Acaricidal efficacy of crude extracts and isolated flavonoids from *Calpurnia aurea* subsp. *aurea* against *Rhipicephalus turanicus*

Submitted in fulfilment of the requirements for the degree of

Philosophiae Doctor (PhD)

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Declaration

This experimental work conducted in the Phytomedicine Programme of the Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria and results described in this thesis have not previously been submitted in any other form to another University or academic institution for consideration. It is my original work in design and in execution, and all aspects contained herein have been duly acknowledged.

I, Olubukola Tolulope Adenubi, declare the above statement to be true.

……..

Olubukola Tolulope Adenubi

Dedication

To my mother, Dr. (Mrs) Janet Ibironke Ogunenika-Makanjuola, who passed on shortly before my academic sojourn to South Africa. In every letter she wrote to me during my primary to tertiary education, she ended it with the phrase “Face your studies, for there your honour lies”. Thank you God for my mother as I am the doctor-doctor that has been made.

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Publications

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Fouche, G., Ramafuthula, M., Maselala, V., Mokoena, M., Senabe, J., Leboho, T., Sakong, B.M, **Adenubi, O.T.,** Eloff, J.N., Wellington, K.W. 2016. Acaricidal activity of the organic extracts of thirteen South African plants against *Rhipicephalus (Boophilus) decoloratus* (Acari: Ixodidae). Veterinary Parasitology 224: 39-43.

Wellington, K.W., Leboho, T., Sakong, B.M., **Adenubi, O.T**., Eloff, J.N., Fouche, G. 2017. Further studies on South African plants: Acaricidal activity of organic plant extracts against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). Veterinary Parasitology 234: 10-12.

Adenubi, O.T., Fasina, F.O., McGaw, L.J., Eloff, J.N., Naidoo, V. 2016. *In vitro* bioassays for plant extracts with tick-repellent and acaricidal properties: A systematic review and meta-analysis. Planta Medica 81 (S01): S1-S381. DOI: 10.1055/s-0036-1596512 (Abstract).

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Adenubi, O.T., Ahmed, A.S., Fasina, F.O., McGaw L.J., Eloff J.N., Naidoo, V. Pesticidal plants as possible alternatives to synthetic, chemical acaricides in tick control: A systematic review and meta-analysis. Industrial Crops and Products.

Adenubi, O.T., McGaw, L.J., Eloff, J.N., Naidoo, V. *In vitro* bioassays for evaluating plant extracts with tick repellent and acaricidal properties: A critical review. Veterinary Parasitology.

Adenubi, O.T., Ali Abdallah, M., Ahmed, A.S.,Njoya, E.M., McGaw, L.J., Eloff, J.N., Naidoo, V. Isolation and characterization of two acaricidal compounds from *Calpurnia aurea* subsp. *aurea* (Fabaceae) leaf extract. Veterinary Parasitology.

Manuscript in preparation:

Adenubi, O.T., Njoya, E.M., Ahmed, A.S., Erhabor, J., Chipangura, J., McGaw, L.J., Eloff, J.N., Naidoo, V. Preliminary studies on the possible mechanism of action of *Calpurnia aurea* subsp. *aurea* (Fabaceae) leaf extract. Prepared for submission to Journal of Ethnopharmacology.

Conferences

Adenubi, O.T., Fasina, F.O., McGaw, L.J., Eloff, J.N., Naidoo, V. Using plant extracts to control ticks and tick-borne diseases: a review. The 41st Annual Conference of the South African Association of Botanists held in Tshipise Forever Resort, Venda, Limpopo Province, South Africa from the 11th-15th January, 2015. Oral presentation.

Sakong, B.M., **Adenubi, O.T.,** Dzoyem, J.P., Naidoo, V., Eloff, J.N. *In vitro* assessment of toxicity and selectivity index of selected South African medicinal plants. The 54th Society of Toxicology Annual Meeting and ToxExpo held in San Diego, California, United States of America from the 22nd-26th March, 2015. Poster presentation.

Sakong, B.M., **Adenubi, O.T.,** Dzoyem, J.P., Naidoo, V., Eloff, J.N. Antibacterial, anti-oxidant and cytotoxic properties of nine South African ethnoveterinary plants. Faculty Day held at the Faculty of Veterinary Sciences, Onderstepoort campus, Pretoria, South Africa on 20th August, 2015. Poster presentation.

Adenubi, O.T., Njoya, E.M., Ramadwa, T., Sakong, B.M., Eloff, J.N., McGaw, L.J., Naidoo, V. Assessment of *in vitro* acaricidal potential and cytotoxicity of crude extract of *Ptaeroxylon obliquum.* The 55th Society of Toxicology Annual Meeting and ToxExpo held in New Orleans, Louisiana, United States of America from the 13th-17th March, 2016. Poster presentation.

Adenubi, O.T., Fasina, F.O., McGaw, L.J., Eloff, J.N., Naidoo, V. *In vitro* bioassays for plant extracts with tick repellent and acaricidal properties: A systematic review and meta-analysis. 9th Joint Natural Products Conference held in Copenhagen, Denmark from the 24th-27th July, 2016. Poster presentation.

Adenubi, O.T., McGaw, L.J., Eloff, J.N, Naidoo, V. Phytochemicals - alternatives to synthetic chemical acaricides? Faculty Day held at the Faculty of Veterinary Sciences, Onderstepoort campus, Pretoria, South Africa on 25th August, 2016. Oral presentation.

Adenubi, O.T., McGaw, L.J., Eloff, J.N, Naidoo, V. Evaluation of the tick repellent and acaricidal properties of some South African ethnoveterinary plant species. Schlumberger Foundation Faculty for the Future Fellows Forum held at Cape Town, South Africa from the 28th-31st August, 2016. Oral and poster presentations.

Abstract

Farmers in developing countries are faced with many diseases that limit the productivity of their animals, several of which are caused by tick infestations. To date, treatment of host animals with synthetic, chemical tick repellents and acaricides remains the method used to reduce the influence of the parasites on animal and human health. Awareness of the environmental health hazards posed by these acaricides, development of tick resistance leading to recurrent ectoparasitism, danger of misuse and presence of toxic residues in food, water and animal by-products has led to the search for safe and environmentally-friendly alternatives, one of which is the use of medicinal plants.

Because there appears to be a need and to contribute to research in this field, extensive literature surveys of published scientific articles were conducted. The following aspects were addressed: the role of ticks in animal health, problems encountered in using synthetic, chemical acaricides, medicinal plants with *in vitro* acaricidal or tick repellent activities against immature and adult stages of ticks and bioassays employed. Veterinary databases (All Databases, CAB Abstracts and Global Health, Medline, Pubmed, Web of Science, BIOSIS Citation Index, Science Direct, Current Content Connect and Google Scholar) were searched. The search words included “acaricidal”, “tick repellent”, “medicinal plants”, “isolated compounds” and “antitick assays”. Meta-analysis was conducted using the Fixed-effect model in an Excel programme to compare the results.

The tick climbing repellency and adult immersion tests were the most commonly used assays to test for repellency and acaricidal activity respectively. Ethanol was the most commonly used extractant and *Rhipicephalus* (*Boophilus*) *microplus* was the most commonly studied tick across all the reviewed papers. More than 200 plant species from several countries globally have tick repellent or acaricidal properties discovered using *in vitro* assays. A large proportion of the published work was done in tropical countries such as Brazil, India and South Africa where ticks cause major problems. The median efficiency values for acaricidal, larvicidal, egg hatching inhibition, inhibition of oviposition, repellency, acaricidal effects of the Lamiaceae and Asteraceae family using a total of 1428, 1924, 574, 281 and 68 events were 80.12 ($CI_{95\%}$: 79.20 - 81.04), 86.05 ($CI_{95\%}$: 85.13 - 86.97), 83.39 ($CI_{95\%}$: 82.47 - 84.31), 53.01 ($CI_{95\%}$: 52.08 - 53.93), 92.00 ($CI_{95\%}$: 91.08 - 92.93), 80.79 ($CI_{95\%}$: 79.87 - 81.71) and 48.34% ($CI_{95\%}$: 47.42 - 49.26) respectively. Extracts of some species including *Azadirachta indica, Gynandropsis gynandra, Lavandula angustifolia, Pelargonium roseum* and *Cymbopogon* species have good acaricidal and larvicidal activities with 90-100% efficacy, comparable to those of currently used acaricides, although, usually at

higher dosages. Compounds with acaricidal activity such as azadirachtin, carvacrol, linalool, geraniol and citronellal were listed.

As a country, South Africa is rich in vascular plant flora, possessing over 10% of the world's vascular floral species. Only a fraction of its plants have been rigorously studied and analyzed for their biological activity against ticks and seventeen plant species based on their ethnoveterinary use in tick control were selected for this study. The plants are *Aloe rupestris* Baker, *Antizoma angustifolia* (Burch.) Miers ex Harv., *Calpurnia aurea* subsp. aurea (Aiton) Benth., *Cissus quadrangularis* L., *Clematis brachiata* Thunb., *Cleome gynandra* L., *Ficus sycomorus* L., *Gnidia deserticola* Gilg., *Hypoxis rigidula* Baker var. *rigidula*, *Maerua angolensis* DC., *Monsonia angustifolia* E. Mey. ex A.Rich., *Pelargonium luridum* (Andrews) Sweet, *Ptaeroxylon obliquum* (Thunb.) Radlk, *Schkuhria pinnata* (Lam.) Kuntze ex Thell., *Sclerocarya birrea* (A.Rich.) Hochst., *Senna italica* subsp. *arachoides* (Burch.) Lock. and *Tabernaemontana elegans* Stapf.

Crude extracts of the above mentioned plants were prepared using four different solvents (acetone, ethanol, ethanol/water and hot water). The extracts at a concentration of 200 mg/ml were screened for their acaricidal efficacy against adult *Rhipicephalus turanicus* ticks using the contact assay. The plant species with the highest acaricidal efficacies for their acetone and ethanol extracts were *C. aurea, S. pinnata* and *S. italica* with mortality of 97, 93, 90% and 93, 93, 87% respectively. The ethanol/water and hot water extracts of many of the plants had low acaricidal activities (<60%). An acaricidal dose-response bioassay of two-fold graded decreasing concentrations (100 to 3 mg/ml) of the acetone and ethanol extracts of *S. pinnata, C. aurea* and *S. italica* was determined using the adult immersion tests. The LC_{50} values for the acetone extracts were 35.75, 111.24 and 42.05 mg/ml respectively and for the ethanol extracts were 37.07, 98.69 and 37.50 mg/ml respectively compared with the positive control (cypermethrin) with LC_{50} of 2.41 mg/ml. In order to evaluate the potential safety of these plants, cytotoxicity against Vero and HepG2 cells was determined. Most of the plant extracts were non-cytotoxic to the two cell lines (LC_{50}>100 µg/ml) and there was a statistically significant higher toxicity to HepG2 cells compared with Vero cells. The ethanol/water and hot water extracts of most of the plants were less toxic to the cells (LC_{50}>1000 µg/ml) compared with their acetone and ethanol extracts. The selectivity indices of *S. pinnata, C. aurea* and *S. italica* were low. Particularly good acaricidal activities were displayed by *C. aurea* subsp. *aurea* extracted using four different solvents on *R. turanicus* ticks. The plant extract also had lower cytotoxicity against the cell lines tested and was selected as the most promising plant species, based on its efficacy and potential safety for further studies.

Solvent-solvent fractionation of the *C. aurea* ethanol extract was undertaken and five fractions, namely hexane, chloroform, butanol, water and methanol/water were obtained. The more non-polar fractions, hexane, chloroform and butanol were tested against *R. turanicus* ticks using adult immersion tests. There were dose-dependent effects with all the fractions and the chloroform fraction was the most active with up to 90% mortality at the highest concentration tested (100 mg/ml). Using bioassay guided silica gel column chromatography, two active flavonoids, apigenin-7-*O*-β-D-glycoside and isorhoifolin were isolated for the first time from *C. aurea*. Acaricidal dose-response bioassay of two-fold graded increasing concentrations (0.03-1 mg/ml) and cytototoxicity tests against Vero and HepG2 cells (5-100 µg/ml) of the isolated compounds were undertaken. Both compounds had relatively low cytotoxicity to the two cell lines (LC_{50}=70-100 µg/ml).

Both isolated compounds had good acaricidal activities and the LC_{50} value of 0.65 mg/ml observed for isorhoifolin compares favourably with some known acaricidal compounds isolated from plants and synthetic, chemical acaricides. The LC_{50} of carvacrol has been reported to be 0.22 and 4.46 mg/ml and that of thymol as 3.86 and 5.50 mg/ml against larvae and engorged females of *R. (B.) microplus* respectively. Other results found are azadirachtin (LC_{50}=5 mg/ml) against engorged females of *R. (B.) microplus;* menthol (LC_{50}=0.13 mg/ml) and synthetic, chemical acaricides such as DDT (LC_{50}=36.8 mg/ml); fluralaner (LC_{50}=0.28 mg/ml); coumaphos (LC_{50}=0.39 mg/ml) and spinosad (LC_{50}=0.11 mg/ml).

Pilot studies to investigate the possible mechanism of action of the crude extract and isolated compounds were undertaken. Ellman's acetylcholinesterase inhibition assay (*in vitro*) was undertaken for the crude extract and fractions of *C. aurea.* There was only 40% acetylcholinesterase inhibition observed for the crude extract, but a promising 90% acetylcholinesterase inhibition for the butanol fraction. The chloroform fraction from which the compounds were isolated did not inhibit acetylcholinesterase. A further attempt was then made to investigate the mechanism of action *ex vivo* (using isolated tissue bath studies) on cholinergic receptors and ion channels in comparison with standard agonists and antagonists. The results tend to suggest that the *C. aurea* crude extract and isolated compounds induced their effect through choline esterase and potassium channel inhibition in the rat ileum assay. These results suggest that synergism of different phytochemicals in *C. aurea* may be responsible for its multiple mechanisms of action.

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List of Abbreviations

^{1}H	Proton
^{13}C	Carbon-13
1D	One dimensional
2D	Two dimensional
AChE	Acetylcholinesterase
AChI	Acetylcholine iodide
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ASF	African Swine Fever
BEA	Benzene/ethanol/ammonia hydroxide
BkCa	Calcium-activated potassium channel
BPTI	Basic Pancreatic Trypsin Inhibitor
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
CCHF	Crimean-Congo haemorrhagic fever
CEF	Chloroform/ethyl acetate/formic acid
CI	Confidence Interval
Cl^{-}	Chloride
CNS	Central Nervous System
CO_2	Carbon dioxide
COSY	Correlation Spectroscopy
COX	Cyclooxygenase
CSIR	Council for Scientific and Industrial Research
DDT	Dichlorodiphenyltrichloroethane
DEET	N, N-diethyl-meta-toluamide
DMEM	Dulbecco's Minimum essential medium
DMSO	Dimethyl sulphoxide
DTNB	5,5'-dithio-bis(2-nitro-benzoic)acid
EC	Effective concentration
EMW	Ethyl acetate/methanol/water
ESI-MS	Electrospray ionisation mass spectroscopy
f	Factor
FCS	Foetal calf serum
Fig	Figure
FUNAAB	Federal University of Agriculture, Abeokuta
GABA	γ- aminobutyric acid
Glu	Glutamate
IGR	Insect growth regulator
HMBC	Heteronuclear multiple bond correlation
HRMS	High Resolution Mass Spectrometry
HRESIMS	High resolution electrospray ionisation mass spectrometry
K^{+}	Potassium ion
KCl	Potassium chloride

KH	Krebs-Henseleit
KH_2PO_4	Potassium phosphate monobasic
LC	Lethal concentration
LD	Lethal dose
LOX	Lipooxygenase
mAchR	Muscarinic acetylcholine receptor
MEM	Minimum essential medium
MEV	Median efficiency value
$MgSO_4$	Magnesium sulphate
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Not active
nAchR	Nicotinic acetylcholine receptor
Na^+	Sodium ion
$NaHCO_3$	Sodium bicarbonate
ND	Not determined
NMR	Nuclear Magnetic Resonance
NSD	Nairobi sheep disease
OH	Hydroxyl
Osp	Outer surface protein
PBS	Phosphate Buffered Saline
Picaridin	1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester
PMD	Para-menthane-3,8-diol
RF	Resistance factor
SEM	Standard error of mean
SI	Selectivity index
SS220	1S,2S-2-methylpiperidinyl-3-cyclohexene-1-carboxamide
TBE	Tick-borne encephalitis
TLC	Thin Layer Chromatography
UPBRC	University of Pretoria Biomedical Research Center
UV	Ultraviolet
WHO	World Health Organisation

CHAPTER 1

Introduction

1.1. Background

Animals are an integral part of human lives, as sources of food, clothing, transportation or companionship (Delgado et al., 1999). Infections of domestic and wild animals that are transmitted directly or indirectly by ticks are major causes of morbidity and mortality (Norval et al., 2004) and the overall production losses in Africa due directly or indirectly to ticks has been estimated at US$720 million per year (Kaaya & Hassan, 2000).

To date, chemical treatment of host animals with acaricides remains the method of choice to reduce the influence of the parasites on animal and human health. Unfortunately, years of use, overuse and in many cases abuse of the available active pharmaceutical compounds have resulted in large scale resistance in these parasites. Other problems with acaricide overuse include accumulation of these chemicals in the environment with resultant pasture and water toxicity (van Wieren et al., 2016). In an attempt to reduce these problems, much focus has been placed on the search for alternative tick control strategies that are devoid of resistance and are environmentally friendly. Among other control measures, the development of medicinal plants as tick repellents/acaricides has been advocated (Gassner et al., 1997).

Plant preparations have been used for treatment of different ailments in animals from time immemorial and form the bulk of the growing field of ethnoveterinary medicine (McCorkle, 1995). South Africa is endowed with a rich flora, with only a fraction of its plants having been rigorously studied and analyzed for their biological activity against ticks. With an estimated 87% of medications used globally against microbial and parasitic infections being derived from natural products, in particular higher plants, the benefits of ethnoveterinary medicine in primary animal healthcare are clearly evident (Martin et al., 2001). Possibly, bioactive products from plant extracts, fractions and isolated compounds may constitute prototypes for the development of promising alternatives to chemical acaricides.

1.2 Hypotheses

- Some plants used traditionally in tick control may contain bioactive compounds.
- The mechanism of action of acaricidal compounds may be different from known pathways.

- It is possible to develop a herbal remedy that is effective against one or more of the economically important ticks of the tropics and sub-tropics.

1.3 Problem statement

Ticks and tick-borne diseases are major causes of morbidity and mortality in livestock and companion animals. Resistance is a serious drawback of available chemical acaricides. The exploration and bioprospecting of plants with acaricidal properties is a potential solution to the problem.

1.4 Aim

To evaluate extracts, fractions and isolated compounds from South African plant species with documented ethnoveterinary use against ticks for their acaricidal properties and cytotoxicity, in a bid to find leads for the development of a safe and effective tick control product.

1.5 Objectives

- To provide an extensive literature survey on the role of ticks in animal health and the problems encountered in using synthetic, chemical acaricides.
- To provide an extensive literature survey on the use of plant extracts as alternatives to combat ticks globally.
- To provide an extensive literature survey and meta-analysis of results of work done on plant extracts used to combat ticks.
- To provide an extensive literature survey on *in vitro* methods used to evaluate plants with tick repellent and acaricidal properties.
- To determine the efficacy against ticks and safety of seventeen plant extracts used in ethnoveterinary medicine using *in vitro* bioassays.
- To select the most promising plant species, isolate the active compound(s) using bioassay-guided fractionation and identify the purified compounds using spectroscopic techniques.
- To confirm the activity of the isolated compound(s).
- To investigate the mechanism(s) of action of the most promising plant species and isolated compound(s) in pilot studies.
- To recommend follow up work to be done.

The objectives are addressed in chapters 2 to 8. Chapter 9 comprises the general conclusions and recommendations for further work. All the references in the thesis have been collated in chapter 10.

CHAPTER 2

The role of ticks in animal health and problems encountered in using synthetic, chemical acaricides

2.1 Economic importance of ticks

In the tropical and subtropical regions, small-scale and emerging farmers own 40% of their national livestock (Keyyu et al., 2003a). While small-scale farmers face many constraints in their farming activities, the most important limiting factor on the productivity of their animals is the prevalence of ticks and the diseases they carry. The latter is particularly important in the wet season as the warm climate of the tropics and sub-tropics enables many species of ticks to flourish, while the large populations of indigenous wild animals also provide a constant reservoir for ticks and infectious organisms. Companion (or pet) animals, in particular dogs, also pay a heavy toll to ticks and tick-borne diseases not only in the tropics, but also in many of the temperate zones of the world (Jongejan & Uilenberg, 2004).

Ticks are haematophagous arthropods ranked close to mosquitoes in their capacity to transmit diseases (viral, bacterial, rickettsial and protozoal) which can be severely debilitating or fatal to livestock, companion animals and humans (Jongejan & Uilenberg, 2004). They are the most economically important ectoparasites of domestic animals and man (Mans et al., 2000). Ixodid ticks such as *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1888), *R.* (*B.*) *decoloratus* (Koch, 1844), *R. turanicus* (Pomerantsev, 1936) and *R. sanguineus* (Latreille, 1806) are among the most economically important parasites in the tropics and subtropics (Rajput et al., 2006). It is estimated that the global economic burden due to ticks and tick-borne diseases on animal health ranges from US$ 13.9 to 18.7 billion annually (De Castro et al., 1997). In Africa, tick-borne diseases kill nearly 1.1 million cattle annually with resultant economic losses of US$168 million (Minjauw & McLeod, 2003). In addition to transmitting diseases, heavy tick infestations cause reduction in animal weights with resultant drops in meat production, damage to hides (Fig. 2.1), severe anaemia in domestic animals (Fig. 2.2) and severe wounds at bite sites (Rajput et al., 2006) (Fig. 2.3).

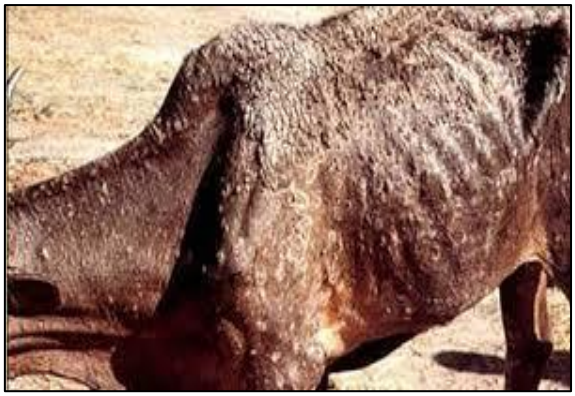

Figure 2.1 Severe tick infestation on an adult bovine with resultant major reduction in live weight and damage to hides. This is evident by the visible ribs and scars on the skin (entnemdept.ufl.edu)

Figure 2.2: Pale mucous membrane of the gums in a dog as a result of heavy tick infestation and subsequent anaemia (www.abc.net.au)

Figure 2.3: Numerous ticks attached to the udder. These ticks can cause severe wounds to the udder with resultant drop in milk production (influentialpoints.com)

2.2 Typical ticks of domestic animals

Ticks belong to the suborder Ixodida which are part of the phylum Arthropoda, of eight joint-legged invertebrate animals (Evans, 1992). They are grouped with spiders, scorpions, whip-scorpions, sun spiders, harvestmen, mites, false scorpions and solifuges in the class Arachnida. They are of the subclass Acari (together with mites) and with the Holotryrida and Mesostigmata, form the order Parasitiformes (Anderson, 2002) (Fig. 2.4). Their bodies are divided into two regions: the anterior region (the capitulum or gnathosoma), which contains the head and mouthparts; and the posterior region (the idiosoma) which has the legs, digestive tract and reproductive organs (Wall & Shearer, 2001).

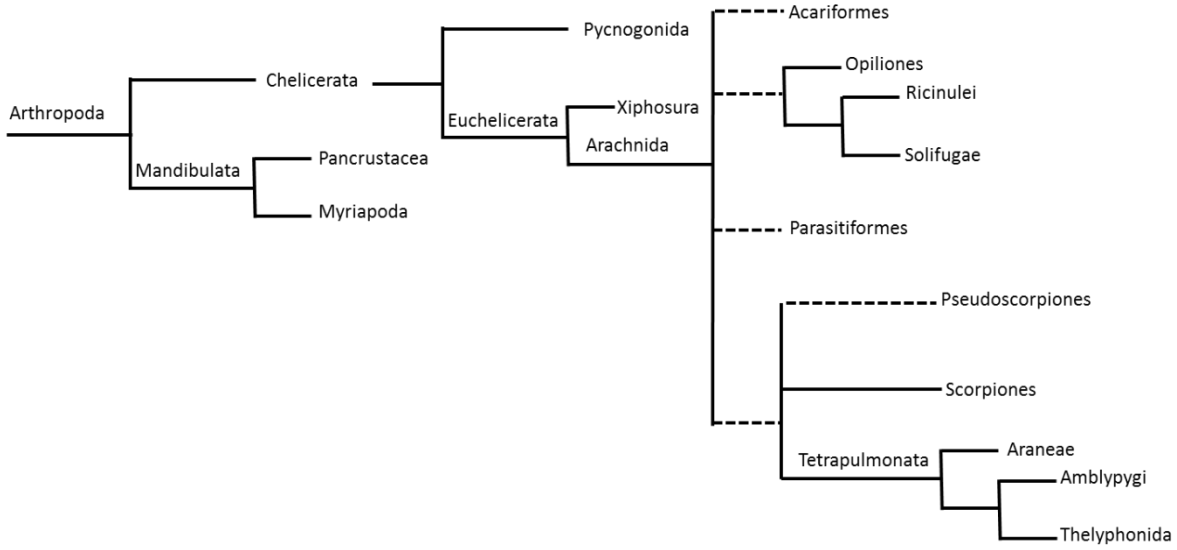

Figure 2.4: Dendrogram of the phylogenetic relationships of ticks in the phylum Arthropoda (Hoogstraal & Aeschlimann, 1982)

There are at least 898 recognized species of ticks, distributed among three families: Ixodidae (C.L. Koch, 1844) (hard ticks - 703 species), Argasidae (C.L. Koch, 1844) (soft ticks - 194 species) and Nuttalliellidae (Schulze, 1935) (intermediate - 1 species) (Norval et al., 2004) (Table 2.1; Fig. 2.5; 2.6).

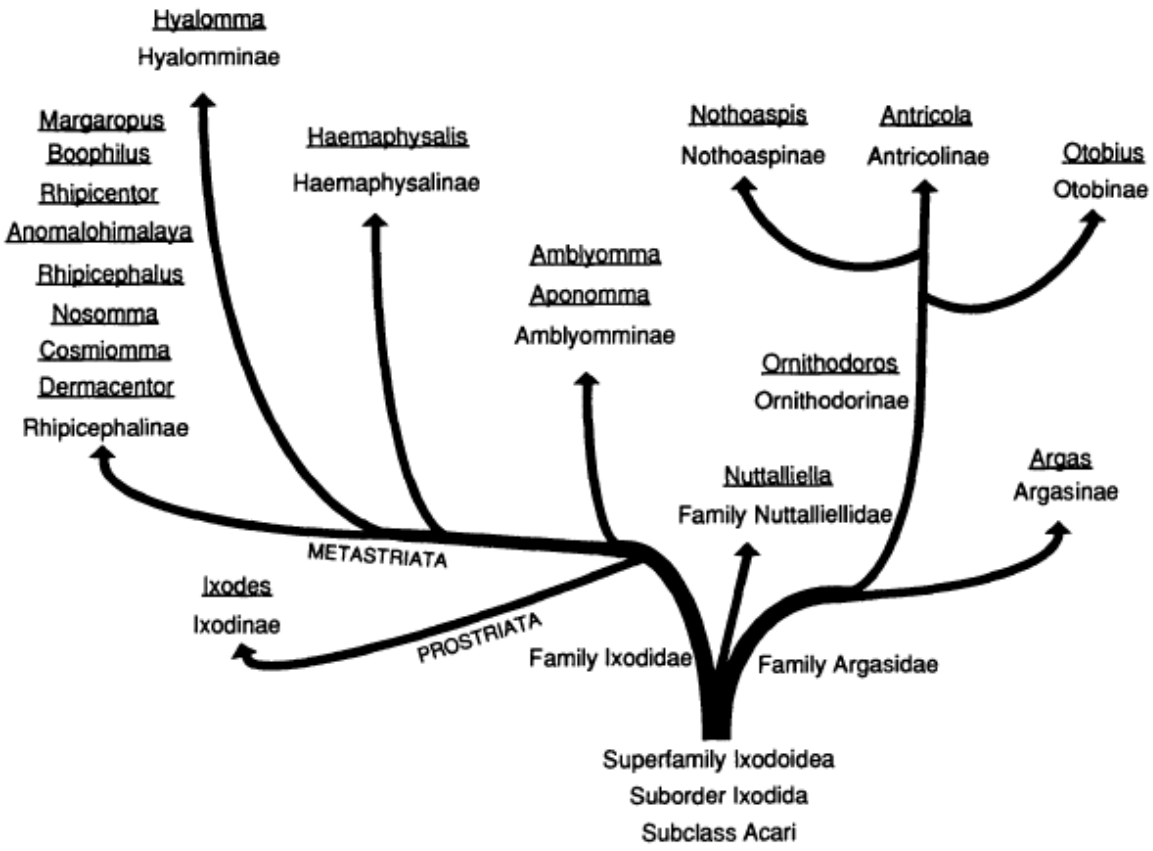

Figure 2.5: Modification of the dendrogram by Hoogstraal & Aeschlimann (1982) showing phylogenetic relationships within the Superfamily Ixodoidea

Table 2.1: General characteristics and differences between Ixodids (hard ticks) and Argasids (soft ticks)

Characteristics		**Ixodids**	**Argasids**
Morphological differences	Scutum (dorsal shield)	The scutum is small in females but covers the entire dorsal surface in males	The scutum is absent (small, soft and leathery cuticle)
	Capitulum (mouthparts)	Anterior, can be seen from the dorsal view	Ventral, cannot be seen from the dorsal view
	Pedipalpi	Strong, non-movable	Movable, active
	Position of spiracles	Behind the base of the fourth pair of legs	Between the third and fourth pair of legs
	Differences of sexes	Evident as the males are visibly smaller	Not evident, the shape of the genital opening distinguishes the sexes. It is circular or crescent-shaped in males and transverse split, wider than long in females
	Salivary gland	Multiple granular acini which develop and change before rapid engorgement	One simple granular acinus which is fully developed before feeding
Life cycle and behavioural differences	Habitat	Live outdoor, search for host during the day (non-nidicolous)	Live in sleeping places of host (nidicolous)
	Host	1-3 hosts	More than 10 hosts
	Mating	On host	Off host
	Nymphal stage	1 instar	Several (5-7 instars)
	Adult feeding	Once, remains attached and completes feeding in days	Drops off a host intermittently to attach to another host, feeding up to 12 times
	Regulation of water balance	Secrete excess water in blood meal via salivary glands	Secrete excess water in blood meal via coxal organs
	Blood meal digestion	Rate of feeding and digestion is slow during the preparatory feeding phase when tick grows to allow rapid engorgement	Feed first, digest later. First stage of digestion includes development of midgut epithelium
	Egg laying events	1 batch	Several batches
	Total eggs laid	3000-8000	400-500
	Life span	2 months-3 years	Up to 16 years

Data obtained from Mans & Neitz (2004a)

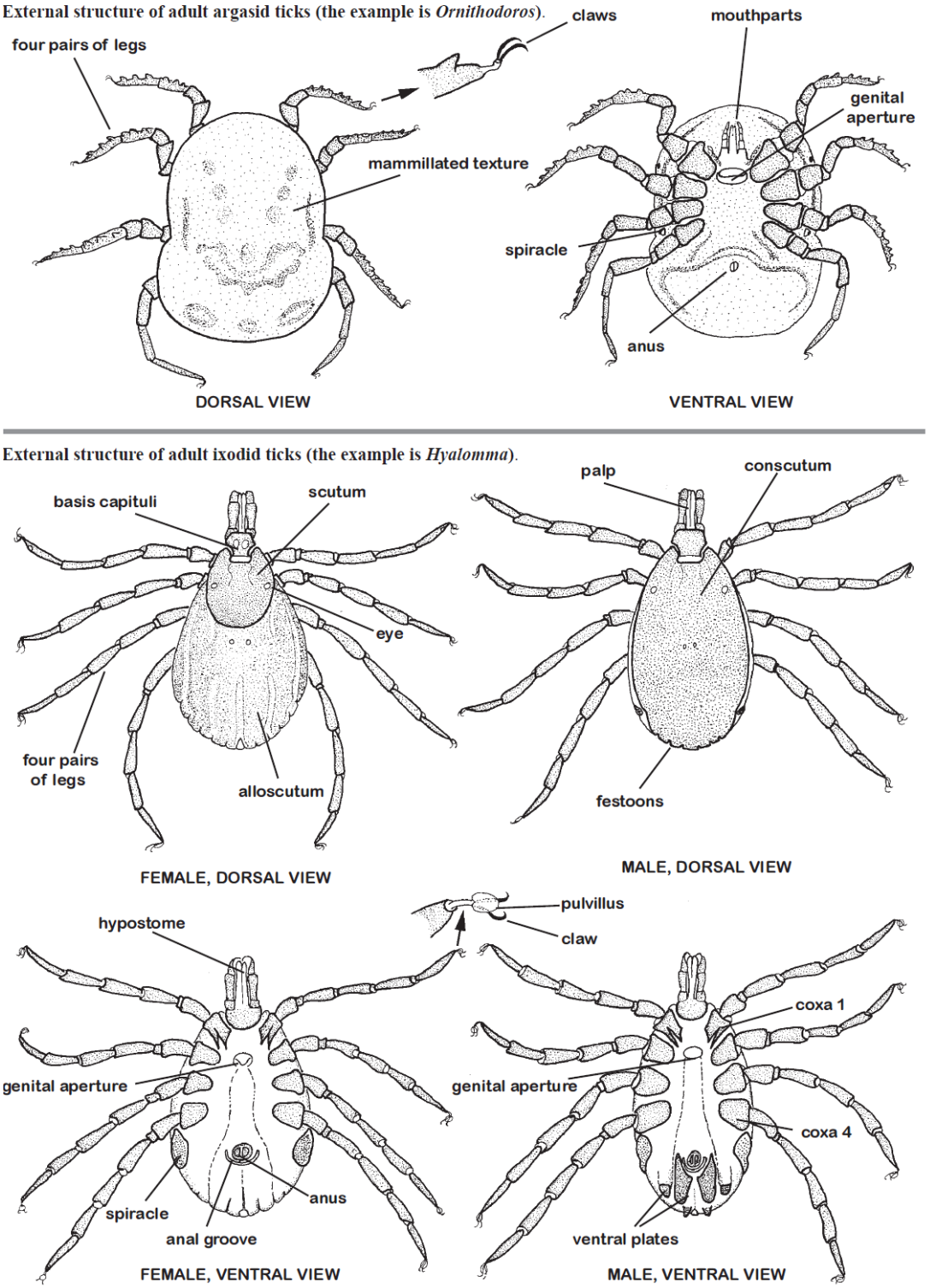

Figure 2.6: Morphological differences between ixodids and argasids. In ixodids, the mouthparts can be seen from the dorsal surface, the scutum is present and adult male ixodids possess the ventral plates (Walker, 2003)

The intermediate family Nuttalliellidae has one species, *Nuttalliella namaqua.* This tick is found in Tanzania and southern Africa from Namibia to South Africa (Guglielmone et al., 2010). It has a partly sclerotized pseudo-scutum, an apical positioned capitulum, a leathery integument with few denticles on its hypostome and has been described as the ‘‘evolutionary missing link’’ between the ixodids and argasids (Mans et al., 2011; Latif et al., 2012). (Fig. 2.7). Although no evidence exists to give definitive information on its life stages, host preference and feeding habits, it has been suggested that rock hyraxes, swallows, rodents and lizards could be preferential hosts. Efforts to feed adult females and nymphs on chickens, pigeons, rabbits, rats or mice have been unsuccessful (Mans et al., 2014).

Figure 2.7: A dorsal view of a fully engorged female *Nuttalliella namaqua* tick showing the pseudo-scutum, visible mouthparts from the dorsal view and leathery integument (blogs.scientificamerican.com)

The Ixodidae is the dominant tick family, with regard to the number of species and their veterinary and medical importance (Tsatsaris et al., 2016). In Africa, of the ten genera of ticks that commonly infest domestic animals, seven are ixodids while three are argasids. Mammals usually serve as hosts more so than birds and reptiles, and at least one species of each tick genus utilizes mammals as hosts. Mammals serve as the chief host for members of the Rhipicephalinae and *Hyalomma*, while a smaller percentage of *Ixodes* and *Haemaphysalis* species parasitize mammals. Many *Amblyomma* species parasitize mammals, birds and reptiles (Turner et al., 2017).

Amblyomma species are large ixodid ticks (6-7 mm), widespread on domestic animals throughout the tropics and sub-tropics. There are 130 *Amblyomma* species which include *A. americanum* (Linnaeus, 1758), the lone-star tick of southern and eastern United States of America and *A. cajennense* (Fabricius, 1787), the Cayenne tick of South America and southern USA. *Amblyomma variegatum* (Fabricius, 1794), the tropical bont tick (bont is the

Afrikaans term for multicoloured) (Fig. 2.8) is one of the most widely distributed ticks on livestock in Africa due to its ability to survive in different environmental conditions, from dry savannahs to more humid regions (Fig. 2.9). It is the most predominant vector of the proteobacterium *Ehrlichia ruminantium* (*Cowdria ruminantium*), the causative agent of cowdriosis (heartwater) in cattle, sheep and goats (Walker, 2003).

Figure 2.8: Female (right) and male (left) ticks of *A. variegatum* with long mouthparts, legs with pale rings and characteristic bright yellow-gold scutum (www.caribvet.net)

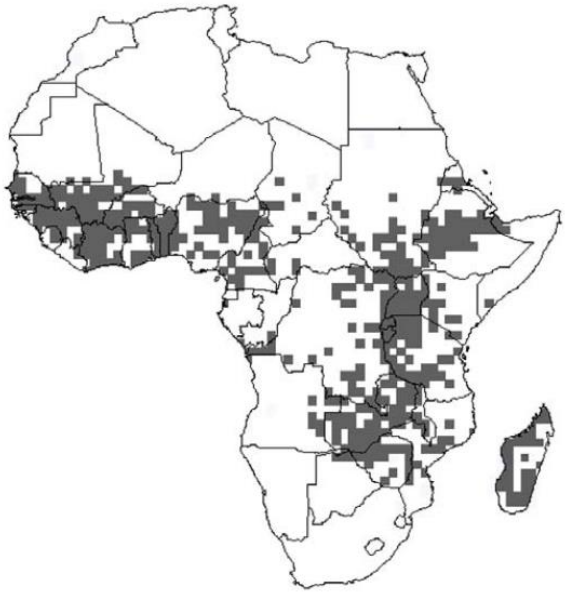

Figure 2.9: *Amblyomma variegatum* tick is widely distributed in Africa and has also been reported in Madagascar (Walker, 2003)

The *Rhipicephalus* species (82 species) is widely distributed in southern Africa, eastern Africa and Madagascar. Typical *Rhipicephalus* species in Africa include *R. turanicus* (Pomerantsev, 1940) which is closely related to *R. sanguineus* (Ioffe-Uspensky et al., 1997) (Fig. 2.10; 2.11), the southern cattle tick, *R.* (*B.*) *microplus* (Canestrini, 1888) (Fig. 2.12; 2.13) and *R. appendiculatus* (Neumann, 1901), commonly called the brown ear-tick, due its colour and preference for feeding on the ears of cattle (Fig. 2.14; 2.15; 2.16).

Figure 2.10: Male (left) and female (right) of *Rhipicephalus turanicus* ticks with broad and long capitulum and scutal punctations (www.saudiwildlife.com)

Figure 2.11: Distribution of *Rhipicephalus turanicus* ticks in Africa. The tick occurs in the northern part of Africa, mainly in Morocco, Algeria and Tunisia; Ethiopia and Somalia in the east and southern Africa (Walker, 2003)

Figure 2.12: Male (left) and female (right) ticks of *Rhipicephalus (B.) microplus*. They possess short, straight capitulum, oval body which is brown to blue-gray in colour and no festoons (nhc.ed.ac.uk)

Figure 2.13: Distribution of *Rhipicephalus (B.) microplus* in Africa. The tick is widely distributed in southern Africa and Madagascar (Walker, 2003)

Figure 2.14: Male (left) and female (right) ticks of *Rhipicephalus appendiculatus* showing the dark, plain scutum, short mouthparts, clear lateral grooves and posterior festoons (influentialpoints.com)

Figure 2.15: *Rhipicephalus appendiculatus* ticks feed on cattle, goats, sheep and dogs. The preferred feeding site of the adults is the outer ear (influentialpoints.com)

Figure 2.16: Distribution of *Rhipicephalus appendiculatus* ticks in Africa, from southern Sudan, through to the south eastern coast of South Africa (www.afrivip.org)

2.3 Behavioural biology of ticks

2.3.1 Life cycle

Ticks have a life cycle consisting of four stages:

- Eggs: After mating and the completion of blood meal, female ticks drop off the host and seek sheltered environments to lay their eggs (Fig. 2.17). The pre-oviposition period [period between engorgement and the commencement of oviposition (egg laying)] varies, depending on the species and environmental temperature. The number of eggs produced is largely dependent on the volume of blood meal taken as females that consume larger blood meals lay more eggs due to the relationship between energy input and output (Sonenshine, 1993).

Figure 2.17: A female ixodid tick laying thousands of eggs in one batch (animals.howstuffworks.com)

- Larvae: Eggs hatch into the first immature stage, the larvae (seed ticks), which have six legs (Fig. 2.18). Unfed larvae climb the vegetation or other surfaces in their natural environment and may seek for a host or enter diapause (a period of suspended development especially during unfavourable environmental conditions such as during winter) until appropriate environmental changes initiate the next state. Because it might prove difficult to find a suitable host, larvae can withstand long periods without feeding as their gut is stretched by remnants of the embryonic yolk, which is slowly digested. For most tick species, following host contact, larvae insert their mouthparts into the host skin, feed rapidly (15-30 minutes) and drop to moult in the sand, cracks and crevices of the natural habitat. Engorgement is completed within several days, depending on the species and host. The engorged larvae moult into unfed eight-legged nymphs (Sonenshine, 1993).
- Nymphs: Nymphs have eight legs like the adult ticks but they do not have a genital opening (Fig. 2.18). They are also able to live without feeding for a long period until a

suitable host is found. Ixodids have only one nymphal instar while argasids may have several (Anderson & Magnarelli, 2008). Male argasids usually emerge sooner than females and require one or two fewer nymphal stages than do females. Nymphs commence questing and the entire cycle of host contact, attachment, feeding, engorgement and detachment is repeated. Detached engorged nymphs drop off and moult into unfed adults (Sonenshine, 1993).

- Adults: Following the nymphal moult, ixodid adults (Fig. 2.18) attach to preferred hosts, feed, mate and the females drop to oviposit in some sheltered environment. However, argasid adults may first mate in the environment before attacking suitable hosts. Following host contact, adults feed rapidly, engorge and drop off, completing the life cycle (Sonenshine, 1993).

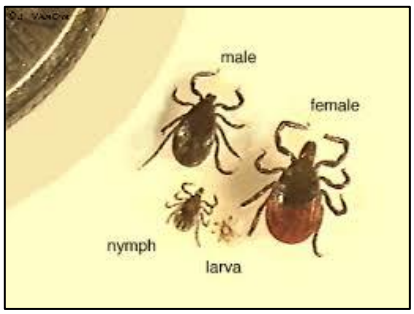

Figure 2.18: Larva (six-legged), nymph (eight-legged, smaller than adult with no genital opening), adult female tick on the right (eight-legged, larger than the male), adult male tick on the left (eight-legged, smaller than the female) (ent.iastate.edu)

The tick life cycle can be further divided into the hosts they feed upon:

- One-host ticks: This occurs in all the *Boophilus* sub-genus of the *Rhipicephalus* genus and in other genera (Fig. 2.19). Eggs are laid on the soil, larvae hatch after several weeks of development and crawl onto vegetation to seek for a host. When they have completed feeding, they remain attached to the host where moulting occurs. The nymphs and adults also feed on the same host. This life cycle is usually rapid and for *R.* (*B.*) *microplus*, it takes three weeks for feeding on the host and two months for egg laying and larval development (Walker, 2003).
- For the two-host life cycle, the larvae and nymphs feed on the same host while the adults feed on another. *Hyalomma detritum* and *R. evertsi evertsi* have two-host life cycles (Walker, 2003).

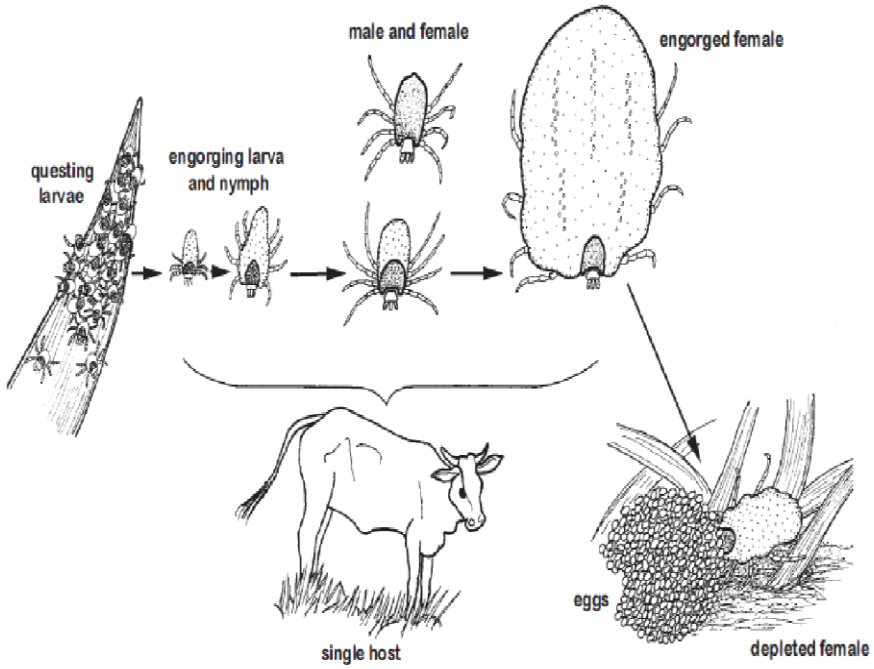

Figure 2.19: One-host life cycle. All the stages of the tick life cycle (larvae, nymphs and adults) feed on the same host (afrivip.org)

- Three-host ticks: This is the most common developmental pattern and is characteristic of the vast majority of tick species. Each life stage (larvae, nymphs and adults) seek for suitable hosts, feed, drop and moult off the host (Fig. 2.20). Under favourable conditions in the natural environment, the life cycle of such three-host ticks, from the time of hatching of the larvae to the hatching of the next generation, can be completed in less than one year. However, climatic conditions and diapause may delay host-seeking behaviour and onset of oviposition, such that only one life stage can be completed in a year. This could extend the duration of the life cycle to as much as three years in *I. ricinus* (Walker, 2003).

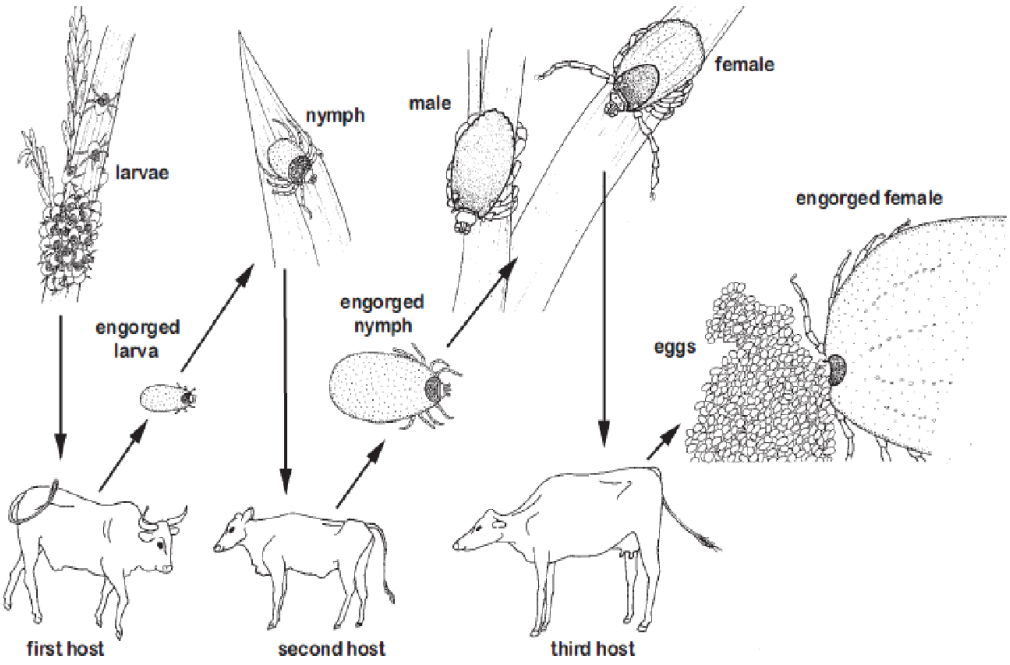

Figure 2.20: Three-host life cycle. Each life stage (larvae, nymphs and adults) of ticks seek for suitable hosts, feed, drop and moult off the host (afrivip.org)

2.3.2 Host-seeking

Ticks find their hosts in two main ways:

1) Exophilic or non-nidicolous behaviour: This is exhibited by tick species that live in the forest, savannah, scrubs or meadow vegetation; sandy soils, under stones and crevices. Most ixodids are non-nidicolous ticks, at least in some stage of their life cycle. Appetence, which is the locomotory hunting for a host or seeking one from a vantage point, initiates the series of behavioural responses that leads to host contact and successful parasitism. Appetence is preceded by hunger, which in turn is influenced by the tick's physiological condition (Kebede, 2004).

Non-nidicolous ticks may be further divided into ambushers and hunters. Ambushers (passive strategy) living in grass, herb or brush covered habitats climb the vegetation and cling to the tips of stems or branches where they wait for direct contact with hosts that brush against these vegetative supports. The ticks rest in the questing pose (Fig. 2.21), sometimes with the anterior end of the body pointed down towards the ground (Bowman & Nuttall, 2008). This behaviour is exhibited by ticks (larvae, nymphs and adults) in the genera *Rhipicephalus*, *Haemaphysalis* and *Ixodes.* The height at which ticks quest is strongly correlated with the specific life stage and size of the most common hosts of each species or life stage (Goddard, 1992). Immature ticks tend to stay near the base of vegetation or leaf

layer, where small mammals and birds are active, while adults generally quest near the tips of vegetation where they attack larger animals.

Adult ticks of the genera *Amblyomma* and *Hyalomma* are however hunter ticks (active strategy). They respond to host-originated stimuli by emerging from their refuges and rapidly seek out their host by crawling or running towards the source of stimuli. Such ticks stay underground and emerge to attack hosts when these animals appear nearby. They may also go across distances of many metres to attack and feed on the hosts for which they are adapted. Vibrations caused by animal movements as well as odours, body heat and shadows from such hosts excite tick responses, causing extension and rapid waving of the forelegs. If contact is made, the excited ticks cling to the bodies of the animals as they brush past (Sonenshine et al., 2002).

Host-finding strategies may also differ in different life stages. The larvae of *A. variegatum* and *A. hebraeum* ticks find their hosts by questing while nymphs and adults are hunter ticks. *Amblyomma americanum*, on the other hand, exhibits both the ambush and hunter strategies.

Figure 2.21: Questing pose. Tick cling to the tip of vegetation with forelegs stretched out waiting for direct contact with hosts that brush against these vegetative supports (santabarbara.realestatetours.com)

2) Endophilic or nidicolous behaviour: Argasids and some ixodids (especially *Ixodes* species) live in secluded enclosures such as caves, burrows and nests of their hosts. They respond to the same spectrum of host stimuli as non-nidicolous ticks, however, the range at which these stimuli are perceived is considerably shorter. The relative importance of different stimuli may vary greatly among nidicolous tick species. In some, such as endophilous nidicoles, host body heat and odours are of paramount importance, since the distance from parasite to host is extremely short. For harborage-infesting parasites, they must migrate over considerable distances to reach their hosts. Gravity, carbon dioxide and even sound serve

as general excitants, bringing the searching ticks to the point where shorter range stimuli lead the parasites to the host body.

2.3.3 Host stimuli

Host-seeking ticks recognise a variety of stimuli from prospective hosts which stimulate their host-finding behaviour. Odours are the most important and best studied stimuli, which provide specific information and when carried on wind currents, also provide directional information for haematophagous arthropods and insects (Barrozo & Lazzari, 2004). Among the most important host-originated odours are carbon dioxide, a component of animal breath and ammonia, from animal wastes. Carbon dioxide and ammonia attraction brings hungry ticks into close proximity to potential hosts, after which the shorter range stimuli such as body heat from the host and odourants characteristic of sweat (lactic acid or butyric acid), become effective (Sonenshine et al., 2002).

Other stimuli which ticks use in host-finding activities include visual cues and vibrations. Visual images are probably most important in hunter ticks, which are believed to discriminate dark shapes against the bright background of the sky. Vibrations are also excitatory as rustling the vegetation on which ticks are perched in ambush will provoke their characteristic "grabbing" behaviour, with the forelegs outstretched to cling to a passing host (Sonenshine et al., 2002). Some species of ticks respond to sounds within a particular range of frequencies. *Rhipicephalus* (*B.) microplus* larvae are highly responsive to sounds in the 80-800 Hz range, frequencies commonly emitted by feeding cattle, while *R. sanguineus* ticks are attracted to the sounds made by barking dogs (Waladde & Rice, 1982). Finally, tactile stimuli come into play only upon host contact, contributing along with short-range odourants to the selection of the feeding site, attachment and the commencement of feeding.

2.3.4 Feeding

Irrespective of the species, all ticks are obligate blood feeders (haematophagous) with 90% of them being specific for particular hosts that normally do not include domestic animals and humans (Sonenshine & Roe, 2013). The remaining 10%, however, are of immense concern due largely to their non-specific parasitic nature and ability to transmit pathogenic organisms to mammals, birds, reptiles, amphibians and humans (who are mainly accidental hosts) (Dantas-Torres et al., 2006).

To feed, ticks crawl onto their host and attach to the skin with their mouthparts which consist of the chelicerae (moveable rods with sharp claws at the end), hypostome and palps. The cheliceral sheath and the toothed hypostome form a tube which cuts a hole and breaks the capillary blood vessels very close to the surface of the skin, forming a feeding lesion

(Walker, 2003) (Fig. 2.22). A material (cement) is secreted in the tick saliva which glues the palps to the epidermis (the surface epithelium of the skin overlying the dermis) and the chelicerae and hypostome to the dermis. The cement consists of two main components: a cortex of carbohydrate-containing protein, stabilized by quinone and disulphide linkages and an internum which is lipoprotein in nature (Moorhouse & Tatchell, 1966).The start of the secretion of cement and the initial cutting of the host tissues by the chelicerae occur simultaneously. Final rapid engorgement of each life stage is preceded by a secondary secretion of cement into a fluid-filled cavity which forms directly beneath the mouthparts and provides additional support (Walker, 2003).

The feeding of female ixodids is slow because their body wall needs to grow and expand to be able take in the very large blood meal required for maturation of the ovaries and to provide nutrition for the thousands of eggs laid in a single batch. Larvae typically take three to five days to fully engorge with blood, nymphs four to eight days and adults five to twenty days. Growing males feed but do not expand like the females, as they only need enough for their reproductive organs to mature. The males in the genus *Ixodes* however, have matured reproductive organs when they moult from the nymphal stage into adults and do not need to feed further (Walker, 2003).

In contrast, argasids feed more rapidly for up to several hours. They only take small, repeated blood meals in each stage of their life cycle as the females are capable of laying a few to several hundred eggs more than once during their lifetime. They also do not form complex attachments to the hosts' skin as they have varied hosts (drop off frequently to re-attach to another host) and mate off the host (Sonenshine, 1993).

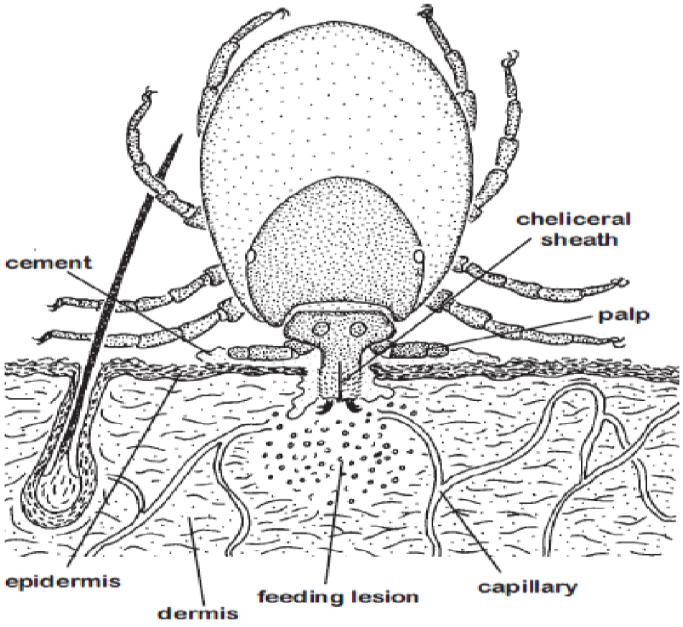

Figure 2.22: The mouth parts of a hard tick cut a hole through the epidermis into the dermis of the host's skin rupturing the capillary blood vessels very close to the surface of the skin, forming a feeding lesion. The tick then feed on the blood and lymph released into this lesion. The cement secreted in the saliva glues the palps to the outer epidermis and the chelicerae and hypostome to the dermis (Walker, 2003)

Several factors favour the success of ticks as ectoparasites. Not the least is a remarkable resilience off the host where the tick can survive for months without taking a blood meal (Needham & Teel, 1991). During non-feeding periods, ticks avoid dehydration and maintain their water balance. The waxy lipids of their cuticle provide a high degree of water proofing and the mechanical spiracle closing devices provide an efficient means of restricting water loss. In ixodid ticks also, blood digestion is a slow, intracellular process divided into three stages; the preparatory stage which is the concentration of the blood meal by elimination of water and sodium ions, the growth stage, which is the phase of intensive digestion and the expansion stage characterized by a very slow rate of digestion during which the tick may endure long periods of starvation (Jones et al., 2015).

2.3.5 Mating

The finding of a suitable mate involves a number of stereotyped behaviours that are triggered by aggregation-attachment pheromones such as 2-nitrophenol and methylsalicylate and sex pheromones such as 2, 6 dichlorophenol. All male ixodids undergo a courting ritual before they can deposit their spermatophores within the female's genital tract. These diverse and prolonged patterns of sexual interaction provide opportunities for interactions between populations and individuals that may be relevant to the role of ticks as vectors of zoonotic pathogens (Kiszewski et al., 2001).

The male reproductive system consists of paired testes which appear as elongate sacs with the distal ends bluntly rounded and joined by a filamentous strand of tissue. Proximally, the testes taper into the paired, convoluted vasa deferentia which join anteriorly forming a single duct, the common vas deferens connecting the testes with the ejaculatory duct. The large, multilobed male accessory glands open into the common vas deferens near the junction of the vasa deferentia. All ticks transfer uncapacitated spermatids via a spermatophore (a shell-like structure formed from a coagulum of mucopolysaccharides and proteins produced by male accessory glands). There are two endospermatophores in each ectospermatophore in argasids and one per ectospermatophore in ixodids. How the spermatophore is moved from the male gonopore to that of the female is not known but the male mouthparts stimulate the female immediately prior to spermatophore transfer. Argasid males fully insert their chelicerae, hypostome and palps into the genital opening of the female while ixodid males insert their chelicerae and hypostome with the palps splayed out to the sides of the female genital opening (Obenchain & Galun, 2013).

Spermiogenesis is divided into two phases: (1) Growth and elongation of spermatids to form prospermia (the form in which sperm is packaged in the spermatophore). (2) Capacitation which occurs in the female genital tract, where the prospermia becomes the spermatozoa. In male ixodids, spermatogenesis (the production of haploid spermatids) begins in the nymphal stage following a blood meal and attains the primary spermatocyte stage at the time of moulting to adult, reaching completion when the adult feeds. In argasids (and *Ixodes* species), sperm development advances to the prospermia stage by the time of moulting to adult, so these ticks are able to mate prior to feeding and while still on the vegetation (off-host) (Fig. 2.24B).

The tick spermatozoon is a flagellate, tubule-shaped cell approximately 5 µ in diameter and 300 µ long (Fig. 2.23A-G). The nucleus and acrosome is found in the tail (as opposed to being in the head in the mammalian spermatozoon). The motile processes "pull" the spermatozoon forward and the tail bearing the nucleus and acrosome follow. In the spermatozoon of most other species, the flagellum pushes the acrosome and nucleus forward, thus the acrosome touches the ovum first. In tick fertilization, it is not known which end of the spermatozoon touches the ovum first (Kiszewski et al., 2001).

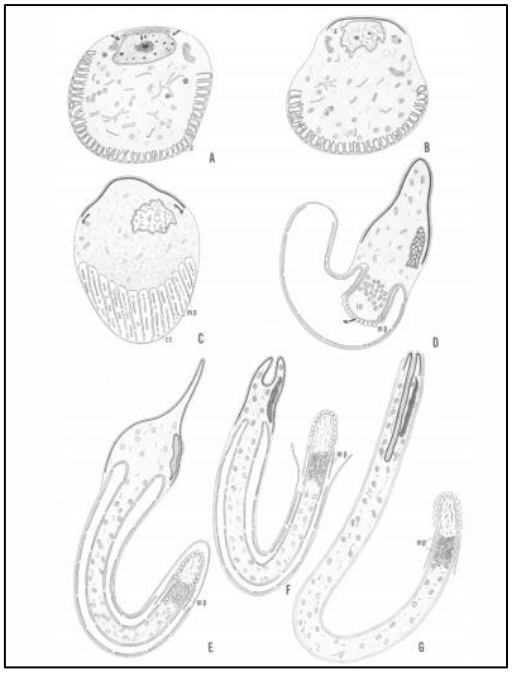

Figure 2.23: The different morphological stages of tick spermatids. (A) The early spermatid is characterized by the presence of circumferentially arranged, subsurface cisternae giving the cell border a striated appearance. As spermiogenesis progresses (B, C, D) the cell and acrosome elongate and the acrosome develops into a thin, electron-dense, disc-shaped structure situated beneath the plasma membrane (E, F). In the mature spermatozoon (G), the acrosome and nucleus are situated at the tail end of the elongate, tubuliform cell. The motile processes are located at the head but extend through the full length of the spermatozoon (Kiszewski et al., 2001)

The female reproductive system of all ticks consists of an ovary with paired oviducts which connect to form a common oviduct or uterus. The uterus connects via a short tube to the vagina which has vestibular and cervical regions having a sac-like extension called the seminal receptacle. Tubular accessory glands open into the vagina at the junction of the cervical and vestibular regions and the vagina opens through the vulva to the exterior (Kiszewski et al., 2001).

For ixodids, mating takes place on the host and once the male and female ticks are in the copulatory position (Fig. 2.24A), they may maintain this position for one to two hours (Allan, 2001). In the vagina, the endospermatophore evaginates into the female genital tract, where it forms a capsule into which the prospermia becomes capacitated and fertilize the ova. The average age at sexual maturity is 29-31 weeks and while male ticks mate with as many females as possible before dying, females mate only once and cannot engorge fully with blood until they have been inseminated. When they finally engorge, the females detach from the host, lay thousands of eggs in a single batch and die. This implies that, the fed and mated adult stage of both males and females is terminal and as such, there is no parental investment after fertilization (Kiszewski & Spielman, 2002). Eggs of all species of ticks are

laid in the physical environment, never on the host, so they can be protected (Dhooria, 2016).

Figure 2.24: Mating of ticks with the male (on top). (A) In Ixodids, the male clasped a partially engorged female, attached to the host. (B) For Argasids and *Ixodes* species, mating can take place off-host (www.visavissymposium.org)

2.3.6 Tick-host specificity

Some tick species only feed on specific hosts (host-specific ticks/specialists) such as *R.* (*B.) microplus*, a one-host tick, which feeds on cattle; while others such as *I. ricinus* and *A. variegatum* are generalists as they feed on mammals, birds and reptiles (Pfaffle et al., 2013).

Ticks locate their host using a simple system of about twenty sensilla (sensory receptors typically hair-shaped) found on the tarsi of the first pair of legs. These sensilla are located within a structure called Haller's organ that comprises an anterior pit that primarily detects humidity and a capsule that contains sensilla used in olfaction (Mcmahon et al., 2003) (Fig. 2.25). There are about 200 olfactory receptor neurones in the sensilla which detect the sex pheromones, aggregation-attachment pheromones (indicating the presence of other ticks) and the semiochemicals emanating from different vertebrates.

The optimal host range of a tick species depends on abiotic parameters (seasonal and daily effects) such as day length, temperature, relative humidity and biotic parameters as biological processes within the tick, biological processes within the host and the interactions between the two (Combes, 2001).

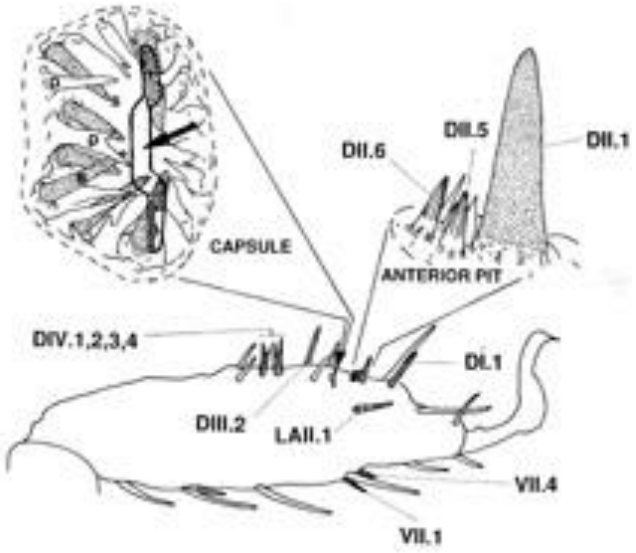

Figure 2.25: Tarsus of the first leg of ticks bearing the olfactory sensilla located within the Haller's organ which has an anterior pit that primarily detects humidity and a capsule that contains sensilla used in olfaction (www2.gsu.edu)

2.4 Tick-host interractions

During haematophagy by ticks, small blood vessels of the hosts' skin are lacerated, cells are ruptured and haemorrhage occurs. This increases the blood volume into the feeding lesion. As the number of ticks that feed on a host over a period of days to months can be very high, this leads to activation of the host's defence mechanisms that include the haemostatic [platelet (thrombocyte) aggregation and blood coagulation] and immune systems to the foreign proteins in tick saliva (Ribeiro & Francischetti, 2003).

The innate immune system which provides an immediate but non-specific response, involves complement, acute phase proteins, neutrophils, macrophages, mast cells, basophils, eosinophils, dendritic cells and natural killer cells (Parham, 2014). Upon vascular damage, platelets are activated by a variety of compounds (adenosine diphosphate, collagen, thrombin, thromboxane A_2, epinephrine, platelet activating factor, thrombospondin) that bind to specific membrane receptors on the platelet surface. Activation is mediated by signal transduction of the different receptors that activate either the cyclooxygenase pathway, phospholipase C pathway, or inhibit adenylyl cyclase, the enzyme which catalyses the conversion of adenosine triphosphate to 3,5-cyclic adenosine monophosphate. This leads to calcium ion mobilization from the platelet open canalicular system and a shape change from discoid to spherical (Mans & Neitz, 2004a). Shape change is accompanied by the extension of numerous pseudopods on the platelet surface. Focal adhesion points form on the pseudopods, with a concomitant activation of the platelet integrin αIIbβ3 that acts as

fibrinogen receptor allowing binding of fibrinogen (a glycoprotein that helps in the formation of blood clots).

The blood coagulation cascade consists of a series of serine proteases that sequentially activate each other (Fig. 2.26). The intrinsic pathway of blood coagulation starts with collagen-induced activation of Factor (f) XII which activates fXI as well as kallikrein. Kallikrein cleaves a precursor to form bradykinin, a peptide causing inflammation, the sensation of pain and irritation (Jelinski, 2016). The extrinsic pathway starts with the release of thromboplastin (tissue factor) from damaged endothelial cells, which activates fVII (Fig. 2.26). Both pathways eventually coalesce in the formation of fXa that in turn produces thrombin. Fibrinogen is cleaved by thrombin to fibrin, which together with platelets and red blood cells is the main constituent of blood clot (Heemskerk et al., 2002). Clotting proteins (fVII, fX and prothrombin) subsequently bind to the membrane through a calcium ion bridge by means of gamma-carboxyglutamic acid and is localized at the site of damage. These events ultimately lead to oedema and associated irritation which facilitates host grooming, an important factor in the reduction of tick burden (Wikel, 2014).

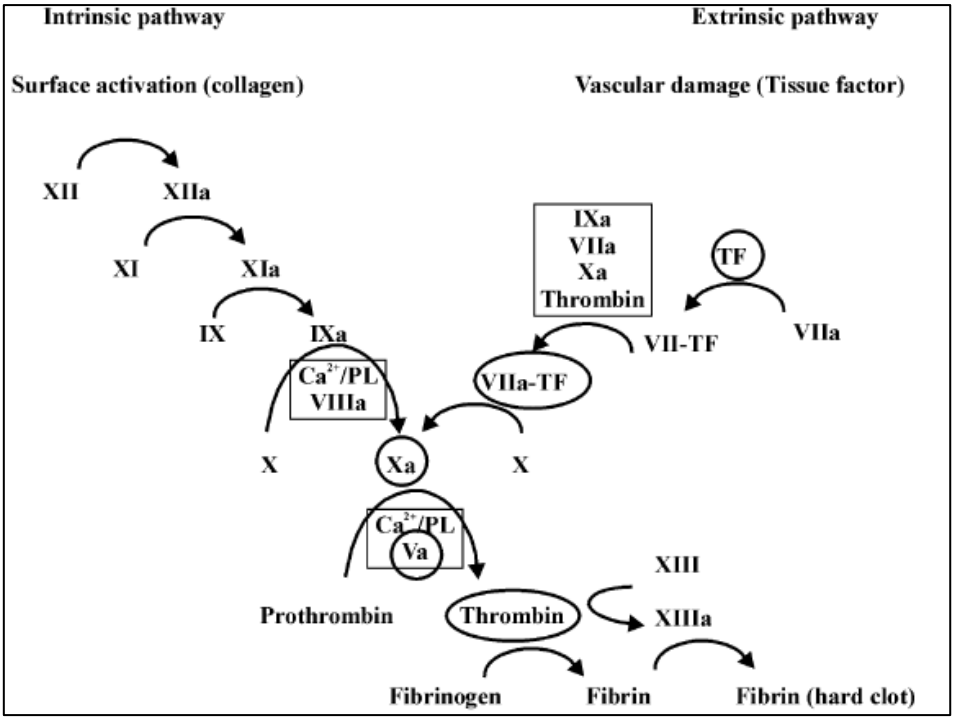

Figure 2.26: Blood coagulation cascade. The intrinsic pathway is initiated by the binding of Factor (f) XII to a negatively charged surface such as collagen or the activated platelet and is then activated by kallikrein. Factor XIIa then activates fXI as well as prekallikrein. This leads to eventual activation of fX. The extrinsic pathway is activated by trauma that releases tissue factor (TF). This binds to fVII, which is activated by thrombin and together activates fX. Prothrombin is activated to thrombin by fXa that cleaves fibrinogen to fibrin that forms a network. Thrombin also activates fXIII that stabilizes the fibrin clot by cross linkage. Coagulation factors for which inhibitors have been identified in ticks are circled (Mans & Neitz, 2004a)

Ticks control the haemostatic system of their hosts by secreting numerous salivary gland proteins with anticoagulation, antiplatelet, vasodilatory, anti-inflammatory and immunomodulatory activities (Kazimirova et al., 2013). These molecules have developed during the host-parasite co-evolution and are crucial to overcoming the haemostatic and immune responses of the host, enabling ticks to complete feeding and development and adventitiously, enhancing the transmission and establishment of tick-borne pathogens (Fontaine et al., 2011).

Apyrase, an ATP-diphosphohydrolase, seen in mammals, has been identified in *I. dammini* (Ribeiro et al., 1985), *O. moubata* (Ribeiro et al., 1991) and *O. savignyi* (Mans et al., 1998) where it functions as an inhibitor of platelet aggregation during the feeding process. Other anti-haemostatics are audoin, metallo dipeptidyl carboxypeptidase and moubatin (Waxman & Connolly, 1993). Vasoconstriction is prevented by vasodilatory agents such as prostaglandins G_2 and nitric oxide secreted by the tick (Ribeiro et al., 1985). Antagonists that prevent the action of different platelet agonists have also been described, among which are

inhibitors of fXa - tick anticoagulant peptide from *O. moubata* (Waxman et al., 1990) and inhibitors of thrombin, such as ornithodorin (van de Locht et al., 1996), savignin (Mans et al., 2002) and boophilin (Macedo-Ribeiro et al., 2008).

Though the anti-haemostatic factors of ixodids and argasids differ in their mechanisms of action and the protein families they belong to, they share common protein folds such as the basic pancreatic trypsin inhibitor (BPTI) and lipocalin in their salivary glands (Mans & Neitz, 2004b). The BPTI-like proteins act as thrombin, fXa and platelet aggregation inhibitors in argasids, while in ixodids, they inhibit the fVIIa/tissue factor complex (Mans et al., 2008). Lipocalins which are abundantly expressed in the salivary glands of argasids, function as anti-complement factors and inhibitors of platelet aggregation (Mans & Ribeiro, 2008). In ixodids, lipocalins that scavenge histamine and serotonin have also been described (Sangamnatdej et al., 2002).

If the host's innate immunity is evaded, the adaptive immune response is activated. This involves numerous cell mediated and humoral components which include but are not limited to homocytotropic antibodies which are capable of binding to receptors on basophils and eosinophils (the prominent infiltrating granulocytes at tick-bite sites); B and T-lymphocytes; Killer and Helper T- cells (McHeyzer-Williams et al., 2006) (Fig. 2.27). Immunosuppression is achieved by impairing the function of the antigen presenting cells, reducing T-lymphocyte function, suppressing cytokine production and action, enzymatic cleavage of antibodies, inhibiting activity of complement components; production of enzymes to counter mediators essential for stimulation of host itch responses; impairing Natural Killer cell function; diminishing *in vitro* proliferative responses of T-lymphocytes to mitogens (substances which trigger cell division), downregulating macrophage pro-inflammatory and Th1-lymphocyte cytokine responses (Mejri et al., 2002).

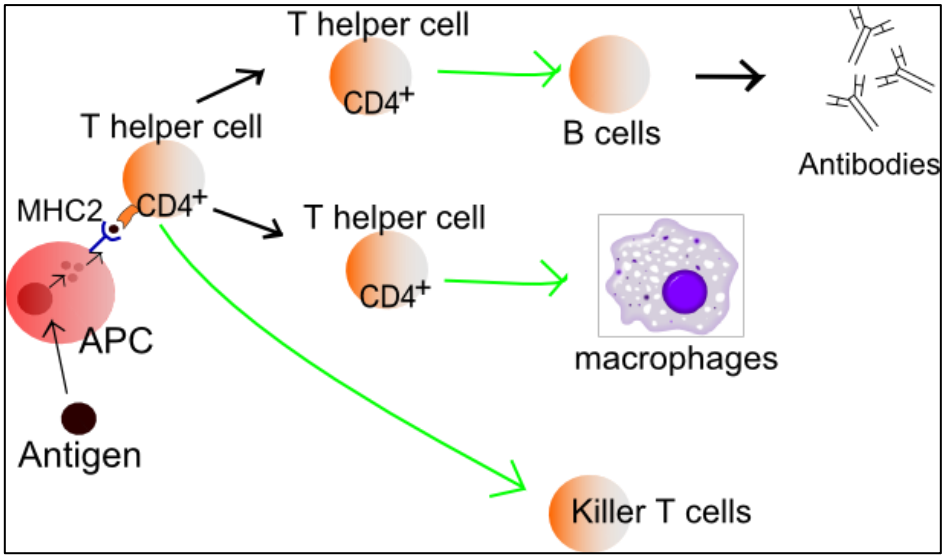

Figure 2.27: Function of T helper cells: Antigen-presenting cells (APC) present antigen on their Class II major histocompatibility complex molecules. Helper T cells recognize these, with the help of their expression of cluster of differentiation 4 (CD4+) co-receptors. The activation of a resting helper T cell causes it to release cytokines and other stimulatory signals (green arrows) that stimulate the activity of macrophages, Natural Killer and B cells (Häggström, 2014)

Several T cell inhibitors have been identified in ticks. Iris, detected in the salivary glands of female *I. ricinus* ticks, suppresses T cell proliferation, induces a Th2 type immune response and inhibits the production of pro-inflammatory cytokines interleukin 6 and tumour necrosis factor-alpha (Leboulle et al., 2002). Salp15, a 15 kDa salivary gland protein is another feeding-induced protein obtained from *I. scapularis.* It specifically binds to the cluster of differentiation 4 molecules, resulting in inhibition of T cell receptor-mediated signaling, reduced interleukin 2 production and impaired T cell proliferation (Garg et al., 2006).

A novel mechanism of tick-induced modulation of host adaptive immunity which may facilitate pathogen transmission has been discovered. Japanin, a salivary gland protein from *R. appendiculatus* belonging to the lipocalins family reprogrammes responses of dendritic cells, thereby altering their expression of co-stimulatory and co-inhibitory transmembrane molecules and secretion of pro-inflammatory, anti-inflammatory and T cell polarizing cytokines (Preston et al., 2013).

2.5 Direct economic losses due to ticks

2.5.1 Biting stress and loss of production

Pruritus (itching) due to the release of histamine from mast cells and pain (caused by bradykinin release) at the numerous feeding sites on the host's skin results in general decrease in food intake (anorexia) of affected animals with resultant weight loss, poor growth and losses in milk production (Fig. 2.1; 2.3). Other effects include chronic blood loss (anaemia) (Fig. 2.2), which over time also contributes to weight loss and poor production from a number of contributing factors of which the main one is from the loss of nutrients to a significant number of ticks (Jonsson, 2006).

2.5.2 Physical damage

All feedings of ticks at each stage of the life cycle are parasitic. During the feeding process, ticks attach to the skin of their host with their mouthparts (Figure 2.22). Argasids feed rapidly with deep penetration of the host's skin and cause considerable damage so that blood loss can still occur long after a tick has stopped feeding. In ixodids which are slow feeders, the feeding lesion develops gradually with the formation of a haematoma (a solid swelling of clotted blood within the tissues). Despite their differing sizes, larval, nymphal and adult mouthparts penetrate to a similar depth towards the base of the Malpighian layer of the skin and this may occur within five minutes of the arrival of the tick on the host causing open wounds (Jones et al., 2015).

The skin attempts to repair itself through an orchestrated cascade of biochemical events: haemostasis, inflammation, tissue growth (proliferation) and tissue remodeling (maturation) (Fig. 2.28).

- Haemostasis: Following injury, the platelets and coagulation factors in the blood prevent further bleeding by clumping and releasing chemical signals to promote clotting that plugs the break in the blood vessel (Versteeg et al., 2013).
- Inflammation: During this phase, the white blood cells engulf the damaged and dead cells, along with pathogens through the process of phagocytosis. Platelet-derived growth factors are released into the wound that cause the migration and division of cells during the proliferative phase (Theoret, 2004).
- Proliferation: In this phase, angiogenesis (formation of new blood vessels by vascular endothelial cells), collagen deposition, granulation tissue formation (fibroblasts grow and form a new, provisional extracellular matrix by excreting collagen and fibronectin), re-epithelialization (epithelial cells proliferate on the wound, providing

cover for the new tissue) and wound contraction (myofibroblasts decrease the size of the wound by gripping the wound edges and contracting) occur (Bainbridge, 2013).

- Maturation (remodeling): During maturation, collagen is realigned along tension lines and cells that are no longer needed are removed by apoptosis (programmed cell death) (Bainbridge, 2013).

This cascade of events produces scars at several feeding sites that remain for years, long after the ticks have detached. When such skins of these livestock are made into leather, these scars remain as blemishes that reduce the value of the leather (Fig. 2.1).

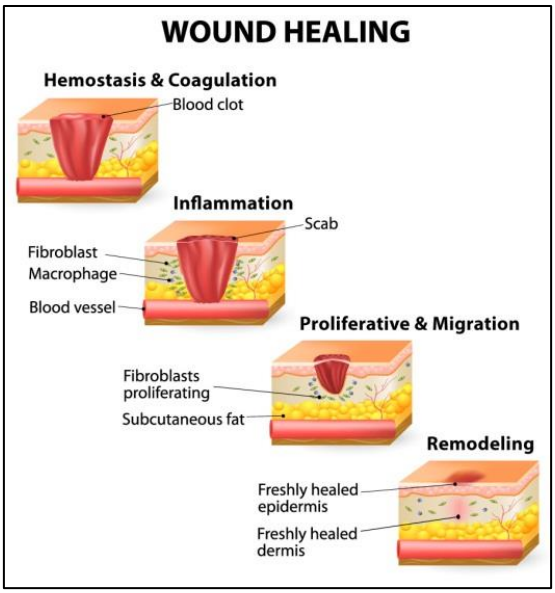

Figure 2.28: Wound healing cascade. The skin attempts to repair itself through a series of biochemical events: haemostasis, inflammation, proliferation and tissue remodelling (surpassinc.com)

2.5.3 Wound infection

The process of tick feeding also results in secondary wound infections with opportunistic bacteria (such as *Staphylococcus aureus*) and fungi (such as *Aspergillus fumigatus)* on the skin. The wound site is also susceptible to infestation with larvae of parasitic flies causing myiasis (the infestation of the body of a live vertebrate animal by larvae of flies that grow inside the host while feeding on its tissue). The adult females of parasitic flies lay their eggs on the animal which hatch in approximately 8-24 hours, depending on the environmental conditions. Once hatched, the larvae tunnel through wounds into the host's subcutaneous tissue. Painful, slow-developing ulcers or furuncle (boil) like sores occur (Fig. 2.29). After about 24 hours, bacterial infection is likely and, if left untreated, could lead to septicaemia (bacteria in the blood) which may be fatal (Mukandiwa et al., 2012a).

Myiasis occurs worldwide, especially in Africa where domestic animals are kept under humid conditions. *Cordylobia arthropophaga* (tumbu fly) affects dogs severely (Adisa & Mbanaso, 2004) and annual losses in the livestock sector from *Cochliomyia hominivorax* (New World screw-worm fly) in USA, were estimated to be more than US$140 million (Mukandiwa et al., 2012a; 2012b).

Figure 2.29: *Cordylobia arthropophaga* (tumbu fly) larvae extracted from a dog. Adult females lay their eggs on the host which hatch into larvae and tunnel through wounds into the subcutaneous tissue (www.dailymail.co.uk)

2.5.4 Poisoning by ticks

Ticks, alongside scorpions and spiders belong to the class Arachnida (Fig. 2.4), notorious for their venom which evolved for defence and predatory purposes (King, 2004). Spider toxins differ structurally from those of scorpions, with many sharing the cystine knot motif suggesting that toxins evolved independently within the class (Escoubas et al., 2000; Craik et al., 2001). The Acari (ticks and mites) are closely related to the Ricinulei or the ‘‘hooded-tickspiders’’, a group of non-toxic arachnids and ticks group closest to the holothyrida, a group of scavengers (Fig. 2.4). It has been suggested that tick paralysis, a major form of tick toxicosis, may be a vestigial function retained in ticks when they evolved a parasitic lifestyle. However, only 81 of the 898 recognized species of ticks have been implicated in tick toxicosis (Mans et al., 2008).

Tick paralysis in animals is caused by the adult female tick during the period of rapid engorgement (days 5-7), although large numbers of larval or nymphal ticks may also cause paralysis. More than sixty species of ticks have been implicated so far to induce tick paralysis. The most noted and dangerous tick in this respect in South Africa is the Karoo paralysis tick (*I. rubicundus*). The adult tick excretes a toxin that causes paralysis in sheep, goats and cattle. The paralysis commonly occurs from February and reaches a peak in April and May. Sudden drops in temperature caused by rain, cold winds and cloudy conditions

seem to stimulate the activity of the adult ticks. Affected animals become paralysed and some may show signs of incoordination and stumbling. Unless ticks are removed, the animal remains paralysed and dies within days (Durden & Mans, 2016).

The feeding of the cattle leg tick, *R. praetextatus,* also causes toxicosis in cattle in Africa resulting in paralysis (Fig. 2.30). *Ixodes holocyclus*, of the eastern coast of Australia, attacks humans, dogs, cats, foxes and livestock while in the USA, dogs suffer from paralysis due to the bite of *Dermacentor variabilis and D. andersoni* (Gordon & Giza, 2004).

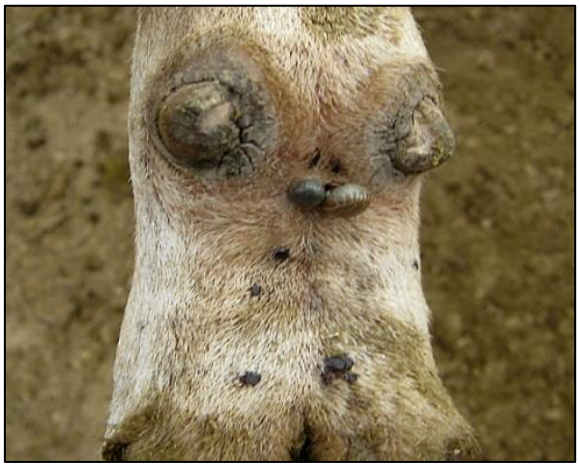

Figure 2.30: The feeding of the cattle leg tick, *Rhipicephalus praetextatus*, can cause toxicosis in cattle, resulting in paralysis (influentialpoints.com)

Tick paralysis affects mainly motor pathways and to a lesser extent, autonomic and sensory pathways of the central nervous system. A neurotoxin in the tick saliva interferes with acetylcholine release at the neuromuscular junction, producing a neuromuscular blockade. Paralysis of oesophageal muscles develops, with or without oesophageal dilatation (megaoesophagus), there is reversible myocardial depression and diastolic failure, leading to cardiogenic pulmonary oedema. In severe cases, increased packed cell volume causes a fluid shift from the general circulation to the lungs. There is progressive pulmonary dysfunction primarily due to oedema, leading to hypoxia, respiratory acidosis and death within 1-2 days of onset of clinical signs. In humans, tick paralysis is mostly seen in children and the symptoms are similar to that seen in dogs (Edlow & McGillicuddy, 2008).

Tick paralysis is similar to botulism, a disease with four naturally occurring syndromes: (1) Foodborne botulism, caused by ingestion of foods contaminated with *Clostridium botulinum* (the most potent toxin known). (2) Wound botulism, caused by colonization of a wound and *in situ* toxin production. (3) Inhalational botulism which results from aerosolization of botulinum toxin. (4) Iatrogenic botulism from injection of toxin for cosmetic or therapeutic

purposes (Sobel, 2005). Botulinum toxin also exerts its action on the cholinergic system at the presynaptic motor neuron terminal by blocking acetylcholine transmission across the neuromuscular junction, resulting in descending, symmetric, flaccid paralysis of voluntary muscles, which may progress to respiratory collapse and death (Sobel, 2005).

2.6 Indirect economic losses due to ticks

In addition to causing direct losses, ticks are vectors of numerous, economically important diseases of livestock and companion animals and as such are key targets for infection control. In this section, a few important diseases are discussed.

2.6.1 Viral diseases

2.6.1.1 Nairobi sheep disease (NSD)

The disease is characterized by fever, haemorrhagic gastroenteritis, abortion and high mortality rate. It was first identified near Nairobi, Kenya, in 1910 and NSD virus was shown to be the causative agent in 1917. The disease is endemic to Kenya, Uganda, Tanzania, Somalia, Ethiopia, Botswana, Mozambique and the Democratic Republic of Congo. Though human infections are rare, accidental infections have been reported among laboratory workers, resulting in fever, joint aches and general malaise (Marczinke & Nichol, 2002).

The NSD virus is classified in the genus Nairovirus, family Bunyaviridae and is possibly the most pathogenic virus known for sheep and goats. In addition, the virus is serologically related to Crimean-Congo haemorrhagic fever virus. It is transmitted transovarially (infection passes from the female, through the eggs to the larvae) and transstadially (infecting one life stage and transmitted to the next stage during moulting) by *R. appendiculatus*, in which it can survive for up to 800 days. The unfed adult tick can transmit NSD virus for more than two years after infection. Other *Rhipicephalus* species and *A. variegatum* ticks can also transmit the disease. The virus is shed in urine and faeces, but the disease is not spread by contact (Marczinke & Nichol, 2002).

2.6.1.2 African Swine Fever (ASF)

African swine fever virus, the causative agent of ASF, is the only member of the Asfarviridae family and the only virus with a double-stranded deoxyribonucleic acid genome transmitted by arthropods which replicates in the cytoplasm of infected cells (Rowlands et al., 2008). It is endemic to sub-Saharan Africa and exists in the wild through a cycle of infection between ticks and wild pigs, bushpigs and warthogs (Denis, 2014).

The ASF virus is transmitted in domestic pigs by the feeding of *O. moubata* ticks. The acute form of the disease (transmitted by highly virulent strains) causes high mortality in pigs. Pigs

may develop a high fever, but show no other noticeable symptoms for the first few days. They then gradually loose their appetite and become depressed. In white-skinned pigs, the extremities turn blueish-purple and haemorrhages become apparent on the ears and abdomen. Groups of infected pigs lie huddled together shivering, breathing abnormally and sometimes coughing. Within a few days post infection, they enter a comatose state and die. In pregnant sows, spontaneous abortions occur and in milder infections, affected pigs lose weight and develop signs of pneumonia, skin ulcers and swollen joints (Howey et al., 2013).

2.6.1.3 Crimean-Congo haemorrhagic fever (CCHF)

Crimean-Congo haemorrhagic fever is endemic to Africa, the Balkan Peninsula, the Middle East and Asia. It is caused by a virus of the Bunyaviridae family isolated from at least 31 tick species from the genera *Haemaphysalis* and *Hyalomma*. The disease is zoonotic and while clinical disease is rare in infected animals, it is severe in infected humans with a mortality rate of 10-40%. Outbreaks have occurred in clinical facilities or in abattoirs where health workers have been exposed to infected human or animal blood and fomites (non-living objects capable of carrying infectious organisms) (Bente et al., 2013).

Typically, 1-3 days following a tick bite or 5-6 days after exposure to infected materials, flu-like symptoms, haemorrhage progressing to respiratory distress, kidney failure and shock occur. Patients usually begin to show signs of recovery 9-10 days post infection. However, 30% of the cases result in death during the second week of illness (Bente et al., 2013).

2.6.1.4 Tick-borne encephalitis (TBE)

This is a viral disease of the central nervous system caused by three subtypes of the TBE virus. It is considered to be the most relevant and dangerous viral zoonosis in North-east Europe. *Ixodes ricinus* and *I. persulcatus*, are thought to be the main vectors of the pathogen which replicates in ticks leading to a constant and high viral titre in them. Rodents from the genera *Apodemus* (true rats and mice) and *Myodes* (red-backed voles), probably also act as maintenance hosts. Larger wild animals are considered not competent for virus transmission but serve as hosts and transporters for the ticks (Kaiser, 2008).

The TBE virus is primarily pathogenic for humans and infection follows the bite of infected ticks, usually in people who visit or work in forests, fields or pastures. In contrast to bacterial and protozoal transmission, the virus enters the mammalian host during early tick feeding. Therefore, use of tick repellents may minimize attachment and subsequent feeding of the tick vector (Kaiser, 2008).

2.6.2 Bacterial and Rickettsial diseases

2.6.2.1 Borreliosis (Lyme disease)

Borreliosis is transmitted to domestic dogs and humans from a natural reservoir among small mammals and birds by *Ixodes* ticks. The causative agents identified are *Borrelia burgdorferi sensu lato; Borrelia burgdorferi sensu stricto* and *Borrelia mayonii*; *Borrelia afzelli* and *Borrelia garinii* in northern Africa, North America, Europe and Asia respectively (Shapiro, 2014).

Most infections are caused by ticks in the nymphal stage and tick bites often go unnoticed because of the small size of the tick as well as tick salivary kininases that prevent the host from feeling any itch or pain from the bite (Tilly et al., 2008). Within the midgut of the tick, the bacteria outer surface protein (Osp) A binds to the tick receptor for OspA. As the tick feeds, OspA is downregulated and OspC is upregulated. After the bacteria migrate from the midgut to the salivary glands, OspC binds to Salp15, which enhances infection. Successful infection of the mammalian host depends on bacterial expression of OspC (Hovius et al., 2007) (Fig. 2.31).

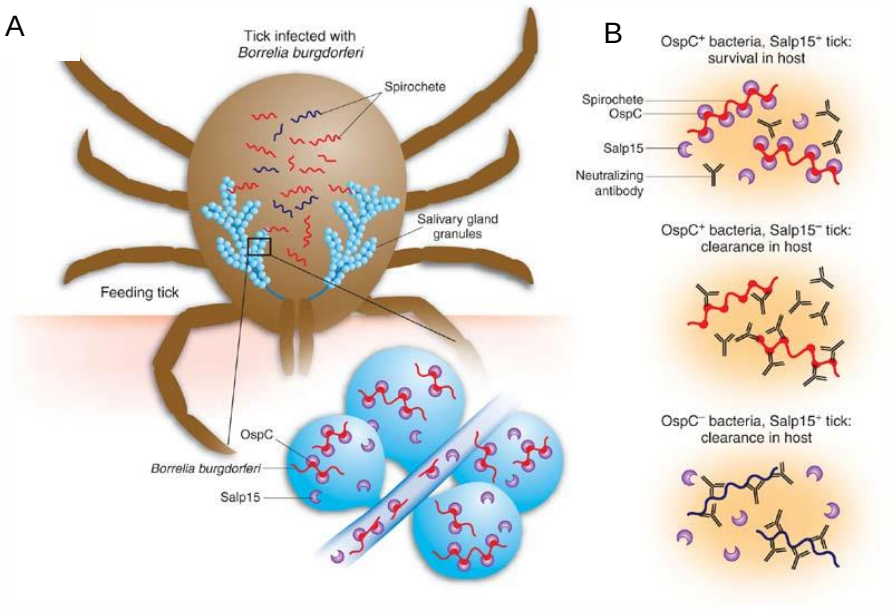

Figure 2.31: Tick salivary protein binds and protects *Borrelia burgdorferi*. (A) As an infected tick feeds on the host, *Borrelia burgdorferi* migrates to the salivary glands and is transmitted through the saliva to the host. The bacterium undergoes a dramatic switch in the major surface protein from OspA (blue) to OspC (red) and binds to the salivary gland protein Salp15. (B) In the presence of neutralizing antibodies from an immune vertebrate host, the bacteria with both OspC and Salp15 on their surfaces preferentially survive, relative to those in which either OspC or Salp15 is missing (www.nature.com)

2.6.2.2 Anaplasmosis (Tick-borne fever, Gall sickness)

Anaplasmosis is a disease of ruminants caused by obligate, intraerythrocytic bacteria of the order Rickettsiales, family Anaplasmataceae and genus *Anaplasma.* This includes *Anaplasma phagocytophilum, Anaplasma marginale, Anaplasma centrale, Anaplasma equi*, *Anaplasma bovis*, *Anaplasma ovis* and *Anaplasma platys.* Up to seventeen tick species have been reported to transmit the pathogen of which *Dermacentor* species have been incriminated as the main vectors in the USA and *Rhipicephalus* species are major vectors in Australia and Africa. Though the term “anaplasmosis” is often associated with animal infection, the disease also occurs in humans (Hartelt et al., 2004).

This disease is of economic significance in the cattle industry and is characterized by progressive anaemia due to extravascular destruction of infected and uninfected red blood cells. Animals with peracute infections die within a few hours of the onset of clinical signs. Acutely infected animals are anaemic and loose condition rapidly, milk production falls, inappetence, loss of coordination, breathlessness when exerted and a rapid pulse are usually evident in the late stages. Pregnant cows may abort and surviving cattle convalesce over several weeks, during which haematologic parameters gradually return to normal (Lew-Tabor & Valle, 2015).

2.6.2.3 Cowdriosis (Heartwater)

The disease is seen only in areas infested by *Amblyomma* ticks. In endemic areas in southern Africa, it is estimated that mortality due to cowdriosis are more than twice that due to babesiosis and anaplasmosis combined. The causative agent is an obligate, intracellular parasite, *Ehrlichia ruminantium* (*Cowdria ruminantium*). Cattle, sheep, goats and antelopes are susceptible (Plans & Plan, 2016).

The name of the disease, “heartwater”, is derived from fluid which accumulates around the heart or in the lungs of infected animals. In peracute cases, animals may drop dead within a few hours of developing a fever, sometimes without any apparent clinical signs; others display dyspnoea (laboured breathing) and/or paroxysmal convulsions. In the acute form, animals often show anorexia and depression along with congested and friable mucous membranes. Dyspnoea slowly develops and nervous signs such as hyperaesthesia, a high-stepping stiff gait, exaggerated blinking and chewing movements. Terminally, prostration with bouts of opisthotonus (pedaling and stiffening of the limbs) and convulsions are seen. In subacute cases, the signs are less marked and the involvement of the central nervous system is inconsistent (Plans & Plan, 2016).

2.6.2.4 Ehrlichiosis (Tropical canine pancytopenia, Canine rickettsiosis, Canine haemorrhagic fever, Canine typhus, Tracker dog disease)

Ehrlichia canis is a rickettsial bacteria belonging to the family Ehrlichiaceae and is transmitted by *R. sanguineus.* It causes ehrlichiosis, a disease of dogs though humans, goats and cats can also become infected after exposure to ticks (Loftis et al., 2008). There are three stages of canine ehrlichiosis, each varying in severity. The acute stage, occurring several weeks post infection and lasting for up to one month, can lead to fever and bone marrow suppression with resultant pancytopenia (lowered peripheral blood cell counts). The second stage (subclinical phase), has no apparent clinical signs and can last through the dog's life-time, during which the dog remains infected with the organism. Some dogs are able to successfully eliminate the disease during this time. In some however, the third and most serious stage of infection, the chronic phase, will commence. Pancytopenia, bleeding, lameness, neurological degeneration, ophthalmic disorders and kidney failure may result which can be fatal. Clinical signs of human ehrlichiosis include fever, headache, eye pain and gastrointestinal upset (Reeves et al., 2008).

2.6.2.5 Rocky Mountain spotted fever (Blue disease, Tick typhus)

This is a potentially fatal and the most frequently reported rickettsial illness in humans in the USA caused by *Rickettsia rickettsii* that is spread to humans (zoonotic) primarily by bites of infected tick species such as *D. variabilis, D. andersoni, A. cajennense* and *R. sanguineus* (Masters et al., 2003). Infections may also occur following exposure to crushed tick tissues, fluids or faeces. Initial symptoms of the disease include sudden onset of fever, headache and muscle pain, followed by development of rash. The disease may be difficult to diagnose in the early stages and can be fatal if prompt and appropriate treatment is not administered. Despite the availability of effective treatment and advances in medical care, approximately 3-5% of infected patients die (Masters et al., 2003).

2.6.2.6 Haemoplasmosis (Haemobartonellosis, Feline infectious anaemia, Feline haemotropic mycoplasmosis)

This disease targets the red blood cells of dogs and cats and there have been no reported cases of haemoplasmosis in humans, although organisms resembling haemotropic mycoplasms have been found in people with suppressed immune systems (Lappin et al., 2006). Previously, it was thought to be caused by *Haemobartonella canis*, an organism transmitted by *R. sanguineus*. Based on genetic analysis however, it is now known to be caused by two distinct bacterial species, *Mycoplasma haemocanis* and *Mycoplasma haematoparvum*, of the family Mycoplasmataceae (Willi et al., 2007).

Clinical signs of the disease depend on the degree of anaemia, the stage of infection and the immune status of the patient. Generally, the disease is clinically inapparent, unless the dog or cat is splenectomised or immunosuppressed. Acute clinical signs include depression, loss of appetite, weight loss and fever. In severe cases, death can occur. A chronic form of the disease has been reported and may cause slight weakness, an increase in appetite and pica (appetite for non-nutrituous substances such as sand, stones) (Lobetti & Lappin, 2012).

2.6.3 Protozoal diseases

2.6.3.1 Babesiosis (Redwater, Texas cattle fever, Piroplasmosis)

Babesia, is thought to be the second most common haemoparasite of mammals, after *Trypanosoma* and can have a major impact on the health of domestic animals and humans. Ticks, especially *R. (B.) microplus, R. sanguineus, R. (B.) decoloratus* and *I. scapularis* transmit several *Babesia* species to cattle (*Babesia bovis, Babesia bigemina*); horses (*Babesia equi, Babesia caballi);* dogs (*Babesia canis*); cats (*Babesia felis, Babesia cati*) and humans (*Babesia microti, Babesia duncani*, *Babesia divergens, Babesia venatorum*) (Gray et al., 2010).

Clinical signs in domestic animals include fever, anorexia, haemolytic anaemia, muscle pain, vomiting, weight loss, enlarged liver, icterus (yellowing of the mucous membrane); general organ failure and death may ensue (Shaw & Day, 2005).

2.6.3.2 Theilerioses

This refers to a group of diseases caused by *Theileria* in domestic and wild animals in tick-infested areas. East Coast fever, an acute disease of cattle is caused by *Theileria parva* and transmitted by the tick, *R. appendiculatus.* It is a serious problem in east and southern Africa. The African buffalo (*Syncerus caffer*) is an important reservoir of the pathogen though infection is asymptomatic. The disease is characterized by fever which occurs 7-10 days post infection and panlymphadenopathy (generalized swelling of the lymph nodes). Anorexia develops and the animal rapidly loses condition, lacrimation (abnormal secretion of tears) and nasal discharge may occur. Terminally, dyspnoea is common and death usually occurs 18-24 days post infection (Katzer et al., 2010).

Theileria annulata, the causative agent of tropical theileriosis, transmitted by *Hyalomma* ticks, is widely distributed in north Africa, the Mediterranean coastal area, the Middle East, India and Asia. It can cause mortality of up to 90%, but strains vary in their pathogenicity. The kinetics of infection and the main clinical findings are similar to those of *Theileria parva*, but anaemia is often a feature of the disease (Pipano et al., 2003).

Theileria lestoquardi (previously known as *Theileria hirci*) causes a disease in sheep and goats similar to that produced in cattle by *Theileria annulata*, with which it is closely related. *Theileria equi* in horses causes equine piroplasmosis and *Theileria lewenshuni* and *Theileria uilenbergi*, transmitted by *Haemaphysalis* ticks, have been identified as the causative agents of a severe disease in sheep in China (Englund & Pringle, 2004).

2.6.3.3 Hepatozoonosis

This is a disease of dogs and cats caused by *Hepatozoon* species, which parasitize white blood cells of the host animal. Dogs in tropical and subtropical regions of the world are commonly infected with *Hepatozoon canis* with *R. sanguineus*, as major vector while *Hepatozoon americanum* transmitted by *A. maculatum* causes disease in dogs in the southern USA (Shaw et al., 2001). There is no clear understanding of the tick species involved for transmission in cats (Baneth, 2011).

Unlike other tick-borne diseases, *Hepatozoon* species are transmitted to new animals by ingestion of an infected tick and no salivary transfer of this parasite has been documented. Once ingested, organisms are released within the intestine of the host, penetrate the intestinal wall, invade mononuclear cells and are carried via the bloodstream or lymphatic system to various tissues. An asymptomatic to mild disease is the most common presentation of the infection, usually associated with low parasitaemia (1-5%), while severe illness presents a high parasitaemia (up to 100%) of the peripheral blood neutrophils. Clinical presentations are variable: fever, emaciation, lethargy, anorexia, anaemia and muscle pain (Baneth, 2011). The name, hepatozoonosis, is a misnomer as it is not a zoonosis.

2.6.3.4 Cytauxzoonosis

This is an emerging, life-threatening infectious disease limited to the family Felidae which means that it poses no zoonotic or agricultural (transmission to farm animals) risk. It is caused by *Cytauxzoon felis* transmitted by *A. americanum*. The natural host is the bobcat (*Lynx rufus*) and reservoir hosts of the parasite include bobcats and domestic cats that survive infection (Brown et al., 2008). Onset of clinical signs is 5-14 days post infection with fever, depression, lethargy and anorexia being the most common clinical signs. Other signs include icterus, enlarged spleen and lymph nodes. Without treatment, death typically occurs within 2-3 days. When first described, mortality due to cytauxzoonosis was reported to be almost 100% (Cohn et al., 2011).

2.7 Classes of acaricides and their mechanisms of action

Tick control programmes are largely based on the use of commercially available chemicals such as the arsenicals, organochlorines, phenylpyrazoles, organophosphates, carbamates, formamidines, pyrethroids, macrocyclic lactones and more recently, the spinosyns, insect growth regulators and isoxazolines on/in the animals or in the environment (Gassel et al., 2014). Several active ingredients with acaricidal and/or tick repellent effects are commercially available for use on companion animals, livestock and humans. These are prescribed in different formulations, including tablets, sprays, soaps, shampoos, powders, impregnated collars, dip solutions, pour-on and spot-on applications. The global parasiticide market was valued at US$ 6 509 million in 2013. This is expected to reach US$8 918 million by 2019, growing at a rate of 5.4% (www.marketsandmarkets.com). In 2013, Africa accounted for 2.7% of the global parasiticide market which was valued at US$173.8 million. Of this, ectoparasiticides accounted for 60.1% (US$96.2 million) and this is expected to reach US$137.9 million growing at a rate of 6.3% by 2019 (www.marketsandmarkets.com).

2.7.1 Arsenicals

Arsenicals (acaricides containing arsenic) (Fig. 2.32) were effectively used globally to control ticks for 30 to 40 years prior to the development of resistance in *Boophilus* ticks (George et al., 2004).

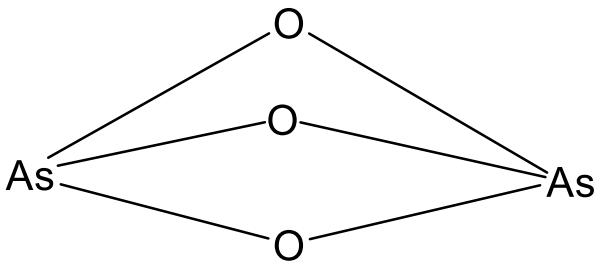

Figure 2.32: Chemical structure of arsenic trioxide (As_2O_3). Arsenic trioxide is a commercially important oxide of arsenic and the main precursor to other arsenic compounds (wwww.researchgate.net)

The arsenicals act by inhibiting adenosine triphosphate production through several mechanisms. At the level of the Kreb's cycle, they inhibit pyruvate dehydrogenase and by competing with phosphate, uncouple oxidative phosphorylation, thus inhibiting energy-linked reduction of nicotinamide adenine dinucleotide, mitochondrial respiration and adenosine triphosphate synthesis leading to death of the parasite (Klaassen & Watkins III, 2003) (Fig. 2.33).

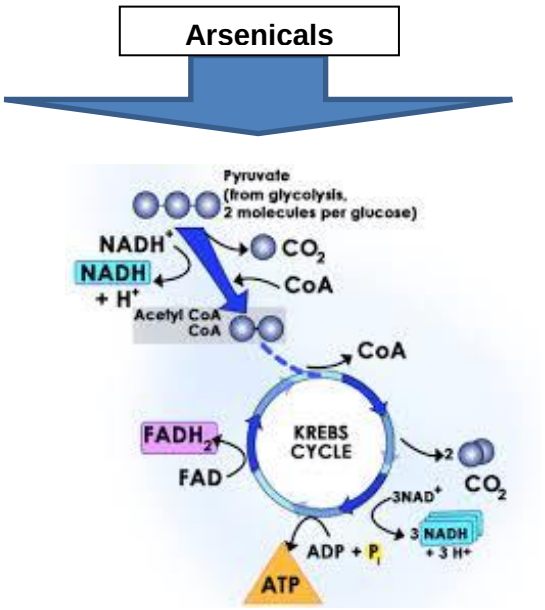

Figure 2.33: Arsenicals inhibit pyruvate dehydrogenase, uncouple oxidative phosphorylation, thus inhibiting energy-linked reduction of nicotinamide adenine dinucleotide (NADH), mitochondrial respiration and adenosine triphosphate (ATP) synthesis. These metabolic interferences lead to death of the parasite (www.biovision.com)

Though arsenicals are inexpensive, stable and water-soluble, they were characterised by short residual effects of less than one or two days and were also environmentally destructive. Extensive arsenic contamination of ground water was reported to cause the world's worst mass poisoning in Bangladesh and neighbouring countries (Meharg, 2005). Epidemiological studies have also suggested a correlation between chronic consumption of drinking water contaminated with arsenic and the increased incidence of cancer (Meliker et al., 2010) (Fig. 2.34). The arsenicals were eventually replaced by the organochlorines.

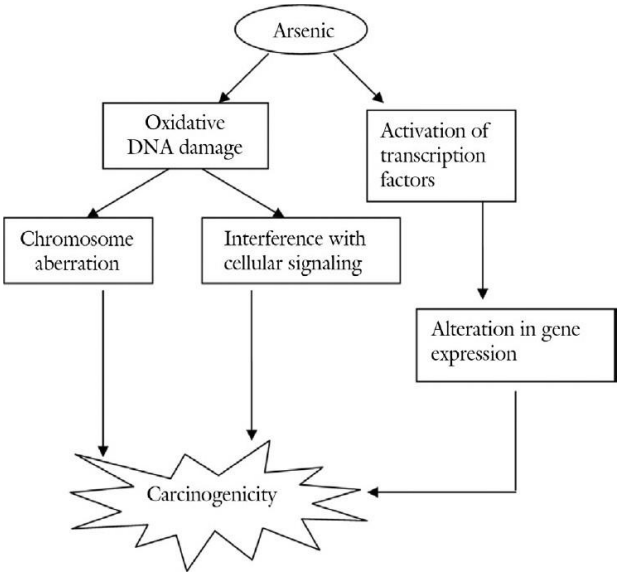

Figure 2.34: Chronic exposure to arsenic leads to oxidative deoxyribonucleic acid (DNA) damage changing the expression of genes involved in cell growth and proliferation thereby causing the induction of carcinogenic cells (toxicologyinternational.com)

2.7.2 Organochlorines (Chlorinated hydrocarbons)

Benzenehexachloride and dichlorodiphenyltrichloroethane (DDT), introduced in 1946 were the first organochlorines to be used as acaricides (Abbas et al., 2014). Later, other chlorinated cyclodienes and chlorinated benzenes such as lindane, endosulphan, methoxychlor, hexachlorocyclohexane, toxaphene, aldrin and dieldrin (Fig. 2.35) were also recognised as acaricides and widely used (Coats, 1990).

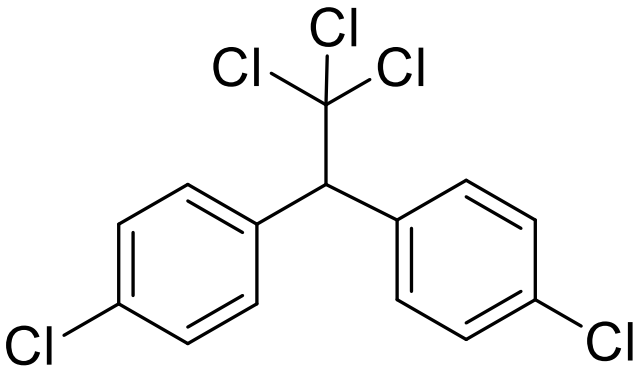

Figure 2.35: Chemical structure of dichlorodiphenyltrichloroethane (DDT), 1,1'-(2,2,2-trichloroethane-1,1-diyl)bis(4-chlorobenzene) Organochlorines are organic compounds containing at least one atom of chlorine covalently bonded which has an effect on the chemical behaviour of the molecule (UNEP nd)

The mechanism of action of the organochlorines is thought to involve binding at the picrotoxinin site in the gamma-aminobutyric acid (GABA) chloride (Cl^-) ionophore complex which inhibits Cl^- flux into the nerve causing hyperexcitation and death of the parasite (Coats, 1990).

The organochlorines have long chemical half life of 7-30 years and they are very effective. Unfortunately, these molecules were very stable and characterised by a long residual effect, persisting in the environment (half life in the soil can be up to 1 year) and tissues of treated livestock for long periods (bioaccumulation) (Connell et al., 1999a; 1999b). The product was also highly toxic to crustaceans, bees, rabbits, lizards, birds, many fishes and aquatic invertebrates prompting their eventual withdrawal (Spickett, 1998) (Fig. 2.36).

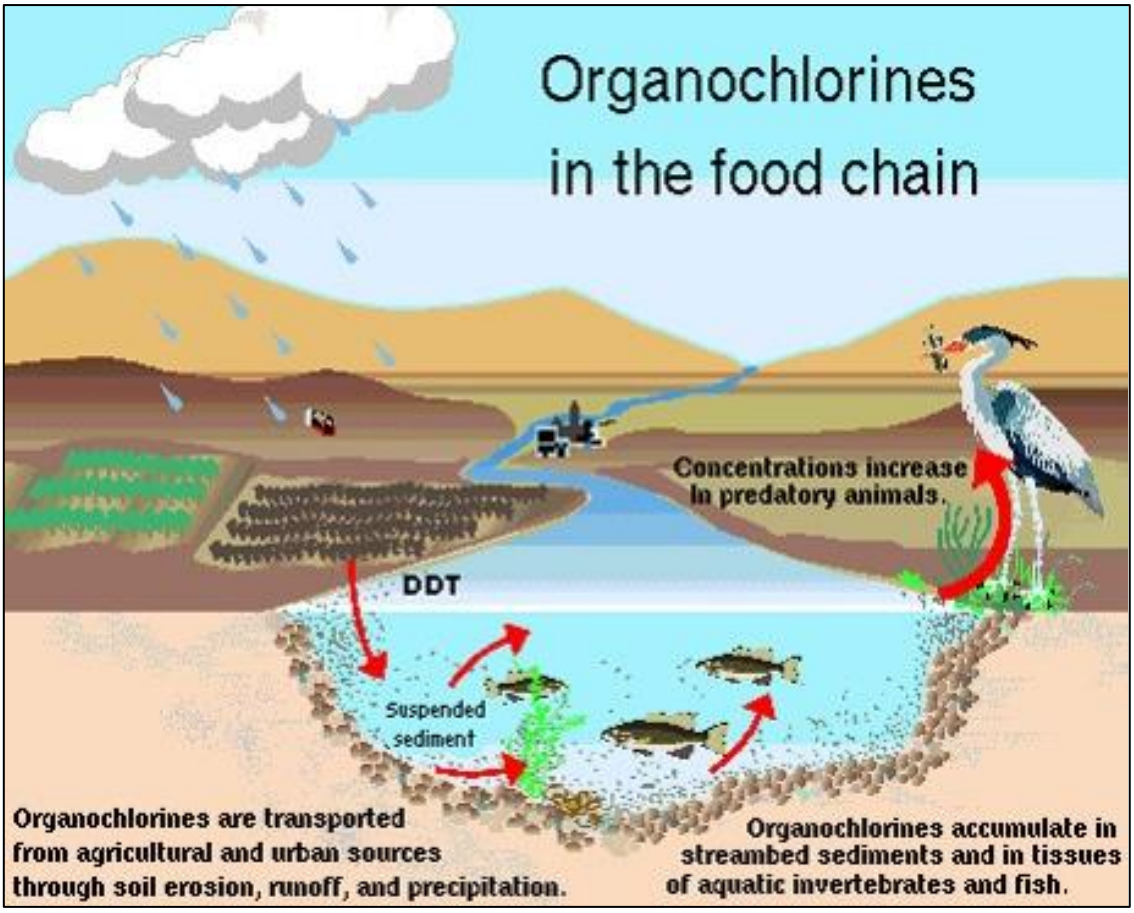

Figure 2.36: The organochlorines are persistent organic pollutants which can be transported from agricultural and domestic sources into water bodies and are highly toxic to many fishes and aquatic invertebrates (pakagrifarming.blogspot.com)

2.7.3 Organophosphates and Carbamates

Organophosphates, esteric compounds from phosphoric acid synthesis (Fig. 2.37), supplemented the use of the organochlorines in the 1955-70s. Coumaphos and diazinon were among the first used to control arachnids. Others include parathion, malathion, chlorpyrifos and dichlorvos. The carbamates on the other hand, have the carbamate ester functional group and examples are aldicarb, carbofuran, carbaryl, ethienocarb, fenobuacrb, oxamyl and methomyl. In contrast to the organochlorines, the organophosphates were characterised by a shorter residual effect, lower environmental persistence, but by 1963, resistance was reported (Metcalf, 1983).

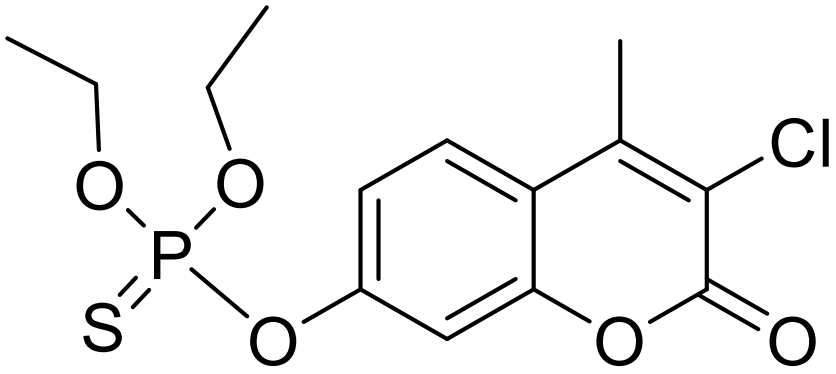

Figure 2.37: Chemical structure of the organophosphate, coumaphos (0, 0-diethyl-0-[3-chloro-4methyl-2-oxo-2h-1-benzopyran-7-yl] phosphorothioate). Organophosphates are esters of phosphoric acid (www.researchgate.net)

Both organophosphates and carbamates act at the synapse of nerve junctions by inhibiting the activity of acetylcholinesterase irreversibly and reversibly respectively (Fig. 2.38). Acetylcholinesterase breaks down the neurotransmitter acetylcholine, which carries impulses across the synapse from one nerve cell to another. Inhibition of this enzyme results in accumulation of acetylcholine and continuous nerve discharges leading to paralysis and death (Barthold & Schier, 2005).

The organophosphates are of concern because even at relatively low levels, they are hazardous to the brain development of foetuses and young children. Though banned in many countries for residential uses, they are still in use for agricultural purposes (sprayed on fruits and vegetables) and can be absorbed through the lungs, skin or by ingestion (Krieger et al., 2012).

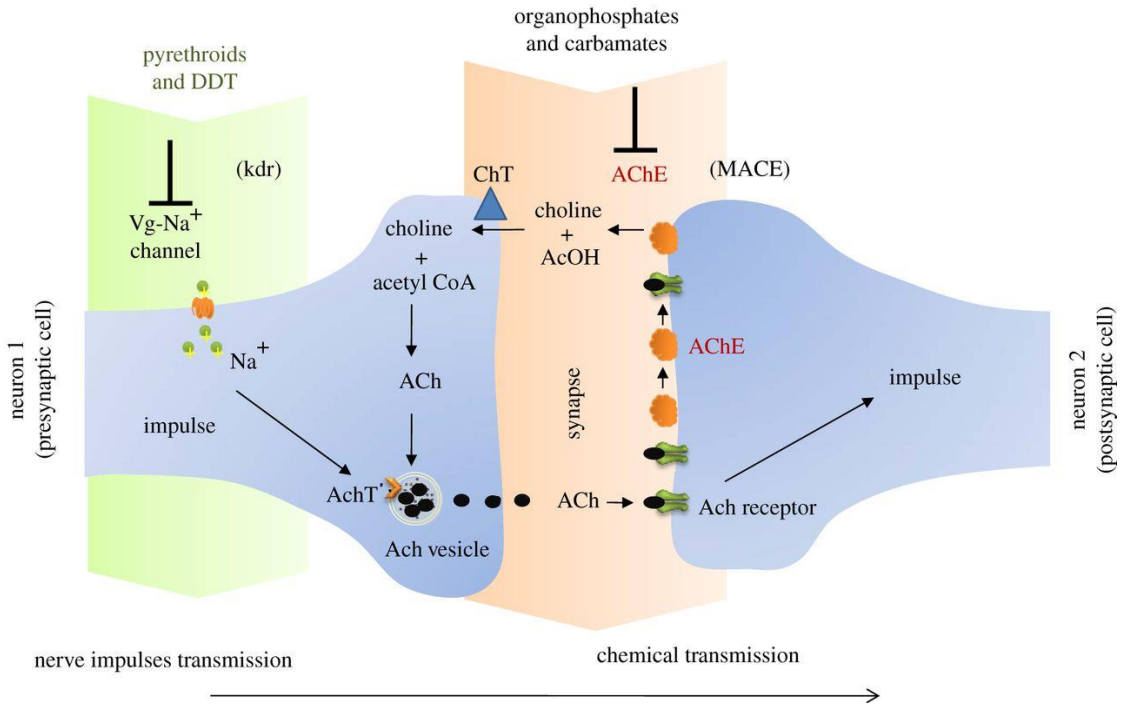

Figure 2.38: Organophosphates and carbamates inactivate acetylcholinesterase (AChE), the enzyme responsible for breaking down the neurotransmitter, acetylcholine (ACh). Acetylcholine accumulates at the nerve junction leading to continuous nerve discharges, paralysis and death of the parasite (rstb.royalsocietypublishing.org)

2.7.4 Formamidines

Formamidines, chlordimeform, clenpyrin and chloromethiuron, are members of a small group of chemicals that are effective against ticks (George et al., 2004). Chlordimeform was introduced in Australia as an additive to organophosphates in dipping vats to restore their efficacy on organophosphate-resistant tick strains but was later withdrawn from the market because of evidence of carcinogenicity (Ware & Whitacre, 2004).

Amitraz (Fig. 2.39), a formamidine, was first successful tested for the control of *R. (B.) microplus* on cattle in Australia with an experimental formulation (BTS 27 419) in 1971 (Palmer et al., 1973). A series of trials executed over a five-year period in South Africa proved the effectiveness of amitraz for the control of *R. (B.) decoloratus, R. appendiculatus, R. evertsi evertsi* and *A. hebraeum* (Stanford et al., 1981). Subsequent trials with commercial amitraz formulations in Australia and in the USA proved the efficacy of the acaricide against *R. (B.) microplus* (George et al., 1998).

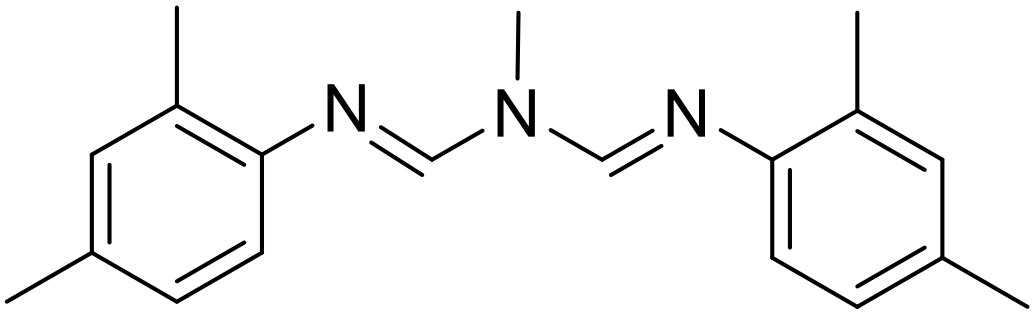

Figure 2.39: Chemical structure of amitraz (N-N-[(methylimino)dimethylidyne]di-2,4—xylidine) (www.newdruginfo.com)

The pharmacological activity of amitraz includes different mechanisms of action, most of which is based on its alpha-adrenergic agonist activity. It also interacts with octopamine receptors in the central nervous system, inhibits monoamine oxidases and prostagladin synthesis (Casida & Durkin, 2013). In contrast to the other acaricides, amitraz is a non-systemic acaricide, which detaches but does not kill ticks (Corta et al., 1999) (Fig. 2.40).

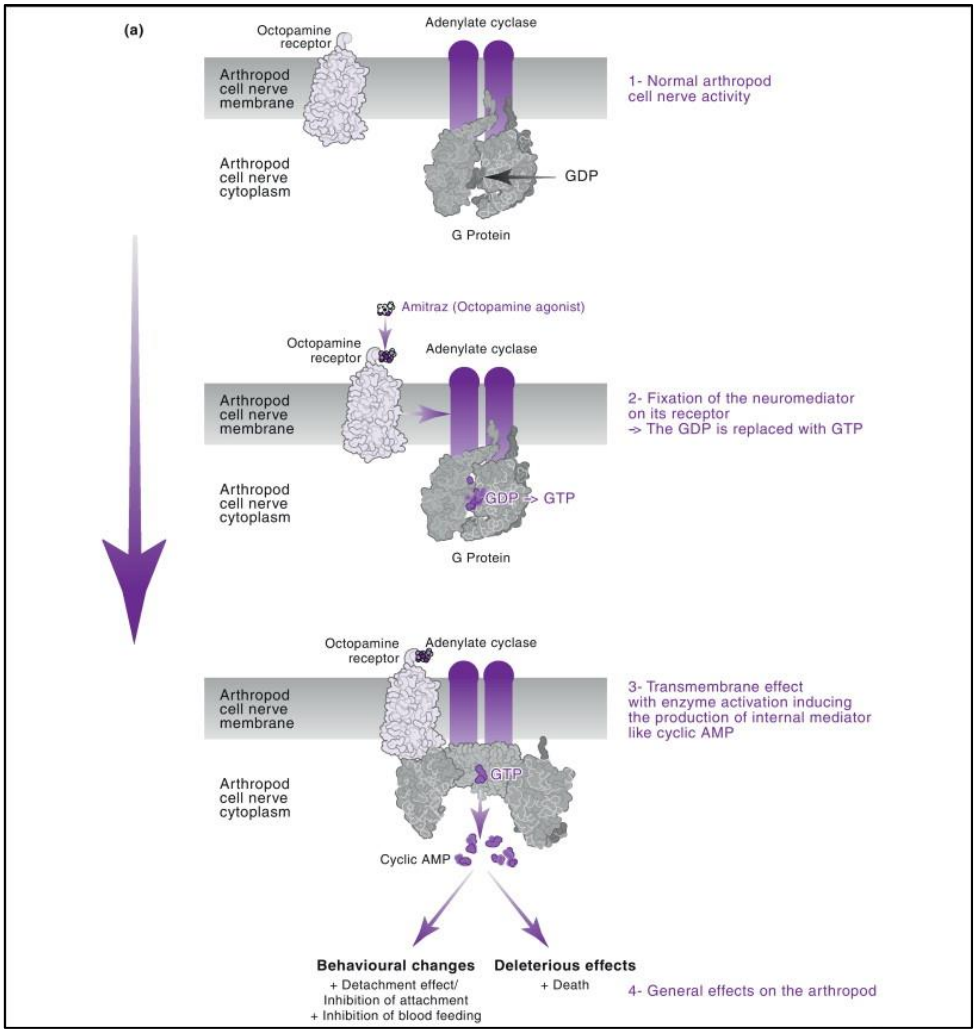

Figure 2.40: Amitraz, an octopamine receptor agonist, competes with octopamine for its receptor site, guanosine diphosphate (GDP) is replaced with guanosine triphosphate (GTP), inducing the production of cyclic adenosine monophosphate (cyclic AMP) leading to inhibition of attachment and ultimately blood feeding with eventual death of the ticks (www.cell.com)

2.7.5 Pyrethrins/Pyrethroids

The pyrethrins are a class of organic compounds derived from the dried flower heads of *Chrysanthemum* species which have been used for centuries for their acaricidal and tick repellent properties (Dhang & Sanjayan, 2014). The pyrethrins also provided the backbone for the synthesis of more potent pyrethroids which are constituents of many commercial household parasiticides. Pyrethroids such as permethrin, flumethrin and cypermethrin (Fig. 2.41), are synthetic adaptations of pyrethrins, specifically designed to be more stable and thus have a longer lasting effect (Dhang & Sanjayan, 2014). Their activity is often enhanced by addition of the synergist piperonyl butoxide, an inhibitor of key microsomal cytochrome P450 enzymes (Ishaaya & Casida, 2013).

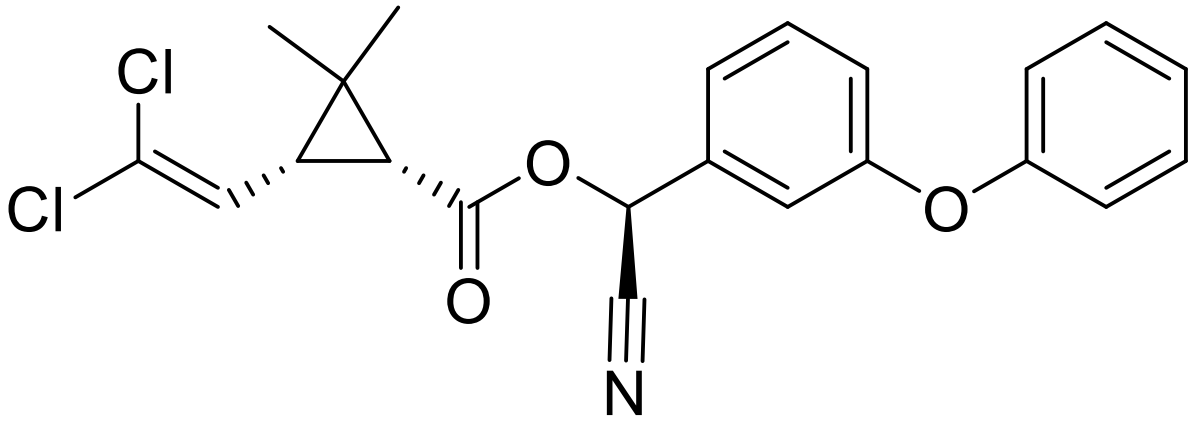

Figure 2.41: Chemical structure of cypermethrin [(Cyano-(3-phenoxyphenyl) methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) (www.alanwood.net)

Pyrethrins and the pyrethroids are lipophilic and this property helps them to act on contact as neurotoxins by blocking sodium ion movement along the axon of the motor nerves in the neuroendocrine and central nervous system of the ticks. This stimulates repetitive nerve discharges that lead to paralysis and death (Ware & Whitacre, 2004) (Fig. 2.42).

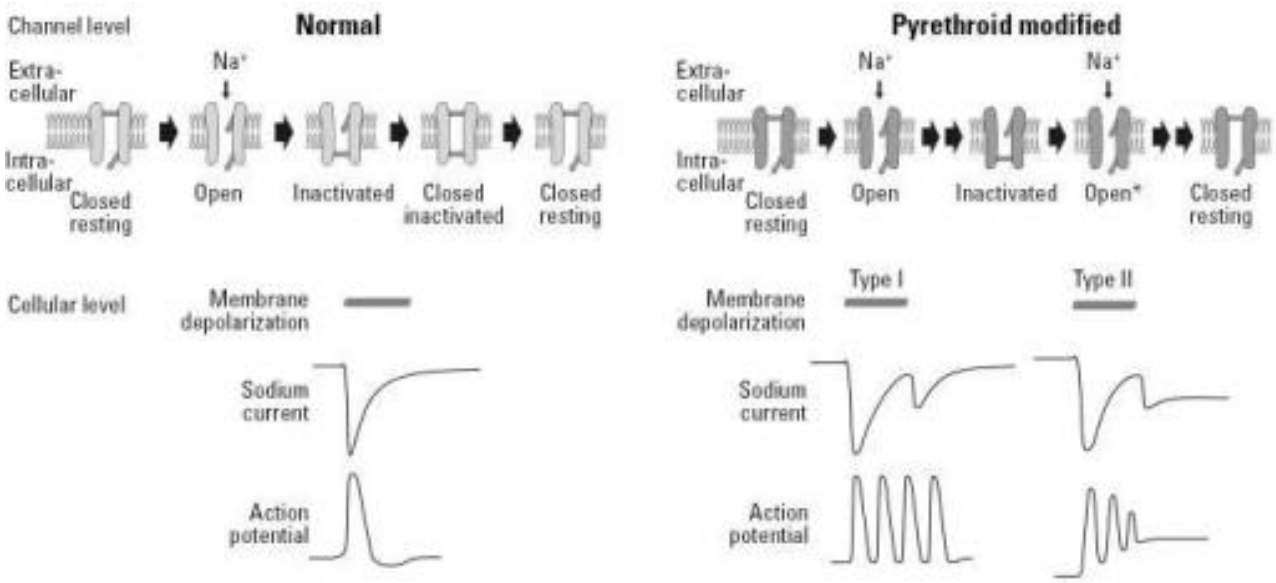

Figure 2.42: Pyrethroid effects on neuronal excitability. Depolarization opens VSSCs (top left) allowing sodium to enter the cell. To limit sodium entry and depolarization length, VSSCs inactivate and must return to a "resting" state before reopening. Pyrethroids inhibit the function of two different "gates" that control sodium flux through VSSCs (top right), delaying inactivation (indicated by double arrows between states) of the channel and allowing continued sodium flux (Open*). If sodium current through an entire cell is measured, depolarization leads to a rapidly inactivating current under normal circumstances (bottom left, Sodium current). Pyrethroid-modified VSSCs remain open when depolarization ends (bottom right, Sodium current), resulting in a "tail" current (the notch at the end of example currents). If membrane voltage is examined, depolarization under normal circumstances generates a single action potential (bottom left). VSSCs modified by type I compounds (bottom right, Action potential) depolarize the cell membrane above the threshold for action potential generation, resulting in a series of action potentials (repetitive firing). Type II compounds cause greater membrane depolarization, diminishing the sodium electrochemical gradient and subsequent action potential amplitude. Eventually, membrane potential becomes depolarized above the threshold for action potential generation (depolarization-dependent block) (openi.nlm.nih.gov)

2.7.6 Macrocyclic lactones (Macrolides)

The macrocyclic lactones are acaricides with potent endo and ectoparasitic properties and were first described in 1978 (Burg et al., 1979). Two classes of macrocyclic lactones are the milbemycins (milbemectin, milbemycin oxime, moxidectin), derived from fermentation products of *Streptomyces hygroscopicus* subsp. *aureolacrimosus* and the avermectins (ivermectin, selamectin, doramectin, abamectin, eprinomectin), which are derivatives of *Streptomyces avermitilis* (Lasota & Dybas, 1991; Nonaka et al., 2000). For the discovery of the avermectins, an extraordinary potent class of compounds, the 2015 Nobel Prize in Physiology or Medicine was awarded to two renowned scientists, William Campbell and Satoshi Omura (www.nobelprize.org).

The structure of the two classes of macrocyclic lactones are closely related and they both share a complex 1, 6-membered macrocyclic lactone ring (Fig. 2.43); however, the milbemycins do not contain the disaccharide substituent in the 1, 3- position of the lactone ring (Nonaka et al., 2000).

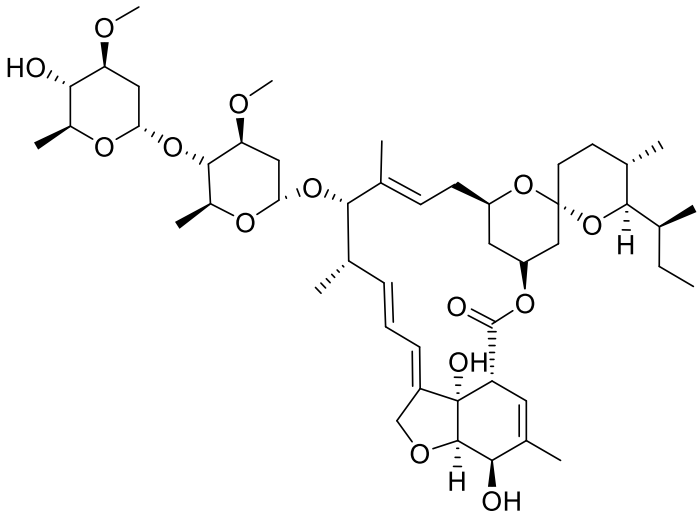

Figure 2.43: Chemical structure of ivermectin (22, 23-dihydroavermectin), a macrocyclic lactone (www.chemicalbook.com)

Macrocyclic lactones bind to multiple channels, including the glutamate-gated Cl^- (GluCl) and GABA-gated Cl^- channels (Fig. 2.44). The Cl^- influx, caused by these compounds opening Cl^- channels in nerves, results in hyperpolarization, subsequent paralysis and death. Comparable doses are not toxic to mammals as they do not possess GluCl channels which accounts for its potency (Raymond & Sattelle, 2002). Though macrocyclic lactones are efficacious, their high cost limits their use (Kemp et al., 1999) and resistance to ivermectin has been reported in *R. sanguineus* (Rodriguez-Vivas et al., 2017).

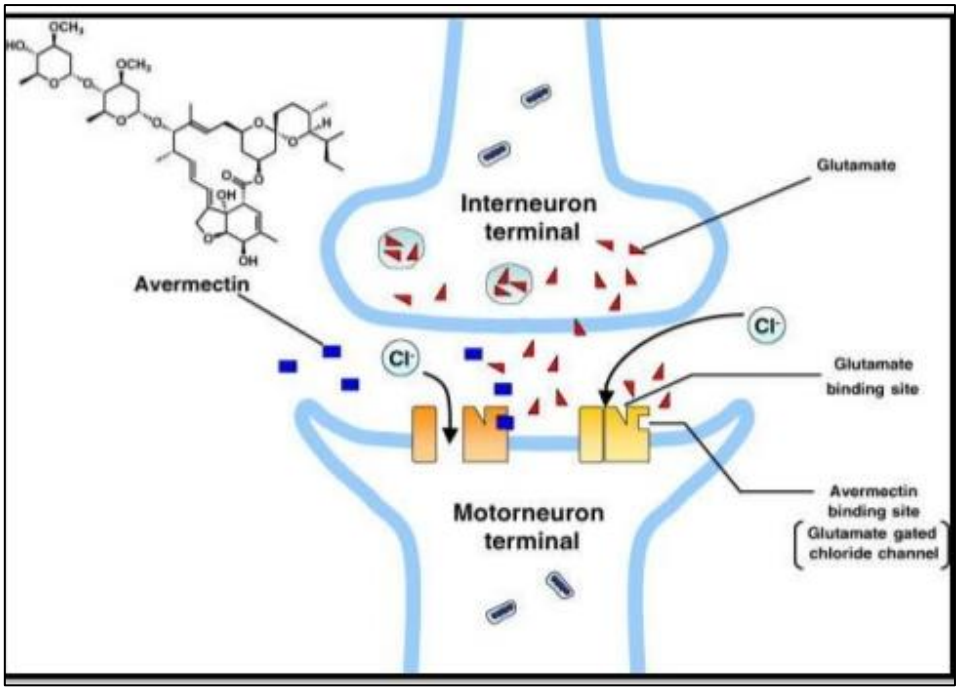

Figure 2.44: The avermectins bind to glutamate-gated chloride channels thereby opening up the channels causing chloride ion flux. This results in disruption of activity and loss of function in these excitable cells (www.slideshare.net)

2.7.7 Phenylpyrazoles

Phenylpyrazoles are a relatively new class of ectoparasiticides with a broad spectrum of insecticidal and acaricidal activity introduced in the early 1990's both for agricultural and veterinary use (Cole et al., 1993). Fipronil (Fig. 2.45), the first phenylpyrazole introduced in the market, is approved for use in dogs and cats while pyriprole is used only in dogs (Singh & Randhawa, 2013).

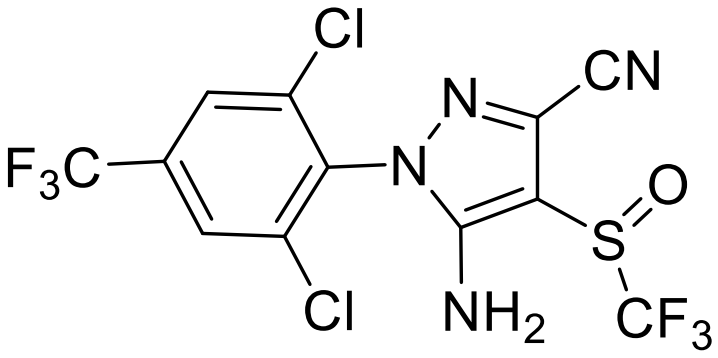

Figure 2.45: Chemical structure of fipronil (RS)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4- (trifluoromethylsulfinyl)-1H-pyrazole-3-carbonitrile) (agrofar.com)

Fipronil, like the avermectins, also acts by binding to $GABA_A$ and GluCl gated channels preventing the opening of Cl^- channels normally encouraged by GABA thereby, reducing the Cl^- ability to lower the neuron's membrane potential. This results in an overabundance of neurons reaching action potential and central nervous system toxicity via over-stimulation (Cole et al., 1993) (Fig. 2.46). Ectoparasiticidal specificity of fipronil may also be because GluCl channels do not exist in mammals (Simon-Delso et al., 2015).

The phenylpyrazoles are quite lipophilic and when applied topically to animals, they are deposited in the sebaceous glands of the skin from where they are slowly released. This allows a rather long residual effect against several ectoparasites which are generally killed before they bite, decreasing the risk of disease transmission.

Fipronil is however highly toxic to fish, certain birds, aquatic invertebrates and bees. In May 2003, the French Directorate-General of Food at the Ministry of Agriculture determined that a case of mass bee mortality observed in southern France was related to acute fipronil toxicity. This necessitated the withdrawal of agricultural products with fipronil in many EU countries (Godfray et al., 2014). Various studies have confirmed cross-resistance between fipronil and the organochlorines (Davari et al., 2007).

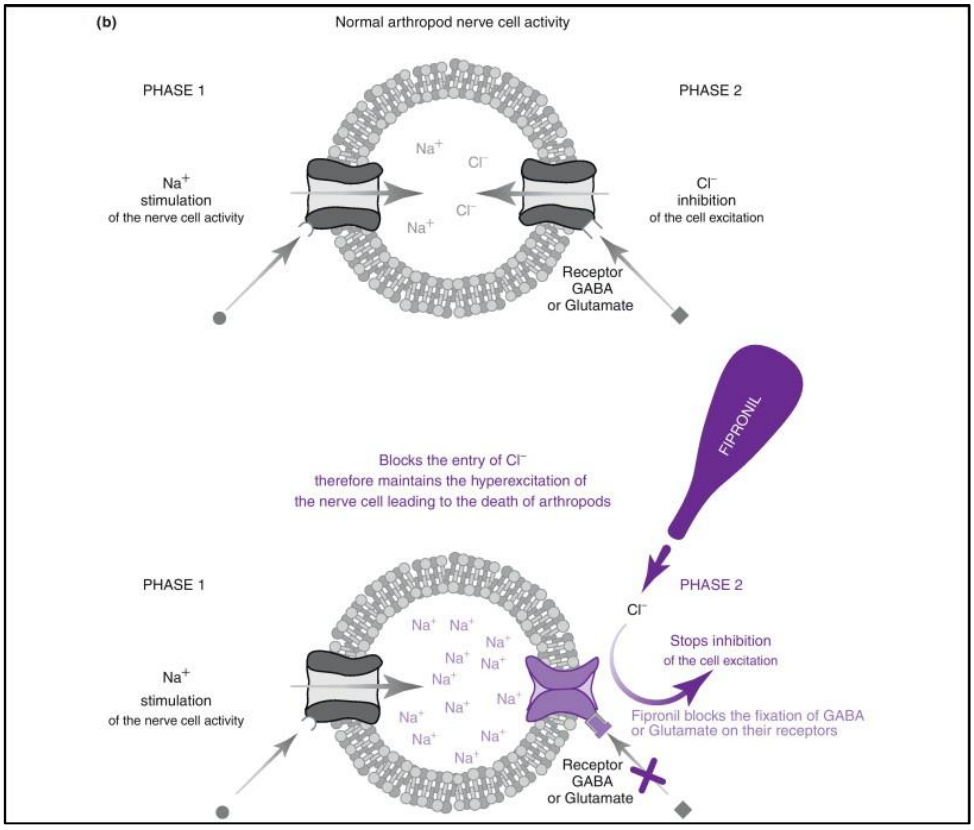

Figure 2.46: Fipronil blocks the fixation of GABA and glutamate on their receptors thereby inhibiting chloride flux into the nerve. This maintains the hyperexcitation of the nerve cell leading to the death of the ticks (www.cell.com)

2.7.8 Spinosyns

Spinosyns are fermentation products of the soil actinomycetes *Saccharopolyspora spinosa* and *Saccharopolyspora pogona*. Spinosad (for livestock), the first of this class, was discovered in the 1990's (Fig. 2.47) and several dozens of natural spinosyns e.g spinetoram (for companion animals) and hundreds of semi-synthetic derivatives have been produced in the laboratory (Bacci et al., 2016).

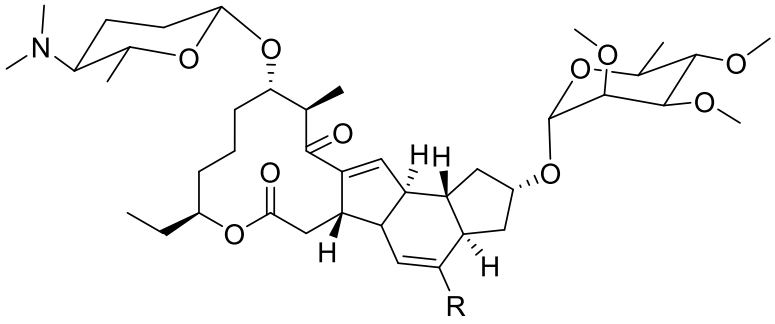

Figure 2.47: Chemical structure of spinosad. Spinosad contains a mixture of two spinosoids, Spinosyn A, R=H and Spinosyn D, R=CH_3 in 17:3 ratio (plantmanagementnetwork.org)

Spinosad is highly active, by both contact and ingestion with a novel mechanism of action that is distinct from those of other acaricides. It primarily targets binding sites on nicotinic acetylcholine receptors of the parasite's nervous system leading to disruption of acetylcholine neurotransmission and also has secondary effects as a GABA neurotransmitter agonist (Bacci et al., 2016) (Fig. 2.48).

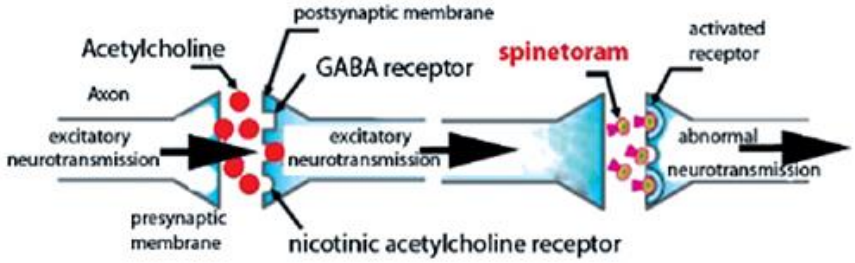

Figure 2.48: The spinosyns such as spinetoram act by binding to nicotinic acetylcholine receptors of the tick's nervous system leading to disruption of acetylcholine neurotransmission (Bacci et al., 2016)

This acaricide has low mammalian toxicity and a good environmental profile; hence, is approved for use in organic agriculture. Recently though, cases of resistance in the field have been reported (Bacci et al., 2016).

2.7.9 Insect Growth Regulators

Insect Growth Regulators (IGRs) prevent insects and ticks from reaching a reproductive stage, thereby reducing the expansion of pest populations. They can be divided into two broad categories; those that disrupt the hormonal regulation of metamorphosis and those that disrupt the synthesis of chitin, a principal component of the exoskeleton (Oberlander & Silhacek, 1998).

Azadirachtin (Fig. 2.49), found in the seeds of the neem tree, *Azadirachta indica* A. Juss., is one of the most widely used botanical IGRs. The compound bears a structural resemblance to the moulting hormone, 20-hydroxyedsyone and thus interrupts moulting, metamorphosis and development of the female reproductive system. Immature insects and arachnids exposed to azadirachtin (mainly by ingestion) may moult prematurely or die before they can complete a properly timed moult. Those that survive are likely to develop into deformed adults incapable of feeding, dispersing or reproducing (Beckage et al., 2000).

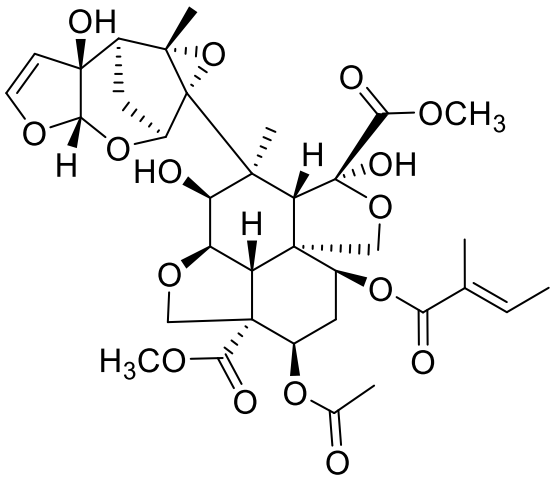

Figure 2.49: Chemical structure of azadirachtin (dimethyl [2aR-[2aα,3ß,4ß(1aR*,2S*,3aS*,6aS*,7S*,7aS*),4aß,5α,7aS*,8ß(E), 10ß,10aα,10bß]]-10-(acetyloxy)octahydro-3,5-dihydroxy-4- methyl-8-[(2-methyl-1-oxo-2-butenyl)oxy]-4-(3a,6a,7,7a)-tetrahydro-6a-hydroxy-7a-methyl-2,7-methanofuro[2,3- b]oxireno[e]oxepin-1a(2H)-yl)-1H,7H-naphtho-[1,8-bc:4, 4a-c']difuran-5,10a(8H)-dicarboxylate) an Insect Growth Regulator (www.odec.ca)

Azadirachtin has very low mammalian toxicity (lethal dose to kill 50% population in rats is greater than 3 540 mg/kg) and is biodegradable (degrades within 100 hours when exposed to light or water). Due to their unique multiple mechanisms of action, the IGRs have played an important role in integrated pest management systems and as effective resistance management tools (Beckage et al., 2000).

2.7.10 Isoxazolines

Isoxazolines (fluralaner, sarolaner, lotilaner, CPD I and afoxolaner) (Fig. 2.50) are the newest class of acaricides introduced in the 2010s for companion animals and have demonstrated remarkable activity against ticks. They are available for oral administration to dogs and topical spot-on for dogs and cats. This has been a major breakthrough as most of the effective acaricides used previously have been topically administered products (Weber & Selzer, 2016). They also are advantageous over the first generation of oral parasiticides available to veterinarians, which had limited ability to effectively kill both ticks and fleas on dogs (Six et al., 2016).

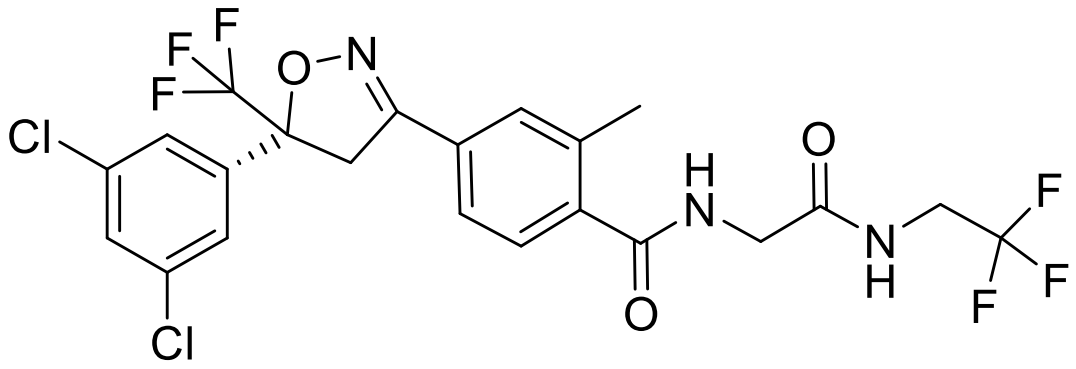

Figure 2.50: Chemical structure of the isoxazoline, fluralaner 4-[(5R/S)-5-(3,5-Dichlorophenyl)-4,5-dihydro-5-trifluoromethyl-1,2-oxazol-3-yl]-N-[2-oxo-2-(2,2,2-trifluoroethylamino)ethyl]-o-toluamide (researchgate.net)

Isoxazolines are non-competitive GABA receptor antagonists, much more selective for ticks than for mammals. They bind to Cl^- channels in nerve and muscle cells blocking the transmission of neuronal channels (McTier et al., 2016). They have a systemic mechanism of action, are rapidly absorbed in blood and distributed throughout the whole body of the host, thus, ticks are killed during their blood meal. Due to their recent introduction, there are no reports of resistance or cross-resistance with other acaricides that act on GABA receptors (Weber & Selzer, 2016).

2.8 Resistance of ticks to commonly used acaricides

Resistance to acaricides is a significant increase in the number within a single population of tick species that can tolerate doses of acaricides that have proved to be lethal for most ticks of the same species. Different life stages of ticks may show different degrees of resistance and eggs are often the most resistant because they have protective envelopes which prevent entry of toxic molecules (Van Leeuwen et al., 2009)**.**

The resistance factor (RF) which describes how strongly resistance has developed, is calculated by dividing the lethal dose needed to kill a population of the resistant parasite strain by the lethal dose for a susceptible reference strain. A RF of 10 means that 10 times more acaricide is needed to kill resistant ticks than to kill susceptible ones. A RF of 2-5 is often considered as tolerance**,** but for macrocyclic lactones, RF of 2-5 may result in product failure because they have a narrow margin of safety and their efficacy at the recommended dose is usually close to the minimum effective concentration needed to kill the parasites (Van Leeuwen et al., 2009).

The RF of many field strains of ticks against the organophosphates and the formamidines is often less than 50. Such products may still provide some level of control, although visibly insufficient. The synthetic pyrethroids have the highest and most widespread resistance (RF>100) among all ectoparasiticides in the world (van Wyk et al., 2016).The severity of tick resistance has reached a level where resistance is expected in ticks within five to ten years of introduction of any acaricide and *R.* (*B.*) *microplus* is the tick species most commonly studied for resistance (Kumar et al., 2016).

2.8.1 Types of resistance

2.8.1.1 Acquired resistance

This is defined as resistance that results from heritable decreases in sensitivity to drugs with time. Long term and repeated use of sublethal doses of acaricides to control tick populations may result in the development of acquired resistance as there is a direct relationship between concentration of a drug and degree of resistance (Rosario-Cruz & Domínguez-García, 2016).

2.8.1.2 Side resistance

If a tick population becomes resistant to a particular compound, it will most likely become resistant to other compounds in the same chemical class. This is because most compounds of the same chemical class have the same mechanism of action at the molecular level, but there are exceptions. Some strains of *R. (B.) microplus* are known to be resistant to cypermethrin and deltamethrin but are susceptible to flumethrin, another synthetic pyrethroid (Rosario-Cruz & Domínguez-García, 2016).

2.8.1.3 Cross resistance

This is the sharing of resistance genes for different acaricides which have similar mechanisms of action. This has been shown among two organophosphates (coumaphos and diazinon) and one carbamate (carbaryl) acaricide in *R. (B.) microplus* (Madder et al., 2011). Rotation of different classes of acaricides that have no cross resistance reduces the selection pressure for resistance to any of the classes e.g., rotating the use of fipronil (a phenylpyrazole) followed by a pyrethroid.

2.8.1.4 Multiple resistance

A parasite population can become simultaneously resistant to two or more chemical classes with different mechanisms of action, termed multiple resistance. This has been reported in *R.* (*B.*) *microplus* and *R.* (*B.*) *decoloratus.* It seems that once a parasite population has developed resistance to a first chemical class, it will likely develop resistance to a second chemical class faster than to the first one. However, research findings on this issue are not conclusive (Rosario-Cruz & Domínguez-García, 2016).

2.8.2 Mechanisms of resistance

Generally, resistance can arise through several mechanisms at the cellular or molecular level in individual ticks. These mechanisms are broadly classified as metabolic, reduced penetration or target site resistance (Rosario-Cruz & Domínguez-García, 2016).

2.8.2.1 Metabolic resistance (enhanced detoxification, excretion or sequestration)

Metabolic resistance to acaricides occurs through changes in the ability of a tick to detoxify, sequester or excrete an acaricide. The enzyme families - cytochrome P450s, esterases and glutathione S-transferases are generally involved (Rosario-Cruz & Domínguez-García, 2016). The oldest reported case was resistance to DDT, an organochlorine due to sequestration of the toxic DDT molecules into the fat bodies and storage organs of many parasites. By this, they prevented the toxic molecules from reaching their target sites.

2.8.2.2 Reduced penetration

Reduced penetration in ticks could arise through alterations in the ability of an acaricide to penetrate a treated tick. This resistance mechanism has been identified in a few arthropods including *R. (B.) microplus* (Rosario-Cruz & Domínguez-García, 2016).

2.8.2.3 Target site insensitivity

Target site insensitivity is the most common mechanism of resistance and exists when an allele of the gene coding for the target molecule attacked by the acaricide has an amino acid mutation that confers resistance to the acaricide (Rosario-Cruz & Domínguez-García, 2016).

The voltage-gated sodium ion channel is the target site for pyrethroid activity and target site resistance to pyrethroids has been studied in many arthropod species. There are at least three target site specific amino acid substitutions reported in *R. (B.) microplus* to date, the domain III mutation (phenylalanine to isoleucine) and domain II mutations (leucine to isoleucine; glycine to valine) (van Wyk et al., 2016).

The organophosphates and carbamates target the acetylcholinesterase enzyme. There is uncertainty about the identity of the transcript encoding acetylcholinesterase that is functionally relevant for acaricide resistance in ticks and more than one acetylcholinesterase might be involved in acaricide responses (Singh et al., 2016). Seven contigs with significant sequence similarity to acetylcholinesterase were reported in the transcriptome of *R. (B.) microplus* and in another study, a malathion-resistant strain of *R. (B.) microplus* was reported to have increased amounts of acetylcholinesterase compared to malathion-susceptible strains (Temeyer & Tuckow, 2016). Thus a target site gene amplification or mutation within the gene promoter region might be the specific resistance mechanism.

The target site of amitraz has not been definitively identified, although candidates such as monoamine oxidase, octopamine receptor and alpha-2 adrenoceptors have been proposed. Resistance to amitraz may arise from modifications of the octopamine receptor. The molecular basis for this has been studied in *R. (B.) microplus* and two nucleotide substitutions in the octopamine receptor have been identified in the resistant tick strains (Chen et al., 2007). It has been suggested that resistance to amitraz is controlled by a recessive inheritance and more than one gene is involved in this process (Guerrero et al., 2012).

Fipronil, a phenylpyrazole, acts on dual targets (GABA and GluCl-gated channels) and probably plays a role in delaying or preventing the buildup of high levels of resistance. However, one of these targets is shared with dieldrin (cyclodiene organochlorine) and low levels of fipronil resistance can be associated with resistance to dieldrin (Buckingham et al., 2005).

The target site for macrocyclic lactones is also believed to be the GABA and GluCl-gated channels. The exact mechanism of resistance against macrocyclic lactones is still unknown in ticks and mites (Perez-Cogollo et al., 2010). Because fipronil and the macrocyclic lactones are both believed to act on the same Cl^- channels, the possibility of cross resistance must be considered.

2.9 Management of resistance

2.9.1 Regular monitoring

Monitoring is very essential in delaying the development of resistance. Though application of acaricides every three weeks during the peak tick season is suggested in areas where tick resistance is common, misuse and overuse of acaricide application is a risk factor for the emergence of resistant strains (Abbas et al., 2014). It is therefore strongly recommended that acaricide treatments should not exceed five per season (Thullner et al., 2007; Jonsson et al., 2000). To reduce the development of resistance, the knowledge of the tick species present in an area and the resistance status should be borne in mind before the selection of acaricides (Sun et al., 2011).

2.9.2 Acaricide combinations

Combination of two acaricides with different mechanisms of action is another attractive approach to delay the emergence of resistance, based on the likelihood that a tick will not have resistant alleles to both (Lovis et al., 2013). This strategy has been tried in South Africa and simulation modeling indicates its promise. Likewise, Fernández-Salas et al. (2012) evaluated the synergistic effect of amitraz and permethrin against a permethrin-resistant *R. (B.) microplus* strain from Mexico. Permethrin alone caused no mortality in the resistant strain even at the highest concentration but addition of amitraz led to a dramatic increase in larval mortality. The chemicals in a combination product must be compatible, of equal persistence on the animal and used at recommended dose.

2.9.3 Rotation of acaricides

Rotation of acaricides having different mechanisms of action reduces the selection pressure for resistance to a particular acaricide group. Thullner et al. (2007) showed that in a *R. (B.) microplus* strain treated with deltamethrin, resistance to deltamethrin was very high (RF=756) after eleven generations. In comparison, in a *R. (B.) microplus* strain treated with deltamethrin then coumaphos in rotation, resistance to deltamethrin was very low (RF=1.6) after ten generations. Further field trials are required to evaluate the beneficial effects of rotation of acaricides of veterinary importance (Adakal et al., 2013). Limitations of this strategy are that it is costly and not easy to practice. There is also no evidence of the duration of time between rotations, although most veterinarians suggest this should not be less than every two years (Maggi et al., 2011). For many parasites, it has been hypothesized that if a drug for which the parasite has developed resistance is withdrawn for some time, the sensitivity to that drug may return (Gharbi et al., 2013).

2.9.4 Vaccination

Enhancing immunity in cattle is an important tool for tick control. Commercially available vaccines that are approved for use including Gavac® (Heber Biotec; Havana, Cuba), TickGARD (Hoechst Animal Health; Australia) and TickGARDPLUS (Intervet, Australia), are based on the recombinant form of the concealed antigen, Bm86, obtained from the mid gut of *R. (B.) microplus* (Freeman et al., 2010). Recent research is providing new indications that the target might be conserved in a number of tick species, resulting in some successes against *R*. (*B). annulatus* (Popara et al., 2013), *H. dromedarii* and *A. cajennense* (Rodríguez-Valle et al., 2012). In the field, promising results have been obtained by using vaccines alone (Carreón et al., 2012; Shahein et al., 2013) or in combination with acaricides (Olds et al., 2012; Cunha et al., 2012).

Field trials of the TickGARD vaccine (produced in Australia) in some areas of Brazil revealed that it was not able to control the target tick. A possible reason for such variation in vaccine efficacy is amino acid sequence divergence between the recombinant Bm86 vaccine component and native Bm86 expressed in ticks from different geographical regions of the world (Ben Said et al., 2012; Said et al., 2012), thus the quest for a universal vaccine continues (Parizi et al., 2012). It is therefore necessary, to do a preliminary screening of vaccine efficacy before launching a vaccine into a new geographical area. Another limitation of vaccination is the short term protection conferred since tick-host interractions mitigate the immune response. The heartwater-infective blood vaccine, frozen Asiatic redwater vaccine, frozen African redwater vaccine and frozen anaplasmosis (tick-borne gall sickness) vaccine (Onderstepoort Biological Products, South Africa) are available in South Africa to immunize livestock against tick-borne diseases (De Waal, 2000).

2.9.5 Genetic manipulation

It is widely known that the Zebu cattle, *Bos taurus indicus* (adapted to hot climates) are more resistant to ticks than *Bos taurus taurus* (typical cattle of Europe, north-eastern Asia and parts of Africa, many of which are adapted to cooler climates) (Bianchin et al., 2007). This resistance is influenced by a number of factors such as the increased levels of histamine released by mast cells at the early stages of ectoparasitic infestation (De Castro & Newson, 1993), presence of specific immunoglobulin patterns, T cells and genes related to the expression of keratins and lipocalins (Ibelli et al., 2012). Hair and coat traits also can be related to the severity of tick infestation, but there is little data available on the relationship of these traits with tick resistance. In a study conducted by Ibelli et al. (2012), it was found that coat traits influenced resistance in two genetic groups, the Senepol×Nelore cross and Nelore

cattle being highly resistant to *R. (B.) microplus* tick when compared with Angus×Nelore cross.

In general, resistant cattle require only one or two topical acaricidal treatments per season compared with three or four in susceptible breeds. It is, therefore, suggested that studies be intensified on cross-breeding, to obtain animals that are more resistant to environmental conditions in tropical countries and are also good meat producers (Ibelli et al., 2012).

2.9.6 Pasture spelling

The theory behind this is based on the tick life cycle. It involves placing cattle in tick-free paddocks for a period of time sufficient to allow all ticks on the animals to mature and fall, but too short a time to allow progeny of the ticks to hatch and reinfest the cattle. The duration of pasture spelling will be determined by the duration of the phases of tick life cycle in the particular area and the climatic season (Harley & Wilkinson, 1971; Canevari et al., 2017).

Pasture spelling combined with acaricide application have proved to be an effective means of tick control (Stachurski & Adakal, 2010). In a number of intergrated pest management strategies for *A. americanum* in forage areas utilized by Zebu cattle and their crossbreeds over a five year period studied, pasture spelling combined with acaricide applications was the most economically feasible, reducing tick burden by 77 to 89% (Jonsson, 2006).

2.9.7 Improving resistance diagnostic tests

Monitoring of ticks is crucial to diagnose resistance at an early stage, to help slow down the spread of resistance and to obtain knowledge of the distribution of acaricide resistance. The Food and Agriculture Organisation currently recommends and provides standardised protocols to evaluate tick resistance using the larval packet test, originally described by Stone & Haydock (1962) and the adult immersion test, developed by Drummond et al. (1973). Standardised methods are needed to assess resistance evolution and allow the comparison of resistance data between laboratories. Ideally, a suitable laboratory test for acaricide resistance should be sensitive enough to identify resistance early in its emergence, cover the full range of chemical classes in use, be simple, inexpensive and provide rapid and reliable results. Additionally, it should require a low number of ticks and small amounts of compounds.

The adult immersion test has the advantage of providing results within seven days after tick collection for all compounds except growth regulators, but it requires high numbers of engorged females, which may become a limiting factor when resistance to several compounds is evaluated or when the objective is to obtain the full dose-response mortality

curve (Lovis et al., 2011). The larval packet test on the other hand, offers the advantage of using limited number of engorged females (to produce the eggs that will hatch into larvae) and is therefore very suitable for the monitoring of resistance. However, the test is labour intensive and time consuming (Lovis et al., 2011). Efforts should be made to develop promising bioassays which will be suitable to assess resistance levels of different field and laboratory tick strains in different countries (Abbas et al., 2014).

2.10 Potential of medicinal plants for alternate control strategies

In addition to the other methods above, herbal remedies may offer an alternative control strategy. This is necessary due to the high cost of developing new drugs and vaccines, development of drug resistance and concerns over drug residues associated with the continuous use of synthetic chemical acaricides (Babar et al., 2012). The healing ability of plants has been exploited for thousands of years, in the treatment of animals and humans. In India, the Ayurvedic medical system which is more than 3 000 years old is still practiced within the field of complementary or alternative medicine while in China, traditional Chinese herbalism has been in existence for more than 2 500 years (Patwardhan et al., 2005). A number of plant-derived antiparasitic drugs have already made significant contributions to animal and human health such as quinine, the oldest antimalarial drug, obtained from the South American plant, *Cinchona officinalis* L. and artemisinin from *Artemisia annua* L. (Elfawal et al., 2015). A host of other natural products have been known to have inspired synthetic drugs such as atropine from *Atropa belladonna* L. and ginkgo from *Ginkgo biloba* L. (Mar & Bent, 1999).

Rural and semi-urban farmers have limited access to veterinary care, information about animal diseases, therapeutic veterinary medicines and vaccines and therefore have to rely heavily on ethnoveterinary medicine in most cases. Ethnoveterinary medicine is a complex system of practices made up of various combinations of folk beliefs, skills, knowledge and animal husbandry practices, with herbal remedies at the core of therapy (Van der Merwe et al., 2001). Ethnoveterinary management of ectoparasites in animals involves spraying of affected animals topically (using commercial or locally improvised hand sprayers) with crude plant concoctions (mixture of various ingredients), decoctions (liquid prepared from boiling plants) or infusions (extract prepared by soaking herbs in liquid). In northern Nigeria, the Fulanis wash their cattle with an infusion of *Sesbania aculeata* (Willd.) Pers. before traversing a tsetse fly belt and for the control of ectoparasites in poultry, ash from the burnt leaves of *Carica papaya* L (pawpaw) or *Nicotiana tabacum* L. and *Nicotiana rustica* L. (tobacco plants) is rubbed on the feathers to prevent infestation (Nwude & Ibrahim, 1980).

While the use of ethnoveterinary medicine is common practice in rural farming areas, it is often questioned for its inherent safety and efficacy by the Western world as the use has developed through trial and error and only rarely via deliberate experimentation for the development of modern pharmaceuticals (Katerere & Naidoo, 2010). Hence, it has been viewed as less systematic, less formalized and at times even questioned for its validity. Nonetheless, there is a growing acceptance that ethnoveterinary medicine has therapeutic value and needs further evaluation not just to justify its use, but also as a potential source of newer medications to combat multi-resistant pests and disease organisms (Lans et al., 2007a; Lans et al., 2007b).

It is important to bear in mind that lack of activity of a plant in an *in vitro* screening system does not automatically translate to lack of efficacy. Many aspects of ethnoveterinary medicine need to be taken into account, for example, methods of preparation and administration of the remedy, as well as management practices to limit the impact of the disease. The shortcomings of ethnoveterinary medicine include seasonal unavailability of plant material, lack of dosing certainty and standardization of remedies which could lead to inefficacy or toxicity of treatments (Martin et al., 2001). Means of overcoming these disadvantages need to be formulated and communicated to the users of ethnoveterinary medicine. The benefits of understanding, evaluating and ultimately integrating ethnoveterinary medicine into primary animal healthcare are clearly evident.

The African continent is still the least explored in terms of available natural and human resources as vast indigenous knowledge on medicinal plants is far from exhaustively explored. However, a rich cultural heritage is readily available in Africa together with its diverse vegetation for potential introduction of new templates for useful drugs in modern medicine. Unfortunately, little effort is being made to harness these opportunities to address the pressing health problems in Africa (Eloff & McGaw, 2014). The exploitation of these medicinal plant resources using simple, low-cost technologies could be a viable tool in alleviating poverty by becoming an additional income-generating activity, thus enhancing the status of local inhabitants and facilitating a self-managed development process (Yongabi et al., 2009).

Pharmaceutical companies are in the process of screening a number of natural compounds derived from plants and microorganisms to develop non-synthetic medications for the veterinary industry. A surge in such research projects is expected to propel the market for natural parasiticides in the coming years. Newer generation acaricides, targeting previously unexplored metabolic pathways, should be generated and these acaricides should be kept in

reserve for any emergency situations caused by multi-acaricide resistant populations in future. Newer methodologies, including combinatorial chemistry and computational biology along with high throughput screening, could yield new acaricides (Abbas et al., 2014).

2.11 Conclusions

The limitations in the use of many synthetic chemical acaricides include their exorbitant costs, development of resistance, hazard to non-target species and the environment hence the need for alternatives. Research on plant extracts for use in tick control has grown in recent years in an attempt to find compounds with tick repellent and/or acaricidal properties and the integration of ethnoveterinary products with synthetic acaricides may produce better effects in controlling ticks and delaying resistance.

CHAPTER 3

Plant extracts to control ticks of veterinary and medical importance: A review

Adenubi O.T., Fasina F.O., McGaw L.J., Eloff J.N., Naidoo V.

Preface

This chapter addresses the second objective of this study which was to provide an extensive literature survey on the use of plant extracts to control ticks in the animal health industry. The text in this chapter has been published: Adenubi O.T., Fasina F.O., McGaw L.J., Eloff J.N., Naidoo V. 2016. Plant extracts to control ticks of veterinary and medical importance: A review. South African Journal of Botany 105, 178-193. Although only published in the latter part of the year, this paper was the second most downloaded paper published in the South African Journal of Botany in 2016 (Personal communication of Managing Editor to JN Eloff).

Abstract

Farmers in developing countries are faced with many diseases that limit the productivity of their animals, many of which are caused by tick infestations. Years of use and overuse of available chemical ectoparasiticides have resulted in the large scale development of resistance in these parasites as well as negative environmental impacts. To reduce these impacts, much focus has been placed on the search for alternative, environmentally friendly parasite control strategies with lower chance of the development of resistance. Many rural farmers have used plants to control ticks. In some cases the traditional use has been confirmed, in other cases, only the traditional use has been documented. A review of published scientific articles was conducted for medicinal plants with *in vitro* acaricidal or tick-repellent activities against immature and adult stages of ticks. Veterinary databases (All Databases, CAB Abstracts and Global Health, Medline, Pubmed, Web of Science, BIOSIS Citation Index, Science Direct, Current Content Connect and Google Scholar) were used. The search words included “acaricidal”, “tick repellent”, “medicinal plants”, “phytomedicine” and “antitick assays”. More than 200 plant species from several countries globally have tick repellent or acaricidal properties using *in vitro* assays. The different extractions and plant parts used as well as the efficacy where available is listed. Species including *Azadirachta indica, Gynandropsis gynandra, Lavandula angustifolia, Pelargonium roseum* and *Cymbopogon* species had good acaricidal and larvicidal effects with 90-100% efficacy, comparable to those of currently used acaricides. A number of active compounds such as azadirachtin, carvacrol, linalool, geraniol and citronellal have been isolated. Based on their wide use by rural livestock farmers, plant-based compounds may be a good source of effective acaricidal preparations either as an extract or as a source of new acaricidal

compounds. The focus may have to be on acaricidal rather than on repellent activities to facilitate control of ticks.

3.1 Introduction

In the tropics and sub-tropics, small-scale and emerging farmers own approximately 40% of the national livestock herds/flocks (Keyyu et al., 2003a). These farmers are faced with many constraints that limit the productivity of their animals. The prevalence of ticks and tick-borne diseases particularly in the wet seasons (Keyyu et al., 2003b) is an important restraint. Ticks, which are haematophagous ectoparasites, have a wide range of hosts and geographic diversity. They transmit protozoan, bacterial, rickettsial and viral diseases and are among the most important vectors of diseases which can be severely debilitating or fatal to livestock, humans and companion animals (Walker et al., 2003; Jongejan & Uilenberg, 2004).

Ixodid ticks such as *Amblyomma variegatum* Fabriscius, *Rhipicephalus appendiculatus* Neumann and *Rhipicephalus (Boophilus) microplus* (Canestrini, 1888) in particular are among the most economically important parasites in the tropics and subtropics (Bram, 1983). Tick-borne protozoan diseases such as theilerioses and babesiosis and rickettsial diseases such as anaplasmoses and cowdriosis are the most common diseases of small and large ruminants affecting the livelihoods of farming communities in Africa, Asia and Latin America (Jongejan & Uilenberg, 2004). In addition to transmitting diseases, heavy infestations of ticks can cause a reduction in live weight, anaemia and losses in milk producton in domestic animals, while tick bites themselves result in damage to hides (Rajput et al., 2006).

Due to severity of the diseases transmitted by ticks, a substantial proportion of the annual input costs by many livestock keepers go into the management and control of ticks and tick-borne diseases (Kaaya & Hassan, 2000). While the true economic losses are not easily quantifiable, losses were estimated at US$720 million, US$100 million and US$1 billion per year for Africa, Australia and South America respectively (Horn, 1987; Cobon & Willadsen, 1990; Kaaya & Hassan, 2000; Minjauw & McLeod, 2003). When losses per disease are looked at, Theileria control in eastern, central and southern Africa was estimated at US$168 million annually, while the annual cost of tropical theileriosis management in India was estimated at US$384.3 million. The Theileria parasite has also been implicated as the cause of annual production losses in excess of US$200 million in small scale and traditional farming communities of Kenya and Tanzania (Mukhebi et al., 1992; Kivaria, 2006). While less substantial than Theileria, losses from heartwater were estimated at US$6 million per annum in Zimbabwe over a 10-year period from the cost of acaricides, milk losses and

treatment costs (Coetzer et al., 1994). Based on this information, it is evident that ticks and the diseases they transmit are a major constraint to the improvement of the livestock industry, particularly in developing countries, where they contribute to food insecurity. Due to financial devastation caused by ticks and tick-borne diseases, animals infected are often treated by the farmer with either an allopathic or herbal remedy.

Current control programmes are largely based on the use of commercially available chemicals such as the arsenicals, chlorinated hydrocarbons, organophosphates, carbamates, formamidines, pyrethroids, macrocyclic lactones, and more recently the insect growth regulators (George et al., 2004). Arsenicals were effectively used globally to control ticks for 30 to 40 years prior to the development of resistance in *Boophilus* ticks (George et al., 2004). While these products were inexpensive, stable and water-soluble, they were characterised by short residual effects of less than one or two days and were also environmentally destructive (Drummond, 1960). The arsenicals were eventually replaced by the chlorinated hydrocarbons between 1945-1955. The chlorinated hydrocarbons were characterised by a long residual effect and were very effective. Unfortunately these molecules were very stable and persisted in the environment and tissues of treated livestock for fairly long periods (Connel et al., 1999). The product also had a major knock-on effect on predators higher in the food chain prompting their eventual withdrawal (Spickett, 1998). Organophosphates, an esteric compound of phosphoric acid synthesis, supplemented organochlorines in the 1955-70s. In contrast to the organochlorines, they were characterised by a shorter residual effect, lower environmental persistence but substantially higher acute toxicity in livestock and by 1963, resistance was reported (Wharton, 1967).

Formamidines, chlordimeform, clenpyrin and chloromethiuron, are members of a small group of chemicals that are effective against ticks (George et al., 2004). Chlordimeform was introduced in Australia as an additive to organophosphates in dipping vats to restore their efficacy on organophosphate-resistant tick strains (Nolan, 1981). It was later withdrawn from the market because of evidence of carcinogenicity (Ware, 2000). Results of successful tests of amitraz for the control of *R. (B.) microplus* on cattle in Australia with an experimental formulation (BTS 27 419) were reported in 1971 (Palmer et al., 1971). Subsequent trials with commercial amitraz formulations in Australia (Roy-Smith, 1975) and in the United States of America (George et al., 1998) proved the efficacy of the acaricide against *R. (B.) microplus*. A series of trials executed over a five-year period in South Africa proved the effectiveness of amitraz for the control of *R*. (*B.) decoloratus, R. appendiculatus, R. evertsi evertsi* and *A. hebraeum* (Stanford et al., 1981).

Macrocyclic lactones are acaricides with potent insecticidal activity which were first described in 1978 (Burg et al., 1979). Two classes of macrocyclic lactones with acaricidal activity are the avermectins (ivermectin, eprinomectin), which are derivatives of the actinomycete *Streptomyces avermitilis* and the milbemycins, derived from fermentation products of *S. hygroscopicus aureolacrimosus* (Lasota & Dybas, 1991). Macrocyclic lactone acaricides are efficacious, but their high cost limits their use (Kemp et al., 1999). Fipronil, a phenylpyrazole compound; fluazuron, a benzoyl phenyl urea; spinosad represents new pesticides, but because of the persistence of residues in fat, it is necessary to withhold treated cattle from human consumption for up to six weeks after use (Bull et al., 1996).

The issues mentioned above have motivated the search for alternative parasite control strategies that are potentially environmentally friendly with fewer negative consequences to the animal being treated. Principal among these alternatives are the plant-based treatment protocols as the healing effect of plants has been explored for thousands of years (Chopra, 2003; Wang & Li, 2005). Other proposals for the full development of medicinal plants as tick repellents/acaricides has been advocated (Gassner et al., 1997) as plants inherently have a number of protective mechanisms to combat predator and pathogen attacks. These include repellency through production of hairs and volatile compounds such as cis-Jasmone (Birkett et al., 2000), 1, 8-cineole (Klocke et al., 1987) and production of chemicals with arthropocidal activities such as I-menthone from *Mentha piperita* L. (Croteau and Winters, 1982; Silva-Aguayo, 2006). These phytochemicals act in different ways, such as counteraction of growth regulatory hormones, inhibition of egg development, disruption of mating and sexual communication and inhibition of chitin formation (Katoch et al., 2007; Chagas et al., 2012). A number of plant-derived novel antiparasitic drugs have already made significant contributions to human and animal health such as quinine, the oldest antimalarial drug, obtained from the South American plant, *Cinchona officinalis* L. and artemisinin from *Artemisia annua* L. (Ronald & Acton, 1987).

Pyrethrum derived from the dried flower heads of *Chrysanthemum cinerariifolium (*Trev.) Vis and *Chrysanthemum coccineum* has been used for centuries as an insecticide and lice remedy in the Middle East (Casida, 1980). More importantly, pyrethrum provided the backbone for the synthesis of more potent synthetic pyrethroids. The first generation pyrethroids (bioallethrin, tetramethrin, resmethrin and bioresmethrin) were developed in the 1960s, following the elucidation of the structures of pyrethrin I and II, its main pesticidal components (Isman & Machial, 2006). The third generation of this class of chemicals, permethrin and fenvalerate, were the first of these products available for control of ticks on cattle (Davey & Ahrens, 1984; Ware, 2000). Cypermethrin and deltamethrin are examples of

fourth generation cyano-substituted pyrethroids that are effective acaricides (Stubbs et al., 1982; Kunz & Kemp, 1994; Aguirre et al., 2000). Pyrethroids now constitute the majority of commercial household insecticides and their activity is often enhanced by addition of the synergist piperonyl butoxide, a known inhibitor of key microsomal cytochrome P450 enzymes (Devine & Denholm, 1998). The insecticidal activity of pyrethrum has relatively low mammalian toxicity and an unusually fast biodegradation hence, it is one of the most commonly used, non-synthetic insecticide allowed in certified organic agriculture (Pottorff, 2010).

In 2007, a new repellent, BioUD, with the active ingredient 7.75% 2-undecanone, originally derived from wild tomato (*Lycopersicon hirsutum* Dunal) plants, was registered by the U.S. Environmental Protection Agency (Gershenzon & Dudareva, 2007; Witting-Bissinger et al., 2008).

3.2 Materials and Methods

This article reviews previous research on plants extracts and essential oils as acaricides/repellents. The keywords used to collect literature for this review were “tick repellent”, “acaricidal”, “medicinal plants”, “phytomedicine” and “antitick assays”. Veterinary databases (All Databases, CAB Abstracts and Global Health, Medline, PubMed, Web of Science, BIOSIS Citation Index, Science Direct, Current Content Connect and Google Scholar) were searched between January and December, 2014. Specifically, *in vitro* antitick assays employed in the last 100 years (1914-2014) were given priority consideration. Plant species tested, the country in which the experiments was/were performed, type of assays used, stage of ticks targeted and method of administration were considered in the filtration. The Medline was filtered down using MeSH Qualifier (Parasitology) and MeSH Headings (Ticks)-in view of the very large returns of titles >15,000- and other filters were applied to other databases as necessary. All documents considered were in English or translated into English.

3.3. Results and Discussion

Acaricidal and insecticidal properties of many plant species have been widely investigated against phytophagous pests and mosquitoes (Calmasur et al., 2006; Mukandiwa et al., 2015), blowflies (Mukandiwa et al., 2012; 2013), mites (Kim et al., 2004; Nong et al., 2013a) and ticks (Lori et al., 2005) with differing results. Many of the earlier studies on acaricidal activities focussed on the *in vitro* and *in vivo* effects and toxicity of chemical acaricides on various insects and acarines (Wilson, 1948; Guilhon, 1950; Arthur, 1951; Hadani et al., 1969).

In the 1970s, more intensive evaluation of plants for their acaricidal activities was started by Khaidarov (1971), who evaluated 84 plant species. Of these, 21 had *in vitro* acaricidal activity against larvae and adults of *Rhipicephalus bursa* C. & F., *Hyalomma anatolicum* Koch and *H. marginatum* Koch. More recently, various plant products, crude extracts and essential oils have been evaluated for their repellent and acaricidal properties against all the stages (adult, nymph, larva and egg) of economically important tick species with encouraging results (Chungsamaryart et al., 1988, 1990, 1991a; Mehlhorn et al., 2005; Coskun et al., 2008; Daemon et al., 2009; Magadum et al., 2009; Monteiro et al., 2009, 2012; Clemente et al., 2010; Kamaraj et al., 2010; Zorloni et al., 2010; Ghosh et al., 2011; Koc et al., 2012; Singh et al., 2014). This has also included numerous review publications of tick repellent and acaricidal properties of plants periodically (Kaaya, 2000; Copping & Menn, 2000; Flamini, 2003; Nerio et al., 2010; gar Ebadollahi, 2011; Zoubiri & Baaliouamer, 2011; Maia & Moore, 2011; Borges et al., 2011; Andreotti et al., 2014; George et al., 2014; Ghosh & Ravindran, 2014).

3.3.1 Taxonomic distribution of activity and countries where the work was done

For this review, a total of thirty families of plant species with acaricidal activity were identified. Sixteen familes had only one species represented and five familes had only two representatives. The Lamiaceae and Asteraceae were the most used with twelve and eight representatives (Table 3.1).

As could be expected, a large proportion of the published work was done in tropical countries where ticks play an important role (Table 3.2). Most of the references were from Brazil (15), India (12) and South Africa (4). The plant species used originated from countries where the eco-climatic conditions are suitable for tick survival. If plants in these tropical environments have compounds that protect them against arachnids, insects and other pests, it is possible that these compounds may also be active against ticks. Because ticks cause major problems in these areas, rural farmers are more likely to use plants for tick control.

Table 3.1 Different plant families and the number of species

S/No	**Lamiaceae**	**Asteraceae**	**Rutaceae**	**Fabaceae**	**Solanaceae**	**Leguminosae**	**Meliaceae**
1	*Anisomeles malabarica*	*Artemisia absinthium*	*Aegle marmelos*	*Calpurnia aurea*	*Capsicum frutescens*	*Cassia didymobotrya*	*Azadirachta indica*
2	*Lavandula angustifolia*	*Baccharis trimera*	*Citrus reticulata*	*Leucaena leucocephala*	*Datura stramonium*	*Copaifera reticulata*	*Carapa guianensis*
3	*Leucas aspera*	*Calea serrata*	*Citrus maxima*	*Lysiloma latisiliquum*	*Solanum trilobatum*	*Tamarindus indica*	
4	*Leucas indica*	*Eupatorium adenophorum*	*Citrus sinensis*	*Piscidia piscipula*	*Withania somnifera*		
5	*Ocimum urticaefolium*	*Matricaria chamomilla*	*Citrus hystrix*				
6	*Origanum minutiflorum*	*Tagetes erecta*					
7	*Origanum onites*	*Tagetes minuta*					
8	*Satureja thymbra*	*Tagetes patula*					
9	*Tetradenia riparia*						
10	*Thymus vulgaris*						
11	*Vitex negundo*						

S/No	**Poaceae**	**Myrtaceae**	**Euphorbiaceae**	**Geraniaceae**	**Verbanaceae**	**Asclepiadaceae**	**Bromeliaceae**
1	*Cymbopogon citratus*	*Corymbia citriodora*	*Jatropha curcas*	*Pelargonium graveolens*	*Lippia javanica*	*Calotropis procera*	*Ananas comosus*
2	*Cymbopogon nardus*	*Melaleuca alternifolia*	*Ricinus communis*	*Pelargonium roseum*	*Lippia sidoides*		
3	*Cymbopogon winterianus*						

S/No	**Acanthaceae**	**Caricaceae**	**Cupressaceae**	**Combretaceae**	**Scrophulariaceae**	**Capparidaceae**	**Guttiferae**
1	*Andrographis paniculata*	*Carica papaya*	*Chamaecyparis nootkatensis*	*Guiera senegalensis*	*Digitalis purpurea*	*Gynandropsis gynandra*	*Hypericum polyanthemum*

S/No	**Ptaeroxylaceae**	**Ericaceae**	**Chrysobalanaceae**	**Lauraceae**	**Alliaceae**	**Piperaceae**	**Simaroubaceae**
1	*Ptaeroxylon obliquum*	*Rhododendron tomentosum*	*Licania tomentosa*	*Lindera melissifolia*	*Allium sativum*	*Piper tuberculatum*	*Simarouba versicolor*

S/No	**Stemonaceae**
1	*Stemona collinsae*

Table 3.2 Number of references in different continents

	ASIA				
S/No	**India**	**Thailand**	**Iran**	**Saudi Arabia**	**China**
1	Elango & Rahuman, 2011	Chungsamarnyart & Jansawan, 1996	Pirali-Kheirabadi & Razzaghi-Abyaneh, 2007	Al-Rajhy et al., 2003	Nong et al., 2013a; 2013b
2	Shyma et al., 2014	Chungsamarnyart & Jiwajinda, 1992	Pirali-Kheirabadi et al., 2009		
3	Zahir et al., 2010	Kongkiatpaiboon et al., 2014			
4	Godara et al., 2014				
5	Srivastava et al., 2008				
6	Singh et al., 2014				
7	Ravindran et al., 2011a; 2011b				
8	Divya et al., 2014				
9	Juliet et al., 2012				
10	Veeramani et al., 2014				
11	Ghosh et al., 2013				
12	Rajakumar et al., 2014				

	AMERICA		
S/No	**Brazil**	**USA**	**Mexico**
1	Domingues et al., 2013	Dietrich et al., 2006	Fernande-Salas et al., 2011
2	Lazaro et al., 2013	Oh et al., 2012	
3	Ribeiro et al., 2008	Tabanca et al., 2013	
4	Vasconcelos et al., 2014		
5	de Freitas Fernandes & Frietas, 2007		
6	Chagas et al., 2014		
7	Ribeiro et al., 2007		
8	Valente et al., 2014		
9	Gomes et al., 2014		
10	Pazinato et al., 2014		
11	Lima et al., 2014		
12	Politi et al., 2012		
14	Gazim et al., 2011		
15	Daemon et al., 2009		

	AFRICA				
S/No	**South Africa**	**Ethiopia**	**Uganda**	**Sudan**	**Kenya**
1	Mkolo & Magano, 2007	Zorloni et al., 2010	Opiro et al., 2013	Osman et al., 2014	Lwande et al., 1999
2	Magano et al., 2011				
3	Moyo & Masika, 2013				
4	Nchu et al., 2012				

	OCEANIA	EUROPE	
S/No	**New Caledonia**	**Turkey**	**Sweden**
1	Hue et al., 2014	Cetin et al., 2009; 2010	Jaenson et al., 2005

3.3.2 Compounds used

Many of the plants reviewed in this study contained terpenes and terpenoids (Table 3.3). These phytochemicals derived from units of isoprene (hemi-, mono-, sesqui-, di- etc) (Moore et al., 2007; Laudato & Capasso, 2013) are structurally a diverse assemblage of compounds that make up the largest group of secondary plant chemicals (Langenheim, 1994) and are involved in defence against herbivorous animals and pathogens (Kappers et al., 2005).

3.3.3 Extractants and bioassays used

A number of solvents including hexane, acetone, ethanol and distilled water were used as extractants in the papers reviewed with ethanol being the solvent most commonly used (Table 3.3). It has been reported previously that many natural products have low water solubility and need to be dissolved in organic solvents or surfactant agents before being used in experimental systems (Domingues et al., 2013). In a study by Goncalves et al. (2007), the effects of solvents and surfactant agents on adult female and larvae of the cattle tick *R.* (*B*.) *microplus* was evaluated. Acetone and methanol were the most toxic solvents while ethanol had moderate toxicity. Ravindran et al. (2011a; 2011b) however noted that methanol can be safely used for dissolving herbal extracts for testing acaricidal properties. While it is recognized that aqueous solvents are widely used in ethnoveterinary medicine, organic solvents may work better in acaricidal bioassays as the cuticle of ticks is formed externally mainly by waxes and internally by proteins (Balashov, 1972). Hence, the more non-polar a chemical compound is, the greater will be its ability to penetrate the cuticle (Chagas et al., 2002).

Different bioassay methods including petri dish method, larvae packet test, tick climbing repellency bioassays using vertical rods or strips of fabric, immersion tests have been used by researchers with immersion tests and larvae packet tests more commonly used (Table 3.3). All species and stages of life cycle of Ixodid ticks have been studied by different researchers and *R.* (*B.*) *microplus* was the tick most commonly studied (Table 3.3). *Rhipicephalus (Boophilus) microplus*, a one-host tick, parasitic mainly on cattle is one of the most widely distributed tick species and is a major threat to the cattle industry in tropical and subtropical areas (Dominguez-Garcia et al., 2010). The tick is also the most important economically as it is responsible for severe losses caused by tick worry, blood loss, damage to hides, injection of toxins and disease transmission. Around the world, extracts from approximately 55 plant species belonging to 26 families have already been evaluated against *R. (B.) microplus* (Borges et al., 2011).

Table 3.3 Medicinal plants with tick repellent and acaricidal properties and their phytochemical constituents

Plant	Family	Plant Part	Extractant	Major Phytochemical constituent(s)	Tick species	Age (ticks)	Bioassay	Summary of Results	Country	References
Aegle marmelos (Linn.) Correa ex Roxb	Rutaceae	L	HX CH EA AC MeOH	Aeglemarmelosine, alkaloids, coumarins	*H. bispinosa* *R.(B.) microplus*	A LV	APT LPT	3 mg/ml and 2 mg/ml MeOH extract caused 100% acaricidal MR for *H. bispinosa* and 100% larvicidal MR for *R. (B.) microplus* at 24 hr PT respectively.	India[a]	Elango & Rahuman, 2011[a] Laphookhieo et al., 2011[b]
Allium sativum L.	Alliaceae	Cl	MeOH	Allicin, terpenoids, steroids	*R. (B.) microplus*	A LV	AIT LPT	100 mg/ml caused 69% larvicidal MR, 85.83% IO, 100% failure of eclosion of eggs and 80% acaricidal MR within 15 days.	India[a]	Aboelhadid et al., 2013 Shyma et al., 2014[a] Reuter & Sendi, 1994[b]
Ananas comosus L. Merr.	Bromeliaceae	Sk	DW	Ananasate, 1-*O*-caffeoylglycerol, caffeic acid, *p*-coumaric acid, β-sitosterol, daucosterol	*R. (B.) microplus*	EF LV	AIT LPT	500 mg/ml caused 39.1% IO, 33.3% EHI, efficacy percentage of 59.4% and 0% larvicidal MR at 24 hr PT.	Brazil[a]	Domingues et al., 2013[a] Ma et al., 2007[b]
Andrographis paniculata (Burm.f.) Wall. ex Nees.	Acanthaceae	L	HX CH EA AC MeOH	Tannins, flavonoids, carbohydrates, proteins	*H. bispinosa* *R.(B.) microplus*	A LV	LPT	3 mg/ml MeOH extract caused 100% acaricidal MR for *H. bispinosa* and 2 mg/ml EA extract caused 100% larvicidal MR for *R. (B.) microplus* at 24 hr PT.	India[a,b]	Tanwer & Vijaguergia, 2010[b] Elango & Rahuman, 2011[a]
Anisomeles malabarica (L) R. Br.	Lamiaceae	L	HX CH EA AC MeOH	Alkaloids, saponins, protein, gum, mucilage	*H. bispinosa*	A	APT	3 mg/ml AC and MeOH extract caused 100% acaricidal MR at 24 hr PT.	India[a,b]	Zahir et al., 2010[a] Nisha & Packialakshmi, 2014[b]
Artemisia absinthium L.	Asteraceae	AP	EtOH CH	Cis-epoxyocimene, sesquiterpenes	*Hyalomma anatolicum* *R. sanguineus*	A E LV	AIT EHT LPT	200 mg/ml caused 100% larvicidal MR, 100% EHI, 59.1% OR and 86.7% acaricidal MR for *H. anatolicum* at 24 hr PT. For *R. sanguineus*, there was 100% larvicidal MR , 100% EHI, 85.1% OR and 93.3% acaricidal MR.	India[a,b]	Bailen et al., 2013[b] Godara et al., 2014a; 2014b[a]
Azadirachta indica A. Juss	Meliaceae	L B S	EtOH	Azadirachtin	*R.(B.) microplus*	EF	AIT	8 mg/ml caused 80% acaricidal MR and 34.0 mg egg mass reduction at 5 hr PT.	India[a]	Williams, 1993 Williams & Mansingh., 1996 Akhila & Rani, 1999[b] Gupta et al., 2000 Choudhury, 2001

										Benavides et al., 2001 Abdel-Shafy & Zayed, 2002 Al-Rajhy et al., 2003 Abdel-Shafy et al., 2006 Alwin et al., 2007 Shyma et al., 2007 Srivastava et al., 2008[a]
Baccharis trimera (Less.) DC	Asteraceae	L	DW	Diterpenes	*R.(B.) microplus*	EF	AIT	150 mg/ml caused 100% EHI 15 days PT.	Brazil[a]	Lago et al., 2008[b] Lazaro et al., 2013[a]
Calea serrata Less	Asteraceae	AP	HX	Eupatorio-chromene, precocene II	*R. (B.) microplus* *R. sanguineus*	EF LV	AIT LIT	6.25 mg/ml caused 100% larvicidal MR of both tick species at 48 hr PT. 50 mg/ml caused 100% EHI and 14.6% ELI in *R. (B.) microplus* after 14 days.	Brazil[a]	Steinback et al., 1997[b] Ribeiro et al., 2008[a]
Calotropis procera (Ait) R.Br	Asclepiadaceae	LX	AC	Stigmasterol, β-sitosterol, digitoxin, calotoxin	*Hyalomma dromedarii*	EF LV	Contact LIT AIT	The contact LC_{50} value against adults and larvae was 9.63 µg/cm^2 and 6.16 µg/cm^2 respectively whereas the dipping LC_{50} values were 1096 mg/L and >20.3 µg/cm^2 respectively.	Saudi Arabia[a]	Al-Rajhy et al., 2003[a] Shyma et al, 2007 Kakar et al., 2012[b]
Calpurnia aurea subsp. *aurea* (Aiton) Benth.	Fabaceae	L	DW HX AC	Calpurmenin, 13a-(2'-pyrrolecarboxylic acid) ester, virgiline, lupanine	*R. pulchellus*	Unfed adult	TCR Contact	Tick attraction was observed. 200 mg/ml AC extract caused 100% acaricidal MR.	Ethiopia[a,b]	Zorloni et al., 2010[a,b] Nana et al., 2010
Capsicum frutescens L.	Solanaceae	F	EtOH	Capsaicin	*R.(B.) microplus*	EF	AIT	75 mg/ml caused 85% MR at 48 hr PT; 25 mg/ml caused 86.8%. There was 99.9% acaricide efficacy.	Brazil[a]	Nazari et al., 2007[b] Vasconcelos et al., 2014[a]
Carapa guianensis Aubl.	Meliaceae	Se	EO	Hexacosanoic acid-2,3-dihydroxy-glyceride, ursolic acid, naringenin, scopoletin	*R. sanguineus*	EF	AIT	200 mg/ml showed 80.17% reproductive efficiency index.	Brazil[a]	Qi et al., 2004[b] Vendramini et al., 2012[a] Roma et al., 2013
Carica papaya L.	Caricaceae	Se	MeOH	Alkaloids, glycosides, phenols and tannins	*R. (B.) microplus*	EF LV	Contact LIT AIT	100 mg/ml, caused 82.2% larvicidal MR, 100% IO and eclosion of eggs, 93.33% acaricidal MR within 15 days.	India[a]	Ocloo et al., 2012[b] Shyma et al., 2014[a]
Cassia didymobotrya (Fresen) Irwin & Barneby	Leguminosae	AP	MeOH DCM HX	Stilbenes, flavones, 7-acetylchrysophanol,biant hrones,	*R. appendiculatus*	LV	FR	0.25 mg/ml MeOH extract showed 87.67% repellency.	Uganda[a]	Delle Monache et al., 1991[b] Opiro et al., 2013[a]

				tetrahydroanthracenes						
Chamaecyparis nootkatensis (D. Don) Spach	Cupressaceae	AP	AC	Carvacrol, nookatene, nookatone	*I. scapularis*	N	Vertical bioassay	Nootkatone and valencene-13-ol had repellent conc. $(RC)_{50}$ values of 0.0458 and 0.0712% respectively at 4 hr PT.	USA[a]	Panella et al., 2005[b] Dietrich et al., 2006[a]
Citrus maxima Burm.	Rutaceae	F	EtOH	Phenol, saponins, alkaloids, tannins, terpenoids	*R. (B.) microplus*	EF LV	AIT LIT	100 mg/ml caused 62.61% larvicidal MR 1-2 hr post dipping and 100% acaricidal MR 24 hr PT.	Thailand[a]	Chungsamarnyart & Jansawan, 1996[a] Chanthaphon et al., 2008[b] Pandey et al., 2010[b]
Citrus reticulata Blanco	Rutaceae	F	EtOH	L-limonene, γ-terpene, β-phellandrene	*R. (B.) microplus*	EF LV	AIT LIT	100 mg/ml. caused 90.77% larvicidal MR 1-2 hr PT and 100% acaricidal MR 24 hr PT.	Thailand[a]	Chungsamarnyart & Jansawan, 1996[a] Sultana et al., 2012 [b]
Citrus sinensis L.	Rutaceae	F	EtOH	Flavonoids, tannins, saponins, phytate, oxalate, limonene	*R. (B.) microplus*	EF LV	AIT LIT	100 mg/ml caused 98.59% larvicidal 1-2 hr PTand 99% acaricidal MR 24 hr PT.	Thailand[a]	Chungsamarnyart & Jansawan, 1996[a] Oluremi et al., 2007 [b]
Citrus hystrix DC (Swangi)	Rutaceae	F	EtOH	Glycerolglycolipids, tannins, tocopherols, furanocoumarins, flavonoids, alkaloids	*R. (B.) microplus*	EF LV	AIT LIT	100 mg/ml caused 90.29% larvicidal MR 1-2 hr PT and 98% acaricidal MR 48 hr PT.	Thailand[a]	Chungsamarnyart & Jansawan, 1996[a] Arumugam et al., 2014 [b]
Copaifera reticulata Ducke	Leguminosae	LX	DMSO DW	Oleoresin	*R. (B.) microplus*	LV	LPT	3.5 mg/ml caused 99% larvicidal MR at 24 hr PT.	Brazil[a]	Prates et al., 1993 Chagas et al., 2002 Fernandes et al., 2005; 2007; 2008 de Freitas Fernandes & Freitas, 2007[a]
Corymbia citriodora (Hook.) K.D. Hill and L.A.S. Johnson	Myrtaceae	AP	EO	Citronellal	*R. (B.) microplus*	LV A	LPT AIT	100 mg/ml caused 100% OR, 100% hatching reduction 100% acaricidal and larvicidal MR at 24 hr PT.	Brazil[a]	Lee & Chang, 2000[b] Clemente et al., 2010 Chagas et al., 2002 Chagas et al., 2014[a]
Cymbopogon citratus (DC) Stapf	Poaceae	L S R	EtOH/EO	Myrcene, α-citral (geranial), β- citral (neral)	*R. (B.) microplus*	EF LV	AIT LIT	125 mg/ml caused 98.78% larvicidal MR 1-2 hr PT and 100% acaricidal MR 5 days PT.	Thailand[a]	Chungsamarnyart & Jiwajinda, 1992[a] Onawunmi et al., 1984[b]
Cymbopogon nardus (Linn) Rendle	Poaceae	L S R	EtOH/EO	Geraniol, trans-citral, cis-citral, geranyl acetate, citronellal, citronellol	*R. (B.) microplus*	EF LV	AIT LIT	125 mg/ml caused 95.78% larvicidal MR 1-2 hr PT and 100% acaricidal MR 24 hr PT.	Thailand[a]	Chungsamarnyart & Jiwajinda 1992[a] Nakahara et al., 2003[b] Clemente et al., 2010
Cymbopogon winterianus Jowitt ex Bor	Poaceae	L	DW EtOH	Geraniol, citronellal, citronellol	*R. (B.) microplus*	EF LV	AIT LPT	50 mg/ml caused 58.01% IO and 10% EHI at 15 days PT.	India[a]	Martins, 2006 Quintans-Júnior et al., 2008[b] Singh et al., 2014[a]
Datura stramonium L.	Solanaceae	L	MeOH	Alkaloids, atropine,	*R. B. microplus*	EF	Contact	100 mg/ml caused	India[a]	Shyma et al., 2014[a]

				scopolamine, tannin, proteins		LV	LIT AIT	73.33% acaricidal MR at 15 day PT, 71.8% larvicidal MR, 77.17% IO and eclosion of eggs.		Sayyed & Shah, 2014[b]
Digitalis purpurea L.	Scrophulariaceae	LX	AC	Digitoxin	*Hyalomma dromedarii*	EF LV	Contact LIT AIT	Contact and dipping LC_{50} values against larvae were 6.16µg/cm^2 and 587.7mg/l.	Saudi Arabia[a]	Al-Rajhy et al., 2003[a]
Eupatorium adenophorum Spreng	Asteraceae	L	EtOH	Sabinene, 1,8-cineole, p-cymene, camphene	*H. longicornis*	LV N	LIT NIT	At a conc. of 1.5 g/ml (w/v), there was 100% MR for both larval and nymphal ticks 6 hr PT.	China[a]	Nong et al., 2013b[a] Padalia et al., 2010[b]
Guiera senegalensis J.F. Gmel.	Combretaceae	L	EtOH PE	Guieranone A, alkaloids	*Hyalomma anatolicum*	All stages	Immersion test	150 mg/ml EtOH extract induced 100% larvicidal MR, 100% feeding inhibition and 100% ELI 48 hr PT.	Sudan[a]	Osman et al., 2014[a] Fiot et al., 2006[b]
Gynandropsis gynandra (L.) Briq	Capparidaceae	AP	EO	Carvacrol, trans-phytol, linalool, trans-2-methylcyclopentanol,β-caryophyllene	*R. appendiculatus*	A	TCR	At 0.1µl conc. there was 98.9% repellency.	Kenya[a]	Dipeolu et al., 1992 Malonza et al., 1992 Ndungu et al., 1995 Lwande et al., 1999[a,b]
Hypericum polyanthemum Klotzsch ex H. Reichardt	Guttiferae	AP	HX MeOH	Xanthones, flavonoids, benzopyrans	*R. (B.) microplus*	EF L	AIT LIT	50 mg/ml HX extract caused 19.2% ELI and 6.25 mg/ml caused 100% larvicidal MR at 48 hr PT.	Brazil[a]	Booth et al., 1986 Rocha et al., 1994[b] Ferraz et al., 2001[b] Borges et al., 2003 Ribeiro et al., 2007[a]
Jatropha curcas L.	Euphorbiaceae	L	EtOH	Stigmasterol, β-sitosterol, campesterol	*R. annulatus*	EF	AIT	50 mg/ml caused 90% EHI at 30 days PT.	India[a]	Neuwinger, 1994[b] Gubitz et al., 1999[b] Juliet et al., 2012[a]
Lavandula angustifolia Mill	Lamiaceae	AP	DW	1,8-cineole, camphor, borneol	*Hyalomma marginatum rufipes*	A	TCR	200 mg/ml caused 100% repellency up to 2 hr PT.	South Africa[a]	Jaenson et al., 2006 Mkolo & Magano, 2007[a] Pirali-Kheirabadi & Teixera da Silva, 2010[b] Azar et al., 2011
Leucaena leucocephala (Lam) De Wit	Fabaceae	AP	DW	Quercetin, mimosine, ficaprenol-11	*R. (B.) microplus*	A LV	AIT LIT	4.8 mg/ml caused 66.79% larval MR at 48 hr PT, 33.14% EHI and 1.8% ELI at 21 days PT.	Mexico[a]	Fernandez-Salas et al., 2011[a] Salem et al., 2011[b]
Leucas aspera (Willd)	Lamiaceae	AP	EtOH	Nicotine, diterpenes, lignans, flavanoids	*R. annulatus*	EF	AIT	100 mg/ml conc. caused 54.16% acaricidal MR and 100% EHI at 15 days PT.	India[a]	Mangathayaru et al., 2006[b] Ravindran et al., 2011b[a]
Leucas indica Spreng	Lamiaceae	L	EtOH	Flavones, diterpenes	*R. annulatus*	EF	AIT	50 mg/ml alkaloid fraction caused 66% adult MR, 55% inhibition of fecundity and 100% hatching within 15 days PT.	India[a]	Mostafa et al., 2007[b] Divya et al., 2014[a]

Licania tomentosa Benth	Chrysobalanaceae	L	HX EtOH	Betulinic acid, licanolide, a new triterpene lactone, oleanolic acid, lupeol, palmitoleic acid, hexadecanoic acid	*R. (B.) microplus*	LV	LPT	600 mg/ml EtOH extract caused larvicidal MR of 40.26% 24 hr PT.	Brazil [a,b]	Castilho et al., 2008[b] Valente et al., 2014 [a]
Lindera melissifolia (Walt.) Blume	Lauraceae	D	EO	β-caryophyllene, α-humulene, germacrene D, β-elemene	*A. americanum* *I. scapularis*	N A	VFP	0.827 mg/cm^2 extract repelled 74% of the *A. americanum* nymphs at 15 min PT and 97.5% of *I. scapularis* adults.	USA[a]	Oh et al., 2012[a,b]
Lippia javanica (Burm. F.) Spreng	Verbernaceae	AP	EO	Myrcene, 1,8-cineole, dihydrotagetone, ipsenone, 2-butanone	*Hyalomma marginatum rufipes*	A	TCR	107 mg/ml caused a repellency index of 100% at 1 hr 30 min PT.	South Africa[a]	Magano et al., 2011[a,b]
Lippia sidoides Cham	Verbernaceae	L	EO	Lippsidoquinone, quercetin, tecomaquinone	*R. sanguineus* *A. cajannense*	LV N	LPT	18.80 mg/ml caused 99% larvicidal MR and 96% nymphal MR (*R. sanguineus*); 100% larvicidal MR and 94% nymphal MR (*A. cajannense*).	Brazil [a]	Costa et al., 2001[b] Gomes et al., 2014[a]
Lysiloma latisiliquum (Tzalam)	Fabaceae	L	AC:DW	Tannins, crude protein, phenols	*R. (B.) microplus*	LV A	LIT AIT	19.2 mg/ml. caused 56% larval MR at 48 hr PT, 69.34% EHI and 36.4% ELI at 21 days PT.	Mexico[a]	Alonzo-Diaz et al., 2006[b] Fernandez-Salas et al., 2011[a]
Matricaria chamomilla L.	Asteraceae	Fl	EtOH	Herniarin, oleanolic acid, stigmasterol	*R. (B.) annulatus*	EF	AIT	80 mg/ml caused 26.67% acaricidal MR at 24 hr PT and 46.67% ELI at 5 days PT.	Iran [a]	Ahmad & Mishra, 1997 Pirali-Kheirabadi & Razzaghi-Abyaneh, 2007[a]
Melaleuca alternifolia (Maiden & Betche) Cheel	Myrtaceae	AP	EO	1,8-cineole, α-pinene, β-pinene	*R. (B.) microplus*	EF	AIT	50 mg/ml and 100 mg/ml showed 100 % reproductive inhibition.	Brazil[a]	Russell & Southwell, 2002[b] Pazinato et al., 2014[a]
Ocimum basilicum L.	Lamiaceae	L	HX CH EA	Linalool, (Z)-cinnamic acid methyl ester, cyclohexene	*R. (B.) microplus*	A	AIT	60 mg/ml, 80 mg/ml and 100 mg/ml crude CH extracts produced 70%, 80% and 100% acaricidal MR respectively.	India[a]	Zhang et al., 2009[b] Veeramani et al., 2014[a]
Ocimum urticaefolium Roth	Lamiaceae	Fl	EO	Eugenol, 1,8-cineole, elemicin, β-Bisabolene, thymol	*R. (B.) microplus*	LV	LPT	50 mg/ml caused 100% larvicidal MR.	New Caledonia[a]	Hue et al., 2014[a]
Origanum minutiflorum O. Schwarz and P.H. Davis	Lamiaceae	AP	EO	Carvacrol, camphene, myrcene	*R. turanicus*	Unfed adult	Vapor phase toxicity bioassays	200 mg/ml caused 100% acaricidal MR at 120 min.	Turkey[a,b]	Cetin et al., 2009[a,b]
Origanum onites L.	Lamiaceae	AP	EO	Cymene, thymol, carvacrol, γ-terpinene	*R. turanicus*	A	APT	250 mg/ml and higher caused 100% MR at 24 hr PT.	Turkey[a]	Coskun et al., 2008[a] Skoula et al., 1999[b]

Pelargonium graveolens L'Her	Geraniaceae	AP	EO	Linallol, citronellol, geraniol	*A.americanum*	N	VFP	0.103 mg/cm^2 repelled >90% of the nymphs.	USA[a]	Hsouna & Hamdi, 2012[b] Tabanca et al., 2013[a]
Pelargonium roseum R. Br.	Geraniaceae	EO	EtOH	β-citronellol, citronellyl formate, geraniol, iso-menthone, linalool	*R. (B.) annulatus*	EF	AIT	50 mg/ml. caused 98.3% acaricidal MR at 6 days PT.	Iran[a]	Jalali-Hevari et al., 2006[b] Pirali-Kheirabadi et al., 2009[a]
Piper tuberculatum Jacq.	Piperaceae	F	HX EA EtOH MeOH	Piplartine, dihydro-piplartine, 3,4,5-trimethoxydihydrocinnamic acid	*R. (B.) microplus*	EF LV	AIT LPT	0.12 mg/ml HX extract showed 100% larvicidal MR at 24 hr PT, 100% OR and 100% acaricidal efficiency.	Brazil[a]	Rodrigues et al., 2009[b] Da Silva Lima et al., 2014[a]
Piscidia piscipula (L.) Sarg.	Fabaceae	L	AC/DW	Alkaloids, glycosides, isoflavones, retonoids	*R. (B.) microplus*	LV A	LIT AIT	19.2 mg/ml caused 88.14% larvicidal MR, no acaricidal effect on adult stages, 15.7% ELI and 39.2% EHI.	Mexico[a]	Fernadez-Salas et al., 2011[a]
Ptaeroxylon obliquum (Thunb.) Radik	Ptaeroxylaceae	B	DW	Saptaeroxylon, pyrogall, resins, alkaloids	*R. sanguineus*	A N	AIT FP	400 mg/ml repelled ticks (100%) for 40 mins PT.	South Africa [a]	Mulholland et al., 2000[b] Moyo & Masika, 2013[a]
Rhododendron tomentosum (Stokes) H. Harmaja	Ericaceae	L	EO	Myrcene, limonene, paklustrol	*I. ricinus*	N	FV	100 mg/ml diluted in AC caused a repellency of 95.1% 5 min PT.	Sweden[a]	Belousova et al., 1991 [b] Jaenson et al., 2003[b] Jaenson et al, 2005[a]
Ricinus communis L.	Euphorbiaceae	L	EtOH	Quercetin, gallic acid, flavone, kaempferol	*R. (B.) microplus*	EF	AIT	100 mg/ml caused 95% acaricidal MR within 14 days PT.	India[a]	Ghosh et al., 2013[a]
Satureja thymbra L.	Lamiaceae	AP	EO	Carvacrol, Γ- terpinene	*Hyalomma marginatum*	Unfed adult	VP	40 µl/L resulted in 100% acaricidal MR 3 hr PT. Conc. between 5 to 20µl/L resulted in 100% acaricidal MR 24 hr PT.	Turkey[a]	Cetin et al., 2010[a,b]
Simarouba versicolor St. Hil.	Simaroubaceae	SB	DCM	Quassinoids, triterpenoids, steroids,the flavonoid kaempferol	*R. (B.) microplus*	EF LV	LPT AIT	100 mg/ml caused larvicidal MR of 30.1% at 24 hr PT.	Brazil[a,b]	Arriaga et al., 2002[b] Valente et al., 2014[a]
Solanum trilobatum L.	Solanaceae	L	DW	Carbohydrates, saponins, phytosterols, tannins	*Hyalomma anatolicum anatolicum* Koch	LV	LIT	10 mg/L caused 100% larvicidal MR.	India[a,b]	Sahu et al., 2013[b] Rajakumar et al., 2014[a]
Stemona collinsae Craib	Stemonaceae	R	MeOH	Stemofoline alkaloids	*R. (B.) microplus*	EF	AIT	250 mg/ml caused 38% acaricidal MR with 24 hr PT.	Thailand[a]	Sastraruji et al., 2005[b] Kongkiatpaiboon et al., 2014[a]
Tagetes erecta L.	Asteraceae	L	HX CH EA AC MeOH	Thiophenes, flavonoids, carotenoids, triterpenoids	*R. (B.) microplus* *H. bispinosa*	LV A	LPT AIT	3 mg/ml and 2 mg/ml MeOH extract caused 70% acaricidal MR for *H. bispinosa* nd 77% larvicidal MR for *R. (B.) microplus* 24 hr PT.	India[a, b]	Elango & Rahuman, 2011[a] Vijay et al., 2013[b]

Tagetes minuta L.	Asteraceae	AP	EO	Tagetone, dihydrotagetone, ocimenones, piperitone	*Hyalomma rufipes*	A EN	TCR GI	Sig. dose repellent response. Delayed moulting in 60% of nymphs after 25 days.	South Africa [a]	Jacobson, 1983[b] Nchu et al., 2012[a,b]
Tagetes patula L.	Asteraceae	AP	EtOH	Kaempferol, patuletin, quercetin-3-O-pentoside	*R. sanguineus*	EF LV	AIT LIT	50 mg/ml showed 21.50% ELI, 10% acaricidal MR and 99.78% larvicidal MR in 5 min PT.	Brazil [a,b]	Politi et al., 2012[a,b]
Tamarindus indica L	Leguminoceae	F	EtOH DW	Crude protein, carbohydrate, fatty acids	*R. (B.) microplus*	EF	AIT	500 mg/ml caused 99% acaricidal MR 7 days PT.	Thailand[a]	Chungsamarnyart & Jansawan, 2001[a] Khanzada et al., 2008[b] De Caluwe et al., 2010[b]
Tetradenia riparia (Hochst) Codd	Lamiaceae	L	EO	Diterpenes, α-pyrones, phytosterols	*R. (B.) microplus*	EF LV	AIT LPT	250 mg/ml caused 100% larvicidal MR at 24 hr PT.	Brazil[a]	Codd, 1985[b] Gazim et al., 2011[a,b]
Thymus vulgaris L.	Lamiaceae	L	EtOH	Thymol, camphor	*R. sanguineus* *D. nitens*	LV	LPT	20 mg/ml conc. caused 98.1% larvicidal MR for *R. sanguineus* and 99.5% larvicidal MR for *D. nitens* 24 hr PT.	Brazil[a]	Rota et al., 2008[b] Daemon et al. 2009[a] Monteiro et al., 2009
Vitex negundo L.	Lamiaceae	L R	DW EtOH	Flavonoids, flavones, glycosides, triterpenes, tannins	*R. (B.) microplus*	EF	AIT	50 mg/ml EtOH extract caused 53.77% IO and DW extract caused 50% EHI 15 days PT.	India[a,b]	Ladda & Magdum, 2012[b] Singh et al., 2014[a]
Withania somnifera Dunal	Solanaceae	L	DW EtOH	Steroids, alkaloids, salts, flavonoids	*R. (B.) microplus*	EF	AIT	50 mg/ml EtOH extract caused 40.22% IO and 50% EHI 15 days PT.	India[a,b]	Singh et al., 2014[a] Monika, 2014[b]

Plant parts: L - Leaves; S - Stem; SB - Stem Bark; B - Bark; R - Root; AP - Aerial parts; D - Drupes; EO - Essential Oil; CV - Cloves; Fl -Flowers; Sk - Skin; Se - Seed
Extract and extractant used: PE - Petroleum ether; MeOH - Methanol; EtOH - Ethanol; CH -Chloroform; AC - Acetone; HX -Hexane; DW - Distilled Water; EA - Ethyl Acetate
Test type: AIT- Adult Immersion Test; LPT- Larvae Packet Test; APT- Adult Packet Test; LIT - Larvae Immersion Test; EHT- Egg Hatchability Test; TCR - Tick Climbing Repellency; FR - Fingertip Repellency; VP - Vapour Phase; VFP - Vertical Filter Paper; FP - Filter Paper; FV - Falcon Vial
Tick Species: R - Rhipicephalus; B - Boophilus; A - Amblyomma; H - Haemaphysalis; I - Ixodes; D - Dermacentor
Others: MR - Mortality Rate; Conc. - Concentration; A - Adult; LV - Larvae; N - Nymph; E - Egg, EF - Engorged adult female; PT - Post Treatment; ELI - Egg Laying Inhibition; EHI - Egg Hatching Inhibition; IO - Inhibition of oviposition; OR - Oviposition Reduction; Ppm - Parts per million; [a]The main contribution; [b]The reference for the phytochemical constituents

3.3.4 Limitations

Though much work had been done on evaluating plants with tick repellent and acaricidal properties, certain limitations have been identified. These ranges from

1) Lack of standardized testing methods or extractants making comparisons among studies very difficult to relate to day to day use of repellents/acaricides for the control of ticks on animals.
2) Reduced efficacy of plant extracts when tested in field trials is undoubtedly a hindrance to development of alternative acaricides. Most assays rely on the use of laboratory-reared non-resistant tick species. Also, many natural products do not persist in the environment, due to degradation caused by photo-oxidation, temperature, pH and microbial action (Mulla & Su, 1999).
3) Differences in climatic conditions, the cultivation and collection of plant materials for extract production may cause differences in results (Heimerdinger et al., 2006). The acaricidal activity of *Melia azedarach* fruits stored for five months at room temperature decreased (de Sousa et al., 2008). There was a 5% reduction in azadirachtin content after one month and 35% reduction after four months of storage of *Azadirachta indica* seeds (Yakkundi et al., 1995). Though the synthesis of chemical compounds is determined by the genetic characteristics of a plant, edaphoclimatic factors may also play a role (Lapa et al., 2002). Thus, the chemical composition of plant extracts may vary depending on the climate and soil type where plants were grown. Such indications were observed by Hue et al. (2014) where the essential oil of *Ocimum gratissimum* from New Caledonia contained high amounts of eugenol and (Z)-β-ocimene as the main components whereas *Ocimum gratissimum* from Cameroun was mainly constituted by thymol and γ-terpinene. This may be more valid for compounds such as essential oils released based on external stimuli than for stable metabolites. Water stress conditions did not materially influence the antimicrobial activity under natural and laboratory conditions (Netshiluvi & Eloff, 2015a; 2015b).
4) Lack of pharmacokinetic studies on the time course of drug absorption, distribution, metabolism and excretion.

3.4. Conclusions

Research on plant extracts for use in tick control has grown in recent years in an attempt to find compounds or extracts with tick repellent and acaricidal properties that can be used in association with or as replacements for synthetic compounds. The success attained with

pyrethrum, the molecule isolated from *Chrysanthenum* species and its derivatives, shows that there is also another approach that may yield good results. In-depth investigation of the large number of plants with good acaricidal activity may be a worthwhile exercise.

CHAPTER 4

Pesticidal plants as possible alternatives to synthetic, chemical acaricides in tick control: A systematic review and meta-analysis

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Preface

An overview of the efficacy of plant extracts against ticks was given in the preceding chapter. In this chapter, further analysis of results found in work done on plant extracts used to combat ticks was done and compared with available, synthetic, chemical acaricides. The text in this chapter has been submitted to the journal Industrial Crops and Products.

Abstract

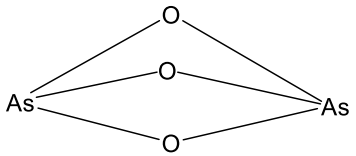
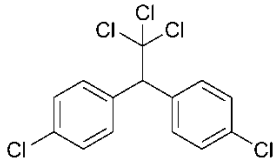
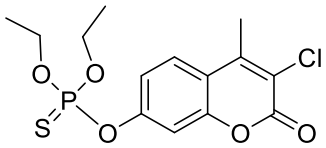
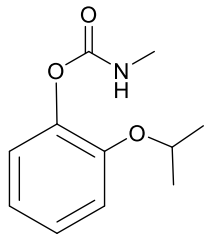
Ticks are a large group of parasitic arthropods which transmit pathogens to animals and humans, causing great economic losses. Chemical-based antitick measures include the use of pyrethroids, carbamates, organophosphates, formamidines and macrocyclic lactones, which all have associated costs, resistance-development and environmental impacts. Some plant-based alternatives may have good efficacy, low toxicity and reduced environmental impacts. A review of published scientific articles was conducted for medicinal plants with *in vitro* acaricidal or tick repellent activities against immature and adult stages of ticks. Veterinary databases (All Databases, CAB Abstracts and Global Health, Medline, PubMed, Web of Science, BIOSIS Citation Index, Science Direct, Current Content Connect and Google Scholar) were used. The search words included “acaricidal”, “tick repellent”, “medicinal plants”, “phytochemical constituents” and “antitick assays”. Assays used in the determination of repellent, acaricidal, larvicidal, inhibition of oviposition and hatchability include tick climbing repellency, petri dish, larval packet and immersion tests amongst others. Significant differences exist in the bioassays and their outcomes. Meta-analysis was conducted using the Fixed-effect model in an Excel programme. Using a total of 1428, 1924, 574, 281 and 68 events, the median efficiency value for acaricidal, larvicidal, egg hatching inhibition, inhibition of oviposition, repellency, acaricidal effects of the Lamiaceae and Asteraceae family were 80.12 ($CI_{95\%}$: 79.20 – 81.04), 86.05 ($CI_{95\%}$: 85.13 – 86.97), 83.39 ($CI_{95\%}$: 82.47 – 84.31), 53.01 ($CI_{95\%}$: 52.08 – 53.93), 92.00 ($CI_{95\%}$: 91.08 – 92.93), 80.79 ($CI_{95\%}$: 79.87 – 81.71) and 48.34% ($CI_{95\%}$: 47.42 – 49.26) respectively. Several plant species used in ethnoveterinary medicine hold vast potential as parasiticides.

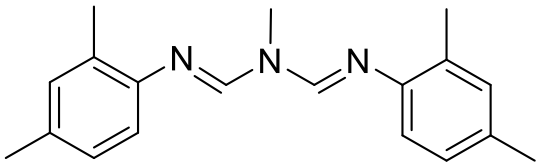
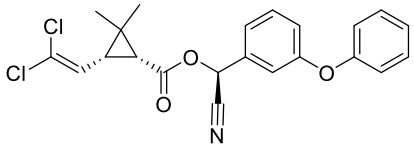
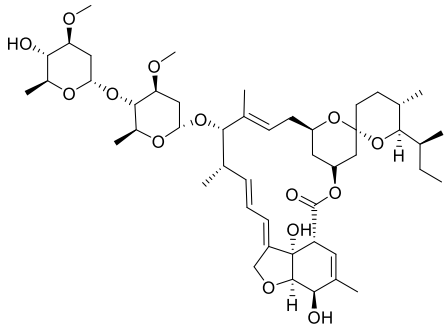
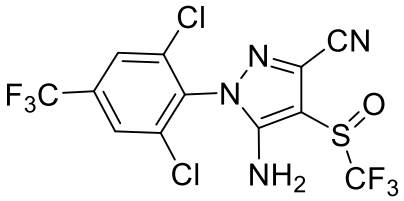
4.1 Introduction

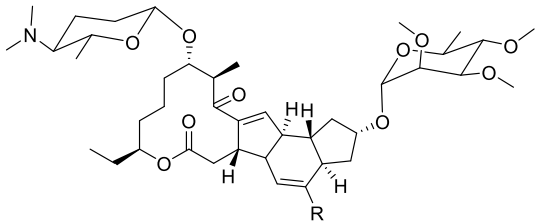
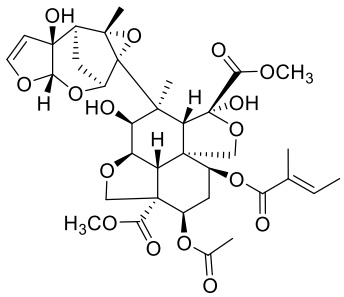
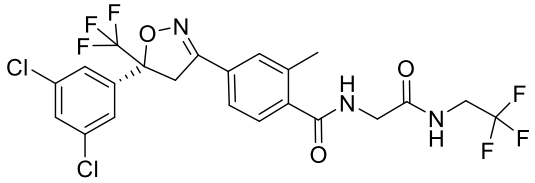
Ticks are a diverse group of haematophagous arthropods, with at least 898 recognized species, distributed among three families: Argasidae (194 species), Ixodidae (703 species) and Nuttalliellidae (1 species) (Norval et al., 2004). They parasitize a wide range of hosts, and are second only to mosquitoes in their capacity to transmit disease agents of importance (protozoa, bacteria, rickettsia and viruses) to livestock, domestic animals and humans (Sonenshine et al., 2002). Ticks are the most economically important ectoparasites of domestic animals and man and the most widespread species include *Amblyomma testudinarium*, *Dermacentor auratus*, *Haemaphysalis bispinosa*, *Rhipicephalus* (*Boophilus*) *microplus*, *Ixodes acutitarsus*, *Ixodes ovatus*, *Nosomma monstrosum*, *Rhipicephalus haemaphysaloides*, *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* (Mans & Neitz, 2004a). Economic loss caused by ticks and tick-borne diseases in cattle is estimated to be more than US$ 7 billion worldwide (Zahir et al., 2010).

Tick control programmes are largely based on the use of commercially available chemicals such as the organochlorines, organophosphates, pyrethroids and more recently, the insect growth regulators and isoxazolines on or in the animals or in the environment (McTier et al., 2016) (Table 4.1). Limiting exposure to tick-infested areas and use of repellents are also considered effective in preventing ticks and tick-borne diseases in companion animals and humans (Cisak et al., 2012). At present, the most commonly used repellents include N, N-diethyl-meta-toluamide (DEET) and 1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1--methylpropylester (picaridin) (Table 4.2).

Table 4.1: Classes of acaricides and their mechanisms of action

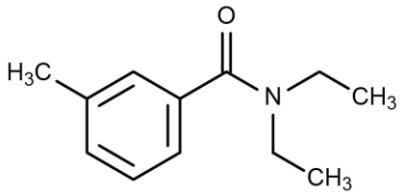
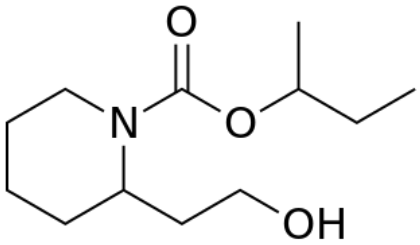
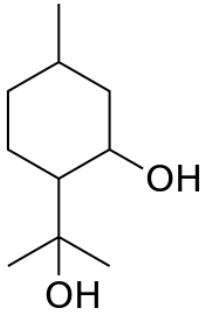
Class of acaricide and examples	Site and mechanism of action	Chemical Structure	LC_{50} (mg/ml)	References
Arsenicals: Arsenic trioxide, potassium arsenite, dihydro-1, 3, 2,-dithiarsenol-2ylmercapto-acetic acid	At the level of the citric acid cycle, they inhibit pyruvate dehydrogenase and by competing with phosphate, uncouple oxidative phosphorylation, thus inhibiting energy-linked reduction of nicotinamide adenine dinucleotide, mitochondrial respiration and adenosine triphosphate synthesis leading to death.	 Arsenic trioxide	-	Klaassen & Watkins III, 2003[a]
Organochlorines: benzenehexachloride, dichlorodiphenyltrichloroethane (DDT), lindane, aldrin, dieldrin, toxaphene, endosulphan, methoxychlor, hexachlorocyclohexane	Binding at the picrotoxinin site in the gamma aminobutyric acid (GABA) chloride (Cl^-) ionophore complex which inhibits Cl^- flux into the nerve causing hyperexcitation and death.	 DDT	36.8	Lawrence & Casida, 1984[a] Camerino, 2015[b]
Organophosphates: coumaphos, chlorfenvinphos, diazinon, parathion, malathion, diaxanthion, oxinothiophos	Act at the synapse of nerve junctions and inhibit the activity of acetylcholinesterase irreversibly. Acetylcholinesterase breaks down the neurotransmitter acetylcholine, which carries impulses across the synapse from one nerve cell to another. Cholinesterase inhibition results in continuous nerve discharges leading to paralysis and death.	 Coumaphos	0.39	Barthold & Schier, 2005[a] Singh et al., 2014[b]
Carbamates: carbaryl, aldicarb, carbofuran, ethienocarb, fenobuacrb, oxamyl, propoxur	Reversibly inhibits the activity of acetylcholinesterase. Cholinesterase inhibition results in continuous nerve discharges leading to paralysis and death.	 Propoxur	0.039	Barthold & Schier, 2005[a] Camerino, 2015[b]

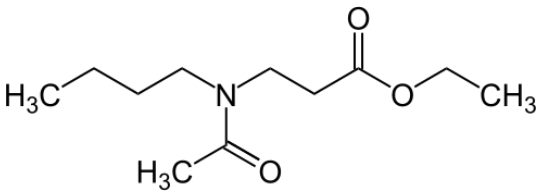
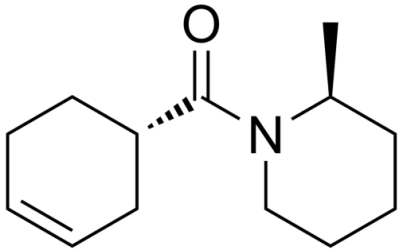
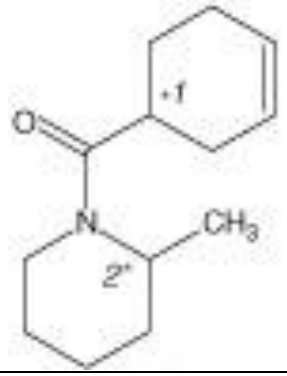
Formamidines: amitraz, chlordimeform, clenpyrin, chloromethiuron	Competes with octopamine for its receptor site, guanosine diphosphate is replaced with guanosine triphosphate, inducing the production of cyclic adenosine monophosphate leading to inhibition of attachment and ultimately blood feeding with eventual death.	 Amitraz	0.001	Beugnet & Franc, 2012[a] Malan, 2015[b]
Pyrethroids: cypermethrin, permethrin, deltamethrin	They block sodium ion movement along the axon of the nerve fibre. This stimulates repetitive nerve discharges that lead to paralysis and death.	 Cypermethrin	0.005	Shafer et al., 2005[a] Singh et al., 2014[b]
Macrocyclic lactones: avermectins (ivermectin, eprinomectin, selamectin, doramectin, abamectin) milbemycins	Bind to GABA and glutamate-gated chloride channels (GluCl) thereby opening chloride channels in nerves, resulting in disruption of activity and loss of function in these cells leading to paralysis and death.	 Ivermectin	0.61	Raymond & Sattelle, 2002[a] Rodriguez-Vivas et al., 2017[b]
Phenylpyrazoles: fipronil, pyriprole	Binds to the allosteric sites of $GABA_A$ and GluCl channels of the parasites as an antagonist (non-competitive inhibition). This prevents the opening of Cl^- channels normally encouraged by GABA, reducing the Cl^- ability to lower the neuron's membrane potential. This results in an overabundance of neurons reaching action potential, CNS toxicity via over-stimulation and eventual death.	 Fipronil	0.00053	Cole et al., 1993[a] Ravindran et al., 2014[b]

Spinosyns: Spinosad (mixture of spinosyn A and D), spinetoram	Primarily targets binding sites on nicotinic acetylcholine receptors of the parasite's nervous system leading to disruption of neurotransmission, paralysis and death.	 Spinosyn A, R=H Spinosyn D, R=CH_3	0.11	Bacci et al., 2016[a] Kovendan et al., 2012[b]
Insect growth regulators: azadirachtin, methoprene, diflubenzuron	Bears a structural resemblance to the moulting hormone, 20-hydroxyecdsyone, and thus interrupts moulting, metamorphosis and development of the female reproductive system. Ticks which survive are unable to produce a progeny.	 Azadirachtin	5	Beckage et al., 2000[a] Al-Raghy et al., 2003[b]
Isoxazolines: afoxolaner, fluralaner, sarolaner, lotilaner, CPD I	Non-competitive GABA receptor antagonists, bind to Cl^- channels in nerve and muscle cells blocking the transmission of neuronal channels, paralysis and death.	 Fluralaner	0.28	McTier et al., 2016[a] Williams et al., 2015[b]

[a]- *Reference for synthetic acaricide and mechanism of action;* [b]- *Reference for LC_{50}; LC_{50} - Lethal concentration killing 50% of the population*

Table 4.2: Insect/Tick repellents and their mechanisms of action

Tick repellent	Site and mechanism of action	Chemical Structure	EC_{50}	References
N, N-diethyl-meta-toluamide (DEET)	Blocks the olfactory receptors for 1-octen-3-ol, a volatile constituent of sweat and breath. A protein, ionotropic receptor 40a, has also been identified as a putative DEET receptor.		0.080 µmol/cm^2	Kain et al., 2013[a,b]
1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester (Picaridin)	The receptors, CquiOR136•CquiOrco and odorant binding protein 1 have been identified.		-	Drakou et al., 2017[a]
Para-menthane-3,8-diol (PMD)	ND		-	Van Lanenhove et al, 2014[a]

Ethyl butylactyloaminopropionate (IR3535)	Has a dual effect on olfactory receptors, both inhibiting (antagonism) and activating (agonism) them, thus, the arthropod can no longer detect attractant cues from a host.		-	Bohbot & Dickens, 2012[a]
1S,2S-2-methylpiperidinyl-3-cyclohexene-1-carboxamide (SS220)	Modality for repellent activity is olfactory.		-	Carroll et al., 2005[a]
Racemic 2-methylpiperidinyl-3-cyclohexene-1-carboxamide (AI3-37220)	Ionotropic receptor 40a could be a putative receptor.		-	Kain et al., 2013[a]

[a]- Reference for tick repellent and mechanism of action; [b]- Reference for EC_{50}; EC_{50} - Effective concentration repelling 50% of the population; ND - Not determined

Commercial acaricidal and/or repellent agents are available for use on companion animals, livestock and humans, in different formulations, including tablets, sprays, soaps, shampoos, powders, impregnated collars, dip solutions, pour-on and spot-on applications (Gassel et al., 2014). The sale and procurement of acaricides/repellents account for a major portion of the annual veterinary market in South Africa (R872 million in 2003 with ectoparasiticides accounting for 22% of the sum) (Peter et al., 2005). In other countries such as Kenya, Zambia, Zimbabwe, Nigeria, Tanzania and Uganda, the annual cost of importing ectoparasiticides had been estimated at US$16 million, $10 million, $9.3 million, $30 million, $26 million and $26 million respectively (Kaaya & Hassan, 2000). The global parasiticide market was valued at US $6509.1 million in 2013. This is expected to reach US$8918.1 million by 2019 growing at a rate of 5.4% (www.marketsandmarkets.com). In 2013, Africa accounted for 2.7% of the global parasiticide market which was valued at US$173.8 millon. Of this, ectoparasiticides accounted for 60.1% (US$96.17 million) and this is expected to reach US$137.86 million growing at a rate of 6.3% by 2019 (www.marketsandmarkets.com).

Appropriate use of these chemicals is beneficial in controlling ticks, but improper application and misuse may lead to poisoning of humans and animals, emergence of resistant strains, issues of drug residues in animal food products (meat and milk) as well as environmental hazards (Babar et al., 2012). To overcome these obstacles, the development of an effective and environmentally friendly alternative of low toxicity to replace the synthetic agents is required. Research and Development orientated towards alternative methods of tick control that are consistent with the principles of sustainable agriculture, includes the use of tick antigens as vaccines (Shahein et al., 2013), entomopathogenic fungi (Nana et al., 2015; Nana et al., 2016) and plant-based alternatives (Benelli et al., 2017).

Plants have long provided mankind with a source of medicinal agents, with natural products once serving as the major provider of all therapeutic drugs (Balandrin et al., 1993). Many plant secondary metabolites are synthesized to provide protection against pathogens, predators and pests. These agents act in one or more of the following ways: counteraction of growth regulatory hormones, anti-feeding effects, inhibition of egg development, disruption of mating and sexual communication, inhibition of chitin formation and repellent action (Benelli et al., 2016). It should be kept in mind that plant-produced chemicals that deter invertebrates and vertebrates primarily target herbivores and not blood feeders, such as ticks. Probably because of their shared arthropod lineage with herbivorous insects, ticks are also susceptible to some plant-produced deterrents. For example, the pyrethrins, which are a class of organic compounds derived from the dried flower heads of *Chrysanthemum cinerariifolium* (Trev.) Vis, have been used for centuries for their acaricidal and tick repellent

properties (Dhang & Sanjayan, 2014). They also provide a structural backbone for more potent synthetic pyrethroids which are components of many house-hold and industrial insecticides (Dhang & Sanjayan, 2014).

In some countries currently, plant-based ectoparasitic formulations are commercially available (Freitag & Kells, 2013). MyggA® Natural (Bioglan, Lund, Sweden), contains 30% of *Corymbia citriodora* (Hook.) oil with a minimum of 50% PMD, Citriodiol®, manufactured by Citrefine International Limited, UK contains 64% PMD, Economist®,a natural alternative to permethrin, which contains pyrethrins and D-limonene, obtained from *Citrus* species is available in South Africa; BioUD®, with the active ingredient 7.75% 2-undecanone, originally derived from *Lycopersicon hirsutum* subsp. *glabratum* C.H. Mull (wild tomato plants), registered by the U.S. Environmental Protection Agency in 2007 and TT302 (Guardian®Wilderness; Tyratech, Inc. Morrisville, NC, U.S.A) containing 5% geraniol (Bissinger et al., 2009; Bissinger et al., 2016).

In an attempt to find safe and efficient compound(s) with tick repellent and/or acaricidal properties, research on plant extracts used traditionally in tick control has grown in recent years as seen in many reviews (Atanasov et al., 2015; Benelli et al., 2016; Pavela et al., 2016; Katz et al., 2016; Benelli et al., 2017). Renewed interest in natural compounds derived from plants and microorganisms to develop non-synthetic medications for the veterinary industry using newer methodologies including combinatorial chemistry and computational biology along with high throughput screening, could yield new acaricides/repellents (Sparks et al., 2016). Acaricides or repellents with mechanisms of action targeting previously unexplored metabolic pathways can be developed that may overcome multi-acaricide resistant populations.

In this review, we provide information from selected studies that include plants used in traditional veterinary medicine globally for tick infestation as repellents or acaricides, including those with antifeedant and growth-inhibition properties. Plant species cited are reviewed for efficacy, bioactive constituents and possible mechanism of action in an attempt to validate their traditional use in animal health. We have summarized the most important results of the tests of plant extract efficacy against different tick species and life stages (eggs, larvae, nymphs or adults) and highlighted plants and compounds therein showing very good efficacy. Bioactive products based on plant extracts or isolated compounds may constitute prototypes for the development of promising alternatives to chemical acaricides.

4.2 Materials and Methods

The keywords used to collect relevant literature for the review were: “tick repellent”, “acaricidal”, “medicinal plants”, “isolated compounds” and “antitick assays”. Veterinary databases (All Databases, CAB Abstracts and Global Health, Medline, PubMed, Web of Science, BIOSIS Citation Index, Science Direct, Current Content Connect and Google Scholar) were searched. Specifically, the plant species tested, the effective concentrations and concentration killing 50% of the population (LC_{50}), extractants, species and life stage of ticks targeted, type of bioassay used and compounds isolated were considered.

4.2.1 Selection criteria applied to published results

One major shortfall in research for new plant-based tick repellents and acaricides is the lack of a standardized testing method. A wide range of methods is employed when testing for tick repellency and acaricidal effects. Studies differ in the time frame in which repellence or toxicity is evaluated, the species and life stages of ticks used, the formulation and amount of active ingredients from crude plant extracts, fractions or essential oils, the use of animal host cues or not, use of different solvents (extractants), measurable standards and variability of tick behaviour (Bissinger & Roe, 2010). These variations in testing methodologies and assay conditions make comparison among studies very problematic and difficult to relate to the daily use of repellents/acaricides for the control of ticks.

Considering the above, some criteria had to be used to select studies suitable for the purpose of this review. The selected papers focusing on *in vitro* tick repellent and/or acaricidal efficacy of plant extracts as listed in the Web of Science database, complied with at least one of the four criteria listed below:

1) Efficacy for the evaluation of larvae, nymph and adult mortalities and/or tick repellency higher than 60%.
2) Efficacy for the evaluation of growth inhibition estimated.
3) The LC_{50} (acaricidal) and/or EC_{50} (repellency) estimated.
4) Compounds isolated from the plant extracts.

4.2.2 Meta-analysis applied to published results

Data for plant extracts with tick repellent and/or acaricidal properties was extracted and compiled from peer reviewed journals obtained from the nine databases mentioned above. The data was quality-checked through data filtration to remove duplicates and harmonized into a single Microsoft Excel® spreadsheet. All concentrations (including LC_{50}/EC_{50}) were harmonised and expressed as mg/ml. Filtered data was coded for use in a Microsoft Excel® programme, including: the plant name, part(s) of the plants used and family; acaricidal

activity, larvicidal activity, inhibition of oviposition, egg hatching inhibition and repellency effects; assay type employed and further *in vivo* studies (if any). The number of events, sample sizes and outcomes were calculated based on the data. All data was analysed using fixed-effect model (precision-based estimates) in the Meta-analyses software on Excel and comparison between individual studies was calculated in WinPepi v11.24 (Neyeloff et al., 2012). Outputs were generated as percentage of acaricidal, larvicidal, inhibition of oviposition, egg hatching inhibition and repellency effects of the plants or of specific families with 95% confidence intervals. Cumulative events with measures of central tendencies were also produced in forest plots.

4.3 Results

4.3.1 Plant species with repellent potential

In total, 27 plant species from 18 families were represented (Table 4.3). The family with the highest frequency was: Asteraceae (15%), followed by Lamiaceae (11%). The Cleomaceae, Poaceae, Rutaceae and Verbenaceae had 7% representation each while the other families were represented by 1 plant species (4%) each (Table 4.3). Most of the studies used essential oils from the aerial parts of the plants (63%). This is not surprising because volatile compounds are required to repel ticks. This was followed by ethanol extracts (22%), methanol and hexane extracts (7%). The leaf was the most used part, followed by the aerial parts, fruits, flowers and drupes in one study. About 56% of the studies used nymphs to test for repellency, 37% used adults while 7% used larvae. Tick climbing repellency, fingertip repellency, vertical filter paper and petri dish repellency assays were commonly employed. Duration of repellency ranged from 1 hour to 35 hours (*Lavandula angustifolia* Mill.) and active phytochemicals include eugenol, β-caryophyllene, linalool, carvacrol, 1, 8-cineole, myrcene and geraniol (Tables 4.3 and 4.6). Only one study progressed to *in vivo* validation of *in vitro* studies.

Table 4.3: Plant species evaluated for repellent activity and their possible bioactives

Plant family and species	Common name	Plant part/ Extractant	Assay	Conc.	Effect (%)	EC_{50} (mg/cm^2)	Some active isolated compounds	References
Asparagaceae								
Convallaria majalis L.	Lily of the valley	L (EO)	PDR using nymphal *I. ricinus*	10%	67	ND	Convallamaroside	Nartowska et al., 2004[b] Thorsell et al., 2006[a]
Asteraceae								
Ageratum conyzoides L.	Billy-goat weed	L (EtOH)	FR using nymphal *A. cajennense*	1.1mg/cm^2	85	0.205	Stigmasterol, β-sitosterol, precocene II, ageratochromene	Soares et al., 2010[a] Kumar et al., 2016[b]
Artemisia abrotanum L.	Southern wormwood	L (EO)	PDR using adult *I. ricinus*	10%	69.1	ND	Coumarin, thujyl alcohol, cinnamyl aldehyde, α-copaene, eugenol, eucalyptol	Tunón et al., 2006 [a, b]
Artemisia absinthium L.	Absinthe wormwood	L (EO)	FVR using nymphal *I. ricinus*	10%	78.1	ND	Sabinene, thujenol, linalool, geranyl acetate	Jaenson et al., 2005 [a, b]
Tagetes minuta L.	Southern marigold	AP (EO)	TCR using adult *H. marginatum rufipes*	-	-	0.07ml/ml	Sabinene, thujenol, linalool, geranyl acetate Cis-ocimene, β-ocimene, 3-methyl-2-(-2-methyl-2-butenyl)-furan, 2-butanone, dihydrotagetone, cis-tagetone	Nchu et al., 2012[a, b] Makanga, 2012[b]
Bignoniaceae								
Kigelia africana (Lam.) Benth	Sausage tree	Fr (MeOH)	FR using larvae *R. appendiculatus*	0.25mg/ml	76	ND	Kiglin, 6-methoxymellein, stigmasterol, lapachol	Gabriel & Olubunmi, 2009[b] Opiro et al., 2013[a]
Burseraceae								
Commiphora holtziana	Myrrh	Re (HX)	PDR using larvae *R. (B.) microplus*	1%	80	ND	Germacrene-D, δ-elemene, β-bourbonene β-selinene, β-elemene, , γ-elemene, α-cubebene	Birkett et al., 2008[a, b]
Caryophyllaceae								
Dianthus caryophyllus L.	Carnation	Fl (EO)	PDR using nymphal *I. ricinus*	10%	100	ND	2-Phenyl-ethanol, eugenol, geraniol, coumarin, α-pinene, β-citronellol	Tunón et al., 2006[a, b]
Chenopodiaceae								
Dysphania ambrosioides (formerly *Chenopodium ambrosioides L.*)	Wormseed	L (EtOH)	FR using nymphal *A. cajennense*	2.2mg/cm^2	100	0.512	Ascaridole, 2-carene, ρ-cymene, isoascaridole, α-terpinene	Soares et al., 2010[a] Chu et al., 2011[b]
Cleomaceae (previously Capparaceae)								

Cleome gynandra L. (*Gynandropsis gynandra* (L.) Briq.	Cat's whiskers	AP (EO)	TCR using adult *R. appendiculatus*	0.1ul	98.9	ND	Carvacrol, transphytol, linalool, trans-2-methylcyclopentanol, β-caryophyllene	Lwande et al., 1999[a, b]
Cleome monophylla L.	Single-leaved cleome	AP (EO)	TCR using adult *R. appendiculatus*	0.1ul	89.9	ND	Terpenolene, 1-α-terpeneol, 2-dodecanone, α-humulene, β-humulene, n-Pentacosane	Ndungu et al., 1995[a, b]
Cupressaceae								
Chamaecyparis nootkatensis (D. Don) Spach (formerly *Cupressus nootkatensis*)	Alaska yellow cedar	AP (EO)	FVR using nymphal *I. scapularis*	-	-	0.048%	Nootkatone, valencene-13-ol, nootkatone 1, 10 epoxide, carvacrol	Dietrich et al., 2006[a, b]
Ericaceae								
Rhododendron tomentosum (Stokes) H. Harmaja (formerly Ledum palustre L.)	Marsh Labrador tea	L (EO)	FVR using nymphal *I. ricinus*	10%	95.1	ND	Myrcene, palustrol, 2,6-dimethyl-1,5,7-octatriene-3-ol, 2-methyl-6-methylene-1,7-octadiene-3-one (myrcenone), alloaromadendrene, ledol, p-cymene, β-caryophyllene	Jaenson et al., 2005[a, b]
Fabaceae								
Senna (Cassia) didymobotrya (Fresen.) Irwin & Barneby	African senna	AP (MeOH)	FR using larval *R. appendiculatus*	0.25mg/ml	87.6	ND	Anthraquinones, terpenoids, flavonoids, phenolic compounds, tannins	Opiro et al., 2013[a] Alemayehu et al., 2015[b]
Geraniaceae								
Pelargonium graveolens L'Her	Rose geranium	L (EO)	VFP using nymphal *A. americanum*	0.103mg/cm^2	90	ND	Citronellol, geraniol, 10-epi-γ-eudesmol	Tabanca et al., 2013[a,b]
Lamiaceae								
Lavandula angustifolia Mill. (syn. *L. officinalis* Chaix ex Vill.)	English lavender	AP (EO)	TCR using adult *H. marginatum rupifes*	20%	100	ND	Linalool, borneol, camphor, eucalyptol	Mkolo & Magano, 2007[a] Fadia et al., 2015[b]
Mentha pulegium L.	Squaw mint	AP (EtOH)	FR using nymphal *A. cajennense*	1.1	85	0.449	1α, 6βdimethyl-5β-hydroxy-4β-(prop-1-en-2-yl)-decahydronaphthalen-2-one, 1-(O-β-D-glucopyranosyl)-2,7-dimethyloct-5-en-3-one	Soares et al., 2010[a] Ibrahim, 2013[b]
Ocimum suave (Willd)	Wild basil	L (EO)	TCR using adult *R. appendiculatus*	-	-	0.024%	1,8-cineole, linalool, pinene, eugenol,	Mwangi et al., 1995[a, b] Pandey et al., 2014[b,]

							camphor, methyl chavicol, ocimene, terpinene, limonene	
Lauraceae								
Lindera melissifolia (Walter) Blume	Pondberry	D (EO)	TCR using nymphal *A. americanum*	0.827mg/cm^2	74	0.668	β-caryophyllene, α-humulene, germacrene D, β-elemene	Oh et al., 2012[a b]
Meliaceae								
Melia azedarach L.	Chinaberry tree	Fr (HX)	FR using nymphal *A. cajennense*	-	-	2.22	3,7,11,15- tetramethyl-2-hexadecen-1-ol, carotene, rhodoxanthin, meliatoxin, melianone, meliantriol, nimbolidin A, nimbolidin B	Soares et al., 2010[a] Krishnaiah & Prashanth, 2014[b]
Myrtaceae								
Syzygium aromaticum L. Merrill & Perry (*Eugenia caryophyllata* Thunb.)	Cloves	EO	PDR using nymphal *I. ricinus*	10%	68	ND	Carvacrol, thymol, eugenol, cinnamaldehyde	Chaieb et al., 2007[b] Thorsell et al., 2006[a]
Poaceae								
Cymbopogon nardus (L.) Rendle	Citronella grass	AP (EtOH)	FR using nymphal *A. cajennense*	0.275mg/cm^2	100	0.089	Linalool, citronellal	Soares et al., 2010[a] Avoseh et al., 2015[b]
Cymbopogon nardus (L.) Rendle		EO	PDR using nymphal *I. ricinus*	10%	89	ND		Thorsell et al., 2006[a]
Rutaceae								
Ruta graveolens L.	Rue	AP (EtOH)	FR using nymphal *A. cajennense*	-	-	4.141	2-undecanone, 2-nonanone, α-limonene, 5, 6-diethenyl-1-methyl-cyclohexane	Soares et al., 2010[a] Haddouchi et al., 2013[b]
Spiranthera odoratissma St. Hil.	Manaca	L (EtOH)	FR using nymphal *A. cajennense*	-	-	8.426	Dictamine, γ-fagarine, skimmianine, 1-methyl-2-phenylquinolin-4-one, limonexic acid, limonin	Soares et al., 2010[a] Terezan et al., 2010[b]
Verbenaceae								
Callicarpa americana L.	American beautyberry	EO	FR using nymphal *A. cajennense*	1.0mg/cm^2	85	0.084	Callicarpenal, intermedeol	Soares et al., 2010 [a, b]
Lippia javanica (Burm. F.) Spreng	Lemon bush	AP (EO)	TCR using adult *H. marginatum rupifes*	5.3%v/v	69.2	ND	Myrcene, 1,8-cineole, dyhrodrotagetone, 2-butanone	Magano et al., 2011[a, b]

Plant parts: AP - Aerial parts; L - Leaves; D - Drupes; Fl - Flowers; Fr -Fruit; Re - Resin; Plant peparations: EO - Essential Oil; EtOH - Ethanol extract; MeOH - Methanol extract; HX - Hexane extract Ticks: A. -Amblyomma; H. - Hyalomma; I. - Ixodes; R. -Rhipicephalus;Test type: FR - Fingertip Repellency; TCR - Tick Climbing Repellency; PDR - Petri Dish Repellency; FVR - Falcon Vial Repellency; VFP- Vertical Filter Paper Repellency; Others: ND - Not determined; Conc. -Concentration; EC_{50} - Effective concentration 50; [a]- Reference for repellent activity; [b]- Reference for isolated compounds

4.3.2 Plant species with acaricidal, larvicidal and growth inhibitory potential

In total, 55 plant species from 22 families were found with acaricidal activities that were in line with the criteria we set (Table 4.4). The families with the highest frequencies were: Lamiaceae (20%), Asteraceae (13%), Rutaceae and Fabaceae (9%) and Solanaceae (7%). The Meliaceae and Poaceae had 6% representation each, Euphorbiaceae and Piperaceae had 4% representation each while the remaining 13 plant families were represented by 1 plant species each (2%) (Table 4.4). Forty plant species from 19 families with larvicidal activity were found (Table 4.5). The family with the highest frequency was Lamiaceae (25%). This was followed by Asteraceae and Poaceae (10% each), Piperaceae (7.5%), Verbenaceae, Solanaceae, Amaryllidaceae (5% each). The other 12 plant families were represented by 1 plant species each (2.5%) (Table 4.5).

Rhipicephalus (B.) microplus was the most studied tick and the adult immersion test was the most commonly employed method. Engorged female ticks obtained from the field were mostly used and 30% of the studies checked for growth inhibition. *Rhipicephalus (B.) microplus* larvae were also mostly studied and the average age was 14 days old (Table 4.4; 4.5).

Most of the studies used essential oils followed by ethanol extracts of the plants, methanol, acetone, hexane, chloroform, aqueous, dichloromethane and ethyl acetate extracts. The number of extracts does not neccesarily indicate which extracts had the highest activity, but rather which extracts were mainly used. The bioactive compounds in the plants were evaluated in 93% of the studies while only 17% attempted to determine the mechanism of action of the plant. Active phytochemicals include geraniol, eugenol, β-caryophyllene, carvacrol, linalool, 1, 8-cineole, azadirachtin, thymol, nicotine and scopolamine (Tables 4.4, 4.5 and 4.6). Only five of the studies progressed to *in vivo* experiments.

Table 4.4: Plant species evaluated for their acaricidal and growth inhibitory activities and their possible bioactives

Plant family and species	Common name	Plant part/ Extractant	Assay	Conc. (LC_{50})	Effect (%)	IO (%)	EHI (%)	Some active isolated compounds	Mechanism of action	References
Acanthaceae										
Andrographis (Justicia) paniculata (Burm.f) Wall. Ex Nees	King of bitters	L (MeOH)	APT using field adult *Haemaphysalis bispinosa*	3000 ppm (327.21 ppm)	100	ND	ND	Andrographolide, andrograpine, panicoline, paniculide-A, B, C	ND	Elango & Rahuman, 2011[a] Hossain et al., 2014[b]
Amaryllidaceae										
Allium sativum L.	Garlic	C (MeOH)	AIT using field EF *R. (B.) microplus*	100 mg/ml	80	85.8	100	Allicin, alliin	ND	Hughes & Lawson, 1991[b] Shyma et al., 2014[a]
		C (DCM)	CB using lab-reared adult *H. marginatum rufipes*	24%w/v (5.9%)	100	ND	ND		ND	Nchu et al., 2005[a]
Annonaceae										
Annona squamosa L.	Sugar apple	FP (Aq)	AIT using field adult *Haemaphysalis bispinosa*	2000 ppm (404.51 ppm)	100	ND	ND	1*H*-cycloprop[e]azulen-7-ol decahydro-1,1,7-trimethyl-4-methylene-[1ar-(1aα,4aα, 7β, 7 a, β, 7bα)], retinal 9-*cis*-3,17-dioxo-4-androsten-11alpha-yl hydrogen succinate, 1-naphthalenepentanol decahydro-5-(hydroxymethyl)-5,8a-dimethyl-y,2-bis(methylene)-(1α,4aβ,5α,8aα), 1-naphthalenemethanol decahydro −5-(5-hydroxy-3-methyl-3-pentenyl)- 1,4a-dimethyl - 6-methylene -(1S-[1α, 4aα, 5α(E), 8aβ], (−)-spathulenol,	ND	Madhumitha et al., 2012[a,b]

								podocarp-7-en-3-one13β-methyl-13-vinyl, 1-phenanthrene carboxaldehyde 7-ethenyl-1,2,3,4,4a,4,5,6,7,9,10,10a-dodecahydro-1,4a,7-trimethyl-[1R-(1α,4aβ.4bα,7β, 10aα)]		
Araceae										
Acorus calamus L.	Sweet flag	Rh (50%EtOH/DW)	AIT using lab-reared EF *R. (B.) microplus; in vivo*	10%	100	100	ND	α-asarone, β-asarone	ND	Ghosh et al., 2011[a, b]
Apocynaceae										
Calotropis procera (Aiton)W.T.Aiton	Apple of Sodom	LX (EtOH)	AIT using field EF *H. dromedarii*	(1096mg/L)	-	ND	ND	Digitoxin, cardenolide	Inhibition of Na+, K+-ATPase of ticks	Al-Rajhy et al., 2003[a, b]
Asteraceae										
Artemisia absinthium L	Absinthe wormwood	AP (CH)	AIT using field EF *R. sanguineus*	20% (8.79%)	93.3	85.1	100	Artemisinin	Reacts with the heme groups of the haemoglobin molecules digested by parasites, altering the cell structure and its functions, thus affecting growth and reproduction	Godara et al., 2014a[a, b]
Calea serrata L.	Snake herb	AP (HX)	AIT using field EF *R. (B.) microplus*	50 mg/ml	14.6	ND	100	Precocene II	Interferes with tick oviposition, development and reproduction	Ribeiro et al., 2011[a, b]
Eupatorium adenophorum Spreng. (syn. *Ageratina*	Sticky snakeroot	AP (EtOH)	AIT using field nymphal *Haemaphysalis longicornis*	1.5 g/ml	100	ND	ND	Quercetagetin 7-β-O-glucoside, 6-methoxykaempferol 7-methyl ether 3- β-	ND	Li et al., 2008[b] Nong et al., 2013b[a]

adenophora)								O-glucoside, quercetagetin 4^-methy lether 7- β-O-glucoside, 6-hydroxykaempferol-7-β-O-glucoside, 6-methoxygenkwanin; umbelliferone; 3-(2^-β-O-pyranoglaucoside)-phenyl-2-trans-trans-propenoic acid, dotriacontanol.		
Matricaria (*Chamomilla*) *chamomilla* L.	Chamolile	Fl (EtOH)	AIT using field EF *R. (B.) annulatus*	8%	26.7	46.7	ND	Angelic acid (2-meyhyl-2-butenoic acid), azolen, chamazulene (1,4-dimethyl-7-etazulene), α-bisablol, sineol, maricarin, matricin	ND	Pirali-Kheirabadi & Razzaghi-Abyaneh, 2007[a, b]
Tagetes erecta L.	Mexican marigold	L (AC)	APT using field adult *Haemaphysalis bispinosa*	3000 ppm	84	ND	ND	Benzaldehyde, limonene, linalool, myroxide, β-ocimene, phenylacetaldehyde, piperitone	ND	Elango & Rahuman, 2011
Tagetes minuta L.	Southern marigold	AP (EO)	NPT using lab-reared nymphal *H. marginatum rufipes*	0.107 ml/ml	60	ND	ND	Cis-ocimene, β-ocimene, 2-butanone, 3-methyl-2-(2-methyl-2-butenyl)-furan, piperitenone	ND	Nchu et al., 2012[a, b]
Tagetes patula L.	French marigold	AP (EtOH)	AIT using lab-reared EF *R. sanguineus*	50 mg/ml	0	21.5	ND	50 - hydroxymethyl-5-(3-butene-1-ynil)-2,20-bithiophene; methyl-5-[4-(3- methyl-1-oxobutoxy)-1-butynyl]-2,2' bithiophene; cholesterol; β-sitosterol (24-R-stigmast-5- ene-3β-ol) (4); stigmasterol [24-(S)-stigmast-5,22E-dien- 3β-ol], lupeol, kaempferol, quercetina, patuletin-7-O-glucoside (patulitrin), patuletin,	ND	Politi et al., 2012[a, b]

								quercetagetin, quercetagetin-7-O-glucoside, luteolin		
Bromeliaceae										
Ananas comosus (L.) Merr.	Pineapple	Sk (Aq)	AIT using field EF *R. (B.) microplus*	500 mg/ml	59.4	39.1	33.3	Bromelain	May be attributed to enzyme complex of bromelain which promote digestion of the cuticle and death of the parasite	Domingues et al., 2013[a, b]
Caricaceae										
Carica papaya L.	Pawpaw	Sd (MeOH)	AIT using field EF *R. (B.) microplus*	100	93.3	100	100	Papain, chymopapain, peptidase A,peptidase B, lysozyme	ND	Shyma et al., 2014 [a, b]
Combretaceae										
Guiera senegalensis J.F. Gmel	Moshi medicine	L (EtOH)	AIT using field EF. *H. anatolicum*	15%	100FI	100	100 (0.508 %)	Catechin, myricitrin, rutin, quartterin, 3,4,5-tri-O-galloylquinic acid	Antifeedant property	Osman et al., 2014[a, b]
Euphorbiaceae										
Jatropha curcas L.	Barbados nut	L (EtOH)	AIT using field EF *R. (B.) annulatus*	100 mg/ml	0	10.1	90	Apigenin 7-O-β-D-neohesperidoside, apigenin 7-O-β-D-galactoside), orientin, vitexin, vicenin II, di-C-β-Dglucopyranoside-methylene-(8, 8')-biapigenin	Could be attributed to apigenin which can cause decrease in the level of active ecdysteroid by inhibiting the P450 enzyme, leading to decreased incorporation	Juliet et al., 2012[a, b]

								of free ecdysteriods into the eggs or interference with the uptake of modified egg yolk protein, vitellin into the oocytes both being important for egg maturation and development.	
Ricinus communis L.	Castorbean	L (EtOH)	AIT using field EF *R. (B.) microplus; in vivo*	9.68%	85	39	ND	Quercetin, gallic acid, flavone, kaempferol, ricin	Inhibition in the development and maturation of oocytes.	Ghosh et al., 2013[a, b]
Fabaceae										
Calpurnia aurea (Ait.) Benth	Wild laburnum	L (AC/DW)	CB using lab-reared adult *R. pulchellus*	20%	100 DA	-	-	Calpurmenin, 13α-(2'-pyrrolecarboxylic acid) ester, virgiline, lupanine	ND	Zorloni et al, 2010[a,b]
Leucaena leucocephala (Lam.) de Wit	White leadtree	L (AC/DW)	AIT using field EF *R. (B.) microplus*	19200 µg/ml	0	7.3	29.0	Tannins, quercetin, caffeic acid, scopoletin	High levels of tannins present probably responsible for activity	Fernández-Salas et al., 2011[a] Von Son-de Fernex et al.,2015[b]
Lysiloma latisiliquum (L.) Benth.	False tamarind	L (AC/DW)	AIT using field EF *R. (B.) microplus*	19200 µg/ml	0	36.4	69.3	Tannins	High levels of tannins present probably responsible for activity	Fernández-Salas et al., 2011[a, b]
Piscidia piscipula (L.) Sarg. (Syn. *Piscidia erythrina* L.)	Fishpoison tree	L (AC/DW)	AIT using field EF *R. (B.) microplus*	19200 µg/ml	0	15.7	39.2	Tannins, 5,7-dihydroxylated isoflavones, coumaronochromones, 5-deoxyisoflavones	High levels of tannins present probably responsible for activity	Fernández-Salas et al., 2011[a] Tahara et al., 1993[b]

Pongamia glabra Vent. (Syn. *Miliettia pinnata* (L.) Panigrahi **Hypericaceae**	Pongam oiltree	L (EtOH)	AIT using field EF *R. (B.) annulatus*	100 mg/ml	17	13	50	Karinjin, pongamol, kaempferol, β-sitosterol	ND	Ravindran et al., 2017[a,b]
Hypericum polyanthemum Klotzch ex Reichardt **Lamiaceae**	St. John's wort	AP (MeOH)	AIT using field EF *R. (B.) microplus*	25 mg/ml	0	12.8	0	Benzopyrans	Disturb the development process and reproduction	Ribeiro et al., 2007[a, b]
Anisomeles malabarica (L.) R. Br. Ex Sims	Malabar catmint	L (MeOH)	APT using field adult *Haemaphysalis bispinosa*	3000 ppm (719.78 ppm)	100	ND	ND	Anisomelic acid, ovatodiolide, pedalitin, acteoside, terniflorin	ND	Rao et al., 2012[b] Zahir et al., 2010[a]
Hesperozygis ringens (Benth.) Epling	Pulegium	L (EO)	AIT using field EF *R. (B.) microplus*	50 µl/ml	ND	76.4	95	Pulegone, limonene, linalool, β-caryophyllene, bicyclogermacrene	Due to chemosterilant effect of pulegone	Ribeiro et al., 2010[a, b]
Hyptis verticillata Jacq.	John Charles	AP (EO)	CB using field EF *R. (B.) microplus*	4.0 uL/g (1.5 uL/g)	45	87.2	90	Cadina- 4,10(15)-dien-3-one (1), aromadendr-1(10)-en-9-one (squamulosone), viridiflorol, hexadecyl acetate	ND	Facey et al., 2005[a] Picking et al., 2013[b]
Leucas aspera (Willd.)	-	AP (EtOH)	AIT using field EF *R. (B.) annulatus*	100 mg/ml	54.2	69.4	100	Nicotine, acacetin, apigenin	Inhibit the action of prostaglandins	Ravindran et al., 2011b[a,b]
Leucas indica (L.)R.Br.ex Vatke	-	L (EtOH)	AIT using field EF *R. (B.) annulatus*	500 mg/ml	66.7	55.2	0	Leucolactone, sitosterol, campesterol, stigmasterol, nicotine	ND	Divya et al., 2014[a, b]
Ocimum basilicum L.	Great basil	L (CH)	AIT using field adult *R. (B.) microplus*	10% (5.46%)	100	ND	ND	Linalool, (*Z*)-cinnamic acid methyl ester, cyclohexene, α- cadinol , 2,4-diisopropenyl-1-methyl-1-vinylcyclohexane, 3,5-pyridine-dicarboxylic acid, 2,6-dimethyl-diethyl ester, β-cubebene ,	ND	Zhang et al., 2009[b] Veeramani et al., 2014[a]

							guaia-1(10),11-diene, cadinene , (*E*)-cinnamic acid methyl ester, β-guaiene		
Origanum minutiflorum O. Schwarz & P.H. Davis	Wild origanum	AP (EO)	VP using field adult *R.turanicus*	10µl/L	100	ND	ND	Carvacrol, p-cymene, borneol, γ-terpinene, myrcene, camphene, α-pinene, thymol	ND	Cetin et al., 2009[a, b]
Origanum onites L.	Turkish oregano	L (EO)	APT using field adult *R.turanicus*	25% (2.34%)	100	ND	ND	Carvacrol, p-cymene, linalool, γ-terpinene, myrcene, camphene, α-pinene, thymol	ND	Coskun et al., 2008[a, b]
Satureja thymbra L.	Savory	AP (EO)	VP using field adult *H. marginatum rufipes*	5µl/L	100	ND	ND	Carvacrol, γ-terpinene	ND	Cetin et al., 2010[a, b]
Tetradenia riparia (Hochst.) Codd	Ginger bush	L (EO)	AIT using field EF *R. (B.) microplus*	1.8% w/v (0.534 g/ml)	100	100 (0.449 g/ml)	100 (0.114g /ml)	14-hydroxy-9-epi-cariophyllene, cismuurolol-5-en-4-a-ol, ledol, a-cadinol, limonene, fenchone	ND	Gazim et al., 2011[a, b]
Vitex negundo L.	Five-leaved chaste tree	L (EtOH)	AIT using field EF *R. (B.) microplus*	5%	(7.02 %)	53.9	100	Terpenoids, irridoids, steroids, phenolic compounds, lignane derivatives, amino acids, fatty acids, aliphatic alcohol	ND	Singh et al., 2014[a, b]
Meliaceae										
Azadirachta indica A.Juss	Neem	L (MeOH)	AIT using field EF *R. (B.) microplus*	100	33.3	20.7	20	Azadirachtin, meliacarpin	Agent having an anti-feedant effect and causing delay in the production of ecdysone	Akhtar et al., 2008[b] Shyma et al., 2014[a]
		S, L, B (EtOH)	AIT using lab-reared EF *R. (B.) microplus; in vivo*	8% (5.12%)	80	ND	ND			Srivastava et al., 2008[a]
Carapa guianensis Aubl.	Andiroba	S (E)O)	AIT using field EF *R. (B.) microplus*	0.625%	ND	ND	93.3	Palmtic acid, oleic acid, stearic acid, α-copaene	ND	Chagas et al., 2012[a, b]
Melia azedarach L.	Chinaberry	Fr (HX)	AIT using field EF *R. (B.) microplus*	0.25%	0	100	100	Azadirachtin, meliacarpin	Due to alterations on the neuroendocrine system of the tick.	Borges et al., 2003[a] Akhtar et al., 2008[b]

Myrtaceae										
Melaleuca alternifolia (Maiden & Betche) Cheel	Narrow-leaved paperback	L (EO)	AIT using field EF *R. (B.) microplus*	5%	ND	100	100	1, 8-cineole, terpinen-4-ol.	ND	Pazinato et al., 2014 [a, b]
Syzygium aromaticum (L.) Merrill & Perry	Clove	L (EO)	AIT using field EF *R. (B.) microplus*	10 mg/ml	100	90.3	ND	Eugenol, trans-β-caryophyllene	ND	Yessinou et al., 2016[a, b]
Papaveraceae										
Argemone Mexicana L.	Mexican poppy	WP (EtOH)	AIT using lab-reared EF *R. (B.) microplus*	10%	90	65.5	ND	Alkaloids, terpenoids, flavonoids, phenolics	ND	Ghosh et al., 2015[a, b]
Phytolaccaceae										
Petiveria alliacea L.	Guinea henweed	S (MeOH)	AIT using field EF *R. (B.) microplus*	20%	86.6	91	17	Benzyltrisulfide, benzyldisulfide	ND	Rosado-Aguilar et al., 2010[a, b]
Piperaceae										
Piper aduncum L.	Spiked pepper	L (EA)	AIT using field EF *R. (B.) microplus*	100 mg/ml	22	46.78	ND	Dillapiole, neorodiol, globulol, spathulenol, croweacin, apiole	Alter development and metabolism producing physiological disturbances that may lead to inhibition of reproduction or death due to interference with feeding and growth	Silva et al., 2009[a, b]
Piper tuberculatum Jacq.	Painful pepper	Fr (HX)	AIT using field EF *R. (B.) microplus*	75 mg/ml (18.4 mg/ml)	100	100	100	Piperine, piplartine	ND	da Silva Lima et al., 2014[a, b]
		L (EA)	AIT using field EF *R. (B.) microplus*	0.625% (3.76%)	ND	ND	81.7		ND	Chagas et al., 2012[a, b]
Poaceae										

Cymbopogon citratus (DC.) Stapf	Lemon grass	L (EO)	AIT using field EF *R. (B.) microplus, in vivo*	1:8	92	ND	ND	Citral	ND	Chungsamarnyart & Jiwajinda, 1992[a, b]
Cymbopogon martinii (Roxb.) Wats.	Ginger grass	L (E)O)	AIT using field EF *R. (B.) microplus*	0.3125% (2.93%)	ND	ND	86.7	Geraniol, geranyl acetate, linalool, trans-ocimene, myrcene, β-caryophyllene	ND	Chagas et al., 2012[a, b]
Cymbopogon nardus (L.) Rendle	Citronella grass	L (EO)	AIT using field EF *R. (B.) microplus, in vivo*	1:8	100	ND	ND	Citronellal, d-limonene	ND	Chungsamarnyart & Jiwajinda, 1992[a, b]
Rutaceae										
Aegle marmelos (L.) Correa	Golden apple	L	APT using field adult *Haemaphysalis bispinosa*	3000 ppm (358.45ppm)	100	ND	ND	Skimmiarepins A, Skimmiarepins C	ND	Elango & Rahuman, 2011[a, b]
Citrus hystrix DC.	Kaffir lime	FP (E)O)	AIT using lab-reared EF *R. (B.) microplus*	1:5	80	ND	ND	Citronellal, β-pinene, sabinene	ND	Chungsamarnyart & Jansawan, 1996[a] Sato et al., 1990[b]
Citrus maxima Merr.	Pomelo	FP (E)O)	AIT using lab-reared EF *R. (B.) microplus*	1:5	100	ND	ND	d-limonene	ND	Chungsamarnyart & Jansawan, 1996[a, b]
Citrus reticulata Blanco	Tangerin	FP (E)O)	AIT using lab-reared EF *R. (B.) microplus*	1:10	100	ND	ND	d-limonene, geranial, neral, geranyl acetate, geraniol	ND	Chungsamarnyart & Jansawan, 1996[a] Chutia et al., 2009[b]
Citrus sinensis (L.) Osbeck	Sweet orange	FP (E)O)	AIT using lab-reared EF *R. (B.) microplus*	1:10	98.59	ND	ND	d-limonene, terpineol, 1,8-cineole, sinsetin	ND	Chungsamarnyart & Jansawan, 1996[a] Favela-Hernández et al., 2016[b]
Solanaceae										
Capsicum frutescens L.	Malagueta pepper	Fr (EtOH)	AIT using field EF *R. (B.) microplus*	50mg/ml	10	25.3	84.6	Capsaicin, dihydrocapsaicin, pentadecanoic acid, hexadecanoic acid, octadecanoic acid	Interference with the conversion of blood ingested by ticks in eggs	Vasconcelos et al., 2014[a, b]
Datura stramonium L.	Devil's snare	L (MeOH)	AIT using field EF *R. (B.) microplus*	100mg/ml	73.33	77.17	70	Scopolamine, hyoscyamine, meteloidine, atropine	Similar to organophosphates	Shyma et al., 2014[a, b]
Datura metel L.	Devil's trumpet	Fr (EtOH)	AIT using lab-reared EF *R. (B.) microplus*	9%	100	100	ND	Yangjinhualine A, megastigmane sesquiterpenes, alkaloids, glycosides	ND	Ghosh et al., 2015[a, b] Kuang et al., 2008[b]
Withania somnifera (L.) Dunal	Indian ginseng	L (EtOH)	AIT using field EF *R. (B.) microplus*	5% (6.1%)	20	21.36	100	Isopelletierine, anaferine, withanolides, withaferins,	Due to the decreased levels of ecdysteroids	Singh et al., 2014[a, b]

								sitoindoside VII, VIII, IX, X	leading to decreased incorporation of free ecdysteroids into the eggs necessary for oocyte maturation	
Stemonaceae										
Stemona collinsae Craib.	-	R (MeOH)	AIT using field engorged adult *R. (B.) microplus, in vivo*	250mg/ml	100	ND	ND	Stemofoline, didehydrostemofoline, stemofurans A-K, dihydrostilbene	Reduce tick attachment	Pacher et al., 2002[b] Kongkiatpaiboon et al., 2014[a, b]
Verbernaceae										
Lippia sidoides Cham.	Pepper-rosmarin	L (EO)	NPT using lab-reared nymphal *R. sanguineus*	18.80mg/ml	96.1	ND	ND	Thymol, o-cymene, E-caryophyllene, myrcene	Blocks the GABA receptors, impairing the flow of Cl- ions, leading to alterations on nerve impulses and death.	Gomes et al., 2014[a, b]

Plant parts: L - Leaves; S - Stem; B - Bark; R - Root; AP - Aerial parts; WP - Whole plant; Sd - Seed, Fl - Flowers; F - Fruit; FP - Fruit peel; Sk - Skin; C - Cloves; Rh - Rhizome
Extractant: EO - Essential Oil; EtOH - Ethanol; MeOH - Methanol; HX - Hexane; AC - Acetone; DW - Distilled Water; CH - Chloroform; Aq- Aqueous; DCM - Dichloromethane; EA - Ethyl acetate
Ticks: H. - Hyalomma; R. - Rhipicephalus
Test: IO - Inhibition of Oviposition; EHI - Egg Hatching Inhibition; AIT- Adult Immersion Test; APT- Adult Packet Test; NPT- Nymphal Packet Test CB- Contact Bioassay; VPB- Vapour Phase bioassay
Others: EF- Engorged adult female; ND - Not determined; Conc. - Concentration; LC_{50} - Lethal concentration 50; DA - Decidedly Affected (dead plus very weak); [a]- Reference for acaricidal activity; [b]- Reference for isolated compounds

Table 4.5: Plant species evaluated for larvicidal activity using the Larval Packet or Larval Immersion Tests and their possible bioactives

Plant family and species	Common name	Plant part/Extractant	Tick species and age of larvae	Conc.	Effect (%)	LC50	Some active isolated compounds	Mechanism of action	References
Acanthaceae									
Andrographis paniculata (Burm.f) Wall. ex Nees	King of bitters	L (MeOH)	7-14 days old LV *R. (B.) microplus*	-	-	207.70 ppm	Andrographolide, andrographine, panicoline, paniculide-A, B, C	ND	Elango & Rahuman, 2011[a] Hossain et al., 2014[b]
Amaryllidaceae									
Allium sativum L.	Garlic	C (MeOH)	12 days old LV *R. (B.) microplus*	100 mg/ml	69	ND	Allicin, alliin	ND	Shyma et al., 2014[a]
		EO	10 days old LV *R. (B.) microplus*	5%	100	ND	Allicin, diallyl trisulfide, diallyl disulphide, ajoene	ND	Martinez-Velazquez et al., 2011[a] Mikaili et al., 2013[b]
		Bl (EO)	15 days old LV *R. (B.) annulatus*	5%	100	ND	Allicin, allin, dially disulfide, methyl alkyl disulphide, diallyl trisulfide.	Interaction with several glycosylated receptor proteins in the mid gut of the parasite leading to inhibition of nutrient absorption and death. Also gets accumulated into the haemolymph and ovarioles thereby interfering with development and reproduction.	Aboelhadid et al., 2013[a,b]
Allium cepa L.	Onion	Bl (EO)	15 days old LV *R. (B.) annulatus*	5%	100	ND	Dipropyl disulphide, methyl propyl disulphide, methyl propyl trisulphide	Chemical reaction with thiol groups of various enzymes which affects the essential metabolism of cysteine proteinase activity involved in virulence of the parasite	Aboelhadid et al., 2013[a,b]
Annonaceae									

Annona squamosa L.	Sugar apple	FP (Aq)	14-21 days old LV *R. (B.) microplus*	2000 ppm	100	548.28 ppm	1*H*- cycloprop[e]azulen-7-ol decahydro-1,1,7-trimethyl-4-methylene-[1ar-(1aα,4aα, 7β, 7 a, β, 7bα)], retinal 9-*cis*- 3,17-dioxo-4-androsten-11alpha-yl hydrogen succinate, 1-naphthalenepentanol decahydro-5-(hydroxymethyl)-5,8a-dimethyl-y,2-bis(methylene)-(1α,4aβ,5α,8aα), 1-naphthalenemethanol decahydro −5-(5-hydroxy-3-methyl-3-pentenyl)- 1,4a-di methyl - 6-methylene -(1S-[1α, 4aα, 5α(E), 8aβ], (−)-spathulenol, podocarp-7-en-3-one13β-methyl-13-vinyl, 1-phenanthrene carboxaldehyde 7-ethenyl-1,2,3,4,4a,4,5,6,7,9,10,10a-dodecahydro-1,4a,7-trimethyl-[1R-(1α,4aβ.4bα,7β, 10aα)]	ND	Madhumitha et al., 2012[a,b]
Apocynaceae									
Calotropis procera (Aiton)W.T.Aiton	Apple of Sodom	AP	14 days old LV *H. dromedarii*	-	-	588 mg/L	Digitoxin, cardenolide	Inhibition of sodium/potassium	Al-Rajhy et al., 2003 [a, b]
		L (MeOH)	12 days old LV *R. (B.) microplus*	100 mg/ml	63.2	ND		ATPase of ticks	Shyma et al., 2014
Asteraceae									
Artemisia absinthium L	Absinthe wormwood	AP (CH)	14 days old LV *R. sanguineus*	-	-	1.11%	Artemisinin	Reacts with the heme groups of the haemoglobin molecules digested by parasites, altering the cell structure and thus affect the growth and reproduction	Godara et al., 2014a[a, b]

Calea serrata L.	Snakeherb	AP	14 days old LV *R. (B.) microplus*	6.25 mg/ml	100	ND	Precocene II, eupatoriochromene	Interfere with tick oviposition, development and reproduction.	Ribeiro et al., 2011[a, b]
Eupatorium adenophorum Spreng.	Sticky snake root	AP (EtOH)	7-14 days old LV *Haemaphysalis longicornis*	1.5 g/ml	100	ND	Cadinene sesquiterpenes	ND	Nong et al., 2013a[a, b]
Tagetes patula L.	French marigold	AP (EtOH)	LV *R. sanguineus*	-	-	7.43 mg/ml	50 - hydroxymethyl-5-(3-butene-1-ynil)-2,20 - bithiophene; methyl-5-[4-(3-methyl-1- oxobutoxy)-1-butynyl]-2,2' bithiophene; cholesterol; β-sitosterol, stigmasterol , lupeol, kaempferol, quercetina, patuletin-7-O-glucoside (patulitrin), patuletin, quercetagetin, quercetagetin-7-O-glucoside, luteolin	ND	Politi et al., 2012 [a, b]
Caricaceae									
Carica papaya L.	Pawpaw	Sd (MeOH)	12 days old LV *R. (B.) microplus*	100mg/ml	82.2	ND	Papain, chymopapain, peptidase A. peptidase B, lysozyme	ND	Shyma et al., 2014 [a, b]
Combretaceae									
Guiera senegalensis J.F. Gmel	Moshi medicine	L (EtOH)	10-14 days old LV *H. anatolicum*	15%	100	0.787%	Catechin, myricitrin, rutin, quartterin	ND	Osman et al., 2014 [a, b]
Euphorbiaceae									
Croton sphaerogynus Baill	Croton	L (DCM)	14-21 days old LV *R. (B.) microplus*	20%	100	6.7%	Abieta-8,11-diene-3-one, abieta-8,11,13-trien-12-ol, podocarp-7-ene,13-methyl-13-vinyl-3-one	ND	Righi et al., 2013 [a, b]
Fabaceae									
Leucaena leucocephala (Lam.) de Wit	White leadtree	L (AC/DW)	LV *R. (B.) microplus*	4800 ug/ml	66.8	ND	Tannins, quercetin, caffeic acid, scopoletin	Tannins are responsible, confirmed by using a specific blocker, polyethylene glycol.	Fernández-Salas et al., 2011 [a, b]
Hypericaceae									

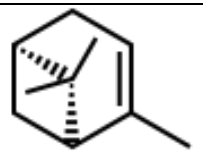
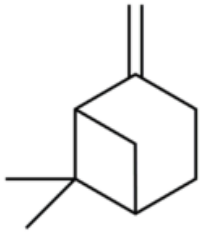
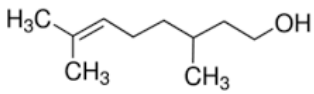
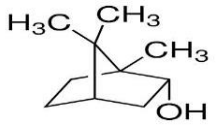
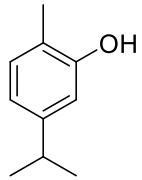
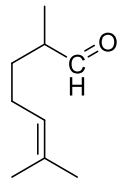
Hypericum polyanthemum Klotzch ex Reichardt	St. John's wort	AP (HX)	14 days old LV *R. (B.) microplus*	6.25 mg/ml	100	ND	6-isobutyryl-5,7-dimethoxy-2,2-dimethylbenzopyran, 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethylbenzopyran and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethylbenzopyran	Affect development and reproduction of the tick	Ribeiro et al., 2007 [a, b]
Lamiaceae									
Cunila angustifolia Benth.	-	AP (EO)	14 days old LV *R. (B.) microplus*	2.5 µl/ml	100	ND	Sabinene, γ- terpinene, limonene	ND	Apel et al., 2009[a, b]
Cunila incana Benth.	-	AP (EO)	14 days old LV *R. (B.) microplus*	2.5 µl/ml	100	ND	α-pinene, β-pinene, β-caryophyllene	ND	Apel et al., 2009[a, b] Agostini et al., 2010[b]
Cunila spicata Benth.	-	AP (EO)	14 days old LV *R. (B.) microplus*	5 µl/ml	100	ND	Menthofurane, borneol	ND	Apel et al., 2009[a, b]
Hesperozygis ringens (Benth.) Epling	Pulegium	L (EO)	14 day old LV *R. (B.) microplus*	0.625µl/ml	100	0.260 µl/ml	Pulegone, limonene, linalool, β-caryophyllene, bicyclogermacrene	Due to chemosterilant effect of pulegone	Ribeiro et al., 2010[a, b]
Ocimum canum L.	Camphor basil	L (aq)	LV *H. a. anatolicum*	25 mg/L	96	15.31 mg/L	α-thujene, myrcene, α-pinene, sabinene, α-phellandrene, α-terpinene, limonene, γ-terpinene, terpinolene, β-caryophyllene, trans-α-bergarmotene, α-caryophyllene, germacrene D,β-seliene, biocyclogermacrene, estragole, thymol, carvacrol	ND	Jayaseelan & Rahuman, 2012 [a, b]
Ocimum suave Willd (syn. *Ocimum gratissimum* L.)	Clove basil	L (EO)	LV *R. appendiculatus; in vivo*	0.2%	100	0.024%	Germacrene-D, β-caryophyllene, β-eudesmol, α-humulene	ND	Mwangi et al., 1995[a] Runyoro et al., 2010[b]
Ocimum urticaefolium Roth	-	L (EO)	14-21 days old LV *R. (B.) microplus*	5%	100	0.90%	Eugenol, elemicin, β-Bisabolene	ND	Hüe et al., 2015[a, b]
Rosmarinus officinalis L	Rosemary	L	10 days old LV *R. (B.) microplus*	10%	89	ND	α-pinene, verbenone,1,8-cineole	ND	Martinez-Velazquez et al., 2011
Tetradenia riparia (Hochst.) Codd	Ginger bush	L (EO)	14-21 days old LV *R. (B.) microplus*	25%	100	1.222g/ml	14-hydroxy-9-epi-cariophyllene, cismuurolol-5-en-4-a-ol, ledol, limonene, fenchone	ND	Gazim et al., 2011[a, b]

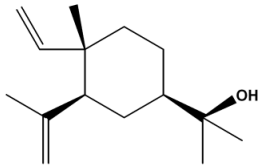
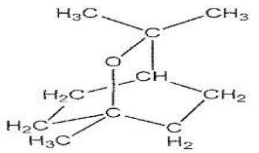
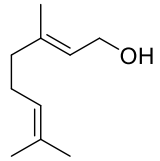
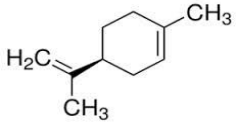
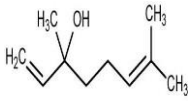
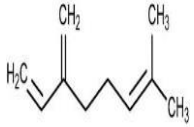
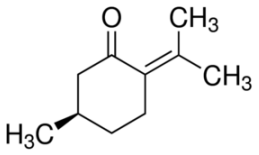
Copaifera reticulata Ducke	Copaiba	L (DMSO/DW)	14-21 days old LV *R. (B.) microplus*	-	-	1579ppm	Oleoresin	ND	de Freitas Fernandes & Freitas, 2007[a, b]
Meliaceae									
Melia azedarach L.	Chinaberry	Fr (HX)	7-21 days old LV *R. (B.) microplus*	0.25%	98	ND	Azadirachtin	Due to alterations on the neuroendocrine system of the tick.	Borges et al., 2003[a, b]
Myrtaceae									
Corymbia citriodora (Eucalyptus citriodora) (Hook.) K.D. Hill & L.A.S. Johnson	Lemon-scented gum	L (EO)	LV *Anocentor nitens*	50%	100	ND	Citronellal	ND	Clemente et al., 2010[a, b]
Phytolaccaceae									
Petiveria alliacea L.	Guinea henweed	S (HX)	7-14 days old LV *R. (B.) microplus*	20%	100	3.88%	Benzyltrisulfide, benzyldisulfide	ND	Rosado-Aguilar et al., 2010[a, b]
Piperaceae									
Piper aduncum L.	Spiked pepper	L (HX)	14-21 days old LV *R. (B.) microplus*	20 mg/ml	70.4	9.3mg/ml	Dillapiole, neorodiol, globulol, spathulenol, croweacin, apiole, β-ocimene	ND	Silva et al., 2009[a, b] Bernuci et al., 2016[b]
Piper mikanianum (Kunth) Steud.	Betel leaf	AP (EO)	14 days old LV *R. (B.) microplus*	-	-	2.33µl/ml	Apiol, dillapiol, myristicin, limonene, bicyclogermacrene, β-caryophyllene, safrole, β-vetivone, (*Z*)-isoelemicin, (*E*)-asarone	ND	de BF Ferraz et al., 2010[a, b] Bernuci et al., 2016[b]
Piper tuberculatum Jacq.	Painful pepper	Fr (HX)	LV *R. (B.) microplus*	0.12 mg/ml	100	0.04 mg/ml	Piplartine, piperine	ND	da Silva Lima et al., 2014[a, b]
		L (EO)	14-21 days old LV *R. (B.) microplus*	-	-	0.41%		ND	Chagas et al., 2012[a, b]
Poaceae									
Cymbopogon citratus (DC.) Stapf	Lemon grass	L (EO)	LV *R. (B.) microplus ; in vivo*	1:8	99	ND	Citral	ND	Chungsamarnyart & Jiwajinda, 1992 [a,b]
Cymbopogon martinii (Roxb.) Wats.	Ginger grass	L (EO)	14-21 days old LV *R. (B.) microplus*	-	-	0.47%	Geraniol, geranyl acetate, linalool, trans-ocimene, myrcene, β-caryophyllene	ND	Chagas et al., 2012[a, b]
Cymbopogon nardus (L.) Rendle	Citronella grass	L (EO)	LV *R. (B.) microplus; in*	1:8	94	ND	Citronellal, d-limonene	ND	Chungsamarnyart & Jiwajinda, 1992[a,b]

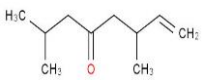
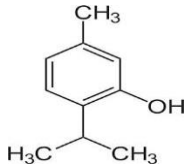
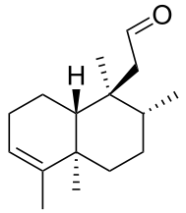
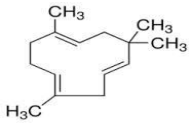
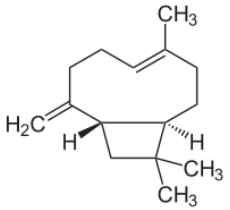
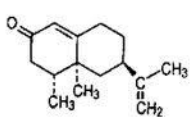
			vivo						
Melinis minutiflora P.Beauv	Molasses grass	L (EO)	LV *R. (B.) microplus*	0.01%	100	ND	Propionic acid, 1,8-cineole, butyric acid, phenylethyl alcohol, hexanal, 9-*E*-eicosene	ND	Prates et al., 1998[a, b]
Rutaceae									
Aegle marmelos (L.) Correa	Golden apple	L (MeOH)	7-14 days old LV *R. (B.) microplus*	2000 ppm	100	134.09 ppm	Skimmiarepin A, skimmiarepin C	ND	Elango & Rahuman, 2011[a, b]
Solanaceae									
Datura stramonium L.	Devil's snare	L (MeOH)	12 days old LV *R. (B.) microplus*	100 mg/ml	71.8%	ND	Scopolamine, hyoscyamine, meteloidine, atropine, terpenoids, flavonoids	May be similar to Organophosphates	Shyma et al., 2014[a, b]
Solanum trilobatum L.	Purple fruited pea eggplant	L (Aq)	LV *H. a. anatolicum*	50 mg/L	55	47.2 mg/L	Solamarine, solaine, solasodine, glycoalkaloid, diosogenin, tomatidine	ND	Shahjahan et al., 2004[b] Rajakumar et al., 2014[a]
Verbenaceae									
Lippia graveolens Kunth	Mexican oregano	L	10 days old LV *R. (B.) microplus*	2.5%	100	ND	Thymol, carvacrol, p-cymene, γ-terpinene	ND	Martinez-Velazquez et al., 2011
Lippia sidoides Cham.	Pepper-rosmarin	L (EO)	15-21 days old LV *A. cajennense*	18.8 mg/ml	100	ND	Thymol, o-cymene, myrcene, E-carophyllene	ND	Gomes et al., 2014[a, b]

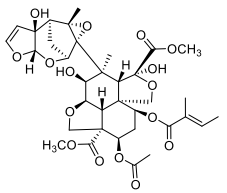
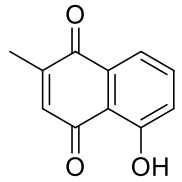
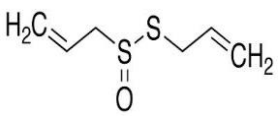
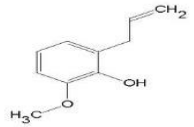
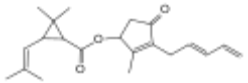
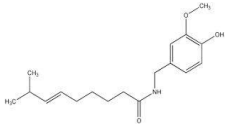
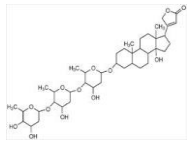
Plant parts: L - Leaves; S - Stem; B - Bark; R - Root; Bl - bulb; AP - Aerial parts; Sd - Seed, Fl - Flowers; F- Fruit; FP - Fruit peel; Sk - Skin; C - Cloves
Extractant: EO - Essential Oil; EtOH - Ethanol; MeOH -Methanol; HX - Hexane; AC - Acetone; DW - Distilled Water; DMSO - Dimethyl sulfoxide; DCM - Dichloromethane
Ticks: A. - Amblyomma; H. - Hyalomma; R. - Rhipicephalus
Others: LV - Larvae; ND - Not determined; Conc. - Concentration; LC_{50} - Lethal concentration 50; [a]- Reference for larvicidal activity; [b]- Reference for isolated compounds

Table 4.6: The lethal concentration (LC_{50}) of some known insecticidal, acaricidal and tick repellent compounds isolated from plants

Class of compound	Compound	Plant species	LC_{50}	Chemical Structure and Formula	References
Monoterpene	α-pinene (insecticidal, acaricidal)	*Plectranthus barbatus* Andrews *Rosmarinus officinalis* *Satureja myrtifolia*	32.09 µg/ml	 $C_{10}H_{16}$	Govindarajan et al., 2016
	β-pinene (repellent)	*Lindera melissifolia* (Walt.) Blume *Stylosanthes humilis* Kunth *Cleome monophylla* *Clausena anisata* *Cannabis sativa*	0.03-6.5 µl/L air	 $C_{10}H_{16}$	Ogendo et al., 2011
	β-citronellol (acaricidal, repellent)	*Pelargonium graveolens* *Dianthus caryophyllus*	78.9%	 $C_{10}H_{20}O$	Pohlit et al., 2011
	Borneol (insecticidal)	*Lavandula angustifolia* *Artemisin abrotanum* *Cunila spinata* *Origanum minutiflorum*	183- >500mg/L	 $C_{10}H_{18}O$	Pohlit et al., 2011
	Carvacrol (acaricidal)	*Chamaecyparis nootkatensis* *Gynandropsis gynandra* *Origanum minutiflorum* *Satureja thymbra* *Lippia gracilis*	4.46 mg/ml	 $C_{10}H_{14}O$	de Oliveira Cruz et al., 2013
	Citronellal (acaricidal)	*Cymbopogon nardus* *Corymbria citriodora* *Citrus hystrix*	21.0%		Pohlit et al., 2011

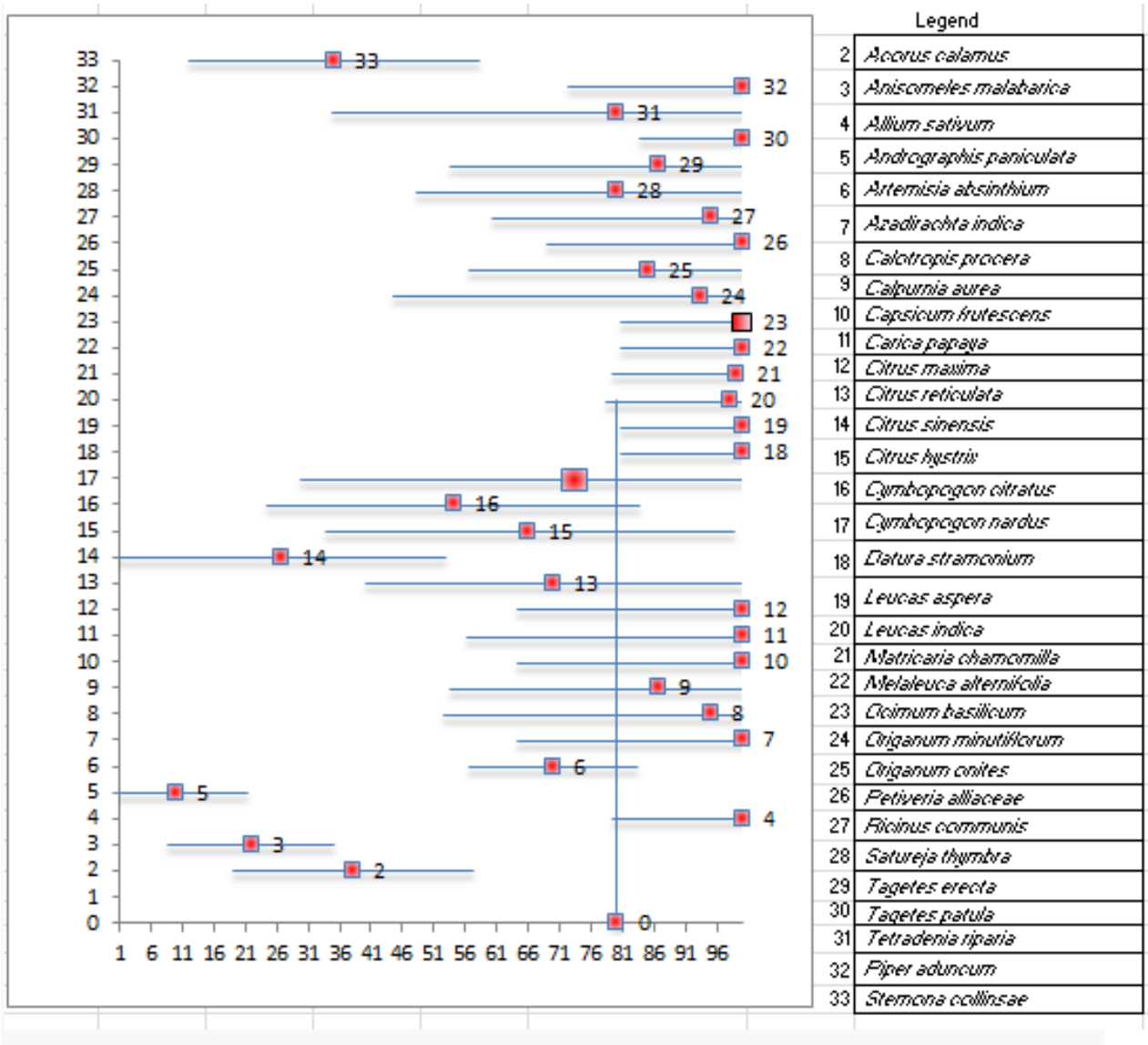
Elemol (repellent)	*Maclura pomifera*	5.16 µg/cm^2	$C_{10}H_{18}O$		Pohlit et al., 2011
Eucalyptol (1,8-cineole) (acaricidal)	*Eupatorium adenophorum* *Lippia javanica* *Ocimum* species	511.47 mg/L	$C_{15}H_{26}O$		Badawy et al., 2010
Geraniol (acaricidal, repellent)	*Pelargonium species*. *Cymbopogon species* *Dianthus caryophyllus*	17.8%	$C_{10}H_{18}O$		Pohlit et al., 2011
Limonene (acaricidal)	*Citrus* species *Copaifera reticulata* *Hesperozygis ringens* *Tetradenia riparia*	255.44 mg/L	$C_{10}H_{18}O$		Badawy et al., 2010
Linalool (acaricidal)	*Tagetes erecta* *Hesperozygis ringens* *Ocimum basilicum* *Origanum onites* *Cymbopogon martinii*	503.43 mg/L	$C_{10}H_{16}$		Badawy et al., 2010
Myrcene (acaricidal)	*Origanum minutiflorum* *Lippia javanica* *Salvia nilotica* Jacq.	>550 mg/L	$C_{10}H_{18}O$		Badawy et al., 2010
Pulegone (acaricidal)	*Mentha suaveolens*	0.321 µl/ml	$C_{10}H_{16}$		Pohlit et al., 2011

	Tagetone (insecticidal)	*Tagetes* species	1.0532 mg/L	$C_{10}H_{16}O$		Gakuubi et al., 2016
	Thymol (acaricidal)	*Thymus vulgaris* *Lippia sidoides* *Lippia gracilis* *Origanum. minutiflorum*	5.50 mg/ml	$C_{10}H_{16}O$		de Oliveira Cruz et al., 2013
Diterpene	Callicarpenal (repellent, acaricidal)	*Callicarpa americana*	0.084 mg/cm^2	$C_{10}H_{14}0$		Carroll et al., 2007
Sesquiterpene	α-humulene (repellent)	*Lindera melissifolia* (Walt.) Blume *Stylosanthes humilis* Kunth *Cleome monophylla*	0.01-4.82 µl/L air	$C_{16}H_{26}O$		Ogendo et al., 2011
	β- caryophyllene	*Syzygium aromaticum* *Canabis sativum*	41.66 µg/ml	$C_{15}H_{24}$		Govindarajan et al., 2016
	Nootkatone (acaricidal)	*Chamaecyparis nootkatensis* (D. Don) Spach *Chrysopogon zizanioides* L. *Citrus grandis* (L.) Osbeck	0.0029% w/v	$C_{15}H_{24}$ $C_{15}H_{22}O$		Panella et al., 2005

Tetranotriterpenoid	Azadirachtin (acaricidal, insecticidal)	*Azadirachta indica* *Melia azedarach*	5000 ppm	$C_{35}H_{14}0_{16}$		Giglioti et al., 2011
Naphthoquinone	Plumbagin (acaricidal)	*Plumbajo zeylanica*	1.37 mg/ml	$C_{11}H_8O_3$		Annan et al., 2009
Organosulfur	Allicin (insecticidal)	*Allium sativum*	0.02-0.25 mg/L	$C_6H_{10}OS_2$		Block, 2004
Phenylpropanoid	Eugenol (insecticidal, acaricidal)	*Ocimum species* *Aetemisia species* *Plectranthus barbatus* Andrews	25.45 µg/ml	$C_{10}H_{12}O_2$		Govindarajan et al., 2016
Pyrethrin	Pyrethrin I (acaricidal, insecticidal)	*Chrysanthemum* species *Pseudoletia unipuncta*	0.4 ppm	$C_{21}H_{28}O_3$		Akhtar et al., 2008
Resin	Oleoresin (acaricidal)	*Copaifera reticulata* Ducke	1579 ppm	$C_{18}H_{27}NO_3$		Fernandes & Freitas, 2007
Steroidal glycoside	Digitoxin (acaricidal)	*Calotropis procera* *Digitalis purpurea*	409.9 mg/L	$C_{41}H_{64}O_{13}$		Al-Rajhy et al., 2003

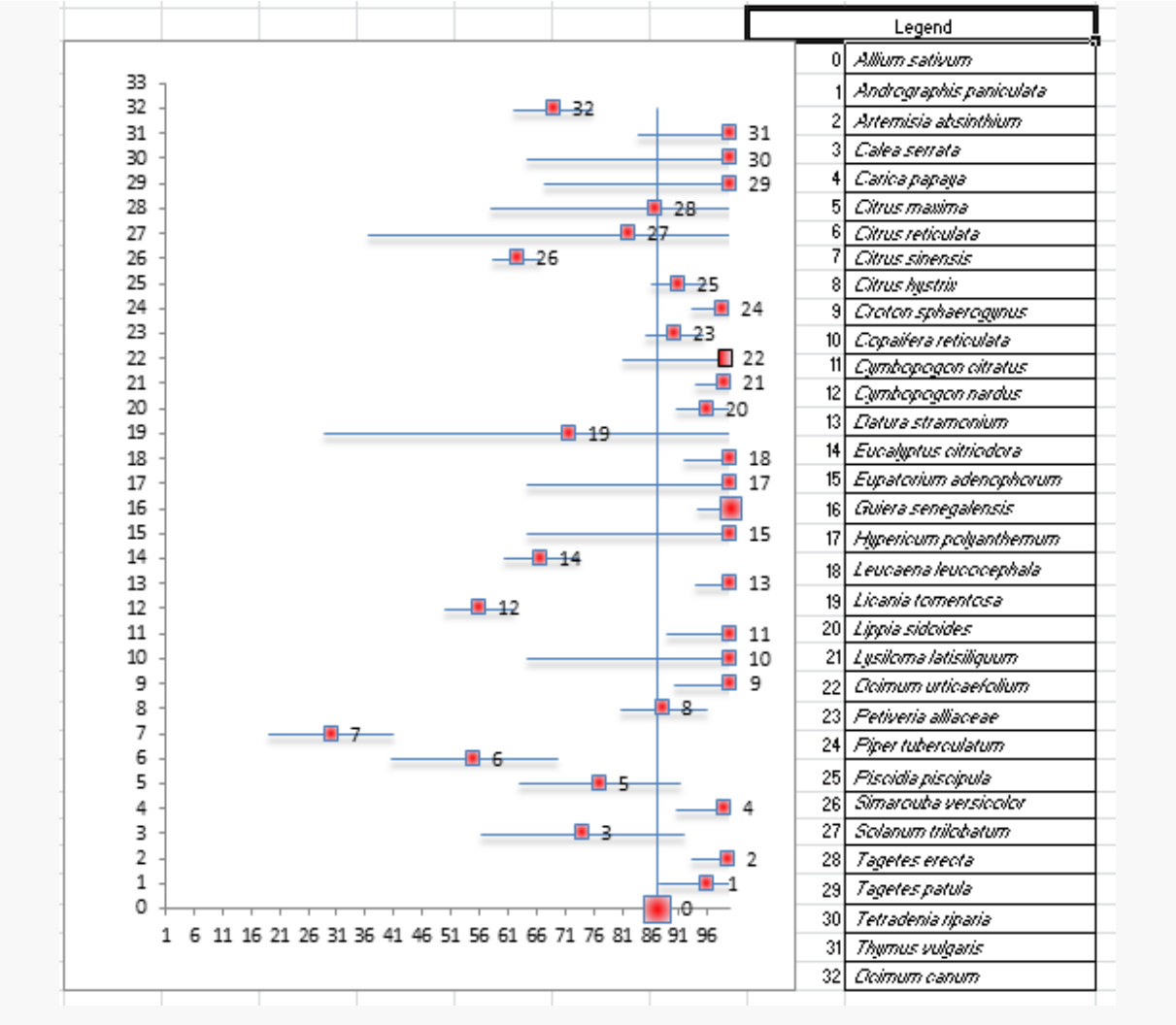
4.3.3 Meta-analysis of plant extracts with tick repellent and/or acaricidal properties

A total of 32, 33, 20, 24, 9, 9 and 4 plants were evaluated separately for acaricidal, larvicidal, egg hatching inhibition, inhibition of oviposition and repellency of various plant extracts, including also the specific acaricidal effects of two selected plant families (Fig. 4.1-4.7). A total of 1428 events were considered for acaricidal activities with median efficiency values (MEV) of 80.12% [95% Confidence Interval ($CI_{95\%}$): 79.20 - 81.04; Fig. 4.1)]; while 1924 events were considered for the larvicidal assays with a MEV of 86.05% ($CI_{95\%}$: 85.13 - 86.97; Fig. 4.2). For the egg hatching inhibition assays, a total of 574 events with a MEV of 83.39% ($CI_{95\%}$: 82.47 - 84.31; Fig. 4.3) while the inhibition of oviposition had the following values: MEV 53.01% ($CI_{95\%}$: 52.08 - 53.93) (Fig. 4.4). The repellency assays had MEV of 92.00% ($CI_{95\%}$: 91.08 - 92.93; Fig. 4.5) while the specific acaricidal effects of the two selected plant families (Lamiaceae and Asteraceae) with 281 and 68 events respectively had MEV of 80.79% ($CI_{95\%}$: 79.87 - 81.71; Fig. 4.6) and 48.34% ($CI_{95\%}$: 47.42 - 49.26; Fig. 4.7) respectively. The efficiency values of various plant extracts showed minimal to wide disparities compared to the MEV for each category. Approximately 63% and 69% of all the plant species evaluated for acaricidal and larvicidal assays respectively surpassed the MEV respectively (Fig. 4.1-4.7).



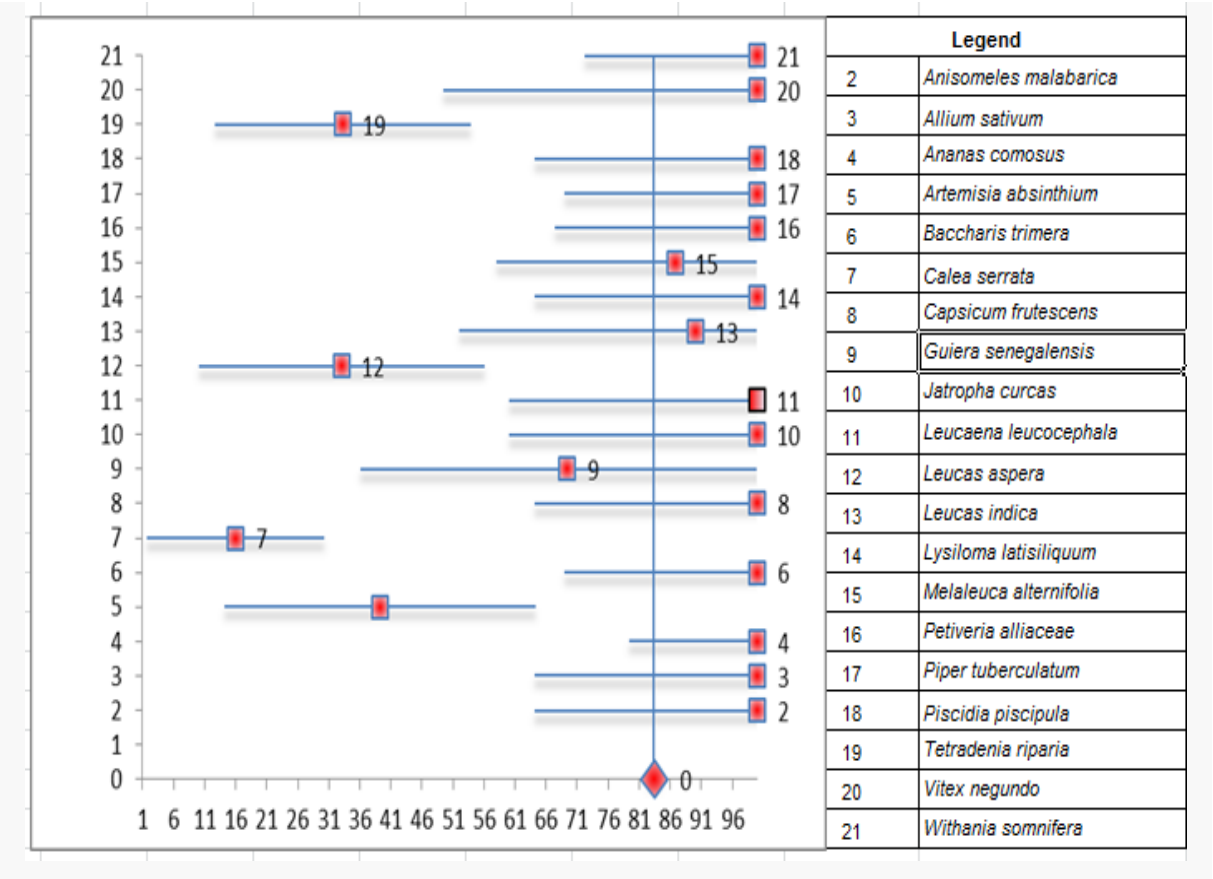
Y axis - Number allotted to the plant extracts evaluated as indicated on the legend; X axis - Percentage adult tick mortality; Red box on the x axis - Median efficiency value (MEV); Lines running perpendicular to the x-axis - Efficiency values of the various plant extracts; Error bars - 95% confidence interval

Figure 4.1: Meta-analyses of acaricidal effects of extracts of some plant species



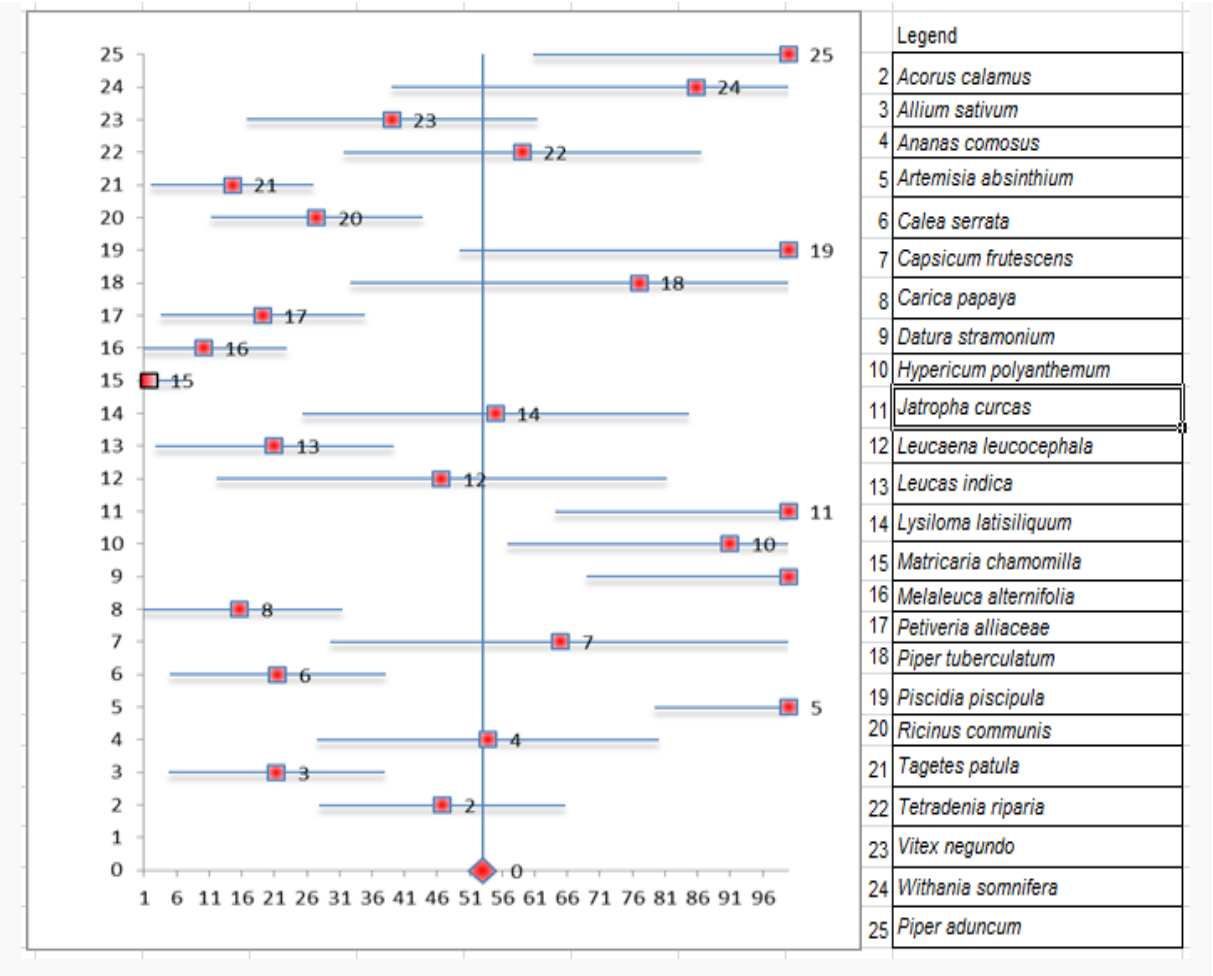
Y axis - Number allotted to the plant extracts evaluated as indicated on the legend; X axis - Percentage larval mortality; Red box on the x axis - Median efficiency value (MEV); Lines running perpendicular to the x-axis - Efficiency values of the various plant extracts; Error bars - 95% confidence interval

Figure 4.2: Meta-analyses of larvicidal effects of extracts of some plant species



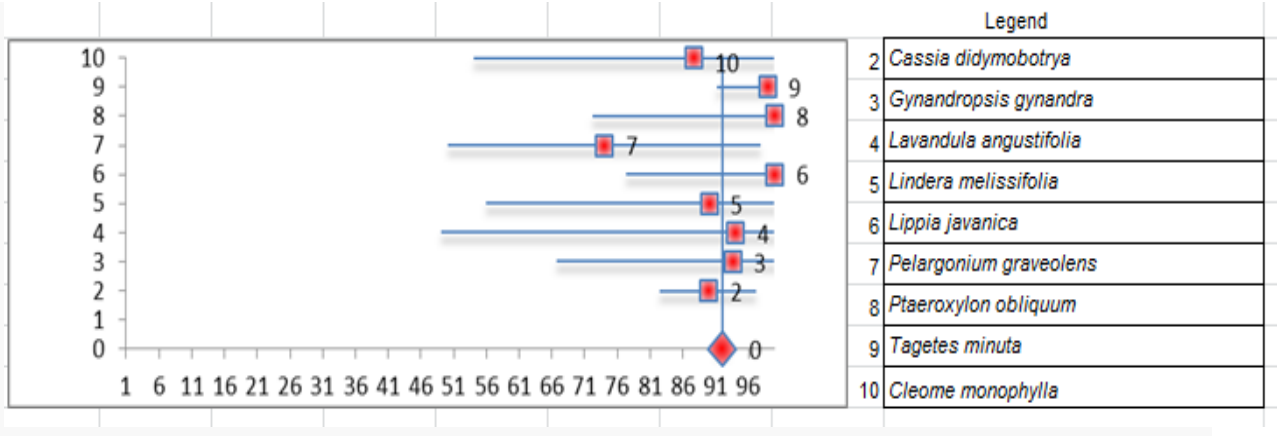
Y axis - Number allotted to the plant extracts evaluated as indicated on the legend; X axis - Percentage inhibition of egg hatching; Red box on the x axis - Median efficiency value (MEV); Lines running perpendicular to the x-axis - Efficiency values of the various plant extracts; Error bars - 95% confidence interval

Figure 4.3: Meta-analyses of egg hatching inhibition effects of extracts of some plant species



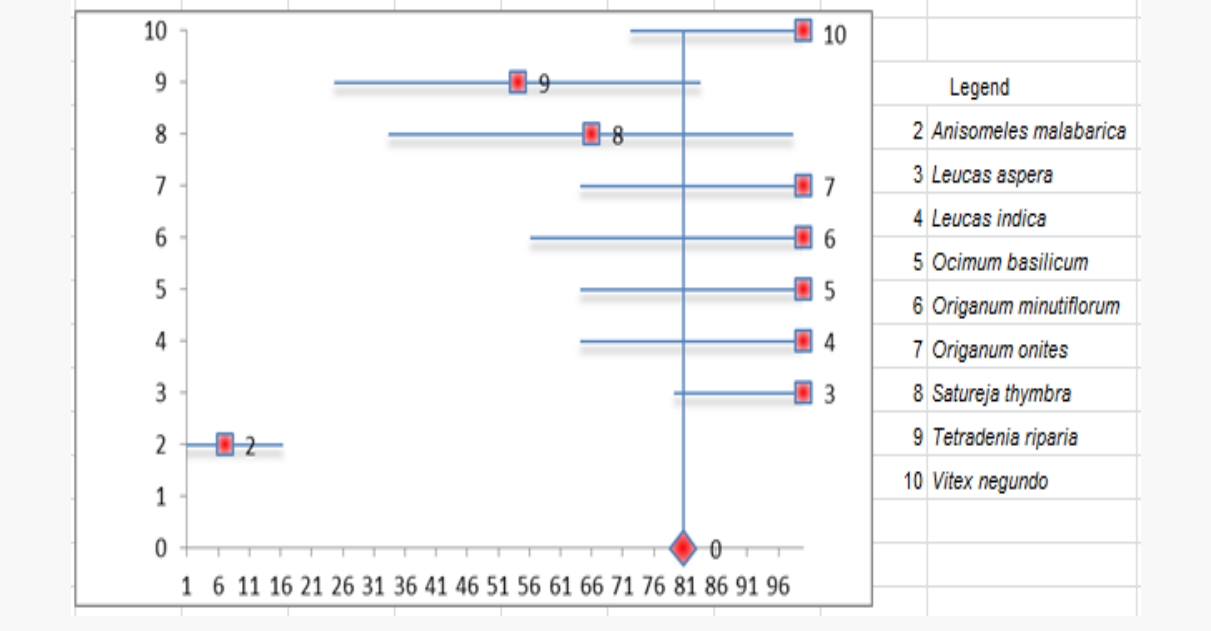
Y axis - Number allotted to the plant extracts evaluated as indicated on the legend; X axis - Percentage inhibition of oviposition; Red box on the x axis - Median efficiency value (MEV); Lines running perpendicular to the x-axis - Efficiency values of the various plant extracts; Error bars - 95% confidence interval

Figure 4.4: Meta-analyses of inhibition of oviposition effects of extracts of some plant species



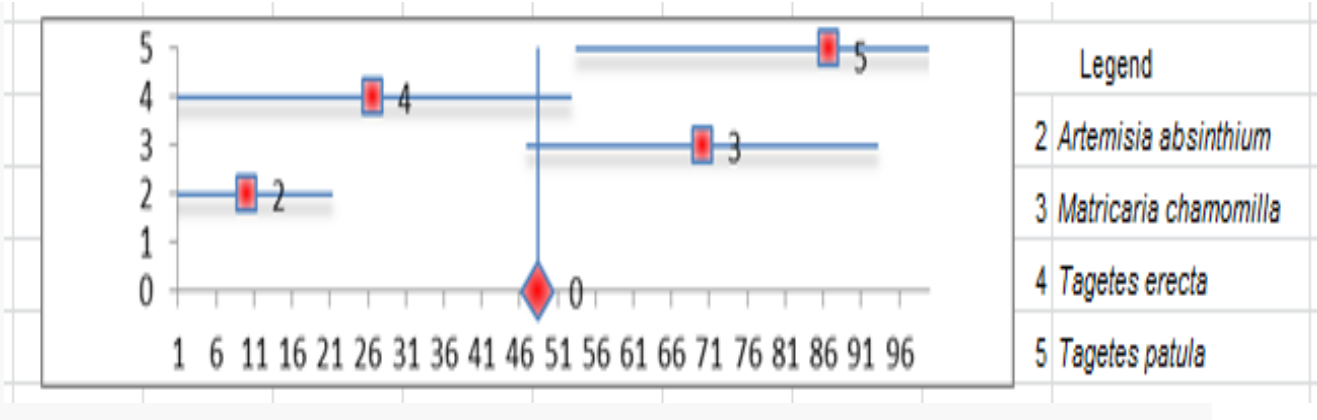
Y axis - Number allotted to the plant extracts evaluated as indicated on the legend; X axis - Percentage tick repellency; Red box on the x axis - Median efficiency value (MEV); Lines running perpendicular to the x-axis - Efficiency values of the various plant extracts; Error bars - 95% confidence interval

Figure 4.5: Meta-analyses of repellency effects of extracts of some plant species



Y axis - Number allotted to the plant extracts evaluated as indicated on the legend; X axis - Percentage tick mortality; Red box on the x axis - Median efficiency value (MEV); Lines running perpendicular to the x-axis - Efficiency values of the various plant extracts; Error bars - 95% confidence interval

Figure 4.6: Meta-analyses of acaricidal effects of extracts of some plant species in the Lamiaceae family



Y axis - Number allotted to the plant extracts evaluated as indicated on the legend; X axis - Percentage tick mortality; Red box on the x axis - Median efficiency value (MEV); Lines running perpendicular to the x-axis - Efficiency values of the various plant extracts; Error bars - 95% confidence interval

Figure 4.7: Meta-analyses of acaricidal effects of extracts of some plant species in the Asteraceae family

4.4 Discussion

The use of botanicals for the control of ticks has a long history as an important component of traditional medicine in Africa and Asia, where most resource-poor farmers use plant materials to treat endo and ectoparasites of livestock (Mondal et al., 2013). Traditional knowledge about the use of these plants is transferred through successive generations, especially in rural communities. Knowledge about the use of individual plant species, however, varies between localities in Africa and scientific validation of their uses may increase the range of plants available for tick control and reduce the burden substantially on plant species that are at risk of extinction.

Pesticidal plant research in veterinary parasitology is a recent area of research globally when compared with screening of plant extracts for the treatment of bacterial diseases. This review indicates that plants have potential for developing acaricides to counteract problems associated with chemical acaricides such as resistance and residues; and for prolonging the useful life of commercial chemical products applied for parasite control through the association of bioactive plant substances with synthetic products (Varma & Dubey, 1999). Plant extracts contain mixtures of substances that can act synergistically, in different ways, which makes the development of parasite resistance more difficult than normally occurs with conventional acaricides. According to Katoch et al. (2007), the efficacy of a single plant can be enhanced by a judicious combination with another plant or active ingredient that has adjuvant properties. This combination of chemicals with different mechanisms of action usually lowers the LD_{50} of the product and hampers the development of resistance of the ticks.

Essential oils are complex mixtures of natural, volatile organic compounds predominantly composed of terpenic hydrocarbons (myrcene, pinene, terpinene, limonene, p-cymene, α- and β-phellandrene), acyclic monoterpene alcohols (geraniol, linalool), monocyclic alcohols (menthol, 4-carvomenthol, terpineol, carveol, borneol), aliphatic aldehydes (citral, citronellal, perillaldehyde), aromatic phenols (carvacrol, thymol, safrol, eugenol), bicyclic alcohol (verbenol), monocyclic ketones (menthone, pulegone, carvone), bicyclic monoterpenic ketones (thujone, verbenone, fenchone), acids (citronellic acid, cinnamic acid) and esters (linalyl acetate) (Nerio et al., 2010).

Different arthropod species respond quite differently to specific plant volatiles. For instance, benzaldehyde and benzyl alcohol are toxic to the storage mite *Tyrophagus putrescentiae* Schranck but are, at lower concentrations, attractants to *Amblyomma* ticks (Yoder et al., 1998). Eucalyptol (1, 8-cineole), which is a characteristic leaf compound of the genus

Eucalyptus, is repellent and toxic to most insects, but exhibits attractant activity for some insects belonging to different orders such as Coleoptera and Lepidoptera (Parra et al., 2009). Linalool, which is ubiquitously present in flowers and induced in wounded plant leaves, is repellent to blood-seeking *Culex pipiens* female mosquitoes (Choi et al., 2002), but is an attractant to the honey bee, *Apis mellifera* L. (Henning et al., 1992). Methyl salicylate, a volatile emitted both from flowers and from stressed plants, is also a component of the aggregation-attachment pheromone of *Amblyomma* ticks and *Sarcoptes scabiei* mites but is highly toxic to house dust mites, *Dermatophagoides* and *Tyrophagus* (Jaenson et al., 2005). Myrcene is repellent to *R. appendiculatus* and the maize weevil *Sitophilus zeamais* but highly toxic to many insects, including house flies, cockroaches and *Culex* mosquito larvae (Koul et al., 2008). These volatiles have different effects on various arthropods as each species seems to have its own chemical niche of positive and negative stimuli. It may be that some of the compounds, in general, act as arthropod attractant stimuli at certain concentrations but as repellent stimuli or toxins at other concentrations. Different concentrations of a substance can certainly change the direction of the behaviour response. Unfortunately, in many studies, the concentrations of particular substances are not clearly stated. Another important consideration is that host-seeking ticks have evolved to respond to particular host odours and other host cues. Most plant volatiles are generally emitted from the plants and not from tick hosts and as such may mask the host odours thereby disrupting the host-seeking behaviour or disorientating the host-seeking ticks rather than acting as true repellents. On the other hand when herbivores graze on plants the release of volatile compounds by the plant indicates the presence of a herbivore.

Results of testing the repellent activity of compounds against ticks vary with respect to the length of protection depending on the mode of testing, the formulation of the product, the concentration of the active ingredient, the developmental stage of the tick species, the fitness of the developmental stage and the tick species tested. Many other aspects of tick repellents are important, e.g. distance of repellency and the duration of the effect, activity against different arthropods and absence of adaptation or resistance are also important. It should preferably also have a pleasant odour, colour, a good consistency and should not affect clothes or other materials. Geraniol, a monoterpene alcohol found in many plants, is an active ingredient in many commercially available insect repellents and has repellent activity against ticks (Chen & Viljoen, 2010). Geraniol appears on the Environmental Protection Agency's FIFRA 25(b) list of compounds that are exempt from federal regulation because they are considered demonstrably safe. Some other compounds such as coumarin, thujyl alcohol and eugenol, might be interesting as lead molecules for the development of effective repellents.

The penetration of a parasiticide varies according to the thickness of the layer of lipids on the cuticle and also to the solubilizing ability or penetration of the active compound.This can vary according to the species and developmental phase of the parasite (Tak, 2015). Therefore, the susceptibility differences between the stages found in many of the studies can be related to the composition and/or thickness of the cuticle. The waxy layer only occurs after nymph ecdysis and is most pronounced in adult ticks. Toxic chemicals may be sequestered within the wax, hampering their toxicity (Sonenshine, 1993). A toxic physical effect through cuticle solubilization by the essential oil cannot be excluded, although terpenes and phenylpropanoids are known to act on the octopaminergic receptor which acts a neurotransmitter, neurohormone and neuromodulator in invertebrates (Regnault-Roger, 2013).

Another approach that could be relevant in the sustainable management of ticks is through the disruption of their life cycle by targeting engorged immature stages. This may result in the reduction of tick infestations to low and controllable levels, hence reducing the tick population during favourable climatic conditions. Though some plant species such as *Melaleuca alternifolia, Piper tubeculatum, Carica papaya, Guiera seneganlensis, Melia azedarach* and *Tetradenia riparia* did not show high acaricidal mortality, they however caused 100% inhibition of oviposition and hatchability, thereby disrupting life stages of the ticks. The bioactive compound azadirachtin present in *Azadirachta indica* and *Melia azedarach* fruit extracts affects tick embryo development and moulting stages. Interestingly, sheep (*Ovis aries* L.) which consumed a feed containing *A. indica* fruit and kernel extracts exhibited no noticeable signs of toxicity (Pohlit et al., 2011). This diet, however, negatively affected the ability of the American dog tick *Dermacentor variabilis* Say to feed on the sheep blood which exhibited plasma levels of azadirachtin of 4.35–4.81 µg/ml over 14 days. This implies that azadirachtin in blood plasma impaired blood feeding by ticks, so *A. indica* extracts as food additives to animals that are resistant may have applications in tick control for use in public health and veterinary applications.

With the identification of plant species having such great potential use in veterinary parasitology, further studies are necessary to isolate active compounds, elucidate their mode of action, side effects and formulation development to improve their efficacy and stability (Maia & Moore, 2011). Factors such as seasonality, circadian rhythm, plant age and development stage, temperature, water availability, ultraviolet radiation and soil nutrients, can affect the concentration of secondary metabolites especially volatile compounds in plants (Gobbo-Neto & Lopes, 2007). Formulations to protect the active compounds from environmental degradation (to maintain stability) and enable fast penetration into ticks are

needed. Formulation plays a crucial role in extending the duration of efficacy of a repellent. For example, a polymer formulation of DEET and cream formulations of Picaridin and SS220 provided almost complete repellency to nymphal *A. americanum* for 12 hours (Carroll et al., 2008). The plant-derived repellent, 2-undecanone provided 74% repellency against *Dermacentor variabilis* 2 hours after application when unformulated compared to 98% repellency from 3 to 3.5 hours after application when formulated in the product BioUD (Bissinger et al., 2011; Kimps et al., 2011).

These studies should be conducted with experts in chemistry, pharmacology and entomology and also with veterinary drug companies. This is necessary in order to determine the most appropriate adjuvant and to develop formulation models that can be adapted according to the nature of the plant extract or bioactive compound isolated. Investigations of formulations based on bioactive plant extracts should also be done as formulations composed of one natural bioactive substance, for example, allow greater control of the efficacy, quality of raw materials and solutions in developing commercial formulations. Simpler formulations based on plant extracts or essential oils, for instance, can be more widely adopted by producers, but tend to have a short residual period due to the instability of the bioactive compounds caused by climatic factors such as ultraviolet light and moisture. In the simpler formulations, the efficacy results may also vary widely, according to plant age and season of harvesting the plant material. This could be circumvented by a study of the main chemical constituents in each new harvest. Therefore, dose adjustments could be recommended to farmers in accordance with this variation. One of the major problems in developing natural formulations is maintaining efficacy. Since active compounds present in any plant species vary with different stages of growth of the plant, season, soil and environmental conditions, identification of quality control marker compound(s) is crucial to maintain the quality of the finished product.

Difficulty in transposing the efficacy obtained from the laboratory to the field is one of the main obstacles of phytotherapy research in animal health. Moreover, toxicity studies to identify risks to animal and human health cannot be neglected. Also, *in vivo* efficacy studies (field trials) using formulated products are clearly essential and the economic feasibility of the products has to be demonstrated. The recommendation for *in vivo* use of the plant extracts depends on careful toxicological studies and pharmacokinetic investigations to ensure that standardized extracts are used. Achieving significant efficacy and adequate residual periods in field conditions are the new challenges of this research area, since the acaricidal activity of various plant species has been well documented *in vitro*.

It is very important to study the most promising species and their main bioactive compounds and to validate the *in vivo* efficacy without toxic effects on the host. Photo-stability and application method are necessary to support the potential use. Attempts to evaluate penetration and cutaneous absorption of the oil components should be done, considering that the bulk of cattle breeding is done to provide meat and dairy products for human consumption.

4.5 Recommendations

Much information has been generated in the past few years on active plant extracts, fractions, essential oils and their isolated components which are responsible for the lethal effects against ticks. Phytochemicals such as azadirachtin, 2-undecanone and limonene already have important commercial applications in a variety of commercial products which are useful for tick control.

For future purposes, some areas of research and further investigation are recommended:

1) The probable differences in enantiomeric purity of essential oil components which are purchased and used in bioassays to confirm the active tick repellent and acaricidal activities in many of the references cited.

2) Deciphering the synergistic, suppressive and other interactions of the components of essential oil (and extracts). In many of the scientific papers reviewed herein, the isolated active components are less active than the plant extracts and essential oils from which they were isolated. It remains unclear whether the compound *per se* is the most important or whether synergism or perhaps a “cocktail of components” is in fact the active agent responsible for the effect of the plant species.

3) Determining the mechanism of action of the bioactives.

4) The standardization of components, extraction techniques, experimental design, mammalian toxicology profiling and excipient development, as well as further investigation into the residual activities and length of shelf-life of these plants are required.

4.6 Conclusions

The use of ethnoveterinary plant species in the control of veterinary ectoparasites is an exciting area which holds much potential for the future. The potential use of this knowledge by pastoral farmers using low level technology also requires serious attention, especially in developing countries. The overview presented in this work should attract necessary funding

for future research in order to present viable alternatives in overcoming the problem of acaricidal drug resistance.

CHAPTER 5

In vitro bioassays used in evaluating plant extracts for tick repellent and acaricidal properties: A critical review

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Preface

This chapter addresses the fourth objective of this study which was to provide an extensive literature survey on *in vitro* methods used to evaluate plants with tick repellent and acaricidal properties. The text in this chapter has been submitted to the journal Veterinary Parasitology.

Abstract

Ticks are haematophagous arthropods which rank closely with mosquitoes in their capacity to transmit disease pathogens of importance to animals and humans. Current control of ticks is based on the routine use of synthetic chemicals administered to animals or their environment. However, years of use and overuse of these chemicals have resulted in the development of resistance in these parasites and negative environmental impacts, hence the need for cheaper, safer and more environmentally friendly alternatives. There has been a large interest in using plants. Peer-reviewed articles on plants evaluated for their tick repellent and/or acaricidal activities against immature and adult stages of ticks were collected from nine scientific databases with the aim of reviewing the bioassays employed. Search words included “acaricidal”, “tick repellent”, “antitick assays” and “phytomedicine”. Many methods were used to determine the repellent and acaricidal activities of plants. These include among a few others petri dish, tick climbing, olfactometer, larval packet and immersion bioassays. Tick climbing repellency and adult immersion bioassays were most commonly used. Ethanol was the most widely used plant extractant and *Rhipicephalus* (*Boophilus*) *microplus* was the most commonly studied tick across all the reviewed papers. It is unclear whether the outcome of these experiments on a one-host tick can be applied to other species of ticks that infest animals and humans. Also, most of the assays on repellency did not discriminate between olfaction and tactile chemoreception-based repellency and though some of the observed methods were similar, results differ significantly. These aspects will need further evaluation. Standardized laboratory methods are required to enable valid comparisons between results from different laboratories.

5.1 Introduction

There are at least 898 recognized species of ticks, distributed among three families: Ixodidae (C.L. Koch, 1844) (hard ticks - 703 species), Argasidae (C.L. Koch, 1844) (soft ticks - 194 species) and Nuttalliellidae (Schulze, 1935) (intermediate - 1 species) (Latif et al., 2012). The Ixodidae is the dominant tick family, regarding the number of species and their veterinary and medical importance (Tsatsaris et al., 2016). In Africa, of the ten genera of ticks that commonly infest domestic animals, seven are ixodids while three are argasids. Mammals rather than birds or reptiles usually serve as hosts for these tick species. At least one species in each tick genus uses mammals as hosts. Mammals serve as the chief host for members of the Rhipicephalinae and *Hyalomma*, whereas a smaller percentage of *Ixodes* and *Haemaphysalis* species parasitize mammals. Many *Amblyomma* species parasitize mammals, birds and reptiles (Turner et al., 2017).

Safe and efficient control of ticks has become pivotal not only for agricultural productivity and human health but also for companion animals in the veterinary sector. The use of acaricides strives to mitigate or prevent tick infestations and consequences associated with them (Weber & Selzer, 2016). Years of use, misuse and overuse of available pesticidal compounds have resulted in development of resistance in ticks as well as negative environmental impacts hence the need for new, preferably cheaper, safer and more environmentally friendly alternatives. In recent years, many researchers have studied the acaricidal properties of many plants species that have been used traditionally to combat tick infestation using different methods (Adenubi et al., 2016). Natural products or extracts may be ideal tick control agents since they are probably environmentally biodegradable. Active compounds can also be modified chemically to develop more potent or safer compounds. More active constituents may be discovered in related plant species, based on the chemotaxonomic relationship of plants (Cole, 1994).

One major shortfall in research for new plant-based tick repellents and acaricides is the lack of a standardized testing method. Early discovery of plant-based products sought to rapidly identify broad-spectrum and non-irritating products with little or no thought given to developing a standardized testing method (Dautel, 2004). Today, a wide range of methods are employed when testing for tick repellents and acaricides. Studies differ in the timeframe in which repellence or toxicity is evaluated, the species and life stages of ticks used, the formulation and amount of active ingredients from crude plant extracts, fractions or essential oils, the use of animal host cues or not, use of different solvents and variability of tick behaviours in the assays (Marchiondo et al., 2013, Pages et al., 2014). These variations in testing methodologies and assay conditions make comparison among studies very

problematic and selecting the best plant species difficult. Since many tick repellent and acaricidal bioassays are frequently relatively expensive, laborious and time-consuming, it is essential to be well-informed beforehand on how to set up the bioassays, how to perform them, how to interpret the data sets that are generated from them and to realise the limitations of a chosen method. Recommendations on the application of the bioassays are also made.

5.1.1 General guidelines for carrying out *in vitro* tick repellent and acaricidal bioassays

The development of animal health products against ticks requires hundreds of vertebrate hosts such as cattle, dogs and rabbits for *in vivo* trials. This would require tens of thousands of animals per year for acaricide research (Kuhnert et al., 1995). Apart from the ethical aspects of using experimental animals, the time and costs of maintaining suitable hosts for ticks are high, hence the urgent need for validated *in vitro* bioassays (Pages et al., 2014).

When performing a bioassay, standardized procedures should be followed throughout. In plant collection, care should be taken that the plant parts are dry because fungal infections could change the chemical composition of extracts drastically. Plant parts should preferably be dried in the shade under good aeration to limit possible photo-oxidative changes (Eloff & McGaw, 2006). Herbarium voucher specimens, preferably containing fertile (flowers, seed or fruit) to facilitate identification should be prepared. Correctly prepared voucher specimens deposited in a recognised herbarium is important to verify the plant identity. The plant material should be extracted, dried and dissolved in a suitable solvent. One problem with drying plant extracts is that it is frequently difficult to dissolve the extract even if the same original extractant is used. The assay chosen should be compatible with the type of extract or compound to be tested and the plant extract should be stored properly to ensure stability.

The aims of *in vitro* assays are to predict the *in vivo* efficacy in whole animal studies. Therefore, the behavioural biology and physiology of ticks should be properly understood and taken into account as the choice of tick used for a bioassay can greatly influence the outcome. The ticks used should preferably be from the same batch throughout the bioassay to reduce the degree of variability that may lead to different responses not attributable to the test samples. Prior to the start of any bioassay, ticks should be allowed 15 to 30 minutes acclimatisation period.

Both a positive control, such as a commercially used acaricide and a negative control, usually the extractant or solvent used should be incorporated into the bioassay design to ensure that the bioassay works and that the solvent has no or little influence. According to

the guidelines recommended by the Food and Agricultural Organisation, if percentage mortality for the negative control is very low (<5%), then the percentage mortality value can be used without a change. If the negative control leads to 5-10% mortality, then the percentage mortality will have to be corrected by applying Abbott's formula (Abbott 1987):

$$Corrected\ mortality(\%) = \frac{\%test\ mortality - \%control\ mortality}{100 - \%control\ mortality} \times 100$$

If percentage mortality is greater than 10% in the negative control, the bioassay will have to be repeated (Osman et al., 2014).

5.1.2 Behavioural biology of ticks

Ticks are haematophagous parasites with a life cycle consisting of four stages: eggs, larvae, nymphs and adults. They could be one-host ticks (all life stages feed on the same host) such as the *Boophilus* sub-genus of the *Rhipicephalus* genus. In the two-host life cycle, the larvae and nymphs feed on the same host while the adults feed on another host (*Hyalomma detritum* and *R. evertsi evertsi*). The vast majority of tick species have the three-host life-cycle where each life stage seeks for a suitable host, feeds, then drops and moults off the host (Walker, 2003). Ticks have a remarkable resilience off the host and can survive for months without taking a blood meal (Needham & Teel, 1991).

Unlike other blood-feeding parasites, ticks lack antennae and locate their host their host using a simple system of about twenty sensilla (sensory receptors typically hair-shaped) found on the tarsi of the first pair of legs. These sensilla are located within a structure called Haller's organ that comprises an anterior pit that primarily detects humidity and a capsule that contains sensilla used in olfaction (Mcmahon et al., 2003). There are about two hundred olfactory receptor neurones in the sensilla which detect the sex pheromones, aggregation-attachment pheromones (indicating the presence of other ticks) and the semiochemicals emanating from different vertebrates.

Ixodid ticks locate their host either by ambushing or hunting. Ambushers following the passive strategy, living in grass, herb or brush covered habitats climb the vegetation and cling to the tips of stems or branches where they wait for direct contact with hosts that brush against these vegetative supports. The ticks rest in the questing pose, sometimes with the anterior end of the body pointed down towards the ground (Bowman & Nuttall, 2008). This behaviour is exhibited by ticks (larvae, nymphs and adults) in the genera *Rhipicephalus*, *Haemaphysalis* and *Ixodes.* Adult ticks of the genera *Amblyomma* and *Hyalomma* are however hunter ticks following the active strategy. Such ticks stay underground and respond to host stimuli when suitable hosts appear nearby, emerging from their refuges and rapidly

crawling or running towards the source of the stimuli. They may also go across distances of many metres to attack and feed on the hosts for which they are adapted (Sonenshine et al., 2002).

The height at which ticks quest is strongly correlated with the specific life stage and size of the most common hosts of each species or life stage (Goddard, 1992). Immature ticks tend to stay near the base of vegetation or leaf layer, where small mammals and birds are active, while adults generally quest near the tips of vegetation where they attack larger animals. Host-finding strategies may also differ in different life stages of the same tick species. Larvae of *A. variegatum* and *A. hebraeum* find hosts by questing while nymphs and adults are hunter ticks. The lone star tick, *A. americanum,* on the other hand, exhibits both the ambush and hunter strategies.

Ticks are known to be attracted to host stimuli such as heat and small volatile molecules including carbon dioxide, ammonia, hydrogen sulphide, acetone, nitric oxide, fatty acids and phenols, which are components of vertebrate hosts' skin or breath (Dautel et al. 2013). Other attractant stimuli include visual cues and vibrations. If the ticks have not fed for a long time, appetence, influenced by the tick's physiological condition, initiates the series of behavioural responses that leads to host contact and successful parasitism (Waladde & Rice, 1982). When on the host, a tick searches for a suitable predilection site; inserts its mouthparts, establishes its feeding site and starts feeding (Walker, 2003).

5.1.3 Tick repellents

Tick repellents are chemical substances that cause a tick to move away from its source while deterrents are chemical substances that inhibit feeding in situations where it would normally take place (Dethier et al., 1960).Tick expellency on the other hand is caused by a stimulus that makes ticks fall off the host animal by disrupting the mechanisms of attachment, either by causing detachment of already attached ticks or by preventing attachment of new infesting ticks (Halos et al., 2012). The concept of expellency includes both the rate at which ectoparasites fall off the host and the disruption of attachment and it is used to assess the impact on tick control (Halos et al., 2012). An ideal repellent should provide protection against a broad spectrum of blood-feeding arthropods for at least eight hours, be non-toxic, non-irritating, odourless and non-greasy (Fradin & Day, 2002). It should influence one, several or all of the behavioural biological aspects of ticks outlined above. Such a repellent is yet to be developed.

Limiting exposure to tick-infested areas and use of tick repellents is considered to be most effective in preventing ticks and tick-borne diseases in livestock, companion animals and

humans (Bissinger & Roe, 2013, Lupi et al., 2013). The earliest commercial arthropod repellents were plant-based (Gerberg et al., 2007). Oil of citronella was first used as an insect repellent in 1882 (Bissinger & Roe, 2010) and was once the standard against which other repellents were tested (Dethier, 1956). After World War I, three synthetic chemical repellents became available; dimethylphthalate, indalone and Rutger's 612 (Strickman, 2006). Dimethylphthalate was used from the 1950s to the 1980s against a broad spectrum of arthropods until it was replaced by other repellents. Indalone was considered more effective than other synthetic repellents, including N, N-diethyl-meta-toluamide (DEET) but it had an unpleasant smell and caused liver and kidney damage in rodents after prolonged exposure while Rutger's 612 was withdrawn from the market in Canada and the United States of America in 1991 after toxicity was noted in laboratory animals (Moore et al., 2007).

At present, commonly used synthetic chemical repellents include DEET, 1-methyl-propyl-2-(hydroxyethyl)-1-piperidinecarboxylate (picaridin), ethyl butylactyloaminopropionate (IR3535), 1S,2S-2-methylpiperidinyl-3-cyclohexene-1-carboxamide (SS220), racemic 2-methylpiperidinyl-3-cyclohexene-1-carboxamide (AI3-37220) and synthethic pyrethroid 3-phenoxybenzylcis-trans-3(2,2 dichlorovinyl)-2,2-dimethylcyclopropancarboxylate (permethrin) - an acaricide with repellent properties. These synthetic chemical repellents have not produced satisfactory long-term result which is not surprising as many of them were primarily developed for protection against mosquito bites rather than tick bites (Schreck et al., 1995).

Plant-based repellents such as pyrethrum derived from *Crysanthemum* species, p-menthane-3, 8-diol (PMD) derived from *Corymbia citriodora* (Hook.) and BioUD, with the active ingredient 7.75% 2-undecanone, originally derived from *Lycopersicon hirsutum* Dunal (wild tomato) are now commercially available (Bissinger et al., 2009, Maia & Moore, 2011).

5.1.4 Acaricides

An acaricide refers to a pesticide that kills mites and ticks. It can be subdivided into a miticide which kills mites and an ixodicide that kills ticks (Mullen & Durden, 2009). These acaricides are available in different formulations (depending on whether they are to be used on humans, animals or plants) including tablets, sprays, soaps, shampoos, powders, impregnated collars, dip solutions, pour-on and spot-on applications (Pfister et al., 2016). Based on the nature of the compound used, the following effects may be obtained, alone or in combination: (1) Disruption of contact between the arthropod parasite and the host. (2) Prevention of feeding. (3) Interference with egg fertility and subsequent development of off-host life cycle stages. (4) Death of the arthropod parasite. Commercially available synthetic acaricides include the arsenicals, organochlorines, organophosphates, carbamates,

phenylpyrazoles, formamidines, pyrethroids, macrocyclic lactones and more recently, the spinosyns, insect growth regulators and isoxazolines (McTier et al., 2016).

5.2 Materials and Methods

In this study, we reviewed published articles of previous research done on pesticidal plants having tick repellent and acaricidal properties with particular focus on the *in vitro* tick repellent and acaricidal bioassays employed. The keywords used to collect relevant literature for this review were “tick repellent”, “acaricidal”, “phytomedicine” and “antitick assays”. Several Veterinary databases (All Databases, CAB Abstracts and Global Health, Medline, PubMed, Web of Science, BIOSIS Citation Index, Science Direct, Current Content Connect and Google Scholar) were searched.

5.2.1 Methods for evaluating plant species for tick repellency

Dethier (1948) grouped repellents into contact and spatial (vapour-based) repellents. For contact repellents, targets must come into contact with a treated surface before being repelled, whereas spatial repellents work at a distance from the site of application and targets do not need to come into physical contact with a treated surface. Spatial repellents should obviously be volatile compounds. Some repellents may fall into both categories. Dautel (2004) however, classified the methods for testing for tick repellency into three: (1) *In vitro* bioassays performed in the absence of host stimuli. (2) *In vitro* bioassays performed in the presence of host stimuli. (3) *In vivo* bioassays using living hosts. The last type is not addressed in this review. Generally, test sample is applied onto a horizontal or vertical path for ticks and the number of ticks walking across the treated area is recorded. Repellence is detected by a significantly reduced number of ticks entering the treated zone compared to the negative control (Dremova & Smirnova, 1970).

5.2.1.1 Petri dish bioassay

This is a two-choice bioassay in which a piece of filter paper is divided into two halves. On one half, a volume of test solution is applied uniformly and on the other half, the same volume of solvent is added as the negative control. These halves are attached again and placed in a petri dish, then a number of ticks is released on the centre of the filter paper. After some minutes to hours, the number of ticks on both halves is recorded and the percentage repellency is calculated. The Petri dish bioassay was conducted at a temperature of 25^{o}C, 65% relative humidity and in complete darkness (except during the approximately 5 seconds needed to monitor tick distribution) using *A. americanum*, *Dermacentor variabilis* and *I. scapularis* adult ticks. Tests were conducted in 63.6 cm^2 petri dish lids lined with two 31.8 cm^2 semi-circle pieces of Whatman no. 1 filter paper, each

treated with 250 ml of either BioUD (test sample) or DEET (positive control) and the other half with 250 ml of the negative control. The filter papers were allowed to dry for 3 hours at room temperature under a fume hood. Six ticks were placed along the line formed by the junction of treated and untreated filter papers and the distribution of ticks was recorded every 5 minutes for 30 minutes. The experiment was repeated six times (Bissinger et al., 2009).

Thorsell et al. (2006) and Tunón et al. (2006) applied 0.5 ml test solution along the periphery of a filter paper, placed six nymphs in the centre and their movement with regards to avoiding the treated area was observed for 5 minutes. Birkett et al. (2008) marked a 10 mm circular patch and concentric rings of 16, 26 and 36 mm diameter on the underside of a glass, crystalline dish. The test solutions were applied to the central surface inside the dish and allowed to dry for 5 minutes, after which the dish was placed upside down into a large petri dish over 1000-2000 *R*. (*B.) microplus* larvae. After about 30 minutes, the number of ticks that had climbed to the dish was counted on the central treated circle and in the three concentric rings at hourly intervals. Del Fabbro & Nazzi (2008) drew two concentric circles of 1 cm (line A) and 2 cm (line B) radii on the inner surface of a petri dish. The test solution was applied outside line B on the outer surface of the petri dish and placed on a wet piece of filter paper inside a larger petri dish. A single nymph was placed in the centre and the time taken for the nymph to go from line A over line B was recorded. There have been modifications to this assay by different researchers (Bissinger et al., 2014b; Choudhury et al., 2015; Ferreira et al., 2017).

5.2.1.2 Tick climbing repellency bioassay

Bioassays that use a vertical climb to test for tick repellency take advantage of the questing behaviour of ambushing ticks. These assays also allow ticks to fall from a vertical surface if they are repelled as would occur in the field (Carroll et al., 2004). Ndungu et al. (1995) and Mwangi et al. (1995) used two vertical aluminium rods attached to an aluminium base 7 cm from each other. Each rod was covered with a glass tube around which a 1 x 4 cm filter paper was attached by a staple, forming a ring around the glass tube. The top of each tube was plugged with wet cotton wool causing the ticks that climbed up to remain there possibly because of the high humidity. Repellents placed on the rod prevent ticks from climbing up and a repellent effect is deduced by a significantly reduced number of ticks climbing the treated rods compared to the untreated one.

Modified versions of this assay have been developed by many researchers. Mkolo & Magano (2007) used ten *Hyalomma marginatum rufipes* ticks (3-5 weeks old), placed on a polystyrene platform and their position on a glass rod was noted at 10 minute intervals for 60

minutes and thereafter at 30 minute intervals until the 120th minute. Ticks on the treatment or control filter papers were considered not repelled while ticks found on the neutral filter paper were repelled. Zorloni et al. (2010) placed ten ticks in the centre of a rectangular (20 cm × 5 cm) polystyrene platform, fixed in the middle of a rectangular basin (filled with water almost to the upper surface of the platform to prevent ticks escaping). At opposite sides of the platform, two glass rods were inserted, each fitted with Whatman no 1 filter paper (5 cm × 3 cm) over the glass rods at the top and at the bottom. One ml of the test solution was applied on the filter papers on one rod, one ml of the positive control or negative control was applied to the other rod. The position of ticks was recorded four times at 30 minute interval.

In a related assay, Tabanca et al. (2013) used a 4 × 7 cm rectangle of Whatman no. 4 filter paper marked into two 1 × 4 cm zones at the far ends of the paper strip and a central 4 × 5 cm zone. Using a pipette, 165 µl of test solution was evenly applied to both sides of the central zone of the filter paper and dried for 10-15 minutes. The paper strip was suspended lengthwise by a bulldog clip from a double clip work holder. Moated petri dishes (a smaller petri dish glued to the centre of a larger petri dish and water added between their walls) were placed directly under the suspended filter paper. Ten *A. americanum* nymphs were placed close to the moated petri dishes, climbed up the rim onto the lower untreated zone of suspended filter paper and the locations of the ticks were recorded at 1, 3, 5, 10 and 15 minutes after all ten nymphs had climbed. The ticks were considered repelled if they were in the lower untreated zone at 15 minutes or if they fell from the filter paper without having crossed the upper boundary of the treated zone. Other researchers that have employed the tick climbing repellency assay include Lwande et al. (1999), Birkett et al. (2011), Oh et al. (2012); Carroll et al. (2016); Meng et al. (2016) and Meade et al. (2017).

5.2.1.3 Fingertip/Palm/Leg/Sock bioassay

The fingertip bioassay is a modified tick climbing repellency bioassay (Carroll et al., 2005; Zhang et al., 2009). The index finger of a volunteer (human subject) is treated with a band of test solution proximal to the distal end of the digit leaving the fingertip untreated. This finger is then positioned vertically with the fingertip touching the centre of an area containing ticks. The ticks that crawl across the treated area on the finger are not repelled while those that retreat or fall off the treated surface are repelled. The test solution may also be applied to the bare leg (Schwantes et al., 2008) or socks. Bissinger et al. (2016) employed the sock bioassay in which each volunteer placed a treated sock on one leg and an untreated sock on the other leg. Volunteers then walked at a pace of approximately 30 steps per minute in an area measuring approximately 5700 m^2 for 15 minutes, taking care not to follow the same path as another volunteer. This process was repeated four times each day using two

volunteers per test solution for a total of eight replicates per treatment. To avoid cross-contamination, volunteers placed the treated sock on the same leg on a given test day. Socks were removed carefully, placed in labelled plastic bags, taken to the laboratory and the number of ticks on each sock counted. Similar tests have been conducted to simulate natural habitats in the laboratory (simulated forest floor method) where the working area may contain grass or dry leaf litter around the ankles of a volunteer who stands in a container and the number of ticks that cross the treated area is recorded as not repelled (Carroll et al., 2008). Other researchers that have employed this method are Schreck et al. (1995), Pretorius et al. (2003), Carroll et al. (2007), Krober et al. (2013) and Opiro et al. (2013).

5.2.1.4 Moving object bioassay

In order to increase the motivation of ticks to move while displaying host-seeking behaviour, the moving object bioassay was developed (Dautel et al., 1999). This assay uses heat and movement as attractant stimuli associated with hosts and allows the ticks to display their natural behaviour of clinging to a passing host in the laboratory. A slowly rotating vertical drum is heated to a surface temperature of 35-36°C. On the drum, there is an elevated surface which serves as a tick attachment site. Ticks approach the drum on a horizontally placed glass rod, ending shortly in front of the drum. The distance between drum and tip of the glass rod is adjusted in such a way that the tick can only reach the elevated attachment site. As the drum rotates, this attachment site periodically passes by and the tick is able to attach to this moving object as if it was a passing animal. Records are made of whether or not the tick approaches the drum (spatial repellence), attaches to the drum and once on the drum, remains on the treated surface or drops off (contact repellence). In addition, the duration of each behavioural step can be measured which can reveal more subtle repellent effects.

For the moving object bioassay to be effective, it requires active ticks ready to move and search for a host. The distance between the tip of the rod and the drum attachment site must be well adjusted to the tick species and life stage examined. The distance must be large enough to prevent the ticks from clinging to other drum surfaces other than the elevated attachment site, but close enough to enable sufficient contact to that site and there must also be a temperature gradient. Other researchers that have employed this method are Dautel (2004), Schwantes et al. (2008), Zakouska et al. (2013) and Pechova (2013).

5.2.1.5 Olfactometer bioassay

Hunter ticks can be investigated in an air stream using an olfactometer. Different types of olfactometers have been used to test candidate repellents against ticks. The apparatus consists of a Y-tube to give the ticks a choice between a test solution on one side of the split

sides and the control treatment on the other side. Air is sucked in at the base of the Y-tube at a reduced speed and ticks are introduced at the base tube. The ticks then move towards the split sides against the air current. Yoder et al. (1998) conducted studies using a Y-tube (1 cm in diameter, 5 cm arm lengths and stem each fit to a 9 cm petri dish). Ten ticks were placed in the stem of the Y-tube and the experiment was replicated until 100 ticks had been exposed. Fresh ticks, Y-tubes and chemicals were used for each run and observations were made after 24 hours. Results were expressed as the percentage of ticks repelled from the test solution. Olfactometer tests can range from these rather simple Y-tube assays to highly sophisticated tracking systems using a locomotion compensator (McMahon & Guerin, 2002; Mcmahon et al., 2003; Carr & Roe, 2016).

5.2.1.6 Falcon vial repellency bioassay

Jaenson et al. (2005) and Dietrich et al. (2006) did a repellency bioassay on unfed *I. ricinus* nymphs using a FalconTM vial. The FalconTM vial is a 50 ml centrifugal tube, 116 x 29 mm, made of transparent plastic. Fifty millilitres (50 ml) each of the test solution, positive control and negative control were applied to separate cotton cloths and attached with a rubber band to the open upper end (660 mm^2) of the vial. Its wall was perforated with to prevent saturation of the air with odours of the substances. In each replicate, five previously unused nymphs were first tested with the positive control for 5 minutes and then immediately afterwards with the test solution for 5 minutes. To simulate host stimuli, the observer held his palm (washed in soap and water between each substance tested) to the outside surface of the cloth during the period. The number of nymphs on the interior surface of the cloth at the end of this period was recorded. For a tick to be regarded as attracted to the cloth, it had to detach all its legs from the vial's surface. Ticks that were clinging to the cloth 5 minutes after the start of the test were recorded as attracted, whereas ticks that did not were recorded as repelled. Other researchers that have employed this method to test for tick repellency include Garboui et al. (2007) and Ashitani et al. (2015).

5.2.2 Methods for evaluating plant species for acaricidal toxicity

Many methods have been employed in testing plants for acaricidal properties. The simplest tests involve dropping the test solution on the ticks or dipping the ticks in the test solution. Effects observed could be morphological, neurological, effect on reproductive parameters (counteraction of growth regulatory hormones, inhibition of egg development, disruption of mating and sexual communication), inhibition of chitin formation or outright mortality (Katoch et al.; 2007; Chagas et al., 2014).

5.2.2.1 Larval packet test

The Food and Agricultural Organisation has adopted the larval packet test, developed by Stone & Haydock (1962) as the preferred assay for assessing test candidates and determining the presence of resistance in a tick population. The acaricidal activity of oleoresin against 14-21 day old *R. (B.) microplus* larvae was determined using a modified larval packet test (Fernandes & Freitas, 2007). Four filter paper envelopes (approximately 327 cm^2) with micropores were treated with 2 ml of different concentrations of test solutions uniformly distributed with a pipette on the internal surfaces. The negative controls were impregnated with dimethyl sulphoxide (DMSO) and distilled water. About 30 or more larvae with good mobility were caught with a no. 4 paintbrush moistened in test solutions, then gently transferred to each envelope. The opening of the envelopes (treated and inoculated with larval ticks) was folded (approximately 10 mm), re-sealed with a metallic clip and labelled. The packets were placed in an incubator at 27-28ºC and 85-95% relative humidity for 24 hours, after which they were inspected using a stereoscope to record the number of live larvae, percentage mortality and any toxicological effects observed. Four replicates were conducted at different dates and larvae used for each replicate were from different engorged *R. (B.) microplus* females. Many authors have employed the Larval Packet Test (Borges et al., 2003; Al-Rajhy et al., 2003; Elango & Rahuman, 2011; Fernández-Salas et al., 2011; Monteiro et al., 2012; Chagas, et al., 2012; Chagas, et al., 2014; Shyma et al., 2014; Ramirez, et al., 2016).

5.2.2.2 Larval tarsal test

Twenty microliters (20 µl) of ethanol: olive oil (1:1) was dispensed into all the wells of a flat bottom 96-well plate and the ethanol was allowed to evaporate for at least 6 hours under a fume hood. Five µl (5 µl) of test solutions were dispensed in the wells to obtain concentrations of 100-0.05 mg/m^2 and 5 µl of DMSO was used as the negative control. Plates were placed for 1 hour in a nitrogen concentrator for complete DMSO evaporation. Fifty eggs were thereafter distributed in each well using a seed counter and the plates were placed uncovered in an environmental chamber with approximately 95% relative humidity at 28±1ºC. One to three days after the start of incubation, the plates were covered with a transparent sealing film and static electricity was removed with a discharging system. Plates were thereafter incubated at 70-80% relative humidity at 28±1ºC. Plates were removed from the environmental chamber 2 weeks after egg hatching and the larval percentage mortality was determined by counting dead or live larvae in each well using a dissecting microscope at 12x magnification. Each test was repeated three times using ticks from different passages (Lovis et al., 2011).

5.2.2.3 Larval immersion test

This method was first developed by Shaw (1966). About 300-500 larvae (14 - 21 days old), were distributed evenly using a no. 3 paintbrush on a Whatman no. 1 filter paper (11 cm diameter) placed in a 15 cm petri dish. About 10 ml of the test solution was poured on the petri dish beneath the filter paper, 4 ml was poured over the larvae on the filter paper and another 11 cm filter paper was placed over the larvae. The remaining 3 ml was poured over the second filter paper to saturate the filter paper sandwich containing the tick larvae for 10 minutes. After this, the sandwich was removed from the petri dish and placed on a dry, double thickness of 4 cm filter paper. About 70-100 larvae were then carefully removed and placed in the apex of a 15 cm filter paper, folded into segments. The open end of the filter paper was sealed, placed in a rack then stored at 80% relative humidity and 27°C for 17-18 hours. The assessment of percentage mortality was made by cutting off the sealed end of the filter paper, opening it and counting the dead and live larvae after 24 hours.

The syringe test is a modified larval immersion test (Jonsson & Iqbal, 2012). The nozzle end of a 3 ml plastic syringe was cut open and the plunger was withdrawn partially. Approximately 200 eggs were placed in the syringe and the open end of the syringe was sealed with a double layer of nylon gauze held tight around the syringe barrel by rubber bands. These syringes were then incubated in the dark at 27ºC and 90% relative humidity. Fourteen days later, 2 ml of test solution was drawn into the syringes containing larvae and shaken for 30 seconds, after which the test solution was discarded by pushing the plunger up to the gauze. A facial tissue paper was placed on the gauze to completely remove the test solution and the plunger was pulled back to the 2 ml mark. All the treated syringes containing the larvae were left in a fume hood for 1 hour, cut end upwards and thereafter placed in an incubator in the dark and incubated at 27ºC and 90% relative humidity for 24 hours and another 6 days. Syringes were opened at the end of incubation period and the dead larvae were counted. Several other modifications have been made to the larval immersion test (Sabatini et al., 2001; Miller et al., 2002; Borges et al., 2003; Souza et al., 2006; Politi et al., 2012; Osman et al., 2014; Ullah et al., 2015).

5.2.2.4 Adult immersion test

This assay has been described by several authors, but the protocol of Drummond et al. (1973) is most commonly cited. It is used for evaluating the efficacy of new acaricides, acaricide resistance testing and toxicity studies and is the most commonly employed bioassay for determining acaricidal properties of many plant species (Adenubi et al., 2016). Laboratory-reared adults or engorged adult females collected in the field are most often used to assess mortality, inhibition of oviposition and egg hatchability. The ticks are immersed in

test solution for varying durations (1-30 minutes) and are then incubated in a chamber. Mortality, inhibition of oviposition or egg hatchability is assessed at different times (24-72 hours). The eggs produced by each group are weighed, incubated up to eclosion of all larvae and compared with the control. Some of the authors that have used this method are Apel et al. (2009),Ghosh et al. (2011), Gazim et al. (2011), Domingues et al. (2013), Singh et al. (2014), Godara et al. (2014a) and Yessinou et al. (2016).

5.2.2.5 Vapour phase bioassay

Cetin et al. (2009) carried out the vapour phase bioassay in which individual cotton wick tampons were soaked in different volumes of test solutions. One wick per concentration was suspended from the inner cap of a 3 L glass jar. An untreated wick served as the negative control. Ten ticks were placed into each jar prior to sealing the jar with the cap and attached wick. Cumulative mortality was recorded from continuous exposure in each jar every 15 minutes, till the 90th minute and then hourly for 3 hours and at 24 hours. Ticks were considered dead if their legs did not move when prodded with a fine pin. Each concentration was replicated three times. During testing all ticks were held at 28±2^{o}C and 60±5% relative humidity with a photoperiod of 12:12 hours (light/dark) conditions.

5.2.2.6 Contact bioassay

One microliter of each test solution, positive and negative controls was dropped on the dorsum of each of ten ticks and the ticks were incubated. The percentage mortality and any toxicological effects observed were recorded after 24 hours (Zorloni et al., 2010; Nchu et al., 2012; Muyobela et al., 2016).

5.2.2.7 Tick feeding bioassay

In contrast to other haematophagous parasites such as tsetse flies and mosquitoes, where the blood meal takes only a few seconds to minutes respectively, ixodid tick feeding can take up to a few days, with each tick firmly anchored to the same feeding site for days. Furthermore, attachment by ticks at feeding sites on the host depends on an appropriate array of chemical and physical stimuli (Kuhnert et al., 1995). During the slow-feeding phase (4-7 days), the female tick feeds up to 10 times her unfed weight and synthesizes the cuticle that serves to accommodate the third phase of the blood meal. Final engorgement occurs during the last 24-36 hours of rapid feeding when the tick imbibes 2-8 times as much blood and can multiply its unfed body mass approximately 100-fold with protein and lipid-rich nutrients for the production of thousands of eggs (Sauer et al., 1995).

Ideally, an *in vitro* feeding assay should permit both the testing of products that affect a tick's capacity to attach for a blood meal and hence growth (developmental disruptors), or restrict

feeding (anti-feedant) and transmission of pathogens once the tick has started to take blood. To be reliable, the *in vitro* system must include an appropriate array of attachment stimuli, a non-biodegradable and elastic membrane and an adequate nutrient supply. Because ticks do not all feed at the same rate, there is an additional challenge to provide a system that permits some ticks to feed and detach when replete, while allowing others to continue to feed to repletion without any bleeding from neighbouring detachment sites as bleeding could cause drowning of the remaining ticks and permit infection of blood.

A feeding unit which consists of a silicone membrane reinforced with cellulose rayon stretched across one end of an acrylic glass tube (44 mm high and 26 mm in diameter) has been described (Kröber & Guerin, 2007). The combination of cellulose rayon fibre and silicone results in a membrane with a low Shore hardness that mimics the elasticity of skin to ensure closure of tick penetration sites on the membrane to prevent bleeding. To improve attachment of ticks to the membrane, a piece of glass fibre mosquito netting (1.4 mm mesh, 24 mm diameter) was glued to the membrane in the feeding unit and left to dry. A plastic cross (2 mm thick tile spacer) was placed on the membrane to create additional borders where ticks prefer to attach. A cow hair extract (0.5 mg lipid extracted from freshly shaven cow hair and dissolved in 75 µl dichloromethane) was applied to the membrane and the solvent allowed to evaporate for 15 to 30 minutes on a hot plate at 40°C. The feeding units were then placed in six-well cell culture plates (34.8 mm diameter) with 3.1 ml test blood and warmed to 37°C using a thermostatic water bath with a tilted acrylic glass cover to keep the air above the feeding units near 100% relative humidity. The six-well plates with the feeding units were positioned on a metal screen submersed 15 mm below the water surface in the bath and the bath kept in a windowless chamber with 16:8 hours light: dark cycle. Ten female and five male *I. ricinus* were placed into each feeding unit.

Artificial feeding of *I. ricinus* on gerbil and rabbit skins obtained from animals previously used for rearing tick colonies in the laboratory have also been described (Bonnet et al., 2007). Other authors that have used modifications of the artificial tick feeding method are Kemp et al. (2012), Tajeri et al. (2016), Trentelman et al. (2017) and Krull et al. (2017).

5.3 Results

5.3.1 Advantages, disadvantages and recommendations for different bioassays

The studies tested a variety of plants, plant parts and extracts but methods and analytic parameters in the studies differed across repellency and acaricidal bioassays. Results obtained from bioassays that differ in seemingly minor ways were surprisingly different, diminishing the value of comparing studies that used similar but not identical methods. The

advantages, disadvantages and recommendations for the different bioassays are outlined (Table 5.1).

Table 5.1: *In vitro* tick repellent and acaricidal bioassays, their advantages, disadvantages and recommendations

Biossay type	Advantages	Disadvantages	Recommendations
Petri dish bioassay	This bioassay is simple and fast as results can be obtained within a few hours. Small quantity of test sample is required.	It is difficult to filter out test samples that are weak repellents, as if the concentration is high enough, even very weak repellents might seem to work. More random movements can be observed as the motivation of a tick to walk into a particular zone will very probably be small. It may not be ascertained if the ticks are in a host seeking mode.	It is important to use different concentrations to determine EC_{50} and compare the results to a known effective positive control. Incorporation of some means that increases the movement of the ticks (host stimuli) in a particular direction such as breath and heat from the observer's palm. Use of automated observation such as video recording.
Tick climbing repellency bioassay	The host seeking behaviour of ticks can be evaluated more empirically.	The ticks may not climb up a particular rod whether the test sample is repellent or not. Extremely time consuming as ticks need to be monitored continuously. Absence of host cues may reduce the motivation of ticks to climb up. Environmental parameters such as temperature and relative humidity will affect the tick movement.	Use of automated observation such as video recording. The use of animal-associated kairomones may aid movements.
Moving object bioassay	Provides a fast test procedure, more precise and predictable. Increases test flexibility, as test sample can be applied to the drum attachment site in pieces of cloth or animal skin, thereby being a good tool to investigate the clinging capabilities or preferences of ticks for different materials. Technically mimics quite closely the situation of a tick being on vegetation and having the sudden opportunity to cling to a host passing by than the tick climbing repellency bioassay. The effect of test sample on several behavioural steps of host-seeking ticks can be investigated. Can be applicable to Ixodid ticks such as *Ixodes, Haemaphysalis*, adult *Dermacentor* and larval *Boophilus*. Allows differentiation of spatial repellents	Aside from warmth and movement as host-associated stimuli, this assay provides no additional attractants from a live host, except perhaps some volatiles from the observer present in the room air. Several tests cannot be run in parallel on the same piece of equipment, so mass screening is not possible. Developed for tick species displaying the ambush strategy, hence it is unsuitable for soft ticks and hunter ticks. The cost of equipment makes the bioassay relatively more expensive.	Innovative methods that may aid the use of the test in parallel to test multiple substances and tick species will need to be developed.

	from contact repellents, because several behavioural steps, including the approach of the tick to the heat source and its walking behaviour on the treated surface can be investigated.		
Olfactometer bioassay	Can identify compounds that act as spatial repellents	Time consuming as only one test can be run per apparatus. The cost of equipment makes the bioassay relatively more expensive. Not suitable for contact repellents. Not very predictive as a tick can make its choice between the treated and the untreated arm only after a walk through the stem where the test sample is usually also present in the gas phase. Difficult to standardize the concentration being volatized.	Method to determine the rate of diffusion of the spatial repellent into the untreated arm of the Y-tube will need to be developed. This may answer the question on why ticks move in a direction within the tube.
Fingertip/Palm/Leg/Sock bioassay	Simple and fast	There is need for ethics approval. There may be differences in potential attractants between different volunteers. Risk of toxicity and infection with tick-borne pathogens to the volunteers. Lack of consistency may introduce error or bias. Highly dependent on the behaviour of the tick chosen for the assay.	The development of dummies to simulate human fingers and legs should be considered. The test should use one slow moving and one rapid moving tick species, to get a better indication of efficacy.
Falcon vial repellency bioassay	Simple and fast. Able to detect spatial repellents.	There is need for ethics approval. Can be laborious holding the palms up.	A method to determine the rate of diffusion of the spatial repellent into the air will need to be developed.
Vapour phase bioassay	Simple and fast.	Only suitable for essential oils or other volatile compounds/extracts. Not suitable for contact repellents.	The assay needs to be standardized in terms of suitable extractant and concentration to ensure reproducibility.
Contact bioassay	Simple and fast. Rules out the possibility of mortality due to blocking of the tick spiracles. Suitable for mass screening of test candidates.	Does not adequately mimic field conditions as animals are drenched in acaricidal preparations.	The assay needs to be standardized in terms of suitable extractant and concentration to ensure reproducibility.
Tick feeding bioassay	Simple and mimic natural skin infestation	Requires an attractive and standardized artificial membrane.	There is a need to standardize methods of feeding between the fast-

	compared to the other methods. Useful for testing novel acaricidal compounds as a high-throughput screening tool.	Requires a reliable means to detect penetration of the membrane by the mouthparts of the tick. Fresh blood must continually be supplied and maintained at appropriate conditions for several days. This requires ethics approval. The assay is susceptible to fungal and bacterial growth which requires the use of microbial inhibitors, which could potentially interfere with tick physiology. This type of assay assumes that the medium on which the test sample and controls are presented are otherwise nutritionally equal. Ticks may not always attach, as they may sense that the membrane is artificial.	feeding and slow-feeding ticks. It will be necessary to develop appropriate membranes and attachment stimuli in order to adapt existing feeding assays for the *in vitro* feeding of the major livestock-infesting tick species. The replacement of blood by an artificial nutrient medium is expedient as this would not only facilitate further applications of *in vitro* feeding assays but could also serve to standardize another parameter of the assay.
Larval packet test	Can be used where insufficient adult ticks are available. More accurate and detailed results are obtained because many more tests can be carried out with the thousands of larvae produced by a few engorged female ticks.	Takes up to 6 weeks to get results. Run-off and evaporation of test sample may occur.	Due to potential tick species differences, it is preferable to use larval species for which the extract is being developed against.
Larval tarsal test	It is a sensitive, highly time-effective test. It allows the investigation of a large number of compounds and doses in a short period of time. It is a suitable test to evaluate the resistance profile of field strains of *R. (B.) microplus.* Suitable to test other ixodid species.	May be expensive to run.	Simplifying the method by avoiding the handling of larvae and using multi-well plates.
Immersion test (larval, nymphal, adult)	Results in 1 week. Different stages of ticks are immersed in the test solution and this usually increases efficacy. Due to direct immersion of ticks in test solutions, the direct drug effect can be observed, even at very low doses	Death need to be differentiated from drug effect, as sticky substances could block the spiracles resulting in death from a non-mechanistic effect. It is laborious. Its results give a poor fit to the log probit model which is a commonly used method for dose determination studies. Poor reproducibility. Standard protocols have not previously been developed making it difficult to compare results obtained from different laboratories.	The assay needs to be standardized in terms of suitable extractant, concentration and period of incubation to ensure reproducibility.

5.4 Discussion

5.4.1 Factors affecting tick repellent and acaricidal bioassays

Behaviour can vary widely among different tick species and this should be taken into consideration when conducting repellency studies. For instance, *I. scapularis* are slow moving and more sedentary than other species of Ixodid ticks (Falco & Fish, 1991). Poor responsiveness of *I. scapularis* in fingertip bioassays has been observed (Schreck et al., 1995; Zhang et al., 2009). In contrast, *A. americanum* is aggressive in host seeking, is fast moving and will readily climb or move onto different surfaces. Ticks should therefore be allowed to acclimatize to the conditions where the bioassay is to be conducted and the difference in behaviour should be considered before the start of the experiment. The use of host cues is encouraged for repellency studies as bioassays without any host stimuli involved have the disadvantage that the behaviour modifying activity of test solution in the presence of host attractants remains unknown.

Not all species and life stages of ticks are amenable to every repellency screening method. For instance, the small size of *I. scapularis* nymphs and adults are ideal for the tick climbing repellency bioassay using filter papers and glass rods. Larger ticks such as *D. variabilis* fall easily from glass rods regardless of whether there is a repellent or not. Also, nymphal ticks are not ideal for the olfactometer bioassay because their small size allows them to escape through the mesh screening that covers the air inlet. In addition, the age of ticks might cause a differential response to repellents (Bissinger et al., 2011). Carroll et al. (2004) recommended that ticks of different ages should not be combined for repellency bioassays as differences in the sensitivity of *I. scapularis* to DEET were found. Nymphs that were 10 - 11 weeks old were significantly more sensitive than were nymphs that were 4 - 6 weeks old. From our previous review, about 56% of the studies reviewed used nymphs to test for repellency, 37% used adults while 7% used larvae (Adenubi et al. 2016). It will be better to use nymphs for repellency studies as this is the most important developmental stage from an epidemiological point of view, being both abundant in the environment and actively involved in animal and human bites.

Results are conflicting about whether or not laboratory repellency studies adequately approximate the results of field trials. Matthewson et al. (1981) found discrepancies between the results of laboratory trials examining repellents against *R. evertsi evertsi* in the absence of a host and those in the presence of a host. In contrast, similar results were observed in field and laboratory trials using the same repellents (Bissinger et al., 2009).

For acaricidal bioassays also, there are concerns as to which is more suitable; field or laboratory-reared ticks. The field ticks best represent the target population but there is the danger of laboratory staff being exposed to tick-borne diseases. Laboratory-reared ticks on the other hand, are pathogen-free but have not been exposed to any acaricide and are not suitable for resistance studies. The latter is important as resistance mechanism may be shared between different classes, as seen with the pyrethroids and fipronil (Eiden et al., 2015). When the response of laboratory-reared versus field-collected nymphal *A. americanum* to different doses of two repellents was examined, results were similar indicating that the use of laboratory-reared ticks for repellency trials is acceptable, at least for this species (Carroll et al., 2008).. Establishment of disease-free, yet acaricidial resistant ticks in the laboratory may be worthwhile. When laboratory-reared ticks are used for these bioassays, it must be ensured that such ticks are in a proper physiological state and show all features of appetence behaviour. Results from *in vitro* acaricidal bioassays may not be useful as a basis for determining the likely field use concentration for a test sample, but may be used as preliminary screening guide prior to dose determination or confirmation studies (Holdsworth et al., 2006). In addition to allowing for more rapid screening, such assays are more ethically acceptable, as only molecules with a higher chance for success would be tested in laboratory animal models and field based studies.

5.4.2 Future prospects and potential of tick repellent and acaricidal bioassays

In vitro behavioural bioassays remain a useful tool in the discovery, development and registration of tick repellents and acaricides. Tick repellent bioassays are generally uncomplicated, but their results can be affected by basic variables (e.g. dimensions of testing materials, substrate, timing, temperature) of the assay. Carroll et al. (2014) investigated the effects of substrate, solvent and drying time on tick responses and recommended that nylon organza or a similar thin cloth may be preferable to filter papers for minimizing solvent-related differences. Also, when a paper substrate is used, acetone may be the most suitable solvent if the solubility of the test compound and other factors allow.

Relatively little research has been conducted to determine how ticks detect repellents. Most repellency assays for ticks do not discriminate between repellency due to olfaction from that due to tactile chemoreception (Carroll et al., 2005). Olfactory sensilla are able to detect vaporized molecules and evidence suggests that olfaction is involved at least in part in repellency (Sonenshine, 1993). Very little is known about chemoreception in ticks at the molecular level. Chemoreception in mammals and insects relies on several families of transmembrane receptors that detect volatile and non-volatile compounds. However, there are differences in the structures of these receptors (Krogh et al., 2001; Benton et al., 2006;

Lundin et al., 2007; Sato et al., 2008). Recent results also show that the functioning of the signalling mechanisms also differs amongst vertebrates and insects (Wicher, 2015).

To date, no published report of a genuine mass screening repellency and acaricidal system involving thousands of candidate substances was founda. When great numbers of substances are to be tested, the assay must be simple, fast and cheap. Such a system will require automation which is particularly difficult with respect to tick handling and is a significant limitation of existing systems. To surmount this barrier, multidisciplinary efforts are needed.

5.5 Conclusions

While it is necessary to develop standardized laboratory tests, the present body of knowledge of available methods is useful in the rapid development of newer generations of plant-based tick repellents and acaricides. Relevant empirical assays that have been developed for *in vivo* drug development and other *in vitro* analyses should be applied to the presently generated datasets. The overview presented in this work could be useful to scientists who are new in the field and in motivating for research funding.

CHAPTER 6

In vitro acaricidal efficacy against *Rhipicephalus turanicus* and cytotoxicity of seventeen South African ethnoveterinary plant species

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[#]Authors contributed equally to this work: GF was responsible for production of the extracts, while OTA ran the *in vitro* tick assay.

Preface

This aspect of the project comprised a collaboration between the Council for Scientific and Industrial Research (CSIR) and the Phytomedicine Programme, Faculty of Veterinary Sciences, University of Pretoria and was funded by the Technology Innovation Agency. The selection of the seventeen plants for this study was based on reports of their use by livestock keepers and traditional healers in South Africa for tick control. Ethnobotanical surveys of these plants were carried out by colleagues at the CSIR over many years (unpublished data) as their part of this study and does not form part of this PhD research. The plants are *Aloe rupestris* Baker, *Antizoma angustifolia* (Burch.) Miers ex Harv., *Calpurnia aurea* subsp. *aurea* (Aiton) Benth., *Cissus quadrangularis* L., *Clematis brachiata* Thunb., *Cleome gynandra* L., *Ficus sycomorus* L., *Gnidia deserticola* Gilg., *Hypoxis rigidula* Baker var. *rigidula*, *Maerua angolensis* DC., *Monsonia angustifolia* E. Mey. ex A.Rich., *Pelargonium luridum* (Andrews) Sweet, *Ptaeroxylon obliquum* (Thunb.) Radlk, *Schkuhria pinnata* (Lam.) Kuntze ex Thell., *Sclerocarya birrea* (A.Rich.) Hochst., *Senna italica* subsp. *arachoides* (Burch.) Lock. and *Tabernaemontana elegans* Stapf.

An extensive literature survey on the seventeen plants was conducted (not included in the thesis) and this information is available on the Phytomedicine database (www.up.ac.za/phyto). The contact bioassay described by Zorloni et al. (2010) was used for the preliminary screening of the test samples. This bioassay is simple and fast hence, it is suitable for the mass screening of test samples. Thereafter, adult immersion tests, the most commonly employed bioassays for determining acaricidal properties of many plant species were used for subsequent experiments. The acaricidal activity of the selected plants against *R. (B.) decoloratus* and *R. (B.) microplus* larvae were also evaluated (Fouche et al., 2016; Wellington et al., 2017). The text in this chapter has been submitted to the journal Parasitology.

Abstract

The menace caused by ticks and tick-borne diseases is a major limitation to the livestock industry in Africa. The high costs and non-availablity of synthetic, chemical acaricides to resource-limited farmers, resistance of ticks to available acaricides and residue problems in meat and milk consumed by humans further complicate matters. The use of plant extracts as a possible source of new acaricides has received much interest in the last decade. Crude extracts of seventeen plants prepared using four different solvents, were screened for their acaricidal efficacy against adult *Rhipicephalus turanicus* ticks (using the contact and adult immersion tests) and cytotoxicity against Vero and HepG2 cells (using the tetrazolium-based colorimetric assay). *Calpurnia aurea, S. pinnata* and *S. italica* showed the highest acaricidal efficacies with 97, 93 and 90%; 93, 93 and 87% mortality for their acetone and ethanol extracts respectively. Dose-response acaricidal bioassay (3-100 mg/ml) was undertaken for these plants and the LC_{50} values were 111.24, 37.75 and 42.05 mg/ml for the acetone extracts and 98.69, 37.07 and 37.50 mg/ml respectively. Many of the plant extracts were non-cytotoxic (LC_{50}>100 µg/ml) to the two cell lines. The result of this study indicates which plant species should be further investigated in-depth for isolation of active compounds.

6.1 Introduction

Ticks are ranked second to mosquitoes as vectors of pathogenic organisms. Although mosquitoes transmit pathogens that infect more people and cause severe diseases of humans and animals (e.g malaria, dengue fever, yellow fever), ticks transmit a greater variety of zoonotic pathogens. In addition, ticks are the direct causes of deadly tick paralysis, allergic reactions and toxicoses (Sonenshine & Roe, 2013). Ixodid ticks such as *Amblyomma* and *Rhipicephalus* species are among the most economically important parasites especially in developing countries in the tropics and subtropics (Kiss et al., 2012).

Rhipicephalus turanicus is a three-host tick largely distributed in the Mediterranean sub-region, Asia and Africa; from North Africa (Morocco, Algeria and Tunisia) to Senegal and Guinea in the west and Ethiopia and Somalia in the east (Chochlakis et al., 2014). The species is closely related to the brown dog tick, *R. sanguineus* and its distribution in the Afrotropical region is underestimated and has to be reappraised, particularly in western and central Africa since recent molecular studies show that many samples previously identified as *R. sanguineus* were *R. turanicus* (Hekimoglu et al., 2016). This tick parasitizes a wide range of hosts including cattle, sheep, dogs and humans. It is sometimes found on horses and in North Africa and the northern Sahel, it is found on camels, water buffalo and goats. Its favoured wild hosts are the larger carnivores, ground-feeding birds, zebras and warthogs. The immature life stages feed on hedgehogs, shrews, gerbils, rodents and hares (Estrada-

Pena et al., 2004). *Rhipicephalus turanicus* is a vector of *Rickettsia massiliae* and *Rickettsia aeschlimannii* (Wei et al., 2015), *Babesia ovis* and *Hepatozoon canis* (Giannelli et al., 2016).

At present, the most common antitick measure used is the application of synthetic chemical acaricides, both to the environment and to animals (Iori et al., 2005). These acaricides are expensive and not easily accessible to rural farmers. Also, toxicity due to overdosing, resistance due to underdosing and misuse as well as food and environmental contamination has been reported (Panella et al., 2005). This has led to the search for safe and environmentally-friendly alternatives and a number of unconventional tick control approaches have been advocated (Mondal et al., 2013). These measures are directed towards averting production losses, dropping tick numbers to minimal levels, decreasing chemical residue risks and reducing the dependence on chemicals (Ghosh et al., 2007). Such methods include pasture spelling (Manjunathachar et al., 2013), vaccination (de la Feunte et al., 2014), biological control (Nana et al., 2015; Nana et al., 2016), genetic manipulation (Kocan et al., 2015) and the use of medicinal plants (Céspedes et al., 2015).

South Africa, a country on the southern tip of the African continent, boasts a unique and diverse botanical heritage with over 30 000 plant species of which about 3 000 are used therapeutically (Steenkamp & Smith, 2006). In addition to this unique botanical heritage, South Africa has a cultural diversity with traditional healing being integral to each ethnic group.Though many medicinal plants in South Africa have been rigorously studied for their biological activities, only a fraction of medicinal plants used traditionally have been scientifically evaluated for their activity against ticks, hence, the aim of this study was to screen seventeen of these plants for their effect on ticks and their cytotoxicity (*in vitro* safety) as a preliminary step towards exploring the possibilities of discovering new plants with antiparasitic properties.

6.2 Materials and Methods

6.2.1 Collection and preparation of plant materials

Seventeen plant species were collected from their natural habitats by the CSIR as their part of the project. To ensure adequate ventilation, the plant materials were transported in open mesh orange bags to the CSIR laboratory and dried in a purpose built oven at 30-60°C, followed by pulverization to fine powder using a hammermill (IKA-Werke, United Scientific, Germany). The plant extracts were prepared using solvents of decreasing polarity [hot water, ethanol/water (30:70), ethanol and acetone]. The ethanol extracts were prepared by pouring 200 ml of 99% ethanol onto 20 g ground plant material which was then stirred for 1 hour. This process was repeated twice and the filtrates were combined and concentrated under a

vacuum using rotary evaporator (Buchi Rotavapor R-200, Switzerland). The acetone extracts were prepared with 99.5% acetone using the same procedure as that used for the ethanol extracts. Hot water extracts were prepared by boiling the pulverised plant material for 30 minutes at 100ºC with continuous stirring, then filtered. The voucher specimens were numbered and kept in the CSIR laboratory for future reference.

6.2.2 Ticks

Adult *R. turanicus* ticks (both sexes) were obtained from Clinvet International, Bloemfontein, South Africa. The ticks were kept at the Phytomedicine laboratory, Faculty of Veterinary Sciences, University of Pretoria in glass humidity chambers at an average temperature of 25ºC±1ºC. Relative humidity was maintained at 75±10% by placing supersaturated sodium chloride solution in the glass chamber. The ticks were stored in vials covered with cotton mesh (to allow for air exchange) and set on a square glass plate placed at the base of the chamber on four small bearings. The four sides of the glass plate were at a distance of 1.5 cm from the wall of the humidity chamber to prevent the ticks from reaching the walls.

6.2.3 *In vitro* acaricidal toxicity bioassays

6.2.3.1 Contact bioassay

Preliminary screening to evaluate the acaricidal activity of the plant species was undertaken using the contact bioassay described by Zorloni et al. (2010). One microlitre (1 µl) of 200 mg/ml of each test sample was dropped on the dorsum of *R. turanicus* ticks (n=10) for 1 minute before storing them in a vial covered with a perforated stopper. The same procedure was followed for the negative control (acetone for the organic extracts and distilled water for the water extracts) and positive control (5 mg/ml cypermethrin). Each experiment was tested in triplicate on each of three different occasions to yield nine replicates. The percentage mortality was determined 24 hours post treatment by viewing the ticks under a stereo microscope (American Optical Corporation). Ticks were recorded as alive and active if they exhibited normal behaviour on exposure to carbon dioxide (CO_2) from human breath (host-associated stimulus) or after being physically stimulated with plastic tweezers. Those showing some difficulty in movement or being able to maintain normal posture were termed weak and those in which there was loss of righting reflex (a reflex that corrects the orientation of the body when it is taken out of its normal upright position) were termed very weak. Ticks were confirmed dead based on signs of cuticle darkness, halted Malpighian tubules movement and haemorrhagic skin lesions.

6.2.3.2 Dose-response bioassay

Two-fold graded decreasing concentrations (100-3 mg/ml) of the acetone and ethanol extracts of the plant species that had very good acaricidal activity (>80% mortality) during

the contact bioassay were used for adult immersion tests (Al-Rajhy et al., 2003). Ten adult ticks were immersed in each test sample for one minute, dried over Whatman no. 1 filter papers and kept at room temperature (25±1^{o}C) and 85% relative humidity in 20 ml glass vials closed with a perforated stopper. Graded two-fold decreasing concentrations of cypermethrin (5-0.2 mg/ml) and acetone served as the positive control and negative control respectively. Each experiment was also tested in triplicate on each of three different occasions to yield nine replicates.

6.2.4 Cytotoxicity (*in vitro* safety) bioassay

6.2.4.1 Maintenance of cell lines

Vero monkey kidney (ATCC® CCL-81™) and HepG2 human liver cancer (ATCC® HB-8065™) cells were purchased from Cellonex, South Africa. Vero cells were maintained in Minimal Essential Medium (MEM, Whitehead Scientific, South Africa) containing 4.5 g/l glucose and 4 mM L-glutamine supplemented with 1% gentamicin and 5% foetal calf serum (FCS, Highveld Biological, South Africa) while HepG2 cells were maintained in Dulbecco's Minimal Essential Medium (DMEM, Whitehead Scientific, South Africa) containing 4.5 g/l glucose and 4 mM L-glutamine supplemented with 10% FCS. Both cells were maintained at 37^{o}C in a 5% CO_2 incubator (Hera Cell 150, Germany). Cells were passaged three times weekly by trypsinization with trypsin/ethylenediaminetetraacetic acid solution (Invitrogen, Cergy-Pontoise, France) into 175 cm^2 culture flasks.

6.2.4.2 Experimental protocol

Viable cell growth after incubation of Vero and HepG2 cells with test samples was determined using the tetrazolium-based colorimetric MTT assay [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] described by Mosmann (1983). The acetone, ethanol, ethanol/water and hot water extracts were first tested on Vero cells, then the ethanol/water and hot water extracts were tested on HepG2 cells in a subsequent experiment. Cells of a subconfluent culture were harvested and centrifuged at 200 x g for 5 minutes and resuspended in a growth medium (MEM for Vero cells and DMEM for HepG2 cells) to 10^5 cells/ml. One hundred microliters (100 µl) of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate, then 200 µl of MEM was added to wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated overnight at 37ºC in a 5% CO_2 incubator to allow cells to attach at the bottom of the plates.

Plant extracts were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 100 mg/ml and then diluted 100-fold in culture medium for the concentration tested (1000 µg/ml). Hundred microliters (100 µl) of decreasing concentrations of plant extracts (1000, 750, 500,

250, 100, 50 µg/ml) were added in the corresponding wells and the plates were again incubated at 37ºC for 48 hours in the 5% CO_2 incubator. Untreated cells served as the negative control while doxorubicin hydrochloride (Pfizer Laboratories, South Africa) served as the positive control.

After incubation, the growth medium was aspirated from the cells which were washed with 200 µl phosphate buffered saline (PBS, Whitehead Scientific) and replaced with 200 µl of fresh medium and 30 µl MTT (Sigma-Aldrich, South Africa; stock solution of 5 mg/ml in PBS). The plates were incubated further for four hours at 37ºC in the 5% CO_2 incubator. After this, the growth medium in each well was carefully removed using a suction pump (Integra, USA), without disturbing the MTT formazan crystals. These crystals were dissolved by adding 50 µl DMSO to each well and shaken gently for 2 minutes. The MTT reduction was measured immediately by detecting absorbance in a spectrophotometer (Biotek Synergy, USA) at a wavelength of 570 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The lethal concentration of test samples resulting in a 50% reduction of absorbance (LC_{50}) compared to untreated cells was calculated. Each extract concentration was tested in quadruplicate and the assays repeated three times.

6.2.5 Data analysis

Data on tick mortality and cytotoxicity were presented as the arithmetic mean values±standard error of mean (Mean±SEM). Significance was analysed using one-way analysis of variance followed by Tukey's multiple comparison test on GraphPad Prism 7.02 (GraphPad Software, San Diego-CA, USA). Values were considered to differ statistically when $p \leq 0.05$. The dose-response graphs of the plants with the highest acaricidal activities were plotted and LC_{50} determined using the linear regression model.

6.3 Results

6.3.1 Extraction and yield

The assayed plants represent fourteen plant families with the Fabaceae, Caparaceae and Geraniaceae families being represented by two plant species (14%) each while the remaining eleven families were represented by one species each (Table 6.1). The leaves were the most commonly used parts followed by the whole plant, stem, bark, root and fruit (Table 6.1).

Four extractants, namely, ethanol, acetone, ethanol/water (30:70) and hot water were used and this gave varied percentage yield (Table 6.1). The percentage yield of each plant extract

was calculated by dividing the mass of plant extract obtained by the mass of ground plant material (20 g) used, the value obtained was multiplied by 100. Of the acetone extracts, the leaves of *M. angolensis* had the highest yield (12%), while the bulb of *H. rigidula* had the lowest yield (3%). *Hypoxis rigidula* bulb however, had the highest yield of the ethanol extracts (12%) while 9 plants had 5% yield each. For most of the plants, ethanol/water and hot water extracts had higher yields than their acetone and ethanol extracts. *Senna italica* and *P. luridum* had the highest yield of the ethanol/water and hot water extracts respectively (12%). On the other hand, *P. luridum* had the lowest yield of the ethanol/water extracts (4%) while *M. angolensis* had the lowest yield of the hot water extracts (3%) (Table 6.1).

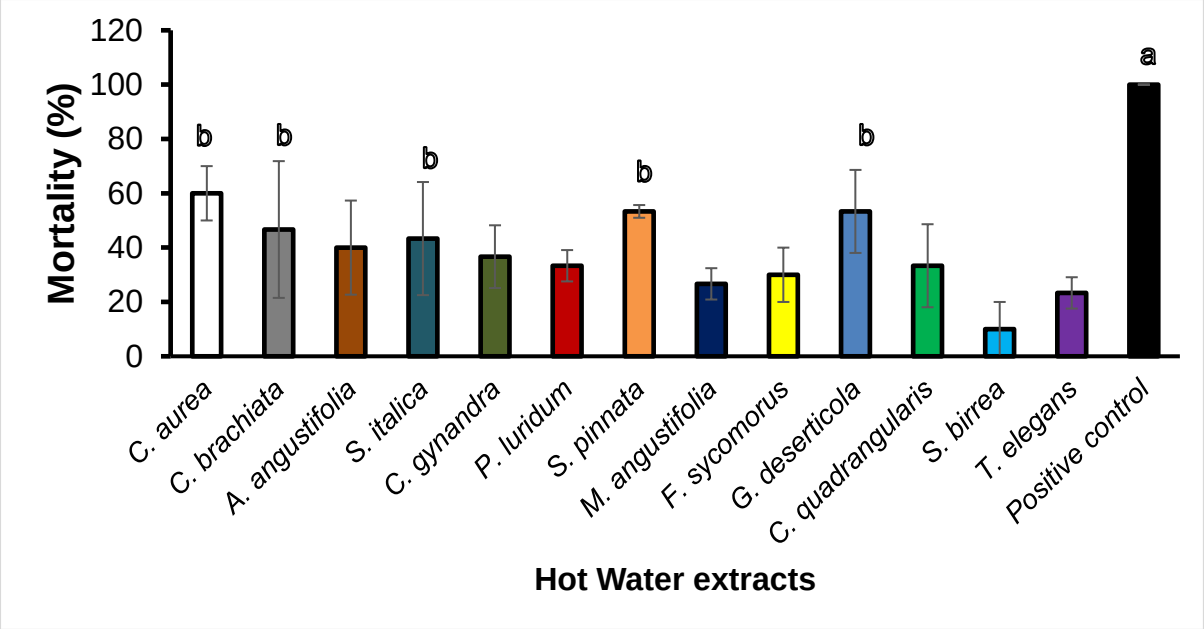
Table 6.1: Plant species and parts used for the solvent extraction, plant family, the mass and percentage yield of plant extract

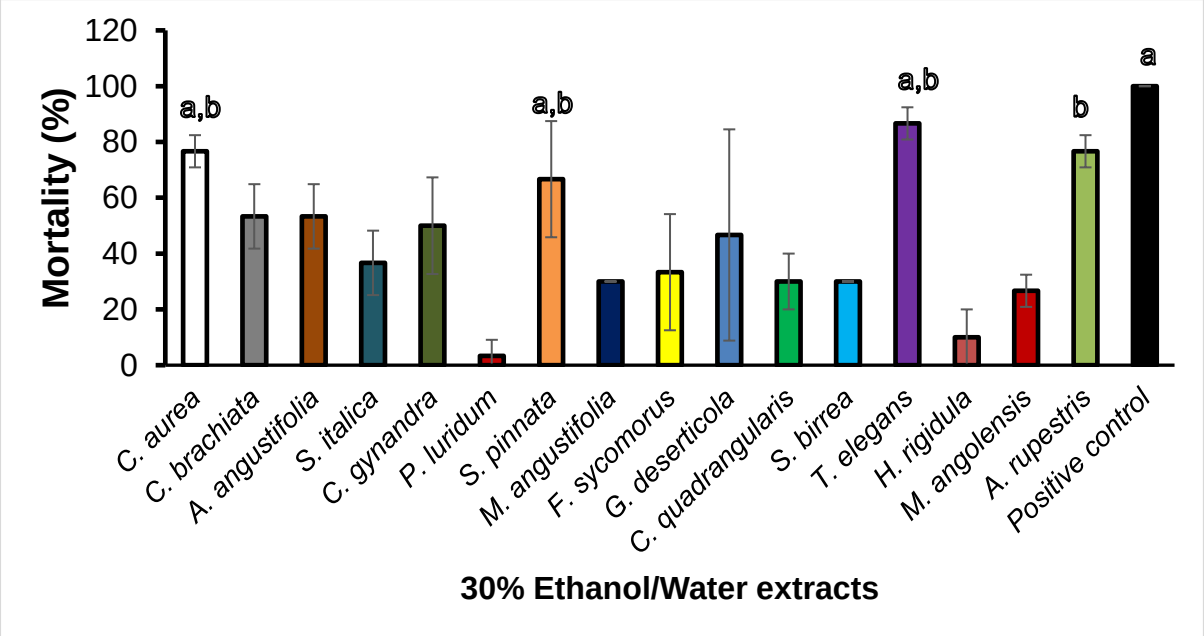
Family	Plant and plant part used	Mass of extract g (%Yield)			
		Acetone	Ethanol	Ethanol/ water	Hot water
Anacardiaceae	*Sclerocarya birrea* (B, R)	0.91 (5%)	1.07 (5%)	2.40 (12%)	2.30 (12%)
Apocynaceae	*Tabernaemontana elegans* (L)	1.00 (5%)	1.03 (5%)	1.12 (6%)	0.93 (5%)
Asphodelaceae	*Aloe rupestris* (L, R)	1.01 (5%)	1.05 (5%)	2.17 (11%)	2.35 (12%)
Asteraceae	*Schkukria pinnata* (WP)	1.11 (6%)	1.16 (6%)	2.42 (12%)	1.77 (9%)
Capparaceae	*Cleome gynandra* (L)	0.97 (5%)	1.07 (5%)	2.35 (12%)	1.91 (10%)
	Maerua angolensis (L)	2.38 (12%)	2.39 (12%)	2.38 (12%)	0.69 (3%)
Fabaceae	*Calpurnia aurea* (L, Fl, S)	0.94 (5%)	1.04 (5%)	2.13 (11%)	2.26 (11%)
	Senna italica (L, R, F)	1.09 (5%)	1.06 (5%)	2.44 (12%)	2.23 (11%)
Geraniaceae	*Monsonia angustifolia* (WP)	1.03 (5%)	1.05 (5%)	2.33 (12%)	2.20 (11%)
	Pelargonium luridum (WP)	1.01 (5%)	1.11 (6%)	0.83 (4%)	2.45 (12%)
Hypoxidaceae	*Hypoxis rigidula* (Bl)	0.50 (3%)	2.44 (12%)	2.37 (12%)	2.25 (11%)
Menispermaceae	*Antizoma angustifolia* (R)	1.22 (6%)	2.23 (11%)	1.33 (7%)	2.27 (11%)
Moraceae	*Ficus sycomorus* (B, S)	1.03 (5%)	1.05 (5%)	2.31 (12%)	1.18 (6%)
*Ptaeroxylaceae	*Ptaeroxylon obliquum* (L)	35.33(9%)	ND	ND	ND
Ranunculaceae	*Clematis brachiata* (WP)	1.04 (5%)	1.06 (5%)	2.36 (12%)	2.33 (12%)
Thymelaeaceae	*Gnidia deserticola* (WP)	1.37 (7%)	2.39 (12%)	2.35 (12%)	1.03 (5%)
Vitaceae	*Cissus quadrangularis* (S)	1.01 (5%)	1.24 (6%)	2.00 (%)	2.32 (12%)

Plant parts: L - Leaves; S - Stem; B - Bark; R - Root; WP - Whole plant; Fl - Flowers; F - Fruit; Bl - Bulb; ND - Not determined.
**Acetone extract only was prepared for Ptaeroxylon obliquum (403.48g in 4 L of acetone)*

6.3.2 Acaricidal activity

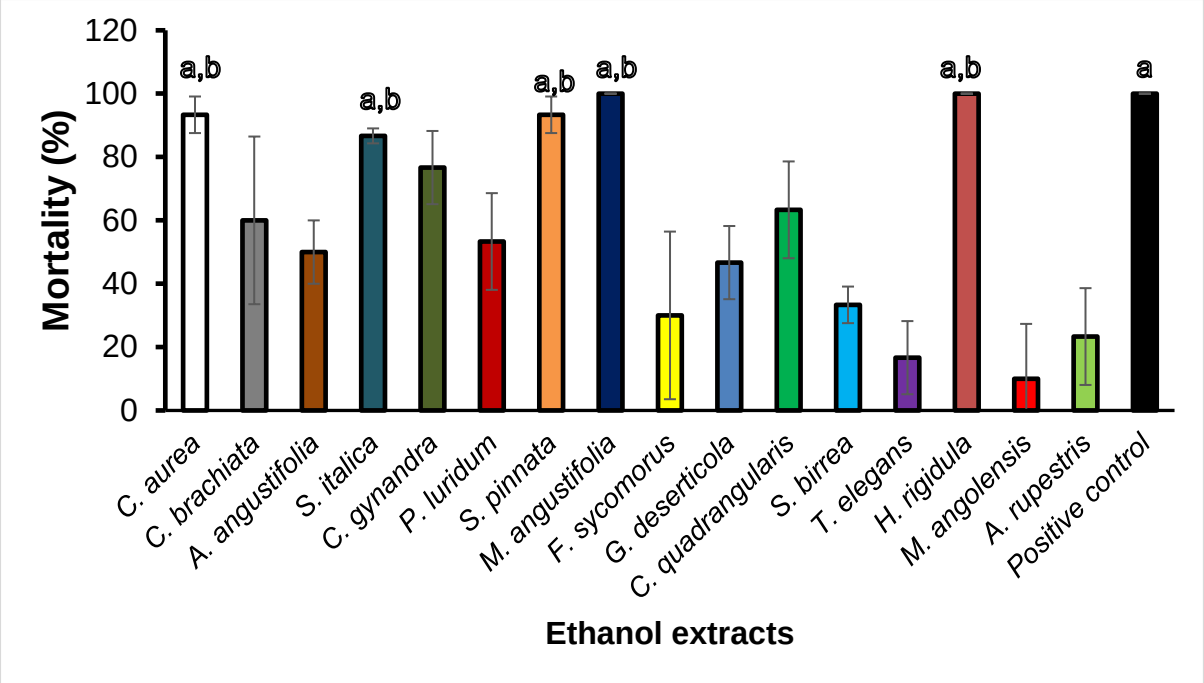
For the evaluation of results, our criteria for a plant extract to be termed as having very good acaricidal activity is tick mortality above 80%; good activity: 70-80%; moderate activity: 60-70%; poor activity: 40-60%; very poor activity: less than 40%. For most of the plants, the ethanol and acetone extracts were more active than the ethanol/water and hot water extracts (Fig. 6.1; 6.2). The ethanol extracts of *M. angustifolia* and *H. rigidula* had the highest acaricidal activity (100%) against *R. turanicus* ticks at 200 mg/ml. Their acetone, ethanol/water and hot water extracts, however, had very poor acaricidal activity (range: 10-30%) (Fig. 6.1; 6.2). Of the acetone and ethanol extracts, *C. aurea, S. pinnata* and *S. italica* had the highest acaricidal activities with mortality values of 97, 93 and 90%; 93, 93 and 87% respectively (Fig. 6.2). The acetone extracts of *F. sycomorus, A. angustifolia, M. angolensis*, *S. birrea* and *P. obliquum* also had very good acaricidal activities (93, 83, 86, 83 and 83% respectively) while their hot water, ethanol/water and ethanol extracts had poor acaricidal activity (tick mortality lower than 60%) (Fig. 6.1; 6.2). The ethanol and acetone extracts of *C. brachiata* and *C. gynandra* and the ethanol/water and acetone extracts of *A. rupestris* had moderate acaricidal activities (range: 60-70%) (Fig. 6.1; 6.2). The ethanol, acetone, ethanol/water and hot water extracts of *P. luridum, G. deserticola* and *C. quadrangularis* had poor acaricidal activities (<60%) against *R. turanicus.* The ethanol/water extract of *T. elegans* had the highest acaricidal activity (87%) followed by *C. aurea* and *A. rupestris* (77%) and *S. pinnata* (67%) (Fig 6.1). The hot water extracts of *C. aurea* had the highest acaricidal activity (60%) followed by *S. pinnata* and *G. deserticola* (53%) with others having very poor acaricidal activities (range: 10-47%) (Fig 6.1). The positive control (5 mg/ml cypermethrin) had acaricidal activity of 100% while the negative control (acetone and distilled water) caused tick mortality less than 5%.

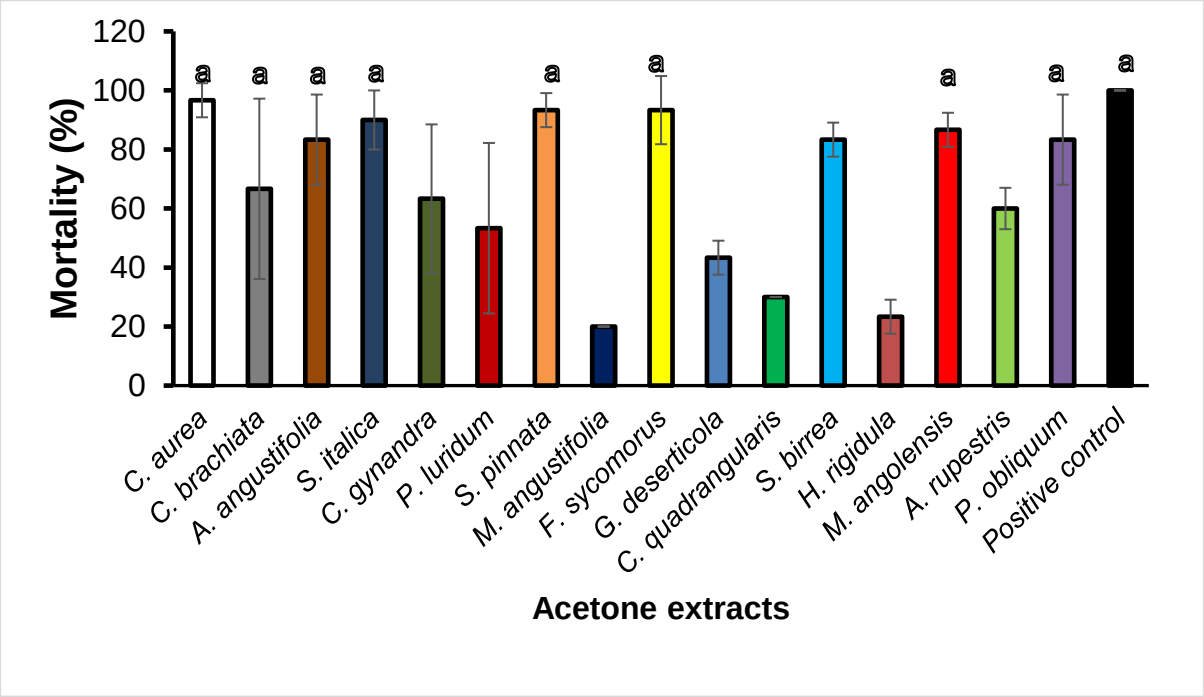




a - Statistically different from positive control ($p<0.05$); b - Statistical difference between the extracts ($p<0.05$); Extracts with the same letter are not statistically different from each other. The acaricidal activities of the hot water extracts of H. rigidula, M. angolensis, A. rupestris and T. elegans were not determined.

Figure 6.1: Acaricidal activity of hot water and 30% ethanol/water extracts of different plant species against *Rhipicephalus turanicus* ticks after 24hrs



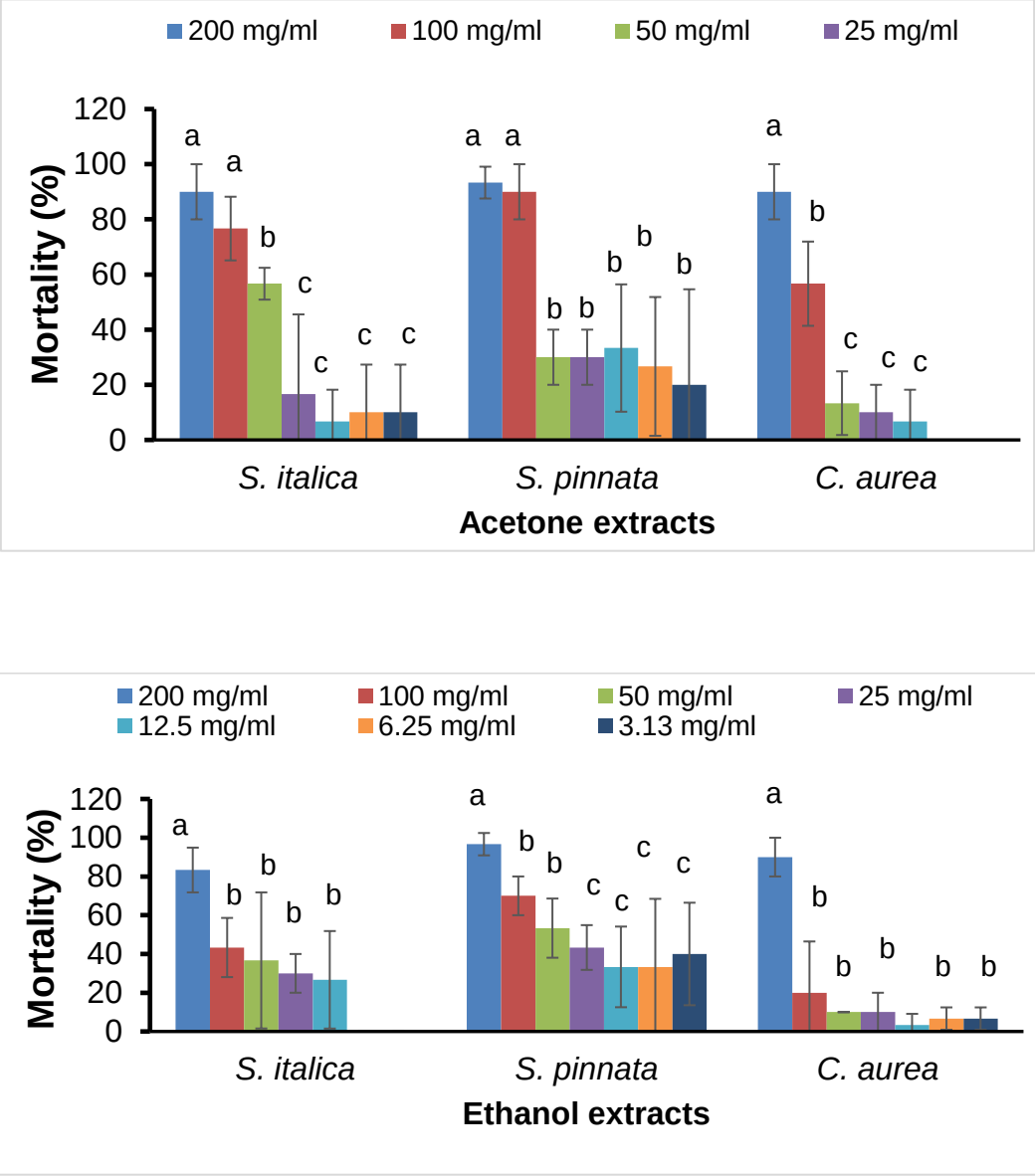


a- Statistically different from positive control ($p<0.05$); b - Statistical difference between the extracts ($p<0.05$); Extracts with the same letter are not statistically different from each other; Positive control is cypermethrin (5 mg/ml).

Figure 6.2: Acaricidal activity of the acetone and ethanol extracts of different plant species against *Rhipicephalus turanicus* ticks after 24hrs

6.3.3 Dose-response effect

The dose-dependent effect was determined for the acetone and ethanol extracts of *S. pinnata, S. italica* and *C. aurea.* The LC_{50} tick mortality values against *R. turanicus* ticks after 24 hours for the acetone extracts were 35.75, 42.05 and 111.24 mg/ml respectively while LC_{50} values for the ethanol extracts were 37.07, 37.50 and 98.69 mg/ml respectively compared with the positive control with LC_{50} of 2.41 mg/ml (Fig. 6.3).



A - Statistically different from positive control (p<0.05); b - Statistical difference between the extracts (p<0.05); Extracts with the same letter are not statistically different from each other; Positive control is cypermethrin (0.16-5 mg/ml)

Figure 6.3: Dose-dependent acaricidal activity of the acetone and ethanol extracts of three plant species against *Rhipicephalus turanicus* ticks after 24hrs

6.3.4 Cytotoxicity

6.3.4.1 Vero cells

For toxicity, we used a cutoff of 100 µg/ml as an indicator for toxicity (Nondo et al., 2015). Based on this criterion, many of the plant extracts had low cytotoxicity to Vero cells (LC_{50}>100 µg/ml) compared with that of the positive control (doxorubicin) which was 1.52 µM (Table 6.2). The ethanol/water and hot water extracts were the least toxic with LC_{50} greater than 1000 µg/ml for many of the plants. Of the acetone extracts, *C. gynandra* was the least toxic of the test samples to Vero cells with an LC_{50} value of 554 µg/ml followed by *S. birrea* with an LC_{50} value of 418 µg/ml. For the ethanol extracts, *S. italica* was the least toxic of the test samples with an LC_{50} value of 551 µg/ml followed by *C. aurea* with an LC_{50} value of 504 µg/ml. Both acetone and ethanol extracts of *S. birrea* were non-cytotoxic with LC_{50} values of 418 and 487 µg/ml respectively. The acetone and ethanol extracts of *P. luridum* (LC_{50}=31 and 33 µg/ml), *S. pinnata* (LC_{50}=40 and 89 µg/ml) and *A. angustifolia* (LC_{50}=44 and 24 µg/ml) were considerably more toxic to Vero cells than their ethanol/water and hot water extracts (LC_{50}=595 and 567; 460 and 655; 163 and 340 µg/ml respectively). The acetone, ethanol, ethanol/water and hot water extracts of *T. elegans* (LC_{50}=32; 40; 49; 69 µg/ml) were cytotoxic to the Vero cells (Table 6.2).

Table 6.2: Cytotoxicity (LC_{50} in µg/ml) of different extracts against Vero cells

Family	Plant species	Toxicity of extracts against Vero cells LC_{50} (µg/ml)±SEM			
		Acetone	Ethanol	Ethanol/water	Hot water
Anacardiaceae	*Sclerocarya birrea*	215±14	487±3	>1000	>1000
Apocynaceae	*Tabernaemontana elegans*	32±1	40±5	49±5	69±2
Asphodelaceae	*Aloe rupestris*	63±11	102±4	153±5	107±1
Asteraceae	*Schkuhria pinnata*	40±2	89±4	460±7	655±32
Capparaceae	*Cleome gynandra*	554±19	40	>1000	>1000
	Maerua angolensis	181±3	74	127±6	>1000
Fabaceae	*Calpurnia aurea*	224±5	504±4	>1000	>1000
	Senna italica	46±3	551±12	>1000	>1000
Geraniaceae	*Monsonia angustifolia*	120±4	35±0.1	241±50	>1000
	Pelargonium luridum	31±3	33±1	595±4	569±6
Hypoxidaceae	*Hypoxis rigidula*	64±3	343±15	>1000	>1000
Menispermaceae	*Antizoma angustifolia*	44±6	24	163±23	340±31
Moraceae	*Ficus sycomorus*	173±9	459±8	641±5	>1000
Ptaeroxylaceae	*Ptaeroxylon obliquum*	148	ND	ND	ND
Ranunculaceae	*Clematis brachiata*	117±4	485±22	857±11	>1000
Thymelaeaceae	*Gnidia deserticola*	90±5	94±6	946±20	>1000
Vitaceae	*Cissus quadrangularis*	41±3	74±4	806±1	>1000
	Doxorubicin			1.52±0.32	

ND - Not determined; Dark green - LC_{50} value greater than 1000 µg/ml (very safe), Green - LC_{50} value 500-1000 µg/ml (safe); Yellow - LC_{50} value 100-500 µg/ml (slightly cytotoxic); Red - LC_{50} value less than 100 µg/ml (cytotoxic)

6.3.4.2 HepG2 cells

The ethanol/water and hot water extracts of the plant species tested on HepG2 cells were non cytotoxic to the cells (LC_{50}>100 µg/ml) except ethanol/water extract of *M. angustifolia, A. angustifolia* and *T. elegans* with LC_{50} values of 3, 37 and 71 µg/ml respectively compared with doxorubicin (positive control) with LC_{50} value of 0.34 µM (Table 6.3). The LC_{50} values were also greater than 1000 µg/ml for many of the plants.

Table 6.3: Cytotoxicity (LC_{50} in µg/ml) of ethanol/water and hot water extracts against HepG2 cells

Family	Plant species	Toxicity of extracts against HepG2 cells LC_{50} (µg/ml)±SEM	
		Ethanol/water	Hot water
Anacardiaceae	*Sclerocarya. birrea*	>1000	>1000
Apocynaceae	*Tabernaemontana elegans*	71±7	418±4
Asphodelaceae	*Aloe rupestris*	496±11	459±12
Asteraceae	*Schkuhria pinnata*	116±40	532±40
Capparaceae	*Cleome gynandra*	>1000	>1000
	Maerua angolensis	>1000	>1000
Fabaceae	*Calpurnia aurea*	>1000	>1000
	Senna italica	>1000	601±36
Geraniaceae	*Monsonia angustifolia*	3	>1000
	Pelargonium luridum	330±24	369±21
Hypoxidaceae	*Hypoxis rigidula*	>1000	740±29
Menispermaceae	*Antizoma angustifolia*	37±8	153±15
Moraceae	*Ficus sycomorus*	708±42	>1000
Ranunculaceae	*Clematis brachiata*	683±17	568±35
Thymelaeaceae	*Gnidia deserticola*	233±1	>1000
Vitaceae	*Cissus quadrangularis*	618±16	>1000
	Doxorubicin	0.34±0.14	

Dark green - LC_{50} value greater than 1000 µg/ml (very safe), Green - LC_{50} value 500-1000 µg/ml (safe); Yellow - LC_{50} value 100-500 µg/ml (slightly cytotoxic); Red - LC_{50} value less than 100 µg/ml (cytotoxic)

6.3.4.3 Comparative cytotoxicity (LC_{50} in µg/ml) of ethanol/water and hot water extracts on Vero and HepG2 cells

The ethanol/water and hot water extracts of many of the plants were more toxic to the HepG2 cells than Vero cells (Table 6.4). Some of the plants in which there was a statistically significant difference are *S. pinnata, S. italica, M. angustifolia, P.* luridum, *H. rigidula* and *A. angustifolia* (Table 6.4).

Table 6.4: Cytotoxicity (LC_{50} in µg/ml) for ethanol/water and hot water extracts on HepG2 and Vero cells

Family	Plant species	Vero cells		HepG2 cells	
		Ethanol/water	Hot water	Ethanol/water	Hot water
Anacardiaceae	*Sclerocarya. birrea*	>1000	>1000	>1000	>1000
Apocynaceae	*Tabernaemontana elegans*	49±5*	69±2*	71±7	418±4
Asphodelaceae	*Aloe rupestris*	153±5*	107±1*	496±11	459±12
Asteraceae	*Schkuhria pinnata*	460±7	655±32	116±40*	532±40
Capparaceae	*Cleome gynandra*	>1000	>1000	>1000	>1000
	Maerua angolensis	127±6*	>1000	>1000	>1000
Fabaceae	*Calpurnia aurea*	>1000	>1000	>1000	>1000
	Senna italica	>1000	>1000	>1000	601±36*
Geraniaceae	*Monsonia angustifolia*	241±50	>1000	3*	>1000
	Pelargonium luridum	595±4	569±6	330±24*	369±21*
Hypoxidaceae	*Hypoxis rigidula*	>1000	>1000	>1000	740±29*
Menispermaceae	*Antizoma angustifolia*	163±23	340±31	37±8*	153±15*
Moraceae	*Ficus sycomorus*	641±5	>1000	708±42	>1000
Ranunculaceae	*Clematis brachiata*	857±11	>1000	683±17*	568±35*
Thymelaeaceae	*Gnidia deserticola*	946±20	>1000	233±1*	>1000
Vitaceae	*Cissus quadrangularis*	806±1	>1000	618±16*	>1000
	Doxorubicin			0.34±0.14	

**Statistically different ($p<0.05$), Graphpad Instat 3.0 Student-Newman-Keuls used to analyse the difference of the same extract on two different cell lines; Dark green - LC_{50} value greater than 1000 µg/ml (very safe), Green - LC_{50} value 500-1000 µg/ml (safe); Yellow - LC_{50} value 100-500 µg/ml (slightly cytotoxic); Red - LC_{50} value less than 100 µg/ml (cytotoxic)*

6.3.5 Selectivity index

Further analysis of the results involved the calculation of the selectivity index (SI) of the plant species based on the ratio of their cytotoxicity to their acaricidal activity. The three plant extracts appear to be more toxic to the cells than to the parasites with low SI values (range: 0.001-0.014) (Table 6.5).

Table 6.5: Selectivity index (LC_{50}/EC_{50}) of the acetone and ethanol extracts of the three plants with very good acaricidal activities

Plant species	Vero cells					
	Acaricidal activity (mg/ml)		Cytotoxicity against Vero cells(µg/ml)		SI (LC_{50}/EC_{50})	SI (LC_{50}/EC_{50})
	EC_{50}	EC_{50}	LC_{50}	LC_{50}		
	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol
Schkuhria pinnata	35.75±18	37.07±11	39.93±2	89.14±4	0.001	0.002
Calpurnia aurea	111.24±11	98.69±8	223.95±5	504.32±4	0.002	0.005
Senna italica	42.05±13	37.50±13	46.31±3	550.67±12	0.001	0.014

6.3.6 Selection of the plant species for further studies

To determine the most promising plant species for further studies, our entire results were compared (Table 6.6). Acetone, ethanol, ethanol/water and hot water extracts of *C. aurea* had good acaricidal activities against adult *R. turanicus* and larvae of *R. (B.) decoloratus and R. (B.) microplus* (Table 6.6). The plant was also safe on Vero and HepG2 cells (Table 6.6).

Table 6.6: Comparison of the three plants with the highest acaricidal activities

Plant species	Acaricidal activity against adult *R. turanicus* (%)				Acaricidal activity against *R. (B) decoloratus larvae* (%)*		Acaricidal activity against *R. (B) microplus larvae* (%)*		Cytotoxicity (LC_{50}) against Vero cells(µg/ml)				Cytotoxicity (LC_{50}) against HepG2 cells(µg/ml)	
	AC	EtOH	EtOH/DW	Hot water	AC	EtOH	AC	EtOH	AC	EtOH	EtOH/DW	Hot water	EtOH/DW	EtOH
Schkuhria pinnata	93	93	67	53	10	22	20	36	40±2	89±4	460±7	655±32	116±40	532±40
Calpurnia aurea	97	93	77	60	11	85	ND	100	224±5	504±4	>1000	>1000	>1000	>1000
Senna italica	90	87	37	43	9	19	ND	97	46±3	551±12	>1000	>1000	>1000	601±36

*AC - Acetone; EtOH - Ethanol; DW - Distilled water; ND - Not determined; Best values highlighted in green; *Fouche et al., 2016; Wellington et al., 2017.*

6.4 Discussion

Plants produce several secondary metabolites in variable concentrations which vary in polarity and solubility in different solvents, hence our use of different extractants. Water was used to evaluate the traditional use of the plants as most of the herbal remedies described in this study are used traditionally in South Africa as water-based infusions or decoctions sprayed on animals or taken orally. Acetone has the ability to extract compounds of a wide polarity range, it is miscible with organic and aqueous solvents and has been reported to be the best extractant for antimicrobial compounds in plants (Eloff, 1998). It was therefore used to evaluate its extraction of acaricidal compounds. Ethanol was used to dissolve water-insoluble volatile compounds and ethanol/water for intermediate polarity. Ethanol (C_2H_6O) appears to be as good as acetone in the extraction of acaricidal compounds in this study and is the most commonly used solvent for many *in vitro* acaricidal bioassays (Adenubi et al., 2016). The polar nature of its hydroxyl (OH) group causes ethanol to dissolve many ionic compounds and because the ethanol molecule also has a non-polar end, it will also dissolve non-polar substances, including most essential oils present in plants (Patel et al., 2015). The higher yield of the hot water and ethanol/water extracts could have resulted in greater dilution of the potential actives.

Both acetone and ethanol extracts of *S. pinnata, S. italica* and *C. aurea* showed high acaricidal efficacies against *R. turanicus* ticks, with an effect comparable with the synthetic chemical acaricide, but at forty times higher concentration. This is not unexpected because active compound(s) in a crude extract constitute only a small proportion of the array of compounds in the extract. Assay of crude extracts at higher concentrations to assess the exact effect of minor constituents is therefore necessary. Other workers have evaluated the acaricidal effects of plants at higher concentration including: 200 mg/ml (Cetin et al., 2009; Zorloni et al., 2010; Vendramini et al., 2012; Godara et al., 2014); 250 mg/ml and above (Coskun et al., 2008; Domingos et al., 2013; Kongkiatpaiboon et al., 2014). If a plant extract is not toxic to animals a dose forty times higher than the positive control, it could still be very useful as a control agent.

Two of the three most promising plants, *S. italica* and *C. aurea* belong to the Fabaceae family. This family, with 630 genera and over 18860 species, is the third largest plant family, after the Orchidaceae and Asteraceae (Judd et al., 2000). It is the most common family found in tropical rainforests in Africa and USA (Burnham & Johnson, 2004). Species of this family frequently contain alkaloids, proanthocyanidins and flavonoids such as kaempferol, quercetin and myricetin.

Both the acetone and ethanol extracts of the roots, leaves and fruits of *S. italica* had good acaricidal effects. This agrees with the study by Magano et al. (2008) where the ethyl acetate root extract (15 g/50 ml=300 mg/ml) of *S. italica* produced 100% mortality of adult *H. marginatum rufipes* ticks with a LC_{50} of 87 mg/ml. Members of the genus *Senna* have high levels of chrysophanic acid which are known to have antifungal, antibacterial and anti-mite properties. They also possess hexadecanoic acid (palmitic acid), identified in the fecal shield of sumac flea beetle, *Blepharida rhois* which serves as a very effective chemical defence (Magano et al., 2008). Given the antitick properties of *S. italica* demonstrated in this study, it is reasonable to attribute these findings, at least in part, to the presence of chrysophanic and hexadecanoic acids in the extracts of this plant.

Twenty-eight plant species used to control ticks on animals in southern Ethiopia were evaluated by Zorloni (2007) who reported that 20% (200 mg/ml) and 10% (100 mg/ml) concentrations of acetone leaf extracts of *C. aurea* either killed or severely compromised the mobility of unfed adult *R. pulchellus* ticks. Differences in climatic conditions, the cultivation and collection of plant materials for extract production may cause differences in results (Heimerdinger et al., 2006). It is encouraging that despite the difference in geographical location, different extractants and tick species used, similar bioactivity was obtained in our study. In a study carried out by Nana et al. (2010), the responses of *R. pulchellus* and *R. appendiculatus* to acetone, aqueous and oil dried leaf extracts of *C. aurea* were investigated. The aqueous extract (25 mg/ml), oil extract (100 mg/ml) and positive control (aggregation-attachment pheromone) at 0.02 mg/ml were attractants to *R. pulchellus* ticks. It was therefore proposed that *C. aurea* extracts can possibly be used as baits to lure ticks to a trap for tick control in the field (Nana et al., 2010). Lure and kill strategies combine attractants with an acaricide into a slow-release formulation or device. The attractant compound(s) lure the tick to some object that contains a contact acaricide such as dichlorvos. This provides a safer means of acaricide application for humans, animals and the environment since the amount of acaricide applied is reduced with restricted bioavailability to the general landscape (Carr et al., 2016).

Schkuhria pinnata (Asteraceae) is used traditionally as a blood cleanser, skin tonic, wound wash, for insect bites and swellings and for the treatment of heartwater, a tick-borne disease of cattle (Luseba et al., 2007).The plant has been reported to have anti-oxidant (León et al., 2009), antibacterial (Wagate et al., 2010) and anti-cancer properties (Alonso-Castro et al., 2011). The acaricidal activity of *S. pinnata* observed in this study confirms results on the insecticidal activity which has been reported previously, in which LC_{50} of 5197 ppm (5 mg/ml) was recorded against mosquitoes (Orozco & Lentz, 2005). The germacranolides, schkuhrin-I

and schkuhrin-II, isolated from the whole plant, exhibited antifeedant activity against the beet armyworm and the Mexican bean beetle (Jacobson, 1989). The genus also contains polyacetylenes, heliangolide, sesquiterpene lactones and phenylpropanoids (León et al., 2009).

The organic extracts of *A. rupestris, P. luridum, T. elegans, H. rigidula, G. deserticola* and *C. quadrangularis* did not meet our criteria for acaricidal activity against *R. turanicus* in this study (acaricidal activity less than 60%). Aqueous extract of the stem of *C. quadrangularis* (50 mg/L) had acaricidal activity of 59% (Santhoskumar et al., 2012) though, *C. quadrangularis* is reported to be effective against cattle ticks and other livestock parasites in Zimbabwe (Nyahangare et al., 2015).

An interesting finding from this study was the relatively poorer activity of the hot water extracts, even though most of the herbal remedies described in this study are used traditionally in South Africa as water-based infusions or decoctions sprayed on animals or taken orally. This was also observed by Ngarivhume et al. (2015) in the survey of plants used for tick control in Zimbabwe where the use of water and leaves was almost a standard practice in most traditional remedies. This use of leaves is ideal as it ensures sustainability of the plants. Despite our efforts to maximise effects by using hot water instead of cold water extract, hot water extract of *C. aurea* showed the highest acaricidal activity (60%) of the plant species tested.

It has been reported previously that many natural products have low water solubility and need to be dissolved in organic solvents or surfactant agents before being used in experimental systems (Azmir et al., 2013). As with other arthropods, the body of ticks is covered by the cuticle which protects the body organs against mechanical pressure, desiccation, pathogens and offers attachment sites for the muscles (Sonenshine et al., 2014). Passage of water and other molecules through the cuticle is restricted by a thin layer of wax (lipid) on the outer surface of the cuticle hence the more non-polar a chemical compound is, the greater will be its ability to penetrate the cuticle (Chagas et al., 2002). Majority of the commonly used synthetic acaricides such as the pyrethroids, fipronil, ivermectin and dieldrin are insoluble in water (lipid-soluble). As such, though, water is a cheap universal solvent, there may be need to use organic solvents to fully optimise the extraction process since water has its polarity limitations (Azmir et al., 2013). It becomes evident that although water-based solvents are widely used in ethnoveterinary medicine, organic solvents may work better in acaricidal bioassays.

In order to verify the safe use of these plant species for a possible acaricidal formulation, cytotoxicity study using two mammalian cell lines; Vero and HepG2 was undertaken. Acetone, ethanol, ethanol/water and hot water extracts were tested on Vero cells, isolated from kidney epithelial cells extracted from an African green monkey (*Cercopithecus aethiops*). These are usually the first line of cells used for cell cytotoxicity studies (Osada et al., 2014). To further evaluate the safety of these plants' use traditionally, we also tested the ethanol/water and hot water extracts (as most herbal remedies are used traditionally as water-based concoctions or water mixed with ethanol, which is the principal type of alcohol found in alcoholic drinks), on HepG2 cells. HepG2, a liver cell line derived from a human hepatoblastoma has been found to express a wide variety of functions that are known as liver tissue-specific pathways which are responsible for xenobiotic (foreign chemical substance) metabolism, detoxification and removal (Gomez-Lechon et al., 2008). HepG2 cell line is thus a suitable model to study liver metabolism, toxicity of xenobiotics and drug targeting (Maurya & Vinayak, 2015).

Many of the plant extracts were not cytotoxic to Vero cells (LC_{50}>100 µg/ml). Nonetheless, the LC_{50} values of 32.35; 40.04; 49.14 and 69.14 µg/ml for the acetone, ethanol, ethanol/water and hot water of *T. elegans* on Vero cells was noted. This cytotoxic activity of extracts of *Tabernaemontana* species has been well documented, can be ascribed to the presence of alkaloids and may account for the use of this plant in the management of cancer (Lee & Houghton, 2005). The ethanol/water and hot water extracts were the least toxic with LC_{50} greater than 1000 µg/ml for many of the plants. We observed that the ethanol/water and hot water extracts of many of the plants were more toxic to HepG2 than Vero cells. This could potentially indicate the possibility of hepatotoxicity in the treated animals. It may even be possible, that due to the metabolic activity of the HepG2 cells, compound(s) within the extract or the pure compound were being metabolised into more toxic metabolites. Since toxicity can be associated with pharmacological activity in lower doses, plants containing some toxic constituents may have useful biological activities (Edwards & Aronson, 2000).

The selectivity index of a plant extract is the ratio of the cytotoxicity to the biological activity of the plant (LC_{50}/EC_{50}). The higher the selectivity index value, the safer the extract. Though the plant extracts were more toxic to the cells than to the parasites, the presence of cellular toxicity is not neccesarily associated with whole animal toxicity especially for topical application to control ticks. In this case, it is possible that the use of an organic extractant may have led to extraction of toxic compounds. For this, chemical fractionation and isolation may help separate out the potential toxic compounds, which may not be the same as the acaricidal compounds. It is also possible that, via *in vivo* interaction with microsomal and

non-microsomal pathways, the molecules may be rendered less toxic due to metabolism, or skin barrier exclusion may play a role.

6.5 Conclusions

The documented uses of many of these plants against ticks have a possible pharmacological basis as indicated by the results of this study. Although the plants had acaricidal activity at a higher concentration than that of the positive control, isolation of the bioactive compounds may improve acaricidal activity.

CHAPTER 7

Isolation and characterization of two acaricidal compounds from *Calpurnia aurea* subsp. *aurea* (Fabaceae) leaf extract

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Preface

In the preceding chapter (chapter 6), preliminary screening was undertaken to evaluate the acaricidal properties of seventeen plant species selected based on their ethnoveterinary use against ticks. Particularly good acaricidal activities were displayed by *Calpurnia aurea* subsp. *aurea.* However, the bioactive compounds responsible for these observations have not been identified. The isolation and structural elucidation of the bioactive compounds are presented. The text in this chapter has been submitted to the journal Veterinary Parasitology.

Abstract

The menace caused by ticks and tick-borne diseases is a major limitation to the livestock industry in Africa. The high costs and non-availability of synthetic, chemical acaricides to resource-limited farmers, resistance of ticks to available acaricides and residue problems in meat and milk consumed by humans further complicate matters. The use of plant extracts as a possible source of new acaricides has received much interest in the last decade. In our endeavour to discover natural acaricidal compounds, the chloroform fraction of *Calpurnia aurea* exhibited good bioactivity in the acaricidal bioassay. Further purification revealed two acaricidal compounds characterized as apigenin-7-*O*-β-D-glycoside (**1**) and isorhoifolin (**2**) by means of NMR spectroscopic and mass spectrometry analysis. Compounds **1** and **2** were isolated from *Calpurnia aurea* for the first time and their acaricidal activity was not investigated previously. The acaricidal LC_{50} value of 0.65 mg/ml obtained for isorhoifolin compares favourably with some known acaricidal compounds isolated from plants and synthetic, chemical acaricides. This makes flavonoids an interesting group of molecules as targets for intensive study regarding their acaricidal properties.

7.1 Introduction

Ticks rank second only to mosquitoes as the most common vectors of diseases to animals and humans (Oberchain and Galun, 2013). In addition, ticks are the direct cause of deadly tick paralysis, allergic reactions and toxicoses (Sonenshine and Roe, 2013). Current tick control involves application of chemical acaricides, usually synthetic, but tick resistance and negative environmental impacts have become problematic from years of overuse (Panella et

al., 2005). The search for alternative control measures such as acaricidal or repellent medicinal plants is strongly advocated (Mondal et al., 2013). According to the World Health Organisation, more than 80% of the world's population rely on herbal remedies to treat various infections and infestations of humans and animals (Zizka et al., 2015). Despite this, research in the use of plant species for the control of animal parasites are scarce, with a lack of further information regarding the conditions of production, harvest time, plant parts and quantities used in the elaboration of products (Politi et al., 2016). The use of plant components, either as pure compounds or as standardized extracts, provides opportunities for new phytotherapics or a source of prototype substances. Among the advantages of herbal medicines that justify their use are synergistic effects of their components acting on different molecular targets, lower risk of side effects and less costs in research (Politi et al., 2016). Plant extracts may also be useful to rural pastoralists that cannot afford chemical acaricides.

Only recently have a number of findings been published on the phytochemistry and biological activities of plant species used in traditional healing (Atanasov et al., 2015). A survey of plant-derived pure compounds used as drugs in WHO Collaborating Centers for Traditional Medicine indicated that, of 122 compounds identified, 80% were used for the same or related ethnomedical purposes and were derived from only 94 plant species (Cragg & Newman, 2013). The success attained with pyrethrin (a compound isolated from *Chrysanthemum* species), which serves as a backbone for the synthesis of pyrethroid, a component of many household parasiticides, shows that in-depth investigation of the large number of plants with good acaricidal activity may be a worthwhile exercise. Aside from the pyrethrins, azadirachtin and limonene, there is little published data on natural products effective for tick control (Adenubi et al., 2016).

Calpurnia aurea (Ait.) Benth. of the family Fabaceae, commonly called wild laburnum, is a small tree up to 4 m, reaching up to 15 m under forest conditions. The plant is widely distributed in Africa from southern Africa (Eastern Cape through to KwaZulu-Natal, Mpumalanga, Gauteng, Limpopo and Swaziland) to Eritrea (the horn of Africa), stretching northwards to as far as Ethiopia and southern India (Getiye et al., 2016). Two subspecies of *C. aurea*, namely subsp. *aureus* (occurs in Ethiopia, Zaire, Zimbabwe, Angola, West and South Africa) and subsp. *indica* (occurs in India) are recognized (Getiye et al., 2016). The plant is easy to cultivate, not toxic to the environment, has the ability to resist drought and overgrazing hence, an ideal component of intergrated pest management systems and organic farming (Gemeda et al., 2014).

In South Africa, the juice of crushed leaves, ground bark and roots of *C. aurea* are used to kill ectoparasites and to relieve itching while unspecified parts are used to destroy maggots. The leaves are used to treat allergic rashes, particularly those caused by caterpillars. In East Africa, the leaf sap of the plant is used to destroy maggots in wounds. In Nigeria, the seeds are used to treat abscesses while in Ethiopia, it is used to for the treatment of stomach complaints, headache, eye diseases, amoebic dysentery, scabies and also as an insecticide (Moyo et al., 2015). The methanol leaf extract of *C. aurea* has antidiarrhoeal and antimicrobial activities (Umer et al., 2013), antimalarial activity (Eyasu et al., 2013), antibacterial and anti-oxidant activities (Adedapo et al., 2008). The seed extract has anti-hypertensive activity (Yohannes, 2014), acaricidal and tick attractant activities (Nana et al., 2010; Nana et al., 2016). Extracts of the stem and bark of the plant have also been studied for their anticancer activity (Korir et al., 2014).

Previous work by other researchers have isolated alkaloids such as 3β, 4α, 13α-trihydroxylupanine and 3β, 4α-dihydroxy 13α-O-(2′-pyrrolylcarbonyl)-lupanine (calpaurine) from the leaves (Asres et al., 1986); a quinolizidine alkaloid (calpurnine), a pterocarpan (3-acetoxy-9-methoxypterocarpan) and isoflavones such as, 4′,5,7-trihydroxyisoflavone, 7,3′-dihydroxy-5′-methoxyisoflavone, 7-hydroxy-4′,8-dimethoxyisoflavone, 7-acetoxy- 4′,8-dimethoxyisoflavone and 3',7-dihydroxy-4′,8-dimethoxyisoflavone from the stem and bark of the plant (Korir et al., 2014).

We previously conducted preliminary screening to evaluate the acaricidal properties of seventeen plant species selected based on their ethnoveterinary use against ticks in South Africa. Extracts of *Calpurnia aurea* subsp. *aurea* had good acaricidal activities against adult *Rhipicephalus turanicus* and larvae of *R. (B.) decoloratus and R. (B.) decoloratus.* However, the bioactive compounds responsible for these observations have not been identified. This study aims to obtain various fractions of *C. aurea* subsp. *aurea*, isolate and characterize the bioactive compounds (in the most efficacious fraction on ticks) using chromatographic and sophisticated spectroscopic techniques respectively.

7.2 Materials and Methods

7.2.1 Plant collection

Calpurnia aurea leaves were collected from the Lowveld National Botanical Garden, Nelspruit, Mpumalanga (GPS coordinates 25.444ºS, 30.970ºE) on 29 January 2015 and a voucher specimen deposited at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria (Specimen no: PRU 121520). The plant material was dried at room temperature (c. 25ºC) for

two weeks in a well-ventilated room. Collection, drying and storage guidelines of the plant material followed were as outlined by McGaw and Eloff (2010).

7.2.2 Plant extraction

Dried and pulverised leaves of *C. aurea* (423.48 g) were extracted with ethanol (4 L) at room temperature with continuous stirring for 1 hour and thereafter filtered through Whatman no. 1 filter paper to remove the residue. This process was repeated twice and the filtrates were combined and concentrated under a vacuum using rotary evaporator (Buchi Rotavapor R-200, Switzerland) to give 44.9 g of dry ethanol extract.

7.2.3 Solvent-solvent fractionation

The ethanol extract was dissolved in a mixture of chloroform and water and fractionated by solvent-solvent extraction to yield hexane (8.7 g), chloroform (26.5 g), butanol (1.3 g), methanol/water (1.8 g) and water (1.2 g) (Eloff, 1998). Each fraction was evaporated to dryness under reduced pressure at low temperature (40-50^{o}C) in a rotary evaporator. The water fraction was dried by vaporisation in an oven at c. 60^{o}C and weighed.

7.2.4 Thin Layer Chromatography (TLC)

Qualitative screening of the *C. aurea* ethanol extract and the fractions obtained was performed to obtain TLC chromatograms (Kotze et al., 2002). Ten microlitres (10 µl) of the extract and fractions were loaded on TLC silicagel 60 F_{254} plates (Merck, Germany) in lines of about 1 cm wide. Separate TLC chromatograms were developed in saturated chambers using mobile phases of varying polarities, namely, ethyl acetate/methanol/water (40:5.4:5) [EMW] (polar/neutral), chloroform/ethyl acetate/formic acid (5:4:1) [CEF] (intermediate polarity/acidic) and benzene/ethanol/ammonia hydroxide (90:10:1) [BEA] (non-polar/basic) (Kotze et al., 2002). Separated components were visualized under ultraviolet (UV) light (wavelength 254 nm; CAMAG universal UV lamp). Subsequently, plates were sprayed with 0.1 g vanillin in 28 ml methanol with 1 ml sulphuric acid and then heated using a heat gun (Steinel type 3484, Romania) at c. 60^{o}C until optimal colour development.

7.2.5 Isolation and characterization of compounds

7.2.5.1 Gravity column chromatography

The chloroform fraction had the highest acaricidal activity against ticks with up to 90% mortality at the highest concentration tested (100 mg/ml) (Fig. 7.7). In order to isolate the active compounds, this fraction was subjected to silica gel column chromatography. The column was eluted with chloroform:methanol gradient (0-100%) and four sub-fractions were collected. Sub-fraction III was refined using further silica gel column chromatography, sub-

fractions collected and combined according to the TLC chromatograms. The process continued until two compounds were obtained in their pure forms.

7.2.5.2 Structural analysis of isolated active compounds

Spectroscopic techniques and High Resolution Mass Spectrometry (HRMS) [heteronuclear multiple bond correlation (HMBC) and H:H correlation spectroscopy (COSY)], were used to elucidate the structures of the two isolated compounds. Compounds **1** and **2** were identified by means of nuclear magnetic resonance (NMR) (1D and 2D) spectroscopic and mass spectrometry data. The proton nuclear magnetic resonance (^{1}H NMR) and two-dimensional NMR (2D NMR) experiments data were acquired on a 400 MHz NMR spectrometer (Bruker Avance III 400 MHz). HPLC-HR-ESI-MS was performed on Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (QTOF) instrument. Chemical shifts were reported with reference to the respective residual solvents or deuterated solvent peaks. Structures of isolated compounds were confirmed by comparison of their NMR data with those in literature.

7.2.6 *In vitro* acaricidal toxicity bioassay

7.2.6.1 Ticks

Adult *Rhipicephalus turanicus* ticks (both sexes) were obtained from Clinvet International, Bloemfontein, South Africa. The ticks were kept at the Phytomedicine laboratory, Faculty of Veterinary Sciences, University of Pretoria in glass humidity chambers at an average temperature of 25^{o}C±1^{o}C. Relative humidity was maintained at 75±10% by placing supersaturated sodium chloride solution in the glass chamber. The ticks were stored in vials covered with cotton mesh (to allow for air exchange) and set on a square glass plate placed at the base of the chamber on four small bearings. The four sides of the glass plate were at a distance of 1.5 cm from the wall of the humidity chamber to prevent the ticks from reaching the walls.

7.2.6.2 Experimental protocol

Two-fold graded increasing concentrations (3-100 mg/ml) of the more non-polar fractions (chloroform, hexane and butanol) were used for adult immersion tests (Al-Rajhy et al., 2003). Ten adult ticks were immersed in each test sample for one minute, dried over Whatman no. 1 filter papers and kept at room temperature (25±1^{o}C) and 85% relative humidity in 20 ml glass vials closed with a perforated stopper. Graded two-fold increasing concentrations of cypermethrin (0.2-5 mg/ml) and acetone served as the positive control and negative control respectively. Each extract concentration was tested in triplicate on each of three different occasions to yield nine replicates. Percentage mortality was determined 24 hours post

treatment by viewing the ticks under a stereo microscope (American Optical Corporation). Ticks were recorded as alive and active if they exhibited normal behaviour on exposure to carbon dioxide (CO_2) from human breath (host-associated stimulus) or after being physically stimulated with plastic tweezers. Those showing some difficulty in movement or being able to maintain normal posture were termed weak and those in which there was loss of righting reflex (the reflex that corrects the orientation of the body when it is taken out of its normal upright position) were termed very weak. Ticks were confirmed dead based on signs of cuticle darkness, halted Malpighian tubules movement and haemorrhagic skin lesions. This bioassay was repeated in a subsequent experiment using the isolated compounds (0.06-1 mg/ml). Each concentration was tested in duplicate and the experiment was done once due to the limited quantity of the isolated compounds available.

7.2.7 Cytotoxicity (*in vitro* safety) bioassay

7.2.7.1 Maintenance of cell lines

Vero monkey kidney (ATCC® CCL-81™) and HepG2 human liver cancer (ATCC® HB-8065™) cells were purchased from Cellonex, South Africa. Vero cells were maintained in Minimal Essential Medium (MEM, Whitehead Scientific, South Africa) containing 4.5 g/l glucose and 4 mM L-glutamine supplemented with 1% gentamicin and 5% foetal calf serum (FCS, Highveld Biological, South Africa) while HepG2 cells were maintained in Dulbecco's Minimal Essential Medium (DMEM, Whitehead Scientific, South Africa) containing 4.5 g/l glucose and 4 mM L-glutamine supplemented with 10% FCS. Both cells were maintained at 37ºC in a 5% CO_2 incubator (Hera Cell 150, Germany). Cells were passaged three times weekly by trypsinization with trypsin/ethylenediaminetetraacetic acid solution (Invitrogen, Cergy-Pontoise, France) into 175 cm^2 culture flasks.

7.2.7.2 Experimental protocol

Viable cell growth after incubation of Vero and HepG2 cells with test samples (*C. aurea* ethanol extract and the five fractions obtained) was determined using the tetrazolium-based colorimetric MTT assay [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] described by Mosmann (1983). Cells of a subconfluent culture were harvested and centrifuged at 200 x g for 5 minutes and resuspended in a growth medium (MEM for Vero cells and DMEM for HepG2 cells) to 10^5 cells/ml. One hundred microliters (100 µl) of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate, then 200 µl of MEM was added to wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated overnight at 37ºC in a 5% CO_2 incubator to allow cells to attach at the bottom of the plates.

Test samples were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 100 mg/ml and then diluted 100-fold in culture medium for the concentration tested (1000 µg/ml). Hundred microliters (100 µl) of decreasing concentrations of the test samples (1000, 750, 500, 250, 100, 50 µg/ml) were added in the corresponding wells and the plates were again incubated at 37ºC for 48 hours in the 5% CO_2 incubator. Untreated cells served as the negative control while doxorubicin hydrochloride (Pfizer Laboratories, South Africa) served as the positive control.

After incubation, the growth medium was aspirated from the cells which were washed with 200 µl phosphate buffered saline (PBS, Whitehead Scientific,) and replaced with 200 µl of fresh medium and 30 µl MTT (Sigma-Aldrich, South Africa; stock solution of 5 mg/ml in PBS). The plates were incubated further for 4 hours at 37ºC in the 5% CO_2 incubator. After this, the growth medium in each well was carefully removed using a suction pump (Integra, USA), without disturbing the MTT formazan crystals. These crystals were dissolved by adding 50 µl DMSO to each well and shaken gently for 2 minutes. The MTT reduction was measured immediately by detecting absorbance in a spectrophotometer (Biotek Synergy, USA) at a wavelength of 570 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The lethal concentration of test samples resulting in a 50% reduction of absorbance (LC_{50}) compared to untreated cells was calculated. Each extract concentration was tested in quadruplicate and the assays repeated three times. This bioassay was repeated in a subsequent experiment using the isolated compounds (5-100 µg/ml).

7.2.8 Data analysis

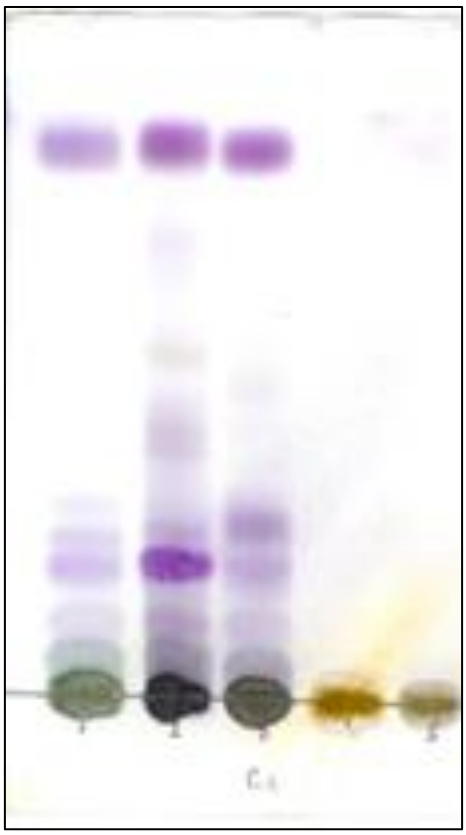
Data on tick mortality and cytotoxicity were presented as the arithmetic mean values±standard error of mean (Mean±SEM). Significance was analysed using one-way analysis of variance followed by Tukey's multiple comparison test on GraphPad Prism 7.02 (GraphPad Software, San Diego-CA, USA). Values were considered to differ statistically when $p \leq 0.05$. The dose-response graphs of the acaricidal efficacies of the fractions and isolated compounds were plotted and LC_{50} determined using the linear regression model.

7.3 Results

7.3.1 Solvent-solvent fractionation and thin layer chromatography

The crude ethanol extract of the leaves of *C. aurea* subjected to solvent-solvent fractionation gave hexane, chloroform, butanol, methanol/water and water fractions with yields of 19, 59, 3, 4 and 3% respectively. Thin layer chromatogram of *C. aurea* crude extract and fractions

developed in BEA system and sprayed with vanillin sulphuric acid showed varied chemical constituents of the extract and fractions (Fig. 7.1).



1 - Crude extract; 2 - Chloroform fraction; 3 - Hexane fraction, 4 - Butanol fraction; 5 - Water fraction

Figure 7.1: Thin Layer chromatogram of *Calpurnia aurea* crude extract and fractions developed in BEA system and sprayed with vanillin sulphuric acid showing varied chemical constituents of the extract and fractions

7.3.2 Isolation of apigenin-7-*O*-β-D-glycoside (1) and isorhoifolin (apigenin-7-O-rutinoside) (2)

Apigenin-7-*O*-β-D-glycoside (**1**) was isolated as a yellow amorphous powder, which gave a strongly UV absorbing band on TLC at 254 nm and turned to yellow with vanillin reagent (Fig. 7.2). Isorhoifolin (**2**) was isolated as a light-yellow, amorphous, compound which gave a strongly UV absorbing band on TLC at 254 nm and turned to yellow with vanillin reagent (Fig. 7.2).

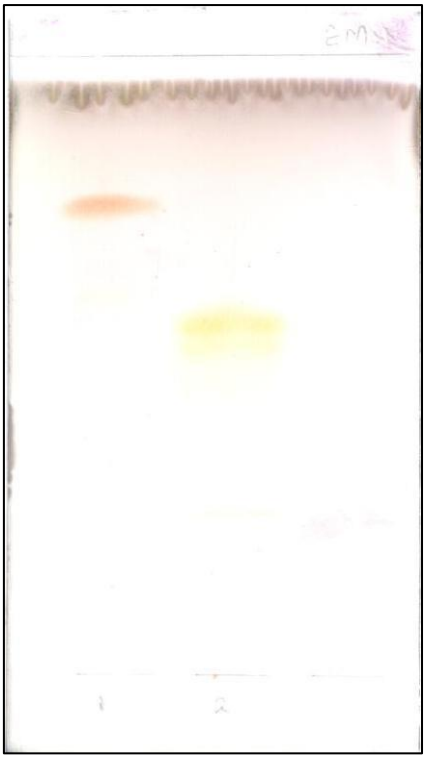


Figure 7.2: Thin Layer chromatogram developed in EMW system and sprayed with vanillin sulphuric acid showing compound (1) with retention factor of 0.81 and compound (2) with retention factor 0.54 isolated from *Calpurnia aurea*

7.3.3 Structural elucidation of apigenin-7-O-β-D-glycoside (1) and isorhoifolin (apigenin-7-O-rutinoside) (2)

7.3.3.1 Apigenin-7-O-β-D-glycoside (1)

The molecular formula of **1** was determined to be $C_{21}H_{20}O_{10}$ as derived from its negative electrospray ionization mass spectrometry (ESIMS) (*m/z* 431.11 $[M-H]^-$,863.2 $[2M-H]^-$) (Fig. 7.3).

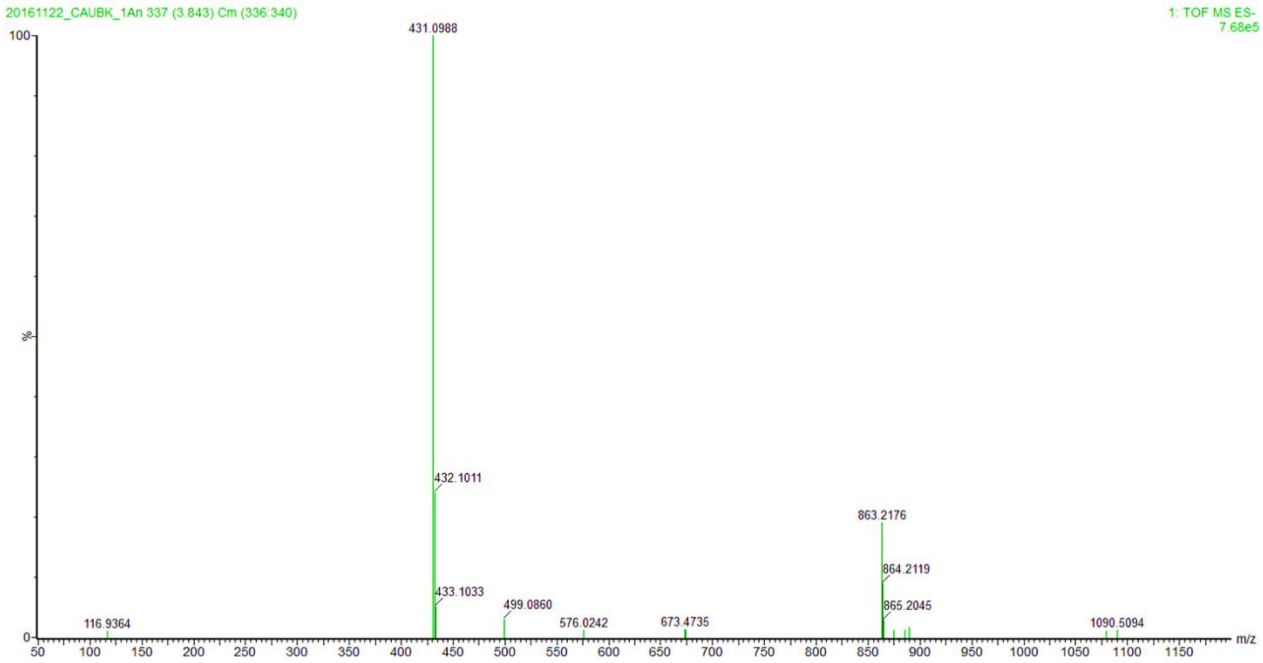


Figure 7.3: Electrospray ionization mass spectrometry spectrum for apigenin-7-*O*-β-D-glycoside (1) isolated from *Calpurnia aurea*

The ^{1}H NMR and ^{13}C NMR spectra showed similar aromatic patterns to compound **2**, where five aromatic proton and carbon signals established rings A, B and C. The ^{13}C NMR spectrum delivered nineteen carbon signals, indicating the presence of twelve degrees of unsaturation. The peak at *m/z* 271.1 $[M+H]^{+}$ from positive ESIMS confirmed the presence of a flavone skeleton. Correspondingly, an anomeric proton at δ 4.92 (H-1") displayed strong 3*J* HMBC with a quaternary carbon at C-7. Further COSY and HMBC correlations as listed in Fig. 7.4 designated compound **1** as apigenin-7-*O*-β-D-glycoside. With respect to the coupling constant of the anomeric proton (*J* = 7.3 Hz), the sugar is β-connected and should have (D)-configuration, according to the Klyne rule (Klyne, 1950).

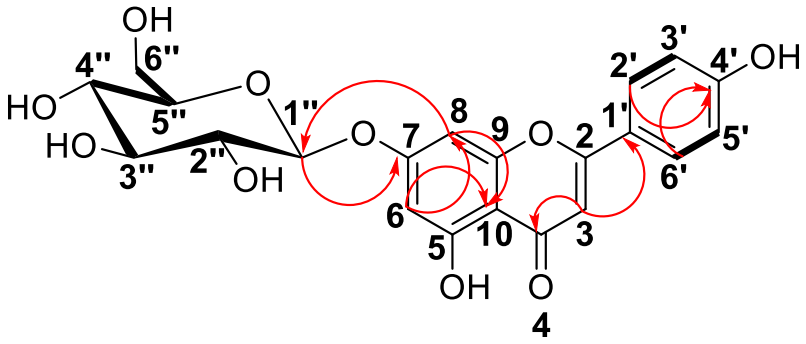


Figure 7.4: Selected H, H COSY (bold lines) and HMBC (arrows) correlations of apigenin-7-*O*-β-D-glycoside (1) isolated from *Calpurnia aurea*

A search in the Dictionary of Natural Products (Chapman & Hall, 2017) and comparing the spectroscopic data with the literature confirmed the structure as apigenin-7-*O*-β-D-glycoside (**1**).

Apigenin-7-*O*-β-D-glycoside (**1**)

Light yellow powder (MeOH); ESIMS m/z: 431.11 $[M-H]^-$; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.95 (2H, d, *J* = 8.7 Hz, H-2', 6'), 6.95 (2H, d, *J* = 8.7 Hz, H-3', 5'), 6.87 (1H, s, H-3), 6.83 (1H, s, H-8), 6.45 (1H, s, H-6), 4.92 (1H, d, *J* = 7.3 Hz, H-1''), 3.71 (1H, d, *J* = 11.2 Hz, Ha-6''), 3.51 (1H, d, *J* = 11.2 Hz, Hb-6''), 3.33 (1H, m, H-5''), 3.31 (m, H-2''), 3.28 (m, H-3''), 3.20 (m, H-4''); ^{13}C NMR (125 MHz, DMSO-d_6, δ (ppm): 182.0 (C-4),165.5 (C-2), 163.5 (C-7), 162.7 (C-4'), 162.5 (C-5), 157.5 (C-9), 129.5 (C-2', 6'), 121.5 (C-1'), 116.5 (C-3', 5'), 106.0 (C-10), 103.5 (C-3), 100.2 (C-1''), 100.0 (C-6), 95.8 (C-8), 77.3 (C-3''), 77.0 (C-5''), 73.6 (C-2''), 70.0 (C-4''), 61.0 (C-6'').

7.3.3.2 Isorhoifolin (apigenin 7-O-rutinoside, 4',5,7-trihydroxyflavone 7-O-rutinoside) (2)

The ^{1}H NMR spectrum (Fig. 7.5) of **2** displayed five aromatic proton signals at δ 7.95 (H-2', 6'), 6.95 (H-3', 5'), 6.87 (H-3), 6.77 (H-8) and 6.45 (H-6). Additionally nine oxymethine, one oxymethylene and one methyl signals appeared at δ 5.06 (H-1''), 4.54 (H-1'''), 3.84 (Ha-6''), 3.64 (bs, H-2'''), 3.59 (H-5''), 3.41 (Hb-6'', H-3'''), 3.46 (m, H-5'''), 3.30 (H-2''), 3.28 (H-3''), 3.15 (H-4''), 3.13 (m, H-4'''), 1.07 (3H-6'''). The ^{13}C NMR spectrum revealed eight quaternary carbons (Cq) at δ 182.0 (C-4), 165.5 (C-2), 163.5 (C-7), 162.0 (C-4'), 161.5 (C-5), 157.5 (C-9), 121.5 (C-1') and 106.0 (C-10), in addition to five aromatic methine signals at 129.5 (C-2', 6'), 116.5 (C-3', 5'), 104.0 (C-3), 100.0 (C-6), 95.5 (C-8). Moreover, nine oxymethine signals were visible at δ 101.0 (C-1'''), 100.5 (C-1''), 77.0 (C-3''), 76.5 (C-5''), 74.0 (C-2''), 72.5 (C-4'''), 71.5 (C-3'''), 71.0 (C-2'''), 70.5 (C-4'') and 69.0 (C-5'''). One oxymethylene carbon appeared at δ 66.0 (C-6'') and one methyl carbon at 17.8 (C-6'''). The ESIMS afforded a pseudomolecular ion peak at 577.15 $[M-H]^-$, which gave the molecular weight 578 and the molecular formula $C_{27}H_{30}O_{14}$ by high resolution electrospray ionization mass spectrometry (HRESIMS). The 1D and 2D data obtained for compound **2** showed typical signals for flavone as well as correlations confirming the flavone skeleton.

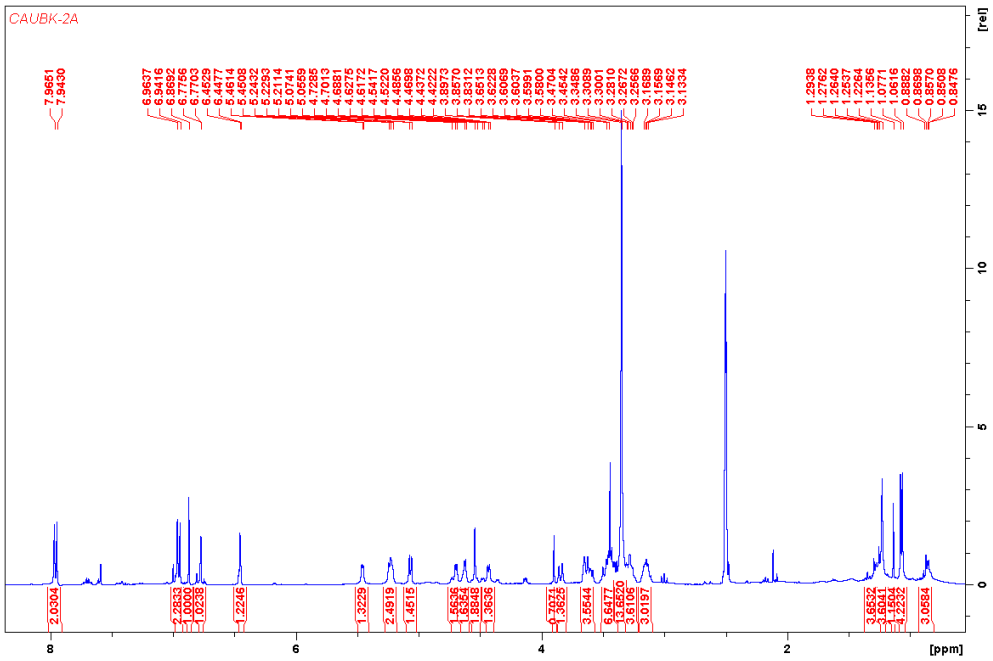


Figure 7.5: 1H NMR spectrum (DMSO-d6, 400 MHz) of isorhoifolin (2) isolated from *Calpurnia aurea*

H,H COSY correlations and the signal pattern indicated the presence of a 1,4-disubstituted benzene ring B (Fig. 7.6). Ring A bears one hydroxyl (OH) group and its position can be distinguished by HMBC correlations (Fig. 7.6): The proton at C-8 in ring A showed a weak 4*J* correlation with C-5 and the carbonyl (at C-4). Moreover, a proton at C-6 displayed strong 3*J* with C-8. Correspondingly, further H,H COSY and HMBC correlations confirmed the presence of two sugar moieties (Fig. 7.6). The proton signal at (H-1'') showed strong 3*J* correlation with quaternary carbon at C-7 and an anomeric proton signal at C-1''' gave strong 3*J* HMBC correlation with a methylene carbon at (C-6''). This resulted in the structure of isorhoifolin (**2**).

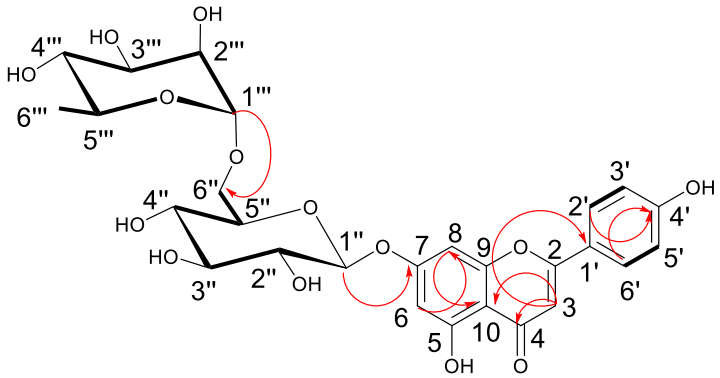


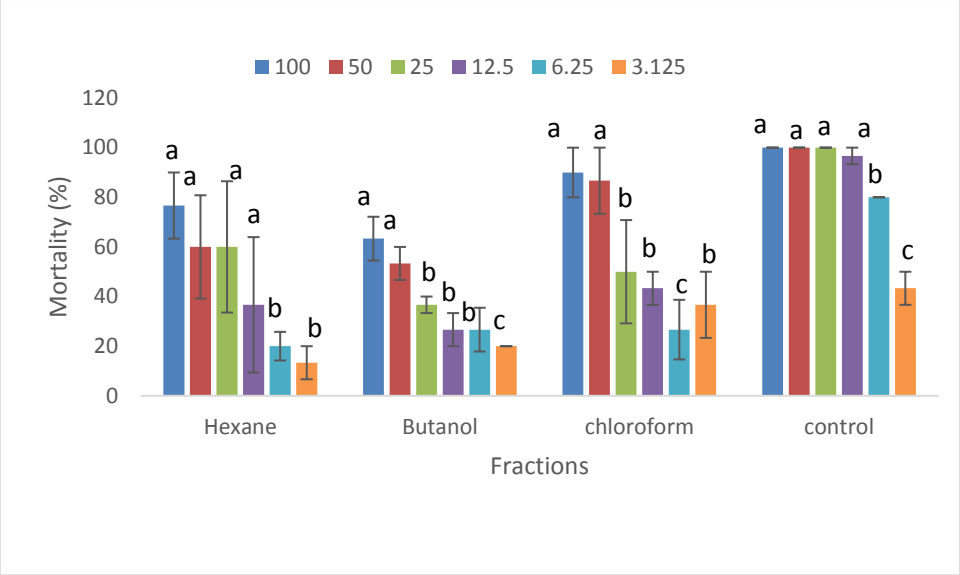
Figure 7.6: Selected H,H COSY (bold lines) and HMBC (arrows) correlations of isorhoifolin (2) isolated from *Calpurnia aurea*

A literature search in the Dictionary of Natural Products (Chapman & Hall, 2017) and the Chemical Abstracts Services (SciFinder) confirmed the structure of isorhoifolin (apigenin-7-O-rutinoside) (**2).**

Isorhoifolin (**apigenin 7-O-rutinoside, 4',5,7-trihydroxyflavone 7-O-rutinoside**)**.** Yellow powder (MeOH); ESIMS m/z: 577 $[M-H]^-$; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.95 (2H, d, *J* = 8.7 Hz, H-2', 6'), 6.95 (2H, d, *J* = 8.7 Hz, H-3', 5'), 6.87 (1H, s, H-3), 6.77 (1H, s, H-8), 6.45 (1H, s, H-6), 5.06 (1H, d, *J* = 7.3 Hz, H-1''), 4.54 (1H, s, H-1'''), 3.84 (1H, d, *J* = 10.9 Hz, Ha-6''), 3.64 (1H, brs, H-2'''), 3.59 (1H, m, H-5''), 3.41 (m, (Hb-6'', H-3'''), 3.46 (m, H-5'''), 3.30 (m, H-2''), 3.28 (m, H-3''), 3.15 (m, H-4''), 3.13 (m, H-4'''), 1.07 (3H, d, *J* = 6.1 Hz, H-6'''); ^{13}C NMR (125 MHz, DMSO-d_6, δ (ppm): 182.0 (C-4),165.5 (C-2), 163.5 (C-7), 162.0 (C-4'), 161.5 (C-5), 157.5 (C-9), 129.5 (C-2', 6'), 121.5 (C-1'), 116.5 (C-3', 5'), 106.0 (C-10), 104.0 (C-3), 101.0 (C-1'''), 100.5 (C-1''), 100.0 (C-6), 95.5 (C-8), 77.0 (C-3''), 76.5 (C-5''), 74.0 (C-2''), 72.5 (C-4'''), 71.5 (C-3'''), 71.0 (C-2'''), 70.5 (C-4''), 69.0 (C-5'''), 66.0 (C-6''), 17.8.0 (C-6''').

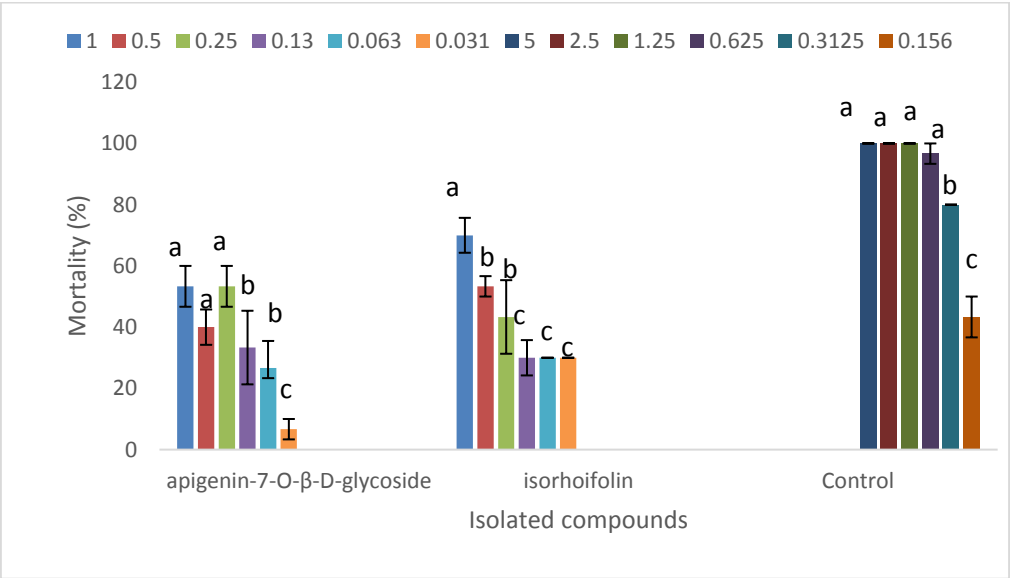
7.3.4 Acaricidal activity

The chloroform fraction of *C. aurea* was the most active fraction with tick mortality of 90% at the highest concentration (100 mg/ml) followed by hexane fraction (77%) and butanol fraction (63%), while the positive control (5 mg/ml cypermethrin) gave 100% mortality (Fig. 7.7). There was a dose-dependent mortality with LC_{50} values of 28.64, 21.53 and 65.09 mg/ml respectively. The two flanonoids, apigenin-7-*O*-β-D-glycoside and isorhoifolin, isolated from the chloroform fraction of *C. aurea* at concentration of 1 mg/ml caused tick mortality of 53% and 70% respectively with LC_{50} values around 1 and 0.65 mg/ml respectively comparable to the positive control (cypermethrin) with LC_{50} value of 0.06 mg/ml (Fig. 7.8).



Positive control - Cypermethrin (0.16-5 mg/ml); fractions (3.13-100 mg/ml); a - Statistically different from positive control (p<0.05); b - Statistical difference between the extracts (p<0.05); Extracts with the same letter are not statistically different from each other.

Figure 7.7: Acaricidal activity of *Calpurnia aurea* fractions against *Rhipicephalus turanicus* ticks after 24 hours



Positive control - Cypermethrin (0.16-5 mg/ml); isolated compounds (0.09-1 mg/ml); a - Statistically different from positive control (p<0.05); b -Statistical difference between the extracts (p<0.05); Extracts with the same letter are not statistically different from each other.

Figure 7.8: Acaricidal activity of the two isolated compounds from *Calpurnia aurea* (apigenin-7-*O*-β-D-glycoside and isorhoifolin) against *Rhipicephalus turanicus* ticks after 24 hours

7.3.5 Cytotoxicity

For toxicity, we used a cutoff of 100 µg/ml as an indicator for toxicity (Nondo et al., 2015). Based on this criterion, most of the fractions and isolated compounds were non-toxic to Vero and HepG2 cells, with the exception of the chloroform fraction with LC_{50} of 76 µg/ml on HepG2. In comparison, the positive control had LC_{50} of 5.03±6.39 and 0.18±0.05 µM against Vero and HepG2 cells respectively (Table 7.1). The crude extract, chloroform fraction, hexane fraction, apigenin-7-*O*-β-D-glycoside and isorhoifolin were more toxic to the HepG2 than the Vero cells (LC_{50}-123 and 623 µg/ml; 76 and 428 µg/ml; 337 and 499 µg/ml; 73 and >100 µg/ml; 72 and >100 µg/ml) respectively (Table 7.1).

Table 7.1: Cytotoxicity (LC_{50} in µg/ml) of the crude extract, fractions and isolated compounds from *Calpurnia aurea* against Vero and HepG2 cells

***Calpurnia aurea* plant**	**Cytotoxicity LC_{50} (µg/ml)±SEM**	
	Vero cells	**HepG2 cells**
Crude extract	623±4	123±11*
Chloroform fraction	428±9	76±13*
Butanol fraction	>1000	>1000
Hexane fraction	499±10	337±5*
Water fraction	>1000	>1000
Methanol/Water fraction	>1000	934±6
Apigenin-7-*O*-β-D-glycoside	>100	73±1*
Isorhoifolin	>100	72±5*
Doxorubicin (positive control)	5.03±6.4	0.18±0.1

**Statistically different ($p<0.05$), Graphpad Instat 3.0 Student-Newman-Keuls used to analyse the difference of the same extract on two different cell lines; Dark green - LC_{50} value greater than 1000 µg/ml (very safe), 100 µg/ml for isolated compounds; Green - LC_{50} value 500-1000 µg/ml (safe), 50-100 µg/ml for isolated compounds; Yellow - LC_{50} value 100-500 µg/ml (slightly cytotoxic); Red - LC_{50} value less than 100 µg/ml (cytotoxic)*

7.4 Discussion

Solvent-solvent fractionation of the crude ethanol extract of the leaves of *C. aurea* gave five fractions (hexane, chloroform, butanol, methanol/water and water) based on solubility characteristics of the constituents. The differences in the chemical constituents of the fractions as seen on the thin layer chromatogram could lead to multiple mechanisms of action if more than one active compound is present. The choroform fraction had the highest number of visible bands, making isolation more possible.

The choroform fraction afforded two acaricidal compounds, **1** and **2**. These compounds were identified as apigenin-7-*O*-β-D-glycoside (**1**) and isorhoifolin (apigenin-7-O-rutinoside) (**2**). Apigenin-7-*O*-β-D-glycoside is a glycosyloxyflavone, that is, apigenin substituted by a β-D-glucopyranosyl moiety at position 7 via a glycosidic linkage. This compound, while identified

for the first time from *C. aurea*, has been previously identified in other plants such as *Pteris multifida* Poir. (Pteridaceae), *Marrubium globosum* Montbret & Aucher ex Benth (Lamiaceae), *Elsholtzia splendens* Nakai ex F.Maek. (Lamiaceae), *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae), *Lonicera gracilipes* var glandulosa (Caprifoliaceae) and *Humulus scandens* L. (Cannabaceae) (Chen et al., 2011; Wölwer-Rieck, 2012; Peng et al., 2016). Isorhoifolin, has been found in many *Citrus* species such as *Citrus paradisi* Macfad (Rutaceae), *Mentha* species (Lamiaceae), *Teurium polium* var *gnapholodes* (Lamiaceae) and *Cynara scolymus* L. (Cynareae) (Nassar et al., 2013; Boghrati et al., 2016; Hawryl et al., 2016). It may be interesting to determine if these plant species also have acaricidal activities.

Flavonoids such as quercetin and luteolin have previously been demonstrated to behave as developmental regulators/signaling molecules as well as interacting with specific proteins central to intracellular signaling cascades (Brunetti et al., 2013). Tereschuk et al. (1997) reported the antimicrobial activity of a flavonoid (quercetagetin-7-arabinosyl-galactoside) isolated from *Tagetes minuta* L. The antifungal activity of a prenylated flavanone (5,7,4 -trihydroxy-8- methyl-6-(3-methyl-[2-butenyl])-(2S)-flavanone) isolated from the shrub *Eysenhardtia texana* Scheele was also reported (Wächter et al., 1999). Critchfield et al. (1996) reported the antiviral properties of the flavonoids, chrysin, acacetin and apigenin via the inhibition of viral transcription. Other biological properties reported are prevention of coronary heart disease, inhibition of cancer cells proliferative activity, anti-oxidant, anti-allergic and anti-inflammatory properties (Chen et al., 2012).

Flavonoids have also been reported to have significant antiparasitic activities. Their effects on arthropods could be repellency, inhibition of oviposition, inhibition of feeding, developmental disorders, deformation, infertility or death (Dantas et al., 2015). The potential role of flavonoids in the modulation of reproductive activity of ticks was reported by Ravindran et al. (2011b) and Juliet et al. (2012), where 1.56 mg/ml of *Leucas aspera* L and 50 mg/ml of *Jatropha curcas* L. produced 100 and 90% failure of eclosion of eggs respectively. This was attributed to acacetin and apigenin isolated from *L. aspera* while the flavones; apigenins (apigenin 7-O-β-D-neohesperidoside, apigenin 7-O-β-D-galactoside), orientin, vitexin, vicenin II and the biflavone di-C-β-Dglucopyranoside-methylene-(8,8')-biapigenin were isolated from the leaves of *J. curcas* (Abd-Alla et al., 2009). Flavonoids such as luteolin, quercetin, apigenin and chrysin were reported to inhibit ecdysone mediated gene expression in an ecdysone responsive cell line, CL8+ (Oberdörster et al., 2001). Recently, quercetin was also reported to produce 50% egg hatching inhibition in *R. (B.) annulatus* ticks (Ravindran et al., 2017). The efficacy of the plant extracts could thus be due to the presence of flanonoids such as apigenin which can cause decreased levels of ecdysteroids into the

eggs by inhibiting the cytochrome P450 isozyme expression and activity. This could lead to interference with the uptake of modified egg yolk protein, vitellin into the oocytes both of which are important for egg maturation and development.The presence of apigenin and luteolin in *Lippia javanica* Burm F. Spreng tested at 50 mg/ml may have contributed to the 92% acaricidal toxicity in adult ticks reported by Madzimure et al. (2011). The synergistic effect of the apigenin derivatives; apigenin-7-*O*-β-D-glycoside and isorhoifolin isolated from *C. aurea* in this study may be responsible for the 93% acaricidal toxicity against *R. turanicus* ticks we observed for the ethanol crude extract.

Chitin and protein make up 95% of the tick cuticle while the remaining 5% is largely made up of lipids which control the movement of water and other molecules through the cuticle. To penetrate the cuticle, chemicals must first dissolve in the lipid (non-polar) layer and then move into the more polar layers. Thus a chemical's lipid solubility and its partition between lipid-water phases determine its rate of absorption through the cuticle (Oberchain & Galun, 2013). The greater acaricidal activity of isorhoifolin may be due to the nature of the attached double sugar moieties at the R_5 unit (Fig. 7.6) as opposed to apigenin-7-O-β-D-glycoside which has a single hexose sugar moiety at the R_5 unit (Fig. 7.4). Also, isorhoifolin is a glycone which appears to be better absorbed through the lipid-water phases than apigenin-7-O-β-D-glycoside, which is a glycoside. Xiao (2017) reported that *O*-glycosylation generally seems to reduce the bioactivity of flavonoids. The presence of the benzene rings and α-L-rhamnopyranose**,** a deoxy sugar, in isorhoifolin, which occurs in nature in its L-form as opposed to most of the naturally occurring sugars, which are in D-form, may also be responsible for the enhanced activity of isorhoifolin. The cell membrane permeabilization property of α-L-rhamnopyranose has been described (Gauthier et al., 2009) and this may explain possible penetration through the lipid and lipid-water phases of the tick cuticle. Existing structure-activity data and genetic manipulation.of flavonoid biosynthetic pathways may soon allow structure-based design of structural analogues for active flavones as acaricidal agents (Gerwick & Sparks, 2014).

Terpenes such as azadirachtin isolated from *Azadirachta indica* Juss. and nootkatone from *Cupressus nootkatensis* D.Don have been reported to have strong repellent, insecticidal and ixodicide effects (Ruiz-Vásquez et al., 2016) and more flavonoids with acaricidal activities such as apigenin, kaempferol, luteolin and quercetin are being discovered. The LC_{50} value of 0.65 mg/ml for isorhoifolin compares favourably with some known acaricidal compounds isolated from plants. The acaricidal activity of carvacrol (LC_{50}=0.22 and 4.46 mg/ml) and thymol (LC_{50}=3.86 and 5.50 mg/ml) against larvae and engorged females of *R. (B.) microplus* respectively (De Oliveira-Cruz et al. 2013); menthol, geraniol, linalool and

eucalyptol (1,8-cineole) with LC_{50} values of 0.13, 0.22, 0.50 and 0.51 mg/ml respectively against the two-spotted spider mite, *Teranychus urticae* Koch. (Badawy et al. 2010) and inhibition of egg hatching of azadirachtin [LC_{50}=5000ppm (5 mg/ml)] (Giglioti et al. 2011) have been reported. This makes flavonoids an interesting group of study molecules for acaricidal activity (Ribeiro et al., 2015). When compared with currently used synthetic acaricides and insecticides, isorhoifolin, a flavone, compares relatively lower. Reported LC_{50} values include DDT (LC_{50}=36.8 mg/ml) (Camerino, 2015); fluralaner (LC_{50}=0.28 mg/ml) (Williams et al., 2015); coumaphos (LC_{50}=0.39 mg/ml) (Singh et al., 2014); spinosad (LC_{50}=0.11 mg/ml) (Kovendan et al., 2012); propoxur (LC_{50}=0.039 mg/ml) (Camerino, 2015); cypermethrin (LC_{50}=0.005 mg/ml) (Singh et al., 2014) and amitraz (LC_{50}=0.001 mg/ml) (Malan, 2015).

In addition to the effect of the compounds on the ticks, another important consideration is physical tick damage. During haematophagy by ticks, small blood vessels of the hosts' skin are lacerated and cells are ruptured. Haemorrhage occurs causing activation of the platelets mediated by signal transduction of the receptors that activate the cyclooxygenase (COX), lipooxygenase (LOX) and phospholipase C pathways, or inhibits adenylyl cyclase leading to blood clot at the site of damage. There is also the activation of the host's defence mechanisms to the foreign proteins in tick saliva (Ribeiro & Francischetti, 2003). These events ultimately lead to inflammation and irritation associated with tick burden. Flavonoids are known to interfere with different stages of the arachidonate cascade via COX or LOX pathways to alleviate inflammatory responses (Politi et al., 2016). The presence of a catechol group is fundamental to the inhibition of COX-2 (main enzyme responsible for inflammation and pain). The number of OH groups on the B ring appears to be related to a molecular conformation that influences the interactions between flavonoids and enzymes such as tyrosine kinase and protein kinase C, which are involved in the transcriptional activity of COX-2 (Hou et al., 2005). Active flavonoids are substituted in B ring, presenting a catechol group or a methoxyl and an OH group and, in general, they are substituted in A ring with a OH group in positions 5 and 7. Apigenin, which possesses a C2-C3 double bond and 5,7-dihydroxyl groups in the A ring has already been described to have inhibitory effects on prostaglandin E_2 production in a macrophage cell line J774A.1 (Raso et al., 2001).

Elisha et al. (2016), reported that *C. aurea* had very good inhibitory activity against the 15-LOX enzyme, with IC_{50} value 34.70 µg/ml which was better than the positive control, quercetin (IC_{50}=53.69 µg/ml). The development of drugs that inhibit both COX-2 and LOX may lead to compounds with enhanced efficacy, fewer side effects and broader spectrum

of activity, when compared with selective COX-2 inhibitors such as non steroidal anti-inflammatory agents (Martel-Pelletier et al., 2003). Flavonoids with a catechol group in B ring, such as luteolin have already proven to be very good inhibitors of human 5-LOX (Ribeiro et al., 2014). This explains that the presence of apigenin derivatives, (apigenin-7-O-β-D-glycoside and isorhoifolin) in *C. aurea* used as decoctions for topical application for tick control may also hasten the resolution of inflammatory processes. Apigenin-enriched formulations have been reported to show great promise as anti-inflammatory agents (Arsić et al., 2011).

In order to verify the safe use of the fractions and isolated compounds from *C. aurea*, cytotoxicity tests were undertaken against two mammalian cell lines. The test samples were tested on Vero cells, isolated from kidney epithelial cells extracted from an African green monkey (*Cercopithecus aethiops*). These are usually the first line of cells used for cell cytotoxicity studies (Osada et al., 2014). HepG2, a liver cell line derived from a human hepatoblastoma was also used. This has been found to express a wide variety of functions that are known as liver tissue-specific pathways which are responsible for xenobiotic (foreign chemical substance) metabolism, detoxification and removal (Gomez-Lechon et al., 2008). HepG2 cell line is thus a suitable model to study liver metabolism, toxicity of xenobiotics and drug targeting (Maurya & Vinayak, 2015). It was observed that the crude extract, chloroform fraction, hexane fraction, apigenin-7-*O*-β-D-glycoside and isorhoifolin were more cytotoxic to the HepG2 than the Vero cells. This could potentially indicate the possibility of hepatotoxicity in the treated animals. It may even be possible, that due to the metabolic activity of the HepG2 cells, compound(s) within the extract or the pure compound were being metabolised into more toxic metabolites.

7.5 Conclusions

New classes of acaricides are urgently required and the flavonoids represent a novel set of leads. The optimization of these compounds through the modification of their structures to increase activity and reduce toxicity may allow for the development of a pharmacologically acceptable natural acaricide. In addition, investigation into the mechanism of action of these compounds is likely to be a productive area of research. Such information will provide a focus for toxicological attention and aid in the anticipation of resistance. Also, characterization of the interaction between acaricidal flavonoids and their target sites could potentially aid in the design of second generation inhibitors.

CHAPTER 8

Preliminary studies on the possible mechanism of action of *Calpurnia aurea* subsp. *aurea* (Fabaceae) leaf extract and isolated compounds

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Preface

The potential mechanisms of action of *Calpurnia aurea* extract, especially the two compounds that were isolated for the first time, needed to be further evaluated to help in understanding the possible mechanism of action. *In vitro* assay using acetylcholinesterase inhibition assay as well as *ex vivo* assay on cholinergic receptors and ion channels using isolated rat ileum as a model were undertaken. The text in this chapter has been prepared for submission to the Journal of Ethnopharmacology.

Abstract

Plant extracts used for tick control are an alternative to chemical acaricides. Previous studies have reported the acaricidal activity of leaf extracts of *Calpurnia aurea*. However, the possible mechanism of action for the acaricidal properties of *Calpurnia aurea* has not been validated. Pilot studies to investigate the mechanism of action of the crude extract and isolated compounds from *Calpurnia aurea* were undertaken *in vitro* using the Ellman's acetylcholinesterase inhibition assay and *ex vivo* using isolated tissue bath studies. The crude extract of *Calpurnia aurea* caused only 40% acetylcholinesterase inhibition while the butanol fraction caused 90% acetylcholinesterase inhibition. The chloroform fraction from which two bioactive compounds, apigenin-7-*O*-β-D-glycoside and isorhoifolin, were isolated did not inhibit acetylcholinesterase. The crude extract and isolated compounds, however, showed a trend towards a dose-dependent inhibition of potassium chloride-induced contractions in the rat ileum. Synergism of different phytochemicals in *Calpurnia aurea* may be responsible for its multiple mechanisms of action.

8.1 Introduction

Despite some novel strategies such as the development of anti-tick vaccines adopted for tick control, topical application of synthetic, chemical acaricides is still the most used method. This is because there is variation in vaccine efficacy in different geographical regions of the world, vaccines offer short term protection and are quite expensive for resource-limited farmers (Said et al., 2012). Many of the synthetic chemical acaricides presently and likely in the future, target components within the central or peripheral nervous system. Several mechanisms of action such as blocking of the neurotransmitter receptors have been

suggested for the acaricidal effects of many phytochemicals (Rattan, 2010). However, the possible mechanism of action for the acaricidal properties of *Calpurnia aurea* has not been validated.

The central nervous system (CNS) of ticks, termed the synganglion, is a highly condensed and fused nerve mass covered by a vascular perignanglionic sheath. The sheath encloses a perignanglionic sinus which controls the supply of fresh, filtered haemolymph from the heart to the synganglion (Lees & Bowman, 2007). The oesophagus runs through the synganglion and divides it into two parts: the supraoesophageal region that lies anterior and dorsal to the oesophagus and the suboesophageal region that lies posterior and ventral to the oesophagus. Neurosecretory cells are present throughout the synganglion and the suboesophageal ganglion contains the larger number of neurosecretory regions and cells (Lees & Bowman, 2007).

The cholinergic system has been amply chemically validated to exist in ticks. As in vertebrates, the cholinergic system is associated with two receptors namely the nicotinic acetylcholine receptor (nAchR) and the muscarinic acetylcholine receptor (mAchR). Historically, *Nicotiana tabacum* L. (tobacco extracts) containing nicotine were used as insecticides, indicating the presence of nAChRs in the tick synganglion (Meinke, 2001). The nAChRs are the primary target of the natural product-derived spinosyns (Sparks et al., 2001). Binnington & Rice (1982) showed that pilocarpine, a mAChR agonist, produced a marked increase in action potential frequency in haemal and salivary nerves in *Rhipicephalus* (*Boophilus*) *microplus,* suggesting the presence of mAChRs in the CNS. Acetylcholinesterase (AChE) is the target site for the organophosphates that act as AChE inhibitors and are potent tick control agents (Lees & Bowman, 2007).

In vertebrates and invertebrates (arthropods), γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter that acts to open the pentameric transmembrane chloride channel within the GABA receptor at neuromuscular junctions and synapses in the CNS (Bloomquist, 2002). It is the site of action of the cyclodiene organochlorines such as dieldrin and aldrin (Bloomquist, 2002). One of the main currently used acaricides on companion animals, fipronil (a phenylpyrazole), is an antagonist of GABA-gated chloride channels demonstrating the chemical validation of this target site (ffrench-Constant et al., 2016).

Glutamate-gated chloride channels (GluCls) are members of the Cys-loop ligand-gated ion channel family that also mediate inhibitory synaptic transmission in the CNS of invertebrates (Wolstenholme, 2012). As they are exclusively found in invertebrates, they represent excellent highly selective acaricidal targets and are the primary target of the macrocyclic

lactones (avermectins and milbemycins). They also play a secondary role in the acaricidal activity of fipronil (Zhao et al., 2004).

Electrical signals in excitable tissues (nerve bundles and muscles) of invertebrates are generated by the synchronized opening and closing of ion channels in cell membranes in response to local changes in transmembrane potential. Because these channels mediate conduction and the release of neurotransmitters across the synapses, they are especially prominent components of the CNS (ffrench-Constant et al., 2016). Ion channels may be classified by gating, into voltage-gated and ligand-gated ion channels or by the type of ions they conduct into sodium, potassium, calcium and chloride ion channels. Ion channels are the molecular targets of the pyrethroids and are frequent targets for drug development (Ding et al., 2014).

Since the acetylcholine receptors, GABA receptors and ion channels are expressed in the enteric system of rats and are important for normal gastrointestinal functions, it is logical to expect that the factors to regulate these neurotransmitters in mammalian intestinal tract may subsequently have the same effects in other parts and organisms where they exist as pharmacological molecules. This study aims to verify the mechanism of action of *C. aurea* crude extract, fractions and isolated compounds (apigenin-7-*O*-β-D-glycoside and isorhoifolin) *in vitro* using AChE inhibition assay as well as *ex vivo* on cholinergic receptors and ion channels using isolated rat ileum as a model.

8.2. Materials and Methods

8.2.1 Acetylcholinesterase inhibition assay

Inhibition of AChE activity was evaluated using Ellman's colorimetric method (Ellman et al., 1961) with some modifications (Dzoyem et al., 2015). Acetylthiocholine iodide (AChI) was used as substrate for this enzymatic reaction. Twenty five microliters (25 µl) of decreasing concentrations (700, 350, 175, 87.5, 43.75, 21.88, 10.94 µg/ml) of test solutions (crude extract and fractions), 125 µl of 3 mM Ellman's reagent [5,5'-dithio-bis(2-nitro-benzoic)acid (DTNB) in Buffer A (50 mM Tris-hydrochloric acid (HCl), pH 8; containing 0.1 M sodium chloride (NaCl) and 0.02 M magnesium chloride hexahydrate ($MgCl_2.6H_2O$)], 50 µl of Buffer B (50 mM Tris-HCl; pH 8, containing 0.1 % bovine serum albumin) were mixed in 96 well microtitre plates. The plates were incubated at room temperature (25°C) for 10 minutes, then 25 µl of AChE (0.2 U/ml) was added to the wells and incubated at 25°C for 5 minutes. The absorbance was read at 405 nm to blank the extract in order to subtract the effect of the colour of the extract. After this, 25 µl of 15 mM AChI in distilled water was added to the wells, incubated at 25°C for 5 minutes to initiate the reaction. The hydrolysis of AChI was

recorded spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine at a wavelength of 405 nm. Eserine (10 mg/ml stock solution; 50ug/ml) and dimethyl sulphoxide (DMSO) served as the positive control and negative control respectively. Percentage inhibition was calculated by comparing the reaction rates for the test solutions to the negative control using the following formula:

$$\frac{(ANC_{after} - ANC_{before}) - (ATS_{after} - ATS_{before})}{(ANC_{after} - ANC_{before})} \times 100$$

Where, ANC_{after} is the absorbance of the negative control after addition of the substrate, ANC_{before} is the absorbance of the negative control before addition of the substrate, ATS_{after} is the absorbance of the test solutions after addition of the substrate and ATS_{before} is the absorbance of the test solutions before addition of the substrate. All experiments were performed in triplicate.

8.2.2 Isolated tissue bath studies

8.2.2.1 Animals

Nine male, four-month-old, Sprague Dawley rats, weighing 290-350 g were purchased from South African Vaccine Producers, Johannesburg, South Africa and ascertained to be pathogen-free. They were housed in pairs at the University of Pretoria Biomedical Research Centre (UPBRC) facility within the Faculty of Veterinary Science in Euro-standard type III cages, on shavings. The animal rooms were maintained at 22±2ºC, 40-70% relative humidity and greater than 10 air changes. The animals had free access to potable water, rodent chow and the standard enrichment (toilet roll cartons, egg boxes and gnaw sticks) offered at the UPBRC. They were monitored twice daily for their habitus and were weighed once weekly for general monitoring purposes. The experiments were approved (Approval no.-V103-16) and carried out according to the guidelines established by the Animal Ethics Committee, University of Pretoria. The carcass at the end of each experiment was disposed of by incineration.

8.2.2.2 Drugs and reagents

Acetylcholine chloride, carbachol, atropine, minoxidil, glibenclamide, DMSO, HCl, isoflurane, NaCl, potassium chloride (KCl), calcium chloride dihydrate ($CaCl_2.2H_2O$), sodium bicarbonate ($NaHCO_3$), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), potassium phosphate monobasic (KH_2PO_4), glucose and Type II distilled water (0.064uS/cm) were used. All the drugs and salts were of analytical grade and were bought from Sigma-Aldrich, South Africa.

8.2.2.3 Preparation of drugs

Fresh solutions of the drugs were prepared on the day of the experiments. Glibenclamide was dissolved in DMSO while the other drugs were dissolved in Krebs-Henseleit solution. The crude extract and isolated compounds (apigenin-7-*O*-β-D-glycoside and isorhoifolin) were also dissolved in DMSO and sonicated before use. Dimethyl sulphoxide was selected as the solvent, as previous studies have indicated that it has no activity on muscle contractility (Yuan et al., 2014).

8.2.2.4 Preparation of solutions

The buffer was Krebs-Henseleit (KH) solution with the following composition (mM): NaCl - 6.95 g; KCl - 0.36 g; KH_2PO_4 - 0.16 g; $NaHCO_3$ - 2.10 g; $MgSO_4.7H_2O$ - 0.30 g; $CaCl_2.2H_2O$ - 0.37 g; glucose - 2.28 g; pH 7.4. Potassium (K^+) free KH solution was prepared by replacing 80 mM K^+ with 73.27 mM sodium (Na^+): NaCl - 7.23 g; NaH_2PO_4 - 0.19 g; $NaHCO_3$ - 2.10 g; $MgSO_4.7H_2O$ - 0.30 g; $CaCl_2.2H_2O$ - 0.37 g; glucose - 2.28 g. Five litres (5 L) of KH solution was prepared fresh on each day of the experiment. The weighed salts were dissolved in distilled water separately before adding the $CaCl_2.2H_2O$ solution to prevent precipitation of salts. The pH of the medium was adjusted to 7.4 (using pH meter HACH, USA) by slow titration with concentrated HCl.

8.2.2.5 Apparatus and software

A circulating water isolated tissue bath with four (25 ml) chambers (Laboratory Thermal Equipment, Greenfield, England) connected to carbogen (5% CO_2 in oxygen) which was bubbled into each chamber was used. The mechanical response of the isolated tissue in each chamber was measured with an isometric force transducer (TR1201AD, Spain) linked to a pre-amplifier (Quad Bridge Amp) and computerized data acquisition system (DAS 6600). The readings were recorded and evaluated in LabChart® 7 Pro (ADInstruments, New Zealand). A tension of 1 g was applied to each tissue and kept constant throughout the experiment.

8.2.2.6 System preparation and set-up

The circulating water tissue bath system was equilibrated to 37°C about 1 hour before the start of each experiment and carbogen constantly bubbled. The force transducers and data acquisition system were also turned on at least 15 minutes prior to the start of the experiment to equilibrate the temperature. The force transducers were calibrated before tissues were placed in the four chambers of the tissue bath and before data recording. The chambers were filled with KH solution and allowed to reach optimal temperature. The aerators were checked to ensure consistent aeration of the KH solution. This oxygenates the

buffer and provides Brownian motion to distribute drugs that will be introduced in the tissue bath during the experiment making sure that bubbles did not cause tissue movement.

8.2.2.7 Tissue preparation

This was done using the method of Costescu et al. (2016). On the day of each experiment, immediately prior to use, the rat which had been fasted overnight, was euthanized using an isoflurane overdose in a saturated bell jar. Immediately, an incision was made on the median line of the abdominal wall, a segment of the ileum was removed and placed directly into a petri dish filled with warmed, carbonated KH solution (37°C) to clean it of intestinal contents, adhering fat and connective tissues. The cleaned tissue was transported in a Schott bottle filled with warm, carbogenated KH solution to the Pharmacology laboratory within 5 minutes where the ileum was cut into segments of 2 cm each and mounted in the equilibrated tissue bath chamber.

8.2.2.8 Experimental protocol

The experiments were conducted according to the protocols described by Carvalho et al. (2009) with some modifications. The procedure and the concentration of drugs used were selected on the basis of previous published work (Janbaz et al., 2013; Jalali-Nezhad et al., 2016), as outlined in Table 8.2. Each concentration of extract/pure compound was allowed a contact time of 2 minutes, prior to dose ascension. Prior to the testing of the next extract/pure compound, the baths were thoroughy washed three times and a resting period of 20 minutes was allowed for the tissues before the next addition of drug for recovery of their spontaneous activity. Each experiment was performed in quadruplicate.

8.2.3 Data analysis

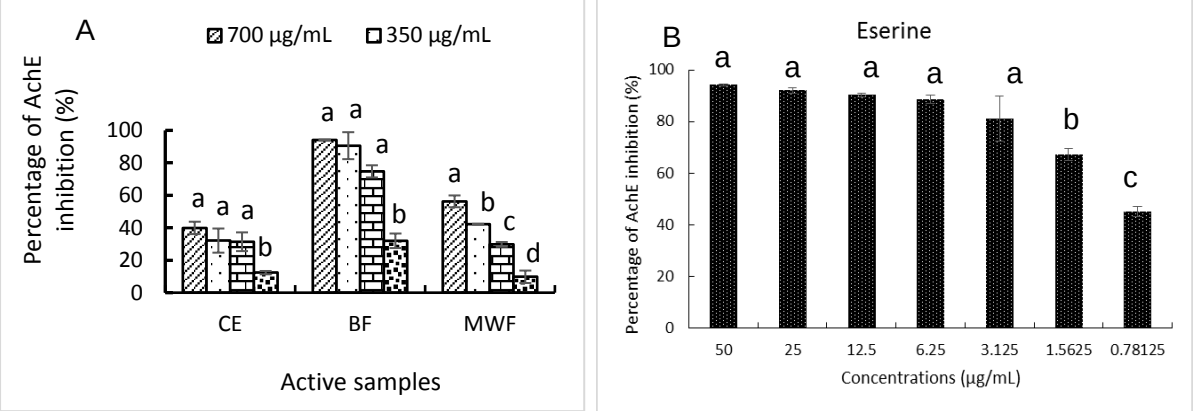
Data were presented as the arithmetic mean values±standard error of mean (Mean±SEM). Significance was analysed using one-way analysis of variance followed by Tukey's multiple comparison test on GraphPad Prism 7.02 (GraphPad Software, San Diego-CA, USA). Values were considered to differ statistically when $p \leq 0.05$. Dose-response graphs were plotted and IC_{50} determined for the AChE inhibition assay using the linear regression model.

8.3 Results

8.3.1 Acetylcholinesterase inhibition assay

There was dose-dependent inhibition of AChE with the highest concentration of *C. aurea* crude extract (700 µg/ml) causing 40% inhibition of AChE while the methanol/water and butanol fractions showed 60 and 90% inhibition of AChE respectively (Fig. 8.1A). This was

comparable with 90% inhibition of AChE produced by 50 µg/ml of the positive control, eserine (Fig. 8.1B). The chloroform, hexane and water fractions were inactive (Table 8.1).



CE - Crude extract; BF - Butanol fraction; MWF - Methanol/water fraction; a - Statistically different from positive control ($p<0.05$); b - Statistical difference between the extracts ($p<0.05$); Extracts with the same letter are not statistically different from each other.

Figure 8.1(A) Acetylcholinesterase inhibitory activity of *Calpurnia aurea* crude extract and active fractions (B) Acetylcholinesterase inhibitory activity of eserine (positive control)

Table 8.1: The acetylcholinesterase inhibitory activity (IC_{50}) of the crude extract and fractions of *Calpurnia aurea*

***Calpurnia aurea* plant**	**IC_{50} (µg/ml)**
	Mean±SEM
Crude extract	> 700
BF	113±8
MWF	503±31
CF	NA
HF	NA
WF	NA
Eserine	1.37±0.01

BF - Butanol fraction; MWF - Methanol/water fraction; CF - Chloroform fraction; HF - Hexane fraction; WF - Water fraction; NA - Not active

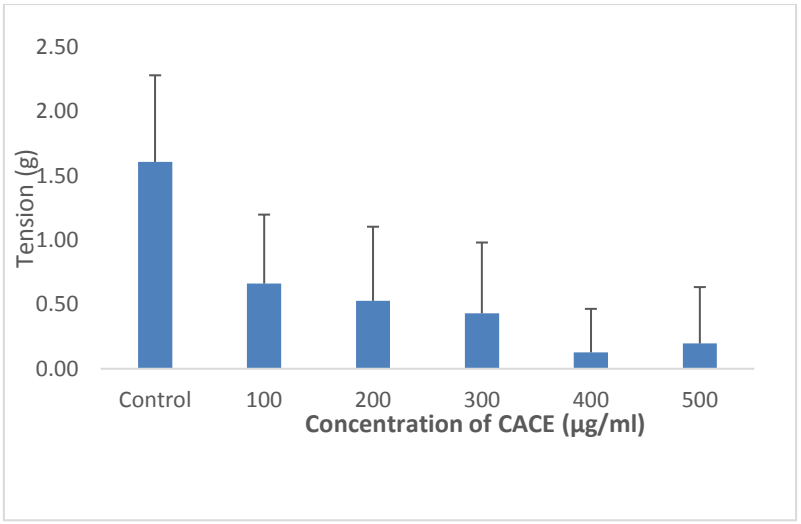
8.3.2 Isolated tissue bath assay

There was dose-dependent relaxation of contractions induced, albeit non-significant, by acetylcholine and carbachol in the rat ileum (Table 8.2; Fig. 8.2-8.4). The crude extract, apigenin-7-*O*-β-D-glycoside and isorhoifolin also appeared to antagonize the contraction of the isolated rat ileum induced by cumulative concentrations of KCl and the effect increased with increasing concentration (Table 8.2; Fig. 8.5-8.9). The results for the latter were also non-significantly different.

Table 8.2: Tissue bath drug concentrations and observed effects

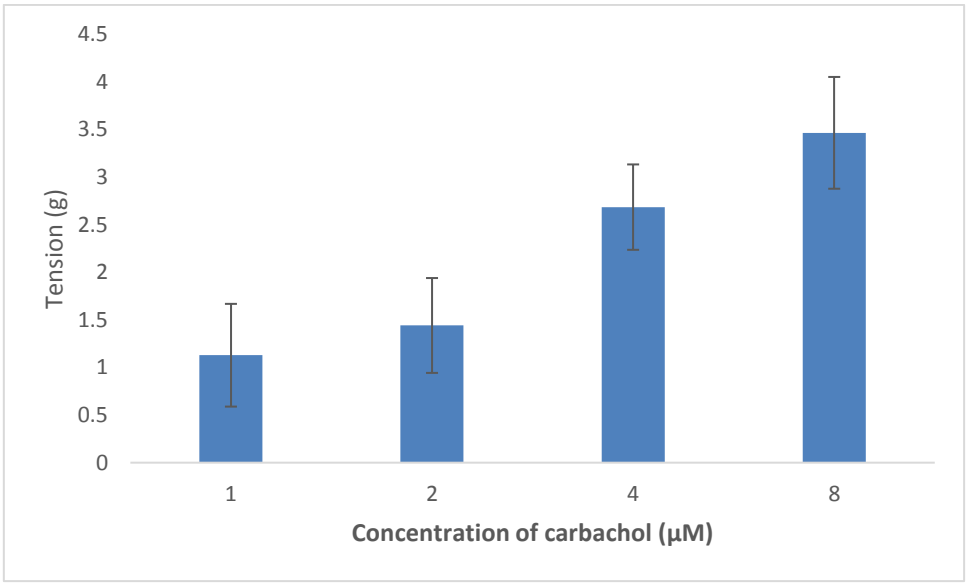
	Step	Drug 1	Drug 2	Observed effect
Cholinergic	1	Increasing concentrations of acetylcholine chloride (mAchR/nAchR agonist) - 0.02; 0.04; 0.054; 0.07µg/ml	-	Contraction
	2	Maximum concentration of acetylcholine chloride (0.07 µg/ml)	Increasing concentrations of *Calpurnia aurea* crude extract- 100; 200; 400; 800; 1600 µg/ml	Relaxation
	3.	Increasing concentrations of carbachol (mAchR/nAchR agonist) - 1; 2; 4; 8; 16 µM	-	Contraction
	4	Maximum concentration of carbachol– 8 µM	Increasing concentrations of atropine (mAchR antagonist) - 0.025; 0.05; 0.1; 0.5; 1 µM	Relaxation
	5	Maximum concentration of carbachol– 8 µM	Increasing concentrations of *Calpurnia aurea*- 100; 200; 400; 800; 1600 µg/ml	Relaxation
K^+ channel	6	Two concentrations of KCl (potassium channel agonist) - 40; 80 mM	-	Contraction
	7	Increasing concentrations of minoxidil (potassium channel agonist) -1; 2; 4; 8; 16; 32 µM	-	Contraction
	8	Increasing concentrations of glibenclamide (potassium channel antagonist) -0.1; 1; 10; 100 µM	-	Relaxation
	9	Maximum concentration of KCl - 80 mM	Increasing concentrations of glibenclamide (potassium channel antagonist) -0.1; 1; 10; 100 µM	Relaxation
	10	Maximum concentration of KCl - 80 mM	Increasing concentrations of *Calpurnia aurea*- 100; 200; 400; 800; 1600 µg/ml	Relaxation
	11	Maximum concentration of KCl - 80 mM	Increasing concentrations of apigenin-7-*O*-β-D-glycoside - 6.25; 12.5; 25; 50 µg/ml	Relaxation
	12	Maximum concentration of KCl - 80 mM	Increasing concentrations of isorhoifolin - 6.25; 12.5; 25; 50 µg/ml	Relaxation

mAchR – Muscarinic acetylcholine receptor; nAchR – Nicotinic acetylcholine receptor; KCl – Potassium chloride



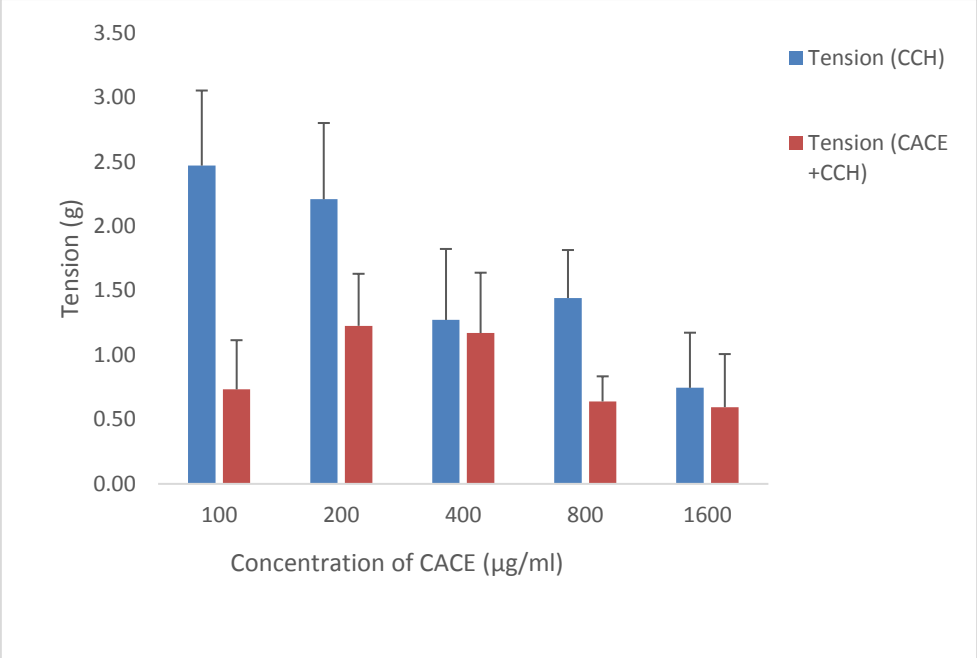
CACE - Calpurnia aurea crude extract; Control - Acetylcholine (0.07 µg/ml) induced contraction; Results are mean±SEM; n=4

Figure 8.2: Effect of increasing concentrations of *Calpurnia aurea* crude extract on acetylcholine induced contraction of isolated rat ileum



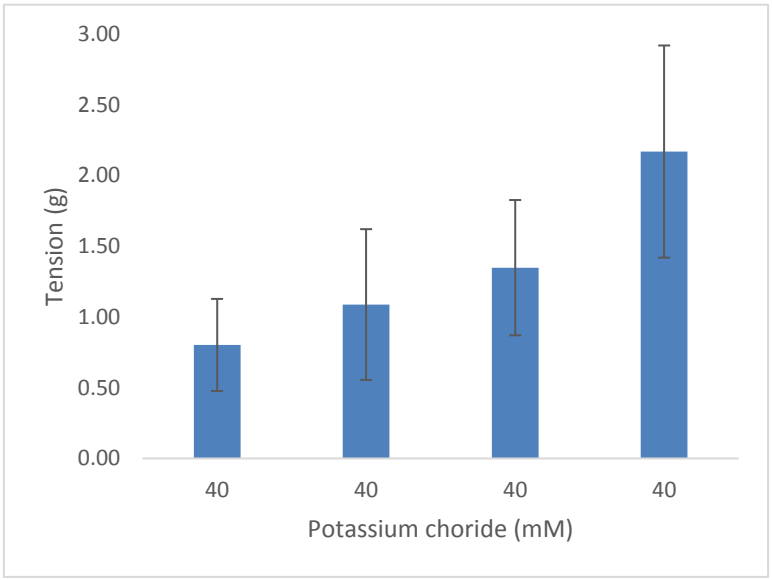
Results are mean±SEM; n=4

Figure 8.3: Dose-response graph of carbachol induced contraction of isolated rat ileum



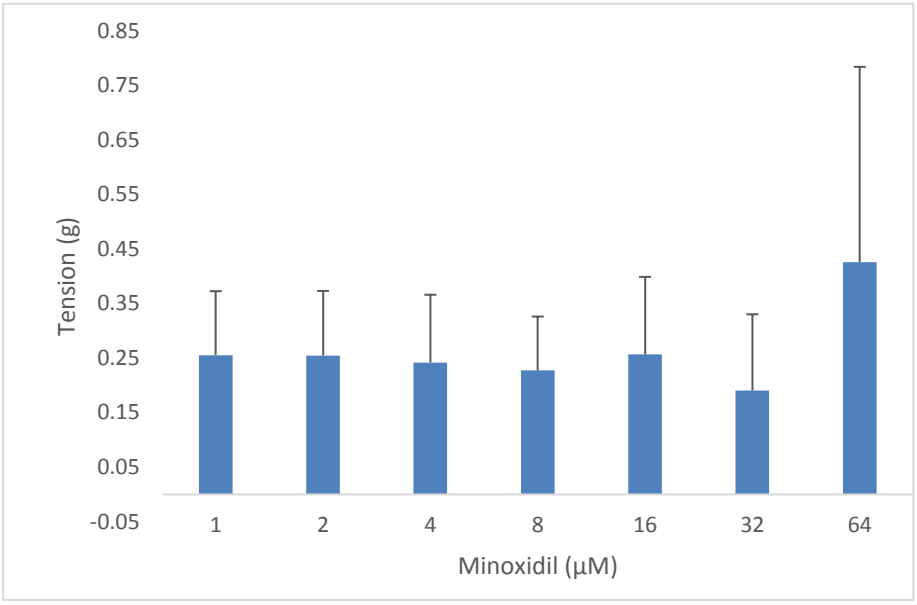
CCH - Carbachol; CACE - Calpurnia aurea crude extract; Control - Carbachol induced contraction in the absence of the extract; Results are mean±SEM; n=4

Figure 8.4: Effect of increasing concentrations of *Calpurnia aurea* crude extract on non-cumulative carbachol induced contraction of isolated rat ileum



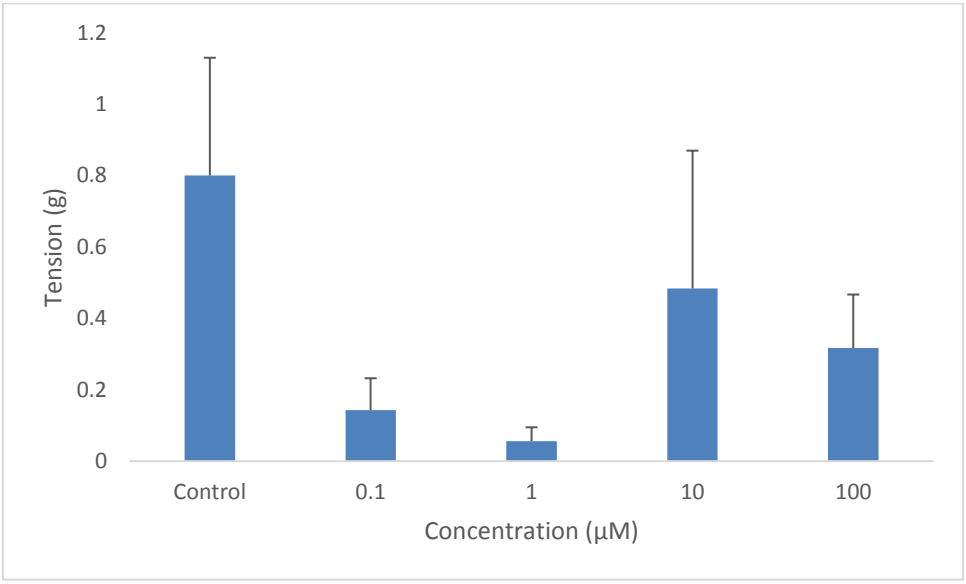
Results are mean±SEM; n=4

Figure 8.5: Effect of increasing concentrations of potassium chloride on isolated rat ileum



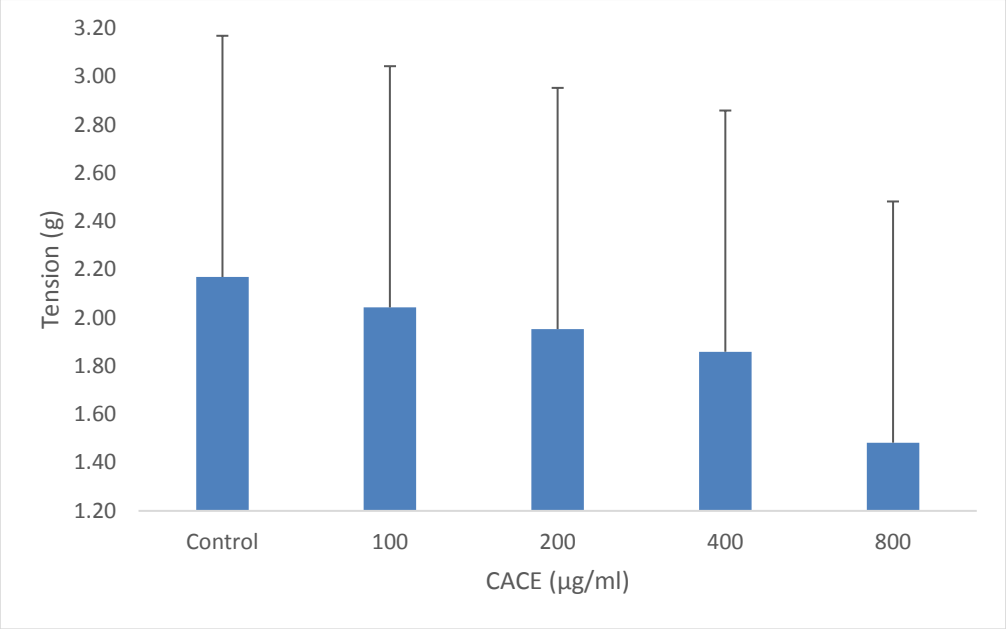
Results are mean±SEM; n=4

Figure 8.6: Effect of increasing concentrations of minoxidil on isolated rat ileum



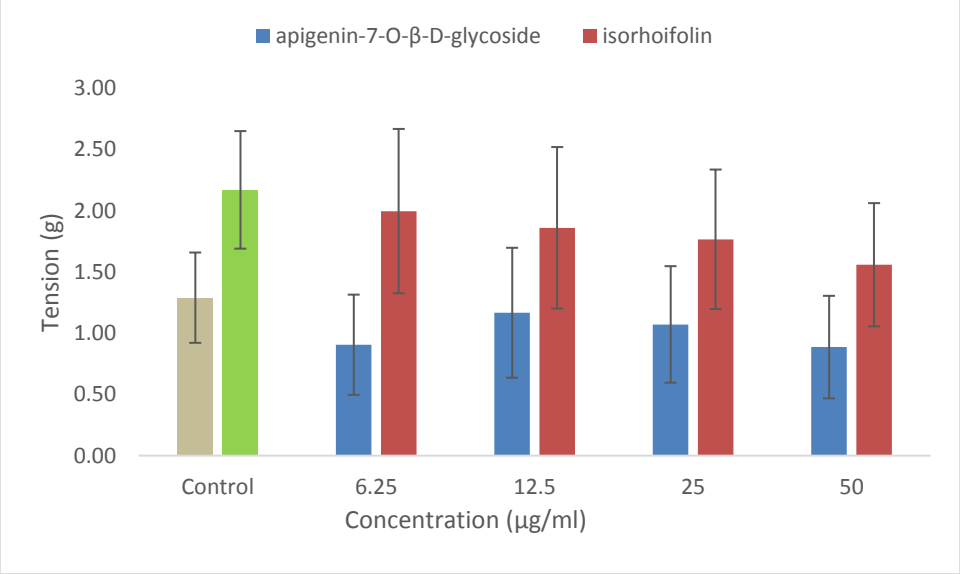
Control - Potassium chloride (80 mM); Results are mean±SEM; n=4

Figure 8.7: Effect of increasing concentrations of glibenclamide on isolated rat ileum



CACE - Calpurnia aurea crude extract; Results are mean±SEM; n=4

Figure 8.8: Effect of increasing concentrations of *Calpurnia aurea* crude extract on potassium chloride induced contraction of isolated rat ileum



Control - Potassium chloride (80 mM), Grey- control for apigenin-7-O-β-D-glycoside**,** *Green- control for* isorhoifolin*; Results are mean±SEM; n=4*

Figure 8.9: Effect of increasing concentrations of apigenin-7-O-β-D-glycoside and isorhoifolin on potassium chloride induced contraction of isolated rat ileum

8.4 Discussion

Acetylcholine is a chemical neurotransmitter found widely in the body of vertebrates and invertebrates. It triggers the stimulation of post-synaptic nerves, muscles and exocrine glands. Acetylcholinesterase is the enzyme that rapidly hydrolyzes acetylcholine to choline and acetate, thereby terminating its effect at cholinergic synapses (Kumar et al., 2015). Certain chemical classes of acaricides, organophosphates and carbamates induce their acaricidal activities by inhibiting AChE irreversibly and reversibly respectively. This results in accumulation of acetylcholine and continuous nerve discharges leading to paralysis and death of the ticks (Barthold & Schier, 2005). These acaricides are routinely and extensively used for tick control of livestock. However, widespread and intensive use has led to resistance in tick populations, contamination of ground water by particle leachates and increasing incidence of animal and human skin, lung and nerve diseases upon exposure (Gemeda et al., 2014). Plants with AChE inhibitory activity may likely produce lesser side effects. To the best of our knowledge, there are no literature data evaluating the AChE inhibitory activity of *C. aurea*.

Ellman's colorimetric method was applied to determine the AChE inhibitory activity of *C. aurea* crude extract and fractions. This method is based on determining the amount of thiocholine released when acetylcholine is hydrolyzed by AChE enzyme. The colouring agent, DTNB, binds with acetylcholine and forms yellow colour, indicating the presence of free acetylcholine which was not hydrolyzed by AChE in the reaction mixture. It was observed that AChE inhibitory activity of *C. aurea* was concentration-dependent and butanol and methanol/water were the active fractions with AChE inhibition higher than that of the crude extract. Butanol (chemical formula-C_4H_9OH) and methanol/water (chemical formula-CH_3OH/H_2O) are polar fractions with tendencies to lose the proton (H^+) ion from the OH group, resulting in a highly water-soluble phenolate anion which can bind with the thiocholine hence the greater AChE inhibition (Gulcin et al., 2016). Chloroform (chemical formula-$CHCl_3$) and hexane (chemical formula-C_6H_{14}) are non-polar fractions and were inactive. Since the two bioactive compounds against ticks, apigenin-7-*O*-β-D-glycoside and isorhoifolin, were isolated from the chloroform fraction of *C. aurea,* which was devoid of AChE inhibitory activity, they were not evaluated further for the AChE inhibitory activity. This was a similar finding to Odonbayar et al. (2017) that apigenin 7-*O*-β-D-glucoronopyranoside and apigenin 7-*O*-β-D-glucoronopyranoside methyl ester isolated from the aerial parts of *Thymus gobicus* Czern. (Lamiaceae) was inactive (IC_{50}> 100µM) against AChE obtained from electric eel, human erythrocytes and horse serum. Another important aspect to this result, would indicate that there are other inhibitory compounds present in the fractions responsible for the action of the plant extract. This may also explain why the crude extract

was more active than the individual compounds as seen in previous tick inhibitory studies (see section 7.3.4).

As a surrogate to determining channel inhibitory activity of the extract/pure compound, we made use of an isolated tissue bath method. The main advantage of this *ex vivo* technique is that the tissue is living and functions as a whole tissue, with a physiological outcome (contraction or relaxation) that is relevant to the body. Several steps such as drug-receptor interaction, signal transduction, second messenger generation, change in smooth muscle excitability and change in tissue function occur at the molecular level and the isolated tissue bath allows for integration of all these steps. Another advantage is that retaining tissue function permits calculation of important pharmacological variables that are more meaningful in a tissue as opposed to a cellular setting which comes closer to how the drugs would work in the body as a whole (Jespersen et al., 2015).

Results from the isolated tissue bath studies indicate that the plant had mixed activity, which is not surprising since the plant extract consists of thousands of different potential compounds. For the crude extract, *C. aurea* induced relaxation in the presence of acetylcholine and carbachol induced contractions. This in itself was an unexpected finding as the AChE inhibitory assay, showed 40% inhibitory activity. Taken together, these data suggest that the activity of *C. aurea* may involve more pathways than the cholinergic system. Perhaps the activity of the crude extract of *C. aurea* as seen with individual fractions is a result of synergistic action of different compounds present in this plant. While apigenin-7-O-β-D-glycoside and isorhoifolin antagonized the KCl induced contractility, the results were not characterised by a significant dose-response relationship, which may have resulted from the sample size used.

Potassium chloride is typically used to analyse the mechanism of action of drugs and plant extracts. Potassium chloride induced contraction is due to depolarization of muscle fibers leading to increased K^+ and opening of L-type calcium ion (Ca^{2+}) voltage-dependent channels, Ca^{2+} influx thereby inducing contraction. Potassium channels are the most widely distributed ion channels found in virtually all living organisms (Chen & Lin, 2012). They rapidly and selectively conduct K^+ down the electrochemical gradient, thereby setting or resetting the resting potential in many cells. There are four major classes of K^+ channels namely: (1) Calcium-activated K^+ channel (BK_{Ca}) which opens in response to the presence of Ca^{2+} or other signalling molecules. (2) Inwardly rectifying K^+ channel which passes current (positive charge) more easily into the cell. (3) Tandem pore domain K^+ channel that possesses high basal activation, such as the resting K^+ channels or leak channels which set

the negative membrane potential of neurons. (4) Voltage-gated K^+ channel that open or close in response to changes in the transmembrane potential (Hallworth et al., 2003).The flux of ions through the K^+ channel pore is regulated by two processes; gating and inactivation. Gating is the opening or closing of the channel in response to stimuli, while inactivation is the rapid cessation of current from an open K^+ channel and the suppression of the channel's ability to resume conducting (Choe, 2002). Potassium channel blockers generally inhibit the flow of K^+ through the channel. They either compete with K^+ binding within the selectivity filter or bind outside the filter to occlude ion conduction. Glibenclamide (K^+ channel blocker) and minoxidil (K^+ channel agonist) used in this study belong to the adenosine triphosphate (ATP)-sensitive subclass of inwardly rectifying K^+ channel which close when ATP is high.

While the activitiy of apigenin-7-*O*-β-D-glycoside and isorhoifolin have not been evaluated before, the activity of other flavonoids has been reported. Activation of different types of K^+ channels has been proposed to explain, at least in part, the relaxant effect of some flavonoids. The ability to activate BK_{Ca} channels is a noteworthy feature and the BK_{Ca} channel opener profile of apigenin has been well documented in the African clawed frog (*Xenopus* species) oocytes and for naringenin (Saponara et al., 2006). As mobilization and increase in Ca^{2+} are required to trigger smooth muscle contraction, activation of BK_{Ca} channels cause a decrease in Ca^{2+} influx through voltage-dependent Ca^{2+} channels, membrane hyperpolarization in the smooth muscles causing relaxation (Rotondo et al., 2009). This hyperpolarization may lead to loss of neuronal activity, paralysis and ultimately death of the ticks.

8.5 Conclusions

This study has increased the understanding of the acaricidal efficacy of *Calpurnia aurea* subsp. *aurea* by demonstrating its high efficacy and multi-target sites (cholinergic system and ion channel) due to the presence of active flavonoids such as apigenin-7-*O*-β-D-glycoside and isorhoifolin in the plant. The results indicate that the plant functions via a combination of effects, which could either be synergistic or alternatively may make it more difficult for resistance to develop.

CHAPTER 9

Overall discussion, recommendations and future research directions

9.1 Synopsis of the study

The aim of this study was to evaluate extracts, fractions and isolated compounds from South African plant species with documented ethnoveterinary use against ticks for their acaricidal properties and cytotoxicity, in a bid to find leads for the development of a safe and effective tick control product. To achieve this aim, nine objectives were set.

To address the first four objectives, extensive literature surveys of published scientific articles were conducted for the role of ticks in animal health, problems encountered in using synthetic, chemical acaricides, medicinal plants with *in vitro* acaricidal or tick repellent activities against immature and adult stages of ticks and bioassays employed. Veterinary databases (All Databases, CAB Abstracts and Global Health, Medline, PubMed, Web of Science, BIOSIS Citation Index, Science Direct, Current Content Connect and Google Scholar) were searched. The search words included “acaricidal”, “tick repellent”, “medicinal plants”, “isolated compounds” and “antitick assays”. Meta-analysis was conducted using the Fixed-effect model in an Excel programme to compare the results. The tick climbing repellency and adult immersion tests were the most commonly used assays to test for repellency and acaricidal activity respectively. Ethanol was the most commonly used extractant and *Rhipicephalus (Boophilus) microplus* was the most commonly studied tick across all the reviewed papers.

South Africa is rich in vascular plant flora possessing over 10% of the world’s vascular floral species. Only a fraction of its plants have been rigorously studied and analyzed for their biological activity against ticks and seventeen plant species selected based on their ethnoveterinary use in tick control were used in this study. Crude extracts of these plants were prepared using four different solvents (acetone, ethanol, ethanol/water and hot water). The extracts (200 mg/ml) were screened for their acaricidal efficacy against adult *Rhipicephalus turanicus* ticks using the contact assay. The plant species with the highest acaricidal efficacies for their acetone and ethanol extracts were *Calpurnia aurea, Schkuhria pinnata* and *Senna italica* with mortality of 97, 93 and 90%; 93, 93 and 87% respectively. The ethanol/water and hot water extracts of many of the plants had low acaricidal activities (<60%). Acaricidal dose-response bioassay of two-fold graded decreasing concentrations (100 to 3 mg/ml) of the acetone and ethanol extracts of *S. pinnata, C. aurea* and *S. italica* was done using the adult immersion tests. The LC_{50} acaricidal mortality values against *R.*

turanicus ticks after 24 hours for the acetone extracts were 35.75, 111.24 and 42.05 mg/ml respectively while LC_{50} values for the ethanol extracts were 37.07, 98.69 and 37.50 mg/ml respectively compared with the positive control with LC_{50} of 2.41 mg/ml. In order to verify the safe use of these plants, cytotoxicity tests against Vero and HepG2 cells were conducted. Most of the plant extracts were non-cytotoxic to the two cell lines (LC_{50}>100 µg/ml) and there was a statistically significant higher toxicity to HepG2 cells compared with Vero cells. The ethanol/water and hot water extracts of most of the plants were less toxic to the cells (LC_{50}>1000 µg/ml) compared with their acetone and ethanol extracts. The selectivity indices of *S. pinnata, C. aurea* and *S. italica* were low. Particularly good acaricidal activities were displayed by *C. aurea* extracted using four different solvents on *R. turanicus* ticks. The plant was also safe on the cell lines tested and was selected as the most promising plant species, based on its efficacy and safety for further studies.

Solvent-solvent fractionation of the *C. aurea* ethanol extract was done and five fractions, namely hexane, chloroform, butanol, water and methanol/water were obtained. The more non-polar fractions, hexane, chloroform and butanol were tested against *R. turanicus* ticks using adult immersion tests. There were dose-dependent effects with all the fractions and the chloroform fraction was the most active with up to 90% mortality at the highest concentration tested (100 mg/ml). Using silica gel column chromatography, two active flavonoids, apigenin-7-*O*-β-D-glycoside and isorhoifolin were isolated for the first time from *C. aurea*. Acaricidal dose-response bioassays of two-fold graded increasing concentrations (0.03-1 mg/ml) and cytototoxicity tests against Vero and HepG2 cells (5-100 µg/ml) of the isolated compounds were undertaken. Both compounds were non-toxic to the two cell lines (LC_{50}=70-100 µg/ml). The two compounds also had good acaricidal activities and the LC_{50} value of 0.65 mg/ml observed for isorhoifolin compares favourably with some known acaricidal compounds isolated from plants and synthetic, chemical acaricides.

The mechanism of action of the crude extract and isolated compounds was then further investigated. Ellman's acetylcholinesterase inhibition assay (*in vitro*) was performed on the crude extract and fractions of *C. aurea.* There was only 40% acetylcholinesterase inhibition observed for the crude extract, 90% acetylcholinesterase inhibition for the butanol fraction and the chloroform fraction from which the compounds were isolated did not inhibit acetylcholinesterase. A further attempt was then made to investigate the mechanism of action *ex vivo* (using isolated tissue bath studies) on cholinergic receptors and ion channels in comparison with standard agonists and antagonists. The results showed a dual effect, with the crude extract showing acetylcholine inhibitory activity, while apigenin-7-*O*-β-D-glycoside and isorhoifolin appeared to function through the inhibition of potassium channels

which tends to indicate that synergism of different phytochemicals in *C. aurea* may be responsible for its multiple mechanisms of action.

9.2 Recommendations and future research directions

For future purposes, some areas of research can be futher investigated:

1) Determining the effect of extracts, fractions and compounds on various ion channels.
2) Formulation development may improve the efficacy and stability of phytoproducts.
3) *In vivo* efficacy studies (field trials) using formulated products are clearly essential.
4) Deciphering the synergistic, suppressive and other interactions of active phytoproducts with synthetic, chemical acaricides is required.

9.3 Final conclusions

Although phytochemicals such as azadirachtin (a tetranortriterpenoid), 2-undecanone (a ketone) and limonene (a cyclic terpene) already have important commercial applications in a variety of commercial products useful for tick control, flavonoids such as apigenin-7-*O*-β-D-glycoside and isorhoifolin isolated from *C. aurea* in this study, are an interesting group of study molecules for acaricidal activity. With the projected increase in worldwide population growth to 9.7 billion (Africa is expected to account for more than half of the world's population growth) and the consequent rise in demand for food, it is imperative that pests such as ticks that have major impacts on animal health be effectively controlled. It is thus essential to invest in developing a pharmaceutical phytotherapy industry, with interdisciplinary approaches towards finding solutions to the menace caused by ticks and tick-borne diseases.

CHAPTER 10

References

Abbas, R.Z., Zaman, M.A., Colwell, D.D., Gilleard, J. & Iqbal, Z. (2014) Acaricide resistance in cattle ticks and approaches to its management: the state of play. *Veterinary Parasitology*, 203. 6-20.

Abbott, W. (1987) A method of computing the effectiveness of an insecticide. *Journal of the American Mosquito Control Association*, 3. 302-303.

Abd-Alla, H.I., Moharram, F.A., Gaara, A.H. & El-Safty, M.M. (2009) Phytoconstituents of *Jatropha curcas* L. leaves and their immunomodulatory activity on humoral and cell-mediated immune response in chicks. *Zeitschrift für Naturforschung C*, 64. 495-501.

Abdel-Shafy, S. & Zayed, A.A. (2002) *In vitro* acaricidal effect of plant extract of neem seed oil (*Azadiracta indica*) on egg, immature and adult stages of *Hyalomma analoticum excavatum* (Ixodoidea: Ixodidae). *Veterinary Parasitology,* 106(1). 89-96.

Abdel-Shafy, S., Soliman, M.M.M. & Salwa, M.H. (2006) *In vitro* acaricidal effect of some crude extracts and essential oils of wild plants against certain tick species. *Acarologia*, 47(1/2). 33-42.

Aboelhadid, S. M., Kamel, A. A., Arafa, W. M. & Shokier, K.A. (2013) Effect of *Allium sativum* and *Allium cepa* oils on different stages of *Boophilus annulatus*. *Parasitology Research*, 112(5). 1883-1890.

Adakal, H., Stachurski, F. & Chevillon, C. (2013) Tick control practices in Burkina Faso and acaricide resistance survey in *Rhipicephalus (Boophilus) geigyi* (Acari: Ixodidae). *Experimental & Applied Acarology*, 59. 483-491.

Adedapo, A.A., Jimoh, F.O., Koduru, S., Afolayan, A.J. & Masika, P.J. (2008) Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of *Calpurnia aurea*. *BMC Complementary & Alternative Medicine.* doi:10.1186/1472-6882-8-53.

Adenubi, O., Fasina, F., McGaw, L., Eloff, J. & Naidoo, V. (2016) Plant extracts to control ticks of veterinary and medical importance: A review. *South African Journal of Botany*, 105. 178-193.

Adisa, C.A. & Mbanaso, A. (2004) Furuncular myiasis of the breast caused by the larvae of the Tumbu fly (*Cordylobia anthropophaga*). *BMC Surgery*, 4. 1.

Agostini, G., Souza-Chies, T., Agostini, F., Atti-Serafni, L. & Echeverrigaray, S. (2010) Essential oil composition of *Cunila incana* Benth. (Lamiaceae). *Journal of Essential Oil Research,* 22. 432-434.

Aguirre, D.H., Vin˜ Abal, A.E., Salatin, A.O., Cafrune, M.M., Volpogni, M.M., Mangold, A.J., & Gugliemone, A.A. (2000) Susceptibility to two pyrethroids in *Boophilus microplus* (Acari: Ixodidae) populations in northwest Argentina: Preliminary Results. *Veterinary Parasitology,* 88. 329-334.

Ahmad, A. & Mishra, L.N. (1997) Isolation of herniarin and other constituents from *Matricaria chamomilla* flowers. *International Journal of Pharmacognosy,* 35(2). 121-125.

Akhila, A. & Rani, K. (1999) Chemistry of the neem tree (*Azadirachta indica* A. Juss.). *In Fortschritte der Chemie organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products*, Springer Vienna 47-149.

Akhtar, Y., Yeoung, Y. & Isman, M. (2008) Comparative bioactivity of selected extracts from Meliaceae and some commercial botanical insecticides against two noctuid caterpillars, *Trichoplusia ni* and *Pseudaletia unipuncta. Phytochemistry Reviews,* 7. 77-88.

Alemayehu, I., Tadesse, S., Mammo, F., Kibret, B. & Endale, M. (2015) Phytochemical analysis of the roots of *Senna didymobotrya*. *Journal of Medicinal Plant Research,* 9. 900-907.

Allan, S.A. (2001) Ticks (Class Arachnida: Order Acarina). *Parasitic Diseases of Wild Mammals, Second Edition*. 72-106.

Alonso-Castro, A.J., Villarreal, M.L., Salazar-Olivo, L.A., Gomez-Sanchez, M., Dominguez, F. & Garcia-Carranca, A. (2011) Mexican medicinal plants used for cancer treatment: pharmacological, phytochemical and ethnobotanical studies. *Journal of Ethnopharmacology*, 133. 945-972.

Alonzo-Diaz, M.A., Rodriguez-Vivas, R.I., Fragoso-Sanchez, H. & Rosario-Cruz, R. (2006) Resistencia de la garrapata *Boophilus microplus* a los ixodicidas. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 68(2). 105-113.

Al-Rajhy, D.H., Alahmed, A.M., Hussein, H.I. & Kheir, S.M. (2003) Acaricidal effects of cardiac glycosides, azadirachtin and neem oil against the camel tick, *Hyalomma dromedarii* (Acari: Ixodidae). *Pest Management Science*, 59. 1250-1254.

Alwin, D., Anbarasi, P. & Latha, B.R. (2007) Synergistic *in vitro* acaricidal effect of *Azadirachta indica* seed oil and camphor on *Rhipicephalus sanguineus. Indian Journal of Animal Science,* 77(5). 353-354.

Annan, K., Dickson, R., Mensah, A. & Fleischer, T.C. (2009) Acaricidal effect of *Plumbago zeylanica* L. against *Amblyoma variegatum.* ir.knust.edu.gh/handle/123456789/5138.

Anderson, J.F. (2002) The natural history of ticks. *Medical Clinics of North America*, 86. 205-218.

Anderson, J.F. & Magnarelli, L.A. (2008) Biology of ticks. *Infectious Disease Clinics of North America*, 22. 195-215.

Andreotti, R., Garcia, M.V., Matias, J., Barros, J.C. & Cunha, R.C. (2014) *Tagetes minuta* Linnaeus (Asteraceae) as a potential new alternative for the mitigation of tick Infestation. *Medicinal & Aromatic Plants,* 3(168). 2167-0412.

Apel, M.A., Ribeiro, V.L.S., Bordignon, S.A., Henriques, A.T. & von Poser, G. (2009) Chemical composition and toxicity of the essential oils from *Cunila* species (Lamiaceae) on the cattle tick *Rhipicephalus (Boophilus) microplus. Parasitology Research* 105. 863-868.

Arriaga, A.M.C., de Mesquita, A.C., Pouliquen, Y.B.M., De Lima, R.A., Cavalcante, S.H., De carualho, M.G., De Siqueira, J.A., Alegrio, L.V. & Braz-Filho, R. (2002) Chemical constituents of *Simarouba versicolor*. *Annals of the Brazilian Academy of Sciences* 74(3). 415-424.

Arsić, I., Tadić, V., Vlaović, D., Homšek, I., Vesić, S., Isailović, G. & Vuleta, G. (2011) Preparation of novel apigenin-enriched, liposomal and non-liposomal, antiinflammatory topical formulations as substitutes for corticosteroid therapy. *Phytotherapy Research*, 25. 228-233.

Arthur, D.R. (1951) Acaricidal control of the tick, *Ixodes ricinus* (L.) on cattle. *Bulletin of Entomological Research,* 41(3). 555-562.

Arumugam, A., Gunasekaran, N. & Perumal, S. (2014) The medicinal and nutritional role of underutilized citrus fruit *Citrus hystrix* (Kaffir lime): A review. *Drug Invention Today* 6(1). 1-5.

Ashitani, T., Garboui, S.S., Schubert, F., Vongsombath, C., Liblikas, I., Pålsson, K. & Borg-Karlson, A.K. (2015) Activity studies of sesquiterpene oxides and sulfides from the plant *Hyptis suaveolens* (Lamiaceae) and its repellency on *Ixodes ricinus* (Acari: Ixodidae). *Experimental & Applied Acarology,* 67(4). 595-606.

Asres, K., Gibbons, W.A., Phillipson, J.D. & Mascagni, P. (1986) Alkaloids of Ethiopian *Calpurnia aurea* subsp. *aurea. Phytochemistry*, 25. 1443-1447.

Atanasov, A.G., Waltenberger, B., Pferschy-Wenzig, E.M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E.H., Rollinger, J.M., Schuster, D., Breuss, J.M., Bochkov, V., Mihovilovic, M.D., Kopp, B., Bauer, R., Dirsch, V.M. & Stuppner, H. (2015) Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances*, *33*(8). 1582-1614.

Avoseh, O., Oyedeji, O., Rungqu, P., Nkeh-Chungag, B. & Oyedeji, A. (2015) *Cymbopogon* species; ethnopharmacology, phytochemistry and the pharmacological importance, *Molecules* 20. 7438-7453.

Azar, P.A., Torabbeigi, M., Sharifan, A. & Tehrani, M.S. (2011). Chemical composition and antibacterial activity of the essential oil of *Lavendula augustifolia* isolated by solvent free microwave assisted extraction and hydrodistillation. *Journal of Food BioSciences & Technology,* 1. 19-24.

Azmir, J., Zaidul, I., Rahman, M., Sharif, K., Mohamed, A., Sahena, F., Jahurul, M., Ghafoor, K., Norulaini, N. & Omar, A. (2013) Techniques for extraction of bioactive compounds from plant materials: a review. *Journal of Food Engineering*, 117. 426-436.

Babar, W., Iqbal, Z., Khan, M.N. & Muhammad, G. (2012) An inventory of the plants used for parasitic ailments of animals. *PakistanVeterinary Journal*, 32. 183-187.

Bacci, L., Lupi, D., Savoldelli, S. & Rossaro, B. (2016) A review of Spinosyns, a derivative of biological acting substances as a class of insecticides with a broad range of action against many insect pests. *Journal of Entomological & Acarological Research*, 48. 40-52.

Badawy, M.E., El-Arami, S.A. & Abdelgaleil, S.A. (2010) Acaricidal and quantitative structure activity relationship of monoterpenes against the two-spotted spider mite, *Tetranychus urticae*. *Experimental & Applied Acarology,* 52. 261-274.

Bailen, M., Julio, L.F., Diaz, C.E., Sanz, J., Martínez-Díaz, R.A., Cabrera, R. & Gonzalez-Coloma, A. (2013) Chemical composition and biological effects of essential oils from *Artemisia absinthium* L. cultivated under different environmental conditions. *Industrial Crops & Products*, 49.102-107.

Bainbridge, P. (2013) Wound healing and the role of fibroblasts. *Journal of Wound Care*, 22.

Balandrin, M.F., Kinghorn, A.D. & Farnsworth, N.R. (1993) Plant-derived natural products in drug discovery and development: An overview. *Human Medicinal Agents from Plants,* 1. 2-12.

Baladrin N.F., Klocke J.A., Wurtle, E.S. & Bollinger, W.H. (1985) Natural plant chemicals: sources of industrial and medical materials. *Science,* 228.1154-1660.

Balashov, Y.S. (1972) Blood sucking ticks (Ixodoidea)-vectors of disease in man and animals. *Miscellaneous publications of the Entomological Society of America*, 8(5).

Bapela, M.J., Meyer, J. M., & Kaiser, M. (2014) *In vitro* antiplasmodial screening of ethnopharmacologically selected South African plant species used for the treatment of malaria. *Journal of Ethnopharmacology*, 156. 370-373.

Baneth, G. (2011) Perspectives on canine and feline hepatozoonosis. *Veterinary Parasitology*, 181. 3-11.

Barrozo, R.B. & Lazzari, C.R. (2004) The response of the blood-sucking bug *Triatoma infestans* to carbon dioxide and other host odours. *Chemical Senses*, *29*(4). 319-329.

Barthold, C.L. & Schier, J.G. (2005) Organic phosphorus compounds-nerve agents. *Critical Care Clinics*, 21. 673-689.

Beckage, N.E., Rechcigl, J. & Rechcigl, N. (2000) Insect growth regulators. *Biological & Biotechnological Control of Insect Pests.* 123-132.

Belousova, N.I., Khan, V.A., Berezovskaya, T.P., Salenko, V.L., Vyalkov, A.I. & Dmitruk, S.E. (1991). Composition of essential oil of *Ledum palustre* L. from Tomsk district. *Rastitel nyeResurs*y, 27. 81–89.

Benavides, O.E., Hernandez, M.G., Romero, N.A., Castro, A.H., & Rodriguez, B.J.L. (2001) Preliminary evaluation of neem (*Azadirachta indica*) extracts as alternative for cattle ticks *Boophilus microplus.* Revista *Colombiana de Entomología,* 27. 1-8.

Ben Said, M., Galai, Y., Mhadhbi, M., Jedidi, M., de la Fuente, J. & Darghouth, M.A. (2012) Molecular characterization of Bm86 gene orthologs from *Hyalomma excavatum, Hyalomma dromedarii* and *Hyalomma marginatum marginatum* and comparison with a vaccine candidate from *Hyalomma scupense*. *Veterinary Parasitology*, 190. 230-240.

Benelli, G., Pavela, R., Canale, A. & Mehlhorn, H. (2016) Tick repellents and acaricides of botanical origin: a green roadmap to control tick-borne diseases? *Parasitology Research*. 1-16.

Benelli, G., Pavela, R., Maggi, F., Petrelli, R., & Nicoletti, M. (2017) Commentary: making green pesticides greener? The potential of plant products for nanosynthesis and pest control. *Journal of Cluster Science*, 1-8.

Bente, D.A., Forrester, N.L., Watts, D.M., McAuley, A.J., Whitehouse, C.A. & Bray, M. (2013) Crimean-Congo hemorrhagic fever: history, epidemiology, pathogenesis, clinical syndrome and genetic diversity. *Antiviral Research*, 100. 159-189.

Benton, R., Sachse, S., Michnick, S.W. & Vosshall, L.B. (2006) Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. *PLoS Biol,* 4(2). e20.

Bernuci, K.Z., Iwanaga, C.C., Fernadez-Andrade, C.M.M., Lorenzetti, F.B., Torres-Santos, E.C., Faiões, V.d.S., Gonçalves, J.E., do Amaral, W., Deschamps, C. & Scodro, Regiane Bertin de Lima, (2016) Evaluation of chemical composition and antileishmanial and antituberculosis activities of essential oils of *Piper* species. *Molecules* 21, 1698.

Beugnet, F. & Franc, M. (2012) Insecticide and acaricide molecules and/or combinations to prevent pet infestation by ectoparasites. *Trends in Parasitology* 28. 267-279.

Bianchin, I., Catto, J., Kichel, A., Torres, R. & Honer, M. (2007) The effect of the control of endo-and ectoparasites on weight gains in crossbred cattle (*Bos taurus taurus*× *Bos taurus indicus*) in the central region of Brazil. *Tropical Animal Health & Production*, 39. 287-296.

Binnington, K.C. & Rice, M.J. (1982) A technique for recording efferent neurone activity from normal and poisoned cattle ticks [*Boophilus microplus* (Canestrini)]. *Journal of the Australian Entomological Society*, 21.161-166.

Birkett, M.A., Campbell, C.A., Chamberlain, K., Guerrieri, E., Hick, A.J., Martin, J.L. & Woodcock, C.M. (2000) New roles for cis-jasmone as an insect semiochemical and in plant defense. *Proceedings of the National Academy of Sciences,* 97(16). 9329-9334.

Birkett, M.A., Al Abassi, S., Kröber, T., Chamberlain, K., Hooper, A.M., Guerin, P.M., Pettersson, J., Pickett, J.A., Slade, R. & Wadhams, L.J. (2008) Antiectoparasitic activity of the gum resin, gum haggar, from the East African plant, *Commiphora holtziana. Phytochemistry,* 69. 1710-1715.

Birkett, M.A., Hassanali, A., Hoglund, S., Pettersson, J. & Pickett, J.A. (2011) Repellent activity of catmint, *Nepeta cataria*, and iridoid nepetalactone isomers against Afro-tropical mosquitoes, ixodid ticks and red poultry mites. *Phytochemistry,* 72(1). 109-114.

Bissinger, B.W. & Roe, R.M. (2013) Tick repellent research, methods, and development, *Biology of Ticks* 2, 382.

Bissinger, B.W. & Roe, R.M. (2010) Tick repellents: past, present, and future. *Pesticide Biochemistry and Physiology* 96, 63-79.

Bissinger, B., Apperson, C., Watson, D., Arellano, C., Sonenshine, D. & Roe, R. (2011) Novel field assays and the comparative repellency of BioUD®, DEET and permethrin against *Amblyomma americanum. Medical & Veterinary Entomology,* 25. 217-226.

Bissinger, B., Apperson, C., Sonenshine, D., Watson, D. & Roe, R. (2009) Efficacy of the new repellent BioUD® against three species of ixodid ticks. *Experimental & Applied Acarology*, 48. 239-250.

Bissinger, B., Kennedy, M. & Carroll, S. (2016) Sustained efficacy of the novel topical repellent TT-4302 against mosquitoes and ticks. *Medical & Veterinary Entomology*, 30. 107-111.

Bissinger, B.W., Schmidt, J.P., Owens, J.J., Mitchell, S.M. & Kennedy, M.K. (2014a) Performance of the plant-based repellent TT-4302 against mosquitoes in the laboratory and field and comparative efficacy to 16 mosquito repellents against *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology,* 51(2). 392-399.

Bissinger, B. W., Schmidt, J. P., Owens, J. J., Mitchell, S. M., & Kennedy, M. K. (2014b). Activity of the plant-based repellent, TT-4302 against the ticks *Amblyomma americanum, Dermacentor variabilis, Ixodes scapularis* and *Rhipicephalus sanguineus* (Acari: Ixodidae).*Experimental & Applied Acarology*, 62(1). 105-113.

Block, E. (2004) Biological activity of allium compounds: recent results. In *IV International Symposium on Edible Alliaceae* 688. 41-58.

Bloomquist, J.R. (2002) Agents affecting chloride channels. In *Handbook of Neurotoxicology* Humana Press (pp. 65-77).

Boghrati, Z., Naseri, M., Rezaie, M., Pham, N., Quinn, R.J., Tayarani-Najaran, Z. & Iranshahi, M. (2016) Tyrosinase inhibitory properties of phenylpropanoid glycosides and flavonoids from *Teucrium polium* L. var. *gnaphalodes. Iranian Journal of Basic Medical Sciences*, 19. 804.

Bohbot, J.D. & Dickens, J.C. (2012) Odorant receptor modulation: ternary paradigm for mode of action of insect repellents. *Neuropharmacology* 62. 2086-2095.

Bonnet, S., Jouglin, M., Malandrin, L., Becker, C., Agoulon, A., L'hostis, M. & Chauvin, A. (2007) Transstadial and transovarial persistence of *Babesia divergens* DNA in *Ixodes ricinus* ticks fed on infected blood in a new skin-feeding technique. *Parasitology,* 134(2). 197-207.

Borges, L.M.F., Ferri, P.H., Silva, W.J., Silva, W.C. & Silva, J.G. (2003) *In vitro* efficacy of extracts of *Melia azedarach* against the tick *Boophilus microplus. Medical & Veterinary Entomology* 17. 228-231.

Borges, L.M.F., Sousa, Lorena Alessandra Dias de & Barbosa, C.D.S. (2011) Perspectives for the use of plant extracts to control the cattle tick *Rhipicephalus (Boophilus) microplus. Revista Brasileira de Parasitologia Veterinária*, 20. 89-96.

Bowman, A.S. & Nuttall, P.A. (2008). Ticks: Biology, Disease and Control. Cambridge University Press.

Bram, A. (1983) Tick-borne livestock diseases and their vectors: The global problem. *In: ticks and tick-borne diseases, FAO animal production and health.* Food and Agriculture Organization, Rome 7-11.

Brown, H.M., Latimer, K.S., Erikson, L.E., Cashwell, M.E., Britt, J.O. & Peterson, D.S. (2008) Detection of persistent *Cytauxzoon felis* infection by polymerase chain reaction in three asymptomatic domestic cats. *Journal of Veterinary Diagnostic Investigation,* 20. 485-488.

Brunetti, C., Di Ferdinando, M., Fini, A., Pollastri, S. & Tattini, M. (2013) Flavonoids as antioxidants and developmental regulators: relative significance in plants and humans. *International Journal of Molecular Sciences*, 14. 3540-3555.

Buckingham, S.D., Biggin, P.C., Sattelle, B.M., Brown, L.A. & Sattelle, D.B. (2005) Insect GABA receptors: splicing, editing, and targeting by antiparasitics and insecticides. *Molecular Pharmacology*, 68. 942-951.

Bull, M.S., Swindale, S., Overend, D. & Hess, E.A. (1996) Suppression of *Boophilus microplus* populations with fluazuron - an acarine growth regulator. *Australian Veterinary Journal,* 74. 468–470.

Burg, R.W., Miller, B.M., Baker, E.E., Birnbaum, J., Currie, S.A., Hartman, R., Kong, Y.L., Monaghan, R.L., Olson, G., Putter, I., Tunac, J.B., Wallick, H., Stapley, E.O., Oiwa, R. & Omura, S. (1979) Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. *Antimicrobial Agents & Chemotherapy*, 15. 361-367.

Burnham, R.J. & Johnson, K.R. (2004) South American palaeobotany and the origins of neotropical rainforests. *Philosophical transactions of the Royal Society of London.Series B, Biological Sciences*, 359. 1595-1610.

Calmasur, O., Aslan, I. & Sahin, F. (2006) Insecticidal and acaricidal effect of three Lamiaceae plant essential oils against *Tetranychus urticae* Koch and *Bemisia tabaci* Genn. *Industrial Crops and Products* 23. 140-146.

Camerino, E. (2015) Fluoromethyl ketone prodrugs: Potential new insecticides towards *Anopheles gambiae*. http://hdl.handle.net/10919/73771.

Canevari, J., Mangold, A., Guglielmone, A. & Nava, S. (2017) Population dynamics of the cattle tick *Rhipicephalus (Boophilus) microplus* in a subtropical subhumid region of Argentina for use in the design of control strategies. *Medical & Veterinary Entomology,* 31(1).6-14.

Carr, A.L., & Roe, M. (2016) Acarine attractants: chemoreception, bioassay, chemistry and control. *Pesticide Biochemistry & Physiology*,131. 60-79.

Carr, A.L., Sonenshine, D.E., Strider Jr, J.B. & Roe, R.M. (2016) Evidence of female sex pheromones and characterization of the cuticular lipids of unfed, adult male versus female blacklegged ticks, *Ixodes scapularis*. *Experimental & Applied Acarology*, 68. 519-538.

Carreón, D., de la Lastra, José M Pérez, Almazán, C., Canales, M., Ruiz-Fons, F., Boadella, M., Moreno-Cid, J.A., Villar, M., Gortázar, C. & Reglero, M. (2012) Vaccination with BM86, subolesin and akirin protective antigens for the control of tick infestations in white tailed deer and red deer. *Vaccine*, 30. 273-279.

Carroll, J., Benante, J., Klun, J., White, C., Debboun, M., Pound, J. & Dheranetra, W. (2008) Twelve-hour duration testing of cream formulations of three repellents against *Amblyomma americanum*. *Medical & Veterinary Entomology,* 22. 144-151.

Carroll, J.F., Babish, J.G., Pacioretty, L.M. & Kramer, M. (2016) Repellency to ticks (Acari: Ixodidae) of extracts of *Nigella sativa* (Ranunculaceae) and the anti-inflammatory DogsBestFriend™. *Experimental & Applied Acarology*, 70(1). 89-97.

Carroll, J., Klun, J. & Debboun, M. (2005) Repellency of deet and SS220 applied to skin involves olfactory sensing by two species of ticks. *Medical & Veterinary Entomology,* 19. 101-106.

Carroll, J.F., Cantrell, C.L., Klun, J.A. & Kramer, M. (2007) Repellency of two terpenoid compounds isolated from *Callicarpa americana* (Lamiaceae) against *Ixodes scapularis* and *Amblyomma americanum* ticks. *Experimental & Applied Acarology,* 41. 215-224.

Carroll, J.F., Carroll, J.F., Kramer, M. & Bedoukian, R.H. (2014) Solvent, drying time, and substrate affect the responses of lone star ticks (Acari: Ixodidae) to the repellents deet and picaridin. *Journal of Medical Entomology,* 51(3). 629-637.

Carroll, J.F., Solberg, V.B., Klun, J.A., Kramer, M. & Debboun, M. (2004) Comparative activity of deet and AI3-37220 repellents against the ticks *Ixodes scapularis* and *Amblyomma americanum* (Acari: Ixodidae) in laboratory bioassays. *Journal of Medical Entomology,* 41(2). 249-254.

Carvalho, A.C.C., Almeida, D.S., Melo, M.G., Cavalcanti, S.C. & Marçal, R.M. (2009) Evidence of the mechanism of action of *Erythrina velutina* Willd (Fabaceae) leaves aqueous extract. *Journal of Ethnopharmacology*, 122. 374-378.

Casida, J.E. (1980) Pyrethrum flowers and pyrethroid insecticides. *Environmental Health Perspectives* 34, 189.

Casida, J. E. & Durkin, K. A. (2013) Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. *Annual Review of Entomology*, *58.* 99-117.

Castilho, R.O., Belo Horizonte, M.G. & Kaplan, M.A.C. (2008) Chemical contistuents of *Licania tomentosa* B. (Chrysobalanaceae). *Inorganic, Organic, Physical & Analytical Chemistry, Quimica Nova 31(1). 66-69.*

Céspedes, C.L., Alarcon, J.E., Aqueveque, P., Seigler, D.S. & Kubo, I. (2015) In the search for new secondary metabolites with biopesticidal properties. *Israel Journal of Plant Sciences*, 62. 216-228.

Cetin, H., Cilek, J.E., Aydin, L. & Yanikoglu, A. (2009) Acaricidal effects of the essential oil of *Origanum minutiflorum* (Lamiaceae) against *Rhipicephalus turanicus* (Acari: Ixodidae). *Veterinary Parasitology*, 160. 359-361.

Cetin, H., Cilek, J.E., Oz, E., Aydin, L., Deveci, O. & Yanikoglu, A. (2010) Acaricidal activity of *Satureja thymbra* L. essential oil and its major components, carvacrol and γ-terpinene against adult *Hyalomma marginatum* (Acari: Ixodidae). *Veterinary Parasitology,* 170(3). 287-290.

Chagas, A.C.S., Passos, M.W.M., Prates, H.T., Leite, R.C., Furlong, J. & Fortes, I.C.P. (2002) Efeito acaricida de oleos essenciais e concentrados emulsionaveis de *Eucalyptus* spp. Em *Boophilus microplus. Brazilian Journal of Veterinary Research & Animal Science* 39. 247-253.

Chagas, A.C.S., de Barros, L.D., Continguiba, F. Furlan, M., Giglioti, R., Oliveira, M.C.S., & Bizzo, H.R. (2012) *In vitro* efficacy of plant extracts and synthesized substances on *Rhipicephalus* (*Boophilus) microplus.* (Acari: Ixodidae). *Parasitology Research,* 110. 295-303.

Chagas, A.C.S., Domingues, L.F., Fantatto, R.R., Giglioti, R., Oliveira, M.C., Oliveira, D.H., Mano, R.A. & Jacob, R.G. (2014) *In vitro* and *in vivo* acaricide action of juvenoid analogs produced from the chemical modification of *Cymbopogon* spp. and *Corymbia citriodora* essential oil on the cattle tick *Rhipicephalus (Boophilus) microplus*. *Veterinary Parasitology,* 205(1). 277-284.

Chagas, A.C.S., Domingues, L.F., Fantatto, R.R., Giglioti, R., Oliveira, M.C., Oliveira, D.H., Mano, R.A. & Jacob, R.G. (2014) *In vitro* and *in vivo* acaricide action of juvenoid analogs produced from the chemical modification of *Cymbopogon* spp. and *Corymbia citriodora* essential oil on the cattle tick *Rhipicephalus (Boophilus) microplus. Veterinary Parasitology*, 205(1). 277-284.

Chaieb, K., Hajlaoui, H., Zmantar, T., Kahla-Nakbi, A.B., Rouabhia, M., Mahdouani, K. & Bakhrouf, A. (2007) The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (Syzigium aromaticum L. Myrtaceae): a short review, *Phytotherapy Research,* 21. 501-506.

Chanthaphon, S., Chanthachum, S. & Hongpattarakere, T. (2008) Antimicrobial activities of essential oils and crude extracts from tropical *Citrus* spp. against food-related microorganisms*. Songklanakarin Journal of Science & Technology,* 30(1). 125.

Chapman & Hall, (2017) Dictionary of Natural Products on CD-ROM, Chemical DataBase.

Chen, A.C., He, H. & Davey, R.B. (2007) Mutations in a putative octopamine receptor gene in amitraz-resistant cattle ticks. *Veterinary Parasitology*, 148. 379-383.

Chen, W. & Viljoen, A. (2010) Geraniol-a review of a commercially important fragrance material. *South African Journal of Botany,* 76. 643-651.

Chen, Z., Ying, X., Meng, S., Zhu, X., Jiang, H., Cao, Q., Li, X. & Meng, F. (2011) High-performance liquid chromatographic determination and pharmacokinetic study of apigenin-7-O-β-D-glucoside in rat plasma after intravenous administration. *Archives of Pharmacal Research*, 34. 741-746.

Chen, Z., Ying, X., Meng, S., Zhu, X., Jiang, H., Cao, Q., Wang, L. & Meng, F. (2012) LC determination of luteolin-7-O-β-D-glucoside and apigenin-7-O-β-D-glucoside in rat plasma after administration of *Humulus scandens* extract and its application to pharmacokinetic studies. *Natural Product Research*, 26. 530-539.

Chen, W., Lin, H. (2012) Identification of voltage-gated potassium channel subfamilies from sequence information using support vector machine. *Computers in Biology & Medicine*, 42(4). 504-507.

Chochlakis, D., Ioannou, I., Papadopoulos, B., Tselentis, Y., & Psaroulaki, A. (2014). *Rhipicephalus turanicus:* from low numbers to complete establishment in Cyprus. Its possible role as a bridge-vector. *Parasites & Vectors*, 7(S1), P11.

Choe, S. (2002) Potassium channel structures. *Nature Reviews Neuroscience* 3. 115-121.

Choi, W.S., Park, B.S., Ku, S.K. & Lee, S.E. (2002) Repellent activities of essential oils and monoterpenes against *Culex pipiens* pallens. *Journal of the American Mosquito Control Association* 18. 348-351.

Chopra, A.S. (2003) Ayurveda. InSelins, Helaine. Medicine Across Cultures: History and Practice of Medicine in Non-Western Cultures. Norwell, M.A. Kluwer Academic Publishers pp 75-83.

Choudhury, M.K. (2001) Toxicity of neem seed oil (*Azadirachta indica*) against the larvae of *Rhipicephalus sanguineus* a three-host tick in dog. *Journal of Parasitic Diseases,* 25. 46-47.

Choi, W.S., Park, B.S., Ku, S.K. & Lee, S.E. (2002) Repellent activities of essential oils and monoterpenes against *Culex pipiens* pallens, *Journal of the American Mosquito Control Association* 18. 348-351.

Choudhury, M.K., Shiferaw, Y., & Hussen, A. (2015) Toxicity of *Millettia ferruginea* Darasana (Family: Fabaceae) against the larvae and adult ticks of *Amblyomma variegatum* Fabricius a three-host tick in cattle. *Journal of Parasitic Diseases*, 39(2). 298-302.

Chu, S.S., Feng Hu, J. & Liu, Z.L. (2011) Composition of essential oil of Chinese *Chenopodium ambrosioides* and insecticidal activity against maize weevil, *Sitophilus zeamais. Pest Management Science* 67. 714-718.

Chungsamarnyart, N. & Jiwajinda, S. (1992) Acaricidal activity of volatile oil from lemon and citronella grasses on tropical cattle ticks. *Kasetsart Journal: Natural Science Supplement,* 26. 46-51.

Chungsamarnyart, N., Jiwajinda, S., Jansawan, W., Kaewsuwan, U. & Buranasilpin, P. (1988) Effective plant crude-extracts on the tick (*Boophilus microplus*) I. Larvicidal action. *Kasetsart Journal: Natural Science Supplement,* 22(5). 37-41.

Chungsamarnyart, N., Jiwajinda, S. & Jansawan, W. (1990) Effect of plant crude-extracts on the tick (*Boophilus microplus*) I. Insecticidal action. *Kasetsart Journal: Natural Science Supplement,* 24. 28-31.

Chungsamarnyart, N., Jiwajinda, S., Ratanakreetakul, C. & Jasawan, W. (1991a). Practical extraction of sugar apple seeds against tropical cattle ticks. *Kasetsart Journal: Natural Science Supplement,* 25. 101-105.

Chungsamarnyart, N., Jiwajinda, S. & Jansawan, W. (1991b). Larvicidal effect of plant-crude-extracts on the tropical cattle tick (*B. microplus*), Thailand. *Kasetsart Journal: Natural Science Supplement,* 25. 80-89.

Chungsamarnyart, N. & Jansawan, W. (1996). Acaricidal activity of peel oil of *Citrus* spp. on *Boophilus microplus*. *Kasetsart Journal: Natural Science Supplement,* 30. 112-117.

Chungsamarnyart, N. & Jansawan, W. (2001) Effect of *Tamarindus indicus* L. against the *Boophilus microplus*. *Kasetsart Journal: Natural Science Supplement,* 35. 34-39.

Chungsamarnyart, N. & Jiwajinda, S. (1992) Acaricidal activity of volatile oil from lemon and citronella grasses on tropical cattle ticks. *Kasetsart Journal: Natural Science Supplement,* 26. 46-51.

Chutia, M., Bhuyan, P.D., Pathak, M., Sarma, T. & Boruah, P. (2009) Antifungal activity and chemical composition of *Citrus reticulata* Blanco essential oil against phytopathogens from North East India. *LWT-Food Science & Technology,* 42. 777-780.

Cisak, E., Wójcik-Fatla, A., Zajac, V. & Dutkiewicz, J. (2012) Repellents and acaricides as personal protection measures in the prevention of tick-borne diseases. *Annals of Agricultural & Environmental Medicine* 19(4).

Clemente, M.A., de Oleveira Monteiro, C.M., Scoralik, M.G., Gomes, F.T., de Azevedo Prata, M.C. & Daemon, E. (2010) Acaricidal activity of the essential oils from *Eucalyptus citriodora* and *Cymbopogon nardus* on larvae of *Amblyomma cajennense* (Acari: Ixodidae) and *Anocentor nitens* (Acari: Ixodidae). *Parasitology Research* 107. 987-992.

Coats, J.R. (1990) Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. *Environmental Health Perspectives*, 87. 255-262.

Cobon, G.S. & Willadsen, P. (1990) Vaccines to prevent cattle tick infestations. *New Generation Vaccines,* 50. 901-917.

Codd, L.E. (1985) The genus *Tetradenia*. *Flora of Southern Africa,* 28(4). 113-116.

Coetzer, J.A.W., Thomson, G.R. & Tustin, R.C. (1994). *Infectious diseases of livestock with special reference to Southern Africa:* Volume 1. Oxford University Press Southern Africa.

Cohn, L., Birkenheuer, A., Brunker, J., Ratcliff, E. & Craig, A. (2011) Efficacy of atovaquone and azithromycin or imidocarb dipropionate in cats with acute cytauxzoonosis. *Journal of Veterinary Internal Medicine*, 25. 55-60.

Cole, L.M., Nicholson, R.A. & Casida, J.E. (1993) Action of phenylpyrazole insecticides at the GABA-gated chloride channel. *Pesticide Biochemistry & Physiology*, 46. 47-54.

Cole, M. (1994) Key antifungal, antibacterial and anti-insect assays-a critical review. *Biochemical Systematics & Ecology,* 22(8). 837-856.

Combes, C. (2001). Parasitism: The ecology and evolution of intimate interactions. University of Chicago Press.

Connell, D., Lam, P., Richardson, B. & Wu, R. (1999) Introduction to Ecotoxicology. Blackwell Science, Oxford.

Connell, D.W., Miller, G.J., Mortimer, M.R., Shaw, G.R. & Anderson, S.M. (1999) Persistent lipophilic contaminants and other chemical residues in the Southern Hemisphere. *Critical Reviews in Environmental Science & Technology*, 29. 47-82.

Copping, L.G. & Menn, J.J. (2000) Biopesticides: a review of their action, applications and efficacy. *Pest Management Science,* 56(8). 651-676.

Cordoves, C., de la Cruz, J., Tamayo, J., Mesejo, J. & Fleites, R. (1986) Experience and perspectives of tick control and eradication in the Republic of Cuba. *Revista Cubana de Ciencias Veterinarias*, 7. 1-13.

Corta, E., Bakkali, A., Berrueta, L., Gallo, B. & Vicente, F. (1999) Kinetics and mechanism of amitraz hydrolysis in aqueous media by HPLC and GC-MS. *Talanta*, 48. 189-199.

Coskun, S., Girisgin, O., Kürkcüoglu, M., Malyer, H., Girisgin, A.O., Kırımer, N. & Baser, K.H. (2008) Acaricidal efficacy of *Origanum onites* L. essential oil against *Rhipicephalus turanicus* (Ixodidae). *Parasitology Research*, 103. 259-261.

Costa, S.M.O., Lemos, T.L.G., Pessoa, O.D.L., Pessoa, C., Montenegro, R.C. & Braz-Filho, R. (2001) Chemical constituents from *Lippia sidoides* and cytotoxic activity. *Journal of Natural Products,* 64(6). 792-795.

Costescu, M., Păunescu, H., Velescu, B., Zugravu, A., Coman, O.A. & Fulga, I. (2016) Experimental research on the effects of serotonin and acetylcholine on mechanical isometric activity of isolated rat intestine after the depletion of endogenous serotonin. *Romania Journal of Biophysics* 26(1). 1-14.

Cragg, G.M. & Newman, D.J. (2013) Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1830. 3670-3695.

Craik, D.J., Daly, N.L. & Waine, C. (2001) The cystine knot motif in toxins and implications for drug design. *Toxicon*, 39. 43-60.

Critchfield, J.W., Butera, S.T. & Folks, T.M. (1996) Inhibition of HIV activation in latently infected cells by flavonoid compounds. *AIDS Research & Human Retroviruses*, 12. 39-46.

Croteau, R. & Winters, J.N. (1982). Demonstration of the intercellular compartmentation of l-menthone metabolism in peppermint (*Mentha piperita*) leaves. *Plant Physiology,* 69(4). 975-977.

Cunha, R.C., Pérez de León, Adalberto Angel, Leite, F.P.L., Pinto, L.d.S., Júnior, S. & Andreotti, R. (2012) Bovine immunoprotection against *Rhipicephalus (Boophilus) microplus* with recombinant Bm86-Campo Grande antigen. *Revista Brasileira de Parasitologia Veterinária*, 21. 254-262.

Daemon, E., Monteiro, C.M., Rosa, L.S., Clemente, M.A. & Arcoverde, A. (2009) Evaluation of the acaricidal activity of thymol on engorged and unengorged larvae of *Rhipicephalus sanguineus* (Latreille 1808) (Acari: Ixodidae). *Parasitology Research,* 105. 495-497.

Dantas, A., Machado, D., Araujo, A., Oliveira-Junior, R., Lima-Saraiva, S., Ribeiro, L., Almeida, J. & Horta, M. (2015) Acaricidal activity of extracts from the leaves and aerial parts of *Neoglaziovia variegata* (Bromeliaceae) on the cattle tick *Rhipicephalus (Boophilus) microplus. Research in Veterinary Science*, 100. 165-168.

da Silva Lima, A., do Nascimento Sousa Filho, J.G., Pereira, S.G., Guillon, G.M.S. P., da Silva Santos, L. & Júnior, L.M.C. (2014) Acaricide activity of different extracts from *Piper tuberculatum* fruits against *Rhipicephalus microplus*. *Parasitology Research,* 113(1). 107-112.

Dautel, H. (2004) Test systems for tick repellents. *International Journal of Medical Microbiology Supplements,* 293. 182-188.

Dautel, H., Dippel, C., Werkhausen, A. & Diller, R. (2013) Efficacy testing of several *Ixodes ricinus* tick repellents: Different results with different assays. *Ticks & Tick-borne Diseases,* 4(3). 256-263.

Dautel, H., Kahl, O., Siems, K., Oppenrieder, M., Müller-Kuhrt, L. & Hilker, M. (1999) A novel test system for detection of tick repellents. *Entomologia Experimentalis et Applicata,* 91(3). 431-441.

Davari, B., Vatandoost, H., Oshaghi, M., Ladonni, H., Enayati, A., Shaeghi, M., Basseri, H., Rassi, Y. & Hanafi-Bojd, A. (2007) Selection of *Anopheles stephensi* with DDT and dieldrin and cross-resistance spectrum to pyrethroids and fipronil. *Pesticide Biochemistry & Physiology*, 89. 97-103.

Davey, R. B. & Ahrens, E. H. (1984) Control of *Boophilus* ticks on heifers with two pyrethroids applied as sprays. *American Journal of Veterinary Research* 45. 1008-1010.

De Caluwé, E., Halamova, K. & Van-Damme, P. (2010) *Tamarindus indica* L.: a review of traditional uses, phytochemistry and pharmacology. *Africa Focus* 23. 53–83

de BF Ferraz, A., Balbino, J.M., Zini, C.A., Ribeiro, V.L.S., Bordignon, S.A. & von Poser, G. (2010) Acaricidal activity and chemical composition of the essential oil from three *Piper* species. *Parasitology Research* 107, 243-248.

De Castro, J., James, A., Minjauw, B., Di Giulio, G., Permin, A., Pegram, R., Chizyuka, G. & Sinyangwe, P. (1997) Long-term studies on the economic impact of ticks on Sanga cattle in Zambia. *Experimental & Applied Acarology*, 21. 3-19.

De Castro, J. & Newson, R. (1993) Host resistance in cattle tick control. *Parasitology Today*, 9. 13-17.

de Freitas Fernandes, F., Freitas & Edméia de Paula Souza (2007). Acaricidal activity of an oleoresinous extract from *Copaifera reticulata* (Leguminosae: Caesalpinioideae) against larvae of the southern cattle tick, *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Veterinary Parasitology* 147. 150-154.

de la Fuente, J.O.S.É. & Kocan, K.M. (2014) Development of vaccines for control of tick infestations and interruption of pathogen transmission. *Biology of Ticks*, *2*. 333-52.

de Oliveira Cruz, Elizangela Mércia, Costa-Junior, L.M., Pinto, J.A.O., de Alexandria Santos, D., de Araujo, S.A., de Fátima Arrigoni-Blank, M., Bacci, L., Alves, P.B., de Holanda Cavalcanti, Sócrates Cabral & Blank, A.F. (2013) Acaricidal activity of *Lippia gracilis* essential oil and its major constituents on the tick *Rhipicephalus (Boophilus) microplus. Veterinary Parasitology* 195, 198-202.

De Waal, D.T. (2000) Anaplasmosis control and diagnosis in South Africa. *Annals of the New York Academy of Sciences*, 916. 474-483.

Del Fabbro, S. & Nazzi, F. (2008) Repellent effect of sweet basil compounds on *Ixodes ricinus* ticks. *Experimental & Applied Acarology,* 45(3-4). 219-228.

Delle Monache, G., Cristina De Rosa, M., Scurria, R., Monacelli, B., Pasqua, G., Dall'Olio, G. & Botta, B. (1991) Metabolites from *in vitro* cultures of *Cassia didymobotrya*. *Phytochemistry,* 30(6). 1849-1854.

Delgado, C.L., Rosegrant, M.W., Steinfeld, H., Ehui, S. & Courbois, C. (1999) The coming livestock revolution. *Background paper n.6, Department of Economic and Social Affairs, Commission of Sustainable Development, Eighth Session*.

Denis, M. (2014) African swine fever: An epidemiological overview. *British Journal of Virology*, 1. 42.

Dethier, V.J. (1948) Chemical insect attractants and repellents. *Soil Science,* 65(2). 204.

Dethier, V. (1956) Repellents. *Annual Review of Entomology,* 1(1). 181-202.

Dethier, V., Browne, B.L. & Smith, C.N. (1960) The designation of chemicals in terms of the responses they elicit from insects. *Journal of Economic Entomology,* 53(1). 134-136.

Devine, G. J. & Denholm, I. (1998) An unconventional use of piperonyl butoxide for managing the cotton whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Bulletin of Entomological Research* 88(6). 601-610.

de Sousa, L.A., Soares, S.F. & Pires, H.B. Jr. (2008) Evaluation of efficacy of ripe and unripe fruit oil extracts of *Melia azedarach* against *Rhipicephalus (Boophilus) microplus* (Acari: ixodidae). *Revista Brasileira de Parasitologia Veterinária* 7(1). 36–40.

Dhang, P. & Sanjayan, K. P. (2014) 15 Plants with pest control properties against urban pests. *Urban Insect Pests: Sustainable Management Strategies*, 216.

Dhooria, M. S. (2016). Feeding, Development and Reproduction. In *Fundamentals of Applied Acarology* Springer Singapore, 161-173.

Dietrich, G., Dolan, M.C., Peralta-Cruz, J., Schmidt, J., Piesman, J., Eisen, R.J. & Karchesy, J.J. (2006) Repellent activity of fractioned compounds from *Chamaecyparis nootkatensis* essential oil against nymphal *Ixodes scapularis* (Acari: Ixodidae). *Journal of Medical Entomology*, 43(5). 957-961.

Ding, H., Deng, E.Z., Yuan, L. F., Liu, L., Lin, H., Chen, W. & Chou, K.C. (2014) iCTX-Type: a sequence-based predictor for identifying the types of conotoxins in targeting ion channels.*BioMed Research International.* http://dx.doi.org/10.1155/2014/286419.

Dipeolu, O.O., Mongi, A.O., Punyua, D.K., Latif, A.A., Amoo, O.A. & Odiambo, T.R. (1992) Current concepts and approach to control of livestock ticks in Africa. *Discovery & Innovation,* 4. 35-44.

Divya, T.M., Soorya, V.C., Amithamol, K.K., Juliet, S., Ravindran, R., Nair, S.N. & Ajithkumar, K.G. (2014) Acaricidal activity of alkaloid fractions of *Leucas indica* Spreng against *Rhipicephalus (Boophilus) annulatus* tick. *Tropical Biomedicine,* 31(1). 46-53.

Domingos, A., Antunes, S., Borges, L. & Rosario, V.E.D. (2013) Approaches towards tick and tick-borne diseases control. *Revista da Sociedade Brasileira de Medicina Tropical*, 46. 265-269.

Domingues, L.F., Giglioti, R., Feitosa, K.A., Fantatto, R.R., Rabelo, M.D., de Sena Oliveira, M.C., Bechara, G.H., de Oliveira, G.P. & de Souza Chagas, A.C. (2013) *In vitro* and *in vivo* evaluation of the activity of pineapple (*Ananas comosus*) on *Haemonchus contortus* in Santa Inês sheep. *Veterinary Parasitology,* 197(1). 263-270.

Dominguez-García, D., Rosario-Cruz, R., García, C., Oaxaca, J. & De la Fuente, J. (2010) *Boophilus microplus*: aspectos biológicos y moleculares de la resistencia a los acaricidas y su impacto en la salud animal. *Tropical & Subtropical Agroecosystems,* 12. 181-192.

Drummond, R.O. (1960) Preliminary evaluation of animal systemic insecticides. *Journal of Economic Entomology* 53(6). 1125-1127.

Drakou, C.E., Tsitsanou, K.E., Potamitis, C., Fessas, D., Zervou, M. & Zographos, S.E., (2017) The crystal structure of the AgamOBP1• Icaridin complex reveals alternative binding modes and stereo-selective repellent recognition. *Cellular & Molecular Life Sciences* 74. 319-338.

Dremova, V. & Smirnova, S. (1970) Effects of repellents on hard (Ixodidae) and soft (Argasidae) ticks. *International Pest Control,* 12(3). 10-14.

Drummond, R.O., Ernst, S.E., Trevino, J.L., Gladney, W.J. & Graham, O.H. (1973) *Boophilus annulatus* and *B. microplus:* laboratory tests of insecticides. *Journal of Economic Entomology*, 66. 130-133.

Durden, L. A., & Mans, B. J. (2016) Tick paralysis: Some host and tick perspectives. *A Century of Parasitology. Wiley, London*, 167-176.

Dzoyem, J.P., Tsamo, A.T., Melong, R., Mkounga, P., Nkengfack, A.E., McGaw, L.J. & Eloff, J.N. (2015) Cytotoxicity, nitric oxide and acetylcholinesterase inhibitory activity of three limonoids isolated from *Trichilia welw i tschii* (Meliaceae). *Biological Research*, 48. 1.

Ebrahimi, A., & Schluesener, H. (2012) Natural polyphenols against neurodegenerative disorders: potentials and pitfalls. *Ageing Research Reviews*, 11(2). 329-345.

Edlow, J.A. & McGillicuddy, D.C. (2008) Tick paralysis. *Infectious Disease Clinics of North America*, 22. 397-413.

Edwards, I.R. & Aronson, J.K. (2000) Adverse drug reactions: definitions, diagnosis, and management. *The Lancet*, 356. 1255-1259.

Eiden, A.L., Kaufman, P.E., Oi, F.M., Allan, S.A., & Miller, R.J. (2015) Detection of permethrin resistance and fipronil tolerance in *Rhipicephalus sanguineus* (Acari: Ixodidae) in the United States. *Journal of Medical Entomology*, 52(3). 429-436.

Elango, G. & Rahuman, A.A. (2011) Evaluation of medicinal plant extracts against ticks and flukes. *Parasitology Research* 108. 513-519.

Elfawal, M. A., Towler, M. J., Reich, N. G., Weathers, P. J., & Rich, S. M. (2015) Dried whole-plant *Artemisia annua* slows evolution of malaria drug resistance and overcomes resistance to artemisinin. *Proceedings of the National Academy of Sciences*, 112(3), 821-826.

Elisha, I.L., Dzoyem, J., McGaw, L.J., Botha, F.S. & Eloff, J.N. (2016) The anti-arthritic, anti-inflammatory, antioxidant activity and relationships with total phenolics and total flavonoids of nine South African plants used traditionally to treat arthritis. *BMC Complementary & Alternative Medicine*, 16. 307.

Ellman, G.L., Courtney, K.D., Andres, V. & Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, 7. 88-95.

Eloff, J. (2001) Antibacterial activity of Marula (*Sclerocarya birrea* (A. rich.) Hochst. subsp. caffra (Sond.) Kokwaro)(Anacardiaceae) bark and leaves. *Journal of Ethnopharmacology*, 76. 305-308.

Eloff, J. (1998) Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*, 60. 1-8.

Eloff, J.N. & McGaw, L.J. 2006 Plant extracts used to manage bacterial, fungal and parasitic infections in southern Africa. *Modern phytomedicine: turning medicinal plants into drugs*, 97-121.

Eloff, J. & McGaw, L. (2014) Using African plant biodiversity to combat microbial infections. *Novel Plant Bioresources: Applications in Food, Medicine & Cosmetics,* 163-173.

Englund, L. & Pringle, J. (2004) New diseases and increased risk of diseases in companion animals and horses due to transport. *Acta Veterinaria Scandinavica Supplementum*, 100. 19-25.

Escoubas, P., Diochot, S. & Corzo, G. (2000) Structure and pharmacology of spider venom neurotoxins. *Biochimie*, 82. 893-907.

Estrada-Pena, A., Bouattour, A., Camicas, J. & Walker, A. (2004) *Ticks of Domestic Animals in the Mediterranean Region,* 131.

Estrada-Peña, A. & de la Fuente, J. (2014) The ecology of ticks and epidemiology of tick-borne viral diseases. *Antiviral Research*, 108. 104-128.

Evans, G.O. (1992) Principles of Acarology. Wallingford: CAB international. (Vol. 18, p. 563).

Eyasu, M., Shibeshi, W. & Gida, M. (2013) *In vivo* antimalarial activity of hydromethanolic leaf extract of *Calpurnia aurea* (Fabaceae) in mice infected with chloroquine sensitive *Plasmodium berghei. International Journal of Pharmacology*, 2. 131-142.

Facey, P.C., Porter, R.B., Reese, P.B. & Williams, L.A. (2005) Biological activity and chemical composition of the essential oil from Jamaican *Hyptis verticillata* Jacq. *Journal of Agriculture & Food Chemistry* 53. 4774-4777.

Fadia, A., Al–Naser, Z. & Al-Hakim, W. (2015) Chemical composition of *Lavandula angustifolia* Miller and *Rosmarinus officinalis* L. essential oils and fumigant toxicity against larvae of *Ephestia kuehniella* Zeller. *International Journal of ChemTech Research* 8. 1382-1390.

Falco, R.C. & Fish, D. (1991) Horizontal movement of adult *Ixodes dammini* (Acari: Ixodidae) attracted to CO2-baited traps. *Journal of Medical Entomology,* 28(5). 726-729.

Falcone Ferreyra, M.L., Rius, S. & Casati, P. (2012) Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Frontiers in Plant Science*, 3. 222.

Favela-Hernández, J.M.J., González-Santiago, O., Ramírez-Cabrera, M.A., Esquivel-Ferriño, P.C. & Camacho-Corona, M.D.R. (2016) Chemistry and Pharmacology of *Citrus sinensis. Molecules,* 21. 247.

Fernández-Salas, A., Rodríguez-Vivas, R.I. & Alonso-Díaz, M.Á. (2012) Resistance of *Rhipicephalus microplus* to amitraz and cypermethrin in tropical cattle farms in Veracruz, Mexico. *Journal of Parasitology*, 98. 1010-1014.

Fernandes, F.F., Freitas, E.P.S., Coats, A.C. & Silva, I.G. (2005) Larvicidal potential of *Sapindus saponaria* to control the cattle tick *Boophilus microplus*. *Pesquisa Agropecuária Brasileira,* 40. 1243-1245.

Fernandes, F. Fernandes, F.F., Leles, R.N., Silva, I.G. & Freitas, E.P.S. (2007) Larvicidal potential of *Sapindus saponaria* (Sapindaceae) against *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae). *Arquivo Brasileiro de Medicina Veterinária e Zootecnia,* 59(1). 145-149.

Fernandes, F. De F., Bessa, P.A.D. & Edmeia de Paula, S.F. (2008) Evaluation of activity of the crude ethanolic extract of *Magonia pubescens* St. Hill (Sapindaceae) against larvae of the cattle tick *Rhipicephalus (Boophilus) micoplus* (Canestrini, 1887) (Acari: Ixodidae). *Brazilian Archives of Biology & Technology,* 51(6). 1147-1152.

Fernandez-Salas, A., Alonso-Dias, M.A., Acosta- Rodriguez, R., Torres-Acosta, J.F.J., Sandoval-Castro, C.A. & Rodriguez-Vivas, R.I. (2011) *In vitro* acaricidal effect of tannin-rich plants against the cattle tick, *Rhipicephalus (Boophilus) microplus* (Acari: ixodidae). *Veterinary Parasitology* 175. 113-118.

Ferreira, L.L., de Oliveira Filho, J.G., Mascarin, G.M., de León, A.A.P. & Borges, L.M.F. (2017) *In vitro* repellency of DEET and β-citronellol against the ticks *Rhipicephalus sanguineus* sensu lato and *Amblyomma sculptum*. *Veterinary Parasitology,* 239.42-45.

Ferraz, A.B., Bordignon, S.A., Staats, C., Schripsema, J. & Lino von Poser, G. (2001) Benzopyrans from *Hypericum polyanthemum*. *Phytochemistry* 57(8), 1227-1230.

Fiot, J., Sanon, S., Azas, N., Mahiou, V., Jansen, O., Angenot, L. & Ollivier, E. (2006) Phytochemical and pharmacological study of roots and leaves of *Guiera senegalensis* JF Gmel (Combretaceae). *Journal of Ethnopharmacology* 106(2). 173-178.

Flamini, G. (2003) Acaricides of natural origin, personal experiences and review of literature (1990-2001). *Studies in Natural Products Chemistry* 28. 381-451.

Fontaine, A., Diouf, I., Bakkali, N., Missé, D., Pagès, F., Fusai, T., Rogier, C. & Almeras, L. (2011) Implication of haematophagous arthropod salivary proteins in host-vector interactions. *Parasites & Vectors*, 4. 1.

Fouche, G., Ramafuthula, M., Maselela, V., Mokoena, M., Senabe, J., Leboho, T., Sakong, B.M., Adenubi, O.T., Eloff, J.N. & Wellington, K.W. (2016) Acaricidal activity of the organic extracts of thirteen South African plants against *Rhipicephalus (Boophilus) decoloratus* (Acari: Ixodidae). *Veterinary Parasitology*, 224. 39-43.

Fradin, M.S. & Day, J.F. (2002) Comparative efficacy of insect repellents against mosquito bites. *New England Journal of Medicine,* 347(1). 13-18.

Freeman, J.M., Davey, R.B., Kappmeyer, L.S., Kammlah, D.M. & Olafson, P.U. (2010) Bm86 midgut protein sequence variation in South Texas cattle fever ticks. *Parasites & Vectors*, 3. 101.

Freitag, J.A. & Kells, S.A. (2013) Efficacy and application considerations of selected residual acaricides against the mold mite *Tyrophagus putrescentiae* (Acari: Acaridae) in simulated retail habitats. *Journal of Economic Entomology*, 106. 1920-1926.

ffrench-Constant, R.H., Williamson, M.S., Davies, T.E., & Bass, C. (2016) Ion channels as insecticide targets. *Journal of Neurogenetics*, 30(3-4). 163-177.

gar Ebadollahi, A. (2011) Iranian plant essential oils as sources of natural insecticide agents. *International Journal of Biological Chemistry* 5(5). 266-290.

Gassner, B., Wuthrich, A., Lis, J., Scholtysik, G. & Solioz, M. (1997) Topical application of synthetic pyrethroids to cattle as a source of persistent environmental contamination. *Journal of Environmental Science & Health* 32. 729-739.

Gabriel, O.A. & Olubunmi, A. (2009) Comprehensive scientific demystification of *Kigelia africana*: A review. *African Journal of Pure & Applied Chemistry* 3. 158-164.

Gakuubi, M.M., Wanzala, W., Wagacha, J.M. & Dossaji, S.F. (2016) Bioactive properties of *Tagetes minuta* L. (Asteraceae) essential oil: A review. *American Journal of Essential Oils & Natural Products* 4(2). 27-36.

Garboui, S.S., Jaenson, T.G., Borg-Karlson, A.K. & Pålsson, K. (2007) Repellency of methyl jasmonate to *Ixodes ricinus* nymphs (Acari: Ixodidae). *Experimental & Applied Acarology*, 42(3). 209-215.

Garg, R., Juncadella, I.J., Ramamoorthi, N., Ashish, Ananthanarayanan, S.K., Thomas, V., Rincon, M., Krueger, J.K., Fikrig, E., Yengo, C.M. & Anguita, J. (2006) Cutting edge: CD4 is the receptor for the tick saliva immunosuppressor, Salp15. *Journal of Immunology*, 177. 6579-6583.

Gassel, M., Wolf, C., Noack, S., Williams, H. & Ilg, T. (2014) The novel isoxazoline ectoparasiticide fluralaner: Selective inhibition of arthropod γ-aminobutyric acid-and L-glutamate-gated chloride channels and insecticidal/acaricidal activity. *Insect Biochemistry & Molecular Biology*, 45. 111-124.

Gassner, B., Wüthrich, A., Lis, J., Scholtysik, G. & Solioz, M. (1997) Topical application of synthetic pyrethroids to cattle as a source of persistent environmental contamination. *Journal of Environmental Science & Health Part B*, 32. 729-739.

Gauthier, C., Legault, J., Girard-Lalancette, K., Mshvildadze, V., & Pichette, A. (2009) Haemolytic activity, cytotoxicity and membrane cell permeabilization of semi-synthetic and natural lupane-and oleanane-type saponins. *Bioorganic & Medicinal Chemistry*, *17*(5). 2002-2008.

Gazim, Z.C., Demarchi, I.G., Lonardoni, M.V.C, Amorim, A.C.L., Howell, A.M.C., Rezende, C.M., Ferreira, G.A., de Lima, E.L. de Cosmo, F.A. & Cortez, D.A.G. (2011) *Tetradenia riparia*. *Experimental Parasitology* 129(2), 175-178.

Gemeda, N., Mokonnen, W., Lemma, H., Tadele, A., Urga, K., Addis, G., Debella, A., Getachew, M., Teka, F., Yirsaw, K., Mudie, K. & Gebre, S. (2014) Insecticidal activity of some traditionally used Ethiopian medicinal plants against sheep ked *Melophagus ovinus. Journal of Parasitology Research.* http:/dx.doi.org/10.1155/2014/978537.

George, J., Davey, R., Ahrens, E., Pound, J. & Drummond, R. (1998) Efficacy of amitraz (Taktic® 12.5% EC) as a dip for the control of *Boophilus microplus* (Canestrini)(Acari: Ixodidae) on cattle. *Preventive Veterinary Medicine*, 37. 55-67.

George, J., Pound, J. & Davey, R. (2004) Chemical control of ticks on cattle and the resistance of these parasites to acaricides. *Parasitology*, 129. S353-S366.

George, D.R., Finn, R.D., Graham, K.M. & Sparagano, O.A. (2014) Present and future potential of plant-derived products to control arthropods of veterinary and medical significance. *Parasite & Vectors* 7, 28.

Gerberg, E.J., Novak, R.J., Debboun, M., Frances, S. & Strickman, D. (2007) Considerations on the use of botanically-derived repellent products. *Insect repellents: Principles, Methods & Uses,* 305-308.

Gershenzon, J. & Dudareva, N. (2007) The function of terpene natural products in the natural world. *Nature Chemical Biology* 3(7). 408-414.

Gerwick, B.C., & Sparks, T.C. (2014) Natural products for pest control: an analysis of their role, value and future. *Pest Management Science*, 70(8). 1169-1185.

Getiye, Y., Tolessa, T. & Engidawork, E. (2016) Antihypertensive activity of 80% methanol seed extract of *Calpurnia aurea* (Ait.) Benth. subsp. aurea (Fabaceae) is mediated through calcium antagonism induced vasodilation. *Journal of Ethnopharmacology*, 189. 99-106.

Gharbi, M., Flegg, J.A., Hubert, V., Kendjo, E., Metcalf, J.E., Bertaux, L., Guérin, P.J., Le Bras, J., Aboubaca, A. & Agnamey, P. (2013) Longitudinal study assessing the return of chloroquine susceptibility of *Plasmodium falciparum* in isolates from travellers returning from West and Central Africa, 2000–2011. *Malaria Journal*, 12. 35.

Ghosh, S., Azhahianambi, P. & Yadav, M. (2007) Upcoming and future strategies of tick control: a review. *Journal of Vector borne Diseases*, 44. 79.

Ghosh, S., Sharma, A.K., Kumar, S., Tiwari, S.S., Ratogi, S., Srivastava, S., Singh, M., Kumar, R., Paul, S., Ray, D.D. & Rawat, A.J.S. (2011). *In vitro* and *in vivo* efficacy of *Acorus calamus* extract against *Rhipicephalus (Boophilus) microplus*. *Parasitology Research,* 108. 361-370.

Ghosh, S., Tiwari, S.S., Srivastava, S., Sharma, A.K., Kumar, S., Ray, D.D. & Rawat, A.K.S. (2013) Acaricidal properties of *Ricinus communis* leaf extracts against organophosphate and pyrethroids resistant *Rhipicephalus (Boophilus) microplus*. *Veterinary Parasitology,* 192(1). 259-267.

Ghosh, S., Tiwari, S.S., Kumar, B., Srivastava, S., Sharma, A.K., Kumar, S., Bandyopadhyay, A., Julliet, S., Kumar, R. & Rawat, A. (2015) Identification of potential plant extracts for anti-tick activity against acaricide resistant cattle ticks, *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Experimental & Applied Acarology* 66. 159-171.

Ghosh, S. & Ravindran, R. (2014) Progress in the development of plant biopesticides for the control of arthropods of veterinary importance. *In Advances in Plant Biopesticides* Springer India, 207-241.

GiannelII, A., Lia, R.P., Annoscia, G., Buonavoglia, C., Lorusso, E., Dantas-Torres, F.I.L.I.P.E., Banet, G. & Otranto, D. (2016) *Rhipicephalus turanicus,* a new vector of *Hepatozoon canis*. *Parasitology*, 1-8.

Giglioti, R., Forim, M., Oliveira, H.N.D., Chagas, A., Ferrezini, J., Brito, L., Falcoski, T., Albuquerque, L.G.D. & Oliveira, M. (2011) *In vitro* acaricidal activity of neem (*Azadirachta indica*) seed extracts with known azadirachtin concentrations against *Rhipicephalus microplus*. *Veterinary Parasitology,* 181. 309-315.

Gobbo-Neto, L. & Lopes, N.P. (2007) Medicinal plants: factors of influence on the content of secondary metabolites. *Quimica Nova*, 30. 374-381.

Godara, R., Parveen, S., Katoch, R., Yadav, A., Verma, P., Katoch, M., Kaur, D., Ganai, A., Raghuvanshi, P. & Singh, N. (2014a) Acaricidal activity of extract of *Artemisia absinthium* against *Rhipicephalus sanguineus* of dogs. *Parasitology Research*, 113. 747-754.

Godara, R., Parveen, S., Katoch, R., Yadav, A., Katoch, M., Khajuria, J.K., Kaur, D., Ganai, A., Verma, P.K., Khajuria, V. & Singh, N.K. (2014b). Acaricidal activity of ethanolic extract of *Artemisia absinthium* against *Hyalomma anatolicum* ticks. *Experimental & Applied Acarology*, 1-8.

Goddard, J. (1992) Ecological studies of adult *Ixodes scapularis* in central Mississippi: questing activity in relation to time of year, vegetation type, and meteorologic conditions. *Journal of Medical Entomology*, 29. 501-506.

Godfray, H.C.J., Blacquière, T., Field, L.M., Hails, R.S., Petrokofsky, G., Potts, S.G., Raine, N.E., Vanbergen, A.J. & Mclean, A.R. (2014) A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proceedings of the Royal Society.* doi: 10.1098/rspb.2014.0558.

Gomes, G.A., Monteiro, C.M.O., Julião, L.D.S., Maturano, R., Senra, T.O.S., Zeringóta, V., Calmon, F., da Silva Matos R., Daemon, E. & Carvalho, M.G.D. (2014) Acaricidal activity of essential oil from *Lippia sidoides* on unengorged larvae and nymphs of *Rhipicephalus sanguineus* (Acari: Ixodidae) and *Amblyomma cajennense* (Acari: Ixodidae). *Experimental Parasitology* 137, 41-45.

Gomez-Lechon, M., Donato, M., Lahoz, A. & Castell, J. (2008) Cell lines: a tool for *in vitro* drug metabolism studies. *Current Drug Metabolism*, 9. 1-11.

Gonçalves, K., Toigo, E., Ascoli, B., von Poser, G. & Ribeiro, V.L.S. (2007) Effects of solvents and surfactant agents on the female and larvae of cattle tick *Boophilus microplus. Parasitology Research*, 100. 1267-1270.

Gordon, B.M. & Giza, C.C. (2004) Tick paralysis presenting in an urban environment. *Pediatric Neurology*, 30. 122-124.

Govindarajan, M., Rajeswary, M., Hoti, S., Bhattacharyya, A. & Benelli, G. (2016) Eugenol, α-pinene and β-caryophyllene from *Plectranthus barbatus* essential oil as eco-friendly larvicides against malaria, dengue and Japanese encephalitis mosquito vectors, *Parasitology Research,* 115. 807-815.

Gray, J., Zintl, A., Hildebrandt, A., Hunfeld, K. & Weiss, L. (2010) Zoonotic babesiosis: overview of the disease and novel aspects of pathogen identity. *Ticks & Tick-borne Diseases*, 1. 3-10.

Gübitz, G. M., Mittelbach, M. & Trabi, M. (1999) Exploitation of the tropical oil seed plant *Jatropha curcas* L. *Bioresource Technology,* 67(1). 73-82.

Guerrero, F.D., Lovis, L. & Martins, J.R. (2012) Acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus*. *Revista Brasileira de Parasitologia Veterinária*, 21. 1-6.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Peña, A., Horak, I.G., Shao, R. & Barker, S.C. (2010) The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. http://hdl.handle.net/2263/17278.

Guilhon, J. (1950) Acaricidal properties of chlorinated insecticides. *Review of Medicine* 32. 83-88.

Gülçin, İ., Scozzafava, A., Supuran, C.T., Akıncıoğlu, H., Koksal, Z., Turkan, F., & Alwasel, S. (2016) The effect of caffeic acid phenethyl ester (CAPE) on metabolic enzymes including acetylcholinesterase, butyrylcholinesterase, glutathione S-transferase, lactoperoxidase, and carbonic anhydrase isoenzymes I, II, IX, and XII. *Journal of Enzyme Inhibition & Medicinal Chemistry,* 31(6). 1095-1101.

Gupta, P.K., Gupta, S. & Khan, M.H. (2000) *In vitro* evaluation of petroleum fractions of different of Neem seed (*Azadirachta indica*) against cattle tick, *Boophilus microplus. Indian Journal of Environment & Toxicology,* 10(1). 38-39.

Hadani, A., Cwilich, R. & Rechav, Y. (1969) Laboratory studies of tick repellents and acaricides. *Proceedings of the 2nd International Congress of Acarology*, Sutton Bonington (England). 524-532.

Haddouchi, F., Chaouche, T.M., Zaouali, Y., Ksouri, R., Attou, A. & Benmansour, A. (2013) Chemical composition and antimicrobial activity of the essential oils from four *Ruta* species growing in Algeria. *Food Chemistry* 141. 253-258.

Häggström, M. (2014) Diagram of the pathways of human steroidogenesis. *Medicine*, 1.

Hallworth, N.E., Wilson, C.J. & Bevari, M. (2003). Apamin-sensitive small conductance calcium-activated potassium channels, through their selective coupling to voltage-gated calcium channels, are critical determinants of the precision, pace and pattern of action potential generation in rat subthalamic nucleus neurons in vitro. *Journal of Neuroscience* 23(20). 7525-7542.

Halos, L., Baneth, G., Beugnet, F., Bowman, A., Chomel, B., Farkas, R., Franc, M., Guillot, J., Inokuma, H. & Kaufman, R. (2012) Defining the concept of 'tick repellency' in veterinary medicine. *Parasitology,* 139(4). 419-423.

Harley, K. & Wilkinson, P. (1971) A modification of pasture spelling to reduce acaricide treatments for cattle tick control. *Australian Veterinary Journal*, 47. 108-111.

Hartelt, K., Oehme, R., Frank, H., Brockmann, S.O., Hassler, D. & Kimmig, P. (2004) Pathogens and symbionts in ticks: prevalence of *Anaplasma phagocytophilum* (*Ehrlichia* sp.), *Wolbachia* sp., *Rickettsia* sp., and *Babesia* sp. in Southern Germany. *International Journal of Medical Microbiology Supplements*, 293. 86-92.

Hawryl, M.A., Niemiec, M.A., Slomka, K., Waksmundzka-Hajnos, M. & Szymczak, G. (2016) Two-dimensional micro-TLC phenolic fingerprints of selected *Mentha* sp. on cyano-bonded polar stationary phase. *Journal of Chromatographic Science*, 54. 64-69.

Heemskerk, J.W., Bevers, E.M. & Lindhout, T. (2002) Platelet activation and blood coagulation. *Thrombosis & Haemostasis-Stuttgart*, 88. 186-194.

Heimerdinger, A., Olivo, C.J., Molento, M.B., Agnolin, C.A., Ziech, M.F., Scaravelli, L.F.B., Skonieski, F.R., Both, J.F. & Charão, P.S. (2006) Extrato alcoólico de Capim-cidreira

(Cymbopogon citratus) no controle do *Boophilus microplus* em bovinos. *Revista Brasileira de Parasitologia Veterinária*, 15. 37-39.

Hekimoğlu, O., Sağlam, İ.K., Özer, N., & Estrada-Peña, A. (2016) New molecular data shed light on the global phylogeny and species limits of the *Rhipicephalus sanguineus* complex. *Ticks & Tick-borne Diseases*, *7*(5). 798-807.

Henning, J.A., Peng, Y., Montague, M.A. & Teuber, L.R. (1992) Honey bee (Hymenoptera: Apidae) behavioral response to primary alfalfa (Rosales: Fabaceae) floral volatiles. *Journal of Economic Entomology* 85. 233-239.

Holdsworth, P., Kemp, D., Green, P., Peter, R., De Bruin, C., Jonsson, N., Letonja, T., Rehbein, S. & Vercruysse, J. (2006) World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of acaricides against ticks (Ixodidae) on ruminants. *Veterinary Parasitology,* 136(1). 29-43.

Hoogstraal, H., Aeschlimann, A. (1982) Tick-host specificity. *Bulletin de la Société Entomologique Suisse*, 55. 5-32.

Hossain, M.S., Urbi, Z., Sule, A. & Rahman, K. (2014) *Andrographis paniculata* (Burm. f.) Wall. ex Nees: a review of ethnobotany, phytochemistry, and pharmacology. *The Scientific World Journal* 2014. http://dx.doi.org/10.1155/2014/274905.

Horn, S. (1987) Ectoparasites of animals and their impact on the economy of South America. *In: Proceedings of the 23rd World Veterinary Congress,* Montreal.

Hou, D., Yanagita, T., Uto, T., Masuzaki, S. & Fujii, M. (2005) Anthocyanidins inhibit cyclooxygenase-2 expression in LPS-evoked macrophages: structure–activity relationship and molecular mechanisms involved. *Biochemical Pharmacology*, 70. 417-425.

Hou, M., Sun, R., Hupe, M., Kim, P.L., Park, K., Crumrine, D., Lin, T., Santiago, J.L., Mauro, T.M. & Elias, P.M. (2013) Topical apigenin improves epidermal permeability barrier homoeostasis in normal murine skin by divergent mechanisms. *Experimental Dermatology*, 22. 210-215.

Hovius, J.W., van Dam, A.P. & Fikrig, E. (2007) Tick-host-pathogen interactions in Lyme borreliosis. *Trends in Parasitology*, 23. 434-438.

Howey, E.B., O'Donnell, V., de Carvalho Ferreira, Helena C, Borca, M.V. & Arzt, J. (2013) Pathogenesis of highly virulent African swine fever virus in domestic pigs exposed via intraoropharyngeal, intranasopharyngeal, and intramuscular inoculation, and by direct contact with infected pigs. *Virus Research*, 178. 328-339.

Hsouna, A.B. & Hamdi, N. (2012) Phytochemical composition and antimicrobial activities of the essential oils and organic extracts from *Pelargonium graveolens* growing in Tunisia. *Lipids in Health & Disease,* 11(1). 167.

Hüe, T., Cauquil, L., Fokou, J.H., Dongmo, P.J., Bakarnga-Via, I. & Menut, C. (2014) Acaricidal activity of five essential oils of *Ocimum* species on *Rhipicephalus (Boophilus) microplus* larvae. *Parasitology Research*, 91-99.

Hughes, B.G. & Lawson, L.D. (1991) Antimicrobial effects of *Allium sativum* L. (garlic), *Allium ampeloprasum* L. (elephant garlic), and *Allium cepa* L.(onion), garlic compounds and commercial garlic supplement products. *Phytotherapy Research,* 5. 154-158.

Ibelli, A., Ribeiro, A., Giglioti, R., Regitano, L., Alencar, M., Chagas, A., Paco, A., Oliveira, H.N.D., Duarte, J. & Oliveira, M. (2012) Resistance of cattle of various genetic groups to the tick *Rhipicephalus microplus* and the relationship with coat traits. *Veterinary Parasitology*, 186. 425-430.

Ibrahim, A.K. (2013) New terpenoids from *Mentha pulegium* L. and their antimicrobial activity. *Natural Product Research,* 27. 691-696.

Ioffe-Uspensky, I., Mumcuoglu, K.Y., Uspensky, I. & Galun, R. (1997) *Rhipicephalus sanguineus* and *R. turanicus* (Acari: Ixodidae): closely related species with different biological characteristics. *Journal of Medical Entomology*, 34. 74-81.

Iori, A., Grazioli, D., Gentile, E., Marano, G. & Salvatore, G. (2005) Acaricidal properties of the essential oil of *Melaleuca alternifolia* Cheel (tea tree oil) against nymphs of *Ixodes ricinus. Veterinary Parasitology*, 129. 173-176.

Ishaaya, I. & Casida, J. E. (2013) Pyrethroid detoxification and synergism in insects. *IUPAC Pesticide Chemistry: Human Welfare and the Environment, ed. J. Miyamoto and PC Kearney*, 3., 307-310.

Isman, M.B. & Machial, C.M. (2006) Pesticides based on plant essential oils: from traditional practice to commercialization. *In: Rai M, Carpinella MC (eds) Advances in phytomedicine: naturally occurring bioactive compounds.* Elsevier, New York, pp 29-44.

Jacobson, M. (1983) Insecticides, insect repellants, and attractants from arid/semiarid-land plants. *In Plants: The Potential for Extracting Protein, Medicines, and other Useful Chemicals Workshop Proceedings,* Congress, Office of Technology Assessment, Washington, DC, OTA-BP-F-23 September pp. 138-146.

Jacobson, M. (1989). Botanical pesticides*. In Anonymous ACS Publications.*

Jaenson, T.G.T., Lindstrom, A. & Palsson, K. (2003) Repellency of the mosquito repellent MyggA (N,N-diethyl-3-methyl-benzamide) to the common tick *Ixodes ricinus* (L.) (Acari: Ixodidae) in the laboratory and field. *Entomologisk Tidskrift,* 124. 245–251.

Jaenson, T.G., Pålsson, K. & Borg-Karlson, A.K. (2005) Evaluation of extracts and oils of tick-repellent plants from Sweden. *Medical & Veterinary Entomology,* 19(4). 345-352.

Jaenson, T.G.T., Garboui, S. & Palsson, K. (2006) Repellency of oils of lemon, eucalyptus, geranium and lavender and the mosquito repellent MyggA natural to *Ixodes ricinus* (Acari: Ixodidae) in the laboratory and field. *Journal of Medical Entomology,* 43. 731-736.

Jalali-Heravi, M., Zekavat, B. & Sereshti, H. (2006) Characterization of essential oil components of Iranian *P. roseum* oil using gas chromatography-mass spectrometry combined with chemometric resolution techniques. *Journal of Chromatography,* 1114. 154-163.

Jalali-Nezhad, A.A., Farajian-Mashhadi, F., Komeili, G. & Barkhordari-Ahmadi, F. (2016) The effect of ginger hydroalcholic extract on rat ileal contraction in vitro. *Zahedan Journal of Research in Medical Sciences*, 18.

Janbaz, K.H., Jan, A., Qadir, M.I. & Gilani, A.H. (2013) Spasmolytic, bronchodilator and vasorelaxant activity of methanolic extract of *Tephrosia purpurea*. *Acta Poloniae Pharmaceutica,* 79. 261-269.

Jayaseelan, C. & Rahuman, A.A. (2012) Acaricidal efficacy of synthesized silver nanoparticles using aqueous leaf extract of *Ocimum canum* against *Hyalomma anatolicum anatolicum* and *Hyalomma marginatum isaaci* (Acari: Ixodidae). *Parasitology Research,* 111. 1369-1378.

Jelinski, J.W. (2016) Painless Hematophagy: The functional role of novel tick metalloproteases in pain suppression. *Honors thesis* 401.

Jespersen, B., Tykocki, N.R., Watts, S.W. & Cobbett, P.J. (2015) Measurement of smooth muscle function in the isolated tissue bath-applications to pharmacology research. *Journal of Visualized Experiments*, (95). e52324, doi:10.3791/52324 (2015).

Jones, C. R., Brunner, J. L., Scoles, G. A., & Owen, J. P. (2015) Factors affecting larval tick feeding success: host, density and time. *Parasites & Vectors*, *8*(1), 340.

Jongejan, F. & Uilenberg, G. (2004) The global importance of ticks. *Parasitology*, 129. S3-S14.

Jonsson, N. (2006) The productivity effects of cattle tick (*Boophilus microplus*) infestation on cattle, with particular reference to *Bos indicus* cattle and their crosses. *Veterinary Parasitology*, 137. 1-10.

Jonsson, N.N. & Iqbal, Z. (2012) Syringe test (modified larval immersion test): A new bioassay for testing acaricidal activity of plant extracts against *Rhipicephalus microplus. Veterinary Parasitology,* 188(3). 362-367.

Jonsson, N., Mayer, D. & Green, P. (2000) Possible risk factors on Queensland dairy farms for acaricide resistance in cattle tick (*Boophilus microplus*). *Veterinary Parasitology*, 88. 79-92.

Judd, W., Campbell, C., Kellogg, E. & Stevens, P. (2000) Plant systematics: a phylogenetic approach. *SIDA, Contributions to Botany* 19(1). 227-232.

Juliet, S., Ravindran, R., Ramankutty, S.A., Gopalan, A.K.K., Nair, S.N., Kavillimakkil, A.K., Bandyopadhyay, A., Rawat, A.K.S. & Ghosh, S. (2012) *Jatropha curcas* (Linn) leaf extract-a possible alternative for population control of *Rhipicephalus (Boophilus) annulatus. Asian Pacific Journal of Tropical Disease*, 2. 225-229.

Kaaya, G.P. (2000) The potential of anti tick plants as components of an intergrated tick control strategy. *Tropical Veterinary Diseases,* 916. 576-582.

Kaaya, G.P. & Hassan, S. (2000) Entomogenous fungi as promising biopesticides for tick control. *Experimental & Applied Acarology*, 24. 913-926.

Kain, P., Boyle, S.M., Tharadra, S.K., Guda, T., Pham, C., Dahanukar, A. & Ray, A. (2013) Odour receptors and neurons for DEET and new insect repellents. *Nature,* 502. 507-512.

Kaiser, R. (2008) Tick-borne encephalitis. *Infectious Disease Clinics of North America*, 22. 561-575.

Kakar, S.A., Tareen, R.B., Kakar, M.A, Jabeen, H. Kakar, S.R., Al-Kahraman, Y.M.S.A., & Shafee, M. (2012) Screening of antibacterial activity of four medicinal plants of Balochistan-Pakistan. *Pakistan Journal of Botany* 44(SI). 245-250.

Kamaraj, C., Abdul Rahuman, A., Mahapatra, A., Bagavan, A. & Elango, G. (2010) Insecticidal and larvicidal activities of medicinal plant extracts against mosquitoes. *Parasitology Research,* 107. 1337-1349.

Kappers, I.F., Aharoni, A., von Herpen, T., Luckerhoff, L.L.R., Dicke, M. & Bouwmester, H.J. (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to Arabodopsis. *Science*, 2070-2072.

Karasov, C. (2001) Who reaps the benefit of biodiversity? *Environmental Health Perspectives* 109, 582-587.

Katerere, D.R. & Naidoo, V. (2010) 17 Herbal Medicines for Pet and Companion Animals. *Ethnoveterinary Botanical Medicine: Herbal Medicines for Animal Health*. 389.

Katoch, P., Katoch, M., Yadav, A. & Srivastava, A.K. (2007) Formulation of herbal ectoparasiticidals. *In compendium of 18th National Congress of Veterinary Parasitology*, September 7-9, Jammu, India, pp 24-31.

Katz, L., & Baltz, R.H. (2016) Natural product discovery: past, present, and future. *Journal of Industrial Microbiology & Biotechnology*, 43(2-3). 155-176.

Katzer, F., Ngugi, D., Walker, A. & McKeever, D. (2010) Genotypic diversity, a survival strategy for the apicomplexan parasite *Theileria parva. Veterinary Parasitology*, 167. 236-243.

Kayser, O., Kiderlen, A.F. & Croft, S.L. (2003) Natural products as antiparasitic drugs. *Parasitology Research,* 90(2). S55-S62.

Kazimírová, M., & Štibrániová, I. (2013) Tick salivary compounds: their role in modulation of host defences and pathogen transmission.*Cellular & Infection Microbiology*, *3*.

Kebede, A.G. (2004) Seasonal dynamics of ticks (*Amblyomma cohaerens* and *Boophilus decoloratus*) and development of a management plan for tick and tick borne diseases control on cattle in Jimma zone, Southwestern Ethiopia. *Doctoral dissertation*.

Kemp, D.H., Mckenna, R.V., Thullner, R. & Willadsen, P. (1999) Strategies for tick control in a world of acaricide resistance. *In Morales, G., Fragosa, H., Garcia, Z. (Eds), Control de la Resistencia en Garrapatas yMoscas de Importancia Veterinaria y Enfermedades quetransmiten,* IV Seminario Internacional de ParasitologiaAnimal Puerto Vallarta, Jalisco, Mexico pp.1-10.

Kemp, D., Stone, B. & Binnington, K. (2012) Tick attachment and feeding: role of the mouthparts, feeding apparatus, salivary gland secretions, and the host response. *Physiology of Ticks,* 119-167.

Keyyu, J., Kassuku, A., Kyvsgaard, N.C. & Willingham Iii, A. (2003a) Gastrointestinal nematodes in indigenous Zebu cattle under pastoral and nomadic management

systems in the lower plain of the Southern Highlands of Tanzania. *Veterinary Research Communications*, 27. 371-380.

Keyyu, J.D., Kyusgaard, N.C., Kassuku, A.A. & Willingham, A.L. (2003b) Worm control practices and anthelminthic usage in traditional dairy cattle farms in the Southern Highlands of Tanzania. *Veterinary Parasitology* 114. 51-61.

Khaidarov, K.M. (1971) Sensitivity of ixodid ticks to some species of plants. *Conference proceedings Problems of Veterinary Sanitation* 40, 341-343.

Khanzada, S.K., Shaikh, W., Sofia, S., Kazi, T.G., Usmanghani, K. & Amina, A. (2008) Chemical constituents of *Tamarindus indica* L. medicinal plant in Sindh. *Pakistan Journal of Botany* 40(6), 2553-2559.

Kim, S.I., Yi, J.H., Tak, J.H. & Ahn, Y.J. (2004) Acaricidal activity of plant essential oils against *Dermanyssus gallinae* (Acari: Dermanyssidae). *Veterinary Parasitology Research* 120. 297-304.

Kimps, N., Bissinger, B., Apperson, C., Sonenshine, D. & Roe, R. (2011) First report of the repellency of 2-tridecanone against ticks. *Medical & Veterinary Entomology* 25. 202-208.

King, G.F. (2004) The wonderful world of spiders: preface to the special Toxicon issue on spider venoms. *Toxicon*, 43. 471-475.

Kiss, T., Cadar, D. & Spînu, M. (2012) Tick prevention at a crossroad: new and renewed solutions. *Veterinary Parasitology*, 187. 357-366.

Kiszewski, A.E., Matuschka, F. & Spielman, A. (2001) Mating strategies and spermiogenesis in ixodid ticks. *Annual Review of Entomology*, 46. 167-182.

Kiszewski, A.E. & Spielman, A. (2002) Preprandial inhibition of re-mating in Ixodes ticks (Acari: Ixodidae). *Journal of Medical Entomology*, 39. 847-853.

Kivaria, F.M. (2006) Estimated direct economic costs associated with tick-borne diseases on cattle in Tanzania. *Tropical Animal Health & Production,* 38(4). 291-299.

Klaassen, C. & Watkins III, J.B. (2003) Casarett and Doull's Essentials of Toxicology.

Klocke, J.A., Darlington, M.V. & Balandrin, M.F. (1987) 1, 8-cineole (Eucalyptol), a mosquito feeding and ovipositional repellent from volatile oil of *Hemizonia fitchii* (Asteraceae). *Journal of Chemical Ecology* 13(12). 2131-2141.

Klyne, W. (1950) The configuration of the anomeric carbon atoms in some cardiac glycosides. *The Biochemical Journal*, 47.

Koc, S., Oz, E., Aydin, L. & Cetin, H. (2012) Acaricidal activity of the essential oils from three Lamiaceae plant species on *Rhipicephalus turanicus* Pom. (Acari: Ixodidae). *Parasitology Research* 111. 1863-1865.

Kocan, K.M., de la Fuente, J., & Coburn, L.A. (2015) Insights into the development of *Ixodes scapularis*: a resource for research on a medically important tick species. *Parasites & Vectors*, 8(1). 592.

Kongkiatpaiboon, S., Pattarajinda, V., Keeratinijakal, V. & Gritsanapan, W. (2014) Effect of *Stemona* spp. against *Rhipicephalus microplus. Experimental & Applied Acarology* 62(1). 115-120.

Korir, E., Kiplimo, J.J., Crouch, N.R., Moodley, N. & Koorbanally, N.A. (2014) Isoflavones from *Calpurnia aurea* subsp. *aurea* and their anticancer activity. *African Journal of Traditional, Complementary & Alternative Medicines*, 11. 33-37.

Kotze, M., Eloff, J. & Houghton, P. (2002) Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). *South African Journal of Botany*, 68. 62-67.

Koul, O., Walia, S. & Dhaliwal, G. (2008) Essential oils as green pesticides: potential and constraints. *Biopesticides International,* 4. 63-84.

Kovendan K., Murugan K., Kumar A.N., Vincent S. & Hwang J.S. (2012) Bioefficacy of larvicidal and pupicidal properties of *Carica papaya* (Caricaceae) leaf extract and bacterial insecticide, spinosad, against chikungunya vector, *Aedes aegypti* (Diptera: Culicidae). *Parasitology Research,* 110(2). 669–678.

Krieger, R., Chen, L., Ginevan, M., Watkins, D., Cochran, R., Driver, J. & Ross, J. (2012) Implications of estimates of residential organophosphate exposure from dialkylphosphates (DAPs) and their relevance to risk. *Regulatory Toxicology & Pharmacology*, 64. 263-266.

Krishnaiah, G.M. & Prashanth, G.K. (2014) Phytochemical studies and GC-MS analysis of the leaf extract of *Melia azedarach* Linn. I*nternational Journal of Advancement in Engineering Technology, Management & Applied Science,* 1(6). 48-54.

Kröber, T. & Guerin, P.M. (2007) An *in vitro* feeding assay to test acaricides for control of hard ticks. *Pest Management Science,* 63(1). 17-22.

Kröber, T., Bourquin, M. & Guerin, P.M. (2013) A standardised *in vivo* and *in vitro* test method for evaluating tick repellents. *Pesticide Biochemistry & Physiology*, 107(2). 160-168.

Krogh, A., Larsson, B., Von Heijne, G. & Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology,* 305(3). 567-580.

Krull, C., Böhme, B., Clausen, P.H., & Nijhof, A.M. (2017) Optimization of an artificial tick feeding assay for *Dermacentor reticulatus*. *Parasites & Vectors*, 10(1). 60.

Kuang, H., Yang, B., Xia, Y. & Feng, W. (2008) Chemical constituents from the flowers of *Datura metel* L. *Archives of Pharmacal Research* 31(9). 1094-1097.

Kuhnert, F., Diehl, P.A. & Guerin, P.M. (1995) The life-cycle of the bont tick *Amblyomma hebraeum in vitro. International Journal for Parasitology,* 25(8). 887-896.

Kumar, D.N., Rajeshwari, A., Alex, S.A., Sahu, M., Raichur, A.M., Chandrasekaran, N. & Mukherjee, A. (2015) Developing acetylcholinesterase-based inhibition assay by modulated synthesis of silver nanoparticles: applications for sensing of organophosphorus pesticides. *RSC Advances,* 5(76). 61998-62006.

Kumar, K.A., Sharma, A.K., Kumar, S., Ray, D., Rawat, A., Srivastava, S. & Ghosh, S. (2016) Comparative *in vitro* anti-tick efficacy of commercially available products and newly developed phyto-formulations against field collected and resistant tick lines of *Rhipicephalus (Boophilus) microplus*. *Journal of Parasitic Diseases*. 1590-1596.

Kunz, S.E. & Kemp, D.H. (1994) Insecticides andacaricides: Resistance and environmental impact. *Revue scientifique et technique Office International des Epizooties,* 13. 1249-1286.

Ladda, P.L. & Magdum, C.S. (2012) *Vitex negundo* Linn: Ethnobotany, Phytochemistry and Pharmacology - A Review. *International Journal of Advanced Research in Pharmacy, Biology & Chemistry* 1(1). 111-120.

Lago, J.H.G., Romoff, P., Favero, O.A., Souza, F.O., Soares, M.G., Baraldi, P.T. & Corrêa, A.G. (2008) Chemical composition of male and female *Baccharis trimera* (Less.) DC. (Asteraceae) essential oils. *Biochemical Systematics & Ecology* 36(9). 737-740.

Langenheim, J.H. (1994) Higher plant terpenoids: a phytocentric overview of their ecological roles. *Journal of Chemical Ecology* 20(6). 1223-1280.

Lans, C., Turner, N., Khan, T. & Brauer, G. (2007a) Ethnoveterinary medicines used to treat endoparasites and stomach problems in pigs and pets in British Columbia, Canada. *Veterinary Parasitology*, 148. 325-340.

Lans, C., Turner, N., Khan, T., Brauer, G. & Boepple, W. (2007b) Ethnoveterinary medicines used for ruminants in British Columbia, Canada. *Journal of Ethnobiology & Ethnomedicine*, 3. 1.

Lapa, N., Barbosa, R., Morais, J., Mendes, B., Méhu, J. & Santos Oliveira, J.F. (2002) Ecotoxicological assessment of leachates from MSWI bottom ashes. *Waste Management,* 22(6). 583-593.

Laphookhieo, S., Phungpanya, C., Tantapakul, C., Techa, S., Tha-in, S. & Narmdorkmai, W. (2011) Chemical constituents from *Aegle marmelos*. *Journal of the Brazilian Chemical Society* 22(1). 176-178.

Lappin, M.R., Griffin, B., Brunt, J., Riley, A., Burney, D., Hawley, J., Brewer, M.M. & Jensen, W.A. (2006) Prevalence of *Bartonella* species, *Haemoplasma* species, *Ehrlichia*

species, *Anaplasma phagocytophilum* and *Neorickettsia risticii* DNA in the blood of cats and their fleas in the United States. *Journal of Feline Medicine & Surgery*, 8. 85-90.

Lasota, J.A. & Dybas, R.A. (1991) Avermectins, a novel class of compounds: implications for use in arthropod pest control. *Annual Review of Entomology*, 36. 91-117.

Latif, A.A., Putterill, J.F., de Klerk, D.G., Pienaar, R. & Mans, B.J. (2012) *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae): first description of the male, immature stages and re-description of the female. *PloS one*, 7. e41651.

Laudato, M. & Capasso, R. (2013) Useful plants for animal therapy. *OA Alternative Medicine* 1(1). 1-6.

Lawrence, L.J. & Casida, J.E. (1984) Interactions of lindane, toxaphene and cyclodienes with brain-specific t-butylbicyclophosphorothionate receptor. *Life Sciences* 35. 171-178.

Lázaro, S.F., Fonseca, L.D., Martins, E.R., de Oliveira, N. J.F. & Duarte, E.R. (2013) Effect of aqueous extracts of *Baccharis trimera* on development and hatching of *Rhipicephalus microplus* (Acaridae) eggs. *Veterinary Parasitology*, 194(1). 79-82.

Leboulle, G., Crippa, M., Decrem, Y., Mejri, N., Brossard, M., Bollen, A. & Godfroid, E. (2002) Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. *The Journal of Biological Chemistry*, 277. 10083-10089.

Lee, C.K. & Chang, M.H. (2000) The chemical constituents from the heartwood of *Eucalyptus citriodora*. *Journal of the Chinese Chemical Society* 47(3). 555-560.

Lee, C. & Houghton, P. (2005) Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. *Journal of Ethnopharmacology*, 100. 237-243.

Lees, K. & Bowman, A.S. (2007) Tick neurobiology: recent advances and the post-genomic era. *Invertebrate Neuroscience*, 7. 183-198.

León, A., Reyes, B.M., Chávez, M.I., Toscano, R.A. & Delgado, G. (2009) Sesquiterpene lactones, acyl phenyl propanoids and other constituents from *Schkuhria pinnata* var. wislizeni: Antioxidant evaluation. *Journal of the Mexican Chemical Society*, 53. 193-200.

Lew-Tabor, A. & Valle, M.R. (2015) A review of reverse vaccinology approaches for the development of vaccines against ticks and tick borne diseases. *Ticks & Tick-borne Diseases.* http://dx.doi.org/10.1016/j.ttbdis.2015.12.012.

Li, Y., Li, Z. & Ye, M. (2008) The chemical compositions and their bioactivities in the different parts of *Eupatorium adenophorum* Spreng. *Journal-Yunnan Agricultural University* 23. 42.

Liang, G., Chen, W. & Liu, T. (2003) Effects of three neem-based insecticides on diamondback moth (Lepidoptera: Plutellidae). *Crop Protection*, 22. 333-340.

Lobetti, R. & Lappin, M.R. (2012) Prevalence of *Toxoplasma gondii, Bartonella* species and haemoplasma infection in cats in South Africa. *Journal of Feline Medicine & Surgery*, 14. 857-862.

Loftis, A.D., Levin, M.L. & Spurlock, J.P. (2008) Two USA *Ehrlichia* spp. cause febrile illness in goats. *Veterinary Microbiology*, 130. 398-402.

Lori, D., Grazioli, E., Gentile, G., Marano, G. & Salvatore, A. (2005) Acaricidal properties of the essential oil of *Melaleuca alternifolia* Cheel (tea tree oil) against nymphs of *Ixodes ricinus*. *Veterinary Parasitology* 129. 173-176.

Lovis, L., Perret, J., Bouvier, J., Fellay, J., Kaminsky, R., Betschart, B. & Sager, H. (2011) A new *in vitro* test to evaluate the resistance level against acaricides of the cattle tick, *Rhipicephalus (Boophilus) microplus*. *Veterinary Parasitology*, 182. 269-280.

Lovis, L., Reggi, J., Berggoetz, M., Betschart, B. & Sager, H. (2013) Determination of acaricide resistance in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) field populations of Argentina, South Africa, and Australia with the Larval Tarsal Test. *Journal of Medical Entomology*, 50. 326-335.

Lundin, C., Käll, L., Kreher, S.A., Kapp, K., Sonnhammer, E.L., Carlson, J.R., von Heijne, G. & Nilsson, I. (2007) Membrane topology of the *Drosophila* OR83b odorant receptor *FEBS Letters,* 581(29). 5601-5604.

Lupi, E., Hatz, C. & Schlagenhauf, P. (2013) The efficacy of repellents against *Aedes, Anopheles, Culex* and *Ixodes* spp.- a literature review. *Travel Medicine and Infectious Disease,* 11(6). 374-411.

Luseba, D., Elgorashi, E., Ntloedibe, D. & Van Staden, J. (2007) Antibacterial, anti-inflammatory and mutagenic effects of some medicinal plants used in South Africa for the treatment of wounds and retained placenta in livestock. *South African Journal of Botany*, 73. 378-383.

Lwande, W., Ndakala, A.J., Hassanali, A., Moreka, L., Nyandat, E., Ndungu, M., Amiani, H., Gitu, P.M., Malonza, M.M. & Punyua, D.K. (1999). *Gynandropsis gynandra* essential oil and its constituents as tick (*Rhipicephalus appendiculatus*) repellents. *Phytochemistry,* 50. 401-405.

Ma, C., Xiao, S.Y., Li, Z.G., Wang, W. & Du, L.J. (2007) Characterization of active phenolic components in the ethanolic extract of *Ananas comosus* L. leaves using high-performance liquid chromatography with diode array detection and tandem mass spectrometry. *Journal of Chromatography A,* 1165(1). 39-44.

Macedo-Ribeiro, S., Almeida, C., Calisto, B.M., Friedrich, T., Mentele, R., Stürzebecher, J., Fuentes-Prior, P. & Pereira, P.J.B. (2008) Isolation, cloning and structural characterisation of boophilin, a multifunctional Kunitz-type proteinase inhibitor from the cattle tick. *PLoS One*, 3. e1624.

Madhumitha, G., Rajakumar, G., Roopan, S.M., Rahuman, A.A., Priya, K.M., Saral, A.M., Khan, F.R.N., Khanna, V.G., Velayutham, K. & Jayaseelan, C. (2012) Acaricidal, insecticidal, and larvicidal efficacy of fruit peel aqueous extract of *Annona squamosa* and its compounds against blood-feeding parasites. *Parasitology Research,* 111. 2189-2199.

Madzimure, J., Nyahangare, E.T., Hamudikuwanda, H., Hove, T., Stevenson, P.C., Belmain, S.R. & Mvumi, B.M. (2011) Acaricidal efficacy against cattle ticks and acute oral toxicity of *Lippia javanica* (Burm F.) Spreng. *Tropical Animal Health & Production*, 43. 481-489.

Magadum, S., Mondal, D.B. & Ghosh, S. (2009) Comparative efficacy of *Annosa squamosa* and *Azadirachta indica* against *Boophilus microplus* Izatnagar isolate. *Parasitology Research* 105(4). 1085-1091.

Magano, S.R., Nchu, F. & Eloff, J.N. (2011) *In vitro* investigation of the repellent effects of the essential oil of *Lippia javanica* on adults of *Hyalomma marginatum rufipes. African Journal of Biotechnology,* 10(44). 8970-8975.

Magano, S., Thembo, K., Ndlovu, S. & Makhubela, N. (2008) The anti-tick properties of the root extracts of *Senna italica* subsp. *arachoides. African Journal of Biotechnology*, 7(4). 476-481.

Maggi, M.D., Ruffinengo, S.R., Mendoza, Y., Ojeda, P., Ramallo, G., Floris, I. & Eguaras, M.J. (2011) Susceptibility of *Varroa destructor* (Acari: Varroidae) to synthetic acaricides in Uruguay: Varroa mites' potential to develop acaricide resistance. *Parasitology Research*, 108. 815-821.

Maia, M.F. & Moore, S.J. (2011) Plant-based insect repellents: a review of their efficacy, development and testing. *Malaria Journal*, 10. 1.

Makang'a, O.B.B. (2012) Composition and repellency of essential oils of *Tagetes minuta* from different zones in Kenya against brown ear tick (*Rhipicephalus appendiculatus*). http://ir-library-ku-ac.ke/handle/123456789/6526.

Malan, R. (2015) Acaricide resistance in *Rhipicephalus (Boophilus)* species at a communal dipping system in the Mnisi community, Mpumalanga Province. http://hdl.handle.net/2263/53302.

Mangathayaru, K., Amitabha, G., Rajeev, R. & Kaushik, V.K. (2006) Volatile constituents of *Leucas aspera* (Willd.) link. *Journal of Essential Oils Research,* 18(1). 104-105.

Manjunathachar, H.V., Saravanan, B.C., Kesavan, M., Karthik, K., Rathod, P., Gopi, M., Tamilmahan, P. & Balaraju, B.L. (2014) Economic importance of ticks and their effective control strategies. *Asian Pacific Journal of Tropical Disease*, 4. S770-S779.

Mans, B.J., Coetzee, J., Louw, A.I., Gaspar, A.R. & Neitz, A.W. (2000) Disaggregation of aggregated platelets by apyrase from the tick, *Ornithodoros savignyi. Experimental & Applied Acarology*, 24. 271-282.

Mans, B.J. & Neitz, A.W. (2004a) Adaptation of ticks to a blood-feeding environment: evolution from a functional perspective. *Insect Biochemistry & Molecular Biology*, 34. 1-17.

Mans, B.J. & Neitz, A.W. (2004b) Exon–intron structure of outlier tick lipocalins indicate a monophyletic origin within the larger lipocalin family. *Insect Biochemistry & Molecular Biology*, 34. 585-594.

Mans, B., Gaspar, A., Louw, A. & Neitz, A. (1998) Apyrase activity and platelet aggregation inhibitors in the tick *Ornithodoros savignyi* (Acari: Argasidae). *Experimental & Applied Acarology*, 22. 353-366.

Mans, B., Gothe, R. & Neitz, A. (2008) Tick toxins: perspectives on paralysis and other forms of toxicoses caused by ticks. *Ticks: Biology, Diseases & Control*. 108-126.

Mans, B., Louw, A. & Neitz, A. (2002) Amino acid sequence and structure modeling of savignin, a thrombin inhibitor from the tick, *Ornithodoros savignyi. Insect Biochemistry & Molecular Biology*, 32. 821-828.

Mans, B.J., De Klerk, D., Pienaar, R. & Latif, A.A. (2011) *Nuttalliella namaqua*: a living fossil and closest relative to the ancestral tick lineage: implications for the evolution of blood-feeding in ticks. *PloS one*, 6. e23675.

Mans, B.J., de Klerk, D.G., Pienaar, R. & Latif, A.A. (2014) The host preferences of *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae): a generalist approach to surviving multiple host-switches. *Experimental & Applied Acarology*, 62. 233-240.

Mans, B.J. & Ribeiro, J.M. (2008) Function, mechanism and evolution of the moubatin-clade of soft tick lipocalins. Insect *Biochemistry & Molecular Biology*, 38. 841-852.

Mar, C. & Bent, S. (1999) An evidence-based review of the 10 most commonly used herbs. *Western Journal of Medicine*, 171. 168.

Marchiondo, A., Holdsworth, P., Fourie, L., Rugg, D., Hellmann, K., Snyder, D. & Dryden, M. (2013) World Association for the Advancement of Veterinary Parasitology (WAAVP): guidelines for evaluating the efficacy of parasiticides for the treatment, prevention and control ofjflea and tick infestations on dogs and cats. *Veterinary Parasitology,* 194(1). 84-97.

Marczinke, B.I. & Nichol, S.T. (2002) Nairobi sheep disease virus, an important tick-borne pathogen of sheep and goats in Africa, is also present in Asia. *Virology*, 303. 146-151.

Martel-Pelletier, J., Lajeunesse, D., Reboul, P. & Pelletier, J.P. (2003) Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. *Annals of the Rheumatic Diseases*, 62. 501-509.

Martin, M., Mathias, E. & McCorkle, C.M. (2001). Ethnoveterinary Medicine: An Annotated Bibliography of Community Animal Healthcare. ITDG publishing. pp 1-611.

Martinez-Velazquez, M., Rosario-Cruz, R., Castillo-Herrera, G., Flores-Fernandez, J., Alvarez, A. & Lugo-Cervantes, E. (2011) Acaricidal effect of essential oils from *Lippia graveolens* (Lamiales: Verbenaceae), *Rosmarinus officinalis* (Lamiales: Lamiaceae), and *Allium sativum* (Liliales: Liliaceae) against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Journal of Medical Entomology,* 48. 822-827.

Martins, R.M. (2006) *In vitro* study of the acaricidal activity of the essential oil from the Citronella of Java (*Cymbopogon winterianus* Jowitt) to the tick *Boophilus microplus*. *Revista Brasileira de Plantas Medicinais* 8(2): 71-78.

Masters, E.J., Olson, G.S., Weiner, S.J. & Paddock, C.D. (2003) Rocky Mountain spotted fever: a clinician's dilemma. *Archives of Internal Medicine*, 163. 769-774.

Matthewson, M.D., Hughes, G., Macpherson, I.S. & Bernard, C.P. (1981) Screening techniques for the evaluation of chemicals with activity as tick repellents. *Pesticide Science,* 12(4). 455-462.

Maurya, A.K. & Vinayak, M. (2015) Anticarcinogenic action of quercetin by downregulation of phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) via induction of p53 in hepatocellular carcinoma (HepG2) cell line. *Molecular Biology Reports*, 42. 1419-1429.

McCorkle, C.M. (1995) Back to the future: Lessons from ethnoveterinary RD&E for studying and applying local knowledge. *Agriculture & Human Values*, 12. 52-80.

McGaw, L.J., & Eloff, J.N. (2010) Methods for evaluating efficacy of ethnoveterinary medicinal plants. *Ethnoveterinary Botanical Medicine: Herbal Medicines for Animal Health*, 1-24.

McHeyzer-Williams, L., Malherbe, L. & McHeyzer-Williams, M. (2006). Helper T cell-regulated B cell immunity. In *From Innate Immunity to Immunological Memory.* Anonymous Springer pp. 59-83.

McMahon, C. & Guerin, P. (2002) Attraction of the tropical bont tick, *Amblyomma variegatum,* to human breath and to the breath components acetone, NO and CO2., *Naturwissenschaften,* 89(7). 311-315.

Mcmahon, C., Kröber, T. & Guerin, P.M. (2003) *In vitro* assays for repellents and deterrents for ticks: differing effects of products when tested with attractant or arrestment stimuli. *Medical & Veterinary Entomology*, 17. 370-378.

McTier, T.L., Chubb, N., Curtis, M.P., Hedges, L., Inskeep, G.A., Knauer, C.S., Menon, S., Mills, B., Pullins, A. & Zinser, E. (2016) Discovery of sarolaner: A novel, orally administered, broad-spectrum, isoxazoline ectoparasiticide for dogs. *Veterinary Parasitology*, 222. 3-11.

Meade, P., Abate, A.L., Pavo, J., Yeung-Cheung, A.K. & Pappas, C.J. (2017) A novel *ex vivo* bioassay suggests DEET is an effective repellent of *Rhipicephalus sanguineus. Journal of Agricultural & Urban Entomology*, 33(1). 19-31.

Meharg, A. (2005) Venomous earth: How arsenic caused the world's worst mass poisoning. http://www.fao.org/library/library-home/en/.

Mehlhorn, H., Schmahi, G. & Schmidt, J. (2005) Extract of the seeds of plant *Vitex angus castus* proven to be highly efficacious as a repellent against ticks, fleas, mosquitoes and biting flies. *Parasitology Research* 95. 363-365.

Meinke, P.T. (2001) Perspectives in animal health: old targets and new opportunities. *Journal of Medicinal Chemistry*, 44.641–659.

Mejri, N., Rutti, B. & Brossard, M. (2002) Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice. *Parasitology Research*, 88. 192-197.

Meliker, J.R., Slotnick, M.J., AvRuskin, G.A., Schottenfeld, D., Jacquez, G.M., Wilson, M.L., Goovaerts, P., Franzblau, A. & Nriagu, J.O. (2010) Lifetime exposure to arsenic in drinking water and bladder cancer: a population-based case–control study in Michigan, USA. *Cancer Causes & Control*, 21. 745-757.

Meng, H., Li, A.Y., Junior, L.M.C., Castro-Arellano, I. & Liu, J. (2016) Evaluation of DEET and eight essential oils for repellency against nymphs of the lone star tick, *Amblyomma americanum* (Acari: Ixodidae). *Experimental & Applied Acarology*, 68(2). 241-249.

Metcalf, R.L. (1983). Implications and prognosis of resistance to insecticides. *In Pest Resistance to Pesticides*. Anonymous Springer pp. 703-733.

Maia, M.F. & Moore, S.J. (2011) Plant-based insect repellents: a review of their efficacy, development and testing. *Malaria Journal,* 10(1). 1.

Middleton, E., Kandaswami, C. & Theoharides, T.C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews*, 52. 673-751.

Miller, R.J., Davey, R.B. & George, J.E. (2002) Modification of the food and agriculture organization larval packet test to measure amitraz-susceptibility against ixodidae. *Journal of Medical Entomology,* 39(4). 645-651.

Mikaili, P., Maadirad, S., Moloudizargari, M., Aghajanshakeri, S. & Sarahroodi, S. (2013) Therapeutic uses and pharmacological properties of garlic, shallot, and their biologically active compounds. *Iranian Journal of Basic Medical Sciences*. 16. 1031-1048.

Minjauw, R., McLeod, A. (2003) Tick borne diseases and poverty. The impact of ticks and tickborne diseases on the livelihoods of small scale and marginal livestock owners in India and Eastern and Southern Africa. *Research report, DFID Animal Health Programme, Centre for Tropical Veterinary Medicine, University of Edinburgh, UK*.

Mkolo, M.N. & Magano, S.R. (2007) Repellant effects of the essential oil of *Lavendula augustifolia* against adults of *Hyalomma marginatum rufipes*. *Journal of the South African Veterinary Association* 78, 149-152.

Mondal, D., Sarma, K. & Saravanan, M. (2013) Upcoming of the integrated tick control program of ruminants with special emphasis on livestock farming system in India. *Ticks & Tick-borne Diseases*, 4. 1-10.

Monika, C. (2014) Steroids- Chemical constituents of *Withania somnifera* Dunal through TLC and HPTLC. *International Journal of Chemistry,* 10-21.

Monteiro, C.M., Daemon, E., Clemente, M.A., Rosa, L.S. & Maturano, R. (2009) Acaricidal efficacy of thymol on engorged nymphs and females of *Rhipicephalus sanguineus* (Latreille 1808) (Acari: Ixodidae). *Parasitology Research,* 105. 1093-1097.

Monteiro, C.M., Maturano, R., Daemon, E., Catunda-Junior, F.E.A., Calmon, F., Senra, T.S., Faza, A. & Carvalho, M.G. (2012) Acaricidal activity of eugenol on *Rhipicephalus microplus* (Acari: Ixodidae) and *Dermacentor nitens* (Acari: Ixodidae) larvae. *Parasitology Research,* 111. 1295-1300.

Moore, S.J., Lenglet, A. & Hill, N. (2007) Plant-based insect repellents. *Insect Repellents: Principles Methods & Uses,* 3-29.

Moorhouse, D. & Tatchell, R. (1966) The feeding processes of the cattle-tick *Boophilus microplus* (Canestrini): a study in host-parasite relations. *Parasitology*, 56. 623-631.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65. 55-63.

Mostafa, M., Nahar, N., Mosihuzzaman, M., Makhmoor, T., Choudhary, M.I. & Rahman, A.U. (2007). Free radical scavenging phenylethanoid glycosides from *Leucas indica* Linn. *Natural Product Research,* 21(4). 354-361.

Moyo, B. & Masika, P.J. (2013). Validation of the acaricidal properties of materials used in ethno-veterinary control of cattle ticks. *African Journal of Microbiology Research,* 7(39). 4701-4706.

Moyo, B., Moyo, B., Moyo, S., & Masika, P.J. (2015) A survey of external parasites of free-range chickens and their ethno-veterinary control remedies used by resource-limited farmers in Eastern Cape, South Africa. *Scientific Journal of Veterinary Advances*, *4*(2), 13-20.

Mukandiwa, L., Eloff, J.N. & Naidoo, V. (2012) Evaluation of plant species used traditionally to treat myiasis for activity on the survival and development of Lucilia cuprina and Chrysomya marginalis (Diptera: Calliphoridae). *Veterinary Parasitology*, 190. 566-572.

Mukandiwa, L., Eloff, J.N., & Naidoo, V. (2012) Extracts of four plant species used traditionally to treat myiasis influence pupation rate, pupal weight and adult fly emergence of *Lucilia cuprina* and *Chrysomya marginalis* (Diptera: Calliphoridae) *Journal of Ethnopharmacology,* 143. 812–818.

Mukandiwa, L., Ahmed, A., Naidoo, V. & Eloff, J.N. (2013) Isolation of seselin isolated from *Clausena anisata* (Rutaceae) leaves and its effects on the feeding and development of *Lucilia cuprina* larvae may explain its the use in ethnoveterinary medicine to treat myiasis. *Journal of Ethnopharmacology,* 150. 886-891.

Mukandiwa, L., Eloff, J.N. & Naidoo, V. (2014) Larvicidal activity of leaf extracts and seselin from *Clausena anisata* (Rutaceae) against *Aedes aegypti*. *South African Journal of Botany,* 100. 169-173.

Mukhebi, A. W., Perry, B. D. & Kruska, R. (1992) Estimated economics of theileriosis control in Africa. *Preventive Veterinary Medicine,* 12(1). 73-85.

Mulholland, D.A., Parel, B. & Combes, P.H. (2000) The chemistry of the Meliaceae and Ptaeroxylaceae of southern and eastern Africa and Madagascar. *Current Organic Chemistry,* 4(10). 1011-1054.

Mulla, M.S. & Su, T.I.A.N.Y.U.N. (1999) Activity and biological effects of neem products against arthropods of medical and veterinary importance. *Journal of the American Mosquito Control Association,* 15(2). 133-152.

Mullen, G.R. & Durden, L.A. (2009) eds. *Medical and Veterinary Entomology,* Academic Press, pp 1-554.

Muyobela, J., Nkunika, P.O.Y., & Mwase, E.T. (2016) *In vitro* acaricidal activity of *Bobgunnia madagascariensis* Desv. against *Amblyomma variegatum* (Fabricius) (Acari: Ixodidae). *Tropical Animal Health & Production*, 48(3). 625-631.

Mwangi, E.N., Hassanali, A., Essuman, S., Myandat, E., Moreka, L. & Kimondo, M. (1995) Repellent and acaricidal properties of *Ocimum suave* against *Rhipicephalus appendiculatus* ticks. *Experimental & Applied Acarology* 19. 11-18.

Nakahara, K., Alzoreky, N.S., Yoshihashi, T., Nguyen, H.T. & Trakoontivakorn, G. (2003) Chemical composition and antifungal activity of essential oil from *Cymbopogon nardus* (citronella grass). *Japan Agricultural Research Quarterly*, 37(4). 249-252.

Nana, P., Ekesi, S., Nchu, F. & Maniania, N. (2016) Compatibility of *Metarhizium anisopliae* with *Calpurnia aurea* leaf extracts and virulence against *Rhipicephalus pulchellus. Journal of Applied Entomology*, 140(8). 590-597.

Nana, P., Maniania, N., Maranga, R., Kutima, H., Boga, H., Nchu, F. & Eloff, J. (2010) Attraction response of adult *Rhipicephalus appendiculatus* and *Rhipicephalus pulchellus* (Acari: Ixodidae) ticks to extracts from *Calpurnia aurea* (Fabaceae). *Veterinary Parasitology*, 174. 124-130.

Nana, P., Nchu, F., Ekesi, S., Boga, H.I., Kamtchouing, P. & Maniania, N.K. (2015) Efficacy of spot-spray application of *Metarhizium anisopliae* formulated in emulsifiable extract of *Calpurnia aurea* in attracting and infecting adult *Rhipicephalus appendiculatu*s ticks in semi-field experiments. *Journal of Pest Science*, 88. 613-619.

Nartowska, J., Sommer, E., Pastewka, K., Sommer, S. & Skopinska-Rozewska, E. (2004) Anti-angiogenic activity of convallamaroside, the steroidal saponin isolated from the rhizomes and roots of *Convallaria majalis* L. *Acta Poloniae Pharmaceutica,* 61. 279-282.

Nassar, M.I., Mohamed, T.K., Elshamy, A.I., El-Toumy, S.A., Lateef, A.M.A. & Farrag, A.H. (2013) Chemical constituents and anti-ulcerogenic potential of the scales of *Cynara scolymus* (artichoke) heads. *Journal of the Science of Food & Agriculture*, 93. 2494-2501.

Nazari, F., Ebrahimi, S.N., Talebi, M., Rassouli, A. & Bijanzadeh, H.R. (2007) Multivariate optimisation of microwave-assisted extraction of capsaicin from *Capsicum frutescens* L. and quantitative analysis by 1H-NMR. *Phytochemical Analysis,* 18(4). 333-340.

Nchu F., Magano S.R. & Eloff J.N. (2012) *In vitro* anti-tick properties of the essential oil of *Tagetes minuta* L. on *Hyalomma rufipes* (Acari: Ixodidae). *Onderstepoort Journal of Veterinary Research,* 79(1). E1-5.

Nchu, F., Magano, S. & Eloff, J. (2005) *In vitro* investigation of the toxic effects of extracts of *Allium sativum* bulbs on adults of *Hyalomma marginatum rufipes* and *Rhipicephalus pulchellus. Journal of the South African Veterinary Association*, 76. 99-103.

Ndungu, M., Lwande, W., Hassanali, A., Moreka, L. & Chhabra, S.C. (1995) *Cleome monophylla* essential oil and its constituents as tick (*Rhipicephalus appendiculatus*) and maize weevil (*Sitophilus zeamais*) repellents. *Entomologia Experimentalis et Applicata,* 76(3). 217-222.

Needham, G.R. & Teel, P.D. (1991) Off-host physiological ecology of ixodid ticks. *Annual Review of Entomology*, 36. 659-681.

Nerio, L.S., Olivero-Verbel, J. & Stashenko, E. (2010) Repellent activity of essential oils: a review. *Bioresource Technology,* 101. 372-378.

Netshiluvhi, T.R. & Eloff, J.N. (2015a) Effect of water stress on antimicrobial activity of selected medicinal plant species. *South African Journal of Botany*. http://dx.doi.org/10.1016/j.sajb.2015.04.005.

Netshiluvhi, T. R. &Eloff, J.N. (2015b) Influence of annual rainfall on antibacterial activity of acetone leaf extracts of selected medicinal trees. *South African Journal of Botany.* http://dx.doi.org/10.1016/j.sajb.2015.04.008.

Nerio, L. S., Olivero-Verbel, J. & Stashenko, E. (2010) Repellent activity of essential oils: a review. *Bioresource Technology,* 101(1). 372-378.

Neuwinger, H.D. (1994) Afrikanische Arzneipflanzen und Jagdgifte. WV GesmH, 450-457.

Neyeloff, J.L., Fuchs, S.C. & Moreira, L.B. (2012) Meta-analyses and Forest plots using a microsoft excel spreadsheet: step-by-step guide focusing on descriptive data analysis, *BMC Research Notes,* 5. 52.

Ngarivhume, T., van't Klooster, C.I., de Jong, J.T. & Van der Westhuizen, Jan H (2015) Medicinal plants used by traditional healers for the treatment of malaria in the Chipinge district in Zimbabwe. *Journal of Ethnopharmacology*, 159. 224-237.

Nisha, N.H.M. & Packialakshmi, N. (2014) Analysis of antibacterial and phytochemical screening by using different *Anisomeles malabraca* samples. *International Journal of Pharmaceutical Research,* 4(1). 22-24.

Nolan, J. (1981) Current developments in resistance toamidine and pyrethroid tickicides in Australia. *In Whitehead, G.B., Gibson, J.D. (Eds), Tick Biology and Control* Tick Research Unit, Rhodes University, Grahamstown, South Africa pp. 109–114.

Nonaka, K., Tsukiyama, T., Okamoio, Y., Sato, K., Kumasaka, C., Yamamoto, T., Maruyama, F. & Yoshikawa, H. (2000) New milbemycins from *Streptomyces hygroscopicus* subsp. *aureolacrimosus.* Fermentation, isolation and structure elucidation. *The Journal of Antibiotics*, 53. 694-704.

Nondo, R. S., Moshi, M. J., Erasto, P., Zofou, D., Njouendou, A. J., Wanji, S., Ngemenye, M.N., Kidukuli, A.W., Masimba, P.J. & Titanji, V. P. (2015) Evaluation of the cytotoxic activity of extracts from medicinal plants used for the treatment of malaria in Kagera and Lindi regions, Tanzania. *Journal of Applied Pharmaceutical Science*, 5(4). 7-12.

Nong, X., Ren, Y.J., Wang, J.H., Fang, C.L., Xie, Y., Yang, D.Y. & Yang, G.Y. (2013a) Clinical efficacy of botanical extracts from *Eupatorium adenophorum* against the scab mite, *Psoroptes cuniculi*. *Veterinary Parasitology* 192(1). 247-252.

Nong, X., Tan, Y.J., Wang, J.H., Xie, Y., Fang, C.L., Chen, L. & Yang, G.Y. (2013b) Evaluation acaricidal efficacy of botanical extract from *Eupatorium adenophorum* against the hard tick *Haemaphysalis longicornis* (Acari: Ixodidae). *Experimental Parasitology,* 135(3). 558-563.

Norval, R., Horak, I., Coetzer, J. & Tustin, R. (2004) Vectors: ticks. *Infectious diseases of livestock*, 1. 3-42.

Nwude, N. & Ibrahim, M. (1980) Plants used in traditional veterinary medical practice in Nigeria. *Journal of Veterinary Pharmacology & Therapeutics*, 3. 261-273.

Nyahangare, E.T., Mvumi, B.M. & Mutibvu, T. (2015) Ethnoveterinary plants and practices used for ecto-parasite control in semi-arid smallholder farming areas of Zimbabwe. *Journal of Ethnobiology & Ethnomedicine*, 11. 30. doi 10.1186/s13002-015-0006-6.

Obenchain, F.D., & Galun, R. (Eds.). (2013) *Physiology of Ticks: Current Themes in Tropical Science*. Elsevier. pp 1-501.

Oberdörster, E., Clay, M.A., Cottam, D.M., Wilmot, F.A., McLachlan, J.A. & Milner, M.J. (2001) Common phytochemicals are ecdysteroid agonists and antagonists: a possible evolutionary link between vertebrate and invertebrate steroid hormones. *The Journal of Steroid Biochemistry & Molecular Biology*, 77. 229-238.

Oberlander, H. & Silhacek, D.L. (1998) Mode of action of insect growth regulators in Lepidopteran tissue culture. *Pesticide Science*, 54. 300-302.

Ocloo, A., Nwokolo, N.C. & Dayie, N.T.K.D. (2012) Phytochemical characterization and comparative efficacies of crude extracts of *Carica papaya*. *International Journal of Drug Research and Technology* 2(5). 399-406.

Odonbayar, B., Murata, T., Matsumoto, N., Batkhuu, J., & Sasaki, K. (2017) Chemical constituents of aerial parts of *Thymus gobicus* and their cholinesterase inhibitory activities. *Mongolian Journal of Chemistry*, *17*(43). 14-17.

Ogendo, J., Deng, A., Kostyukovsky, M., Ravid, U., Matasyoh, J., Omolo, E., Kariuki, S. & Kamau, A. (2011) Biocontrol potential of selected plant essential oil constituents as fumigants of insect pests attacking stored food commodities. *Health,* 10. 287-318.

Oh, J., Bowling, J.J., Caroll, J.F., Demirci, B., Can Baser, K.H., Leininger, T.D., Bernier, U.R. & Hamann, M.T. (2012) Natural product studies of U.S. endangered plants: Volatile components of *Lindera mellisfolai* (Lauraceae) repel mosquitoes and ticks. *Phytochemistry,* 80. 28-36.

Olds, C., Mwaura, S., Crowder, D., Odongo, D., van Oers, M., Owen, J., Bishop, R. & Daubenberger, C. (2012) Immunization of cattle with Ra86 impedes *Rhipicephalus appendiculatus* nymphal-to-adult molting. *Ticks & Tick-borne Diseases*, 3. 170-178.

Olivo, C. J., Heimerdinger, A., Ziech, M. F., Agnolin, C. A., Meinerz, G. R., Both, F. & Charão, P.S. (2009). Rope tobacco aqueous extract on the control of cattle ticks. *Ciência Rural,* 39(4). 1131-1135.

Oluremi, O.I.A., Ngi, J & Andrew, A.I. (2007) Phytonutrients in citrus fruit peel meal and nutritional implication for livestock production. *Livestock Research for Rural Development,* 19(7). 345-346.

Onawunmi, G.O., Yisak, W.A. & Ogunlana, E.O. (1984) Antibacterial constituents in the essential oil of *Cymbopogon citratus* (DC.) Stapf. *Journal of Ethnopharmacology,* 12(3). 279-286.

Opiro, R., Osinde, C., Okello-Onen, J. & Akol, A.M. (2013) Tick-repellent properties of four plant species against *Rhipicephalus appendiculatus* Neumann (Acarina: Ixodidae) tick species. *Journal of Agricultural Research & Developmen*t 3(2). 17-21.

Orozco, O.L., & Lentz, D.L. (2005) Poisonous plants and their uses as insecticides in Cajamarca, Peru. *Economic Botany*, 59(2). 166-173.

Osada, N., Kohara, A., Yamaji, T., Hirayama, N., Kasai, F., Sekizuka, T., Kuroda, M. & Hanada, K. (2014) The genome landscape of the african green monkey kidney-derived vero cell line. *DNA Research*, 21. 673-683.

Osman, I. M., Mohammed, A. S. & Abdalla, A. B. (2014) Acaricidal properties of two extracts from *Guiera senegalensis* JF Gmel. (Combrataceae) against *Hyalomma anatolicum* (Acari: Ixodidae). *Veterinary Parasitology* 199(3). 201-205.

Pacher, T., Seger, C., Engelmeier, D., Vajrodaya, S., Hofer, O. & Greger, H. (2002) Antifungal stilbenoids from *Stemona collinsae. Journal of Natural Products* 65. 820-827.

Padalia, R.C., Verma, R.C. & Sundaresan, V. (2010) Volatile constituents of three invasive weeds of Himalayan Region. *Records of Natural Products* 4(2). 109-144.

Pages, F., Dautel, H., Duvallet, G., Kahl, O., de Gentile, L. & Boulanger, N. (2014) Tick repellents for human use: prevention of tick bites and tick-borne diseases. *Vector-Borne & Zoonotic Diseases,* 14(2). 85-93.

Palmer, B.H., McCarthy, J.F., Kozlik, A. & Harrison, I.R. (1973). A new chemical group of cattle acaricides. *Proceedings of the 3rd International Congress of Acarology*, Prague pp. 687- 691.

Pamo, E.T., Tendonkeng, F., Kana, J.R., Tapondjou, A.L. & Fongang, M.D. (2004). The acaricidal effect of the essential oils of *Ageratum houstonianum* Mill. Flowers on ticks (*Rhipicephalus lunatus*) in Cameroun. *South African Journal of Animal Science,* 34(1). 244-247.

Pamo, E.T., Tendonkeng, F., Kana, J.R., Payne, V.K., Boukila, B., Lemoufouet, J., Khasawneh, M., Montenier, J.A. & Maupin, G.O. (2005) A study of the acaricidal properties of an essential oil extracted from the leaves of *Ageratum houstonianum* Mill. *Veterinary Parasitology,* 12. 319-323.

Pandey, R.R., Dubey, R.C. & Saini, S. (2010) Phytochemical and antimicrobial studies on essential oils of some aromatic plants. *African Journal of Biotechnology,* 9(28). 4364-4368.

Panella, N.A., Dolan, M.C., Karchesy, J.J., Xiong, Y., Peralta-Cruz, J., Khasawneh, M., Montenieri, J.A. & Maupin, G.O. (2005) Use of novel compounds for pest control: insecticidal and acaricidal activity of essential oil components from heartwood of Alaska yellow cedar. *Journal of Medical Entomology*, 42. 352-358.

Parra, L., Mutis, A., Ceballos, R., Lizama, M., Pardo, F., Perich, F. & Quiroz, A. (2009) Volatiles released from *Vaccinium corymbosum* were attractive to *Aegorhinus superciliosus* (Coleoptera: Curculionidae) in an olfactometric bioassay. *Environmental Entomology,* 38. 781-789.

Parham, P. (2014) *The immune system*. Garland Science.

Parizi, L.F., Githaka, N.W., Logullo, C., Konnai, S., Masuda, A., Ohashi, K. & da Silva Vaz, I. (2012) The quest for a universal vaccine against ticks: cross-immunity insights. *The Veterinary Journal*, 194. 158-165.

Patel, S., Behara, R., Swanson, G.R., Forsyth, C. B., Voigt, R.M. & Keshavarzian, A. (2015) Alcohol and the intestine. *Biomolecules,* 5(4). 2573-2588.

Paterna, A., Gomes, S.E., Borralho, P.M., Mulhovo, S., Rodrigues, C.M., & Ferreira, M.J.U. (2016) Vobasinyl–Iboga alkaloids from *Tabernaemontana elegans*: Cell cycle arrest and apoptosis-inducing activity in HCT116 colon cancer cells. *Journal of Natural Products*, *79*(10). 2624-2634.

Patwardhan, B., Warude, D., Pushpangadan, P. & Bhatt, N. (2005) Ayurveda and traditional Chinese medicine: a comparative overview. *Evidence-based Complementary & Alternative Medicine,* 2. 465-473.

Pavela, R., Canale, A., Mehlhorn, H. & Benelli, G. (2016) Application of ethnobotanical repellents and acaricides in prevention, control and management of livestock ticks: a review. *Research in Veterinary Science*, 109. 1-9.

Pazinato, R., Klauck, V., Volpato, A., Tonin, A.A., Santos, R.C., de Souza, M.E., Vaucher, R.A., Raffin, R., Gomes, P., Felippi, C.C., Stefani, L.M. & Da Silva, A.S. (2014) Influence of tea tree oil (*Melaleuca alternifolia*) on the cattle tick *Rhipicephalus microplus*. *Experimental & Applied Acarology,* 63(1). 77-83.

Pechová, T. (2013). Metody pro testování repelentů proti klíšťatům (*Doctoral dissertation*, Masarykova univerzita, Přírodovědecká fakulta).

Peng, H.Y., Zhang, X.H. & Xu, J.Z. (2016) Apigenin-7-O-beta-D-glycoside isolation from the highly copper-tolerant plant Elsholtzia splendens. *Journal of Zhejiang University.Science.B*, 17. 447-454.

Perez-Cogollo, L., Rodriguez-Vivas, R., Ramirez-Cruz, G. & Miller, R. (2010) First report of the cattle tick *Rhipicephalus microplus* resistant to ivermectin in Mexico. *Veterinary Parasitology*, 168. 165-169.

Peter, R., Van den Bossche, P., Penzhorn, B.L. & Sharp, B. (2005) Tick, fly, and mosquito control—lessons from the past, solutions for the future. *Veterinary Parasitology*, 132. 205-215.

Pfäffle, M., Littwin, N., Muders, S.V., & Petney, T.N. (2013) The ecology of tick-borne diseases. *International Journal for Parasitology*, 43(12). 1059-1077.

Pfister, K. & Armstrong, R. (2016) Systemically and cutaneously distributed ectoparasiticides: a review of the efficacy against ticks and fleas on dogs. *Parasites & Vectors,* 9(1). 436.

Picking, D., Delgoda, R., Boulogne, I. & Mitchell, S. (2013) *Hyptis verticillata* Jacq: A review of its traditional uses, phytochemistry, pharmacology and toxicology. *Journal of Ethnopharmacology,* 147. 16-41.

Pirali-Kheirabadi, K. & Razzaghi-Abyaneh, M. (2007) Biological activities of chamomile (*Matricaria chamomile*) flowers extract against the survival and egg laying of the cattle tick (Acari Ixodidae). *Journal of Zhejiang University Science B* 8(9). 693-696.

Pirali-Kheirabadi, K., Razzaghi-Abyaneh, M. & Halajian, A. (2009) Acaricidal effect of *Pelargonium roseum* and *Eucalyptus globulus* essential oils against adult stage of *Rhipicephalus (Boophilus) annulatus in vitro*. *Veterinary Parasitology,* 162(3). 346-349.

Pirali-Kheirabadi, K. & Teixeira da Silva, J.A. (2010) *Lavandula angustifolia* essential oil as a novel and promising natural candidate for tick *Rhipicephalus (Boophilus) annulatus* control. *Experimental Parasitology* 126(2). 184-186.

Pipano, E., Alekceev, E., Galker, F., Fish, L., Samish, M. & Shkap, V. (2003) Immunity against *Boophilus annulatus* induced by the Bm86 (Tick-GARD) vaccine. *Experimental & Applied Acarology*, 29. 141-149.

Plans, S. & Plan, S. (2016) AU-IBAR Current Publications. *Bulletin of Animal Health & Production in Africa*.

Pohlit, A.M., Rezende, A.R., Lopes Baldin, E.L., Lopes, N.P., de Andrade Neto, Valter Ferreira, (2011) Plant extracts, isolated phytochemicals, and plant-derived agents which are lethal to arthropod vectors of human tropical diseases-A review. *Planta Medica-Natural Products &. Medicinal Plant Research* 77. 618.

Politi, F.A.S., Figueira, G.M., Araújo, A.M., Sampieri, B.R., Mathias, M.I.C., Szabó, M.P.J., Bechara, G.H., dos Santos, L.C., Vilegas W. & Pietro, R.C.L.R. (2012) Acaricidal activity of ethanolic extract from aerial parts of *Tagetes patula* L. (Asteraceae) against larvae and engorged adult females of *Rhipicephalus sanguineus* (Latreille, 1806). *Parasite & Vectors,* 5. 295-306.

Politi, F.A., Queiroz-Fernandes, G.M., Rodrigues, E.R., Freitas, J.A. & Pietro, R.C. (2016) Antifungal, antiradical and cytotoxic activities of extractives obtained from *Tagetes patula* L. (Asteraceae), a potential acaricide plant species. *Microbial Pathogenesis*, 95. 15-20.

Popara, M., Villar, M., Mateos-Hernández, L., de Mera, Isabel G Fernández, Marina, A., del Valle, M., Almazán, C., Domingos, A. & de la Fuente, J. (2013) Lesser protein degradation machinery correlates with higher BM86 tick vaccine efficacy in *Rhipicephalus annulatus* when compared to *Rhipicephalus microplus*. *Vaccine*, 31. 4728-4735.

Pottorff, L.P. (2010) Some pesticides permitted in organic gardening. *Colorado State University Cooperative Extension*. http://www. colostate. edu/Dept/CoopExt/4DMG/VegFruit/organic. htm.

Prates, H.T., Oliveira, A.B., Leite, R.C. & Craveiro, A.A. (1993) Ativadade carrapaticida e composicao quimica do oleo essencial do capim-goidura. *Pesquisa Agropecuária* Brasileira, 28. 621-625.

Preston, S.G., Majtán, J., Kouremenou, C., Rysnik, O., Burger, L.F., Cruz, A.C., Guzman, M.C., Nunn, M.A., Paesen, G.C. & Nuttall, P.A. (2013) Novel immunomodulators from hard ticks selectively reprogramme human dendritic cell responses. *PLoS Pathog*, 9. e1003450.

Pretorius, A.M., Jensenius, M., Clarke, F. & Ringertz, S.H. (2003) Repellent efficacy of DEET and KBR 3023 against *Amblyomma hebraeum* (Acari: Ixodidae). *Journal of Medical Entomology,* 40(2). 245-248.

Qi, S.H., Wu, D.G., Zhang, S. & Luo, X.D. (2004) Constituents of *Carapa guianensis* Aubl. (Meliaceae). *Die Pharmazie*, 59(6). 488-490.

Quintans-Júnior, L.J., Souza, T.T., Leite, B.S., Lessa, N.M.N., Bonjardim, L.R., Santos, M.R.V., Alves, P.B., Blank, A.F. & Antoniolli, A.R. (2008) Phytochemical screening and anticonvulsant activity of *Cymbopogon winterianus* Jowitt (Poaceae) leaf essential oil in rodents. *Phytomedicine,* 15(8). 619-624.

Rajakumar, G., Rahuman, A. A., Jayaseelan, C., Santhoshkumar, T., Marimuthu, S., Kamaraj, C. & Jose, S. (2014) *Solanum trilobatum* extract-mediated synthesis of titanium dioxide nanoparticles to control *Pediculus humanus capitis,*

Hyalommaanatolicum anatolicum and *Anopheles subpictus*. *Parasitology Research,* 113(2). 469-479.

Rajput, Z.I., Hu, S., Chen, W., Arijo, A.G. & Xiao, C. (2006) Importance of ticks and their chemical and immunological control in livestock. *Journal of Zhejiang University Science B*, 7. 912-921.

Ramírez, C., Ibarra, F., Pérez, H.I., Manjarrez, N., Salgado, H.J., & Ortega, L. (2016) Assessment and determination of LC 50 of carvacrol and salicylic acid analogues with acaricide activity in larvae and adult ticks of *Rhipicephalus (Boophilus) microplus*. *Parasite Epidemiology & Control*, 1(2). 72-77.

Rao, Y.K., Lien, H., Lin, Y., Hsu, Y., Yeh, C., Chen, C., Lai, C. & Tzeng, Y. (2012) Antibacterial activities of *Anisomeles indica* constituents and their inhibition effect on *Helicobacter pylori*-induced inflammation in human gastric epithelial cells. *Food Chemistry,* 132. 780-787.

Raso, G.M., Meli, R., Di Carlo, G., Pacilio, M. & Di Carlo, R. (2001) Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A. 1. *Life Sciences*, 68. 921-931.

Rattan, R.S. (2010) Mechanism of action of insecticidal secondary metabolites of plant origin. *Crop Protection*, 29. 913-920.

Ravindran, R., Chithra, N.D., Deepa, P.E., Ajithkumar, K.G., Chandrasekhar, L., Sreelekha, K., Nair, S.N., Juliet, S. & Ghosh, S. (2017) *In vitro* effects of caffeic acid, nortriptyline, precocene I and quercetin against *Rhipicephalus annulatus* (Acari: Ixodidae). *Experimental & Applied Acarology*, 71. 183-193.

Ravindran, R., Amithamol, K.K., Sunil, A.R., Soorya, V.C., Nair, S.N., Juliet, S., Arunkumar, N.S., Kumar, K.G.A. & Ghosh, S. (2014) Determination of LC50 and LC95 values of fipronil against *Haemaphysalis bispinosa* based on adult immersion test. *Journal of Parasitic Diseases,* 38. 132-134.

Ravindran, R., Juliet, S., Kumar, K.A., Sunil, A., Nair, S.N., Amithamol, K., Rawat, A.K.S. & Ghosh, S. (2011a) Toxic effects of various solvents against *Rhipicephalus (Boophilus) annulatus. Ticks & Tick-borne Diseases*, 2. 160-162.

Ravindran, R., Juliet, S., Sunil, A., Kumar, K.A., Nair, S.N., Amithamol, K., Shynu, M., Rawat, A.K.S. & Ghosh, S. (2011b) Eclosion blocking effect of ethanolic extract of *Leucas aspera* (Lamiaceae) on *Rhipicephalus (Boophilus) annulatus*. *Veterinary Parasitology*, 179. 287-290.

Raymond, V. & Sattelle, D.B. (2002) Novel animal-health drug targets from ligand-gated chloride channels. *Nature Reviews Drug Discovery*, 1. 427-436.

Reeves, W.K., Loftis, A.D., Nicholson, W.L. & Czarkowski, A.G. (2008) The first report of human illness associated with the Panola Mountain Ehrlichia species: a case report. *Journal of Medical Case Reports*, 2. 1.

Regnault-Roger, C. (2013) Essential oils in insect control. *In: Anonymous Natural Products*. Springer, pp. 4087-4107.

Reuter, H.D. & Sendi, A. (1994) *Allium sativum* and *Allium ursinum*. Chemistry, pharmacology and medicinal applications. *Economic & Medicinal Plant Research,* 6. 56-113.

Ribeiro, V.L.S., dos Santos, J.C., Bordignon, S.A., Apel, M.A., Henriques, A.T. & von Poser, G.L. (2010) Acaricidal properties of the essential oil from *Hesperozygis ringens* (Lamiaceae) on the cattle tick *Rhiphicephalus (Boophilus) microplus. Bioresource Technology,* 101. 2506-2509.

Ribeiro, V.L.S., dos Santos, J.C., Martins, J.R., Schripsema, J., Siqueira, I.R., von Poser, G.L. & Apel M.A. (2011) Acaricidal properties of the essential oil and precocene II obtained from *Calea serrata* (Asteraceae) on the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Veterinary Parasitology,* 179. 195-198.

Ribeiro, V.L.S., Toigo, E., Bordignon, S.A.L., Goncalves, K. & von Poser, G. (2007). Acaricidal properties of extracts of the aerial parts of *Hypericum polyanthemum* on the cattle tick *Boophilus microplus. Veterinary Parasitology,* 147(1/2). 199-203.

Ribeiro, V.L.S., Avacini, C., Goncalves, K. & Poser, G.V. (2008) Acaricidal activity of *Calea serrata* (Asteraceae) on *Boophilus microplus* and *Rhipicephalus sanguineus. Veterinary Parasitology,* 151. 351-354.

Ribeiro, D., Freitas, M., Tomé, S.M., Silva, A.M., Laufer, S., Lima, J.L. & Fernandes, E. (2015) Flavonoids inhibit COX-1 and COX-2 enzymes and cytokine/chemokine production in human whole blood. *Inflammation*, 38. 858-870.

Ribeiro, D., Freitas, M., Tomé, S.M., Silva, A.M., Porto, G., Cabrita, E.J., Marques, M.M.B. & Fernandes, E. (2014) Inhibition of LOX by flavonoids: a structure-activity relationship study. *European Journal of Medicinal Chemistry*, 72. 137-145.

Ribeiro, J.M. & Francischetti, I.M. (2003) Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annual Review of Entomology*, 48. 73-88.

Ribeiro, J.C., Endris, T.M. & Endris, R. (1991) Saliva of the soft tick, *Ornithodoros moubata,* contains anti-platelet and apyrase activities. *Comparative Biochemistry & Physiology Part A: Physiology*, 100. 109-112.

Ribeiro, J.M., Makoul, G.T., Levine, J., Robinson, D.R. & Spielman, A. (1985) Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, *Ixodes dammini*. *The Journal of Experimental Medicine*, 161. 332-344.

Righi, A.A., Motta, L.B., Klafke, G.M., Pohl, P.C., Furlan, C.M., Santos, D.Y., Salatino, M.L., Negri, G., Labruna, M.B. & Salatino, A. (2013). Chemical composition and efficacy of dichloromethane extract of *Croton sphaerogynus* Baill. (Euphorbiaceae) against the cattle tick *Rhipicephalus microplus* (Acari: Ixodidae). *Veterinary Parasitology,* 192. 292-295.

Rios, M.Y. (2005) Terpenes, coumarins and flavones from *Acacia pennatula*. *Chemistry of Natural Compounds,* 41(3). 297-298.

Rocha, L., Marston, A., Kaplan, M.A.C., Stoeckli-Evans, H., Thull, U., Testa, B. & Hostettmann, K. (1994) An antifungal gamma-pyroneand xanthones with monoamine oxidase inhibitory activity from *Hypericum brasiliense*. *Phytochemistry,* 36. 1381-1385.

Rodrigues, R.V., Lanznaster, D., Longhi Balbinot, D.T., Gadotti, V.D.M., Facundo, V.A. & Santos, A.R.S. (2009) Antinociceptive effect of crude extract, fractions and three alkaloids obtained from fruits of *Piper tuberculatum*. *Biological & Pharmeutical Bulletin,* 32(10). 1809-1812.

Rodríguez-Valle, M., Taoufik, A., Valdés, M., Montero, C., Hassan, I., Hassan, S.M., Jongejan, F. & de la Fuente, J. (2012) Efficacy of *Rhipicephalus (Boophilus) microplus* Bm86 against *Hyalomma dromedarii* and *Amblyomma cajennense* tick infestations in camels and cattle. *Vaccine*, 30. 3453-3458.

Rodriguez-Vivas, R., Ojeda-Chi, M., Trinidad-Martinez, I. & de León, A.P. (2017) First documentation of ivermectin resistance in *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae). *Veterinary Parasitology*, 233. 9-13.

Roma, G.C., Mathias, M.I.C., De Faria, A.U., De Oliveira, P.R., Furquim, K.C.S. & Bechara, G.H. (2013). Morphological and cytochemical changes in synganglion of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) female ticks from exposure of andiroba oil (*Carapa guianensis*). *Microscopy Research & Technique,* 76(7). 687-696.

Ronald, J.R. & Acton, N. (1987) Isolation of arteannuic acid from *Artemisia annua. Planta Medica,* 53. 501–502.

Rosado-Aguilar, J.A., Aguilar-Caballero, A., Rodriguez-Vivas, R.I., Borges-Argaez, R., Garcia-Vazquez, Z. & Mendez-Gonzalez, M. (2010) Acaricidal activity of extracts from *Petiveria alliacea* (Phytolaccaceae) against the cattle tick, *Rhipicephalus (Boophilus) microplus* (Acari: ixodidae). *Veterinary Parasitology,* 168(3). 299-303.

Rosario-Cruz, R. & Domínguez-García, D.I. (2016) Biological and biochemical bases of pesticides resistance in *Rhipicephalus (Boophilus) microplus.* http:dx.doi.org/10.5772/61839.

Rota, M. C., Herrera, A., Martínez, R. M., Sotomayor, J. A. & Jordán, M.J. (2008) Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. *Food Control,* 19(7). 681-687.

Rotondo, A., Serio, S. & Mule, F. (2009) Gastric relaxation induced by apigenin and quercetin: Analysis of the mechanism of action. *Life Sciences,* 85(1-2). 85-90.

Rowlands, R.J., Michaud, V., Heath, L.E., Hutchings, G., Oura, C., Vosloo, W., Dwarka, R.M., Onashvili, T., Albina, E. & Dixon, L.K. (2008) African swine fever virus isolate, Georgia, 2007. http://hdl.handle.net/2263/10192.

Roy-Smith, F. (1975). Amitraz – Australian field trials against the cattle tick (*Boophilus microplus*). *Proceedings of the 8th British Insecticide and Fungicide Conference* pp. 565-571.

Ruiz-Vásquez, L., Olmeda, A.S., Zúñiga, G., Villarroel, L., Echeverri, L.F., González-Coloma, A. & Reina, M. (2016) Insect antifeedant and ixodicidal compounds from *Senecio adenotrichius*. *Chemistry & Biodiversity* http:/dx.doi.org/10.1002/cbdv.201600155.

Runyoro, D., Ngassapa, O., Vagionas, K., Aligiannis, N., Graikou, K. & Chinou, I. (2010) Chemical composition and antimicrobial activity of the essential oils of four *Ocimum* species growing in Tanzania. *Food Chemistry,* 119. 311-316.

Russell, M. & Southwell, I. (2002) Monoterpenoid accumulation in *Melaleuca alternifolia* seedlings. *Phytochemistry,* 59(7).709-716.

Sabatini, G.A., Kemp, D.H., Hughes, S., Nari, A. & Hansen, J. (2001). Tests to determine the LC50 and discriminating doses for macrocystic lactones against the cattle tick, *Boophilus microplus. Veterinary Parasitology,* 95. 53-63.

Sahu, R. K., Kar, M. & Routray, R. (2013) DPPH free radical scavenging activity of some leafy vegetables used by tribals of Odisha. *Indian Journal of Medicinal Plants,* 1(4). 21-27.

Said, M.B., Galai, Y., Canales, M., Nijhof, A.M., Mhadhbi, M., Jedidi, M., de la Fuente, J. & Darghouth, M.A. (2012) Hd86, the Bm86 tick protein ortholog in *Hyalomma scupense* (syn. *H. detritum*): expression in *Pichia pastoris* and analysis of nucleotides and amino acids sequences variations prior to vaccination trials. *Veterinary Parasitology*, 183. 215-223.

Salem, A.Z.M., Salem, M.Z.M, Gonzalez-Ronuillo, M., Camacho, L.M. & Cipriano, M. (2011) Major chemical constituents of *Leucaena leucocephala* and *Salix babylonica* leaf extract. *Journal of Tropical Agriculture,* 49(1-2). 95-98.

Sangamnatdej, S., Paesen, G., Slovak, M. & Nuttall, P. (2002) A high affinity serotonin-and histamine-binding lipocalin from tick saliva. *Insect Molecular Biology*, 11. 79-86.

Santhoshkumar, T., Rahuman, A.A., Bagavan, A., Marimuthu, S., Jayaseelan, C., Kirthi, A.V., Kamaraj, C., Rajakumar, G., Zahir, A.A. & Elango, G. (2012) Evaluation of stem aqueous extract and synthesized silver nanoparticles using *Cissus quadrangularis* against *Hippobosca maculata* and *Rhipicephalus (Boophilus) microplus*. *Experimental Parasitology*, 132. 156-165.

Saponara, S., Testai, L., Lozzi, D., Martinotti, E., Martelli, A., Chericoni, S., Sparagli, G., Fusi, F. & Calderone, V. (2006) (+/-)- Naringenin as large conductance Ca^{2+}-activated $K^{+}(Bk_{Ca})$ channel opener in vacsular smooth muscle cells. *British Journal of Pharmacology,* 149(8). 1013-1021.

Sastraruji, T., Jatisatienr, A., Pyne, S. G., Ung, A. T., Lie, W. & Williams, M.C. (2005) Phytochemical studies on *Stemona* plants: isolation of stemofoline alkaloids. *Journal of Natural Products,* 68(12). 1763-1767.

Sato, A., Asano, K. & Sato, T. (1990) The chemical composition of *Citrus hystrix* DC (Swangi). *Journal of Essential Oils Research* 2(4). 179-183.

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B. & Touhara, K. (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature,* 452 (7190). 1002-1006.

Sauer, J., McSwain, J., Bowman, A. & Essenberg, R. (1995) Tick salivary gland physiology. *Annual Review of Entomology,* 40(1). 245-267.

Sayyed, A. & Shah, M. (2014) Phytochemistry, pharmacological and traditional uses of *Datura stramonium* L. *Journal of Pharmacognosy & Phytochemistry*,2(5). 123-125.

Schmeda-Hirschmann, G. & de Arias, A.R. (1992) A screening method for natural products on triatomine bugs. *Phytotherapy Research*, 6. 68-73.

Schreck, C., Fish, D. & McGovern, T. (1995) Activity of repellents applied to skin for protection against *Amblyomma americanum* and *Ixodes scapularis* ticks (Acari: Ixodidae), *Journal of the American Mosquito Control Association-Mosquito News,* 11(1). 136-140.

Schwantes, U., Dautel, H. & Jung, G. (2008) Prevention of infectious tick-borne diseases in humans: Comparative studies of the repellency of different dodecanoic acid-formulations against *Ixodes ricinus* ticks (Acari: Ixodidae). *Parasites & Vectors,* 1(1). 1.

Shafer, T.J., Meyer, D.A. & Crofton, K.M. (2005) Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environmental Health Perspectives,* 123-136.

Shah, K.A., Patel, M.B., Patel, R.J. & Parmar, P.K. (2010) *Mangifera indica* (mango). *Pharmacognosy Reviews,* 4(7). 42.

Shahein, Y.E., Abouelella, A.M., Hussein, N.A., Hamed, R.R., El-Hakim, A.E., Abdel-Shafy, S. & Tork, S.E. (2013) Identification of four novel *Rhipicephalus annulatus* upregulated salivary gland proteins as candidate vaccines. *The Protein Journal*, 32. 392-398.

Shahjahan, M., Sabitha, K., Jainu, M. & Devi, C.S. (2004) Effect of *Solanum trilobatum* against carbon tetrachloride induced hepatic damage in albino rats. *Indian Journal of Medical Research,* 120. 194.

Shapiro, E.D. (2014) Lyme disease. *New England Journal of Medicine*, 370. 1724-1731.

Shaw, R. (1966) Culture of an organophosphorus-resistant strain of *Boophilus microplus* (Can.) and an assessment of its resistance spectrum. *Bulletin of Entomological Research,* 56(3). 389-405.

Shaw, S.E., Day, M.J., Birtles, R.J. & Breitschwerdt, E.B. (2001) Tick-borne infectious diseases of dogs. *Trends in Parasitology*, 17. 74-80.

Shaw, S. & Day, M. (2005). Arthropod-borne infectious diseases of the dog and cat. CRC Press. pp 110-119.

Shyma, K.P., Kumar, S., Sharma, A.K., Ray, D.D. & Ghosh, S. (2012) Acaricidal resistance status in Indian isolates of *Hyalomma anatolicum*. *Experimental & Applied Acarology*, 58. 471-481.

Shyma, K.P., Gupta, J.P., Ghosh, S., Patel, K.K. & Singh, V. (2014) Acaricidal effect of herbal extracts against cattle tick *Rhipicephalus (Boophilus) microplus* using *in vitro* studies. *Parasitology Research*, 113(5). 1919-1926.

Silbering, A.F. & Benton, R. (2010) Ionotropic and metabotropic mechanisms in chemoreception: 'chance or design'? *EMBO Reports,* 11. 173-179.

Silva-Aguayo, G. (2006) Botanical insecticides. *Radcliffes IPM World Textbook*. University of Minnesota.

Silva, W.C., de Souza Martins, João Ricardo, de Souza, Hellen Emília Menezes, Heinzen, H., Cesio, M.V., Mato, M., Albrecht, F., de Azevedo, J.L. & de Barros, N.M. (2009) Toxicity of *Piper aduncum* L. (Piperales: Piperaceae) from the Amazon forest for the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Veterinary Parasitology*, 164. 267-274.

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J., Chagnon, M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C. & Girolami, V. (2015) Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental Science & Pollution Research*, 22. 5-34.

Singh, N.K., Vemu, B., Nandi, A., Singh, H., Kumar, R. & Dumka, V. (2014) Acaricidal activity of *Cymbopogon winterianus, Vitex negundo* and *Withania somnifera* against synthetic pyrethroid resistant *Rhipicephalus (Boophilus) microplus*. *Parasitology Research,* 113. 341-350.

Singh, N.K., Singh, H., Singh, N.K. & Rath, S.S. (2016) Multiple mutations in the acetylcholinesterase 3 gene associated with organophosphate resistance in

Rhipicephalus (Boophilus) microplus ticks from Punjab, India. *Veterinary Parasitology*, 216. 108-117.

Singh, N. & Randhawa, S. (2013) Chemotherapeutics for control and treatment of ectoparasites in companion animals. *Intas Polivet*, 14. 257-263.

Six, R.H., Geurden, T., Packianathan, R., Colgan, S., Everett, W.R., Grace, S., Hodge, A., Mahabir, S.P., Myers, M.R. & Slootmans, N. (2016) Evaluation of the effectiveness of a novel oral formulation of sarolaner (Simparica™) for the treatment and control of fleas on dogs. *Veterinary Parasitology*, 222. 18-22.

Skoula, M., Gotsiou, P., Naxakis, G. & Johnson, C.B. (1999) A chemosystematic investigation on the mono-and sesquiterpenoids in the genus *Origanum* (Labiatae). *Phytochemistry*, 52(4). 649-657.

Soares, S.F., Borges, L.M.F., de Sousa Braga, R., Ferreira, L.L., Louly, C.C.B., Tresvenzol, L.M.F., de Paula, J.R. & Ferri, P.H. (2010) Repellent activity of plant-derived compounds against *Amblyomma cajennense* (Acari: Ixodidae) nymphs. *Veterinary Parasitology*, 167. 67-73.

Sobel, J. (2005) Botulism. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 41. 1167-1173.

Soberanes, C.N., Santamaria, V.M., Fragoso, S.H. & Garcia, V.Z. (2002) Primer caso de Resistencia al amitraz en la garrapata del Ganado *Boophilus microplus* en Mexico. *Tecnica Pecuaria en Mexico*, 40. 81-92.

Sonenshine, D. (1993) The Biology of ticks, vol. II. Oxford University Press, New York

Sonenshine, D., Lane, R. & Nicholson, W. (2002) Ticks (Ixodida). *Medical and Veterinary Entomology*. 517-558.

Sonenshine, D.E. & Roe, R.M. (2013) Overview: ticks, people and animals. In: Sonenshine DE, Roe RM (eds) The biology of ticks, vol 1. Oxford University Press, New York.

Sonenshine, D., Roe, R., Sonenshine, D. & Roe, R. (2014) Chapter 1. Overview. *Biology of Ticks*, 1. 3-16.

Soto-Trejo, F., Schilling, E.E., Solórzano, S., Oyama, K., Lira, R., & Dávila, P. (2015) Phylogenetic relationships in the genus *Florestina* (Asteraceae, Bahieae). *Plant Systematics & Evolution*, 301(9). 2147-2160.

Souza, A., Veiga, L., Sartor, A., Bellato, V., Nunes, A. & Cardoso, H. (2006) Teste carrapacida por imersão de larvas de *Boophilus microplus*: avaliação em cipermetrina e amitraz. *Proceedings of 14 Congresso Brasileiro de Parasitologia Veterinária, Ribeirão Preto, Brazil*, pp. 3.

Sparks, T.C., Crouse, G.D., & Durst, G. (2001) Natural products as insecticides: the biology, biochemistry and quantitative structure–activity relationships of spinosyns and spinosoids. *Pest Management Science*, *57*(10). 896-905.

Sparks, T.C., Hahn, D.R. & Garizi, N.V. (2016) Natural products, their derivatives, mimics and synthetic equivalents: role in agrochemical discovery. *Pest Management Science*, 73(4). 700-715.

Spickett, A.M. (1998) Acaricides and resistance. *Veterinary Ectoparasitology and Protozoology,* 1. 1-13.

Srivastava, R., Ghosh, S., Mandal, D.B., Azhahianambi, P., Singhal, P.S., Pandey, N.N. & Swarup, D. (2008) Efficacy of *Azadirachta indica* against *Boophilus microplus. Parasitology Research*, 104.149-153.

Stachurski, F. & Adakal, H. (2010) Exploiting the heterogeneous drop-off rhythm of *Amblyomma variegatum* nymphs to reduce pasture infestation by adult ticks. *Parasitology*, 137. 1129.

Stanford, G.D., Baker, J.A.F., Ratley, C.V. & Taylor, R.J. (1981) The development of a stabilized amitraz cattle dip for control of single and multi-host ticks and their resistant strains in South Africa. *In Whitehead, G.B., Gibson, J.D. (Eds), Proceedings of a Conference on Tick Biology and Control*, Rhodes University, Grahamstown, South Africa pp. 143-181.

Steenkamp, Y. & Smith, G. (2006) Introduction to a checklist of South African plants. *A checklist of South African plants.Southern African Botanical Diversity Network Report*, 41.

Steinback, C., Spitzer, V., Starosta, M. & von Poser G. (1997) Identification of two chromenes from *Calea serrata* by semiautomatic structure elucidation. *Journal of Natural Products*, 60. 627-628.

Stone, B. & Haydock, K. (1962) A method for measuring the acaricide-susceptibility of the cattle tick *Boophilus microplus* (Can.). *Bulletin of Entomological Research*, 53. 563-578.

Strickman, D. (2006) PMD (p-menthane-3, 8-diol) and quwenling. *Insect repellents. Principles, methods and uses.CRC Press, Boca Raton,* pp. 347-351.

Stubbs, V.K., Wilshire, C. & Webber, G. (1982) Cyhalothrin – a novel acaricidal and insecticidal synthetic pyrethroid for the control of the cattle tick (*Boophilus microplus*) and the buffalo fly (*Haematobia irritans exigua*). *Australian Veterinary Journal,* 59.152-155.

Sultana, H.S., Ali, M. & Panda, B.P. (2012) Influence of volatile constituents of fruit peels of *Citrus reticulata* Blanco on clinically isolated pathogenic microorganisms under *in-vitro*. *Asian Pacific Journal of Tropical Biomedicine*, S1299-1302.

Sun, M., Ren, Q., Liu, Z., Guan, G., Gou, H., Ma, M., Li, Y., Liu, A., Yang, J. & Yin, H. (2011) *Beauveria bassiana*: Synergistic effect with acaricides against the tick *Hyalomma anatolicum anatolicum* (Acari: Ixodidae). *Experimental Parasitology*, 128. 192-195.

Tabanca, N., Wang, M., Avonto, C., Chittiboyina, A.G., Parcher, J.F., Carroll, J.F., Kramer, M. & Khan, I.A. (2013) Bioactivity-guided investigation of geranium essential oils as natural tick repellents. *Journal of Agricultural & Food Chemistry,* 61(17). 4101-4107.

Tahara, S., Moriyama, M., Ingham, J.L. & Mizutani, J. (1993) Diverse oxygenated isoflavonoids from *Piscidia erythrina*. *Phytochemistry,* 34(1). 303-315.

Tajeri, S., Razmi, G., & Haghparast, A. (2016) Establishment of an artificial tick feeding system to study *Theileria lestoquardi* infection. *PloS one*, 11(12). e0169053.

Tak, J.H., & Isman, M.B. 2015 Enhanced cuticular penetration as the mechanism for synergy of insecticidal constituents of rosemary essential oil in *Trichoplusia ni*. *Scientific Reports*, *5*. doi:10.1038/srep12690.

Tanwer, B.S. & Vijaguergia, R. (2010) Phytochemical evaluation and molluscicidal activity of *Andrgraphis paniculata*. *Herba Polonica*, 56(4). 71-77.

Temeyer, K.B. & Tuckow, A.P. (2016) Tick salivary cholinesterase: A probable immunomodulator of host-parasite Interactions. *Journal of Medical Entomology*. 53(3). 500-504.

Tereschuk, M.L., Riera, M.V., Castro, G.R. & Abdala, L.R. (1997) Antimicrobial activity of flavonoids from leaves of *Tagetes minuta*. *Journal of Ethnopharmacology*, 56. 227-232.

Terezan, A.P., Rossi, R.A., Almeida, R.N., Freitas, T.G., Fernandes, J.B., Silva, M., Vieira, P.C., Bueno, O.C., Pagnocca, F.C. & Pirani, J.R. (2010) Activities of extracts and compounds from *Spiranthera odoratissima* St. Hil.(Rutaceae) in leaf-cutting ants and their symbiotic fungus. *Journal of the Brazilian Chemical Society*, 21. 882-886.

Theoret, C.L. (2004) Update on wound repair. *Clinical Techniques in Equine Practice*, 3. 110-122.

Thorsell, W., Mikiver, A. & Tunon, H. (2006) Repelling properties of some plant materials on the tick *Ixodes ricinus* L. *Phytomedicine,* 13. 132-134.

Thullner, F., Willadsen, P. & Kemp, D. (2007) Acaricide rotation strategy for managing resistance in the tick *Rhipicephalus (Boophilus) microplus* (Acarina: Ixodidae): laboratory experiment with a field strain from Costa Rica. *Journal of Medical Entomology*, 44. 817-821.

Tilly, K., Rosa, P.A. & Stewart, P.E. (2008) Biology of infection with *Borrelia burgdorferi. Infectious Disease Clinics of North America*, 22. 217-234.

Trentelman, J.J., Kleuskens, J.A., van de Crommert, J., & Schetters, T.P. (2017) A new method for *in vitro* feeding of *Rhipicephalus australis (*formerly *Rhipicephalus microplus*) larvae: a valuable tool for tick vaccine development. *Parasites & Vectors*, 10(1). 153.

Tsatsaris, A., Chochlakis, D., Papadopoulos, B., Petsa, A., Georgalis, L., Angelakis, E., Ioannou, I., Tselentis, Y. & Psaroulaki, A.(2016) Species composition, distribution, ecological preference and host association of ticks in Cyprus. *Experimental & Applied Acarology*, 70(4). 523-542.

Tunón, H., Thorsell, W., Mikiver, A. & Malander, I. (2006). Arthropod repellency, especially tick (*Ixodes ricinus*), exerted by extract from *Artemisia abrotanum* and essential oil from flowers of *Dianthus caryophyllum. Fitoterapia*, 77. 257-261.

Turner, W. C., Küsters, M., Versfeld, W., & Horak, I. G. (2017) Ixodid tick diversity on wild mammals, birds and reptiles in and around Etosha National Park, Namibia. *African Journal of Ecology*. doi:10.1111/aje.12369.

Ullah, S., Khan, M.N., Sajid, M.S., Iqbal, Z. & Muhammad, G. (2015) Comparative efficacies of *Curcuma longa, Citrullus colocynthis* and *Peganum harmala* against *Rhipicephalus microplus* through Modified Larval Immersion Test. *International Journal of Agriculture & Biology*, 17(1). 216-220.

Umer, S., Tekewe, A. & Kebede, N. (2013) Antidiarrhoeal and antimicrobial activity of *Calpurnia aurea* leaf extract. *BMC Complementary & Alternative Medicine*, 13. 21.

Valente, P.P., Amorin, J.M., Castilho, R.O., Leite, R.C. & Ribeiro, M.F.B. (2014) *In vitro* acaricidal efficacy of plant extracts from Brazilian flora and isolated substances against *Rhipicephalus microplus* (Acari:Ixodidae). *Parasitology Research*, 113. 417-423.

van de Locht, A., Stubbs, M.T., Bode, W., Friedrich, T., Bollschweiler, C., Hoffken, W. & Huber, R. (1996) The ornithodorin-thrombin crystal structure, a key to the TAP enigma? *The EMBO Journal*, 15. 6011-6017.

Van der Merwe, D., Swan, G. & Botha, C. (2001) Use of ethnoveterinary medicinal plants in cattle by Setswana-speaking people in the Madikwe area of the North West Province of South Africa. *Journal of the South African Veterinary Association*, 72. 189-196.

Van Leeuwen, T., Vontas, J., Tsagkarakou, A. & Tirry, L. (2009). Mechanisms of acaricide resistance in the two-spotted spider mite *Tetranychus urticae. In Biorational Control of Arthropod Pests.* Anonymous Springer pp. 347-393.

van Wieren, S.E., Braks, M.A. & Lahr, J. (2016). Effectiveness and environmental hazards of acaricides applied to large mammals for tick control. *In Ecology and Prevention of Lyme Borreliosis.* Anonymous pp. 75-89. Wageningen Academic Publishers.

van Wyk, R.D., Baron, S. & Maritz-Olivier, C. (2016) An integrative approach to understanding pyrethroid resistance in *Rhipicephalus microplus* and *R. decoloratus* ticks. *Ticks & Tick-borne Diseases*, 7. 586-594.

Van Langenhove, L. & Paul, R. (2014) Insect repellent finishes for textiles, Functional finishes for textiles: improving, comfort, performance and protection. *Elsevier, Cambridge*, 333-360.

Varma, J. & Dubey, N. (1999) Prospectives of botanical and microbial products as pesticides of tomorrow. *Current Science-Bangalore*, 76. 172-178.

Vasconcelos, V.O., Martins, M.A.D., de Oliveira, N.J. & Duarte, E.R. (2014) Effect of ethanolic extract of *Capsicum frutescens* L. on adult female of *Rhipicephalus microplus* (Ixodidae). *Parasitology Research,* 113(4). 1389-1394.

Veeramani, V., Sakthivelkumar, S., Tamilarasan, K., Aisha, S.O. & Janarthanan, S. (2014) Acaricidal activity of *Ocimum basilicum* and *Spilanthes acmella* against the ectoparasitic tick, *Rhipicephalus (Boophilus) microplus* (Arachinida: Ixodidae). *Tropical Biomedicine*, 31(3). 414-421.

Vendramini, M.C.R., Mathias, M.I.C., De Faria, A.U., Furquim, K.C.S., De Souza, L.P., Bechara, G.H. & Roma, G.C. (2012) Action of andiroba oil (*Carapa guianensis)* on *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females:

Morphophysiological evaluation of reproductive system. *Microscopy Research & Technique*, 75. 1745-1754.

Versteeg, H.H., Heemskerk, J.W., Levi, M. & Reitsma, P.H. (2013) New fundamentals in hemostasis. *Physiological Reviews*, 93. 327-358.

Vijay, K.P., Laxman, B.C., Balasaheb, S.R., Vuvraj, N.R. & Janardhan, P.M. (2013) Pharmacognistic, physicochemical and phytochemical investigation of *Tagetes erecta* Linn. Flowers (Asteraceae). *Journal of Biological Sciences,* 1(1). 21-24.

von Son-de Fernex, E., Alonzo-Diaz, M.A., Mendoza-de Gives, P., Valles-de la Mora, B., Gonzalez-Cortazar, M. & Zamilpa, A. (2015) Elucidation of *Leucaena leucocephala* antihelminthic-like phytochemicals and the ultrastructural damage generated to eggs of *Cooperia* species. *Veterinary Parasitology*, 214(1-2). 89-95.

Wächter, G.A., Hoffmann, J.J., Furbacher, T., Blake, M.E. & Timmermann, B.N. (1999) Antibacterial and antifungal flavanones from *Eysenhardtia texana*. *Phytochemistry*, 52. 1469-1471.

Wagate, C.G., Mbaria, J.M., Gakuya, D.W., Nanyingi, M.O., Kareru, P., Njuguna, A., Gitahi, N., Macharia, J.K. & Njonge, F.K. (2010) Screening of some Kenyan medicinal plants for antibacterial activity. *Phytotherapy Research*, 24. 150-153.

Waladde, S. & Rice, M. (1982) The sensory basis of tick feeding behaviour. *The Physiology of Ticks*. 71-118.

Walker, A.R., Bouattour, A., Camicas, J.L., Estrada-Pena, Horak, I.G., Latif, A.A., Pegram, R.G. & Preston, P.M. (2003). Ticks of domestic animals in Africa: a guide to identification of species. *Biosciences Reports* Edinburgh pp 44-221.

Wall, R. & Shearer, D. (2001) Veterinary ectoparasites: Biology, Pathology and Control, second ed.Blackwell Science, London.

Wang, S. & Li, Y. (2005) Traditional chinese medicine. Complementary and alternative medicine therapies for epilepsy. *Devinsky, O., Schacter, S., Pacia, S. (Eds). Demos Medical Publishing,* 386 Park Avenue South, New York, NY 10016.

Ware, G.W. (2000) The Pesticide Book, 5th edn. Thomson Publications, Fresno, California.

Ware, G.W. & Whitacre, D.M. (2004) An introduction to insecticides. *The Pesticide Book.Meister Pub.Willoughby, Ohio*.

Waxman, L. & Connolly, T.M. (1993) Isolation of an inhibitor selective for collagen-stimulated platelet aggregation from the soft tick *Ornithodoros moubata*. *The Journal of Biological Chemistry*, 268. 5445-5449.

Waxman, L., Smith, D.E., Arcuri, K.E. & Vlasuk, G.P. (1990) Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. *Science,* 248. 593-596.

Weber, T. & Selzer, P.M. (2016) Isoxazolines: a novel chemotype highly effective on ectoparasites. *ChemMedChem*, 11. 270-276.

Wei, Q.Q., Guo, L.P., Wang, A.D., Mu, L.M., Zhang, K., Chen, C.F., Zhang, W.J. & Wang, Y. Z. (2015) The first detection of *Rickettsia aeschlimannii* and *Rickettsia massiliae* in *Rhipicephalus turanicus* ticks, in northwest China. *Parasites & Vectors*, 8(1). 631.

Wellington, K.W., Leboho, T., Sakong, B.M., Adenubi, O.T., Eloff, J.N. & Fouche, G. (2017) Further studies on South African plants: Acaricidal activity of organic plant extracts against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Veterinary Parasitology*, 234. 10-12.

Wharton, R.H. (1967) Acaricide resistance and cattle tick control. *Australian Veterinary Journal*, 43(9). 394-398.

Wharton, R.H. (1974) Ticks with special emphasis on *Boophilus microplus. In Pal, R., Wharton, R.H. (Eds). Control of anthropods of medical and veterinary importance*. New York, Plenum Publishing Corporation pp 36-52.

Wicher, D. (2015) Chapter Two-olfactory signaling in insects. *Progress in Molecular Biology & Translational Science*, 130. 37-54.

Wikel, S. K. (2014) Tick-host interactions. *Biology of ticks*, *2*, 88-128.

Willi, B., Boretti, F.S., Tasker, S., Meli, M.L., Wengi, N., Reusch, C.E., Lutz, H. & Hofmann-Lehmann, R. (2007) From *Haemobartonella* to *Hemoplasma*: molecular methods provide new insights. *Veterinary Microbiology*, 125. 197-209.

Williams, H., Zoller, H., Roepke, R.K.A., Zschiesche, E., Anja, R.H. (2015) Fluralaner activity against life stages of ticks using *Rhipicephalus sanguineus* and *Ornithodoros moubata* in *in vitro* contact and feeding assays. *Parasites & Vectors,* 8(90). 1-5.

Williams, L.A.D. (1993) Adverse effects of extracts of *Artocarpus altilis* Park. and *Azadirachta indica* (A. Juss) on the reproductive physiology of the adult female tick, *Boophilus* (Canest.). *Invertebrate Reproduction & Development,* 23(2-3). 159-164.

Williams, L.A. & Mansingh, A. (1996) The insecticidal and acaricidal actions of compounds from *Azadirachta indica* (A. Juss.) and their use in tropical pest management. *Integrated Pest Management Reviews*, 1(3). 133-145.

Wilson, S.G. (1948) A method for assessing the acaricidal properties of DDT and Gammexane preparations. *Bulletin of Entomological Research*, 39. 269-279.

Winkel, B.S. (2006). The biosynthesis of flavonoids. *In The Science of Flavonoids*. Anonymous, Springer.71-95.

Witting-Bissinger, B.E., Stumpf, C.F., Donohue, K.V., Apperson, C.S. & Roe, R.M. (2008) Novel arthropod repellent, BioUD, is an efficacious alternative to DEET. *Journal of Medical Entomology*, 45(5). 891-898.

Wolstenholme, A.J. (2012) Glutamate-gated chloride channels. *Journal of Biological Chemistry*, 287(48). 40232-40238.

Wölwer-Rieck, U. (2012) The leaves of *Stevia rebaudiana* (Bertoni), their constituents and the analyses thereof: a review. *Journal of Agricultural & Food Chemistry*, 60. 886-895.

Wulff, H., & Yarov-Yarovoy, V. (2015). Small molecule modulation of voltage-gated ion channels. *Biophysical Journal*,108(2).

Xiao, J. (2017). Dietary flavonoid aglycones and their glycosides: Which show better biological significance? *Critical Reviews in Food Science & Nutrition*, 57(9). 1874-1905.

Yakkundi, S.R., Thejavathi, R. & Ravindranath, B. (1995). Variation of azadirachtin content during growth and storage of neem (*Azadirachta indica*) seeds. *Journal of Agricultural & Food Chemistry,* 43(9). 2517-2519.

Yessinou, R.E., Adinci, J., Sessou, P., Adehan, S., Tonouweha, A., Akpo, Y., Adoligbe, C., Assogba, M.N., Koutinhouin, B. & Youssao, A.K.I. (2016) *In vitro* acaricidal effect of *Syzygium aromaticum* and *Cymbopogon citratus* essential oil on engorged female of cattle tick *Rhipicephalus microplus* in Benin. *Scientific Journal of Veterinary Advances,* 5. 80-86.

Yoder, J., Atwood, A. & Stevens, B. (1998) Attraction to squalene by ticks (Acari: Ixodidae): First demonstration of a host-derived attractant, *International Journal of Acarology,* 24 143-147.

Yohannes, G. (2014) Evaluation of *in vivo* antihypertensive and *ex vivo* vasodepressor activities of the seed extract of *Calpurnia aurea* (Ait.) Benth.subsp.*aurea* (Fabaceae). (*Doctoral dissertation, AAU*).

Yongabi, K., Harris, P. & Lewis, D. (2009) Poultry faeces management with a simple low cost plastic digester. *African Journal of Biotechnology*, 8. 1560.

Yuan, Y., Tan, Y., Xu, P., Li, H., Li, Y., Chen, W. Y., Zhang, J.Q., Chen, F. & Huang, G. (2014) Izalpinin from fruits of *Alpinia oxyphylla* with antagonistic activity against the rat bladder contractility. *African Journal of Traditional, Complementary & Alternative Medicines*, 11(4). 120-125.

Zahir, A.A., Rahuman, A.A., Bagavan, A., Santhoshkumar, T., Mohamed, R.R., Kamaraj, C., Rajakumar, G., Elango, G., Jayaseelan, C. & Marimuthu, S. (2010) Evaluation of botanical extracts against *Haemaphysalis bispinosa* Neumann and *Hippobosca maculata* Leach. *Parasitology Research,* 107. 585-592.

Žákovská, A., Nejezchlebová, H., Bartoňková, N., Rašovská, T., Kučerová, H., Norek, A. & Ovesná, P. (2013) Activity of the tick *Ixodes ricinus* monitored in a suburban park in

Brno, Czech Republic, in association with the evaluation of selected repellents. *Journal of Vector Ecology*, 38(2). 295-300.

Zhang, J.W., Li, S.K. & Wu, W.J. (2009) The main chemical composition and *in vitro* antifungal activity of the essential oils of *Ocimum basilicum* Linn. var. pilosum (Willd.) Benth. *Molecules,* 14(1). 273-278.

Zhao, X., Yeh, J.Z., Salgado, V.L., & Narahashi, T. (2004) Fipronil is a potent open channel blocker of glutamate-activated chloride channels in cockroach neurons. *Journal of Pharmacology & Experimental Therapeutics*, 310(1). 192-201.

Zizka, A., Thiombiano, A., Dressler, S., Nacoulma, B.M., Ouédraogo, A., Ouédraogo, I., Ouédraogo, O., Zizka, G., Hahn, K. & Schmidt, M. (2015) Traditional plant use in Burkina Faso (West Africa): a national-scale analysis with focus on traditional medicine. *Journal of Ethnobiology & Ethnomedicine*, 11. 9.

Zoghbi, M.D.G.B., Andrade, E.H.A. & Maia, J.G.S. (2002) Volatile constituents from *Adenocalymma alliaceum* Miers and *Petiveria alliacea* L., two medicinal herbs of the Amazon. *Flavour Fragrance Journal,* 17(2). 133-135.

Zorloni, A., Penzhorn, B.L. & Eloff, J.N. (2010) Extracts of *Calpurnia aurea* leaves from southern Ethiopia attract and immobilise or kill ticks. *Veterinary Parasitology*, 168. 160-164.

Zorloni, A. (2007). Evaluation of plants used for the control of animal ectoparasitoses in Southern Ethiopia (Oromiya and Somali Regions). *MSc thesis*.

Zoubiri, S. & Baaliouamer, A. (2011) Potentiality of plants as source of insecticide principles. *Journal of Saudi Chemical Society*, 18(6). 925-938.