Isolation and Characterisation of Tick (*Rhipicephalus appendiculatus*) Attractive Compounds from *Calpurnia aurea*

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DECLARATION

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

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DEDICATION

I dedicate this work to my mother, Yunes Sese Mogwambo and my late father, Samson Mogwambo.

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ABBREVIATIONS

BCED	Behavioural and Chemical Ecology Department
DCM	Dichloromethane
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
icipe	International Centre of Insect Physiology and Ecology
ICTTBD	Integrated Control of tick and tick-borne diseases
IR	Infra-red
LCMS	Liquid Chromatography coupled Mass Spectrometry
NMR	Nuclear Magnetic Resonance
R_f	Retention factor
RI	Repellency Index
TBD	Tick-borne diseases
TLC	Thin Layer Chromatography
TTBD	Ticks and tick-borne diseases
UV	Ultraviolet
WFS	World Federation of Scientists

ABSTRACT

Ticks pose a major challenge and economic burden to livestock rearing as they inflict physical injury and transmit diseases resulting in heavy losses of livestock. Their control has relied mainly on the use of synthetic acaricides which are expensive and are hazardous to the environment and non target organisms. Intensive use of these acaricides has also resulted in tick resistance. Plant extracts could provide a viable alternative to tick control and management strategies. *Calpurnia aurea* (Aiton) Benth is an example of a plant whose extracts have been used to treat various internal and external parasites in both livestock and humans. However, despite its extensive use, little is known about its chemical composition and bio-active components. This study describes the isolation and characterisation of tick (*Rhipicephalus appendiculatus*) attractive compounds from *C. aurea* using chromatographic and spectroscopic techniques. The results show that acetone extract of the plant significantly attracted ticks at lower concentrations than the hexane extract in tick climbing bioassays. Using 1 mg/ml of acetone extract, showed 77% attractancy while the fractionated acetone extract at the same concentration, attracted 81.3% of the ticks. Further fractionation of the acetone leaf extract increased tick attraction by 4.7%. Lupanine, Calpurnine and Virgiline Pyrrolcarboxylic acid ester are some of the probable compounds that contribute to the activity of *C. aurea* extract. The results of this study suggest that the acetone leaf extract of *C. aurea* may be used for the control of *R. appendiculatus*.

CHAPTER ONE

1.0 INTRODUCTION

1.1 General Introduction

Ticks are important ectoparasites of wild and domestic animals and affect about 800 million cattle and a similar number of horses, sheep and goats around the world. They affect their hosts by damaging hides, reducing their growth rates and milk production, transmitting diseases that are often lethal, and by causing paralysis and injuries leading to secondary infections. Ticks and tick-borne diseases (TTBD) have impacted negatively on the development of livestock industry in Africa (Walker *et al.*, 2003). The economic losses caused by ticks and tick-borne diseases in cattle are estimated at be about US$ 20 billion annually worldwide (Dantas-Torres, 2007).

The important tick genera vary from country to country but the most important ones in East and Central Africa include *Amblyomma* and *Rhipicephalus* (Bram, 1983). They transmit pathogens that cause tick-borne diseases such as East Coast Fever (Theileriosis), heartwater (*Cowdria ruminantium*), Tick-bite Fever (*Rickettsia conori*), Nairobi Sheep Disease (*Nairovirus*), Q Fever (*Coxiella burnettii*), and dermatophylosis (*Dermatophilus congolensis*).

The use of chemical acaricides still remains the main approach to management of ticks and tick-borne diseases (De Castro and Newson, 1993). Although acaricides have been shown to reduce tick population and their incidences, their main disadvantages have been; they are expensive and their heavy use has resulted in development of tick resistance (Kagaruki, 1991). In addition, concern is growing over the effects of widespread use of chemical acaricides on humans and the

environment. This has prompted a search for alternative methods of tick control that can be used alone or in combination with other tick control methods in an integrated tick management strategy (Jongejan, 1998). The use of entomopathogenic fungi (EPF) is one of the alternatives being explored (Maniania *et al.*, 2007). Inundative and augmentative releases are the main methods employed for introduction of EPF into the ecosystem (Lacey and Goettel, 1995). However, new strategies are currently being considered, whereby insect pathogens are disseminated among target pest populations using devices that attract host pests into a focus of the pathogens (Vega *et al.*, 2009). Sex pheromone or lure is generally used to attract target insects. The findings by Hess and De Castro (1986) and Maranga *et al.* (2006) that *A. variegatum* is attracted to attraction-aggregation-attachment pheromone (AAAP) has prompted the idea of using EPF in combination with pheromone (Maranga *et al.*, 2006). A *Metarhizium anisopliae*-treated AAAP-baited trap was tested in the field and resulted in reduction of 64% of *A. variegatum* ticks relative to the control (Nchu *et al.*, 2010). However, AAAP was found not to be compatible with *M. anisopliae* (Nana *et al.*, 2010) and could not be, therefore, used in spot-spray strategy. Several plant extracts have been reported to have acaricidal/repellent effects (Castrejon *et al.*, 2003) while others exhibit attraction properties (Hassan *et al.*, 1994; Zorloni *et al.*, 2010; Nana *et al.*, 2010). They include *Acalypha fruticosa* Forssk (Euphorbiaceae), *Ipomoea spathulata* Hallier (Convolvulaceae), *Solanum incanum* Linnaeus (Solanaceae) and *Calpurnia aurea* Benth.

1.2 Statement of the Problem

Tick infestation causes serious economic losses in cattle, including, *inter alia,* reduction in weight gain and milk yield, reduced fertility and draught power, damage to hides and skin and predisposition to fungal, bacterial and parasitic diseases (Mukhebi and Perry, 1992, L'Hostis and Seegers, 2002; Peter *et al.*, 2005). It has been calculated that every adult female *R. appendiculatus* that completes her feeding can provoke a loss in live-weight gain of 4g and milk loss of 7g in cows (Norval, 1990; Norval *et al.,* 1988). The value of hides is also depreciated by ticks (De Castro, 1997; Mersie and Bekele, 1994). Bekele (2002) estimated an annual loss of US$500 000 from hide and skin downgrading from ticks, and approximately 65.5% of major defects of hides in eastern Ethiopia are from ticks.

Besides economic loss caused by their blood sucking habits, wounds caused by their mouthparts, are carriers of pathogens, which they transmit from host to host during blood sucking and cause a large variety of diseases (FAO, 1998). These diseases include babesiosis, anaplasmosis, heartwater and East Coast Fever which cause severe economic losses to the livestock industry (Drummond, 1983; Bram, 1983). The important species include *Amblyoma hebraeum* which transmits *Ehrlichia ruminantium,* the cause of heartwater in cattle, sheep and goats (Norval and Horak, 2004), *Rhipicephalus microplus* species which transmit *Babesia bigemina* and *Babesia bovis* the cause of babesiosis in cattle and *Anaplasma marginale* the cause of anaplasmosis in cattle (Norval and Horak, 2004; Peter *et al.*, 2005); *R. appendiculatus,* which transmits *Theileria parva,* the cause of East Coast Fever (Norval and Horak, 2004). The estimation of global costs of ticks and tick borne

diseases (TTBDs) in cattle is between US$13.9 and US$18.7 billions annually (De Castro, 1997; and Minjauw and Mcleod, 2003). These effects seriously limit livestock production and improvement (Latif and Jongejan, 2002).

1.3 Justification

The control of ticks in Africa has relied mainly on commercial acaricides and repellents (Dipeolu and Ndungu, 1991). Access to these chemicals and money to purchase them is curtailed by uncertain economic conditions, resulting in escalating prices of imported goods in remote areas. Furthermore, incorrect administration of acaricides enhances development of resistance, environmental pollution and residues in meat and milk (Latif, 1992). These shortcomings have prompted the search for new alternatives such as biological control with EPF as a component of integrated pest management that are ecologically sound and have no detrimental effect on non target organism (Nana *et al.*, 2010). The role of ethno-veterinary plants in integrated tick control have been the focus of attention in the last two decades (Wanzala *et al.*, 2005; Abduz Zahir *et al.*, 2009). Although attraction of some tick species to plants have been reported (Hassan *et al.*, 1994; Zorloni *et al.*, 2010; Nana *et al.*, 2010), no tick attractant compound from plant origin that could be used in a trap is available. The purpose of this study is to isolate tick attractant compounds from *C. aurea* that could be used as a trap system to control ticks on and off host, hence economical and less pollution to the user, livestock products and environment.

1.4 Hypothesis

There are no tick (*R. appendiculatus*) attractive compounds in leaves of *Calpurnia aurea.*

1.5 Objectives

1.5.1 General objective

To isolate and characterize tick (*R. appendiculatus*) attractive compounds from the leaves *C. aurea.*

1.5.2 Specific objectives

i) To screen for activity of different crude leaf extracts of *C. aurea* against *R. appendiculatus.*

ii) To isolate and characterise tick (*R. appendiculatus*) attractive compounds from *C. aurea* leaf extracts.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Classification of Ticks

Ticks are members of the arthropod class Arachnida, sub-class Acarina, order Parasitiformes. There are two families of ticks, *Ixodidae* (hard ticks) and *Argasidae* (soft ticks) (Eaton, 2009). *Ixodidae* which include *R. appendiculatus, R. pulchelus* and *Amblyoma variegatum* infest a variety of mammalian hosts. *Argasidae* are parasites of birds and they include Argas radiatus raillet (Guerrero, 2009). The two families differ from each other remarkably in appearance, habits and development (Pegram *et al.,* 1986).

Ixodid ticks of the genera of *Hyalomma, Boophilus, Rhipicephalus* and *Amblyomma* are of great economic importance as they cause great losses in livestock production (Frans, 2000). They are very important to man and his domestic animals, and should, therefore, be controlled if livestock production is to meet world needs for animal protein. Knowledge of the nature and habits of the tick and the disease agents it transmits helps in its control (Stewart *et al.*, 1981).

Ixodidae (hard ticks) are subdivided into three groups according to the number of hosts they require to complete their life cycle, and these groupings also determine the manner in which they will transmit pathogens. One-host ticks, such as *Rhipicephalus decoloratus* remain on the same animal from the time they attach to it as larvae until they finally drop off as fully-fed adults (Soneshine, 1992; Wakelin, 1984). Two-host ticks, like *Rhipicephalus evertsi evertsi* attach as larvae, feed and then stay on the same animal after moulting into nymphs (Walker *et al.*, 2003). The nymphs re-

attach, feed and then drop to the ground to moult. When the adults emerge they have to find a second host on which to feed. Three-host ticks require three animals to enable them complete their life cycle because each of the two immature stages drops from the host after feeding and then moults to the next stage, with the adults feeding on a third host (Walker *et al.*, 2003). Most ixodid ticks belong to this group, for example *A. hebraeum* and *R. appendiculatus.* These ticks are parasites of cattle, but their immature stages and sometimes adults will feed on goats and sheep. Nymphs and larvae frequently feed on the same hosts as the adults as well as on small animals such as hares, among others, (Howell *et al.*, 1978; Horak *et al.*, 1987a; Fourie and Horak 1991; Walker *et al.*, 2000).

2.1.1 Life-cycle of ticks

The developmental cycle of a tick typically includes 4 stages namely: egg, larvae, nymph and adult.

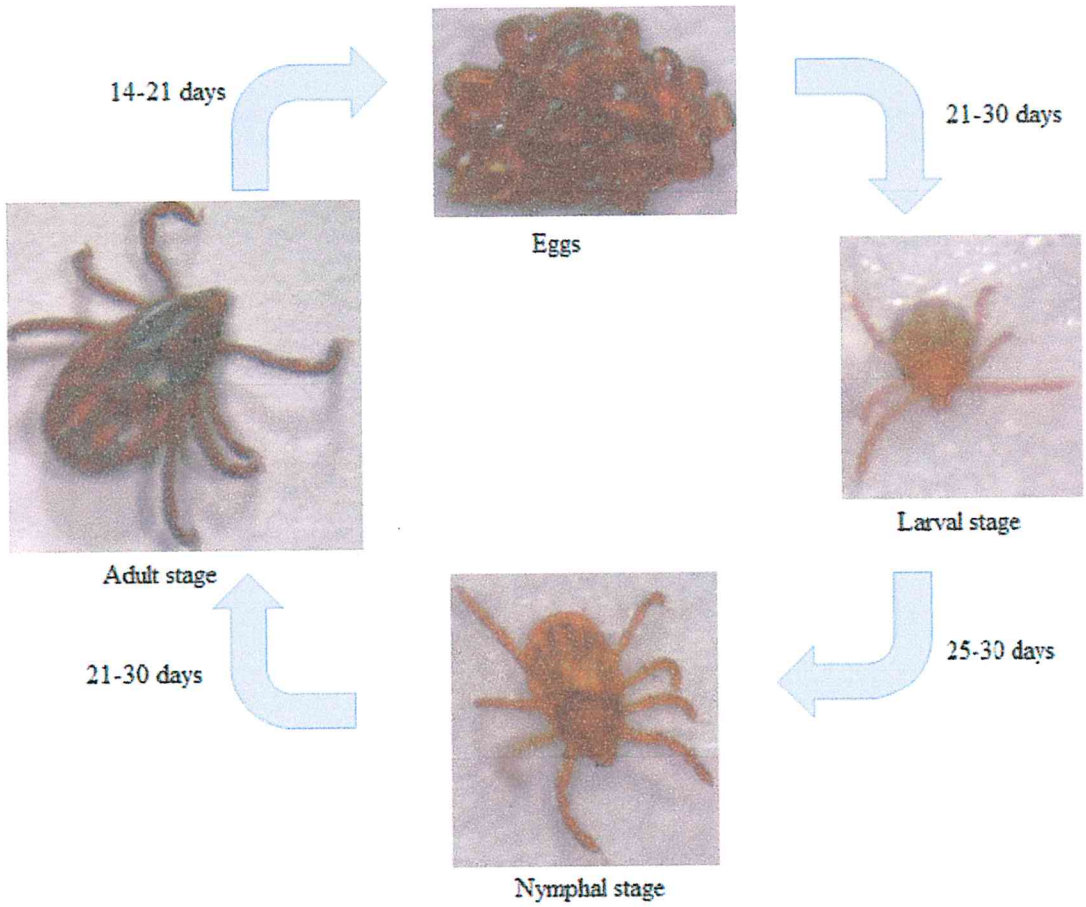


Plate 1: The life-cycle of ticks (photo by Mogwambo, 2012)

Most Ixodid ticks have 3 hosts, one for each stage of life cycle (larvae, nymph and adult) but in some species, this has been reduced to one- or two-hosts (Wakelin, 1984; Oliver, 1989). Ixodid ticks contain only one nymphal stage which develops into an adult (Walker *et al.*, 2003). They need several days to feed, and once the

female is engorged she drops, lays eggs, and then dies. The male, also, dies after mating. They have remarkable longevity and have been observed to live for many years (about 20 years) and long periods of starvation (Soneshine, 1991).

2.1.2 Host seeking behaviour of hard ticks

Hard ticks seek hosts by a behaviour called 'questing'. Questing ticks crawl up the stems of grass or perch on the edges of leaves on the ground in a typical posture with front legs extended, in response to a host passing by. Subsequently, these ticks climb onto a potential host, which has brushed against their extended legs (Soneshine, 1991). Ticks detect their host through several host odours (including CO_2, NH_3, lactic acid, and other specific body odours), body heat, moisture, vibrations, and for some, visual cues like a shadow (Stafford, 2004).

2.2 Mitigation measures

The Current control measures have mainly been use of acaricides and integrated pest management.

2.2.1 Use of acaricides in the control of ticks

Several control interventions have been employed to mitigate perennial losses caused by ticks. The most popular practice has been the periodic application of acaricides which may be directed against the free living stages of ticks in the environment or against the parasitic stages on the host. They can be applied by dipping, hand-held sprayers and spray races, washes, pour-on, or spot-on. Dipping is expensive but is desirable when a large number of cattle are to be treated (Hungerford, 1990). The acaricides used include arsenical, chlorinated hydrocarbons, organophosphates, carbamates and synthetic pyrethroids. The effectiveness of an acaricide applied to

cattle for the control of ticks depends not only on the degree of toxicity of a chemical, but also on the quality, quantity and degree of dispersal of active ingredient deposited on cattle or delivered internally. Whatever the treatment method, adherence to procedures developed by the manufacturer is essential for maximising the degree of tick control (George, 2000).

Arsenic acaricides, like sodium arsenate, were the first to be developed, in 1896, in Australia. However, after 1935, *Boophilus microplus* and *B. decoloratus* developed resistance to them. Relief was not available until the mid-1940s when the first organochlorine products (BHC, DDT, aldrin and lindane) were available (Shaw, 1970). These were banned and therefore not available or have been withdrawn from the market (Kunz and Kemp, 1994) because they are persistent in the environment and are prone to accumulate in the body fat (Ware, 2000).

Unlike the persistent organochlorines, the organophosphate compounds that replaced them are chemically unstable and non-persistent. The organophosphates are generally categorized as the most toxic of all pesticides to vertebrates (Ware, 2000). The development of organophosphate acaricides was for the control of organochlorine-resistant *Boophilus* ticks which had become common throughout much of the cattle-producing areas of the tropics and subtropics (Shaw, 1970). Ethion, diazinon, dimethoate and coumaphos are some of the most widely used organophosphates for the treatment of tick-infested cattle. Carbamate acaricides (e.g. Carbaryl and promacyl), like the organophosphates, function by inhibiting the target's cholinesterase, but have low mammalian and dermal toxicity (Roulston *et al.,* 1968; McDougall and Machin, 1988). Resistance to organophosphates and

carbamates has eliminated or minimised their use in Australia, Africa and parts of Latin America (Kunz and Kemp, 1994).

The formamidines, Chloromethiuron and amitraz, are members of a small group of chemicals that are effective against ticks. A series of trials executed over a 5-year period in South Africa proved the effectiveness of amitraz for the control of *B. decoloratus, R. appendiculatus, R. evertis* and *Amblyoma hebraeum* (Stanford *et al.*, 1981). Amitraz is unstable in dipping vats, but adding sufficient calcium hydroxide or hydrated lime to raise and maintain the pH of the vat solution to 12 ensures the stability of the active ingredient (Stanford *et al.*, 1981; George *et al.*, 1998).

Pyrethroids, from pyrethrum flowers, were developed in 1949. Permethrin and fenvalerate were the first of these chemicals available for the control of ticks on cattle (Ware, 2000), and cypermethrin, deltamethrin and cyhalothrin are examples of next generation cyano-substituted pyrethroids that are effective acaricides (Kunz and Kemp, 1994; Aguirre *et al.*, 2000). The next generation of pyrethroids contains a-cyano group that enhances insecticidal activity and molecular stability ensuring effectiveness. Mixtures of pyrethroids are also available in the market (Schnitzerling *et al.*, 1989), as well as a combination of pyrethroids and other products (Johnson and Matschoss, 1998). Mixtures of acaricides control both ticks and horn fly (Furlong, 1999) and reduce the rate of evolution of acaricide resistance based on the assumption that resistance to each acaricide is monogenic (Tabashnik, 1990).

2.2.2 Integrated Pest Management (IPM)

Integrated pest management (IPM) has been defined as a control system that, in the context of the associated environment and the population dynamics of the pest

species, uses all suitable techniques and methods in as compatible manner as possible and maintains the pest population at levels below those causing economic injury (Flint and van den Bosch, 1981). It involves the systematic application of two or more methods to control pest populations which adversely affect the host species (Bram, 1994; De Castro, 1997). The ultimate aim is to achieve pest or parasite control in a more sustainable, environmentally compatible and cost effective manner than is achievable with a single method. According to Jonsson *et al* (2000b), IPM programs not only have the direct benefits of reduced chemical usage, but also the expected longer-term benefit of reducing the risk of chemical resistance. There is an association between the frequency of acaricide application and the likelihood of resistance. Sangster (2001) notes that IPM schemes must manage chemical use because of concerns about chemical residues in meat, eggs, wool scouring and milk. In controlling animal parasitism, IPM works to improve host resistance using non-chemical means to control parasites, using chemicals judiciously, improving monitoring of infection and resistance and understanding host-parasite relationship. Consequently, some level of parasitism and production loss might have to be tolerated.

Tick control in the habitat and vegetation requires modification of the plant cover by removal of vegetation, which shelters ticks, and by burning. This improves pasture and eradicates ticks (Wilkinson, 1979; Baars, 1999).

Rotational grazing is one of the options in an integrated control scheme (Hernandez *et al*., 2000). The aim of periodically withdrawing domestic hosts is to cause the ticks to disappear through inanition since the only available hosts are cattle (McCosker,

1979). Harley and Wilkinson (1971) proposed a rotation based on a group of fenced pastures, with acaricidal treatment during movement between pastures. Wharton *et al* (1969) found out that a summer rotation of 14 weeks without cattle could reduce the need for acaricidal treatments from 19 per year to 7 per year. The challenge for most farmers is to know how long the rotation should be on their pastures in a specific environment.

In biological tick control, entomopathogens are used (Nana *et al.*, 2010). Predators are most effective, especially ants and birds (guinea fowl). Depending on conditions, they can consume a large number of ticks (Samish and Alekseev, 2001).

Rearing of cattle breeds that exhibit a high degree of resistance to ticks like the Zebu reduces the degree of tick infestation. Hand picking of ticks, restriction on the movement of livestock, improved quarantine, and mandatory systematic and sequential acaricide treatments of livestock by the farmers (Kabede, 2004) are some of the integrated methods of controlling ticks.

2.3 *Calpurnia aurea*

C. aurea is a member of the sub-family Papilionoidea of the Family Fabaceae (Coates, 1983). It is a fast-growing small, multi-stemmed tree or shrub, 3-4 m tall, found in overgrazed areas and is easily cultivated (Germishuizen and Meyer, 2003; Pooley, 1993). It is an indigenous plant of South Africa, growing along the Coastal regions from the south-eastern Cape northwards and inland to the Central Transvaal. It also grows northwards into tropical Africa as far as Ethiopia, and Southern India (Asres *et al.*, 1986b). In Zimbabwe, it is known as wild laburnum, a native plant that grows along the margins of evergreen and riverine forest, 900 m above sea level. In

Kenya, *C. aurea* is found in Lake Nakuru National Park, along the banks of River Athi, at Nairobi National Park, the Nairobi Aboretum; Muringato in Nyeri; parts of Western Kenya, Oldonyo Rock Hills, Kajiado district and Limuru, Kiambu County (*Kenya National Museum, East African Herbarium).*

Plate 2: *C. aurea* leaves and flowers (photo by Mogwambo, 2012)

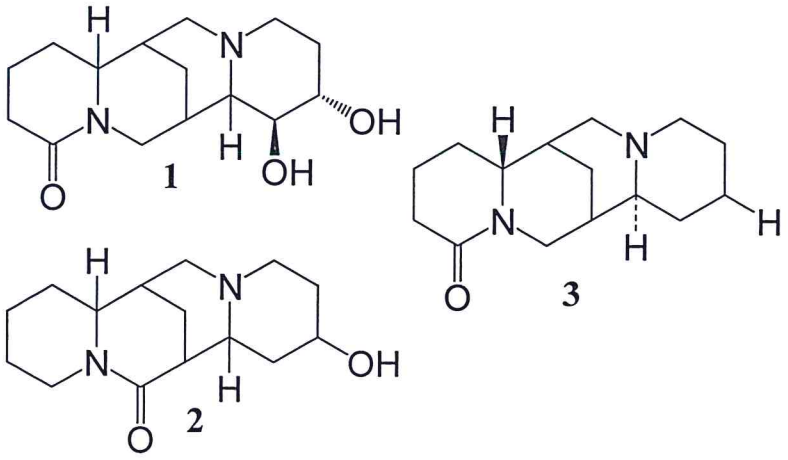
2.3.1 Ethno-botanical uses of *C. aurea*

C. aurea is commonly used in traditional medicine to treat diverse health conditions and parasitic infestation, both in humans and animals (Hutchings, 1996). Extracts of *C. aurea* have been used in South Africa to treat maggot-infested wounds (Watt and Breyer- Brandwijk, 1962), and in Ethiopia to treat scabies (Jansen, 1981). Powdered seeds are pasted with honey and swallowed to treat syphilis and the ground fruits are

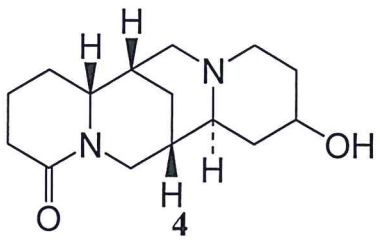
mixed with butter and applied topically for treating scabies (Fullas, 2001) in Ethiopia. It is also used, in Ethiopia, to treat stomach disorders, amoebic dysentery and eye diseases (Abate, 1989). In south western Ethiopia, the leaves of *C. aurea,* mixed with other plant species, are crushed and squeezed to obtain a juice, which is applied through the auricular route for two days to treat earache in humans (Yineger and Yewhalaw, 2007). The plant is traditionally used to treat rheumatism (Yineger *et al.,* 2008), in the same area. Antibacterial and antioxidant activity of *C. aurea* have been reported (Adedapo *et al.,* 2008), and the plant has been used for the treatment of bacterial infections of the skin in Ethiopia (Tadeg *et al.,* 2005). It has also been used as a natural pesticide to improve grain storage (Blum and Bekele, 2002). In north-western Ethiopia, it is used to treat ascaris and gastric ulcers. The leaves are boiled in water, filtered and a cup of this concoction is administered orally on an empty stomach, in the morning for a week (Ragunathan and Solomon, 2009). In Kenya, the leaves are used for the treatment of diarrhea, leishmaniasis, wounds, and as an insecticide. The seeds are used for toothache while the roots are used for lung tuberculosis, swelling, cough and snake bite (Omino and Kokwaro, 1993). In western Ethiopia, the juice of crushed leaves and bark is used for tick control (Regassa, 2000), and in southern Ethiopia, extracts of the leaves attract and immobilise or kill ticks (Zorloni *et al.,* 2010). The Borana people of north eastern Kenya and southern Ethiopia soak the leaves of *C. aurea* in cold water to treat louse infestations in humans and calves (Heine and Brenzinger, 1988).

2.4 Previous Phytochemical works

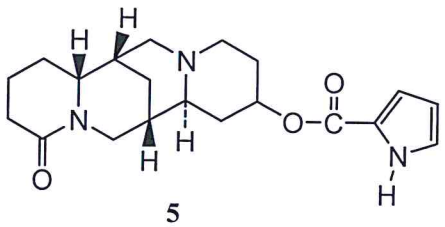
The main phytochemical compounds of *C. aurea* are the alkaloid calpurmenine (**1**) and its 13α-(2^1-pyrrolcarboxylic acid) ester (Vermin *et al.,* 1979). The alkaloids virgiline (**2**) and lupanine (**3**), as well as their carboxylic acid esters, have also been recorded (Van Wyk *et al.,* 1991).



Investigations on *C. aurea* have resulted in the isolation of a series of quinolizidine alkaloids. The leaves and twigs of Ethiopian *C. aurea* yielded 13-hydroxylupanine **(4)**.



The South African species yielded the well known alkaloids: hydroxylupanine, calpurnine **(5)**, virgiline and its pyrrolylcarboxylic acid ester **(6)** as found in Ethiopian sample.



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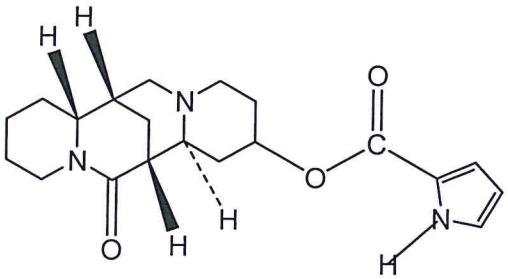

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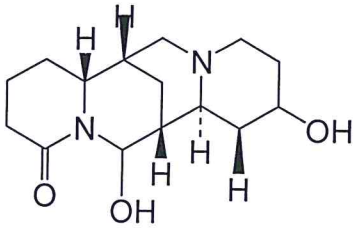
In addition, the alkaloid 10, 13-dihydroxylupanine **(7)** was found in dichloromethane (CH_2Cl_2) extract of the pods.



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This compound, also occurring in *Cadia purpurea,* was absent from the Ethiopian species. The alkaloids (calpurmenine and 13-(2^1-pyrrolylcarboxyl) calpurmenine), specifically present in the South African material were isolated from the extracts of leaves and pods (Asres *et al.,* 1986a*;* Radema *et al.,* 1979*; and* Asres *et al.,* 1986b*).*

The antibacterial, antioxidant activities and phenolic contents of the methanol extracts of the leaves and stems of *C. aurea* were evaluated using *in vitro* standard methods (Adedapo *et al.*, 2008). Spectrophotometry was the basis for the determination of the total phenol, total flavonoids, flavonols, and proanthocyanidins.

Tannins, quercetin and catechin equivalents were used for these parameters. Isao *et al* (1984) also found out that the alkaloid virgiline isolated from *C. aurea* possesses a potent molluscicidal activity against *Biomphalaria glabrata.* It is also reported to have lecitins, non-protein amino acids and tannins (Fullas, 2001).

As part of a larger ethnoveterinary survey to assess plants traditionally used for tick control in southern Ethiopia, Zorloni *et al* (2007) investigated the attractant/repellent and acaricidal effects of *C. aurea* extracts (Zorloni, 2007; Zorloni *et al.*, 2010). He found out that organic solvent extracts of *C. aurea* exhibited tick toxicity. Information on the reputed acaricidal properties of the plant was provided by a group of semi-nomadic pastoralists belonging to the Borana ethnic group (Zorloni, 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 General experimental procedure

All glassware was washed in hot water and soap, rinsed with distilled water and acetone, and then dried in the oven at 100°C for 50 minutes. All the solvents and reagents used were obtained from Aldrich Chemical Co. Ltd, England and Merck, Germany.

3.2 Ticks

R. appendiculatus used in this study was obtained from the Animal Rearing and Quarantine Unit, International Centre of Insect Physiology and Ecology (*icipe*). The stock originated from cattle from the Marsabit area of Kenya in 2006. All life stages of the ticks were fed on New Zealand White rabbits. Three to four-month old unfed adults were used in this study. The ticks were stored in sample vials stoppered with cotton wool and kept in a metallic chamber at room temperature (24 ± 2°C). The temperature was maintained by keeping the chamber in the incubator whose temperature was maintained at 25°C and were kept that way until when required.

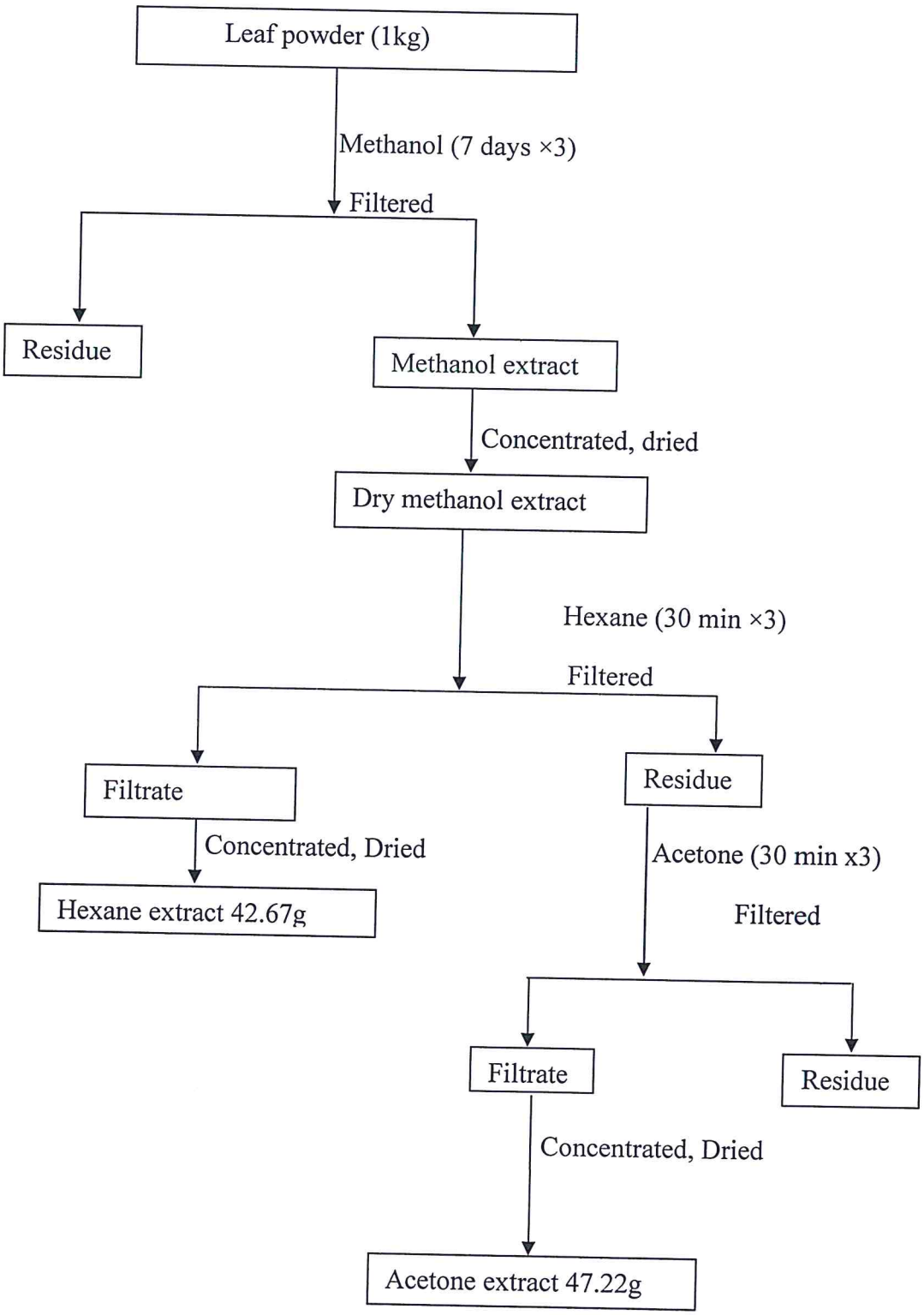
3.3 Plant material

The *C. aurea* leaves were collected from their natural habitat, Brackenhurst botanical garden, Tigoni, in Limuru, Kiambu County-Kenya, and given voucher number 2010/348. Sample specimen has been kept in the Herbarium of the Botany Department, University of Nairobi, Kenya. The leaves were air dried under shade before grinding into fine powder.

3.4 Extraction Procedure

Extraction was done by soaking 1 kg of the fine powder in 4 litres of methanol, shaken and kept in the dark, and shaken thereafter every day for a week. The mixture was then filtered on Whatman filter paper (No.1) (Whatman®, England) and the filtrate concentrated using a vacuum rotary evaporator, at 40°C, to remove the organic solvent. Extraction was done three times on the same plant material. The concentrated filtrate (crude extract) was dried in the hood, weighed and then extracted sequentially using hexane and acetone (Eloff, 1998) (Scheme 1). The extracts were then decanted, filtered using Whatman filter paper (No.1) and concentrated under reduced pressure. The dry samples were then stored in the freezer at -4°C. Hexane was used to extract the non-polar organic compounds while acetone extracted the polar ones. The hexane and acetone crude extracts were subjected to tick attraction bioassays.

Scheme 1: Solvent extraction of the leaves of *C. aurea*



3.5 Bioassays

3.5.1 Preparation of extract for bioassay

Crude extract (48 mg) was dissolved in 4 ml of the solvent that was used for extraction (100% extract) of which 2 ml was mixed with the same volume of the solvent (50% extract). Serial dilution was performed to obtain up to a dilution of 12.5% extract. For each extract, 0.5 ml was used for bioassay (Nana *et al.*, 2010). For the fractions, a solution of 1 mg/ml was prepared and for each fraction, 0.5 ml was used for bioassay.

3.5.2 Tick climbing bioassay

The technique developed by Nchu *et al.* (2005) was used. Ten ticks were placed in the centre of a rectangular (15 cm × 13 cm) polystyrene platform, fixed in the middle of a rectangular basin, which was filled with water to the upper surface of the platform, to prevent ticks escaping. At opposite sides of the platform, two glass rods were inserted, each fitted with a piece of cotton wool over the glass rods at the top. A test tube was inverted over this rod. The piece of cotton wool on one rod was moistened with the test solution (extract plus solvent) while that of the other rod was moistened with the solvent only (control experiment).

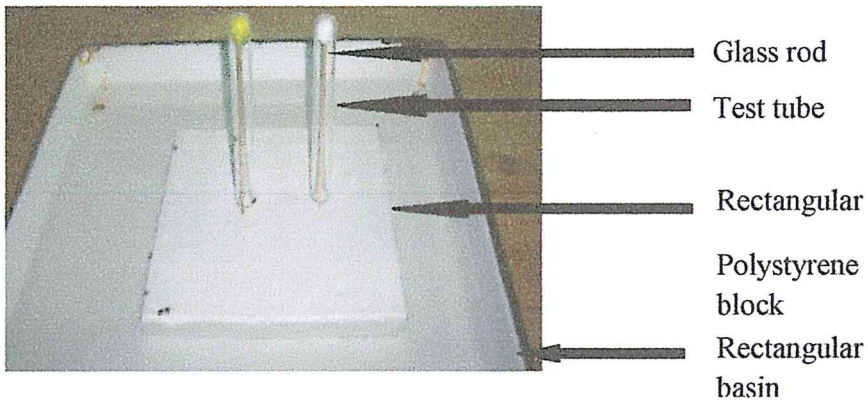


Plate 3: Tick climbing bioassay set-up (Nchu *et al.*, 2005)

The experiment was based on the climbing behaviour of the ticks, which have a natural tendency to ascend grass stalks seeking a favourable position to find a vertebrate host (Nchu *et al.*, 2004). This behaviour was maintained under laboratory conditions (Browning, 1976). The position of ticks was recorded after 30 minutes. Four replicates were done with each extract using fresh ticks each time.

3.5.3 Standard tick attractant Pheromone

A standard tick attractant, Attraction-Aggregation-Attachment-Pheromone (AAAP), was included in the tick climbing bioassays as a positive control. It was made up of 8 mg of ortho-nitrophenol, 4 mg of methyl salicylate and 32 mg nonanoic acid. The mixture was dissolved in acetone and the volume made up to 4 ml of solution (Schoni *et al.*, 1984). This was serially diluted to give various doses (Nchu *et al.*, 2009; Nana *et al.*, 2010). Bioassays were done using 0.5 ml. The synthetic compounds used to prepare AAAP were obtained from sigma-Aldrich Chemie GmbH, Steinheim, Germany.

3.6 Isolation and Purification

The active crude extract was column chromatographed on silica gel (230-400 mesh) using a glass column of 81 cm long × 4.5 cm diameter. Slurry-packing method was employed and elution done with hexane/ethyl acetate gradient solvent system. Fractions collected were of 10 ml each. Separations were monitored using preparative TLC plates of silica gel coated on aluminium, F_{254}nm and thickness 0.2 mm. For visualisation, the TLC plates, 5 cm x 4 cm, were developed with hexane/ethyl acetate, sprayed with 3% sulphuric acid/methanol, and then allowed to dry at room temperature before heating at 100°C for 10 minutes. Fractions that showed spots with the same retention factor (R_f) were combined, concentrated and subjected to tick attraction bioassay. A total of 18 combined fractions were obtained. The active fractions were further chromatographed on silica gel (230-400 mesh) using 10% hexane/acetone, 5% hexane/acetone and 100% acetone. The size of the glass column used was 55 cm long x 3 cm diameter. The various combined fractions were subjected to tick attraction bioassay.

The active fraction was further purified using preparative HPLC (Shimadzu ACE-C_{18} reverse phase column, 250 × 4.6 mm, 5 μm particle size) under gradient conditions using methanol (B)/water (A) as the eluent with the run time of 65 minutes (Table 1). Both solvents were acidified with 5% formic acid, for better resolution. The samples were 1mg/ml and elution was carried out at a flow rate of 1 ml/min. The compounds were detected by UV at 270 nm, using a Photodiode Array (PDA) detector. The HPLC fractions were evaporated to dryness in a rotary evaporator at 40°C, and then

each was dissolved in methanol/water 5% formic acid and re-injected to check for purity.

Table 1: HPLC gradient elution program

Time (min)	B (%) Methanol
0	5
3	15
13	25
25	30
35	55
45	55
46	95
58	5
60	5
65	5

3.7 Characterisation of the isolated compounds

3.7.1 Test for alkaloids

The HPLC pure fractions were dissolved in methanol and 0.5 ml of the solution was put in a test tube. A similar volume of Dragendorff's reagent was added, and colour of precipitate formed recorded.

3.7.2 Ultraviolet-Visible spectroscopy

This was achieved through the Photodiode Array (PDA) detector coupled with HPLC during purity check.

3.7.3 Liquid Chromatography coupled Mass Spectrometry (LCMS)

The HPLC pure fractions were dissolved in 10% methanol solution then injected into the LCMS (Agilent Technologies, ZORBRAX Eclipse plus C_{18} reverse phase column, 100 × 4.6mm, 3.5µm particle size) under isocratic elution at 40% and 50% using methanol 1% formic acid/water 1% formic acid. Electrospray ionisation (ESI) was used with quadrupole ion analyser. The mass spectrometry detector (MSD) analysis was in scan, using positive ionisation mode. Fragmentation was done at 60 and 120 volts, and mass range of 100 - 800 units. In all cases, the run time was 20 minutes and the flow rate was 0.75 ml/min.

3.8 Data analysis

For each test, the total number of ticks responding to plant extract was pooled across replicates. Data was analysed using Chi-Square.

Attraction was expressed in terms of percentage activity and was obtained by the formula modified from Lwande *et al.*,(1999)

$$A = \{(N_t - N_c) / (N_t + N_c)\} \text{ X } 100$$

Where A is activity

N_t is the total number of ticks on the test rod and

N_c is the total number of ticks on the control rod

Percentage activity above zero meant attraction while below zero was repellency or no attraction. Since there were no significant differences in response between male and female ticks, data for the two sexes were pooled together. Ticks that did not respond (no response group) were excluded from the analysis.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Introduction

In this chapter, the results of the study are presented. They include: extraction, isolation by chromatographic methods, bioassay results and characterisation of the isolated compounds by spectroscopic methods.

4.2 Extraction

The methanol extract yielded a brown solid which was sequentially extracted with hexane and acetone, respectively (Table 2). Methanol was the preferred extraction solvent because it extracts both polar and non-polar organic compounds.

Table 2: The percentage yield of extracts from C. *aurea*

Extracts	**Yield (gm)**	**% Yield**
Methanol	256.58	25.65
Hexane	42.67	4.27
Acetone	47.22	4.72

4.3 Activity of crude extracts of *Calpurnia aurea* against *R. appendiculatus*

The results of the tick climbing bioassay showed that both hexane and acetone extracts did not show activity to *R. appendiculatus* at high concentrations (12.0 mg/ml) but did at lower concentrations (Figure 1). As the concentration of both extracts was decreased, activity increased up to maximum levels (3.0 mg/ml for acetone extract, $p < 0.05$ and 1.5 mg/ml for hexane extract, $p > 0.05$) (Figure 1). Further decrease in concentration resulted in decreased activity.

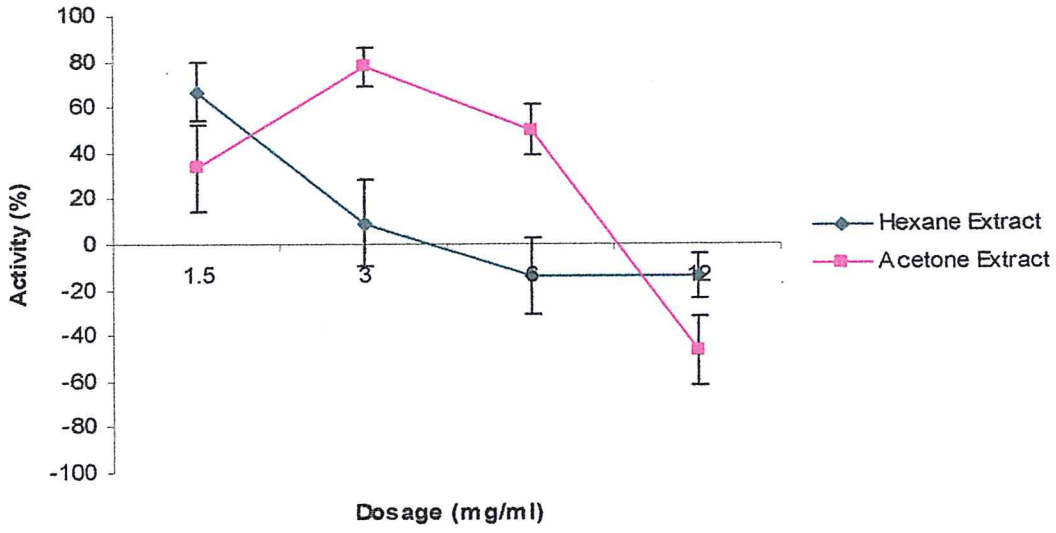


Figure 1: Activity of crude extracts of *C. aurea* against *R. appendiculatus*

The composition of the extract was the same regardless of whether the concentration was high or low. However, at higher concentrations, the various compounds could have resulted in antagonistic interaction with one another (Nwodo *et al.*, 2010).

Hexane extract showed repellence between the concentration of 12.0 mg/ml ($\chi^2 = 0.0714$, df = 1, $p > 0.05$) and 6.0 mg/ml ($\chi^2 = 0.1905$, df = 1, $p > 0.05$). Further

decrease in concentration resulted in a gradual increase in activity. At a concentration of 1.5 mg/ml activity of hexane extract was 66% (χ^2 = 1.5, df = 1, p > 0.05). However, that activity was not statistically significant as compared to acetone extract, when you consider their p-values (Nana *et al.*, 2010, Zorloni *et al.*, 2010). Similarly, acetone extract showed no activity at the higher concentration of 12.0 mg/ml (χ^2 = 2.4, df = 1, p > 0.05). However, increased activity was observed at lower doses of 6.0 and 3.0 mg/ml. At a concentration of 3.0 mg/ml activity of acetone extract was highest, 77.8% (χ^2 = 4, df = 1, p > 0.05), which was comparable with that of the standard AAAP that had optimum activity of 85% at a concentration of 0.02 mg/ml (Figure 2) (Nana *et al.*, 2010). Further decrease in the concentration of the extract resulted in a decreased activity. These results agree with the previous findings which indicate that active compounds in crude extracts (acetone extract in this study) are in very small amounts and therefore do not show high specific activity at higher dilutions (Nwodo *et al.*, 2010).

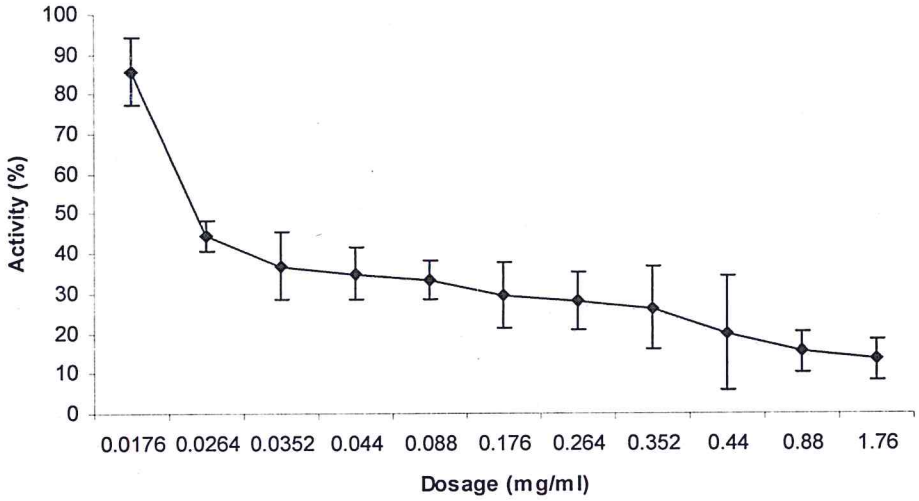


Figure 2: Activity of AAAP standard against *R. appendiculatus*

4.4 Activity of hexane/ethyl acetate fractions

The acetone extract was further separated by column chromatography using hexane/ethyl acetate gradient solvent system. After drying, each of the fractions was screened for activity against ticks. Climbing bioassay results showed that of the 18 fractions, only four showed activity while the rest did not (Figure 3).

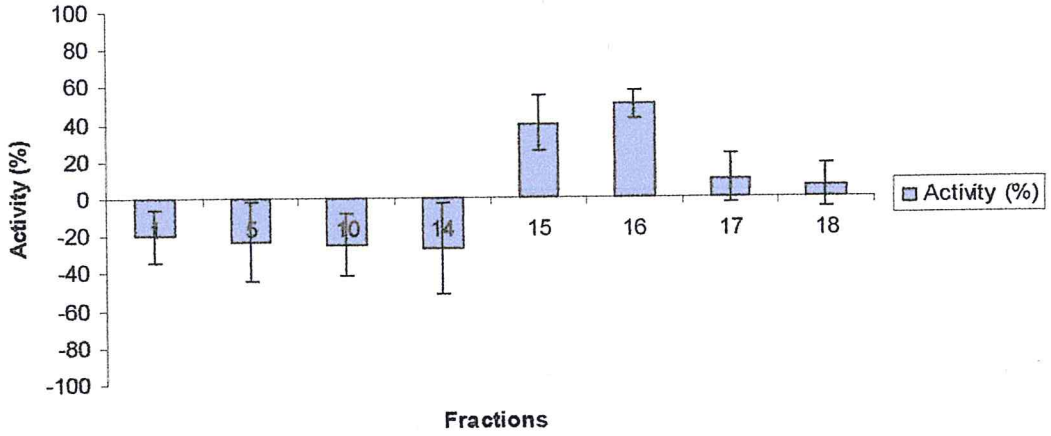


Figure 3: Activity of hexane/ethyl acetate fractions of *C. aurea* against *R. appendiculatus*

Hexane/ethyl acetate gradient solvent system eluted fractions 1-14. The components of these fractions are mainly non-polar while some are weakly polar, and all are not showing any activity towards ticks, as shown by their activities being below zero.

The active fractions (15, 16, 17 and 18) had the activity of 40%, 50%, 10% and 6.3%, respectively. They all eluted at 100% ethyl acetate. These compounds were mid-polar to polar since they were eluted by 100% ethyl acetate implying that the active compounds found in *C. aurea* are either mid-polar or polar.

Fractions 15 and 16 being more active than the other two mean that they contain more active ingredients or they contain compounds acting together synergistically or additively.

4.5 Activity of hexane/acetone fractions

Retention factor (R_f) showed that the TLC profiles of the active fractions, above, were similar. Consequently, they were combined, subjected to column chromatography and eluted with hexane/acetone, 10:90, 5:95 and 0:100. Four fractions were obtained with only one of them showing activity (-4%, -15.8%, 81.25% and -11%) (Figure 4).

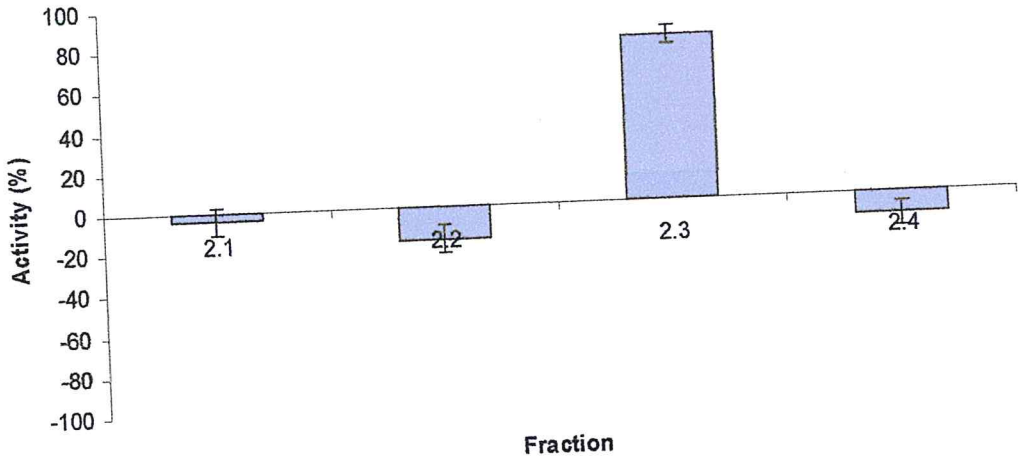


Figure 4: Activity of hexane/acetone fractions of *C. aurea* against *R. appendiculatus*

The active fraction, 2.3, eluted at 100% acetone while fractions 2.1 and 2.2 which showed deterrence were eluted by hexane/acetone, 10:90 and 5:95, respectively, and fraction 2.4 eluted at 100% acetone.

The compounds eluted by hexane/acetone were mid-polar and not active. The fact that fraction 2.3 was active while fraction 2.4 was not, suggests that the active

ingredients were all eluted in fraction 2.3 implying that the tick attractive compound(s) were polar.

Fraction 2.3, prepared at a concentration of 1mg/ml showed an activity of 81.25% (χ^2 = 19.5312, df = 1, $p > 0.05$). The activity of that fraction was comparable with that of the standard AAAP which had an optimum activity of 85.7% ($\chi 2$ = 8.6429, df = 1, $p < 0.05$) at a concentration of 0.02 mg/ml (Figure 2). The activity of this fraction was higher than the activity of the crude extract at the optimum concentration (77.8%) and that of the most active fraction (50%) obtained from the elution of the crude extract with hexane/ethyl acetate above. Evidently, fractionation concentrates the active ingredients in the extract which corroborates with the findings of the previous studies (Marjorie, 1999; Nwodo *et al.*, 2010).

4.6 Characterisation of the isolated Compounds

The compounds **8-14** were obtained by preparative high performance liquid chromatography (HPLC) (Figure 5) of *C. aurea* active fraction. When tested with dragendorff's reagent, they all formed a reddish-orange precipitate implying that they are alkaloids (Plate 4). Compound **8**, **9** and **14** were obtained as white crystals, while **10**, **11** and **12** were brown crystals and **13** was a yellow oily solid. Purity of the compounds was confirmed using analytical HPLC while characterisation was achieved by LC-MS.

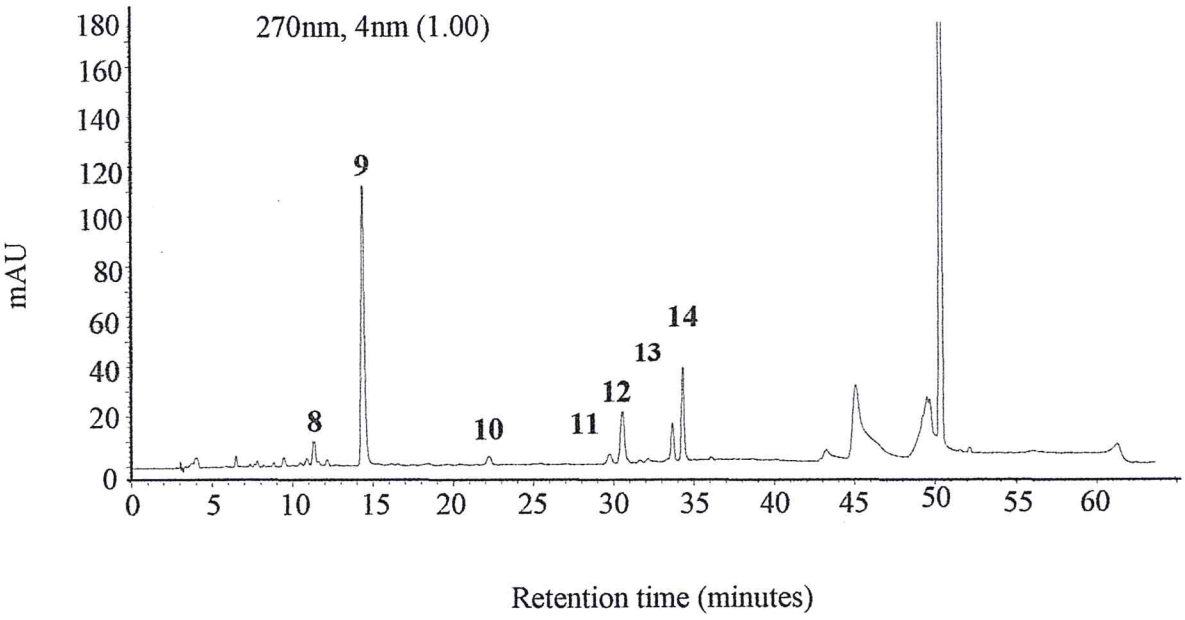


Figure 5: HPLC chromatogram of acetone crude extract of *C. aurea*

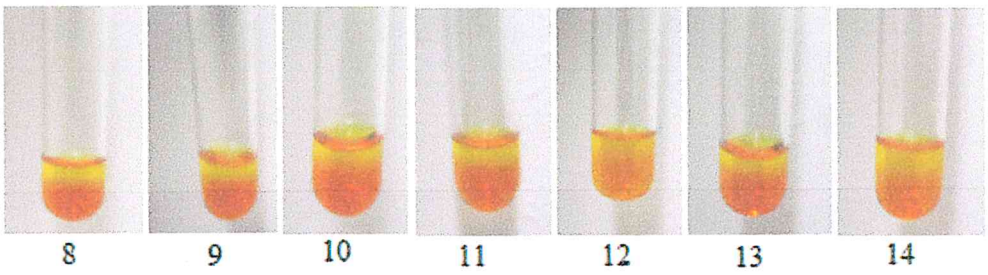


Plate 4: Test for alkaloids (Photo by Mogwambo, 2012)

4.6.1 Characterisation of Compound 8

Compound **8** had a retention time of 10.909 min in the HPLC (Figure 6). It was an alkaloid (plate 4), had UV absorption band at λ_{max} 217.5 nm implying that it is non-conjugated (Figure 13c) and its spectrum shows that it is aromatic.

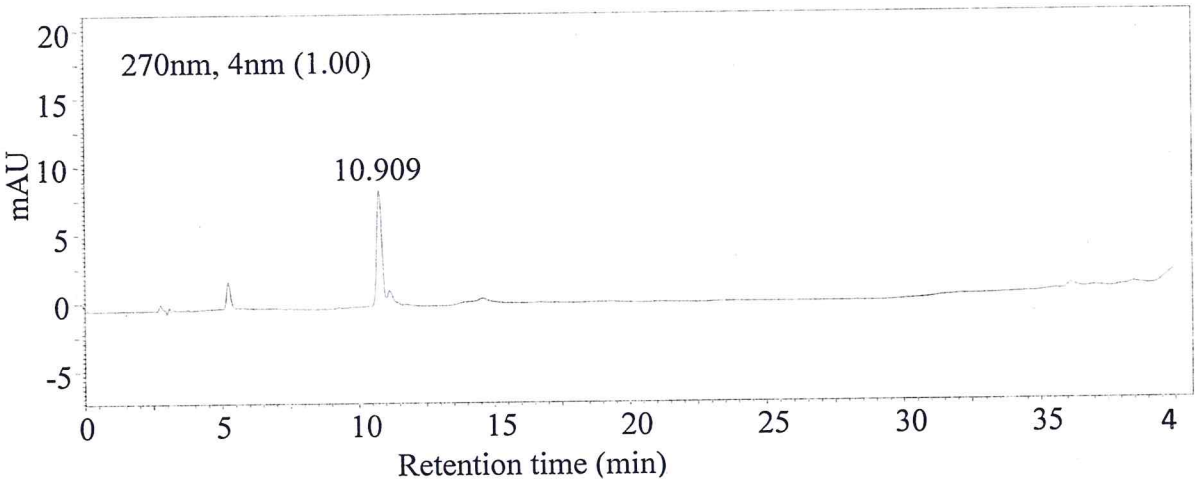
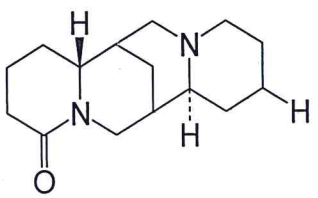


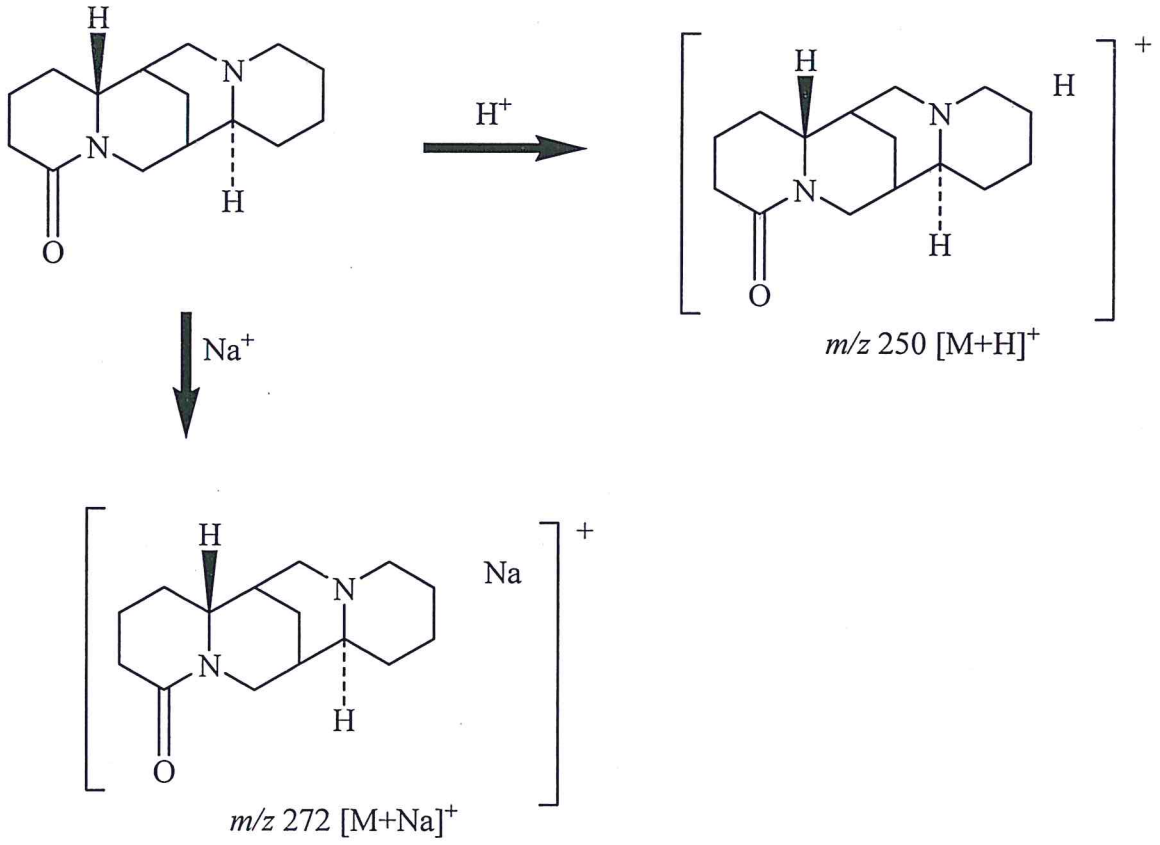
Figure 6: HPLC chromatogram of compound **8**

In the LCMS, compound **8** had a molecular ion peak $[M+H]^+$ at *m/z* **250** and a sodium adduct, $[M+Na]^+$, at *m/z* **272,** at a cone voltage of 60v (Figure 13a). Similar fragments were formed at cone voltage 120v (Figure 13b). Its molecular weight (M.W.) is **249**. Though structure determination has not been done in the present study, compound **8** could, probably, be lupanine (Van Wyk *et al.*, 1991). Lupanine with a molecular weight 249, had been reported isolated from *C. aurea*. The molecular formula of Lupanine is $C_{15}H_{24}N_2O$



Proposed compound **8**

Possible fragmentation patterns for formation of *m/z* 250 and 272.



4.6.2 Characterisation of Compound 9

Compound **9** had a retention time of 14.525 min in the HPLC (Figure 7). It was an alkaloid (plate 4), had UV absorption band at λ_{max} 218 nm implying that it is non-conjugated (Figure 14c) and its spectrum shows that it is aromatic.

In the LCMS, compound **9** showed only one peak at *m/z* 358 $[M+H]^+$ at a cone voltage of 60v (Figure 14a). However, at the higher cone voltage of 120v (Figure 14b) it gave more peaks. The fragment peak at *m/z* 380 $[M+Na]^+$ formed as a result

of the formation of a sodium adduct. It lost a fragment with a mass of 111, probably pyrrolcarboxylic acid, giving *m/z* 247. The molecular weight of compound **9** is 357.

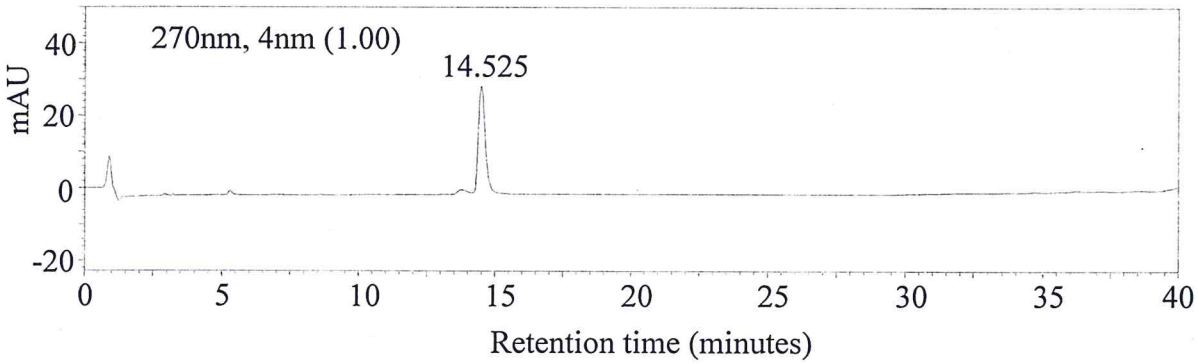
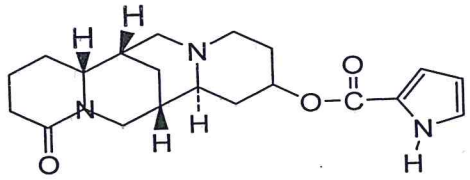


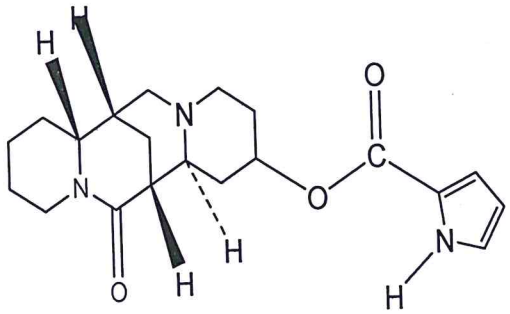
Figure 7: HPLC chromatogram of compound **9**

Although the structure of compound **9** was not determined in the study, it was most likely to be compound **5** (Calpurnine) or compound **6** (Virgiline Pyrrolcarboxylic acid ester) which have been reported in reviewed literature of having been isolated from *C. aurea* (Asres *et al.*, 1986a; Radema *et al.*, 1979; and Asres *et al.*, 1986b). Firstly, Compound **9** was likely to be compound **5** or **6** because its molecular weight was **357** which were similar to that of these two compounds. Secondly, it was a metabolite of *C. aurea*, just like these two. The two compounds had the same molecular formula, $C_{20}H_{27}N_3O_3$, and hence molecular weight but differ in the position of C=O bond in the aromatic system. Probably the molecular formula of compound **9** was $C_{20}H_{27}N_3O_3$ and its structure was likely to be



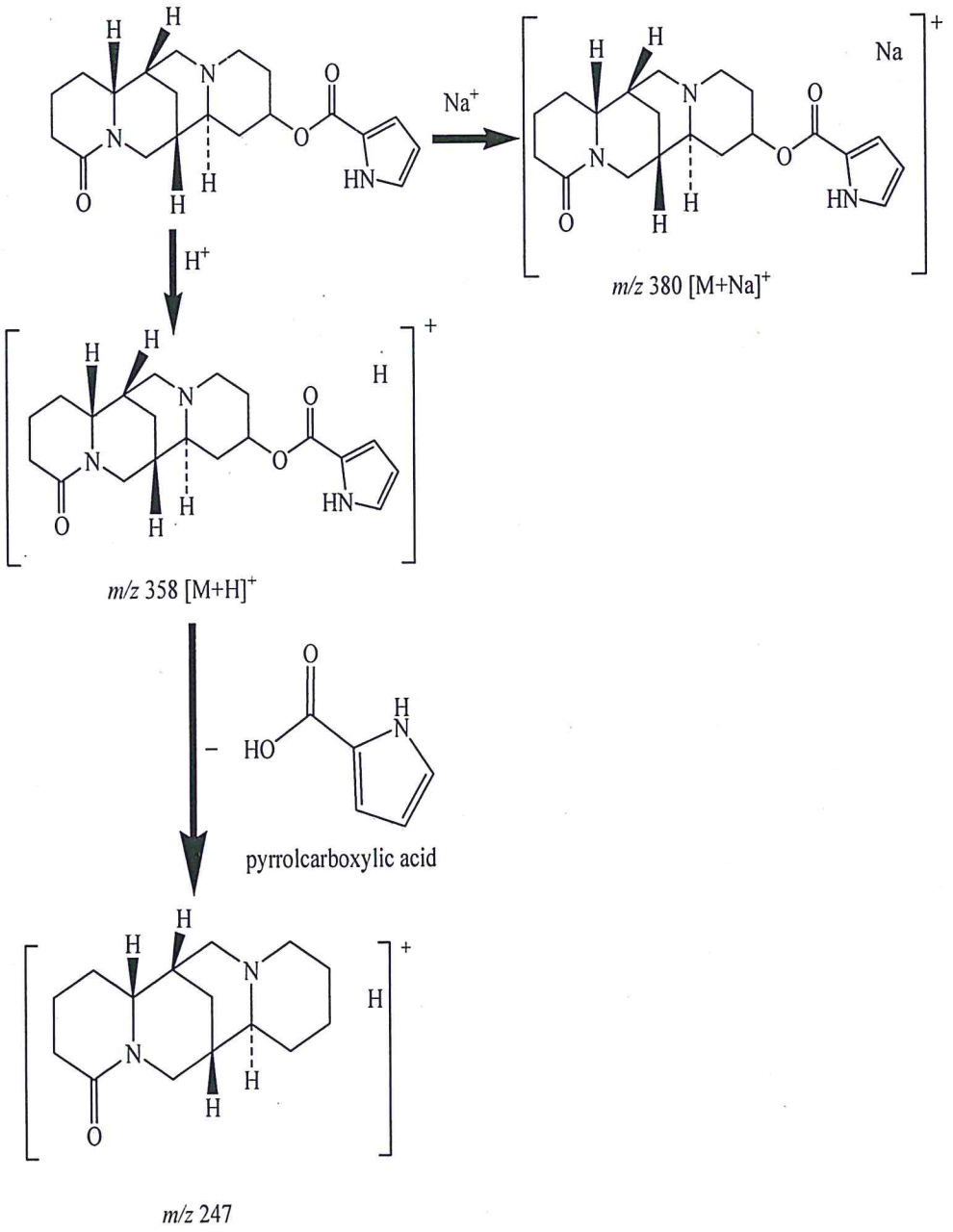
Proposed compound **9**

or

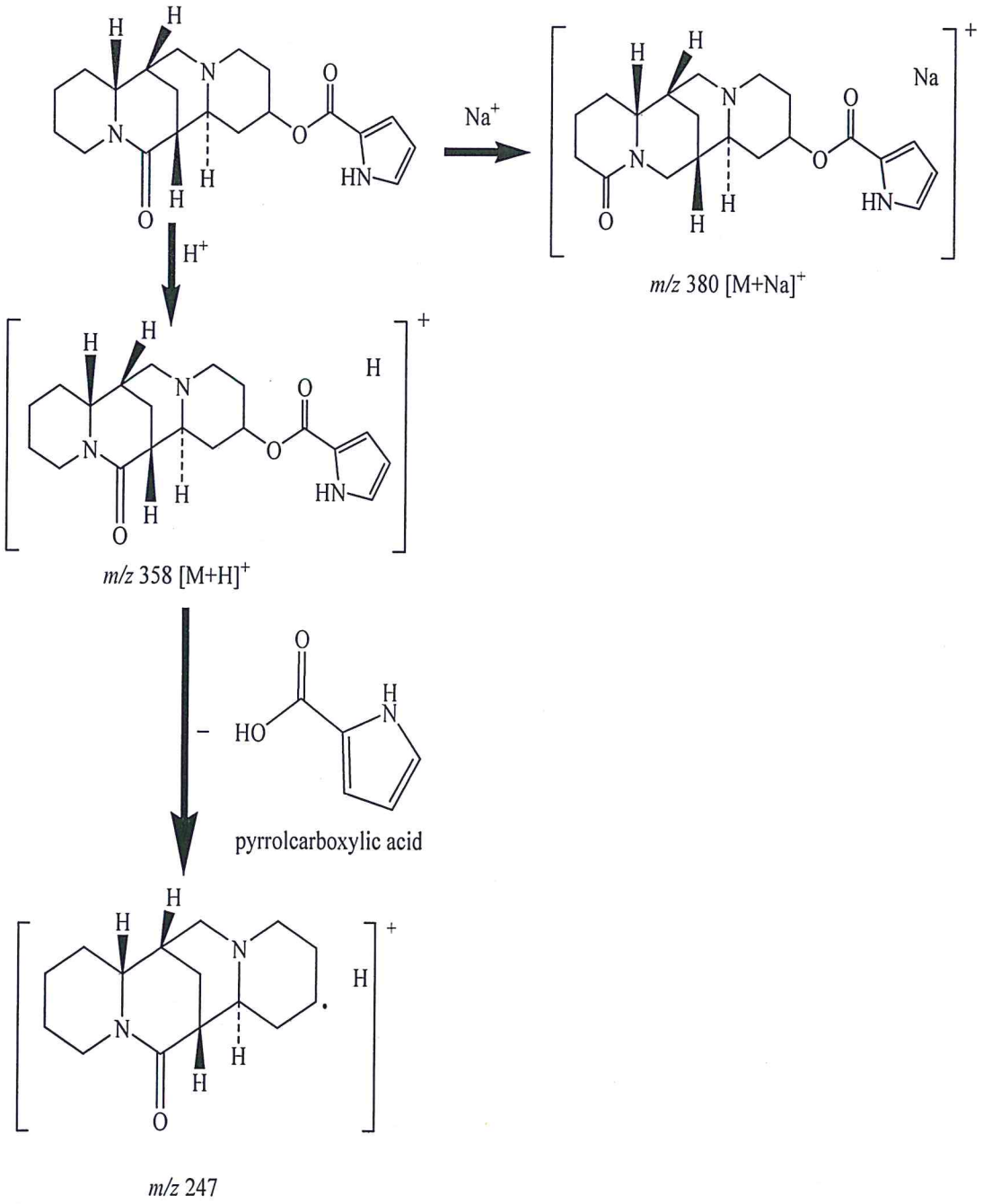


Proposed compound **9**

Possible fragmentation patterns for formation of *m/z* 358 and 247.



or



4.6.3 Characterisation of Compound 10

Compound **10** had a retention time of 21.481 min in the HPLC (Figure 8). It was an alkaloid (plate 4), had UV absorption band at λ_{max} 216 nm implying that it is non-conjugated (Figure 15c) and its spectrum shows that it is aromatic.

In the LCMS, at cone voltage of 60v, compound **10** gave a molecular ion peak $[M+H]^+$ at *m/z* 472 (Figure 15a). At the higher cone voltage of 120v, it gave a fragment peak at *m/z* 372 and another at *m/z* 494 $[M+Na]^+$ (Figure 15b), which was a sodium adduct. The Molecular weight of compound **10** was 471.

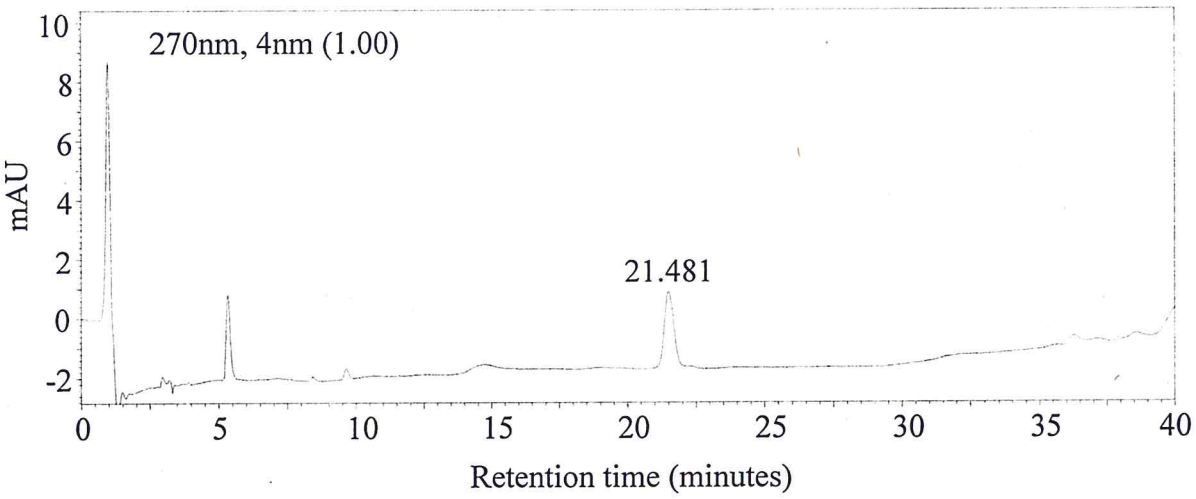


Figure 8: HPLC chromatogram of compound **10**

4.6.4 Characterisation of Compound 11

Compound **11** had a retention time of 28.959 min in the HPLC (Figure 9). It was an alkaloid (plate 4), had UV absorption band at λ_{max} 217 nm implying that it is non-conjugated (Figure 16c) and its spectrum shows that it is aromatic.

In the LCMS, compound **11** gave fragment ion peaks at *m/z* 581, molecular ion $[M+H]^+$, *m/z* 603 $[M+Na]^+$, sodium adduct, and *m/z* 273 at cone voltage of 60v (Figure 16a). Fragmentation at cone voltage of 120v (Figure 16b) gave a fragment at *m/z* 273 as the base peak. The molecular ion peak $[M+H]^+$ at *m/z* 581 was also formed but not the base peak as at the lower cone voltage. Other prominent fragment peaks were at *m/z* 419 and *m/z* 435 respectively. The molecular weight of compound **11** was 580.

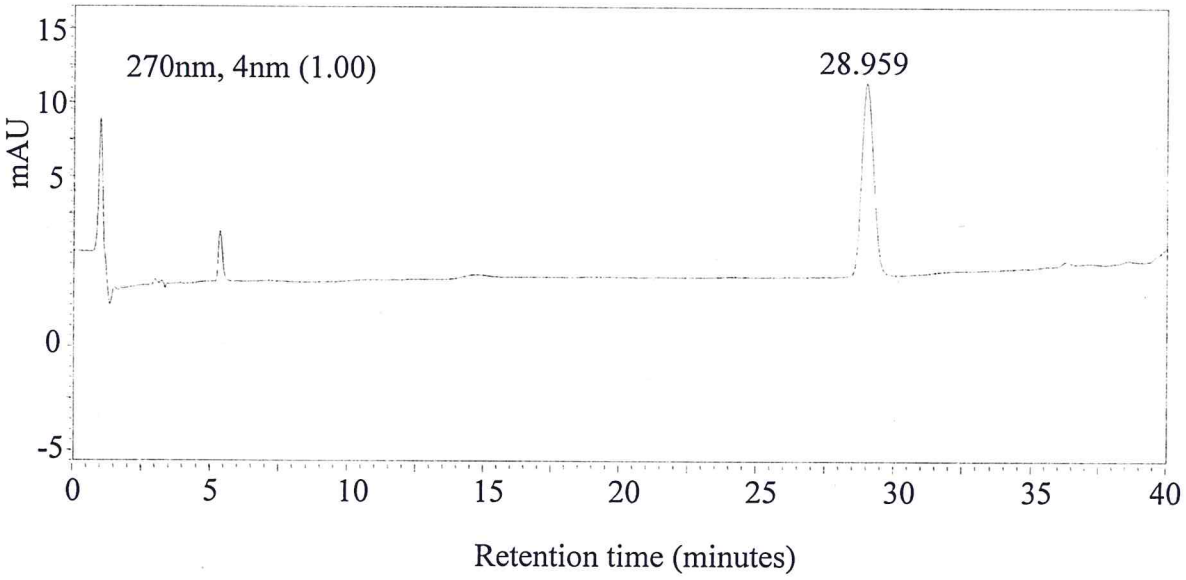


Figure 9: HPLC chromatogram of compound **11**

4.6.5 Characterisation of Compound 12

Compound **12** had a retention time of 30.475 min in the HPLC (Figure 10). It was an alkaloid (plate 4), had UV absorption band at λ_{max} 219 nm implying that it is non-conjugated (Figure 17c) and its spectrum shows that it is aromatic.

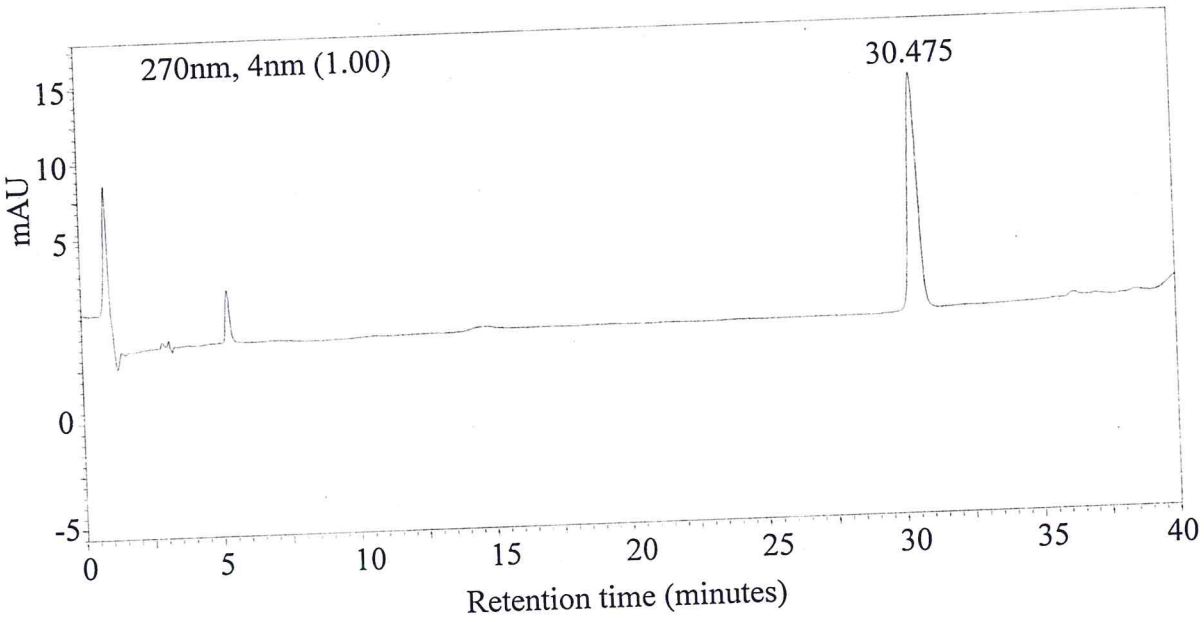


Figure 10: HPLC chromatogram of compound **12**

In the LCMS, compound **12** had fragment peaks at *m/z* 449 $[M+H]^+$ and at *m/z* 471 $[M+Na]^+$ as the only fragments at cone voltage of 60v (Figure 17a). The same peaks were observed at cone voltage of 120v (Figure 17b). The molecular weight of compound **12** was 448.

4.6.6 Characterisation of Compound 13

Compound **13** had a retention time of 33.890 min in the HPLC (Figure 11). It was an alkaloid (plate 4), had UV absorption band at λ_{max} 218 nm implying that it is non-conjugated (Figure 18c) and its spectrum shows that it is aromatic.

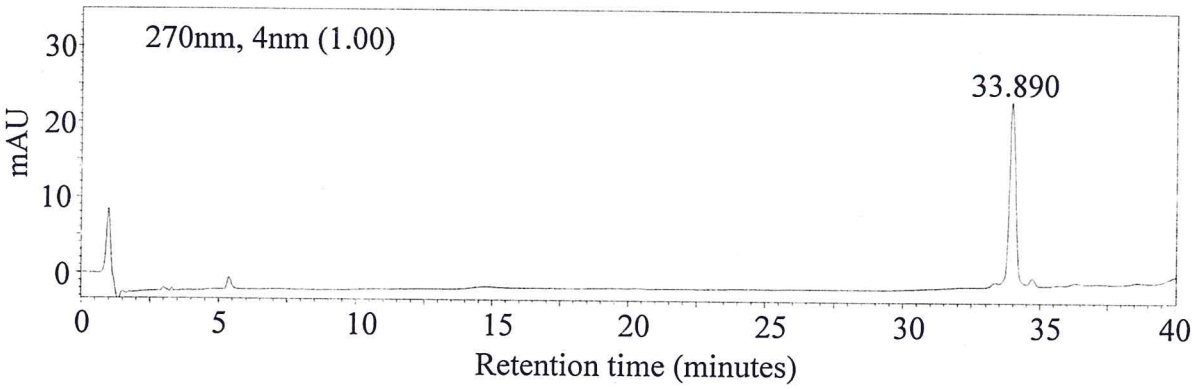


Figure 11: HPLC chromatogram of compound **13**

In the LCMS, compound **13** had a molecular ion peak at *m/z* 433 $[M+H]^+$, as the base peak at cone voltage of 60v (Figure 18a). At cone voltage of 120v the compound had fragment peaks at *m/z* 433 $[M+H]^+$, *m/z* 455 $[M+Na]^+$ and *m/z* 271 (Figure 18b). The molecular mass of compound **13** was 432.

4.6.7 Characterisation of Compound 14

Compound **14** had a retention time of 34.674 min in the HPLC (Figure 12). It was an alkaloid (plate 4), had UV absorption band at λ_{max} 216 nm implying that it is non-conjugated (Figure 19c) and its spectrum shows that it is aromatic.

In the LCMS, compound **14** had fragment peaks at *m/z* 579 $[M+H]^+$ and at *m/z* 601 $[M+Na]^+$ (Figure 19a). At the higher cone voltage of 120v an extra fragment peak at *m/z* 271 was observed (Figure 19b). In both cone voltages the molecular ion was the base peak. The molecular weight of compound **14** was 578.

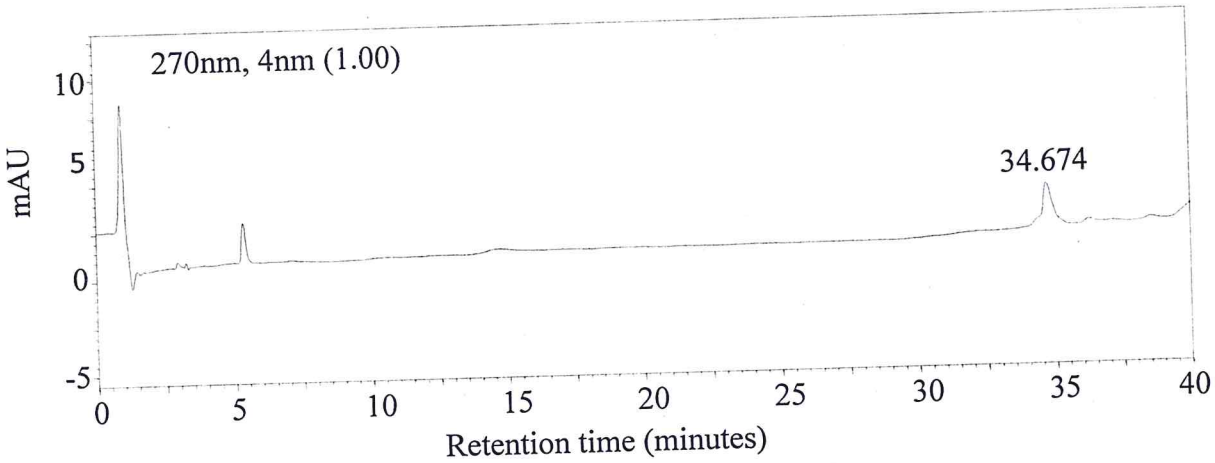


Figure 12: HPLC chromatogram of compound **14**

In the LCMS, compound **14** was eluted by isocratic 50% methanol solution, unlike the rest which were eluted by isocratic 40%. This showed that it was less polar than the rest. Compound **8** and **9** are known because they seem to have been isolated before from the same plant. It is not certain but further research has to be done to characterise these new compounds further to confirm if they were the same ones that have earlier been isolated or not. Compounds **10-14** seem not to have been isolated before. However, they need to be characterised by other spectroscopic methods like FTIR, NMR or solid probe MS to establish their functional groups and structure.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

- The results show that the acetone extract of *C. aurea* leaves significantly attracted ticks in the tick climbing bioassays.
- Fractionation of the crude extract made it more active by concentrating the active ingredients. It increased tick attraction by 4.7%.
- Lupanine, $C_{15}H_{24}N_2O$, Calpurnine and Virgiline Pyrrolcarboxylic acid ester, $C_{20}H_{27}N_3O_3$, were some of the probable compounds that contributed to the activity of *C. aurea* extract. Other alkaloids belonging to this category of tick attracting compounds are compound **10**, **11**, **12**, **13** and **14** with molecular weights of 471, 580, 448, 432, and 578, respectively. Some or all of these compounds could be working synergistically or one, two or more are active.
- The results of this study suggest that the acetone leaf extract of *C. aurea* may be used for the control of *R. appendiculatus*.

5.2 RECOMMENDATIONS

1. Further research need to be done to determine the activity of the isolated and characterised compounds against ticks.
2. Extensive study has to be done to determine whether these bioactive compounds are found in other parts of the plant like roots, stem, seedpods and seeds.

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APPENDICES

Figure 13a: LCMS chromatogram of compound **8** at 60v

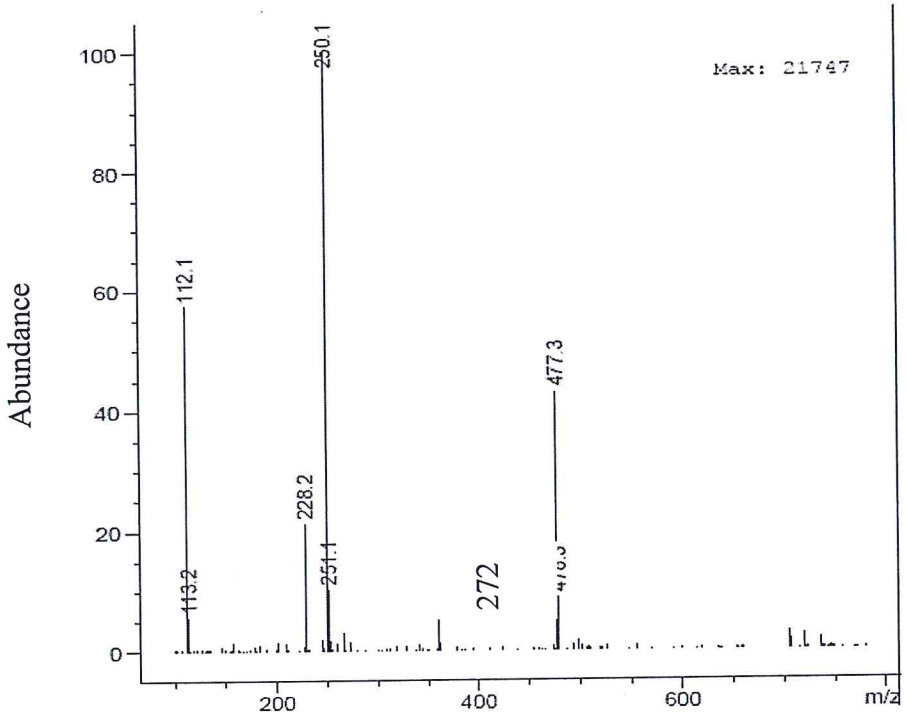

Figure 13b: LCMS chromatogram of compound **8** at 120v

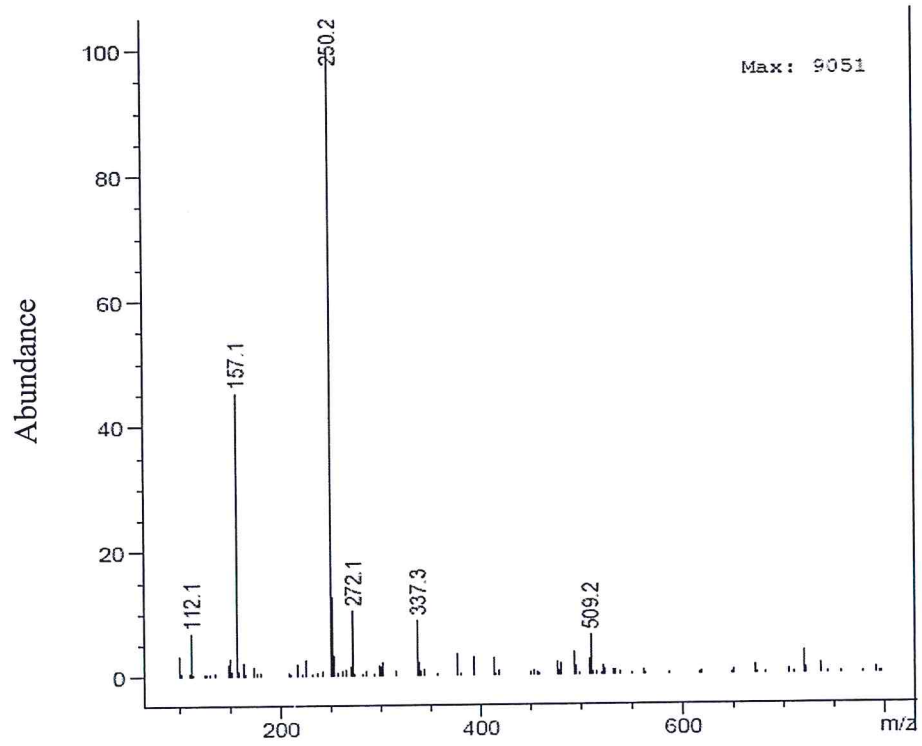

Figure 13c: UV Chromatogram of compound **8**

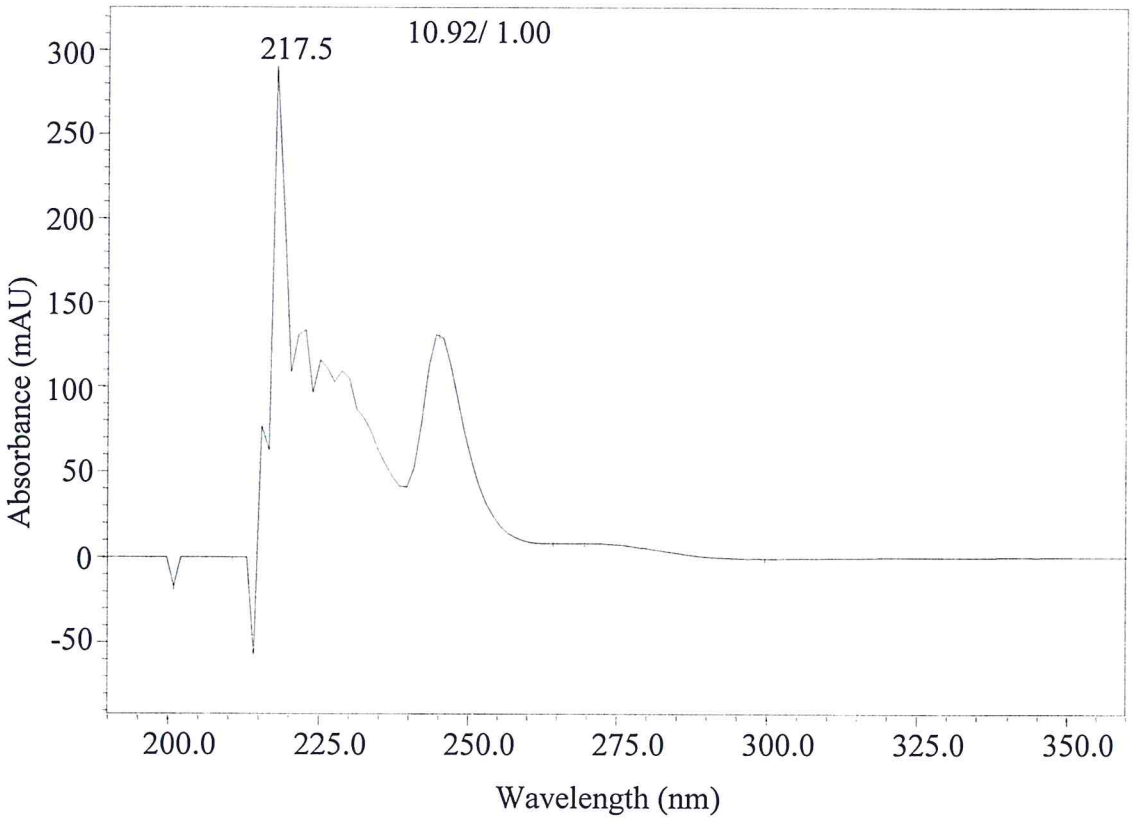

Figure 14a: LCMS Chromatogram of compound **9** at 60v

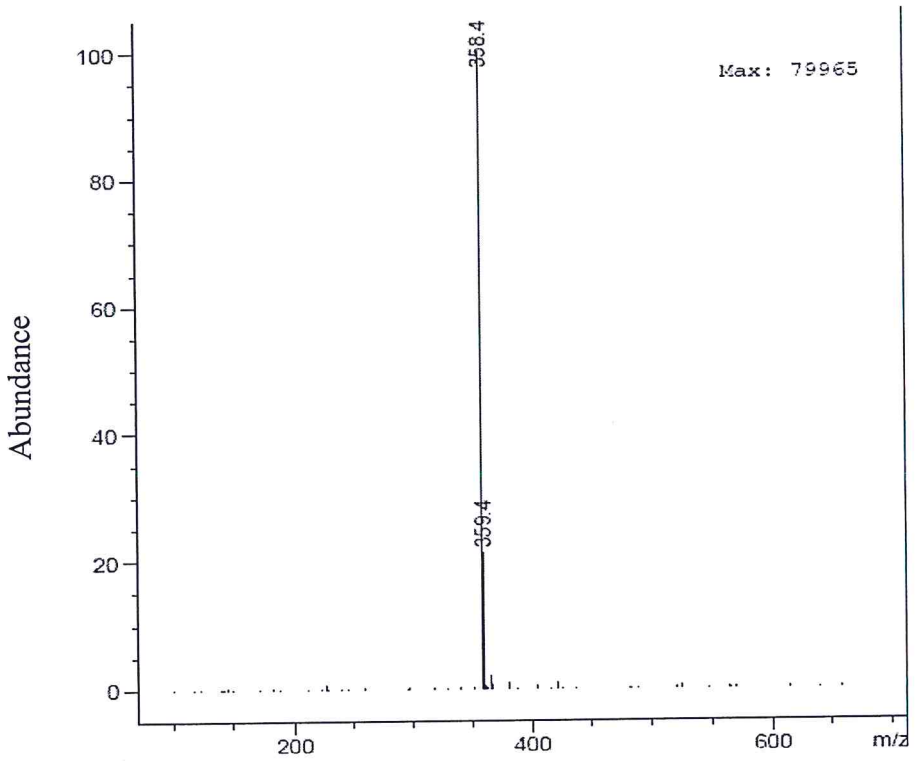

Figure 14b: LCMS chromatogram of compound **9** at 120v

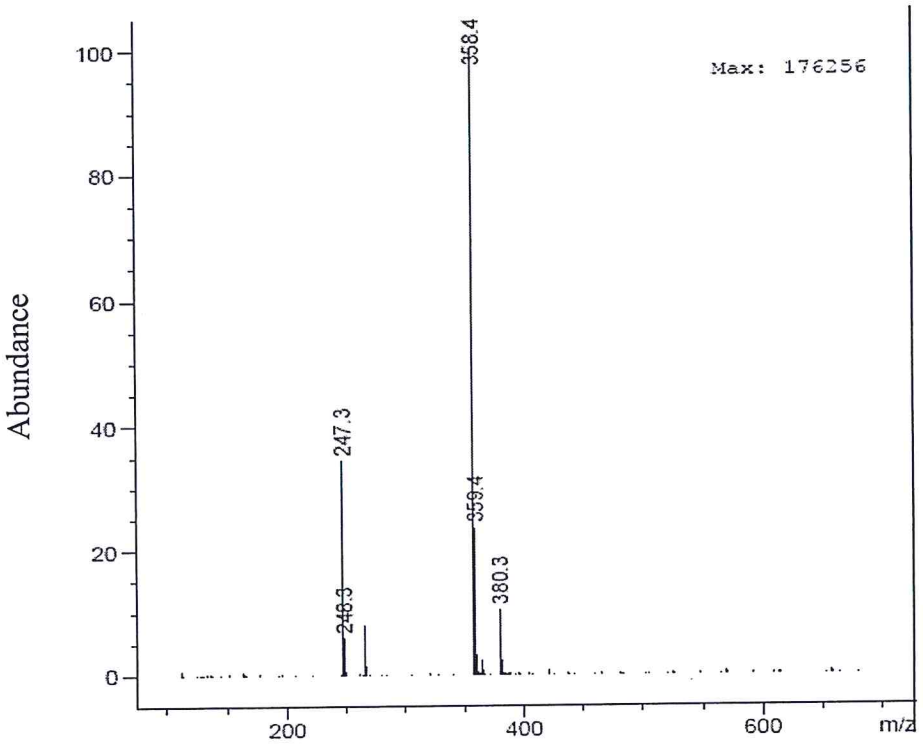

Figure 14c: UV Chromatogram of compound **9**

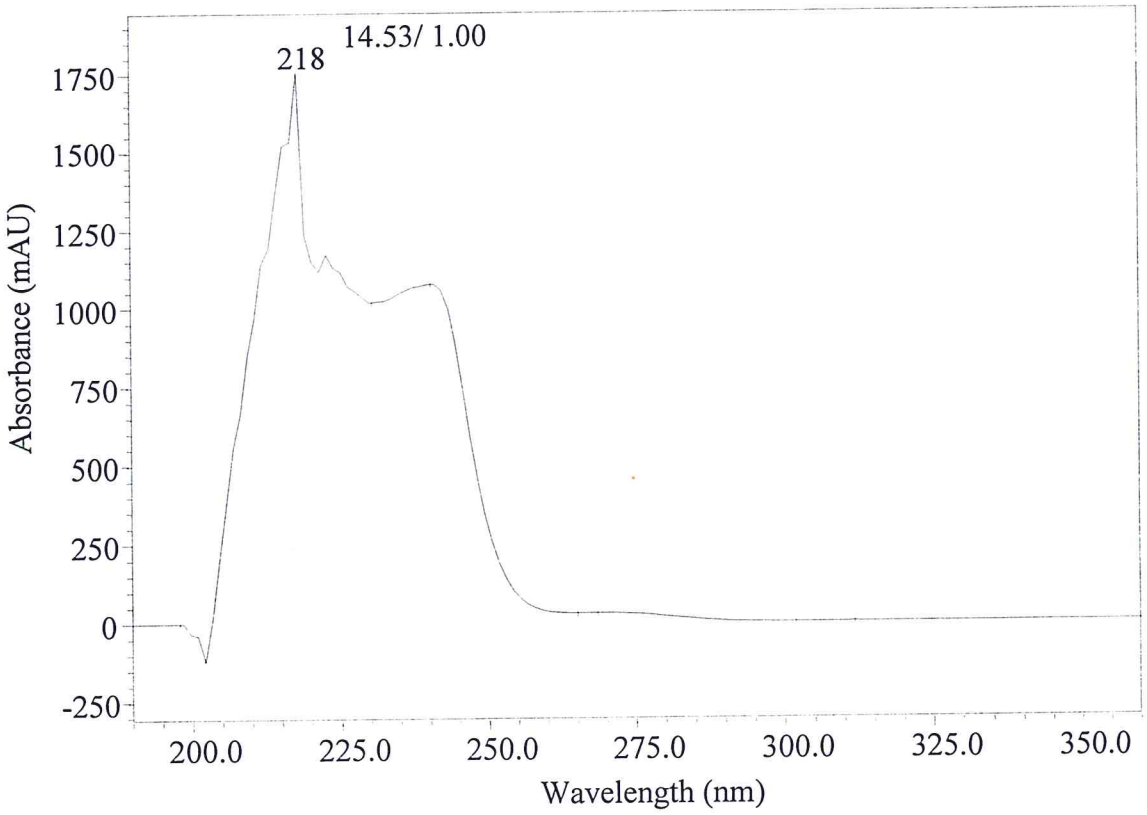

Figure 15a: LCMS chromatogram of compound **10** at 60v

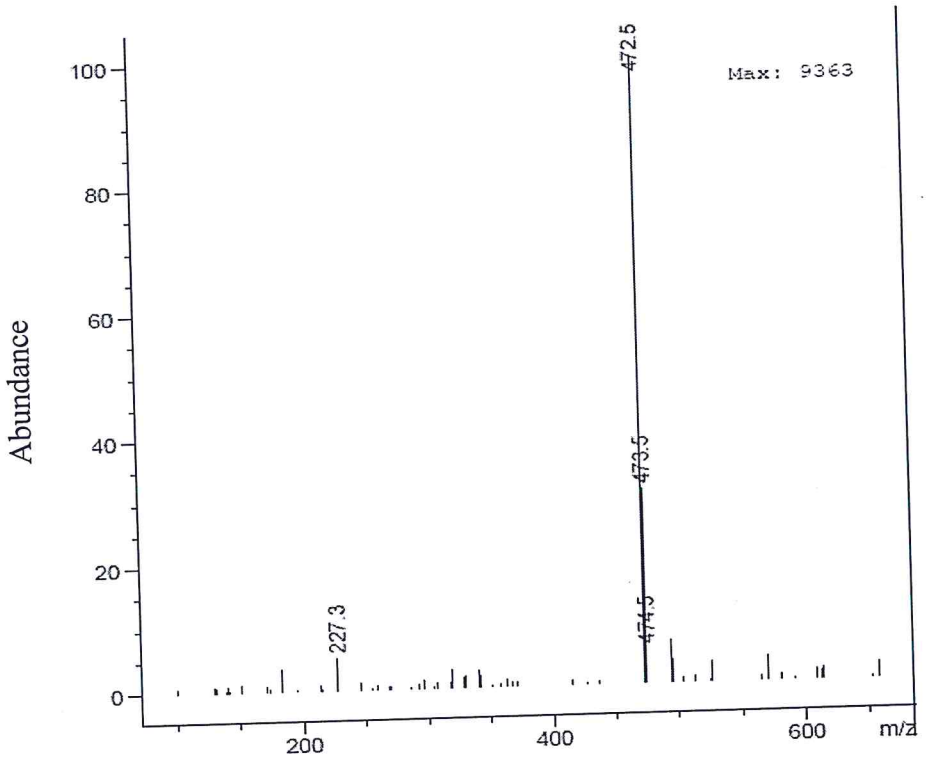

Figure 15b: LCMS chromatogram of compound **10** at 120v

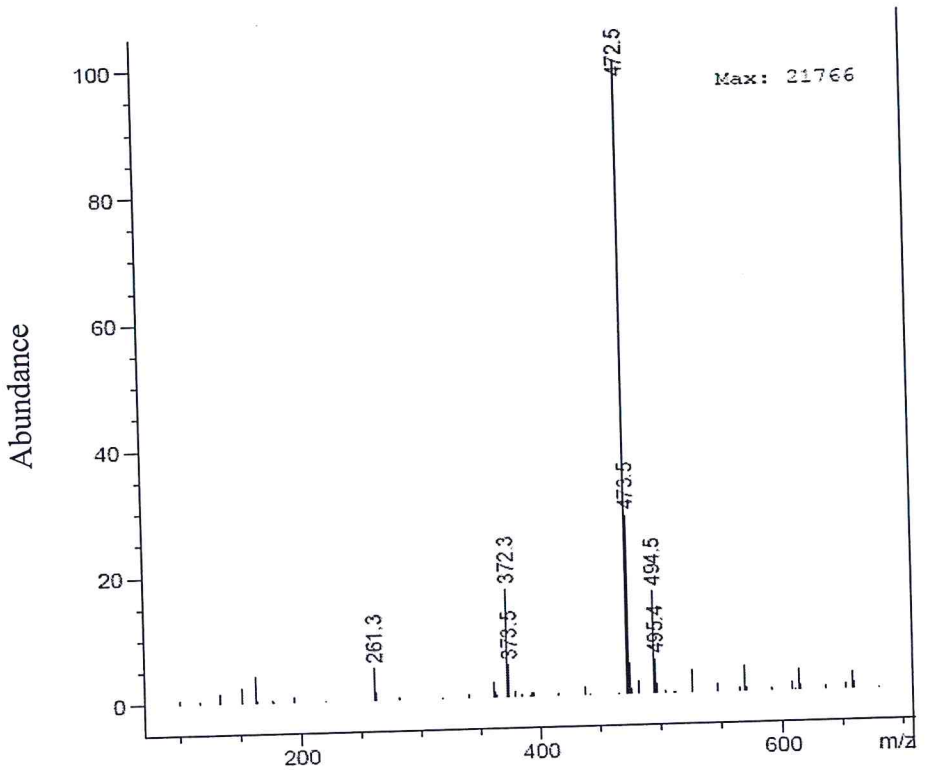

Figure 15c: UV Chromatogram of compound **10**

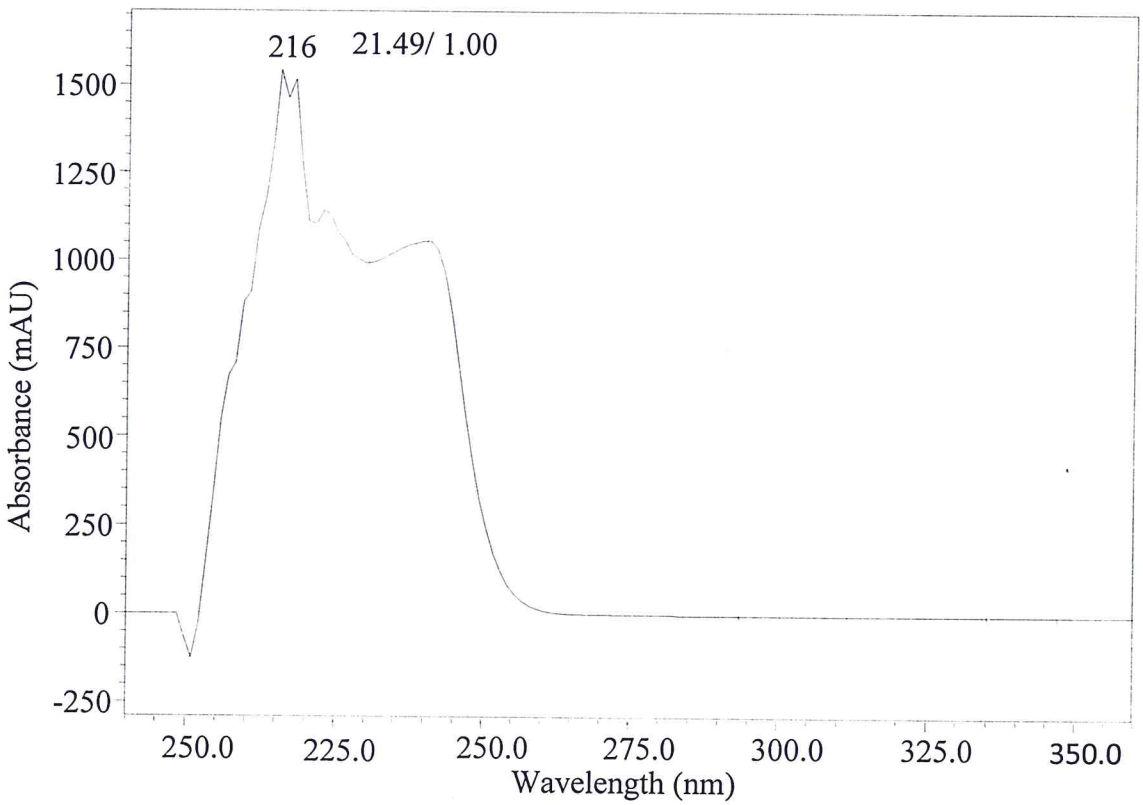

Figure 16a: LCMS chromatogram of compound **11** at 60v

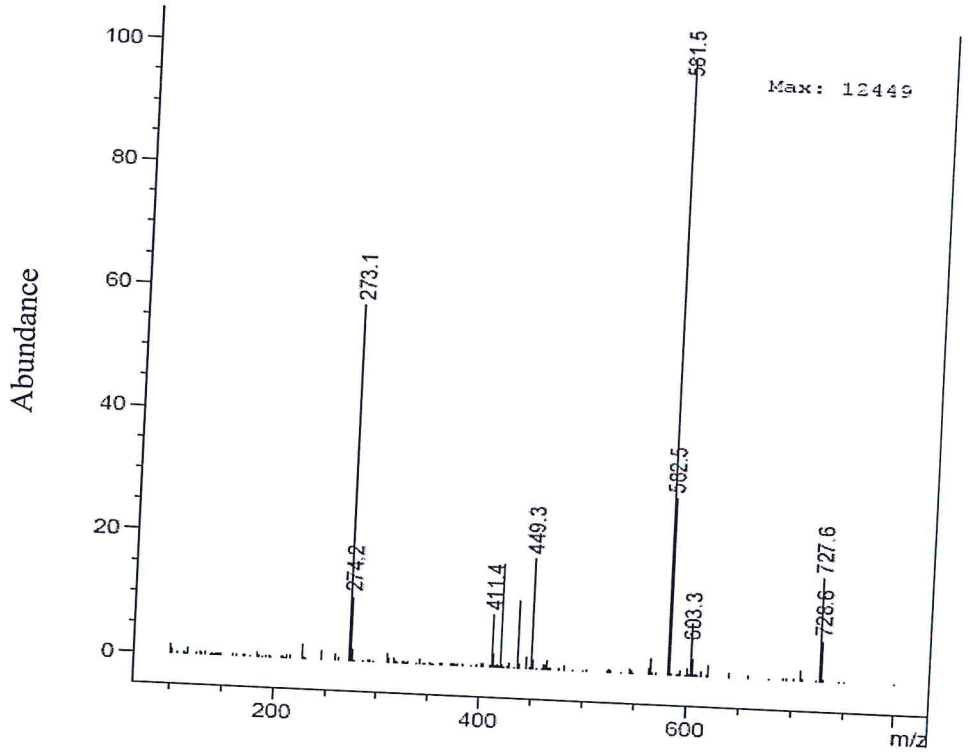

Figure 16b: LCMS chromatogram of compound **11** at 120v

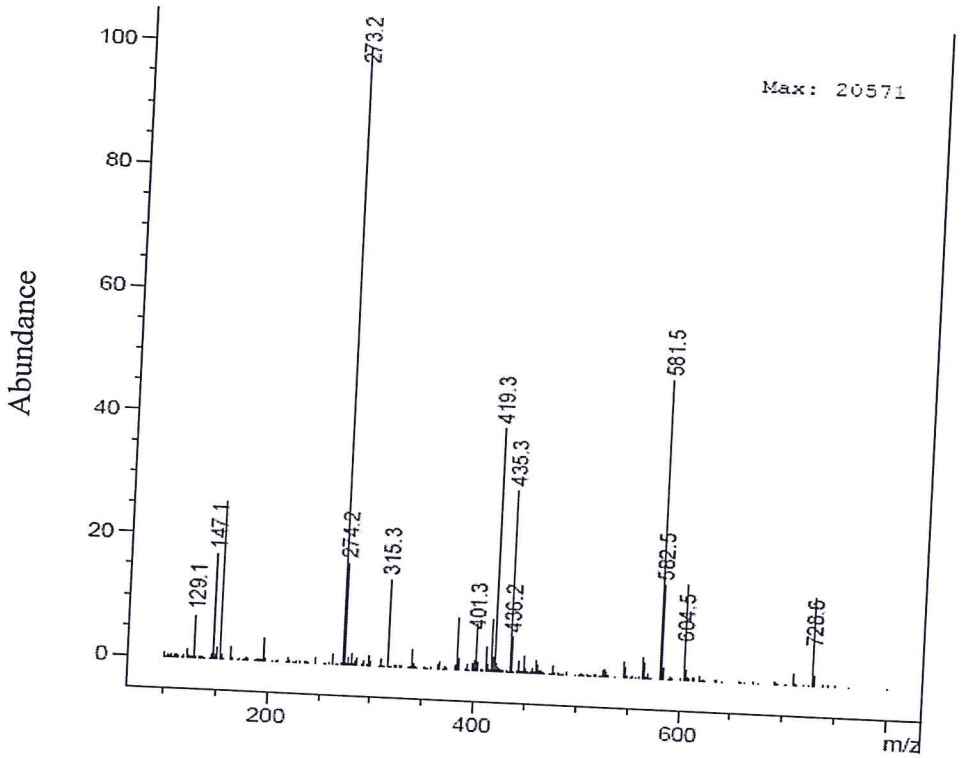

Figure 16c: UV chromatogram of compound **11**

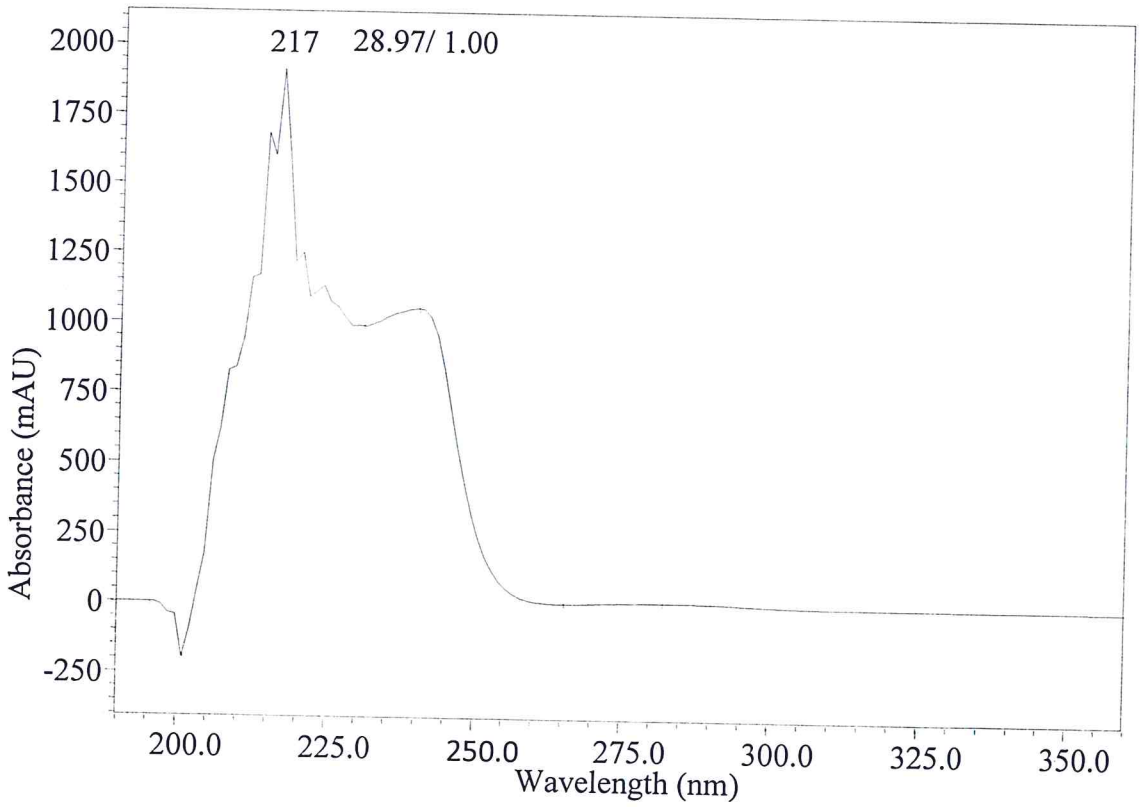

Figure 17a: LCMS chromatogram of compound **12** at 60v

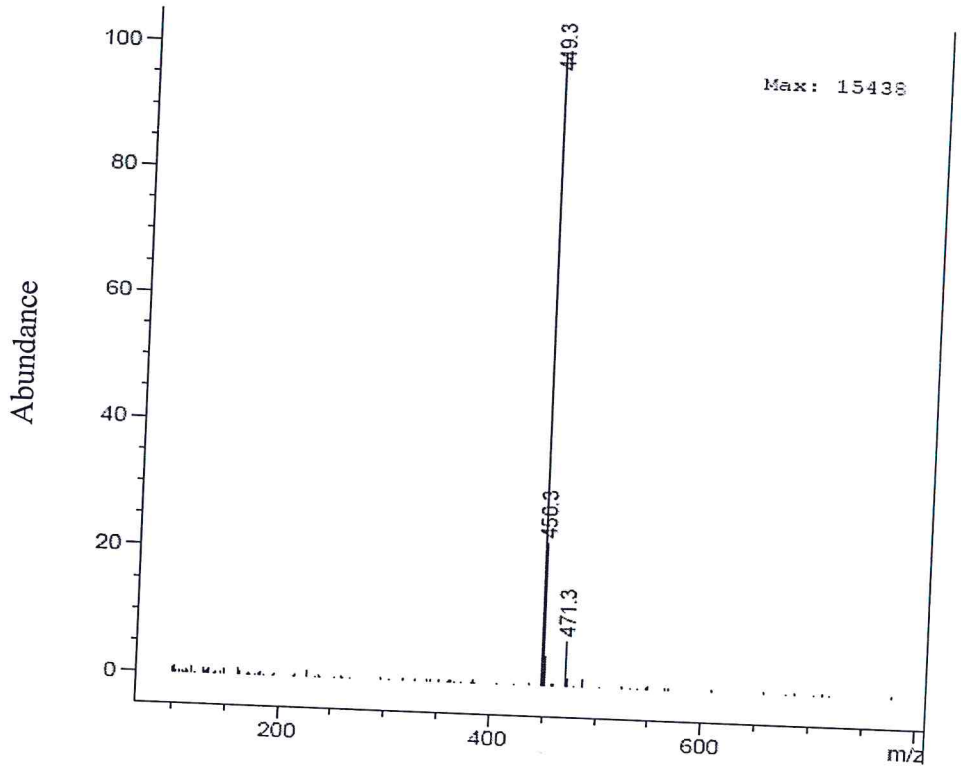

Figure 17b: LCMS chromatogram of compound **12** at 120v

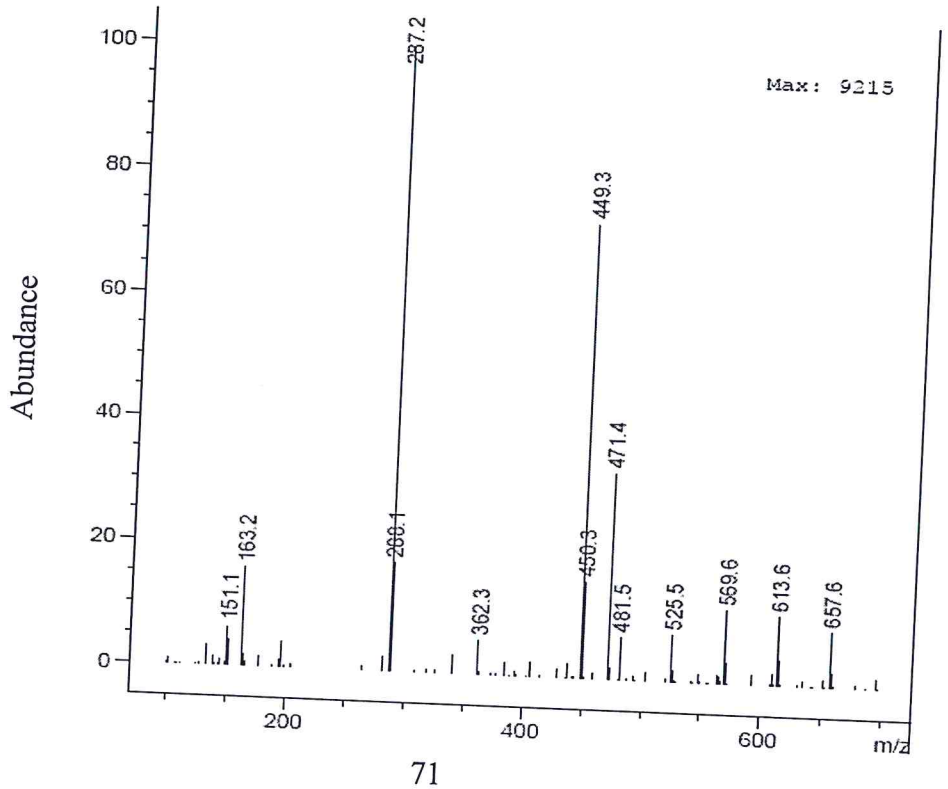

Figure 17c: UV Chromatogram of compound **12**

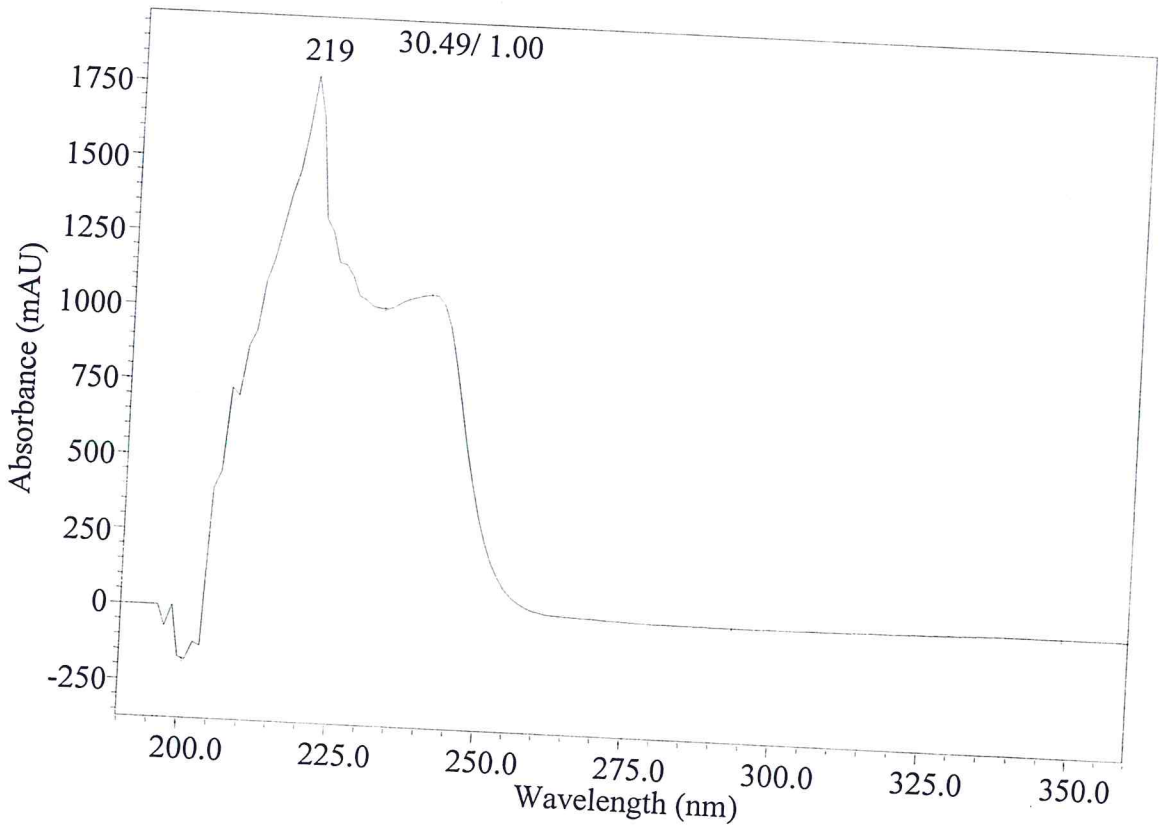

Figure 18a: LCMS chromatogram of compound **13** at 60v

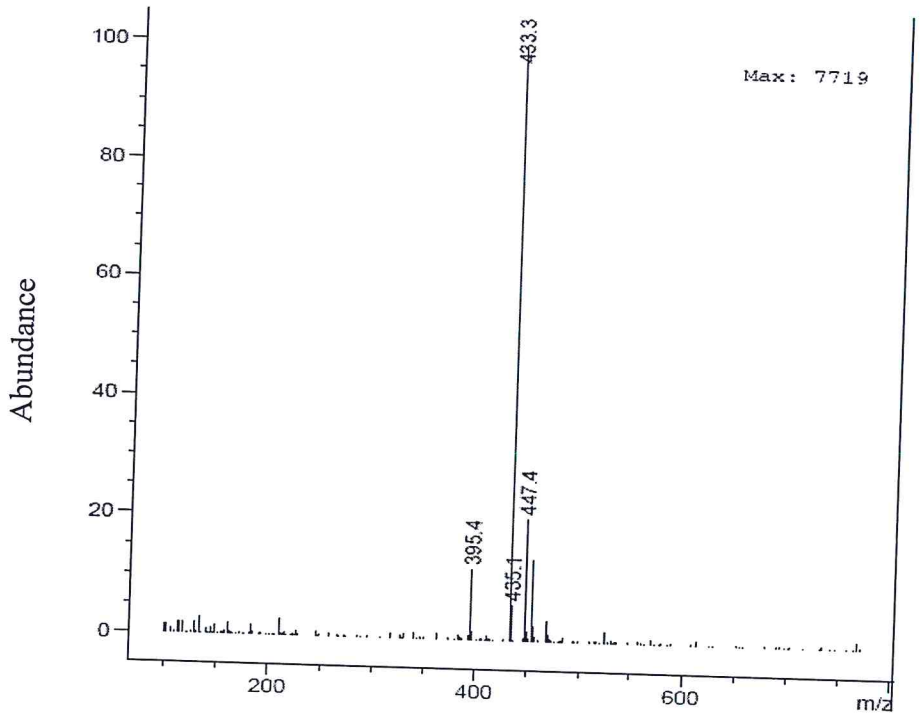

Figure 18b: LCMS chromatogram of compound **13** at 120v

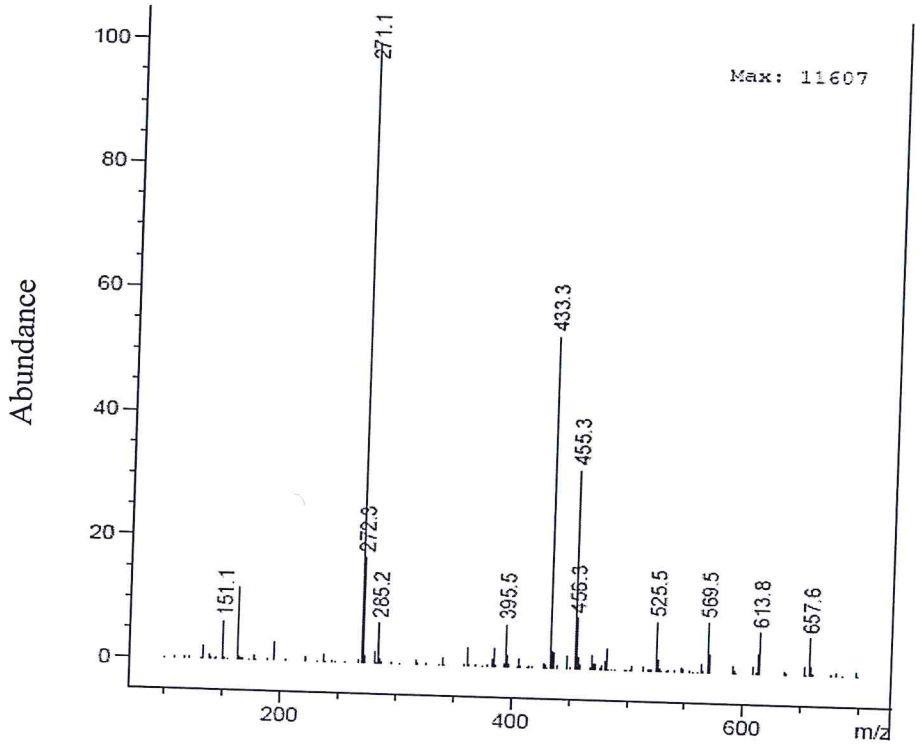

Figure 18c: UV Chromatogram of compound **13**

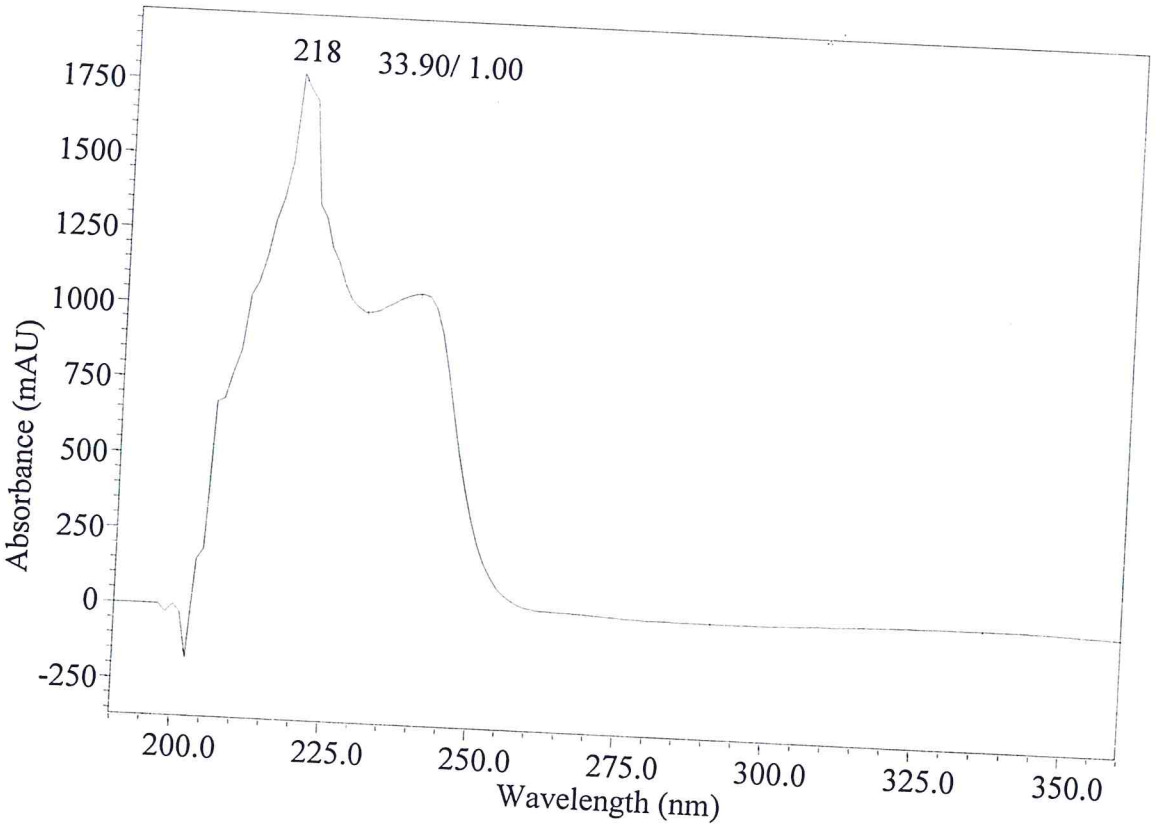

Figure 19a: LCMS chromatogram of compound **14** at 60v

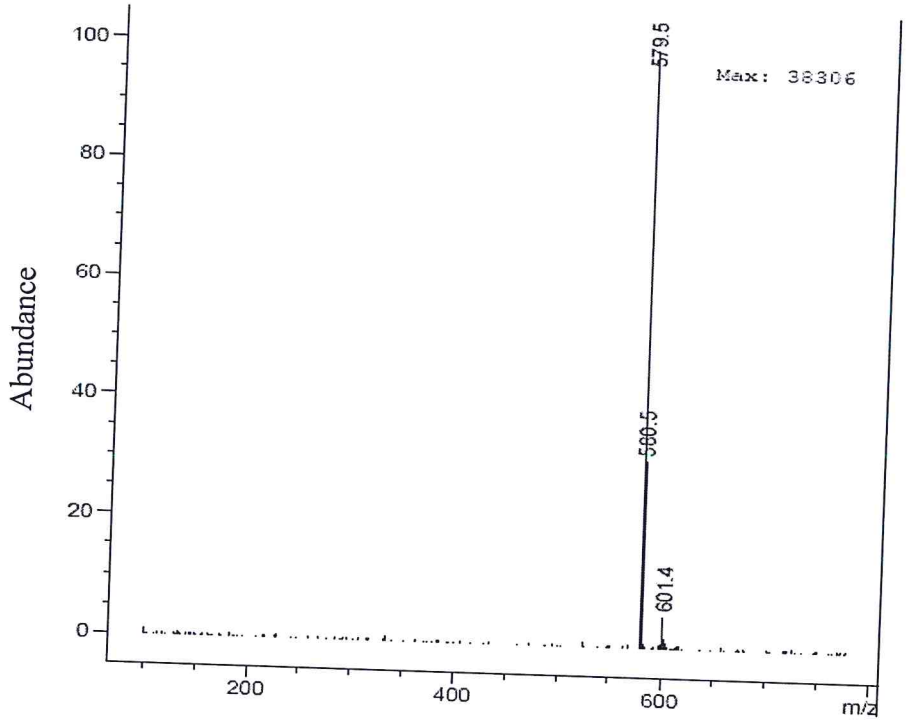

Figure 19b: LCMS chromatogram of compound **14** at 120v

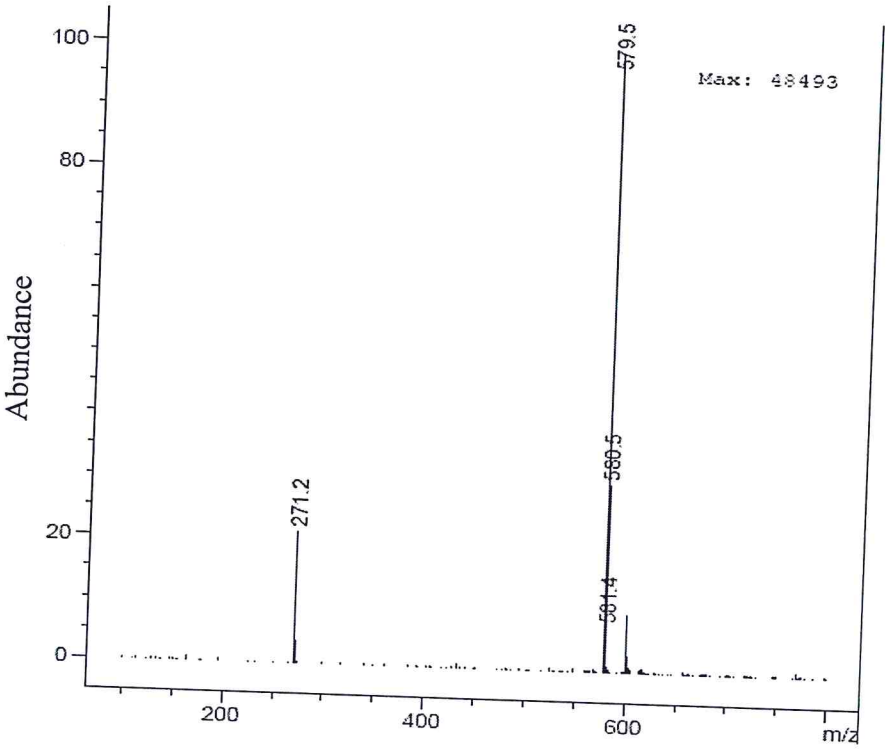

Figure 19c: UV Chromatogram of compound **14**