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Toxic and repellent activity of selected monoterpenoids (thymol, carvacrol and linalool) against the castor bean tick, *Ixodes ricinus* (Acari: Ixodidae)

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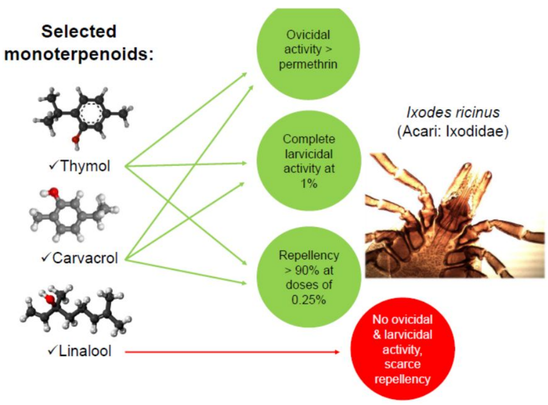
Graphical abstract



Highlights

- The effective and eco-friendly control of ticks is a major challenge for modern parasitology
- We studied the toxicity and repellent potential of several plant-borne monoterpenoids on *Ixodes ricinus*
- Thymol and carvacrol tested at low doses showed high ovicidal and larvicidal activity Both compounds tested at 0.25% led to repellency rates >90% on *I. ricinus* larvae.
- Linalool failed to show any toxic activity against ticks, repellency was limited

Abstract

The castor bean tick, *Ixodes ricinus*, is a species of medical and veterinary importance. The use of synthetic acaricides for tick control has led to development of resistance, residue in the environment and animal products, and public health concerns. In this regard, plant essential oils and their main constituents represent an appealing alternative strategy to combat ticks. The phenols thymol and carvacrol and the alcohol linalool are monoterpenoids occurring in essential oils of several aromatic and medicinal plants, such as thyme, oregano, savory, lavender and coriander. Recent studies have shown toxicity of these monoterpenoids against several mosquito vectors and other arthropod pests. However, information on their bioactivity on *I. ricinus* is not available. On this basis, here we evaluated the ovicidal, larvicidal and repellency effects of these compounds against *I. ricinus*. Concentrations of 0.25, 0.5, 1, 2 and 5 % were sprayed on the egg masses, then hatching rates were noted. Larvicidal assays were conducted on unengorged larvae, following the larval packet technique. The repellency was determined by measuring the vertical migration behavior of ticks. Carvacrol and thymol at all concentrations tested led to a significant hatching decrease, showing an efficacy higher than permethrin ($P<0.01$), whereas linalool did not cause any significant effect. In the larvae treated with carvacrol and thymol (1, 2 and 5%), mortality rates reached 100% after 24 h, showing a larvicidal efficacy higher than permethrin, whereas no effect was seen in the larval groups treated with linalool. Carvacrol and thymol at all concentrations tested showed >90% repellency on *I. ricinus*. Linalool was scarcely effective (50.24% repellency) only at the concentration of 5%. Overall, based on these results, the phenols carvacrol and thymol can be considered as candidate ingredients for the development of novel acaricidal formulations to control the spread of *I. ricinus* and related tick-borne diseases.

Keywords: acaricide; eco-friendly control; larval packet test; larvicidal toxicity; monoterpene; repellent activity test

1. Introduction

Hard ticks belonging to the genus *Ixodes* act as vectors of many pathogens of public health relevance, including Lyme disease, babesiosis, human granulocytic anaplasmosis, and Russian spring-summer encephalitis (Iori et al., 2005; Willadsen, 2006; Lupi et al., 2013; Pfäffle et al., 2013; Benelli et al., 2016). *Ixodes ricinus* is one of the most abundant ticks in the forest areas of north of Iran (Nabian et al., 2007). This species led to hyperkeratosis and strong itching in infested livestock, the blood sucking behavior also led to anemia, loss of weight and, most importantly, the transmission of tick-borne infections caused by protozoa belonging to the genus *Babesia* and bacteria of the genus *Anaplasma* (de Freitas Fernandes and Freitas, 2007; Mehlhorn et al., 2011).

Therefore, it is necessary to protect the humans and animals from tick infestation (Bissinger and Roe, 2010; Abdel-Ghaffar et al., 2015; Benelli, 2016; Pavela et al., 2016). To date, the use of synthetic acaricides is the most common method for tick control (Piesman and Eisen, 2008; Benelli et al., 2016). On the other hand, the indiscriminate overuse of synthetic acaricides has led to concerns related to environmental pollution, non-target toxicity, and development of resistance in targeted tick populations (Naqqash et al., 2016). Therefore, environmentally friendly and cost-effective methods to protect humans and domestic animals from ticks would be welcome and attractive to global market of acaricides (Marimuthu et al., 2011, 2013; Velayutham et al., 2012; Lupi et al., 2013; Rajakumar et al., 2013; Khater et al., 2016; Banumathi et al. 2017).

Monoterpenoids are important chemical constituents of plant essential oils, endowed with a skeleton of ten carbon atoms, and seem to possess promising effects against mite parasites (Badawy et al., 2010). Thymol and carvacrol are strictly biogenetically correlated monoterpene phenols (Dall'Acqua et al., 2017), with molecular formula $C_{10}H_{14}O$, occurring in essential oils from aromatic and medicinal plants such as oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.), savory (*Satureja montana* L.) and ajwain (*Trachyspermum ammi* (L.) Sprague) where they constitute the major oil fraction (Vitali et al., 2016; Benelli et al., 2017; Morshedloo et al., 2017; Nabavi et al., 2015). Both carvacrol and thymol were reported to have broad acaricidal activity against a number of arthropod pests of medical and veterinary importance (Barimani et al., 2016; Concepción et al., 2013; Senra et al., 2013; Tabari et al., 2015; da Silva Mendes et al., 2011; Daemon et al., 2009; de Oliveira Monteiro et al., 2009; de Oliveira Monteiro et al., 2010; Masoumi et al., 2016). Linalool is a linear monoterpene alcohol, with molecular formula $C_{10}H_{18}O$, reported to be the main volatile component of the essential oils of several aromatic species such as lavender (*Lavandula angustifolia* Mill.), coriander (*Coriandrum sativum* L.), ligurian yarrow (*Achillea ligustica* All.) and *Cinnamosma madagascariensis* Danguy (Lis-Balchin and Hart, 1999; Telci et al., 2006; Cecchini et al., 2012; Pavela et al., 2017). Several studies have demonstrated toxicity, antifeedant, and repellent activity of linalool against insect pests, with special reference to house flies and fruit flies (Chang et al., 2009; Lopez et al., 2012; Maganga et al., 1996).

Bearing in mind the harmful effects of tick parasitism, the resistance to synthetic acaricides, and the strict legislation for residue-free products (Pavela and Benelli, 2016), this study has been conducted to evaluate the efficacy of the above monoterpenoids for the control of this Ixodidae economically important species in terms of ovicidal, larvicidal and repellency effects. Our findings, may give new insights into the possible application of these terpenes in the preparation of effective acaricidal and repelleny formulations with low impact on the environment as well as on human and animal health.

2. Material and Methods

2.1. Ticks

One hundred engorged females of *I. ricinus* were collected from 30 naturally infested cattle in the region of Babolkenar, Babol city, Mazandaran Province, Iran. The farm was free of acaricidal residues for at least 120 days prior to the experiment. The females were taken to the laboratory, washed, dried and then kept at 27°C and R.H. > 80% for oviposition. The eggs were collected and kept at the same temperature and humidity conditions to obtain larvae.

2.2. Chemicals

Carvacrol, thymol and linalool were obtained from Sigma (Sigma-Aldrich, Germany) and permethrin was provided by Chimie-Ghahraman Company (Gilan, Iran). All other materials were of analytical grade and commercially available (Sigma Aldrich, Germany). Since the test compounds are not easily soluble in water, dimethyl sulfoxide (DMSO) 1 % was used as emulsifier. DMSO (1 %) in water was used as negative control. Permethrin, because of its proved acaricidal activity was tested as a positive control, to compare its efficacy with the tested compounds.

2.3. Ovicidal activity

Egg masses were gathered and weighed; 50 mg of eggs were placed on a 4 × 4cm filter paper and considered as standard egg mass; 1 mL of carvacrol, thymol, linalool and permethrin solutions at concentrations of 0.25, 0.5, 1, 2 and 5 % were sprayed on the eggs. Control received sprays of water + DMSO 1 %. 10 replicates for each group were performed. The egg masses were kept at the previously mentioned conditions to evaluate hatching rates (%).

2.4. Larvicidal activity

Larvicidal assays were conducted on unengorged larvae, following the larval packet technique (LPT) (Miller et al., 2002). One hundred larvae (age: 10 days old) were placed on the 2 × 2 cm filter papers impregnated with 40 µL of the test solutions at concentrations of 0.25, 0.5, 1, 2 and 5 %, then placed on the larger sheets of filter paper (6 × 6 cm) and enclosed to form packets. Control packets received 40 µL of water + DMSO 1 %. 10 replicates for each tested concentration were performed. All the test packets were kept in the controlled conditions as previously described and after 24 h were opened for mortality calculation. To assess the mortality rates of treated ticks, we used a vacuum pump with a pipette tip attached to the end of a rubber tube.

2.5. Repellent activity

The bioassay was developed according to the method by Wanzala et al., (2004) with minor modifications. The repellency measurement apparatus was designed based on the vertical migration behavior of ticks. It consisted of two aluminum rods (0.7 cm diameter), filter paper treated with the test solution (concentrations: 0.25, 0.5, 1, 2 and 5 %) was stapled to one rod while the other one contained solvent-treated filter paper, serving as control. One hundred larvae of *I. ricinus* were placed at the base of the rods and observed for 60 min. Then, the repellence (%) of each tested compound was calculated comparing the number of larvae climbed on the treated and untreated rods (Wanzala et al., 2004). 10 replicates were performed for each tested concentration.

2.6. Data analysis

Data from ovicidal, larvicidal and repellent activity experiments were analyzed by ANOVA followed by Tukey's HSD test. Values of *P*<0.05 were considered significant. All the statistical analyses were carried out using SPSS (SPSS Inc. IL, Chicago) Version 16.

3. **Results**

The results of ovicidal activity are presented in Table 1. Carvacrol and thymol at all concentrations led to a significant decrease in *I. ricinus* egg hatching, if compared with the control (*P*<0.001). The highest decrease in hatching rates (%) was observed in thymol-treated *I. ricinus* eggs. On the other hand, treatments with linalool, even at higher concentrations, did not lead to significant toxicity against eggs of *I. ricinus*. Permethrin showed significant toxicity on *I. ricinus* eggs, especially at the higher concentrations (2 and 5%) (*P*<0.05), whereas at the lower concentrations, thymol and carvacrol showed significantly higher ovicidal activity (P<0.05) and reduction in hatching rates (P<0.01) (Table 1).

Data obtained from larvicidal activity assays are presented in Table 2. Treatment of *I. ricinus* larvae with carvacrol and thymol at all concentrations resulted in significant mortality in comparison to the control (*P*<0.001). In the groups of *I. ricinus* larvae treated with the higher concentrations of carvacrol and thymol (1, 2 and 5%), mortality rates reached 100% and complete larval mortality was obtained after 24 h. Mortality rates in the *I. ricinus* larval groups treated with linalool did not show any significant difference in comparison with the control. Permethrin did not result in significant mortality rates in comparison with the control, and only at the concentration of 5% led to significant mortality in *I. ricinus* larvae (*P*<0.05) (Table 2).

Repellent activity of carvacrol and thymol was significant against *I. ricinus* larvae (Figure 1). At all concentrations tested, they showed over 90% repellency on *I. ricinus*, which was statistically significant in comparison with the control (P<0.001). Linalool at the concentration of 5% showed 50.24% repellency on *I. ricinus* ticks, which was significant in comparison with the control (P<0.01). Also, permethrin, except for the concentration of 0.25 %, achieved significant repellent activity (P<0.05) (Figure 1).

4. Discussion

Arthropod vector management by synthetic pesticides has become progressively hindered by fast-developing vector resistance (Naqqash et al., 2016) as well as the increasing consumer demand for safer and residue-free foodstuffs. This has created a considerable market opportunity for alternative products, including botanical pesticides (Semmler et al., 2011; Pavela and Benelli, 2016). Plant-derived compounds, in comparison with the synthetic chemicals, are of great interest due to several key advantages. Indeed, botanical pesticides are obtained from auto-sustainable natural resources. They are easily degradable and do not leave residues in food and environment. Moreover, the development of resistance to these substances is unlikely to happen (de Oliveira Monteiro et al., 2010), due to their multiple mechanisms of action (Pavela and Benelli, 2016). On the other hand, there are some significant challenges to face in this research field, including the standardization of their chemical composition (Heng et al., 2013), as well as the improvement of botanical pesticide stability in the field (Turek and Stintzing, 2013).

The results obtained in the present study clearly indicate that two of the tested monoterpenoids, namely carvacrol and thymol, can be regarded as highly effective candidate ovicides and larvicides against *I. ricinus*. Our experiments highlighted a significant decrease in hatching rate of *I. ricinus* eggs in thymol and carvacrol treated groups. In agreement with our results, the ovicidal effect of thymol on eggs of *Rhipicephalus (Boophilus) microplus* ticks has been demonstrated by de Oliviera Monteiro et al. (2010), who showed high mortality rates for this compound. Considering larvicidal activity, both carvacrol and thymol at all concentrations led to a significant mortality in larvae of *I. ricinus*. In line with these findings, da Silva Mendes et al. (2011) have reported the acaricidal activity of thymol on larvae and nymphs of *Amblyomma cajennense,* demonstrating that thymol at 1% concentration led to mortality > 90% in unengorged larvae. The larvicidal effect of thymol on immature stages of ticks was reported by Daemon et al. (2009) and Novelino et al. (2007), who found high mortality rates in larvae of *Rhipicephalus sanguineus* and *R. (B.) microplus*, respectively. Furthermore, combinations of thymol and carvacrol on the same tick species led to synergistic effects (Araújo et al., 2016).

The toxicity of thymol on engorged nymphs and females of *R. sanguineus* has been described by de Monteiro et al. (2012). The acaricidal activity of carvacrol and linalool was studied on *R. (B.) microplus* and *Dermacentor nitens* larvae by Senra et al. (2013). These authors have reported that carvacrol at the lowest concentration (2.5 µL/mL) caused 100% mortality on larvae of both species. Very recently, it has been noted that – when encapsulated with yeast cell walls – carvacrol maintained its acaricidal efficacy against *R. (B.) microplus,* showing a LC_{50} (i.e. lethal concentration killing the 50% of exposed individuals) of 0.71 mg/ml versus LC_{50} of 1.82 mg/ml of carvacrol alone (da Silva Lima et al., 2017). Thymol and carvacrol were highly toxic to the larvae of *Amblyomma sculptum* and *D. nitens* with LC_{50} of 2.04 and 3.49, and 2.17 and 3.33 mg/ml, respectively. Furthermore, their combination treatment gave a synergistic effect, especially on *D. nitens* (Novato et al., 2015).

More generally, the antimicrobial properties of carvacrol and thymol are well-known and utilized on a large scale. To our mind, some of their properties apply to the tick model. From a mechanistic point of view, carvacrol and thymol are endowed with similar effects. As an example, their lipophilicity allows them to cross easily the cuticle and enter the tick body. At the cellular level, they can cause perturbation of cell membrane and mitochondria (Burt, 2004). In addition, they can interact with the cholinergic system of which is an important target for the development of acaricides (Jukic et al., 2007). Carvacrol and thymol possess the GRAS (Generally Recognized as Safe) status by FDA (US Food and Drug Administration) (Guarda et al., 2011) so that safety concerns related to their use as acaricides can be regarded as minimal. In addition, it should be also noted that carvacrol and thymol tested on *Dermanyssus gallinae* (De Geer) (Acarina: Dermanyssidae) good duration of the repellent and toxic effects over time. In particular, carvacrolthymol tested in 4:1 ratio at 2 % concentration showed good residual toxicity and was able to control *D. gallinae* until 14 days post spraying (Masoumi et al., 2016). This point out the highly promising potential concerning the long-lasting effects of the tested monoterpenoids against mites, allowing us to candidate them for further development of tick acaricidal and repellent formulations. Generally, both thymol and carvacrol, when applied to control harmful arthropods, showed negligible effects on non-target organisms such as honey bees, mealworm beetles, shellfish and the mosquito fish *Gambusia affinis* (Mattila et al., 2000; George et al., 2009; Lahlou,

2002). Therefore, they can be considered as relatively safe to non-target organisms (see also

Pavela and Benelli, 2016).

In our study, linalool failed to show any toxic activity against *I. ricinus* ticks, and repellency was limited. Our results support earlier studies, where the mortality rates of linalool at the highest concentration (20 µL/mL) were significantly lower than those of carvacrol and thymol, reaching 14.5 and 8.4% on larvae of *R. microplus* and *D. nitens*, respectively (Senra et al., 2013). Moreover, it has been demonstrated that the linalool-rich essential oil of *Ocimum basilicum* L. did not exert any toxicity on *R. (B.) microplus* at any of the tested concentrations (Martinez-Velazquez et al., 2011).

On the other hand, linalool and *Lavandula angustifolia* essential oil were effective against adults of *Rhipicephalus* (*B.*) *annulatus* with a dose-dependent effect (Pirali-Kheirabadi and da Silva, 2010). Although linalool was not sufficiently toxic on eggs and larvae of *I. ricinus* to be considered as a potential acaricide, it showed some beneficial characteristics which can be subjected for further investigations. Indeed, it has been shown that linalool facilitates skin and membrane penetration of other drugs (Letizia et al., 2003). Therefore, this substance can be added to contact acaricide formulations as a penetration enhancer of active compounds through the cuticle of ticks. Surely, studies on efficacy of combination of linalool with carvacrol and thymol will give us new insights into the application of this substance for the development of acaricidal formulations.

The climbing behavior of *I. ricinus* larvae in pastures is a strategy to locate and attach to hosts. So, inhibition of this behavior is an indication of repellents, which can be considered as effective means for the control of *I. ricinus*. In the present study, carvacrol and thymol at all concentrations tested showed over 90% repellency on *I. ricinus.* Linalool, only at the highest concentration (5%) caused significant repellency on larvae. Earlier, Del Fabbro and Nazzi (2008) evaluated the repellency activity of eugenol and linalool against *I. ricinus* ticks, and demonstrated high repellent activity of eugenol and low repellency of linalool on nymphs. In agreement with our observations, significant repellent activity for carvacrol and thymol on the poultry red mite *Dermanyssus gallinae* has been previously reported (Barimani et al., 2016).

In conclusion, the results of our study, along with the low toxicity of the tested products for human health and the environment, as well as low residue level in animal products, highlighted the promising potential of these monoterpenoids, especially carvacrol and thymol, to develop newer and safer control tools against *I. ricinus* ticks. However, we would like to point out that other tick stages such as nymphs and adults should also be tested, especially since these stages more frequently infest larger mammals. Further studies including field trials, encapsulation assays (Pavela, 2016) and nano-formulation (Benelli et al. 2017b,c) improving the acaricidal efficacy of these components, are urgently needed.

Conflict of Interest

The Authors declare no competing interest.

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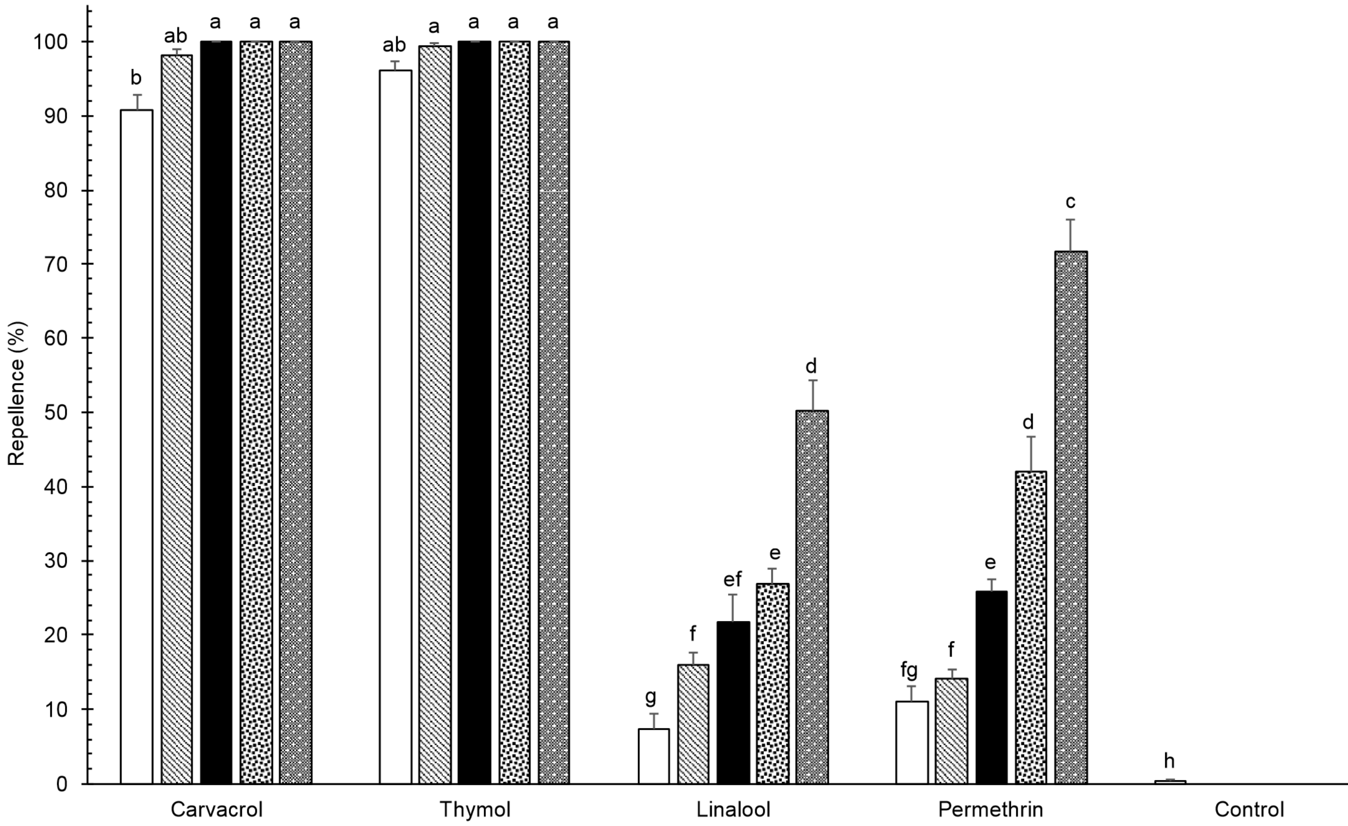
Figure Caption

Figr-1**Figure 1.** Repellent activity of carvacrol, thymol, linalool and permethrin against larvae of *Ixodes ricinus* under laboratory conditions (27±1°C and R.H. ≥ 80%).



T-bars represent standard errors. Above each column, different letters indicate significant differences (ANOVA followed Tukey's HSD test, P<0.05).

Table 1. Hatching rates of *Ixodes ricinus* eggs treated with different concentrations of carvacrol, thymol, linalool and permethrin under laboratory conditions (27±1°C and R.H. ≥ 80%).

Treatment	Concentration (%)	Hatching rate (%)
Carvacrol	0.25	18±1.53[c]
	0.5	16.4±1.77[c]

	1	8.3±1.26[bc]
	2	2±0.73[ab]
	5	0±0[a]
Thymol	0.25	13±1.24[c]
	0.5	9.8±0.85[bc]
	1	4.6±1.26[b]
	2	0±0[a]
	5	0±0[a]
Linalool	0.25	100±0[g]
	0.5	100±0[g]
	1	100±0[g]
	2	99.8±0.2[g]
	5	97.8±1.16[g]
Permethrin	0.25	67.1±3.50[f]
	0.5	46.4±2.72[e]
	1	23.5±1.96[d]
	2	13.5±1.45[c]
	5	8.7±1.29[bc]
Control	100±0[g]	

Data are presented as means ± standard errors; different superscript letters indicate significant differences at $P<0.05$ (ANOVA followed by Tukey's HSD test).

Table 2. Mortality of unengorged larvae of *Ixodes ricinus* treated with different concentrations of carvacrol, thymol, linalool and permethrin under laboratory conditions (27±1°C and R.H. ≥ 80%).

Treatment	Concentration (%)	Mortality (%)
Carvacrol	0.25	60.1±2.03^{d}
	0.5	79.8±1.93^{e}
	1	100±0^{f}
	2	100±0^{f}
	5	100±0^{f}
Thymol	0.25	67.9±1.73^{de}
	0.5	85.6±1.68^{e}
	1	100±0^{f}
	2	100±0^{f}
	5	100±0^{f}
Linalool	0.25	0±0^{a}
	0.5	0±0^{a}
	1	0±0^{a}
	2	0±0^{a}
	5	0.9±0.5^{ab}
Permethrin	0.25	0±0^{a}
	0.5	0±0^{a}
	1	2.5±0.61^{ab}
	2	7±1.93^{b}
	5	23.2±3.6^{c}

Control	0±0[a]

Data are presented as means ± standard errors; different superscript letters indicate significant differences at $P<0.05$ (ANOVA followed by Tukey's HSD test).