ATTRACT-AND-KILL STRATEGY FOR SUSTAINABLE CONTROL OF TICKS USING ENTOMOPATHOGENIC FUNGUS, *Metarhizium anisopliae* AND EXTRACTS OF *Senna didymobotrya*

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DECLARATION AND RECOMMENDATION

Declaration:

This thesis is my original work and has not been presented in this University or any other for the award of a degree.

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DEDICATION

I dedicate this work to my parents, Mr. and Mrs. George Kinyua, and my siblings, Laban, Manasseh, Elias and Boaz. Thank you for your constant and unending love, concern, support and inspiration throughout this period.

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ABSTRACT

The increasing prevalence of acaricide resistance and the expanding range of invasive tick species necessitate the use of alternative interventions, such as semiochemicals and biopesticides, for sustainable tick control. The current study employed an olfactory-based bioassay-guided approach to screen and identify tick attractants in methanol leaf, flower, seedpod and twig branches extracts of *Senna didymobotrya*, a plant that has been established to alter the behaviour of ticks. Gas Chromatography-Mass Spectrometry (GC-MS) analysis was conducted to characterize the chemical composition of the extracts and fractions. In addition, the effect of various concentrations of the identified attractants on the viability and virulence of *Metarhizium anisopliae* ICIPE 7, a potent tick biopesticide, was assessed by exposure of the fungus to headspace volatiles of the attractants and immersion tests, respectively. Floral extracts elicited the highest attraction to *Amblyomma variegatum* ($P< 0.001$) and *Rhipicephalus appendiculatus* ($P = 0.060$) adults. Fractionation of the floral extract using solvents of increasing polarity revealed that hexane and ethyl acetate fractions were most attractive to *A. variegatum* ($P< 0.001$) and *R. appendiculatus* ($P< 0.001$) respectively. However, the hexane fraction also attracted *R. appendiculatus* ($P = 0.040$). Chemical analysis of the active extract and fractions, combined with a literature search, identified squalene and linoleic acid as potential attractants. Attraction bioassays conducted with these two compounds and their blend (1:1) demonstrated that *A. variegatum* ($P< 0.010$) and *R. appendiculatus* ($P< 0.001$) were significantly attracted to the two-component blend. The crude floral extract and squalene did not affect the viability of *M. anisopliae* ICIPE 7. Linoleic acid, squalene: linoleic acid blend (1:1) and the attraction aggregation attachment pheromone (AAAP) negatively affected the viability of ICIPE 7. Squalene: linoleic acid (1:1) (10%) combined with ICIPE 7 resulted in the shortest lethal time response to mortality of 50% of the tick population (LT_{50}) in both *A. variegatum* and *R. appendiculatus* (9.05 and 10.3 days, respectively). These findings suggest that the (10%) squalene: linoleic acid blend (1:1) can be combined with *M. anisopliae* ICIPE 7 for the sustainable control of *A. variegatum* and *R. appendiculatus* populations in an "attract-and-kill" strategy leading to control of ticks and tick-borne diseases.

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LIST OF ABBREVIATIONS AND ACRONYMS

2, 6 – DCP	2, 6 - dichlorophenol
AAAP	Attraction-aggregation-attachment pheromone
ANOSIM	Analysis of similarities
AP	Arrestment pheromone
APU	Arthropod Pathology Unit
ARQU	Animal Rearing and Quarantine Unit
ASL	Above Sea Level
ATVs	Anti-tick Vaccines
BLASTn	Nucleotide Basic Local Alignment Search Tool
bp	base pairs
CBOL	Consortium for the Barcode of Life
EAC	East African Committee
ECF	East Coast Fever
EI	Electron Impact
EPF	Entomopathogenic Fungi
FAO	Food and Agriculture Organisation of the United Nations
GC-MS	Gas Chromatography Mass Spectrometry
GDP	Gross Domestic Product
GLM	Generalised Linear Models
icipe	International Centre of Insect Physiology and Ecology
IJ	Infective Juveniles
L: D	Light: Dark photoperiod
LT_{50}	Lethal time response to mortality of 50% of the population
m/z	mass to charge ratio
MAFFT	Multiple Alignment using Fast Fourier Transform
matK	maturase K
NACOSTI	National Commission for Science, Technology and Innovation
NCBI	National Centre for Biotechnology Information
NIST	National Institute of Standards and Technology
NMDS	Non-metric Multidimensional Scaling
PAST	Paleontological Statistics Software
PCR	Polymerase Chain Reaction
PSMs	Plant Secondary Metabolites

rbcL	ribulose-1,5-bisphosphate carboxylase large subunit
RH	Relative Humidity
SDA	Sabouraud Dextrose Agar
SIMPER	Similarity Percentage Analysis
TBDs	Tick-borne Diseases
TLC	Thin Layer Chromatography
TTBDs	Ticks and Tick-borne Diseases

CHAPTER ONE
INTRODUCTION

1.1 Background information

Livestock farming plays a vital role in the global economy, offering jobs to more than 1.3 billion individuals worldwide and serving as a cornerstone of the livelihoods of 600 million small-scale farmers in developing countries. This industry accounts for a minimum global asset value of $1.4 trillion (World Bank Group, 2021). Approximately 40 - 80% of Africa's poverty-stricken population engages in livestock keeping (Guthiga *et al*., 2021). According to the East African Committee (EAC) Secretariat (2016), the livestock sector typically makes up around 10% of the Gross Domestic Product (GDP) and contributes between 30% and 50% to the agricultural GDP in Eastern Africa. In Kenya, the sector accounts for 4.4% of the GDP and approximately 14.2% of the added value in agriculture (FAO, 2019). Humans depend on livestock for sustenance (meat, milk, fats, and other value-added products). Livestock also provide hides and skins, organic fertilizers, energy through biogas production, draft power, a means of transportation, and act as indicators of wealth and social status in certain regions (Guthiga *et al.*, 2021). Regrettably, the livestock industry encounters a multitude of challenges that hinder its advancement; notably, ticks and tick-borne diseases (TTBDs) emerge as prominent and formidable obstacles (Kasaija *et al.*, 2021).

Regarded as the most important disease vectors, ticks are obligate hematophagous acarine pests that rank second after mosquitoes in terms of their significance to human and animal health (Kasaija *et al.*, 2021; Nicholson *et al*., 2018). Apart from the physical harm they cause by directly biting and damaging the skin and hides, ticks result in substantial agricultural and economic losses by transmitting infectious tick-borne pathogens that are of veterinary and zoonotic significance, including bacteria (such as rickettsia and spirochaetes), fungi, viruses, and protozoa (Hurtado & Giraldo-Ríos, 2018; Jongejan & Uilenberg, 2004; Nicholson *et al*., 2018). Babesiosis, theileriosis, anaplasmosis, and heartwater, transmitted by *Rhipicephalus* and *Amblyomma* species, are the predominant tick-borne diseases (TBDs) of veterinary importance in Africa, Asia, Latin America, and Australia (Domingos *et al*., 2013). On a global scale, approximately 80% of the cattle population faces the threat of TTBDs, resulting in significant financial losses. These losses encompass both production-related setbacks, such as livestock deaths, as well as expenses associated with pest and disease control, amounting to approximately US$ 7.3 per animal per year (Hurtado & Giraldo-Ríos, 2018). Globally, the annual economic losses caused by TTBDs exceed US$ 22 billion (Lew-Tabor & Rodriguez

Valle, 2016), while in Kenya alone, these losses are estimated to surpass KES 30 billion (US$ 221 million) each year (Kanduma, 2018).

Tick control relies on synthetic acaricides such as arsenic preparations, chlorinated hydrocarbons, organophosphorus compounds, and pyrethroids (Gonzaga *et al*., 2023; Nicholson *et al*., 2018). However, the successful use of acaricides to control ticks is impeded by the emergence of resistance by some tick species to existing acaricides, the persistence of chemical residues in livestock products and the environment, and their toxicity to non-target vertebrates (de la Fuente *et al*., 2023). As a result, there is a growing need for research into innovative and desirable methods of tick management. One such promising approach is the utilization of environmentally friendly, effective, and sustainable semiochemicals and bio acaricides. These control strategies can be implemented either on their own or in combination, in an "attract-and-kill" strategy for tick management.

Tick attractant semiochemicals are information-bearing chemical signals which when detected trigger changes in the recipient(s) behaviour and/or physiology, in this case, causing ticks to make oriented movements towards them (Allan, 2010; Mweresa *et al*., 2020; Sonenshine, 2004). The attractant can be combined with a lethal substance, trapping devices, a sprayable formulation for application, or a disseminated bait station to facilitate the detection, monitoring, surveillance, and control of ticks, in an attract-and-kill strategy (Gregg *et al*., 2018). Thus far, the attraction-aggregation-attachment pheromone (AAAP), 2,6-dichlorophenol (2,6-DCP) sex pheromone and arrestment pheromones (AP) are the commonly known tick attractant cues that have been combined with the kill agents, either acaricides (Carr & Roe, 2016; Sonenshine, 2004) or entomopathogenic fungi (Nchu *et al*., 2009, 2010), for management and control of ticks. Nevertheless, the effectiveness of these semiochemicals is limited by the fact that the targeted ticks will only respond to the attractants once they have reached a specific physiological and developmental stage (in the case of AAAP and 2,6-DCP), are of a particular sex or gender (2,6-DCP), or upon physical contact (in the case of AP) (Sonenshine, 2004).

Entomopathogenic fungi (EPF) are microorganisms that infect arthropods, cause diseases and ultimately their death (Ebani & Mancianti, 2021). *Metarhizium anisopliae*, *Beauveria bassiana* and *Akanthomyces lecanii* (formerly, *Lecanicillium lecanii*) are the best candidate EPF for tick control (Alonso-Díaz & Fernández-Salas, 2021). *Metarhizium anisopliae* (Metsch.) Sorok. (Hypocreales: Clavicipitaceae) strain ICIPE 7 is a potent biopesticide that reduced populations of *Amblyomma variegatum* Fabricius in field trials (Nchu

et al., 2009), was virulent against *R. appendiculatus* and *R. pulchellus*, ticks (Nana *et al*., 2012; Nana *et al*., 2016), and amitraz-resistant *Rhipicephalus decoloratus* ticks (Murigu *et al*., 2016). Interestingly, certain plants such as *Acalypha fruticosa* Forssk (Euphorbiaceae) (Hassan *et al.*, 1994) and *Calpurnia aurea* Benth (Nana *et al*., 2010) have been shown to attract various developmental stages (larvae and nymphs) and both male and female adult ticks, respectively. However, the specific bioactive compounds underpinning this tick-attractive phenomenon in these plants are not known. Logan and Birkett (2007) proposed the use of botanicals as a financially feasible and efficient source of semiochemicals, especially in developing nations. In addition, Nana *et al*. (2010) suggested substituting chemical attractants employed in attract-and-kill strategies with plant-based attractants for ticks due to their minimal toxicity to mammals and lower risk of environmental pollution due to their short persistence; potential synergistic effects and limited development of resistance owing to their intricate chemical compositions; and relatively affordable procurement costs (George *et al*., 2014).

Noteworthy amidst these botanical candidates is *Senna didymobotrya* (Fresenius), whose extracts have been scientifically acknowledged to modulate tick behaviour (Opiro *et al*., 2013; Wanzala *et al*., 2014). This facet extends to other hematophagous arthropods as evidently shown by the plant's capacity to attract mosquitoes (Nikbakhtzadeh *et al*., 2016), introducing an additional dimension of intrigue to its potential to attract ticks. Commonly known as African senna, the plant is an evergreen distinctive-smelling small tree or large shrub that grows to a height of 0.5 – 9 m. It is native to East and Central Africa and its habitat spectrum includes disturbed areas, roadsides, urban open spaces, wastelands, savannahs, grasslands, woodlands and riparian vegetation (Witt & Luke, 2017). Traditionally, farmers in Central Kenya used boiled leaves of the plant to manage livestock diseases and remove ticks (Njoroge & Bussmann, 2006). Although *S. didymobotrya* is an extensively used medicinal plant, available literature focuses on its morphological identification (Jeruto *et al*., 2017), which can be constrained by factors such as incorrect identification due to almost identical external morphological characteristics, for instance, with *Senna italica*, and environmental and geographical variations (Ho *et al*., 2021). These challenges can be overcome by identification using molecular barcodes.

1.2 Statement of the problem

Currently, attraction-aggregation-attachment pheromone (AAAP), 2,6-dichlorophenol (2,6-DCP) sex pheromone, and arrestment pheromones (AP) are tick attractants that have been integrated, for instance, with entomopathogenic fungi, in tick control schemes. However, the

effectiveness of these attractants is limited by their high costs and the fact that recipient ticks will only respond to the attractants when they have reached specific physiological and developmental stages, are of a particular sex, or upon direct contact (Sonenshine, 2004). Though researchers have advocated for the replacement of these synthetic attractants with affordable and renewable plant extracts, the compounds that confer the attraction to ticks within them are not known. When combined with entomopathogenic fungi, the plant extracts tend to either act synergistically with the fungus to kill the arthropod, or to negatively affect the growth parameters of the fungus and consequently, their ability to kill the arthropods. In this context, the current study sought to identify tick attractant cues in *S. didymobotrya* and to assess their influence on the viability and virulence of *M. anisopliae* ICIPE 7 against *A. variegatum* and *R. appendiculatus* ticks in a laboratory setting.

1.3 Objectives

1.3.1 General objective

To contribute to improved animal health by developing a sustainable tick control method that incorporates *Metarhizium anisopliae* and tick attractants from *Senna didymobotrya* based on "attract-and-kill" strategy.

1.3.2 Specific objectives

i. To identify and infer evolutionary relatedness of putative *S. didymobotrya* samples collected from various climatic zones in Kenya using molecular barcodes.
ii. To identify tick attractants in *S. didymobotrya* using olfactory-based bioassay-guided activities.
iii. To determine the effect of identified tick attractants on the viability and virulence of *M. anisopliae* ICIPE 7.

1.4 Null hypotheses

i. Putative *S. didymobotrya* samples collected from various climatic zones in Kenya are not identified and their evolutionary relatedness inferred using molecular barcodes.
ii. *Senna didymobotrya* does not have any tick attractant compounds.
iii. Tick attractants identified in *S. didymobotrya* do not influence the viability and virulence of *M. anisopliae* ICIPE 7.

1.5 Justification

In Kenya, the livestock industry contributes 4.4% of the national GDP and 14.2% of the agricultural GDP (FAO, 2019). It sustains a vast majority of rural households and a sizable number of urban households through the sale of livestock products, such as milk, meat, hides and skins, and as a source of employment in the livestock-related agro-processing industries

However, this sector faces some impediments, the major one being ticks and tick-borne diseases. Ticks are the most critical veterinary parasites of domestic animals. They damage livestock's skin as they bite to suck blood leading to host irritation, anaemia, weight loss, and reduction in milk yield. Additionally, they act as vectors of viral, protozoal, and bacterial pathogens, which cause debilitating diseases in livestock and humans. *Amblyomma variegatum* is implicated in heartwater, benign bovine anaplasmosis, benign bovine theilerioses in animals; *R. appendiculatus* has been associated with East Coast Fever (ECF), benign bovine theilerioses and ehrlichiosis (Horak *et al*., 2018). 65% of cattle mortalities are attributed to ECF, whereas babesiosis and anaplasmosis account for 5.1% and 4.5% of cattle mortalities (Chepkwony *et al*., 2020). These TTBDs account for more than KES 30 billion (US$ 221 million) each year in financial losses per annum (Kanduma, 2018). Therefore, it is crucial to prospect and develop new sustainable tick control strategies, which can be used alone or together with other strategies in integrated pest management. Incorporating plants and plant-derived products into the tick management toolkit is advantageous because they are biodegradable, less harmful to the environment, including non-target organisms and consist of many compounds that may act synergistically, therefore reducing chances of development of resistance by ticks (George *et al.*, 2014; Logan & Birkett, 2007; Nana *et al*., 2010).

CHAPTER TWO
LITERATURE REVIEW

2.1 Biology of ticks

2.1.1 Taxonomy of ticks

Ticks are obligate hematophagous ectoparasitic arachnids of birds, reptiles, and mammals. They belong to the superorder Parasitiformes, subclass Acari. They are classified into the following three families; Argasidae (soft ticks), Ixodidae (hard ticks), and monotypic Nutalliellidae, which has features attributed to both soft and hard ticks (Estrada-Peña, 2015). The current study focused on ixodid ticks.

2.1.2 Life cycle of hard ticks

The development cycle of the family Ixodidae comprises the following four developmental stages; egg, larva, a single nymph, and adult in both the male and female ticks (Apanskevich & Oliver, 2014). Based on the location of the tick, either on or off its host, as it moults from larva into nymph and adult, hard ticks are categorised as 1-, 2-, or 3- host. Most ticks of the family Ixodidae have a 3-host life cycle. In this, the eggs on the ground spawn into larvae that climb up on grass or other objects as they quest for their host. Once on the host, they feed on it, drop -off, and moult into the nymph. Unfed nymphs search for a suitable host (the same or a different one), climb onto it, feed on blood, and then drop off to the land, where they moult into adults. The adult ticks quest for an appropriate host, climb onto it, feed, and mate. The males die while the females fall off the host, oviposit successively for several weeks, and then die. Most species of *Amblyomma*, *Ixodes*, *Anomalohimalaya*, *Haemaphysalis*, *Bothriocroton*, *Dermacentor*, and *Rhipicephalus* are obligate 3-host ticks (Basu & Charles, 2017).

2.1.3 Morphology and adaptations of ixodid ticks

Ticks' body consists of the capitulum (gnathosoma) and the body (idiosoma). The gnathosoma comprises the basis capituli and mouthparts. The idiosoma consists of the legs, spiracles, and genital pores (Nicholson *et al.*, 2018). The chitinous scutum of female ticks encloses the anterior third of the idiosoma's dorsal side, whereas it covers the entire male' idiosoma's dorsum (Mehlhorn *et al*., 2016). The sensory organs of the ticks include the chemosensilla, photosensilla, and mechanosensilla. The Haller organ – the tick's sensory organ, containing both the olfactory and gustatory chemosensilla, is found on the dorsal surface of the tarsi of every foreleg. Olfactory chemosensilla recognise volatiles, whereas the gustatory chemoreceptors perceive stimulus following contact (Carr & Roe, 2016).

2.2 Tick ecology

Ticks mostly live on or close to the ground as they await an appropriate host. Ticks climb onto suitable objects like tall grass or weeds. They wait for appropriate hosts to pass by from these favourable positions. On detecting vibrations and chemical cues, for instance, host odours or breathed-out carbon dioxide, the ticks fall off from their perch or extend out (holding onto their perch with only 2 or 4 hind legs) and hop to affix themselves onto a passing host (Nicholson *et al*., 2018). Ticks can also detect shadows of hosts passing by (Estrada-Peña, 2015).

2.3 Semiochemicals

Semiochemicals are organic signalling molecules or chemicals that transmit chemical messages emitted by one organism to induce a behavioural or physiological response in another (Mweresa *et al*., 2020). They enable insects to find hosts, mates, and food, keep competition at bay, escape their natural enemies and overpower their hosts' defence system. There are two types of semiochemicals: pheromones, which mediate communication between organisms of the same species (Sonenshine, 2004); and allelochemicals, which mediate the transfer of information between organisms of different species.

Different types of pheromones, including attraction-aggregation-attachment pheromones, arrestment/ assembly pheromones, sex pheromones and primer pheromones have been documented in ticks (Sonenshine, 2004). Arrestment pheromones signal a pause in movement and a decrease in distance among individuals detecting the stimulus. These pheromones lead to groups of ticks forming in their natural habitat (Carr & Roe, 2016). Attraction-aggregation-attachment pheromones entice hungry ticks to a host, prompting them to gather before attaching and exploring the host's skin. Their purpose is to encourage feeding in a localized area (Sonenshine, 2006). Sex pheromones are emitted by one gender and shape the sexual actions of the opposite gender. They contribute to mate attraction, choice, and the eventual merging of reproductive cells between mates (Logan & Birkett, 2007). Though knowledge on primer pheromones is currently limited, they manage physiological functions in ticks. For instance, they can affect tick reproduction under crowded conditions.

Allelochemicals include kairomones, allomones, and synomones. Kairomones are chemical signals emitted to benefit the receiver by eliciting a behavioural or physiological response but have no effect (positive or negative) on the emitter, for example, hosts’ body secretions, various odours, carbon dioxide emissions (Sonenshine, 2008) and some plant extracts (Nana *et al*., 2010). Allomones benefit the emitter. For instance, ticks secrete

hydrocarbons, which keep off ants. Synomones favour both the emitting and receiving organisms (Mulenga, 2013; Sonenshine, 2004, 2006, 2008).

2.3.1 Attraction-Aggregation-Attachment Pheromones (AAAP)

These pheromones are secreted by feeding male ticks, and attract both the unfed male and female ticks from their natural habitats and are made up of various organic volatiles (methyl salicylate, *o*-nitrophenol (=2-nitropenol), and nonanoic acid in a ratio of 1: 2: 8, respectively – with *o*-nitrophenol eliciting host searching and tick aggregation, while the other two enabled attachment) (Carr & Roe, 2016). They also induce aggregation of the attracted ticks on their vertebrate hosts and enable them to feed near each other. Usually, the AAAP attracts ticks up to 3 meters away from a tick-infested host; however, for it to be maximally effective, carbon dioxide secreted by the host is needed to activate the ticks (Norval *et al*., 1989).

2.4 *Amblyomma variegatum* Fabricius, 1794

The tropical bont tick (Figure 1) is a three-host ixodid tick found in the Savanna biome. The adult ticks are prevalent on cattle, sheep, goats, wild bovids, warthogs, and rhinoceroses. *A. variegatum* is the vector for *E. ruminantium* – causes heartwater; *Anaplasma bovis* – implicated in benign bovine anaplasmosis; *Theileria mutans* and *Theileria velifera*, the aetiology for benign bovine theilerioses (Horak *et al*., 2018).

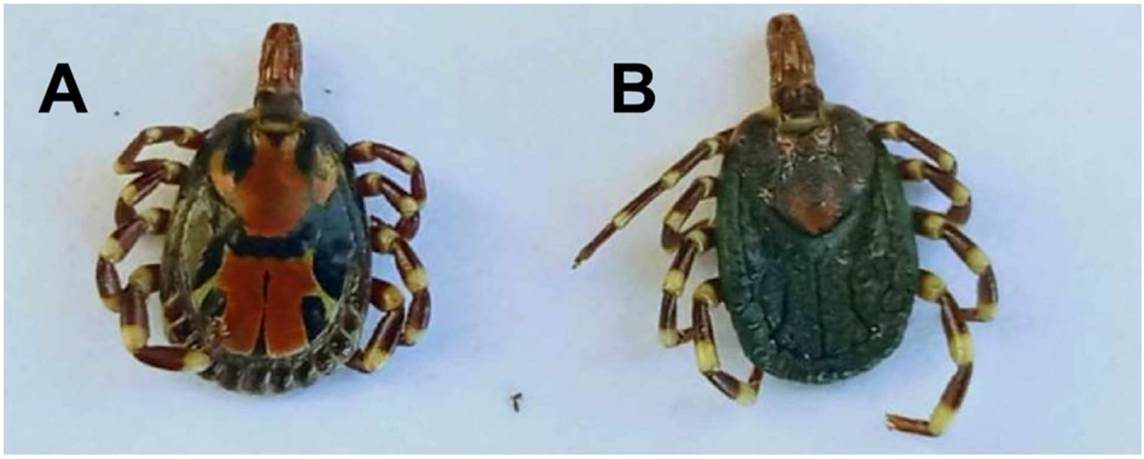


Figure 1: Adult male (A) and female (B) of *A. variegatum*

2.5 *Rhipicephalus appendiculatus* Neumann, 1901

The brown ear tick (Figure 2) is a three-host ixodid tick prevalent in the east, central, and southeast of Africa. It transmits *Theileria parva*, which causes East Coast Fever, the Nairobi sheep disease and the Thogoto viruses. It is also implicated as a vector for *Theileria taurotragi*, the aetiology of benign bovine theileriosis, and *Anaplasma bovis*, the aetiology for

ehrlichiosis in cattle. Its hosts are cattle, buffaloes, large antelopes and occasionally, hares, warthogs, and dogs (Horak *et al*., 2018).

Figure 2: Adult male (A) and female (B) of *R. appendiculatus*

2.6 Economic importance of ticks

Tick bites result in tick worry – characterised by irritation, anaemia, anorexia, weight loss and reduction in milk production; severe dermatitis in heavy tick infestations (Mehlhorn *et al*., 2016); the introduction of systemic toxins into the hosts' system (tick bite paralysis and sweating sickness) (Rajput *et al*., 2006); formation of abscesses following secondary bacterial infections at sites of the bite; and the lowered value of livestock hides and skins for the leather industry (Hurtado & Giraldo-Ríos, 2018).

Additionally, ticks vector pathogens that cause various diseases from one host to another during a blood meal (Mehlhorn *et al*., 2016). Most tick-borne diseases affect the lymphatic system and/or the blood. They include East Coast Fever, Babesiosis, heart-water, Anaplasmosis, Theileriosis and other diseases that cause economic losses (Estrada-Peña, 2015). Ticks get infected with pathogens when obtaining a blood meal from an infected animal. Transmission of tick-borne pathogens from one developmental stage to the next occurs transstadially or transovarially (Rajput *et al*., 2006). Transovarial transmission refers to the process where pathogens are passed from an infected female tick to her eggs, ensuring the continuation of infection from one generation to the next. In the case of ticks, this phenomenon leads to the transmission of pathogens from the female tick to the hatched larvae (Domingos *et al*., 2013). Transstadial transmission involves the transfer of pathogens from one life stage of a tick to the next stage during the moulting process (Domingos *et al*., 2013).

2.7 Tick management strategies

2.7.1 Chemical control

The use of acaricides is the most relied-upon method for tick control due to its efficacy. The downside of using acaricides is that they are toxic, leave residues of the drugs in livestock meat and milk products, and pollute the environment. Additionally, there is resistance to acaricides, which is linked to mutations in genes that are implicated in drug susceptibility due to an increase in the metabolism of the acaricide, its sequestration or a decline in the acaricide's ability to permeate the tick's outer protective layers (de la Fuente, 2018; Salman & Tarrés-Call, 2013).

2.7.2 Vaccines

Anti-tick vaccines (ATVs) represent an ecologically conscientious and efficient approach for managing cattle ticks and tick-borne diseases (TBDs) by targeting the vector (ticks) (Kasaija *et al*., 2021). These vaccines are designed to decrease both tick populations and the prevalence of TBDs. They achieve this by diminishing tick feeding, reproduction, and development through antibodies that specifically target tick antigens. These antibodies impact tick protein functionality and engage other immune mechanisms (de la Fuente & Contreras, 2022).

The ATVs, Gavacplus and TickGard, both formulated from antigens originating in *Rhipicephalus microplus*, specifically the glycoprotein Bm86 expressed on mid-gut cell surfaces, represent the sole commercially registered ATVs. These vaccines, whose effectiveness spans from 10% to 89%, have been used in Australia, Cuba, Mexico, and Venezuela, targeting *R. microplus*, *Rhipicephalus australis*, and *Rhipicephalus annulatus* cattle ticks (Kasaija *et al*., 2023). Despite this, commercially registered ATVs have not been embraced for tick control in tropical Africa, mainly due to their inadequacy in safeguarding economically significant tick species within Africa and the dearth of comprehensive research in this context (Kasaija *et al*., 2023).

2.7.3 Biological control

Based on random observations, beetles, ants, and spiders have been reported to consume ticks, especially the engorged ones, through predation. The amphibians, toads (*Bufo paracnemis*), and tortoises (*Pelomedusa subrufa*) feed on engorged female ticks (Samish & Rehacek, 1999). Birds and mammals predate on ticks as they self-groom. Birds implicated as tick predators include oxpeckers, cattle egrets, domestic fowls, and ravens (Samish & Rehacek, 1999).

Parasitoids refer to small insects whose premature stages grow within or are attached to the outside surface of other insects, typically their hosts. Ultimately, the parasitoids kill their hosts (Raupp *et al*., 2021). Chalcid wasps of the family Encyrtidae and the genus *Ixodiphagus* are the only tick parasitoids that have thus far been characterised. *Ixodiphagus hookeri* (Khachatourians, 1996) has been characterised in *Rhipicephalus*, *Amblyomma*, *Dermacentor*, *Ixodes*, *Hyalomma*, *Ornithodoros*, and *Haemaphysalis* species (Gaye *et al*., 2020). Irrespective of the fact that *I. hookeri* has previously been utilised to control ticks, its efficacy is still controversial, and there are still sizeable knowledge gaps concerning its biology (Gaye *et al*., 2020).

2.7.4 Entomopathogenic bacteria

Some bacterial species have displayed pathogenic traits towards ticks, making them valuable for biological control applications. A notable instance is *Bacillus thuringiensis*, extensively researched for its ability to manage ticks and its incorporation in commercial insecticide formulations. *Bacillus thuringiensis* functions post-ingestion, its insecticidal *δ*-endotoxins specifically interacting with midgut epithelial cell receptors in ticks (Bravo *et al*., 2007). The efficacy against ticks has been shown to differ based on species and developmental stages (Fernández-Ruvalcaba *et al*., 2010). For instance, two out of four environmental *B. thuringiensis* strains effectively controlled *Dermacentor reticulatus* and *I. ricinus* ticks, with up to 80% tick mortality, while Vectobac, the commercially available product had limited effectiveness (Szczepańska *et al*., 2018). Additionally, the pathogenic potential of *B. thuringiensis* variety *kurstaki* was observed against engorged larvae of *I. scapularis* (the blacklegged tick) with an LC_{50} of 107 spores/mL (Zhioua *et al*., 1999).

Though *Proteus mirabilis* demonstrated pathogenicity against all developmental stages of a laboratory colony of *Dermacentor andersoni* ticks (Brown *et al*., 1970), it is not recommended for tick control due to its propensity for causing opportunistic infections in both humans and animals (Drzewiecka, 2016).

2.7.5 Entomopathogenic nematodes

Infective juvenile stages (IJs) of entomopathogenic nematodes *Heterorhabditis* and *Steinernema* nematodes have been used for pest control. On encountering appropriate susceptible hosts, the IJs use enzymes and mechanical force to permeate the haemocoel, releasing symbiotic bacteria such as *Xenorhabdus* spp in sternernematids and *Photorhabdus* spp by heterorhabditids, which proliferate within and kill the hosts (Samish & Glazer, 2001). The nematodes invade their hosts through natural orifices (mouth, spiracles, anus) or via the cuticle and kill the host in 24 – 72 hours (Samish *et al*., 2006). Filgueiras *et al*. (2022) showed

a significant reduction of 73.1% in *Rhipicephalus* (*Boophilus*) *microplus* larvae populations using *Heterorhabditis bacteriophora* under field conditions. This highlights the potential of entomopathogenic nematodes as an effective tool for controlling economically important tick species. The "HB+SF Beneficial Nematode Mix" combines *H. bacteriophora* (HB) and *Steinernema feltiae* (SF) is commercially designed to target different soil-dwelling pests, including ticks.

2.7.6 Entomopathogenic fungi

Entomopathogenic fungi (EPF) are microbes that infect, bring about deadly diseases, and kill arthropods. Their primary function is the control of insect populations in the environment. They naturally inhabit the soil; however, they are principally isolated from arthropod carcasses (Behie & Bidochka, 2014). There are six classes of EPF: *Ascomycota*, *Basidiomycota*, *Oomycetes*, *Microsporidia*, *Chytridiomycota*, and *Entomophytoromycota*, with *Ascomycota* and *Entomophytoromycota* being the most prevalent (Litwin *et al*., 2020). Species of the class *Ascomycota* belonging to the genera *Metarhizium*, and *Beauveria*, among others, are illustrated in the literature (Litwin *et al*., 2020).

Entomopathogenic fungi infect arthropods when spores penetrate their cuticle and less frequently on ingestion of infective propagules (Skinner *et al*., 2014). Infection begins by the adhesion of the fungal spores to the cuticle of the arthropod, which occurs in two phases: In the first, it relies on hydrophobic action and electrostatic forces, and in the second, it involves enzymes and hydrophobins (Skinner *et al*., 2014). At optimum temperature (20 to 30 ºC) and humidity, the spores germinate in the existence of sources of energy and carbon on the pest's cuticle (Litwin *et al*., 2020). Appressoria then emerge, imparting substantial mechanical tension on the cuticle, and lytic enzymes (chitinases, lipases, and proteases) are produced, which break down the body shell of the arthropod (Skinner *et al*., 2014). On penetrating the insect's haemocoel, the EPF's hyphae start to grow. Some EPF form blastopores, which get into the host's haemolymph and give rise to secondary hyphae, which reside in the host's tissues. At this point, the EPF releases secondary metabolites, which lead to numbness and disturbance of the host's physiology, especially its immune system (Donzelli & Krasnoff, 2016). As the disease develops, the destruction of the arthropod's body results from mechanical injury of the pest's internal organs by the hyphae and exhaustion of nutrients (Donzelli & Krasnoff, 2016).

Bioprospecting at *icipe* identified *Metarhizium anisopliae* isolate ICIPE 7 as a potent EPF against various tick species found in Kenya such as *A. variegatum* (Nchu *et al*., 2009, 2010), *R. appendiculatus* and *R. pulchellus* (Nana *et al*., 2012, 2016). Moreover, Murigu *et al*.

(2016) demonstrated the efficacy of *M. anisopliae* ICIPE 7 in reducing populations of both amitraz-resistant and -susceptible ticks in the field. Based on these promising results, an innovative formulation of *M. anisopliae* ICIPE 7 has been developed into a commercial product known as Mazao Tickoff by Real IPM for use in controlling tick populations.

2.7.7 The use of plants for ethnoveterinary control of ticks

Plants, since time immemorial, have been used to control agricultural pests; but their use, though promising, was overtaken and replaced by synthetic chemicals (Nyahangare, 2019). However, interest in them has recently been rekindled owing to the shortcomings mentioned above of synthetic acaricides. Locally available plants with toxic, repellent, attractive, antifeedant and/or growth-regulating properties towards ticks utilised by specific communities can be integrated into tick control programmes (Wanzala, 2017). The major stumbling block facing the application of plants in tick control is the lack of scientific validation, which is thus considered backward and ineffective (Wanzala *et al*., 2005).

Some of the plants that control ticks in Kenya include *Nicotiana tabacum* L. (Solanaceae), whose leaves are mixed with Magadi Soda or *Solanum incanum*; *Olea europaea* subsp. *cuspidate* (Oleaceae), which is mixed with *Cadia purpurea*; *Margaritaria discoidea* (Baill) G.L. (Phyllanthaceae), used as a latex; *Azadirachta indica* Adr. Juss. (Meliaceae) – its seed oil; and *Solanum incanum* L. (Solanaceae), used as a fruit juice or in a concoction with *Nicotiana tabacum* (Nchu *et al*., 2020). *Acalypha fruticosa* Forssk (Euphorbiaceae), *Solanum infantum* Linneaus (Solanaceae), *Ipomoea spathulata* Hallier (Convolvulaceae), and *Calpurnia aurea* Benth are reported to have attractant properties towards ticks (Nana *et al*., 2010). The plant, *Senna didymobotrya* also stands out for its ability to influence tick behaviour (Opiro *et al*., 2012; Wanzala *et al*., 2014) and attract hematophagous arthropods such as mosquitoes (Nikbakhtzadeh *et al*., 2016).

The following is the scientific classification of *Senna didymobotyra* (Fresen.) H. S. Irwin & Barneby:

Kingdom: Plantae
Division: Spermatophyta / Angiospermae
Class: Magnoliopsida
Order: Magnoliopsidales / Fabales
Family: Caesalpiniaceae / Fabaceae
Sub-family: Caesalpinioideae
Genus: *Senna*
Species: *Senna didymobotyra* (Fresen.) H. S. Irwin & Barneby

Synonyms: *Cassia didymobotrya* Fresen (1839), *Cassia nairobensis* L. H. Bailey (1941)

Senna didymobotrya (Fresenius), also known as African senna, popcorn senna, popcorn cassia, peanut butter cassia, candelabra tree, or bush encroacher, is a fragrant evergreen tree or shrub with a recognizable scent that typically grows to a height of 0.5 to 9 meters. This plant is indigenous to East and Central Africa and is frequently encountered in areas that have been disturbed, such as roadsides, open urban spaces, wastelands, savannahs, grasslands, woodlands, and riparian vegetations (Witt & Luke, 2017).

In Eastern Africa, the decoction obtained from *S. didymobotrya*'s roots has been used to treat malaria, intestinal worms, ringworms, fevers and jaundice (Nagappan, 2012). Further, the plant is beneficial in the treatment of fungal and bacterial infections, hypertension, haemorrhoids, sickle cell anaemia, as well as a range of women's ailments including inflammation of the fallopian tubes, fibroids, and backaches. Moreover, it can promote lactation, initiate uterine contractions, and assist in inducing abortion (Nyamwamu *et al*., 2015).

In addition to the treatment of skin conditions in humans and livestock, farmers in Central Kenya boil the plant's leaves to manage livestock ailments and eliminate ticks (Njoroge & Bussmann, 2006). The plant's root decoction is also utilized as a remedy to counteract poisoning, facilitate the expulsion of a retained placenta, and treat East Coast fever and blackleg (Njoroge & Bussmann, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of African senna (*Senna didymobotrya*) samples

Onsite, a taxonomist identified the target plant, *S. didymobotrya* (Figure 3), using morphological characteristics. Samples of leaves, flowers, seedpods and twig-branches of the plant were collected at random from four Kenyan climatic zones (Figure 4). The various zones were chosen to determine the effect of environmental factors on the phylogenetic diversity of the study plant.

Figure 3: *Senna didymobotrya*

At 1158-5199 m elevation above surface level (ASL) and 0.6 °S, 37.33 °E, Kirinyaga County is typified by a tropical wet and dry or savanna climatic condition. It has annual temperatures of 14 °C – 25 °C and an average annual rainfall of 1100 – 1250 mm. Nairobi County experiences a cool and temperate climatic condition with an annual temperature of 14.78 - 24.9 °C and an average yearly rainfall of 610 mm. It is located at 1.3 °S, 36.82 °E and 1660 – 1800 m ASL. With a tropical wet and dry or savanna climate, Kajiado County is located at 1582 m ASL and 2.1 °S, 36.78 °E. It experiences an average annual rainfall of 400 mm and annual temperatures of 15.96 - 23.89 °C. Homabay County, which has a tropical rainforest climate is situated at an elevation of 1193 m ASL and 0.5 °S, 34.45 °E. It has an annual temperature of 15.55 - 25.05 °C and its yearly rainfall is estimated at 1200 - 2500 mm. Migori

County (1394 m ASL, 1.1 °S, 34.48 °E) experiences a tropical monsoon climate, mean annual rainfall of between 700 and 1800 mm, and annual temperatures of 15.55 °C to 25.05 °C.

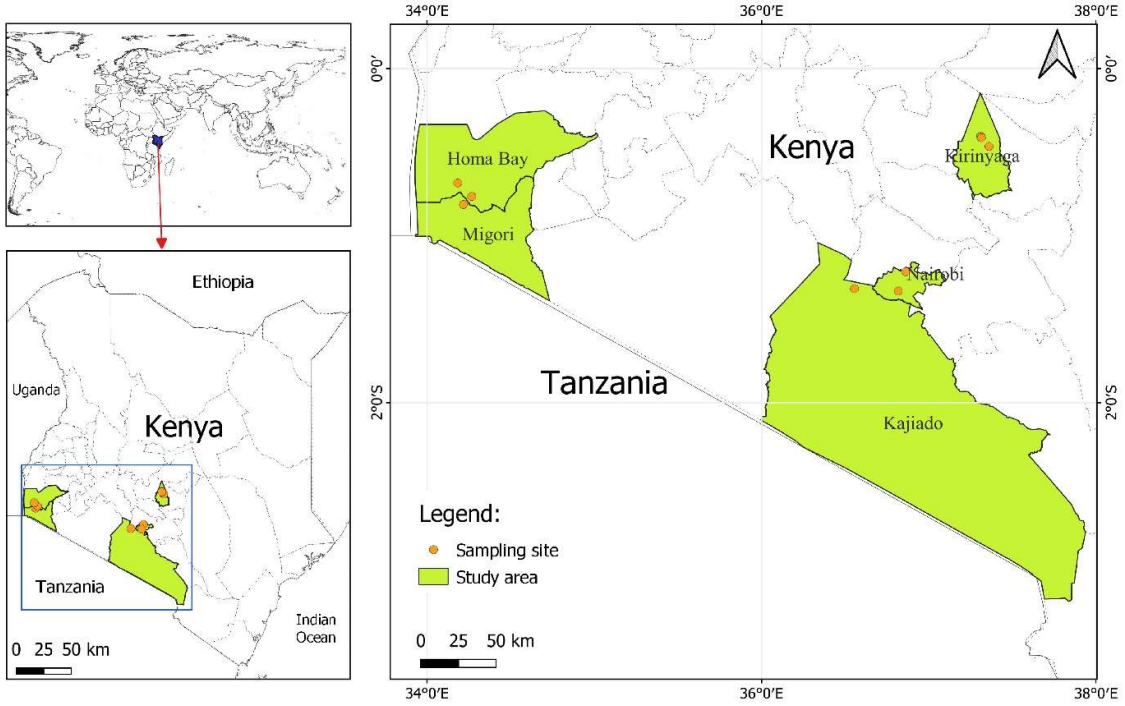


Figure 4: Map displaying sampling locations of *S. didymobotrya* plant in Kenya

The freshly sampled plant parts (leaves, flowers, seedpods and twig-branches – 100 kg of each) were packaged in separate well-labelled gunny bags and ferried to the laboratory at the International Centre for Insect Physiology and Ecology (*icipe*), Duduville Campus, on the same day.

3.2 Identification of *S. didymobotrya* samples using molecular barcodes

An ISOLATE II Plant DNA Kit (Bioline, London, UK) was used to extract DNA from the presumed *S. didymobotrya* leaf samples collected from Kirinyaga, Nairobi, Homabay, Migori, Kajiado and Homabay counties of Kenya, following the manufacturer's directions. Polymerase Chain Reaction (PCR) amplification of the extracted DNA was done by targeting the ribulose-1,5-bisphosphate carboxylase large subunit (*rbcL*) and maturase K (*matK*) genes. The primers used in amplification are presented in Table 1.

Amplification reactions were conducted in a ProFlex PCR systems thermocycler (Applied Biosystems, Foster City, CA, USA) in 20 µL reaction volumes comprising 4 µL 5× HOT FIREPOL Blend Master Mix (Solis BioDyne, Tartu, Estonia), 0.6 µL of forward and reverse primers, 3 µL of DNA template, and PCR grade water. PCR cycling conditions for *rbcL* amplification were as follows: initial denaturation at 95 °C for 15 min; 30 cycles of

denaturation at 95 °C for 20 sec, annealing at 66 °C for 1 min, and extension at 72 °C for 2 min 30 sec; and a final extension at 72 °C for 7 min. Determination of successful amplifications was accomplished by observing 5 µL of the amplicons on 2% (w/v) agarose gels that were stained with ethidium bromide (Sigma-Aldrich, GmbH, Germany), and they were compared to a 100 bp DNA ladder (Solis BioDyne, Tartu, Estonia) under ultraviolet light with a Kodak Gel Logic 200 Imaging System (SPW Industrial, Laguna Hills, CA, USA). ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) was used to clean the remaining volumes of positive amplicons, as indicated by the manufacturer. Macrogen Inc. (Seoul, South Korea) performed Sanger sequencing of the purified amplicons.

Table 1: Primers used in PCR amplifications

Primer name	**Target gene**	**Sequence (5'- to -3')**	**Amplicon size**	**Reference**
*rbcL*1F	*rbcL*	ATGTCACCACAAACAGAGACTAAAGC	702 bp	(Fay *et al*., 1997)
*rbcL*724R		TCGCATGTACCTGCAGTAGC		
*matK*3F	*matK*	CGTACAGTACTTTTGTGTTTACGAG	792 - 798 bp	Ki-Joong Kim, unpublished
*matK*1R		ACCCAGTCCATCTGGAAATCTTGGTTC		

3.3 Workflow for identification of probable tick attractants in *S. didymobotrya*

Figure 5provides a schematic overview of the processes involved in the bioassay-guided identification of probable tick attractant compounds in *S. didymobotrya*.

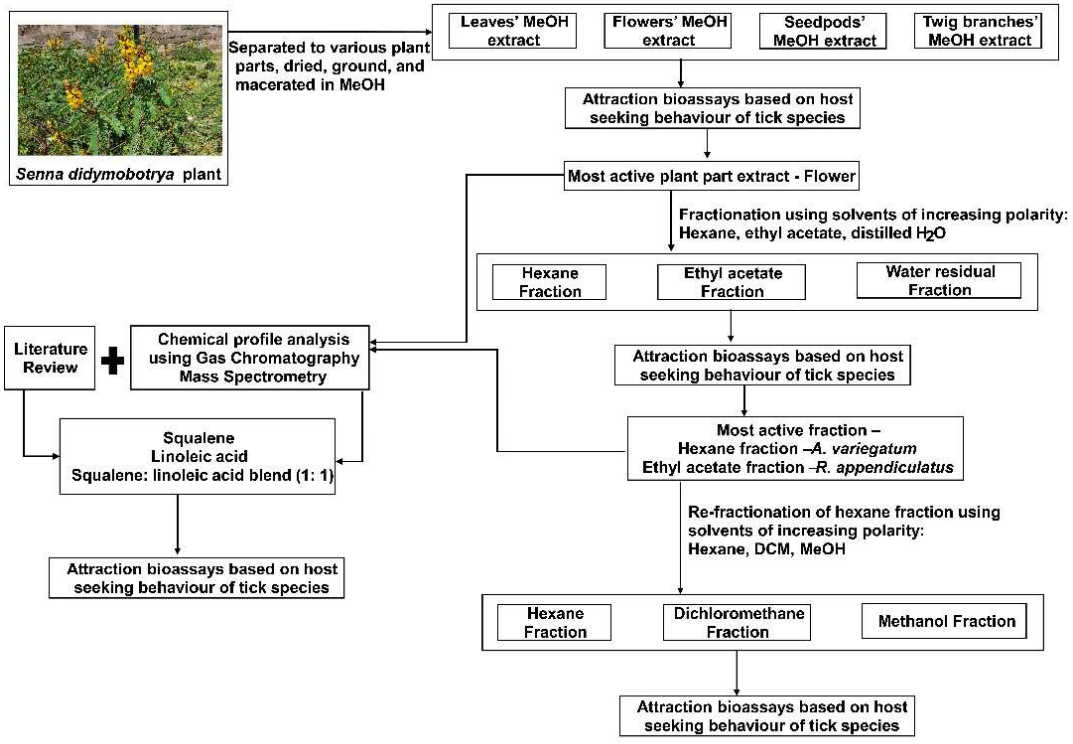


Figure 5: Flow chart for the bioassay-guided fractionation and identification of probable attractants in *S. didymobotrya*

3.4 Preparation of the crude extract

Based on the phylogenetic analysis, *S. didymobotrya* samples from Nairobi, Homabay, Migori, Kajiado and Kirinyaga counties were combined for chemical analysis. The plant samples were allowed to air-dry in a screen house (19 -35 °C, 30 – 90% RH) to a constant weight. An SM 100 cutting mill (Retsch GmbH, Haan, Germany) was used to reduce the plant materials to a fine powder. Each powder sample was soaked thrice in analytical grade methanol (Sigma Aldrich, St. Lois, USA) (0.25 kg/L) for 72 h with occasional shaking. Whatman® filter paper (#1) was used to filter the resulting extract. The three filtrate volumes were combined, and the solvent removed *in vacuo*, weighed and stored at -20 °C until needed.

3.5 Attraction-Aggregation-Attachment Pheromone (AAAP)

In the tick climbing and wind tunnel attraction bioassays, the AAAP pheromone was used as a positive control. It was made by mixing nonanoic acid (0.8 mg), *o*-nitrophenol (0.2 mg), and methyl salicylate (0.1 mg), all of which were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. The bioassays were carried out using a 0.02 mg/mL concentration, which attracts *A. variegatum* (Nchu *et al*., 2009) and *R. appendiculatus* (Nana *et al*., 2010) adult ticks.

3.6 Ethics statement

Tick rearing on rabbits was done following standard operating procedures at the International Centre for Insect Physiology and Ecology's (*icipe*) Animal Rearing and Quarantine Unit (ARQU), which has been approved by Kenya's National Commission for Science, Technology, and Innovation (License No: NACOSTI/P/20/4253).

3.7 Experimental ticks

The hard ticks, *Amblyomma variegatum* and *Rhipicephalus appendiculatus*, known to transmit *Ehrlichia ruminantium* causing heartwater and *Theileria parva* causing East Coast Fever - the most prevalent tickborne diseases in the Kenyan livestock industry (Chiuya *et al*., 2021), were utilized in the study. Adult ticks were obtained from colonies raised at *icipe*'s ARQU following Levin and Schumacher (2016). The *R. appendiculatus* and *A. variegatum* colonies were initiated from samples collected from the field in Rusinga Island, Kenya, in 2006 and 2008, respectively, and have since been reared and maintained in the ARQU. All active developmental stages (larvae, nymphs, and adults) were bred on New Zealand white rabbits. Adult ticks were identified to species level before use in the bioassays using morphological keys (Okello-Onen *et al*., 1999). Thirty ticks were kept in each 10 mL glass vial with a nylon mesh and a tube stopper (Figure 6A). The vials were maintained over saturated sodium chloride solution to ensure a relative humidity (RH) of 85 ± 5% in aluminium tins (20.5 cm d, 20 cm h) (Figure 6B) contained in a Sanyo MIR-153 incubator under 16:8 Light: Dark (L: D) hr photoperiod at 25 ± 1 °C.

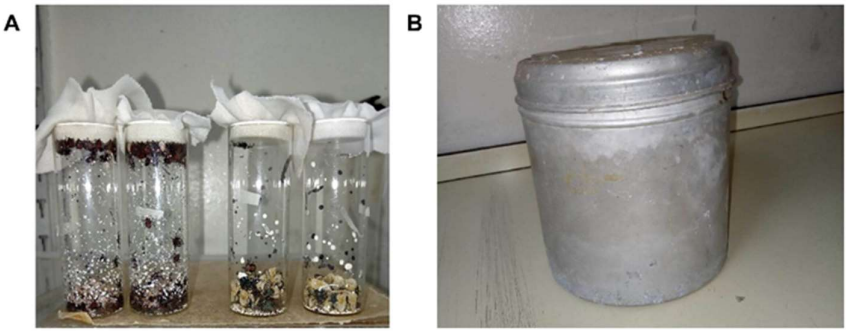


Figure 6: Ticks in glass vials with nylon mesh and tube stopper (A). Aluminium tins in which the glass vials containing ticks were maintained (B)

3.8 Behavioural responses of ticks to crude methanolic extracts of *S. didymobotrya*

3.8.1 Tick climbing bioassay

Based on *R. appendiculatus*'s ambushing host-seeking approach (Wanzala *et al*., 2014), a two-choice tick climbing assay with slight modifications from that used by Nana *et al*. (2010) was adopted (Figure 7). The setup comprised two aluminium rods (26 cm l × 0.7 cm d) opposite

each other and 7 cm apart on an aluminium base (14.5 × 14.5 × 1.7 cm). The equipment was put in a tray (32 × 25 × 3.5 cm) filled with water to the upper surface of the aluminium base to stop ticks from escaping.

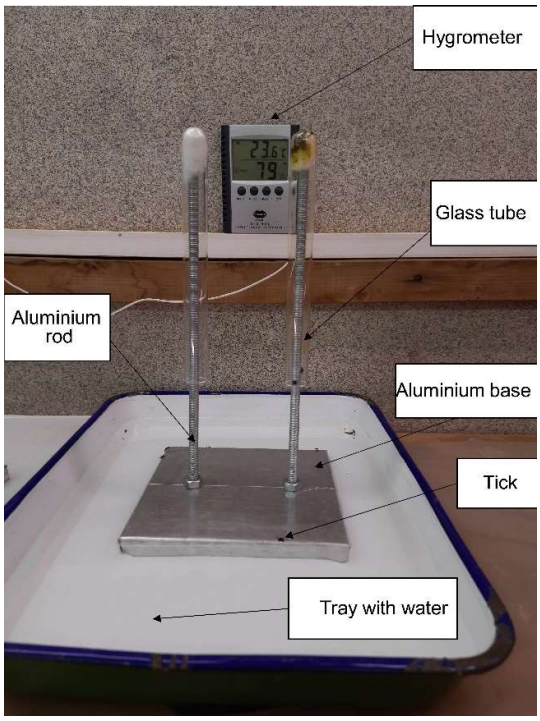


Figure 7: Tick climbing bioassay

Aliquots (0.5 mL) of treatments and control (solvent used to reconstitute the treatment) were spread on sterile cotton wool plugs (0.2 g) prepared by autoclaving before the assays. To prepare the treatments, each crude extract was reconstituted in methanol to give different concentrations - 100, 50, 25 and 1 mg/mL. After waiting 5 minutes for the solvent to evaporate, the plugs were placed on top of the aluminium rods. On each aluminium rod, a test tube was placed over the cotton wool plug. The treatment cotton wool plug was applied to one rod, while the control was applied to the other.

The activity of ticks was established before each assay by blowing human breath against them (Kimps *et al*., 2011); only responsive ticks were used in the experimental assays. Ten *R. appendiculatus* ticks of the same sex were placed between the rods on the aluminium base. The experiment was carried out in the bioassay room for 30 minutes at 23-26 °C and 70 ± 10% RH. The number of ticks that ascended the test and control rods was counted at the end of each session. The number of ticks that did not ascend any rod was also recorded but was omitted from the data analysis.

After each test run, the equipment was washed with soap under running tap water, rinsed with distilled water, followed by ethanol (70%), and then dried at 40 °C. To avoid behavioural response due to positional bias, treatment and control rods were switched between experiments. The treatments were assigned at random, and each assay was repeated eight times, totalling 80 adult ticks (40 male and 40 female) tested for each concentration.

3.8.2 Olfactometer bioassay

Based on *A. variegatum*'s hunting strategy of host location (Nchu *et al*., 2009), an olfactometer bioassay (Figure 8) was used to assess its responses to *S. didymobotrya* methanolic extracts. The experimental setup comprised a clear, cuboid Perspex tube (31.5 × 5.7 × 4 cm) with a hole at the centre. Two removable 50 mL Falcon tubes cut at the bottom were linked to the tube's extreme ends, one on the right and one on the left. The removable Falcon tubes sufficed as sites for the placement of test substances. Using Teflon tubes, holes in the corks of the Falcon tubes were connected to a portable field air delivery and vacuum pressure pump (Sigma Scientific, Micanopy, FL, United States). The wind tunnel tube was partitioned into three: two response zones (10 cm zones on each side of the tube); and a no-response zone in the middle (10 cm). The hole at the centre of the tube was the release point for the ticks and the port for attachment to the vacuum. At a flow rate of 5 mL/s, air entered the wind tunnel through holes in the lids of the Falcon tubes at the extreme ends of the tube (Nana *et al*., 2010; Nchu *et al*., 2009), and the vacuum sucked out the air at the same rate.

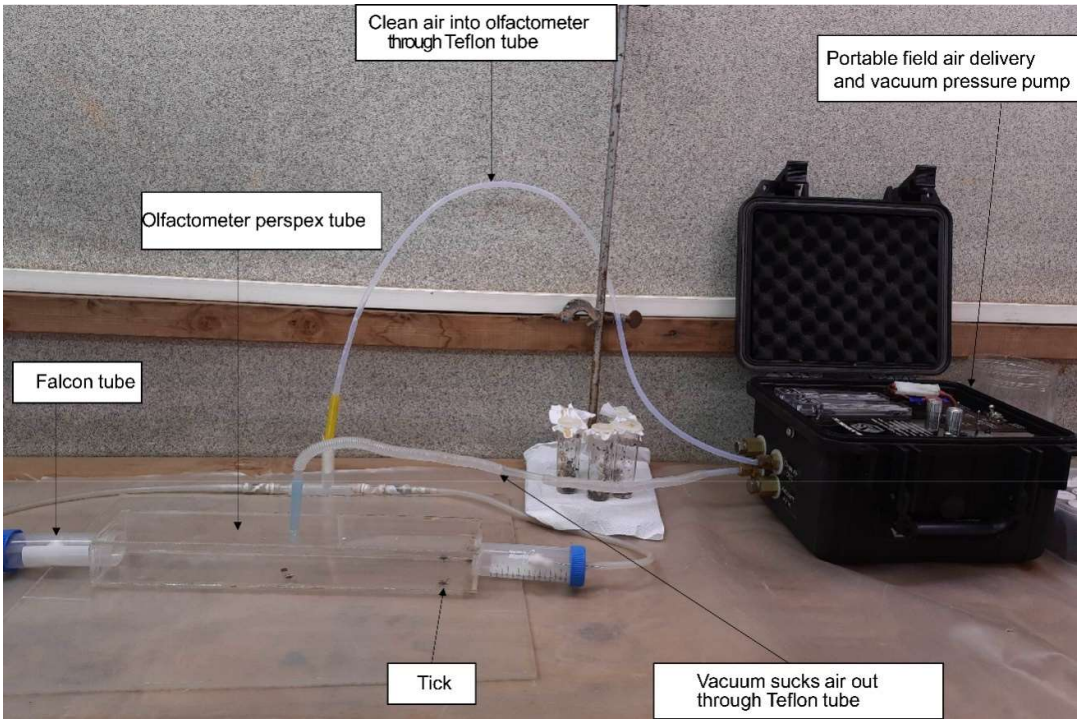


Figure 8: Olfactometer bioassay

Aliquots (0.5 mL) of the treatments (crude methanolic extracts of the flowers, twig branches, leaves, and seedpods at 100, 50, 25 and 1 mg/mL concentrations, one at a time) and control (solvent used to reconstitute the treatment, in this case, methanol) were spread on sterile cotton wool plugs (0.2 g). The concentrations used were selected because they corresponded to ranges of plant extracts that are physiologically significant to ticks (Nana *et al*., 2010). The plugs were allowed to air-dry for 5 minutes before being introduced into the wind tunnel, with one arm receiving the treatment and the other receiving the control.

The activity of ticks was established before each assay by blowing human breath against them (Kimps *et al*., 2011); only responsive ticks were used in the experimental assays. To avoid overcrowding in the olfactometer tube, five ticks were introduced into the wind tunnel at a time, and given a maximum of 5 minutes to choose an arm (Carr *et al*., 2013). Ticks that did not choose an arm were regarded as unresponsive to both the treatment and the control. The experimental equipment was cleaned as in section 3.8.1. To avoid response due to positional bias, the treatment and control arms were alternated between assay replicates. For each test substance and concentration, tests were conducted using 80 adult ticks (40 males and 40 females).

3.9 Fractionation of the crude flower extract

The most active crude methanolic extract, the flower extract, was separated into different fractions by liquid-liquid partitioning using solvents of increasing polarity (Figure 9A). In summary, 15 g of the crude flower extract was dissolved in 200 mL distilled water and then separated with hexane (4 × 100 mL) and ethyl acetate (4 × 100 mL) in a separating funnel. The resultant water part was freeze-dried to obtain the aqueous fraction. Solvents were removed from the hexane and ethyl acetate fractions *in vacuo*. The fractions (hexane, ethyl acetate and aqueous) were then weighed and stored at -20 °C before being used in bioassays. Various test concentrations (50, 25 and 1 mg/mL), prepared by reconstituting the fractions in the fractionation solvent, were subjected to tick attraction bioassays as defined in sections 3.8.1 and 3.8.2 above. The concentrations evaluated were selected based on previously reported physiologically relevant plant extract doses for ticks (Nana *et al*., 2010).

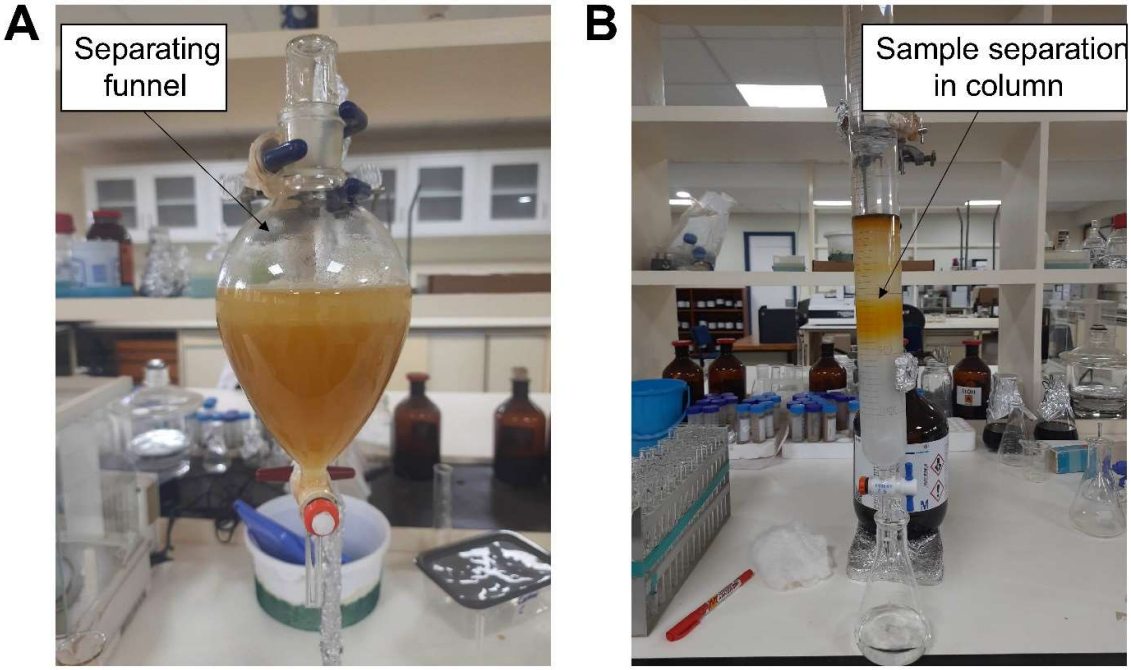


Figure 9:Fractionation of the active flower extract in a separating funnel (A) and hexane fraction via gradient column chromatography (B)

3.10 Column chromatography of the hexane fraction

The most potent fraction from liquid-liquid partitioning (the hexane fraction) was fractionated by gradient elution in column chromatography (Jandera & Churacek, 1985) (Figure 9B). Briefly, 2 g of hexane fraction extract was mixed with 4 g of silica gel to yield a powder that was loaded onto the vertical glass column packed with silica gel (200 g, Kiesegel S [230 - 400 mesh]), Macherey-Nagel GmbH & Co. KG, Germany), which was pre-

conditioned using analytical grade hexane (Sigma Aldrich, USA). The column was eluted with solvents of increasing polarity using hexane, dichloromethane, and methanol (from 100% hexane with increasing amounts of dichloromethane to 100% of it and finally with increasing amounts of methanol to 100% of it) to give 140 sub-fractions. These sub-fractions were concentrated *in vacuo*.

The sub-fractions were subjected to preparative thin layer chromatography (TLC) using aluminium sheets (20 × 20 cm) coated with silica gel F254 (Macherey-Nagel GmbH & Co., Duren, Germany) using hexane: DCM (90:10), hexane: DCM (10:90), and DCM: MeOH (10:90). The plates were visualised by dipping in 95% sulphuric acid then heating to 120°C in an oven. Based on their chemical profile, as demonstrated by their retention factor values, the 140 fractions were pooled into six sub-fractions that were then subjected to tick attraction bioassays as detailed in sections 3.8.1 and 3.8.2 above.

3.11 GC-MS Analysis

Gas chromatography-mass spectrometry (GC-MS) was used to profile the chemical composition of the attractive crude extracts (methanolic flower) and solvent fractions (hexane and ethyl acetate). To prepare the samples for analysis, 1 mL of GC-grade dichloromethane (DCM) (Sigma Aldrich, St. Lois, USA) was added to each 5 mg of methanolic crude extract and ethyl acetate fraction samples. The samples were vortexed for 1 min, ultra-sonicated for 10 min, and centrifuged at 14 000 rotations per minute (rpm) for 5 min. The resultant supernatant was dried over anhydrous sodium sulphate, filtered, diluted to a final concentration of 100 ng/µL in DCM, and then analysed by GC-MS. The hexane fraction samples were prepared similarly, the only exception being the use of GC-grade hexane in place of DCM. Hexane and DCM solvent blanks were also analysed.

A 7890A gas chromatograph and a 5975C mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA) were used in the GC-MS analysis system. Sample injections were done using a 7683B autosampler (Agilent Technologies, Inc., Beijing, China). The instrument was operated using a Hewlett-Packard (HP Z220 SFF intel Xeon) workstation equipped with MSD Chemstation software B.02.02. The GC was fitted with a mid-polar (5%-phenyl)-methylpolysiloxane (HP5 MS) low bleed, fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) (J&W, Folsom, CA, USA). Helium, at a 1.2 mL/min flow rate, was used as a carrier gas. Sample aliquots (1 µL) were injected into the instrument in spitless mode. The inlet temperature was set at 270 °C. The oven temperature was kept at 35 °C for 5 min, programmed to increase at a rate of 10 °C/min to 280 °C for 10.5 min, then programmed to 285 °C at a rate of 50 °C/min for 9.9 min. The mass selective detector's ion

source and quadrupole temperatures were kept at 230 °C and 180 °C, respectively. The mass spectrometer was run in full scan mode over a 38 – 550 m/z mass range with the electron impact (EI) acquired at an acceleration energy of 70 eV. A 3.3 min solvent delay time was set. Compound identification was done by comparing their gas chromatographic retention times and mass fragmentation spectra to reference spectra published in Adams2, Chemecol, and the National Institute of Standards and Technology (NIST) 11 databases. A linear calibration curve was generated by analysing serial dilutions of the authentic standard squalene (≥ 98%) (Sigma-Aldrich, St. Louis, MO) in full scan mode using GC-MS. The resulting curve correlated peak areas with concentration, and had a regression equation (1):

$$y = 1E + 07x - 4E + 08 \quad (1)$$

and a determination coefficient of $R^2 = 0.9923$. This equation was used to quantify detected compounds in the analysed samples.

3.12 Fungal culture and viability assessment

Metarhizium anisopliae (Metschnikoff) Sorokin isolate ICIPE 7 (Figure 10) used in this study was obtained from *icipe*'s Arthropod Pathology Unit (APU) germplasm Centre. It was isolated from *Amblyomma variegatum* (Fabricius) in Kenya in 1996 (Nana *et al*., 2016). The isolate's pathogenicity against *A. variegatum* and *R. appendiculatus* has been reported (Nana *et al*., 2012; Nchu *et al*., 2010), and it is currently being commercialized as Mazao TickOff® by Real IPM Ltd, Kenya, for biological control of ticks (http://www.realipm.com).

Figure 10: *Metarhizium anisopliae* isolate ICIPE 7

Since Sabouraud Dextrose Agar (SDA) (Oxoid, Hampshire, UK) has been shown to favour sporulation of *M. anisopliae* ICIPE 7, it was cultured on SDA in 90 mm Petri dishes

and incubated at 25 ± 2 °C and 0:24 L:D photoperiod (Opisa *et al*., 2019). Fungal conidia were harvested from 14 – 21 days old sporulated cultures by scraping the surface using a sterile spatula. The harvested conidia were suspended in 10 mL sterile distilled water amended with 0.05% (w/v) Triton X-100 (EMD Millipore Corporation, USA) in universal bottles containing five 3 mm glass beads per bottle. The conidial suspension was vortexed at 700 rpm for 5 minutes to break up clumps of conidia and ensure its homogeneity. Conidial concentrations were ascertained using a Neubauer haemocytometer (Marienfeld, Lauda-Konigshofen, Germany) under a Leica DMLB light microscope (Goettel & Inglis, 1997).

Conidial viability was assessed prior to each bioassay. Briefly, a 0.1 mL aliquot of the conidial suspension titrated to 3×10^6 conidia/mL was spread-plated on SDA in 9 mm Petri dishes. The plates were sealed with Parafilm and incubated at 25 ± 2 °C and 0:24 L:D photoperiod. After 16 – 20 h, lactophenol cotton blue stain (Hardy Diagnostics, Santa Maria, CA) was added to the plates to halt germination and ease the counting of spores. Four sterile microscope coverslips were placed at random on the surface of each stained plate. The percentage of conidial germination was determined from 100 conidia selected at random under each cover slip under a Leica DMLB light microscope (× 400 magnification). Conidia were deemed viable when the length of the germinating germ tube was at least twice the diameter of the conidium (Inglis *et al*., 2012). Four replicate plates were used each time and only plates with 99 – 100% conidial germination were utilised for subsequent assays.

3.13 Preparation of tick attractants for compatibility and virulence assays

Crude flower (methanol) extract (5 g) of *S. didymobotrya* (prepared in section 3.4) was reconstituted in methanol (50 mL). Various concentrations (10%, 5%, and 2.5%) of the flower extract were prepared by dissolving the correct volumes of the reconstituted methanol extract in appropriate volumes of sterile distilled water prepared by autoclaving before the bioassays. The presumed potential attractants, synthetic squalene (≥ 98% purity) and linoleic acid (≥ 99% purity) (identified in section 3.11), both from Sigma-Aldrich (St. Louis, MO), were reconstituted by dissolving 5 g of the compound in 50 mL hexane. The concentrations used in the study (10%, 5%, and 2.5%) were obtained by serial dilution of the stock solutions in sterile distilled water. Similarly, the two-component synthetic blend was prepared by reconstituting 2.5 g squalene and 2.5 g linoleic acid in 50 mL of hexane, and the different concentrations (10%, 5%, 2.5%) acquired by serial dilution of the blend's stock solution in sterile distilled water. The AAAP, at a 0.02 mg/mL concentration (initially prepared in section 3.5) was

prepared through a serial dilution in sterile distilled water to obtain various concentrations (10%, 5% and 2.5%) for use in the bioassays.

3.14 Compatibility of tick attractants with *M. anisopliae* ICIPE 7

The method employed by Mfuti *et al*. (2016) was used to examine the antifungal property or fungitoxic effect of various concentrations (10, 5, 2.5 %) of the tick attractants and their effect on the viability of *M. anisopliae* ICIPE 7 conidia in a compatibility test when the fungus and attractants are not mixed but placed separately (Figure 11). Conidial suspensions of ICIPE 7 were prepared and titrated to 1×10^8 conidia/mL in 10 mL distilled water containing 0.05% Triton X-100. The spores were filtered and retained on a nitrocellulose filter membrane (diameter 47 mm, pore size 0.45 µm, Sigma Chemicals) by pouring 10 mL of the prepared suspension through a filter holder unit (MFS) under aspirator vacuum (Maniania, 1994). The filter membranes were dried in a laminar flow cabinet for 30 minutes. Five nitrocellulose membranes were placed in single glass desiccators (2.5 L) for exposure to the attractants.

Figure 11: Experimental set-up of the compatibility bioassays

Sterile cotton plugs prepared prior to the bioassays by autoclaving were impregnated with sterile distilled water amended with various concentrations of the attractants (up to 10 ml volumes) and placed in a desiccator to allow for diffusion of the volatile components. Fungus-treated nitrocellulose membranes were exposed to the attractants for 1, 2, 3, 6 and 8 days, respectively. One fungus-treated nitrocellulose filter membrane was removed from each desiccator after the appropriate exposure time and transferred into 10 mL sterile distilled water with 0.05% (w/v) Triton X-100 and vortexed for 5 minutes to dislodge the conidia. A 0.1 mL aliquot of the suspension was plated on SDA using a glass spreader. Plates were incubated at

25 ± 2 °C, 12: 12 L: D photoperiod and examined after 18 - 20 h for conidial germination assessment (as described in section 3.12 above) after the different exposure times. A similar procedure, but without any attractant was applied for the control treatment (consisting of only sterile distilled water). Each of the five nitrocellulose filter membranes was considered a treatment for fungal viability at each exposure time. The treatments for fungal viability were randomized, and the experiment was repeated four times.

3.15 Virulence of formulations consisting of *M. anisopliae* ICIPE 7 and tick attractants against *A. variegatum* and *R. appendiculatus* adults

For the bioassay, harvested conidia were titrated to 1×10^9 conidia/mL in 10 mL sterile distilled water containing 0.05% Triton X-100 and pure canola oil (9:1) in universal glass bottles containing sterile 3 mm glass beads. Various concentrations (2.5%, 5% and 10%) of either the crude flower (methanol) extract of *S. didymobotrya*, squalene, linoleic acid, squalene: linoleic acid (1:1) or AAAP were added to the conidial suspension and vortexed vigorously for 5 minutes for homogeneity of the suspension. A control, consisting of 0.05% Triton X-100 and pure canola oil (9:1) only was included in the study.

Evaluation of the effect of the selected attractants on the virulence of *M. anisopliae* ICIPE 7 was carried out as previously described by Frazzon *et al*. (2000). Twenty adult ticks were immersed in each treatment suspension for approximately 30 sec and then placed on a piece of paper towel to absorb the excess (Frazzon *et al*., 2000). The ticks were thereafter transferred into glass vials (1.5 × 12 cm) and kept in an incubator over sodium chloride at 25 ± 2°C and 75% RH. Ticks were monitored for 21 days and mortality was recorded daily. Three replicate trials were done for each test substance. Dead ticks were removed, surface-sterilised with 2.5% sodium hypochlorite and 70% alcohol, rinsed in double distilled water and then put in Petri dishes (90 mm diameter) lined with moist filter paper to assess for mycosis of the cadaver, which was observed under a microscope.

3.16 Data analysis

Chromatograms of the *rbcL* and *matK* region's forward and reverse sequences were put into Geneious Prime software version 2020.2.2 (developed by Biomatters, Auckland, New Zealand) and visually inspected, trimmed, modified, and aligned to generate their consensus sequences (Kearse *et al*., 2012). The consensus sequences for each sample were then compared to known sequences in the GenBank database using BLASTn (Altschul *et al*., 1990). Only sequences with greater than 98% similarity to reference sequences in GenBank were considered candidate plant species.

To conduct the phylogenetic analysis, *rbcL* sequences of other *Senna* species were obtained from the NCBI GenBank database and used as reference sequences. The study sequences were aligned using the MAFFT plugin in Geneious Prime software version 2022.2 (Kearse *et al*., 2012). The generated multiple alignment file was then exported into MEGA 7 (Kumar *et al*., 2016) for use in the phylogenetic analyses. Maximum-likelihood phylogenies were inferred using automatic model selection based on the Akaike Information Criterion with 1000 bootstrap replicates. Fig Tree v. 1.4.4 (Rambaut, 2014) was used to visualise the final phylogenetic tree. *Calpurnia aurea* sequences from *icipe* were incorporated into the trees as an outgroup.

Due to no considerable difference in behavioural response of male and female ticks to the test substances, data from both sexes was combined. Ticks that did not respond to either the treatment or control substances were left out of the analysis. For each test substance, the number of ticks that responded to the test substance and the control was pooled across replicates. The formula (2) was used to calculate the relative attraction index of ticks in the olfactometer and tick climbing bioassays (Nana *et al*., 2010):

$$RAI = (Nt - Nc)/(Nt + Nc) \times 100 \quad (2)$$

Where:

RAI is relative attraction index,

Nt is the total number of ticks in test, and

Nc is the total number of ticks in control.

The percentage of ticks attracted to the test substances was analysed using a Chi-Square goodness of fit test.

Heatmap clustering was employed to display differences in the chemical profiles across replicates of the flowers, twig branches, leaves and seedpods of *S. didymobotrya*. The chemical profiles of the various plant parts were compared using a one-way analysis of similarity (ANOSIM) test based on a dissimilarity matrix built using Bray-Curtis measure. In addition, a non-metric multidimensional scaling (NMDS) was conducted to create a visual representation of the variations in the chemical profiles of the extracts. Similarity percentage breakdown (SIMPER) analysis was used to identify compounds that contributed to the dissimilarity between plant parts based on their peak areas. The statistical analyses were conducted using two software programs, R version 1.4.1717 (R Core Team, 2024) and PAST version 4.06 (Hammer *et al*., 2001).

Proportional data on conidial viability was analysed using the binomial logistic regression model of the Generalised Linear Model (GLM) to evaluate the effect of treatments on germination in each data collection day, and the effect of time/days on germination in each treatment. The means of the various treatments were separated using Sidak's multiple comparisons test. Daily percentage mortality of ticks was subjected to probit regression using the ecotox package (Hlina, 2023). The analysis produced the estimates for lethal time-response mortality to 50% (LT_{50}) of the exposed tick population. Differences in LT were determined by comparing the LT_{50} estimates. The packages, survival (Therneau & Lumley, 2023) and survminer (Kassambara *et al*., 2023) were used to analyse tick survival data. Tick survival was modelled with Cox mixed-effect regression model in the coxme package (Therneau, 2023). Experimental replicate was utilised as a random factor in this model. Means were separated using the lsmeans package (Lenth, 2023). To generate survival curves, the Kaplan-Meier estimator was employed.

CHAPTER FOUR

RESULTS

4.1 Molecular identification and phylogenetic analysis of plant samples

Both *rbcL* and *matK* sequences of sample representatives from various locations in Homabay, Kirinyaga, Kajiado, Migori and Nairobi counties were successfully amplified and sequenced. Based on the BLASTn homology search, the 13 *rbcL* query sequences were unanimously identified as *Senna didymobotrya* with 99-100 % identity with reference sequences from the GenBank nr database (see Appendix C: Figure C 1). The study's plant *rbcL* sequences were all deposited in GenBank (gene accessions OP524161-OP524173).

On the contrary, BLAST homology search of the 12 *matK* query sequences identified the plant samples as either *Senna didymobotrya* and/or *Senna italica*, both with 99 and/or 100% identity from the algorithm (see Appendix C: Figure C 2). Due to these inconsistencies, the *matK* query sequences from this study were not submitted to GenBank but have been included in Appendix C (Table C 1).

Employing the Maximum Likelihood tree building method, the 13 *rbcL* accessions generated from this study formed a monophyletic clade that also corresponded to a reference *S. didymobotrya* sequence (Figure 12). Contrary to this, phylogenetic analysis using the *matK* gene grouped the study sequences into two clades, and grouped them with *S. italica*, *S. didymobotrya*, and *S. longiracemosa* reference sequences (Figure 13).

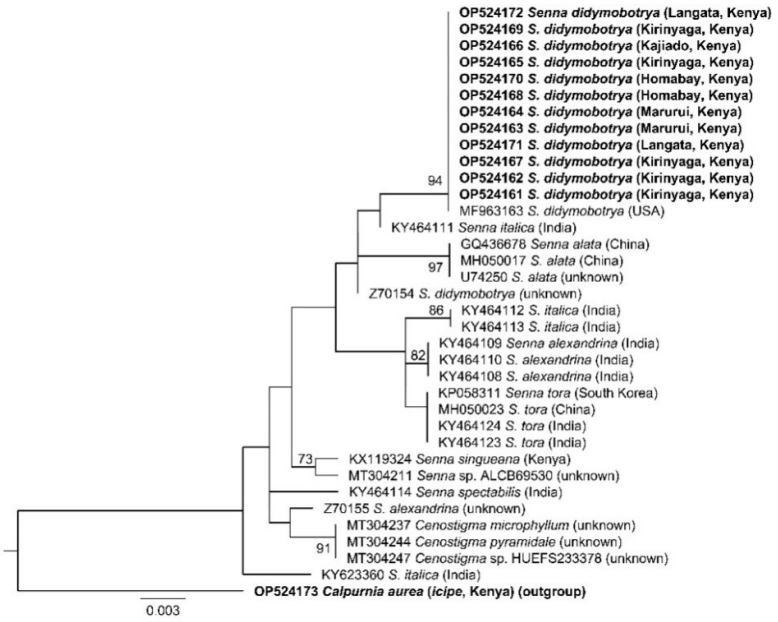


Figure 12: Maximum likelihood phylogenetic tree of representative *rbcL* gene sequences from plant samples collected from different locations in Kenya

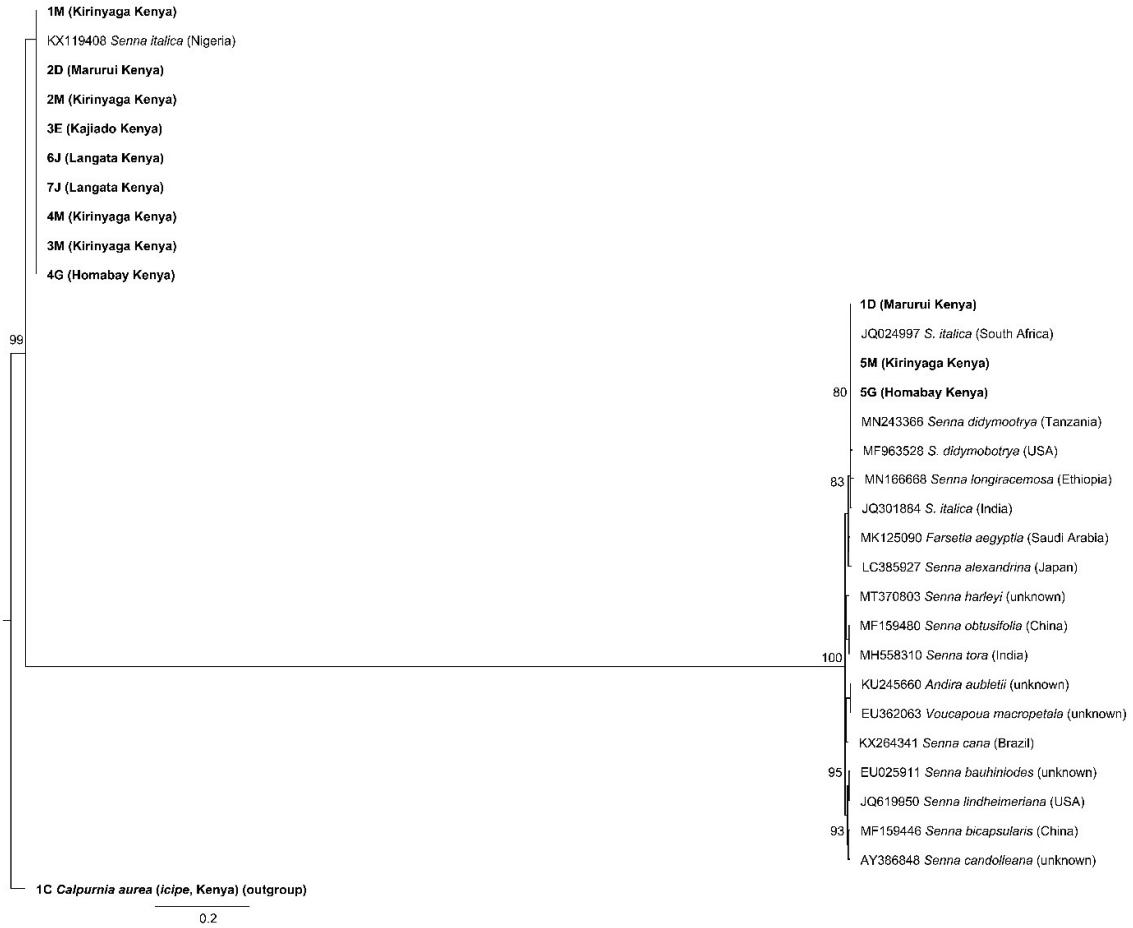


Figure 13: Maximum likelihood phylogenetic tree of representative *matK* gene sequences from plant samples collected from different locations in Kenya

4.2 Extraction yield of *S. didymobotrya*'s plant parts

Methanol extracted the highest and the lowest masses from the flowers and twig branches, respectively (Figure 14). The percentage yield ranged from 6.7 to 22.9%.

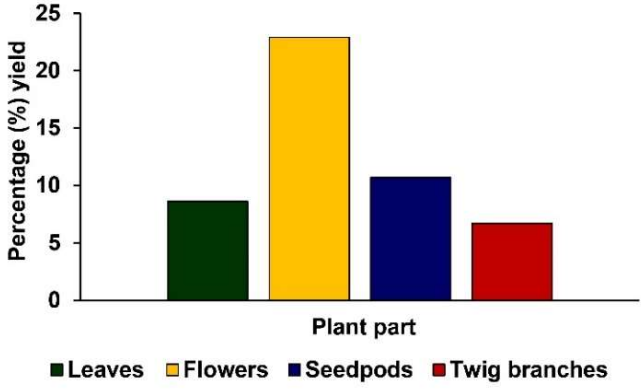


Figure 14: Percentage yield (w/v) of the leaves, flowers, seedpods and twig branches of *S. didymobotrya* extracted using methanol

4.3 Response of *A. variegatum* and *R. appendiculatus* to crude methanol extracts of *S. didymobotrya*

In both species, the behavioural response of adult male and female ticks was not significantly different (p-value > 0.05) (Table 2 and Table 3). Additionally, these adult ticks responded differently to the plant parts and concentrations tested (Figure 15 and Table 4). The flower extract significantly attracted *A. variegatum* adults over the control at 100 mg/mL ($\chi 2$ = 33.15, df = 1, P< 0.001), 50 mg/mL ($\chi 2$ = 6.93, df = 1, P< 0.01), and 25 mg/mL ($\chi 2$ = 5.54, df = 1, P = 0.02) (Figure 15A). The twig branches crude extract also attracted *A. variegatum* adult ticks at 100 mg/mL - $\chi 2$ = 5.10, df = 1, P = 0.02 (Figure 15A). Furthermore, at the highest concentration tested (100 mg/mL), *A. variegatum* were attracted to the different plant parts over the control - the twig branches ($\chi 2$ = 5.10, df = 1, P = 0.02), and the seedpods ($\chi 2$ = 4.00, df = 1, P = 0.05)(Figure 15A). Contrastingly, *A. variegatum* adults were significantly repelled by the leaf at 1 mg/mL ($\chi 2$ = 13.80, df = 1, P< 0.001) and the flower at 1 mg/mL ($\chi 2$ = 7.11, df = 1, P< 0.01) (Figure 15A). The relative attraction index of *A. variegatum* to the flower extract was greater at 100 mg/mL (57.58%) than in the AAAP treatment (51.52) (Table 4).

Similarly, the flower extract attracted *R. appendiculatus* at all tested concentrations, although not significantly, except at 1 mg/mL, where they were repelled (Figure 15B). Adults of *R. appendiculatus* were significantly attracted to the control over the seedpods at 25 mg/mL ($\chi 2$ = 11.85, df = 1, P< 0.001) and the twig branches at 1 mg/mL ($\chi 2$ = 8.01, df = 1, P < 0.01) (Figure 15B). At 100 mg/mL, twig branches extract significantly attracted *R. appendiculatus* ($\chi 2$ = 5.98, df = 1, P = 0.01) (Figure 15B).

Table 2: Chi-squared- ($\chi 2$) and *p*-values of adult male and female *A. variegatum* ticks that were attracted to the crude methanol extracts of *S. didymobotrya*'s plant parts

Plant part	Concentration (mg/mL)	Male (%)	Female (%)	χ^2-value	Degrees of freedom	*p*-value
	100	45	55	1.00	1	0.32
	50	47	53	0.35	1	0.56
	25	47	53	0.44	1	0.51
Leaf	1	41	59	3.31	1	0.07
	100	50	50	0.00	1	1.00
	50	48	52	0.17	1	0.68
Flower	25	55	45	0.91	1	0.34
	1	45	55	0.83	1	0.36
	100	57	43	2.04	1	0.15
	50	50	50	0.00	1	1.00
Seedpods	25	48	52	0.23	1	0.63
	1	53	47	0.35	1	0.56
	100	42	58	2.49	1	0.11
Twig	50	47	53	0.31	1	0.58
branches	25	54	46	0.51	1	0.48
	1	50	50	0.00	1	1.00
AAAP	0.02	54	46	0.64	1	0.42

Male (%) and female (%) stand for the proportion of adult male and female ticks, respectively, that were attracted to the various crude extracts of *S. didymobotrya* treatments.

Table 3: Chi-squared- ($\chi 2$) and *p*-values of adult male and female *R. appendiculatus* ticks that were attracted to the crude methanol extracts of *S. didymobotrya*'s plant parts

Plant part	Concentration (mg/mL)	Male (%)	Female (%)	χ^2-value	Degrees of freedom	*p*-value
	100	52	48	0.16	1	0.69
	50	47	53	0.39	1	0.53
Leaf	25	50	50	0.00	1	1.00
	1	48	52	0.12	1	0.73
	100	56	44	1.23	1	0.27
	50	49	51	0.08	1	0.78
Flower	25	57	43	1.78	1	0.18
	1	48	52	0.19	1	0.66
	100	52	48	0.19	1	0.66
	50	47	53	0.39	1	0.53
Seedpods	25	50	50	0.00	1	1.00
	1	58	42	2.78	1	0.10
	100	58	42	2.37	1	0.12
Twig	50	46	54	0.59	1	0.44
branches	25	52	48	0.14	1	0.71
	1	53	47	0.28	1	0.60
AAAP	0.02	48	52	0.17	1	0.68

Male (%) and female (%) stand for the proportion of adult male and female ticks, respectively, that were attracted to the various crude extracts of *S. didymobotrya* treatments.

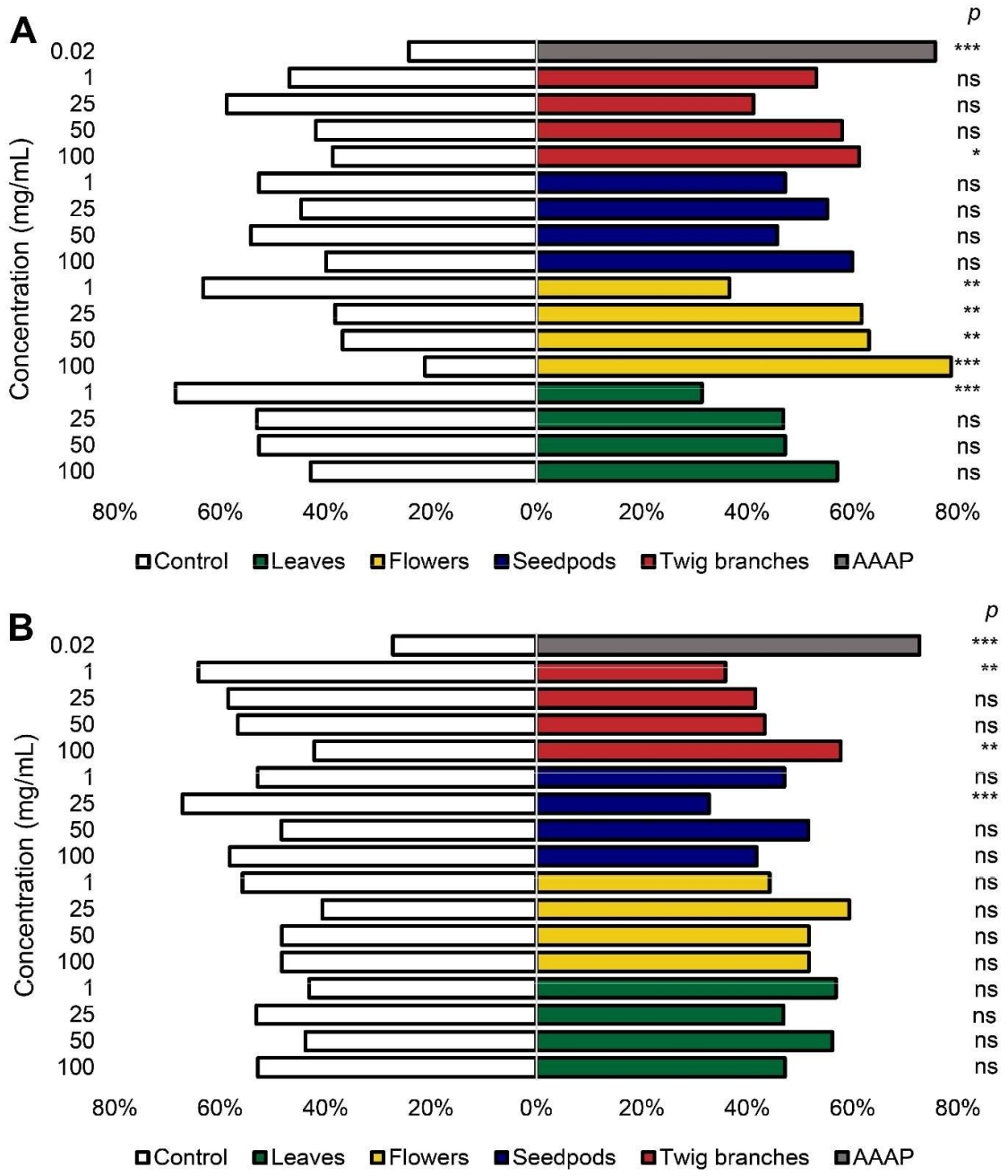


Figure 15: Responses (%) of adult *A. variegatum* (A) and *R. appendiculatus* (B) to methanol extracts of *S. didymobotrya* and AAAP. Statistical significance: ns ($P > 0.05$); * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$) from $\chi 2$ test at $\alpha = 0.05$.

Table 4: Relative attraction of adult *A. variegatum* and *R. appendiculatus* to methanol extracts of *S. didymobotrya* and AAAP

Plant part	Concentration (mg/mL)	Relative percentage (%) attraction index	
		A. variegatum	***R. appendiculatus***
Leaf	100	14.29	-5.66
	50	-5.56	12.28
	25	-6.25	-6.25
	1	-37.14***	13.73
Flower	100	57.58***	3.33
	50	26.32**	3.33
	25	23.53**	18.75
	1	-26.67**	-11.54
Seedpods	100	20.00	-16.36
	50	-8.57	3.23
	25	10.53	-34.43***
	1	-5.56	-5.88
Twig branches	100	22.58*	24.44*
	50	16.13	-13.33
	25	-17.65	-16.92
	1	6.25	-28.30**
AAAP	0.02	51.52***	45.45***

*, **, *** stands for significant differences at $P< 0.05$; $P< 0.01$; and $P< 0.001$, respectively, from χ^2 test at $\alpha = 0.05$. Negative attraction index depicts repellence of the ticks by the test substance, whereas positive attraction index depicts attraction of the ticks to the test substance.

4.4 Fractionation yield of the crude flower (methanol) extract by separating funnel

The hexane, ethyl acetate and water residual fractions weighed 3.9 g, 1.2 g, and 6.7 g, respectively.

4.5 Response of *A. variegatum* and *R. appendiculatus* adults to fractions from solvent–solvent fractionation in a separating funnel

There was no significant difference in the response to the treatments by adult male and female ticks of both species (p-value > 0.05) (Table 5 and Table 6). Adult ticks of both tick species behaved differently when exposed to the hexane, ethyl acetate and water residual

fractions of the crude methanol flower extract against the control (Figure 16 and Table 7). More *A. variegatum* adults were attracted to the hexane fraction at all concentrations when tested against the solvent (control) (Figure 16A). However, attraction to the hexane fraction was only statistically significant at 50 mg/mL ($\chi 2 = 15.52$, df = 1, $P < 0.001$) (Figure 16A). These ticks exhibited a significant preference for the solvent (control) over water residual fraction at 1 mg/mL ($\chi 2 = 4.94$, df = 1, $P = 0.03$) (Figure 16A).

Similarly, *R. appendiculatus* adults were also significantly attracted by the hexane fraction at 25 mg/mL ($\chi 2 = 4.34$, df = 1, $P = 0.04$) (Figure 16B). Notably, the ethyl acetate fraction at 1 mg/mL ($\chi 2 = 17.90$, df = 1, $P< 0.001$) significantly attracted *R. appendiculatus* adults (Figure 16B), and had an almost comparable relative attraction index to the standard AAAP (Table 7). Nonetheless, the ethyl acetate fraction was the most repellent to *R. appendiculatus* adults, at 50 mg/mL ($\chi 2 = 8.65$, df = 1, $P = 0.003$) (Figure 16B).

Table 5: Chi-squared- ($\chi 2$) and *p*-values of adult male and female *A. variegatum* ticks that were attracted to various fractions obtained from solvent-solvent partitioning of *S. didymobotrya*'s flower extract

Fraction	Concentration (mg/mL)	Male (%)	Female (%)	χ^2-value	Degrees of freedom	*p*-value
	50	46	54	0.76	1	0.38
Hexane	25	58	42	2.49	1	0.11
	1	50	50	0.00	1	1.00
Ethyl	50	53	48	0.25	1	0.62
acetate	25	50	50	0.00	1	1.00
	1	43	57	1.78	1	0.18
Water	50	53	47	0.35	1	0.56
residual	25	55	45	1.00	1	0.32
	1	54	46	0.51	1	0.48
AAAP	0.02	54	46	0.64	1	0.42

Male (%) and female (%) stand for the proportion of adult male and female ticks, respectively, that were attracted to the various fractions obtained from solvent-solvent partitioning of *S. didymobotrya*'s flower (methanol) extract.

Table 6: Chi-squared- ($\chi 2$) and *p*-values of adult male and female *R. appendiculatus* ticks that were attracted to various fractions obtained from solvent-solvent partitioning of *S. didymobotrya*'s flower extract

Fraction	Concentration (mg/mL)	Male (%)	Female (%)	χ^2-value	Degree of freedom	*p*-value
	50	50	50	0.00	1	1.00
Hexane	25	52	48	0.12	1	0.73
	1	48	52	0.09	1	0.76
Ethyl	50	44	56	1.23	1	0.27
acetate	25	58	42	2.78	1	0.10
	1	49	51	0.07	1	0.79
Water	50	52	48	0.14	1	0.71
residual	25	46	54	0.69	1	0.40
	1	52	48	0.16	1	0.69
AAAP	0.02	48	52	0.17	1	0.68

Male (%) and female (%) stand for the proportion of adult male and female ticks, respectively, that were attracted to the various fractions obtained from solvent-solvent partitioning of *S. didymobotrya*'s flower (methanol) extract

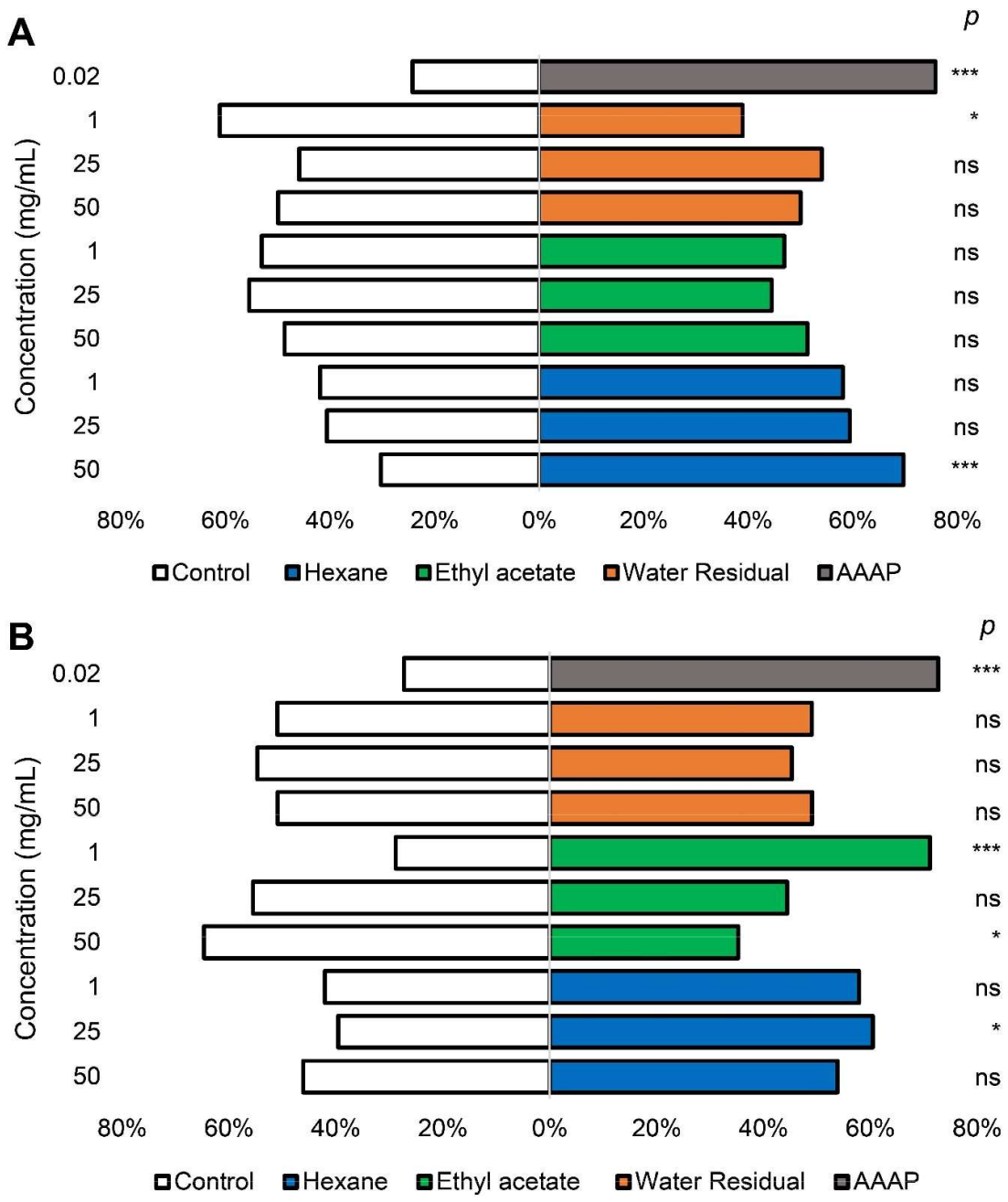


Figure 16: Responses (%) of *A. variegatum* (A) and *R. appendiculatus* (B) to fractions of crude methanol extracts from *S. didymobotrya* flowers and AAAP. Statistical significance: ns ($P > 0.05$); * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$) from $\chi 2$ test at $\alpha = 0.05$

Table 7: Relative attraction of *A. variegatum* and *R. appendiculatus* to fractions of *S. didymobotrya*'s crude methanol flower extracts and AAAP

Fraction	Concentration (mg/mL)	Relative percentage (%) attraction index	
		A. variegatum	***R. appendiculatus***
Hexane	50	39.39***	7.69
	25	18.75	20.83*
	1	16.13	15.79
Ethyl acetate	50	2.56	-29.41**
	25	-11.11	-11.11
	1	-6.25	42.31***
Water residual	50	0	-1.82
	25	8.11	-9.43
	1	-22.22*	-1.96
AAAP	0.02	51.52***	45.45***

*, **, *** stand for significant differences at $P< 0.05$; $P< 0.01$; and $P< 0.001$, respectively, from $\chi 2$ test at $\alpha = 0.05$. Negative attraction index depicts repellence of the ticks by the test substance, whereas positive attraction index depicts attraction of the ticks to the test substance.

4.6 Response of *A. variegatum* and *R. appendiculatus* adults to column chromatography fractions

There was no significant attraction of either the male or female ticks of either species to all the fractions at the tested concentrations (p-value > 0.05) (Table 8 and Table 9). No significant attraction to either the fractions or the solvent (control) for all the six resulting fractions tested at 1 mg/ml was observed in both *A. variegatum* and *R. appendiculatus* adult ticks (Figure 17 and Table 10). Attraction, or lack thereof of the ticks to either the treatment or solvent control was more pronounced in *R. appendiculatus* in comparison to *A. variegatum* (Figure 17).

Table 8: Chi-squared- ($\chi 2$) and *p*-values of adult male and female *A. variegatum* ticks that were attracted to various fractions obtained by column chromatography of the active hexane fraction

Fraction	Concentration (mg/mL)	Male (%)	Female (%)	χ^2-value	Degree of freedom	*p*-value
Hexane fraction 1	1	44.83	55.17	1.07	1	0.30
Hexane fraction 2	1	51.61	48.39	0.10	1	0.75
Hexane fraction 3	1	52.63	47.37	0.28	1	0.60
DCM fraction 1	1	50.00	50.00	0.00	1	1.00
DCM fraction 2	1	43.75	56.25	1.56	1	0.21
MeOH fraction	1	55.26	44.74	1.11	1	0.29

Male (%) and female (%) stand for the proportion of adult male and female ticks, respectively, that were attracted to various fractions obtained column chromatography of the active hexane fraction

Table 9: Chi-squared- ($\chi 2$) and *p*-values of adult male and female *R. appendiculatus* ticks that were attracted to various fractions obtained by column chromatography of the active hexane fraction

Fraction	Concentration (mg/mL)	Male (%)	Female (%)	χ^2-value	Degrees of freedom	*p*-value
Hexane fraction 1	1	52	48	0.19	1	0.66
Hexane fraction 2	1	46	54	0.59	1	0.44
Hexane fraction 3	1	50	50	0.00	1	1.00
DCM fraction 1	1	42	58	2.37	1	0.12
DCM fraction 2	1	53	47	0.35	1	0.56
MeOH fraction	1	54	46	0.51	1	0.48

Male (%) and female (%) stand for the proportion of adult male and female ticks, respectively, that were attracted to various fractions obtained column chromatography of the active hexane fraction

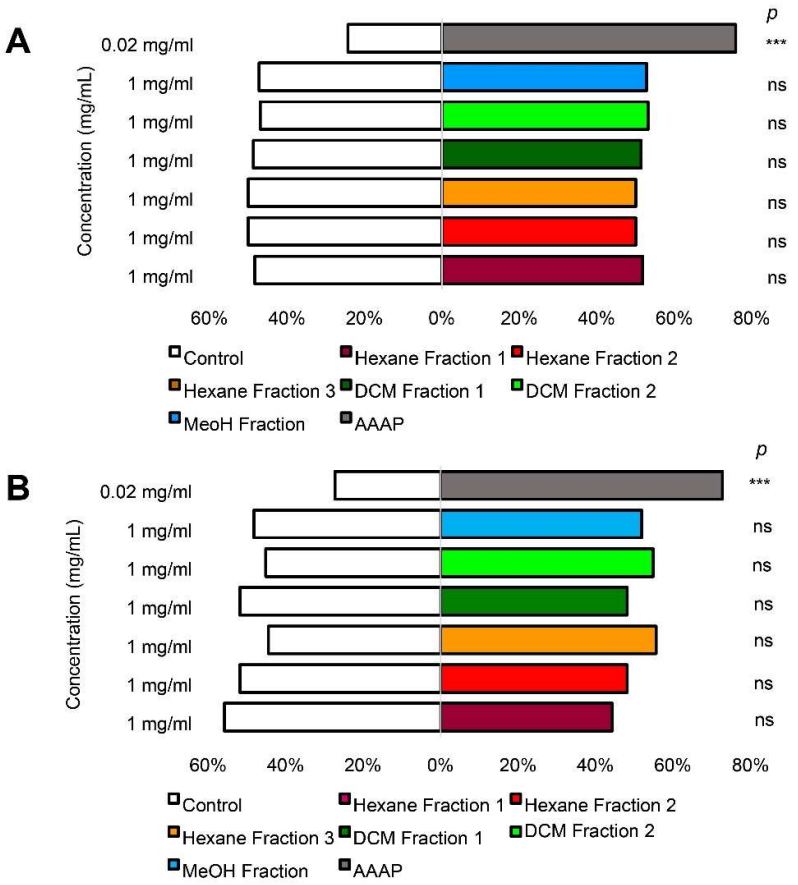


Figure 17*:* Responses (%) of *A. variegatum* (A) and *R. appendiculatus* (B) to column chromatography fractions of the active hexane fraction and AAAP. Statistical significance: ns ($P > 0.05$); * ($P< 0.05$); ** ($P< 0.01$); *** ($P< 0.001$) from $\chi 2$ test at $\alpha = 0.05$

Table 10: Relative attraction of *A. variegatum* and *R. appendiculatus* to column chromatography fractions of the active hexane fraction and AAAP

Fraction	Concentration (mg/mL)	Relative (%) Attraction Index	
		A. variegatum	***R. appendiculatus***
Hexane Fraction 1	1	3.45	-11.54
Hexane Fraction 2	1	0.00	-3.70
Hexane Fraction 3	1	0.00	11.11
DCM Fraction 1	1	2.70	-3.70
DCM Fraction 2	1	6.25	9.68
MeOH Fraction	1	5.56	3.70
AAAP	0.02	51.52***	45.45***

*, **, *** stand for significant differences at $P< 0.05$; $P< 0.01$; and $P< 0.001$, respectively, from $\chi 2$ test at $\alpha = 0.05$. Negative attraction index depicts repellence of the ticks by the test substance, whereas positive attraction index depicts attraction of the ticks to the test substance.

4.7 Chemical profile of extracts of *S. didymobotrya* plant parts

Differences in both the quantity and quality of the chemical constituents found in the extracts of *S. didymobotrya* were detected. The chemical analysis revealed a total of 34, 40, 36 and 32 compounds in the extracts obtained from the flower, leaf, seedpod, and twig branches of *S. didymobotrya* (Figure 18 and Appendix D). Twenty-two (22) and nineteen (19) compounds were identified in the hexane and ethyl acetate fractions, respectively (Appendix D).

Overall, there were qualitative and quantitative variations in the chemical composition of the different plant parts; however, these differences were not statistically significant according to the one-way ANOSIM test based on Bray-Curtis dissimilarity with 9999 permutations ($R = 0.6178$, $p = 0.0014$)) (Figure 19A). The four plant parts were successfully categorised into four distinct groups based on their chemical profiles (Figure 19A).

In the NMDS analysis, the Shepard plot (Figure 19B) demonstrated a stress value below 0.2, indicating a high level of ordination and excellent representation of the data. The SIMPER analysis combining all plant parts revealed a dissimilarity of 65.48%. The compounds that significantly contributed to this dissimilarity were (3β, 5β)-stigmast-7-en-3-ol (11.15%), phytol (9.63%), hexadecanoic acid (7.32%), stigmasterol (4.42%), and methyl lineolate (4.32%) (Figure 19C). Overall, the least average dissimilarity with the flower was seen between it and

the twig branch extracts (62.8%), followed by the seedpod (64.86%) and lastly the leaf (69.04%) extracts. The compounds, phytol (17.7%), (3*β*, 5*β*)-stigmast-7-en-3-ol (11.77%) and hexadecanoic acid (8.85%) majorly accounted for the dissimilarity between the flower and leaf extracts.

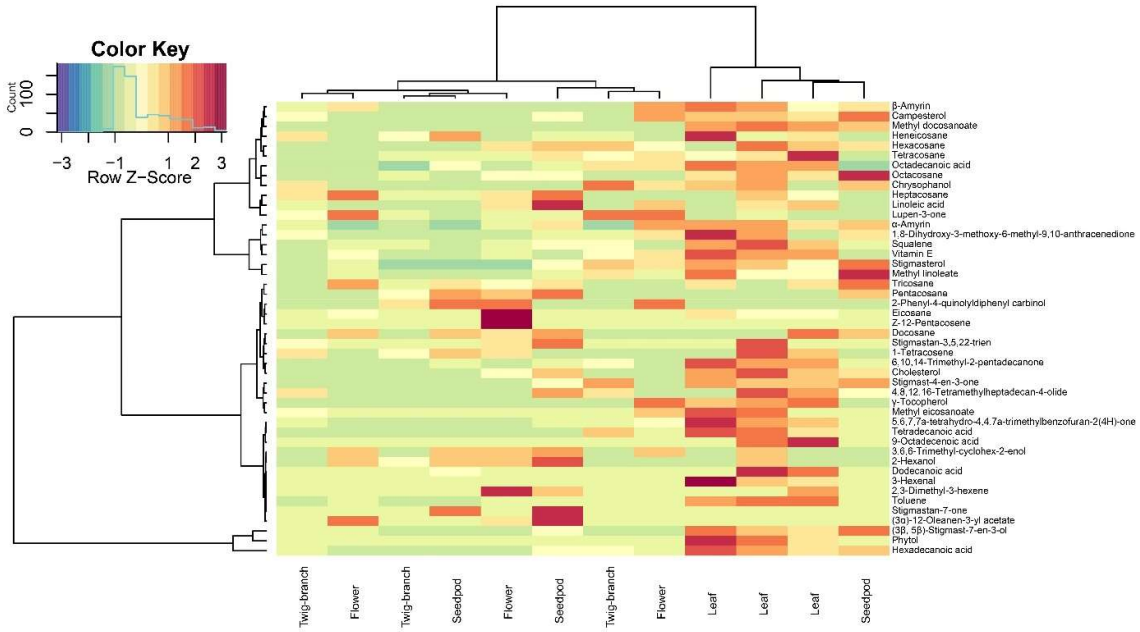


Figure 18: Heatmap clustering showing differences in the chemical composition of *S. didymobotrya* leaf, flower, seedpod, and twig branch extracts. Compound abundance is represented by colour intensity across replicates of each plant part

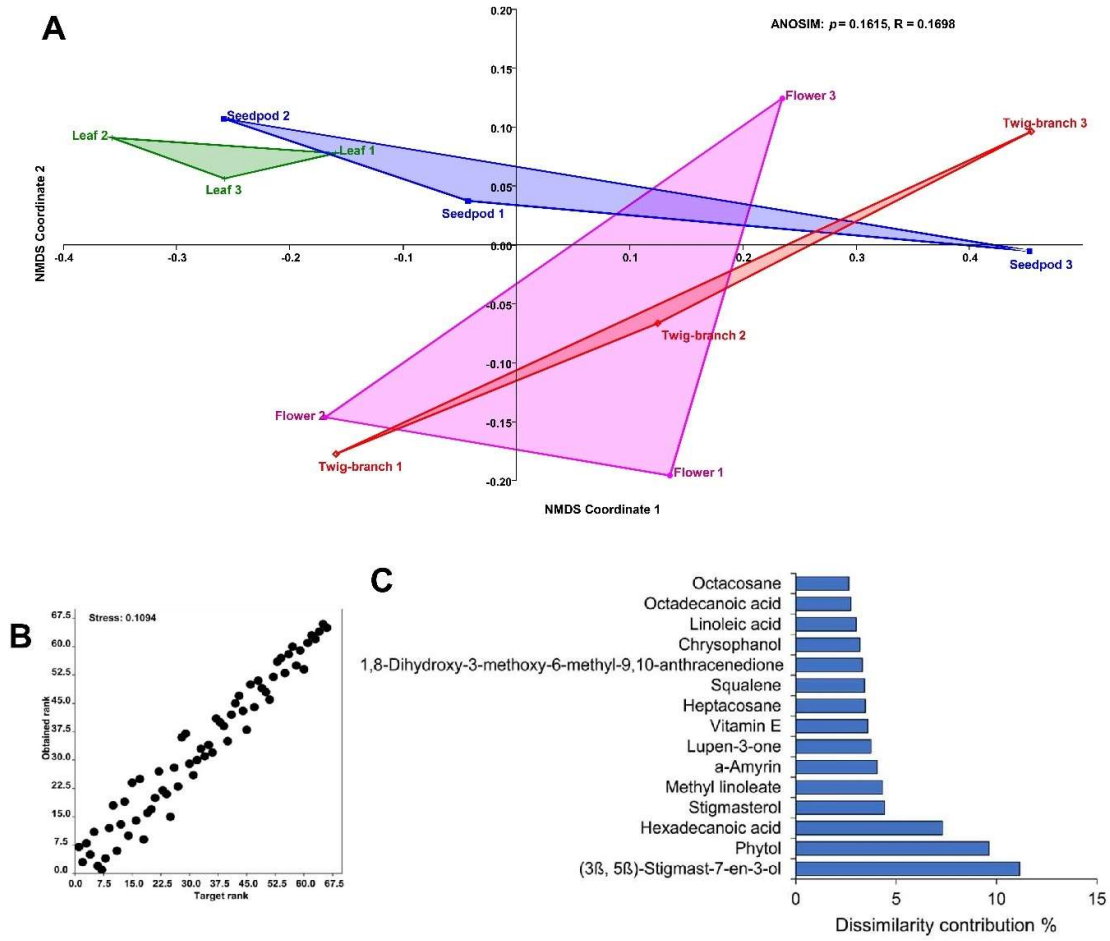


Figure 19: Non-metric multidimensional scaling (NMDS) (A), shepard plot (B) and histogram from similarity percentages (SIMPER) (C) analysis of *S. didymobotrya*'s plant parts.

4.8 Responses of *A. variegatum* and *R. appendiculatus* to selected synthetic compounds

No significant differences were observed between male and female adult ticks of both species in their response to the compounds and synthetic blend (p-value > 0.05) (Table 11 and Table 12). Adult ticks of the two species exhibited different responses to the concentrations of compounds tested but similar responses to the tested two-component synthetic blend (Figure 20 and Table 13). Squalene ($\chi 2 = 22.15$, df = 1, $P < 0.001$), linoleic acid ($\chi 2 = 4.50$, df = 1, $P = 0.03$), and the squalene: linoleic acid (1:1) blend ($\chi 2 = 13.57$, df = 1, $P < 0.01$) at 1 mg/mL significantly attracted *A. variegatum* adult ticks (Figure 20A). However, 10 mg/mL of the squalene: linoleic acid (1:1) blend ($\chi 2 = 8.65$, df = 1, $P < 0.01$) significantly repelled *A. variegatum* adults (Figure 20A).

Adults of *R. appendiculatus* were significantly attracted by the squalene: linoleic acid (1:1) blend ($\chi 2 = 23.85$, df = 1, $P < 0.001$) at 1 mg/mL but significantly repelled by the squalene:

linoleic acid (1:1) blend ($\chi 2 = 6.72$, df = 1, $P < 0.01$) at 10 mg/mL (Figure 20B). The relative attraction index of *R. appendiculatus* adults to the squalene: linoleic acid (1:1) blend (48.84) was greater than their relative attraction to the positive control used, AAAP (45.45) (Figure 20B and Table 13).

Table 11: Responses (%) of (A) *A. variegatum* and (B) *R. appendiculatus* to squalene, linoleic acid, a 1:1 blend of squalene and linoleic acid, and AAAP. Significance levels: ns ($P > 0.05$); * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$) from $\chi 2$ test at $\alpha = 0.05$

Test	Concentration (mg/mL)	Male (%)	Female (%)	χ^2-value	Degrees of freedom	*p*-value
Squalene	0.1	44	56	1.23	1	0.27
Linoleic acid	0.1	52	48	0.21	1	0.65
Squalene	1	44	56	1.44	1	0.23
Linoleic acid	1	53	48	0.25	1	0.62
Squalene	10	50	50	0.00	1	1.00
Linoleic acid	10	56	44	1.56	1	0.21
Squalene:	0.1	56	44	1.49	1	0.22
linoleic acid	1	54	46	0.59	1	0.44
	10	42	58	2.78	1	0.10
AAAP	0.02	54	46	0.64	1	0.42

Male (%) and female (%) stand for the proportion of adult male and female ticks, respectively, that were attracted to squalene, linoleic acid and a squalene: linoleic acid blend (1: 1)

Table 12: Chi-squared- ($\chi 2$) and *p*-values of adult male and female *R. appendiculatus* ticks that were attracted to squalene, linoleic acid and squalene: linoleic blend (1: 1)

Test	Concentration (mg/mL)	Male (%)	Female (%)	χ^2-value	Degrees of freedom	*p*-value
Squalene	0.1	52	48	0.10	1	0.75
Linoleic acid	0.1	46	54	0.51	1	0.48
Squalene	1	52	48	0.10	1	0.75
Linoleic acid	1	48	52	0.14	1	0.71
Squalene	10	48	52	0.19	1	0.66
Linoleic acid	10	56	44	1.56	1	0.21
Squalene:	0.1	45	55	1.00	1	0.32
linoleic acid	1	47	53	0.39	1	0.53
	10	55	45	1.00	1	0.32
AAAP	0.02	48	52	0.17	1	0.68

Male (%) and female (%) stand for the proportion of adult male and female ticks, respectively, that were attracted to squalene, linoleic acid and a squalene: linoleic acid blend (1: 1)

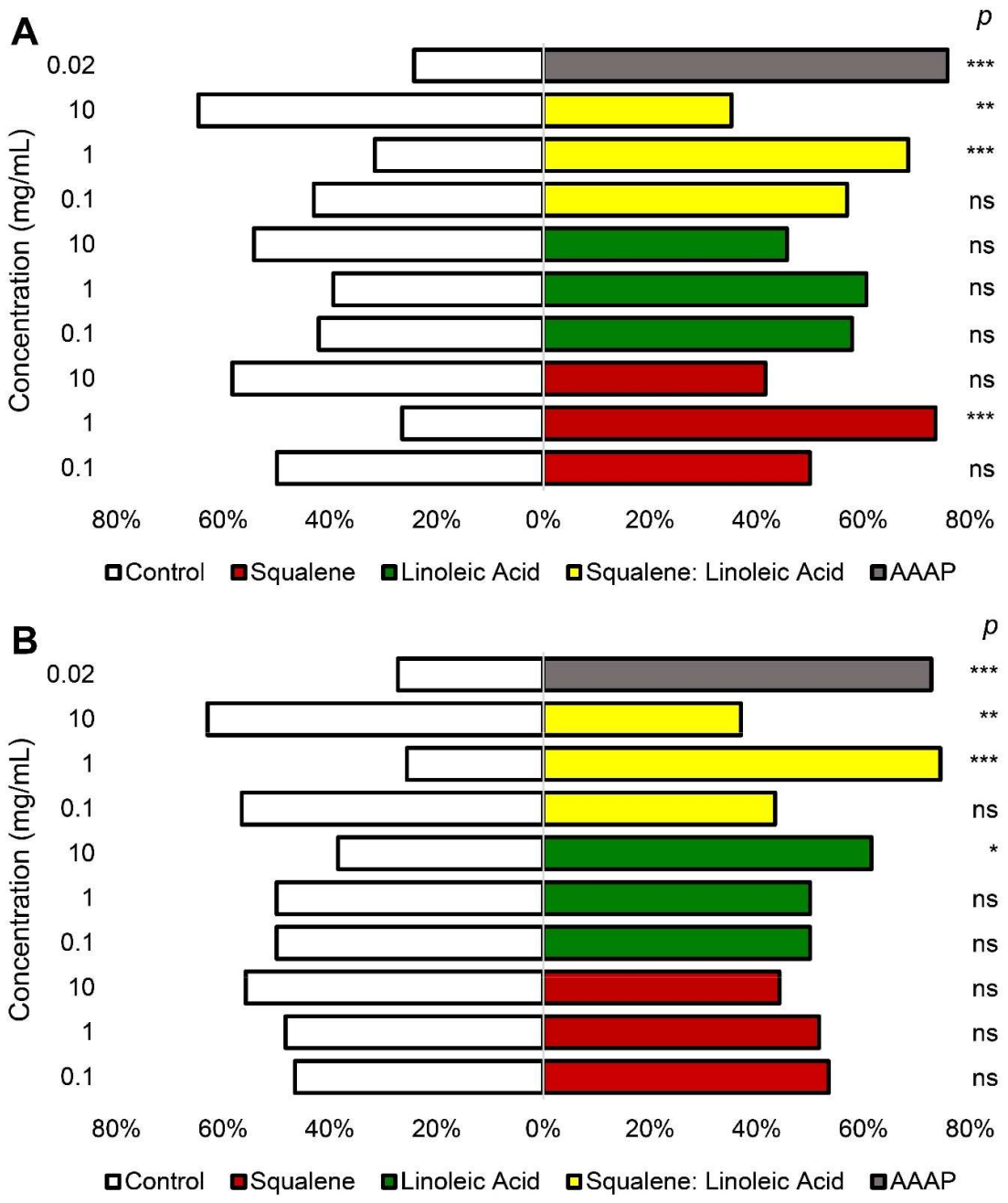


Figure 20: Responses (%) of (A) *A. variegatum* and (B) *R. appendiculatus* to squalene, linoleic acid, a 1:1 blend of squalene and linoleic acid, and AAAP. Significance levels: ns ($P > 0.05$); * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$) from $\chi 2$ test at $\alpha = 0.05$

Table 13: Relative attraction of adult *A. variegatum* and *R. appendiculatus* to squalene, linoleic acid, squalene: linoleic acid blend (1:1), and AAAP

Compound/Blend	Concentration (mg/mL)	Relative percentage (%) attraction index	
		A. variegatum	***R. appendiculatus***
Squalene	0.1	0	6.90
Linoleic acid	0.1	15.79	0.00
Squalene	1	47.06***	3.33
Linoleic acid	1	21.21*	0.00
Squalene	10	-16.67	-11.54
Linoleic acid	10	-8.57	23.08*
Squalene: linoleic acid (1:1)	0.1	13.89	-13.04
	1	36.84***	48.84***
	10	-29.41**	-25.93**
AAAP	0.02	51.52***	45.45***

*, **, *** stand for significant differences at $P< 0.05$; $P< 0.01$; and $P< 0.001$, respectively, from $\chi 2$ test at $\alpha = 0.05$. Negative attraction index depicts repellence of the ticks by the test substance, whereas positive attraction index depicts attraction of the ticks to the test substance.

4.9 Effect of tick attractants on the viability of *M. anisopliae* ICIPE 7 conidia

In general, the effects of tick attractants on germination of ICIPE 7's conidia varied significantly between the treatments ($\chi 2 = 5232.60$, df = 15, $p < 0.0001$) and time of exposure (days) ($\chi 2 = 3442.30$, df = 4, $p< 0.0001$). The interaction treatment: days was also statistically significant ($\chi 2 = 410.50$, df = 60, $p< 0.0001$). Exposure time had significant effects on the viability of *M. anisopliae* ICIPE 7's conidia on day 1 ($\chi 2 = 579.28$, df = 15, $p< 0.0001$), day 2 ($\chi 2 = 1324.8$, df = 15, $p< 0.0001$), day 3 ($\chi 2 = 1384.8$, df = 15, $p < 0.0001$), day 6 ($\chi 2 = 1403.00$, df = 15, $p< 0.0001$), and day 8 ($\chi 2 = 951.33$, df = 15, $p< 0.0001$). The highest conidial germination rate on day 8 was observed in the treatments: 10% flower extract + ICIPE 7 (85.21 ± 1.35%), control consisting of ICIPE 7 only (83.73 ± 1.52%), 2.5% squalene + ICIPE 7 (82.54 ± 1.17%), 5% flower extract + ICIPE 7 (82.42 ± 1.94%) and 5% squalene + ICIPE 7 (80.98 ± 1.45%) (Table 14). At 8 days, conidial viability of ICIPE 7 was lowest in fungus exposed to 5% AAAP + ICIPE 7 (53.27 ± 1.57%), 10% AAAP + ICIPE 7 (56.13 ± 1.19%), 2.5% AAAP

+ ICIPE 7 (56.73 ± 1.64%), 10% linoleic acid + ICIPE 7 (68.17 ± 2.21%) and 5% squalene: linoleic acid blend + ICIPE 7 (72.31 ± 1.78%) (Table 14).

Generally, the order of compatibility of the attractants with *M. anisopliae* ICIPE 7 over the five days of data collection was as follows: crude methanol flower extract of *S. didymobotrya*> squalene > squalene: linoleic acid (1: 1) blend > linoleic acid > AAAP (Table 14). The flower extracts and squalene were highly compatible as shown by the germination of conidia exposed to these treatments, whose means were similar to those of the control (consisting of ICIPE 7 fungus only) (Table 14). Squalene: linoleic acid blend and linoleic acid had a slight negative effect on conidial germination (Table 14). The AAAP pheromone negatively affected conidial viability as shown by the low proportion of conidia that germinated over the five data collection days (Table 14).

With regards to the concentrations of each attractant, the following were highly compatible with the fungus as shown by the high conidial germination rate over the data collection days (Table 14): 10% crude methanol flower extract of *S. didymobotrya*, 2.5% squalene, 2.5% linoleic acid, 2.5%squalene: linoleic acid (1: 1) blend, and 2.5% AAAP (Table 14).

Table 14: Effect of different tick attractants and their various concentrations on the viability of conidia of *Metarhizium anisopliae* ICIPE 7 over time.

Treatment	Days after exposure					ANOVA	Treatment composition
	1	2	3	6	8		
	% Germination						
Cntrl	$98.92 \pm 0.13a^{A}$	97.58 ± 0.24^{abA}	95.71 ± 0.48^{bA}	89.58 ± 0.68^{cA}	83.73 ± 1.52^{dAB}	$\chi^2 = 305.31$, $p<0.0001$	ICIPE 7 only
A1	98.19 ± 0.16^{aABC}	95.90 ± 0.37^{bAB}	92.92 ± 0.57^{cAB}	88.35 ± 1.18^{dA}	85.21 ± 1.35^{dA}	$\chi^2 = 212.86$, $p<0.0001$	10% Flower extract + ICIPE 7
A2	98.15 ± 0.19^{aABC}	95.35 ± 0.44^{bAB}	93.94 ± 0.47^{bAB}	88.54 ± 0.81^{cA}	82.42 ± 1.94^{dABC}	$\chi^2 = 243.89$, $p<0.0001$	5% Flower extract + ICIPE 7
A3	98.52 ± 0.12^{aAB}	95.25 ± 0.45^{bAB}	93.60 ± 0.49^{bcAB}	90.81 ± 0.51^{cA}	79.73 ± 1.98^{dABCD}	$\chi^2 = 316.11$, $p<0.0001$	2.5% Flower extract + ICIPE 7
B1	$95.98 + 0.32^{aCD}$	92.75 ± 0.48^{bBC}	91.38 ± 0.64^{bcB}	89.46 ± 0.79^{cA}	79.71 ± 1.68^{dABCD}	$\chi^2 = 187.64$, $p<0.0001$	10% Squalene + ICIPE 7
B2	96.65 ± 0.30^{aBCD}	93.54 ± 0.46^{bB}	90.08 ± 0.71^{cBC}	89.46 ± 0.69^{cA}	80.98 ± 1.45^{dABC}	$\chi^2 = 186.12$, $p<0.0001$	5% Squalene + ICIPE 7
B3	96.88 ± 0.25^{aABCD}	94.85 ± 0.48^{aB}	91.17 ± 0.84^{bB}	90.29 ± 0.65^{bA}	82.54 ± 1.17^{cABC}	$\chi^2 = 181.98$, $p<0.0001$	2.5% Squalene + ICIPE 7
C1	92.00 ± 0.83^{aEF}	84.92 ± 1.19^{bDE}	83.46 ± 1.43^{bDE}	78.83 ± 1.59^{cBC}	68.17 ± 2.21^{dG}	$\chi^2 = 250.86$, $p<0.0001$	10% Linoleic acid + ICIPE 7

C2	90.75 ± 1.37^{aFG}	84.35 ± 1.42^{bDE}	83.02 ± 1.64^{bcDE}	79.69 ± 1.23^{cBC}	74.58 ± 1.60^{dDEF}	χ^2 = 121.20, p< 0.0001	5% Linoleic acid + ICIPE 7
C3	94.92 ± 0.67^{aDE}	85.71 ± 0.96^{bDE}	80.42 ± 1.17^{cE}	76.58 ± 1.51^{cdC}	73.56 ± 1.31^{dEFG}	χ^2 = 270.29, p< 0.0001	2.5% Linoleic acid + ICIPE 7
D1	90.88 ± 0.97^{aFG}	81.04 ± 1.22^{bE}	78.50 ± 1.13^{bcE}	78.38 ± 1.93^{bcC}	75.65 ± 1.51^{cCDE}	χ^2 = 121.53, p< 0.0001	10% Blend + ICIPE 7
D2	91.98 ± 1.03^{aEF}	85.23 ± 1.29^{bDE}	80.67 ± 1.11^{cE}	77.33 ± 0.95^{cC}	72.31 ± 1.78^{dFG}	χ^2 = 191.91, p< 0.0001	5% Blend + ICIPE 7
D3	92.21 ± 1.30^{aEF}	89.08 ± 1.17^{abCD}	86.04 ± 1.08^{bcCD}	83.15 ± 1.31^{cdB}	78.92 ± 1.26^{dBCDE}	χ^2 = 105.58, p< 0.0001	2.5% Blend + ICIPE 7
E1	87.23 ± 1.51^{aGH}	73.13 ± 1.56^{bF}	68.13 ± 1.66^{bF}	59.19 ± 1.32^{cD}	56.13 ± 1.19^{cH}	χ^2 = 368.85, p< 0.0001	10% AAAP + ICIPE 7
E2	85.25 ± 1.71^{aH}	68.17 ± 1.11^{bF}	60.77 ± 1.45^{cG}	56.83 ± 1.57^{cdD}	53.27 ± 1.57^{dH}	χ^2 = 366.95, p< 0.0001	5% AAAP + ICIPE 7
E3	89.96 ± 1.50^{aFG}	73.92 ± 1.84^{bF}	69.15 ± 1.89^{bF}	61.92 ± 1.49^{cD}	56.73 ± 1.64^{cH}	χ^2 = 421.67, p< 0.0001	2.5% AAAP + ICIPE 7
ANOVA	χ^2 = 579.28, p< 0.0001	χ^2 =1324.80, p< 0.0001	χ^2 = 1384.80, p< 0.0001	χ^2 = 1403.00, p< 0.0001	χ^2 = 951.33, p< 0.0001		

Means (± SE (standard error)) followed by the same capital letters within a column are not statistically different based on Sidak's multiple comparisons test.

Means (± SE (standard error)) followed by the same small letters within a row are not statistically different based on Sidak's multiple comparisons test.

4.10 Time response mortality of *A. variegatum* and *R. appendiculatus* to *M. anisopliae* ICIPE 7 amended with tick attractants

Percentage mortalities of *A. variegatum* and *R. appendiculatus* adult ticks 21 days post-exposure is presented in (Figure 21). Mortality ranged from 77 – 100% and 73 – 98% in *A. variegatum* and *R. appendiculatus* ticks, respectively.

Twenty-one days after exposure of ticks to ICIPE 7, the highest mortality rates in A. *variegatum* were observed in ticks that received the following treatments: 10% squalene: linoleic acid blend, and ICIPE 7 (100%), 10% AAAP and ICIPE 7 (98%), 2.5 % squalene: linoleic acid blend and ICIPE 7 (97%), 10% linoleic acid and ICIPE 7 (97%), and 2.5% squalene and ICIPE 7 (97%) (Figure 21A). The treatments – 5% flower methanol extract and ICIPE 7 (77%), 10% squalene and ICIPE 7 (77%), 5% AAAP and ICIPE 7 (77%), and 5% linoleic acid and ICIPE 7 (78) – resulted in the lowest mortality rates (>70%) of adult *A. variegatum* ticks (Figure 21A).

In *R. appendiculatus* ticks, the highest mortality rates were induced by 10% AAAP and ICIPE 7 (98%), 10% squalene: linoleic acid and ICIPE 7 (98%), 2.5% linoleic acid and ICIPE 7 (98%), ICIPE 7 only (control) (98%), and 10% flower methanol extract and ICIPE 7 (97%) treatments after 21 days post-exposure (Figure 21B). The treatments, 2.5% squalene and ICIPE 7 (73%), 10% squalene and ICIPE 7 (87%), 2.5% squalene and ICIPE 7 (92%), 10% linoleic acid and ICIPE 7 (92%), 5% squalene: linoleic acid blend and ICIPE 7 (92%), and 5% AAAP and ICIPE 7 (93%) slightly reduced the mortality rates of *R. appendiculatus* adults exposed to *M. anisopliae* ICIPE 7 in comparison to other treatments (Figure 21B).

The treatments that elicited slightly low mortality rates in both *A. variegatum* and *R. appendiculatus* adults were 10% squalene with ICIPE 7 and 5% AAAP with ICIPE 7 (Figure 21). The treatments - 10% squalene: linoleic acid blend with ICIPE 7 and 10% AAAP with ICIPE 7, contributed to high mortality rates in both species of ticks (Figure 21).

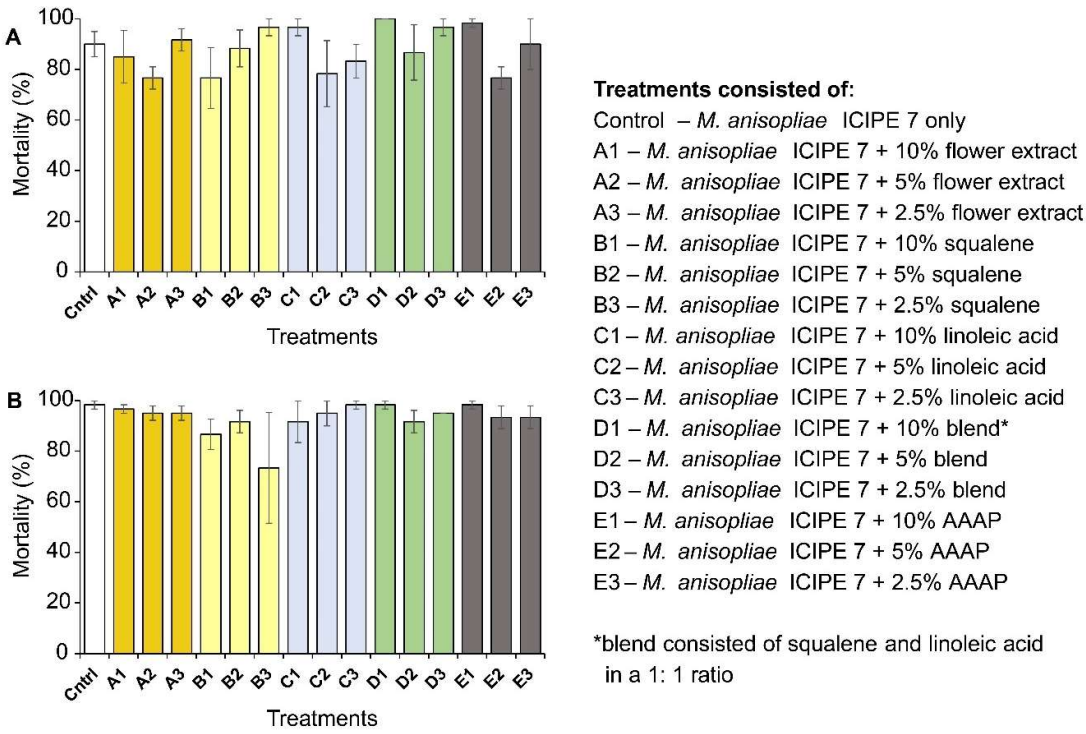


Figure 21: Mortality (%) of *A. variegatum* and *R. appendiculatus* adult ticks exposed to *M. anisopliae* ICIPE 7 amended with various concentrations of tick attractants twenty-one days post-exposure

Cumulative mortalities of the two tick species monitored for 21 days after their exposure to *M. anisopliae* ICIPE 7 are also presented - Figure 22 for *A. variegatum* and Figure 23 for *R. appendiculatus* adult ticks. Generally, mortality rates were low (<10%) in all treatments in the early days post-exposure in both *A. variegatum* (days 1 – 5) (Figure 22) and *R. appendiculatus* (days 1 – 4) (Figure 23). In *A. variegatum*, the treatments consisting of the highest concentration (10%) of the attractants, linoleic acid, squalene: linoleic acid and AAAP was high between days 6 and 13 (Figure 22). Mortality rates of *R. appendiculatus* exposed to the treatments consisting of *M. anisopliae* ICIPE 7 and 2.5% of the flower extract, squalene: linoleic acid blend (1: 1), and AAAP quite comparable to mortalities observed in the control treatments (ICIPE 7 only) throughout the 21 days post-exposure (Figure 23).

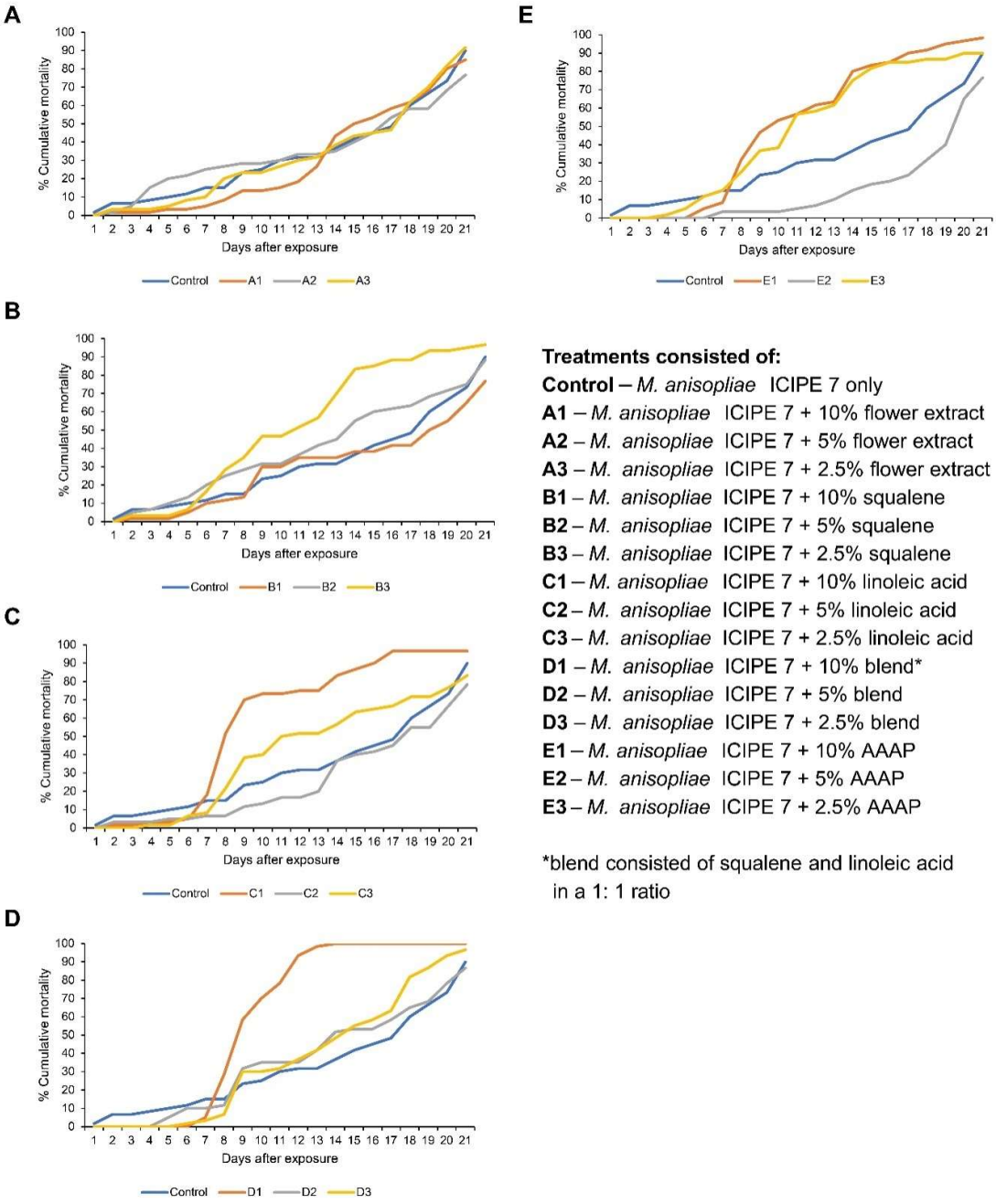


Figure 22: Cumulative mortality of *A. variegatum* adult ticks exposed to *M. anisopliae* ICIPE 7 amended with various concentrations of tick attractants twenty-one days post-exposure

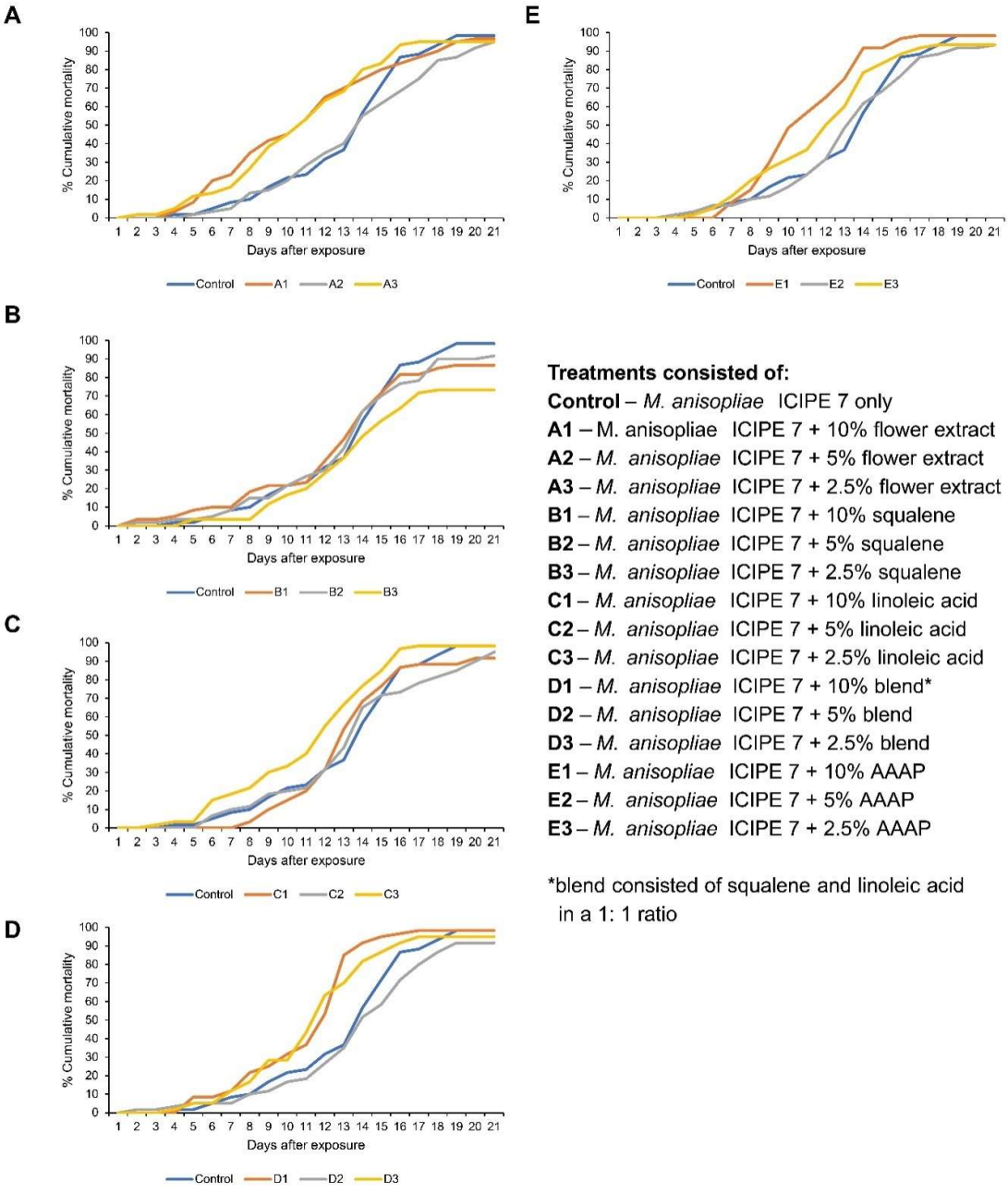


Figure 23: Cumulative mortality of *R. appendiculatus* adult ticks exposed to *M. anisopliae* ICIPE 7 amended with various concentrations of tick attractants twenty-one days post-exposure

The lethal time-response mortality to 50% (LT_{50}) and the corresponding regression slopes of the ticks exposed to *M. anisopliae* ICIPE 7 and attractants after twenty-one days post-exposure are presented in (Table 15). Generally, the lethal time-response to mortality of 50% of the two tick species were different, with the LT_{50} estimates for all treatments, except in 10% squalene: linoleic acid blend + ICIPE 7 and 2.5% AAAP + ICIPE 7, being shorter for *R. appendiculatus* adults than *A. variegatum* adults.

In bioassays with *A. variegatum*, the shortest LT_{50} estimates were obtained in the treatments consisting of 10% linoleic acid and ICIPE 7 (8.8 days), 10% squalene: linoleic acid blend and ICIPE 7 (9.05 days), 2.5% squalene and ICIPE 7 (9.51 days), 10% AAAP and ICIPE 7 (10.4 days), and 2.5% AAAP and ICIPE 7 (10.8 days) (Table 15). However, the longest LT_{50} estimates for *A. variegatum* ticks were recorded in the treatments: 5% AAAP and ICIPE 7 (19.9 days), 5% linoleic acid and ICIPE 7 (18 days), 10% squalene and ICIPE 7 (16.6 days), 5% flower methanol extract and ICIPE 7 (15.9%), and control comprising ICIPE 7 only (15.8 days) (Table 15).

Adult *R. appendiculatus* exposed to the 2.5% flower methanol extract and ICIPE 7, 10% flower methanol extract and ICIPE 7, 10% squalene: linoleic acid blend, 2.5% linoleic acid and ICIPE 7, and 10% AAAP and ICIPE 7 treatments had the shortest LT_{50} estimates (9.77, 9.88, 10.3, 10.3 and 10.4 days, respectively) (Table 15). However, the longest LT_{50} estimates for *R. appendiculatus* were observed in the ticks that received the 2.5% squalene and ICIPE 7 treatment (14.6 days), 5% squalene: linoleic acid blend (13.2 days), 5% flower methanol extract and ICIPE 7 (13.1 days), 10% linoleic acid and ICIPE 7 (13 days), 5% linoleic acid and ICIPE 7 (12.8 days), 5% AAAP and ICIPE 7 (12.7 days) (Table 15).

Short LT_{50} estimates were observed in the 10% squalene: linoleic acid blend with ICIPE 7 and 10% AAAP with ICIPE 7 treatments in both *A. variegatum* and *R. appendiculatus* ticks (Table 15). Both tick species also experienced the longest LT_{50} estimates in the 5% AAAP with ICIPE 7, 5% linoleic acid with ICIPE 7 and 5% flower methanol extract with ICIPE 7 treatments (Table 15).

Table 15: Median lethal time (LT_{50}) of *A. variegatum* and *R. appendiculatus* twenty-one days after exposure to *M. anisopliae* ICIPE 7 amended with various concentrations of tick attractants

	A. variegatum		***R. appendiculatus***		
Treatment	**LT_{50}**	**Slope + SE**	**LT_{50}**	**Slope + SE**	**Composition of treatment**
Control	15.8	2.51 ± 0.00832	12.4	7.18 ± 0.0172	ICIPE 7 only
A1	15.5	4.52 ± 0.0134	9.88	4.89 ± 0.0113	10% Flower extract + ICIPE 7
A2	15.9	2.18 ± 0.00749	13.1	6.32 ± 0.0158	5% Flower extract + ICIPE 7
A3	15.1	3.37 ± 0.0103	9.77	5.05 ± 0.0116	2.5% Flower extract + ICIPE 7
B1	16.6	2.94 ± 0.00969	12.1	4.27 ± 0.0110	10% Squalene + ICIPE 7
B2	12.5	2.74 ± 0.00804	12.5	5.27 ± 0.0132	5% Squalene + ICIPE 7
B3	9.51	4.56 ± 0.0106	14.6	5.41 ± 0.0150	2.5% Squalene + ICIPE 7
C1	8.8	5.38 ± 0.0122	13	8.66 ± 0.0211	10% Linoleic acid + ICIPE 7
C2	18	3.33 ± 0.0113	12.8	6.16 ± 0.0153	5% Linoleic acid + ICIPE 7
C3	12.6	4.13 ± 0.0109	10.3	6.14 ±0.0141	2.5% Linoleic acid + ICIPE 7
D1	9.05	12.9 ± 0.0390	10.3	7.37 ± 0.0171	10% Blend + ICIPE 7
D2	13.9	4.04 ± 0.0113	13.2	5.38 ± 0.0138	5% Blend + ICIPE 7
D3	13.3	6.05 ± 0.0154	10.8	6.59 ± 0.0152	2.5% Blend + ICIPE 7
E1	10.4	6.22 ± 0.0143	10.4	8.72 ± 0.0209	10% AAAP + ICIPE 7
E2	19.9	6.11 ± 0.0232	12.7	6.39 ± 0.0157	5% AAAP + ICIPE 7
E3	10.8	5.01 ± 0.0119	11.3	6.49 ± 0.0152	2.5% AAAP + ICIPE 7

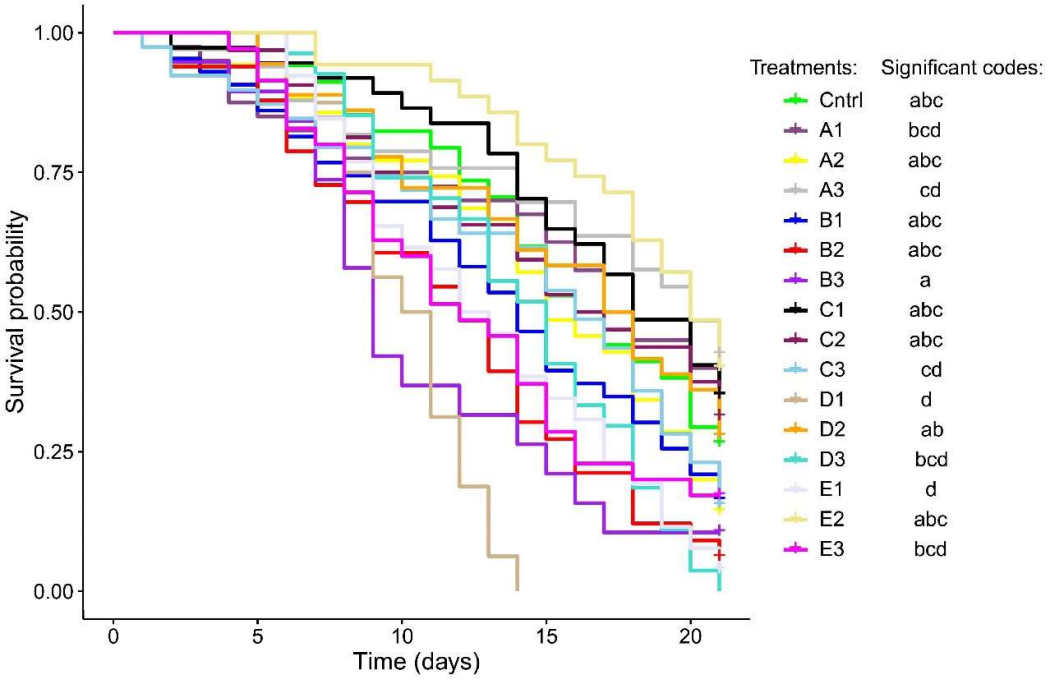
LT_{50} denotes lethal time response to mortality of 50% of the tick population

SE stands for standard error of the regression slope

4.11 Survival of *A. variegatum* and *R. appendiculatus* after exposure to *M. anisopliae* ICIPE 7 amended with tick attractants

The 21-day postexposure survival rates of *A. variegatum* and *R. appendiculatus* are summarised using the Kaplan-Meier survival curves (Figure 24 and Figure 25, respectively). Survival between the two tick species following exposure to the various attractants and their concentrations was significantly different ($\chi 2 = 87.00$, df = 1, $p < 0.0001$) in all bioassays. In addition, the survival of both *A. variegatum* and *R. appendiculatus* ticks was significantly affected by treatments (various concentrations of different tick attractants) ($\chi 2 = 81.77$, df = 15, $p < 0.0001$ and $\chi 2 = 97.04$, df = 15, $p < 0.0001$, respectively). There were significant interactions between experimental replicates and treatments in assays with *A. variegatum* ($\chi 2 = 131.57$, df = 30, $p < 0.0001$) and *R. appendiculatus* ($\chi 2 = 147.09$, df = 30, $p < 0.0001$).

The lowest survival probabilities were observed in *A. variegatum* ticks that received the following treatments: ICIPE 7 amended with 10% squalene: linoleic acid blend (1: 1), ICIPE 7 with 2.5% squalene: linoleic acid blend and ICIPE 7 amended with10% AAAP (Figure 24). However, the highest survival probability was in *A. variegatum* that were exposed to 2.5% flower extract in ICIPE 7, 2.5% AAAP with ICIPE 7 and ICIPE 7 containing 10% linoleic acid (Figure 24). In bioassays with *R. appendiculatus*, survival rates were lowest in ticks treated with 10% flower extract with ICIPE 7, 2.5% squalene: linoleic acid blend with ICIPE 7 and ICIPE 7 amended with 10% squalene: linoleic acid (Figure 25). Conversely, survival rates were highest in *R. appendiculatus* ticks that were exposed to: 5% squalene and ICIPE 7, 10% AAAP with ICIPE 7, and 2.5% squalene in ICIPE 7 (Figure 25).



Treatments consisted of:

Control – *M. anisopliae* ICIPE 7 only
A1 – *M. anisopliae* ICIPE 7 + 10% flower extract
A2 – *M. anisopliae* ICIPE 7 + 5% flower extract
A3 – *M. anisopliae* ICIPE 7 + 2.5% flower extract
B1 – *M. anisopliae* ICIPE 7 + 10% squalene
B2 – *M. anisopliae* ICIPE 7 + 5% squalene
B3 – *M. anisopliae* ICIPE 7 + 2.5% squalene
C1 – *M. anisopliae* ICIPE 7 + 10% linoleic acid
C2 – *M. anisopliae* ICIPE 7 + 5% linoleic acid
C3 – *M. anisopliae* ICIPE 7 + 2.5% linoleic acid
D1 – *M. anisopliae* ICIPE 7 + 10% blend*
D2 – *M. anisopliae* ICIPE 7 + 5% blend
D3 – *M. anisopliae* ICIPE 7 + 2.5% blend
E1 – *M. anisopliae* ICIPE 7 + 10% AAAP
E2 – *M. anisopliae* ICIPE 7 + 5% AAAP
E3 – *M. anisopliae* ICIPE 7 + 2.5% AAAP

*blend consisted of squalene and linoleic acid in a 1: 1 ratio

Figure 24: Kaplan-Meier survival curves of *A. variegatum* exposed to *M. anisopliae* ICIPE 7 amended with various concentrations of tick attractants.

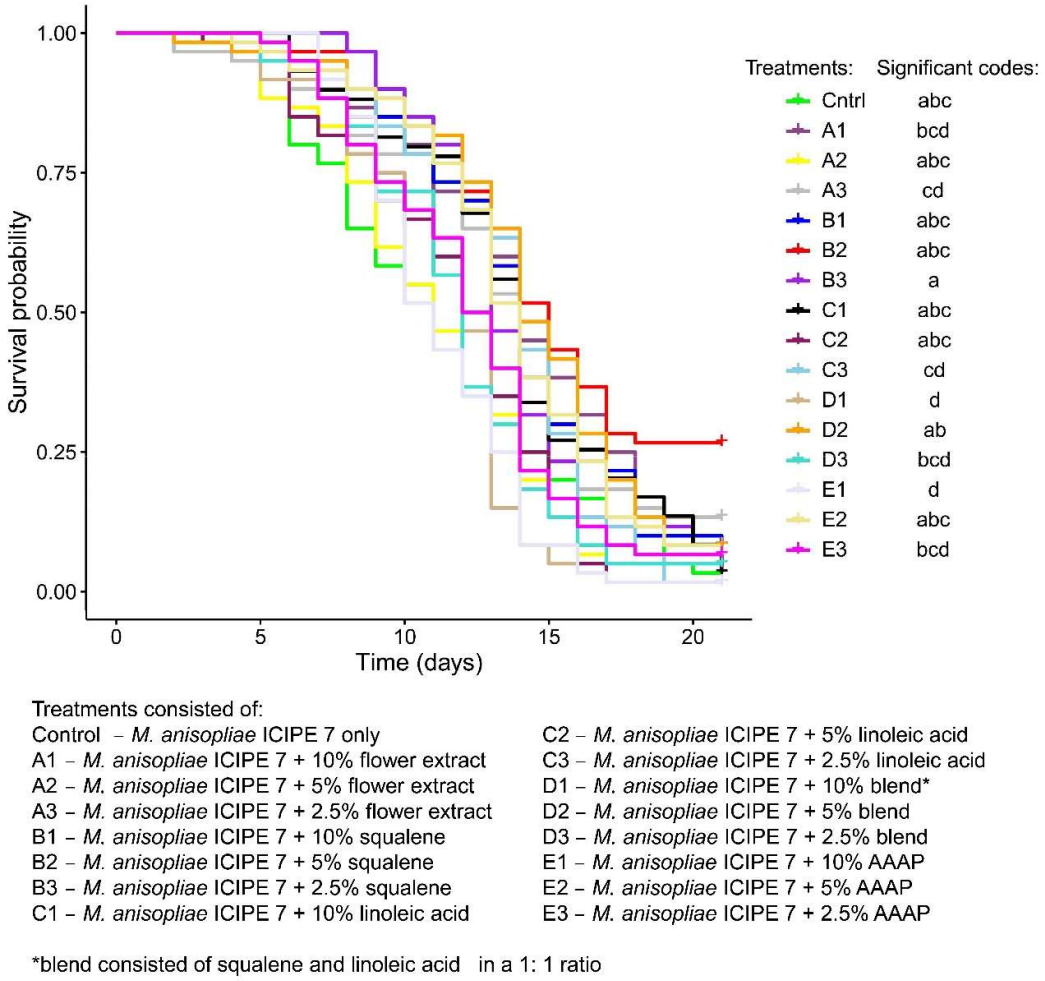


Figure 25: Kaplan-Meier survival curves of *R. appendiculatus* exposed to *M. anisopliae* ICIPE 7 amended with various concentrations of tick attractants.

CHAPTER FIVE
DISCUSSION

The current study identified plant samples collected from Homabay, Kajiado, Kirinyaga, Migori and Nairobi counties of Kenya as *Senna didymobotrya* using molecular barcodes and illustrated their evolutionary relatedness. Olfactory-based bioassay revealed that the crude methanol extract of the plant's flower, the hexane and ethyl acetate fractions of the flower's extract, and a two component-blend of squalene and linoleic acid (1: 1) attracted ticks. The study also demonstrated that these identified attractants modified the viability and virulence of *M. anisopliae* ICIPE 7.

5.1 Molecular identification of *S. didymobotrya*

An initial objective of this study was to complement the morphological identification of the plant samples with molecular identification using DNA barcodes. The *rbcL* and *matK* coding regions in plant samples from various locations in this study were successfully amplified and sequenced. These findings are in accord with the stipulated characteristics of plant DNA barcodes – universality (the primer's ease of amplification and sequencing) (Cowan & Fay, 2012; Hollingsworth *et al*., 2011). Another important criterion for the success of a primer for use as a barcode in plant identification is its discriminatory power (Hollingsworth *et al*., 2011). In this study, plant identification using the *rbcL* primer was highly effective as it resolved, distinguished and identified the samples unanimously as *Senna didymobotrya*. These results support the CBOL recommendation for its application as a universal plant DNA barcode (CBOL Plant Working Group, 2009). However, the *matK* primer had low discriminating power as demonstrated by its inefficiency to differentiate between *Senna italica* and *S. didymobotrya*. These findings are somewhat surprising given the fact that previously, *matK* has been deemed to possess more discriminatory power than *rbcL* (CBOL Plant Working Group, 2009; Hollingsworth *et al*., 2011).

The evolutionary relationship of the *S. didymobotrya* samples obtained from different locations in Kenya was determined using the Maximum Likelihood method. Unlike the *matK* locus which led to the formation of two clades, the *rbcL* locus grouped the study's sequence into a monophyletic clade. As such, it can be inferred that the *rbcL* locus is an excellent candidate for ascertaining evolutionary relatedness from this taxon (Sikdar *et al*., 2018). According to Piasecka *et al*. (2015) specific plant secondary metabolites (PSMs) are often found in particular lineages, such as a specific genus or family, suggesting a phylogenetic occurrence. Moreover, members within a monophyletic clade tend to have similar PSMs, and plants within a taxonomic group generally have similar metabolite content and bioactive

properties (Liu *et al*., 2017; Zhang *et al*., 2021). This informed the pooling of plant samples from the various locations in the study for behavioural response assays.

5.2 Behavioural response of ticks to test substances

Ticks, unlike other blood-feeding arthropods like mosquitoes that depend on plants for obtaining sugars, do not have a clear ecological rationale for being attracted to plants or their extracts, as ticks do not prey upon or consume plants (Nana *et al*., 2010). Nevertheless, there is speculation that plant odours might imitate tick semiochemicals or serve as favourable environments for ticks to cluster (Carr & Roe, 2016) or as ambushing ticks await the passage of potential hosts (Nicholson *et al*., 2018). To advocate for the inclusion of plants and their extracts in tick management programs, it is worth noting that they pose minimal risks to non-target organisms and the environment. They are also biodegradable and contain a wide range of chemical compounds that work together to influence both the behaviour and physiological processes of ticks, reducing the likelihood of target arthropods developing resistance to these plants or their extracts (George *et al*., 2014).

The findings of the behavioural assays conducted in this study demonstrate that both *A. variegatum* and *R. appendiculatus* adult ticks, irrespective of their sex, exhibit varying responses to the methanol extracts derived from different plant parts (flowers, leaves, seedpods, and twig branches), as well as their respective concentrations. The variation in response can be attributed to the ticks' capability to detect subtle distinctions in the composition of the extracts, as they belong to various genera and species. This ability enables the ticks to avoid the formation of aggregations involving different tick species or genera in regions where they coexist (Sonenshine, 2004, 2008). This notion is further reinforced by the observation that despite both tick species feeding on the same hosts, namely cattle, they exhibit distinct preferences for feeding sites on the host's body. Specifically, *A. variegatum* tends to favour areas such as the udder, abdomen, and dewlap, while *R. appendiculatus* shows a preference for feeding on the inner ears (Horak *et al*., 2018). Corroborating this, Mulenga (2013) suggested that the ability of ticks to discern various physical and chemical cues ensures that tick species, which feed on the same host, select distinct feeding sites. Wanzala *et al*. (2004) further support the idea of ticks' sensitivity to different chemical profiles by showing that specific odours from hosts, which can attract or repel ticks, direct the sympatric *R. appendiculatus* and *Rhipicephalus evertsi* ticks to their respective feeding sites on bovids, namely the inner ears and anal regions.

During the dual choice assays conducted to assess the response of ticks to crude extracts obtained from leaves, flowers, seedpods, and twig branches, both *A. variegatum* and *R. appendiculatus* adult ticks displayed a preference for and moved towards the crude methanol flower extract in comparison to the control. These findings suggest that the ticks likely detected specific chemical signals within the flower extract, which potentially influenced their behaviour and prompted them to move in a directed manner towards the odour emitted by the flower extract. Furthermore, the vibrant yellow or golden-yellow colour of the flower extract may have served as a visual cue that attracted the ticks, stimulating their movement towards it. This finding aligns with the study conducted by Stjernberg and Berglund (2009), which determined that ticks were attracted to light colours, as evidenced by the presence of a greater number of ticks (20.8 more ticks) on individuals wearing light-coloured clothing compared to those wearing dark-coloured clothing. In this particular case, the bright yellow colour of the flower extract may have indicated a secure habitat or potential host for these tick species. Interestingly, a similar olfactory preference for the flowers of *S. didymobotrya* has been observed in another blood-feeding arthropod, *Anopheles gambiae*, which is a known malaria vector (Nikbakhtzadeh *et al*., 2016).

The significant attraction of *A. variegatum* ticks to the extracts derived from flowers and twig branches can be attributed to the similarity in the chemical composition of these plant parts, as evidenced by the findings of the NMDS and SIMPER analyses. The preference of adult *A. variegatum* ticks to the extracts obtained from the flower, twig branches, seedpods, and leaves of *S. didymobotrya* at the highest tested concentration (100 mg/mL) aligns with the findings of Nana *et al*. (2010). Nana *et al*. reported that a dose of 100 mg/mL of *Calpurnia aurea* oil extract exhibited the greatest relative attraction (52.4% and 65.9%, respectively) for *Rhipicephalus pulchellus* and *R. appendiculatus* ticks. Since ticks respond to volatiles in a dose-dependent manner (Ferreira *et al*., 2020), this suggests that at high extract concentrations, there exists an optimum concentration/dose of attractant volatiles that activates the ticks responses.

The results of this study further demonstrated that both *A. variegatum* and *R. appendiculatus* ticks were repelled by the extracts obtained from the leaves and seedpods, albeit at different concentrations. Likewise, Opiro *et al*. (2013) documented the avoidance behaviour of *R. appendiculatus* larvae (87.7%) towards the methanol extracts of *S. didymobotrya*. In vitro experiments conducted by Wanzala *et al*. (2014) demonstrated that the essential oil extracted from the aerial parts of *S. didymobotrya* exhibited repellent properties against *R. appendiculatus* ticks – with approximately 35% and 90% repellence at doses of 0.1

mg and 50 mg, respectively. This tendency of the ticks to exhibit a preference for the control over the plant parts' extracts can be attributed to the presence of repellent compounds present within them. The chemical analysis of the crude methanol extracts obtained from the leaves and seedpods of S. *didymobotrya* confirmed the presence of repellent compounds within these plant parts. It is well-known that phytol is a strong repellent of *R. appendiculatus* ticks (Ndungu *et al*., 1999). Likewise, similar repellent properties have been observed in other arthropod genera, including *Anopheles gambiae*, the primary mosquito vector for malaria, and *Aedes aegypti*, the vector responsible for transmitting chikungunya, dengue, yellow fever, and Zika viruses (Cantrell *et al*., 2016). Dodecanoic acid (Bissinger & Roe, 2013) and octacosane (Ndungu *et al*., 1999) are other compounds found in the leaf and seedpod extracts of *S. didymobotrya* that are known to act as repellents against ticks. The findings reported by Faraone *et al*. (2020) support this study's results, indicating that when antagonistic compounds, including both attractants and repellents, are present simultaneously, as observed in the leaf and seedpod extracts, the presence of repellents diminishes the attractant stimuli, thereby deterring the tick responses.

Fractionation of the methanol crude flower extract using solvents of increasing polarity allowed for separation of the extract into fractions made up of several compounds based on their polarity. The observed preference of *A. variegatum* adults for the hexane fraction after fractionation using a separating funnel indicated that the attractant compounds present in the extract were of non-polar nature (Abubakar & Haque, 2020). The significant attraction of *R. appendiculatus* adult ticks to the ethyl acetate fraction obtained through the separation funnel method indicated that the active compounds responsible for the attraction were of polar or mid-polar nature (Abubakar & Haque, 2020). Remarkably, the chemical analysis of the ethyl acetate fraction revealed the presence of 2,4-bis(1,1-dimethylethyl) phenol, a compound that was likely responsible for triggering the attraction behaviour observed in the *R. appendiculatus* adult ticks. Ticks belonging to the genus *Rhipicephalus*, such as the cattle tick *R. microplus*, demonstrate a generic attraction behavioural response to phenolic compounds (Yoder *et al*., 2008). However, the response of ticks to this compound should be ascertained using coupled gas chromatography-single sensillum recording (GC-SSR).

Similarly, Nana *et al*. (2010) observed that *R. appendiculatus* adults were significantly attracted to the polar extracts (specifically, aqueous and acetone) in the tick climbing bioassays and that higher concentrations of these extracts tended to reduce the attraction of ticks, as was also observed in this study.

Adult ticks of *A. variegatum* exhibited a strong attraction response to squalene (2,6,10,15,19,23-hexamethyl-6,6,10,14,18,20-tetracosahexane) (Appendix E). Squalene, a triterpenoid lipid naturally found in mammalian skin and blood, has been shown to strongly attract larvae, nymphs, and adults of *Amblyomma americanum* (L.) and adults of *Dermacentor variabilis* (Say) in previous studies (Yoder *et al*., 1998). In the present study, squalene demonstrated high attraction at a low concentration (1 mg/mL) and repellent effects at a higher concentration (10 mg/mL). These results agree with the findings reported by Allan (2010) and Bissinger and Roe (2013). Yoder *et al*. (1999) documented a similar pattern where ticks showed a high attraction to low concentrations of squalene and reduced attraction at high concentrations of squalene – with 1%, 10% and 100% squalene attracting 72%, 62% and only 21% of *A. americanum* ticks, respectively.

In contrast, the present study found that *R. appendiculatus* adults exhibited a preference for squalene over the solvent control, but this preference was not statistically significant, even at the lowest tested dose. Furthermore, no clear dose-response relationship was observed in terms of behavioural response to squalene. This observation could be explained by the fact that squalene functions as an arrestant rather than an attractant as previously demonstrated by the clustering of *Rhipicephalus* ticks at the point of contact with squalene (Carnohan *et al*., 2016; Yoder *et al*., 2008). The lack of aggregation observed in *R. appendiculatus* ticks in the current study can be attributed to their inability to come into direct contact with the squalene-treated cotton plugs, due to the placement of the plugs on top of the aluminium rods. The differences in the response of *A. variegatum* and *R. appendiculatus* adults to squalene can be attributed to their various host-searching strategies. Squalene may act as an attractant for ticks that actively search for hosts (hunters), while serving as an arrestant for ticks that lie in wait for passing hosts (ambushers) (McMahon & Guerin, 2002).

Strikingly, linoleic acid (9Z,12Z-octadecadienoic acid) (Appendix E), at certain tested concentrations, evoked behavioural responses in *A. variegatum* and *R. appendiculatus* adults. Linoleic acid, which is naturally found on the skin surface of humans (Pappas, 2009), cattle (Poon *et al*., 1978), and other mammals parasitized by ticks, could potentially stimulate ticks to initiate host-searching behaviours. According to a study by Dunn *et al*. (2019), the sheep scab mite *Psoroptes ovis* (Acari: Psoroptidae) exhibited a significant attraction to linoleic acid when tested using filter paper discs in a Petri dish in a 1:4 test: control choice test assay. Earlier research has provided evidence that linoleic acid functions as an attractant signal for arthropods, either when presented alone or in conjunction with other compounds (Bomar & Lockwood, 1994). Similar to the attraction bioassays conducted in the present study using

crude extracts and fractions, there was a varied response of the ticks to linoleic acid. Adults of *A. variegatum* exhibited a non-significant attraction to a lower dose (1 mg/mL) of linoleic acid, while *R. appendiculatus* displayed a significant attraction to the highest tested dose of linoleic acid. One plausible hypothesis is that linoleic acid, a lipid found on the skin surface of mammals, may act as an attractant semiochemical for various tick species, although further validation is required to confirm this.

Both *A. variegatum* and *R. appendiculatus* ticks were significantly attracted to a synthetic blend of squalene and linoleic acid (1: 1), though at different concentrations. Sonenshine (2008) previously highlighted that combining two or more compounds in a specific ratio could sometimes elicit the highest behavioural response.

Overall, *A. variegatum* adult ticks exhibited a more pronounced and distinct response to the test substances, including plant extracts, fractions, and synthetic compounds, compared to *R. appendiculatus* adults. This may have been influenced by the variability in the physiological state of the two tick species at the time the bioassays were carried out (Allan, 2010). The dissimilarities in the results between the two tick species could also be attributed to limitations associated with the tick climbing bioassay used for *R. appendiculatus* ticks. Unlike the controlled environment of the wind tunnel where ticks were exposed to two volatile choices at a time, the climbing bioassay was more susceptible to environmental factors that could have influenced the outcomes. However, previous studies have reported similar disparities in the sensitivity of different tick species to various odour profiles within the same study (Carr *et al*., 2013; Osterkamp *et al.*, 1999).

The observed attraction of ticks to the crude methanol flower extract of *S. didymobotrya*, as well as the synthetic compounds and their blend, provides a basis for further exploration of their potential integration with killing or infecting agents (such as acaricides or entomopathogenic fungi). This opens up possibilities for the development of an off-host "attract-and-kill" strategy, which could contribute to sustainable tick control methods. Consequently, it is necessary to assess the compatibility of these attractants with the chosen infecting or killing agents. The findings of this study offer the potential for the development of an alternative tick control method, the off-host push-pull strategy. In this approach, the repellent leaf extracts can be utilized to repel ticks from areas where livestock are present, while the attractant flower extracts, combined with a trap treated with an infecting or a killing agent, can be used to lure the ticks towards a specific location for effective elimination.

5.3 Effect of identified tick attractants on the viability and virulence of *M. anisopliae* ICIPE 7

Attractants could affect the germination and consequently, the virulence of entomopathogenic fungi (Mfuti *et al*., 2016; Nana *et al*., 2012; Nana *et al*., 2016; Opisa *et al*., 2019). The effect an attractant could have on conidial germination is therefore the most critical determinant of fungus-attractant compatibility in pest management (Nawaz *et al*., 2022). This study demonstrated that there was no significant difference in the germination of conidia exposed to the headspace volatiles of the crude (methanol) flower extract and squalene. However, there was a slight reduction in the germination of conidia exposed to headspace volatiles of linoleic acid and the squalene; linoleic acid (1: 1) blend. Germination was significantly reduced in the conidia that were exposed to the headspace of the AAAP. Additionally, when various concentrations of the tick attractants were incorporated in a suspension with the fungus, they also influenced the ability of ICIPE 7 to kill *A. variegatum* and *R. appendiculatus* adults differently.

Viability bioassays with the crude methanol flower extract of *S. didymobotrya* at the three tested concentrations revealed conidial germination that was significantly similar to that in the control treatment consisting of *M. anisopliae* ICIPE 7 only throughout the exposure time. This indicates that the bona fide volatiles released in the headspace of the fungus by the *S. didymobotrya* flower extracts either had no negative effect on conidial germination, or were released in negligible quantities that were not enough to affect conidial germination. These findings are similar to the reports of Nana *et al*. (2012, 2016) who mixed extracts of *C. aurea* with *M. anisopliae* ICIPE 7 and reported that the aqueous extract and emulsifiable formulation (in corn oil) of *Calpurnia aurea* at all tested concentrations (1.2%, 2.5%, 5%, and 10%) were compatible with ICIPE 7 by their failure to negatively affect fungal growth parameters (mycelial dry weight, radial growth and spore production). In addition, some other plant extracts, such as the leaf (benzene) and root (methanol) extracts of *Ocimum sanctum* (Borgio *et al*., 2008); the aqueous rhizome extracts of *Curcuma longa* (Zingiberaceae), aqueous leaf extracts of *Corymbia citriodora* (Myrtaceae), *Cymbopogon citratus* (Poaceae), and *Rosmarinus officinalis* (Lamiaceae) (Formentini *et al*., 2014); and the essential oils of fennel (*Foeniculum vulgare*) and lavender (*Lavandula angustifolia*) (Lak *et al*., 2022) have also been reported to be compatible with *M. anisopliae*. Nonetheless, some plant extracts are known to be toxic to *M. anisopliae* – essential oil of tarragon (*Artemisia dracunculus*) (Lak *et al*., 2022), cashew nutshell extraction waste (Putra *et al*., 2023); and to the entomopathogenic fungus, *Beauveria bassiana* – neem oil (Depieri *et al.*, 2005).

The results of this study also revealed that the triterpene squalene identified in *S. didymobotrya* extract as a compound that attracts ticks, was also compatible with *M. anisopliae* ICIPE 7 as shown by the conidial germination throughout the exposure time; where the viability of fungal conidia in the fungus exposed to squalene was statistically similar to that of the control (ICIPE 7 only) at days six and eight post-treatment. Previously, a study established that squalene had no effect on the growth of *M. anisopliae* cultured on Potato Dextrose Agar (PDA) media supplemented with squalene (Benoit *et al*., 2005). This was determined by the similar radial growth rates of the fungus in media supplemented with various concentrations of squalene (0.1, 0.01, and 0.001) and the control (not supplemented PDA) media. It is worth noting that the compatibility between squalene and *M. anisopliae* cannot be attributed to squalene being a triterpene, as evidenced by the toxic effects demonstrated by the triterpenes cucurbitacin (Gothro, 1993) and azadirachtin (Kiruthiga *et al*., 2022) on *M. anisopliae*.

The current study also indicated that the linoleic acids and squalene: linoleic acid (1: 1) blend treatments slightly reduced the germination of *M. anisopliae* ICIPE 7's conidia in comparison to the control (ICIPE 7 only) treatment. A possible explanation for this decrease in viability of conidia exposed to these treatments may be due to the presence of the unsaturated fatty acid, linoleic acid (C18:2). This agrees with previous studies that have established the ability of linoleic acid to either inhibit germination of conidia, growth of hyphae and/or the pathogenicity of several entomopathogenic fungi, including *Beauveria bassiana*, *Paecilomyces fumosoroseus* (Saito & Aoki, 1983) and *Conidiobolus coronatus* (Boguś *et al*., 2010).

Another interesting finding from this study is that the AAAP attractant at all tested concentrations (10%, 5% and 2.5%) significantly reduced the germination of *M. anisopliae* ICIPE 7's conidia in comparison to the control and other attractant treatments. These results are consistent with those of Nana *et al*. (2012) who found that AAAP significantly inhibited ICIPE 7's growth parameters, including conidial germination and yield, radial growth and mycelial weight, when it was plated on SDA media amended with various concentrations of AAAP (0.005, 0.01, and 0.02 mg/mL). This inhibition effect might be due to the synthetic nonanoic acid and o-nitrophenol, which are components of the AAAP pheromone (Schöni *et al.*, 1984). A previous study evaluated the effect of various concentrations of nonanoic acid on spore germination and mycelial growth of the cacao fungal pathogens, *Moniliophthora roreri* and *Crinipellis perniciosa*, and reported that spore germination and mycelial growth were highly inhibited by nonanoic acid (Aneja *et al*., 2005). Further, Verdcourt (1952) reported that the presence of two nitro groups (as is the case in dinitrophenols and *o*-nitrophenols) increases the toxicity of these substituted phenols to fungi. The similar findings from this study and that

of Nana *et al*. (2012) are compelling due to the different methods used to expose the fungus to the attractant pheromone - although this study exposed the fungus to the headspace volatiles of AAAP, whereas Nana and colleagues plated the fungus on SDA media amended with AAAP. These findings support the successful non-combined use of AAAP with ICIPE 7 in field conditions for the control of ticks (Nchu *et al*., 2009, 2010). A possible explanation for this may be that the distance of separation between AAAP and the ICIPE 7 fungus plays a crucial role on the influence of the attractant pheromone on the fungal growth parameters.

The two tick species, *A. variegatum* and *R. appendiculatus* exhibited different percentage mortalities and varied susceptibility levels, where *R. appendiculatus* recorded shorter LT_{50} response time estimates when they were exposed to a suspension consisting of the fungus and either of the various concentrations of the tick attractants. Mortality was expected in all treatment groups as they all consisted of *M. anisopliae* ICIPE 7, a proven virulent pathogen of both *A. variegatum* (Nchu *et al.,* 2009, 2010) and *R. appendiculatus* (Nana *et al*., 2012) adult ticks. However, the differences in percentage mortalities and LT_{50} estimates between the two species may be linked to their morphological differences, such as body size (Abe & Ikegami, 2005). Smaller ticks, in this case *R. appendiculatus* adults, are expected to be more susceptible to the various fungal treatments within a shorter time, compared to the bigger *A. variegatum* ticks. Conversely, the different mortality rates and LT_{50} estimate values within each species may be related to the effect of the attractant components on the viability, pathogenicity and virulence of the fungus to the ticks (Ambethgar, 2009).

An interesting finding that emerged from the analysis is that both 10% squalene with ICIPE 7 and 5% AAAP with ICIPE 7 were treatments that exhibited the lowest mortality rates and longest LT_{50} response times in both *A. variegatum* and *R. appendiculatus* species. A possible explanation for the low mortality in the treatment with 10% squalene is that squalene affected vital growth parameters of the fungus when they were directly combined. Gnamusch *et al*. (1992) demonstrated that high quantities of squalene form hydrophobic lipid droplets that partition into fungal cellular membranes, disturbing their structure and consequently interfering with vital membrane functions. This finding corroborated with Davis and Papich (2014) assessment who reported that a high concentration of extracellular squalene inhibits function of the fungal cell wall consequently leading to its toxicity to fungal cells. The finding that 5% AAAP and ICIPE 7 treatment caused the lowest mortality and longest LT_{50} response of ticks may be explained by the fact that AAAP is made up of nonanoic acid and *o*-nitrophenol, which possess antifungal properties (Aneja *et al*., 2005; Verdcourt, 1952), and that the 5% concentration was most optimum to negatively affect the germination/viability of the conidia,

and consequently the infection of the ticks by the fungus. The pheromone, AAAP might therefore have a high inhibition rate to *M. anisopliae* ICIPE 7 compared to other treatments. Furthermore, the results showed that the highest mortality, shortest LT_{50} response time, and lowest probability of survival in both *A. variegatum* and *R. appendiculatus* ticks were credited to the 10% squalene: linoleic acid blend with ICIPE 7 and 10% AAAP with ICIPE 7 treatments. This unanticipated response exhibited by ticks exposed to these treatments contradicts previous literature where the treatments consisting of either squalene (Davis & Papich, 2014), linoleic acid (Boguś *et al*., 2010), nonanoic acid (Aneja *et al*., 2005) and/or *o*-nitrophenol (Verdcourt, 1952) are expected to negatively influence the growth parameters of *M. anisopliae*, and ultimately, its virulence. These findings are quite significant as they indicate the possibility of a synergistic effect of either the squalene: linoleic acid blend (1: 1) or AAAP at the 10% concentration with *M. anisopliae* ICIPE 7 pending validation.

This study provided fundamental knowledge regarding the compatibility of *M. anisopliae* ICIPE 7 with selected tick attractants. The crude flower (methanol) extract of *S. didymobotrya* and squalene did not affect the germination of ICIPE 7's conidia, demonstrating their compatibility with the fungus when not combined. The extract of *S. didymobotrya* and squalene could therefore be combined with *M. anisopliae* ICIPE 7 to suppress the population of ticks in livestock using an "attract-and-kill" approach. However, linoleic acid, the two-component blend squalene: linoleic acid (1: 1) and the attraction-aggregation-attachment pheromone (AAAP) negatively affect the germination of ICIPE 7's conidia, with higher fungal inhibition rate compared to the other treatments, translating to the lack of compatibility between the fungus and these attractants when they are not combined (exposure to headspace volatiles). Additionally, the squalene: linoleic acid (1: 1) (10%) mixed with ICIPE 7 increased the overall mortality, and reduced both the lethal time response to mortality of 50% of the exposed tick population and as well as the survival probabilities of both *A. variegatum* and *R. appendiculatus* adult ticks. Nevertheless, the reason mechanism behind this observed result ought to be further explored.

In summary, this study confirmed that *M. anisopliae* ICIPE 7 is a potent biological control agent for *A. variegatum* and *R. appendiculatus* adult ticks. The headspace volatiles of the crude flower (methanol) extract of *S. didymobotrya* and squalene were compatible with ICIPE 7, and could be used in an integrated approach to tackle ticks in livestock through "attract-and-kill". The squalene: linoleic acid blend (1: 1) (10%) increased the virulence of ICIPE 7, shortened the mortality time and lowered the survival probabilities of the two tick species, *A. variegatum* and *R. appendiculatus*. In addition, the study showed that the squalene:

linoleic acid (1: 1) blend (10%) could be therefore combined with *M. anisopliae* ICIPE 7 in an "attract-and-kill" strategy for the biological control of ticks and tick-borne diseases.

CHAPTER SIX
CONCLUSIONS & RECOMMENDATIONS

6.1 Conclusions

i. From this study, molecular barcoding using the *rbcL* locus effectively identified *S. didymobotrya* and elucidated the evolutionary relationships among samples from various climatic zones in Kenya. This indicates that the genetic diversity *within S. didymobotrya* can be documented accurately, supporting that molecular barcodes are efficient for species identification and phylogenetic inference in *S. didymobotrya* populations.

ii. The olfactory-based bioassay-guided activities revealed that the methanolic extract of *S. didymobotrya*'s flower, its hexane and ethyl acetate fractions contain tick attractant compounds, including squalene and linoleic acid.

iii. The study demonstrated that the headspace volatiles of the crude methanol extract of *S. didymobotrya*'s flower and squalene are compatible with *M. anisopliae* ICIPE 7, and can therefore be used separately in an attract-and-kill strategy to control tick populations. The squalene: linoleic acid blend (1: 1) can be directly combined with *M. anisopliae* ICIPE 7 and used to control ticks.

6.2 Recommendations

This study recommends that:

i. In future studies, sample collection should be expanded to more climatic locations to increase the feasibility of molecular barcoding in *S. didymobotrya*.

ii. The activity of identified tick attractants should be tested and validated under field conditions, and practical concentrations that maximize tick attraction while minimizing non-target effects should be established.

iii. An attract-and-kill device consisting of a slow-release mechanism for identified tick attractants, alongside a suitable formulation of *M. anisopliae* ICIPE 7 that protects the fungus from environmental factors such as rain and sunlight, should be developed. Further, field studies should be done to assess the effect of this device on non-target organisms. This could lead to a sustainable and ecologically friendly solution for tick control.

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APPENDICES

Appendix A. Peer-reviewed Publication

Veterinary Parasitology 329 (2024) 110210

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Attractant and repellent properties of *Senna didymobotrya* plant extracts to *Amblyomma variegatum* and *Rhipicephalus appendiculatus*

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ABSTRACT

The growing challenge of acaricide resistance and geographical range expansion of invasive tick species demands other interventions, like plant-based alternatives, for sustainable tick control. Leaves, flowers, seedpods, and twig branch extracts of *Senna didymobotrya* were analyzed using coupled gas chromatography mass spectrometry (GC-MS). Response of adult *Amblyomma variegatum* and *Rhipicephalus appendiculatus* to extracts was evaluated. The most attractive plant extract was fractionated and ticks' responses to its fractions assessed. Potential tick attractants in the attractive plant part extract and its fractions were identified by GC-MS analysis. Non- significant qualitative and quantitative differences were observed in the plant parts' extract composition ($R = 0.6178$). Flower extracts attracted both species, with a 0.1-fold higher attraction in *A. variegatum* compared to the standard attraction aggregation attachment pheromone (AAAP). Leaf and seedpod extracts repelled ticks at various concentrations. Bioassays after fractionating flower extracts identified hexane and ethyl acetate fractions as most attractive to *A. variegatum* ($P < 0.001$) and *R. appendiculatus* ($P < 0.001$), respectively. Chemical analysis of the most attractive extracts and fractions identified compounds, including documented acarine attractants, squalene and linoleic acid. A squalene and linoleic acid blend (1:1) at 1 mg/mL significantly attracted adult *A. variegatum* ($P < 0.01$) and *R. appendiculatus* ($P < 0.001$). The results of this study broaden comprehension of how ticks respond to plants in nature, and showcase the promising potential for integrating these insights into effective tick management programs.

1. Introduction

Amblyomma variegatum and *Rhipicephalus appendiculatus* are prominent tick species in sub-Saharan Africa (Jongejan and Uilenberg, 2004). With an extensive range across tropical sub-Saharan Africa and introduction in the Caribbean, *A. variegatum* is highly adaptable to domestic livestock (Jongejan and Uilenberg, 2004; Walker et al. 2003). It is a key vector for transmitting *Ehrlichia ruminantium*, the causative agent of heartwater, and significantly promotes the occurrence of dermatophilosis in cattle (Pavis et al., 1994; Walker, 1996). *Rhipicephalus appendiculatus* is of paramount importance in East and Southern Africa, where in its adult stage, it prefers to feed on the ears of various domestic and wild ruminants (Jongejan and Uilenberg, 2004; Walker et al. 2003). This tick species transmits *Theileria parva* and *Theileria taurotragi*, the causative agents of East Coast Fever (ECF) and benign bovine theileriosis, respectively, in cattle (Minjauw and Mcleod, 2003).

Acaricides have traditionally been central in tick control, but their overuse, often at suboptimal doses, has led to challenges, including resistance development, chemical residue persistence, limited effectiveness duration, non-target vertebrate toxicity, and high costs (de la Fuente, 2018; Kasaija et al., 2021; Nwanade et al., 2020). In response to these issues, there is a growing interest in eco-friendly alternatives, particularly plants as a sustainable and effective tick control solution.

Various plants, their extracts, and essential oils exhibit multifaceted behavioral effects on ticks, serving as attractants (Hassan et al., 1994; Nana et al., 2010; Zorloni et al., 2010), acaricides or repellents (Adenubi

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Appendix B. Research Permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **909776**

Date of Issue: **19/May/2021**

RESEARCH LICENSE

This is to Certify that Ms.. Diana Wairimu Kinyua of Egerton University, has been licensed to conduct research in Kajiado, Kwale on the topic: ATTRACT AND KILL STRATEGY FOR SUSTAINABLE CONTROL OF TICKS USING ENTOMOPATHOGENIC FUNGUS, Metarhizium anisopliae AND EXTRACTS OF Calpurnia aurea UNDER FIELD CONDITIONS for the period ending : 19/May/2022.

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Appendix C: Multiple sequence alignments of sequences amplified using *rbcL* and *matK* primers

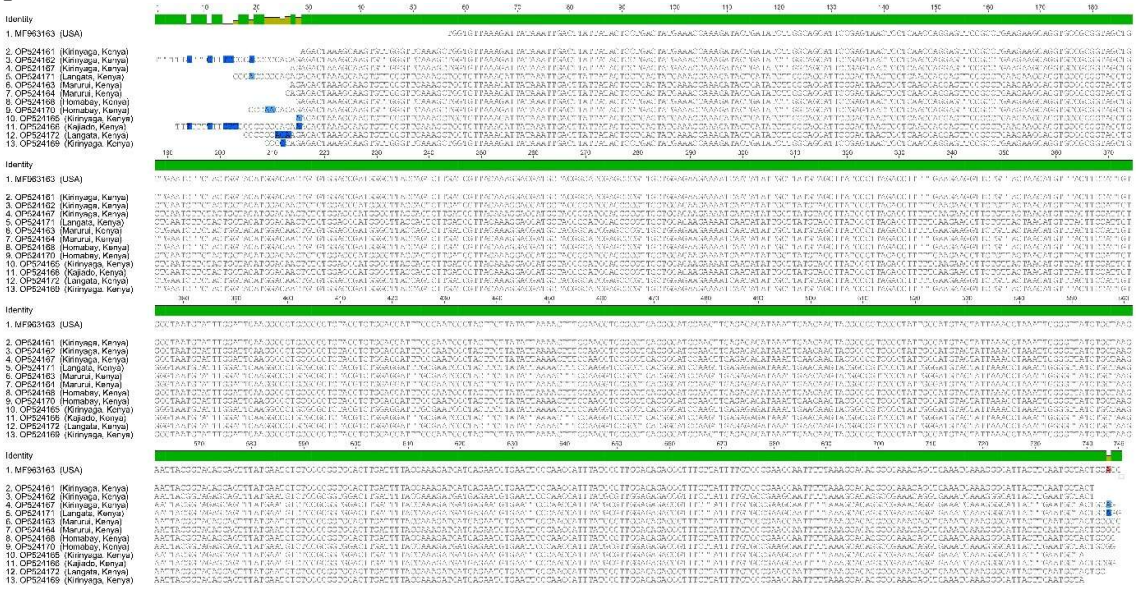

Figure C 1: Multiple sequence alignment (MAFFT; Geneious 8.1.8 software) showing the relationship between the ribulose-1,5-bisphosphate carboxylase large subunit (*rbcL*) gene fragment sequences of *Senna didymobotrya* from various locations in Kenya and the United States of America

Figure C 2: Multiple sequence alignment (MAFFT; Geneious 8.1.8 software) showing the relationship between the maturase K (*matK*) gene fragment sequences of *Senna didymobotrya* from various locations in Kenya and other Senna species from various places

Table C 1: Maturase K (*matK*) consensus sequences of *Senna didymobotrya* samples obtained from various locations in Kenya

Sample	Consensus Sequence
1C	GAATCTTGGTTCAAACCTTTCGATACTGGGTGAAAGATGCCCCTTTCTT
	TCATTTATTAAGGTTGTTTCTTTATGAGTATTCTAATTGGAATAGTCTTA
	TTACTCCAAAAAAATCGATTTCTACCTTTTCAAAAAGTAATCCAAGATT
	TTTCTTGTTCCTATATAATTTTTATGTATGTGAATACGAATCTATCTTCC
	TTTTTTTACGTAAGAAATCCTCTTATTTACGATTAATATCTTTTAACGTT
	CTTTTTGAGCGAATCTATTTCTATGGAAAAATGGAACATTTTGTAGAAG
	TCTTTGCTAAGGATTTTTCGTCTACCTTATCATTCTTCAAGGATCCTTTC
	ATTCATTATGTTAGATATCAAGGAAAATCCATTTTGGCTTCAAAGAATG
	CGCCCTTTTTGATGAATAAATGGAAATACTATCTTATCCATTTATGGCA
	ATCTCATTTTGATGTTTGGTCTCAGCCAGGAACGATCCAAATAAACCAA
	TTCCCCGAGCATTCATTTCACCTTTTGGGCTATTTTTCAAATGTGCGGTT
	AAATCTTTCAGCGGTACGAAGTCAAATGCTGGAAAATTCATTTCTAATT
	GAAATTGTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTCTAA
	TTAGATCATTGGCTAAAGCGAAATTTTGTAATGTATTAGGACACCCCAT
	TAGTAAGCTGGTCTGGGCCGATTCATCCGATTTTGATATTATTGACCGA
	TTTTTGCGGATATGTAGAAATCTTTCTCATTATTACAATGGATCCTCAA
	AAAAAAAGAGTTTGTATCGAATAAAATATATACTTCGACTTTCGTGTAT
	TAAAACTTTGGCTCGTAAA
1D	GAATCTTGGTTCAAACCCTTCGATACTGGGTGAAAGATGCCTCTTCTTT
	TCATTTATTAAGGCTCTTTCTTTATGAGTATTTTAATTGGAATAGTCTTA
	TTACTCCAAAAAAATGGATTTCTACTTTTTCAAAAAGGAATCCAAGATT
	CTTCCTGTTCCTATATAATTTTTATGTATGTGAATACGAATCTATCTTTC
	TTTTTCTCCGTAACAAATCTTCTTATTTACGATTAACATCTTCTAGAGTC
	CTTTTTGAGCGAATCTATTTCTATGCAAAAATAGAACATTTTGTAGAAG
	TCTTTGATAAAGATTTTCCGTCCACCCTATGGTTCTTCAAGGACCCTTTC
	ATTCATTATGTTAGATATCAAGGAAAATCCATTTTGGCTTCAACGAATA
	CGCCCTTTTTGATGAATAAATGGAAATACTATCTTATCCGTTTATGGCA
	ATGTCATTTTTCTGTTTGGTCTCAACCAGAAAAGATCCATATAAACCAA
	TTATCTGAGCATTCATTTTACTTTTTGGGCTATTTTTCAAATGTGCGGTT
	AAATCCTTCAGTGGTACGGAGTCAAATGCTGGAAAATTCATTTCTAATT

GAAAATGTTATGAAAAGGCTTGATACAATAATTCCAATTATTCCACTAA
TTAGATCATTGGCTAAAGCGAGATTTTGTAATGTATTAGGGCATCCCAT
TAGTAAGCCGGTCTGGGCCGATTCATCCGATTTGGATATTATTGACCGA
TTTTTGCGGAGATGCAGAAATCTTTCTCATTATTACAATGGATCCTCAA
CAAAAAAGAGTTTGTATCGAATAAAATATATACTTCGGCTTTCTTGTAT
TAAAACTTTAGCTCGTAACACAG

1M TTCTGGTTACGAGCTAAAGTTTTAATACAAGAAAGCCGAAGTATATATT
TTATTTCGATACAAACTCTTTTTTGTTGAGGATCCATTGTAATAATGAG
AAAGATTTCTGCATCTCCGCAAAAATCGGTCAATAATATCCAAATCGG
ATGAATCGGCCCAGACCGGCTTACTAATGGGATGCCCTAATACATTAC
AAAATCTCGCTTTAGCCAATGATCTAATTAGTGGAATAATTGGAATTAT
TGTATCAAGCCTTTTCATAACATTTTCAATTAGAAATGAATTTTCCAGC
ATTTGACTCCGTACCACTGAAGGATTTAACCGCACATTTGAAAAATAGC
CCAAAAAGTAAAATGAATGCTCAGATAATTGGTTTATATGGATCTTTTC
TGGTTGAGACCAAACAGAAAAATGACATTGCCATAAACGGATAAGATA
GTATTTCCATTTATTCATCAAAAAGGGCGTATTCGTTGAAGCCAAAATG
GATTTTCCTTGATATCTAACATAATGAATGAAAGGGTCCTTGAAGAACC
ATAGGGTGGACGGAAAATCTTTATCAAAGACTTCTACAAAATGTTCTAT
TTTTGCATAGAAATAGATTCGCTCAAAAAGGACTCTAGAAGATGTTAA
TCGTAAATAAGAAGATTTGTTACGGAGAAAAAGAAAGATAGATTCGTA
TTCACATACATAAAAATTATATAGGAACAGGAAGAATCTTGGATTCCTT
TTTGAAAAAGTAGAAATCCATTTTTTTGGAGTAATAAGACTATTCCAAT
TAAAATACTCATAAAGAAAGAGCCTTAATAAATGAAAAGAAGAGGCA
TCTTTCACCCAGTATCGAAGGGTTTGAACCAGATT

2M CGAGCTAAAGTTTTAATACAAGAAAGCCGAAGTATATATTTTATTCGAT
ACAAACTCTTTTTTGTTGAGGATCCATTGTAATAATGAGAAAGATTTCT
GCATCTCCGCAAAAATCGGTCAATAATATCCAAATCGGATGAATCGGC
CCAGACCGGCTTACTAATGGGATGCCCTAATACATTACAAAATCTCGCT
TTAGCCAATGATCTAATTAGTGGAATAATTGGAATTATTGTATCAAGCC
TTTTCATAACATTTTCAATTAGAAATGAATTTTCCAGCATTTGACTCCGT
ACCACTGAAGGATTTAACCGCACATTTGAAAAATAGCCCAAAAAGTAA
AATGAATGCTCAGATAATTGGTTTATATGGATCTTTTCTGGTTGAGACC
AAACAGAAAAATGACATTGCCATAAACGGATAAGATAGTATTTCCATT

	TATTCATCAAAAAGGGCGTATTCGTTGAAGCCAAAATGGATTTTCCTTG
	ATATCTAACATAATGAATGAAAGGGTCCTTGAAGAACCATAGGGTGGA
	CGGAAAATCTTTATCAAAGACTTCTACAAAATGTTCTATTTTTGCATAG
	AAATAGATTCGCTCAAAAAGGACTCTAGAAGATGTTAATCGTAAATAA
	GAAGATTTGTTACGGAGAAAAAGAAAGATAGATTCGTATTCACATACA
	TAAAAATTATATAGGAACAGGAAGAATCTTGGATTCCTTTTTGAAAAA
	GTAGAAATCCATTTTTTTGGAGTAATAAGACTATTCCAATTAAAATACT
	CATAAAGAAAGAGCCTTAATAAATGAAAAGAAGAGGCATCTTTCACCC
	AGTATCGAAGGGTTTGAAC
2D	TGGTTACGAGCTAAAGTTTTAATACAAGAAAGCCGAAGTATATATTTTA
	TTCGATACAAACTCTTTTTTGTTGAGGATCCATTGTAATAATGAGAAAG
	ATTTCTGCATCTCCGCAAAAATCGGTCAATAATATCCAAATCGGATGAA
	TCGGCCCAGACCGGCTTACTAATGGGATGCCCTAATACATTACAAAAT
	CTCGCTTTAGCCAATGATCTAATTAGTGGAATAATTGGAATTATTGTAT
	CAAGCCTTTTCATAACATTTTCAATTAGAAATGAATTTTCCAGCATTTG
	ACTCCGTACCACTGAAGGATTTAACCGCACATTTGAAAAATAGCCCAA
	AAAGTAAAATGAATGCTCAGATAATTGGTTTATATGGATCTTTTCTGGT
	TGAGACCAAACAGAAAAATGACATTGCCATAAACGGATAAGATAGTAT
	TTCCATTTATTCATCAAAAAGGGCGTATTCGTTGAAGCCAAAATGGATT
	TTCCTTGATATCTAACATAATGAATGAAAGGGTCCTTGAAGAACCATA
	GGGTGGACGGAAAATCTTTATCAAAGACTTCTACAAAATGTTCTATTTT
	TGCATAGAAATAGATTCGCTCAAAAAGGACTCTAGAAGATGTTAATCG
	TAAATAAGAAGATTTGTTACGGAGAAAAAGAAAGATAGATTCGTATTC
	ACATACATAAAAATTATATAGGAACAGGAAGAATCTTGGATTCCTTTTT
	GAAAAAGTAGAAATCCATTTTTTTGGAGTAATAAGACTATTCCAATTAA
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Appendix D: Chemical composition of *Senna didymobotrya*

Table D 1: Mean amount (µg per gram of plant powder for the flower, leaf, seedpod and twig branch, and ng per milligram of hexane and ethyl acetate fractions) of compounds identified in *S. didymobotrya* extracts (n = 3)

RT (min)	Compound Identity	Chemical Class	Flower	Leaf	Seedpod	Twig branch	Hexane Fraction	Ethyl acetate Fraction
5.39	Toluene	Benzene	8.0 ± 0.00	8.7 ± 0.09	8.1 ± 0.04	8.0 ± 0.00	458.1 ± 31.02	
6.46	3-Hexenal	Aldehyde		10.0 ± 0.78				
6.63	2-Hexanol	Alcohol	10.9 ± 0.07	10.9 ± 0.00	11.9 ± 1.02	9.7 ± 0.00		
8.60	2,3-Dimethyl-3-hexene	Alkene	8.6 ± 0.59	8.3 ± 0.26	8.3 ± 0.26			
9.43	3,6,6-Trimethyl-cyclohex-2-enol	Alcohol	11.7 ± 1	9.5 ± 1.38	11.0 ± 1.47	8.1 ± 0.01		
18.51	2,4-bis(1,1-Dimethylethyl)-phenol	Phenol						1027.9 ± 135.99
19.24	5,6,7,7a-Tetrahydro-4,4,7a-trimethylbenzofuran-2(4H)-one	Ketone	11.9 ± 0.00	19.1 ± 2.58	9.3 ± 0.43	9.7 ± 0.32		
19.50	Dodecanoic acid	Fatty acid		11.4 ± 0.69	8.8 ± 0.00			

21.67	Tetradecanoic acid	Fatty acid				16.9 ± 2.24		10.2 ± 0.00		15.5 ± 0.00		726.9 ± 72.74				
22.53	6,10,14-Trimethyl-2-pentadecanone	Sesquiterpene				40.3 ± 3.91		11.8 ± 1.21		18.8 ± 0.00		505.9 ± 465.64				
23.33	7,9-Dimethyl-hexadecane	Alkane												408.8 ± 292.41		
23.42	Nonadecane	Alkane												429.5 ± 388.77		
23.49	9-Octadecenoic acid	Fatty acid				19.2 ± 2.10										
23.64	Ethyl hexadecanoate	Ester												2657.2 ± 1316.99		
23.72	Hexadecanoic acid	Fatty acid	46.9 ± 22.86			277.3 ± 59.82		148.3 ± 61.42		82.1 ± 38.52						
24.02	Eicosane	Alkane	40.4 ± 22.60			19.6 ± 1.57		11.1 ± 0.00		9.9 ± 0.55				2153.9 ± 1506.51		
24.60	Methyl octadeca-9,12,15-trienoate	Ester												2775.5 ± 0		
24.96	Methyl linoleate	Ester	21.9 ± 3.61			111.8 ± 45.21		106.1 ± 79.43		35.2 ± 27.11				45.2 ± 0		
25.12	Phytol	Alcohol				479.2 ± 124.25		16.4 ± 4.81		19.4 ± 0.00						
25.14	Ethyl linoleate	Ester												2940.6 ± 0		

25.28	Methyl octadecanoate	Ester											878.6	±	60.63			
25.55	Linoleic acid	Fatty acid	50.5	±	23.05	46.6	±	3.96	56.5	±	45.37	14.7	±	4.24	4323.0	±	712.58	2791.8	±	2749.44
25.55	Octadecanoic acid	Fatty acid	37.3	±	14.11	103.6	±	4.68	35.8	±	13.23	47.6	±	25.57						
25.86	Heneicosane	Alkane	14.7	±	0.00	58.1	±	23.65	51.3	±	22.97	37.0	±	4.74	3391.6	±	100.88			
25.90	Docosane	Alkane	22.6	±	2.82				27.1	±	1.43				5050.3	±	528.64			
26.72	Tricosane	Alkane	27.2	±	12.36	34.8	±	1.65	41.4	±	8.30	15.6	±	0.00	1726.2	±	1677.10	1888.0	±	958.55
26.97	Methyl eicosanoate	Ester	18.9	±	10.87	45.7	±	19.04	8.1 ± 0.05			14.1	±	6.03						
27.25	4,8,12,16-Tetramethylheptadecan-4-olide	Isoprenoid γ-lactone	8.0	±	0.00	41.8	±	17.27	37.5	±	11.51	31.2	±	1.01	3773.8	±	425.01			
27.34	Chrysophanol	Anthraquinone	71.7	±	0.00	93.8	±	7.92	96.8	±	0.00	103.6	±	33.72						
27.62	1-Tetracosene	Alkene				48.3	±	9.71	37.4	±	0.00	24.1	±	1.97						

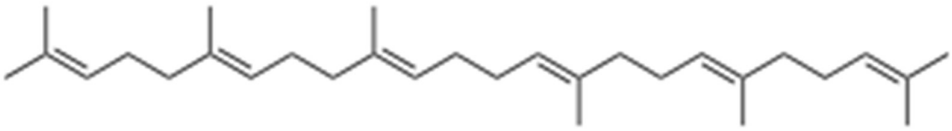
27.83	Tetracosane	Alkane	27.6 ± 12.47	77.0 ± 31.08	37.6 ± 13.99	32.5 ± 12.23	10509.2 ± 1424.65	4205.9 ± 4162.50
28.59	Methyl docosanoate	Ester	8.1 ± 0.01	86.6 ± 6.80	26.4 ± 18.27	8.1 ± 0.02		
28.62	Pentacosane	Alkane	19.7 ± 11.64	8.1 ± 0.01	52.8 ± 5.95	14.0 ± 5.94	68.1 ± 3.09	10199.9 ± 5108.83
29.25	2-Phenyl-4-quinolyldiphenyl carbinol	Alcohol	42.5 ± 0.69		40.6 ± 0.00	25.6 ± 0.00		
29.36	Hexacosane	Alkane	48.0 ± 3.38	81.5 ± 15.49	61.3 ± 7.72	62.4 ± 0.00	6652.3 ± 1334.47	
29.69	Z-12-Pentacosene	Alkene	34.2 ± 25.91					
29.76	1,8-Dihydroxy-3-methoxy-6-methyl-9,10-anthracenedione	Anthraquinone	47.5 ± 36	174.9 ± 38.51	61.7 ± 19.73	40.0 ± 10.12		
29.86	Heptacosane	Alkane	66.8 ± 33.42	72.3 ± 17.12	59.8 ± 39.76	36.2 ± 19.88		44.3 ± 2.59
30.70	Octacosane	Alkane	28.1 ± 20.04	71.3 ± 9.34	98.3 ± 46.49	17.0 ± 8.94		
31.04	Squalene	Triterpene	27.1 ± 6.62	157.2 ± 23.93	46.2 ± 11.87	43.5 ± 13.37	7525.0 ± 292.56	5179.2 ± 2490.63
32.99	*β*-Tocopherol	Tocopherol						4429.7 ± 0.00

33.74	γ-Tocopherol	Tocopherol	25.7 ± 17.32		51.4 ± 4.66		8.2 ± 0.17		8.1 ± 0.04		2521.5 ± 1050.64								
34.21	Stigmastan-3,5,22-trien	Sterol	12.1 ± 3.92		19.6 ± 11.03		18.1 ± 9.70		10.7 ± 2.30										
34.88	Cholesterol	Sterol	10.5 ± 2.40		33.3 ± 3.40		18.6 ± 5.35		8.5 ± 0.34										
35.00	Vitamin E	Tocopherol	56.2 ± 16.55		136.5 ± 16.52		16.3 ± 3.40		24.6 ± 12.05		19223.1 ± 1746.47		3139.0 ± 3095.56						
36.79	Campesterol	Sterol	30.8 ± 22.60		56.8 ± 4.27		41.9 ± 20.21		16.9 ± 7.45		5108.3 ± 103.77								
37.43	Stigmasterol	Sterol	73.8 ± 27.15		133.7 ± 23.33		100.3 ± 54.99		68.8 ± 36.66		9904.3 ± 395.62								
37.44	Stigmastan-3,5-diene	Steroid											1229.8 ± 1189.04						
38.69	(3β, 5β,24*S*)-Stigmast-7-en-3-ol	Sterol	56.9 ± 28.76		366.8 ± 77.54		231.8 ± 135.64		42.5 ± 22.21		2245.8 ± 171.15								
38.94	γ-Sitosterol	Sterol									18759.1 ± 1602.13								
39.50	β-Amyrin	Triterpene	48.1 ± 19.97		69.5 ± 14.87		26.7 ± 12.84		15.2 ± 6.63		2617.5 ± 192.95								

39.51	(3α)-12-Oleanen-3-yl acetate	Triterpene	17.5	±	3.43				25.7	±	0.00					
39.92	Stigmastan-7-one	Triterpene							10.0	±	0.41					
40.05	Lupen-3-one	Triterpene	95.0	±	29.51	16.1	±	7.97	19.4	±	11.30	65.5	±	30.24		
40.54	α-Amyrin	Triterpene	68.4	±	43.48	126.9	±	15.12	75.2	±	32.74	30.8	±	13.64	3982.3	± 1952.37
41.87	Stigmast-4-en-3-one	Terpene				47.8	±	2.14	29.7	±	11.51	58.4	±	0.00	3227.0	± 491.72

RT (min) = retention time in minutes

Appendix E: Compound structures

Compound	Structure
Squalene	
Linoleic acid