REPELLENCE OF CATTLE ANAL ODOUR CONSTITUENTS AND SELECTED ANALOGUES AGAINST *Rhipicephalus appendiculatus*

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ABSTRACT
A study was conducted to determine repellence of anal odour constituents, blends and selected analogues against *Rhipicephalus appendiculatus* control. The odours were trapped, eluted and analyzed by gas chromatography-linked mass spectrometry. Constituents were identified by comparing their mass spectra with those in the National Institute of Standards and Technology libraries. Major constituents were *o*-xylene, 4-hydroxy-4-methyl-2-pentanone, 4-methyl-2-methoxyphenol, ethyl benzene, 2,6,6-trimethyl-[1S(1α,β,5α)]bicycloheptanes, 5-ethoxydihydro-2(3H)-furanone, 3-methylene-2-pentanone, 5-methyl-2-phenyl-1H-indole, and 3-pentanone. Repellency of the odours, selected constituents and blends was evaluated using dual choice tick climbing assay. The 4-methyl-2-methoxyphenol was most repellent (RD_{75} =0.56) and 3-pentanone least active (RD_{75} = 622.7). Two blends that were more repellent than the anal odour were: made up of 4-methyl-2-methoxyphenol, 3-pentanone, 3-methyl-2-pentanone, and 4-hydroxy-4-methyl-2-pentanone, with RD_{75} of 0.032, and another without 3-pentanone, with RD_{75}= 0.019; $p \leq 0.05$, SNK. Bioassays of analogues of 4-methyl-2-methoxyphenol (4-Methylguaiacol) was done: guaiacol, eugenol, 3,4-methylenedioxytoluene and 2,4-dimethylphenol, where 3,4-Methylenedioxytoluene was inactive, while 2,4-dimethylphenol was most repellent (RD_{75}= 0.0089) compared to all compounds and blends tested. The 2,4-dimethylphenol analogue of 4-methylguaiacol may represent a promising additional tool in the arsenal of techniques in East Coast Fever control.
Key words: *East Coast Fever, Repellence, 4-Methylguaiacol, 2,4-dimethylphenol*

INTRODUCTION
Ticks transmit the widest variety of pathogens of any blood sucking arthropod including bacteria, rickettsiae, protozoa and viruses. Tick bites and tick-borne diseases continue to be a serious public health problem throughout the world (Carroll, 2005). Ticks are responsible for substantial losses to livestock industry (Willadsen *et al.,* 1988). Cattle tick borne infections are widespread in Africa and they present a great constraint (Walker *et al.,* 2003).

The tick *R. appendiculatus* Neumann 1901, *R. zambeziensis* (Walker *et al*., 2003, Norval *et al*., 1991) and to a lesser extent *R. duttoni* Neumann 1907, are the only known field vectors of *Theileria parva* Theiler 1904, the causative agent of cattle disease, East Coast fever (ECF) (Lawrence,1991). East Coast fever is one of the most devastating livestock diseases in east, central and southern Africa, and remains the major health hindrance to the development and improvement of the livestock industry (Norval *et al.,* 1992). East Coast fever causes economic losses to individual farmers and governments. Such losses can be classified into direct and indirect production losses, losses through costs incurred for controlling the disease and costs for providing research, training and extension services pertaining to the disease. Such economic losses vary widely within and among countries in both time and space, due to differences in livestock production systems, cattle types, level of disease risk, disease control policies and programmes cost and price structures (Mukhebi *et al*., 1992).

The disease is prevalent across the eastern, central, and southern parts of Africa, and has been reported in 11 countries in the region: Kenya, Uganda, Tanzania, Burundi, Rwanda, Malawi, Mozambique, southern Sudan, Democratic Republic of Congo (DRC), Zambia and Zimbabwe (Lawrence et al., 1992). About 28 million cattle in the region are at risk and the disease kills at least 1 million cattle per year. Economic losses are concentrated on small-scale resource-poor households (De Deken *et al*., 2007).The disease further prevents the introduction of the ECF-susceptible but more productive exotic breeds of cattle, hampering the development of the livestock sector considerably. This loss is termed "lost potential (De Deken *et al*., 2007).

ECF is thus a major limiting factor working against small-scale farmers attempting to climb out of poverty by moving from subsistence to market oriented enterprises as the demand for meat and milk in developing countries is expected to double by 2020, the majority which will be provided by small holders (VIE, 2002). This puts further pressure on the need to control ECF.

Ticks are commonly controlled using conventional acaricides which has been considered as the best method of controlling ticks, however it has certain shortcomings. Conventional acaricides are expensive or unaffordable to rural farmers. In some studies done by Garcia-Garcia *et al*. (2000) and Laffont *et al*. (2001), residues of conventional acaricides were noticed in meat and dairy products and also caused contamination of environment via the cattle dung. Some reports have shown that ticks have developed resistance against a range of conventional acaricides (George, 2000).

In most areas of the world, ticks have become resistant to arsenic and organophosphorus acaricides (Drummond, 1983). Chlorinated hydrocarbon acaricides have also been withdrawn from the market (Spickett, 1998), because of their high toxicity and long residual (lifespan). Carbonates are more toxic than organophosphates for mammals and are much more expensive (Spickett, 1998). Existence of alternative wild hosts increases the tick population and also the diseases, (Young *et al.*, 1988).

The limitation associated with the current methods of ECF control and the opportunities for reducing reliance on intensive acaricides use in region have prompted the search for new, safer, cheaper and more sustainable control strategies. The use of semiochemicals represents one such strategy.

MATERIAL AND METHODS

Stocks of *R. appendiculatus* ticks were obtained from colonies at International Centre of Insects Physiology and Ecology (ICIPE) Nairobi, Kenya. Rearing conditions were as described by Irvin and Brocklesby (1970).

The odour trapping was carried out on Friesian steers (Figure 6) at Kenyatta University cattle shed using adsorbent sachets (4 x 4 cm) made of filter papers containing 0.2g of either reversed-phase C_{18} –bounded silica, Porapak Q or Super Q. Six such traps were be placed in an oven bag (An oven bag is a special plastic bag used for the roasting of meat or other food in an oven) and this bag was attached to the anal region of the cattle as shown in Figures 3.1 using metallic clips. Prior to use, traps were cleaned first by putting them into a 200 ml soxhlet extractor for 3 days, dried, and then flushed of any contaminant with a stream of dry nitrogen at 60 ˚C for 3 hours.

The oven bags were heated in the oven at a temperature of 100˚C to remove any volatiles that may have been present. The sachets were held on the anal region for 6 hrs. The trappings were wrapped with clean aluminium foil, and in metallic tins. These tins were carried in a flask containing dry ice, Then transferred into a Pasteur pipette and eluted with distilled dichloromethane (4ml, > 99.9%).elution was carried out under dry ice. Elution from trapping cycles was pooled, concentrated and stored at -20˚C until required for analysis and bioassay. For bioassay, volumes of 100 µl, 200µl and 300µl were used.

A dual-choice tick climbing assay apparatus was used for screening the repellency of the odours, synthetic chemical compounds the blend, all at doses of 0.001, 0.01, 0.1, and 1 mg respectively. DEET which was a positive control was also screened in a similar manner. The bioassays were done using a tick climbing assay in a laboratory at ICIPE (Wanzala *et al*., 2004). The assay protocol exploited the behavior of the adult ticks, *R. appendiculatus,* which climb up grass stems to the stem tip to wait for any potential passing host (Chiera *et al.,* 1985). An aluminum base of area

105 cm^2 with two stands of 26 cm in height and 7.0 cm apart was put in a basin of water 1.5 cm deep (to retain the ticks at the base) this was as describe by Wanzala *et al*., 2004. A strip of filter paper (Whatman No 7, 4.25 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 aluminum 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) alone to serve as control. The solvent was allowed to evaporate for about 10 minutes after which these tubes was shielded with wider tubes (4.5 cm diameter) from 4 cm above the aluminum base to shield the inner ones and limit the diffusion of the test material laterally and facilitate relatively uniform vertical gradients of the odors along the 3.7 cm gap between two tubes. The upper ends of the larger tubes were plugged with dry cotton wool. The top of the smaller tubes was plugged with wet cotton wool to ensure relatively high relative humidity (>75 %) within the columns.

Ten adult ticks were be placed at the centre of the aluminum base and observed for 60 minutes. The recording was done after every 15 minutes. Each assay was replicated 6 times. The number of ticks that climbed on treated and control columns were counted. Mean % repellency was calculated using the formula

Percentage repellency $(PR) = \frac{Nc-Nt}{Nc+Nt} \times 100$

Where Nc = the number of ticks that climbed on the untreated glass rod tubes and
Nt = the number of ticks that climbed on and or above the filter paper collar strip on the treated glass tube respectively.

Anal and dung odours were analysed using gas-chromatography (GC) and gas chromatography-linked mass spectrometry (GC-MS) techniques (Tholl *et al*., 2006*)*. One microlitre of the eluent sample was injected into the HP 6890 series gas chromatography interfaced to a 5973 Mass Selective Detector (MSD) and controlled by HP chemstation software (version b.02.05, 1989-1997). The chromatographic separation was achieved using a DB-5 MS capillary column (30.0 m x 0.25 mm x 0.25 µm). The column stationary phase comprised of 5%-diphenyl- 95% dimethylpolysiloxane.

The operating GC condition was an initial oven temperature of 50 °C then programmed to 300 °C at the rate of 10 °C/minute and then kept constant at 300 °C for 3 minutes. The injector and detector temperatures were set at 250 °C. The mass spectrometer was operated in the electron impact mode at 70 eV. Ion source and transfer line temperature was kept at 300 °C. The mass spectra were obtained by centroid scan of the mass range from 40 to 800 amu. The constituents of the odours was identified by analysis of their mass spectra, direct comparison of their mass spectra to the Wiley NBS and NIST database of library of mass spectra, on the GC equipment.

Two blends of anal odour constituents were tested. Blend 1 was made up of 4-methyl-2-methoxyphenol, 3-pentanone, 3-methyl-2-pentanone, and 4-hydroxy-4-methyl-2-pentanone. Blend 2 comprised of 4-methyl-2-methoxyphenol, 3-methyl-2-pentanone, and 4-hydroxy-4-methyl-2-pentanone (i.e. without 3-pentanone).

Data Analysis

The repellency data obtained at different concentrations were subjected to analysis of variance (ANOVA) for a completely randomized design. Treatment means were separated using Student-Newman-Keuls (SNK) at $p < 0.05$ significance level. Dose-response relationship was determined using probit analysis and repellent concentrations at RD_{75} values obtained from the regression model.

RESULTS

Anal odour constituents

The anal odour was analyzed using GC –MS. The analysis of the odours revealed a complex mixture of constituents. A total of 43 compounds were identified in the anal odours. These compounds included ketones, phenols, amine and alcohols. Figure 1. The order of occurrence of the 10 major compounds was as follows: 3-methylene-2-pentanone, 5-methyl-2-phenyl-1H-indole, 4-hydroxy-4-methyl-2-pentanone, ethylbenzene, *o*-xylene), , 4-methyl-2-methoxyphenol, 5-ethoxydihydro-2(3H)-furanone, 3-pentanone , thymol and 2,6,6-trimethyl-[1S(1α,β,5α)]bicycloheptane(Table 1).

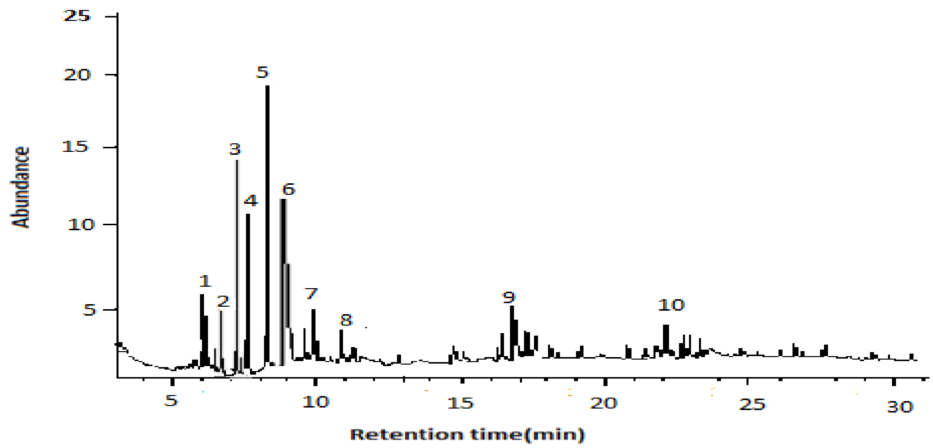


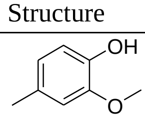
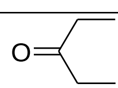
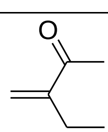
Figure 1: GC-MS profile of the anal odour

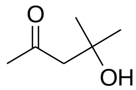
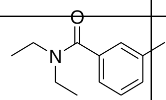
Table 1: Major identified constituents of the anal odours

No.	Compound	Relative (%)
1	3-Methylene-2-pentanone,	5.8
2	5-Methyl-2-phenyl 1H-Indole	4.9
3	4-Hydroxy-4-methyl-2-pentanone	14.1
4	Ethyl benzene	11.2
5	*O*-Xylene	19.2
6	2-Methoxy-4-methyl phenol	12.4
7	5-Ethoxydihydro 2(3H)-furanone	4.8
8	3-Pentanone	4.3
9	Thymol	4.5
10	2,6,6-Trimethyl-1S-bicyclo[3.1.1]heptane	3.1

The data in Table 2 summarizes the repellent effects of the synthetic compounds. At the smaller doses (0.001 and 0.01 mg), 3-methyl-2-pentanone had a negative repellency, i.e. it was attractant to the tick. However, at higher dose, it was significantly repellent against *R. appendiculatus.* Of the 5 compounds tested, 3-pentanone was least repellent (RD_{75}=622.7) as compared to 3-methylene-2-pentanone (RD_{75}=1.34) and 4-hydroxy-4-methyl-2-pentanone (RD_{75}=4.93). Further work needs to be done to ascertain whether the position of the carbonyl group has any effect on repellence. 4-Methylguaiacol had the highest repellency (RD_{75}=0.56) against *R. appendiculatus*. The repellency of the compounds against *R. appendiculatus* was compared with those obtained with DEET. The repellence of DEET (RD_{75}=0.0014) proved to be better compared to compounds identified in the anal region.

Table 2: Repellency of synthetic compounds at different doses

Dose (mg)		0.001	0.01	0.1	1	
Compound	Structure	Mean(±SE)	Mean(±SE)	Mean(±SE)	Mean(±SE)	RD_{75}
4-Methylguaiacol		33.9 ± 3.9^{Db}	41.4 ± 8.4^{Cbc}	53.3 ± 4.8^{Bb}	89.7 ± 6.65^{Aa}	0.56^{b}
3-Pentanone		18.3 ± 2.4^{Ccd}	$31.0 \pm 5.2B^{Cc}$	31.1 ± 4.3^{BCcd}	50.2 ± 6.4^{Ad}	622.7^{f}
3-Methylene-2-pentanone		-22.2 ± 9.2^{Ce}	-13.65 ± 5.2^{Ce}	37.5 ± 13.3^{Bc}	76.6 ± 11.4^{Abc}	1.34^{c}

4-Hydoxy-4-methyl-2-pentanone		14.1±3.5^{Ccd}	28.9±6.4^{BCcd}	36.8±7.9^{BCc}	81.5±8.5^{Ab}	4.93^{d}
DEET		76.7 ±4.8^{Ba}	83.1 ±5.8^{Aa}	87.8 ±5.1^{Aa}	97.0±3.3^{Aa}	0.0014^{a}

Mean (±SE) with the same lowercase letter in each column and uppercase letters in each row are not significantly different at α=0.05 (Student-Newman-Keuls test), respectively.

Repellent effects of synthetic blends against *R.appendiculatus*

One of the blends (made up of 4-methyl-2-methoxyphenol, 3-pentanone, 3-methyl-2-pentanone, and 4-hydroxy-4-methyl-2-pentanone) tested gave RD_{75} of 0.032. The other blend (without 3-pentanone) was more repellent (RD_{75}= 0.019; $p \leq 0.05$, SNK). The repellency data are given in Table 3. There was no overall significant difference between the repellency of blend 1 and blend 2 as reflected in the RD_{75} values.

Table 3: Repellency of selected blends

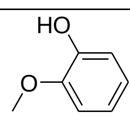
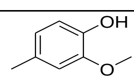
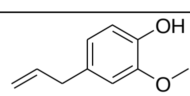
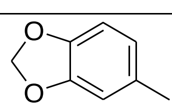
Dose(mg)	0.001	0.01	0.1	1		
SAMPLE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	p-value	RD_{75}
Blend 1	56.9±5.6^{Ca}	67.1±1.7^{BCab}	83.0±7.7^{ABa}	95.2±4.8^{Aa}	0.001	0.0322^{b}
Blend 2	64.1±4.6^{Ca}	64.2±3.9^{Cabc}	81.2±6.1^{Ba}	100.0±.0^{Aa}	6.03 x10^{-6}	0.0196^{a}
P value	0.343	0.509	0.859	0.341		

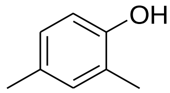
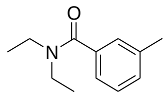
Mean±SE with the same upper case letters in each row and lower case letter in each column are not significantly different at α=0.05

Repellent of selected 4-methylguaicol analogues against *R. appendiculatus*

4-Methylguaiacol having been the most repellent of the compounds emanating from the anal region, some analogues were also tested against *R. appendiculatus.* These included guaiacol, eugenol (4-allyl-2-methoxyphenol), 3, 4-methylenedioxytoluene and 2, 4-dimethylphenol. Their repellent effects against R.appendiculatus are as shown in Table 4. At the lower concentration (0.001 and 0.01 mg), guaiacol and 3,4-methylenedioxy toluene had a negative repellency, i.e. they were attractant to the tick. The repellent Dose of 2,4-dimethylphenol at RD_{75} (0.0089) (Table 6) are the lowest. This implies that 2,4-dimethylphenol was more effective as a repellent compared to the other analogues. 3,4-Methylenedioxytoluene had an extremely low activity and therefore would not be considered as an effective repellent. In this study, 2, 4-dimethylphenol elicited the best response of all the analogues tested.

Table 4: Repellency by 4-methylguaicol analogues

	Dose mg	0.001	0.01	0.1	1	
Compound	structure	Mean(±SE)	Mean(±SE)	Mean(±SE)	Mean(±SE)	RD_{75}
Guaiacol		-38.9±3.5^{Dd}	38.7±5.5^{Cb}	46.1±11.9^{Cb}	95.2±4.8^{Aa}	0.66^{e}
4-Methylguaiacol		33.9±3.9^{Db}	41.4±8.4^{Cb}	53.3±4.8^{Bb}	89.6±6.6^{Aa}	0.56^{d}
Eugenol		13.7±3.3^{Cc}	16.9±4.4^{Cb}	56.1±2.9^{Bb}	100.0±.0^{Aa}	0.21^{c}
3,4-Methylenedioxy toluene		-2.6±10.1^{Cc}	-11.1±8.7^{Cc}	6.6±3.0^{ABc}	29.2±7.1^{Ac}	10>^{f}

2,4-Dimethylphenol		61.7±7.9^{Da}	76.7±10.5^{Ca}	87.8±7.8^{Ba}	100.0±.0^{Aa}	0.0089 b
DEET		76.7 ±4.8^{Ba}	83.1 ±5.8^{Aa}	87.8 ±5.1^{Aa}	97.0±3.3^{Aa}	0.0014 a

Mean±SE with the same lower case letter in each column and upper case letters in each row are not significantly different α=0.05)

DISCUSSION

Of the anal odour constituents tested against, 4-methylguaiacol had the highest repellency (RD_{75}=0.56) against *R. appendiculatus*. Interestingly, previous structure-activity studies involving the repellency of a series of guaiacol analogues against tsetse, showed 4-methylguaiacol to be most repellent (Saini and Hassanali, 2007). 3-methyl-2-pentanone had a negative repellency at lower doses but was repellent at higher doses. However; this is not strange to insect behavior as demonstrated by locust which had been found to prefer to be within phenylacetonitrile (PAN) -permeated air column at low relative doses of the pheromone, but away from PAN at high relative doses in a choice assay (Rono *et al*., 2008).

The choice of the analogues was based on the fact that, 4-methylguaiacol was the most repellent anal odour constituent. In the present study, some of the analogues were significantly the repellent against *R. appendiculatus.* 2 ,4-dimethylphenol and eugenol showed high repellence. 2 ,4-Dimethylphenol was the most effective as a repellent compared to the other analogues. These results suggest that 2 ,4-dimethylphenol represents a promising tool for protecting individual cattle against East coast fever.

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