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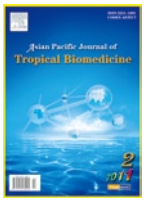

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Synergistic effect of *Croton caudatus* (fruits) and *Tiliacora acuminata* (flowers) extracts against filarial vector *Culex quinquefasciatus*

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ABSTRACT

Objective: To investigate the synergistic effect of crude and solvent extract of *Croton caudatus* (*C. caudatus*) (fruits) and *Tiliacora acuminata* (*T. acuminata*) (flowers) against the larval form of *Culex quinquefasciatus* (*Cx. quinquefasciatus*). **Methods:** Crude and solvent [chloroform: methanol (1:1 v/v), benzene and ethyl acetate] extracts of two plants, *C. caudatus* (fruits) and *T. acuminata* (flowers) were examined separately against filarial vector *Cx. quinquefasciatus* larvae with gradually increasing concentration *i.e.* from 0.1% to 0.5% of crude extract and 25 ppm to 75 ppm of solvent extracts. To observe the synergistic effect, if any, extracts of these two plant parts were mixed at different concentrations and treated against mosquito larvae. Phytochemical analyses of extracts of both the plant parts were carried out. **Results:** In a 72-h bioassay experiment with plant extracts, highest mortalities were recorded at 0.5% (crude) and 75 ppm (solvent) concentration for fruits of *C. caudatus* and flowers of *T. acuminata* individually. For synergistic effect, only 0.2% of the mixture of these two crude extracts and 75 ppm concentration of chloroform: methanol (1:1 v/v) and ethyl acetate extracts showed 100% mortality after 24 h and 48 h of exposure respectively. **Conclusions:** In the field of mosquito control, insecticides of plant origin may serve as suitable alternative to the toxic chemicals. Some secondary metabolites in combination may be responsible for better larvicidal activity.

1. Introduction

Mosquito, the living dipteran creature is the important vector of many of the vector borne diseases having the potentiality to kill more than a million victims annually around the world[1]. In tropical developing countries the pathogen of filariasis is transmitted by *Culex quinquefasciatus* (*Cx. quinquefasciatus*) mosquito. *Cx. quinquefasciatus* is widely distributed in tropical and subtropical countries infecting around 120 million people, of which 40 million people show chronic manifestation of lymphatic filariasis[2]. To reduce the incidence of different mosquito borne diseases such as malaria, filariasis, Japanese encephalitis, dengue *etc*., regulation of mosquito population is a very important and effective step.

Mosquito in the larval stage is attractive target for control activity because they breed in aquatic ecosystem and are easy to control in this habitat. But over and injudicious application of persistent synthetic insecticides, such as DDT, resulted in accumulation of non-biodegradable chemical in ecosystem, biomagnifications through food chain, development of insecticide resistance among vector population and toxic effect in human health and other non-target organisms. Persistent exposure of pesticides causes adverse effect on human health, such as immune dysfunction, cancer and birth defect[3]. Now the top priority in finding a new insecticide is to see whether it is biodegradable and does not have any ill effect on non-target organisms[4]. The use of easily biodegradable phytochemicals is one of the best alternatives for mosquito control programmes because they do not have any adverse effect on natural ecosystem[5,6]. In the present study an attempt was made to establish the synergistic effect of crude and solvent extracts [chloroform: methanol (1:1 v/v), benzene and ethyl acetate] of two plant parts of *Croton caudatus* (*C. caudatus*) (fruits) and *Tiliacora acuminata* (*T. acuminata*) (flowers) against *Cx. quinquefasciatus* larvae because some time individual plant extract are active only at high concentration which are economically

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less beneficial for field use. Phytochemical analyses of both plant parts were also done in search of bio-active compound responsible for mosquito larvicidal properties. The effect of appropriate lethal concentration of mixed crude and solvent extracts were also studied on non-target organisms such as *Diplonychus annulatum* (*D. annulatum*) (Heteroptera: Belostomatidae) and *Chironomus circumdatus* (*C. circumdatus*) (Diptera: Chironomidae).

2. Materials and methods

2.1. Rearing of mosquito larvae

The present study was conducted at Burdwan (23° 16′, 87° 54′) West Bengal, India during July and August 2010. *Cx. quinquefasciatus* eggs were collected from drains surrounding the university campus and reared in mosquito research laboratory of Burdwan University, Burdwan, West Bengal, India. The larvae were fed with artificial food (powder of dig biscuits and dried yeast powder in the ratio of 3:1). They were maintained at (33 ± 1) ℃ with 85% relative humidity.

2.2. Preparation of crude phyto-extract

Fresh mature fruits of *C. caudatus* (voucher No. 114) and flowers of *T. acuminata* (voucher No. 115) were randomly harvested from the bank of the river Ganga, at Hooghlyghat, Hooghly, West Bengal, India. The harvested plants were identified properly. The fruits of *C. caudatus* and flowers of *T. acuminata* were initially rinsed with tap water followed by distilled water and dried on paper towel. The crude phyto extract was prepared by grinding the fruits of *C. caudatus* and flowers of *T. acuminata* in mortar and pestle individually and filtered through Whatman No.1 filtered paper. Clear filtrate was used as stock solution for bioassay experiments. Different concentrations of crude extract were prepared by mixing the stock solution with variable amounts of distilled water.

2.3. Preparation of plant extracts in different solvent systems

Fresh mature fruits of *C. caudatus* and flowers of *T. acuminata* were harvested, rinsed with distilled water and dried in shed. Dried materials of both plants were put in different Soxhlet apparatus and the plant extracts were prepared using three solvents, *viz.* benzene, ethyl acetate and chloroform: methanol (1:1) applying one after another (extraction period 72 hour for each solvent and the temperature was < 40 ℃). The column of the Soxhlet apparatus after each type of solvent extraction procedure was washed with distilled water and similar solvent as an eluent. Each extract was concentrated by evaporation in a rotary evaporator. The solid residues of the extracts was weighed and then dissolved in suitable amount of sterilized distilled water for the preparation of graded concentrations.

2.4. Larvicidal bioassay

The larvicidal bioassay was done by following the World Health Organization standard protocol with suitable modification[7]. Five concentrations of crude phyto-extracts of *C. caudatus* fruits and *T. acuminata* flowers (0.1% to 0.5%) were individually transferred into sterile glass Petri dishes (9 cm diameter and 150 mL capacity). Ten first, second, third and fourth instars larvae of *Cx. quinquefasciatus* were separately treated with each concentration. Ten larvae of different instars were introduced separately in petri-dishes containing gradually increasing concentration of crude extract (0.1% to 0.2%) of *C. caudatus* fruits and *T. acuminata* flowers mixed at different ratio. Similar types of bioassay were conducted with chloroform: methanol (1:1 v/v), benzene and ethyl acetate solvent (concentrations of 25, 50 and 75 ppm) extract of *C. caudatus* (fruit) and *T. acuminata* (flower) individually and their combined form on the 3rd instar larval form of the mosquito. Mortality rates were recorded after 24, 48 and 72 h of exposure and data of mortality at 48-h and 72-h were expressed by addition of mortality at 24 -h and 48 -h, respectively. The dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region. The experiments were replicated three times on three different days and conducted under laboratory condition at (33 ± 1) ℃ and 85% relative humidity.

2.5. Effect on non- target organisms

The effect of the crude and solvent extract of both the plant parts were tested against two non-target organisms *e.g. D. annulatum* and *C. circumdatus* which are usually present in the same habitat of mosquito larvae. Both the insects were exposed to individual and combined concentration of plant extracts which is similar to its LC_{50} value at 24 h against *Cx. quinquefasciatus* larvae. The mortality and other abnormalities was observed up to 72 h of exposure.

2.6. Phytochemical analysis of plants extract

Phytochemical analyses of extracts of both the plant parts were carried out according to the method of Harborne and Stahl to get an assumption on active ingredient responsible for larval mortality[8,9]. The phytochemicals include under study were saponins, terpenoids, flavonoids, cardiac glycosides, free amino acid and glycoside bound anthroquinone.

2.7. Determination of synergistic factors (SF)

Synergistic factor = LC_{50} value of the insecticide alone/

LC_{50} value of the insecticide with the assumed Synergist = LC_{50} value of the individual plant extract/ LC_{50} value of the combined plant extracts or assumed synergist[10].

If the value of SF > 1, so indicates synergism, or SF <1, indicates antagonism. The different value of SF was tabulated.

2.8. Statistical analysis

Statistical analyses of the experimental data were performed using the computer software "STAT PLUS 2007 (trial version)" and "MS EXCEL 2002" to find out the LC_{50} and LC_{90} lethal values, regression equations and regression co efficient values.

3. Results

Means of three experiments on larval mortality of different instars exposed separately to *C. caudatus* fruits and *T. acuminata* flowers (crude extracts of different concentrations) are presented in Table 1. Mean larval mortality (three experiments) of different instars exposed to 1:1 and other different combinations of crude extracts of *C. caudatus* fruits and *T. acuminata* flowers when applied at different concentrations are presented in Table 2 and 3 respectively.

Results of log-probit analysis (at 95% confidence level) revealed that LC_{50} values gradually decreased with the exposure period having the lowest value at 0.2% combined extracts at 1:1 ratio to third-instars larvae followed by second and fourth instars larvae after 72-h exposure period. The rate of mortality (Y) was positively correlated with the concentration (X) of the plant extract as evident from established regression equations (Table 4). The values of SF of crude extract from Table 5 indicate that all results

Table 1
Mortality of larvae of *Cx. quinquefasciatus* exposed separately to *T. acuminata* flower and *C. caudatus* fruit.

Plant extrat	Conc. (%)	1st Instars			2nd Instars			3rd Instars			4th Instars		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
TA	0.1	4.33±0.33	5.67±0.33	6.33±0.33	4.67±0.33	5.33±0.67	7.00±0.00	4.33±0.33	6.67±1.33	8.00±0.58	2.67±0.33	5.67±0.33	6.33±0.67
	0.2	5.33±0.67	5.67±0.67	6.33±0.67	5.33±0.67	5.67±0.33	7.67±0.67	6.33±0.67	7.33±0.33	7.67±0.33	3.67±0.89	5.67±0.33	7.00±0.00
	0.3	6.67±0.67	8.00±1.00	8.00±0.00	6.67±0.33	7.67±1.20	8.33±0.33	6.33±0.33	7.67±0.33	8.33±0.33	5.33±0.33	6.33±0.33	7.00±0.58
	0.4	7.00±0.58	7.33±0.67	8.33±0.33	7.33±0.67	7.67±0.89	9.33±0.33	7.33±0.67	8.67±0.67	9.33±0.33	5.33±0.67	6.33±0.67	8.33±0.33
	0.5	7.67±0.33	9.00±0.00	9.33±0.67	7.67±0.33	8.00±0.00	9.67±0.33	8.00±0.58	9.33±0.33	10.00±0.00	6.67±0.33	8.33±0.33	9.67±0.33
CC	0.1	3.33±0.33	4.33±0.67	5.00±0.00	2.67±0.33	4.67±0.33	6.67±0.67	2.67±0.67	6.00±1.00	8.33±0.33	3.33±0.33	5.67±0.67	7.00±0.00
	0.2	3.67±0.33	5.67±0.33	7.67±0.67	3.67±0.67	5.67±0.67	8.00±1.00	3.33±0.67	6.67±0.33	8.33±0.33	4.00±0.58	5.67±0.67	7.33±0.33
	0.3	5.00±0.00	6.33±0.67	7.67±0.33	5.00±0.58	6.33±0.67	8.33±0.67	5.67±0.67	7.33±0.33	8.33±0.33	3.67±0.33	5.00±0.00	8.67±0.67
	0.4	7.00±0.58	9.33±0.33	9.33±0.33	5.00±0.58	8.00±0.00	9.33±0.33	5.67±0.89	8.00±0.58	8.67±0.67	5.00±1.15	7.33±0.89	8.33±0.33
	0.5	7.33±0.33	8.33±0.33	9.33±0.33	6.33±0.67	8.33±0.67	9.67±0.33	7.67±0.33	8.67±0.33	9.33±0.33	5.67±0.67	8±0.58	9.67±0.33

TA- *T. acuminata*, CC- *C. caudatus*; Conc.- Concentration. Values are expressed as Mean ± SEM.

Table 2
Mortality of *Cx. quinquefasciatus* larvae exposed to mixture (1:1) of crude extracts of *T. acuminata* flower and *C. caudatus* fruit (Mean ± SEM).

Conc. (%)	1st instars			2nd instars			3rd instars			4th instars		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
0.1	5.67±0.33	7.00±0.58	9.00±0.00	6.00±0.58	7.33±0.33	8.67±0.33	7.00±0.58	9.00±0.58	9.67±0.33	7.00±0.58	7.67±0.33	9.00±0.58
0.2	9.00±0.58	9.67±0.33	10.00±0.00	9.33±0.33	10.00±0.00	10.00±0.00	9.67±0.33	10.00±0.00	10.00±0.00	9.00±0.58	9.33±0.33	10.00±0.00

Table 3
Mortality of *Cx. quinquefasciatus* larvae exposed to combined percentage crude extract of *T. acuminata* flower and *C. caudatus* fruit (Mean ± SEM).

Conc. (%) TA+CC	1st instars			2nd instars			3rd instars			4th instars		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
0.25+0.75	5.67±0.33	7.00±0.00	8.67±0.67	5.67±0.67	6.67±0.67	8.33±0.67	7.00±0.00	7.33±0.33	7.67±0.33	4.33±0.33	6.33±0.67	7.33±0.67
0.04+0.16	5.67±0.33	6.33±0.33	9.00±0.58	6.33±0.33	7.00±0.58	8.67±0.33	7.33±0.33	7.67±0.33	8.67±0.33	4.67±0.67	7.00±0.58	7.67±0.89
0.10+0.10	9.00±0.58	9.67±0.33	10.00±0.00	9.33±0.33	10.00±0.00	10.00±0.00	9.67±0.33	10.00±0.00	10.00±0.00	9±0.58	9.33±0.33	10.00±0.00

TA- *T. acuminata*, CC- *Croton caudatus*.

Table 4
Log–probit analysis and regression analysis of larvicidal activity of *T. acuminata* flower crude extract, *C. caudatus* fruit crude extract and combined crude extract on different instars larvae of *Cx. quinquefasciatus* (*n* = three experiments).

Plant extracts	Instars	Hour	Regression equation	LC_{50}	R^2
C. caudatus	1st instars	24 h	Y=11.333X + 1.8667	0.2257	0.857
		48 h	Y=11.667X + 3.3	0.1461	0.724
		72 h	Y=10.333X + 4.7	0.1006	0.755
	2nd instars	24 h	Y=8.6667X + 1.9333	0.3257	0.668
		48 h	Y=9.6667X + 3.7	0.1325	0.745
		72 h	Y=7.3333X + 6.2	0.0742	0.545
	3rd instars	24 h	Y=12.333X + 1.3	0.2626	0.736
		48 h	Y=6.6667X + 5.3333	0.0691	0.571
		72 h	Y=2.3333X + 7.9	0.0018	0.170
	4th instars	24 h	Y=5.6667X + 2.6333	0.4327	0.380
		48 h	Y=6.3333X + 4.4333	0.0977	0.384
		72 h	Y=6.3333X + 6.3	0.0484	0.654
T. acuminata	1st instars	24 h	Y=8.333X + 3.7	0.1467	0.685
		48 h	Y=8.333X + 4.6333	0.1013	0.552
		72 h	Y=8X + 5.2667	0.0828	0.702
	2nd instars	24 h	Y=8X + 3.9333	0.1395	0.702
		48 h	Y=7.3333X + .6667	0.1167	0.451
		72 h	Y=7X + 6.3	0.0558	0.750
	3rd instars	24 h	Y=8.3333X + 3.9667	0.1312	0.656
		48 h	Y=6.6667X + 3.9667	0.0548	0.460
		72 h	Y=5.6667X + 6.9667	0.0352	0.628
	4th instars	24 h	Y=9.6667X + 1.8333	0.2934	0.720
		48 h	Y=6X + 4.6667	0.0827	0.547
		72 h	Y=8X + 5.2667	0.0754	0.702
Combined crude extract	1st instars	24 h	Y=45X + 4.2333	0.022	0.6956
		48 h	Y=38.333X + 5.5	0.012	0.6679
		72 h	Y=18.333X + 8.3	0.005	0.4201
	2nd instars	24 h	Y=43.333X + 4.5333	0.019	0.6680
		48 h	Y=43.333X + 5.4	0.014	0.7042
		72 h	Y=21.667 + 7.766	0.005	0.4356
	3rd instars	24 h	Y=35X + 6.0333	0.010	0.6764
		48 h	Y=35X + 6.5	0.011	0.6806
		72 h	Y=28.333X + 7.3667	0.009	0.7448
	4th instars	24 h	Y=61.0667X + 2.7667	0.031	0.8188
		48 h	Y=36.667X + 5.4667	0.013	0.6368
		72 h	Y=33.333X + 6.4667	0.009	0.5618

Combined crude extrat– *T. acuminata* flower and *C. caudatus* fruit.

Table 5
Estimation of SF of *T. acuminata* flower and *C. caudatus* fruit crude extract against the combined form.

Instars	*C. caudatus*			*T. acuminata*		
	24 h	48 h	72 h	24 h	48 h	72 h
1st instar larvae	10.25	11.68	17.96	6.66	8.10	14.78
2nd instar larvae	17.14	9.07	14.84	7.34	7.99	11.16
3rd instar larvae	24.00	6.28	0.18	12.03	4.98	3.66
4th instar larvae	13.82	7.34	4.88	22.56	6.21	7.61

Table 6
Mean mortality of *Cx. quinquefasciatus* larvae exposed to different concentration of *C. caudatus* and *T. acuminata* solvents extract (chloroform: methanol, ethylacetate and bengene). Each solvent extract of both plant were applied individually and 1:1 combination at different concentrations.

Plant	Conc. (ppm)	Chloroform: methanol extract			Ethylacetate extract			Benzene extract		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
CA	25	2.33±0.33	3.33±0.33	4.33±0.33	1.33±0.33	2.33±0.33	3.00±0.58	0.33±0.33	1.33±0.33	1.67±0.33
	50	7.00±0.58	7.33±0.33	7.67±0.33	1.67±0.33	2.67±0.67	3.67±0.33	3.33±0.33	3.67±0.33	4.33±0.33
	75	8.67±0.33	9.00±0.00	9.67±0.33	4.67±0.33	5.67±0.33	6.33±.33	5.67±0.33	6.67±.33	7.33±0.33
TA	25	1.67±0.89	2.33±0.33	3.67±0.33	1.33±0.67	1.67±0.33	2.67±0.33	0.00±0.00	0.00±0.00	0.00±0.00
	50	4.00±0.58	4.67±0.33	6.00±0.58	4.67±0.67	5.33±0.89	6.67±0.33	0.00±0.00	0.00±0.00	0.33±0.33
	75	6.33±0.33	6.67±0.33	8.00±0.58	5.67±0.33	7.33±0.33	8.67±0.33	0.33±0.33	0.67±0.33	0.67±0.33
CC: TA (1:1)	25	6.33±0.67	7.67±0.33	8.33±0.33	4.33±0.33	4.67±0.33	5.67±0.33	0.00±0.00	0.00±0.00	0.33±0.33
	50	9.00±0.00	10.00±0.00	10.00±0.00	7.67±0.33	8.67±0.33	9.67±0.33	1.33±0.33	1.67±0.33	2.33±0.33
	75	10.00±0.00	10.00±0.00	10.00±0.00	10.00±0.00	10.00±0.00	10.00±0.00	2.67±0.33	3.33±0.33	3.67±0.33

CC- *C. caudatus*, TA- *T. acuminata*.

Table 7
Estimation of SF of *T. acuminata* flowers and *C. caudatus* fruits solvent extracts (Chloroform: methanol, (1:1/v: v) at 24 h of exposure against third instars larvae of *Cx. quinquefasciatus*.

Solvent used	LC_{50} of *C. caudatus*	LC_{50} of *T.acuminata*	LC_{50} of combined extract	SF value against *C. caudatus*	SF value against *T.acuminata*
Chloroform: methanol, (1:1/v:v)	49.49	96.48	22.11	2.23*	4.36*
Ethyl acetate	98.05	60.34	28.68	3.41*	2.10*
Benzene	66.51	67.67	104.03	0.63**	0.65**

* synergism, ** antagonism.

Table 8
Qualitative analysis of secondary phytochemicals of *Tiliacora acuminata* flowers and *Croton caudatus* fruits.

Plants	Plant parts used	Saponin	Tanin	Flavonoid	Alkaloid	Steroid	Terpenoid	Cardiacglycoside	Freeboundamino acids
T. acuminata	Flower	++	++	++	--	++	--	++	++
C. caudatus	Fruit	--	--	--	++	++	--	++	--

show synergism because their SF values were greater than 1 except *C. caudatus* fruits against third instars larvae at 72-h of post- exposure.

Mean mortalities (three experiments) due to the application of chloroform: methanol (1:1) extracts of above mentioned two plant parts applied individually and combinedly (1:1) against third instars larvae are presented in Table 6. Similarly, mortalities for ethyl acetate and benzene extracts are also presented in Table 6. The value of SF of three solvent extracts (chloroform: methanol (1:1), ethyl acetate and benzene) are presented in Table 7.

The result of preliminary phytochemical analysis of the *C. caudatus* fruits and *T. acuminata* flowers showed the presence of some secondary phytochemicals (Table 8). No change in the survival rate and swimming activity of the non-target organisms, *e.g. D. annulatum* and *C. circumdatus*, were observed within 72-h of post exposure to 0.5% crude extract of individual plants and 0.2% combined form.

4. Discussion

From ecological point of view, insecticides of plant origin are efficient, biodegradable as well as suitable alternative for mosquito control. Shaalan *et al*[11] reviewed on different mosquito larvicidal plant species with growth retarding, reproduction inhibiting, ovicides, synergistic, additive and antagonistic action of botanical mixture. Several secondary metabolites produced by some plants for their own defense from their enemies have good mosquito larvicidal activity, such as titerpenes, Isoflavonoids, tannins, saponine, phenolics *etc*[16-22].

In the present study crude extracts of *C. caudatus* fruits and *T. acuminata* flowers showed highest mortality at 0.5% concentration when applied separately. 100% mortality was recorded when 0.2% crude mixed extract of *C. caudatus* fruits and *T. acuminata* flowers at 1:1 combination was applied and it was found to be the best combination when compared to other combinations. Second and third instars larvae of *Cx. quinquefasciatus* are more susceptible to crude extract mixture (1:1) of *C. caudatus* fruits and *T. acuminata* flowers

because 100% mortalities were recorded at 48 h of exposure. 100% mortality was recorded at 75 ppm when mixture (1:1) of chloroform: methanol (1:1) extract of *C. caudatus* fruits and *T. acuminata* flowers was applied to third instars larvae of *Cx. quinquefasciatus* indicating synergistic effect. Similar result was observed in case of ethyl acetate extracts. But antagonistic activity was noted with benzene extract. If we see the spectrum of secondary phytochemicals six and three such chemicals are present in *T. acuminata* flowers and *C. caudatus* fruits respectively but only steroids and cardiac glycosides are present in both plant parts. Some secondary metabolites in combination may be responsible for better effect of larvicidal activity. In conclusion, the combined form of *C. caudatus* fruits and *T. acuminata* flowers show good bioactive potentiality against *Cx. quinquefasciatus* larvae due to synergism of plant extracts. No mortality was recorded in the non target organisms *i.e.* the plant extract is ecologically safe to use in the field condition.

Conflict of interest statement

We declare that we have no conflict of interest.

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