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LARVICIDAL ACTIVITY OF SILVER NANOPARTICLE SYNTHEZED BY THE LEAF EXTRACTS OF *ADHATODA VASICA* AGAINST *CULEX QUINQUEFASCIATUS* (SAY) (DIPTERA: CULICIDAE).

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ABSTRACT

In the present study the Larvicidal activity of Silver nanoparticle synthezed by the leaf extracts of Adhatoda vasica against Culex quinquefasciatus was carried out in the laboratory conditions. The result of the present study indicated that the LC_{50} values of the methanolic leaf extract of A. vasica along with silver nanoparticle was 450.03 ppm against late third larva of Cx. quinquefasciatus. The LC_{90} and regression equation were 870.80 ppm, Y=05.98+0.22X. The 95% lower and upper confidence limit of LC_{50}, LC_{90} (LCL-UCL) were (435.44-603.05) and (792.21-958.25) ppm respectively. The chi –square value 0.30 was significant at p<0.05 level. However, LC_{50} value of crude obtained by Soxhlet extraction showed higher larval mortality. The Soxhlet method to be more effective for extraction of larvicidal components. Some time the Hot extraction does not show any appreciable mortality even at 250 ppm concentration. Further, in the present study, the methanolic leaf extract of A. vasica along with silver nanoparticle was significantly inhibits the adult emergence. The percentage of adult emergence inhibition were 08.0±1.3, 14.0±1.3, 30.0±1.5, 45.5±1.5, 58.0±3.2, 76.0±2.4 and 96.0±2.3 % at different concentration

viz., 250, 500, 750, 1000, 1,250,1,500 and 1,750 ppm against the larvae of Cx.quinquefasciatus. The LI_{50} value was 623.12 ppm and the EI_{90} value was 1254.35. The regression equation was Y=12.15+0.615X. The 95% lower and upper confidence limit of LC_{50}, LC_{90} (LCL-UCL) were (510.23-750.40) and (1115.15-1452.05) ppm respectively. The chi –square value 0.03.65 was significant at p<0.05 level.

Key word: Larvicidal activity, silver nanoparticles, leaf extracts, A*dhatoda vasica, culex quinquefasciatus.*

Introduction

Mosquitoes are an ancient group of insects, which have persisted for millions of years. Human malaria is transmitted only by females of the genus *Anopheles*. Of approximately 430 species of *Anopheles*, only 30-40 transmit malaria in nature (National Centre for Infectious Diseases (NCID) (2004). The diseases spreading vectors are: *Culex*, the ordinary mosquitoes found in houses, carrier of encephalitis and filariasis in tropical and sub-tropical climates, with life cycle of 10-14 days. *Aedes* is responsible for Yellow fever, Dengu fever, Encephalitis, etc., with life cycle of about 10 days to one month that affect millions of people worldwide (WHO, 1996). In general, *Cx. quinquefasciatus* breeds in water polluted with organic debris such as rotting vegetation, breeding in pit latrines and blocked drains and ditches.

It is very clear that over two billion people in tropical countries are at risk from mosquito borne diseases such as dengue fever, haemorrhagic fever, malaria and filariasis. The search for effective vaccines against these diseases is still in progress. *Cx. quinquefasciatus* (Say.) acts as a vector for filariasis in India. Human filariasis is a major public health hazard and remains a challenging socioeconomic problem in many of the tropical countries (WHO, 1975). Lymphatic filarasis caused by *Wuchereia bancrofti* and transmitted by mosquito *Cx. quinquefasciatus* is found to be more endemic in the India and its subcontinent. It is reported that *Cx. quinquefasciatus* infects more than 100 million individuals worldwide annually (National Centre for Infectious Diseases (NCID) (2004). *W. bancrofti* is the most predominant filarial nematode, which is usually characterized by progressive debilitating swelling at the extremities, scrotum or breast (elephantiasis) in an infected individual (WHO, 1996).

One of the approaches for control of mosquito-borne diseases is the interruption of disease transmission by either killing, preventing mosquitoes to bite human beings (by using repellents) or by causing larval mortality in a large scale at the breeding centres of vectors. Plant products have been used traditionally by human communities and application of easily degradable plant compounds is considered to be one of the safest methods of control of insect pests and vectors (Alkofahi *et al.* 1989). Many medicinally important plant extracts have been studied for their efficacy as mosquito agent against different species of vector mosquitoes (Nandita *et al.* 2008). Not only can medicinal plant extracts be effective but also they may greatly reduce the risk of adverse ecological effects and do not induce pesticide resistance in mosquitoes. Therefore, the present study had been carried out to evaluate the larvicidal activity with *Adhatoda vasica* leaf extracts along with silver nanoparticles against the filarial vector *Culex quinquefasciatus (*Say).

Development of resistance by pests and vectors against the botanicals has not been reported (Sharma *et al.* 1995) because botanical insecticides are generally pest specific, readily biodegradable, target specificity, lower bioaccumulation and lack toxicity to higher animals (Sharma *et al.* 1989). Many medicinally important plant extracts have been studied for their efficacy as mosquito agent against different species of vector mosquitoes (Saxena *et al.* 1993). Not only can medicinal plant extracts be effective but also they may greatly reduce the risk of adverse ecological effects and do not induce pesticide resistance in mosquitoes. More than 2000 plant species have been known to produce chemical factors and metabolites of value in pest control programmes. Members of the plant families-Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Miliaceae, Oocystaceae and Rutaceae have various types of larval, adulticidal or repellent activities against different species of mosquitoes (Shaalan *et al.* 2005).

According to the latest WHO statistics, the parasitic disease caused by mosquitoes infects from 300 to 500 million persons per year in the world and kills more than a million and a half each year, mainly African Children. Control measures used against mosquitoes include elimination of breeding sites, application of surface films of oil to clog the breathing tubes of wrigglers and the use of larvicides. Many strains of the mosquito are resistance to conventional insecticides. From the foregoing account, it is very clear that the larvicidal activity of Silver Nanoparticle Synthezed by the leaf extracts of *A. vasica* against *Cx. quinquefasciatus* is essentially unstudied. Therefore, the present study was conducted to evaluate the larvicidal

activity of crud extract of *A. vasica* along with mixture of Silver nanoparticle against the larvae of *Cx. quinquefasciatus*.

Materials and Methods

In the present study the Larvicidal activity of Silver nanoparticle synthezed by the leaf extracts of *Adhatoda vasica* against *Culex quinquefasciatus* was carried out in the laboratory conditions.

Collection of plant materials

The plant *A. vasica* leaf (Plate I) was collected from in and around the Arignar Anna Government College, Musiri, Tiruchirappalli district of Tamil Nadu. The plant was taxonomically identified at the Department of Botany, Arignar Anna Government College, Musiri and the voucher specimen was preserved at the Department of Zoology, Arignar Anna Government College, Musiri.

Preparation of plant extract

The leaves of *A. vasica* were carefully examined and old leaf, insect damaged, and fungus infected leaves were carefully removed. The fresh and healthy leaves were washed with tap water and shade dried at room temperature (27-31°C) for 5 to 10 days or until they broke easily by hand. Once completely dry, plant material (1.0 kg) was ground to a fine powder using electrical blender (Plate III). The methanol was used for the extraction of 1.0 kg in the Soxhlet apparatus (Plate I) followed by the standard procedure (Vogel, 1978). The plant material was loaded in the inner tube of the Soxhlet apparatus and then fitted into a round bottomed flask containing ethanol. The solvent was boiled gently (40°C) over a heating mantle using the adjustable rheostat. The extraction was continued until complete extraction was effected (8 hrs) and the solvent was removed at the reduced pressure with the help of rotary vaccum evaporator to yield a viscous dark green residue (12.5 g) of leaf extracts.

Preparation of Silver Nanoparticle

0.017 mg of Silver nitrate was mixed with 100 ml of distilled water.

Laboratory colonization of Mosquitoes

The egg rafts of *Cx. quinquefasciatus* were collected in locally in drainage of Musiri town (Plate II). The mosquito colony maintained at 70-85% RH, 28±2ºC temperature and 14:10 light and dark photoperiod cycle. The larvae were fed on powdered mixture of dog biscuits and yeast tablets in 3:1 ratio. The blood meal was given to the female adult mosquitoes (Plate II) and 5.0% glucose solution and honey were given to the male adult mosquitoes.

Test for larvicidal activity

Testing of the plant extracts along with silver nanoparticle for larvicidal activity was carried out at different concentration by preparing the required stock solution by following the standard procedure (WHO, 1996). The desired concentration of the test solution (Plate III) was achieved by adding 1.0 ml of an appropriate stock solution to 249 ml of dechlorinated water. Six replicates for each concentration were maintained (Plate III). Six test tubes were taken and 0.5, 1.0, 1.5, 2.0 and 2.5 ml of silver nanoparticle were introduced in each tube. Then twenty numbers of late third larvae were introduced into the beaker were obtained from the laboratory colony (Plate III). Acetone was used as control. The larval mortality in both treated and control was recorded after 24 hrs and the percentage of mortality was calculated using Abbott's formula (Abbott, 1925).

$$\% \text{ Mortality} = \frac{\text{Mortality at treatment} - \text{Mortality at control}}{100 - \text{Mortality at control}} \times 100$$

The statistical evaluation of LC_{50}, LC_{90}, regression equation and 95 percent confidence limit LCL and UCL were calculated from the data, which was carried out by Probit analysis (Finney, 1971).

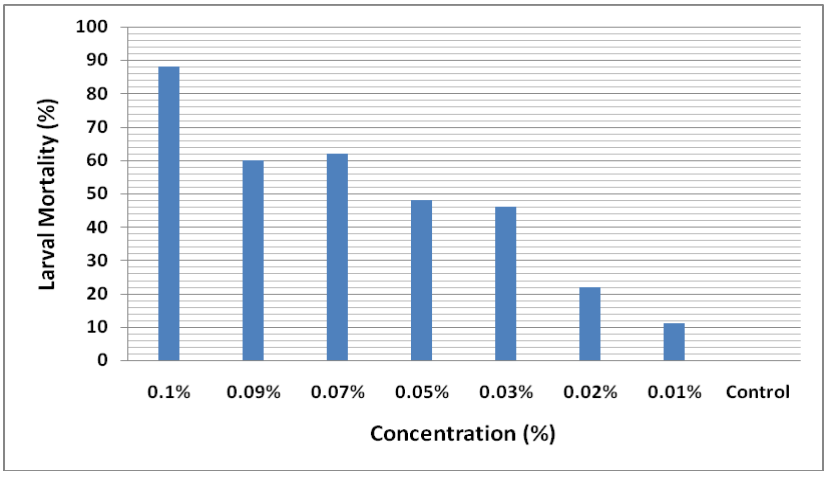
Observations and Result

The result of the larvicidal activity of methanolic extract of leaf of *Adhatoda vasica* along with silver nanoparticle against larval mosquitoes of *Culex quinquefasciatus* is presented in Table 1, 2 and 3 ; Fig 1.

Table. 1 Larvicidal activity leaf extract of *Adhatoda vasica* along with silver nanoparticle against *Culex quinquefasciatus* in 24 hrs.

% solution	Mortality of larva in 24 hrs (Replica N-5)					Average	% Mortality
	1	2	3	4	5		
0.1 %	15	18	20	20	14	17.6	88 %
0.09 %	12	14	12	12	10	12.0	60 %
0.07 %	14	11	13	09	15	12.4	62 %
0.05 %	10	10	10	09	09	09.6	48 %
0.03 %	08	10	10	09	09	09.2	46 %
0.02 %	05	05	04	04	04	04.4	22 %
0.01 %	03	03	02	0	03	02.2	11 %
Control	0	0	0	0	0	0	0%

Fig. 1 Larvicidal activity leaf extract of *Adhatoda vasica* along with silver nanoparticle against *Culex quinquefasciatus* in 24 hrs.



The LC_{50} values of the methanolic leaf extract of *Adhatoda vasica* along with silver nanoparticle was 450.03 ppm against late third larva *Cx. quinquefasciatus* (Table 2). The LC_{90} and regression equation were 870.80 ppm, Y=05.98+0.22X. The 95% lower and upper confidence limit of LC50, LC90 (LCL-UCL) were (435.44-603.05) and (792.21-958.25) ppm respectively. The chi –square value 0.30 was significant at p<0.05 level.

In the present study one leaf extract was chosen with ethanol extract along with silver nanoparticle (0.017 mg in 100 ml distilled water). Some other technique like reflux extraction method may be applied to get 100% mortality at various ppm in different hours like 24, 42, and 72 hrs. However, LC_{50} value of crude obtained by Soxhlet extraction showed higher larval mortality. The Soxhlet method to be more effective for extraction of larvicidal components. Some time the Hot extraction does not show any appreciable mortality even at 250 ppm concentration.

Table. 2 Larvicidal activity leaf extract of *Adhatoda vasica* along with silver nanoparticle against *Culex quinquefasciatus* in 24 hrs.

Concentration (%)	Larval mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	Regression equation	Chi square
0.1 %	88 %	450.03 (435.44-603.05)	870.80 (792.21-958.25)	Y=05.98+0.22 X	0.30*
0.09 %	60 %				
0.07 %	62 %				
0.05 %	48 %				
0.03 %	46 %				
0.02 %	22 %				
0.01 %	11 %				
Control	0%				

*significant at P<0.05

Values in parenthesis represent 95% confidence interval

Table 3 Probit analysis of Larvicidal activity leaf extract of *Adhatoda vasica* along with silver nanoparticle against *Culex quinquefasciatus* in 24 hrs.

Observed and Expected Frequencies

CON	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
.10	20.0	17.6	16.021	1.579	.80105
.09	20.0	12.0	14.875	-2.875	.74373
.07	20.0	12.4	12.158	.242	.60792
.05	20.0	9.6	9.147	.453	.45736
.03	20.0	9.2	6.255	2.945	.31275
.02	20.0	4.4	4.974	-.574	.24871
.01	20.0	2.2	3.848	-1.648	.19241

Leaf Extract

Culex quinquefasciatus

The methanolic leaf extract of *A. vasica* along with silver nanoparticle was significantly inhibits the adult emergence. The percentage of adult emergence inhibition were 08.0±1.3, 14.0±1.3, 30.0±1.5, 45.5±1.5, 58.0±3.2, 76.0±2.4 and 96.0±2.3 % at different concentration viz., 250, 500, 750, 1000, 1,250,1,500 and 1,750 ppm against the larvae of *Cx.quinquefasciatus* (Table 4, Fig 2). The LI_{50} value was 623.12 ppm and the EI_{90} value was 1254.35. The regression equation was Y=12.15+0.615X. The 95% lower and upper confidence limit of LC_{50}, LC_{90} (LCL-UCL) were (510.23-750.40) and (1115.15-1452.05) ppm respectively. The chi –square value 0.03.65 was significant at p<0.05 level (Table 4).

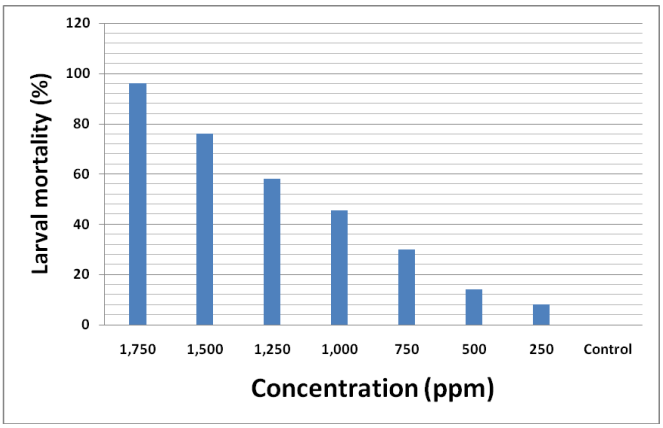
Table. 4 Insect growth regulator activity leaf extract of *Adhatoda vasica* along with silver nanoparticle against *Culex quinquefasciatus*

Concentration (ppm)	Larval mortality (%)±SD	LC_{50} (ppm)	LC_{90} (ppm)	Regression equation	Chi square
1,750.0	96.0±2.3				
1,500.0	76.0±2.4				
1,250.0	58.0±3.2				
1,000.0	45.5±1.5	623.12	1254.35		
750.0	30.0±1.5	(510.23-	(1115.15-	Y=12.15+0.615X	0.03.65*
500.0	14.0±1.3	750.40)	1452.05)		
250.0	08.0±1.3				
Control	00.0± 0.00				

*Significant at P<0.05

Values in parenthesis represent 95% confidence interval

Fig. 2 Insect growth regulator activity leaf extract of *Adhatoda vasica* along with silver nanoparticle against *Culex quinquefasciatus*



Discussion

Mosquitoes are the most deadly vector for several of these disease causing organisms. In many parts of the world, plant-derived natural products have traditionally been used against mosquitoes (Cavalcanti *et al.* 2004). In the search for safer insecticide technologies, more selective modes of action and reduced risks for non-target organisms and the environment, progress has been made in the last twenty years with the development of natural compounds capable of interfering with the processes of growth, development and metamorphosis of the target insects (Abahussain, 1999). According to Boivin, (1990) the screening of locally available medicinal plants for mosquito control would reduce dependence on expensive imported products and stimulate local efforts to enhance public health. The results of present study are comparable with earlier reports. The toxicity to the late third instar larvae of *Culex quinquefasciatus* by methanolic leaf extract of *Memordica charantia*, *Trichosanthus anguina, Luffa acutangula, Benincasa cerifera* and *Citrullus vulgaris* showed the LC_{50} values of 465.85, 567.81, 839.81, 1189.30 and 1636.04 ppm respectively (Pushplatha and Muthukrishnan, 1995).

The present study clearly proved the bioefficacy of *Adhatoda vasica* extracts on *Cx. quinquefasciatus*. *A. vasica* extracts might lead to better application of botanical derivatives during the suitable developmental period and could also be helpful in usage of a natural mosquitocide which in future might be used directly as a larvicidal agent in small-volume aquatic habitats or breeding sites of limited size around human dwellings. Further studies such as mode of action, synergism with the biocides under field condition are needed.

Crude extracts of saporium from fruit pods of *Sivartzia madagascarinsis* produced high mortality in *Anopheles gambiae* larvae (Mathur, 2010). The phytochemicals may also be responsible for the growth abnormalities and delay in the pupal emergence observed in the course of the study. Srivastava and Srivastava (1993) reported prolonged pre- imago period, for *Aedes aegypti* larva exposed to methanol extract of *Nerium indicum.* The test concentrations recorded larvicidal activities in concentrations from 40 mg/ml and above while concentrations below 40mg/ml were sub lethal. The LC_{50} and LC_{90} for ethanolic extracts were 60.9mg/ml and 464. 1 mg/ml respectively while those for methanol extracts were 73.6mg/ml for LC50 and 1021mg/ml for LC90. Some plant extracts have been shown to exhibit larvicidal activity or insect growth regulatory activity against mosquito larvae at concentration above 10mg/ml (Cetin *et al.* 2006).

Suganya (2013) reported LC_{50} values less than 0.2mg/ml for methanolic extracts of *Atlantia monophylla* against larvae and pupae of *Anopheles aegypti* and *Cx. quinquefasciatus*. Nevertheless, the LC_{50} of 60.9mg/ml of ethanolic extracts of *O. gratissimum* can be compared to LC_{50} of 55-65mg/ml for some Neem extracts as reported by Assar and El-Sobky (2003). Recent report highlights botanical extracts not only having mosquitocidal properties and also have water purification properties (Arjunan *et al.* 2012).

The leaf extract of *A. vasica* tested in the present study is known to be eco-friendly and is not toxic to vertebrates and other related animals. Moreover, it is clearly proved that crude or partially purified plant extracts are less expensive and highly efficacious for the control of mosquitoes rather than the purified compounds or extracts (Cavalcanti, *et al.* 2004 ; Jang, *et al.* 2002). The present study showed high bioactivity of the studied plant which is grown widely all over the world. Such results may offer an opportunity for developing alternatives to rather expensive and environmentally hazardous organic insecticides. Results of the mortality, biology, repellency and biting deterrence effects of the present plant extracts on *Cx. quinquefasciatus* as discussed latter confirm their potential for control of the mosquito populations. Sukumar *et al.* (1991) reported that the toxicity values of tested ethanolic extracts of different plant parts based on LC_{50} values were arranged in a decreasing order as follows: leaves › roots › stems. The toxicity of petroleum ether extracts based on LC_{50} was roots › stems › leaves.

In the present study the larvicidal activity was recorded in different concentrations of leaf extract of *A. vasica* along with silver nanoparticle. Several plant extracts other than those used in the present study had been tested against different species of mosquitoes by many authors worldwide. The tested plant extracts on larval mortality of *C. pipiens* were in agreement with many other results (Cetin, *et al.* 2006 ; Dharmshaktu, *et al.* 1987 ; Hamouda, *et al.* 1996). Using the water extracts of *Echhornia crassipes* and *Atemisia monosperma* against the 2nd instar larvae of *C. pipiens* at concentrations of 0.5, 1.0 and 2.0%, Assar and El-Sobky (Assar and El-Sobky, 2003) recorded a significant effect on the larval mortality at concentrations 1.0 and 2.0%. However, the present study showed that the ethanol extracts of *Adhatoda vasica* (leaves) plant extract were effective on larval mortality of *Culex quinquefasciatus*, whereas caused 100% larval mortality at concentrations 100, 200 and 25 ppm, respectively.

In the present study the LC_{50} values of the methanolic leaf extract of *Adhatoda vasica* along with silver nanoparticle was 450.03 ppm against late third larva *Cx. quinquefasciatus*. The LC_{90} and regression equation were 870.80 ppm, Y=05.98+0.22X. The 95% lower and upper confidence limit of LC_{50}, LC90 (LCL-UCL) were (435.44-603.05) and (792.21-958.25) ppm respectively. The chi –square value 0.30 was significant at p<0.05 level. Sing *et al.* (2003) pointed out that the mosquito larvicidal properties of the leaf extract of mosquito larvicidal properties of the leaf extract of a herbaceous plant, *Ocimum canum* against *Aedes aegypti*. The LC_{50} values for 2nd, 3rd and 4th instars larvae were 177.82, 22.08 and 331.13ppm respectively. Kalyanasundaram and Das (1985) reported that the plant extracts of *Vinca rosea*, *Calatropis* sp.and *Adathoda sp.* posses larvicidal activity against *Ae.aegypti*. The LC_{50} values of 34.06, 35.18 and 34.30ppm respectively.

All the concentrations of plant extracts used in the present study exhibited repellence activity against the starved female adults of *Cx. quinquefasciatus*. The repellent or antifeeding activity depends on the plant species, plant part, solvent used in extraction and the dose of the extract. The present study indicates that the ethanol extraction of the plants used was effective in exhibiting the repellent action against the mosquito tested. Many plant extracts and essential oils manifest repellence activity against different mosquito species. The present results are in accordance with such results obtained by Sharma *et al.* (1995) using neem oils against mosquito bites of *Anopheles* spp., *Culex* spp., and *Aedes* spp., Govere *et al.* (2000) using extracts of fever tea (*Lippia javanica*) rose geranium (*Pelargonium reniforme*) and lemon grass (*Cymbopogon excavatus*) against *Anopheles arabiensis*, Jeyabalan *et al.* (2003) using methanolic extracts of *Pelargonium citrosa* against *Anopheles stephensi.*

In the present study that the methanolic leaf extract of *A. vasica* along with silver nanoparticle was significantly inhibits the adult emergence. The percentage of adult emergence inhibition were 08.0±1.3, 14.0±1.3, 30.0±1.5, 45.5±1.5, 58.0±3.2, 76.0±2.4 and 96.0±2.3 % at different concentration viz., 250, 500, 750, 1000, 1,250,1,500 and 1,750 ppm against the larvae of *Cx. quinquefasciatus*. The LI_{50} value was 623.12 ppm and the EI_{90} value was 1254.35. The regression equation was Y=12.15+0.615X. The 95% lower and upper confidence limit of LC_{50}, LC_{90} (LCL-UCL) were (510.23-750.40) and (1115.15-1452.05) ppm respectively. The chi –square value 0.03.65 was significant at p<0.05 level. Nurie Misganaw *et al.* (2012) reported in their study that the percentage mortality of immature mosquitoes was significantly greater in petroleum ether,

acetone and benzene extract at above 125 ppm; 100% mortality was observed at 1000 ppm in all the tested solvent extracts. Similar findings was observed by Govindarajan *et al.* (2011a) they have confirmed significant larvicidal properties of crude benzene, acetone and methanol extracts of *C. fistula* leaf against *An. subpictus* and *Cx. tritaeniorthynchus*. The highest larval mortality was also found in the benzene extract of *E. coronaria* against the larvae of *An. stephensi*, *Ae.aegypti* and *Cx. quinquefasciatus* (Govindarajan *et al.* 2011b).

Furthermore, the results of the present study may contribute to a reduction in the application of synthetic insecticides, which in turn increases the opportunity for natural control of various medically important pests by botanical pesticides. In the present study one leaf extract was chosen with ethanol extract along with silver nanoparticle (0.017 mg in 100 ml distilled water). Some other technique like reflux extraction method may be applied to get 100% mortality at various ppm in different hours like 24, 42, and 72 hrs. However, LC_{50} value of crude obtained by soxhlet extraction showed higher larval mortality. The soxhlet method to be more effective for extraction of larvicidal components. Some time the Hot extraction does not show any appreciable mortality even at 250 ppm concentration. The present study suggested that *A. vasica* leaf extract along with silver nanoparticle act as larvicides against *Cx. quinquefasciatus* larvae. Moreover, these results could be useful in the search for newer, more selective and biodegradable larvicidal natural compounds. Further investigations are needed to isolate these active compounds. However, large scale implementation needs to develop effective formulation. The application of these plant extract along with silver nanoparticle on mosquito breeding places surely prevent environmental pollution and also protect the earth from toxic chemical pollutants.

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PLATE I

PLANT (*ADHATODA VASICA*) WITH LEAVES

SOXHLET APPARATUS

PLATE II

FEMALE MOSQUITO *CULEX QUINQUEFASCIATUS*

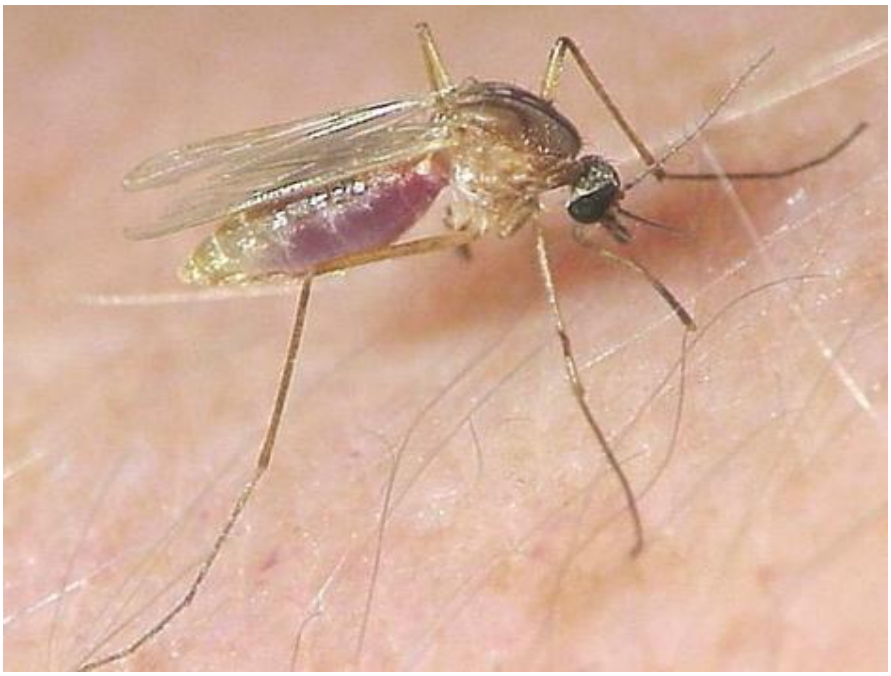
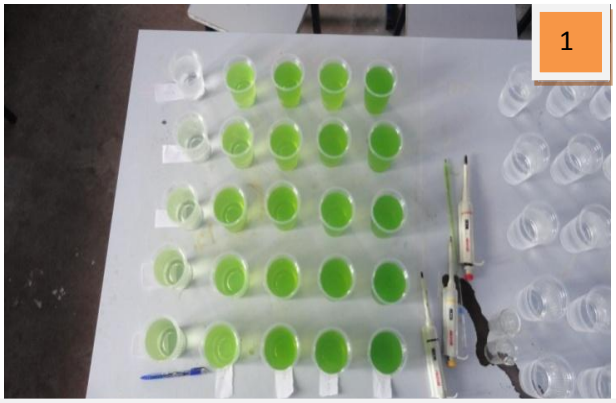


PLATE III

ADHATODA VASICA LEAF POWDER & POWDER EXTRACT

1. PLANT (*ADHATODA VASICA*) EXTRACT APPLIED IN DIFFERENT CONCENTRATION AGAINST MOSQUITO LARVA



2. MORTALITY OF MOSQUITO LARVA RECORDED AGAIST *ADHATODA VASICA* PLANT EXTRACT