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# Toxicity and sexual behavior in fruit fly *Drosophila Melanogaster* [Diptera: Drosophilidae] treated with the fruit extract of *Citrullus Colocynthis*

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## ABSTRACT

Currently, biological control through the use of plants is of paramount importance to limit the nuisance of several insect pests. In this study we evaluated the different effects of *Citrullus colosynthis* aqueous extract on *Drosophila melanogaster*; we started with determining the toxicity of this fruit against the second instar of *D. melanogaster* larvae by using 3 different concentrations (50g/l, 100g/l, 150g/l). 50% mortality among the tested individuals was reported after 48 hours after treatment and reaches 93% on the 12$^{th}$ day. Thereafter we studied the delayed effects of sublethal concentrations on the sexual behavior in adults emerging from sublethal concentration treatment. We noticed a disturbance in some sequences of the courtship such as allied vibration, licking, mating, and a decrease in successful mating rate.

**KEYWORDS:** *Citrullus colosynthis*, *Drosophila melanogaster*, sublethal concentration, sexual behavior.

## INTRODUCTION

The fight against 'harmful' insects is a global challenge as far as human health, animal and plant production are concerned [1]. The uninterrupted and widespread use of even third-generation insecticides has not only led to the development of resistant strains of insects, but also the presence of toxic residues throughout the environment. The chemical arsenal used in the fight against insects, although very diverse, has not succeeded in completely eradicating certain pests [2]. In addition, it has increased the environmental impact by poisoning humans and livestock, and by depleting and destroying useful fauna, vegetation cover and the ecosystem [3]. Taking care of environmental and ecological problems has prompted research organizations and institutions to move towards biological control in its various forms to combat these nuisances. In the research for new methods of control, the possibilities of substances to protect plants from certain pests such as insects has attracted a lot of interest, and some studies have documented the wide variety of biological activities of plant-based preparations [1].

Indeed, the field of plant extracts offers means of control against insects in particular, with better protection of the environment from contamination by chemicals products. The use of plant extracts as insecticides has been known for a long time; pyrethrum, nicotine and rotenone are already known as insect control agents [4, 5]. The use of spontaneous plants by man and his dietary needs (medicinal, therapeutic and pharmaceutical) has

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been well reported for several years [6]. In Africa, traditional medicine contributes to meeting the health needs of more than 80% of the population [7]. There are about 500,000 plant species on Earth, 80,000 of which have medicinal properties [8]. They contribute to phytotherapy that is appreciated by the population of certain countries in the world, especially developing countries [9].

We chose the fruit fly *D. melanogaster* as a model to test the effect of aqueous extract from the *Citrullus colosynthis* plant. *D. melanogaster* occupies a central place in scientific research. This is one of the best-studied models currently used for biological research, particularly in the areas of genetics and development [10]. At the same time, little work has been done on the effects of insect repellents on the fruit flies; therefore, this study focuses on the toxic effects of aqueous extracts of *Citrullus colosynthis* on the vinegar fly *D. melanogaster*.

## MATERIALS AND METHODS

### Presentation of the insect

*Drosophila melanogaster* is a cosmopolitan insect that measures about 3 mm long. Its life cycle is very short and lasts only about 2 weeks at 25 °C [11]. After fertilization the females lay about 300 eggs (the egg is 0.05 mm long) and after hatching they give birth to larvae. After 5 or 6 days and 3 larval stages, they will transform into pupae. It is inside this cocoon that the metamorphosis takes place, giving rise to a winged adult insect. Adult flies are sexually dimorphic but both sexes have yellow to light brown body coloration with dark bands on the tergum of the abdomen [12].

### Strain and medium maintenance

For this study, we used the wild Annaba strain of *D. melanogaster*. The flies were recovered from ripe apples. The rearing was carried out in vials capped with a foam buffer and containing a semi-solid nutritive medium based on corn semolina, agar-agar yeast feed, and an antifungal (acide benzoique + alcohol) agent for preservation [13]. It is necessarily essential to maintain the rearing at a well-determined temperature of 24 ± 1 °C and a humidity of 65-75% to ensure that biological cycles remain predictable [13].

### Presentation of the plant

*C. colocynthis* (L.) Schrad is a vegetable plant that is cultivated and geographically dispersed in the desert of the Middle East, Asia, North Africa, and Southern Europe [14, 15]. It is a perennial plant with long creeping stems that spread over the ground and can exceed 1 m in length. The leaves are large, alternate, and indented. Depending on the stage of maturity, the fruits were spherical, and smooth resembling small watermelons, colored dark green or yellow [16, 17].

### Preparation of the plant extract

The fruits of *C. colocynthis* was collected from the Laghouat region (33° 48′ 24″ N,2° 52′ 56″ E) (Algeria) in March 2021. Fruits were washed with distilled water and then dried in an oven at 40 °C for 48 to 72 hours. It was then crushed using a grinder until it is reduced to powder. A quantity of 150 g of plant powder was diluted in one liter of distilled water previously brought to a boil, then left to cool under magnetic stirring for 30 minutes. The resulting mixture was filtered through Whatman paper (3 mm). The filtrate recovered represents an initial stock solution at 150 g/liter of 10% [16].

### Toxicity tests

The toxicity test consists of exposing the second instar larvae of *D. melanogaster* to different concentrations (50g/l, 100g/l, 150g/l) (choosing after preliminary tests) in the nutrient medium (10 ml of extract in 40 g of nutrient medium). Different concentrations were administered in adults by contact and by ingestion. The larvae tested for each concentration were in batches of 20 individuals divided into 3 replicates plus an untreated control batch. The variable quantified was the mortality rate as a function of concentrations and time. The mortality was recorded every day for 15 days, which allowed us to calculate the different toxicological parameters and sub-lethal concentrations. The sub-lethal concentration was administered to second instar larvae of *D. melanogaster* to study its effects on sexual behavior.

### Mating tests

The behavioral tests were carried out in a darkened room, covering as much external noise as possible,

with stable temperature of 24 ± 1 °C and a humidity of 65-75%. The experiments were performed by direct observation; we applied the technique proposed by Elens and Wattiaux, 1960 [18]. The flies used were virgin and aged 3 to 5 days to ensure that the flies were sexually mature. They were treated with a sub-lethal concentration of the aqueous extract. The courtship tests consisted of recording the start and end of each courtship sequence by the male to the female during a 30-minute observation period, which is sufficient time for the male to complete its parade.

The male was first introduced into the behavioral cell to become accustomed to its new environment, followed by the female ten minutes later. The moment the female was introduced into the cell corresponds to the time $T_0$ when the test starts [10].

A series of crosses were performed:

Control male / Control female (20 pairs).
Control male / Treated female (20 pairs).
Treated male / Control female (20 pairs).
Treated male / Treated female (20 pairs).

### Statistical analysis

The lethal concentrations (LC50, LC90) were calculated according to the mathematical procedures of Finney. The data were transformed and normalized according to Bliss tables [19]. The calculations and descriptive statistics as well as the chi-square test and the One-Way ANOVA were calculated by using Spss v23.0 software (IBM Corp., 2015).

## RESULTS

### Direct effect of treatment on mortality

The results of the mortality recorded for the second instar larvae showed that the mortality correlates positively with the increase in the concentration used and the exposure time (Figure 1). It reaches its maximum value (30% to 93%) after the 12$^{th}$ day of exposure for all concentrations used.

Table 1 shows the different toxicological parameters of the aqueous extract of *C. colocynthis* according to the different concentrations used. The treatment with aqueous extract of *C. colocynthis* allowed to eliminate 50% of *D. melanogaster* individuals with a concentration of 112 g /l only after 3 days of exposure; on the other hand, it takes 67 g/l and 12 days to eliminate 50% of the controlled specimens of the tested population. Furthermore, for the concentration 150 g/l, the necessary time was 2 days to eliminate 50% of the individuals. There is a strong correlation between the mortality rate and the time of exposure of flies to different concentrations of the treatment used.

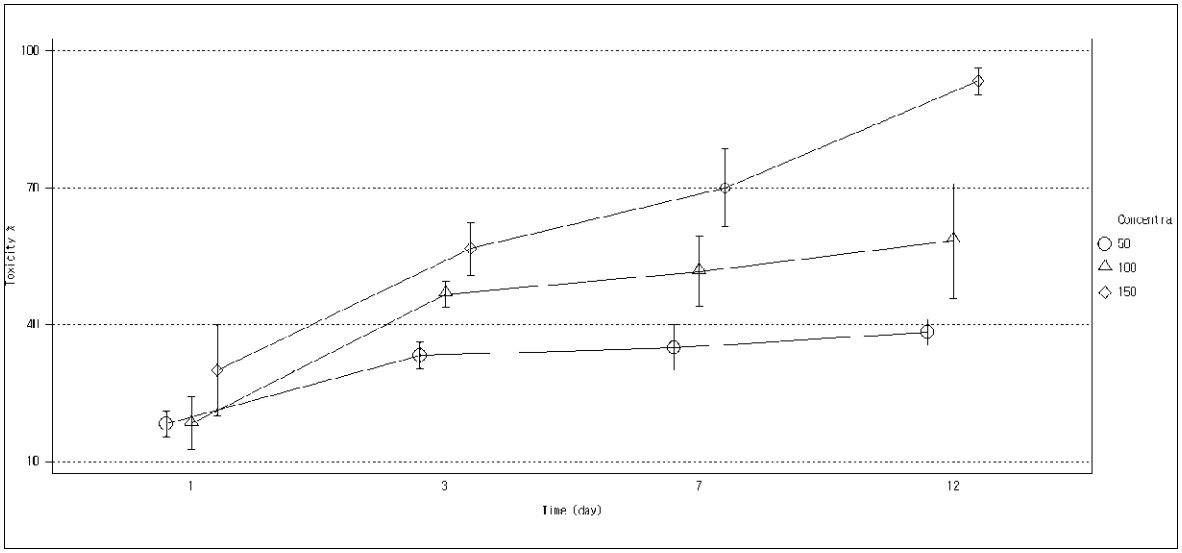


**Figure 1.** Variation of the mortality rate of *D. melanogaster* exposed to different concentrations of the aqueous extract of *C. colocynthis* at different exposure times.

**Table 1.** Toxicological parameters and their confidences intervals applied on D.*melanogaster treated with C. colocynthus.*

| Days | 1 day | 3 days | 7 days | 12 days |
|---|---|---|---|---|
| **linear regression** | Y = -2.16 + 0.71X | Y = -2.49 + 1.21X | Y = -3.57 + 1.85X | Y = -6.46 + 3.54X |
| | $R^2$ = 0.61 | $R^2$ = 0.99 | $R^2$ = 0.97 | $R^2$ = 0.85 |
| **LC50% [g/l]** | 1000 | 112.27 | 84.56 | 67.68 |
| | | [85.20-186.45] | [68.24-102] | [47.21-84.16] |
| **LC90% [g/l]** | 54061 | 1273.04 | 417.52 | 170.9 |
| | | [461.84-72937] | [262-1203] | [126.95-363.42] |
| **Concentration g/l** | **50g/l** | **100g/l** | **150g/l** | |
| **linear regression** | Y = -0.83 + 0.54X | Y = -0.79 + 1.01X | Y = -0.63 + 1.75X | |
| | $R^2$ = 0.87 | $R^2$ = 0.77 | $R^2$ = 0.86 | |
| **LT50% [Days]** | 36.09 | 6.04 | 2.31 | |
| **Confidence interval** | [19.21-127.63] | [4.16-10.09] | [1.62-3.03] | |
| **LT90% [Days]** | 371 | 127.4 | 14.56 | |
| **Confidence interval** | [276-565] | [45.10-1436] | [9.72-28.72] | |

**Table 2.** Latency time [second] of the beginning of each sexual parade sequence.

| Sequences | M C x F C | M C x F T | M T x F C | M T x F T | F | P |
|---|---|---|---|---|---|---|
| **Orientation** | 6.90 ± 2.10 | 10.65 ± 2.18 | 12.55 ± 2.96 | 13.55 ± 3.18 | 1.74 | 0.16 |
| **Contact** | 9 ± 2.15 | 12.95 ± 2.87 | 15.20 ± 3.03 | 17.55 ± 3.45 | 1.05 | 0.37 |
| **Vibration** | 60.4 ± 29.13 | 157.58 ± 93.78 | 161.52 ± 76.38 | 210.75 ± 84.31 | 5.4828 | 0.002 |
| **Licking** | 74 ± 29.77 | 141.15 ± 74.87 | 173.93 ± 63.48 | 242 ± 87.93 | 3.8873 | 0.013 |
| **Attempt** | 86.3 ± 35.60 | 167.75 ± 82.50 | 202.90 ± 92.87 | 287.25 ± 61.25 | 4.8335 | 0.004 |
| **Successful mating** | 109.63 ± 44.66 | 185.77 ± 48.00 | 210.42 ± 44.91 | 337.4 ± 106.70 | 2.576 | 0.06 |

[M: Male, F: Female, C: control, T: treated]

## Effect of treatment on sexual behavior

### Effect of the treatment on latency time of the beginning of each sexual parade sequence

Table 2 summarizes the latency times for the execution of each sequence of the sexual parade according to the different crossings made. In the control pairs, it took 6.90 ± 2.10 s to start their courtship, while in the other types of crosses it took 10.65 ± 2.18 s or more to start their courtship. To record the first instance of vibration, licking, or the first mating attempt, it took a long time in the treated couples compared to the control couples. The variance analysis showed that there are significant differences (Table 2). Treated couples took almost the double time to complete a successful mating compared to control couples.

### Effect of treatment on mating duration in the crosses studied

The mating duration is the time between the start of copulation and the disengagement of the two partners. In the control couples it is 1086.52 ± 152.63 s. The control pairs have the longest mating time, unlike the treated pairs, which show a significant decrease especially in the crossing where both partners are treated 416.20 ± 175.98 s (Figure 2).

### Effects of treatment on the number of repeats of the different courtship sequences

The number of repeats varies between the different crosses applied from one sequence to another of the courtship. Treated couples have a high number of repeats compared to the controls. The cross control

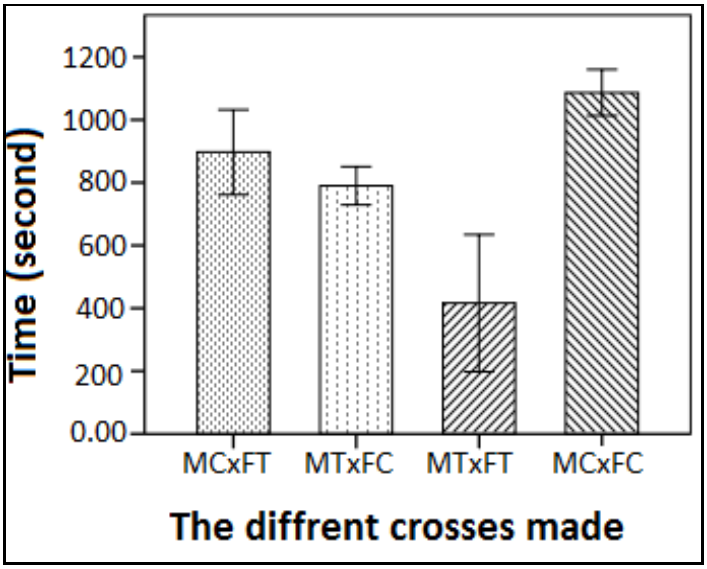


**Figure 2.** The duration of mating according to the different crosses.

**Table 3.** Effect of treatment on the number of repetitions of the different courtship sequences.

| Sequences | M C x F C | M C X FT | M T X F C | M T x F T | F | P |
|---|---|---|---|---|---|---|
| **Orientation** | 6 ± 1.58 | 11.8 ± 2.11 | 6.85 ± 2.10 | 7.65 ± 1.79 | 0.67 | 0.57 |
| **Contact** | 4 ± 1.07 | 9.2 ± 2.37 | 4.95 ± 1.39 | 6 .9 ± 1.20 | 7.18 | 0.0003 |
| **Vibration** | 3.7 ± 1.17 | 8.35 ± 2.05 | 5.23 ± 1.34 | 4.12 ± 1.02 | 3.59 | 0.01 |
| **Licking** | 3.15 ± 1.03 | 7.69 ± 2.46 | 4.93 ± 1.83 | 4.35 ± 1.15 | 2.67 | 0.05 |
| **Attempt** | 3.25 ± 2.02 | 6 ± 3.61 | 4.18 ± 1.47 | 4 ± 1.27 | 3.32 | 0.02 |

[M: Male, F: Female, C: control, T: treated]

**Table 4.** Effect of treatment on sexual behavior of *D. melanogaster*.

| | Orientation | Contact | Vibration | Licking | Attempt | Successful mating |
|---|---|---|---|---|---|---|
| **M CXF C** | 100% | 100% | 100% | 100% | 100% | 95% |
| **M C XF T** | 100% | 100% | 85% | 65% | 60% | 45% |
| **M T X F C** | 100% | 100% | 85% | 75% | 55% | 35% |
| **M C X FT** | 100% | 100% | 80% | 70% | 60% | 25% |
| **Chi-square $x^2$[df]** | 0.000 [3].NS | 0.000 [3].NS | 2.57 [3].NS | 9.35 [3].S | 19.18 [3].S | 58.3 [3].S |

[M: Male, F: Female, C: control, T: treated]

male X treated female recorded the highest number of repeats in all the crosses performed. We record a significant difference between the number of repeats and the different crosses applied (Table 3).

**Effect of treatment on different sequences of sexual behavior of *D. melanogaster***

Exposure of the second instar larvae to a sublethal concentration (50 g/l) of *Citrullus colocynthis*

aqueous extract shows that the adults, as a result of this treatment, show great disturbance in their sexual parade compared to the control individuals (Table 4). The first two sequences (orientation, contact) are passed by all the couples tested. Chi-square test showed no significance (X2 = 0,000: DF = 3, P = 1); further, the vibration was not affected by the treatment (X2 = 2,57: DF = 3, P = 0,45). However, the aqueous extract of *C. colocynthis* has a significant effect on the different types of crosses released, where the couples are unable to ensure the licking, attempted sequence, and successful mating (Table 4).

## DISCUSSION

With a population of more than 50% of the animal kingdom, some insects are a source of nuisance for humans and cause damage [20]. Insecticides are one of the most powerful tools that humans have been able to develop to control insect pests, but the frequent and indiscriminate use of synthetic insecticides leads to environment issues and pest resistance [21]. The use of chemical insecticides has adverse effects on the environment and human health. Another source of natural insecticides used by humans for centuries is plant-derived chemicals that are environmentally safe, less toxic to natural enemies and do not persist for long periods in nature [22]. Therefore, biological control takes various forms, but the ones that are currently attracting the attention of researchers is biological control using natural substances of plant origin as insecticides [23].

In this study, we chose *C. colocynthis* which is known for its toxic effects. The biological activity of *C. colocynthis* as a natural insecticide has been investigated against many insect pests like *Aphis craccivora, Schistocerca gregaria, Spodoptera litura, Tetranychus urticae, Sitophilus oryzae, S. zeamais,* and *Rhopalosiphum padi* [24, 25]. *C. colocynthis* had deterrent, anti-feeding, growth regulating and fecundity reducing properties on insects like *S. gregaria, S. litura* and *A. craccivora* [26].

The results obtained from the different concentrations tested (50 g/l, 100 g/l, 150 g/l) indicate a mortality rate that varies from one concentration to another and increases with exposure time. After 12 days, we noted a mortality rate of over 90% for the highest concentration (150 g/l). This shows the insecticidal effects of this plant.

Many works also show the toxic effects of aqueous extracts of *C. colocynthis* on Diptera. According to Merabti *et al.*, [16], aqueous extracts of *C. colocynthis* fruits have larvicidal effects on L4 larvae of *Culex pipiens L.* and *Culiseta longiareolata L.* and C*ulex quinquefasiatus* [27, 28]. On Lepidoptera Gulzar *et al.* [44] used different concentrations to determine its toxicities on *Helicoverpa armigera* [29]. The aqueous extract of different parts of *C. colocynthis* significantly reduces the population of *Rhopalosiphum padi* [24]. In 2020, Kandibane *et al*. [25] showed the anti-insect activity of ethyl acetate extract of *C. colocynthis [L.] Schrad* against *Spodoptera litura [Fab.] [Noctuidae: Lepidoptera]*. *C. colocynthis* had deterrent, anti-feeding, growth regulating, and fecundity reducing properties on insects like *S. gregaria, S. litura* and *A. craccivora* [26, 30].

Current research is focused on the side effects of new plant molecules. Exposure to insecticides at sublethal doses can lead to abnormalities in the behavior of insects. Different behaviors can be altered such as mobility foraging and feeding behavior [31].

*Drosophila* has proven to be an interesting model for toxicity studies. Many studies have been conducted using *D. melanogaster* under laboratory conditions to well reveal and define the effects of various insecticides and pesticides on the life cycle, hatchability, and emergence of the fly [32, 33].

After determining the toxicological parameters of aqueous extracts of this plant, we evaluated its effects on the sexual behavior of *Drosophila melanogaster* by treating L2 larvae with a sublethal concentration of 50 g/l. Sublethal effects can be defined as physiological changes that occur in individuals that survive exposure to a pesticide at a sublethal concentration [22, 34]. Various studies on the effects of sublethal concentrations of various insecticides have been conducted to determine the harmful and non-lethal effects of insecticides on insect life span, fecundity, fertility, and olfactory learning [33, 35].

Sexual courtship in *D. melanogaster* has been the subject of a large number of works since the first description by Sturtevant [36] to the present day. Mate choice is a complex process that affects

fitness and involves both genetic factors [37, 38]. The initiation and conduct of sexual behavior in this species and Diptera, in general, is characterized by the use of various sensory modalities: visual [39, 40] olfactory [41, 42], acoustic [42, 43] and tactile [44].

The adults resulting from this treatment show great disturbance in their sexual display, whatever the crosses made, and we recorded a very great decrease in the rate of successful mating in the treated pairs [25% of successful mating]. Sexual display, involving a multitude of sensory information, is also characterized by a complex constellation of behaviors with qualitative, quantitative, and sequential properties on which its success depends [45]. The first two sequences were successful for all pairs tested; the disturbance occurred during the vibration where we recorded some pairs unable to vibrate. Courtship in *Drosophila* is a form of multimodal communication, involving chemosensory, auditory, tactile, and visual signals [46]. Often, male signals are more visible and easily observable, and are therefore more widely studied; for example, males of many species produce a courtship song by extending and vibrating their wings (vibration) [47].

The structure and function of the olfactory system in Drosophila have been studied in several species of insects by several scientists; olfactory perception and behavioral change have been tested using several molecules of different nature [48]. Another signal that can be easily quantified, and has been widely studied, is the variation in chemotactic pheromones [49, 50]. These pheromones, present on the fly cuticle, sometimes act as sex- and species-specific identifiers that stimulate courtship between conspecific pairs but suppress courtship between heterospecific pairs in some species [51, 52].

In *Drosophila*, pheromones play a major role in sex or species recognition [41]. The volatile pheromones of *D. melanogaster* are produced by the females and are species-specific, attracting both sexes from a distance [48]. The completion of the different mating sequences in treated pairs is delayed compared to their control counterparts. Males have difficulty detecting females. The number of orientations in the crosses of treated pairs is higher than in controls, and orientation is based on visual perception. In the case of visual cues, for *Drosophila melanogaster*, visual perception of a moving courtship target is necessary for males to initiate and maintain courtship [43, 52]. Locomotion is sexually antagonistic and is thought to have a common genetic basis in males and females [53]. *D. melanogaster* males prefer larger females [54, 55].

In treated pairs, the number of vibrations and matings is higher in treated pairs, males have difficulty detecting females; any deviation in the structure and/or timing of the species-specific male courtship would be followed, in one way or another, by the male's inability to adequately stimulate female receptivity [43].

The treatment also influenced the mating latency; the realization of the different sequences of courtship in the treated males is delayed compared to the controls. The physiological state of the male increases the duration of the latency of each sequence. The duration of the coupling is the time between the start of copulation and the disengagement of the partners; this duration reduced in the treated couples [52].

It appears that the aqueous extracts of the plant act as neurotoxins, which is reflected in the disruption of sexual behavior involving pheromonal perception, movement, and vision. This toxicity is due to the cucurbitacins and their glycosides present throughout the plant, particularly in the fruit and seeds [24, 26]. Neurotoxins remain the most important class, accounting for more than 75% of the global insecticide market. They act on the nervous system of insects by disrupting synaptic transmission [56].

## CONCLUSION

*D. melanogaster* has served as a valuable organism for studies on insecticide toxicology for decades. In this study, we showed the direct and indirect efficacy of aqueous extract of *C. colocynthis* as an insecticide on *D. melanogaster*. It allowed to eliminate 50% of *D. melanogaster* after 3 days of exposure.

The treatment has shown that it has effects on the sexual behavior of the adults treated by a sub-lethal concentration; it causes a big disturbance in the sequences of the courtship and decreases the rate of successful mating. The treatment with aqueous extract of *C. colocynthis* seems to act at the level of the nervous system of the adults.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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