STRUCTURE-REPELLENCE STUDIES ON SYNTHESIZED STEREOISOMERS OF MENTHANE-DIOL AND ANALOGUES AGAINST THE *Rhipicephalus appendiculatus*

BY

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DECLARATION

I hereby declare that this is my original work and has not been presented for the award of a degree or any award in any other university.

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DEDICATION

This work is dedicated to my father Peter Muthengi, my mother Elizabeth Kabaara and all the family members for their support.

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ABBREVIATIONS AND ACRONYMS

ABP	Applied Bio-prospecting Programme
BCED	Behavioral and Chemical Ecology Department
CL	Confidence Limit
DCM	Dichloromethane
GC-MS	Gas Chromatography-Mass Spectrometry
HP	Hewlett Packard
LC-MS	Liquid Chromatography-Mass Spectrometry
MS	Mass Spectroscopy
NIST	National Institute of Standards and Technology
PMD	*p*-Menthane-3,8-diol
QUISTOR	Quadruple Ion Storage Trap Mass Spectrometer
RD	Repellence Dose
R_f	Retardation Factor
SAS	Statistical Analysis Software
TBD	Tick-Borne Diseases
TIC	Total Ion Chromatogram
t_r	Retention Time

ABSTRACT

Tick-borne infections of livestock are wide-spread in Africa and present a great constraint to livestock development, particularly in the improvement of local breeds. This problem is compounded by the high susceptibility of foreign breeds of livestock being used to improve livestock productivity in many African countries. Brown Ear tick, *Rhipicephalus appendiculatus,* is the most economically important of the 40-70 African tick species. This is due to the fact that it is a highly efficient vector of *Theileria parva*, that causes East Coast Fever (ECF), the tick also causes cutaneous effects such as focal dermal necrosis and irritation, such it's the pathogen of the most important and complex tick–borne disease, (ECF). A commonly used commercial arthropod repellent N,N-diethyl-3-methylbenzamide (DEET) is still considered the best available product for repelling arthropods. However, this repellent is harmful to human beings and it has been found to cause considerable environmental pollution. In search for effective alternatives to DEET against different hematophagous arthropods, there has been renewed interest in repellents of botanical origin. A monoterpene of relatively low volatility, *p*-menthane-3,8-diol, obtained from lemon eucalyptus leaves (*Eucalyptus citriodora*) has shown potent repellence against mosquitoes. This study sought to undertake structure-activity studies of synthesized *p*-menthane-3,8-diol stereoisomers and analogues against the Brown ear tick in order to identify structural features associated with high repellency. The essential oil of lemon eucalyptus was extracted by hydrodistillation. Commercial standards of (+) and (−)-isopulegol were hydrated at C-8 using the oxy-mercuration/demercuration procedure to obtain (+) and (–)-*trans*-*p*-menthane-3,8-diol respectively. (±)-*Cis*-*p*-menthane-3,8-diol stereoisomers were prepared from (±)-citronellal via the Zimmermann and English procedure that involved acid catalyzed cyclization of (±)-citronellal. GC-MS was used to identify the constituents of *E. citriodora* oil and the structure of the synthesized *p*-menthane-3,8-diol (PMD) stereoisomers, LC-MS was used to analyze the aqueous fraction. The *E. citriodora* oil, menthane diol stereoisomers, its analogues and DEET were screened for their repellent activity against *R. appendiculatus*, through subjecting them to a dual choice tick climbing bioassay. The data obtained was analyzed using (SAS® Institute, 2002-2003, version 9.0) and R-statistical package. Menthane diols were potently repellent (RD_{75} (1.21– 2.04) $\times10^{-3}$ mg)) against *R. appendiculatus* and comparable to that of DEET, 1.43×10^{-3} mg. Racemates of *cis*, (RD_{75} 1.42×10^{-3} mg) and *trans* 1.42×10^{-3} mg were as repellent as (+) and (−)-*trans* diols, (2.03– 2.04) $\times10^{-3}$ mg. Thus, repellency was neither stereospecific nor stereoselective. PMD analogues of the diol (L-menthol, 1-α-terpineol) showed much lower repellency, RD_{75}, 1734.12 mg and 6960.34 mg against *R. appendiculatus* compared to *p*-menthane-3,8-diol stereoisomers. *E. citriodora* oil had much lower repellency than PMD stereoisomers, but significantly higher repellency than L-menthol and 1-α-terpineol. The yield of *E. citriodora* oil was 1.84% and the major constituents in *E. citriodora* oil were citronellal (32.03%), citronellol 19.41%), cineole (9.87%) and isopulegol (5.97%). The aqueous fraction of the hydro-distillate was least repellent to the Brown ear tick and it was found to contain aromadendrene oxide, linalool, citronellic acid and ursolic acid, which have been shown to be weakly repellent to arthropods. Therefore, the structural features required for repellency against *R. appendiculatus,* according to this study is a saturated menthane skeleton with two hydroxyl functions at C-3 and C-8. The findings of this study lay down the groundwork for search for new user-approved, efficacious tick repellents.

CHAPTER ONE: INTRODUCTION

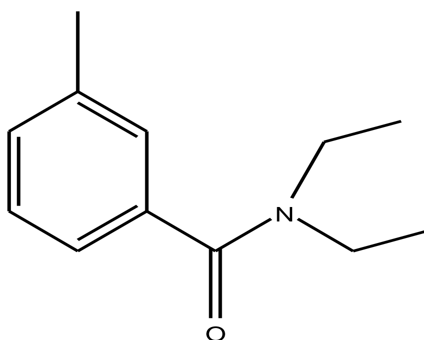
1.1 Background information

In sub-Sahara Africa, East Coast Fever (ECF), caused by the protozoan parasite, *Theileria parva* (Theiler, 1904) and transmitted by brown ear tick, *Rhipicephalus appendiculatus*, is one of the major constraints to the development of the livestock industry (Neumann, 1901; Norval *et al*., 1992; Olwoch *et al*., 2008). Of the estimated 12·7 million heads of cattle (both indigenous and exotic), 76% is at risk to ECF (East Africa, 2003). The disease is associated with up to 10% morality in zebu calves in ECF endemic areas and can cause up to 100% mortality in susceptible exotic and indigenous breeds (Mbogo *et al*., 1995; East Africa, 2003).

Control and management of both vector and pathogen have continued to rely heavily on the application of synthetic chemical acaricides on the host. This has proved to be unsuitable in many ways (Norval *et al*., 1992). The acaricides can eliminate ticks from the host, but do not prevent continued re-infections from the source environment where ticks spend 90% of their life. Secondly, these acaricides are costly and out of reach to many poor livestock farmers. For effective management of ticks, there is need to look for a mechanism to control ticks on individual hosts as well as in the host environment in order to control re-infections during grazing. One possible strategy towards achieving this would be use of tick's repellents on the host and tick-repellent plants in the pasture.

A commonly used commercial arthropod repellent, N͵N-diethyl-3-methylbenzamide (DEET) (**1**), is still considered the best available product repelling a wide variety of insects, ticks and mites (Fai and Xin͵ 2007). In humans, however, the repellent may cause insomnia, mood disturbances,

impaired cognitive functions, seizures, toxic encephalopathy and allergic reactions (Robbins and Cherniack, 1986; Qui *et al*., 1998; Lewis *et al*., 2000). Though DEET (**1**) is not expected to be bio-accumulative, it has been found to cause considerable environmental pollution (Seo *et al*., 2005). More recent research has shown that mosquitoes, as well as being 'blinded' by the chemical, actively dislike the smell of DEET (**1**) (Syed and Leal, 2008). Studies have also shown that plant-based repellents can be as effective as DEET (**1**) or even better (Panda, 2004; Trongtokit *et al*., 2004). The practical application of tick-repellent plants and essential oils and their integration with other measures either on the host or in the pasture land could be a practical and economical way of controlling not only livestock ticks but other arthropod vectors (Gupta and Rutledge, 1994).



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1.2 Tick Control Methods

Tick control is practiced in a wide variety of circumstances involving different tick and host species. The main reasons for tick control are to protect hosts (livestock) from irritation and production losses, formation of lesions that can become secondarily infested, damage to hides and udders, toxicosis, paralysis, and of greatest importance, infection with a wide variety of

disease agents (Norval *et al.,* 1992). Control also prevents the spread of tick species and the diseases they transmit to unaffected areas, regions, or continents (Aiello and Mays, 2003). The resultant product being the increased livestock production manifested in improved livelihood and economic development and growth of livestock holders (Mukhebi *et al.*, 1991). Some of the tick control methods include; chemical control using synthetic acaricides, handpicking, biological control, cultural control and vaccination against the vectors.

1.2.1 Chemical control

It involves use of synthetic acaricides including arsenicals, organochlorides, organophosphates, carbamates, amidenes, pyrethrines and synthetic pyrethroids (Mitchell, 1996). The modes of application of these acaricides have included the use of tanks knapsack sprayer, hand sprayer, hand dressing, and dusting as well as cattle dipping in acaricide-treated artificial pools (Awumbila, 1996).

Acaricides are pesticides that kill members of the arachnid subclass *Acari*, which includes ticks and mites. Acaricides are used both in medicine and agriculture, although the desired selective toxicity differs between the two fields. The first application of ixodicides to control ticks on cattle was made by treating the infested cattle with various oils-including paraffin but without much success (Harrison *et al.,* 1973). An effective chemical control of livestock ticks began with the introduction of arsenical solutions as cattle dips in South Africa in 1893 and in Australia in 1895 (Mitchell, 1996). The use of chemical ixodicides against livestock ticks has continued until today. In Africa, seven basic acaricide groups have been indiscriminately used to control livestock ticks and these are: (i) arsenicals, (ii) organochlorines, (iii) organophosphates, (iv)

carbamates, (v) amidines, (vi) pyrethrins and (vii) synthetic pyrethroids (Mitchell, 1996). In recent years, several other methods of acaricide application have been tested, including the slow release of systemic acaricides from implants and boluses; the slow release of conventional acaricides from impregnated ear-tags; 'pour-ons', which are applied on the backs of livestock and spread rapidly over the entire body surface; and 'spot-ons', which are similar to 'pour-ons' but have less capacity to spread (Norval, 1989).

Acaricide application may either be directed against the free-living stages in the environment or the parasitic stages on the hosts. In either case, the problems associated with the use of acaricides in livestock industry are very challenging without imminent solutions. Acaricides are costly and out of reach of poor rural livestock farmers who also do not have sufficient technical know-how of managing and handling them (Norval *et al.,* 1992). The high costs of acaricides have become a major problem for smallholder farmers who constitute the majority in the livestock industry on the continent of Africa (Kariuki, 1996). The control of ixodid ticks by acaricidal treatment of vegetation has been done in specific sites to reduce the risk of tick infestation to susceptible hosts. This method is, however, not sustainable and has not been recommended for wider use because of associated environmental pollution problems and the high costs involved in the treatment of large areas (Aiello and Mays, 2003). In addition, inappropriate drainage of dip liquid causes water pollution and indiscriminate damage to the flora and fauna in the environment. Few quantitative data are available on the impact of these acaricides on flora and fauna but it can be assumed to be substantial at the local level (De Haan *et al.,* 1996). Acaricides are a health hazard as well. They cause food poisoning (through meat, blood and milk) and

residual toxicity. For instance, organochlorine products have been demonstrated to leave residues in meat and milk (Mitchell, 1996).

Intensive application of acaricides to livestock creates an enzootically unstable disease situation in the population (Norval *et al.,* 1992). When tick control breaks down, large losses can occur. An example of this was in Zimbabwe, where a compulsory dipping policy had been in force since 1914 and when dipping infrastructure broke down during the war of independence between 1974 and 1979, an estimated one million cattle died, mainly of tick-borne diseases (Lawrence *et al.,* 1980). The ticks have consistently shown themselves to possess a genetic pool containing the potential to resist a wide range of chemical poisons. This has been compounded by illegal cattle movement, civil unrest in some areas, poor management and inadequate maintenance of cattle dips and poor use of manufacturers' instructions (Matthewson, 1984; Nolan, 1990).

It has been shown that the indiscriminate use of acaricides may affect future tick control as this has the consequence of facilitating rapid development of tick resistance to the active compounds used in the acaricide formulations (Fraga *et al.,* 2003). There is therefore a very real danger that unless new acaricides of different chemical structures are forthcoming, tick resistance to existing compounds will spread. But the problem is that the development of new acaricides is a long and very expensive process (Graf *et al.,* 2004).

1.2.2 Mechanical Control / Hand picking

The livestock is communally organized, brought together and held in a crutch facility, then tick are picked off the animals one by one and either buried or thrown into the fire (Marina *et al.*,

2001). This practice was also conducted during milking and cleaning of livestock sheds by women (Marina *et al*., 2001). Some ticks, after being picked from the respective host animals, were given to chickens/birds at home as a food supplement. However, this method is tedious, time consuming and involves much labour in order to serve a big herd of cattle, hence not a sustainable method.

1.2.3 Biological control

Biological control of ticks is the use of natural enemies (parasitoids, pathogens, parasites and predators) that can reduce the density of the target population or even eliminate it. The development of biological control of ticks is more neglected than that of most other animal pests (Rutz and Patterson, 1990) and lags behind that of plant pests by several decades (Samish, 2000).

In the literature, however, more than 257 tick bio-control agents are mentioned, comprising 100 species of pathogens, seven parasitoids and 150 predators (Samish and Alekseev, 2001). The subject has been extensively reviewed by Hu *et al.,* (1998) and Samish (2000), who shed some light on the way forward and reflected on the previous failures and successes. Only a few studies have as yet been conducted on pathogens, parasitoids, and predators of ticks. A first remarkable field trial was made with the introduction of parasitic wasps originating from France, in the late 1920s and early 1930s in the USA and in the early 1940s in Russia (Hu *et al.,* 1998). However, in both the USA (Smith and Cole, 1943) and Russia (Alfeev, 1946) they were unsuccessful in controlling target tick populations, similar to a previous trial conducted in the USA between the years 1927 and 1932 (Cooley and Kohls, 1934). During the past decades, interest in developing biological methods for tick control using birds (Couto, 1994), parasitoids (Hu *et al.,* 1998),

entomopathogenic nematodes (Samish, 2000), entomopathogenic fungi, arthropods (Samish and Alekseev, 2001) have gained momentum worldwide, because of the limited impact of these organisms on the environment.

1.2.4 Host resistance

Host resistance is a measure of the host's ability to limit the establishment, growth rate, fecundity and/or persistence of a parasite population (Coop and Kyriazakis, 1999). It has been reported from numerous studies that zebu (*Boophilus indicus*) and sanga (*B. taurus* and *B. indicus* cross-breed) cattle, the indigenous breeds of Asia and Africa, usually carry significantly fewer ixodid ticks than exotic European (*B. taurus*) breeds of cattle (Utech and Wharton, 1982; Aiello and Mays, 2003).

It has been shown that tick infestation increases as the proportion of European genes in an animal increases (Lemos *et al.,* 1985). Studies have also shown that the magnitude of losses due to tick infestation varies with the genotype of cattle (Lemos *et al.,* 1985). Within a genotype, losses per tick unit increase with the number of attached ticks (Pegram *et al.,* 1989a; b). The tick resistance of zebu breeds and their crosses is increasingly being considered for exploitation as a means of control of tick ectoparasitic stages on livestock. The introduction of zebu cattle to Australia has positively revolutionized the control of *B. microplus* on that continent as zebu breeds were successfully exploited in cattle breeding programmes to develop tick resistant cattle breeds that limited the impact of *B. microplus* infestation (Seifert, 1984).

Use of resistant cattle as a means of tick control is also becoming important in Africa, Asia and America (Fraga *et al*., 2003; Silva *et al*., 2007). Host resistance, reviewed by Latif and Pegram (1992), manifests itself as the rejection of ticks that attach to the host because of host-specific physiological and immunological reactions. Highly resistant cattle keep overall tick populations very low in contrast to cattle with low resistance in the same herd that harbour more ticks in certain seasons (Solomon and Kaaya, 1996). Cattle can be ranked for resistance on the basis of natural tick counts, and about 10% of cattle ranked as of low resistance carrying 50% of the total tick population infesting the herd (Latif *et al*., 1991).

Comparison of the respective tick infestation on calves and cows showed that calves had a lower tick infestation than cows (Jongejan *et al*., 1987). In most parts of Africa studied, zebu and sanga cattle are found to be considerably more resistant to tick-borne diseases (Tatchell and Easton, 1986; Bakheit and Latif, 2002) and the application of intensive dipping with the purpose to increase weight gain is therefore not justifiable (Norval *et al*., 1992). The use of naturally tick-resistant cattle biotypes should be incorporated in tick control schemes as a means to contribute to the control of tick infestations on livestock (Silva *et al*., 2007). Although assessing the levels of host resistance in different breeds of cattle by selection, breeding or gene alteration (De Castro, 1991) may take some time, the feasibility of this method has been demonstrated at the International Centre of Insect Physiology and Ecology, Kenya (Latif, 1992).

1.2.5 Host grooming

There is evidence to strongly support the concept that grooming (cleaning the fur of an animal), which occurs amongst animal communities, is beneficial (Hart, 2000; Park, 2008). In Africa it

has been observed that many species of wild bovids live in tick infested environments but usually with low levels of tick loads. This is the result of a behavioural defense against infestations with ectoparasites such as ticks as was experimentally demonstrated and observed in free-ranging adult female impala (Mooring and Hart, 1995). It is reasonable to assume that much of the vulnerability of cattle to ticks reflects a relatively reduced predisposition to groom, a behaviour that in turn stems from their derivation from European (and more recently from North American) stock, where the environment has been relatively free from ticks (Hart *et al*., 1996).

Thousands of years of low exposure to ticks may have led to a selection against frequent grooming in favour of reducing the cost associated with frequent grooming. The grooming in many wild bovids observed in a number of studies provide evidence that systemic chemical cues (chemical signals affecting the whole body) may be modulating the grooming rate (Hart, 1997). Thus, there might be some way to enhance the grooming of European-derived cattle through appropriate administration of biological substances that are found to increase grooming in wild bovids. Given the potential value of grooming in the removal of attached ticks, another possibility is that cattle might be selectively bred (or genetically engineered) to show high rates of grooming and, hence, be less susceptible to tick-borne diseases. However, before embarking upon a costly and time consuming breeding programme, effective and protective values of grooming for tick control first needs to be ascertained (Hart, 1997).

In Trinidad, studies by Smith in 1974 showed that grooming was effective in reducing the infestation of cattle with the tick, *Amblyomma cajennense* Fabricius (Smith, 1975). In Kenya, adult female *R. appendiculatus* have been observed to die as a result of increased grooming by

cattle because of increased dermal irritation by the ticks (Essuman *et al.,* 1991). There have been studies to show the interspecific and intraspecific determinants that modulate grooming in animals, some of the intraspecific determinants appear to be mediated by systemic physiological influences, suggesting there may be ways to improve grooming activity in weak groomers, such as cattle (Hart, 2000).

1.2.6 Cultural control

This involves habitat interference and host removal, directed against both the free-living and parasitic stages of ticks. Alteration of the environment by removal of certain types of vegetation has been used in the control of the *Amblyomma americanum* in recreational areas and *Ixodes rubicundus* in South Africa (Neumann, 1901). Habitat interference and host removal can be directed against both the free-living and parasitic stages of ticks (Aiello and Mays, 2003). Removal of alternate host or hosts of a particular stage of the life cycle can also reduce the abundance of tick species as this may starve ticks to death depending on starvation period (Aiello and Mays, 2003). This approach has occasionally been advocated for the control of three-host ixodid ticks such as *R. appendiculatus*, *A. hebraeum* and *A. rubicundus* in Africa, and *Hyalomma spp* in south-eastern Europe and Asia (Norval, 1975).

In Kenya, the Somali community uses this method as one of their tick control strategies. Rotation of pastures or pasture spelling has been used in the control of the one-host ixodid tick *Boophilus microplus* (Canestrini), in Australia (Sutherst *et al*., 1979). The method could also be applied to other one-host ticks in which the duration of the spelling period is determined by the relatively short life span of the free-living larvae. However, it has minimal application to multi-host ixodid

ticks or argasid ticks because of the long survival periods of the unfed nymphs and adults. Burning grazing pastures directly kills ticks, while ploughing grazing fields buries them and eventually they die (Sutherst *et al*., 1979).

1.2.7 Vaccination

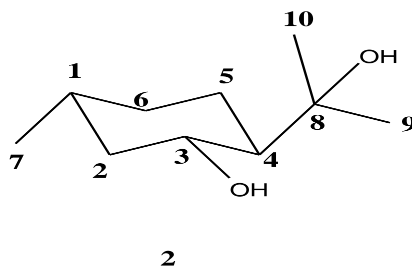
A novel approach of tick control is to make vaccines against the vectors rather than against all the individual disease agents they carry (Labuda *et al.,* 2006). Previous studies have shown this approach to be feasible (Burke *et al.,* 2005; Labuda *et al.,* 2006). Research has focused on identifying proteins from the whole tick macerates, the tick's salivary glands and digestive tract as candidate broad-spectrum tick vaccine targets (Trimnell *et al.,* 2005).

Efforts have so far yielded potential vaccines against *Boophilus spp.* (Willadsen *et al*., 2006). Prospects of developing similar vaccines against other ixodid ticks of major veterinary importance have not been forthcoming. *Boophilus spp.* are one-host ticks and show a marked preference for bovine hosts, which act as the principal reservoir of perhaps the most important group of disease agents (*Babesia spp.*) that *Boophilus* ticks transmit. By contrast, most other ticks of medical importance are three-host ticks, which infest not only cattle but also wild ungulate species. For these reasons, vaccines against non-boophilid ticks may not be feasible and a development of the near future. Anti-tick vaccines, however, remain one of the most promising prophylactic measures against tick bites and transmission of tick-borne pathogens (De la Fuente and Kocan, 2006).

1.3 Problem Statement and Justification

Due to the rapid development of tick resistance to synthetic chemical acaricides and the potential risk posed by these chemicals to non-target species, efforts are intensifying towards the development of alternative tick control strategy. ECF is the cause of great economic loss due to its high mortality in livestock especially cattle, and the cost of disease surveillance, control and treatment. According to United States Department of Agriculture USDA (2012), developing regions like Sub-Saharan Africa, livestock producers are often faced with the challenge of managing diseases that infect animals, making it difficult to sustain herds and farm productivity.

A commonly used commercial arthropod repellent N¸N-diethyl-3-methylbenzamide (DEET) (**1**) has various side effects to livestock and human. This study seeks to look for an alternative, user-friendly repellent, with high potency, *p*-menthane-3,8-diol (**2**) from *Eucalyptus citriodora* Hook leaves has been shown to be repellant against mosquitoes (Barasa *et al.*, 2002), naturally derived PMD is as effective as DEET when used in equal doses (Yang *et al.,* 1974). The current study seeks to determine the potency of stereoisomers of *p*-menthane-3,8-diol and analogues on Brown ear tick, and ascertain the requirements for repellency.



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1.4 Hypothesis

p-Menthane-3,8-diols and specific analogues may be good repellent(s) to the Brown ear tick, *R. appendiculatus*.

1.5 Objectives

1.5.1 General objective

To undertake structure-activity studies on stereoisomers of *p*-menthane-3,8-diol and analogues in order to identify structural features associated with high repellency against the Brown ear tick.

1.5.2 Specific objectives

i. To extract essential oil from *Eucalyptus citriodora* Hook, whose minor constituent is *p*-menthane-3,8-diol, determine its repellence on adult *R. appendiculatus* and its chemical composition.

ii. To synthesize stereoisomers of *p*-menthane-3,8-diol, and determine the stereochemical features responsible for tick repellency.

iii. To compare the repellency of some selected menthane diol analogues and DEET with stereoisomers of *p*-menthane-3,8-diol.

1.6 Scope and limitation of the study

Because of time constraints and limited number of ticks, only the laboratory climbing bioassay will be used to evaluate the repellency of stereoisomers of *p*-menthane-3,8-diol and the analogues. These compounds will not be tested on cattle and only a selected number of analogues will be assayed.

CHAPTER TWO: LITERATURE REVIEW

2.1 *Rhipicephalus appendiculatus*

R. appendiculatus (Figure 2.1) is a three-host tick *Amblyomma hebraeum, Rhipicephalus (Boophilus*) *spp*., and *Rhipicephalus appendiculatus*, found on most animal species and belongs to a genus of ticks in the family *Ixodidae* (hard ticks), also called Brown ear tick. It transmits *Theileria parva*, *Babesia spp*. and other protozoan and viral diseases (including Nairobi sheep disease and louping ill) in cattle, other livestock, and antelope. It belongs to the class of hard ticks and they are found in the ears of cattle, other livestock, and antelope. Heavy infestations on cattle can result in severe damage to the ears, a potentially fatal toxemia, or the loss of resistance to some infections (Konnai *et al.,* 2007).

Figure 2.1: *Rhipicephalus appendiculatus* (Neumann, 1901)

R. appendiculatus (Figure 2.1) prefers cool, shaded shrubby or woody savannas with at least 24 inches of annual rainfall. It is endemic from southern Sudan and eastern Zaire to South Africa and Kenya and can be found from sea level to 7400 feet (2300 meters).

2.1.1 Life Cycle of Brown Ear Tick

R. appendiculatus (Figure 2.1) is the principal vector of East Coast Fever and becomes infected with *T. parva* when feeding on an infected host having piroplasms in erythrocytes (Konnai *et al*.,

2007). Piroplasm-infected erythrocytes are ingested by ticks of the larval or nymphal stages and undergo a sexual development cycle in the gut of the replete tick to produce zygotes, which in turn develop into motile kinete stages that infect the salivary gland acini of the next instars, the nymph or adult (Fawcet *et al*., 1985). In the salivary glands of the tick, the kinete develops into infective sporozoites (sporogonic phase) and this repeats the cycle when the infected tick with infective sporozoites in its salivary glands takes a blood meal from a susceptible cattle host (Figure 2.2).

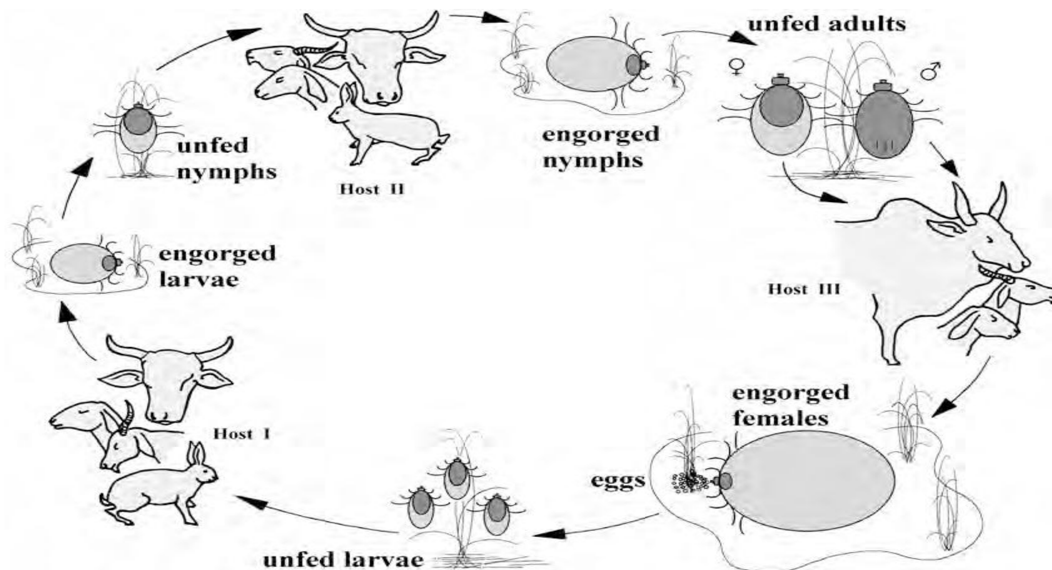


Figure 2.2: The life cycle of *Rhipicephalus appendiculatus* displaying the teletropic type of behavior (the three-host cycle) (Speybroeck *et al*., 2003).

Theileria parva only mature and enter the saliva after the tick attaches to a host for a considerable period of 3 − 4 days (Martin *et al.,* 1964). This period has been recently shown to be between 24 and 72 hours (Ochanda *et al.,* 1988; Konnai *et al.,* 2007). However, if environmental temperatures are high, infective sporozoites can develop in ticks on the ground

and may enter the host within hours of attachment (Ochanda *et al.,* 1988). The time from entry in the tick to sporozoite development in the salivary glands is on average 19 − 20 days for feeding nymphs and 20 − 21 days for adult females (Ochanda *et al.,* 1996; Watt and Walker, 2000).

2.1.2 Behaviour of *Rhipicephalus appendiculatus*

2.1.2.1 Host seeking behavior of *Rhipicephalus appendiculatus*

Blood-feeding arthropods such as ticks have over time developed a complex relationship with their mammalian hosts. Broad variations occur in host specifically of ticks, duration and multiplicity of contacts and in host location behavior (Gibson and Torr, 1999). The results obtained from analysis of a quantitative data set of 43,615 individual collection records of ticks in Africa suggest the existence of a spectrum in host-specificity but with the edges of this spectrum readily demarcated (Cumming, 1998). From this broad range of a spectrum in host-specificity, generalists and specialists in host/prey location can be discerned (Steidle and Van Loon, 2003). Nevertheless, what induces host-seeking behavior of the vector and its subsequent finding and selection of suitable hosts by different tick species has not been fully understood. This behavior has been considered either as the result of evolutionary adaptation processes to the host-derived stimuli (Cupp, 1991; Steidle and Van Loon, 2003), pathogen-induced behaviour in the vector, normal feeding habits, visual cues, host food and or its products such as faeces, urine or exuviates (Steidle and Van Loon, 2003) or combinations of these factors. In other arthropods such as mosquitoes, the role of olfaction in host-seeking behaviour has been explained (Takken, 1991), including cues as human breath and body odours (Mukabana, 2002). It is assumed that host-seeking behaviour of ticks is affected by similar cues. The combined knowledge demonstrates that the host-vector-parasite relationship is complex in nature, whose pattern of

responses and sequence of behavioral events, particularly of the vector, have to be clearly understood and strategically integrated in epidemiological tools in order to achieve sustainable control and management of vector-borne diseases. Previous studies indicated that various attractive host-derived stimuli (for example, host texture, host skin humidity, host body temperature and chemical factors (kairomones/allomones/synomones)) such as skin emanations, breath, urine and faeces, influence host-seeking behaviour in ticks (Sika, 1996). Kairomones are the main sensory cues used by haematophagous organisms to find their hosts (Mordue. and Mordue, 2003).

Environmental factors complement these kairomones in influencing host-seeking behaviour in ticks (Speybroeck *et al*., 2003). Adult *R. appendiculatus* search their hosts for a blood meal when they are active early in the day. They become active under specific sets of temperature, rainfall, humidity, length of the rainy season, number of rainy and cloudy days, and day length (Pegram *et al*., 1989b). Numbers of adult *R. appendiculatus* on the host increase after the onset of the rains (Berkvens *et al.,* 1998). The main factor responsible for this phenology is thought to be day length, where a long photoperiod terminates the state of diapauses and induces host-seeking behaviour in the wet months (Madder *et al*., 2002). Diapauses in ticks are considered to be a pre-adaptive behaviour to allow the ticks to survive unfavorable conditions of a given season. Near the equator, ticks are non-diapausing and may usually feed throughout the year and their numbers vary less (Speybroeck *et al*., 2004).

Adult *R. appendiculatus* ticks prefer to feed inside and around the ears of their hosts (their predilection sites). Combination of a repellent blend from the anal region and an attractive blend

at the ear have been shown to play natural "push" and "pull" roles, respectively, to guide these ticks to the cattle ears. In the laboratory, odour trapped from cattle ears attracted *R. appendiculatus* but repelled *R. eversis* whereas that from the anal region had an opposite effect (Wanzala *et al*., 2004). This odour-based push-pull pair of stimuli may largely account for efficient orientation behaviour of the two tick species to their respective feeding sites (Wanzala *et al*., 2004).

2.2 The Genus *Eucalyptus*

The genus *Eucalyptus*, a native of China and Australia, consist of over 600 species. The planted eucalypts are used mainly for pulpwood, poles, fuel wood, charcoal, and more recently sawn timber. Less well known is their use in the production of non-timber forest products such as floral nectar for honey, bulk for tannin and leaf extracts for pharmaceutical and industrial purposes (Boland *et al*., 1991). The leaves of most species of *Eucalyptus* yield an essential oil, known in trade as eucalyptus oil. While 300 species have been shown to contain volatile oils in their leaves, less than 20 species are grown commercially for oil. The major species are *Eucalyptus polybractea* (Blue Melle), *E. citriodora* Hook (Lemon scented gum), *E. viridis* (Green Mellee), *E. dives* Schau (Broad leafed peppermint), *E. radiata* Hook (Narrow leafed peppermint), *E. smithii* Hook (Gully gum) and *E. globulus* Hook (Tasmanian Blue gum) (Boland *et al*., 1991).

2.2.1 *Eucalyptus citriodora* Hook

Eucalyptus citriodora Hook (Figure 2.3), also known as lemon gum, spotted gum or citron-scented gum, is a tall tree attaining a height of 40 m. The essential oil of *E. citriodora*, with its

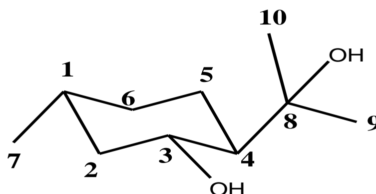
high content of citronellal, is one of the three perfumery oils distilled on commercial scale from the *Eucalyptus* species (Mwangi *et al*., 1982). In addition to yielding an important essential oil from its leaves, *E. citriodora* is grown for its sawn timber. The wood has also been reported to be resistant to termite attack (FAO, 1953; 1974).

Figure 2.3: *Eucalyptus citriodora* Hook (Hill and Johnson, 1995)

Chemical investigations by Mwangi *et a*l., (1981) showed that the three principal components of the oil of the Kenyan grown *E. citriodora* are citronellal (65-88%), citronellol (2-25%) and isopulegol (2-19%). It has long been shown that oil of lemon *Eucalyptus* has repellent effects on mosquitoes and chemical studies have shown that its principal constituents are citronellal, isopulegol and α-pinene (Curtis *et al*., 1991).

2.3 *p*-Menthane-3, 8-diol

p-Menthane-*3,*8-diol (PMD) (**2**), is an active ingredient used in insect repellents (Othmer, 1981). It smells similar to menthol and acts as a coolant. PMD is found in the essential oil within leaves of the *E. citriodora* tree. This tree is native to Australia, but is now cultivated in many warm places around the world. When refined, the oil known as oil of lemon Eucalyptus or, more commonly, citriodiol is used as insect repellent. Typically, Citriodiol contains 64% PMD (a mixture of the *cis* and *trans* isomers of *p*-menthane-3,8-diol). It is the only natural ingredient that is used as an insect repellent (Othmer, 1981).



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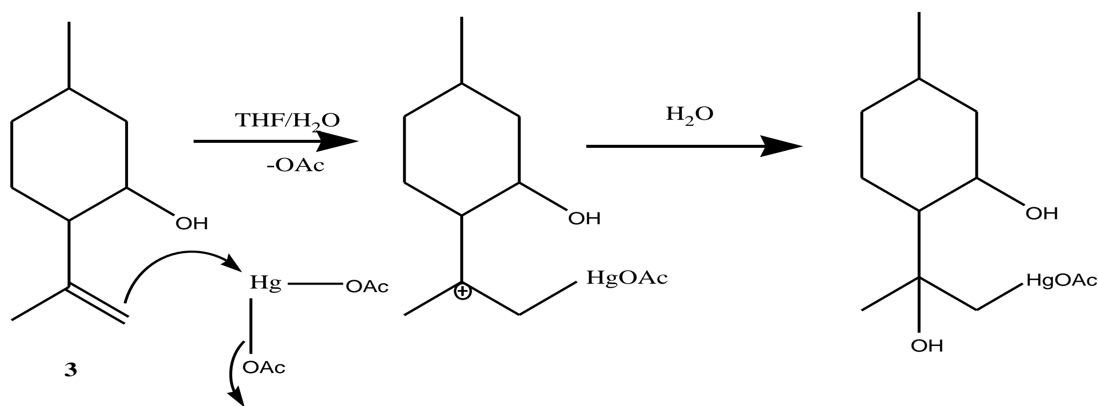
The Center for Disease Control has recognized PMD as the only effective, natural substance for deterring mosquitoes carrying West Nile Virus. PMD can be synthetically manufactured; however, one study has found that repellents containing synthetic PMD are not as effective as

those containing naturally derived PMD (oil of lemon Eucalyptus). *p*-Menthane-3,8-diol has 3 chiral centers and hence eight possible stereoisomers (Yang *et al.,* 1974).

2.4 Methods of synthesis of *p*-menthane-3,8-diol stereoisomers

2.4.1 Oxymercuration-demercuration of olefins

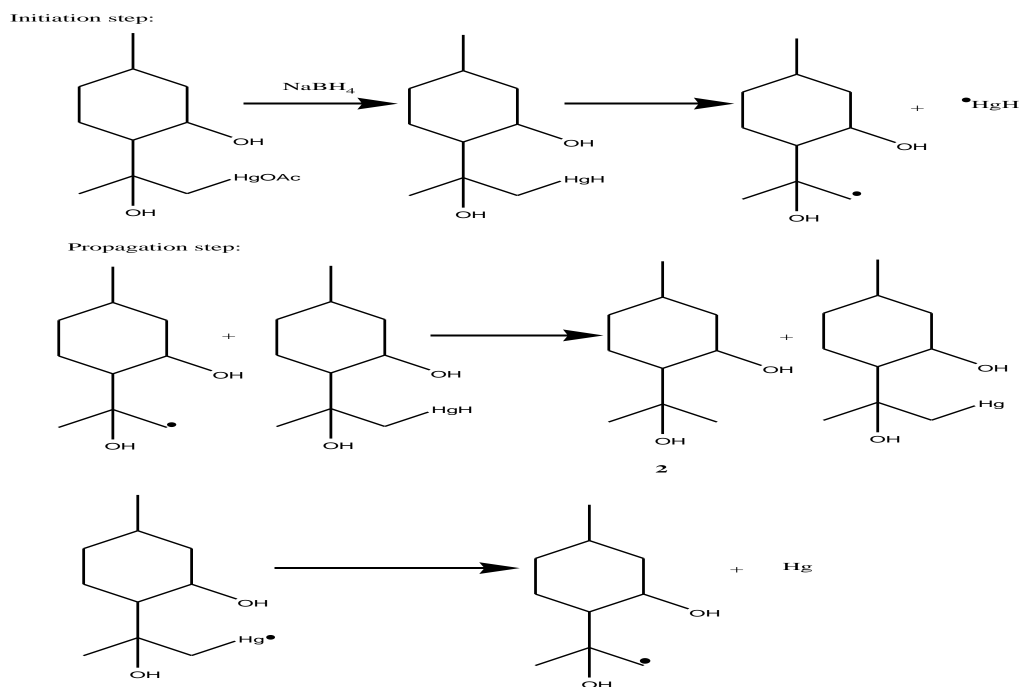
The oxy-mercuration reaction, combined with the reduction of the oxy-mercurial intermediate by sodium borohydride *in situ,* provides a convenient mild method to achieve the Markovnikov hydration of carbon-carbon double bonds without observable rearrangements. The mechanism of the oxy-mercuration step is thought to proceed as shown in scheme 2.1 (Brown and Geoghegan, 1967). Isopulegol (**3**) gets hydrated at C-8 using the oxy-mercuration/demercuration procedure; this leads to introduction of a hydroxyl group at C-8 of (**3**) and does not create a new chiral center in the product.



Scheme 2.1: Oxy-mercuration step

The reactivity of different alkenes towards mercuration spans a considerable range and is governed by a combination of stereo-electronic factors. Terminal double bonds are more reactive than internal ones. Disubstituted terminal alkenes, however are more reactive than monosubstituted ones (Brown and Geoghegan, 1967).

This method which is applicable to mono-, di-, tri-, as well as phenyl substituted olefins gives Markovnikov addition. Hydroxy, methoxy, acetoxy, halo and other groups may be present in substrate without causing difficulties (Larock, 1986). Apart from water, other nucleophiles that can be used for synthetic purposes includes; alcohols, carboxylate ions, hydroperoxides, amines and nitriles. The reductive replacement of mercury by hydrogen with the use of sodium borohydride is a free-radical process (Scheme 2.2) (Norman and Coxon, 1993).



Scheme 2.2: Demercuration step

The evidence for the free-radical mechanism including the course of the reaction can be diverted by oxygen, an efficient radical scavenger. Also consistent with the occurrence of a free-radical intermediate is the formation of cyclic products when 5-hexenylmercury compounds are reduced with sodium borohydride (Carey and Sundberg, 1990). The simplicity and speed of the present procedure suggest that it should provide a highly convenient synthetic route for the Markovnikov hydration of the carbon-carbon double bond.

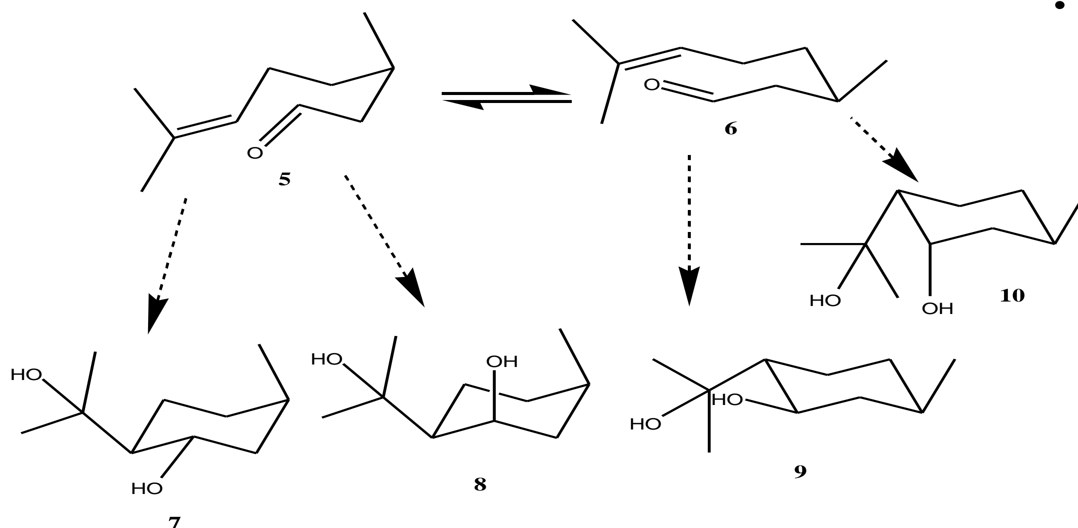
2.4.2 End-Game Tactics: Combination of the Felkin-Anh Rule and the Zimmerman-Traxler model

Many addition reactions to carbonyl compounds proceeds via cyclic six-membered transition states. The course of such reactions can be understood using the Zimmerman-Traxler model, which requires the arrangement of the reaction partners in a chain-like transition state, placing the large substituent as equatorial as possible (Mengel and Reiser, 1999).

Different enantiomers of citronellal (**4**) could be used as candidate reactants for the synthesis of pure enantiomeric *p*-menthane-3,8-diol (**2**). A chair-like transition state is expected to form. Under hydrative (kinetic or thermodymanic) conditions, the tertiary carbocation is expected to undergo attack as illustrated in scheme 2.3.

The conformation of the transition state in which the methyl group is axial is of high energy and hence unfavorable. Thus, it is expected that the conformation in which the methyl group is equatorial will be favored. It is therefore expected that if an enantiomerically pure starting

material is used, then two diastereoisomers should be afforded in significant yields. Thus it is predicted that (R)-citronellal (**5**) should afford two diastereomers. Similarly, (S)-citronellal (**6**) should provide the other two antipodes. This is due to the fact that the π-electrons can attack the carbonyl from either below or above the plane.



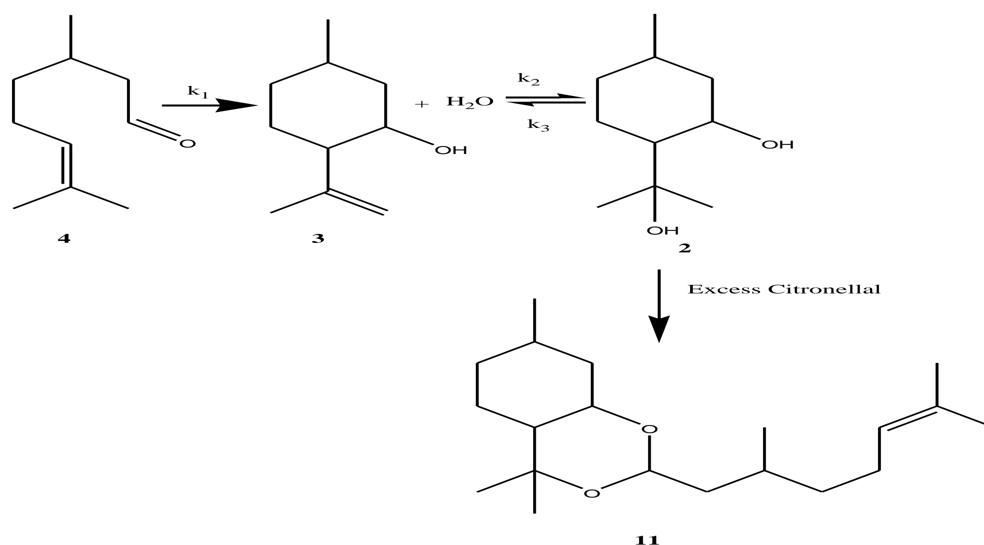
Scheme 2.3: Zimmerman-Traxler model

2.4.3 Cyclization of citronellal to *p*-menthane-3,8-diols in water and carbon dioxide

A clean process was developed for the synthesis of *p*-menthane-3,8-diols from cyclization of citronellal (**10**) in CO_2–H_2O medium without any additives (Haiyang *et al.*, 2009).. With the addition of CO_2, the reaction rate could be enhanced about 6 times for the cyclization of citronellal in H_2O, because CO_2 dissolved into water and formed carbonic acid inducing an increase of the acidity. Although, the reaction conversion in CO_2–H_2O was slightly lower compared to that obtained with sulfuric acid as catalyst, CO_2–H_2O could replace the sulfuric acid at a relative higher reaction temperature. The reaction kinetics studies showed that the hydration

of isopulegol (**3**) to *p*-menthane-3,8-diol (**2**) was a reversible reaction. The equilibrium constant and the maximum equilibrium yield obtained in CO_2–H_2O at a range of CO_2 pressures were similar to that with sulfuric acid catalyst (Haiyang *et al*., 2009).

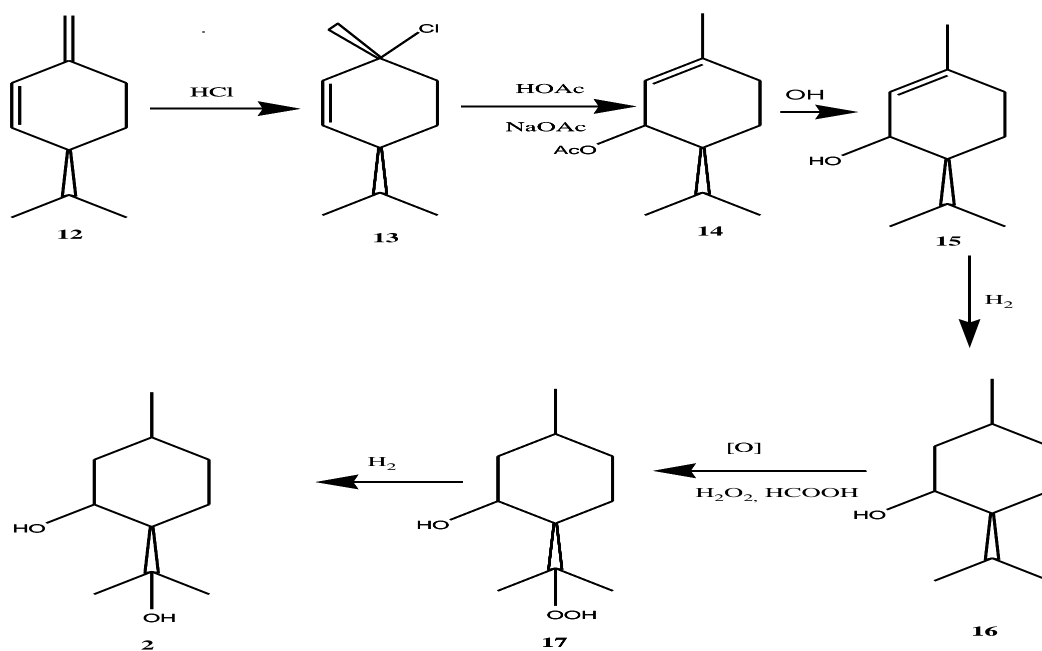
The CO_2 pressure and temperature are the key factors affecting reaction rate and selectivity. The reaction rate in water has been enhanced largely in the presence of CO_2 for the formation of carbonic acid. Kinetic analysis indicates the hydration of isopulegols to *p*-menthane-3,8-diols is a reversible reaction. Compared to sulfuric acid catalyst, the CO_2–H_2O system is an environmentally friendly and easy to operate process for the cyclization of citronellal to *p*-menthane-3,8-diol (**2**) (Scheme 2.4) (Haiyang *et al*., 2009). The cyclization of citronellal as shown in Scheme 2.4 involves the isomerization of citronellal firstly to isopulegols, and then isopulegols hydration to *p*-menthane-3,8-diols, as well as citronellal and *p*-menthane-3,8-diols condensation to *p*-menthane-3,8-diol citronellal acetals (**11**), if product (**2**) is not separated from the unreacted (**4**).



Scheme 2.4: The cyclization of citronellal to *p*-menthane-3,8-diol

2.4.4 Synthesis of *p*-menthane-3,8-diol from phellandrene

Phellandrene (12) is available from the turpentine of the *Pinus contorta*, with the common names *Lodgepole pine* and Shore pine, and also known as twisted pine (mainly β-phellandrene) where it is the major constituent and from Eucalyptus dives (mainly α-phellandrene). After converting the phellandrene to menthol (**20**), the synthesis follows hydroperoxidation route (Leffingwell and Shackelford, 1974) (Scheme 2.5). Reduction to form (**13**) takes place on the exo double bond rather than the endo one, because when the pi-electrons of the alkenes form a bond with a proton from HCl, a carbocation and a halide ion are formed, the exo reaction forms a more stable, tertiary carbocation, while an endo one forms a secondary one. The halide ion reacts with the carbocation by donating an electron pair to form (**13**).



Scheme 5: Synthesis of *p*-menthane-3,8-diol from phellandrene

2.5 Analytical Techniques

2.5.1 Gas chromatography - mass spectrometry (GC-MS)

Gas Chromatography-Mass Chromatography (GC-MS) is the synergistic combination of two powerful analytical techniques. The GC separates the components of a mixture in time and the MS provides information that aids in structural identification of each component (Fulton *et al.,* 1996).

GC-MS in the present work was used as a technique for identification of the *E. Citriodora* oil and synthesized stereoisomers of *p*-menthane-3,8-diol. This is because the mass spectra of the various components eluting during GC separation are recorded, and the mass spectrum of a compound is characteristic of the identity of the compound confirmed by derivatization. So long as there is a library equipped with the mass spectra of all the compounds, any compound in the sample analyzed can be identified. Hence identification is not limited to the availability of authentic samples.

2.5.2 Sample Introduction

Once the GC separates the constituents of the original sample, the individual components enter the mass spectrometer through an interface between the GC and the MS. The MS is held at extremely low pressure by use of a specialized vacuum which is coupled to the MS manifold. Within the manifold is housed the ion source. As the compounds elute from the GC column, the stream of molecules enters this source, where a metallic filament discharges electrons into the oncoming path (Raymond and Scott, 2003).

Several different interface designs are used to connect these two instruments as listed below:

2.5.3 Interfacing techniques

Interfacing techniques include:

- Direct interface: The end of GC column is fitted directly into the ion source.
- Open slit interface: The capillary transfer column is split in the middle and purge gas used to introduce the effluent to MS.
- Molecular jet separator: The GC carrier gas passes through a jet and expands into a partial vacuum. Only the heavier molecules pass through into the capillary jet.
- Effusion separator: Consist of a tube through which the carrier gas flows at slightly reduced pressure. It then diffuses through the porous material and is removed.
- Diffusion separator: The carrier gas passes through a thin membrane supported on a fine metal mesh .The inorganic carrier gas passes onto the exit while organic analyte is attracted to the membrane and diffuses through to the low pressure ion source.

The most common GC/MS interface now uses a capillary GC column. Since the carrier gas flow rate is very small for these columns, the end of the capillary is inserted directly into the source region of the mass spectrometer. The entire flow from the GC enters the mass spectrometer (Raymond and Scott, 2003).

2.5.4 Ion Sources

A variety of ionization techniques are used for mass spectrometry. Most ionization techniques excite the neutral analyte molecule which then ejects an electron to form a radical cation ($M^{\bullet+}$). Other ionization techniques involve ion molecule reactions (MH^+). The most important considerations are the physical state of the analyte and the ionization energy. Electron ionization and chemical ionization are only suitable for gas phase ionization. The ionization energy is significant because it controls the amount of fragmentation observed in the mass spectrum. Although this fragmentation complicates the mass spectrum, it provides structural information for the identification of unknown compounds (Ernst, 2004).

2.5.4.1 Electron ionization

The electrons used for ionization are produced by passing a current through a wire filament. The amount of current controls the number of electrons emitted by the filament. An electric field accelerates these electrons across the source region to produce a beam of high energy electrons. When an analyte molecule passes through this electron beam, a valence shell electron can be removed from the molecule to produce an ion. EI produces positive ions by knocking a valence electron off the analyte molecule. As the electron passes close to the molecule the negative charge of the electron repels and distorts the electron cloud surrounding the molecule. This distortion transfers kinetic energy from the fast-moving electron to the electron cloud of the molecule (Ernst, 2004).

If enough energy is transferred by the process; the molecule will eject a valence electron and form a radical cation ($M^{\bullet+}$). A mass spectrum is produced by ionizing many molecules; the

spectrum is a distribution of the possible product ions. Intact molecular ions are observed from ions produced with little excess energy. Other molecular ions have more energy and undergo fragmentation in the source region. The abundance of the resulting fragments, often called product ions is determined by the kinetics of the fragmentation pathways and the ionization energy. This distribution provides the structural information for interpreting mass spectra (Ernst, 2004).

2.5.4.2 Chemical ionization

Chemical Ionization (CI) is a “soft” ionization technique that produces ions with little excess energy. As a result, less fragmentation is observed in the mass spectrum. Only slight modifications of an EI source region are required for CI experiments. In Chemical ionization the source is enclosed in a small cell with openings for the electron beam, the reagent gas and the sample. In the CI source, analyte molecules undergo many collisions with a reagent gas which is ionized with an electron beam to produce a cloud of ions. The reagent gas ions in this cloud react and produce adduct ions which are excellent proton donors (Ernst, 2004).

When analyte molecules (M) are introduced to a source region with this cloud of ions, the reagent gas ions donate a proton to the analyte molecule and produce MH^+ ions. The energetic proton transfer is controlled by using different reagent gases. The most common reagent gases are methane, isobutane and ammonia. Methane is the strongest proton donor commonly used. For softer ionization, isobutane and ammonia are frequently used. The reagent gas must be a strong enough Brønsted acid to transfer a proton to the analyte. Fragmentation is minimized in

CI by reducing the amount of excess energy produced by the reaction. Because the adduct ions have little excess energy and are relatively stable, CI is very useful for molecular mass determination. Other ionization techniques include: field ionization, fast atom bombardment and liquid secondary ion mass spectrometry, field desorption, plasma desorption, laser desorption, matrix-assisted laser desorption ionization, thermospray, atmospheric pressure ionization, electron spray, atmospheric pressure chemical ionization, atmospheric pressure photoionization, atmospheric pressure secondary ion mass spectrometry; (desorption electron spray ionization, direct analysis in real time), inorganic ionization sources; (spark source, glow discharge source, inductively coupled plasma source) and gas-phase ion-molecule reactions (Ernst, 2004).

2.5.5 Mass analyzers

After ions are formed in the source region they are accelerated into the mass analyzer by an electric field. The mass analyzer separates these ions according to their m/z value. The selection of a mass analyzer depends upon the mass range, scan rate and detection limits required for an application. There are two common mass analyzers or separators commercially available for GC/MS these are: quadruple analyzer and the ion trap analyzer (Ernst, 2004).

2.5.5.1 Quadruple

The quadruple mass spectrometer is the most common mass analyzer. Their compact sizes, fast scan rate, high transmission efficiency, and modest vacuum requirements are ideal for small inexpensive instruments. Most quadruple instruments are limited to unit m/z resolution. In the mass spectrometer, an electric field accelerates ions out of the source region and into the

quadruple analyzer. The analyzer consists of four rods or electrodes arranged across from each other (Thomas and Chasteen, 1998).

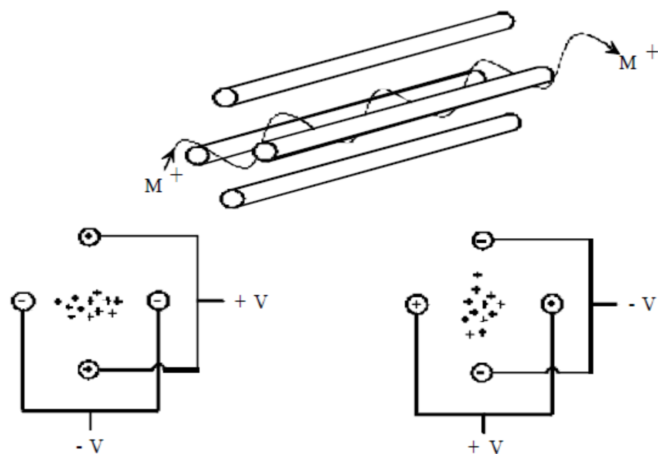


Figure 2.4: Quadruple Mass Analyzer

As the ions travel through the quadruple they are filtered according to their m/z value so that only a value ion can strike the detector single m/z. The m/z value transmitted by the quadruple is determined by the Radio Frequency (RF) and Direct Current (DC) voltages applied to the electrodes. These voltages produce an oscillating electric field that functions as a band pass filter to transmit the selected m/z value. The RF voltage rejects or transmits ions according to their m/z value by alternately focusing them in different planes. The four electrodes are connected in pairs and the RF potential is applied between these two pairs of electrodes. During the first part of the RF cycle the top and bottom rods are at a positive potential and the left and right rods are at a negative potential. This squeezes positive ions into the horizontal plane (Thomas, 1998).

During the second half of the RF cycle the polarity of the rods is reversed. This changes the electric field and focuses the ions in the vertical plane. The quadruple field continues to alternate

as the ions travel through the mass analyzer. This causes the ions to undergo a complex set of motions that produces a three dimensional wave. The quadruple field transmits selected ions because the amplitude of this three dimensional wave depends upon the m/z value of the ion, the potentials applied, and the RF frequency. By selecting an appropriate RF frequency and potential, the quadruple acts like a high pass filter, transmitting high m/z ions and rejecting low m/z ions (Thomas and Chasteen, 1998).

2.5.5.1.1 Quadruple ion trap

The Quadruple ion storage trap mass spectrometer (QUISTOR) is a recently developed mass analyzer with some special capabilities. Several commercial instruments are available and this analyzer is becoming more popular. QUISTORs are very sensitive, relatively inexpensive, and scan fast enough for GC/MS experiments. The sensitivity of the QUISTOR results from trapping and then analyzing all the ions produced in the source. Other analyzers include: electrostatic trap or ‘orbitrap’, time-of-flight analyzers, linear time-of-flight mass spectrometer, magnetic and electromagnetic analyzers, ion cyclotron resonance and Fourier transform mass spectrometry (Thomas and Chasteen, 1998).

2.5.6 Ion detection

Detection of ions is based upon their charge or momentum. For large signals a faraday cup is used to collect ions and measure the current. Most detectors currently used amplify the ion signal using a collector. The gain is controlled by changing the high voltage applied to the detector. A detector is selected for its speed, dynamic range gain, and geometry. Some detectors are sensitive enough to detect single ions (Thomas and Chasteen, 1998).

2.5.7 Library analysis

The most popular computerized method for determining the structure of unidentified, but not novel compound is to search through a library of low-resolution mass spectra for a match between its spectrum and that of the compound in the library usually stores as many as 50,000 electron impact mass spectra. The matching routine involves calculation by the data system of a similarity index, match factor or purity between the unknown spectrum and the library (reference) spectra. Common scales are 0 for complete mismatch to 1, 100 or 1000 for a perfect match. To avoid time-consuming calculations for the purity against all entries in a large library, there is usually a pre-search or a filter which rapidly eliminates dissimilar spectra. Often at each *m/z* value in turn, the ratio (R) of the mass peak heights in the normalized unknown and reference spectra is calculated. The individual R values are summed and the average value is normalized to produce the final purity for a particular reference spectrum (Rose and Johnstone, 1982).

The entries with the highest figures are displayed as the results of the library search. The entries are ranked in order of highest purity, and the fit values for each match calculated. Whatever the fit or purity values, the result of library search should be assessed by careful visual examination of the matching spectra when presented for the highest ranked library entry. All peaks in the reference spectrum should be present also in the spectrum of the sample (Rose and Johnstone, 1982).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Sampling and extraction of essential oils

Eucalyptus citriodora Hook plant leaves were collected from Arboretum, Nairobi County in Kenya (Appendix 1) and identified by Simon Mathenge of National Museums of Kenya. A voucher specimen (**EC/AM/04/2012**) was deposited at Kenyatta University herbarium. The plants materials were left in a well-ventilated room for 1 − 2 weeks before hydrodistillation. The materials were cut into small pieces and 1 kg of the air-dried leaves was hydrodistillated using a Clevenger-type apparatus for 8 hours after the first drop of oil. The essential oil was collected on water layer in the Clevenger apparatus (Jeremy *et al*., 2011), and separated from the water layer.

3.1.1 Purification of essential oil

50 ml of distilled water was added to the hydrodistilled oil and shaken, it was then rinsed on the sides with n-hexane and left to settle. Using a separating funnel, the organic layer (essential oil + n-hexane) was separated from the water layer. Anhydrous Na_2SO_4 was added to the organic layer until no clamps formed and filtered into a round bottomed bottle for rotor evaporation under vacuum at 40^oC. The cleaned and dried essential oil was put in the vials, weighed and stored at −20 °C in a freezer.

3.1.2 Separation and purification of chloroform extract

The aqueous distillate after steam distillation was extracted with chloroform (3×25 ml), dried with anhydrous Na_2SO_4. The undistilled fraction remaining after steam distillation was filtered to remove the leaf residue, saturated with NaCl and extracted with chloroform (4×100 ml). The combined chloroform extracts was similarly dried with anhydrous Na_2SO_4 (drying agent) and

concentrated in vacuo at 40°C to afford 28.277 g of a yellowish concentrate. The chloroform concentrate was further extracted using water (3×50 ml). The water extract was put in ten different plastic vials and freeze-dried for 48hrs. After freeze-drying, 1.02 g of white powder was obtained, stored at −20°C until LC-MS analysis and tick climbing bioassay.

3.2 Synthetic chemicals

Synthetic standards; (+) and (−)-isopulegol, (±)-citronellal were obtained from Sigma - Aldrich Chemical Company (USA) and Fluka respectively

3.3 Experimental ticks

The ticks used (*R. appendiculatus*), were obtained from colonies at the International Livestock Research Institute (ILRI) and bred at International Centre of Insect Physiology and Ecology ICIPE, Nairobi County in Kenya. Rearing conditions were as described by Bailey (1960) and Irvinand and Brocklesby (1970).

3.4 Tick repellency assay - dual-choice climbing assay

The tick climbing bioassay design exploited the well known predisposition of ticks to climb up and aggregate on grass stem to await passing host (Browning, 1976; Chiera, 1985). This experiment was set up at ICIPE, Nairobi County, Kenya. An aluminium base of area 105 cm^2 with two stands of 26 cm in height and 7.0 cm apart was put in a basin of water, 1.5 cm deep (the water restricts the movement of the ticks to the aluminium base). Two sets of glass tubes were used; one of 4.5 cm (outer one) and the other one 0.8 cm (smaller inner tube) in diameter. A strip

of filter paper (Whatmann No 7, 2 cm wide) was stapled to form a collar around the upper parts of each smaller inner glass tubes at a distance of 15 cm from the aluminium base to provide the source of either test odours or pure solvent. A set up consisting of an aluminium base (15 × 7 × 1.5 cm) with a pair of aluminium rods (26 cm l × 0.7 cm d) 7 cm apart covered with glass tubes (0.8 cm d) was used (Figure 3.1). One collar on the pair of tubes was treated with test sample solution and the other with the solvent (dichloromethane) to serve as control. After the solvent was allowed to evaporate (10 min), these tubes were shielded with wider tubes (4.5 cm d) from 4 cm above the aluminium base to facilitate relatively uniform vertical gradients of the test sample along the 3.7 cm gap between two tubes. Wet cotton wool plugs on the top of these tubes ensured relatively high humidity (>75 %) within the columns. Ten ticks of mixed age and sex were placed at the centre of the aluminium base and observed for 60 minutes. The apparatus was placed in a tray with shallow water, which prevented the dispersal of test ticks from the base.

Initial comparison of the responses of the ticks in the set up with and without residual dichloromethane on one and both sides, showed no bias for either side and no effects of the residual solvent. A piece of whatman paper measuring 4 cm by 1 cm, folded and stapled to make a ring, was impregnated and placed on the glass covering about 12 cm from the bottom. The test materials (synthesized *p*-menthane-3,8-diol stereoisomers, analogues, Eucalyptus citriodora oil and the aqueous fraction) and the solvent were dispensed using a calibrated Eppendorf pipette and equilibrated for 30 min before ten adult ticks of mixed ages and sexes were released at the centre of the aluminium base. Prior to each bioassay, ticks were kept at high relative humidity (>85% RH) for 24 hrs in containers with moist cotton wool, so that they were not dehydrated and as a result would have less tendency to drown in the water surrounding the aluminium base

(Figure 3.1). All bioassays were conducted in a room kept at 28 ± 1 °C and 75 ± 5% RH, which had an exhaust fan running continuously. The assays were run for 60 minutes, and the number of ticks above the filter paper strip on the control glass tube (Nc) and on the treated glass tube (Nt) were recorded at 15, 30, 45 and 60 minutes. After each test, the apparatus was thoroughly cleaned and dried at 100 °C. Each dose of the test material was tested 12 times; each time with a fresh, naive adult tick. The repellent effect of the essential oils was evaluated according to the formula adopted by Ndung'u *et al*., (1995) and Lwande *et al*., (1999) on equation 1.0 below.

$$\text{Percentage repellency} = \frac{\text{Nc} - \text{Nt}}{\text{Nc} + \text{Nt}} \times 100\% \quad \ldots \ldots \ldots \; 1.0$$

Where Nt and Nc represent the number of ticks that climbed on or passed the treated and control collar of filter papers on the glass tubes, respectively.

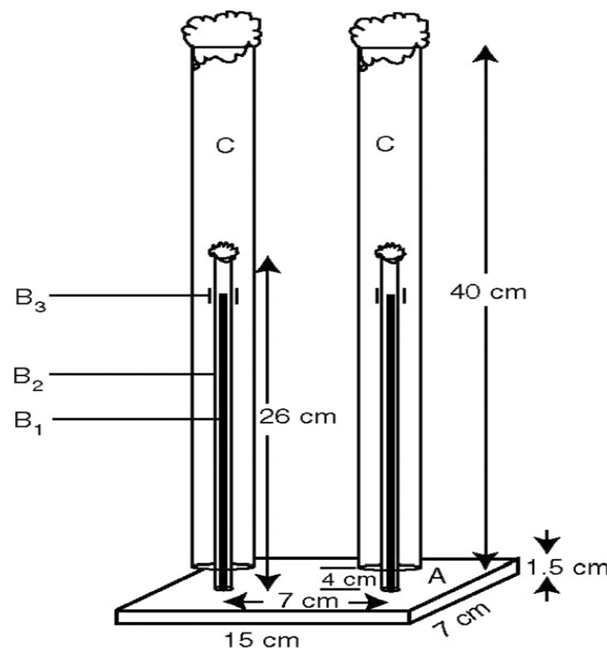


Figure 3.1: Tick climbing bioassay apparatus (Wanzala *et al*., 2004)

Tick climbing bioassay apparatus (placed in a tray with shallow water, not shown): A, aluminium base; B_1, aluminium rod (26 cm l × 0.7 cm d); B_2, 0.8 cm d glass tube; B_3, filter paper collar; C, 4.5 cm d glass tube plugged with dry cotton wool. The two aluminium rods, Bi on the aluminium base, A, (15 x 7cm), were 7 cm apart. The outer tubes, C are held in position, 4 cm above the aluminium base, A by a retort stand clamp. The ten ticks were introduced on the aluminium base, A, at a position marked with a star, 3.5 cm from the base of the aluminium rods, Bi (modified from Browning, 1976).

3.5 Synthesis of *p*-menthane-3,8-diol

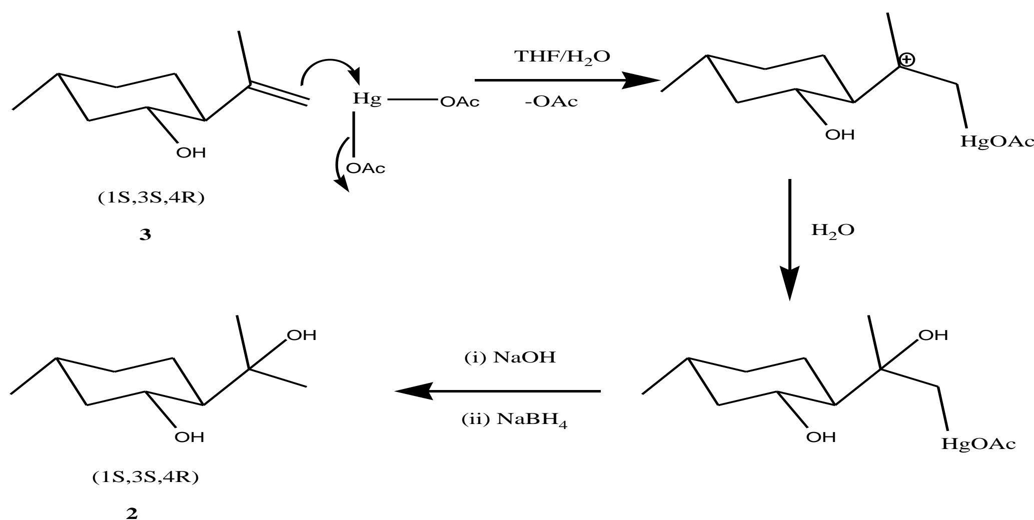
3.5.1 Synthesis of (+)-*trans*-*p*-menthane-3,8-diol from (+)(1S, 3S, 4R)-isopulegol

This synthesis was achieved according to Brown and Geoghegan (1967). Mercury (II) acetate (1.3 g, 4.1×10^{-3}moles) was weighed and put in a100 ml three necked flask fitted with an efficient magnetic stirrer, a dropping funnel and a thermometer. 9 ml of water was then added and stirred until the acetate dissolved. 9 ml of tetrahydrofuran (THF) was then run in rapidly; an orange yellow suspension was formed after several hours. After stirring overnight [18 hrs], 0.5 g, 3.24×10^{-3}moles of (+) (1S, 3S, 4R)-isopulegol (Sigma-Aldrich) was added whereupon the colour was slowly discharged. The mixture was stirred at room temperature for 24 hrs to ensure completion of the oxy-mercuration step. Next, 5 ml of 3 M NaOH followed by 0.16 g, 4.23×10^{-3}moles of $NaBH_4$ in 3.5 ml of 3 M NaOH was added slowly. The rate of addition of both solutions was controlled to maintain the temperature at 25°C, through cooling the reaction mixture from time to time. Demercuration occurred with the separation of elementary Hg, this reduction step was carried out for 15 hours.

Using a separating funnel, the organic (THF) layer was separated from the aqueous alkaline layer. The aqueous layer was saturated with NaCl and extracted with tetrahydrofuran (3×10 ml) to extract the organic fraction, dried (anhydrous Na_2SO_4) and filtered. The solvent was removed from the filtrate by evaporating in vacuo at 45°C; further concentration was done by blowing the solvent off under white spot nitrogen. This resulted to a white thick opaque liquid.

This crude *p*-menthane-3,8-diol was purified on a silica gel column (230 – 240 mesh); using the eluent system of 20 − 40% ethyl acetate in hexane. Further purification of the PMD product was achieved by crystallization from the hexane-ethyl acetate mixture to get white crystals, 495 mg (100%) yield. (+)-isopulegol was hydrated at C-8 using the oxy-mercuration/demercuration procedure, leading to synthesis of (+)–*trans* –*p*-menthane-3,8-diol (**2**). GC-MS analysis gave a single sharp peak at t_r 17.1 minutes.

The introduction of a hydroxyl group at C-8 of the starting material [(+)(1S,3S,4R)-isopulegol], does not create a new chiral center in the product, implying that the product obtained from the oxy-mercuration/demercuration reaction had the same stereochemistry as the starting material (Scheme 3.1).



Scheme 3.1: Synthesis of (+)-*trans*-*p*-menthane-3,8-diol from (+)-isopulegol

3.5.2 Synthesis of (−)-*trans*-*p*-menthane-3,8-diol from (−) (1R,3R,4S)-isopulegol

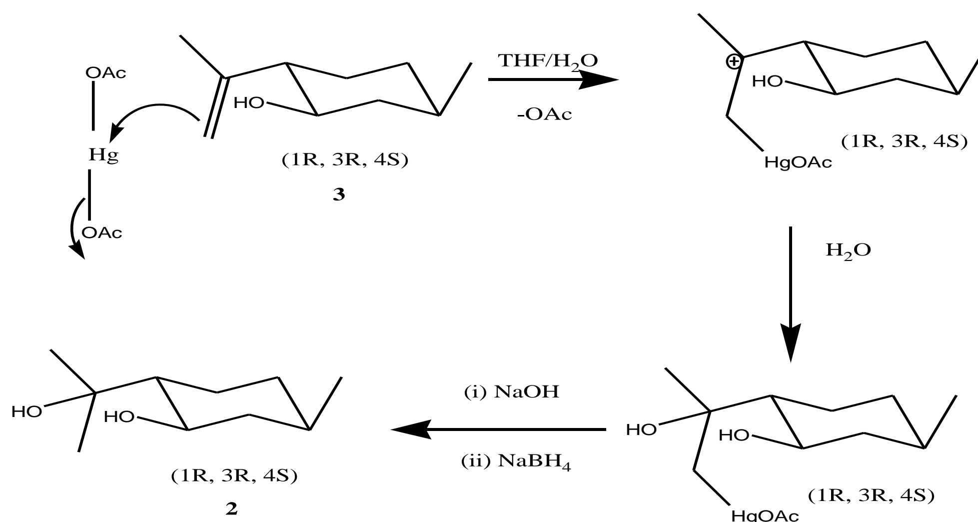
This synthesis was achieved according to Brown and Geoghegan (1967). Mercury (II) acetate (1.3 g, 4.1×10^{-3}moles) was weighed and put in a 100 ml three necked flask fitted with an efficient magnetic stirrer, a dropping funnel and a thermometer. 9 ml of water was then added and stirred until the acetate dissolved. While stirring, 9 ml of tetrahydrofuran (THF) was then run in rapidly; an orange yellow suspension was formed after several hours.

After stirring overnight [18 hrs], 0.5 g, 3.24×10^{-3}moles of (-) (1R,3R,4S)-isopulegol (Sigma-Aldrich) was added whereupon the colour was slowly discharged. The mixture was stirred at room temperature for 24hrs to ensure completion of the oxy-mercuration step. Next, 5 ml of 3 M NaOH followed by 0.16 g, 4.23×10^{-3}moles of $NaBH_4$ in 3.5 ml of 3 M NaOH was added slowly. The rate of addition of both solutions was controlled to maintain the temperature at 25°C, through cooling the reaction mixture from time to time. Demercuration occurred with the

separation of elementary Hg, this reduction step was carried out for 15 hours to ensure completion of demercuration step.

Using a separating funnel, the organic (THF) layer was separated from the aqueous alkaline layer. The aqueous layer was saturated with NaCl and extracted with tetrahydrofuran (3×10 ml) to extract the organic fraction, dried (anhydrous Na_2SO_4) and filtered. The solvent was removed from the filtrate by evaporating in vacuo at 45°C; further concentration was done by blowing the solvent off under white spot nitrogen. This resulted to a white thick opaque liquid.

This crude *p*-menthane-3,8-diol (PMD) was purified on a silica column (230 – 240 mesh); using the eluent system of 20 − 40% ethyl acetate in hexane. Further purification of the PMD product was achieved by crystallization from the hexane-ethyl acetate to get white crystals, 494 mg (99.8%) yield. (−)-Isopulegol was hydrated at C-8 using the oxy-mercuration/demercuration procedure, leading to the synthesis of (–)-*trans*–*p*-menthane-3,8-diol (**8**) (Scheme 3.2). GC-MS analysis gave a single sharp peak at t_r 17.1 minutes.



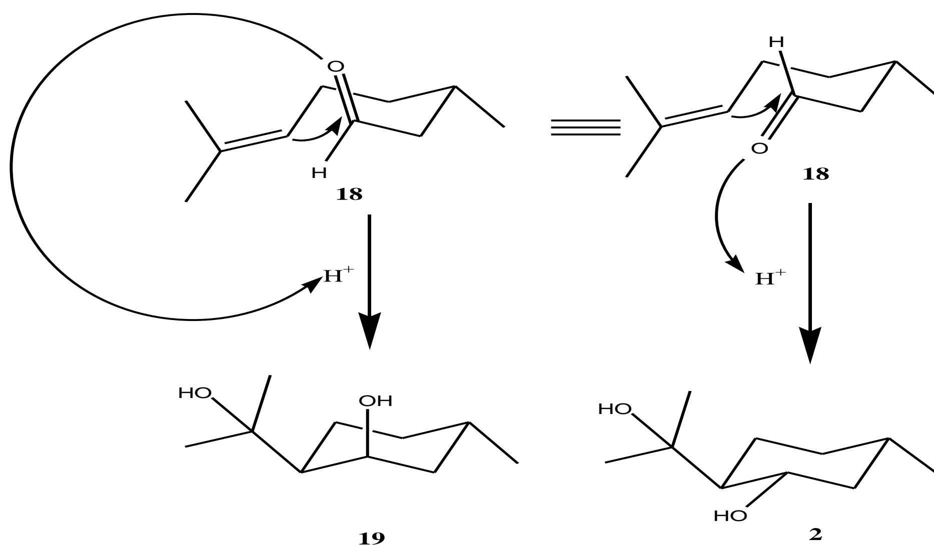
Scheme 3.2: Synthesis of (−)-*trans*-*p*-menthane-3,8-diol from (−)-isopulegol

3.5.3 Synthesis of (±)-*cis*-*p*-menthane-3,8-diol and (±)-*trans*-*p*-menthane-3,8-diol from (±)-citronellal

(±)-*cis*-*p*-menthane-3,8-diol and (±)-*trans*-*p*-menthane-3,8-diol were prepared from (±)-citronellal using the procedure of Zimmermann and English (1953). 100 ml, 5% sulfuric acid solution was put in a 500 ml quickfit round bottomed flask covered with a quickfit male joint. This solution was then stirred for 8 hrs using a magnetic stirrer whereupon 2 g, 1.296×10^{-2} moles of (±)-citronellal (Fluka) was added and stirred for a further 30 hrs.

The reaction was stopped and the organic layer separated from the aqueous layer. To quench the residual acid sodium bicarbonate was added to the organic layer until the effervescence stopped. The aqueous layer was extracted with dichloromethane (5×20 ml), after saturating the solution with NaCl. The dichloromethane extract was dried using anhydrous Na_2SO_4 and filtered. The solvent was then removed by evaporating in vacuo at 40^oC.

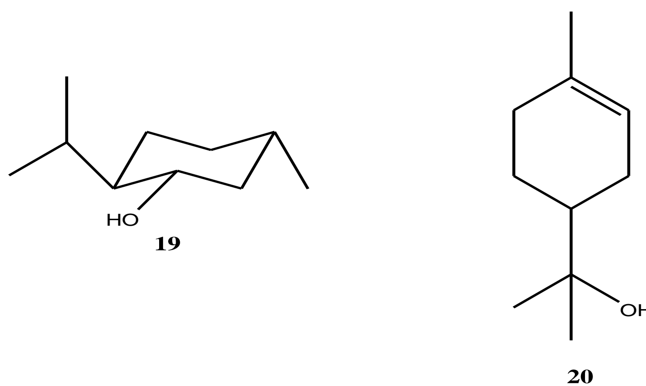
Purification was done on a column packed with silica gel (230 − 240 mesh, eluent 20 − 40% ethyl acetate in hexane to separate the target *cis*-diols from the *trans* products and un-reacted starting material, TLC analysis revealed two spots at R_f 0.12 and 0.16. Out of 15 fractions obtained, fractions 5 − 7 had R_f 0.12 and were pooled, solvent removed by evaporating in vacuo at 40°C to afford a white waxy compound, 400 mg, 17.9% yield (**19**). Fractions 8-14 were similarly treated to afford 900 mg of a white waxy substance (**2**), in 40.3% yield. Further purification of the PMD product was achieved by crystallization from the 20% hexane-ethyl acetate to get white crystals. The crystallized diol products were analyzed by GC-MS, to obtain two sharp peak at t_r 14.0 and 17.1 minutes. Separation of the target *cis*-diols from the *trans*-diols was done by preparative HPLC, separation was achieved using a Supercosil ™ LC-NH_2 (nucleosil amine) column (25 cm × 5 µm) with guard column (Supelco High Chrom, Bellefonte, Pennyslvania, USA), followed by crystallization from the hexane-ethyl acetate. The separated *cis*-diol and *trans*-diol were analyzed by GC-MS; *cis*-diol gave a single sharp peak at t_r 14.0 minutes, while *trans*-diol gave a single sharp peak at t_r 17.1 minutes.



Scheme 3.3: Synthesis of (±)-*cis*-*p*-menthane-3,8-diol (**19**) and (±)-*trans*-*p*-menthane-3,8-diol (**2**) from (±)-citronellal (**4**)

3.6 Menthane-diol analogues

L-Menthol (1**9**) and 1-α-terpeneol (**20**) were obtained from Sigma-Aldrich Chemical Company (USA). They were used for bio-assay to assist in determining the structural components responsible for tick repellency.



3.7 Chemical Analysis

3.7.1 Structure Determination using Gas Chromatography – Mass Spectrometry

Structure determination of the components in the *E. citriodora* oil, the synthesized *p*-menthane-3,8-diol stereoisomers were identified and confirmed using GC-MS. GC-MS analyses were performed with a VG Masslab 12-250 quadruple gas chromatography- mass spectrometer. Chromatographic separations were achieved using a fused silica capillary column (Hewlett Packard, 50 m x 0.32 mm ID) coated with Carbowax 20M (0.3 μm film thickness) with helium as the carrier gas. Injections were made in the splitless mode with helium as the carrier gas. Compounds were identified by their electron impact (El) mass spectral data, order of elution and relative GC retention times, and by comparison of their mass spectra and GC retention times to those of authentic samples. The computer on the GC-MS system records a mass spectrum for each scan and has a National Institute of Standards and Technology (NIST) library of spectra that can be used to identify an unknown chemical in the sample. The library compares the mass spectrum from a sample component with mass spectra in the NIST library. Identification of compounds in *E. citriodora* oil and synthesized *p*-menthane-3,8-diol were verified by comparison with authentic samples.

3.7.2 Structure determination using Liquid Chromatography-Mass Spectrometry

For the LC-MS analysis, the white powder obtained from aqueous fraction of the hydro-distillate was dissolved in the mobile phase, an aliquot of the solution (1 ml) was further diluted ten times. The LC-MS used consisted of a quaternary LC pump (Model 1200) coupled to Agilent MSD 6120-Single quadruple MS with electron spray source (Palo Alto, CA). The system was controlled using ChemStation software (Hewlett-Packard). Separation was performed on a reversed-phase liquid chromatography on Agilent technologies 1200 infinite series, Zorbax SB

C_{18} column, 2.1 x 50 mm, 1.8 µm (Phenomenex, Torrance, CA, USA). An isocratic mode with 40% **A** (5% formic acid in LC-grade ultra pure H_2O): 60% **B** (LC-grade ACN) (Sigma, St. Louis, MO) at a flow rate of 1 ml min^{-1}. Injection volume was 10 µl and data was acquired in a full-scan positive-ion mode using a 100 to 800 *m/z* scan range. The dwell time for each ion was 50 min. Other parameters of the mass spectrometer were as follows: capillary voltage, 3.0 kV; cone voltage, 70 V; extract voltage, 5 V; RF voltage, 0.5 V; source temperature at 110ºC; drying gas, nitrogen, temperature for desolvation at 380ºC; and nitrogen gas flow for desolvation, 400 L/h. The injection volume was 10 µL.

3.8 Statistical analysis

The mean % repellency presented in tables of results was computed from the original untransformed data using (R – software, version 2.15.2 (2012-10-26)). Data were normalized by logarithmic (log (n+1)) transformation before being subjected to an analysis of variance (ANOVA). Means between treatments were separated using the Student-Newman – Keuls test (Sokal and Rohlf, 1995) at $P \leq 0.05$ with (SAS® Institute, 2002 − 2003, version 9.0). During analysis, percentage repellency (PR) values were converted to repellency probabilities ranging from 0 to 1 in order to fit into a probit model. Dose-response relationships were determined using probit analyses and repellent doses (RD) at RD_{75} using (SAS® Institute, 2002-2003, version 9.0).

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Composition of *Eucalyptus citriodora* oil

Air-dried *E. citriodora* leaves (1 kg) yielded 1.84% (18.4g) of essential oil. Gas chromatography-mass spectrometry (GC-MS) showed the presence of thirty eight (38) compounds in the oil fraction (Figure 4.1). The mass spectra of some of the compounds identified in *E. citriodora* oil are shown in Appendix 3a - 3j. Previously, chemical investigations by Mwangi *et al*., (1981) had shown that the three principal components of the oil of the Kenyan grown *E. citriodora* are citronellal (65-88%), citronellol (2-25%) and isopulegol (2-19%). However, in this study the principal components of the oil of *E. citriodora* sampled from Arboretum, Nairobi County were citronellal (**4**) (32.03%), citronellol (**25**) (19.41%), 1,8-cineole (**26**) (9.87%) and isopulegol (**3**) (5.97%), as listed in Table 4.1, their mass spectra are shown in Appendix 3f, 3e, 3d and 3h respectively. The difference in composition of the oil between this study and that of Mwangi *et al*., (1981) could be attributed to the differences in ecology and temperature.

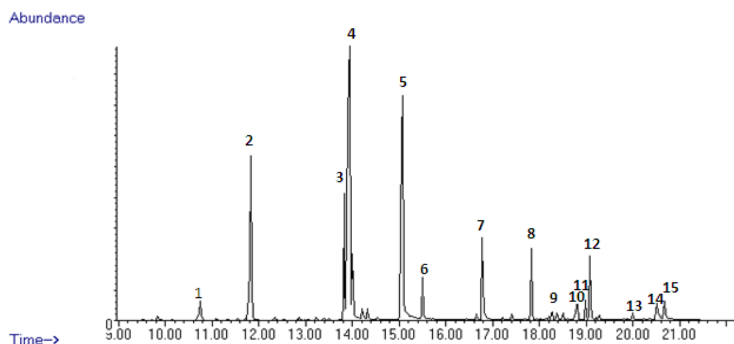


Figure 4.1: Total ion chromatogram showing *E. citriodora* oil profile

Table 4.1: Major constituents in the essential oil of *E. citriodora* sampled in Aboretum, Nairobi County in the month of June

Peak No.	RT	Area %	Compound Identity
1	10.75	1.32	β-Pinene
2	11.82	9.87	1,8-Cineole
3	13.84	5.92	Neo-isopulegol
4	13.95	32.03	Citronellal
5	15.07	19.41	Citronellol
6	15.52	2.32	Methyl citronellate
7	16.79	5.07	Citronellyl acetate
8	17.82	2.96	Caryophyllene (E-)
9	18.27	0.77	α-Humulene
10	18.81	1.59	Amorpha-4,7(11)-diene
11	19.01	0.92	γ-Cadinene
12	19.10	2.99	*Cis*-calamenene
13	20.51	1.48	Dauca-5,8-diene
14	20.67	1.39	γ-Cadinene
15	20.83	0.82	*trans*-dauca-4(11),7-diene

The water soluble fraction was analyzed by LC-MS (Appendix 2) and some of the compounds found in it included; linalool (**27**), aromadendrene oxide (**28**), citronellic acid (**29**) and ursolic acid (**30**). However, *p*-menthane-3,8-diol was not found in *E. citriodora* oil nor in the water soluble fraction of the hydro-distillate.

Yield and composition of the essential oil of *E. citriodora* leaves is affected by season (Manika *et al.*, 2011). Seasonal variation of the essential oil of *E. citriodora* Hook grown under subtropical conditions of North Indian plains for commercial cultivation showed that the oil yield ranged between 1.0 to 2.1% during different months, high during April to September, when temperature was high and low during November to March, when the temperature was relatively

low. However, during the rainy season, when both temperature and humidity were high, oil yield was also observed to be quite high (1.8 to 2.1%), and the major constituent of oil was citronellal (69.7 to 87.4%), citronellol (5.1 to 9.9%), linalool (2.1 to 6.4%), isopulegol (0.9 to 3.1%) and citronellyl acetate (0.4 to 1.2). Concentration of citronellal decreased during the summer and rainy seasons, while those of others increased during this period (Manika *et al*., 2011).

4.2 Synthesis of *p*-menthane-3,8-diol stereoisomers

4.2.1 Synthesis of (+)-*trans*-*p*-menthane-3,8-diol (from (+)(1S,2R,5S)-isopulegol)

Oxy-mercuration/demercuration procedure (Brown and Geoghegan, 1967) was used to synthesize (+)-*trans*-*p*-menthane-3,8-diol by hydration of (+)-isopulegol (0.5 g, 3.24×10^{-3}moles) at C-8. TLC results of the THF layer revealed one spot at R_f 0.16 using 20% ethyl acetate in hexane solvent system. Purification on a silica gel column (230 − 240 mesh) using the eluent system of 20 − 40% ethyl acetate in hexane and crystallization from the hexane-ethyl acetate afforded white waxy crystals (495 mg, 100%). GC-MS analysis gave a single sharp peak at t_r 17.1 minutes (Figure 4.2) with mass spectrum (Figure 4.3) identical with that of *trans*-*p*-menthane-3,8-diol from NIST library. Since pure starting material, with known optical activity, (+)-isopulegol was used for synthesis, and the reacting reagents are achiral, the final product remains with the same optical activity as the starting material.

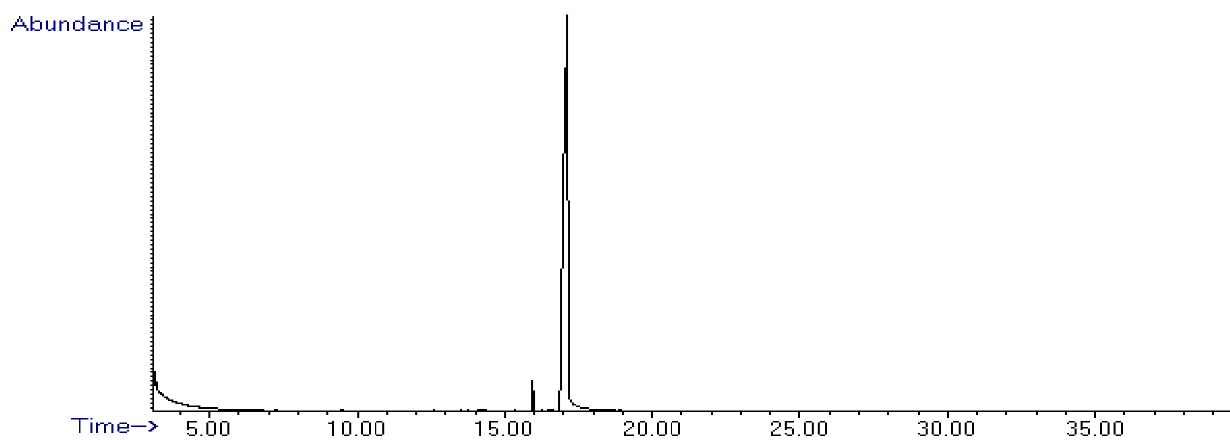


Figure 4.2: Total ion chromatogram of (+)-*trans*-*p*-menthane-3,8-diol (**22**) synthesised from (+) – isopulegol(**3**)

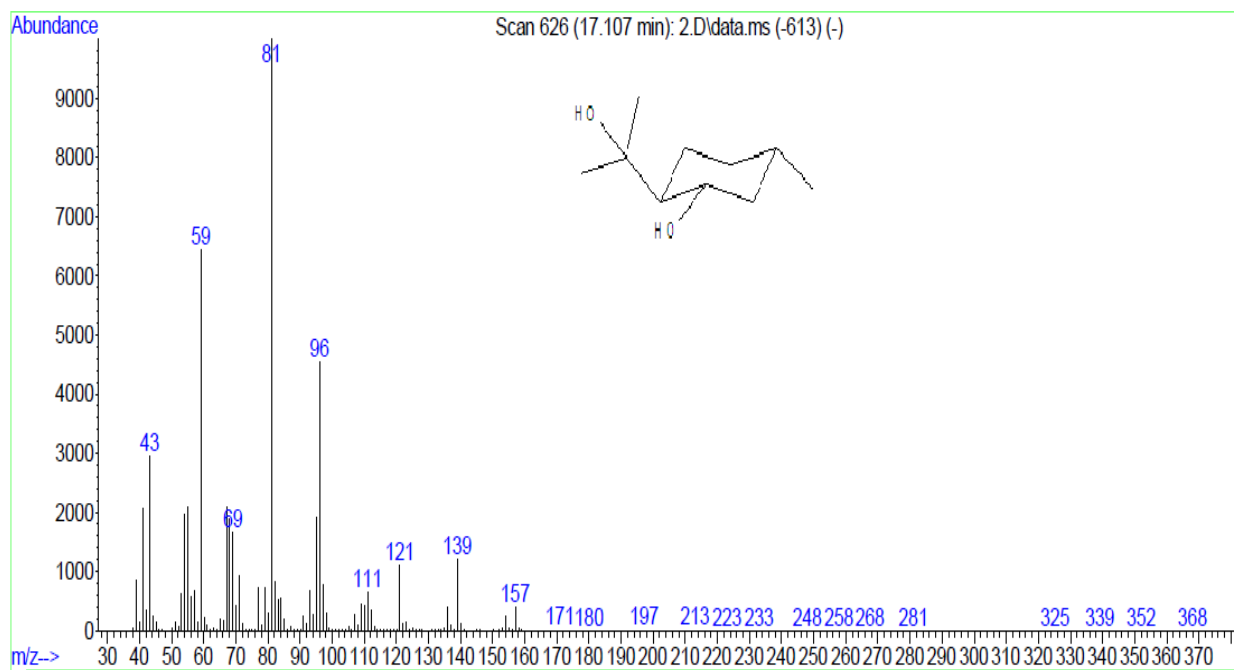


Figure 4.3: MS and structure of (+)-*trans*-*p*-menthane-3,8-diol (**22**)

4.2.2 Synthesis of (−)-*trans*-*p*-menthane-3,8-diol from (−) (1R, 3R, 4S)-isopulegol

Oxy-mercuration/demercuration procedure (Brown and Geoghegan, 1967) was used to synthesize (−)-*trans*-*p*-menthane-3,8-diol by hydration of (−)-isopulegol (0.5 g, 3.24×10^{-3}moles) at C-8. TLC results of the THF layer revealed one spot at R_f 0.16 using 20% ethyl acetate in hexane solvent system. Purification on a silica gel column (230 − 240 mesh) using the eluent system of 20 − 40% ethyl acetate in hexane and crystallization from the hexane-ethyl acetate afforded white waxy crystals (494 mg, 99.8%). GC-MS analysis gave a single sharp peak at t_r 17.1 minutes (Figure 4.4) with mass spectrum (Figure 4.5) identical with that of *trans*-*p*-menthane-3,8-diol from NIST library. Structures (**22**) and (**23**) are enantiomers. Since pure starting material, with known optical activity, (−)-isopulegol was used for synthesis, and the reacting reagents are achiral, the final product remains with the same optical activity as the starting material.

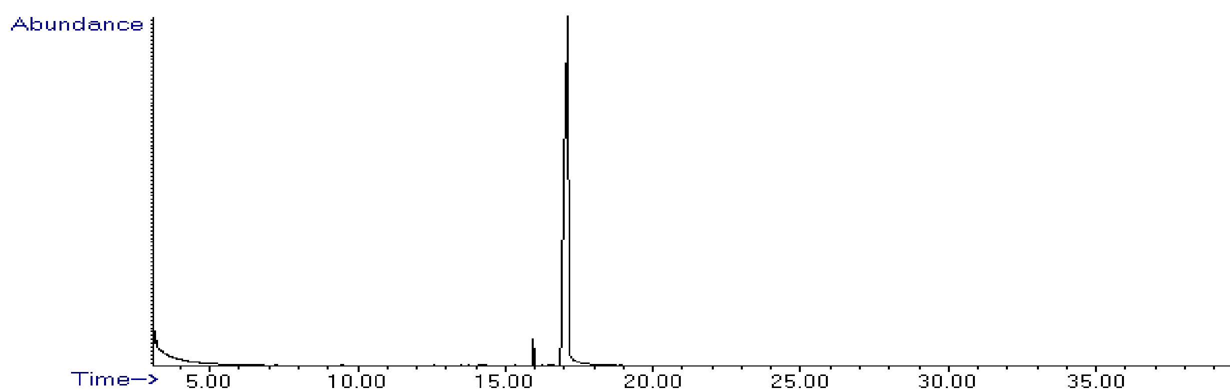


Figure 4.4: Total ion chromatogram of (−)-*trans*-*p*-menthane-3,8-diol (**23**) synthesised from (−) - isopulegol (**3**)

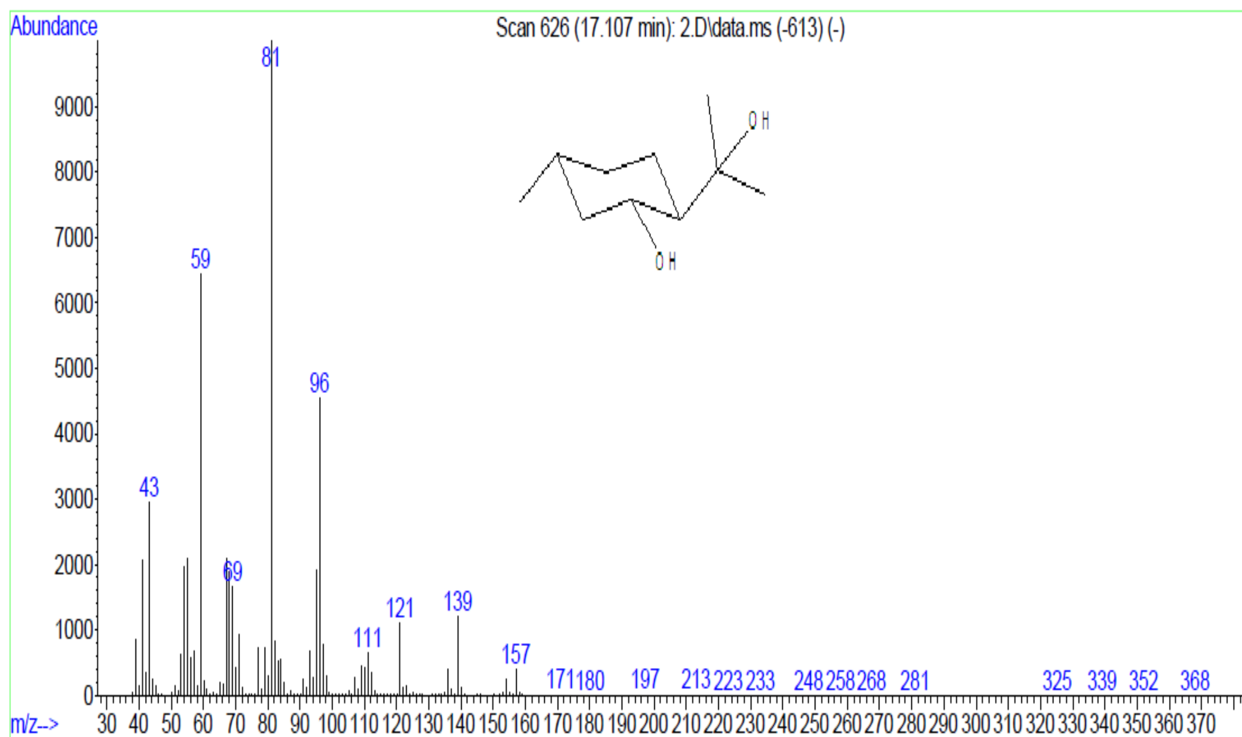


Figure 4.5: MS and structure of (−)-*trans*-*p*-menthane-3,8-diol (**23**)

4.2.3 Synthesis of (±)-*cis*-*p*-menthane-3,8-diol and (±)-*trans*-*p*-menthane-3,8-diol from (±)-citronellal

Acid catalysed cyclisation of (±)-citronellal (**18**) was performed at room temperature. TLC analysis of the dichloromethane extract gave two spots at R_f 0.12 and 0.16 using 20% ethyl acetate in hexane solvent system. Purification was done on a silica gel column (230 − 240 mesh) using the eluent system of 20 − 40% ethyl acetate in hexane. GC-MS analysis and spectral matching (library search) gave two sharp peaks at t_r 14.0 and 17.1 minutes (Figure 4.6) in 17.9% and 40.3% yield, respectively.

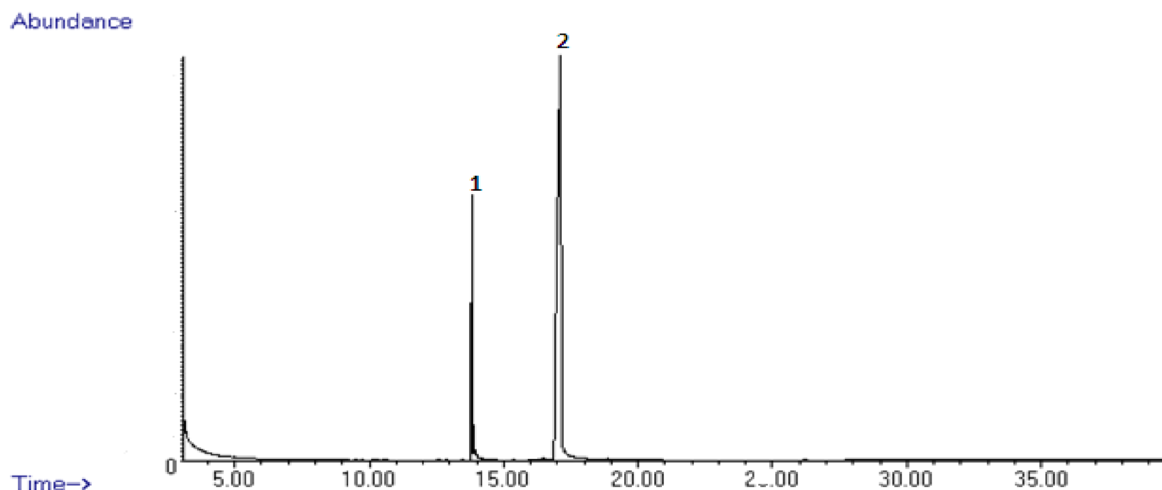


Figure 4.6: Total ion chromatogram showing (±)-*cis*-*p*-menthane-3,8-diol (**19**) and (±)-*trans*-*p*-menthane-3,8-diol (**24**) profile obtained from (±)-citronellal synthesis

Separation of the target *cis*-diols from the *trans*-diols was done by preparative HPLC followed by crystallization from the hexane-ethyl acetate. The separated *cis*-diol and *trans*-diol were analyzed by GC-MS; *cis*-diol gave a single sharp peak at t_r 14.0 minutes (Figure 4.7), while *trans*-diol gave a single sharp peak at t_r 17.1 minutes (Figure 4.8), with mass spectra depicted in Figure 4.9 and 4.10 respectively. These were identical respectively with those of (±)-*cis*-*p*-menthane-3,8-diol and (±)-*trans*-*p*-menthane-3,8-diol from NIST library. Racemic citronellal was used as the starting material for synthesis, to form racemic diastereoisomers; (±)-*cis*-*p*-menthane-3,8-diol and (±)-*trans*-*p*-menthane-3,8-diol.

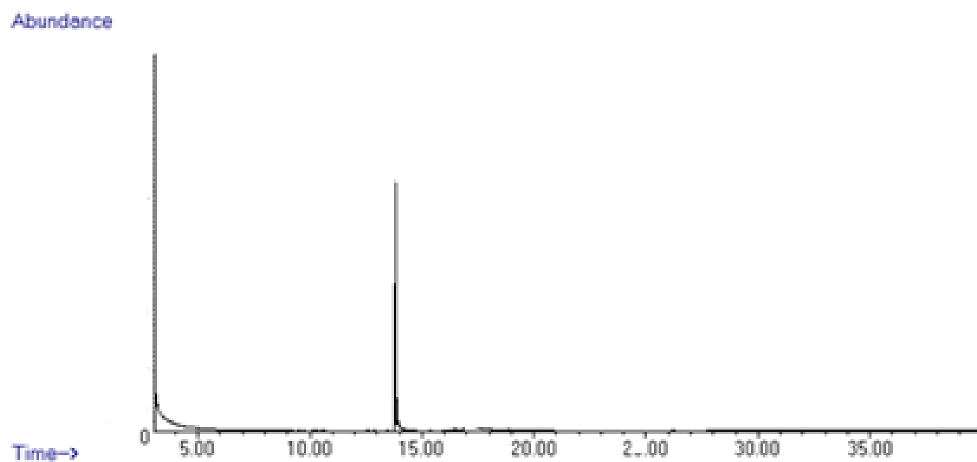


Figure 4.7: Total ion chromatogram of (±)-*cis*-*p*-menthane-3,8-diol (**19**) separated by prep HPLC

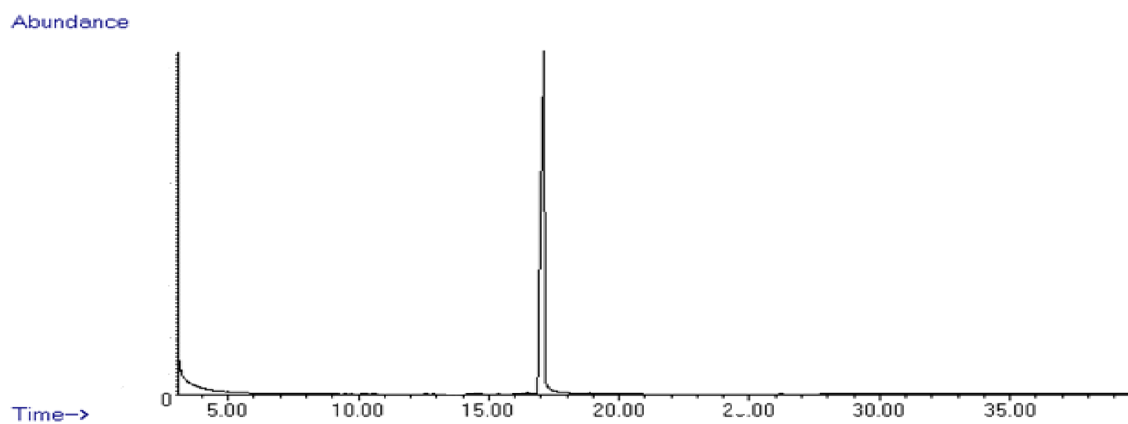


Figure 4.8: Total ion chromatogram of (±)-*trans*-*p*-menthane-3,8-diol (**24**) separated by prep HPLC

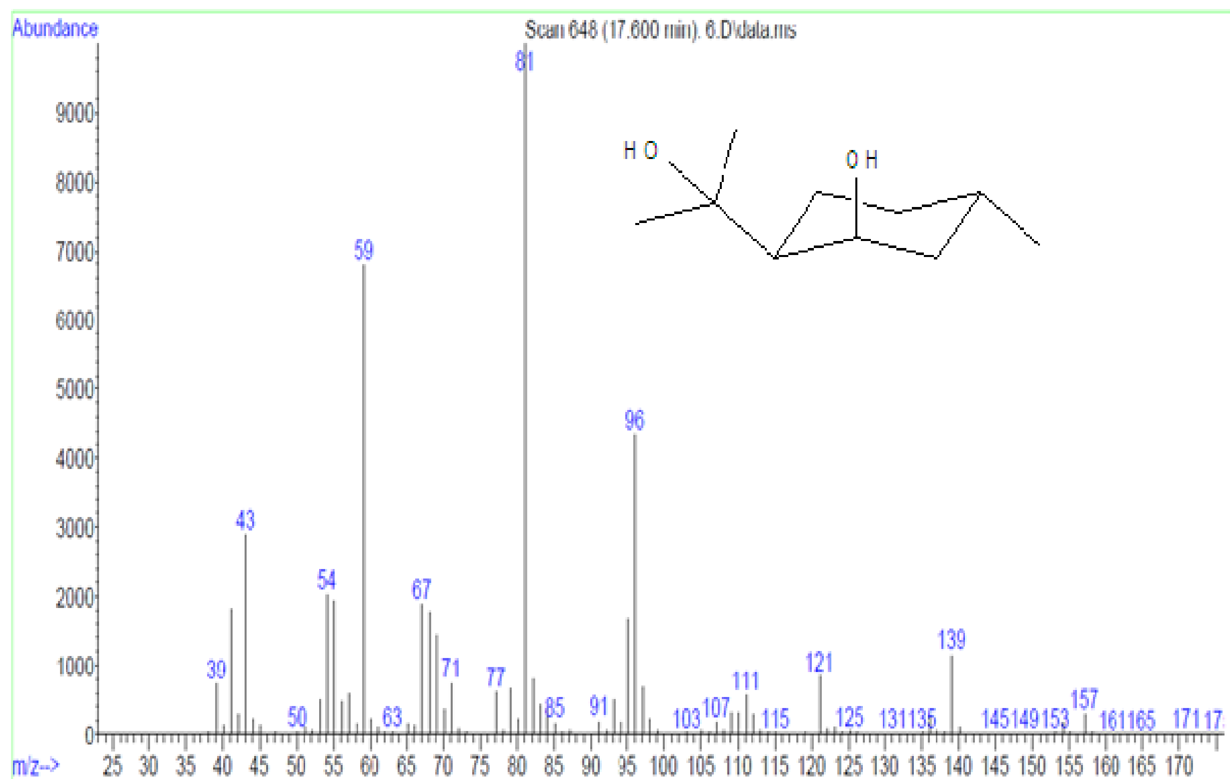


Figure 4.9: MS and structure of (±)-*cis*-*p*-menthane-3,8-diol (**19**) separated by prep HPLC

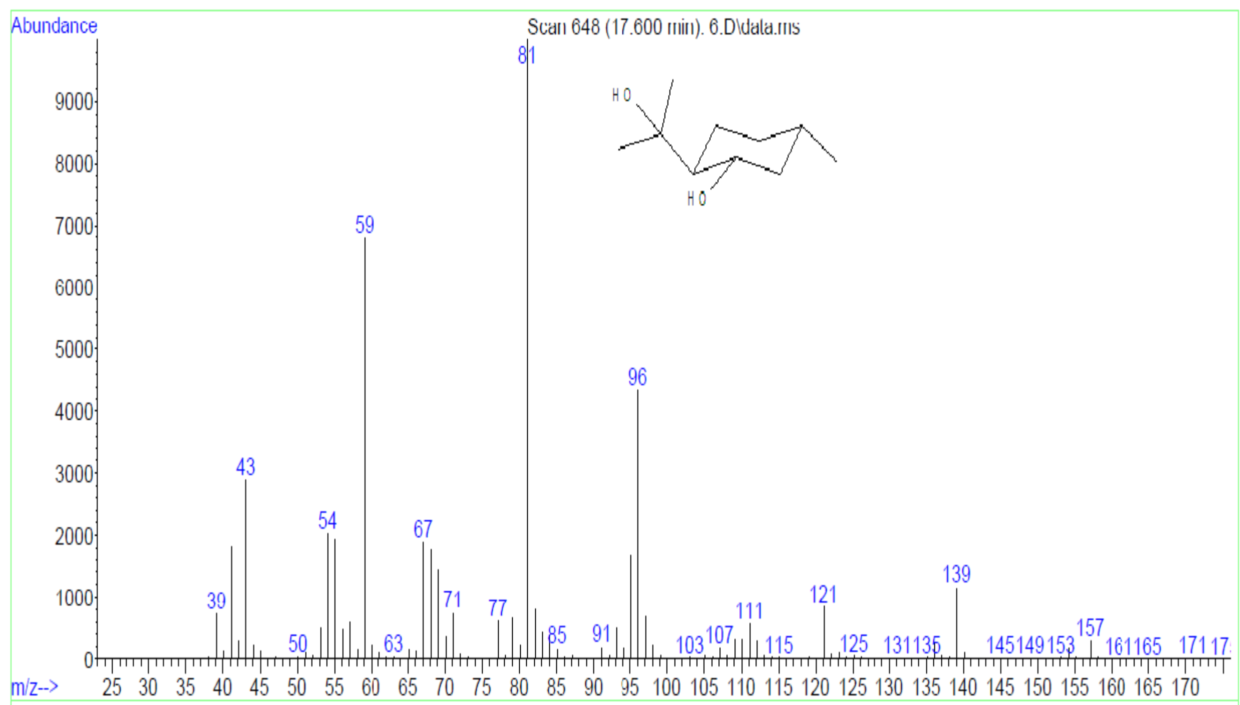


Figure 4.10: MS and structure of (±)-*trans*-*p*-menthane-3,8-diol (**24**) separated by prep HPLC

4.3 Repellency of PMD stereoisomers, analogues, *E. citriodora* oil, DEET and aqueous fraction

Table 4.2 provides results of percentage repellency of PMD stereoisomers and DEET, and Table 4.3 provides those of PMD analogues, *E. citriodora* oil and the aqueous fraction of the hydro-distillate. Table 4.4 summarizes RD_{75} of *p*-menthane-3,8-diol stereoisomers, PMD analogues, DEET, *E. citriodora* oil and aqueous fraction of the hydro-distillate. Menthane diols were potently repellent against *R. appendiculatus* and comparable to that of DEET (Table 4.4).

Two interesting structural effects on repellence may be highlighted. First, racemates of *cis* and *trans* were as repellent as (+) and (−)-*trans* diols (Table 4.2). Thus, repellency was neither stereospecific nor stereoselective. This shows that the precise orientation of the hydroxyl groups (at C-3 and C-8) in menthane diol skeleton is not important in conferring repellency against *R. appendiculatus.* From the practical standpoint, these results mean that one can obtain synthetic or semi-synthetic products of optimal repellent action against *R. appendiculatus* without regard to the stereochemical form of the starting material or the diol product. Interestingly, a similar result was also obtained against *Anopheles gambie s.s.* (Barasa *et al*., 2002). This suggests some similarity in the chemoreceptors and odorant-binding proteins (OBPs) of mosquitoes and ticks.

Secondly, analogues of the diol (L-menthol, 1-α-terpineol) showed much lower repellency against *R. appendiculatus* compared to *p*-menthane-3,8-diol stereoisomers. Menthol (**20**) has the menthane ring as well as a hydroxyl group at C-3, while 1-α-terpineol (**21**) has a menthene ring and a hydroxyl group at C-8. Thus, the presence of a second hydroxyl group in menthane diol is important for repellency against the Brown ear tick. Moreover, the presence of a double bond,

which changes the shape of the molecule, further reduces the repellence of 1-α-terpineol. Previously, these PMD analogues were found to be attractive to *Anopheles gambiae*, particularly at higher doses (Barasa *et al*., 2002). This indicates that although there are common features in the odorant-binding proteins (OBPs) of mosquitoes and ticks, as reflected in their behavioural responses to PMD stereoisomers, there are also significant differences. Unlike mosquitoes, ticks lack antennae and they detect host cues using sensilla located on the tarsi of the front legs. Their olfactory reception neurons may be narrowly tuned to specific odors (Hess and Vlimant, 1986).

The presence of two hydroxyl groups in PMDs raises their polarity, which would lower their volatility. This may be of particular advantage in substantially extending the rate of their evaporation, thus enhancing the longevity of their performance.

Table 4.2: % Repellency (±SE) of *p*-menthane-3,8-diol stereoisomers and DEET at different doses (mg)

Test Compound	% Repellency (±SE)				
	0.0001	**0.001**	**0.01**	**0.1**	**1**
(±)-*cis*-*p*-menthane-3,8-diol	70.5±3.9[a]	77.1±5.2[a]	81.5±5.8[a]	86.2±5.2[a]	95.8±3.9[a]
(±)-*trans*- *p*-menthane-3,8-diol	70.7±4.0[a]	76.7±5.7[a]	80.7±6.0[a]	93.2±4.8[a]	95.8±3.9[a]
(−)-*trans*- *p*-menthane-3,8-diol	73.0±5.7[a]	75.3±5.3[a]	83.4±5.8[a]	85.1±5.7[a]	96.6±3.7[a]
(+)- *trans*- *p*-menthane-3,8-diol	73.5±5.7[a]	75.9±5.1[a]	79.0±5.1[a]	82.8±5.3[ab]	95.5±4.1[a]
DEET	73.4 ±5.6[a]	76.9 ±5.6[a]	83.1 ±5.8[a]	87.8 ±5.1[a]	97.0±3.3[a]

Means in columns followed by the same letters are not significantly different (P≤0.05; SNK test).

Table 4.3: % Repellency (±SE) of *p*-menthane-3,8-diol analogues and *E. citriodora* oil (g)

Test Compound	**Mean % Repellency (±SE) at different doses (g)**				
	0.0001	**0.001**	**0.01**	**0.1**	**1**
L-Menthol	62.3±4.2[b]	75.5±7.7[b]	79.5±7.4[b]	91.2±5.5[b]	95.6±4.1[b]
1-α-Terpineol	33.8±8.3[c]	60.7±7.5[c]	77.8±7.8[b]	89.2±6.1[b]	96.5±3.8[b]
E.citriodora oil	81.1±7.9[a]	89.9±6.4[a]	98.9±2.2[a]	100±0[a]	100±0[a]
Aqueous Fraction	41.5±2.9[c]	57.0±2.5[c]	62.0±4.8[c]	66.3±4.4[c]	70.4±5.2[c]

Means in columns followed by the same letters are not significantly different (P≤0.05, SNK test).

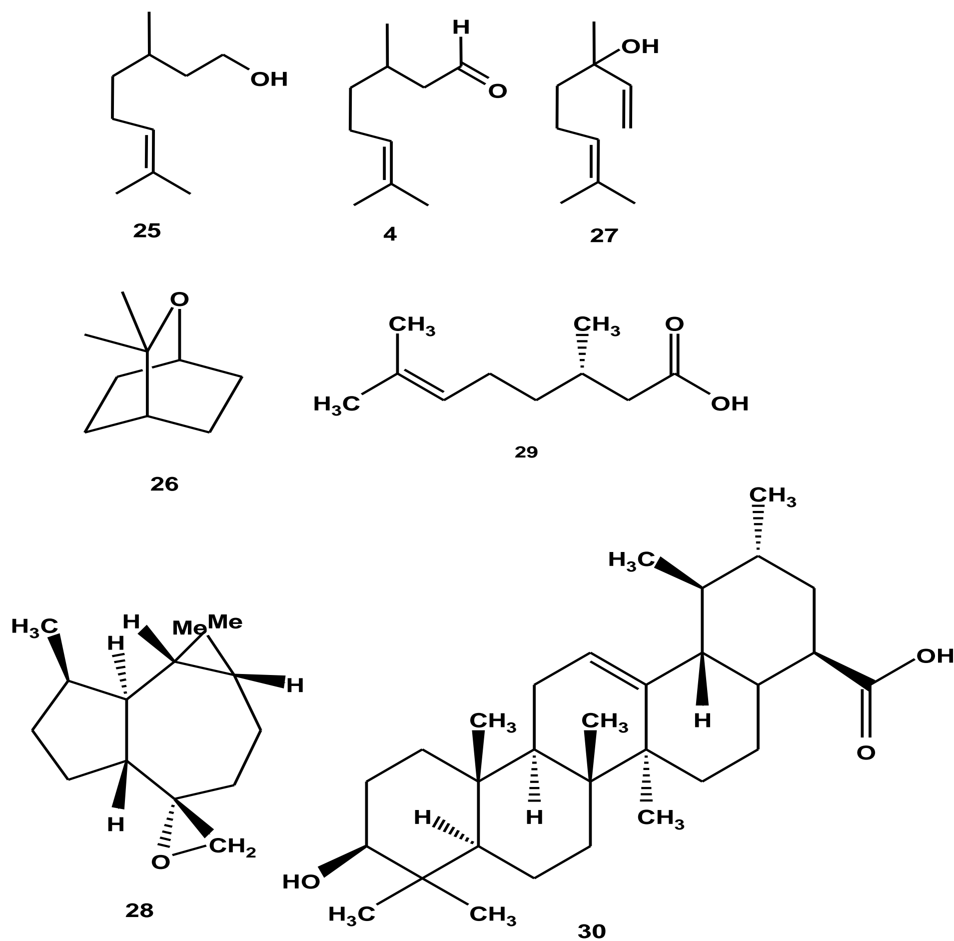
Table 4.4: RD_{75} of *p*-menthane-3,8-diol stereoisomers, analogues, DEET and *E. citriodora* oil

RD_{75} of *p*-menthane-3,8-diol stereoisomers, analogues, DEET and *E. citriodora* oil	
Compounds	RD_{75} ($\times10^{-3}$ mg)
(−)-*Trans*- *p*-menthane-3,8-diol	2.03[d]
(+)-*Trans*- *p*-menthane-3,8-diol	2.04[d]
(±)-*Trans*- *p*-menthane-3,8-diol	1.21[d]
(±)-*Cis*- *p*-menthane-3,8-diol	1.42[d]
DEET	1.43[d]
E. citriodora oil	189.33[c]
L-Menthol	1734.12[b]
1-α-Terpineol	6960.34[a]
Aqueous Fraction	9425.44[a]

Means in columns followed by the same letters are not significantly different (P≤0.05; SNK test).

Two phytochemical blends were assayed in the study, *E. citriodora* oil and water extract of the hydro-distillate. *E. citriodora* oil had much lower repellency than PMD stereoisomers, but significantly higher repellency than L-menthol and 1-α-terpineol (Table 4.2 and 4.3). The major constituents in *E. citriodora* oil were citronellal (**4**) (32.03%), citronellol (**25**) (19.41%), 1,8-cineole (**26**) (9.87%) and isopulegol (**3**) (5.97%), their mass spectra are shown in Appendix 3f, 3e, 3d and 3h respectively. Racemic citronellal (**4**) and isopulegol (**3**) are present in relatively large amounts in the terpenoid fraction of *E. citriodora* oil and are easily convertible to menthane diol. The aqueous fraction of the hydrodistillate was least repellent to the Brown ear tick (Table

4.4). It was found to contain aromadendrene oxide (**28**), linalool (**27**), citronellic acid (**29**) and ursolic acid (**30**), which have been shown to be weakly repellent to arthropods (Barnard and Xue, 2004).



CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

(i) Repellency tests of individual isomers obtained by stereospecific synthetic procedures showed that they are equally active as repellents against *R. appendiculatus* at different doses. Racemates of *cis* and *trans* were as repellent as (+) and (−)-*trans* diols. Therefore, repellency was neither stereospecific nor stereoselective. Of special interest is that menthane diols are potently repellent against *R. appendiculatus* and comparable to that of DEET.

(ii) The much lower repellency of 1-α-terpineol and L-menthol gives useful insights on the structural and stereochemical requirements for repellency of menthane diol. Menthol has the menthane ring as well as a hydroxyl group at C-3, while 1-α-terpineol has a menthene ring and a hydroxyl group at C-8. Thus, the presence of a second hydroxyl group in menthane diol is important for repellency against the Brown ear tick. Moreover, the presence of a double bond, which changes the shape of the molecule, further reduces the repellence of 1-α-terpineol. Therefore, the structural features required for repellency against *R. appendiculatus* is a saturated menthane skeleton with two hydroxyl functions at C-3 and C-8.

(iii)*E. citriodora* oil had much lower repellency than PMD stereoisomers, but significantly higher repellency than L-menthol and 1-α-terpineol. The major constituents in *E. citriodora* oil were citronellal (32.03%), citronellol 19.41%), cineole (9.87%) and isopulegol (5.97%). The results show potential for use of menthane-diol stereochemical blend prepared by cyclization of (±)-citronellal obtainable in good quantities from citronella grass.

5.2 RECOMMENDATIONS

The following recommendations can be made from this study:

(i) Behavior of Brown ear ticks on cattle with ears treated with different doses of PMD stereoisomeric blend should be compared with cattle anal odor blend and essential oils of different plants which have been found to be repellent to the tick.

(ii) Large scale synthesis of *p*-menthane-3,8-diol stereoisomeric blend from citronellal obtained from citronella glass should be initiated, followed by its formulation with different bases.

(iii) The adult *R. appendiculatus* ticks prefer to feed inside and around the ears of their hosts (their predilection sites). Odour trapped from cattle ears attracted *R. appendiculatus* whereas that from the anal region had an opposite effect (Wanzala *et al*., 2004). Thus, a combination of a repellent blend from the anal region and an attractive blend at the ear play natural "push" and "pull" roles respectively to guide these ticks to the cattle ears. Therefore, commercial synthesizes of known attractants and repellents and application of "Push" and "Pull" to eradicate loss of livestock by East Coast Fever should be initiated.

(iv) Further work should be carried out to ascertain if the precise positions of the two hydroxyl groups in menthane diol is important for repellency.

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APPENDICES

Appendix 1: Map of Nairobi County showing the Sampling Area

Appendix 2: LC-MS profile for Aqueous fraction

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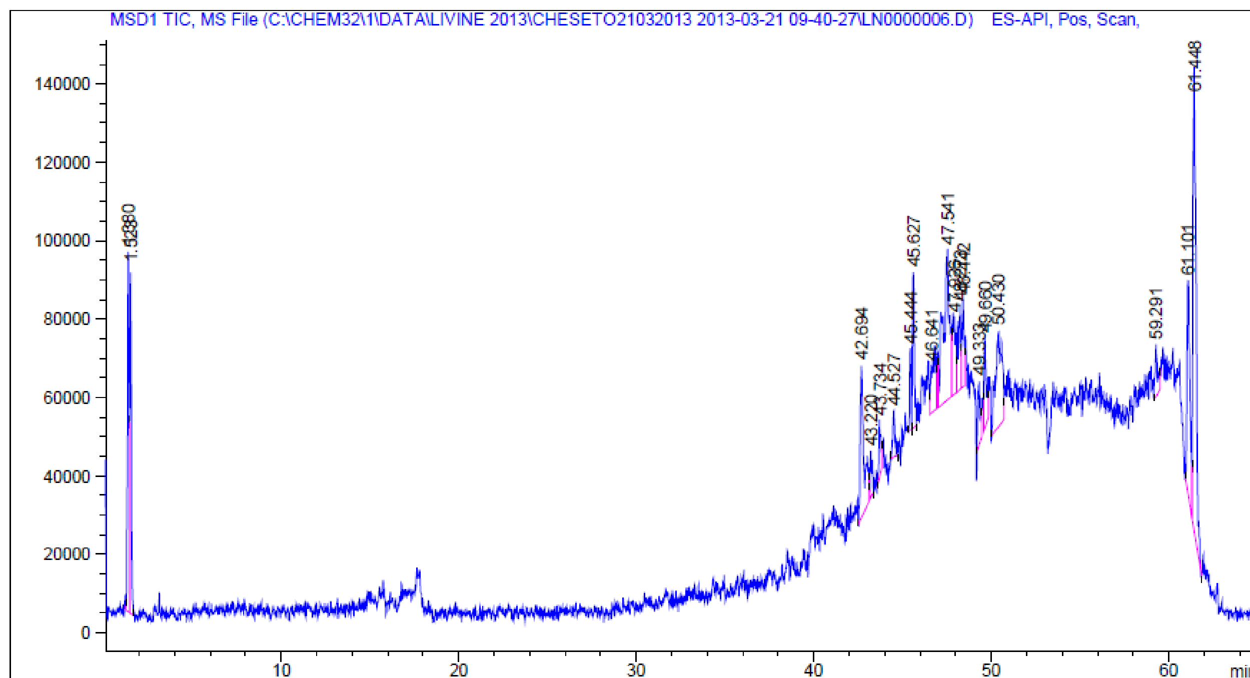
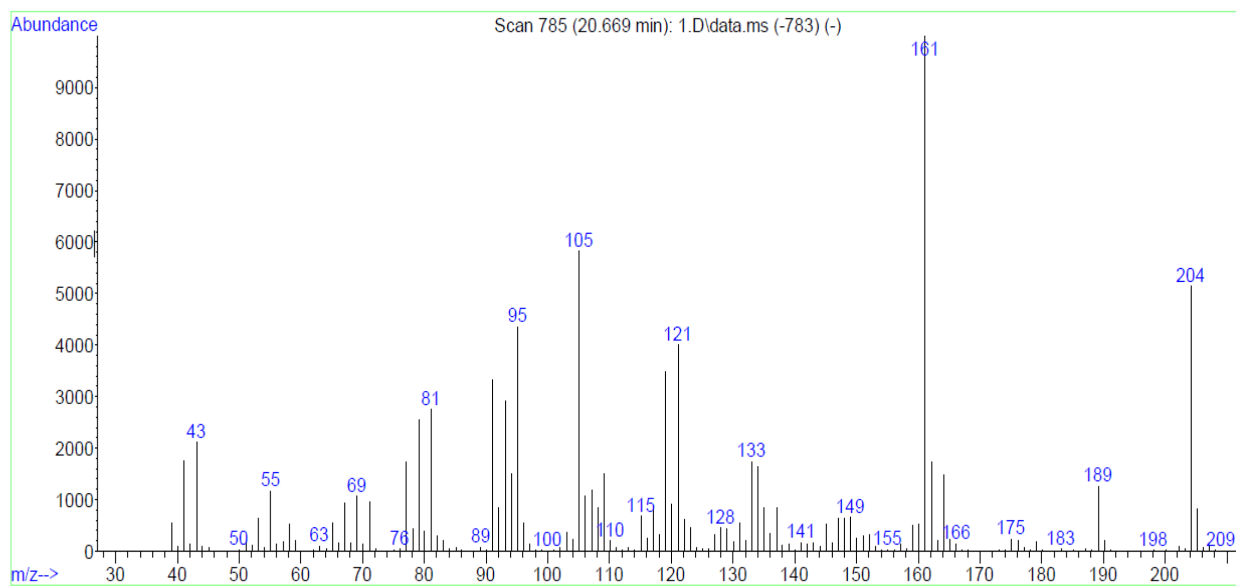
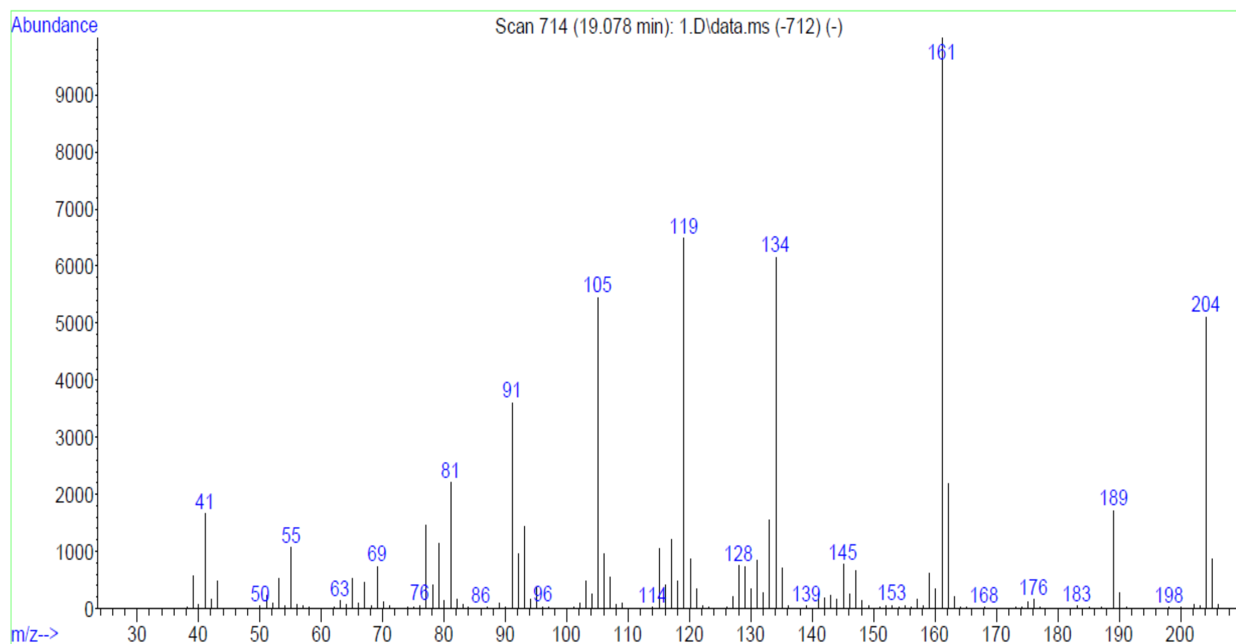


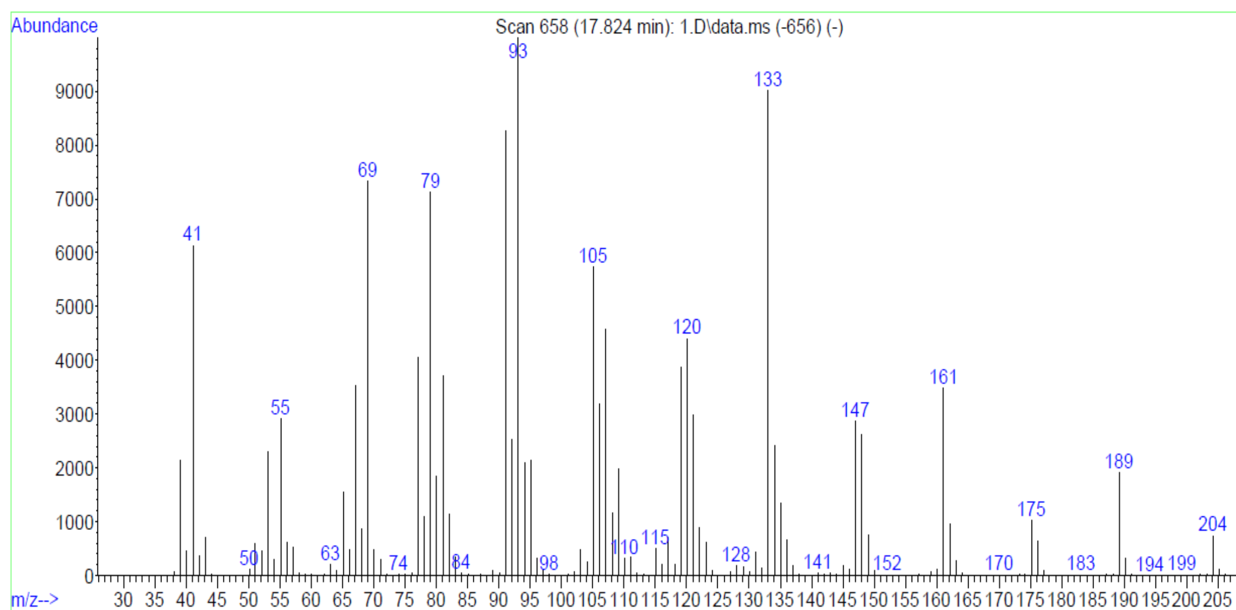
Appendix 3a: MS profile of γ-Cadinene



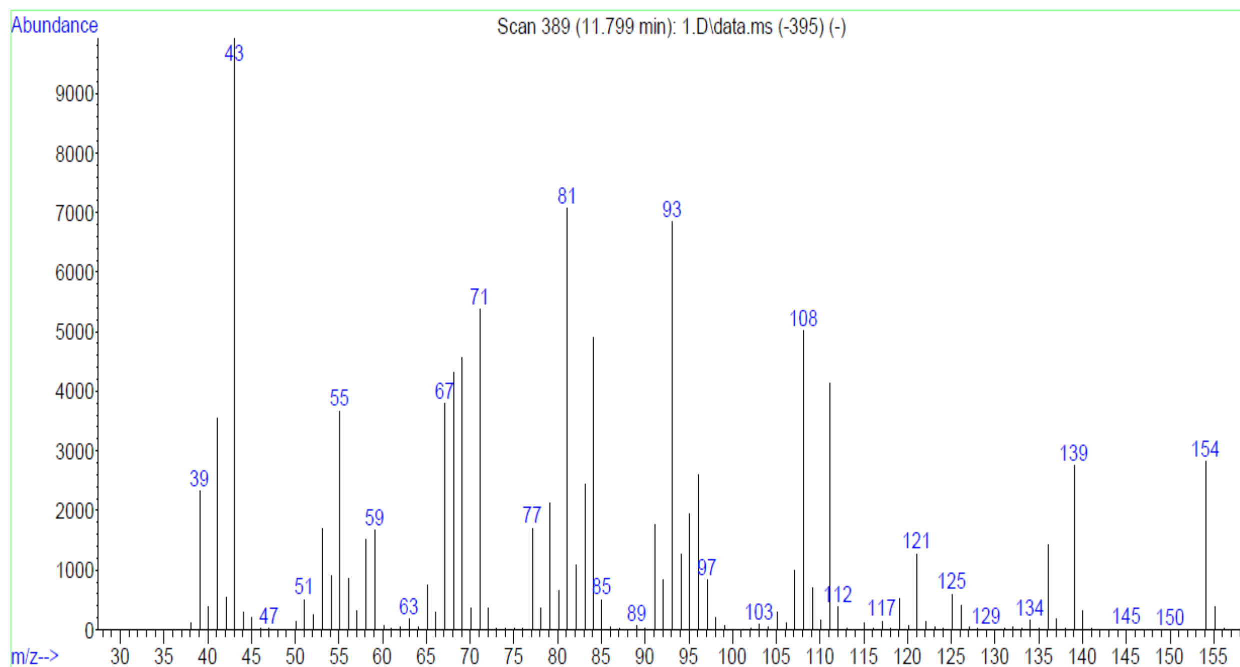
Appendix 3b: MS profile of *trans*-Cadina-1(6), 4-diene



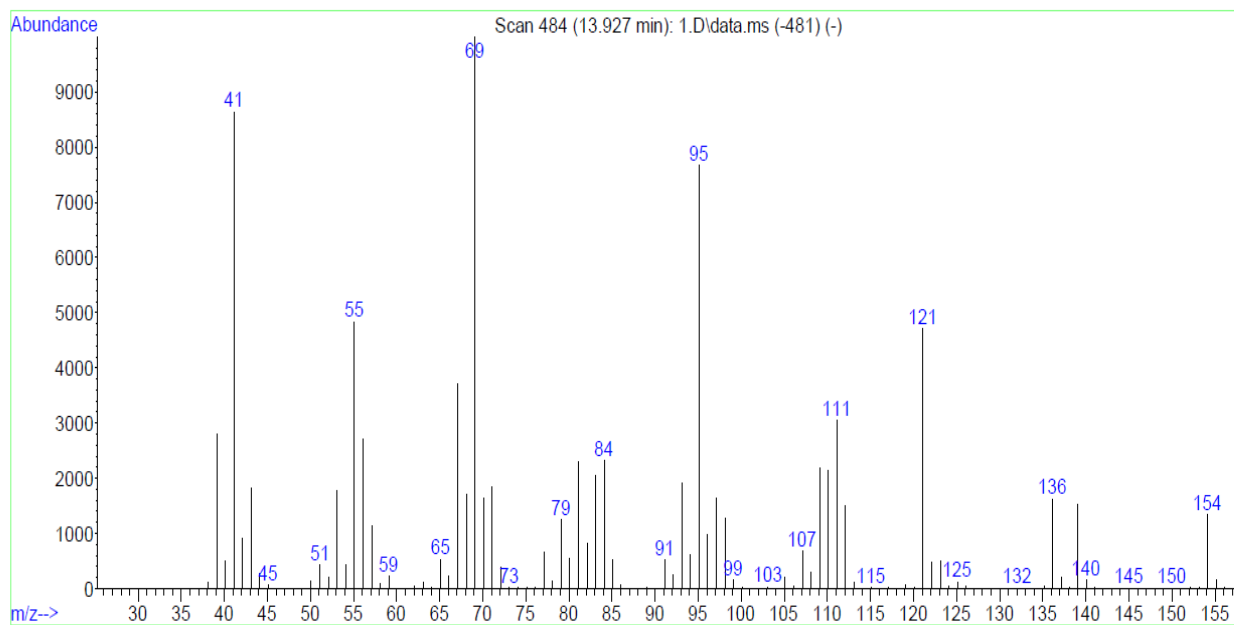
Appendix 3c: MS profile of Caryophyllene (E-)



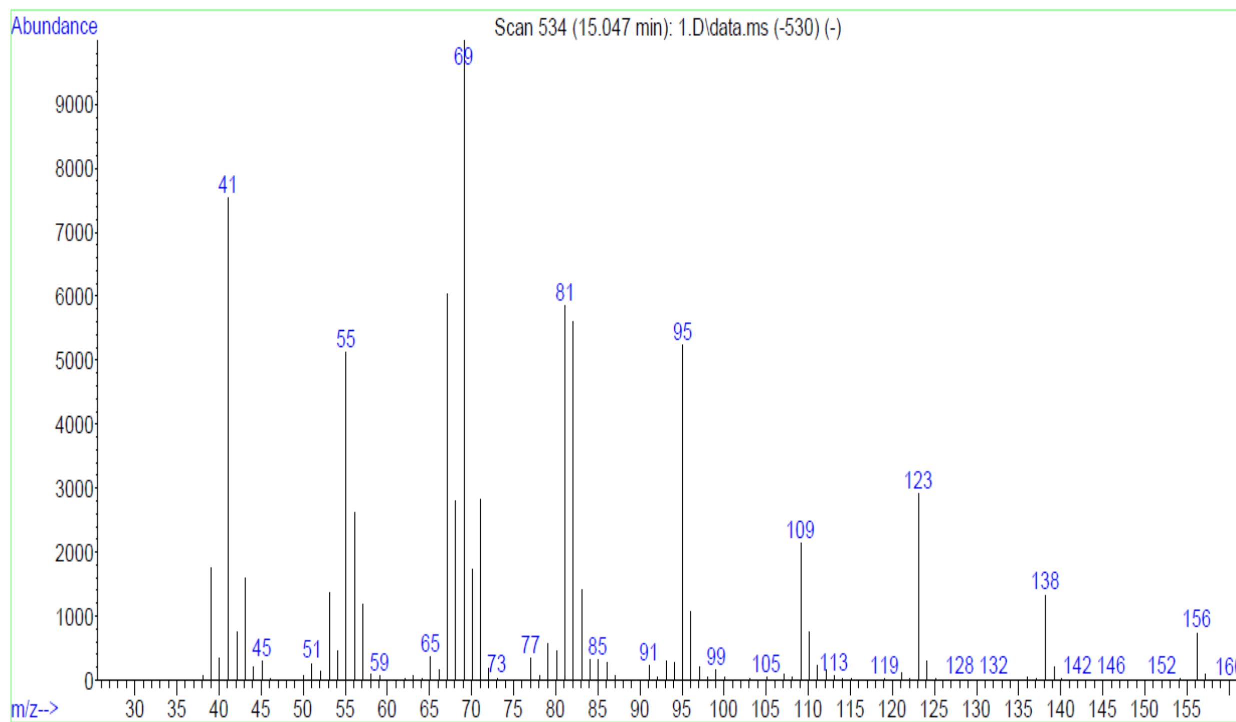
Appendix 3d: MS profile of 1,8-Cineole



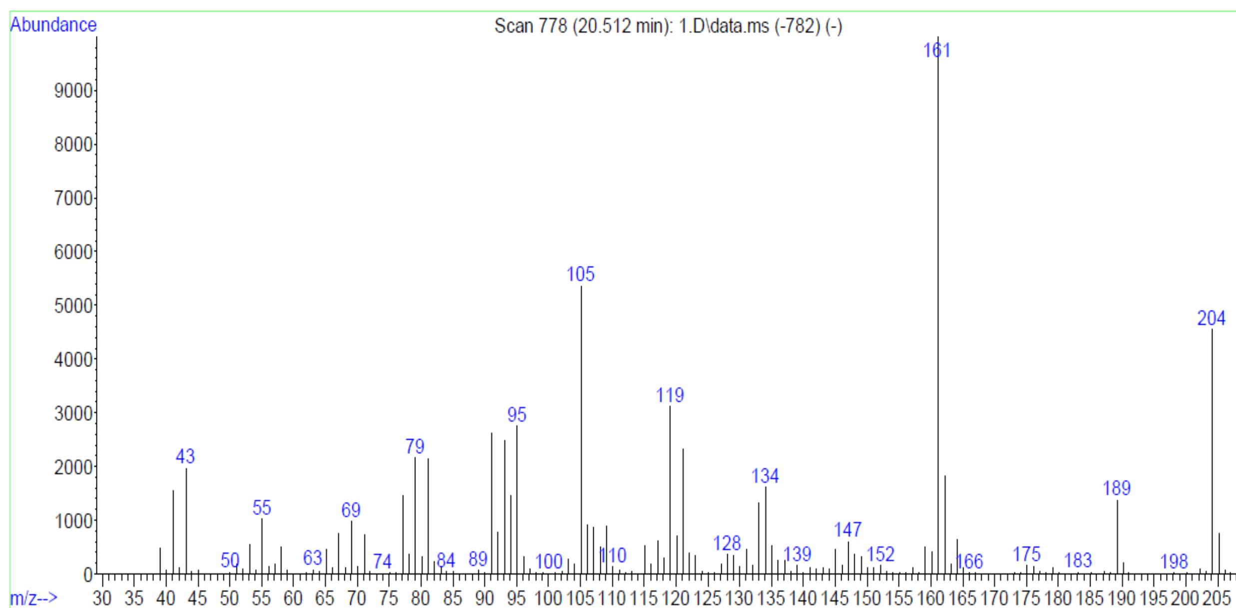
Appendix 3e: MS profile of Citronellal



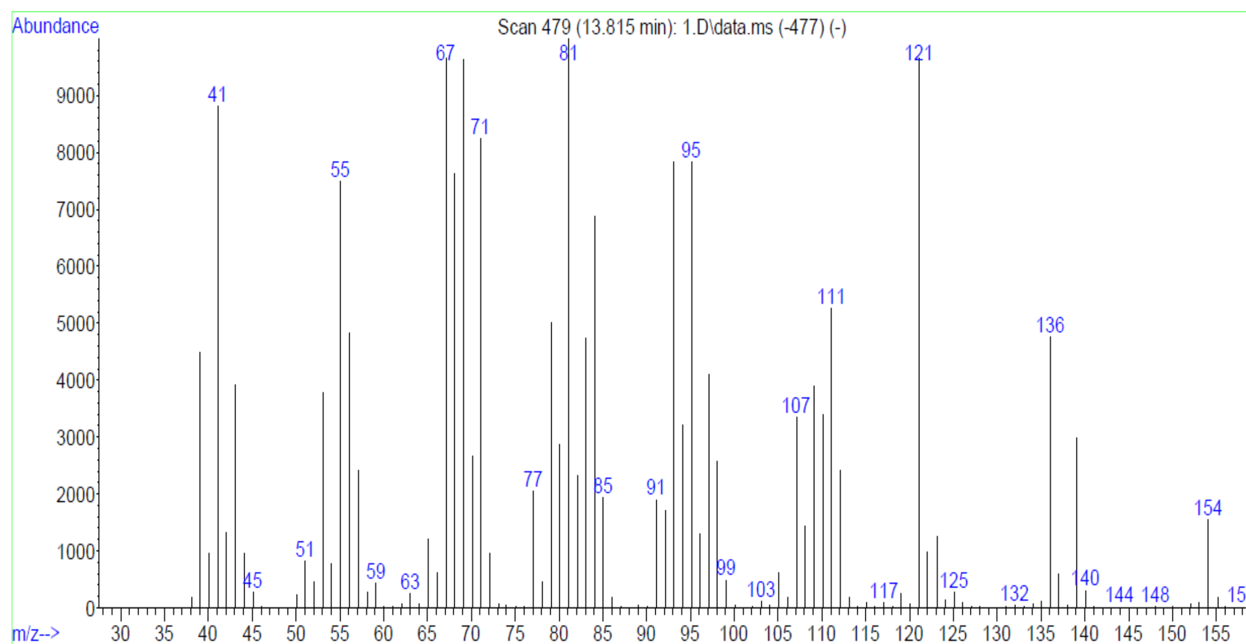
Appendix 3f: MS profile of Citronellol



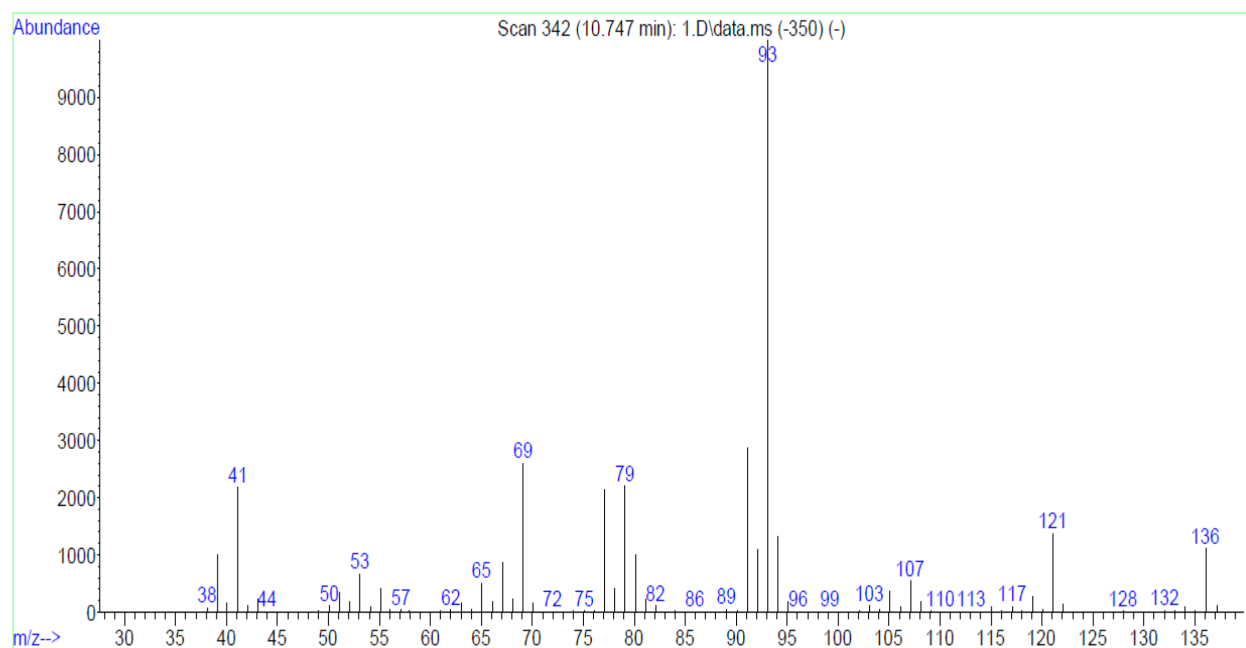
Appendix 3g: MS profile of Dauca-5,8-diene



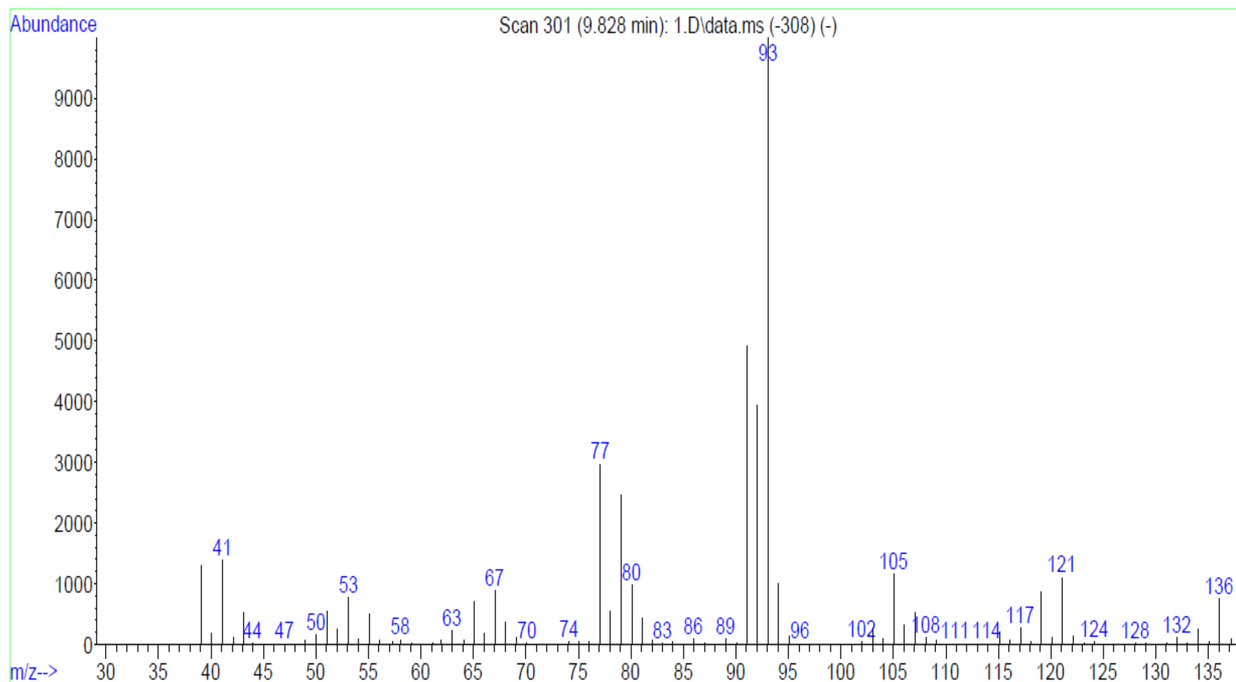
Appendix 3h: MS profile of Isopulegol



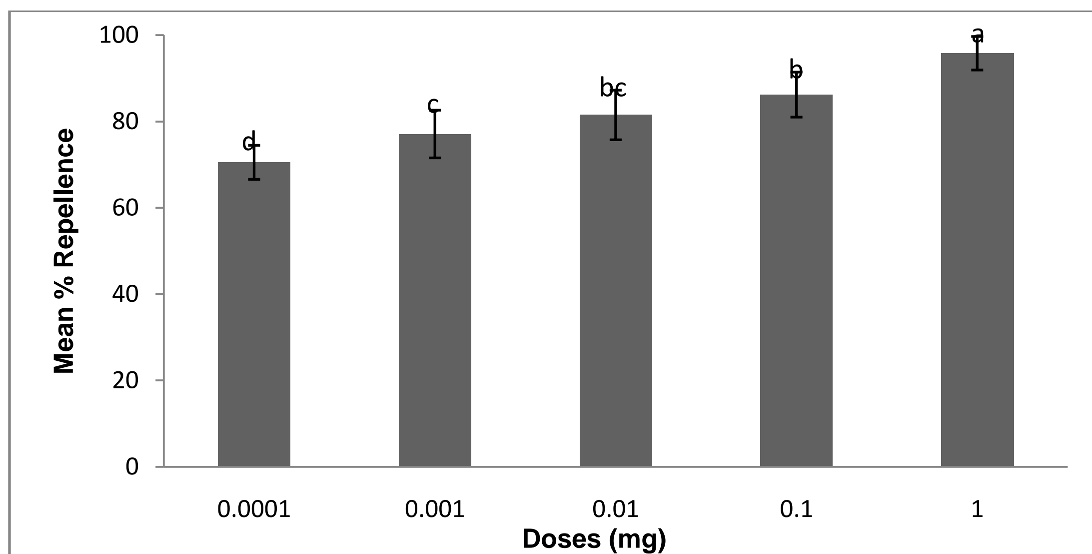
Appendix 3i: MS profile of β-Pinene



Appendix 3j: MS profile of α-Pinene

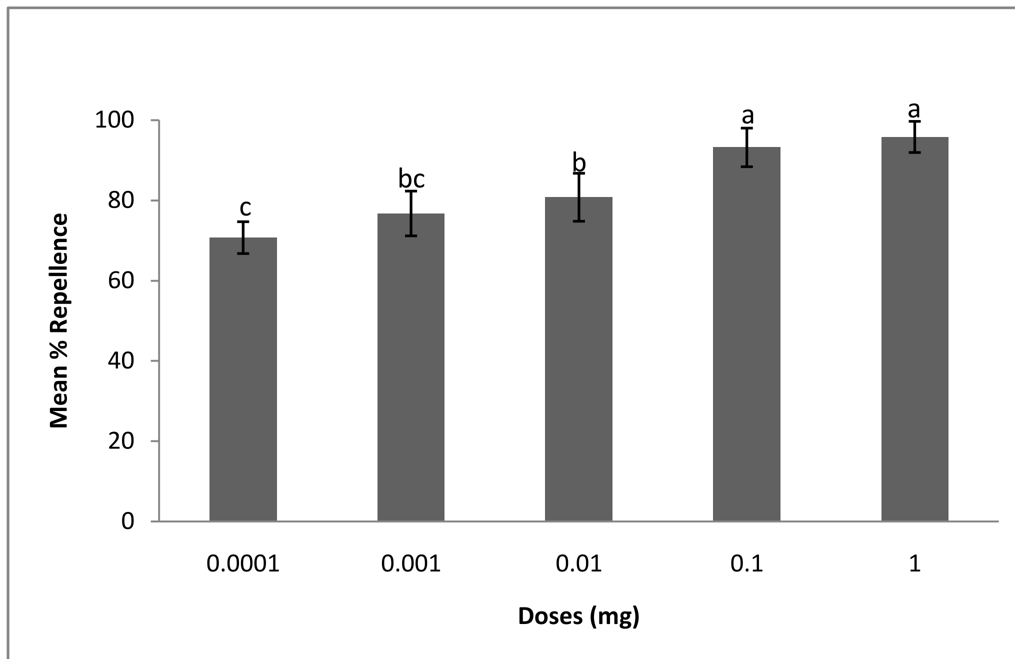


Appendix 4a: Mean repellence of synthesized (±)-*cis*-PMD against *R. appendiculatus*



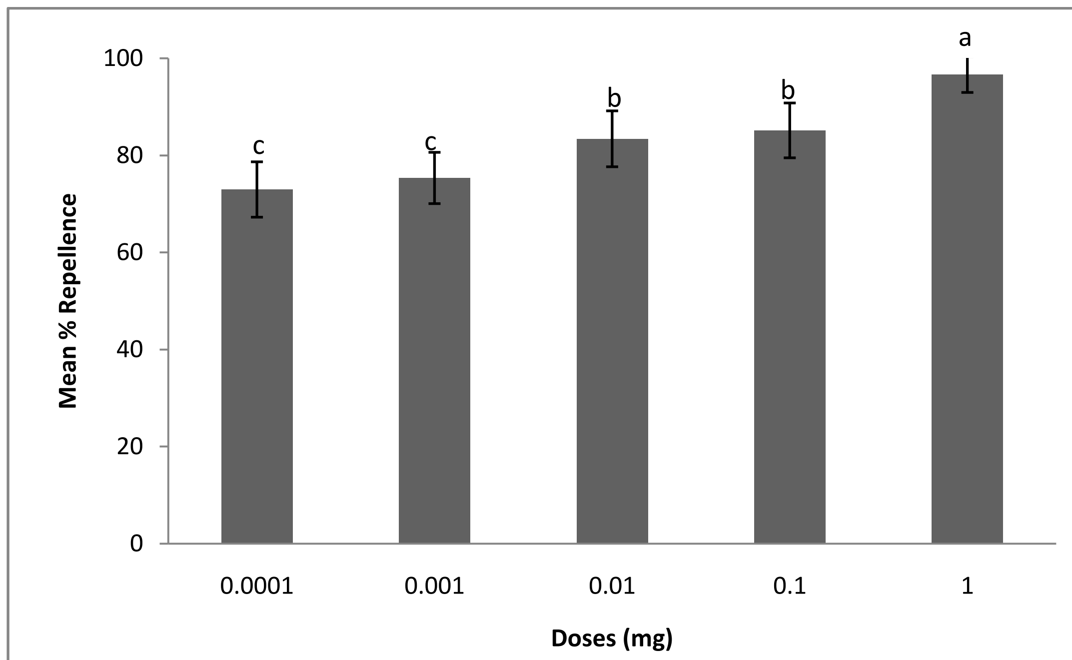
Mean (±SE) followed by the same letters are not significantly different (P≤0.05; Student-Newman – Keuls test).

Appendix 4b: Mean repellence of synthesized (±)-*trans*-PMD against *R. appendiculatus*



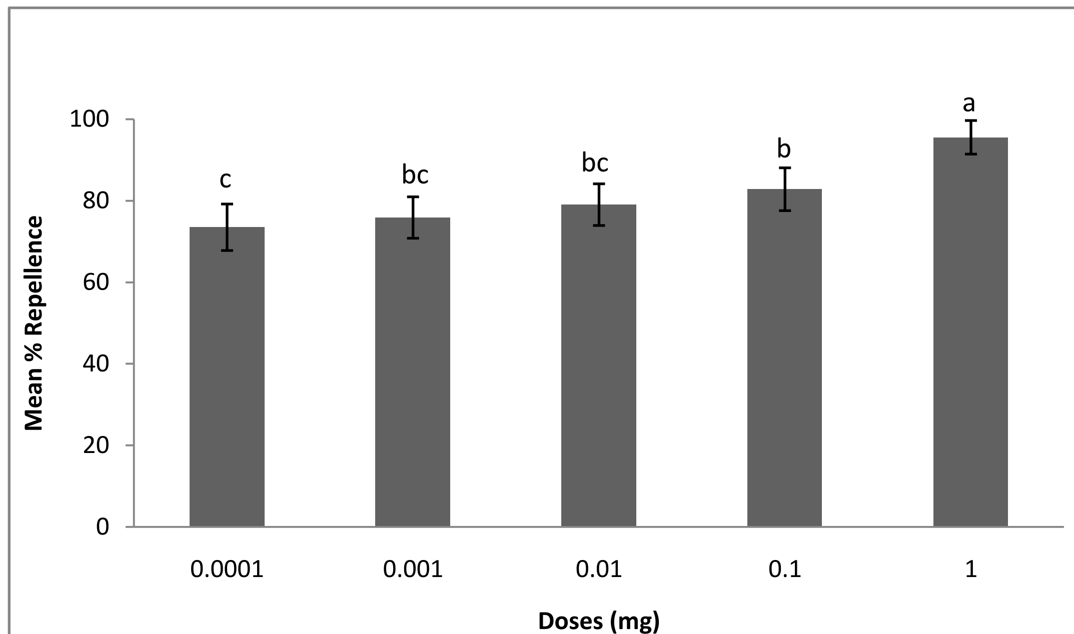
Mean (±SE) followed by the same letters are not significantly different (P≤0.05; Student-Newman – Keuls test).

Appendix 4c: Mean repellence of synthesized (−)-*trans*-PMD against *R. appendiculatus*



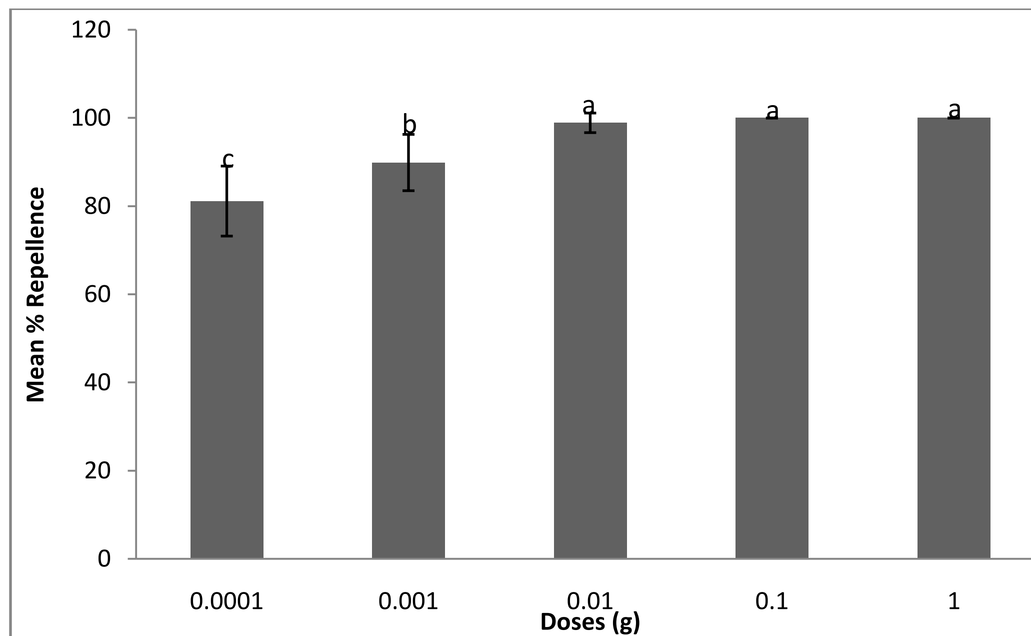
Mean (±SE) followed by the same letters are not significantly different (P≤0.05; Student-Newman – Keuls test).

Appendix 4d: Mean repellence of synthesized (+)-*trans*-PMD against *R. appendiculatus*



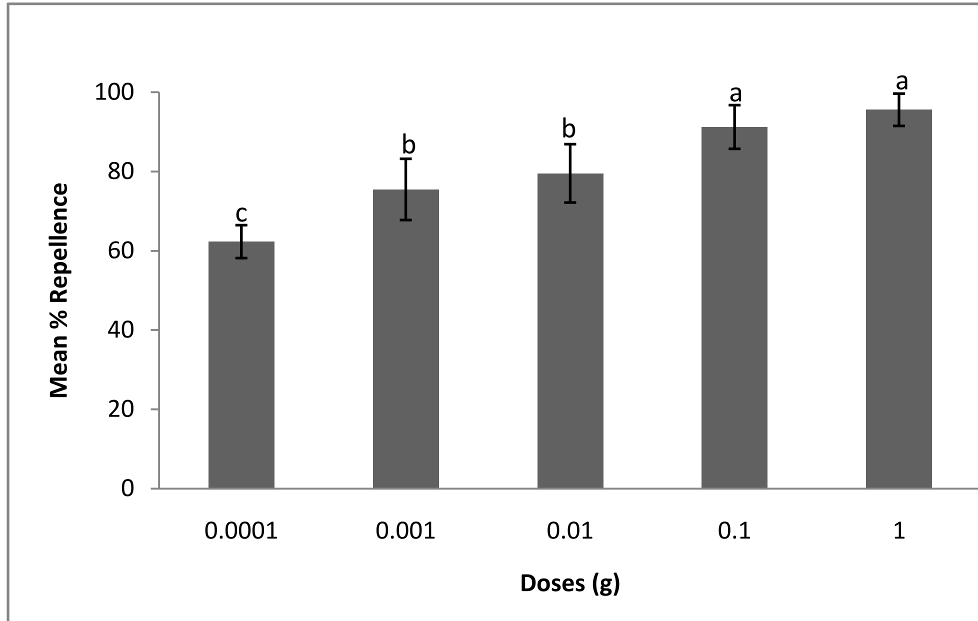
Mean (±SE) followed by the same letters are not significantly different (P≤0.05; Student-Newman – Keuls test).

Appendix 4e: Mean repellence of *Eucalyptus citriodora* oil against *R. appendiculatus*.



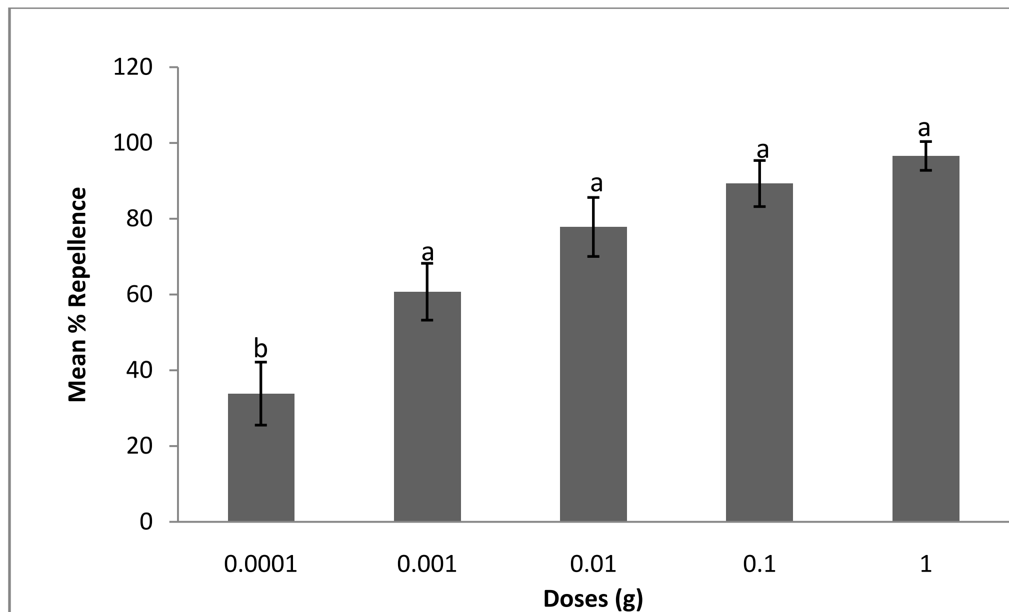
Mean (±SE) followed by the same letters are not significantly different (P≤0.05; Student-Newman – Keuls test).

Appendix 4f: Mean repellence of L-menthol against *R. appendiculatus*.



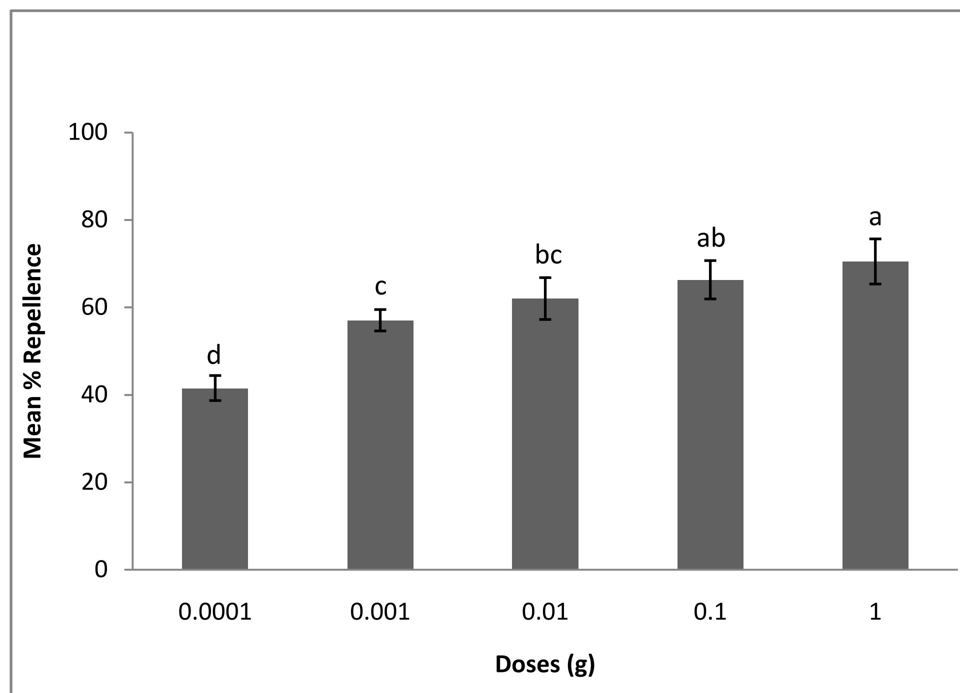
Mean (±SE) followed by the same letters are not significantly different (P≤0.05; Student-Newman – Keuls test).

Appendix 4g: Mean repellence of 1-α-Terpineol against *R. appendiculatus*.



Mean (±SE) followed by the same letters are not significantly different (P≤0.05; Student-Newman – Keuls test).

Appendix 4h: Mean repellence of aqueous fraction against *R. appendiculatus*.



Mean (±SE) followed by the same letters are not significantly different (P≤0.05; Student-Newman – Keuls test).

Appendix 5: Dose-response relationship of synthesized *p*-menthane-3,8-diol stereoisomers, analogues, DEET and *E.citriodora* oil.

Probit analysis of dose-response relationship of synthesized *p*-menthane-3,8-diol stereoisomers, analogues, DEET and *E.citriodora* oil					
		Time			
Compounds	Repellence probability	15	30	45	60
(−)-Trans-PMD	0.75	0.0003[a]	0.003[b]	0.001[b]	0.002[b]
(+)-Trans-PMD	0.75	0.0007[a]	0.003[b]	0.002[b]	0.002[b]
(±) Cis-PMD	0.75	0.0004[a]	0.001[b]	0.002[b]	0.001[b]
(±) Trans-PMD	0.75	0.0004[a]	0.001[b]	0.001[b]	0.001[b]
DEET	0.75	0.0005[a]	0.001[b]	0.001[b]	0.001[b]
E.citriodora Oil	0.75	0.0934[a]	0.106[b]	0.327[b]	0.231[b]
L-Menthol	0.75	0.1102[a]	1.743[b]	3.013[b]	2.07[b]
1-α-Terpineol	0.75	0.7213[a]	5.436[a]	8.138[b]	13.53[a]
Aqueous Fraction	0.75	0.7213[a]	5.454[a]	18.02[a]	13.53[a]

Means in columns followed by the same letters are not significantly different (P≤0.05; SNK test).