BIO-PROSPECTING FOR ANTI-MOSQUITO COMPOUNDS FROM *TURRAEA ABYSSINICA* AND *TURRAEA CORMICORPIA*

By: Joseph Odero Owino

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DECLARATION

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

Signed..... Date.....16/05/05

Joseph Odero Owino
Jomo Kenyatta University of Agriculture and Technology

This thesis has been submitted for examination with our approval as supervisors.

Signed..... Date.....16/5/05

Dr Mary Wambui Ndung'u
Chemistry Department
JKUAT
NAIROBI

Signed..... Date.....16/05/05

Prof. Ahmed Hassanali
Chemical Ecology Department
ICIPE
NAIROBI

Signed..... Date.....19/05/05

Prof. George Thuku Thiong'o
Chemistry Department
JKUAT
NAIROBI

DEDICATION

To the memory of my late sister Hellen Lilian Amolo

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LIST OF ABBREVIATIONS

BCE…Behavioural and Chemical Ecology

$CDCl_3$…Deuterated chloroform

$CHCl_3$…Chloroform

COSY…Correlation Spectroscopy

DDT…Dichloro-diphenyl-dichloroethane

H_2O…Water

HMBC…Heteronuclear Multiple Bond Correlation

HMQC…Heteronuclear Multiple Quantum Correlation

HPLC… High Perfomance Liquid Chromatography

HREIMS…High Resolution Electron Impact Mass Spectroscopy

ICIPE…International Centre of Insect Physiology and Ecology

KBr… Pottasium Bromide

MeOH… Methanol

MIM…Multilateral Initiative on Malaria

MS…Mass Spectroscopy

NMR..Nuclear Magnetic Resonance

NOE…Nuclear Overhauser Effect

NOESY… Nuclear Overhauser Effect Spectroscopy

TDR…Tropical Disease Research

TLC…Thin Layer Chromatography

WHO… World Health Organization

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ABSTRACT

Malaria, transmitted by mosquitoes of the genus *Anopheles*, is the most important parasitic disease worldwide. It affects 40% of the global population mostly within the tropical world. Yearly, over one million children under the age of five die in Africa as a result of malaria.

Environmental hazards associated with presently used synthetic insecticides, development of drug resistance by the malaria parasite and insecticide resistance by the vectors have greatly hampered malaria control. The search for alternative control measures for the parasite and vector is of significant importance.

The genus *Turraea* belongs to the family Meliaceae. Meliaceae is characterised by the presence of tetranortriterpenoids (limonoids), a group of compounds that exhibit a wide variety of biological properties including anti-insect, anti-protozoa, anti-bacterial and anti-fungal activities

In the present study, the root bark of *T. abyssinica* and *T. cormicorpia* were extracted with methanol and partitioned between water and chloroform. The activity of crude methanol and chloroform extracts was tested against larvae and adults of *Anopheles gambiae sensu stricto* (Diptera: Culicidae). As larvicides, the methanol extract of *T. cormicopia* was the most active (LD_{50} 202 ppm). The chloroform extract of *T. cormicopia* was the most active as an adulticide (LD_{50} 302.1 ppm).

Partitioning of the methanolic extracts between chloroform and water, followed by silica gel chromatography of the organic extract gave limonoids-rich fractions that had larvicidal activity higher than those of the corresponding crude extracts.

Column chromatography of the chloroform fraction followed by semi-preparative HPLC yielded a new limonoid, 1α-12α-diacetoxy-1,2-dihydro-7-deacetyl-3β-7α-dihydroxyazadiron, naturally occurring 12α-acetoxy-7-deacetylazadiron together with the known mzikonone.

The structures of these compounds were elucidated using spectroscopic methods (IR UV, MS, ^{1}H-, ^{13}C- NMR, gradient COSY, and gradient HMBC experiments). Stereo chemical assignments were made by gradient NOE spectroscopy.

CHAPTER ONE

1.0 INTRODUCTION

1.1 General introduction

Malaria is by far the most devastating and deadly parasitic disease in the world. It is a public health problem in more than 90 countries inhabited by over 2.4 billion people (approximately 40% of the world's population). The disease is estimated to cause up to 500 million clinical cases and 2.7 million deaths each year (WHO, 2002). Of the people infected, Africa accounts for over 90 % of the reported cases. Experts foresee as much as 20% annual increase in Africa's rate of malaria related illnesses and deaths (WHO, 2002). The mortality rate is high in children under five years and pregnant women (WHO, 2002).

The cost of malaria when viewed in economic terms is enormous. In most African countries, over a quarter of a family's income goes towards the cost of malaria treatment (WHO, 2002). This is in addition to the cost of prevention or the opportunity cost of labour lost during illness. Each bout of malaria causes its victim to forego an average of twelve days productive output. People are more at risk during the warm and rainy season. This is usually when there is most agricultural activity. Malaria and fear of malaria prevents investment and tourism in new regions. The global effects of malaria threaten public health and productivity on a broad scale and impede economic progress in many countries (WHO, 2002).

Malaria, which had been effectively controlled in many parts of the world through the use of antimalarials like quinine and chloroquine, insecticides like *p,p*-dichloro-2,2-diphenyl-1,1,1-trichloroethane (DDT) and pyrethrins among others, is undergoing resurgence (WHO, 1997). Most epidemics have been linked to climatic change, (Mouchet *et al.,* 1998; Bouma *et al.,* 1997; Lindsay and Birley, 1996; Jetten *et al.,* 1996) and drug and insecticide resistance (WHO, 1999). Demographic changes have resulted in more people moving into already densely populated areas thereby increasing transmission. In areas where development projects like agro forestry, irrigation projects, road construction

and mining have been introduced, new breeding sites are created (Lindsay & Martens, 1998). In many regions malaria control programs have deteriorated or been abandoned due to high costs of sustaining them. Renewed efforts in malaria control are required.

In a bid to develop environmentally safe and biodegradable insecticides against mosquitoes and other pests, the search for such compounds has been directed towards the plant kingdom. Plants serve as vast reservoirs of naturally bio-degradable chemicals, many of which may have evolved in the defence to herbivores (Champagne, 1989). Plant-derived extracts and phytochemicals, which once formed the basis of pest control technology, are again being scrutinized for potentially useful products or as models for new classes of synthetic insecticides (Isman *et al.,* 1995).

Botanical pesticides are plant natural products that belong to the group of secondary metabolites, examples of which include alkaloids, terpenoids and phenolics (Torto and Hassanali, 1997). The biological activity of these products on insects, nematodes and phytopathogenic fungi, among other organisms is well documented in the recent explosion of literature in chemical ecology (Arnason *et al.,* 1993). Phytochemicals derived from various botanical sources have provided numerous beneficial uses ranging from pharmaceuticals to insecticides. These chemicals comprise of repellents, feeding and oviposition deterrents, growth inhibitors, sterilants and toxicants (Arnason *et al.,* 1993).

Perhaps within the large group of natural substances, many new alternatives can be found to reduce the reliance on expensive synthetic insecticides for the control of insect pests and, more importantly for the control of vectors of disease such as malaria in Africa.

1.2 Malaria parasite

Ross (1897) first observed the oocyst and all stages of the parasite *(Plasmodium)* in mosquitoes. Grassi and Felleti (1900) described the developmental cycles of *P. falciparum* and *P. vivax*. They showed that only *Anopheles* mosquitoes transmit human malaria. Malaria in humans has since been shown to be caused by one or more of the four species of *plasmodium: P. vivax, P. ovale, P. malariae* and *P. falciparum*. However, *P.*

falciparum is the most virulent species and predominates in sub-Saharan Africa, Asia, Oceania and Amazons (WHO, 1997).

1.3 Malaria disease

Malaria attack is caused by innoculation of the parasite (*Plasmodium*) by an infected female *Anopheles* mosquito. The parasite undergoes a life cycle constituted of two phases: sexual (sporogony) and asexual (shizogony). Asexual phase occurs in man. Sporozoites innoculated by an infected mosquito develop and multiply in the liver after which they develop into merozoites. The merozoites enter the red blood cells, multiply, rupture the erythrocytes and invade other red blood cells. The incubation period lasts between 6-16 days depending on the parasite species.

The sexual phase of parasite life cycle takes place in female *Anopheline* mosquitoes. A mosquito sucking blood from an infected person picks up the gametocytes that fuse to form a zygote. The zygote penetrates the mosquito stomach to form an oocyst within which large numbers of slender sporozoites develop. Some of these sporozoites pass through the salivary gland. When an infected mosquito feeds on a new host, it inoculates the infective sporozoites from its salivary gland into the host and the asexual life starts again. Incubation period of the parasite in mosquitoes varies with temperature and the *Plasmodium* species.

1.4 Malaria vectors

All human malaria vectors belong to the genus *Anopheles* within the family Culicidae that consists of two major sub-families, the Anophelinae and Culicinae. Some 60 species of *Anopheles* are vectors of human malaria and are found in the tropical and sub-tropical regions below 2000 m (Manson and Bell, 1987).

However, there are only three efficient vectors of malaria; *A. gambiae, A. arabiensis* and *A. funestus* (Temu *et al.*, 1998; Palsson, 1999). In sub-Saharan Africa, there are only two important Anopheles species, *A. gambiae* and *A. funestus*. These two species are largely anthropophilic while *A. arabiensis* also feeds on cattle besides humans. *A. gambiae* is the most ubiquitous and breeds in quiet water bodies exposed to sunlight whereas *A. funestus* is confined to grassy edges of slow flowing streams

(Wigglesworth, 1976). Anopheline larvae are found in a variety of aquatic habitats such as fresh and salt marshes, lakes, mangrove swamps, rice fields, streams, ponds and even small temporary water bodies like puddles, tree holes, hoof prints, tyre marks, wells and domestic water storage pots. In the tropics, larval development takes seven days while pupal stage lasts 3 days. Both female and male mosquitoes feed on nectar and other plant fluids; however, female mosquitoes also feed on blood from vertebrates (Clement, 1992). One blood meal is required to produce a batch of 30-150 eggs. In the family Culicidae, *Anopheles* species have the most regular gonotrophic cycle (blood feeding and egg laying), which takes 2-3 days depending on temperature. Different *Anopheles* species have different cycles of activity, some attack at dusk, some during the day and some at night. Most species are nocturnal. Some species have a strong attraction to man (anthropophilic) and prefer feeding on man (anthropophagic). Zoophilic and zoophagic species are attracted to and feed on animals respectively. Endophilic and endophagic species normally rest and feed indoor respectively. Exophilic and exophagic species rest and feed outdoor. Garret-Jones *et al.*, (1980) found out that some species may feed indoors on man and fly outside to rest before oviposition in water.

1.5 Malaria control strategies

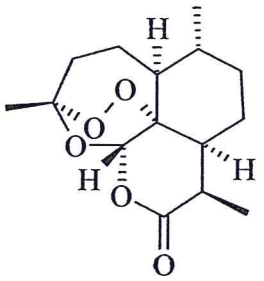
Various strategies have been adopted towards malaria control. These include chemotherapy, vaccine development and vector control {personal protection (insecticide treated nets (ITNs) and repellents), insecticides and larvicides}.

1.5.1 Chemotherapy

A limited number of drugs for the treatment of malaria are available today. Because of the worsening problems of drug resistance in many parts of the world (Trigg *et al.*, 1997), adequate treatment of malaria is becoming increasingly difficult. Although some new drugs (mefloquine, halofantrin, malaron, atavavaquone and artemisinin derivatives) have appeared in the last 20 years, new drugs (especially inexpensive and affordable ones) are badly needed (WHO, 1998). Chloroquine is an extremely safe and cheap drug; however in Africa, Asia and areas of South America, the resistance levels are

high. 4-amino quinoline-based dugs such as quinine have been employed successfully in efforts to treat malaria (WHO, 1999).

Artemisinin (**1**) isolated from the Chinese medicinal plant, *Qing hao* (*Artemisia annua*) (Asoka *et al.*, 1993), represents one of the most remarkable success stories of antimalarial compounds from plants. This metabolite is a sesquiterpene lactone with an unusual endoperoxide group essential for its activity. Schizontocidal activity of artemisinin and several of its derivatives against *Plasmodium* strains resistant to all known antimalarial drugs, with virtually no toxicity, have been well evaluated in clinical tests performed in clinical tests in China (Klayman, 1993). However, artemisinin derivatives require long treatment courses and when used alone, recrudescence may occur (WHO, 1998).



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1.5.2 Malaria vaccine

In the past decade considerable progress has been made in the search for a malaria vaccine. An effective vaccine would constitute a powerful addition to malaria control tools. The three main types of vaccines being developed are:

Anti-sporozoite vaccines are designed to prevent severe infection (Franke *et al.*, 1999). Anti-asexual blood stage vaccines are designed to prevent severe manifestations of the disease and the transmission blocking vaccine are designed to arrest the development of the parasite in the mosquito (WHO, 1998). The hope is that an effective vaccine will be available within the next 7-15 years; however, development of such vaccines is complicated by the parasites ability to change their immunological identity and thereby conceal themselves from the immune responses that might otherwise be stimulated by a vaccine. Mapping of the malaria parasite genome is being done by a consortium of partners (WHO, 1998). Knowledge of the genome will open more rational ways of

discovering new vaccines. The development of a vaccine still may not be an easy task because a different set of genes is probably switched on at each of the four stages of the parasites extraordinary complex life cycle.

1.5.3 Vector control

In the absence of an effective vaccine or chemotherapeutic agent, renewed emphasis must be given to vector control.

1.5.3.1 Use of insecticide treated nets (ITNs)

The use of insecticide treated bed nets (ITNs) over the last decade has been shown to be an effective tool to control childhood mortality and morbidity due to malaria. The distribution and use of ITNs for the control of *A. gambiae*, successfully shown in five large scale studies, carried out in four African countries, Gambia, Ghana, Burkina Faso and Kenya (Lengeler, 1996), has provided renewed hope for the development of alternative malaria control tools. In Gambia an overall mortality reduction of 63% was observed (Alonso *et al.*, 1991). In coastal Kenya where transmission pressure is in the order of 10-30 infectious bites per person per year, and coverage of the population high, reduction of overall mortality was in the order of 3% (Neville *et al.*, 1996). In Ghana, with the highest transmission pressure of 100-300 infectious bites per person per year on average, overall mortality was in the order of 17% (Binka *et al.*, 1996).

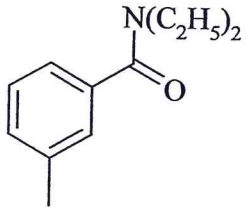
At present, synthetic pyrethroids (SPs) are the most commonly used anti-mosquito agents on ITNs. However, even though bed nets are now available in many rural areas, the insecticide is not. Under dosing and untimely re-impregnation may increase the risk of susceptibility of malaria vectors to SPs.

Development of resistant strains of mosquitoes and the public fear of environmental hazards of the insecticides themselves is a greater barrier to the success of malaria elimination. Steadily rising costs of materials and labour have made current control efforts increasingly difficult. Thus the evaluation of the potential of using local plants as repellents, insecticides or natural insect growth regulators (IGRs) on ITNs will contribute novel approaches in providing cheap and reliable control strategies for mosquitoes in the rural communities. Naturally occurring bioactive compounds, which

may be less likely to cause ecological damage, provide a multifactorial selective pressure that slows the development of resistance in pests (Arnason *et al.*, 1989).

1.5.3.2 Repellents

Repellents are substances that are not acceptable to pests and disturb their sensory receptors or are sensed as alarm signals. Repellents may be mildly poisonous to the target organism (Mafong and Kaplan, 1997). So far, a limited number of synthetic or plant-based compounds have been shown to provide satisfactory protection against mosquitoes. The most commonly used compound is N, N-diethyl-*m*-toluamide (DEET) (**2**).



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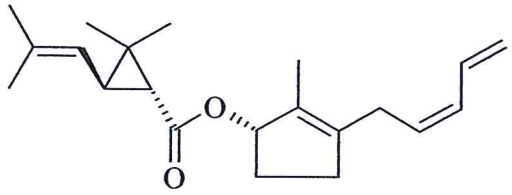
Questions have been raised about its safety, effectiveness against some species of Anopheles mosquitoes in addition to attacking paint and some hard plastics (Stinecipher and Shah, 1997).

1.5.3.3 Insecticides

In mid 1940s to mid 1960s, *p,p*-dichloro-2,2-diphenyl-1,1,1-trichloroethane (DDT), was the most successful insecticide (Collins and Paskewitz, 1995). By 1964, malaria was controlled from most parts of India by the use of DDT house spraying (Sharma, 1987). Vector control by synthetic pesticides has now experienced serious limitations. Many vector populations are now resistant to organochlorines, organophosphates and organocarbamates insecticides. Many insecticides in particular the persistent organochlorine compounds have caused serious adverse effects on non-target organisms. There have been logistical problems, including difficulties to organize and

pay trained spray teams and to provide transport to cover the whole region in time before the malaria transmission season starts (Curtis, 1991).

Currently the most abundantly used insecticides are organophosphates; organocarbamates and natural/synthetic pyrethroids (**3** and **4**, respectively). Their mode of action is based on either disruption of acetylcholine esterase activity or the voltage sensitive sodium channel. This limited number of working mechanisms has resulted in the development of resistance to these pesticides in insect population and poses a serious limitation to their continuous use. As an example, *Anopheles gambiae* has been reported to be resistant to permethrin with decreased susceptibility to deltamethrin and lambdacyhalothrin (WHO, 1996).



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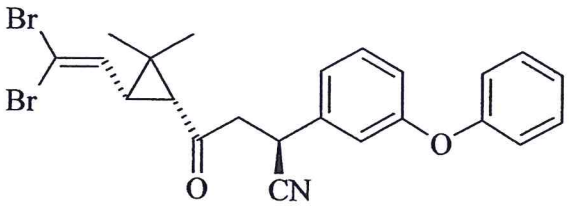

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1.5.3.4 Larval control

Mosquito larval control has proved to be the most effective means of controlling anopheline densities. The mosquito life cycle is cut before the emergence of adults, which bite and transmit malaria. Besides, habitats of adult mosquitoes are less likely to be accessible than larval breeding sites (Jamieson *et al.*, 1994; Rattanarithkul *et al.*, 1995). Use of classical larvicides such as kerosene to control mosquito population in small pools was rampant before the discovery of other synthetic larvicides (Wigglesworth, 1976).

Some of the oil enters the tracheal system and act as poisons that presumably affect the nervous system (Wigglesworth, 1976). *Anopheles* larvae below such film at 24^{O}C should all be dead in two to three hours.

Environmental management in mosquito control may involve covering all open water surfaces, removal of non-essential water containers from around houses, such as jars storage pots, and tins. This protects breeding places and prevents adult mosquitoes from laying eggs in these places. Draining the breeding sites into the sea has controlled mosquito larvae (Macdonald, 1939). Biological control implies the use of predators, parasites or entomo-pathogens, against a given vector or parasite. This form of control has taken three major dimensions; larvivorus fish, bacteria, nematodes, algae and fungi.

Attempts have been made to control mosquito larvae by these means. Larvivorous fish such as *Gambusia affinis* (Meisch, 1985) have been used. Previously, other fishes such as Amargosa pupfish (*Cyprinoden nevadensis amargosae*) and guppies (*Poecillia reticulata*) were used (Moyle, 1976). These fish species feed on the larvae and thus control their level at the points of introduction. Predatory activity of *Fundulus zerinus*, a North American indigenous fish has also been studied extensively and found to be comparable to the activity of the mosquito-fish, *Gambusia affinis* (Nelson and Keenan, 1992). Laboratory experiments also showed that a cyprinodontid fish, *Aphanius mento*, could be used to control *Culex pipiens* (Blaustein and Byard, 1993). Although use of mosquito feeding fish has succeeded experimentally, large-scale application in the field has experienced a number of setbacks. During rainy seasons, which are also peak of malaria transmission periods, there are countless rainwater pools that furnish mosquitoes with adequate and favorable breeding sites, which are less favourable for fish survival. Besides, some of these fish (*G. affinis*) are unable to survive in flooded muddy-water (Mahmoud, 1985). *G. affinis* give birth to live fingerlings and therefore have small fecundity. Difficulties in their mass rearing represent a major obstacle in increasing their effectiveness (Joseph and Alison, 1987). Agricultural practices have contributed to fish kills, thus requiring continuous need for restocking. To date, there is no mosquito-feeding fish production adequate for stocking on a large operational scale (James, 1985). These fishes may have negative effect on other fish species when introduced in areas outside their normal habitat (Nelson and Keenan, 1992).

Toxins produced by certain *Bacillus* species have shown excellent results in attempt to control mosquito larvae. The *Bacillus* species that have been used include *Bacillus thuringiensis H-4* and *Bacillus sphaericus* (WHO, 1996). Commercially available formulations of *Bacillus thuringiensis* var *israelensis* include Bactimos; Vectoac and Tenkar. Major isolates of *B. sphaericus* are 1593 (1f-119) and 2362 (1f-118). These formulations and isolates were tried against *A. gambiae* larvae in Ouagadougou (Giancarlo *et al.*, 1987). These bacteria species have high molecular weight proteins; this leads to their sinking at the bottom. Since mosquito larvae are surface feeders, efficacy of these bacterial formulations is questionable, unless a good base is used to suspend them at the surface. Studies have shown that *B. sphaericus* die quickly in hot sun and have short persistence thus requiring regular application. As a result of this, high levels of resistance have been reported in field population of *Culex quinquefasciatus*. This is likely to spread among other mosquito species due to continued exposure (Barbazan *et al.*, 1997; Rao *et al.*, 1995).

Laboratory tests of a fungus, *Tolypocladium clindrosporum*, against *Aedes aegypti*, *A. vexans* and *A. triseratus*, gave interesting results (Goettel, 1987; Nadeau and Boisvert, 1994). Suspension and supernatant of algae, *Chlorella ellipsoide*, were found to be toxic to *C. quinquefasciatus* under laboratory conditions. This indicates the potential candidacy of this algae for use in mosquito control (Dhillon and Mulla, 1981). In the rice fields where mosquitoes are a great menace, use has been made of a mermithid nematode *(Romanomermis culicivorax)* and fungus (*Lagenidium giganteum*). The mermithid nematode and entomo-pathogenic fungi have demonstrated little or no adverse effects on populations of non-target vertebrate and invertebrate organisms (Lawrence and Cynthia, 1990). The potentials of this method in large-scale mosquito larvae control are yet to be realized.

1.5.3.5 Insect Growth Regulators (IGRs)

Many chemicals exhibiting insect growth inhibiting properties have been developed. Successful laboratory tests have been done with IGRs such as S-31183 [1-(4-phenoxyphenoxy)-2-(2-pyridoxy) propane] and S-21149 [O-(2-4-phenoxyphenoxy)ethyl propionaldoxime]. A carbamate, fenoxycarb (RO13-5223) [ethyl-p-phenoxyphenoxy-

ethylcarbamate] has also been tested with interesting results (Mulla *et al.*, 1986). Currently, the most widely used IGR in Europe is Altosid that contains methoprene as the active ingredient. Methoprene is claimed to have low toxicity to fish and birds. Altosid, a methoprene based product is applied to ditches, ponds, marshes or flooded areas (Knepper *et al.*, 1992). It is only applied to non-drinking water sources. Most of the compounds discovered so far are grouped as juvenile hormone mimics or chitin synthesis inhibitors. These compounds in general have a high margin of safety to humans and little or no toxicity to most non-target organisms. They are primarily active against immature stages of mosquitoes, flies and other insects, but some IGRs induce sterility and other reproductive anomalies in the adult stage (WHO, 1996).

1.6 Significance of the Study

Vector control by spraying houses with residual insecticide initially had some success especially in Asia (Sharma, 1987), but the development of resistance to insecticide has caused serious difficulties. The synthetic insecticides have proved detrimental to a variety of animal lives including man. These factors have created the need for environmentally safe, biodegradable and target specific insecticides against mosquitoes. The search for alternative insecticides has been directed towards the plant kingdom. Co-evolution of plants and insects has led to the production of secondary plant compounds that have insect growth inhibitory and feeding deterrent properties. Plant parts containing such compounds are sometimes used in indigenous methods of insect control (Mwangi and Mukiama, 1988). Plants of the family Meliaceae were first reported to contain insecticidal properties by Chopra in 1928. Besides the use of phytochemicals in agricultural insect pest control, their use in mosquito larvae control is an interesting prospective.

This work is therefore, undertaken to investigate the potential of plants belonging to the family Meliaceae, known from previous research (Champagne *et al.*, 1992) to posses anti-arthropod properties, in the control of malaria vectors.

1.7 Hypothesis

Limonoids from some Meliaceae species exhibit insecticidal, antifeedant and insect growth regulation (IGR) effects on arthropods (Schmutterer and Ascher, 1987; Jacobson, 1988). Extracts and some of the limonoid constituents of the neem tree *Azadirachta Indica* A. Juss, a traditional anti-malarial plant (Kokwaro, 1993), have also been shown to exhibit IGR effects on *A. aegypti* (Nagui, 1987). The active principle in the genera *Azadirachta* and *Melia* responsible for bioactivity against insects are mostly limonoids. In view of these empirical findings, it is hypothesised that species within the genus *Turraea* may contain anti-mosquito limonoids.

1.8 Justification

The biological activity against arthropods in Meliaceae species has been attributed to the presence of limonoids. These limonoids are stable solids and dissolve easily in water miscible organic solvents, which make them suitable for use as contact insecticide on treated nets and as mosquito larvicides. For extracts of the plant species that exhibit potent anti-mosquito activity a botanical crude extract could possibly be formulated and standardized based on the active limonoid content.

1.9 Objectives of the Study.

General Objective

To screen for, isolate and characterize anti-mosquito compounds from *Turraea abyssinica* Hochst and *T. cormicorpia* Styles & F.White.

Specific Objectives

(i) To screen for larvicidal and adulticidal activities of methanol and chloroform extracts of *T. abyssinica* and *T. cormicorpia*

(ii) To carry out bioassay-guided chromatographic separation of the most active extracts.

(iii) To isolate and characterize the active compounds from selected column chromatographic fractions by spectroscopic methods (IR, UV, MS, NMR).

(iv) To carry out larvicidal bioassays of the characterized compounds using the vector, *Anopheles gambiae sensu stricto.*

CHAPTER TWO

2.0 LITERATURE REVIEW

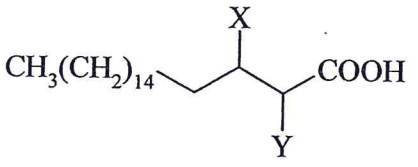
2.1 Uses of natural products in mosquito control

Human beimgs since before the time of ancient Romans, have used extracts of plants as insecticides, a practice that continues to the present with many of the 2000 species of plants known to have insecticidal properties (Crosby, 1966). Many plants have been extracted and tested for mosquito larvicidal activity (Saxena *et al.*, 1993; Sukumar *et al.*, 1991; Minjas and Sarda, 1986). Some of these plants have been investigated and the isolated compounds show potent activity. However, many remain un-investigated and the chances of finding potent larvicides remain high. In as much as this approach has not been used widely, its potential in mosquito control is high.

Traditionally, use was made of plants such as *Ocimum spp* (Labiatae) (Chogo and Crank, 1981; White, 1973*), Hyptis suaveolens* (Lamiaceae), *Daniella olivera* (Caesalpinaceae), *Lantana spp* (Verbenaceae), *Ajuga remota*, and *Nepata cataria* (Labiatae) among others, to repel mosquitoes from houses (Anonymous, 2001; Palsson, 1999; Sharma *et al.*, 1993).

Until 1938, oil of citronella, an extract of *Andropogon* and *Cymbopogon nardus* Rendle was the most widely available mosquito repellent (Fletcher, 1974). Crude methanol extract and the oil fraction from dehydrated minced garlic are larvicidal against third instar larvae of *Culex pipiens*, *C. tarsalus*, *A. aegypti*, *A. trisenatus* and *A. sierrensis* and third and fourth stage larvae of insecticide-resistant strains of *A. nigromaculis* (Amonkar and Reeves, 1970).

Some branched chain fatty acids, 2-ethyloctadecanoic acid **(5)**, 3-methyloctadecanoic acid **(6)**, and 2,3-dimethyloctadecanoic acid **(7)**, have displayed good larvicidal activity against *Culex pipiens* and *C. quinquefasciatus* (Ikeshoji and Mulla, 1974).



X	Y	
H	CH_3CH_2	**5**
CH_3	H	**6**
CH_3	CH_3	**7**

1,2,4-Trialcohols of long chain hydrocarbons isolated from unripe avocado fruit, *Persea americana* (Lauraceae), showed larvicidal activity against *A. aegypti*. These include 1,2,4-trihydroxynonadecane **(8)**, 1,2,4-trihydroxyhepta-dec-16-ene **(9)**, and 1,2,4-trihydroxyheptadec-16-yne **(10)** (Oberlies *et al.*, 1998).

$CH_3(CH_2)_{14}CH(OH)CH_2CH(OH)CH_2OH$

8

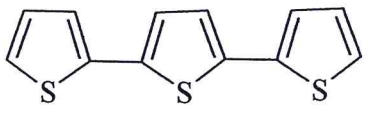
$CH_2CH(CH_2)_{11}CH(OH)CH_2CH(OH)CH_2OH$

9

$HCC(CH_2)_{11}CH(OH)CH_2CH(OH)CH_2OH$

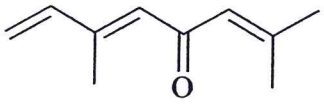
10

α-Terthienyl **(11)**, a naturally occurring polyacetylene isolated from *Ajuga remota* (Labiatae), has been reported to exhibit high phototoxic larvicidal activity against mosquito larvae (Arnason *et al.*, 1981; Kagan *et al.*, 1987).



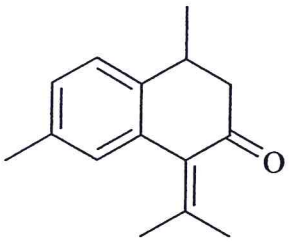
11

The larvicidal activity of a terpenoid, 5-*E*-ocimenone (2,6-dimethyl-2,5,7-octatrien-4-one) **(12)**, isolated from *Tagetes minuta* (Compositae) has been reported against *A. aegypti* (Maradufu *et al.*, 1978).

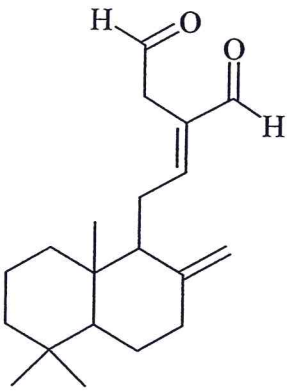


12

Larvicidal activity of *ar*-turmerone **(13)**, fractionated from the volatile oil of rhizomes of *Curcuma longa* (Zingiberaceae) against *A.aegypti* has also been reported (Roth *et al.*, 1998). The leaves also yielded λ-8 (17), 12-diene-15, 16-dial **(14)** with larvicidal activity against the same species (Roth *et al.*, 1998).

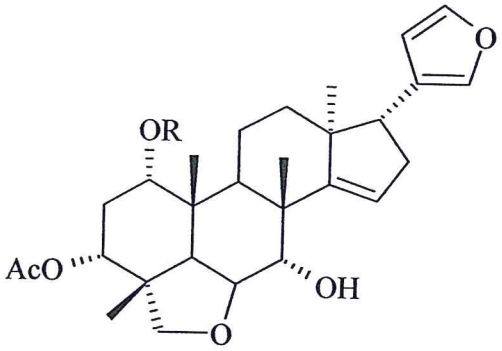


13

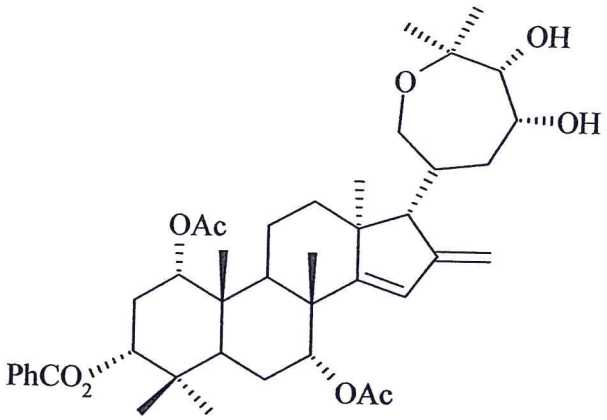


14

Several triterpenoids have also been reported to exhibit larvicidal activity. Meliavolkinin **(15)**, 1,3-diacetylvilasinin **(16)** and melianin B **(17)** have been reported to possess mosquito larvicidal activity against *A. aegypti*. These compounds were isolated from *Melia volkensii* (Meliaceae) (Rogers *et al.*, 1998).



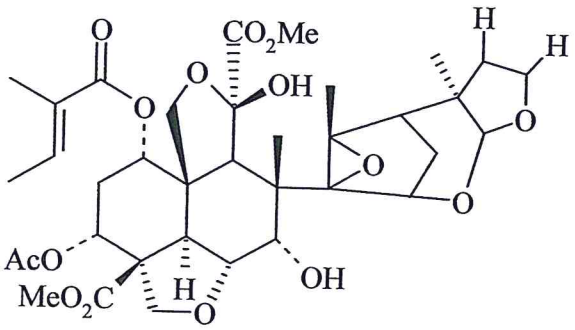
R=PhCO **15**
R=Ac **16**



17

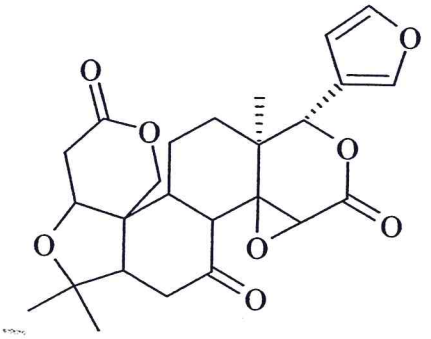
Extracts from *M. volkensii* fruits dried at 60^{0}C had enough activity to cause mortality and clearly demonstrated growth inhibiting activity against second instar larvae of *A. arabiensis* (Mwangi and Mukiama, 1988).

Azadirachtin **(18),** a limonoid isolated from *Azadirachta indica* (Meliaceae) was reported to have larvicidal activity against various mosquito species (Zebitz, 1984; Lingling *et al.*, 1998).



18

Three Limonoids, namely limonin **(19)**, nomilin **(20)**, and obacunone **(21)**, isolated from the seeds of *Citrus reticulata* Blanco Coorg Mandarin, inhibited adult emergence of fourth instar larvae of *C. quinquefasciatus*. The EC_{50} for inhibition of adult emergence was 6.31, 26.61 and 59.57 ppm for obacunone, nomilin and limonin, respectively (Jayaprakasha *et al.*, 1997).



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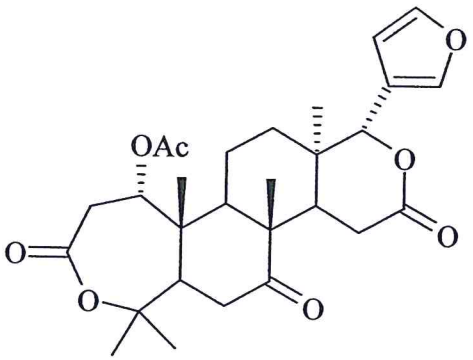

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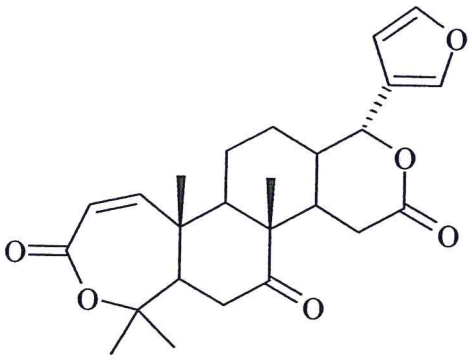
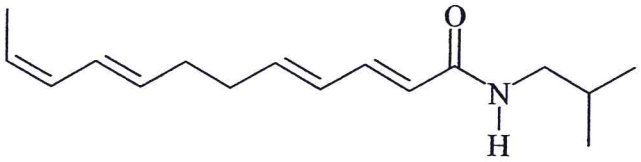


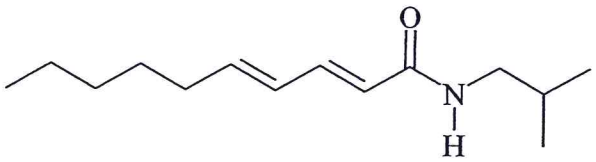
21

N-isobutyl-2 *E*, 4 *E*, 8 *E*, 10 *Z*-dodeca-2,4, 8,10-tetraenamide **(22)** isolated from wet stem and leaves of *Spilanthes mauritiana* was found to elicit 100% mortality at 0.003 g/ml after 7 hrs of application against third instar larvae of *A. aegypti* (Jondiko, 1986).



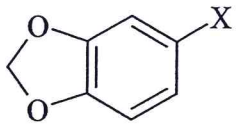
22

Larvicidal activity of some long chain fatty acid amides has been reported. Pellitorine [N-(2-methylpropyl)-(*E,E*)-2,4-decadienamide] **(23)** isolated from *Fagara macrophylla* (Rutaceae), was found to be active against *C. pipiens* larvae (Kubo *et al.*, 1984). The same compound was isolated from *Achillea millefolium* (Compositae) and found to be active against *A. triseriatus* (Lalonde *et al.*, 1980).



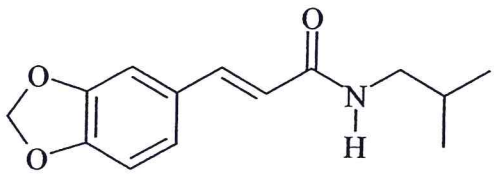
23

Other isobutyl-amides isolated from *F. macrophylla*, piperlongumine **(24)**, fagaramide **(25)**, and 4, 5-dihydropiperlongumine **(26)** and N-isobutyl-2*E*, 4*E*,-octadienamide **(27)** have also demonstrated good activity against *C. pipiens* larvae (Kubo *et al.*, 1984).



$X=(CH)_4CONHCH_2CH(CH_3)_2$ **24**

$X=(CH_2)_2(CH)_2CONHCH_2CH(CH_3)_2$ **26**



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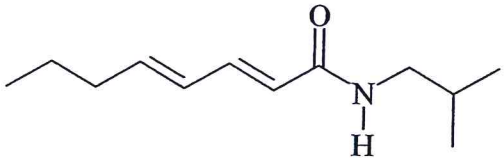
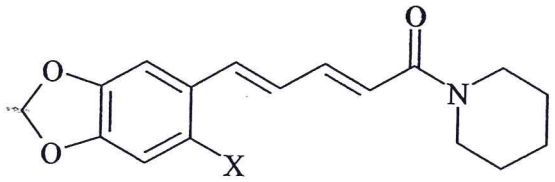


27

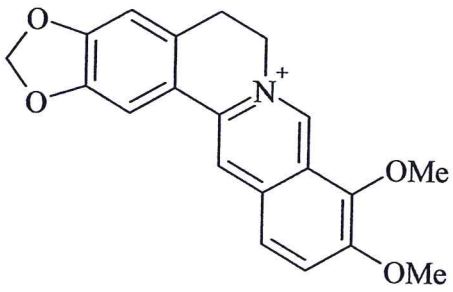
Piperine **(28)** and wisanine **(29)** are piperidine alkaloids that were isolated from *Piper guineense* (Piperaceae) and found to be effective against *A. aegypti* larvae (Addae-Mensah and Achieng, 1986).



X=H **28**

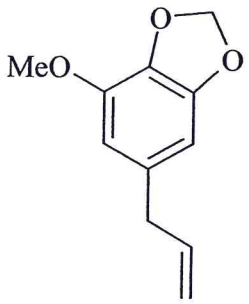
X=OMe **29**

Berberine **(30),** a naturally occurring isoquinoline alkaloid, is present in at least nine botanical families; Annonaceae, Berberidaceae, Jungladaceae, Magnoliaceae, Menispermaceae, Papaveraceae, Ranunculaceae, Rubiaceae and Rutaceae. Its activity against *A. atropalpus* in the presence of near UV has been reported (Philogene *et al.*, 1984).

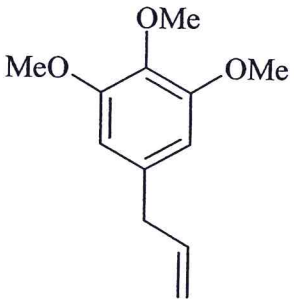


30

Phenylpropanoids, myristicin **(31)**, elemicin **(32)**, and *trans*-isoelemicin **(33)** together with a furanocoumarin, oxypeucedanin **(34)**, isolated from the leaves of *Diplolophium buchanani* (Umberlliferae), exhibited larvicidal activity against *A. aegypti* (Marston *et al.*, 1995).



31



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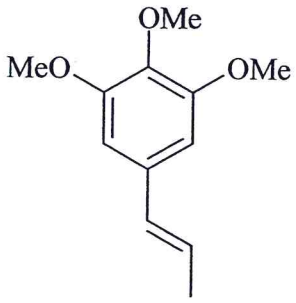
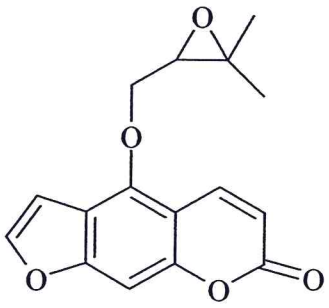
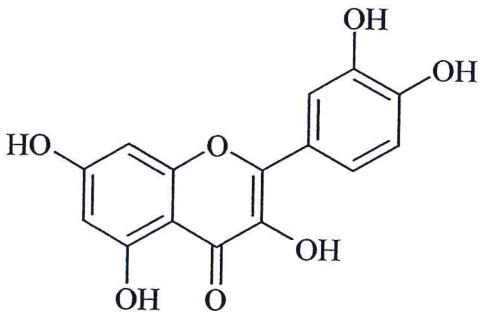


33

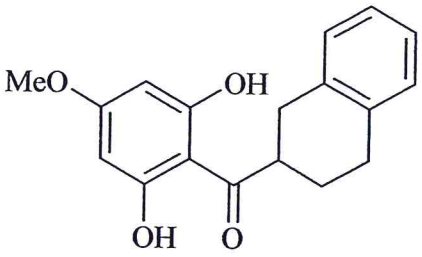


34

Flavonoids such as quercetin **(35)** and 2′,6′-dihydroxy-4′-methoxydihydrochalcone **(36)** isolated from *Polygonum senegalense* (Polygonaceae) have been reported to posses larvicidal activity on *A. aegypti* larvae (Gikonyo *et al.*, 1998).

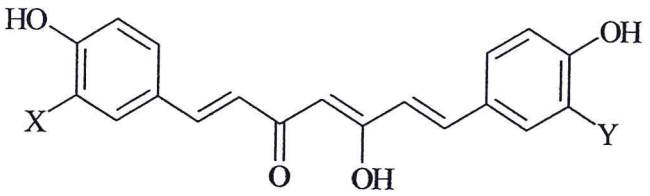


35



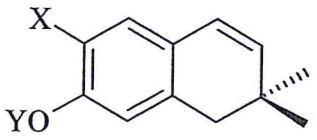
36

Curcuminoids isolated from the rhizomes of *Curcuma longa* (Zingiberaceae), exhibited larvicidal activity against *A. aegypti*. These include curcumin I **(37)**, curcumin II **(38)**, and curcumin III **(39)** (Roth *et al.*, 1998).



X	Y	
OMe	OMe	**37**
H	OMe	**38**
H	H	**39**

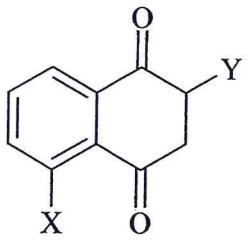
Chromenes isolated from volatile oil *Hermizonia fitchii* (Asteraceae), have shown larvicidal activity against *C. pipiens*. These include encecalin **(40)**, chromene (desmethyl-encecalin) **(41)** and 6-vinyl-7-methoxy-2, 2-dimethylchromene **(42)** (Klocke *et al.*, 1985).



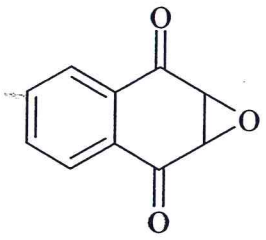
X	Y	
CH_3	Ac	**40**
H	Ac	**41**
CH_3	CH_2CH_2	**42**

Some quinones isolated from *Plumbago zeylanica* (Plumbaginaceae) displayed effective larvicidal activity against *A. aegypti*. These include plumbagin **(43)**, juglone **(44)**, 2-methyl-1,4-napthoquinone **(45)**, 1,4-napthoquinone **(46)**, 2-hydroxy-1,4-

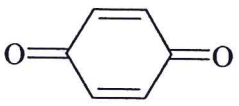
napthoquinone **(47)**, 2,3-epoxy-1,4-benzoquinone **(48)**, 1,4 benzoquinone **(49)** and 1,2-napthquinone **(50)** (Hassanali and Lwande, 1989; Chapya, 1984).



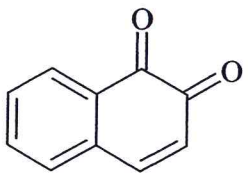
X	Y	
OH	CH_3	**43**
OH	H	**44**
H	CH_3	**45**
H	H	**46**
H	OH	**47**



48

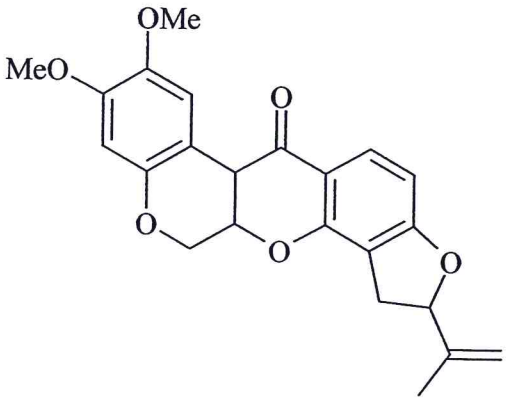


49



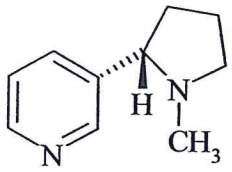
50

Roots of *Derris elliptica*, *D. malaccensis*, *Lonchocarpus utilis*, *L. urucu* and *Tephrosia virginiania* have yielded rotenone **(51)**. This compound has been known to be a good mosquito larvicide (Kirk and Othmer, 1981; Metcalf *et al.*, 1962).



51

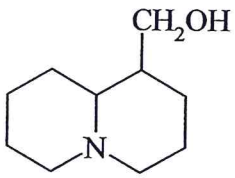
Nicotiana tobacum and *N. rustica* (Solanaceae) have yielded two well-known insecticides, nicotine **(52)** and anabasine **(53)**. Nicotine has been used for a long time in mosquito larvae control (Campbell and Sullivan, 1933).



52

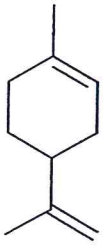
53

The quinolizidine alkaloid lupinine **(54)** has been reported to have larvicidal activity against *C. pipiens*; *C. territans* and *C. quiquefasciatus*. Quinolizidine alkaloids are known to have non-specific insecticidal activity (Campbell and Sullivan, 1933).



54

Essential oils from *Lippia wilmsii* H. H. W. Pearson, *L. dauensis* Chiov and *L. javanica* Burm.f. showed moderate mosquito larvicidal activity, causing between 55 – 68% mortality to third instar larvae of *A. aegypti* at 75 ppm while the larvicidal activity of limonene **(55)**, a major constituent of the oil of *L. wilmsii*, was 77% at 15 ppm (Torto and Hassanali, 1997). The other major component of the oil, piperitone **(56)** required ten times this dose to produce a similar effect. The larvicidal activity of limonene was also shown by similar hydrocarbon monoterpenes such as *p*-cymene **(57)** and ocimene **(58)**, while the rather low larvicidal activity of piperitone was found in other oxygenated terpenes such as linalool **(59)**, 1,8-cineole **(60)** and camphor **(61)** (Torto and Hassanali, 1997).



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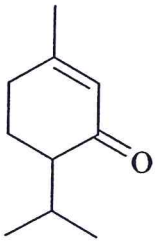
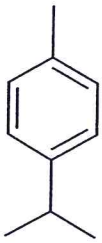


56



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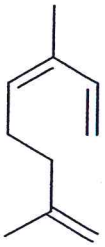

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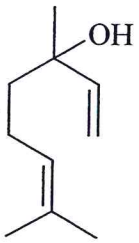

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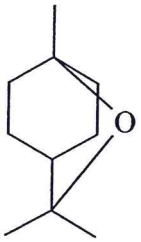

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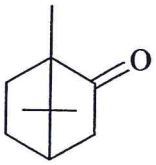


61

These results suggest that the mosquito larvicidal activities recorded for the oils from the different *Lippia* species may not be due to the activity of any individual component but to the activities of the different components acting synergistically or additively (Torto and Hassanali, 1997).

2.2 The Family Meliaceae

Meliaceae is a family of woody tropical plants comprising approximately 50 genera and a total of 500-550 species (Pennington and Styles, 1975). Chemically, the family Meliaceae is characterized by its biosynthesis of limonoids, which are modified triterpenes with or derived from a precursor with a 4,4,8-trimethyl-17-furanyl steroid skeleton (Champagne *et al.,* 1992; Taylor, 1981). Over 300 limonoids have been isolated to date, and they are more diverse and abundant in Meliaceae than in any other family.

Traditionally in certain parts of Africa, some Meliaceae species are used for treatment of fevers and malaria (Iwu, 1993). In west and east Africa, the Meliaceae species *A. indica* is used for treatment of malaria (Ayensu, 1981; Kokwaro, 1993). In tropical America, members of the Meliaceae family, *Cedrela odorata, Carapa guianensis* and *Swietenia mahagoni* have been used in traditional medicine for the treatment of fevers, a characteristic symptom of malaria (Mackinnon *et al.,* 1997).

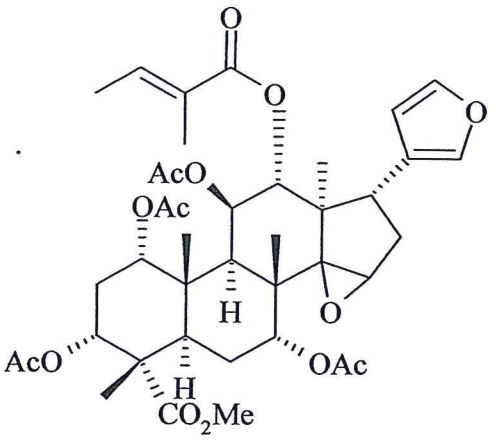
Melia azedarach Linn. (China berry or Persian lilac tree) has long been recognized as a medicinal and insecticidal plant (Dey, 1973). One of the constituents from *M. azedarach* is azadirachtin, a highly potent limonoid insect antifeedant and ecdysis inhibitor (Nakatani *et al.*, 1993). The roots of *Chukrasia tabularis* (Meliaceae) have been reported to be active against chloroquine resistant strains of *P. falciparum* (Taylor, 1981).

Extracts of the leaves of *A. indica, Cedrela salvadorensis* and the wood of *C. odorata* and *Dysoxylum fraseranum* are reported to have high activity against chloroquine sensitive *P. falciparum* (Mackinnon *et al.,* 1997).

2.3 The Genus Turraea

The genus Turraea (Meliaceae) is comprised of a group of 60-70 species of shrubs and small trees occurring in the Indian Ocean region (Bentley *et al.*, 1995). Several *Turraea* species have been used in folk medicine in East Africa for the treatment of variety of ailments (Kokwaro, 1993). In Tanzania a decoction of the roots of *T. nilotica* Kotschy &Peyr is taken for toothaches and stomach upsets while in Kenya, a decoction of the roots of *T. mombassana* Hiern ex C.D.C. is used for the treatment of malaria and other fevers (Kokwaro, 1993). Other ethno-medical uses have been reported from various parts of Africa. In South Africa, a decoction of the bark and dried root bark of *T. floribunda* is used to treat dropsy, cardiac diseases, urinary infections and rheumatism. The bark alone is used by Zulu witchdoctors to induce a trance prior to their divining dances. An overdose is said to be poisonous (Bhat and Jacobs, 1995). In Ivory Coast, hot water extract of the root of *T. heterophylla* is used as an aphrodisiac (Bouquet and Debray, 1974) while the leaf juice of *T. leonensis* is applied as an eye drop and also for treatment of sleeping sickness (Kerharo, 1974). A decoction of the bark of *T. laciniata* is used for the treatment of migraines and headaches in Rodrigues islands (Gurib *et al.*, 1996).

Other species are reported to show insect antifeedant, larvicidal, antifungal and insect growth inhibiting properties (Bentley *et al.*, 1995). Nilotin **(62)** a limonoid isolated from the root bark of *T. nilotica* displayed significant activity as an antifeedant against larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Bentley *et al.*, 1995). Champagne (1989) found that extracts of foliage of *T. holstii* Guerke were extremely inhibitory to larval growth of the Cutworm *Peridroma saucia* (Lepidoptera: Noctuidae) being comparable in potency to extracts of *A. indica* foliage. The *T. holstii* extracts also strongly deterred feeding of migratory grasshopper (*Melenoplus sanguinipes*) nymphs under no choice conditions (Champagne, 1989). Investigations of the stem and root bark of *T. holstii* yielded several limonoids that included 11-epi-toonacilin **(63)**, 11β, 12α-diacetoxycedrelone **(64)**, 12α-acetoxyneotrichilenone **(65)** and 11β-acetoxy-7-acetyl-12α-hydroxy-1, 2-dihydroneotrichilenone **(66)** (Mulholland *et al.*, 1998).



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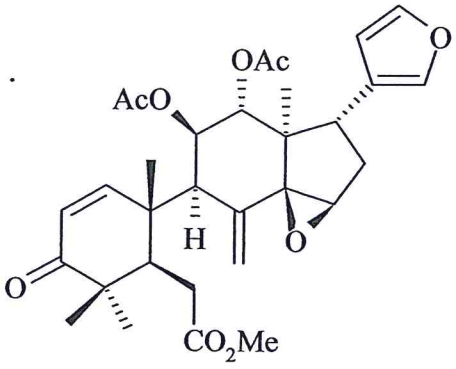

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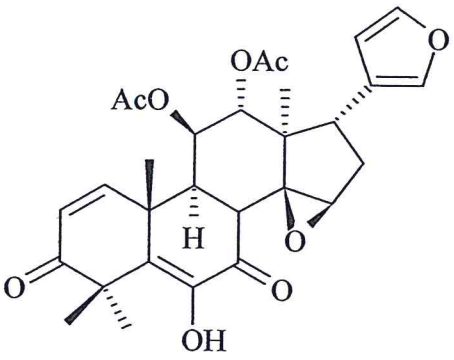

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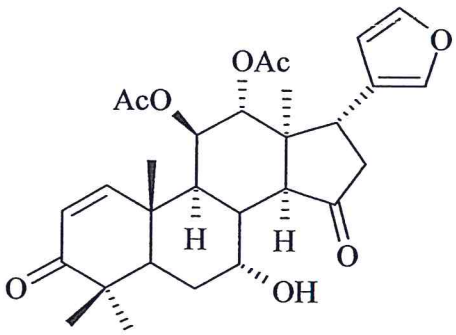

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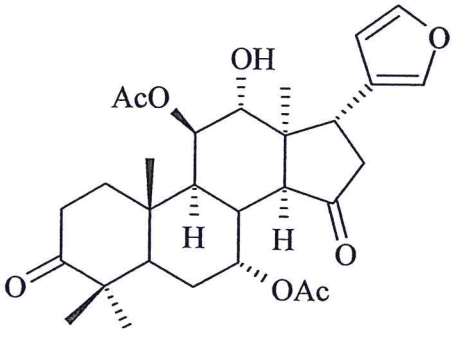
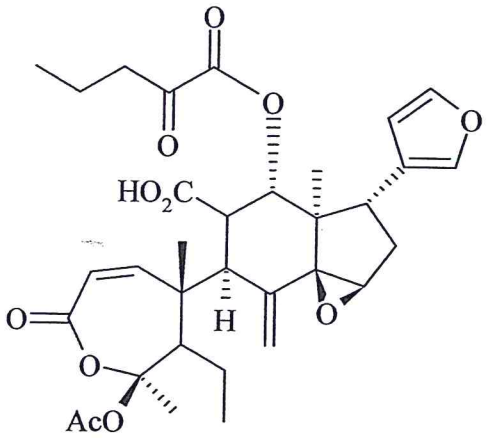


66

Several species studied have been shown to produce limonoids of prieurianin and havanensin classes (Rajab *et al.*, 1988a). *T. obtusifolia* Hochst has been reported to contain limonoids of the prieurianin class (Garcez *et al.*, 1996). Two limonoids, mombasone **(67)** and mombasol **(68)** characterised from *T. mombassana* are highly oxidized and related to the characteristics B-ring cleaved limonoid, prieurianin (Adul *et al.*, 1993).



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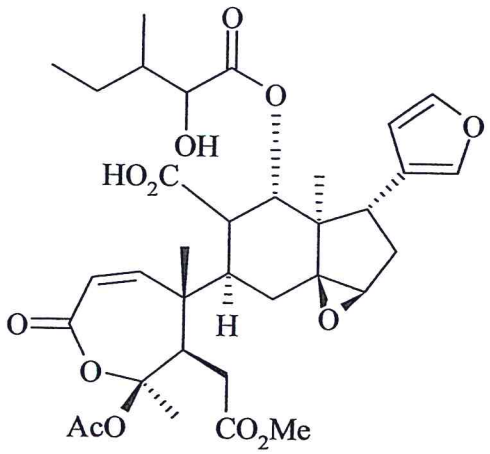
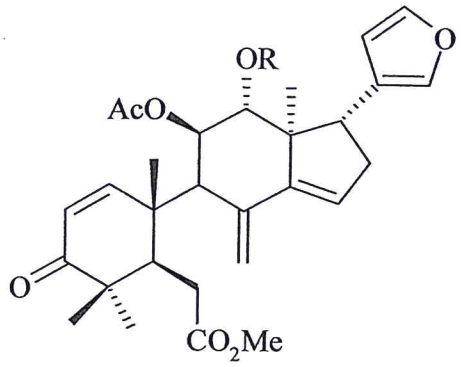
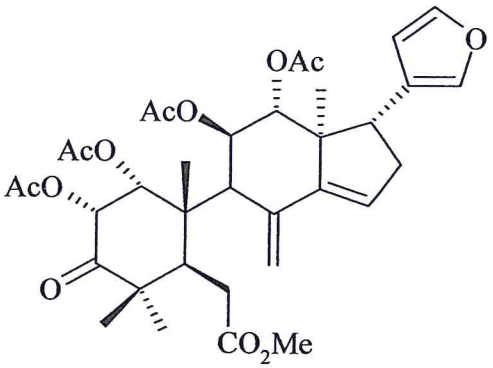


68

T. floribunda Hochst is known to contain B- seco limonoids (Taylor, 1981) related to the toonacilins reported to have bioactivity against insects (Kraus *et al.*, 1991). The seeds of *T. floribunda* contain limonoids of the havenensin class: turraflorins A **(69)**, B **(70)** and C **(71)** (Fraser *et al.*, 1994). Extracts of the root bark of *T. floribunda* have yielded several limonoids of the havanensin class which include 28-*nor*-4α-carbomethoxy-11β-acetoxy-12α-(2-methylbutanoyloxy)-14,15-deoxyhavanensin-1,7-diacetate **(72)**, 28-*nor*-4α-carbomethoxy-11β-hydroxy-12α-(2-methylbutanoyloxy)-14,15-deoxyhavanensin-1-acetate **(73)** and 18-nor-4α-carbomethoxy-11β-acetoxy-12α-(2-methylbutanoyloxy)-14,15-deoxyhavanensin-1-acetate **(74)** (Torto *et al.*, 1995).



R=Ac **69**
R=H **70**



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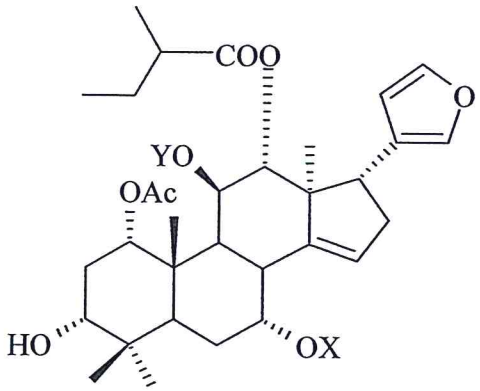
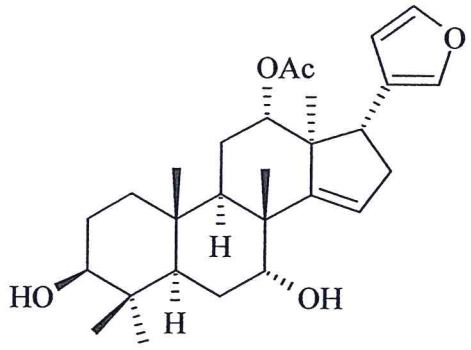


Y=Ac, X=Ac **72**
Y=H, X=H **73**
Y=Ac, X=H **74**

A series of apo-euphol-type limonoids have been isolated from *T. robusta.* They include mzikonol **(75)** mzikonone **(76),** azadirone **(77)**, 1,2-dihydroazadirone **(78)** and nimbolinin B **(79)** (Bentley *et al.,* 1992).



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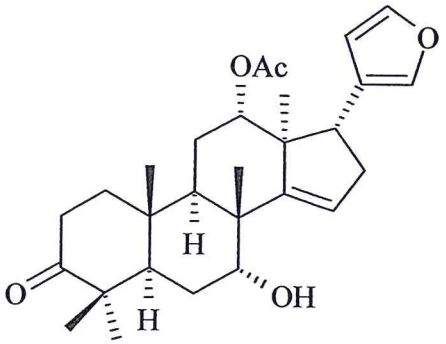

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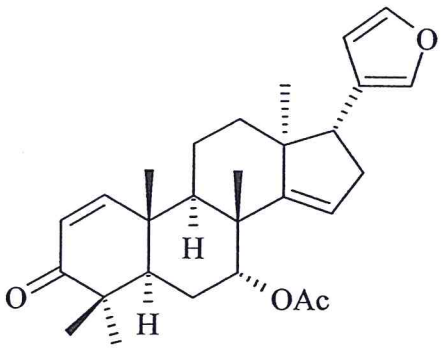

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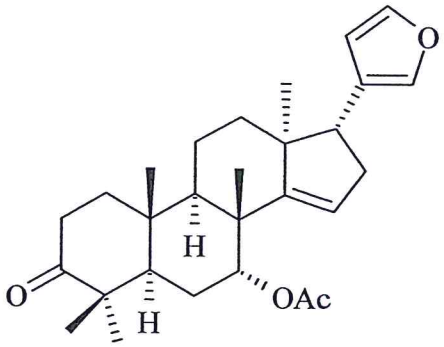

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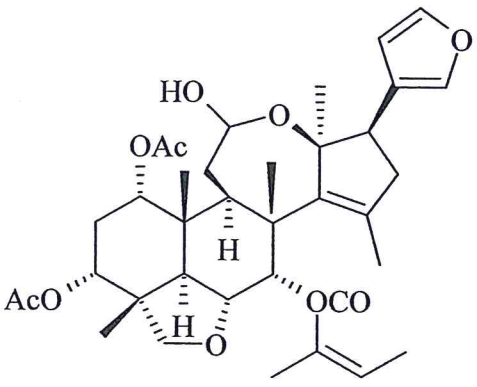
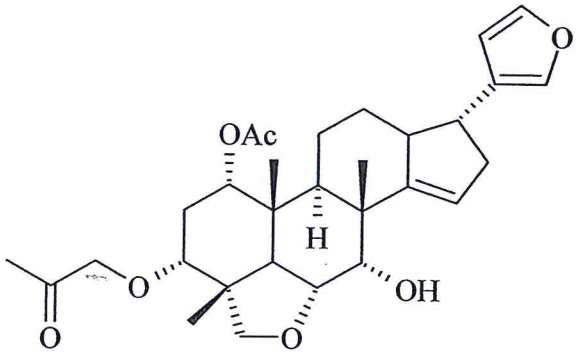


79

Thirteen limonoids have been isolated from *T. wakefieldii* and classified as vilasinin, tecleanin and azadiron-derivative types. They include 1α–acetoxy-3α-propanoyloxy vilasinin **(80),** 11β,12α–diacetoxy-7-dehydroxy-7-neotecleanin **(81)** and 11β,12α–diacetoxy-7-diacetyl-7α-hydroxyazadiron **(82). (81)** showed potent larvicidal activity against *A. gambiae* with an LD_{50} value of 7.83 ppm (Ndung'u, 2002).



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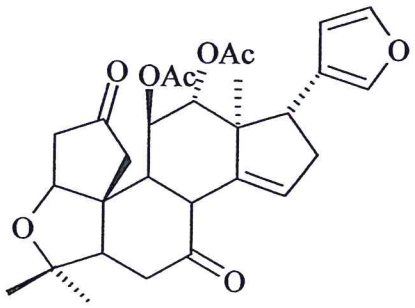

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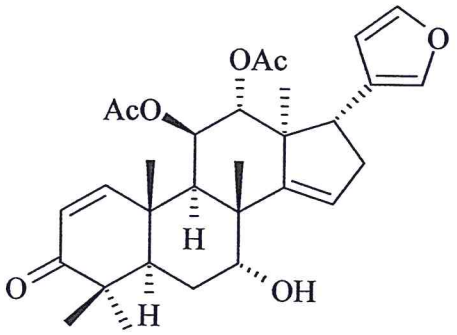
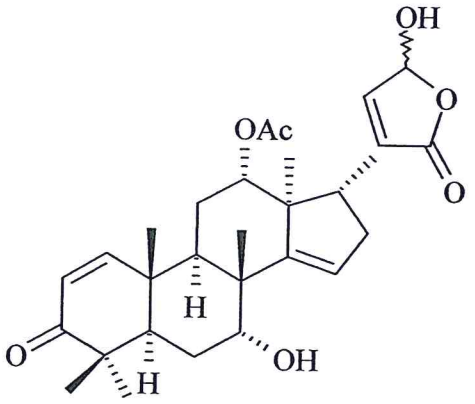


82

The methanol extract of the seeds of *T. parvifolia* Defl. yielded triterpenoid derivatives: Turraparvins A **(83)**, B **(84)**, C **(85)**, and tetranortriterpenoid lactam, turraparvin D **(86)** (Cheplogoi and Mulholland, 2003).



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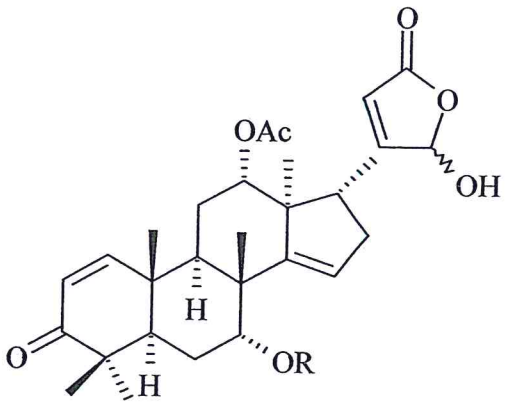
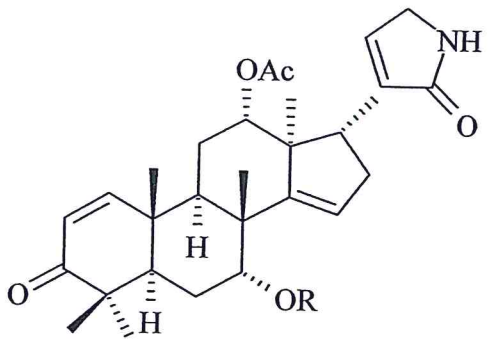


R=H **84**
R=Ac **85**



86

Phytochemical screening of the leaves and stem bark of *T. casimiriana* showed the presence of glycoflavones, saponins, sterols and tannins (Forgacs *et al.*, 1981). Apart from limonoids, other compounds present in the leaves and stem of *T. nilotica* include alkaloids, flavonoids, saponins and sterols (Ayoub and Kingston, 1982).

2.4 Limonoids

Limonoids are described as modified triterpenes, having a 4,4,8 trimethyl-17 furanyl steroid skeleton. Arrangements of subgroups and ring structures within this basic building block provide a host of characteristics that have generated interest in this plant product. Limonoids appear to be a plant chemical response to insect herbivory. Studies in recent years have revealed insecticidal effects of many different limonoids. The specific effects studied include growth inhibition, feeding inhibition, molt inhibition, and insect growth regulation. Most studies have focussed on the insect orders of Coleoptera, Diptera, Heteroptera, Lepidoptera, and Orthoptera (Huang *et al.*, 1996; Kumar *et al.*, 1996).

Some of the most exciting applications of limonoids and compounds derived from them are their use in treatments of specific cancers. Limonin (**19**) and nomilin (**20**) have been shown to be successful in treatments with in vitro bioassays on human tumour cell lines (Ahn, 1994).

Table 1 gives a summary of the biological activity of limonoids from different groups.

Table 1. Effects of limonoids on insect feeding and growth

Limonoid	Test insect	Order	Effective concentration	References
18	Azadirachtin			
	Epilachna varivestis	C	FI_{50}=0.0014%	Schwinger *et al.*, 1984
	Oncopeltus fasciatus	H	MID_{50}=5 ng/nymph	Champagne, 1989
	Melanoplus sanguinipes	O	MID_{50}=10.8 μg g^{-1}	Champagne, 1989

89	Azadiron			
	Epilachna varivestis	C	FI_{50}=0.66%	Schwinger *et al.*, 1984
90	Azadiradione			
	Heliothis zea	L	EC_{50}=250 ppm	Kubo and Klocke 1986
	Epilachna varivestis	C	FI_{50}=0.033%	Schwinger *et al.*, 1984
91	14-Epoxyazadiradione			
	Epilachna varivestis	C	FI_{50}=0.14%	Schwinger *et al.*, 1984
92	Gedunin			
	Epilachna varivestis	C	FI_{50}=0.1%	Schwinger *et al.*, 1984
	Heliothis zea	L	EC_{50}=50 ppm	Kubo and Klocke 1986
93	7-Deacetylazadiradione			
	Heliothis zea	L	EC_{50}=3500 ppm	Kubo and Klocke 1986
	Pectinophora gossypiella	L	EC_{50}=290 ppm	Kubo and Klocke 1986
97	Anthothecol			
	Spodoptera frugiperda	L	EC_{50}=3 ppm	Kubo and Klocke 1986
	Pectinophora gossypiella	L	EC_{50}=8 ppm	Kubo and Klocke 1986

99	Nimocinolide	
	Aedes aegypti	
100	Isonimocinolide	
	Aedes aegypti	
103	Methylangolensate	
	Heliothis zea	
	Spodoptera frugiperda	
105	Tecleanine	
	Spodoptera frugiperda	
	Pectinophora gossypiell	
111	Volkensin	
	Spodoptera frugiperda	
112	Volkensin hydroxylacto	
	Spodoptera frugiperda	

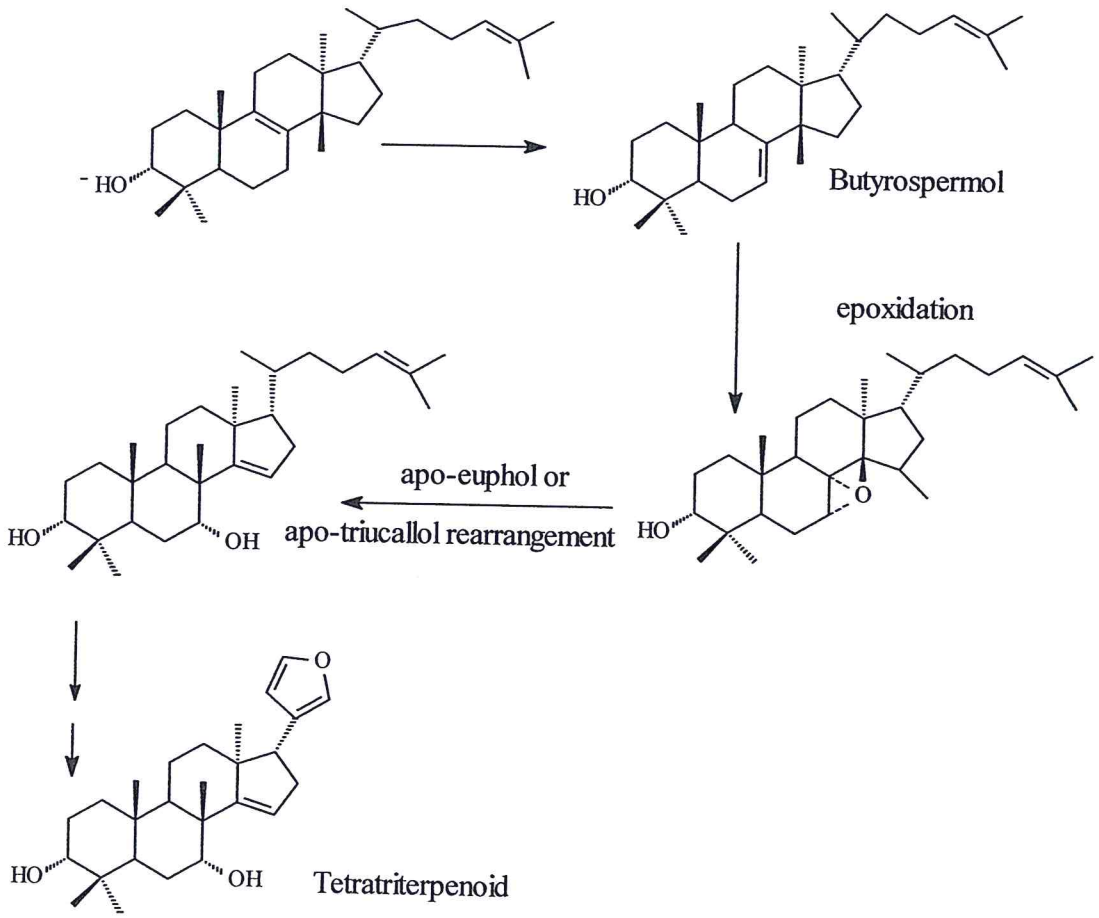
Abbreviations: EC_{50}, concentra
inhibition MID_{50}, dose producir
insect growth regulator.

Order abbreviations are; L, Lep
D, Diptera.

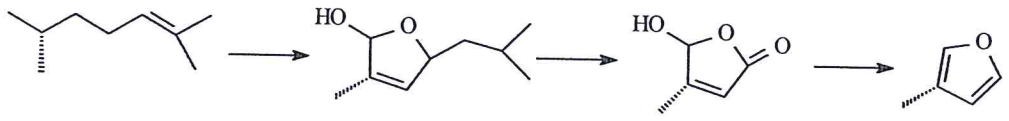
2.4.1 Biosynthesis of limonoids

According to the prevailing view, (Dreyer, 1977; Siddiqui *et al.*, 1988), the triterpene alcohols Δ^7-tirucallol [H-20=α] or Δ^7-euphol [H-20=β] are considered to be the general precursors of the degraded triterpenes in the Rutales (Scheme 1). In *A. indica* leaves, the [H-20=β] euphol is converted to the limonoid nimbolide more efficiently than is tirucallol (Champagne *et al.*, 1992). Epoxidation of the Δ^7-bond to a 7α -epoxide, which opens with a concurrent Wagner-Meerwin shift of the 14β methyl to 8β, associated with oxidative cyclization of the side chain and loss of the four terminal carbons may lead to the

Scheme 1: Biosynthetic pathway leading to the formation of a simple apo-euphol type limonoid



Scheme 2 : Side chain cylisation to form a furan ring



hypothetical precursors of limonoids (Scheme 2) (Dreyer, 1977; Siddiqui *et al.*, 1988). That the cyclization of the side chain is accomplished after the formation of the 4,4,8-trimethyl steroid skeleton is indicated by the occurrence of several protolimonoids, 4,4,8-trimethyl steroid compounds with intact C-8 side chain, such as meliantriol (**87**) from *A. indica* and niloticin (**88**) from the stem bark of *T. nilotica* (Kraus and Grimminger, 1980; Mulholland and Taylor, 1988).

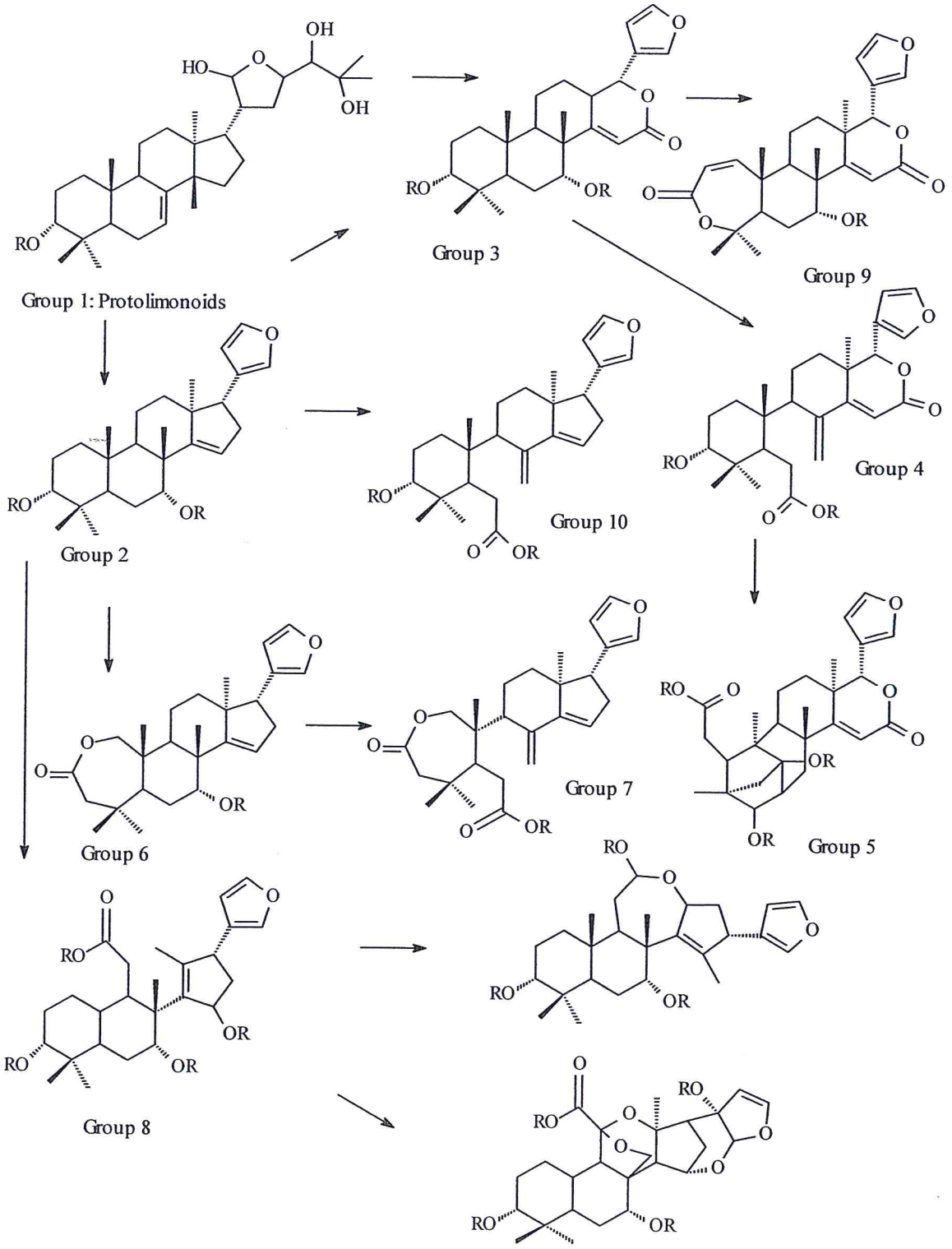
Limonoids are usually grouped according to the changes they undergo in one or more of their four-ring structures. This is brought about by a variety of oxidations and skeletal rearrangements. (Das *et al.*, 1987) (Scheme 3). Commonly the D-ring is oxidized to a lactone (D-*seco* limonoids). The process involves allylic oxidation of the 14-15 double bond to give a Δ^{14}-16-keto derivative. This compound through a Baeyer-Villiger oxidation would then lead to the desired epoxylactone. The series azadiron (**89**) /azadiradion (**90**)/14-epoxyazadiradion (**91**)/gedunin (**92**) isolated from *A. indica* (Lavie *et al.*, 1971), illustrates the process involved (Scheme 4). The A-ring may be oxidized by a similar mechanism. A, D-*seco* limonoids (Scheme 5) are found in all four families of the Rutales. Rearrangement of both A and B rings results in the highly derived limonoids characteristic of the Cneoraceae. In the sub-family Swieteniodeae of the Meliaceae, D-*seco* limonoids are further oxidized to B, D-*seco* structures such as methyl angolensate (**103**) (Scheme 6) (Dreyer, 1977; Siddiqui *et al.*, 1988). In contrast, members of the subfamily Meliodeae produce limonoids through a variety of pathways, leading to B-, A-, A, B-, and C-*seco* compounds (Scheme 7-10) (Dreyer, 1977).

In the C-*seco* class of limonoids, an 8α-epoxide gives rise to a $\Delta^{7,9\,(11)}$-diene; the Δ^{7} function generates the OH-7, and the $\Delta^{9\,(11)}$ function activates C-12, leading to oxidative fission of the C-ring (Siddiqui *et al.*, 1988; Ekong and Ibiyemi, 1985). Taylor (1984) proposed that cleavage of the C-12/C-13 bond is accompanied by the simultaneous opening of a 14, 15-epoxide to generate CHO-12 and OH-15 functions; rotation about the 8-14 bond would allow the OH-15 to recyclize with the CHO-12 to form a lactol C-ring, as in volkensin (**111**). This latter mechanism has been supported by Rajab *et al.*, (1988a), who isolated salanin (**109**) and volkensin (**111**) from *Melia volkensii* and described simple, presumably bio mimetic and thermodynamically favoured *in vitro* transformations for the synthesis of salanin from volkensin. Synthesis of the C-

seco limonoids occurs only in those Meliaceae belonging to the tribe Melieae (restricted to the genera *Azadirachta* and *Melia*) (Taylor, 1984; Dreyer, 1977).

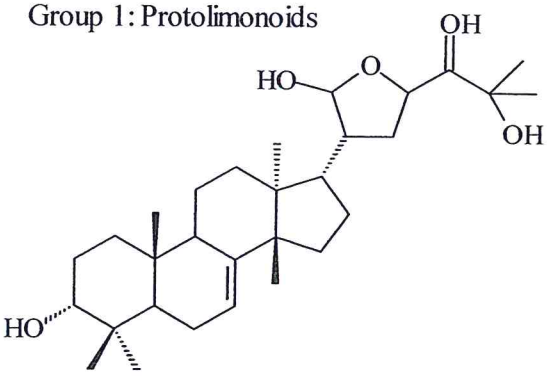
All pathways of limonoid biosynthesis are characterized by diversification through skeletal rearrangement and increasing oxidation (Gottlieb, 1989; Das *et al.*, 1987).

Scheme 3 : Major biosynthetic pathways leading to formation of limonoids in the Rutales

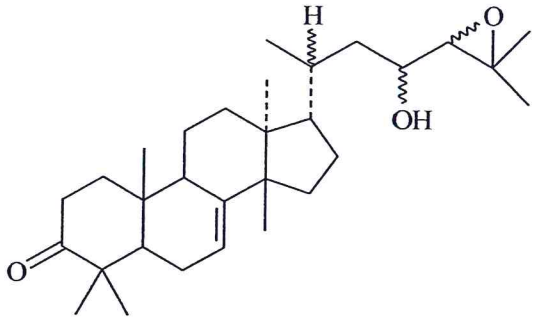


Scheme 4: Group 1 protolimonods and Apo-euphol limonoids

Group 1: Protolimonoids

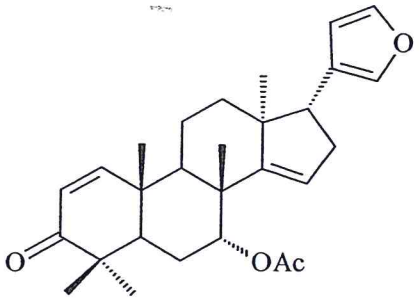


Meliantriol (**87**)

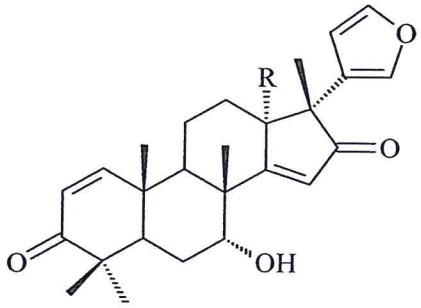


Niloticin (**88**)

Group 2:Apo-euphol limonoids



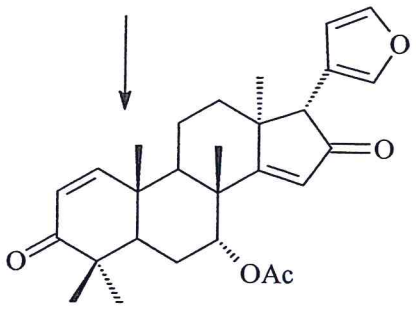
Azadiron (**89**)



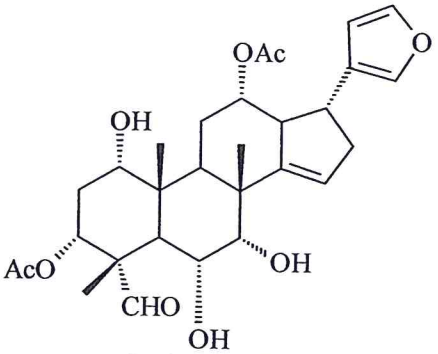
R

H 7-Deacetylazadiradione (**93**)

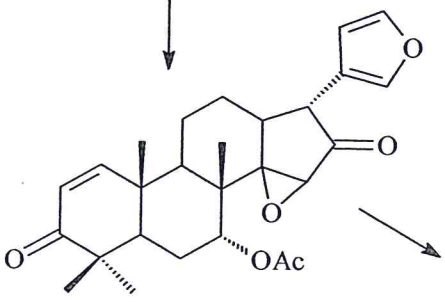
OH 7-Deacetyl-17-*B*-hydroxyazadiradione (**94**)



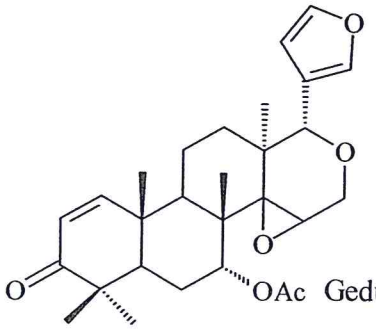
Azadiradione (**90**)



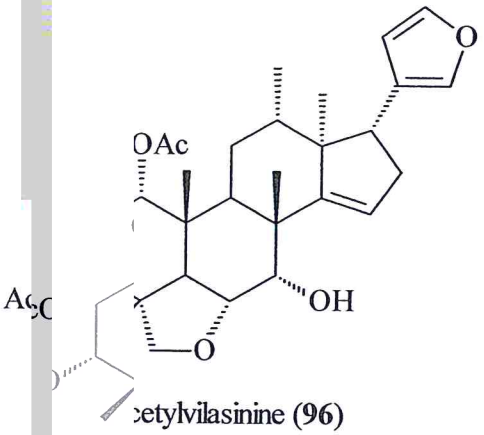
Sedanal (**95**)



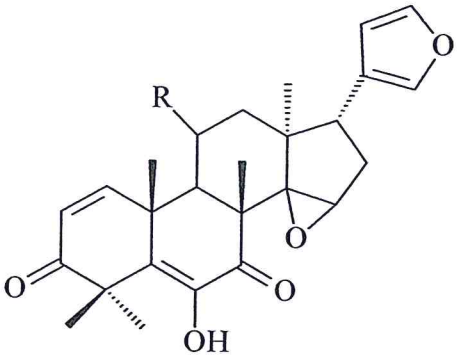
14-epoxyazadiradione (**91**)



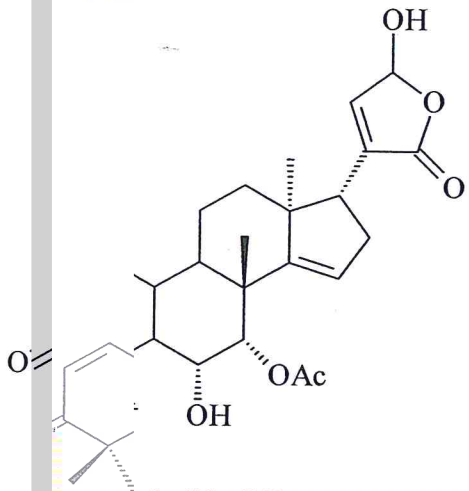
Gedunin (**92**)



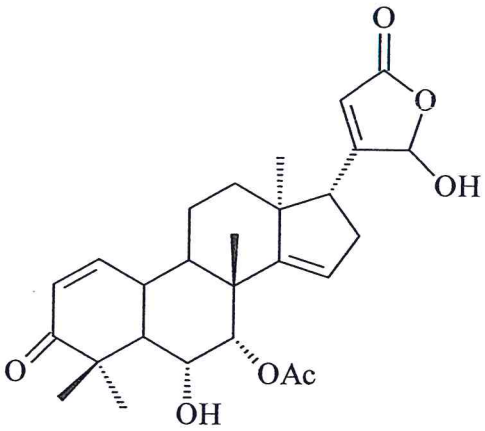
cetylvilasinine (**96**)



R
OAc Anthothecol (**97**)
H Cedrelone (**98**)



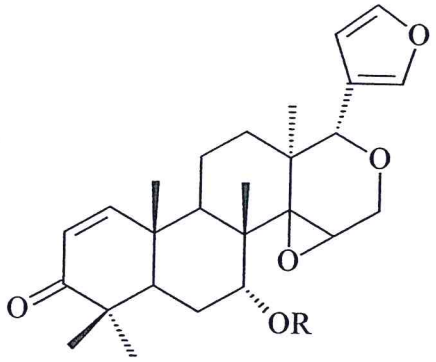
cinolide (**99**)



Isonimocinolide (**100**)

Scheme 5: Examples of Group 3 and 9 limonoids

Group 3 : D-*seco* limonoids

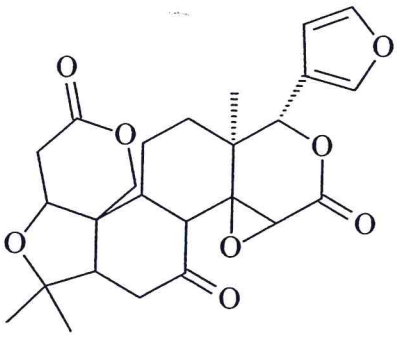


R

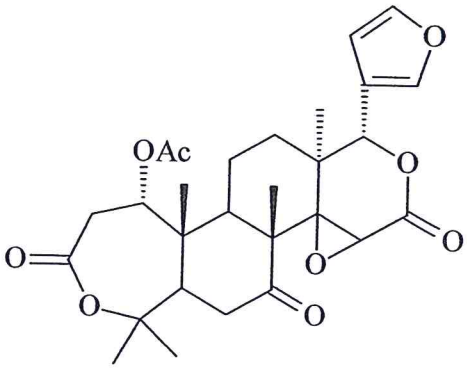
Ac Gedunin (**92**)

\- 7 -Ketogedunin (**101**)

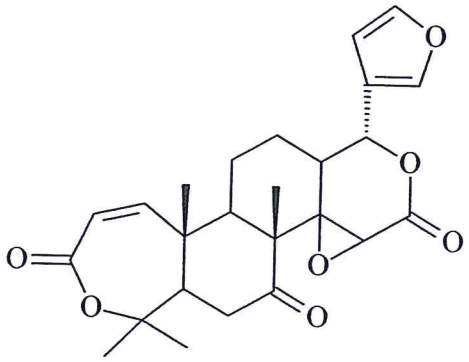
Group 9 : A,D- *seco* limonoids



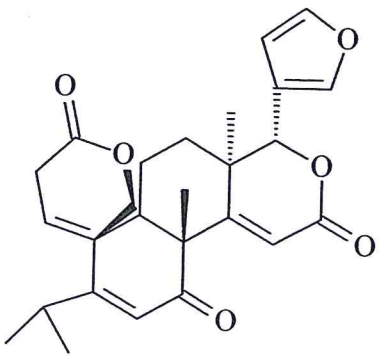
Limonin (**19**)



Nomilin (**20**)

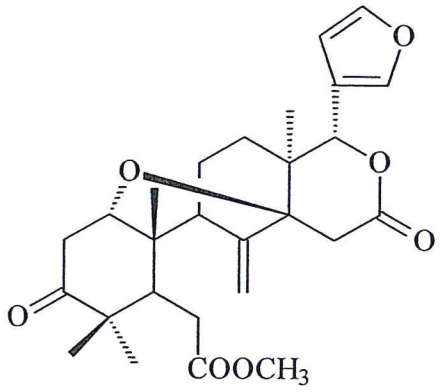


Obacunone (**21**)

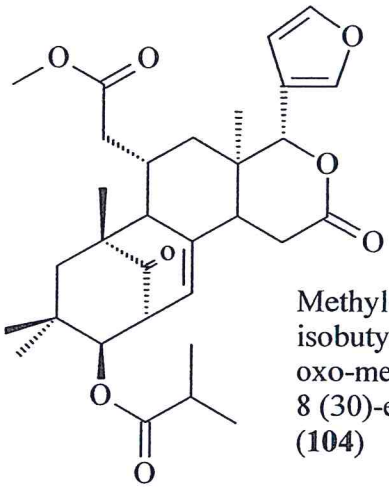


Citrolin (**102**)

Scheme 6: Examples of Group 4 and 5 (B,D-*seco*) limonoids

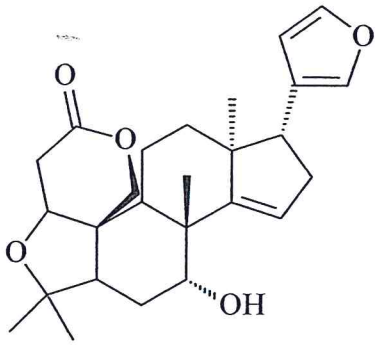


Methyl angolensate (**103**)

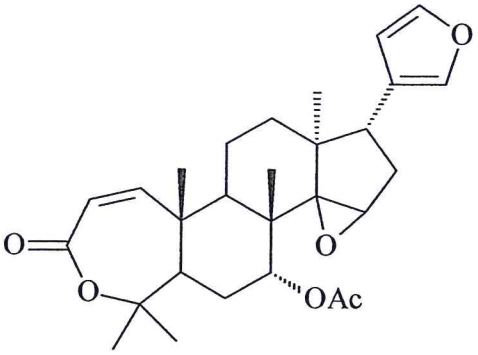


Methyl-3-isobutyryloxy-oxo-meliac-8 (30)-enate (**104**)

Scheme 7: A-*seco* limonoids (Group 6)

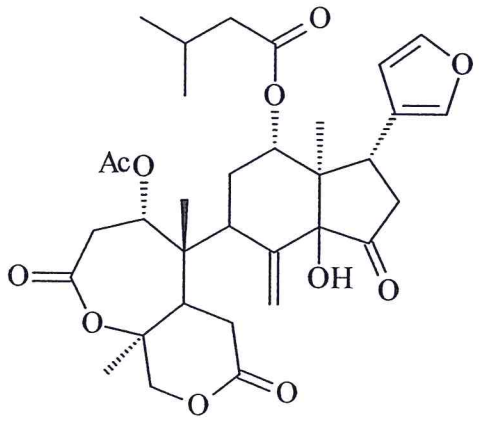


Tecleanin (**105**)

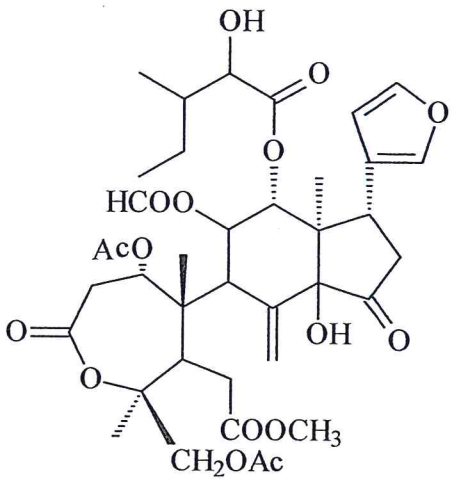


Evodulone (**106**)

Scheme 8: A,B-*seco* limonoids (Group 7)

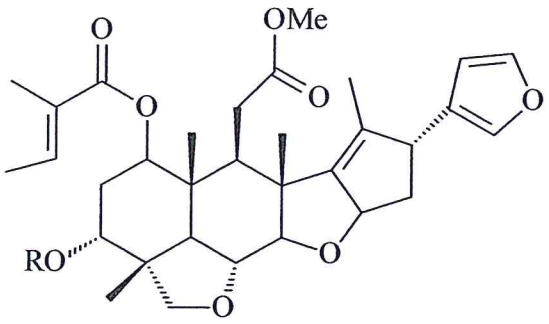


Rohitiukin (**107**)



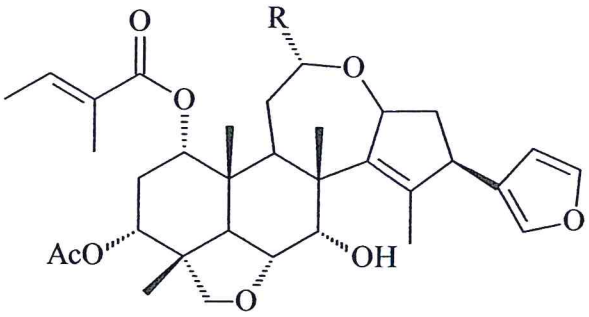
Prieurianin (**108**)

Scheme 9 : C-*seco* limonoids



R=Ac Salanin (**109**)

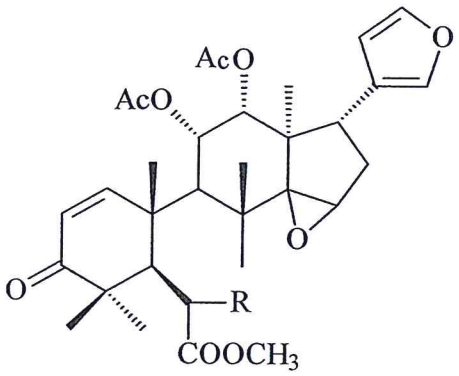
R=H 3-Deacetylsalanin (**110**)



R=OH Volkensin (**111**)

R=O Volkensin hydroxylactone (**112**)

Scheme 10: B-*seco* limonoids



R=H Toonacilin (**113**)

R=OAc 6-Acetoxytoonacilin (**114**)

CHAPTER THREE

3.0 EXPERIMENTAL

3.1 General Experimental procedures

All recyclable glassware used was washed in hot water and soap and rinsed with distilled acetone. The glassware was then dried at 100^0C for one hour. All the solvents and reagents used were obtained from Aldrich Chemical Co. Ltd, England and Merck, Germany.

3.2 Plant Materials

T. abyssinica was collected from Kijabe while *T. cormicorpia* was collected from Ngong forest both in Rift Valley Province. They were identified by Mr S.G. Mathenge of the Botany Department, University of Nairobi. Voucher specimen number 2003/203 for *T. cormicorpia* and 2003/211 for *T. abyssinica* have been deposited in the Herbarium of that Department.

3.3 Mosquito Larvae and Adults

Anopheles gambiae s.s larvae were reared under standard laboratory conditions. This strain of mosquitoes originates from Mbita, and has been reared under laboratory conditions in the ICIPE mosquito insectary. Eggs were allowed to emerge in plastic containers filled with distilled water, and were transferred to larger pans at densities of 200-300 at L2 (second larvae instar) stage. Water temparature was kept constant by heating the insectary and varied only slightly between 28-30^0C. Larvae were fed on Tetramin fish food (Tetr Werke Germany) and were used for experimental purposes upon reaching the late L3 or early L4 developmental stage.

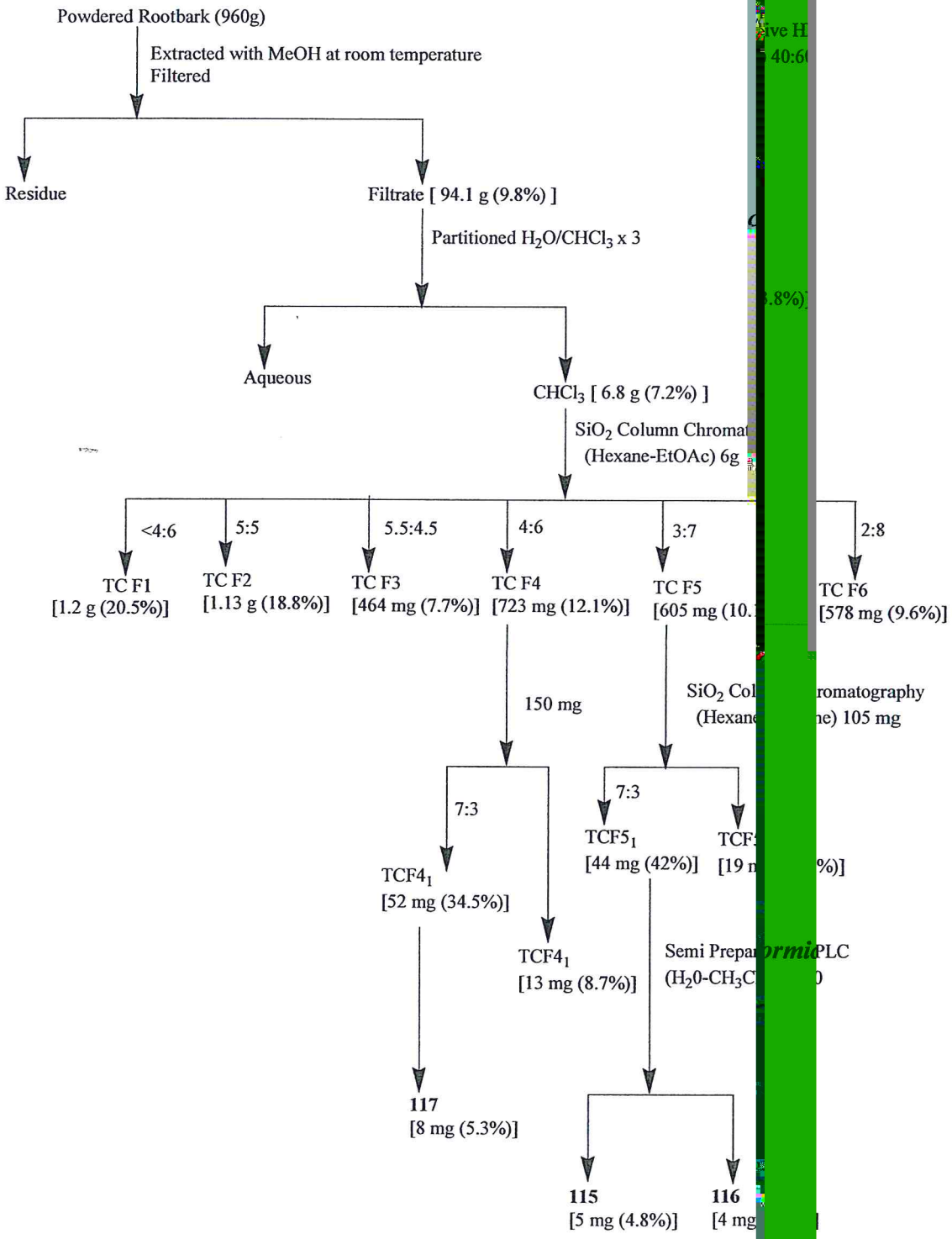
The adults have been reared under laboratory conditions in the ICIPE mosquito insectary. Room temperature was kept constant between 26-28^0C. 2-5 Days old mosquito adults were used for experimental purposes.

3.4 Extraction procedures

The air-dried root bark of *T. cormicorpia* was powdered (960g) and extracted in the dark by soaking for three weeks with methanol. The methanol extract was concentrated *in vacuo* to yield viscous red oils (94g). The methanol filtrate was partitioned between water and chloroform (1:2.5 (three times). The combined organic layer was then concentrated to dryness (6.6g). The chloroform extract (6g) was column chromatographed on silica gel (81x 4.5 cm; 230-400 mesh glass column) using hexane: ethyl acetate gradient (100:0 to 0:100). Separation was monitored by thin layer chromatography (TLC). The TLC plates were developed with hexane-ethyl acetate (2:1), sprayed with Ehrlich's reagent (2% 4-dimethylaminobenzaldehyde in ethanol) and developed in a hydrogen chloride gas chamber. Typical pink/reddish colored spots were obtained for limonoids- rich fractions eluted at 50-100% ethyl acetate (Maier and Edward, 1970). The non limonoid fractions were pooled into one fraction, TC F1 (1.2g). Other fractions were TC F2 (1.13g), TC F3 (464 mg) TC F4 (723 mg), TC F5 (605 mg) and TC F6 (578mg).

The active fractions, TC F4 (150 mg) and TC F5 (105 mg) were rechromatographed on another silica gel column (50 x 2 cm; 230-400 mesh) using 30% acetone-hexane eluent to yield fractions TC $F4_1$ (52 mg) and TC $F5_1$ (44mg) which were further purified by preparative HPLC (Ultrasphere ODS reverse phase column (250 x 10 mm. 5 μm particle size). The elution was carried out at a flow rate of 2.0 ml per minute under isocratic conditions using acetonitrile-water eluent (60:40). The compounds were detected by UV absorption at 215 nm (Ozaki *et al.*, 1991).The HPLC fractions were evaporated to dryness *in vacuo*. Three pure compounds **115** (5mg), **116** (4 mg) and **117** (8 mg) were obtained. The purity of the isolated compounds was confirmed by analytical HPLC (Ultrasphere ODS reverse phase column (250 x 4.6 mm, 5 μm particle size).

Fig.1. Bioassay guided fractionation of the root bark extracts of *T.* *copia*



3.5 Biological Activity Tests

3.5.1 Larvicidal assays

Larvicidal activities of both the methanol and chloroform extracts were subjected to standardized WHO bioassays for larvicidal activity (WHO, 1996). The stock solution was made by dissolving 500 mg of crude methanol extract in 1 ml of DMSO and 9 ml of distilled water to obtain a concentration of 50 mg/ml. Subsequent lower concentrations (1000, 750, 500, 250, 100 and 50) ppm were made by diluting the stock solution 50, 67, 100, 200, 500 and 1000 fold respectively by making to 100 ml with distilled water in a 250 ml beaker. For the chloroform extract, 500 mg of the extract was dissolved in 10 ml of acetone and the same procedure repeated as for the methanol extract. Twenty late L3 or young L4 instar larvae were used. Mortality was observed after 24 hours. In concentrations where mortality was delayed for more than 24 hours, mortality was assessed every 24 hours up to emergence of the adults or death of the last larva or pupa. During the experiment, larvae were fed with Tetramin® fish food. From the results, the probit mortality/log dose regression, and hence the LD_{50} was computed for the respective test materials.

3.5.2 Adulticidal assays

Adulticidal assays were conducted with the same series of doses as those for larvicidal bioassay. The test material was applied to filter paper (Whatman No.1) 12 x 15 cm. The papers were air dried for one hour and then inserted into sterile petri dishes. The lids of the petri dishes were perforated for ventilation to avoid suffocation of the mosquitoes. Groups of 20 non-blood-fed female *A. gambiae*, 2 to 5 days old, were placed in each petri dish, exposed to the treated paper for twenty minutes and returned to holding cages (12 x 12 cm) for the determination of the 24 hour dosage/mortality relationship. The dosage/mortality curve was replicated on three separately reared batches to allow for inter-batch variability. From the results, the probit mortality/log dose regression, and hence the LD_{50} was computed for the respective test materials.

3.6 Data Analysis

The larvicidal and adulticidal effects of different extracts with varying concentrations were tested on *A. gambiae* larvae and adult against a standard.

Repeated –measures analysis of variances (ANOVA) was applied so as to test the effects of different dosages (DOSE), different extracts (CHEM) and their interaction (DOSE* CHEM) on the response variable (PERCENT MORTALITY) on *A. gambiae* larvae and adults.

Abbot's formula was used to adjust mortality in treatment with mortality in control, that is

$$\% \text{ Mortality (adjusted)} = \frac{\% \text{ Alive in control} - \% \text{ Alive in treatment}}{\% \text{ Alive in control}}$$

The adjusted mortality was transformed to a scale to fit a general linear model (GLM)

Tukey test was used for comparison of means. Log_{10} probit analysis was used to compute the LD_{50} values.

Statistical analysis system (SAS) version 8.2 was used.

3.7 Structure Elucidation Experiments

3.7.1 Chromatography

Column chromatography was performed on silica gel 60 (0.40-0.063 mm, 230-400 Mesh ASTM) and thin layer chromatography (TLC) on precoated silica gel $60F_{254}$ plates (0.2 mm thickness, Merck). Semi- preparative HPLC work was done on Beckman Ultrasphere ODS column, 250 x 10 mm, on Varian 5000 liquid Chromatograph. Analytical HPLC was done on Beckman Ultrasphere ODS column, 250 x 4.6 mm, on Beckman System Gold Chromatograph.

3.7.2 Ultra Violet Spectroscopy

The ultra violet spectra of purified compounds was determined using an online diode array detector (Module 168) on a Beckman HPLC (System Gold), $UV\lambda_{max}$ was determined in acetonitrile.

3.7.3 Infra-Red (1R) Spectroscopy

The IR spectra of the compounds were recorded using Shimadzu Fourier Transform infrared spectrophotometer (FT-IR-8400). The samples were prepared in KBr discs. The spectrum was recorded after background correction in the range 4000-400 cm^{-1}

3.7.4 Nuclear Magnetic Resonance (NMR) Experiments

NMR data was recorded at room temperature on Bruker Avance 500 (500 MHz). The spectra were recorded in $CDCl_3$ solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS).

3.7.5 Melting point

Melting points (Mpt) were determined using a Yanaco micro melting point apparatus model MP500D and the values were uncorrected.

3.7.6 Physical and Spectroscopic data of the limonoids

3.7.6.1 12α-acetoxy-1, 2-dihydro-7-deacetylazadiron (115) ($C_{28}H_{38}O_5$). White powder, 5 mg (4.8%), m.p. 99-100°C; IRν_{max} KBr cm^{-1} 3423, 1737, 1702, 1652, 1248; HREIMS, m/z: 454. Eluted at 60% acetonitrile (MeCN). UV λ_{max} 201 nm. ^1H and ^{13}C NMR spectrum data is shown on Table 18.

3.7.6.2 12α-acetoxy-7-deacetylazadiron (116) ($C_{28}H_{36}O_5$), white powder, 4 mg (3.8%), m.p.110-112°C; IRν_{max} KBr cm^{-1} 3449, 1740, 1729, 1665, 1248; HREIMS, m/z: 452. Eluted at 60% acetonitrile (MeCN). UV λ_{max} 216 nm.^1H and ^{13}C NMR spectrum data is shown on Table 19.

3.7.6.3 1α-12α-diacetoxy-1, 2-dihydro-7-deacetyl-3β-7α-dihydroxyazadiron (117) ($C_{30}H_{42}O_7$), white powder, 8 mg (5.3%), m.p. 227-228°C; IRν_{max} KBr cm^{-1} 3558, 1740, 1728, 1245 HREIMS, m/z: 514. Eluted at 60% acetonitrile (MeCN). UV λ_{max} 200 nm.^1H and ^{13}C NMR spectrum data is shown on Table 20.

CHAPTER FOUR

4.0 RESULTS AND DICUSSION

4.1 Extraction

The powdered root bark of each plant under study was extracted with methanol for three weeks to yield thick red oils that were then partioned between water and chloroform to give two soluble fractions.

Table 2: %Yield of MeOH Extracts and $CHCl_3$ soluble fractions

	Yield (%)	
Plants	MeOH extract	$CHCl_3$ extract
T. abyssinica	130 g (11.8)	7.9 g (6.1)
T. cormicopia	94.1g (9.8)	6.8 g (7.2)

4.2.1 Larvicidal bioassays for the methanol and chloroform extracts

The crude extracts displayed potent larvicidal activity. The results of the activities for the extracts are given in Table 3. The results show that the mortality of larvae increased as doses of the extracts were increased. High mortality (> 90% mortality) values were observed within 24 hours of treatment with 750-1000 ppm dose of all the methanol extracts. It was also observed that the methanol extracts of *T. abyssinica* and *T. cormicopia* had the same level activity as azadiractin at this dose. The activity of methanol extracts at 50 and 100 ppm were not significantly different at 95% confidence interval for *T. abyssinica.* For *T. cormicopia*, the activity of the methanol extracts at 500, 750 and 1000 ppm were not significantly different at 95% confidence level. At the 250 ppm dose, *T. cormicopia* extract was the most active giving a mortality value of 58%. At

100 ppm dose the activities of both *T. abyssinica* and *T. abyssinica* extracts were not significantly different. The methanol extracts were more active than the standard azadiractin at the 50 ppm dose.

Table 3: Mean percent larvicidal activity ± standard error, in 24 hours, of the methanol, chloroform extracts and azadirachtin against *A. gambiae.*

Extracts	Dose (ppm)						
	50	100	250	500	750	1000	LD_{50}
MeOH							
T. abyssinica	8±1.2^{aD}	15±1.6^{bD}	45±2.2^{bcC}	62±2.6^{bB}	92±2.6^{bA}	99±1.0^{aA}	265
T. cormicopia	1±1.0^{bD}	14±1.9^{bC}	58±4.6^{abB}	96±1.9^{aA}	100±0.0^{aA}	100±0.0^{aA}	202
$CHCl_3$							
T. abyssinica	1±1.0^{bD}	8±4.1^{bcD}	23±3.4^{dC}	60±5.0^{bB}	97±2.0^{abA}	100±0.0^{aA}	350
T. cormicopia	0±0.0^{bC}	0±0.0^{cC}	35±3.5^{cdB}	95±2.2^{aA}	100±0.0^{aA}	100±0.0^{aA}	281
Azadirachtin	0±0.0^{bE}	42±1.2^{aD}	72±2.5^{aC}	93±1.2^{aB}	100±0.0^{aA}	100±0.0^{aA}	145

Mean values with the same capital letters within the same row (dose level) are not significantly different at 5% ($p>0.05$).

Mean values with the same small letters within the same column (extracts) are not significantly different at 5% ($p>0.05$).

Extracts; D F=4, F=75.8 $p<0.0001$

Dose; D F=5, F=1944.5 $p<0.0001$

Extracts*dose; D F=20, F=27.73 $p<0.0001$

D F (Degree of Freedom)

For the methanol extract, *T. cormicopia* displayed an LD_{50} value of 202 ppm while *T. abyssinica* had an LD_{50} value of 265 ppm. For the chloroform extract, *T. abyssinica* presented an LD_{50} value of 350 ppm while *T. cormicopia* showed LD_{50} value of 281 ppm. Azadirachtin had an LD_{50} value of 145 ppm.

The differences in activity between azadirachtin and the crude extracts at the lower doses could be attributed to the fact that the total blend of compounds in the crude extracts may have synergistic effect on the level of activity as opposed to the pure azadirachtin.

The chloroform extracts showed the same activity at the highest dose, 1000 ppm. At 750 ppm dose, after 24 hours larval mortality was100% for all the chloroform extracts, except for the *T. abyssinica* extract which had a mortality of 97%. At 500 ppm dose, *T. abyssinica* extract exhibited an activity of 60% while *T. cormicopia* extract had an activity of 95% that was not significantly different from that of azadirachtin. At 250 ppm dose, *T. cormicopia* extract was the most active giving mortality value of 35%. At 100 ppm dose, *T. cormicopia* extract exhibited no activity while *T. abyssinica* extract had an activity of 8%. There was no significant difference between the larvicidal activities of the extracts at the 50 ppm dose.

Table 4 gives the results of the mean percent mortaliy of *A. gambiae* larvae in rearing water treated with methanol and chloroform extracts of *T. abyssinica.*

Larval mortality was dose dependent across the dose range tested for both the methanol and chloroform extracts. The methanol extract showed enhanced larvicidal activity as compared to the chloroform extract. The methanol extract showed some delayed larval mortality. At 100 ppm, mortality was low (15%) in 24 hours, but increased on prolonged exposure of the larvae to the treatment, upto 91% at day seven. A dose of 50 ppm had larvicidal activity of 91% on the eighth day. The surviving larvae moulted into pupae that emerged into adults.

The chloroform extracts showed a clear pattern of dose dependent effects. After 24 hours mortality was 100% at 1000 ppm. This reduced to 60% at 500 ppm and only 1% at 50 ppm. Seven days after treatment, mortality increased considerably to 49% for the 50 ppm dose.

Table 4: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with methanol and chloroform extracts of *T. abyssinica*

Days after Treatment	Dose (ppm)					
	50	**100**	**250**	**500**	**750**	**1000**
MeOH						
1	8.0±1.2	15.0±1.6	45.0±2.2	62.0±2.6	92.0±2.6	99.0±1
2	27.0±2.0	31.0±3.3	59.0±2.9	86.0±1.9	99.0±1.0	100±0
3	33.0±2.6	52.0±3.4	72.0±3.0	98.0±1.2	100.0±0.0	
4	39.0±3.7	73.0±4.4	100±0.0	100±0.0		
5	57.0±3.4	84.0±5.3				
6	81.0±2.9	86.0±4.9				
7	89.0±2.9	91.0±4.6				
8	91.0±2.9					
$CHCl_3$						
1	1.0±1.0	8.0±4.1	23.0±3.4	60.0±5.0	97.0±2.0	100±0
2	3.0±2.0	8.0±4.1	43.0±4.1	75.0±3.5	100±0.0	
3	4.0±2.9	8.0±4.1	62.0±4.1	94.0±2.9		
4	11.0±4.3	16.0±3.8	78.0±3.4	100.0±0.0		
5	28.0±4.1	33.0±5.1	87.0±3.4			
6	39.0±4.6	49.0±8.6	89.4±2.4			
7	49.0±2.9	69.1±7.2				
8	53.0±2.6	71.0±6.7				
9	72.7±5.0					

Results of the mean percent mortality of *A. gambiae* larvae in rearing water treated with different chloroform fractions of *T. abyssinica* are given in table 5-9.

Table 5: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TAF1 of *T. abyssinica*

	Dose (ppm)			
	500	**250**	**100**	**LD_{50}**
Days after treatment				
1	95±2.9	28.3±6.0	1.7±1.7	293.3
2	100±0.0	35±5.0	3.3±1.7	
3		45±7.6	10±2.9	
4		55±10.4	13.3±1.7	
5		73±4.4	23.0±3.3	
6		80±2.9	33.3±6.0	
7			48.3±6.0	

Table 6: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TAF2 of *T. abyssinica*

	Dose (ppm)			
	500	**250**	**100**	**LD_{50}**
Days after treatment				
1	100±0.0	43.3±6.0	16.7±4.4	252.9
2		53.3±8.8	33.3±3.3	
3		66.7±7.2	40.0±2.9	
4		78.3±6.0	61.7±3.3	
5		81.7±4.4	70.0±5.8	

Table 7: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TAF3 of *T. abyssinica*

	Dose (ppm)			
	500	**250**	**100**	**LD_{50}**
Days after treatment				
1	96.7±1.7	26.7±4.4	8.8±3.3	279.19
2	100±0.0	35±2.9	25±5.0	
3		55±5.6	41.7±4.4	
4		76.7±3.3	58.3±3.3	
5		100±0.0	76.7±4.4	
6			100±0.0	

Table 8: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TAF4 of *T. abyssinica*

	Dose (ppm)			
	500	250	100	LD_{50}
Days after treatment				
1	100±0.0	56.7±10.3	21.7±4.4	185.4
2		73.3±7.3	41.7±6.7	
3		96.0±3.3	73.3±4.4	
4		100±0.0	83.3±4.4	
5			96.0±3.3	

Table 9: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TAF5 of *T. abyssinica*

	Dose (ppm)			
	500	250	100	LD_{50}
Days after treatment				
1	86.7±4.41	46.7±10.9	11.7±1.7	265
2	100±0.0	55.0±10.4	13.3±4.4	
3		70.0±8.7	38.3±8.8	
4		78.3±4.4	56.7±8.8	
5			66.7±4.4	

Fractionation of the chloroform soluble fraction of *T. abyssinica* yielded four limonoids-rich fractions. The non-limonoid fractions were combined to make one fraction TAF1 that induced the least activity with an LD_{50} of 293.3 ppm. Fraction TAF4 eluted at 85% ethyl acetate elicited the highest mortality with an LD_{50} 185.4 ppm. The larvicidal activities of all the fractions increased on prolonged exposure of larvae to the treatment.

Table10: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with methanol and chloroform extract *T. cormicopia*

Days after Treatment	Dose (ppm)					
	50	**100**	**250**	**500**	**750**	**1000**
MeOH						
1	1.0±1.0	14.0±1.9	58.0±4.6	96.0± 1.9	100.0±0.0	100.0±0.0
2	16.0±2.9	39.0±4.3	76.0±3.7	100.0±0.0		
3	35.0±3.5	71.0±4.8	98.0±2.0			
4	54.0±4.3	90.0±4.2	100.0±0.0			
5	72.0±4.6	98.0±2.0				
6	97.0±2.0					
7	100.0±0.0					
$CHCl_3$						
1	0.0	0.0	5.0± 3.5	90.5±2.0	100.0±0.0	100.0±0.0
2	0.0	8.0±4.1	72.3±5.4	100.0±0.0		
3	4.0±2.5	16.0±4.0	84.1±1.9			
4	11.0±2.5	25.0±3.5	89.3±2.2			
5	15.0±2.2	32.0±3.0	100.0±0.0			
6	20.0±1.6	38.0±2.6				
7	25.0±2.4	51.0±1.9				
8	32.0±2.5	56.0±1.9				
9	40.0±1.6	58.0±1.2				
10	45.0±1.6	60.0±1.6				

At the 50 ppm dose of the methanolic extract, larval mortality was only 1% after 24 hours of exposure. Mortality increased to 100% after 7 days of exposure of larvae to the treatment at the same dose. 1000 and 750 ppm doses showed larvicidal activity of 100% after 24 hours. Larval mortality at 500 and 250 ppm dose was 96% and 58%, respectively after 24 hours.

Larvicidal activity of the chloroform soluble fractions was comparable to the methanol extracts at higher doses but showed delayed larval mortality at lower doses. A dose of 50 ppm had little larvicidal activity which increased to 45% on the tenth day. The surviving larvae moulted into pupae that emerged into adults.

Results of the mean percent m y of *A. gambiae* larvae in rearing water treated with different chloroform solubl tions of *T. cormicopia* are given in Table 11-19.

Table 11: Cumulative mean percent m ity of *A. gambiae* larvae in rearing water treated with chloroform soluble fracti F1 of *T. cormicopia*

	Dose (ppm)			
	500		100	LD_{50}
Days after treatment				
1	60.0±7.6	)±2.3	0.0±0.0	425.9
2	81.7±3.3	3±4.4	5.0±2.9	
3	96.7±3.3	)±5.0	16.7±4.4	
4	100±0.0	:7.6	28.3±4.4	
5		0±5.8	35.0±5.0	
6		7±3.3	50.0±2.9	
7		.7±1.7	55.0±2.9	
8			63.3±1.7	

Table 12: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TCF2 of *T. cormicopia*

	Dose (ppm)			
	500	**250**	**100**	**LD_{50}**
Days after treatment				
1	66.7±6.0	21.7±4.4	1.7±1.7	392.4
2	76.7±6.0	26.7±7.3	6.7±1.7	
3	95.0±5.0	53.3±7.3	33.3±4.4	
4	100±0.0	75.0±5.8	51.7±6.0	
5		76.7±3.3	63.3±1.7	

Table 13: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TCF3 of *T. cormicopia*

	Dose (ppm)			
	500	**250**	**100**	**LD_{50}**
Days after treatment				
1	83.3±7.3	15.0±2.9	5.0±2.9	347.6
2	95.0±5.0	18.3±4.4	10.0±2.9	
3	100±0.0	36.7±4.4	20.0±2.9	
4		61.7±4.4	36.7±1.7	
5		71.7±4.4	65.0±5.7	
6			66.7±6.0	

Table 14: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TCF4 of *T. cormicopia*

	Dose (ppm)			
	500	250	100	LD_{50}
Days after treatment				
1	100±0.0	95.0±5.0	33.3±4.4	118.7
2		100±0.0	41.7±1.7	
3			65.0±2.9	
4			80.0±2.9	
5			88.3±1.7	

Table 15: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TCF5 of *T. cormicopia*

	Dose (ppm)			
	500	250	100	LD_{50}
Days after treatment				
1	100±0.0	90.0±5.0	75.0±2.9	56.6
2		100±0.0	81.7±3.3	
3			91.7±1.7	
4			100±0.0	
5				

Table 16: Cumulative mean percent mortality of *A. gambiae* larvae in rearing water treated with chloroform soluble fraction TCF6 of *T. cormicopia*

	Dose (ppm)			
	500	**250**	**100**	**LD_{50}**
Days after treatment				
1	83.3±6.0	31.7±6.0	15.4±2.9	270.8
2	93.3±3.3	50.0±2.9	18.3±1.7	
3	100±0.0	56.7±4.4	40.0±2.9	
4		75.0±2.9	58.3±6.0	
5			61.7±1.7	
6			73.3±4.4	

Fractionation of the chloroform soluble fraction of *T. cormicopia* yielded five limonoids–rich fractions. The non-limonoid fractions were combined to make one fraction TCFI that was the least active with LD_{50} value of 425.9 ppm. Fraction TCF5 eluted at 70% ethyl acetate induced the highest mortality (LD_{50} of 56.6 ppm)

Table 17: Mean percent mortality of adult *A. gambiae* treated with methanol and chloroform extracts

Extract	Dose (ppm)				
	100	250	500	1000	LD_{50}
MeOH					
T. abssynica	8.3 ± 3.3^{bB}	16.7 ± 4.4^{bB}	20.0 ± 2.9^{cB}	43.3 ± 7.3^{cA}	1569
T. cormicopia	26.7 ± 1.7^{aC}	36.7 ± 4.4^{abBC}	50.0 ± 2.9^{abB}	66.7 ± 4.4^{abcA}	451.8
$CHCl_3$					
T. abssynica	10.0 ± 0.0^{bC}	16.7 ± 4.4^{bBC}	38.0 ± 6.0^{bcAB}	50.0 ± 7.6^{bcA}	945.5
T. *cormicopia*	31.7 ± 3.3^{aB}	38.3 ± 6.0^{aB}	61.7 ± 4.4^{aA}	76.7 ± 4.4^{aA}	302.1
Azadirachtin	20.0 ± 2.9^{aC}	33.3 ± 1.7^{abB}	45.0 ± 2.9^{abB}	70.0 ± 2.9^{abA}	552.2

Mean values with the same capital letters within the same row (dose level) are not significantly different at 5% ($p>0.05$).

Mean values with the same small letters within the same column (extracts) are not significantly different at 5% ($p>0.05$).

Extracts; D F=4, F=32.6 $p<0.0001$

Dose; D F=3, F=91.3 $p<0.0001$

Extracts*dose; D F=12, F=0.96 $p<0.0001$

D F (Degree of Freedom)

4.2.2 Adulticidal activity

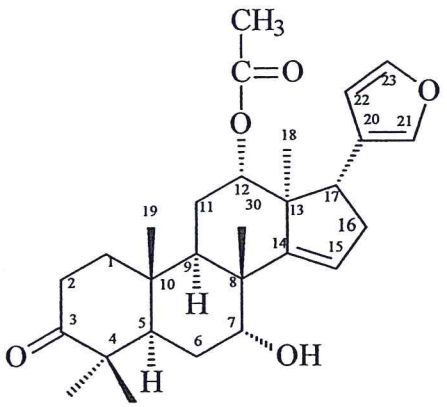
The adulticidal activity for all the extracts was dose dependent. The chloroform extracts exhibited higher activity than the corresponding methanol extracts. As an adulticide, the chloroform extract was most potent, with an LD_{50} value of 302.1 ppm which was lower than that of azadirachtin at 552.2 ppm. The methanol extract was the least active as an adulticide with an LD_{50} value of 1569 ppm. At 100 ppm dose, there was no significant difference between azadirachtin and the methanol and chloroform extracts of *T. cormicopia* at 95% confidence interval.

4.3 Structure Elucidation for Isolated Compounds

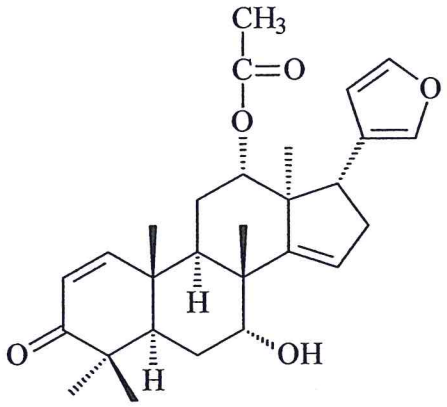
In light of the larvicidal and adulticidal activities of these extracts, the most active fractions were analysed to identify the individual constituents in order to find out which ones are responsible for the activity.

The compounds **115-117** were obtained from semi-preparative HPLC of the respective silica gel column chromatography fractions of *T. cormicopia.* All were obtained as white powders. The purity of the isolates was confirmed by analytical HPLC. The compounds were characterized by analysis of their NMR, IR, UV and MS spectra data.

4.3.1 Compound 115



115



116

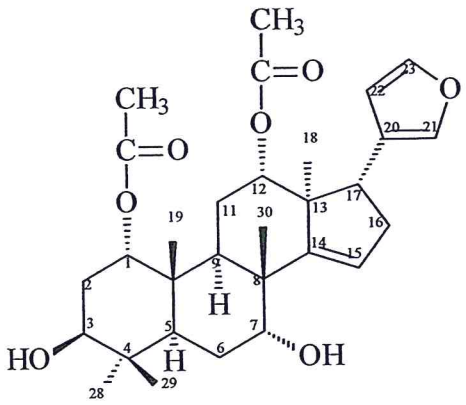
High resolution mass spectrometry of compound **115**, showed molecular ion peak at 454 (Calculated value of 454.6052). Fragment ion peaks were observed at m/z 394 $[M-CH_3COOH]^+$ and 376 $[M-CH_3COOH-H2O]^+$ due to the loss of acetic acid and water. The molecular formula of $C_{28}H_{38}O_5$ was deduced from the mass spectrum in conjugation with the ^1H and ^{13}C NMR spectrum. The infrared spectrum (KBr) indicated the presence of a hydroxyl (3423 cm^{-1}), an ester (1737 cm^{-1}), a ketone (1702 cm^{-1}), C-O (1248 cm^{-1}) and an olefinic double bond (1652 cm^{-1}). The presence of the ketone and ester was further substantiated by ^{13}C NMR absorptions at δ 216.9 and δ 170.8 respectively. The limonoid

β-substituted furan ring proton occurred at δ 7.34, 7.21 and 6.26 in the 500 ^{1}H NMR spectrum. A proton resonance at δ 5.68 that corresponds to a methine carbon resonance at δ 122.6 in the HMQC spectrum confirmed the C-14/C15 double bond. A hydroxy and an acetate group were present at C-7α and C-12α respectively. The H-7 resonance at δ 4.0 was assigned based on HMBC correlation with C-5 and 3H-30. The H-12 resonance at δ 5.11 showed HMBC correlations with C-18 and C-17. The acetate methyl group proton singlet occurred at δ 1.89. ^{1}H NMR COSY techniques verified coupling of H-17 and H-15 with H-16, H-9 and H-12 with H-11, H-22 with H-21 and H-23 and H-6 with H-5 and H-7. Stereochemistry was assigned on basis of the NOE experiments. The H-7 had a NOE correlation with 3H-30 confirming that H-7 was β and thus, the hydroxy group was α. The H-12 had a NOE correlation with 3H-30 confirming that H-12 was β and thus the acetate group was in the α orientation. The 3Hβ-29 showed NOEs to H-6β, H-7β, H-12β and H-15β. Further assignments were determined on the basis of HMQC and HMBC correlations (Table 18). The ^{1}H, ^{13}C NMR and Mass spectra are given in fig 3-18. Compound **115** was characterized as 12α-acetoxy-1,2-dihydro-7-deacetylazadiron. It has previously been isolated from *Turraea robusta* (Rajab *et al.*, 1988 b).

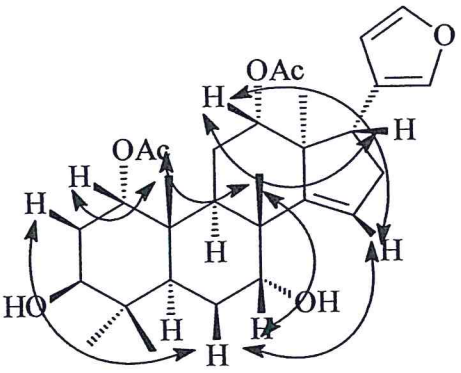
4.3.2 Compound 116

Compound **116** was found to be α, β-unsaturated ketone of **115**. This was indicated by a pair of doublets at δ 7.03 (J=10.2 Hz, H-1) and δ 5.83 (J=10.2 Hz, H-2) and resonances at δ 157.3, δ 125.7 and δ 204.8 ascribed to C1, C2, and C-3 respectively (^{1}H and ^{13}C NMR data on table 19). The ^{1}H, ^{13}C NMR and Mass spectra are given in fig 19-32. It has been isolated as a synthetic derivative by reacting compound **115** with benzene seleninic anhydride and refluxing for thirty minutes in chlorobenzene (Rajab *et al.*, 1988b). This is the first report of its isolation from a natural source.

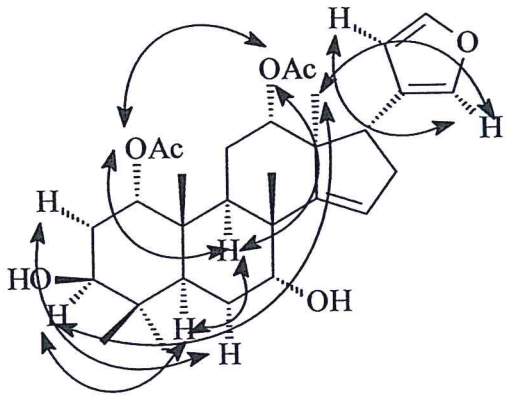
4.3.3 Compound 117



117



117-top face



117-back face

Figure 2: NOESY correlations of compound **117**

Compound **117** had an elemental formula of $C_{30}H_{42}O_7$ (HREIMS m/z 514.1). Calculated value was found to be 514.6574. Peaks at m/z 454 $[M\text{-}CH3COOH]^+$, 394 [M-2 $(CH3COOH)]^+$ and 376 [M-2 (CH3COOH)-H2O]$^+$ indicated the presence of two acetate groups and a hydroxyl in the molecule. Infrared (KBr) spectroscopy indicated the presence of hydroxyl (3558 cm^{-1}), ester (1740 cm^{-1}), ketone (1728 cm^{-1}) and C-O (1245 cm^{-1}). A $^{\Delta}14$ –double bond was indicated by resonance at δ 158.6 and δ 122.6 for C-4 and

C-15 respectively, and resonances ascribed to H-15 at δ 5.68. The limonoid β-substituted furan ring proton occurred at δ 7.33, 7.20 and 6.24 in the 500 ^{1}H NMR spectrum. Two hydroxyl groups were present at C-3β (δ 77.2) and C-7α (δ 71.8) respectively. The H-3 resonance at δ 2.26 was assigned based on HMBC correlation with C-1. The H-7 resonance at δ 3.96 was assigned based on HMBC correlation with C-5.

Two acetate groups were present C-1α (δ 74.7) and C-12α (δ 76.9) respectively. The H-1 resonance at δ 4.7 was assigned based on HMBC correlation with C-10 (δ 35.4) and C-5 (δ 40.4). The H-12 resonance at δ 5.08 was assigned based on HMBC correlation with C-17(δ 50.1) and C-18 (δ 21.4). The relative stereochemistry at C-1, C-5, C-9 and C-12 was assigned based on the NOE experiments. The H-9α resonance was seen to correlate with 3H-18 and 3H-28 (which are α) and H-5 also showed a NOE correlation with the 3H-28. The H-1 and H-12 showed a NOE correlation with 3Hβ-19 confirming the α orientation of both the acetate groups at C-1 and C-12 respectively. The H-7 had a NOE correlation with 3H-30 confirming that H-7 was β and thus, the hydroxy group was α. The H-3 had a NOE correlation with 3H-18 confirming that H-3 was α and thus, the hydroxy group was β. The H-17 resonance showed a NOE correlation with H-12β confirming the β-orientation of H-17. ^{1}H NMR COSY techniques verified coupling of H-17 and H-15 with H-16, H-9 and H-12 with H-11, H-22 with H-21 and H-23, H-6 with H-5 and H-7 and H-2 with H-1 and H-3. Further assignments were done based on the HMQC and HMBC correlations (Table 20). The ^{1}H, ^{13}C NMR and Mass spectra are given in fig 33-46. Compound **117** is new and was characterised as 1α-12α-diacetoxy-1,2-dihydro-7-deacetyl-3β-7α-dihydroxyazadiron.

Due to the small amounts of the compounds, bioassay experiments were not carried out.

Table 18: ^{1}H and ^{13}C NMR spectrum data for compound (115)

Proton	δ (Multiplicity J, Hz)	HMQC	HMBC
1β	1.89,m	38.4	C-2, C-5, C-10
1α	1.60,m		C-19
2 β	2.45,m	33.8	C-10, C-1
2 α	2.58,m		C-1
3		216.9	
4		43.9	
5	2.1,m	46.3	C-1, C-6, C-10
6 β	1.86,m	24.8	C-5, C-8, C-10
6 α	1.98,m		
7	4.0,bs	71.8	C-5
8		46.9	
9	2.36,m	42.6	C-5, C12
10		37.0	
11 β	2.42,m	25.3	C-9, C-10
11 α	1.59,m		C-9, C-12
12	5.11,t (8.6)	77.2	C-13, C-17, C18, Ac CO
13		51.3	
14		158.8	
15	5.68,d (1.7)	122.6	C-13, C-14, C-16
16 β	2.54,m	36.8	C-14, C-15, C-17, C-20
16 α	2.55,m		
17	3.06,t (7.9)	50.2	C-12, C-13, C-15, C-16, C21, C-22
18	1.15, 3H,s	21.1	C-12, C-13, C-14, C-17
19	1.04,3H,s	15.6	C-1, C-5, C-9, C-10
20		124.3	
21	7.21,s	140.3	C-20, C-22, C-23
22	6.26,s	111.7	C-20, C-21, C-23
23	7.34,s	142.1	C-20, C-21, C-22
28	1.14,3H,s	26.1	C-4, C-5
29	1.02,3H,s	14.8	C-4, C-5
30	1.16,3H,s	27.4	C-7, C-8, C-9, C-14
Ac (Me)	1.89,s	21.3	
Ac (CO)		170.8	

Table 19: ^{1}H and ^{13}C NMR spectrum data for compound (116)

Proton	δ (Multiplicity J, Hz)	HMQC	HMBC
1	7.03,d, (10.2)	157.3	C-2, C-3, C-10
2	5.83,d, (10.2)	125.7	C-10, C-5
3		204.8	
4		44.1	
5	2.43,m	46.3	C-1, C-4, C-6, C-10 C-28, C-29
6 β	1.87,m	24.3	C-5, C-8,
6 α	1.94,m		
7	3.99,bs	71.3	C-5
8		44.7	
9	2.39,m	40.0	C-5, C-10, C12
10		38.3	
11 β	2.44,m	25.3	C-8, C-10, C-12
11 α	1.61,m		C-10, C-12, C-13
12	5.16,t (8.8)	77.2	C-13, C-17, C18, Ac CO
13		51.3	
14		158.6	
15	5.69,d (2.0)	122.6	C-13, C-16
16 β	2.54,m	36.8	C-14, C-13, C-20
16 α	2.55,m		
17	3.08,t (8.0)	50.2	C-12, C-13, C-16, C-22
18	1.16, 3H,s	21.1	C-12, C-13, C-14, C-17
19	1.03,3H,s	15.6	C-9, C-10
20		124.3	
21	7.20,s	140.3	C-20, C-22, C-23
22	6.26,s	111.7	C-20, C-21, C-23
23	7.22,s	142.1	C-20, C-21, C-22
28	1.16,3H,s	27.0	C-4, C-3
29	1.02,3H,s	15.6	C-3, C-4, C-5, C-28
30	1.16,3H,s	27.9	C-7, C-9, C-14
Ac (Me)	1.89,s	21.4	
Ac (CO)		170.8	

Table 20: ^{1}H and ^{13}C NMR spectrum data for compound (117)

Proton	δ (Multiplicity J, Hz)	HMQC	HMBC
1	4.0,s	74.7	C-5, C-10
2 β	1.97,d, (16.2)	28.3	C-1, C-4, C-10,
2 α	2.08,d, (10.3)		
3	3.35,t, (10.0)	77.2	C-1
4		37.3	
5	2.26,m	40.4	C-6, C-7, C-9, C-19, C-29
6 β	1.89,m	23.5	C-5, C-8,
6 α	1.87,m		
7	3.96,bs	71.8	C-5
8		44.3	
9	2.67,m	36.7	C-1, C-5, C-8, C-10, C12, C-14, C-19, C-30
10		35.4	
11 β	1.00,m	24.8	C-8, C-10, C-12
11 α	0.99,m		
12	5.08,t, (8.7)	76.9	C-11,C-13, C-17, C18, Ac CO
13		51.3	
14		158.8	
15	5.68,s	122.7	C-13, C-16, C-14
16 β	2.55,m	34.3	C-14, C-13, C-17, C-20
16 α	2.52,m		
17	3.03,t, (2.2)	50.1	C-12, C-13, C-16, C-20, C-21, C-22
18	1.01, 3H,s	21.4	C-12, C-13, C-14, C-17
19	0.95,3H,s	15.6	C-1, C-5, C-9, C-10
20		124.2	
21	7.20,s	140.3	C-20, C-22, C-23
22	6.24,s	111.6	C-20, C-21, C-23
23	7.33,s	142.1	C-20, C-21, C-22
28	1.02,3H,s	27.7	C-4, C-3
29	0.84,3H,s	15.6	C-3, C-4, C-28
30	1.12,3H,s	27.8	C-7, C-8, C-9, C-14
Ac (Me)	1.89,s	21.4	
Ac (Me)	2.07	22.1	
Ac (CO)		168.7	
Ac (CO)		170.8	

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

- Two Meliaceae species were studied; *Turraea abyssinica* and *T.cormicopic* is the first time that *T.cormicopia* has been studied phytochemically.
- Three limonoids were isolated from *T. cormicopia* and classified as azadiro derivative type.
- The methanol and chloroform extracts exhibited potent larvicidal and adu activities against *A. gambiae*.
- Delayed mortality was observed at low doses for all the methanol and chlo extracts.
- These plants offer a significant potential for mosquito control as larvici adulticides. Due to the stability of the constituent limonoids against U oxygen, the extracts could be potential bed net impregnants

5.2 Recommendations

- From this study, it was not possible to attribute the activity of the mo fractions to the isolated compounds, though they were the major consti the respective active fractions. Bioassays could be carried out using characterised constituents to determine whether bioactivities are due to sy or additive effects of the minor compounds.
- Further bioassays are recommended in order to observe delayed effec extracts and isolated compounds on the adult mosquito, i.e. delayed r fecundity, egg viability and survival rates of the next filial generation view of developing non-knock down mosquito control products.
- The isolated compounds could be derivatized and structure activity rela carried out on *A. gambiae* to come up with more potent larvicides and ac from the synthetic derivatives.

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APPENDICES

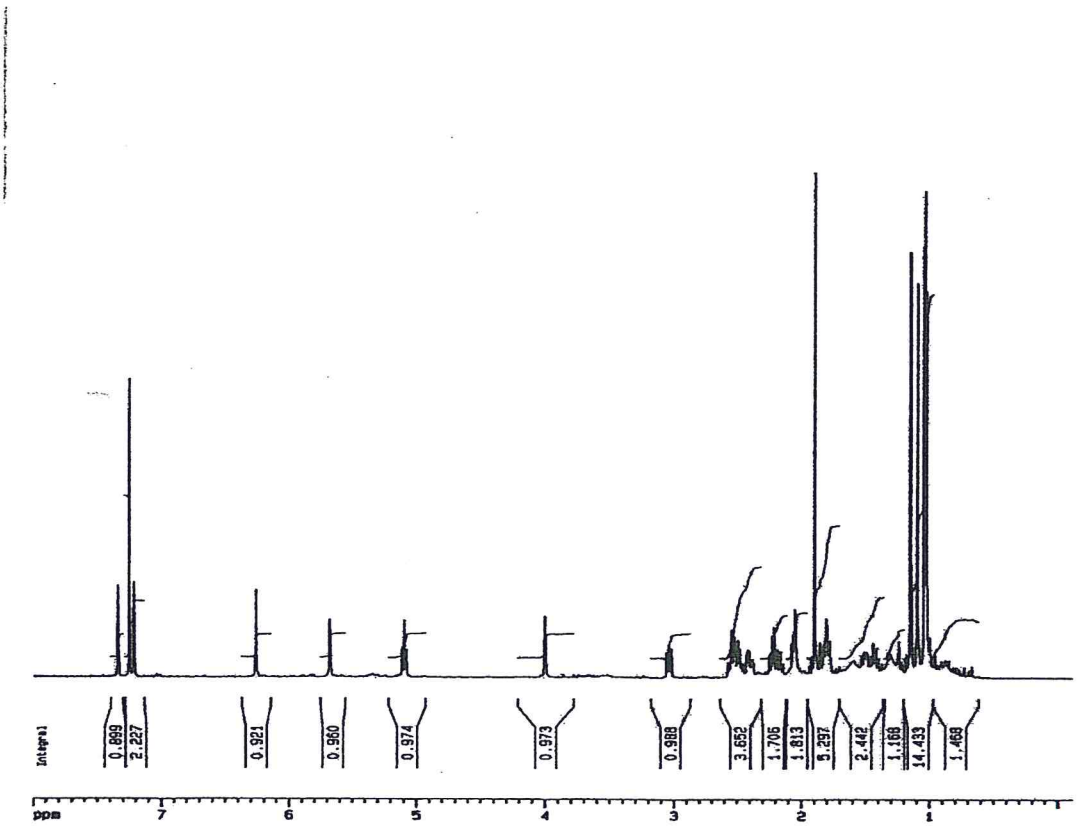

Figure 3:500 MHz ^{1}H NMR spectrum of compound **115**

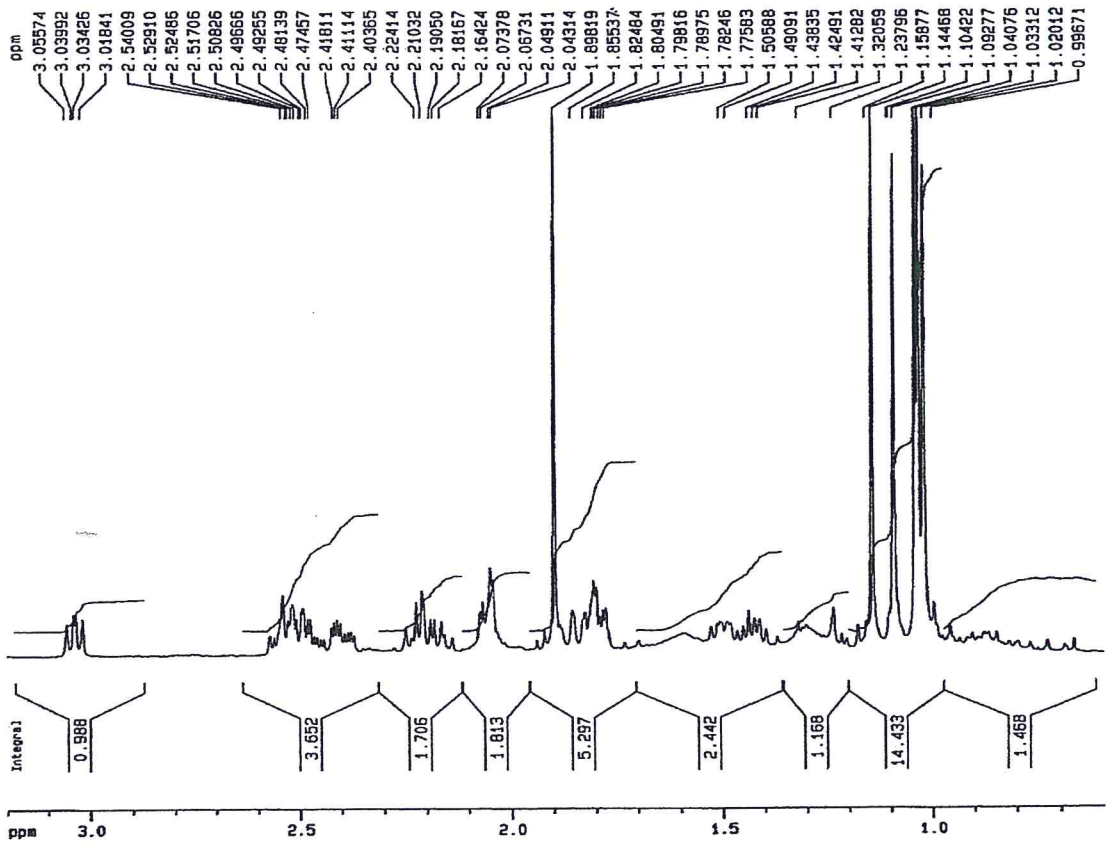

Figure 4: Expanded section of 500 MHz ^{1}H NMR spectrum of compound **115**

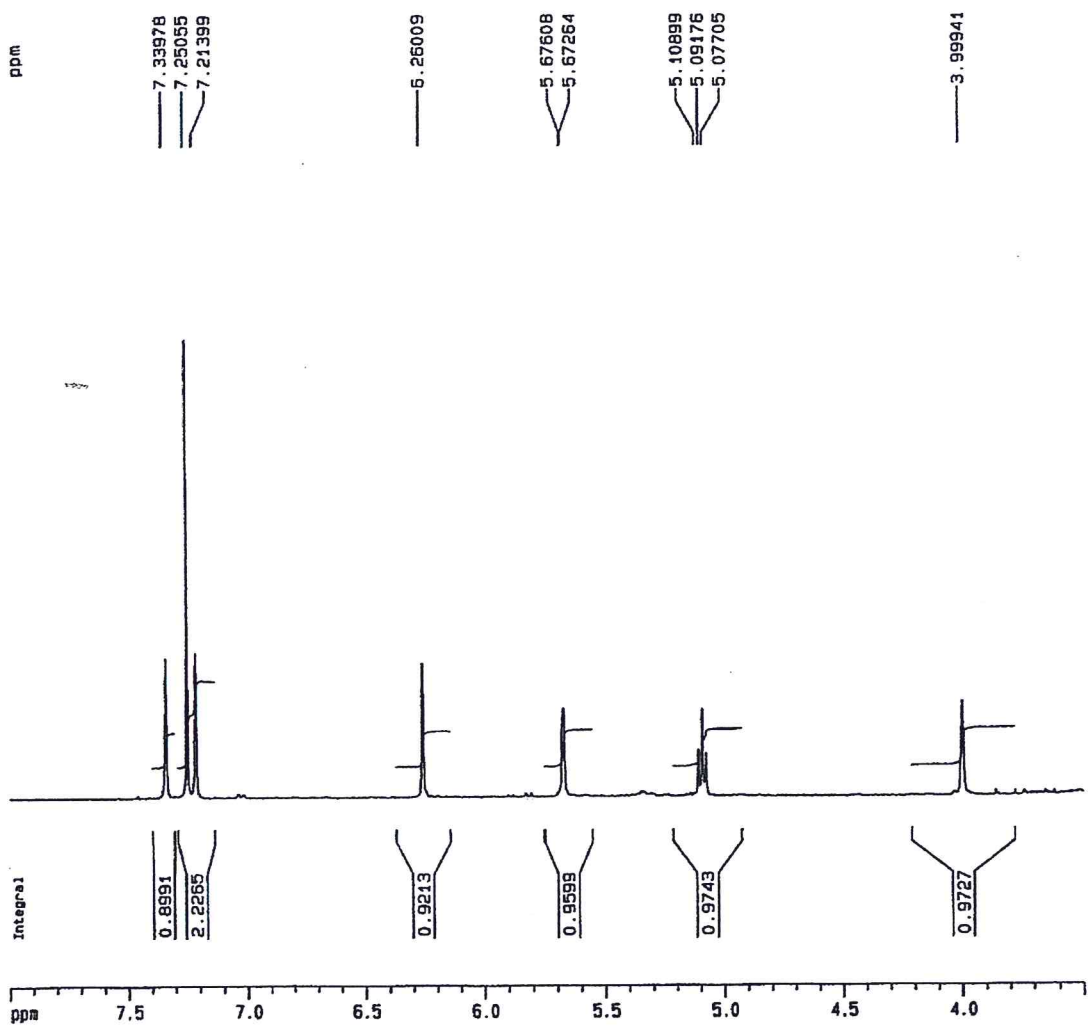

Figure 5: Expanded section of 500 MHz ^{1}H NMR spectrum of compound **115**

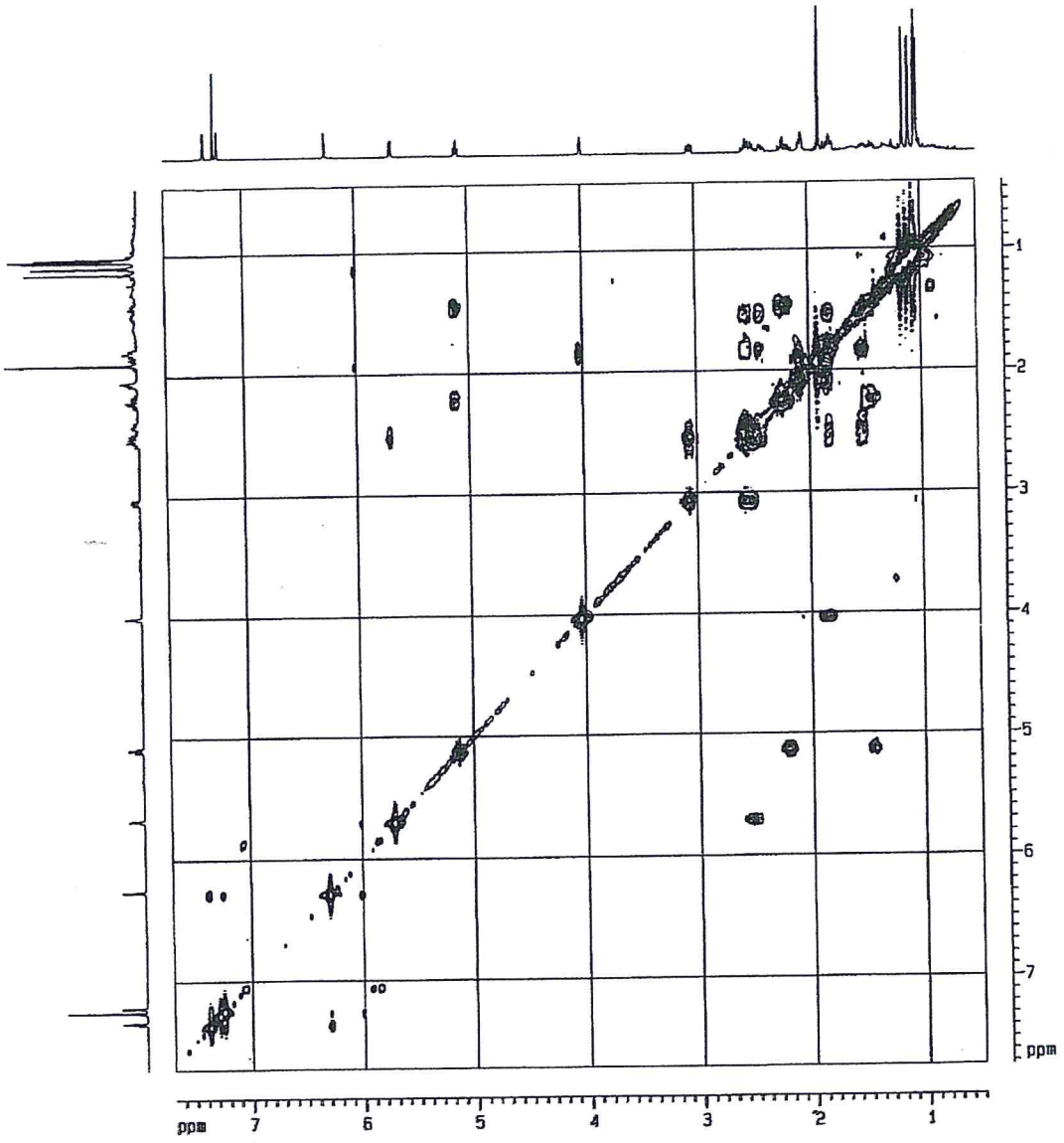

Figure 6: 500MHz COSY spectrum of compound **115**

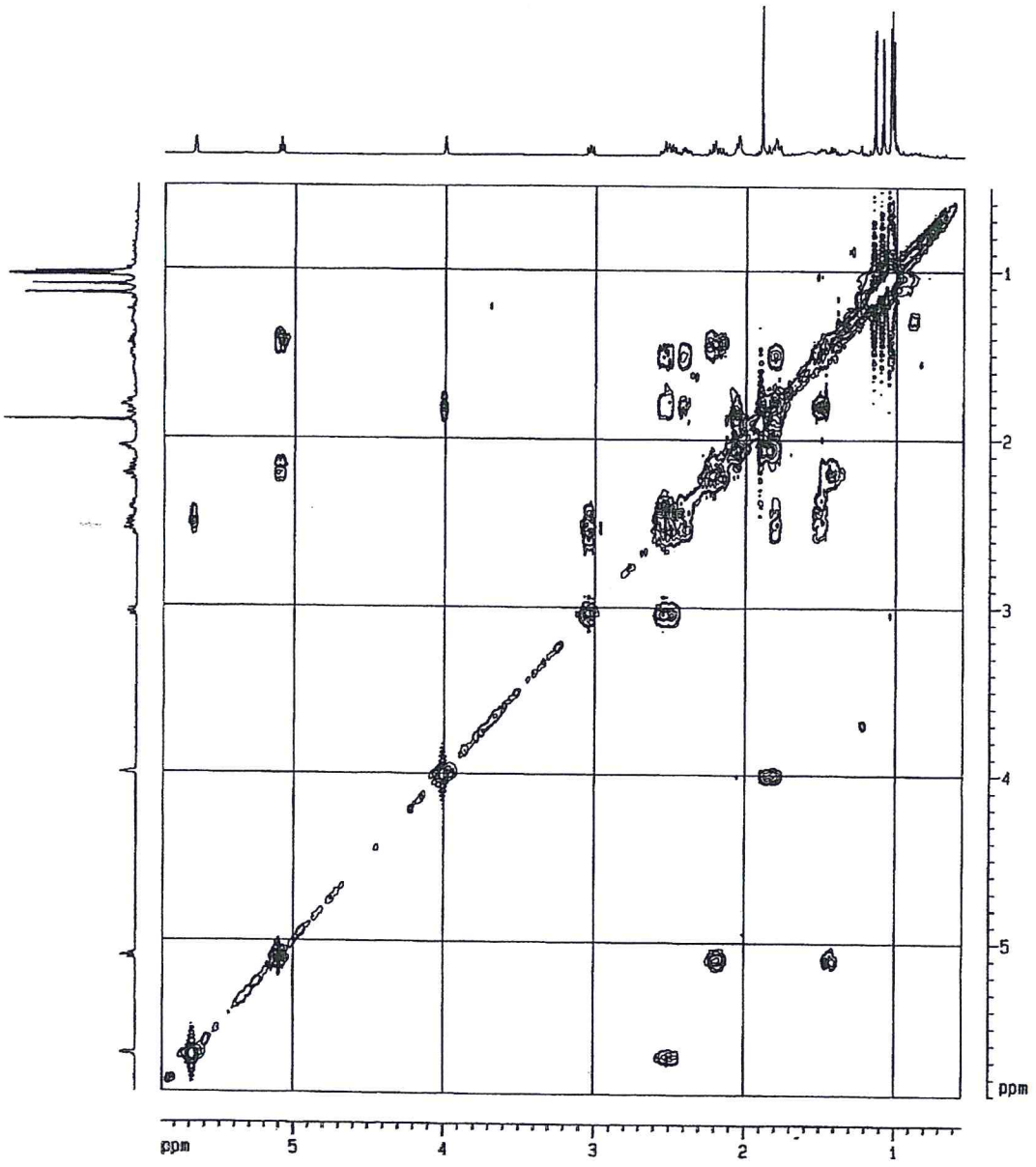

Figure 7: Expanded section of 500 MHz COSY spectrum of compound **115**

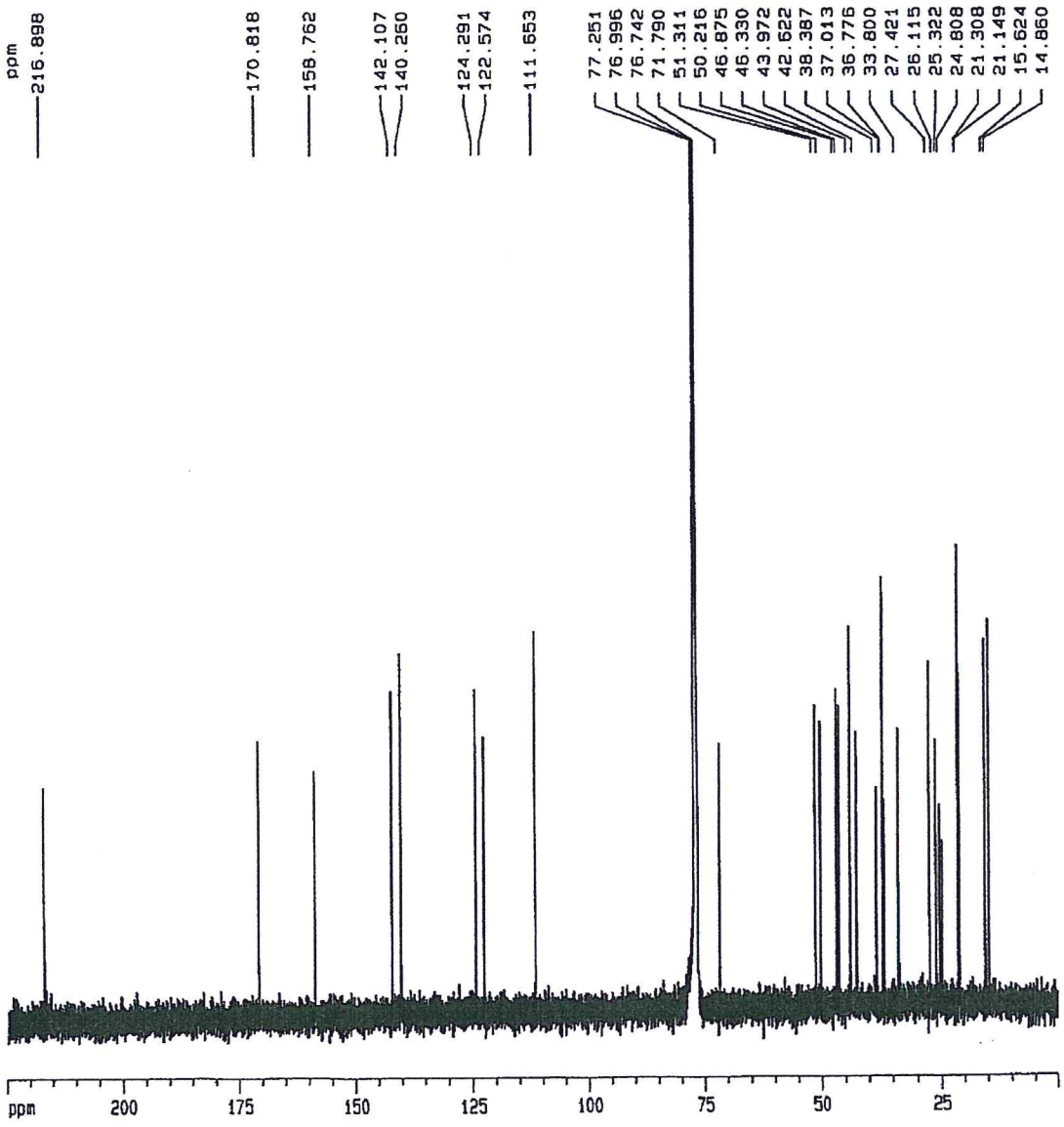

Figure 8: 125 MHz ^{13}C spectrum of compound **115**

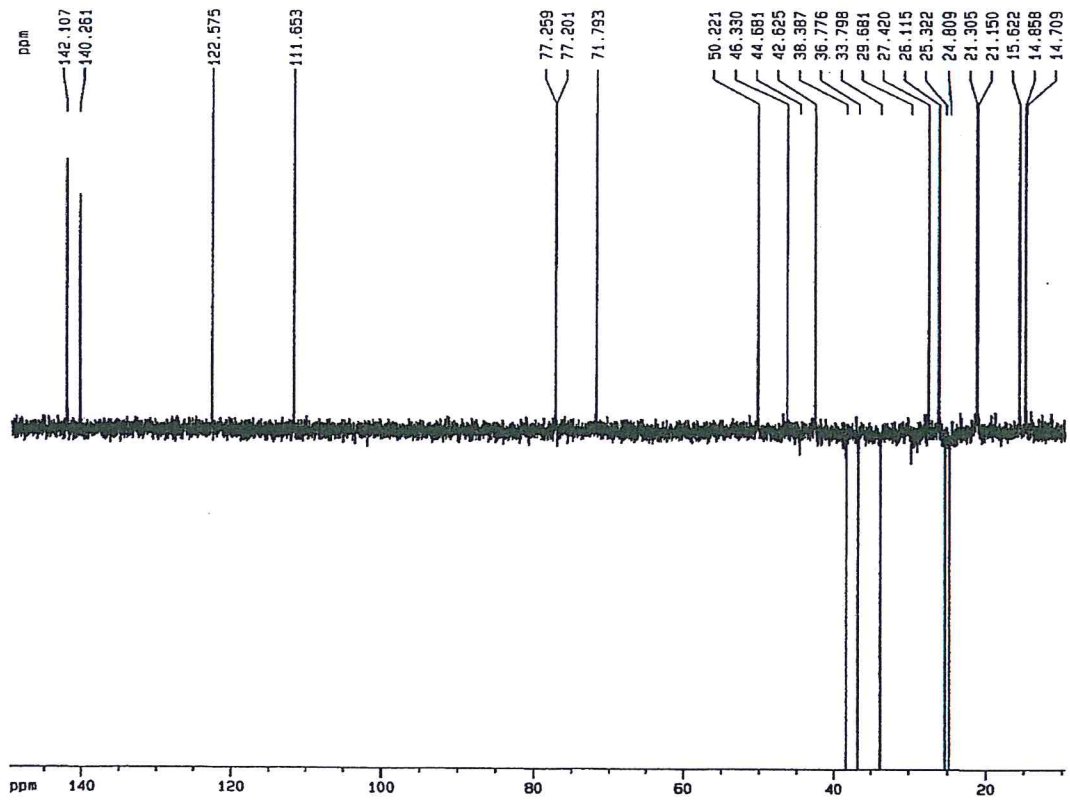

Figure 9: 125 MHz ^{13}C DEPT spectrum of compound **115**

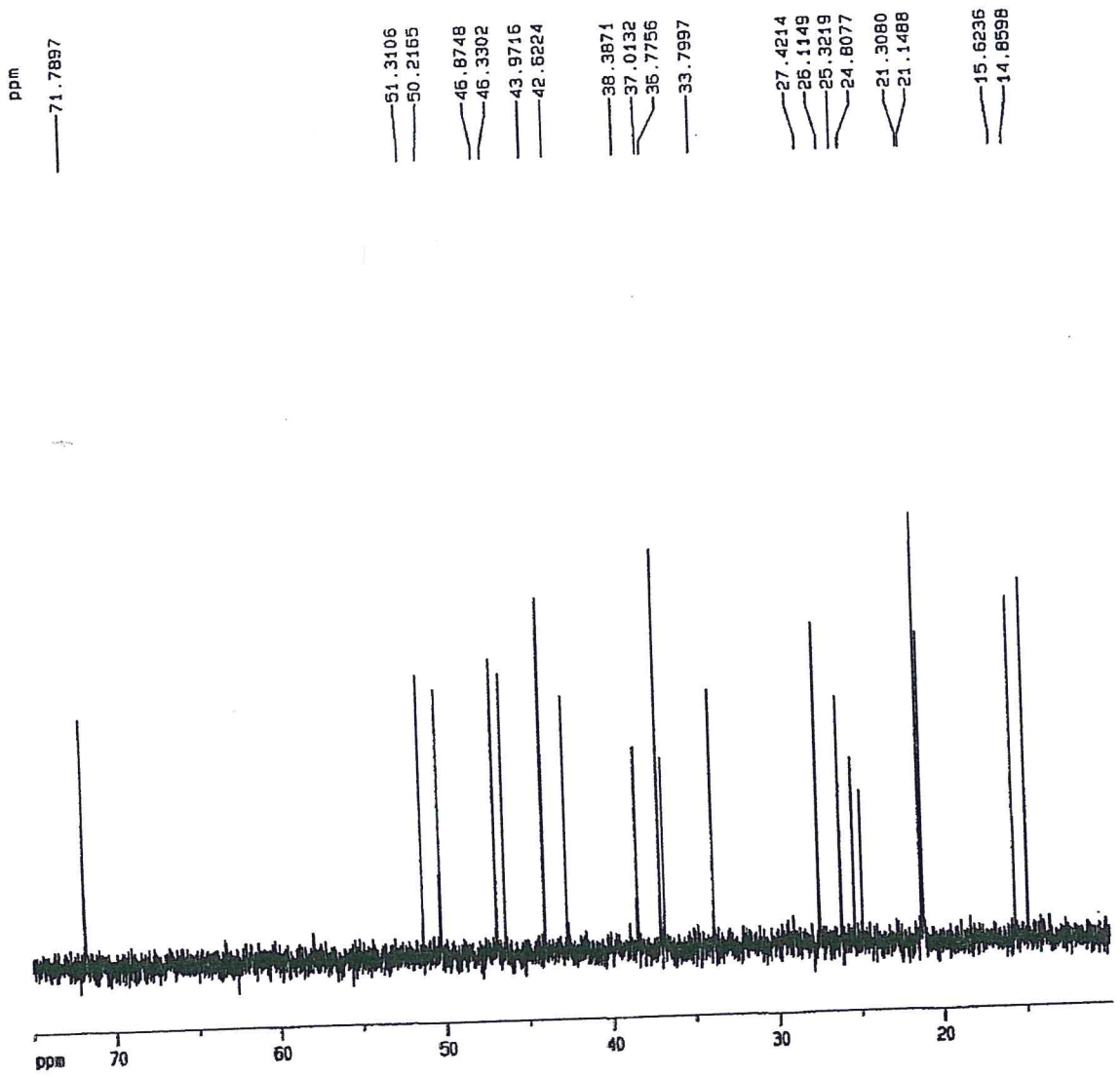

Figure10: Expanded section of 125 MHz ^{13}C spectrum of compound **115**

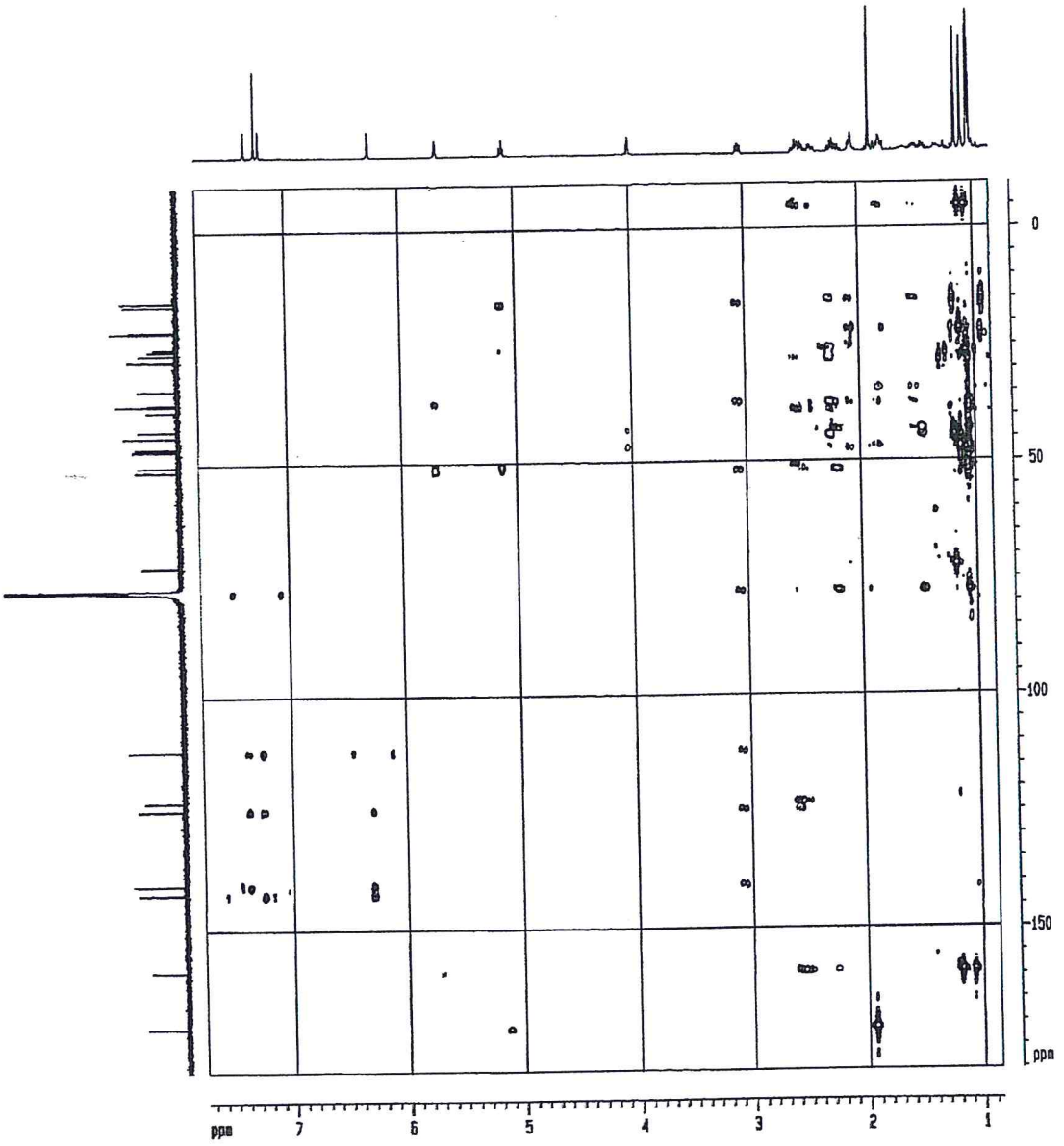

Figure 11: 500 MHz HMBC spectrum of compound **115**

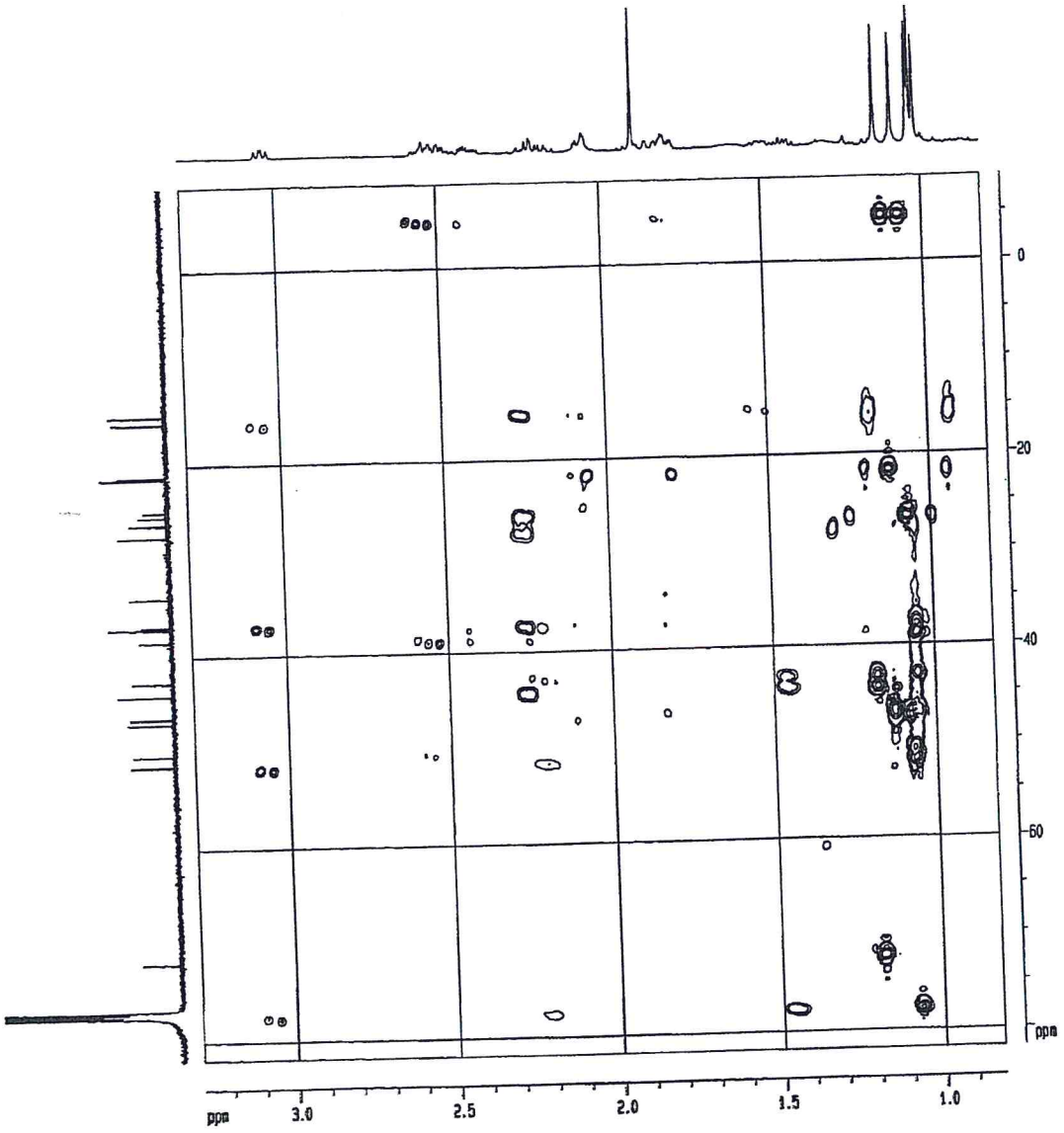

Figure 12: Expanded section of 500 MHz HMBC spectrum of compound **115**

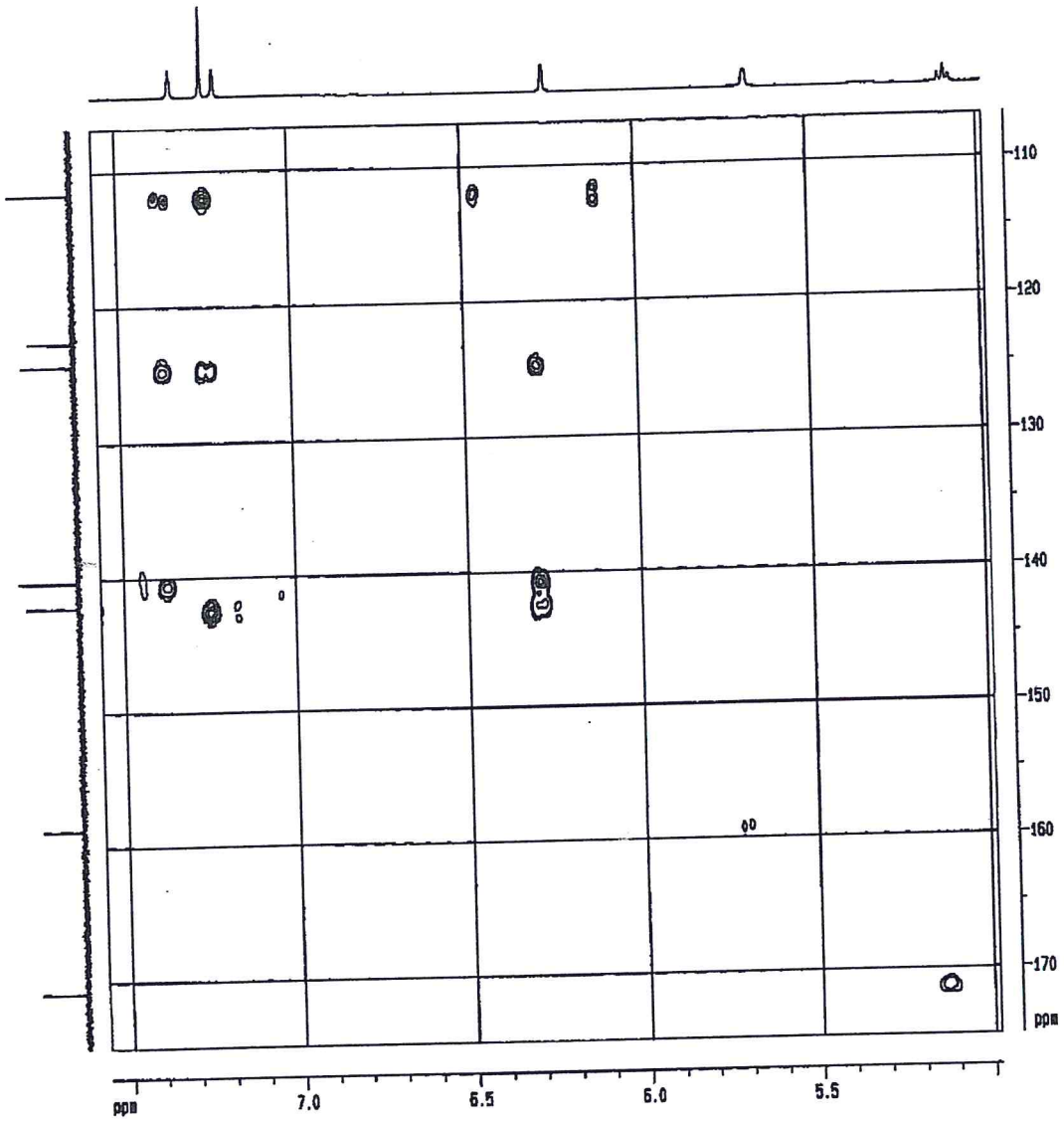

Figure 13: Expanded section of 500 MHz HMBC spectrum of compound **115**

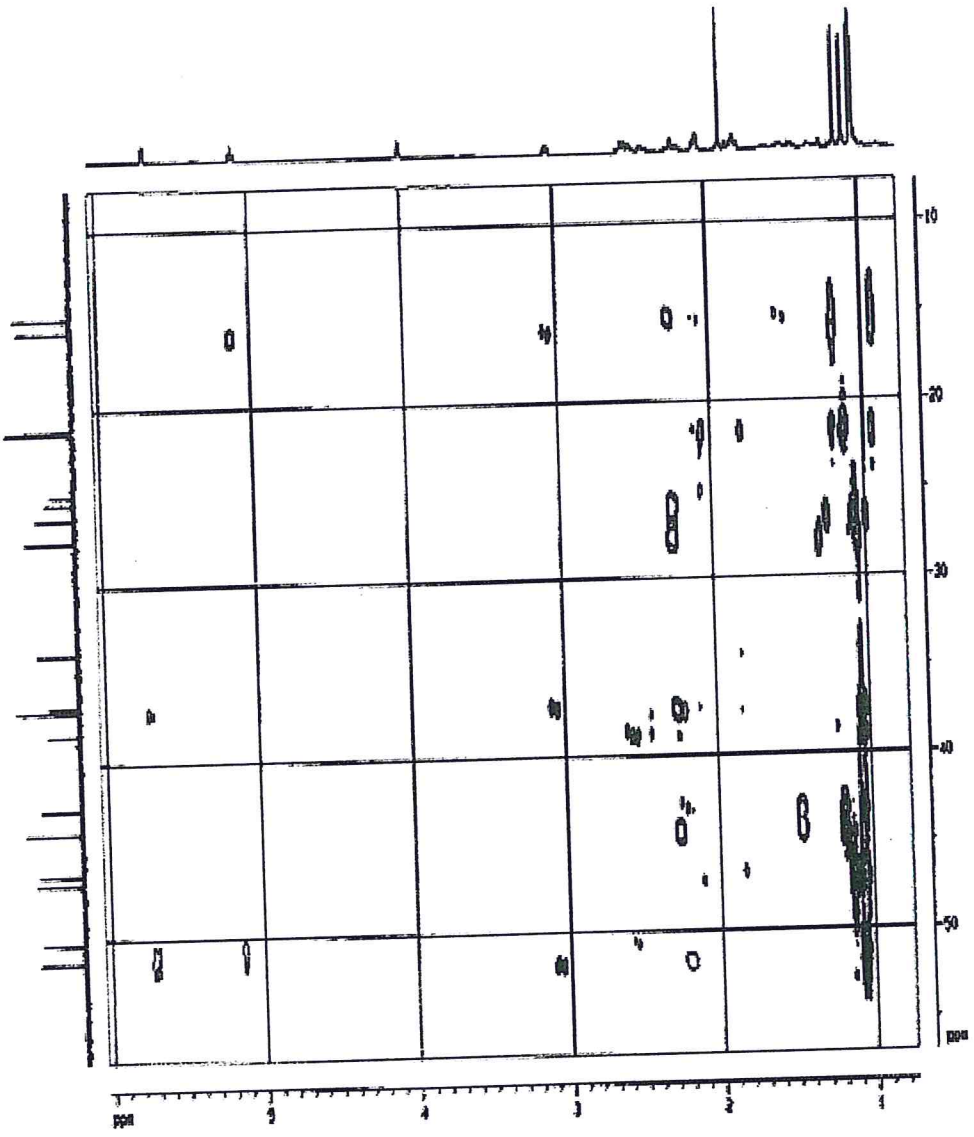

Figure 14: Expanded section of 500 MHz HMBC spectrum of compound **115**

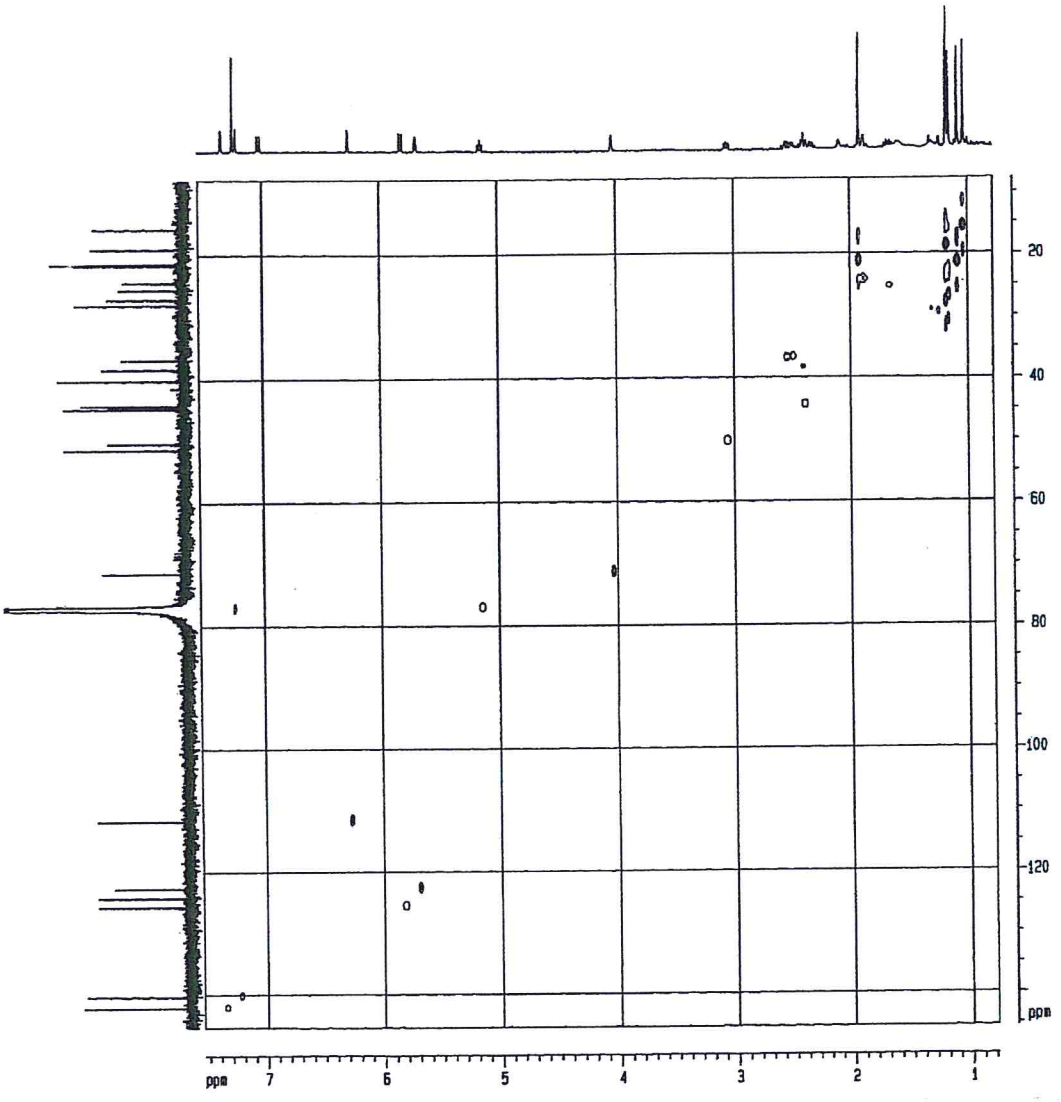

Figure 15: Expanded section of 500 MHz HMBC spectrum of compound **115**

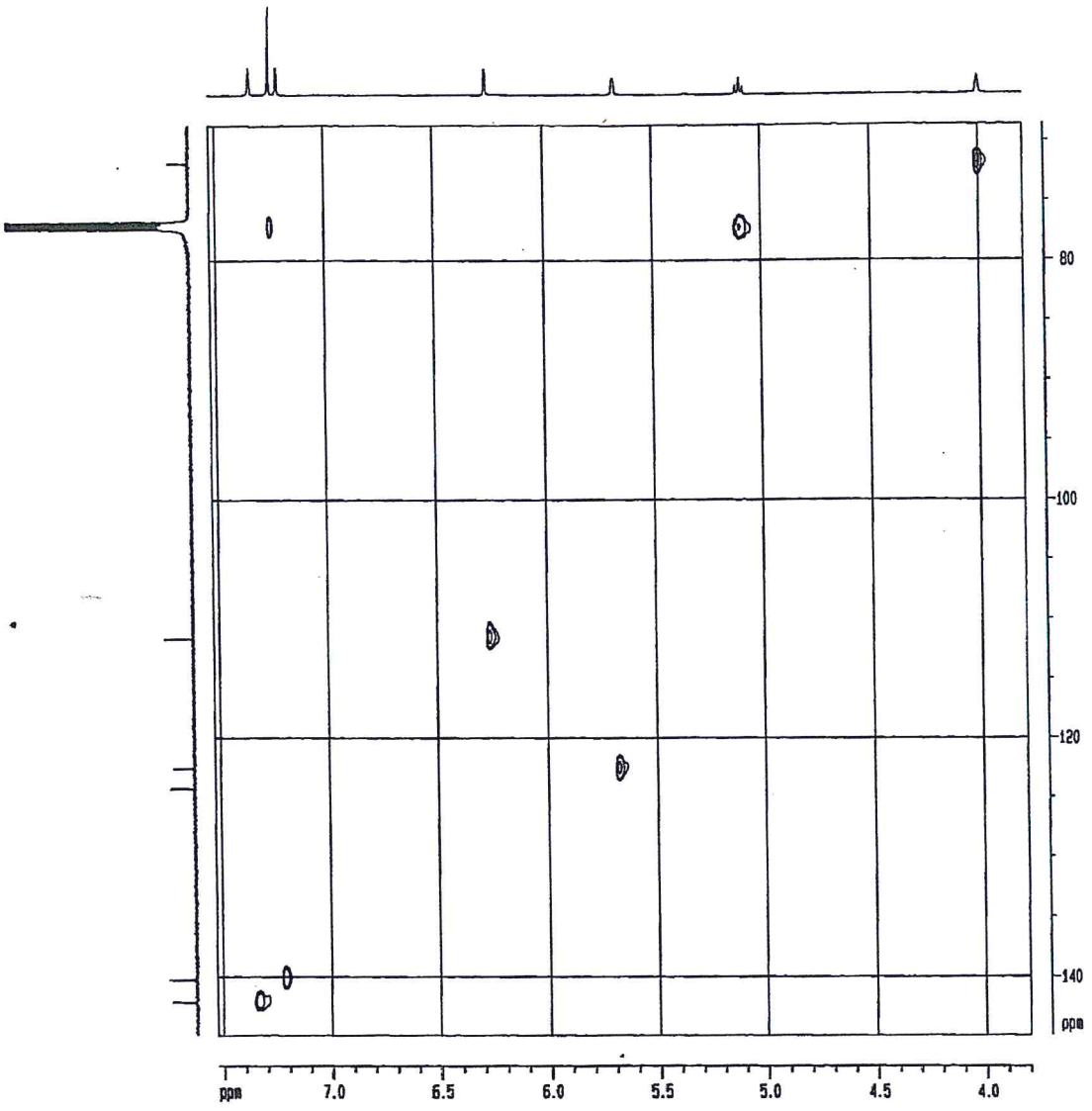

Figure 16: Expanded section of 500 MHz HMQC spectrum of compound **115**

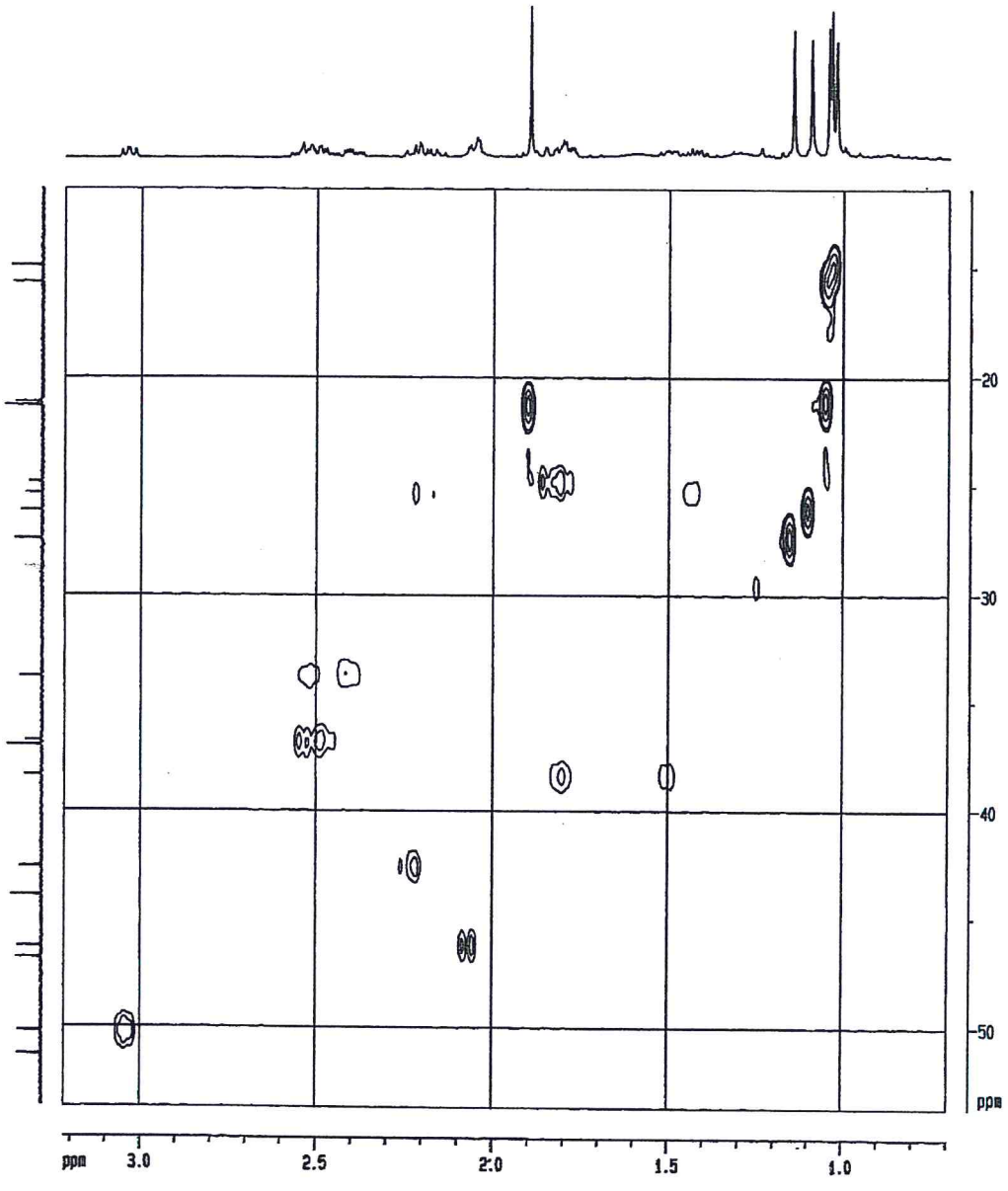

Figure 17: Expanded section of 500 MHz HMQC spectrum of compound **115**

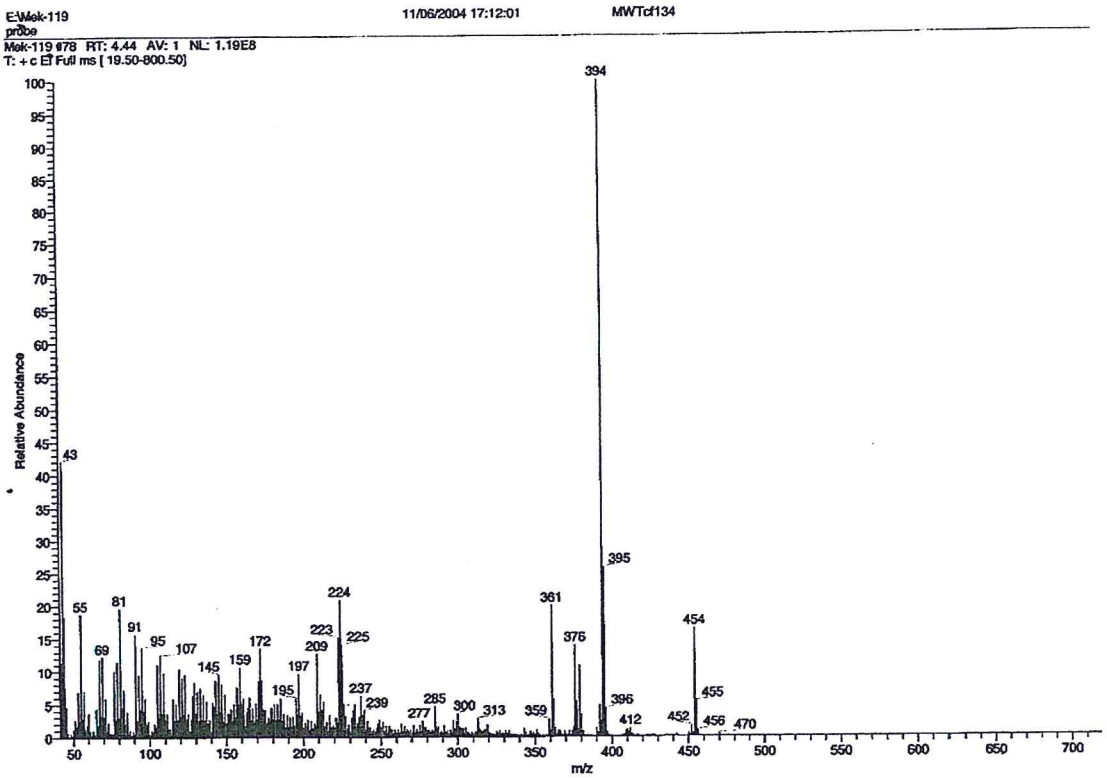

Figure 18: HREIMS spectrum of compound **115**

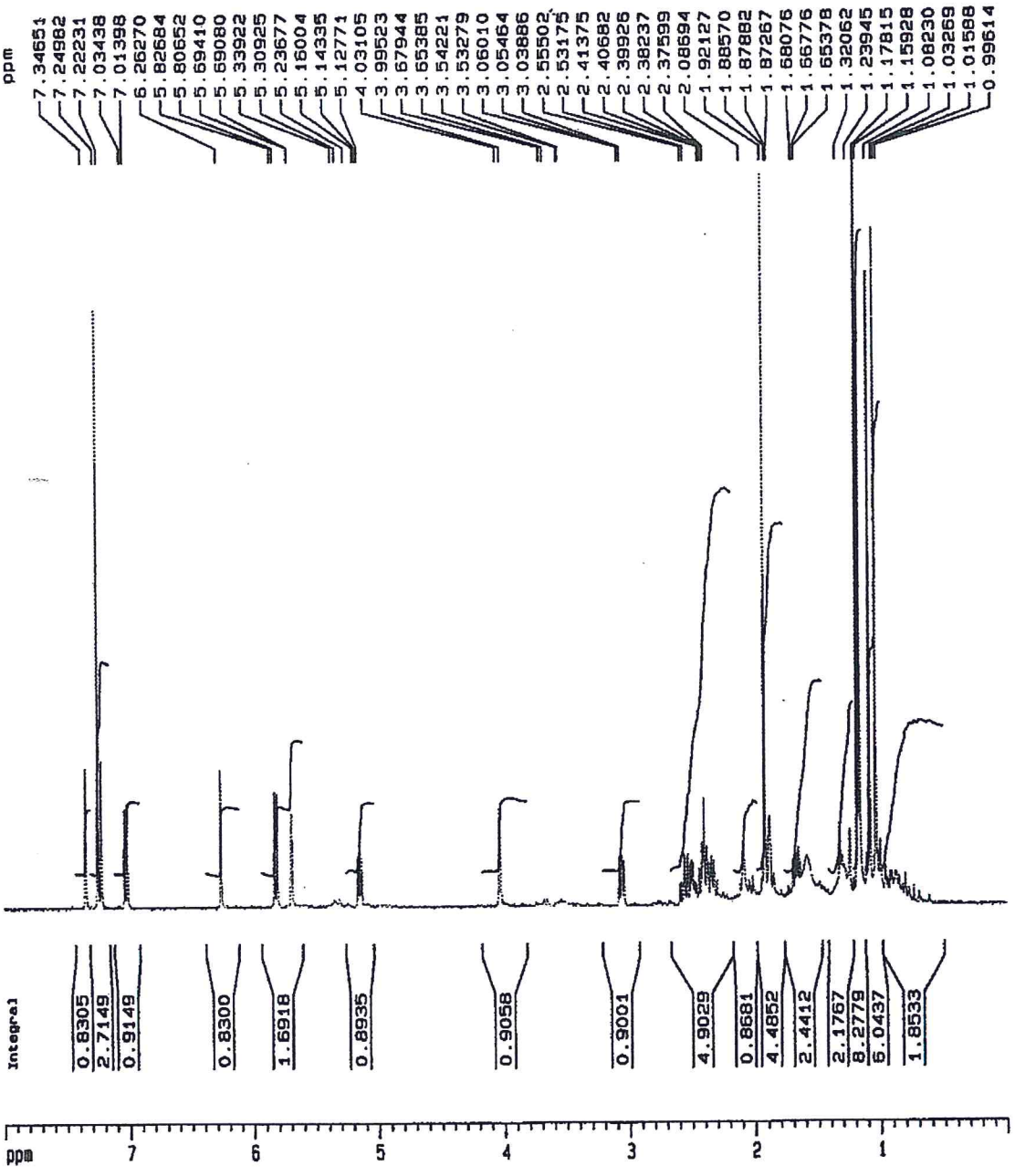

Figure 19:500 MHz ^{1}H NMR spectrum of compound **116**

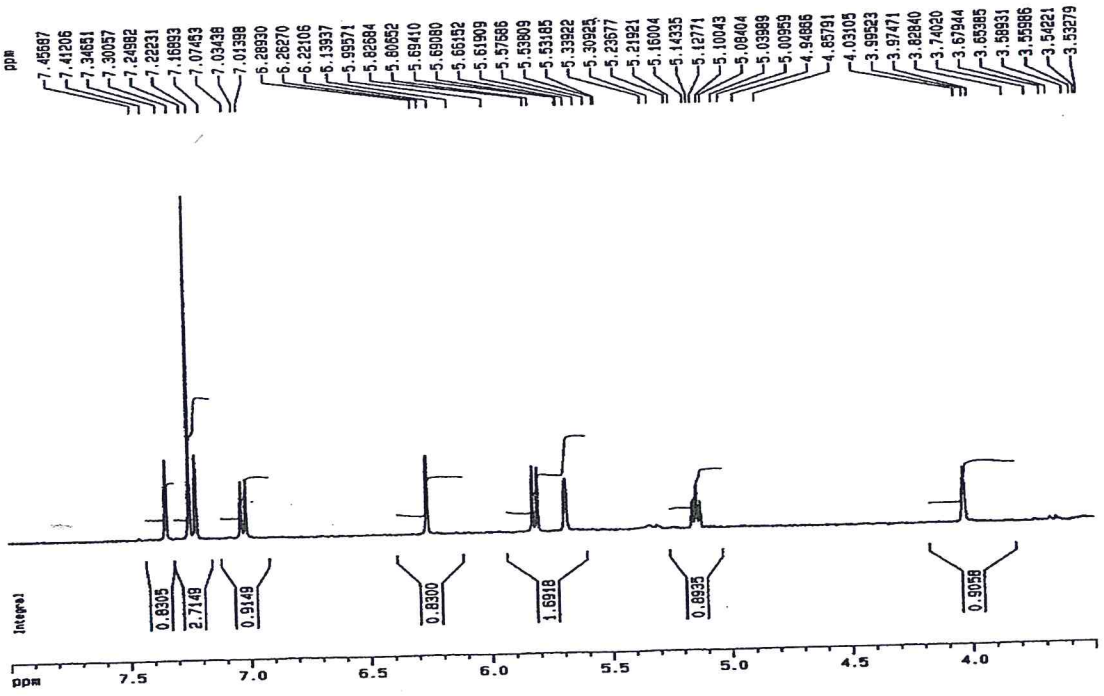

Figure 20: Expanded section of 500 MHz ^{1}H NMR spectrum of compound **116**

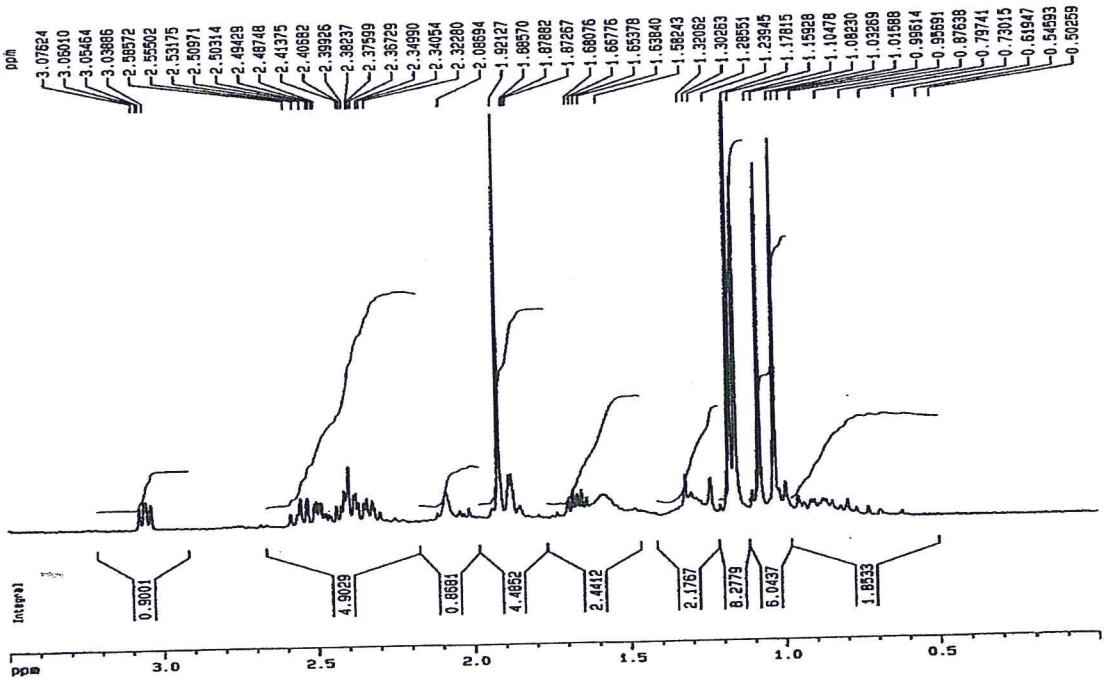

Figure 21: Expanded section of 500 MHz ^{1}H NMR spectrum of compound **116**

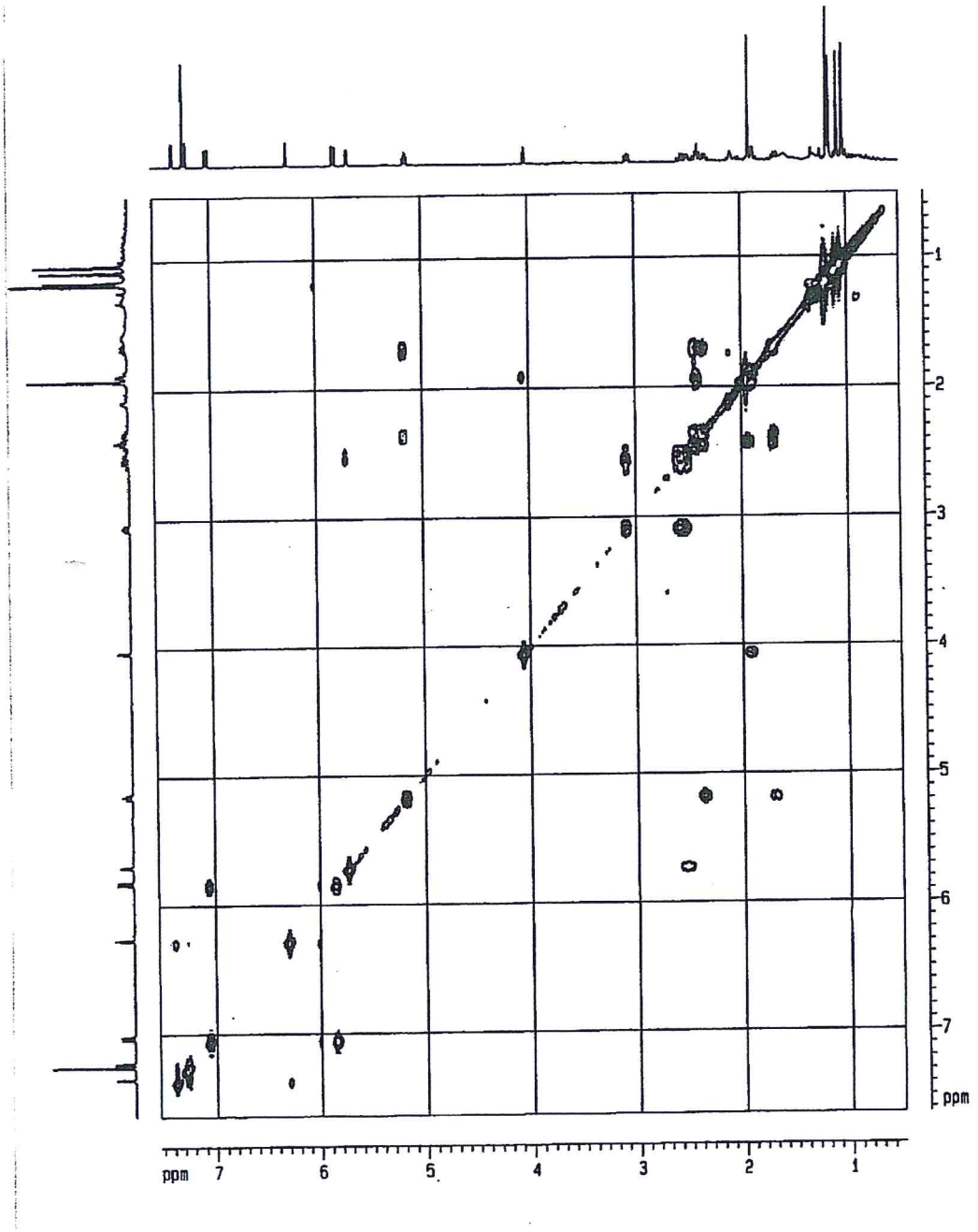

Figure 22: 500 MHz COSY spectrum of compound **116**

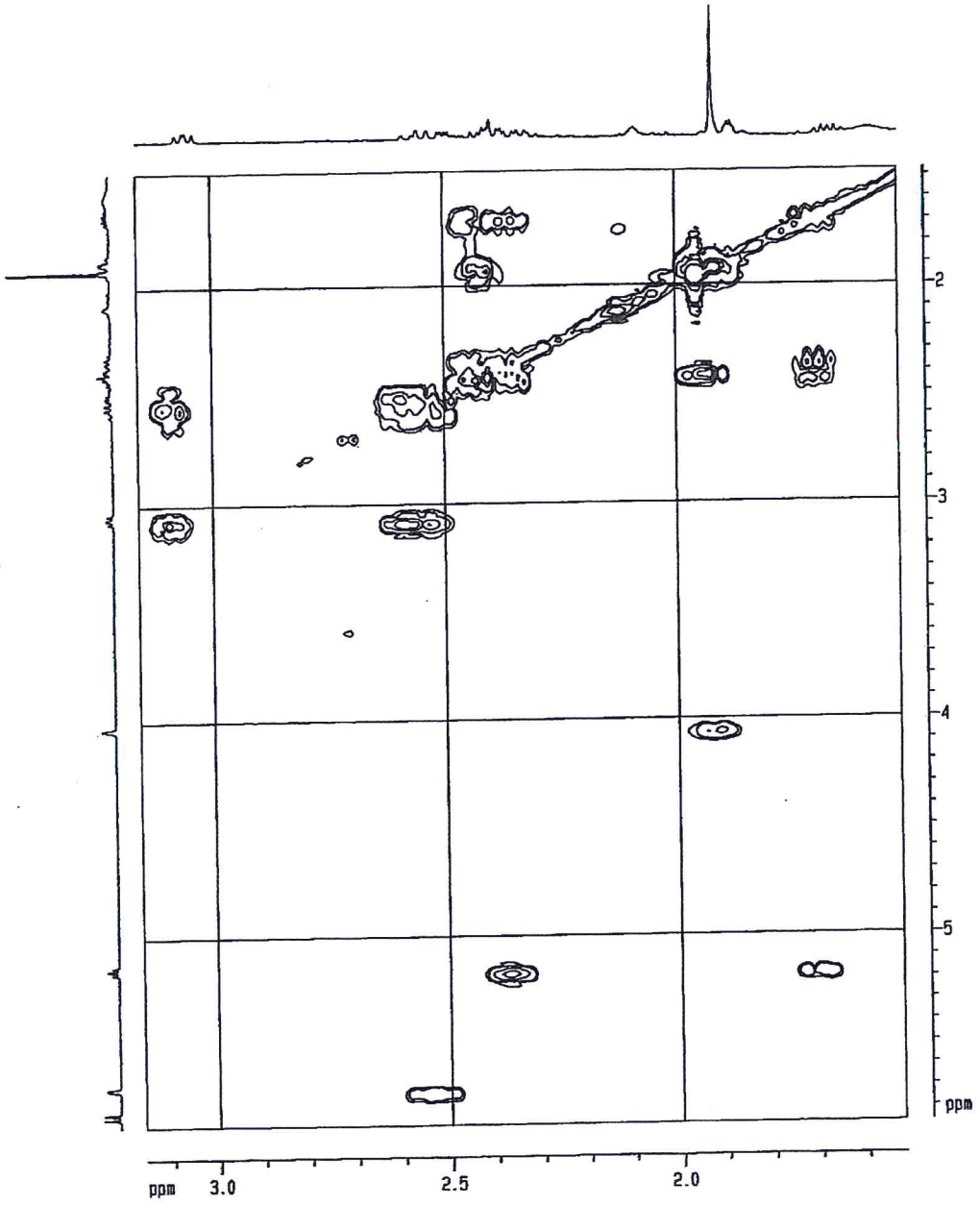

Figure 23: Expanded section 500 MHz COSY spectrum of compound **116**

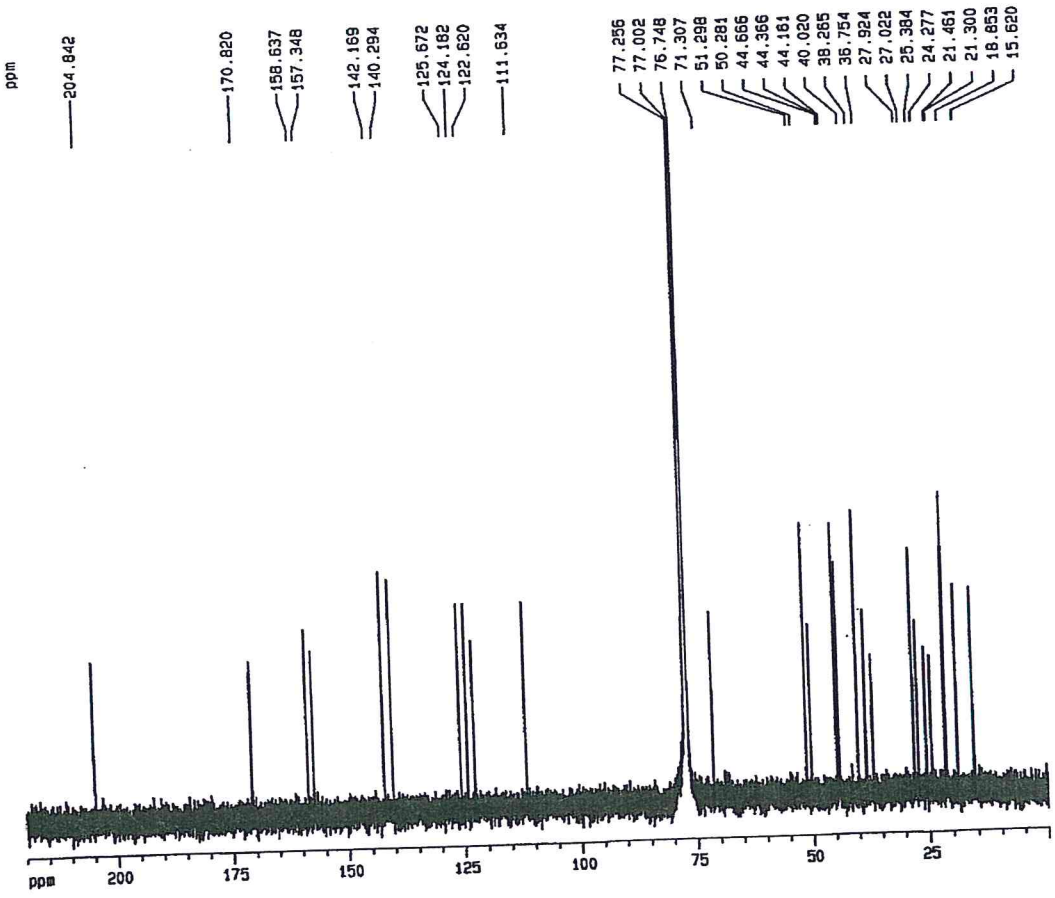

Figure 24: 125 MHz ^{13}C spectrum of compound **116**

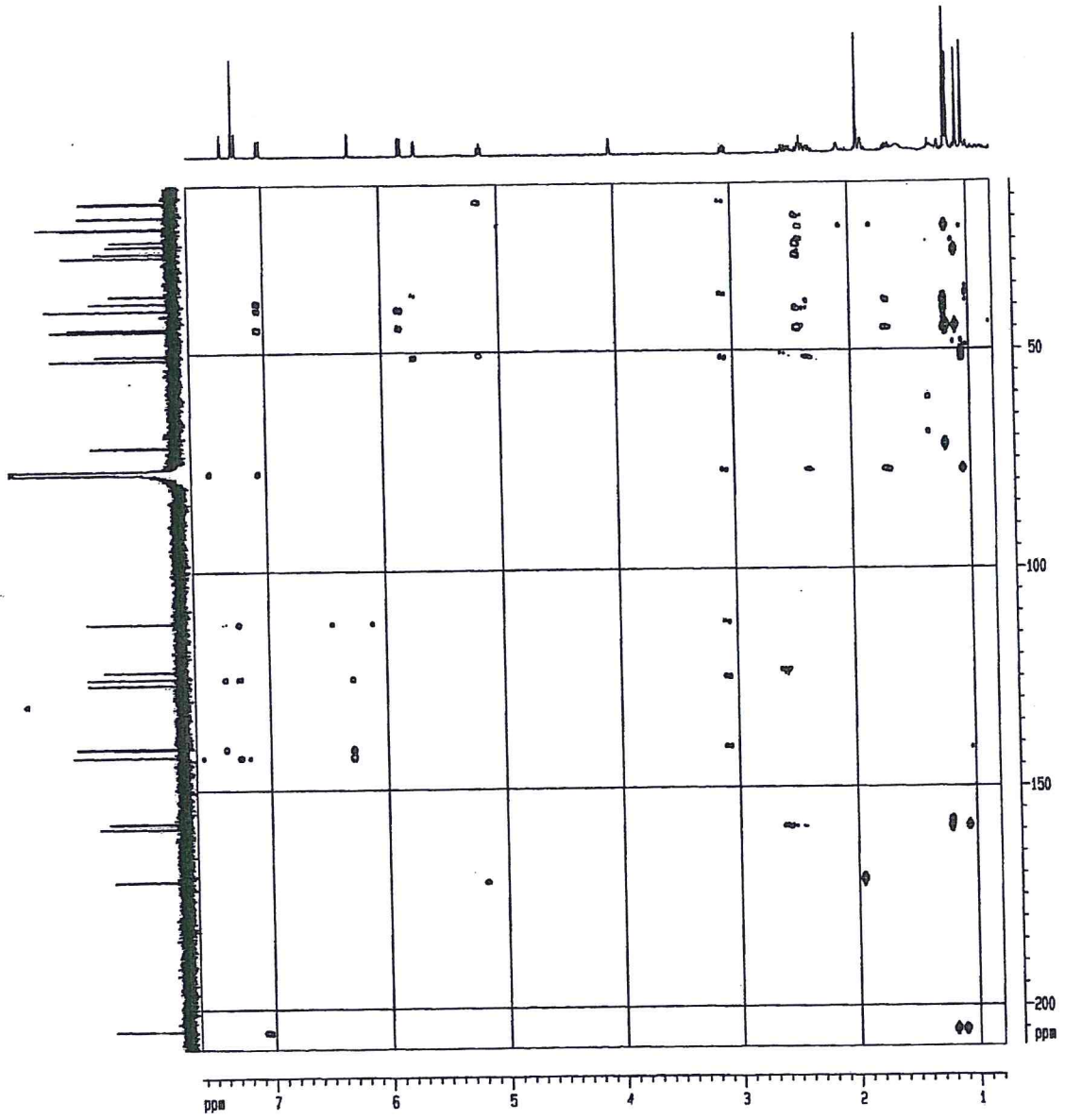

Figure 25: 500 MHz HMBC spectrum of compound **116**

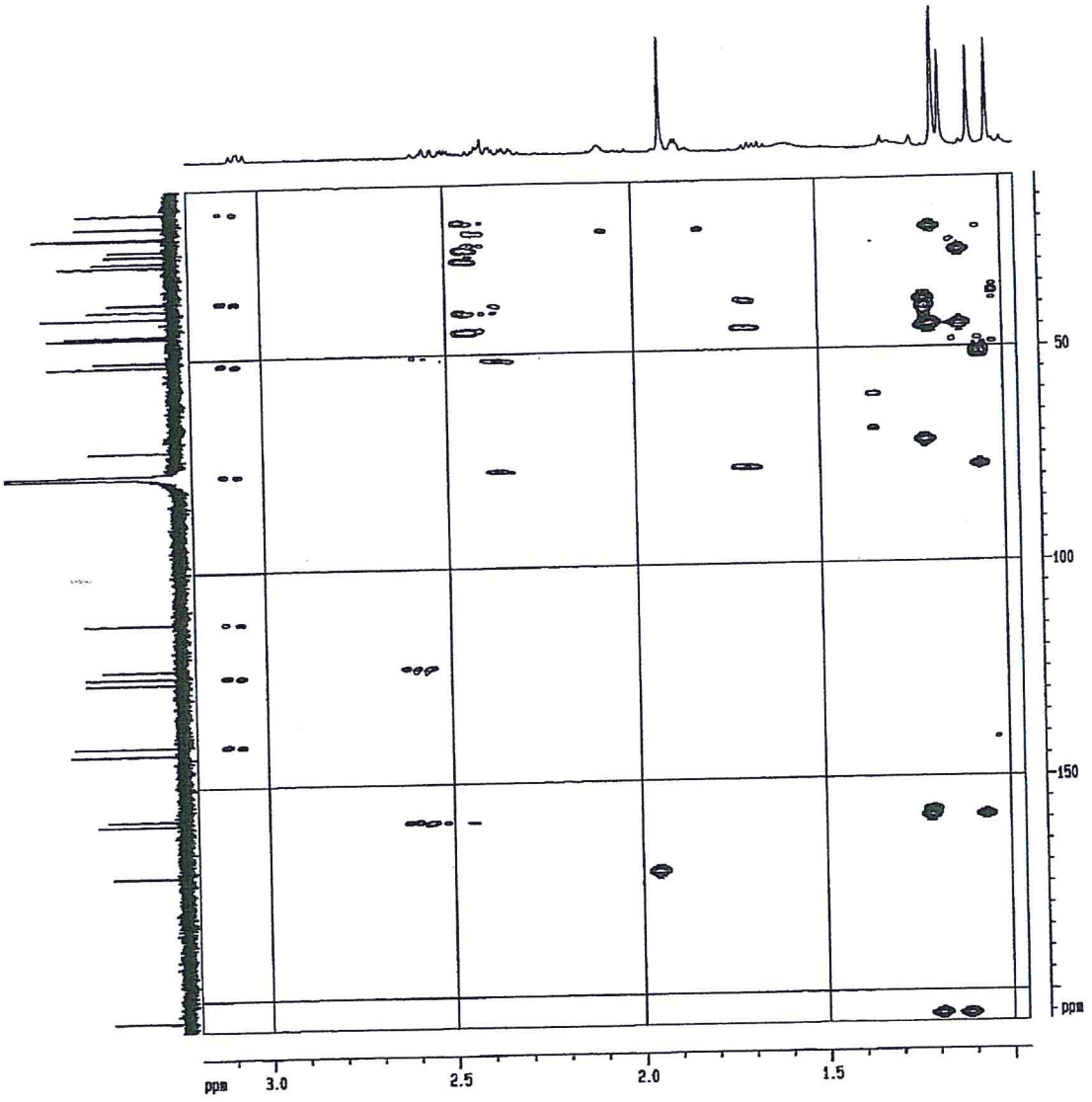

Figure 26: Expanded section of 500 MHz HMBC spectrum of compound **116**

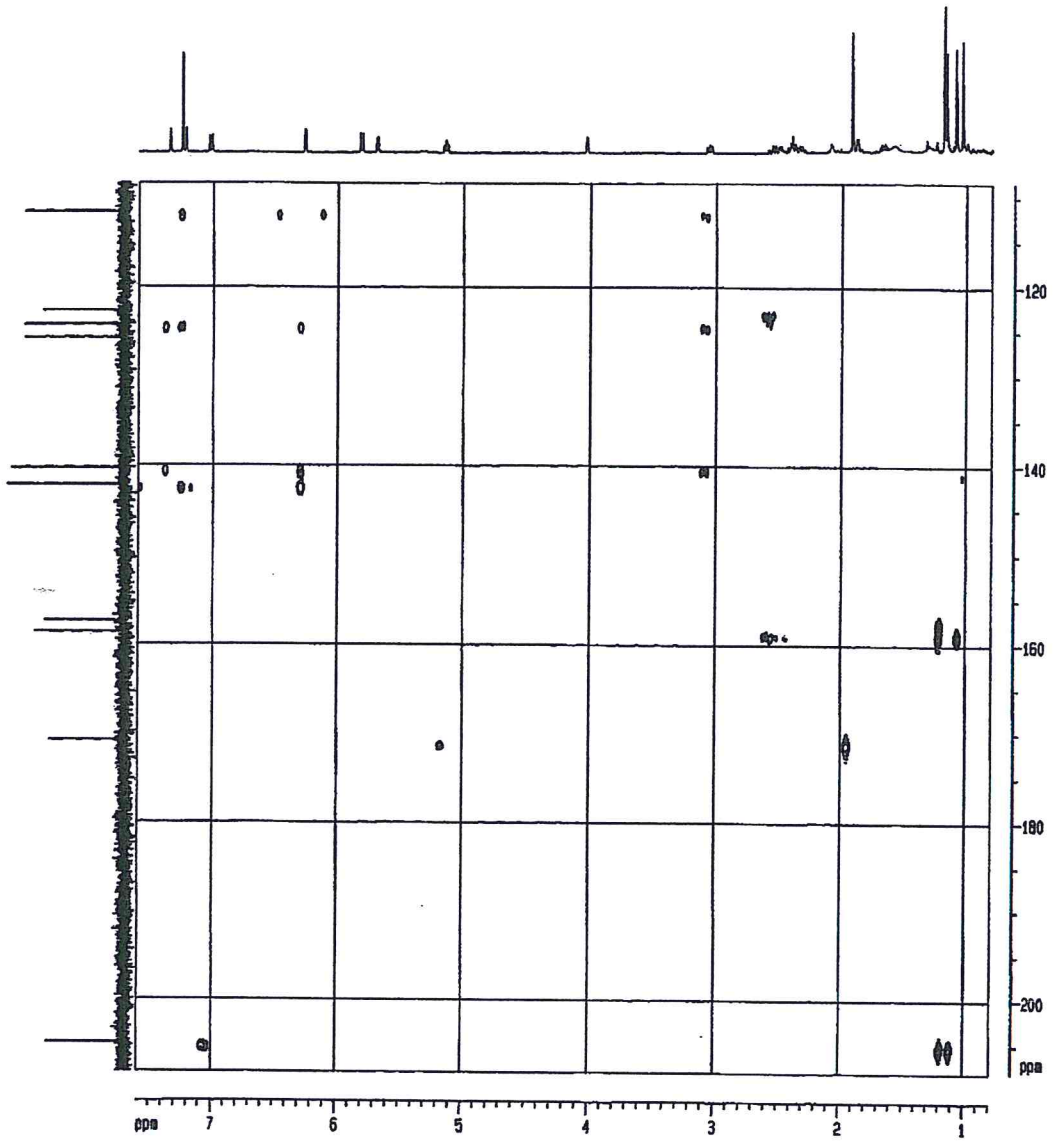

Figure 27: Expanded section of 500 MHz HMBC spectrum of compound **116**

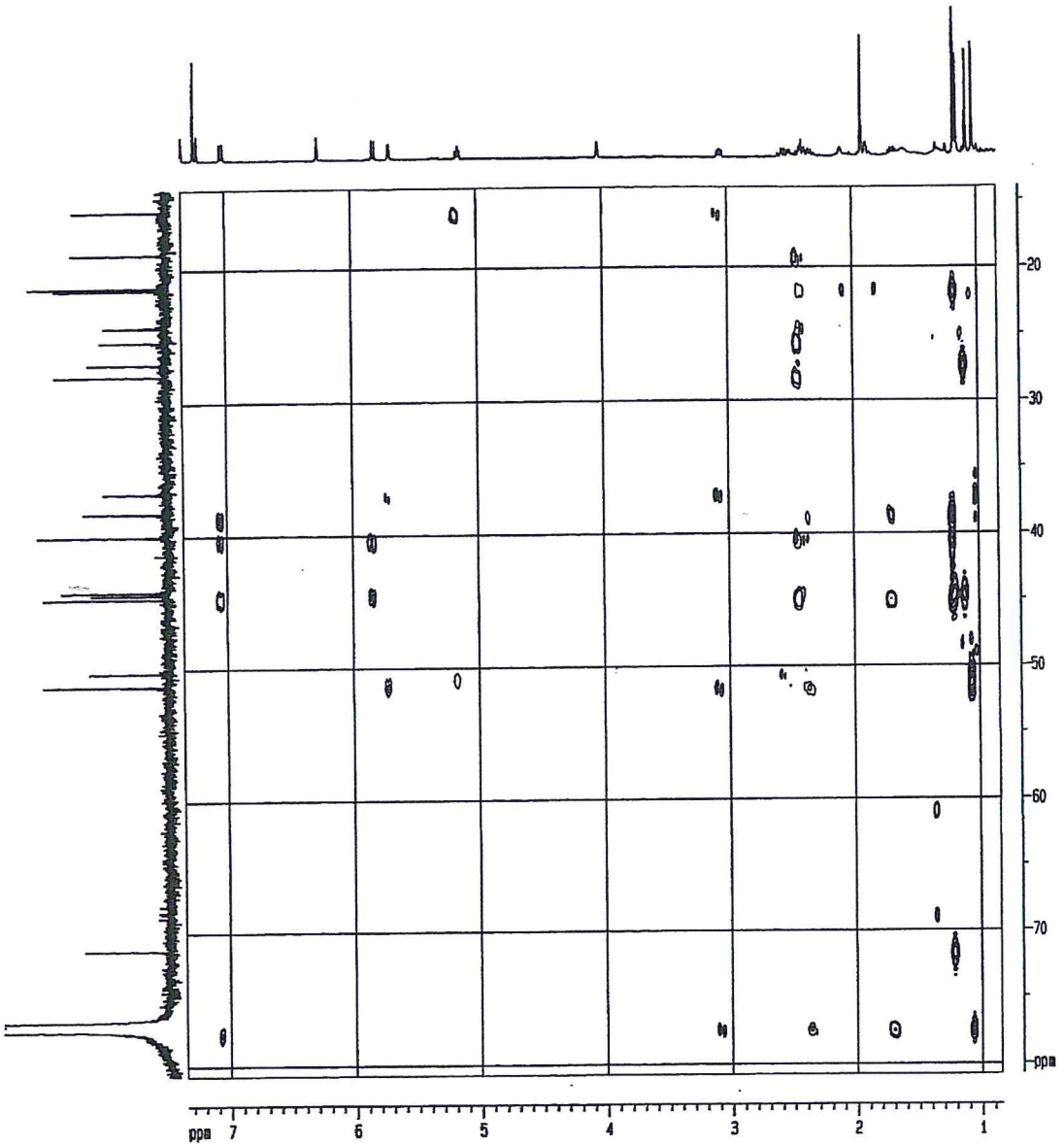

Figure 28: Expanded section of 500 MHz HMBC spectrum of compound **116**

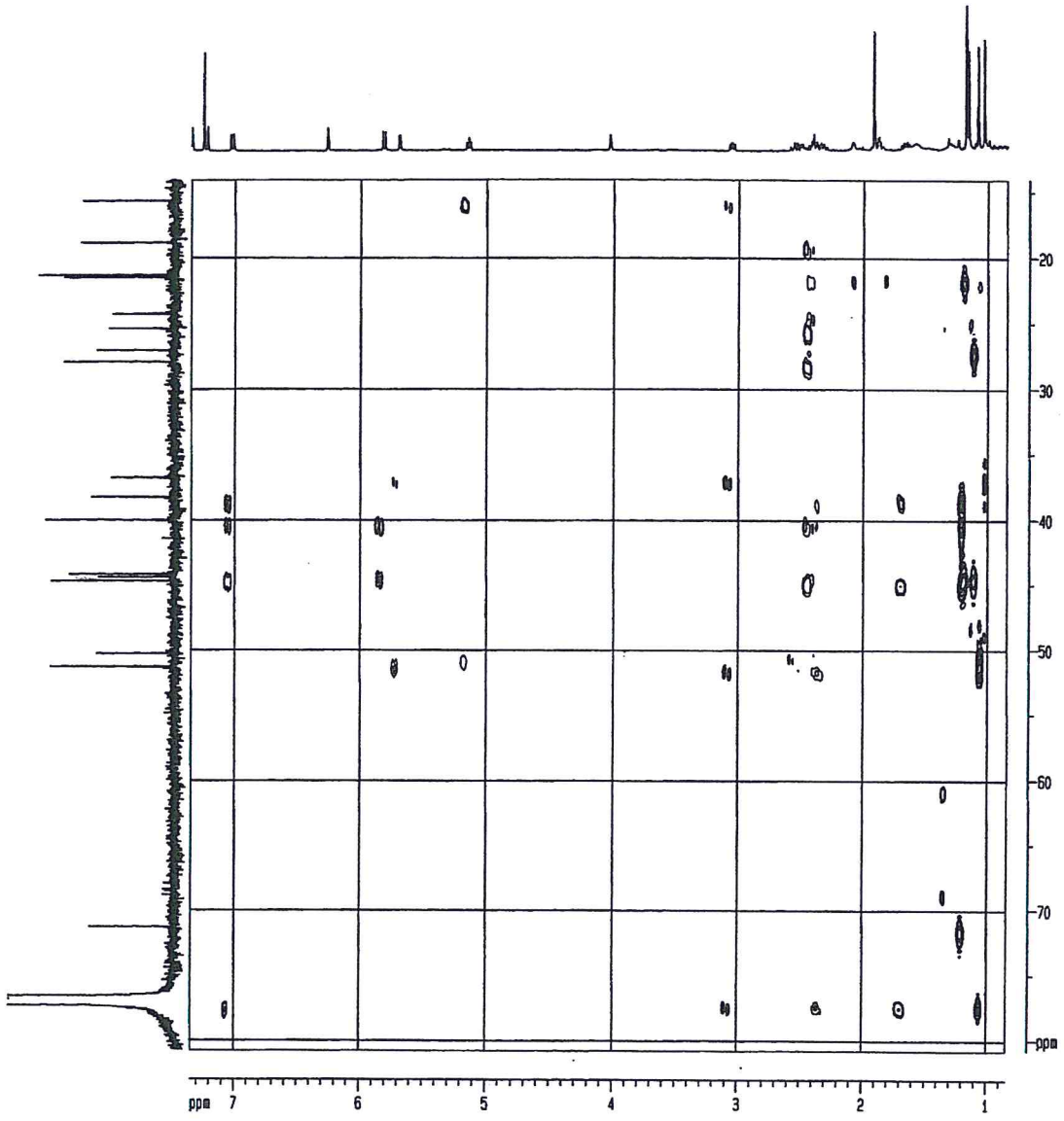

Figure 29: Expanded section of 500 MHz HMBC spectrum of compound **116**

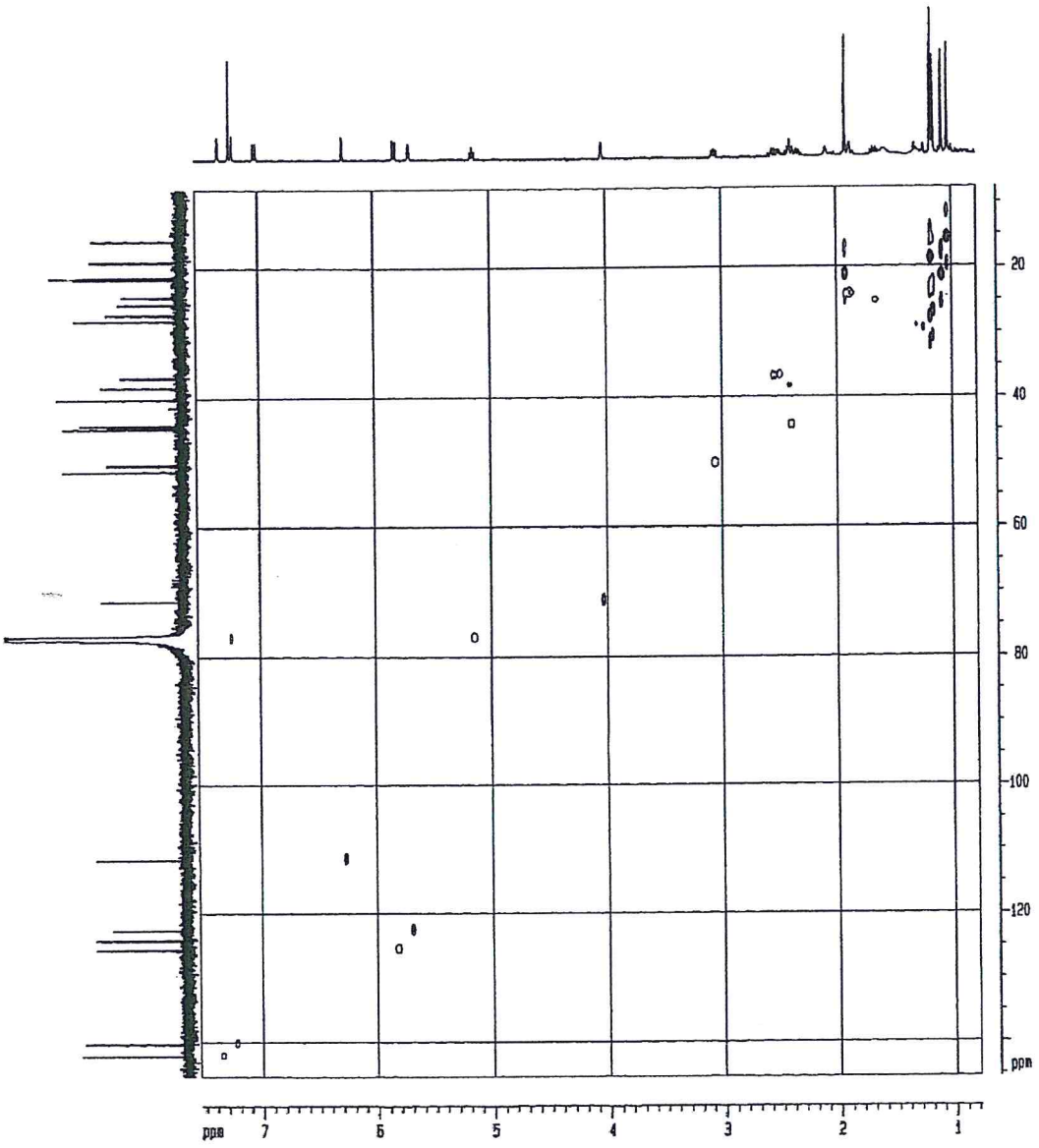

Figure 30: 500 MHz HMQC spectrum of compound **116**

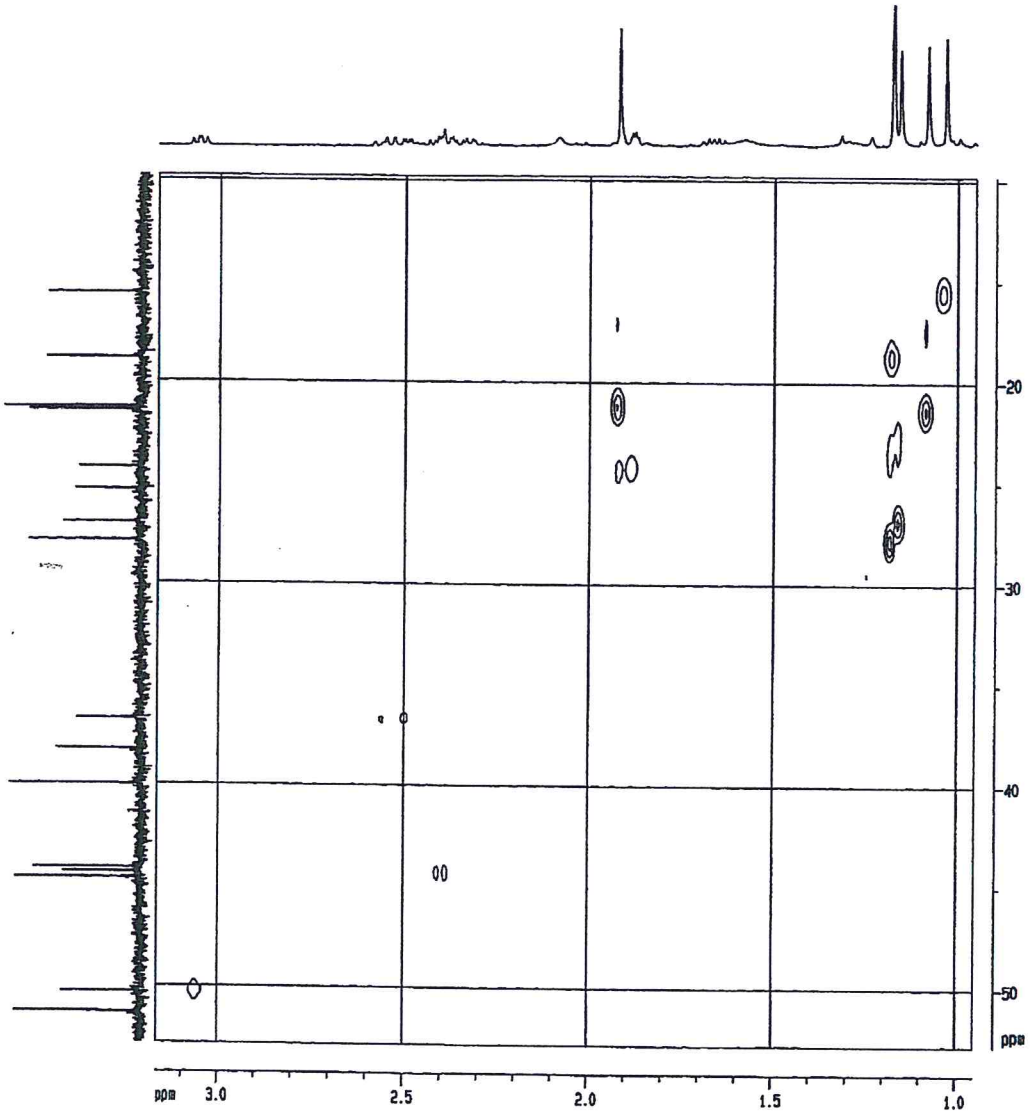

Figure 31: Expanded section of 500 MHz HMQC spectrum of compound **116**

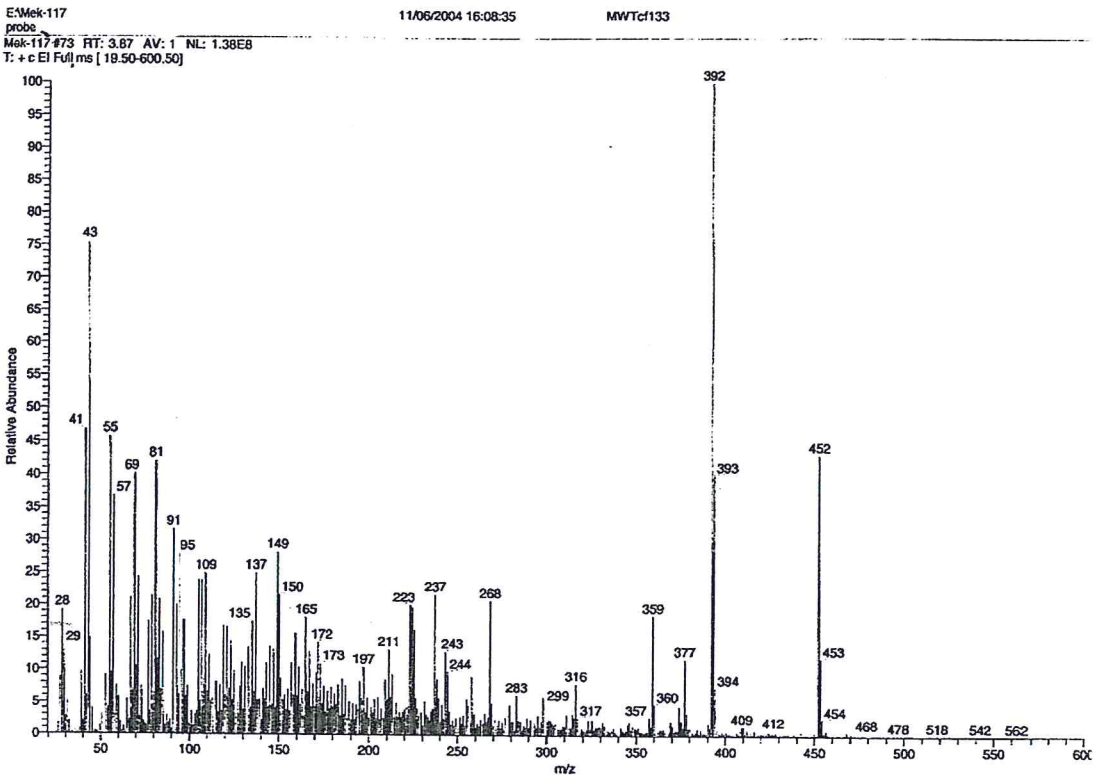

Figure 32: HREIMS spectrum of compound **116**

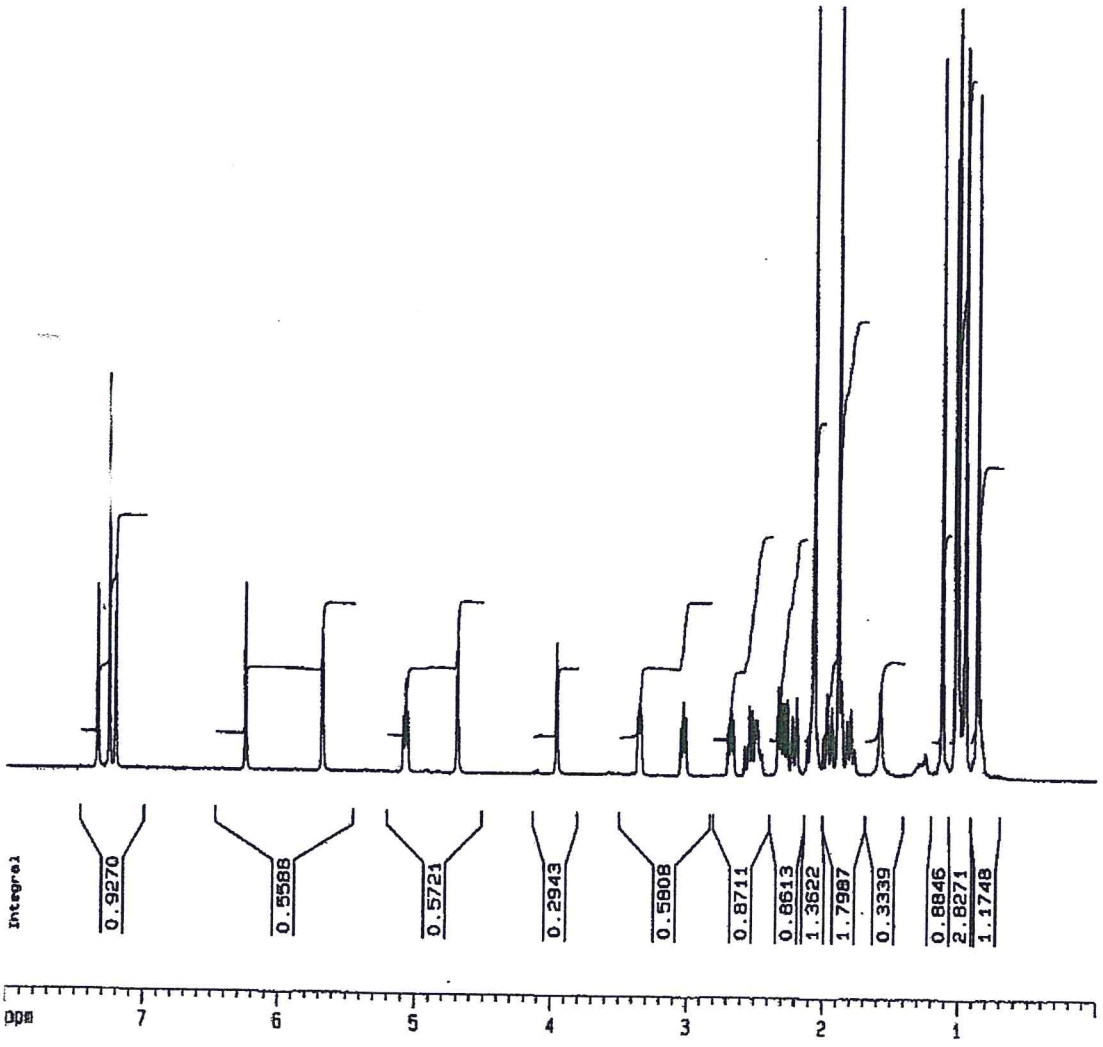

Figure 33:500 MHz ^{1}H NMR spectrum of compound **117**

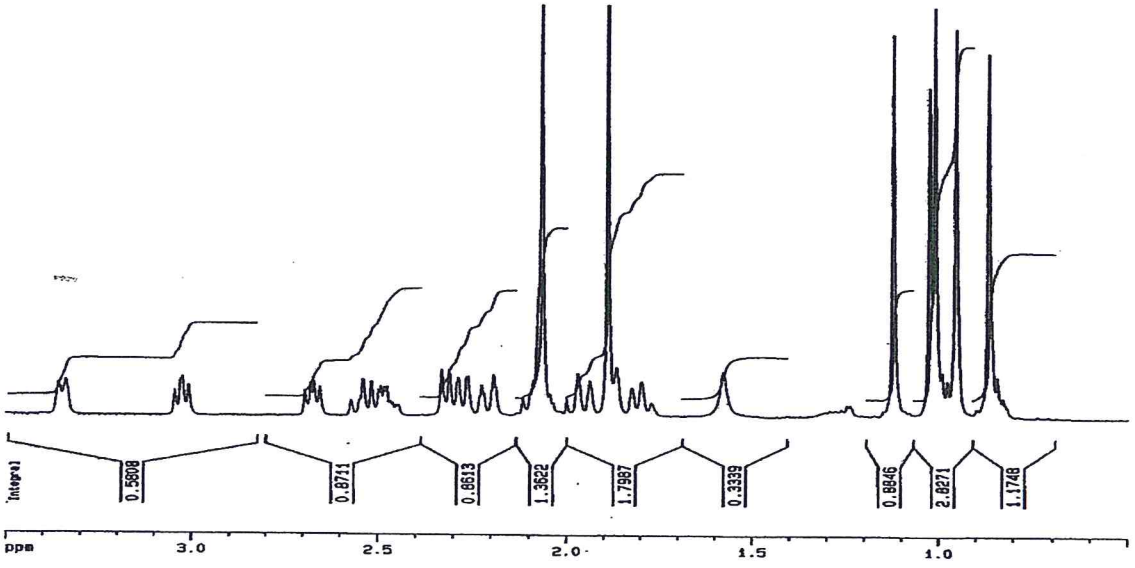

Figure 34: Expanded section of 500 MHz ^{1}H NMR spectrum of compound **117**

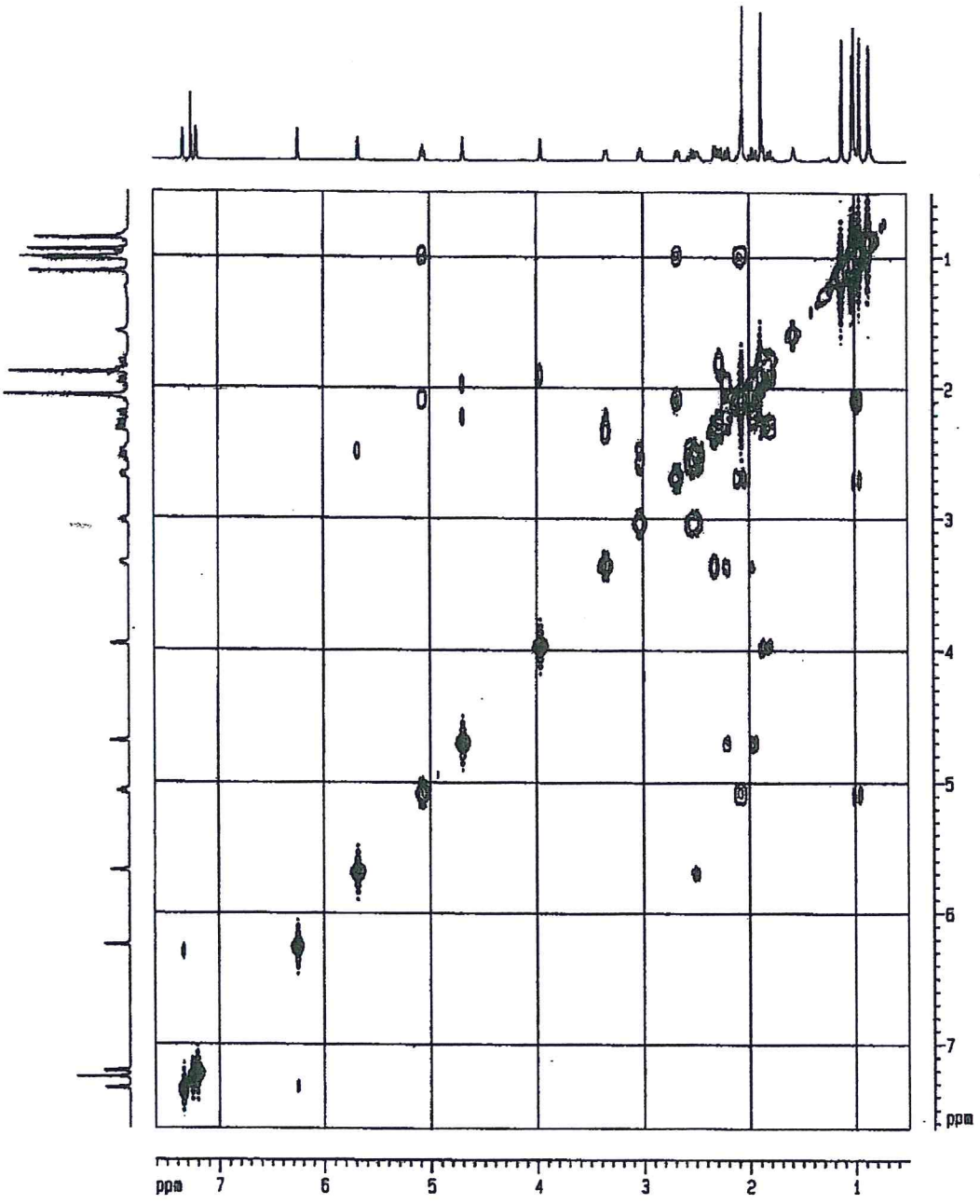

Figure 35: 500 MHz COSY spectrum of compound **117**

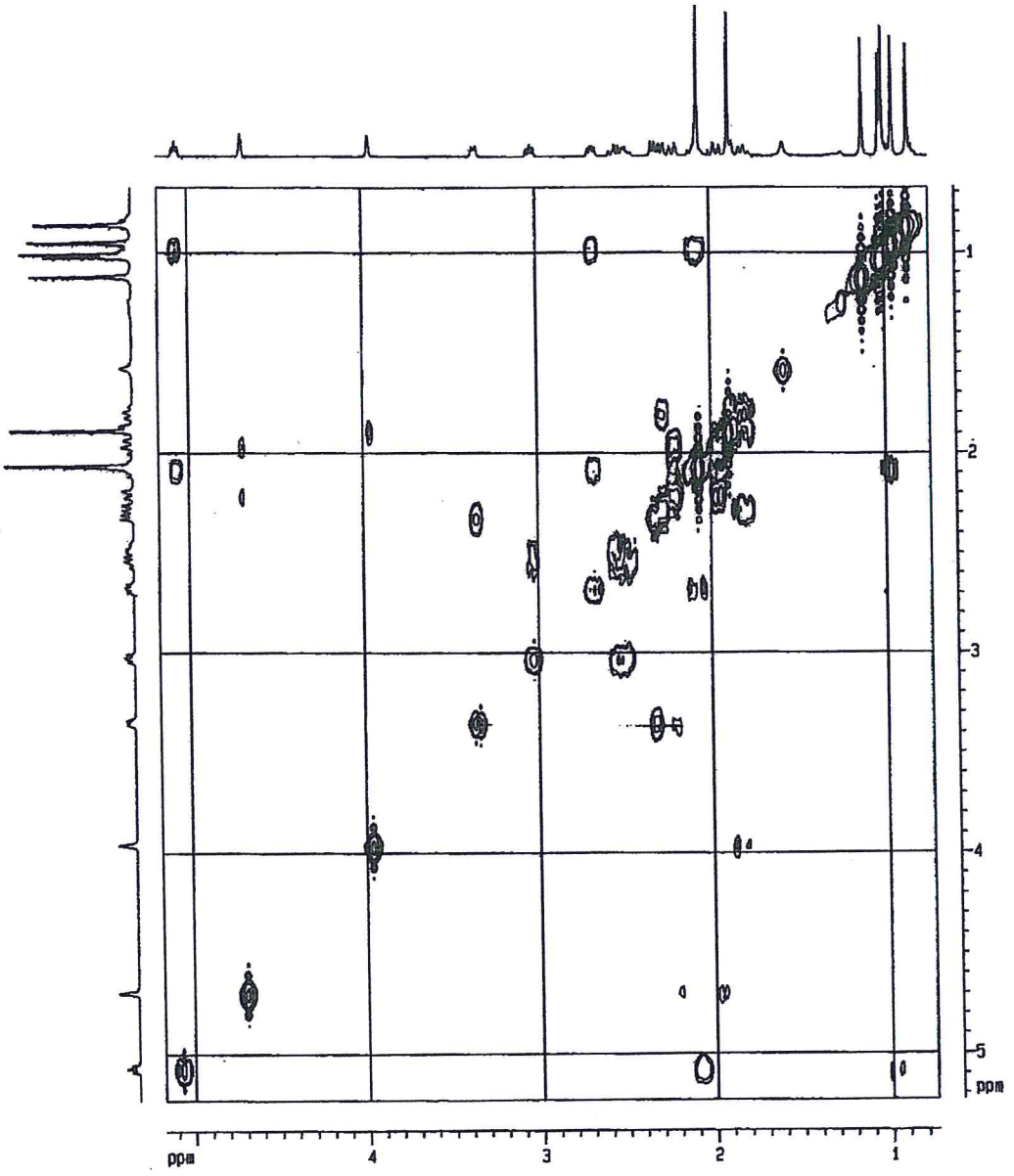

Figure 36: Expanded section of 500 MHz COSY spectrum of compound **117**

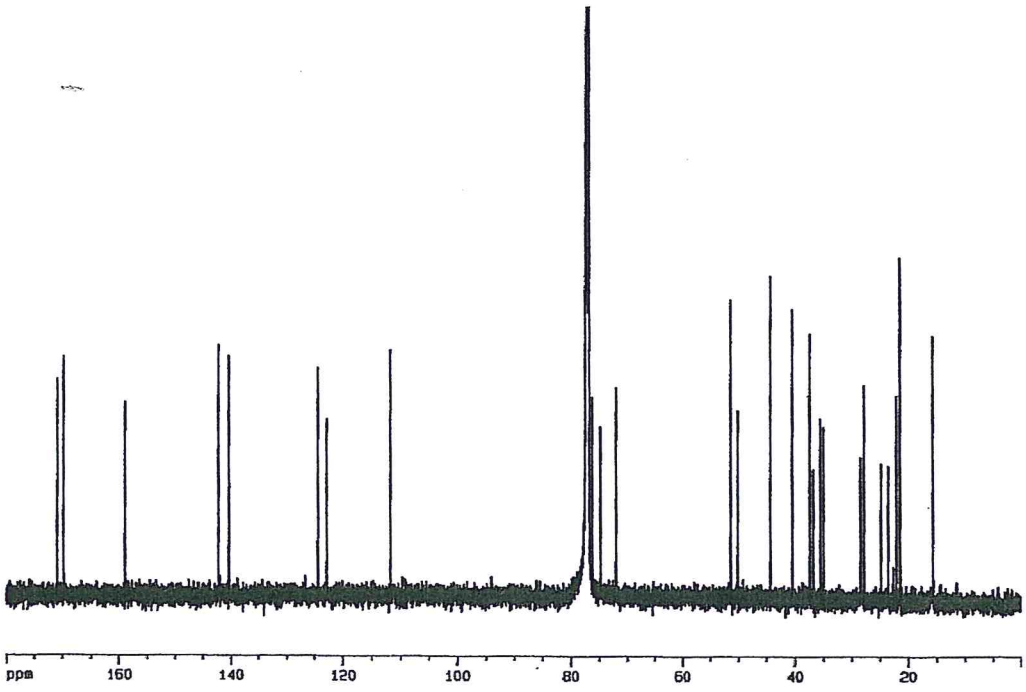

Figure 37: 125 MHz ^{13}C spectrum of compound **117**

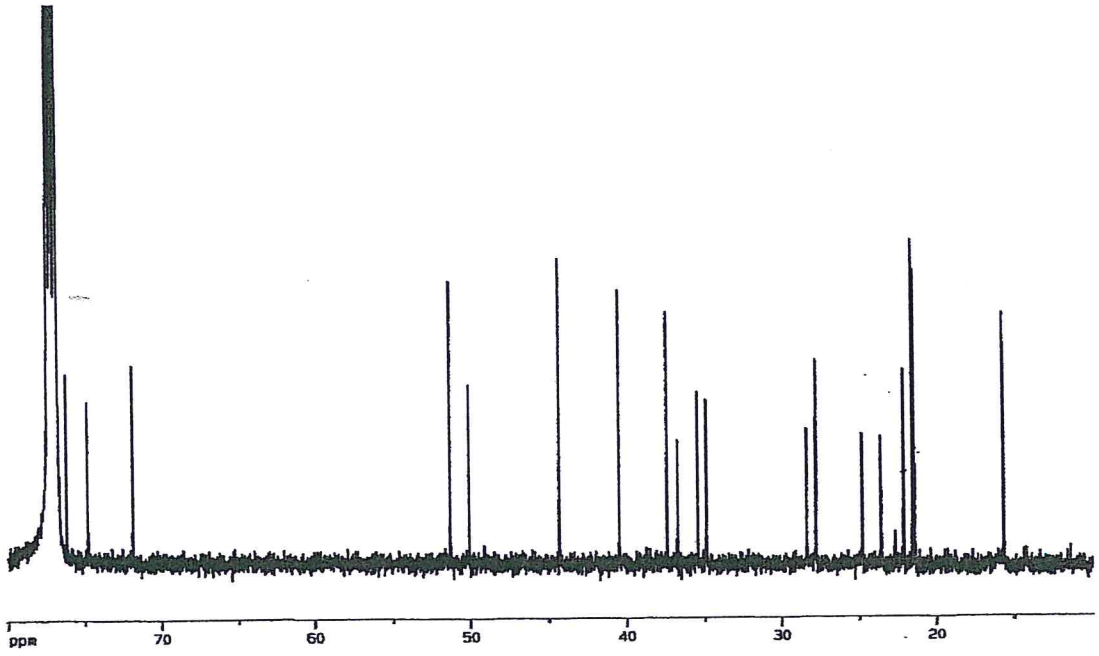

Figure 38: Expanded section of 125 MHz ^{13}C spectrum of compound **117**

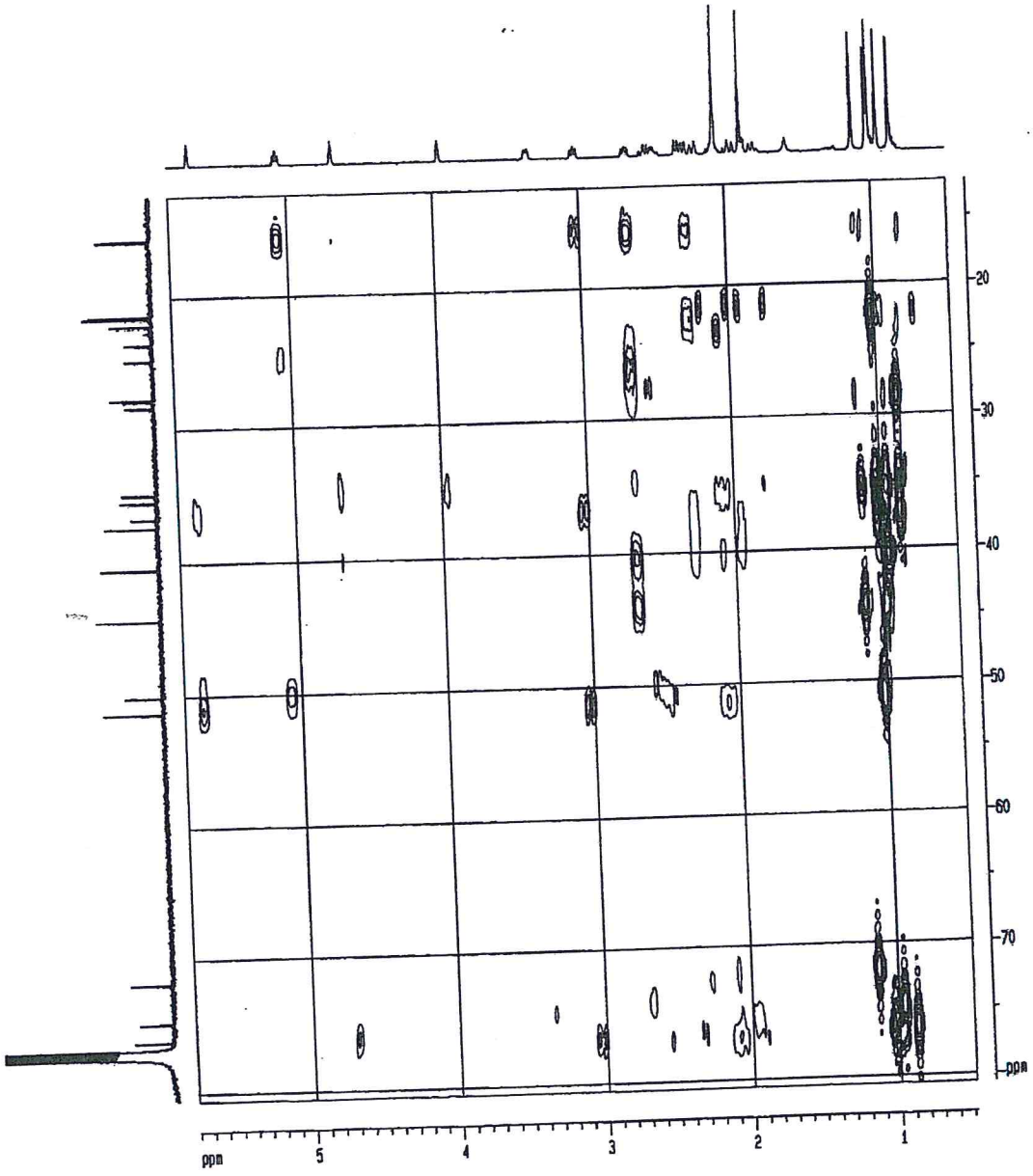

Figure 39: Expanded section of 500 MHz HMBC spectrum of compound **117**

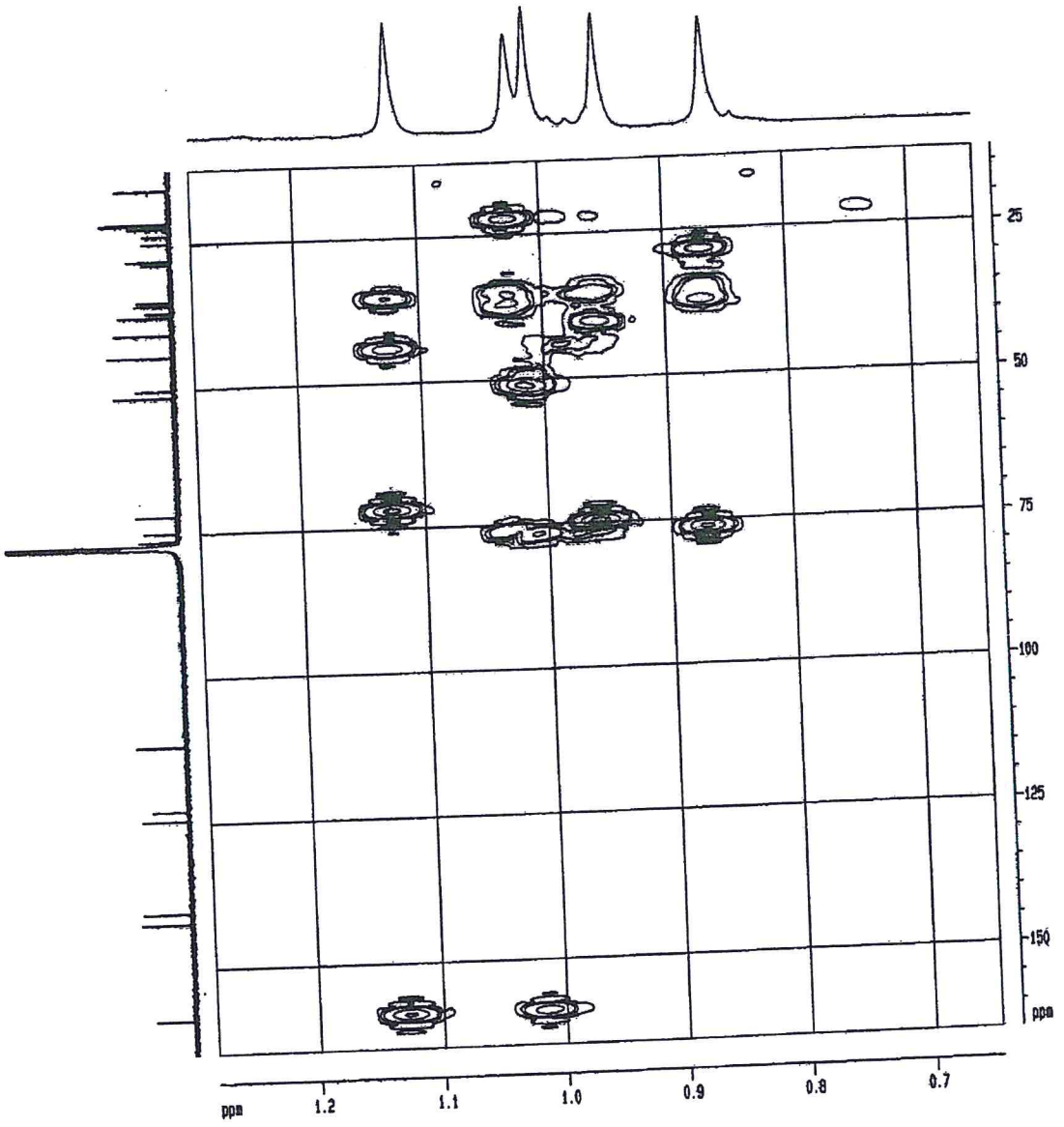

Figure 40: Expanded section of 500 MHz HMBC spectrum of compound **117**

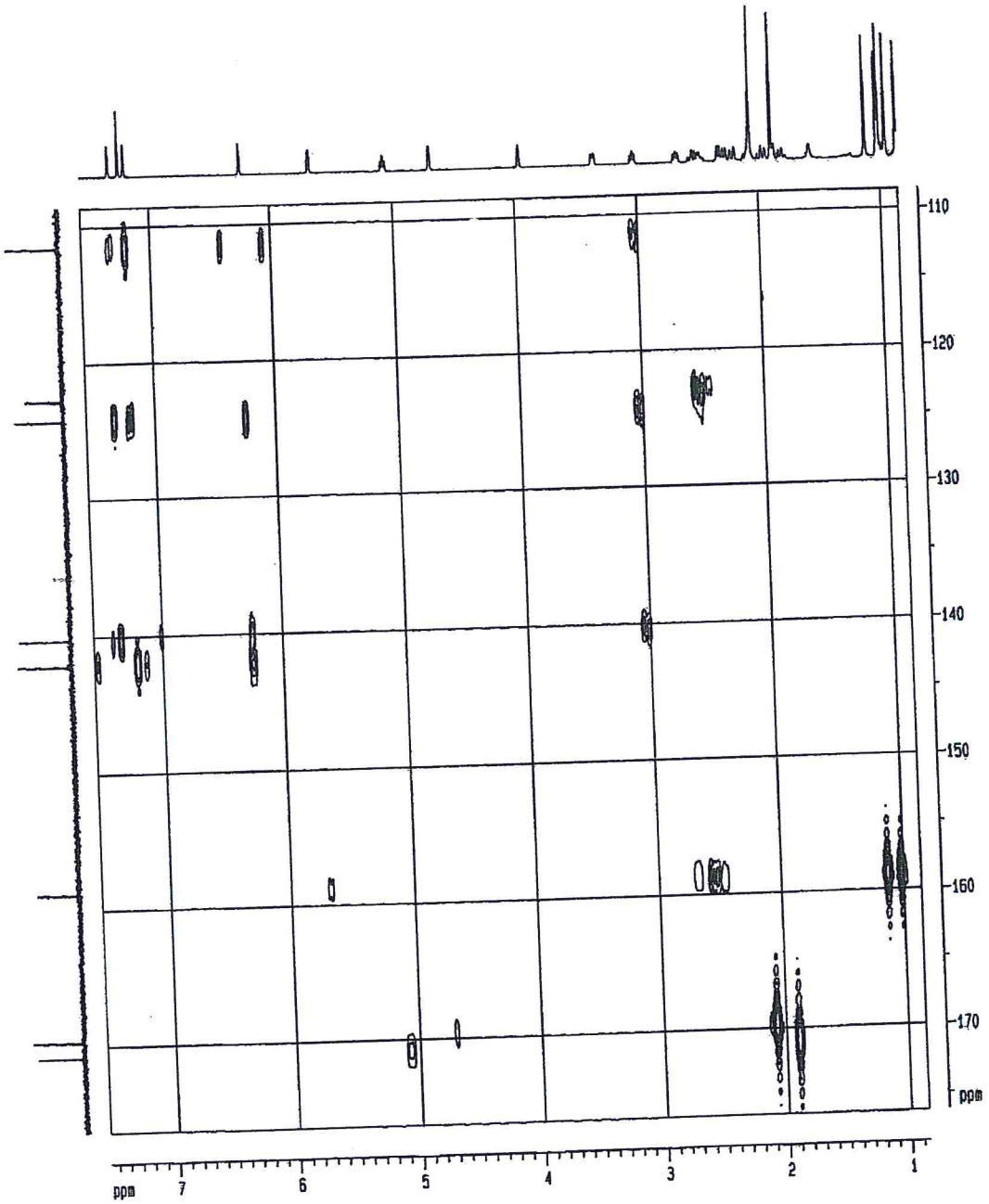

Figure 41: Expanded section of 500 MHz HMBC spectrum of compound **117**

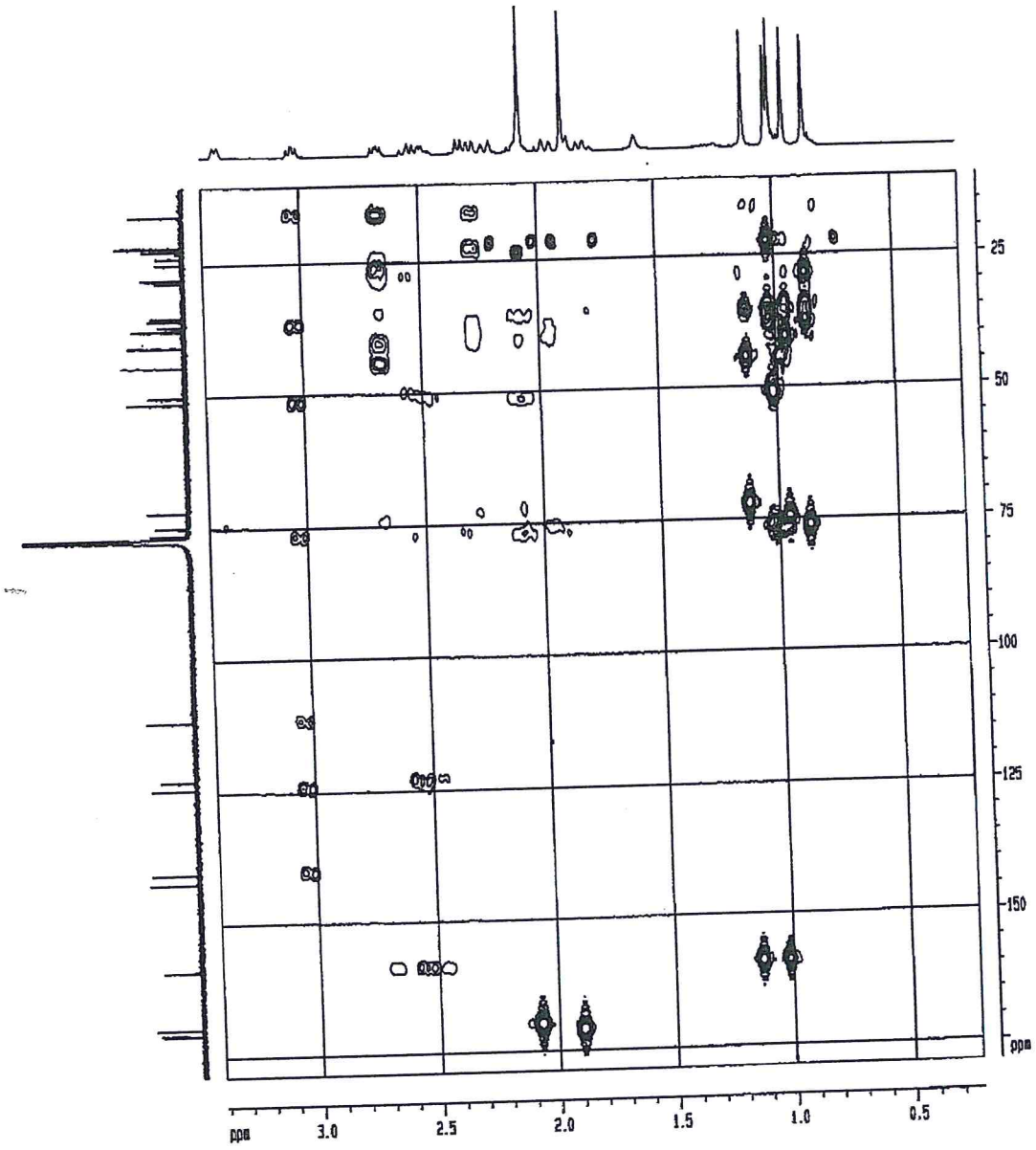

Figure 42: Expanded section of 500 MHz HMBC spectrum of compound **117**

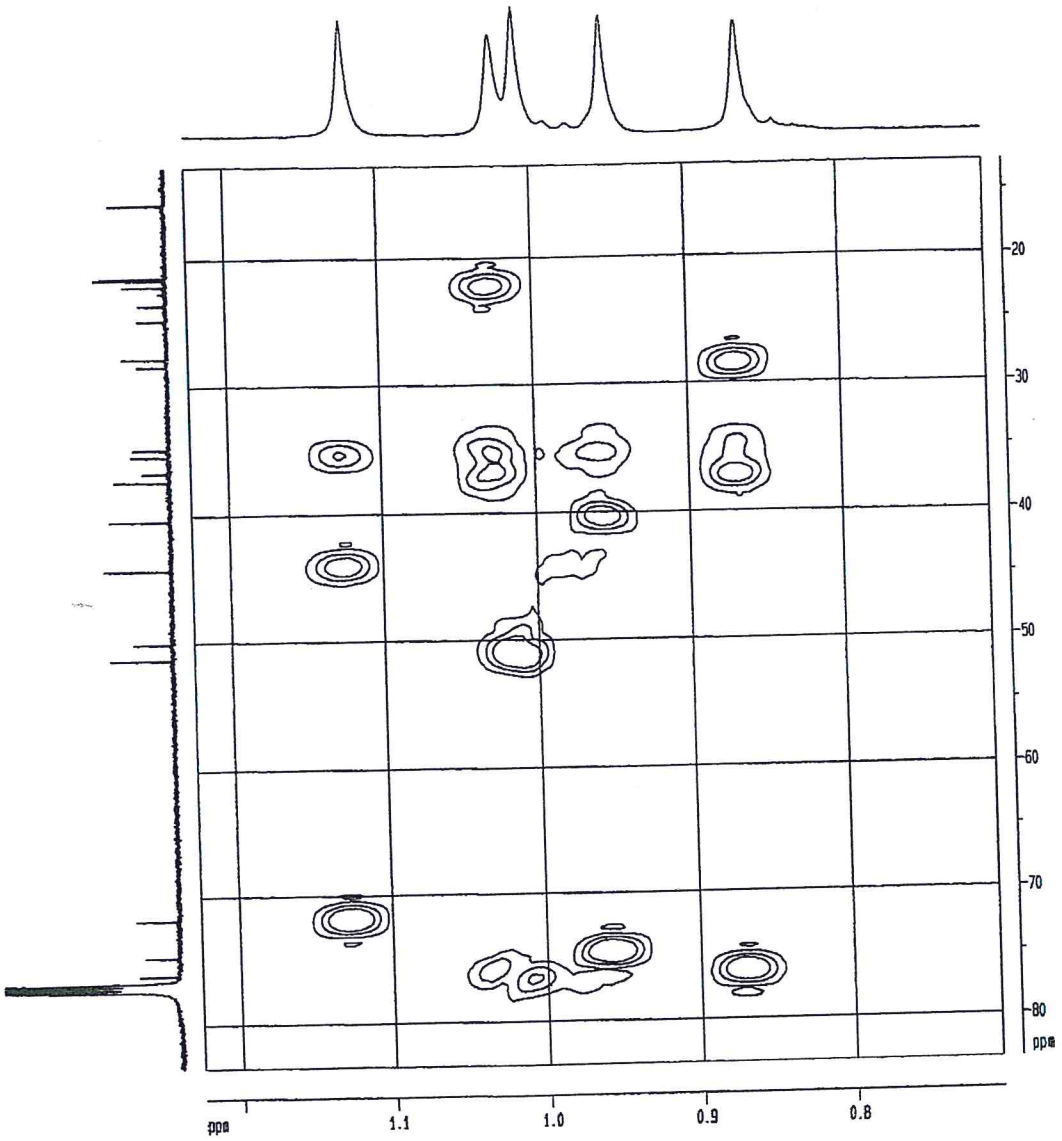

Figure 43: Expanded section of 500 MHz HMBC spectrum of compound **117**

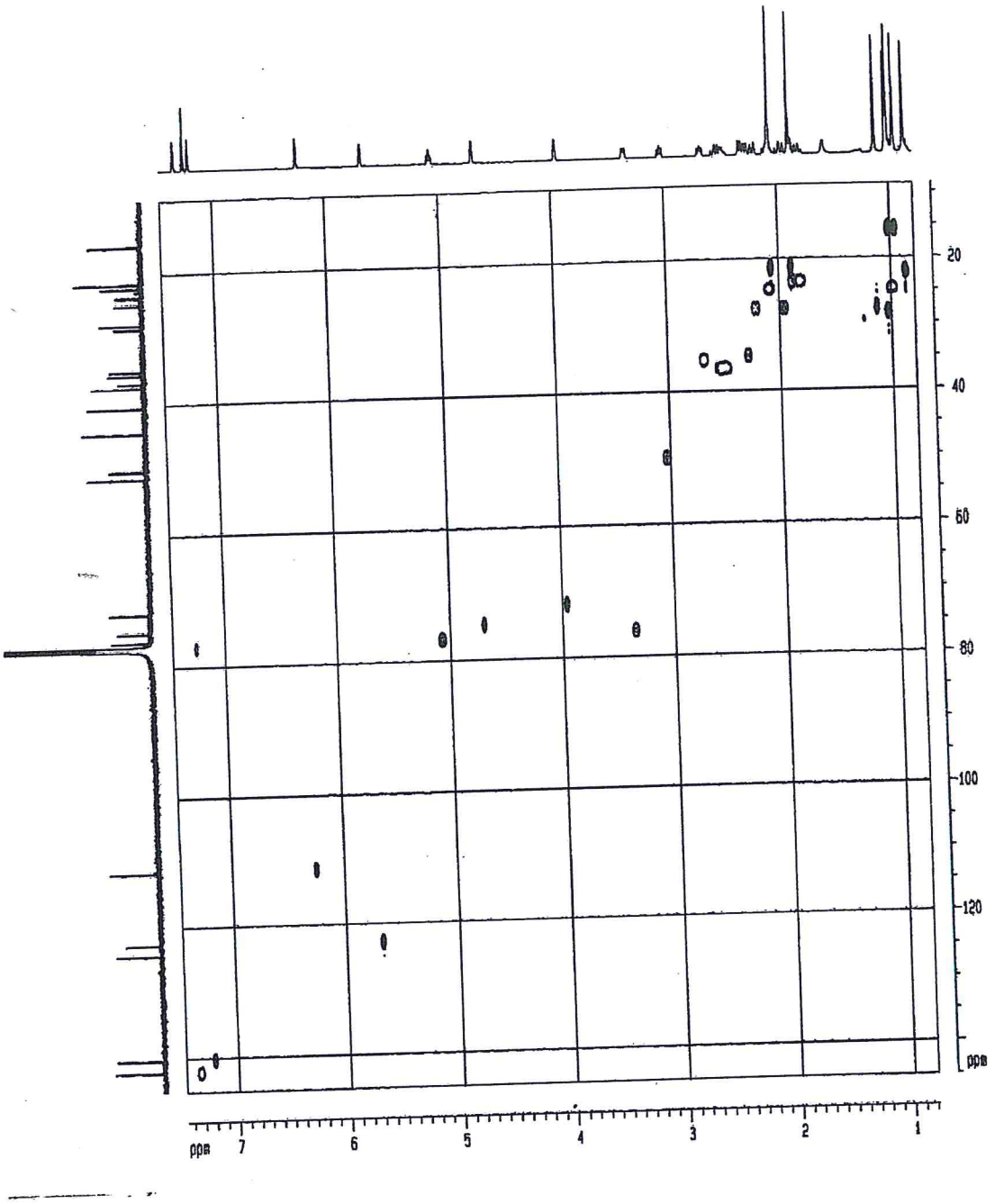

Figure 44: 500 MHz HMQC spectrum of compound **117**

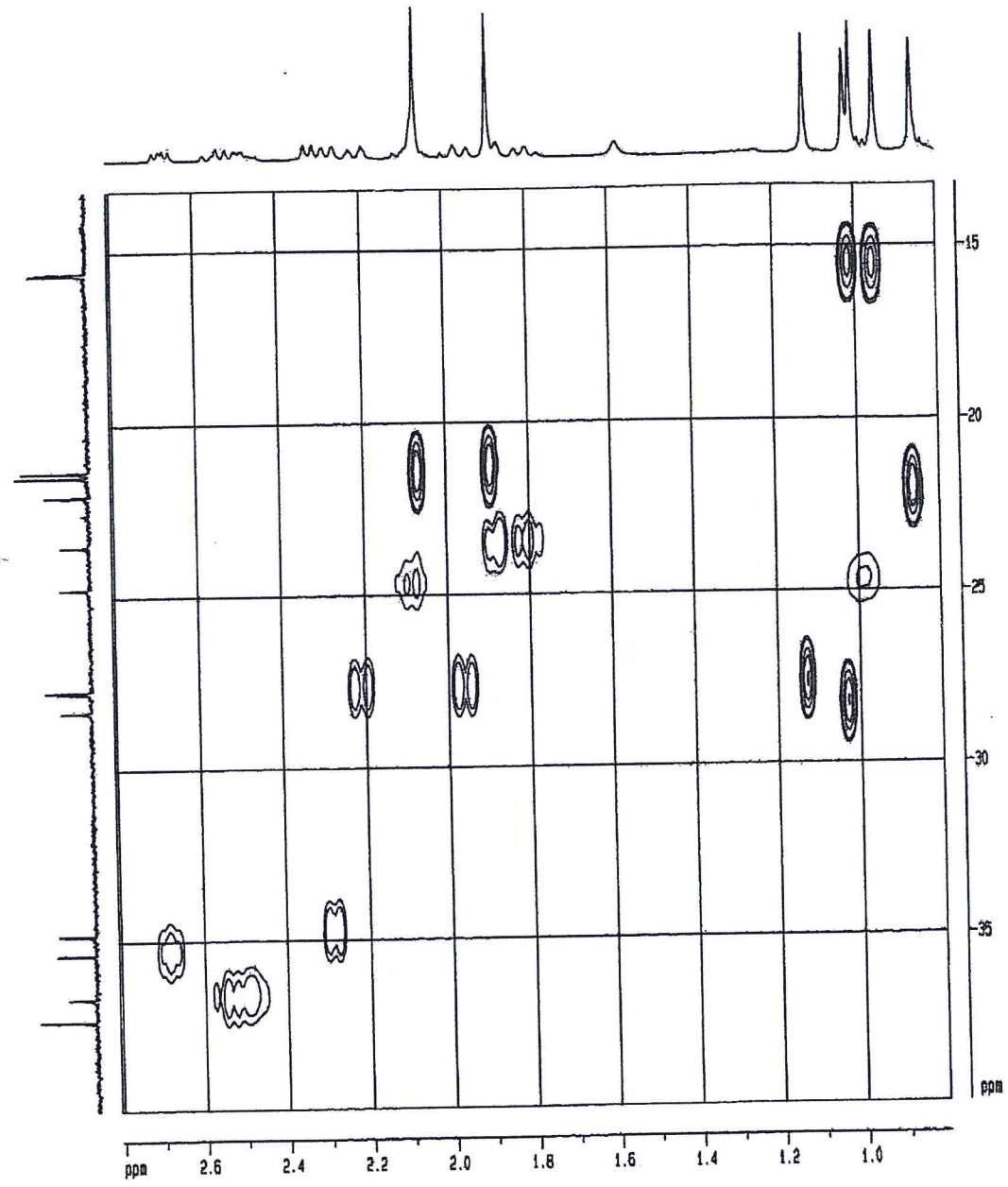

Figure 45: Expanded section of 500 MHz HMQC spectrum of compound **117**

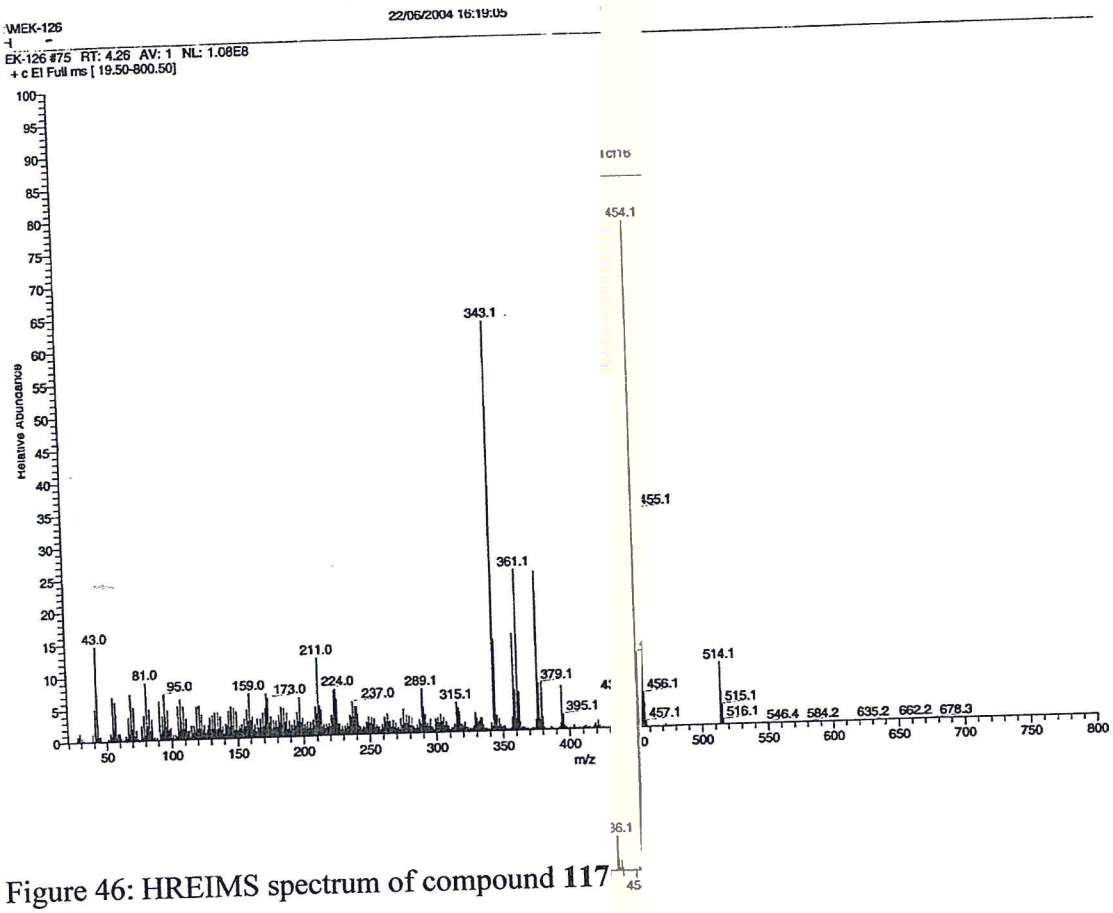

Figure 46: HREIMS spectrum of compound **117**