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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

EFFICACY OF ESSENTIAL OILS OF *Thymbra capitata* L. AND *Mentha pulegium L.* COLLECTED IN TUNISIA ON LARVAE OF *Galleria mellonella L.*

Tunus'ta Toplanan *Thymbra capitata* L. ve *Mentha pulegium* L. Esansiyel Yağlarının *Galleria mellonella* L. Üzerine Etkisi

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ABSTRACT

The wax moth is one of the honeybee pests that cause a lot of damage and loss for the beekeepers in Tunisia. The use of insecticides is more and more used although they are known to contaminate wax and honey. This study aims to test the essential oils of two North African common plants *Thymbra capitata* L. and *Mentha pulegium L.* efficacy as alternative method by fumigation on instars of *Galleria mellonela* L. We also determined the duration of the development stages of the great wax moth (GWM) presented in Tunisia. Results showed that under a temperature ranged between 30 and 33°C, the total duration from eggs to adults of GWM lasted 51 days. The fumigant test showed the toxicity of both oils on larvae instars tested. The second larvae instar was more susceptible than the 4th instar. Moreover, *M. pulegium* was more toxic against the 2nd larvae instar than *T. capitata* with an LC50 at 48h of 41.82 and 456.27 µl/L air, respectively. The essential oils present a good alternative to the insecticides to control wax moths.

Keywords: *Galleria mellonella*, *Thymbra capitata, Mentha pulegium*, fumigation, development stages, LC_{50}.

ÖZ

Balmumu güvesi, Tunus'taki arıcılar için çok fazla zarara ve kayba neden olan bal arısı zararlılarından biridir. Balmumu ve balı kirlettikleri bilinmesine rağmen böcek öldürücülerin kullanımı giderek daha fazla kullanılmaktadır. Bu çalışmada, iki Kuzey Afrika ortak bitkisi *Thymbra capitata* L. ve *Mentha pulegium* L.'nin büyük mum güvesi (GWM) *G. mellonella* üzerine etkisinin araştırılması amaçlanmıştır. Ayrıca büyük balmumu güvesinin gelişme aşamalarının sürelerini de belirlenmiştir. Sonuçlar, 30 ila 33°C arasında değişen bir sıcaklık altında, yumurtalardan GWM'li yetişkinlere kadar olan toplam sürenin 51 gün sürdüğünü göstermiştir. Fumigant testi, test edilen larva dönemlerinde her iki yağın da toksisitesini göstermiştir. İkinci larva dönemi, 4. evreye göre daha hassastır. Dahası, *M. pulegium*, 2. larva dönemine karşı, sırasıyla, 48. saatte 41.829 ve 456.276 µl / Lair'de bir LC50 ile *T. capitata*'dan daha toksik olduğu belirlenmiştir. Bu bulgulara göre uçucu yağlar, mum güvelerini kontrol altına almak için böcek ilaçlarına iyi bir alternatif sunmaktadır.

Anahtar Kelimeler: *Galleria mellonella*, *Thymbra capitata, Mentha pulegium*, tütsüleme, gelişme evreleri, LC_{50}.

GENiŞLETiLMiŞ ÖZET

Amaç: Balmumu güvesi, Tunus'taki arıcılar için çok fazla zarara ve kayba neden olan bal arısı zararlılarından biridir. Balmumu ve balı kirlettikleri bilinmesine rağmen böcek öldürücülerin kullanımı giderek daha fazla kullanılmaktadır. Bu çalışma, iki Kuzey Afrika ortak bitkisi Thymbra capitata L. ve Mentha pulegium L.'nin uçucu yağlarının alternatif bir yöntem olarak Galleria mellonela L. Tunus'ta sunulan güve (GWM).

Materyal ve Metot: G. mellonella'nın larvaları, istila edilmiş bal arısı kolonilerinden elde edildi ve laboratuvarda yetiştirildi. Büyük mum güvesinin (GWM) gelişme aşamalarının süresi belirlendi. Uçucu yağlar, bir Clevenger tipi kullanılarak Thymbra capitata L. ve Mentha pulegium'dan çıkarıldı. Uçucu yağların toksisitesi, fümigasyon yoluyla değerlendirildi. Bunun için çap filtre kağıtlarına (Whatman No.1) 4, 8, 12, 14, 16, 20, 25, 30 ve 50 µl'lik farklı yağ dozları emprenye edilmiş ve 10 larva içeren pleksiglas şişelerin vidalı kapaklarına takılmıştır. İkinci ve dördüncü dönem olmak üzere iki evre test edildi. Mortalite 2, 4, 6, 8, 12, 24 ve 48 saat sonra değerlendirildi. 48 saatte ölümcül konsantrasyon LC50 de değerlendirildi.

Sonuç ve Tartışma: Sonuçlar, 30 ila 33°C arasında değişen bir sıcaklık altında, yumurtalardan GWM'li yetişkinlere kadar olan toplam sürenin 51 gün sürdüğünü gösterdi. Fumigant testi, test edilen larva dönemlerinde her iki yağın da toksisitesini göstermiştir. İkinci larva dönemi, 4. evreye göre daha hassastır. Dahası, M. pulegium, 2. larva dönemine karşı, sırasıyla, 48. saatte 41.829 ve 456.276 µl / Lair'de bir LC50 ile T. capitata'dan daha toksikti. Uçucu yağlar, mum güvelerini kontrol altına almak için böcek ilaçlarına iyi bir alternatif sunar.

INTRODUCTION

The wax moth is one of the most serious threats of honeybee colonies and stored combs. (Ritter and Akratanakul 2006). This pest is found throughout the world. *Achroia grisella Fabricius 1794* called the lesser wax moth (Lepidoptera: Pyralidae) and *G. mellonella* L., 1758 (Lepidoptera: Pyralidae) called the greater wax moth (GWM) are both common in Tunisia but *Galleria mellonella* is more widespread and causing more damage (Pirk et al. 2016).

The damage of wax moths is caused only during their larval stage.The larvae attack stored combs and those inside the honeybee colonies, burrow into the combs and feed on skins of pupae, on pollen, on wax and other debris found in the beewax (Jindra and Sehnal 1989). If the colonies are strong the honey bees defend themselves and the chance of infestation is low. However, larvae of GWM can destroy the weak colonies (Shimanuki 1981) The larvae when digging into the combs, make silken tunnels in the middle of the comb that become covered with a mass of webbing, a condition described as “Galleriasis” (Ritter and Akratanakul 2006).

Several chemicals and non-chemical have been used to control the wax moth on stored beeswax combs (Williams 1997, Ritter and Akratanakul 2006). Para-dichlorobenzene (1,4-dichlorobenzene, PDCB) is a fumigant insecticide used to control the wax moth during the storage of beeswax combs. Its toxicity was reported by the US Department of Labor: NIOSH/OSHA/DOE (Bogdanov et al. 2004). In Germany for example 36 to 50% of German honey contained from 3 to 50 µg PDCB /kg (Wallner 1992).

Alternative methods such as the essential oils supposedly do not present the same risks as synthetic insecticides (Jemâa et al. 2012, Isman 2016, Ncibi et al. 2020). Plants may present potential alternatives to control insects because they are composed of many bioactive chemicals (Wink 1993). Oils are a mixture of several molecules, and their biological activities have been shown to act as potent acute or chronic insecticides (Owayss and Abd-Elgayed 2007, Elbehery et al. 2016, Bisht et al. 2017, Almadani and Hiware 2020).

In our study, we attempted to study for the first time the duration of development stages of the GWM in TUNISIA under laboratory conditions on one hand and on another hand to evaluate the toxicity of essentials oils extracted from two local plants (*Thymbra capitata L* and *Mentha pulegium*) on the second and the fourth larvae instars. The choice of these instars was made after survival analyses on all the instars. The larvae of the first instar were too small to handele and the larvae of the last insars were too bigs and cause already a lot of damage. For this reason we choosed the second and the fourth instars.

MATERIALS AND METHODS

Wax moth rearing

Larvae of *G. mellonella* were obtained from infested honey bee colonies collected from the National Agronomic Institute of Tunisia apiary (36.84536, 10.19184). The wax moths were reared based on the methods described by Ellis et al. (2013). Briefly, around 50 to 60 larvae were placed into containers with artificial diets (Marilleau 1978) and fed until the pupal stage. Once the adult moths emerged, the males and females were transferred together into other plastic containers to mate. Once the females laid eggs, the eggs were collected and transferred to artificial diets used as food by the newly emerged larvae.

Essential oils extraction

Fresh aerial parts of the two spontaneous plants *T. capitata L* and *M. pulegium. L* were collected from two regions Ras Jbal (37, 1254; 10,0726) Menzel Abdrahmen 37.1413; 9.5146) respectiveley, situated in the Bizerte Govornate in Northen Tunisia, and Essential oils (thyme and mint oils respectively) were obtained from 1 kg of fresh aerial parts by steam distillation for 3 hours using a Clevenger-type apparatus. Brievely, the vessel of apparatus is heated and temperature is controlled with a thermometer. Evaporation under atmospheric pressure occurs at 100°C, and the vapors charged in essential oils flow through the distillation column, enrich the concentration of essential oil, and flow further into the condenser where both the water and essantial oil drop into the Clevenger apparatus (Périno et al. 2019).

Fumigant activity bioassay

To assess fumigant toxicity of two plants, 4 cm diameter filter papers (Whatman No.1) were impregnated with the different oil doses of 4, 8, 12, 14, 16, 20, 25, 30 and 50 µl. Control received water. The impregnated filter papers were then attached to the screw caps of 115 ml plexiglas bottles. The calculated fumigant concentrations were respectively 34.78; 69.56; 104.34; 121.73;139.13; 260. 86; 434.78; 869.56 and 1304.34 µl/l air. Each bottle contained 10 larvae. Two instars were tested, the second and the fourth instars of GWM. Each concentration and control were replicated four times. Mortality was recorded after 2, 4, 6, 8, 12, 24 et 48 hours. Bioassays were designed to assess respectively median lethal concentration LC_{50} (dose that kills 50% of the exposed insects) at 48h.

Statistical Analysis

Probit analysis (Finney 1971) was conducted to estimate lethal concentrations (LC_{50}) and with its 95% fiducial limits using SPSS version 23 (IBM Corporation, USA).

RESULTS

Development stages of *Galleria mellonella*

The rearing of GWX under laboratory conditions revealed that at a temperature ranging from 30 to 33°C and a 40 to 45% RH, the egg stage lasts 10 days while the larvae stage lasts 30.5 days almost (Table 1). The color of the eggs is white close to light pink (Fig.1a). Upon hatching, the larvae are creamy and white, and their length is variable (Fig. 1B). In this study, we did not follow the number of molting stages. But we noted that the increase in growth and size of larvae happens during the final instars where the length reached approximately 20.5±0.26 mm. This last instar ceases feeding and starts the formation of the cocoon and finally became a pupa (Fig.1c). From pupae to adult moth took 10.5 days. The mean life span of adults was around 11 days (Table 1).

The entire life cycle from eggs to adult emergence took approximately 51 days.

Table 1: Duration of development stages of *G.mellonella*

Stage	Duration (days)
Egg	10 ± 0.33
Larvae	30.5 **±** 2.38
Pupae	10.5 **±** 0.57
Adult (female)	11 **±** 0.81

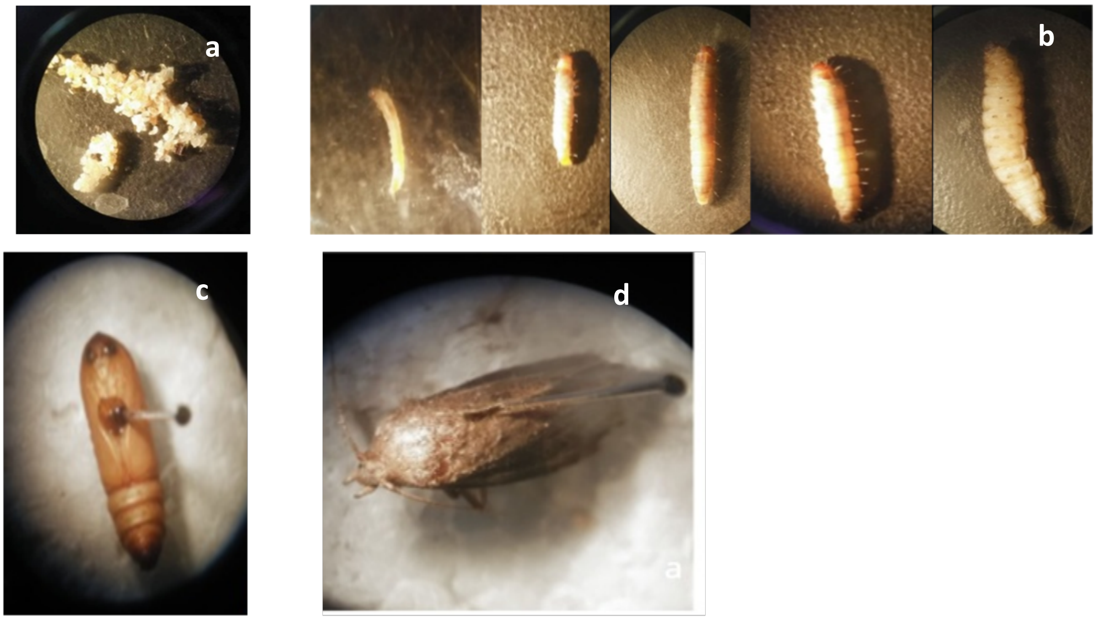

Figure 1. Different developmental stages of *Galleria mellonella:* eggs (a), larval (b), pupae (c), adult moths (d)

Fumigant test

The effect of thyme oil on wax moth larvae is variable depending on the stage exposed. The mortality of second instar larva exposed to thyme oil started after 2h at the highest concentration (1304.34µl/l air) to reach 100% at 24h (Fig. 2A). As the concentration gradually decreases, the time to record a larva mortality increases. At the lowest concentration (34.78 µl/l air), the mortality of larvae was only 2.5 % only after 48 hours. However, with the fourth instar larvae, we started to record the mortality after 24h (Fig. 2B). Moreover, with the highest concentration, we reached only 50% of mortality after 48h.

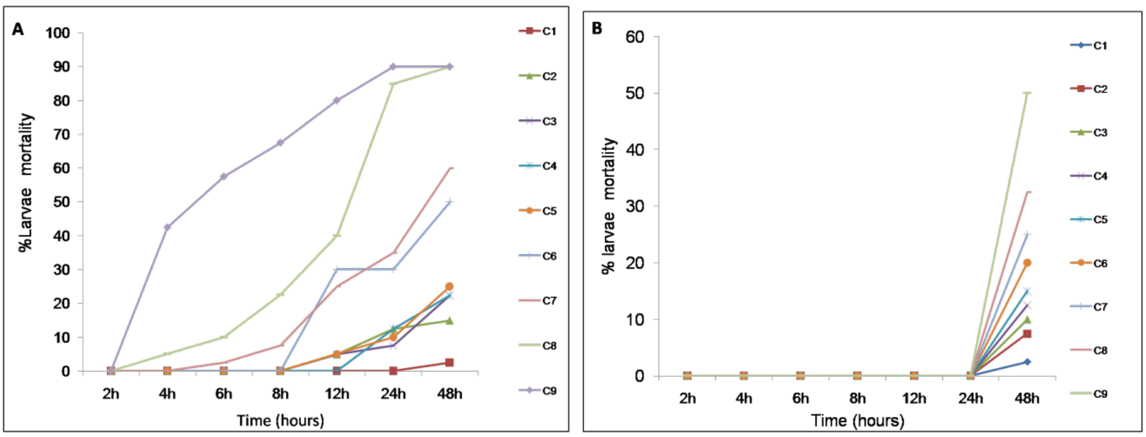

Figure 2. Percentage of mortality of L2 (A) and L4 (B) instar over time exposed to *Thymbra capitata L* oil.

Concerning the mint oil, the mortality of L2 instar at1304.34 µl/l air after 4h of exposure was 17% and reached 100% after 48h. And at the lowest concentration, 20% of mortality was recorded at 12h and more than 60% was recorded after 48h for all the concentrations (Fig. 3A).

However, when L4 instar was exposed to the same concentrations, only 7.5% of mortality was recorded after 48h. The six first concentrations did not induce mortalities with the L4 instar (Fig. 3B)

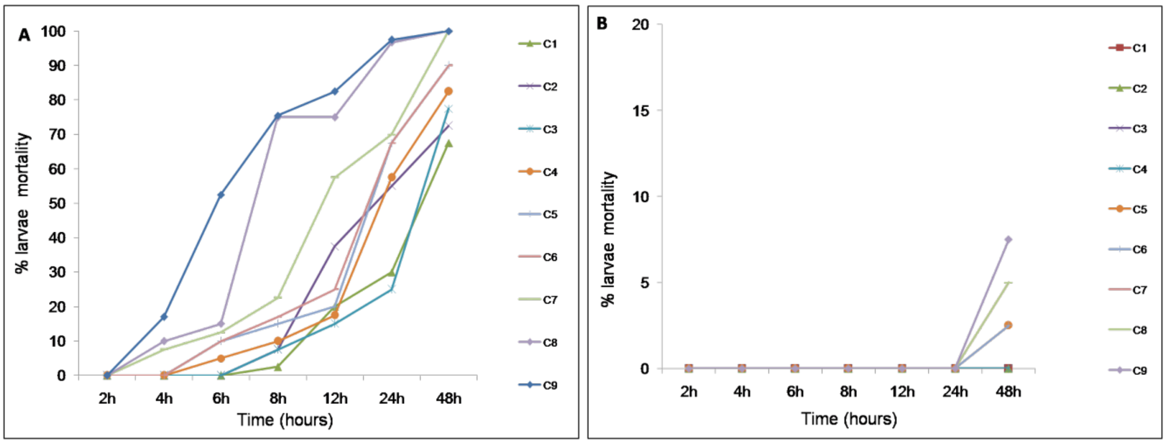

Figure.3. Percentage of mortality of L2 (A) and L4 (B) instar over time exposed to *Mentha pulegium* oil

When calculated in terms of concentration (LC_{50}), probit analysis showed that the 2nd larvae instar is more susceptible to both oils than the 4th larvae instar (Table 2). Moreover, mint oil seems to be more toxic than thyme oil when applied on larvae of the second instar. While thyme oil is more toxic than mint oil when applied on the 4th instar larvae.

Table 2. The LC 50 (µl /ml) for essential oils of *Mentha.pulegium* and *Thymbra.capitala* against the 2nd and the 4th larval instars of GWM

Larvae instar	plant	Lethal concentration LC50 (µl/L air)	Model fitness		
			(X^2) [a]	df	p
L2	*M. pulegium L.*	41.8 (-27.92 – 75.92)	12.79	9	0.119
	T. capitata L	456.2 (334,49– 637.23)	8.63	8	0.374
L4	*M. pulegium L.*	1677 (695.70 – 43.35)	0.34	7	1.000
	T. capitata L	957.7 (678.57-1701.15)	5.58	8	0.664

Units LC_{50} = µl/l air, applied for 48 h

df; degree of freedom

p; significance

95% lower and upper confidence limits are shown in parenthesis.

[a]Chi-square value

DISCUSSION

Various investigators have noted the biological behavior of *G. mellionella* all around the world (Paddock 1918, Jindra and Sehnal 1989, Williams 1997, Ellis et al. 2013, Kwadha et al. 2017) but no information has been published for Tunisia.

Paddock (1918) reported the egg stage ranged from an average of 7.2 to 21.8 days while Shimanuki (1981) and Charriere and Imdorf (1999) reported a period between 3and 30 days before eggs hatching into larvae. In this study, the eggs stage lasts 10 days at a temperature ranging from 30 to 33°C. According to Williams (1997), the duration of egg development is faster at warm temperatures (29°C-35°C) and more slowly at cold temperatures reaching 30 days at 18°C.

Larval development also depends on temperature and humidity. It could last 24-28 days at 29-32°C and high humidity (Ellis et al. 2013). In this study, larvae development lasts 30.5 days while from pupae to adults, it tooks 10.5 days. This result is close to those found by Warren and Huddleston (1962) who reported a duration of 32.9 and 8 days for larvae and pupal development, respectively.

Moreover, the total duration of 51 days from eggs to adults of *G. mellonella* did not differ from some previous studies (Paddock 1928, Warren and Huddleston 1962). The cycle can last from 6 weeks to 6 months depending on temperature and food available (Charriere and Imdorf 1999).

The wax moth is highly destructive and can cause important losses to combs and hive materials. Many essential oils and their components were tested for control wax moth larvae such as clove and eugenol (Williams 1997).

Elbehery et al. (2016) found that the treatment of beeswax with neem oil at different concentrations caused the mortality of larvae all stages of *G. mellonella* and that the mortality was concentration dependent. These results agree with our findings. Indeed, the fumigant test showed that the mortality of GWM larvae is increasing with the increase of the concentrations and exposure time.

Comparing the LC50 values of the present investigation with different studies indicated different results. The LC50 of thyme recorded for the 5^{th} instar larvae was 4.5384 ml/Lair in the study of Almadani and Hiware (2020) which was much higher than the LC_{50} of the 4^{th} instar recorder in this study. The LC_{50} of peppermint oil-1% in acetone of 5th instar larvae of GWM was 2920 µl/ml (Bisht et al. 2017) while the LC_{50} of the 4th larvae instar of mint oil in this study was 1677 µl/ml air.

Results of this research illustrated that larvae varied in their susceptibility to the two essential oils. This variability might be explained by the insecticidal ability of the active constituents of oils but also to the age of exposed larvae (Owayss and Abd-Elgayed 2007). The essential oils of *M. pulegium* and *T. capitata* were widely used to control stored grain insects in Tunisia (Salem et al. 2017, Ncibi et al. 2020).

Also the *Thymus. algeriensis* oil from two sites in Tunisia was found to possess strong insecticidal activity after 24 hours with LC_{50} ranged from 44.25 to 112.75 µl/l air) against *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) larvae third instar (Ben El Hadj Ali et al. 2015). The lethal concentration (LC_{50}) value of *M. pulegium* was 0.3 µL/L on some Lepidopteran species adults was demonstrated (Chaaban et al. 2019). Other studies showed that larvicidal activity of M. pulegium was more efficient on the 3rd instar fourth and fifth instars larvae of *Orgyia trigotephras* (Lepidoptera) (Ezzine et al. 2018).

This work is an attempt to study the biological cycle of the wax moth and its control using a North African endemic species. *T. capitata L.* and *M. pulegium L.* have an insecticide activity against the larvae of *G.mellonella* and they are more toxic to the second instar than the fourth instar.

CONCLUSION

G. mellonella has a large geographic distribution and causes serious problems for the apicultural industry. Botanical insecticides particularly essential oils supposedly do not pose the same risks on human health and environment, as synthetic insecticides, the interest in and utilization of such control tools have become increasingly relevant in the control of insect pests (Almadani and Hiware 2020). Essential oils seem to be a safe alternative to control the wax moth, but further studies should be carried out using different plants in Tunisia.

Author contribution

Faten Ben Abdelkader and Abir Ben Amor designed the study. Sarra Ncibi and Abir Ben Amor carried out the experiments. Faten Ben Abdelkader wrote the manuscript. All authors read and approved the manuscript.

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Ethical issue

Not applicable

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