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Mosquito Larvicidal Potentiality of *Holoptelea integrifolia* Leaf Extract against Japanese Encephalitis Vector, *Culex vishnui* Group

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Abstract Vector control is facing a threat due to the emergence of resistance to synthetic insecticides. Insecticides of botanical origin may serve as suitable alternative in future. *Holoptelea integrifolia* distributed in many parts of India is a medicinal plant. During the present study, larval mortality of *Culex vishnui* group was observed after 24 h, 48 h and 72 h of exposure with five concentrations of crude extract (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) and four concentrations (100 ppm, 200 ppm, 300 ppm and 400 ppm) of acetone extract of leaf of the plant. Respective lethal concentrations were determined by log-probit analysis (at 95% confidence level). Effects of acetone extract of *H. integrifolia* leaves were tested against non-target predatory fishes and insect larvae. During the present study, the mortality rates of all larval instars at 0.5% concentration were significantly higher ($p < 0.05$) than at 0.1%, 0.2%, 0.3% and 0.4% concentrations of crude leaf extract. Highest mortality was observed at 400 ppm concentration of acetone extract. Phytochemical analysis of crude extract revealed the presence of tannin, saponin, steroid and phenol as major bioactive secondary metabolites. No nontarget mortality was observed.

Keywords Japanese encephalitis; *Culex vishnui* group; *Holoptelea integrifolia*; Larvicidal activity

Introduction

Japanese encephalitis (JE) is a disease caused by a flavivirus that affects the membranes around the brain. Fatality rate can be as high as 60% among those with disease symptoms; 30% of those who survive suffer from lasting damage to the central nervous system. The virus causing Japanese encephalitis is transmitted by mosquitoes belonging to *Culex vishnui* group *(Cx. tritaeniorhynchus, Cx. vishnui* and *Cx. pseudovishnui)* which breed exclusively in flooded rice fields in India and other tropical countries (Hati, 1981). Through different strategies have been developed to reduce the incidence of mosquito borne disease through out the world, the only efficacious approach of minimizing the incidence of these diseases is to control mosquito population by application of insecticides at larval habitats. Mosquitoes in the larval stage are attractive target for control operation due to their low mobility in the breeding habitats and are easy to control them in these habitats (Howard et al., 2007). In recent years, the top priority in finding a new insecticide is that, they must be of plant origin and does not have any ill effect on ecosystem such as biomagnifications through food chain, development of insecticides resistance, toxic effect in human and other non target organisms (Redwane et al., 2002).

Holoptera integrifolia belonging to the family Urticaceae is a deciduous tree, found throughout world. Petroleum ether, benzene, chloroform, methanol and aqueous extracts of bark of this plant were evaluated for anti microbial activity (Paarakh et al., 2011). The methanolic extracts of *H. integrifolia* leaves and stem bark were studied for the wound-healing and antioxidant potential (Srinivas et al., 2008). The antitumour and antidiarrhoeal activities of ethanol extract of leaves of *H. integrifolia* (EHI) have been evaluated (Lakshmi et al., 2010; Sharma et

al., 2009). The objective of the present study was to observe mosquito larvicidal activity of crude and acetone extracts of *H. Integrifolia* leaves against *Cx. vishnui* group. The qualitative and chromatographic analyses of the acetone extract of mature leaves were also done to isolate the active principle responsible for larval mortality. The effect lethal concentrations were also tested against non target organisms like *Gambusia affinis*, *Poecilia reticulata* (predatory fishes), *Diplonychus annulatum* (predatory water-bug) and *Chironomus circumdatus* larvae (insect).

1 Result and Discussion

Results of the present study indicate that the mortality rate of all larval instars of *Cx. vishnui* group at 0.5% concentration was significantly higher ($p < 0.05$) than the mortality rates at 0.1%, 0.2%, 0.3% and 0.4% concentrations of crude plant extract at 24, 48 and 72 hours of exposure (Table 1). Higher mortality rate was also recorded in 72 h bioassay than those in 24 h and 48 h. The results of regression analysis revealed that the mortality rate (Y) was positively correlated with the period of exposure (X) having a regression coefficient close to one in each case (Table 2). The results of log probit analysis (95% confidence level) revealed that LC_{50} values gradually decreased with the exposure period (Table 2).

The result of all instars larval mortality with acetone extracts was presented in (Table 3). Higher mortality was observed at 400 ppm concentration of acetone extract than the mortality rates at 100 ppm, 200 ppm and 300 ppm concentrations. LC_{50} value and regression analysis of acetone extract are presented in Table 4.

The result of preliminary phytochemical analysis of the crude or acetone extract of the mature leaves showed the presence of tannin, saponin, steroid and phenol as major phytochemicals and absence of free glycoside-bound anthraquinones, terpenoid, cardiac glycosides, alkaloid and flavonoid (Table 5).

When the isolated compounds from the TLC plates were further bioassay against the third-instars larvae, the mortality was recorded in the compound Rf=11/14 which are presented in Table 6. IR analysis of active compound and its respective functional group is shown in Figure 1 that proves that several functional groups are associated with the active principle. Solvent extract did not cause any mortality of the non-target organisms; even no abnormality was noted among them.

Mosquito control is mostly directed against larvae and only against adults when necessary. Larval control can be an effective control tool due to the low mobility of larval mosquitoes, especially where the principal breeding habitats are artificial and can be easily identified (Lee, 2000). From ecological point of view, insecticides of plant origin are efficient biodegradable as well as suitable alternative to synthetic products for mosquito control activity. Botanicals have prime importance over synthetic chemicals as these are target specific, cheap and easily available. Secondary metabolites such as alkaloids (Carvalho et al., 2003), steroids (Ghosh et al., 2008; Chowdhury et al., 2008a), phenolics (Tripathi et al., 2001), triterpenes (Rahuman et al., 2008), protein (Chowdhury et al., 2008b) etc. have good mosquito larvicidal properties.

In conclusion it is the first report of *H. integrifolia* as larvicide of *Cx. vishnui* group, crude and acetone extract can be effectively used. The plant extracts were safe to those non-target organisms that share the same habitat of *Cx. vishnui* group of mosquito larvae. Further study is needed to know the chemical structure of the active principle involved in larvicidal activity.

Table 6 Efficacy of *H. integrifolia* leaf acetone extract TLC fraction with Rf 11/14 value on third instar larvae of *Cx. vishnui* group at different concentrations.

Concentrations (ppm)	Mortality rate (%, Mean±standard errors)		
	24h	48h	24h
10	27.78±1.11	32.22±1.11	41.11±2.22
15	54.45±2.22	60.00±5.09	72.22±2.22
20	74.44±2.22	88.89±1.11	100.00±0.00

Table 1 Efficacy of *H. integrifolia* leaf crude extract at different concentration on different larval instars forms of *Cx. vishnui* group

Larval instars	Concentrations (%)	Mortality rate (%, Mean±standard errors)		
		24h	48h	72h
First	0.1	5.56±1.11	7.78±1.11	12.22±1.11
	0.2	17.78±1.11	24.44±2.22	30.00±0.00
	0.3	46.67±1.92	51.11±1.11	58.89±1.11
	0.4	64.44±1.11	68.89±1.11	78.89±1.11
	0.5	93.30±3.33	97.78±1.11	100.00±0.00
Second	0.1	4.44±1.11	6.67±0.00	11.11±1.11
	0.2	18.89±1.11	25.56±1.11	28.89±1.11
	0.3	44.44±1.11	48.89±2.94	55.56±1.11
	0.4	62.22±1.11	68.89±2.22	77.78±1.11
	0.5	90.00±5.09	98.98±1.11	100.00±0.00
Third	0.1	3.33±0.00	4.44±1.11	8.89±1.11
	0.2	18.89±2.22	26.67±1.93	27.78±2.22
	0.3	42.22±1.11	47.78±2.22	55.56±2.94
	0.4	62.22±2.22	68.89±1.11	75.56±2.94
	0.5	92.22±2.22	100.00±0.00	100.00±0.00
Fourth	0.1	2.22±1.11	4.44±1.11	7.78±1.11
	0.2	15.56±1.11	21.11±2.22	26.67±1.92
	0.3	40.00±1.92	44.44±1.11	54.45±2.22
	0.4	58.89±1.11	65.55±2.22	74.45±2.22
	0.5	88.89±1.11	97.78±1.11	100.00±0.00

Table 2 Log probit analysis and regression analysis of larvicidal activity of *H. integrifolia* leaf crude extract against different larval instars forms of *Cx. vishnui* group

Larval instars	Period of bioassay (h)	LC_{50} (% extract)	LC_{90} (% extract)	Regression equations	R value
First	24	0.2983	0.5820	Y=222.22X+21.109	0.9780
	48	0.2713	0.5460	Y=224.44X－17.332	0.9885
	72	0.2374	0.4905	Y=224.45X－11.335	0.9940
Second	24	0.3075	0.6100	Y=214.44X－20.334	0.9774
	48	0.2736	0.5342	Y=227.77X－18.553	0.9875
	72	0.2445	0.5002	Y=226.67X－13.334	0.9954
Third	24	0.3097	0.5876	Y=221.12X－22.559	0.9824
	48	0.2765	0.5168	Y=233.34X－20.446	0.9894
	72	0.2521	0.4999	Y=230.00X－15.444	0.9885
Fourth	24	0.3267	0.6075	Y=216.00X－23.891	0.9816
	48	0.2920	0.5449	Y=231.12X－22.669	0.9824
	72	0.2580	0.5005	Y=232.22X－16.998	0.9916

Table 3 Efficacy of *H. integrifolia* leaf acetone extract at different concentration on different larval instars forms of *Cx. vishnui* group

Larval instars	Concentrations (ppm)	Mortality rate (%, Mean ± standard errors)		
		24h	48h	72h
First	100	17.78±1.11	21.11±1.11	26.67±1.92
	200	31.11±1.11	38.89±1.11	45.56±1.92
	300	42.22±1.11	53.33±1.92	57.78±1.11
	400	67.78±2.22	75.56±1.11	84.44±1.11
Second	100	12.22±1.11	17.78±1.11	22.22±1.11
	200	30.00±1.92	37.78±2.22	38.89±1.11
	300	38.89±2.22	51.11±2.22	57.78±2.22
	400	62.22±2.22	77.78±1.11	91.11±2.22
Third	100	17.78±2.22	22.23±1.11	24.44±2.94
	200	28.89±2.22	35.56±1.11	41.11±2.22
	300	42.22±1.11	45.56±1.11	51.11±2.94
	400	75.56±2.94	91.11±2.22	100.00±0.00
Fourth	100	12.22±1.11	16.67±1.93	23.33±1.93
	200	26.67±1.93	28.89±1.11	37.78±2.22
	300	36.67±1.93	47.78±2.22	53.33±1.93
	400	67.78±1.11	71.11±2.22	83.33±1.93

Table 4 Log probit analyses and regression analysis of larvicidal activity of *H. integrifolia* leaf acetone extract against different larval instars forms of *Cx. vishnui* group

Larval instars	Period of bioassay (h)	LC_{50} (% extract)	LC_{90} (% extract)	Regression equations	R value
First	24	303.2008	1166.6181	Y=0.1611X－0.5533	0.9498
	48	241.1423	844.6025	Y=0.1778X+2.7767	0.9839
	72	201.9232	680.1275	Y=0.1856X+7.2233	0.9706
Second	24	336.4225	1190.386	Y=0.1589X－3.8941	0.9514
	48	247.5779	758.1138	Y=0.1933X－2.2217	0.9710
	72	210.3340	557.8027	Y=0.2256X－3.8900	0.9616
Third	24	283.7960	932.3710	Y=0.1867X－5.5560	0.9069
	48	230.2690	661.3331	Y=0.2167X－5.5560	0.8695
	72	202.2580	521.9790	Y=0.2367X－5.0017	0.8729
Fourth	24	327.1664	1034.2895	Y=0.1767X－8.3367	0.9276
	48	283.7680	922.1800	Y=0.1822X－4.4433	0.9647
	72	226.1716	722.4882	Y=0.1956X+0.5550	0.9496

Table 5 Qualitative phytochemical analysis of plant (*H. integrifolia)* leaf crude extract

Tannin	Saponin	Steroid	Phenol	Terpenoid	Cardiac glycosides	Alkaloid	Free glycoside-bound Anthraquinones	Flavonoid
++	++	++	++	–	–	–	–	–

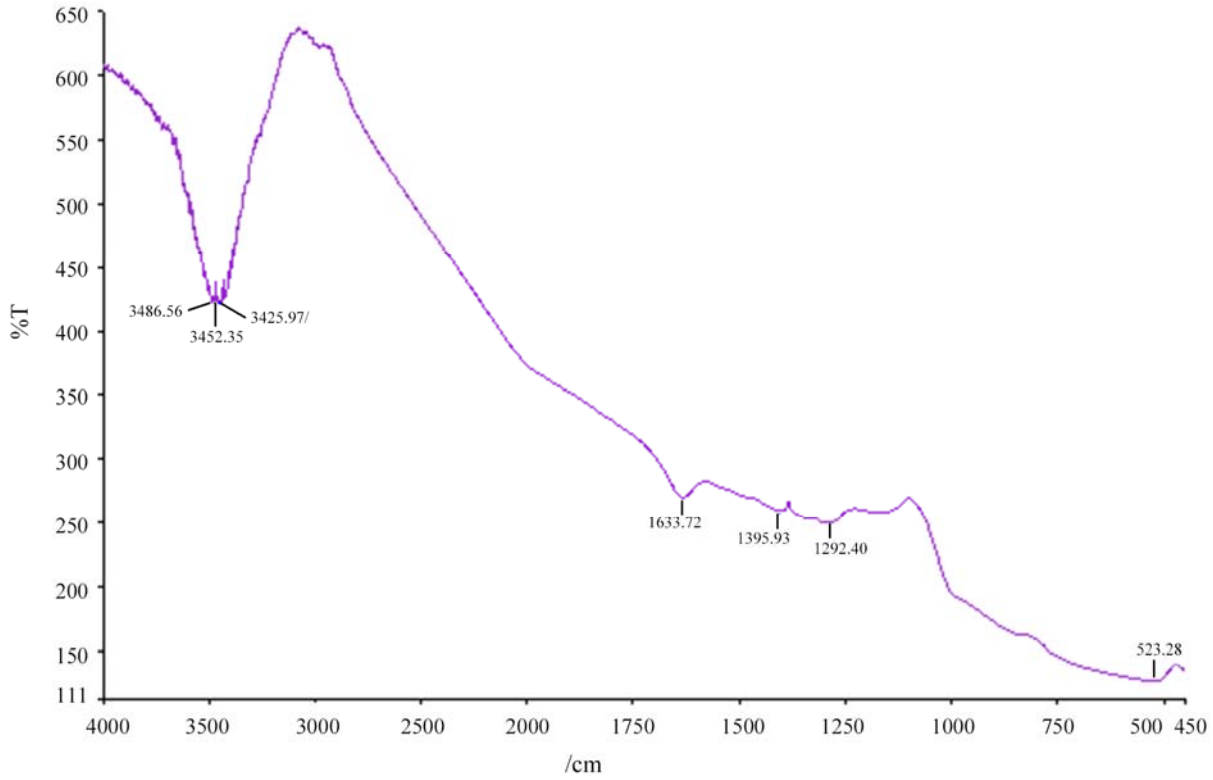

Figure 1 Interpretation of IR spectra of the compound having Rf 0.785

Note: Frequency range and probable functional groups of the compound (Rf 0.785): 3452.35/cm alcohol, amine, phenol; 3486.56/cm alcohol, phenol; 3425.97/cm alcohol, phenol; 1633.72/cm amide, amine; 1395.93/cm carboxylic acids, sulfate; 1292.40/cm alkyl halides, amine, carboxylic acids, ester; 523.28/cm alkyl halides, disulfide linkage.

2 Materials and Methods

2.1 Preparation of crude extract

Fresh mature and green leaves of *H. integrifolia* were randomly harvested from Debipur, Burdwan district, west Bengal, India and the voucher specimen was deposited in the herbarium of the department of Zoology, the University of Burdwan (voucher no: 119). Leaves were initially rinsed with distilled water and dried on paper towel. Crude extract of plant leaves were prepared in an electric blender and the plant juice was filtered by passing through the Whatman no. 1 filter paper. The filtrate was used as stock solution and required concentration (0.1%, 0.2%, 0.3%, 0.4%, and 0.5%) were prepared through mixing of stock extract with variable amount of distilled water.

2.2 Preparation of solvent extract

Semidried leaves were at first crushed by hand and then ground by an electronic blender. The dried leaves were put in a Soxhlet apparatus and the plant extract was prepared using solvent namely acetone. The solid residue of extract was used for preparation of graded concentrations of 100 ppm, 200 ppm, 300 ppm and 400 ppm.

2.3 Mosquito culture

The present study was conducted at Mosquito and Microbiology Research Units, Department of Zoology, The University of Burdwan, Burdwan (23°16′N, 87°54′E) West Bengal, India. Larvae of *Cx. vishnui* group were collected from rice fields surrounding the university campus and the larval colony was maintained in laboratory condition for further bioassay experiments. Larvae of mosquito were fed with artificial food i.e. mixture of dog biscuits and dried yeast powder at the ratio of 3:1. Colonies were kept free from exposure to pathogen, insecticides or repellents.

2.4 Larvicidal bioassay

The bioassay experiments were conducted according to

standard WHO procedure (1981) with slight modifications. During experiment with crude and acetone extract, all larval instars of *Cx. vishnui* group were used. The larvae were put in glass Petri-dishes (9 cm diameter/150 ml capacity) containing 100 ml of tap water. Five concentrations of crude extract (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) and four concentrations of solvent extract (100 ppm, 200 ppm, 300 ppm and 400 ppm) were applied into separate Petri-dishes with three replications to study the rate of larval mortalities. Tap water was used in the control treatment without any extract. No food was provided to the larvae during experimental procedure. Larval mortalities were recorded after 24 h, 48 h and 72 h of exposure. The data of mortality in 48 h and 72 h were expressed by the addition of the mortality at 24 h and 48 h, respectively.

2.5 Effect on non-target organisms

The effect of acetone extract of *H. integrifolia* leaves were tested against non-target organisms like *Gambusia affinis*, *Poecilia reticulata* (predatory fishes), *Diplonychus annulatum* (predatory water-bug) and *Chironomus circumdatus* larvae (insect). These were collected from field and maintained for past few days in cemented tanks in the laboratory. The predators were exposed to appropriate lethal concentrations (LC_{50}) of solvent extract for 24 h to observe the mortality and other abnormalities such as sluggishness and reduced swimming activity up to 72 h of exposure.

2.6 Phytochemical analysis

Phytochemical analysis of acetone or crude extract of *H. integrifolia* leaves was carried out according to the methods of Harbone (1984) and Stahl (1989) to get an assumption of active ingredient responsible for larval mortality.

2.7 Isolation and IR analysis of the active ingredient

The acetone stock extract of mature leaves of *H. integrifolia* was further chromatogrammed and each of the spot was separately scrapped from TLC plates according to their ratio of front (i.e. Rf, 11/14 or 0.785) values. Each spot represents separate phytochemical. From 30 plates chromatogrammed each spot with similar Rf (0.785), values was combined and dissolved in absolute alcohol. The silica gel was dissolved in alcohol. Now the solid fraction containing active ingredient was deposited at the bottom of beaker. When the absolute alcohol was evaporated, the dried compound was measured and dissolved in required amount of distilled water for bioassay experiment. The spot (Rf 11/14) that showed positive response in larval mortality of bioassay experiments underwent (IR) spectroscopy analysis using KBr plates (JASCO FT_IR Model-420) with a scanning speed of 2 mm/s.

Author's Contributions

SS performed the larvicidal bioassay with crude and solvent extracts. UA carried out the isolation of active ingredient and statistical analysis. AG performed the IR analysis of the active ingredient and spectral analysis. GC supervised the whole work and prepared the manuscript. All authors read and approved the final manuscript.

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