Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2016; 4(6): 638-642

Received: 26-09-2016
Accepted: 27-10-2016

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Sublethal effects of *Peganum harmala* (Zygophyllaceae) on sexual behavior and oviposition in fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae)

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Abstract
Peganum harmala (Zygophyllaceae) is an herbaceous plant of the mediterranean boards, rich in indolic alkaloids that confer it an insecticidal activity (biopesticide). In this study, the deferred toxic effects of *P. harmala* were evaluated on sexual behavior in the fruit fly, *Drosophila melanogaster*. Treatment was applied on 2nd instar larvae (L_2), by ingestion of a sublethal concentration (300 µg/ml). The harmel affected negatively the series of behavioral sequences leading to mating in adults (courtship elements), causing an abortion rate of 90%. In addition, it has a repellent effect expressed by the choice of oviposition site by females causing a reduction the number of laid eggs.

Keywords: *Peganum harmala*, deferred effects, sublethal concentration, mating, oviposition, *Drosophila melanogaster*

1. Introduction

In recent decades, environmental problems have incited organizations and research institutions to develop more biological methods, in order to limit the use of chemical pesticides. One of its forms is the use of secondary compounds from plants to control harmful insects [1].

Peganum harmala (L.) (Zygophyllaceae), is a one of the most important sources of natural substances due to the structural diversity of alkaloids that are present in the plant and their bioactivity. It is abundant in Mediterranean arid areas (Oriental Morocco, Septentrional Sahara, Algerian highlands, Tunisia, Libya and Egypt) [2]. Many studies have showed the toxic effects of the substances extracted from *P. harmala* in control of various insect species, such as *Schistocerca gregaria* [3-5], *Plodia interpunctella* [6], *Spodoptera littoralis* [7] and *Tribolium castaneum* [8, 9].

Dipteran regroup a large number of insects that are a causing a major risk to human health [10], including the fruit fly, *Drosophila melanogaster* (M.) (Diptera, Drosophilidae). It is a species of tropical origin (East Africa), worldwide distributed due to human activities and its abundance is correlated to the degree of urbanization [11, 12]. Its nuisance comes from the fact that it is responsible for moisture and infestation of fruits via fungi and bacteria that it transports, producing also hundreds of insects resistant to the different living conditions in a more or less important duration. The larvae can cause intestinal irritation and diarrhea if swallowed by eating infested fruit [13, 14]. It also remains a choice material for biological essays.

Many behaviors of *Drosophila* based on recognition of chemicals and especially pheromones representing intraspecific chemical signals, which play an important role in attracting and discrimination of sexual partners [15]. Recent studies describe that pesticides may also interfere with the chemical communication (olfactory and gustatory senses) of exposed species and therefore cause inadequate behavioral responses [16]. Some studies have shown the effect of sublethal doses of insecticides on the behavior of harmful or beneficial insects [17, 18], including a decrease in behavioral responses to sex pheromones in treated individuals [19, 20].

The sexual behavior of the fly can be used as an important parameter in order to understand the effects of plant extracts. The aim of the present work is to evaluate the secondary effects of aqueous leaves extract of *P. harmala*, at a sublethal concentration (300 µg/ml), on the series of behavioral sequences leading to mating, reproductive potential and olfactory attractiveness in choosing of oviposition sites in *Drosophila*.

2. Materials and Methods
2.2 Insect
D. melanogaster is a holometabolous insect, characterised with a short time of the developmental cycle (from egg to adult imago, passing through three larval stages and pupal stage in duration of 10-12 days, at 25 °C) and its prolific reproduction mode induces a large number of descendants in each generation (300 descendants per female). The female lays eggs on the flesh of ripe and wounded fruits. For experiment, a stock of a wild strain, derived from fermented apples in region of Annaba (Algeria), was conserved in vials containing a standard agar medium (cornmeal and brewer's yeast base), at a temperature of 25 ± 1 °C, a humidity of 70% and a 12 : 12 h light : dark cycle. Rearing of insects was conducted in laboratory of Applied Neuro-Endocrinology, Faculty of Sciences, University Badji Mokhtar of Annaba (Algeria) in 2016.

2.2 Plant aqueous extract preparation
Fresh leaves of *P. harmala* (500g) were collected in September 2016 from the Laghouat region Algeria (33 ° 47'59 "N, 2 ° 51'54" E). Leaves were rinsed (with water) and carefully cut into pieces of 2 to 5 cm, then they are boiled in a liter of distilled water for 1 hour of time until obtaining an initial stock solution of 1000 µg/ml, filtered with filter paper (Whatman). The used concentration (300 µg/ml) was obtained after dilution with distilled water.

2.3 Effects of *P. harmala* on the behavior of *D. melanogaster*:
2.3.1 Description of courtship elements: Courtship in *Drosophila* is a succession of predetermined and invariable actions [21, 22]. The male, first, is moving towards a potential female and tap her on the cuticle with his fore legs; if the female moves, the male follows and vibrates one wing. Then, when the female stops, the male running in circles around her, lick her genitalia with his proboscis and attempts to copulate [23, 24].

2.3.2 Treatment: To evaluate the activity of *P. harmala* on adults courtship, a treatment by ingestion was performed on 2nd instar larvae. 40g of culture medium containing 10ml of product, is distributed in four tubes where we put the larvae. An untreated medium was used as control. The newly emerged adults are kept in isolation (individually) in small tubes containing culturing medium. Insects used must be aged 3 to 5 days so that they are sexually mature.

2.3.3 Ethological tests: Concerning coupling tests, watch glasses of 3 cm in diameter were used. The male is introduced first in order to get used to his new environment, then the female five minutes later will be placed. When the female is introduced into the cell corresponds to the time T_0; test start. Contacts, wings vibrations, lickings, attempts and duration mating are noted separately in the control and treated groups. All experiments are conducted in a quiet room, dark and lit by a red light. It allows significantly to limit the influence of visual stimuli [25]. Four types of crosses are performed (n= 30) : a/ Control Males x Control Females ; b/ Control Males x Treated Females ; c/ Treated Males x Control Females ; d/ Treated Males x Treated Females. 48 hours after isolation of mated females, we observe the culture medium, placed in watch glasses, under a stereomicroscope to realize a count of the eggs laid. The mediums used in the olfactory attraction in oviposition test are: control medium and treated medium with harmel (300 µg/ml).

2.4 Statistical analysis
The various parameters obtained were analyzed by descriptive metric methods then compared using a variance analysis (ANOVA). All calculations are made on XLStat 2014 (Add in soft, New York, NY). Regarding the results of the tests of choice (oviposition), data were analyzed by Monte Carlo simulation based on a Chi2 test at significance level α = 0.05 [26].

3. Results
3.1 Effect on sexual behavior
Except touching (contacts), where all the males were moving towards their females and tap them (100%) (Tab. 1), our results show that the treatment acts differently on other behavioral sequences of courtship, depending on cross type.
Only 40 to 46.67% of the treated male paraded (wings vibrations), while 100% of controls performed this act (Tab. 1). Moreover, only 23.33 to 50% of these latters licked females secretions, in couples where at least one individual is treated (Tab. 1). Regarding mating attempts, 26 males in the control pairs have attempted to mate, leading to a successful coupling rate of 83.33% (25 pairs). On the other hand, only 4 to 5 couples were able to attempt copulation, when one sex or both partners are treated. The aborted coupling rate in treated couples is equivalent to 90% (Tab. 1).

Table 1: Number of successful mating behavioral sequences in treated adults with leaves aqueous extracts of *P. harmala* (n = 30) :

	Contacts	Vibrations	Lickings	Attempts	Successful coupling
♂C x ♀C	30	30	26	26	25
♂C x ♀T	30	19	15	14	12
♂T x ♀C	30	12	7	5	4
♂T x ♀T	30	14	7	4	3

[♂, Male; ♀, Female; C, Control; T, Treated]

We recorded different behavioral sequences from those usually observed in untreated individuals. The mean number of touching differs according to the couple tested, with over 12.47 ± 1.39 contacts are registered in treated males (treated couples). Indeed, the analysis of variances shows that there are highly significant differences (F_{obs}= 5.18; p <0.002) (Tab. 2). Thus, their vibrations wings is repeated 20.64 times, higher than those recorded in the control couples (F_{obs}= 17.73; p <0.0001) (Tab. 2). The mean number of licks and of mating attempts when at least one of the two adults is treated, can reach up respectively to 12 and 6 times. At significance level α = 0.05, the analysis of variances shows that there are highly significant differences comparing to control pairs (Tab. 2).

Table 2: Effect of treatment on the number of repetitions of each behavioral sequence (n = 30) (M ± SEM) :

	♂C x ♀C	♂C x ♀T	♂T x ♀C	♂T x ♀T	F_{obs}	*p*
a	4.17 ± 0.49	6.7 ± 0.94	6.5 ± 0.88	12.47 ± 1.39	5.18	0.002**
b	8.3 ± 0.90	8.32 ± 1.43	11.08 ± 2.56	20.64 ± 6.89	17.73	0.0001***
c	4.65 ± 0.76	6.53 ± 1.28	8 ± 1.38	12 ± 2.66	2.50	0.07*
d	2.64 ± 0.47	2.54 ± 0.78	3.2 ± 0.58	6 ± 2.48	2.70	0.06*

[*a*, Number of contacts; *b*, Number of vibrations; *c*, Number of lickings; *d*, Number of attempts; ♂, Male; ♀, Female; C, Control; T, Treated; M, Mean; SEM, Standard error of the mean; * : Significant differences]

Once of the two is treated individual, the time needed for the male to recognize his female and makes the first contact increases sharply (32.8 to 81.33 seconds). The variance analysis showed that there are highly significant differences (F_{obs}= 16.64; *p* <0.0001) (Tab. 3). In addition, the mean time of the first song, first lick and of mating attempt, also remain very high compared with those of control adults. Indeed, the coupling latency is quite high and reach up to 427.17 to 1199.67 seconds (F_{obs}= 7.25; *p* <0.0005) (Tab. 3). The mean time to make the adults to achieve a successful mating, differs depending on the subject. Highly significant differences were revealed by the analysis of variances F_{obs}= 6.38 and *p* <0.0005 (Tab. 3).

Table 3: Effect of treatment on the time required (in seconds) for the completion of each behavioral sequences (n = 30) (M ± SEM):

	♂C x ♀C	♂C x ♀T	♂T x ♀C	♂T x ♀T	F_{obs}	*p*
a	9.33 ± 1.44	53.63 ± 7.89	81.33 ± 8.79	32.8 ± 5.16	16.64	0.0001***
b	67.37 ± 11.06	298.95 ± 56.32	305.08 ± 45.76	159.14 ± 29.89	7.26	0.0002***
c	98.69 ± 17.35	283 ± 56.07	445.14 ± 76.90	206.43 ± 73.57	2.45	0.07*
d	192.85 ± 36.61	362.14 ± 72.46	480.8 ± 123.01	717.75 ± 302.61	8.05	0.0002***
e	286.8 ± 44.51	427.17 ± 51.62	445.25 ± 126.31	1199.67 ± 349.95	7.25	0.0005***
f	720.9	491.73	176.30	1039.33	6.38	0.0005***

[*a*, Number of contacts; *b*, Number of vibrations; *c*, Number of lickings; *d*, Number of attempts; ♂, Male; ♀, Female; C, Control; T, Treated; M, Mean; SEM, Standard error of the mean; * : Significant differences]

3.2 Effect on oviposition

The results in table 4 show that about 25 control couples tested, all females prefer to lay their eggs significantly in control medium (*p* <1.000) (Tab. 4). While, treated females have a very low positive reaction (2 of 11), even nil (0 of 3) to the same odor (Tab. 4).

By analyzing their behavior towards treated medium with *P. harmala* (300 µg/ml), treated females appear attracted by the smell of this medium (9 females with *p* <0.968 and 3 females with *p* <0.885), whereas that controls are not (Tab. 4).

Table 4: Effect of *P. harmala* on the choice of laying in *D. melanogaster*:

	N	Control medium			Traited medium		
		A	NA	*p*	A	NA	*p*
♂C x ♀C	25	25	0	1,000 (S)	7	18	<0.828 (NS)
♂C x ♀T	11	2	9	<0.968 (NS)	9	2	0.968 (S)
♂T x ♀C	4	4	0	<0.923 (NS)	1	3	<0.923 (NS)
♂T x ♀T	3	0	3	<0.885 (NS)	3	0	<0.885 (NS)

[♂ : Male ; ♀, Female ; C, Control ; T, Treated ; A : Attracted ; NA : Not attracted ; S, Significance ; NS : No significance]

The mean number of eggs laid by control females (in control pairs) was 35.94 ± 3.54 with a minimum of 3 and a maximum of 74, while the one filed by the treated females was 13.33 ± 2.40, the maximum of which is 18 eggs (Tab. 5). Analysis of variance shows that there are highly significant differences at α = 0.05 level (F_{obs}= 7.41; *p* <0.0003) (Tab. 5).

Table 5. Effect of *P. harmala* on the number of eggs:

	♂C x ♀C	♂C x ♀T	♂T x ♀C	♂T x ♀T	F_{obs}	*p*
N	25	11	4	3	7.41	<0.0003***
Moy ± SEM	35.94 ± 3.54	24.18 ± 1.90	20.80 ± 5.19	13.33 ± 2.40		
Min - Max	(3 - 74)	(15 - 35)	(4 - 34)	(10 - 18)		
Total	1150	266	104	40		

[♂ : Male ; ♀, Female ; C, Control ; T, Treated ; M, Mean ; SEM, Standard error of the mean ; Min, Minimum ; Max, Maximum, ***, Very highly significant differences]

4. Discussion

The *Drosophila* courtship by its complexity is the best example of behavior requiring a range of sensory perceptions mobilizing a large number of organs, it consists of a series of actions quite accompanied by exchanges of visual, auditory, chemical, touch signals... and it involves a lot of upstream genes to achieve this courtship [27, 28]. This behavior has been the subject of intense research [29, 30]. The male mate choice experiment was not simply a measure of male preference; successful copulation also requires the target female be receptive [31].

In our study, we tested the hypothesis that low doses of an insecticide, such as *P. harmala*, might influence the traits of fitness and reproductive performance of adult, disturbing smell necessary for reproduction. They have shown a remarkable deficiency causing abortion of the coupling process. Clearly, a long couplation latency indicates the non-responsiveness of the female despite the dynamism of the male. This sexual inappetence is related to the nature of epicuticular pheromones of female and male courtship behavior. A non-receptive female will give pawed the male, raising and lowering her abdomen, leak or extrude completely her ovipositor [29]. If accepted, during copulation, the male sperms are transferred into the reproductive tract of the female [32], *Drosophila* females are fertilized for only 20 minutes [33]. Our results show that the duration of copulation, in treated adults, signify of their difficulty, they take a long time for the females are fertilized.

Note that no study of Sublethal effects of *P. harmala* or other toxic plants has been realised on the sexual behavior of *Drosophila*. However, they have also been described in experiments with other insecticides. The exposure to imidacloprid, a neonicotinoid, at Sublethal doses in chronic intoxication induced effects on mating and fertility of fruit flies [34]. Similar results are obtained by the use of insecticides: dithiocarbamate, Dithane M 45 [32], spinosad (2.5 mg/l) and azadirachtin (0.05 mg/l) [23], *Bacillus thuringiensis* (5 g/l) [35]. In addition, other results indicate that spiromesifen disrupts lipid metabolism in *Drosophila*, confirming also its primary mechanism of action on cuticular hydrocarbons lipid synthesis [36].

After mating, the second essential phase for the reproduction of the insect oviposition, which is also under neuronal control leading to selective egg-laying on suitable substrates [37]. The choice of the substrate is the result of a sensory evaluation expressed in search-like behavior, preceding the stereotypical ovipositioning program [38]. The repellent effect of insecticides can also induce a diet or a drop in food to the insect which can lead to reduced fertility [39, 34]. Our study confirms that *P. harmala* has a repellent effect on *Drosophila* causing changes its olfactory responses. A significant decrease in the number of eggs was recorded in treated couples comparing to controls. Furthermore, the neurotoxic alkaloids of this plant would be cause of blocking ovarian development and vitellogenesis in females and the absence of sexual maturity in males [4].

Previous works, concerning the anti-fertilizing effect and anti-ovipositing of different extracts of *P. harmala*, match with our results as observed in *Callosobruchus chinensis* [40], peach fly, *Bactrocera zonata* [41, 42] and the olive fruit fly, *Bactrocera oleae* [43]. Other work demonstrated that these latter can affect the incubation period and the hatching rate of *Trialeurodes vaporariorum* [44]. Similar results are reported in *Tribolium castaneum* [45], *Blattella germanica* [46] and *Culex pipiens* [47]. The negative impact of the same plant species has also been reported on reproduction, oogenesis and egg laying capacity of the desert locust *S. gregaria,* under laboratory conditions [4].

5. Conclusion

The exposure to sublethal concentrations of *P. harmala* has allowed us to observe abnormal or unusual pre-copulatory behavior in *Drosophila*, and detect deficient parades. Which signify that this biopesticide probably as a disruptive information by altering the chemical communication, which leads to reduced reproduction chances of target insects. It also acts as an anti-fertilizing agent. The repellent effect of the plant also influences the choice of ovipositing site and the number of laid eggs.

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