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Activity of neem oil in *Drosophila melanogaster*: toxicity and delayed effect on the progeny

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Abstract
Azadirachtin, derived from the neem tree, has been used successfully against insect pests; however, their effects on non-target organisms remain contradictory. The current study, examined the effect of neem oil, a commercial formulation of azadirachtin, on biological model of reference, *D. melanogaster*. In first bioassays, various doses of neem oil were tested, by topical application, on newly ecdysed pupae and the inhibition doses (ID) of adult emergence were determined (ID_{25}: 0.68 µg; ID_{50} 1.17 µg). Then, sublethal effects of neem oil have been specified on the weight of the pupae and on the fertility of adults that survived from treated pupae. Results showed that neem oil reduced, significantly, pupal weight at the two tested doses. Fertility and fecundity of various formed couples (between control and treated) were affected significantly by the insecticides excepted when only males were treated with the lowest dose. Thus, neem oil acts with delayed effects.

Keywords: *D. melanogaster*; Toxicity; Azadirachtin; Development; Reproduction.

1. Introduction
The use of pesticides has been the major approach in crop protection for decades to prevent or control pests, diseases, weeds, and other plant pathogens in an effort to reduce or eliminate yield losses and maintain high product quality [1, 2]. Therefore, the preservation of the agroecosystems is important and necessary to establish an effective safe control, avoiding environmental pollution, resistance and negative impact on non-target organisms caused by conventional pesticides [3, 4]. Natural product-based pesticides such as the nicotine, spinosad and azadirachtin offer a more sustainable solution to pest control than synthetic alternatives [5, 6, 7]. Azadirachtin, a tetranortriterpenoid, is the predominant insecticidal active ingredient derived from neem seed kernel (*Azadirachta indica* A. Juss) [8]; it has been thought to be as an excellent alternative to synthetic insecticides for crop protection [9] because of it's low toxicity in environment, rapid biodegradability and no resistance problem [10]. It possesses a strong toxic activity against many insect pests, such as Diptera [11, 12], Lepidoptera [13, 14] and Dictyoptera [15]. Azadirachtin acts as a growth regulator with an antagonistic action with juvenile hormone and ecdysteroids [11, 16]. Azadirachtin had moderate to strong cytotoxicity, neurotoxic, antimitotic effects in insect [16, 17, 18, 19], but the mechanism of action of this pesticide remains still unknown [20]. In addition, varied effects were noted according to azadirachtin formulation tested [21, 22]. Several studies have questioned the safety of neem products against non-target insects but the results remain contradictory [3, 23, 24, 25, 26, 27]. In a recent report we showed that Neem Azal exhibited toxicity and affected reproduction in *D. melanogaster* [28]. Furthermore, few studies have been assessed the delayed effects of azadirachtin on the reproduction. Therefore, the objectives of the current study were to determine the lethality parameters of neem oil, applied topically, on newly ecdysed pupae of *Drosophila melanogaster* and to examine its sublethal effect on the growth of pupae and on the progeny of surviving adults by assessing the number of eggs, larvae, pupae and adults obtained during the following F1 generation.

2. Material and methods
2.1 Insect
D. melanogaster, Meigen 1830 (Canton S) was reared in plastic vials on artificial medium, consisting of corn meal, agar, yeast, and methyl-4-hydroxy-benzoate as previously described [29]. Flies were maintained in an incubator at 25 ± 2 °C under a photoperiod of 12 h light: 12 h dark and a relative humidity of 70%.

2.2 Insecticide and Toxicity

A commercial formulation containing neem oil (Azadirachtin 0.3%; EC; H3D Terraneem, France) was used in all experiments. The product was dissolved in acetone and applied topically (1µl per insect) on newly ecdysed pupae (< 6 h). Control insects were treated with acetone alone (1 µl). Five doses (0.5, 1, 1.62, 3.25 and 6.5 µg) were tested and the bioassay was conducted with 3 replicates per dose were done each consisting of 30 insects. The inhibition percentages of adult emergence were corrected in accordance with Abbott [30]. The inhibition doses (ID) ID_{25} and ID_{50} (doses causing inhibition of adult emergence in 25% and 50% of the treated insects, respectively) were determined together with their corresponding 95% fiducial limits (95% FL) by a non linear regression.

2.3 Effects of neem oil on pupal weight

Neem oil was administrated by topical application at its ID_{25} (0.68 µg) and ID_{50} (1.17 µg) on newly ecdysed pupae (< 6 h). The pupal weight was recorded all days after treatment up to the emergence of adults. Twenty biological repeats were done for each series.

2.4 Effects on the number of descendants from surviving adults

Newly molted (< 6 h) *D. melanogaster* pupae were treated topically with the neem oil at its ID_{25} (0.68 µg) and ID_{50} (1.17 µg). Subsequently, the surviving adults from control and treated series were collected and males and females coupled following different combinations: $Male_{control}$ x $Female_{control}$; $Male_{control}$ x $Female_{ID25}$; $Male_{ID25}$ x $Female_{control}$; $Male_{ID25}$ x $Female_{ID25;}$ $Male_{control}$ x $Female_{ID50}$; $Male_{ID50}$ x $Female_{control}$ and $Male_{ID50}$ x $Female_{ID50}$. Each combination was conducted with nine repeats and each couple was reared individually, during 48 hours, in a Petri Dish containing a nutritive medium. After this time, the different couples formed were withdrawn and the number of individuals for all developmental stages was evaluated (eggs, larvae (L3), pupae and adults).

2.5 Statistical analysis

Results were presented as means and standard deviation (S.D). The homogeneity of variances was checked by a Levene's test. The significance between different series was tested using Student's *t*-test at 5% level. Data were subjected to one-way or two-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey test HSD. All statistical analyses were performed using graphpad prism software v6.01 (for Windows) and $p < 0.05$ was considered statistically different.

3. Results

3.1 Toxicity

Neem oil applied topically on newly ecdysed pupae causes an inhibition of adult emergence with a dose-response effects (Figure 1). Indeed, our data revealed that the corrected inhibition values varied from 10.80 ± 3.35 % for the lowest dose (0.5 µg) to 95.67 ± 7.49 % for the highest tested dose (6.5 µg). The inhibition recorded in controls was 10.36 ± 3.39 %. Statistical analysis indicated that the compound was toxic with a significant ($p < 0.001$) effect of doses. The Tukey HSD test indicates that there was a significant relationship between the different tested doses (Figure 1). The ID values with 95% fiducial limits (95%FL, R^2 =0.99) were recorded of 0.68 (0.52-0.89) and 1.17 (0.98-1.38) µg, respectively for ID_{25} and ID_{50} (Table 1).

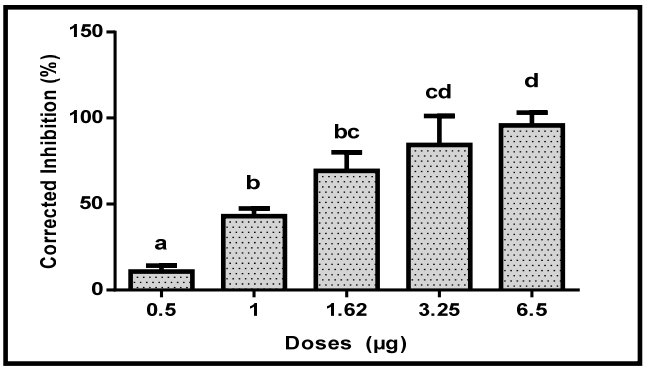

Fig 1: Effect of neem oil (µg), topically applied, to newly ecdysed pupae of *D. melanogaster* on the adult exuviation inhibition (%) (Mean ± SD; n=3 replicates containing each 30 pupae; values affected by different letters are significantly different by HSD test at p<0.001).

Table 1: Inhibition doses (ID) with their corresponding 95% fiducial limits [FL] of neem oil topically applied to newly ecdysed pupae of *D. melanogaster*.

	ID_{25} (95 % FL) µg	ID_{50} (95 % FL) µg	Hill Slope (95 % FL)
Neem oil	0.68 [0.528-0.893]	1.17 [0.988 -1.384]	2.066 [1.331-2.802]

3.2 Weight of pupae

In control series the weight of pupae at 0 day was 1.34 ± 0.04 mg and remains stable for all duration of the pupal stage $p > 0.05$. Data showed that topical application of neem oil at their respective ID_{25} and ID_{50} caused a significant ($p < 0.05$) reduction, compared to controls, in the weight at day 2 ($p < 0.0001$), and 3 ($p < 0.0001$) during the pupal stage. Furthermore, there was no significant ($p > 0.05$) difference between the two tested doses (Table 2).

Table 2: Effect of neem oil, topically applied, on newly ecdysed pupae of *D. melanogaster* on the pupal weight (mg), as function the time (Days) following treatment (Mean± SD; n= 20 repeats; for each exposure time values affected by different letters are significantly different by t-test at $p < 0.05$).

Time (Days)	Control	ID_{25}	ID_{50}
0	1.34 ± 0.04 a	1.33 ± 0.04 a	1.35 ± 0.04 a
1	1.33 ± 0.04 a	1.31 ± 0.10 a	1.32 ± 0.05 a
2	1.35 ± 0.04 a	1.21± 0.04 b	1.18 ± 0.10 b
3	1.35 ± 0.06 a	1.17 ± 0.09 b	1.14 ± 0.09 b

3.3 Effects on the number of descendants of the F1 generation

Progeny from surviving adults after treatment of pupae has been evaluated and different developmental stages (eggs, larvae, pupae and adults) of different couples from controls and treated series have been considered. Statistical analysis of data showed a decrease in the number of eggs, larvae, pupae and adults of the F1 generation for all treated series as compared to controls (Table 3) except when only males were treated with the lowest dose. The comparison between treated series for every developmental stage revealed a significant dose effect between the different couples ($p < 0.0001$) given in Table 3; the test HSD revealed four groups of couples for eggs, larvae and pupae (M_C x F_C; M_{ID25} x F_C < M_C x F_{ID25}; M_{ID50} x F_C < M_C x $F_{ID50;}$ M_{ID25} x F_{ID25} < M_{ID50} x F_{ID50}) and five groups of couples for adults stage (M_C x Fc; M_{ID25} x F_C < M_{ID25} x F_C ; M_C x F_{ID25}< M_C x F_{ID25}; M_{ID50} x F_C< M_C x F_{ID50}; M_{ID25} x F_{ID25} < M_{ID50} x F_{ID50}).

Table 3. Effect of neem oil topically applied on newly ecdysed pupae of *D. melanogaster* on the number of descendants (F1 generation) of adults that survived from treated pupae (Mean ± SD; n= 9 repeats; for each stage of development the mean values followed by the same letter are not significantly different at 5% level by test of multiple comparison of Tukey.

	Eggs	Larvae (L3)	Pupae	Adults
Control	71.00 ± 3.50 a	70.10 ± 5.25 a	69.33 ± 4.33 a	68.50 ± 3.44 a
M_{ID25} x F_C	70.10 ± 5.12 a	68.00 ± 2.33 a	66.13 ± 3.88 a	62.10 ± 8.10 ab
M_C x F_{ID25}	59.33 ± 4.22 b	57.13 ± 3.20 b	56.33 ± 3.13 b	54.25 ± 9.10 bc
M_{ID50} x F_C	56.00 ± 6.44 b	54.55 ± 6.88 b	52.95 ± 6.88 b	50.44 ± 6.33 c
M_C x F_{ID50}	40.99 ± 7.10 c	39.22 ± 4.55 c	38.40 ± 4.14 c	35.22 ± 7.00 d
M_{ID25} x F_{ID25}	37.13 ± 6.10 c	35.33 ± 2.33 c	33.00 ± 3.40 c	31.25 ± 5.39 d
M_{ID50} x F_{ID50}	26.89 ± 3.55 d	24.33 ± 2.10 d	22.66 ± 2.99 d	20.10 ± 3.13 e

Eggs: $F_{(6, 56)} = 91.77$, $P<0.0001$; Larvae: $F_{(6,56)} = 155.2$, $P< 0.0001$; Pupae: $F_{(6, 56)=149.9,}$ $p< 0.0001$; Adults $F_{(6, 56)=} 67.69$, $p<0.0001$.

4. Discussion

In this study, the topical toxicity of neem oil on newly emerged pupae of *D. melanogaster* caused lethal effects during the formation of pupae leading to inhibition of adult formation and various morphological types such as partial ecdysed, moults blocked and malformed adults (absence of the wings) were observed.

The ID_{25} and ID_{50} values were estimated to be 0.68 and 1.17 µg, respectively corresponding to inhibition concentration (IC) IC_{25}= 687 ppm and IC_{50} = 1170 ppm. A previous report of Anjum *et al.* [31] indicated that the larvae of *D. melanogaster* were found to be melanized and failed to pupate when a compound of neem extract was applied by ingestion at an IC_{50} of 24000 ppm. Commercial neem formulations such as Neem-Azal had been reported in other dipteran species as mosquito and an higher pupicidal activity was noted; ID_{50} of adult emergence, tested by contact in the larvae, was found 0.398, 0.249, 0.048 and 0.006 ppm respectively in *Anopheles albimanus* [32], and *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi* [33]. However, in the literature, strong variations in susceptibility to azadirachtin changes as a function of the insect order, species, formulation and method of application [12, 13, 14, 15, 34]. These adverse developmental effects may be due to the action of azadirachtin on the endocrine and neuroendocrine system regulating the developmental processes of insects [11]. For instance, in literature, molt inhibition to be due to a blockage of ecdysteroid synthesis and release [11]. Moreover, the neurotoxic action of azadirachtin could affect the neuroendocrine system which controls the hormonal regulation of the development processes [19]. On the other hand, azadirachtin can affect cuticular protein genes of *Drosophila* [10] which are structural constituents of chitin-based larval cuticle, have crucial functions for synthesis of a new cuticle and ecdysed (the shedding of the cuticle of the preceding instar or stage) and is an important part of molt succession.

The results obtained on the weight during pupal development reveal that the neem oil disrupts the growth of *D. melanogaster*, which translates by a reduction of weight of pupae often described in other insects such as *Helicoverpa armigera* treated with Bioneem [35, 36]. Antimitotic action of azadirachtin can disturb the formation of new adult organs like the eyes and wings, during the metamorphosis in *D. melanogaster* [37]. In addition this decrease of weight in the treated series could be explained by the use of pupae of *D. melanogaster* (which do not feed) of energy reserves acquired during the larval stages for detoxify the toxic product.

In this study the deferred effects of neem oil on the reproduction of surviving adult of *D. melanogaster* showed a reduction in number of progeny; the depressive effects seem more marked when female was treated than male this decrease in the progeny presents a dose-response relationship and is directly related to treated sex. This impact of azadirachtin on fecundity and/or fertility is also found in *Locusta migratoria* [11], *Lobesia botrana* [34] and *Habrobracon hebetor* [21]. In *Drosophila*, as in all insects, juvenile hormone (JH) and ecdysteroids play a gonadotropic role in control reproduction [38]. It is known that azadirachtin causes deep effects on reproductive system of both male and female insects by causing alterations and morphological changes of oocytes and spermatocytes [11, 39, 40, 41, 42]. For the normal progress of oogenesis and spermatogenesis, a proper balance between JH and 20-hydroxyecdysone (20E) is of a paramount importance; consequently, the reduction of reproductive capacity could be explained by the antagonist action of azadirachtin on these two essential hormones [11]. Reduced fecundity and fertility could be due to the interference of azadirachtin with yolk protein synthesis (fat body and ovaries) in females and/or its uptake into oocytes [43]; these effects on vitellogenesis could be related to the disruption of juvenile hormone levels and ovarian ecdysteroid production [11]. In males, fertility reduction could be due to a degeneration of spermatocytes and an inhibition of cell division in developing spermatocytes leading to significantly smaller mature testes [11]. Similarly, the reduced fecundity and fertility observed in *Ephestia kuehniella* treated with ecdysteroid agonists could be due to interference with the vitellogenesis process and also to alteration of the quality of sperm and the sexual behavior [44, 45]. The decrease of the fertility and fecundity observed in *D. melanogaster* may be explained also, by the neurotoxic action of azadirachtin [19], which can affect secondarily the endocrine regulation.

5. Conclusion

The current results showed that neem oil applied topically on *D. melanogaster* pupae presented delayed effects as evidenced by a decrease in the number of descendants of the F1 generation. Further research on the effect of azadirachtin on other reproductive parameters such as sexual attraction, number of mating and reproductive potential of the progeny over several generations can be evaluated to give further additional information.

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