"Studies on the effect of methanol extract of *Adhatoda vasica* Nees (Family Acanthaceae) on the II instar larvae of mosquito *Culex quinquefasciatus* (Culicidae) Diptera."

Pankaj Kumar Neeraj, Arti Saxena, Manisha Singh, Vinay Kumar Singh
Department of Zoology Govt. Science College, Rewa (M.P.), India

Abstract

Mosquitoes are becoming increasingly resistant to traditional chemical pesticides. They constitute a major public health menace and serve as vectors for transmitting diseases to humans. Control of mosquito-borne diseases is becoming more and more difficult because they are increasing resistance to pesticides occurs due to excessive and indiscriminate use of insecticides for vector and pest control. Insecticides of plant origin have been extensively used on agricultural pests, and to a very limited extent, against insect vectors of public health importance, which deserve careful and thorough screening. The use of plant extracts for mosquito control has several appealing features, as these are generally more biodegradable, less hazardous, and rich store house of chemicals of diverse biological activity. In the present study it was observed that the leaf extract of *Adhatoda vasica* proved to be a very effective mosquito larvicide and can provide eco-friendly alternative to synthetic insecticides. The methanol and chloroform fractions of *Adhatoda vasica* proved effective on *Culex quinquefasciatus*. More intensive studies are needed to find out the eco-friendly and safe methods for control of mosquito vectors.

KEYWORD: Larvicide, *Culex quinquefasciatus*, *Adhatoda vasica*, Eco-friendly.

Introduction

Mosquitoes are well known for their public health importance, since they act as vectors of many tropical and subtropical diseases, such as malaria, dengue, chikungunya, lymphatic filariasis and Japanese encephalitis. *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae) are the major urban vectors of malaria, dengue and lymphatic filariasis respectively in India. Malaria alone kills three million people annually, including one child every 30 as is reported in **Malaria Fact sheet No.94 Geneva (1999)** and **Hugh (1994**).

The resurgence of these diseases is mainly due to the ever increasing urbanization and associated anthropogenic activities. One of the effective methods to control these diseases has been to target the vectors for interrupting the transmission. Though such measures could target all stages of the mosquito life cycle, main focus was almost on adult stages by using conventional insecticides based on indoor residual house spraying **(Manzava *et al.*, 1993)** and insecticides treated nets (**Ukpong *et al.*, 2007**). Control of mosquito at the larval stage is also in practice in integrated mosquito management, as they are relatively immobile in the adult stage **(Rutledge *et al.*, 2003).** However, the indiscriminate application of synthetic insecticides has created multifarious problems such as environmental pollution, insecticides resistance and toxic hazards to humans. Globally there have been many efforts to overcome these problems and great emphasis has been placed recently on eco-friendly and economically viable methodologies for vectors control. Thus, in recent years various studies on natural plant products against mosquito vector revealed it as possible

alternatives to synthetic chemical insecticides (**Maria *et al.*, 1997; Mittal** and **Subbarao, 2003; Nazar *et al.*, 2009)**.

The bio-pesticides derived from natural products are quite acceptable on the grounds that:

(i) They are biodegradable.

(ii)They are non-hazardous to the non- target organisms.

(iii)They are eco-friendly and leave no by or lend products in the form of polled out.

Plants have evolved a variety of secondary compounds, some of them for providing protection from phytophagus insects. These plant extracts with insecticidal properties are used in the form of biological eco-friendly pesticides.

In the last few years, petroleum ether extract from 41 indigenous plants found in India have been studied for the larvicidal activity against culicine mosquitoes **Latha *et al*., (1999)**.

The chemicals derived from plants have been projected as weapons for future mosquito control programmes. They function as general toxicants, growth and reproductive inhibitors, repellents and deterrents, **Sukumar et al., (1991)**.

Das *et al*., (2003) analysed repellent properties of extracts of two plants viz. *Zanthoxylum limonella* and *Citrus aurantifolia*.

Larvicidal efficacy of *Capsicum annum* against *Anopheles stephensi* and *Culex quinquefasciatus* was observed by **Madhumathy *et.al* (2007)**.

The methanolic extract of *Hydrocotyle javanica* exhibited larvicidal activity against *Culex quinquefasciatus* **Venkatachalam *et al*., (2001)**.The toxicity of *Solanum torvum* extract was found effective against the early fourth instar larvae of *Culex quinquefasciatus* **(Rahuman *et.al.*, 2008)**. Acetone, chloroform, ethyl acetate, hexane and methanol dried leaf extracts of *S. trilobatum* were tested against the fourth instar larvae of *Anopheles subpictus* and *Culex quinquefarciatus* **(Zahir *et al*., 2009)**. A number of plants have been tested against the mosquito vectors during the last decade.

In the present work extract of *Adhatoda vasica* leaves was tested for mosquito control. Important chemical constituents of leaf include phenoxy vasicolinone and phenyl adhatodine. Besides this, the health benefits they provide are as protective against asthma, leprosy, skin diseases and piles.

Materials and methods

Collection of plant material-

In the present study an indigenous plant *Adhatoda vasica* of family *Acanthaceae* was selected to obtain biologically active chemical compounds from it.

The collection was done in the winter season. Identification of the collected plant was carried out at the Department of Botany of Govt. Science College Rewa.

The taxonomic position of the experimental plant

Phylum	:	Angiosperm
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Lamiales
Family	:	Acanthaecae
Genus	:	*Adhatoda*
Species	:	*Vasica* (Nees)
Popular name	:	Adulsa or Malabar nut.

Commonly known as:-

Sanskrit	:	Vasaka, Vasa, Adulsa
Bengali	:	Baksa, Vasaka
Gujarati	:	Aradusi
Hindi	:	Arusa
Malayalam	:	Atalotakam
Marathi	:	Adulsa
Kannada	:	Adusoge
Tamil	:	Adathodai
Telugu	:	Addasaramu

Characteristic features of *Adhatoda vasica*

Malabar nut or *Adhotoda vasica* is a small evergreen, sub herbaceous bush. The leaves of *Adhotoda vasica* are rich in vitamin C and carotene and yield an essential oil. The shrub is the source of a drug well known in indigenous systems of medicine for its beneficial effects, particularly in bronchitis. For phytochemical analysis of plant the collected material after identification was used. The cold percolation method as mentioned by **Abbott (1925)** is used for fresh plant leaves.

Chemical analysis and identification of the compounds

First the crude extract of plant was defatted in n-hexane and extracted with methanol. The concentrated solution was allowed to stand when a green yellow deposit was obtained. The deposit was repeatedly crystallized from a mixture of chloroform and methanol, till a single spot was obtained by paper and thin layer chromatography.

Test of Phenols: To 1ml of the extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of green colour indicated the presence of phenols.

Test for Tannins: To 1ml plant extract, 2ml of 5% ferric chloride was added. Formation of greenish black indicated the presence of tannins.

Test for Flavonoids: To the test compound solution, add few drops of NaOH solution. Intense yellow colour is formed which turn to colourless an addition of few drops of dilute HCL, this indicate presence of flavonoids.

Identification and Structural elucidation of extracted bioactive compounds of the plant

For further identification and structural elucidation of plant extract, the purified sample of experimental plant fraction $AsMf_5$ was sent to SAIF, CDRI Lucknow for spectral analysis, where IR spectrum, UV spectrum, NMR and Mass spectrum were done.

Laboratory colonization of Culicine mosquitoes

The aquatic stages of the larvae were collected with the help of long hand dippers and other manual methods especially behind Govt. Science College ground and sewage water from gutter and brought to the insectary with a daily photoperiod (L:D) 14 hours of light and 10 hrs of darkness at 27± 2^0C temperature and 75-85% relative humidity (RH). The third and fourth instar stages were sorted out and transferred to enamel trays of size (30×25×5) and fed with a diet of finely ground Brewer's yeast and dog biscuits (3:1). The adults, which emerged, were transferred to insect iron cages covered with muslin cloth. For mass production, standard wooden as well as iron insect cages of (30×30×30 cm.) size were used. Hatching of eggs started within 48 hrs and newly hatched larvae were held in the enamel trays.

Pupae that emerged from the larvae were separated in beakers containing water and were placed in iron cages.

Selection and preparation of different concentrations of the plant extract

First 11% stock solutions (w/v) of the extract was prepared. The crude extract and the fractions were screened using a concentration of 100 to 1000 ppm respectively from the stock solution. Secondly, those extracts that produced a high larval mortality and other biological activities were further tested at lower concentrations of 50-250 ppm for purified fractions.

Different concentrations ranging 50-250 ppm were prepared by adding desired volumes of stock solutions to distilled water in beakers followed by vigorous stirring. Acetone was used as a solvent to dilute the extract of the plant for experimental bioassay.

Test insects were taken out from the field collected and laboratory cultured stock per replicate. Population of test insects exposed to an insecticidal activity of the plant extract under bioassay experiments were II^{nd} and IV^{th} instar larvae.

Mortality was observed after 24 hrs. interval at each level of concentration.

One ml of stock solution of appropriate concentration was added in 499 ml of water in enamel trays (15×10×5) having 50 II^{nd} or IV^{th} instar larvae. Four replicates were set for each concentration along with one controlwhich were run simultaneously. The experiment was performed at $27+2^0$ C and R.H. about 80% ± 5%.

Dead larvae, pupae, partly emerged and precocious adults were regularly removed and counted. Live pupae were collected and observed till emerged into the adults. LC_{50} value of each concentration of each of the plant extract was calculated. For morphogenetic abnormalities, dead larvae, pupae supernumerary larvae and precocious adults were collected.

Larvicidal activity

The larvicidal potency of plant extracts (crude and purified) were evaluated by exposing II^{nd} and IV^{th} instar *Culex quinquefasciatus* larvae of laboratory origin in batches of 25. The larvae were strained with the help of insecticides free strainer and delivered into 500 ml beaker containing 250 ml of water.

The required volume of the stock solution was added in the beaker to give the desired test concentration. Each concentration was tested at four replicates with one control. Routine food schedule to larvae was followed for both treated and untreated beakers following **Ansari *et al.*, (1978)**.

Observations were recorded at an interval of 24 hrs till the emergence of adults. Dead and moribund larvae, pupae and partly emerged adults were regularly removed. LC_{50} values were determined by probit-analysis..

Bio-statistical evaluations of experimental data

As the biological observations were measured or counted, it becomes clear that some statistical methods were necessary to help in presenting and verifying the research data. For this, purpose different methods and tests were applied in the present work,which are mentioned below:

A. Percentage of mortality

Percentage of mortality was based on the number of larvae, pupae died after the treatment of plant extract in each set of experiments. **Abbott (1925)** formula was applied to correct the percentage mortality as given below:

$$\text{Correct Percentage Mortality} = \frac{\text{Observed mortality\%} - \text{Control mortality\%}}{100 - \text{Control mortality\%}} \text{x} 100$$

B. Growth Index (G.I.)

In the present investigation, the effect of plant extracts on different developmental stages of the vector was assessed in terms of growth index. It is the product of the percentage adult emergence 'a', at a given set of experiment divided by the average developmental period of the larvae (in days) 'b' :

$$G.I. = \frac{a}{b}$$

Where,

a = percentage adult emergence

b= average developmental period (days)

C. Level of significance

Level of significance between two variables was calculated at different degrees of freedom by student 't' test mentioned as under:

$$t = \frac{O - E}{E} \sqrt{n - 1}$$

Where,

O	=	observed value
E	=	expected value
n-1	=	number of degrees of freedom
n	=	standard deviation
t	=	student't'

D. Regression line

Regression analysis was carried out to calculate the lethal concentration from the mortality in the experiments against plant extracts tested. Corrected percentage mortality was plotted on log probit scale against concentration.

In the present study, the independent variable 'log' concentration was plotted on the horizontal axis (x-axis) while the dependent variable percentage mortality on vertical axis (y-axis). The line of the best fit (regression line) was drawn and the values of 'a' and 'b' obtained by using regression equation, $y = a \pm bx$.

To calculate the LC_{50} values of the plant extracts, the corrected percent mortality was plotted on log-probit scale against its concentration as described above. The LC_{50} values were calculated from the graph by projecting a line to meet the regression line on it and the values were calculated.

E. The X^2 test of goodness of fit for the regression line

The formula for calculating X^2 for individual difference in expected mortality and observed mortality corrected for a particular dose or concentration was as follows:

$$X^2 = \frac{\text{Observed} - \text{expected mortality}}{\text{expected mortality \% x 100} - \text{expected mortality}}$$

The individual X^2 values were summed up and multiplied by the average number of insect tested for each concentration. The values of X^2were compared with X^2values at 'a' level of significance of 0.5, 0.01 and 0.001 from the table as given by **Fisher and Yates (1983)** at the n-1 degrees of freedom. If the X^2computed regression line is less than the values obtained from the table of X^2, it may be concluded that the line is a good fit and data is not significantly heterogeneous.

F. Confidence limit of LC_{50}

(a) The expected LC_{16} and expected LC_{84} were read from regression line.

(b) Number of insect listed at concentration whose expected effects were between 16% and 84% is ascertained. The number is denoted by N.

(c) The slope function (S) is then computed by the formula

$$S = \frac{LC_{80}/LC_{50} + LC_{50}/LC_{16}}{2}$$

(d) The factor (FLC_{50}) is obtained from the formula (FLC_{50}) = S x 77/N.

(e) Fiducial limits were obtained from the under mentioned equation.

(1) Confidence limit (upper) = LC_{50} x FLC_{50}

(2) Confidence limit (lower) = LC_{50} x FLC_{50}

G.Probit analysis

Experimental data were analysed for X^2, Regression equation, LC_{50}, standard deviation and fiducial limit as suggested by **Finney** probit analysis method (1971). Probit analysis was performed with the help of statistical software SPSS 16.0.

Result and discussion

The observations taken from the experiments are as follows-

When the experiment was conducted on *Culex quinquefasciatus* IInd instar larvae with methanol extract of *Adhatoda vasica* leaves, in fraction ($AvMF_2$) larval mortality was observed to be highest in 250 ppm conc. and the LC_{50} value was calculated as 172.579 ppm while lower and upper fiducial limits were calculated to be 148.570 and 208.092 respectively (Table No.1). For fraction the regression equation ($y=a\pm bx$), chi square, variance and S.E were also recorded.

Table No. 1 reports the statistical analysis of experimental data of methanol purified extracts of *Adhatoda vasica*. It was observed that the mortality of IInd instar larvae is dose dependent. Experimental statistical data of purified fraction of *Adhatoda vasica* ($AvMF_2$) methanol extract against Culicine mosquitoes (*Culex quinquefasciatus*). The mortality range of IInd instar larvae was found to be 18 to 64.

These values were found quite significant over the control ($p< 0.001$). It was observed that 500 ppm concentration caused high mortality rates.

The regression equation ($y=a\pm bx$), chi square, variance and S.E for each fraction were also calculated.

Long before the advent of synthetic insecticides, plants and their derivatives were used to kill pests of agriculture, veterinary and public health. **Sosan *et al*** reported larvicidal activities of essential oils of *Ocimum gratissium, Cymbopogon citrus* and *Ageratum conyzoides* against *Aedes Aegypti* and achieved 100% mortality at 120, 200 and 300 ppm concentrations respectively. Similarly, it was reported that the essential oil of *Ipomoea cairica* Linn. possesses remarkable larvicidal properties as it could produce 100% mortality in the larvae of *Culex tritaeniorhnchus, Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes at concentrations ranging from 100 to 170 ppm. **Dwivedi & Kawasara** found acetone extract of *Lantana camara* to be most effective against *Culex quinquefasciatus* larvae at the dose of 1ml/100 ml. **Latha *et al*** reported *Piper longum* and *Zingiber wightianum* extracts at 80 mg/1 causing complete mortality in *Culex quinquefasciatus* and 60mg/1 for *Culex sitiens*.

Singh *et al*., (2013) observed the larvicidal activity of leaf powder of *Annona squamosa* against second instar larvae of *Anopheles stephensi*.

In the present work the leaves of *Adhatoda vasica* (Family Acanthaceae) have been proved effective an alternative to conventional synthetic insecticides for the control of *Culex quinquefasciatus*.

The results of present investigation indicate that IInd instar larvae were more susceptible to the purified extract. The outcome of this piece of work will contribute to a great extent in reduction of synthetic insecticides, which in turn will increase the opportunity for natural control of various medically important pests by botanical pesticides. This could lead to the development of new classes of economic & safe insect control agents.

References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. *J. Eco. Entomology*, 18: 265-267.

Ansari MA, Sharma VP, Razdan RK (1978) Mass rearing procedures for *Anopheles stephensi* Liston. *J Com Dis*. 10(2):131-135.

Das, N.G.; Baruah, I.; Talukdaar, P.K. and Das, S.C. (2003). Evalution of botanicals as repellents against mosquitoes. J. Vect. Borne Dis. 40: 49-53.

Dwivedi SC, Karwasara K. Larvicidal activity of five plants extracts against *Culex quinquefasciatus.* Indian J Entomol 2003; 65(3): 335-8.

Finney, D. J. (1971). Probit analysis, III Ed. Cambridge University Press, London, 235.

Fisher, R. A and Yates, F, (1983). Statistical tables for biological, agriculture and medical research, 6th Edt. Published by Oliver and Boyd Ltd. Edinburgh, 138.

Hugh C.P. (1994). Arthropods: vectors of disease agents. *Lab Med*; 25: 42937.

Latha C, Vijaykumar PD, Velasudhan S, Ammini J (1999) Biological activity of indigenous plant extracts as mosquito larvicides. *Ind J Exp Biol.* 37: 206-208.

Madhumathi AP, Aivazi AA, Vijayan VA (2007) Larvicidal efficacy of *Capsicum annum* against *Anopheles stephensi* and *Culex quinquefasciatus*. *J Vect Borne Dis.* 44: 223-226.

Manzava, A.E., R.T. Rwegoshora, M. Tanner, F.H. Msuya, C.F. Curtis and S.G. Irare, 1993. The house spraying with DDT or Lambda-cyhalothrine against *Anopheles stephensi* on measures of malaria morbidity in children in Tanzania. Acta Trop., 54: 141-151.

Maria, E.M., A.G.B.C. Rotraut, S.M.G. Telma, M.G.A. Antonoi and A.B. de Oliveira et al., 1997. *Aedes fluviatilis* (Diptera: Culicidae). Mem. Inst. Oswalado Cruz., 92: 565-570.

Mittal, P.K. and S.K. Subbarao, 2003. Propects of using herbal products in the control of mosquito veectors. ICMR. Bull., 33: 1-12.

Malaria (1999). Fact Sheet No. 94. Geneva: Word Health Organization.

Nazar, S., S. Ravikumar, G.P. Williams, M. Syed Ali and P. Suganthi, 2009. Screening of Indian coastal plant extracts for larvicidal activity of *Culex quinquefasciatus*. Indian J.Sci. Technol., 2: 24-27.

Rutledge, C.R., F. Clarke, A. Curtis and S. Sacket, 2003. Larval mosquito control. Tech. Bull. Florida Mosquito Control Assoc., 4: 16-19.

Rahuman, A.A., Gopalkrishnan, G., Venkatesan, P. & Geetha K. (2008). Isolation and identification of mosquito larvicidal compound from *Abutilon indicum* (Linn) Sweet. Parasitology Research 102: 981-988.

Singh, Amrita, Ashok Kumar and Veena P. Swami (2013). Laboratory evaluations of leaf of *Annona squamosa* to suppress the population of malaria vector *Anopheles stephensi* (Diptera: Culicidae). *CIB Tech Journal of Pharmaceutical Sciences* 2319-389.

Sosan MB, Adewunmi CO. Larvicidal properties of three indigenous plant oils on the mosquito *Aedes aegypti*. Nigerian Prod Med 2001; 5: 30-3.

Sukumar K, Michael JP, Boobar LR (1991) Botanical derivatives in mosquito control. *J Amer Mosq Cont Aso.* 7(2): 210-217.

Ukpong, I.G., K.N. Opara, L.P.E. Usip and F.S. Ekpu, 2007. Community perceptions about malaria, mosquito and insecticide treated nets in a rural community of the Niger delta Nigeria: Implications for control. Res. J. Parasitol., 2: 13-22.

Venkatachalam, M.R. & Jebanesan, A. (2001). Larvicidal activity of *Hydrocotyl javanica* Thumb. (Apiaceae) extract against *Culex quinquefasciatus*. Journal of Experimental Zoology 4: 99-101.

Zahir, A.A., Rahuman, A.A., Kamaraj, C., Bagavan, A., Elango, G., Sangaram, A. & Kumar, B.S. (2009). Laboratory determination of efficacy of indigenous plant extracts for parasites control *Parasitology Research* 105: 453-461.

Table No. 1

Statistical data of purified fraction of *Adhatoda vasica* ($AvMf_2$) methanol extract against Culicine mosquito (*Culex quinquefasciatus*)

Instar	Concentration (ppm)	24hr. larval mortality	Regression equation (y=a±bx)	Chi square χ^2(n-1)	LC_{50}	Variance (V)	S.E.	Fiducial limits (ppm)
	50	18						
	100	33						
II	150	44	y= 3.972±1.776x	.626	172.579	0.0630	.251	L=148.570 U=208.092
	200	53						
	250	64						
	Control	04						

25 each second instar larvae were taken in average of four replicates.
Values are significantly different from control (Duncan's multiple range test, $p<0.05$)