JAP&T
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Journal of Agroalimentary Processes and Technologies 2023, *29* (4), 280-287

Journal of Agroalimentary Processes and Technologies

The toxic fumigant activity of *Thymus syriacus* essential oil against the greater wax moth *Galleria mellonella*

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Abstract

The present study aims to assess the insecticidal activity of *Thymus syriacus* essential oil against early third larvae and adults of the greater wax moth *Galleria mellonella* L. Also to test the insecticidal activity of thymol against the insect third larval stage. *T. syriacus* essential oil was extracted by a Clevenger-like distillation system. The effect of essential oil vapor was tested on larvae and adults of *G. mellonella*. Various concentrations of the essential oil were tested for various incubation times. To estimate the significance of differences between treatments ($P<0.05$) mortality percentage values were compared using ANOVA. LC_{50} of the essential oil was estimated using Probit analysis. The results showed that the vapor of the Syrian thyme essential oil has toxic fumigation activity against the two tested life stages of *G. mellonella* in varying degrees. Mortalities varied according to the concentration and exposure time. Adults were more sensitive than the larvae. Treatment of wax combs with thymol protected the combs from infestation with third larval stage of *G. mellonella*. The present work showed that the vapor of *T. syriacus* essential oil and thymol showed insecticidal activities against the greater wax moth *G. mellonella*.

Keywords: *Thymus syriacus***,** Essential oil, greater wax moth**.**

1.Introduction

Galleria mellonella L. is a member of the Galleriinae, a subfamily of the Pyralidae. The females of this species lay their eggs in beehives. Galleriinae sufamily has two pest species the greater wax moth *G. mellonella* and the less harmful wax moth *Achroia grisella* [17] Being one of the serious pests of wax comb of honey beehives, the larval stage of *G. mellonella* causes extensive damage to beehives all over the world. Several methods were suggested to control wax moth infestation of stored combs. The use of gamma irridation [35] is an example. Exposure to carbon dioxide [29], sulfur dioxide [23], and methyl bromide [15], is another. Or by the direct application of paradichlorobenzene [39]. Those methods aim at killing all the different life stages of the moth. However, the extensive use of such insecticides is detrimental to public health, the environment and biological diversity including pollinating insects such as honeybees. Several researchers investigated the use of biological control methods to control *Galleria mellonella*., such as studying the potential of *Apanteles galleriae*, *Bacillus thuringiensis Nosema galleriae* and a nuclear polyhedrosis virus [52,16,14,21] as biological control agents, A strain of *B. thuringiensis* was available for use inside beehives as a biological control agent [57]. Natural products are considered as one of the good substitutes of synthetic pesticides in the quest for reducing their negative effects on human health and the environment [31, 27]. Studies have shown that plant essential oils could serve as a means for controlling pest infestation in stored products [1, 44]. A large number of essential oils and other plant extracts were studied for their insecticidal effect on several agricultural pests [12, 54, 42]. Despite the screening of many plants in search of insecticidal bioactive compounds, and although some of these compounds were found to possess effective insecticidal activities against many insect pests, only a few of these compounds are available as products in use as insecticides [3,8,30,47,34]. Currently, botanicals comprise a mere 1% of the world insecticide market [28]. Thymus is a genus that contains at least 350 different documented species [19] over five species

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were identified in the Syrian flora. One of those is *T. syriacus* (local name Za'atar). An aromatic plant belonging to the family Lamiaceae, thymus is used for food flavoring. *T. syriacus* leaves are also used for the treatment of many ailments [5]. Leaves and flowers contain an essential oil that can be harvested by distillation. The objective of this work is to assess the insecticidal effect of the vapor of *T. syriacus* essential oil against early third larvae and adults of the greater wax moth *G. mellonella*.

2. Materials and methods

2.1. Insect rearing technique

Insects of *G. mellonella* used in this study were obtained from infected beehives, and reared in our laboratory under standard culture conditions. (Temperature 26 ± 1°C, RH 65 ± 5%, light 16:8, L:D). Larvae were reared in plastic containers on an artificial diet and under rearing conditions as described earlier [4].

2.2. Volatile oils

Essential oil used in this study was extracted from leaves of wild *Thymus syriacus* Boiss. Plants were collected at the flowering stage in May 2018, from the Deir Al-Hajar area, (Damascus suburb, Syria). Prof. M. Oudat (a plant taxonomist, AECS) identified the collected plants. Leaves were left to dry in the shadow at room temperature before they were ground into a fine powder. Essential oil was obtained by steam extraction of leaf powder (100 g) using a Clevenger-type apparatus [18]. Extraction time was 3 hours, and the obtained oil represented 2.5% of the extracted powder (volume/weight) .

Earlier The author, (Tayoub and others, unpublished data), conducted a study on the chemical constituents of the essential oil of *T. syriacus* and found that it contains about 29 compounds. The monoterpene compounds represented 95.63%, mainly Thymol (77.32%), Isoborneol (6.48%), Ç-Terpinen (4.87%), and p-Cymene (4.15%).

2.3. Fumigation experiments

Experiments were performed to examine the insecticidal action of *T. syriacus* essential oil on the greater wax moth. Various concentrations of the essential oil, in three replicates for each concentration, were tested on the third instar larvae and adult insects to determine the concentration that achieved a 100% mortality and the lowest none-effective concentration.

2.3.1. Fumigation of adults

Groups of 30 individual adult insects, (<24 h of age), were placed in 1000 ml glass jars. They were exposed to different concentrations of thyme oil, namely: 1, 2, 3, 4, 5, 6, 7 and 8 µl/l air. Filter paper strips (3x3 cm) hanging inside the jars attached to their lids were treated with the essential oil. The jars were then kept in the incubator (temperature 26 ± 1°C, RH 65 ± 5%, light 16:8, L:D). Mortality was determined after 24, 48, 72 and 168 h of exposure. Untreated groups (controls) were subjected to the same conditions.

2.3.2. Fumigation of larvae

Third larval instars were chosen according to the calculated age of the larva, width of the head capsule and/or its weight. They were divided into 21 groups with three replicates per group, and 20 larvae per replicate. One group represented control. Each group was put into a 1000 ml glass Jar that contained an artificial feeding diet. Essential oils were then applied using the same method applied to adult insects. Oil concentrations tested were 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19, 20, 25 and 30µl. Jars were incubated for 7 days, then paper strips were removed, and the development of larvae to adults was monitored.

In a separate experiment, the effect of the essential oil on third- instar larvae was tested for a 24 and 38-hour exposure periods. The tested oil concentrations were 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 19, 21, 25, 30, 35, 40, 45, 50, 55, and 60 ul/ml air (jar). The experiments were performed as indicated above except that the jars were incubated for 24 and 48 hours after which the paper strips that were treated with the essential oil were removed by the end of the exposure period.

2.4. Treatment of wax frames with synthetic Thymol

Bee wax frames were sprayed with the acetone solution of thymol compound (10%) until the full coverage of the frame (approx. 50 ml solution per frame). Control bee wax frames were sprayed with acetone solution only in addition to untreated wax frames. Treated wax frames were left to dry in the air to ensure that the acetone was completely blown away. The frames, in three replicates, were then placed in plastic jars and 50 third instar larvae were put on each frame. The jars were incubated under suitable culture conditions (temperature 26 ± 1°C, RH 65 ± 5%, light 16:8, L:D), and observed daily to score dead larvae and monitor their nutrition and development.

2.5. Statistical analysis

Mortality percentages were estimated and corrected mortalities were estimated using Schneider-Orelli's formulae. The significance of differences between means of percentages was estimated using a one-way analysis of variance (ANOVA) (SPSS software, version 17). Probit was used to calculate the LC_{50} and LC_{90} [26].

3. Results and Discussion

The vapor of *T. syriacus* essential oil showed a prominent toxicity to the different life phases of *G. mellonella*. The insecticidal effect was proportional to oil concentration and exposure time. Figure 1 shows the mortalities of *G. mellonella* larvae exposed to various concentrations of the essential oils. Exposure of larvae to 30 µl/l air for 7 days resulted in a 98% mortality. Whereas, the lowest concentration used, 3 µl/l air, caused only 2% mortality. Percentages of mortality increased with the increase in essential oil concentration for the range of tested doses. The estimated lethal concentrations (LC_{50} and LC_{90}) values obtained are shown in Table 1. LC_{50} was 8.5 µl/l air,and LC_{90} was 20.6 µl/l air after 7 days-exposure period. To compare the insecticidal effect of *T. syriacus* essential oil vapor on larvae with its corresponding effect on adults we ran a separate experiment on larvae whereby the larvae were exposed to the essential oil for 24 and 48 hours. The estimated lethal concentrations (LC_{50} and LC_{90}) values were much higher than those of adults. LC_{50} values were 95.3 and 99.53 following 24 and 48 h-exposure periods, respectively (Table 1). No mortality was observed for treatments up to 19 ul/l air, and only 21.6% and 23.3% mortalities were observed at concentrations 60 ul/l air for 24 and 48 h-exposure periods, respectively.

Tabel 1. The calculated LC50 and LC90 values (µl/l air) for *T. syriacus* essential oil against the 3[th] larval and adults of *G. mellonella.*

G. mellonella	LC_{50} µl/l air	LC_{90} µl/l air	Slope±SE	F_{cal}	F_{05}	χ^2
Larva (after 7 days)	8.5	20.6	3.34±0.3	4.6	113.7	23.68
Larva (after 24h)	95.3	228.5	3.37±0.8	33.27	-	138.63
Larva (after 48h)	99.53	284.77	2.87±0.57	47.06	-	180.99
Adult (after 48h)	0.98	3.7	2.21±0.48	13.8	5.99	12.6
Adult (after 24h)	1.69	3.7	2.26±0.43	16.3	5.99	12.59

Adult insects of *G. mellonella* were more sensitive to the vapor of *T . syriacus* essential oil. Exposure of adult insects to 8 µl/l air of *T . syriacus* essential oil for 24 h resulted in a 100% mortality. Even the lowest dose used, i.e. 1 µl/l air, had a significant effect and caused a 20%, 40%, and 74%mortality at 24h, 48h, 72h and 196 h-exposure period, respectively (Figure 2).

Calculated LC_{50} for *G. mellonella* adults exposed to *T. syriacus* essential oil for 24 h was 1.69 µl/l air, whereas LC_{90} was 6.2 µl/l air. However, for the 48 h-exposure period, LC_{50} was 0.98 µl/l air and LC_{90} was 3.7 µl/l air. LC_{50} values were insignificant after 72 h and 168 h of treatment, (Table 1).

Treatment of wax frames with synthetic thymol was effective in killing *G. mellonella* larvae. The death rate of *G. mellonella* larvae ranged between 94-100%. Thymol acted as a feed suppressor and prohibited larvae from having a large feed on wax frames. Larvae fed on untreated frames or on frames treated with acetone survived. 88 % of the resulting adults were alive and active in terms of flight, mating and egg laying, noting the presence of a large feeding on wax frames (Figure 3).

Many studies have reported the insecticidal effects of essential oils. Ayvaz et al. [10], demonstrated that the essential oils of *Origanum onites, Satureja thymbra* and *Myrtus communis* were highly effective against *Ephestia kuehniella* Zeller, *Plodia interpunctella* (Hübner) and *Acanthoscelides obtectus* (Say). Ebadollahi and Ashouri, [22]. In addition, essential oils of *Achillea millefolium*, *Artemisia dracunculus* and *Heracleum persicum* were found to have toxic effects on adults of *Plodia interpunctella*.

The mechanism of the insecticidal effect of volatile oils on insects was investigated by many researchers [10, 50, 32, 24, 20, 33, 55]. The insecticidal effect is due to the monoterpenoids found in these essential oils. These compounds are volatile and can enter into the insect's body and disrupt their physiological functions.

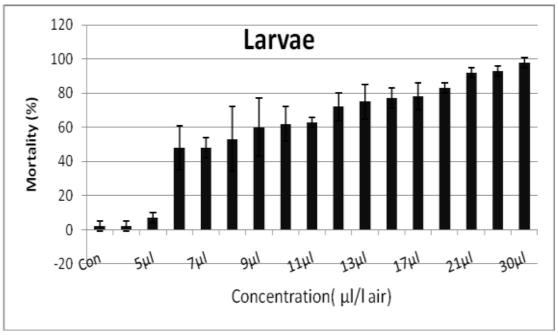

Figure 1: Mortality percentages of *G. mellonella* larvae exposed to eight different concentrations of *Thymus syriacus* essential oil.

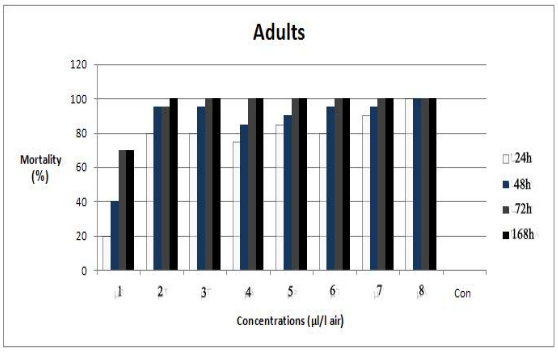

Figure 2: Mortality percentages of *G. mellonella* adults exposed to eight different concentrations of *Thymus syriacus* essential oil, after 24h, 48h, 72h and 196h exposure period.

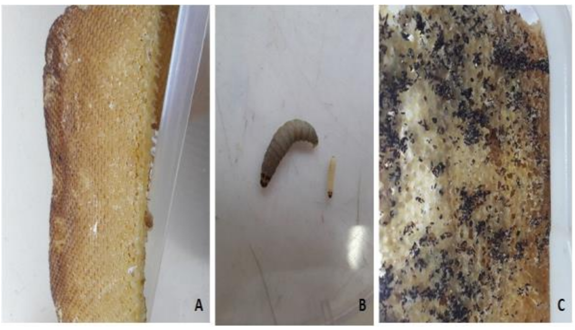

Figure 3: Bee wax frames treated with acetone solution of thymol (10%), (A), or acetone solution only (C). developed and undeveloped larvae of *G. mellonella* (B) at the end of incubation period.

Some researchers associated differences in mortality rates that result from exposure of insects to various bioactive molecules in the essential oil of a plant with an accumulation of these molecules in the digestive tract of the insects. A group of microsomal oxidase enzymes is found in the central digestive tract of insects. Those enzymes are capable of neutralizing any toxic effect that a natural compound found in a plant might impose on the feeding insect. Any compound that affects those enzymes leads to the death of the insect.

Many investigators have tested the insecticidal effect of essential oils and monoterpenes on greater wax moth. Ayman and Atef, [9], tested the efficacy of 24 plant volatile oils against *G. mellonella*. and found that the volatile oils of some of those plants showed a high level of deterrence against adults of this insect. Zaitoun, [57], studied the `deterrence effect of the volatile oils of seven plant species namely: *Eugenia aromatic*, *Ocium bacilicum*, *Thymus vulgaris*, *Eucalyptus spp*., *Mentha viridis*, and *Cymbogon cittratus* to determine the lethal doses to the fifth larvae of the wax worm. Al-Omairy, [6], evaluated the efficacy of some essential oils of *Rosmarinus officinalis*, *Cuminum cyminum*, *Pimpinella anisum*, *Thymus vulgaris*, *Coriandrum sativum* and *Carum carvi* on egg laying and on the percentage of egg hatch of *G. mellonella*. They concluded that the volatile oils targeted the development of the eggs of greater wax moth and their hatchability. The volatile oil extracted from caraway seeds, in particular, was the most effective. Arango et al. [7], tested the potential insecticidal activity of sesquiterpenes found in *Dalea coerulea*. The residual mixture had a significant toxic effect against pupae of *Spodoptera sunia*. It also had larvicidal effects on *G. mellonella* and *A. grisella*. The crude twig and leaf extract of *Aglalia odorata* were active as a stomach poison against the larvae of *G. mellonella* [46]. Abdul-Jabbar [2], studied the effects of *Eucalyptus camaldulensis* Dehnh on the biology of *G. mellonella*. He found that treating eggs with a 10% concentration of the oil extract reduced egg hatching rate by 1.33%. Mohamed [37], studied the effect of different concentrations of Neem extract, namely: 0, 10, 15, 20, 25, 50, 75 and 100 ppm, on mortality percentage of *G. mellonella*. He found that the concentration of 20ppm caused 63.34% larval mortality after 20 days of treatment. Mohamed et al. [36], tested the toxic effect of volatile oils extracted from *Origanum majorana* and *Cymbopogon proximus* on the 4th instar larvae of *G. mellonella* and calculated the values of LC_{50} which were 1.0166 %, and 1.7824%, respectively. Basedow et al. [11], evaluated the effects of XenTari® (*B. thuringiensis* aizawai), NeemAzalT/S® and their combinations on the survival of *G. mellonella*. and *Spodoptera exigua*. The results showed that Neem AzalT/S had higher efficacy and both were harmless to bees [51, 40]. Thymus species are known to possess strong insecticidal effect against various insects such as *Tribolium castaneum* and *Callosobruchus maculates* [38], *Sitophilus oryzae* [53] *Aedes* *aegypti* L. [49], *Acanthoscelides obtectus* (Bruchidae) [48], and the essential oil of this species is known to have bioactivity on insects when exposed to its of *T. syriacus* essential oil resulted in toxicity to the exposed insects, and that the insecticidal activity of the essential oil varied with insect stage at the time of exposure.

In addition, the present study showed that the insecticidal activity of the *T. syriacus* essential oil was significant considering the high mortality observed in treated *G. mellonella* insects. 100 % of *G. mellonella* adults were killed due to their exposure to 8µ/l air (LC_{50}: 1.69 and 0.98 µl/l air after 24 and 48h-exposure period, respectively) and larvae were killed due to their exposure to 30 µl/l air (LC_{50}: 8.5µl/l air). Generally, adults of *G. mellonella* were more susceptible to the essential oil or their components than larvae. LC_{50} and LC_{90} of larvae were much higher than that of adults (see Table 1) even after 7 days of larvae exposure to the vapor of the essential oil. Comparison of the toxicity values for larvae and adults following their exposure to the essential oil for 24 and 48 hour-exposure periods revealed that larvae were not affected by the vapor of the oil at the concentrations that caused total mortality to adults (8 ul/L air, for 24 and 48hour-exposure period). In fact, death of larvae after 24 and 48 hour-exposure periods was only seen at concentrations not less than 21 and 25 ul/l air, respectively (Table 1). A possible explanation is that at low concentrations, the effect observed at 7 day-exposure periods larvae was due to the larvae refraining from eating which could have resulted in the death that was observed at 7 day-exposure periods, but not at 24 and 48 hour-exposure The actual and expected mortality increased with the increase in essential oil concentration. The significant toxic effect was most likely due to one or more of the monoterpenes found in the essential oil of *T. syriacus*. Monoterpenes found in *T. syriacus* oil represent 95.6 %of the total compounds, (Tayoub et al., unpublished data). The main monoterpenes found in *T. syriacus* oil is Thymol, (77.32%), which is used as a natural antiseptic [25], and as an insecticide [43, 45, 41].

Indeed, the level of toxicity exhibited by *T. syriacus* as reported by this study was much higher than the toxicities of essential oils extracted from other plants. Bisht et al. [13], found that *G. mellonella* larval mortality percentages due to treatment with essential oils extracted from Neem, Karang, Clove, and Peppermint were 65.33%, 2.33%, 29.5%, and 35.33%, respectively, after 7 day-exposure period. In a study on the effect of essential oil extracted from *Origanum majorana* and *Cymbopogon proximus* on the greater wax moth.

Mohamed et al. [36], found that *O. majorana* was more toxic to wax moth than *C. proximus* at the tested concentrations 0.625, 1. 250, 2.500 and 5% achieving 100% and 90% mortality rates for *O. majorana* and *C. proximus*, respectively at 96 h exposure period. The LC_{50} value for *T. syriacus* essential oil as revealed by this study (LC_{50}= 8.5) was higher than LC_{50} values for the essential oil of *Origanum majorana* (LC_{50}= 1.0166) and *Cymbopogon proximus* (LC_{50}= 1.7824) which means that the latter two essential oils are more toxic to *G. mellonella* than the essential oil of *T. syriacus*.

Since thymol is the main component in the essential oil of *T. syriacus* we propose that the treatment to wax frames with an acetone solution of synthetic thymol could protect wax frames and inhibit the development of the greater wax moth larvae on them.

To conclude, thyme essential oil had a potent fumigant toxicity against *G. mellonella.* Further studies should concentrate on the isolation of the main bioactive ingredients present in thyme. Essential oil and to investigate their toxic effects on the studied insect.

Acknowledgements: The authors would like to express their thanks and gratitude to Professor Ibrahim Othman, Director General of Atomic Energy Commission of Syria and Professor Nizar Mir Ali for support and encouragement.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

Conflicts of interest: The authors declare that they have no potential conflicts of interest.

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