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Enhancement of acaricide activity of citronella oil after microemulsion preparation

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Abstract

The objectives of this study were to evaluate the preparation of microemulsions for citronella oil and subsequently to compare their acaricide efficacy as against conventional citronella oil. The chemical analysis of the commercial citronella oil used was carried out using an Agilent 6890 gas chromatograph (GC) coupled to an electron impact ionization (EI, 70 eV) with an HP 5973 mass selective detector (MSD). In comparison among the emulsifier systems, the mixture of Tween 20 with propylene glycol in ratios 3 : 1, 2 : 1, 1 : 1, 1 : 2, and 1 : 3, the pseudoternary phase diagrams were conducted and used to define the most suitable system for preparing citronella oil microemulsions and using them in acaricide efficacy testing. The citronella microemulsions in concentrations ranging from 0.39–25% w/w were prepared and tested for their acaricide efficacies using Adult Immersion Test and Larval Package Test. The acaricide efficacies were determined by percentages of mortalities of larval and adult ticks and the values of LC_{50} and LC_{99}. Results show that the most suitable emulsifier system was in a ratio of 3 : 1. The physicochemical characteristics indicated that the size of the microemulsions were in the range of 19.6 $\pm$ 0.4 nm to 47.3 $\pm$ 2.3 nm with moderate polydispersity index (0.3–0.7). The microemulsions had stronger acaricide efficacy than the conventional citronella oil, indicated by significant lower LC_{50} and LC_{99} values. The LC_{99} of the microemulsions against larval and adult ticks at 24 h were 0.78% w/w (0.56–1.02) and 28.4% w/w (23.27–37.43), respectively. These results demonstrated that microemulsion preparation can be used to improve the acaricide efficacy of citronella oil.

Key Words: microemulsion, citronella oil, acaricide efficacy, *Rhipicephalus microplus*

Introduction

In tropical countries, tick-borne diseases, especially bovine babesiosis and anaplasmosis, can cause sudden death of severely infected animals. The cattle tick *Rhipicephalus microplus* (*R. microplus*) is a significant vector of these deadly diseases[11)]. In general practice, many dairy farmers use synthetic insecticides, such as pyrethroids, organophosphates, carbamates, and macrocyclic lactones, to control external parasites[9)]. However, the extensive use of these insecticides increases the risk of insecticide residues in dairy products and the environment[22,24)]. From 96 milk samples collected from Mexico, 39.6% contained detectable level of organophosphorus

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pesticide residues[24]. In Brazil, 20% of milk samples were contaminated with organophosphate, 16.7% with carbamate and some samples with both pesticides[6]. Similarity, approximate 14% of 132 raw milk samples from dairy farms in Minas Gerais State, Brazil was contaminated with pyrethroids and 20% with macrocyclic lactones[22]. In response to the insecticides residue problems, many researchers attempted to develop bioinsecticide products especially from the essential oil of *Cymbopogon winterianus,* the so-called citronella oil[14,15,23], that possesses sufficient biological properties, including repellent, acaricidal, and larvicidal[8,16,17] and which in particular acts against *R. microplus*[4,16]. However, the acaricide efficacy of the natural oil has not been sufficient for it to replace synthetic insecticides in livestock industries.

Microemulsions, isotropic mixtures of oil, water and surfactant providing thermodynamic stability, are potential drug delivery vehicles. In pharmaceutical applications, the vehicles were used to improve acaricide efficacies in both some natural products[3,28] and synthetic pesticides[10,20]. However, knowledge on both preparation of microemulsions for citronella oil and its acaricide efficacies were not reported. Therefore, the objectives of this study were to evaluate the preparation of microemulsions for citronella oil and subsequently to compare their acaricide efficacy with citronella oil with conventional preparation.

Materials and Methods

Chemical analysis of citronella oil: Citronella oil was obtained from Thai-China Flavors and Fragrances Industry Co., Ltd., Bangkok, Thailand. The chemical analysis of the oil was carried out using an Agilent 6890 gas chromatograph (GC) coupled to an electron impact ionization (EI, 70 eV) with an HP 5973 mass selective detector (MSD). An HP-5MS fused silica capillary column (30.0 m × 250 μm, i.d. 0.25 μm film thickness, HP, USA) was used. The carrier gas was helium (grade 5.0) with a flow rate of 1.0 mL/min. The analytical conditions used were as follows: the injector temperature was set at 250°C; the oven temperature was initially held at 70°C for 3 min, and then raised by 3°C/min to 188°C, further raised to 280°C with a heating rate of 20°C/min (isothermal 3 min). For quality assurance of GC-MS, the programmed- temperature Kováts retention indices (RI) was determined. RI was obtained by GC-MS analysis of an aliquot of the citronella oil spiked with an *n*-alkanes mixture containing each homologue from *n*-C_{11} to *n*-C_{27}. The active compounds were identified by comparing their mass spectra with reference compounds from the WILEY version 2007 and NIST version 2005 databases. The amount of each active compound was proportional to the area under the peak (% area) when compare with all other substances which found in the same sample. The RI is based on the retention of n-alkanes with an even number of carbon atoms, the calculation as follows[13]:

$$I = 100Z + 100\left(\frac{\log t'_{R(x)} - \log t'_{R(Z)}}{\log t'_{R(Z+1)} - \log t'_{R(Z)}}\right)$$

where I = Kovats retention index (RI)
t'_R = retention time
x = the compound of interest
Z = number of carbon atoms in the *n*-alkane
Z + 1 = number of carbon atoms in the *n*-alkane + 1

Microemulsion preparation: For comparison among the emulsifier systems, the mixture of Tween 20 with propylene glycol in ratios 3 : 1, 2 : 1, 1 : 1, 1 : 2, and 1 : 3, the pseudoternary phase diagrams were conducted and used to define the most suitable system for preparing citronella oil microemulsions and testing their acaricide efficacy. Briefly, the solutions were prepared by mixing the appropriate amount of oil with the surfactant mixtures of emulsifier systems and subsequently titrated with water under moderate

agitation using a vortex mixer until homogenous. The solutions were designated as microemulsions when they were flowable and transparent. The pseudoternary phase diagrams were drawn using the Origin 8 program, and the system with the largest microemulsion region was identified as the most suitable form. Microemulsions of concentrations ranging from 0.039% w/w to 25% w/w were prepared according to the selected system.

Characterization and stability testing of microemulsion: The microemulsions obtained were characterized for their particle sizes and size distributions (polydispersity index: PDI) using Zetasizer® version 5.00 (Malvern Instruments, Malvern, UK). The particle size measurements were performed at 25°C with a scattering angle of 173°. Each sample was measured 13 times. The flow characteristics and viscosity of the formulations were measured in triplicate using a Brookfield viscometer. The pH values of the microemulsions were measured using the Mettler Toledo 320 pH meter (Switzerland). The stability of the microemulsions was assessed using the heating–cooling cycle method[3]. The microemulsions were kept at 4°C for 24 h and moved to 45°C for another 24 h for 12 cycles. The physical appearance and the characteristics of all microemulsions were determined after their last cycles.

Collection and identification of R. microplus: Engorged female ticks were collected from naturally infested cattle at a smallholder dairy farm in Chiang Mai Province, Thailand. They were placed in plastic boxes with orifices, and immediately transported to a laboratory for identification. All ticks were specific identified using stereomicroscope based on their morphologies for *R. microplus* as described by Walker *et al*[27]. Briefly, adult female *R. microplus* has broad oval porose areas. The hypostomal teeth are in 4 + 4 column arrangement. The internal margin of palp article has no protuberance and is short and distinctly concave. Coxa I spurs have distinct, whereas coxae 2 and 3 spurs are present. The genital aperture posterior lips form a broad U shape.

Preparation of R. microplus adults and larvae: After identification, all female ticks were transferred into small plastic boxes with holes and incubated at 27–28°C with 80–85% relative humidity for 14–18 days until eggs were laid. The eggs laid were separated and transferred to polypropylene tubes with orifices on the lid. Unfed live larvae of 14–21 days of age after hatchability were used for acaricide efficacy testing.

Acaricide efficacy test: Citronella oil was serially diluted in 2% Tween 20 to obtain concentrations of 25.0, 12.5, 6.25, 3.125, 1.5625, 0.78, and 0.39% w/w. The acaricide efficacies against ticks of citronella oil and citronella microemulsion were evaluated using the Larva Package Test (LPT) and Adult Immersion Test (AIT), according to Drummond *et al.*[5], with some modifications. For LPT, approximately 100 larvae were placed onto each filter paper in 4 cm^2 envelopes (Whatman, 80 g, No. 4) moistened with 2 ml each of either citronella oil or citronella microemulsion. The borders of the envelopes were then folded over and fastened with metallic clips. The packages were incubated at 27–28°C and 85–95% relative humidity for 48 h.

For AIT, 10 female ticks were immersed for 10 min in each of the respective concentrations of citronella oil and citronella microemulsion. Tween 20 at 2% w/w in distilled water was used for the negative control group. Cypermethrin was used for the positive control. After immersion, the ticks were dried on tissue paper before being placed in plastic boxes with holes and incubated at 27–28°C with 70–80% relative humidity for 48 h.

Statistical analyses: The acaricide efficacies were determined in 2 ways as 1) mean ± standard

Table 1. Characteristics of chemical components of citronella oil in this study

Peak No.	RT.[a]	RI. (Lit.)[b]	RI.(Oil)[c]	Compounds	% Area	% QA
1	10.53	1153	1159	citronellal	31.4	98
2	13.64	1228	1237	L-citronellol	12.1	97
3	14.74	1255	1264	geraniol	19.4	97

[a]Retention time (minutes)
[b]Kovát Retention Index from literature data
[c]Kovát Retention Index obtained from this study
QA = %similarity or quality match with NIST and WILEY databases

deviation (SD) of mortality rate of adult and larval ticks at 24 and 48 h after treatment and 2) the value of lethal concentrations (LC) of conventional citronella oil and citronella microemulsion that killed 50% (LC_{50}) and 99% (LC_{99}) of larvae and adult ticks. The dead larvae were reconfirmed as dead using a stereomicroscope. Each treatment was performed in three replicates. The percentage of mortality was calculated as follows:

$$\% \text{ mortality} = \frac{\% \text{ mortality of experimental group} - \% \text{ mortality of control group}}{100 - \% \text{ mortality of control}} \times 100$$

Comparisons of acaricide efficacies were evaluated in two ways including 1) comparisons within the same formulation and same time and 2) comparison within the same concentration, using analysis of variance with Tukey test comparison. The lethal concentrations (LC) of citronella oil and citronella microemulsion that killed 50% and 99% of larvae and adult ticks were calculated by probit analysis using SAS program. Significant levels in all tests were defined at $P < 0.05$.

Results

The main chemical components of citronella oil, kovats retention index (RI) and % quality match (QA) are shown in Table 1. RI is useful references for tentative compound identification by GC-MS. In this study, RI of main compounds found in citronella oil nearly the same value on any GC system under any set of parameters from references. Mass spectra of main compounds match closely with the corresponding standard library of 97–98%.

The chromatogram of citronella oil from GC-MS is shown in Fig. 1. Three main chemical compounds of citronella oil were separately identified: citronellal (31.4%), geraniol (19.4%), and L-citronellol (12.1%) (Table 1). The microemulsion regions are indicated by the dark areas in the phase diagrams shown in Fig. 2. Among the tested systems in this study, system containing Tween 20 and propylene glycol with a weight ratio of 3 : 1 exhibited the largest microemulsion region (34.62%), indicating it was the most suitable form of citronella microemulsion. Therefore, this system was selected for the acaricide efficacy testing. Overall characteristics of all formulations were transparent yellow with a pH of 5.0. Means and SD of particle sizes were in between 19.6 ± 0.4 nm and 47.3 ± 2.3 nm, with moderate PDI (0.3–0.7) (n = 13). All formulations exhibited good stability and low viscosity (0.035–0.0642 mPS), with Newtonian flow behavior.

Percentages of larval mortality from all concentrations of citronella oil and citronella microemulsions are shown in Table 2. Mortality rates with citronella oil of 0.39% w/w were significant lower than the rate obtained from microemulsion at the same concentration at 24 h. A concentration of microemulsion (0.78%) causing 100% larval mortality rate at 24 h was less than that of conventional citronella oil (3.125%), indicating higher acaricide efficacies of the microemulsion. The acaricide efficacies against

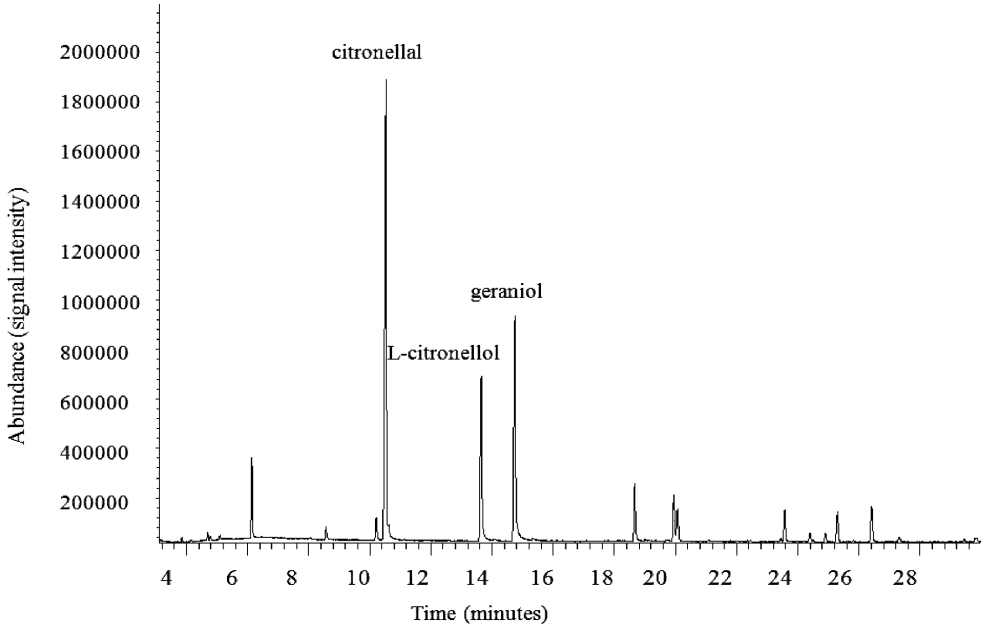

Fig. 1. Chromatogram of citronella oil.

adult cattle ticks are shown in Table 3. Mortality rates with microemulsion at 25% w/w at both 24 and 48 h were higher than the rate obtained from cypermethrin significantly. Values of LC_{50} and LC_{99} and their 95% confidence intervals of both citronella oils are shown in Table 4. For AIT, LC_{99} of conventional citronella oil (54.29% w/w) were significantly higher than that of citronella microemulsion (28.44%). However, no significant difference of LC_{99} for LPT was observed between conventional citronella oil and citronella microemulsion.

Discussion

The three main active compounds–citronellal, geraniol, and L-citronellol–found in this study (Fig. 1) were classified as monoterpenes that are known to have acaricide properties especially citronellal[15,25] and geraniol[15,23]. The variation in the main compound of citronella oil is due to differences of environment and plant management across regions[19].

In order to obtain the suitable system for generated citronella microemulsion, different weight ratios of Tween 20 and propylene glycol were used. In this study, the ratio of 3 : 1 was the most suitable system since it could produce the largest region of microemulsion. It was noted that surfactant plays an important role in the formation of microemulsions. The role of the surfactants is to reduce the interfacial tension between the oil and the water phase which is crucial for the formation of microemulsions, hence, the higher proportion of surfactant in the surfactant mixtures could produce the larger region of microemulsion in the pseudoternary phase diagram[3,28].

This study obtained the ticks (*R. microplus*) from small holder dairy farms that have routinely used insecticides, especially cypermethrin, for a long time. Improper use of cypermethrin, including incorrect dosage, frequency and duration, can lead to cypermethrin resistance in *R. microplus*[7]. According to Veiga *et al.*[26], the mortality rate of ticks less than 95% was considered as ineffectiveness of acaricide. Therefore, the approximately 50% mortality of adult ticks using cypermethrin (Table 2) indicated some degree of resistance to insecticides by the ticks used in this study. This agreed with Mendes *et al.*[18] and

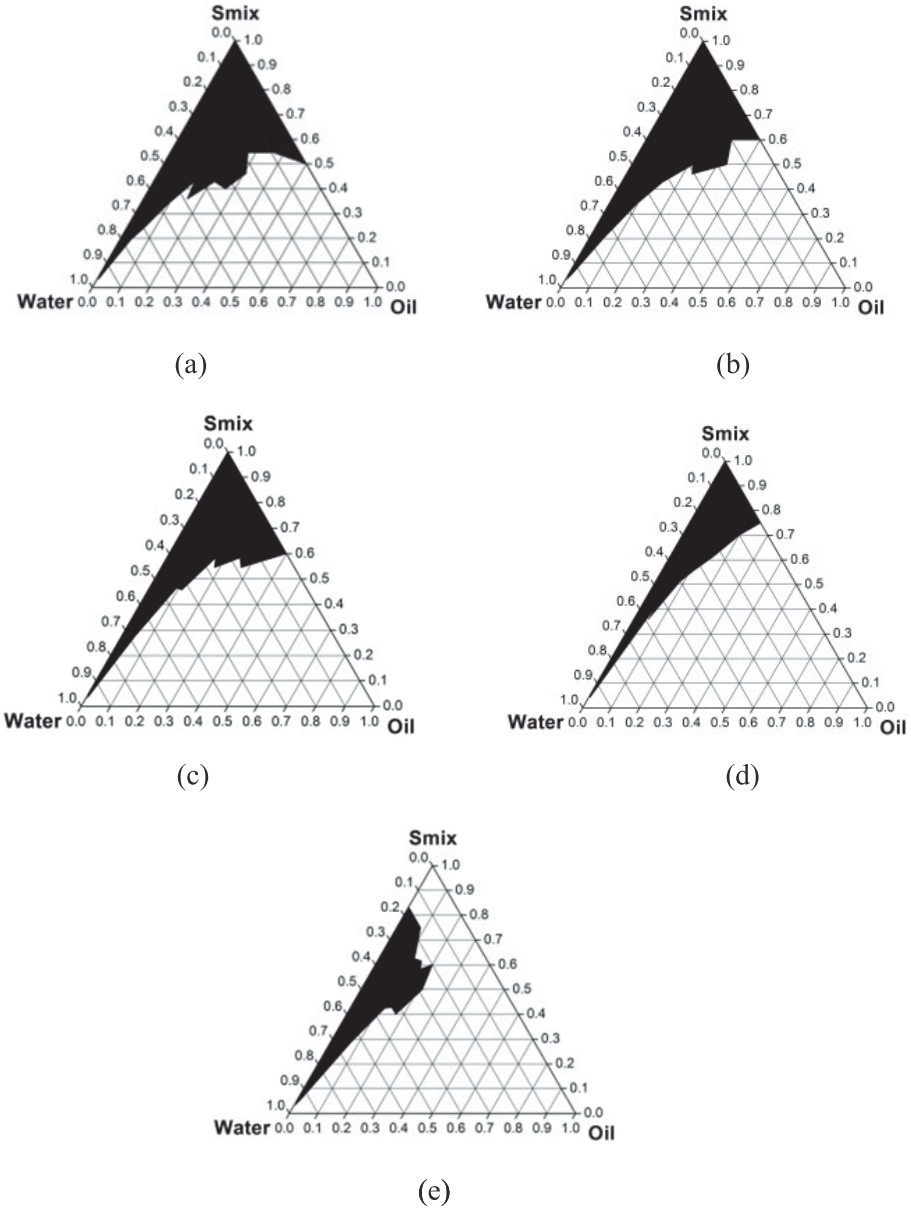

Fig. 2. The pseudoternary phase diagrams of the system of Tween 20 mixed with propylene glycol (Smix) in the ratios of 3 : 1 (a), 2 : 1 (b), 1 : 1 (c), 1 : 2 (d), and 1 : 3 (e).

Veiga *et al.*[26], who also found resistance to cypermethrin by *R. microplus*.

The standard citronella oil prepared in this study had similar ranges of acaricide activity for larval ticks as reported by a previous study[2], and was in between the acaricide efficacies for adults ticks reported by two other studies[16,17]. For larvae with the concentrations of citronellal substance (35%) in citronella oil at 25%, Chagus *et al.*[2] reported a 97% mortality rate of *R. microplus*, but they found a low acaricide efficacy for adult ticks. Martin[16] reported 100% acaricide efficacy against adult ticks, more than 85% found in this study, using 12% to 50% w/w of citronella oil enriched by citronellal (41.15%). In contrast, Mello-Peixoto *et al.*[17] reported lower acaricide efficacy (killing 87% of gravid female ticks) using 100% w/w citronella oil with citronellal at 39.42%. The lower acaricide efficacy found in our study might relate to the use of suspected

Table 2. Percentages of mortality of larval ticks in either conventional citronella oil or citronella microemulsion

Concentration of oil (% w/w)	Citronella oil		Citronella microemulsion	
	24 h	48 h	24 h	48 h
2% Tween	$0.0 \pm 0.0^{a, x}$	$32.7 \pm 14.04^{a, y}$	$0.0 \pm 0.0^{a, x}$	$32.7 \pm 14.04^{a, y}$
0.39	$30.3 \pm 15.82^{b, x}$	$79.3 \pm 18.10^{b, y}$	$96.7 \pm 1.55^{b, y}$	$100.0 \pm 0.0^{b, y}$
0.78	82.5 ± 17.73^{c}	98.3 ± 2.17^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{b}
1.5625	99.6 ± 0.63^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{b}
3.125	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{b}
6.25	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{b}
12.5	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{b}
25	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{b}
Cypermethrin*	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}

Data are expressed as the mean ± SD (n = 100 larval ticks/group)
[a, b, c]Different letters within the same column indicate statistically significant ($P < 0.05$)
[x, y]Different letters within the same row indicate statistically significant ($P < 0.05$)
*Cypermethrin data were used to compare both Citronella oil and Citronella microemulsion

Table 3. Percentages of mortality of adult ticks in either conventional citronella oil or citronella microemulsion

Concentration of oil (% w/w)	Citronella oil		Citronella microemulsion	
	24 h	48 h	24 h	48 h
2% Tween	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
1.56	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	3.7 ± 6.42^{a}	4.2 ± 7.22^{a}
3.125	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	18.5 ± 12.83^{ab}	18.9 ± 12.45^{ab}
6.25	$7.4 \pm 12.83^{ab, x}$	$11.1 \pm 11.11^{ab, xy}$	$33.3 \pm 11.11^{abc, xy}$	$38.4 \pm 5.61b^{c, y}$
12.5	31.9 ± 28.66^{ab}	42.5 ± 28.51^{bc}	59.3 ± 16.97^{cd}	61.1 ± 14.70^{c}
25	85.2 ± 16.97^{bc}	88.4 ± 11.14^{d}	88.9 ± 11.11^{d}	92.1 ± 6.85^{d}
Cypermethrin*	51.7 ± 20.71^{c}	62.0 ± 8.83^{cd}	51.7 ± 20.71^{bc}	62.0 ± 8.83^{c}

Data are expressed as the mean ± SD (n = 10 adults ticks/group)
[a, b, c, d]Different letters within the same column indicate statistically significant ($P < 0.05$)
[x, y]Different letters within the same row indicate statistically significant ($P < 0.05$)
*Cypermethrin data were used to compare both Citronella oil and Citronella microemulsion

Table 4. Lethal Concentrations (50% and 99%) of conventional citronella oil and citronella microemulsion for larval ticks at 24 h, with a 95% confidence interval

	LC_{50}		LC_{99}	
	Mean	Confidence Interval	Mean	Confidence Interval
LPT				
Citronella oil	0.53	0.39–0.69	1.13	0.89–1.78
Microemulsion	–*	–	0.75	0.58–1.10
AIT				
Citronella oil	19.73^{a}	15.19–25.99	54.29^{a}	43.18–75.99
Microemulsion	11.46^{b}	9.35–14.48	28.44^{b}	23.27–37.43

[a, b]Different letters within the same column indicate statistically significant ($P < 0.05$)
*LC_{50} could not be calculated because the tested concentrations can kill ticks more than 50%

acaricide resistant adult ticks, as previously mentioned. In addition, differences in drug absorption due to variations in the content and the composition of a tick's cuticular lipids in its different life stages may contribute to the differing efficacies reported[21].

In this study, microemulsion preparations had higher acaricide efficacies than conventional citronella oil in all parameters, including larval mortality (Table 1), adult mortality (Table 2), LC_{50} and LC_{99} (Table 3). In a study of mites on sheep, neem oil prepared as a microemulsion had higher acaricide efficacy than that with a standard preparation[28]. In addition, Chagus *et al.*[1] reported enhanced activity of oils when formulated in small particle sizes or in emulsifiable concentrates. Xu *et al.*[28] have reported that the higher acaricide efficacy of a microemulsion was probably due to the synergistic effect of the surfactants contained in the formulation. In addition, a microemulsion might interrupt the continuity of the waxy layer of the tick's epicuticle, which is comprised of a complex mixture of lipids, and reduce the resistance of its skin to drug penetration[12,21].

Conclusion

The microemulsion of citronella oil prepared in this study exhibited higher acaricide efficacies against both larval and adult cattle ticks than pure citronella oil. The emulsifier system that generated the largest microemulsion in this study was composed of citronella oil, Tween 20 and propylene glycol in the ratio 3 : 1.

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