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Larvicidal Activity and Influence of Azadirachtin (Neem Tree Extract) on the Longevity and Fecundity of Mosquito Species

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Abstract: The toxic effect of the commercial product azadirachtin (a chemical compound extracted from the plant *Azadirachta indica*) on larvae (L4) of two species of mosquitoes (*Culex pipiens* and *Culiseta longiareolata* isolated from Southern Algeria) is evaluated under laboratory conditions. Five doses (3, 6, 12, 24 and 48 mg/L) of this product are used. The LD50 and LD90 values after treatment are 7.66 mg/L, with a confidence interval of 6.14–9.33 mg/L for LD50, and 19.70 mg/L, with a confidence interval of 4.66–130 mg/L for LD90 for *Culex pipiens*, and LD50 = 7.60 mg/L (6.91–8.49 mg/L) and LD90 = 23.76 mg/L (4.38–90.80 mg/L) for *Culiseta longiareolata.* We record reduction in fertility in adult females emerging from treated larvae and an increase in sterility of treated males as compared to the controls. The results demonstrate that azadirachtin is promising as a larvicidal agent against larvae of *Culex pipiens* and *Culiseta longiareolata* and could be an alternative of chemical insecticides.

Keywords: *Azadirachta indica*, *Culex pipiens*, *Culiseta longiareolata*, fecundity

Introduction

The important role of mosquitoes for public health has made them a good research model in biology (ecology, entomology, etc.). The control of these insects is a current concern, with the aim of finding an alternative of chemical techniques. The modern control includes the use of natural products. Studies of the bioinsecticidal effect, in general and especially on the Culicidae, have become very frequent, e.g. Lacey & Orr (1994) and Kreutzweiser et al. (1994) used the HR-5992 and Soltani et al. (1999) used triflumuron. Mosquito control is essential as many species of this group are vectors of malaria, filariasis and arboviral diseases and constitute an intolerable biting nuisance (Youdeowei & Service 1983, Curtis 1994, Collins 1995). Oil extracted from seeds of neem (*Azadirachta indica* A. Juss.) has been a subject of studies starting in the 1960s (Butterworth & Morgan 1968). The effect of azadirachtin on insects has been clearly demonstrated (Ivbijaro 1990, Naqvi et al. 1991, Mordue & Blackwell 1993, Sundaram 1996, Greenberg et al. 2005, Rehimi et al. 2011, Huang et al. 2012).

Our study was conducted over six years and presented c. 20 types of habitats in the region of Biskra (South-eastern Algeria), demonstrating that *Culex pipiens* L., 1758 and *Culiseta longiareolata* Macquart, 1838 are the most widespread mosquito species in the region. According to several studies, these two species are widespread throughout Algeria. Boudjelida *et al*. (2008) studied the effect of *Bacillus thuringiensis* on their larval stages L1 and L4.

In this article, we present the results of studies on the activity of commercial product of azadirachtin at different concentrations (3–48 mg/L) against L4 of *Culex pipiens* and *Culiseta longiareolata*. We also

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quantify the effect of azadirachtin on fertility and fecundity of females.

Materials and Methods

Larval sampling and breeding. Larvae of *Culex pipiens* and *Culiseta longiareolata* were collected from different cottages in the region of Biskra (34°51′00″N, 5°44′00″E). The larvae were reared in storage jars containing 500 ml of stored tap water and maintained at a temperature of 25–30°C, 85% RH and a photoperiod of 14 : 10 (L : D). Larvae were fed daily with fresh food consisting of a mixture of biscuit-dried yeast (75:25 by weight) and water was changed every four days (Rehimi & Soltani 1999). The feeding continued until the larvae developed into pupae. The pupae were transferred from the trays to a cup containing tap water and placed in screened cages (30 × 30 × 30 cm) where the adults emerged. After emergence, female mosquitoes obtained blood meal from caged pigeons, while male mosquitoes were fed a 10% sucrose solution. Egg masses were kept to continue the next generation.

Bioassays and larval mortality. We used a standard technique of the World Health Organization (WHO) in order to measure the sensitivity of the mosquito population (larvae and adults) of the insecticide during a given period (WHO 1963). Our bioassay was performed with L4 of *Culex pipiens* and *Culiseta longiareolata* using five concentrations (3, 6, 12, 24 and 48 mg/L) of azadirachtin. We had 25 larvae in a beaker containing 200 ml of spring water. The lots were observed after 24-120 hours for each beaker. The mortality rate was calculated using formula (1), whereas the corrected mortality was calculated according to the formula (2) of Abbot (1925):

Percentage of Mortality = (Number of dead larvae / Number of larvae introduced) × 100 (1)

Corrected Percentage of Mortality = (n in T after treatment / n in C after treatment) × 100, (2)

where n = number of larvae, T = treated, and C = control.

For estimation of fecundity and sterility, the experiment was designed to estimate the effect on azadirachtin of some adult reproductive parameters of emerged larvae that were treated with sub-lethal concentrations of the product. The experiment was conducted with a series of repeats, and each series was formed by an equal number of males and females (n = 10) of L4. For all the series, the tests were performed in a cage (20 × 20 × 20 cm). The duration of study was one month, after which nacelles were recovered, and the number of eggs in each nacelle was counted under stereomicroscope. The percentage of hatching (E), the reduction of the rate of hatching (RE), the fecundity (F) and the reduction of the fecundity (RF) were calculated after Chugh et al. (2011):

E = (Number of hatched eggs / Total number of eggs) × 100 (3)

RE = [(Number of hatched eggs in the control females – Number of hatched eggs in the treated females) / Number of eggs in the control females] × 100 (4)

F = (Total number of hatched eggs / Total number of females) × 100 (5)

RF = [(Number of laid eggs in the control females – Number of laid eggs in the treated females) / Number of laid eggs in the control females] × 100 (6)

The Sterility Index (SI) was calculated after Saxena et al. (1993):

SI = 100 – [(Fecundity of the treated larvae × Hatchability %) / (Fecundity of the control larvae × Hatchability %)] (7)

Statistical analysis. The observed mortality for the two species in various concentrations was corrected by the Abbott (1925) formula and subjected to probit analysis (Finney 1971). The method of Swaroop et al. (1996) permits the calculation of the confidence interval (95%) of the LD50, LD90 (mg/L) and regression equations. The mortality and fertility results were subjected to an Analysis of variance (ANOVA of square root arcsine transformed percentages). The descriptive statistics and test statistics were calculated by Statistica Version 8.0.

Results

The biological tests showed that azadirachtin has a high toxicity against larvae of the two species *Culex pipiens* and *Culiseta longiareolata.* The observed mortality rate (Fig. 1) is proportional to the concentration and exposure time (24 to 120 h). For *Culex pipiens*, the mortality rate was between 5 and 55% to reach the maximum value (100%) for the last two concentrations (24 and 48 mg/L). The ANOVA (for the doses used) showed that the difference was highly significant ($P \leq 0.0001$ and $F = 66.0$) while, for the observation time, the difference was significant ($P = 0.0037$ and $F = 2.68$). Larvae of *Culiseta longiareolata* were weakly sensitive compared to *Culex pipiens*. The ANOVA (for the doses used) showed that the difference was highly significant ($P \leq 0.0001$ and $F=86.4$) and that for observation time showed a significant difference ($P=0.082$ and $F=2.14$).

The number of eggs that were produced by the

Table 1. Lethal doses with confidence intervals for the two mosquito species examined

Culex pipiens **(95% confidence interval) (mg/L)**		*Culiseta longiareolata* **(95% confidence interval) (mg/L)**	
LD50	LD90	LD50	LD90
6.914 < 7.62 > 8.49	4.38 < 19.70 > 59.32	6.14 < 7.57 > 9.35	4.66 < 21.52 > 40.88

Table 2. Comparison of the number of eggs that were laid by *Culex pipiens* and *Culiseta longiareolata* - female control groups and treated females (n = 10)

P (0.05)	**F**	**Max**	**Min**	**Mean ± s.e**		
**≤0.0001	4.41	102	82	91.5 ± 6.014	Control	*Culex pipiens*
		87	21	53.4 ± 18.37	3 mg/L	
		59	20	39.8±10.27	6 mg/L	
**≤0.0001	3.45	111	87	97 ± 8.56	Control	*Culiseta longiareolata*
		91	29	60.6 ± 21.42	3 mg/L	
		70	31	42.5±12.98	6 mg/L	

Table 3. Effect of azadirachtin on the reproductive potential of adult females of *Culex pipiens* and *Culiseta longiareolata* after treatment of (L4) larvae (n = 10 females, duration 30 days)

	Concentration	**Number of laid eggs**	**% of hatching**	**Reduction of hatching (%)**	**Reduction of Fecundity (%)**
Culex pipiens	Control	945	87.86±3.5	/	/
	3 mg/L	606	75.15±7.44	41.43±18.57	34.04±21.19
	6 mg/L	425	59.66±23.75	63.7±16.82	51.09±14.52
Culiseta longiareolata	Control	978	85.87±5.37	/	/
	3 mg/L	582	94.7±50.93	39.50±15.85	38.49±23.74
	6 mg/L	536	56.13±37.60	50.12±16.82	42.19±13.32

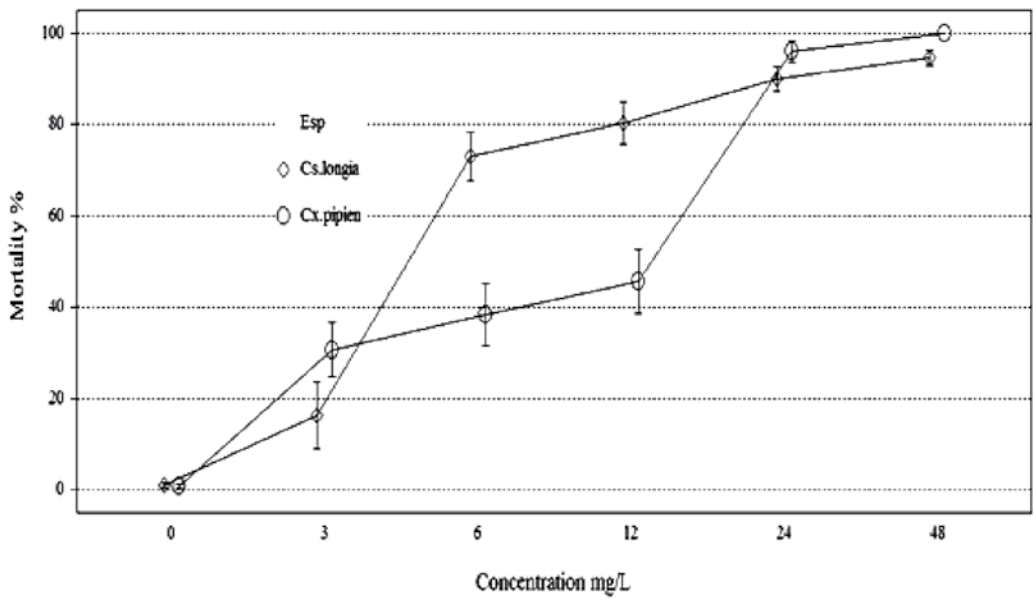

Fig. 1. Toxicity of azadirachtin against larvae of *Culex pipiens* and *Culiseta longiareolata*

treated and control females of *Culex pipiens* and *Culiseta longiareolata* was different (Table 2). As demonstrated by ANOVA), the difference was highly significant (p≤0.0001). It is clear that the number of eggs laid by the females of *Culex pipiens* was low compared to that of *Culiseta longiareolata.*

The mean number of eggs laid was inversely proportional to the concentration that was used. For

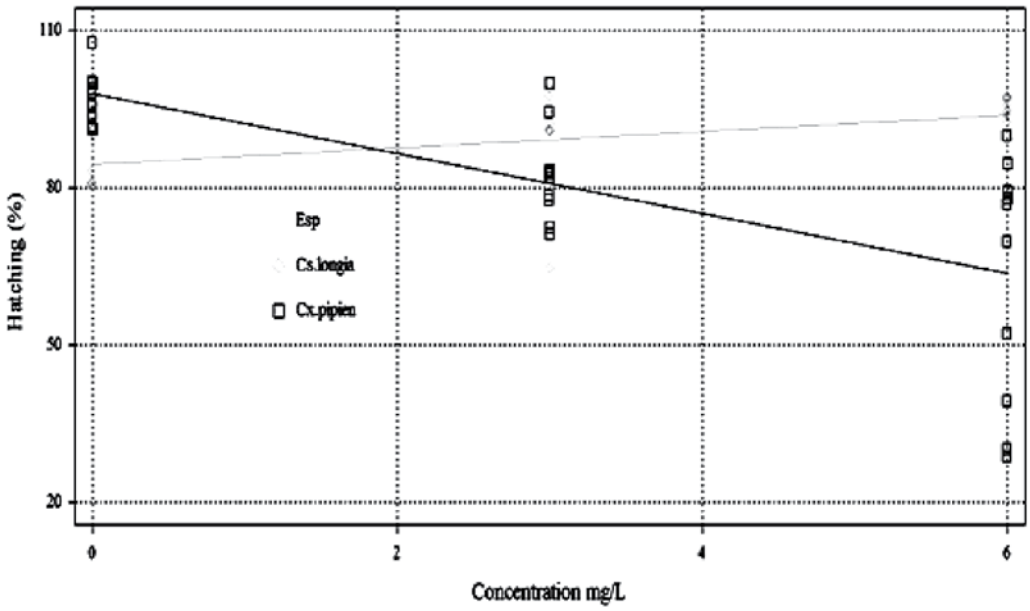

Fig. 2. Rate of hatching of of *Culex pipiens* and *Culiseta longiareolata*

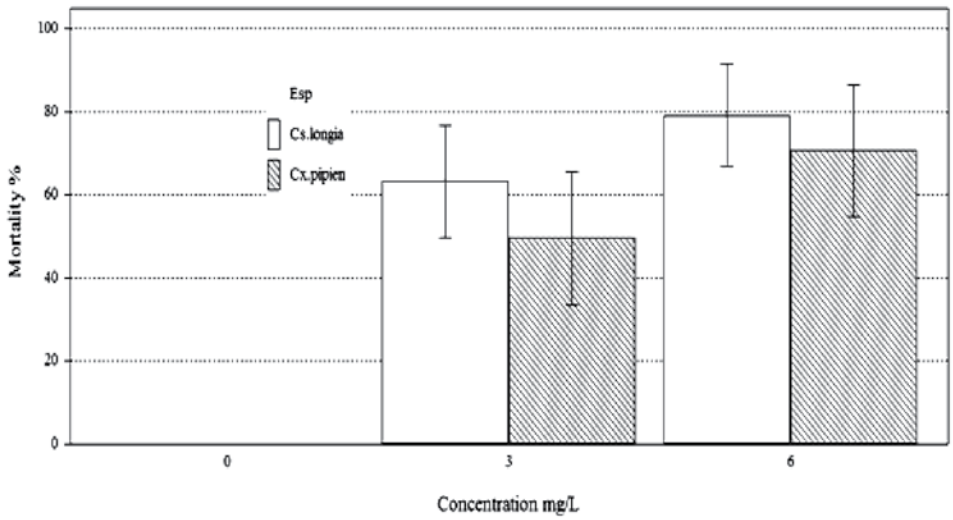

Fig. 3. Sterility Index of *Culex pipiens* and *Culiseta longiareolata*

Culex pipiens, the average number of eggs laid decreased from 91 in the controls to 53 and 39, respectively, at concentrations of 3 and 6 mg/L. The same observation was recorded for *Culiseta longiareolata,* where the mean number of eggs decreased from 97 in the control to 60 and 42, respectively, under treatments of 3 and 6 mg/L (Table 2).

Concerning the decrease in the number of eggs laid (Table 3), in control females, the number was 945 eggs, whereas it was 606 and 425 eggs, respectively, in treated females treated with the two treatment doses. The same reduction was also observed during hatching. The percentage of hatching of the eggs of the treated females of *Culex pipiens* was significantly reduced ($p \leq 0.0001$) for the concentrations 3 and 6 mg/L and had rates of 41% and 63%, respectively. For *Culiseta longiareolata*, the reduction in the number of eggs was estimated to be 978 eggs in the controls and 582 and 536 eggs for 3 and 6 mg/L, respectively, and the percentage of hatching eggs of the treated females decreased significantly ($p = 0.0001$) for both 3 and 6 mg/L at rates or 39 and 63%, respectively (Fig. 2). The effect of treatment (3 and 6 mg/L) on fertility (Tables 3) showed a clear decrease. The calculated percentage reduction showed a decrease of 75% at 3 mg/L and 59% at 6 mg/L for *Culex pipiens*. The fertility of *Culiseta longiareolata* markedly decreased by approximately 94% and 56%, respectively (at 3 and 6 mg/L). The ANOVA for female fertility between control and treated females indicated that the difference is very significant between the two species of mosquito

($p \leq 0.000$). Thus, fertility in females of both species also declined but more distinct in *Culex pipiens* than in *Culiseta longiareolata* ($r=0.83$, $r=0.68$) (Fig. 2).

The index of sterility increased in the treated individuals, indicating that the treatment affected adult sterility. We noticed that the index of sterility increased according to the concentration used. The values of this index were inferior for *Culex pipiens* that for *Culiseta longiareolata* where they reached almost 80% (Fig. 3).

Discussion

Culex pipiens and *Culiseta longiareolata*, which have been examined in this study, are very widespread in the zone of Biskra (Merabti & Ouakid 2011). By this reason, we tested the efficiency of azadirachtin on the last stage of larvae (L4) of the two species. The intense use of pesticides produced side effects on insects, many of which were acute or chronic (Abudulai et al. 2001). Recently, biopesticides of plant origin have been used against several insect vectors, particularly because plant-derived compounds are safer to use, without phytotoxic properties that can harm other species (Schmutterer 1990, Senthil et al. 2004, 2005a, b).

The phytotoxic substances of several families with active ingredients may have direct lethal effects, delay embryo development or affect egg shape (Champagne et al. 1986). Extracts of raw or partially purified plants are cheaper and very efficient for mosquito control compared to purified compounds (Jang et al. 2002). The results of our study show that the tested bioinsecticide was interesting in terms of toxicity. Azadirachtin has a good action against the two tested species. Indeed, LD50 and LD90 of *Culex pipiens* were lower than those of *Culiseta longiareolata.* Our results indicate that plant-based compounds, such as azadirachtin, may be an efficient alternative to conventional synthetic insecticides for the control of *Culex pipiens* and *Culiseta longiareolata.* The treated larvae were morphologically normal but showed a great reduction in fertility. The same results were noted when *Bacillus sphaericus* was tested against the malaria vector *Anopheles stephensi* (Kumar et al. 2013). Pushpalatha (2015) confirmed the effect of the extract of plants on the fecundity and fertility of *Culex quinquefasciatus*.

Khan et al. (2007) demonstrated decreased fecundity of the pest dipterans *Bactrocera cucurbitae* and *B. dorsalis* exposed to a neem compound as a result of blocking ovarian development. Likewise, the mixing of a commercial formulation of neem in the adult diet reduced the fecundity of the dipteran *Ceratitis capitata* by interfering with oogenesis (Di Ilio et al. 1999). Many studies using extracts of the family Meliaceae (e.g. azadirachtin) plants have shown prolonged larval development, reduced pupal weight, oviposition and delayed phenology in the surviving larvae under the effect of sublethal doses (Murugan et al. 1996, Su & Mulla 1999). E. g., 50% inhibition of the emergence of the adult mosquitoes was observed by the use of many plants leaf extracts (Zebitz 1986). The plant family Meliaceae is used as a growth regulator against many insects due to casing prolonged larval development, reduced pupal weight and reduced oviposition (Saxena et al. 1984, Schmutterer 1990, Hammad et al. 2001, Gajmer et al. 2002, Banchio et al. 2003, Wandscheer et al. 2004).

The product examined by us is generally thought to be suitable for inclusion into integrated pest management programs (Ruckin 1992). The effect of the extract on fecundity and hatchability reported in the present study prompts to speculate that its effects are produced through their influence on the neuro-endocrine system. Further studies on the mechanism of action of the extracts and their efficiency are required for the development of a more potent biocontrol agent. The disturbance of the parameters of the reproduction provoked by this product can be explained by the sexual confusion due to the disturbance of the hormonal system of the insect (Stockel et al. 1994) by the active substance being bound on the specific nuclear receivers of the hormone of metamorphosis (Swevers & Latrou 2003). Alternatively, a decrease of the rate of the metabolic processes may occur, which can affect the maturation of eggs and percentage of hatching because most of larvae are developed inside the shell of eggs; however, they do not hatch due to their weak embryonic cuticle, which has no mechanical force to cross the shell of egg.

Conclusion

The present study examines the effect of the azadirachtin on the toxicity of larvae (L4) of *Culex pipiens* and *Culiseta longiareolata* and has shown that this molecule has a remarkable efficiency for control of mosquitoes. We have shown that the mortality is influenced by both dose used and exposure time. The effects of this bioinsecticide on the reproductive parameters of the two mosquito species show that the azadirachtin lowered fertility of females and increased the sterility of males following the dose used.

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