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In vitro acaricidal activity of essential oils and their binary mixtures against *ixodes scapularis* (Acari: Ixodidae)

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A B S T R A C T

Ixodes scapularis ticks are vectors of infectious agents that cause illness in humans, including Lyme disease. Recent years have seen a surge in tick-borne diseases (TBD) resulting in a high demand for tick management products. Plants offer a valuable source of active compounds for the development of novel, eco-friendly tick control products, reducing potential risks to human and animal health. Essential oils (EOs) have emerged as potential acaricides and repellents against ticks providing an alternative to synthetic chemicals and aiding in the prevention of TBD by lowering the risk of tick bites. We investigated the acaricidal activity of EOs from lemongrass (*Cymbopogon citratus*), geranium (*Pelargonium x asperum*), savory thyme (*Thymus saturejoides*), and white thyme (*Thymus zygis*) on *I. scapularis*. The interactions (i.e., synergistic, antagonistic, or additive) of their binary mixtures were also evaluated. EO samples were analyzed via gas chromatography-mass spectrometry to determine their chemical composition. The adult immersion test was used to determine the lethal concentration (LC_{50}) of each EO alone and in mixtures. Quantitative assessment of synergistic, additive, or antagonistic effect of the binary mixtures was performed by calculating the combination index. Strong acaricidal activity was recorded for savory thyme and white thyme EOs, with LC_{50} values of 28.0 and 11.0 μg/μL, respectively. The LC_{50} of lemongrass and geranium EOs were 49.0 and 39.7 μg/μL, respectively. Among the tested EOs, savory thyme and white thyme had a strong acaricidal effect on *I. scapularis*, which might be linked to the presence of carvacrol (26.05 % ± 0.38) and thymol (53.6 % ± 2.31), main components present in savory thyme and white thyme EOs, respectively. The tick killing efficacy of lemongrass and geranium EOs was lower when mixed than when used separately (LC_{50} of 65.3 μg/μL). The same happened with savory thyme and white thyme EOs, except at 9.75 μg/μL where they had a synergistic effect (LC_{50} of 58.3 μg/μL). Lemongrass and savory thyme EOs had a synergistic effect at low concentrations, and an antagonistic effect at higher concentrations (LC_{50} of 95.4 μg/μL). Lemongrass and white thyme EOs had a synergistic effect against ticks from 15 to 120 μg/μL (LC_{50} of 18.5 μg/μL) similar to white thyme EO. Geranium and savory thyme EOs had an antagonistic effect at all concentrations, with an LC_{50} of 66.8 μg/μL. Geranium and white thyme EOs also had an antagonistic effect, except at 12.7 μg/μL where they had a synergistic effect (LC_{50} of 66.8 μg/μL). The interaction observed when combining selected essential oils suggests promising potential for developing acaricidal formulations aimed at controlling ticks and curbing the transmission of tick-borne disease agents.

1. Introduction

In North America, the blacklegged tick, or deer tick, *Ixodes scapularis* Say, 1821 (Acari: Ixodidae), is a serious vector of disease agents affecting humans and animals, including those causing Lyme disease (*Borrelia burgdorferi* sensu lato spirochetes), animal and human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), human babesiosis (*Babesia microti*), cervid babesiosis (*Babesia odocoilei*), and Powassan encephalitis (Powassan virus) (Eisen and Eisen, 2018). In the absence of vaccines against major tick-borne disease (TBD) agents, controlling ticks using acaricides and preventing tick bites by applying repellent products remain the main strategies to protect animals and

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humans against TBD infections (Bakshi et al., 2019; Cisak et al., 2012; Faraone et al., 2020; Luker et al., 2023). One of the most widely used methods in tick management in the United States is the application of synthetic chemical acaricides (Jordan and Schulze, 2020). Synthetic pyrethroids such as bifenthrin effectively control *I. scapularis*; however, they might negatively impact nontarget organisms, such as pollinators, and other terrestrial and aquatic wildlife (Qualls et al., 2010; Schulze and Jordan, 2020; Shamim et al., 2008; Zhang et al., 2019). Therefore, in areas with a high incidence of TBDs, the implementation of environmentally-friendly, efficient integrated pest management tactics to combat ticks is essential (Ogden et al., 2010; Slatculescu et al., 2020; Sperling and Sperling, 2009).

The interest in the development of new acaricides and repellents against ticks using natural products such as essential oils (EO) has increased in recent decades, due to their environmentally friendly and biodegradable characteristics (Anholeto et al., 2017; Gonzaga et al., 2023; Lima-de-Souza et al., 2022; Luker et al., 2023; Pickett et al., 2023; Silva et al., 2023; Wang et al., 2022). EOs are organic compounds produced by the secondary metabolism of aromatic plants, consisting in a mixture of 20 to 60 volatile, lipophilic and highly aromatic molecules and they are responsible for attracting pollinators and seed dispersers, and repelling and protecting the plant against parasites, pathogens and predators (Gonzaga et al., 2023). The compounds in EOs can be mainly classified into two groups based on their biosynthesis: terpenes/terpenoids (e.g., monoterpenes and sesquiterpenes) and aromatic and aliphatic compounds, like phenylpropanoids (Gonzaga et al., 2023). Lemongrass (*Cymbopogon citratus* (D.C.) Stapf., Poaceae), geranium (*Pelargonium x asperum* Ehrh. ex Spreng., Geraniaceae), savory thyme (also known as thyme borneol) (*Thymus saturejoides* Coss., Lamiaceae) and white thyme (*Thymus zygis* (L.) Kuntze, Lamiaceae) are plants with diverse biological and pharmacological properties (Ćavar and Maksimović, 2012; Kasrati et al., 2014; Santos and Vogel, 2012), and their EOs contain an abundance of active components suitable for creating products categorized as minimum risk pesticides by the United States Environmental Protection Agency, presenting minimal or no threat to human well-being and the environment (United States Environmental Protection Agency, 2023). Acaricidal activity of lemongrass, geranium, thyme and white thyme has been reported against different tick species, such as *Rhipicephalus microplus* Canestrini, 1888 (Singh et al., 2022), *Rhipicephalus annulatus* Say, 1821 (Aboelhadid et al., 2016; Ibrahium et al., 2022), *Haemaphysalis longicornis* Neumann, 1901 (Agwunobi et al., 2020), and *Hyalomma scupense* Schulze, 1918 (Djebir et al., 2019) and the acaricidal activity is attributed to their chemical constituents, such as citral, geraniol, thymol and carvacrol.

One strategy to developing acaricidal products is combining different synthetic compounds (Tielemans et al., 2014), synthetic compounds with EOs (Teixeira et al., 2023), or different EOs (Jyoti et al., 2019; Lazcano Díaz et al., 2019). This approach aims to promote possible synergies, enhance pest mortality rate, and decrease the amount used for each included active ingredient. In addition, it might reduce the production cost, and minimize the risk of environmental contamination (Jyoti et al., 2019; Vale et al., 2021).

In the effort to find more effective and environmentally friendly tick control compounds, we investigated the acaricidal activity of EOs from lemongrass, geranium, savory thyme, and white thyme on *I. scapularis* female adult ticks. Furthermore, we investigated the interaction (i.e., synergistic, antagonistic, or additive) of their binary mixtures on the same tick species.

2. Materials and methods

2.1. Chemicals and essential oils

Hexanes (CAS No.: 110–54–3), ethanol (CAS No.: 64–17–5), and Tween 80 (CAS No.: 9005–65–6) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). The commercial acaricide Safer's® End All® Miticide/Insecticide/Acaricide (Woodstream Canada Corporation, Brampton, Ontario, Canada) was used as positive control. This acaricide product includes 20 % potassium salts of fatty acids (CAS No.: 67,701–09–1) and 0.2 % pyrethrins (CAS No.: 8003–34–7). Lemongrass (*Cymbopogon flexuosus*) (CAS No.: 8007–02–1 /91,844–92–7), geranium (*Pelargonium x asperum*) (CAS No.: 8000–46–2), savory thyme (*Thymus saturejoides*) (CAS No.: 934–977–8), and white thyme (*Thymus zygis*) (CAS No.: 85,085–75–2) EOs were purchased from New Directions Aromatics (Mississauga, Ontario, Canada).

2.2. Analysis of essential oils

Samples of EOs were analyzed in triplicate via gas chromatography-mass spectrometry (GC–MS) according to Pickett et al. (2023) to determine the qualitative EO composition. Briefly, one μL of each sample at the concentration of 100 ng/μL was injected using a Maestro Autosampler (Gerstel, Mülheim an der Ruhr, Germany) at 250 °C with a 1:5 split ratio into a Bruker 456 GC (Bruker Chemical Analysis, Goes, The Netherlands), equipped with a (5 %-phenyl)-methylpolysiloxane DB-5 capillary column (Agilent, Santa Clara, CA, USA) and a mass spectrometer Scion 456 MS (Scion Instruments, Livingston, UK). The MS was operated at an electron ionization (EI) energy of 70 eV with the ion source temperature at 250 °C. The mass scan range was *m/z* 35–350. The carrier gas was helium, at a flow rate of 1 mL/min. Column temperature was initially 50 °C for 5 min, then gradually increased to 220 °C at 7 °C/min for 29.9 min, and finally increased to 280 °C at 30 °C/min. The compounds were identified by comparing the retention indices and mass spectra from NIST Mass Spectral Search Program (NIST/EPA/NIH EI Mass Spectral Library v. 2.4, 2020, Scion Instruments). The percentages of the EO constituents were obtained by normalizing the peak areas.

2.3. Ticks

Unfed adult female *I. scapularis* were purchased from Oklahoma State University (OSU)'s Tick Rearing Facility (Stillwater, OK, USA). The *I. scapularis* colony at OSU has been maintained since 1991. It started with engorged females collected from the natural population of *I. scapularis* in Stillwater, Payne County, Oklahoma. Engorged females were introduced yearly, collected from cattle which are owned by OSU. The resulting larvae, nymphs, and adults are fed in the laboratory on sheep and rabbits. Since 2015, pathogen screening (*Borrelia burgdorferi* and *Anaplasma phagocytophilum*) is regularly conducted on a subset of each group of adult *I. scapularis* using standardized PCR assays. Ticks are kept at 22 °C, with a photoperiod of 15 h light/9 hweakly dark, and relative humidity of 90 % or higher. They must be held in a humidity chamber until they are put on a host to prevent desiccation and enhance survival. Once received, ticks were stored in containers with moistened Kimwipe (Fisher Scientific, Burlington, ON, Canada) at 4 °C in the dark (Pickett et al., 2023). Before the bioassays, ticks were acclimated to room temperature (20–22 °C) for at least 20 min. Unfed *I. scapularis* adult females that were no older than 5 months from the moult were used in the bioassays. The 5-month window of tick age may represent a potential study limitation and be linked to the acaricidal responses recorded. Previous studies on wild adults of *Ixodes persulcatus* Schulze, 1930 and *Ixodes ricinus* (Linnaeus, 1758) indicated positive associations between physiological age (from 1 to 4 months post-molt) and susceptibility to various pesticides, including DDT and fenthion (Uspensky and Ioffe-Uspensky, 2006). However, from our preliminary tests conducted on lab-reared, infection-free ticks, we have observed no change in acaricidal compound susceptibility in relation to various tick ages up to 5 months post-molt (unpublished data).

2.4. Adult immersion test

The mortality of ticks exposed to individual EOs and their binary combinations was determined using the Adult Immersion Test (AIT) described by Pickett et al. (2023), with modifications based on Drummond et al. (1973). EOs were solubilized in a mixture of 5 % (v/v) of Tween-80 and 50 % (v/v) ethanol. Deionized water and a mixture of 5 % (v/v) Tween-80 and 50 % (v/v) ethanol were used as the negative control for all the experiments. A commercial acaricide (Safer's® End All® Miticide/Insecticide/Acaricide) at 5 % (v/v) was used as the positive control.

Adult ticks were immersed for 5 min at 5, 10, 20, 30, 40, 50, 60, 70, and 80 μg/μL of each EO alone to determine the Lethal Concentration $(LC)_{50.}$ For the experiments, we used 5 ticks per treatment and each experiment was repeated 5 times (n = 25). Afterwards, ticks were removed with fine tweezers and transferred to absorbent paper to dry. Then, ticks were transferred to labelled Petri dishes with moist filter paper. Petri dishes were sealed with parafilm and kept at room temperature (25 ± 1 °C). After 24 h, adult tick mortality was assessed by exposing the ticks to the breathing of the observer and gently poking them with the tip of a paintbrush to stimulate movement. Ticks that were not able to move after 5 min of stimulation were considered dead.

2.5. *In vitro acaricidal effect of binary combinations of essential oils*

After determining the LC_{50} for each EO, the acaricidal activity of EO binary combinations was assessed in unfed female *I. scapularis* using the AIT according to the Chou-Talalay method (Chou, 2010, 2006). EO treatments were tested at equipotency constant ratios as described in Table 1, determined by dividing the LC_{50} of EO 1 by LC_{50} of EO 2 (based on data from Table 3) (Chou, 2010, 2006; Chou and Talalay, 1984), so that the contributions of effects of each active ingredient to the combination would be roughly equal. Chou and Talalay (1983, 1984) introduced the concept of a combination index (CI) based on the physicochemical principle of the median effect equation of the mass-action law for the quantitative definition of synergism (CI<1), additive effect (CI=1), and antagonism (CI>1). The experimental results were subjected to the computerized determination of synergism, additive effect, or antagonism using CompuSyn (ComboSyn, Inc., Paramus, NJ, USA, Version 1.0, 2004), by calculating the CI through the following formula:

$$\mathrm{CI} = \frac{D_1}{(Dx)_1} + \frac{D_2}{(Dx)_2}$$

Where $(Dx)_1$ represents the dose of EO 1 (D_1) alone that kills x% of ticks and $(Dx)_2$ is the dose of EO 2 (D_2) alone that kills x% of ticks. Dx can be calculated using the following equation: $D_x = D_m[f_a/(1\text{-}f_a)]^{1/m}$. D_x corresponds to the dose of the EO, D_m is the median-effect dose, f_a is the fraction of ticks affected (e.g., killed) by the dose, and m is the exponent defining the shape of the dose–effect curve.

Table 1
Binary combinations of essential oils based on corresponding acaricidal effect (LC_{50} values) against *Ixodes scapularis* adult females.

Ratio* $((LC_{50})_1/(LC_{50})_2)$	Concentrations (μg/μL)				
	Lemongrass	Geranium	Savory thyme	White thyme	Total
[1.2:1]	12.3	9.9	–	–	22.2
	24.5	19.9	–	–	44.4
	49.0	39.7	–	–	88.7
	98.0	79.4	–	–	177.4
	196.0	158.8	–	–	354.8
[2.5:1]	–	–	7.0	2.75	9.75
	–	–	14.0	5.5	19.5
	–	–	28.0	11.0	39.0
	–	–	56.0	22.0	78.0
	–	–	112.0	44.0	156.0
[1.75:1]	12.0	–	6.9	–	18.9
	24.5	–	14.0	–	38.5
	49.0	–	28.0	–	77.0
	98.0	–	56.0	–	154.0
	196.0	–	112.0	–	308.0
[4.5:1]	12.2	–	–	2.8	15.0
	24.5	–	–	5.5	30.0
	49.0	–	–	11.0	60.0
	98.0	–	–	22.0	120.0
	196.0	–	–	44.00	240.0
[1.4:1]	–	9.9	7.1	–	17.0
	–	19.8	14.2	–	34.0
	–	39.7	28.3	–	68.0
	–	79.4	56.7	–	136.1
	–	158.8	113.4	–	272.2
[3.6:1]	–	9.9	2.8	–	12.7
	–	19.9	5.5	–	25.4
	–	39.7	11.0	–	50.7
	–	79.4	22.1	–	101.5
	–	158.8	44.2	–	203.0

*The equipotently constant ratio was determined by dividing the LC_{50} of EO 1 by the LC_{50} of EO 2 according to the Chou and Talalay method (based on data from Table 3).

2.6. *Data analysis*

Probit analysis was conducted on bioassay results data using MedCalc version 20.218 (MedCalc Software Ltd., Ostend, Belgium). This analysis included Probit transformation of percentage mortality and logarithm transformation of concentration. Lethal concentrations at 50 % (LC_{50}) with 95 % confidence intervals (CIs) were estimated. Comparisons between the treatments were deemed to show significant differences when the calculated 95 % CIs of the LC_{50} did not overlap. The software CompuSyn (ComboSyn, Inc., Paramus, NJ, USA, Version 1.0, 2004) was used for determining the effects of interactions (i.e., synergistic, additive, or antagonistic) of EO mixtures (Chou and Martin, 2005; Jyoti et al., 2019).

3. Results

3.1. *Analysis of essential oils*

The chemical characterization of lemongrass EO identified a total of 6 compounds, representing 97.22 % of the total chemical volatile composition. The main compounds present in lemongrass EO were geranial (48.36±0.14 %) and neral (40.59±0.15 %) (Table 2; Fig. 1A Supplementary material). In geranium EO, 11 compounds were identified, representing 97.31 % of the total volatile composition; citronellol (54.38±0.26 %) was the major compound (Table 2; Fig. 1B Supplementary material). Twelve compounds were identified in savory thyme EO (93.23 % of total); carvacrol (26.50±0.38 %) and borneol (24.63 ±0.36 %) were the major components (Table 2; Fig. 1C Supplementary material). In white thyme, it was possible to identify 10 compounds, representing 97.02 % of the total volatile compounds with thymol (53.26±2.31 %) and *o*-cymene (20.89±1.45 %) as major compounds (Table 2; Fig. 1D Supplementary material).

3.2. *In vitro acaricidal effect of individual essential oils against ixodes scapularis adult females*

The acaricidal activity of lemongrass, geranium, savory thyme, and white thyme EOs was assessed against unfed *I. scapularis* adult females by recording mortality after 24 h. A concentration-dependent mortality response was recorded for all EOs, whereas no mortality was observed in negative control groups exposed to distilled water and to 5 % (v/v) Tween-80 + 50 % (v/v) ethanol only, therefore no Abbott's correction for natural mortality was applied to the data. One hundred percent mortality was observed when ticks were exposed to the manufacturer-recommended dose at 5 % (v/v) of Safer's® End All® Miticide/Insecticide/Acaricide used as the positive control. LC_{50} values for the EOs evaluated on unfed female *I. scapularis* are shown in Table 3. Among the four EOs, white thyme showed the strongest acaricidal activity with the

Table 2
Main components and relative abundance (% ± SEM) in *Cymbopogon flexuosus* (lemongrass) (LG), *Pelargonium x asperum* (geranium) (G), *Thymus saturejoides* (savory thyme) (ST), and *Thymus zygis* (white thyme) (WT) essential oils. Analysis was performed by GC–MS. Major compounds are in bold.

Chemical group	Compound*	No. C atoms	RT[†]	% abundance ± SEM			
				LG	G	ST	WT
Aromatic alcohol	phenylethyl alcohol	8	13.42	–	6.22±0.12	–	–
Monoterpenes	α-phellandrene	10	8.39	–	–	2.01±0.01	1.27±0.14
	α-pinene	10	8.60	–	–	4.11±0.16	1.62±0.23
	camphene	10	9.08	–	–	6.58±0.21	–
	β-myrcene	10	10.26	–	–	–	1.33±0.10
	α-terpinolene	10	10.99	–	–	–	1.47±0.10
	o-cymene	10	11.20	–	–	10.71± 0.42	**20.89±1.45**
	limonene	10	11.33	3.52 ± 0.126	–	–	–
	α-terpinene	10	12.09	–	–	–	9.13±0.79
Oxygenated monoterpenes	linalool	10	13.12	–	6.10±0.16	5.71±0.05	4.09±0.10
	trans-rose oxide	10	13.37	–	1.35±0.06	–	–
	camphor	10	14.25	–	–	0.97±0.02	–
	L-menthone	10	14.45	–	2.98±0.11	–	–
	isomenthone	10	14.66	–	1.29±0.05	–	–
	borneol	10	14.84	–	–	**24.63±0.36**	–
	terpinen-4-ol	10	15.02	–	–	1.31±0.18	–
	α-terpineol	10	15.34	–	–	4.05±0.18	–
	citronellol	10	16.01	–	**54.38±0.26**	–	–
	neral	10	16.25	**40.59±0.15**	–	–	–
	geraniol	10	16.50	–	9.80±0.54	–	–
	nerol	10	16.50	1.32±0.02	–	–	–
	geranial	10	16.87	**48.36±0.14**	–	–	–
	thymol	10	17.34	–		1.73±0.13	**53.26±2.31**
	carvacrol	10	17.51	–		**26.05±0.38**	1.96±0.48
	R-citronellene	10	18.47	–	1.38±0.04	–	–
Terpenes	citronellyl formate	11	16.95	–	11.20±0.29	–	–
	geranyl formate	11	17.47	–	1.37±0.05	–	–
	geranyl isovalerate	15	19.02	1.38±0.19	–	–	–
Sesqui terpenes	β-caryophyllene	15	19.93	2.05±0.08	–	5.35±0.07	2.00±0.10
	cis-β-farnesene	15	22.81	–	1.23±0.11	–	–

*NIST: NIST Mass Spectral Library 2020.
[†] RT: retention time.

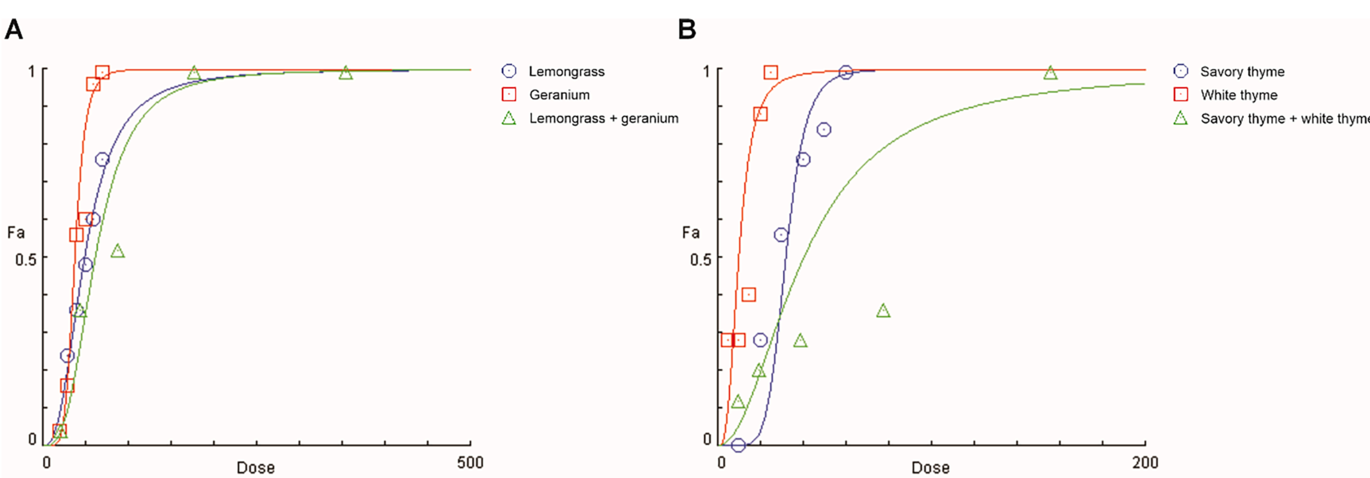

Fig. 1. Dose-response curve for binary combinations of lemongrass and geranium (A) and savory thyme and white thyme (B) essential oils on unfed *Ixodes scapularis* adult females. Fa= fraction affected (percentage of mortality). The dose is expressed in μg/μL.

lowest LC_{50} value of 11.1 μg/μL, with 95 % CIs not overlapping with the other three tested EOs. Savory thyme EO, with a LC_{50} of 28.0 μg/μL, showed stronger acaricidal activity in comparison with lemongrass and geranium EOs (LC_{50} values of 39.7 and 49.0 μg/μL, respectively).

3.3. *In vitro acaricidal effect of binary combinations of essential oils against ixodes scapularis adult females*

The binary combination of lemongrass and geranium EOs had an antagonistic effect in terms of tick killing efficacy in the range of 22.2 to 354.8 μg/μL (Table 4; Fig. 1), with CI values >1. Tick mortality of 100 % was observed only when ticks were exposed to 177.4 μg/μL of the mixture. The LC_{50} value obtained for the mixture was 65.3 μg/μL, being higher in comparison with the LC_{50} of each EO alone (Table 3). Similarly, the savory thyme and white thyme EO binary combination had antagonistic effects on the range of 19.5 to 156 μg/μL (Table 4; Fig. 1), with CI values >1, but showed synergism at 9.75 μg/μL of the mixture (CI <1) exhibiting 12 % of mortality; 100 % mortality was achieved only when the ticks were exposed to 156 μg/μL of the savory thyme and white thyme binary combination (Table 4; Fig. 1). The LC_{50} value obtained for the mixture was 58.3 μg/μL, similar to the LC_{50} value for savory thyme EO alone, with CIs overlapping, but higher than for white thyme

Table 3
Toxicity (LC_{50}) of single and combined essential oils against unfed *Ixodes scapularis* adult females under laboratory conditions.

Essential oil(s)	Total no. of ticks tested	Slope	LC_{50} (μg/μL)	95 % confidence interval
Lemongrass	150	3.7 ± 0.93	49.0	41.7–58.0
Geranium	150	7.24 ±0.98	39.7	36.1–43.1
Savory Thyme	150	5.06 ±0.73	28.0	24.3–31.5
White Thyme	150	3.10 ±0.54	11.1	8.6–14.4
Lemongrass + Geranium	150	3.7 ± 0.57	65.3	53.1–79.8
Savory Thyme + White Thyme	150	1.99 ±0.34	58.3	18.8–1096.5
Lemongrass + Savory Thyme	150	0.93 ±0.28	95.4	43.4–210.0
Lemongrass + White Thyme	150	3.87 ±0.86	18.5	13.6–22.8
Geranium + Savory Thyme	150	3.19 ±0.47	66.8	53.7–82.9
Geranium + White Thyme	150	1.98 ±0.33	36.9	26.4–49.5

(Table 3).

The lemongrass and savory thyme EO combination had a synergistic effect (CI <1) at low concentrations (18.9 μg/μL), being able to kill 40 % of ticks (Table 4; Fig. 2). At 38.5 μg/μL, the effect was additive (CI =1), with 40 % mortality (Table 4; Fig. 2) while at higher concentrations (77 and 154 μg/μL) the observed effect was antagonistic (CI >1), killing only 24 and 28 % of ticks, respectively (Table 4; Fig. 2); 100 % mortality was achieved when the ticks were exposed to 308 μg/μL, but an antagonistic effect (CI >1) was observed (Table 4; Fig. 2). The LC_{50} value obtained for the mixture was 95.4 μg/μL, being similar to the value obtained for lemongrass, with CIs overlapping, but higher than the LC_{50} value for savory thyme (Table 3).

The combination of lemongrass and white thyme EOs had a synergistic effect (CI <1) against ticks exposed to the range of 15 to 120 μg/μL (Table 4; Fig. 2). Tick mortality increased from 40 % at 15 μg/μL to 100 % at 60 μg/μL or higher. Only at 240 μg/μL, the effect was antagonistic (CI >1) (Table 4; Fig. 2). The LC_{50} obtained for the mixture was 18.5 μg/μL, being similar to the value obtained for white thyme EO alone, with CI 95 % weakly overlapping, but being lower than the LC_{50} value for lemongrass (Table 3).

The binary combination of geranium and savory thyme EOs (LC_{50} of 66.8 μg/μL) (Table 3) had antagonistic effects (CI >1) on ticks at all concentrations and tick mortality increased from 27 % at 34 μg/μL to 100 % at 272.2 μg/μL (Table 4; Fig. 3). The interaction between geranium and white thyme EOs was antagonistic (CI >1) at concentrations from 25.4 to 203 μg/μL, and 100 % of tick mortality was only achieved at 203 μg/μL (Table 4; Fig. 3). However, a synergistic effect (CI <1) occurred when the concentration was low, 12.7 μg/μL (Table 4; Fig. 3). The LC_{50} value of 66.8 μg/μL was higher in comparison to the LC_{50} obtained for each EO alone (Table 3).

No mortality was observed in the negative control groups exposed to distilled water and diluents only. One hundred percent mortality was observed when ticks were exposed to the manufacturer-recommended dose at 5 % (v/v) of Safer's® End All® Miticide/Insecticide/Acaricide (pyrethrins (0.2 % v/v) used as the positive control.

CI = 1, <1, and >1 indicates additive effect, synergism, and antagonism, respectively. Data was analyzed according to the Chou-Talalay method (Chou, 2006). * The ratio was determined by dividing the LC_{50} of EO 1 by the LC_{50} of EO 2 (Tables 1, 3).

4. Discussion

We investigated the acaricidal activity of EOs from lemongrass, geranium, savory thyme, and white thyme on *I. scapularis* adult females, and the synergistic, antagonistic, or additive interactions of their binary mixtures on the same tick species. Each EO alone had acaricidal activity, and selected binary combinations, depending on the concentration used and the type of EO, increased this activity (e.g., synergism).

Table 4
Effects of essential oil (EO) binary combinations on unfed *Ixodes scapularis* adult females.

EO Binary combinations	Ratio* $[(LC_{50})_1/(LC_{50})_2]$	Concentrations (μg/μL)	% Mortality	Combination index	Effect
Lemongrass + Geranium		22.2	4	1.29821	Antagonism
		44.4	36	1.20858	Antagonism
	1.2:1	88.7	52	2.01856	Antagonism
		177.4	100	1.33038	Antagonism
		354.8	100	2.66076	Antagonism
Savory Thyme + White Thyme		9.75	12	0.83072	Synergism
		19.5	20	1.41850	Antagonism
	2.5:1	39.0	28	2.53261	Antagonism
		78.0	36	4.61233	Antagonism
		156.0	100	2.78082	Antagonism
Lemongrass + Savory Thyme		18.9	40	0.51576	Synergism
		38.5	40	1.05302	Additive
	1.75:1	77.0	24	2.60400	Antagonism
		154.0	28	4.90520	Antagonism
		308.0	100	2.47315	Antagonism
Lemongrass + White Thyme		15.0	40	0.61214	Synergism
		30.0	72	0.75994	Synergism
	4.5:1	60.0	100	0.42161	Synergism
		120.0	100	0.84322	Synergism
		240.0	100	1.68644	Antagonism
Geranium + Savory Thyme		17.0	0	3.02030	Antagonism
		34.0	27	1.14061	Antagonism
	1.4:1	68.0	42	2.05148	Antagonism
		136.1	80	3.13259	Antagonism
		272.2	100	3.77747	Antagonism
Geranium + White Thyme		12.7	24	0.72898	Synergism
		25.4	32	1.31702	Antagonism
	3.6:1	50.7	60	1.97153	Antagonism
		101.5	73	3.41447	Antagonism
		203.0	100	2.97051	Antagonism

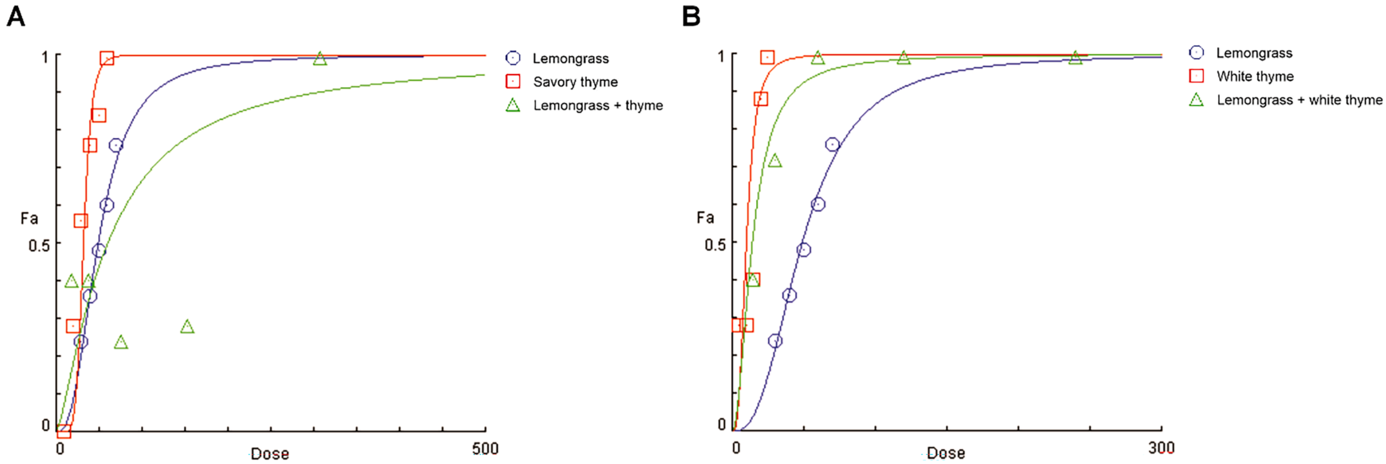

Fig. 2. The dose-effect curve for combinations with lemongrass and savory thyme (A) and lemongrass and white thyme (B) essential oils on unfed *Ixodes scapularis* adult females. Fa= fraction affected (percentage of mortality). The dose is expressed in μg/μL.

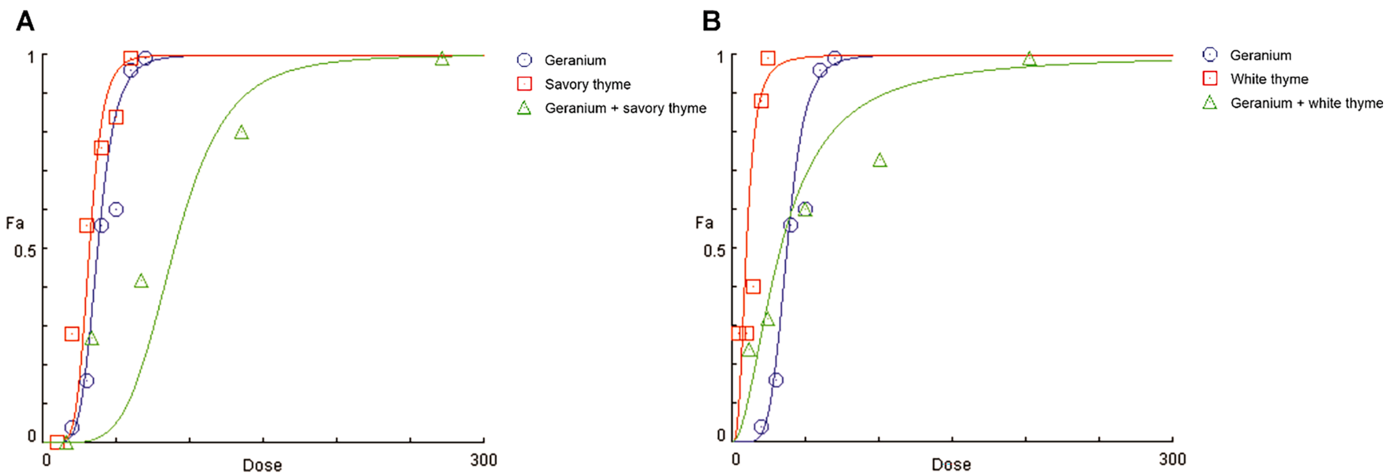

Fig. 3. The dose-effect curve for combinations with geranium and savory thyme (A) and geranium and white thyme (B) essential oils on unfed *Ixodes scapularis* adult females. Fa= fraction affected (percentage of mortality). The dose is expressed in μg/μL.

Our first goal was to determine the individual activity of each essential oil against *I. scapularis* adults. Savory thyme and white thyme EO had higher acaricidal activity on the ticks than lemongrass and geranium EO, as shown by their LC_{50} values of 28.0 and 11.1 μg/μL, respectively. The acaricidal activity of savory thyme and white thyme EOs can be mainly attributed to the monoterpenes thymol and carvacrol, major compounds found in the EO composition. These compounds can be present in variable amount according to the plant chemotypes and the different geographical sources (El Yaagoubi et al., 2021) contributing in potential variability in term of observed bioactivity. These compounds have been reported to be effective against various tick species, such as *Rhipicephalus sanguineus* sensu latu (Coelho et al., 2020; Lima-de-Souza et al., 2022), *R. microplus* (Sousa et al., 2022) and *I. ricinus* (Tabari et al., 2017). Panella et al. (2005) showed the acaricidal activity of carvacrol present in the Alaska yellow cedar (*Chamaecyparis nootkatensis*) EO against *I. scapularis* nymphs. The authors reported an LC_{50} value of 0.0068% (w/v) after 24 h of treatment, and after one week of treatment, the LC_{50} value obtained was 0.035 % (w/v), reinforcing our findings of carvacrol as a promising compound for tick management.

We observed that savory thyme EO, with carvacrol as a major compound, exerts weaker acaricidal activity than white thyme EO, with thymol as a major compound. Similarly, carvacrol has been shown to have weaker acaricidal activity compared to thymol against larvae of *Amblyomma sculptum* Berlese, 1888 (LC_{50} values of 3.49 and 2.04 μg/μL, respectively) and *Dermacentor nitens* Neumann, 1897 (Acari: Ixodidae) (LC_{50} values of 3.33 and 2.17 μg/μL) (Novato et al., 2015). This difference can be related to the mode of action exerted by each compound. In arthropods, thymol can disrupt synaptic transmission by binding GABA receptors on the postsynaptic neuronal membrane and resulting in an inhibitory effect on neuronal and muscular activity (Priestley et al., 2003; Silva et al., 2023). Carvacrol affects the central nervous system of arthropods by interacting with nicotinic acetylcholine receptors (nAChR) (Tong et al., 2013). These receptors are a common target for many insecticides, such as neonicotinoids, that can bind to the nAChR, causing excessive stimulation of the nervous system, which results in the death of the arthropod (Tong et al., 2013). The presence of nAChR in ticks was reported by Lees et al. (2014) when they isolated a full-length functional nAChR a-subunit from the brown dog tick, *R. sanguineus* sensu latu. Functional characterization of nAChRs in *I. ricinus* was achieved by Le Mauff et al. (2020) through microtransplantation of tick synganglion membranes into *Xenopus laevis* oocytes, demonstrating the existence of these receptors in the tick species. Another possible mode of action for thymol and carvacrol is by stimulating the production of reactive oxygen species, causing oxidative stress, which can result in oxidative damage in some tissues and increase the mortality of exposed organisms (Tavares et al., 2022). Tavares et al. (2022) studied the effects of thymol and carvacrol on the enzymatic activity of superoxide dismutase, catalase, glutathione S-transferase, and glutathione peroxidase in

R. microplus populations with different degrees of resistance to synthetic compounds. The authors found that the monoterpenes were capable of increasing the activity levels of those enzymes in the ticks, in an attempt to minimize the damage caused by the compounds, contributing to redox balance and detoxification.

The interaction of lemongrass and geranium resulted in antagonistic effects when *I. scapularis* ticks were exposed to the mixture. The combination of savory thyme and white thyme EOs, which have acaricidal activity against ticks, showed antagonistic effects against *I. scapularis* for concentrations ranging from 19.5 to 156 μg/μL. However, a synergistic effect was observed when ticks were exposed to 9.75 μg/μL of the mixture. At low concentrations (18.9 μg/μL), the lemongrass and savory thyme EO mixture had a synergistic effect. At medium concentration (38.5 μg/μL), the compounds had an additive effect. At high concentrations (77, 154, and 308 μg/μL), the compounds had an antagonistic effect. The combination of lemongrass and white thyme EOs showed a synergistic effect at concentrations ranging from 15 to 120 μg/μL. Only at the highest concentration of 240 μg/μL, the effect was antagonistic. The observed antagonistic and synergistic activities observed in the binary combinations suggests that these molecules have different mechanisms of action. Plant essential oil components often have multiple effects on arthropods and may work together in complex ways affecting different targets or using different mechanisms (Agwunobi et al., 2020; Tak and Isman, 2016). The combination of lemongrass and white thyme EO may enhance the neurotoxic action or impacting other target sites in ticks due to the presence of *E*- and *Z*-isomers of citral and thymol in lemongrass and white thyme EOs, respectively (Cardoso et al., 2020). Inhibition of acetylcholinesterase (AChE) is reported as one of the main actions of organophosphates and carbamates in ticks (Temeyer, 2018). This causes excessive accumulation of acetylcholine in the synapses, resulting in muscle spasms, seizures, and death of the arthropod pest. Cardoso et al. (2020) reported that the terpenes thymol, carvacrol, eucalyptol, citral, and *R*-(-)-carvone inhibited *R. microplus* AChE, and the most potent AChE inhibitors were carvacrol and thymol. According to the same authors, citral, however, had low AChE inhibition, indicating that it may have other acaricidal mechanisms of action. The relationship between AChE inhibition and terpene pesticidal activity is still unclear, as it may depend on various factors such as different biological activities, multiple AChEs in ticks, and different targets for different terpenes (Temeyer, 2018; Temeyer et al., 2013). It also may be suggested that other mechanisms are involved in the synergistic effect of EO binary combinations, such as the decrease in metabolic detoxification of xenobiotics through the inhibition of the enzymatic system (cytochrome P450, monooxygenases, and carboxylesterases). For example, citronellal (the main compound of lemongrass EO) was found to interfere with cytochrome P450-mediated oxidation in the mosquito *Aedes aegypti* (Waliwitiya et al., 2012). These different mechanisms of action may be responsible for the synergistic effects observed in the current trial.

Another possible mechanism for the synergistic effect observed specifically in the binary mixture of lemongrass and white thyme EOs against *I. scapularis* is the enhancement of penetration by the EOs through the tick cuticle. Tick cuticle is a complex structure that serves as a protective barrier against water loss, mechanical damage, and external agents. It also acts as an exoskeleton that supports the tick body (Remedio et al., 2015). The cuticle is composed of different layers, including a protein-rich inner layer, a lipid-rich intermediate layer, and a wax-rich outer layer produced by dermal glands (Remedio et al., 2015; Ribeiro-Silva et al., 2022). The permeability of the cuticle depends on the polarity of the substances that interact with it. For example, 1, 8-cineole, a major component of rosemary EO, has been shown to increase the insecticidal activity of camphor against the cabbage looper, *Trichoplusia ni*, by disrupting the lipid organization of the cuticle (Tak and Isman, 2017, 2015). However, more studies are needed to confirm this hypothesis for our combination of EOs.

The binary mixture of geranium and savory thyme EOs had an antagonistic effect on ticks at all concentrations tested. The antagonism was also evident for the combinations of geranium and white thyme EOs at concentrations from 25.4 to 203 μg/μL. Only at 12.7 μg/μL, the mixture of geranium and white thyme EOs exhibited a synergistic effect on *I. scapularis* adults.

According to Yin et al. (2014), designing synergistic drug combinations remains a challenge despite active experimental and computational efforts, since drugs manifest their action via their receptors on the cell surface, and the effects of drug combinations depend on the interaction of their receptors on the cell surface. The negative interaction between the EOs tested here is not completely understood since the action of EO in ticks may involve multiple mechanisms. Vale et al. (2021) found that the mixture of thymol and carvacrol had an antagonistic effect on *A. sculptum* larvae when the ticks were exposed from 0.3125 μg/μL to 1.25 μg/μL. Similarly, Coelho et al. (2020) observed antagonist effects when engorged nymphs of *R. sanguineus* sensu latu were exposed to 0.625 and 1.25 μg/μL of a combination of thymol and eugenol. The authors attributed the antagonistic effect at the lowest concentrations to the small amount of each substance, below the threshold that causes an increase in the acaricidal activity, which might be the case in our combinations.

One of the main goals of using synergistic combinations is to enhance the biological activity against the target organism by lowering the concentration of each substance. Among the mixtures we tested, the one that showed strong synergy was lemongrass and white thyme. Considering that lemongrass have demonstrated significant tick repellent properties (Faraone et al., 2019; Hogenbom et al., 2021), the lemongrass and white thyme mixture might have both acaricidal and repellent effects on *I. scapularis* ticks. This is a promising possibility that requires further verification through repellency bioassays with the mixtures in the future.

In conclusion, our results suggest that lemongrass and white thyme have a promising potential to be used in acaricide formulations against *I. scapularis* ticks. It is crucial to conduct screening on binary combinations of EOs since not all of them are suitable candidates able to induce a synergistic effect. Future studies on the effects of each major EO component when delivered in a mixture and the efficacy of such products in field conditions are required for the development of novel plant-based formulation for controlling *I. scapularis* ticks.

CRediT authorship contribution statement

Luís Adriano Anholeto: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Sophia Blanchard:** Investigation, Methodology, Writing – original draft. **Haozhe Vincent Wang:** Methodology. **Ana Carolina de Souza Chagas:** Conceptualization, Data curation, Funding acquisition. **Neil Kirk Hillier:** Formal analysis, Funding acquisition, Investigation, Methodology. **Nicoletta Faraone:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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