# LARVICIDAL ACTIVITY OF NEEM (*Azadirachta indica* A. Juss) LEAF EXTRACTS AGAINST THE MOSQUITO LARVAE OF *Culex quinquefasciatus* (Say) (DIPTERA: CULICIDAE)

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## Abstract

The toxicity of two different solvent (viz. acetone and carbon tetrachloride) extracts of *Azadirachta indica* A. Juss (Family: Meliaceae) leaves was studied on the 4$^{th}$ instar larvae of *Culex quinquefasciatus* (Say) (Diptera: Culicidae). For bioassay test, five concentrations (viz. 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) of crude extracts of neem leaves were prepared. Two ml of di-methyl sulfoxide (DMSO) were used to solubilize the crude extracts in water. A set of control was also used taking 2.0% DMSO in 100 ml of water for comparison. Data were taken after 24 hours exposure. The test larvae showed 37.33, 45.33, 61.33, 72.00 and 81.33 percent mortalities at 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml dose concentrations of acetone leaf extract, respectively, and 46.66, 60.00, 73.33, 80.00 and 85.33 percent mortalities at the same dose concentrations for $CCl_4$ leaf extract, respectively. The $LC_{50}$ and $LC_{90}$ values ranged from 0.700 to 1.008 mg/ml and from 3.489 to 9.860 mg/ml, respectively for acetone leaf extract; and those for $CCl_4$ leaf extracts ranged from 0.397 to 0.779 mg/ml and 2.707 to 7.145 mg/ml, respectively. The $CCl_4$ leaf extracts were found to be slightly more toxic against the larvae than the acetone leaf extracts.

***Key words*:** *Azadirachta indica*, neem, leaf extract, larvae, mosquito, *Culex quinquefasciatus.*

## INTRODUCTION

Mosquitoes are the most prominent blood-sucking arthropods causing annoyance and are of great concern for the public health throughout the tropical regions of the world. In Dhaka city more than 90% of the mosquitoes belong to *Culex quinquefasciatus* (Ameen *et al.* 1994). Recently, Khan *et al*. (2014 and 2015) reported 13 species of mosquitoes in five wards of Dhaka metropolitan city of which *Cx. quinquefasciatus* was the predominant one. *Culex* mosquitoes are well known for transmitting diseases like filariasis, encephalitis and West Nile fever. As the population of *Cx. quinquefasciatus* is increasing in an alarming rate, which is a serious threat to human health, it needs to be controlled.

At present, various kinds of synthetic pesticides, such as pyrithroids, carbamates, and hydrochloride and organophosphate compounds are frequently used for mosquito control. But, these synthetic pesticides have increased toxicity to human health and have adverse effects on the environment, causing soil, water and air pollution because of their non-biodegradable nature. Moreover, mosquitoes have developed resistance to these synthetic pesticides. So, an alternative way should be found out, which will be effective in the mosquito management strategies, and also it will focus on public health, monitoring and surveillance, source reduction and environmentally least-toxic larval control. These factors have resulted in an approach to look for environment friendly, cost-effective, biodegradable and target specific insecticides against mosquito species.

Natural products of plant origin with insecticidal properties have been tried in the recent mosquito control. One of the most important plant species which have effective larvicidal and insecticidal properties is *Azadirachta indica* (Neem), belonging to the Family Meliceae. This tree abundantly grows in the tropical and sub-tropical parts of Asia, including Bangladesh. Various parts of neem tree, such as leaves, seeds, bark, flower etc. have been used to control pests from ancient time. Different studies show that *A. indica* have a variety of effects on mosquitoes, such as antifeedant, growth retardation, reduced fecundity, molting disorder and changes of behavior (Schmidt *et al.* 1998).

Nowadays, the formulation of neem oil has been effective in controlling mosquito larvae in different breeding sites under natural field conditions (Hossain *et al*. 2013). Khan *et al.* (2016a) studied the effect of neem (*Azadirachta indica*) and mohaneem (*Melia azadirachta*) leaf extracts on the larvae of *Cx. quinquefasciatus*. The efficacy of *Cassia fistula* fruit extracts on the 4$^{th}$ instar larvae of the same mosquito species was also studied by Khan *et al*. (2016b). Ndione *et al*. (2007) studied the treatment and comparative analysis of the properties of aqueous extracts of seed kernel of *A. indica* on *Aedes aegypti* larvae.

The present study was conducted in the laboratory to observe the toxicity of two different solvent crude extracts of the leaves of *Azadirachta indica* A. Juss against the mosquito larvae *Culex quinquefasciatus* (Say).

## MATERIAL AND METHODS

*Test insects*

The larvae of mosquito species *Culex quinquefasciatus,* were collected from the drains, pits etc. of the Curzon Hall premises. These larvae were reared at the Entomological Laboratory of the Department of Zoology, University of Dhaka and later bioassay tests were conducted in an ambient environment (27±2ºC and 75-85% RH) of the laboratory. The larvae of *Cx. quinquefasciatus* were identified following the identifying key suggested by Bram (1967). During rearing, the larvae were served with yeast powder while the newly emerged adults were provided with 10% glucose solution as food. After 3-4 days of emergence, the adult female mosquitoes were given blood meal from a pigeon, *Columba livia* for egg development and maturation.

*Test plant material*

The fresh leaves of neem, *A. indica,* were collected from Curzon Hall and Kabi Sufia Kamal Girls' residential Hall premises of the University of Dhaka in November 2015. The extraction procedure was conducted in the Entomological Laboratory of the Department of Zoology and the Centre for Advanced Research Science (CARS) of the University of Dhaka.

*Preparation of plant extracts*

The collected fresh leaves of neem were washed thoroughly with water and shed dried for five days at room temperature (25-30 $^{o}$C). Later on the leaves were ground into fine powder with the help of an electric grinder. Four conical flasks (500 ml) were rinsed, two with acetone and two with carbon tetrachloride ($CCl_4$) separately. Fifty gram of neem leaf powder were taken separately in each of the four flasks. Three hundred ml of acetone and $CCl_4$ were added separately to the respective flasks containing the neem leaf powder and mixed properly by shaking with hands. Then, those four flasks were kept inside an orbital shaker machine for 24 hours at 30 $^{0}$C temperature and 100 RPM for periodic shaking. After 24 hours, the solvent extract was collected. This process was repeated three times with fresh volume of solvents. Finally, the extracts were concentrated by evaporating the solvents through a rotary evaporator and further drying under open air. The complete evaporation of solvent was done at 40 $^{0}$C temperature and 80 RPM.

*Dose preparation*

Five test dose concentrations for each solvent extract were prepared, based on some previously conducted preliminary tests, for larvicidal bioassay. Different amount of the leaf extracts, viz. 50, 100, 150, 200 and 250 mg of acetone and $CCl_4$ were taken separately in small screw capped vials. As the crude extracts were insoluble in water, Di-methyl sulfoxide (DMSO) was used to solubilize the extract

in water, as suggested by Nour *et al.* (2012). The dissolved extract was then added to 100 ml water in a glass beaker. Thus, dose concentration of the plant extracts were 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml, respectively for each of the solvent extracts. Three replicates of each dose concentration were prepared.

*Bioassay tests*

A larviciadal bioassay method, as suggested by Dua *et al*. (2009), was followed with slight modification. Twenty five actively swimming 4$^{th}$ instar mosquito larvae was taken into a conical flask (250 ml) containing 100 ml water along with one of the five doses of the plant extracts. This method was repeated for each replication of dose concentration. The flasks were stored at an ambient room environment (29±2ºC, 80 ±5% RH and 14L: 10D photoperiod). The mortality of the larvae was recorded after 24 hour exposure and the moribund larvae were counted as dead. A set of control was prepared using 2.0% DMSO in 100 ml of water.

The toxicity of the extracts was calculated as $LC_{50}$ and $LC_{90}$ representing 50% and 90% mortality of the test larvae for 24 hour exposure, respectively. The number of larvae died at each of the dose concentrations at the end of the stipulated exposure period was recorded and the percentage mortality was calculated using the formula-

$$\text{Percentage of mortality} = \frac{\text{Number of larvae died}}{\text{Number of test larvae}} \times 100$$

When the mortality in control was more than 5%, the percentage mortality was corrected using Abbott's (1925) formula-

$$\text{Corrected mortality} = \frac{\text{Larval mortality in treatment} - \text{Larval mortality in control}}{100 - \text{control mortality}} \times 100$$

*Statistical analysis*

The $LC_{50}$ and $LC_{90}$ values at 95% confidence intervals and lower and upper confidence limits were determined by the probit analysis method suggested by Finney (1971). Chi-square values, regression at 95% confidence intervals of upper confidence limits and lower confidence limits and t-tests were calculated using the IBM SPSS statistics 20 (Statistical Package of Social Science) software, where significance level was set at $p< 0.05$.

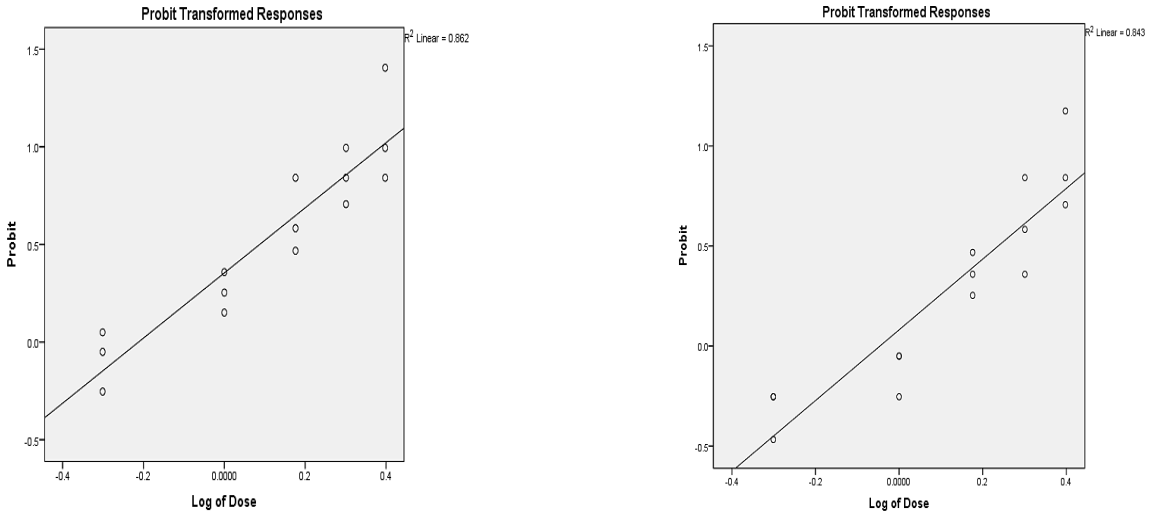
## RESULTS AND DISCUSSION

The larvicidal activity of acetone and $CCl_4$ based extracts of the leaves of *A. indica* as potential toxicant, was observed on the 4$^{th}$ instar larvae (n=75) of *Cx. quinquefasciatus*. The larval mortality against different dose concentrations (viz. 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) is shown Table 1.

**Table 1. Mortality of the 4$^{th}$ instar larvae (n=75) of *Cx. quinquefasciatus* against different doses of acetone-based *A. indica* leaf extracts after 24 hours exposure.**

| Solvents | Dose (mg/ml) | Larvae survived | | | Larval mortality | | |
|---|---|---|---|---|---|---|---|
| | | Number | (Mean±SD) | Percentage | Number | (Mean±SD) | Percentage |
| Acetone | 0.5 | 47 | 15.67±1.15 | 62.66 | 28 | 9.33±1.16 | 37.33 |
| | 1.0 | 41 | 13.67±1.16 | 54.66 | 34 | 11.33±1.16 | 45.33 |
| | 1.5 | 29 | 9.00±1.00 | 38.66 | 46 | 16.00±1.00 | 61.33 |
| | 2.0 | 21 | 7.00±2.00 | 28.00 | 54 | 18.00±2.00 | 72.00 |
| | 2.5 | 14 | 4.60±1.53 | 18.66 | 61 | 20.33±1.53 | 81.33 |
| $CCL_4$ | 0.5 | 40 | 13.33±1.53 | 53.33 | 35 | 11.67±1.53 | 46.66 |
| | 1.0 | 30 | 10.00±1.00 | 40.00 | 45 | 15.00±1.00 | 60.00 |
| | 1.5 | 20 | 6.67±1.53 | 26.66 | 55 | 18.33±1.53 | 73.33 |
| | 2.0 | 15 | 5.00±1.00 | 20.00 | 60 | 20.00±1.00 | 80.00 |
| | 2.5 | 11 | 3.67±1.53 | 14.66 | 64 | 21.33±1.53 | 85.33 |
| Control | | 75 | 25.00±0.00 | 100.00 | 0 | 0.00±0.00 | 00.00 |

The probit mortality lines of acetone and carbon tetra chloride based neem leaf extracts at different dose concentrations indicating different LC values and predicted regression line is shown in Fig. 1.




A B

Fig. 1. Plot of adjusted probit and predicted regression lines of acetone-based (A) and $CCl_4$-based (B) *A. indica* leaf extract against the 4th instar larvae of *Cx. quinquefasciatus*.

Based on the percentages of larval mortalities, $LC_{50}$ and $LC_{90}$ values, Chi-square, parameter estimation and 95% confidence limits were calculated and are shown in the Table 2. The calculated $LC_{50}$ and $LC_{90}$ values of the acetone and $CCl_4$-based neem leaf extracts were 0.906 and 5.043 mg/ml, and 0.609 and 3.784 mg/ml, respectively at 95% confidence limit (Fig. 1 and Table 2).

**Table 2. Efficacy of acetone and $CCl_4$ solvent based extract of *Azadirachta indica* leaves against the 4th instar larvae of *Culex quinquefasciatus*.**

| Base solvent | $LC_{50}$ value (Lower-Upper values (mg/ml) | $LC_{90}$ value (Lower-Upper values) ( mg/ml) | Parameter estimation | | Chi-square value (significance level) |
|---|---|---|---|---|---|
| | | | Concentration (Lower-Upper values) (mg/ml) | Intercept (Lower-Upper values) (mg/ml) | |
| Acetone | 0.906 (0.700-1.008) | 5.043 (3.489-9.860) | 1.721 (1.178-2.263) | 0.074 (0.001-0.147) | 6.983 (0.903[a])* |
| $CCl_4$ | 0.609 (0.397-0.779) | 3.784 (2.707-7.145) | 1.615 (1.065-2.166) | 0.348 (0.274-0.422) | 4.624 (0.983[a])* |

*Since the significance level is greater than 0.15, no heterogeneity factor is used in the calculation of confidence limits.

To compare the effectiveness of two different extracts, a paired sample t-test was done (Table 3). The mean mortality of larvae against $CCl_4$ based extract is greater than that of acetone based extract indicating more efficacy of the $CCl_4$ based extract. The standard error value of $CCl_4$ based extract is smaller than acetone based extract (Table 2) which also indicates that $CCl_4$ based extract is more efficacious.

The acetone leaf extract of *A. indica* showed 100% mortality at 1mg/ml concentration against the 2nd and 3rd instar larvae of *Aedes aegypti* after 24 hours exposure (Nour *et al.* 2012). In the present study, the 4th instar larvae of *Cx. quinquefasciatus* showed 45.33% mortality against the same dose concentration after 24 hour exposure (Table 1). This difference of mortality at the same dose concentration (1 mg/ml) might be due to the fact that dried neem leaf powder was used to prepare

solvent extract in the present study, while Nour *et al*. (2012) used fresh neem leaves. And also different species and larval stages might also be the reason of difference in larval mortality. In the present study, the dead larval bodies showed abnormalities like contraction of the larval bodies. This similar kind of abnormality was also observed by Schumtter (1990).

**Table 4. Paired sample t-test showing the comparison between two different solvent extracts of neem leaf against the 4th instar larvae of *Cx. quinquefasciatus*.**

| Base solvent | Mean mortality (%) | SD | SE | Correlation | Significance | t-value | df | Significance (2-tailed) |
|---|---|---|---|---|---|---|---|---|
| Acetone | 14.80 | 4.568 | 2.003 | 0.989 | 0.001 | − 4.707 | 4 | 0.009 |
| $CCl_4$ | 17.20 | 3.701 | 1.655 | | | | | |

El-Mahmood *et al.* (2010) reported that the chloroform extract of neem leaf showed 87% mortality at 1mg/ml concentration when tested against the 3rd instar larvae of *Cx. quinquefasciatus*. In the present study, the $CCl_4$ extract showed the highest 84% mortality at 2.5mg/ml dose concentration. The similar larval mortality at different dose concentrations might be due to different larval instars of mosquito. Aliero (2003) reported that 0.02% *A. indica* seed oil caused 100% mortality both in *Aedes aegypti* and *Culex quinquefasciatus*.

Chakkaravarthy *et al.* (2011) reported that the $LC_{50}$ and $LC_{90}$ values of the chloroform extract of *A. indica* were 198.32 ppm (0.198 mg/ml) and 1147.5 ppm (1.15 mg/ml), respectively, against the 3rd and 4th instar larvae of *Cx. quinquefasciatus* after 24 hour exposure. In the present study, the $LC_{50}$ and $LC_{90}$ values are greater than the values obtained by Chakkaravarthy *et al.* (2011). This difference might be due to the use of different solvents.

The neem has been reported to contain several biologically active constituents, such as azadirachtin (Naganishi 1975), meliantriol (Lavie *et al.* 1965) etc. In addition to these, compounds, such as salannol, salannolacetate, 3-deacetylsalannin, azadiradion, 14-epoxyazadiradion, gedunin and deacetylnimbinen, were isolated from oil extracted from neem seed (Schmutter 1990); whereas nimocinolide and isonimicinolide were obtained from fresh neem leaves (Siddiqui *et al*. 1986).

This study reveals that the crude extract of *Azadirachta indica* has larvicidal properties. Comparing present work with other researcher's works it is suggested that the insecticidal properties of *Azadirachta indica* (neem) varies due to the origin of tree in different habitats, different parts of the tree, different solvents used for extraction of plant materials, different methods of extraction, formulation of extracts, and also on the insect species on which the extracts are bioasayed. Further, extensive study should be carried out to establish the larvicidal properties of *A. indica* with appropriate formulation and doses, in order that the insecticidal ingredients in the extracts may be isolated, bioassayed and test its feasibility in the field with a view for commercial production in future.

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