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# Post-application temperature as a factor influencing the insecticidal activity of essential oil from *Thymus vulgaris*

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ABSTRACT

Essential oils (EOs) have found applications in many industries, including the development and production of botanical insecticides. However, little information is available about the effect of post-application temperature on the insecticidal efficacy of EOs. This study therefore explores the effect of temperature on the insecticidal efficacy of an essential oil (EO) obtained from *Thymus vulgaris* and its major constituents – thymol, *p*-cymene and carvacrol, constituting 60.2%, 19.9% and 10.3% of the EO, respectively – in terms of acute toxicity against the larvae of *Spodoptera littoralis* and *Culex quinquefasciatus*. As shown by a comparison of lethal doses, the lethal doses significantly decreased with rising temperature upon topical application of the EO against *S. littoralis* larvae, with $LD_{50}$ at 15 °C and 30 °C estimated as 52 and 32 µg/larva, respectively. Thymol ($LD_{50}$ = 31 and 19 µg/larva, for 15 °C and 30 °C, respectively) and carvacrol ($LD_{50}$ = 73 and 41 µg/larva, for 15 °C and 30 °C, respectively) exhibited similar effects. The opposite effect was found upon EO application to the larvae of *C. quinquefasciatus*, with $LC_{50}$ = 19.3 and 24.4 µg $L^{-1}$ for 15 °C and 30 °C, respectively. Just like the EO, thymol and carvacrol exhibited significantly higher efficacy at the lower temperature. $LC_{50}$ at 15 °C was estimated as 16.9 and 19.4 µg $L^{-1}$, and at 30 °C it was estimated as 33.9 and 27.1 µg $L^{-1}$, respectively, for thymol and carvacrol. The effect of temperature on the efficacy of *p*-cymene was minimal. As shown in this study, the EO from *T. vulgaris* exhibits a significant positive as well as negative gradient of toxicity depending on the application mode.

## 1. Introduction

In recent decades, environmental and health risks associated with frequent applications of synthetic pesticides, as well as the development of pathogen and pest populations resistant to active ingredients of pesticide products (Fantke et al., 2012; Casida, 2017) have provided a major impulse to search for not only new alternatives for the protection of plants and agricultural products (Isman, 2015), but also insecticidal substances suitable for the protection of people and livestock against parasites and medically important vectors (Pavela, 2015a; Pavela and Benelli, 2016).

Botanical insecticides are a potential alternative for protection against harmful or noxious insects (Benelli, 2015; Pavela, 2017). These products utilize the insecticidal effects of secondary plant metabolites synthesized by plants in the scope of their natural defences against pathogens and pests (Pavela, 2016). Botanical insecticides are composed of plant extracts that usually contain complex mixtures of several active ingredients with different mechanisms of action, oftentimes exhibiting synergistic relationships (Pavela, 2015b). For the purposes of producing botanical insecticides (BIs), the active ingredients are usually isolated using various extraction methods (Pavela et al., 2008) from medicinal and aromatic plants. These substances naturally occur in nature, and as demonstrated many times, they show only very low toxicities for non-target organisms, including humans, and are therefore considered to be products that are safe for the environment and for personal health (Isman, 2015; Pavela, 2014a; Pavela and Benelli, 2016).

Essential oils (EOs), usually obtained through distillation or supercritical extraction from aromatic plants, are also highly promising plant metabolites (Pavela et al., 2008). However, although excellent insecticidal and repellent effects have been demonstrated in many studies (Bakkali et al., 2008; Pavela, 2015a), few commercial products have been marketed, and lately this fact has been frequently criticized (Isman and Grieneisen, 2014; Pavela and Benelli, 2016). Mass use of EOs as active ingredients in BIs is hindered for several reasons. In addition to problems with costly and lengthy authorisation processes, the low persistence of essential oil (EO) effects is a major cause of the current situation, characterised by rapid degradation or evaporation of EOs in

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the environment (Turek and Stintzing, 2013). Currently, this problem has been managed using various encapsulation methods that may result in a significant reduction of EO evaporation to the environment as well as of biodegradation of the substances, thereby extending biological efficacy to the necessary periods of time (Majeed et al., 2015). Although the insecticidal efficacy of EOs has been studied in many insect species, very little information is available about other limiting factors of the post-application setting that may have a significant impact on the insecticidal efficacy of BIs based on EOs.

Environmental temperature after application is one such limiting factor that could have an important effect on EO efficacy and has not yet been sufficiently studied. Understanding of the relationship between post-application temperature and the insecticidal efficacy of EOs is important, particularly in terms of practical recommendations for application of BIs produced based on EOs. This is why this study tests the insecticidal efficacy of an essential oil obtained from *Thymus vulgaris* L. (Lamiaceae), applied at different environmental temperatures. EO from *T. vulgaris* was chosen intentionally because, compared to other EOs, it provides significantly better insecticidal efficacy and has therefore been considered a highly promising active ingredient for some potential as well as already-manufactured BIs (Pavela, 2016). In order to evaluate the effect of temperature on acute toxicity, we selected two target organisms – larvae of the Egyptian cotton leafworm *Spodoptera littoralis* Boisd (Lepidoptera: Noctuidae), a polyphagous pest in tropical and subtropical regions that causes huge economic losses to producers of cotton, cereals, potatoes, vegetables and ornamental plants (Baldwin and Graves, 1991), and *Culex quinquefasciatus* Say (Diptera: Culicidae) larvae, an important vector of protozoan, viral, parasitic and helminthic diseases whose spread represents an important factor in increasing the disease burden, death poverty and social debility in tropical countries (Benelli et al., 2016; Vadivalagan et al., 2017).

## 2. Materials and methods

### 2.1. Chemicals

The EO was obtained from aerial parts of *Thymus vulgaris* cv. Varico 3. Plants were collected in June 2016 from the experimental field of the Crop Research Institute (Prague, Czech Republic). The plants were dried at 40 °C, and the dried plants were then ground and used for isolation of the EO using the hydro-distillation method. 100 g of dry matter were distilled using a Clevenger-type apparatus for 3 h. Anhydrous sodium sulphate was used to remove water after extraction. The extracted oils were stored in Eppendorf safe-lock tubes and stored at 5 °C.

The substances used in biological assays (dimethyl sulfoxide, acetone, *p*-cymene, thymol and carvacrol) were purchased from SigmaAldrich, Czech Republic and have a chemical purity of at least 99%.

### 2.2. GC/MS analysis

We analysed *T. vulgaris* essential oil through gas chromatography/mass spectrometry (GC/MS) using an Agilent 7890A GC coupled to an Agilent 5975C single-quadrupole mass detector fitted with an Agilent HP-5MS capillary column (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 μm film thickness; J &W Scientific, Folsom). After being diluted in hexane at a ratio of 1:100, the samples were injected by volumes of 1 μL, in splitless mode, into an injector that had been heated to 250 °C. The oven temperature was set at 60 °C for the first 3 min and was programmed to increase at a rate of 3 °C $min^{-1}$ until it reached 250 °C. It then remained constant for 10 min. The carrier gas was helium at a flow rate of 1 mL $min^{-1}$. Full-scan mode was used for analysis of the EO composition, and the electron ionisation energy was established at 70 eV. We identified the constituents of the EOs by comparing their mass spectra and Kovats retention indices (Adams, 2007). Correction factors were not used when calculating the percentage compositions from peak areas.

### 2.3. Insects

The study used early 3rd instar *Culex quinquefasciatus* larvae, which were reared in a laboratory colony (Crop Research Institute, Prague, Czech Republic). Food for the larvae consisted of dog biscuits and yeast powder in a 3:1 ratio. A 1-week-old chicken (for blood feeding) and a 10% sucrose solution (w.$v^{-1}$) were given to the adults.

Larvae of the tobacco cutworm *Spodoptera littoralis* were acquired from an established laboratory population (more than 20 generations; outcrossed once) at the Crop Research Institute, and fed on an artificial insect diet (Stonefly Industries, Bryan, TX, USA). We used early 3rd instar *S. littoralis* larvae for the study.

All used insects were kept under controlled conditions: 25 ± 1 °C, 70 ± 3% relative humidity and 16:8 h (L:D).

### 2.4. Bioassay

With a few minor adjustments, the mosquito larvicidal trials were executed according to WHO (1996) standard procedures (Pavela, 2015b). Thyme EO, carvacrol, thymol and *p*-cymene were diluted in dimethyl sulfoxide (DMSO) in order to make a serial dilution of the test dosage. For the purpose of producing a homogeneous test solution for experimental treatment, we added 1 mL of serial dilution to 224 mL of distilled water in a 500 mL glass bowl, which we shook gently. Twenty-five *C. quinquefasciatus* larvae per beaker were transferred in water into a bowl of the prepared test solution, whose final surface area was 125 $cm^2$. Four duplicate trials were executed for each sample concentration. Every trial featured a negative control that used distilled water containing DMSO in an amount equal to the test sample. Using a different series of concentrations of essential oil or compounds that resulted from the prior screening, mortality between 10% and 90% was obtained. We chose five or more concentrations when calculating the lethal doses, and mortality was determined after 24 h of exposure; no food was offered to the larvae during this time.

Determination of acute toxicity, measured as mortality after 24 h of exposure, was made through topical application of the thyme EO, carvacrol, thymol and *p*-cymene to the 3rd instar of *S. littoralis* larvae. The EO or compounds were diluted in acetone at various concentrations that resulted from the prior screening. Using a repeating topical dispenser attached to 100 μL syringes, we treated the dorsum of each larva's body with different doses of EO or compounds (1 μL/larva). The lethal dose was calculated using five doses of the EO and, using four duplicate measurements of 20 larvae were tested for each dose. The larva were transferred to plastic boxes (10 × 10 × 7 cm, sealed with perforated caps to eliminate fumigation effects) following treatment and given free access to the appropriate diet. These boxes were then moved into a growth chamber for 24 h. Larval mortality was indicated when the larvae were unresponsive to stimulation with forceps.

Growth rooms with constant relative humidity 70 ± 3%, a 16:8 h (L:D) photoperiod and varied temperatures of 15; 20; 25 and 30 ± 1 °C were used to house both experiments.

### 2.5. Statistical analysis

It was shown through experimental testing that over 20% of the controlled mortality was discharged and repeated. When the level of controlled mortality was at 1–20%, the observed mortality was corrected by Abbott's formula (Abbott, 1925). Using probit analysis, the $LC_{50}$ and $LC_{90}$ regression equations, as well as a 95% confidence limits, were then calculated (Finney, 1971).