

## Valorisation of the Effects of Bioactive Compounds of the Ethanolic Extract of *Ramalina Farinacea* (Ramalinaceae) on the Development, Eating and Pupation Behavior of *Drosophila Melanogaster* (Diptera: Drosophilidae)

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## Cover Page Footnote

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## VALORISATION OF THE EFFECTS OF BIOACTIVE COMPOUNDS OF THE ETHANOLIC EXTRACT OF *RAMALINA FARINACEA* (*RAMALINACEAE*) ON THE DEVELOPMENT, EATING AND PUPATION BEHAVIOR OF *DROSOPHILA MELANOGASTER* (DIPTERA: DROSOPHILIDAE)

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### ABSTRACT

Plants are rich in bioactive chemical secondary metabolites and have proven insecticidal activity by killing or repelling insects. In this work, we aim to evaluate the direct and delayed effects of ethanolic plant extracts on the vinegar fly (*Drosophila melanogaster*). The treatment was performed by ingestion on second instar larvae (L2) to evaluate the impact of the ethanolic extract on development for 15 days and subsequently on the feeding behavior of the larvae. The results of this study indicate a slowing down of pupal growth until the adult stage, at the three concentrations (0.25 µg/ml, 0.5 µg/ml, 1.5 µg/ml, 2 µg/ml) used. The results also showed that after three days of treatment, third instar *D. melanogaster* larvae lost the ability to detect the odors of their nutrient environments. Other numbers of larvae (34 %) do not make a choice in the different tests performed. This study indicates that the ethanolic extract of *Ramalina farinacea* has a neurotoxic property our results confirmed the presence of toxic secondary metabolites which have bioinsecticidal activities in this extract.

**Keywords:** *Drosophila melanogaster*, *Ramalina farinacea*, Bioactive Compound, Food Behavior, Pupation Behavior.

### INTRODUCTION

Plants produce active substances with insecticidal, aseptic or plant and insect growth regulating properties. Most often, these active substances are secondary metabolites that originally protect plants from herbivores (Schmutterer, 2002). Biopesticides, which are based on natural plant extracts, are the best alternative means of controlling insects. They are less expensive, effective and without risk for the environment and human health. In fact, to reduce the dependence of the agricultural sector on chemical pesticides, the use of biopesticides is becoming more and more

effective and recommended (Schmutterer, 2002).

The aim of this work is to test the effects of ethanolic extract of *Ramalina farinacea* which is a species of lichenized fungi fruticose fairly widespread on tree trunks, the most tolerant of air pollution. Its secondary metabolites contain cortex with usnic acid (Nash et al., 2004) which effects the fruit fly, *Drosophila melanogaster*, in particular, the effects on the development, food and pupation behavior. The most widespread species in the world is considered a dreaded pest both for the inconvenience caused by parasitic diseases that it can inoculate (Jolivet, 1980; Joly, 2006; Habbachi et al., 2013). It is an important vector for various infectious microorganisms for crops

(yeasts and phytopathogenic bacteria) (Klopper et al., 1979; Corby-Harris et al., 2007; Nadarasah and Stavrinides, 2011; Becher et al., 2012).

## MATERIALS AND METHODS

### *Insect Rearing*

*D. 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 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  $25 \pm 1$  °C, a humidity of 70 to 80 %, and a 12-hour scotophase.

### *Ramalina farinacea (Ramalinaceae)*

*Ramalina farinacea* is a bush-shaped epiphytic lichen (small tree) (fruticose) common to regions with 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, 1999).

### *Ramalina Collection Site*

The present work concerns the Séraïdi region. Part of the Edough massif,

Séraïdi is perched on an altitude of 840 m, bordering the Northwest of Annaba city. The plant is collected in the oak zeen forests (36°54'27.02" North latitude, 7°39'49.95" East longitude) in November 2019.

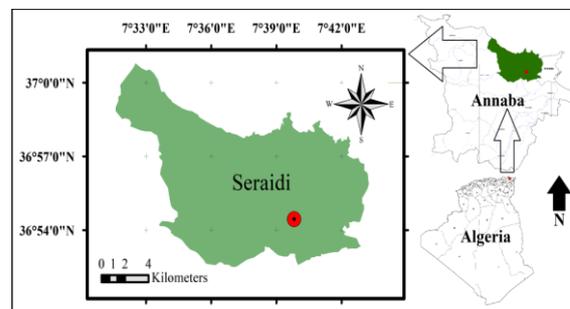


Figure 1: Séraïdi region map.

### *Preparation of the Plant Ethanolic Extract*

For the extract we macerated 57 g 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 (1.30 g) was dissolved in 200 ml of distilled water, giving a mother concentration of *R. farinacea* (6.5 g/l). Store it in the refrigerator at 4°C until use. The preparation date, the extraction mode and concentrations are noted on each bottle.

### *Treatment of Larvae with R. farinacea Extract*

We have prepared three different concentrations of the ethanolic extract 0.25 µg/ml, 0.5 µg/ml and 1.5 µg/ml. The treatment is done by ingestion; each concentration is mixed with food (40 g) which will be distributed in four different tubes. In these tubes, 20 larvae of the second instar larvae (L2) were placed from the initial mass rearing. In a fifth tube containing no treatment, 20 larvae were placed as a control. The monitoring of

larval development was done for 15 days (time needed to finish development).

### ***Effect of the Plant Extract on Food Behaviour***

The larvae of the second larval instar (L2) were used in the treatment with a sub-lethal concentration 0.12 µg / ml for two day. These larvae were easy to handle and will be used for our food attractiveness tests. 50 untreated larvae are used for the observation of witnesses. The principle of these tests carried out on 3rd instar larvae of *D. melanogaster* was to better understand the olfactory acuity in larvae and how they can detect the presence of food.

In order to carry out these tests, the following material is used: plastic petri dishes (100 mm diameter), Whatman filter paper (15 mm diameter), fine tweezers, small needles (about 10 mm long), stainless steel spatulas, stopwatches, glass cups.

The test arena used is a plastic Petri dish (100 mm diameter) containing 2 % agar, the bottom of which is covered with a paper on which 2 circular zones (Zone "A" and "B") have been drawn in a line with a pencil; each zone represents 10 % of the total surface of the arena

### **Preparation of Petri Dishes containing 2% Agar**

The artificial medium used ensures larval movement and provides a smooth surface that facilitates larval movement. In the laboratory, we prepared the medium based on agar and water. To prepare 8 dishes, 5 g of agar is dissolved in 250 ml of water, then the pan is placed on the hot plate while stirring continuously so that the agar product dissociates completely in the water. The mixture is brought to a boil until a dense layer appears under the mixture. The prepared amount is spread on the Petri dishes to a thickness of about 5 mm and left for about 3 hours so that the

medium cools and the surface becomes smooth.

Then we introduce the filter papers (already prepared) in tubes containing the control culture medium mixed with the ethanolic extract of *Ramalina farinacea* and we leave them for 2 hours so that the filter papers keep the smell of the food.

After 2 hours, the papers are removed and the medium is removed by rubbing with a spatula both sides of the filter papers. These papers are then put in the petri dishes in zone A and zone B. We note the choice of each larva and the time it takes to reach the chosen zone. The test is followed for 30 minutes.

The larvae are transferred from the rearing tubes to a glass dish with distilled water and then they are taken with tweezers in another glass dish also containing demineralized water. The filter papers soaked with nutritional medium are introduced into the petri dish containing agar (2 %). They are handled only with forceps. Each filter paper is fixed with a small needle.

A larva is taken (with a pair of forceps) and placed in the center (point "L") of the petri dish containing the filter papers. At this moment, we start the stopwatch and we note the choice of larva and the time it takes to make its choice.

The tests are done with control larvae and treated larvae (N = 50) and we tested the choice between the media: treated vs treated, control vs treated and control vs control

### ***Analyse des donnees***

The toxicological parameters (LC50 %, LC90 %, LT50 %, and LT90 %) were calculated according to Finney's mathematical methods (Finney, 1971). Regarding eating 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

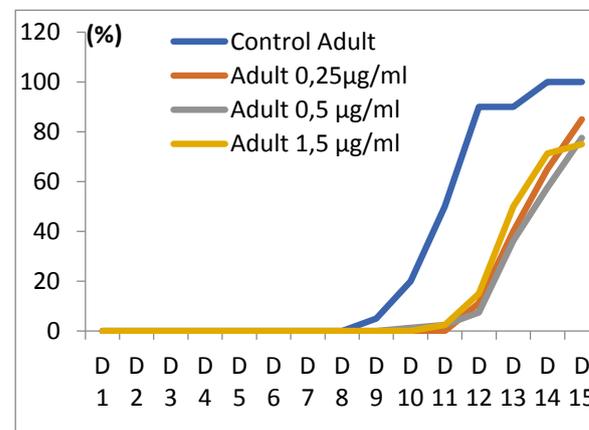
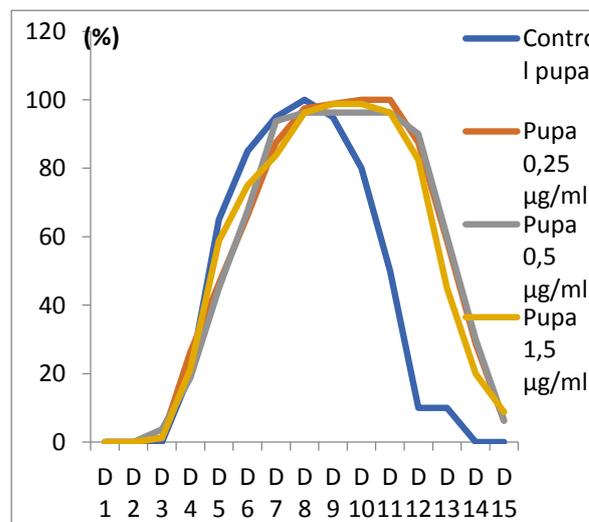
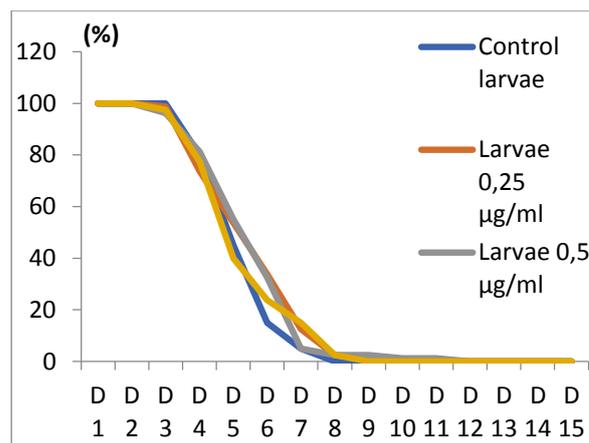
### *Effect on Development:*

*R. farinacea* had a significant effect on fly development by inducing a slowing down in pupae growth to adult, at all three concentrations. We observe that only 15 to 20 % of treated population with ethanolic extracts of *R. farinacea* did not reach the adult stage (Fig.2).

### *Time of Detection*

In the presence of two different odors (control vs. treated), control larvae move faster to the control medium with  $405.316 \pm 95.660$  with *R. farinacea* ethanolic extract averaging  $475.320 \pm 85.069$  seconds (Fobs = 0.435;  $p$ : 0.513) (Tab.1). Once control larvae are exposed to the same odorant sources (control vs. control or treated vs. treated), the attraction is still faster for their initial developmental medium with  $225.233 \pm 46.283$  seconds on average (Fobs = 2.400;  $p$ : 0.130) (Tab.1). However, they take more than  $273.360 \pm 64.811$  seconds to detect the odor coming from the treated medium (Fobs = 0.269;  $p$ : 0.606) (Tab.1).

In treated larvae, the recorded detection times are on average  $345.964 \pm 64.780$  and  $251.238 \pm 61.503$  seconds to locate, respectively, the papers soaked in the control and *R. farinacea* ethanolic extract ( $0.12 \mu\text{g/ml}$ ) (Fobs = 0.324;  $p$  = 0.572 not significant) (Tab.1). In addition, they take between less than  $441.381 \pm 82.054$  seconds to localize the control odor (Fobs = 0.002;  $p$  = 0.961), and  $354.190 \pm 80.788$  seconds for the treated odor (Fobs = 2.516;  $p$  = 0.120), when using two similar media in the test acen (Tab.1).



**Figure 2: Effect of *R. farinacea* ethanolic extract on *D. melanogaster* development (A: Larval development; B: Pupal development; C: Adult development).**

**Table 1: Detection time in control and treated larvae in response to the different odors of the tested medium [control medium; medium treated with ethanolic extract of *R. farinacea*].**

	Medium	Choice	N	Mean ± SEM	Min	Max	Var	F <sub>obs</sub>	p	
	Control Larvae	Control X Treated ( <i>R.f</i> )	Control medium	19	405,316±95,660	6,000	1300,000	173865,784	0,435	0,513
		<i>Ramalinamedium</i>	25	475,320±85,069	3,000	1673,000	180918,810			
Control X Control		A	9	180,778±20,450	108,000	256,000	3763,944	2,400	0,130	
		B	30	225,233±46,283	39,000	1054,000	64264,047			
Treated ( <i>R.f</i> ) X Treated ( <i>R.f</i> )		A	25	273,360±64,811	6,000	1213,000	105010,990	0,269	0,606	
		B	24	230,792±47,716	3,000	756,000	54644,607			
<i>Ramalina</i> Larvae		Medium	Choice	N	Mean ± SEM	Min	Max	Var	F <sub>obs</sub>	p
		Control X Treated ( <i>R.f</i> )	Control medium	28	345,964±64,780	43,000	1551,000	117501,517	0,324	0,572
	<i>Ramalina medium</i>		21	251,238± 61,503	17,000	1053,000	79435,490			
	Control X Control	A	21	441,381±82,054	30,000	1170,000	141388,848	0,002	0,961	
		B	27	377,667±78,330	3,000	1455,000	165659,077			
	Treated ( <i>R.f</i> ) X Treated ( <i>R.f</i> )	A	21	354,190±80,788	14,000	1389,000	137060,262	2,516	0,120	
		B	24	223,917±44,853	9,000	731,000	48282,080			

[Mean : Mean ; SEM : Standard deviation of the mean ; Min : Minimum ; Max : Maximum ; Var : Variance ; F<sub>obs</sub> : F observed ; P: p-unilateral value :]

### Pupation Behaviour

For control larvae, concerning the observation of the pupation of control larvae in the presence of the control and treated medium at the same time we noted that 16 % of the larvae choose while 8 % chooses the pupation in the treated medium, on the other hand 76 % of these last ones do not make their choice in the presence of the control and treated medium at the same time (Tab.2).

10 % of the control larvae marked the presence of their pupa on the soaked papers of the control culture medium and 90 % of them did not make their choice when both medium were controls (Tab.2). For pupation in boxes containing two treated media, 56 % of the control larvae chose pupation in the tested

culture medium as we note the absence of 44 % of pupation after these tests (Tab.2).

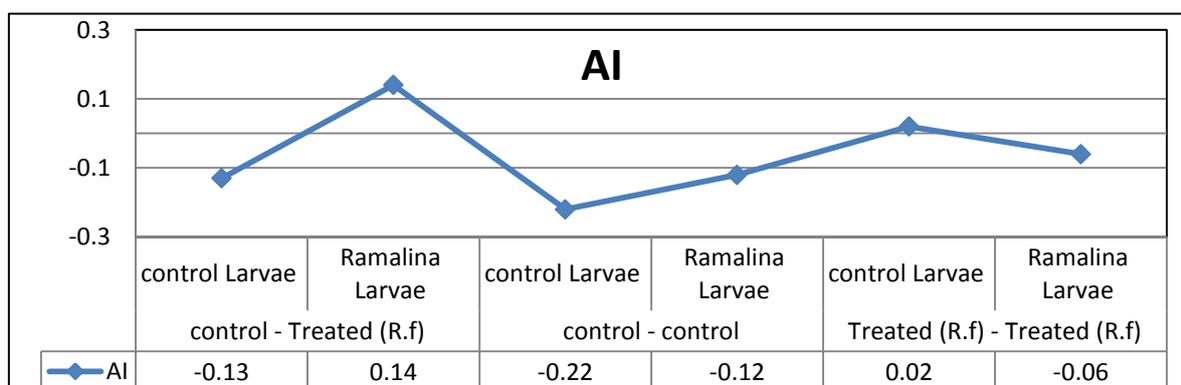
For the treated larvae, the observation of the pupation of the treated larvae after the feeding behavior tests showed that 2 % of the larvae made their choice for the control medium and only 12% for the treated medium and on the other hand 86% of the latter did not make their choice between the two control and treated medium.(Tab.2)

For the observation of pupation choice in treated larvae between the two control medium, 42 % marked the presence of their pupa on the control medium and 58 % made no choice (Tab.2). Regarding the observation of treated larvae pupation in arenas containing two treated medium 22 % marked the presence of their pupa in the treated medium and 78 % made no choice (Tab. 2).

**Table 2: Pupation of control and treated larvae with the different odors of the medium tested by the ethanolic extract of *R. farinacea*.**

	Control Larvae				Ramalina Larvae		
	C x C	C x R.f	R.f x R.f		C x C	C x R.f	R.f x R.f
<b>control medium</b>	10%	16%	0%	<b>Control medium</b>	42%	2%	0%
<b>Ramalina medium</b>	0%	8%	56%	<b>Ramalina medium</b>	0%	12%	22%
<b>No choice</b>	90%	76%	44%	<b>No choice</b>	58%	86%	78%

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



**Figure 3: Graphical representation of the attraction index of *D. melanogaster* larvae (control and treated) towards the different media tested by the ethanolic extract of *R. farinacea*.**

### **Attraction Index**

The calculation of the attraction index (AI) confirms the results obtained in the previous table and shows positive values which indicates that the control larvae present an attractive effect towards the odor of the medium treated with the *R. farinacea* ethanolic extract (Fig.3).

### **DISCUSSION**

In recent years, efforts to create biological insecticides against pests using various extracts and components of lichen have gained popularity. Cetin et al., (2008) studied the insecticidal effects of (-)-usnic acid and secondary metabolites of (+)-usnic acid obtained from *Cladonia foliaceae* (Huds.) Willd. Silva et al., (2009) examined the potential insecticidal effects of the lectin isolated from *Cladonia verticillaris* (Raddi) Fries on the termite *Nasutitermes corniger* (Motschulsky). As a result of their work, it was stated that preparations of *C. verticillaris* may be able to control termites (or other insects) that are economically linked to agriculture and the wood industry. The anti-feedant and lethal effects of four of the lichen metabolites, (-)- and (+)-usnic acid, vulpinic acid and stictic acid on larvae of *Spodoptera littoralis Boisduval* have been determined by Emmerich et al., 1993.

The insecticidal effects of *Ramalina farinacea* (L.) Ach, were studied against the larvae of *Culex pipiens* L. (mosquito) under laboratory conditions and obtained 100% results at certain biological periods of these organisms. In this work, we have shown that the lichen studied causes a disruption of feeding behavior and attractiveness of larvae, is due to the existence of molecules that act on the olfaction of the larvae and the chemical signal. Research on the use of lichens as an insecticide has noted that two types of usnic acid as well as vulpinic acid have a lethal effect at high levels and a delay in growth (Emmerich et al., 1993).

### **CONCLUSION**

This study indicates that the ethanolic extract of *R. farinacea* has a neurotoxic property. The plant had a significant effect on fly development by inducing acceleration, disrupts food behavior (attraction of larvae) and Pupation behaviour; it acts on the chemical signal as it disrupts the ability to detect odors (effect on larval olfaction) and the choice of larvae is random. The results of this work suggest the presence of toxic secondary metabolites in the studied extract, which may lead to the development of bio-insecticides based on *R. farinacea* to be used in agriculture and sold on the pesticide market.

### **CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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