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Biological Control Test of Ethanol Extracts Of Peganum Harmala (L.) on The Mortality And Development of Culex Pipiens (Diptera)

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ABSTRACT

The Culicidae family constitutes a plague of health risks. These biting insects are harmful to human populations and pass on infectious diseases such as malaria. Since chemical control has limited effectiveness against mosquitoes, biological control has become more prevalent. We conducted toxicological tests with an ethanolic extract of fresh leaves of Peganum harmala on Culex pipiens larvae (L4). The results show a larvicidal effect that resulted in an LD 50% in 10.17 days for a 2 g/L dose and an LD 50% in 5.76 days for a 4 g/L dose. A sub-lethal dose was tested on the different stages of the studied species, and a significant difference was observed between the duration until hatching in female eggs from the control group and eggs from the treated group (P = 0.01). There was a considerable prolongation of each stage of the development cycle in the treated group, in the egg-laying period, in the hatching period, and also in the duration until the emergence of the adults. These results show that P. harmala causes retardation and inhibition of moulting and development of Culex pipiens.

Keywords: *Culex pipiens; Peganum harmala; ethanolic extract; toxicity; development; fertility.*

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INTRODUCTION

Among the different groups of insects that exist, mosquitoes are one of the most dangerous and most feared both for the inconveniences they cause as well as for the diseases that they can inoculate during blood meals. To control these vector diseases, very large quantities of larvicides, in the form of synthetic chemicals, are discharged into the aquatic environment. However, some chemicals used in this struggle have become less effective due to the resistance developed by some mosquitoes (OMS 1963, Chandre et al. 1997). Scientists have turned to the use of alternative natural controls such as bactericides, fungicides, acaricides, and especially plant extracts (Tabashnik 1994). The potential for plant extracts to be used as insecticides has been known for a long time, and some plant extracts such as pyrethrum, nicotine and rotenone are already known insect control agents (Crosby et al. 1966). We will cite for this purpose the work of (Jang et al. 2002) on A. aegypti and C. pipiens in which the larvicidal activity of certain legumes was tested and the work of (Alaoui Slimani 2002) in which the toxicity of Mentha pulegium (Lamiaceae) was confirmed on Culicidae larvae. In addition to the direct effects of these molecules on mortality, the indirect effects of these substances on the development, reduction of fertility in females and infertility in males has attracted the attention of several researchers (Pushpalatha 2015). It is in this context that we studied the effects of ethanolic extracts of fresh leaves of P. harmala on the L4 stage of Culex pipiens and the effects of sub-lethal doses on the life cycle and the reproductive performance of this species.

MATERIALS AND METHODS:

1.1. Larval sampling and breeding:

Larvae of C. pipiens were collected from different cottages in Annaba, Algeria (36° 47′53, 84″ N 7° 39′ 46, 66″ E). The larvae were reared in storage jars containing 500 ml of spring water and maintained at 25-30°C, 85% RH and a photoperiod of 14:10 (light;dark). Larvae were fed daily with fresh food consisting of a mixture of biscuit-dried yeast (75:25 by weight), and the water was changed every four days (Benhissen et al. 2016). The feeding continued until the larvae transformed into the pupal stage. The pupae were transferred from the trays to a cup containing spring water and placed in screened cages (30×30×30 cm) where the adults emerged. After emergence, female mosquitoes obtained a blood meal from caged pigeons, while male mosquitoes were fed a 10% sucrose solution. Then, egg masses were kept to continue the next generation (Merabti et al. 2016).

1.2. Peganum harmala (Harmal):

Leaves of P. harmala were collected in the southeast region of Algeria (34° 25′ 44″ N, 5° 03′ 51″ E, Ouled Djellal, Biskra) in the spring. This plant grows in semi-arid rangeland. The plant is used traditionally as an emmenagogue and as an abortifacient agent in North Africa. All parts of the plant are thought to be toxic, and severe intoxication occurs in domestic animals after consumption (Mahmoudian et al. 2002).

1.3. Ethanol extraction:

Fresh leaves of Peganum harmala (300 g) were macerated in ethanol in the shade within 24 hours. After filtration, the solvent was evaporated to dryness under a vacuum with a Rotavapor at 60 °C, and the residues obtained were kept cold

until the tests were carried out. The initial concentration used was 300 g/L.

1.4. Toxicological tests:

The treatment of C. pipiens was inspired by the technique of standardized sensitivity tests conducted by the World Health Organization (OMS 1963). The tests were carried out in 200 ml beakers, each containing 20 C. pipiens larvae (L4) in 100 ml of spring water and 50 ml of extract. Each concentration was applied three times, with a preparation of 20 larvae of C. pipiens as a control. The number of dead individuals (L4 larvae, nymphs and adults) was measured daily for 15 days.

1.5. Effects of P. harmala on the development of Culex pipiens:

2.5.1 Hatching time: Eggs spawned by the females of the treated and control groups were separated to observe the exact time to hatch, measured as the day of laying to the day of the appearance of the first larva.

2.5.2 Duration of each stage of development: After egg-laying, hatching is the first larval stage, and it includes several moults (L2, L3, and L4). The duration of each stage was measured in days, beginning at the start of the larval stage (L1) immediately after hatching and ending at the end of the last moult of the larval stage (L4). We also observed how many days it took for the two groups (control and treated) to transition from the larval stage to the nymph stage. The passage from the nymph to the adult remains the most important moult because it ends the aquatic phase and begins the aerial phase. This duration was measured in days from the day of the appearance of the nymph to the day of the emergence of the adult.

2.5.3 Data analysis: Lethal concentrations and lethal times (LC 50%, LC 90%, LT 50% and LT 90%) were calculated using Finney's mathematical methods (Finney 1971). Data were normalized and processed according to the tables of Bliss, and calculations were performed with XLStat 2009 Software. Also, fecundity and female fertility were subject to statistical description and a comparison of variances using XLSTAT2009 Software.

RESULTS:

1.6. Effects of the ethanolic extract of P. harmala on the mortality of Culex pipiens:

The results of the treatment of C. pipiens larvae show that the ethanolic extract of P.harmala affects larval mortality as a function of the applied concentration and the time of exposure. A low larvicidal activity was recorded for a concentration of 1 g/L, and 90% of the treated individuals died after 15 days of treatment at a concentration of 4 g/L. There is no significant difference between the recorded mortality rates and the concentrations applied ($p<0.000$).

Table 1: Mortality rate (%) of C. pipiens treated with the ethanolic extract of P. harmala

Time / Concentration	2 days	5 days	10 days	15 days	F obs	*P*
1 g/l	1,7	0,0	17,6	60,8	0,03	0,99
2 g/l	16,7	35,1	41,2	64,7	3,92	0,05*
4 g/l	11,7	35,1	68,6	92,2	2,49	0,13
F obs	1,42	3,61	4,16	1,12		
P	0,31	0,09	0,07	0,39		

(*: Significant difference**)**

3.2. Toxicological parameters of the ethanolic extract of P. harmala:

After two days of treatment, the 50% lethal concentration was 16.57 g/L. It decreased to 5.64 g/L in five days. In 10 days, it was 2.49 g/L, and on the fifteenth day of treatment, 90% mortality of the larvae was caused by a concentration of between 0.89 g/L and 4.07 g/L (Table 2). There is also a strong correlation between mortality and larval exposure time at different concentrations of P. harmala ethanol extract (R^2 = 0.31-0.96).

Lethal times ranged from 5.76 to 29.92 days for 50% mortality and from 16.03 to 84.73 days for LT 90% (Table 2).

Table 2: Toxicological parameters of the ethanolic extract of P. harmala:

Lethal concentrations				
Times (days)	**2**	**5**	**10**	**15**
Linear regression	Y=3,11+1,55X R^2=0,58	Y=0,77+7,68X R^2=0,75	Y=4,07+2,35X R^2=1,00	Y=5,11+1,92X R^2=0,82
LC50% (g/l)	16,57 g/l	5,64 g/l	2,49 g/l	0,89 g/l
LC90% (g/l)	110,96 g/l	8,28 g/l	8,72 g/l	4,07 g/l
Lethal times				
Concentrations (g/l)	**1**	**2**	**4**	
Linear regression	Y=0,45+3,30X R^2=0,31	Y=3,60+1,39X R^2=0,92	Y=2,81+2,88X R^2=0,96	
LT50% (day)	29,92 J	10,17 J	5,76 J	
LT90% (day)	58,43 J	84,73 J	16,03 J	

Y: probits of percentage mortality **X:** logarithm of concentration or time**.**

3.3. Effect of extract on fertility and infertility:

After treating fourth-instar larvae with a sub-lethal concentration of 1 g/L, the test revealed a disturbance of fertility in adults resulting from the P. harmala treatment. Treated individuals yielded an average of 47.50 ± 5.89 eggs with a minimum of 40 eggs and a maximum of 61 eggs. However, control females laid between 31 and 123 eggs. Comparison of these two averages shows that there is a significant difference between the fertility of the two groups ($t = -2.73$, $p = 0.01$; Table 3).

Table 3: Comparison of Culex pipiens eggs laid by control females and females treated with ethanol extract of P. harmala (n = 15)

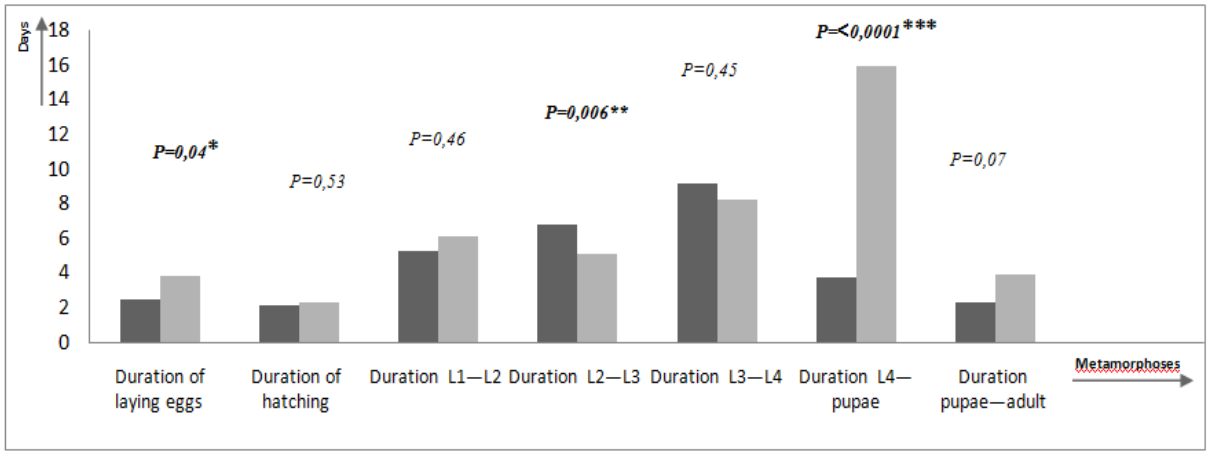
	Mean ± SEM	**Min**	**Max**	***t obs***	***P***
A	70,60 ± 26,08	31	123	-2,73	0,01*
B	47,50 ± 5,89	40	61		

A: Control B: Treated

3.4. Effects of the extract on the development of Culex pipiens:

The control females began to lay on the first day, and this laying was spread over 5 days, with the average time being 2.5 ± 1.08 days. Females from the treated group take longer (3.80 ± 1.47 days) with a minimum of 2 days and a maximum of 6 days (figure1). Eggs laid by control females take slightly less time to hatch than those spawned by treated females, with an average of 2.1 days for controls and 2.3 days for treated animals (figure1).

Figure 1: Effects of *P. harmala* on egg, larva, pupae and adults of *Culex pipiens*.



The hatching rate of control females was 95.11%, which is slightly higher than that of treated females (94.20%; Figure 1). Larvae from treated females take less time to pass through the larval stages (L1, L2, L3, and L4) with an average of 19.4 days. Larvae from female control eggs take more time, with an average of 21.3 days (Figure 1). Passage from the final larval stage (L4) to the pupal stage takes much less time in generations from the eggs of Control females, with an average of 3.7 ± 1.89 days, as opposed to generations from the eggs of treated females, which have an average of 16 ± 5.02 days (Figure 1).

The passage from the nymph stage to the adult stage lasts, on average, 2.3 days with a minimum of 1 day and a maximum of 5 days in the control group, while the average for treated individuals is 3.9 days with a minimum of 2 days and a maximum of 8 days (Fig 1).

Finally, there are significant differences (P = <0.0001***) in the duration of the passage from the aquatic to the aerial phase between the two groups studied (Figure 1).

DISCUSSION:

Culex pipiens is the most abundant species in our study area and was described (Bendali 2006) as the typical species of the suburbs and neighbourhoods of the city of Annaba and the surrounding communities. Because of the discomforts it causes and the diseases it can inoculate, control of this pest is necessary. The intensive use of conventional pesticides has produced undesirable effects (Abudulai et al., 2001). Recently, biopesticides of plant origin have been used successfully against several insect vectors (Schmutterer 1990, Senthil et al.2004, 2005a). Phytotoxic substances from several families with active ingredients can have direct lethal effects, delay embryonic development or affect the shape of the egg (Champagne et al., 1986). This led us to emphasize in our study the use of natural products such as plant extracts. Raw or partially purified plant extracts are cheaper and highly effective at controlling mosquitoes relative to purified compounds (Jang et al.2002). We have recorded the larvicidal activity of the ethanolic extracts of fresh leaves of P. harmala on Culex pipiens. This activity results in high mortality rates (50%) in a period not exceeding 5.76 days of treatment. These mortality rates are correlated with the dosages used and the time exposed to the product. The results also indicate LC 50% values of 2.49 g/L and 0.89 g/L after 10 and 15 days of treatment, respectively. The insecticidal activity of P. harmala is probably attributable to toxic substances (alkaloids). Our results confirm the results of (Mahmoudian et al. 2002). Several studies indicate the toxic effect of P harmala on insects. (Benhissen 2016) showed the toxic effect of P. harmala aqueous extracts on the larvae of the fourth stage of Culex pipiens, and Habbachi et al. (2013, 2014) indicated a larvicidal effect on D. melanogaster and C. pipiens (Diptera). (Idrissi-Hassani et al. 2008, Abbasi et al. 2003) and (Idrissi-Hassani & Hermas 2008) evaluated the acaricid potential of the plant against Shistocerca gregaria. In the life of a mosquito, only the act of oviposition determines the survival or death of the next generation (Subra 1971). Phytotoxic substances from several families with active ingredients may have direct lethal effects, delay the development of the embryo or affect the shape of the egg (Champagne et al. 1986, Dris et al. 2017). Our study showed that adults treated with a sub-lethal dose of ethanolic P. harmala extracts are less fertile, with an average of only 47.50 eggs laid, compared to the average of 70.60 eggs laid by their control counterparts. Thus, it appears that ethanolic extracts of P. harmala cause a decrease in the fertility of female Culex pipiens. This effect, also reported by (Benhissen 2016) in Culex pipiens, shows that a sub-lethal dose of P. harmala significantly decreased the fertility of treated females by 32%, as opposed to 58% of eggs in the controls. Similarly (Alouani et al. 2009), Azadirachtin was shown to cause sterilization in treated adult Culex pipiens. Other studies have shown that the treatment of female Anopheles stephensi with Neem causes delayed oocyte development (Lucantoni et al. 2006, Dhar et al. 1996) indicated that exposure to Neem extracts suppressed oviposition in mosquitoes. We found that female Culex pipiens treated with sub-lethal doses of ethanolic extracts of P. harmala take longer to lay (an average of 3.8 days) relative to control females, which take an average of only 2.5 days. The extracts used seem to act as an inhibitor and retardant of hatching. Many studies using other molecules have focused on this axis, such as (Ganesan et al. 2006) who reported that boric acid and palmitoleic acid are laying stimulants for Aedes aegypti females and are present in their eggs. Our results concur with those of Hwang et al. (Hwang et al. 1984) who describe these fatty acids as egg-laying inhibitors in pregnant female Culex quinquefasciatus.

During our study, several variables were collected, including the duration until the hatching of the eggs laid by Culex pipiens females from the control group and the group treated with ethanolic extracts of P. harmala. Our results show that eggs laid by the control females take slightly less time to hatch (an average of 2.1 days) than the eggs laid by the treated females (an average of 2.3 days), which suggest that the ethanolic extract of P.harmala contains active substances that retard not only the duration of laying but also the duration until hatching of the eggs laid by the Culex pipiens females.

Other works in which aqueous plant extracts have been used have conflicting results, such as (Benhissen 2016), who tested the aqueous extracts of P. harmala and Daphne gnidium on the rate of egg-laying in Culex pipiens females and found that the eggs obtained from the treatment group take slightly less time to hatch than the eggs from control females.

We also recorded an increase in the duration of development of the larval stage of Culex pipiens in individuals from the eggs laid by treated females. Indeed, we noticed not only delays during the passage through various stages (L1, L2, L3, L4, nymph) but also during embryonic development, with an average of 35-42 days for the treated group compared to an average of only 25 days for the control group. Extracts of P. harmala appear to possess inhibitory properties of growth and development. The same conclusions have been reported by (Benhissen 2016) who found that after the treatment of fourth-instar larvae with sub-lethal concentrations of bioinsecticides such as P. harmala and D. gnidium, C. pipiens exhibited an extension of the larval life, an extension in the development of the various stages and a fertility disruption of the adults. Concerning the elongation of the development of the embryonic stage, other members of the plant family of Meliaceae are used as regulators of growth against numerous insects; a prolonged embryonic development and a reduction of the weight of chrysalises and of laying number is reported by (Saxena et al. 1984, Schmutterer 1990, Hammad et al. 2001, Gajmer et al. 2002, Banchio et al. 2003, Wandscheer et al. 2004).

The ethanolic extract of P. harmala used in our study shows effects on the duration of the pupal stage of the treated group relative to the control group. These effects include a developmental delay in which it is noted that the duration of the nymphal stage in the control group is 2.3

days, on average, while the development of the treatment group takes longer, with an average of 3.9 days. Our results concur with those of (El Bahri & Chemli 1991), which demonstrated that the extract of the leaves of P. harmala and its active ingredients act as a single mode of action to induce insect paralysis, produce disorders in the locomotion of the insect and intervene in its life cycle.

CONCLUSION:

The results obtained in this study on the toxic effect of P. harmala on the four stages of Culex pipiens larvae showed a remarkable efficacy of the molecules of this plant. The mortality rates correlate with the dose and exposure time to the extract. This plant also has an effect on the development and fertility of individuals tested lindeed P.harmala retards and inhibits moulting and fertility of Culex pipiens.

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