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COMPARATIVE ANALYSIS OF DUAL AND NO-CHOICE REPELLENCY BIOASSAYS OF *TAGETES MINUTA* ESSENTIAL OIL AGAINST *RHIPICEPHALUS APPENDICULATUS* CLIMBING RESPONSE BEHAVIOUR

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ABSTRACT:

The study compared two sets of bioassays designed to evaluate repellency of *Tagetes minuta* essential oil against climbing response behavior of adult, *Rhipicephalus appendiculatus*, the vector of deadly livestock disease, East Coast fever. The study aimed at evaluating the appropriate bioassay set up suitable for screening repellent essential oils that may become applicable in preventive measures for managing arthropod vectors and vector–borne diseases. All bioassays were conducted under the same laboratory conditions. In both bioassays, repellency was dose–dependent and significant differences between doses remained the same at $P<0.0001$. However, for the same doses, mean per cent repellency was lower in no–choice bioassay (ranging from 39.30±2.53% to 69.5±3.00%) than in dual–choice bioassay (ranging from 57.92±7.11% to 100.00%). This difference was significant ($P = 0.047$) but its underlying mechanism however, remained unknown. In contrast to my initial predictions, using a no-choice tick climbing assay did not increase perceptions of treatment accuracy or a sense of self-efficacy; instead, the assay appeared costly and the repellent effect was comparatively lower. Probit analysis showed that to achieve the same repellent effect, a higher repellent dose is required in no–choice bioassay than in a dual–choice bioassay, hence the former proving unsuitable for screening purposes. Although the dual-choice assay appears to be an ideal method for testing tick repellent products, it requires that during statistical analysis of data generated by the repellency equation, a statistical model that includes all the existing variations and factors that are currently not considered in order that absolute repellency is estimated. These choice bioassays

however, provide baseline data against which novel tick repellents/attractants may be evaluated for development into agents suitable for providing prophylactic measures in integrated pest management. Nevertheless, the dual-choice assay proved a more sensitive assay than the no-choice assay.

KEY WORD: *Rhipicephalus appendiculatus, livestock ticks, repellency, Tagetes minuta essential oil, dual–and no–choice bioassays, integrated pest management.*

INTRODUCTION:

Livestock ticks are increasingly becoming not only a big nuisance but a serious animal and human health risk (Tonbak *et al*., 2006; Vial *et al*., 2006; Salit, 2007; Jongejan, 2007). Among prophylactic measures used against them, botanical prodglass tubeucts (repellents, deterrents, toxicants, stimulants, arrestants and attractants) have, in the recent past, proven to offer a sustainable approach toward integrated livestock tick control and management. In particular, plant–based repellents have been shown to protect vertebrates against tick bites (Weldon and Carroll, 2007) and have been recommended as an effective prophylactic measure against tick bites and/or tick-borne infection (Schreck *et al*., 1995; Okahl, 1996; Jaussaud *et al*., 2001; Jensenius *et al*., 2004; Roch *et al*., 2008). In the course of development of such repellents, an effective and reliable bioassay is essential for rapid screening of candidate products (Dautel *et al*., 1999) before selection. The rationale for developing a reliable bioassay is to help screen large populations of candidate tick repellent botanical products accurately and identify effective ones for incorporation into tick control strategies (McMahon *et al*., 2003).

Worldwide, research laboratories have developed different assay apparatus to evaluate repellent/attractant properties of candidate plant products against livestock ticks (Dautel *et al*., 1999; Jaenson *et al*., 2006; Carroll *et al*., 2003; 2005; Garboui *et al*., 2006). The assay methods employed vary a great deal but generally target the behaviour of ticks during questing for a host (Alekseev *et al*., 2000). In the course of these endeavours, both choice and no–choice assays are widely used to study the behavioural responses of arthropods towards botanicals (repellents, deterrents, arrestants, stimulants and attractants) and synthetic products. Nevertheless, these assays are also popular with research studies involving non–arthropod organisms (Howard *et al*., 1976; Mondy *et al*., 1998; Papachristos and Stamopoulos, 2002; Rodriguez–Saona *et al*., 2006). For instance, a dual–choice assay apparatus with the same scientific rationale as the one shown in Fig. 1A was used to test the ability of a termite to discriminate between two test chemicals and further used to show that trail pheromones in *Reticulitermes virginicus* Banks, 1907*, R. flavipes* Kollar, 1837 and *R. tibialis* Banks and Snyder, 1920 were species specific (Howard *et al*., 1976).

Basically, three types of assay methods are commonly used for testing tick repellents. First, test substances are applied onto vertebrate hosts, which are subsequently exposed to hungry ticks and the percentage of

feeding ticks and that of protection afforded are estimated (Bar–Zeev and Gothilf, 1973; Mount and Snoddy, 1983; Mehr *et al*., 1986; Carroll *et al*., 1989; Kumar *et al*., 1992; Solberg *et al*., 1995; Mwangi *et al*., 1995a). Secondly, test material is applied onto a horizontal or vertical walking path of ticks in the absence of any host cues and the percentage of ticks entering or passing the treated area is recorded and the protection percentage estimated (Dremova and Smirnova, 1970; Mathewson et al., 1981; Lane and Anderson, 1984; Kaaya *et al*., 1995; Malonza *et al*., 1992; Mwangi *et al*., 1995a, b; Ndungu *et al*., 1995). Thirdly, the test material is applied onto a horizontal or vertical walking path of ticks in the presence of host cues and the number of ticks entering or passing the treated area is recorded, from which the degree of protection afforded can be calculated (Alekseev *et al*., 2000). Using vertebrates as experimental hosts is unsuitable for routine tests with ticks because of the large number of animals required and because of the time– and cost–intensive procedures involved. On the other hand, assays without any host stimuli involved have the disadvantage that the behaviour-modifying activity of the tested material in the presence of host cues remains unknown (Schreck, 1977). Of particular interest too, is tick orientation behaviour under abiotic environmental parameters such as relative humidity, temperature and light as described by Okulova (1978) in the presence of candidate repellents/attractants and other host cues (Alekseev *et al*., 2000). Therefore, a suitable test system should be one that recognizes all these conditions. For example, in the present study, the test system used took into account relative humidity and temperature, which have been described as attractive host–derived and environmental cues that determine the questing behaviour of ticks such as *Ixodes ricinus* L. (McLeod, 1935; Leez amd Milne, 1951; Arthur, 1962; Alekseev *et al*., 2000). Furthermore, the test system examined the repellent activity of the essential oil of *Tagetes minuta* L. during critical behavioural steps of host finding.

Because the dual-choice assays more closely represent the natural situation in the field, they are preferred to no–choice assays and are widely used (Ryan, 2002). For example, in the USA, a dual–choice filter paper assay was successfully used by Roe *et al*. (2006) to develop a botanical tick repellent (BioUD) against the American dog tick, *Dermacentor variabilis* Say. Similar to a dual–choice filter paper assay is a vertical assay, in which ticks are allowed to climb a vertical strip of filter paper whose central portion is treated with a repellent. This method was also used by Carroll *et al*. (2003) to compare the repellent properties of N, N–diethyl–3–methylbenzamide (DEET) and 2–methylpiperidinyl–3–cyclohexene–1–carboxamide (AI3–37220) and to determine their relative effectiveness against host–seeking nymphs of the blacklegged tick, *Ixodes scapularis* Say, and the lone star tick *Amblyomma americanum* L.

In South Africa, Nchu et al. (2004) compared three types of tick climbing repellent assay methods, that is, a no-choice assay, an avoidance assay and a dual-choice assay using essential oils from *T. minuta* and *Lippia javanica* (Burm.f.) Spreng. Although more ticks avoided the essential oil of *L. javanica* than that of *T. minuta*, there was no significant difference in the abilities of the three assays to test the repellency of the

two oils against *Hyalomma marginatum rufipes* Koch ticks. In both no–and dual–choice assays, there was a significant increase ($P < 0.05$) in repellency with increasing concentration for both *T. minuta* and *L. javanica* essential oils.

In Kenya, the dual–choice assay apparatus has been used to study the repellent effects of some botanicals against livestock ticks (Malonza *et al*., 1992; Mwangi *et al*., 1995a, b; Ndungu *et al*., 1995). The assay apparatus was recently modified by inclusion of wider tubes to shield the inner climbing tubes, avoid diffusion of test materials laterally, and facilitate their more uniform gradients along the set–up (Wanzala *et al*., 2004). However, it was realized that in the dual–choice assay, the choice of the first tick to climb the glass tube fitted with either an essential oil–treated or control filter paper collar may influence the final score of the test as the choice of one tick may affect that of the others. The no–choice assay was therefore proposed, as it does not have this effect and results from the essential oil treatment and control can be compared as independent data sets. In the present paper therefore, we describe experiments that compare and contrast a dual–choice with a no–choice assay using the essential oil of *T. minuta* as the test substance to examine the climbing behavioural responses of *R. appendiculatus* adult ticks.

METHODS:

Legal framework of animal use

All experiments were conducted at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya and any procedures requiring experimental animals were approved by ICIPE's Institutional Animal Care and Use Committee and were performed in compliance with the guidelines published by the Kenya Veterinary Association and the Kenya Laboratory Animal Technician Association (KVA and KLATA, 1989).

Ticks

The tick species used (*R. appendiculatus*) was obtained from colonies at the International Livestock Research Institute (ILRI) and transferred to laboratories at the ICIPE, Nairobi, Kenya, for rearing and management. The rearing and management conditions were as described previously (Bailey, 1960; Irvin and Brocklesby, 1970).

Extraction of *Tagetes minuta* essential oils

Fresh aerial parts of *T. minuta* were cut into small pieces and about 1 kg was hydrodistilled using a Clevenger–type distillation apparatus for 8 h (Sereshti and Samadi, 2007). Pure oil was collected into 2 ml–glass vials, sealed and stored at –20 ^{o}C until required for analysis and assay studies.

The tick climbing assay apparatus

The two sets of tick climbing assay apparatus used included: – (1) a double stranded dual–choice (Fig. 1A) and (2) a no–choice climbing assay apparatus (Fig. 1B), both being a modification from Browning (1976) at ICIPE laboratories.

Double stranded dual–choice tick repellent climbing assay apparatus

A dual–choice tick climbing assay apparatus was used to test for tick repellency of essential oil of *T. minuta* (Fig. 1A). The assay apparatus exploited the behaviour of the ticks, *R. appendiculatus*, which climb up grass stems and settle for a period near the stem tip to wait for any passing potential hosts to go on board and start looking for predilection feeding sites (Browning, 1976; Chiera, 1985). This experiment was done according to the specification set up in the laboratories at ICIPE, Nairobi, Kenya. An aluminium base of area 105 cm^2 with two stands of 26 cm each in height and 7.0 cm apart were put in a basin of water, 1.5 cm deep (the water restricts the movement of the ticks to the aluminium base). The two sets of glass tubes were used, one of 4.5 cm (outer tube) and the other one 0.8 cm (smaller inner tube) in diameter. A strip of filter paper (Whatmann No 7, 2 cm wide) was stapled to form a collar around the upper parts of each smaller inner tube at a distance of 20 cm from the aluminium base to provide the source of either test odours or pure solvent. One collar on the pair of the tubes was treated with test odour solution and the other one with the same amount of pure solvent (dichloromethane–DCM) alone to serve as control. After the solvent was allowed to evaporate (for about 10 min), these tubes were shielded with wider tubes (4.5 cm d) from 3 cm above the aluminium base to shield the inner ones and limit the diffusion of the test material laterally and facilitate relatively uniform vertical gradients of the odours along the 3.7 cm gap between two tubes. The upper ends of larger tubes were plugged with dry cotton wool. Wet cotton wool plugging the top of the smaller tubes ensured relatively high relative humidity (>75%) within the columns. The test materials and the solvent were dispensed by a calibrated eppendorf pipette, equilibrated for 30 minutes and then five adult ticks of mixed ages and sexes were released at the centre of the aluminium base. Prior to each assay, ticks were kept at high relative humidity (RH) (>85% RH) for 24 h in containers with moist cotton wool.

All assays were conducted in a room of 28 ± 1 °C and 75 ± 5% RH. The room was continuously exhausted of air using a fan. The assays were left to run for 1 h, during which the number of ticks above the filter paper strip on the control glass tube (Nc) and on the treated glass tube (Nt) were counted and recorded after 15, 30, 45 and 60 minutes. After each test, the apparatus was thoroughly cleaned and dried at 100 °C. Initial comparison of the responses of ticks in the setup with and without residual dichloromethane on one and both sides, showed no bias for either side and no effects of the residual solvent. The repellent effect of the essential oil of *T. minuta* in dual choice assay was evaluated according to the formula adopted by

Ndung'u *et al*. (1995) and Lwande *et al*. (1999) namely: percent of repellency (PR) = [(Nc-Nt)/(Nc+Nt)] x 100, where Nt and Nc represent the number of ticks that climbed on or passed the treated and control collar of filter papers on the glass tubes, respectively.

Single stranded no–choice tick repellent climbing assay apparatus

Except for the experimental design, laboratory conditions and specifications of the single–stranded climbing apparatus were as described for the dual–choice assay (Fig. 1B). Two climbing rods were placed on separate bases at a distance of 27.5 cm within a tray (D) filled with tap water up to 1.5 cm deep. In each experiment, five newly–hatched and 24–h–hydrated adult ticks of mixed sexes were placed at a distance of 3.5 cm from the base of aluminium rod, B_1 (Fig. 1B). Here, the assays were also left to run for 1 h and the number of ticks above the filter paper strip on the control experiment glass tube (Nc) and on the treated experiment glass tube (Nt) were counted and recorded after 15, 30, 45 and 60 min.

The repellent effect of the essential oil of *T. minuta* was expressed in terms of non-climbing ticks as a suitable formula was not available. The non–climbing ticks are those that did not climb the glass tube fitted with a stapled collar of filter paper strip treated with either the essential oil of *Tagetes minuta* or dichloromethane (control) during the observation. These are ticks that deviated from the expected normal behaviour of climbing up the stems of vegetations to aggregate at their tips (Browning, 1976; Chiera *et al*., 1985).

Choice of the type and dose(s) of the essential oils used

In preliminary dose response assay studies with the essential oils of *T. minuta* and *Tithonia diversifolia* (Hemsl.) A. Gray, which had been selected from a group of eight plants for the sources of the essential oils (Wanzala *et al*., 2012), the former showed higher repellent effect against adult *R. appendiculatus* than the latter (Wanzala, 2009). The essential oil of *T. minuta* was therefore selected for comparative analysis of dual and no–choice repellency bioassays at the 0.025, 0.1 and 1 mg doses following preliminary studies.

Data management and analysis

The ticks'climbing behavioural responses data were entered into a Statistical Products and Service Solutions (SPSS version 16.0 for windows) spreadsheet database and analysed. A one–way analysis of variance (ANOVA) and Univariate analysis of SPSS were used to compare means between doses and over time. The means were separated using Student–Newman Keuls test at $\alpha = 0.05$ (Zar, 2009). Whereas data collected from the comparison of dual–choice and no–choice assays, an independent samples t–test was used to evaluate the difference between the mean percentage of non–climbing ticks of the essential oil–treated experiment and the control treated with dichloromethane in a no–choice assay (Dixon and Massey, 1969).

RESULTS:

Dual-and no-choice assay results

Data for no-choice and dual-choice assays were analysed differently and thus the results presented were unique to each bioassay type.

Repellent effect of *T. minuta* essential oil using a dual–choice assay

The results of the dose–dependent response of newly emerged adult *R. appendiculatus* to the essential oil of *T. minuta* in a dual–choice climbing assay apparatus were shown in Table 1. For a given dose and period of time of observation, there were varying degrees of dose–and time–dependent responses, respectively. In the first 15 minutes, there was a significant difference between mean percentage of repellencies caused by different doses of essential oil of *T. minuta* ($P = 0.006$). Thereafter, with the exception of 45th minute's observation ($P = 0.036$), there was no significant difference between the mean percentage of repellencies caused by different doses of the essential oil of *T. minuta* ($P > 0.05$). There was no significant difference between the mean percent of repellencies caused by lower doses of essential oil of *T. minuta* over time ($P > 0.05$). In the highest dose of essential oil of *T. minuta* (1 mg), there was a significant difference between the mean percentage of repellencies caused by the dose over time ($P = 0.017$) (Student-Newman-Keuls test) (Table 1). This assay did not show a clear trend of time-dependent responses of adult *R. appendiculatus* to the essential oil of *T. minuta*.

The overall mean percentage of tick repellency of *T. minuta* essential oil results obtained by the dual–choice assay are presented in Table 2.

Repellent effect of essential oil of *T. minuta* using a no–choice assay

The results reporting the mean percent of non–climbing ticks with respect to the doses of the essential oil of *T. minuta* and over a period of time of observation in the no–choice assay are shown in Fig. 2. These results suggest a time–dependent response in which the average number of ticks climbing up the glass tube in the presence of essential oil or dichloromethane was a function of the time taken for observation. In the first 15 minutes after release of ticks on the aluminium base, the proportion of non–climbing ticks was relatively high, but decreased with time as more ticks climbed the glass tube (Fig. 2). At any one given time, more ticks climbed up the control glass tube than the essential oil–treated glass tube for every observation made at all doses of essential oil of *T. minuta* (Fig. 2). This difference was significant ($P < 0.05$) (Table 3). Using an independent samples t–test, the mean per cent of non–climbing ticks in the essential oil–treated experiment was compared with the ticks not climbing the control glass tube whose collar of filter paper was treated with dichloromethane. The results of this comparison showed a significant difference between the control and the essential oil–treated experiment ($t_{(0.05)}(638) = 16.31$; $P < 0.05$). At every concentration, and for a given period of time of observation, the mean per cent of ticks not climbing

the glass tube fitted with a collar of filter paper treated with the essential oil of *T. minuta* was significantly higher than the mean percent of ticks not climbing the control glass tube treated with dichloromethane ($P < 0.05$) (Fig. 2 and Table 3).

The patterns of the graphs resulting from the treatment with the essential oil and dichloromethane for control were very similar, particularly the trend from low to high doses (Fig. 2). The mean per cent of ticks not climbing the glass tube fitted with a collar of treated filter paper increased significantly with increasing concentration of the essential oil of *T. minuta* ($P < 0.05$) (Fig. 2 and Table 3). At the highest dose of the essential oil, the repellent effect on *R. appendiculatus* was maintained for a longer period of time than with the other doses (Fig. 2(d)). However, at the lower doses of the essential oil, there was an increasing number of ticks climbing up the essential oil–treated glass tube over time. The gap between the control and essential oil–treated experiments widened with increasing concentration of the essential oil over time for all doses except 0.025 mg. Thus the variables, time, dose and repellent effect of the essential oil are thus interactive. The trend of the average number of ticks climbing up the glass tube increasing over time was also noted in the controls (Fig. 2). This trend of climbing ticks increasing in number over time in the control experiment was significantly higher than in the essential oil–treated experiment at all times of observation for all doses ($P< 0.05$) (Table 3).

Between doses of the essential oil of *T. minuta*, mean percent of ticks not climbing the glass tube fitted with a collar of treated filter paper were significantly different from each other ($P < 0.05$) (Table 3). With the 0.025 mg dose of essential oil, the mean percent of ticks not climbing the glass tube after the first 15 minutes was significantly different ($P = 0.041$) from the subsequent mean percent of ticks not climbing the glass tube thereafter, between 30 and 60 minutes (Fig. 2). Within a 60–minute observation period, the mean percent of ticks not climbing the glass tube due to the doses, 0.1 mg, 1 mg and 2.25 mg of the essential oil of *T. minuta*, were not significantly different from one another at $P = 0.087$, $P = 0.279$ and $P = 0.106$, respectively (Fig. 2). While in the control more ticks climbed up the glass tube than in the essential oil–treated experiment, there were some significant differences in the percent of ticks climbing up the glass tube between the observation periods ($P <0.05$) except for 0.025 mg dose ($P = 0.067$).

Comparative independent samples t-test and probit analyses

For corresponding doses (Tables 2 and 3), the mean percentage of repellency is lower in the no-choice assay than in the dual-choice assay. At the corresponding dose level, for all the three doses (0.025, 0.1 and 1 mg) used for comparison in independent samples t-test, there was significant difference between the overall mean percentage of tick repellency of *T. minuta* essential oil results obtained by the two assays ($t_{(0.05)}$ (438) = 2.757; p = 0.006). While probit analysis showed that to achieve the same

repellence rate, a much higher repellent dose of *T. minuta* essential oil is required in no–choice bioassay than in a dual–choice bioassay (Table 4).

DISCUSSION:

The two assay methods investigated in this study independently showed a significant repellent effect of the essential oil of *T. minuta*, even though the oil was not tested in the presence of host–derived stimuli as suggested by Dautel (2004). The repellent effect of the essential oil of *T. minuta* in the presence of host cues was demonstrated and concluded that the oil affected adult *R. appendiculatus* under various circumstances (Wanzala, 2009). Koschier and Sedy (2003) also showed a significant repellent effect of the essential oils from plants within the Lamiaceae family against *Thrips tabaci* Lindeman using the dual–and no–choice assays. The effect of the oils, though, was less in the no–choice assay compared to the dual–choice assay, particularly with low doses, implying that this assay method may be less suitable for screening purposes (Ryan, 2002). This is because the no–choice assay may not be able to identify plants whose essential oils have low repellent activity and is therefore less discriminatory. In the no–choice assay, high values of repellency comparable to those obtained in the dual–choice assay were only achieved with a high dose (2.25 mg) of the essential oil of *T. minuta*. Given that, in addition to the high doses of essential oils required, more ticks are used in a no–choice assay than in a dual–choice assay, a no–choice assay may be a more costly method than its counterpart. In contrast to our predictions, therefore, using a no–choice tick climbing apparatus to study the effects of repellent oils did not increase treatment accuracy; instead, it was time consuming and the mean repellent effects were comparatively low.

Although the two experimental set–ups are two different designs, the mechanism by which these assays exert their influence on tick behaviour, however, remains unknown. The advantage of the no–choice assay is that there was no interaction between the treated and control rods, as each rod was offered separately to the ticks (as an independent experiment). Because ticks in the treatment with the essential oil and in the controls were tested independently, the overall result of the assay should reflect the true effect of the oils on the ticks better than in the dual–choice assay, where ticks responding to the essential oil could affect each other and interfere with the end–result. It was realized that to obtain a better estimate of this behaviour, in the no–choice essay the behaviour of the ticks should ideally be examined singly as potential aggregating effects of ticks when examined in a group may affect the end–result (Sonenshine, 2006).

In a no–choice assay set–up, the rationale of giving a single tick at a time the chance to choose the glass tube to climb on as designed originally, was not there, and therefore, one would contemplate if the equation used to calculate the percentage of repellence caused by treatment as explained in materials

and methods section, was still valid for use or another one to be developed. An equation possibly more suited for estimating the percentage of effectiveness (PE) of a tick repellent is PE = 100 x (T_P – T_R)/ T_P), where T_P and T_R represent the average number of ticks per hour spent in wooded areas for the placebo and repellent groups, respectively (Staub *et al*., 2002). However, this equation also has shortcomings as all ticks involved in the interaction are not used in the evaluation of the end result.

The equation used in this study to generate the data for the dual–choice assay did not, however, recognize (a) the varied interactions involved such as: - (1) influence of the test odour from the treated filter paper collar and (2) test organisms that keep on climbing on the control and the treated glass tubes and vice–versa before making final decision, (b) test organisms that show the test material to confer either true repellent effects or excitorepellent/irritant effects, (c) test organisms that drop off in water and drown and (d) non–responding test organisms, which were initially part of the entire interaction and original population (n = 5). Although these factors are not considered in the equation, they influenced the number of ticks that climbed the filter paper collar on either the control or the essential oil–treated filter paper collar. In a no–choice assay apparatus, the interaction (1) above was minimized while interaction (2) was completely removed. Nevertheless, factors b, c and d mentioned above equally affected the results obtained by both tick climbing assay apparatus. This could be the reason why the results showed the same pattern of increase in repellent effects with increasing concentration, just as in the results obtained by Nchu *et al*. (2004; 2005). But this small difference emanating from interaction (2) above may not be sufficient to explain the low values obtained when a no–choice assay apparatus alone was used.

It is possible that from the experimental–design point of view, in both assays, there might be an aggregation effect amongst interacting ticks due to pheromones (Sonenshine 2006). This pheromonal influence may be having an effect on the climbing behaviour and other intraspecific interactions of *R. appendiculatus* ticks in both dual–and no–choice assays (Sonenshine, 1985). This aggregation behaviour of *R. appendiculatus* has also been observed in the laboratory (Browning, 1976) and field (Chiera, 1985) and was attributed to pheromones (Sonenshine, 2006). However, the pheromonal effect may be suppressed by the essential oil in both assays. This suppression may be greater in the dual–choice assay than in the no–choice assay due to the fact that the two glass tubes fitted with treated filter paper collar in the former assay were close to one another. This pheromonal effect could be removed if ticks were observed one at a time and between observations, the assay apparatus was rinsed with 99.98% alcohol and allowed to dry before being used again.

Although the dual–choice assay set up appears to be effective, it requires, during statistical analysis of data generated by the equation (PR = [(Nc – Nt)/(Nc + Nt)] x 100), a statistical model that includes all the existing variations and factors mentioned in the paragraphs above in order that the absolute

repellency is estimated with respect to these variations and factors. However, in a dual–choice assay, data are pooled after employing the equation (PR = [(PR) = [(Nc – Nt)/(Nc + Nt)] x 100), and therefore, this does not put into consideration the time over which the observations were made. A no–choice set up generates data that gives more comprehensive information on the behaviour of the ticks in response to the essential oil of *T. minuta* over time and within doses than the dual–choice set up, especially about the interaction of the variables selected for analysis. However, the no–choice assay does not simulate a natural field situation of freedom of choice (Ryan, 2002), thus changing the behaviour of test organisms, a situation that makes it a less efficient testing device (Huang *et al*., 2003; Adebowale and Adedire, 2006).

In the no–choice assay, more ticks climbed the control glass tube than the essential oil–treated glass tube for all doses of the essential oil of *T. minuta* over time, and this difference was significant. This suggests a significant repellent effect of the essential oil on climbing behaviour of *R. appendiculatus*. Over time, the gap between the number of ticks climbing the control and the essential oil–treated filter paper collar in the no–choice assay widened with increasing concentration of the essential oil of *T. minuta*. This implied significant dose–and time–dependent responses of *R. appendiculatus* adult ticks to the essential oil. Such consistent significant responses were not obtained with the dual–choice assay, except for dose responses. Thus, in the no–choice assay, the variables "time'' and "repellency" were interactive. The average number of ticks repelled was a function of the time of exposure of ticks to the repellent oil. In the first 15 minutes, the repellent effect was relatively high and thereafter this reduced with time. The reduction in repellency was greater with lower doses than with higher ones. The higher doses tended to maintain a higher repellent effect against ticks for a longer period of time than the lower ones. This trend of the results is comparable to that obtained by Dolan *et al* (2008) when testing essential oil (lemon, picaridin and nootkatone) against *I. scapularis* in vertical, finger and horizontal assays. However, these results were different from the results obtained with a dual–choice assay, implying that the two assays manifest different patterns of behavioural responses of ticks to the essential oil. The trend in which the average number of ticks climbing up the glass tube increased over time in the no–choice assay was also noted in the controls. Whether this trend reflected the natural tick climbing behaviour or not, is yet to be confirmed as the control material (dichloromethane) was not shown to cause any behavioural effects. It is possible that this behaviour was caused by increasing degrees of nutritional depletion, resulting in stronger behavioural responses.

CONCLUSION:

It was concluded that both bioassay methods tested in this study however, provided baseline data against which novel tick repellents may be examined and selected for field–testing and subsequent development

into agents suitable for providing prophylactic measures in integrated arthropod pest management. However, the dual–choice assay proved a more sensitive assay than the no–choice assay.

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Table 1. The mean (±SE) percent repellency caused by *Tagetes minuta* essential oil over time and at different doses using the dual–choice assay (n = 5).

Doses of essential oil of *T. minuta* (mg)	Time (minutes)				P–values
	15	30	45	60	
0.025	46.7±15.07^{b1}	66.7±14.05^{a1}	56.7±15.24b^{c1}	61.7±13.94^{a1}	0.795
0.1	51.7±13.71^{b1}	72.0±12.27^{a1}	72.0±12.27^{b1}	78.0±12.09^{a1}	0.483
1	100.0±00^{a1}	100.0±00^{a1}	100.0±00^{ab1}	88.0±6.11^{a2}	0.017
P–values	0.006	0.81	0.036	0.263	

Within a column, means with the same superscript letter(s) and across a given row, means with the same superscript number(s) after the alphabetical letter(s) are not significantly different at the level of significance, α = 0.05 (Student–Newman–Keuls test), respectively.

Table 2. The overall mean (±SE) percent repellency of the essential oil of *Tagetes minuta* obtained using a dual–choice assay.

Doses of essential oil of *Tagetes minuta* (mg)	Mean per cent repellency
0.025	57.9±7.11^{b}
0.100	68.4±6.26^{b}
1.000	97.0±1.69^{a}
P–values	< 0.05

Within a given column, means (±SE) with the same superscript letter(s) are not significantly different at the level of significance, α =0.05 (Student–Newman–Keuls test).

Table 3. The mean(±SE) percent of ticks not climbing a glass tube fitted with a collar of filter paper strip treated with either the essential oil of *Tagetes minuta* or dichloromethane (control) in a no–choice assay apparatus.

Doses of essential oil of *Tagetes minuta* (mg)	Mean percent of ticks not climbing a glass tube		
	Essential oil treatment	Control	P–values
0.025	39.3±2.53^{d1}	16.0±2.03^{c2}	< 0.05
0.100	50.3±2.59^{c1}	29.0±2.71^{b2}	< 0.05
1.000	69.5±3.00^{b1}	26.8±2.39^{b2}	< 0.05
2.250	99.5±0.35^{a1}	43.8±2.62^{a2}	< 0.05
P–values	< 0.05	< 0.05	

Within a given column, means (±SE) with the same superscript letter(s) are not significantly different at the level of significance, α =0.05 (Student–Newman–Keuls test). In a given raw, means (±SE) with the same superscript number(s) after the alphabetical letter(s) are not significantly different at the level of significance, α =0.05 (Student–Newman–Keuls test).

Table 4. Probit analysis of dose–response relationship of *Tagetes minuta* essential oil at RD_{50} and RD_{75} in a dual–choice (2c) and no–choice (Nc) bioassays, together with 95% fiducial limits.

Bioassay type	Repellence probability	Repellent dose (RD) (mg)	Lower confidence limit at 95%	Upper confidence limit at 95%
2c	0.50	0.20864	0.23762	0.18211
2c	0.75	0.22049	0.20312	0.23816
Nc	0.50	0.8873	0.8595	0.9168
Nc	0.75	1.6547	1.5992	1.7149

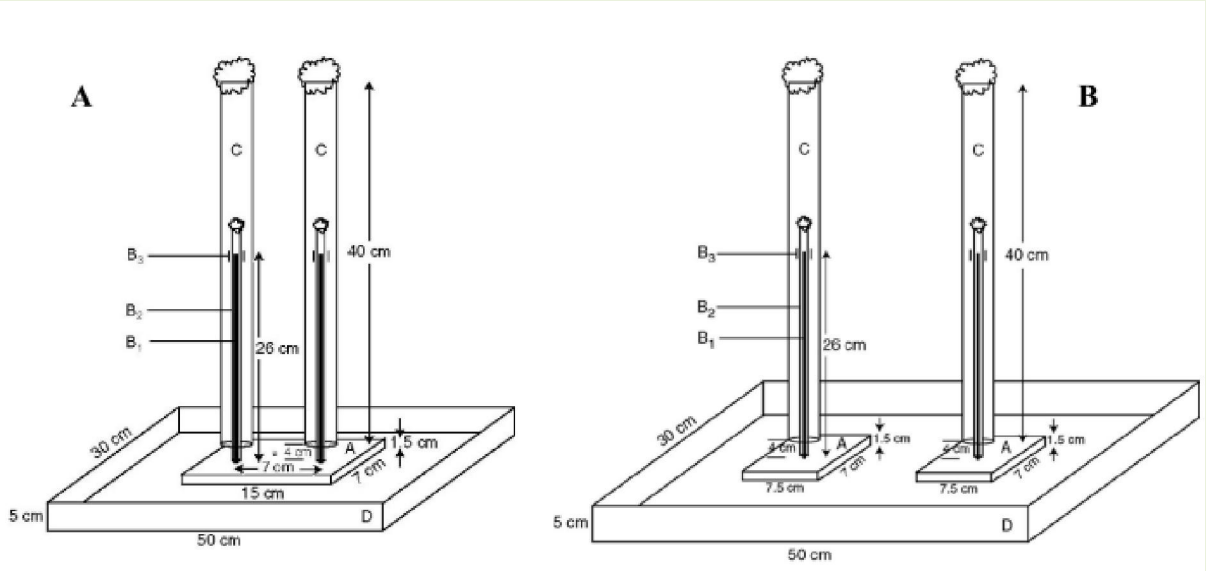

Fig. 1. Two different bioassay devices to study the impact of putative repellents on tick climbing response. **A.** Dual-choice climbing assay apparatus (placed in a tray with water (D) (50 cm l x 30 cm w x 5 cm h) up to 1.5 cm deep); A, alluminium base; B_1, aluminium rod (26 cm l x 1.7 cm d); B_2, 0.8 cm glass tube plugged with wet cotton wool; B_3, filter paper collar; C, 4.5 cm d glass tube plugged with dry cotton wool. The middle star (*) on alluminium base, A, indicates the centre where the 5 ticks were introduced (modified from Browning, 1976). The trough D is filled with water up to a depth of 1.5 cm so that it does not flood the aluminium base (B_1) on which 5 ticks are introduced at equidistance between the two aluminium rods (B_1) that are 7 cm apart. The ticks are given the freedom to choose the rod to climb on; the one bearing filter paper collar treated with the test material or the one bearing filter paper collar treated with the solvent alone (control). **B.** The 2 aluminium rods (B_1) on the aluminium base, A, (15 cm l x 7 cm w), are separated by water. The 5 ticks were introduced on each of the two aluminium bases at a distance of 3.5 cm from the base of aluminium rod, B_1 (modified from Browning, 1976).

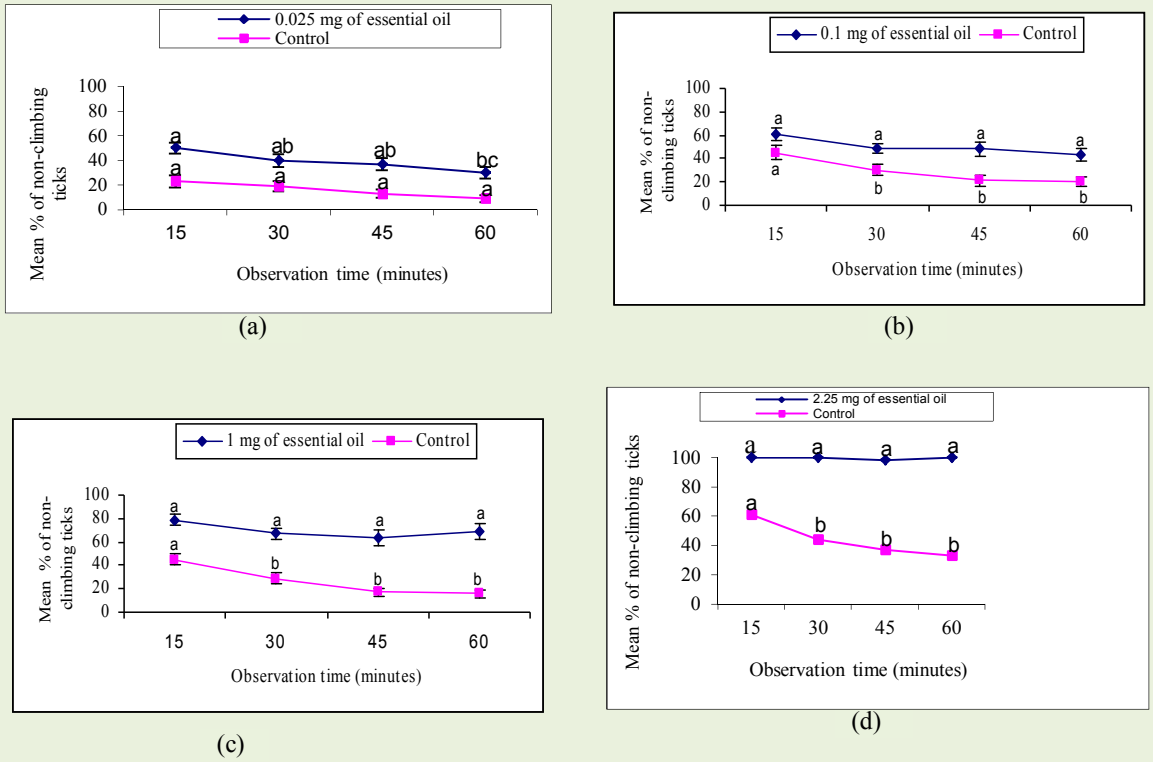

Fig. 2. Mean percentage of ticks not climbing a glass tube fitted with a collar of filter paper treated with either the essential oil of *Tagetes minuta* or dichloromethane (control). The effect of treatment on climbing response of *Rhipicephalus appendiculatus* is considered over time for different doses of essential oil of *Tagetes minuta* with adult *Rhipicephalus appendiculatus* using a no-choice assay. The Figures from **(a)** to **(d)** represent doses of essential oil of *Tagetes minuta* exposed to newly emerged *Rhipicephalus appendiculatus* in a no-choice climbing assay (n = 5). For either control or essential oil treatment, the means (±SE) with the same letters are not significantly different from one another at the level of significance, $\alpha = 0.05$ (Student-Newman Keuls test).