Potential of Integrating *Calpurnia aurea* with Entomopathogenic Fungus *Metarhizium anisopliae* for the Control of *Rhipicephalus appendiculatus* and *Rhipicephalus pulchellus*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

To my wife **Justine Hidzem Manedji** and my daughter **Karen Djamen Nana**, I dedicate this dissertation, fruit of many months of hard work and sacrifices. Thanks for your love and support.

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LIST OF ABREVIATIONS AND ACRONYMS

ANOVA   Analysis of Variance

BCA   Biological Control Agent

DDT   Dichlorodiphenyltrichloroethane

DEET   N, N-Diethyl-meta-Toluamide

Df   Degree of Freedom

EPN   Entomo Pathogenic Nematodes

ESM   Error Standard Mean

IJ   Infective Juvenile

ICIPE   International Centre of Insect Physiology and Ecology

IPM   Integrated Pest Management

JKUAT   Jomo Kenyatta University of Agriculture and Technology

L : D   Light-Darkness photoperiod proportion

RH   Relative Humidity

TBD   Tick-borne diseases

SAS   Statistical Analysis System

SDA   Sabouraud Dextrose Agar

SE   Standard Error

SNK   Student-Newman-Keuls

ABSTRACT

Ticks of the genus *Rhipicephalus* are important parasites of livestock in the world. *Rhipicephalus* ticks cause huge economic losses to cattle and have a great capacity to develop resistance to chemical acaricides. There is the need therefore to develop alternative strategies that could complement the existing control methods of this ectoparasite. Entomopathogenic fungi are being considered as a promising option for the control of ticks on-host and off-host. The aim of this study was to assess the potential of using extracts from *Calpurnia aurea* as attractant in association with *Metarhizium anisopliae* for the control of *Rhipicephalus* spp. as an affordable and environmentally friendly technology. Experiments were first carried out to assess the response of two tick species, *Rhipicephalus pulchellus* and *R. appendiculatus* to different concentrations of extracts (acetone, aqueous and oil) of dry leaves of *C. aurea* in both an inverted glass tube and a dual choice T-olfactometer. The oil extract at the concentrations of 50 and 100 mg/ml attracted 46.7 and 65.9% of *R. appendiculatus,* respectively, in the inverted glass tube assay, which was comparable to the attraction-aggregation-attachment pheromone (AAAP) used as a check (47.8%). The attraction of both tick species to plant extract was also tested in semi-field plot experiments using a trap baited with different concentrations of emulsifiable extract of *C. aurea.* A dose of 100 mg/ml attracted 52.2% of *R. pulchellus* and 44.4% of *R. appendiculatus* from a distance of 1 m, respectively, while 14.4% of *R. pulchellus* and 12.2% of *R. appendiculatus* were attracted from 5 m distance. Addition of CO_2 to the plant extract-baited trap at the dose of 100 mg/ml

increased the range of attraction of adult *R. pulchellus* to 44.4% and to 33.3% of adult *R. appendiculatus* tick from a distance of 5 m. The compatibility of the fungus *M. anisopliae* with emulsifiable extract of *C. aurea*, and AAAP was evaluated in the laboratory in terms of fungus vegetative growth, conidia production and viability. Compared to AAAP which inhibited fungus vegetative growth and conidial viability, emulsifiable formulation of the plant extract was compatible with the fungus at all the concentrations tested. The prospects of attracting and infecting *R. appendiculatus* ticks were evaluated in semi-field experiments. Ticks were released at various distances and then attracted to the trap baited with a mixture of emulsifiable extract and fungal conidia. Half of these ticks exposed to *M. anisopliae* were brought to the laboratory and incubated at 26 ± 2 ^{o}C and $85 \pm 5\%$ RH. The other half was allowed to feed on rabbit in order to evaluate the reproduction potential. Eighty three (83%) per cent of the ticks brought to the lab died of fungal infection. Male *R. appendiculatus* ticks were attracted and exposed to conidia in a plant extract-baited trap. These males were collected and placed together for five weeks with uncontaminated females in proportions of 1:1. This study showed that autodissemination of fungal inoculum between *R. appendiculatus* ticks did occur under semi-field conditions. The results of the current study revealed that *C. aurea* extracts with *M. anisopliae* in a trap system can potentially be used as an environmentally friendly and low cost strategy to control *Rhipicephalus* ticks in the field.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Introduction

Livestock rearing is an important source of income for many small- and large-scale farmers worldwide (Herani *et al*., 2008). Marketed livestock products include meat, milk, skins and hides while some animals e.g. oxen also help alleviate labour constraints by assisting in transport and land cultivation. However, there are a number of constraints that limit livestock production and one of the major is the infestation by ticks (Hlatshwayo and Mbati, 2005). They cause direct loss through blood sucking and injury, and are also vectors of important livestock and several human diseases. Global losses to livestock due to tick and tick-borne diseases are estimated at US$ 13.9-18.7 billion annually. In Africa, ticks and tick-borne diseases (TBD) are considered the single most important animal disease problem. In Tanzania alone for example, the total annual national loss due to tick-borne diseases is estimated at 364 million US dollars including an estimated mortality of 1.3 million cattle (Kavaria, 2006).

Ixodid ticks such as *Rhipicephalus appendiculatus* Neumann, 1901 and *R. pulchellus* Gerstäcker, 1887 in particular, are among the most economically important parasites in tropics and subtropics (Bram, 1983). They transmit pathogens that cause tick-borne diseases such as East Cost Fever (Theileriosis), heartwater (*Cowdria ruminantium*), Tick-bite Fever (*Rickettsia conori*), Nairobi Sheep Disease

(*Nairovirus*), Q Fever (*Coxiella burnettii*), and dermatophylosis (*Dermatophilus congolensis*).

The use of synthetic acaricides including chlorinated hydrocarbons, pyrethroids, organophosphate and formamidines still remains the main approach to management of tick and tick-borne diseases (Davey *et al.*, 1998; George *et al.*, 2004). However, excessive use has resulted in development of tick resistance to most of the major products (Alonsa-diaz *et al.*, 2006) and led to point concerns over the effects of widespread use of chemical acaricides on humans and the environment (Jonsson *et al.*, 2000; Karralliede *et al.*, 2003). In addition, synthetic acaricides are expensive to African farmers who mainly practice subsistence farming.

These shortcomings have prompted the search for alternative tick control methods that are cheap and environmentally friendly. They include the use of entomopathogenic fungi and nematodes (Maniania *et al.*, 2007; Reis-Menini *et al.*, 2008), vertebrate predators (Samish and Alekseev, 2001), tick vaccines (Boue *et al.*, 1998) and plant extracts (Wanzala *et al.*, 2005; Zorloni *et al.*, 2010). There have been also attempts to use entomopathogenic fungi or acaricides in combination with semiochemicals (pheromone and kairomone) (Norval *et al.*, 1996; Maranga *et al.,* 2006; Nchu *et al.*, 2009; 2010).

The potential of pheromone-mediated tactics for the control of ticks was demonstrated by Norval *et al.* (1989a) who used a crude extract of *Amblyomma hebraeum* Koch, 1844 male-produced attraction-aggregation-attachment pheromone

(AAAP). A device treated with conidia of the entomopathogenic fungi and baited with AAAP and carbon dioxide for infection of ticks in vegetation was tested by Maranga *et al*. (2006) and Nchu *et al*. (2009). Recently, Nchu *et al*. (2010) demonstrated the potential of a *Metarhizium anisopliae* (Metsch.) Sorokin (Hypocreales: Clavicipitaceae)-treated pheromone-baited traps in reducing *Amblyomma variegatum* Fabriscius populations in the field.

1.2 Justification

Ticks are obligatory bloodsucking arthropods, capable of transmitting diseases in humans, domestic and wild animals, thereby inflicting great economic losses in livestock in many regions of the world. In Africa, ticks and tick-borne diseases are considered the single most important animal disease problem.

The management of this pest has mainly relied on the use of synthetic acaricides and repellents (Jernigan *et al*., 2000). However, the excessive use and misuses of synthetic acaricides have led to negative effects such as environmental pollution and destruction of beneficial insects. In addition, ticks have developed resistance to many of these synthetic acaricides (Beugnet and Chardonnet, 1995; Jonsson *et al*., 2000; Mekonnen *et al*., 2002). The cost of these chemical pesticides is high and cannot be easily afforded by small-scale farmers. There is the need therefore for alternatives to acaricides that are sustainable and environmentally-friendly. Pathogens and plants extracts are among the alternatives being considered.

Among the pathogens, entomopathogenic fungi have been the most widely evaluated. They infect the host through the cuticle and their conidia can be applied as sprays in total cover on the vegetation (Kaaya *et al*., 1996; Kaaya and Hassan, 2000) and on host (Kaaya *et al*., 1996; Polar *et al*., 2005). However, a new strategy is currently being considered, whereby fungal conidia are disseminated among target pest populations by using devices that attract insects into a focus of the pathogens (Vega *et al.,* 2000). The system relies on visual cues (Maniania *et al*. 1998, 2002; Migiro *et al*., 2010), sex pheromone (Pell *et al.,* 1993; Vega *et al*., 1995; Klein and Lacey, 1997) or kairomones (Dimbi *et al.* 2003) and the pathogen.

Members of certain genera of ticks such as *Amblyomma* actively respond to the attraction-aggregation-attachment-pheromones (AAAP), which is secreted by feeding male ticks, and to carbon dioxide exhaled by their host (Norval *et al*., 1989b). AAA pheromone in *A. variegatum* is composed of a blend of three phenols; *o*-nitrophenols, methyl salicylate and perlagonic acid (Schoni *et al*., 1989). Hess and deCastro (1986) and Maranga *et al*. (2003) were able to demonstrate the attraction of *A. variegatum* to the AAA pheromone. Subsequently a device treated with conidia of the entomopathogenic fungi and baited with AAAP and carbon dioxide for infection of ticks in vegetation was developed and tested by Maranga *et al.* (2006) and Nchu *et al*. (2009).

A number of plants extracts have been reported to attract ticks of the genera *Rhipicephalus* and could be used as substitutes to AAAP in a device to infect ticks with entomopathogenic fungi. These include *Acalypha fruticosa* Forssk

(Euphorbiaceae)*, Ipomoea spathulata* Hallier (Convolvulaceae)*, Solanum incanum* Linnaeus (Solanaceae) (Hassan *et al*., 1994) and *Calpurnia aurea* Benth (Zorloni *et al*., 2010). The latter is also used for tick control in western Ethiopia (Regassa, 2000). The present study was motivated by the attraction response of ticks to extracts of *C. aurea* leaves collected in Ethiopia (Zorloni *et al*. 2010). *Calpurnia aurea* is a small tree occurring widely all over Africa and India. *C. aurea* is used to destroy lice and to relieve itches, to destroy maggots and to treat allergic rashes, particularly those caused by caterpillars.

The role of ethno-veterinary plants in integrated tick control have been the focus of attention in the last two decades (Wanzala *et al*., 2005; Abduz Zahir *et al*., 2009). Although attraction of some tick species to plants have been reported (Hassan *et al*., 1994; Zorloni *et al*., 2010), no product combining an attractant plant extract with a biological control agent in a trap is available. This may be attributed to the lack of adequate information on tick attraction responses at species level, and the absence of suitable application technique for a mycoacaricide. The aim of this study was to address the different aspects that could play a role in developing effective product for tick control by integrating ethno-veterinary plant as attractant with entomopathogenic fungus *Metarhizium anisopliae*.

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1.3 Objectives

1.3.1 Overall objective

To assess the potential of integrating the ethno-veterinary plant *Calpurnia aurea* with *Metarhizium anisopliae* for the control of adult *Rhipcephalus appendiculatus* and *Rhipicephalus pulchellus*.

1.3.2 Specific objectives

1. To assess the potential of *C. aurea* extracts in attracting adult *R. appendiculatus* and *R. pulchellus* ticks

2. To assess the compatibility between *M. anisopliae* isolate ICIPE and *C. aurea*

3. To evaluate the effectiveness of an attractant trap for infection and autodissemination of *M. anisopliae* amongst adult *R. appendiculatus*

4. Bioassay guided fractionation of *C. aurea* leaf extracts responsible for attraction of *R. appendiculatus*.

1.4 Null hypotheses

1. *Rhipicephalus appendiculatus* and *R. pulchellus* are not attracted to *C. aurea* leave extracts.

2. *Calpurnia aurea* extract is not compatible with *M. anisopliae.*

3. The attractant trap for infection and autodissemination of *M. anisopliae* amongst adult *R. appendiculatus* is not effective.

4. There are no active components in *C. aurea* extracts responsible for attraction *R. appendiculatus*.

1.5 Alternative hypotheses

1. *Rhipicephalus appendiculatus* and *R. pulchellus* are attracted to *Calpurnia aurea* leave extracts.

2. *Calpurnia aurea* extract is compatible with *M. anisopliae.*

3. The attractant trap for infection and autodissemination of *M. anisopliae* amongst adult *R. appendiculatus* is effective.

4. There are active components in *C. aurea* extracts responsible for attraction *R. appendiculatus*.

2.1 Ticks biology

2.1.1 Tick classification

Ticks are obligate haematophagous ectoparasites of vertebrates (Mans and Neitz, 2004). They belong to the subclass Acari and sub-order Ixodida which comprises of three families; Argasidae (soft ticks), Ixodidae (hard ticks) and Nutalliellidae (Sonenshine, 1991). According to Kranz (1978), the major behavioural difference between ixodids and soft ticks is that ixodids spend several days feeding on the host while argasids ticks feed rapidly and the feeding usually lasts less than an hour. Ixodid ticks also differ from argasid ticks in the number of nymphal stages; with ixodid ticks having only one nymphal stage while argasid ticks have at least two nymphal stages (Sonenshine, 1991). For the purpose of this study, focus was on ixodid ticks.

2.1.2 Life cycle of ixodid ticks and development patterns

Ticks have four stages in their life cycle: egg, larva, nymph and adult. Mating usually occurs while adult ticks are on the body of the host animal. The female then drops to the ground and deposits her eggs. Adult female hard ticks feed only once and lay one large batch of eggs, often containing as many as 10,000 or more. Some adult female ticks will feed several times and lay 20 to 50 eggs after each

meal. Depending on such conditions as temperature and humidity, larvae will hatch from the eggs in anywhere from two weeks to several months (Sonenshine, 1991).

The first immature stages (larvae, which are many times called seed ticks) have only six legs. These larvae must find and attach themselves to a host in order to get a blood meal. After obtaining this blood meal they usually drop to the ground, shed their skin and emerge as 8-legged nymphs. Larvae of some ticks which feed only on one host remain on the host to molt. Because of the difficulty of finding a suitable host, larvae can withstand long periods without feeding (Bedford, 1934).

Nymphs resemble the adult tick in that they have eight legs. They do not, however, have a genital opening. Like the larva, the nymph must be able to live without feeding for long periods of time until it finds a suitable host. After finding a host and feeding, the nymph molts and becomes an adult tick. Hard ticks have only one nymphal instar while soft ticks may have several. A few ticks, such as the cattle, *Rhipicephalus annulatus* Say, 1821 have only one host and molt on it, while three-host ticks such as *Amblyomma hebraeum* Koch, 1844 needs three hosts to complete their life cycle. Each life stage seeks a host (Sonenshine, 1991). Adult ticks may require several days of feeding before they are able to reproduce. Male hard ticks usually die soon after mating, and females die soon after laying their eggs.

2.1.3 Morphology of ixodid ticks and adaptation

The body of tick comprises of two main regions i.e. gnathosoma and idiosoma. Gnathosoma includes basis capituli and mouthparts. The mouthparts consist of a pair of four-segmented palps, a pair of two-segmented chelicerae and a hypostome. Ticks use the chelicerate to penetrate the epidermis of their host and insert the hypostome with retrograde teeth into the wound. The retrograde teeth on the hypostome, together with the cement material secreted by tick's salivary glands, enhances attachment of tick to its host (Sonenshine, 1991). The idiosoma bears the legs, genital pores and spiracles. The chitinous scutum in female ticks covers the anterior third of the dorsal side of the idiosoma while in males it extends over the entire dorsum of the idiosoma (Kranz, 1978).

Hard tick have evolved and successfully adapted to a blood-feeding mode of life (Mans and Neitz, 2004). In order to allow the flow of blood during feeding, the salivary gland of ticks secretes anticoagulants and vasodilators (Sauer *et al*., 2000). Tick salivary gland also secretes immuno-modulators, which can suppress the immune system of the host (Barriga, 1999). Ticks have highly efficient sensory organs, which consist of chemosensilla, mechanosensilla and photosensilla. The tick's sensory organ, the Haller's organ, is situated on the dorsal surface of the tarsi of each foreleg and it has both olfactory and gustatory chemosensilla (Sonenshine, 1991). Olfactory chemoreceptors or sensilla perceive volatiles while gustatory chemoreceptors perceive stimulus on contact (Perritt *et al*., 1993). Also, Caroll (1998) demonstrated that aqueous wipes of metatarsal gland of White-tailed deer

elicited an arrestant response in *Ixodes scapularis* Say, 1821. Some Ixodid ticks such as *Hyalomma truncatum* Koch, 1844 possess eyes equipped with photoreceptors and capable of perceiving visual signals (Bergermann and Gothe, 1997).

The mouthparts of hard ticks have three visible components: the two outside jointed parts are the highly mobile palps; between these are paired chelicerae, which protect the centre rod-shaped structure, the hypostome. The palps move laterally while the tick is feeding and do not enter the skin of the host.

2.2 Tick habitat

Most ticks spend the bulk of their life on or near the ground, waiting for a suitable host animal. Since they cannot run, hop, fly or move quickly, ticks must climb onto an appropriate object such as tall grass or weeds or up onto fences and siding of buildings. It is from these advantageous positions that they wait for a suitable host to pass by. When they detect vibrations and chemical cues such as host odours or exhaled carbon dioxide, ticks will fall from their perch or stretch out (holding on to their perch with only 2 or 4 of their rear legs) and hop to snag or attach onto a passing host (e.g., a mammal with a fur coat or pants and socks worn by humans). Ticks are also capable of detecting shadows cast by a passing host. These tick behaviours are important to understand and recognize in order to make effective control (Sonenshine, 1991).

2.3 Semiochemicals

A semiochemical or infochemical is any chemical compound used in communication, whether between species (as in symbioses) or between members of the same species (Hölldobler and Wilson, 1990; Meyer, 2006). The signals transmitted between individuals of different species are called allelochemicals, while those mediating behaviour between individuals of the same species are known as pheromones. Pheromones are glandular secretions which when released by one individual trigger a behavioural response from other individuals upon tasting or smelling it (Hölldobler and Wilson, 1990; Howse *et al.*, 1998). Allelochemicals are subdivided into three broad classes: allomones, synomones and kairomones (Nordlund, 1981; Hölldobler and Wilson, 1990). Allomones are signals that benefit the emitter while being of negative or no significance to the receiver, e.g. a lure used by a predator to attract a prey. A kairomone, benefits the receiver by either evoking a behavioural or physiological reaction. Synomones benefit both the emitter as well as the receiver.

2.3.1 Semiochemical communication in ticks

Communication in ticks is very diverse and includes mostly streaking of chemicals that evoke various responses from simple recognition to recruitment and alarm (Hölldobler and Wilson, 1990). Chemical signals mediating communication in ticks are usually complex mixtures of substances with considerable variation in molecular composition and in the relative proportions of components. Such multicomponent

signals can be produced in single exocrine glands, but they can also be blends composed of secretions from several glands. Chemical signals can be further combined with cues from other sensory modalities, such as vibrational or tactile stimuli. The various kinds of accessory signals usually serve in modulatory communication, lowering the response threshold in the recipient of the actual releasing stimulus. Comparative studies suggest that modulatory signals evolved through ritualisation from actions originally not related to the same behavioural context, and modulatory signals may further evolve to become independent releasing signals (Hölldobler, 1995).

2.3.2 The role of odours in behaviour of ticks

Secretions from dermal glands of both prostriate and metastriate ticks have been widely reported (Walker *et al*., 1996). Particular attention has been paid to the aggregation–attachment pheromone of some *Amblyomma* spp. (Diehl *et al*., 1991) and the suspected tick pheromone product 2, 6-dichlorophenol (De Bruyne and Guerin, 1994). It is known that several metastriate ticks transiently release cuticular droplets in response to physical disturbance, for which a defensive role has been suggested. Yoder *et al.* (1993) found that *Dermacentor andersoni* Stiles, 1908, *D. variabilis* Say, 1821, *A. americanum* Linnaeus, 1758 and *A. maculatum* Kock, 1854 release a "pungent odour" associated with the appearance of droplets on the cuticle. Pavis *et al*. (1994) observed the release of droplets by *A. variegatum* after disturbance, and Walker *et al.* (1996) reported the release of droplets with a "sour, nut-like smell" from unfed and fed *R. appendiculatus* adults after handling. Those

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different chemicals play an important role in the process of attraction, aggregation and attachment for feeding.

2.4 *Rhipicephalus appendiculatus* and *R. pulchellus*

These two tick species are economically important in tropical regions including Kenya (Figure 2.1) and were selected for the present study.
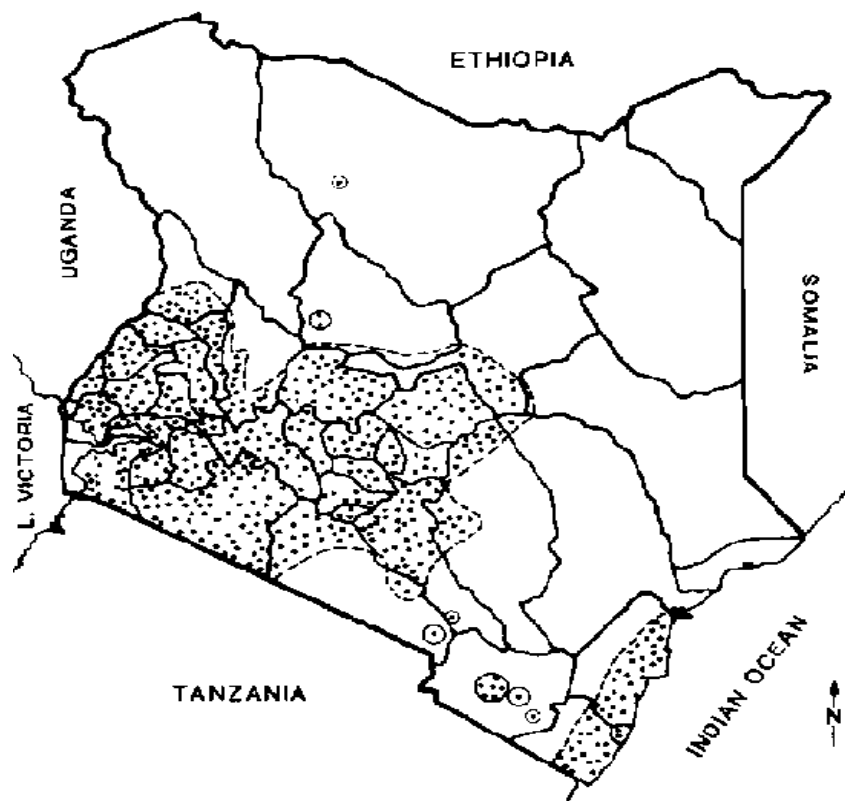
Distribution of *Rhipicephalus* ticks in Kenya

Figure 2. 1: **The distribution of *Rhipicephalus* spp., vector of *Theileria parva* in Kenya (FAO, 1992)**

2.4.1 *Rhipicephalus appendiculatus* Neumann, 1901

R. appendiculatus, is widespread in Africa (Figure 2.2), feeding on many species of vertebrate hosts (Walker, 1974). At certain times of the year, particularly after rain, adults can be found stationed at the top of the flowering heads of grasses, sometimes with more than ten ticks per stem. It is noticeable that this may occur even in areas densely covered with grass, and where few stems carry any ticks. *Rhipicephalus appendiculatus* (Plate 2.1) feeds in each of its three instars (larva, nymph and adult). Ticks ready to feed climb vertical objects, usually the flowering heads of grasses, but sometimes grass leaves or, rarely, other plants. There they wait, and if a host approaches they usually adopt 'questing' attitude with their fore-legs extended horizontally (Lees & Milne, 1951). Should the host touch them, they immediately cling on and feed. After feeding, each individual detaches and falls to the ground, where the larvae and nymphs moult to the next instar, and the adult females lay their eggs and die. Thus the adults observed on the stems arise from nymphs that have previously engorged and fallen. A host may carry large numbers of each instar, and these fall wherever the host happens to be at the time that the ticks detach. The common hosts are large active ungulates, and although there may be a 'preferred' time of day for detachment (George, 1964, 1971; Hadani and Ziv, 1974) with many ticks detaching over a short period, the likelihood of two or more falling so close together that they will later climb the same grass stem by chance seems small. Moreover, the period that elapses between the falling of the nymphs and the appearance of adults on the stems may be several months in cool weather and the

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chances of survival are probably small in the rather arid regions where the species is abundant (George, 1971).

Plate 2. 1: **Adult female (left) and male (right) of *Rhipicephalus appendiculatus* (Kibuka, 2006)**

Rhipicephalus appendiculatus
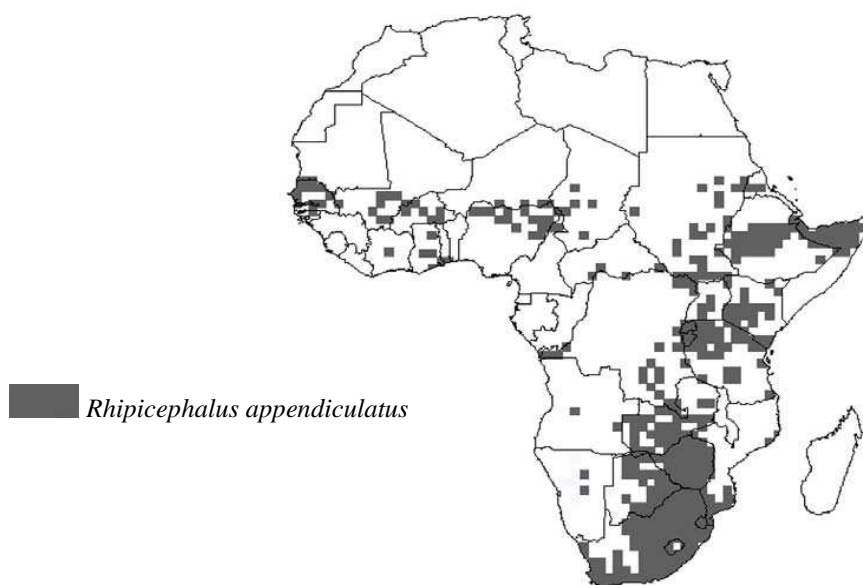
Figure 2. 2: **Distribution of *Rhipicephalus appendiculatus* in Africa (Walker *et al*., 2003)**

2.4.2 *Rhipicephalus pulchellus* Gerstäcker, 1873

This tick is commonly known as the Zebra tick with a three-host life cycle and can transmit the protozoan *Trypanosoma theileria* to cattle (Kibuka-Sebitosi, 2006). *Rhipicephalus pulchellus* (Plate 2.2) is found in the savannah, steppe, and desert regions and is the commonest tick in North East Africa and Rift valley (Figure 2.3). It has a wide variety of hosts including Zebra, Black rhinoceros and Eland (Walker *et al*., 2003).

Plate 2. 2: **Adult female (left) and male (right) of *Rhipicephalus pulchellus* (Kibuka, 2006)**
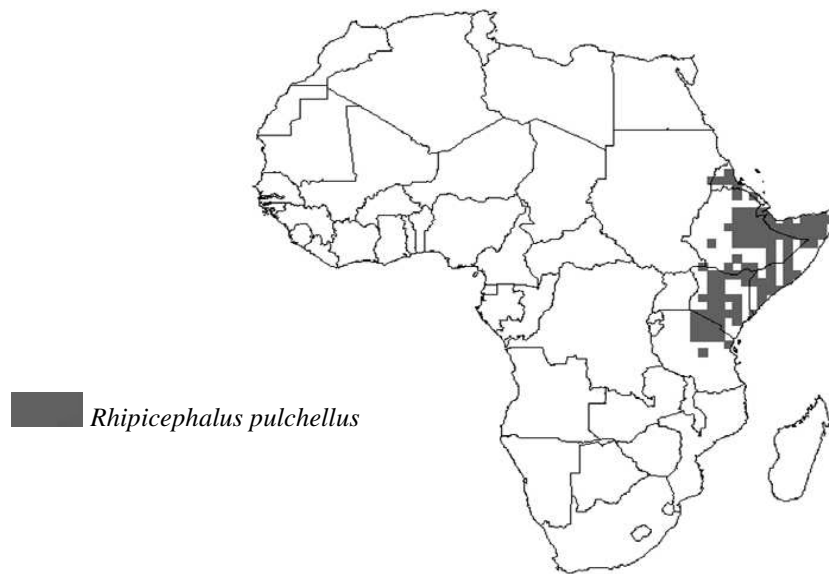
Figure 2. 3: **Distribution of *Rhipicephalus pulchellus* in Africa (Walker *et al.,* 2003)**

2.5 Economic importance of ticks

The economically most important ixodid ticks of livestock in tropical regions belong to the genera of *Hyalomma*, *Boophilus*, *Amblyomma* and *Rhipicephalus* (Frans, 2000). In many areas of the world, tick-induced productivity and mortality losses inflict large costs on beef and dairy industries, and the problem remains especially severe in Africa. Ticks are responsible for severe economic losses both through direct effect of blood sucking and indirectly as vector of pathogens and toxins.

2.5.1 Direct effect on the host

Feeding by large numbers of ticks causes reduction in live weight and anaemia among domestic animals, while tick bites also reduce the quality of hides. Apart from

irritation or anaemia in case of heavy infestations, tick can cause severe dermatitis (FAO, 1998). These parasites generate direct effects in cattle in terms of milk production and reduce weight gain (L'Hostis and Seegers, 2002; Peter *et al.*, 2005). Tick paralysis is characterized by an acute ascending flaccid motor paralysis caused by the injection of a toxin by certain ticks while feeding. Examples are paralysis caused by the feeding of *Dermacentor andersoni*, sweating sickness caused by *Hyalomma truncatum*, Australian tick paralysis caused by *Ixodes holocylus*, and tick toxicosis caused by *Rhipicephalus* species (Drummond, 1983). Tick paralysis can occur at any time if the weather is warm and humid (Stewart and de Vos, 1984). Ticks are attached to the body for a blood meal and may cause irritation and serious physical damages to livestock including "tick worry", irritation, unrest, and weight loss due to massive infestation of ticks, direct injury to hides due to tick bites, and loss of blood due to the feeding of ticks (Drummond, 1983).

2.5.2 Vector of pathogens

Ticks can be carrier of pathogens which they transmit from host to host during blood sucking and cause a large variety of diseases (FAO, 1998). The major diseases include Babesiosis, Anaplasmosis, Theileriosis, and heart-water, East Coast fever; in addition, other diseases of lesser importance cause severe economic losses to the livestock industry (Drummond, 1983; Bram, 1983). The presence, dynamics and amount of parasite stock in ticks exert a major influence on the kinetics of transmission of tick-borne parasitic diseases (Morel, 1980).

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2.6 Tick Control

2.6.1 Chemical control

Farmers mostly rely on the use of chemical acaricides and repellents to limit production losses. In order to reduce contact between ticks and vertebrate hosts, chemical repellents such as N,N-diethyl-m-toluamide (DEET) and permethrin are extensively used (Faulde *et al.*, 2003; Klun *et al.*, 2003). Acaricides typically are highly lethal to ticks, and field applications generally are quite effective in reducing tick numbers (Sonenshine, 1993; Stafford and Kitron, 2002). Jernigan *et al.* (2000) demonstrated the acaricidal efficacy of Selamectin against experimentally induced ticks (*Rhipicephalus sanguineus* and *Dermacentor variabilis*) infestation on dogs. Witchey-Lakshman (1999) outlined the various class of ectoparasiticide: Organophosphate (diazinon, fampur, phosmet, dichlorvos), synthetic pyrethroids (resmethrin, deltamethrin, permethrin), carbamates (carbofuran, propoxur), growth regulator (fenoxycarb, methroprene), amitraz, fipronil and methandiol that are currently being used for tick control. Although clearly effective at reducing transmission of tick-borne pathogens to livestock, repeated heavy applications of pesticides to hosts can cause considerable mortality in non-target arthropods through environmental contamination (Gassner *et al.*, 1997). Moreover, evolved resistance to acaricides, which is a well-known problem with mosquitoes, is a persistent issue for tick species such as *Rhipiephallus microplus* that are chronically exposed by virtue of their close association with cattle to which the acaricides are applied (Foil *et al.*, 2004, George *et al.*, 2004).

2.6.1.1 Problems posed by synthetic acaricides

Tick resistance to acaricides is on the rise due especially to increased frequency in the application of acaricides (Jonsson *et al*., 2000). For instance, *R. microplus* has developed resistance to synthetic pyrethroids and amitraz (Beugnet and chardonnet, 1995; Jonsson *et al*, 2000), amitraz, chlorfenvinphos and cypermethrin against *Boophilus decoloratus* (Mekonnen *et al.,* 2002). The resistance mechanism of ticks such as *R. microplus* to acaricides (coumaphos and diazinon) has been linked to an enhanced cytochrome P 450 monooxygenase-mediated detoxification (Li *et al*., 2003).

Environmental pollution is a serious problem posed by the use of synthetic acaricides in tick control. Chemical compound such as DDT, endosulfan and endosulfan sulphate are toxic and bioaccumulate in nature (Bhattacarya *et al*., 2003). Accumulation of these contaminants in water, soil and animals has been reported in Jamaica (Mansingh and Wilson, 1995). In 1961, the breeding number of peregrine falcons fell drastically and this was correlated with abnormally high residual levels of metabolites of DDT and dieldrin found in both the tissue and the carcasses of birds that fed on seeds treated with these compounds (Jarvis, 2000). The accumulation of toxic chemicals obviously has an amplified effect on the food chain leading to magnification of toxic residues in animals occurring at higher levels of the food chain (Boudou and Ribeyre, 1997). Acaricides were also identified in honey bees using reversed-phase high performance liquid chromatography (Martel and Zeggane, 2002). It is obvious that such a situation is potentially dangerous to humans.

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Organophosphate accumulation in fatty tissue of mammals can lead to poisoning in man (Karalliede *et al*., 2003). According to Selim *et al*. (1995), DEET can be absorbed through the skin of humans. Recently, permethrin that was used to repel arthropods has also been implicated in Gulf war related diseases (Riviere *et al*., 2002).

2.6.2 Vaccination

Ticks, in the process of feeding produce antigens which facilitate the acquisition of blood from their hosts and that can activate the production of antibodies in the hosts against internal organs of the ticks (da Silva vaz *et al*., 1996). These antigens might be useful in the development of anti-tick vaccines (Imamura *et al.,* 2008). A vaccine (Gavac^{TM}) effective against *R. microplus* was recently developed in Cuba (Boué *et al.,* 1998), but its efficacy against the other species could not be demonstrated. According to Willadsen (2005), current vaccines could be improved by inclusion of additional tick antigens. Research in tick vaccine development is fairly recent and it opens interesting opportunities for future research.

2.6.3 Ethno-veterinary plants

Ethno-veterinary medicine is very important in Africa and other developing countries since a greater proportion of livestock farmers are small-scale and most of these are in rural areas where cultural practices are still preserved (Madge, 1998). Plant extract preparations are developed by famers rather than scientists due to lack of finance to purchase synthetic acaricides which force them to depend on traditional methods of

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tick control. Furthermore, products from plant such as neem (*Azadirachta indica*) are easily biodegradable (Liang *et al*., 2003); thus making them likely less toxic to the environment and non-targeted species (Castagnoli *et al*., 2002).

Traditional knowledge on the use of ethno-veterinary plants for tick control is fast disappearing due to the lack of documentation since this type of knowledge has been transferred orally (Mathias, 2001). Furthermore, the efficacy of most plants that have been traditionally used has not been scientifically tested. Due to the economic and medical importance of ticks, it is necessary to screen some ethno-veterinary plants that have acaricide properties and could be used widely. Some of the advantages of promoting research on ethno-veterinary include the development of plant-derived semiochemicals which may be easily accessible by the rural communities, their low toxicity and biodegradability; thus, the need for their conservation.

2.6.4 Biological control of ticks

Biological control is the use of natural enemies (predators, parasitoids, and pathogens) for the control of insect pests and is one of the alternatives to synthetic chemical pesticides.

2.6.4.1 Predators

Predators such as spiders, ants, beetles, rodents, shrews, birds and reptiles have been reported to significantly contribute to natural control of tick populations (Samish and Rehacek 1999). Fleetwood *et al*. (1984) found that predators are efficient in

biocontrol of ticks in different habitats and may reach up to 100% control in some cases. In southern Queensland, ants and the house mouse (*M. musculus*) were found to be the only significant predators of engorged females of the cattle tick *B. microplus* (Sutherst *et al*. 2000). Nine genera of spiders were identified as predators of five genera of hard ticks and two of soft ticks (Carroll, 1995). However, their potential as biological control agents in inundative release is very limited.

2.6.4.2 Parasitoids

The most widespread parasitoid of ticks is *Ixodiphagus hookeri* (=*Huterelius hookeri*, *I. caucurtei*) (Hymenoptera: Encyrtidae) (Trjapitzin, 1985) which has been recorded from Asia, Africa, North America and Europe (Hu *et al*., 1993). Unfortunately, their mass production for inundative releases is prohibitively expensive, in addition to the high costs of maintaining tick colonies, necessary for raising these obligate tick parasitoids.

2.6.4.3 Entomopathogens

2.6.4.3.1 Bacteria

Although several bacterial species have been reported pathogenic to ticks in the laboratory (Samish, 1999), no attempt to develop them as microbial biocontrol agents of ticks has been done. They need to be ingested to cause infection in the host.

2.6.4.3.2 Nematodes

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are known to be obligatory parasites of arthropods. The only free-living stage of the nematode, the third/infective juvenile (IJ), actively locates and enters the host via natural openings, and then releases symbiotic bacteria that kill the host insect within 24-72 h. These nematodes have never been reported to parasitize ticks in nature. However, it was demonstrated that they efficiently kill engorged *R. annulatus* females in petri dishes (Samish and Glazer, 1992). Moreover, engorged females of numerous other tick species were also killed by these nematodes (El-Sadawy, 1994; Samish *et al*., 1996; Zhioua *et al*., 1995; Kocan *et al*., 1998; Samish *et al.,* 1999).

2.6.4.5 Fungi

2.6.4.5.1 General biology

The taxonomy of the entomopathogenic fungi has increased interest the last past ten years leading to a new phylogenetically based classification of fungi (Humber, 2008). Entomopathogenic fungi widely studied for biological control belong to Clavicipitaceae Family (Order Hypocreales) and include species like *Metarhizium, Beauveria, Isaria* (=*Paelomyces*), *Hirsutella, Lecannicillium* (formerly *Verticillium*), *Culicinomyces, Tolypocladium* and *Nomuraea* (Vega *et al*., 2009)*.* Ekesi and Maniania (2007) reviewed the use of entomopathogenic fungi in biological pest management. *Metarhizium*, *Beauveria* and *Isaria* genera are considered non specific

pathogens for Acari (Chandler *et al.*, 2000) and they are however the most studied for biological control of ticks.

2.6.4.5.2 Mode of infection

The fungi invade hosts by a process which involves the adhesion of conidia to the cuticle, conidia germination, formation of appressoria and penetration through the cuticle, with penetration occurring 72 hrs post-inoculation (Arruda *et al.*, 2005). This penetration is helped by secretion of the chitinolytic enzyme CHIT 30 by *M. anisopliae* (Da silva *et al.*, 2005). Other enzymes implicated in the degradation of the tick cuticle by *M. anisopliae* during the infection process include chitinases and proteases (de Moraes *et al.*, 2003). According to Goettel *et al.* (1989), penetration of the epicuticle primarily occurs by enzymatic degradation, while penetration of the procuticle involves both enzymatic degradation and mechanical separation of the lamella. Differences in tick cuticular lipids and hydrocarbons can influence tick susceptibility to fungal infection, which may account for differential susceptibility of *A. maculatum* Koch and *A. americanum* to *M. anisopliae* and *B. bassiana* (Kirkland *et al.*, 2004).

2.6.4.5.3 Potential of fungal pathogens for tick control

The pathogenicity to various fungal species against ticks has already been demonstrated in the laboratory. For example, Samish *et al.* (2001) showed that *M. anisopliae*, *B. bassiana, M. flavoviride* and *I. fumosorosea* were pathogenic *against R. sanguineus* L. (Acari: Ixodidae). In an in vitro bioassay comparison of the efficacy

of *M. anisopliae, M. flavoviride, B. bassiana, I. fumosorosea and L. lecanii* against engorged female *R. annulatus* ticks, Gindin *et al*. (2001) found that *M. anisopliae* and *B. bassiana* were the most effective, killing 85 to 100% of ticks within 7 to 10 days of treatment and preventing egg production before death. An isolate of *B. bassiana* (Bb 28) caused high larval mortality and low hatching of the eggs among fungus-treated *R. microplus* eggs and larvae (Fernandes *et al*., 2003). Investigating the effect of 12 isolates of *M. anisopliae*, infected and non-infected by dsRNA viruses, on engorged *R. microplus* females, Frazzon *et al*. (2000) observed that the most pathogenic isolates caused 100% mortality in engorged females of *R. microplus* 13 days post-treatment at test concentration of 10^7 conidia ml^{-1}. They also found that dsRNA mycovirus-free isolates of *M. anisopliae* were more infective than the ones taken from experimentally infected ticks. Isolates of *M. anisopliae* killed laboratory populations of engorged females, eggs and larvae of *R. microplus* and affected their oviposition behaviour (Bittencourt *et al*., 1992; Bittencourt *et al*., 1994a,b).

The potential of entomopathogenic fungi for tick control has also been demonstrated in the field. When ticks were treated with entomopathogenic fungi and maintained in potted grass, oil-based formulations of *B. bassiana* and *M. anisopliae* of 10^9 conidia ml^{-1} induced 100% mortality in *R. appendiculatus* and *A. variegatum*, larvae, between 80 and 100% mortality in nymphs and 80 and 90% mortality in adults (Kaaya and Hassan, 2000). In a more recent study, post engorgement and egg mass weight were 33 and 55% lower, respectively, in females of *Ixodes Scapularis* treated with *M. anisopliae* in the field before they were allowed to feed on the rabbits

(Hornbostel *et al*., 2004). Kaaya (2000a) and Benjamin *et al.* (2002) reduced tick populations of *R. appendiculatus* larvae and *I. scapularis* unfed adults respectively in vegetation by spraying with aqueous suspension of *M. anisopliae*. Benjamen *et al*. (2002) determined the efficacy of the fungal suspension by counting the number of *I. scapularis* collected in both the fungus-treated and the control treated plots after 4 weeks by the drag sampling method. Spray treatments of *M. anisopliae* and *B. bassiana* to *R. decoloratus* Koch on cattle induced tick mortality and resulted in a significant reduction in egg viability (egg hatchability). In the control group, egg viability was 98% compared with 30% and 50% in *B. bassiana* and *M. anisopliae*-treated groups, respectively (Kaaya and Hassan, 2000). In another study conducted by Alonso-Diaz *et al.* (2007), *R. microplus* population on naturally-infested cattle was significantly reduced following multiple applications of an aqueous suspension of *M. anisopliae* titrated at 10^8 conidia ml^{-1} compared to the control on days 0, 1, 2, 3, 5, 7 and 14 post-treatment.

2.6.4.5.4 Abiotic factor affecting the efficacy of entomopathogenic fungi

Several climatic factors may influence the infectivity of entomopathogenic fungi for tick. These factors include temperature, humidity and solar radiation. Temperature is one of the principal factors affecting the effectiveness of entomopathogenic fungi. It not only regulates the physiology of the fungus and insect, but also the ability of the fungus to infect the host. It also affects the progression of disease and the time of death (Inglis *et al*., 2001). In general, optimum temperatures for the germination, growth, sporulation and virulence of the entomopathogenic mitosporic fungi range

between 20 and 30 ^{o}C (Hall and Papierock, 1982). High humidity is essential for spores of entomopathogenic fungi to germinate, penetrate the cuticle and sporulate on cadavers (Inglis *et al*., 2001). However, Fargues *et al.* (1997) reported that *M. anisopliae* var. *acridum* can infect Desert locust at relative humidity as low as 13% and the fungus can even produce spores within cadavers under dry weather conditions. Solar radiation constitutes one of the most important factors affecting propagules persistence in the environment. Conidia of entomopathogenic fungi are susceptible to solar radiation, especially ultra-violet radiation (Inglis *et al*., 2001). For instance, exposure of conidia formulated in oil for 2 hours to radiation below 320 nm reduced germination from 99.0% to 37.5% after incubation for 48 hours (Moore *et al*., 1993). However, in spite of the detrimental effects of ultra-violet radiation, light has been reported to stimulate mycelial growth, intensity of sporulation and germination of spores of *B. bassiana* (Benz, 1987). Tang and Hou (2001) reported that the virulence of *Nomurea rileyi* Farlow against the larval stage of *Helicoverpa armigera* Hub was higher when incubated under full (24 hours) and half-light (12 hours) than under full darkness.

2.6.4.5.5 Biotic factor affecting the efficacy of entomopathogenic fungi

The characteristics that are needed before an entomopathogenic fungus can be considered as potential microbial pesticide include: high virulence, rapid mode of action, a broad host range, stability in culture and storage, amenability to submerge fermentation, amenability to quantitative bioassay and safety to workers (McCoy, 1990). Although these characteristics vary with different types of pathogens,

virulence and pathogenicity are essential elements in the selection of a suitable candidate for microbial control (Tanada and Fuxa, 1987). The susceptibility of arthropods to entomopathogenic fungi can be influenced by different factors such as developmental stages of the host (Tanada and Fuxa, 1987; Inglis *et al.*, 2001). For example, Kirkland *et al.* (2004) reported that *A. manculatum* and *A. americanum* nymphs are more susceptible to conidia and blastospores of *B. bassiana* and conidia of *M. anisopliae* than adults.

2.7 *Calpurnia aurea* (Aiton) Benth

C. aurea is a member of the subfamily Papilionoideae of the Family Fabaceae (Coates Palgrave, 1983). It is a small, multi-stemmed tree, 3–4 m tall, widespread in bushland and grassland areas in sub-Saharan Africa and India. In southern Ethiopia, it is called cheka by the Borana people. It is often found in overgrazed areas and is easily cultivated (Germishuizen and Meyer, 2003; Pooley, 1993). The leaflets are oblong 2.5-5 cm long with a lopsided base and a rounded or notched apex. They are a fresh light green, graceful and drooping. *C. aurea* is the most widespread of the genus.

Extracts of *C. aurea* have been used in South Africa to treat maggot-infested wounds (Watt and Breyer-Brandwijk, 1962) and in Ethiopia to treat scabies (Jansen, 1981). In western Ethiopia, the juice of crushed leaves and bark is used for tick control (Regassa, 2000). The Borana people of northern Kenya and southern Ethiopia soak leaves of *C. aurea* in cold water to treat lice infestations in humans and calves (Heine

and Brenzinger, 1988) and to control ticks on cattle. The plant is also used in Ethiopia to treat stomach disorders, amoebic dysentery and eye diseases (Abate, 1989). In south-western Ethiopia, the leaves of *C. aurea* (Plate 2.3) are mixed with other plant species, crushed and squeezed to obtain a juice, which is applied through the auricular route to treat earache in humans (Yineger and Yewhalaw, 2007). The plant is also used to treat rheumatism (Yineger *et al*., 2008). Antibacterial and antioxidant activity of *C. aurea* have been reported (Adedapo *et al*., 2008; Tadeg *et al*., 2005). It has also been used as a natural pesticide to improve grain storage (Blum and Bekele, 2002).

The main active compounds of *C. aurea* known to date are the alkaloid *calpurmenin* and its 13a-(20- pyrrolecarboxylic acid) ester (Vermin *et al*., 1979, Van Wyk *et al* 1991). The alkaloids virgiline and lupanine, as well as their carboxylic esters, have also been recorded (Van Wyk *et al*., 1991).

Plate 2. 3: **Fresh leaves and flowers of *Calpurnia aurea* (Nana, 2009)**

Plants are important to most terrestrial arthropods, and this may apply even to those whose major habitat is not vegetation. Ticks demonstrate this; newly hatched larvae move up vegetation to assist contact with a passing mammalian host. This tick behaviour can be exploited with success for off-host semiochemical-assisted tick control strategy (Sonenshine, 2006).

CHAPTER THREE

3.0 GENERAL MATERIALS AND METHODS

3.1 Study area

Laboratory work was undertaken in the Arthropod Pathology Unit (APU) and Behavioral and Chemical Ecology Department (BCED) at ICIPE. Semi-field plot experiments were also undertaken at ICIPE's Headquarters, Duduville, Nairobi.

3.2 Plant material

Dry powder of *C. aurea* leaves (Plate 3.1) was obtained from the Department of Paraclinical Science, Phytomedicine Unit, University of Pretoria, South Africa, in 2007.

Plate 3. 1: **Dry powder of *C. aurea* leaves (Nana, 2009)**

3.3 Preparation of extracts

Aqueous extract was prepared by infused of leaf powder (100 g) in 100 ml distilled water maintained at room temperature (23-26° C) for 24 hr. For oil extract, leaf powder (100 g) was soaked in 100 ml of corn oil (Elianto$^®$) all maintained in a warm water bath at 40° C for 2 hr. The mixture was later filtered and different concentrations; 12.5, 25, 50 and 100 mg/ml were prepared by serial dilution in distilled water. Acetone extract was prepared by maceration leaf powder (100 g) in acetone (1:2 w/v) for 6-8 hrs at room temperature. The extract was evaporated to dryness in vacuo and stored at 4° C. The dried extract (1 g) was re-suspended in 10 ml of a mixture of acetone, Tween 80, distilled water in a ratio of 1:2:7 respectively and the above concentrations prepared by double dilution. An emulsifiable formulation of the plant extract was used for the field experiment. The dry powder (100 g) of *C. aurea* was macerated in 500 ml of corn oil (Elianto$^®$) and 100 ml distilled water for 6 hours and then placed in a water bath at 40° C for 2 hrs. The mixture was later filtered and the different concentrations (12.5, 25, 50 and 100 mg/ml) were obtained by serial dilution with solvant.

3.4 Preparation of dichlorometane extract of *C. aurea*

Seventy grams of the plant powder were soaked in 0.8 l of 95% acetone for 3 days at room temperature (26 $\pm$ 2° C). It was later filtered and the organic filtrate concentrated under reduced pressure in a rotor evaporator at 50° C, to yield 5.6 g of crude organic extract (8%). 4.6 g of this extract was suspended in 500 ml distilled water and extracted by constant shaking with 500 ml 95% dichloromethane in a

separation flask. Both organic and aqueous extracts were concentrated using rotor evaporator at 556 and 72 mbar, respectively. Acetone, dichloromethane and aqueous extracts were used to perform a comparative thin layer chromatography.

3.4.1 Preparation of the fractions of *C. aurea*

Two grams of the organic extract were subjected to flash chromatographic fractionation on a silica gel column eluted with hexane followed by gradient mixtures of hexane-ethyl acetate. Several fractions obtained were combined based on the comparison of their RF values (Sigma silica gel precoated TLC plates).

3.5 Tick rearing

Engorged females of *R. appendiculatus* and *R. pulchellus* ticks used to start the colony originated from cattle from the Marsabit area of Kenya in 2006. Ticks were reared at the Animal Rearing and Quarantine Unit of ICIPE. All life stages of the tick were fed on New Zealand white rabbits. The different instars were maintained in Perspex chambers at 26 ± 1° C and $85 \pm 5\%$ RH under 12:12 L : D photoperiod. Three to four-week old unfed adults were used in this study.

3.6 Fungus

Metarhizium anisopliae isolate ICIPE 07 used in this study was obtained from the ICIPE's Arthropod Germplasm Centre. The strain was isolated from an engorged female *A. variegatum* collected from Rusinga Island, Kenya in 1996 and was previously reported to be virulent against *R. appendiculatus* (Kaaya *et al.,* 1996). The

fungus was stored under mineral oil before being used in the experiment. The virulence of the isolated fungus was restored by passaging twice through adult *R. appendiculatus*.

3.6.1 Fungal culture

Blastospores were cultured in liquid medium containing glucose (30 g/l), peptone (10 g/l) and yeast extract (30 g/l) in a 250 ml Erlenmeyer flask maintained in a shaker at 100 rpm and 26 ± 2° C for 3 days (Plate 3.4) and conidia were mass produced using rice as a substrate (Milner R.J., unpubl.). Glucose, peptone and yeast extracts were obtained from Sigma. The contents of the flask were autoclaved for 30 minutes at 121°C and allowed to cool before inoculation with the conidia. Two kilograms of rice per plastic bag was autoclaved for 1 h at 121° C, transferred to polyethylene autoclavable bags and inoculated with the 3-day old culture of blastospores (50 ml). The blastospores suspension was thoroughly mixed with the rice to distribute the inoculum throughout the substrate and incubated between 26 and 30° C and 60–75% R.H. for 21 days. The polyethylene bag was then opened and the culture was allowed to dry for 5 days at room temperature to approximately 10-15% moisture content (Plate 3.5 and 3.6). Conidia were harvested by sifting the substrate through a sieve (295 μm mesh size) and approximately 200 g of spores was produced per bag. Conidia were then put into glass container with silica gel to dry. Dry conidia were stored in a refrigerator (4–6°C) for at most 1 week prior to use.

Plate 3. 2: Starter cultures of *M. anisopliae* in orbital shaker incubator (Ouna, 2009)

Plate 3. 3: Mass production of *M. anisopliae* on rice substrate in Milner bags (Ouna, 2009)

Plate 3. 4: **Mass produced conidia of *M. anisopliae* in plastic basin undergoing drying at room temperature (Ouna, 2009)**

3.6.2 Preparation of conidial suspensions

Conidia were harvested from 3 week-old culture plates by scrapping the surface of the plate using a sterile spatula. Spores were suspended in 20 ml sterile 0.05% Triton X-100 water solution in universal bottles containing 3 mm glass beads. Bottles were then vortexed for 5 min to produce a homogenous conidial suspension. Conidial concentration was determined by using a haemocytometer and the desired concentration was obtained by serial dilutions. Viability of conidia was determined before every bioassay by spread-plating 0.1 ml of conidial suspension (titrated to 3×10^6 conidia.ml^{-1}) on SDA plates. A sterile cover slip was placed on each plate and incubated at 26 ± 2 ^{o}C. The percentage of germination was determined after 15 hours from 100 - spore count on each plate, using a compound microscope at $40\times$ magnification.

CHAPTER FOUR

4.0 RESPONSE OF ADULT *RHIPICEPHALUS APPENDICULATUS* AND *RHIPICEPHALUS PULCHELLUS* (ACARI: IXODIDAE) TICKS TO EXTRACTS OF *CALPURNIA AUREA* (FABACEAE)

4.1 Introduction

Rhipicephalus appendiculatus Neumann, 1901 and *Rhipicephalus pulchellus* Gerstaäker, 1873 (Acari: Ixodidae) are blood sucking arthropods endemic to tropical and sub-tropical regions. They feed on a variety of hosts including cattle, sheep and goat, and transmit theileriosis caused by the protozoan *Theileria parva* Theiler, 1911 (Billiouw *et al*., 2002). The disease is fatal in cattle and has been reported in 11 countries in eastern, central and southern Africa (Norval *et al*., 1992). Estimated direct economic loss associated with theileriosis is enormous with 1.1 million cattle deaths due to the disease annually (Mukhebi, 1992).

Existing methods of tick control rely heavily on chemical acaricides and repellents. Although they have been effective in suppressing tick populations and incidences of tick-borne diseases, their main disadvantages have been the high costs relative to the value of cattle and cattle products, and the development of tick resistance to various ranges of acaricides (Martins *et al.,* 1995; George, 2000). This has prompted a search for alternative methods of tick control that can be used alone or in combination with other tick control methods in an integrated tick management strategy (Jongejan, 1998). These include the use of predators (Samish and Alekseev, 2001),

entomopathogenic nematodes (Reis-Menini *et al*., 2008), and entomopathogenic fungi (Kaaya and Hassan, 2000; Maranga *et al*., 2006; Maniania *et al*., 2007).

The potential of pheromone-mediated tactics for the control of ticks was demonstrated by Norval *et al.* (1989a) who used a crude extract of *Amblyomma hebraeum* Koch, 1844 male-produced attraction-aggregation-attachment pheromone (AAAP). A device treated with conidia of the entomopathogenic fungi and baited with AAAP and carbon dioxide for infection of ticks in vegetation was tested by Maranga *et al.* (2006) and Nchu *et al*. (2009).   Recently, Nchu *et al*. (2010) demonstrated the potential of a *Metarhizium anisopliae* (Metsch.) Sorokin (Hypocreales: Clavicipitaceae)-treated pheromone-baited traps in reducing *Amblyomma variegatum* Fabriscius populations in the field.

A number of plants extracts have been reported to attract ticks of the genera *Rhipicephalus* and could be used as substitutes to AAAP in a device to infect ticks with entomopathogenic fungi. These include *Acalypha fruticosa* Forssk (Euphorbiaceae)*, Ipomoea spathulata* Hallier (Convolvulaceae)*, Solanum incanum* Linnaeus (Solanaceae) (Hassan *et al*., 1994) and *Calpurnia aurea* Benth (Zorloni *et al*., 2010). The latter is also used for tick control in western Ethiopia (Regassa, 2000). *Calpurnia aurea* is a small tree occurring widely all over Africa and into India. Adedapo *et al*. (2008) lists many uses of extracts or sap against ailments in man and animals.

In the present study, we investigate the attraction of *R. appendiculatus* and *R. pulchellus* adults to different extracts of the leaves of *C. aurea* in order to use them in a baited-trap treated with entomopathogenic fungi for autodissemination of the pathogen.

4.2 Materials and methods

4.2.1 Plant material

Calpurnia aurea leaves were collected in September 2007 in the Lowveld National Botanical Garden in Nelspruit, South Africa. The voucher specimens of the tree are available in the Botanical Garden herbarium. Leaves were dried in the shade and ground to a fine powder with a McSalib mill. The powder was stored in a closed glass container in the dark.

4.2.2 Preparation of extracts

Aqueous extract

The leaf powder (100 g) was soaked for infusion in 100 ml distilled water and maintained at room temperature (23-26 °C) for 24 hr. The mixture was filtered and different concentrations; 12.5, 25, 50 and 100 mg/ml were prepared by serial dilution in distilled water.

Acetone extract

The leaf powder (100 g) was extracted with 200 ml of acetone (1:2 w/v) for 6-8 hrs. The extract was evaporated to dryness in vacuo and stored at 4 $^\circ$C. The dried extract (1 g) was re-suspended in 10 ml of a mixture of acetone, Tween 80, distilled water in a ratio of 1:2:7 respectively. Different concentrations; 12.5, 25, 50 and 100 mg/ml were prepared by serial dilution in distilled water.

Oil extract

The leaf powder (100 g) was extracted in 100 ml of corn oil (Elianto$^{®}$) all maintained in a warm water bath at 40 °C for 2 hr. The mixture was filtered and the same concentrations as for the aqueous extract were prepared by dilution with corn oil.

Emulsifiable formulation

An emulsifiable formulation of the plant extract was used for the field experiment. The dry powder (100 g) of *C. aurea* was soaked in 500 ml of corn oil (Elianto$^{®}$) and 500 ml distilled water for 6 hours and then placed in a water bath at 40° C for 2 hrs. The mixture was later filtered and the different concentrations (12.5, 25, 50 and 100 mg/ml) were obtained by serial dilution with vehicle.

4.2.3 Pheromone

Attraction-aggregation-attachment-pheromone (AAAP) was included bioassays as a check. The pheromone was prepared by mixing 0.2 mg of ortho-nitrophenol, 0.1 mg of methyl salicylate and 0.8 mg of nonanoic acid. Tests were carried out using a

pheromone concentration of 0.02 mg/ml which significantly attracts *A. variegatum* (Nchu *et al*., 2009). The synthetic compounds used to prepare AAAP were obtained from Sigma–Aldrich Chemie GmbH, Steinheim, Germany.

4.2.4 Ticks

Two- to three- month-old unfed *R. appendiculatus* and *R. pulchellus* ticks were obtained from the Rearing and Quarantine Unit of ICIPE. They were kept in glass vials covered with cotton wool and placed in aluminium tins and maintained at 75% relative humidity and 26 ± 2 ℃ temperature.

4.2.5 Bioassays

4.2.5.1 Inverted glass tube assay

The device was a modification of bioassay previously described by Lwande *et al*., 1999 to evaluate tick repellency. Two test tubes (12 cm long and 1.5 cm diameter) were mounted (with the open end at the bottom), on a wooden stem and fixed on 8×8 cm platform made of polystyrene (Plate 4.1). The platform was placed inside a plastic lunch box ($20 \times 10 \times 8$ cm) surrounded by water to prevent ticks to escape. Approximately 1.5-cm space was left between the mouth of the test tube and the platform. The two test tubes were placed 6-cm apart. The plant extract (0.5 ml) was applied to a piece of cotton wool using a micropipette. Equal quantity of the solvent used to make the extract was also applied on the control test tube. AAAP at the dose of 0.02 mg/ml was also tested as a positive control. Ticks were introduced in a group

of 10 (5 males and 5 females) on the platform between the test tubes. Based on their climbing behaviour, ticks that did not climb after 30 minutes were considered as "no choice" and excluded from the experiment. The number of ticks that climbed the test tubes containing the extract and the controls were counted. After each experiment, the tubes were washed with running tap water and soap, and subsequently rinsed with 70% ethanol and dried. Treatment and control tubes were alternated after every assay by alternating control and test cotton wool. Treatments were randomized and the experiment was replicated 10 times. The experiment was conducted in the laboratory at 23-26 °C and 70 ± 5% RH.
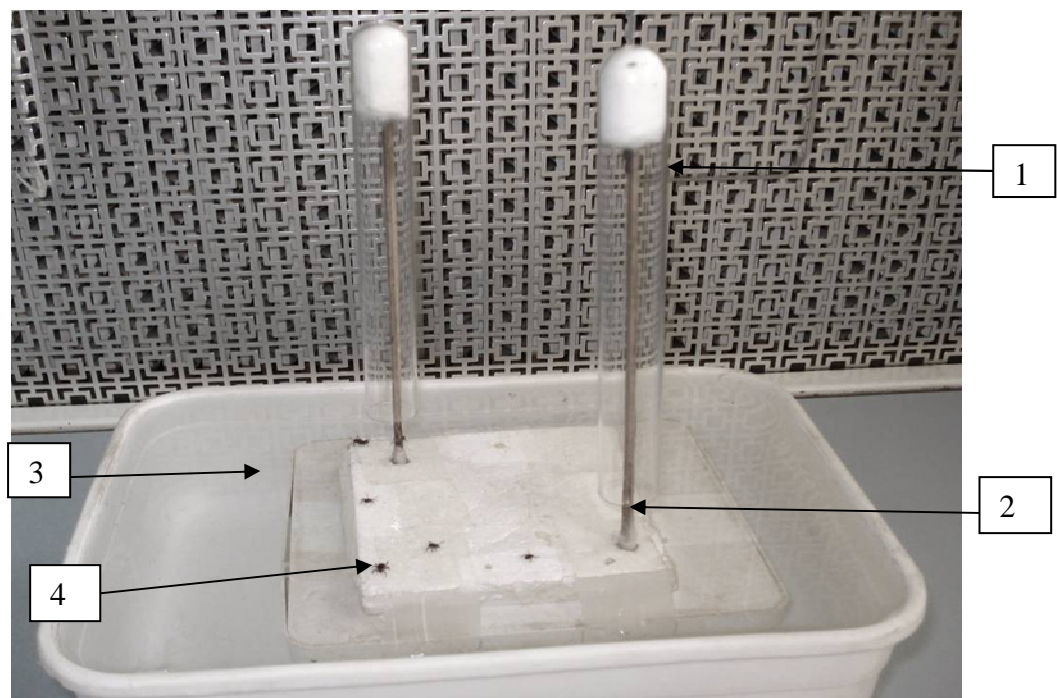
Plate 4. 1: **Experimental arena made up of inverted glass tubes (Nana, 2009)**

(1)   Glass tube, (2) Stem, (3) water surrounding the platform (4) Tick on the plaform

4.2.5.2 Dual choice T-tube olfactometer assay

This assay was adopted from previous work described by Nchu *et al.* (2009). Briefly, two cubicle glass arms (1 cm^3 × 10 cm length each) and a stem (1 cm^3 × 5 cm length) were connected tightly using hard glue. The extreme end of the arms and stem was connected to a cubicle glass chamber (3 cm^3) (Plate 4.2). The chamber of the stem served as the release point. Air entered each arm of the olfactometer from the respective odour source chamber at a flow rate of 5 ml/sec. The plant extract (0.5 ml) and the AAAP (0.5 ml) were applied to 2-cm^2 filter paper using a micropipette. In the negative control treatments, only solvents were applied to the filter paper. One tick was placed in the release chamber at a time and allowed a maximum of 5 min to make a choice in the assay chamber. Any tick that failed to respond was removed after the maximum allowed period. Twenty ticks (10 males and 10 females) were assayed for each dose and were used only once. The olfactometer was rinsed with 70% ethanol and dried at 40 °C for 10 min after each bioassay. The treatment and control chambers were switched between assays to avoid positional bias. All the assays were conducted in the laboratory at 23-26 °C and 70 ± 5% RH.
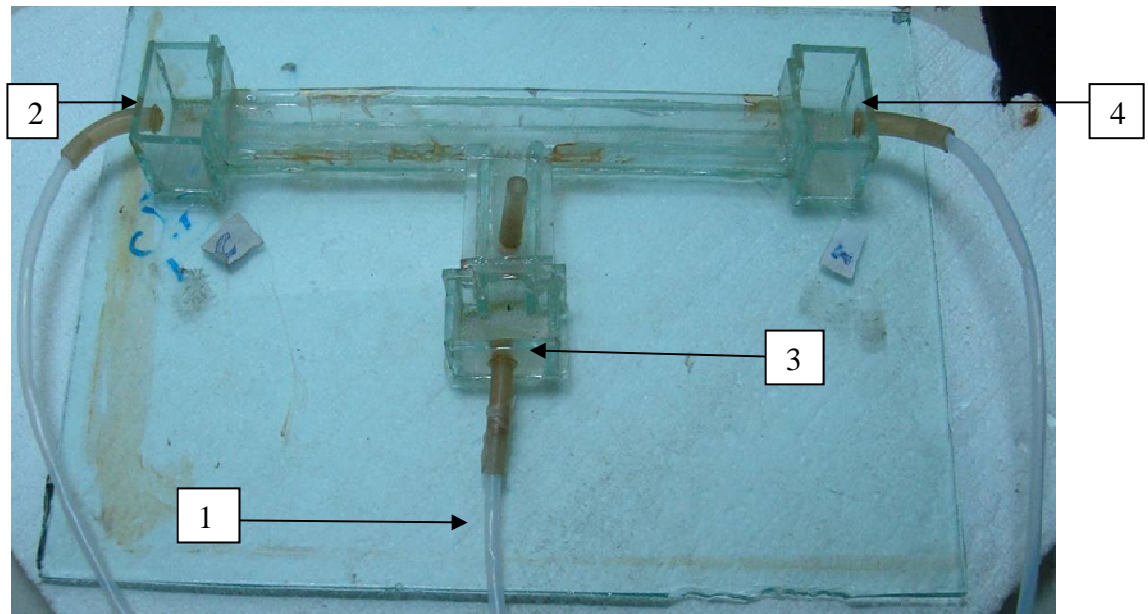
Plate 4. 2: **Dual choice T-Olfactometer (Nana, 2009)**

(1) Pipe for air flow, (2) Control side, (3) Entrance of the olfactometer (4) Test side

4.2.6 Attraction of ticks to plant extract-baited trap in semi-field plot experiments with and without CO_2

This experiment was carried out at *icipe*'s Headquarters, Duduville, Nairobi, Kenya, during the month of February 2009 using the protocol described by Nchu *et al*. (2009). The grass within each plot was cut to a height of about 5 cm. The plot was marked at l m interval using wooden pegs from the centre up to 5 m. The extract was placed in a bare 10-cm diameter circle, prepared at the centre of the plot. A 2-cm^2 rubber sponge impregnated with 1 ml of each dose of emulsifiable formulation of the plant extract was fixed on the top of each of the four wooden pegs per trap. The direction of wind was monitored using a thread attached to a wooden stick (l m)

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placed outside the circular plot in a position where the wind directed the thread roughly towards the centre of the plot. For the experiment with CO_2, plastic beakers with tops opened containing approximately 70 g of solid CO_2 were placed in the centre of the trap to serve as CO_2 source. Baited-traps with and without CO_2 were placed upwind on the chosen sites to allow ticks to move upwind. Ticks were released at 1, 2, 3, 4 and 5 m downwind from the odour source at an angle of 90° midway between the test and control traps. Ten ticks (five males and five females) were used for each distance. Ticks were marked on their dorsal shell with an artist's paint (Rowney Georgian oil colour, London Graphic Centre, London, UK) which had been diluted with linseed oil. Ticks were given a distinct colour spot by painting them topically depending on the distance from the trap. The experiment was replicated three times and was carried out in the morning between 0700 h and 1100 h. The temperature above ground during the experimental period ranged between 25 and 29° C (under the sun) and the relative humidity between 50 and 70%.

4.2.7 Statistical analysis

For each test, the total number of ticks responding to plant extract was pooled across replicates. The percentage attraction was determined using the following formula: (number of ticks in test - number of ticks in control)/(number of ticks in control + number of ticks in test) x 100. The McNemar chi-square test for the significance of change in frequencies between control and test substance was used (Sokal and Rohlf, 1981). Since there were no significant differences in response between male and female ticks, data for the two sexes were pooled together. For semi- field plot

experiments, the total numbers of ticks responding to plant extract was pooled across replicates and then analysed using ANOVA. Mean separation was carried out using Student-Newman-Keuls test. Ticks that did not respond were excluded from the analysis.

4.3 Results

4.3.1 Attraction of *R. appendiculatus* and *R. pulchellus* in inverted glass tubes assay

In the inverted glass tube assay, both sexes of *R. appendiculatus* were attracted to different formulations of *C. aurea* extracts and the AAAP used as a positive control (Table 4.1). The highest attraction values were obtained with the oil extract at concentrations of 50 mg/ml ($X^2 = 19.6; df = 1; P = 0.05$) and 100 mg/ml ($X^2 = 40.8; df = 1; P = 0.01$), aqueous extract at a concentration of 25 mg/ml ($X^2 = 8.9; df = 1; P = 0.05$), acetone extract at the concentrations of 25 mg/ml ($X^2 = 5.1; df = 1; P = 0.05$) and 50 mg/ml ($X^2 = 5.1; df = 1; P = 0.05$), and AAAP ($X^2 = 6.4; df = 1; P = 0.05$). At lower concentrations of oil extract *R. appendiculatus* was repelled. With *R. pulchellus* there was no evidence of repellency in any of the extracts with this bioassay. The concentration of 100mg/ml significantly ($X^2 = 12.6; df = 1; P = 0.05$) attracted 38.1% of *R. pulchellus*. In the case of the two other extracts there was a variable dose related reaction (Table 4.2).

4.3.2 Attraction of *R. pulchellus* in the dual choice T-tube olfactometer

Rhipicephalus appendiculatus ticks did not exhibit any response to the odour source in the dual choice T-tube olfactometer assay and were therefore excluded in this experiment. With exception to the acetone extract at the concentration of 12.5 mg/ml which was repulsive, both sexes of *R. pulchellus* were attracted to all the concentrations of the three formulations of *C. aurea* extract and the AAAP (Table 4.3). Oil extract at the concentration of 100 mg/ml ($X^2 = 23.1; df = 1; P = 0.01$) and aqueous extract at 25 mg/ml ($X^2 = 19.0; df = 1; P = 0.01$) were the most attractive (Table 4.3).

4.3.3 Attraction of adult ticks to plant extract-baited trap in semi-field plots

4.3.3.1 *Rhipicephalus pulchellus*

The response of *R. pulchellus* adult ticks to the plant extract varied with the dose and distance of release, in the absence or presence of CO_2 (Table 4.4). Significant number of *R. pulchellus* ticks were attracted from 1 m distance than from 5 m at all the doses tested. For example at the concentration of 12.5 mg/ml without CO_2, 34.4% of ticks were attracted from 1 m while no single tick was attracted from a distance of 5 m at the same concentration (Table 4.4). At a concentration of 100 mg/ml in the absence of CO_2, 52.2% and 14.4% of ticks were attracted from 1 and 5 m, respectively (Table 4.4). A similar trend in tick response was observed when CO_2 was added to the baited-trap, but there was a significant increase in the number of ticks attracted to the trap compared with responses obtained with no CO_2 added. For

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example at 100 mg/ml, 1.2 and 3 times increase in tick attraction was recorded from

1 and 5 m, respectively (Table 4.4).

Table 4. 1: **Percent relative attraction of adult *R. appendiculatus* (pooled data over replicates) to *C. aurea* extracts and AAA pheromone in inverted glass bioassays**

Substances (mg/ml)	N° of ticks attracted			
	N	*Control*	*Test*	% relative attraction[a]
Aqueous extract				
12.5	100	28	24	-7.7
25	100	30	58	31.8*
50	100	36	32	-5.9
100	100	40	56	16.6
Oil extract				
12.5	100	28	20	-16.7
25	100	46	34	-15.0
50	100	24	66	46.7*
100	100	16	78	65.9**
Acetone extract				
12.5	100	32	34	3.0
25	100	30	52	26.8*
50	100	36	56	19.1*
100	100	44	44	0
AAAP				
0.02	100	24	68	47.8*

[a] Relative attraction= (number of ticks attracted to test - number of ticks attracted to control)/Total x 100. The McNemar chi-square test for the significance of change in frequencies between control and test substance was used

* represents significance at $P < 0.05$; ** represents significance at $P < 0.01$.

Table 4. 2: **Percent relative attraction of adult *R. pulchellus* (pooled data over replicates) to *C. aurea* extracts and AAA pheromone in inverted glass bioassays**

Substances (mg/ml)	N of ticks attracted			
	N	*Control*	*Test*	% relative attraction[a]
Aqueous extract				
12.5	100	22	30	15.4
25	100	30	36	9.1
50	100	36	48	14.3
100	100	38	50	13.7
Oil extract				
12.5	100	36	38	2.7
25	100	34	40	8.1
50	100	30	42	16.7
100	100	26	58	38.1*
Acetone extract				
12.5	100	30	36	9.1
25	100	36	48	16.2
50	100	40	44	4.8
100	100	34	44	12.8
AAAP				
0.02	100	24	62	44.2*

[a] Relative attraction= (number of ticks attracted to test - number of ticks attracted to control)/Total x 100. The McNemar chi-square test for the significance of change in frequencies between control and test substance was used

* represents significance at $P < 0.05$

Table 4. 3: **Percent relative attraction of adult *R. pulchellus* (pooled data over replicates) to *C. aurea* extracts and AAA pheromone in T-tube olfactometer bioassay**

Substances (mg/ml)	N° of ticks attracted			
	N	*Control*	*Test*	% relative attraction[a]
Aqueous extract				
12.5	100	24	46	20.0*
25	100	20	52	44.4**
50	100	24	54	38.5*
100	100	36	56	22.2*
Oil extract				
12.5	100	32	42	13.4
25	100	26	50	31.6*
50	100	28	56	34.1*
100	100	20	64	52.4**
Acetone extract				
12.5	100	32	30	-3.2
25	100	38	46	9.5
50	100	42	42	0.0
100	100	34	40	8.1
AAAP				
0.02	100	28	70	34.9*

[a] Relative attraction= (number of ticks attracted to test - number of ticks attracted to control)/Total x 100. The McNemar chi-square test for the significance of change in frequencies between control and test substance was used

* represents significance at $P < 0.05$; ** represents significance at $P < 0.01$.

4.3.3.2 *Rhipicephalus appendiculatus*

The percentages of adult *R. appendiculatus* attracted to the plant extract are summarized in Table 4.5. As with *R. pulchellus,* the attraction of *R. appendiculatus* adults varied with dose and distance of release. More ticks were attracted from 1 m distance than from 5 m at all the doses tested. At 12.5 mg/ml of extract without CO_2, 23.3% of ticks were attracted at 1 m compared to 2.2% from 5 m distance. At 100 mg/ml in the absence of CO_2, 55.5% and 27.8% of ticks were attracted from 1 and 5 m, respectively (Table 4.5). On the other hand, addition of CO_2 to the plant extract baited-trap significantly increased the attraction in all the treatments (Table 4.5).

4.4 Discussion

Both *R. pulchellus* and *R. appendiculatus* adults were attracted to extracts from *C. aurea* in the inverted glass tube bioassay, which is in accordance to their climbing behaviour. In nature, *Rhipicephalus* ticks must climb onto an appropriate object such as grass or weeds where they can encounter a host. However, in the T-tube olfactometer assay, only *R. pulchellus* was attracted to the plant extracts, but not *R. appendiculatus*. There is no clear explanation to this differential behaviour between the two tick species. It was however observed that *R. pulchellus* ticks were more active than *R. appendiculatus*. In the inverted glass tube bioassays, *R. pulchellus* often clumped on top of the stem, suggesting a type of aggregation behaviour. Similar observations were made by Browning (1976) when comparing aggregation

behaviour of *R. pulchellus* and *R. appendiculatus* on grass stems in the field in Kenya.

The role of plants in integrated tick control has been the focus of attention in the last two decades (Kaaya, 2000; Wanzala *et al*., 2005; Abduz Zahir *et al*., 2009; Zorloni *et al.*, 2010). Some plants have strong acaricidal and/or repellent properties (Kaaya *et al*., 1995; Mwangi *et al*., 1995; Zorloni *et al*., 2010), while others elicit attraction in some tick species (Hassan *et al*., 1994; Zorloni *et al*., 2010). Plants produce several compounds including secondary metabolites in variable concentrations in different plant parts, the primary role of which is chemical defence to arthropods (Chadwick and Marsh, 1990). Because ticks do not attack plants, the effect of plant extracts on ticks does not have an ecological basis. Since these metabolites vary in polarity, their solubility in different solvents will also vary; hence the use of different solvents (distilled water, acetone and oil) to extract these components for screening in the bioassays. Water was used to evaluate the traditional use of the plant, acetone was used because it is the best extractant for antimicrobial compounds in plants (Eloff, 1998), and oil was used because volatile compounds are usually non-polar and would be extracted by the oil.

Table 4. 4: Attraction of adult *Rhipicephalus pulchellus* (pooled data for males and females) to different concentrations of *Calpurnia aurea* in semi-field plot experiments in absence (-) and presence (+) of CO_2

Distance (m)	Mean percentage (X ± ESM) tick attraction							
	12.5 mg/ml		25 mg/ml		50 mg/ml		100 mg/ml	
	- CO_2	+ CO_2	- CO_2	+ CO_2	- CO_2	+ CO_2	- CO_2	+ CO_2
1	34.4 ± 2.2cA	34.4 ± 4.0cA	35.5 ± 7.8dAB	52.2 ± 6.1bCE	44.4 ± 4.0dBC	57.8 ± 2.2cDE	52.2 ± 6.2cE	61.1 ± 6.1bE
2	14.4 ± 4.8bA	32.2 ± 4.0cB	25.5 ± 4.8dB	40.0 ± 3.8bC	27.8 ± 6.2cB	47.7 ± 6.1aD	36.7 ± 7.7bBC	57.8 ± 4.8aD
3	7.8 ± 2.9bA	22.2 ± 4.0bBC	15.5 ± 2.9cB	34.4 ± 2.9abD	28.9 ± 5.9cCD	43.3 ± 6.9aDE	34.4 ± 8.0bD	54.4 ± 6.1aE
4	1.1 ± 1.1aA	24.4 ± 2.2bD	5.5 ± 1.1bB	27.8 ± 4.4aD	14.4 ± 2.2bC	42.2 ± 4.0aE	20.0 ± 3.3aD	47.8 ± 10.5aE
5	0.0 ± 0.0aA	12.2 ± 6.7aB	1.1 ± 1.1aA	24.4 ± 4.0aD	8.9 ± 1.1aB	37.8 ± 5.6aE	14.4 ± 2.9aC	44.4 ± 9.7aE

Means (X ± ESM) within column followed by the same lowercase letter and within row bearing the same uppercase letter are not significantly different by Student-Newman-Keul's test (P = 0.05).

Table 4. 5: Attraction of adult *Rhipicephalus appendiculatus* (pooled data for males and females) to different concentrations of *Calpurnia aurea* in semi-field plot experiments in absence (-) and presence (+) of CO_2

Distance (m)	Mean percentage (X ± ESM) tick attraction							
	12.5 mg/ml		25 mg/ml		50 mg/ml		100 mg/ml	
	- CO_2	+ CO_2	- CO_2	+ CO_2	- CO_2	+ CO_2	- CO_2	+ CO_2
1	23.3 ± 3.8cA	27.7 ± 2.9cA	22.2 ± 1.1cA	41.1 ± 4.0cB	37.8 ± 4.8dB	48.9 ± 6.2bcB	44.4 ± 1.1cB	55.5 ± 7.2cC
2	7.9 ± 1.1bA	26.6 ± 1.9bcB	13.3 ± 5.1bA	36.6 ± 3.7bcC	24.4 ± 2.9bcD	51.1 ± 2.9bcC	34.4 ± 7.3bcC	47.8 ± 5.9bcD
3	3.3 ± 1.9aA	26.7 ± 7.0abcD	8.9 ± 1.1bA	36.7 ± 5.1bcE	18.9 ± 2.2abE	41.1 ± 8.9bC	28.9 ± 2.2bD	45.6 ± 4.8bE
4	2.2 ± 1.1aA	22.2 ± 11.2abcBC	4.4 ± 1.1aA	25.6 ± 6.2abBC	5.6 ± 2.9aA	26.7 ± 9.6aBC	14.4 ± 2.2aB	33.3 ± 3.3abC
5	2.2 ± 2.2aA	11.1 ± 2.9aB	3.3 ± 2.0aA	20.0 ± 6.9aBC	7.8 ± 1.1aA	26.7 ± 6.7aC	12.2 ± 2.2aB	27.8 ± 2.9aC

Means (X ± ESM) within-column followed by the same lowercase letter and within row bearing the same uppercase letter are not significantly different by Student-Newman-Keul's test ($P < 0.05$).

In the present study, *R. appendiculatus* adults were significantly attracted to aqueous extract at the dose of 25 mg/ml and acetone extract at the doses of 25 and 50 mg/ml in glass tube assay, while *R. pulchellus* were significantly attracted to oil extract at the concentration of 100 mg/ml. There was however a decrease in attractancy at higher concentrations (50 and 100 mg/ml) of the aqueous and acetone extracts. Topical application of acetone and aqueous extracts from *C. aurea* at 10% and 20% concentration resulted in 100 and 50% mortalities of *R. pulchellus* adult ticks (Zorloni *et al*., 2010). These concentrations correspond to 12.5 and 25 mg/ml used in our study, but interestingly, no mortality was observed in the treated ticks. This could be explained by dissimilarity in the method of application. In topical application, the plant extract permeates through the cuticle and if the plant extract content has toxic compounds, it might lead to the death of the tick; while in our case ticks were tested for their sensory perception/response to attractants in the extracts. There was a dose related response between the concentration of the oil extracts and the attraction for both tick species. Although no attempts were made in this study to identify the compound(s) responsible for attraction at this level, *C. aurea* contains considerable amounts of phenolic compounds (Adedapo *et al*., 2008), which are believed to play a key role in the attraction behaviour of over 12 species of ixodid ticks including *R. appendiculatus* and *R. pulchellus* (Wood *et al*., 1975; Mcdowell and Wallade, 1986; Yoder and Stevens, 2000).

The relative attraction of 52.4% and 65.9%, respectively, of *R. pulchellus* and *R. appendiculatus,* observed at the concentration of 100 mg/ml of oil extract was not

significantly different from the one obtained with AAAP at optimal dose of attraction (0.02 mg/ml) reported for *A. variegatum* (Nchu *et al*., 2009). It might therefore be conceivable to use high concentrations of plant extracts of *C. aurea* as substitutes to AAAP to attract and infect *R. pulchellus* and *R. appendiculatus* adult ticks in the auto-dissemination approach (Nchu *et al.,* 2009; 2010). In the semi-field plot experiments, there was a dose related attraction of both tick species at all the distances from the odour source. In all cases, the presence of CO_2 significantly increased the number of ticks attracted over the 4-hr observation period. Carbon dioxide has been reported to stimulate certain tick species such as *A. variegatum* to move, sometimes towards the source (Norval *et al*., 1989b; Maranga *et al*., 2003).

The extracts of *C. aurea* leaves did not perform as well as AAAP in attracting ticks in the *in vitro* studies at the same dose level. AAAP at the dose of 0.02 mg/ml showed attraction to both species of ticks and the most effective dose with the plant extract was 100 mg/ml oil extract. This dose could be further optimized by varying the formulation. In the semi-field plot experiments *C. aurea* suspensions work well and they could be used as a cheap way of controlling ticks because the cost of preparing the plant extract is affordable in rural settings. However, there might also be the need to screen for more potent plant extracts. For instance, Hassan *et al*. (1994) observed natural attraction of ticks to the leaves of *Acalypha fruticosa* Forsk. var. *villosa* Hutch and laboratory bioassays were able to demonstrate the attraction of ticks to the odour from the plant. Ethno-veterinary leads could be valuable in selecting species to be screened. Zorloni (2008) evaluated the activity of 28 plant

species used to control ticks on animals in southern Ethiopia and in most cases there were good activities. *Calpurnia aurea* was one of the most interesting species. It is pleasing that similar activities were found in *C. aurea* collected from Ethiopia and South Africa growing under widely different environmental conditions.

Tick control using only acaricides is costly for the resource-poor African farmer. As such, application of fungi in combination with a tick-attractant (pheromone or kairomone) seems to be a promising low-cost alternative that can be used by such farmers to control ticks (Nchu *et al*., 2009, 2010). A low quantity of inoculum is required and treatments could be applied in spot-sprays instead of total cover.

The present study has provided evidence of attraction of *R. pulchellus* and *R. appendiculatus* to *C. aurea* leaf extracts, thereby the prospects of using them in combination with entomopathogenic fungi in a trap system for auto-dissemination of fungal conidia in the field. Safety of this plant for the environment makes it an ideal component of integrated pest management systems (Liang *et al*., 2003). Future research will concentrate on the screening for more potent plant extracts attractive to ticks, and identification and compatibility studies of active components with entomopathogenic fungi.

CHAPTER FIVE

5.0 COMPATIBILITY BETWEEN THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* AND *CALPURNIA AUREA* LEAF EXTRACTS AND THEIR COMBINED EFFECTS AGAINST *RHIPICEPHALUS APPENDICULATUS*

5.1 Introduction

Rhipicephalus appendiculatus Neumann, 1901 (Acari: Ixodidae) is a serious pest in livestock production. It is one of the world's most widely distributed and damaging tick. It transmits a wide range of devastating, even fatal diseases of livestock including East cost fever, Corridor disease, and theileriosis (Razmi *et al.,* 2003). These diseases cause high mortality and morbidity, and are considered to be an important constraint to the improvement of the livestock industry in Africa (Zahid Iqbal *et al*., 2006).

Current tick control methods are still heavily dependent on the use of synthetic acaricides and repellents but several problems which have been generated by these chemicals including resistance in tick populations (Beugnet and Chardonnet, 1995; Jacobson *et al,*. 1999; Dagli and Tunc, 2001), safety risks for humans and domestic animals, contamination of ground water and effects of non-target organisms, have prompted interest in the development of more environmentally friendly alternative strategies such as biological control based the use on entomopathogenic fungi (Inglis *et al.,* 2001; Feng *et al.*, 2004; Faria and Wraight, 2007; Nchu *et al*., 2010).

A new approach whereby ticks are attracted to semiochemicals such as attraction-aggregation-attachment pheromone (AAAP) and infected with entomopathogenic fungi is also being investigated (Maranga *et al*., 2006; Nchu *et al*., 2009; 2010). Recently, Nchu *et al*. (2010) demonstrated reduction of *A. variegatum* populations in the field by infecting them with *M. anisopliae* applied in semiochemical-baited traps.

A number of plants have been reported to be attractive to ticks of the genera *Rhipicephalus* and these could be used as substitute to AAAP. They include *Acalypha fruticosa* Forssk (Euphorbiaceae)*, Ipomoea spathulata* Hallier (Convolvulaceae)*, Solanum incanum* Linnaeus (Solanaceae) (Hassan *et al*., 1994) and *Calpurnia aurea* Benth (Fabaceae) (Zorloni *et al*., 2010).

In a system where entomopathogenic fungus is used in combination with chemicals or ethno-veterinary plant extracts, it is of paramount importance to study the compatibility between the two agents (Hirose *et al*., 2001). Some plant extracts have been reported to affect negatively entomopathogenic fungi and subsequently the control of the pest (Duarte *et al*., 1992; Malo, 1993). For instance, seed aqueous extract from *Azadirachta indica* (Meliaceae) commonly known as neem have been reported to reduce conidial vegetative growth and production, but did not affect the viability of spores (Rogerio *et al*., 2005). The present study was therefore initiated to evaluate *in vitro* the compatibility of the fungus *M. anisopliae* and *C. aurea* leaf extract, and AAAP. I also evaluated the virulence of *M. anisopliae* against different developmental stages (larvae, nymphs and adults) of *R. appendiculatus* when formulated in emulsifiable extract of *C. aurea*.

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5.2 Materials and methods

5.2.1 Plant material

Calpurnia aurea leaves from South Africa was described in chapter 2, section 2.7.

5.2.2 Preparation of *C. aurea* emulsifiable formulation

An emulsifiable formulation of the plant extract was used for the field experiment. It was prepared as described in chapter 3, section 3.3.

5.2.3 Preparation of the pheromone

Attraction-aggregation-attachment-pheromone (AAAP) was prepared by mixing 0.2 mg of ortho-nitrophenol, 0.1 mg of methyl salicylate and 0.8 mg of nonanoic acid. Tests were carried out using pheromone at the concentrations of 0.005, 0.01 and 0.02 mg/ml. This dose was previously shown to significantly attract *R. appendiculatus*. The synthetic compounds used to prepare AAAP were obtained from Sigma–Aldrich Chemie GmbH, Steinheim, Germany.

5.2.4 Fungus

Metarhizium anisopliae isolate ICIPE 07 was described in Chapter 3.6.

5.2.5 Tick colony

Different stages (larvae, nymphs and adults) of *R. appendiculatus* ticks were used. They were obtained from the *icipe*'s Animal and Quarantine Rearing Unit. The ticks were counted in batches of 20. Each batch was then placed in a vial with a cotton

wool plug and the ticks were stored in darkness at RH 75% and 25 ± 2° C until further use.

5.2.6 Mycelia dry weight assessment

A conidial suspension (0.1 ml of 1×10^{6} conidia ml^{-1}) was spread plated on SDA plates. Plates were then incubated at 25° C for three days in order to obtain mycelial mats (Dimbi *et al*., 2004). The unsporulated mycelial mats were then cut from the culture plates into round agar plugs using a 4mm-diameter cork borer. Each agar plug was then transferred singly onto the centre of a fresh SDA agar plate of 90-mm diameter amended with 0%, 1.2%, 2.5%, 5% and 10% *C. aurea* emulsifiable extracts. Agar plug was also transferred singly onto the centre of a fresh SDA agar plate of 90-mm diameter amended with AAAP at the concentrations of 0.005%, 0.01% and 0.02%. Control plates were amended with respective solvents (water and oil). Four replicate Petri dishes were sealed with Parafilm and incubated in complete darkness at $25\ ^{o}$C for 7 days. After a week, the mycelial mat was taken with sterile spatula, placed in sterile dishes containing filter paper. The initial weight of the paper was recorded. The Petri dishes were kept in hot air oven at $50°$ C for 30 minutes and the final weight of the fungal mat along with the filter paper was recorded immediately. The difference between the final and initial weight was considered as dry weight of mycelium.

5.2.7 Radial fungal growth

The unsporulated mycelial mats were cut and transferred singly onto the centre of a fresh SDA agar plate of 90-mm diameter amended with 0%, 1.2%, 2.5%, 5% and 10% *C. aurea* emulsifiable extracts and SDA agar plate of 90-mm diameter amended with AAAP at the concentrations of 0.005%, 0.01% and 0.02%. Control plates were amended with respective solvents. Four replicate Petri dishes were sealed with Parafilm and incubated upside down in complete darkness at 25 ^{o}C. Radial growth was recorded daily for 6 days using two cardinal diameters, through two orthogonal axes previously drawn on the bottom of each petri dish to serve as a reference (Plate 5.1).
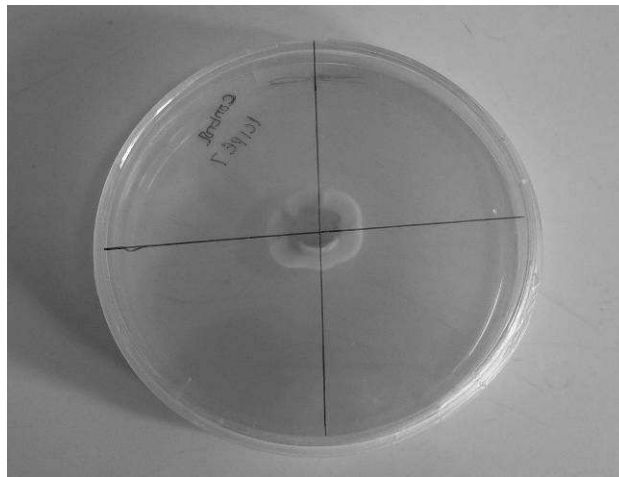
Plate 5. 1: **Radial fungal growth of a pure culture of *M. anisopliae* (Nana, 2009)**

5.2.8 Spore production assessment

A conidial suspension (0.1 ml of 1×10^6 conidia ml^{-1}) was spread plated on a fresh SDA agar plate of 90-mm diameter amended with 0%, 1.2%, 2.5%, 5% and 10% *C. aurea* emulsifiable extracts and SDA agar plate of 90-mm diameter amended with AAAP at the concentrations of 0.005%, 0.01% and 0.02%. Plates were then incubated at 25 ^{o}C for seven days. The sporulated mycelial mats were then cut from the culture plates into round agar plugs using a 4mm-diameter cork borer. Each agar plug was then transferred singly onto the universal bottle containing 10 ml of sterile distilled water with 0.02% sterile Tween 20. It was then vortexed and the conidial concentration was determined using a Neubauer counting chamber. This experiment was replicated four times.

5.2.9 Compatibility Calculations

Compatibility was calculated by using the formula proposed by Alves *et al.* (1998) to classify chemical products according to their toxicity to entomopathogenic fungi *in vitro*. This classification is based on calculations of the T factor, which relates vegetative growth (VG) and sporulation values (conidiogenesis) (SP) to the control (%): $T = [20 \ (VG) + 80 \ (SP)]/100$. In this model, values for mycelia dry weight (MDW) and sporulation count (SC) are given in relation to the control (100%). "T" values between 0 and 30 classify products as very toxic; from 31 to 45 as toxic; from 46 to 60, moderately toxic; and above 60, products are considered compatible with the fungus being studied.

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5.2.10 Susceptibility of larvae, nymphs and adults stages of *R. appendiculatus* to *M. anisopliae* formulated in emulsifiable extract of *C. aurea*

Conidia of *M. anisopliae* isolate ICIPE 07 were harvested from a 3-week old culture by scrapping the surface of sporulating culture. Conidia were suspended in sterile distilled water containing 0.05% Triton X-100 amended with 0, 1.25, 2.5, 5 and 10% of emulsifiable extracts in universal bottles with glass beads. The suspension was vortexed for 5 minutes to produce homogenous conidial suspension. The viability of conidia was then determined by spray-platting 0.1 ml of the suspension (titrated to 3.0×10^6 conidia ml^{-1}) on SDA plates. Plates were incubated at 26 ± 2 ^{o}C for 18 hours. Sterile microscope cover slip was then placed on each plate and the percentage of germination was determined by counting 100 spores for each plates. Ten millilitres (10 ml) of standard concentration of 1.0×10^9 conidia ml^{-1} of each treatment was sprayed on larvae, nymphs and adults *R. appendiculatus* using the Burgerjon's spray tower (Burgerjon, 1956) (INRA, Dijon, France). Each treatment group had two different controls: one received sterile distilled water containing 0.05% Triton X-100 only and the other received sterile distilled water containing 0.05% Triton X-100 with 10% emulsifiable extract without fungus. Twenty ticks were used for each treatment and the experiment was replicated five times. Tick-tests were transferred in the vials (1.5 cm $\times$ 12 cm) and maintained in an incubator at $25 \pm$ 2 ^{o}C with 75% RH. Mortality was recorded daily for 14 days. Dead ticks were surface-sterilized with 2.5% sodium hypochlorite and 70% alcohol, rinsed twice in

sterile distilled water, and then placed into 9-cm diameter Petri dishes lined with moistened filter paper to allow the growth of fungus on the cadaver.

5.2.11 Data analysis

Analysis of variance (ANOVA procedure of SAS) was used to analyze percentage germination, radial growth and mortality data (SAS institute, 1990) after arcsine transformation to normalize the data. Percentage mortality (at 14 day post-treatment) was also adjusted for natural mortality in controls using Abbott (1925) formula before analysis and was then analyzed using two-way analysis of variance for a completely randomized design. Tukey test was used for post hoc analysis. A value of $P < 0.05$ was considered significant.

5.3 Results

5.3.1 Compatibility of emulsifiable extract of *C. aurea* and AAAP with *M. anisopliae*

Compared to the control, emulsifiable formulation of the extract of *C. aurea* did not affect the vegetative growth, conidial yield, dry weight and viability of the *M. anisopliae* at all the concentrations (Tables 5.1 and 5.3). On the hand, AAAP had negative effects on all the growth parameters of *M. anisopliae* at all the concentrations tested (Tables 5.2 and 5.4).

5.3.2 Virulence of *M. anisopliae* formulated in emulsifiable extract of *C. aurea* against different developmental stages of *R. appendiculatus*

Mortality in the controls was 1.7 ± 0.8, 2.8 ± 0.9 and $2.3 \pm 1.8\%$ in larvae, nymphs and adults, respectively (Table 5.5). In viability tests, approx. 98% of conidia germinated. *M. anisopliae* caused mortalities in all the developmental stages of *R. appendiculatus*. However, mortality varied according to the developmental stage. For instance, mortality of 100% was observed in larvae, between 74.8-79.1% in nymphs and 68.9-74.1% in adults (Table 5.5). No significance difference in virulence was observed between *M. anisopliae* applied alone and *M. anisopliae* formulated in different concentrations of *C. aurea* extract. All the ticks that died in fungus-treated treatments developed mycosis.

Table 5. 1: **Effects of emulsifiable formulation of *C. aurea* leaf extract on radial growth, mycelial dried weight, conidial yield and viability of *M. anisopliae* isolate ICIPE 07**

Emulsifiable leaf extract/concentration	Colony diameter (mm)		Mycelial dry weight (mg)	Yield ($\times 10^8$ conidia m^{-1})	(%) conidial germination
	3 d post-inoculation	6 d post-inoculation			
	mm	mm			
Control	$17.2 \pm 1.0a$	$33.0 \pm 0.5a$	$81.1 \pm 1.1a$	$11.2 \pm 1.7a$	$98.4 \pm 1.7a$
SDA+1.2%	$15.2 \pm 1.0a$	$31.4 \pm 1.1a$	$74.9 \pm 7.6a$	$11.1 \pm 0.6a$	$97.2 \pm 1.1a$
SDA + 2.5%	$16.8 \pm 1.7a$	$32.2 \pm 0.5a$	$78.0 \pm 5.1a$	$10.6 \pm 1.4a$	$98.4 \pm 2.8a$
SDA + 5%	$17.2 \pm 1.5a$	$31.4 \pm 0.5a$	$80.5 \pm 12.8a$	$10.8 \pm 0.8a$	$97.8 \pm 2.6a$
SDA + 10%	$16.8 \pm 1.0a$	$31.8 \pm 0.5a$	$75.2 \pm 9.7a$	$9.7 \pm 4.8a$	$97.8 \pm 2.1a$
F value	2.06	2.85	0.59	0.27	0.26
P value	0.12	0.06	0.67	0.89	0.89

Means followed by the same letter on same column are not significantly different by ANOVA ($P < 0.05$)

Table 5. 2: Effects of AAAP on radial growth, mycelial dried weight, conidial yield and viability of *M. anisopliae* isolate ICIPE 07

AAAP concentrations	Colony diameter (mm)		Mycelial dry weight (mg)	Yield ($\times 10^8$ conidia m^{-1})	(%) conidial germination
	3 d post-inoculation	6 d post-inoculation			
	mm	mm			
SDA + 0%	$16.4 \pm 1.5a$	$31.6 \pm 0.5a$	$77.9 \pm 1.3a$	$10.6 \pm 1.5a$	$99.0 \pm 1.0a$
SDA+0.005%	$6.6 \pm 1.0b$	$16.8 \pm 1.1b$	$8.3 \pm 1.1b$	$3.9 \pm 0.5b$	$13.2 \pm 16.0b$
SDA + 0.01%	$6.8 \pm 1.0b$	$16.6 \pm 1.5b$	$7.9 \pm 1.0b$	$0.4 \pm 0.4b$	$10.0 \pm 12.5b$
SDA + 0.02%	$6.8 \pm 2.0b$	$14.2 \pm 1.1b$	$6.7 \pm 1.2b$	$0.0 \pm 0.0b$	$0.0 \pm 0.0 b$
F value	56.24	239.68	4278.45	166.79	101.99
P value	0.0001	0.0001	0.0001	0.0001	0.0001

Means followed by the same letter on the same column are not significantly different by Tukey test ($P < 0.05$)

Table 5. 3: **Values and compatibility classification of various concentrations of emulsifiable extract from *C. aurea* on *M. anisopliae***

Emulsifiable plant extract	*M. anisopliae*	
	"T" Values	Classification[1]
SDA + 1.25%	96.3	HC
SDA + 2.5%	94.9	HC
SDA + 5%	96.5	HC
SDA + 10%	87.9	C

[1] HC = Highly Compatible, C = Compatible Alves et al. (1998)

Table 5. 4: **Values and compatibility classification of various concentrations of AAAP on *M. anisopliae* isolate**

AAAP concentrations	*M. anisopliae*	
	"T" Values	Classification[1]
SDA + 0.005%	31.5	T
SDA + 0.01%	5.4	VT
SDA + 0.02%	1.7	VT

[1] T = Toxic, VT = Very Toxic; Alves et al. (1998)

Table 5. 5: **Percent mortality of larvae, nymphs and adult *R. appendiculatus* ticks caused by *M. anisopliae* (Ma) alone or in association with different concentrations of emulsifiable extract of *C. aurea***

Treatments	Mortality (Mean % ± SD)		
	Larvae	Nymphs	Adults
Control (no extract, no fungus)	$1.7 \pm 0.8a$	$2.8 \pm 0.9a$	$2.3 \pm 1.8a$
Control (10% extract no fungus)	$2.5 \pm 4.0a$	$1.9 \pm 1.0a$	$3.2 \pm 0.7a$
Ma 10^9 + 0%	$100.0 \pm 0.0b$	$77.3 \pm 7.5b$	$72.5 \pm 5.6b$
Ma 10^9 + 1.25%	$100.0 \pm 0.0b$	$79.1 \pm 9.2b$	$70.8 \pm 7.6b$
Ma 10^9 + 2.5%	$100.0 \pm 0.0b$	$74.8 \pm 9.7b$	$74.1 \pm 8.5b$
Ma 10^9 + 5%	$100.0 \pm 0.0b$	$75.7 \pm 9.5b$	$68.9 \pm 9.9b$
Ma 10^9 + 10%	$100.0 \pm 0.0b$	$74.9 \pm 9.8b$	$69.7 \pm 6.2b$
F value	2952.26	74.82	85.21
P value	0.0001	0.0001	0.0001

Means followed by the same letter in the same column are not significantly different by Tukey test ($P < 0.05$)

5.4 Discussion

The main objective of the present study was to evaluate the compatibility of the *M. anisopliae* and AAAP and emulsifiable formulation of *C. aurea* in order to use them in autodissemination approach, whereby ticks that are attracted to kairomone/pheromone will be infected with fungus. The results indicate that emulsifiable extract from *C. aurea* incorporated into SDA media did not affect

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fungal growth parameters regardless of the concentrations. Similar results have been reported with extracts from *Ocimum sanctum* Linn. (Lamiaceae) with *M. anisopliae* (Borgio *et al*., 2008). Formulation of conidia of *M. anisopliae* in *C. aurea* emulsifiable formulation did not also affect the virulence of the pathogen against the developmental stages of *R. appendiculatus*. Although all the stages were susceptible to fungal infection, larval stage was more susceptible than nymphal and adult stages. Similar results were reported by Kirkland *et al*. (2004) and Hartelt *et al.* (2008) when studying entomogenous fungi as promising biopesticides for the control of ticks.

Contrary to *C. aurea,* AAAP significantly inhibited all the growth parameters of *M. anisopliae.* This inhibition could be due to nonanoic acid and synthetic phenolic compounds that are part of the tick pheromone. Nonanoic acid produced by *Trichoderma* spp. has been reported to inhibit the spore germination and the mycelia growth of two cocoa pathogens (Madhu *et al*., 2005). On the other hand, phenolic compounds have been documented to inhibit the growth of entomopathogenic fungi (Lopez-Ilorca and Olivares-Bernabeu, 1997). These results suggest that conidia of *M. anisopliae* cannot be mixed with AAAP but used separately (Nchu *et al*. 2009; 2010).

It can be therefore concluded that emulsifiable formulation of *C. aurea* does not have any effect on *M. anisopliae* and could be mixed together for a spotted spray as a tick control method in grazing field while AAAP should be used separately.

CHAPTER SIX

6.0 AN ATTRACTANT TRAP FOR INFECTION AND AUTODISSEMINATION OF *METARHIZIUM ANISOPLIAE* AMONGST ADULT *RHIPICEPHALUS APPENDICULATUS* TICKS

6.1 Introduction

Ticks are blood-sucking ectoparasites of mammals, birds and reptiles throughout the world, so they are potentially involved in transmission of the widest variety of pathogens including bacteria, protozoa and viruses between different animals in nature (Rak, 1976). Despite the major role in control of hard ticks, synthetic acaricides suffer from a large limitation. Because of the hard delayed degradation, their residues usually remain in agricultural environment where they adversely affect the life of living organisms in natural ecosystem. As well, they are able to induce the production of resistant strains of ticks (Drummond, 1977; Baxter and Barker, 2002; Ducornez *et al.,* 2005). Besides of their adverse effects of synthetic acaricides on ecosystem, some recent studies have shown that chemical substances widely used for pest control have a considerable genotoxic and cytotoxic effect on human target cells (Undeger and Basaran, 2005), so it is necessary to look for alternative methods which are adaptable, safer and cheaper than chemical substance.

The interest in alternative methods for the control of ticks has been considerably increased in recent years, in accordance with increasing demands for safer animal

products and environmental protection (Georghiou, 1986; Georghiou and Lagunes-Tejada, 1991). Entomopathogenic nematodes and entomopathogenic fungi are being considered among the most promising agents for biological control of ticks (Hassanain *et al*., 1997; Zhioua *et al*., 1999; Maniania *et al.*, 2007). Entomopathogenic fungi are generally applied in inundative and augmentative approach (Lacey and Goettel, 1995). For instance, Kayaa and Hassan (2000) showed that spraying fungi in grazing pastures seeded with *R. appendiculatus* larvae significantly reduced the populations of these tick species on cattle. However, a new strategy is currently being investigated and by which pests are infected after being attracted into a focus of the pathogen (Vega *et al.,* 2007). High levels of control of *Amblyomma varigatum* have been demonstrated in the field using a combination of tick pheromone and entomopathogenic fungi (Maranga *et al* 2006; Nchu *et al*., 2010)**.**

Some ethnoveterinary plants have been reported to attract ticks of the genus *Rhipicephalus* in the laboratory (Hassan *et al*., 1994; Zorloni *et al*., 2010). I previously demonstrated the attraction of *R. appendiculatus* to emulsifiable leaf extract of *C. aurea* in a semi-field plot experiment. In the present study, I evaluated an attractant trap for infection and autodissemination of *M. anisopliae* amongst adult *R. appendiculatus* ticks in semi-field plot experiments and assessed the prospects for tick control.

6.2 Materials and methods

6.2.1 Tick colony used

Two to three month-old unfed *R. appendiculatus* ticks were used in the experiments. They were obtained from the *icipe*'s Animal and Quarantine Rearing Unit. The ticks were counted in batches of 30 consisting of 15 males and 15 females. Each batch was placed in a vial with a moist cotton wool plug. Males and females were marked on the dorsal side using a camel brush and artist paint. Ticks of different batches were marked with different colours. They were returned to the vials which were then plugged with cotton wool. Marking was done a day before field experiments, and the ticks were stored in darkness at RH 75% and 25 ± 2° C.

6.2.2 *Calpurnia aurea* extract

C. aurea leave extract was prepared as described in the section 4.2.2.

6.2.3 Fungus

The *M. anisopliae* isolate ICIPE 07 used in this study was obtained from the *icipe*'s Arthropod Germplasm Centre. The strain was isolated from an engorged female *A. variegatum* collected from Rusinga Island, Kenya in 1996 and was previously reported to be virulent against *R. appendiculatus* (Kaaya *et al*., 1996). Conidia were mass produced using long rice as a substrate (Milner R.J., unpubl.). The liquid medium was made up of glucose (30 g/l), peptone (10 g/l) and yeast extract (30 g/l) in a 250 ml Erlenmeyer flask. The content of the flask was autoclaved for 30 minutes at 121°C and allowed to cool before inoculation with the conidia. The culture was

maintained in a shaker at 100 rpm and $26 \pm 2^{\circ}$ C for 3 days. Two kilograms of rice per plastic bag was autoclaved for 1 h at 121°C, transferred to polyethylene autoclavable bags and inoculated with 50 ml 3-day old culture of blastospores. The substrate was thoroughly massaged to distribute the inoculum throughout and incubated in room conditions ($26\text{-}30^{\circ}$ C and 60-75% R.H.) for 21 days. The polyethylene bag was then opened and the culture was allowed to dry for 5 days at room temperature to approximately 10-15% moisture content. Conidia were harvested by sifting the substrate through a sieve (295 μm mesh size) and approximately 200 g of spores was produced per bag. Dry conidia were stored in a refrigerator ($4\text{-}6^{\circ}$ C) for at most 1 week prior to use. The viability of conidia was determined using the technique described by Goettel and Inglis (1997) before being used in the field trials. Germination rates > 90% after 24 h on Sabouraud dextrose agar was considered adequate for use in the field trials. One litre of emulsifiable formulation (consisting of 49.5% sterile distilled water, 49.5% corn oil [Elianto, BIDCO Oil Refineries Ltd] and 1% Tween 80) of *C. aurea* extract with conidial suspension containing 1×10^{9} conidia ml^{-1} was prepared for the trials. One litre of a control solution was also prepared in a similar manner without the fungus.

6.2.4 Attraction and infection of *R. appendiculatus* with *M. anisopliae* formulated in emulsifiable extract of *C. aurea* in semi-field plot experiments

This experiment was carried out at ICIPE's Headquarters, Duduville, Nairobi, Kenya, during the month of December 2009 using the protocol described by Nchu *et al*. (2009). The grass within each plot was cut to a height of about 5 cm. The plot was

marked at l-m interval using wooden pegs from the centre up to 5 m. The centre of the plot (30 cm radius) was the trap area (Figure 6.1) in the fungal test plots were treated with 250 ml of emulsifiable formulation of plant extract and fungal conidia using a 1 L hand sprayer at a output of approximately 150 L/hectare. Plastic beaker containing approximately 70 g of solid CO_2 obtained from Carbacid Kenya was placed at the centre of the trap area to serve as CO_2 source. Trap areas with CO_2 in the centre were placed upwind on the chosen sites to allow ticks to move upwind. In the control plots, trap area were treated with an emulsifiable plant extract carrier without the fungus. Ticks were released at 1, 2, 3, 4 and 5 m downwind from the odour source. Twenty ticks (ten males and ten females) were used for each distance. Ticks were marked with an artist's paint (Rowney Georgian oil colour, London Graphic Centre, London, UK) which had been diluted with linseed oil. Ticks were given a distinct colour spot by painting them topically and ventrally depending on the distance from the trap. The experiment was replicated three times and was carried out in the morning between 0700-1100 h. The temperature above ground during the experimental period ranged between 25 and 29° C (under the sun) and the relative humidity between 50 and 70%.
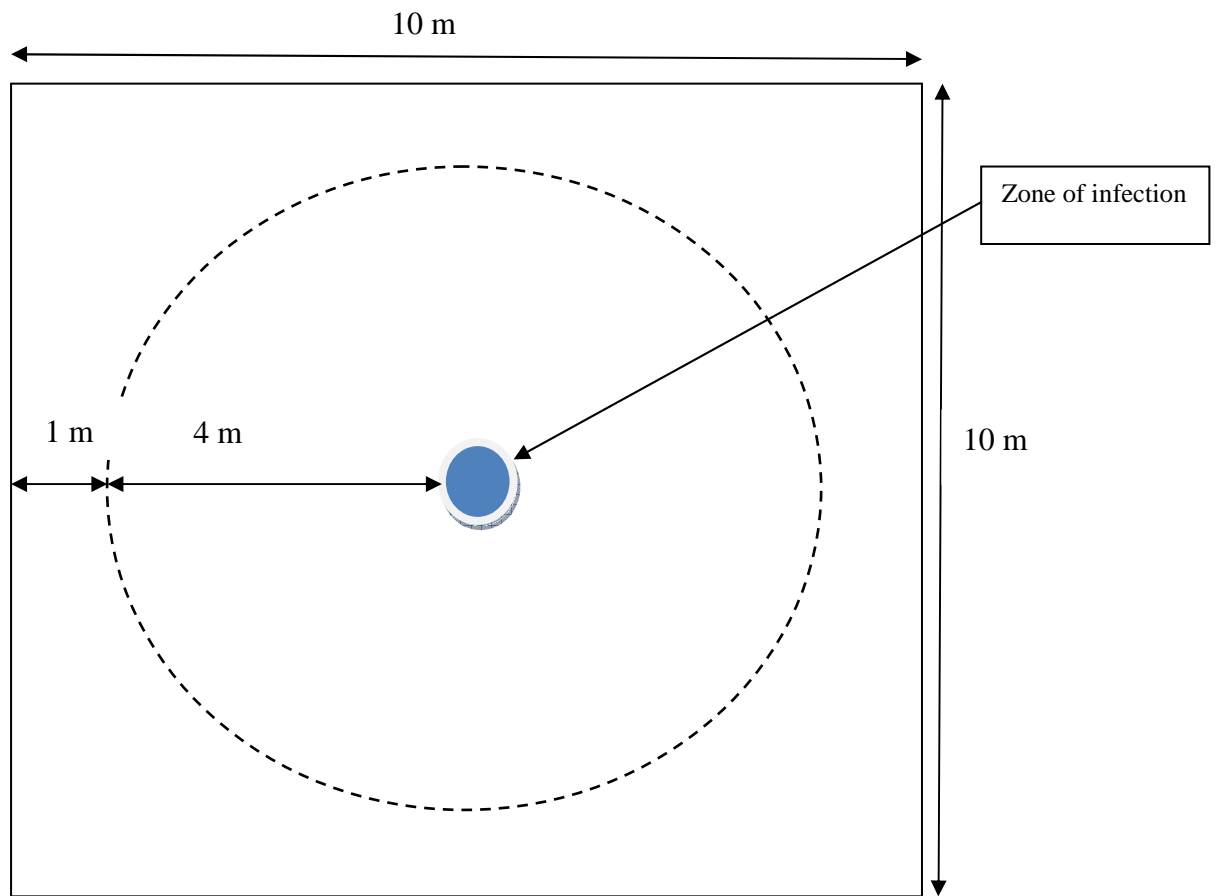
Figure 6. 1: Illustration of the semi-field plot for attraction and infection of *R. appendiculatus*

6.2.5 Assessing the inoculum uptake by ticks

The objective was to estimate the number of conidia that a single tick picked up while visiting plant extract-baited trap after treatment. Four ticks per replicate and per treatment were selected at random and each was transferred separately into 10-ml vial and brought to the laboratory. Vials were filled with 5 ml 0.05% Triton X-100 and subjected to vigorous shaking by a vortex shaker 5 min to dislodge conidia from the tick surface. The number of conidia was determined using the improved

Neubauer counting chamber (Nchu *et al.,* 2009). The treatment consisted of five replicates of four ticks each.

6.2.6 Evaluation of the efficacy of treatments

Ticks collected in each treated plot were transferred to labelled 2-cm diameter glass vials and brought to the laboratory where 50% were maintained at 26 ± 2° C, 85 ± 5 % RH and 12:12 h L:D photoperiod for two weeks. Mortality was recorded after two weeks. Dead ticks were surface-sterilized with 2.5% sodium hypochlorite and 70% alcohol, rinsed twice in sterile distilled water, and then placed into 9-cm diameter Petri dishes lined with moistened filter paper to allow the growth of fungus on the cadaver. The other 50% were transferred to cotton cloth sleeves (ear-bags) and attached to the back of naïve rabbits using leucoplast. Ticks were allowed to attach and feed. The period of feeding was recorded. Ticks detaching were recovered daily by opening the free ends of the ear-bags. The ticks were then taken to the laboratory to determine engorgement weights and incubated at $26 \pm 2°C$ and 70-90% RH during which mortality, egg mass, egg counts, and egg hatchability were determined.

6.2.7 Efficacy of *M. anisopliae* formulated in emulsifiable extract of *C. aurea* in reducing the number of ticks and transmission of inoculum in potted grass

Conidia of *M. anisopliae* were formulated in emulsifiable extract of *C. aurea.* The latter served as attractant. The suspension was then sprayed in the centre of a 900 cm^2–trap using a hand sprayer. In the control plots, traps were treated with an emulsifiable plant extract carrier without the fungus. Twenty-four (24) male ticks per

replicate were released at 4-m away from the traps. CO_2 was used to increase the attraction. Ticks that were attracted to the *M. anisopliae*-baited trap were collected and transferred to plastic basins (41 cm top diameter × 29 cm bottom diameter × 24 cm height) previously planted with *Pennisetum clandestinum* (kikuyu grass) (Plate 6.1). The height of the grass was approximately 12 cm. Same number of non-treated female ticks was introduced in the basins to have a ratio of 1:1 in order to assess horizontal transmission of inoculum between fungus-infected male and non-treated female ticks. The top of the basin was sealed with mosquito net (supplied by Amiran Kenya) using rubber bands to prevent ticks from escaping. The experiment was allowed to run for 5 weeks, after which the number of ticks was recorded. Ticks that survived infection after 5 weeks in the field were transferred to 9-cm diameter Petri dish and brought to the laboratory where they were maintained at $26 \pm 2\ ^{o}C$, $75 \pm 5\%$ RH and 12:12 h L:D photoperiod for 3 weeks, after which mortality was recorded. Dead ticks were surface-sterilized with 2.5% sodium hypochlorite and 70% alcohol, and rinsed twice in sterile distilled water. They were then placed into 9-cm diameter Petri dish lined with moistened filter paper to favour development of mycosis on the surface of the cadaver. Petri dishes were sealed with paraffin and maintained at room temperature ($26 \pm 2\ ^{o}C$). Five plastic basins were used for each treatment, which represented five replicates. The experiment was conducted from March to April 2010, which corresponded to rainy season. The average daytime range of temperatures and relative humidity within buckets were $24.8 \pm 0.7 – 29.3 \pm 0.5\ ^{o}C$ and $86.3 \pm 2.4 – 89 \pm 1.8\%$, respectively.

Plate 6. 1: **Potted grass seeded with male fungus-infected and untreated female**
R. appendiculatus **(Nana, 2010)**

6.2.8 Data analysis

Since there were no significant differences in response between male and female
ticks, data for the two sexes were pooled together. For each test, the total numbers of
ticks responding to the plant extract was pooled across replicates and then analysed
using ANOVA. Mean separation was carried out using Student-Newman-Keuls test.
A student's t-test was used to compare the following: (i) percentage of ticks
recovered from control and fungus treated semi-field plots; (ii) percentage tick
mortality in the laboratory of ticks recovered from the control and fungus-treated
plots. The relative (%) reduction of tick populations in treated plots was calculated

using the formula [(number of surviving ticks recovered from control basins -number of surviving ticks recovered from fungus-treated basins)/number of surviving ticks recovered from control basins] × 100 (European Medicines Agency, 2004). All analyses were performed using the SAS (2001) package.

6.3 Results

6.3.1 Attraction and mortality of *R. appendiculatus*

No significant differences in attraction of ticks were observed between the control (plant extract without fungus) and treated plots (conidia formulated in emulsifiable plant extract). However, significant differences were observed in the attraction of groups of ticks released between 1 and 5 m away from the trap. The mean percentage of ticks attracted from 5 m was significantly lower ($F = 27.67; P = 0.001$) than the one attracted from 1 m (Figure 6.2). Mortality of ticks that were collected from the field plots was significantly higher ($F = 13.34; P < 0.001$) among those collected from fungus-treated than the control treatments (Figure 6.3). Forty nine (49%) per cent of the ticks released were attracted and exposed to the fungus and of these, eighty three (83%) per cent of the ticks succumbed to fungal infection in fungus-treated plots compared to 3% in the controls. All the ticks that died in fungus-treated plots developed mycosis (Plate 6.2). No mycosis was observed on dead ticks collected from control semi-field plots.
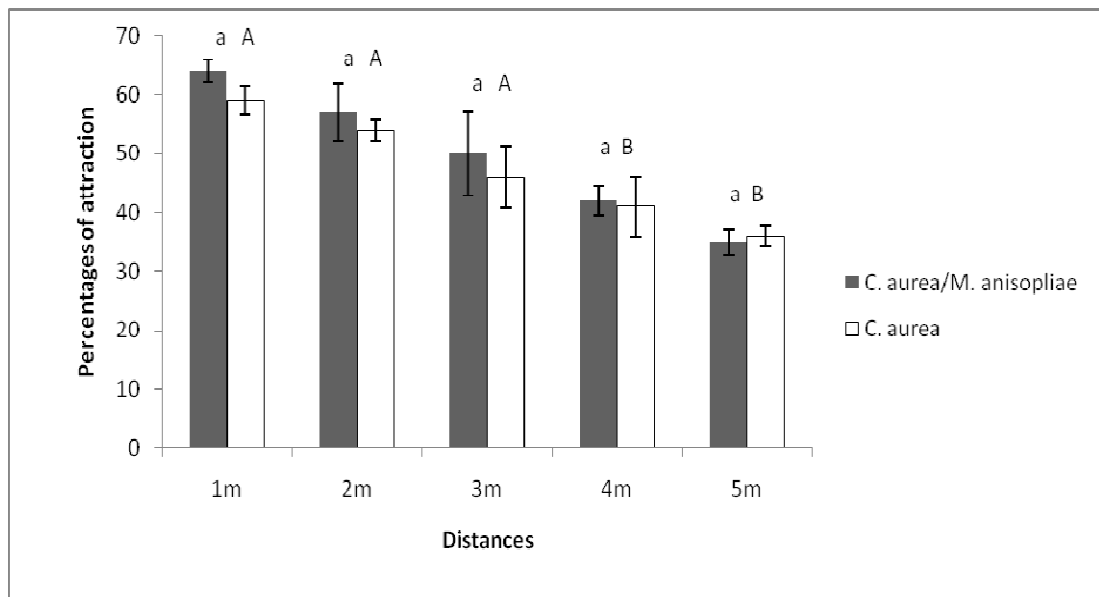
Figure 6. 2: **Mean percentage (± SD)** *R. appendiculatus* **attracted to** *M. anisopliae/C. aurea***-baited trap and** *C. aurea***-baited trap in semi-field plots. (Means with the same letter are not significantly different by SNK).**
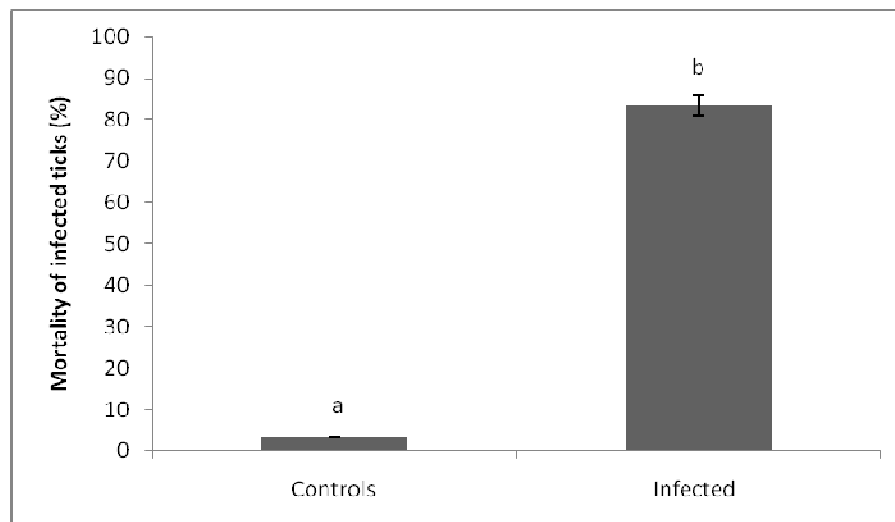
Figure 6. 3: **Mean percentage mortality (± SD)** *R. appendiculatus* **due to** *M. anisopliae* **following infection through fungus/plant extract-baited trap. (Mean bearing the same letter are not significant)**
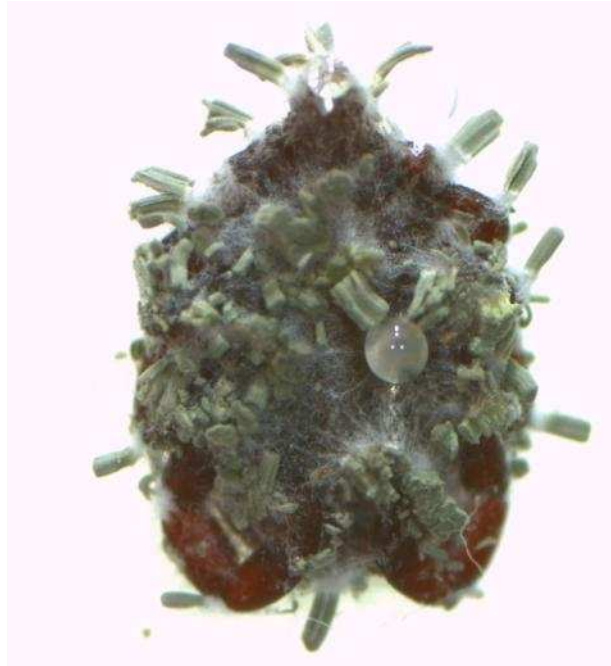
Plate 6. 2: **Mycosis by *M. anisopliae* on *R. appendiculatus* cadaver; viewed from the ventral side. (Leica Microscope, 16 ×)**

6.3.2 Effects of fungal infection on *R. appendiculatus* feeding and reproduction potentials

Tick mortality in the control group was 6.6%. *M. anisopliae* formulated in emulsifiable extract of *C. aurea* caused mortality of 40% (Table 6.1). The reproductive and feeding potentials of female ticks that survived infection in the field were significantly affected by the treatment. Fungus-infected ticks had significantly long period of engorgement and reduced blood meal intake as compared to the controls (Table 6.1). Pre-oviposition, oviposition and post-oviposition period was significantly longer in fungus-treated than in the control treatment (Table 6.1). There

were also significant reduction (P < 0.0001) in body weight and egg mass of fungus-infected ticks. For instance in the control, the average weight of the engorged ticks and egg masses was 333.1 ± 40.9 and 234.1 ± 29.3 mg, respectively, while in *M. anisopliae* infected ticks the average weight was 232.2 ± 50.4 and 147.9 ± 45.7 mg, respectively (Table 6.1). Significant reductions (P < 0.0001) in viability of eggs produced by surviving infected ticks were also observed; with the control group having 90% egg viability compared to 55% in fungus-treated group. Batch of eggs that failed to hatch and incubated at 26 ± 2° C and 85 ± 5% RH developed mycosis on the surface (Plate 6.3). No mycosis was observed on the egg that failed to hatch on the control group.

6.3.3 Efficacy of *M. anisopliae* formulated in emulsifiable extract of *C. aurea* in reducing tick populations and transmission of inoculum in potted grass

The number of conidia picked by individual tick (data pooled across replicates) immediately after attraction and contamination in the trap was 3.2 - 4.1$\times$ 10^5. No conidium was found in the control ticks. The mean percentage of male ticks recovered in the basins 5 weeks after exposure to the *M. anisopliae* treatment was (53.4%), which was significantly lower (F = 37.57; P < 0.0001) than the one of male ticks recovered from the control basins (94.7%) (Figure 6.4). Similarly, the percentage of live untreated female ticks in basins containing fungus-infected male ticks was significantly lower (F = 16.47; P < 0.004) than the one of females recovered from the control treatment (Figure 6.4), implying horizontal transfer of inoculum from fungus-inoculated male to untreated female ticks. Subsequently,

mortality was significantly higher in male (F = 62.51; P < 0.0001) and female (F=18.45; P < 0.003) in fungus than in control treatments (Figure 6.5).

Table 6. 1: **Effects of infection by *M. anisopliae* on the reproductive parameters of female *R. appendiculatus* following exposure to conidia formulated in emulsifiable extract of *C. aurea* in semi-field plot experiments**

Parameters	Controls (X ± SD)	Infected ticks (X ± SD)	Statistics
Mortality (%)	6.6	40.0	$F = 13.34$; $df = 1$; $P < 0.001$
Engorgement period (days)	8.5 ± 0.6	11.7 ± 2.1	$F = 27.36$; $df = 1$; $P < 0.0001$
Weight of engorged ticks (mg)	333.0 ± 40.9	232.2 ± 50.4	$F = 26.75$; $df = 1$; $P < 0.0001$
Pre-oviposition period (days)	12.6 ± 2.4	17.5 ± 2.5	$F = 9.48$; $df = 1$; $P < 0.01$
Oviposition period (days)	13.5 ± 0.9	16.8 ± 1.0	$F = 59.34$; $df = 1$; $P < 0.0001$
Post-oviposition period (days)	22.7 ± 2.4	31.3 ± 4.5	$F = 32.57$; $df = 1$; $P < 0.0001$
Eggs mass weight (mg)	234.1 ± 29.2	147.8 ± 45.6	$F = 29.33$; $df = 1$; $P < 0.0001$
Number of eggs	4213.6 ± 526.7	2660.8 ± 822.2	$F = 27.65$; $df = 1$; $P < 0.0001$
Number of larvae emerged	3792.3 ± 474.0	1490.8 ± 882.0	$F = 26.17$; $df = 1$; $P < 0.0001$
Egg viability (%)	90.0 ± 2.9	55.0 ± 24.9	$F = 25.73$; $df = 1$; $P < 0.0001$

Data were analysed using one way ANOVA, $P < 0.05$ was considered significant
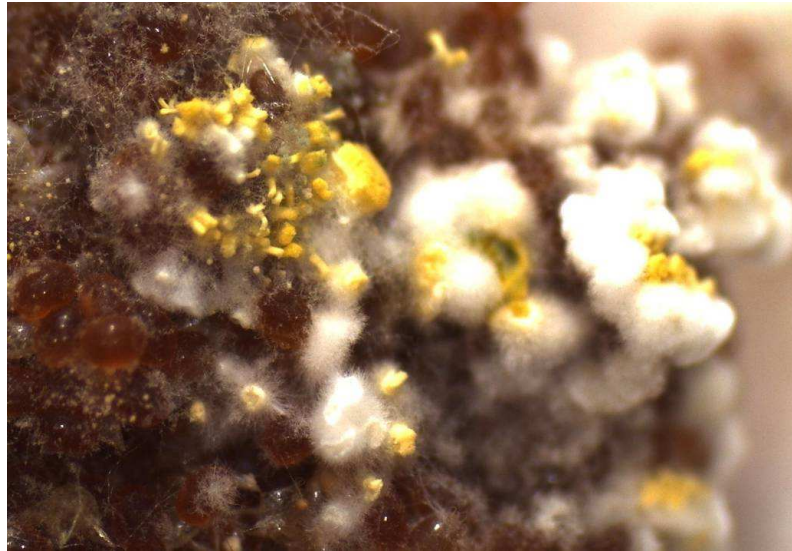
Plate 6. 3: **Mycosis by *M. anisopliae* on *R. appendiculatus* eggs that failed to hatch (Nana, 2010)**
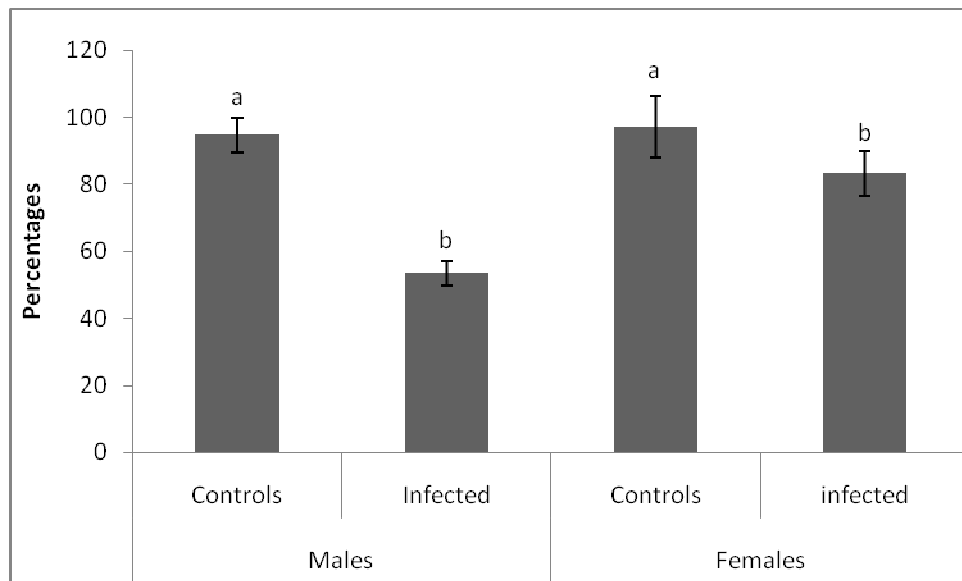
Figure 6. 4: Mean percent male *R. appendiculatus* recovered alive following exposure to fungal inoculum and untreated female transferred in the grass containing infected males for 5 weeks. (Means between treatment bearing the same letter are not significantly different (P < 0.05) by ANOVA).
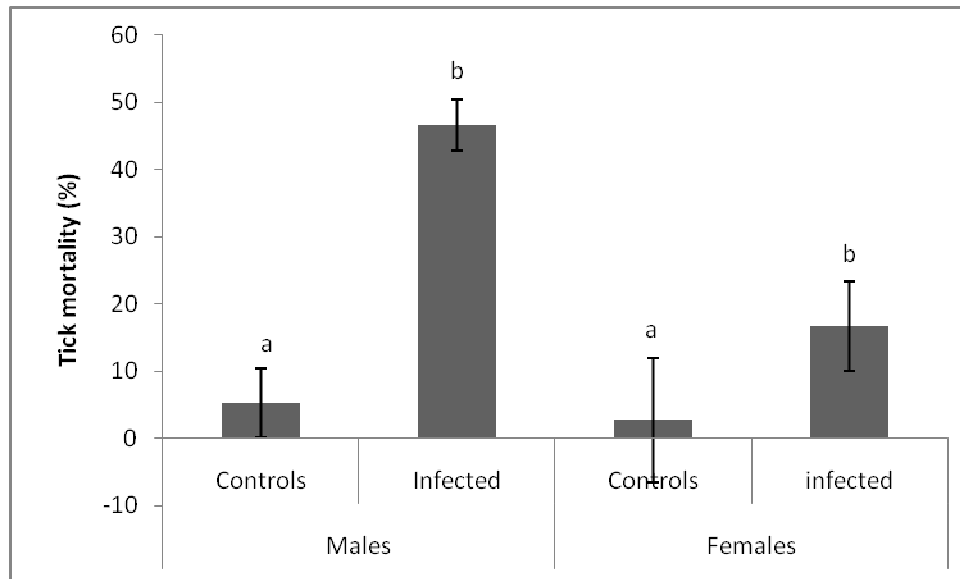
Figure 6. 5: Percent mortality in male *R. appendiculatus* initially exposed to fungal inoculum and in untreated female transferred in the grass containing infected males for 5 weeks. (Means between treatment bearing the same letter are not significantly different (P < 0.05) by ANOVA).

Ticks that survived after 5 weeks post-infection in the grass were brought in the laboratory where they were maintained for 3 weeks in conditions described above. Ninety three (93%) of male ticks succumbed to infection in fungus treatment compared to 10% in the control. Among untreated females that were introduced along with fungus-treated males, 56.2% of them died from fungal infection, which was significantly higher (F = 45.33; P < 0.0001) than in the control treatments where only 7.1% mortality was recorded (Figure 6.6). All dead male ticks recovered from fungus-treated treatment developed mycosis; whereas 85% of dead female ticks had mycosis, thus confirming horizontal transmission. No fungal growth was observed on dead ticks collected from the control treatment.
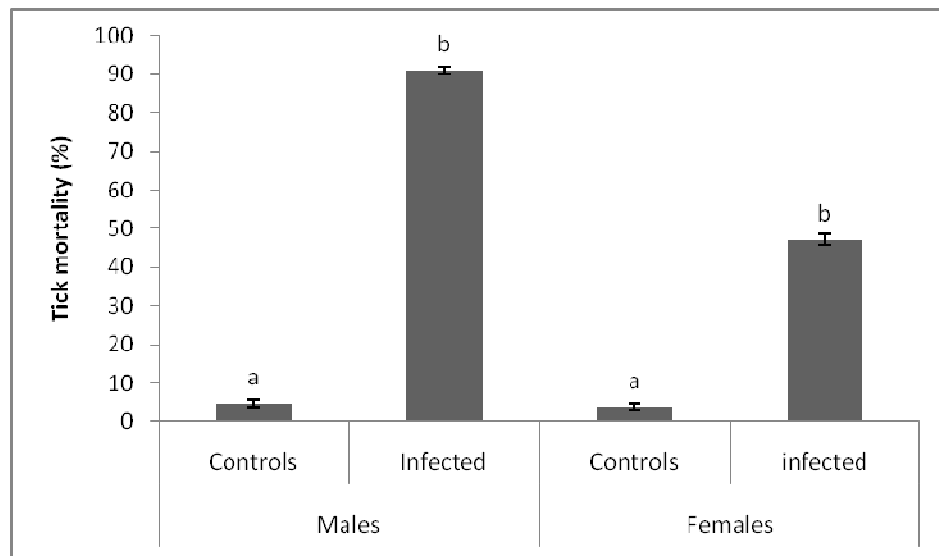
Figure 6. 6: **Percent mortality in male *R. appendiculatus* initially exposed to fungal inoculum and in untreated female transferred in the grass containing infected males for 5 weeks and later maintained in the laboratory for 3 weeks after removal from the grass. (Means between treatment bearing the same lowercase letter are not significantly different (P < 0.05) by ANOVA).**

6.4 Discussion

This study demonstrated that emulsifiable formulation of *C. aurea* extract mixed with conidia of *M. anisopliae* is effective in attracting and infecting adult *R. appendiculatus*, resulting in high mortality of ticks that were brought to the laboratory. The attraction and infection strategy is consistent with the aims of integrated pest management. *C. aurea* extract attracted *R. appendiculatus* adult ticks in agreement with my previous observations. Ethno-veterinary plants play an important role in integrated tick control in rural areas in Africa (Kaaya, 2000; Wanzala *et al*., 2005; Abduz Zahir *et al.,* 2009; Zorloni *et al*., 2010). Some plants

demonstrated strong acaricidal and/or repellent properties (Kaaya *et al*., 1995; Mwangi *et al*., 1995) while others showed attraction in some tick species (Hassan *et al*., 1994; Zorloni *et al*., 2010). Data from field plot experiments suggest that *C. aurea* suspensions could be used as an affordable way of controlling ticks in rural settings because the cost of preparing the plant extract is relatively low.

Kaaya and Hassan (2000) in their study showed that spraying fungi in grazing pastures seeded with *R. appendiculatus* larvae significantly reduced the populations of this tick species. However, this approach is likely to be very expensive in view of the special scale of application that would be needed. Moreover, such an approach has high risks of non-target effects. *C. aurea* emulsifiable extract mixed with *M. anisopliae* sprayed by spot in semi-field experiments were more economical. Furthermore, this tool would minimize the area treated with a mycoacaricide, thus reducing the probable non target contamination. The present approach is more target-oriented and more environmentally friendly.

The fungus could not prevent adult *R. appendiculatus* female ticks from engorging. However, the feeding potential and consequently the egg masses of infected female ticks were significantly reduced. Furthermore, the viability of larvae was reduced by 45% involving a significant decrease in the size of the progeny. The reduction in egg hatchability of *R. appendiculatus* following infection with *M. anisopliae* observed in this study is comparable to the one reported by other authors (Kaaya *et al*., 1996; Maniania *et al*., 2007). Fungal infection of engorged female ticks have been reported to often result in longer periods of pre-oviposition, oviposition time, egg-incubation,

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and egg-hatching of the egg rnass, as well as in lowered egg production (Gorshkova, 1966; Bittencourt, Massard & Lima, 1994; Barci, 1997; Gindin *et al.*, 2001). This suggests that there is a relatively long-lasting sub-lethal influence of the fungus on their tick hosts. Infection by *M. anisopliae* has also been reported to reduce egg hatchability in other arthropods such as mite *Tetranychus urticae* (Irigaray *et al*., 2003) and stem borer *Chilo partellus* (Maniania, 1991).

A high number of ticks was recovered from the control basins (94%) than from the fungus-treated basins (54%), implying horizontal transmission of the inoculum from fungus-infected males to untreated female ticks. Fungal dissemination within a host population generally occurs during activities such as mating, grooming and movements of the host (Watanabe, 1987; Andreadis, 1987; Schmid-Hempel, 1988; Scholte *et al.*, 2005). Horizontal transmission of fungal inoculum has been reported in many insects (Kaaya and Okech, 1990; Maniania, 1994; Sholte *et al*., 2005). Recently, Brooks and Wall (2005) demonstrated the horizontal transmission of *M. anisopliae* in parasitic mite, *Psoroptes ovis* (Hering) syn. *Psoroptes cuniculi*) (Acari: Psoroptidae) by allowing live uninfected mites to walk over a piece of filter paper from which an infected cadaver had been removed. The aggregation behaviour of questing ticks in the presence of vegetation cover (Browning, 1976) could explain autodissemination of *M. anisopliae* from male to female *R. appendiculatus*.

The use of attraction and infection approach presents several advantages including targeted delivery of a pest control agent; consequently, limited detrimental effects on non-target organisms; less costly and more effective than conventional methods of

application (Vega *et al*., 2007). The success of this approach relies on the availability of pheromones and kairomones like CO_2. A major advantage of using ethno-veterinary plant extract such as *C. aurea* to attract ticks is that, it is a shrub occurring in tropical areas; its preparation and formulation is simple and it may facilitate contamination of *Rhipicephalus* ticks with the fungus. Most of the ticks that survived infection in the field and brought to the laboratory succumbed to fungal infection, indicating that further mortality could occur beyond the 5-week experimental period.

This study represents the first attempt to evaluate the use of an ethno-veterinary plant in combination with entomopathogenic fungus for the control of livestock ticks. Similar approaches have been previously tested using pheromone-baited trap with entomopathogenic fungi (Maranga *et al*., 2006; Nchu *et al.,* 2009, 2010) and pheromone/acaricide mixture for tick control on host (Norval *et al*., 1996). The use of mycoacaricide for tick control has an advantage over ordinary acaricides because fungi can be readily produced locally.

The use of ethno-veterinary plant *C. aurea* and entomopathogenic fungus *M. anisopliae* in a trap has demonstrated potential for off-host tick control. Fungal conidia formulated in emulsifiable plant extract-baited traps can be a valuable tool to enhance the infection, autodissemination of *M. anisopliae* and thereby reducing the populations of *Rhipicephalus* ticks. The results of experiments to develop an attractant-infection strategy for the cattle tick *R. appendiculatus* are promising and ideal for tick IPM programme. Field testings are needed to demonstrate the efficacy of the approach in controlling tick populations on large scale.

CHAPTER SEVEN

7.0 BIOASSAY GUIDED FRACTIONATION OF *CALPURNIA AUREA* LEAF EXTRACTS RESPONSIBLE FOR ATTRACTION OF *RHIPICEPHALUS APPENDICULATUS*

7.1 Introduction

Control of *Rhipicephalus appendiculatus* Neumann (Acari: Ixodidae) vector of many viral and rickettsial diseases continues to rely heavily on application of residual acaricides (George *et al*., 2004). This approach has been highly effective in reducing tick populations and their incidence. However, associated problems regarding environmental pollution such as contamination of soil and ground water, adverse effect on non-target species, and cost (Chaton *et al*., 2002) and the now the continuing development of resistance (Ducornez *et al.,* 2005) give emphasis to the need for alternative strategies, such as vector control with biological agents (Samish and Alekseev, 2001; Maniania *et al*., 2007; Reis-Menini *et al*., 2008) and the use of ethnoveterinary plants (Kaaya, 2000).

Several tropical pasture legumes of the genus *Stylosanthes* (Fabaceae) have acaricidal/repellent effects (Castrejon *et al*., 2003) while others exhibit attraction properties. These include *Acalypha fruticosa* Forssk (Euphorbiaceae)*, Ipomoea spathulata* Hallier (Convolvulaceae)*, Solanum incanum* Linnaeus (Solanaceae) (Hassan *et al*., 1994) and *C. aurea* Benth (Zorloni *et al*., 2010). *C. aurea*, a member of the subfamily Papilionoideae of the family Fabaceae (Coates Palgrave, 1983), is a

small, multi-stemmed tree, 3–4m tall, occurring widespread in bushland and grassland in sub-Saharan Africa and India. Phytochemical investigations of *C. aurea* has led to the isolation of different alkaloids including calpurmenine (Isao *et al*., 1984), virgiline and virgiline pyrrolecarboxylic acid ester (Alonso *et al*., 2000), digittine and its amino alcohol (Radema *et al.,* 1979), lupanine, calpaurine, lupinine, calpurmenine and its pyrolecarboxylic acid ester, 13-hydroxylupanine and its tiglate, and calpurnine (Asre and polo, 1986). It is also reported to have lectins, non-protein amino acids and tannins (Fullas, 2001).

The aim of this study was to perform a bioassay guided fractionation of *C. aurea* leaf extracts responsible for attraction of *R. appendiculatus* and *R. pulchellus.*

7.2 Materials and methods

7.2.1 Tick colony used

Engorged *R. appendiculatus* females used to start the colony originated from cattle from the Marsabit area of Kenya in 2006. Ticks were reared at the Animal Rearing and Quarantine Unit of ICIPE. All life stages of the tick were fed on New Zealand white rabbits. The different instars were maintained in perspex chambers at $26 \pm 2\ ^{o}C$ and $85 \pm 5\%$ RH under 12:12 L:D photoperiod. Three to four-week old unfed adults were used in this study.

7.2.2 Plant material

Calpurnia aurea leaves originated from South Africa was described in chapter 2, section 2.7.

7.2.3 Extraction of *C. aurea*

7.2.3.1 Dichloromethane and aqueous extracts

Seventy grams of the plant powder were macerated in 0.8 litre of 95% acetone for 3 days at room temperature. It was later filtered, and concentrated under reduced pressure in a rotor evaporator at 50° C, to yield 5.6 g of crude organic extract. A 4.6 g portion of this extract was suspended in distilled water and extracted with 95% dichloromethane. Both organic and aqueous extracts were concentrated using rotary evaporator at 556 and 72 mbar respectively. Acetone, dichloromethane and aqueous extracts were used to perform a comparative thin layer chromatography (TLC).

7.2.3.2 Preparation of the fractions

Two grams of the organic extract were subjected to flash chromatographic fractionation on a silica gel column of 40 mL eluted with hexane followed by gradient mixtures of hexane-ethyl acetate-methanol. Several fractions were obtained and combined based on the comparison of their RF values. Ten major fractions were obtained: F_1 (Hexane 100%), F_2 (Hexane-EtOAc 95:5), F_3 (Hexane- EtOAc 90:10), F_4 (Hexane- EtOAc 75:25), F_5 (Hexane- EtOAc 50:50), F_6 (Hexane- EtOAc 25:75), F_7 (EtOAc-MeOH 95:5), F_8 (EtOAc-MeOH 90:10), F_9 (MeOH-EtOAc 90:10), F_{10} (MeOH 100%).

7.2.3.3 Coupled Gas Chromatography-Mass Spectrometric (GC-MS) analysis

The analysis was carried out on an Agilent technology 7890A GC- with 5975 MSD. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV and emission current of 34.6µA. The temperature of the source was held at 230 ºC (ion source), 150 ºC (Quadrupole) and multiplier voltage was 1106 V. The pressure of the ion source was held at 7 x10-6 mBar. The spectrometer had a scan cycle of 3 scans per 2 seconds. Oven program was 35 °C for 5 min then 10 °C/min to 280 °C for 5.5 min. The mass range was set at m/z 1-1050 and scan range for the compounds from m/z 38-550. The instrument was calibrated using heptacosa (Perfluorotributylamine) $[CF_3(CF_2)_3]_3N$ (Apollo scientific Ltd. UK). HP-5 GC capillary column, 30 m x 0.25mm (i.d) x 0.25 µm (film thickness) supplied by J & W Scientific was used. The GC-MS was linked to a computer with MS library (NIST &WILEY). The compounds were identified by comparing their MS with those of authentic samples or with library data and their fragmentation pattern.

7.2.4 Inverted glass tube bioassays

The inverted glass tube assays used to test attraction of ticks was previously described in chapter 4, section 4.2.5.1.

7.2.5 Data analysis

For each test, the total number of ticks responding to plant extract was pooled across replicates. The percentage attraction was determined using the following formula: (number of ticks in test - number of ticks in control)/(number of ticks in control +

number of ticks in test) × 100. The percentage of attraction was analysed using Chi Square Goodness of fit test (G-test) (Sokal and Rohlf, 1981). All analyses were performed using the SAS (2001) package.

7.3 Results

7.3.1 Attraction bioassays of crude plant extracts and fractions

In inverted glass tube bioassays, dichloromethane and aqueous extracts significantly attracted more ticks than did the control. Their relative attractions were 80% and 68.4% respectively (Table 7.1). Ticks responded variably to the ten fractions of DCM extract tested at a singly dose of 10 mg/ml. Fraction 1, 2, 3 and 4 significantly attracted *R. appendiculatus* while the other fractions were unattractive to the ticks (Table 7.1).

7.3.2 Compounds identified from GC-MS

GC-MS analysis revealed that fraction 2 and 3 were made up of complex mixture of fatty acids, alkenes and alcohols. Twenty compounds were identified in fraction 2, the highest values in relative abundance were Oleic acid (64%), Methyl linoleate (58%) and the others compounds ranging in relative abundance from 9% to 38% (Table 7.2). In Fraction 3, the highest relative abundant compounds were Hexadecanoic acid (98%) and Decanoic acid (46%), the others substances ranging from 1% to 10% of relative abundance (Table 7.3).

Table 7. 1: **Responses of *R. appendiculatus* in inverted tube glasses to different extracts and fractions from *C. aurea***

Substances	N^o of tick attracted				
	N	Doses(mg/ml)	Control	Test	% of relative attraction
DCM extract	50	50	4	36	80.0**
Aqueous extract	50	50	6	32	68.4**
F_1 (Hex 100%)	50	10	18	30	28.5*
F_2 (Hex-EtAc 95:5)	50	10	13	33	43.4*
F_3 (Hex-EtAc 90:10)	50	10	9	30	54.5**
F_4 (Hex-EtAc 75:25)	50	10	12	27	38.5*
F_5 (Hex-EtAc 50:50)	50	10	13	17	13.3
F_6 (Hex-EtAc 25:75)	50	10	16	20	11.1
F_7 (EtAc-MeOH 95:5)	50	10	17	17	0.0
F_8 (EtAc-MeOH 90:10)	50	10	23	27	8.0
F_9 (MeOH-EtAc 90:10)	50	10	18	23	1.2
F_{10} (MeOH 100%)	50	10	17	22	12.5

* represents significance at $P < 0.05$; ** represents significance at $P < 0.01$.

The McNemar chi-square test for the significance of change in frequencies between control and test substance was used.

Table 7. 2: **Compounds identified from Hexane fraction 2 of *C. aurea.***

	Compound	RI[a]	RA[b](%)
1.	Oleic acid	2141	64
2.	Methyl linoleate	2095	58
3.	Linoleic acid	2132	41
4.	Citronellene<beta->	942	38
5.	Nonen-1-ol<3Z>	1152	38
6.	Nonenol<6Z>	1164	25
7.	Undecyne<1->	1122	22
8.	Heptenol<3Z->	947	22
9.	Decadienal<2E,4Z>	1292	14
10.	Octadienal<2E,4E->	1102	10
11.	Prenyl formate	825	10
12.	Musk ambrette	1929	10
13.	Hexenol<3E->	844	10
14.	Cyclopent-2-en-1-one<2-pentyl->	1288	10
15.	Geranyl linalool<Z,E->	1998	10
16.	Undec-9E-en-1-al	1311	10
17.	Cyclohexyl formate	954	10
18.	Sabina ketone	1154	9
19.	Fenchol<exo->	1118	9
20.	Undec-9Z-en-1-al	1322	9

[a] Retention index

[b] RA: Relative abundance

Table 7. 3: Compounds identified from Hexane fraction 3 of *C. aurea.*

	Compounds	RI[a]	RA[b](%)
1.	Hexadecanoic acid	1959	98
2.	Decanoic acid	1364	46
3.	Butanoic acid	763	10
4.	Valeric acid<3-methyl->	939	9
5.	Octanoic acid	1167	9
6.	Dodecanoic acid	1565	9
7.	Hexanoic acid<5methyl->	1033	6
8.	Decenoic acid<9->	1359	4
9.	Nonanoic acid	1267	4
10.	Hexanoic acid	967	2
11.	Pentanoic acid	933	2
12.	Isovaleric acid	827	2
13.	Isopropyl hexadecanoate	876	1

[a] Retention index
[b] RA: Relative abundance

7.4 Discussion

The present study demonstrated that ticks were more attracted (80%) to dichloromethane extract than to aqueous extract (68%). Preliminary phytochemistry of *C. aurea* revealed the presence of phenolic compounds and hydroxy-phenolic compounds with acids, alcohols, sugars or glycosides, as reported by Adedapo *et al.* (2008). Based on the results obtained on liquid chromatography, it is possible that ticks are attracted to one or many of these compounds present in *C. aurea* extracts. For instance, phenolic components are believed to play a key role in the attraction

behaviour of over 12 species of ixodid ticks (Wood *et al*., 1975; Mcdowell and Wallade, 1986; Yoder and Stevens, 2000). Furthermore, sesquiterpenes, terpenoids, and hydrocarbons compounds are also reported to serve as tick pheromones/kairomones (Sonenshine, 1985).

The partition of the dichloromethane (DCM) extract from the leaves of *C. aurea* with different solvents resulted in ten distinct fractions; evidence that DCM leaf extract of *C. aurea* is rich in both apolar and polar compounds. The present study demonstrated that ticks were mostly attracted to non-polar compounds which is in accordance with my previous observations. Many infochemicals of different origin are susceptible to affect the behaviour of ticks. Host-emitted odours provoke orientation in ticks that walk towards their prey (Donze *et al*., 2006). However, natural attraction of some *Rhipicephalus* tick species to certain plants was observed (Hassan *et al*., 1994; Zorloni *et al*., 2010). Plant secondary metabolites have been reported to attract many species of hard ticks including *R. appendiculatus* (Donze *et al*., 2004). The results of this study show that carboxylic acids (hexadecanoic, butanoic, oleic or valeric) predominate in the most effective fractions. Previous investigations show that ticks possess in the surface of tarsus different fatty acid receptors (Steullet and Gnerin, 1993). The behaviourally active compounds identified such as hexadecanoic acid have been found to be attractant to Ixodid tick (Allan *et al*., 1998; Price Jr *et al.,* 1994), to American house dust mites (Glass *et al.,* 2001) and to male fruit flies (Chuah *et al.,* 1997).

This study focused on bioassay guided fractionation of *C. aurea* leaf extracts responsible for attraction of *R. appendiculatus*. The whole extract and fractions of the legume *C. aurea*, demonstrated attraction properties. The major source of attraction was found to be in fraction 2 where most of active compounds are located. To fully understand the attraction of tick to this plant, additional research is needed to characterize the active compounds or blend.

CHAPTER EIGHT

8.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMANDATIONS

8.1 General discussion

Ticks are considered to be a major pest in the cattle and other livestock, buffalo and antelope. Heavy infestations can cause anaemia, severe damage to the ears, or a toxicosis that result in the loss of resistance to some tick-borne infections. *Rhipicephalus appendiculatus* can transmit a number of pathogens including *Theileria parva*, Nairobi sheep disease virus and Thogoto virus. Control of livestock ticks has depended almost entirely on the large-scale administration of toxic substances onto the external body surfaces of these hosts or by environmental spray. Used as acaricides, these toxicants also have been dispersed in large quantities in the environment where they indiscriminately kill ticks and other non-target organisms. The discovery of insecticidal compounds with low mammalian toxicity such as pyrethroids or avermectins and improvements in the methods of delivery has enhanced their efficacy for tick control but at greatly increased cost, in addition to development of resistance by ticks (Baxter and Barker, 2002; Ducornez *et al*., 2005). These setbacks have led to the search of alternative methods of tick control that can be applied alone or integrated with acaricides. These include the use of tick resistant animals (Sutherst, 1983; Jongejan *et al.*, 1989; Rechav *et al.*, 1990); use of tick-killing plants (Sutherst *et al.*, 1982; Norval *et al.*, 1983; Wanzala *et al.*, 2005); use of pheromones (Norval *et al.*, 1991; Maranga *et al*., 2006; Nchu *et al*., 2010) and the

use of entomopathogens (Kaaya and Hassan, 2000; Gindin *et al*., 2009; Nchu *et al*., 2010).

The most commonly investigated entomopathogenic fungi are species of *Metarhizium* and *Beauveria,* as they have a wide geographic spread and host range. These entomopathogenic fungi have been widely considered for biological control of agricultural pests (Gillespie and Moorhouse, 1989; Van der Geest *et al*., 2000), particularly ticks (Kaaya *et al*., 1996; Kirkland *et al*., 2004; Gindin *et al* 2009). For entomopathogenic fungi to become feasible biological control agents for ticks, considerable amount of research is required to promote product development. This includes appropriate bioassays to ensure compatibility of the selected strain with semiochemicals, and development of suitable formulations and delivery systems. An attractant-infection and autodissimination strategy for the control of ticks is a promising and ideal component for tick IPM programme. The current study was initiated to address these aspects and to generate information relevant to the practical use of fungi in tick management strategies. The aim of this study was therefore to investigate the prospect of integrating *C. aurea* plant extracts with entomopathogenic fungus *M. anisopliae* for the control of *Rhipicephalus* ticks off-host.

This study has demonstrated the attraction of *R. pulchellus* and *R. appendiculatus* to emulsifiable extract of *C. aurea* in the laboratory and field conditions. The importance of plants in integrated tick control has been the focus of attention in these recent years (Kaaya, 2000; Wanzala *et al*., 2005; Abduz Zahir *et al*., 2009; Zorloni *et al*., 2010). A number of plants have strong acaricidal and/or repellent properties

(Kaaya *et al*., 1995; Mwangi *et al.,* 1995; Zorloni *et al.,* 2010), while others elicit attraction in some ticks (Hassan *et al*., 1994; Zorloni *et al*., 2010). On the other hand, entomopathogenic fungi have been used successfully to control various agricultural and pasture pests. No effect of *C. aurea* leaf extract was observed on *M. anisopliae* in the present study. Plants extracts have been reported to affect sometimes the growth and germination of conidia of an entomopathogenic fungus. For instance, Rogerio *et al*. (2005) reported that seed extracts from Neem have negative effect on *Beauveria bassiana*, inhibiting germination, reducing colony diameter and conidiogenesis.

The present study also established that emulsifiable formulation of *C. aurea* extract mixed with conidia of *M. anisopliae* was effective in attracting and infecting *R. appendiculatus* adult ticks, resulting in high mortality of the ticks. Previous research has demonstrated that emulsifiable formulations of conidia can enhance fungi activity against arthropods (Nchu *et al.*, 2009).

Previous studies focused on the control of *R. appendiculatus* by spraying fungi in grazing pastures (Kaaya and Hassan, 2000). However, this approach is very expensive in view of the scale of application that would be needed. Moreover, such an approach has high risks of non-target effects. *M. anisopliae* formulated in emulsifiable extract of *C. aurea* applied in spot sprays would be more economical by reducing the area to treat with a mycoacaricide. The present approach is more target-oriented and more environmentally friendly. Such an approach was recently tested by Nchu *et al*. (2010) with success.

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Considering the fact that ticks communicate with each other by means of physical contact and chemical stimuli (Sonenshine, 1985), autodissemination of entomopathogenic fungi within populations of pests, using attractant traps as the initial source of infection, could successfully be used to control ticks in the field (Nchu *et al*., 2010).

8.2 Conclusions

With regard to the results obtained in the laboratory and in the semi-field plots, it was concluded that:

1. *Rhipicephalus pulchellus* and *R. appendiculatus* are attracted to *C. aurea* leaf extracts both in the laboratory and in the semi-field conditions.

2. Oil formulation of *C. aurea* was more attractive to *Rhipicephalus* ticks compared to aqueous and acetone formulations.

3. *Calpurnia aurea* emulsifiable formulation revealed compatibility with *M. anisopliae* when mixed together and not with AAAP.

4. The combination of an ethno-veterinary plant with an entomopathogenic fungus has demonstrated the prospects of the approach in attracting and infecting ticks; thus, resulting in high mortality of ticks.

5. This study demonstrates that *M. anisopliae* formulated in emulsifiable plant extract-baited traps could be a valuable approach in enhancing autodissemination of inoculum for the control of *Rhipicephalus* ticks.

8.3 Recommendations

In the course of this study, various questions arose, which may be subject for future studies:

1. *C. aurea* used in this study originated from South Africa. Surveys should be conducted in other parts of Africa to identify this plant and investigate whether the attractant properties to ticks are conserved across regions.

2. Attractive response studies of *C. aurea* extracts was primarily evaluated on *R. appendiculatus* and *R. pulchellus* and should be extended to other species of ticks in order to assess their potential.

3. Field testings are needed to demonstrate the efficacy of the approach in controlling tick populations on large scale

4. Screen other ethno-veterinary plants for repellent/attractant properties in order to integrate them with other control agents.

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