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Acaricidal and Repellent Efficacy of *Cinnamomum verum* Essential Oil Against *Rhipicephalus microplus* Ticks

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ABSTRACT

The emergence of issues like non-target toxicity, drug residues and drug resistance has stressed the need to search for effective alternatives such as botanicals. Hence, the current research was aimed at the evaluation of acaricidal and repellent efficacies of the *Cinnamomum verum* bark essential oil against the *Rhipicephalus microplus* ticks of cattle. Moreover, the *C. verum* essential oil was also analysed using GC-FID analysis which revealed the cinnamaldehyde as its major component. The acaricidal and the repellent efficacies were evaluated at five concentrations (1, 2.5, 5, 10 and 20%) of the *C. verum* essential oil. Different parameters like adult tick mortality, fecundity index, egg hatchability, oviposition reduction, reproductive estimation, product effectiveness, larval mortality and tick repellency were determined. The results indicated a dose-dependent effect of *C. verum* essential oil showing 20% concentration to be the most effective in view of the acaricidal and the repellent activities. Hence, this essential oil can be considered as a potential candidate for the effective control of cattle tick (*R. microplus*).

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INTRODUCTION

Parasitism with particular emphasis on tick infestation is the most important constraint towards profitable livestock rearing especially of large ruminants (Salman *et al*., 2020). Almost 80% of cattle population across the globe is at risk of contracting tick infestations and their associated diseases (Burrow *et al*., 2019). Ticks are the blood-sucking parasitic arthropods which are prevalent all over the world. However, the most important genus of hard ticks affecting large ruminants is the *Rhipicephalus* (Rooman *et al*., 2021). *Rhipicephalus* species inflict heavy financial losses on the large ruminant industry. They suck blood from their hosts causing a direct impact on the economics of the livestock production system in terms of reduced milk production, increased weight loss and physical damage to hides whereas indirect losses are associated with ticks-vectored protozoal, viral, and bacterial diseases (Saleem *et al*., 2019; Calvano *et al*., 2021; Ceylan *et al*., 2021; Hussain *et al*., 2021). Among these species, *R. microplus* is the most important tick in large ruminants. Its importance can be understood from the fact that an engorged female of *R. microplus* ticks can cause a 0.6g reduction in weight gain of beef calves (Zaman *et al*., 2012). Moreover, 22-30 billion dollars annual loss is estimated to be caused by alone *R. microplus* infestation globally (Lew-Tabor and Valle, 2016).

Control of ticks is mainly dependent upon synthetic acaricidal drugs. These drugs are also used prophylactically and serve as the epicentre for the control and eradication measures against ticks. These drugs provide an effective and relatively quick control over tick populations (Selles *et al*., 2021). However, the long-term and extensive use of these drugs has caused some serious problems including the development of drug resistance, the presence of these drugs' residues in meat and milk of animals, and their toxicity to non-target organisms (Nath *et al*., 2018; Singh *et al*., 2019; Srisanyong *et al*., 2021; Goswami *et al*., 2022). Moreover, vaccination against tick infestation is another preventive strategy to overcome this problem but again there are some constraints limiting its effectiveness. Firstly, no single vaccine can prevent all the prevalent tick species and, secondly, there are antigenic strain variations which also undermine the vaccination's significance.

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Furthermore, the higher cost of vaccine development is another limiting factor (De La Fuente and Estrada-Pena, 2019; Ndawula *et al*., 2019).

Hence, there is a need for other effective alternative methods which can help us overcome the tick problem. Among these alternatives, botanicals can prove to be a promising solution. More than 2000 plant species are estimated to have some sort of pest control potential (Mamun and Ahmed, 2011). Many plants or their products are known to have antiparasitic and anti-tick activities (Shnawa *et al*., 2017; Selles *et al*., 2021; Eltaly *et al*., 2022). Of these products, essential oils are very important. A number of investigations carried out on essential oils have proven their repellent and acaricidal efficacies against various tick genera such as *Rhipicephalus*, *Dermacentor* and *Amblyomma* (Benelli and Pavela, 2018; Salman *et al*., 2020; Selles *et al*., 2021). Similar experiments using essential oils from various species of the *Cinnamomum* genus have revealed these oils to possess potent acaricidal activities. For example, the essential oils of *C. cassia* and *C. verum* have known acaricidal activities against the *Haemaphysalis longicornis* ticks (Qiao *et al*., 2021; Nwanade *et al*., 2023). However, the repellent and the acaricidal efficacies of the essential oil extracted from the bark of *C. verum* against the cattle ticks have not been investigated so far. Hence, the current study was undertaken for the estimation of the repellent and the acaricidal efficacies of the *C. verum* essential oil against the *R. microplus* ticks of large ruminants.

MATERIALS AND METHODS

Essential oil

The essential oil was extracted from the bark of *C. verum* using the hydro-distillation technique with the help of a clevenger apparatus. After extraction, the essential oil was analysed through the gas chromatography-flame ionization detection (GC-FID) procedure. The procedure was carried out at the University of Agriculture Faisalabad, Pakistan using the gas chromatograph apparatus of Shimadzu, GC-17A of the Central Hi-Tech Laboratory. This apparatus was attached to the flame ionization detector equipped with column DB WEX (30m×0.25) using nitrogen as its mobile phase having 20 ml/min flow rate. The oven temperature was maintained at 90°C for 2 min, 180°C for 2 min with the maximum temperature reaching 240°C for 3 min. The temperatures of the injector and detector were 250 and 270°C, respectively. The identification of the essential oils constituents was made on the basis of a comparison of retention times of both the samples' constituents and the standards used (Belhachemi *et al*., 2022).

Ticks

Only naturally-infested buffaloes and cattle with strict compliance of no acaricidal treatment in the past 30 days were selected for ticks collection. The ticks were collected from the district of Faisalabad, Punjab, Pakistan during the summer season. The collected ticks were transported to the Laboratory of Parasitology in special aerated plastic bottles having cotton swabs soaked in water for provision of the moisture. These ticks were washed, dried, and identified with the help of a stereomicroscope (Olympus SZ61) at 10X magnification using the Walker (2003) identification guide.

Test compounds

Five different concentrations (1, 2.5, 5, 10 and 20% v/v) of the *C. verum* essential oil were prepared using acetone as the solvent and the results were compared to the negative and the positive control groups. The negative control was the acetone for both the acaricidal and the repellent experiments while 0.1% cypermethrin acted as the positive control for the acaricidal bioassays whereas the diethyltoluamide (DEET) was the positive control in the repellent bioassay.

Acaricidal experiment

Dipping test

The test was conducted following the procedure described by Koc *et al*. (2013). During the test, 10 adult ticks of mixed sexes were selected at random and subjected to each dilution for five min of dipping. After this, these ticks were then taken out, placed in the clean jars and put into the biological oxygen demand incubator at 27°C and 90% relative humidity for 24 h. After 24 h, the ticks were observed, and the mortality was recorded by counting the live and dead ticks. Each treatment was replicated thrice in this experiment. The following formula was used for the determination of percent mortality (Sousa *et al*., 2022):

$$\text{Adult tick mortality (\%)} = \frac{\text{No. of dead ticks}}{\text{Total no. of ticks}} \times 100$$

Adult immersion test

The adult immersion test was conducted on the engorged female ticks following the procedure of Drummond *et al*. (1973) for the estimation of efficacy of *C. verum* essential oil on the reproductive capability of ticks. The female ticks were selected on the basis of their size, mobility, and body integrity. The ticks were weighed and dipped in the respective test solutions for 30 sec. After dipping, these ticks were taken out and dried gently using tissue paper. Then, these ticks were put into the biological oxygen demand incubator having a relative humidity of 90% at 27°C for 20 days. After this period, the laid eggs

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were separated from dead female ticks to avoid microbial contamination. These eggs were weighed and again incubated at 27°C for 30 days at the same relative humidity level of more than 80% as mentioned above. Each of the test treatments comprised ten engorged female ticks and was replicated thrice. The parameters of fecundity index (FI), egg hatchability (EH), oviposition reduction (OR), reproductive estimation (RE), and product effectiveness (PE) were calculated as described previously (FAO, 2004; Castro *et al*., 2018; Jia *et al*., 2018):

$$FI = \frac{\text{Oviposited eggs weight}}{\text{Female ticks weight}}$$

$$EH\ (\%) = \frac{\text{No. of larvae hatched from eggs}}{\text{Total no. of incubated eggs}} \times 100$$

$$OR\ (\%) = \frac{\text{Control FI} - \text{Treatment FI}}{\text{Control FI}} \times 100$$

$$RE = \frac{\text{Oviposited eggs weight (g)}}{\text{No. of engorged females}} \times \%\ EH \times 20000$$

$$PE\ (\%) = \frac{(\text{RE of control} - \text{RE of treatment})}{\text{RE of control}} \times 100$$

Syringe test

This test was conducted following the procedure of Sindhu *et al*. (2012). For this test, 3ml plastic syringes were cut and opened at their nozzle ends with the plunger partially pulled back. Around 200 (10 mg) eggs were placed in these syringes and their open ends were closed using organza fabric with tightly round rubber bands along the syringes barrels. These syringes were incubated at 27°C in the biological oxygen demand incubator having 90% relative humidity in the dark. After hatching, 14 days old larvae were used for estimation of the acaricidal potential of the essential oils. The syringes with larvae were filled with two millilitres of the test concentration and shaken for 30 sec. The test substance was then completely discarded and removed by pushing the plunger and with the help of tissue paper. Again, the plunger was drawn back to the marked 2 ml point. These syringes were then placed for one hour in the fume hood and then shifted to the biological oxygen demand incubator set at 27°C and 90% relative humidity under the dark conditions. After 24 h, the syringes were opened, and the mortality of larvae was recorded by counting the live and dead larvae. Only those larvae were considered live which had the ability of walking. The larval mortality (LM) was calculated using the following formula (FAO, 2004):

$$\text{Corrected LM}\ (\%) = \frac{\text{Test LM} - \text{Control LM}}{100 - \text{Control LM}} \times 100$$

Repellency experiment: Tick climbing test

This experiment exploits the climbing behaviour of *R. microplus* ticks for the test of their repellence towards particular substances. The repellency test was conducted following the procedure of Ndungu *et al*. (1995). For this test, a specific apparatus was used consisting of aluminium base attached to aluminium rods. The aluminium rods were slide over with the glass tubes and the ends of these glass tubes were covered with moist cotton wool plugs. The purpose of these plugs was to block the ticks' movement which climbed up to the top of these rods. These glass tubes provide the benefit of easy cleaning. These glass tubes were then wrapped around with filter paper strips of 1 cm width at 10 cm height from the base. This whole apparatus was then put into a tray having water in such a way that the upper surface of the base remained above water level. Ten ticks were then placed onto the base and the tubes were separated with a glass plate lifted 3 cm above base level to avoid mixing of odours. The test concentrations of the substances were then applied onto the filter paper strips. All this experiment was conducted at 27°C and almost 80% relative humidity and run for 1 hour. After one hour, the number of ticks above and below the filter paper strips were counted and the percentage repellence was calculated using the formula given below. This apparatus was cleaned thoroughly after running each experiment.

$$\text{Tick Repellency}\ (\%) = \frac{CN - TN}{CN + TN} \times 100$$

Where, CN is the number of ticks above the filter paper in the control while TN is the number of ticks above the filter paper in the respective treatment.

Statistical analysis

The obtained results for the experiments were analysed through the statistical procedures of the analysis of variance (ANOVA), Probit Analysis and Tukey's means comparisons test. All these procedures were conducted by keeping a confidence level of 95% and the results were considered significant at $P<0.05$ (Barrios *et al*., 2022; Park *et al*., 2022). These statistical procedures were applied using the IBM SPSS statistical software.

RESULTS AND DISCUSSION

The *R. microplus* ticks are the most important tick species throughout the globe, especially in the tropics, in terms of economic damage (Jabeen *et al*., 2022; Sultan *et al*., 2022). They are principally controlled by synthetic chemical acaricides. However, over the last few years, the research on botanicals including essential oils for investigation of their potential for control of ticks has been intensified. These botanicals are mixtures of a large number of active ingredients which possess diversified modes of action (Salman *et al*., 2020; Selles *et al*., 2021).

Essential oils, as the secondary metabolites, are the mixtures of different aliphatic and aromatic compounds

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produced by different families of the plant kingdom (Sharmeen *et al*., 2021). These essential oils may be extracted from the plants using different extraction techniques. However, in the current experiment, the essential oil was obtained from the *C. verum* bark through the process of hydro-distillation. When subjected to the phytochemical analysis, the *C. verum* essential oil was shown to be composed of a number of different components. These components were detected on the basis of their retention times using specific voltages. The major component revealed to have the highest concentration was cinnamaldehyde present at 33.6% concentration. These results were similar to the previous studies in the sense of cinnamaldehyde being the major component (Alizadeh *et al*., 2020; Pathak and Sharma, 2021). However, the difference observed in the detected concentration of cinnamaldehyde in the current experiment may be attributed to various factors like the age, soil nature, and the cultivar of the *C. verum* plant (Moghaddam and Mehdizadeh, 2017). Moreover, the chemical composition of the essential oils is also affected by the technique used for the essential oil's extraction (Ayub *et al*., 2023). All the detected components along with their respective concentrations and retention times are listed in Table I.

Table I. GC-FID analysis of *Cinnamomum verum* essential oil.

Name of the component	Concentration (%)	Retention time (min.)
Acetaldehyde	1.9	2.317
Benzaldehyde	7.8	22.567
Cinnamaldehyde	33.6	28.750
Citral	4.7	37.300
Ethyl acetate	1.7	1.883
Eugenol	4.6	25.700
Gamma terpinene	0.8	19.367
Gamma undecalactone	1.1	7.783
Geraniol	9.5	5.150
Isopropyl acetate	9.3	13.050
Limonin	4.6	32.967
Linalool	2.7	31.133
Nerol	4.7	40.550
Octanal	8.9	16.567
Unknown	1.7	1.433
Valerolactone	1.4	44.750

Table II. Lethal concentrations for *Cinnamomum verum* essential oil against adult ticks and larvae.

	χ^2	LC_{50}	95% CI	LC_{90}	95% CI
Adult tick	1.950	6.258	4.912-8.090	22.284	15.495-40.036
Larvae	3.706	5.009	4.658-5.385	16.646	14.829-19.009

As far as the acaricidal effectiveness of the *C. verum* essential oil is concerned, it exhibited a dose-dependent effect on the *R. microplus* ticks. The LC_{50} and LC_{90} values for the adults and larvae calculated using the probit analysis at 24 h are mentioned in Table II. The results revealed the larvae to be substantially more susceptible to the essential oil than the adult ticks as per the discovery of Ellse and Wall (2014). This acaricidal effect could have been exerted mainly by the cinnamaldehyde which was found to be the major component of this essential oil. Cinnamaldehyde is reported to be very effective against the *R. microplus* and other ticks exhibiting 100% mortality at 2.5-20 µL/mL concentrations (Nwanade *et al*., 2021). However, the varied response of the *C. verum* essential oil from that of the cinnamaldehyde is the reflection of overall antagonistic or synergistic interactions of both its major and minor components (Akhtar *et al*., 2012; Soares *et al*., 2016; Abbas *et al*., 2018).

Table III. Effect of different treatments on fecundity index and oviposition reduction of adult female ticks.

Treatment	Fecundity index	Oviposition reduction (%)	Egg hatchability (%)	Reproductive estimation (×20000)
A	51.78 ± 8.31^{A}	2.76 ± 15.60^{A}	80.1 ± 4.1^{A}	41.70 ± 8.81^{A}
B	48.69 ± 4.18^{AB}	8.56 ± 7.86^{AB}	77.5 ± 3.9^{AB}	37.78 ± 4.48^{A}
C	34.57 ± 8.59^{B}	35.08 ± 16.13^{B}	67.2 ± 2.5^{B}	23.37 ± 6.66^{B}
D	15.12 ± 2.03^{C}	71.61 ± 3.81^{C}	40.2 ± 3.1^{C}	6.12 ± 1.29^{C}
E	5.75 ± 1.13^{C}	89.20 ± 2.12^{C}	18.9 ± 4.0^{D}	1.07 ± 0.20^{C}
F	53.25 ± 7.84^{A}	0.00 ± 14.73^{A}	84.1 ± 6.1^{A}	44.50 ± 3.75^{A}
G	1.14 ± 0.83^{C}	97.86 ± 1.56^{C}	11.2 ± 5.7^{D}	0.10 ± 0.02^{C}

A: *C. verum* oil 1%; B: *C. verum* oil 2.5%; C: *C. verum* oil 5%; D: *C. verum* oil 10%; E: *C. verum* oil 20%; F: Negative control; G: Positive control. Mean values (±SD) with same superscript letters within the column differ non-significantly from each other (P>0.05).

In the current experiment, the *C. verum* essential oil also had a negative impact on the reproductive performance of the *R. microplus* ticks. This reproductive effect had a direct relation with the essential oil's concentration and was reflected in the reduced fecundity index, oviposition

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reduction, egg hatchability, and reproductive estimation (Table III). For most of these parameters, the obtained results for the 10% concentration varied non-significantly (P> 0.05) from those of the positive control group.

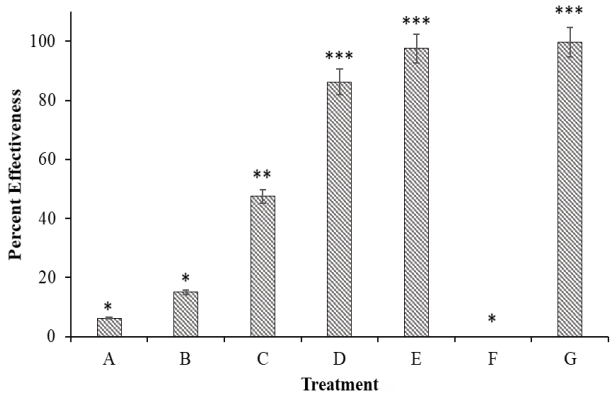

Fig. 1. Product effectiveness for *Cinnamomum verum* essential oil.
A, *C. verum* oil 1%; B, *C. verum* oil 2.5%; C, *C. verum* oil 5%; D, *C. verum* oil 10%; E, *C. verum* oil 20%; F, Negative control; G, Positive control. Bars with same superscript symbols differ non-significantly from each other (P > 0.05).

Moreover, the overall reproductive effect of the *C. verum* essential oil estimated through product effectiveness indicated the minimum 10% concentration of the essential oil to have similar results (P>0.05) to that of the positive control treatment (Fig. 1). The mechanism behind the acaricidal and reproductive effects of essential oils is very complicated. As the essential oils come into contact with the cuticle of ticks, these penetrate the haemolymph, thus, affecting the internal organs. Normally, ticks concentrate the ingested blood with the help of their salivary glands. Thus, any damage to these salivary glands leads to the poor absorption of nutrients. There exists a direct correlation between the ticks' reproductive and digestive systems. Hence, the impaired digestive system results in the reduced reproductive performance by the ticks (Remedio *et al*., 2016). As the *R. microplus* ticks possess a high biotic capability, hence, the products which can affect the reproductive performance of these ticks are of great importance for tick control (De Oliveira *et al*., 2016). These substances affect the ovaries, thus, reducing the female tick's capability to lay viable eggs (Remedio *et al*., 2015; Wang *et al*., 2020). Moreover, these oils may block the respiratory spiracles of ticks and disintegrate their cuticular waxes, thus, leading to suffocation and water stress (Agwunobi *et al*., 2020). Additionally, the central nervous system of the ticks may also get affected, thus, leading to the neurotoxic effect (Selles *et al*., 2021).

Similarly, the *C. verum* essential oil also exhibited a dose-dependent repellent effect against the *R. microplus* ticks. The probit analysis revealed the EC_{50} and the EC_{90} concentrations for *C. verum* essential oil to be 7.575 and 27.098%, respectively. The probit repellency graph for different concentrations of the *C. verum* essential oil is shown as Figure 2.

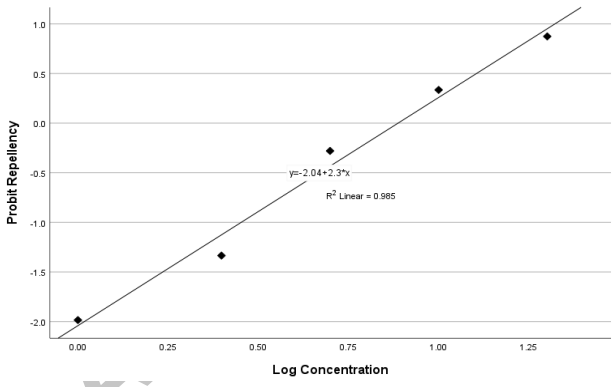

Fig. 2. Probit repellency graph for *Cinnamomum verum* essential oil.

As mentioned earlier in the discussion, the current repellent activity of the *C. verum* essential oil may also be attributed to the cinnamaldehyde which is already known to have repellent activity against insects (Deletre *et al*., 2019; Wahab *et al*., 2020). The volatile chemical substances of the essential oils are responsible for the exertion of repellent effects owing to their vapour barrier. They produce a driving impact on their target arthropod species which are then forced to move away from these odorous substances, thus, imparting a repellent potential to these substances. However, this volatile nature of the essential oils leads to the faded activity with time (da Silva Lima *et al*., 2016; Chen *et al*., 2019; Salman *et al*., 2020; Gupta *et al*., 2022).

CONCLUSION

From the results of the current experiment, it is very clear that the *C. verum* essential oil is capable of both killing and repelling the *R. microplus* ticks. However, trials on animals are suggested before recommending the use of this essential oil under the field conditions. Moreover, research should also be conducted on improving the techniques for essential oils extraction and prolonging the residual life of these volatile oils.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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