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Larvicidal Effect of Eucalyptus Essential Oils (*E. globulus*, *E. radiata* and *E. citriodora*) on L_3 and L_4 Larvae of the Tomato Leaf Miner *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae)

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Abstract
The abuse of chemical substances used as pesticides harms the environment affected animal and human health. An alternative control proposal using three different essential oils of Eucalyptus tested on the larvae of the last stages of the tomato leaf miner Tuta absoluta, whose damage to Solanaceae crops is still relevant. Essential oils are extracted from the leaves of Eucalyptus globulus, E. radiata and E. citriodora and are tested by inhalation at different doses on L_3 *and* L_4 *larvae of T. absoluta. The average toxicity of these three essential oils increases proportionally with the doses as well as the duration of exposure. The values obtained for the LD50 after 24 hours of exposure enabled us to conclude that the three oils tested had a larvicidal effect against the third and fourth stage larvae of this pest.*

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1. INTRODUCTION

Chemicals are used in an abusive and improper way in most African countries. Farmers often divert pesticides for uses other than their intended use and use toxic pesticides without adequate protective measures due to high illiteracy rates (Isman, 2006).

Thus, the intensive use of these products has caused contamination of the biosphere and the food chain, the eradication of non-target species such as auxiliary fauna and the appearance of resistant microorganisms (Magan and Olsen, 2004). Although some forms of resistance of *T. absoluta* to pesticides are reported in Brazil (Siquiera and *al.*, 2000) and Argentina (Lietti and *al.*, 2005), the management of *T. absoluta* is done by spraying with chemical insecticides from various families, the most important of which are organophosphates, pyrethroids and avermectins (Diattes *et al.*, 2015). Other chemical fights are tested by using active substances such as abamectin (Polack, 1999) or spinozades (Cácares, 2000).

The abuse of these chemicals harms the environment and human health. A proposal for an alternative control by essential oils is presented in this study, with a view to replacing these chemical molecules with products of vegetable compositions, therefore natural, better tolerated by the environment and above all less harmful to animal and human health. In this context, the objective of this study is to evaluate the toxic effect of essential oils of three species of eucalyptus (*E. globulus*, *E. radiata* and *E. citriodora*)) by inhalation with regard to the larvae of the third and fourth stage of the tomato leaf miner *T. absoluta* which is an insect pest of tomato (*Lycopersicon esculentum*).

2. MATERIAL AND METHODS

The effect of three essential oils of *Eucaluptus globulus*, *E. radiata* and *E. citriodora*, is tested on third and fourth stage larvae of *T.absoluta* in the laboratory. To carry out our work we used a graduated pipette for the dosage of the oils, as well as a micro pipette for the micro-doses, filter paper on which the essential oil will be deposited, acetone is used to allow the distribution homogeneous essential oil on the filter paper hermetically sealed glass bottles of 125 ml volume.

2.1. Biological material

2.1.1. Insect pest

The species studied is the tomato leaf miner *T. absoluta*. This micro lepidopteran is polyvoltine, whose larvae develop at the expense of the leaves and fruits of its host plant, the tomato. The larvae are harvested early in the morning in leafminer-infested greenhouses. Armfuls of leaflets are torn from the tomato plants and then brought back to the laboratory for the rest of the experiment. The larvae are then removed manually using a needle and then sorted according to their stage of development L_3 or L_4.

2.1.2. Essential oils

Three essential oils are tested, which are extracted by hydro distillation from the leaves of three eucalyptus species *Eucalyptus globulus*, *E. radiata* and *E .citriodora*.

2.1.2.1. Description of the Eucalyptus plant

Eucalyptus are large trees, some of which can exceed 10m in height, but the average of the most common species is 40 to 50 m, others have smaller dimensions (Traore *et al*., 2013). The trunk is smooth, the bark of which is easily detached in long strips (Koziol, 2015). Eucalyptus bear evergreen, leathery, glabrous but different leaves depending on the age of the branches. The older ones have aromatic, short-stalked, alternate and vertically hanging leaves (Goetz and Ghedira, 2012). The flowers, visible in spring, the calyx in the shape of a bumpy top whose wide part is covered by an operculum which detaches at the time of flowering, revealing numerous stamens (Beloued, 1998). The woody fruit is a large glaucous capsule taking on a brown color when ripe, hard, angular, warty, and opening slightly by three, four or five slits to release numerous dark and tiny seeds (Goetz and Ghedira, 2012).

- **Systematic**

According to Ghidira and *al*. (2008), Eucalyptus is classified as follows:
Kingdom: Plantae
Subkingdom: Tracheobionta
Division: Magnoliophyta
Class:Magnoliopsida
Subclass: Rosidae
Order: Myrtales
Family: Myrtaceae
Genus: *Eucalyptus*
Species: *Eucalyptus globulus*
Eucalyptus radiata
Eucalyptus citridora

- **Chemical composition**

The main compounds of the essential oils of *Eucalyptus globulus*, *E. radiata* and *E. citriodora* obtained by hydrodistillation and analyzed by CG/SM, are presented in the tables below.

Table 1: Main components of *Eucalyptus globulus* essential oil in percentage identified by CG/MS (Original)

Compounds	Concentrations %
Estragol	28.14
Terpinolene	7.12
Linalool	5.54
Furfural	4.56
Alpha-longipinene	2.64
Terpinene-4-ol	2.07
Carvone	1.59
P-menth-8	1.39
Cyclohexane	0.61

Table 2: Main components of *Eucalyptus radiata* essential oil in percentage identified by CG/SM (Original)

Compounds	Concentrations%
Eucalyptol	75.49
Alpha-terpineol acetate	9.26
Carene	4.58
Alpha-pinene	2.52
Terpinene-4-ol	1.43
Beta-phellandrene	1.15

Table 3: Main components of *Eucalyptus citriodora* essential oil in percentage identified by CG/MS (Original)

Compounds	Concentrations %
Menthol	75.86
Citronellal	7.28
Citronellyl propionate	4.52
Bicyclo-nonane	2.05
2,6-dimethyl 2,6-octadien	1.52
P-menthane	1.07
Eucalypthol	0.68
Alpha-pinene	0.68

2.2. Method of application of bioassays

All the essential oils are tested separately at different doses (1µl, 3µl, 5µl, 10µl) against third and fourth stage larvae of *T.absoluta*. The test undertaken in this study is the inhalation toxicity test. Ten larvae L_3 and L_4 are placed separately and quickly in each jar. Four repetitions are carried out for each dose and for each control batch. Dead individuals are counted on a regular basis from the beginning of the exposure of the tests until the death of all the larvae.

2.3. Statistical analysis

The data obtained are subjected to analysis of variance according to several classification criteria, using the Stat Box software, version 6.4. When this analysis reveals significant differences, it is supplemented by the Newman and Keuls test at the 5% threshold, which makes it possible to determine the homogeneous groups by ascending hierarchical classification.

2.4. Estimation of the LD50 by the regression line method

The lethal dose for 50% of the population of insects LD50, is the dose from which we obtain 50% mortality of a population, the corrected mortalities are transformed into probits, and the doses into decimal logarithm, which allow establishing the equations of the regression line. The LD50 allows us to compare the toxicity of the essential oils tested. The percentages of observed mortalities are corrected by Abbott's formula which makes it possible to eliminate natural mortality and to know the real toxicity of the biopesticide by probit analysis (Finney, 1971).

Abbott's formula is:

$$\mathbf{Mc\% = (MB - Mt)/ (100 - Mt) \times 100}$$

Mc%: Mortality corrected in percentage.

Mo: Mortality observed in the treated population.

Mt: Mortality observed in the control population.

3. Results

3.1. Toxicity of *Eucalyptus globulus* essential oil

The results obtained for the action of the essential oil of *E. globulus* with regard to the third and fourth stage larvae of *T. absoluta* by inhalation are illustrated in the following figure:

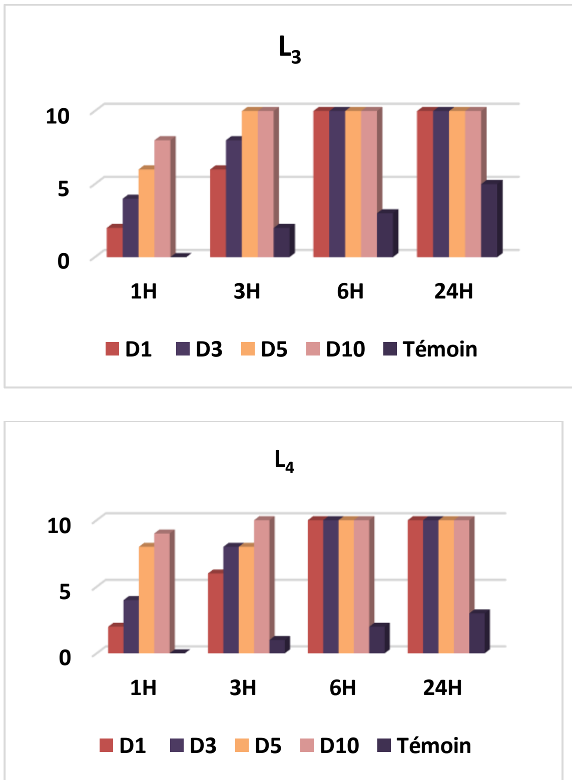


Figure 1: Average mortality rate of third and fourth stage larvae of *T. absoluta* treated by inhalation with different doses of *E. globulus* essential oil.

3.2. Toxicity of *Eucalyptus radiata* essential oil

The results obtained for the action of the essential oil of *E. radiata* against third and fourth instar larvae of *T. absoluta* by inhalation are illustrated in the following figure:

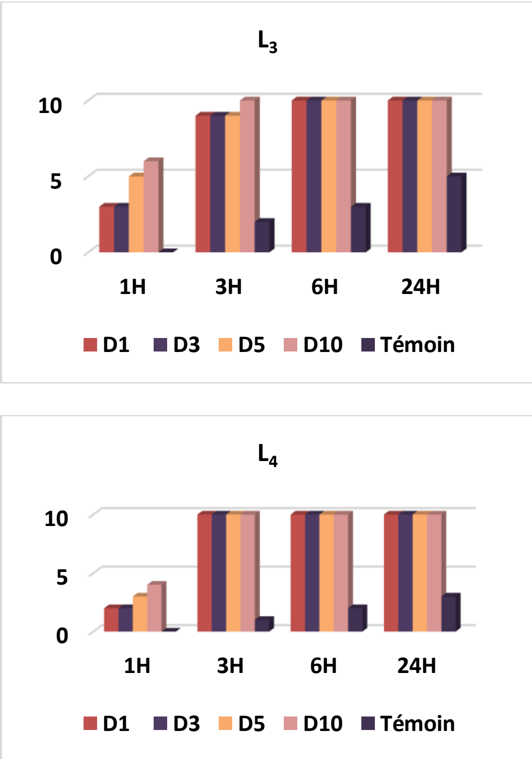
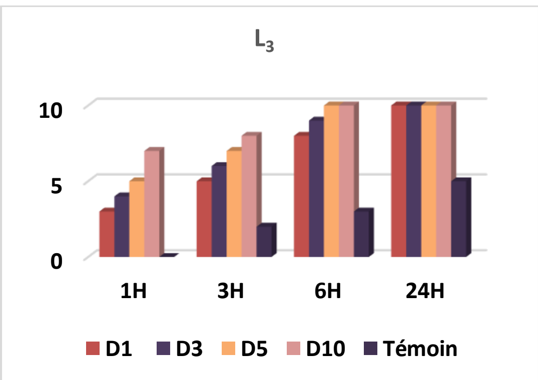


Figure 2: Average mortality rate of third and fourth instar larvae of *T.absoluta* treated by inhalation with different doses of *E.radiata* essential oil.

3.3. Toxicity of *Eucalyptus citriodora* essential oil

The results obtained for the action of the essential oil of *E. citriodora* against third and fourth instar larvae of *T.absoluta* by inhalation are illustrated in the following figure:



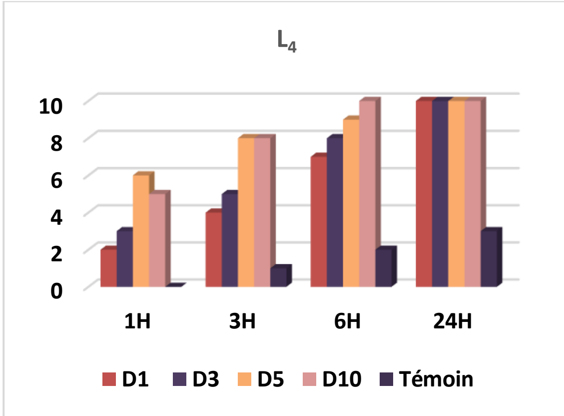


Figure 3: Average mortality rate of third and fourth instar larvae of *T. absoluta* treated by inhalation with different doses of *E. citriodora* essential oil.

The average sensitivity of L_3 and L_4 larvae of *T. absoluta* increases proportionally with the doses and essential oils used as well as the duration of exposure.

A reduction in the average longevity of L_3 and L_4 larvae is observed from the lowest dose of 1µl, varying according to the oils used after 1 hour of exposure. It varies on average from 1.75 for L_3 and 1.25 for L_4 for *Eucalyptus globulus* essential oil; 2 for L_3 and 2.5 for L_4 for the essential oil of *E. radiata*; 2.75 for L_3 and 2 for L_4 for the essential oil of *E. citriodora*. The highest dose of 10µl reduces the average longevity to 0 days after 24 hours of exposure and those for all the oils used and for the two larval stages.

Analysis of variance with two classification criteria for third instar larvae shows no significant difference for the dose factor (P=0.57), and shows highly significant differences for the oil factor (P=0.06).

The Newman and Keuls test, at the 5% significance level, shows 4 homogeneous groups for the dose factor characterized by group A for dose 1, group B for dose 2, group C for dose 3 and group D for dose 4 (Table 4). For the oil factor, 3 groups are reported characterized by group A for *Eucalyptus globulus* essential oil of, group B for that of *Eucalyptus radiata* and group C for *Eucalyptus citriodora* essential oil of (Table 5).

Table 4: Results of the classification of the Newman and Keuls test concerning the dose factor.

F1	Labels	Averages	Homogeneous Groups			
4.0	d4	9.07	A			
3.0	d3	8.297		B		
2.0	d2	7.539			C	
1.0	d1	7				D

Table 5: results of the classification of the Newman and Keuls test concerning the oil factor.

F2	Labels	Averages	Homogeneous groups		
1.0	H1	8.313	A		
2.0	H2	8.188		B	
3.0	H3	7.,297			C

The variance analysis with two classification criteria concerning the fourth stage larvae shows no significant difference for the dose factor (P=0.3097) as well as for the oil factor (P=0.7295). The Newman and Keuls test, at the 5% significance level, shows no significant differences.

3.4. Estimation of the LD50 of essential oils tested on *T. absoluta* larvae

In order to determine the lethal doses on 50% of the individuals of the *T. absoluta* larvae and thus to classify the essential oils according to their toxicity, we used the method of Finney (1971), which makes it possible to transform the percentages of mortality of the larvae after 24 hours of exposure in probits, and the regression lines of these data according to the decimal logarithm of the doses of essential oils made it possible to obtain the equations which are configured in the figure 4, 5 and 6.

The regression lines show a strong correlation between mortality and the doses of essential oils used, the more the dose increases the more the mortality rate increases.

The correlation coefficients are close to 1 for the third instar larvae as well as the fourth instar larvae, which means that there is a significant correlation between the two quantitative variables (doses and average mortality). The equations obtained by the regression lines allowed us to determine the lethal doses that kill 50% of individuals (LD 50) and thus classify the essential oils according to their order of effectiveness or toxicity. The values obtained for the LD50 calculated after 24 hours of exposure, allowed us to conclude that the essential oils tested present the following order of effectiveness:
The three species of eucalyptus (*E. globulus*, *E. radiata*, *E. citriodora*), with LD50 of 1.17, 1.17, 1.17 µl/l of air respectively for third instar larvae and LD50 of 1.53, 1.53, 1.53µl / l of air respectively in fourth-stage larvae (Table 6).

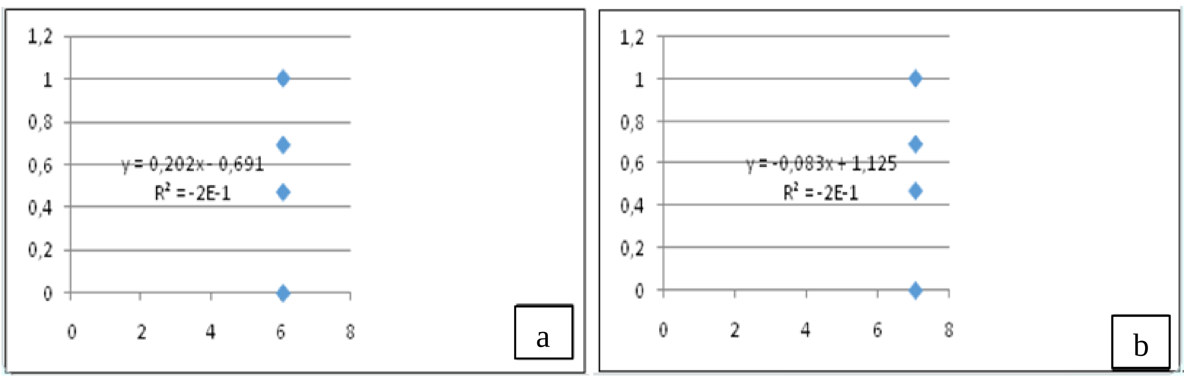


Figure 4: Log dose/mortality (probit) regression lines in *T. absoluta* larvae subjected to the action of essential oils by inhalation after 24 hours. (a) *E. globulus* L_3; (b) *E. globulus* L_4

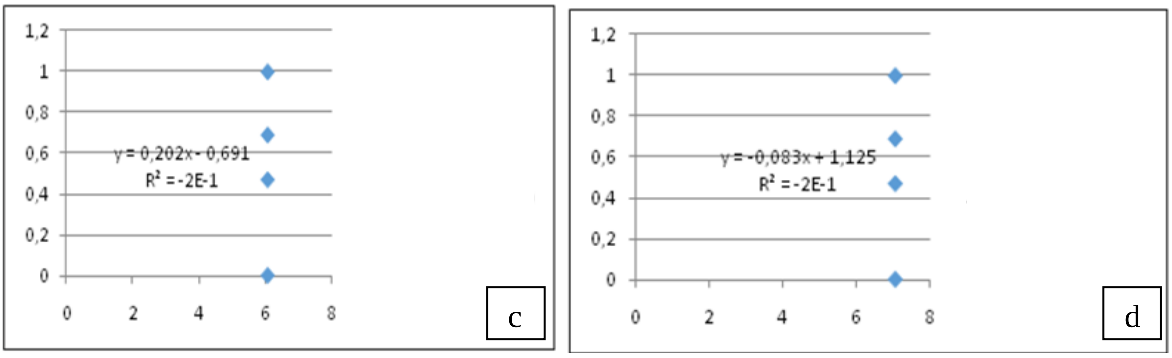


Figure 5: Log dose/mortality (probit) regression lines in *T. absoluta* larvae subjected to the action of essential oils by inhalation after 24 hours. (c) *E. radiata* L_3; (d) *E. radiata* L_4;

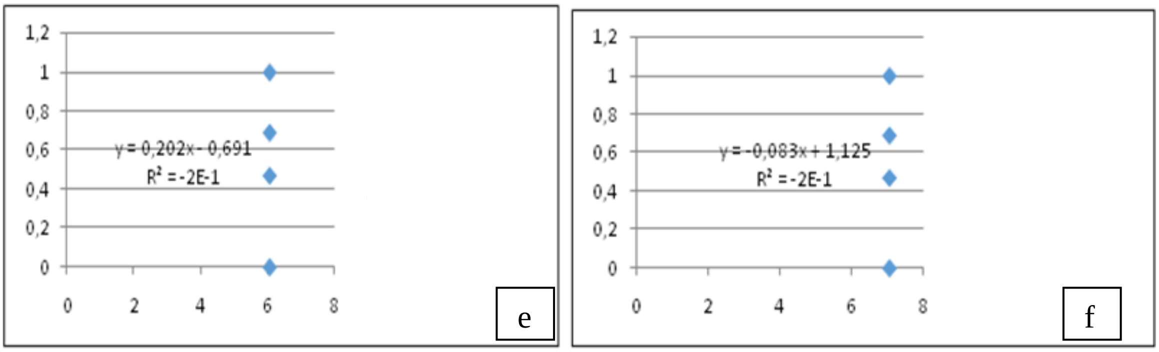


Figure 6: Log dose/mortality (probit) regression lines in *T. absoluta* larvae subjected to the action of essential oils by inhalation after 24 hours. (e) *E. citriodora* L_3; (f) *E. citriodora* L_4

Table 6: Classification of essential oils according to their order of effectiveness on the larvae of *T. absoluta*

Essential Oils Tested	DL 50 L3	DL50 L4
Eucalyptus globulus	1.17	1.53
Eucalyptus radiata	1.17	1.53
Eucalyptus citriodora	1.17	1.53

4. DISCUSSION

A decrease in the average longevity of L_3 and L_4 larvae was observed from the lowest dose (1µl), varying according to the oils used after 01 hour of exposure.

This toxicity varies on average from 1.75 for L_3 and 1.25 for L_4 for the essential oil of *Eucalyptus globulus*; 2 for L_3 and 2.5 for L_4 for *Eucalyptus radiata* essential oil; 2.75 for L_3 and 2 for L_4 for *Eucalyptus citriodora* essential oil.

The highest dose of 10µl reduces the average longevity to 0 days after 24 hours of exposure and those for all the oils used and for the two larval stages. The analysis of variance with two classification criteria shows no significant difference for the dose factor (P=0.3097) or for the oil factor (P=0.7295). The Newman and Keuls test, at the 5% significance level, shows no significant difference.

In order to counter the abuse of chemicals in phytosanitary pest control, several works aimed at the use of natural products are being carried out. Indeed, the alternative of replacing these pesticides with essential oils that are less harmful to the environment and human health has been the subject of several trials (Belmain *et al*., 2001; Kellouche and Soltani, 2004; Sanon *et al*., 2006)

The results obtained by combining gas chromatography analysis and mass spectrometry (CG/SM) carried out on the leaves of the essential oils of our eight samples, enabled us to highlight the main constituents for each oil: 28.14% estragole for *Eucalyptus globulus*, 75.49% eucalyptol for *Eucalyptus radiata* and 75.86% Menthol for *Eucalyptus citriodora*. The richness of *Eucalyptus globulus* essential oil is reported in 2015 by Taleb-Toudert.

Bruneton (1993) recalls that essential oils contain volatile active principles contained in plants, which are however modified during their preparation. Figueredo (2007) joins the idea of the previous author by attesting that the composition of essential oils is very fluctuating. Indeed, it depends on a certain number of natural factors (genetics, soil, climate, etc.) or technological factors (cultivation methods, storage or extraction).

In this present study, three essential oils extracted from eucalyptus leaves were tested by inhalation against the larvae of the L_3 and L_4 stages of *T. absoluta*, in order to observe the insecticidal effect that would have these oils on the caterpillars of the last larval stages of this microlepidoptera. The test could not have been carried out on the first larval stages. Indeed, the first and the second stage larvae are very small in size, their vulnerability would make this test very difficult, if not impossible, to perform. Different doses are used for each of the oils, they are 1, 3, 5 and 10µl.

Our results agree with those of Benchouikh and *al*. (2006) who used the essential oil of *Syzzygium aromaticum* against the larvae of *T. absoluta* and who recorded after two hours of exposure to different doses of this oil, an effect 75% repulsion generated by the larvae of this pest. These same authors certify that the essential oil of this plant has a strong insect repellent activity and is observed a high mortality according to the doses.

The average toxicity of the eight essential oils used with regard to the third and fourth stage larvae of the tomato leaf miner *T. absoluta* increases proportionally with the doses as well as the duration of exposure.

Casida (1990) recalls the volatile nature of essential oils. According to the same author, the principle would be a volatile constituent.

However, the composition of essential oils is generally very complex, possibly due to the considerable diversity of their structure and the high number of constituents. The work of Taadaouit *et al.* (2011) perfectly illustrate what is mentioned above. Indeed, the evaluation of the toxicity of the methalonic and ethalonic extracts of the plant, including *Argania spinosa* on the larvae of *T. absoluta* show a difference in toxicity within the same plant.

Thus *Thymus vulgaris* caused a remarkable mortality rate which exceeds 90% mortality for the two solvents while *A. spinosa* only presented a low toxicity not exceeding 30% for the ethalonic extracts and 44% for the extracts metallonic. Along the same lines, Tchoumbougnang *et al.* (2007) link the toxicity of *Thymus vulgaris* to its richness in terpene molecules. The insecticidal activity of essential oils of the lamiaceae family, for example *Mentha peperita*, on the mortality of *T.absoluta* larvae may be due to their richness in menthol, phenol, which are components known for their insecticidal properties thanks to the monoterpenes they contain. (Regnault-Roger *et al*., 2012). The same authors add that at the level of essential oils there are monoterpene mixtures which are neurotoxic.

For example, linanool and estragole are essential constituents of *O.basilicum* that disrupt neuronal activity in insects. Goucem-Khelfane (2014) confirms the action of linalool which induces high mortality on adults of *Acanthocelides obtectus* subjected to the action of essential oils of Myrtaceae. Hedjal-Chebheb (2014) reports the toxic effect of eucalyptol on *Callosobruchus maculatus* larvae tested by inhalation of Eucalyptus oil. Cassida (1990) explains that the toxic and repellent effects of essential oils could depend on their chemical compositions and the level of insect sensitivity.

Other work has been carried out around the world on the larvicidal effects of essential oils on tomato leaf miner caterpillars. Cajas (2013) in Chile reports that the essential oil of *Lonchocarpus guarencis* had a strong larvicidal activity with a high percentage on *T.absoluta* larvae. In the western Algerian region, Bouayad Alam (2015) recorded good larvicidal activity of extracts of *Thymus capitatus*, *Daucus crinitus* and *Tetraclinis articulata* on the tomato leaf miner with a mortality rate of 70% and 90% respectively.

Moreover, among the five oils that Bitter and *al.* (2008) tested on *S. zeamis* the essential oils extracted from *Eucalyptus globulus* and *Thymus vulgaris* proved to be toxic on this insect with high mortality at the highest doses tested.

The regression lines show a strong correlation between mortality and the doses of essential oils used, the more the dose increases the more the mortality rate increases. The correlation coefficients are close to 1 for the third instar larvae as well as the fourth instar larvae, which means that there is a significant correlation between the two quantitative variables (doses and average mortality).

The results obtained show that the eight oils tested by inhalation exhibit a larvicidal effect on the caterpillars of the latest development of *T. absoluta*. The correlation coefficients in association with the adjustment equation R2 oscillate between 0.53% and 0.87%, indicating that the mortality rates of the larvae increase as the doses of the oils used increase. These results agree with those of Hamani-Aoudjit (2019) who tested the essential oils of *Eucalyptus globulus*, and *Origanum vulgare*, *Thymus vulgaris*, and *Mentha peperita* by inhalation on adults of *Bruchus rufimanus*.

The equations obtained by the regression lines allowed us to determine the lethal doses that kill 50% of individuals (LD 50) and thus classify the essential oils according to their order of effectiveness or toxicity.

The values obtained for the LD50 calculated after 24 hours of exposure, allowed us to conclude that the essential oils tested present the following order of effectiveness:

E. globulus, *E. radiata* and *E. citriodora*, with LD50 of 1.17, 1.17, 1.17µl/l of air respectively for third instar larvae and LD50 of 1.53, 1.53, 1.53µl/l of air respectively for fourth instar larvae.

Our results agree with those obtained on the bean bruchid *A.obtectus* obtained by many authors including Regnault-Roger and Hamraoui (1995), Bouchikhi Tani and *al.* (2011); Goucem-Khelfane (2014), who showed that the essential oils of many aromatic plants have an insecticidal effect when inhaled. According to Regnault-Roger and Hamraoui (1993) the various oils extracted by hydrodistillation from plants of the Lamiaceae, Myrtaceae, Lauraceae and grasses families present an inhalant toxicity on *A. obtectus*, in particular the essential oils of *T. serpyllum*, *T. vulgaris* and *L.angustifolia* (Lamiaceae) which cause 95% mortality after 24 hours of exposure and 100% mortality after 48 hours of exposure for respective concentrations of 160mg/dm3, 136.1mg/dm3 and 145mg/dm3. Similarly, these authors have reported that males have a high sensitivity to essential oils compared to females. The same sensitivity of males was confirmed by the work of Papachristos and Stamopouoso (2002) on the bean weevil *A. obtectus*, tested with essential oils of *Citrus sinensis*, *Pistacia terebinthus* and *Thuja orientalis*.

Bouchikhi Tani *et al*. (2011) for their part studied and compared the biocidal effect of aromatic plants of six essential oils including Lamiaceae, Rutaceae, Cistaceae and Asteraceae in ascending order in their effectiveness and is presented as follows, on adult bean weevil (*A. obtectus*) and moth (*T. bisselliela*). They demonstrated that the essential oils extracted from the three plants *Rosmarinus officinalis* (Lamiaceae), *Origanum glandulosum* (Lamiaceae), and *Artemesia herba-alba* (Asteraceae) are the most toxic on the two treated insects compared to the other oils. According to the same authors, these essential oils are ranked in ascending order in their effectiveness and are as follows, on adults of *A. obtectus*: *A. herba-alba*, *O. glandulosum*, *R. officinalis* (LD50 of 1.69µL/30g seed, 1.44µL/30g seed, 0.59µL/30g seed respectively). On the adults of *T. bisselliella*: *R. officinalis*, *A. herba-alba*, *O. glandulosum* (LD50 of 1.26µL/50.24cm², 1.25µL/50.24cm², 1.23µL/50.24cm² respectively).

It emerges from this work that to fight against *T. absoluta*, the essential oils that have been used can be an interesting alternative to the chemical fight

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