Essential oils of Tagetes minuta and Tithonia diversifolia affect host location behaviour and on-host attachment site preference of the brown ear tick, Rhipicephalus appendiculatus in the semi-field studies

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Abstract: Effects of essential oils of Tagetes minuta and Tithonia diversifolia (Asteraceae) on orientation behaviour and on-host attachment site preference of newly hatched adult Rhipicephalus appendiculatus were evaluated. Host animals were treated at the ear pinna (by smearing the oil directly on the ear or suspending a tube containing the oil on the ear pinna) and legs + tail in semi-field plots. The legs + tail sites of the essential oil application showed the lowest mean percentage of ticks observed on host body (16.5 ± 1.9% and 26.0 ± 2.8%) and the highest mean percentage reduction of attaching ticks (76.5 ± 3.9% and 67.0 ± 0.8%) for essential oils of Tagetes minuta and Tithonia diversifolia, respectively. The control animals had the highest mean percentage of ticks observed (93.0 ± 2.1%). The ear tube resulted in the highest mean percentage of ticks on the host (47.5 ± 5.1% and 55.8 ± 5.1%) and a lowest mean percentage reduction of attaching ticks (44.8 ± 5.1% and 36.5 ± 7.4%) for the essential oils of Tagetes minuta and Tithonia diversifolia, respectively. For both the essential oils, legs + tail sites of essential oil application, followed by ear smear and then ear tube, had significant effects on orientation to the host and attachment site preference of adult R. appendiculatus on the host, in that order. We, therefore, recommend the ear smear site for treating hosts with essential oils whose performance may improve upon increasing concentration and formulating them in a carrier material that stabilizes the active ingredients.

Key words: Host-seeking behaviour; Rhipicephalus appendiculatus; Semi-field repellent studies; Essential oils of Tagetes minuta and Tithonia diversifolia; Cattle

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Introduction

Ticks and tick-borne diseases continue to be a major constraint to livestock production and development, particularly in the tropics (McCosker 1993; Slingenbergh et al. 2002; Moyo and Masika 2008). The infestation of ticks on livestock and the resulting tick-borne diseases pose serious socio-economic problems (Jonsson et al. 1998; D'Haese et al. 1999; Makala et al. 2003; Peter et al. 2005; Muchenje et al. 2008).

The current conventional method of tick control relies mainly on the application of chemical acaricides. However, this method is associated with a number of problems including environmental pollution, chemical residues in food products as well as in wool, development of tick resistance and high costs of acaricides. The use of acaricides does not prevent the ticks already attached from transmitting the disease-causing pathogens. In addition, acaricide application only targets 5% of the total tick population in any given environment, while 95% of ticks, which are present on the vegetation, are left unaffected. Adult ticks can survive for more than a year without a blood meal, particularly in dense bush and rainforest (Lans 2001). These ticks become a source for re-infestation of host animals during grazing. A method and product that continuously keep ticks away from the hosts are therefore desirable. Among tick control strategies under consideration to that effect are prophylactic measures that prevent contact between ticks and host animals, i.e., repellents. Repellents provide a practical means of protection against tick bites and along with vaccines, they may be a fundamental resource for minimizing the transmission of tick-borne diseases at an individual level (Staub et al. 2002).

Repellent compounds in various commercial formulations are available for tick bite prevention. There is a concern however, about possible adverse effects of some of these compounds on human health and non-target species in the environment (Abdel-Rahman et al. 2001). For these reasons, the development of novel repellents can be of great value.

Repellent effects of some plants and plant products on the brown ear tick Rhipicephalus appendiculatus have been demonstrated (Dipeolu and Ndungu 1991; Malonza et al. 1992; Mwangi et al. 1995a,b; Lwande et al. 1999; Ndung'u et al. 1999). A 10% concentration of the essential oil of Ocimum suave in liquid paraffin was found to protect rabbits against the attachment larvae of Rhipicephalus appendiculatus and repelled more than 70% of adult R. appendiculatus for 5 days (Mwangi et al. 1995a; ICIPE 1998/99). A neem oil concentration of 25% applied directly to the skin of hosts repelled all stages of R. appendiculatus and showed some antifeedant activities (ICIPE 1997). Tagetes minuta and Tithonia diversifolia have been used traditionally to control livestock ticks in Central Kenya (Njoroge and Bussmann 2006).

In the present study, an approach providing protection against Rhipicephalus appendiculatus bites on hosts was explored. The effects of essential oils of Tagetes minuta and Tithonia diversifolia on host location and attachment site preference of newly hatched adult R. appendiculatus on hosts treated on the ear (smearing oil on the ear or suspending a tube containing oil on the ear) and legs + tail were evaluated.

Materials and Methods

Experimental host animals

A group of nine indigenous zebu cattle (Bos indicus) was purchased from livestock farmers in Bungoma County, western Kenya. Details of the area, such as the tick population, cattle diseases, climate and vegetation were previously described (Wanzala et al. 2014). Theileriosis is endemic in the study area and there are large populations of alternative wild animal hosts for cattle ticks and diseases. The experimental cattle used had been reared under traditional management in Bungoma County. Some of the animals were purchased during a market day from the Bukusu livestock farmers as steers aged between 16½ to 18 months, while others were oxen used in the farms for ploughing. According to farmers' records, these animals had been immersed weekly in various types of acaricides used indiscriminately in the area for the control of ticks. Records at Bungoma County Veterinary Office (DCO) indicated that acaricides used included organochlorine compounds [lindane (0.5% to 25% v/v), dieldrin (0.55% w/v) and toxaphene (0.25-0.3% w/v)], organophosphate compounds [chlorpyrifos (0.02% v/v), chlorfenvinphos (0.05% v/v), coumaphos (0.1% v/v), dioxathion (0 075% w/v) etc.], carbamate acaricides [e.g., carbaryl (0.2% w/v), promacyl (0.15% w/v)], pyrethrins (0.02% w/v), pyrethroids (permethrin, decamethrin, deltamethrin, cyhalothrin, cyfluthrin, flumethrin etc.) (0.02% - 0.05% w/v), and the commonly used formamidine (amitraz (0.025% w/v)].

Experimental ticks

The tick species used (Rhipicephalus appendiculatus) was obtained from a colony reared in the insectary at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. This colony was initially obtained from the rearing unit at the International Livestock Research Institute (ILRI), Nairobi, Kenya. The colony at ILRI had been established from collections at

Muguga in Kiambu County, Kenya and reared under laboratory conditions since 1952 using the methods described by Bailey (1960). At ICIPE, R. appendiculatus were bred on New Zealand white rabbits as described by Bailey (1960). Rearing conditions and management were as described previously (Bailey 1960; Irvin and Brocklesby 1970). The newly hatched adult R. appendiculatus were transported in a coolbox on moist sand from ICIPE to Bungoma (a distance of about 500 km), and used within a 48-h period after hatching.

Essential oils

Essential oils of Tagetes minuta and Tithonia diversifolia were obtained by the hydrodistillation method using Clevenger type distillation apparatus. Essential oils were diluted to 10% in odourless vaseline petroleum jelly (BP-USP 100% Grade) (Unilever Kenya Limited). This carrier material contained no colour, fragrance or irritants and was used for control experiments. The essential oils were transported from ICIPE to Bungoma in a cool box on ice and stored in a freezer at –4 ^{o}C until required for use on hosts. After being taken out of the freezer, the oils were used within 12 hours. The formulation used in this study was adopted from the previous assays in the laboratory and field at ICIPE while evaluating the essential oil of Ocimum suave against Rhipicephalus appendiculatus (Mwangi et al. 1995a; ICIPE 1998/99).

Experimental pasture plots

Adult Rhipicephalus appendiculatus that had been starved for 24 h were released in a demarcated pasture plot measuring 4m l x 2m w, between 09:00 and 11:00 hours (Fig. I). The release time coincided with the time when most livestock tick species leave their hideout to start hunting for suitable hosts for a blood meal (Norval et al. 1992). After this time, when it gets hotter, ticks hide under existing vegetation and/or in burrows to avoid desiccation and subsequent death. Within the demarcated experimental plots, any plants known to be used for protection against biting insects (Kokwaro 1993) and livestock ticks (Malonza et al. 1992; Lwande et al. 1999; Ndung'u et al. 1995,1999; Mwangi et al. 1995a, b) were removed to avoid interference with the results.

A ditch measuring 30 cm wide × 15 cm deep was dug round the demarcated plot (Fig.I). The ditch was lined with aluminium foil and filled with water to prevent the ticks from leaving the plot during and after the experiment. The plot was then infested with 200 adult R. appendiculatus. Freshly collected, tick-free Napier grass was evenly spread over the entire plot as fodder, to make the experimental animal walk around the demarcated plot comfortably while feeding on this and some other grasses found within the plot. The treated host, having either known levels of tick infestation or no prior exposure to ticks, was allowed to stay in the plot for 3 h in order for ticks to attach to it and move to their preferred feeding sites. Three hours was, in preliminary observations, found to be enough time for the ticks to infest the treated animals and reach the preferred feeding site. After 3 h, ticks were recovered from the exposed animal and counted. The plot was then burnt down using paraffin in order to kill any ticks that had not climbed on the host, and thus avoid tick infestation in the study area. A new plot was used for each of the 27 replicates.

Treatments

The nine host animals used were randomly divided into three groups of three, one for each of the treatment sites on the host: (A) Eppendorf tube with oil, suspended on the inner side of both ear pinnae of each animal (ear tube), (B) oil smeared on the inner side of both ear pinnae (ear smear), and (C) oil smeared on legs and tail (leg + tail smear) (Fig. I). In each group, one animal was randomly selected to be treated with petroleum jelly (control), one was treated with the essential oil of Tagetes minuta and the third host was treated with the essential oil of Tithonia diversifolia both essential oils diluted to 10% in petroleum jelly. Once used in this order, the hosts were not interchanged until the end of the experimental period in order to avoid cross contamination.

There were three independent replicates for each treatment. Each replicate was conducted in its own plot. For each treatment site and within each treatment application, one animal was used to make observations three times successively. Between the replicates, the entire body of the animal was thoroughly cleaned using 99% alcohol. Thorough cleaning was meant to remove any residues of previous applications of petroleum jelly or essential oils and any odours that may have been produced by previously attached ticks, which could have affected the results of successive trials. This precaution was taken because it is known that ticks themselves may play a role in guiding other ticks of the same species to preferred feeding sites, as has been observed in Amblyomma ticks (Schoni et al. 1984; Diehl et al. 1991; Norval et al. 1991) and Rhipicephalus appendiculatus (Sika 1996). After cleaning, the host was allowed to stay in the open for about 30-45 min before being used again, allowing its fur to dry by evaporation.

Recovery of ticks and determination of the impact of treatment on tick attachment

Ticks were observed and recovered on the body of the host at five specific sites. The five sites were: 1) Head-eyes/nose/ears/horn-base (Hd), 2) Dewlap (Dp), 3) Forelegs (Fl), 4) Hind legs (Hl), and 5) Tail (T). The five sites were chosen as recovery sites following preliminary field observations of grazing animals that showed them to be the most preferred areas of not only alightment by the ticks from their questing positions on the vegetation (Browning, 1976) but also of temporary attachment (Wanzala et al. 2004). The following formula was used to estimate the percentage of reduction of adult R. appendiculatus found attached on the host, caused by each essential oil type:

$$\text{\% reduction of ticks on host} = \frac{(\text{No. ticks released-No. attached in treatment})-(\text{No. ticks released-No. attached in control})}{\text{No. ticks released}} \times 100$$

Statistical analyses

Data of the number of ticks observed on the body of hosts were analysed by one-way analysis of variance (ANOVA) using the general linear model (GLM) procedure for SAS system for PC (SAS® Institute, 2002-2003). The effect of essential oils delivered on hosts at the ear (smear or tube) and legs + tail and the mean percentage of reduction of attaching ticks were compared by Students-Newman-Keuls test (Sokal and Rohlf 1995) at $\alpha = 0.05$. The effects of the essential oils of Tagetes minuta and Tithonia diversifolia on the number of ticks that located the host and attached to it was examined by paired samples t-test statistics using the t-test procedure of the Statistical Products and Service Solutions (SPSS) version 15 for windows. Data regarding the comparisons of the three essential oil application sites were analysed by one-way analysis of variance (ANOVA) and univariate analysis using the general linear model (GLM) procedure for SPSS. The mean differences were compared and separated using Students-Newman-Keuls H test at $\alpha = 0.05$ (Sokal and Rohlf 1995).

Legal use of field experimental animals

All procedures requiring the use of experimental animals in the field were approved by County Veterinary Office of Bungoma County, western Kenya. The importance and risk-free nature of the project was further explained to the Bukusu community by the DVO and agricultural extension officers working within the study area. The field experiments were performed in compliance with guidelines published by the Kenya Veterinary Association and the Kenya Laboratory Animal Technician Association, regarding the ethical use and handling of laboratory and farm animals in the field (KVA and KLATA 1989). Informed consent was obtained from the livestock farmer from whom we rented the experimental field plots.

Results

Treatment of hosts revealed a significant effect on the percentage of the released ticks counted on their bodies ($P < 0.05$) (Table I). More ticks were found on the control hosts than on the animals treated with the essential oils of either Tagetes minuta or Tithonia diversifolia ($P < 0.05$). (Table I). The mean percentage of released ticks counted on the hosts treated with the essential oil of Tagetes minuta was significantly lower than the mean percentage of released ticks counted on the hosts treated with the essential oil of Tithonia diversifolia for corresponding treatment sites ($t_{0.05}$ (8) = 3.438, P = 0.009).

The site of delivery of the essential oil on the hosts had a significant effect on the mean percentage of released ticks counted on their bodies ($P < 0.05$) (Table I). In a group of the hosts treated with the essential oil of Tagetes minuta, the method of essential oil delivery using the ear tube had a significantly higher mean percentage of released ticks counted on the host than the ear and legs + tail smears ($P < 0.05$). The legs + tail smear method had the lowest mean percentage of released ticks counted on the host. In the group of the hosts treated with the essential oil of Tithonia diversifolia , the mean percentage of released ticks counted on the host were significantly different for the three methods of delivery of the essential oil on the hosts with the ear tube method having the highest percentage of ticks counted on the host while the legs + tail smear method having the lowest percentage of ticks counted on the host ($P < 0.05$).

Treatment of hosts had a significant effect on the percentage of reduction of ticks on the host after 3 h of host exposure ($P < 0.05$) (Table I). There was a significant difference in percentage reduction of ticks on the hosts after 3 h exposure between the hosts treated with the essential oil of Tagetes minuta and Tithonia diversifolia for corresponding treatment sites ($t_{0.05}$ (8) = 3.438, P = 0.009). The percentage reduction of ticks on the hosts was significantly higher for animals treated with the essential oil of Tagetes minuta than the essential oil of Tithonia diversifolia ($P < 0.05$). The control animals had the highest mean percentage of ticks (percentage of ticks released experimentally) (93.0 ± 2.1%).

The site of treatment on the hosts had a significant effect on the percentage of reduction of ticks on the host ($P < 0.05$). In a group of the hosts treated with the essential oil of Tagetes minuta, the ear tube treatment had a significantly lower mean percentage of reduction of ticks on the hosts than the legs + tail smear method but the ear smear treatment was not significantly different from the other two ($P < 0.05$). In the group of the hosts treated with the essential oil of Tithonia diversifolia, the ear tube and smear treatment sites had a significantly lower mean percentage of reduction of ticks on the host than the legs + tail smear treatment site ($P < 0.05$). Corresponding values for mean percentage reduction of attaching ticks were significantly higher for the essential oil of Tagetes minuta, however, than those for the essential oil of Tithonia diversifolia ($P < 0.05$).

Of the three application sites of essential oils, smearing of essential oils of either Tagetes minuta or Tithonia diversifolia on the legs and tail of the hosts caused a significantly greater repellent effects on orientation to the host and attachment site preference of adult R. appendiculatus on the host than treatment of the ear with the tube or smearing the essential oils on the ear (Table I). In both the groups of host animals treated with either essential oil of Tagetes minuta or Tithonia diversifolia, the ear smear site was second to that of smearing essential oils on the legs + tail, and the ear tube third, in terms of preventing ticks from attaching to the host at their preferred feeding site (Table I). The three sites of application of essential oils had a significant effect on the mean number and percentage of adult R. appendiculatus observed on the hosts ($P < 0.05$) (Table I). The legs + tail and ear smear sites (with the exception of the sites treated with the essential oil of Tithonia diversifolia) were not significantly different from one another but the former was significantly different from the ear tube method ($P < 0.05$).

Site of tick attachment

The control hosts, treated with petroleum jelly only, were used as indicators for identifying preferential sites for attachment during the observation period and also as a reference point for comparing the behavioural effect of the two essential oils (Table I). The consideration was based on the mean number of ticks observed at different sites on the body of the host mean (Table II). The essential oil of Tagetes minuta delivered by the tube on the ear as well as all methods used to deliver the essential oil of Tithonia diversifolia, did not affect the choice of ticks for any of the five body sites (Dp, Fl, Hl, T and Hd) ($P > 0.05$) (Table II). This implies that using the three delivery methods, the essential oil of Tithonia diversifolia affected the attachment of R. appendiculatus to the five body sites equally, whereas with the essential oil of Tagetes minuta, there was a significant difference between the choice of ticks for body sites when using ear smear and legs + tail methods of delivering the essential oil ($P < 0.05$) (Table II). During the delivery of the essential oil of Tagetes minuta using the ear smear method, R. appendiculatus preferred attaching at the hind legs and then the fore legs, followed by the remaining three sites which were equally selected. When the essential oil of Tagetes minuta was delivered by the legs + tail method, the tail site was the least preferred for attachment by R. appendiculatus while the remaining four body sites were equally selected.

Comparison of the main repellent effects of the essential oils of Tagetes minuta and Tithonia diversifolia

The mean percentage of tick reduction on the host by the essential oil of Tagetes minuta (60.8 ± 4.9%) was higher than that by the essential oil of Tithonia diversifolia (49.3 ± 5.3%). The mean percentage of ticks observed (calculated as a percentage of the number of ticks released experimentally) on the host treated with the essential oil of Tithonia diversifolia (41.2 ± 4.8%) was higher than that of the essential oil of Tagetes minuta (29.7 ± 4.9%). This implies therefore that the essential oil of Tagetes minuta had a greater repellent effect on adult R. appendiculatus than the essential oil of Tithonia diversifolia. The means were compared by paired samples t-test and the two differences were found significant, $t_{0.05}$ (2) = 4.486; P = 0. 046.

Discussion and conclusion

In this study, all three treatment sites on the host gave some degree of protection against host-seeking Rhipicephalus appendiculatus, but the essential oils of Tagetes minuta and Tithonia diversifolia varied considerably in their effects. Rhipicephalus appendiculatus were more affected by the essential oils of Tagetes minuta and Tithonia diversifolia when smeared round the legs + tail of the host than when applied on the ear by either smearing or suspending a tube containing the oil on the inner side of the ear. However, treatment of the legs + tail is tedious, time consuming, and requires more essential oil, and hence may be uneconomical for use by the target group, the rural livestock farmers. Suspending the tube on the ear had the least effect on host-seeking R. appendiculatus, and its difference with the legs + tail treatment was significant for both essential oils (P < 0.05). The ear tube could possibly offer slow release of the odour, which would make it likely to affect ticks over a longer period of time than the other two sites. However, this has yet to be evaluated. Smearing the inner side of the ear of the host with the essential oil seems to offer the best compromise of ease of application and good repellency, and is therefore the technique that was considered and adopted for future use by the local communities during field experimentation in Bungoma County, western Kenya.

In the control hosts (with the exception of the ear smear method), the dewlap was the most preferred site for R. appendiculatus attachment followed by head, forelegs, hind legs, and tail in that order. These observations, however, appear to contradict the known fact that R. appendiculatus ticks have a marked preference for attachment to the ears and head region of cattle (Yeoman and Walker 1967). This contradiction may be due to the specific direction to which the odour coming from the ear initially flows in the highest concentration, as this would be more important to the 24 h-starved host-seeking ticks than tracing the actual source of the odour. This of course will depend on the positioning of the host ear and the direction of the breeze blowing around the entire ear of the host. Nevertheless, the odour intensity fluctuates considerably and gradually decreases after several hours compared to the source and this eventually results in the directional movement of ticks to the source (ear) (Sika 1996; Wanzala et al. 2004).

A number of ticks that were observed on the legs of the host were found between the hooves. This part of the leg provides the first soft part of the host that the newly-hatched, host-seeking ticks come into contact with upon arrival on the host body. These soft parts act as stimulants for the ticks to start preparing to feed (Sika 1996). This also accounts for the attachment of more ticks on the nose, eyelids and base of the horns, upon alighting on the host body from their questing positions on the vegetation. These parts are also soft and/or contain the soft waste residues of the host. By the time ticks were being located on the hosts, most of them were still scanning and therefore had not moved away from the entry site. However, the ticks that were removed from the host body while scanning, in the process of attaching, or attached, did not show any evidence of feeding; when crushed between white paper towels there was no blood apparent. Nor had the females increased in size, a physical manifestation of feeding

Due to the itching and irritation caused by tick movement and attachment on the host, a number of ticks are removed from the body of the host using tail, tongue and scratching with the mouth, more particularly on the forelegs and tail (Hadani et al. 1977). The hind legs are also used to scratch and remove ticks attached on the head. This may have reduced the number of ticks that were observed on the body of the host. From the previous studies (Sika 1996; Wanzala et al. 2004), there is no doubt that the efficacy of the essential oils was also affected by host-derived repellents (from the anal region) and attractants (from the ear) and other body emanations (Akinyi 1991; Sika 1996). Although it was hypothesized that the effects of host-derived repellents and attractants were masked by the essential oils, it is not yet known to what extent this happens. It is therefore possible that these host-derived repellents and attractants may be contributory factors to some of the observed discrepancies in the results, especially in the controls. The efficacy of essential oils was also influenced by several other factors, such as their chemical constituents, environmental conditions at the time of application, and the persistence and the spread of the active ingredients on the host body (Mwangi et al. 1995a).

This study has shown that the essential oils of Tagetes minuta and Tithonia diversifolia may provide a useful prophylactic means of reducing vector (tick)-host (livestock) contact, thereby reducing the number of tick bites and subsequent tick-borne infections, physical damage, and resulting secondary infections. Different formulations, particularly those that stabilize the active ingredients and perhaps higher concentrations than the one used (10%) should be tested in multi-locational field trials in order to determine whether these essential oils are appropriate for use on a large scale. The use of plant extracts with known effects on livestock ticks in this way may be a useful complementary or alternative tick control method to the heavy use of classical acaricides. This may decrease the quantity of toxic acaricide residues periodically released in the environment. In addition, the essential oils have the added advantage that they may provide a certain degree of control of flies and other ectoparasites that disturb and bite the target hosts (Toledo et al. 2003; Adedire and Akinneye 2004; Taiwo and Makinde 2005).

Competing Interests

The authors declare that there is no conflict of any interests regarding the publication of this manuscript in any ways.

Declaration

Authors declare that this manuscript is our own original research work and that have read and confirmed the content. Authors further confirm that the content of the manuscript has not been presented for publication in any other Journal and in any other form of this nature and that all sources of materials used for the research have been fully acknowledged accordingly.

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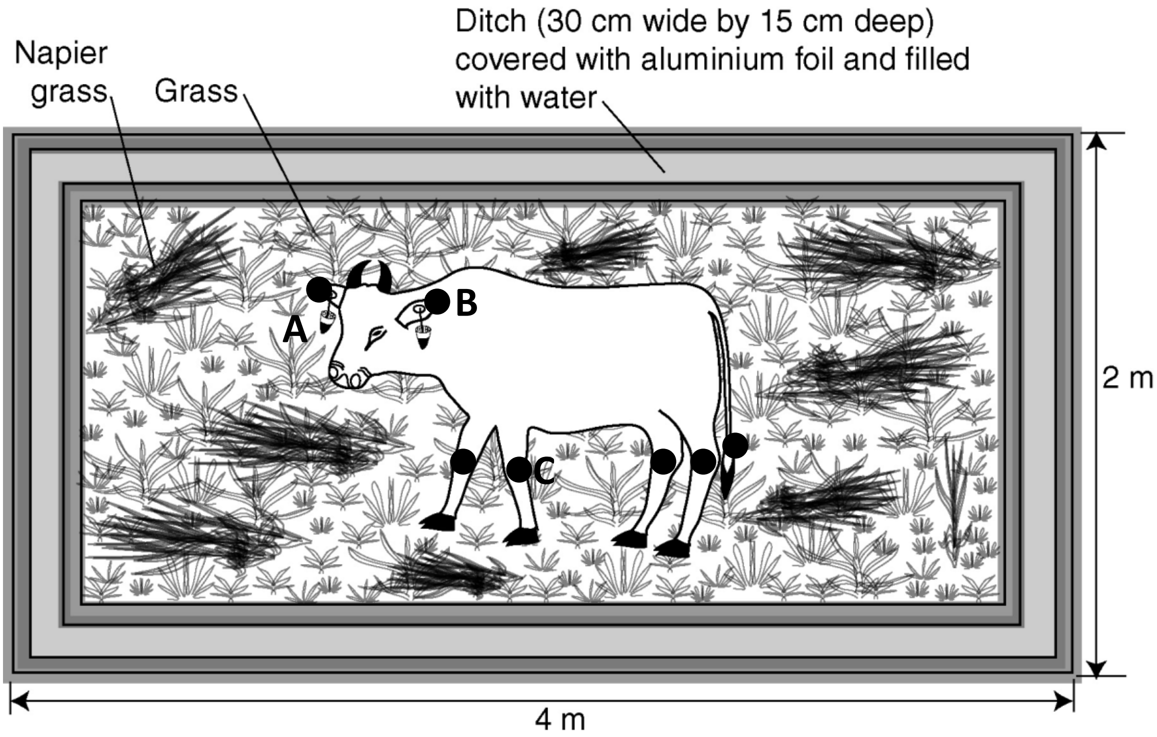

Fig.I. Experimental plot in which 200 adult Rhipicephalus appendiculatus were released, indicating various experimental treatments: (**A**) tubes with petroleum jelly (control) or essential oils of Tagetes minuta or Tithonia diversifolia; (**B)** ears treated by smearing petroleum jelly (control) or essential oils of Tagetes minuta or Tithonia diversifolia; and **(C)** legs and tail treated by smearing with petroleum jelly (control) or essential oils of Tagetes minuta or Tithonia diversifolia.

Table I. The mean (± SE) number and percentage of adult Rhipicephalus appendiculatus ticks observed on the host treated on the ear (smear and tube) and legs + tail with petroleum jelly (control) and the essential oils of either Tagetes minuta (Tm) or Tithonia diversifolia (Td) and % reduction in numbers on hosts of the 200 ticks released.

Animal code	Essential oil	Treatment site of the essential oil on the host	Mean tick counts on the host body after 3 h of host exposure (± SE)	Mean % of released ticks counted on the host after 3 h of host exposure	% reduction in ticks on host after 3 h of host exposure
Cet	Control	Ear tube	184.7±4.67d	92.3±2.33d	-
Ces	Control	Ear smear	172.3±3.18d	86.2±1.59d	-
Ctl	Control	Legs + tail smear	186.0±4.16d	93.0±2.08d	-
Tmet	Tm	Ear tube	95.0±10.21bc	47.5±5.11bc	44.8±5.10bc
Tmes	Tm	Ear smear	50.3±2.85a	25.2±1.42a	61.0±0.87ab
Tmtl	Tm	Legs + tail smear	33.0±3.79a	16.5±1.89a	76.5±3.91a
Tdet	Td	Ear tube	111.7±10.14c	55.8±5.07c	36.5±7.40c
Tdes	Td	Ear smear	83.3±9.39b	41.7±4.69b	44.5±5.80bc
Tdtl	Td	Legs + tail smear	52.0±5.69a	26.0±2.84a	67.0±0.76a

– not applicable, as the control values were used to calculate the mean % reduction in ticks on host in the equation described in the materials and methods.

For a given column, means followed by the same letter(s) are not significantly different from one another at $\alpha = 0.05$ level of significance (t-test, SAS).

Table II. The impact of treatment of the hosts with petroleum jelly (control) and the essential oils of Tagetes minuta (Tm) and Tithonia diversifolia (Td) on the site on the host where adult Rhipicephalus appendiculatus were observed to have attached (Dewlap, Dp; Forelegs, Fl; Hind legs, Hl; Tail, T; and Head-eyes/nose/ears/horn-base, Hd) (n = 200).

Treat-ment	Site of application of essential oil on the host animal	Mean (±SE) number of ticks observed at different body sites					P-value
		Dp	Fl	Hl	T	Hd	
Co	Ear tube	73.6±9.87a3	41.0±2.08b1	11.7±6.17c1	15.0±2.52c1	43.3±3.28b2,3	P<0.05
	Ear smear	63.3±12.02a2,3	36.7±12.24ab1	18.0±6.25b1	16.0±2.08b1	38.3±2.85ab2,3	0.015
	Legs + tail smear	77.7±4.91a3	24.7±5.90c1	22.0±0.58c1	8.0±3.79d1	53.7±1.333b	P<0.05
Tm	Ear tube	32.7±1.76a1,2	26.0±12.54a1	7.0±1.53a1	3.7±1.67a1	25.7±15.68a1,2	0.172
	Ear smear	3.3±0.88c1	8.7±1.86b1	26.7±1.20a1	7.0±0.58c1	4.7±0.88c1	P<0.05
	Legs + tail smear	7.0±0.58ab1	6.3±0.67ab1	5.7±0.33ab1	4.3±0.67b1	9.7±1.76a1	0.026
Td	Ear tube	56.0±24.01a2,3	19.0±5.69a1	19.7±13.67a1	8.7±6.67a1	8.3±6.36a1	0.145
	Ear smear	4.7±1.20a1	19.0±15.04a1	37.0±21.50a1	18.3±9.84a1	4.3±1.20a1	0.393
	Legs + tail smear	17.0±9.29a1	5.3±1.20a1	4.3±0.33a1	2.7±1.20a1	22.7±5.84a1,2	0.062
P-value		P<0.05	0.058	0.242	0. 154	P<0.05	

In a given row, means followed by the same letter(s) are not significantly different from one another at α = 0.05 (Students-Newman-Keuls H-test).

For a given column, means followed by the same number(s) after the alphabetical letter(s) are not significantly different from one another at α = 0.05 (Students-Newman-Keuls H-test).