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Full Length Research Paper

Effects of an aqueous extract of *Azadirachta indica* on the growth of larvae and development of pupae of *Culex quinquefasciatus*

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The neem tree *Azadirachta indica* Juss (Meliaceae) is one of the most studied plant species for pest control, including mosquitoes. However, the effect of aqueous neem seed extracts (ANSE) on each of the 4 instars of mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) is unknown. In order to determine the effect of applying ANSE on the growth of each of the 4 larval instars and pupal stage of *C. quinquefasciatus*, 8 bioassays with ANSE concentrations ranging from 30 to 1000 ppm were applied to larvae of 1st, 2nd, 3rd and 4th instars. The experimental unit consisted of a plastic cup with 100 ml of water and 20 larvae. 1 ml of the different concentrations of ANSE was applied. Mortality was assessed when 93 to 98% of the larvae reached the pupae stage in the control. In the surviving population the duration of larval stages and pupae viability were recorded. The growth of larvae and development of pupae was recorded to determine a growth inhibition index (GII) and a relative growth index (RGI), which was used to calculate the EC_{50} (concentration of ANSE that caused 50% reduction in RGI) for each larval stage. The concentrations of 460, 440, 410 and 550 ppm inhibited the growth of 50% the population of larvae of 1st, 2nd, 3rd and 4th instars, respectively; concentrations ≥ 500 and 700 ppm applied to larvae of 1st and 2nd instar, respectively, prevented the formation of pupae and concentrations ≥ 700 ppm prevented the emergence of adults in 4th instar larvae. The results showed that the different treatments inhibited the growth of larvae and pupae, prevented the formation and inhibited the development of pupae and reduced larvae and pupae viability.

Key words: *Azadirachta indica, Culex quinquefasciatus*, insecticide plant, instars, mosquitoes.

INTRODUCTION

Organo-synthetic insecticides have been traditionally used for mosquito control, which have caused pollution, resistance in mosquitoes and poisoning of the organisms exposed. Therefore, it is important to implement environment-friendly control alternatives to decrease the populations of mosquitoes, which besides are vectors of diseases and also cause much discomfort to humans. Amongst such alternatives, aqueous herbal extracts are an option to reduce the use of chemical insecticides, as more than 344 species of plants have been reported to be effective for mosquito control (Sukumar et al., 1991). Because of its multiple effects, one of the most oustanding has been *Azadirachta indica* (Obomanu et al., 2006). The seed is the most widely used plant part, as it contains a higher proportion of active substances against pests. Several authors reported that the neem extracts of acetone, water, ethanol and methanol caused mortality in the larval and pupal stages, prolonged their development, inhibited the formation of pupae and adults and inhibited larval molting in mosquitoes (Zebitz, 1984; Sagar and Sehgal, 1997; Elhag et al., 2001). The inhibitions of growth considered the negative effects on larval growth only and were classified according to the multiple effects

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observed as: strong, moderate, mild or false (Rodriguez and Vendramim, 1999). However, the effects of aqueous seed extract on the growth and development of mosquito larvae has not been investigated.

Therefore, in the present research the following objecttives were sought to determine the effect of different concentrations of an aqueous extract of neem seeds (ANSE) on larval growth and pupal development by means of an growth inhibition index (GII) and a relative growth index (RGI) in each of the 4 larval instars of *Culex quinquefasciatus*, and to determine the susceptibility of the 4 larval instars to ANSE throughout the estimation of mean lethal times and mean lethal dosages.

MATERIALS AND METHODS

This study was conducted at the Laboratory of Botanical Insecticides of the Postgraduate College, Campus Montecillo, Texcoco, Mex., Mexico and in the laboratory for mass production of mosquito larvae located at the Center for Interdisciplinary Research on Regional Development (CIIDIR), National Polytechnic Institute (IPN), Oaxaca, Oax., Mexico. Using automatic electric heaters, mean temperatures during the bioassays were maintained at 27ºC.

Mosquito larvae population and preparation of seed extracts

The larvae of *C. quinquefasciatus* were obtained from the insectarium located at CIIDIR-IPN-Oaxaca. Here, a colony of the insects, which are nourished by a live chicken, is kept year round.

The neem fruits were collected in Santa Rosa de Lima Tututepec, Oaxaca. After the fruits were de-pulped and dried indoors, 20 g were placed in 200 ml distilled water, liquefied for a minute and placed in an amber glass bottle of 500 ml (Mya et al., 2002; Pérez et al., 2004). After for 24 h, the mixture was strained through a cheese cloth and the remnant solids were discarded; this 1000 ppm aqueous extract, which represented the stock solution, was used to prepare the different concentrations evaluated in the experiments. To prepare the 100 ppm concentration, 1 mL of the stock solution, was mixed with 9 mL of distilled water. Using a similar procedure, other 6 different solution concentrations, in the range of 10 to 0.0001 ppm were obtained and were evaluated in a first approximation bioassay. During a second phase, concentrations of 700, 500, 300, 200, 150, 100 and 30 ppm were evaluated.

Bioassays

Using larvae of 1st, 2nd, 3rd and 4th instars, independent bioassays were conducted. The experimental unit consisted of a 125 mL plastic cup containing 100 ml of distilled water and 20 larvae of the same instar. Each experimental unit received 1 mL of the different concentrations of ANSE evaluated and the treatments were repeated 5 times. During the first phase of the study, aimed at determining the range of action of ANSE, the following concentrations were used: 1000, 100, 10, 1.0, 0.1, 0.01, 0.001 and 0.0001 ppm, while at the second phase 1000, 700, 500, 300, 200, 100 and 30 ppm were evaluated.

Each bioassay included a control, where no ANSE were applied. When this treatment reached a 93 - 98% of insects at the pupae stage, the numbers of larvae at different instars and the larvae and pupae live or dead, as well adults insects, were determined. Larvae or pupae were considered dead when they did not reacted to punctures made with a dissection needle. Using the formula of Zhang et al. (1993), these data were used to estimate the growth inhibition index(GII):

$$GII = \frac{\sum_{i=5} (\text{No. alive insects per instar}) + \sum_{i=5} ((\text{No. dead insects} \times (\text{Insect instar-1})}{(\text{Total no. of insects} \times \text{Total no. of instars})}$$

Where 1, 2, 3, 4 and 5 correspond to instars 1st, 2nd, 3rd, 4th and pupa, respectively. The number of insects tested was 100, the total number of instars of the insect was 5 (4 larval and 1 pupal). The relative growth index (RGI) was determined by RGI = GII of treatment/GII of the control data on length and viability of larvae and pupae survivors were taken; larval duration was considered the number of days since the beginning of treatment until they reached the pupal stage, while the duration of the pupal stage was from pupal stage to adult emergence. The formation of pupae and adults of the initial population was recorded as percent of the individuals reaching a particular stage.

Statistical analysis

The experiments were established under a completely randomized design. The data were tested to verify the normality of errors (Shapiro-Wilks test) and homogeneity of variances (Bartlett test). If these were met, analysis of variance and comparison of means by Tukey test ($p \leq 0.05$) were carried out by the program JMP version 7 (SAS Inc., 2008). When these assumptions were not met, a nonparametric analysis, the Kruskal-Wallis test, was carried out, as well as multiple comparisons by ranks (STATISTIX version 9, 2008). RGI data for the 1st and 2nd instars were adjusted to a fourth-order polynomial model with the JMP program, while data for 3rd and 4th instars were processed by simple linear regression analysis.

The JMP program was also used to obtain the LD_{50}'s for the 4 larval instars. A mortality data for concentration and time were applied probit analysis (SAS Inc., 2002).

Through linear regression analysis, RGI was plotted against the concentrations of ANSE for obtain EC_{50} (concentration of ANSE that caused 50% reduction in RGI) for *C. quinquefasciatus* larvae.

By means of regression techniques, data on days required to kill individuals by a particular treatment were used to estimate mean lethal times (LT_{50}).

RESULTS

Effects of ANSE on the growth of larvae of first and second instars and pupae development

In the first phase of research, the ANSE at 1000 ppm prevented the formation of pupae when applied to larvae of 1st and 2nd instars, but at 100 ppm, 73 and 63% of the larvae at those stages transformed to pupae, respecttively. Thus, the concentration that inhibits growth in 50% of the larvae exposed must be in the range of 100 to 1000 ppm.

During research phase 2, the ANSE at 1000 and 700 ppm inhibited the formation of pupae in larvae of 1st and 2nd instars. The concentration of 700 ppm had a TL_{50} of 2.6 and 1.9 days and a TL_{95} of 5.7 and 6.2 days. GII values for 700 ppm were 0.09 and 0.04, respectively. These last values were significantly different (Tukey, p ≤

Table 1. Percentages of larvae and pupae mortality and growth inhibition index (GII) of 1st and 2nd instar *C. quinquefasciatus* larvae treated with an extract of *A. indica*.

Concentration (ppm)	No. of larvae In 5 rep.	Mortaliy (%) and instar					GII[b]	
		1st	2nd	3rd	4th	Pupa		
1st instar	**100**							
1000	100	53	47	0	0	0	0.09	b
700	100	58	40	1	0	0	0.09	b
500	100	27	19	10	43	0	0.34	ab
300	100	4	1	0	0	0	0.90	ab
200	100	0	2	0	1	0	0.95	a
100	100	0	2	0	2	0	0.96	a
30	100	1	2	0	1	0	0.90	ab
Control	100	0	1	0	0	0	0.98	a
LD_{50}(ppm) = 460(450 - 470)								
2nd instar								
1000	100		95	5	0	0	0.01	c
700	100		84	16	0	0	0.04	c
500	100		63	2	25	0	0.21	b
300	100		2	0	3	1	0.95	a
200	100		0	1	3	1	0.97	a
100	100		0	0	5	0	0.97	a
30	100		1	0	1	1	0.97	a
Control	100		0	0	1	0	0.99	a
LD_{50}(ppm) = 440(430 - 450)								

[b]Means produced by non-parametric analysis (Kruskal-Wallis, p ≤ 0.05), values with different letters are significantly different (p ≤ 0.05).

0.05) from other treatments (Table 1).

The ANSE at 500 ppm caused the death of 99 and 90% of the larvae of 1st and 2nd instars, respectively. The respective LT50's were of 4.4 and 2.5, while observed LT95's were of 19.4 and 15.2 days; GII values were 0.34 and 0.21. The ANSE at concentrations ≤ 300 ppm caused only a slight reduction in growth with values of GII from 0.90 to 0.97 in larvae of both stages. It was found that concentrations of ANSE of 460 and 440 ppm in larvae of instars 1st and 2nd, respectively, inhibited growth of 50% of the larvae population, with no overlap in the fiducial limits. These data indicate a greater susceptibility of larvae at the 2nd instar (Table 1).

At the end of the life cycle, with the emergence of adults, it was found that the concentrations of 700 to 1000 ppm applied to larvae of 1st and 2nd instars, respectively, inhibited the formation of pupae. The larval and pupal viability were statistically similar to values observed in the control at concentrations ≤ 300 ppm. The larval duration increased with concentrations of 30, 100 and 200 ppm, as the duration was 12.2, 10.8 and 11.1 days, respectively. These values were statistically different (Tukey, p ≤ 0.05) from those observed in the control, which had a duration of 9.9 d. The pupal period lasted 13.8 and 12.6 days with 30 and 200 ppm, respectively, which were significantly different (Tukey, p ≤ 0.05) from the 11.6 recorded in the control (Table 2).

ANSE effect on the growth of larvae of third and fourth instars and pupae development

In the first phase, the application of 100 and 1000 ppm of ANSE to 3rd instar larvae caused a mortality of 25 and 93% and GII values of 0.84 and 0.38, respectively. In 4th instar larvae, concentrations of 100 and 1000 ppm caused 0 and 100% mortality and GII values of 1.0 and 0.14, respectively. Thus, the LD_{50} for both instars must be between 100 and 1000 ppm.

In the second phase, concentrations ≥ 500 ppm caused 76 to 86% mortality to 3rd instar larvae and mortalities of 52 to 64% in the 4th instar; both instars were inhibited significatively (Tukey, p ≤ 0.05). Concentrations of 300 to 100 ppm affected larvae growth to a lesser extent, as survivorships from 44 to 85% were observed. This was reflected in the GI values of 0.56 to 0.68 in 3rd instar larvae and values of 0.67 to 0.90 in 4th instar larvae (Table 3).

According to the data obtained, the ANSE applied at a concentration of 410 ppm inhibited growth in 50% of the population of 3rd instar larvae, while a concentration of 550 ppm was required to get the same effect on 4th

Table 2. Larval and pupal duration and pupae and adults of 1st and 2nd instar *C. quinquefasciatus* larvae treated with an extract of *A. indica*.

Concentration (ppm)	Duration (days)				Formed (%)			
	Larvae		Pupae		Pupae Þ		Adults	
1st instar								
1000	††		¶¶		0.0	b	¶¶	
700	††		¶¶		0.0	b	¶¶	
500	††		¶¶		0.0	ab	¶¶	
300	10.5	bc	12.1	bc	78.0	ab	78.0	ab
200	11.1	b	12.6	b	93.0	ab	90.0	ab
100	10.8	b	12.3	bc	96.0	a	96.0	b
30	12.2	a	13.8	a	91.0	ab	89.0	a
Control	9.9	c	11.6	c	99.0	a	99.0	c
2nd instar						Þ		
1000	††		¶¶		0.0	b	¶¶	
700	††		¶¶		0.0	b	¶¶	
500	9.0	a	12.0	a	2.0	b	1.0	b
300	7.8	b	9.6	b	92.0	ab	90.0	a
200	7.7	b	9.4	b	94.0	ab	89.0	a
100	7.7	b	9.5	b	94.0	ab	92.0	a
30	8.2	ab	9.9	ab	95.0	ab	92.0	a
Control	8.1	ab	9.9	ab	99.0	a	99.0	a

†† No one of the individuals completed its larval stage. ¶¶ There were no pupae.
Þ Averages produced by non-parametric analysis (Kruskal-Wallis, $p \leq 0.05$). Values with different letters are significantly different ($p \leq 0.05$).

instar larvae, which showed that 3rd instar larvae had a greater susceptibility.

At the end of the life cycle, when the emergence of adults occurred, it was observed, that the ANSE applied to 3rd instar larvae at concentrations ≥ 500 ppm, caused that only 0 - 3% of pupae and 0 - 3% adults were formed. Concentrations ≤ 300 ppm data produced significant differences as 30 - 55% of pupae were formed and only 11 - 35% reached the adult stage (Table 4). When the ANSE was applied at concentrations ≥ 700 ppm to 4th instars only 4 - 20% of pupae were formed, but no adults were observed. However, at concentrations ≤ 500 ppm, 55 - 95% of pupae and 3 - 73% of adults were recorded.

The duration of the larval and pupal stages of 3rd instars treated with 500 and 700 ppm of ANSE was increased to 6 and 8 d, respectively. The control showed a duration of 4.9 days for larvae and 6.3 days for pupae. Larvae treated in the 4th instar with 700 and 1000 ppm showed slight reductions in larval duration (3.1 and 3.8 d, respectively) as compared with the control (4.3 d). Similar results were found in the pupal stage where concentrations of 100 to 300 ppm reduced the pupal duration to 5.1 to 5.6 d, respectively. The duration for the control was 6.1 days (Table 4).

In summary, the LD_{50} of ANSE applied to 3rd instars were 498, 316 and 262 ppm at 2, 3 and 4 days after treatment application, respectively, while the LD_{95} were 26495, 9543 and 3643 ppm. In 4th instars the LD_{50} was 509 ppm, which was observed 7 days after treatment initiation Here, the LD_{95} was of 1615 ppm. The $LT_{50's}$ for 3rd instars for concentrations of 700, 500, 300 and 200 ppm were 1.9, 2.1, 4.1 and 4.4 d, respectively, larvae at 4th instar treated with 700 ppm had an LT_{50} of 5 d, while the LT_{95} was 10.8 d.

DISCUSSION

The results indicated that the ANSE applied at different concentrations to the 4 larval instars of *C. quinquefasciatus* inhibited the growth of larvae, avoided the formation of pupae and adult emergency and decreased larval and pupal viability, which agrees with results of several studies on extracts of neem seeds. These studies have reported a wide range of effects in more than 200 species of arthropods, including toxicity, repellency, feeding and oviposition inhibition, inhibition of reproducetion and regulation of growth (Gaaboub and Hayes, 1984; Schmutterer, 1990, 1995; Elhag et al., 2001).

The extension in the duration of the larval stage observed with the application of ANSE concentrations ≥ 300 ppm, > 500 ppm and 500 - 700 ppm to 1st, 2nd and 3rd instars, showed significant inhibition of larvae growth, while the extension of the duration of the pupal stage obtained with concentrations ≥ 300, 500 and 500 ppm in 1st, 2nd and 3rd instars indicated inhibition of develop-

Table 3. Percentages of larvae and pupae mortality and growth inhibition index (GII) obtained by the application of *A. indica* to 3rd and 4th instar larvae of *C. quinquefasciatus.*

Concentration (ppm)	No. of larvae In 5 rep.	Mortaliy (%) and instar			GII[b]	
		3rd	4th	Pupa		
3rd instar						
1000	100	25	61	0	0.30	c
700	100	24	55	0	0.32	bc
500	100	3	73	0	0.41	bc
300	100	0	54	2	0.56	abc
200	100	3	51	3	0.58	abc
150	100	6	45	0	0.59	abc
100	100	2	39	0	0.68	ab
Control	100	0	0	0	0.99	a
LD_{50} (ppm) = 410 (340 - 480)						
4th instar						
1000	100		49	3	0.26	d
700	100		43	16	0.31	cd
500	100		34	30	0.44	bcd
300	100		23	12	0.67	abcd
200	100		20	7	0.76	abcd
150	100		13	11	0.81	abc
100	100		5	10	0.90	ab
Control	100		0	0	0.99	a
LD_{50} (ppm) = 550 (500 - 600)						

[b]Means produced by non-parametric analysis (Kruskal-Wallis, $p \leq 0.05$), values with different letters are significantly different ($p \leq 0.05$).

ment; similar effects were reported by Sagar and Sehgal (1997) whom used an acetone extract of the of neem seeds which resulted in the prolongation of the larval and pupal stages of *C. pipiens* and *A. aegypti*.

Singh (1996), using methanol extracts of neem seeds, an azadiractine extract and an ANSE, was only able to identify effects on the prolongation of the larval stages of *C. quinquefasciatus*. These effects were attributed to one or more toxic alelochemicals present at low concentrations in the extracts used (Tanzubil and McCaffery, 1990), such as meliantriol or salanina (El-Ela et al., 1998), which affect the endocrine mechanism (Benerjee and Rembold, 1993; Barnby and Klocke, 1987) by causing a hormonal or nutritional imbalance or a combination of both. Likewise, one must consider that azadiractine interferes with the synthesis and release of the molting hormone, slowing the ecdisis and extending the duration of the larval stages and inhibiting larval growth (Isman, 2006). Champagne et al. (1988) also reported that azadiractine affects growth and metamorphosis of insects, interfering with the production of β-ecdisona and the juvenile hormone.

In this investigation, the application of 1000 and 700 ppm of ANSE to 1st instar larvae resulted in total mortality of 2nd instars; the application of 1000 and 700 ppm to 2nd instar larvae produced 100% mortality of 3rd instars and dosages of 1000 and 700 ppm applied to 3rd instar larvae caused total mortality of larvae reaching the 4th instar. Using an ANSE (100 g/500 mL of water), Mya et al. (2002) reported similar results as after 4 h obtained total mortality of 1st and 2nd instar larvae. Sagar and Sehgal (1997) reported a 100% mortality of 1st instar larvae with the application of an acetone extract of neem seeds at a concentration of 20 ppm. The LT_{50}'s determined for a concentration of 1000 ppm of ANSE applied to the larval instars 1st, 2nd, 3rd and 4th were 2.6, 1.4, 1.8 and 4.8 d, respectively; this showed the effectivity of the ANSE evaluated, as well as the higher resistance of 4th instar mosquito larvae.

The dosages of ANSE *A. indica,* which controlled 50% the population (LD_{50}) of 1st, 2nd, 3rd and 4th instar larvae of *C. quinquefasciatus*, were 460, 440, 410 and 550 ppm, respectively; these dosages affected significantly the duration and viability of larval and pupal stages and prevented the formation of pupae, decreasing adult emergency. Therefore, ANSE should be considered as an alternative control method of mosquito larvae.

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Table 4. Larval and pupal duration and pupae and adults of 3rd and 4th instar *C. quinquefasciatus* larvae treated with an extract of *A. indica*.

Concentration (ppm)	Duration (days)				Formed (%)			
	Larvae		Pupae		Pupae[Þ]		Adults[Þ]	
3rd instar								
1000	††		¶¶		0.0	c	¶¶	
700	6.0	a	¶¶		1.0	bc	0.0	b
500	6.0	a	8.0	a	3.0	bc	3.0	b
300	5.2	ab	6.7	ab	30.0	abc	11.0	ab
200	5.1	b	6.6	ab	38.0	abc	21.0	ab
150	5.1	b	6.1	b	42.0	abc	22.0	ab
100	5.1	b	6.3	b	55.0	ab	35.0	ab
Control	4.9	b	6.3	b	100.0	a	100.0	a
4th instar		Þ						Þ
1000	3.8	b	¶¶		4.0	e	0.0	c
700	3.1	b	¶¶		20.0	e	0.0	c
500	3.8	ab	6.3	a	55.0	d	3.0	bc
300	3.8	ab	5.6	b	72.0	cd	24.0	abc
200	4.0	ab	5.5	bc	79.0	bc	48.0	abc
150	3.9	ab	5.3	bc	86.0	abc	71.0	ab
100	3.7	ab	5.1	c	95.0	ab	73.0	ab
Control	4.3	a	6.1	a	100.0	a	100.0	a

†† No one of the individuals completed its larval stage. ¶¶ There were no pupae.Þ Averages produced by non-parametric analysis (Kruskal-Wallis, $p \leq 0.05$). Values with different letters are significantly different ($p \leq 0.05$).

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