ISOLATION OF ACARICIDAL COMPOUNDS FROM *Acokanthera schimperi* WITH ACTIVITY AGAINST *Rhipicephalus appendiculatus*

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DECLARATION AND RECOMMENDATION

DECLARATION

I, Jared Odhiambo Owino , declare that this researchs thesis is my original work and has not been submitted wholly or in part for any award in any institution.

Jared Odhiambo Owino

Signature _____ Date _____

RECOMMENDATION

We wish to confirm that this thesis has been prepared under our supervision andis submitted for examination as per the Egerton Universityregulations and with our approval.

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DEDICATION

To

My wife Mrs. Caroline Odhiambo and family for the financial and moral support they offered to me throughout my studies.

And

My former principal, Mr. Elly Owiti Mingusa for having allowed me toproceed for the Master's program, his constant moral support he always offered me. You are a source of my strength Mr Elly.

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ABSTRACT

Tick borne diseases have severe consequences on the health of millions of cattle worldwide and cause serious economic losses. Synthetic drugs have been effective acaricideshowever they are expensive, show side effects and develop resistance.This has generated interest in the use ofplant based acaricides, which however seem to offer a reliable, cheap and cost effective methods. In this research, the acaricidal activityof *Acokanthera schimperi* secondarymetabolites against*Ripicephalus. appendiculatus* is reported.These secondary metabolites were active against the larvae of *R. appendiculatus*. The study aimed to isolate, purify and elucidate the structures of acaricidal compounds from *Acokanthera schimperi*. The collected leaves were air dried under a shade, ground into powder and exhaustively extracted with methanol and then suspended in water. Sequential extraction using hexane and then ethyl acetate was done. Methanol, hexane, ethyl acetate crudes and the water phase were screened for acaricidal activity after 48hours. Methanol crude extract registered (LC_{50}42.26/ LC_{90} 79.14 mg/ml), Hexane crude (LC_{50} 36.49/ LC_{90}58.34 mg/ml), Ethyl acetate crude (LC_{50} 47.11/ LC_{90} 69.48 mg/ml). The fractionation of ethyl acetate crude extract yielded fractions FA11, FA21(LC_{50} 31.94 / LC_{90} 66.93 mg/ml) and FA30 (LC_{50} 29.85 /LC_{90} 87.65 mg/ml). Fraction FA21 subjected tofurther, fractionation and purification led to fractions FA21a (LC_{50} 5.88 /LC_{90} 11.19 mg/ml), FA21b (LC_{50}5.88 /LC_{90} 11.19 mg/ml), $FA21b_1$ (LC_{50} 4.53/LC_{90} 6.92mg/ml), $FA21b_2$ (LC_{50} 2.96/ LC_{90} 6.09mg/ml),FA21a (LC_{50} 5.88/ LC_{90} 11.19 mg/ml), $FA21b_1$ (LC_{50} 4.53/ LC_{90} 6.92mg/ml) and FA21b2 (LC_{50} 2.96/ LC_{90} 6.09mg/ml). Fraction $FA21b_2$ subjected to further HPLCpurification yielded two pure compounds (**23)** and(**24)**.Separate acaricidal mortality tests could not be determined for the pure compounds (**23)** and (**24)** due to the minute quantities of each after HPLC fractionation and purification. However, the compound mixture$FA21b_2$had (LC_{50} 2.96/ LC_{90} 6.09mg/ml).These two new compounds were successfully identified through analysis of 1D and 2D nuclear magnetic resonance spectroscopy and mass spectrometry data, as well as comparing with literature data. The two newlyisolated compounds were8-hydroxy-2H -chromen -2- one (**23)** and (E)-methyl-4-hydroxyl -7- oxo-5- (2-oxo-2H-chromen -8-yloxy) oct-2- enoate(**24)**.These findings show that active principles from *A. schimperi*are likely to provide new, biodegradable, environmentally friendly biological active constituents that will serve as an alternativeto presently less effective and high cost synthetic acaricides.

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LIST OF ABBREVIATIONS AND ACRONYMS

ASALs	Arid and Semi-arid lands
COSY	^{1}H- ^{1}H correlation
DEPT	Distortion less Enhancement of Polarization Transfer
DMSO	Dimethyl sulfoxide
ECF	East Coast Fever
GDP	Growth domestic product
HMBC	Heteronuclear Multi-bond correlation
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
LC_{50}	The concentration at which 50% of the population responds
LC_{90}	The concentration at which 90% of the population responds
LWG	Live weight gain
MS	Mass spectrometry
nm	Nanometer
ppm	Parts per million
PTLC	Preparative thin layer chromatography
RH	Relative humidity
SPSS	Statistical package for social sciences
TBD	Tick borne disease
TLC	Thin layer chromatography
v/v	Volume by volume

CHAPTER ONE
INTRODUCTION

1.1 Background information

Livestock sector has an important role in the economy andlivelihoods ofa large proportion of the rural aswell as urban households in Kenya. Contribution oflivestock sector toKenya'seconomy, often given as 12% of the country's GDP and 42% of agricultural sector(SNV-2008). About 28 million cattle in the region are at risk and diseases kill at least 1 million cattle per year. Economic losses are higher in small-scale resource-poor households (Gachohi *et al.*, 2012).

With pressure from an increasing human population and declining per-capita food production in Africa, there is an urgent need to develop appropriate technologies so as to optimize livestock production. These technologies must be socially acceptable and provide effective remedies from reasonably inexpensive sources that can complement modern practices. While pharmacotherapy is one of the most important means of managing livestock diseases, it is only viable when the livestock owners can afford to cover the cost of treatment. Prevention of diseases by control of ecto-parasites and vectors is a viable alternative, though limited by the high cost of commercially available acaricides and insecticides.Chemical acaricides such as synthetic pyrethroids, organophosphates and amitraz have played a pivotal role in the control of ticks (Martins *et al*., 1995).

Development of resistance to commercial acaricides by tick has stimulated the search for new control strategies (Rosado-Arguilar*et al*., 2009). It is without doubt that there are other costs not easily measured related to the development of chemical resistance, since conventional acaricides are toxic products, very low degradation and not selective thatare harmfulto beneficial species and non-target to organisms including human (Romo-Martinez *et al.*, 2013). The problems of acaricides resistance, chemical residues in food and environment and the unsuitability of the resistance cattle for all production systems, make the current situation unsatisfactory. That is why there is need to develop alternative absolute control methods. Chemical- vaccine synergies have been demonstrated and a combination of chemicals and vaccine for tick and tick borne disease control has been identified as a suitable option (Oliver, 1989).Ethno-botanical studies are often significant in revealing locally important plant species especially for the discovery of crude drugs. The documentation of traditional knowledge, especially on the medicinal uses of plants, has provided many important drugs of modern day (Teklehaymanot *et al.*, 2007). Moreover, the discovery of effective natural products among native plants will introduce new, high value crops for

farming and open increased job opportunities for agricultural workers in the extractionand processing industry of the ethno medicinal drugs . The scientific rationalization of local plant species as an alternative ethno-veterinary acaricides will add value and contribute to increased farmer income and poverty alleviation among the rural peasants (Teklehaymanot *et al.*, 2007).

1.2 Statement of the problem

Livestock farming is constrained by ticks and tick borne diseases, which are a global problem and considered a major obstacle in the health and performance of animals. East Africa, an important livestock rearing region is particularly constrained by this problem. Ecto -parasites can significantly diminish the productivity of domestic animals through their biting, blood sucking and nuisance behaviour which leads to constant interruption of feeding by the animal. Their predation seriously reduces animal growth and development, curtailing profits to farmers. Ecto-parasites can also serve as vectors of animal diseases, causing extensive mortality and morbidity. In this region, animal diseases such as the East Coast Fever (ECF) causedby*Theileria parva*and transmitted by *Rhipicephalus appendiculatus* remain one of the principal causes of poor livestock performance. This problem leads to poverty in the rural areas, where livestock is one of the main sources of income. Reliance on conventional veterinary services cannot ensure complete coverage in preventive and curative health care because of inadequate trained personnel, logistical problems, erratic supply and the high cost of drugs.Besides, continued uses of synthetic acaricides or insecticides have resulted into environmental pollution.The use of plant based ethno-veterinary products is the available alternative. However, there has been little research effort to scientifically rationalize and validate the potency of the plant based ethno-veterinary products against major ecto-parasites as well as their bio-safety.Moreover, natural products from botanical sources used in traditional medicine may combat multiple drug resistant infectious diseases, a part from being environmentally friendly. Necessity to elucidate andvalidate biologically active components in such plants comes in handy. This may offer lasting solution to the emerging problems in the livestock farming.

1.3 Objectives

1.3.1 General objectives

To determine the acaricidal activity ofthe extract indigenousplant *Acokanthera schimperi* and isolate active compounds against *R. appendiculatus*.

Specific objectives

i. To screen crude extracts from *Acokanthera schimperi* for acaricidal activity

ii. To isolatephyto-chemicals and evaluate the bioactivity against *R. appendiculatus*

iii. To chemically characterise and validate the bio-active phyto-chemicals responsible for acaricidal activity

1.3 Hypotheses

i. Crude extracts from *Acokanthera schimperi* do not have acaricidal activity

ii. Isolated phyto-chemicals are not biologically active against *R. appendiculatus*

iii. The characterized bioactive compounds have no acaricidal activity

1.5 Justification

It is known thatplants naturally employ a variety of secondary metabolites (phyto-chemicals) to protect themselves frominsect predation and disease. These metabolites may also provide a valuable resource for developing efficacious plant based ethno-veterinary products. Historically, plant extractives and products from *A. schimperi* have been used successfully for insect pest control by the Samburupastoralists'.With pressure from an increasing human population and declining per-capita food production in Africa, there is an urgent need to develop appropriate technologies so as to optimize livestock production. The proposed study aims to promote the sustainable use of affordable plant based ethno-veterinary products for the management of livestock ecto-parasites. These technologies must be socially acceptable and provide effective remedies from reasonably inexpensive sources. It is therefore necessary to develop effective, cheap and environmentally friendly remedies for managing this problem. The use of plant based ethno-veterinary products will lead to reduced disease control; increased livestock productivity and more so improved house hold livelihoods.

CHAPTER TWO
LITERATURE REVIEW

2.1 Ecto-parasite and plant based ethno-veterinary

Animal ecto-parasites are those arthropods that live at the expense of their hosts and on the outside of the animals. Most important ecto-parasites are those which feed on the blood of their host. Ticks are ecto-parasites and are known to be parasitic; they also transmit diseases to man and livestock. They transmit viruses, rickettsias, bacteria, protozoa and paralytic toxins. Ticks act not only as vectors, but also serve as reservoirs of some infectious agents(Dominguez-Penafiel *et al.*, 2011).Theileriosis or East Coast fever, babesiosis or red water and cowdriosis or heart-water are among the major cattle diseases transmitted by ticsk.They take considerable amount of blood leading to anaemia, and their wounds are subject to secondary bacterial infection and myiasis. The feeding actions of ecto-parasites significantly reduce animal growth and development affecting their productivity and thereby curtailing profits to farmers and ranchers. Tick bites also lead to economic loss through damage to hides and skins (Gashar and Marsher, 2013). Historically, plants and plant extracts have been used to repel insects such as mosquitoes and other blood sucking arthropods well before the use of synthetic repellents (Brooke *et al.*, 2009).

Several plant-derived compounds, especially terpenes, with insect repellency are currently in use as food protectants (Hansen and Heins, 1992; Bowers, 1996). However, none are currently being used widely against animal pests. It is well known that plants employ a variety of secondary metabolites (phyto-chemicals) to protect themselves from insect predation and disease.These metabolites therefore provide a valuable resource for developing efficacious plant based ethno-veterinary products. Phyto-chemical based pesticides usually exhibit very low toxicity to humans and domestic animals and also rapidly breakdown in the environment thereby minimizing accumulation of harmful residues (Suresh *et al*., 2014).

Among the secondary plant metabolites known to contain compounds with insecticidal and acaricidal activities include essential oils, terpenes and flavonoids. Essential oils and pentacyclic triterpenes have been demonstrated to exhibit antimicrobial activity (Matasyoh *et al*., 2004; Matasyoh *et al*., 2007) as well as insecticidal activity (Matasyoh *et al.*, 2006). Four bio-active monoterpenes namely piperitone, 4-terpineol (**4)** and linalool (**2)** have been isolated from the Chinese prickly ash tree *Zanthoxylumbungeanum* (Bowers *et al.*, 1993). From the folklore of the Maori in New Zealand, an active sesquiterpenoid repellant (2*S*, 3*R*) – 1, 2-Dimethyl-3-3(4-methyl-3-

3 pentenyl) norbornanol (**1)**(Refer to figure 1)has been isolated from the tree *Dysoxylum spectabile (*Russel *et al.,* 1994). Another essential oil constituent β-caryophyllene has showed a highly significant effect on mortality of *Spodoptera exigua*. Li *et al.* (1978) isolated and identified as p-menthane-3, 8-diol(**3)** from leaves of Eucalyptus and demonstrated that it is a highly effective pest repellent. (Shaaya *et al.,* 1991) examined the effects of 28 essential oils on four species of stored product beetle pests and identified the most toxic oils.

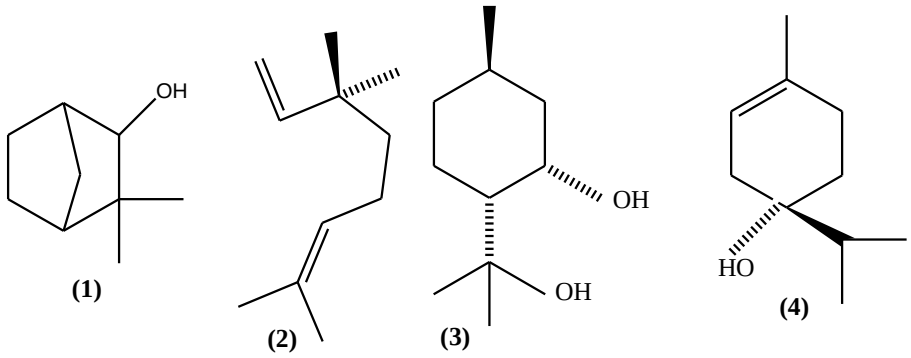


Figure 2.1: Compounds isolated from chinese pricky ash tree *zathoxylum bungueanum*

2.1.1 Argasid ticks

Argasid ticks of medical and veterinary importance belong to the generaArgas, Ornithodoros and Otobius. The Argasidae live near their favorite host and the parasitic stages feed for a short period only on the host and then go back to their hiding place. Exceptions are the larvae of certain Argas spp. that attach and feed for some days on domestic poultry, and the immature stages of Otobius which are parasitic for long periods in the external ear canals of their hosts (Schwan *et al*., 1992).

2.1.2 Ixodid ticks

Ixodid ticks may be one-, two-, or three host species depending on the number of host animals they attach during their life cycle. One-host ticks moult twice on the same host animal, from larva to nymph and from nymph to adult. Two-host ticks moult once on the host, from the larval to the nymph stage; the engorged nymph drops off, moults off the host and the resulting adult have to find a second host animal (which may or may not be of the same species as the first). Three-host ticks do not moult on the host; the engorged larva drops off, moults to a nymph, which then has to find a second host animal on which it engorges and drops off again to moult to the adult stage, which attaches to a third host animal. Amongst the ixodid ticks are Amblyomma*, Boophilus, Dermacentor,*

*Haemaphysalis, Hyalomma, lxodesand Rhipicephalus.*Tick Ixodidae is a hard tick that plays a significant role as vectors of pathogens of domestic animals. Adults of all Ixodid except specie of Ixodide require a blood meal to initiate the gognotrophic cycle. The female usually lays several thousands of eggs in one continuous cycle (Sonenshine *et al*., 1969).

2.1.3 Rhipicephalus

The genus *Rhipicephalus* comprises 70species. These small to medium sized ticks with short , broad palp that are usually inornate and have eyes and festoons .Most *Rhipicephalus spp*are found on the African continent. They are usually three host ticks although others havetwo host cycle (Walker *et al*., 2000).

Figure 1.2:Male and female *Rhipicephalus appendiculatus*

2.2 Life cycle and seasonal occurrence of*R. appendiculatus*

Ripicephalus appendiculatus is a hard tick found in the ears of domestic livestock,and other wildanimals like, buffalo and antelope. The *R. appendiculatus* feed on three hosts, during each life stage. They drop off and reattach to a new host during each life stage, until finally the female lays a batch of eggs (See Figure 2.2). Immature ticks may also be seen on small antelope, carnivores, hares and other species (Arthur, 1961).*R. appendiculatus*prefers relatively cool, shaded, shrubby or woody savannas or woodlands with at least 24 inches of annual rainfall. This tick occurs in parts of Eastern, Central and Southeastern Africa, and can be found from sea level 7400 feet. Its distribution within this area is limited to suitable environments with appropriate hosts. The pattern of seasonal occurrence of *R. appendiculatus* isdetermined by climate (Floyd *et al.,* 1987a).

The seasonal cycle is determined by the adults, which are only active under warm, wet conditions when the photo-phase (day length) exceeds approximately 11 hours. This means in locations near the equator, such as Entebbe- Uganda, adults can be active throughout the year if there is no prolonged dry season. As a consequence, larvae and nymphs will also be continuously present and the tick will probably pass through two or more generations each year. If there are two wet seasons, as in the highlands of Kenya, there will be two periods of adult activity and probably two generations each year. Research has shown that exposure of adult tickto high temperatures (26and 37°C) prior to feeding stimulates the maturation of *Thelaria parva parva* parasites in the salivary glands to mature sporozoites. It isthought that adults ticks exposed to high temperatures in the field would transmit infection to cattle more rapidly than would otherwise occur (Floyd *et al.*,1987a; Young*et al*., 1979; 1984 and 1987;and Ochanda *et al*., 1988).

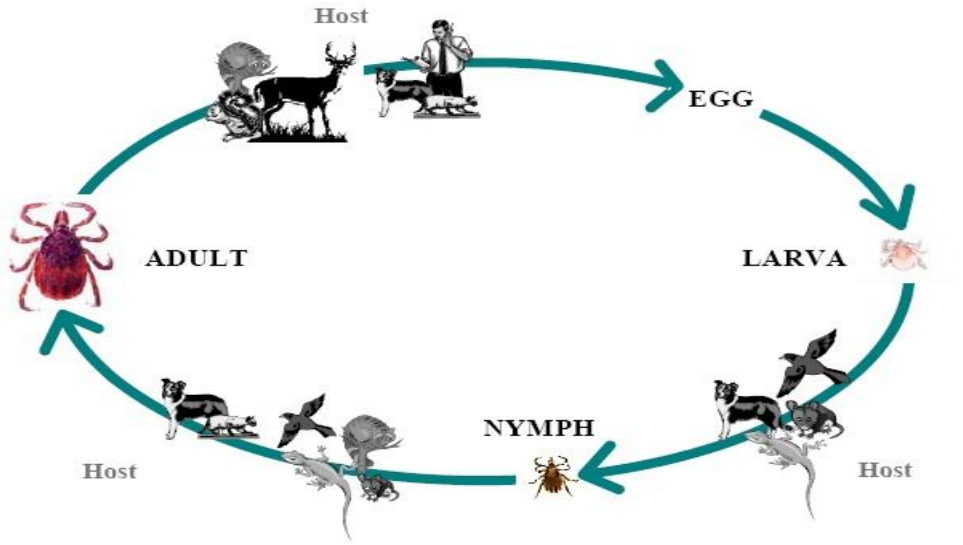


Figure 2: Life cycle of *Rhipicephalus appendiculatus*

2.3 Tick borne diseases

Approximately 80 % of the world's cattle population of 1281 million are at risk from ticks and tick borne diseases (TBD). In Africa, with 186 million heads of cattle, ticks and TBDs are the most serious constraints to increased production. About 28 million cattle in the region are at risk and the disease kills at least 1 million cattle per year. Economic losses are concentrated on small-scale resource-poor households. In Kenya, *Theilaria parva* infection poses a significant threat to the livestock sector in two ways; through the economic impact of the disease from cattle morbidity and mortality and production losses in all production systems, as well as from the costs of the measures

taken to control ticks and diseases. The costs of acaricide application, which is the primary means of tick control, was estimated to range between US$6 and US$36 per adult animal in Kenya, Tanzania and Uganda (Gachohi *et al.*, 2012).

2.3.1 Production losses caused by *R. appendiculatus*

The rapidly rising costs of tick control make it increasingly important to consider the economics of strategies for the control of ticks and tick-borne diseases. An important economic factor is the effect of ticks *per se* on cattle productivity, particularly where the diseases are controlled by immunization. In these situations the cost of tick control can be weighed against the benefit of increased productivity. The effects of tick infestation on the growth of Sanga and *Bos taurus* cattle in Zimbabwe was studied (Norval *et al*., 1988). Groups of young cattle were infested with high, moderate and low numbers of larvae, nymphs and adult *R. appendiculatus.* The numbers of each stage completing feeding and the live weight gain (LWG) of the cattle were recorded. Larvae and nymphs had no significant effect on LWG, but each adult female that completed feeding caused a loss of approximately 4grams. Cattle *Bos taurus* had a low resistance to the tick and consequently suffered large losses from adult infestations. The losses in Sanga cattle, which were very resistant to the tick, were insignificant. The effect of adult *R. appendiculatus* on milk production in Sanga cows was small but statistically significant (Norval *et al.,* 1991)

2.4 Control methods of ticks

2.4.1 Chemical control of *R. appendiculatus*

The main weapon for the control of *R. appendiculatus* at present is the use of chemical acaricides. The acaricides used to control ticks on livestock are applied in such a manner that *R. appendiculatus* are killed, but will not harm livestock or applicators, the tissue of the treated animals will not contain chemical residues, and environment will not be adversely affected. Synthetic chemical repellents are also commonly accepted means of personal protection against tick bites. These ticks are active host seekers that are strongly attracted to host producing carbon dioxide (CO_2) (Nurhayat*et al.,* 2013). Arsenical acaricides have been used for at least 50 years in most areas before tick resistance became a problem. Subsequently, organochlorine, organophosphate, carbamate, amidine and synthetic pyrethroid acaricides have been introduced, in that order, to most countries in the region. Tick resistance to organo-chlorines is now widespread and these compounds have largely been phased out. Organophosphates are currently the most widely used acaricides, but problems with

tick resistance are increasing and so their use is likely to decline in the future. The amidines and synthetic pyrethroids are becoming more widely used and have a much longer residual effect than the other acaricide groups but are considerably more expensive. A potential problem with the pyrethroids is cross-resistance between them and the organo-chlorines; evidence of this has already been reported in *Boophilus decoloratus* in South Africa (Coetzee *et al.,* 1987). The uses of acaricides have disadvantages, such as the presence of residues in milk and meat and development of chemical resistant strains (Willadsen, 1988)

The development of acaricides is a long and expensive process which reinforces the need for an alternative approaches to controlling *R. appendiculatus* infestation (Graft*et al*., 2004). The modeling approach has indicated that the most effective control strategies for *R. appendiculatus* are those directed against the adult stage (Floyd *et al*., 1987b). These strategies would also reduce the severity of challenge with the *T. parva* group of diseases, because adults are the most important vectors. In view of these problems, there has been an increasing interest in searching for alternative sustainable control method of ticks in recent years. Numerous pathogens and predators of ticks have been known for decades, but few bio control programs have been developed for ticks. Some studies have used herbal medicine such as *Margaritaria discoidea*plant extracts against*R.appendiculatus andHyaloma varigatum*or *Matricania achmomile* flower extracts against the adult stage of *R. Boophilus annulatus* (Piralie*t al.,* 2011) .

2.4.2 Traditional control methods of ticks

The animal health care systems, otherwise known as ethno-veterinary knowledge and practices play an important role in complementing modern approaches in management of diseases and their vectors in Kenya and possibly elsewhere in East Africa. However, there has been little research effort to scientifically rationalize and validate the potency of the plant based ethno-veterinary products, against major ecto-parasites as well as their bio-safety (Tamboura *et al*., 2000). Ethno-veterinary medicine includes use of medicinal plants, surgical techniques and management practices (Wanyama, 1997)

With the knowledge of adverse effects of synthetic pesticides worldwide,due to accumulation of unwanted residues in food, water and the environment, attention is rapidly shifting to non-synthetic safer options. The non-synthetic options developed should ideally reduce parasite populations, be target specific; for instance, kill the parasite and not other organisms, breakdown quickly and have low toxicity to man and other mammals. Most phyto-chemicals are known to

degrade rapidly in air, in sunlight and in moisture and hence are less persistent and have reduced risks to non-target organisms. Although phyto-chemicals may be promising as pesticides, there is need to generate useful information and knowledge on their bio-safety aspects (Tamboura *et al*., 2000).

Natural products from botanical sources used in traditional medicine may combat multiple drug resistant infectious diseases (Barbara *et al*., 2008), through elucidation and validation of biological compounds with novel mechanisms of action. Cultural acceptability of the traditional practices, along with perceptions of affordability, safety and efficacy play a role in stimulating scientific research and validation of traditional medicine. There are ethno-medicinal and ethno-veterinary studies which is being carried out to realize the benefit of traditional medication to promote the cheap and safe disease management. The outcome of these researches has immense contribution to attitude change and adaptation, though there are very little in light with Kenya's biodiversity application (Michael, 1992).As an example, the Samburu pastoralists in Kenya are still among the communities of the country that have retained most of their knowledge about use of a large part of the plants in the environment for a wide variety of purposes. This knowledge is however dwindling rapidly, due to changes towards a western lifestyle, overgrazing and over exploitation of plant resources leading to rapid decline of plant material available (Mark *et al*., 2008).

Ethno pharmacology and natural products drugs still remains a significant hope in improving the livelihoods of the rural communities. Many modern pharmaceuticals have their origin in the ethno-medicine and ethno-veterinary medicine, which relies upon the local pharmacopoeia (Tamboura *et al*., 2000).

2.5 Acokanthera schimperi

2.5.1 Ecological distribution of *Acokanthera schimperi*

*Acokanthera schimperi*shown in Figure 2.4,one of the medicinal plants frequently used by the Samburu people,belongs to a family of Apocynaceae, which is a small tree native in East Africa and Yemen.*Acokanthera Schimperi* occurs at the margins of dry forest, in relict forest, thickets, grass lands and bush lands, at 1100- 2400m altitude and with 600- 1000mm rainfall. It is drought resistant and prefers well drained, red or black soils, but also grows on black cotton soil and poor soil of dry sites. The distribution of the plant away from its natural habitat appears to be associated with human introduction. (Maundu andTengnas, 2005).

Figure2.3: Picture of *Acokanthera schimperi* plant

2.5.2 Uses of Acokanthera schimperi

The back of wood and roots of *Acokanthera Schimperi* is an important ingredient of arrow poison in Africa. All plant parts contain acovenoside A and ouabain which are cardiotonic glycosides (Sisay *et al*., 2012). These are prepared by traditional methods, for example *A.schimperi* containing acologifloroside K as its major active principle as well as smaller amounts of ouabain acovenoside A in the Maasai plains of Kenya **(**Tatjana *et al.,* 2007). Its fruits are edible and eaten as food. When ripe they are sweet but also slightly bitter. Unripe fruits have caused accidental poisoning as they are highly toxic. The plant*A. Schimperi* is not equally poisonous throughout the year. The toxic potential of the trees is sometimes established by observation of dead birds under the tree (Maundu, 2005). Cardiac glycosides, digitoxin from *Digitalis purpurerea L (Scrophuriaceae*), a cardiac glycosidal extract from *calotropis procesa* were tested for their effects against larvae and adult stages of the camel ticks (Salwa, 2010).

Roots and bark of *A. schimperi* (Apocynaceae) trees are gnawed, masticated and slavered onto highly specialized hairs that wick up the compound, to be delivered whenever the animal is bitten or mouthed by a predator. The poison is a cardenolide, closely resembling ouabain, one of the active components in a traditional African arrow poison, long celebrated for its power to kill

Elephants (Jonathan *et al.*, 2012). In traditional African medicinal practices, it is used for treatment of snake bite and tape worm infection (Sisay *et al.*, 2012).

2.5.3 Chemical composition of *Acokanthera schimperi*

*A. schimperi*is a genus in which some species are known to contain cardiotonic glycosides for example ouabaine (EFSA, 2009). All plants of *Acokanthera schimperi*, except the pulp of ripe fruits contain large amounts of Cardiac glycosides, of which nearly 20 have been identified. The glycosides are responsible for the activity as an arrow poison, but also act as cardiac stimulant. The main compounds are; acovenoside A, (**7)**. (0.3- 1.8%), with acovenosigenin as glycone, followed by ouabain, 6 and Oeandrin **5**, and traces of acovenisigenin A,(**8)**. *Acokanthera Schimperi* fromNairobi region in Kenya contain the highest amounts *acovenoside A*, and lowest amounts of ouabain. Plants from the coastal region of Kenya contain ouabain, while plants from Eritrea contain only half as much acovenoside A. A methanol extract from the leaves showed significant antiviral activity against influenza virus A, Coxsackievirus B3and HSV- 1 by inhibiting their replication. The extract also exhibit significant antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and significant antifungal activity against *Trichophyton mentaqgraphytes*(Tadeg *et al.*, 2005).

Cardiotonic streroids (CSs) or cardiotonic glycosides represent a group of compounds that share the capacity to bind to the extra-cellular surface of the main transport protein in cell, the membrane inserted sodium pump. These compounds have long been used and continued to be used in the treatment of congestive heart failure as positive inotropic agent. Several plants (more particularly those belonging to *Asclepiadacea*, *Apocynaceae* and *Ranuneulaceae* families) are recognized to contain CSs (Tatjana *et al.*, 2007).

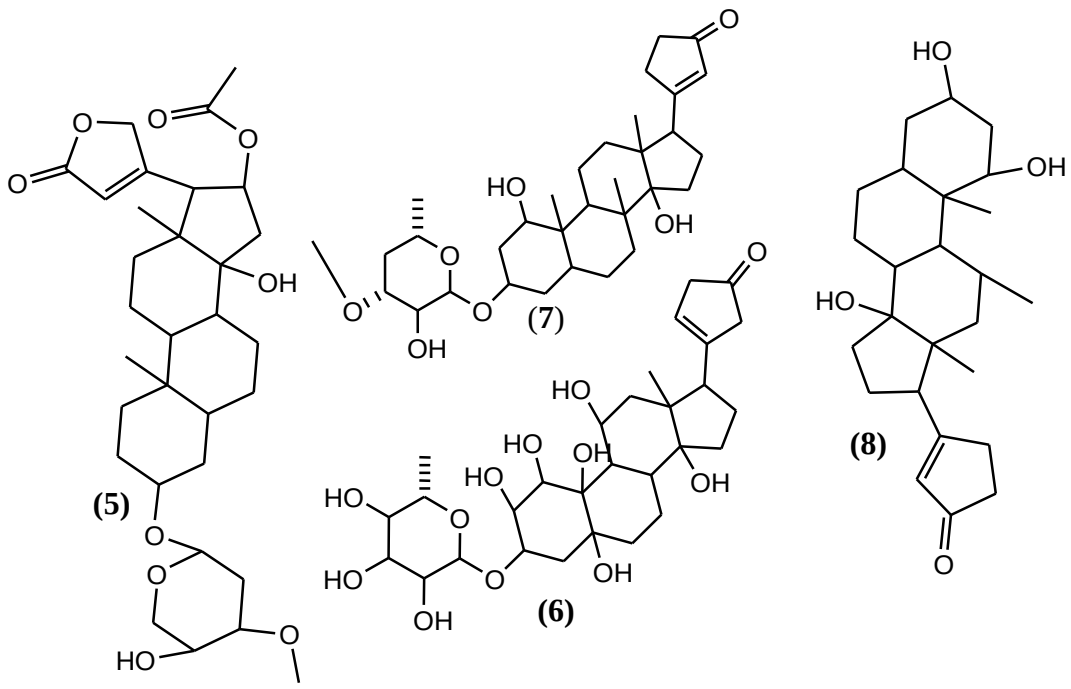


Figure2.4: Some of the compounds isolated from *A. schimperi*

2.6 Coumarin compounds

From theaerial part of *Launaea resedifolia* , four coumarin compounds were isolated from the methylene chloride- methanol (1:1),namely cichoriin(**9)**, esculetin(**10)** , scopoletin(**11)** and isocopoletin(**12)**. These compounds showed high antibacterial activity against some Gram –positive bacteria as Bacillus cereus and staphylococcus aureus in minimum inhibitory concentration of200 and 400µg/ml(Ashraf and Abdel, 2006).

Three coumarins have been isolated from the roots and stem bark of *Clausena pentaphylla* and analysis of their spectral data confirmed their structures as 3,10-bis (1,1 –dimethyl prop -2-en-1-yl)- 5,6,7- trihydroxy-8,8-dimethyl-7,8-dihydro pyranchromen -2-one(**13)**, bergapten(**14)** and Xanthotoxin(**15)**(Javed and Mohammed , 2008).New coumarin diol namely 6-(2'3'- dihydroxy-3-methylbutyl)-8-prenylumbelliferone (**16)** was isolated along with three known coumarin compounds 6,8-diprenyl umbelliferon(**17)**, bergapten(**14)** and isopimpinellin (**18)** from a chloroform fraction of the leaves of the plant, *chloroxylon swietenia DC*(Venkateswara *et al* ., 2009*).*Extraction of plant seed *Zosima absinnihifolia* afforded three furanocoumarin named imperatorin(**20)** and two coumarins,7-prenyloxy coumarin(**21)** andaurapten(**22)**. These compounds especially imperatorin exhibited fungi toxic activity against*Sclerotinia sclerotiorum*, a common plantpathogen (Seyed *et al*., 2010)

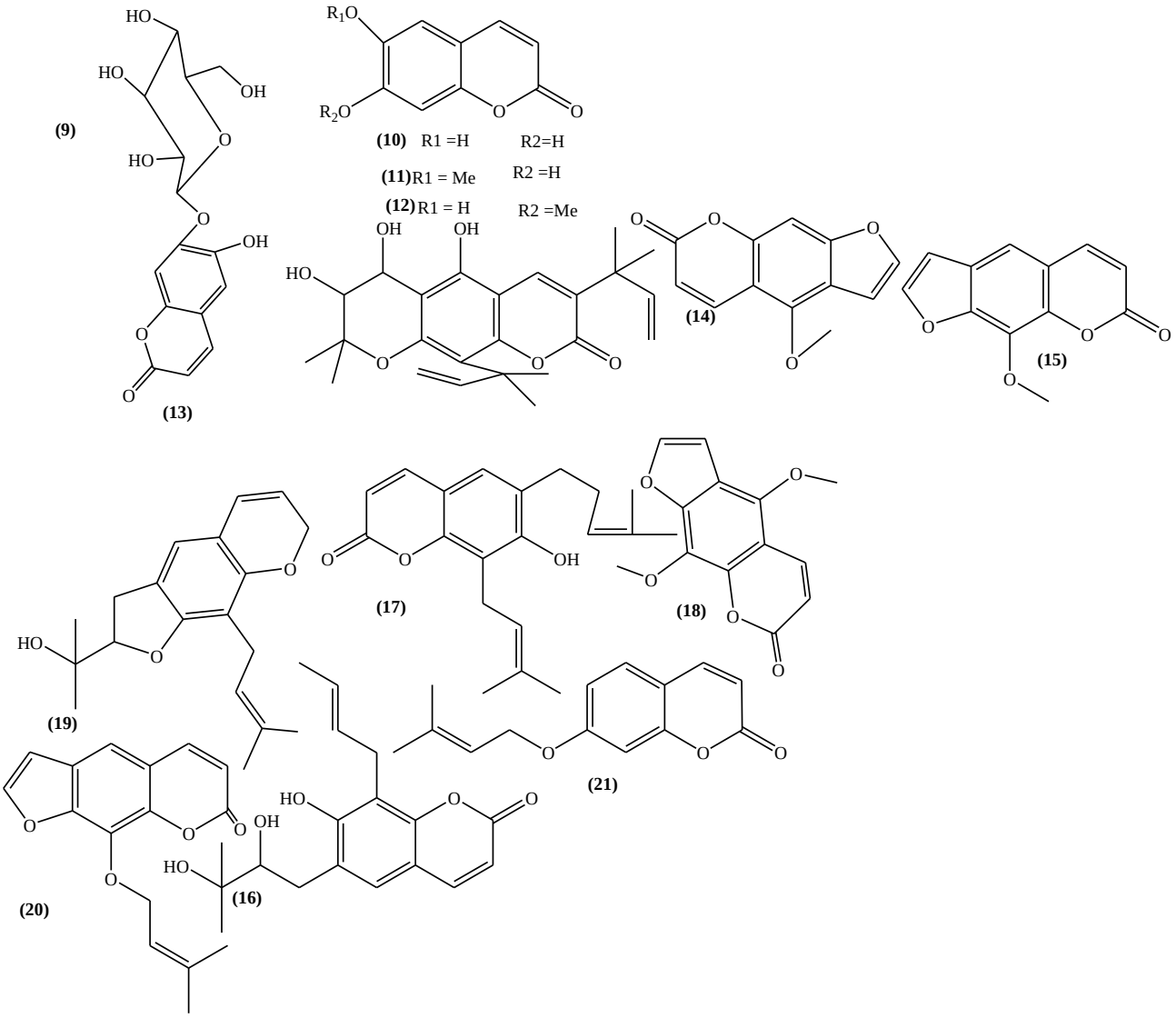


Figure2.5: Coumarin compounds

The control of ectoparasites of veterinary importance relies heavily on the use of chemicals and the effective pest control, around the world. It is necessary to have a range of compounds with different modes of action to enable the rotation of these chemicals and so help to manage existing problems of acaricidal resistance. Tick control by use of chemical acaricides is also fraught with various problems like residues, environmental pollution and high cost, clearly demanding the need for alternative approaches. Even though many plants extracts with promising acaricidal effects have been reported in literature, the feasibility of many of these extracts for the control of ticks infesting animals, has not been adequately studied (Sanin*et al*.,2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant identification and collection

The leaves of *Acokanthera schimperi* were collected from Kapkimolwa Longisa area in Bomet county, at 00º 52᾽ S and 035º25᾽ E and 1871m above sea level.The plant was identified by a taxonomist at the Department of Biological Sciences of Egerton University , where a voucher specimen was deposited .

3.2 Sample preparation

Leaves of the selected plant materials were then taken to the center for Herbal research in Egerton University,where they were dried in doors under shade for a period of one month, to retain their active ingredients and ground to a fine powder to increase the surface area during the extraction process. The duration of drying however depended greatly on the moisture content of the leaves and the season during which collection was done. The material (1500g) was then ground to a fine powder using Wiley mill model 4 at the Kenya Agricultural Research institute – Njoro.

3.3 Chemicals used and the working conditions

Ethyl acetate, hexane and methanol used were acquired from the commercial suppliers. All did nothave analytical reagent grades andwere purified by distillation method before use.

3.4 Extraction and isolation of non-volatile compounds

The powdered leaf material (1500g) was extracted exhaustively with 5 liters of methanol repeatedly fora period of two days. The filtrate obtained was then concentrated to dryness using a rotavapor machine (BUCH Rota vapor R- 205). The oily yellow crude extract obtained was suspended in distilled water, to remove any available sugars and then extracted sequentially with hexane and ethyl acetate as indicted in Figure 3.1.

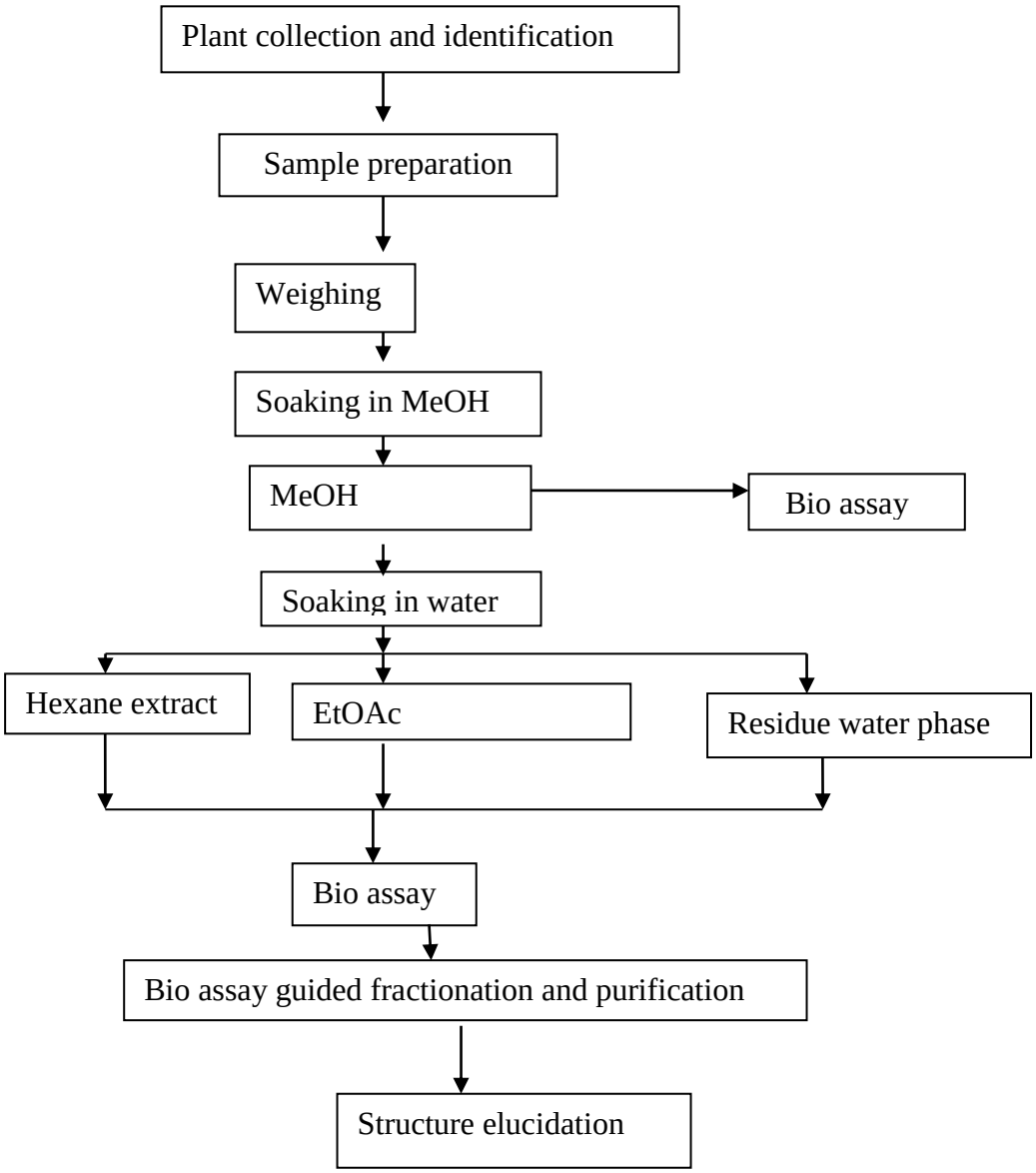


Figure 3.1: Flow chart on extraction of compounds from *A. schimperi*

3.5.1Thin layer chromatography analysis

To obtain an appropriate solvent system of separation, several solvent mixtures were tried and a mixture of ethyl acetate and ethanol in the ratio 6:4 v/v was found to give clear separation. On further purification, 100% ethyl acetate was also used.

3.5.2Fractionation of ethyl acetate extract

A glass tube with diameter 2cm and a height of 50cm fitted with a tap at the bottom was used for column chromatography. It was packed using silica gel (70-230 mesh) as the stationary phase and ethyl acetate - methanol solvent mixture in the ratio 6:4(v/v) as the mobile phase. The ethyl acetate crude extract was fractionated using the solvent system (Refer to figure 3.2). This led to 14 fractions that were combined according to their TLC patterns to two major fractions namely, FA11 and FA21. Fraction FA11 showed very low acaricidal activity during the screening process and was therefore left out for further purification (See apendix16). Further fractionation of FA21 using PTLC as explained in section3.7, with the same solvent system led to two fractions F21a and FA21b. Fraction FA21a had brown coloration and was visible at 254nm on the multiband UV- 254/365nm lamp coated (UV GL- 58) when developed on pre- coated silica gel 60 F254 aluminium TLC plate with fluorescence indicator. Fraction F21b was fluorescesing at 254 nm (purple spot) and 254nm (brown spot). Also further fractionation of FA21b (1.376 grams) fraction using 100% ethyl acetate solvent gave two sub fractions labelled $FA21b_1$ and $FA21b_2$. Fractions F21a (5.67grams), $FA2b_1$ (0.196grams) and $F21b_2$(0.129grams) were purified using preparative thin layer chromatography. Their retardation factors (RFs) were calculated as the ratio of the distance covered by the compound to the distance covered by the solvent, along the chromatogram. The obtained retention factor (RF) values for the compounds FA21a, $FA21b_1$ and $FA21b_2$ as developed on the TLC plate under UV light were 0.55, 0.73 and 0.86 respectively.

3.5.3 Purification of compounds

The fractions of interest were purified using preparative thin layer chromatographic techniques. The preparative TLC (PTLC) plate measuring 20cm by 20cm by 0.2 cm glass plates were used. The plates were prepared by mixing the adsorbent silica gel with a small amount of calcium sulphate which acted as the inert binder and water. Silica gel (in powder form) was weighed, where 180g was mixed with 45g of calcium sulphate, which was to help bind the slurry on the glass. Distilled water (400ml) was used to make the slurry and a magnetic stirrer to enable obtain uniform slurry. The glasses were placed on a flat surface; this was to allow slurry spread evenly. The prepared plates were allowed to dry overnight (12 hours) and then activated by heating in the oven forduration of one hour at a pre-set temperature of 140^{o}C. The plates were allowed to cool gradually to reduce possible breakages during the development process. The prepared dry PTLC plates were uniformly loaded with the sample at the base of the plate. The sample was allowed to dry and then

afterwards put in the development tank using EtOAc: MeOH 6:4 (v/v) as eluent. After the solvent front had covered 75% of the plate distance, the plates were removed and allowed to dry. The compounds were then scrubbed from the plates based on the separation patterns and the compounds extracted from the silica gel using ethyl acetate: methanol 6:4(v/v). The filtrate obtained was then concentrated to dryness using a rotavapor machine (BUCH Rota vapor R- 205). This process enabled the purification of the said pure compounds. Further purification of $FA21b_2$ using reversed phased high performance liquid chromatography (HPLC) with water and acetonitrile resulted into two compounds (**23)**and (**24)**.

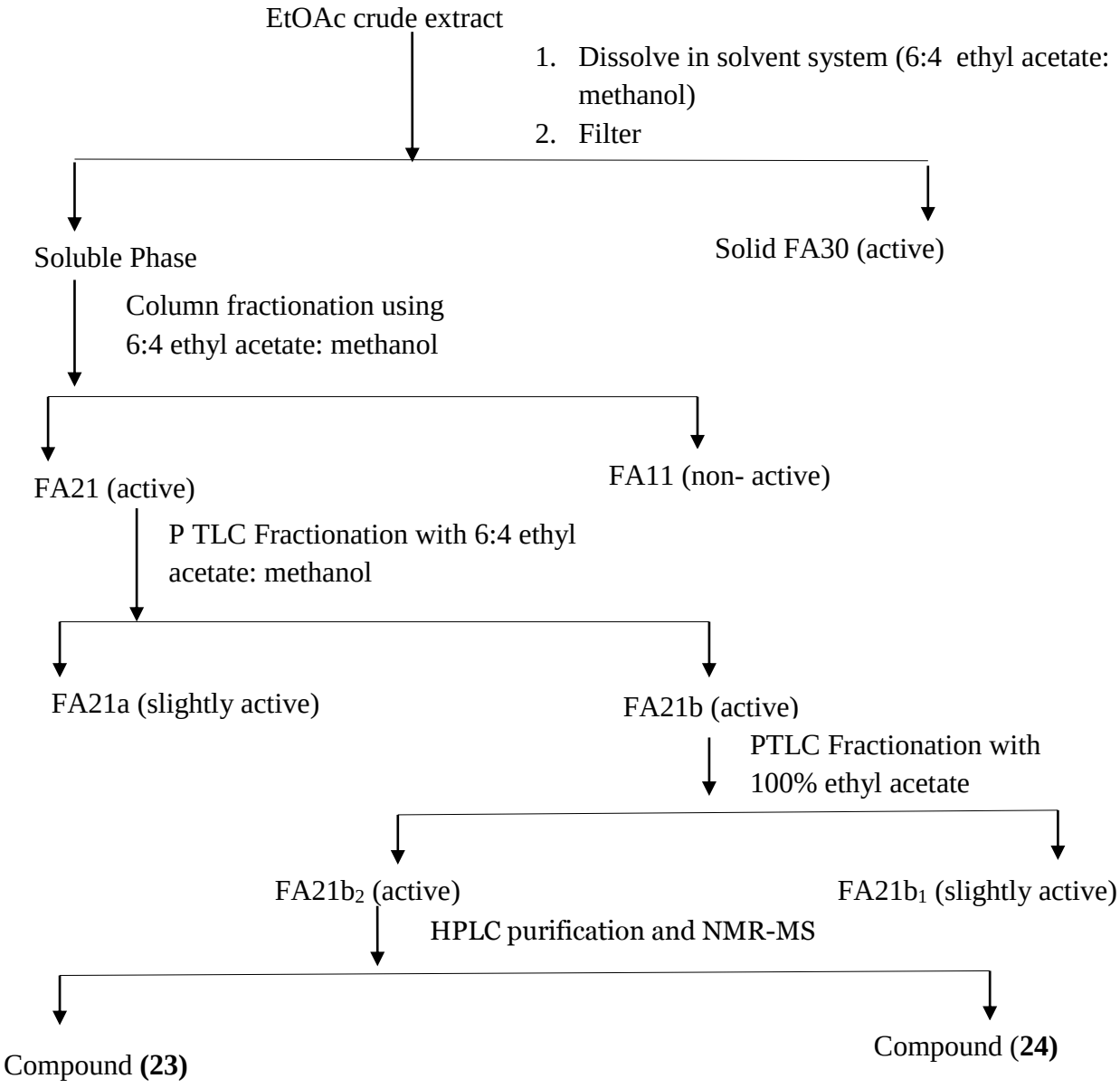


Figure 3.2: Schematic diagram showing fractionation and purification of compounds

3.5.4 Bio-assay guided fractionation

The crude extracts and all the fractions were screened for their acaricidal activities against the larvae species of *R. Appendiculatus*. The result of this screening is in Table 4.3. For all the samples, concentration used was 50mg/ml. At lower concentration of 4mg/ml the larvae only had knock down effect. Methanol crude extracts, Hexane and Ethyl acetate crude extract was found to be active against larvae of *R. Appendiculatus*. Considering the duration of exposure, hexane extract had the highest mortality rate at 24hour while water phase had the least activity at 48hours of exposure. This meant that all the three; methanol crude extract, hexane crude extract and ethyl acetate crude extract qualified for further bio assay guided fractionation.

3.5.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H, DEPT, HSQC, COSY and HMBC spectra were recorded on the Bruker advanced 500MHz NMR spectrometer at the Technical University of Berlin, Germany. All the readings were done in DMSO and chemical shifts assigned by comparing with the residue proton and carbon resonance of the solvent. Tetramethylsilane (TMS) was used as the internal standard and the chemical shifts were given as δ (ppm). The structures were simulated using ACD NMR manager program to obtain the chemical shifts of proton,1D and 2D high field NMR spectroscopy and mass specdroscopy.

The mass spectra of the compounds were recorded on Finnigan Triple Quadrupol spectrometer (TSQ-70) with electro spray ionization (ESI) method. The Thermo Xcalibur Qual computer software was used in the analysis of the mass chromatograms.

3.6Rearing of R. appendiculatus

The larvae that were used for bio assay were reared according to (Pirali-Kheirabiadi, *et al.*, 2011). A circumference of approximately 22cm of hair from the back of the rabbit was first shaved. This allowed porcelain cloth that was folded cylindricallyattached at the back of the rabbitat the shaved area using conta glue. Male and female *R. appendiculatus* wereplaced inside the folded porcelain cloth at the back of the rabbit. The rabbit was the placed at the cage and then fed with rabbit pellets and water. A collar like object was also placed at the neck of the rabbit to prevent it from rubbing the back, which was due to possible irritation from the tick bites. After the ticks had fed for about 6days, adult male and female *R. appendiculatus* mate. Complete engorgement of the female tick then followed, as the tick fed on the rabbit for aperiod of 4 days. Once fully engorged the females dropped off the rabbit and were placed on the glass vials covered with net cloth.Within 2-

5days later, the engorged female laid eggs. The eggs were then incubated at 25-27^{0}C and 80% relative humidity for 21-30 days followed by hatching to larvae. The larvae are able to stay for a period of six months without having a meal and only moult to the next stage (nymph) once they are fed. The vials with larvae were wrapped in cotton net cloth for oxygen supply and transported to the University laboratory for bioassay within 24hours to perform subsequent bio assay experiments.

3.7 Bio assay

Methanol, hexane, ethyl acetate, water phase crude extracts, fraction 11, fraction 21, and fraction 30 and purified compounds FA21a, FA21b$_1$ and FA21b$_2$ were solubilised in Dimethyl sulfoxide (DMSO) and diluted with distilled water to give 50mg/ml of stock solution. According to (Sanin *et al*., 2012), a series of seven concentrations of the bio-active phyto chemicals ranging from 50, 45, 40, 35, 30, 25 and 20 mg/ml were prepared by serial dilution, where DMSO was kept at an optimised concentration of 4% v/v, at which the concentration did not affect the acaricidal mortality. A series of concentrations of both the positive control (0.2%v/v amitraz) and negative control (DMSO and distilled water), was also prepared. The insect bioassay was carried out by the dipping method where the larvae were sprayed with test sample. A filter paper was dipped in the test solution. Ten larvae were placed at the centre of a filter paper and then allowed to move around. Larvae were also placed on filter paper dipped in DMSO and distilled water. Mortality was observed after 48hours (Fernando *et al.*, 2007). The larvae were examined under a microscope and those that did not respond to human breath (CO_2) and tactile stimulus for each test solution was considered dead. Mortality of larvae was reported for methanol, hexane, ethyl acetate crude extracts, fraction 11, fraction 21, and fraction 30 and purified compounds FA21a, FA21b$_1$ and FA21b$_2$. A series of concentrations of both positive control – 0.2%v/v (amitraz), and negative control – 4%v/v DMSO and water, were used and the bioassay was performed at 27 $\pm$1^{O}C, RH $\geq$ 80%.

3.8 Data analysis

The data analysis was performed using statistical packages SPSS 20 computer software. The acaricidal mortality was subjected to probit regression analysis. Once a regression was run, the output of the probit analysis was compared to the amount of chemical required to create the same response in each of the various amounts of chemical. The goodness of fit of the points to a straight line was tested, and analysis of LC_{50} and LC_{90} values was determined.

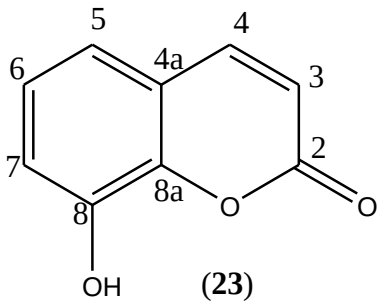
CHAPTER FOUR

RESULTS AND DICUSSION

4.1 Structure elucidation of the isolated compounds

4.1.1 Structure elucidation of compound(23)

Two dimensional NMR ^{1}H-^{1}H correlation (COSY), hetero nuclear single quantum coherence (HSQC) and hetero nuclear multiple bond correlation (HMBC)spectroscopic techniques were used. The ^{1}H NMR spectrum of compound (**23)**showed five aromatic proton resonances in the molecule.The doublet at δ 6.20 ($J_{3,4}$=9.52 Hz) and δ 7.93 ($J_{4,3}$= 9.5Hz) are characteristic of H-3 and H-4 Coumarin moety (Venkateswara *et al.,* 2009). The rather up field chemical shift of the H-3was due to the possible shielding influence of the C=O function, while the relatively down field chemical shift of the 4-H may be attributed to the de shielding effect of C=O function operating atC-4. The H-7 aromatic proton appeared at δ 6.72 as a doublet ($J_{5,7}$= 1.8Hz). The value of coupling constant showed the presence of H-7meta to H- 5. A doublet of doublet at δ 6.79 ($J_{5,6}$=8.52Hz and $J_{5,7}$=2.2 Hz) was assigned to the H-5 aromatic proton. The larger coupling constant (J=8.52 Hz was due to the H-6 ortho proton while the smaller coupling constant J=2.2Hz was due to H-7 meta coupling (Silva *et al.*, 2012; Metin, 2005). The H-5 on the other hand showed COSY interaction with the H-6.The appearance of H-5 as a doublet doublet with one of the coupling constant J=2.18Hz could only result if the OH was placed at C-8. From the literature, Coumarin compounds are found to havesubstituents at C-7 and C-8, whether glycoside moiety or hydroxyl group (Ashraf *et al*., 2006; Seyed *et al*., 2010; Lozhkin and Skanyan, 2006; Mohamed *et al.,* 2007; Trong – Tuan *et al*., 2012; Renmin *et al*.,2004). A doublet at δ 7.52 and J= 8.5 Hz signified the presence of proton at the ortho positionat C-6(δ 130.2).



(**23**)

Figure 6: Structure of compound**(23)**

The ^{13}C NMR spectrum of the compound (**23)**showed nine carbonresonances in the molecule. The nine signals corresponding to basic coumarin skeleton (δ 102.6, 111.8, 111.9, 113.6, 130.2, 155.9, 160.9 and 161.8) (Venkateswara *et al.*, 2009; Zaffer *et al*., 2012). The C-2 lactone carbon appeared at δ 161.8. The C-3 and C-4 resonated at δ 111.8 and δ 144.9 respectively. The down field chemical shift noted on the 4-C was due to the resonance of the lactone carbonyl. The C-5 appeared at δ113.6, while C-6 appeared at δ130.2. The C-7 appeared at δ102.6 and the C-8 appeared at δ160.9. The down field chemical shift of C-8 showed the resonance of oxygen function on the carbon having the chemical shifts at δ160.9. The quaternary carbon signals155.9 and 111.9 were assigned to the carbon 4a and 8a respectively.

The ^{1}H-^{13}C connectivities were established through HSQC spectrum. The H-4 (δ7.93) showed cross peak with C-4 (δ144.3) while H-3(δ6.20) in the HSQC spectrum showed cross peak with C-3(δ 111.8). The H-5 (δ6.79) showed a cross peak with the C-5 signal at (δ 113.6), while the H-6 (δ 7.52) showed a cross peak with C-6 signal at (δ130.2) and H- 7 (δ 6.72) showed cross peak with C-7(δ 102.6).

The HMBC spectral data showed correlations between H-3(δ 6.20) and the carbonyl at (δ 161.8). It also exhibited correlation with C-4a (δ 111.9). The doublet signal of H-4 (δ 7.93) showed correlation with C-2(δ 161.8), C-3(δ 111.8) C- 4a (δ 111.9) C-6(δ 130.2), C-7(δ 102.6))and C-8a (δ 155.9). The doublet doublet signal of H-5 showed correlation with C-4a (δ 111.9), C-6(δ 130.2), C-7(δ 102.6), C-8(δ 160.9) and C-8a (δ 155.9). The doublet signal of H-6 showedcorrelation with C-4a (δ 111.9), C-5(δ 113.6), C- 7(δ 102.6) and C-8(δ 160.9). The doublet signal of H-7 (δ 6.72) also showed correlation with C-4a (δ 111.9), C-8 (δ 160.9), C-5 (δ 113.6), and C-8a (δ 155.9). (Silva*et al*., 2012; Metin, 2005). ^{1}H-^{13}C (HMBC)and^{1}H-^{1}H (COSY) correlations in the molecule are illustrated in Table 1 below as obtained from their spectra shown in the appendices 20 and 21.

^{1}H-^{1}H Cosy spectrumconnectivitie showed correlation of proton with signal at δ 6.20(H-3) and that of δ 7.93 (H-4) and vice versa. Similarly, proton signal at δ 7.52 (H-6)showed correlation with proton at δ 6.79(H-5). There was a Meta coupling between the proton with signal δ 6.79 (H-5) with acoupling constant J= 2.2Hz and δ 6.72 (H-7) with coupling constant J=1.8Hz.

Compound **23** was identified as with molecular ion peak m/z with molecular ion peak m/z 163.04 $[M+H]^{+}$, calculated for $[C_9H_6O_3 +H]^{+}$ (m/z 163.143) , using high resolution positive electron impact mass spectrometry (HREIMS) at 1.82 minutes retention time. The mass spectrum of the

compound is as shown in the Figure **8**. The compound was identified as 8-hydroxy-2H -chromen -2-one with a molecular formula $C_9H_6O_3$. The structure of compound **23** is shown in Figure **9**.

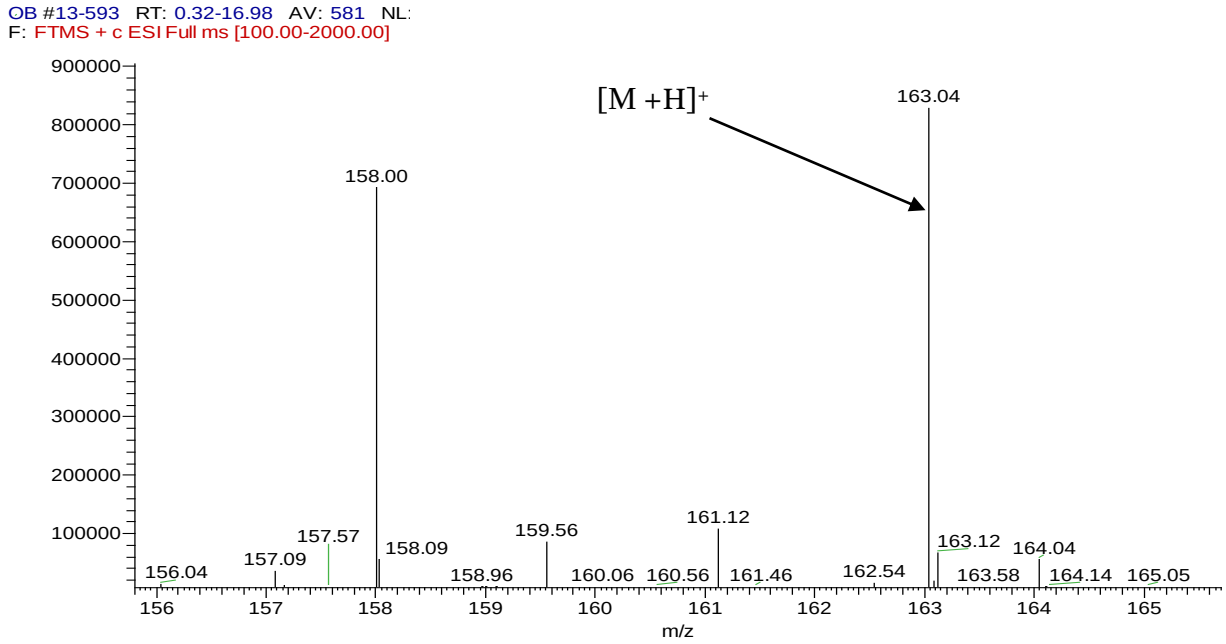


Figure 7: Mass spectrum of compound **23**

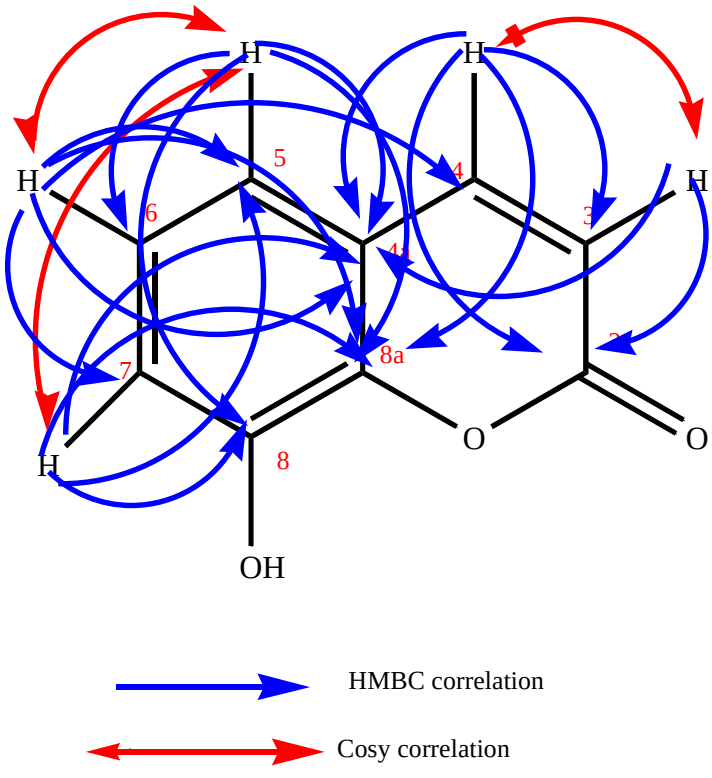


Figure 8: Structure of compound **(23)** showing COSY and HMBC correlations

Table 4.1: Summary of 1D and 2D NMR data values for compound**(23)**

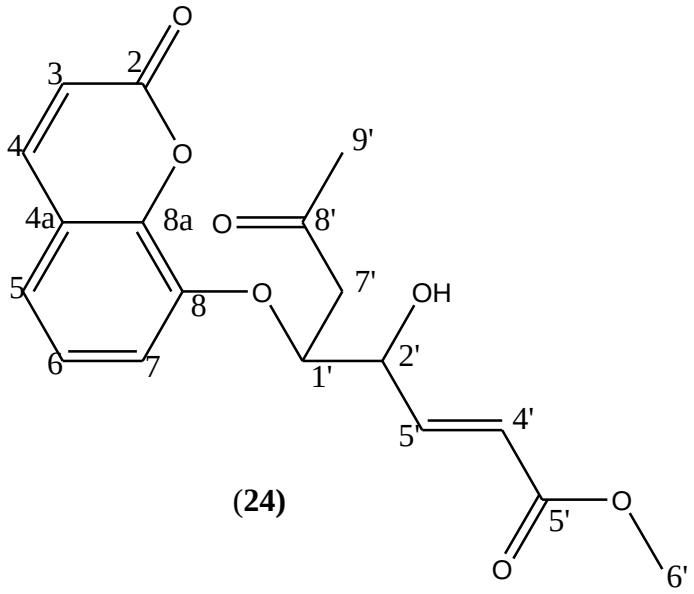
Carbon No.	^{13}C (ppm)	^{1}H (ppm)	DEPT	COSY (δ)	HMBC(δ)
2	161.8	-	Cq	-	-
3	111.8	6.20 (d,J=9.5Hz)	CH	4	2,4a
4	144.9	7.93 (d,J=9.5Hz)	CH	3	2, 3, 4a,7,6, 8a
4a	111.9	-	Cq	-	-
5	113.6	6.79(d,d ,J=2.2,8.5Hz)	CH	6,7	4a, 6 ,8, 8a
6	130.2	7.52(d,J=8.5H)	CH	5	4,4a,5,7,8a
7	102.6	6.72 (d,J=1.8Hz)	CH	5	4a ,5 ,8, 8a
8	160.9	-	Cq	-	-
8a	155.9	-	Cq	-	-

From structural elucidation and comparing with the literature data, indicated that compound (23) has never been isolated and termed as new compound.

4.1.2 Structure elucidation of compound (24)

The ^{1}H NMR spectrum of compound (**24)** showed 18 proton resonances in the molecule;five aromatic proton resonances and 13 proton resonances attached on the prenyl group. There was a proton broad peak signal at δ 3.56, which isassociated with a methoxy proton (Ahmed *et al*, 2012). Two broad signals at δ 5.39 and δ 5.77 were attributed to H-4' and H-3' associated with methine protons attached tovinylic carbons (Seyed et *al*., 2010). A quartet signal at δ 5.01 was accounted to C-1' methine proton. And a doublet signal at δ 1.76 was due to C-7' methylene protons. The doublets at δ 6.13 ($J_{3,4}$=15.93Hz) and δ 7.38($J_{4,3}$=15.67 Hz) were assigned to H-3 and H-4, respectively. The rather up field chemical shift of the H-3 was due to the possible shielding influence of the CO function and the relatively down field chemical shift of the H-4 is attributed to the de shielding effect of the CO function, which is operating at C-4. The H-5 aromatic proton appeared at δ 6.76 as a doublet ($J_{6,5}$=8.2 Hz). The value of the coupling constant showed the presence of H-5 ortho protons. This showed a possibility of a substituent at C-8. A doubletof doublets at δ 6.96 ($J_{5,7}$ = 2.1 Hz) and δ 6.76 ($J_{6,5}$= 8.2 Hz) was assigned to the H-5 aromatic proton. The larger coupling constant J= 8.2 Hz was due to the H-6 ortho proton and the smaller coupling constant J=2.1 Hz was due to H-7 meta proton (Silva and Maria, 2012).The H-5 on the other hand

showed COSY interaction with H-6. The Appearance of H-5 as a double of a doublet with one of the coupling constant J= 2.06 Hz could only result if there was a substituent group placed at C-8. A doublet at δ 6.76, J= 8.22Hz showed presence of proton at the ortho position. Substitution of a substituent group on C-7 leaves the proton attached at C-8 to be a singlet ,which was however not observed in 1H –NMR spectrum.



(**24**)

Figure4.1: Structure of compound**(24)**

The ^{13}C NMR spectrum of compound (**24)** showed 18 carbon resonances in the molecule, nine being typical for umbeliferone skeleton and the other nine signals were ascribed to a prenyl group. The DEPT experiments classified the carbon signals to eight methines, including five for umbeliferone moiety at C-3(δ 114.3), C-4 (δ 145.6), C-5(δ 121.9), C-6 (δ 116.3) and C-7(δ 114.9) and the other three were C-1' (δ 71.5) , C-3'(δ 125.8) and C- 4'(δ 132.0). One signal for methylene was attributed to C-7' (δ 37.6). The C-2 lactone carbon appeared at δ 165.9. The C-3and C-4 showed resonance at δ 114.3 and δ 145.6 respectively. The down field chemical shift that was noted on the C-4 was due to the resonance of the lactone carbon.The C-5 appeared at δ 121.9 while C-6 appeared at δ 116.3. The C-7 appeared at δ 114.9 and the C-8 appeared at δ 146.1. The down field chemical shift of C-8 showed the resonance of oxygen.The C-5' appeared at δ 174.1 which is associated with chemical shift of methyl estersand C-8' appeared at δ 207.8 which is associated with ketone (Erno *et al*., 2008).

^{1}H-^{13}C connectivities were established through HSQC spectrum. The H-3 (δ6.13) showed cross peak with C-3(δ114.3), while H-4 (δ7.38) showed cross peak with C-4 (δ145.6). The H-7(δ7.03) showed cross peak withC-7signal at δ 114.9, while 5-H(δ 6.96) and H-6(δ 6.76) showed cross peak with C-5 and C-6 signal at δ 121.9 and δ 116.3, respectively .

The HMBC spectraldata showed correlations between H-3 (δ 6.13) and the carbonyl at C-2 (δ 161.8). The doublet signal of H-4 (δ 7.38) showed correlation with C-2 (δ 161.8), C-3(δ 114.3)and C-5 (δ 121.9).The doublet signal of H-7(δ 7.03) also showed correlation with C-4 (δ 145.6), C-8(δ 146.1), C-5(δ 121.9) and C-8a (δ 148.9). The doublet doubletsignal of H-5(δ 6.96) also showed correlation with C-4(δ 145.6), C-6 (δ 116.3), C-8(δ 146.1), and C-7 (114.9) (Silva *et al*., 2012); (Metin, 2005). And the doublet signal of H-6 (δ 6.76) showed correlation with C-8 (δ 146.1) and C-8a(δ 148.9). The quartet signal of H-1'(δ 5.01) showed correlation with C-1'(δ 71.5); the triplet signal of H-3'(δ 125.8)showed correlation with C-2'(δ 73.5) and C-5'(δ 174.1). The singlet signal of H-6' (δ 3.56) exhibited correlation with C-5' (174.1),while the singlet of H-9' (δ 2.08) also showed correlation with C-8' (δ 207.5).

^{1}H-^{1}H Cosy spectrum connectivitie showed correlation of proton with signal at δ 6.13(H-3) and that of δ 7.38 (H-4) and vice versa. Similarly, proton signal at δ6.76 (H-6) showed correlation with proton at δ 6.96 (H-5). There was a Meta coupling between the proton with signal δ 6.96 (H-5) with acoupling constant J= 2.06Hz and δ 7.03 (H-7) with coupling constant J=2.06Hz.

From structural elucidation and comparing with the literature data, indicated that compound (**24**) has never been isolated and termed as new compound.

Table 4.2: Summary of 1D and 2D NMR data values for compound**(24)**

Carbon No.	^{13}C(ppm)	^{1}H(ppm)	DEPT	COSY	HMBC
2	165.9	-	Cq	-	-
3	114.3	6.13(d,J=15.9)	CH	4	2
4	145.6	7.38(d,J=15.7)	CH	3	2,3,5
4a	115.8	-	Cq	-	-
5	121.9	6.96(d,d, J=2.06, 8.2)	CH	6,7	4,6,7,8,8a
6	116.3	6.76(d, J=8.2)	CH	5	8, 8a
7	114.9	7.03(d,J =2.1)	CH	5	5,8,8a
8	146.9		Cq	-	-
8a	148.9	-	Cq	-	-
1’	71.5	5.01 , q	CH	-	2’,
2’	73.5	3.17 , brd	CH	-	-
3’	125.8	5.77, t	CH	-	2’,5’
4’	132.0	5.39 , d,d	CH	-	-
5’	174.1	-	Cq	-	-
6’-OCH_3	52.3	3.56 ,s	-	-	5’
7’	37.6	1.76 , d	CH	-	-
8’	207.5	-	Cq	-	-
9’	31.1	2.08 , s	CH_3	-	8’

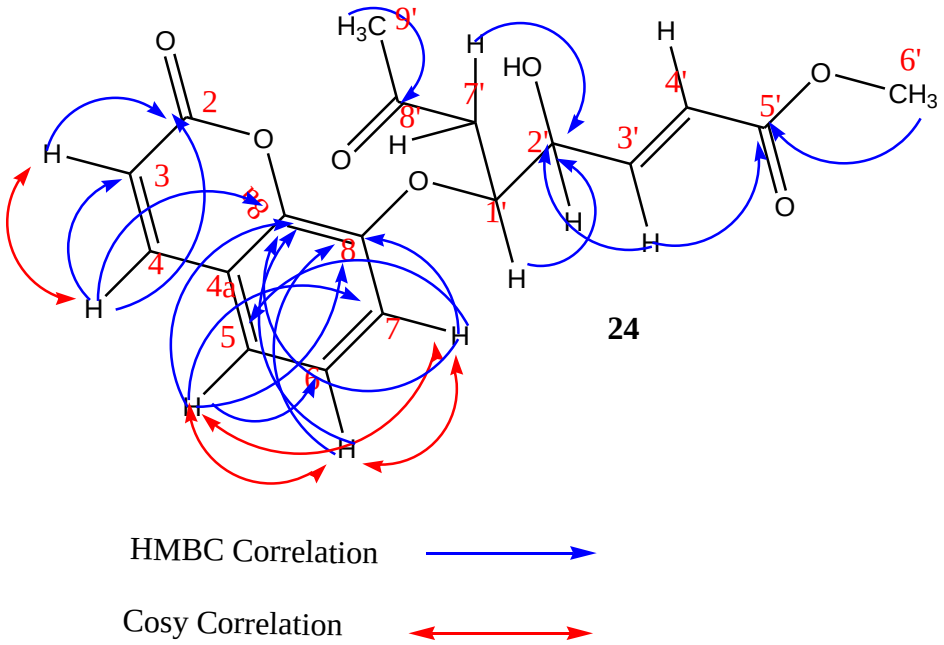


Figure 4.2: Structure of compound (24) showing COSY and HMBC correlations

Compound (**24)** was identified with molecular ion peak m/z [M+Na-2H] ⁻ 367.04 calculated for [$C_{18}H_{18}O_7$ + Na-2H] ⁻367.329, using high resolution negative electron impact mass spectrometry (HREIMS) at 0.24 minutes retention time. The compound was identified as (*E*)-methyl-4- hydroxyl -7- oxo-5- (2-oxo-2H-Chromen -8-yloxy) oct-2-enoate with a molecular formula $C_{18}H_{18}O_7$. The mass spectrum of the compound (**24**) isas shown in Figure 14whereas the structure of compound (**24)** is shown in Figure12.

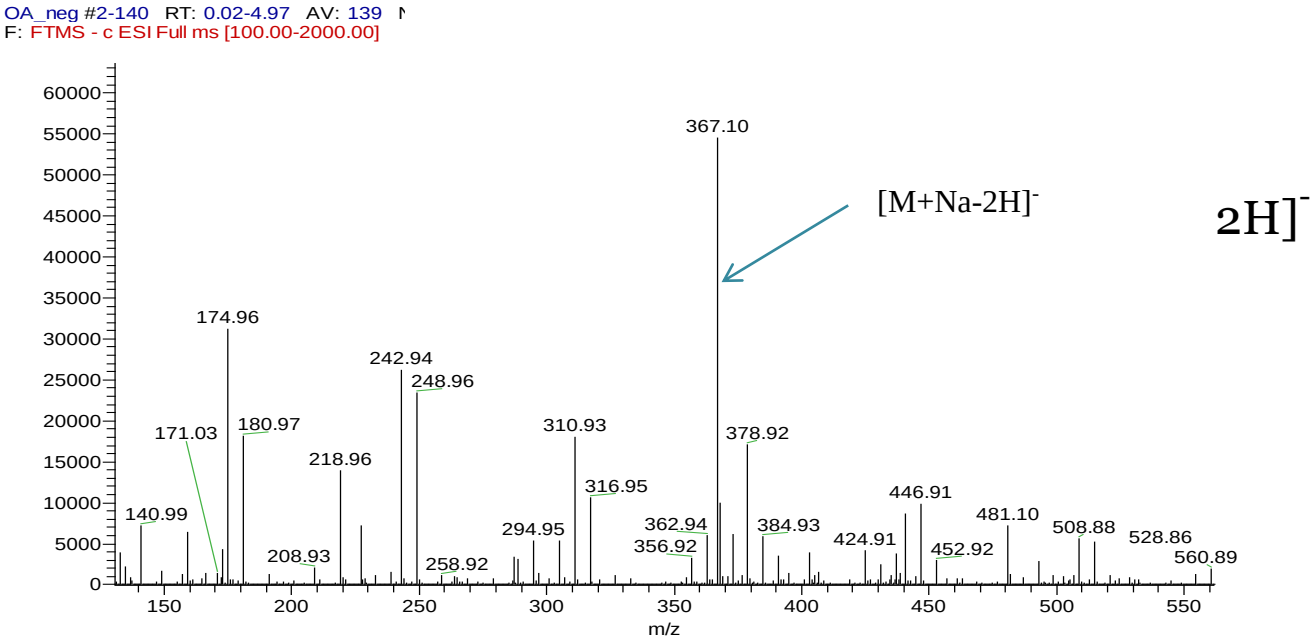


Figure 9.3: Mass spectrum of compound**(24)**

Compound(**23)**and (**24)** were confirmed to be in a class called the Coumarins by comparing the spectras obtained with those in literature.The two compounds were confirmed to be actual compounds by comparing their spectroscopic data and their mass spectrometric data with that from the literature (Kupranova, 1997;Chun-ching et al., 2010; Seyed *et al*., 2010. A number of coumarin compounds have been isolated from varied family of plants (Iyer *et al*.,2014; Golfakhrabadi *et al*.,2014; Jaraslaw *et al*.,2009;Venkateswara *et al*., 2009 and Ashraf *et al*., 2006). Compound (**23)** was identified as 8-hydroxyl–chromen-2-one. The present study reports the isolation of Coumarins for the first time from the genus *Acokanthera*and species*schimperi*.A new coumarin derivative identified as (E)-methyl-4- hydroxyl -7- oxo-5- (2-oxo-2H-Chromen -8-yloxy) oct-2- enoate (**24)**is being reported for the first time.Similar compounds have been isolated from the seeds of *Zosiman absinthifolia* including imperatorin, 7-prenyloxy coumarin and aurapten (Seyed *et al*., 2010). Apart from its activity against *Rhipicephalus appendiculatus*, investigation of Coumarin compounds revealed awide spectrum of medicinal plant extracts,subsequent analysis of scientific literature revealed numerous reports of anti-proliferative (Mirunalini and Krishnaveni, 2011) and anti tumor activities of varied Coumarin compounds, for example both Coumarin and 7- hydroxyl coumarin have been reported to inhibit the proliferation of anumber of human malignant cell lines in vitro against several types of animal tumor.Most of the coumarin compounds isolated have been majorly used as antihyperlipidermic antitumor activities (Iyer *et al*.,2014), anticoagulant (Golfakhrabadi *et al*.,2014), antibacterial activities (Jaraslaw *et al*.,2009; Ashraf *et al*.,2006), allelopatic activity (Seyed *et al*., 2010),acetylcholinesterase activity inhibitoryactivity (Young*et al*.,2001). Coumarin dyes are widely employed in chemistry, medicine andengineering aseffective lasermedia generating radiation in the greenish–blue region. Therefore these compounds are important objects of investigation (Kupravanova, 1997).

4.2 Acaricidal activity of *A. schimperi* from methanol crude extract

The preliminary screening of methanol crude extract of*A. schimperi* against *R. appendiculatus* resulted in mean larval mortalities shown in Table 4.3 shows detailed larval bio assay results at 50mg/ml. The mean larval mortalities (%) that was observed at different concentrations were in the increasing order from 10 to 70% at 48 hour of exposure. No mortalities were observed within 12 and 24 hours, the larvae only had knock down effects. No mortalities were observed in the negative control within 48hours while in the positive control (Amitraz) 100 % mortality was observed at 48hours. After 48hours where there was significant mortality, the $LC_{50}$was

42.26mg/ml and LC_{90} was 79.14 mg/ml. The larvae in the positive control displayed tragic knock down effect within 12 hours through the 48hour where mortality was at 100%. This indicated the possibilityof activity in the plant.

Table 4.3: Screening results of methanol, hexane, and ethyl acetate and aqueous of *A. Schimperi* extracts

Extracts at 50 mg/ml	**Test**	**No of larvae per petri dish**	**No of larvae ticks dead at the hour indicated**			
			6hours	**12hours**	**24hours**	**48hours**
Methanol extract	1	10	0	1	2	9
	2	10	0 (0%)	0 (3%)	0 (7%)	7 (80%)
	3	10	0	0	0	8
Hexane extract	1	10	0	2	5	8
	2	10	0 (0%)	3 (20%)	7 (60%)	9 (83%)
	3	10	0	1	6	8
Ethyl acetate extract	1	10	0	2	3	8
	2	10	0 (0%)	0 (10%)	1 (20%)	5 (67%)
	3	10	0	1	2	7
Aqueous extract	1	10	0	1	1	5
	2	10	0 (0%)	1 (7%)	3 (20%)	5 (37%)
	3	10	0	0	2	4
FA11	1	10	0	1	3	4
	2	10	0 (0%)	1 (7%)	3 (27%)	4 (47%)
	3	10	0	0	2	3
FA21	1	10	0	2	6	8
	2	10	0 (0%)	3 (23%)	7 (60%)	8 (80%)
	3	10	0	2	5	8
FA30	1	10	0	1	5	7
	2	10	0 (0%)	3 (20%)	7 (57%)	8 (80%)
	3	10	0	2	5	7
Amitraz (0.125mg/ml)[1]	1	10	0	10	10	10
	2	10	0 (0%)	9 (90%)	10 (97%)	10 (100%)
	3	10	0	8	9	10
(Distilled water+ DMSO)[2]	1	10	0	0	0	0
	2	10	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	3	10	0	0	0	0

[1]Positive control; [2] Negative control

Table 4.4: Acaricidal activity of *A. Schimperi* from methanol crude extract after 48 hours

Concentration (mg/ml)	**% Larval mortality ±SD after 12 hours**	**% Larval mortality±SD after 24 hours**	**% Larval mortality±SD after 48 hours**
20	0.0*	0.0*	10±5.8
25	0.0*	0.0*	13.3±5.8
30	0.0*	0.0*	23.3±5.8
35	0.0*	0.0*	27.0±5.8
40	0.0*	0.0*	47.0±5.8
45	0.0*	0.0*	53.0±5.8
50	0.0*	0.0*	70.0±10.0
Amitraz(0.125mg/ml)[1]	50.0±0.0	100±0.0	100±0.0
(Distilled water+DMSO)[2]	0.0*	0.0*	0.0*
LC_{50}	-	-	42.26(36.07-58.62)
LC_{90}	-	-	79.14(57.57-243.58)

[1]Positive control, [2]Negative control * No response

4.2.1 Acaricidal activity of *A. schimperi* hexane crude extract

Hexane crude extract obtained from partitioningthe methanol crude extracts of *A. schimperi* resulted in mean larval mortalities displayed in Table 4.4. At 48hours of exposure, the highest concentration of hexane extract had killed more than 70% of the larvae. The behavioralobservation in larvae coming into contact with the extract was similar to what was observed with methanol crude extract.The $LC_{50}$was 36.49mg/ml while the LC_{90} was 79.14mg/ml. The LC value generated from probit regression analysis of bioassay within 48hours is shown in appendix 2.

Table 4.5: Acaricidal activity of *A. Schimperi* from hexane crude extract after 48 hours

Concentration (mg/ml)	**% Larval mortality ± SD after 12 hours**	**% Larval mortality±SD after 24 hours**	**% Larval mortality±SD after 48 hours**
20	0.0*	0.0*	0.0±0.0
25	0.0*	0.0*	10.0±5.8
30	0.0*	0.0*	43.3±5.8
35	0.0*	0.0*	56.7±5.8
40	0.0*	0.0*	60.0±5.8
45	0.0*	0.0*	63.3±5.8
50	0.0*	0.0*	76.7±5.8
Amitraz(0.125mg/ml)[1]	100±0.0	100±0.0	100±0.0
(Distilled water+DMSO)[2]	0.0	0.0±0.0	0.0±0.0
LC_{50}	-	-	36.49(32.01-42.13)
LC_{90}	-	-	58.34(48.37-93.42)

[1]Positive control,[2]Negative control * No response

4.2.2 Acaricidal activity of ethyl acetate crude extract

Ethyl acetate extract obtained from partitioning the methanol crude extracts of *A. schimperi* resulted in mean larval mortalities displayed in Table 4.5. At 48hours of exposure, the highest concentration of ethyl acetate extract had killed more than 70% of the larvae. Similar behavioral observation in larvae coming into contact with the extract was also observed as was the case of methanol and hexane crude extracts. The LC_{50} was 47.11mg/ml while LC_{90} was 69.48mg/ml. The LC value generated from probit regression analysis of bioassay within 48hours is shown in appendices 3 and 4 .

Table 4.6: Acaricidal activity of ethyl acetate crude extract after 48 hours (Appendix3)

Concentration (mg/ml)	**% Larval mortality ± SD after 12 hours**	**% Larval mortality±SD after 24 hours**	**% Larval mortality±SD after 48 hours**
20	0.0*	0.0*	0.0*
25	0.0*	0.0*	3.0±5.8
30	0.0*	3.0±5.8	10.0±5.8
35	0.0*	6.0±5.8	13.0±0.0
40	0.0*	10.0±10.0	20.0±5.8
45	0.0*	13.0±5.8	50.0±5.8
50	0.0*	20.0±0.0	60.0±5.8
Amitraz(0.125mg/ml)[1]	100±0.0	100±0.0	100±0.0
(Distilled water+DMSO)[2]	0.0±0.0	0.0±0.0	0.0±0.0
LC_{50}	-	48.58(42.80-68.16)	47.11(41.87-61.99)
LC_{90}	-	72.68(57.04-178.61)	69.48(55.72-147.31)

[1]Positive control,[2]Negative control * No response

From table 4.4, it is evident that methanol crude extract is active against *R. appendiculatus* larvae. There is a positive correlation between the percentage mortality and the crude extract concentrations. Thus percentage mortality values depend on the concentration of the crude extract. Seventy percent larval mortality was achieved at a concentration of 50 mg/ml. The lowest concentration 20mg/ml that was used gave a percent mortality of 10% larval tick mortality. According to the log probit analysis (Table3 and appendix1) the crude extract had an LC_{50} value of 42.26 mg/ml and LC_{90} value of 79.14 mg/ml at 95% confidence limit. Going by the fact that the crude extract gave a positive result on the acaricidal assay, it is therefore presumed that there are compounds in the methanol crude extract that were responsible for the acaricidal activity against *R. appendiculatus*. This formed the basis of further fractionation of methanol crude extract. According to the observed mortality in methanol crude extract, it is found to compare well with studies already done on the same species (Apocynacae). For instance it has been shown that the same crude extract from *Acokanthera schimperi* induce acaricidal effect against *R. appendiculatus* (Mark *et al*., 2008).

In previous studies, on methanol extracts of *G. surperba* and*P. embilica* showed the activity against *H. bispinosa* tick with LC_{50} 225.27 and 256.08 ppm respectively(Bagavana *et al*., 2009).

Methanol extracts of different plants have been studied previously for their acaricidal activity. In previous studies, on methanol extracts of *G. communis* demonstrated acaricidalactivity against thelarvae of R. microplus tick with LC_{50} of 181.49 ppm and LC_{90} 1,829.94 ppm respectively(Zahir et al., 2009). Cardiac glycosides that were isolated from *Calotropies procera* have been shown to be potent against Camel tick *Hyalomma drometarii*that was shown byits lower LC95 value of 2539mg/ml compared to Azadirachtin and the neem oil which both had LC95 of above 500mg/l(Al- Rahg *et al* ., 2003).

Apart from being polar, methanol is known as a broad spectrum solvent which extracts all compounds including possible glycoside compounds. All these possible molecules present in the methanol extract may have worked synergistically (Akn *et al.,* 2010) or working individually causing larval mortalities and the observed knock down effects.

Both hexane and ethyl acetate crude fractions were found to be active to different extents against the larvae of *R. appendiculatus*. Referringto Tables 4 and 5, it is evident that the percentage mortalities of hexane and ethyl acetate crude extracts are concentration defendant. Hexane crude extract had 76.7% mortality at 50mg/ml while ethyl acetate crude extract had60% mortality at the same concentration.The LC_{50} values for hexane and ethyl acetate crudeextracts were 36.49mg/ml and 47.11mg/ml respectively. The LC_{90} valuesfor the same extractswere 58.34mg/ml and 69.48mg/ml respectivelyHexane crude extract had lower LC values than ethyl acetate crude extract however,the ethyl acetate crude extract was subjected to further fractionation and purification. Following the preliminary separation and purification, hexanecrude extract could not give clear TLC plate separation and development, therefore isolation and purification was notpossible with the humble apparatus available in the University at the time.

4.2.3 Acaricidal assay of fractions from Ethyl acetate Extracts

The ethyl acetate crude extract was subjected to further bioactive guided fractionation, leading to three fractions namely FA11, FA21 and, FA30. All the fractions were subjected to acaricidalassays against *R. appendiculatus* larvaein triplicates and the data obtained istabulated in tables 7 and8.The LC_{50} and LC_{90} values for each of the three fractions were thensubjected to a Regressionanalysis and the generatedprobit transformed mortalities were plotted against the log of the extracts dose to determine LC_{50} and LC_{90}. The LC values for FA11 could not be determined due to its low larvicidal activity.

Table4.7: Acaricidal activity of *A. Schimperi*fraction FA30 crude extract after 48 hours

Concentration (mg/ml)	**% Larval mortality ±SD after 12 hours**	**% Larval mortality±SD after 24 hours**	**% Larval mortality±SD after 48 hours**
20	0.0*	10.0± 5.8	36.0±0.0
25	0.0*	13.3±5.8	43.0±5.8
30	0.0*	26.7±5.8	47.0±5.8
35	0.0*	23.3±5.8	50.0±0.0
40	0.0*	26.7±5.8	60.0±5.8
45	0.0*	36.7±5.8	70.0±5.8
50	0.0*	63.3±0.0	80.0±5.8
Amitraz(0.125mg/ml)[1]	100±0.0	90.0±0.0	100±0.0
(Distilled water +DMSO)[2]	0.0*	0.0*	0.0*
LC_{50}	-	50.70(40.58-141.48)	29.85(12.73-40.46)
LC_{90}	-	112.46(67.69-2674.25)	87.65(53.99-21302.7)

[1]Positive control,[2]Negative control * No response

Table 4.8: Acaricidal assay result for fraction FA21 after 24hours

Concentration (mg/ml)	**% Larval mortality±SD after 12 hours**	**% Larval mortality±SD after 24 hours**	**% Larval mortality±SD after 48 hours**
20	0.0*	0.0*	16.7.0±5.8
25	0.0*	3.0±5.8	43.3±5.8
30	0.0*	10.0±5.8	46.7±5.8
35	0.0*	13.3±5.8	53.3±5.8
40	0.0*	20.0±5.8	46.7±5.8
45	0.0*	30.0±5.8	73.3±5.8
50	0.0*	67.0±5.8	83.3±5.8
Amitraz(0.125mg/ml)[1]	100±0.0	90.0±10.0	100±0.0
(Distilled water +DMSO)[2]	0.0*	0.0*	0.0*
LC_{50}	-	48.58(42.80-68.16)	31.94(24.62-39.08)
LC_{90}	-	72.68(57.04-178.61)	66.93(49.58-204.37)

[1]Positive control,[2]Negative control * No response

4.2.4 Bioassay of FA21a, FA21b$_1$ and FA21b$_2$fractions from*A. schimperi*

Following the bio assay guided fractionation and purification procedures discussed in section3.5.3, three fractions were isolated namely FA21a,FA21b$_1$ andFA21b$_2$. Each of the three fraction was subjected to acaricidal tests against the larvae of *Rhipicephalus appendiculatus*. A triplicate test for each concentration during the experiment was set and4% v/vDMSO in water, whichactually showed no activity, was designed as negative control. Their respective activity results are then reported in tables 9-11(see also appendix 9-15)

Table 4.9 : Acaricidal assay result for FA21a after 48 hours

Concentration (mg/ml)	**% Larval mortality ± SD after 12 hours**	**% Larval mortality± SD after 24 hours**	**% Larval mortality± SD after 48 hours**
20	0.0*	0.0*	0.0
25	0.0*	0.0*	7.0±5.8
30	0.0*	0.0*	10.0±0.0
35	0.0*	0.0*	16.0±5.8
40	0.0*	0.0*	20.0±5.8
45	0.0*	0.0*	23.0±0.0
50	0.0*	0.0*	33.3±5.8
Amitraz (0.125mg/ml)[1]	100±0.0	100±0.0	100±0.0
(Distilled water+DMSO)[2]	0.0*	0.0*	0.0*
LC_{50}	-	-	5.88(4.53-122.52)
LC_{90}	-	-	11.1(6.56-13699.35)

[1]Positive control, [2]Negative control * No response

Table4.10: Acaricidal assay result for FA21b1 after 48 hours

Concentration (mg/ml)	**% Larval mortality ± SD after 12 hours**	**% Larval mortality± SD after 24 hours**	**% Larval mortality± SD after 48 hours**
20	0.0*	0.0*	0.0*
25	0.0*	0.0*	7.0±5.8
30	10.0±0.0	10.0±5.8	23.0±5.8
35	13.3±5.8	13.0±5.8	27.0±5.8
40	16.0±5.8	23.00±5.8	37.0±5.8
45	20.0±5.8	26.0±5.8	40.0±0.0
50	33.3±5.8	40.0±5.8	67.0±5.8
Amitraz(0.125mg/ml)[1]	100±0.0	100±0.0	100±0.0
(Distilled water+DMSO)[2]	0.0*	0.0*	0.0*
LC_{50}	5.86 (4.82-29.71)	5.33(4.61-10.45)	4.53(4.07-5.72)
LC_{90}	8.67(6.34-362.27)	7.94(5.95-40.28)	6.72(5.45-13.40)

[1]Positive control, [2]Negative control * No response

Table 4.11: Acaricidal assay result for FA21b2 after 48hours

Concentration (mg/ml)	**% Larval mortality±SD after 12 hours**	**% Larval mortality±SD after 24 hours**	**% Larval mortality±SD after 48 hours**
20	10.0±0.0	14.0±5.8	23.0±5.8
25	13.3±5.8	23.3±5.8	37.0±5.8
30	23.3±5.8	40.0±10.0	57.0±5.8
35	30.0±10.0	50.0±5.8	60.0±5.8
40	37.0±5.8	57.0±5.8	67.0±5.8
45	63.3±15.3	73.3±5.8	80.0±0.0
50	70.0±0.0	83.3±5.8	90.0±5.8
Amitraz(0.125mg/ml)[1]	100±0.0	100±0.0	100±0.0
(Distilled water+DMSO)[2]	0.0*	0.0*	0.0*
LC_{50}	4.39(3.70-6.12)	3.53(2.93-4.29)	2.96(2.22-3.55)
LC_{90}	8.67(6.67-26.45)	6.85(5.25-14.63)	6.09(4.70-13.32)

[1]Positive control, [2]Negative control

The three isolated fractions obtainedfrom *A. schimperi* leaves showed acaricidal activity after 48hours of exposure. The highest acaricidal mortality was noted in compound $FA21b_2$ against the larvae of *R. appendiculatus* withLC_{50}(2.96mg/ml) and LC_{90}(6.09mg/ml). And the least larval mortality was noted in compound FA21awithLC_{50}(5.88mg/ml) and (LC_{90} 11.19mg/ml).From tables 4.4-4.10 it is evident that the purified compounds are more active against *R. appendiculatus* than the crude extracts (Methanol, ethyl acetate andhexane extracts). This could be attributed to the fact that the purified compounds had more concentration of the active compounds than it is in the unpurified grades. Also, the LC values for $FA21b_2$ was noted to be high at 12hour duration, LC_{50} 4.39 mg/ml LC_{90} 8.67mg/ml (see table 13 and appendix 11). This attests to the fact that fraction$FA21b_2$ indeed had very active compounds. In reference to Figure 3.2, it is evident that fraction FA21b2 resulted into two compounds (**23)** and (**24)**and so the fraction is a mixture of two compounds as already stated. In regards to the findings so far discussed it is therefore possible to concludethat the activity was due to either of compound(**23)** and (**24)** or both.

Crude ethanolic extract (CEE) of the stem of peel of *sapindus saponoria* has been evaluated against *Rhipicephalus sanguineus* and showed larvicidal potential . The CEE of *S. saponria* gave

anLC_{50} value of 1994 ppm and an LC_{99} value of 3922ppm (Fernades *et al*., 2007). The larvicidal activity of plant extracts of *Aloe pirottae*and *Acokanthera schimperi* leaves gave 100% mortality at 160ppm and 480 ppm respectively against *Anopheles arabiensis*fourth instar (Damtew *et al*, 2014).

Other literature also indicates that *Acokanthera schimperi* at 5mg/ml produced 63% mortality to *B. decoloratus* larvae and 53% on *R. appendiculatus*, while 1mg/ml produced 33% and 7% mortality*to Psiadia punctulata* and 5mg/ml produced 90% mortality to larvae of *B. decoloratus* and 60% mortality to*R. Appendiculatus*(Mark *et al.*, 2008). Extracts of *A. schimperi*demonstrated larvididal activity by producing LC_{50} 4.50 mg/ml and LC_{99}8.84 mg/ml on *R. appendiculatus* while LC_{50} 2.78 mg/ml andLC_{99}8.945 mg/ml,*B.decoloratus*respectively(Mark *et al.*, 2008). As indicated in tables 3- 15,sthe study compares well with some other work already done, indicating presence of activity of the plant against larvae.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

Methanol, hexane and ethyl acetate crude extracts from *A. schimperi* leaves were active against*R. appendiculatus* larvae.Hexane extract had LC_{50} 36.47 mg/ml and LC_{90} 58.34 mg/ml.The methanol extract had LC_{50} and LC_{90} of 42.26mg/ml and 79.14mg/mlrespectively.While ethyl acetate had LC_{50} 47.11 mg/ml andLC_{90} 69.48mg/ml.

Sepaartion of ethyl acetate crude extract over silica gel column chromatography yielded three fractions FA21, FA30 and FA11 and two were active against *R. appendiculatus*larvae. FractionFA11which was inactive.

Fractionation of $FA21b_2$ from *A. schimperi*yielded two new compounds which were successfully identified through analysis of their MS, NMR, HREIMS as well as making reference to literature data. Compound 8-hydroxy-2H -chromen -2- one **(23)** and (E)-methyl-4- hydroxyl -7- oxo-5- (2-oxo-2H-chromen -8-yloxy) oct-2- enoate (**24)**are coumarin derivatives. Themixture of **(23)** and (**24)**was active against *R. appendiculatus* larvae and registered LC_{50} and LC_{90} of 2.96 mg/ml and 6.09 mg/ml respectively.

Results from this study indicate that, the two isolated naturally occurring acaricidal compounds may have potential application in the control of ticks. Such findings avail an opportunity for developing newer and more selective biodegradable and natural acaricidal compounds more potent against *R. apendiculatus.*The two new compounds isolated from the plant will add value to the agricultural sector as it will complement the already existing acaricides.

5.2 Recommendations

1. The toxicity tests towards the non-targetorganisms and field test evaluation should be carried out for the two isolated compounds.
2. Further advanced methods of purification such as HPLCneed to be used on the unidentified compounds of the compounds which showed acaricidal activity.
3. Further acaricidal bio assayshould be done on the separate isolated compounds**.**
4. The plant need to be safe guarded from over exploitation from the masses

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APPENDICES

Appendix 1 : Generated LC values for *A. Schimperi* methanol crude extract at 48 hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	13.529	3.451	19.762	1.131	.538	1.296
	.020	15.460	4.727	21.577	1.189	.675	1.334
	.030	16.827	5.768	22.827	1.226	.761	1.358
	.040	17.933	6.696	23.824	1.254	.826	1.377
	.050	18.887	7.558	24.676	1.276	.878	1.392
	.060	19.739	8.376	25.432	1.295	.923	1.405
	.070	20.517	9.164	26.122	1.312	.962	1.417
	.080	21.240	9.929	26.761	1.327	.997	1.428
	.090	21.919	10.678	27.364	1.341	1.028	1.437
	.100	22.564	11.414	27.937	1.353	1.057	1.446
	.150	25.441	14.990	30.545	1.406	1.176	1.485
	.200	27.987	18.495	33.006	1.447	1.267	1.519
	.250	30.373	21.958	35.581	1.482	1.342	1.551
	.300	32.689	25.319	38.510	1.514	1.403	1.586
	.350	34.992	28.465	42.061	1.544	1.454	1.624
	.400	37.328	31.297	46.484	1.572	1.495	1.667
	.450	39.736	33.807	51.958	1.599	1.529	1.716
PROBIT	**.500**	**42.257**	**36.071**	**58.623**	**1.626**	**1.557**	**1.768**
	.550	44.938	38.191	66.654	1.653	1.582	1.824
	.600	47.837	40.261	76.342	1.680	1.605	1.883
	.650	51.030	42.363	88.159	1.708	1.627	1.945
	.700	54.626	44.577	102.874	1.737	1.649	2.012
	.750	58.791	47.000	121.771	1.769	1.672	2.086
	.800	63.804	49.768	147.177	1.805	1.697	2.168
	.850	70.189	53.119	183.836	1.846	1.725	2.264
	.900	**79.139**	**57.570**	**243.574**	**1.898**	**1.760**	**2.387**
	.910	81.466	58.689	260.752	1.911	1.769	2.416
	.920	84.072	59.925	280.808	1.925	1.778	2.448
	.930	87.034	61.310	304.667	1.940	1.788	2.484
	.940	90.465	62.890	333.744	1.956	1.799	2.523
	.950	94.544	64.736	370.343	1.976	1.811	2.569
	.960	99.572	66.966	418.546	1.998	1.826	2.622
	.970	106.121	69.804	486.554	2.026	1.844	2.687
	.980	115.499	73.749	594.481	2.063	1.868	2.774
	.990	131.989	80.395	815.505	2.121	1.905	2.911

a. Logarithm base = 10.

Appendix 2:Generated LC values for *A. Schimperi* hexane crude extract at 48hours Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	15.571	7.253	20.701	1.192	.861	1.316
	.020	17.205	8.746	22.205	1.236	.942	1.346
	.030	18.330	9.844	23.223	1.263	.993	1.366
	.040	19.224	10.759	24.025	1.284	1.032	1.381
	.050	19.983	11.563	24.703	1.301	1.063	1.393
	.060	20.653	12.292	25.298	1.315	1.090	1.403
	.070	21.259	12.968	25.835	1.328	1.113	1.412
	.080	21.817	13.603	26.329	1.339	1.134	1.420
	.090	22.337	14.205	26.789	1.349	1.152	1.428
	.100	22.826	14.782	27.223	1.358	1.170	1.435
	.150	24.969	17.406	29.135	1.397	1.241	1.464
	.200	26.815	19.774	30.821	1.428	1.296	1.489
	.250	28.507	22.004	32.428	1.455	1.342	1.511
	.300	30.117	24.146	34.046	1.479	1.383	1.532
	.350	31.691	26.220	35.750	1.501	1.419	1.553
	.400	33.259	28.227	37.612	1.522	1.451	1.575
	.450	34.851	30.157	39.712	1.542	1.479	1.599
PROBIT	.500	36.492	32.005	42.130	1.562	1.505	1.625
	.550	38.210	33.775	44.947	1.582	1.529	1.653
	.600	40.038	35.491	48.250	1.602	1.550	1.683
	.650	42.020	37.193	52.148	1.623	1.570	1.717
	.700	44.216	38.932	56.804	1.646	1.590	1.754
	.750	46.713	40.778	62.483	1.669	1.610	1.796
	.800	49.661	42.827	69.655	1.696	1.632	1.843
	.850	53.332	45.243	79.241	1.727	1.656	1.899
	.900	58.339	48.368	93.408	1.766	1.685	1.970
	.910	59.618	49.141	97.219	1.775	1.691	1.988
	.920	61.038	49.991	101.546	1.786	1.699	2.007
	.930	62.639	50.937	106.537	1.797	1.707	2.027
	.940	64.477	52.009	112.413	1.809	1.716	2.051
	.950	66.639	53.253	119.527	1.824	1.726	2.077
	.960	69.271	54.744	128.481	1.841	1.738	2.109
	.970	72.651	56.625	140.438	1.861	1.753	2.147
	.980	77.400	59.210	158.114	1.889	1.772	2.199
	.990	85.523	63.496	190.687	1.932	1.803	2.280

a. Logarithm base = 10.

Appendix 3:Generated LC values for ethyl acetate crude extract at 48hrs

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	23.261	11.105	28.921	1.367	1.046	1.461
	.020	25.266	13.411	30.593	1.403	1.127	1.486
	.030	26.627	15.106	31.725	1.425	1.179	1.501
	.040	27.699	16.513	32.620	1.442	1.218	1.513
	.050	28.602	17.746	33.380	1.456	1.249	1.523
	.060	29.395	18.862	34.053	1.468	1.276	1.532
	.070	30.107	19.891	34.666	1.479	1.299	1.540
	.080	30.760	20.853	35.235	1.488	1.319	1.547
	.090	31.366	21.762	35.772	1.496	1.338	1.554
	.100	31.934	22.626	36.284	1.504	1.355	1.560
	.150	34.399	26.472	38.652	1.537	1.423	1.587
	.200	36.493	29.752	40.967	1.562	1.474	1.612
	.250	38.390	32.583	43.466	1.584	1.513	1.638
	.300	40.179	35.004	46.300	1.604	1.544	1.666
	.350	41.910	37.063	49.547	1.622	1.569	1.695
	.400	43.621	38.839	53.232	1.640	1.589	1.726
	.450	45.344	40.417	57.370	1.657	1.607	1.759
PROBIT	**.500**	**47.105**	**41.873**	**61.993**	**1.673**	**1.622**	**1.792**
	.550	48.935	43.263	67.169	1.690	1.636	1.827
	.600	50.868	44.636	73.015	1.706	1.650	1.863
	.650	52.945	46.033	79.711	1.724	1.663	1.902
	.700	55.227	47.496	87.535	1.742	1.677	1.942
	.750	57.799	49.080	96.937	1.762	1.691	1.986
	.800	60.804	50.862	108.693	1.784	1.706	2.036
	.850	64.505	52.977	124.309	1.810	1.724	2.095
	.900	**69.484**	**55.715**	**147.313**	**1.842**	**1.746**	**2.168**
	.910	70.743	56.391	153.497	1.850	1.751	2.186
	.920	72.136	57.132	160.515	1.858	1.757	2.206
	.930	73.701	57.956	168.612	1.867	1.763	2.227
	.940	75.487	58.888	178.146	1.878	1.770	2.251
	.950	77.578	59.964	189.691	1.890	1.778	2.278
	.960	80.109	61.251	204.228	1.904	1.787	2.310
	.970	83.334	62.864	223.655	1.921	1.798	2.350
	.980	87.822	65.067	252.403	1.944	1.813	2.402
	.990	95.392	68.679	305.473	1.980	1.837	2.485

a. Logarithm base = 10.

Appendix 4:Generated LC values for *A. Schimperi* ethyl acetate crude extract at 24hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	23.377	10.306	29.234	1.369	1.013	1.466
	.020	25.469	12.694	30.968	1.406	1.104	1.491
	.030	26.892	14.476	32.147	1.430	1.161	1.507
	.040	28.015	15.972	33.083	1.447	1.203	1.520
	.050	28.962	17.292	33.880	1.462	1.238	1.530
	.060	29.794	18.494	34.590	1.474	1.267	1.539
	.070	30.543	19.608	35.238	1.485	1.292	1.547
	.080	31.230	20.654	35.844	1.495	1.315	1.554
	.090	31.868	21.646	36.418	1.503	1.335	1.561
	.100	32.467	22.591	36.969	1.511	1.354	1.568
	.150	35.068	26.810	39.570	1.545	1.428	1.597
	.200	37.283	30.386	42.223	1.572	1.483	1.626
	.250	39.294	33.406	45.210	1.594	1.524	1.655
	.300	41.192	35.910	48.691	1.615	1.555	1.687
	.350	43.034	37.987	52.719	1.634	1.580	1.722
	.400	44.857	39.759	57.293	1.652	1.599	1.758
	.450	46.694	41.335	62.422	1.669	1.616	1.795
PROBIT	**.500**	**48.575**	**42.796**	**68.156**	**1.686**	**1.631**	**1.834**
	.550	50.533	44.201	74.598	1.704	1.645	1.873
	.600	52.603	45.597	81.911	1.721	1.659	1.913
	.650	54.831	47.025	90.341	1.739	1.672	1.956
	.700	57.282	48.527	100.270	1.758	1.686	2.001
	.750	60.049	50.159	112.310	1.779	1.700	2.050
	.800	63.289	52.000	127.525	1.801	1.716	2.106
	.850	67.286	54.191	147.990	1.828	1.734	2.170
	.900	**72.677**	**57.035**	**178.611**	**1.861**	**1.756**	**2.252**
	.910	74.042	57.737	186.930	1.869	1.761	2.272
	.920	75.554	58.509	196.415	1.878	1.767	2.293
	.930	77.253	59.366	207.409	1.888	1.774	2.317
	.940	79.195	60.336	220.427	1.899	1.781	2.343
	.950	81.470	61.458	236.287	1.911	1.789	2.373
	.960	84.227	62.799	256.400	1.925	1.798	2.409
	.970	87.743	64.482	283.512	1.943	1.809	2.453
	.980	92.646	66.782	324.081	1.967	1.825	2.511
	.990	100.936	70.558	400.220	2.004	1.849	2.602

a. Logarithm base = 10.

Appendix 5: Generated LC values for *A. Schimperi* FA30 fraction at 48hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	4.226	.000	11.089	.626	-4.412	1.045
	.020	5.313	.000	12.580	.725	-3.755	1.100
	.030	6.145	.000	13.633	.789	-3.338	1.135
	.040	6.855	.001	14.486	.836	-3.024	1.161
	.050	7.492	.002	15.222	.875	-2.769	1.182
	.060	8.082	.003	15.880	.907	-2.552	1.201
	.070	8.636	.004	16.481	.936	-2.362	1.217
	.080	9.165	.006	17.042	.962	-2.192	1.232
	.090	9.674	.009	17.570	.986	-2.037	1.245
	.100	10.168	.013	18.072	1.007	-1.894	1.257
	.150	12.494	.050	20.336	1.097	-1.305	1.308
	.200	14.716	.145	22.389	1.168	-.838	1.350
	.250	16.935	.365	24.384	1.229	-.438	1.387
	.300	19.212	.830	26.429	1.284	-.081	1.422
	.350	21.594	1.769	28.648	1.334	.248	1.457
	.400	24.127	3.588	31.247	1.383	.555	1.495
	.450	26.860	6.966	34.704	1.429	.843	1.540
	.500	**29.852**	**12.729**	**40.455**	**1.475**	**1.105**	**1.607**
	.550	33.177	20.504	53.496	1.521	1.312	1.728
	.600	36.936	27.263	86.746	1.567	1.436	1.938
	.650	41.268	31.965	163.696	1.616	1.505	2.214
	.700	46.385	35.730	338.161	1.666	1.553	2.529
	.750	52.621	39.323	758.120	1.721	1.595	2.880
	.800	60.556	43.195	1886.685	1.782	1.635	3.276
	.850	71.329	47.809	5504.457	1.853	1.680	3.741
	.900	**87.646**	**53.994**	**21302.765**	**1.943**	**1.732**	**4.328**
	.910	92.117	55.570	29556.451	1.964	1.745	4.471
	.920	97.233	57.324	42192.433	1.988	1.758	4.625
	.930	103.187	59.304	62415.486	2.014	1.773	4.795
	.940	110.269	61.584	96674.382	2.042	1.789	4.985
	.950	118.941	64.277	159267.907	2.075	1.808	5.202
	.960	130.005	67.573	286399.766	2.114	1.830	5.457
	.970	145.027	71.836	589396.287	2.161	1.856	5.770
	.980	167.716	77.889	1539032.176	2.225	1.891	6.187
	.990	210.897	88.418	6991242.666	2.324	1.947	6.845

a. Logarithm base = 10.

Appendix 6:Generated LCvalues for *A. Schimperi* FA30 at 24hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	11.939	.560	19.521	1.077	-.252	1.291
	.020	14.144	1.053	21.624	1.151	.022	1.335
	.030	15.749	1.570	23.096	1.197	.196	1.364
	.040	17.076	2.120	24.285	1.232	.326	1.385
	.050	18.237	2.705	25.313	1.261	.432	1.403
	.060	19.287	3.326	26.237	1.285	.522	1.419
	.070	20.258	3.984	27.089	1.307	.600	1.433
	.080	21.168	4.682	27.890	1.326	.670	1.445
	.090	22.032	5.419	28.654	1.343	.734	1.457
	.100	22.858	6.195	29.392	1.359	.792	1.468
	.150	26.620	10.692	32.954	1.425	1.029	1.518
	.200	30.047	16.145	36.875	1.478	1.208	1.567
	.250	33.337	22.131	42.193	1.523	1.345	1.625
	.300	36.597	27.666	50.561	1.563	1.442	1.704
	.350	39.901	31.994	63.581	1.601	1.505	1.803
	.400	43.313	35.311	82.192	1.637	1.548	1.915
	.450	46.891	38.074	107.507	1.671	1.581	2.031
PROBIT	**.500**	**50.701**	**40.581**	**141.481**	**1.705**	**1.608**	**2.151**
	.550	54.820	43.000	187.286	1.739	1.633	2.273
	.600	59.348	45.440	249.953	1.773	1.657	2.398
	.650	64.423	47.989	337.668	1.809	1.681	2.528
	.700	70.240	50.739	464.450	1.847	1.705	2.667
	.750	77.109	53.807	656.085	1.887	1.731	2.817
	.800	85.551	57.375	965.015	1.932	1.759	2.985
	.850	96.565	61.765	1514.742	1.985	1.791	3.180
	.900	**112.459**	**67.691**	**2674.245**	**2.051**	**1.831**	**3.427**
	.910	116.675	69.196	3068.228	2.067	1.840	3.487
	.920	121.434	70.865	3562.469	2.084	1.850	3.552
	.930	126.891	72.743	4198.519	2.103	1.862	3.623
	.940	133.277	74.895	5044.339	2.125	1.874	3.703
	.950	140.953	77.421	6219.320	2.149	1.889	3.794
	.960	150.538	80.490	7954.656	2.178	1.906	3.901
	.970	163.219	84.422	10766.211	2.213	1.926	4.032
	.980	181.745	89.934	16101.289	2.259	1.954	4.207
	.990	215.306	99.331	30373.324	2.333	1.997	4.482

a. Logarithm base = 10.

Appendix 7: Generated LC values for *A. Schimperi* FA21 fraction at 48hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	8.336	.882	14.425	.921	-.055	1.159
	.020	9.757	1.325	15.938	.989	.122	1.202
	.030	10.782	1.715	16.984	1.033	.234	1.230
	.040	11.623	2.082	17.820	1.065	.319	1.251
	.050	12.355	2.438	18.534	1.092	.387	1.268
	.060	13.015	2.787	19.166	1.114	.445	1.283
	.070	13.622	3.134	19.741	1.134	.496	1.295
	.080	14.189	3.481	20.273	1.152	.542	1.307
	.090	14.726	3.829	20.771	1.168	.583	1.317
	.100	15.238	4.179	21.242	1.183	.621	1.327
	.150	17.555	6.000	23.341	1.244	.778	1.368
	.200	19.645	7.979	25.214	1.293	.902	1.402
	.250	21.634	10.163	27.011	1.335	1.007	1.432
	.300	23.593	12.586	28.834	1.373	1.100	1.460
	.350	25.565	15.270	30.779	1.408	1.184	1.488
	.400	27.589	18.216	32.978	1.441	1.260	1.518
	.450	29.700	21.375	35.636	1.473	1.330	1.552
PROBIT	**.500**	**31.935**	**24.622**	**39.080**	**1.504**	**1.391**	**1.592**
	.550	34.338	27.772	43.769	1.536	1.444	1.641
	.600	36.964	30.687	50.227	1.568	1.487	1.701
	.650	39.891	33.380	59.016	1.601	1.523	1.771
	.700	43.226	35.978	70.917	1.636	1.556	1.851
	.750	47.139	38.642	87.283	1.673	1.587	1.941
	.800	51.914	41.565	110.723	1.715	1.619	2.044
	.850	58.094	45.025	146.839	1.764	1.653	2.167
	.900	**66.925**	**49.575**	**210.373**	**1.826**	**1.695**	**2.323**
	.910	69.252	50.717	229.566	1.840	1.705	2.361
	.920	71.872	51.980	252.445	1.857	1.716	2.402
	.930	74.867	53.397	280.288	1.874	1.728	2.448
	.940	78.360	55.015	315.084	1.894	1.740	2.498
	.950	82.543	56.909	360.141	1.917	1.755	2.556
	.960	87.743	59.205	421.468	1.943	1.772	2.625
	.970	94.588	62.138	511.491	1.976	1.793	2.709
	.980	104.521	66.239	661.849	2.019	1.821	2.821
	.990	122.335	73.212	994.122	2.088	1.865	2.997

a. Logarithm base = 10.

Appendix 8: Generated LC values for A. Schimperi FA21 fraction at 48hours

Confidence Limits

	Probability	95% Confidence Limits for Conc			95% Confidence Limits for log(Conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	23.377	10.306	29.234	1.369	1.013	1.466
	.020	25.469	12.694	30.968	1.406	1.104	1.491
	.030	26.892	14.476	32.147	1.430	1.161	1.507
	.040	28.015	15.972	33.083	1.447	1.203	1.520
	.050	28.962	17.292	33.880	1.462	1.238	1.530
	.060	29.794	18.494	34.590	1.474	1.267	1.539
	.070	30.543	19.608	35.238	1.485	1.292	1.547
	.080	31.230	20.654	35.844	1.495	1.315	1.554
	.090	31.868	21.646	36.418	1.503	1.335	1.561
	.100	32.467	22.591	36.969	1.511	1.354	1.568
	.150	35.068	26.810	39.570	1.545	1.428	1.597
	.200	37.283	30.386	42.223	1.572	1.483	1.626
	.250	39.294	33.406	45.210	1.594	1.524	1.655
	.300	41.192	35.910	48.691	1.615	1.555	1.687
	.350	43.034	37.987	52.719	1.634	1.580	1.722
	.400	44.857	39.759	57.293	1.652	1.599	1.758
	.450	46.694	41.335	62.422	1.669	1.616	1.795
PROBIT	**.500**	**48.575**	**42.796**	**68.156**	**1.686**	**1.631**	**1.834**
	.550	50.533	44.201	74.598	1.704	1.645	1.873
	.600	52.603	45.597	81.911	1.721	1.659	1.913
	.650	54.831	47.025	90.341	1.739	1.672	1.956
	.700	57.282	48.527	100.270	1.758	1.686	2.001
	.750	60.049	50.159	112.310	1.779	1.700	2.050
	.800	63.289	52.000	127.525	1.801	1.716	2.106
	.850	67.286	54.191	147.990	1.828	1.734	2.170
	.900	**72.677**	**57.035**	**178.611**	**1.861**	**1.756**	**2.252**
	.910	74.042	57.737	186.930	1.869	1.761	2.272
	.920	75.554	58.509	196.415	1.878	1.767	2.293
	.930	77.253	59.366	207.409	1.888	1.774	2.317
	.940	79.195	60.336	220.427	1.899	1.781	2.343
	.950	81.470	61.458	236.287	1.911	1.789	2.373
	.960	84.227	62.799	256.400	1.925	1.798	2.409
	.970	87.743	64.482	283.512	1.943	1.809	2.453
	.980	92.646	66.782	324.081	1.967	1.825	2.511
	.990	100.936	70.558	400.220	2.004	1.849	2.602

a. Logarithm base = 10.

Appendix 9: General LC values for *A. Schimperi* FA21b2 at 48hrs

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	.797	.134	1.353	-.099	-.872	.131
	.020	.929	.189	1.498	-.032	-.725	.175
	.030	1.024	.234	1.598	.010	-.631	.203
	.040	1.102	.275	1.677	.042	-.561	.225
	.050	1.170	.314	1.746	.068	-.504	.242
	.060	1.231	.351	1.806	.090	-.455	.257
	.070	1.287	.387	1.861	.110	-.412	.270
	.080	1.339	.422	1.912	.127	-.374	.281
	.090	1.389	.457	1.959	.143	-.340	.292
	.100	1.436	.492	2.005	.157	-.308	.302
	.150	1.649	.665	2.205	.217	-.177	.343
	.200	1.840	.844	2.382	.265	-.074	.377
	.250	2.022	1.033	2.551	.306	.014	.407
	.300	2.200	1.236	2.719	.343	.092	.434
	.350	2.380	1.455	2.893	.377	.163	.461
	.400	2.564	1.692	3.081	.409	.228	.489
	.450	2.755	1.947	3.293	.440	.289	.518
PROBIT	**.500**	**2.957**	**2.217**	**3.545**	**.471**	**.346**	**.550**
	.550	3.174	2.497	3.858	.502	.397	.586
	.600	3.411	2.778	4.264	.533	.444	.630
	.650	3.674	3.053	4.803	.565	.485	.682
	.700	3.974	3.325	5.525	.599	.522	.742
	.750	4.325	3.602	6.505	.636	.557	.813
	.800	4.752	3.900	7.874	.677	.591	.896
	.850	5.304	4.248	9.911	.725	.628	.996
	.900	**6.090**	**4.700**	**13.324**	**.785**	**.672**	**1.125**
	.910	6.296	4.813	14.321	.799	.682	1.156
	.920	6.529	4.937	15.492	.815	.693	1.190
	.930	6.794	5.077	16.895	.832	.706	1.228
	.940	7.103	5.236	18.616	.851	.719	1.270
	.950	7.473	5.422	20.800	.874	.734	1.318
	.960	7.933	5.648	23.704	.899	.752	1.375
	.970	8.536	5.935	27.844	.931	.773	1.445
	.980	9.410	6.338	34.507	.974	.802	1.538
	.990	10.973	7.022	48.431	1.040	.846	1.685

a. Logarithm base = 10.

Appendix 10: Generated LC values for compound FA21b2 at 24hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	1.060	.288	1.627	.025	-.540	.211
	.020	1.220	.385	1.791	.087	-.415	.253
	.030	1.335	.462	1.903	.125	-.335	.280
	.040	1.428	.531	1.994	.155	-.275	.300
	.050	1.508	.593	2.070	.178	-.227	.316
	.060	1.580	.652	2.138	.199	-.186	.330
	.070	1.646	.709	2.200	.216	-.149	.342
	.080	1.707	.763	2.258	.232	-.117	.354
	.090	1.765	.817	2.311	.247	-.088	.364
	.100	1.820	.869	2.362	.260	-.061	.373
	.150	2.066	1.121	2.589	.315	.050	.413
	.200	2.285	1.368	2.793	.359	.136	.446
	.250	2.491	1.619	2.990	.396	.209	.476
	.300	2.692	1.875	3.192	.430	.273	.504
	.350	2.893	2.136	3.409	.461	.330	.533
	.400	3.097	2.402	3.654	.491	.381	.563
	.450	3.309	2.667	3.941	.520	.426	.596
PROBIT	**.500**	**3.531**	**2.926**	**4.290**	**.548**	**.466**	**.633**
	.550	3.768	3.176	4.722	.576	.502	.674
	.600	4.026	3.417	5.257	.605	.534	.721
	.650	4.310	3.654	5.923	.634	.563	.773
	.700	4.632	3.896	6.761	.666	.591	.830
	.750	5.006	4.155	7.839	.699	.619	.894
	.800	5.458	4.445	9.279	.737	.648	.968
	.850	6.036	4.792	11.335	.781	.681	1.054
	.900	**6.852**	**5.250**	**14.629**	**.836**	**.720**	**1.165**
	.910	7.065	5.365	15.564	.849	.730	1.192
	.920	7.304	5.492	16.651	.864	.740	1.221
	.930	7.577	5.635	17.935	.879	.751	1.254
	.940	7.893	5.798	19.490	.897	.763	1.290
	.950	8.269	5.988	21.433	.917	.777	1.331
	.960	8.734	6.218	23.968	.941	.794	1.380
	.970	9.343	6.512	27.507	.970	.814	1.439
	.980	10.217	6.922	33.043	1.009	.840	1.519
	.990	11.764	7.617	44.143	1.071	.882	1.645

a. Logarithm base = 10.

Appendix 11: Generated LC values for compound FA21b2 at 12 hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	1.279	.325	1.917	.107	-.488	.283
	.020	1.478	.449	2.111	.170	-.347	.325
	.030	1.620	.552	2.246	.209	-.258	.351
	.040	1.736	.644	2.354	.239	-.191	.372
	.050	1.836	.729	2.446	.264	-.137	.389
	.060	1.926	.811	2.529	.285	-.091	.403
	.070	2.008	.889	2.604	.303	-.051	.416
	.080	2.085	.966	2.674	.319	-.015	.427
	.090	2.157	1.041	2.741	.334	.018	.438
	.100	2.226	1.115	2.804	.348	.047	.448
	.150	2.535	1.477	3.092	.404	.169	.490
	.200	2.811	1.833	3.364	.449	.263	.527
	.250	3.072	2.188	3.649	.487	.340	.562
	.300	3.327	2.536	3.970	.522	.404	.599
	.350	3.581	2.867	4.353	.554	.457	.639
	.400	3.841	3.171	4.826	.584	.501	.684
	.450	4.110	3.446	5.408	.614	.537	.733
PROBIT	**.500**	**4.394**	**3.700**	**6.117**	**.643**	**.568**	**.787**
	.550	4.697	3.940	6.974	.672	.595	.844
	.600	5.026	4.176	8.014	.701	.621	.904
	.650	5.390	4.417	9.289	.732	.645	.968
	.700	5.803	4.672	10.886	.764	.670	1.037
	.750	6.284	4.953	12.948	.798	.695	1.112
	.800	6.867	5.276	15.737	.837	.722	1.197
	.850	7.615	5.668	19.788	.882	.753	1.296
	.900	**8.672**	**6.193**	**26.446**	**.938**	**.792**	**1.422**
	.910	8.949	6.325	28.371	.952	.801	1.453
	.920	9.260	6.472	30.624	.967	.811	1.486
	.930	9.614	6.637	33.311	.983	.822	1.523
	.940	10.025	6.825	36.594	1.001	.834	1.563
	.950	10.516	7.046	40.740	1.022	.848	1.610
	.960	11.123	7.313	46.220	1.046	.864	1.665
	.970	11.918	7.654	53.986	1.076	.884	1.732
	.980	13.064	8.131	66.381	1.116	.910	1.822
	.990	15.097	8.940	91.982	1.179	.951	1.964

a. Logarithm base = 10.

Appendix 12: Generated LC values for compoundFA21b1 at 48hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	2.210	1.055	2.763	.344	.023	.441
	.020	2.404	1.272	2.924	.381	.104	.466
	.030	2.536	1.431	3.033	.404	.156	.482
	.040	2.640	1.563	3.119	.422	.194	.494
	.050	2.727	1.679	3.191	.436	.225	.504
	.060	2.804	1.784	3.255	.448	.251	.513
	.070	2.873	1.881	3.313	.458	.274	.520
	.080	2.936	1.972	3.366	.468	.295	.527
	.090	2.995	2.057	3.416	.476	.313	.534
	.100	3.050	2.139	3.464	.484	.330	.540
	.150	3.290	2.507	3.680	.517	.399	.566
	.200	3.494	2.826	3.884	.543	.451	.589
	.250	3.678	3.109	4.098	.566	.493	.613
	.300	3.853	3.358	4.339	.586	.526	.637
	.350	4.021	3.572	4.617	.604	.553	.664
	.400	4.188	3.757	4.939	.622	.575	.694
	.450	4.357	3.920	5.305	.639	.593	.725
PROBIT	**.500**	**4.529**	**4.068**	**5.719**	**.656**	**.609**	**.757**
	.550	4.708	4.209	6.184	.673	.624	.791
	.600	4.897	4.346	6.712	.690	.638	.827
	.650	5.100	4.486	7.316	.708	.652	.864
	.700	5.323	4.631	8.023	.726	.666	.904
	.750	5.576	4.789	8.871	.746	.680	.948
	.800	5.870	4.967	9.930	.769	.696	.997
	.850	6.234	5.177	11.336	.795	.714	1.054
	.900	**6.723**	**5.451**	**13.401**	**.828**	**.736**	**1.127**
	.910	6.847	5.518	13.956	.836	.742	1.145
	.920	6.984	5.592	14.585	.844	.748	1.164
	.930	7.138	5.675	15.310	.854	.754	1.185
	.940	7.314	5.768	16.163	.864	.761	1.209
	.950	7.520	5.875	17.195	.876	.769	1.235
	.960	7.770	6.004	18.494	.890	.778	1.267
	.970	8.088	6.166	20.227	.908	.790	1.306
	.980	8.531	6.387	22.788	.931	.805	1.358
	.990	9.279	6.750	27.505	.967	.829	1.439

a. Logarithm base = 10.

Appendix 13: Generated LC values for compound FA21b1 at24hours

Confidence limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	2.593	.817	3.212	.414	-.088	.507
	.020	2.822	1.089	3.390	.451	.037	.530
	.030	2.977	1.305	3.513	.474	.116	.546
	.040	3.100	1.494	3.611	.491	.174	.558
	.050	3.203	1.666	3.697	.506	.222	.568
	.060	3.294	1.826	3.774	.518	.262	.577
	.070	3.376	1.978	3.846	.528	.296	.585
	.080	3.451	2.123	3.915	.538	.327	.593
	.090	3.520	2.262	3.982	.547	.354	.600
	.100	3.585	2.396	4.049	.555	.379	.607
	.150	3.868	2.991	4.407	.588	.476	.644
	.200	4.109	3.453	4.871	.614	.538	.688
	.250	4.328	3.779	5.486	.636	.577	.739
	.300	4.534	4.011	6.237	.656	.603	.795
	.350	4.734	4.191	7.103	.675	.622	.851
	.400	4.931	4.344	8.085	.693	.638	.908
	.450	5.131	4.481	9.193	.710	.651	.963
PROBIT	**.500**	**5.334**	**4.612**	**10.454**	**.727**	**.664**	**1.019**
	.550	5.546	4.740	11.903	.744	.676	1.076
	.600	5.770	4.869	13.596	.761	.687	1.133
	.650	6.011	5.002	15.610	.779	.699	1.193
	.700	6.276	5.143	18.067	.798	.711	1.257
	.750	6.575	5.297	21.165	.818	.724	1.326
	.800	6.925	5.472	25.256	.840	.738	1.402
	.850	7.356	5.680	31.047	.867	.754	1.492
	.900	**7.937**	**5.950**	**40.276**	**.900**	**.774**	**1.605**
	.910	8.084	6.016	42.892	.908	.779	1.632
	.920	8.247	6.089	45.927	.916	.785	1.662
	.930	8.429	6.171	49.515	.926	.790	1.695
	.940	8.638	6.263	53.856	.936	.797	1.731
	.950	8.883	6.369	59.275	.949	.804	1.773
	.960	9.179	6.496	66.346	.963	.813	1.822
	.970	9.557	6.655	76.208	.980	.823	1.882
	.980	10.084	6.872	91.633	1.004	.837	1.962
	.990	10.973	7.227	122.542	1.040	.859	2.088

a. Logarithm base = 10.

Appendix 14: Generated LC values for compound FA21b1 at 12 hours

Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	2.533	.287	3.231	.404	-.541	.509
	.020	2.795	.488	3.434	.446	-.311	.536
	.030	2.974	.682	3.575	.473	-.166	.553
	.040	3.117	.875	3.692	.494	-.058	.567
	.050	3.239	1.070	3.796	.510	.029	.579
	.060	3.346	1.268	3.894	.524	.103	.590
	.070	3.442	1.469	3.989	.537	.167	.601
	.080	3.531	1.672	4.085	.548	.223	.611
	.090	3.614	1.876	4.184	.558	.273	.622
	.100	3.692	2.080	4.289	.567	.318	.632
	.150	4.033	3.016	5.030	.606	.479	.702
	.200	4.327	3.622	6.387	.636	.559	.805
	.250	4.596	3.962	8.386	.662	.598	.924
	.300	4.851	4.190	10.975	.686	.622	1.040
	.350	5.101	4.371	14.217	.708	.641	1.153
	.400	5.349	4.530	18.259	.728	.656	1.261
	.450	5.602	4.677	23.317	.748	.670	1.368
PROBIT	**.500**	**5.861**	**4.819**	**29.708**	**.768**	**.683**	**1.473**
	.550	6.133	4.960	37.891	.788	.695	1.579
	.600	6.422	5.103	48.555	.808	.708	1.686
	.650	6.735	5.253	62.778	.828	.720	1.798
	.700	7.082	5.412	82.338	.850	.733	1.916
	.750	7.475	5.588	110.385	.874	.747	2.043
	.800	7.940	5.788	153.054	.900	.762	2.185
	.850	8.518	6.027	224.108	.930	.780	2.350
	.900	**9.305**	**6.340**	**362.270**	**.969**	**.802**	**2.559**
	.910	9.506	6.417	406.851	.978	.807	2.609
	.920	9.729	6.502	461.531	.988	.813	2.664
	.930	9.980	6.597	530.187	.999	.819	2.724
	.940	10.269	6.704	619.023	1.012	.826	2.792
	.950	10.608	6.828	738.672	1.026	.834	2.868
	.960	11.020	6.977	909.146	1.042	.844	2.959
	.970	11.550	7.164	1173.611	1.063	.855	3.070
	.980	12.293	7.419	1648.039	1.090	.870	3.217
	.990	13.563	7.839	2814.628	1.132	.894	3.449

a. Logarithm base = 10.

Appendix 15: Generated LC values for compound FA21a at 48hours

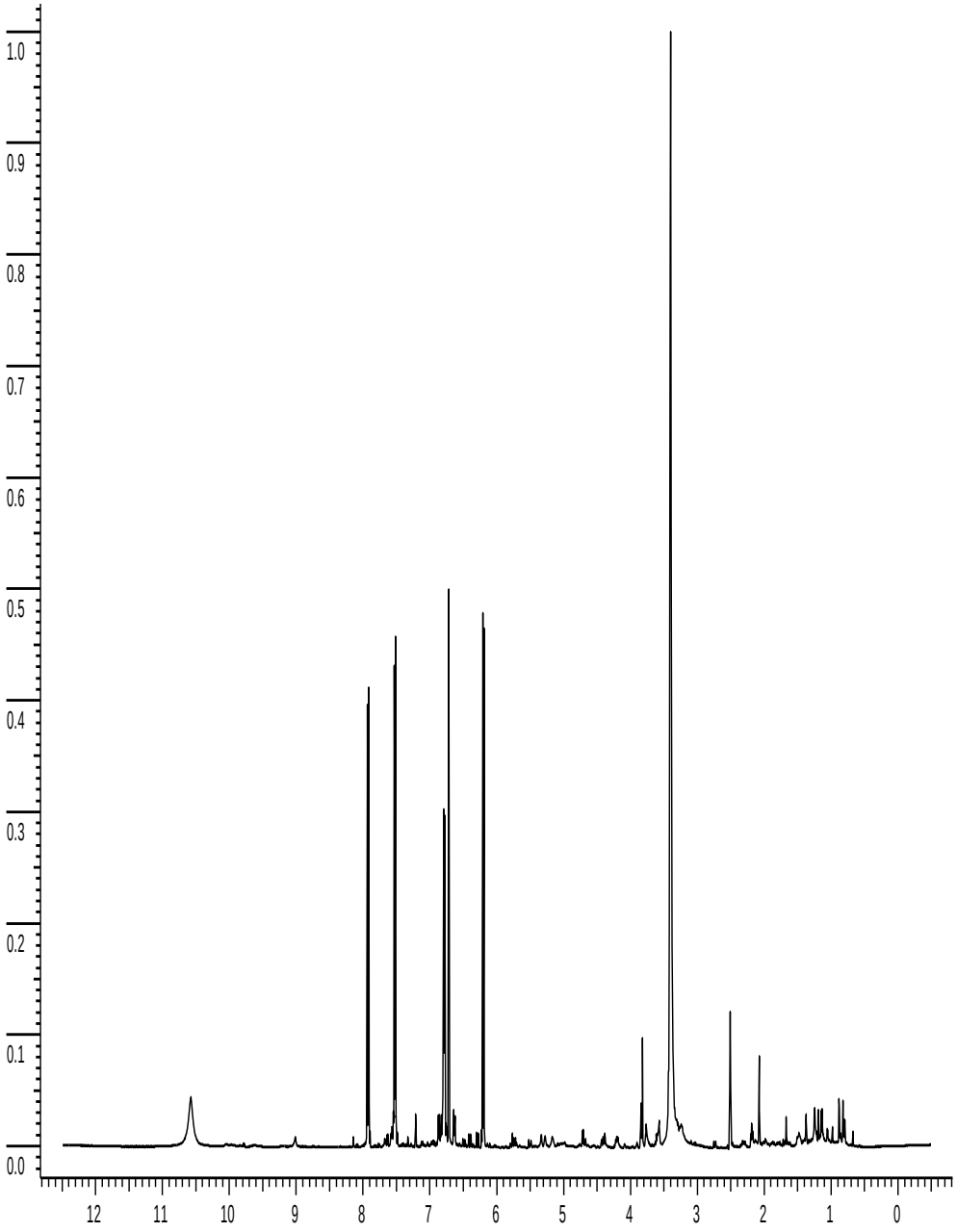
Confidence Limits

	Probability	95% Confidence Limits for conc			95% Confidence Limits for log(conc)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
	.010	1.831	.021	2.605	.263	-1.681	.416
	.020	2.099	.057	2.823	.322	-1.246	.451
	.030	2.290	.107	2.977	.360	-.971	.474
	.040	2.444	.172	3.104	.388	-.765	.492
	.050	2.577	.252	3.216	.411	-.598	.507
	.060	2.696	.349	3.321	.431	-.457	.521
	.070	2.805	.464	3.422	.448	-.334	.534
	.080	2.907	.597	3.523	.463	-.224	.547
	.090	3.002	.748	3.626	.477	-.126	.559
	.100	3.093	.918	3.736	.490	-.037	.572
	.150	3.497	1.994	4.552	.544	.300	.658
	.200	3.856	2.948	6.671	.586	.470	.824
	.250	4.194	3.444	11.085	.623	.537	1.045
	.300	4.522	3.743	18.509	.655	.573	1.267
	.350	4.848	3.971	30.300	.686	.599	1.481
	.400	5.180	4.169	48.730	.714	.620	1.688
	.450	5.523	4.353	77.466	.742	.639	1.889
PROBIT	**.500**	**5.882**	**4.532**	**122.521**	**.770**	**.656**	**2.088**
	.550	6.265	4.711	194.069	.797	.673	2.288
	.600	6.680	4.895	310.011	.825	.690	2.491
	.650	7.137	5.089	503.472	.854	.707	2.702
	.700	7.653	5.299	839.837	.884	.724	2.924
	.750	8.251	5.532	1459.638	.917	.743	3.164
	.800	8.973	5.800	2702.540	.953	.763	3.432
	.850	9.894	6.126	5544.016	.995	.787	3.744
	.900	**11.189**	**6.558**	**13699.348**	**1.049**	**.817**	**4.137**
	.910	11.526	6.667	17046.017	1.062	.824	4.232
	.920	11.904	6.786	21615.022	1.076	.832	4.335
	.930	12.334	6.920	28065.237	1.091	.840	4.448
	.940	12.833	7.073	37570.596	1.108	.850	4.575
	.950	13.426	7.250	52401.517	1.128	.860	4.719
	.960	14.158	7.464	77469.172	1.151	.873	4.889
	.970	15.113	7.736	125281.155	1.179	.889	5.098
	.980	16.483	8.111	237373.611	1.217	.909	5.375
	.990	18.899	8.738	650067.417	1.276	.941	5.813

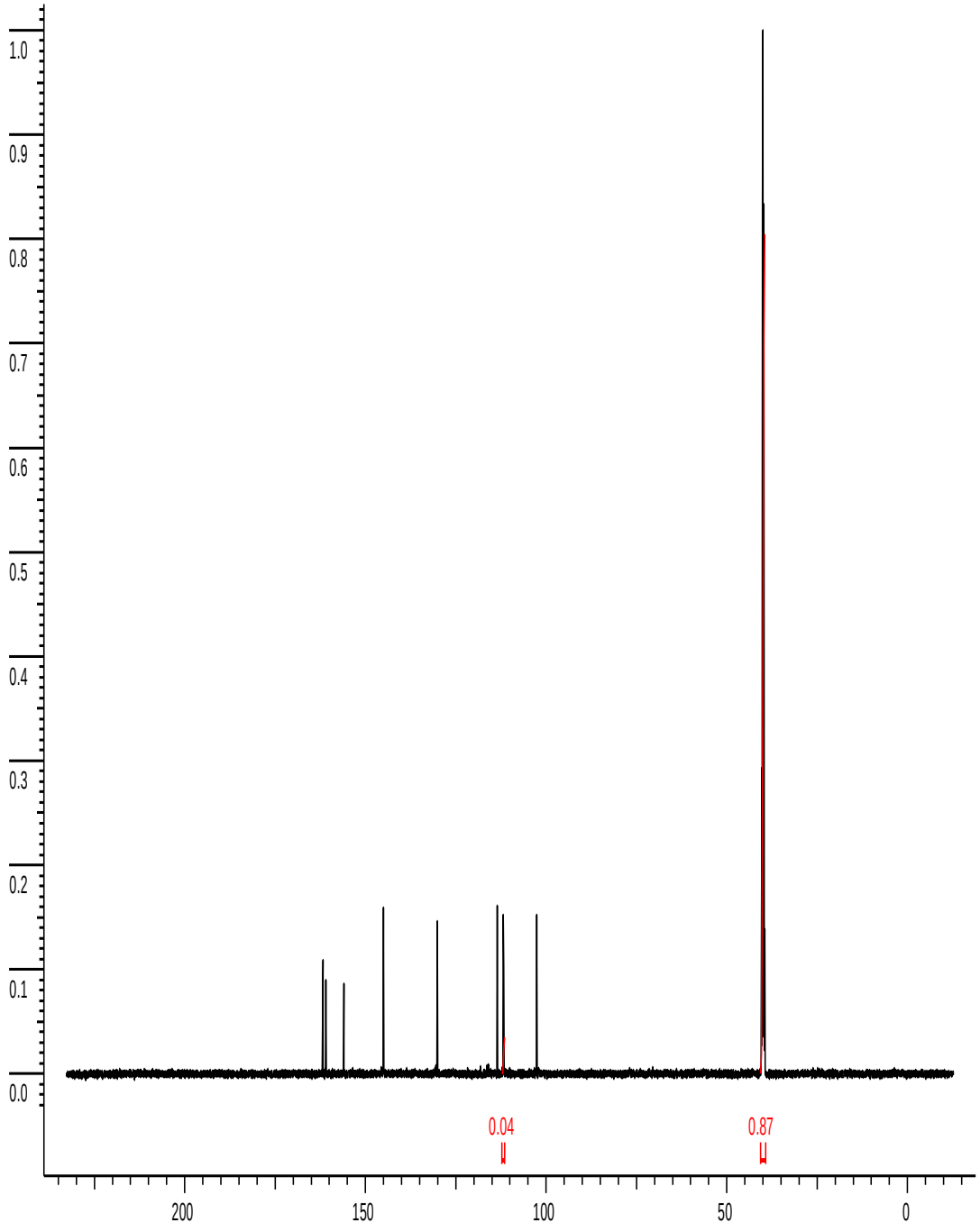
a. Logarithm base = 10.

[1]Positive control;[2] Negativecontrol

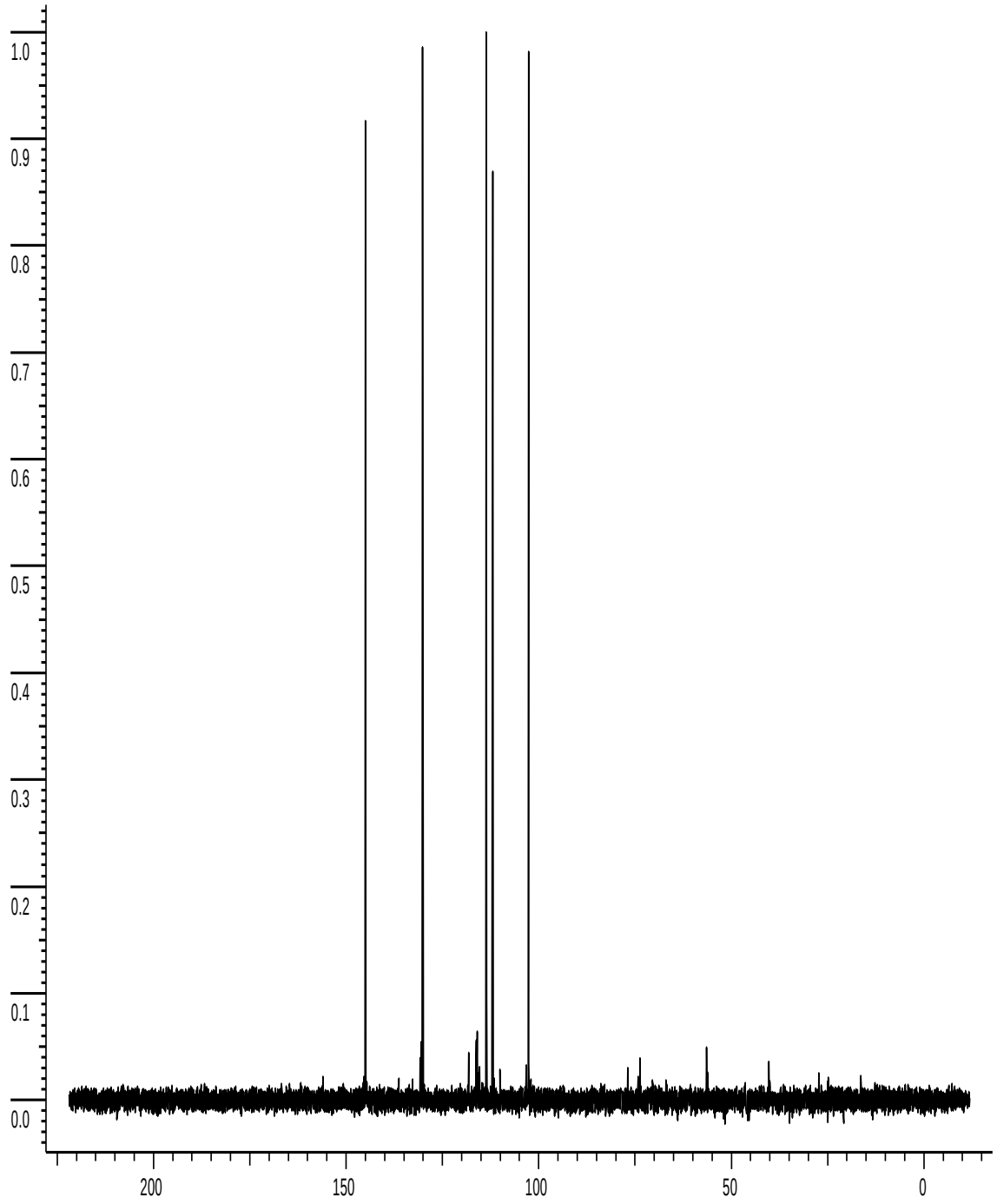
Appendix 16:[1]H NMR spectrum for compound **23**



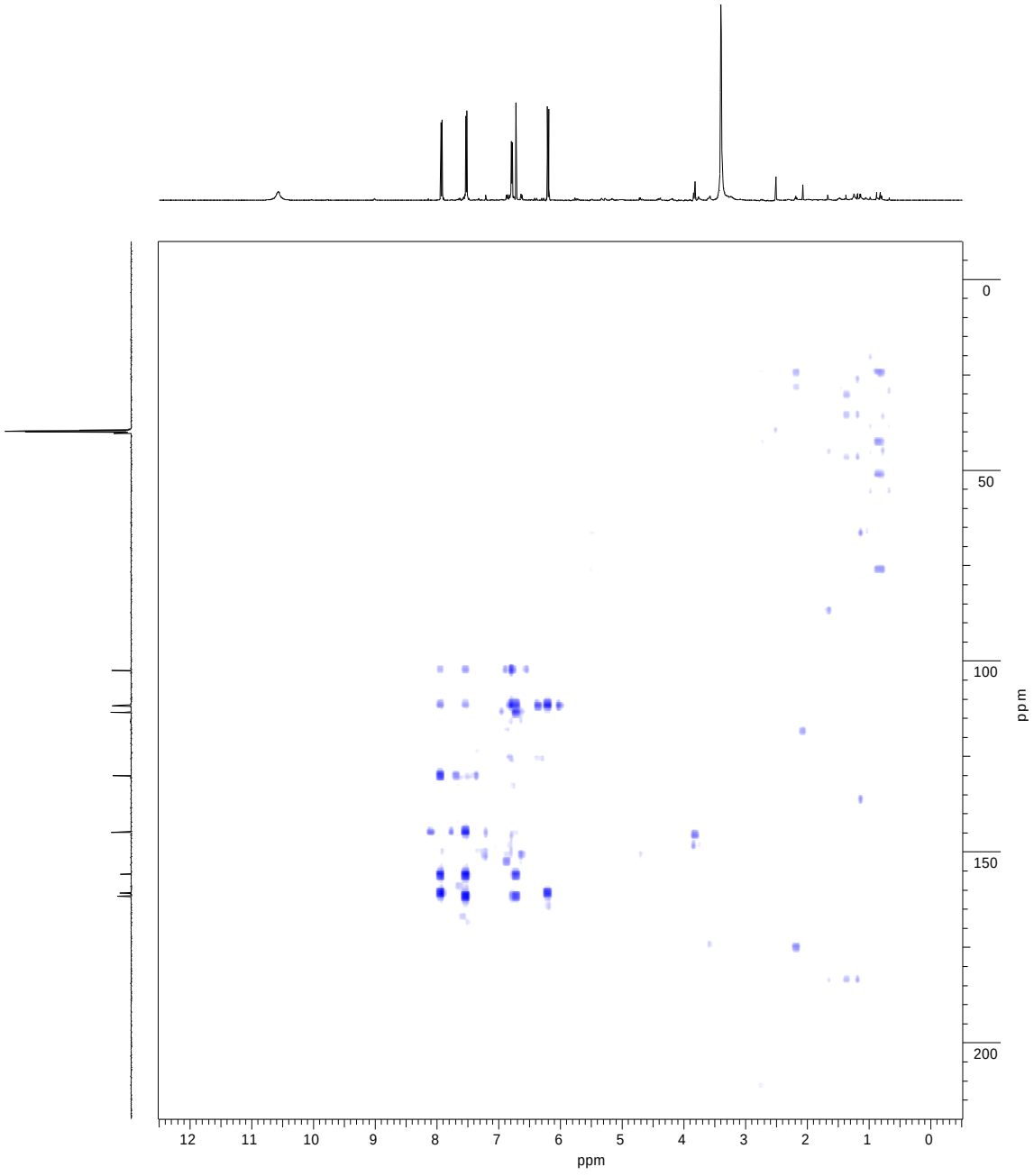
Appendix 17: 500MHz, ^{13}C NMR spectrum for compound (23) (DMSO)



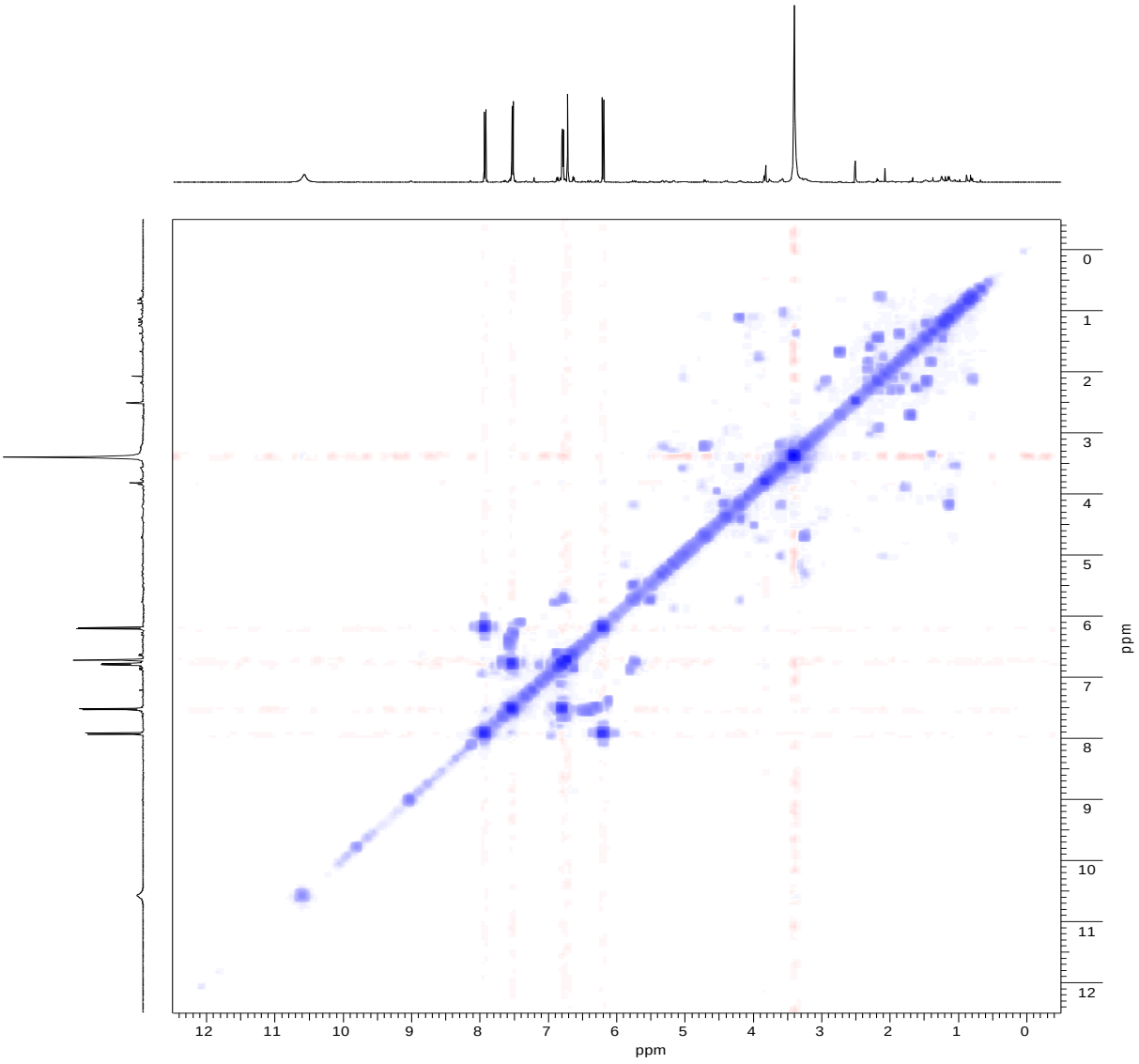
Appendix 18: 500MHZ, DEPTNMR spectrum for compound**(23)(**DMSO**)**



Appendix 19:500HMZ, HMBCNMR spectrum for compound**(23)(**DMSO**)**



Appendix 16: 500MHz COSY NMR spectrum for compound (23)(DMSO)


12
11
10
9
8
7
6
5
4
3
2
1
0
ppm
0
1
2
3
4
5
6
7
8
9
10
11
12
ppm

Appendix 17 :500HMZ, HSQCNMR spectrum for compound**(23)(**DMSO**)**