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

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Abstract

Background The brown dog tick is globally distributed and harms the host in terms of blood feeding and pathogen transfer. Chemicals are traditionally used for the control, but herbal plants have been investigated mainly due to their natural components with killing and repellant effects. Previously, the role of thymol has been described for the bio-control of ticks at different stages. Therefore, a study was conducted 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 performing 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 the great activity of the nano EO and thymol on live larva. The nano form caused 98.7% larval mortality at a low concentration of 0.25%. This effect reached 100% at 0.5% concentration, while the promising results for the EO was observed at 1 and 2% concentration showing 95.3 and 100% mortality, respectively. The nanoemulsion 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 by a statistically significant reduction in female oviposition rate ($p < 0.05$), however at its utmost concentrations of 20 and 40 mg/mL.

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

Keywords *Thymus vulgaris*, Nano essential oil, Larvicidal activity, Repellent effect, Tick control

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]. *R. sanguineus* also considered to act as a vector of prevalent

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pathogens of the genus *Ehrlichia*, *Babesia*, *Rickettsia*, *Coxiella* and etc. [2]. Therefore, using effective chemical acaricides has been the conventional way against different developmental stages. Despite their significant effects, synthetic chemicals may cause intoxication and also the selection of genotypic resistant ticks [3, 4]. Also, the number of commercially available tick repellents is limited. For these reasons, the essential oils (EO) of natural plants and their purified compounds have been studied as safe and attractive alternatives for pest management programs [5].

According to the literature, levels of acaricide activity were demonstrated for the botanical EOs. Among many of the well-studied medicinal plants, *Thymus vulgaris* has been known for its therapeutic features and health benefits [6]. Different species of this plant is indigenous in parts of Europe, Africa, and Asia with a wide range of medicinal aims. According to qualitative and quantitative variations in the volatile compounds found in the plant and the growth condition, a number of chemotypes have been described for *T. vulgaris* [7]. The high level of terpenoids and phenolic derivatives detected in the extracted EO composition in all species are likely to be associated with its biological properties [8].

The *T. vulgaris* EO have demonstrated to enhance the function of pesticides [9]. This extract also has potential for inactivation and repellent effects against tick species of *Dermacentor reticulatus* [10] and *Hyalomma lusitanicum* [11], as well as some insects such as *Aedes aegypti* [12], housefly [13] and mites [14]. Nevertheless, the advantages of using this product have not properly investigated for the control of the prevalent ticks worldwide.

In recent years, nanocompounds have become increasingly used to enhance the activity of herbal products. Different compounds have been made by various methods in nanotechnology, all aiming to achieve high inhibitory and repellent functions [15]. A variety of nanodelivery systems have been developed to entrap the EO materials. The result has to improve the efficacy and enhance the functionality of the bioactive compound [16]. The promising effectiveness of metallic nanoparticles synthesized from several leaf extracts against *Rhipicephalus microplus* was reviewed by Banumathi et al. (2017) [17]. In addition, nanoemulsions made from herbal EOs are considered to be accompanied by prolonged mosquitocidal activity [18], increased insecticidal potential and significant alterations in morphology of the larvae [19]. The available knowledge on the advantage of nanotechnology to strength the plant-derived EOs against prevalent ticks is still rare and needs to be more expanded. Therefore, in the current study, we evaluated and compared the effects of EO and nano EO of *T. vulgaris* on larva and engorged females of *R. sanguineus*.

Materials and methods

Collection of the plant material

The aerial parts of Garden thyme (*Thymus vulgaris* L.) were collected at the Flowering stage on the 22nd of October 2021, from a field located in Shahrak-e Abraj Region (Marvdasht, Iran) at an altitude of 1643 m above sea level, and was geographically coordinated at 30°10′34" north and 52°33′15" east. It was identified and authenticated by Prof. Ahmad R. Khosravi, a plant taxonomist at Shiraz University, Shiraz, Iran. The voucher specimen (No: 55146) was deposited in the herbarium. All of the harvested materials were air-dried at room temperature (at less than 25 °C) in the shade for 14 days.

Extraction of the EO

The shade-dried aerial parts of Garden thyme were subjected to water distillation (hydro distillation) for 3 h using an all-glass Clevenger-type apparatus to extract EO according to the method outlined by the European Pharmacopoeia [20]. The extracted oil was then dried over anhydrous sodium sulphate and stored in sealed vials at 4 °C before acaricide assessments, gas chromatography (GC) and gas chromatography- mass spectrometric (GC–MS) analyses.

EO analysis procedure

The components of the volatile oil from the plant aerial parts were identified using GC and GC–MS analyses. The GC analysis was performed using an Agilent gas chromatograph series 7890-A (Agilent, USA) equipped with a flame ionization detector (FID). The analysis was carried out on fused silica capillary HP-5 column (30 m × 0.32 mm i.d.; film thickness 0.25 µm). The sample volume injected into the GC was 0.2 µl pure EO. The temperature of injector and detector was set at 250 °C and 280 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 1 mL/min; the oven temperature program was 60–210 °C at the rate of 4 °C/min, which was then programmed to 240 °C at a rate of 20 °C/min, and finally, the temperature was held isothermally for 8.5 min. The split ratio was 1:50. The GC–MS analysis was carried out by the use of an Agilent gas chromatograph equipped with a fused silica capillary HP-5MS column (30 m × 0.25 mm i.d.; film thickness 0.25 µm) coupled with 5975-C mass spectrometer. The sample volume injected into the capillary column was 0.1 µl pure EO in the split mode (1:50). Helium was used as carrier gas with the ionization voltage of 70 eV. The temperature of ion source and interface was 230 °C and 280 °C, respectively. Mass range was from 45 to 550amu. The oven temperature program was the same as for the GC. The retention indices for all components were determined according to the method by which n-alkanes are used as standard. (The n-alkanes

in the present study were in the range of C8-C28 (C8, C9, C10, C11, C12, C13…C28). In gas chromatography for calculating Kovats retention index, the normal hydrocarbons (n-alkanes) are used.

Identification of the EO components

The compounds were identified by comparing their retention indices (RI, HP-5) with those reported in the literature and also by comparing their mass spectra with the Wiley GC–MS Library, Adams Library and Mass Finder 2.1 Library data, as well as comparisons with the published mass spectra data [21].

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) [22]. 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+Tween 80 as 2% tween 80 (v/v))/EO ratio was 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.

Determination of nanoemulsion droplet size

The Emulsion droplet size and polydispersity index (PDI) were measured by dynamic light scattering (DLS) using NANO-flex® 180° (USA). To prevent the effect of multiple scattering, samples were diluted to 10% with deionized water prior to size determination. The emulsion droplet size was calculated using the average of three measurements [23, 24]. In this study, the average of NE droplet size was 130.6±2.5 nm at the time of application.

Preparation of test concentrations

The extracted pure EO was dissolved in 2% tween 80 (v/v) (in distilled water) to obtain final concentrations: 0.125, 0.25, 0.5, 1, 2 and 4% (v/v). The concentrations were selected based on investigations about the effects of herbal EOs on *Rhipicephalus sanguineus* [25] and our preliminary study.

According to our results and also previous reports [26], 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; CAS No. 89-83−8) and p-cymene (CAS No. 99-87−6) 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). 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 engorged female ticks were collected from naturally infected dogs referred to the Veterinary Clinic of Pet Animals, Shiraz University, Iran. Tick collection was performed on animals with no recent contact with acaricide products and were done after taking the consent of their owners. The animal ethics and all protocols for sampling were approved by the animal welfare and ethics committee in Faculty of Veterinary Medicine, Shiraz University, Iran (letter No. 14013/46/6).

The Ticks were cleaned with a soft paintbrush and identified according to the taxonomic keys given by Walker et al. (2000) [27]. 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 (Memmert®, Germany) at a temperature of 27°C and relative humidity of 80% for oviposition. After about 10 days, eggs were weighed into approximately 10 mg aliquots which were placed in 2 mL microtubes and kept in the incubator for hatching. The microtube 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 the scent of any test substance. The experiments were started when the hatched Larvae aged 7–15 days.

Larva bioassays

The effectiveness of all prepared test solutions was evaluated on tick larva using bioassays that are described as follow:

Larvicidal activity in microtubes

In this study, we developed a method that applies larvae containing microtubes (Fig. 1) instead of routine paper pockets explained first by Stone and Haydock (1962) [28] and the immersion technique [29]. At the time of the experiment, the tube cap was opened, filled with a cotton plug and moistened immediately with 250 µL of the prepared solutions. Then the cap was closed and the tubes were incubated for about 24 h at 27°C and relative humidity of 80%. After this time, movements of larvae in microtubes were evaluated using a stereomicroscope. Those larva with no motility, or incapable of maintaining normal posture were assigned as dead. The total larva count is difficult because live ticks change their position and run quickly. Therefore, the larva mass was

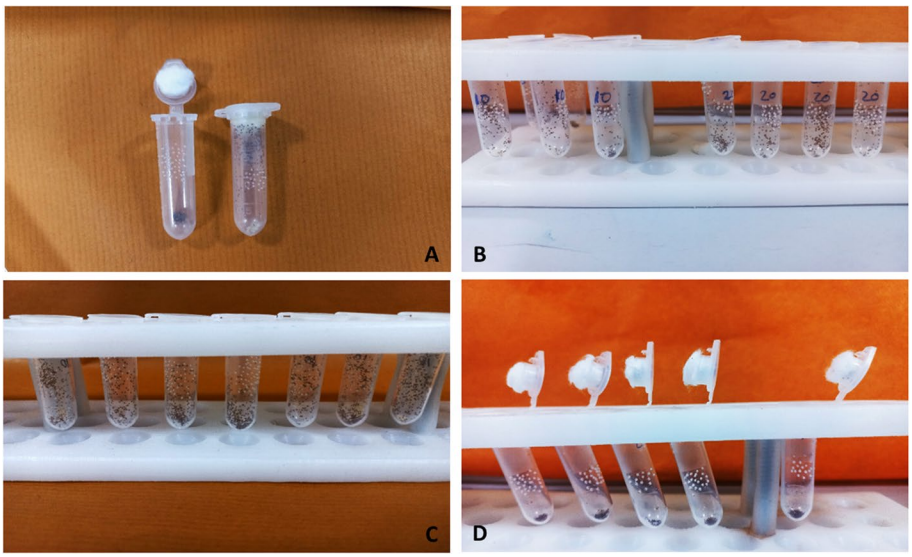

Fig. 1 Larval mortality test in 2 mL microtubes. **A** Test tubes prior to hatching (left) and the control test with 2% tween 80 (right); **B** and **C** Test with different concentrations of test solutions with no significant lethal activity; **D** Complete mortality effect that is indicated by all larva dead

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%.

The negative control was established with Tween 80 (2%) and each treatment was repeated 6 times.

Repellent activity on larva

The repellency bioassay was done on the basis of the 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 was assigned to each test odor and the other used for its control. The lower side (horizontal part) was fixed at its center to the upper opening of 10 cc laboratory glass bottle standing itself on a square watch glass (3cm × 3cm × 1cm) at its base. 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

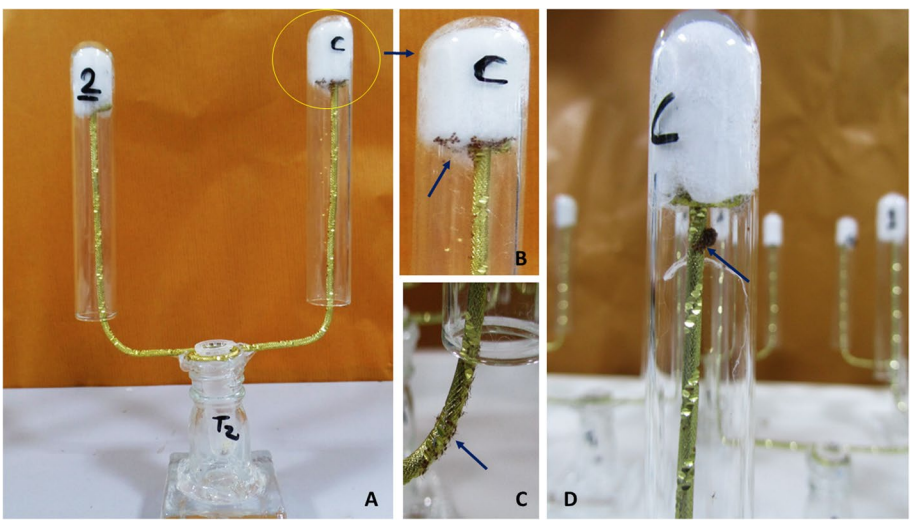

Fig. 2 Larval repellency assay (**A**). The arrows indicate to larva aggregation (**B**, **D**) or escape (**C**) to the control side when exposed to 2% *T. vulgaris* nano emulsion

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 the 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 larva was placed in the glass bottle and the cap was cut to allow larva 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 were attracted to the control and treatment parts, respectively. Grade 2 was defined for groups showing more than half (50 %) of larva were attracted to the control rod. Grade 1 was referred to groups if > 50% of larva ran toward the treatment part. Grades 1 and 2 imply to dispersal of larva between control and treatment groups.

Tween 80 (2%) was used as the negative control and each treatment was replicated 6 times.

Adult immersion test

Tests on the adult female ticks were done by a method previously described by Drummond et al. (1973) [29], 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 towels. Then they were transferred, individually, to a type of glass bottle 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.

All the prepared test solutions were used and the effects of any substances on female reproduction were calculated using formulas as follows:

$$\text{Egg Reproductive index (RI)} = (\text{Egg mass weight} / \text{weight of tick before oviposition}) \times 100$$

$$\text{Percent Reduction of Oviposition (OR)} = \left(\text{mean RI in control group} - \text{mean RI in treated group} / \text{mean RI in control group}\right) \times 100.$$

As a positive control, a commercial synthetic cypermethrin was purchased containing 20% active ingredient (Saman®, Iran). Three concentrations were made in distilled water to obtain 3.75, 7.5 (the recommended dose), and 15 mg(powder)/mL dilutions. Ticks in the negative control group were examined by Tween 80 (2%).

Statistical analysis

Data obtained from larva and adult tick bioassays were measured as percentage and presented as mean±SEM. The statistical differences between treatment and control values in each group were done using ANOVA followed by Tukey test ($p<0.05$ and $p<0.01$). 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. The analyses were done using the SPSS software (IBM Inc., Armonk, NY), version 27. In addition, in order to have an estimation on the overall comparison between the effect of different substances on larva and adult ticks, the values were analyzed with GraphPad Prism® version 8 (GraphPad software Inc., La Jolla, CA, USA) using Dunnett's and Tukey multiple comparison tests and the results were presented as column drawings.

Results

Effects of oils on the larva

A total of 55 compounds were identified in the EO of *T. vulgaris*, equivalent to 99.9% of the total oil (Additional 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 rate and repellent activities of different substances are represented in Table 1. The larvicidal activity of *T. vulgaris* EO was seen from the concentration of 0.5% ($p<0.05$). However, it was significantly effective when the concentration increased to 1%, representing of about 95% mortality. In comparison, the nano form has a remarkable killing effect (of 98.7%) at its lower concentration (0.25%) and caused complete mortality at the concentration of 0.5%. Thymol, as the major EO component, was effective on larvae with 100% mortality at the concentration of 10 mg/mL. These results were not true for p-cymene because the larva population remained alive unless its concentration was increased more to 60 µL/mL. Multiple comparisons on the overall

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 ± SEM

Substance	Concentrations						
	Effect*	**0.125%**	**0.25%**	**0.5%**	**1%**	**2%**	**4%**
***T. vulgaris* EO**	LM	1.1 ± 1.0 [a]	6.1 ± 2.3 [a]	8.4 ± 4.03 [ab+]	95.3 ± 2.1 [b++]	100 [b++]	100 [b++]
	Rep	**1.6 ± 0.2**	**1.8 ± 0.2**	**1.8 ± 0.2**	**2 ± 0.0**	**2.2 ± 0.2**	**2.8 ± 0.2**
***T. vulgaris* Nano**	LM	88.9 ± 3.4 [b++]	98.7 ± 0.8 [b++]	100 [b++]	100 [b++]	100 [b++]	100 [b++]
	Rep	**2.6 ± 0.2**	**2.8 ± 0.2**	**3 ± 0.0**	**2.2 ± 0.2**	**3 ± 0.0**	**3 ± 0.0**
		2.5 †	**5**	**10**	**20**	**40**	**60**
Thymol	LM	3.3 ± 0.8 [a]	14.4 ± 1.5 [b+]	100 [c++]	100 [c++]	100 [c++]	NE
	Rep	**1 ± 0.0**	**2 ± 0.0**	**2 ± 0.0**	**3 ± 0.0**	**3 ± 0.0**	-
P-cymene	LM	1.34 ± 0.3 [a]	0.6 ± 0.3 [a]	3.3 ± 1.2 [a]	5.8 ± 1.3 [ab+]	6.2 ± 1.1 [ab+]	86.9 ± 3.6 [b++]
	Rep	**1.4 ± 0.2**	**2.2 ± 0.2**	**2 ± 0.0**	**2.2 ± 0.2**	NE	NE

* The control value for larval mortality was 0.85 ± 0.4. All data in each group compared to the control value. Note that different letters in each row shows the significance of difference (+ refers to $p < 0.05$ and ++ point to $p < 0.01$)

† The values are represented as mg/ml for thymol and µl/ml for p-cymene

Table 2 Lethal concentrations of *T. vulgaris* oils and its major constituents against *R. sanguineus* larva

Substance	LC50[a] (95% CI)	LC90[a] (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)

[a] Calculated as (%) for EO and nano EO; and mg/ml for Thymol and p-cymene

effects of these substances are represented on Fig. 3A. The graph confirms the considerable potency of nano EO for deactivation of tick larva ($p<0.01$). The measurement of the LC50 and LC90 values (Table 2) revealed a noticeable distance between the values obtained for EO and the nano substance and also the thymol and p-cymene groups were remarkable.

The grades corresponding to the repellency effect (RE) were approximately similar to those obtained in the 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. 2) and 4% 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 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 totally different when compared to the control value. Among the chemicals examined, thymol had only a statistically negative effect on egg laying (Table 3 and Fig. 3B). This occurred at higher concentrations of 20 and 40 mg/mL (p=0.013) with the OR value of 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 the 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).

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. For a long duration of time, the paper pocket test has been commonly employed in many similar experiments. This method was proposed by Stone and Haydock (1962) [28] and subjected to some modifications [30]. 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 airflow. This condition would not occur naturally on the ground that tick larva are often accommodated. In our method, larva are placed in a space with the possibility of movement and direct access to air and moisture exchange through pores on the tube wall. Due to frequent displacements, the most critical or difficult stage

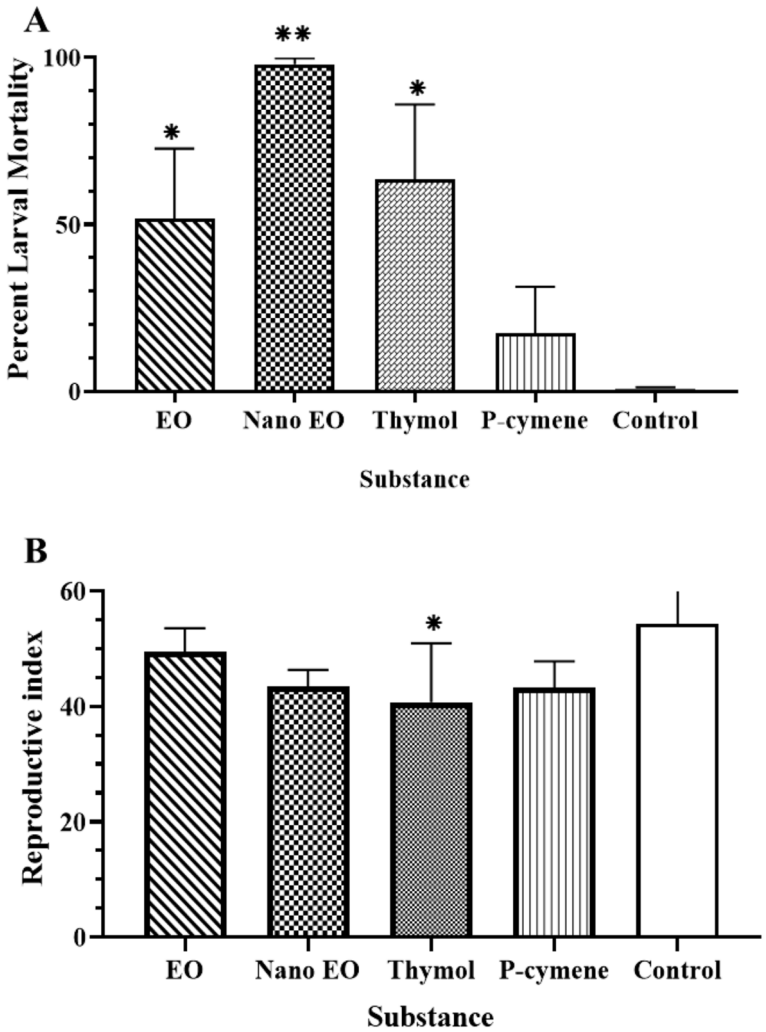

Fig. 3 Multiple comparison tests between the overall effects of different substances on the larva (**A**) and adult ticks (**B**). Figure A indicates that *T. vulgaris* nano EO had a highly significant activity on larva ($p < 0.01$; assigned by **), followed by the EO and thymol ($p < 0.05$; as showed by *). In contrast, only thymol had a notable decrease ($p < 0.05$) on the reproductive index of the adult ticks (Fig. 3B)

of such assays is the counting of living and dead larva. According to our personal experience, counting, particularly of dead larva, was easier in the microtubes compared to the 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 direct contact with test solutions, the immersion method has been suggested [29, 31]. Klafke et al. (2006) [32] 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 [33] which is a good simulation of a dipping bath for ticks. Although those methods appear more efficient to assay larval mortality, some larvae may be inactivated during shaking of the container, tube or syringe, or discarding of the solution. In addition, larval access to airflow is limited. In this study, we made an attempt to resolve this problem by using microtubes. However, our method is suitable for the examination of plant EOs with volatile molecules and not feasible for other types of chemicals like methrin derivatives. In addition, it is recommended to execute this methodology after conducting further research on its accuracy and the validity of the results.

In the past two decades, different extractions of medicinal plants have been investigated to fight against ticks. Among available herbs, we selected *T. vulgaris* mainly based on documents on high thymol content in its oil extract [26]. Thymol is known for acaricide action [34, 35]

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 ± SEM

	Concentrations					
Substance	**index**	**0.5%**	**1%**	**1.5%**	**2%**	**4%**
T. vulgaris EO	RI *	49.08 ± 2.5 [a]	45.4 ± 3.2 [a]	54.1 ± 1.3 [a]	53.1 ± 1.9 [a]	45.4 ± 6.9 [a]
	OR	**9.6**	**16.4**	**0.36**	**2.2**	**16.4**
T. vulgaris Nano	RI	44.5 ± 1.5 [a]	46.0 ± 7.3 [a]	NE	39.3 ± 5.9 [a]	43.9 ± 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 ± 2.9 [a]	48.4 ± 2.1[a]	44.5 ± 2.7 [a]	29.9 ± 4.2 [b+]	29.9 ± 5.1 [b+]
	OR	**5.8**	**10.8**	**18.0**	**44.9**	**44.9**
P-cymene	RI	45.4 ± 2.88 [a]	43.7 ± 3.9 [a]	47.9 ± 1.3 [a]	42.1 ± 3.3 [a]	43.6 ± 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 ± 10.1 [b+]	24.6 ± 15.7 [b+]		
(Positive control)	**OR**	**100 (0/6)†**	**66.3 (3/6)**	**54.7 (2/6)**		

* The control value for RI was 54.3 ± 3.2. All data in each group compared to the control value. Note that different letters in each row shows the significance of difference (refers to + $p < 0.05$ and point to ++ $p < 0.01$)

† In each cypermethrin group, this sign indicates to "the number of ticks produced eggs /all examined engorged females"

and evidenced to have cytotoxic effects [36]. 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 *T. vulgaris* found to be potent against *R. sanguineus* larva. 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 *T. vulgaris* 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) [11] observed the strong larvicidal effects of *T. vulgaris* and *T. zygis* EO against *Hyalomma lusitanicum* larva with respective 49 and 74% thymol content. Among insects, promising adulticidal activity was also evidenced on *Aedes aegypti* using EO of *T. vulgaris* with thymol estimation of 40% [12]. In many investigations on the use of *T. vulgaris* and thymol-enriched plants against ticks and insects, the possible role of thymol has often been 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 [37]. 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% [38].

In this study, we observed high mortalities in larva exposed to EO nanoemulsion. In addition, nanomaterial revealed complete repellency at the concentration of 0.5% while no other materials caused total or near complete larval escape at low concentrations. Therefore, nanoformulations 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 the use of nanoemulsions as suitable carriers. In addition, the production of nano EO provides several advantages including higher thermodynamic stability [39].

Opposed to our expectations, the effects of *T. vulgaris* EO and the nano EO on adult tick oviposition were not in agreement with that occurred on larva. The egg production of females was finally reduced by 16.4 and 27.6%. This value increased to 44.9% by using thymol at 20 and 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 [40, 41]. In line with our results, nanostructured forms of *Eucalyptus globulus* were not effective on the reproduction of *Rhipicephalus microplus* [42]. Similar data was reported after using Tea tree and lemon nano oils on adult females of *R. annulatus* [43] and nano EO of *Cinnamomum verum* on *R. microplus* [44]. In contrast, geranium oil nanoemulsion displayed a greater impact on *R. annulatus*, compared to the pure form [45]. The nanoformulation enhances the contact surface with droplets that

contain the active agent and many features that offers some advantages to control ticks and insects (reviewed in [46]). 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 repellant activity [47]. Therefore, it can be suggested to investigate a hypothesis that the *T. vulgaris* 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. A Perforated tube wall with free air exchange and preparation of space for the 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 was 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
GC–MS	Gas chromatography–mass spectrometry
RI	Egg reproductive index
RO	Percent reduction of oviposition
RE	Repellant effect

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04609-y.

Additional file 1. Details of chemical composition of the essential oil extracted from Thymus vulgaris including the Retention index (RI) and percents of each composition.

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Authors' 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.

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Data availability

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

Declarations

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.

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