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Bioefficacy of Azadirachtin in Controlling *Culex pipiens pipiens* (Diptera: Culicidae)

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Abstract

Azadirachtin was tested for its effects against First and second instars larvae of mosquito *Culex pipiens pipiens* (Diptera: Culicidae), laboratory reared larvae were exposed to 0.125 ; 0.250; 0.500 and 0.750 mg/ L of azadirachtin in laboratory of biology animal application, biology Department , university of Badji Mokhtar, Annaba, Algeria. Larvicidal assays were conducted according to standard World Health Organization (WHO) .The results have been exploited according to classic statistical methods. A linear correlation was revealed between concentration and larval mortality. At first stage, larval mortality increased from 45.83 % at 0.125 mg /L to 94.44 % at concentration 0.750mg /L of Azadirachtin in direct effect. The lethal concentration LC_{16}, LC_{50} and LC_{90} in direct effect was measured as 0.056; 0.166 and 0.663 mg/L respectively. Cumulate mortality increased from 54.28% to 95.71% at 0.125mg/L and 0.750mg/L respectively. The LC_{16}, LC_{50} and LC_{90} values for Azadirachtin were 0.040 ; 0,127 and 0.555mg/L respectively. At second stage, larval mortality increased from 39.66 % at 0.125 mg /L to 87.66 % at concentration 0.750mg /L of azadirachtin in direct effect, the LC_{16}, LC_{50} and LC_{90} values was 0.063; 0.190 and 0.891 mg/L respectively. In indirect effect the mortality increased from 49.27% to 91.54% at 0.125mg/L and 0.750mg/L respectively, the LC_{16}, LC_{50} and LC_{90} values was 0.041; 0.141 and 0.724 mg/L respectively. After a comparison between the two stages showed that the first stage is the most sensitive than the second stage .The results show that the azadirachtin is promising as a larvicidal agent against *Culex pipiens pipiens* naturally occurring biopesticide could be an alternative for chemical pesticides.

Keywords: Mosquito, *Culex pipiens pipiens,* Azadirachtin, Insecticide.

1. Introduction

Various neem products have been investigated extensively for their phytochemistry and exploitation in pest control programmes. A number of bioactive components have been isolated from various parts of the neem tree *(Azadirachta indica:* Meliacae*).* The Meliaceae plant family is known to contain a variety of compounds, which show insecticidal, antifeedant, growth regulating and development modifying properties (Greger *et al*., 2001; D'Ambrosio and Guerriero, 2002; Nakatani *et al*., 2004). The effects of the compounds extracted from *M. azedarach* on insects have been reviewed by Ascher *et al*., (1995) and reported by Saxena *et al*., (1984), Schmidt *et al*., (1998), Juan *et al*., (2000), Carpinella *et al*., (2003) , Senthil Nathan and Saehoon, (2005). Control of mosquito is essential as many species of mosquitoes are vectors of malaria, filariasis, and many arboviral diseases and they constitute an intolerable biting nuisance (Collins and Paskewitz, 1995). The development of insect's growth regulators (IGR) has received considerable attention for selective control of insect of medical and veterinary importance and has produced mortality due to their high neurotoxic effects (Wandscheer *et al*., 2004; Senthil Nathan *et al*., 2005a). Although, biological control has an important role to play in modern vector control programs, it lacks in the provision of a complete solution by itself. Irrespective of the less harmful and ecofriendly methods suggested and used in the control programmes, there is still need to depend upon the chemical control methods in situations of epidemic out break and sudden increase of adult mosquitoes. Recent studies stimulated the investigation of insecticidal properties of plant derived extracts and concluded that they are environmentally safe, degradable, and target specific (Senthil Nathan and Kalaivani, 2005). Muthukrishnan and Puspalatha (2001) evaluated the larvicidal activity of extracts from *Calophyllum inophyllum* (Clusiaceae), *Rhinacanthus nasutus* (Acanthaceae), against *Anopheles stephensi* (Senthil Nathan *et al*., 2006) were studied for their larvicidal action on fourth instar larva of *Culex quinquefasciatus* (Kalyanasundaram and Dos, 1985). Murugan and Jeyabalan (1999) reported that *Leucas aspera*, *O. santum*, *Azadirachta. indica*, *Allium sativum* and *Curcuma longa* had strong larvicidal, anti-emergence,

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adult repellency and anti-reproductive activity against *A. stephensi*. In addition, *Pelargonium citrosa* (Jeyabalan *et al*., 2003), *Dalbergia sissoo* (Ansari *et al*., 2000a) were shown to contain larvicidal and growth inhibitory activity against *A. stephensi.* With these backgrounds this present study was aimed to assess the larvicidal activity of azadirachtin on first and second instar larvae of *Culex pipiens pipiens* under laboratory condition.

2. Materials and Methods

2.1. Mosquitoes

Culex pipiens pipiens eggs were collected from cellarage tribes (region sidi amar - Annaba) and readed in the laboratory of biology animal application, university of Annaba- Algeria. Larvae were placed in Pyrex storage jars (80 by 100mm) containing 200 ml of tap water. They were maintained at 25-27°C, 75-85% relative humidity under 14:10 light and dark photo period cycle. The larvae were fed with fresh food consisting of a mixture of Biscuit Petit Regal-dried yeast (75:25 by weight). Pupae were transferred from the trays to a cup containing tap water and placed in screened cages (20x20x20cm) where the adult emerged. After emergence, female mosquitoes obtained blood meal from caged pigeons while male mosquitoes were fed on a 10% sucrose solution. Then egg-masses were kept to continue next generation.

2.2. Laboratory Bioassay procedure

The selected insecticides were evaluated against the against the 1st and 2nd instars larvae of mosquito *Culex pipiens pipiens (Diptera: Culicidae)* using the standard bioassay technique (WHO). The Bioassays were performed with using concentration from 0.125; 0,250; 0,500 and 0,750mg/L of the azadirachtin. For mortality studies, 25 larvae of 1st and 2nd instars were introduced in Pyrex storage jars (80 by 100mm) containing various concentrations of the azadirachtin. A control was maintained. The treatments were replicated five times and each replicate set contained one control. The percentage mortality was calculated by using the formula (1) and corrections for mortality when necessary were done using Abbot's (1925) formula (2).

$$\text{Percentage of mortality} = \frac{\text{Number of dead larva}}{\text{Number of larvae introduced}} \times 10 \quad (1)$$

$$\text{Corrected percentage of mortality} = 1 - \frac{n \text{ in T after treatment}}{n \text{ in C after treatment}} \times 100 \quad (2)$$

Where n = number of larvae, T = treated, C = control.

2.3. Statistical Analysis

For larval bioassay under laboratory conditions, the differences between the LC_{16}, LC_{50} and LC_{90} values are considered significant if their fiducial limits (95%) did not overlap as mentioned by Litchfield and Wilcoxin (1949).

In addition, statistical analysis was carried out for all the estimated measurements of treatments and compared with the control values by test ANOVA and Student's *t*-test using the computer program (MINITAB, version 13).

3. Results

3.1. Insecticidal activity

Results of treating early the first and second instar larvae of *C. pipiens pipiens* with different concentrations follow: 0.125; 0.25; 0.50 and 0.75 mg/L of azadirachtin exhibited insecticidal activity with a dose – response relationship. Moreover, this compound presented toxicity by direct action on the treated larval instars but also by differed action (cumulate mortality) on t he other following stages of development

3.1.1. Effect Direct

For first stage, the highest concentration tested 0,750mg/L in direct effect, caused 94,44% mortality and under concentration caused 45,83% mortality presented in Figure 1. With probit, the regression equation as Y = 2,13X+0,90, the LC_{50} was calculated as 0,166 mg/L (95% Cl=0,139 – 0,197 mg/L), LC_{16} as 0,056mg/L and LC_{90} was 0,663 mg/L presented in Table 2. After treatment the second stage, in direct effect, the highest concentration, caused 87,66% mortality and less concentration caused 39.66% mortality of larvae (Figure 3) , the LC_{50} was 0,190 mg/L (95% Cl=0,157-0,230 mg/L) , LC_{16} as 0,063 mg/L and the LC_{90} was 0,891 mg/L presented in Table2. After a comparison between the two stages showed that the first stage is the most sensitive than the second stage, because the percentage of mortality at the first stage is high of mortality at the second stage, and the LC_{50} and LC_{90} of first stage is less that the LC_{50} and LC_{90} of second stage presented in Figure 5.

3.1.2. Effect Indirect (Cumulated Effect)

Dose response relationship was determined for azadirachtin applied for first and second instar larvae, the mortality was scored up t o adult formation. After treatment the first stage, the highest concentration tested 0,750 mg/L in indirect effect, caused 95,71% mortality and under concentration caused 54,28% mortality, presented in Figure 2. The LC_{50} was calculated as 0,127 mg/L (95% Cl=0,106-0,152 mg/L), LC_{16} as 0.040mg/L and the LC_{90} was 0,555 mg/L, presented in table1. After treatment the second stage, in indirect effect, the highest concentration, caused 91,54% mortality and less concentration caused 49,27% mortality of larvae (Figure 4) , the LC_{50} was 0,141 mg/L (95% Cl=0,114-0,173mg/L) , LC_{16} as 0,041mg/L and the LC_{90} was 0,724 mg/L presented in Table2. After a comparison between the two stages showed that the first stage is the most sensitive than the second stage, because observes an increase of mortality always of the first larval stage compared to the second stage and lethal concentrations at the first stage is less that the second stage, presented in Figure 6.

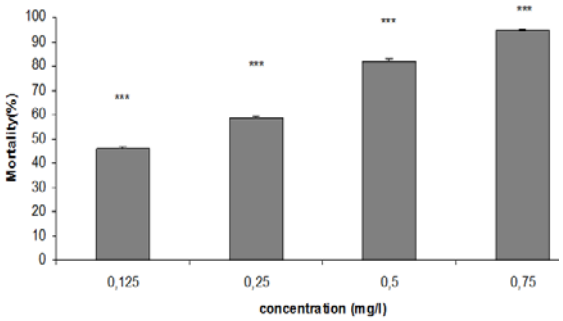

Figure 1. Larvicidal activity of azadirachtin against the first instars larvae of *Culex pipiens pipiens (effect direct)* (data following by *** are significantly different to concentrations, $p \leq 0.001$)

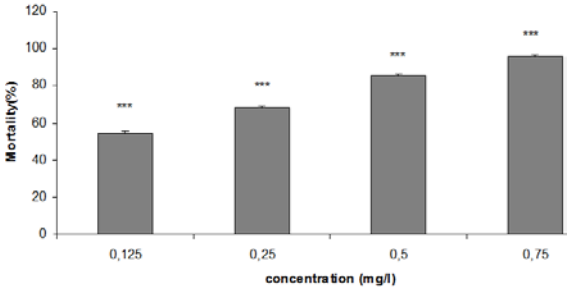

Figure 2. Larvicidal activity of azadirachtin against the first instars larvae of *Culex pipiens pipiens (effect indirect)* (data following by *** are significantly different to concentrations, $p \leq 0.001$).

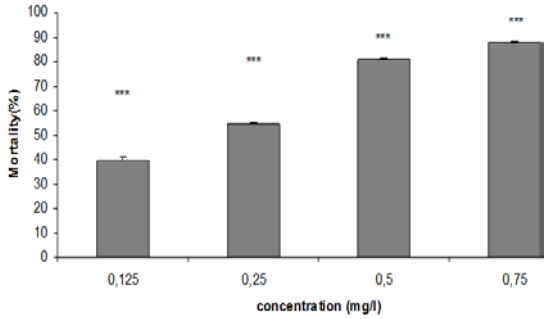

Figure 3. Larvicidal activity of azadirachtin against the second instars larvae of *Culex pipiens pipiens (effect direct)* (data following by *** are significantly different to concentrations, $p \leq 0.001$)

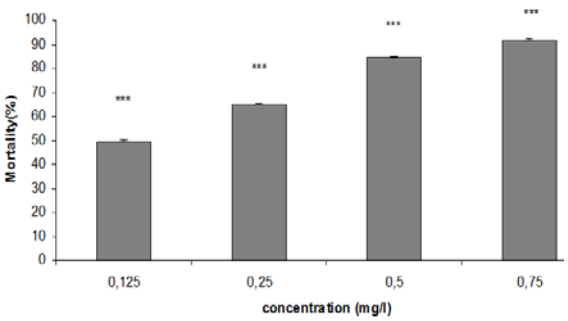

Figure 4. Larvicidal activity of azadirachtin against the second instars larvae of *Culex pipiens pipiens (effect indirect)* (data following by *** are significantly different to concentrations, $p \leq 0.001$)

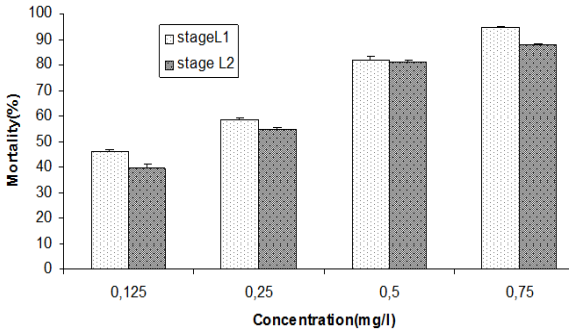

Figure 5. Effect of the azadirachtin on the two stages: comparison of mortality between the first and second instars larvae of *Culex pipiens pipiens (effect direct)*

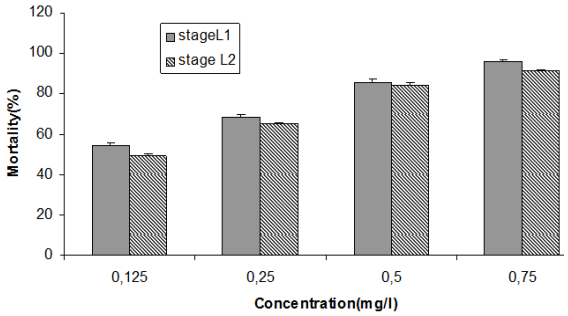

Figure 6. Effect of the azadirachtin on the two stages: comparison of mortality between the first and second instars larvae of *Culex pipiens pipiens (effect indirect).*

Table 1. Larvicidal activity of azadirachtin at various concentrations applied on first instar larvae of *Culex pipiens pipiens* at 24 hrs exposure period.

Effects	LC_{50} (mg/l)	95% Confidence limits(mg/l)		LC_{16} (mg/l)	LC_{90} (mg/l)	Regression equation	χ^2
		Lower	Upper				
Direct	0,166	0,139	0,197	0,056	0,663	Y=2,13X+0,27	2.54
Indirect	0,127	0,106	0,152	0,040	0,555	Y=2,00X+0,79	0.81

Table 2. Larvicidal activity of azadirachtin at various concentrations applied on second instar larvae of *Culex pipiens pipiens* at 24 hrs exposure period.

Effects	LC_{50} (mg/l)	95% Confidence limits(mg/l)		LC_{16} (mg/l)	LC_{90} (mg/l)	Regression equation	χ^2
		Lower	Upper				
Direct	0,190	0,157	0,230	0,063	0,891	Y=1,89X+0,69	4.11
Indirect	0,141	0,114	0,173	0.041	0,724	Y=1,80X+1,13	2.22

4. Discussion

In the present study azadirachtin have displayed varied toxicity on first and second instar larvae of *Culex pipiens pipiens.* The results showed that an increase in mortality with the increase in concentration and the early instar larvae are much susceptible than the later ones.

Neem products are capable of producing multiple effects on a number of insect species, such as antifeeding effects, growth regulation, fecundity suppression and sterilization (Mulla and Su, 1999; Vatandoost and Vaziri, 2004; Kondo *et al.*, 2004). Azadirachtin proved to be highly efficient to larva of *C. pipiens pipiens.* At first stage, larval mortality increased from 45,83 % at 0,125 mg /L to 94,44 % at concentration 0,750mg /L of Azadirachtin in direct effect. The lethal concentration LC_{16}, LC_{50} and LC_{90} in direct effect was measured as 0,056; 0,166 and 0,663 mg/L respectively. Cumulate mortality increased from 54,28% to 95,71% at 0,125mg/L and 0,750mg/L respectively. The LC_{16}, LC_{50} and LC_{90} values for Azadirachtin were 0,040; 0,127 and 0,555mg/L respectively.At second stage, larval mortality increased from 39.66 % at 0,125 mg /L to 87,66 % at concentration 0.750mg /L of azadirachtin in direct effect, the LC_{16}, LC_{50} and LC_{90} values was 0,063; 0,190 and 0,891 mg/L respectively. In indirect effect the mortality increased from 49,27% to 91,54% at 0,125mg/L and 0.750mg/L respectively, the LC_{16}, LC_{50} and LC_{90} values was 0,041; 0,141 and 0,724 mg/L respectively. However, the results which reflect the high toxicity of azadirachtin to the developmental stages (larva, pupa and adult). The results confirmed other studies concerning this compound (Alouani *et al*., 2009). In insecticidal experiment conducted on mosquitoes with compounds extracted from *Az. Indica* using a co mmercial preparation Neemarin showed mortality for fourth instar larvae of *An. stephensi*, with LC_{50} values of 60 and 43 ppm, respectively (Ruskin, 1992) .This compares with the LC_{50} in our study of 0.184 and 0.125 mg /Liter respectively for first instar larvae of *C. pipiens pipiens.* Our results were comparable with findings from other researchers as shown. The variation in LC_{50} is due to mosquito species, formulation, climate and method of application. Neem extracts act like insect growth regulators, so the mortality at different stages were considered. Mortality of the pupae stage was significantly higher than the larvae and adult stages. In addition, the mortality of *Cx. quinquefasciatus* was significantly lower than *An. Stephensi* (Vatandoost and Vaziri, 2004). In another study, Ndung'u *et al.* (2004) reported that (LC_{50} =57,1 mg/Liter) of Azadirachtin when tested against larvae of *Anopheles gambiae* (Essam *et al*., 2006). Azadirachtin the extract of neem tree, tested in the present study is reported to be eco-friendly and not toxic to vertebrates (Al- Sharook *et al*., 1991). It is clearly proved that crude or partially purified plant extract are less expensive and highly efficacious for the control of mosquitoes rather than the purified compounds or extract (Jang *et al*., 2002; Cavalcanti *et al*., 2004). The larvae of a number of mosquito species (*Aedes spp., Anopheles spp*.) are sensitive to neem products and show antifeedant and growth regulating effects (Zebitz, 1987; Murugan *et al*., 1996). The effect of these crude plant extract on the biology, reproduction and adult emergence of the mosquitoes are remarkably greater than those reported for other plant extracts in the literature. For example 50% inhibition of the emergence of the adult mosquitoes was observed by the use of *C. inophyllum*, *S. suratense*, *S. indica* and *Rhinocanthus nasutus* leaf extracts (Muthukrishnan and Puspalatha, 2001). Similarly 88% of the adult mortality was observed by the use of *P. citrosa* leaf extracts at 2% concentration (Jeyabalan *et al*., 2003). The Meliaceae plant family is used a g rowth regulator against many insect pests (Saxena *et al*., 1984; Jacobson, 1987; Schmutterer, 1990; Gajmer *et al*., 2002; Banchio *et al*., 2003; Wandscheer *et al*., 2004).

The growth regulatory effect is the most important physiological effect of *M. azedarach* on insects. It is because of this property that family Meliaceae has emerged as a potent source of insecticides. The results of this study indicate the plant-based compounds such as azadirachtin (compounds present in the Meliaceae plant family seed) may be effective alternative to conventional synthetic insecticides for the control of *Culex pipiens pipiens* Undoubtedly, plant derived toxicants are a valuable source of potential insecticides. These and other naturally occurring insecticides may play a more prominent role in mosquito control programs in the future (Mordue and Blackwell, 1993). The results of this study will contribute to a great reduction in the application of synthetic insecticides, which in turn increase the opportunity for natural control of various medicinally important pests by botanical pesticides. Since these are often active against a limited number of species including specific target insects, less expensive, easily biodegradable to non-toxic products, and potentially suitable for use in mosquito control programme (Alkofahi *et al*., 1989), they could lead to development of new classes of possible safer insect control agents.

Plant allelochemicals may be quite useful in increasing the efficacy of biological control agents because plants produce a large variety of compounds that increase their resistance to insect attack (Berenbaum, 1988; Murugan *et al*., 1996; Senthil Nathan *et al*., 2005a). Recently, bio-pesticides with plant origins are given for use against several insect species especially disease- transmitted vectors, based on the fact that compounds of plant origin are safer in usage, without phytotoxic properties; also leave no s cum in the environment (Schmutterer, 1990; Senthil Nathan *et al*., 2004, 2005a, b).

The intensive use of pesticides produces side effects on many beneficial insects and also poses both acute and chronic effects to the *milieu* (Abudulai *et al*., 2001). The most interesting observation in the present study was the deformations observed in the Azadirachtin treatment larvae, pupa and adult of *C. pipiens pipiens* are in accord with the characteristic manifestation of exposure to other insect growth regulators and insect growth inhibitors such Flucycloxuron (Andalin), and Triflumuron (Alsystin) realized at the same conditions for treatment and laboratory conditions (Rehimi and Soltani, 1999). The present study clearly proved the efficacy of Azadirachtin on larvae, of *Culex pipiens pipiens* further studies such as mode of action, synergism with the biocides under field condition are needed.

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