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Acaricidal activity of the essential oils from *Citrus hystrix* (Rutaceae) and *Cymbopogon citratus* (Poaceae) on the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* larvae (Acari: Ixodidae)

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Abstract. *Rhipicephalus* (*Boophilus*) *microplus* serves as an important ectoparasite of livestock and a vector of several pathogens resulting in diseases, subsequently affecting the agricultural field as well as the economy. The extensive use of synthetic acaricides is known to cause resistance over time and therefore a much safer, effective and environmentally friendly alternative to overcome tick infestation should be implemented. Larval immersion tests (LIT) were done to evaluate the effects of *Citrus hystrix* (Family: Rutaceae) and *Cymbopogon citratus* (Family: Poaceae) essential oils (EOs) for their individual and combined (1:1) acaricidal activity against the cattle tick. Results showed that LC_{50} and LC_{90} values in 24 and 48 hours for *Cit. hystrix* EO were 11.98% and 24.84%, and 10.95% and 21.71% respectively. LC_{50} and LC_{90} values for *Cym. citratus* EO were 1.21% and 6.28%, and 1.05% and 6.12% respectively. The mixture of EOs from two plants in 1:1 ratio (*Cit. hystrix* 50%: *Cym. citratus* 50%) was found to exhibit antagonistic effect (synergistic factor < 1). The 24 hours and 48 hours LC_{50} and LC_{90} values for combined EOs were 1.52% and 2.84%, and 1.50% and 2.76% respectively. Individual and combined essential oils were subjected to qualitative analysis using gas chromatography-mass spectrometry (GC-MS) to screen the chemical components present in EOs. Our results showed that the combination *of Cit. hystrix* and *Cym. citratus* at 1:1 ratio resulted in an antagonistic effect and the use of *Cym. citratus* alone is more toxic to *R.* (*B.*) *microplus*, making it a better alternative to chemical based acaricide.

INTRODUCTION

Livestock can be defined as animals which are kept for the purpose of gaining profit where they play an important role in sustaining and providing economic growth. These livestock are at risk of many parasitic diseases caused by both endo- and ecto-parasites. Unlike endoparasites, ticks live on the surface of the host where they depend for shelter, food and other necessities (Rechav & Nuttall 2000). The common cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is a one-host-tick and a potential vector for various protozoan species such as *Babesia bovis* and *B. bigemina* and bacteria such as *Coxiella burnetii* (Tongjura *et al.*, 2012). Tick infestation will not only result in blood loss, but will severely affect milk production as well as the quality of the skin (Ducornez *et al.*, 2005).

The use of synthetic acaricide is the main approach for tick control. However, the use of chemical acaricide could not only result in resistance within 5-10 years of use (Wharton 1983), but it could also potentially be risky to non-target organisms, leaving traces of the chemical residue in milk and meat of livestock (George *et al.*, 2014). Therefore, an alternative approach using plant-derived products can be promising. The use of plant extracts will lower the probability for the development of resistance. In addition, plant-based acaricides have lower toxicity on mammals and are water-soluble (Chungsamarnyart *et al.*, 1988, 1991).

Citrus hystrix of the Rutaceae family, also known as *limau purut* (Malay name) *makrut* (Thai name), kaffir lime or leech-lime is well known not only for its distinct aroma used in Southeast Asian cuisines but also its medicinal properties. The genetic pool of citrus plants is believed to have come from both tropical and subtropical regions of South-Eastern Asia before spreading to the other parts of the continent (Webber 1967; Chapot 1975). *Citrus hystrix* has been studied for its antibacterial (Nanasombat & Lohasupthawee 2005; Chanthaphon *et al.*, 2008; Sreepian *et al.*, 2019) and antifungal activities (Soffian *et al.*, 2017). The plant has also been studied on various insects and acari including *Dermatophagoides pteronyssinus* (mites) (Veeraphant *et al.*, 2011), adult *Blatella germanica* (German cockroach) and *Periplaneta americana* (American cockroach) (Thavara *et al.*, 2007; Chooluck *et al.*, 2019), *Spodoptera litura* (cotton leafworm) (Loh *et al.*, 2011), *Musca domestica* (house fly), *Chrysomya megacephala*, *Chrysomya rufifacies* and *Lucilia cuprina* (blow flies) (Suwannayod *et al.*, 2018), as well as *Ae. aegypti* mosquito larvae (Sutthanont *et al.*, 2010).

Cymbopogon citratus or lemongrass is a perennial grass plant whose origin has been linked to Asia, Africa and the Americas. This plant has a worldwide distribution especially in tropical and subtropical countries (Francisco *et al.*, 2011). The plant extracts are used not only as part of the ingredients in culinary, but traditionally they are made into tea with beneficial health effects (Figueirinha *et al.*, 2010; Olorunnisola *et al.*, 2014; Boukhatem *et al.*, 2014). In addition to having antifungal (de Bona *et al.*, 2008) and antibacterial (Zulfa *et al.*, 2016) effects, there are also reports on the plant being studied on various insects including *Sitophilus zeamais* (maize weevil) and *Oryzaephilus surinamensis* (saw-toothed grain beetle) (Hernandez-Lambraño *et al.*, 2015), *Megalurothrips sjostedti* (bean flower thrips) (Diabate *et al.*, 2019), repellency against sandflies and silverfish (Kimutai *et al.*, 2017; Kumar *et al.*, 2017), as well as larvicidal effects against *Ae. aegypti* and *Anopheles arabiensis* mosquito larvae (Cavalcanti *et al.*, 2004; Karunamoorthi & Ilango 2010). Combination of plant extracts are seen to have a synergistic effect and able to have an increase in toxicity even at lower concentrations (Poonia & Kaushik 2013). In this study, *Cit. hystrix* (kaffir lime) and *Cym. citratus* (lemongrass) essential oils were used separately and in combination to observe the potential acaricidal effects on *R.* (*B.*) *microplus* larvae.

MATERIALS AND METHODS

Tick collection and preparation

Tick collection was approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (Approval number: PARAST/PP/2017/SYAMSA/27-SEPT./876-SEPT.-2017-SEPT.-2018-NAR-CAT2). The collection was conducted in a farm located approximately 18 kilometers from Universiti Kebangsaan Malaysia, Cheras. The farm has an area of 1.2 km^2 and it's primarily used for teaching, research as well as practical purposes for university students. The breeds involved for tick collection were from Kedah-Kelantan and Brangus cattle. These cattle were already vaccinated for foot-and-mouth diseases (FMD), haemorrhagic septicaemia (HS) and also RB51 for brucellosis prior to tick collection. In addition, both ivermectin and bomectin were used alternately as commercial acaricides once every four to six months depending on the needs. Eight engorged, adult, female *R.* (*B.*) *microplus* ticks were collected and maintained in the laboratory at 27°C and 80% relative humidity. The ticks were kept in closed containers, providing a space for them to lay eggs. Once the eggs have hatched, the larvae were separated into a different container. Seven to fourteen days old larvae were used for larval immersion test (LIT).

Essential oil preparation

Citrus hystrix and *Cym. citratus* essential oils were purchased from Tropical Bioessence (Tropical Bioessence Sdn. Bhd., Malaysia). Tween-20 was diluted in distilled water at 2% concentration. This stock solution was used to dilute each essential oil at different concentrations. For combined essential oils, this process involved preparing another set of essential oils at different concentrations by using the stock solution. The essential oil was prepared at 1:1 ratio using nine concentrations at 0.5%, 0.7%, 1.0%, 1.5%, 2.5%, 5.0%, 15%, 30% and 40% (final volume of 10 mL in each concentration).

Larval immersion test (LIT)

A modified larval immersion test was used to determine the acaricidal activity of essential oils when they were tested separately and in combination against seven to fourteen days old *R.* (*B.*) *microplus* larvae (Soberanes *et al.*, 2002). Thirty tick larvae were used per replicate and carefully placed onto a Whatman no. 1 filter paper in a petri dish (90 mm) using a soften wooden applicator/brush. This was followed by dropping 3 mL of the diluted essential oil onto the larvae and let them immersed for one minute before transferring them into a new container and sealed using parafilm. Distilled water was used as the negative control, while an undiluted commercial formulation of ivermectin (Biomectin 1%) was used as the positive control.

This process was done in triplicate for both the treated larvae as well as controls. The containers were kept in laboratory conditions at room temperature of 27°C. The counting for the number of dead and live larvae was done manually after 24 and 48 hours. It was determined through observation where the larvae which were able to move forward were considered as alive, and vice versa (Rosado-Aguilar *et al.*, 2010).

Data analysis

LC_{50} and LC_{90} values were calculated using log-probit (Finney 1952). Abbott's corrected mortality formula (Abbott 1925) and Microsoft Excel® 2016 were used to calculate the percentage mortality, mean and standard deviation of larval mortality rate. The significance of larval mortality was determined by Wilcoxon signed-rank test ($p<0.05$) using IBM SPSS Statistics version 20. Synergistic factor (SF) was calculated using the formula of Kalyanasundaram and Das (1985) with a slight modification. A synergistic factor of > 1 shows synergism, while the value of < 1 shows antagonism. Synergistic factor value of = 1 shows no significant effect.

a) Abbott's corrected mortality (CM)

$$CM = \frac{\text{\% test mortality} - \text{\% control mortality}}{100 - \text{\% control mortality}} \times 100$$

b) Synergistic factor (SF)

$$SF = \frac{LC_{50} \text{ value of the individual plant extract}}{LC_{50} \text{ value of the combined plant extract}}$$

Gas chromatography-mass spectrometry

The analyses were carried out using Thermo Scientific Trace GC Ultra equipped with TSQ Quantum XLS operating in EI mode (70 µA). Chromatographic separations were performed with a GC Thermo Scientific TraceGOLD TG-5MS column (30 mm x 0.25 mm, 0.25 µm film thickness) using helium as carrier gas with the following temperature programme: 50–350°C (10°C min^{-1}), isothermal at 350°C. The peak was identified in the basis of comparison between the mass spectra with mass spectra data from the search program of National Institute of Standards and Technology (NIST) version 2.3. This analysis is based on fragmentation pattern obtained from comparison on retention index using a system software Xcalibur version 2.1.0.

RESULTS

Both plants exhibit acaricidal effects on larvae of *R.* (*B.*) *microplus*. Table 1 shows the mean, standard deviation and percentage mortality of tick larvae. Individually, the lowest mortality value for *Cit. hystrix* EO was observed at 2.50% concentration with

1.11% mortality, while 100% tick mortality was achieved at 30% concentration. *Cymbopogon citratus* EO has higher acaricidal effect in which 100% mortality was achieved at 5% concentration and 26.67% tick mortality was observed at a much lower concentration (0.5%) (Table 1).

Combination of these essential oils at 1:1 ratio showed 100% tick mortality at 5% concentration while 0.7% concentration resulted in 2.22% tick mortality. Similarly, no significant difference was observed in the mortality values between different concentrations of EO ($p > 0.05$) and the time of exposure ($p > 0.05$). This shows that the effects of *Cit. hystrix*, *Cym. citratus* and combination of these two essential oils on tick larvae did not depend on the time of exposure.

Table 2 depicts the lethal concentrations (LC) for individual plant essential oils and combination. LC_{50} and LC_{90} values for *Cit. hystrix* EO at 24 hours were 11.98% and 24.84% respectively. *Cymbopogon citratus* shows significantly higher acaricidal effects with LC_{50} and LC_{90} values at 24 hours of 1.21% and 6.28% respectively. Combination of these essential oils at 1:1 ratio resulted in LC_{50} and LC_{90} values of 1.52% and 2.84% respectively. LC_{50} and LC_{90} values for *Cit. hystrix* at 48 hours were 10.95% and 21.71% respectively. In contrast, LC_{50} and LC_{90} values for *Cym. citratus* at 48 hours were 1.05% and 6.12% respectively. Combination of these essential oils at 1:1 ratio resulted in LC_{50} and LC_{90} values of 1.50% and 2.76% respectively. This shows that individual use of *Cym. citratus* essential oil is the most toxic compared to *Cit. hystrix* and in combination at 1:1 ratio.

Synergistic factor value from the combination of these essential oils at 1:1 ratio is shown in Table 3. Synergistic factor against *Cit. hystrix* post 24 and 48 hours exhibited synergistic effect at 7.90 and 7.32

Table 1. Mean and standard deviation of the mortality rate of tick larvae treated with *Cit. hystrix*, *Cym. citratus* and combination of EO

Plant essential oil	Concentration (%)	Mean mortality		Percentage mortality (%)	
		24 hours	48 hours	24 hours	48 hours
Citrus hystrix	0.50	0.00±0.00	0.00±0.00	0.00	0.00
	1.00	0.00±0.00	0.00±0.00	0.00	0.00
	2.50	0.33±0.58	0.33±0.58	1.11	1.11
	5.00	0.67±0.58	0.67±0.58	2.22	2.22
	7.50	4.00±5.29	4.00±5.29	13.33	13.33
	10.00	4.33±0.58	6.67±2.52	14.44	22.22
	12.50	13.33±8.02	16.67±8.33	44.44	55.56
	15.00	25.67±7.51	26.00±6.93	85.56	86.67
	30.00	29.67±0.58	30.00±0.00	98.89	100.00
	40.00	30.00±0.00	30.00±0.00	100.00	100.00
Cymbopogon citratus	0.50	7.00±7.21	8.00±7.00	23.33	26.67
	1.00	12.67±10.07	15.00±11.14	42.22	50.00
	2.50	19.00±10.15	19.33±10.07	63.33	64.44
	5.00	30.00±0.00	30.00±0.00	100.00	100.00
	15.00	30.00±0.00	30.00±0.00	100.00	100.00
	30.00	30.00±0.00	30.00±0.00	100.00	100.00
	40.00	30.00±0.00	30.00±0.00	100.00	100.00
Citrus hystrix + Cymbopogon citratus	0.50	0.00±0.00	0.00±0.00	0.00	0.00
	0.70	0.67±0.58	0.67±0.58	2.22	2.22
	1.00	13.00±10.82	13.00±10.83	43.33	43.33
	1.50	13.67±5.51	13.67±5.51	45.56	45.56
	2.50	23.67±3.51	24.33±4.04	78.89	81.11
	5.00	30.00±0.00	30.00±0.00	100.00	100.00
	15.00	30.00±0.00	30.00±0.00	100.00	100.00
	30.00	30.00±0.00	30.00±0.00	100.00	100.00
	40.00	30.00±0.00	30.00±0.00	100.00	100.00

Table 2. LC_{50} and LC_{90} of essential oils on *Rhipicephalus (Boophilus) microplus* at 24 and 48 hours of exposure

Essential oil	24 hours	
	LC_{50} (%v/v) (CI 95%)	LC_{90} (%v/v) (CI 95%)
Citrus hystrix	11.98 (9.63 – 14.89)	24.84 (19.97 – 30.89)
Cymbopogon citratus	1.21 (0.69 – 2.13)	6.28 (3.56 – 11.08)
Combination (1:1)	1.52 (1.21 – 1.89)	2.84 (2.28 – 3.56)
Essential oil	**48 hours**	
	LC_{50} (%v/v) (CI 95%)	LC_{90} (%v/v) (CI 95%)
Citrus hystrix	10.95 (8.92 – 13.44)	21.71 (17.69 – 26.65)
Cymbopogon citratus	1.05 (0.57 – 1.93)	6.12 (3.32 – 11.26)
Combination (1:1)	1.50 (1.20 – 1.86)	2.76 (2.22 – 3.43)

Table 3. Synergistic factor for plant essential oils

Plants	Essential oil combination	Synergistic factor (SF) against *Cit. hystrix*		Synergistic factor (SF) against *Cym. citratus*	
		24 hours	48 hours	24 hours	48 hours
Cit. hystrix and *Cym. citratus*	Ch 50%:Cc 50%	7.90*	7.32*	0.80**	0.70**

*: synergism, **: antagonism, SF: synergistic factor, SF > 1: synergism, SF < 1: antagonism, SF = LC_{50} individual extract/LC_{50} combined extract.

respectively, while antagonistic effect was observed against *Cym. citratus* post 24 and 48 hours at 0.80 and 0.70 respectively. This shows that the combination of 1:1 ratio, addition of *Cym. citratus* into *Cit. hystrix* resulted in a higher acaricidal effect, while the addition of *Cit. hystrix* lowers the effect of *Cym. citratus*. This indicated an antagonistic effect in the combination of these plants at 1:1 ratio.

Qualitative analysis of all three prepared essential oils is shown in Table 4. The components with medical importance screened in *Cit. hystrix* include α-myrcene, linalool, α-pinene, citronellal, neral and citronellol. Similarly, the components found in *Cym. citratus* include linalool, eucalyptol, neral, camphene, α-pinene and citronellal. Combination of essential oils at 1:1 ratio also resulted in similar chemical components which are neral, linalool, α-pinene, camphene and citronellal. Combination of these essential oils also resulted in two new components which are 2,4-undecadien-1-ol and 1,7-nonadien-4-ol,4,8-dimethyl-. Although 2,4-undecadien-1-ol has been described as an aromatic agent, the importance of both of these components in the medical field have not yet been reported (Purkayastha & Modifier 2013).

DISCUSSION

According to Drummond (1983), there are a few guidelines that need to be adhered in tick control. This includes using acaricides in a way that the target organism is killed without injuring the livestock or applicators. Chemical residues should also be absent in the tissues of the animals, as well as not affecting the environment. The disadvantages of using chemical acaricides include resistant tick populations, negative effects on human beings, the environment as well as the treated animals (García-García *et al.*, 2000). Due to the lengthy and costly process for the development of new chemical acaricides, there is a need for alternative means of tick control (Graf *et al.*, 2004). Plant

Table 4. Screening of chemical components in essential oils using GC-MS

Chemical composition	*Cit. hystrix*	*Cym. citratus*	Combination 1:1
3-Hexen-1-ol	√	–	√
α-Pinene	√	√	√
Camphene	–	√	√
1-Hepten-6-one, 2-methyl-	–	√	√
α-Myrcene	√	–	–
Eucalyptol	–	√	–
1,5-Cyclooctadiene,3,4-dimethyl-	√	–	√
Linalool	√	√	√
Citronellal	√	√	√
(–)-trans-Isopiperitenol	–	√	–
2,4-Undecadien-1-ol	–	–	√
Citronellol	√	–	–
Neral	√	√	√
3-Methyl-3-nitrobut-1-ene	√	–	–
2,6-Nonadienal, (E,Z)-	√	√	√
2-Pentenoic acid,2-methyl	√	–	–
1,7-Nonadien-4-ol,4,8-dimethyl-	–	–	√
2-Pentanol	–	√	√
1,11-Dodecadiyne	–	√	–
1,6-Octadiene,3,7-dimethyl-	–	√	√
12-Methyl-E,E-2,12-octadecadien-1-ol	–		

extracts have been broadly used against phytophagous pests in the last two decades as some of these plants have minimal or no consequence on non-target organism (Balandrin *et al.*, 1985; Valladares *et al.*, 2003).

To the best of our knowledge, there is no study reported on the acaricidal effects on the combination of *Cit. hystrix* and *Cym. citratus* on cattle ticks. However, the acaricidal potential for both plant essential oils in this study is supported by a research done by Veeraphant *et al.* (2011) who studied 24 plant essential oils including *Cit. hystrix* and *Cym. citratus* on *Dermatophagoides pteronyssinus* (house dust mite). It was found that *Cym. citratus* has a higher acaricidal effects towards tick larvae compared to *Cit. hystrix* with LC_{50} value of 0.0088% compared to *Cit. hystrix* at 0.0218%. Citronellal, limonene and α-pinene were reported as the main components in *Cit. hystrix* using GC-MS (Veeraphant *et al.*, 2011). However, some chemical components were absent in the EO of *Cit. hystrix* and *Cym. citratus* in the present investigation. This could be attributed to the components of essential oil which are easily converted into each other through the process of oxidation, cyclization, dehydrogenation as well as isomerization. Due to the nature of essential oils being highly volatile when being subjected to high heat, it is likely that the chemical compositions are highly dependent on multiple conditions including plant processing and storage, distillation as well as the way the oil is handled (Turek & Stintzing 2013).

Similarly, a study done by Politi *et al.* (2012) using plant extract of *Tagetes patula* (French marigold) on a different species of tick, *Rhipicephalus sanguineus* (brown dog tick) larvae showed an LC_{50} value of 7.43%. This plant exhibits higher acaricidal effects in comparison to *Cit. hystrix* on *R.* (*B.*) *microplus* used in current study with LC_{50} value of 11.98%. However, *T. patula* is seen to have a lower LC_{50} value post 48 hours compared to *Cym. citratus* (1.21%). The use of another plant extracts, *Cymbopogon winterianus* (Java citronella), on *R.* (*B.*) *microplus* was also reported whereby the LC_{50} value is better than *Cit. hystrix* at 4.1%, but lower compared to *Cym. citratus*. The main components of *Cym. winterianus* consist of citronellal, geraniol and citronellol, as reported by Martins (2006), were also found in the current study. The main components were tested individually in which both citronellal and geraniol were reported to have significantly better results compared to citronellol.

Synergism occurs when the combination of components produce a greater effect than the sum of their separate effects at the same level of exposure that occurs in the mixture. Antagonism refers to an interaction that occur between compounds where it reduces the toxicity of the active compounds (Singh *et al.*, 2017). The results of the lethal concentrations suggested that the combination of EOs of *Cit. hystrix* and *Cym. citratus* essential oils at 1:1 ratio resulted in antagonism. Although the mechanism is not fully understood, some of the main constituents in the essential oils may have individual antagonistic activity and contribute to overall antagonistic activity of the essential oils when they coexist. Other than concentration, it can be observed that the combination of ratio plays a role in the efficacy of combined essential oils. In this study, combination of EOs of *Cit. hystrix* and *Cym. citratus* at 1:1 ratio produced antagonistic effect (synergistic factor <1).

According to Aqil *et al.* (2006) and Kamatou *et al.* (2006), addition or synergism effects are important in bioactivity in plants. However, there are also reports of the activity's loss in filtered fractions (Cos *et al.*, 2006). Even though this study only use a single ratio of 1:1 in the combination of essential oil, there are several similar study reports on other ratios. A study was done by Tadtong *et al.* (2014) in Thailand using three different combinations of *Alpinia galanga* (Thai galangal) and *Cym. citratus* essential oils at 1:1, 3:7 and 7:3 ratios; and the result showed that the ratio of 3:7 (*A. galanga*: *Cym. citratus*) was the most toxic. In India, Poonia and Kaushik (2013) used three different ratios of *Pongamia pinnata* (Indian beech) and *Kigelia africana* (sausage tree) plant extracts at 1:1, 1:2 and 2:1 on *Aedes aegypti.* It is found that individual use of *P. pinnata* has a higher toxicity compared to *K. africana*. Combination of these plants at 2:1 (*P. pinnata*: *K. africana*) resulted in higher toxicity, while 1:2 combination (*P. pinnata: K. africana*) resulted in antagonism.

CONCLUSION

In this study, both *Cit. hystrix* and *Cym. citratus* EOs are toxic on *R.*(*B.*) *microplus* larvae. However, a combination of these EO at 1:1 ratio showed antagonistic effect (SF < 1). It can be concluded that *Cym. citratus* EO is the most toxic compared to both *Cit. hystrix* in individual use and also combination of these essential oils at 1:1 ratio, and can be proposed as a potential alternative for chemical acaricide. In addition, isolation and purification of the active compounds which might contribute to the acaricidal activity against *R.* (*B.*) *microplus* could be a vital step in the development of novel acaricidal agents.

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