Lethal Toxicity of *Thymus capitatus* Essential Oil Against *Planococcus citri* (Hemiptera: Pseudococcidae) and its Coccinellid Predator *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae)[1]

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Abstract Botanical extracts, including essential oils, are promising alternatives to synthetic insecticides for pest control. In this study, we evaluated the fumigant toxicity of an essential oil extracted from *Thymus capitatus* (L.) Hoffmanns. & Link against the citrus mealybug, *Planococcus citri* Risso (Hemiptera: Pseudococcidae), and its coccinellid predator *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) under laboratory conditions. Gas chromatography–mass spectrometry analysis indicated that the major chemical compounds identified from *T. capitatus* were carvacrol (65.15%), followed by p-cymene (11.79%) and γ-terpinene (7.48%). High mortality levels were registered for *P. citri* larvae (up to 100%) and adults (up to 96%) when exposed to the tested essential oil. The median lethal concentration values calculated for *P. citri* adults were higher than for larvae. *Thymus capitatus* essential oil applied at 10 and 20 $\mu L/L_{air}$ showed high toxicity towards *C. montrouzieri* adults. These results highlighted the efficacy of *T. capitatus* essential oil as a promising tool to control *P. citri* in Tunisia. However, the adverse effects of this oil towards *C. montrouzieri* should be taken into consideration to enhance its practical implication in integrated pest management.

Key Words citrus mealybug, natural extracts, fumigant toxicity, biocontrol agent

The citrus mealybug, *Planococcus citri* Risso (Hemiptera: Pseudococcidae), is a major pest of a variety of host plants due to its direct feeding on the plant and as a vector of plant pathogens (Blaisdell et al. 2020). This pest is known as one of the most serious polyphagous pests throughout the Mediterranean basin attacking plants belonging to 70 families (Franco et al. 2009, Mishra et al. 2021). It thrives under the mild and tropical climate of the region (Goldasteh et al. 2009, Uygun and Satar 2008, Yayla et al. 2020), mainly in citrus orchards and nurseries (Bodenheimer 1951, Rao et al. 2006, Rung et al. 2009). *Planococcus citri* has a strong negative impact on yields by reducing plant vigor (Morandi Filho et al. 2015, Zappalà 2010), which may lead to lowering fruit quality and marketable value (Jaouad et al. 2020). High economic losses have been reported, with defoliation

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and fruit drop reaching 80% and 100%, respectively, in citrus crops (Zappalà 2010). Management of *P. citri* has largely depended on the use of a few selective insecticides, including methidathion (Brück et al. 2009, Mansour et al. 2010), malathion (Jacas 2010, Kerns et al. 2001), spirotetramat, and buprofezin (Satar et al. 2013). However, problems of resistance development to some active ingredients have already been reported worldwide (Mansour et al. 2010, Venkatesan et al. 2016). Implementation of effective and sustainable control tools by application of biopesticides or release of natural enemies are recommended as alternatives for pest control (Afifi et al. 2010, Kairo et al. 2013, Pérez-Rodríguez et al. 2019, Raveau et al. 2020, Regnault-Roger et al. 2012, Saljoqi et al. 2015). Essential oils are considered as a promising tool for pest management thanks to their properties as fumigants, antifeedants, contact insecticides, and repellents (Raveau et al. 2020). These natural compounds may also influence the growth rate, behavior, and reproduction of insect pests (Isman et al. 2008, Wang et al. 2006).

In this context, the aim of this work was to explore the chemical composition of essential oil extracted from *Thymus capitatus* (L.) Hoffmanns. & Link collected from the north of Tunisia. We further aimed to evaluate its fumigant toxicity against *P. citri* and a coccinellid predator, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae), under laboratory conditions.

Materials and Methods

Insects. *Planococcus citri* eggs, larvae and adults were collected from infested citrus, *Citrus sinensis* (L.) Osbeck var. 'Thomson' located in Mornag (northeastern Tunisia; N 36°40′54″, E 0°15′30″) and transported to the laboratory. The pest was reared for several generations on sugar pie pumpkin, *Cucurbita pepo* Grebensh-chikiv, (weighing 2–4 kg) placed in a cage (50 × 50 × 50 cm) under controlled climatic conditions (28 ± 2°C; 65 ± 5% relative humidity [RH]; 12:12 h light:dark [L:D]). New pumpkins were added whenever necessary to avoid possible contamination by mites or fungi.

Cryptolaemus montrouzieri Mulsant beetles (72 h old) were purchased from Nutriplant (Tunisia) and reared for several generations on *P. citri* population under laboratory conditions (25 ± 2°C; 65 ± 5% RH; 16:18 h L:D).

Essential oil extraction and chemical identification. *Thymus capitatus* aerial parts (flower, leaves, and stems) were collected from the north of Tunisia (Governorate of Zaghouan, N 36°20′25″, E 10°03′46″) during June 2019. The essential oil was extracted by steam distillation of 1 kg of fresh aerial parts for 3 h using a Clevenger-type apparatus. The extracted essential oil was stored at 4°C.

The essential oil was analyzed and quantified by gas chromatography–mass spectrometry (GC-MS) and fast GC. GC-MS analyses were conducted on a thermo trace MS Finnigan mass-selective detector equipped with an Optima 5 MS (Macherey-Nagel) capillary column (30 m × 0.25 mm internal diameter [I.D.], 0.25-μm film thickness) and a split/splitless injector (splitless mode) at 250°C. The oven temperature was programmed from 40 to 210°C. Helium was the carrier gas at 1 ml/min. Volatile compounds were identified by comparing the obtained mass spectra with those from the Wiley 275-L spectral library and with their retention indices. Retention indices were determined relative to the retention times of a series of n-

alkane standards (C9-C30, Sigma-Aldrich, 0.025 μg/μl in n-hexane), measured under the chromatographic conditions described above, and compared with literature values (Adams 2001).

Fast GC analyses were conducted on a Thermo Ultra Fast Trace GC gas chromatograph operated with a split/splitless injector and a Thermo AS 3000 autosampler (Thermo Electron Corp.). The GC system was equipped with an ultrafast module (UFM) incorporating a direct resistively heated column (Thermo Electron Corp.): UFC-5, 5% phenyl, 5 m × 0.1 mm I.D., 0.1-μm film thickness. The following chromatographic conditions were used for obtaining suitable peak resolution. The UFM temperature program was as follows: initial temperature at 40°C, held for 0.1 min, ramp 1 at 30°C/min to 95°C, ramp 2 at 35°C/min to 155°C, ramp 3 at 200°C/min to 280°C, held for 0.5 min. Injection temperature was 240°C; injection volume, 1 μl; carrier gas was helium, at constant flow rate of 0.5 ml/min; and split ratio, 1:100. The GC unit has a high-frequency fast flame ionization detector (300 Hz), at 250°C. Hydrogen gas flow was 35 ml/min; air flow, 350 ml/min; makeup gas flow (N_2), 30 ml/min. Data processing was by Chromcard software (version 2.3.3).

Toxicity bioassays. The insecticidal activity of *T. capitatus* essential oil was determined by fumigation method using Whatman filter papers impregnated with various concentrations of the essential oil. *Planococcus citri* or *C. montrouzieri* specimens were transferred from citrus leaves maintained on watered cotton and placed individually in 1-L plastic bottles using a soft brush. Five concentrations (1, 5, 7, 10, and 20 $\mu l/L_{air}$) were tested for *P. citri*. However, only two high concentrations (10 and 20 $\mu l/L_{air}$) were tested for *C. montrouzieri*. Tap water was used as the untreated control. Five replications were conducted for each concentration as well for the untreated control for both trials. For the *P. citri* trial, 20 larvae (L1) and 20 adults were used separately for each concentration. However, only 20 *C. montrouzieri* adults were used in that trial for both concentrations. Bottles serving as the bioassay arenas were maintained in a controlled climatic room (28 ± 2°C, 65 ± 5% RH, and 16:8 h L:D). Mortality was recorded after 4, 10, 24, 48, and 72 h of treatment. Insects were considered dead when neither leg nor antennae showed movement when probed with a fine hair brush while being observed under a binocular microscope (Leica® model MS5).

Statistical analysis. SPSS statistical software, version 21.0, was used to perform all statistical analysis. Data, already checked for their homogeneity (Levene test) and normality (Shapiro-Wilk test), were subjected to repeated measures analysis followed by one-way analysis of variance to assess the effect of *T. capitatus* essential oil on insect survival. Means were separated using the Tukey HSD test at $P = 0.05$. A probit test was performed to calculate the concentration-mortality responses (Finney et al. 1971).

Results

Characterization of the essential oil. The volatile compounds in the *T. capitatus* essential oil obtained by hydrodistillation are shown in Table 1. The yield of the extracted oil from the aerial part was 0.93%. Ten representative components

Table 1. Major chemical compounds of *Thymus capitatus* and relative proportions in the pure oil identified by gas chromatography–mass spectrometry and quantified by gas chromatography–flame-ionization detection.

No.	Components	Retention Index (Measured)	Compound Percentage
1	α-Pinene	931	0.91
2	Myrcene	990	2.19
3	P-cymene	1,025	11.79
4	γ-Terpinene	1,061	7.48
5	Linalool	1,099	1.32
6	Borneol	1,172	0.56
7	terpinen-4-ol	1,182	0.75
8	Carvacrol	1,299	65.15
9	Thymol acetate	1,376	0.42
10	E-caryophyllene	1,434	4.44

were identified by GC-MS and GC–flame-ionization detection, representing 94.76% of the total essential oil constituents. Carvacrol was the most abundant compound (65.15%), followed by p-cymene (11.79%), γ-terpinene (7.48%), E-caryophyllene (4.44%), myrcene (2.19 %), and linalool (1.32%).

Insecticidal activity against P. citri larvae and adults. Obtained data fit the linear model and indicated the suitability of the model to estimate the median lethal concentration (LC_{50}) of the essential oil against *P. citri* (Table 2). The LC_{50} calculated for *P. citri* adults was higher than for larvae (Table 2). Based on these results, high levels of *T. capitatus* essential oil were required to kill 50% of exposed larvae and adults after 4 and 10 h of treatment (Table 2).

Fumigant toxicity. The *T. capitatus* essential oil was toxic towards *P. citri* larvae and adults. The highest levels of mortality were obtained at highest concentrations (10 and 20 $\mu l/L_{air}$) tested for both larvae and adults during the entire study period. Our results indicate that larval and adult mortality numbers depended on oil concentration.

Repeated-measures analysis indicated that the application rate ($F = 21.94$; df = 1; $P < 0.0001$) and the insect stage ($F = 4.79$; df = 1; $P = 0.03$) had a significant effect on the mortality obtained at the five time intervals (4, 10, 24, 48, and 72 h) after treatment. There was a significant difference between the control and all tested rates for larvae ($F = 10.52$; df = 5, 149; $P < 0.0001$) (Table 3) and adults ($F = 24.42$; df = 5, 149; $P < 0.0001$) (Table 4). Moreover, statistical analysis indicated that the mortality was influenced by the insect stage ($F = 5.16$; df = 1, 299; $P = 0.02$).

Table 2. Mortality-concentration response of *Planococcus citri* larvae and adults to *Thymus capitatus* essential oil.

Time After Treatment (h)	Stage	Toxicity Regression Equation	R^2	LC_{50} (mg ai/L) (95% CI)*	χ^2 (df)	*P* Value
4	Larva (L1)	$-2.04 + 0.08x$	0.88	27.79 (19.75–75.74)	0.62 (3)	0.89
	Adult (♀)	$-2.06 + 0.06x$	0.67	33.01 (21.98–221.49)	2.13 (3)	0.54
10	Larva (L1)	$-1.44 + 0.08x$	0.89	17.23 (13.27–27.28)	1.90 (3)	0.59
	Adult (♀)	$-1.99 + 0.11x$	0.76	20.27 (15.64–33.81)	4.14 (3)	0.24
24	Larva (L1)	$-1.06 + 0.15x$	0.96	7.02 (5.11–9.02)	1.67 (3)	0.64
	Adult (♀)	$-1.29 + 0.12x$	0.88	10.67 (8.27–13.84)	3.88 (3)	0.27
48	Larva (L1)	$-1.1 + 0.3x$	0.91	3.70 (1.86–4.96)	2.03 (3)	0.56
	Adult (♀)	$-0.94 + 0.13x$	0.95	7.00 (4.82–9.16)	1.78 (3)	0.61
72	Larva (L1)	$-0.38 + 0.25x$	0.98	1.32 (2.39–2.96)	0.21 (3)	0.97
	Adult (♀)	$-0.6 + 0.12x$	0.89	4.92 (2.12–6.95)	3.94 (3)	0.26

* LC_{50} indicates median lethal concentration; ai, active ingredient; CI, 95% confidence interval.

Response of C. montrouzieri adults to essential oil. *Thymus capitatus* essential oil showed high toxicity against *C. montrouzieri* adults at the two tested concentrations (10 and 20 $\mu l/L_{air}$). Three days after treatment, the oil applied at 20 $\mu l/L_{air}$ killed more than 90% of exposed beetles.

Repeated-measures analysis indicated that the applied concentrations ($F = 9.51$; df = 1; $P < 0.001$) had a significant effect on the mortality at the five time intervals (4, 10, 24, 48, and 72 h) after treatment. There was a significant difference between the control and the two concentrations in mortality response at 10 $\mu l/L_{air}$ ($F = 14.76$; df = 1, 49; $P < 0.0001$) and at 20 $\mu l/L_{air}$ ($F = 17.22$; df = 1, 49; $P < 0.0001$).

Table 3. Mortality of *Planococcus citri* larvae following treatment with various concentrations of *Thymus capitatus* essential oil.

Concentration	Mean No. of Larvae*
Control	0.12a
1 $\mu l/L_{air}$	2.00b
5 $\mu l/L_{air}$	3.08bc
7 $\mu l/L_{air}$	3.40c
10 $\mu l/L_{air}$	3.80c
20 $\mu l/L_{air}$	3.84c

* Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey HSD test).

Table 4. Mortality of *Planococcus citri* adults following treatment with various concentrations of *Thymus capitatus* essential oil.

Concentration	Mean No. of Adults*
Control	0.00a
1 $\mu l/L_{air}$	1.16b
5 $\mu l/L_{air}$	1.44c
7 $\mu l/L_{air}$	2.84d
10 $\mu l/L_{air}$	3.28de
20 $\mu l/L_{air}$	3.92e

* Means followed by the same letter are not significantly different at $P = 0.05$ (Tukey HSD test).

Discussion

In this study, 10 compounds were identified from the essential oil extracted from *T. capitatus*. Yield of the extracted essential oil was 0.93%. Previous studies reported that the yield of *T. capitatus* essential oil in Tunisia may differ according the regions from which plants grew (Akrout et al. 2010, Ben Ghnaya-Chakroun et al. 2015, Bounatirou et al. 2007, Mkaddem et al. 2010, Tammar et al. 2018, Zaïri et al. 2019). In the north of Tunisia, the yields of *T. capitatus* essential oil collected from three regions—Jendouba and Ain Tounine (Governorate of Jandouba) and Haouaria (Governorate of Nabeul)—were 3.4%, 2.6%, and 2.8%, respectively (Bounatirou et al. 2007). Also, Ben Ghnaya-Chakroun et al. (2015) reported that *T. capitatus* yield was 1.46% in Wed Ezzarga (Governorate of Beja). Recently, Tammar et al. (2018) indicated that the yield of *T. capitaus* obtained from the governorates of Bizerte, Kef, Nabeul, and Ben arous was 2.37 $\pm$ 0.18%, 1.64 $\pm$ 0.18%, 1.39 $\pm$ 0.19%, and 1.39 $\pm$ 0.11%, respectively. In the south of Tunisia, previous studies indicated that the yield of essential oil from *T. capitatus* leaves from Matmata (Governorate of Gabès) was 1.2% (Mkaddem et al. 2010) and 2.6% from Beni-Khedache (Governorate of Medenine) (Akrout et al. 2010). Zaïri et al. (2019) showed that *T. capitatus* yield was 0.82% in the Governorate of Kairouan in the center of Tunisia. The yield of *T. capitatus* essential oil varied among countries. For example, in Libya, El-Jalel et al. (2018) showed the yield of this oil varies depending on the latitude of the region. They demonstrated that the yield of the oil was higher at lower elevations (1.5%) compared to higher ones (1.06%). In Algeria, Goudjil et al. (2020) reported that the yield of *T. capitatus* essential oil was 1.56%. In Egypt, the yield of *T. capitatus* extracted plant oil was 0.5% (Salama et al. 2012).

Here, carvacrol was the major component (65.15%) identified from the extract. In fact, in Tunisia and regardless of the region, *T. capitatus* essential oil was characterized by a predominance of phenols due to the high amount of carvacrol (Akrout et al. 2010, Ben Ghnaya et al. 2015, Bounatirou et al. 2007, Mkaddem 2010, Moujahed et al. 2011, Tammar et al. 2018, Zaïri et al. 2019). In Tunisia, according to Hosni et al. (2013), the chemical composition of *T. capitatus* collected from Mograne (Governorate of Zaghouan) was carvacrol (58.66–81.49%; major

component), p-cymene (3.83–13.17%), and γ-terpinene (7.81–3.16%). However, Mkaddem et al. (2010) showed that thymol was the major component of *T. capitatus* essential oil collected from Matmata (Governorate of Gabes), with 89.06%, followed by p-cimene with 5.04%, and γ-terpinene with 3.19%. In Algeria (Tiaret, western Algeria), thymol was the major component with 51.22%, followed by carvacrol with 12.59% and γ-terpinene with 10.3% (Goudjil et al. 2020). In Libya, Giweli et al. (2016) demonstrated that the most abundant composition of *T. capitatus* essential oil was carvacrol (68.19%), followed by thymol (12.29%) and γ-terpinene (3.09%). In Morrocco, Aissaoui et al. (2018) showed that *T. capitatus* is composed mostly of carvacrol (55.59%), followed by p-cymene (11.23%), and α-pinene (0.56%). Variation in the chemical composition of essential oils could be related to various factors including the environmental conditions (e.g., climatic conditions) and the time of harvest (Fidan et al. 2019, Msaada et al. 2012). The fluctuation in carvacrol percentage may be due to many bioclimatic factors such as the elevation, longitude, and latitude (Mkaddem 2010, Tammar et al. 2018) as well the origin of plant (Santiago et al. 2014).

The present study determined the insecticidal activity of the *T. capitatus* essential oil against *P. citri* and *C. montrouzieri*. Mortality of both stages of the two insect species were directly related to concentration of the oil. Several studies report the toxicity of *Thymus* genus against hemipteran pests worldwide. In this context, Khaled et al. (2017) demonstrated that *T. capitatus* essential oil showed a fumigant toxicity against *Myzus persicae* Sulzer (Hemiptera: Aphididae), with an LC_{50} of about 20.01 $\mu l/L_{air}$, after 24 h of treatment. Al-Mazra'awi and Ateyyat (2009) demonstrated that *T. capitatus* essential oil was repellent towards *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) adults. According to Attia et al. (2012) these variations in toxicity between different species of plants could be explained by the proportion of each component present in each essential oil. The genus *Thymus* is frequently applied on *P. citri*. For example, Erdemir and Erler (2017) reported that *Thymus vulgaris* (L.) essential oil had a repellent activity against *P. citri* adults with 88.8% at a concentration of 5 ml/L water after 96 h following application.

Our data clearly demonstrated high toxicity of *T. capitatus* essential oil against *C. montrouzieri* adults. High levels of mortality occurred with the two applied concentrations (10 and 20 $\mu l/L_{air}$). Recently, Bakkali-Aissaoui and Elamrani (2020) demonstrated that *T. capitatus* essential oil was less toxic to adults of *Phytoseiulus persimilis* Athias-Henroit (Acari: Phytoseiidae) at a concentration of 1% with 16.82% mortality. Santiago et al. (2014) showed that extracts of *Chenopodium ambrosioides* L. exhibited an insecticidal effect on *C. montrouzieri* adults after 144 h of exposure with a LC_{50} of 1.4×10^{3}. Bibi et al. (2021) indicated that the citrus oil was safe to *C. montrouzieri* adults with low mortality of 15–25% after 24 h of exposure, reporting a LC_{50} of 0.10 μl/ml. Fand et al. (2012) reported that aqueous garlic extract applied at concentrations of 0.5%, 1.0%, and 1.5% was also safe with less than 20% mortality to second-instar *C. montrouzieri* after 24, 48, and 72 h of treatment.

In conclusion, the biological activity of *T. capitatus* is likely related to its chemical composition. Our data showed promising insecticidal potential of *T. capitatus* essential with lower LC_{50} values against *P. citri* larvae and adults under laboratory conditions; this oil also caused high mortality levels of adults of the predatory beetle *C. montrouzieri*. Further studies should be conducted to confirm the efficacy of

extracted *T. capitatus* essential oil under field conditions. Possible integration into integrated pest management programs should be considered.

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