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Larvicidal Activity of a Neem Tree Extract (Azadirachtin) Against Mosquito Larvae in the Republic of Algeria

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Abstract

An insecticide containing azadirachtin, a tree (*Azadirachta indica*) extract, was tested against *Culex pipiens* mosquito larvae and pupae in east of the Republic of Algeria under laboratory conditions. First, after treatment of larval stage, LC_{50} and LC_{90} values for Azadirachtin were 0.35 and 1.28 mg/L in direct effect and 0.3-0.99 mg/l in indirect effect, respectively. Second, after treatment of the pupal stage, the LC_{50} and LC_{90} in direct effect were measured as0.42 -1.24mg/l and in indirect effect was 0.39mg/l-1.14mg/l respectively. Mosquito adult fecundity were markedly decreased and sterility was increased by the Azadirachtin after treatment of the fourth instar and pupal stage. The tretment also prolonged the duration of the larval stage. The results show that the Azadirachtin is promising as a larvicidal agent against *Culex pipiens*, naturally ocurring biopesticide could be an alternative for chemical pesticides.

الملخص

تناول هذا البحث دراسة تأثير مبيد نباتي Azadirachtin المستخلص من شجرة Azadirachta indica ، على يرقات وعذارى البعوض في شرق جمهورية الجزائر في شروط المختبر، حيث حسبت الجرعة المميتة للنصف (LC_{50}) والجرعة المميتة لتسعين بالمئة (LC_{90}) بالنسبة لبعوض Culex pipiens وهي أكثر العوامل نقلا لبعض الأمراض الإلتهابية على الصعيد المحلي ،حيث قدرت بعد معاملة الطور الرابع LC_{50} =0.35 ملغ /ل وLC_{90}= 1.28ملغ/ل هذا بالنسبة إلى التأثير المباشر، أما التأثير الغير مباشر فكانت 0.30 ملغ/ل و 0.99 ملغ/ل على التوالي، أما عند معاملة طور العذراء كانت النتائج بالنسبة للتأثير المباشر (LC_{50} =0.42 ملغ /ل وLC_{90}= 1.24ملغ/ل) أما التأثير الغير مباشر فكانت 0.39 ملغ/ل و 1.14 ملغ/ل على التوالي. كذلك لوحظ انخفاض كبير في نسبة الخصوبة وزادة مؤشر العقم للإناث الناتجة عن معاملة الطور الرابع والعذارى للبعوض ، وزيادة في مدة الطور. حيث نقول في الأخير أنه يمكن استعمال هذا المبيد كمبيد طبيعي بدل المبيدات الكيميائية.

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

1. Introduction

The Meliaceae plant family is known to contain a variety of compounds that show insecticidal, antifeedant, growth-regulating, and development-modifying properties (Nugroho et al., 1999; Greger et al., 2001; D'Ambrosio and Guerriero, 2002; Nakatani et al., 2004). *Melia azedarach* L. and *Azadirachta indica* (Sapindales: Meliaceae), commonly known as Chinaberry or Persian lilac tree, are deciduous trees that are native to northwestern India; and have long been recognized for their insecticidal properties. These trees typically grow in the tropical and subtropical parts of Asia, but nowadays they are also cultivated in other warm regions of the world because of their considerable climatic tolerance. Fruit extracts of *Melia azedarach and Azadirachta indica* elicit a variety of effects in insects such as antifeedant, growth retardation, reduced fecundity, moulting disorders, morphogenetic defects, and changes of behavior (Schmidt et al., 1998; Abou Fakhr Hammad et al., 2001; Gajmer et al., 2002; Banchio et al., 2003; Wandscheer 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 (Youdeowei and Service, 1983; Curtis, 1994; Collins and Paskewitz, 1995). Biotechnologists and entomologists agree that mosquito control efficiency should be with selectivity for a specific target organism. New control methodologies aim at reducing mosquito breeding sites and biting activity by using a combination of chemical–biological control methods soothing several advocated biocontrol methods to reduce the population of mosquito and to reduce the man–vector contact (Service, 1983). Recently, there has been a major concern for the promotion of botanicals as environment friendly pesticides, microbial sprays, and insect growth regulators amidst other control measures such as beneficial insects and all necessitate an integration of supervised control (Ascher et al., 1995; Senthil Nathan et al., 2004, 2005b, c, and d). The development of insects' growth regulators (IGR) has received considerable attention for selective control of insect for medical and veterinary importance and has produced mortality due to their high neurotoxic effects (Wandscheer et al., 2004; Senthil Nathan et al.,

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2005a). Although, biological control has an important role to play in modern vector control programs, it lacks the provision of a complete solution by itself. Irrespective of the less harmful and eco-friendly methods, suggested and used in the control programmers, there is still a need to depend upon the chemical control methods in situations of epidemic outbreak and sudden increase of adult mosquitoes. Hence, insecticides are known for their speedy action and effective control during epidemics. Nonetheless, they are preferred as effective control agent to reduce the mosquito population irrespective of their side effects. 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), *Solanum suratense* (Solanaceae) and *Samadera indica* (Simaroubaceae), *Myriophyllum spicatum* (Haloragaceae) against *Anopheles stephensi* (Senthil Nathan et al., 2006). Several indigenous plants in India and subtropical parts of Asia, such as *Ocimum basilicum*, *Ocimum santum*, *Azadirachta indica*, *Lantana camera*, *Vitex negundo* and *Cleome viscose* (Senthil Nathan et al., 2006) were studied for their larvicidal action on the field which collected fourth instar larva of *Culex quinquefasciatus* (Kalyanasundaram and Dos, 1985). Chavan (1984), Zebitz(1984,1986), Schmutterer (1990), Murugan and Jeyabalan (1999) reported that *Leucas aspera*, *O. santum*, *Azadirachta. indica*, *Allium sativum* and *Curcuma longa* had a strong larvicidal, antiemergence, adult repellency and antireproductive activity against *A. stephensi*. In addition, *Pelargonium citrosa* (Jeyabalan et al., 2003), *Dalbergia sissoo* (Ansari et al., 2000a) and *Mentha piperita* (Ansari et al., 2000b) were shown to contain larvicidal and growth inhibitory activity against *A. stephensi.* The present investigation was conducted to study the effect of Azadirachtin, a neem tree *Azadirachta indica* extract, against larvae and pupae of *Culex pipiens* mosquitoes in east of the Republic of Algeria.

2. Materials and Methods

2.1. Mosquito Rearing

Culex 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 reared in plastic and enamel trays in 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. Bioassays and Larval Mortality

Bioassays were performed with fourth larvae stages and pupae of *C. pipiens* using concentration from 0.125; 0.250; 0.500; 0.750 and 1mg/l of the Azadirachtin. A minimum of 25 larvae/concentration were used for all the experiments. And these experiment were replicated five times. For mortality studies, 25 larvae each of fourth instar and pupae were introduced in 250 ml plastic beaker 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)

$$\text{Percentage of Mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \text{ X100} \quad (1)$$

$$\text{Corrected percentage of mortality} = 1 - \frac{n \text{ in T after treatment}}{n \text{ in C after treatment}} \text{ X100} \quad (2)$$

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

2.3. Fecundity and Sterility

The fecundity experiments were conducted by taking equal number of male and female *C. pipiens* which had emerged from the control and treated sets of each concentration. They were mated in the cages of (20 x 20 x 20) cm dimension individually to each concentration. Three days after the blood meal, eggs were collected daily from the small plastic bowls containing water kept in ovitraps in the cages. The fecundity was calculated by the number of the eggs laid in the ovitraps divided by the number of female let to mate (The death of the adult in the experiment was also considered) The Sterility Indices experiments were conducted by the formula (3) of Sexina et al ,(1993):

$$SI = 100 - \frac{\text{Total number of eggs of females treated x percentage of hatch}}{\text{Total number of eggs of control x percentage of hatch}} \quad (3)$$

2.4. Total Larval and Pupal Duration

To assay the growth factors of *C. pipiens*, test solution of concentration of Azadirachtin extract (0.125; 0.250; 0.500; 0.750 and 1mg/l) were used. A known number of eggs were made to hatch and the total larval duration (days) was calculated from hatching to pupation period, the pupa was placed in a small container closed with a transparent mesh, so that the adults were kept trapped. The pupal duration (days) was calculated from the pupal molt to the emergence of imago.

2.5. Statistical Analysis

The analysis program Probit (Finney, 1971), the lethal concentrations (µg/ml) for 50%, and 90% of the mortality, LC_{50} and LC_{90}, respectively, were at 24h after treatment.

Table 1. Larvicidal activity of Azadirachtin at various concentration applied for 24h to newly ecdysed fourth instars of *Culex pipiens.*

Effects	Concentration (mg/l)	Mortality (%)	LC_{50}(mg/l)	95%Confidancelimits (µg/ml)		LC_{90} (mg/l)	Regression equation	χ^2
				Lower	Upper			
Direct	Control	9.6						
	0.125	17.39						
	0.250	33.62	0.357	0.307	0.414	1.280	Y=2.20x-0.61	0.615
	0.500	60.17						
	0.750	76.39						
	1	85.24						
Indirect	Control	15.2						
	0.125	19.80						
	0.250	35.52	0.304	0.286	0.335	0.992	Y=2.63x-1.60	3.69
	0.500	65.40						
	0.750	81.12						
	1	93.70						

Table 2. Larvicidal activity of Azadirachtin at various concentration, applied for 24h to newly ecdysed pupae of *Culex pipiens*.

Effects	Concentration (mg/l)	Mortality (%)	LC_{50}(mg/l)	95%Confidancelimits (mg/l)		LC_{90} (mg/l)	Regression equation	χ^2
				Lower	Upper			
Direct	Control	4.33						
	0.125	7.57						
	0.250	28.78	0.426	0.379	0. 479	1.243	Y=2.76x-2.2	1.1625
	0.500	45.45						
	0.750	66.66						
	1	87.87						
Indirect	Control	4						
	0.125	7.93						
	0.250	31.74	0.398	0.355	0.445	1.141	Y=2.82x-2.3	1.485
	0.500	49.20						
	0.750	71.42						
	1	92.05						

The 95% confidence intervals, values, and degrees of freedom of the χ^2 goodness of fit tests, and regression equations, were recorded .Whenever the goodness of χ^2 was found to be significant (p <0.05) , a heterogeneity correction factor was used in the calculation of confidence limits. Data from biology, mortality, fecundity deterrence and effective concentration were subjected to analysis of variance (ANOVA of arsine square root transformed percentages).

3. Results

3.1. Insecticidal Activity

Dose-response relationship was determined for Azadirachtin applied for 24h to newly ecdysed fourth instar larvae and pupae. The mortality was scored up to adult formation.

For fourth stage, the highest concentration tested 1 mg / l in direct effect caused 85.24% mortality (Figure1). With probit, the LC_{50} was calculated as 0.35 mg/l (95% CI=0.30-0.41mg/l; n=75; Slope=2.83) and the LC_{90} was 1.28 mg/l, (Table1). For indirect effects, the highest concentration caused 93.70% mortality (Figure2), the LC_{50} was 0.32 mg/l (95% CI=0.28-0.33 mg/l; n=75; Slope=2.46) and LC_{90} was calculated as 0.99 mg/l, respectively, (Table1). After treatment, during the pupal stage and in direct effect, the highest concentration caused 87.87% mortality (Figure1), the $LC_{50}$was 0.42 mg/l (95% CI=0.37-0.47mg/l; n=75; Slope=2.83) and the LC_{90} was calculated as 1.24 mg/l, (Table 2). For indirect effects the LC_{50} was 0.39 mg/l (95% CI=0.35-0.44 mg/l; n=75; Slope=2.46) and LC_{90} was calculated as 0.99 mg/l respectively, the highest concentration tested caused 92.05% mortality (Table2, Figure2).

3.2. Effect on Fecundity, Sterility and growth

Adults fecundity also was markedly decreased by the Azadirachtin treatment the fourth instar larvae and pupal stages (Figure 3, 4). Adults' sterility indices were markedly increased by the Azadirachtin treatment, (Figure 5, 6). An adverse sub-lethal effect in pupa exposed to Azadirachtin

was evident. In addition to significantly lower survivorship and protracted development, larval duration was reduced markedly. The plant extracts (Azadirachtin) drastically reduced the fecundity of the females, and only few adults survived. The duration of larval instars and the total developmental time were prolonged. (Table3). In the present study, application of Azadirachtin greatly affected the growth of *Culex pipiens* .

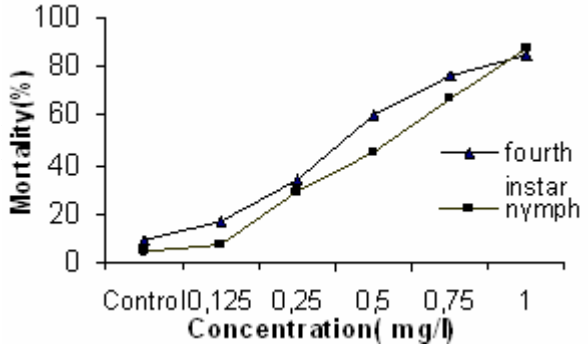

Figure1. Dose-response relationship for treatment of Azadirachtin, applied for 24h to newly ecdysed fourth instar larvae and pupae of *Culex pipiens* (*effect direct*).

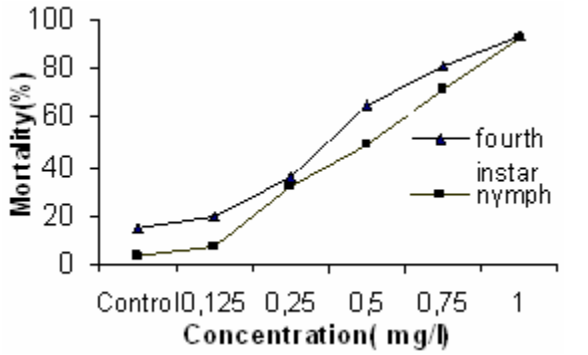

Figure 2. Dose-response relationship for treatment of Azadirachtin, applied for 24h to newly ecdysed instar larvae and pupae instars of *(Italique) Culex pipens*(effect indirect).

4. Discussion

Azadirachtin, the extract of neem tree, was tested in the present study, and 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 extracts are less expensive and highly efficacious for the control of mosquitoes rather than the purified compounds or extracts (Jang et al., 2002; Cavalcanti et al., 2004).

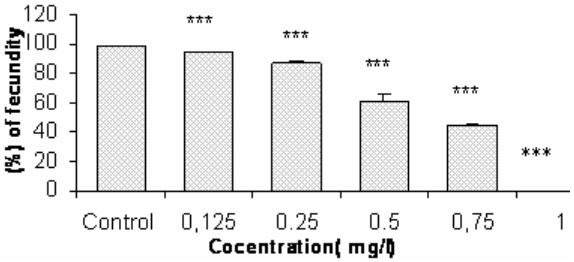

Figure 3. Fecundity of *C. pipiens* females after treated the fourth instars with Azadirachtin.(Data following by *** are significantly different from control, p<0.001)

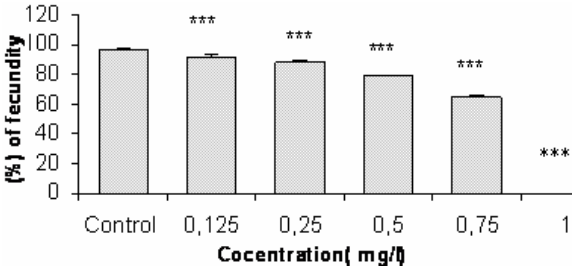

Figure 4. Fecundity of *(Italique) Culex pipens* females after treating the pupae stages with Azadirachtin.(Data following by *** are significantly different from control, p<0.001).

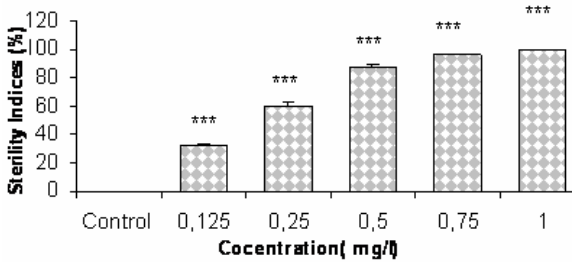

Figure5. Sterility Indices of *C. pipiens* females after treating the fourth instars with Azadirachtin.(Data following by *** are significantly different from control, p<0.001).

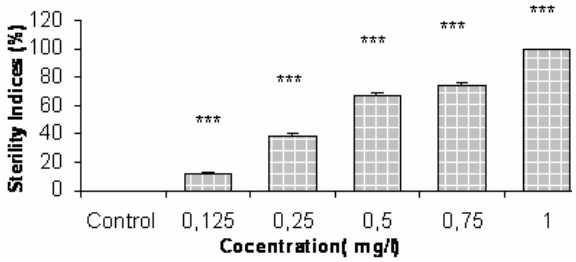

Figure 6**.** Sterility Indices of *C. pipiens* females after treating the pupae with Azadirachtin. (Data following by *** are significantly different from control, p<0.001)

Table 3. Effect of Azadirachtin applied to newly ecdysed 4th instar larvae of C. *pipiens* on the duration of development.

Concentration (mg/l)	Duration (days)Mean±SE	
	4th larval instar	Pupal stage
0	8.75±2.99	2.61±0.65
0.125	15.24±1.28	3.81±0.84
0.250	16.00±2.82	3.54±0.55
0.500	17.94±1.54	3.42±0.87
0.750	18.45±2.80	3.33±0.28
1	19.75±2.22	3.33±0.46

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 as growth regulator against many insect pests (Saxena et al., 1984; Jacobson, 1987; Schmutterer,

1990; Hammad et al., 2001; 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. Exposure of *A. stephensi* larvae to sub-lethal doses of neem leaves extract in the laboratory prolonged larval development, reduced pupal weight and ovipostion (Murugan et al., 1996; Su and Mulla, 1999). In the field, delayed phenology of surviving larvae and reduced pupal weight are common occurrence after treatment with neem (Zebitz, 1984, 1986). The direct and indirect contribution of such effects to treatment efficacy through reduced larval feeding and fitness need to be properly understood in order to improve the use of *M. azedarach* for management of *A. stephensi*. The results of this study indicate the plant-based compounds such as Azadirachtin (compounds present in the Meliaceae plant family seed) may be an effective alternative to conventional synthetic insecticides for the control of *Culex pipiens*. Undoubtedly, plant derived toxicants are valuable sources 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 will 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). 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). 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 scum in the environment (Schmutterer, 1990; Senthil Nathan et al., 2004, 2005a, d). Large alterations in the fecundity and sterility of insects exposed to neem have been extensively reported, such as those in the fly, *Ceratatis capitata* (Steffens and Schmutterer 1982); banana root borer, *Cosmopolites sordidus* (Germar) (Musabyimana et al., 2001); and mosquitoes, *A. stephensi* and *A. culicifacies* (Dhar et al., 1996). The work published by Khan et al., (2007) microscopically demonstrated that the decrease in fecundity of *Bactocera cucurbitae* and *Bactocera dorsalis* exposed to neem compound was due to the block of ovarian development. Likewise, mixing of a commercial formulation of neem in the adult diet caused reduction in the fecundity of *C. capitata* by interfering with oogenesis (Di Ilio et al., 1999). The block in the ovarian activity of *C. capitata,* resulting from neem compound, was verified by histological observation (Di Ilio et al., 1999). Results from the study of Lucantoni et al., (2006) clearly indicated that the neem treated female mosquito, *A. stephensi*, displayed a delay in oocyte development in the vitellogenesis. As discussed by Weathersbee III and Tang (2002), the disruption of reproductive capability could lead to substantial population decline over time. Furthermore, Dhar et al., (1996) revealed that exposure to neem extract suppressed rather than inhibited oviposition in mosquitoes. The present study clearly proved the efficacy of Azadirachtin on larvae, pupae, and adult of *Culex pipiens*. Further studies such as mode of action and synergism with the biocides under field condition are needed.

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