

The acaricidal and repellent activity of the essential and nano essential oil of *Thymus vulgaris* against the brown dog tick, *Rhipicephalus sanguineus* (Acari: Ixodidae)

Zohreh Alibeigi

Shiraz University

Ehsan Rakhshandehroo

Rakhshandehroo@Shirazu.ac.ir

Shiraz University

Mohammad Jamal Saharkhiz

Shiraz University

Amir Mootabi Alavi

Shiraz University

Research Article

Keywords: *Rhipicephalus sanguineus*, *Thymus vulgaris*, Nano essential oil

Posted Date: November 2nd, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3522907/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BMC Veterinary Research on March 3rd, 2025. See the published version at <https://doi.org/10.1186/s12917-025-04609-y>.

Abstract

Background

The brown dog tick is globally distributed and harms the host in terms of blood feeding and pathogen transferring. Chemicals are traditionally used for the control, but herbal plants has been investigated mainly due to their natural components with killing and repellent effects. Previously, the role of thymol has been described for the biocontrol of ticks at different stages. Therefore, was conducted a study to evaluate the effects of a thymol-rich herbal plant, *Thymus vulgaris* L., and its major constituents on *Rhipicephalus sanguineus*.

Results

In this work, we suggested to perform the larval mortality test using 2 ml microtubes instead of previously described pocket and immersion methods. This method seems to be closer to the environmental condition. The results represented a great activity of the nano EO and thymol on live larva. The nano form was highly effective starting from the lowest concentration (0.125%) showing 88.9% mortality. In comparison, the EO revealed similar effects but at higher concentrations. The nano emulsion and thymol showed also a complete repellency effect against larva at the concentrations of 0.5% and 20 mg/ml, respectively. In adult tick bioassays, thymol was the only substance that accompanied with a statistically significant reduction in female oviposition rate ($p < 0.05$), however at its utmost concentrations.

Conclusion

According to the results, the nano EO of *T. vulgaris* is recommended for the biologic control against *R. sanguineus* larva. In addition, further studies should be done on the nano material to enhance its effect on the adult female tick reproduction.

Background

The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806), is a prevalent ectoparasite with a global distribution. The tick, from larva to the adults, is of great importance mainly due to the harm to animal by bites, blood feeding, clinical manifestations in heavy infestations and high reproductive rate [1]. Therefore, using effective chemical acaricides have been the conventional way against different developmental stages. Despite their significant effects, chemicals may cause intoxication and also evolution of genotypic resistant ticks (Li et al., 2007; Becker et al., 2019). Also, they often do not show any repellent effects on the ticks. For these reasons, the essential oils (EO) of natural plants and its purified compounds have been studied as the safe and attractive alternatives for pest management programs (Karabörklü and Ayvaz, 2023).

According to the literature, levels of acaricide activity was demonstrated for the botanical EOs. But many of promising results have reported using those plants that contain high levels of thymol in their composition (de Oliveira Monteiro et al., 2010). Among many of the well-studied medicinal plants, *Thymus vulgaris* contains predominantly a high level of thymol in its EO composition and have utilized to enhance the function of pesticides (Pavela, 2016). This plant is indigenous in parts of Europe, Africa and Asia with a wide range of medicinal aims. In addition to thymol, the EO of this plant species is rich in carvacrol, p-cymene and some other monoterpenes [7]. The killing effect and repellent feature of thyme EO was documented against tick species of *Dermacentor reticulatus* [8] and *Hyalomma lusitanicum* [9], some insects like *Aedes aegypti* [7] and housefly [10] and also mites [11]. Nevertheless, studies on the advantages of using this product has not properly investigated for controlling of the prevalent ticks worldwide.

In recent years, the application of nano formulations was investigated for enhancing the activity of the herbal products. Different compounds have been made by various methods in nanotechnology, all aiming to achieve high inhibitory and repellent function. The promising effectiveness of metallic nano particles synthesized from several leaf extracts against *Rhipicephalus microplus* was reviewed by Banumathi et al. (2017) [12]. In addition, nanoemulsions made from herbal EOs are considered to be accompanied with prolonged mosquito-cidal activity [13], increased insecticidal potential and significant alterations in morphology of the larvae [14]. The effects of nano materials on different stages of ticks are still not clear and need to be more investigated. In the current study, we evaluated and compared the effects of EO and nano EO of *T. vulgaris* on larva and engorged female of *R. sanguineus*.

Materials and Methods

Essential oil (EO) preparation and analysis

Aerial part of *Thymus vulgaris* was purchased from local markets from Shiraz city, Fars province, Iran. The plant species was identified and confirmed using the taxonomic features at college of Agriculture, Shiraz University, Iran. The leaves were air dried at room temperature (less than 25°C) and the EO was provided by all-glass Clevenger-type apparatus through approximately 4h hydrodistillation on 500 gr powder as described by the European Pharmacopoeia (1983)[15] and also some previous studies [16, 17]. The EO samples were dried over anhydrous sodium sulphate, sealed and stored at 4°C for further analyses. It was then analyzed by gas chromatography–mass spectrometry (GC-MS) using a GC (Agilent Technologies-7890A-GC) equipped with a mass spectrometer (Agilent Technologies-5975C). Separations were done by a HP-5 MS capillary column (30mm (length) × 0.25mm (Internal Diameter) and film thickness: 0.25 micron). The Oven temperature initiated from 60 °C and increased to 210 °C with the rate of 3 °C/min, then increased to 240 °C with the rate of 20 °C/min and the final temperature kept isothermally for 8.5 min. The pure EO sample was injected into the GC/MS in split mode (split ratio: 1/50). The detector and injector temperatures were both set at 280°C. The carrier gas was Helium with a flow rate of 1 mL/min. The temperature of interface and ion source were set at 280°C and 230°C, respectively. The electron ionization energy was 70eV in the electronic ionization (EI) mode and mass

range was from 50–480 amu. Finally, the Chemstation Software (Agilent Technologies, Santa Clara, CA) was used to analyze mass spectra and chromatograms.

Preparation of nanoemulsion from *T. vulgaris* EO

The nanoemulsion was made through an optimized high energy ultrasound emulsification method described by Hashtjin and Abbasi (2015)[18]. The nanoemulsions were prepared in two phases. The mixture of Tween 80 and deionized water (with final concentration of 2%, v/v), as the components of the aqueous phase, were first stirred at 700rpm using a magnetic stirrer for 15 min. Then, the EO as the organic phase was added to the aqueous phase, further stirred at 700rpm for 15 min and mixed using a homogenizator at 13500 rpm for 5 min. (Water + Tween80)/EO ratio were adjusted to have final EO concentrations of 0.125, 0.25, 0.5, 1, 2 and 4% v/v. The pre-emulsions were finally sonicated for 5 min with a 20 kHz ultrasonic processor (Bandelin, Sonopuls HD 4200, Berlin, Germany) with 150watt output.

Preparation of test concentrations

The extracted pure EO was dissolved in 2% tween 80 (v/v) to obtain final concentrations: 0.125, 0.25, 0.5, 1, 2 and 4% v/v.

According to our results and also previous reports [17], thymol and p-cymene are two major constituents of the *T. vulgaris* EO. In order to test the effects of these substances on ticks alone, thymol (in the form of crystals) and p-cymene were purchased from Sigma-Aldrich® with 99% purity. Thymol was serially diluted in 2% tween 80 to give final concentrations of 2.5, 5, 10, 20 and 40 mg/ml (w/v) and the same concentrations were also prepared as µl/ml for p-cymene. Additional concentrations of 1.5% of *T. vulgaris* EO and 60 µg/ml of p-cymene were also examined on larva and adults, respectively.

Tick samples

The female engorged ticks were collected from naturally infected dogs referred to the Veterinary Clinic of pet animals, Shiraz University, Shiraz, Iran. Collections were done after taking the consent of their owner. The sampled animals had not recent contact with acaricide products. Ticks were cleaned with a soft paintbrush and identified according to the taxonomic keys given by Walker et al. (2000)[19]. In order to test on tick larvae, each 1 to 2 engorged ticks were placed in one plastic jar with half closed lid and kept in an incubator (Mettmert®, Germany) at a temperature of 27°C and relative humidity of 80% for oviposition. After about 10 days, eggs were weighted into approximately 10 mg aliquots which placed in 2 ml microtubes and kept at the incubator for hatching. The microtubes wall was perforated using a fine needle in order to help air exchange with the surrounding environment and to prevent saturation of the air with scent of any test substance. The experiments were started when the hatched Larvae aged 7–15 days.

Larva bioassays

Larvicidal activity in microtubes

In this study, we developed a method which applies larvae containing microtubes instead of routine paper pockets or immersion technique (Fig. 1). At the time of experiment, the tube cap was opened, filled with a cotton plug and moisten immediately with 250 µl of the prepared solutions. Then the cap was closed and the tubes were incubated for about 24 h. After this time, movement of larvae in microtubes were evaluated using a stereomicroscope. Those larva with no motility, or incapable of maintaining normal posture were assigned as dead. For live ticks, counting is difficult because they constantly change their position and run quickly. Therefore, the larva mass was then inactivated by placing tubes at 4°C for about 1 h and enumerated totally using line graded sheet papers. Finally, the Mortality rate was calculated as (number of dead larvae/total number of larvae) × 100%.

Repellent activity on larva

The repellency bioassay was done on the basis of climbing feature of the ticks. An apparatus was set up using an aluminum wire (2mm diameter) bended to form a pair of vertical rods measuring about 8cm in height and 9 cm distance between them (Fig. 2). One side assigned to each test odor and the other used for its control. The lower side (horizontal part) was fixed at its center to upper opening of 10 cc laboratory glass bottle standing itself on a square watch glass (3cm × 3cm × 1cm) at its base. In order to provide the source of drug odor, two laboratory glass tubes (1.5cm diameter, 10cm length) were filled with cotton plug at the bottom covering about 2 cm of the end (Fig. 2). The apparatus was then set down on a tray with shallow water to prevent tick dispersal and to provide humidity. At the time of experiment, the cotton mass was moistened with 1 ml of any substances and the glass tubes were placed upside down on the rods. Then, a microtube containing larvae was placed in the glass bottle and the cap was cut to allow larvae to escape. After about 2h, rods of any side were observed and the number of larvae attracted to each side were recorded. In contrast to adult ticks, larva are of small size and may be aggregated in some parts of rods, and/or hidden under the cotton plug. So, final evaluation could be accompanied with errors. Therefore, the repellency values were transformed to numeric grades using a criterion; Grades 3 (complete repellency) and 0 (complete attraction) were assigned when 100% of larva have attracted to the control and treatment parts, respectively. Grade 2 was defined for groups showing more than half (50%) of larvae were attracted to the control rod. Grade 1 was referred to groups if > 50% of larvae run toward the treatment part. Grades 1 and 2 implies to dispersal of larvae between control and treatment groups.

For all larva bioassays, the negative control was established with Tween 80 (2%) and each treatment was repeated for 6 times.

Adult immersion test

Tests on the adult female ticks was done by a method previously described by Drummond et al. (1973) [20], with modifications. For each test, a group of 5 to 7 engorged females were first weighed and immersed individually in at least 3 ml of dilutions in small glass bottles for 5 min. Ticks were recovered from the solutions with the aid of a sieve and dried with paper towel. Then they were transferred, individually, to a type of glass bottles with perforated plastic lid (5 cm diameter, 2.5 cm height) for

oviposition. After about 10 days of incubation, ticks were removed and the laid Eggs were weighed and put back to the same condition for larval hatching.

The effects of any substances on female reproduction were calculated using formulas as follows:

Egg Reproductive index (RI) = (Egg mass weight / weight of tick before oviposition) × 100

Percent Reduction of Oviposition (OR) = (mean RI in control group – mean RI in treated group/ mean RI in control group) × 100.

As a positive control, a commercial synthetic cypermethrin was purchased containing 20% active ingredient (Saman®, Iran). Three concentrations were made in distilled water in order to obtain 3.75, 7.5 (the recommended dose) and 15 mg(powder)/ml dilutions.

Statistical analysis

Data obtained from larva and adult tick bioassays were measured as percentage. The statistical difference between treatment and control values were done using ANOVA followed by Tukey test ($P < 0.05$). The Kruskal–Wallis and the Student-Newman-Keuls tests were used whenever data did not show normal distribution. The lethal concentration estimations, LC50 and LC90, were identified by Probit analysis. All the analyses were done using the SPSS software (IBM Inc., Armonk, NY), version 27.

Results

Effects of oils on larva

A total of 55 compounds were identified in the EO of *T. vulgaris*, equivalent to 99.9% of the total oil (Supl. file 1). According to the GC-MS analysis, Thymol (38.37%), γ -Terpinene (15.09%) and p-Cymene (18.54%) were found to be the major EO components. Other chemical compositions were found to be in low concentrations.

The percentage larval mortality (LM) rate and repellent (rep.) activities of different substances are represented in Table 1. The larvicide effect of *T. vulgaris* was seen from the concentration of 0.5% ($p < 0.05$). But it was highly effective when the concentration increased to 1%, representing of about 95% mortality. In comparison, the nano form has remarkable killing effect (88.9%) at its lowest concentration (0.125%) and caused complete LM at the concentration of 0.5%. Thymol, as the major EO component, showed a significant effect on larvae at 5% concentration but reached to 100% at the concentration of 10 mg/ml. These results were not true for p-cymene because larva population remained survived unless its concentration was increased more to 60 μ /ml. The LC50 and LC90 were calculated and depicted in Table 2. As the table shows, the differences between the values obtained for EO and the nano substance and also the thymol and p-cymene groups were remarkable.

Table 1

Larval mortality (LM) and larval repellency (Rep) effects of *T. vulgaris* EO and nano EO, Thymol and p-cymene on *R. sanguineus* larva. The results are represented as Mean $\pm$ SEM.

Substance	Concentrations*						
	Effect	0.125%	0.25%	0.5%	1%	2%	4%
T. vulgaris EO	LM	1.1 $\pm$ 1.0 ^a	6.1 $\pm$ 2.3 ^a	8.4 $\pm$ 4.03 ^{ab}	95.3 $\pm$ 2.1 ^b	100 ^b	100 ^b
	Rep	1.6 $\pm$ 0.2	1.8 $\pm$ 0.2	1.8 $\pm$ 0.2	2 $\pm$ 0.0	2.2 $\pm$ 0.2	2.8 $\pm$ 0.2
T. vulgaris Nano	LM	88.9 $\pm$ 3.4 ^b	98.7 $\pm$ 0.8 ^b	100 ^b	100 ^b	100 ^b	100 ^b
	Rep	2.6 $\pm$ 0.2	2.8 $\pm$ 0.2	3 $\pm$ 0.0	2.2 $\pm$ 0.2	3 $\pm$ 0.0	3 $\pm$ 0.0
		2.5 †	5	10	20	40	60
Thymol	LM	3.3 $\pm$ 0.8 ^a	14.4 $\pm$ 1.5 ^b	100 ^c	100 ^c	100 ^c	NE
	Rep	1 $\pm$ 0.0	2 $\pm$ 0.0	2 $\pm$ 0.0	3 $\pm$ 0.0	3 $\pm$ 0.0	-
P-cymene	LM	1.34 $\pm$ 0.3 ^a	0.6 $\pm$ 0.3 ^a	3.3 $\pm$ 1.2 ^a	5.8 $\pm$ 1.3 ^{ab}	6.2 $\pm$ 1.1 ^{ab}	86.9 $\pm$ 3.6 ^b
	Rep	1.4 $\pm$ 0.2	2.2 $\pm$ 0.2	2 $\pm$ 0.0	2.2 $\pm$ 0.2	NE	NE
* The control value for larval mortality was 0.85 $\pm$ 0.4 . All data in each group compared to the control group. Note that different letters in each row shows the significance of difference (p < 0.05).							
† The values are represented as mg/ml for thymol and μ l/ml for p-cymene.							

Table 2

lethal concentrations of *T. vulgaris* oils and its major constituents against *R. sanguineus* larva.

substance	LC50 * (95% CI)	LC90 * (95% CI)
T. vulgaris EO	0.69 (0.63–0.74)	0.98 (0.91–1.1)
T. vulgaris nano EO	0.09 (0.05–0.13)	0.27 (0.22–0.36)
Thymol	6.4 (5.5–7.6)	8.8 (7.6–11.02)
P-cymene	50.5 (45.8–56.5)	75.33 (67.5–86.8)
* Calculated as (%) for EO and nano EO; and mg/ml for Thymol and p-cymene.		

The grades corresponding to the repellency effect (RE) was approximately similar to that obtained in mortality test (Table 1). Among oils used, the nano EO represented a strong RE at all concentrations evaluated. Complete RE was seen at 0.5, 2 (Fig. 1) concentrations of the nano EO. In contrast, complete or near complete RE was only achieved for Thymol and *T. vulgaris* at their highest concentrations (4%

and 20 mg/ml, respectively). P-cymene oil did not show complete RE but dispersed larva further to the control part at higher concentrations (5, 10 and 20 mg/ml).

Effects on the female reproduction

T. vulgaris, in the forms of EO and nano EO, caused no significant effect on the egg production at all concentrations ($P > 0.05$) (Table 3). In the nano group, the average oviposition estimates reduced for 27.6% and 19.1% at the concentration of 2% and 4%, respectively; but the collected data were not overall different when compared to the control value. Among materials examined, thymol had a statistically negative effect on egg laying. This occurred at only higher concentrations of 20 and 40 mg/ml ($p < 0.05$). The respective OR were 38.8 and 44.9 percent. Compared to the controls, ticks treated with p-cymene had a normal egg production rate even at high level dilutions. In addition, cypermethrin inhibited oviposition efficiently at 3.75 mg/ml as no egg mass was seen in this group; however, in other 2 concentrations (7.5 and 15 mg/ml) the egg production was completely suppressed in 50 (3/6) and 66.7% (4/6) of female ticks, respectively (Table 3).

Table 3

The effects of *T. vulgaris* oils and its major constituents on adult females of *R. sanguineus*. The egg reproductive index (RI) and percent reduction of Oviposition (OR) values are represented as Mean $\pm$ SEM.

Concentrations						
Substance	index	0.5%	1%	1.5%	2%	4%
<i>T. vulgaris</i> EO	RI *	49.08 $\pm$ 2.5 ^a	45.4 $\pm$ 3.2 ^a	54.1 $\pm$ 1.3 ^a	53.1 $\pm$ 1.9 ^a	45.4 $\pm$ 6.9 ^a
	OR	9.6	16.4	0.36	2.2	16.4
<i>T. vulgaris</i> Nano	RI	44.5 $\pm$ 1.5 ^a	46.0 $\pm$ 7.3 ^a	NE	39.3 $\pm$ 5.9 ^a	43.9 $\pm$ 9.3 ^a
	OR	18.0	15.3	—	27.6	19.1
		2.5 mg/ml	5 mg/ml	10 mg/ml	20 mg/ml	40 mg/ml
Thymol	RI	51.1 $\pm$ 2.9 ^a	48.4 $\pm$ 2.1 ^a	44.5 $\pm$ 2.7 ^a	33.2 $\pm$ 2.9 ^b	29.9 $\pm$ 5.1 ^b
	OR	5.8	10.8	18.0	38.8	44.9
P-cymene	RI	45.4 $\pm$ 2.88 ^a	43.7 $\pm$ 3.9 ^a	47.9 $\pm$ 1.3 ^a	42.1 $\pm$ 3.3 ^a	43.6 $\pm$ 3.7 ^a
	OR	16.4	19.5	11.7	22.4	19.7
		3.75 mg/ml	7.5 mg/ml	15mg/ml		
Cypermethrin	RI	0.00 ^c	18.3 $\pm$ 10.1 ^{ab}	24.6 $\pm$ 15.7 ^{ab}		
(positive control)	OR	100 (0/6) [†]	66.3 (3/6)	54.7 (2/6)		
* The control value for RI was 54.3 $\pm$ 3.2 . All data in each group compared to the control value. Note that different letters in each row shows the significance of difference (p < 0.05).						
[†] In each cypermethrin group, this sign indicates to "the number of ticks produced eggs /all examined engorged females".						

Discussion

In this study, microtubes were applied to evaluate the activity of different materials on larva. Those tubes were used to prepare a place more closely to the environmental condition. From a long duration of time, the paper pocket test has been commonly employed in many of such experiments. This method was proposed by Stone and Haydock (1962)[21] and subjected to some modifications [22]. In the pocket bioassay, larvae are captured in a small amount of space with direct contact to test material for a long time with limited air flow. This condition would not occur naturally on the ground that tick larva are often

accommodated. In our method, larvae are placed in a space with possibility of movement and direct access to air and moisture exchange through pores on the tubes wall. Due to frequent displacements, the most critical or difficult stage of such assays is counting of living and dead larva. According to our personal experience, counting, particularly of dead larva, was easier in the microtubes compared to pocket method. One reason is that larva cannot escape when the microtube lid is closed but they run away quickly after opening of the pockets.

In order to establish a direct contact with test solutions, the immersion method have also suggested [20, 23]. Klafke et al. (2006)[24] performed immersion of larva in 1.5 mL microcentrifuge tubes filled with 1 mL of each solution and then subjected to pocket test. This assay was also modified to the syringe test [25] that is a good simulation of dipping bath for ticks. Although those method seems more efficient to assay larval mortality but some larvae may be inactivated during shaking the container, tube or syringe, or discarding of the solution. In addition, larval access to air flow is limited. In this study we made attempt to resolve this problem by using microtubes. However, it should be stated that our method is suitable for examination of plant EOs with volatile molecules and not feasible for other type of chemicals like methrin derivatives.

In the past two decades, different extractions of medicinal plants have investigated to fight against ticks. Among available herbs, we selected *Thymus vulgaris* (Tv) mainly on the basis of documents on high thymol content in its oil extract [7, 17]. Thymol is known for acaricide action [26, 27] and evidenced to have cytotoxic effects [28]. Here, our results also showed that thymol was the only substance leading to significant results in all bioassays on both larva and adult ticks.

In our study, the EO of Tv found to be potent against *R. sanguineus* larvae. This effect was accompanied with 90% mortality (LC90) at the concentration of 0.98%. The EO repellency grade was also remarkable at 4% concentration. Investigations on Tv oil on ticks is relatively rare but the results are in line with the outcome of our study. In a recent study, Valcárcel et al. (2021)[9] observed the strong larvicidal effects of *T. vulgaris* and *T. zygis* EO against *Hyalomma lusitanicum* larvae with respective 49 and 74% thymol content. Among insects, promising adulticidal activity was also evidenced on *Aedes aegypti* using EO of Tv with thymol estimation of 40% [7]. In many of investigations on the use of of Tv and thymol enriched plants against ticks and insects, the possible role of thymol has often discussed. For example, *Lippia sidoides* with 67.6% thymol revealed 100% mortality on larvae of *Dermacentor nitens* and *Rhipicephalus microplus* at the concentration of 20 µl/ml [29]. Thymol concentration of 1% (10 mg/ml) caused 100% death rate on unengorged larvae of *Ixodes Ricinus*. Thymol also evidenced a significant repellent activity at the concentration of 0.5% [30].

In this study, we observed high mortalities in larva exposed to EO nanoemulsion. In addition, nano material revealed complete repellency at the concentration of 0.5% while no other materials caused total or near complete larval escape at low concentrations. Therefore, nano formulations can be recommended to be considered for the control of *R. sanguineus* larva. This suggestion is according to our results, low cost of preparation and possibility of industrial production. In recent years, scientific data

revealed and emphasized on the use of nanoemulsions as suitable carriers. In addition, production of nano EO provides several advantages including higher thermodynamic stability [31].

Opposed to our expects, the effects of *Tv*EO and the nano EO on adult tick oviposition was not in agreement with that occurred on larva. The egg production of females was only reduced for 16.4 and 27.6%. This value increased to 44.9% by using thymol at 40 mg/ml and > 50% by the commercial cypermethrin. This contradiction needs more investigation, but there is one possibility that the above substances could show their potentiation on the adult ticks if employed with higher concentrations. Also, some previous works showed that adult ticks are significantly more resistant than larva [32, 33]. In line with our results, nanostructured forms of *Eucalyptus globulus* was not effective on the reproduction of *Rhipicephalus microplus* [34]. Similar data reported after using Tea tree and lemon nano oils on adult female of *R. annulatus* [35] and nano EO of *Cinnamomum verum* on *R. microplus* [36]. In contrast, geranium oil nanoemulsion displayed greater impact on *R. annulatus*, compared to the pure form [37]. The nano formulation enhances the contact surface with droplets that contains the active agent and many of features that offers some advantages to control ticks and insects (reviewed in [38]). These contradictory data may imply that the nano EOs are more applicable against larval stage. On the other side, the combination of acaricide compounds is a way to increase the ovicidal and repellent activity [39]. Therefore, it can be suggested to investigate a hypothesis that the *Tv*EO and nano EO may be effective on the egg production of adult ticks when used with higher concentrations or combined with other compounds like thymol.

Conclusion

In this study, we used microtubes for mortality bioassay on *R. sanguineus* larva. Perforated tube wall with free air exchange and preparation of a space for displacement of larva can close the *in vitro* condition to the real environment. In this work, the nanomaterial made from *T. vulgaris* EO revealed a high significant mortality and repellency on larva population at lower concentrations. In contrast, treatment of adult female ticks with the nano EO did not accompanied with significant reductions we expected before. This result may be enhanced using the higher concentrations of the nano substance. On the other side, Thymol was effective against larva and also influenced the productivity of engorged female ticks. Together, our data showed the potency of *T. vulgaris* nano EO and thymol for the control of the brown dog tick.

Abbreviations

EO: essential oil

***Tv*:** *Thymus vulgaris*

GC-MS: gas chromatography–mass spectrometry

RI: egg reproductive index

RO: percent reduction of oviposition

RE: Repellant effect

Declarations

Acknowledgements

The authors acknowledge the help of technicians of the departments of Veterinary Parasitology and Horticultural Science at Shiraz University. Also we should thank Fars Agricultural and Natural Resources Research and Education Center (FANREC) for essential oil analysis.

Author contributions

ZA performed conceptualization, tick sample collection and project administration. ER was involved in tick bioassays, data analysis and drafting the manuscript. MJS helped for the plant preparation and taxonomic identification, oil and nano material extraction. AMA contributed to tick bioassays and setting up methods for repellant and mortality tests. All the authors have reviewed and confirmed the present manuscript.

Funding

This research was supported by a grant from Shiraz University (Grant No: ZA1401). The assigned funding was used for material preparations.

Data Availability

Details of data generated and/or analyzed during the current study are available on request from the corresponding author.

Ethics approval and consent to participate

All experiments were performed in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines. This study has been approved by the Ethics Committee of the Faculty of Veterinary Medicine, Shiraz University, Iran. Informed permission/consent was obtained from dog owners before collection of ticks.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Conflict of Interest

The authors declare no competing of interest.

References

1. Dantas-Torres F. Biology and ecology of the brown dog tick, *Rhipicephalus sanguineus*. Parasit Vectors. 2010;3:26.
2. Li AY, Chen AC, Davey RB, Miller RJ, George JE. Acaricide resistance and synergism between permethrin and amitraz against susceptible and resistant strains of *Boophilus microplus* (Acari: Ixodidae). Pest Manag Sci. 2007;63:882-889.
3. Becker S, Webster A, Doyle RL, Martins JR, Reck J, Klafke GM. Resistance to deltamethrin, fipronil and ivermectin in the brown dog tick, *Rhipicephalus sanguineus sensu stricto*, Latreille (Acari: Ixodidae). Ticks Tick borne dis. 2019;10(5):1046-1050.
4. Karabörklü S, Ayvaz A. A comprehensive review of effective essential oil components in stored-product pest management. J Plant Dis Protect. 2023;130:449-481.
5. de Oliveira Monteiro CM, Daemon E, Silva AMR, Maturano R, Amaral C. Acaricide and ovicide activities of thymol on engorged females and eggs of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). Parasitol Res. 2010;106:615-619.
6. Pavela R. History, presence and perspective of using plant extracts as commercial botanical insecticides and farm products for protection against insects - a review. Plant Protect Sci. 2016;52:229-241.
7. de Oliveira AA, França LP, Ramos ADS, Ferreira JLP, Maria ACB, Oliveira KM, Araújo Jr ES, da Silva JN, Branches AD, Barros GDA, da Silva NG. Larvicidal, adulticidal and repellent activities against *Aedes aegypti* L. of two commonly used spices, *Origanum vulgare* L. and *Thymus vulgaris* L. S Afr J Bot. 2021;140:17-24.
8. Štefanidesová K, Škultéty L, Sparagano OA, Špitalská E. The repellent efficacy of eleven essential oils against adult *Dermacentor reticulatus* ticks. Ticks Tick borne dis. 2017;8(5):780-786.
9. Valcárcel F, Olmeda AS, González MG, Andrés MF, Navarro-Rocha J, González-Coloma A. Acaricidal and insect antifeedant effects of essential oils from selected aromatic plants and their main components. Front Agron. 2021;3:662802.
10. Pavela R. Lethal and sublethal effects of thyme oil (*Thymus vulgaris* L.) on the house fly (*Musca domestica* Lin.). J Essent Oil-Bear Plants. 2007;10(5):346-356.
11. Wu L, Huo X, Zhou X, Zhao D, He W, Liu S, Liu H, Feng T, Wang C. Acaricidal activity and synergistic effect of thyme oil constituents against carmine spider mite (*Tetranychus Cinnabarinus* (Boisduval)). Molecules. 2017;22(11):1873.
12. Banumathi B, Vaseeharan B, Rajasekar P, Prabhu NM, Ramasamy P, Murugan K, Canale A, Benelli G. Exploitation of chemical, herbal and nanoformulated acaricides to control the cattle tick, *Rhipicephalus (Boophilus) microplus*—A review. Vet Parasitol. 2017;244:102-110.

13. Ferreira TP, Haddi K, FT Corrêa R, Zapata VL, Piau TB, Souza LF, Santos SMG, Oliveira EE, Jumbo LO, Ribeiro BM, Grisolia, CK. Prolonged mosquitocidal activity of *Siparuna guianensis* essential oil encapsulated in chitosan nanoparticles. PLoS Neglect Trop Dis. 2019;13(8), :e0007624.
14. Gupta P, Preet S, Ananya, Singh N. Preparation of *Thymus vulgaris* (L.) essential oil nanoemulsion and its chitosan encapsulation for controlling mosquito vectors. Sci Rep. 2022;12(1):4335.
15. European Pharmacopoeia. Part 1. Editors: Maisonneuve SA, Sainte Ruffine. 1983. p.V.4.5.8.
16. Golmakani MT, Rezaei K. Comparison of microwave-assisted hydrodistillation with the traditional hydrodistillation method in the extraction of essential oils from *Thymus vulgaris* L. Food Chem. 2008;109:925-930.
17. Ramezani A, Azadi M, Mostowfizadeh-Ghalefarsa R, Saharkhiz MJ. Effect of *Zataria multiflora* Boiss and *Thymus vulgaris* L. essential oils on black rot of 'Washington Navel'orange fruit. Postharvest Biol Technol. 2016;112:152-158.
18. Hashtjin AM, Abbasi S. Optimization of ultrasonic emulsification conditions for the production of orange peel essential oil nanoemulsions. J Food Sci Technol. 2015;52(5);2679-89.
19. Walker JB, Keirans JE, Horak IG. The genus *Rhipicephalus* (Acari, Ixodidae): a guide to the brown ticks of the world. Cambridge: Cambridge University Press. 2000. pp, 643.
20. Drummond RO, Ernest SE, Trevino JL, Gladney WJ, Graham OH. *Boophilus anulatus* and *Boophilus microplus*: laboratory test of insecticides. J Econ Entomol. 1973;66:130-133.
21. Stone BF, Haydock KP. A method for measuring the acaricide-susceptibility of the cattle tick *Boophilus microplus* (Can.). Bull Entom Res. 1962;53(3):563-578.
22. de Monteiro CM, Maturano R, Daemon E, Catunda-Junior FEA, Calmon F, de Souza Senra T, Faza A, Geraldo de Carvalho M. Acaricidal activity of eugenol on *Rhipicephalus microplus* (Acari: Ixodidae) and *Dermacentor nitens* (Acari: Ixodidae) larvae. Parasitol Res. 2012;111:1295-1300.
23. Shaw RD. Culture of an organophosphorus-resistant strain of *Boophilus Microplus* (Can.) and an assessment of its resistance spectrum. Bull Entomol Res. 1966;56:389.
24. Klafke GM, Sabatini GA, Albuquerque TA, Martins JR, Kemp DH, Miller RJ, Schumaker TTS. Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from state of São Paulo, Brazil. Vet Parasitol. 2006;142:386-390.
25. Sindhu ZD, Jonsson NN, Iqbal Z. Syringe test (modified larval immersion test): a new bioassay for testing acaricidal activity of plant extracts against *Rhipicephalus microplus*. Vet Parasitol. 2012;188(3):362-367.
26. Novelino AMS, Daemon E, Soares GLG. Evaluation of acaricide effect of thymol, menthol, salicylic acid and methyl salicylate on *Boophilus microplus* (Canestrini, 1887) (Acari:Ixodidae) larvae. Parasitol Res. 2007;101:809-811.
27. Daemon E, de Oliveira Monteiro CM, dos Santos Rosa L, Aparecido Clemente M, Arcoverde A. Evaluation of the acaricide activity of thymol on engorged and unengorged larvae of *Rhipicephalus sanguineus* (Latreille, 1808)(Acari: Ixodidae). Parasitol Res. 2009;105:495-497.

28. Matos RS, Delmonte C, Marchesini P, Monteiro CMO, Sampieri B, Daemon E, Camargo-Mathias ML. Thymol action on cells and tissues of the synganglia and salivary glands of *Rhipicephalus sanguineus* sensu lato females (Acari: Ixodidae). Ticks Tick Borne Dis. 2019;10:314-320.
29. Gomes GA, Monteiro CMD, Senra TDOS, Zeringota V, Calmon F, Matos R.D.S., Daemon E, Gois RWDS, Santiago GMP, de Carvalho MG. Chemical composition and acaricidal activity of essential oil from *Lippia sidoides* on larvae of *Dermacentor nitens* (Acari: Ixodidae) and larvae and engorged females of *Rhipicephalus microplus* (Acari: Ixodidae). Parasitol Res. 2012;111: 2423-2430.
30. Tabari MA, Youssefi MR, Maggi F, Benelli G. Toxic and repellent activity of selected monoterpenoids (thymol, carvacrol and linalool) against the castor bean tick, *Ixodes ricinus* (Acari: Ixodidae). Vet Parasitol. 2017;245:86-91.
31. De Campos VEB, Ricci-Júnior E, Mansur CRE. Nanoemulsions as delivery systems for lipophilic drugs. J Nanosci Nanotechnol. 2012;12(3):2881-2890.
32. Ribeiro VLS, Avancini C, Gonçalves K, Toigo E, Von Poser GL. Investigation of the acaricidal activity of *Calea serrata* (Asteraceae) on *Boophilus microplus* and *Rhipicephalus sanguineus*. Vet Parasitol. 2008;151:351-354.
33. de Oliveira Monteiro CM, Daemon E, Aparecido Clemente M, dos Santos Rosa L, Maturano R. Acaricidal efficacy of thymol on engorged nymphs and females of *Rhipicephalus sanguineus* (Latreille, 1808)(Acari: Ixodidae). Parasitol Res. 2009;105:1093-1097.
34. Galli GM, Volpato V, Santos RCV, Gebert RR, Quatrin P, Ourique AF, Klein B, Wagner R, Tonin AA, Baldissera MD. Effects of essential oil of *Eucalyptus globulus* loaded in nanoemulsions and in nanocapsules on reproduction of cattle tick (*Rhipicephalus microplus*). Arch Zootec. 2018; 67:494-498.
35. Ibrahim SM, Wahba AA, Farghali AA, Abdel-Baki AAS, Mohamed SA, Al-Quraishy S, Hassan AO, Aboelhadid SM. Acaricidal Activity of Tea Tree and Lemon Oil Nanoemulsions against *Rhipicephalus annulatus*. Pathogens. 2022;11(12):1506.
36. Monteiro IN, dos Santos Monteiro O, Costa-Junior LM, da Silva Lima A, de Aguiar Andrade EH, Maia JGS, Mouchrek Filho VE. Chemical composition and acaricide activity of an essential oil from a rare chemotype of *Cinnamomum verum* Presl on *Rhipicephalus microplus* (Acari: Ixodidae). Vet Parasitol. 2017;238:54-57.
37. Ibrahim SM, Aboelhadid SM, Wahba AA, Farghali AA, Miller RJ, Abdel-Baki AA., Al-Quraishy S. Preparation of geranium oil formulations effective for control of phenotypic resistant cattle tick *Rhipicephalus annulatus*. Sci Rep. 2022;12:11693.
38. Echeverría J, Duarte Galhardo de Albuquerque RD. Nanoemulsions of essential oils: new tool for control of vector-borne diseases and in vitro effects on some parasitic agents. Medicines. 2019;6(2):42.
39. Arafa WM, Aboelhadid SM Moawad A, Shokeir KM, Ahmed O. Toxicity, repellency and anti-cholinesterase activities of thymol-eucalyptus combinations against phenotypically resistant *Rhipicephalus annulatus* ticks. Exp Appl Acarol. 2020;81:265-277.

Figures

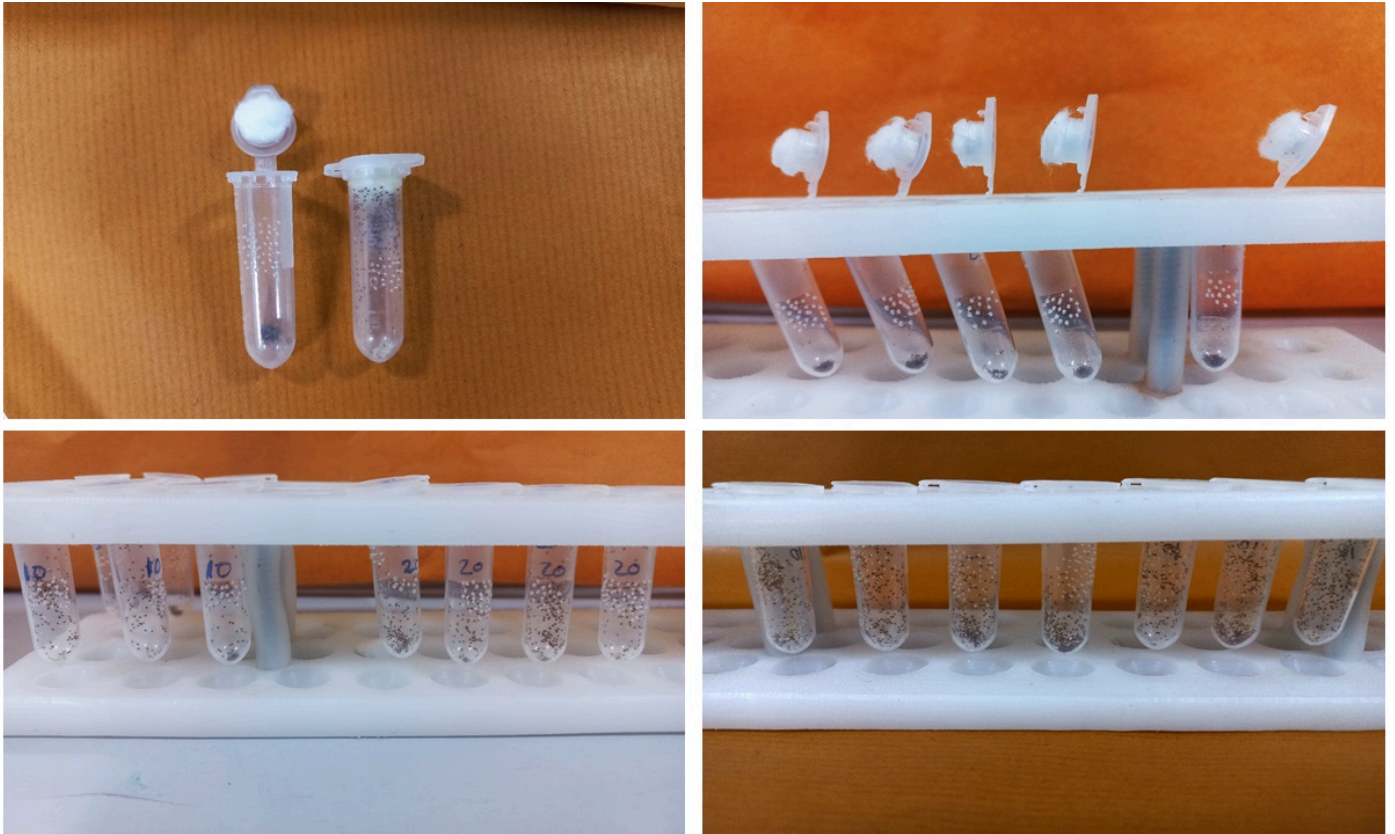


Figure 1

Larval mortality test in 2 ml microtubes. Tubes with opened lids corresponds to those materials with complete mortality effect.

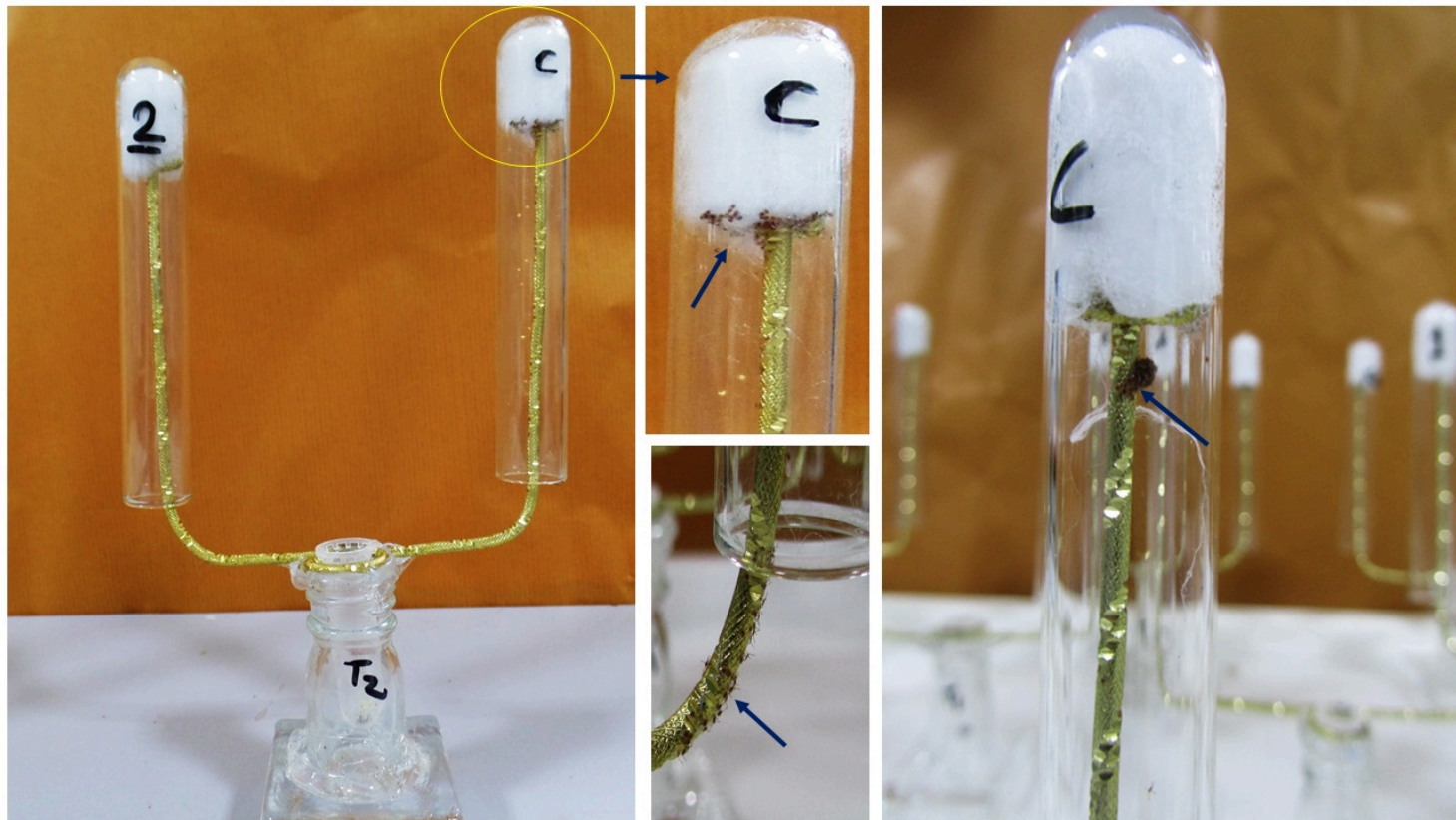


Figure 2

Larval repellency assay. The arrows indicate to larva aggregation or escape to the control side when exposed to 2% *T. vulgaris* nano emulsion.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.docx](#)