Effects of phytochemicals on fecundity and hatchability of *Culex quinque fasciatus* and *Anopheles stephensi*

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Abstract

Culex quinque fasciatus Say. and Anopheles stephensi L. larvae were reared in de-chlorinated water at different concentrations less than the critical concentration. Inhibition of adults emergence in 50% of treated larvae (Ec_{50}) of Tephrosin (1,2a β-hydroxydeguelin) from Tephrosia purpurea L. root extract, Kaemferol (3,5,7,4 tetra hydroxyflavone) from Callistemon lanceolatus Dc. leaves extract and Cycasin (methylazoxymethanol β-D-glucoside) from Cycas revolute Thunb. Female cone extract from hatching to emergence significantly decreased the fecundity of the mosquitoes and the hatchability of their eggs. At the highest concentration of 50% Ec_{50} of the compounds reared decrease in the fecundity and hatchability over control ranged between 74.62 -85.78% and 51.87 - 65.62% respectively. Sterility index of the mosquitoes reared in the media with the different compounds at this concentration ranged between 89.12 and 95.11%.

Keywords: *Tephrosia purpurea, Callistemon lanceolatus, Cycas revoluta,* Mosquitoes, Anti-fertility, Sterility Index.

Introduction

Plant compounds producing negative effects on fecundity are very valuable for vector control as they prevent rapid buildup of population. Bioactive compounds in the neem kernel extracts have been reported to induce male sterility, oviposition repellency and suppress fecundity in *Culex tarsalis* and *Cx. quinque fasciatus*[1]. A few plant compounds present in the extracts may indirectly decrease fecundity by deterring feeding and decreasing growth. A few of them may directly influence egg production through endocrines. For instance, Aristolochic acid from *Aristolochia bracteata* is reported to induce sterility in mosquitoes[2,3].

Callistemon lanceolatus extract treated III instar larvae of *Cx. quinque fasciatus* resulted in adults with significantly smaller ovary[4,5] anti-fertility activity[6]. Leaf extract of the plant significantly affected the growth and development of *Helicoverpa armigera* larvae[7]and decreased the fecundity of mollusca[8]. *C.lanceolatus* leaves contain a volatile oil that inhibited feeding in the third instar larvae of *Spodoptera litura*[9]. Although considerable information is available on plants (*Annona squamosa* alkaloid extract) inducing sterility, anti-fertility effect in mosquitoes[10]. The lipophyllic extracts of *Tagetes* sp. (Asteraceae) inhibited emergence of treated larval population of *Aedes intrudues* have been reported[11]. Besides interfering with development, growth and emergence, any biologically active compound, which hampers fecundity and/or hatchability, would also contribute very much to check the growth of vector populations.

In the present article we evaluate the effects of three plant compounds of root fraction of *Tephrosia purpurea* L. leaf fraction of *Callistemon lanceolatus* Dc. and fraction from female cone of *Cycas revolute* Thunb. To interfere with fecundity and hatchability of two important species of mosquitoes.

Materials and Methods

Extraction and purification of active compounds: Root of *Tephrosia purpurea,* leaves of *Callistemon lanceolatus* and female cone of *Cycas revolute* were collected from Nagamalai, Madurai District, India and were identified with voucher specimen. The plant parts were dried under shade and powdered. The powdered material was weighed and extracted overnight in analytical grade methanol (MeOH) in the case of *C. lanceolatus* and *T. purpurea* and ethyl acetate (EtOAc) in the case of *C. revoluta* thrice in the ratio 1:10 W/V over a magnetic stirrer. The MeOH/EtOAc extracts were filtered and concentrated in a vacuum evaporator at 45°C under low pressure. The residues of the extracts were defatted by washing them thrice with equal volume of petroleum ether (PE) in a separating funnel. The two fractions were collected separately and concentrated to dryness.

The residue of the defatted MeOH fraction was dissolved in EtOAc and the EtOAc soluble and EtOAc insoluble fractions were separated. The residue of the defatted EtOAc fraction was redissolved in EtOAc. The EtOAc soluble fractions were washed with equal volume of double distilled water. These active fractions constituted 0.56, 3.24 and 2.5% of dry weight of

the respective starting materials. Preliminary bioassay revealed that partially purified EtOAc fractions of these extracts were more active than the other fractions. Based on the preliminary results EtOAc fractions of *T. purpurea* root, *C. lanceolatus* leaves and *C. revoluta* female cone were chosen for further purification and screening.

T. purpurea EtOAc fraction was further fractionated using semi-preparatory thin layer chromatography in glass plates. Silica gel-G served as the stationary phase. Using benzene (C_6H_6), $CHCl_3$ and MeOH in the ratio of 14:14:6 as mobile phase, the partially purified fraction was further separated. *C. lanceloatus* EtOAc fraction was further separated using analytical thin layer chromatography (Merck: 1.05554: 20 × 20cm aluminum sheet coated with 0.25mm thick silica gel-60 F_{254}).

Petroleum ether (PE), C_6H_6 and EtOAc in the ratio of 4:2:1 was used as the mobile phase and CH Cl_3, EtOAc in the ratio of 5:3 for the separation of crude EtOAc fraction of *C. revoluta*. The different fraction/compounds obtained from the plates were further screened for larvicidal activity of *Cx. quinque fasciatus*.

The compound with an Rf value of 0.239 from the EtOAc fractions of *T. purpurea* root, an Rf value of 0.941 from the *C. lanceolatus* leaf and that with an Rf value of 0.364 from the *C.revoluta* female cone displayed highest larvicidal activity. The three active plant compounds were further analyzed by GC-MS, ^{1}H NMR (400.138 MHz) and ^{13}C NMR (100.624 MHz) spectrum. The three known compounds of tephrosin (1,2a β-hydroxydeguelin), kaemferol (3,5,7,4 tetra hydroxyflavone) and cycasin (methylazoxymethanol β-*D*-glucoside) were identified with literature cited. Yield of tephrosin, kaemferol and cycasin from the crude EtOAc extract and MeOH extract respectively averaged to 0.5%, 0.4% and 0.7%. Therefore, further studies were conducted with these compounds.

Estimation of fecundity and hatchability: Egg rafts of *Cx. quinque fasciatus* and *An. stephensi* were obtained from the Center for Research in Medical Entomology, Indian Council for Medical Research, Madurai – 625 002 and hatched in enamel trays. The culture was maintained as described[12]. Fifty early III instar larvae of the selected species of mosquitoes were reared separately in 300 ml of de-chlorinated water containing different sub-lethal concentration of the active compounds of the selected plant compounds. Normal control (water alone) and methanol solvent control (water with maximum volume of methanol in the sample) were also maintained. Triplicates were maintained for control and different treatment concentrations.

The larvae were provided with dry yeast powder and dog biscuit in the ratio of 3:1. The levels of water in the test plastic cups were maintained by adding required volume of de-chlorinated water. After the larvae metamorphosed into pupae they were transferred to emergence cages (1 ft^3) and allowed to emerge. Number of males and females that emerged from different treatments and controls were counted.

The ratio between male and female was calculated. The male mosquitoes were provided with 10% sugar solution and females were provided with blood meal from an immobilized chicken kept overnight inside the cage. The cages were covered with wet cloth to maintain constant humidity (85 ± 5%). One small bowl containing de-chlorinated water was also kept inside the cage to facilitate oviposition by females.

The egg rafts/eggs oviposited by females were removed from the cage next morning, counted and allowed to hatch in enamel trays. Fecundity was monitored for 25 days. Number of eggs that successfully hatched into first instar larvae in each concentration was counted. Relating fecundity and hatchability of the treated female to those of control female, reduction in fecundity and hatchability due to treatment was calculated in percentage.

The sterility index (SI) was calculated as follows: SI = 100 - [fecundity (number of eggs in treatment × percentage of hatchability)]/[fecundity (number of eggs in control ×percentage of hatchability)] × 100. The slopes of the regressions equations and the correlation coefficients were statistically analyzed by Origin 7.5 (Origin lab scientific graph and data analysis software, Northampton, USA).

Results and Discussion

Fecundity: On an average twenty *Cx. quinque fasciatus* females in the control series deposited 12 to 25 egg rafts containing 250 to 375 eggs each in about 25 days after emergence. Average realized fecundity of a female in the control series pertaining to the tests of the three active compounds ranged from 134 to 145 eggs. Treatment of the freshly hatched *Cx. quinque fasciatus* larvae with the active compounds at 5 to 50% of Ec_{50} concentrations significantly decreased the fecundity in a dose dependent manner. For instance, fecundity of *Cx. quinque fasciatus* female treated with 10 and 50% of tephrosin decreased from143 eggs/female in the control series to 74 and 20.3 eggs/female (Table-1).

Figure-1a shows the relationship between treatment dose and average fecundity of *Cx. quinque fasciatus* treated with tephrosin. The inverse relationship between the two variables is statistically highly significant (r = - 0.958; N= 15; P< 0.01). Similar significant inverse relationship was also obtained between fecundity and treatment concentration of the females treated with kaemferol and cycasin (r = - 0.963; N= 15; P< 0.01, r = - 0.969; N= 15; P< 0.01) (Figure-1b, c).

The effect of kaemferol and cycasinon the fecundity of *Cx. quinque fasciatus* was comparatively less than that of tephrosin. For instance, at the highest tested concentration of 50% of Ec_{50}, the fecundity was decreased to 32.2 and 35.3 eggs/female treated with the kaemferol and cycasin. Whereas the decrease in the fecundity of the female treated with 50% Ec_{50} of tephrosin with that of control was 85.8% that of the female treated with

50% Ec_{50} of kaemferol or cycasin was 76.3% or 74.6% (Table-1). It is pointed out that the Ec_{50} of tephrosin(0.0075 ppm) was several folds less than those of kaemferol (1.990 ppm) and cycasin (4.478 ppm).

An. stephensi untreated female in the control series deposited 100 to 102 eggs in a period of about 25 days. Treatment with the active compounds of tephrosin, kaemferol and cycasin significantly decreased the fecundity. For instance fecundity of the female treated with 25 and 50% Ec_{50} of tephrosin compound decreased to 39 and 19 eggs/female (Table-2).

Fecundity of the females treated with 25 and 50% Ec_{50} of kaemferol and cycasin were 41 and 22 and 43 and 23eggs/female (Table-2). Comparison of the fecundity of the females treated with 50% Ec_{50} of the active compounds with that of the females in the respective control series pointed out that the decrease in the fecundity over control was (81%) far higher than those in the kaemferol (79%) and cycasin (77%) series.

Figures-2a-c show the relationship between treatment concentrations of tephrosin, kaemferol and cycasin and fecundity of *An. Stephensi* (r = - 0.958; N= 15; P < 0.01, r = - 0.960; N= 15; P< 0.01, r = - 0.968; N= 15; P< 0.01). The slopes of the regressions equations and the correlation coefficients were statistically highly significant. The negative effect of tephrosin on the fecundity of *An. stephensi* was far higher than that of kaemferol or cycasin, despite the fact that the Ec_{50} of tephrosin (0.370 ppm) was several folds less than that of kaemferol (1.505 ppm) or cycasin (4.640ppm).

Hatchability: About 91 to 92% of the eggs deposited by *Cx. quinque fasciatus* female in the control series hatched successfully. Treatment of the freshly hatched larvae from hatching to emergence at < Ec_{50} concentrations not only affected the fecundity but also the hatchability of the eggs deposited by the adults.

Like fecundity, hatchability of eggs deposited by the females treated with the active compounds also displayed significant inverse relationship with treatment concentrations (r = - 0.981; N= 15; P< 0.01, r = - 0.974; N= 15; P< 0.01, r = - 0.973; N= 15; P< 0.01) (Figures-3a to c). Treatment with 50% Ec_{50} of tephrosin, kaemferol and cycasin decreased the hatchability to 31.7, 36.2 and 37.8% respectively; these decreases were 65.6, 60.7 and 58.4% less than the hatchability in the respective control series (Table-1).

Figures-4a, c show the inverse relationship between hatchability of *An. stephensi* eggs and treatment concentrations (r = - 0.988; N= 15; P< 0.01, r = - 0.983; N= 15; P< 0.01, r = - 0.980; N= 15; P< 0.01). At the highest tested concentration (50% Ec_{50}) of tephrosin, kaemferol and cycasin, hatchability of *An. stephensi* eggs were 32.3, 39.1 and 43.9% compared with 89 to 91% hatchability observed in the control series; these decreases were 64.1, 56.5 and 51.9% less than the hatchability in the respective control series (Table-2). The negative effect of the active compounds on hatchability decreased in the order of tephrosin>kaemferol>cycasin.

Sterility Index: Consequent to the significant decrease in fecundity of the females treated with the active compounds and decrease in the hatchability of the eggs deposited by them, the active compounds rendered the *Cx. quinque fasciatus* and *An. stephensi* females sterile to various extents. Sterility index of *Cx. quinque fasciatus* and *An. stephensi* linearly increased with treatment concentration.

For instance, it increased from 33.3% for *Cx.quinque fasciatus* treated with 5% of Ec_{50} of tephrosinto 95.1% in that treated with 50% Ec_{50} (Table-1). At the highest tested concentration of kaemferol and cycasin, sterility index of *Cx. quinque fasciatus* was 90.7 and 89.5%.

In general sterility index of *An. stephensi* at any chosen treatment concentration was less than that of *Cx. quinque fasciatus* indicating that the sterilizing effect of the compounds on *Cx. quinque fasciatus* was more than that on *An. stephensi.* At 5% Ec_{50} of tephrosin, kaemferol and cycasin, sterility indices of *An. stephensi* were 29.1, 26.3 and 23.5% respectively. These values increased to 93.3, 90.7 and 89.0% respectively in those treated with 50% Ec_{50} of the active compounds (Table-2). Briefly, the active compounds of all the tested extracts significantly decreased the fecundity and hatchability of the *Cx. quinque fasciatus* and *An. stephensi.*

Discussion: *T. purpurea, C. lanceolatus* and *C. revoluta* compounds exerted dose dependent negative effect on fecundity of females that emerged from larvae treated with the compounds; in addition hatchability of these fewer eggs deposited was also very low. Decrease in fecundity and hatchability of *Cx. quinque fasciatus* and *An. stephensi* treated with tephrosin, kaemferol and cycasinat respective 50% Ec_{50} concentrations has been compared with the data reported for different plants[13].

It is pointed out that despite the fact that 50% Ec_{50} concentration of tephrosinfor *Cx. quinque fasciatus* is several folds lesser than that of the plants reported, its negative effect on fecundity and hatchability of *Cx. quinque fasciatus*is significantly greater than that of *Rhinacanthus nasutus* extract. Crude extract of seeds of *Millettia dura* in chloroform was highly toxic (24 hr Lc_{50}: 3.5ppm) to second instar *Aedes aegypti* larva[14].

The rotenoids, deguelin and tephrosin, isolated from the seeds of *Millettia dura* also showed potent mosquitocidal activities[14]. The negative effects of tephrosin and kaemferol compounds of present study are greater than those of *Eichhornia crassipes, Ageratum conyzoides, Cleome icosandra, Imperata cylindrica* and *Annona squamousa*[9,16,17]. Irrespective of the species of mosquito tested tephrosin and kaemferol affected the fecundity and hatchability to a considerable extent.

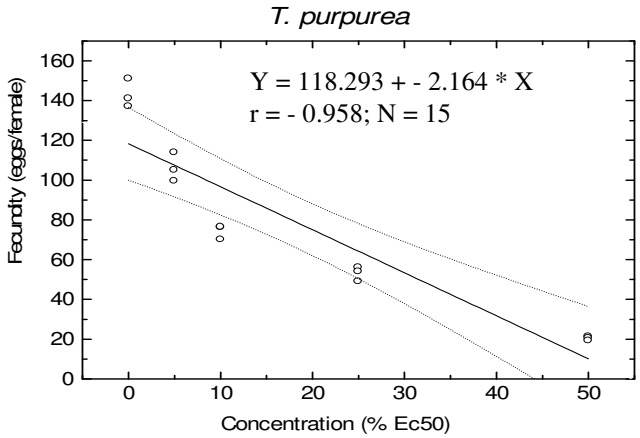
Table-1
Fecundity and hatchability of *Cx. quinque fasciatus* treated with sub-lethal concentrations of the tephrosin (a), kaemferol (b) and cycasin (c). The treatment lasted from commencement of early III instar larva to emergence. 20 males and 20 females that emerged from each treatment were allowed to mate and deposit eggs in breeding cage. Each value represents the mean (X ± SD) of three observations

Concentration			Fecundity	Hatchability	Decrease over control (%)		Sterility Index
	%Ec_{50}	ppm	(eggs/female)	(%)	Fecundity	Hatchability	(SI)
a	0	0	143.00 ± 5.89	92.40 ± 2.35	--	--	--
	5	0.0004	106.17 ± 5.98	82.71 ± 1.08	25.76	10.48	33.54
	10	0.0008	74.25 ± 3.01	74.23 ± 2.52	48.08	19.66	58.28
	25	0.0019	53.00 ± 2.94	46.02 ± 0.48	62.94	50.20	81.54
	50	0.0038	20.33 ± 0.82	31.70 ± 3.26	85.78	65.62	95.11
b	0	0	135.92 ± 7.45	92.32 ± 2.12	--	--	--
	5	0.100	106.58 ± 4.70	84.54 ± 0.74	21.58	7.34	27.34
	10	0.199	82.17 ± 4.44	76.18 ± 1.88	39.55	17.47	50.11
	25	0.498	59.92 ± 2.43	48.25 ± 6.56	55.92	47.73	76.96
	50	0.995	32.17 ± 3.02	36.24 ± 3.16	76.33	60.75	90.71
c	0	0	137.05 ± 6.52	91.15 ± 2.71	--	--	--
	5	0.236	111.00 ± 4.10	87.66 ± 0.85	19.01	3.83	22.11
	10	0.478	85.88 ± 1.24	76.75 ± 2.90	37.33	15.80	47.24
	25	1.195	63.10 ± 1.08	50.57 ± 6.37	53.96	44.52	74.46
	50	2.390	34.78 ± 0.62	37.85 ± 5.13	74.62	58.47	89.46

Table-2

Fecundity and hatchability of *An.stephensi* treated with sub-lethal concentrations of the tephrosin (a), kaemferol (b)andcycasin (c). The treatment lasted from commencement of early III instar larva to emergence. 20 males and 20 females that emerged from each treatment were allowed to mate and deposit eggs in breeding cage. Each value represents the mean (X ± SD) of three observations

Concentration			Fecundity	Hatchability	Decrease over control (%)		Sterility
	%Ec_{50}	ppm	(eggs/female)	(%)	Fecundity	Hatchability	Index SI)
a	0	0	101.02 ± 4.17	90.07 ± 0.89	--	--	--
	5	0.019	78.28 ± 4.80	82.45 ± 3.76	22.50	8.46	29.06
	10	0.037	56.02 ± 1.38	73.94 ± 1.25	44.55	17.91	54.48
	25	0.093	38.65 ± 1.03	50.06 ± 0.71	61.74	44.43	78.74
	50	0.185	19.00 ± 1.54	32.31 ± 0.97	81.19	64.12	93.25
b	0	0	101.55 ± 3.72	89.78 ± 0.89	--	--	--
	5	0.076	80.98 ± 3.58	82.97 ± 2.54	20.25	7.58	26.30
	10	0.151	58.25 ± 1.92	75.92 ± 1.29	42.64	15.44	51.50
	25	0.377	40.83 ± 1.94	52.97 ± 0.24	59.79	41.00	76.28
	50	0.753	21.78 ± 0.93	39.08 ± 4.18	78.55	56.47	90.66
c	0	0	99.57 ± 2.88	91.17 ± 0.50	--	--	--
	5	0.232	82.08 ± 4.05	84.59 ± 3.02	17.56	7.22	23.51
	10	0.464	60.62 ± 2.10	78.80 ± 0.67	39.12	13.57	47.38
	25	1.160	42.90 ± 2.11	54.77 ± 2.35	56.91	39.92	74.11
	50	2.320	22.5 ± 1.23	43.88 ± 1.22	77.40	51.87	89.12



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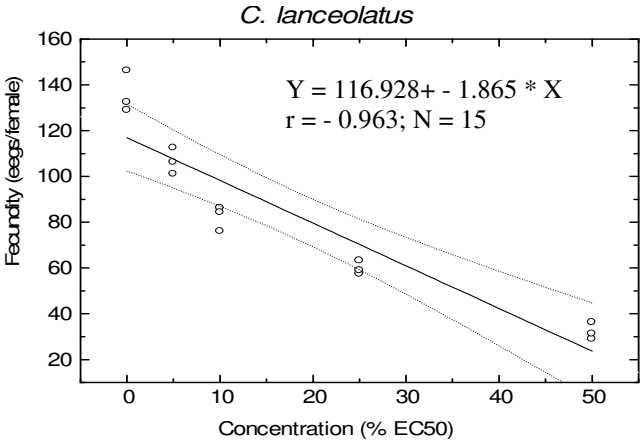

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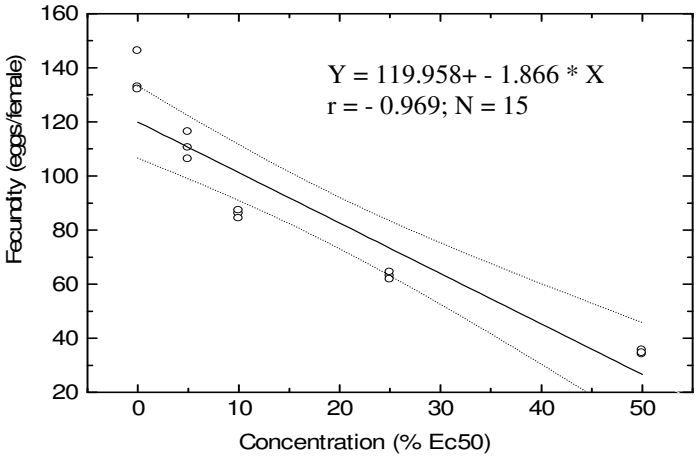
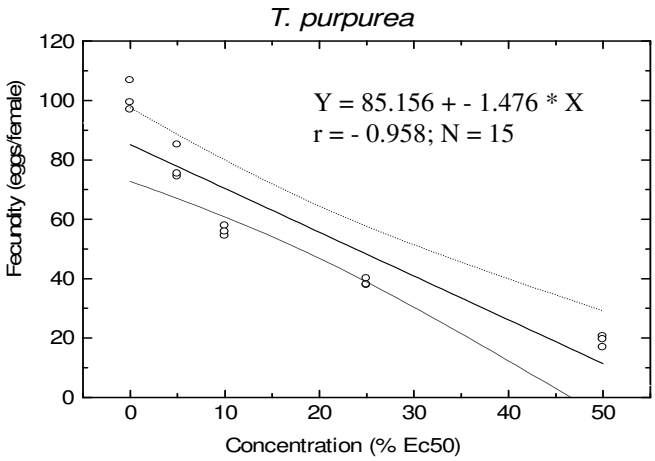


c

Figures-1a–c

Fecundity (eggs/female) of *Cx. quinque fasciatus* as functions of concentrations of active compounds of selected plants. The lines were drawn using regression equations shown in the figures. Broken lines indicate 95% fiducial limits



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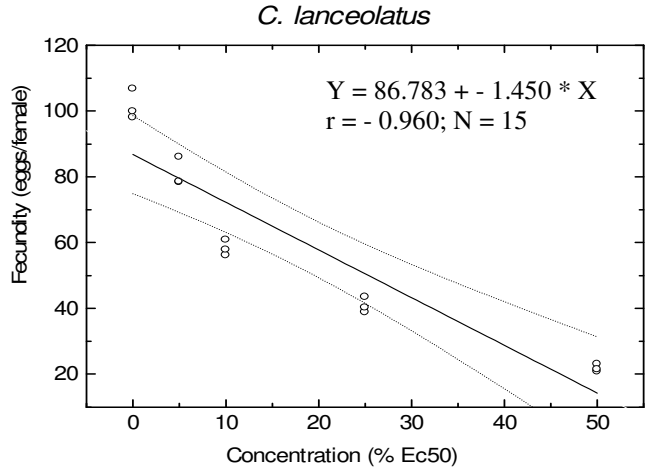

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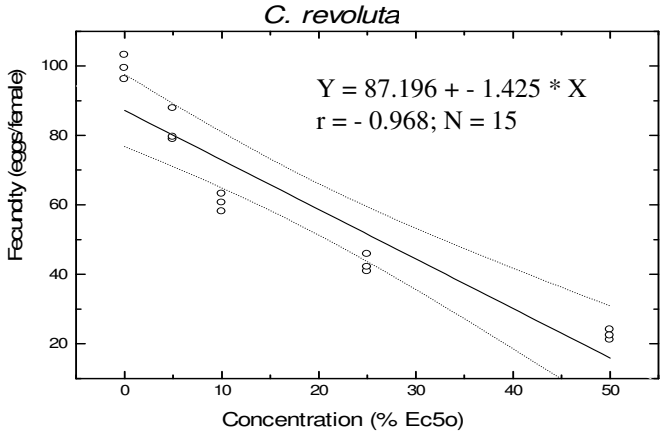
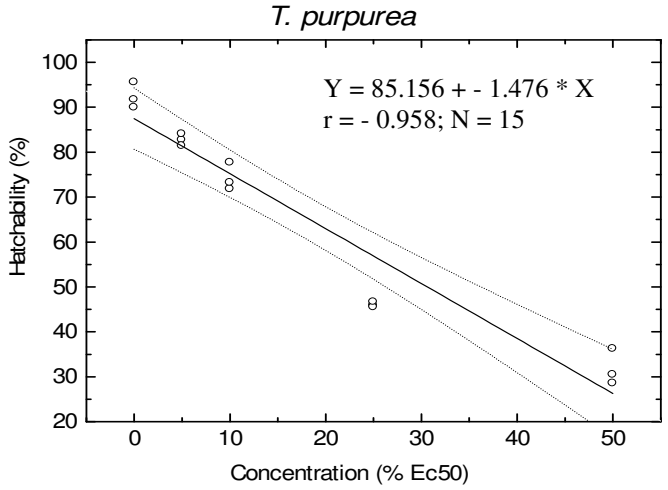


c

Figures-2a–c

Fecundity (eggs/female) of *An. stephensi* as functions of concentrations of active compounds of selected plants. The lines were drawn using regression equations shown in the figures. Broken lines indicate 95% fiducial limits



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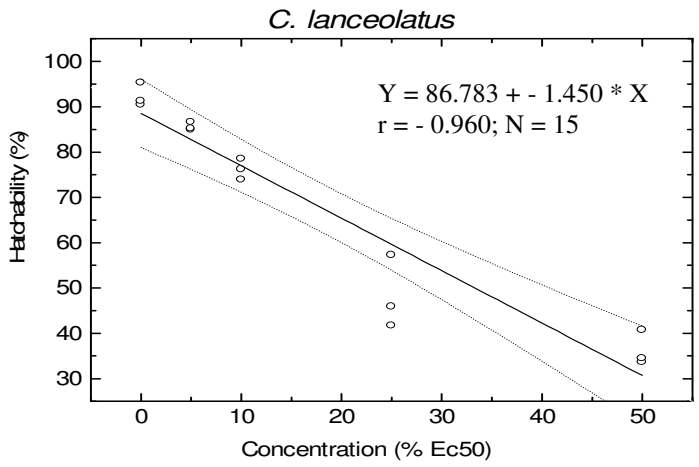

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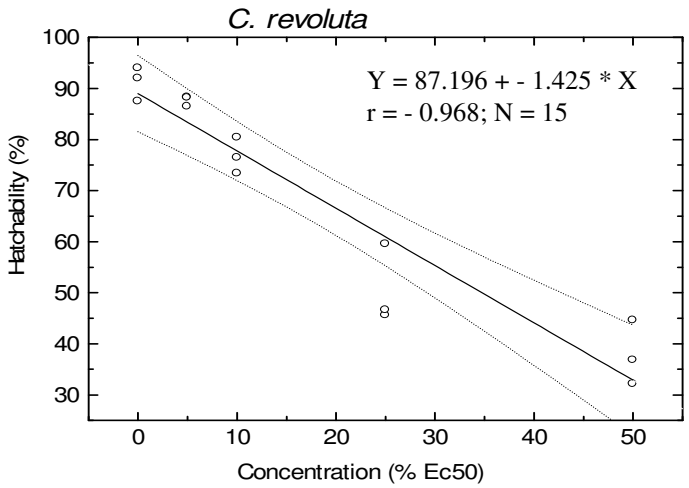
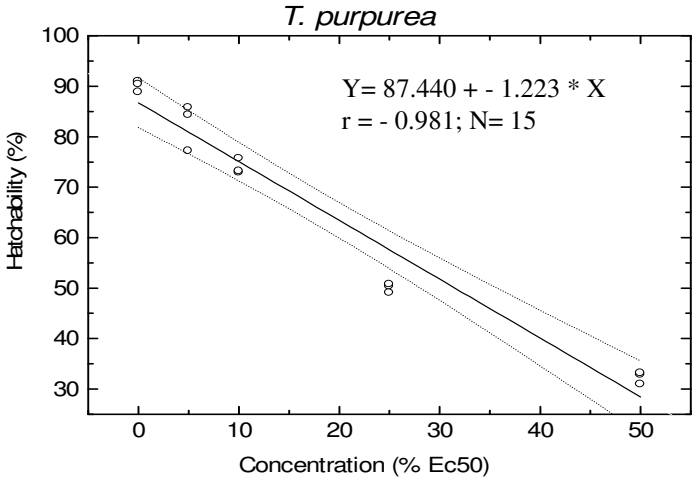


c

Figures-3a–c

Hatchability (%) of *Cx. quinque fasciatus*as functions of concentrations of active compounds of selected plants. The lines were drawn using regression equations shown in the figures. Broken lines indicate 95% fiducial limits



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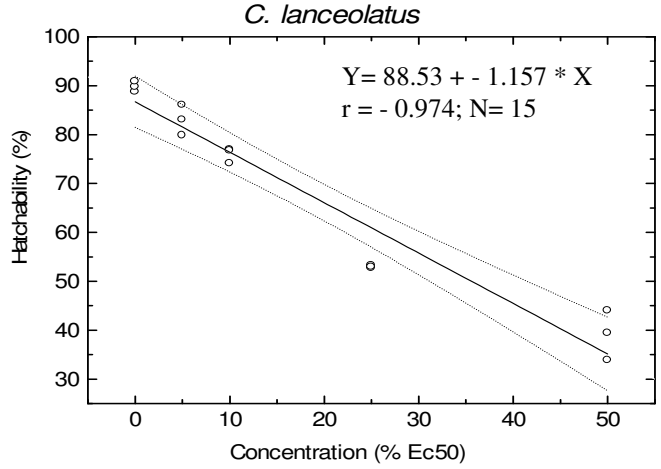

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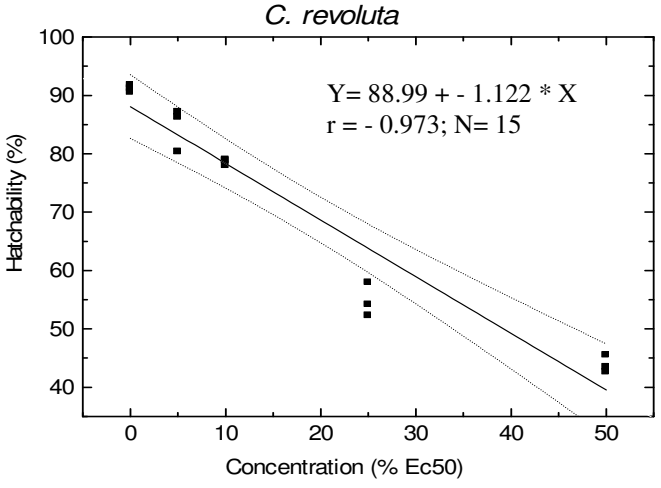

c

Figures-4a–c

Hatchability (%) of *An. stephensi* as functions of concentrations of active compounds of selected plants. The lines were drawn using regression equations shown in the figures. Broken lines indicate 95% fiducial limits

Discussion: It is pointed out that despite the fact that 50% Ec_{50} concentration of tephrosinfor *Cx. quinque fasciatus*is several folds lesser than that of the plants reported, its negative effect on fecundity and hatchability of *Cx. quinque fasciatus*is significantly greater than that of *Rhinacanthus nasutus* extract. Crude extract of seeds of *Millettia dura* (Leguminosae) in chloroform was highly toxic (24 hr Lc_{50}: 3.5ppm) to second instar *Aedes aegypti* larva[14]. The rotenoids, deguelin and tephrosin, isolated from the seeds of *Millettia dura* also showed potent mosquitocidal activities[14]. The negative effects of tephrosin and kaemferol compounds of present study are greater than those of *Eichhorniacrassipes, Ageratum conyzoides, Cleome icosandra, Imperatacylindrica* and *Annona squamousa*[9,16,17]. Irrespective of the species of mosquito tested tephrosin and kaemferol affected the fecundity and hatchability to a considerable extent.

The insecticidal activity of *Kunzea ambigua* and *Kunzea baxterii* belonging to Family Myrtaceae to a mixture of flavonone isomers attributed[18]. The presence of C-methylated flavones such as 3-methyl-tetradec-2-en-7-ol,5-hydroxy-7,4'-dimethoxy-6-methylflavone, 5-hydroxy-7,4'-dimethoxy-6,8-dimethylflavone, 5-hydroxy-3,8,4'-trimethoxy-6-C-methylflavone in *C. lanceolatus* and other members of genus *Callistemon* were reported[19-20]. Essential oils plants such as *Eucalyptus* sp. belonging to family Myrtaceae are useful as mosquito repellents also emphasize the role of flavones for insect control[21,22] and antifeedant activity against *Spodoptera litura*[9]. Therefore, the anti-fertility effects of kaemferol observed in the present study have been attributed to the presence of insecticidal compounds such as C-methylated flavones.

Although the effects of cycasin are not as high as that of tephrosin and kaemferol, they are not closer to those of *C.inophyllum* seed extract[13]. The effects of tephrosin, kaemferol and cycasinon *An. stephensi* are more or less similar to the trends described above for *Cx. quinque fasciatus*. Consequent to the negative effects of the compounds on fecundity and hatchability, fertility of the female is very much affected. The compounds rendered the treated mosquitoes sterile to different extent. Sterility indices of *Cx. quinque fasciatus* and *An. stephensi* treated with 50% Ec_{50} of tephrosin, kaemferol and cycasin ranged 89.0 - 95 and are comparable with the range of 86 to 90% for the various plants reported.

Conclusion

Very little information is available on insecticidal activity of plants belonging to Family Cycadaceae or its repellency to mosquitoes known from ethnopharmacological reports. Cycasin and neocycasin present in the seeds of *Cycas* are toxic to insects[23]. Mosquitocidal activity of cycasin has been attributed to these chemicals in *C. revolute* female cone. The anti-fertility activity of cycasin from EtOAc extracts of *C. revoluta* cone reported in the present study is lower than that of many other plants. Briefly, the active compounds tested in the present study against *Cx. quinque fasciatus* and *An. stephensi* inhibited a greater percentage of the treated larvae from emerging as adults, extended their developmental duration and rendered them sterile to a greater extent. Therefore, these compounds hold a promising role in mosquito control programs.

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