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RESEARCH ARTICLE

Tick Control Potential of *Thymus vulgaris* essential oil: Repellency, Acaricidal Activity, and Enzyme-Modulating Effects Against *Haemaphysalis doenitzi* (Acari: Ixodidae)

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ABSTRACT

The increasing resistance of ticks to chemical acaricides has shifted attention toward plant-derived alternatives as effective control agents. This study evaluated the repellent, acaricidal, and enzyme-modulating properties of *Thymus vulgaris* essential oil (EO) and its main components against *Haemaphysalis doenitzi*. Gas chromatography–mass spectrometry (GC–MS) analysis identified p-cymene (41.25%) and thymol (39.91%) as the predominant EO components. Thymol exhibited stronger repellent activity than *T. vulgaris* EO, with a 90% repellency percentage (EC_{90}) of 19.1ng/ml, and its prolonged use showed comparable efficacy to N,N-diethyl-3-methylbenzamide (DEET). Immersion bioassays demonstrated that thymol exhibited greater toxicity than p-cymene against both nymphs and adults, with LC_{50} values of 15.2mg/ml and 31.2mg/ml, respectively. Enzyme assays showed that *T. vulgaris* EO, thymol, and p-cymene significantly inhibited Na^+/K^+-ATPase and GST activities. *T. vulgaris* EO achieved 51.8% and 27.2% inhibition against Na^+/K^+-ATPase and GST, respectively, thereby disrupting tick energy metabolism and detoxification defenses. Gene expression analysis showed that thymol significantly upregulated *HD-ABCE1* and *HD-GSTa*, while *T. vulgaris* EO promoted *HD-GSTa* and *HD-CYP450a* expression, indicating differential modulation of detoxification pathways. Molecular docking results further confirmed thymol's strong binding affinities to GST (–4.11kcal/mol), CYP450 (–3.10kcal/mol), and ATP-binding cassette transporter proteins (–3.51kcal/mol), mediated by hydrophobic interactions and hydrogen bonds with Ile residues. These findings demonstrate that *T. vulgaris* EO (61.3% overall control) and thymol alone (67.1%) offer potent natural alternatives or supplements to synthetic acaricides for sustainable tick management.

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INTRODUCTION

Plant essential oils (EOs), also known as volatile or aromatic oils, are key secondary metabolites produced by plants. Depending on the plant species and extraction method, essential oils are complex mixtures of terpenes, phenols, aldehydes, esters, alcohols, and other compounds, thereby conferring diverse biological properties (Ma *et al*., 2025). Natural plant extracts exhibit multiple antibacterial, anti-inflammatory, and antioxidant activities (Yuan *et al*., 2016). Essential oils degrade rapidly in soil and water, leaving minimal residue and presenting lower risks to food chains and the environment than conventional pesticides (Hossain *et al*., 2025). Highly renewable and widely available, they are now gaining momentum as cornerstone green solutions for

eco-friendly crop protection. Within this context, the development of plant-derived tick repellents is both urgent and promising, attracting increasing attention in livestock pest management (Chargui *et al*., 2025; Gao *et al*., 2025). Recent studies have highlighted the tick repellent potential of spruce-derived volatile mixtures against *Hyalomma excavatum* and *Ixodes ricinus* (Wood *et al*., 2026), as well as essential oil components from Lamiaceae against *Rhipicephalus sanguineus* sensu lato (Koc *et al*., 2025). *Mentha piperita* EO exhibited both contact acaricidal and repellent effects against *R. microplus*, underscoring the promise of plant-based compounds in tick control (Ahmad *et al*., 2025).

Ticks are obligate blood-feeding ectoparasites that infest a wide range of hosts, including mammals, birds, and reptiles (Taylor *et al*., 2025; Groenevelt *et al*., 2025). During blood-feeding, ticks transmit numerous pathogens, which can impair livestock survival and reproductive performance, while also causing zoonotic diseases, thus posing a dual threat to animal production and public health (Kimera *et al*., 2025). *Haemaphysalis doenitzi* (Acari: Ixodidae) is widely distributed throughout East Asia, including China, Japan, and South Korea, and is a vector of medical and veterinary importance, transmitting pathogens such as *Babesia* and *Rickettsia* (Zhang *et al*., 2025a). It spreads efficiently via avian hosts and parasitizes livestock, particularly cattle and sheep, where its blood-sucking activity not only compromises host health but also reduces meat quality, leading to significant economic losses (Zhang *et al*., 2024a). This species can easily spread between different habitats through migratory bird movements, with a dispersal range far exceeding that of many tick species associated with mammals (Chitimia *et al*., 2024).

The intensifying resistance of ticks to chemical acaricides, together with concerns over pesticide residues, has accelerated research into sustainable plant-derived alternatives (Lu *et al*., 2021; Malak *et al*., 2024). Among them, thyme (*Thymus* spp.) EO shows strong potential, with multiple bioactivities including repellency, contact toxicity, and inhibition of oviposition, making it a promising candidate for environmentally friendly tick control (Lazarević *et al*., 2020). The genus *Thymus* (Lamiaceae) comprises perennial aromatic herbs widely distributed across Europe, northern Africa, and Asia (Castagliuolo *et al*., 2023). Representative species include *Thymus vulgaris*, *Thymus citriodorus*, and *Thymus serpyllum* (Taghouti *et al*., 2020; Perez *et al*., 2022; Jalil *et al*., 2024). Their EOs, rich in bioactive constituents, have been studied extensively for insecticidal and repellent effects (Coimbra *et al*., 2022). For example, wild thyme EO has shown larvicidal activity against mosquitoes and repellency against mosquitoes and cockroaches (Barnard, 1999; Gaire *et al*., 2017), while *T. vulgaris* EO has been shown to exert lethal effects on *Amblyomma americanum* larvae (Showler *et al*., 2020). Thymol, the primary component of *T. vulgaris* EO, is known to disrupt nervous and physiological functions in ticks, leading to repellency and acaricidal effects (Waliwitiya *et al*., 2010). However, the precise mechanisms of action of *T. vulgaris* EO on ticks remain poorly understood, and experimental data are still limited.

Given the growing challenges of acaricide resistance and environmental contamination, the exploration of efficient, low-risk, plant-based alternatives has become an important direction in tick control. *T. vulgaris* EO is of particular interest owing to its broad-spectrum anthelmintic and acaricidal properties, pharmacological potential, and eco-friendly properties (Oliveira *et al*., 2025). This study is the first to compare the acaricidal and repellent activities of *T. vulgaris* EO, thymol, and p-cymene against *H. doenitzi* using dual *in vitro* assays. We further examined their biochemical effects on detoxification- and nerve-related enzymes, including glutathione S-transferase (GST), carboxylesterase (CarE), acetylcholinesterase (AchE), and Na^+/K^+-ATPase. In addition, gene expression analyses of *HD-GSTa*, *HD-CYP450a*, and *HD-ABCE1*, along with molecular docking studies, were performed to elucidate the molecular mechanisms underlying their acaricidal action. Finally, the tick control effect of the essential oil and its key constituents against ticks was thoroughly investigated. These findings not only provide a theoretical basis for developing plant-derived tick repellents and acaricides but also contribute to understanding the molecular responses of ticks to phytochemicals.

MATERIALS AND METHODS

Chemicals and Ticks: Thymol (purity≥98.0%) and p-cymene (purity≥98.0%) were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China), and DEET (purity≥99.3%) was obtained from Wuhan Weishi Environmental Protection Technology Co., Ltd. (Wuhan, China). *Thymus vulgaris* is native to northern China, and the essential oil was extracted from the dried aerial leaves of this plant. After 2h of continuous steam distillation, the essential oil yield was approximately 3.0% based on dry weight. The resulting essential oil was dried over anhydrous sodium sulphate and stored in amber vials at 4°C (Abdelrazek *et al*., 2026).

Unfed nymphs and adults of *H. doenitzi* were collected by flag-dragging and direct sampling in Cangxi County, Sichuan Province, China. The ticks were identified using a Motic SMZ-140 series stereomicroscope, manufactured in Xiamen, China (Yang *et al*., 2025b). Ticks were subsequently maintained under laboratory conditions (27±1°C, 80% relative humidity) and propagated on New Zealand White rabbits. The engorged females were then incubated to lay their eggs. The larvae hatched, fed on rabbits, and molted into nymphs during incubation. The nymphs were then refed, collected, and incubated to molt into adults. The resulting nymphs and adults were then used for experiments.

Analysis of EO composition: The chemical composition of *T. vulgaris* EO was determined by gas chromatography–mass spectrometry (GC–MS) using an Agilent 7890A-5975C system equipped with a HP-5MS capillary column (30m×0.25mm×0.25μm) (Zhang *et al*., 2025c). For GC-MS analysis, the essential oil was diluted to 1mg/mL in n-hexane (HPLC grade); 1μL of this solution was injected into the GC at a split ratio of 100:1, giving an on-column load of 10ng. The injector temperature was maintained at 300°C. The oven

temperature was initially held at 60°C for 3min, then increased to 300°C at a rate of 30°C/min, and maintained at 300°C for 5min.

Electron ionization (EI) at 70eV was employed, with the ion source temperature set at 230°C and the quadrupole temperature at 150°C. Mass spectra were recorded in full scan mode over the range 40–550amu. Compounds were identified by comparison with the NIST 08 mass spectral library, and their relative contents were quantified using the area normalization method.

Nymph repellency tests: DEET was used as the positive control, while 2% Tween 80 solution served as the negative control. Test solutions of DEET, *T. vulgaris* EO, thymol, and p-cymene were prepared at different concentration gradients using 2% Tween 80 as the diluent. The repellency assay followed the standardized procedure described by Carroll *et al*. for the *Ixodes scapularis* and the *Amblyomma americanum* (Carroll *et al*., 2004).

Briefly, three concentric circles (inner circle=1.6cm, middle circle=3.2cm, outer circle=6.0cm) were drawn on a 7cm diameter Whatman No.4 filter paper. A micropipette was used to evenly apply 160μL of the test solution to the annular zone between the inner and outer circles. After air-drying, the treated filter paper was placed inside a Petri dish (7cm diameter), which was then fixed at the center of a larger Petri dish (15cm diameter). Water was added to the space between the two dishes to form a barrier preventing tick escape.

For each replicate, 10 unfed nymphs were released into the center of the treated zone (1.6cm circle). Tick behavior was recorded at 0, 4, 8, and 12min. Nymphs that turned back or stopped at the boundary and remained in the untreated area for at least 60s were considered "effectively repelled," whereas those that crossed the treated zone were recorded as “ineffective.” Escaped ticks were immediately removed from analysis. Each treatment was replicated three times. A total of 30 ticks were used per experimental group, divided into three separate replicates. Each chemical treatment comprised 240 ticks in total. Repellency (%)=(number of repelled ticks/total number of ticks)×100%.

Nymph and adult immersion test: The acaricidal activity of the test compounds was evaluated using the immersion method (Zhang *et al*., 2025b). Working solutions of different concentrations were prepared in 2% Tween 80. Groups of 10 unfed nymphs or adults in good condition were placed in 5mL centrifuge tubes and completely submerged in the test solution for 5min. After immersion, the solution was aspirated, and ticks were transferred onto clean filter paper to air-dry. 10 ticks per group, with 3 replicates per group, for a total of 30 ticks per treatment. Each chemical treatment covers 180 ticks.

Treated ticks were then maintained under controlled conditions (27±1°C, 80% RH) for 24h. Individuals showing complete loss of mobility after 24h were considered dead. Each concentration was tested in triplicate, and a control group treated only with 2% Tween 80 solution was included.

Enzyme activity: Treatment groups were established at the LC_{50} concentrations of *T. vulgaris* EO, thymol, and p-cymene, with 2% Tween 80 as the control (Zhang *et al*., 2024b). Thirty unfed adult ticks were selected from each group, frozen in liquid nitrogen for 24h, and 10 ticks per replicate were then finely ground in a pre-cooled mortar. Three biological replicates per group. Pre-cooled PBS buffer (0.1M, pH7.2) was added at a ratio of 1:10 (m/v), and the mixture was vortexed to obtain a homogenized suspension. The homogenate was centrifuged at 12,000×g for 15min at 4°C, and the supernatant was collected as the crude enzyme extract and stored in cryotubes for analysis. The four enzyme activity assay kits were purchased from Solarbio Life Sciences (Beijing, China); enzyme activities are expressed as U/g protein. GST activity (Number: BC0355) was determined by adding 100μL of enzyme solution and measuring absorbance at 340nm, with activity calculated as [(Acontrol–Asample)/(Astandard–Aempty)]×standard concentration×dilution× incubation time÷(sample volume×protein amount). CarE activity (Number: BC0845) was assessed using 5μL of enzyme solution with absorbance measured at 412nm, and activity was calculated as (ΔA sample–ΔA null)×total V÷(protein×Vsample×t). AchE activity (Number: BC2025) was measured with 30μL of enzyme solution at 412nm, and activity was calculated as [(ODsample–ODnull)/(ODlabel–ODnull)]×amount of label÷protein. Na^+/K^+-ATPase activity (Number: BC0065) was determined using 50μL of enzyme solution at 37°C with absorbance at 340nm, and activity was calculated as (ΔA sample–ΔA null)/(ΔA label–ΔA null)×Clabel×Vtotal÷(Cprotein×V sample×t).

Gene expression analysis by RT-Qpcr: Unfed adult ticks were treated with 2% Tween 80 (control) or *T. vulgaris* EO for 5min and incubated for 24h at 27±1°C and 80% RH, after which total RNA was extracted using TRIzol reagent. Ticks were frozen and homogenized in a pre-cooled mortar with 1ml of TRIzol, transferred to RNase-free tubes, incubated for 5min at room temperature, and mixed with 200μL chloroform. The mixture was vortexed for 30s and centrifuged at 12,000×g for 10min at 4°C, after which the aqueous phase was collected, mixed with an equal volume of isopropanol, incubated at –20°C for 10min, and centrifuged at 12,000×g for 15min at 4°C to precipitate RNA. The pellet was washed with 75% ethanol, centrifuged at 12,000×g for 10 min at 4°C, air-dried, and dissolved in 30μL RNase-free water. RNA purity and concentration were determined using a NanoDrop spectrophotometer. Reverse transcription was performed in a 20μL reaction containing 2×TS Reaction Mix (10μL), Anchored Oligo(dT) Primer (1μL), TransScript RT/Enzyme Mix (1μL), gDNA Remover (1μL), nuclease-free water, and 1μg RNA. RT-qPCR was conducted in a 20μL mixture consisting of 2×Power Taq PCR MasterMix (10μL), ddH_2O (8μL), cDNA (2μL), and 0.5μL each of forward and reverse primers, using an annealing temperature of 60°C. The primers were: *β-actin* (70bp) F: CGTTCCT GGGTATGGAATCG, R: TCCACGTCGCACTTCATG AT. *HDABCE1* (215bp) F: GGCTTCGTGCCAACTGA GA, R: TGTCTTCCCCGTGCCGTT. *HD-CYP450a* (157bp) F: GCATGGGTCCTCGAAACTG, R: GCCGG AACAGCGAAGATAG. *HD-GSTa* (191bp) F: GGCCG TGGGAGCTGTACAA, R: TGATGGTCGGCACCGTAT.

All primer pairs exhibited amplification efficiencies ranging from 90% to 100%. Melt curve analysis confirmed the presence of a single, specific peak for each amplicon, indicating the absence of non-specific amplification or primer dimer formation. PCR efficiency values and R^2 were ≥0.98 for all assays (Zhang *et al*., 2025c). Gene expression was normalized to *β-actin* and quantified using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software, USA). Three biological replicates were established for each treatment group, each comprising a pool of 10 ticks, with three technical replicates per sample.

Molecular modelling and docking: The structures of GST (GenBank: PQ657472), CYP450 (GenBank: XCD23281.1), and ATP-binding cassette transporter (GenBank: XOD50192.1) proteins from *H. doenitzi* were modeled using SWISS MODEL (https://swissmodel.expasy.org/interactive/) and AlphaFold (https://alphafold.ebi.ac.uk/) for modeling. The obtained protein structures were processed in PyMOL to remove water molecules, delete excess ions, and repair missing amino acid residues. The 3D structures of thymol and p-cymene were downloaded from PubChem https://pubchem.ncbi.nlm.nih.gov/. AutoDock 4.2 software was used to prepare the ligands and receptors by adding hydrogen atoms and charges, performing molecular docking simulations, and calculating binding affinities. The docking calculations were performed using the Lamarckian Genetic Algorithm implemented in AutoDock 4.2, with all other parameters set to default values. The docking complexes were subsequently visualized and analyzed using PyMol.

Tick control efficiency: Acaricidal efficacy was assessed at the LC_{50} of *T. vulgaris* EO, p-cymene, and thymol prepared in 2% Tween 80 (Zhang *et al*., 2025b). In total, 1,568 unfed adults (784♂, 784♀) were allocated to four replicates of three treatments (EO, p-cymene, thymol, and control). Ticks were immersed for 1min, dried, and held at 27±1°C for 24h. After counting surviving individuals, the minimum number required was randomly allocated to each replicate group. Each rabbit received 49 ticks, and four rabbits were used per treatment group. Four New Zealand White rabbits were used per group (n=4); surviving females were placed on rabbit ears for engorgement, after which attachment number, egg mass, and hatching rate were recorded. All animal experiments involving ticks and New Zealand white rabbits were approved by the Animal Ethics Committee of Hebei Normal University (approval no. LLSC2025008).

Statistical analysis: The half repellency concentration (EC_{50}) and half lethal concentration (LC_{50}) were estimated using PoloPlus 1.0 software. Chi-square (χ^2) and regression equations were determined using SPSS 22.0. All data were analyzed using one-way ANOVA followed by Tukey's HSD post-hoc test. Prior to ANOVA, normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. When assumptions were violated, data were log-transformed or analyzed using the Kruskal–Wallis test with Dunn's post-hoc test. Significance was set at α=0.05. All analyses were performed in Prism 8.0.

RESULTS

Chemical composition of the EO: A total of 13 chemical compounds were identified and six of which had a concentration above 1% (Table 1). These included 3-carene (2.55%), p-cymene (41.25%), d-limonene (1.73%), linalool (2.94%), thymol (39.91%), and carvacrol (6.03%). Among them, p-cymene and thymol were the dominant constituents, together accounting for 81.16% of the total oil content, and are considered the main bioactive components of *T. vulgaris* EO.

Table 1: Chemical composition of *T. vulgaris* EO.

No.	RT (min)	Constituent	RIa	RIb	Area (%)
1	3.53	3-carene	945	948	2.55
2	4.25	α-pinene	944	943	0.63
3	5.09	P-cymene	1050	1048	41.25
4	5.20	D-Limonene	1050	1052	1.73
5	5.29	Eucalyptol	1050	1050	0.74
6	6.49	α-Terpinolene	1050	1052	0.24
7	6.76	Linalool	1038	1070	2.94
8	8.58	Borneol	1054	1088	0.33
9	8.81	endo-Borneol	1044	1038	0.54
10	9.44	α-Terpineol	1140	1043	0.68
11	12.25	Thymol	1255	1262	39.91
12	12.50	Carvacrol	1245	1262	6.03
13	16.31	Caryophyllene	1454	1446	0.50
Total					98.34

Note: RT=retention time. RIa=Retention index calculated by linear interpolation relative to retention times of a standard mixture of n-alkanes (C7–C40) using a HP-5MS column. RIb= Retention index from literature. Area (%)=Percentage of the chemical compound.

Nymph repellency tests of EO: Repellency assays with unfed nymphs demonstrated a clear time- and concentration-dependent increase in repellency (Fig. 1). At 12min, all tested concentrations produced significant repellency, and therefore, data at this time point were used for standardized comparison. The EC_{50} values of *T. vulgaris* EO, thymol, and p-cymene were 3.2ng/ml, 3.2ng/ml, and 8.5ng/ml, respectively, compared with 1.7ng/ml for DEET (Table 2). Thymol exhibited stronger repellency than p-cymene, and its repellent efficacy at 90% (EC_{90}=19.1ng/ml) exceeded that of DEET (P<0.05). However, there was no significant difference in the repellency of each treatment group at the EC_{50} level (P>0.05). These results highlight the promising potential of *T. vulgaris* EO, particularly thymol, as an effective tick repellent.

Table 2: Repellent activity of DEET, *T. vulgaris* EO, thymol and p-cymene against *H. doenitzi* unfed nymphs at 12min.

Treatment	N	DF	Regression eq. (Y=)	EC_{50}ng/ml (CI95%)	EC_{90}ng/ml (CI95%)	χ^2	Slope ±SE
DEET	240	5	0.0853x+0.3	1.7^{b} (1.1-3.1)	29.0^{a} (10.2-346.4)	22.2	1.0±0.2
T. vulgaris	240	5	0.0507x+0.3	3.2^{ab} (1.9-12.4)	102.2^{a} (20.8-13761.8)	14.6	0.9±0.2
Thymol	240	5	0.1525x+0.1	3.2^{ab} (2.0-9.0)	19.1^{a}(7.4-186.1)	28.5	1.7±0.4
P-cymene	240	5	0.0499x+0.2	8.5^{a} (4.6-30.7)	158.8^{a} (39.6-4139.0)	25.6	1.0±0.2

Note: EC=effective concentration (ng/ml); CI=confidence interval; N=sample capacity; DF=degree of freedom; χ^2=Chi-square value. In a one-way ANOVA, columns with different letters indicate significant differences (P<0.05).

Nymph and adult immersion test: In the immersion assay, thymol showed the strongest contact toxicity against unfed nymphs of *H. doenitzi*, with an LC_{50} of 15.2mg/mL ($P<0.05$) (Fig. 2D–F). As a single component, p-cymene displayed weaker toxicity (LC_{50}=62.0mg/mL), whereas the whole *T. vulgaris* EO exhibited intermediate activity. Against unfed adults, thymol again demonstrated strong contact toxicity with an LC_{50} of 31.2mg/mL ($P>0.05$) (Fig. 2A–C). In comparison, *T. vulgaris* EO had an LC_{50} of 58.9mg/mL, while p-cymene was the least effective, with an LC_{50} of 119.7mg/mL (Table 3).

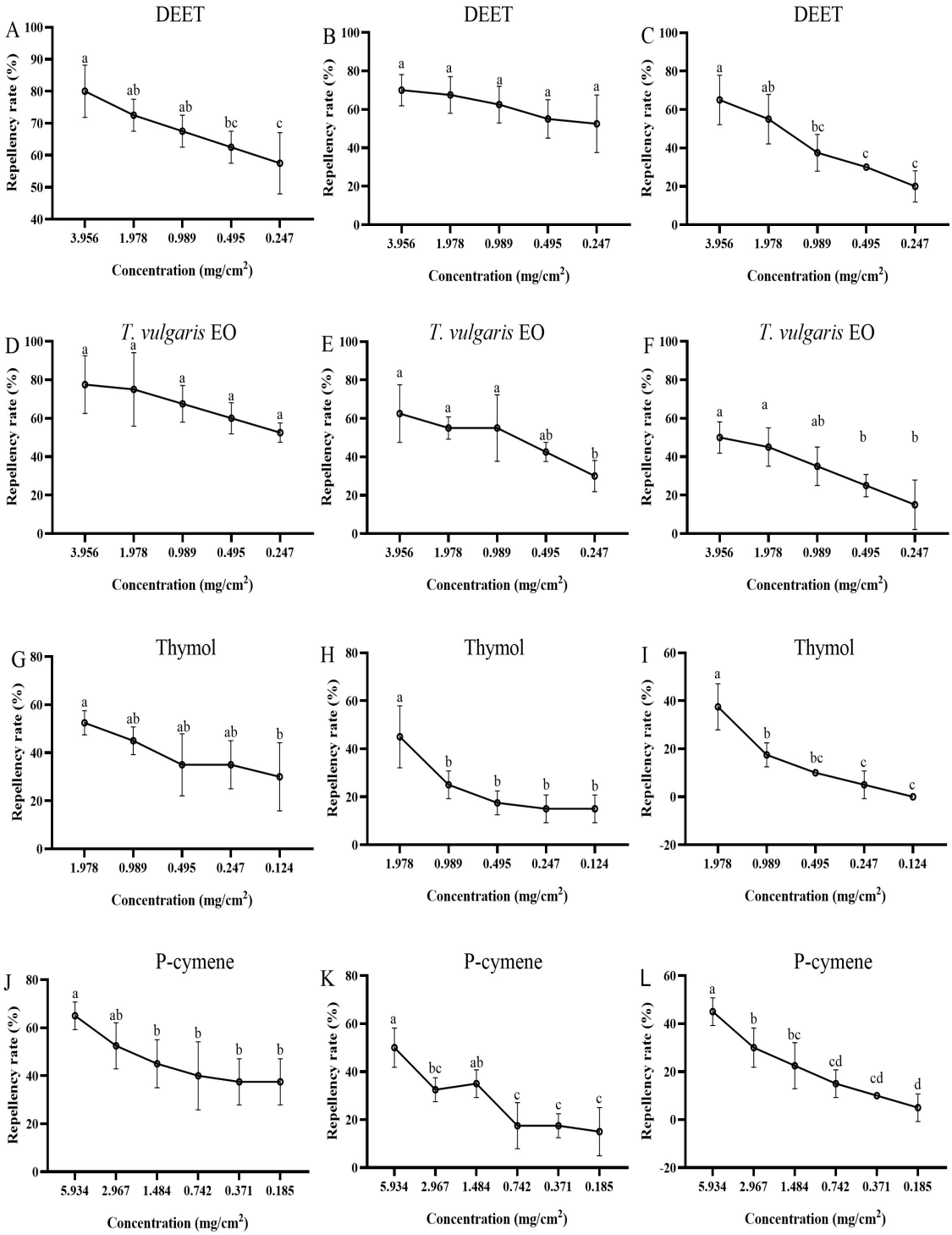

Fig. 1: Repellent activity of DEET, *T. vulgaris* EO, thymol, and p-cymene against *H. doenitzi* unfed nymphs. (A–C): DEET at 4, 8, and 12min; (D–F): *T. vulgaris* EO at 4, 8, and 12min; (G–I): Thymol at 4, 8, and 12min; (J–L): P-cymene at 4, 8, and 12min. Columns with different letters indicate significant differences ($P<0.05$).

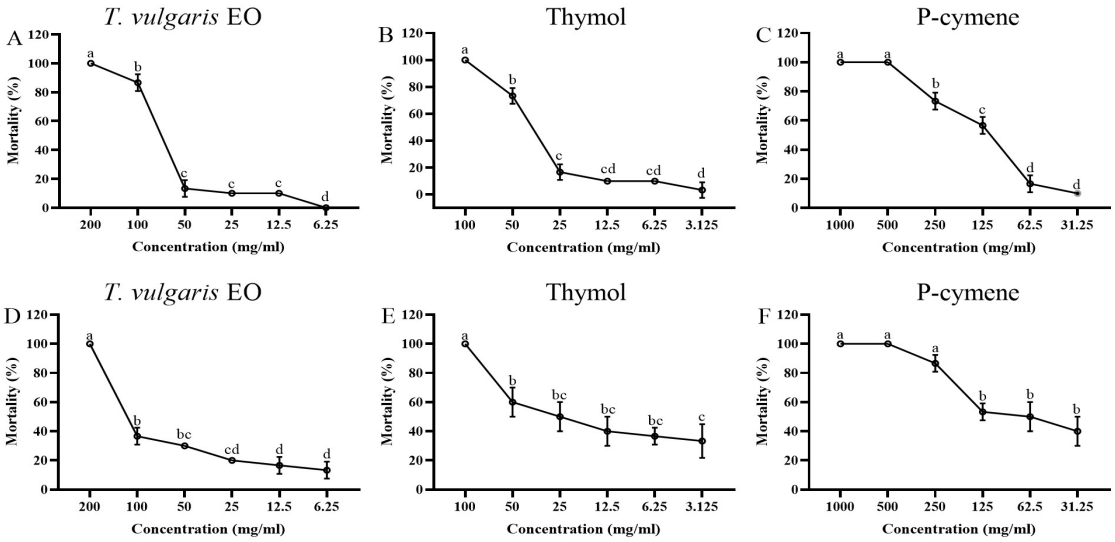

Fig. 2: Mortality rates of *H. doenitzi* unfed nymphs and unfed adults following treatment with *T. vulgaris* EO, thymol, and p-cymene, respectively. (A–C): Unfed adult mortality; (D–F): Unfed nymph mortality. Columns with different letters indicate significant differences (P<0.05).

Table 3: Toxicity of *T. vulgaris* EO, thymol, and p-cymene against *H. doenitzi* unfed nymphs and adults.

Stage	Treatment	N	DF	Regression eq. (Y=)	LC_{50}mg/ml (95%CI)	LC_{90}mg/mL (95%CI)	χ^2	Slope±SE
Unfed nymphs	*T. vulgaris*	180	5	0.0087x+0.07	70.1[a] (45.3–131.0)	489.7[a] (220.8–3038.8)	68.6	1.5±0.2
	Thymol	180	5	0.0067x+0.31	15.2[b] (9.1–23.8)	181.2[a] (84.5–964.0)	37.2	1.2±0.2
	P-cymene	180	5	0.0006x+0.51	62.0[a] (41.4–83.7)	333.3[a] (227.9–621.0)	51.8	1.8±0.3
Unfed adults	*T. vulgaris*	180	5	0.0109x+0.02	58.9[b] (44.4–80.9)	149.9[a] (103.8–292.7)	126.9	3.2±0.4
	Thymol	180	5	0.0107x	31.2[b] (21.9–47.8)	99.3[a] (61.1–263.9)	112.2	2.6±0.3
	P-cymene	180	5	0.0009x+ 0.31	119.7[a] (96.9–147.4)	341.8[a] (260.1–511.1)	96.6	2.8±0.4

Note: LC=lethal concentration (mg/mL); CI=confidence interval; N= sample capacity; DF=degree of freedom; χ^2=Chi-square value. In a one-way ANOVA, columns with different letters indicate significant differences (P<0.05).

Enzyme activity: After 24h post-treatment of unfed adult ticks with LC_{50} *T. vulgaris* EO, thymol, and p-cymene, the enzyme activities of treated groups were compared with those of the control group (Fig. 3). These four enzymes were selected to simultaneously monitor the tick's detoxification system, nerve conduction, and energy metabolism, thereby comprehensively assessing the disruption by *T. vulgaris* EO to the physiological homeostasis of *H. doenitzi*. The results showed that Na^+/K^+-ATPase activity was significantly inhibited in all treatment groups, with the thymol-treated group reduced to about 0.3-fold of the control level (P<0.0001). GST activity was also markedly decreased by both p-cymene and thymol, with the p-cymene-treated group reduced to approximately 0.5-fold of the control (P=0.0024). In contrast, AchE activity was significantly increased in ticks treated with *T. vulgaris* EO and p-cymene (P=0.0012). Furthermore, CarE activity was significantly enhanced following *T. vulgaris* EO treatment, whereas both thymol and p-cymene treatments caused significant inhibition of CarE activity compared with the control (P<0.0001).

Gene expression analysis by RT-qPCR: The relative expression levels of three genes, *HDABCE1*, *HD-CYP450a*, and *HD-GSTa* were quantified using RT-qPCR (Fig. 4). For *HDABCE1*, expression was highest in the thymol-treated group, being significantly greater than in both the *T. vulgaris* EO and p-cymene groups (P<0.0001), and approximately 9-fold higher than in the p-cymene group. The relative expression levels of *HDABCE1* in the *T. vulgaris* EO, thymol, and p-cymene treatment groups were 3.2-, 113.7-, and 12.5-fold higher than those of the control group, respectively. The relative expression levels of *HD-GSTa* in the *T. vulgaris* EO, thymol, and p-cymene treatment groups were 9.9-, 18.1-, and 4.1-fold higher than that of the control group, respectively (P<0.0001). In contrast, *T. vulgaris* EO treatment significantly upregulated *HD-CYP450a* expression to 2.5-fold of the control level, which was markedly higher than that observed in the thymol and p-cymene treatment groups (P<0.0001).

Molecular docking: To further investigate the interaction of volatile terpene and phenolic compounds with detoxification-related proteins in *H. doenitzi*, molecular docking was performed between thymol or p-cymene and the target proteins HDABCE1, HD-CYP450a, and HD-GSTa (Table 4). Docking analysis revealed that thymol exhibited the strongest binding to GST with a binding energy of –4.11kcal/mol, followed by the ATP-binding cassette transporter (–3.51kcal/mol) and CYP450 (–3.10kcal/mol). In comparison, p-cymene displayed relatively uniform binding energies with GST (–3.82kcal/mol), CYP450 (–3.72kcal/mol), and the ATP-binding cassette transporter (–3.75kcal/mol). Visualization of docking patterns (Fig. 5) showed that thymol formed stable hydrophobic interactions with residues within the binding pockets of GST, CYP450, and the ATP-binding cassette transporter. In addition, thymol established hydrogen bonds with ILE-26, ILE-205, ASP-47, and LYS-21, which anchored it firmly within the binding pocket and enhanced the stability of the protein–ligand complex.

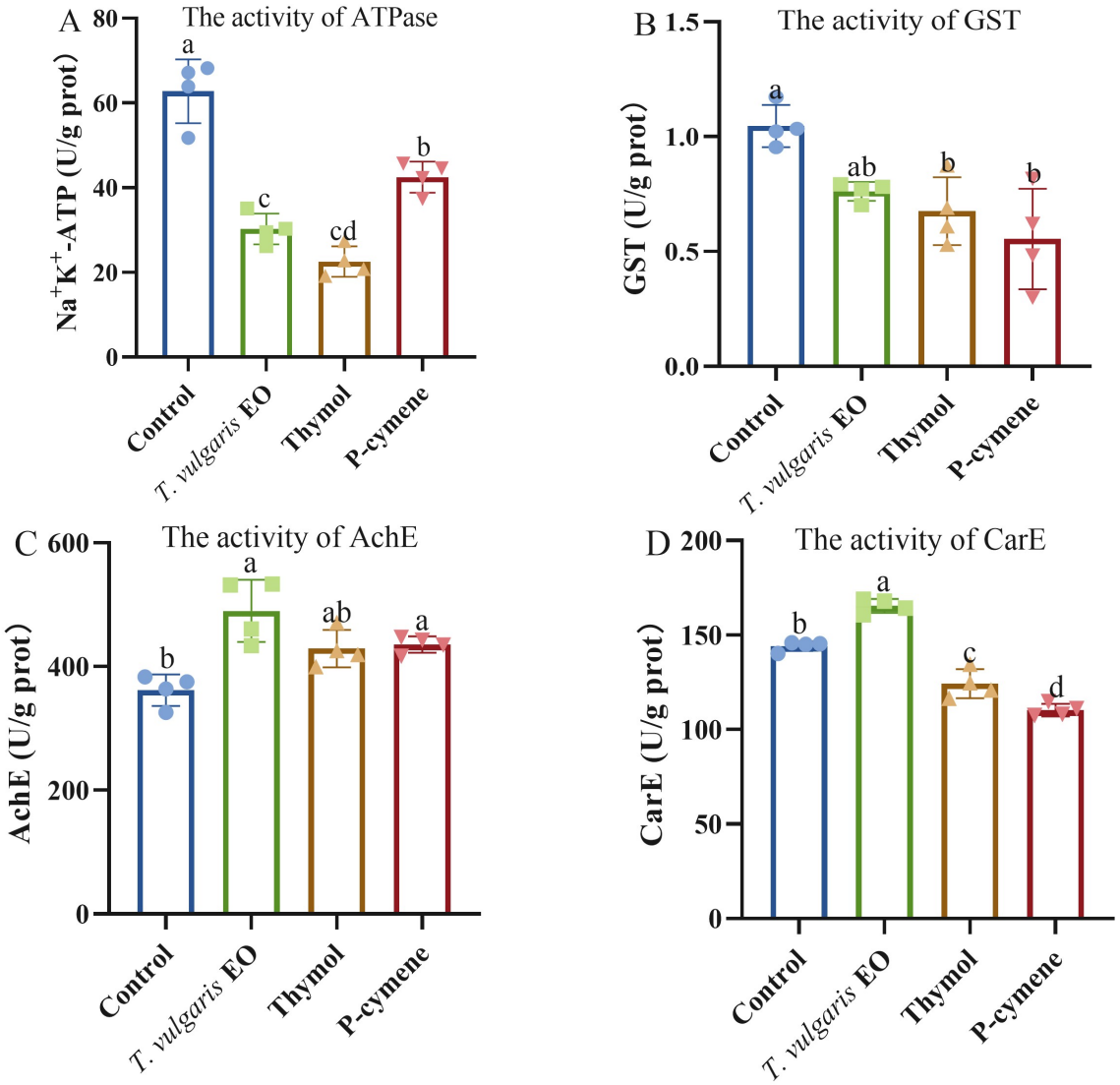

Fig. 3: Effects of *T. vulgaris* EO, thymol, and p-cymene at their respective LC_{50} concentrations on enzyme activities in *H. doenitzi*: (A) Na^+/K^+-ATPase (ATPase), (B) glutathione S-transferase (GST), (C) acetylcholinesterase (AchE), and (D) carboxylesterase (CarE). Columns with different letters indicate significant differences (P<0.05).

Table 4: Affinity of thymol and p-cymene docking with three receptor protein molecules.

Protein Molecules	Small compound ligands	Affinity (kcal/mol)
GST	Thymol	−4.11
	P-cymene	−3.82
CYP450	Thymol	−3.10
	P-cymene	−3.72
ATP-binding cassette transport	Thymol	−3.51
	P-cymene	−3.75

Tick control efficiency: Table 5 shows that at the LC_{50} of *T. vulgaris* EO, thymol, and p-cymene all markedly suppressed the blood-feeding and reproductive performance of *H. doenitzi*. Relative to the control, the three treatments reduced female attachment by 41–46% (P<0.05), decreased engorgement weight by 9–11%, lowered egg mass by 11–21% and cut hatchability by 17–21%. The resulting overall control efficiencies were 61.3% (EO), 67.1% (thymol), and 60.0% (p-cymene). The visibly smaller engorged females further confirmed the integrated control effect of the oil and its key constituents on tick feeding, egg hatching rate, and offspring viability.

Table 5: Physiological parameters of of *H. doenitzi* as affected by *T. vulgaris* EO, p-cymene, and thymol.

Group	Control	*T. vulgaris* EO	Thymol	P-cymene
The total number of ticks	196	196	196	196
Average number of attatched ticks	40.8[a]	21.8[b]	22.8[b]	23.8[b]
Average engorged weight (mg)	200.0[a]	181.7[b]	177.8[b]	179.0[b]
Average egg weight (mg)	105.3[a]	94.0[b]	83.0[b]	91.1[b]
Average egg hatching rate (%)	82.8[a]	67.1[b]	61.8[b]	65.6[b]
EW (%)	/	53.4	55.9	58.3
EM (%)	/	89.3	78.8	86.5
EH (%)	/	81.0	74.6	79.2
Tick control efficiency (%)	/	61.3	67.1	60.0

Note: EW=number of ticks attached in the experimental group/number of ticks in the control group, EM=average egg weight in the experimental group/average egg weight in the control group, EH=average egg hatching rate in the experimental group/average egg hatching rate in the control group. Tick control efficacy (E) was calculated as: E (%)=100×[1−(EW×EM×EH)]. Different letters indicate significant differences between the experimental group and the control group (P<0.05).

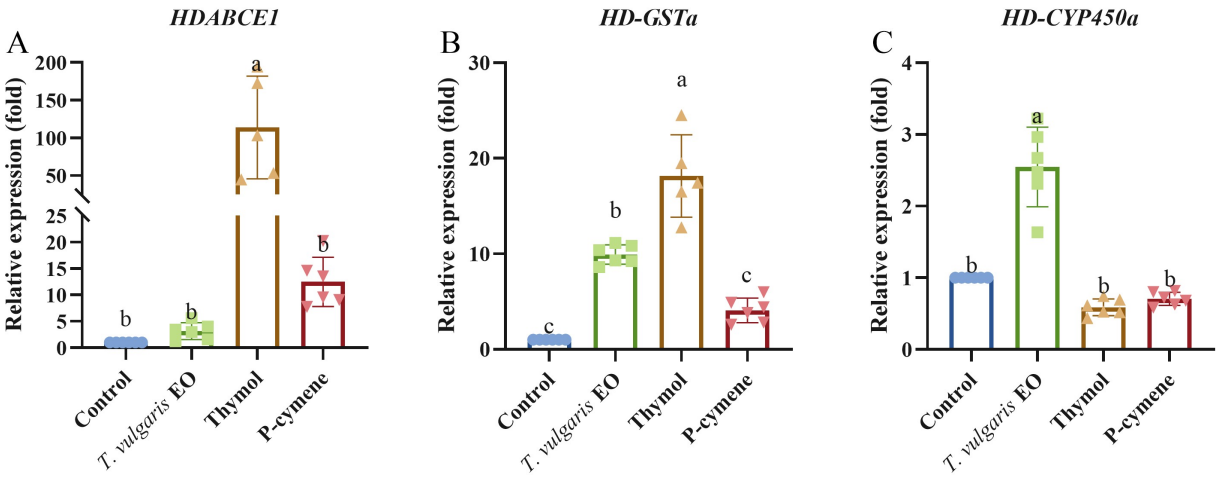

Fig. 4: Relative expression levels of detoxification-related genes in *H. doenitzi*: (A) *HDABCE1* (ATP-binding cassette transporter), (B) *HD-GSTa* (glutathione S-transferase), and (C) *HD-CYP450a* (Cytochrome P450). Columns with different letters indicate significant differences (P<0.05).

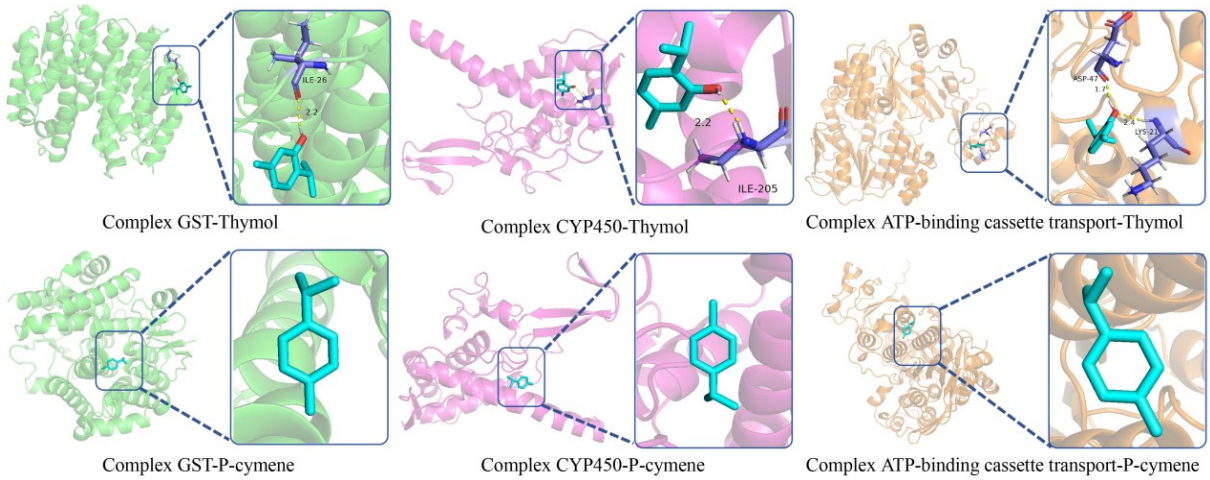

Fig. 5: Molecular docking representation showing the interaction of ligand with the proteins of *H. doenitzi*.

DISCUSSION

Plant essential oils have demonstrated considerable potential as natural alternatives in integrated pest management in animal husbandry (Changbunjong *et al*., 2022). To our knowledge, this is the first systematic investigation of the biological activity of *T. vulgaris* EO against *H. doenitzi*. The EO analyzed in this study was dominated by thymol (39.91%) and p-cymene (41.25%), with carvacrol (6.03%), linalool (2.94%), and 3-carene (2.55%) present in smaller amounts, thereby forming its distinctive chemical profile. The thymol content was slightly lower than the 47.59% reported by Borugă and the 52.33% reported by Bakó, but higher than the 37.45% reported by Chen. (Borugă *et al*., 2014; Chen *et al*., 2020; Bakó *et al*., 2023). Thus, a thymol concentration around 40% appears to be typical and biologically relevant. Interestingly, the p-cymene content was unusually high compared with previous reports, being two to three times greater than the values reported by Galovičová (11.7%) and Darrag et al (17.3%) (Galovičová *et al*., 2021; Darrag *et al*., 2021). Such differences in EO composition are largely attributable to the plant part used, environmental conditions, and extraction methods.

Plant volatiles serve as crucial chemical cues that guide insects in host-seeking, oviposition, and mate location, and they also provide eco-friendly tools for pest repulsion (Zhu *et al*., 2015; Xu and Turlings, 2018; Gong *et al*., 2023). Both thymol and p-cymene are natural monoterpenes released from thyme leaves and inflorescences, with thymol being mainly responsible for antimicrobial and insecticidal activity, while p-cymene functions as both a defensive signal and a biosynthetic precursor of aromatic compounds (Marchese *et al*., 2016; da Silva Costa *et al*., 2024; Yang *et al*., 2022). DEET, the widely used synthetic repellent, remains the benchmark for comparison. At 1.978mg/cm², thymol, p-cymene, and *T. vulgaris* EO exhibited lower repellency than DEET, consistent with previous studies on *Amblyomma americanum* nymphs (Meng *et al*., 2016; Mauff *et al*., 2023). Nonetheless, thymol displayed repellency comparable to *T. vulgaris* EO and significantly higher than p-cymene, suggesting that thymol is the main contributor to repellent efficacy. This aligns with reports of *T. vulgaris* EO repelling *Rhipicephalus sanguineus* nymphs and eliminating engorged adults (Alibeigi *et al*., 2025). Moreover, thymol has been shown to repel a wide range of arthropods, including *Culex pipiens pallens*

mosquitoes, yellow mealworms (*Tenebrio molitor*), and fire ants (*Solenopsis invicta*), with activities approaching those of DEET (Park *et al*., 2005; Bumbulytė *et al*., 2023; Paudel *et al*., 2023). Collectively, these findings highlight thymol as the principal active constituent of *T. vulgaris* EO, providing both repellent and toxic effects against ticks.

In vitro immersion assays further confirmed the acaricidal potential of thymol, p-cymene, and *T. vulgaris* EO against *H. doenitzi* nymphs and adults. Thymol demonstrated the strongest activity, with LC_{50} values of 15.2mg/ml for nymphs and 31.2mg/ml for adults, indicating the lowest effective concentrations among the compounds tested. *T. vulgaris* EO followed, achieving 100% adult tick mortality at 200mg/ml within 24h, while p-cymene was the least effective, requiring up to 1000mg/ml for similar results. These patterns mirrored those observed in repellency assays, reinforcing the role of thymol as the dominant toxicant. Comparable acaricidal effects of thymol have been reported against *R. microplus* larvae (LC_{50}=1.21mg/mL) and against *R. sanguineus*, where thymol disrupted female reproductive organ development and impaired reproduction (Tavares *et al*., 2022; Matos *et al*., 2020) Although p-cymene alone is a weak acaricide, it possesses notable insecticidal activity against pests such as the red flour beetle (*Tribolium castaneum*), cigarette beetle (*Lasioderma serricorne*), book louse (*Liposcelis bostrychophila*), and cotton bollworm (*Helicoverpa armigera*) (Xie *et al*., 2023; Gong and Ren, 2020). The present findings extend the acaricidal spectrum of *T. vulgaris* EO, previously confirmed against *R. microplus* and *R. sanguineus*, to *Haemaphysalis* ticks for the first time (Teixeira *et al*., 2023). Furthermore, this study systematically evaluated the control efficacy of the EO and its two main constituents against *H. doenitzi*. Experimental data revealed that both compounds markedly reduced tick engorged weight, egg mass, and hatching rate, demonstrating a potent inhibitory effect on tick reproduction.

In the context of coevolution, plants have developed diverse secondary metabolites as chemical defenses against insects and arthropods, while their counterparts have evolved detoxification enzyme systems to cope with these xenobiotics (Chamani *et al*., 2025; Giraldi *et al*., 2025). Despite increasing evidence, the mechanism of action of *T. vulgaris* EO on ticks remains poorly understood. In this study, *T. vulgaris* EO significantly altered the biochemical parameters of *H. doenitzi*, highlighting the pivotal role of detoxification enzymes in maintaining physiological stability under toxic stress (Janadaree and Parakrama, 2017).

Ester hydrolases (ESTs), including acetylcholinesterase (AchE) and carboxylesterase (CarE), are critical first-line enzymes capable of hydrolyzing ester bonds in synthetic and natural pesticides. At LC_{50}, *T. vulgaris* EO induced both AchE and CarE activity, thereby potentially enhancing its toxic effects. AchE, localized at the synaptic cleft of the neuromuscular junction, hydrolyzes acetylcholine to terminate synaptic transmission and sustain normal neuromuscular function (Bilal *et al*., 2021). CarE, by contrast, has broad substrate specificity and is a central mechanism of insecticide resistance due to its ability to detoxify ester-based compounds (Feng *et al*., 2018). Interestingly, while EO as a mixture strongly induced both enzymes, thymol and p-cymene alone inhibited CarE, with only p-cymene moderately activating AchE. This divergence suggests that minor EO constituents may act in concert to amplify detoxification responses (Spinozzi *et al*., 2024). Elevated AchE activity may represent a compensatory mechanism against EO-induced neurotoxicity, consistent with reports that *Thymus serpyllum* EO significantly upregulates AchE activity (Wang *et al*., 2024).

Beyond esterases, glutathione S-transferases (GSTs), ATP-binding cassette (ABC) transporters, and cytochrome P450 monooxygenases (CYP450s) form the triad of core detoxification systems in ticks and other arthropods, acting in concert to metabolize, conjugate, and excrete foreign compounds (Zhao *et al*., 2025). Among these, CYP450 enzymes, abundant in the midgut, fat body, and nervous tissues, catalyze oxidative reactions such as hydroxylation to convert EO toxins into more soluble metabolites, and their transcriptional induction is a hallmark of tolerance (Dermauw *et al*., 2020). Our findings showed that LC_{50} *T. vulgaris* EO markedly upregulated CYP450 expression, whereas thymol and p-cymene alone were insufficient to trigger such responses.

Molecular docking analyses supported these observations, showing that both p-cymene (−3.72kcal/mol) and thymol (−3.10kcal/mol) could occupy the active pocket of tick CYP450 proteins, with thymol forming stabilizing hydrogen bonds with ILE-205. These findings suggest that each compound possesses independent binding potential toward the target enzymes; however, their combined effects within the essential oil mixture may be amplified through synergistic interactions, a possibility that warrants further experimental validation (Elhidar *et al*., 2019). The strong transcriptional activation of CYP450 by EO suggests that these enzymes are the first metabolic line of defense against plant volatiles. Consistently, knockdown of CYP450 genes in insects reduces chemical tolerance and accelerates mortality (Liu *et al*., 2022).

GST is highly expressed in the Malpighian tubules and ovaries of ticks, where it catalyzes the conjugation of glutathione with exogenous toxins to form easily excretable complexes and removes lipid peroxides to alleviate oxidative stress (Yang *et al*., 2025a). ATP-binding cassette (ABC) transporters are ATP-dependent pumps that expel metabolic and xenobiotic toxins from cells, and overexpression of their genes is central to drug resistance (Pohl *et al*., 2011). Together, GST, CYP450, and ATP-binding cassette transporter constitute a highly efficient and flexible multidrug resistance network. In this study, LC_{50} concentrations of *T. vulgaris* EO, p-cymene, and thymol significantly inhibited GST and Na^+/K^+-ATPase activity in ticks. Interestingly, LC_{50} thymol has been reported to increase GST activity in larvae of *R. microplus* while LC_{50} *Elettaria cardamomum* EO inhibited GST activity in *Hyalomma anatolicum* larvae (Alanazi *et al*., 2022), and exposure of engorged female *R. microplus* to 10µl/ml trans-anethole also reduced GST activity (Reis *et al*., 2025). These observations suggest that both tick species and physiological state influence the direction of GST response. Given the limited research on Na^+/K^+-ATPase in ticks, the observed decrease in GST

activity may have coincided with a reduction in the energy required to maintain ion gradients, consistent with reports showing that LC_{50} *Citrus sinensis* EO inhibits Na^+/K^+-ATPase activity in *Callosobruchus maculatus* and *Sitophilus zeamais* (Oyedeji *et al*., 2020).

At the genetic level, LC_{50} concentrations of *T. vulgaris* EO, p-cymene, and thymol significantly induced the expression of tick GST and ATP-binding cassette transporter genes, suggesting that ticks may compensate for acute chemical stress through transcriptional activation. Exposure to fluconazole and dichlorvos similarly resulted in elevated GST expression in *Haemaphysalis longicornis* (Hernandez *et al*., 2018). High GST gene expression enhances arthropods' tolerance to xenobiotics, increasing survival (Jin *et al*., 2024; Jin *et al*., 2023). Elevated GST gene expression induced by insecticide stress has also been observed in *Spodoptera frugiperda* (Wang *et al*., 2026). Likewise, *Cinnamomum cassia* EO and its major component (E)-cinnamaldehyde can significantly upregulate ATP-binding cassette transporter genes (Nwanade *et al*., 2024). Overexpression of ATP-binding cassette transporter in *Plutella xylostella* has been associated with enhanced insecticide resistance (Shi *et al*., 2026). As observed by Xu et al. in *Eocanthecona furcellata*, a synchronous decrease in GST and Na^+/K^+-ATPase activity may indicate impaired transcription-translation coupling or insufficient substrate/energy supply (Xu *et al*., 2024).

Molecular docking revealed binding energies of −3.82 and −3.75kcal/mol for p-cymene with GST and ATP-binding cassette transporter proteins, respectively, whereas thymol showed stronger binding at −4.11 and −3.51kcal/mol, forming hydrogen bonds at the ILE-26 site of GST and the ASP-47–LYS-25 site of the ATP-binding cassette transporter (Xu *et al*., 2025). Thymol binding may block substrate efflux via ATP-binding cassette transporter, while p-cymene lacks polar interactions, does not form hydrogen bonds, and exhibits weaker binding, highlighting the structural basis for the superior thermodynamic stability, repellent activity, and acaricidal effects of thymol. These results offer computational support for differential target engagement by EO components, pending experimental validation.

This study provides laboratory evidence supporting the acaricidal activity of *T. vulgari*s EO. However, several limitations must be acknowledged for field application. The volatile constituents of the EO are susceptible to photodegradation and evaporation, necessitating the development of stabilized delivery formulations to ensure adequate residual efficacy. Furthermore, findings derived from a single tick population require validation across different geographic regions and tick populations. Ecological safety also remains to be confirmed through in vivo studies. Overall, *T. vulgaris* EO may serve as a rotational alternative in integrated tick management programs and is suitable for localized applications; however, semi-field trials should be conducted prior to any large-scale deployment.

Conclusions: This study systematically investigated the acaricidal and repellent activities of *T. vulgaris* EO and its major constituents, thymol and p-cymene, against *H. doenitzi*. The potent toxic and repellent properties of thymol are the primary drivers of the comprehensive tick-suppressing effects of *T. vulgaris* EO. At LC_{50} concentrations, *T. vulgaris* EO significantly induced the expression of GST, CYP450, and ATP-binding cassette transporter genes, while also enhancing AchE and CarE enzyme activities. Molecular docking demonstrated that thymol forms stable hydrogen bonds with CYP450, GST, and ATP-binding cassette transporter, thereby enhancing the overall toxicity and repellent efficacy of this EO. In conclusion, *T. vulgaris* EO exhibits dual efficacy as both a potent repellent and acaricide, achieving tick control rate of 61.3%. Future research should prioritize developing stabilized formulations, such as microencapsulated or nanoemulsion formulations, to enhance the field persistence of essential oils. These formulations should be used in rotation or in combination with chemical acaricides to delay the emergence of resistance and reduce pesticide dependence. Comprehensive assessments of cost-effectiveness and accessibility for farmers in resource-limited settings should be conducted prior to broader implementation.

Ethics Statement: All animal experiments involving ticks and New Zealand white rabbits were approved by the Animal Ethics Committee of Hebei Normal University (approval no. LLSC2025008).

Data availability: Data will be made available on request.

Conflict of interest: The authors declare no competing interests.

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