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EVALUATION OF *RAMALINA FARINACEA* (RAMALINACEAE) INSECTICIDE EFFECTS: IMPACT ON THE LETHALITY, SEXUAL BEHAVIOUR AND REPRODUCTION OF *DROSOPHILA MELANOGASTER* (DIPTERA: DROSOPHILIDAE)

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Abstract: Recently, biological control is based on the use of secondary molecules of plant extracts. In this work, ethanolic extracts of the plant *Ramalina farinacea* were tested for their effects on mortality, sexual behavior, and reproduction of the vinegar fly (*Drosophila melanogaster*). The results showed the presence of the toxic molecules with mortality and incomplete courtship as well as a significant decrease in the number of eggs laid and larvae treated.

Keywords: fruit fly, lichen, toxicity, mating, oviposition.

INTRODUCTION

The preferred applications for pest control are the use of chemical insecticides. However, the high level of these insecticides' secondary effects has led researchers to turn to other areas of study. The insecticides production by natural products, who entitled biological control, is very common now (Emsen and Aslan, 2018).

Natural substances used against insects are compounds that are obtained by various methods from plants, with insecticidal properties. They can come in different forms, such as unprocessed plant compounds, plants extracts, and pure vegetal compounds isolated. While some natural insecticides are used directly as killers, others have repellent and nutrition-inhibiting properties (Murugesan et al., 2016).

The mechanisms of insecticides action are generally located on the nervous and digestive body system. Insecticides of plant origin would have penetrated into the insect's organism by contact and poisoned the nervous-muscular system through secondary metabolites (Denecke et al., 2015). It has also been found that plant-based insecticides affect insect physiology by acting in different forms at various receptor sites (Buckingham et al., 2017).

Most plants used as pesticides are superior plants. Few works value the insecticidal effect of lower plants like lichens (Bachi, 2018; Cetin et al., 2008; Cetin et al., 2012; Emmerich et al., 1993; Giez et al., 1994; Kathirgamanathar et al., 2006; Pöykkö, 2006). The study of lichens in Algeria began more than a century ago, but in the form of explorations by naturalists who were collecting lichen species collected along the way and identified by Nylander. Several botanists followed one another in this direction and were able to mark the history of Algerian lichenology (Boutabia, 2000).

The lichens used in therapeutics go back to antiquity among ancient civilizations: Chinese, Hindu; in Egypt, they were used in anesthesia and mummification, especially for uterine obstruction and treatment of boils (Lev and Amar, 2008).

Lichens still preserve their role in pharmacological preparations, they are found today in the form of anti-tussive syrups and lozenges (Arnaud-Dieu, 2015).

We called lichens all plants presenting a well-marked individuality which result from the Eumycète symbiosis' with a "cyanobacterium" or a "chlorophyte" (Ozenda and Clauzade, 1970).

Lichens are also materials for natural insecticides sources because of their unique constituents in construction. For many years, the lichens toxic effects used in many fields on the different insect species have been tested through their extracts and pure components (Emsen and Aslan, 2018).

Our study is based on the lichen *Ramalina farinacea* which is a species of lichenized fungi, fruity in the form of a grey yellow-greenish strip of the same color on the underside. The lobes are of small width of zero point five to two mm flattened with numerous soralies (tearing of the thallus upper cortex) save marginal. The thallus is up to seven cm long with a variable size from three to six cm long for three mm width at the maximum for the widest ramification (Nash et al., 2004).

The most widespread species in the world is considered a dreaded nuisance both for the inconveniences caused by the parasitic diseases it can inoculate (Jolivet, 1980; Joly, 2006; Habbachi et al., 2013). It is also an important vector for various infectious microorganisms, including phytopathogenic yeasts and bacteria that attack not only vegetable and fruit crops but also cereal and sunflower crops (Kloepper et al., 1979; Corby-Harris et al., 2007; Nadarasahand Stavrinides, 2011; Becher et al., 2012).

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Within the framework of biological control, the objective of this work is to seek new alternative solutions to reduce the excessive use of synthetic pesticides, among these alternatives that nature presents, the products of plant origin.

We aim to evaluate the direct and indirect toxic effects of the lichen ethanolic extract (*R. farinacea*) on *D. melanogaster*. We seek to determine the sublethal concentration activities (0.12 µg / ml) in particular, the effects on mortality, sexual behavior, and egg-laying. Some studies have shown that sublethal-doses insecticides affect the behavior of harmful or beneficial insects (Haynes, 1988; Lûrning and Scheffer, 2007, Benhissen et al., 2018, Benhissen et al., 2019, Bekhakhche et al., 2018, Habbachi et al., 2019, Habbachi, 2020, Saadane et al., 2021a, Saadane et al., 2021b) in particular they induce a decrease in behavioral responses to sex pheromones in treated individuals (Wei and Du, 2004; Zhou et al., 2005).

MATERIALS AND METHODS

Insect rearing

Drosophila melanogaster was described by Johann Wihelm Meigen in 1830. Its reproduction is very fast. Its life cycle is very short and includes three larval instars and a pupal stage from which an adult emerges that can fly and reproduce. A wild strain harvested from rotten apples in the Annaba region (Algeria), was used. The culture was carried out in vials (250 ml) capped with a foam pad and containing an agar-based nutrient medium of cornmeal and brewer's yeast. The culture was maintained at 25±1°C, with a humidity of 70 to 80%, and a 12-hour scotophase (Habbachi et al., 2013; Habbachi, 2020). The use of *Drosophila* in biological research began in the early 20th century (Stephenson and Metcalfe, 2013). At present, it is one of the most popular, extensively studied, and cost-effective model organism for biological experiments (Feala, 2008). It significantly contributed to several key research like genetics, embryonic development, behavior, and disease-related signaling studies (Beckingham et al., 2005; Apidianakis and Rahme, 2011).

Ramalina farinacea (Ramalinaceae)

Ramalina farinacea is a bush-shaped epiphytic lichen (small tree) (fruticose) common to regions with the Mediterranean, subtropical, or temperate climate, mostly corticole species (no restriction concerning the support species), rarely saxicolous and occasionally on sand (Léonardo et al., 2011). *R. farinacea* does not have a fruiting body but uses soredies to reproduce and has oval organs that create soredies called soralia that form on the slender lobes. *Farinacea* means "flour-like", the name referring to the structure of the soredies (Hanus et al., 2008). This species of lichen is characterized by its long and narrow branches (less than two to three millimeters wide) and a clearly defined marginal soralia. It is most often found at low altitudes on trees and shrubs (Howard –Trevor, 1999). For the present study the plant is collected in the Seraïdi region (Annaba, Algeria) (36°54'27.02" north latitude, 7°39'49.95" east longitude) in November 2019.

Preparation of the plant ethanolic extract

For the extract, we macerated 57g of dry bulb powder in 500 ml of 70% ethanol for 24 hours at room temperature and in the shade. After filtration using Whatman filter paper, the filtrate obtained was evaporated in the shade using a magnetic stirrer heated to a temperature of 45°C to remove the solvent ethanol. The concentrated extract (1.30g) was dissolved in 200ml of distilled water, giving a mother concentration of *R. farinacea* (6.5g/l). Store in the oven at °C until use. The date of preparation, the type of extraction, and the concentration are noted on each bottle.

Treatment of larvae with plant extract

We have prepared four different concentrations 0.25µg/ml, 0.5µg/ml, 1.5µg/ml and 2µg/ml. The treatment is done by ingestion; each concentration is mixed with 40 g of food which will be distributed in four different tubes. In each tube, 20-second stage larvae from the mass rearing are placed. In a fifth tube containing no treatment, 20 larvae are placed as a control. The monitoring of mortality and development of the larvae is done during 15 days (time necessary to finish the development).

Effect of the extract on sexual behavior

Sexual parade in fruit flies is an eventful behavior (Clynen et al., 2011; Chardonnet, 2013), goes through six essential, predetermined, and invariable steps (Terhzaz, 2003; Dickson, 2008; Revadi et al., (2015) and requires many sensory signals, of which chemical signals (cubic hydrocarbon pheromones) play an important role (Greenspan and Ferveur, 2000; Sokolowski, 2001). The male first walks up to a potential female and taps her on the cuticle with his front legs; if the female moves, the male follows and vibrates a wing. Then, when the female stops, the male runs in circles around her, lick her genitals with his proboscis, and try to hang her up until mating (Revadi et al., 2015).

In this work, we treated a group of larvae with the sublethal concentration of *R. farinacea* extract (0.12 µg / ml) and then recovered the adults as soon as they emerged. 48 hours after emergence these adults will be used for sexual behavior tests where we note the time and number of contacts, the time and number of wing vibrations, the time and number of licks, the time and number of mating attempts as well as the time and duration of mating if successful. These tests are carried out according to four types of crossings: control male X control female, treated male X treated female, control male X treated female, and treated male X control female.

Effect of the plant extract on oviposition behavior and reproduction

To the purpose of determining the egg-laying site choice, we observed mated females and we noted the site eggs hatching, as well as eggs and larval numbers.

Data analysis

The toxicological parameters (LC50%, LC90%, LT50%, and LT90%) were calculated according to Finney's mathematical methods (Finney, 1971). Regarding sexual and oviposition behavior tests, results were analyzed statistically by descriptive metric methods then an analysis of variance (ANOVA) was performed on XLSTAT 2009 software (Addinsoft, New York, NY).

RESULTS

Effect on mortality

The results show that ethanol extracts of *R. farinacea* act on larval mortality depending on the concentration applied. The four concentrations used showed larvicidal activity at the end of the mortality monitoring (15 days after treatment) (Tab.1). 16% of the population was killed after 15 days of treatment (Tab.1). There are significant differences between the mortality rates recorded as a function of concentrations and exposure times (*p:* 0.037; 0.019; 0.001**) (Tab. 1).

Table 1.

Insecticidal effects of *R. farinacea* on *D. melanogaster*

	0,25 µg/ml	0,5 µg/ml	1,5µg/ml	2µg/ml	F_{obs}	*p*
5 days	0.00%	0.00%	1.25%	0.00%	***9.000***	***0.002***
10 days	0.00%	1.25%	1.25%	0.00%	***9.000***	***0.002***
15 days	8.75%	16.25%	16.25%	*12.50%*	***0.651***	***0.597***
F_{obs}	***3.917***	***4.866***	***2.540***	***10.897***		
p	***0.037****	***0.019****	***0.106***	***0.001*****		

(*P* <0.05) *: significant; (*P*<0.01) **: highly significant; (*P* <.001) ***: very highly significant

Table 2.

Toxicological parameters of *R. farinacea* ethanolic extracts on *D. melanogaster*

A									
	regression	**LC $_{50\%}$**	**LC $_{90\%}$**	**LC$_{84\%}$**	**LC$_{16\%}$**	**SLOPE**	**ʃLC50 %**	**lim.Inf**	**lim.Sup**
15 days	Y=6,05+2,29X (R=0,87)	0,00001	0,004	2,82 * 10^9	2,81	1,41* 10^{14}	338,75	0,000	0,003
B									
	regression	**LT$_{50\%}$**	**LT$_{90\%}$**	**LT$_{84\%}$**	**LT$_{16\%}$**	**SLOPE**	**ʃLT50 %**	**lim,Inf**	**lim,Sup**
0,25 µg/ml	Y=-1,60+3,17X (R=0,67)	*120,22*	*302*	245,47	*57,54*	2,07	1,138	105,60	136,87
0,5 µg/ml	Y=-2,12+4,91X (R=0,90)	*28,18*	*51,28*	*44,66*	*17,37*	1,60	1,088	25,90	30,66
1,5 µg/ml	Y=-0,66+3,99X (R=0,87)	*25,7*	*54,95*	*54,95*	*50,11*	1,33	1,052	24,44	27,03
2 µg/ml	Y=-1,7+3,35X (R=0,67)	*100*	*240*	*194,98*	*50,11*	1,97	1,000	100,00	100,00

(**A**: larval exposure time; **B**: concentrations used; **y**: probits of mortality rates; **X**: the decimal logarithm of concentrations and/or times).

Effect on *D. melanogaster* sexual behavior

Effect on the mating success rate

The results show that *R. farinacea* ethanolic extract, administered at a sublethal concentration of 0.12 µg / ml, decreases the successful mating rate in the fly, regardless of the sex treated within a couple (dyad) (Tab. 3). The mating success rate is 100% in controls; whereas it reaches 30% when one of the two partners is treated with the Mediterranean plant (Tab. 3). The aborted mating rate (couples attempting to mate unsuccessfully) is 30 to 60% while the null mating number (neither attempt nor mating) is higher in couples (control males X treated females) (Tab. 3).

Table 3.

R. farinacea (0.12 µg / ml) effects on successful mating rate

	% mating		
	Successful	**aborted**	**null**
♂C x ♀C	100	0	0
♂C x ♀*R.f*	25	30	45
♂*R.f* x ♀C	30	40	30

♂**R.f** x ♀**R.f**	40	60	0

[**C**: *Control*; **R.f**: *Treated with R. farinacea ethanolic extract*]

Effect on nuptial courtship and mating

We have recorded that couples where the male is treated (composed of control males and females) take longer to establish the first contact between the two partners. The study shows that there are highly significant differences between the time of this mutual recognition (Fobs=4.990;p: 0.004**) (Tab. 4). The same result is observed for the different times recorded during the sexual parade of *D. menlanogaster* (time of first vibration p: 0.001**; time of first licking *p:* 0.155; time of first attempt p: 0.022*; mating time *p*: 0.333) (Tab. 4).

The *R. farinacea* plant extract does not influence the contacts number between flies (Fobs=1.899; p: 0.139), the wing vibrations number (F_{Fobs}= 9.716; *p*: < 0.0001***) and the licks number (Fobs= 18.746; *p*:< 0.0001***) (Tab. 4).

Table 4.

Effect of *R. farinacea* ethanolic extracts on different sequences of sexual behavior of *D. melanogaster* (Moy ±SEM)

	First contact time	First vibration time	First licking time	First attempt time	Mating time
♂**C** X♀**C**	201.150±30.910	211.150±31.177	284.450±33.620	3.200±0.506	485.150±64.382
♂***R.f***X♀***R.f***	255.500±59.130	249.650±68.777	366.722±74.973	5.500±1.172	91.650±31.746
♂**C**X♀***R.f***	290.286±54.107	431.556±79.943	433.083±85.286	4.100±0.836	122.600±68.954
♂***R.f***X♀**C**	409.059±111.010	417.167±107.622	352.846±108.353	8.615±1.849	122.750±62.777
F_{obs}	**4.990**	**5.806**	**1.811**	**3.470**	**1.155**
p	***0.004****	***0.001****	***0.155***	***0.022****	***0.333***
	Contacts number	**Vibrations number**	**Lickingnumber**	**Attemptsnumber**	**Mating duration**
♂**C** X♀ **C**	3.000±0.653	14.050±2.929	3.150±0.499	3.200±0.506	1058.100±93.620
♂***R.f***X♀***R.f***	5.188±1.272	28.700±5.056	9.944±1.587	5.500±1.172	1340.375±89.901
♂**C** X♀***R.f***	3.000±0.524	12.667±2.775	5.833±1.370	4.100±0.836	1069.000±225.865
♂***R.f***X♀**C**	3.235±0.559	42.667±7.192	13.615±3.178	8.615±1.849	714.000±217.351
F_{obs}	**9.136**	**9.716**	**18.746**	**3.470**	**1.089**
p	**1.899**	***< 0.0001**** ***	***< 0.0001**** ***	***0.022****	***0.367***

[Mean: Mean; SEM: Standard deviation of the mean; C: Control, R.f: Treated with *R. farinacea* ethanolic extract] [(*P* < 0.05) *: *significant*; (*P* < 0.01) **: *highly significant*; (*P* < 0.001) ***: *very highly significant*]

The follow-up of 20 mated females for the different crosses types allowed us to determine the laying choice, the fecundity, and fertility of these females after treatment.

The results in Table 5 show that the eggs laid number is low in couples where one of the two partners is treated (about one to nine eggs). This result is subsequently observed at the level of emerged larvae; the larvae number in these pairs is 8,800 ± 1,9703 larvae to 11,25±1,820 larvae (Tab. 5). The eggs laid number in the control and treated environment is the same in all four types of crosses (p: ≥0.05).

Table 5.

Effect of *R. farinacea* ethanolic extract on the total number of eggs laid by females from the four crosses

Couples	Laid eggs number in control medium	Laid eggs number in *Ramalina* medium	t_{obs}	*P*	Larvae number in control medium	Larvae Number in *Ramalina* medium	t_{obs}	*p*
♂**C X** ♀**C**	84.190 ± 40.243	/	***4 .375***	***0.043****	32.55 ± 3.6190	/	***56.916***	***<0.0001**** ***
♂***R.f*** **X** ♀***R.f***	8.800 ± 1.9703	11.25±1.820	***0.223***	***0.640***	2.050 ± 0.766	2.500±1.333	***0.342***	**0.562**
♂**C X** ♀***R.f***	1.600 ± 1.054	4.000±1.577	***1.426***	***0.240***	8.800 ± 1.9703	11.25±1.820	***0.223***	***0.640***
♂***R.f*** **X** ♀**C**	8.800 ± 1.9703	11.25± 1.820	***0.223***	***0.640***	2.050 ± 0.766	2.500±1.333	***0.342***	**0.562**

Oviposition Preference Index (OPI)

Couples in which the female controls and the male is control prefer the control environment with a negative preference index (-1) (Tab. 6). The results show that there is a repellent effect of the *R. farinacea* ethanolic extract (Tab. 6).

An OPI of (+0.12) was recorded in treated couples (Tab. 6). The couples where one of the two partners is treated have an OPI of (+0.20) which shows that the females of these couples are attracted by the treated environment (Tab. 6).

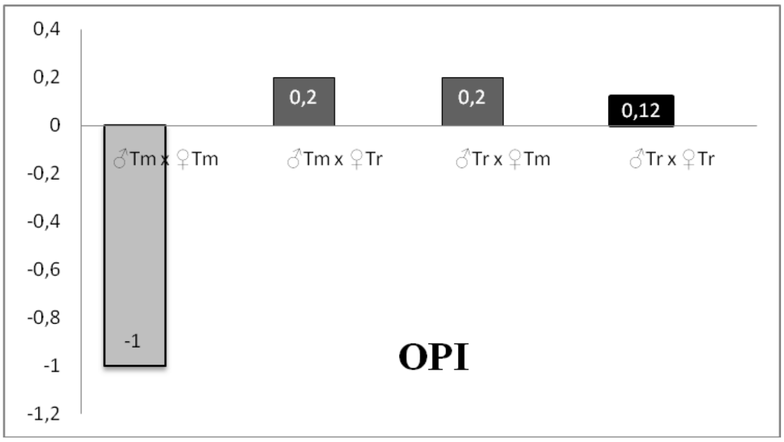

Fig. 1. Oviposition preference index (OPI) of *D. melanogaster* females to *R. farinacea* ethanolic extract (0.12µg/ml).

DISCUSSION

In recent years, natural products have drawn the researchers' attention to find new solutions alternatives where the aims are to reduce the excessive use of synthetic pesticides. Among these alternatives in nature, products of plant origin are, in particular, more interesting because they are less toxic, biodegradable, and target-specific (Dua et al., 2010; Subramaniam et al., 2012).

The insecticidal activity of aromatic plant extracts were also confirmed by Jang et al., (2002); Habbachi et al., 2014; Mahmoudian et al., 2002; Habbachi et al., 2013; Idrissi-Hassani et al., 1998; Abbasi et al., 2003; Abbasi et al., 2003; Idrissi-Hassani and Hermas, 2008; Abbasipour et al., 2010; Masna et al., 2016; Benhissen et al., 2018; Habbachi et al., 2019; Kheroubi et al., 2020 ; Saadane et al., 2021).

Lichens represent the association of a fungal element and an algal element, only what should be emphasized and indisputably, that lichen is a biomorphosis, that is to say, a new form that results from the interactions between its constituents. It does not really resemble any more one or the other of the associates, so much the morphological, physiological, chemical, and genetic integration has been pushed forward (Van Haluwyn and Lerond, 1993).

Giez, Lange, and Proksch (1994) showed that the lichen compounds, oxyphysodic acid, and vulpinic acid, were toxic to the herbivorous polyphagous insect *Spodopteralittoralis* (Boisd) (*Lepidoptera: Noctuidae*). The results of the Evaluation of Lichen Metabolites showed very high potential as a natural insecticide resource against *C. longiareolata* (Cetin et al., 2012).

All metabolites are compared on the basis of their LC50 values. The order of toxicity is as follows: gyrophoric acid (0.41 ppm) 4(þ)-usnic acid (0.48 ppm) 4atranorine (0.52 ppm) 43-hydroxyphysodium acid (0.97 ppm), however, based on the estimated LC90 values, the order of toxicity is (þ)-usnic acid (1.54 ppm) 4 gyrophoric acid (1.93 ppm) 43-hydroxyphysodium (4.33 ppm) 4atranorine (5.63 ppm) (Cetin et al., 2008).

In this study, we highlighted the direct and delayed toxicological effects of the lichen *R. farinacea*. Its maceration in ethanol allows the recovery of an extract that has insecticidal activity, leading to different mortality rates in the fruit fly. Fly mortality is mainly a function of the exposure time.

Other studies show that all secondary metabolites of lichen have a larvicidal activity to varying degrees, (þ)- usnic acid was isolated from *R. farinacea* (Cetin et al., 2012) which is toxic to polyphagous herbivores (Emmerich et al., 1993).

Several studies have reported that lichen extracts can have intense insecticidal activity on fruit flies, such as the study by Cetin et al., (2008) when using the main lichen compounds, ((_)- and (þ)-usnic acid) against the larvae of the house mosquito, *Culex pipiens L*, These metabolites were found to be highly toxic at concentrations of 2.5, 5 and 10 ppm, so (þ)-usnic acid had a high lethal effect on second and third instar larvae of *Culiseta longiareolata*.

Similarly, the larvicidal activity of certain lichen metabolites against second instar larvae of *Aedes aegypti (L)* was also reported by (Kathirgamanathar et al., 2006). Larval mortality of the four metabolites was concentration-dependent; (þ)-usnic acid, gyrophoric acid and 3-hydroxyphysodium acid showed the greatest larval toxicity, with complete mortality at 5 ppm (Cetin et al., 2012).

In addition, several researches have highlighted the effective insecticidal effect on adults of *Ceratitis capitata* since the average cumulative mortality of adults increases up to 100% according to the doses tested and the time of exposure compared to the control with the extract of *R. farinacea* (Bachi, 2018).

Plants may not kill insects, but they block their reproduction. This effect type was observed in *D. melanogaster* fly after treatment with *P. harmala* (El-Bah et al., 2016). The study by Habbachi et al., (2019) shows that the aqueous extract of *Cleome arabica* (*Capparidaceae*) causes up to 50% mortality in

Drosophila but has a significant effect on fly sexual behavior. The bulbs of our plant act in the same way; a disturbance of the different sequences leading to adult mating is observed after ingestion of the ethanolic extract of *R. farinacea*. Blockage of sexual parade or mating is especially noticeable when one of the two partners is treated; this may be due to the mutual non-recognition between the two insects especially as the plant acts significantly on the contact sequence in the fly. The contacts' role in the mutual partners' recognition has been demonstrated in different insect species such as cockroaches (Roth and Willis, 1952; Smyth, 1963; Farine et al., (1993); Gropeaux, 1994). One in control means that works in better harmony with the environment are the use of plant-derived toxicants, some of which are involved in the neuroendocrine regulation, metamorphosis, and reproduction of insects (Philogene, 1991; Rembold, 1994).

In the third part of the present study, we showed that oviposition site choice in *Drosophila* females (oviposition behavior) is not modified after treatment; females can also choose the treated culture medium as an oviposition site to ensure their generations development and the "OPI" choice index confirms this result.

The same results have been reported in females of *Cleorodes lichenaria* that tend to lay eggs on host lichens that provide the shortest development at the expense of a higher growth rate in the early larval period without decreasing survival or pupal size (Pöykkö, 2006).

On the other hand, *R. farinacea* extract acts on eggs laid and larvae numbers; the larvae number decreases significantly after treatment, which indicates that *R. farinacea* has blocked fecundity and fertility of *D. melanogaster*. El-Bah's (2016) study indicated the same results as when using the toxic plant *P. harmala*.

Against undesirable species, biological control is an effective solution, although it requires observation of the environment, which is much more subtle and difficult. Biological control can be part of a sustainable development perspective, provided that it benefits from rigorous scientific monitoring. More widespread use of this practice will lower the production costs of auxiliaries, which will lead to a new virtuous circle (Web Site).

CONCLUSION

This study indicates that *R. farinacea* ethanolic extract has a neurotoxic property, the sublethal concentration (0.12 µg / ml) shows that the treated individuals are unable to present a complete nuptial courtship. Chemical analysis of treated and control flies can provide information on any changes in adults' nuptial courtship, pheromone secretions, mating duration, and various sexual sequences for adults. This work results suggest the presence of toxic secondary metabolites in the extract studied, which may lead to the development of bioinsecticides based on *R. farinacea* to be used in agriculture and sold on the pesticide market.

AUTHORS CONTRIBUTION

All authors are equally contributed to this study. Nour El Imene BOUBLATA, Sarra HABBACHI, Abir BOUZAR, Wafa HABBACHI, Abderachid SLIMANI, Abdelkrim TAHRAOUI, designed and carried out the experimental study and writing of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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