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EFFECT OF TOXICITY OF NEEM (AZADIRACHTA INDICA A. JUSS) AND MOHANEEM (MELIA AZEDARACH LINNAEUS) ON THE LARVAE OF MOSQUITO CULEX QUINQUEFASCIATUS (SAY) (DIPTERA: CULICIDAE)

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Abstract

The larvicidal activities of three solvent extracts, viz. ethanol, chloroform and water of two plants neem *Azadirachta indica* (A. Juss) and mohaneem *Melia azedarach* (Linn.) against the fourth instar larvae of mosquito *Culex quinquefasciatus* (Say) (Diptera: Culicidae) were studied in the laboratory at 27 ± 2 °C and 75-85% RH. No larval mortality was observed in control treatment. The larval mortalities by the ethanol extracts of *A. indica* at the five dose concentrations were 37.33, 64.00, 64.00, 76.00 and 97.33%, respectively; by the chloroform extracts were 24.00, 54.66, 80.00, 96.00 and 100%, respectively; by the water extracts were 32.00, 56.00, 62.66, 68.00 and 81.33%, respectively. The larval mortalities by the ethanol extracts of *M. azedarach* at the five dose concentrations were 29.33, 58.66, 64.00, 74.66 and 89.33%, respectively; by the chloroform extracts were 40.00, 49.33, 61.33, 73.33 and 84.00%, respectively; by the water extracts were 29.33, 58.66, 64.00, 74.66 and 89.33%, respectively. In case of *A. indica*, LC_{50}, LC_{90} and LC_{99} values for the ethanol extracts were 1.805, 3.581 and 6.261 mg/ml, respectively; for the chloroform extracts were 0.686, 1.112, and 1.648 mg/ml, respectively; and for the water extracts were 3.002, 5.584 and 9.262 mg/ml, respectively. In case of *M. azedarach*, LC_{50}, LC_{90} and LC_{99} values for the ethanol extracts were 1.949, 3.89 and 6.835 mg/ml, respectively; for the chloroform extracts were 0.695, 2.256, and 5.886 mg/ml, respectively; and for the water extracts were 3.536, 6.662 and 10.866 mg/ml, respectively.

Key words: Azadirachta indic, Melia azedarach, Cx. Quinquefasciatus, Toxicity, Lethal concentration

Introduction

Mosquitoes transmit more diseases than any other group of arthropods affecting millions of people throughout the world (Ghosh *et al*. 2012) and causing millions of deaths every year (Kamaraj *et al*. 2011). The World Health Organization (WHO) has declared mosquito as "*public enemy number one*" (Ghosh *et al*. 2012) because it is the principal vector of many of the "vector-borne" diseases affecting human beings and other animals (Ravichandran *et al.* 2014). Several mosquito species of the genera *Aedes*, *Anopheles* and *Culex* are vectors for the pathogen of various diseases like malaria, yellow fever, dengue, chikungunya, Zika, West Nile, Japanese encephalitis and filariasis (Kazembe and Makusha 2012).

In Bangladesh Ahmed (1987) reported 25 species of *Culex* and recorded 22 species of mosquitoes related to medico-veterinary importance among which eight species belonged to

Culex. In Dhaka city, the population of *Cx. quinquefasciatus* is peaked during the dry season from November to December (Khan *et al*. 2015). Begum *et al*. (1996) studied the larval population of *Cx. quinquefasciatus* in Dhaka city and its suburbs. Recently, Khan *et al*. (2014 and 2015) reported 13 species of mosquito in five wards of Dhaka city of which *Culex quinquefasciatus* was the predominant one.

Botanicals as potential insecticides were studied for the first time in the country by Ameen *et al*. (1985) and Ameen *et al*. (1983a and b); they bioassayed the solvent based root extracts of *Derris elliptica* plant on the larvae of two mosquito species of the genera *Aedes* and *Culex*. The principal toxicant of *D. elliptica* as insecticide is rotenone. Neem, *Azadirachta indica,* is a member of the family Meliaceae, which has been reported to contain several biologically active constituents, such as azadirachtin, meliantriol, salanin, nimbin and nimbidin (Naganishi 1975 and Aliero 2003). Mohaneem, *M. azedarach,* is another member of the family Meliaceae which contains azadirachtin, meliantriol steroids, tarpenoids, saponins and tannins (Azam *et al.* 2013 and Ahmed *et al*. 2012). Among the chemicals, azadirachtin showed maximum biological activity (95%) against the larvae, pupae and adult of *A. stephensi* (Nathan *et al*.2005). Nour *et al.* (2012) tested acetone, chloroform and ethanolic extracts of the bark, root, leaf and seeds of *A. indica* against the larvae of *Ae. Aegypti*. Batabyal *et al*. (2007) tested petroleum ether, carbon tetra-chloride and methanol extracts from the seeds of *A. indica* against the larvae of *An. stephensi*. The methanolic leaf and seed extracts of *M. azedarach* were tested against *An. stephensi* for its larvicidal, pupicidal, adulticidal, oviposition deterrent and repellent activities by Nathan *et al*. (2006). The fruit extracts of *M. azedarach* and *A. indica* elicit a variety of effects in insects, such as antifeedant, growth retardation, reduced fecundity, moulting disorders, morphogenetic defects and changes of behavior (Wandscheer *et al*. 2004). Alouani *et al*. (2009) studied the effects of azadirachtin of neem on the fourth instar larvae of *Cx. pipens* at different concentrations and reported that *A. indica* was potentially more effective to mosquito control than that of *M. azedarach*.

Since neem and mohaneem plants are easily available in the country and since they have immense potentiality as the source of botanical insecticides, the objective of the present paper was, therefore, to assess the three solvent extracts of each of the two plant species *A. indica* and *M. azedarach* on the larvae of *Cx. quinquefasciatus* (Say).

Materials and Methods

After the collection of larvae of *Cx. quinquefasciatus,* rearing of the larvae into adults, collection of the leaves of neem and mohaneem, extraction process of the leaves, and bioassay tests were conducted from September 2015 to March 2016 at the Entomology Research Laboratory of the Department of Zoology, University of Dhaka and also in the Center for Advanced Research in Sciences (CARS), University of Dhaka.

Collection and rearing of larvae and adults in the laboratory: The larvae of the mosquito *Cx. quinquefasciatus* were collected from some potential breeding places in Dhaka city, such as the drains of Curzon Hall area, Dhaka University. These were then reared in the laboratory at 27±2 °C and 75-85% RH. The procedure of rearing of *Cx. quinquefasciatus* was done following Bilkis (1997). During rearing the larvae were served with yeast powder while the emerged adults were provided with 10% glucose solutions as their food. After pupation, the

pupae were transferred to small plastic containers and then to an adult rearing cage (30 cm x 30 cm x 30 cm) for emergence. After 3-4 days of emergence, the adult female mosquitoes were given a blood meal from a pigeon, *Columba livia* for egg maturation. A petridish containing tap water was placed inside the cage for the females to oviposit. After couple of days, the females laid egg rafts on the surface of water of the petridish. Then the egg masses were allowed to hatch into the first instar larvae which were subsequently moulted into 2nd, 3rd and 4th instar larvae and pupae, and finally emerged into adults. In this way the rearing process continued for several generations for obtaining adequate number of larvae for bioassay tests with plant extracts of neem and mohaneem.

Preparation of plant extract: The leaves of neem *A. indica* and mohaneem *M. azedarach* were collected from these trees located in the premises of Curzon Hall, Dhaka University. Both the plant species were authenticated from the Botany Department, Dhaka University. The collected leaves were washed with tap water, sun-dried for seven days, and then powdered by using an electrical blender. Each 50g of the leaf powder of neem and mohaneem was dissolved separately in 300 ml of ethanol, chloroform and water, and kept for 24 hours with periodic shaking in a Shaking Orbital Machine at 100 rpm and 30°C, then filtered and the sample solutions were collected. This procedure was repeated three times with fresh volume of respective solvents. The total volumes of the samples were concentrated separately in a Rotary Vacuum Evaporator machine. The water in the samples was completely evaporated and dried at 200 rpm and 60^0C, and the ethanol and chloroform samples were at 100 rpm and 40 ^{0}C; these dried extracts were then stored at 4°C in an air tight white glass bottle for future use in dose preparation.

Dose preparation and bioassay test: The fourth instar larvae of *Cx. quinquefasciatus* were exposed to test doses of 150, 200, 250, 300 and 350 mgs each of the ethanol based extracts, 50, 75, 100, 125 and 150 mgs each of the chloroform based extracts, and finally 250, 300, 350, 400 and 450 mgs each of the water based extracts of the leaves of both *A. indica* and *M. azedirach.* The concentrations of the above doses calculated were: 1.5, 2.0. 2.5, 3.0 and 3.5 mg/ml, respectively for the ethanol extracts; 0.5, 0.75, 1.0, 1.25 and 1.50 mg/ml, respectively for the chloroform extracts; and 2.5, 3.0, 3.5, 4.0 and 4.5 mg/ml, respectively for the water extracts. For each of the dose concentrations, 25 fourth instar larvae of *Cx. quinquefasciatus* were exposed and three replicates were maintained for each case. The measured amount of the extracts was dissolved in 2 ml of dimethyl sulfoxide (DMSO) which was used to solubilize the plant extracts in water as suggested by Nour *et al.* (2012), but the water extracts were dissolved directly in water and no DMSO was required to add into it. Each of the dissolved plant extracts in DMSO was added to 100 ml water in a beaker. A set of control, using 2.0% DMSO as Control 1 and an untreated set of larvae in water (tap) as Control 2, were also used for comparison. The larvae were fed with dry yeast powder sprinkled on the surface of water at the rate of 50 mg/ml.

The mortality of the larvae was recorded after 36 hours of exposure and moribund larvae were counted as dead. The toxicity of the plant extracts was calculated in the form of LC_{50}, LC_{90} and LC_{99} values, which indicate 50, 90 and 99 per cents death of test larvae, respectively. The recorded mortality percentage values were calculated by using the formula-

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

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

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

Statistical analysis: $LC_{50,}$ LC_{90} and LC_{99} values at 95% confidence intervals of lower and upper confidence limits were calculated by following the probit analysis method suggested by Finney (1971). Other statistics like chi-square values, regression at 95% confidence intervals of upper and lower confidence limits and t-tests were calculated using the IBM SPSS statistics 20 (Statistical Package of Social Science) software; here significance levels were set at $p < 0.05$.

Results and Discussion

After 36 hours of exposure, the larval mortalities at five different concentrations of three solvent extracts of *A. indica* and *M. azedarach* are presented in Table 1 and no mortality was observed in control treatments.

LC_{50}, LC_{90} and LC_{99} values in relation to the larval mortalities of *Cx. quinquefasciatus* due to the effects of solvent extracts of neem and mohaneem leaves and their lower and higher confidence limits at 95%, and chi-square test values were calculated and the results are presented in Table 2.

Comparison of toxicity of different solvents based extracts of neem and mohaneem: The values of $LC_{50,}$ LC_{90} and LC_{99} show that the water extracts of neem (*A. indica*) and mohaneem (*M. azedarach*) were least toxic to the mosquito larvae followed by the ethanol extracts which was again followed by Chloroform extracts (Fig. 1). For neem and mohaneem leaf extracts, the grading of LC_{50} for these three solvent extracts on the basis of their toxicity is as follows: neem, Chloroform (0.686) > ethanol (1.805) > water (3.002); mohaneem, Chloroform (0.695) > ethanol (1.949) > water (3.536). The grading

Table 1. Mean percentage mortality of larvae of *Cx. quiquefasciatus* exposed to different concentrations of three solvent leaf crude extracts of *A. indica* and *M. azedarach.*

Solvens		*Azadirachta indica*		*Melia azedarach*	
	Concentration (mg/ml)	Mean no. of larvae died (Mean±SD)	Mean % of larvae died	Mean no. of larvae died (Mean±SD)	Mean % of larvae died (Mean±SD)
Ethanol	Control	0.00±0.00	0.00	0.00±0.00	0.00
	1.50	9.33±1.33	37.33	7.33±1.33	29.33
	2.00	16.00±1.00	64.00	14.66±0.33	58.66

	2.50	16.00±0.00	64.00	16.00±1.00	64.00
	3.00	19.00±1.00	76.00	18.66±0.33	74.66
	3.50	24.33±1.33	97.33	22.33±0.33	89.33
Chloroform	Control	0.00±0.00	0.00	0.00±0.00	0.00
	0.5	6.00±1.00	24.00	10.00±1.00	40.00
	0.75	13.66±2.33	54.66	12.33±0.33	49.33
	1.00	20.00±1.00	80.00	15.33±0.33	61.33
	1.25	24.00±1.00	96.00	18.33±0.33	73.33
	1.50	25.00±0.00	100	21.00±1.00	84.00
Water	Control	0.00±0.00	0.00	0.00±0.00	0.00
	2.50	8.00±1.00	32.00	6.00±1.00	29.33
	3.00	14.00±1.00	56.00	9.00±1.00	58.66
	3.50	15.66±0.33	62.66	12.66±2.33	64.00
	4.00	17.00±1.00	68.00	14.33±1.33	74.66
	4.50	23.00±2.33	81.33	17.66±0.33	89.33

SD standard deviation.

Table 2. LC_{50}, LC_{90}, LC_{99} and chi square values of the larvicidal activities of leaf extracts of *A. indica* and *M. azaderach* on the larvae of *Cx. quinquefasciatus*.

Plant and Solvents	LC_{50} (mg/ml) (LCL-UCL)	LC_{90} (mg/ml)(LCL-UCL	LC_{99} (mg/ml) (LCL-UCL)	x^2(df13) (Significant value)
Neem:	(LCL-UCL)	(LCL-UCL)	(LCL-UCL)	nt value)
Ethanol	1.805(1.606-1.962)	3.581(3.188-4.299)	6.261(5.024-9.042)	14.384 (0.347[a])
Chloroform	0.686(0.638-0.731)	1.112(1.029-1.229)	1.648(1.454-1.962)	7.564 (0.871[a])
Water	3.002(2.736-3.208)	5.584(4.888-7.130)	9.262(7.224-14.852)	4.605(0.983[a])
Mohaneem:				
Ethanol	1.949(1.761-2.107)	3.890(3.433- 4.740)	6.835(5.43010.006)	5.149(0.972[a])

Chloroform	0.695(0.576-0.792)	2.256(1.1763-3.551)	5.886(3.694-14.356)	3.553(0.995[a])
Water	3.536(3.315-3.792)	6.662(5.574-8.891)	10.866(8.216-18.459)	2.513(0.999[a])

LCL lower confidence limits; UCL upper confidence limits; x^2 chi-square; df degrees of freedom, [a]Since the significance level is greater than 150, no heterogeneity factor is used in the calculation of confidence limits.

of LC_{90} for these three solvent extracts as follows: neem, Chloroform (1.112) > ethanol (3.581) > water (5.584); mohaneem, Chloroform (2.256) > ethanol (3.890) > water (6.662). The grading of LC_{99} for these three solvent extracts as follows: neem, Chloroform (1.648) > ethanol (6.261) > water (9.262); mohaneem, Chloroform (5.886) > ethanol (6.835) > water (LC_{99} = 10.866). So, for three solvents, chloroform extract of both plants showed highest mortality rate when compared with two other solvents.

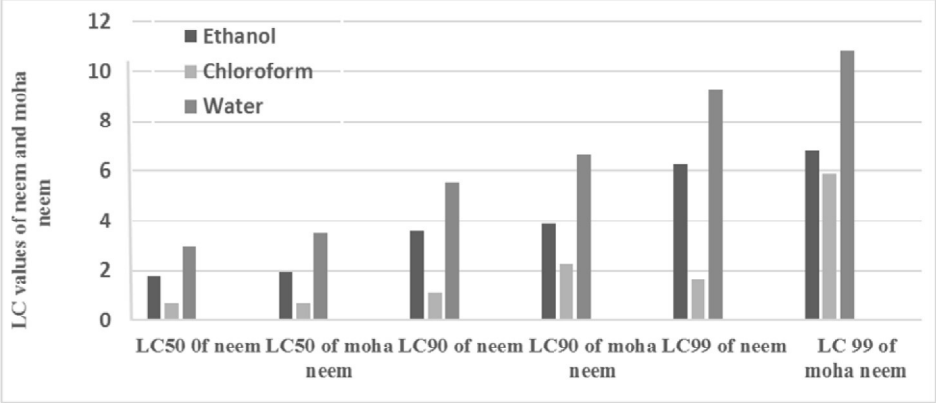

Fig 1. Comparision of lethal concentrations (LC) at three solvent extracts of *A. indica* and *M. azedarach.*

*Comparison of toxicity following paired t-test for neem and mohaneem***:** From the above discussion it is apparent that the extracts of the mohaneem (*M. azedarach*) is less effective than the extracts of the neem (*A. indica*). To compare between neem and mohaneem solvent extracts, significant paired t-test was followed (Table 3). For the ethanol, chloroform and water extracts of neem and mohaneem, the mean difference was 0.944, 1.944 and 2.556 for which the calculated t-test value is 3.183, 2.331 and 5.569 which were significant (Table 3).

Table 3. Paired t-test with the ethanol, chloroform and water extracts of *A. indica* and *M. azedarach* leaves bioassayed on the fourth instar larvae of *Cx. quinquefasciatus.*

Solvents	Dead at each level		Paired samples				
	Mean of neem	Mean of Mohaneem	Mean difference	SD (df17)	Standard error mean	t	Significant (2tailed)
Ethanol	14.11	13.17	0.944	1.259	0.297	3.183	0.005
Chloroform	14.78	12.83	1.944	3.539	0.834	2.331	0.032

Water	12.5	9.94	2.556	1.947	0.459	5.569	0

SD standard deviation, df degrees of freedom, t test.

The ethanol extracts of neem *A. indica* at 3.5 mg/ml dose concentration caused 97.33% 4th instar larval mortality (Table 1). Maragathavalli *et al.* (2012) reported that the ethanol extracts of *A. indica* leaves at 200 mg/ml dose concentration caused 100% mortality of the 3rd and 4th instar larvae of *Cx. quinquefasciatus* and 90% mortality of *Ae. aegypti.* The ethanol extracts of mohaneem *M. azedarach* at 0.5 mg/ml (500 ppm) caused 45% larval mortalities of *Cx. quinquefasciatus* in 24 hours (Ravichandran *et al.* 2014). After 36 hours of exposure, the larval mortalities of the mosquito at 2.0 mg/ml and 3.5 mg/ml dose concentrations of ethanol leaf extracts of *M. azedarach* were 58.66 and 89.33%, respectively (Table 1). The LC_{50} and LC_{90} values of the ethanol extracts of neem leaves bioassayed on the 4th instar larvae of *Cx quinquefasciatus* mosquito were 1.805 and 3.581 mg/ml, respectively (Table 2) while LC_{50} and LC_{90} values of the same extracts of neem bioassayed on the same mosquito species were found to be 0.565 mg/ml and 2.39 mg/ml, respectively (Ravichandran *et al.* 2014). The above findings indicate that the toxicity of the neem leaves used in the present study seem to have less toxic potentiality than the neem leaves used by Ravichandran *et al.* (2014), but seem to have higher toxic potential than the neem leaves used by Maragathavalli *et al.* (2012).

The chloroform extracts of *A. indica* leaves at 1.50 mg/ml dose concentration caused 100% of 4th instar larval mortality of *Cx quinquefasciatus* (Table 1.). Chakaravarthy *et al.* (2011) reported that the chloroform extracts of the same plant *A. indica* produced maximum 87% mortality of *Cx. quinquefasciatus* larvae at 1 mg/ml (1000 ppm) in 24 hours; LC_{50} and LC_{90} were 0.198 mg/ml (198.32 ppm) and 1.15 mg/ml (1147.5 ppm), respectively. In the present study the chloroform extracts of mohaneem *M. azedarach* at 1.50 mg/ml caused 84% mortality of the 4th instar larvae of *Cx quinquefasciatus*.in 36 hours (Table 1). The chloroform extracts of *M. azedarach* caused 35% mortality of *Cx. quinquefasciatus* larvae at 0.5 mg/ml (l500 ppm) in 24 hours (Ravichandran *et al.* 2014).

The LC_{50} and LC_{90} values of chloroform extracts of mohaneem *M. azedarach* leaves bioassayed on the 4th instar larvae of *Cx quinquefasciatus* mosquito were 0.695 mg/ml and 2.256 mg/ml, respectively (Table 2) while LC_{50} and LC_{90} values of the same extracts of mohaneem bioassayed on the same mosquito species were found to be 0.93 mg/ml mg/ml and 5.65 mg/ml mg/ml, respectively (Ravichandran *et al.* 2014).

The water extracts of neem *A. indica* at. 2.50, 3.0, 3.50, 4.0 and 4.50 mg/ml dose concentrations caused 32.00, 56.00, 62.66, 68.00 and 81.33% larval mortalities of *Cx. quinquefasciatus*, respectively (Table 1). Aliero (2003) reported that the aqueous leaf extract of *A. indica* was 83% mortality of *Anopheles* mosquito when the larvae were treated with 20 ml extract while 75% and 68% mortality were recorded with 10 and 5 ml extracts, respectively after 12 hours.

Kubmarawa *et al.* (2008) reported that the most important substance present in *A. indica* include alkaloids, glucocides, sterids, flavonoids, fatty oils, resins, mucilages, tannins, gums, phosphorus and calcium for cell growth, replacement and body building. Among the limnoids of *M. azedarach*, azadirachtin has been found to be the main ingredient for fighting insects

and pests, being upto 90% effective in most instances (Azam *et al*. 2013). It can also be suggested that the two plants have a number of chemical components, which may be responsible for the many pharmacological actions. Furthermore, the crude extracts may be more effective compared to the individual active compounds, due to natural synergism that discourages the development of resistance in the vectors (Maurya *et al*. 2007). Finally, it may be suggested that the leaf extracts of neem (*A. indica)* and mohaneem (*M. azedarach)* act as a natural larvicidal for controlling mosquitoes. Both these plants are easily available, environmentaly friendly and less expensive for controlling mosquitoes. More research is needed to develop an easy, economically viable and sustainable method to isolate the main toxic ingredients particularly azadirachtin from both neem and mohaneem in order that these insecticidal ingredients may be commercially produced and effectively applied in the country for controlling the insect pests.

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