

The toxicity and repellent properties of plant extracts used in ethnoveterinary medicine to control ticks

By

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ABSTRACT

The toxicity and repellent properties of plant extracts used in ethnoveterinary medicine to control ticks

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Six plant species (*Aloe ferox*, *Aloe marlothii*, *Clerodendrum glabrum*, *Jatropha curcas*, *Ricinus communis* and *Strychnos madagascariensis*) used by communities in the northern parts of South Africa to control ticks on livestock were selected from the database of the Onderstepoort Veterinary Institute. The leaves of these species were extracted with organic solvents ranging from polar to non-polar (methanol, acetone and dichloromethane). Infusions (soap-water-paraffin) and decoctions traditionally used were also prepared. The tick repelling and toxicity activities of the extracts were evaluated against the livestock tick, *Rhipicephalus appendiculatus*.

Initial results of the tick-climbing repellency bioassay illustrated that a 30% concentration of crude acetone extracts of *A. ferox*, *A. marlothii* and *C. glabrum*; DCM extracts of *R. communis* and *A. marlothii* and MeOH extract of *J. curcas* repelled the ticks to a statistically significant level. Of all the aqueous extracts only the decoction of *S. madagascariensis* repelled ticks significantly. At lower concentration of 10%, only acetone extracts of *C. glabrum* were effective.

Three types of bioassays were conducted for toxicity i.e., dipping, topical and dry extract applications. Organic extracts were not effective. Infusions of *A. ferox* and *S.*

madagascariensis had strong topical application toxicity at a concentration of 35.5 % while the same concentration of infusions of *A. ferox* and *J. curcas* had a strong dipping toxicity effect. Both *A. ferox* and *S. madagascariensis* extracts were still toxic at the concentration of 30% in topical and dipping bioassays.

In general acetone was a good extractant with lower yields but higher percentage repellency. Polar aqueous extracts had high yields but lower percentage repellencies. It may be that volatiles present in organic extracts are repellents and non-volatiles from aqueous extracts are toxic.

Some of the fractions of *C. glabrum* had a higher tick repellency activity than the commercial tick control agents used as positive controls. Unfortunately the activity decreased after 2.5 hours probably due to a volatility of the biologically active compound.

Attempts were made to isolate the repellent compound from the acetone extract of *C. glabrum*. The process gave very good results up to a late stage of the bioassay guided fractionation. At that stage the repellent activity was lost. When two fractions were combined the repellent activity was present again. This is strong evidence for the existence of a synergistic effect. Further attempt to isolate the compounds at this stage and characterize them by nuclear magnetic resonance spectroscopy failed probably through decomposition of the compounds. This aspect can probably be followed up in more detail.

The results substantiated the rationale behind the use of these plant extracts to control ticks. Even a simple acetone extract of leaves of some species has excellent tick repelling activities. If the duration of this effect could be increased it could very well develop into a commercially useful product.

The tick repelling activity of *S. madagascariensis* decoctions and tick killing activity of *A. ferox* soap infusion holds great promise for the control of ticks by rural farmers. *In vivo* trials under controlled conditions should provide useful results.

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LIST OF ABBREVIATIONS AND ACRONYMS

aceto-hex	Hexane in acetone
ARC	Agricultural Research Council
	<i>Aloe ferox</i>
Alom	<i>Aloe marlothii</i>
Amit	Amitix
Bay	Bayticol
BuOH	butanol
Clero	<i>Clerodendrum glabrum</i>
conc.	concentration
contr	control
CHCl₃	chloroform
CCl₄	carbon tetrachloride
DCM	dichloromethane
EC₅₀	effective concentration to repel 50 %
EtOAc	ethyl acetate
EVM	Ethnoveterinary medicine
g	gram
hex	hexane
hrs	hours
H₂O	water
Jap	<i>Jatropha curcas</i>
LD₅₀	lethal dose to kill 50 %

M_c	corrected or accrued mortality rate
MeOH	methanol
mg	milligram
min	minutes
ml	millilitre
M_o	Mortality rate in treatment
M_t	Mortality rate in control
no.	number
OVI	Onderstepoort veterinary institute
p	probability
% rep	percentage repellency
Ric	<i>Ricinus communis</i>
Stry	<i>Strychnos madagascariensis</i>
subfr	subfraction
var	variance

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Tick biology

1.1.1 Tick classification

Ticks are obligate haematophagous external parasites of mammals, birds and reptiles throughout the world (Parola & Raoult 2001; Rajput *et al.* 2006). They belong to the phylum, Arthropoda. They are divided taxonomically into two main families, Argasidae and Ixodidae (Simlinger 2005). A third family, the Nuttalliellidae is represented by only a single species that is confined to southern Africa (Parola & Raoult 2001). Argasids, for example, *Otobius megnini* and *Ornithodoros moubata* are often called soft ticks because they do not have hard chitinous plates on the dorsal surface of their bodies but a flexible cuticle. On the other hand the ixodids, for example, *Hyalomma excavatum* and *Rhipicephalus pulchellus* have sclerotised dorsal plates and are commonly called hard ticks (Walker *et al.* 2003).

The characteristic features of the hard ticks differ from those of soft ticks. Hard ticks which are the subject of this study have a number of attributes that enhance their parasitism. They feed for extended periods of time varying from several days to weeks. The length of the feeding period is influenced by factors such as life cycle, host type and tick species (Sonenshine 1991). Their bites are usually painless and may go unnoticed for a long time (Parola & Raoult 2001).

Soft ticks feed briefly and usually on a single host species (Sonenshine 1991). Many argasids have an uncanny resistance to starvation and can survive for many years without starvation (Furman & Loomis 1984). The cuticle of soft ticks expands, but does not grow to accommodate the large volume of blood ingested, which may be anywhere from 5 to 10 times their unfed body weights (Rajput *et al.* 2006).

1.1.2 Life cycle of ixodid ticks and development patterns

Ixodids consist of 4 stages in their life span, namely egg, six-legged larva, eight-legged nymph and eight-legged adult, and each of the post-embryonic stages requires a blood meal (Horak *et al.* 2002) for further development. They exhibit varied developmental patterns during their life cycle and as a result are grouped as one-, two- and three-host ticks (Fletcher 1984). A one-host tick requires only one host to complete its life cycle. All three post-embryonic stages in a one-host life pattern feed and mate on one host and the engorged adult ultimately drops off. Moulting between stages, that is larvae-nymphs and nymphs-adult takes place on one tick.

Two-host ticks such as *Rhipicephalus evertsi evertsi* and *Hyalomma marginatum rufipes* require two hosts to complete their life cycle. The larvae and nymphs of a two-host tick feed on the same individual host, and the adults feed on another host. A three-host tick requires three hosts to complete the life cycle and each instar requires its own single host (Walker *et al.* 2003). Each instar seeks out a host, attaches, feeds to fully engorgement and then drops off. After dropping off, a three-host tick e.g., *Rhipicephalus appendiculatus* finds a resting place where it can digest its bloodmeal and moult to the next instar or enter diapause, a state characterised by reduced metabolism and delayed development (Parola & Raoult 2001).

1.1.3 Host seeking behaviour

Ticks appear not to rely on eyes for perception of the environment. Instead, they have a variety of peripheral sensory organs which include the hair-like structures on the body, legs, mouthparts and a sensory complex located on the dorsal surface of the tarsus of leg I. The latter contains a cluster of olfactory and gustatory receptors (Haller's organ). Ticks are able to locate their hosts and communicate with other ticks through these sensory organs (Parola & Raoult 2001).

Ticks have an instant response to chemical stimuli such as carbon dioxide (CO₂) and ammonia (NH₃) gases that indicate the presence of hosts (Parola & Raoult 2001).

Other stimuli include humidity, airborne vibrations and body temperatures which are associated with warm-blooded animals (Parola & Raoult 2001). Upon receiving stimuli ticks are ready for attack. There are two typical host-seeking behaviour patterns among the ticks: the ambush strategy and the hunter strategy. In the ambush strategy, the ticks climb up vegetation when questing for hosts, with their front legs held out (e.g., *Rhipicephalus sanguineus* and *Dermacentor variabilis*). In the hunter strategy, ticks attack hosts by emerging from their habitat and running towards their hosts when these animals appear nearby (e.g., *Amblyomma hebraeum* and *Amblyomma variegatum*) (Sonenshine 1991; Sonenshine 1993). Ticks demonstrate varying levels of host-specificity, for instance *R. sanguineus* has high affinity for dogs and *Amblyomma americanum* usually feed on different host species, particularly mammals and birds (Hillyard 1996).

1.2 Health and economic importance of ticks

Tick bites and tick-borne diseases continue to be a serious public health problem throughout the world (Carroll 2004). Ticks are responsible for substantial economic losses to the livestock industry (Willadsen & Kemp 1988). The East Coast Fever caused by *Rhipicephalus appendiculatus* species has been estimated to cost Africa US\$168 million in 1993 (Eisler *et al.* 2003). More and more chemical acaricides have been synthesised and tick resistance poses a serious threat to most farmers worldwide (Lane & Crosskey 1996). The high expenditure of these acaricides, food and environmental contamination prompted many farmers across the globe to spend a large percentage of their time and money on management of ticks and tick-borne diseases (Kaaya & Hassan 2000).

Ticks transmit viral, rickettsial, bacterial and protozoal diseases affecting wild, domestic animals and human (Lane & Crosskey 1996). Heavy infestations of ticks cause reduction in live weight and anaemia in domestic animals, while tick bites also reduce the quality of hides. Apart from irritation or anaemia, severe infestations can cause dermatitis (Rajput *et al.* 2006). *Hyalomma* species infest camels, cows, buffaloes, horses and sheep in Egypt. *Hyalomma anatolicum excavatum* is a debilitating parasite in various countries of the world and is a vector of a variety of

diseases. It also constitutes a major problem to the dairy and beef industry inducing decrease in weight gain, milk production and transmitting *Theileria annulata* (Abdel-Shafy & Zayed 2002).

Tick feeding is a major threat to the host. During the feeding process, the salivary secretions produced include the cement to anchor the mouthparts to the skin of the host as well as enzymes, vasodilators, anti-inflammatory, antihemostatic and immunosuppressive substances (Parola & Raoult 2001). In some tick species such as *Ixodes rubicundus* found in South Africa, the toxic substances present in the saliva causes tick paralysis which may lead to death in man and animals (Drummond 1983; Walker *et al.* 2003).

Salivary glands are the organs of osmoregulation, and excess water from the bloodmeal is returned via saliva into the host. Host blood must continue to flow into the feeding lesion as well as remain fluid in the tick mouth parts and gut. Ticks salivary components suppress the immune and inflammatory response of the host permitting the ticks to remain on the host for an extended period of time and, adventitiously, enhancing the transmission and establishment of tick-borne pathogens (Bowman *et al.* 1997). Most of the tick-borne diseases are transmitted by Ixodid ticks with 694 species and more than 80 of these occur in South Africa (Sonenshine 1991; Horak *et al.* 2002). Various tick-borne diseases and their vectors have been identified. Heartwater or cowdriosis is transmitted by the *Amblyomma hebraeum* with *Ehrlichia (Cowdria) ruminantium* as a causative organism (Coetzer & Tustin 2004). Gallsickness and redwater are diseases transmitted by *Boophilus species*, sweating sickness is transmitted by bont legged tick (*Hyalomma species*) and tick toxicosis is transmitted by most ticks (Fletcher 1984).

A good example of diseases infecting humans is the South African tick-bite fever which is transmitted by infected *Amblyomma* species and the infecting organism is *Rickettsia africae* (Magee 2003). Tick borne diseases generally affect the blood and or lymphatic system (Rajput *et al.* 2006). Therefore ticks and tick borne diseases necessitate intensive use of chemical acaricides in many parts of the world (Willadsen & Kemp 1988).

1.3 *Rhipicephalus appendiculatus* Newmann (Acari: Ixodidae)

Rhipicephalus appendiculatus was selected for this project (Fig. 1.1) because it climbs up vegetation when questing for hosts. It is referred to as a Brown Ear Tick because of its colour and preference for feeding on the ears of cattle. *R.*

appendiculatus is also a livestock pest of major economic importance in Africa where it is a chief vector of the pathogen, *Theileria parva* (Apicomplexa: Theileridae) which causes East Coast Fever (Lwande *et al.* 1999; Walker *et al.* 2003). East Coast Fever is a non-contagious, febrile, lymphoproliferative disease in cattle that leads to high mortality and morbidity rates, high costs of controlling the vector tick and high cost of the chemotherapeutic drugs used to treat it (Mbogo & Kariuki 1995). The disease was eradicated in South Africa by 1954 but still exists in numerous countries in Central, East and North Africa (Fletcher 1984).



Figure 1.1 A picture of a female (right) and male (left) *Rhipicephalus appendiculatus*.

Ticks are vectors of various pathogens that are a major source of diseases in animals. Different strains of *Theileria parva* have been identified and cause Corridor or Buffalo disease and Zimbabwean theileriosis. *T. taurotragi* causes benign bovine theileriosis. *Anaplasma bovis* (*Ehrlichia bovis*) is a bacterium responsible for bovine ehrlichiosis and *Rickettsia conori* causes tick typhus in human. When the host immunity is suppressed massive infestations may lead to Tzaneen disease caused by *T. parva* (Walker *et al.* 2003).

A plant extract is considered a good repellent if it prevents a tick from contacting a host at all (Dautel 2004). To ascertain the effect of plant-based repellents, very few *in vitro* bioassays have been developed to test for tick repellency. So far there is no widely accepted standard procedure. This is a pity since different repellency bioassays produce contrasting results even when using the same tick species (Carroll 2004).

Chemical compounds used to control ticks simply by terminating their feeding process are classified as antifeedants (Danielson 2006). Antifeedants are substances leading to temporary or permanent cessation of feeding when tasted by arthropods (Klocke *et al.* 1989). Deterency and toxicity are the two main modes of action by which secondary plant compounds may act. Deterrents affect the peripheral nervous system and ultimately prevent the arthropod from further feeding whereas toxicants disrupt cellular, biochemical and physiological processes once digested by an insect (Danielson 2006).

Definitions of important concepts used in this project

Repellent: an extract that prevents ticks from being in close contact with it (Dautel 2004). According to the definition from the Internet (Wikipedia), a repellent is a chemical substance that repels animals.

Toxicant: an extract that kills ticks after having been in contact with it (Dautel 2004). According to the definition from the Internet (Wikipedia), a toxicant is any chemical or mixture of chemicals that presents a risk of death, disease or injury in organisms that ingest or absorb it.

1.4 Tick control

Synthetic acaricides appeared in the 1940's but problems like environmental contamination, residues in food and feed, high costs relative to the values of cattle and cattle products, development of acaricide resistance in tick stimulated research on new safe methods for tick control (Kaaya & Hassan 2000; Silva-Aguayo 2006).

Tick resistance to chemical acaricides has been on the rise (Lane & Crosskey 1996). Resistance to one or more pesticides has been documented in more than 440 species of insects (Lane & Crosskey 1996). There are strains of ticks resistant to arsenicals, a wide range of organophosphates, carbamates, amidines and synthetic pyrethroids (Willadsen & Kemp 1998).

Fox and Leon (1986) reported the failure of carbaryl-based products to control *Rhipicephalus sanguineus* in dogs. These carbaryl-based products were in the form of a powder containing carbaryl plus piperonyl butoxide and pyrethrins as well as carbaryl, pyrethrins, piperonylbutoxide and N-octylbicycloheptenedicarboxamide [2-(2-ethylhexyl) 3a,4,7,7a-tetrahydro-4,7-dimetheno-1H isoindole-1,3 (2H)-dione]. Strains of *Boophilus microplus* had developed resistance to organophosphorus compounds and carbamates (Villalba *et al.* 1982).

Boophilus microplus has developed resistance to a succession of chemical acaricides, which has led to the investigation of alternative approaches for eradication of ticks (Floyd *et al.* 1995). Johnston *et al.* (1986) considered the use of biological control such as “pasture spelling” and tick resistant cattle to combat tick resistance. The development of resistance by ticks also stimulated interest in tick control by immunological means. A vaccine against ticks which enhances the natural immunity often acquired by animals in response to tick infestation was developed (Willadsen & Kemp 1988).

Other methods of tick control used in southern Africa include slaughter and quarantine (Fletcher 1984). However, the former type of tick control was not as effective as the latter since it was effective on specific tick species. It was able to eradicate *Theileria parva* successfully and not *Babesia bigemina* and this triggered a concern by most scientists who evaluated the most appropriate methods to use for management of ticks (Fletcher 1984).

Plant extracts used in ethnoveterinary medicine represent a cheaper and easily accessible method for tick control. It is an answer to which most rural farmers turn to when financial circumstances do not allow them to purchase modern western medicines (Nchu *et al.* 2005). It is an approach that offers sustainable strategies

directed towards developing sound and appropriate animal health care systems suitable and relevant to rural communities in improving livestock performance and production (Wanzala *et al.* 2005). It has been developed by farmers in the fields rather than by scientists in laboratories and it is dynamic and changing (Mathias 2001).

Botanical arthropocides are amongst the end products of ethnoveterinary medicine that probably evolved simultaneously with the evolution of human beings (Wynn 2001). Botanicals have different properties from plant to plant. They are processed into various forms which include: preparation of crude plant materials, plant extracts or resins and pure chemicals isolated from plants (Johnson 1998). Before the organised search for arthropocides, plants species with acaricidal constituents include tobacco, camphor, derris and turpentine (Berger & Mugoya 1995). Extracts of tropical and subtropical plants have acaricidal effects on *Boophilus microplus* and *Rhipicephalus appendiculatus* (Abdel-Shafy & Zayed 2002). There are also other sources for the control of ticks. Burg *et al.* (1979) reported the acaricidal properties of avermectins from the actinomycete *Streptomyces avermitilis*.

Pyrethrins from *Chrysanthemum cinerariaefolium* have also been studied for acaricidal properties (Mathewson 1984). Microencapsulation of pyrethrin protects it from sunlight and increases its persistence as a toxicant and repellent to ticks (Simpkin & Galun 1983). A ground mixture of dried tobacco leaves and a mineral called "Magadi soda" from Kenya killed and prevented the completion of all feeding phases of *Rhipicephalus appendiculatus* (Ixodidae) (Dipeolu & Ndungu 1991).

Some ingredients obtained from plant extracts, seeds, leaves and barks of trees, tubers and roots of various trees are considered valuable in animal health (Akut *et al.* 2002). Such ingredients are mainly secondary metabolites which include thousands of alkaloids, terpenoids, phenolics and minor secondary chemicals (Arnason *et al.* 1988). Rotenone is an example of a flavonoid extracted from roots of two plants: *Derris species* (Fabaceae) and *Lonchocarpus species* (Fabaceae). It is a contact and ingestion compound which also acts as a repellent.

The most common secondary metabolites specifically with protective action against arthropods include: non-protein amino acids, steroids, phenols, flavonoids, glycosides, alkaloids, glucosinolates, quinines, tannins and terpenoids (Silva-Aguayo 2006). Arthropods may be repelled by different plant species on the basis of the plant's secondary metabolites.

Although many plant species have been used traditionally to control ticks, the efficacy of extracts of plant species have not been investigated widely. Zorloni (2008) evaluated acetone and hexane extracts of many plant species used in Ethiopia to control ticks on camels on toxicity and repellency activity against *R. pulchellus*.

This project aimed at validating the efficacy of plant species used to control ticks in ethnoveterinary medicine. Two different types of *in vitro* bioassays were used, namely, repellency and toxicity bioassays.

1.5 Motivation

Many of Africa's resource-poor farmers fail to dip their cattle regularly due to the increased cost of the synthetic acaricides used in tick control. Natural products may offer these farmers a cheaper alternative to synthetic acaricides and farmers have experimented on the use of plants over many years. There is also a potential for the use of plants to develop sources of acaricides to reduce the cost of tick control, being locally available and potentially being able to be produced and used by farmers themselves or by cottage industries (Kaposhi 1992).

The widespread use of acaricides has led to many ecological, medical and financial difficulties including the high cost and contamination of the environment and food with toxic residues. Tanzania and Uganda, for instance each spends US\$26 million annually on the importation of acaricides, Kenya spends US\$6-10 million, Zambia US\$10 million and Burundi US\$159 000 (Kaaya 2000). The overall loss in Africa due to ticks is estimated at US\$720 million per year. Therefore it is worthwhile to look for alternative methods of tick control which are relatively cheap, environmentally friendly and which directly involve farmers in tick management. The use of effective

chemicals from natural sources such as plants, fungi and bacteria may be a viable alternative (Berger & Mugoya 1995).

1.6 Aim

The main aim of this project is to evaluate the efficacy of plant extracts used in ethnoveterinary medicine to control ticks in the northern parts of South Africa in an attempt to develop a plant product or products that can be used by farmers.

1.7 Objectives

The objectives to attain the aim of this study are :

- ✓ To select the plants species to investigate
- ✓ To extract the selected plant species with different extractants and evaluate the efficacy of different extracts
- ✓ To screen the repellency of selected plant extracts on *Rhipicephalus appendiculatus*
- ✓ To screen the toxicity of selected plant extracts on *R. appendiculatus*
- ✓ To attempt to isolate the chemical compounds with anti-tick activity
- ✓ To assess the potential use of these extracts by rural farmers.

CHAPTER 2

SELECTION OF PLANT SPECIES TO INVESTIGATE

2.1 Selection of plant species for investigation

In many rural areas of the Republic of South Africa, traditional healthcare is the only available alternative to expensive or unavailable modern orthodox health care for the management of both human and animal health (Luseba & van der Merwe 2006)

During their evolution, plants have developed a number of protective mechanisms to combat predator and pathogen attack. These include repellency through production of hairs and volatile compounds, and production of chemicals with arthropocidal activity (Silva-Aguayo 2006). Although plants are not attacked by ticks, the compounds developed to protect plants against other arthropods may also have activity against ticks. The interest in using plant-based repellents is on the rise and some plant species have so far been screened for tick repellency (Palsson & Jaenson 1999; Kaaya 2003).

Zorloni (2008) evaluated various plant species from southern Ethiopia for tick toxicity and repellency activities. There were no plant extracts which showed both good repellency and toxicity activities. *Aloe calidophila*, *Croton macrostachys*, *Croton megalocarpus*, *Lantana camara*, *Maerua tryphilla*, *Ocimum suave* and *Tagetes minuta* had good repellency activity. Plants which have been used as acaricides against *Boophilus microplus*, *Rhipicephalus haemaphysaloides* and *Hyalomma anatolicum anatolicum* include extracts of *Cedrus deodara*, *Azadirachta indica* and *Embelia ribes* (Maske *et al.* 1996). Some oils extracted from various plants species such as *Eucalyptus*, *Pongamia* and *Ocimum suave* oils were effective in the control of *Boophilus microplus* and *Rhipicephalus appendiculatus* (Mwangi *et al.* 1995; Sivaramakrishnan *et al.* 1996).

Based on the ethnoveterinary use as documented in the database of the Agricultural Research Council /Onderstepoort Veterinary Institute (ARC/OVI) a number of plant species were selected for evaluation (Table 2.1).

Table 2.1 The selected plant species with their traditional names, part of plant used, indications, preparations, routes of administration and quoted references obtained from ARC-OVI / EVM database.

Plant name	Common name	Plant part used	Indications	Preparation	Route of administration	References
<i>Aloe marlothii</i> Berger (Asphodelaceae)	Tshikhopha (Venda) Mogopa (Tswana) Umhlaba (Zulu) Mountain aloe (Eng) Bergaalwyn (Afr)	Leaves	Ticks, chickens, diarrhoea, ataxia, blood cleaning, blowfly, constipation, dystocia and hasten weaning	Decoction – crushed leaves in boiled water	Topical	Hutchings <i>et al.</i> 1996; Roberts 1997
				Infusion or leaf sap directly squeezed out of leaves and applied immediately	Topical and per os	
				Decoction—one measure chopped leaves boiled in 4 measures of water for 10 minutes	Per os	Roberts 1997
<i>Clerodendrum glabrum</i> E. Mey. var. <i>glabrum</i> (Verbenaceae)	Munukha-tshilongwe (Venda) Umqangazane (Zulu) Mohlokohloko (Sotho) Tinderwood tree (Eng) Kaster boon (Afr)	Leaves Roots Bark	Ticks, diarrhoea, heartwater, helminths and insect repellent	Infusion – leaves are crushed and mixed with water	Per os	Hutchings <i>et al.</i> 1996; Mabogo 1990
				Decoction – ¼ cup of leaves in 2 cups of water is boiled for 15 minutes, strained and applied	Per os	Roberts 1997
				Decoction – leaves and twigs are boiled in water to cover it for 20 minutes, then strained and applied	Topical	Roberts 1997; Hutchings <i>et al.</i> 1996

<i>Aloe ferox</i> Mill (Asphodelaceae)	Mokopa (Tswana) Umhlaba (Zulu) Hlaba (Sotho) Bitter/red aloe (Eng) Bitteraalwyn (Afr)	Leaves	Ticks, wounds, sores, redwater, purgative and abortifacient	Infusion from the leaves and water which is left to stand overnight	Topical	Roberts 1997
				Fresh leaf juice is squeezed out and applied	Per os	Hutchings <i>et al.</i> 1996; Roberts 1997
<i>Ricinus communis</i> L. (Euphorbiaceae)	Mokhura(N. Sotho) Inhlakuva (Zulu) Castor oil (Eng) Kasterolieboom (Afr)	Leaves	Helminths and insects, sores wounds, stomach ache, afterbirth bleeding, facilitate expulsion of placenta and hasten parturition	Infusion from leaves and water is prepared and applied orally	Topical	Johns <i>et al.</i> 1990; Hutchings <i>et al.</i> 1996
<i>Jatropha curcas</i> L. (Euphorbiaceae)	Matlhapametse (Tswana) Inhlakuva (Zulu) Mafuredonga (Venda) Purging nut (Eng) Purgeerboontjie (Afr)	Leaves	Purgative, insect repellent and toxicant, jaundice, fevers, diarrhea and ringworm	Leaf infusions made from leaves and water	Per os	Hutchings <i>et al.</i> 1996
<i>Strychnos madagascariensis</i> (Strychnaceae)	Mukwakwa (Venda) Black monkey orange (Eng)	Leaves	Various insects	Leaves and water infusion of <i>S. madagascariensis</i>	Topical	Karlsson 1995

2.2 Medicinal and other uses of phytochemicals of selected plant species

General background of the selected plant species was discussed below and the origin of photos was stated in case the photo was not personally taken. For the purpose of this project, only the leaf portions of the selected plant species were investigated.

2.2.1 *Aloe ferox* and *A. marlothii*



Aloe ferox Mill

Family : Asphodelaceae

(www.succulents.co.za)



Aloe marlothii Berger

Family : Asphodelaceae

(www.succulents.co.za)

Various species of aloes are extensively used as cure-alls since ancient times in many areas across the globe (van der Merwe 2000). Aloe species form the basis of a large commercial industry in health-care products (van Wyk & van Wyk 1997). *Aloe ferox* is characterized by the presence of polysaccharides accumulating in the leaves as well as anthranoids and anthraglycosides (Harborne *et al.* 1997). Infusions prepared from the leaves of *A. ferox* are used to treat wounds, sores, redwater and also as a purgative, abortificant and to control ticks (Roberts 1997).

An oxymethylantraquinone derivative nataloin has been isolated from the leaves of *Aloe marlothii* (Watt & Breyer-Brandwijk 1962). *A. marlothii* is the most

important ethnoveterinary medicinal plant utilized by the Tswana speaking people. Van der Merwe (2000) studied the diverse range of indications for which *A. marlothii* was utilised by the Tswana people. He noted some contradictions such as its use for both diarrhoea and constipation. Decoctions and infusions of *A. marlothii* have been used to treat chickens, ataxia, for blood cleaning, blowfly, dystocia, hasten weaning and for tick control (Hutchings *et al.* 1996; Roberts 1997).

2.2.2 *Clerodendrum glabrum*



Clerodendrum glabrum E. Mey. var. *glabrum*,
Family : Verbenaceae

Leaves of *Clerodendrum glabrum* are well known of their insect repellency (Mabogo 1990; Hutchings *et al.* 1996). They are also used by the Vhavenda (Limpopo province) to counter the effects of witchcraft and their smell is believed to repel pole cats and hyenas used by witches (Mabogo 1990; Hutchings *et al.* 1996). Said *et al.* (1998) found a vast quantity of saponins in the *Clerodendrum* species. Saponins are triterpenoids or steroids with one or more sugar molecules attached to them, making them water soluble and they form a foamy solution when shaken (van Wyk *et al.* 2002; Gurib-Fakim 2006).

2.2.3 *Jatropha curcas*



Jatropha curcas L.
Family : Euphorbiaceae

Jatropha curcas (Euphorbiaceae) is a popular Tswana traditional medicine and a common cause of human poisoning in South Africa (van Wyk *et al.* 2002). It has insecticidal properties (Malaya & Banda 1995; Hutchings *et al.* 1996). Its secondary metabolites include the diterpenoids and lectins which are toxic (Adolf *et al.* 1984;

Joubert *et al.* 1984; Mampane *et al.* 1987; van Wyk *et al.* 2002). Lectins (haemagglutinins, phytotoxins) are proteins or glycoproteins that are able to attach themselves to the red blood cells of a specific blood group and cause their precipitation or agglutination (Gurib-Fakim 2006).

2.2.4 *Ricinus communis*



Ricinus communis L.
Family : Euphorbiaceae
(2002 Floridata.com)

Ricinus communis commonly known as castor oil plant is familiar for its purgative properties. It also contains ricin, one of the most toxic compounds known (van Wyk & Wink 2004). Toxalbumin and ricin are contained in the seed extracts of *R. communis*. The decoctions are used by Zambian farmers as powerful poisons and to control a wide range of insects (Malaya & Banda 1995). The insecticidal and acaricidal properties of *R. communis* have also been noticed in Democratic Republic of Congo, Kenya, and Nigeria (Lindblom 1920; Adjanohoun 1991; Cihyoka 1994; Defour 1994).

2.2.5 *Strychnos madagascariensis*



Strychnos madagascariensis

Family : Strychnaceae

The fruit flesh of *Strychnos madagascariensis* is edible, but the pips are very bitter because they contain poisonous indole alkaloid strychnine. It also contains, a curare alkaloid C-Toxiferine I and many other structurally related alkaloids that have been used as rodent and ordeal poisons. (Verdoorn 1963; Neuwinger 1996; van Wyk & Wink 2004).

Malaya and Banda (1995) reported that *S. madagascariensis* contains saponins which are pesticidal, repellent and toxic to several insect pests especially termites. Infusion made from leaves of *Swartzia madagascariensis* was used to control various insects (Karlsson 1995).

2.3 Discussion

Based on the literature there is some rationale in the plants used to control ticks, but there are many difficulties associated with using poisonous plant species. If some of the toxins are absorbed by the animals it could cause intoxication of the animal or lead to residues of toxins in the animal products. It may also have a negative effect on the environment. If extracts or compounds are discovered that are selectively toxic to ticks it would be a great advantage.

CHAPTER 3

EXTRACTION OF PLANT MATERIALS

3.1 Introduction

Different parts of the plant species are used as medicine. In some cases the plants are used directly but usually extracts are used. Extraction is the process of dissolving the biologically active substances of plant material. Either fresh or dry plant material may be used to extract the biologically active components. The disadvantage of using fresh plant material is that it is tedious to remove water from the extract subsequently. Therefore most scientists prefer to dry plant material prior to extraction since membranes of plant organelles containing different secondary compounds are destroyed, making extraction more efficient (Eloff & McGaw 2006). Some components may be degraded by oxidation or photo-oxidation or by microbes therefore quick drying in drying cabinets or freeze drying may be employed (Gurib-Fakin 2006). Otherwise drying can take place under the shade at room temperature.

The dry plant material is normally ground to finer particles for better extraction yields. There are numerous extraction techniques available such as cold extraction, hot percolation, supercritical fluid extraction and soxhlet extraction. In this project only cold extraction and hot percolation were used. Plant extracts were prepared using organic solvents ranging from polar to non-polar (methanol, acetone and dichloromethane). Because these extractants are not always available to rural people infusions using a mixture of water and soap and decoctions using water were also prepared. The main motivation was to evaluate different extracts in repellency and toxicity bioassays to determine its potential use and also to facilitate identifying active compounds found in plant species.

For cold extraction, the plant material was extracted in methanol, acetone and dichloromethane at room temperature whereas for hot percolation, the plant is heated in the solvent usually under reflux to allow extraction of large number of metabolites (Gurib-Fakin 2006).

3.2 Materials and methods

3.2.1 Plant drying and processing

The six plant species selected belong to four different plant families. Members of the Euphorbiaceae, Strychnaceae and Verbenaceae were collected from Venda (Vhembe region) whereas the two *Aloe* spp., members of the Asphodelaceae family were collected from the Onderstepoort (ARC/OVI) medicinal plant garden.

The plant species were dried at room temperature in the ethnoveterinary medicine (EVM) laboratory at Agricultural Research Council/Onderstepoort Veterinary Institute (ARC/OVI). They were ground to fine particles using Büchi Grinder B-400 (Labotec). The ground plant materials were stored in brown closed bottles to avoid oxidation and stored in the laboratory at room temperature.

3.2.2 Cold extraction

The leaves of the selected plant species were extracted with dichloromethane (non-polar), acetone (intermediate polar) and methanol (polar) (Eloff 1998a). Three grams of the dry finely ground leaves in 30 ml of solvent was placed in a centrifuge tube and shaken vigorously for 15 minutes. Two ml of the extract was removed from the top and placed in a separate 40 ml glass beaker. This was dried and weighed to determine the concentration of the extract.

The remaining 28 ml solution was centrifuged for 5 minutes at 3500 rpm, filtered through Whatman no. 1 filter paper and the supernatant was collected into a 50 ml glass beaker. The process was repeated and the supernatant collected from the second extraction was combined with the first collected supernatant to complete extraction and stored in a fridge at 4°C to avoid fungal growth (Eloff & McGaw 2006).

3.2.3 Hot percolation

3.2.3.1 Preparation of the decoction

Five grams of the dry finely ground leaves and 30 ml of water were placed in a 100 ml glass beaker and boiled for 10 minutes at 80-100°C. The mixture was allowed to cool, filtered through a Whatman no. 1 filter paper into a clean 250 ml conical flask as

adopted from Berger and Mugoya (1995). The prepared extract was then stored in a fridge at 4°C to avoid fungal growth (Eloff & McGaw 2006).

3.2.3.2 Preparation of soap infusion

Five grams of the dry finely ground leaves were weighed into a 100 ml glass beaker and 0.2 g of Surf © (a commercial detergent containing biodegradable silicates, soda ash and phosphates) that acts as a water softener, was added to 30 ml of warm water (55-65°C) and the mixture was left standing for 30 minutes on the bench. The solution was then strained with a clean dry cloth into a 250 ml conical flask. Paraffin (1.2 ml) and 0.15 g of sodium bicarbonate of soda was added. The solution was incubated for two weeks in an oven at 30°C (Berger & Mugoya 1995). The prepared extract was then stored in a fridge at 4°C to avoid fungal growth (Eloff & McGaw 2006).

The rationale for adding paraffin and sodium bicarbonate to the extract and then incubation after the plant material was removed is not clear. It may have been added to protect the extract from fungal attack. To yield comparable results I followed the method as published.

3.3 Results

3.3.1 Percentage of dry weight extracted

The calculated percentages of dry weight extracted from the different plants are shown in Table 3.1. Most of the observations in the table lie close to the mean since the variances were quite small. The data points of soap infusion of *S.*

Madagascariensis and the decoction of *A. ferox* exhibit a fair amount of variability in the sense that the variances lie far from the mean (Milton 1992). Graphical representations are listed in Fig. 3.1A, B and C. High extract yields were obtained from aqueous solvents followed by methanol, dichloromethane and acetone.

The high percentage extracted by polar solvents from the *Aloe* spp can be explained by the mucilaginous interior part of the *Aloe* leaves. In general the plants parts extracted appeared to have a higher percentage of polar compounds than generally found in the Combretaceae (Kotze & Eloff 2002).

Table 3.1 Percentage of dry weight extracted from leaves of six plant species using five different extractants.

Plant species	Level of extraction	DCM		Acetone		MeOH		Decoction		Soap infusion	
		%	var (x10 ⁻³)	%	var (x10 ⁻³)	%	var (x10 ⁻³)	%	var (x10 ⁻³)	%	var (x10 ⁻³)
<i>A. ferox</i>	1 st extraction	2.53	38.4	0.66	0.100	2.65	2.63	12.04	331	15.80	41.6
	2 nd extraction	1.93	2.00	0.25	2.50	0.95	7.00	-	-	-	-
	Total	4.46		0.91		3.50		12.04		15.80	
<i>A. marlothii</i>	1 st extraction	0.80	38.1	0.40	9.23	2.23	12.1	9.7	62.2	9.20	122
	2 nd extraction	0.24	0.433	0.18	0.700	0.91	0.100	-	-	-	-
	Total	1.04		0.58		3.14		9.1		9.20	
<i>C. glabrum</i>	1 st extraction	1.73	8.13	1.07	57.7	6.47	78.6	4.46	60.7	3.22	6.03
	2 nd extraction	1.32	115	0.74	11.1	3.45	0.400	-	-	-	-
	Total	3.05		1.81		9.92		4.46		3.22	
<i>J. curcas</i>	1 st extraction	3.78	46.2	3.12	0.233	6.06	6.43	4.24	1.73	1.08	6.43
	2 nd extraction	0.22	0	1.61	152	3.03	0.233	-	-	-	-
	Total	4.00		4.73		9.09		4.24		1.08	
<i>R. communis</i>	1 st extraction	1.14	25.6	1.47	32.4	1.88	8.10	6.98	44.1	2.49	2.23
	2 nd extraction	0.82	25.6	0.58	4.90	1.31	28.9	-	-	-	-
	Total	1.96		2.05		3.19		6.98		2.49	
<i>S. madagascariensis</i>	1 st extraction	0.33	25.6	0.53	3.03	3.80	24.1	8.31	10.3	9.05	520
	2 nd extraction	0.11	2.87	0.24	4.90	1.66	112	-	-	-	-
	Total	0.44		0.77		5.46		8.31		9.05	

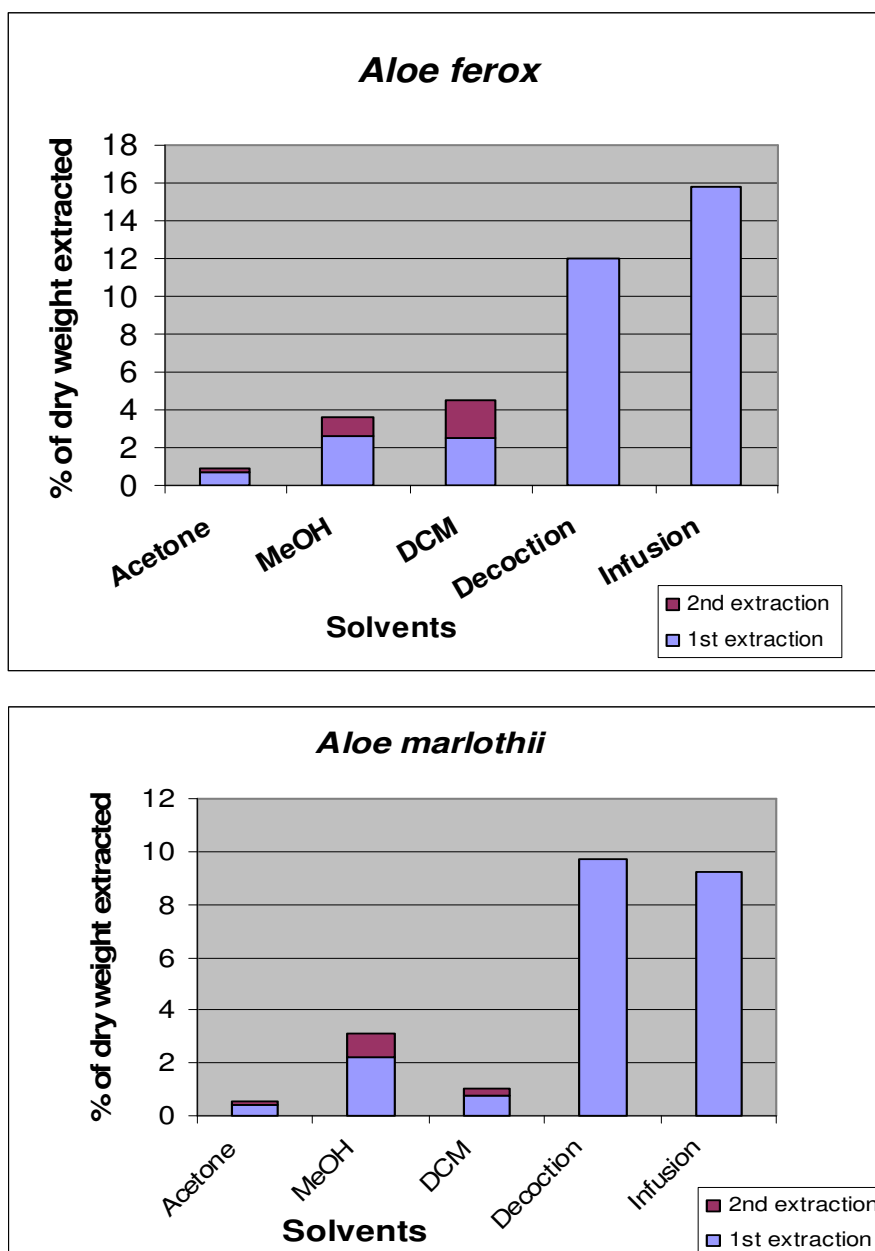


Figure 3.1A Percentage of dry weight extracted from *A. ferox* and *A. marlothii* using five different solvents. Value for first extraction at bottom and second at top.

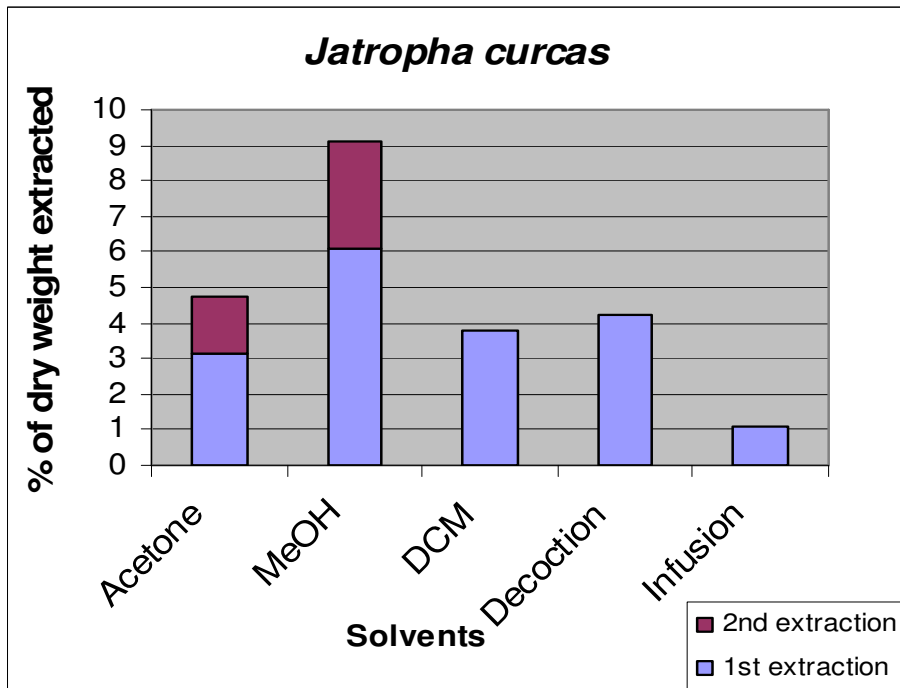
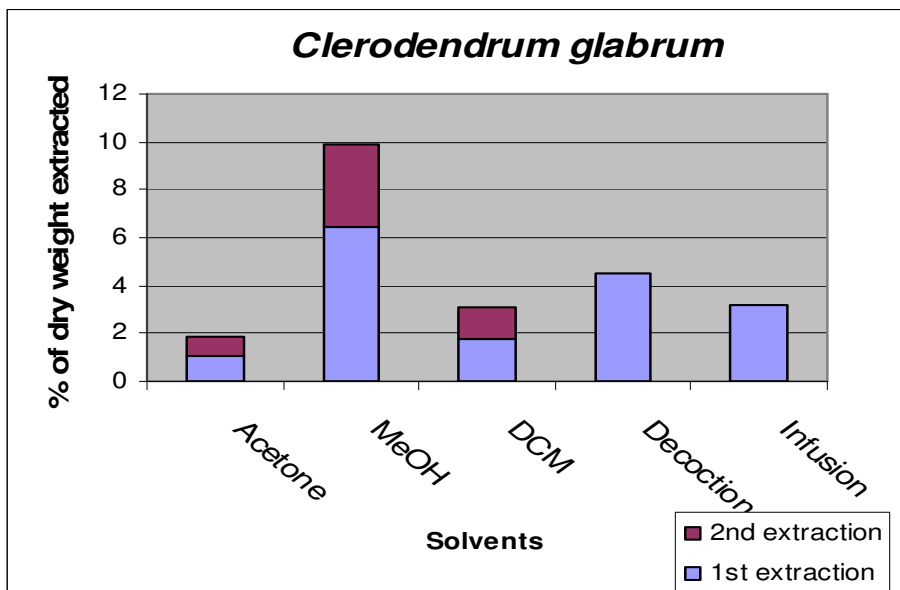


Figure 3.1B Percentage of dry weight extracted from *C. glabrum* and *J. curcas* leaves using five different solvents.

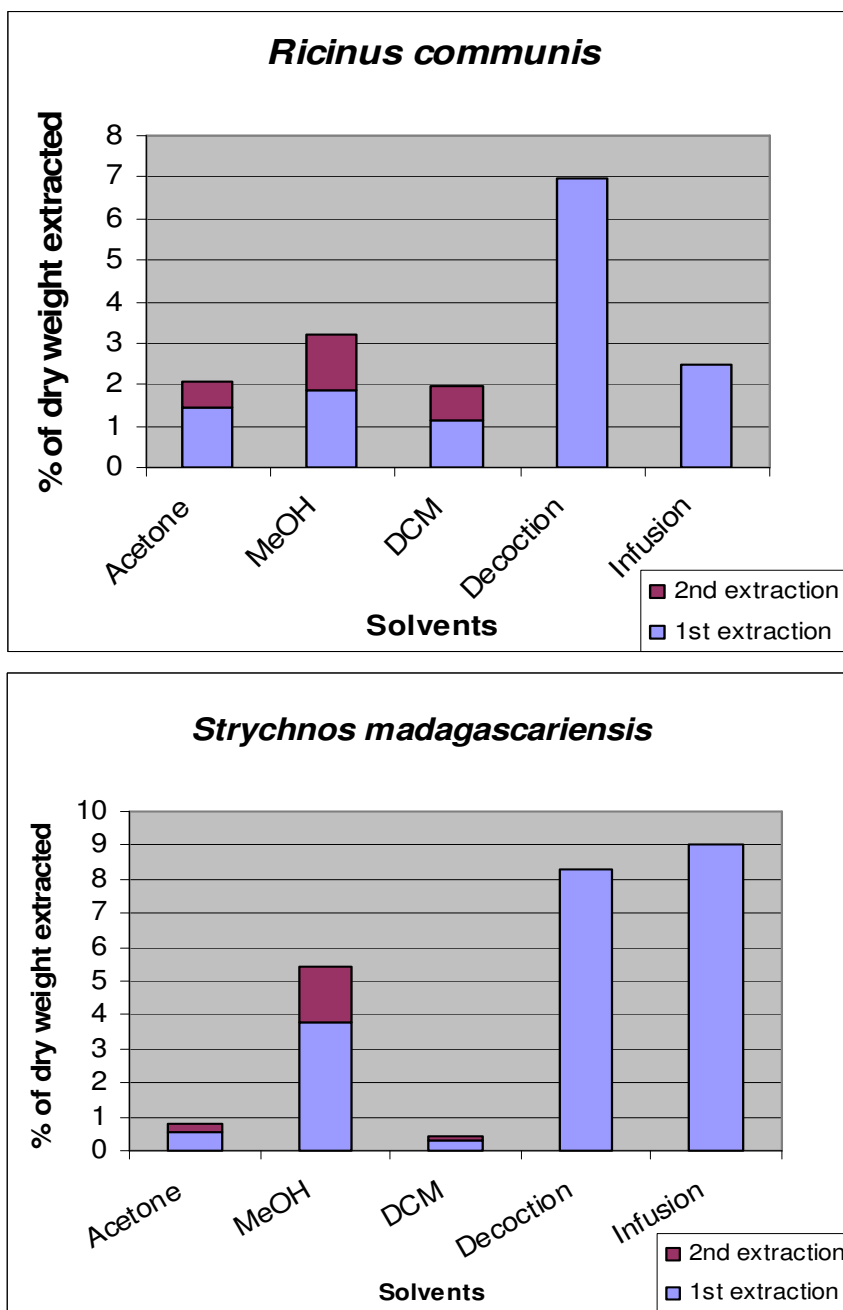


Figure 3.1C Percentage of dry weight extracted from *R. communis* and *S. madagascariensis* using five different solvents.

3.4 Discussion

Aqueous extracts in all plant species except in *J. curcas* and the soap infusion of *R. communis* had high percentage yields of dry weight extracted. The presence of water softeners found in surf soap (detergent) and the boiling water played a major role in increasing the extractability of the soap infusion and decoction respectively. They also have an influence in further destroying the membranes of the plant organelles left during the drying and finer grinding of the plant materials, making extraction more efficient (Eloff & McGaw 2006). If all the Surf components are non-volatile, residues left in the dried extract may be responsible for up to 4% of the dried extract obtained.

In the cold extraction technique, methanol was the best extractant compared to the intermediate polarity acetone and the non-polar dichloromethane. This is comparable to the previous results where methanol extracted a greater quantity of plant material from six *Terminalia* species than acetone, hexane and DCM (Masoko *et al.* 2005). As expected, most materials were extracted in the first extraction from all the plant species. The highest percentage of dry weight extracted was 15.8% of the soap infusion of *A. ferox*. All the organic extracts were below 7% when compared with the previous findings wherein the six solvents extracted in a range of 8-34% in the first extract of *Anthocleista grandiflora* and *Combretum erythrophyllum* (Eloff 1998a). The differences may be explained by different types of compounds present in the extracts or there may have been a difference in the mesh size of the ground material. In the next part the repellency of the extracts will be determined.

CHAPTER 4

REPELLENCY BIOASSAY OF EXTRACTS

4.1 Introduction

R. appendiculatus is a vector of tick-borne diseases as described in Section 1.3 of this thesis. Even though it has been eradicated in South Africa by 1954 it still forms part of the health and economic loss throughout the world and needs to be dealt with immediately (Fletcher 1984).

Different methods have been used to control ticks including repelling ticks from the host. Ticks follow a certain behavioural pattern when questing for hosts. Some may crawl on the ground and some prefer climbing up vegetation (Sonenshine 1991; Parola & Raoult 2001). Therefore it is of utmost importance to know the behaviour of the tick species before applying the bioassay. The selected three-host *Rhipicephalus appendiculatus* would prefer climbing up plants when questing for hosts. Thus, a climbing repellency bioassay would be suitable for this tick species.

In chapter six attempts to isolate and identify the compounds with activity against ticks are described. In this chapter tick repellency of extracts and of different fractions obtained in chapter six are presented.

4.2 Materials and methods

4.2.1 Rearing of developmental stages of ticks

Rabbits that served as hosts for the ticks were handled and cared for according to the norms established and accepted by the Medunsa Animal Care and Ethics Committee. One thousand nymphs and 600 adults of *Rhipicephalus appendiculatus* bought from Clinvet Ltd were bred at the Department of Biology (University of Limpopo - Medunsa campus). In the laboratory, the *R. appendiculatus* were kept at $25 \pm 1^\circ\text{C}$ and $75 \pm 5\%$ relative humidity (RH) in glass humidity chamber.

The breeding was performed at the Production Unit (formerly veterinary building) at the University of Limpopo. The rabbits were infested with the nymphs and after they were fully engorged, the engorged nymphs were collected from the hosts and incubated in the glass humidity chambers where moulting into the adult stage took place. The engorged nymphs moulted into adult *R. appendiculatus* within four days.

Some of the *R. appendiculatus* adults were taken to the ARC/OVI and kept at the quarantine's room with a relative humidity of $78 \pm 5^\circ\text{C}$ in glass humidity chamber with natural day and night regimen.

4.2.2 Development of repellency bioassay

In this project, a modified climbing repellency bioassay described by Mkolo and Magano (2007) was adopted to test for the repellent properties of *A. ferox*, *A. marlothii*, *C. glabrum*, *J. curcas*, *R. communis* and *S. madagascariensis* against adults of *R. appendiculatus*.

4.2.2.1 Climbing repellency bioassay set-up

An 80 ml glass beaker filled with polystyrene was firmly inserted in the centre of a 250 ml glass beaker. The polystyrene provided support to the vertically inserted glass rod (length 22.1 cm) and also served as a platform on which ticks were placed. Additional polystyrene was used to support the 80 ml glass beaker, preventing it from falling in the 250 ml glass beaker (Fig. 4.1). Water was poured into the 250 ml glass beaker to completely surround the 80 ml glass beaker and to almost reach its height. This was done to discourage ticks from crawling away from the polystyrene platform and to stabilise humidity. The top 5 cm of the glass rod was covered with Whatman number 1 filter paper (2.5 x 5 cm) on which the plant extract was released. The second filter paper of the similar kind and size was fixed just below the top one to serve as neutral filter paper (Fig. 4.2). No extract was released on this filter paper. The neutral filter paper provided an alternative questing place comparable with the treatment filter paper. Similarly, a control was set up with the appropriate solvent on the top filter paper. The solvent in both the treatment and control filter papers was allowed to evaporate for 15 minutes before the start of the experiment.



Figure 4.1 Repellency bioassay system composed of six 80 ml glass beakers fixed with glass rods inserted in the centre of six 250 ml glass beakers.

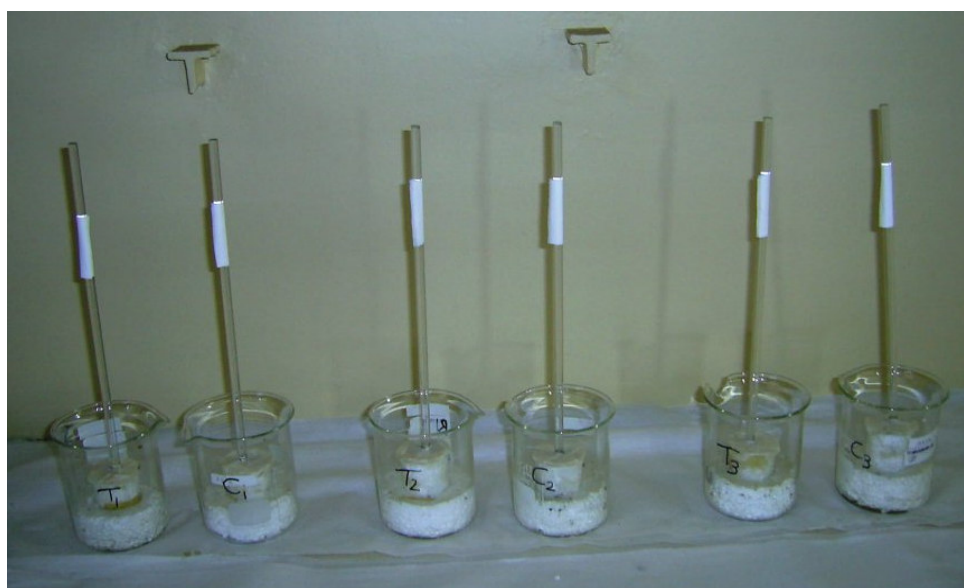


Figure 4.2 Repellency bioassay system showing the position of the neutral filter papers on the glass rods and the top 5 cm blank space for the treatment or control filter papers.

4.2.2.2 Experimental procedure

The climbing repellency bioassay was conducted as follows:

Ten unsexed *R. appendiculatus* adults were placed on a platform of the treatment apparatus and subsequently the same was done on the control apparatus. Prior to

the start of the experiment the ticks were allowed a 15 minute acclimatisation period, following which their position on the glass rod was noted at 10 minute intervals up to 60 minutes. After each 10 minute interval ticks on the glass rods were moved back to the polystyrene platform.

Ticks on the extract filter paper were considered not to be repelled by the extract and ticks found on the neutral filter paper or glass rod below the extract were considered to be repelled. Similarly ticks found on the negative solvent control were considered not repelled and ticks found on the neutral filter paper or glass rod below the negative solvent control were considered to be repelled by the solvent. The same argument held for the positive controls that were used. Ticks that dropped into surrounding water were dried using Kimberly-Clark paper towel and replaced onto the platform using forceps.

Different concentrations of the extracts (in the original solvent) and positive controls (Amitix and Bayticol dissolved in water) from 30% down to 5% based on activity found were used. Three replications were carried out for each concentration of the extracts.

4.2.3 Repellency data analysis

The number of ticks climbed up the glass rod within an hour during the repellency bioassay was counted and their mean number calculated and recorded. The percentage repellency was calculated using the following formula as adopted from Jantan and Mohd (1998).

$$\% \text{ Repellency} = 100 - \frac{[\text{mean number of ticks on test}]}{[\text{mean number of ticks on control}]} \times 100$$

The average percentage repellency was calculated per hour.

A non-parametric Mann-Whitney U test was selected as the main statistical analysis on the grounds that the data obtained were quantitatively discreet and distribution-free (Bland 1987).

4.3 Results

4.3.1 General results

The reaction of ticks to an acetone extract of *A. ferox* and decoction of *A. marlothii* illustrates typical results of extracts with repellent and non-repellent effects respectively (Fig 4.3A and B).

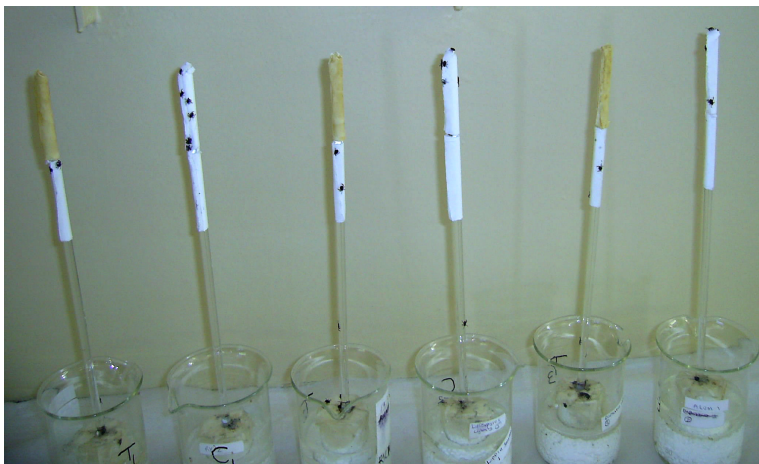


Figure 4.3A Example of a repellent extract. Treatment bioassays, the top orange colours are the filter papers coated with an *A. ferox* acetone extract. All the bottom filter papers are neutral with no extract or acetone applied. Control bioassays, uncoloured filter papers at the top were treated with acetone. Ticks were repelled from the treated filter papers to the neutral filter papers. No repellency was observed in control bioassays, i.e., ticks climbed up the rods to the top filter papers.



Figure 4.3B Example of a non-repellent *A. marlothii* decoction. Ticks were mainly found at the top filter papers in both control and treatment bioassays.

The average repellent response of *R. appendiculatus* subjected to five solvents used as negative controls were determined (Fig. 4.4). Ten ticks were subjected to repellency bioassay in three replicates but not all the ticks climbed up the rods. For instance, the average total number of ticks which climbed up the DCM bioassay rod was 4.165. An average number of 3.61 of these were not repelled and an average number of 0.555 were repelled as shown on the graph. For the rest of the controls, it can be seen that the average number of ticks which were repelled were less than the average number of ticks which were not repelled. Therefore, all the negative controls had a limited influence on tick repellency. In any case the repellency due to the solvent *per se* was deducted from the value obtained with the extracts in later analyses. DCM had the lowest average repellency followed by acetone, decoction, infusion and methanol. This may be explained by the volatility of the different solvents used and traces that may have remained on the filter paper.

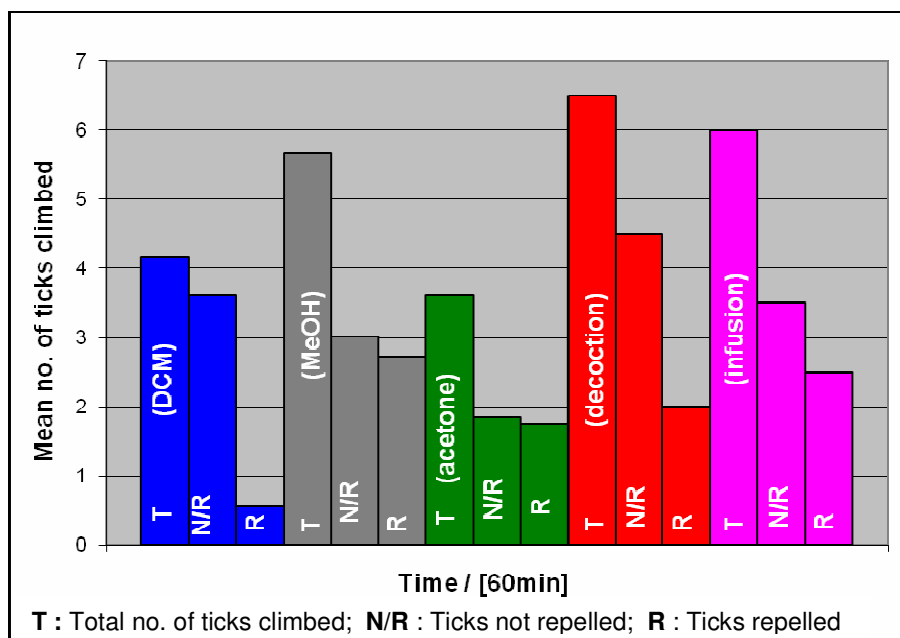


Figure 4.4A Average repellent response of *R. appendiculatus* adults to DCM, MeOH, acetone, decoction and soap infusion as negative controls.

Colour legends: Blue=DCM control, grey=MeOH, green=acetone, red=decoction and pink=infusion.

The repellent response of *R. appendiculatus* species subjected to the positive controls was also determined. The repellent response was presented in percentage repellency form because water was used as a control and also for dilution purposes in preparation for the 30% concentration. Amitix® had a 45% and Bayticol® had 89% percentage repellency (Fig. 4.4B). The variation found over the one hour time span is probably due to natural variation and there was little evidence of increased or decreased repellency within this period. The results found validates the efficacy of the method that we have used.

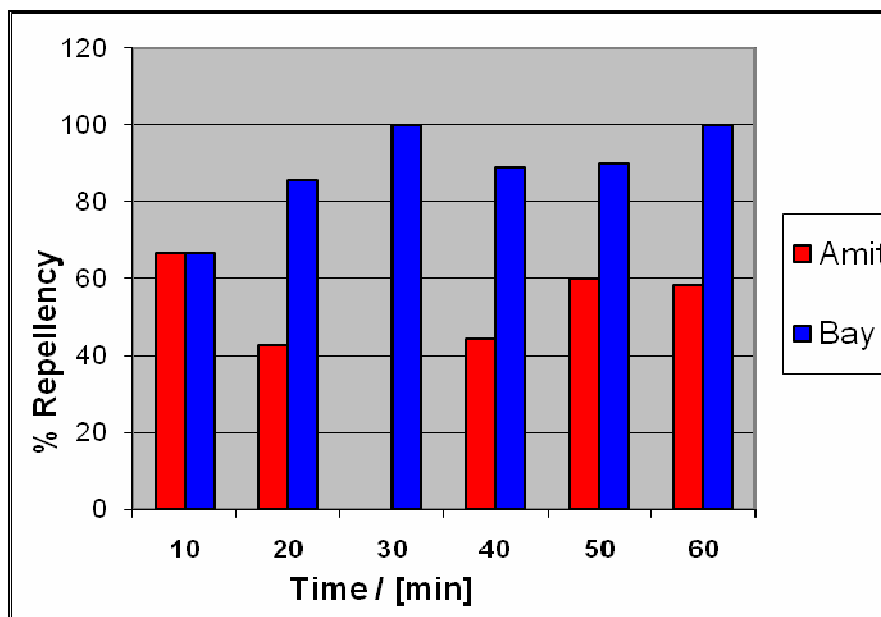


Figure 4.4B Percentage repellencies of a 30% concentration of the positive controls Amitix® and Bayticol® against *R. appendiculatus* adults as calculated by the formula adopted from Jantan and Mohd (1998), 89% for Bayticol® and 45% for Amitix®.

Colour legends: Red = Amitix® and blue = Bayticol®.

The repellency effects, variances and statistical analysis obtained with extracts of the six plant species is summarised in Table 4.1. The average percentage repellencies were shown by the red shaded area. The soap infusion had no repellency for any of the six plants. The average repellency of *S. madagascariensis* was 45.2% and the highest variance (47×10^{-3}) in the table suggested the inconsistency of the data points (Milton 1992). The Mann – Whitney U test proved that the difference is statistically significant at $P \leq 0.05$ (Table 4.1).

Table 4.1 Summary of the repellency effects and statistical results obtained with a 30% concentration of six plant species. The red shaded area show the average percentage repellencies of the ticks which were repelled from the top filter paper strips with $p \leq 0.05$. No statistically significant repellency was obtained with the soap water extract.

Plant species		Decoction	Infusion	MeOH	Acetone	DCM
<i>A. ferox</i>	Effect	Does not repel	Does not repel	Does not repel	Repels	Does not repel
	Ave % repellency	17.83	34.83	12.38	60.67	-112.83
	Variance ($\times 10^{-3}$)	2.00	12.0	9.88	8.0	18.2
	Mann-Whitney U test	$P \geq 0.05$	$P \geq 0.05$	$P \geq 0.05$	$P \leq 0.05$	$P \geq 0.05$
<i>A. marlothii</i>	Effect	Does not repel	Does not repel	Repels	Repels	Repels
	Ave % repellency	25.1	27.8	50.2	58.9	73
	Variance ($\times 10^{-3}$)	20.1	11.0	11.0	30.0	4.50
	Mann-Whitney U test	$P \geq 0.05$	$P \geq 0.05$	$P \leq 0.05$	$P \leq 0.05$	$P \leq 0.05$
<i>C. glabrum</i>	Effect	Does not repel	Does not repel	Does not repel	Repels	Does not repel
	Ave % repellency	33.7	8.95	11	87.3	5.92
	Variance ($\times 10^{-3}$)	2.90	1.23	24.2	18.1	8.40
	Mann-Whitney U test	$P \geq 0.05$	$P \geq 0.05$	$P \geq 0.05$	$P \leq 0.05$	$P \geq 0.05$
<i>J. curcas</i>	Effect	Does not repel	Does not repel	Repels	Does not repel	Does not repel
	Ave % repellency	25.5	3.67	79.7	15.2	20.5
	Variance	6.01	6.21	11.0	9.00	8.21
	Mann-Whitney U test	$P \geq 0.05$	$P \geq 0.05$	$P \leq 0.05$	$P \geq 0.05$	$P \geq 0.05$
<i>R. communis</i>	Effect	Does not repel	Does not repel	Does not repel	Does not repel	Repels
	Ave % repellency	-1.33	-27.4	19.6	-50.33	87.5
	Variance ($\times 10^{-3}$)	2.00	7.80	9.88	45.2	22.0
	Mann-Whitney U test	$P \geq 0.05$	$P \geq 0.05$	$P \geq 0.05$	$P \geq 0.05$	$P \leq 0.05$
<i>S. madagascariensis</i>	Effect	Repels	Does not repel	Does not repel	Does not repel	Does not repel
	Ave % repellency	45.2	1.33	-38	17	-54.33
	Variance ($\times 10^{-3}$)	47.0	4.66	1.00	6.20	0.89
	Mann-Whitney U test	$P \leq 0.05$	$P \geq 0.05$	$P \geq 0.05$	$P \geq 0.05$	$P \geq 0.05$

4.3.2 Description of results

4.3.2.1 *Aloe ferox*

Five different crude extracts of *A. ferox* made using different solvents with a 30% concentration were tested on repellency bioassay (Fig. 4.5). The repellent response of *R. appendiculatus* to all extracts subjected to repellency bioassay is shown in Fig. 4.6. Ticks were able to tolerate any remaining odour of extracts except for the DCM extract. Ticks spent most of their times in the DCM extract as illustrated in Fig. 4.5 and this would connote an attraction. Ticks were initially attracted to the decoction from the first 20 minutes of the experiment and they were ultimately repelled (Fig. 4.5). Acetone extract had an efficacy with an average repellency of 60.67% (Table 4.1). The Mann – Whitney U test statistical results showed a significant difference between the effects of an acetone extract and a control ($P \leq 0.05$). The rest of the extracts did not have any statistical significant difference. As the concentration of acetone extract decreased to 20%, repellency activity was lost and the percentage repellency dropped to 37% (Fig. 4.7; Table 4.1).

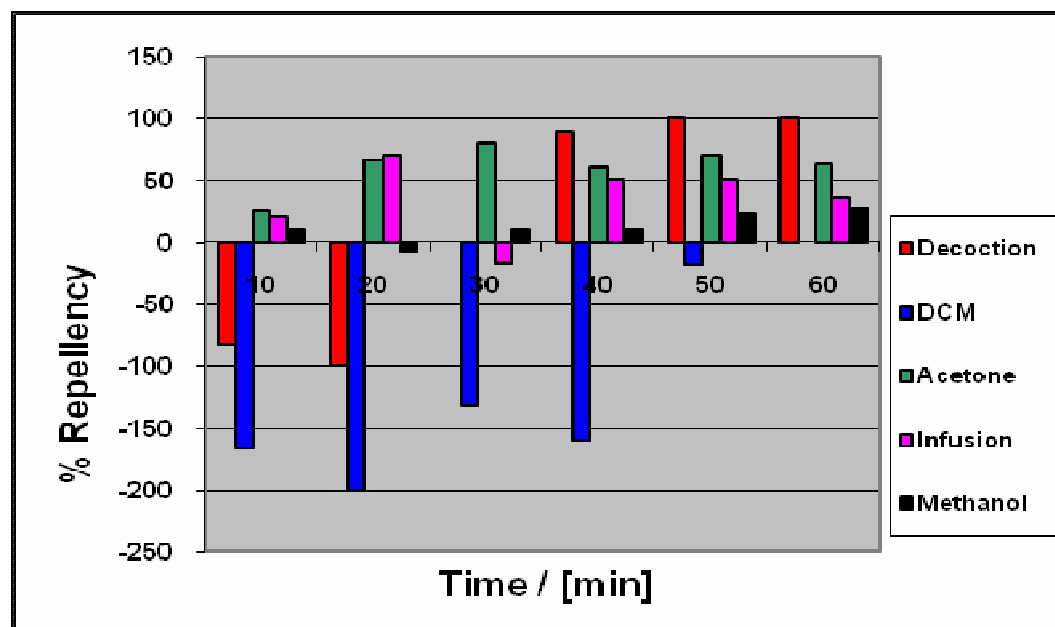


Figure 4.5 The repellent response of *R. appendiculatus* adults to different 30% crude extracts of *A. ferox* as calculated by the formula adopted from Jantan and Mohd (1998).

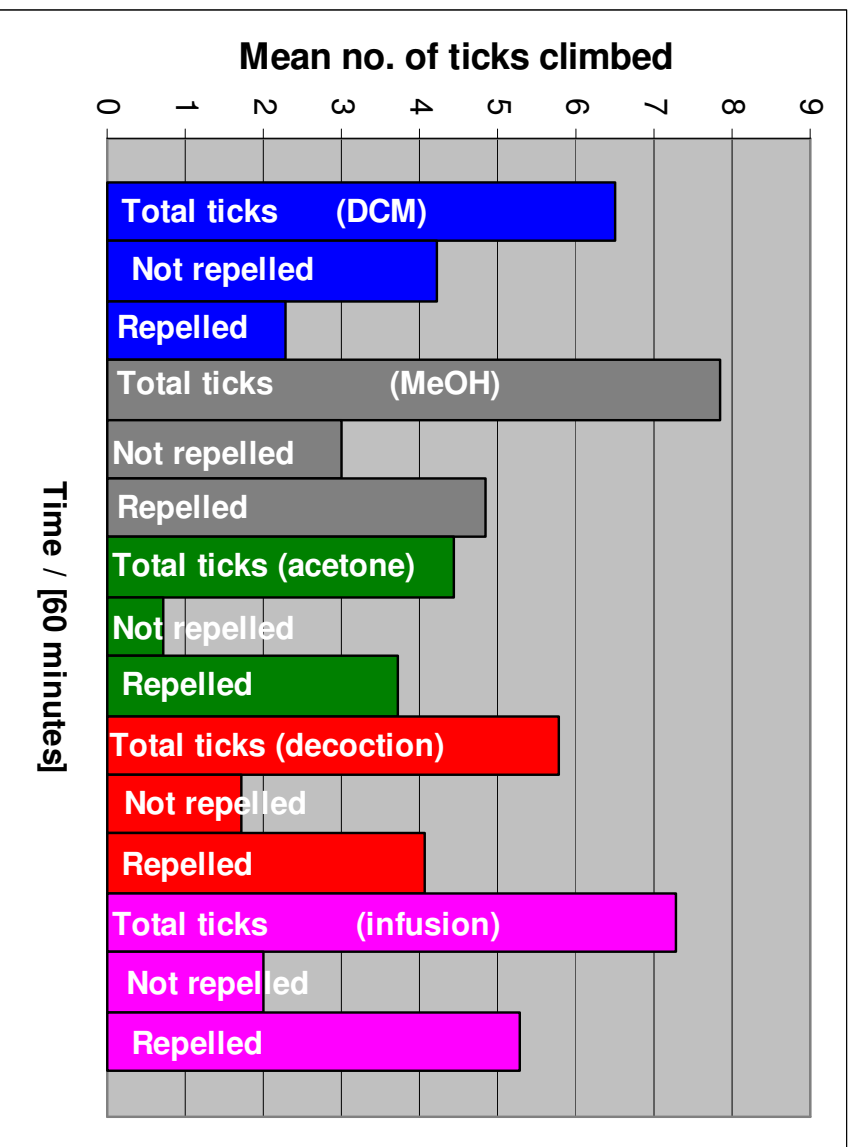


Figure 4.6 Graphical representation of the average number of *R. appendiculatus* adult ticks repelled by 30% *A. ferox* extracts.

Colour legends: Blue=DCM, grey=MeOH, green=acetone, red=decoction and pink=infusion.

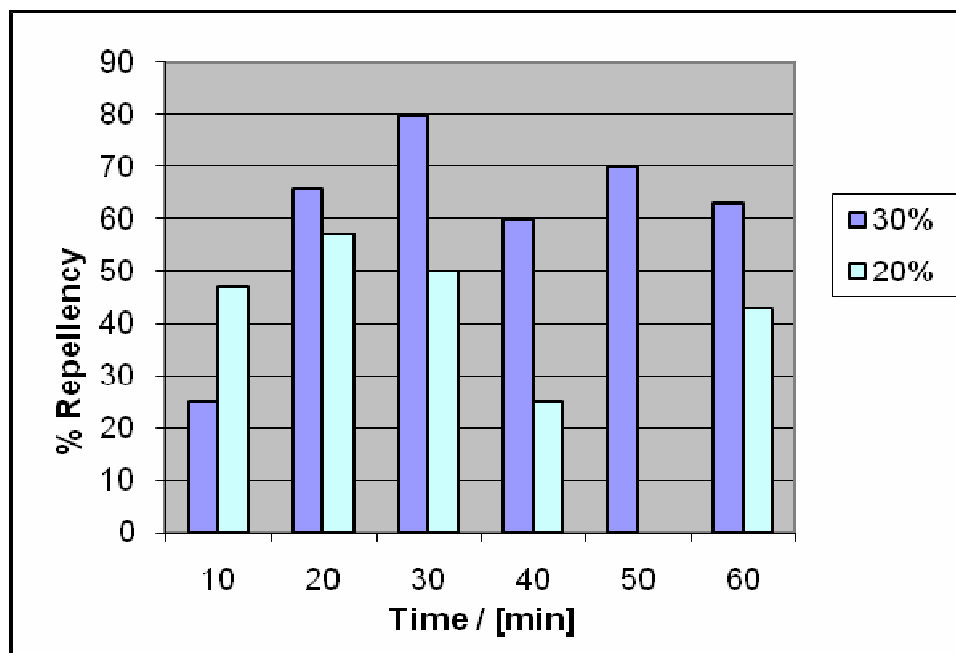


Figure 4.7 Graphical representation of the repellent response of *R. appendiculatus* adults to 20 and 30% *A. ferox* crude acetone extracts at different times.

4.3.2.2 *Aloe marlothii*

All the organic 30% crude extracts of *A. marlothii* were active in repelling the ticks as shown from their average number climbing up the glass rods Fig. 4.8A. The percentage repellencies of all 30% crude extracts were determined (Fig. 4.8B). The active extracts had 58.9, 50.2 and 73% repellency for acetone, MeOH and DCM extracts respectively (Table 4.1). The Mann-Whitney U test showed that there was a significant difference ($P \leq 0.05$, two-tailed test) for the active 30% extracts.

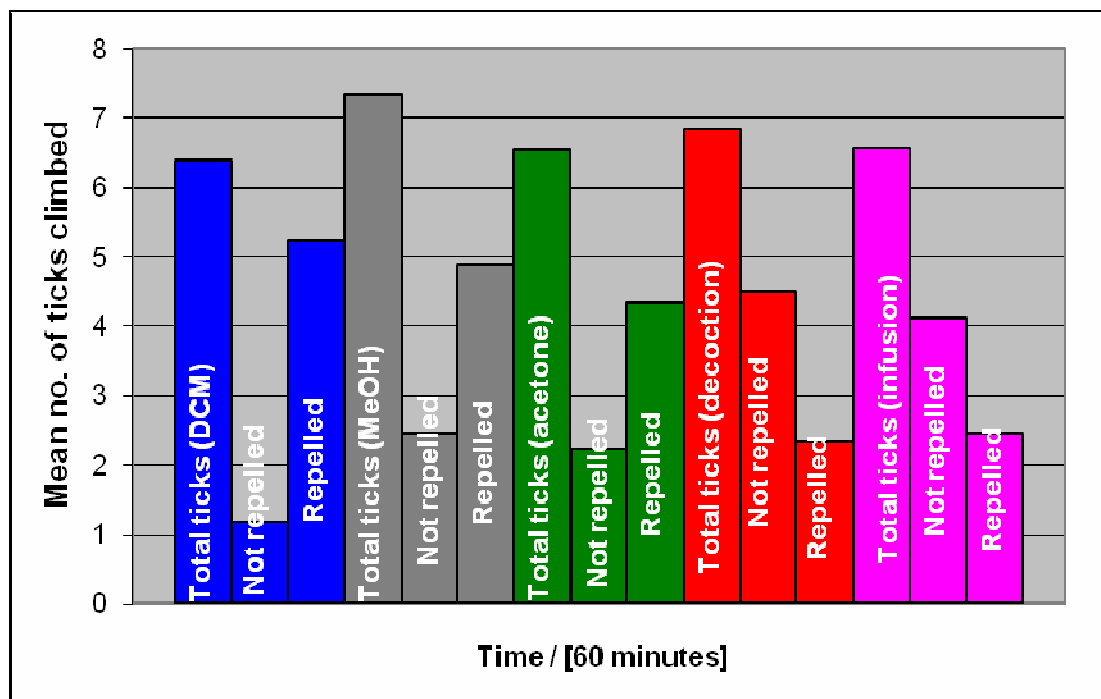


Figure 4.8A Graphical representation of the average number of *R. appendiculatus* adult ticks repelled by 30% crude extracts of *A. marlothii*.

Colour legends: Blue=DCM, grey=MeOH, green=acetone, red=decoction and pink=infusion.

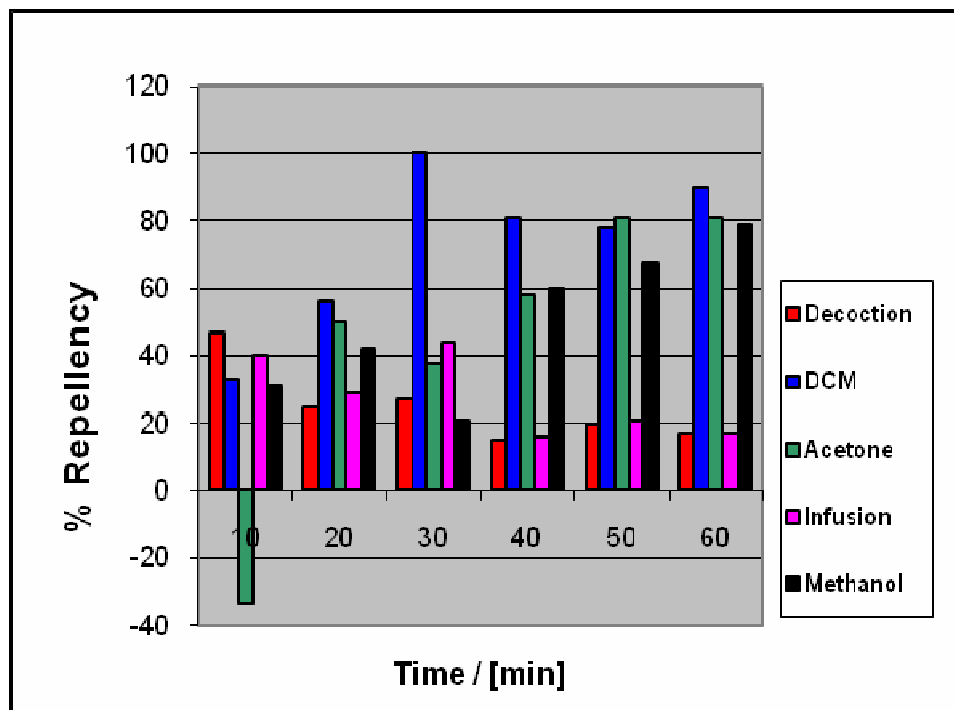


Figure 4.8B Repellent percentages of 30% crude extracts of *A. marlothii* on *R. appendiculatus* adults (Jantan & Mohd 1998)

The acetone extract lost activity at 5% concentration level with a calculated percentage repellency of 42. A trendline graph was drawn and the effective concentration to repel about 50% ticks was extrapolated, $EC_{50} = 8.5\%$ (Fig. 4.9).

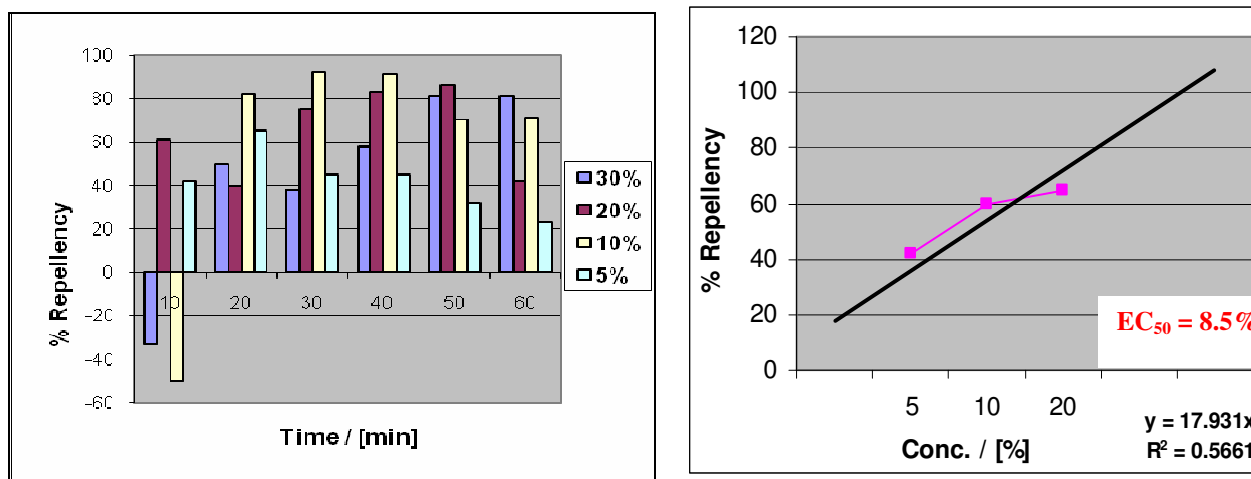


Figure 4.9 Representation of repellent percentages of three different concentrations of *A. marlothii* crude acetone extracts and the trendlined graph where the EC_{50} value of 8.5% was extrapolated.

The crude DCM extract lost repellency activity at 10% with percentage repellency of 33 and EC_{50} value of 16% (Fig. 4.10).

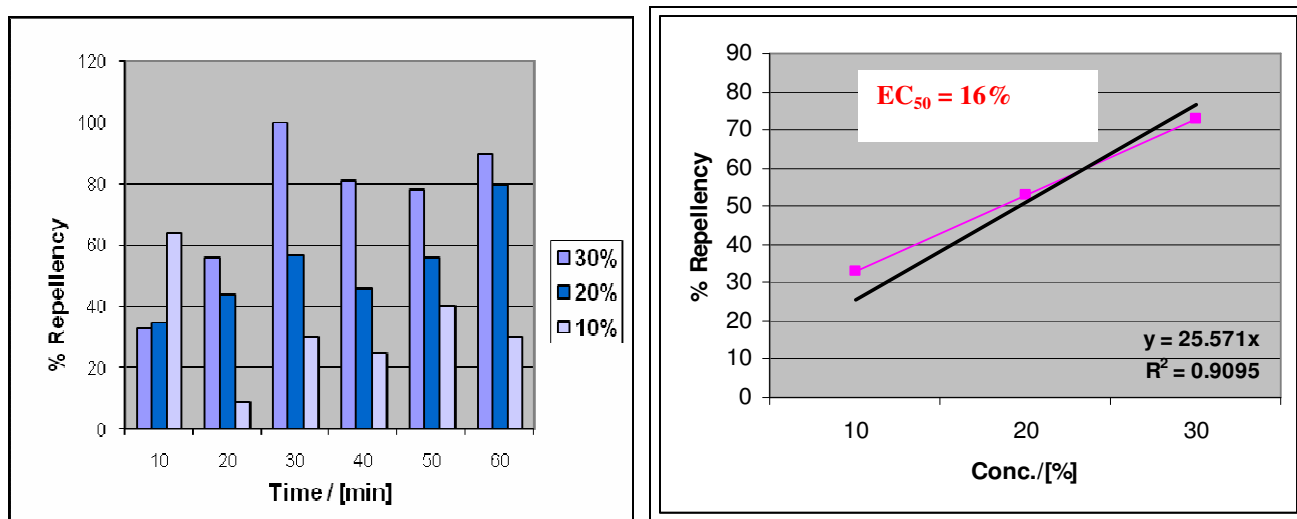


Figure 4.10 Repellent percentages of three different concentrations of *A. marlothii* crude DCM extracts and the trendlined graph where the EC_{50} value of 16% was extrapolated

Both the acetone and dichloromethane extracts had tick repellency properties. The repellency effect was more pronounced from the acetone extract ($EC_{50} = 8.5\%$) than from the DCM extract ($EC_{50} = 16\%$). This provides some information on the nature of the repellents present in *A. marlothii* extract. Since acetone is more polar than DCM, and the compounds found in the acetone extract have a stronger activity, the repellents are possibly slightly polar or some slightly polar compounds have a synergistic effect with the non-polar compounds.

The excellent LD_{50} of 8.5% for the acetone extract is very promising. Unfortunately at higher concentration the inhibition tapers off. This makes the acetone extract from *A. marlothii* less interesting to test in the field although the EC_{50} was higher.

4.3.2.3 *Clerodendrum glabrum*

Crude extracts

Due to the good activity obtained extracts of *C. glabrum* were evaluated in much more detail.

Repellency response of *R. appendiculatus* adults to all 30% crude extracts of *C. glabrum* was determined (Figure 4.11A). DCM and acetone extracts had an average repellency efficacy of 5.92% and 87.3% respectively (Fig. 4.11B; Table 4.1). The acetone extract differed significantly ($P \leq 0.05$, two-tailed test) but not the DCM extract ($P \geq 0.05$ (two-tailed test)) (Table 4.1).

It was surprising that the methanol extract attracted the ticks 100% after one hour. It was also noteworthy that the decoction of *C. glabrum* initially had a good repellency against the ticks Figure 4.11B. Because this decreased after 50 minutes, the differences were not statistically significant (Table 4.1). These results may substantiate the ethnoveterinary use of *C. glabrum*. The excellent repellency of the 30% crude acetone extract led to evaluating activities at lower concentrations after different times.

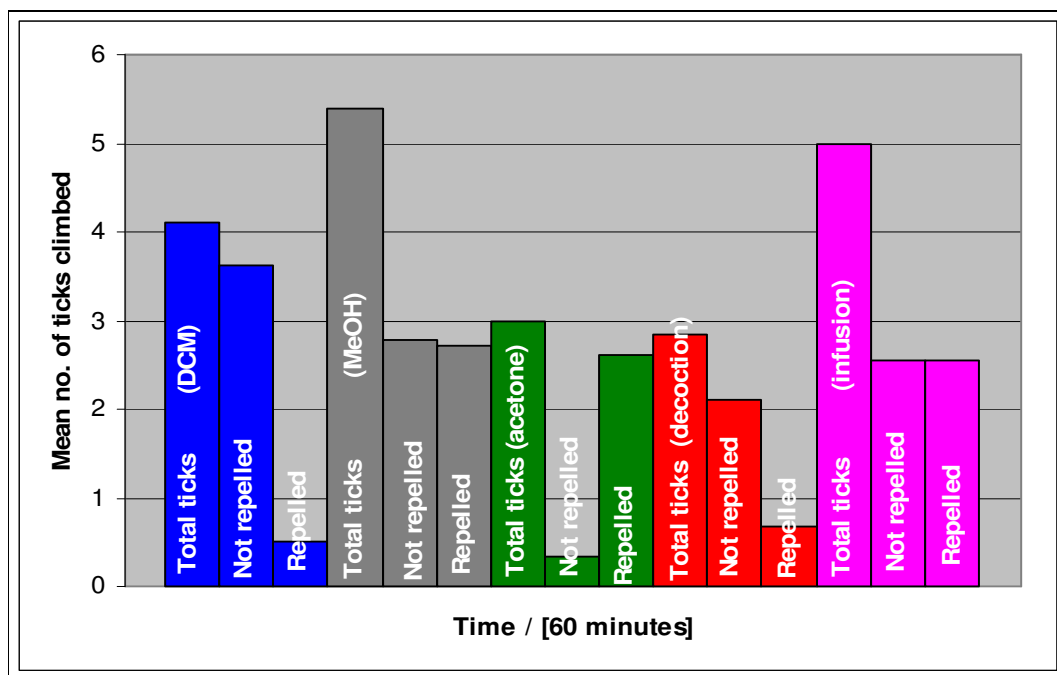


Figure 4.11A Graphical representation of the average number of *R. appendiculatus* adult ticks repelled by 30% crude extracts of *C. glabrum*.

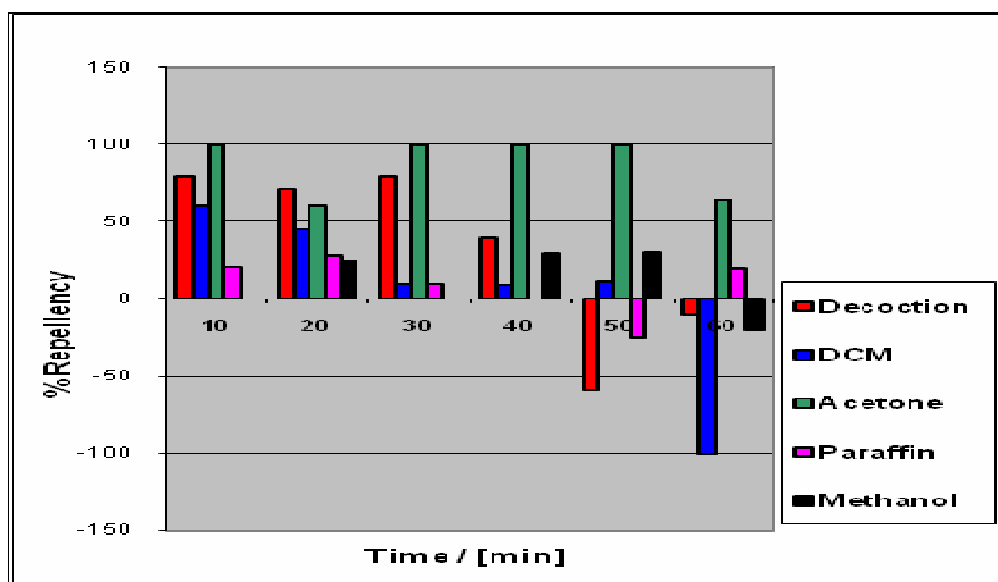


Figure 4.11B Percentage repellency of 30% crude extracts of *C. glabrum* against *R. appendiculatus* adults (Jantan & Mohd 1998)

The 30% acetone extract retained 100% repellency up to 50 minutes. As the concentration decreased to 5%, the extract lost its average repellency activity. The EC_{50} of 4.9% was calculated from the trend line graph (Fig. 4.12).

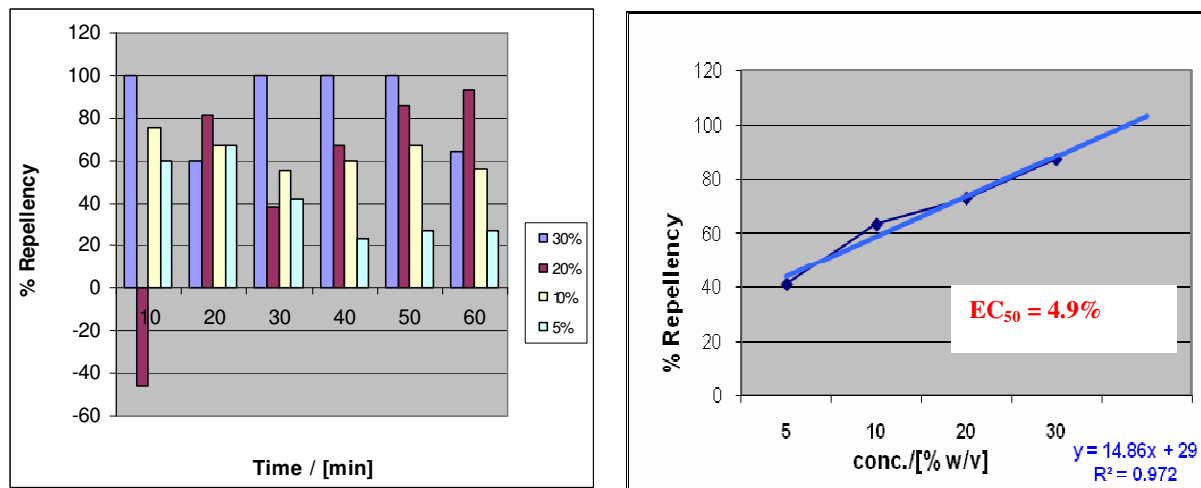


Figure 4.12 Repellent effects of four different concentrations of *C. glabrum* crude acetone extracts and the trendlined graph where EC_{50} value of 4.9% was determined.

Activity of acetone fraction subjected to solvent-solvent fractionation

Percentage repellencies of six different fractions of *C. glabrum* acetone extract were determined (Fig. 4.13A). The hexane solvent-solvent fraction had an average repellency of 96.2%, followed by 35% water in methanol fraction (36.8%), chloroform (24.0%), butanol (18%), water (14.3%) and carbon tetrachloride (9.27%) (Fig. 4.13B). From the data in Fig. 4.13B it is clear that the active principles are highly non polar. The value for the hexane fraction was also much better than the repellency for the two positive controls, Bayticol (89%) and Amitix (45%) (Fig. 4.4B).

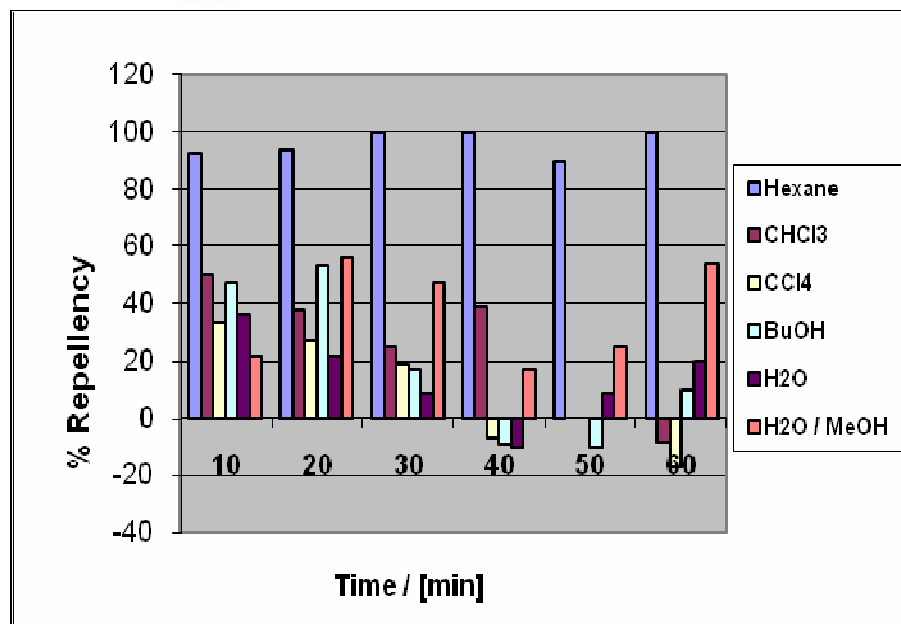


Figure 4.13A Percentage repellencies of 30% solvent-solvent fractions of *C. glabrum* acetone extract against *R. appendiculatus* adult ticks.

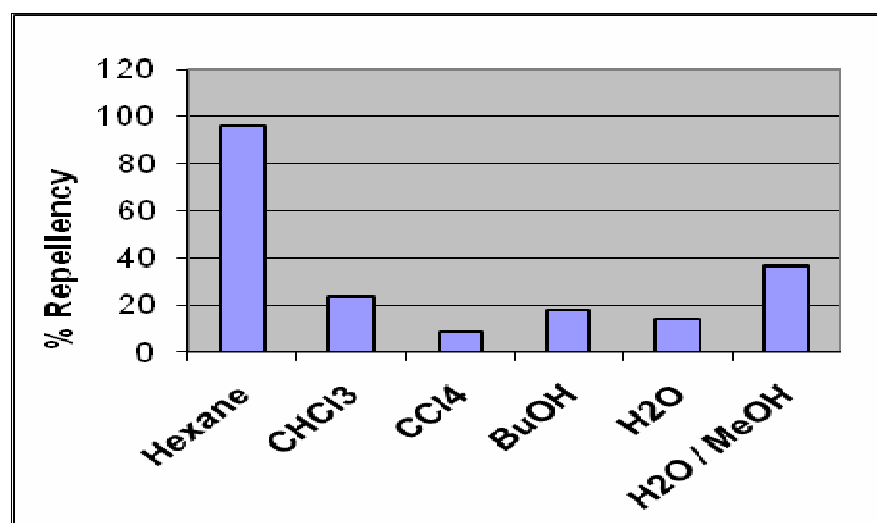


Figure 4.13B Average repellency percentage of 30% solvent-solvent fractions of *C. glabrum* acetone extract against *R. appendiculatus* adult ticks.

To determine the effectivity of lower concentrations the experiment was repeated with a 20% concentration. The average repellency dropped to 40% (Fig. 4.14).

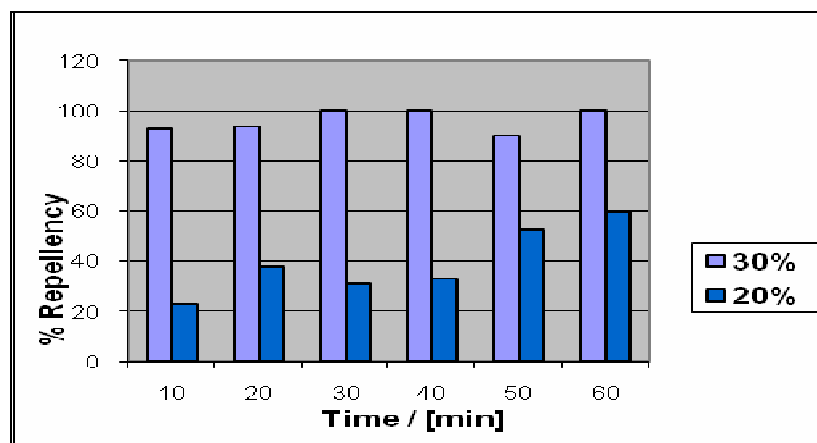


Figure 4.14 Percentage repellencies of two concentrations of hexane fraction of *C. glabrum* against *R. appendiculatus* adults.

Evaluating volatility of the extracts

Another aspect to be considered was how long the repellency activity would last. Five different drying times up to three hours of the filter paper coated with 30% hexane fraction were explored and the percentage repellencies were determined at the end (Fig. 4.15A). The average percentage repellencies were calculated (Fig. 4.15B). As could be expected the 0.5 h drying time gave the highest repellency of 96.17% dropping to 58% after 2.5 h and 18% after 3 h.

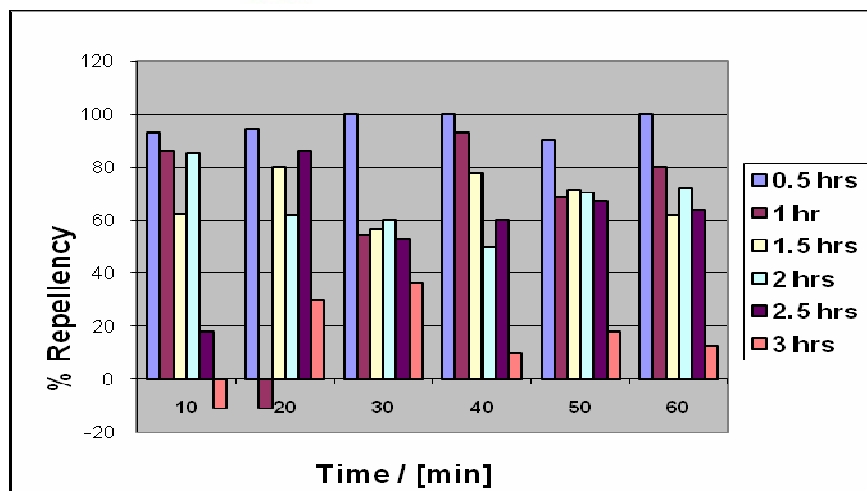


Figure 4.15A Percentage repellency of 30% hexane solvent-solvent fractions of *C. glabrum* acetone extract against *R. appendiculatus* adults when the experimental filter papers were dried at different drying times.

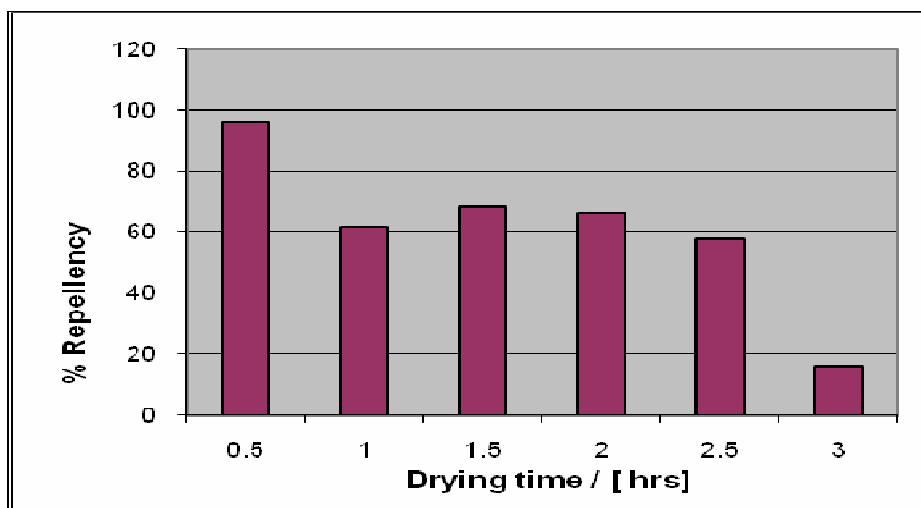


Figure 4.15B Average percentage repellencies of *C. glabrum* hexane fractions against *R. appendiculatus* adults.

Evaluation of vacuum liquid chromatographic separation fractions

The hexane fraction was further subjected to vacuum liquid chromatography (VLC).

The four subfractions obtained were tested on repellency bioassay and the percentage repellencies were determined (Fig. 4.16). Ethyl acetate gave the highest

repellency activity of 73.6% followed by acetone in hexane (65.9%), hexane (15.0%) and methanol (12.5%).

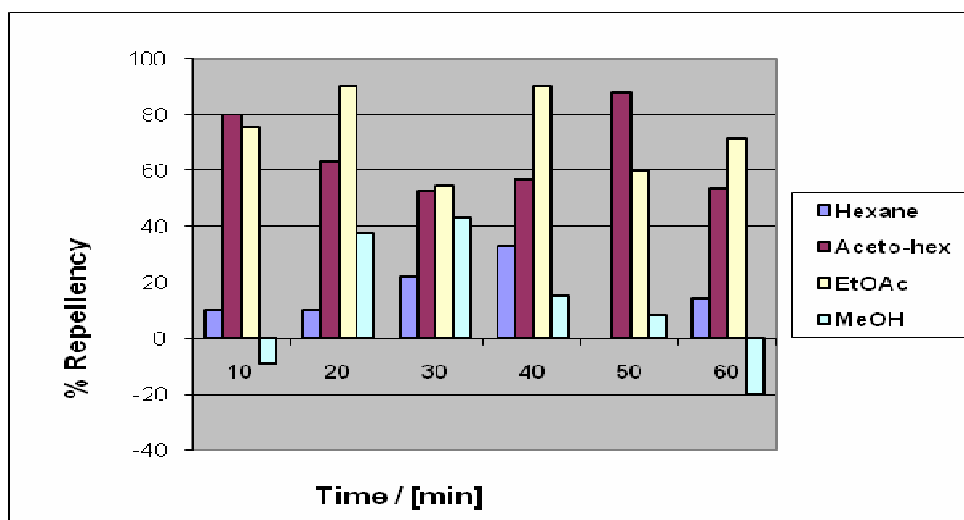


Figure 4.16 Percentage repellencies of 30% hexane subfractions of *C. glabrum* against *R. appendiculatus* adults.

Acronyms: Aceto-hex = acetone in hexane, EtOAc = ethyl acetate and MeOH = methanol.

Further fractionation

Repellencies were determined after further fractionation of the ethyl acetate subfraction from the previous round by VLC (Fig. 4.17). All the next subfractions had some activity; DCM had the highest repellency activity of 73.6% followed by hexane (65.5%), 50% DCM/ethyl acetate (58.7%) and ethyl acetate (57.2%).

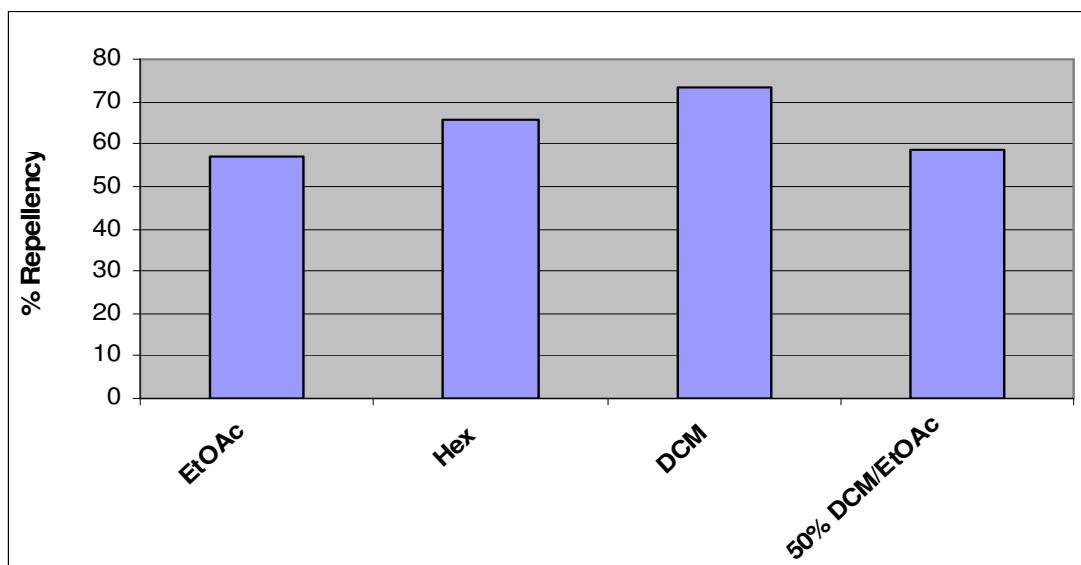


Figure 4.17 Percentage repellencies of 30% hexane second subfractions of *C. glabrum* against *R. appendiculatus* adults.

Acronyms: Hex = hexane, DCM = dichloromethane and 50% DCM/EtOAc = 50% dichloromethane in ethyl acetate.

Summary of discussion

To summarise this section, ticks were repelled by the acetone extract of *C. glabrum*. Six different fractions were made from the acetone extract and the hexane fraction gave the highest activity. It was the only fraction active at a concentration of 30%. Five different drying times of the fraction-coated filter paper strips were explored to detect potency and duration of activity of the hexane fraction. At 2½ hours, the fraction still remained active. Because of their volatility, repellents decrease in concentration for a period of 2½ hours after extract application. This indicates a limitation in the practical use of extracts although the activities were very much higher than that of the commercially used products.

Fractionation of the hexane fraction was conducted and hexane sub-fraction still retained the activity, meaning that the non-polar compounds of the *C. glabrum* are the active repellents.

4.3.2.4 *Jatropha curcas*

Only the methanol extract had an interesting activity (Figs. 4.18A, B; Table 4.1). This is interesting because it means that the less polar i.e., less volatile compounds are involved. Unfortunately at a lower concentration 20% there was no repellency and actually a substantial attraction of the ticks after 30 minutes (Fig. 4.19).

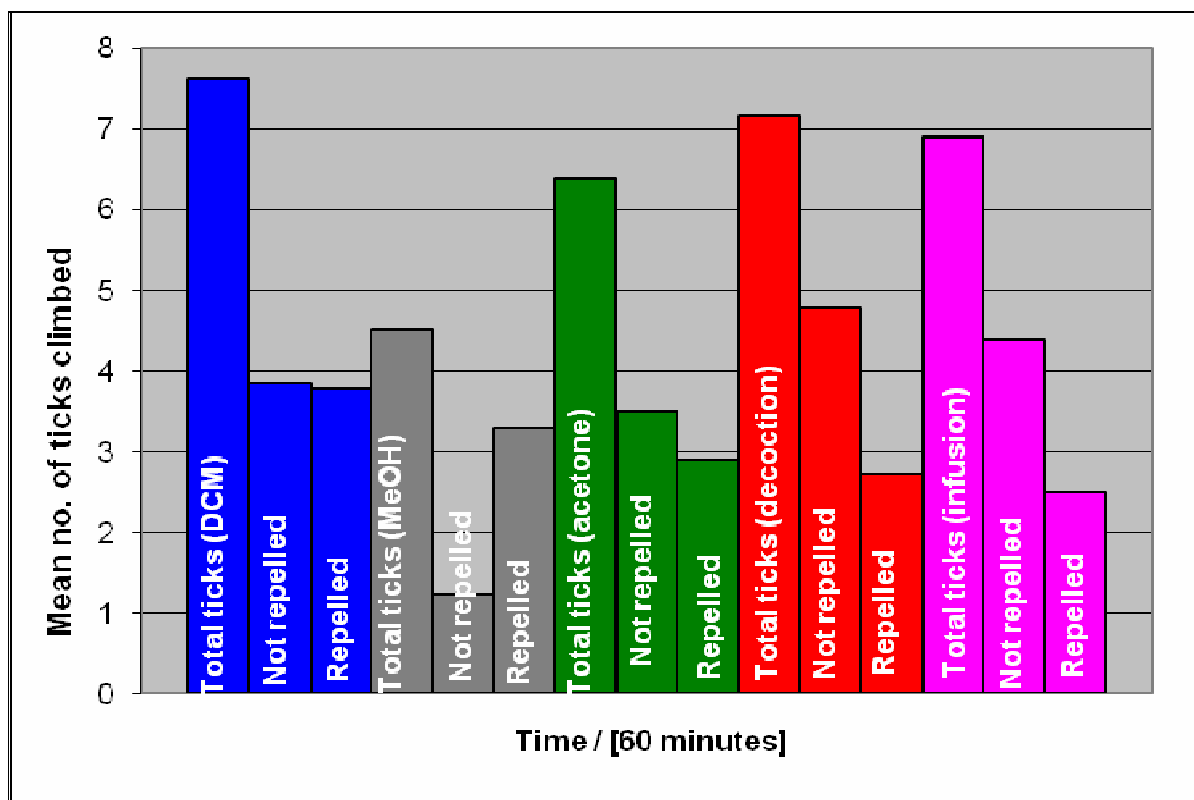


Figure 4.18A Graphical representation of the average number of *R. appendiculatus* adults repelled by 30% crude extracts of *J. curcas*.

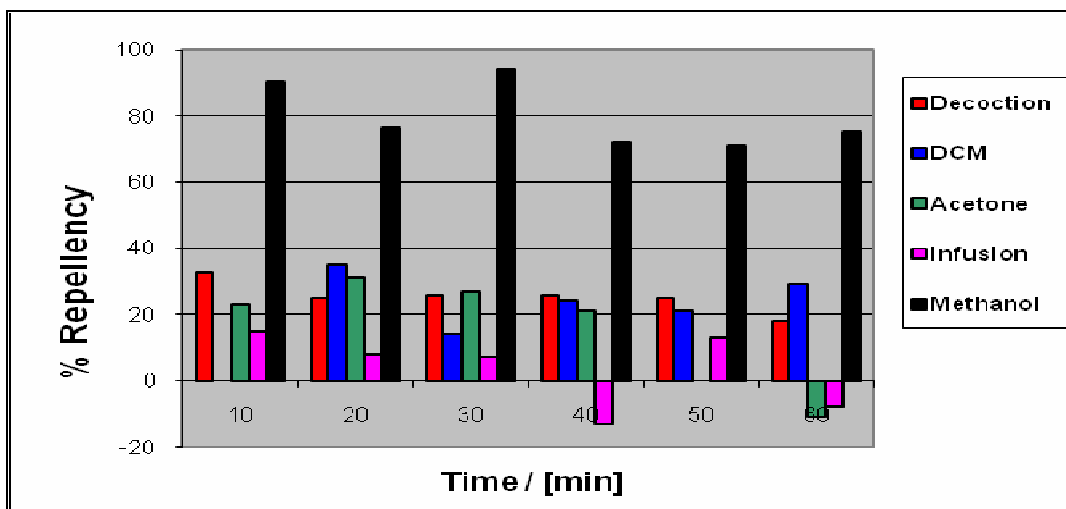


Figure 4.18B Repellent percentages of 30% crude extracts of *J. curcas* against *R. appendiculatus* adults (Jantan and Mohd 1998).

Statistical results of MeOH extracts have a significant difference i.e., $P \leq 0.05$. As the concentration of the methanol extract decreased to 20%, the extract lost its activity and the percentage repellency dropped to -21% (Fig. 4.19).

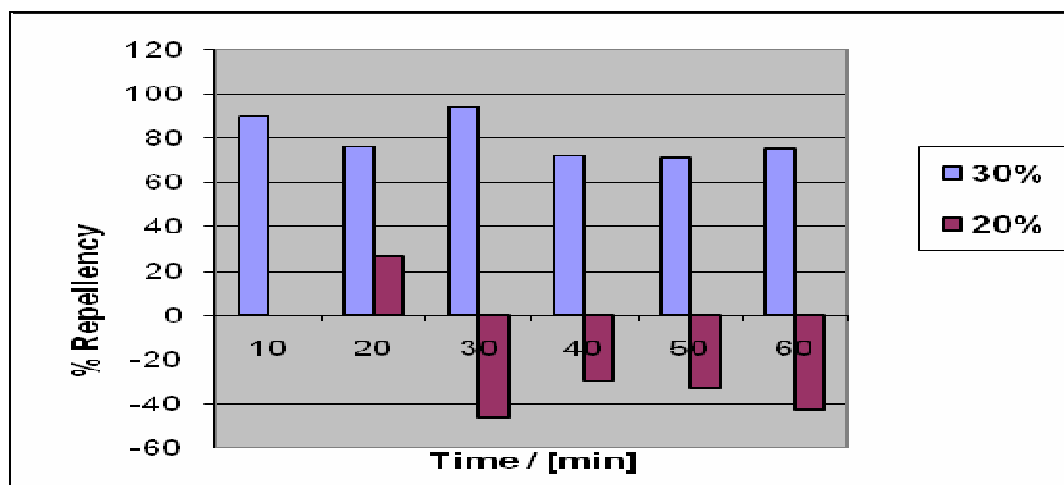


Figure 4.19 Repellent percentages of the crude MeOH extracts of *J. curcas* against *R. appendiculatus* adults.

4.3.2.5 *Ricinus communis*

The results with *R. communis* extracts were surprising because acetone extract had an attraction to the ticks within the first half hour and especially within the first 10 minutes (Figs. 4.20A, B). The DCM extract had the highest activity and the repellency was retained up to an hour and the 20% concentration was also effective (Fig. 4.21).

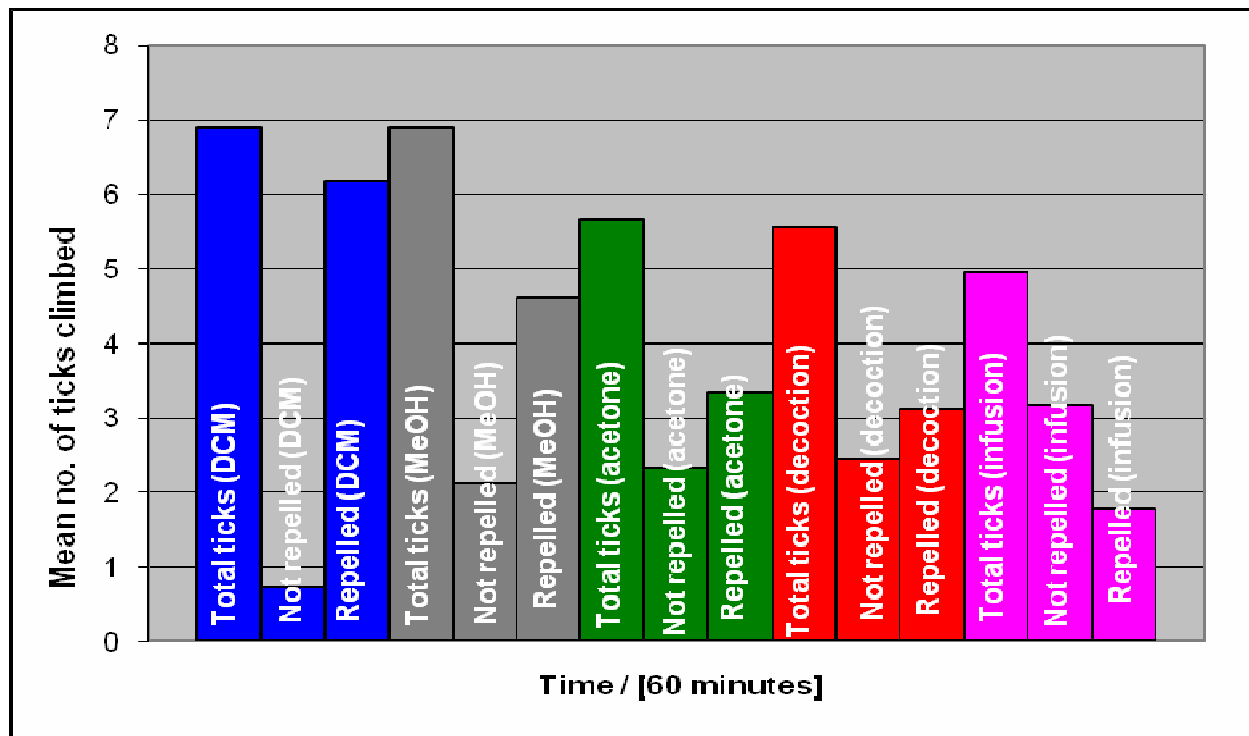


Figure 4.20A Graphical representation of the average number of *R. appendiculatus* adults repelled by 30% crude extracts of *R. communis*

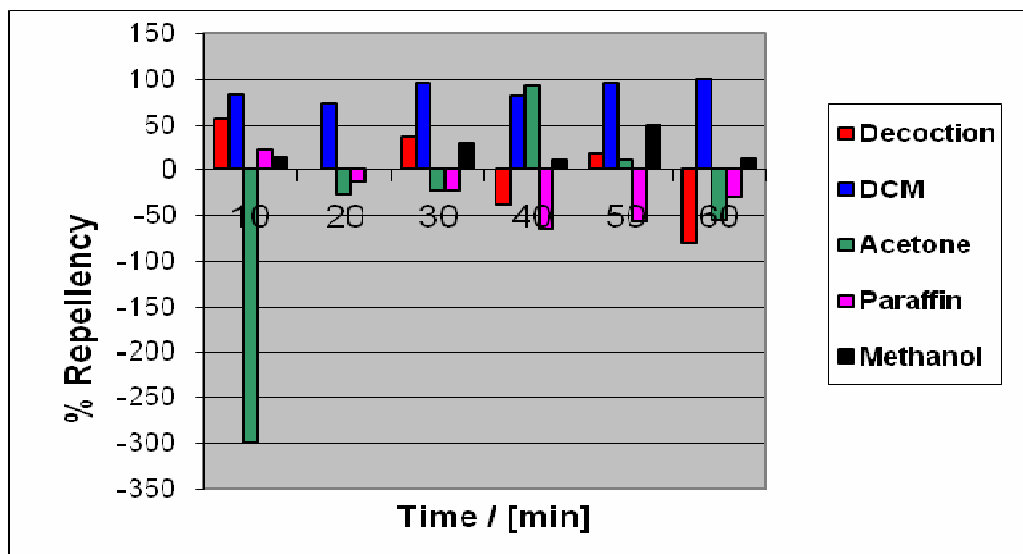


Figure 4.20B Repellent percentages of 30% crude extracts of *R. communis* against *R. appendiculatus* adults

The p-value of the DCM extract showed a significant difference between the control and test, i.e., $P \leq 0.05$, two-tailed test. The DCM crude extract lost its activity at 10% and the percentage repellency dropped to 40.83% (Fig. 4.21).

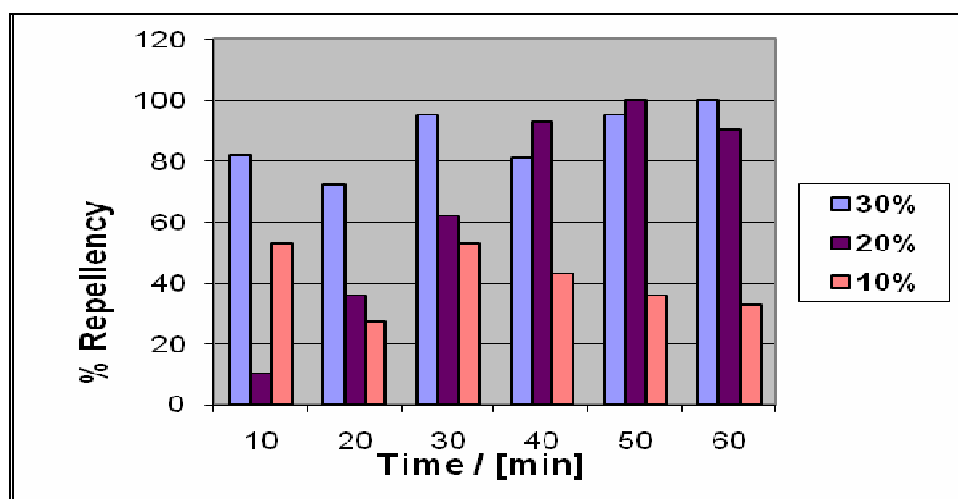


Figure 4.21 Repellent percentages of three different concentrations of *R. communis* crude DCM extracts. Repellent activity was lost at 10%.

4.3.2.6 *Strychnos madagascariensis*

The average number of *R. appendiculatus* adults repelled by 30% crude extracts of *Strychnos madagascariensis* are presented in Fig. 4.22A. In this case DCM and methanol extracts attracted the ticks. The best repellency results were obtained with the decoction of the species investigated followed by the acetone extract. This is the only plant where the traditional method of extraction gave the best results. The decoction in an aqueous medium would probably extract non volatile polar compounds.

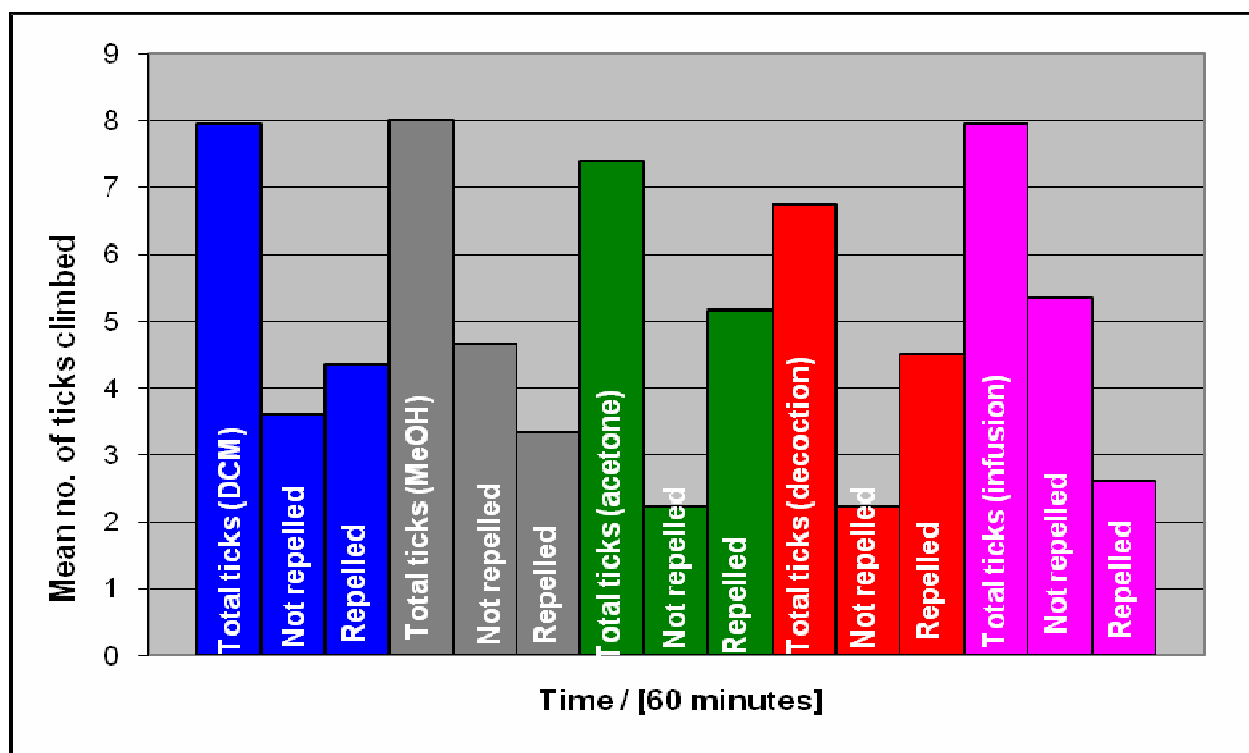


Figure 4.22A The average number of *R. appendiculatus* adult ticks repelled by 30% crude extracts of *S. madagascariensis*.

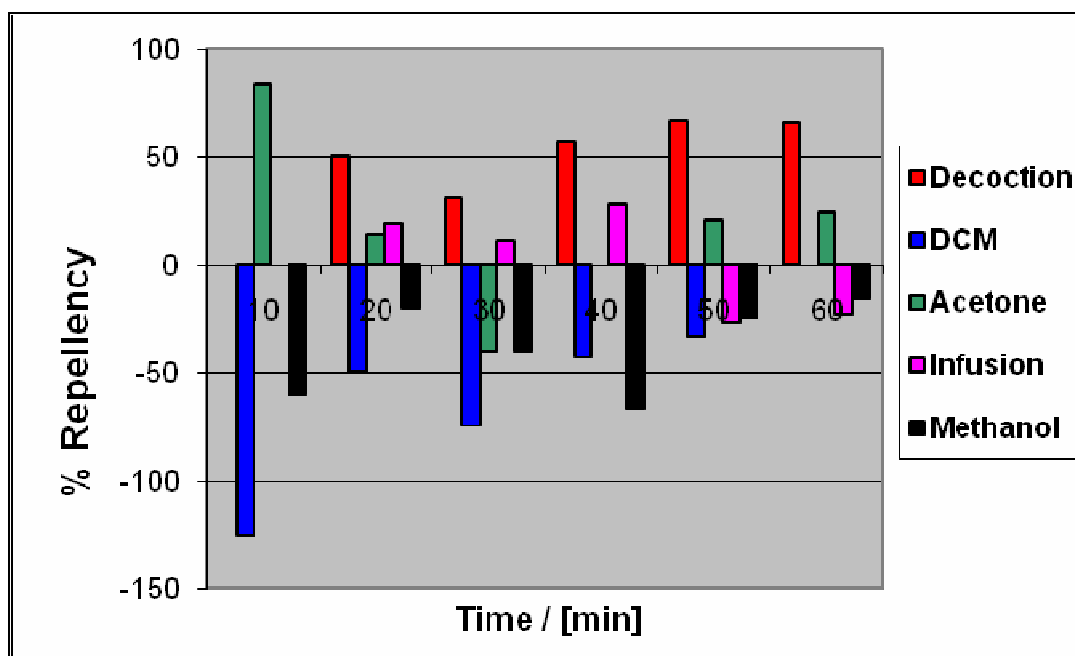


Figure 4.22B Repellent percentages of 30% crude extracts of *S. madagascariensis* against *R. appendiculatus* adults.

4.4 Discussion

Acetone extracts of *A. ferox*, *A. marlothii* and *C. glabrum* were the most effective. In the case of *J. curcas* only the methanol extracts had a good repellency activity. The DCM extract of *R. communis* had good activity in tick repellency. Secondary metabolites of this species probably include the ricinine which is the pyridine alkaloid and ricin. The alkaloids are well known for their insecticidal properties (Silve-Aguayo 2006). Malaya and Banda (1995) extracted toxalbumin and ricin from the seed extract of *R. communis* and decoctions were used as powerful poisons and to control a wide range of insects. Because ricin is water soluble and does not occur in widely used castor it is unlikely that DCM would extract the water soluble ricin and ricin is unlikely to be the repellent compound.

The acetone extract of *C. glabrum* was the only extract prepared from the plant with very potent repellent activity. A possibility was that the activity could be due to the vast amount of saponins as evaluated by Said *et al.* (1998). Saponins are readily soluble in aqueous extracts and in this case the activity was all in the non-polar fractions that rules out the possibility that saponins present in *C. glabrum* is responsible for the anti-tick activity. Mabogo (1990) and Hutchings *et al.* (1996) also reported insect repellency from *C. glabrum*. The hexane fraction of the solvent-solvent fractionation of the acetone extract had an excellent repellent activity, much higher than the commercially used products. Unfortunately it appears that the activity is lost after a few hours. It is worthwhile trying to isolate the active compound from this extract after the toxicity of the extracts were determined. This work is reported in the next chapters.

In comparing the repellent activity of the different extracts it appeared that non polar compounds are frequently responsible for repellent activity. This is not unexpected because the compounds would require a degree of volatility for the ticks to sense the presence.

The activity of the decoctions of *S. madagascariensis* validates the traditional use of decoctions to protect animals against ticks.

CHAPTER 5

TOXICITY BIOASSAY OF PLANT EXTRACTS

5.1 Introduction

Plant extracts possess different secondary metabolites which characterize them in various aspects especially in defence mechanism against various arthropods. Ticks and tick-borne diseases are a major problem to many farmers across the globe and they have massive economic implications. Many rural farmers have used plant extracts to control ticks.

Ticks have been controlled using various methods and one of the best one was to kill them immediately before they could do any harm. Many scientists have developed or used bioassays to investigate eradication of ticks. Toxicity bioassay is one of the methods being used in the laboratories. In toxicity bioassay, ticks are exposed to toxins. Once ticks feed on a toxin, it terminates their feeding process immediately (Klocke *et al.* 1989). Termination of feeding may be due to the toxicants that affect the peripheral nervous system or the disruption of the cellular, biochemical and physiological processes of the arthropods (Klocke *et al.* 1989; Danielson 2006).

5.2 Materials and methods

Whereas the previous chapter investigated the repellent activities of plant extracts in this chapter the toxicity extracts to ticks were examined. Ticks were exposed to three different types of contact toxicity bioassays, namely dipping, topical and dry extract application. With the dipping and topical bioassays, coverage is the important factor when considering the impact of extracts on adult *R. appendiculatus*. Whereas the fumes, vapours and volatiles play a role in dry extract application.

5.2.1 Types of contact toxicity bioassay

5.2.1.1 Dipping application

Ticks were dipped in the aqueous plant extract for one minute and percentage mortality recorded after 1 hour until mortality was complete. For control, ticks were dipped in the corresponding aqueous solvent in case of the decoction and infusion.

Three replications with ten unsexed ticks per replication were carried out (Nchu *et al.* 2005).

5.2.1.2 Topical application

This method is described by Nchu *et al.* (2005). Each tick in the treatment group was topically applied with 10 µl of the standardized plant extract and the corresponding solvents were used as controls on other ticks. The application was on the dorsal surface of the ticks. Three replications for each concentration were made with each replication having 10 ticks per tick species.

Death of ticks was determined by touching, shaking and breathing on them to increase carbon dioxide concentration. If no response was obtained the tick was considered dead. Dead ticks were counted every hour and mortality rate was calculated using Abbott formula (see Section 5.2.2). Data was recorded as percentage mortality and the lethal concentration needed to kill 50% (LC₅₀) of the ticks was determined.

5.2.1.3 Dry extract application

One gram of the dry extract was placed in an empty vial. Ten unsexed *R. appendiculatus* were inserted into the vial. The vial was sealed with a plug of clean cotton wool to ensure that ventilation is maintained and ticks could not escape. Dead ticks were counted every hour and the data recorded.

In case of the controls, one ml of the corresponding solvent was placed in an empty vial and evaporated at room temperature to complete dryness. The rest of the procedure is the same as the extract above.

Three replications with ten unsexed ticks per replication were done.

5.2.2 Mortality data analysis

Corrected mortality rates were calculated using the Abbott formula as shown below;

$$M_c = \frac{M_o - M_t}{100 - M_t} \times 100$$

M_c = corrected mortality rate

M_o = Mortality rate in treatment

M_t = Mortality rate in control (Pamo *et al.* 2004)

5.2.3 Statistical analysis

For statistical analysis, a parametric test had been selected from the normal distribution of the population. A simple Student's t test of percentage mortality data was used to determine if there was a significant difference between the control and test treatments.

5.3 Results

The results of the toxicity bioassays were recorded and analysed. The positive controls used so far are classified as strong toxicants since they killed all ticks within 24 hours and achieved 100% tick mortality within two weeks. With the plant extracts ticks were classified as being strong if they killed 100% of the ticks within 2 days, moderate 100% within 3-14 days and weak if it took more than 15 days to kill all the ticks. The summary of the toxicity effects obtained from six selected plant species are shown in Table 5.1. The effect of time on the mortality of the extracts, the positive and negative controls are presented in Figs. 5.2A to 5.10.

Table 5.1 Summary of the toxicity effect obtained from six selected plant species. Numericals in parenthesis represent the average number of days it took to achieve 100% mortality

Conc. [% (mg/ml)]	Extract – (bioassay)	Strong (0 - 2 days)	Moderate (3 - 14 days)	Weak (≥ 15 days)
35.5	Decoction- (Dipping)	-	-	All plant species (15 - 19)
	Decoction- (Topical)	-	<i>R. communis</i> (12)	<i>A. ferox</i> (17), <i>C. glabrum</i> (17), <i>S. madagascariensis</i> (17), <i>A. marlothii</i> (17), <i>J. curcas</i> (19)
	Infusion – (Topical)	<i>A. ferox</i> (2), <i>S. madagascariensis</i> (2)	<i>J. curcas</i> (3), <i>C. glabrum</i> (7)	<i>R. communis</i> (15), <i>A. marlothii</i> (16)
	Infusion- (Dipping)	<i>A. ferox</i> (2), <i>J. curcas</i> (2)	<i>C. glabrum</i> (4), <i>S. madagascariensis</i> (4), <i>R. communis</i> (7)	<i>A. marlothii</i> (18)
35	Infusion – (Topical)	-	<i>A. ferox</i> (5), <i>J. curcas</i> (6), <i>S. madagascariensis</i> (6), <i>C. glabrum</i> (8) <i>R. communis</i> (9)	<i>A. marlothii</i> (15)
	Infusion – (Dipping)	-	<i>A. ferox</i> (5), <i>R. communis</i> (8), <i>S. madagascariensis</i> (8), <i>J. curcas</i> (9), <i>C. glabrum</i> (9)	<i>A. marlothii</i> (16)
30	Infusion – (Topical)	-	<i>R. communis</i> (8), <i>J. curcas</i> (10), <i>S. madagascariensis</i> (12), <i>C. glabrum</i> (13)	<i>A. ferox</i> (16), <i>A. marlothii</i> (18)
	Infusion – (Dipping)	-	<i>R. communis</i> (12), <i>J. curcas</i> (12), <i>S. madagascariensis</i> (14), <i>C. glabrum</i> (14), <i>A. ferox</i> (13)	<i>A. marlothii</i> (18)
-	Acetone – (Dry extract)	-	<i>R. communis</i> (11)	<i>J. curcas</i> (18), <i>C. glabrum</i> (32), <i>A. ferox</i> (>53), <i>A. marlothii</i> (>53), <i>S. madagascariensis</i> (>53)
-	MeOH – (Dry extract)	-	<i>R. communis</i> (11)	<i>C. glabrum</i> (32), <i>J. curcas</i> (39), <i>S. madagascariensis</i> (46), <i>A. ferox</i> (>53), <i>A. marlothii</i> (>53)
-	DCM – (Dry extract)	-	<i>J. curcas</i> (7)	<i>S. madagascariensis</i> (32), <i>A. ferox</i> (>53), <i>C. glabrum</i> (53), <i>R. communis</i> (>53), <i>A. marlothii</i> (>53)

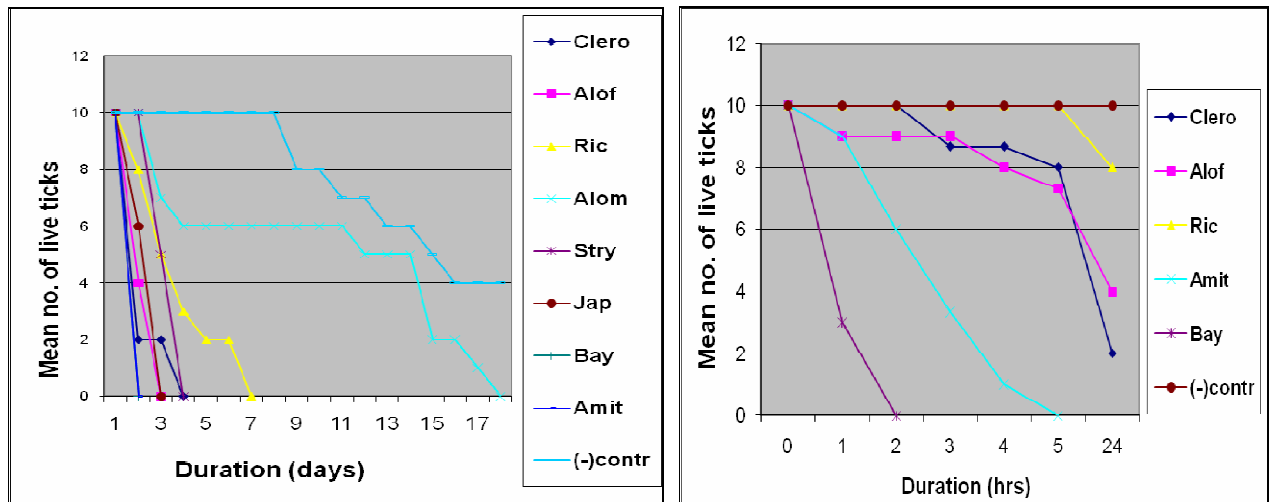


Figure 5.1A Dipping toxicity effects of plant soap infusions at the concentration of 35.5 mg/ml recorded in days and hours against *R. appendiculatus* adults.

Stry – *S. madagascariensis*, Alom - *A. marlothii*, Alof – *A. ferox*, Ric – *R. communis*, Jap – *J. curcas*, Clero – *C. Glabrum*, (-) contr – negative control, Bay- Bayticol and Amit - Amitix.

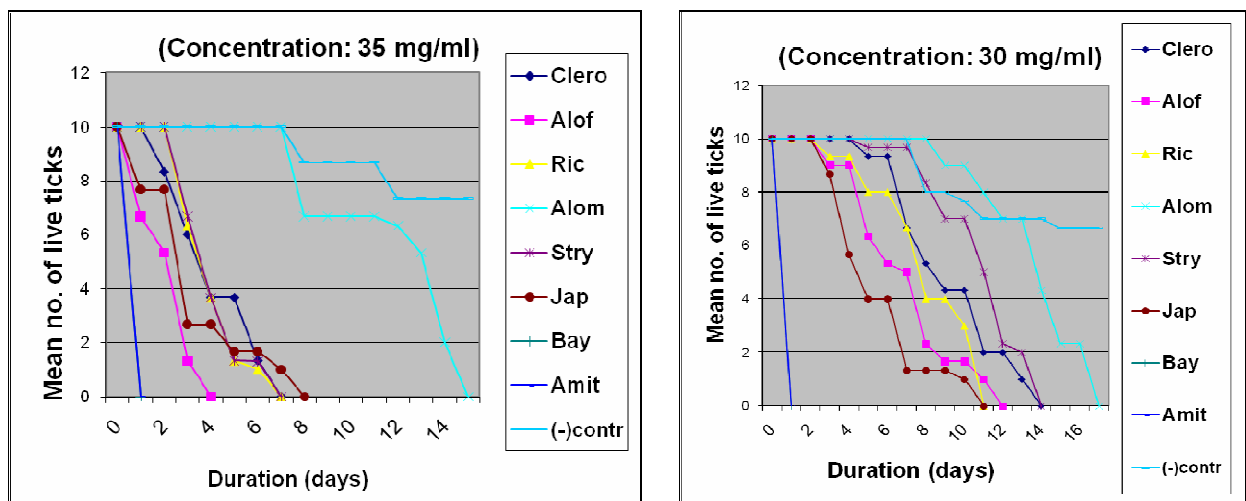


Figure 5.1B Dipping toxicity effects of plant soap infusions at 35 and 30 mg/ml concentrations against *R. appendiculatus* adults.

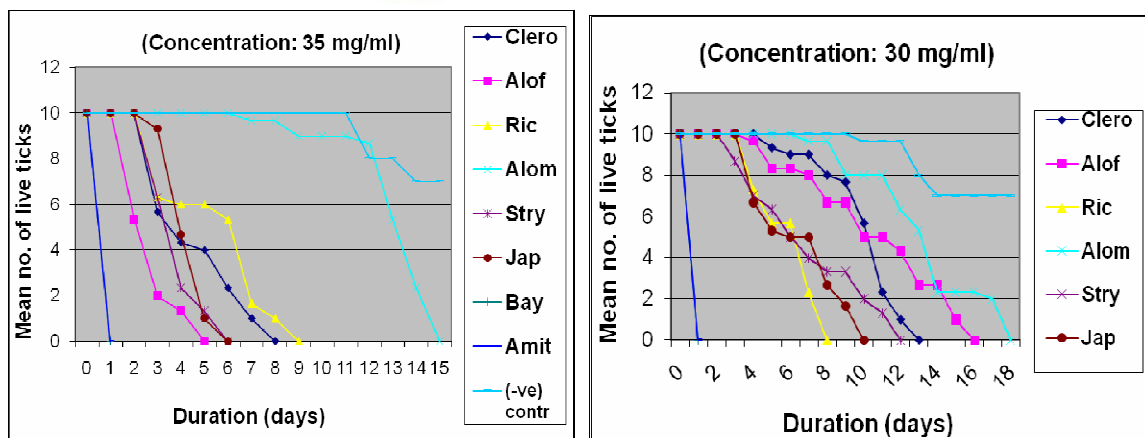


Figure 5.2A Topical toxicity effects of plant soap infusions at 35 and 30 mg/ml concentrations against *R. appendiculatus* adults

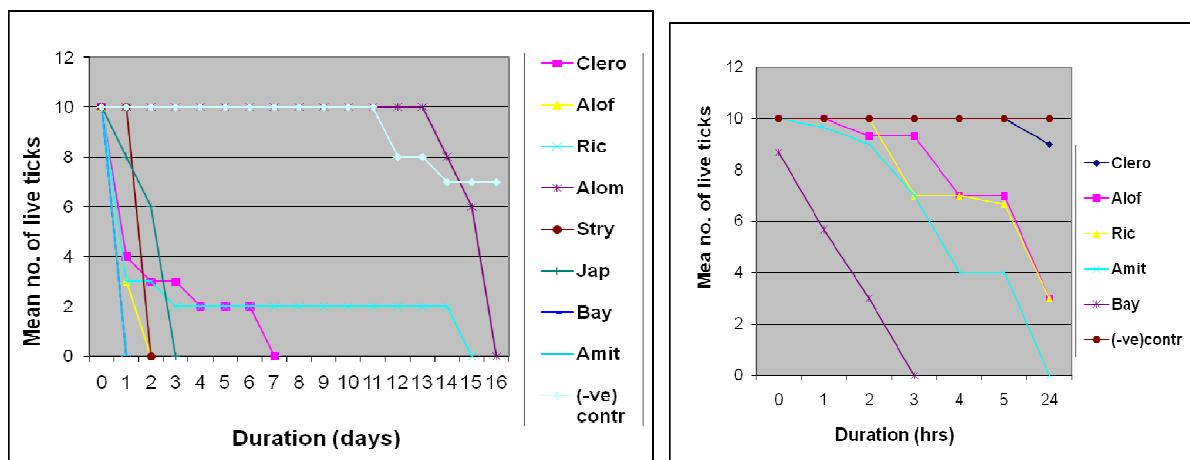


Figure 5.2B Topical toxicity effects of plant soap infusions at 35.5 mg/ml recorded in days and hours against *R. appendiculatus* adults

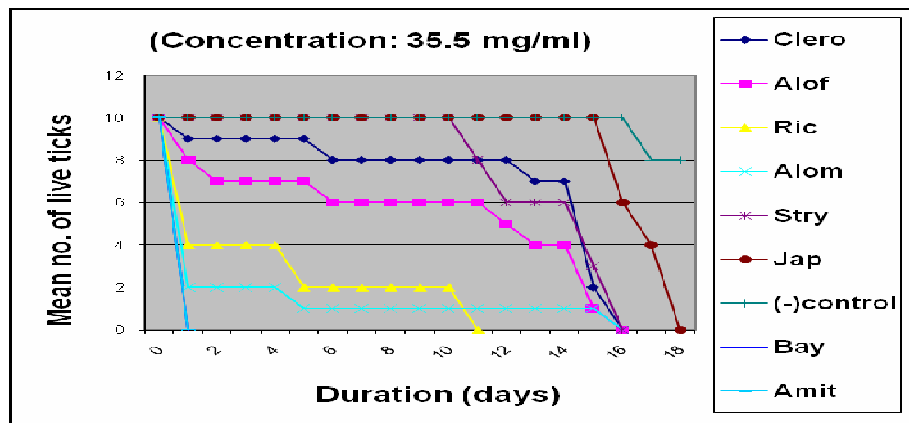


Figure 5.3 Dipping toxicity effects of plant decoctions on *R. appendiculatus* adults.

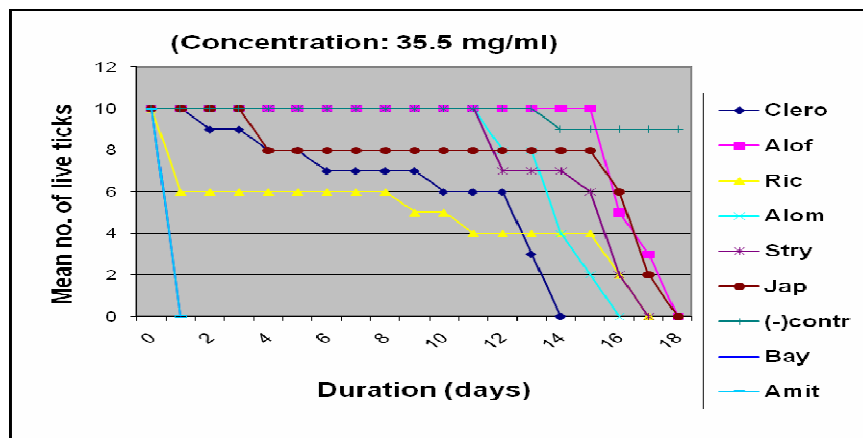


Figure 5.4 Topical toxicity effects of plant decoctions on *R. appendiculatus* adults.

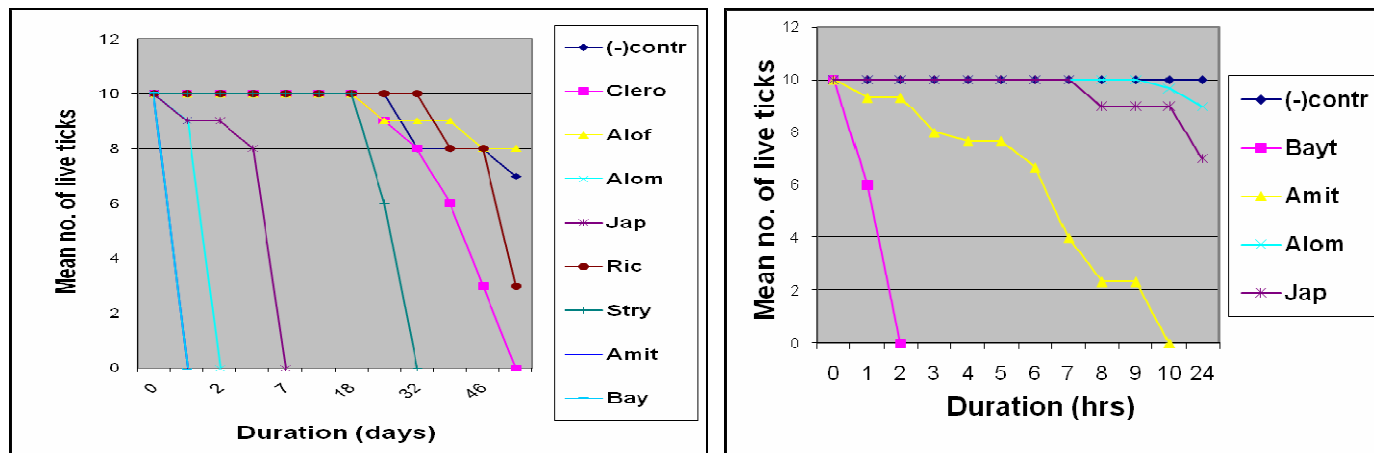


Figure 5.5 Contact toxicity effects of DCM extracts recorded in days and hours against *R. appendiculatus* adults.

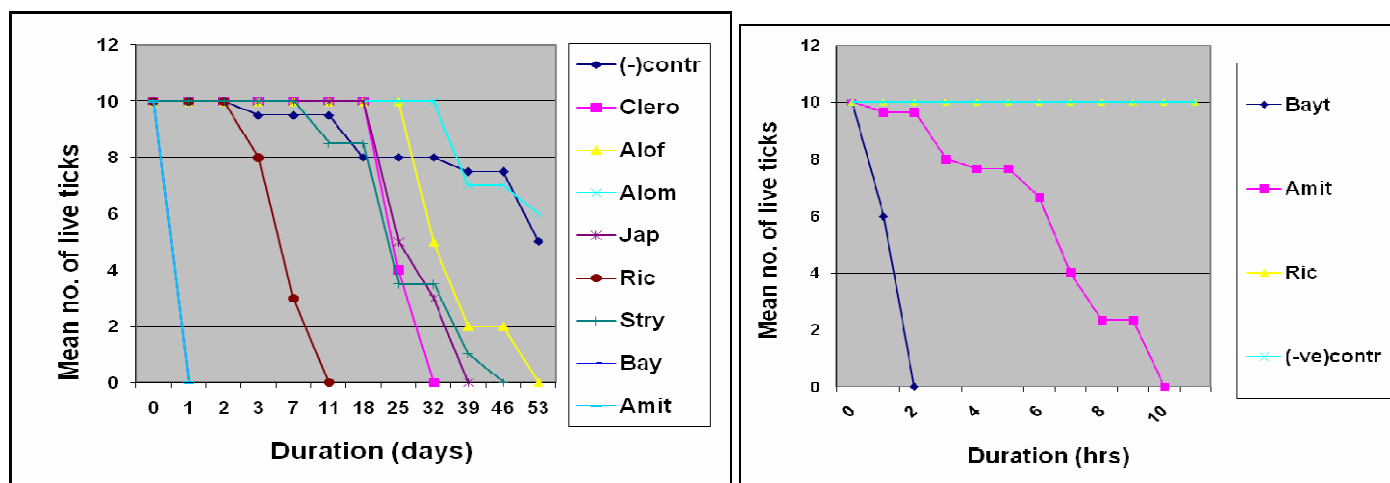


Figure 5.6 Contact toxicity effects of MeOH extracts recorded in days and hours against *R. appendiculatus* adults.

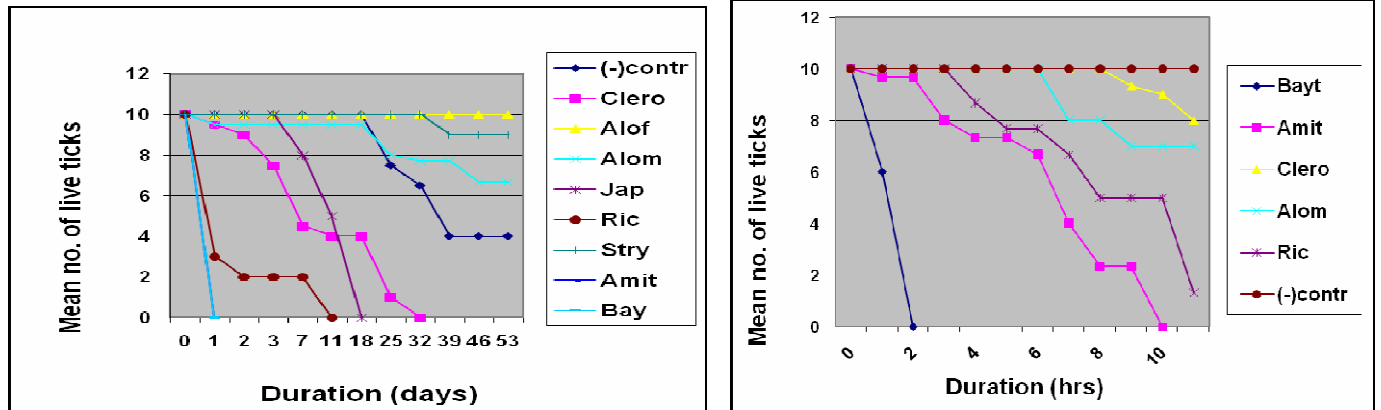


Figure 5.7 Contact toxicity effects of acetone extracts recorded in days and hours against *R. appendiculatus* adults

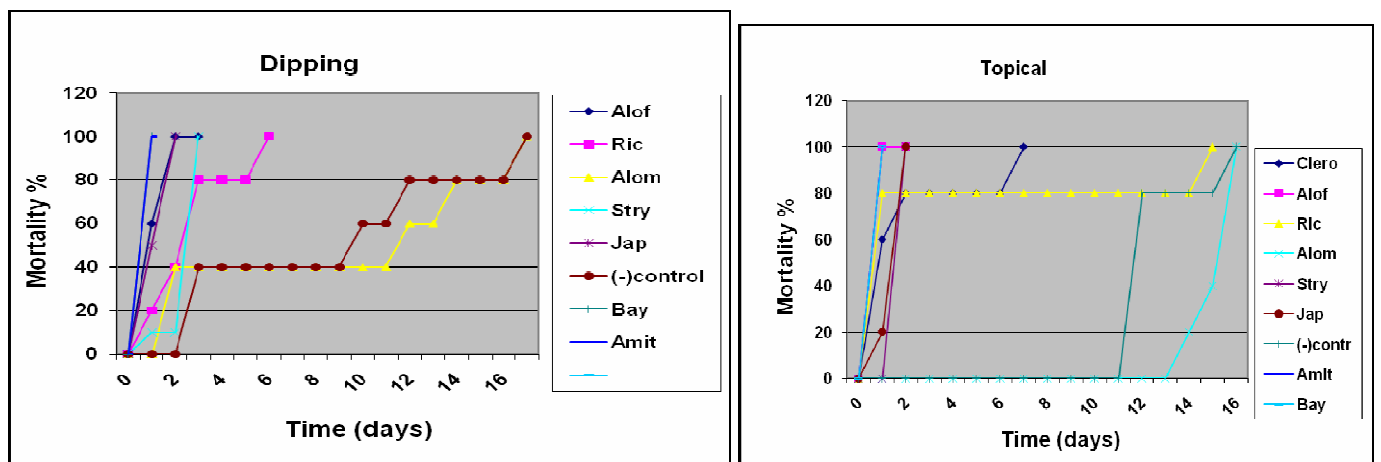


Figure 5.8 Mortality percentages of 35.5 mg/ml infusion preparations on *R. appendiculatus* adults by dipping and topical application.

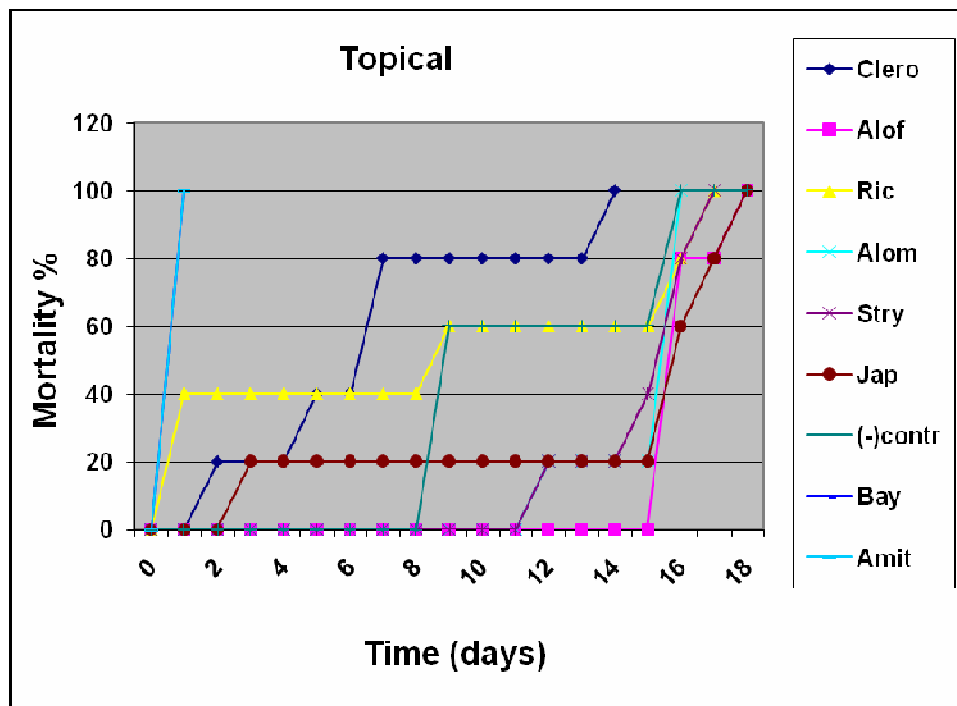
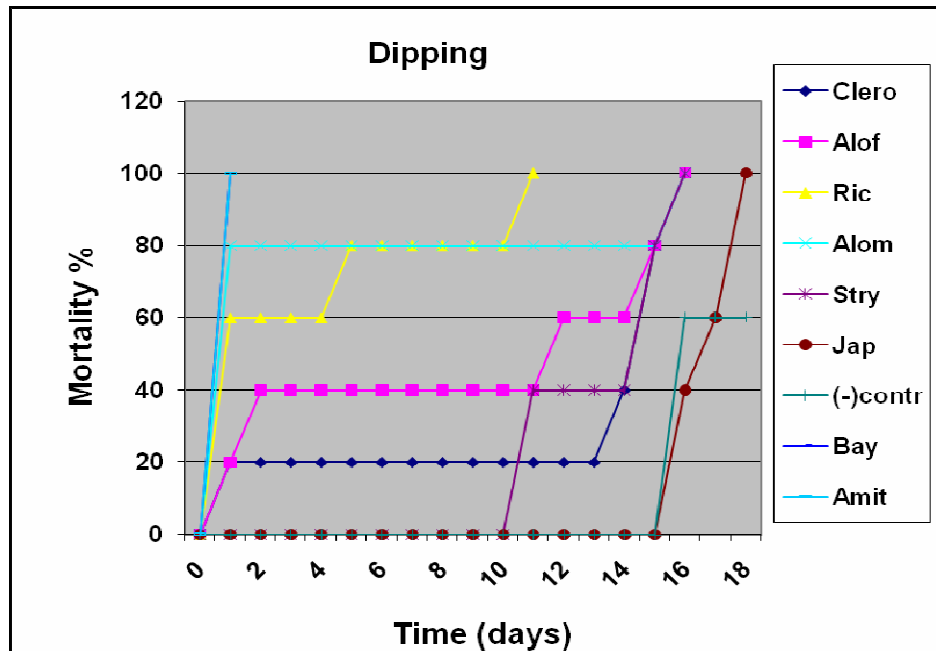


Figure 5.9 Mortality percentages of the 35.5 mg/ml decoctions on *R. appendiculatus* adults by dipping and topical application

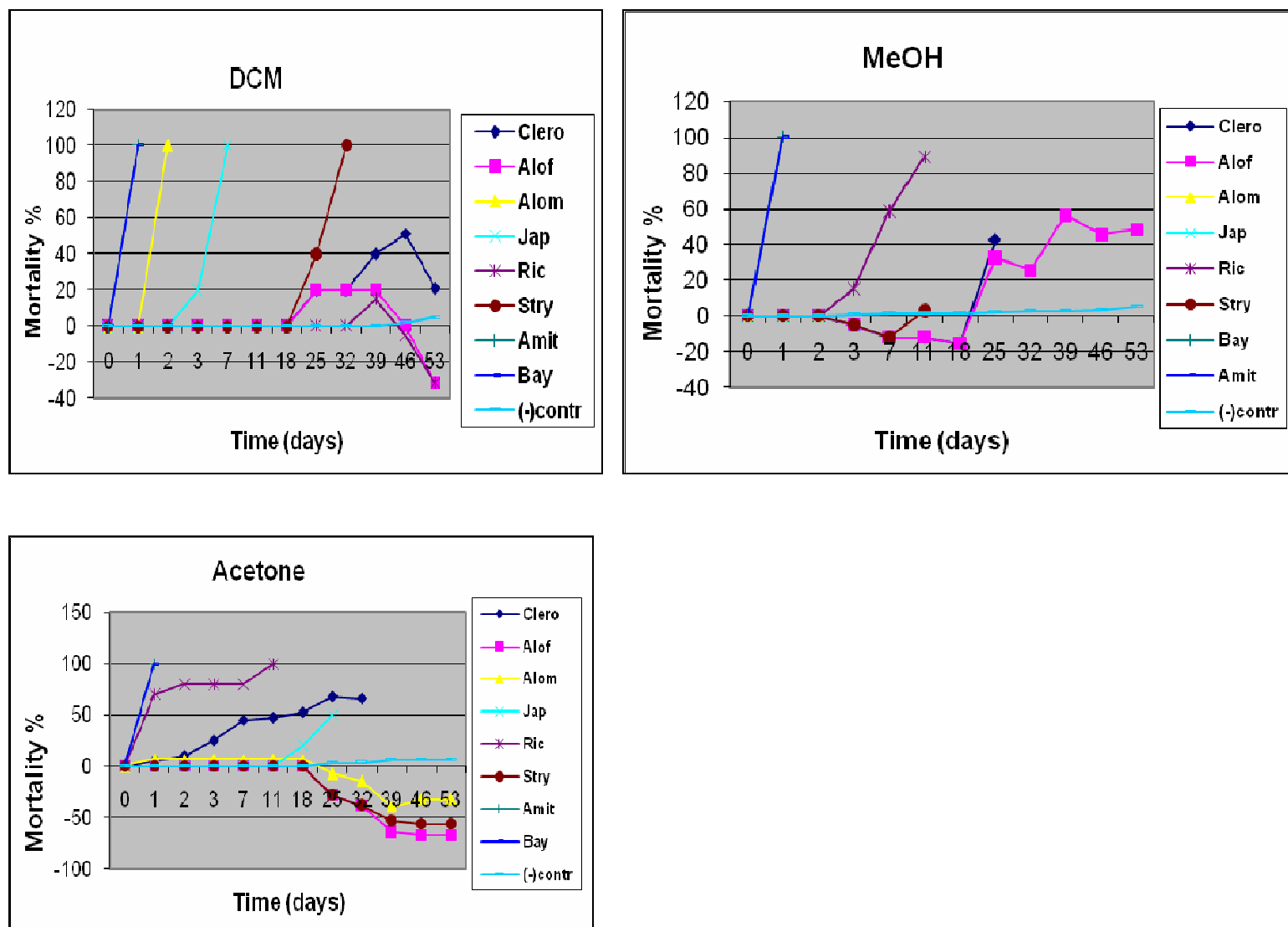


Figure 5.10 Mortality percentage of the organic extracts against *R. appendiculatus* adults.

5.3.1 Description of results

5.3.1.1 *Aloe ferox*

The results of the dry extract application indicated that all the crude extracts were weak toxicants to *R. appendiculatus* species, thus the ticks only died after 15 days (Table 5.1; Figs. 5.5, 5.6 and 5.7). Topical and dipping applications of the decoctions were also not toxic (Figs. 5.3 and 5.4). Topical and dipping applications of the infusions had better results at 35.5% (Figs. 5.1A, B, 5.2A and B). As the concentrations decreased from 35.5 to 30%, the moderate toxicity effect was retained but the duration to complete 100% mortality was extended (Figs. 5.1A, B, 5.2A and B; Table 5.1). A statistical significant difference was exhibited ($p = 0.0083$) as α -value was set at 1%. The LD_{50} value was 25% (Fig. 5.11).

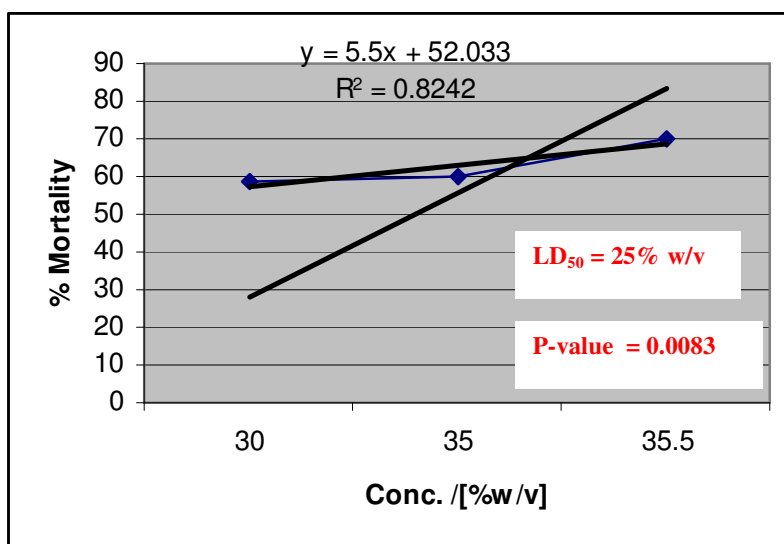


Figure 5.11 Trendlined graph showing % mortality at three concentrations of *A. ferox*

The infusion extracts were toxic to ticks, thus a large number of metabolites extracted contributed to the toxicity effect of the plants (Gurib-Fakim 2006).

5.3.1.2 *Aloe marlothii*

All concentrations of the crude extracts of *A. marlothii* were weakly toxic to *R. appendiculatus* species (Fig. 5.1A – Fig.5.10; Table 5.1).

A. marlothii extracts had a low toxicity to *R. appendiculatus*, maybe it is due to the method or the type of tick species used. The infusion prepared in this project is different from the one reported by Hutchings *et al.* (1996) and Roberts (1997).

5.3.1.3 *Clerodendrum glabrum*

Topical and dipping applications of the plant infusions of *C. glabrum* showed a moderate toxicity effect at all concentrations (Fig. 5.1 –5.9; Table 5.1).

5.3.1.4 *Jatropha curcas*

The dry extract application showed a moderate toxicity effect of DCM extract with 7 days duration to complete 100% mortality. Topical and dipping applications had strong (2 days) and moderate (4 days) toxicity of the *J. curcas* infusions respectively at the concentration level of 35.5% (Fig. 5.1A, B, 5.2A and B). As the concentration decreased, the toxicity effects remained moderate (Fig. 5.1A, B, 5.2A and B). The lethal dose was, $LD_{50} = 29.3\%$ and the probability was 6.9% which is considered statistically significant when α – value was set at 10% (Fig. 5.12).

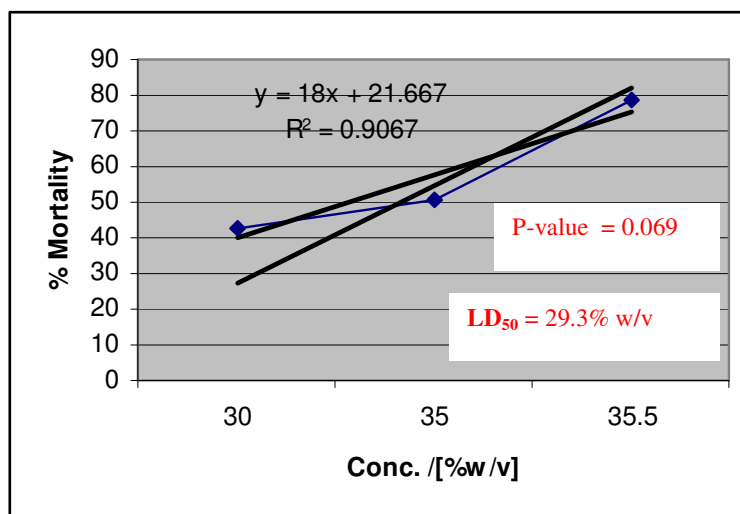


Figure 5.12 Trendlined graph showing % mortality versus three concentrations of *J. curcas* infusions

5.3.1.5 *Ricinus communis*

The decoction, infusion (dipping application), MeOH (dry extract application) and acetone (dry extract application) extracts had a moderate toxicity with duration to complete 100% mortality of 12, 6, 11 and 11 days respectively (Figs. 5.1, 5.3, 5.6 and 5.7). As the concentration of the infusion decreased, the toxicity effects remained moderate but the duration to complete 100% mortality was extended (Fig. 3.5).

5.3.1.6 *Strychnos madagascariensis*

The infusion of topical application had a strong toxicity with 2 days duration to complete 100% mortality (Figs. 5.2A and B). The infusion of the dipping application showed a moderate toxicity effect with duration to complete 100% mortality of 4 days (Figs. 5.1A and B). As the concentration decreased for topical and dipping applications, the duration to complete 100% mortality was extended but retained the moderate toxic activity (Figs. 5.1A, B, 5.2A and B). The LD₅₀ was determined as 26.5% (p = 0.152) (Fig. 5.13).

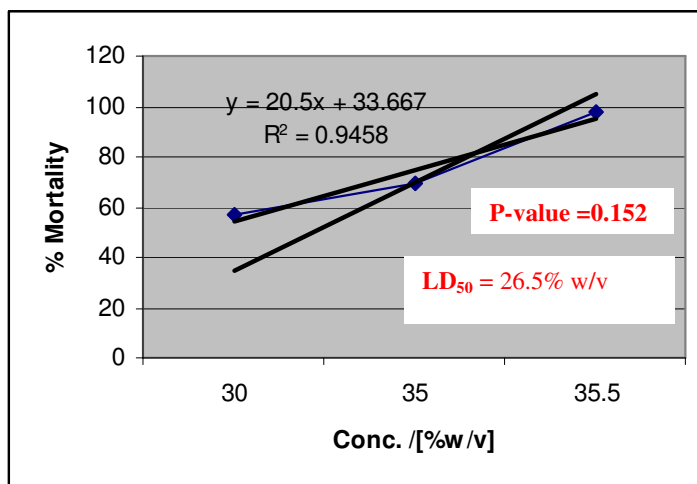


Figure 5.13 Trendlined graph showing % mortality versus three concentrations of *S. madagascariensis* infusions.

5.4 Discussion

The two Aloes have polysaccharides accumulating in the leaves as well as anthranoids and anthraglycosides with phenolic groups (Harborne *et al.* 1997; van Wyk & Wink 2004). Oxymethylantraquinone was isolated from the leaves of *A. marlothii* (Watt & Breyer – Brandwijk 1962). These secondary metabolites give the plants a defense mechanism against insects (Silva-Aguayo 2006). Hutchings *et al.* (1996) and Roberts (1997) reported the infusion and decoction to control ticks. In the current study, the ticks were controlled by the infusion of *A. ferox*. The fact that the infusion and the decoction of *A. marlothii* did not have any activity may be due to the season and the environment at which the plants were collected which may have an influence in the diversity of secondary metabolites. Or perhaps the extracts were prepared differently.

The infusion of *J. curcas* was active in killing ticks. Most of the active secondary metabolites are being extracted and these may include poisonous diterpenoids and lectins (Adolf *et al.* 1984; Joubert *et al.* 1984; Mampane *et al.* 1987; van Wyk *et al.* 2002). Terpenoids are found to have insecticide properties (Silva-Aguayo 2006). *R. communis* has been evaluated and reported for insect repellency and also considered insecticidal (Malaya & Banda 1995; Hutchings *et al.* 1996).

The infusion of *S. madagascariensis* had strong toxicity effect. Since *S. madagascariensis* bears edible fruits, some of the toxic strychnine related alkaloids may be found spread in the leaves and played a role in giving the plant an activity (Verdoorn 1963; Neuwinger 1996; van Wyk & Wink 2004).

The results of the toxicity assays provide evidence for the efficacy of the traditional use of plants to control ticks.

CHAPTER 6

PHYTOCHEMISTRY OF PLANTS

6.1 Introduction

Active components of the extracts may be identified by bioassay-guided fractionation of the crude extract, fractionated extracts and ultimately individual compounds separated by chromatography. This frequently requires a preliminary separation to simplify subsequent steps. Partitioning is one of the simplest preliminary separation methods which are widely used as an initial extract purification step. All the phytochemicals within the extract may be separated into groups based on polarity by a combination of immiscible solvents (Gurib-Fakim 2006). The components are grouped according to their polarities.

TLC is a practical, appropriately sensitive analytical approach to a broad spectrum of chemical analyses (Butler 2006). It is a powerful, fast and widely used chromatographic technique which separates compound mixtures based upon their polarity and interaction with silica gel and the elution solvent (Eloff 2001). It is also used to assess the purity of compounds. Separation of the compounds in an extract is a function of their different affinities for a stationary phase and their differential affinities for a mobile phase.

Column chromatography is widely used for preparative purposes. By determining the activity of the different fractions and repeating the process with subsequent fractionation of the active extract (s) the biologically active compound may be isolated. In isolating compounds that can be identified on a TLC chromatogram e.g., by bioautography or antioxidant activity, the process is relatively simple because the R_f value of the active compound can easily be determined.

Isolating the compound responsible for the activity against the ticks is much more challenging. In the previous chapter it was shown that *C. glabrum* had the most promise. In this chapter isolation of the compound(s) responsible for the tick repellency will be attempted. If only a single compound is responsible for the activity it may be possible to isolate it. If more than one compound is responsible for the

activity the isolation of more than one synergistic compound is beyond the scope of an MSc, focus will be on bioassays. The results of the bioassays of the crude extracts and fractions obtained in this chapter were presented in chapter 4. The results obtained there will only be listed here in the bio-assay guided fractionation.

6.2 Materials and methods

6.2.1 Thin layer chromatography

The extracted material and fractions were submitted to TLC using Merck TLC F254 plates with different standardized solvent mixtures routinely used in the Phytomedicine Programme at the University of Pretoria.

6.2.1.1 Standard mobile phases

The standardized mobile phases used for TLC are as follows;

- (EMW) - ethyl acetate (10 ml), methanol (1.35 ml) and distilled water (1 ml)
- (CEF) – chloroform (10 ml), ethyl acetate (8 ml) and formic acid (2 ml)
- (BEA) – benzene (18 ml), ethanol (2 ml) and ammonia (0.2 ml)
- (HE) – different proportions of ethyl acetate and hexane solutions.

6.2.1.2 Development of plates

Three 20 x 20 cm aluminium plates were cut in half and used for each of the solvents systems described above. To each plate, 10 µl of a 10 mg / ml solution of the extract was applied in a line of about 1.3 cm long. The plates were put into the TLC tanks to develop for 20 minutes.

The tanks were saturated before development by lining with filter paper moistened with the eluent. The separated components were visualized under UV light at 240 nm and 360 nm.

6.2.1.3 Application of vanillin spray reagent

The vanillin spray reagent is widely used to detect separated compounds on TLC chromatograms. It was prepared as follows;

Vanillin powder (0.1g) was placed in 40 ml glass beaker and 28 ml of methanol was added to dissolve it. About 2 ml of concentrated sulphuric acid was added cautiously (Serage 2003).

The developed plates were sprayed with vanillin solution in the fumehood using a spray gun. The sprayed plates were heated at 100°C to optimal colour development.

6.2.2 Solvent – solvent fractionation process (group separation)

Subsequent to the results obtained from repellency bioassays, the acetone extract of *C. glabrum* was selected and subjected to a solvent-solvent fractionation process. The separation is undertaken with immiscible solvents to fractionate components with different polarities. The procedure used for the group separation process is based on a procedure of the National Cancer Laboratory in the USA and was slightly altered as described below and illustrated in Fig. 6.1 (Eloff 1998b).

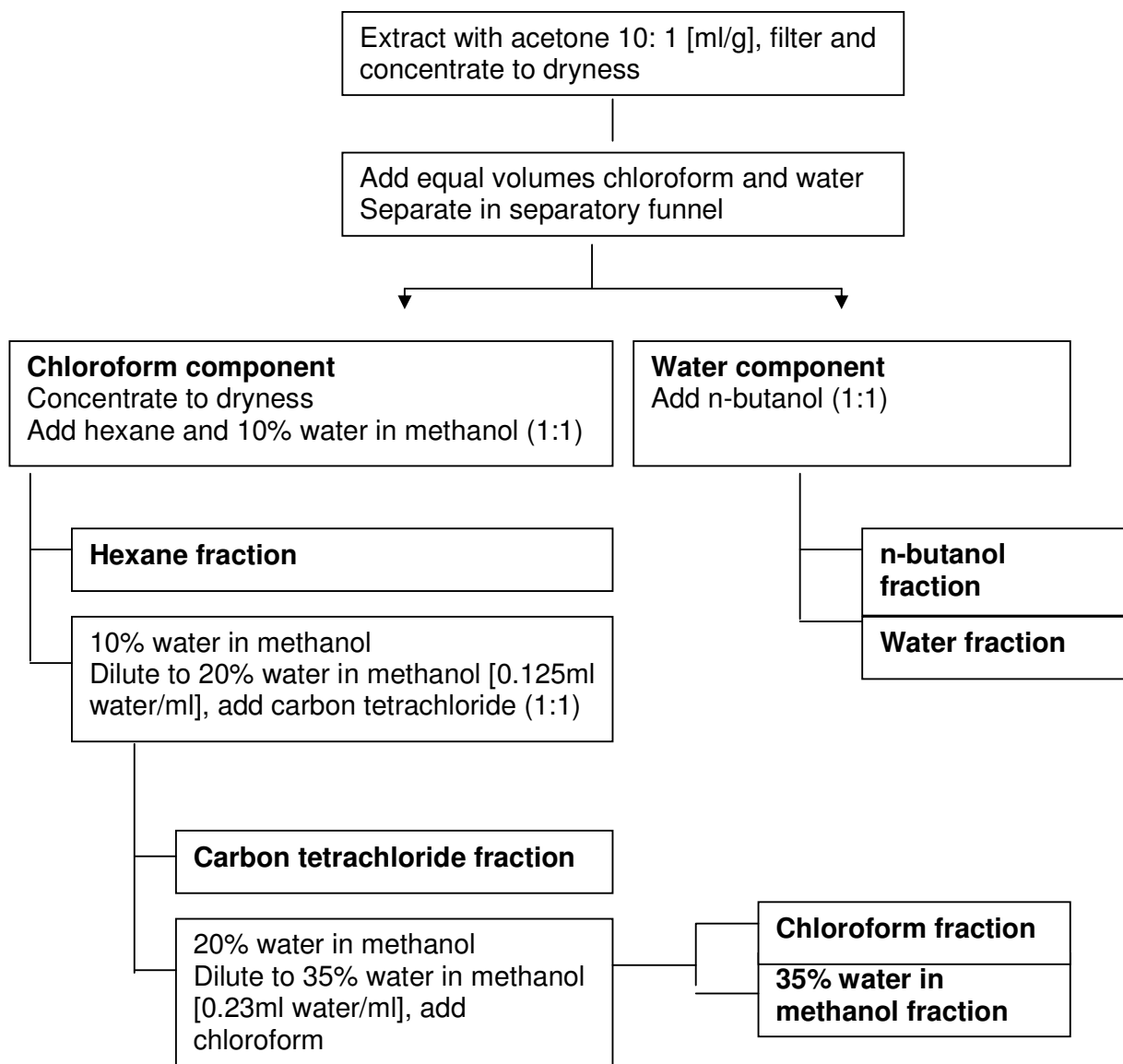


Figure 6.1 Solvent-solvent fractionation process used

6.2.2.1 Chloroform – water fractionation

Working in a fumehood, equal volumes of chloroform (added first) and water was added to the plant extract. The minimum volume necessary to dissolve the extract was used, and after thorough mixing, the contents were poured into a 500 ml separatory funnel. The contents were rotated carefully to avoid formation of emulsions. The components were left to separate, resulting in the water fraction on top and the heavier chloroform fraction at the bottom. The water fraction was extracted with additional small volumes of chloroform until the observed colour

disappeared. The fractions were collected separately in 250 ml Erlenmeyer flasks that were then sealed with aluminium foil to avoid inhalations.

6.2.2.2 Water – butanol fractionation

The water fraction was extracted with an equal volume of n-butanol in a separatory funnel to yield water and butanol fractions which were collected and evaporated under fan at 37°C.

6.2.2.3 Hexane and water in methanol fractionation

The chloroform fraction from the first separation was evaporated under reduced pressure in a rotary evaporator to dryness. The weighed dry product was obtained and dissolved in 1:1 mixture of hexane and 10% water in methanol. The 10% water/methanol fraction was separated from hexane fraction in a 250 ml separatory funnel. Hexane fraction was on top and the heavier water fraction was at the bottom. The 10% water/methanol fraction was extracted with equal volumes of hexane until the observed colour disappeared.

6.2.2.4 Carbon tetrachloride and water in methanol fractionation

The 10% water in methanol fraction was placed in a separatory funnel and further diluted to 20% water in methanol by adding 0.125 ml of water for every ml of the 10% water in methanol fraction. An equal volume of carbon tetrachloride was then added to obtain 20% water in methanol fraction and a carbon tetrachloride fraction after separation. The 20% water in methanol fraction was extracted with equal volumes of carbon tetrachloride three times. The combined carbon tetrachloride fractions were evaporated under reduced pressure in a rotary evaporator to obtain a dry product.

6.2.2.5 Chloroform and water in methanol fractionation

The 20% water in methanol fraction was further diluted to 35% water in methanol by adding 0.23 ml of water for every ml of the 20% water in methanol fraction. Equal volumes of chloroform were used for extraction. The combined chloroform fractions were collected in a 250 ml Erlenmeyer flask and evaporated under reduced pressure

in a rotary evaporator to obtain dry product. The water fraction was evaporated under fan at 37°C.

6.2.3 Column chromatography by vacuum liquid chromatography (VLC)

The fraction of interest as obtained in section 2.5.2 was further fractionated on silica gel.

Procedure for VLC

About 500 g of silica gel was packed in a sintered funnel with hexane as an eluent and 5.0 g dry hexane fraction of *C. glabrum* was loaded. A 500 ml conical flask was used to collect the yellow solution which eluted first in the presence of hexane. As the yellow solution faded away, the conical flask was removed and the solution contained was transferred into a 1000 ml round bottom flask and concentrated under reduced pressure to obtain 1.5 g of dry hexane subfraction.

A 50% acetone in hexane solution was introduced as the second eluent. A green solution was collected in the conical flask. As the green solution disappeared, the eluent was no longer added. The conical flask was removed and the solution was concentrated under reduced pressure to obtain 0.89 g of dry acetone-hexane subfraction.

Ethyl acetate (100%) was used as the third eluent to remove another green layer as observed through the sintered funnel. The green solution collected was also concentrated under reduced pressure to obtain 2.61g of dry ethyl acetate subfraction.

A 30% methanol in ethyl acetate solution was used to strip the VLC column whereby most polar compounds retained in the silica gel were removed.

6.2.4 Gravity based column chromatography packed with silica gel

A ratio of 100: 1 (silica gel: fraction) was used. The column used was 55 cm long with a diameter of 5 cm. About 100% hexane was used as the first eluent. This was

followed by a gradual increase of DCM in hexane starting from 10 to 80% where no more components eluted. The column was finally eluted with 100% DCM.

6.2.5 Bioassay of extracts and fractions

All extracts and fractions were assayed for repellency activity after making up to a concentration of 30% in acetone.

6.3 Results and discussion

6.3.1 Diversity of compounds

The TLC of all plant extracts in three solvents systems were recorded to show the diversity of compounds and their polarity characteristics (Figs. 6.2A – 6.4B). The BEA solvent system did not separate compounds of the water and methanol extracts (Figs. 6.2A and B). Probably because the compounds were too polar to be separated by the non polar solvent system. Fewer compounds were separated by *A. marlothii* methanol extract (Fig. 6.2A). Several compounds were separated from the acetone and DCM extracts.

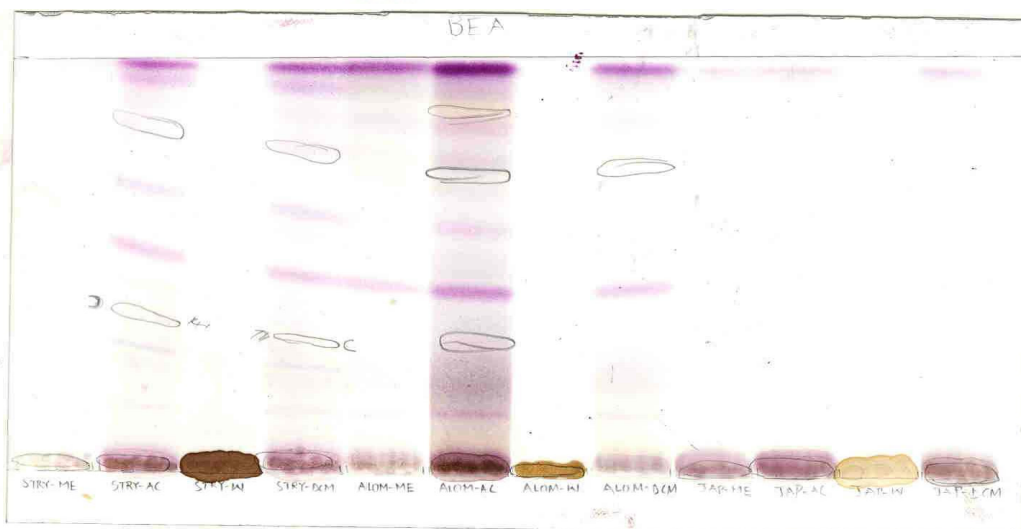


Figure 6.2A Chromatograms of plant extracts separated by BEA mobile phase and sprayed with vanillin reagent to observe colour derivatives.

Lanes from left to right of the chromatogram: Stry-ME, Stry-AC, Stry-W, Stry-DCM, Alom-ME, Alom-AC, Alom-W, Alom-DCM, Jap-ME, Jap -AC, Jap -W and Jap -DCM.

(Stry = *S. madagascariensis*, Alom = *A. marlothii*, Jap = *J. curcas*, ME = MeOH, AC =acetone, W = water extract and DCM = dichloromethane).

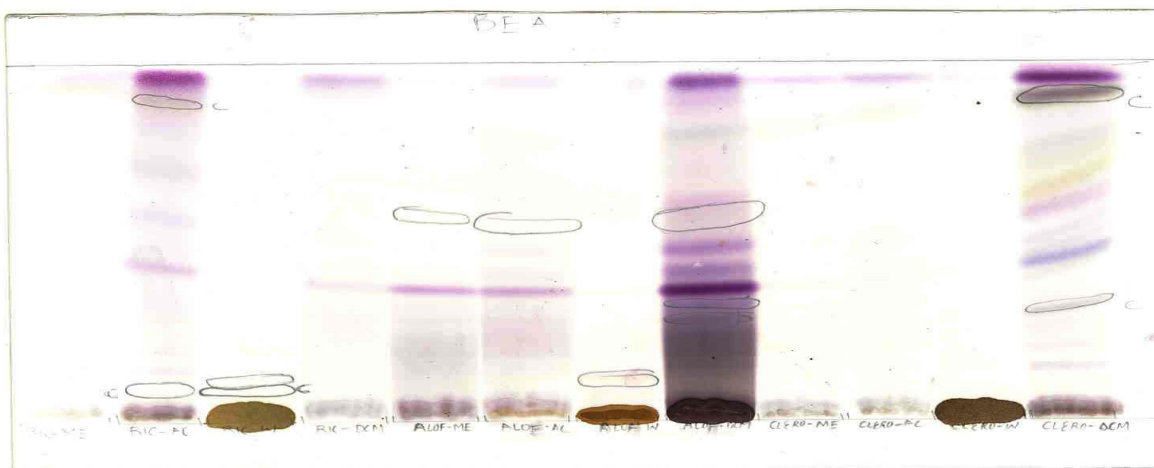


Figure 6.2B Chromatograms of plant extracts separated by BEA mobile phase and sprayed with vanillin reagent to observe colour derivatives.

Lanes from left to right of the chromatogram: Ric-ME Ric-AC, Ric-W, Ric-DCM, Alof-ME, Alof-AC, Alof-W, Alof-DCM, Clero-ME, Clero-AC, Clero-W and Clero-DCM.

(Ric = *R. communis*, Alof = *A. ferox*, Clero = *C. glabrum*, ME= MeOH, AC = acetone, W = water extract and DCM =dichloromethane).

The CEF solvent system separated fewer compounds regardless of type of extract (Figs. 6.3A and B).

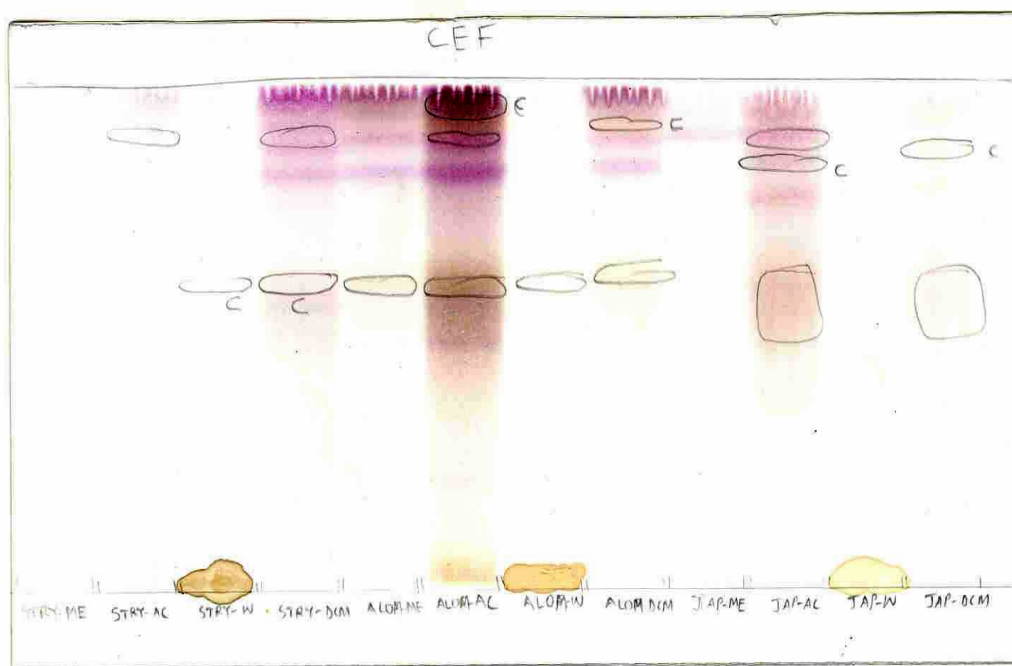


Figure 6.3A Chromatograms of all plant extracts separated by CEF mobile phase and sprayed with vanillin reagent to observe colour derivatives.

Lanes from left to right of the chromatogram: Stry-ME Stry-AC, Stry-W, Stry-DCM, Alom-ME, Alom-AC, Alom-W, Alom-DCM, Jap-ME, Jap -AC, Jap -W and Jap -DCM.

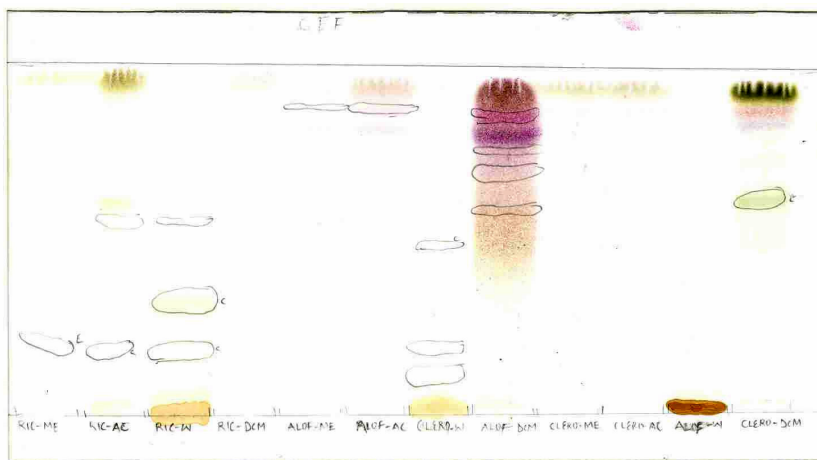


Figure 6.3B Chromatograms of all plant extracts separated by CEF mobile phase and sprayed with vanillin reagent to observe colour derivatives.

Lanes from left to right of the chromatogram: Ric-ME Ric-AC, Ric-W, Ric-DCM, Alof-ME, Alof-AC, Alof-W, Alof-DCM, Clero-ME, Clero-AC, Clero-W and Clero-DCM.

The EMW solvent system separated all the compounds from DCM, acetone and some methanol extracts in close proximity to the solvent front whereas the water extracts are found half-way the solvent front (Figs. 6.4A and B).

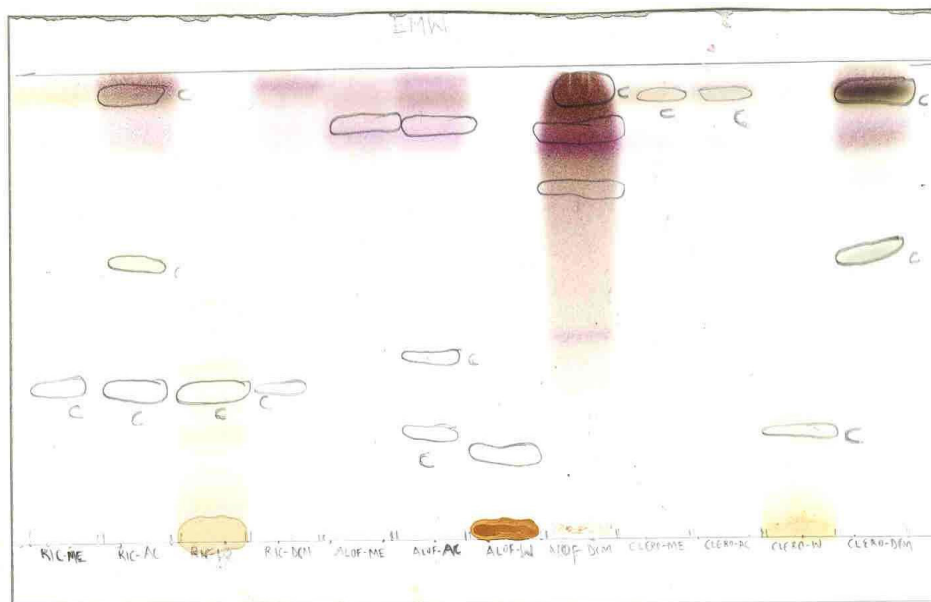


Figure 6.4A Chromatograms of all plant extracts separated by EMW mobile phase and sprayed with vanillin reagent to observe colour derivatives.

Lanes from left to right of the chromatogram: Ric-ME Ric-AC, Ric-W, Ric-DCM, Alof-ME, Alof-AC, Alof-W, Alof-DCM, Clero-ME, Clero-AC, Clero-W and Clero-DCM.

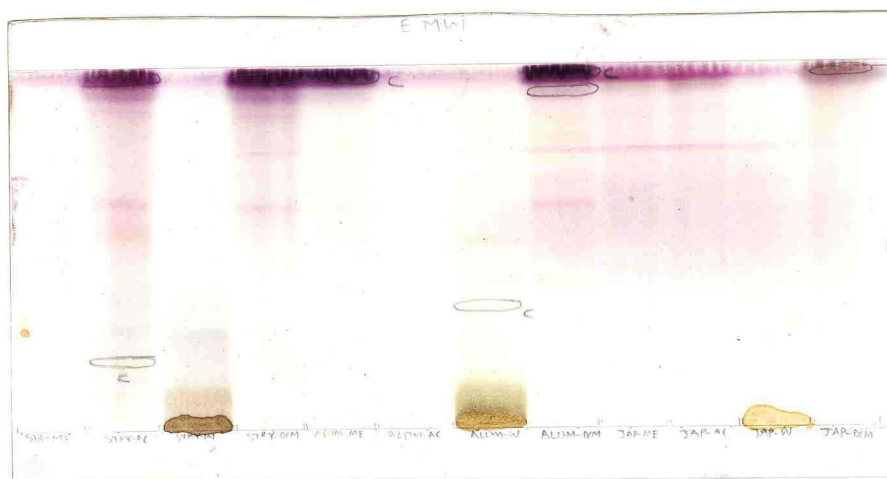


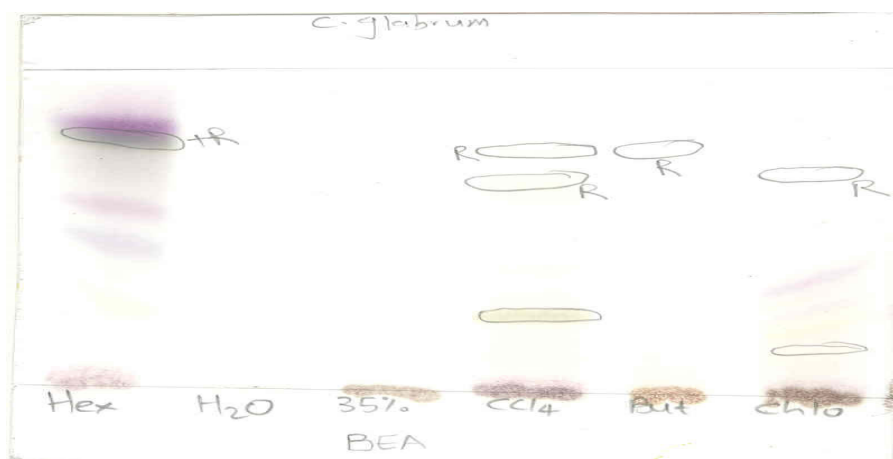
Figure 6.4B Chromatograms of all plant extracts separated by EMW mobile phase and sprayed with vanillin reagent to observe colour derivatives.

Lanes from left to right of the chromatogram: Stry-ME Stry-AC, Stry-W, Stry-DCM, Alom-ME, Alom-AC, Alom-W, Alom-DCM, Jap-ME, Jap -AC, Jap -W and Jap -DCM.

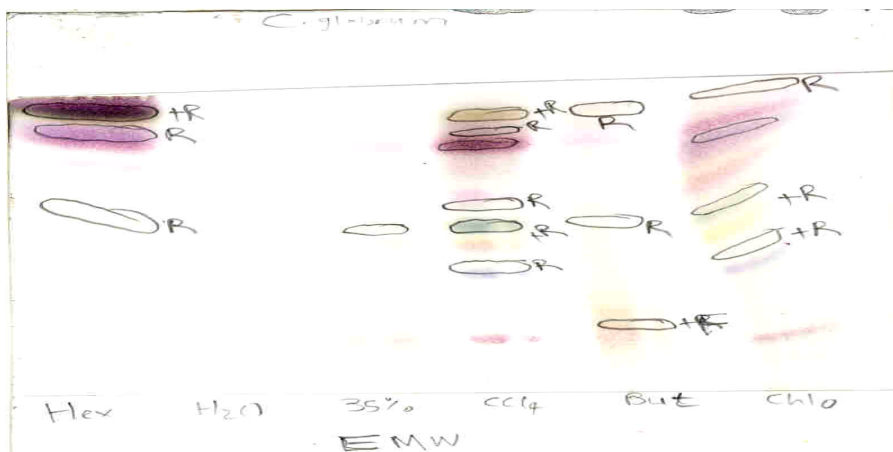
6.3.2 Solvent solvent fractionation

Solvent-solvent fractionation process was conducted on *A. marlothii* and *C. glabrum* since their acetone extracts were found to be active on tick repellency bioassay. All fractions of *A. marlothii* were inactive therefore the results reported in this section are basically of *C. glabrum*. Three TLC chromatograms of *C. glabrum* separated the compounds (Fig. 6.5)

1. BEA system



2. EMW system



3. CEF system

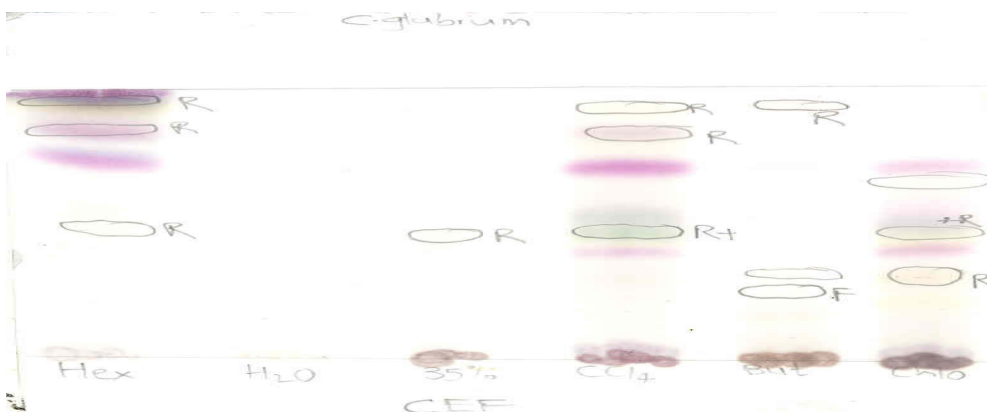


Figure 6.5 Chromatograms of six fractions of *C. glabrum* separated by BEA, CEF and EMW mobile phases and sprayed with vanillin reagent. Lanes from left to right of the

chromatogram: Hexane, water, 35% water / methanol, carbontetrachloride, butanol and chloroform

(Hex =hexane, H₂O = water, 35% = 35% water in methanol, CCl₄ = carbotetrachloride, But = butanol and chlo =chloroform).

The physical properties of all fractions are listed in Table 6.1. The chloroform and hexane fractions contributed a quantity of 62.2 and 46.6% respectively. The R_f values of each component from all fractions were calculated and recorded per solvent system (Table 6.2). The compounds active against the ticks are relatively non polar because they were present in the non polar fractions.

The chloroform fraction was a major component of the acetone extract and contained quite a number of compounds compared to the other fractions. The hexane fraction being the second major component had approximately the same number of compounds compared to the rest of the component fractions and few compounds compared to chloroform fraction.

Table 6.1 Physical properties of six fractions obtained after solvent-solvent fractionation process of *A. marlothii* and *C. glabrum* were conducted

Plant species	Colour	Weight of crude extract	Physical properties	Six fractions							
				H ₂ O component	CHCl ₃ component	Hexane	n-BuOH	H ₂ O	CCl ₄	CHCl ₃	35% H ₂ O in MeOH
<i>A. marlothii</i> sap	Yellow	4.26 g									
			Weight obtained / [g]	-	0.218	0.084	1.306	0.145	0.159	0.065	0.007
			Extraction volume / [ml]	-	3x50	3x50	3x60	-	3x50	3x50	-
			Colour	Dark brown	Yellow	Light yellow	Dark brown	Dark brown	Greenish brown	Yellow	Coffee brown
			% Fraction	-	5.1	1.97	30.7	3.4	3.73	1.53	0.16
<i>C. glabrum</i>	Green	4.79	Weight obtained/[g]	-	2.98	2.23	0.11	0.15	0.98	0.14	0.19
			Extraction volume/[ml]	-	100	3x100	3x100	-	3x100	3x100	-
			Colour	Green	Green	Green	Green	White	White	Green	Green
			% Fraction	-	62.2	46.6	2.3	3.13	20.5	2.92	3.97

Table 6.2 R_f values of six fractions of *C. glabrum* in three different solvent systems

(The letters R and F denote the colour observed under fluorescence light, R stands for red and B for blue)

		CEF system - R _f values						EMW system - R _f values						BEA system - R _f values					
		Hexane	n-Butanol	Carbon tetrachloride	Chloroform	Water	35% water in methanol	Hexane	n-Butanol	Carbon tetrachloride	Chloroform	Water	35% water in methanol	Hexane	n-Butanol	Carbon tetrachloride	Chloroform	Water	35% water in methanol
<i>C. glabrum</i>	1	0.47 R	0.18 F	0.39	0.21		0.45 R	0.61 R	0.15 R	0.18	0.20		0.16	0.44	0.77 R	0.24	0.15		
	2	0.71 R	0.25	0.46 R	0.31 R		0.69	0.83	0.71 R	0.43 R	0.50 R		0.52	0.54		0.66 R	0.22		
	3	0.84	0.33	0.52	0.42			0.88 R	0.91 R	0.54 R	0.53		0.81	0.77		0.74 R	0.26		
	4	0.93 R	0.70	0.70	0.48 R			0.94 R		0.62 R	0.59 R			0.80			0.40		
	5		0.93R	0.82 R	0.66					0.81	0.71						0.69 R		
	6			0.91 R	0.70					0.85 R	0.84								
	7									0.90 R	0.88								
	8										0.98 R								

The hexane fraction was very active with a repellency of 96.2%. This fraction was further fractionated with vacuum liquid chromatography according to the guidelines outlined in Fig. 6.6A and B. The hexane and DCM subfractions had repellency activity of 65.5% and 73.6% respectively (Fig. 6.6A). This indicates that the active compounds must be highly non polar.

The VLC was conducted with the active DCM subfraction but repellency activity was lost. Repellency of 58.7% was regained when some of the DCM subfractions i.e., 50% DCM in ethyl acetate were recombined and two layers were formed (Fig. 6.6B). The two layers were inactive when separated from each other.

Three TLC chromatograms of hexane sub-fraction and other steps followed to separate the components (Fig. 6.7). The individual components lost their activity when subjected to repellency bioassay.

There has been much speculation that in many cases different plant compounds have synergistic activities. There has not been too much direct proof for this hypothesis. These results prove that more than one compound is involved in the repellency of ticks by the hexane fraction of *C. glabrum* acetone extracts.

A final attempt was made by separating the compound present in the hexane fraction by preparative TLC and subjecting the fractions obtained to LC-MS (results not shown). The results were very poor. It appeared that the compound isolated may have decomposed or possibly they were lost due to volatility.

To continue with the isolation of the different compounds responsible for the repellency is beyond the scope of an MSc, but poses an interesting challenge for other scientist to follow up.

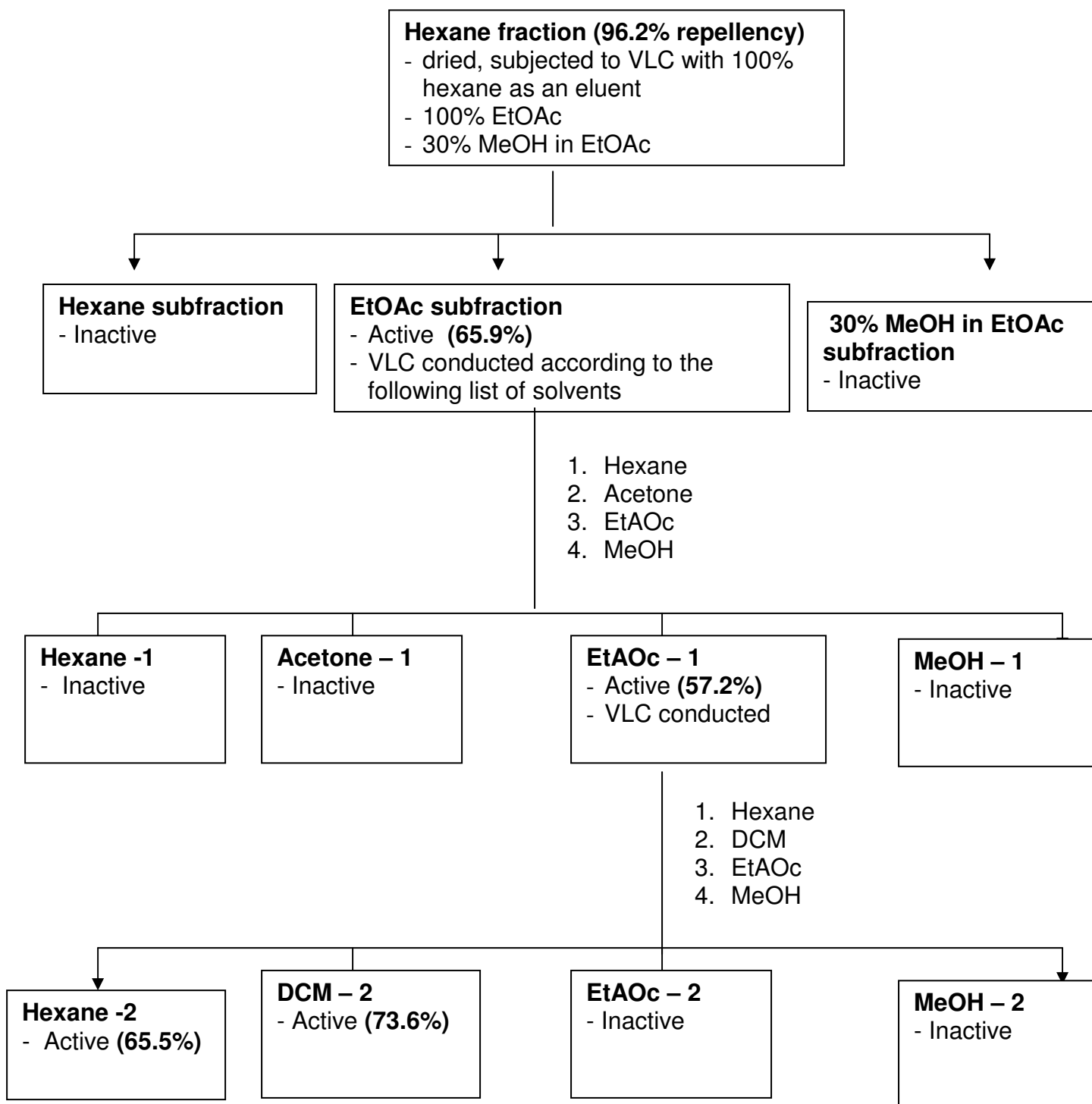


Figure 6.6A Results obtained after vacuum liquid chromatography of *C. glabrum* hexane fraction was conducted

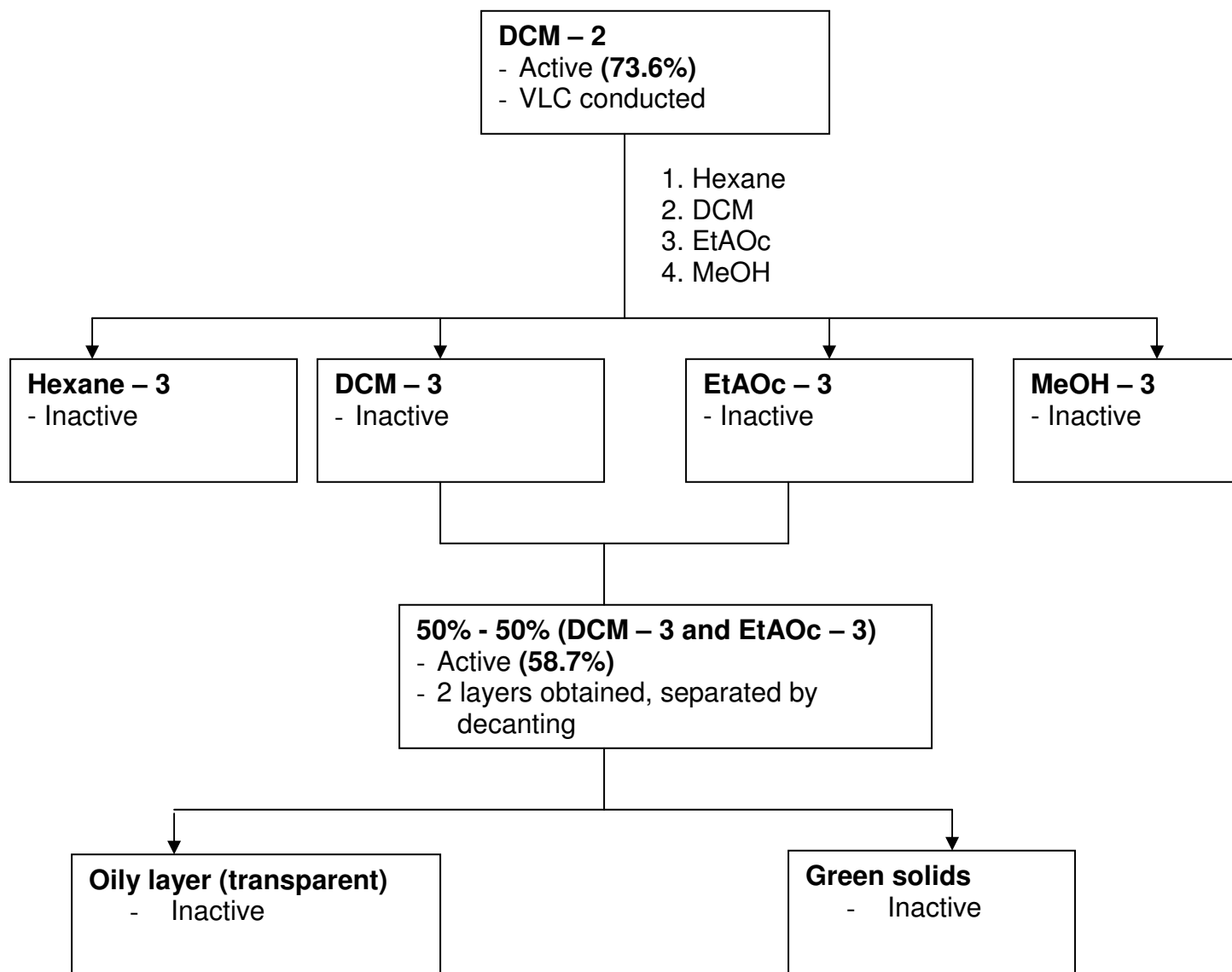


Figure 6.6B Results obtained after vacuum liquid chromatography of *C. glabrum* DCM fraction was conducted

CHAPTER 7

DISCUSSION AND CONCLUSION

The aim of this study was to evaluate six different plant species used traditionally by rural farmers to protect livestock against ticks. There were major differences between the different plant species examined.

Traditional concoctions

Where the activity of decoctions prepared in the traditional way was tested, only the *S. madagascariensis* leaf extracts had substantial tick repellent properties. This is important because it not only validates the ethnoveterinary use, but the extraction system used is available to rural farmers. Furthermore the aqueous extract would not contain mainly volatile compounds that may evaporate and become ineffective within a short period. It is important that the efficiency under field conditions and also against other ticks should be tested. The positive results should be brought under the attention of rural farmers.

Because the compounds extracted are so polar it was not amenable to analysis by normal phase TLC and was not investigated in more depth. These results could explain the wide use of *S. madagascariensis* to control ticks on livestock (Karlsson 1995; Malaya & Banda 1995).

Soap infusions with paraffin added

The soap infusions did not have any significant effect on the repellency assays. All plant soap infusions were however, toxic to *R. appendiculatus*. *A. ferox*, *S. madagascariensis* and *J. curcas* were the most toxic. The dipping and topical applications of all plant paraffin-soap infusion showed almost the same results for instance, *A. ferox* with strong to moderate toxicity at concentrations of 35.5% to 30%. Complete and incomplete coverage by dipping or topical application of the extract on *R. appendiculatus* also had the same impact in tick mortality. There

may have been an effect of the plant compounds, but the paraffin present may have been a common denominator in all of the assays.

Activity of organic extracts

The 30% DCM extract of *R. communis* and 30% acetone extract of *C. glabrum* had excellent average repellency activities of 87.5 and 87.3% compared to values of 89% for the same concentrations of the commercial products Bayticol® (89%) and Amitix® (45%). At lower concentrations of the two plant extracts *R. communis* lost most of its activity at a 10% concentration (average repellency 40.8%). *C. glabrum* lost most of its activity at a concentration of 5% (average repellency 41%).

Attempts to isolate the active compounds

Mabogo (1990) and Hutchings *et al.* (1996) reported that the leaves of *C. glabrum* as used for insect and tick control. Based on the results obtained the acetone extract of *C. glabrum* was selected as the best extract to attempt to isolate the tick repellent compounds. Further fractionation of acetone extract of *C. glabrum* by solvent-solvent fractionation process followed by VLC and silica gel column chromatography was conducted to determine the exact tick repellent responsible in this plant species. Some of the fractions had very high repellency activity. The process of bioassay guided fractionation went very well up to a point when repellency activity was lost. This could be due to decomposition of the bioactive compounds. When two of the fractions were combined however, lost activity was re-established. This is one of the few cases where unambiguous proof for synergism between different compounds was found. Attempts to isolate and characterize the compounds at this last stage by preparative TLC followed by NMR spectroscopy were not successful possibly because the compounds decomposed by oxidation on the silica gel TLC plate in contact with the atmospheric oxygen.

Potential use of results

There are two potential products that can come out of this study. In the first place the high repellency found with extracts compared to commercial products opens the possibility that a low cost commercial product could be developed from these results. Unfortunately comparative time and concentration studies on the commercial products were not carried out. The acetone extract of *C. glabrum* had excellent repellent activities, but the activity was lost after a few hours. If the loss of activity is in the same order for the commercial products, the *C. glabrum* extract could play role in the market. If the commercial product has a longer efficiency, some work could be done on the extract to increase its duration of activity.

A second product that could come out of this work is the fine tuning of decoctions or aqueous extracts with good activity. This could involve evaluating the activity of e.g. different species of *S. madagascariensis* as well as plants from different areas. One would also have to evaluate the activity against other tick species. If it could be shown that extracts are active under field conditions and does not for example quickly decompose by photo-oxidation, this information could be a boon to rural stock farmers where these species occur.

Further research on these lines could have some merit.

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