ANTI-LARVAL COMPOUNDS FROM *VITEX SCHILIEBENII* AND *VITEX PAYOS*

BY

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Declaration by Candidate

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

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Dedication

This thesis is dedicated to my husband Geoffrey M. Maroko for being my mentor and to our children, Brenda, Marian, Allan and Emmanuel for persevering an ever absent mother during my studies.

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List of abbreviations

ANOVA	Analysis of Variance
B.t	*Bacillus thuringiensis*
CC	Column Chromatography
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
CSA	Circumsporozoite Surface Antigen
CSP	Circumsporozoite Surface Proteins
C N B	*Culex nigripalpus baculovirus*
DDT	Dichlorodiphenyl-trichloroethane
DEET	*N, N*-diethyl-*m*-toluamide
DEPT	Distortionless Enhancement by Polarisation Transfer
EI_{50}	Emergence Inhibition
EIMS	Electron Impact Mass Spectrometry
HCH	Hexachlorocyclohexane
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
^{1}H NMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IGR	Insect Growth Regulator
IR	Infrared
IVM	Integrated Vector Control
ITN	Insecticide Treated Net
LC_{50}	Lethal Concentration
m.p.	Melting Point
MS	Mass Spectrometer
M/z	Mass charge ratio
NMR	Nuclear Magnetic Resonance
PPM	Parts per million
PTLC	Preparative Thin Layer Chromatography
RBC	Red Blood Cells
SIDA	Swedish International Development Agency
TDR	Tropical Diseases Research
TLC	Thin Layer Chromatography
ULV	Ultra-low-volume
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
DMSO	Dimethylsulphuroxide

Abstract

Over two billion people in tropical countries are at risk from mosquito-borne diseases such as dengue fever, hemorrhagic fever, malaria and filariasis. The search for effective vaccines against these diseases is still in progress. It is estimated that US $ 2 billion is spent on malaria control and treatment programmes in Africa annually. The problem has become increasingly difficult to manage because of the spread of resistance to anti-malarial drugs by the parasites resulting in increased severity of the disease. Protection from mosquito bites is still the most important measure to control the disease. However, vector resistance to insecticides is a recurring problem and a threat to malaria control programmes. The safety and efficacy of *N, N*-diethyl-*m*-toluamide (DEET), which is the most potent of the modern synthetic repellents, is questionable. Controlling mosquitoes at larval stage is an additional effort that man has put in place to try and combat malaria menace. During the past two decades, considerable progress has been made in the development of natural and synthetic compounds, which are capable of interfering with the process of growth, development and metamorphosis of the target mosquito species: insect growth regulators (IGRs). Several phytochemicals such as azadirachtin have been shown to posses larvicidal/IGR activities. World Health Organization (WHO) has recommended IGR compounds such as methroprene, diflubenzuron and triflumuron. These compounds have been approved by World Health Organization Pesticide Evaluation Scheme (WHOPES) for use against immature mosquitoes. In this study, we carried out phytochemical investigation of anti-larval compounds from *Vitex payos* and *Vitex schilliebenii* using late third instar *Anopheles gambiae* larvae using WHO bioassay procedures. Monitoring their larval mortality after every 24 hours and inhibition of adult emergence to assess the impact was done. The crude extracts with larvicidal or anti-larval activity were subjected to chromatographic seperation techniques to isolate the active compounds, whose structures were elucidated by the conventional spectroscopic techniques. Four compounds: 20-hydroxyecdysone (2, 3, 14, 20, 22, 25-hexahydroxycholest-7-en-6-one (**80**) (VSCE 2), 22-*epi*-20-hydroxyecdysone (**82**) (VSCE 3), stigmast-5-en-ol (**118**) (VSH 2), stigmaterol (**133**) (VSH 1), were isolated from *Vitex schiliebenii*, while five compounds: VPH 1, VPH 2, stigmasterol (**133**) (VPH 3), secoisolariciresonol (**134**) GF/CE/1 and GF/CE/2 were isolated from *Vitex payos*. Four compounds VSCE 2, VSCE 3, VSH 1 and VSH 2, were assayed for IGR activity. They exhibited 100 % inhibition of emergence and caused morphological deformation in some pupae of *Anopheles gambiae* at 50 ppm, resulting in larval-pupal intermediates with the heads of pupae and the abdomens of larvae. Most adults from larvae treated with the test compounds died within 48 hours of emergence. The compounds showed delayed larval mortality at lower doses (1-5 ppm), which indicated effective developmental inhibition potential.

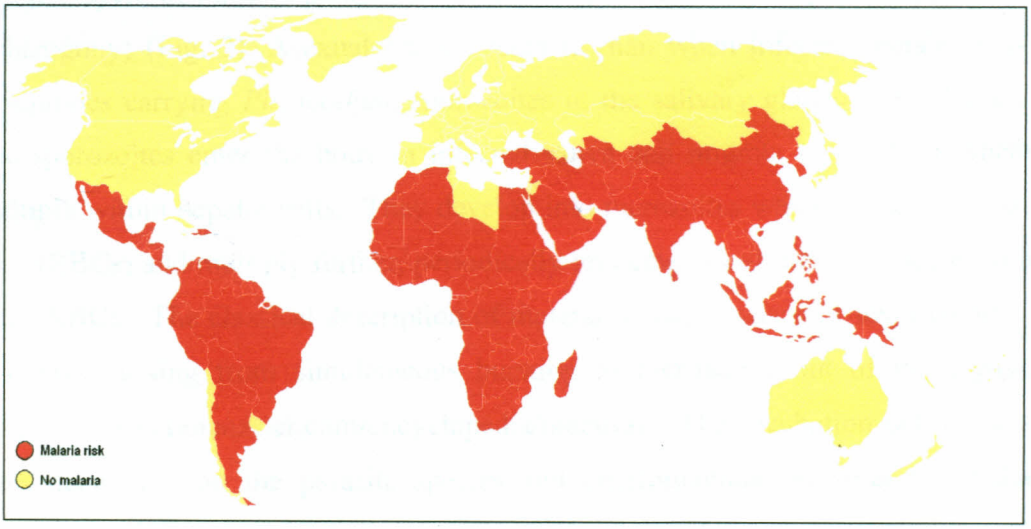
CHAPTER 1: INTRODUCTION

1.1 Background

Malaria is a common and potentially fatal parasitic disease. Typical symptoms of malaria are flu-like and include: fever, fatigue, muscle aches, dizziness, vomiting, headache, arthralqia (joint pain), and convulsions (Anon., 2004). There may be a feeling of tingling in the skin, particularly with *Plasmodium falciparum* inflicted malaria. Complications of malaria include spleenomegaly, renal failure, anaemia and cerebral damage leading to coma and death if left untreated (http://www.nationmaster.com/encyclopedia/malaria). While rapid diagnosis and treatment of the disease is essential to prevent complications, malaria is often misdiagnosed (Kain *et al.*, 1998).

Malaria is caused by a parasitic infection, transmitted by female anopheline mosquitoes (TDR, 1995). Four different *Plasmodium* species: *P. falciparum, P. vivax, P. ovale* and *P. malariae* are responsible for the human form (WHO, 1997a). In the disease endemic regions like sub-Saharan Africa, Asia, Oceania and the Amazons (Fig. 1), *P. falciparum* is the most virulent (WHO, 1997a).

Figure 1: Global distribution of malaria



Source: http://upload.wikimedia.org/wikipedia/en/d/d5/Malaria_map.PNG

Malaria is a major threat to health in most African countries. In Kenya, the disease is a major public health problem, which threatens poverty reduction efforts and contributes to

retarded development. About 70% of the population (~ 20 million) are at risk of the disease, which claims 34,000 children annually (~ 93 children per day) (Anon., 2004). Endemic regions such as Nyanza, Coast and Western Provinces bear the greatest burden of malaria. Recently, the malaria epidemic has been reported in the highlands of Kenya which were initially non-endemic (http: www.mdtravelhealth.com/destinations/africa/kenya.html). The high risk groups include: non-immunes, comprising of young children from 6 months to 5 years; pregnant women; populations in borderline transmission areas and people moving from malaria free to endemic zones for reasons of work, migration, refuge, war or tourism (TDR, 1995).

Due to the frequent attacks of malaria, there is need for the use of innovative interventions to prevent and cure the disease (WHO, 2002). The complete knowledge of genome sequences of *P. falciparum*, *Homo sapiens* and the *Anopheles gambiae* have been elucidated and need to be fully understood for the design and development of effective vaccine and insecticide (Mauro, 2002).

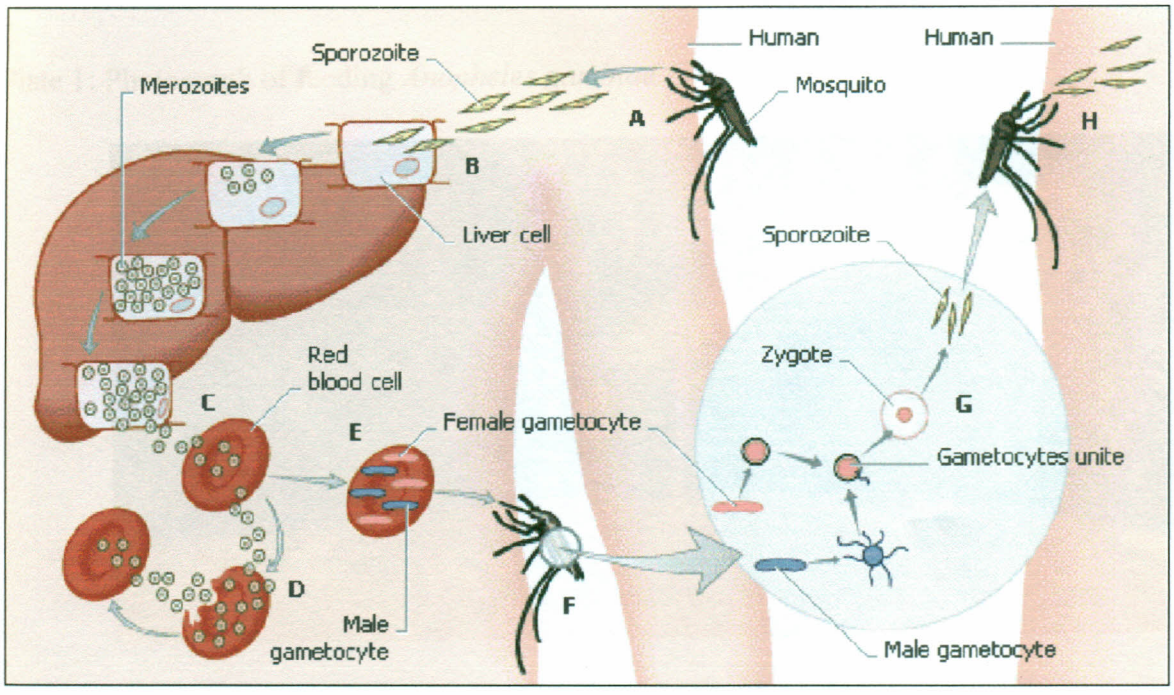
1.2 Life cycle of malaria parasite

The life cycle of malaria parasite involves two phases: sexual (sporogony) and asexual (schizogony) (Fig. 2). Asexual phase occurs in man when infected female anopheline mosquitoes carrying *Plasmodium* sporozoites in the salivary glands bite a human host. The sporozoites enter the body in infected saliva and migrate to the liver where they multiply within hepatic cells. They develop into merozoites which invade the red blood cells (RBCs) and multiply further, periodically breaking out of the erythrocytes to invade other RBCs. The classical description of malaria includes waves of fever coming every 3-4 days, arising from simultaneous bursting of merozoites out of the erythrocytes (http://www.nationmaster.com/encyclopedia/malaria). The incubation period lasts 6-16 days depending on the parasite species and environmental temperature (Wallace & Herbert, 1995).

The sexual phase of the mosquito parasite life cycle takes place in female anopheline mosquitoes when some merozoites turn into male and female gametocytes. A mosquito feeding on infected blood from a host picks up the gametocytes. The male and female

gametes fuse to form zygotes. The zygotes penetrate the stomach to form an oocyst, within which large numbers of slender sporozoites develop. Some of these sporozoites pass through the body fluid cavities while others enter the salivary gland of the insect. When an infected mosquito feeds, it innoculates the sporozoites from its salivary gland into the host and the asexual life cycle starts again (http://www.cdc.gov/malaria/biology life-cycle.htm).

Figure 2: Life cycle of malaria parasite



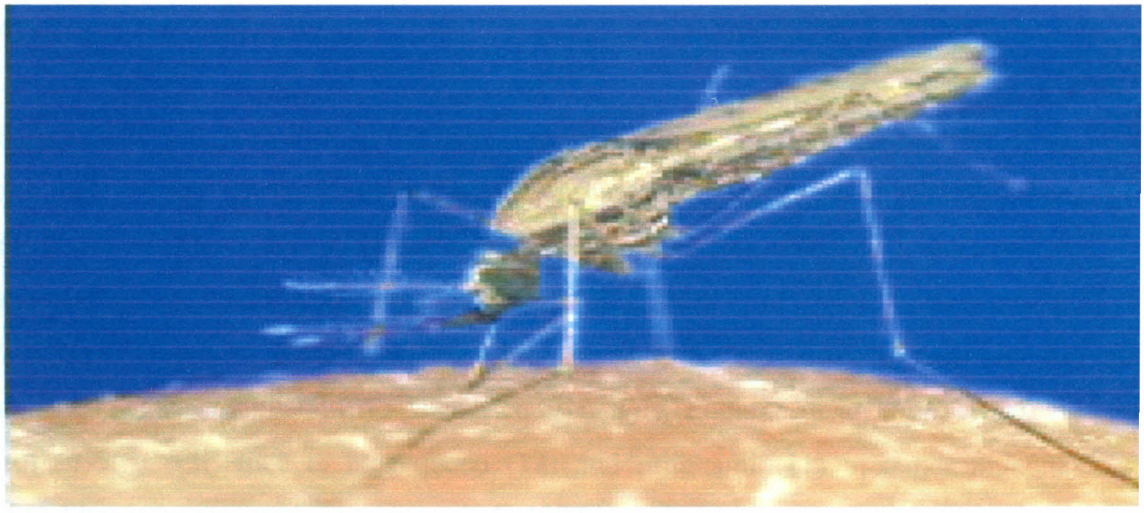
Source: http://www.cdc.gov/malaria/biology

1.3 Malaria vectors

Mosquitoes of the genus *Anopheles* are the exclusive vectors of malaria in humans (Robert & Frank, 1996). About 70 species of *Anopheles* are known vectors of malaria (Rao, 1984), and the number is expected to increase as more sibling species of various complexes continue to be identified within the genus. Most of the malaria cases in tropical Africa are transmitted by three malaria vectors: *Anopheles gambiae sensu stricto* (Plate 1), *An. funestus* and *An. arabiensis* (Hunt & Coetzee, 1995). *Anopheles gambiae s.s.* is the most efficient vector due to its high anthropophilic character (Garret-Jones *et al.*, 1980). Due to the high densities of *An. gambiae* during wet seasons, it is an

important vector in specific locations (Charlwood & Jolly, 1984). *Anopheles funestus* is important in malaria transmission during dry periods since it breeds in permanent water bodies (Didier *et al.*, 1997). The combination of anthropophilicity and endophilicity of *An. gambiae* and *An. funestus* puts them in an advantageous position over other malaria vectors (Gillies & Coetzee, 1987). *Anopheles arabiensis* is known to vary from being anthropophilic to zoophilic depending on geographical location, climatic conditions and host availability. It has lower vectorial capacity than *An. gambiae s.s* (Brack *et al.*, 1994).

Plate 1: Photograph of feeding *Anopheles gambiae*



Source: http://phil.cdc.gov/Phil/

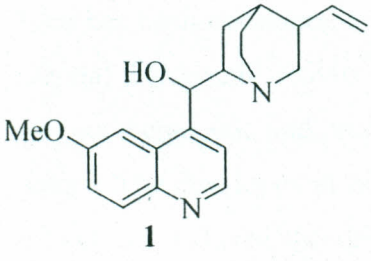
1.4 Malaria control

1.4.1 Chemotherapy

Various anti-malarial drugs are available for curative or prophylactic purposes. These include: quinine (**1**), chloroquine (**2**), mefloquine (**3**), amodiaquine (**4**), primaquine (**5**), mepacrine (**6**), sulfadiazine (**7**), sulfadoxine (**8**), dapsone (**9**), pyrimethamine (**10**), proguanil (**11**), atovaquone (**12**), halofantrine (**13**), lumefantrine (**14**), quinacrine (**15**), artemisinin (**16**) and its derivatives (**17-19**). However, the synthetic anti-malarial drugs (**2-15**) have not eradicated malaria due to increased cases of drug resistance (Childs *et al.*, 1991), high costs and side effects. Resistance to chloroquine (**2**) has been observed in

Nigeria (Serpa *et al.*, 1988), Rwanda (Giboda & Dennis, 1988), Ivory Coast (Charmot *et al.*, 1988), and Kenya (Brandling-Bennet *et al.*, 1988). Resistance to mefloquine (**3**) in non-immune individuals has also been reported in Thailand (Boudreau, *et al.*, 1982), Tanzania (Bygbjerg *et al.*, 1983) and West Africa (Ringwald *et al.*, 1990). Resistance to amodiaquine (**4**) was reported in the early 1990s (Philips-Howard & Björkman, 1990). Halofantrine (**13**) has been effective against *P. falciparum* with multiple drug-resistances (Watt *et al.*, 1994; Del Nero *et al.*, 1994), but recent studies in Thailand revealed lower cure rates than expected (Olliaro & Trigg, 1995). Lumefantrine (**14**) has been reported as an effective anti-malarial drug but has the disadvantage of being slow in action during monotherapy (Wendsorfer, 1994).

Resistance of *P. falciparum* to sulfadoxine-pyrimethamine (SP) has been reported from various parts of the world, including sub-Saharan Africa (Ogutu *et al.*, 2000). The potential use of atovaquone (**12**) for treatment of multi-drug-resistant *P. falciparum* malaria has been reported in Thailand and Brazil (Blanchard *et al.*, 1994; Looareesuwan *et al.*, 1996). However, there is evidence that the parasites may develop resistance to atovaquone (**12**) and proguanil (**11**) when used separately (Looareesuwan *et al.*, 1996). A case of *in vivo* resistance to atovaquone (**12**) and proguanil (**11**) in a non-immune individual has been reported (Fivelman *et al.*, 2002).



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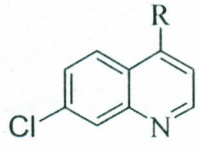

R = $HNCH(CH_3)(CH_2)_3N(C_2H_5)_2$

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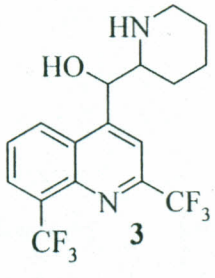

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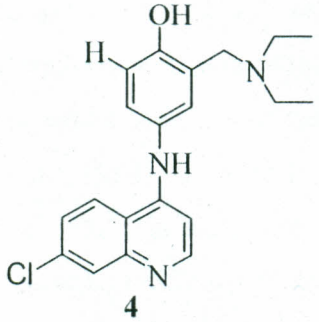

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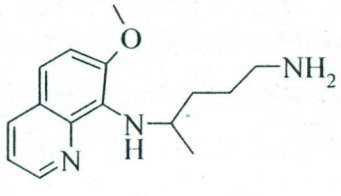

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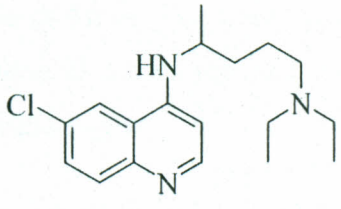

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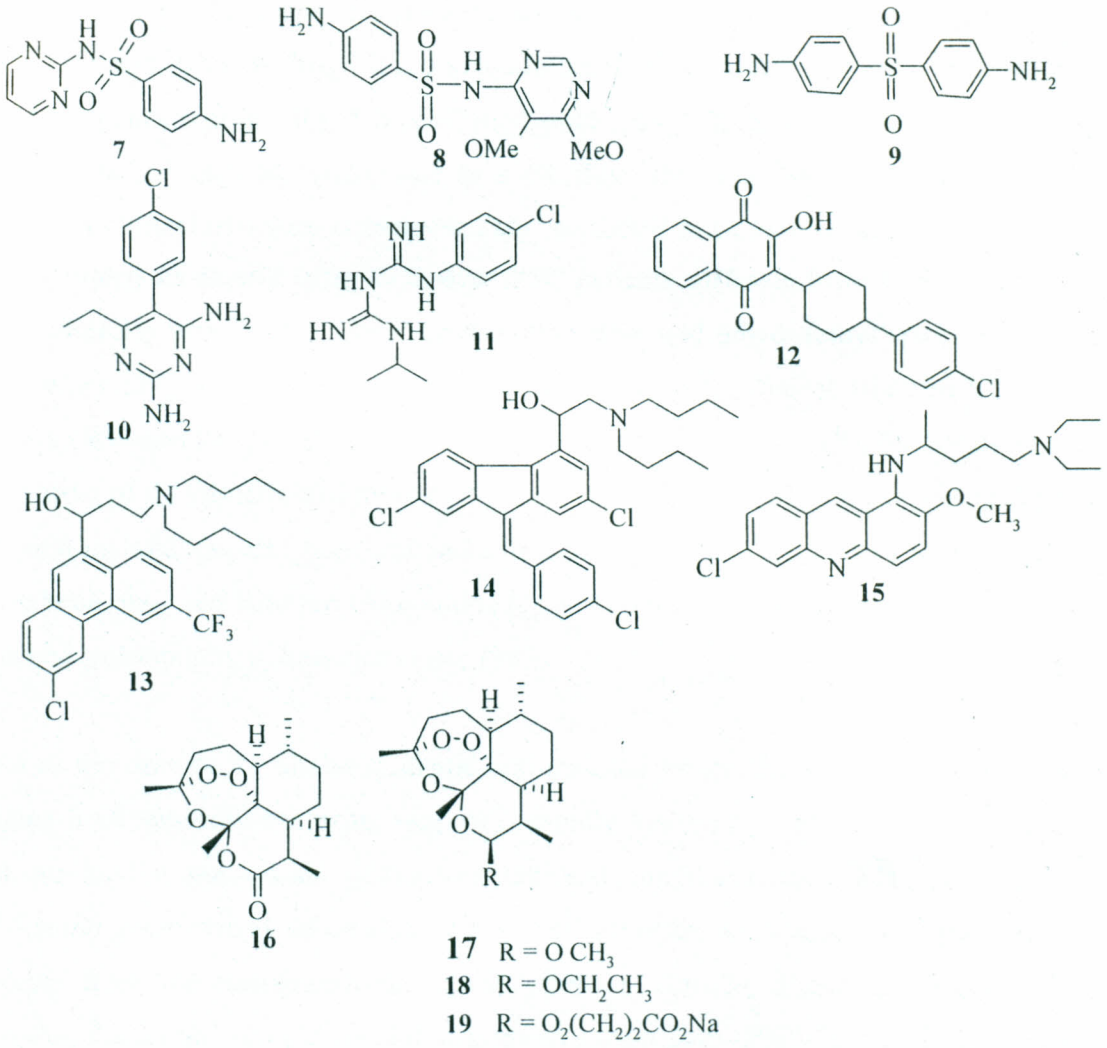
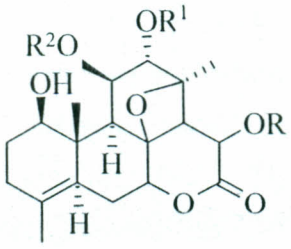
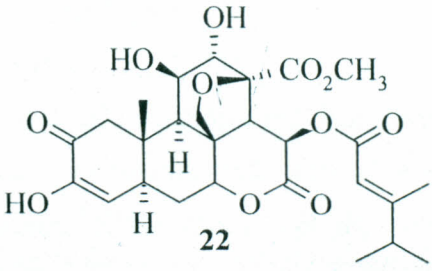


There has been considerable progress in the identification of new natural products with potential therapeutic activity like quinine (**1**) and artemisinin (**16**). Quinine (QN) (**1**) has been the therapeutic and prophylactic of choice for malaria since its discovery in the 17th century. The development of chloroquine (**2**), primaquine (**5**) and quinacrine (**15**) in the 20th century reduced the reliance on quinine. These anti-malarial drugs can be taken preventively, and are recommended for travelers to endemic regions (http://www.nationmaster.com/encyclopedia/Malaria). Quinine (**1**) still remains an important drug for the treatment of severe *falciparum* malaria in Africa (Pukrittayakamee *et al.*, 1994). However, cases of resistance to quinine (**1**) have also been reported from Vanuatu (Bastein, 1987), Solomon Islands (Isaacs & Ellis-Pegler, 1987), Zambia (Bisseru, 1988) and Malawi (Warhurst *et al.*, 1985).

Artemisinin (**16**) is perhaps one of the most significant natural products to emerge in the past few years as a new drug. It is a sesquiterpene lactone with an unusual endoperoxide moiety, isolated from the Chinese medicinal plant *Artemisia annua* (Qinghaosu) (Compositae), which has been used as a febrifuge for thousands of years (Liu *et al.*, 1979). A clinical trial with artemisinin (**16**) conducted in China between 1973 and 1980, demonstrated substantial efficacy in over 2000 patients suffering from *P. falciparum* and *P. vivax* (Klayman, 1985). The dihydromethyl ether and dihydroethyl ether derivatives, artemether (**17**) and arteether (**18**), have also undergone clinical trials in China and Burma (Woerdenberg *et al.*, 1990; Li, 1991). Sodium artesunate (**19**), the hemisuccinate derivative of dihydroartemisinin that, demonstrated considerable clinical success against celebral malaria has also been evaluated in China (Li, 1991). Strong synergistic effects have been observed between chloroquine (**2**) and artemisinin (**16**) such that doses can be reduced substantially (Chatterjee *et al.*, 1989).

Due to the drawbacks of the synthetic anti-malarial drugs, the search for anti-malarial agents from naturally occurring sources is rapidly increasing. This has led to two new anti-plasmodial quassinoids: gutolactone (**20**) and simalikalactone D (**21**), from the bark of *Simaba guianensis* (Carbral *et al.*, 1993). Several researchers have investigated various species from the Simaroubaceae family (*Brucea, Castela, Picrasma, Ailanthus* and *Simaba*) due to the anti-plasmodial quassinoid constituents (Phillipson & Wright, 1991). Considerable success has been achieved in the bioactivity-guided fractionation of the active principles and the identification of some of the features that are key to the potentiation of the activity. Unfortunately, these features are similar to those that have been associated with cytotoxicity. The most active compound, bruceantin (**22**), is also the most cytotoxic (Angerhofer *et al.*, 1992). Kanyanga *et al.* (1997) reported the *in vivo* and *in vitro* anti-plasmodial activity of cryptolepine (**23**) and related alkaloids from *Cryptolepis sanguinolenta.* Nimbolide (**24**) and gedunin (**25**), from *Azadirachta indica* (Meliaceae) have been reported to exhibit plasimodial activity against *P. falciparum* (Bray *et al.*, 1990). *Dichroa febrifuga* (Saxifragaceae), used in Chinese traditional medicine, yielded febrifugine (**26**) as the active principle (Suping *et al.*, 2005). However, it has not been exploited as a malaria drug because of its hepatoxicity (Haruisa *et al.*, 2006).





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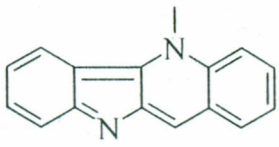

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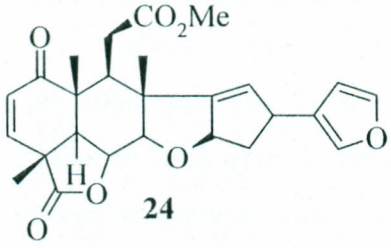
20 R = Me_2C = CHCO, R^1 = R^2 = H

21 R = $MeCH_2CH(Me)CO$, R^1 = R^2 = H



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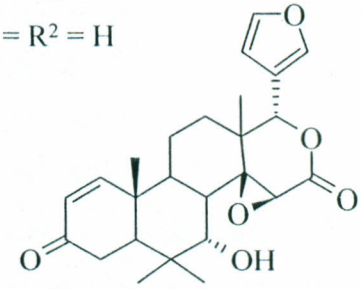

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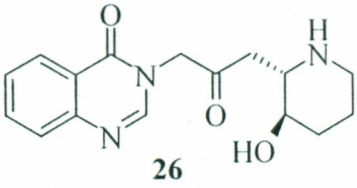

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Most natural products remain unused because their toxicological profiles have not been studied. In addition, only a few of the plants used traditionally to manage malaria have been investigated for efficacy and safety of their active principles. Whereas curative methods must be used against disease, prevention of the infection is the best strategy.

1.4.2 Vaccine development

Vaccines for malaria are under development with no effective operational vaccine yet available. Currently, four types of anti-malarial vaccines are under development, classified according to the stages of the life cycle of the parasite they target (Targett, 1989).

(i) Pre-erythrocytic vaccines - reduce the number of the successful inoculations by neutralising sporozoites or liver forms (Targett, 1989).

(ii) Asexual blood stage vaccines - expected to prevent or reduce the incidence of disease in the same way as chemoprophylaxis (Targett, 1989).

(iii) Transmission blocking vaccines - expected to prevent the development of the parasite in mosquitoes after biting an infected individual (Targett, 1989).

(iv) The anti-toxic vaccines act against exo-toxins to prevent serious pathological complications of the disease (Playfair *et al.*, 1990).

Volunteers immunised with irradiated sporozoites were shown to be protected against *P. falciparum* and *P. vivax* (Clyde *et al.*, 1975). However, it has been reported that subsequent recombination of synthetic vaccine candidate derived from pre-erythrocytic stage antigens, aimed at protection against infection of malaria was not successful (Ballou *et al.*, 1987). The vaccine, SPf66 (Alonso *et al.*, 1994) has undergone trials in children between ages 1-5 years. The vaccine is based on pre-erythrocytic and asexual blood stage proteins of *P. falciparum*. It was reported to have no protection against infection but reduces clinical episodes (Valero *et al.*, 1993). Its protective efficacy was found to be 30% in Tanzania and 90% in Thailand (Alonso *et al.*, 1994; Migasena *et al.*, 1997). However, it has been found to cause mild tenderness at site of injection (Alonso *et al.*, 1994; Migasena *et al.*, 1997). SPf66 vaccine has been reported to be partially effective in field trials in South America,, South-East Asia and Africa. Sophisticated biochemical methods are now being used to improve its potency (Philippe *et al.*, 2002).

Development of an effective malaria vaccine could greatly contribute to disease control. RTS,S/AS02A is a pre-ethrocytic malaria vaccine candidate based on *P. falciparum* circumsporozoite surface antigen (CSA). The vaccine has been reported to be safe, well tolerated and immunogenic (Alonso *et al.*, 2004). Thus, development of an effective vaccine against malaria is feasible. However, genetic features and the intensity of malaria transmission may modify the safety and immune response of a vaccine (Macete *et al.*, 2007). RTS,S/AS02A has been reported to confer partial protection in African children aged 1-4 years living in rural endemic areas against a range of clinical diseases caused by *Plasmodium falciparum* for at least 18 months. This confirms the pontential of malaria vaccines in becoming credible control tools for public-health use (Alonso *et al.*, 2005). RTS,S/SBAS2 vaccine has been reported to induce no significanct toxicity in semi-immune population and it produces significant increases in antibody titers to circumsporozoite surface proteins (CSP) (Doherty *et al.*, 1999).

Recently partially successful field trials for RTS,S/AS02A, a vaccine which reduces infection risk by 30% and severity of infection by over 50% was reported. The phase 2b trials of RTS,S in Mozambique show that the vaccine candidate is effective in children of ages 1-4 years (WHO, 2004). However, further research may delay this vaccine from

commercial use until around 2010 (http://www.malariavaccine.org/mal-vac2-challenge.htm).

The recent discovery of an antibody that protects against the disease (Fiona, 2005) is exciting and it is hoped that the genome sequence of *P. falciparum*, which was completed in 2002, will provide targets for new drugs and vaccines (Ito *et al.*, 2002).

Many factors make malaria vaccine development difficult and challenging. First, the size and genetic complexity of the parasite mean that each infection presents thousands of antigens to the human immune system. Understanding which of these antigens are useful target for vaccine development is necessary. To date at least 40 promising antigens have been identified. Second, the parasite changes through several life stages even while in the human host presenting a different subset of molecules for the immune system to combat at each stage. Third, the parasite has evolved a series of strategies that allow it to confuse, hide and misdirect the human immune system. Fourth, it is possible to have multiple infections of not only different species but also of different strains at the same time (http://www.malariavaccine.org/mal-vac2-challenge.htm). Finally, the cost of production is another limiting factor to vaccine development. It should be noted that vaccine development is a slow process and other control tools such as vector control should be employed.

1.4.3 Vector control

Traditionally vector control has been the main tool for the reduction of malaria transmission in endemic areas. The control strategy targets both adult and larval stages. It involves self protection, adulticides and larvicides.

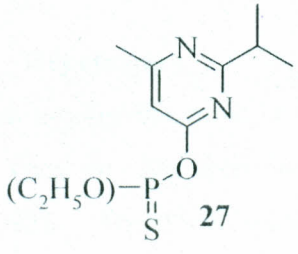
1.4.4.1 Adult control

1.4.4.1.1 Insecticides

Adulticides are residual insecticides, like diazinon (**27**), dichlorodiphenyltrichloroethane (DDT) (**28**), hexachlorocyclohexane (HCH) (**29**), malathion (**30**) and fenitrothion (**31**), are becoming increasingly important for household applications. DDT (**28**) was developed during World War II and initially used with great effect on malaria-

transmitting mosquitoes. It was banned for use in many countries in the 1970s due to the negative environmental impact. There is great controversy regarding the impact and use of DDT (**28**) to control human disease vectors. Some claim that its ban is responsible for the increase in malaria deaths in tropical countries where the disease had been previously under control (http://www.nationmaster.com/encyclopedia/malaria).

Mekuria *et al.* (1991) reported insecticide resistance in *Aedes aegypti* in Dominican Republic. Generally, ultra-low-volume insecticide sprays were used in low concentration or diluted in light oil (Groves *et al.*, 1994). With increasing environmental concerns, synthetic pyrethroids have replaced organochlorines, organophosphates and organocarbamates in mosquito control (WHO, 1997b). Use of permethrin (**32**) treated bed-nets has had tremendous impact on malaria cases (MacIntyre *et al.*, 2003). Other examples of synthetic pyrethroids include; cyfluthrin (**33**), deltamethrin (**34**) and λ-cyhalothrin (**35**) (WHO, 1997b).



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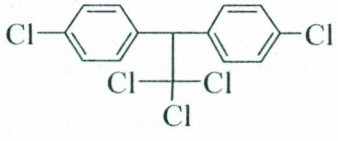

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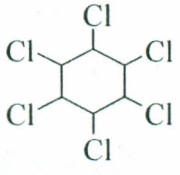

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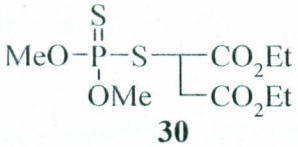

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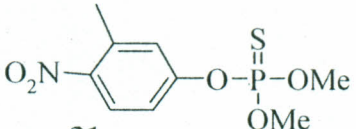

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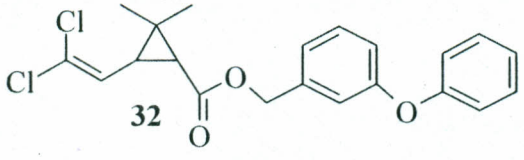

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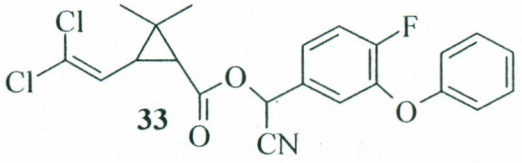

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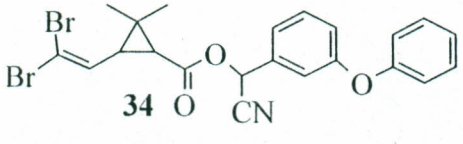

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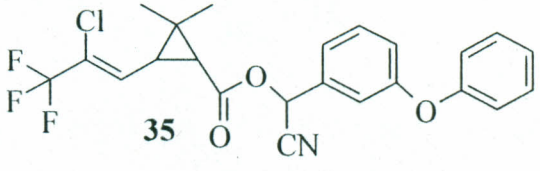

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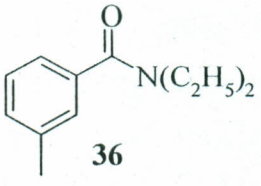
1.4.4.1.2 Insecticides for reduction of man-vector contact

The approach used here includes reduction of man-vector contact through clothing, netting, screening, use of repellents and insecticides. Clothing, bed nets and curtains impregnated with pyrethroids have been used against bitting insects in various parts of the world (Choi *et al.*, 1995). The effectiveness of permethrin (**32**) impregnated bed nets in Tanzania has been documented (Lines *et al.*, 1987). Permethrin (**32**) deposits have a long shelf life but frequent washing and handling reduce efficacy (Curtis & Lines, 1988). Use of insecticide treated nets (ITNs) reduced malaria inoculation rate by 90% in Tanzania (Magesa *et al.*, 1991). Similarly, 95% reduction in the number of indoor resting *An. gambiae* has been reported in Guinnea Bissau (Jaensen *et al.*, 1994). Although pyrethroid impregnated bed-nets and curtains are widely employed to reduce the risk of malaria transmission, its resistance is becoming more prevalent among malaria vectors (Vulule *et al.*, 1994).

1.4.4.1.3 Repellents

Repellents are among the most commonly used methods to prevent adult mosquitoes from biting. They provide short-lived protection due to their volatile nature (Gupta & Rutledge, 1992). The most widely used commercial repellent is *N, N*-diethyl-*m*-toluamide (DEET) (**36**). It repels a wide variety of insects, ticks and mites, and lasts longer than other repellents (Curtis *et al.*, 1987).

Other synthetic repellents include: dimethyl phthalate (**37**), 2-ethyl-2-butyl-1, 3-propanediol (**38**), *o*-chloro-*N*, *N*-diethylbenzamide (**39**), 2-ethyl-1, 3-hexanediol (**40**) and *n*-propyl-*N*, *N*-diethyl-succinamate (**41**).



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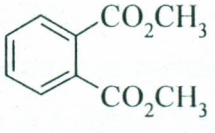

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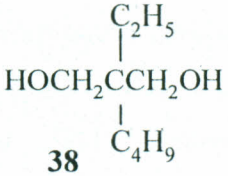

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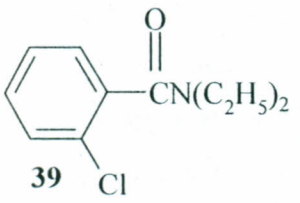

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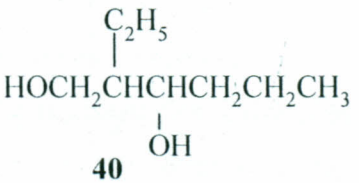

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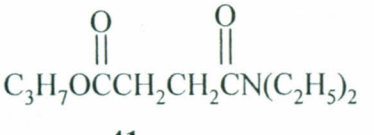

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Allergy and other serious reactions to DEET (**36**) have rarely been reported (Robbins & Cherniak, 1986). Since children are more sensitive, it is recommended that their skin exposure be kept to a minimum and DEET (**36**) should be applied to clothing and not skin (WHO, 1991a). Other modes of administration of repellents include use of mosquito coils, which is popular in many tropical countries (Stephens *et al.*, 1995), candles, incence, creams, foams, soap, lotion, sprays among others. Housing improvements that reduce access of mosquitoes to the occupants such as screening of windows and doors may have a major impact on malaria transmission if rigorously promoted (WHO, 1997b).

Traditionally, plants and their derived substances were used to repel or kill mosquitoes (Curtis, 1991). Smoke from burning cattle or goat dung was used in some communities to keep mosquitoes away from human dwellings. It has been reported that repellent effect of smoke may be increased by burning certain materials such as aromatic wood containing resins (Snow *et al.*, 1987). In Southern India, leaves of *Vitex negundo* are burned to repel mosquitoes from houses (Curtis, 1991). In Africa, Asia and Latin America, leaves of the neem tree (*A. indica*) are sometimes burned providing unpleasant odour for the mosquitoes (WHO, 1997b). It is believed that the neem tree keeps mosquitoes away but there is no scientific evidence for this. The repellent effect of *Lantana camara* flowers has been evaluated against *Aedes* mosquitoes. *Lantana* flower extract in coconut oil provided 94.5% protection from *Aedes albopictus* and *Aedes aegypti* (Dua *et al.*, 1996). Studies have shown *Ocimum* sp to be repellent against mosquitoes, either by burning or hanging fresh leaves in occupied houses or by brushing foliage on the exposed arms and legs of humans (Waka *et al.*, 2004). *Ocimum forskolei* has been reported as a beneficial repellent in reducing vector biting if used in communities in areas with partially zoophilic mosquito species such as *Anopheles stephensi* and where animals are present (Waka *et al.*, 2006). Ethnobotanical survey in two communities in western Kenya revealed that the most commonly known repellent

plants were *Ocimum americanum*, *L. camara*, *Targetes minuta*, *A. indica*, *Hyptis suaveoleus* and *Ocimum basilicum*. Direct burning of the plants is the most common method of application. The repellency of these plants was evaluated against the *An. gambiae* s.s Giles in experimental huts within a screenwalled green house (Seyoum *et al.*, 2002). Plant essential oils and terpenoids have also been reported to show repellency to adult mosquitoes (Curtis *et al.*, 1989). Sharma *et al.* (1993) reported the effectiveness of neem oil as an alternative and safe method of protection from mosquitoes.

1.4.4.2 Larval control

This involves controlling juvenile mosquitoes while in larval and pupae life stages which only occur in water. To safely alter the aquatic environments, even temporarily, for the purpose of controlling mosquitoes requires thorough knowledge of both target species and larvicides, which include commercial pesticides and natural predators. Methods used for larval control include: environmental management, synthetic chemical larvicides, biological control agents, botanical anti-pest agents and insect growth regulators (IGRs).

1.4.4.2.1 Environmental management

Environmental management approach to vector control aims at modifying the environment to deprive the target vector of its requirements for survival (mainly breeding, resting and feeding). This reduces human-vector contact and renders the conditions less conducive for disease transmission (WHO, 1991b).

In Malaysia, the breeding of the *Anopheles maculata* in streams has been controlled by periodical flushing of small dams with siphons and sluice gates. In Indonesia, changing the salinity of the breeding habitats of *Anopheles sundaicus* has been used as an environmental management tool for vector control (WHO, 1995). In India, community-based environmental management projects for vector control have been undertaken (Sharma, 1993). However, most of these environmental control strategies are not universally applicable and should be designed with close attention to the local ecological, socio-economic, political and cultural factors in mind. Environmental management practices are labour intensive, repetitive and costly. There is need to use other strategies like larvicides for effective mosquito control.

1.4.4.2.2 Biological larvicides

The biological control of mosquitoes involves introduction of natural enemies, such as parasites, pathogens and predators into the environment. *Bacillus thuringiensis* serotype H-14 (B.t. H-14) produces toxins (δ-toxin) which kill larvae after ingestion and is effective against mosquito strains resistant to chemical larvicides (Reuben, 1991). A major drawback of *B.t.* is lack of residual activity (McLaughlin *et al.*, 1982). *Bacillus sphaericus* var *israeliensis* also produces a larvicidal toxin but is more effective in polluted water while B.t. H-14 is more effective in clean water (Mulla *et al.,* 1984; De Barjac & Sutherland, 1990). Inert spores of the fungus, *Beauveria bassiana*, kill mosquitoes when sprayed on walls and bed nets (Donald, 2005). Another fungus, *Tolypocladium cylindrosporum* Gams, is a potential microbial control agent of mosquitoes (Weiser & Pillai, 1981). It is effective against *Culex* and *Psorophoria* with variable activity against *Aedes* species (Mulla *et al.,* 1985). So far mosquitoes have not shown resistance to fungal infections (Donald, 2005).

Recently there have been tremendous advancements in the ability to transmit some mosquito pathogenic viruses as well as new molecular tools and capabilities to understand and manipulate viruses at the molecular level. *Culex nigripalpus* baculovirus, (CNB) is being studied for possible mosquito control. It is highly pathogenic to C. *nigripalpus* and *Culex quinquefascitus* both of which are important vectors of St. Louis and Eastern encephalitis virus. It is also responsible for the reduction of field populations of *C. nigripalpus* larvae (Becnel, 2006).

Romanomermis culicivorax, an entomopathogenic nematode, parasitizes *C. quinquefasciatus* larvae (Mitchell *et al.*, 1974). However, *C. quinquefasciatus* can develop resistance to *R. culicivorax* after about 300 generations which might reduce the possibilities of large scale use (Petersen, 1978). Larvae of *Toxorhynchites*, *Culex* and *Lutzia* species predate on other mosquito larvae thus reducing their populations. This may be a way that natural population control may be taking place (Kuldip *et al.*, 1984).

Genetically manipulated insect vectors have also been proposed as bio-control agents (Collins, 1994). However, its application may present serious operational problems.

Proposed improvements in sterile insect techniques include release of insect carrying dominant lethal genes that may make it a practical biocontrol method (Thomas *et al.*, 2000). However, mass application may present serious operational problems.

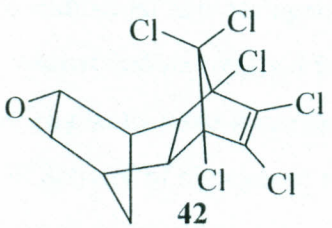
Vertebrate bio-control agents include fishes, like *Gambusia affinis* and *Poecilia reticulate*, which feed on mosquito larvae (Sasa & Kurihara, 1983). However, mass breeding and introduction in unstable habitat present serious challenges. Bio-control agents being members of a balanced ecosystem cannot be an alternative to insecticidal control. However, they can be used as adjuncts in logical and necessary components of integrated vector management (IVM) measures as a population stabilising agents. They are slow acting and cannot be used in emergencies. Alternative control strategies can be sought from chemical larvicides.

1.4.4.2.3 Chemical larvicides

Larvicides are applied to mosquito breeding sites to kill larvae. They act as stomach poisons, which must be ingested by the larvae while feeding or as contact poisons, which penetrate the body wall or the respiratory tract. Application of petroleum oil on stagnant water is one of the oldest methods of killing mosquito larvae (Thevasagayam *et al.*, 1979). The larvae are killed in two ways when they rise to the surface to breath: by suffocation and by poisoning with toxic vapour. Because of the relatively high cost of petroleum oils compared with some other larvicides and environmental pollution, their use for mosquito control has decreased. They are of special interest in situations where mosquitoes have developed resistance to insecticides. However, for small-scale applications they offer the advantage of wide availability.

Monomolecular organic surface films alter water surface tension and thus disrupt behaviour and normal development of immature mosquitoes. The role of surface tension agents (surfactants) and their effects on immature stages of mosquitoes has been studied by several workers. White *et al.* (1978) reported how changes in surface tension of water by the addition of surfactants and monomolecular organic films produce substantial mortality in mosquito larvae and pupae or results in the drowning of emerging adults in treated water.

Organochlorines like DDT (**28**) and dieldrin (**42**), organophospates such as temephos (**43**), malathion (**30**), parathion (**44**), fenitrothion (**31**), chlorpyrifos (**45**) and pirimiphos methyl (**46**); and organocarbamates such as propoxur (**47**) have been used to control insect larvae. (WHO, 1997b). Resistance of anopheline mosquitoes to organochlorines (DDT (**28**) and dieldrin (**42**)) has been reported (WHO, 1997b). Organochlorines are persistent in the soil, plant and animal tissues (Schoof *et al.*, 1961). Consequently, organophosphates and organocarbamates have been recommended for many situations including household use because of low toxicity to non-target organisms and low effective dosage (Thevasagayam *et al.*, 1979). Due to biodegradability, low soil persistence and low toxicity to fish, phosphates and carbamates have also found use as mosquito larvicides. However, resistance to phosphates and carbamates have been reported (WHO, 1997b).



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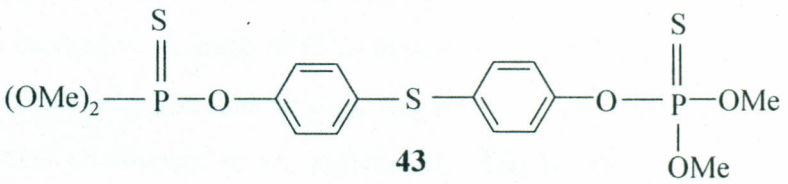

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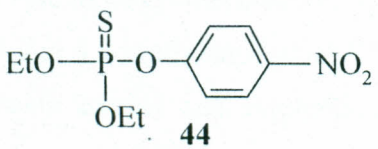

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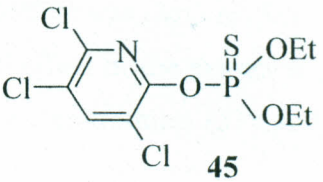

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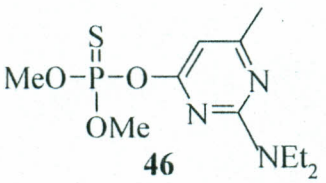

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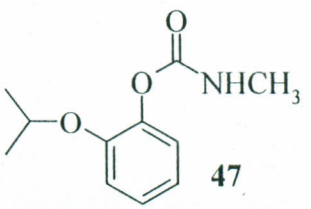

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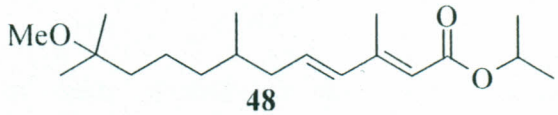
1.4.4.2.4 Botanical larvicides

In response to strong selective pressures of herbivorous insects, plants produce toxic secondary metabolites that often affect insect nerve function and behaviour (Sharma *et al.*, 1998). Studies on the natural plant products for larvicidal activity suggest potential sources of compounds for mosquito control (Sukamar *et al.*, 1991). In view of the fact that mosquitoes develop genetic resistance to synthetic larvicides (Wattal *et al.*, 1981)

and even bio-pesticides such as *B. sphaericus* (Tabashnik, 1994), the application of easily degradable botanicals for larval control is recommended (Alkofahi *et al.*, 1989). Recently, studies from North America (Bergeron *et al.*, 1996), Argentina, Bolivia, Brazil and Peru (Ciccia *et al.*, 2000), Trinidad and Tobago (Chariandy *et al.*, 1999), Mali (Diallo *et al.*, 2001), Negev Desert (Sathiyamoorthy *et al.*, 1997) and Africa (Marston *et al.*, 1993) among others revealed numerous examples of plant extracts, representing diverse taxonomic groups, that are active against *Ae. aegypti*. More systematic and directed studies have revealed many active plant extracts, essential oils, and isolated larvicidal phytochemicals (Park *et al.*, 2002).

Plants belonging to Asteraceae, Cladophoraceae, Labiatae, Meliaceae, Oocystaceae and Rutaceae appear to have potential for mosquito control (Sukumar *et al.*, 1991). Methanolic extract of the leaves of *Atlantia monophylla* (Rutaceae) was evaluated for mosquitocidal activity against immature stages of three species, *C. quinquefasciatus, An. stephensi* and *Ae. aegypti* in the laboratory. Larvae of *C. quinquefasciatus* and pupae of *An. stephensi* were more susceptible (LC_{50} 140 and 50 µg/l, respectively) to the extract. IGR activity of the extract was more pronounced in *Ae. aegypti* (EI_{50} 2 µg/l). The extract was safe to larval predators: *G. affinis*, *P. reticulata* and *Diplonychus indicus* (LC_{50} 23.4, 21.3 and 5.7 mg/l) respectively. The larvicidal effect of the extract is comparable to that of neem extract and synthetic chemicals like fenitrothion (**31**) and methoprene (**48**) (Sivagnanam & Kalyanasundaram, 2004).



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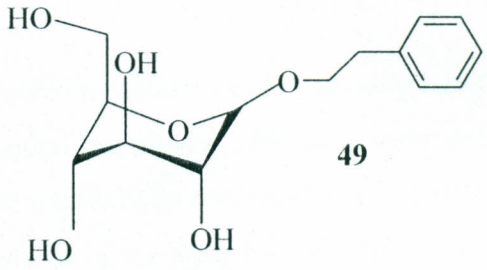
Thagam & Kathiresan (1988a & b) reported that acetone extracts of several species of marine angiosperms and algae posses high mosquito larvicidal activity with lethal concentrations (LD_{50}) ranging from 17.0 to 95.5 ppm. The leaf extract of *Vitex negundo, Nerium oleander* and seed extract of *Syzgium jambolanum* exhibited larvicidal activity against *C. quinquefasciatus* and *An. stephensi* (Pulshpalatha & Muthukrishnan, 1995). Crude extracts containing saponins from fruits pods of *Swartzia madagascariensis* produced higher mortality in *An. gambiae* larvae than *Ae. aegypti* and no effect in *C.*

quinquefasciatus (Minijas & Sarda, 1986). Ethanolic extracts of *Haplophyllum tuberculatum* exhibited high larvicidal activity on the 1st-instar larvae of *C. quinquefasciatus* (Mohsen *et al.*, 1989).

Alcoholic extracts of leaves and stems of *Vanilla fragrans* fractionated with ethyl acetate and aqueous butanol exhibited mosquito larvicidal activity (Sun *et al.*, 2001). Neem oil is a potential mosquito larvicide (Dhar *et al.*, 1996). Petroleum ether extract of thyme plant, *Thymus capitatus*, was found to be toxic to the larvae and adults of *Culex pipiens* (Mansour *et al.*, 2000). Flowers of *L. camara* extracted in methanol and mixed with coconut oil have been reported to exhibit larvicidal activity (Mittal *et al.*, 1995).

Several larvicidal compounds have been isolated from various plants. *O*-phenylethyl-*D*-glucopyranoside (**49**) isolated from the stem bark of *Sida rhombifolia*, was evaluated for larvicidal activity against common filarial vector, *C. quinquefasciatus*, under laboratory conditions. The activity (LC_{50} 36.22, 43.94; 44.92, 58.34; and 60.40, 63.32, 70.72, 82.52 ppm) of the compound against 1st, 2nd, 3rd and 4th instar larvae after 24 and 48 h, respectively, has been reported (Ekramul *et al.*, 2003).



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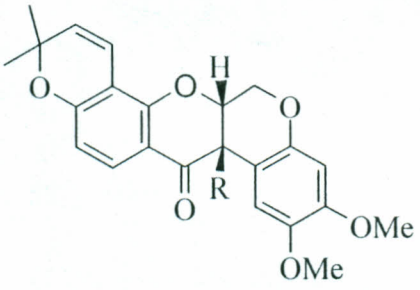
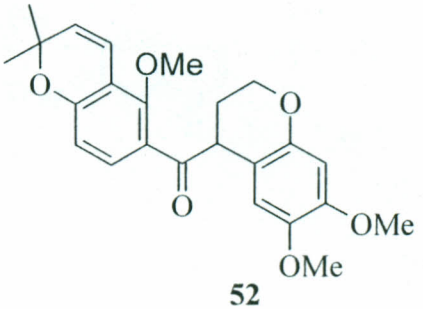
Chloroform extract of seeds of *Millettia dura* Dunn (Leguminosae) showed high larvicidal activity (LC_{50} 3.5 µg/ml after 24 h) against 2nd instar larvae of *Ae. aegypti* (Yenesew *et al.*, 2003). Deguelin (**50**) and tephrosin (**51**), isolated from the extract showed potent larvicidal acivity (LC_{50} 1.6 and 1.4 µg/ml, respectively, after 24 h). Saturation at the B/C ring junction and the presence of the methoxy groups at C-2 and /or C-3 in deguelin (**50**) and tephrosin (**51**) were suggested to be important for the observed larvicidal activity. Acetone extract of the seeds of *Derris trifolia* (Leguminosae) has also been reported to exhibit high toxicity against second instar larvae of the mosquito *C. quinquefasciatus* (LC_{50} 1.35 µg/ml) (Yenesew *et al.*, 2005). From this extract, a novel

isoflavonoid derivative (7a-*O*-methyldeguelol) (**52**) together with three known compounds [(-)-rotenone (**53**), (-)-deguelin (**50**) and (-)-α-toxicarol (**54**)] were isolated (Yenesew *et al.*, 2005).



50 R = H
51 R = OH



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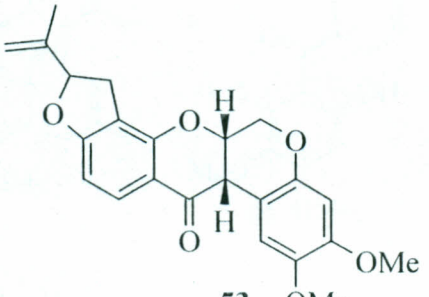

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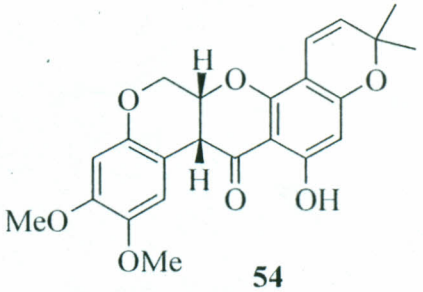
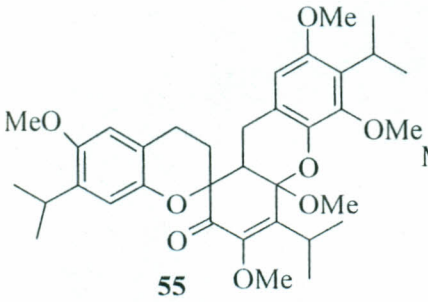


54

From the root bark of *Uvaria scheffleri*, a mild mosquito larvicide (±)-schefflone (**55**) and the anti-protozoal compound (espintanol) (**56**) have been isolated (Nkunya *et al.*, 2004). *Uvaria scheffleri* is used traditionally for treating fevers (Kokwaro, 1993), and previous phytochemical investigations of the stem bark yielded 3-farnesylindole (**57**) as the anti-malarial constituent and the condensed chalcones (±)-schefflerion (**58**) and (±)-isoschefflerin (**59**) (Nkunya *et al.*, 1990). From *Cryptotaenia canadensis*, an umbellifer frequently encountered in moist woodlands in North America, two acetylenic compounds: falcarinol (**60**) and falcarindiol (**61**) (LC_{50} 3.5 and 6.5 ppm, respectively, against *C. pipiens*), were isolated (Eckenbach *et al.*, 1999).

Isobutyl amides like fagaramide (**62**) and pellitorine (**63**) (LC_{50} 14.92 and 3.69 μg/ml, respectively have been reported to display good mosquito larvicidal activity against *An.*

gambiae (Weenen *et al.*, 1990). Sesamine (**64**) and lupeol (**65**) have also been reported to exhibit some larvicidal activity against *An. gambiae* (Okinyo, 2002).



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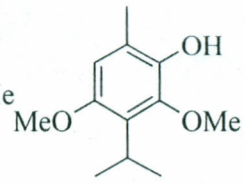

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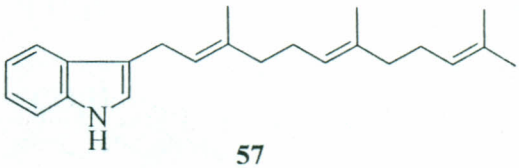

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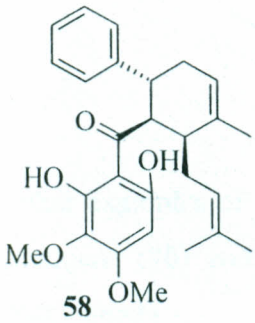

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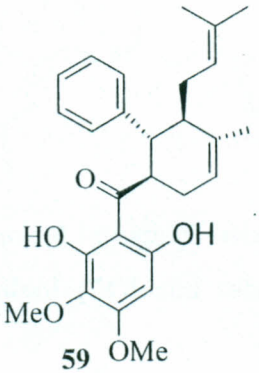

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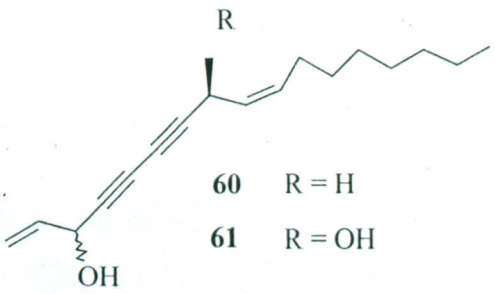
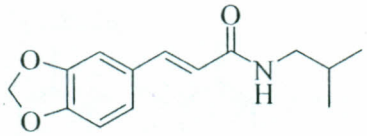


60 R = H

61 R = OH



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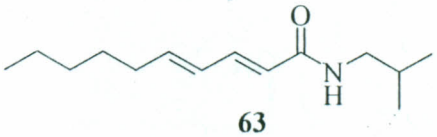

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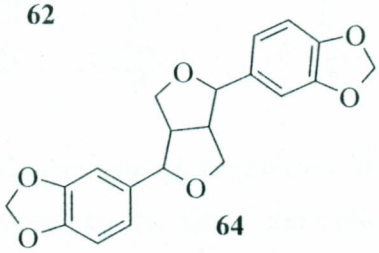

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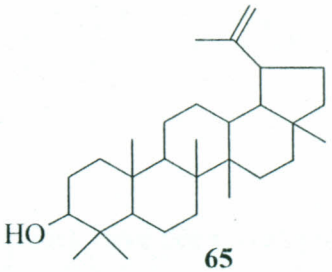

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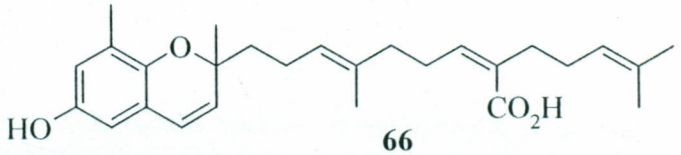
Limonoids from the Meliaceae have anti-insect activity and low toxicity. Gedunin (**25**), isolated from *A. indica* has good larvicidal properties against (Cespedes *et al.*, 2000). Methanol extract from the aerial parts of *Roldana barba-johannis* (Asteraceae) afforded sargachromenol (**66**), sargahyroquinoic acid (**67**), and sargaquinolic acid (**68**). These

compounds show insecticidal and IGR activities against *Spodoptera frugiperda* (LC_{50} 20-35 ppm) (Cespedes *et al.*, 2004).



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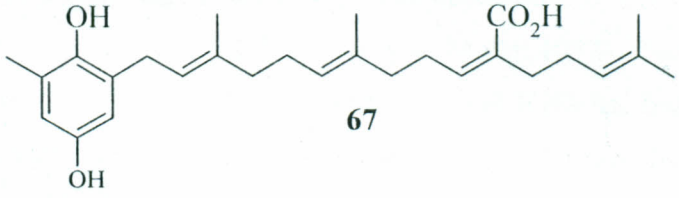

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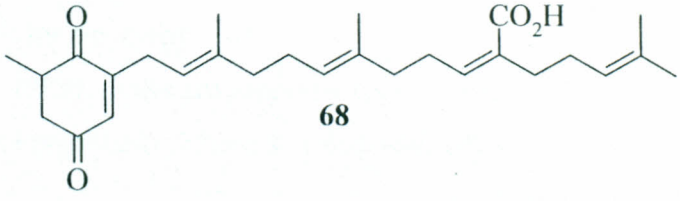

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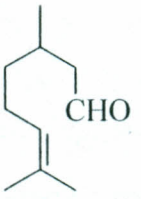
Other examples of botanical larvicides used include: citronellal (**69**), citrus oil extracts limonene (**70**) and linalool (**71**)] and sabadilla (**72**) (http://hgic.clemson.edu) among many others.



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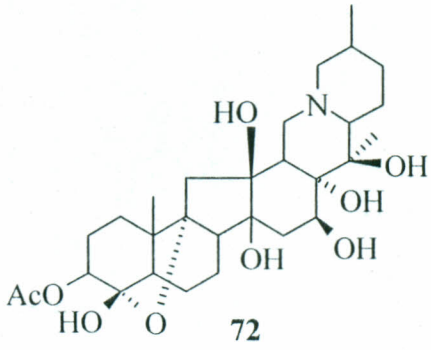

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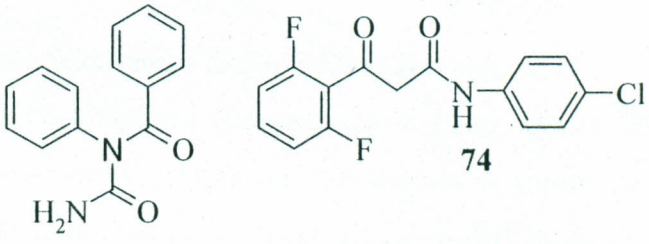
1.4.4.2.5 Insect growth regulators (IGRs)

The problems posed by the use of conventional larvicides have been overcome by IGRs. Compounds containing growth-regulating properties are found in terpenoids, benzamides, carbamates, triazines, benzylurea and several other classes of organic compounds (Mulla, 1995; Quraishi, 1977; Itoh, 1981; Worthing & Walker, 1987; Mulla *et al.*, 1991). Synthetic IGRs including juvenoids and chitin synthesis inhibitors have shown promising results for the control of insects of public health importance (Williams, 1967). The efficacy of many IGRs has been studied against various mosquito species (Tyagi *et al.*,

1987; Amalraj *et al.*, 1988). Laboratory and field investigations with methroprene (**48**) have shown high efficacy in controlling the immature stages of the dipterans of medical importance (Das *et al.*, 1981).

Benzoyl phenylurea (**73**) causes various morphogenetic abnormalities in larvae, pupae and adult mosquitoes (Amalraj *et al.*, 1988). The application of diflubenzuron (**74**) has shown high larval mortality in blackflies (Lacey & Mulla, 1977). Successful control of DDT-resistant strains of mosquitoes by the application of IGRs has been demonstrated in the laboratory (Schaefer *et al.*, 1975). Methoprene (**48**) and diflubenzuron (**74**) have low mammalian toxicity (WHO, 1984). Fenoxycarb (**75**), a non-neurotoxic carbamate, exhibits IGR activity on many insects (Dorn *et al.*, 1993; Grenier & Grenier 1993; Retnakaran *et al.*, 1985). IGRs are generally species specific, biodegradable, non-toxic to man and non-target organisms (Miura & Takahashi, 1973).



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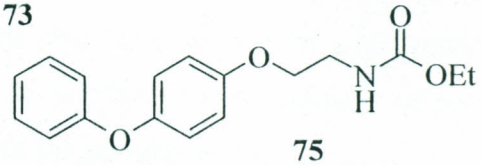

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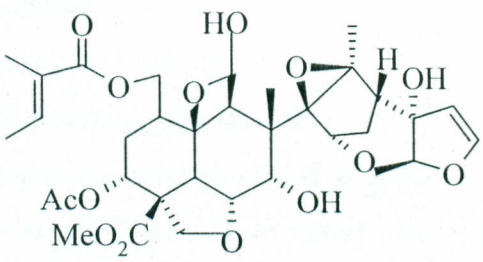
Previous studies on six plant extracts (*Acorus calamus, Ageratum conyzoides, Annona squamosa, Bambusa arundanasia, Madhuca longifolia* and *Citrus medica*) showed remarkable IGR activity, at 5 ppm, against larvae of major malaria vector species (Sujatha *et al.*, 1988). Plant-derived IGR substances are known to be safe to man and environment, and have a potential in intergrated pest management programmes (Williams, 1967).

So far, azadirachtin (**76**) is the most potent tetranotriterpenoid IGR isolated from neem tree (*A. indica*) that is being commercialised and marketed for managing insect pests (Schmutterer, 1988; Mordue & Blackwell, 1993). It was isolated from the seeds of *A.*

indica (Butterworth & Morgan, 1968) and the structural determination completed 17 years later (Broughton *et al.*, 1995; Kraus *et al.*, 1985). 23-*O*-methylnimocinolide [7α-acetoxy-6α-hydroxy-23ξ-methoxy-3-oxo-24, 25, 26, 27-tetranorapotirucalla (apoeupha)-1, 14, 20-trieno-21, 23-lactone] (**77**) and 7-*O*-acetyl-23-*O*-methyl-7α-*O*-senecioylnimotrieno-21, 23-lactone (**78**) isolated from the methanolic extract of the fresh leaves of *A. indica* (neem) show IGR effect on *Ae. aegypti* (LC_{50} 53 and 2.14 ppm, respectively). The senecioyloxy substituent at C-7 in **78** results in a significant increase of activity (Bina *et al.*, 1999).



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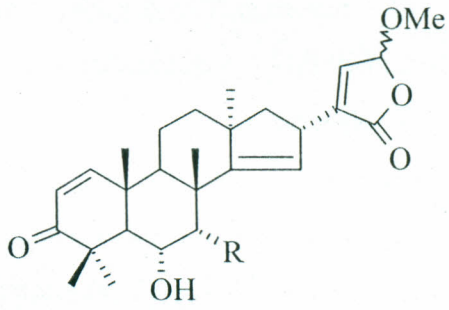
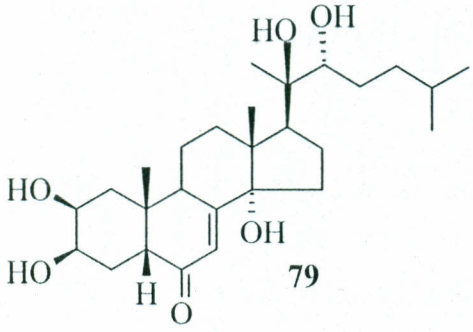


77 R = OAc
78 R=$O_2CCHC(CH_3)_2$

Ecdysteroids represent a widespread family of steroids found in both animal (zoosteroids) and plant (phytosteroids) kingdoms (Bergamasco & Horn, 1980). They were initially defined as moulting hormones. Howerver, this definition appears to be too restricted, since they are present at all stages of insect development: in newly laid eggs, during embryonic and post-embryonic development, and in adult insects (Whiting & Dinan, 1988). The fortuitous discovery in 1966 of large amounts of ponasteroid A (**79**) in the bark of *Podocarpus nakaii* was the starting point for highly fruitful research which has led to the discovery of more than 100 ecdysteroids in plants (Camps, 1992). In plants, ecdysteroids are widely distributed as secondary metabolites, and often reach concentrations of several orders of magnitude greater than in insects (Bergamasco & Horn, 1980). For example, flowers of *Serratula inermis* contain up to 2% of 20-hydroxyecdysone (**80**) (Yatsyuk & Segal, 1970). The roots of the Chines herb, *Cyanotis arachnoidae*, afforded 2.9% of 20-hydroxyecdysone (**80**), while the mature stem of *Disploclisia glaucesens* yieled 3.2% of 20-hydroxyecdysone (**80**). Ecdysteroid concentrations vary with the plant part, climatic conditions, the season and habitat of the

plant (Chou & Lu, 1980). The presence of ecdysteroids in plants represents a clear case of defense mechanism against insects using chemical mimickry.

From the results of phytochemical screening (Bergamasco & Horn, 1980), ecdysteroids have been found in vascular plants: 27 families of *Pteridophyta*, 10 families of the *Gymnospermae*, and 74 families of the *Angiospermae*. The probability of finding ecdysteroids in ferns is high. The probability of finding active plant species in *Angiospermae* is typically much lower than in *Pteridophyta* or *Gymnospermae*. Among the Angiosperms: Verbenaceae, Labiatae (*Ajuga*), Asteraceae (*Serratuea*), Amaranthaceae (*Achyranthes*, *Cyathula*), Ranuculaceae (*Helleborus*) and Caryophyllaceae are more likely sources than others. Sileneoside H (**81**), a phytoecdysteroidglycoside, was isolated from the roots of *Silene brahuica* (Caryophyllaceae) and identified as 22-*O*-α-*D*-galactosylintegristerone A 25-acetate (**81**) (Sadikov *et al.*, 2000). Two minor plant ecdysteroids, 22-*epi*-20-hydoxyecdysone (**82**) and gerardiasterone (**83**), were isolated from *Serratula tinctoria* L (Compositae) (Bathori *et al.*, 1998). Punisterone [(20R, 24S)-25-deoxy-11α, 20, 24-trihydroxyecdysterone] (**84**) has been isolated from the seeds of *Blandfordia punicea* (Satyajit *et al.*, 1996) while cyasterone, (**85**), 25-*epi*-cyasterone (**86**) and 25-*epi*-28-*epi*-cyasterone (**87**) were recently isolated from *Cyathula officinalis* (Okuzumu *et al.*, 2005). From *Ajuga* (Lamiaceae), 20-hydroxyecdysone (**80**), cyasterone (**85**) and ajugasterone C (**88**) were isolated (Santos *et al.*, 2001).



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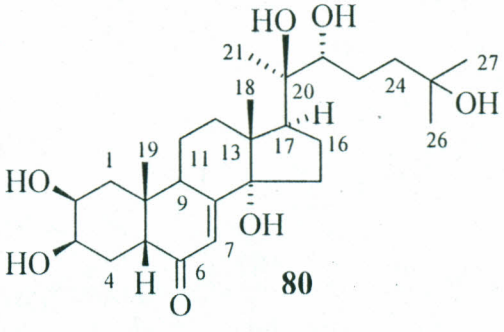

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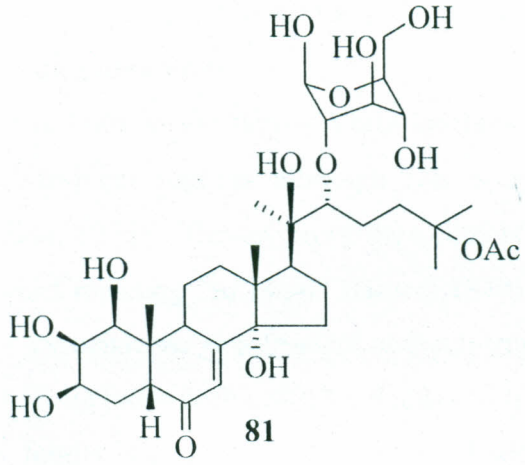

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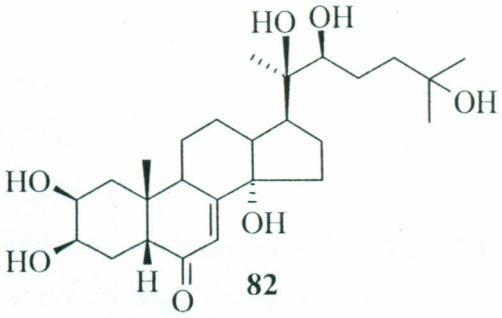

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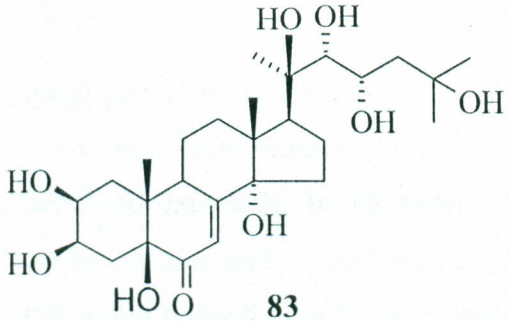

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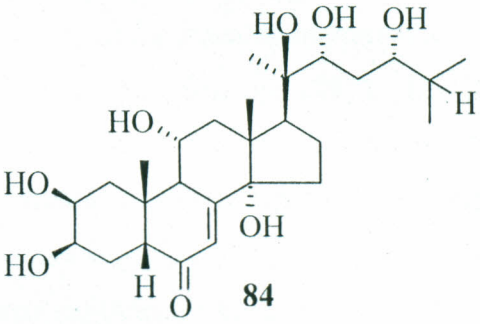

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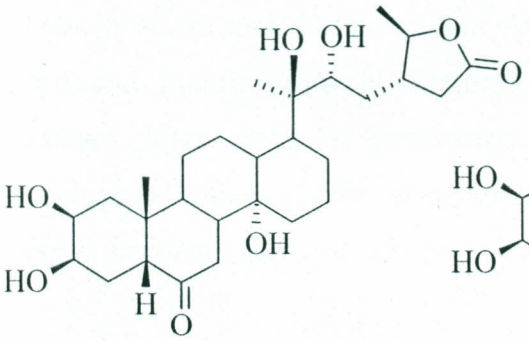

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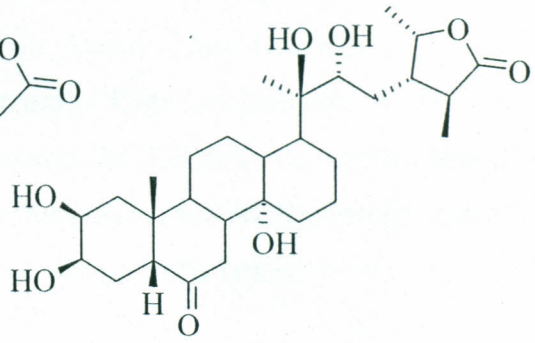

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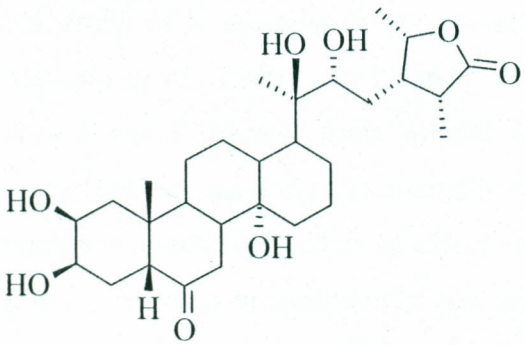

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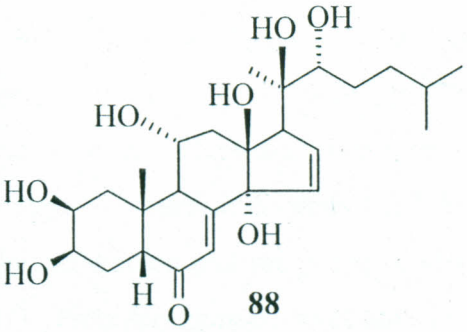

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In this study, larvicidal and insect growth regulatory (IGR) compounds from *Vitex schilliebenii* and *Vitex payos* (Verbenaceae) against *An. gambiae* were investigated.

CHAPTER 2: LITERATURE REVIEW

2.1 The genus *Vitex*

The genus *Vitex* in the family Verbenaceae consists of shrubs found mainly in tropical and sub-tropical regions although few species may be found in temperate zones (McMillan, 1976). The origin of the genus is obscure (Eagles, 1986) or may be from a Latin word meaning "to blind" (Bruce, 1970) referring to the flexible twigs (Mathews, 1962). Verbenaceae is a tropical and sub-tropical family of about 75 genera and 3000 species. It includes herbs, shrubs, lianas and trees (Poole & Nancy, 1994). Many species of this family are used for a number of medicinal, timber and food purposes. For instance, timber from *Citharexylum* species is used to make musical instruments while *Vitex agnus castus* is renowned for its medicinal properties (Salmon, 1991). The local members of this genus include: *Vitex doniana* (black plum, mfudu in Kiswahili, mufuku in Gikuyu and mutahuru in Ekegusii); *Vitex mombassae* (mfudumaji in Kiswahili, mfududu in Giriama and mfudukoma in Digo); *Vitex payos* (chocolate berry, mfufu in Kiswahili and mfudu in Digo); *Vitex ambonienesis* (mufudu in Giriama); *Vitex fischeri* (mohutu in Nandi and mufutumwe in Ateso); *Vitex keniensis* (Meru Oak, muhuru in Gikuyu and muuru moru in Kimeru); *Vitex schiliebenii; Vitex madiensis*; *Vitex ferruginea*; *Vitex strickeri* (mukichano in Giriama, mvumbain in Kiswahili and Mukakinga in Gikuyu), *Vitex zanzibariensi* (vi fuu in Kiswahili) and *Vitex tangensis* (mgegi in Kiswahili and mfududu/mufudumaji in Giriama).

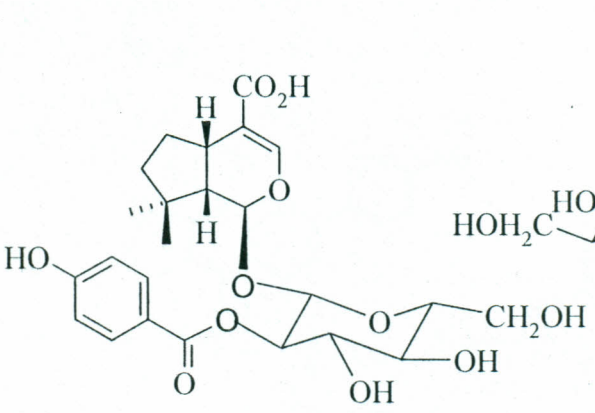
2.2 Traditional uses of *Vitex*

In Kenya, fruits of *V. doniana, V. ferruginea, V. mombassae* and *V. payos* are used as food (Maundo *et al.*, 1999). In Uganda, *V. doniana, V. madiensis, V. ferruginea, V. ambonienesis* and *V. fischeri* fruits are also used as food (Katende *et al.*, 1995). *Vitex mombassae* roots are used to treat infertility and to control vomiting. The roots of *Vitex buchananii* have been reported as an effective cure for venereal diseases while the roots of *V. doniana* are used in treatment of backaches in women and the young tender leaves are a remedy for eye infections (Kokwaro, 1993). *Vitex ferruginea* leaves have been used in treatment of sore throat. The boiled leaves of *V. strickeri* are used to reduce inflammation in cases of snake bite and the roots for the treatment of influenza (Kokwaro, 1993).

In Uganda and Ethiopia, roots, leaves and bark of *V. doniana* are used as medicine (Katende *et al.*, 1995; Azene *et al.*, 1993). The stem and root of *V. glabrata* have been used as astringents while the stem bark is also been used as an anti-helmintic and for gastro-intestinal disorders (Werawattanametin *et al.*, 1986). *Vitex negundo* is well known for its medicinal value (Chawla *et al.*, 1992). The fruits of *V. agnus castus* have been used traditionally against premenstrual disorder and the symptoms of menopause because of its hormone-like effect (Lucks *et al.*, 2002). In Anatolia folk medicine, the plant is used as diuretic, digestive, and anti-fungal and also against anxiety, early birth and stomach ache (Honda *et al.*, 1996). *Vitex rotundifolia* is used as folk medicine for headache, colds, migraine and eye pain (But *et al.*, 1996). In Japan, seeds of the plant are used as remedy for cold and raw material for Chinese traditional medicine. *Vitex mollis* is a remedy for dysentery, as well as an analgesic and anti-inflammatory medicine. Other folk uses include the treatment of scorpion stings, diarrhoea and stomachache (Argueta *et al.*, 1994).

2.3 Phytochemistry of *Vitex* species

The ethanolic extract of *V. negundo* leaves yielded 2′-*p*-hydroxybenzoylmussaenosidic acid (**89**) (Sehgal *et al.*, 1982) and 6′-*p*-hydroxybenzoylmussaenosidic acid (**90**) (Sehgal *et al.*, 1983) while 3-formyl-4, 5-dimethyl-8-oxo-5H-6, 7-dihydronaphtho-(2, 3-b)-furan (**91**) and nishindaside (**92**) (Dutta *et al.*, 1983) have been isolated from the roots (Surendra *et al.*, 1983).



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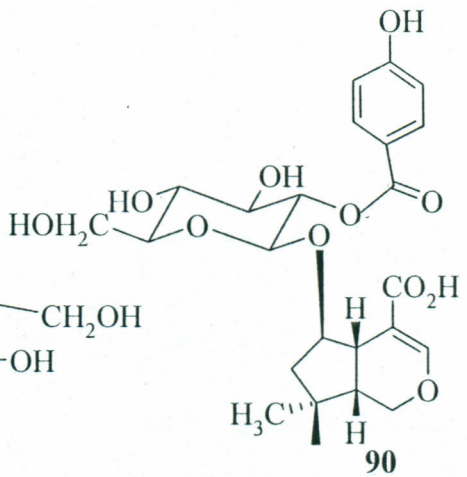

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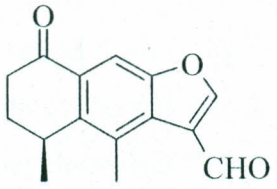

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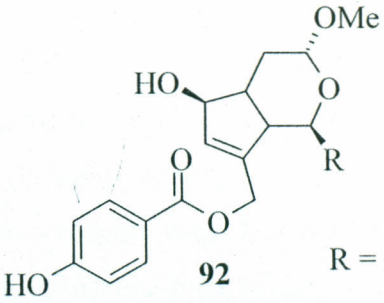
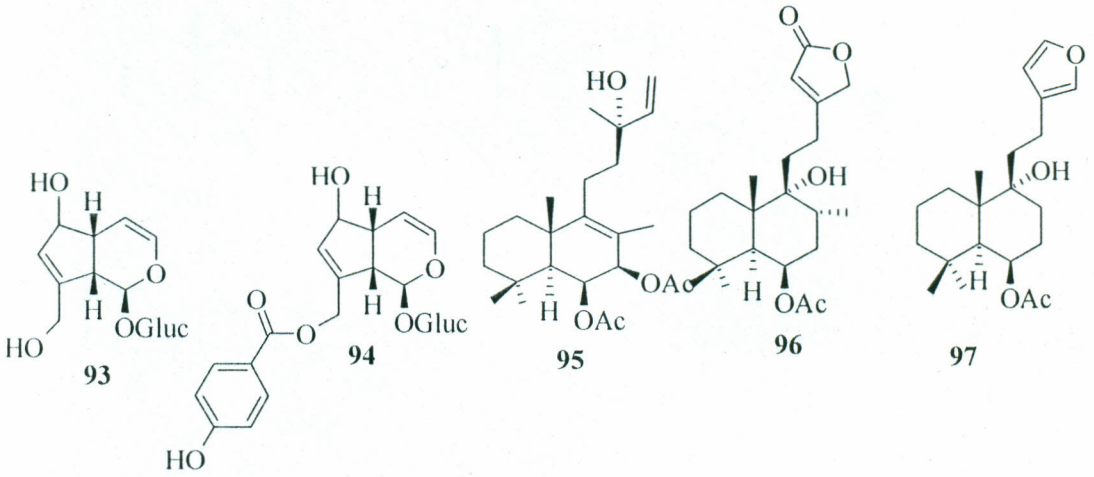


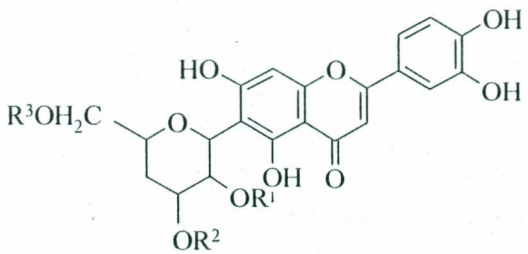
92

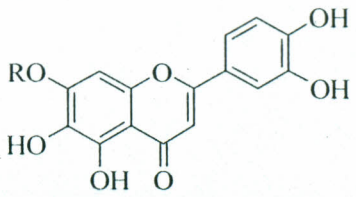
R = *O*-Beta-Gluc

Vitex agnus castus contains iridoids, aucubin (**93**) and agnuside (**94**) (Gomaa *et al.*, 1978) in the leaves, flavonoids (Hirobe *et al.*, 1997), essential oils (Sorensen & Katsiotis, 2000), diterpenoids (Li *et al.*, 2002) and ketosteroids (Saden-Krehula *et al.*, 1990). 6β, 7β–Diacetoxy-13-hydroxy-8λ, 14-diene (**95**), vitexilactone (**96**) and rotundifuran (**97**) have been isolated from the fruits (Eva *et al.*, 1999). Luteolin 6-*C*-(4-methyl-6-*O*-*trans*-caffeoylyglucoside) (**98**), luteolin 6-*C*-6-*O*-*trans*-caffeoylglucoside (**99**), luteolin 6-*C*-2-*O*-*trans*-caffeoylglucoside (**100**) and luteolin 7-*O*-(6-*p*-benzoylglucoside) (**101**), with potent anti-tumor properties have been isolated from the root bark (Hirobe *et al.*, 1997).



93 **94** **95** **96** **97**





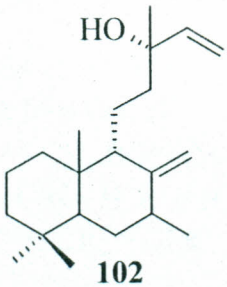
98 R^1 = H, R^2 = CH_3, R^3 = *trans* caffeoyl

99 R^1 = R^2 = H, R^3 = *trans* caffeoyl

100 R^1 = *trans* caffeoyl, R^2 = R^3 = H

101 R = *p*-benzoylglucoside

Some diterpenoids and sesquiterpenoids, including: vitexfolin A (**102**), vitexfolin B (**103**), vitexfolin C (**107**), vitexfolin D (**105**), vitexfolin E (**106**) and vitexfolin D (**104**), were obtained from fruits of *V. rotundifolia* (Asaka *et al.*, 1999; Ono *et al.*, 2002). 1-(3, 4-Dimethoxyphenyl)-7-hydroxy-8-methoxynaphthalene-3-carbaldehyde (**108**), 1-(3, 4-dimethoxyphenyl)-2, 7-dihydroxy-8-methoxynaphthalene-3-carbaldehyde (**109**) and 2, 7-dihydroxy-1, 9, 10-trimethoxy-7H-benzo[c]fluorene-6-carbaldehyde (**110**) were isolated from the roots of *V. rotundifolia* (Kazuyoshi *et al.*, 1999).



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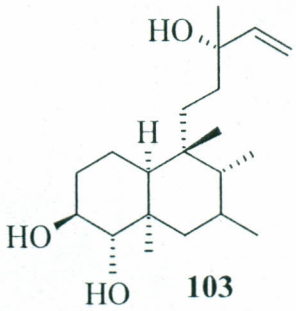

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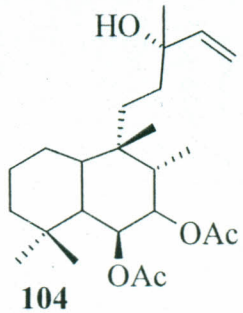

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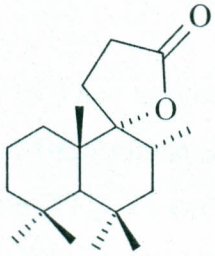

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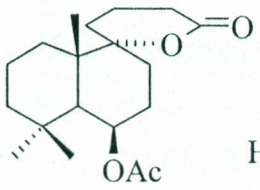

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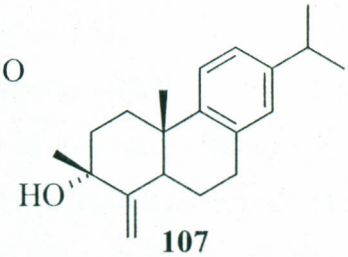

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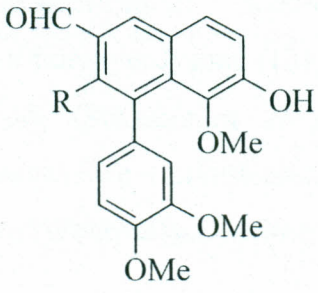
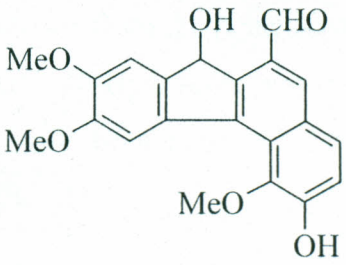


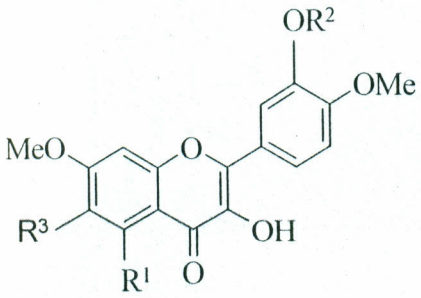
108 R = H
109 R = OH



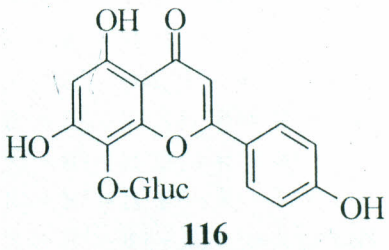
110

Compounds isolated from *Vitex trifolia* leaf and fruit extracts include casticin (**111**), artemetin (**112**), 5-methylartemetin (**113**), luteolin (**114**) (Nair *et al.*, 1975), isoorientin (**115**) and vitexin (**116**) (Ramesh *et al.*, 1986). Friedelin (**117**) and β-sitosterol (**118**) have

also been isolated from *V. trifolia* (Zeng *et al.*, 1996). Essential oils from the leaves have also been reported (Pan *et al.*, 1989).



111 $R^1 = R^2 = H$, $R^3 = OMe$
112 $R^1 = H$, $R^2 = CH_3$, $R^3 = OMe$
113 $R^1 = R^2 = CH_3$, $R^3 = OMe$
114 $R^1 = OH$, $R^2 = H$, $R^3 = H$
115 $R^1 = R^2 = H$, $R^3 = Gluc$



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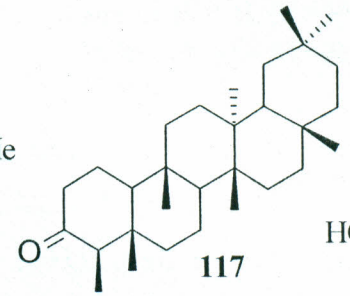

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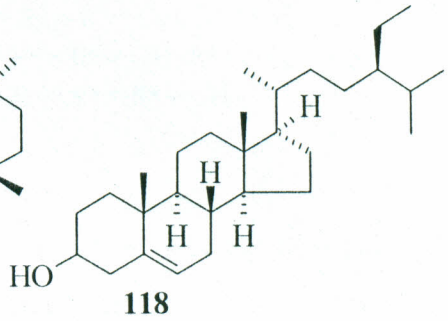
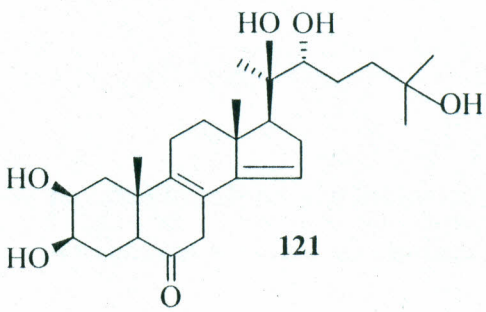
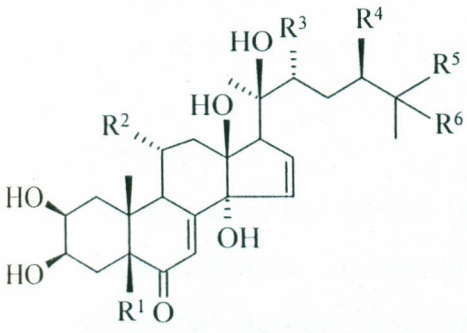


118

A number of *Vitex* species have been investigated for ecdysteroids (Suksamrarn *et al.*, 2000). 24-*Epi*-pinnatasterone (**119**), scabrasterone (**120**), calonysterone (**121**), pterosterone (**122**), 24-*epi*-makisterone A (**123**), 20-hydroxyecdysone (**80**), polypodine B (**124**), ajugasterone C (**88**), pinnasterone (**125**), 11-α-hydroxyecdysone (**126**), 24-*epi*-abutasterone (**127**), 20, 26-dihydroxyecdysone (**128**) and turkesterone (**129**) were isolated from the stem bark of *V. scabra*. (24*R*)-11α, 20, 24-Trihydroxyecdysone (**130**) and 11α, 20, 26-trihdroxyecdysone (**131**) were isolated from the polar fraction of *Vitex canescens* root bark (Suksamrarn *et al.*, 2002). 20-Hydroxyecdysone (**80**) and 11α, 20-dihydroecdysone (turkesterone) (**129**), have been isolated from the stem bark of *V. glabrata* (Werawattanametin *et al.*, 1986).



121

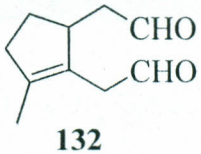


119 $R^1 = R^2 = R^3 = H$, $R^4 = R^6 = OH$, $R^5 = CH_3$
120 $R^1 = R^3 = R^4 = H$, $R^2 = R^6 = OH$, $R^5 = CH_3$
122 $R^1 = R^6 = H$, $R^2 = R^3 = R^4 = OH$, $R^5 = CH_3$
123 $R^1 = R^2 = H$, $R^3 = R^4 = R^6 = OH$, $R^5 = CH_3$
124 $R^1 = R^6 = OH$, $R^2 = R^3 = R^4 = H$, $R^5 = CH_3$
125 $R^1 = R^2 = R^3 = H$, $R^4 = R^6 = OH$, $R^5 = CH_3$
126 $R^1 = R^2 = H$, $R^3 = R^4 = R^6 = OH$, $R^5 = CH_3$
127 $R^1 = R^2 = H$, $R^3 = R^4 = R^6 = OH$, $R^5 = CH_3$
128 $R^1 = R^4 = R^6 = H$, $R^2 = R^3 = OH$, $R^5 = CH_3$
129 $R^1 = R^4 = H$, $R^2 = R^3 = R^6 = OH$, $R^5 = CH_3$
130 $R^1 = R^2 = H$, $R^3 = R^4 = R^6 = OH$, $R^5 = CH_3$
131 $R^1 = H$, $R^2 = R^3 = R^4 = R^5 = R^6 = OH$

2.4 Pharmacology of *Vitex* species

Vitexin (**116**), from *Vitex lucens* has been reported to be a potent inhibitor of thyroid peroxidase (Harbone & Baxter, 1993). Anti -malarial, anti-microbial and anti-fungal properties have been reported from *Vitex gaumeri*, *V. agnus castus* and *V. negundo*, respectively. *Vitex negundo* is also used as an anti-inflammatory agent, and in the treatment of colds and coughs (Damayanti *et al.*, 1996).

Besides their popular use as traditional medicine in many countries, plants in the genus *Vitex* exhibit insecticidal activities. *Vitex negundo* extracts exhibit larvicidal activity against *C. quinquefasciatus* and *An. stephensi* (Pulspalatha & Muthukrishnan, 1995), and act as a feeding deterrent to *Ae. aegypti* larvae (Hebbalkar *et al.*, 1992). *Vitex rotundifolia* also shows feeding deterrent properties towards *Ae. aegytpi* larvae while insecticidal substances were reported to be present in the leaf tissue (Watanabe *et al.*, 1995). Rotundial (**132**) isolated from *V. rotundifolia* exhibited insect repellent properties. *Vitex trifolia* has insecticidal properties and anti-feedant activity in the seeds (Hosozawa *et al.*, 1974).



132

Compounds **119** and **120** were reported to exhibit low biological activity (Suksamrarn *et al.*, 2002). *Vitex payos* and *Vitex schiliebenii* have anti-mosquito properties (Musyoka,

unpublished). The genus *Vitex* is therefore a good source of IGR compounds. No phytochemical investigations have been reported on *V. payos* and *V. schiliebenii*.

2.5 *Vitex payos*

Vitex payos (chocolate berry) (Plate 2) is a deciduous tree that may grow up to 8 m high with a low rounded crown. Its bark is grey-brown, deeply fissured. Younger stems have a rusty, woolly bark and the leaves have 5 leaflets. In Kenya, it grows in Kitui, Embu, Machakos, Kilifi and Kwale in low hot and semi-arid lands at 0-1600 m above sea level (Maundu *et al.*, 1999). A decoction of the root has been reported as a remedy for stomach problems, and the pounded bark is administered to treat threadworm and skin problems while boiled leaves are taken by patients to improve appetite for food (Christopher *et al.*, 2002).

Plate 2: *Vitex payos* (chocolate berry)

2.6 *Vitex schiliebenii*

Vitex schiliebenii (Plate 3) is a branched shrub with multiple stems and low height (4-8 m). The leaves have 3-leaflets with a smooth surface, about 10-12 cm long. In Kenya, it grows in the coastal region at Watamu which is a low and semi-arid land.

Plate 3: *Vitex schiliebenii*

2.7 Statement of the Problem

The IGR principles of *Vitex payos* and *Vitex schiliebenii* that may be useful for the control of mosquito larvae have not been identified.

2.8 Hypothesis

Vitex schiliebenii and *V. payos* are rich in anti-larval compounds that are stable enough to be extracted, isolated, assayed and identified.

2.9 Objectives

The general objective was to investigate the larvicidal and IGR potential of *V. schiliebenii* and *V. payos* against *An. gambiae*. The specific objectives were:

(a) To extract and carry out anti-larval assay of the crude extracts;

(b) To fractionate crude extracts, isolate and assay the anti-larval compounds;

(c) To identify the isolated anti-larval compounds.

2.10 Justification for the study

Mosquitoes are responsible for more human diseases than any other group of arthropods (Cepleanu, 1993). Malaria has been a real concern for centuries and it threatens the well being of more than 40 % of the world's population (Batcher *et al.*, 2000). It kills between 1.5 and 2.7 million people each year while 300-500 million others fall ill with malaria

(WHO, 1998). Futhermore, the parasites have become increasingly resistant to most commonly used and affordable anti-malarial drugs. An effective malaria vaccine would constitute a powerful control tool but none is currently available. Vector control based on chemical insecticides is a major technique to combat malaria in many parts of the world. However, the emergence and spread of insecticide resistance along with the high cost of new types of chemical insecticides and environmental pollution have necessitated the search for affordable, safe, biodegradable and effective alternatives. In the last 30 years of research, only two IGRs have been registered for use in mosquito control, a situation indicating the need for further evaluation and development of others. The findings of this study will therefore contribute towards the development of more natural products-based IGRs that may be used as commercial products for larval control.

CHAPTER 3: BIOASSAYRESULTS AND DISCUSSIONS

Bioassay of the crude extracts from both plants was carried out under laboratory conditions. Cummulative larval mortality and lethal concentrations (LC) of the extracts were determined. The IGR activity of the isolated compounds was also determined.

3.1 Bioassay of crude extracts of *Vitex payos*

The stem bark of *Vitex payos* and *V. schiliebenii* gave semi solid solvent extracts that were subjected to larvicidal assays using 3rd-instar *An. gambiae* larvae (Table 1). The average yields ranged between 0.08-0.89 % (w/w). The yields of *n*-hexane, chloroform and ethyl acetate extracts were fairly low as compared to that of methanol.

Table 1: Yield (%) of the plant solvent extracts

	Extract yield (%)			
Plant	C_6H_{14}	$CHCl_3$	EtOAc	MeOH
V. payos	0.08	0.33	0.48	0.89
V. schiliebenii	0.09	0.18	0.40	0.75

The larvicidal data for *Vitex payos* is presented in table 2.

Table 2: Cummulative larval mortality for *n*-hexane extract of *Vitex payos*

	Cummulative Mean mortality (%± SE)							
Conc. (ppm)	24 h	48 h	72 h	96 h	120 h	144 h	168 h	192 h
25	85.0 ± 2.9^{a}	85.0 ± 2.9^{a}	85.0 ± 2.9^{a}	85.0 ± 2.9^{a}	85.0 ± 2.9^{a}	90.0 ± 2.9^{a}	90.0 ± 2.9^{a}	90.0 ± 2.9^{a}
12.5	1.7 ± 1.7^{fg}	25.0 ± 2.9^{cg}	30.0 ± 0^{cf}	78.3 ± 1.7^{a}	83.3 ± 1.7^{a}	83.3 ± 1.7^{a}	85.0 ± 2.9^{a}	85.0 ± 2.9^{a}
6.25	-	-	20.0 ± 2.9^{cg}	46.7 ± 1.7^{bc}	66.6 ± 1.7^{ab}	75.0 ± 2.9^{ab}	75.0 ± 2.9^{ab}	75.0 ± 2.9^{ab}
2.5	-	-	15.0 ± 5.8^{dg}	26.7 ± 1.7^{cg}	33.4 ± 3.3^{ce}	36.3 ± 1.7^{cd}	36.3 ± 1.7^{cd}	36.3 ± 1.7^{cd}
1.25	-	-	-	5.0 ± 2.9^{dg}	11.7 ± 3.3^{dg}	13.3 ± 6.0^{dg}	18.3 ± 6.7^{cg}	21.6 ± 1.7^{cd}

Means with the same letter are not significantly different

The *n*-hexane crude extract produced substantial mortality of 85.0 % at the highest concentration (25 ppm) 24 h after treatment with a maximum of 90.0 % after 144 h. At the lowest concentration (1.25 ppm), the extract was relatively less toxic to the larvae, with no mortality in the first 72 h. However, there was observed increase in cumulative mortality from 5.0 to 11.7 % at this concentration between 96 and 120 h. The highest mortality was 21.6 %, 192 h after treatment of the larvae. The cumulative mortality at 12.5 ppm increased from 1.7 to 25.0 % after 24 h reaching a maximum of 85.0 % after 168 h. This revealed the slow activity of the extract at the lower concentrations.

Cumulative mortalities of 90.0 and 85.0 % for the concentratioins 25 and 12.5 ppm respectively, were not significantly different (P = 0.05). At concentrations 2.5 and 1.25 ppm, cumulative mortalities of 36.3 and 21.6 %, respectively, were not also significantly different (P =0.05). At the highest and lowest concentrations, the cumulative mortalities were significantly different at (P = 0.05). The rest of the larvae pupated.

The larvicidal data of *V. payos* chloroform extract against *An. gambiae* 3rd-instar larvae is summarized in table 3.

Table 3: Cummulative larval mortality for chloroform extract of *Vitex payos*

	Cumulative mean mortality (%± SE)						
Conc. (ppm)	24 h	48 h	72 h	96 h	120 h	144 h	168 h
25	86.7 ± 1.7^{b}	96.7 ± 1.7^{ab}	98.4 ± 1.7^{ab}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}
12.5	-	25.0 ± 2.9^{dg}	30.3 ± 2.9^{de}	33.0 ± 2.9^{d}	48.3 ± 3.3^{c}	53.3 ± 0.0^{c}	55.0 ± 2.9^{c}
6.25	-	13.3 ± 1.7^{gi}	13.3 ± 1.7^{ghi}	16.7 ± 1.7^{eh}	25.0 ± 0.0^{dg}	28.3 ± 3.3^{deg}	28.3 ± 3.3^{dg}
2.5	-	-	3.33 ± 1.7^{ij}	11.7 ± 3.3^{gi}	18.3 ± 3.3^{eh}	25.0 ± 0.0^{dg}	28.3 ± 4.4^{df}
1.25	-	-	3.33 ± 1.7^{ij}	6.7 ± 1.7^{hj}	8.3 ± 1.7^{hj}	15.0 ± 2.9^{gi}	15.0 ± 2.9^{gi}

Means with the same letter are not significantly different

After 24 h application of the plant extract, the percentage mortality at 25 ppm was 86.7 % while no mortality was recorded after 24 h of exposure at 12.5, 6.25, 2.5 and 1.25 ppm. However, there was a general increase in mortality with time. The cummulative mortality increased from 0.0 to 25.0 % after 48 h; 0.0 to 13.3 % at 6.25 ppm and 0.0 to 3.3 % each at 72 h at 12.5, 2.5 and 1.25 ppm, respectively. This revealed the slow activity of the extract at lower concentrations. Significantly different cumulative mean mortalities of 100.0, 55.0 % were observed for the concentrations 25 and 12.5 ppm, respectively after 96 and 168 h. Non significant cumulative mortalities of 28.3, 28.3 and 15.0 % were recorded at 144 and 168 h, at 6.25 2.5 and 1.25 ppm, respectively. These results revealed that the extract was most toxic at the high concentrations. The toxicity increased with concentration and exposure time.

The cumulative larval mortality data for 3rd-instar *An. gambiae* larvae treated with *V. payos* ethyl acetate extract is given in table 4.

Table 4: Cummulative larval mortality data for ethylacetate extract of *V. payos*

Conc. (ppm)	Cumulative mean mortality (%± SE)						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
25	71.7 ± 4.0^{ab}	75.0 ± 4.0^{ab}	80.0 ± 5.0^{ab}	83.3 ± 1.7^{a}	85.0 ± 2.9^{a}	85.0 ± 2.9^{a}	85.0 ± 2.9^{a}
12.5	5.0 ± 2.9^{f}	6.7 ± 1.7^{f}	10.0 ± 2.9^{ef}	26.7 ± 1.7^{ce}	63.3 ± 1.7^{b}	65.0 ± 1.7^{b}	67.7 ± 1.7^{ab}
6.25	3.3 ± 1.7^{f}	5.0 ± 0.0^{f}	5.0 ± 0.0^{f}	10.0 ± 2.9^{ef}	15.7 ± 2.5^{eg}	28.0 ± 1.79^{cd}	33.3 ± 1.7^{c}
2.5	1.7 ± 1.7^{f}	1.7 ± 1.7^{f}	3.3 ± 1.7^{f}	6.7 ± 1.7^{f}	6.7 ± 1.7^{f}	10.7 ± 1.7^{ef}	25.3 ± 2.9^{ce}
1.25	-	-	-	10.00 ± 2.9^{eg}	15.0 ± 2.9^{eg}	15.0 ± 2.9^{eg}	15.0 ± 2.9^{eg}

Means with the same letter are not significantly different

Significant mortality of 71.7 % (P = 0.05) was recorded after 24 h of exposure to the extract at the highest concentration (25 ppm). Non significant mortalities of 5.0, 3.3 and 1.7 % (P = 0.05) were recorded after 24 h at 12.5, 6.25 and 2.5 ppm, respectively. It was observed that mortality increased after 72 h at the low concentrations. Cumulative mortalities of 85.0, 67.7, 33.3, 25.3 and 15.0 % were recorded after 120 and 168 h, at 25, 12.5, 6.25 and 2.50 ppm, respectively. The rest of the larvae pupated.

The cumulative mortality data for 3rd-instar *An. gambiae* larvae treated with *V. payos* methanol extract is shown in table 5.

Table 5: Cummulative larval mortality data for methanol extract of *Vitex payos*

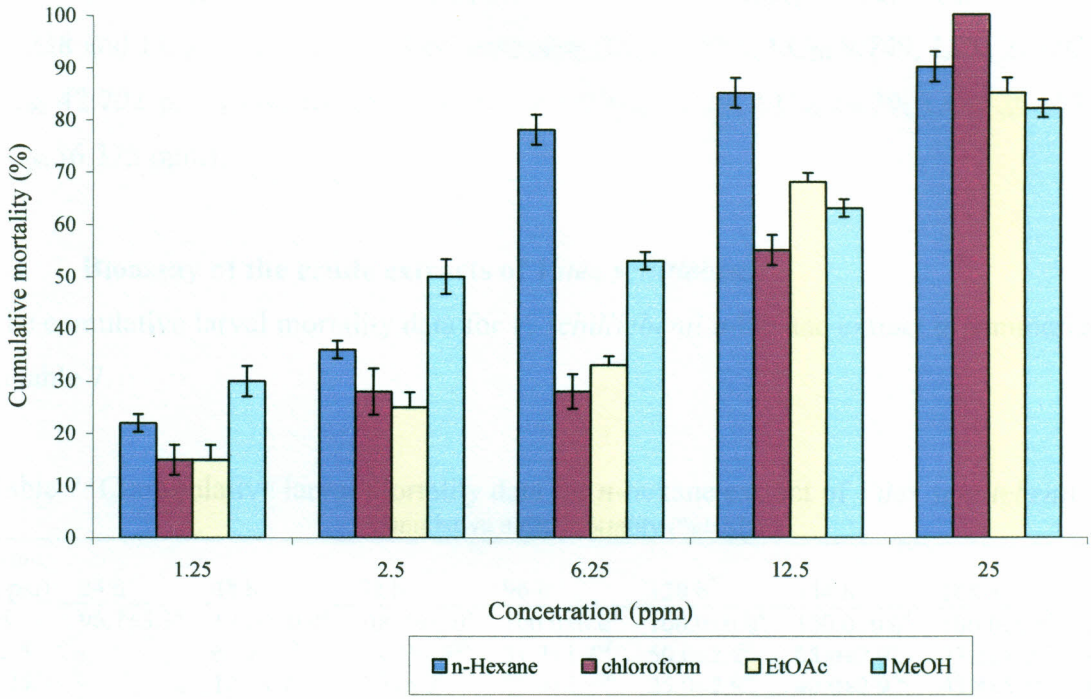
Conc. ppm)	Cumulative mean mortality (%± SE)						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
25	80.0 ± 5.0^{a}	81.7 ± 1.7^{a}	81.7 ± 1.7^{a}	81.7 ± 1.7^{a}	81.7 ± 1.7^{a}	81.7 ± 1.7^{a}	81.7 ± 1.7^{a}
12.5	16.7 ± 1.7^{iim}	16.7 ± 1.7^{ilm}	21.7 ± 1.7^{gm}	38.0 ± 2.9^{ei}	41.7 ± 2.9^{bg}	56.7 ± 1.7^{bd}	62.7 ± 1.7^{ab}
6.25	10.0 ± 2.9^{jm}	10.0 ± 2.9^{jm}	11.7 ± 4.4^{jm}	13.3 ± 3.3^{im}	13.3 ± 3.3^{im}	31.7 ± 1.7^{ei}	53.3 ± 1.7^{bd}
2.5	1.7 ± 1.7^{m}	1.7 ± 1.7^{m}	1.7 ± 1.7^{m}	8.3 ± 1.7^{lm}	25.0 ± 2.9^{ek}	40.7 ± 2.9^{ch}	50.3 ± 3.3^{be}
1.25	1.7 ± 1.7^{m}	1.7 ± 1.7^{m}	1.7 ± 1.7^{m}	3.3 ± 1.7^{lm}	13.7 ± 3.3^{jm}	18.3 ± 13.3^{hm}	30.0 ± 2.9^{ek}

Means with the same letter are not significantly different

Mortality after 24 h was higher and significantly different (P = 0.05) at the highest concentration than at the lowest concentration. Treatment of the larvae with the highest concentration (25 ppm) of *V. payos* methanol extract resulted in 81.7 % larval mortality after 48 h. Mortalities of 16.7, 10.0 and 1.7 % were recorded at 12.5, 6.25, 2.5 and 1.25 ppm, respectively, 24 h after treatment. Mortality was instantaneous at 25 ppm but increased with exposure time at low concentrations. The extract was most toxic at 25 ppm. Cumulative mean mortalities of 81.7, 62.7, 53.3, 50.3 and 30.0 % were recorded at 25, 12.5, 6.25 2.5 and 1.25 ppm after 48 and 168 h, respectively. The rest of the larvae pupated.

A summary of the 8 day-cummulative mortality of 3rd-instar *An. gambiae* larvae for the different extracts of *V. payos* is summarized in fig 3.

Fig 3: Eight-day cumulative mortality data for 3rd-instar *An. gambiae* larvae treated with *V. payos* extracts



The data indicates that the cumulative % mortality increased with the concentration of the extracts.

Probit analysis was used to determine the regression equation that enabled the calculation of LC values of *V. payos* extracts against 3rd-instar *An. gambiae* larvae after 72 h of exposure as described by Finney, 1971 and SAS, 2000. The larvicidal activity data for *V. payos* extracts is presented in table 6.

Table 6: Larvicidal activity data (LC) for *Vitex payos* extracts

	Lethal concentration (LC) (ppm) after 72 h			
Extract	LC_{25}	LC_{50}	LC_{75}	LC_{90}
C_6H_{14}	2.436	3.093	11.622	19.29
$CHCl_3$	5.591	8.915	12.238	15.229
EtOAc	3.437	14.79	26.153	36.375
MeOH	7.595	9.749	27.092	42.702

n-Hexane extract showed the highest larvicidal activity (LC_{25} 5.436, LC_{50} 3.093, LC_{75} 11.622 and LC_{90} 19.29 ppm) followed by chloroform (LC_{25} 5.591, LC_{50} 8.915, LC_{75} 12.238 and LC_{90} 15.229 ppm), then methanol (LC_{25} 7.595, LC_{50} 9.749, LC_{75} 27.092 and LC_{90} 42.702 ppm) and finally ethyl acetate (LC_{25} 3.437, LC_{50} 14.79, LC_{75} 26.153 and LC_{90} 36.375 ppm).

3.2 Bioassay of the crude extracts of *Vitex schiliebenii*

The cumulative larval mortality data for *V. schilliebenii n*-hexane extract is summarized in table 7.

Table 7: Cummulative larval mortality data for *n*-hexane extract of *Vitex schiliebenii*

Conc.	Cumulative mean mortality (%± SE)							
(ppm)	24 h	48 h	72 h	96 h	120 h	144 h	168 h	192 h
25	96.7 ± 3.3^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
12.5	-	6.7 ± 1.7^{j}	20.0 ± 2.9^{gh}	31.7 ± 1.7^{ef}	50.0 ± 2.9^{d}	65.0 ± 2.9^{c}	73.3 ± 1.7^{bc}	80.0 ± 1.7^{b}
6.25	-	1.7 ± 1.7^{j}	3.3 ± 1.7^{j}	20.0 ± 2.9^{gh}	25.0 ± 2.9^{fh}	40.0 ± 2.9^{de}	43.4 ± 3.3^{d}	43.4 ± 3.3^{d}
2.5	-	-	3.3±1.7j	18.3 ± 1.7^{hi}	20.0 ± 2.9^{gh}	26.7 ± 1.7^{fh}	30.0 ± 2.9^{eg}	30.0 ± 2.9^{eg}
1.25	-	-	3.3 ± 1.7^{j}	18.3 ± 5.8^{hi}	20.0 ± 2.9^{gh}	21.7 ± 1.7^{fh}	21.7 ± 1.7^{fh}	21.7 ± 1.7^{fh}

Means with the same letter are not significantly different

The percentage larval mortality was 96.7 % 24 h after application of 25 ppm of the extract, this was significantly different (P = 0.05) while no mortality was observed at 12.25-1.25 ppm during this period. Similarly, the percentage larval mortality was 100.0, 6.7 and 1.7 % at 25, 12.5, and 6.25 ppm, respectively, 48 h after application of the extract. The mortality of larvae treated with low concentrations of extract increased with exposure time. Cummulative mean larval mortalities of 100.0, 80.0, 43.4, 30.0 and 21.7 % were recorded at 25, 12.5, 6.25, 2.5 and 1.25 ppm, after 48, 192, 168, 168 and 144 h, respectively. The rest of the larvae pupated.

The cumulative larval mortality data for *V. schilliebenii*, chloroform extract is summarized in table 8.

Table 8: Cummulative larval mortality data for chloroform extract of *Vitex schilliebenii*

Conc. ppm)	Cumulative mean mortality (%± SE) 24 h	48 h	72 h	96 h	120 h	144 h	168 h	192 h
25	91.6 ± 3.3^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
12.25	3.3 ± 1.7^{ef}	3.3 ± 1.7^{ef}	6.7 ± 1.7^{ef}	18.3 ± 1.7^{be}	25.0 ± 2.9^{bd}	26.6 ± 1.7^{bc}	33.3 ± 1.7^{b}	33.3 ± 1.7^{b}
6.25	1.7 ± 1.7^{f}	3.3 ± 1.7^{f}	3.3 ± 1.7^{f}	10.0 ± 0.0^{df}	25.3 ± 4.4^{bc}	27.0 ± 1.7^{bc}	27.0 ± 1.7^{bc}	27.0 ± 1.7^{bc}
2.5	-	-	-	15.0 ± 2.9^{ef}	18.3 ± 1.7^{be}	23.0 ± 1.7^{bd}	23.0 ± 1.7^{bd}	23.0 ± 1.7^{bd}
1.25	-	-	-	10.0 ± 5.8^{df}	13.3 ± 1.3^{cf}	18.3 ± 4.4^{be}	18.3 ± 4.4^{be}	18.3 ± 4.4^{be}

Means with the same letter are not significantly different

The extract achieved 91.6, 3.3 and 1.7 % mortality at 25, 12.5 and 6.25 ppm, respectively, after 24 h. No mortality was recorded at 2.5 and 1.25 ppm after the same duration. The activity of the extract was found to increase with concentration of extract and exposure time. At 25 ppm, 100.0 % mortality was achieved after 48 h and this was significantly different (P = 0.05), while 18.3 % mortality was achieved after 144 h at 1.25 ppm. The rest of the larvae pupated.

Table 9 gives a summary of the cumulative mortality data for *V. schilliebenii* ethyl acetate extract.

Table 9: Cummulative larval mortality data for ethylacetate extract of *Vitex schiliebenii*

Conc. ppm)	Cumulative mean mortality (%± SE) 24 h	48 h	72 h	96 h	120 h	144 h	168 h	192 h
25	86.7 ± 7.3^{ab}	88.4 ± 6.0^{a}	90.1 ± 5.0^{a}	93.4 ± 3.3^{a}	95.1 ± 2.9^{a}	95.1 ± 2.9^{a}	95.1 ± 2.9^{a}	95.1 ± 2.9^{a}
12.5	15.0 ± 2.9^{dh}	15.0 ± 2.9^{dh}	15.0 ± 2.9^{dh}	61.7 ± 1.7^{c}	70.0 ± 2.9^{bc}	80.0 ± 2.9^{ab}	90.0 ± 2.9^{a}	90.0 ± 2.9^{a}
6.25	3.3 ± 3.3^{gh}	3.3 ± 3.3^{gh}	8.3 ± 1.7^{eh}	13.0 ± 1.7^{dh}	18.3 ± 1.7^{dg}	21.3 ± 4.4^{df}	26.7 ± 3.3^{d}	28.3 ± 4.4^{d}
2.5	3.3 ± 1.7^{gh}	3.3 ± 1.7^{gh}	3.3 ± 1.7^{gh}	5.0 ± 2.9^{fh}	8.3 ± 3.3^{eh}	8.3 ± 3.3^{eh}	20.0 ± 2.9^{dg}	23.3 ± 4.9^{de}
1.25	-	-	-	-	-	3.3 ± 1.7^{gh}	15.0 ± 3.9^{dh}	20.0 ± 2.9^{dg}

Means with the same letter are not significantly different

The extract induced 86.7, 15.0, 3.3 and 3.3 % larval mortality at 25, 12.5, 6.25 and 2.5 ppm, respectively, after 24 h of application. No mortality was recorded at the lowest concentration (1.25 ppm) in the first 120 h. Generally, larval mortality was observed to increase with concentration and exposure time. Cumulative mean larval mortalities of 95.1, 90.0 at 25, 12.5 ppm, respectively after 120 and 168 h were not significantly (P = 0.05) different. Non significant cumulative mean mortalities of 28.3, 23.3 and 20.0 % (P

= 0.05) at concentrations 6.25, 2.5 and 1.25 ppm, respectively were recorded after 192 h. The rest of the larvae pupated.

Table 10 summarizes the cumulative mortality data for *V. schilliebenii* methanol extract.

Table 10: Cummulative larval mortality data for methanol extract of *Vitex schiliebenii*

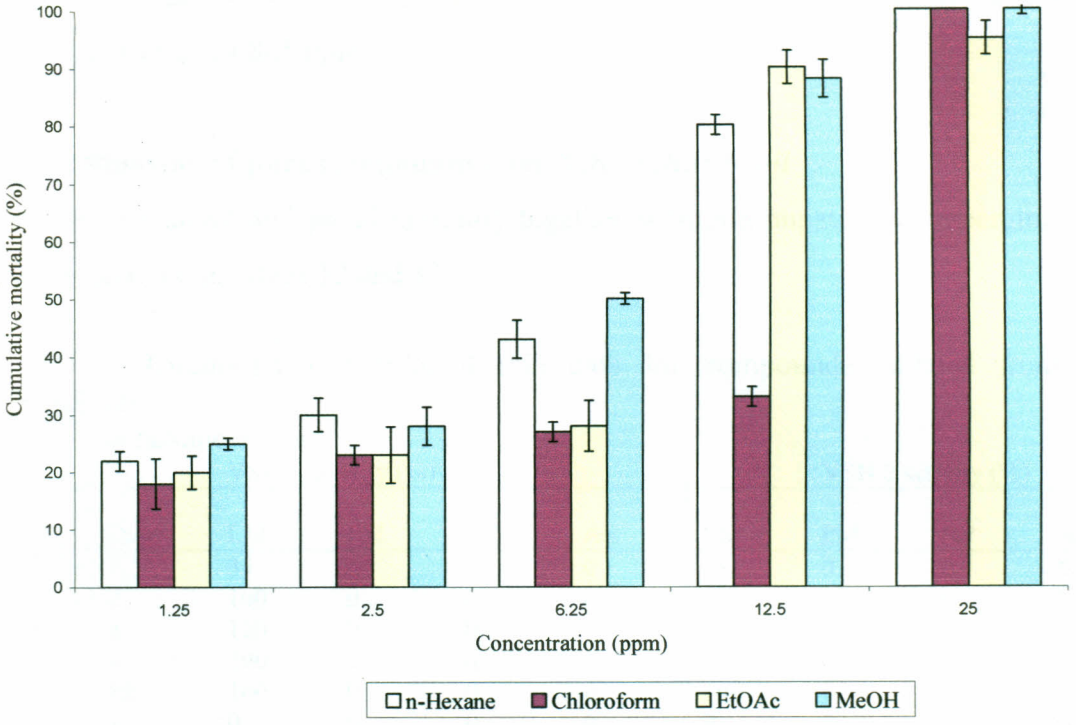
Conc. (ppm)	Cumulative mean mortality (%± SE)							
	24 h	48 h	72 h	96 h	120 h	144 h	168 h	192 h
25	55.0 ± 5.0^{cd}	73.3 ± 4.4^{bd}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
12.25	48.3 ± 4.4^{eg}	56.6 ± 4.4^{dc}	76.6 ± 0.0^{ac}	86.6 ± 0.0^{ab}	88.3 ± 3.3^{ab}	88.3 ± 3.3^{ab}	88.3 ± 3.3^{ab}	88.3 ± 3.3^{ab}
6.25	5.0 ± 2.9^{gh}	5.0 ± 2.9^{gh}	13.3 ± 3.3^{gh}	48.3 ± 10.9^{df}	48.3 ± 10.9^{df}	50.0 ± 10.0^{de}	50.0 ± 10.0^{de}	50.0 ± 10.0^{de}
2.5	-	-	-	5.0 ± 2.9^{gh}	20.0 ± 2.9^{gh}	20.0 ± 2.9^{gh}	23.3 ± 3.3^{fh}	28.3 ± 3.3^{eg}
1.25	-	-	1.7 ± 1.7^{h}	15.0 ± 0.0^{gh}	18.3 ± 6.0^{gh}	18.3 ± 6.0^{gh}	25.0 ± 0.0^{eh}	25.0 ± 0.0^{eh}

Means with the same letter are not significantly different

Larval mortalities of 55.0, 48.3 and 5.0 % were recorded at 25, 12.5 and 6.25 ppm, respectively, after 24 h and these were significantly different (P = 0.05). No mortality was recorded at 2.5 and 1.25 ppm after the same duration. Larval mortality of 100.0 % was achieved after 72 h at 25 ppm. Cumulative mean larval mortalities of 100.0, 88.3, 50.0, 28.3 and 25.0 % were recorded after 72, 120, 144, 192 and 168 h at 25, 12.5, 6.25, 2.5 and 1.25 ppm, respectively. The rest of the larvae pupated. Larval mortality increased with concentration of extract and exposure time.

The 8 day-cummulative mortality of 3rd-instar *An. gambiae* larvae for different *V. schiliebenii* extracts is summarized in fig 4.

Fig 4: Eight-day cumulative mortality data for 3rd-instar An. gambiae larvae treated with *V. schiliebenii* extracts



The data indicates that the cumulative mortality (%) increased with the concentration of the extracts.

Probit analysis of the larval mortality data for *V. schilliebenii* extracts provided the regression equation which was used to calculate the lethal concetration (LC) values of *V. schilliebenii* extracts against *An. gambiae* 3rd-instar larvae after 72 h exposure. The larvicidal activity data is summarized in table 11.

Table 11: Larvicidal activity data for *Vitex schilliebenii* extracts

	Lethal concentrations (LC) (ppm) after 72 h			
Extract	LC_{25}	LC_{50}	LC_{75}	LC_{90}
C_6H_{14}	0.755	5.453	11.660	17.246
$CHCl_3$	2.756	10.386	18.017	24.885
EtOAc	3.707	9.919	16.131	21.720
MeOH	1.108	4.859	8.609	11.984

From the results, it was revealed that methanol extract had the highest activity (LC_{25} 1.108, LC_{50} 4.859, LC_{75} 8.609 and LC_{90}11.984 ppm) followed by hexane (LC_{25} 0.755, LC_{50} 5.453, LC_{75} 11.660 and LC_{90}17.246 ppm), ethyl acetate (LC_{25} 3.707, LC_{50} 9.919,

LC_{75} 16.131 and LC_{90} 21.720 ppm) and chloroform (LC_{25} 2.756, LC_{50} 10.348, LC_{75} 18.017 and LC_{90} 24.885 ppm).

3.3 Bioassay of pure compounds from *Vitex schiliebenii*

The data for larval and pupal mortality together with non-pupated and emerging adults are summarized in tables 12 and 13.

Table 12: Cummulative anti-larval (%) data for compounds isolated from *Vitex schiliebenii*

n-hexane extract

		VSH 1 activity (%)				VSH 2 activity (%)			
Conc (ppm)	Days	LM	PM	NP	AE	LM	PM	NP	AE
50	1	35	0	0	0	43	0	0	0
	2	100	0	0	0	100	0	0	0
	4	100	0	0	0	100	0	0	0
	8	100	0	0	0	100	0	0	0
	10	100	0	0	0	100	0	0	0
10	1	0	0	0	0	0	0	5	0
	2	10	0	23	0	5	5	25	0
	4	15	5	63	18	15	20	45	5
	8	15	30	78	33	25	25	60	20
	10	15	40	85	38	30	35	70	25
5	1	0	0	0	0	0	0	0	0
	2	5	0	35	0	0	0	10	0
	4	5	10	58	25	8	5	38	5
	8	5	23	78	35	13	18	68	20
	10	5	35	95	43	15	30	85	38
1	1	0	0	0	0	0	0	0	0
	2	0	0	38	0	0	0	13	0
	4	0	8	63	30	0	5	48	8
	8	0	20	75	43	5	13	73	35
	10	5	22	88	53	10	23	90	50

LM-% larval mortality; PM-% pupal mortality; NP-% normal pupae; AE-% adult emerged

Table 13: Cummulative (%) anti-larval data for compounds isolated from *V. schliebenii* chloroform and ethyl acetate mixture extract

		VSCE 2activity (%)				VSCE 3 activity (%)			
Conc (ppm)	Days	LM	PM	NP	AE	LM	PM	NP	AE
50	1	65	0	0	0	65	0	0	0
	2	100	0	0	0	100	0	0	0
	4	100	0	0	0	100	0	0	0
	8	100	0	0	0	100	0	0	0
	10	100	0	0	0	100	0	0	0
10	1	0	0	0	0	5	0	13	0
	2	0	5	5	5	18	3	36	10
	4	8	5	5	10	20	14	58	22
	8	13	13	55	28	25	20	75	38
	10	15	40	85	45	25	30	75	45
5	1	0	0	10	0	0	0	18	0
	2	0	0	10	0	5	5	40	13
	4	5	0	45	10	5	18	58	30
	8	5	15	85	30	7	23	90	43
1	10	10	35	90	55	10	30	90	60
	1	0	0	35	0	3	0	25	0
	2	0	10	48	25	3	5	38	15
	4	0	15	70	33	3	15	60	23
	8	5	22	90	48	7	15	93	43
	10	10	20	90	70	5	30	95	65

LM: % larval mortality; PM: % pupal mortality; NP: % normal pupae; AE: % adult emerged

At 50 ppm, all the larvae were killed within 48 h of treatment with the test compounds. At 10 ppm, survival of the larvae was relatively high ($\leq$ 4 days) but adult emergence was low (38, 25 45 and 45 % for the compounds VSH 1, VSH 2, VSCE 2 and VSCE 3, respectively, after 10 days). The cumulative larval mortality after 10 days was 15, 30, 15 and 25 % for VSH 1, VSH 2, VSCE 2 and VSCE 3, respectively at 10 ppm. Pupae arising from larvae treated with VSH 1, VSH 2, VSCE 2 and VSCE 3 were also affected resulting in 40, 35, 40 and 30 % cumulative mortality, respectively, after 10 days. The cumulative adult emergence of larvae treated with VSH 1, VSH 2, VSCE 2 and VSCE 3 were 38, 25, 45 and 45 % for the test compounds, respectively, after 10 days. At 5 and 1 ppm, larval mortality was low (< 20 %). IGR effect was observed on larvae treated with the compounds since the developmental period was >10 days (Tables 12-13) while that of the control averaged 8 days. At high concentrations, the compounds were toxic while at low concentrations they were growth inhibiting. Only 5 % mortality occurred in control larvae, 95 % pupated and mortality of corresponding pupae was 5 % only. Adult emergence for untreated larvae (control) was high at 90 %.

3.4 Symptomatological and morphological observations

All larvae were normal and active immediately (Plate 4) after exposure to each test compound at all the concentrations tested, and the feeding process and normal zigzag motions of larvae could be seen. However, after 24 h exposure to 1-10 ppm of compounds, some abnormal sluggishness was observed in the test experiments with the larvae sinking down and floating again. After 48-192 h, some larvae were excited, restless and while others died (Plate 5).

Plate 4: Photograph of untreated larva (control)

Plate 5: Photograph of treated dead Larva

Observations of the morphological deformation of the treated larvae revealed that 15 % of the emerged pupae did not have well defined normal pupal features. Infact, they were larval-pupal intermediates, with the heads of a pupae and the abdomen of a larvae (Plate 6). The deformed larvae were found in the medium treated with 10 ppm of the isolated compounds and increased in number to 20 % as the concentration of the compounds reduced to 1 ppm. The larval-pupal intermediates were not observed in the control experiments (Plate 4). Most adults from the test experiments (75 %) died within 48 h of emergence. Morphological disruption of the extension on the terminal segment was observed in the treated (Plate 6) and dead larvae (Plate 5). The treated larvae also showed signals of swollen thorax and abdomen.

Plate 6: Photograph of larval-pupal intermediate treated from treated larvae

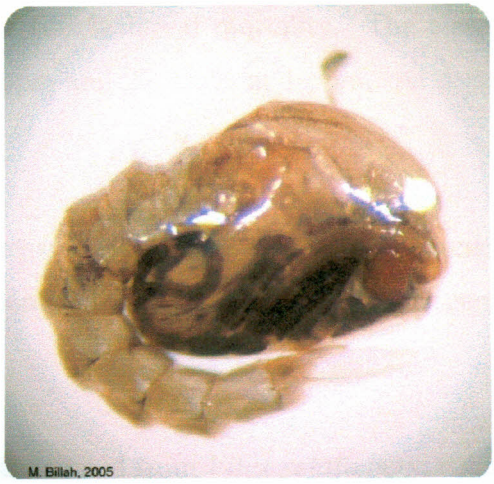


Plate 7: Photograph of pupa from larvae emerging to adults

It may be concluded that the isolated compounds exhibited IGR activity at lower doses but had acute toxicity at high doses.

The cumulative anti-larval data for the isolated compounds against 3rd-instar larvae *An. gambiae* under laboratory conditions is given in table 14.

Table 14: Cummulative anti-larval data (%) for compounds isolated from *Vitex schiliebenii*

Compound	Conc (ppm)	Cumm. Mortality (%)	Total Pupae	Mean Emerged Adults	% Inhibition of Emergence
VSH 1	50	100	0	0	100
	10	55	17	8	53
	5	50	19	10	47
	1	40	19	12	37
VSH 2	50	100	0	0	100
	10	65	18	7	61
	5	45	13	9	31
	1	35	18	13	28
VSCE 2	50	100	0	0	100
	10	55	17	9	45
	5	45	18	11	39
	1	30	18	14	22
VSCE 3	50	100	0	0	100
	10	55	15	9	38
	5	40	19	12	37
	1	35	19	13	32

At 50 ppm, all the test compounds achieved 100 % larval mortality. The cumulative larval mortality ranged from 55-65 % at 10 ppm and 27-35 % at 1 ppm. At 5 ppm the range was 40-45 %.

Probit analysis was used to determine the regression equation of the adult emergence data. The regression equation was used to calculate the inhibition of adult emergence for each compound (Table 15).

Table 15: Anti-emergence data for compounds isolated from *Vitex schiliebenii*

	Inhibition of Emergence (IE) values (ppm)		
Compound	IE_{50}	IE_{75}	IE_{90}
VSH 1	2.773	13.980	24.060
VSH 2	5.619	10.568	17.713
VSCE 2	7.972	13.778	22.161
VSCE 3	8.239	15.805	26.720

The concentrations required for 50 % emergence inhibition (IE_{50}), were 2.773, 5.619, 7.972, and 8.239 ppm; (IE_{75}), were 13.980, 10.568, 13.778 and 15.805ppm, and (IE_{90}), were 24.060, 17.713, 22.161 and 26.720 ppm for VSH 1, VSH 2, VSCE 2 and VSCE 3, respectively (Table 15). These results show high efficacy of the compounds against *An. gambiae*.

3.5 Discussion

3.5.1 Larval mortality

In this study, crude extracts of the plants *V. payos* and *V. schilliebenii* showed larvicidal activity against laboratory reared 3rd-instar *An. gambiae*. The efficacy of the plants was not significantly different (p= 95 %). The larvicidal activity of the two plant materials may therefore be due to the similarity in the larvicidal principles. The similarity in the family may have contributed to the similarity in the lethal effects. Crude extracts of the two plants have not been studied previously.

From the results, mortality of larvae of *An gambiae* exposed to the plant extracts increased with time of exposure and concentration (1.25-25 ppm) of the extracts as previously reported for larvae of *C. quinquefasciatus* and other mosquito species exposed to extracts of several other plants such as *Nerium indicum* and *Euphorbia royleana*

(Choochote *et al.*, 2004). The phenomenon has been attributed to the high toxicity at the high concentrations. The increase of mortality with exposure time suggests that the larvae fed on the toxins continuously over time without inhibition but the larvicides was slow in action at low concentrations. Consequently, high emergence rates were observed at the low concentrations. It was also observed that 20-40 % of the larvae treated with the low concentration of the extract successfully pupated but often died before emerging into adults. This is an advantage for malaria control programmes since adult *An. gambiae* mosquitoes are the prime vectors of malaria. The low adult emergence rates can reduce the entomological inoculation rates and parasite transmission.

Considerable research on the larvicidal potential of natural products for controlling mosquitoes has been carried out but with varied results. Several plants in the genus *Vitex* sp have been reported to exhibit larvicidal activity against the mosquito species (Pushpalatha & Muthukrishnan, 1995). Extracts of *V. negundo* and *V. rotundifolia* are feeding deterrent to *Ae. aegypti* larvae (Hebbalkar *et al.*, 1992; Watanabe *et al.*, 1995). Several other *Vitex* species have been investigated for their pest control potential (Sudarsanan *et al.*, 1995). Essential oils extracted from nine plants commonly found in northeastern Brazil exhibited various degrees of larvicidal activity against *Ae. aegypti* (LC_{50} 60-538 ppm) (Cavalcanti *et al.*, 2004). Investigation of seventeen Brazilian plants revealed that five oils derived from *Anacardium occidentalis, Copaifera langsdorffi, Carapa guinanensis, Cymbopogon winterianus* and *Ageratum conyzoides*, and one ethanolic extract of *Annona glabra* showed high larvicidal activities against *Ae. aegypti* larvae, (LC_{50} 14.5, 41, 57, 98 148 and 27 mg/l), respectively (De Mendonca *et al.*, 2005). Compounds extracted from *Az. indica* showed mortality for 4th-instar larvae of *An. stephensi* (LC_{50} 43 ppm) (Vatandoost & Vaziri, 2004).

Obomanu *et al.*, (2006) reported the effect of aqueous extract of *Lepidagathis alopecuroides* and *A. indica* against *An. gambiae* and *C. quinquefasciatus*. The toxicity of the extract was found to increase with time and concentration. With increase in exposure time, LC_{50} of *Lepidagathis alopecuroides* on *C. quinquefasciatus* decreased from 384.69 mg/l (10 min) to 0.87 mg/l (20 min), while that of neem on the same organism decreased from 1479.43 mg/l (105 min) to 47.19 mg/l (225 min). For *An.*

gambiae, the LC_{50} decreased from 409.77 mg/l (30 min) to 26 mg/l (110 min). Comparison with results mentioned above reveals that the larvicidal potential of *Vitex* spp. tested in this study was comparable to that of previously described natural products. The slight differences may be due to differences in insect species and the active ingredients contained in the plants and the method of extraction.

3.5.2 Adult emergence

The number of adults emerging from the breeding site determines the significance of the threat of mosquitoes in disease transmission. In control experiments, 95% adult emergence was observed. More adults emerged from the experiments treated with low concentrations than at high concentration. Although high pupal mortality was recorded in treated experiments, 25-70% emerged as adults especially at the low concentrations. The reduction in the adult emergence suggests the IGR action of the test compounds on pupae. It also indicates high pupal mortality at high concentrations. The low pupal mortality at low concentrations may explain why there were a high number of adults emerging from experiments treated with low concentrations.

3.5.3 Symptomatic and morphological observations

Besides the direct toxicity of the IGRs, morphological abnormalities were observed in 15-20% of the larvae from the test experiments as compared to the controls. Larval-pupal intermediates, with pupal heads and larval abdomens were observed. Similar observations were reported by Ndung'u *et al.* (2004) using chloroform extract of *Melia volkensii* on *An. gambiae*. They reported larvae with intermediary stages plus discoloured and longer pupae. Saxena *et al.* (1994) also reported similar results with *Aedes aegypti* larvae. The observation suggests that the test compounds act as IGRs against the *An. gambiae* larvae.

Under light microscope, both treated and control larvae showed similarities in physical morphological architecture. However, distinct differences were observed in the structure alteration of the extension on the terminal segment and the swelling of the abdomen. The extension was observed to be darker in treated than in normal (control) larvae. This suggested some damage on the anal gills which indicated that the toxic effect of the test

samples was predominantly on the abdomen leading to its morphological deformation. The findings correspond to those from earlier studies on the effect of plant derived compounds on some mosquito species, where morphological disruption of anal gills was observed in dead *C. quinquefasciatus* larvae (Insun *et al.* 1999). Damaged anal gills with shrunken cuticle border and destroyed surface with loss of ridge-like reticulum were observed in *C. quinquefasciatus* after treatment with ethanolic extract of *Kaempferia galangal*. Structural deformation of the abdomen and consequently the anal gills probably led to their dysfunction, which may be intrinsically associated with the death of the mosquito larvae (Clements, 1992). In fresh-water mosquito larvae, uptake and elimination of most ions occur via the anal gills, while the process of ion conservation is mainly located in the alimentary canal (Clements, 1992). The dysfunction of the anal gills probably led to an interruption of the osmotic and ion regulation which was attributed to the swelling of the abdomen (Clements, 1992).

CHAPTER 4: STRUCTURE ELUCIDATION

4.1 Introduction

The chemical structures of the isolated compounds were deduced by extensive IR, NMR and mass spectral analysis. The structure elucidation of the isolated compounds VSCE 2 and VSCE 3 (ecdysteroids), VSH 2 (β-sitosterol), VSH 1 and VPH 3 (stigmasterol) and GF/CE/1 (secoisolariciresinol) are extensively discussed in this chapter.

4.2 2R, 3R, 14S, 20R, 22R, 25-hexahydroxycholest-7-en-6-one (20-Hydroxyecdysone) (80) (VSCE 2)

This compound was isolated as a crystalline solid with melting point of 234-236 °C (lit. 230-233 °C, Kavel *et al.*, 1998). The IR spectrum indicated strong absorption bands for hydroxyl group (3381 cm^{-1}) and α, β-unsaturated ketone (1651 cm^{-1}).

The ^1H NMR data for VSCE 2 is summarized in table 16.

Table 16: ^1H NMR (400 MHz, DMSO/CD_3OD) data for VSCE 2

Position	$\delta_{(obs.)}$	*$\delta_{(lit.)}$	†$\delta_{(lit.)}$
2	3.65 (1H, m)	3.83	-
3	3.75 (1H, m)	3.95	-
4a	Not observed	-	-
4b	Not observed	-	-
5	2.10 (1H, m)	2.38	2.11
7	5.65 (1H, s)	5.81	5.64
9	2.98 (1H, m)	3.15	2.81
12a	2.05 (1H, m)	2.14	2.03
12b	1.75 (1H, m)	1.85	1.75
15a	1.85 (1H, m),	1.60	1.48
15b	1.50 (1H, m)	1.95	1.80
16a	1.65 (1H, m)	1.75	1.65
16b	1.87 (1H, m)	1.95	1.85
17	2.27 (1H, m)	2.38	2.27
18	0.75 (3H, s)	0.90	0.78
19	0.86 (3H, s)	0.98	0.88
21	1.09 (3H, s)	1.19	1.07
22	3.15 (1H, t, 5.1)	3.33	3.13
23a	1.48 (1H, m)	1.28	1.12
23b	1.11 (1H, m)	1.62	1.47
24a	1.25 (1H, m)	1.78	1.65
24b	1.68 (1H, m)	1.45	1.28
26	1.05 (3H, s)	1.19	1.07
27	1.05 (3H, s)	1.19	1.08

*Literature data for 20-hydroxecdysone in CD_3OD taken from Roussel *et al.* (1997)

†Literature data for 2, 3-*O*-Isopropylidene-20-hydroxecdysone in DMSO taken from Roussel *et al.* (1997)

The ^{1}H NMR spectrum of VSCE 2 and 20-hydroxyecdysone (**80**) (Roussel *et al.*, 1997) were similar. Methyl singlets were observed at δ 0.75 (18-Me), 0.86 (19-Me), 1.09 (21-Me), 1.05 (26-Me), and 1.05 (27-Me). The signal at δ 5.65 (s, 1H), was assigned to an olefinic proton at C-7 due to the double bond between C-7 and C-8. The signal at δ 3.15 (m, 2H) suggested the presence of a hydroxylated carbon.

^{13}C NMR revealed 27 signals (Table 17).

Table 17: ^{13}C NMR (400 MHz, DMSO) data for VSCE 2

Position	$\delta_{(obs.)}$	$^{*}\delta_{(lit.)}$	$^{\dagger}\delta_{(lit.)}$	DEPT	Position	$\delta_{(obs.)}$	$^{*}\delta_{(lit.)}$	$^{\dagger}\delta_{(lit.)}$	DEPT
1	38.1	37.3	37.3	CH_2	15	29.9	31.7	30.2	CH_2
2	67.3	68.6	71.4	CH	16	20.5	21.5	20.4	CH_2
3	67.1	68.5	71.2	CH	17	48.4	50.5	48.7	CH
4	31.3	32.7	26.0	CH_2	18	17.4	18.1	17.1	CH_3
5	50.6	51.7	50.5	CH	19	24.1	24.4	23.2	CH_3
6	203.6	206.6	201.6	C	20	76.3	78.0	75.7	C
7	120.9	122.1	120.1	CH	21	21.1	21.1	21.9	CH_3
8	165.9	168.1	164.8	C	22	76.8	78.4	77.6	CH
9	33.7	35.0	33.9	CH	23	26.5	27.3	26.1	CH_2
10	36.9	39.3	37.2	C	24	41.6	42.3	41.4	CH_2
11	20.7	21.5	20.1	CH_2	25	69.5	71.4	68.7	C
12	29.9	32.4	30.9	CH_2	26	29.1	29.1	29.0	CH_3
13	47.4	48.6	47.1	C	27	29.1	29.7	29.9	CH_3
14	83.6	85.2	82.9	C					

*Literature data for 20-hydroxecdysone in CD_3OD (Roussel *et al.*, 1997) †Literature data for 2, 3-isopropyl-20-hydroxecdysone in DMSO taken from Roussel *et al.*, (1997)

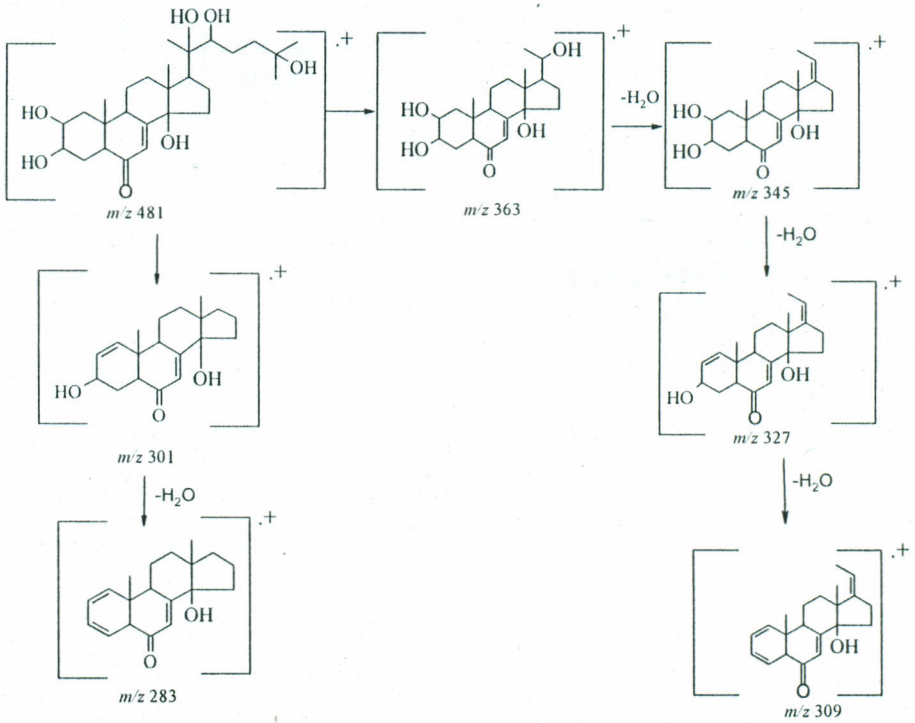
^{13}C NMR revealed 27 signals. Two olefinic carbon signals observed at δ 120.9 (C-7) and 165.9 (C-8) and one carbonyl at δ 203.6 (C-6) suggested an α, β unsaturated system. Presence of two quartenary hydroxyl groups was confirmed by the signals at δ 83.6 and 76.3. ^{13}C NMR further suggested the presence of secondary hydroxyl groups by signals at δ 67.1, 67.3, 69.5 and 76.8. The signal at δ 29.1 was assigned to the two equivalent methyl groups at C-26 and C-27.

DEPT analysis revealed five methyl groups at δ 21.1, 29.1, 29.1 24.1 and 17.4, eight methylene carbons at δ 38.1, 41.6, 31.3, 29.9, 29.9, 26.5, 20.5 and 20.7, six methine carbons at δ 121.3, 77.1, 67.4, 50.9, 48.7 and 34.0. Signals at δ 67.3 (C-2), 67.1 (C-3), 69.5 (C-25), 76.3 (C-20), 76.8 (C-22) and 83.6 (C-14) suggested oxygenation on the carbons. The signals at δ 36.9 and 47.4 suggested quaternary carbons.

The ^{1}H NMR, ^{13}C NMR spectral data and melting points of VSCE 2 and 20-hydroxyecdysone (**80**) were almost identical (Roussel *et al.*, 1997). It was thus concluded that VSCE 2 is 20-hydroxyecdysone (**80**).

The positive ion mass spectrum of the compound revealed the molecular ion at *m/z* 481 and suggested the formula $C_{27}H_{45}O_7$. The EI mass spectral peak at *m/z* 363 (8%), which is consistent with the formula $C_{21}H_{31}O_5$, was associated with a fragmentation between C-20 and C-22 resulting in an ion containing an ecdysteroid ring system and the side chain. Loss of one, two or three molecules of water (Scheme 1) from the above fragment ions gave rise to the peaks at *m/z* 345 (22 %), 327 (20 %) and 309 (5 %), respectively. The peaks at *m/z* 301 and 283 were attributed to fission between C-17 and C-20 and with loss of one and two water molecules, respectively. The ions in the mass spectrum of **80** can be accounted for by the fragmentation pattern in scheme 1.

Scheme 1: Mass spectral fragmentation pattern of 20-hydroxyecdysone (**80**)



4.3 (20R,22S)-20-hydroxyecdysone [22-*epi*-20-hydroxyecdysone (82)] (VSCE 3)

This compound was obtained as a white solid of melting point 240-242 °C. The ^{1}H NMR data for VSCE 3 is summarized in table 18

Table 18: ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) data for VSCE 3 (**82**)

Position	$\delta_{(obs.)}$	$^{*}\delta_{(lit.)}$	$^{\dagger}\delta_{(lit.)}$
2	3.56♣ (1H, br t, 4.6)	3.83	4.25
3	3.67♣ (1H, br s)	3.95	4.25
4a	1.29 (2H, t, 7.8)	1.65	-
4b	1.67 (1H, m)	1.75	-
5	2.10 (1H, dd; 4.4, 4.4)	2.38	2.32
7	5.53 (1H, s)	5.81	5.83
9	2.80 (1H, t, 5.4)	3.15	2.84
10	1.11 (t, 10.2)	-	-
11a	1.42 (1H, m)	1.65	-
11b	1.52 (1H, m)	1.78	-
12a	1.44 (1H, m)	2.14	-
12b	1.57 (1H, m)	1.85	-
15a	1.80 (1H,ddd; 3.6, 3.6, 3.6)	1.60	-
15b	2.80 (1H, m)	1.95	-
17	2.05 (1H, t, 6.8)	2.38	2.32
18	0.57 (3H, s)	0.90	0.87
19	0.68 (3H, s)	0.98	1.00
21	0.98 (3H, s)	1.19	1.22
22	3.05 (1H, s)	3.33	3.48
23a	1.01 (1H, m)	1.28	-
23b	1.37 (1H, m)	1.62	-
24a	1.17 (1H, m)	1.78	-
24b	1.47 (1H, m)	1.45	-
26	0.91 (3H, s)	1.19	1.27
27	0.91 (3H, s)	1.19	1.27

*Literature data for 20-hydroxecdysone in CD_3OD (Roussel *et al.*, 1997); †Literature data for 2, 3-O-isopropyl-20-hydroxecdysone in $CDCl_3$ (Roussel *et al.*, 1997); ♣Assignment may be reversed

Methyl singlets were observed at δ 0.57 (C-18), 0.68 (C-19) 0.98 (C-21), 0.91 (C-26) and 0.91 (C- 27). The signal at δ 5.53 (s, 1H), was assigned to an olefinic proton at C-7. The signals at δ 3.67 (1H, br *s*, C-3), 3.56 (1H, br t, C-2) and 3.05 (1H, *s*, C-22) suggested the presence of hydroxylated carbons.

^{13}C NMR revealed 27 signals (Table 19).

Table 19: ^{13}C NMR (400 MHz, $CDCl_3$/CD3OD) data for VSCE 3 (**82**)

Position	$\delta_{(obs.)}$	*δ (lit.)	†$\delta_{lit.}$	HMQC (δ)	HMBC(δ)	Position	δ (obs.)	*δ (lit.)	†δ (lit.)	HMQC (δ)	HMBC(δ)
1	38.4	37.3	38.6	1.49	0.68	15	31.4	31.7	31.6	1.80, 2.80	1.20
2	67.8	68.6	67.4	3.67	0.68	16	20.6	21.5	20.5	1.68	2.05
3	67.4	68.5	67.8	3.54	0.68	17	49.4	50.5	49.1	2.05	3.05
4	31.0	32.7	32.7	1.29	-	18	17.4	18.1	17.4	0.57	1.67, 2.05
5	50.6	51.7	50.8	2.10, 1.67	5.52, 3.05	19	24.0	24.4	23.6	0.68	1.10
6	205.8	206.6	206.6	-	2.10	20	77.9	78.0	76.9	-	-
7	121.3	122.1	121.5	5.52	2.10, 2.78	21	20.4	21.1	20.7	0.90	-
8	166.9	168.1	168.1	-	2.78, 1.10	22	78.1	78.4	78.4	3.05	3.05
9	34.1	35.0	34.6	1.10	0.68, 1.10	23	26.2	27.3	26.1	1.01, 1.37	3.05
10	36.5	39.3	36.8	-	0.68	24	41.3	42.3	40.8	1.17, 1.47	3.05
11	20.7	21.5	20.6	1.42, 1.52	-	25	70.6	71.4	70.6	-	-
12	31.6	32.4	31.2	1.44, 1.57	0.57	26	29.0*	29.1	29.3	0.91	-
13	47.6	48.6	47.7	-	0.57, 1.57	27	28.8*	29.7	30.0	0.91	
14	84.3	85.2	84.8	-	0.57, 5.52, 1.57						

*Literature data for 20-hydroxecdysone in CD_3OD taken from Roussel *et al.* (1997); †Literature data for 2, 3-O-Isopropyl-20-hydroxecdysone in $CDCl_3$ taken from Roussel *et al.*, (1997); *Assignment may be reversed

^{13}C NMR showed 27 signals. The presence of a signal at δ 166.9 suggested an α, β-unsaturated carbonyl system. Two olefinic carbon signals appeared at δ 121.3 (CH) and 166.9 and one carbonyl at δ 205.8. The ^{1}H NMR spectrum of the tetracyclic ring system of VSCE 3 was very similar to that of the 20-hydroxyecdysone (**80**).

The HMBC and HMQC analysis of this compound revealed that the signals resonating at δ 34.1, 70.6, 77.9, 84.6, 167.2 and 205.9 ppm had no protons. The signal at δ 84.3 correlated to proton signals at δ 0.57, 5.52, and 1.57, the signals δ 50.6, 121.3, 167.2, 34.1, 49.4 and 17.4 correlated to two protons each.

A comparison of the melting point, ^{1}H NMR and ^{13}C NMR spectral data for VSCE 3 was consistent with the reported spectral data for 22-*epi*-20-hydroxyecdysone (**82**) (Bathori *et al.*, 1998, 2000). It was thus concluded that VSCE 3 is 22-*epi*- 20-hydroxyecdysone (**80**). 20-hydroxyecdysone (**80**) is a common ecdysteroid which occurs widely in many *Vitex* species (Werawattanametin *et al.*, 1986).

4.4 Stigmast-5-en-ol (β-sitosterol) (118) (VSH 2)

This compound was isolated as a colorless crystalline solid (27 mg) with a melting point of 134-6 °C (lit. 130-134°C, McCarthy *et al.*, 2005).

The ^{1}H NMR is summarized in table 20.

Table 20 ^{1}H NMR (400 MHz, $CDCl_3$) data for VSH **(118)**

Position	$\delta_{(obs.)}$	$\delta_{(lit.)}$	Position	$\delta_{(obs.)}$	$\delta_{(lit.)}$
1	1.01 (2H, m)	1.01	16	1.30 (2H, s)	-
2	1.40 (2H, m)	1.37	17	1.75 (1H, m)	1.74
3	3.50 (1H, m)	3.51	18	0.70 (3H, s)	0.67
6	5.35 (1H, d; 5.1)	5.35	19	0.98 (3H, s)	0.98
7	1.90 (2H, m)	1.93	20	1.90 (1H, m)	1.90
8	1.55 (1H, m)	1.54	21	0.90 (3H, s)	0.92
9	1.75 (1H, s)	-	26	0.82 (3H, s)	0.82
11	1.42 (2H, m)	1.44	27	0.80 (3H, s)	0.80
12	1.70 (2H, m)	1.69	29	0.85 (3H, s)	0.84

Literature data taken from Saxena & Sosanna (2005) and McCarthy *et al.* (2005)

^{1}H NMR revealed a signal at δ 5.35 (d, 1H), which was assigned to an olefinic proton at C-6 owing to the double bond C-5 and C-6. The signal at δ 3.50 (t, 1H) suggested the presence of an α-proton typical of sterols hydroxylated at C-3. Some methyl singlets at δ 0.98, 0.9, 0.82, 0.85, 0.8 and 0.7 were observed and assigned to H-18, H-21, H-26, H-29, H-27 and H-18, respectively. The doublet at δ 5.35 and the broad tripplet at δ 3.50 were assigned to olefinic proton and hydroxyl group, respectively. The rest of the protons were in complex continuous multiplets spread between δ 1.0-2.2.

^{13}C NMR revealed 29 signals (Table 21).

Table 21: ^{13}C NMR (400 MHz, $CDCl_3$) data for VSH 2 (**118**)

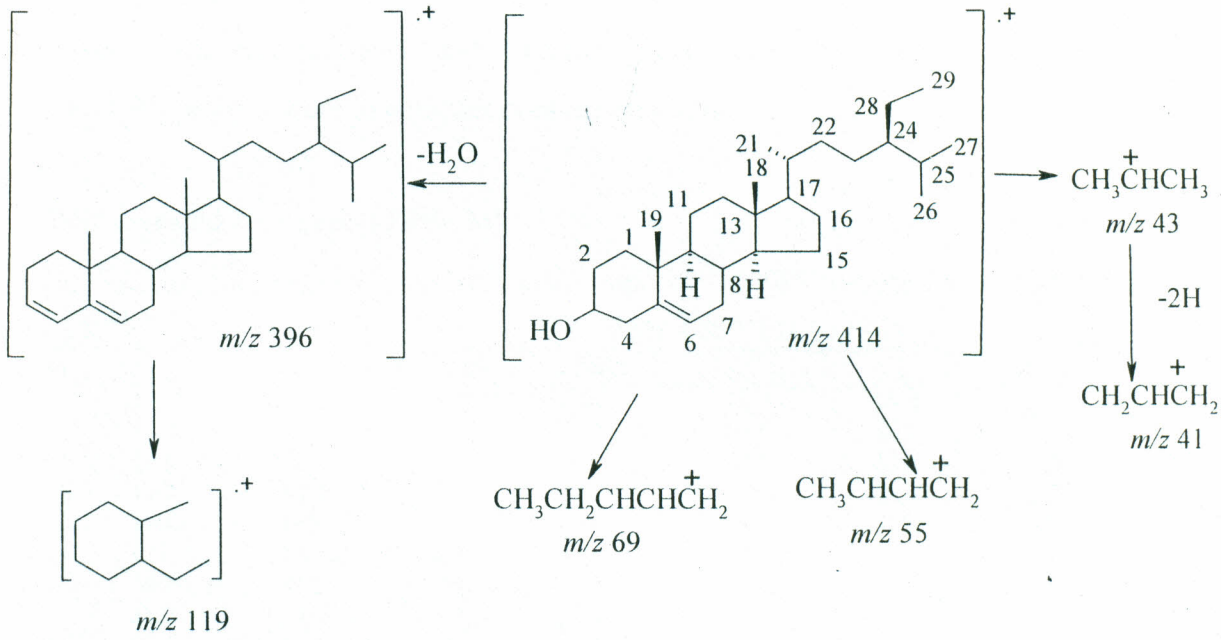
Position	$\delta_{(obs.)}$	$\delta_{(lit.)}$	DEPT	Position	$\delta_{(obs.)}$	$\delta_{(lit.)}$	DEPT
1	37.3	37.0	CH_2	16	39.8	39.8	CH_2
2	29.4	29.5	CH_2	17	56.1	56.1	CH
3	71.8	71.8	CH	18	11.9	12.2	CH_3
4	42.3	42.3	CH_2	19	18.8	18.8	CH_3
5	140.8	140.8	C	20	34.0	34.0	CH
6	121.7	121.7	CH	21	19.1	19.1	CH_3
7	31.7	31.9	CH_2	22	37.3	37.3	CH_2
8	29.2	29.2	CH	23	26.2	26.6	CH_2
9	50.2	50.2	CH	24	50.2	50.1	CH
10	36.5	36.5	C	25	28.2	28.3	CH
11	21.1	21.1	CH_2	26	19.4	19.4	CH_3
12	26.1	26.1	CH_2	27	19.8	19.8	CH_3
13	45.9	45.9	C	28	23.3	23.3	CH_2
14	56.8	56.7	CH	29	11.8	12.0	CH_3
15	24.3	24.1	CH_2				

Literature data taken from Saxena & Sosanna (2005) and McCarthy *et al.*, (2005)

From ^{13}C NMR, two olefinic carbons appeared at δ 121.7 (C-6) and 140.8 (C-5). The carbon signal at δ 71.8 (C-3) suggested oxygenation. From DEPT analysis, six methyls at δ 11.9, 11.8 18.8, 19.1, 19.4 and 19.8, eleven methylenes at δ 21.1, 23.3, 24.3, 26.1 26.2, 29.4, 31.7, 37.3, 37.3, 39.8 and 42.3, nine methines at δ 28.2, 29.2, 34.0, 50.2, 50.2, 56.1, 56.8, 71.8 and 121.7 were noted. The other three remaining signals at δ 36.5 (C-10), 45.9 (C-13) and 140.8 (C-5) were due to quaternary carbons. From ^{1}H and ^{13}C NMR analysis, the structure stigmast-5-en-ol (**118**) was proposed.

EIMS revealed molecular ion peak $[M]^+$ at *m/z* 414 (35 %), which is consistent with the formula $C_{29}H_{50}O$. Other peaks were observed at *m/z* 396 (20 %) $[C_{29}H_{48}]^+$, 119 (25 %) $[C_9H_{11}]^+$, 69 (37 %) $[C_5H_9]^+$, 55 (77 %) $[C_4H_7]^+$, 43 (100 %) $[C_3H_7]^+$, 41 (53 %) $[C_3H_5]^+$. The ions may be accounted for by the fragmentation pattern in scheme 2.

Scheme 2: Mass spectral fragmentation pattern of β-sitosterol (**118**)



4.5 Stigmasterol (133) (VSH 1 & VPH 3)

The compounds were isolated as white crystalline solids with melting point of 165-167 °C, (lit. mpt. 163-6 °C, Greca *et al.*, 1990). The IR spetra of VSH 1 & VPH 3 revealed the presence of a hydroxyl group at 3398.3, a weak C=C absorption at 1652 and a C-H stretch at 2923 cm^{-1}.

The ^1H NMR data is summarized in table 22.

Table 22: ^1H NMR (400 MHz, $CDCl_3$) data for VSH 1 & VPH 3 (**133**)

Position	δ (obs.)	δ (lit.)	Position	δ (obs.)	δ (lit.)
1	1.00 (2H, m)	-	19	1.05 (3H, s)	1.00
2	1.50 (2H, m)	-	21	1.20 (3H, s)	-
3	3.50 (1H, m)	3.51	22	5.35 (1H, dd; 8.5, 15.2)	5.40
6	5.30 (1H, d; 5.2)	5.35	23	5.05 (1H, dd; 8.4, 15.2)	5.04
7	1.90 (2H, m)	-	26	0.85 (3H, s)	0.84
8	1.50 (1H, m)	-	27	0.80 (3H, s)	0.80
11	1.40 (2H, m)	-	29	0.82 (3H, s)	0.82
18	0.65 (3H, s)	0.67			

Literature data taken from Mohammad *et al.*, (2005)

The ^1H NMR spectra of VSH 1 and VPH 3 revealed a doublet at δ 5.30 (1H, d, H-6) which was assigned to an olefinic proton. Another signal at δ 3.50 (1H, m) suggested the

presence of α-proton typical of sterols hydroxylated at C-3. Some methyl singlets at δ 0.65, 0.85, 0.80, 1.05 and 1.20 were observed and assigned to H-18, 26, 27, 19 and 21, respectively. The multiplet at δ 5.0-5.2 (2H) suggested presence of 2 olefinic protons. The rest of the protons were in complex continuous multiplets spread between δ 1.5-2.2.

^{13}C NMR revealed 29 signals (Table 23).

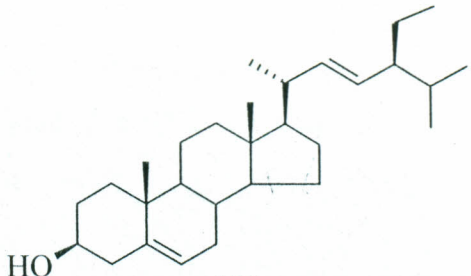
Table 23: ^{13}C NMR (400 MHz, $CDCl_3$) data for VSH 1 & VPH 3 (**133**)

Position	$\delta_{(obs.)}$	$\delta_{(lit)}$	DEPT	Position	$\delta_{(obs.)}$	$\delta_{(lit.)}$	DEPT
1	37.3	37.2	CH_2	16	28.2	28.3	CH_2
2	31.7	31.8	CH_2	17	56.0	56.0	CH
3	71.8	71.5	CH	18	12.2	12.1	CH_3
4	42.2	42.2	CH_2	19	19.4	19.4	CH_3
5	140.8	140.7	C	20	40.5	40.3	CH
6	121.7	121.6	CH	21	19.8	20.5	CH_3
7	34.1	33.6	CH_2	22	138.3	138.5	CH
8	29.2	29.6	CH	23	129.3	129.4	CH
9	50.1	50.1	CH	24	51.2	51.2	CH
10	36.1	36.4	C	25	31.7	31.9	CH
11	21.1	21.1	CH_2	26	21.0	21.2	CH_3
12	39.7	39.7	CH_2	27	19.4	19.8	CH_3
13	42.3	42.2	C	28	25.4	25.4	CH_2
14	56.1	56.1	CH	29	11.9	11.9	CH_3
15	24.3	24.1	CH_2				

Literature data taken from Maria *et al.* (2004).

From ^{13}C NMR, four olefinic carbons at δ 122.1 (C-6), 129.7 (C-23), 138.7 C-22) and 140.8 (C-5) suggested presence of two double bonds. The carbon signal at δ 71.8 suggested oxygenation. From DEPT analysis, there were six methyls at: δ 12.3, 11.9, 19.2, 19.4, 19.8 and 20.2; nine methylenes: at δ 21.1, 24.3, 25.4, 28.2, 31.7, 34.1, 37.3, 39.7 and 42.2; and eleven methines at δ 29.2, 29.6, 40.3, 50.1, 51.2, 56.1, 56.2, 71.8, 121.7, 31.7 and 138.3. The other three signals at: δ 36.1, 42.3 and 140.8 ppm due to quaternary carbons were assigned to C-10, 13 and 5, respectively.

From mpt, NMR analysis and comparison with published data, compouind **133** is stigmasterol) (Maria *et al.*, 2004) was proposed.



133

The spectral data obtained was comparable to that published by Maria *et al.*, (2004).

4.6 Secoisolariciresinol (134) GF/CE/1

The compound was isolated as light-brown solid with melting point of 113-115°C (lit. 112-114 °C, http://www.seqchem.com.). The ^{1}H NMR data is summarized in table 24.

Table 24: ^{1}H NMR (200 MHz, CD_3OD) data for GF/CE/1 (**134**)

Position	δ (obs.)	δ (lit.)	Multiplicity	Integral
2, 3	1.89	1.86	m	2H
1, 4	3.51	3.54	m	4H
7′, 7″	2.48	2.50	m	4H
2′, 2″, 5′, 5″, 6′, 6″	6.55-6.67	6.49-6.61	m	6H
3′, 3″-OCH_3	3.73	3.68	s	6H

Literature data taken from Li *et al.*, (2003)

The signal at δ 1.89 suggested a methine whereas the signal at δ 3.73 suggested methoxy protons. The aromatic protons resonated at δ 6.55-6.67.

^{13}C NMR revealed 10 signals (Table 25).

Table 25: ^{13}C NMR (400 MHz, CD_3OD) data for GF/CE/1 (**134**)

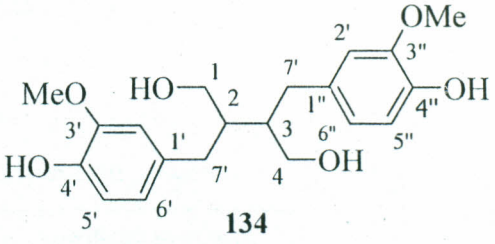
Position	δ (obs.)	δ (lit.)	DEPT
C-1′, C-1″	133.9	133.9	C
C-2′, C-2″	113.4	113.4	CH
C-3′, C-3″	148.9	148.8	C
C-4′, C-4″	145.5	145.5	C
C-5′, C-5″	115.8	115.8	CH
C-6′, C-6″	122.8	122.7	CH
C-7’, C-7’’	36.1	36.0	CH
C-1, C-4	62.2	62.1	CH_2
C-2, C-3	44.0	44.1	CH_2
3′, C-3″-OMe	56.2	56.2	CH_3

Literature data taken from Li *et al.*, (2003)

From ^{13}C NMR, three signals 148.9, 145.5 and 133.9 were shown to be aromatic and qurternary as shown from DEPT. The signals at δ 148.9 and 145.5 suggested

oxygenation at the aromatic ring. Protonated aromatic carbons were revealed at δ 122.8, 115.8 and 113.4. The signal at δ 56.2 suggested the presence of a methoxy group on an aromatic nucleus. The signal at δ 62.2 suggested oxygenation while the one at 36.1 suggested a methine carbon.

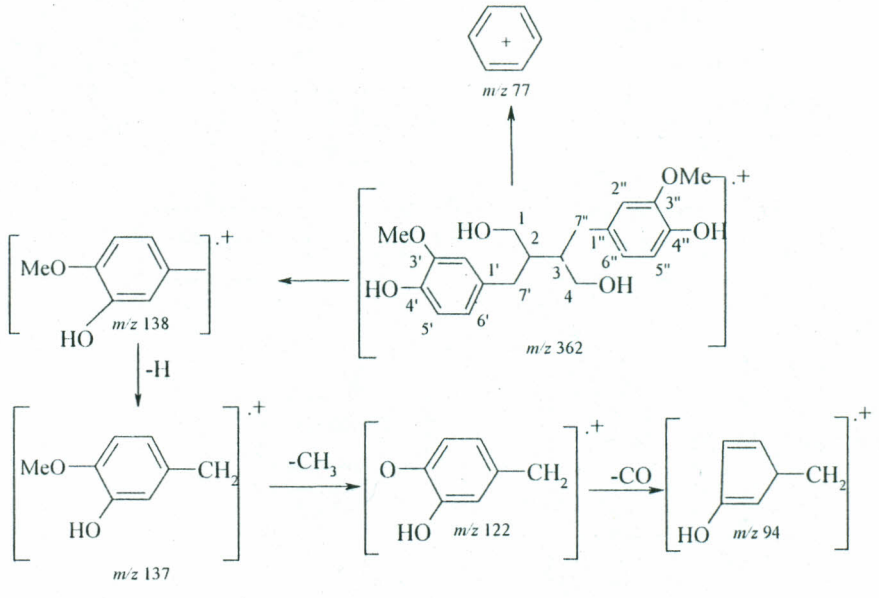
A comparison of the observed ^{1}H NMR and ^{13}C NMR spectral data and literature information confirmed that GF/CE/1 is secoisolariciresinol (**134**) (Li *et al.*, 2003). The reported melting point (112-114 °C) (http://www.seqchem.com) also confirm that GF/CE/1 is secoisolariciresinol.



134

EIMS revealed molecular ion peak $[M]^{+}$ at *m/z* 363 (15 %), which is consistent with the formula $C_{20}H_{27}O_6$. The base peak at *m/z* 137 (100 %) was attributed to the ion $[C_8H_9O_2]^{+}$. Other peaks were observed at *m/z* 138 (22 %) $[C_8H_{10}O_2]^{+}$, 122 (12 %) $[C_7H_6O_2]^{+}$, 94 (8 %) $[C_6H_6O]^{+}$, 77 (6 %) $[C_6H_5]^{+}$. The ions were accounted for by the fragmentation pattern in scheme 3.

Scheme 3: Proposed spectral fragmentation pattern of GF/CE/1 (**134**)

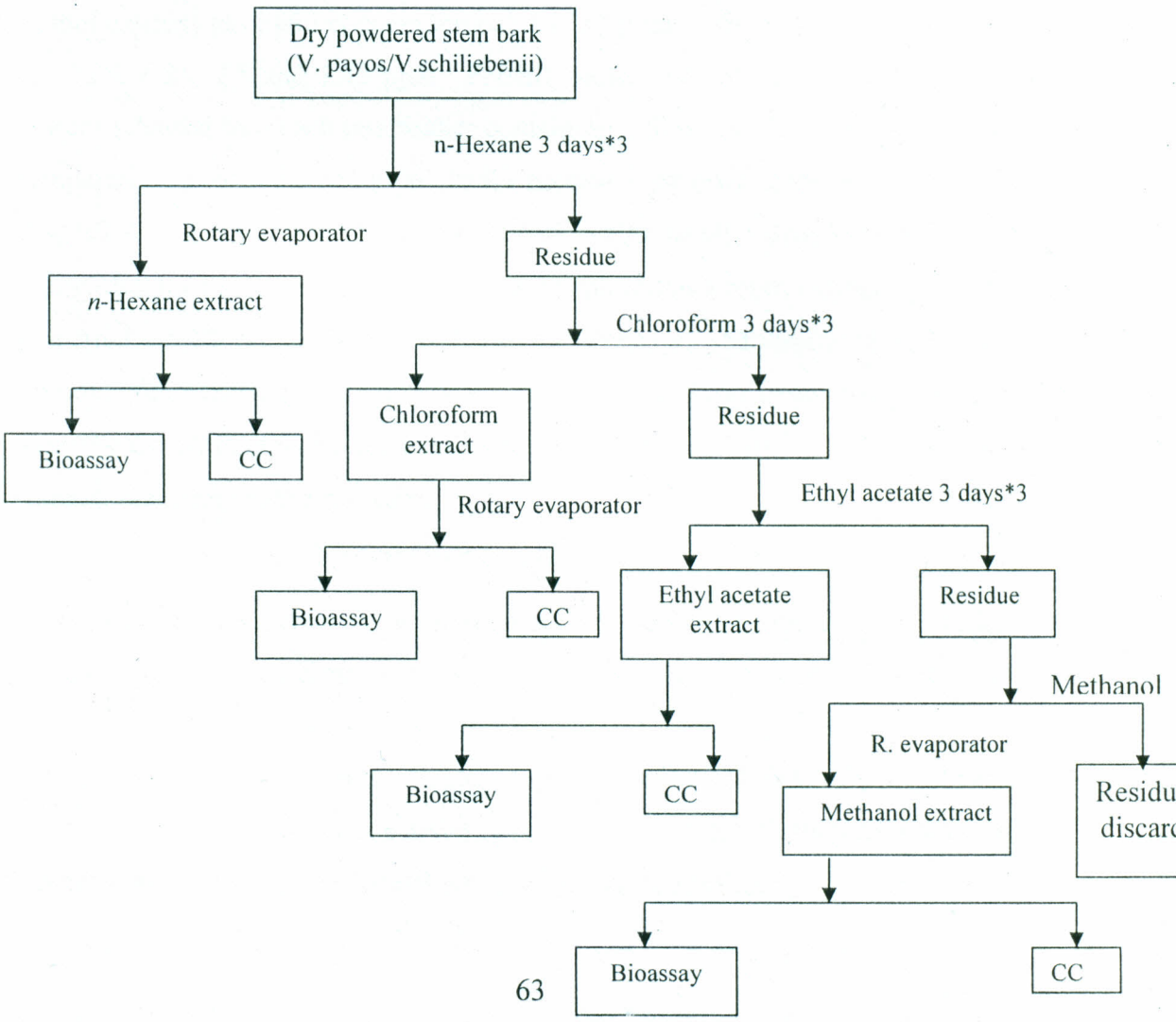


CHAPTER 5: MATERIALS AND METHODS

5.1 Collection and extraction of plants

The stem barks of *V. schiliebenii* and *V. payos* were collected from Watamu (Malindi District) and North Coast (Kilifi District), respectively. Voucher specimens of the plant species were deposited at University of Nairobi Herbarium at the Department of Botany for future reference. The collected plant materials were dried under shade for 7 days and ground into powder. The dry powder (2 kg) was soaked in 5 litres of the solvent for 3 days, with occasional stirring. The solution was decanted and the process repeated 3 times. The solvent extracts were combined, filtered and concentrated at low temperature and pressure in a rotary evaporator. The extraction was done sequentially in solvents of increasing polarity (*n*-hexane, chloroform, ethyl acetate and methanol) to obtain different extracts (Scheme 4).

Scheme 4: Plant material extraction process



5.2 Larvicidal assay

Crude extracts of *n*-hexane, chloroform and ethyl acetate (25 mg) were dissolved in 20 ml of DMSO in acetone (5 %) to make 1250 ppm stock solutions. For methanol extract, 25 mg of the extract was dissolved in 20 ml of water to make 1250 ppm stock solution for bioassay. The stock solutions were serially diluted appropriately to achieve the desired concentrations ranging from 1.25-25 ppm using the dilution formula:

$$C_1V_1=C_2V_2$$

Where: C_1 = concentration of stock solution, C_2 = concentration of sample solution, V_1 =Volume of stock solution and V_2 = Volume of sample solution (http://www.ruf.rice.edu/bioslab/methods/solution/dilutions.html).

5.3 Preliminary bioassay

A stock solution was prepared by dissolving 1.25 mg of each extract in 1 ml of solvent (5 % DMSO in acetone for hexane, chloroform and ethyl acetate extracts, and water for methanol extract) to obtain concentrations of 1250 ppm. The stock solution was diluted to 25, 12.5, 6.25, 2.5 and 1.25 ppm. Freshly molted late 3rd-instar *An. gambiae* larvae (20) were released into each test beaker containing 100 ml of distilled water treated with the respective sample concentration. In the control experiments, the larvae were exposed to 2.0, 1.0, 0.5, 0.2 and 0.1 ml acetone diluted to 100 ml with distilled water. Fish food was provided for the larvae, and the test beakers placed in a room maintained at 26°C in a photoperiod of 12 hours light and 12 hours darkness. Mortality of the larvae was monitored after every 24 hours until the death of the last larva or pupa or emergence into adults. The experiment was replicated 5 times for each concentration. Mortality (%) was calculated according to the formula:

$$\% \text{ Mortality}=\frac{Y \times 100}{Z} \text{ (WHO, 1996).}$$

Where: Z=Initial number of larvae introduced into each test beaker and Y=Mean death defined by the difference between the mean test deaths and the mean control deaths.

Probit analysis was employed to calculate LC_{50} (Finney, 1971; SAS, 2000). The extracts that exhibited high anti-larval activity were considered for further chemical analysis, isolation, purification and identification of the active principles.

IGR activities of pure compounds were tested against *An. gambiae* mosquito larvae. Twenty 3rd-instar larvae were transferred into 250 ml beaker containing some distilled water and then added to 100 ml. The compounds were tested at concentrations ranging from 1 to 50 ppm. Each concentration was replicated 3 times. Two replicates of control were maintained. Mortality of the larvae and pupae were recorded at 24-hour intervals. The treated and untreated beakers containing pupae were kept separately for adult emergence. Morphological abnormalities in pupae and adults were noted, and partially emerged adults recorded as dead. Percentage of emergence inhibition (% EI) in the treated and control were monitored so that the EI_{50} and EI_{90} values determined by probit analysis (Busvine, 1971):

$$\% \text{ EI} = 100 - \frac{\text{Pupae emerged to adult X 100}}{\text{Total No. of emerged pupae}}$$

5.4 Statistical analysis

The anti-larval data for each extract was subjected to log-probit analysis (SAS, 2000). The lethal concentration (LC) was estimated by linear regression analysis (Finney, 1971; SAS, 2000). Overall activity was assessed from the LD_{50} or IE_{50} of the extracts for larvicidal and IGR activity, respectively. Pairwise comparisons of different treatments were made using the t-test and multiple comparisons by analysis of variance (ANOVA).

5.5 General procedures

All solvents used were analytical grade. The active crude extracts were fractionated by column chromatography (CC) on silica gel (0.040-0.063 mm, 230-400 mesh, Merck) and eluted with different solvent systems. The fractions were monitored by TLC and those with same R_f values combined. Analytical TLC was performed on Merck pre-coated silica gel 60 F_{254} (5 x 10 cm x 0.20 mm film thickness) and the compounds located by UV light and/or sprayed with 5% sulphuric acid in methanol, *p*-anisaldehyde and oven dried at 105 °C for 2 minutes. The fractions were assayed for IGR activity and those that exhibited high anti-larval activity subjected to further fractionation, isolation and chemical analysis.

The anti-larval fractions were purified using PTLC or HPLC followed by recrystallization. Structural elucidation of the active compounds was done using, infrared

(IR) ultraviolet (UV), mass spectrometery (MS), and nuclear magnetic resonance (NMR) spectroscopy.

5.6 Isolation of larvicidal compounds from *Vitex payos* stem bark

5.6.1 *n*-Hexane extract of *Vitex payos*

The extract was fractionated by column chromatography (CC) on silica gel 60 G (180 g). Crude *n*-hexane extract (6 g) was dissolved in 10 ml hexane and 10 g silica gel added to this solution. The solvent was evaporated using a rotary evaporator before introducing the mixture of dry silica gel and the extract onto the column. Cotton wool was put on top to provide a stable bed and eluted with varying mixtures of *n*-hexane and ethyl acetate in increasing polarity to give 70 portions which were pooled together based on the R_f values and concentrated *in vacuo* to give 15 fractions. Five fractions were selected for further purification using PTLC on precoated TLC. The result is presented on table 26.

Table 26: The selected fractions of the *n*-hexane extract of *Vitex payos*

Fraction No.	Fractions	Weight (mg)	R_f
FPH I	10-19	107	0.85
FPH 2	20-30	70.3	0.80
FPH 3	31-42	11.2	0.45, 0.73
FPH 4	43-55	2.3	0.3
FPH 5	56-70	5.2	0.32, 0.40, 0.43

5.6.1.1 VPH 1

The first combined fraction (10-19) was chromatographed, (silica gel 120 g), eluting with hexane, hexane-ethyl acetate with gradual increase in solvent polarity. After elution, a white solid (103 mg) was obtained and recrystallized from hexane-chloroform to give 100 mg of VPH 1 (R_f 0.85, C_6H_{14}-$CHCl_3$, 9:1).

5.6.1.2 VPH 2

The second combined fraction (20-30) was chromatographed (silica gel 105 g) eluting with hexane and hexane-ethyl acetate mixtures with gradual increase in the solvent polarity to give a white solid (70 mg) which was recrystallized from hexane-chloroform to yield 69 mg of VPH 2 (R_f 0.80, C_6H_{14}-$CHCl_3$, 8.5:1.5).

5.6.1.3 Stigmasterol VPH 3 and VSH 1 (113)

The third fraction (31-42) was purified using PTLC by precoated TLC plates, eluting with hexane-chloroform (7:3). Colorless crystals (11.2 mg) were obtained and recrystallized from methanol-chloform (1:1) to yield 10 mg of colorless crystalline solid (VPH 3) (R_f 0.45, C_6H_{14}-EtOAc, 7:3) with a melting point of 165-7 °C (lit. 163-6 °C, Greca *et al.*, 1990). A similar compound (VSH 1) was also isolated. The second fraction (13-30) was purified on sephadex LH_{20} and eluted with 1:1 methanol-dichloromethane to give a solid (23 mg), which recrystalized to 21 mg of colorless crystals (VSH 1), with a melting point of 165-7 °C (lit. 163-6 °C, Greca *et al.*, 1990). It was isolated as a colorless crystalline solid (10 mg, R_f 0.45, C_6H_{14}-EtOAc, 7:3): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.00 (2H, *m*, C-1), 1.50 (2H, *m*, C-2), 3.50 (1H, *m*, C-3), 5.30 (1H, *d*, C-6), 1.90 (2H, *m*, C-7), 1.50 (1H, *m*, C-8), 1.40 (2H, *m*, C-11), 0.65 (3H, *s*, C-18), 1.05 (3H, *s*, C-19), 1.20 (3H, *m*, C-21), 5.35 (1H, *dd*, C-22), 5.05 (1H, d*d*, C-23), 0.85 (3H, *s*, C-26), 0.80 (3H, *s*, C-27), 0.82 (3H, *s*, C-29); ^{13}C NMR (400 MHz, $CDCl_3$) δ 12.2 (*q*, C-18), 11.9 (*q*, C-29), 19.4 (*q*, C-19), 19.4 (*q*, C-27), 19.8 (*q*, C-21), 21.0 (*q*, C-26), 21.1 (*t*, C-11), 24.3 (*t*, C-15), 25.4 (*t*, C-28), 28.2 (*t*, C-16), 31.7 (*d*, C-25), 29.2 (*d*, C-8), 31.7 (*t*, C-2), 34.1 (*t*, C-7), 36.1 (*s*, C-10), 37.3 (*t*, C-1), 39.7 (*t*, C-12), 40.5 (*d*, C-20), 42.2 (*t*, C-4), 42.3 (*s*, C-13), 50.1 (*d*, C-9), 51.2 (*d*, C-24), 56.0 (*d*, C-17), 56.1 (*d*, C-14), 71.8 (*d*, C-3), 121.7 (*d*, C-6), 129.3 (*d*, C-23), 138.3 (*d*, C-22), 140.8 (*s*, C-5); EIMS *m/z* 414 (35 %), 396 (20 %), 119 (25 %), 69 (37 %), 55 (100 %), 43 (96 %) and 41 (53 %).

The fourth and fifth combined fractions, (43-70), were not investigated further due to the small amount of the available samples.

5.6.2 Ethyl acetate-chloroform extract of *Vitex payos*

Column chromatography of combined ethyl acetate (3 g) and chloroform (4 g) extracts of *V. payos* yielded three fractions. The first and second fractions, VPCEA 1 and 2 were purified using HPLC on a reverse phase column (C_{18}, water-acetonitrile, 9:1) to yield 5.0 and 4.5 mg of GF/CE/1 and 2, respectively. The third fraction, VPCEA 3 contained a single compound (3 mg, R_f 0.71, C_6H_{14}-EtOAc, 7:3). GF/CE/1: melting point 113-115°C (lit. 112-114°C, http://www.seqchem.com.); ^{1}H NMR (200 MHz, CD_3OD) δ 3.73 (-OCH_3, *s*, C-3', 3''), 1.89 (2H, *m*, C-2,3), 3.51 (4H, *m*, C-1, 4), 2.48 (4H, *m*, C-7', 7''),

6.55-6.67 (6H, *m*, C-2', 2'', 5', 5'', 6', 6''); ^{13}C NMR (200 MHz, CD_3OD) δ 56.2 (s, OCH_3), 36.1 (C-7', 7''), 44.0 (C-2, 3), 62.2 (C-1, 4), 113.4 (C-2', C-2''), 115.8 (C-5', C-5''), 122.8 (C-6', 6''), 133.9 (C-1', 1''), 145.5 (C-4', 4'') and 148.9 (C-3', 3''); IR υ_{max} $(CH_3)_2CO$) 1222.8, 1361.7, 1714.6, and 3004.9 cm^{-1}; EIMS *m/z* at 362 (15 %), $[C_{20}H_{27}O_6]$, 138 (22 %), $[C_8H_{10}O_2]$, 137 (100 %), $[C_8H_9O_2]$, 122 (12 %), $[C_7H_6O_2]$, 94 (8 %) $[C_7H_6O]$ and 77 (6 %) $[C_6H_5]$.

5.7 Isolation of larvicidal compounds from *Vitex schilliebenii* stem bark

5.7.1 *n*-Hexane extract of *Vitex schilliebenii*

The extract (6 g), was first subjected to fractionation using silica gel chromatography. Elution was done with *n*-hexane and *n*-hexane-ethyl acetate mixture with increasing polarity. The portions were collected into 6 fractions, analyzed by TLC and 2 fractions were selected for further purification (Table 27).

Table 27: The selected fractions of the *n*-hexane extract of *Vitex schilliebenii*

Fraction No.	Fractions	Weight (mg)	Rf
FSH 2	9-12	30	0.35
FSH 1	13-30	23	0.45

5.7.1.1 β-sitosterol (118) (VSH 2)

The first fraction (9-12) was purified on silica gel eluted with hexane and hexane-ethyl acetate mixture with increasing solvent polarity to give a solid (28 mg) which was recrystallized from methanol-chloroform (1:1) to yield 27 mg white crystalline material (VSH 2) (R_f, 0.35, C_6H_{14}-EtOAc, 6:4) with melting point of 134-6 °C (lit. 130-134 °C, McCarthy *et al.*, 2005). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.01 (2H, *m*, C-1), 1.40 (2H, *m*, C-2), 3.50 (1H, *m*, C-3), 5.35 (1H, *d*, C-6), 1.90 (2H, *m*, C-7), 1.55 (1H, *m*, C-8), 1.75 (1H, *s*, C-9), 1.42 (2H, *m*, C-11), 1.70 (2H *m*, C-12), 1.30 (2H, *s*, C-16), 1.75 (1H, *m*, C-17), 0.70 (1H, *s*, C-18), 0.98 (3H, *s*, C-19), 1.90 (1H, *s*, C-20), 0.90 (3H, *s*, C-21), 0.82 (3H, *s*, C-26), 0.8 (3H, *s*, C-27), 0.85 (3H, *s*, C-29); ^{13}C NMR ($CDCl_3$) δ 11.9 (*q*, C-18), 11.8 (*q*, C-29), 19.1 (*q*, C-21), 18.8 (*q*, C-19), 19.4 (*q*, C-26), 19.8 *q*, C-27), 21.1 (*t*, C-11), 23.3 (*t*, C-28), 24.3 (*t*, C-15), 26.1 (*t*, C-12), 26.2 (*t*, C-23), 28.2 (*d*, C-25), 29.4 (*t*, C-2), 29.2 (*d*, C-8), 31.7 (*t*, C-7), 34.0 (*d*, C-20), 37.3 (*t*, C-1), 36.5 (*s*, C-10), 39.8, (*t*, C-

16), 42.3 (*t*, C-4), 45.9 (*s*, C-13), 37.3 (*s*, C-22), 50.2 (*d*, C-24), 50.2 (*d*, C-9), 56.1 (*d*, C-17), 56.8 (*d*, C-14), 71.8 (*d*, C-3), 121.7 (*d*, C-6), 140.8 (*s*, C-5)

5.7.2 Combined chloroform and ethyl acetate extract of *Vitex schilliebenii*

Chloroform extract (3 g) and ethyl acetate (3 g) mixture was first subjected to fractionation using silica gel chromatography. Elution was done with increasing polarity of hexane and ethyl acetate mixture starting from hexane to pure ethyl acetate. Fractions (50) were collected, analyzed by TLC and combined into 5 fractions of which 3 were selected for further purification (Table 28)

Table 28: The selected fractions of the chloroform-ethyl acetate extract of *Vitex schilliebenii*

Fraction No.	Fractions	Weight (mg)	R_f
FSCEA 1	20-27	15	0.77
FSCEA 2	28-36	50	0.54
FSCEA 3	37-48	40	0.63

5.7.2.1 VSCE 1

The first fraction (20-27) contained mainly chlorophyll. No pure compound was isolated from this fraction.

5.7.2.2 20-Hydroxyecdysone (80) (VSCE 2)

The second fraction (28-36) was purified using sephadex eluted with 1:1 methanol-dichloromethane to give a white solid (48 mg) that was recrystallized from methanol-chloroform (1:1) to yield VSCEA 2 with melting point of 234-236 °C (lit. 230-233 °C, Kavel *et al.*, 1998). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.65 (1H, *m*, C-2), 3.75 (1H, *m*, C-3), 2.10 (1H, *m*, C-5), 5.65 (1H, *s*, C-7), 2.98 (1H, *m*, C-9) 2.05 (1H, *m*, C-12a), 1.75 (1H, *m*, C-12b), 1.85 (1H, *m*, C-15a), 1.50 (1H, *m*, C-15b), 1.65 (1H, *m*, C-16a), 1.87 (1H, *m*, C-16b), 2.27 (1H, *m*, C-17), 0.75 (3H, *s*, C-18), 0.86 (3H, *s*, C-19), 1.09 (3H, *s*, C-21), 3.15, (1H, *t*, C-22), 1.05 (3H, s, C-26), 1.05 (3H, *s*, C-27), 0.86 (3H, *s*, C-19), 1.09 (3H, *s*, C-21), 3.15 (1H, t, C-22), 1.48 (1H, *m*, C-23a), 1.11 (1H, *m*, C-23b), 1.25 (1H, *m*, C-24a), 1.68 (1H, *m*, C-24b), 1.05 (3H, s, C-26), 1.05 (3H. *s*, C-27); ^{13}C NMR ($CDCl_3$) δ 17.4 (C-18), 21.1 (C-21), 20.7 (C-11), 20.5 (C-16), 24.1 (C-19), 26.5 (C-23), 29.1 (C-27), 29.1 (C-26), 29.9 (C-12), 29.9 (C-15), 31.3 (C-4), 33.7 (C-9), 41.6 (C-24), 36.9 (C-10), 38.1

(C-1), 47.4 (C-13), 48.4 (C-17), 50.6 (C-5), 67.1 (C-3), 67.3 (C-2), 69.5 (C-25), 76.3 (C-20), 76.8 (C-22), 83.6 (C-14), 120.9 (C-7), 165.9 (C-8), 203.6 (C-6); IR υ_{max} (KBr) 3398.3, 1651.0 cm^{-1}; EIMS *m/z* at 363 (8 %), 345 (22 %), 327 (20 %), 309 (5 %), 301 (25 %) and 283 (20 %).

5.7.2.3 22-*Epi*-20-hydroxyecdysone (82) VSCE 3

The third fraction (37-48) was purified using HPLC on a reverse phase column (C_{18}, water-acetonitrile, 8:2, Rt was 7.29 min.) to yield a white solid (38 mg) that was recrystallized from methanol-chloroform (1:1) to give VSCEA 3 (36 mg) of melting point 240-242 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.56 (1H, *br, t* C-2), 3.67 (1H, *s*, C-3), 1.29 (1H, *t*, J = 7.8, C-4a), 1.67 (1H, *m*, C-4b), 2.10 (1H, *dd*, 4.4, 4.4, C-5), 5.53 (1H, *s*, C-7), 2.80 (1H, *t*, J = 5.4, C-9), 1.11 (*t*, 10.2, C-10), 1.44 (1H, *m*, C-11a), 1.52 (1H, *m*, C-11b), 1.44 (1H, *m*, C-12a), 1.57 (1H, *m*, C-12b), 1.80 (1H, *ddd*, 3.6, 3.6, 3.6, C-15a), 2.80 (1H, *m*, C-15b), 2.05 (1H, *t*, 6.8, C-17), 0.57 (3H, *s*, C-18), 0.68 (3H, *s*, C-19), 0.98 (3H. *s*, C-21), 3.05 (1H, *s*, C-22), 1.01 (1H, *m*, C-23a), 1.37 (1H, *m*, C-23b), 1.17 (1H, *m*, C-24a), 1.47 (1H, *m*, C-24b), 0.91 (3H, *s*, C-26), 0,91 (3H, *s*, C-27); ^{13}C NMR ($CDCl_3$) δ 20.4 (C-21), 17.4 (C-18), 20.6 (C-16) 20.7 (C-11), 24.0 (C-19), 26.3 (C-23), 29.0 (C-26), 28.8 (C-27), 31.4 (C-15), 31.6 (C-12), 31.0 (C-4), 34.1 (C-9), 41.3 (C-24), 38.4 (C-1), 36.5 (C-10), 49.4 (C-17), 47.6 (C-13), 50.6 (C-5), 67.4 (C-3), 67.8 (C-2), 70.6 (C-25), 77.9 (C-20), 78.1 (C-22), 84.3 (C-14), 121.3 (C-7), 166.9 (C-8), 205.8 (C-6).

CHAPTER 6: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The purpose of this study was to investigate the larvicidal and IGR potential of *V. schiliebenii* and *V. payos* against *An. gambiae*. The following findings were made:

1) The yield of the crude extracts of *V. payos* and *V. schiliebenii* was found to increase with the polarity of the extraction solvents.
2) *Vitex payos* and *V. schiliebenii* extracts were found to exhibit larvicidal and IGR activity against 3rd instar *An. gambiae* larvae.
3) *Vitex payos n*-hexane extract exhibited the highest larvicidal activity followed by chloroform and methanol while ethyl acetate had the least activity.
4) *Vitex schiliebenii* methanol extract exhibited the highest larvicidal activity followed by *n*-hexane and ethyl acetate while chloroform showed the least activity.
5) Five compounds were isolated from *V. payos* (3 from *n*-hexane, 2 from chloroform-ethyl acetate mixture, and 1 from methanol extract) but none was bioassayed for IGR activity due to small quantities available.
6) From *V. schiliebenii*, four compounds were isolated (2 from *n*-hexane and 2 from chloroform-ethyl acetate mixtrure) and assayed for IGR activity. All the isolated compounds exhibited IGR activity.
7) Stigmasterol and secoisolariciresinol were isolated from *V. payos* and identified while two other compounds were isolated in too small amounts for spectroscopic analysis. Stigmasterol, stigmast-5-en-3-ol, and two ecdysteroids (20-hydroxyecdysone and 22-*epi*-20-hydroxecdysone) were isolated from *V. schiliebenii*. .

6.2 Conclusions

From the findings of this study, the following conclusions were made:

1) *Vitex payos* and *V. schiliebenii* extracts exhibit larvicidal activity against 3rd-instar *An. gambiae* larvae under laboratory conditions. Larval mortality was high at the high concentrations (25 ppm), suggesting toxicity concentration. The larvicidal activity increased with exposure

time at the lower concentrations suggesting the IGR activity of the extracts.

2) Extracts from both plants did not exhibit any significant difference in their efficacy hence may be having similar larvicidal principles.

3) The four compounds isolated and assayed were toxic to the 3rd-instar larvae at 50 ppm, induced morphological abnormalities, delayed growth and emergence at lower concentrations suggesting IGRs activity against *An. gambiae* larvae.

Generally, results in this study show that *V. Payos* and *V. schiliebenii* have potential for *An. gambiae* control.

6.3 Implications of the study

The larvicidal and IGR activity exhibited by the two plants need to be investigated further for the control of mosquitoes at the larval stage. This will be of importance in the control of the malaria vectors which have developed insecticide resistance. Fewer IGR compounds have been evaluated against mosquitoes, compared to the conventional larvicides. Only two IGR compounds: methroprene (a juvenoid-JH mimic) and diflubenzuron (chitin synthesis inhibitor), are available for use in the control of vectors of public health importance. However, they are not recommended for use in drinking water sources. Consequently, evaluation of more IGRs against malaria vectors is essential for the development of new tools for vector control programme.

6.4 Recommendations for further research

The following recommendantions are made based on the findings and conclusions:

1) Since the plants have shown larvicidal and IGR activity in the laboratory, it would be important to carry out field trials in small pools, ponds, swamps streams and drainages to evaluate their efficacy in mosquito control.

2) There is need for field assessment of both crude extracts and the active compounds to enable formulation of suitable mosquito control products.

3) The effect of the plant materials on non-target aquatic organisms and mammals need to be investigated for the establishment of the toxicity profiles and assessment of their suitability for mosquito control.

4) The mode of their action as larvicides and IGRs need to be investigated and understood to ensure safety and inspire structure-activity relationship studies to enhance efficacy.

5) To study assay properties of the pure compounds VPH1, VPH2, GF/CE/1 and GF/CE/2.

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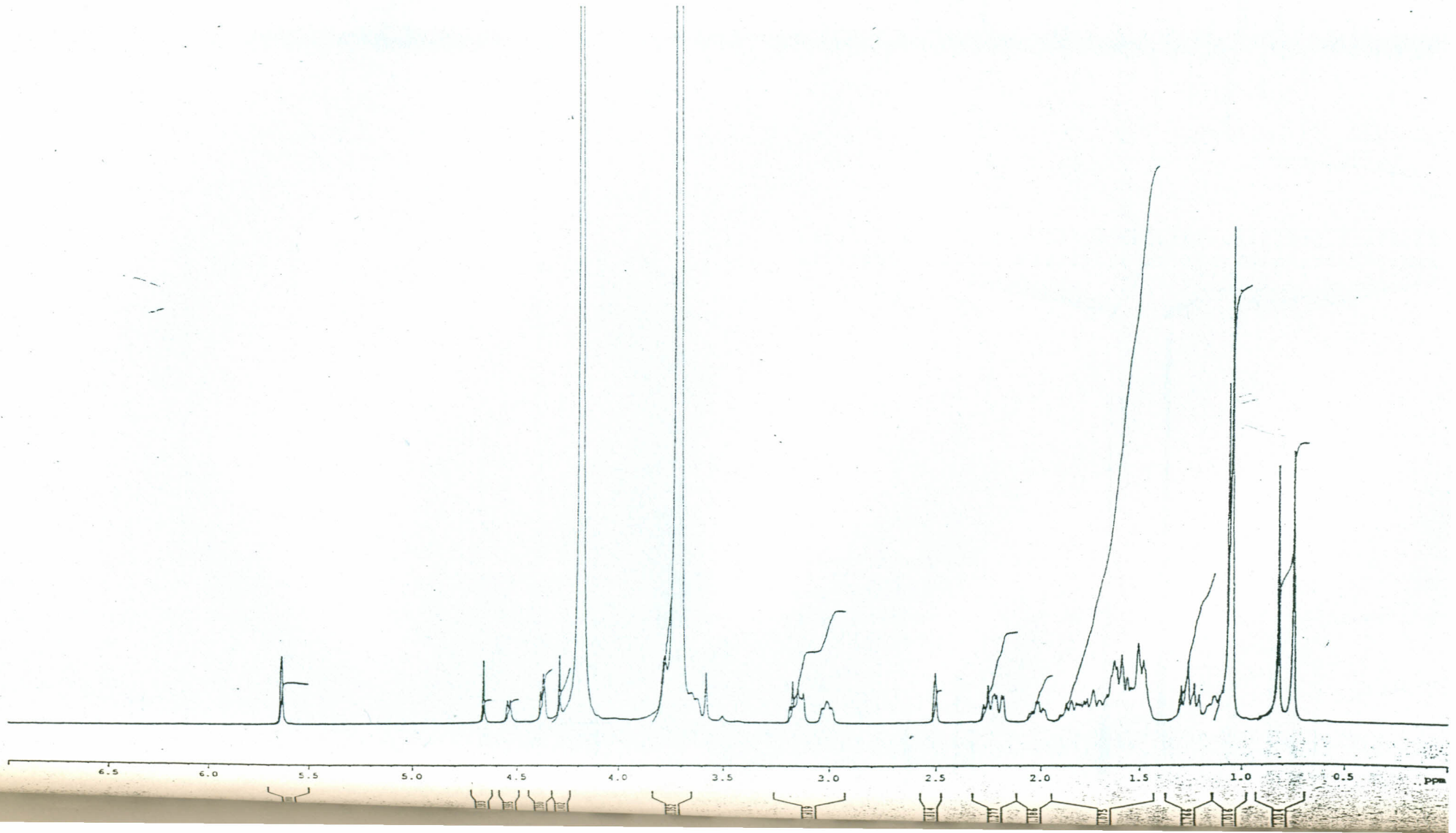
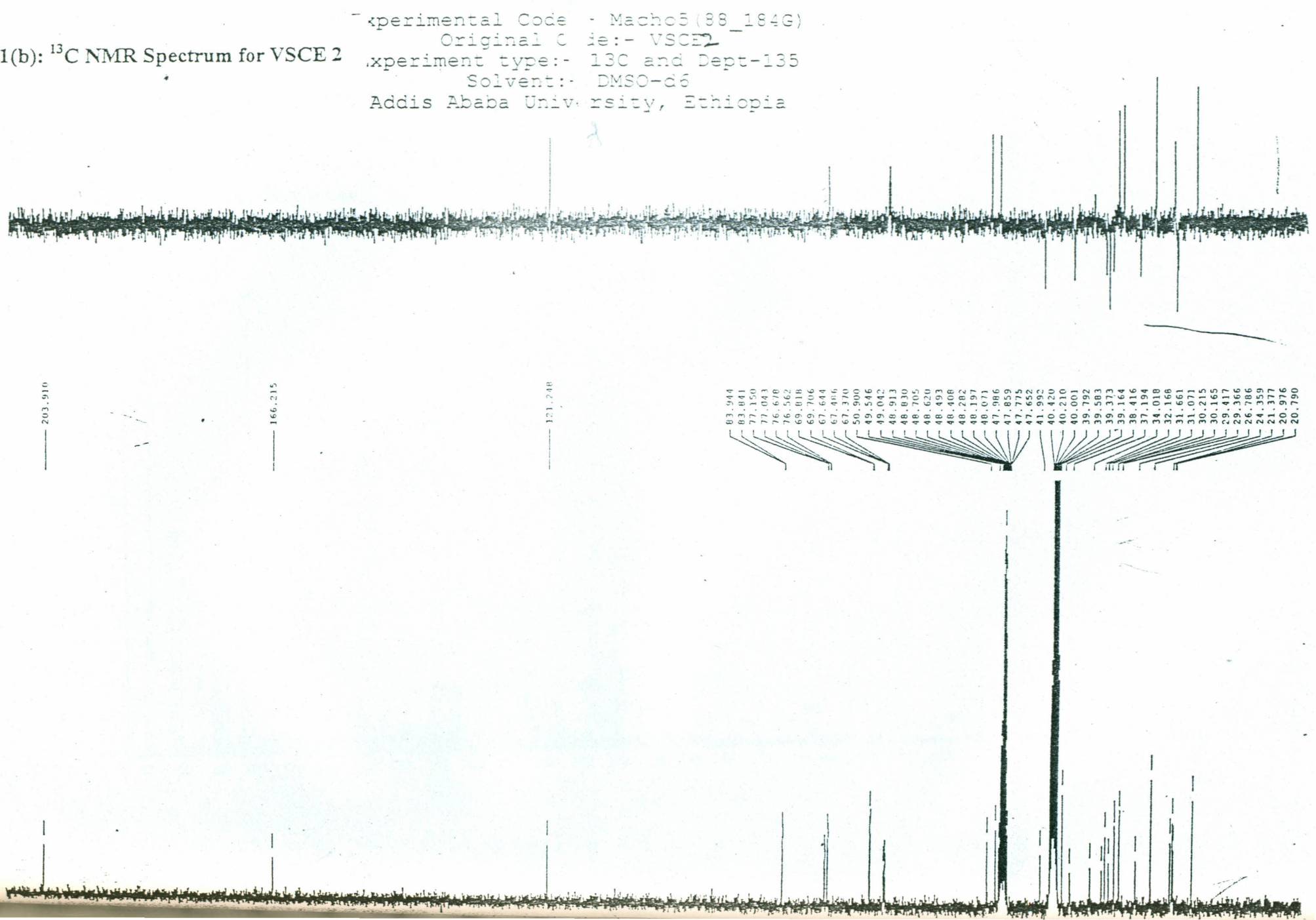
Appendix 1(a): ^{1}H NMR Spectrum for VSCE 2

Experimental Code:- Macho5(88_184G)
Original Code:- VSCE2
Experiment type:- 1H
Solvent:- DMSO-d6
Addis Ababa University, Ethiopia



Appendix 1(b): ^{13}C NMR Spectrum for VSCE 2



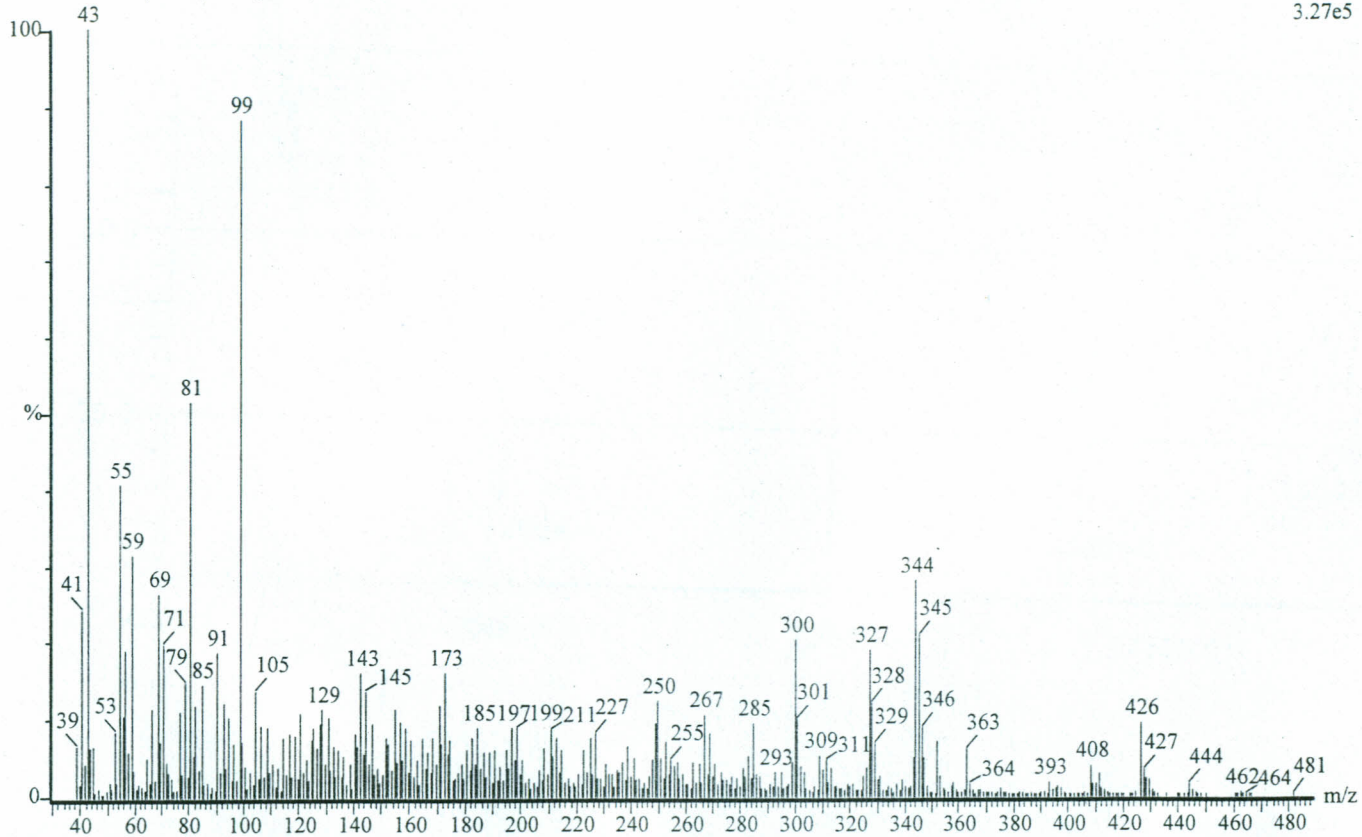
Appendix 1(c): Mass spectrum for VSCE 2

INS: VG 12-250 UPGRADE
BpM: 43
Sample VSCE 2 155mg by SOLID PROBE

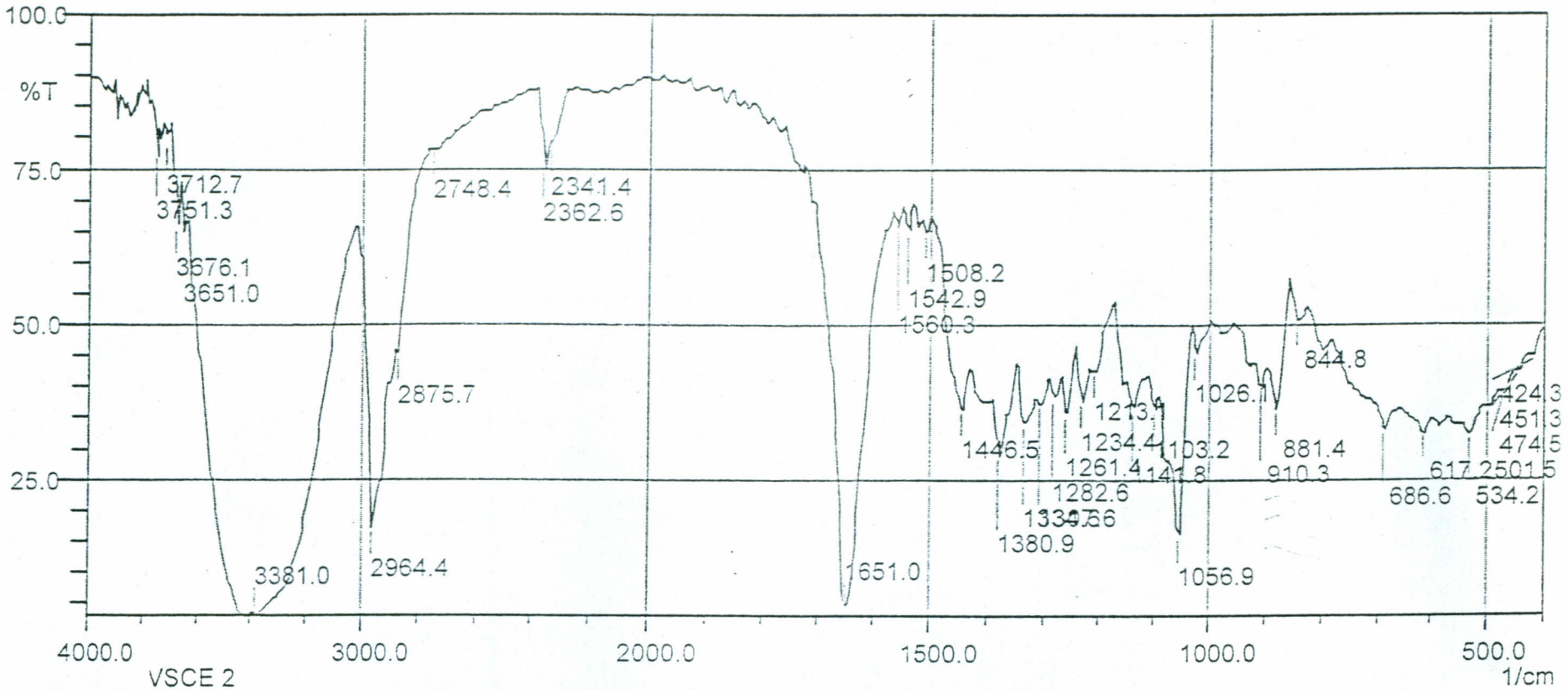
Date: 26-Oct-2005 Time: 14:06:54
BpI: 326592

GK26105B 53 (1.792) Cm (15:53)

Scan EI+
3.27e5



IR SPECTRUM FOR VSCE 2



VSSEA2
1H

1.00	5.530
12.35	4.310
0.37	3.909
0.97	3.666
0.99	3.558
0.05	3.542
1.42	3.535
0.97	3.369
1.97	3.061
0.98	3.040
1.95	2.820
9.90	2.802
1.96	2.786
1.05	2.119
8.92	2.108
2.91	2.096
2.99	2.085
	2.066
	2.049
	2.032
	1.834
	1.825
	1.808
	1.799
	1.782
	1.773
	1.708
	1.684
	1.671
	1.654
	1.628
	1.199
	1.190
	1.169

0 500 1000 1500 2000 2500

V55EA2

3.909
3.666
3.558
3.542
3.535
3.369
3.061
3.040
2.820
2.802
2.786

1000
500
0

0.37
0.97
0.99
0.05
1.42
0.97

2.119
2.108
2.096
2.085
2.066
2.049
2.032
1.834
1.825
1.808
1.799
1.782
1.773
700
600
500
400
300
200
100
0
-100
1.97
0.98
2.20

VSS E4 1H
1.95
9.90
1.708
1.684
1.671
1.654
1.628
1.583
1.564
1.558
1.528
1.520
1.502
1.492
1.479
1.467
1.442
1.419
1.393
1.387
1.367
1.343
1.323
1.310
1.291
1.270
-100
0
100
200
300
400
500
600
700

1.96
1.05
8.92
1.199
1.190
1.169
1.151
1.142
1.132
1.106
1.081
1.043
1.033
1.022
1.013
0.996
0.986
0.974
0.964
0.908
0.900
-100
0
100
200
300
400
500
600
700

VSSEA2

H

0.908

0.900

0.681

0.573

4000

3000

2000

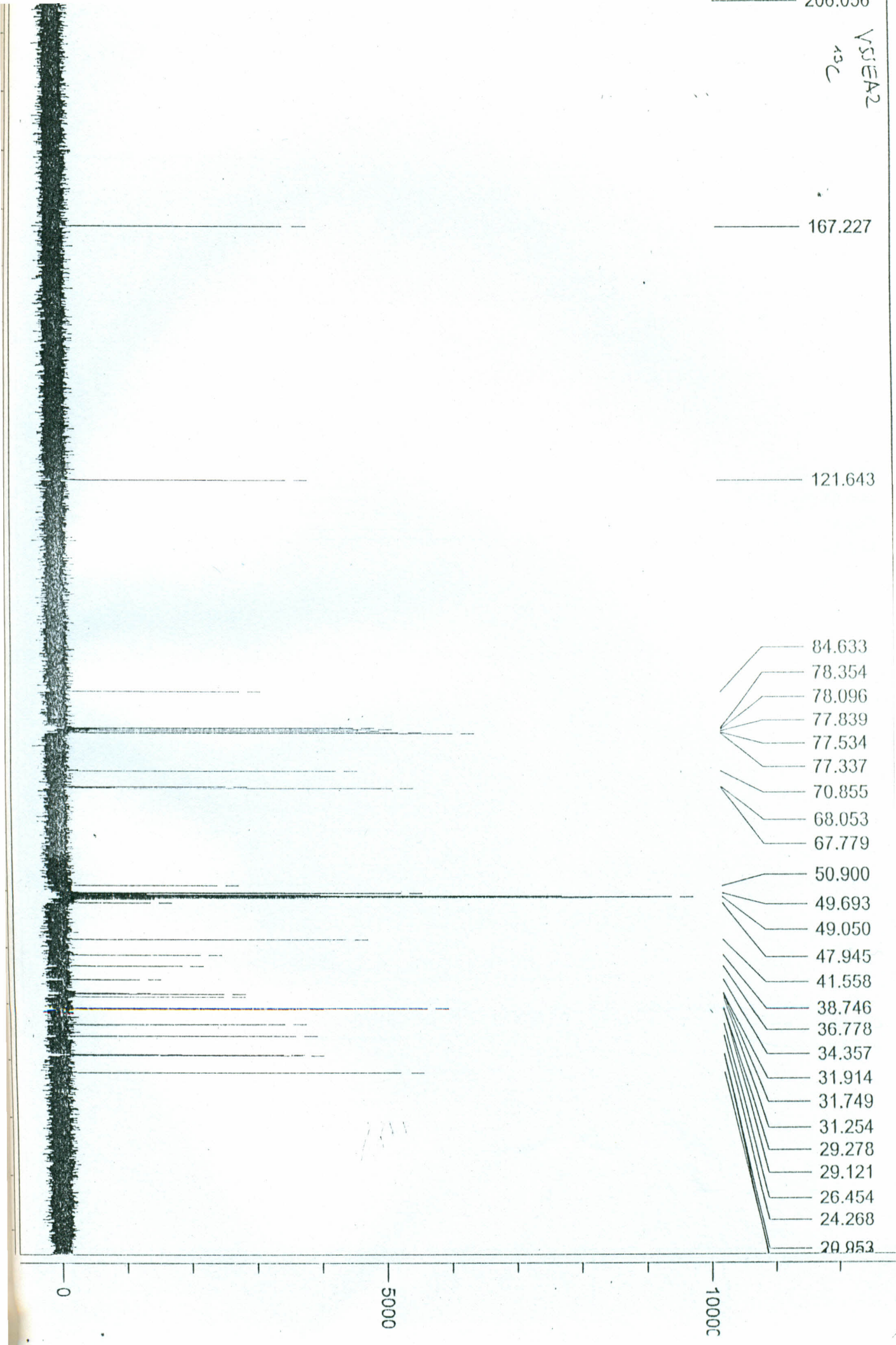
1000

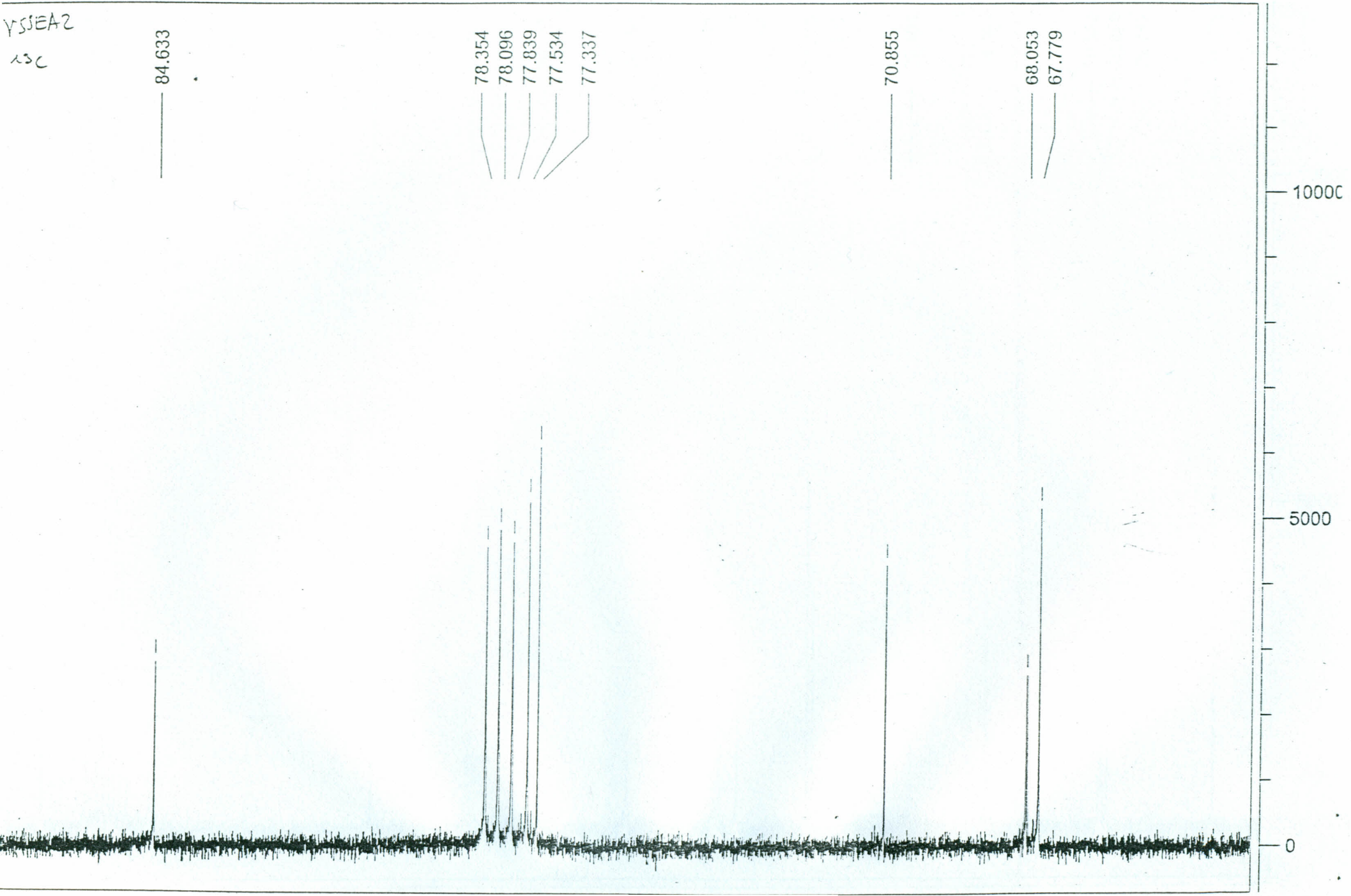
0

8.92

2.91

2.99


206.056
VSJEA2
13C
167.227
121.643
84.633
78.354
78.096
77.839
77.534
77.337
70.855
68.053
67.779
50.900
49.693
49.050
47.945
41.558
38.746
36.778
34.357
31.914
31.749
31.254
29.278
29.121
26.454
24.268
20.953
0
5000
10000


YSSEA2
13C
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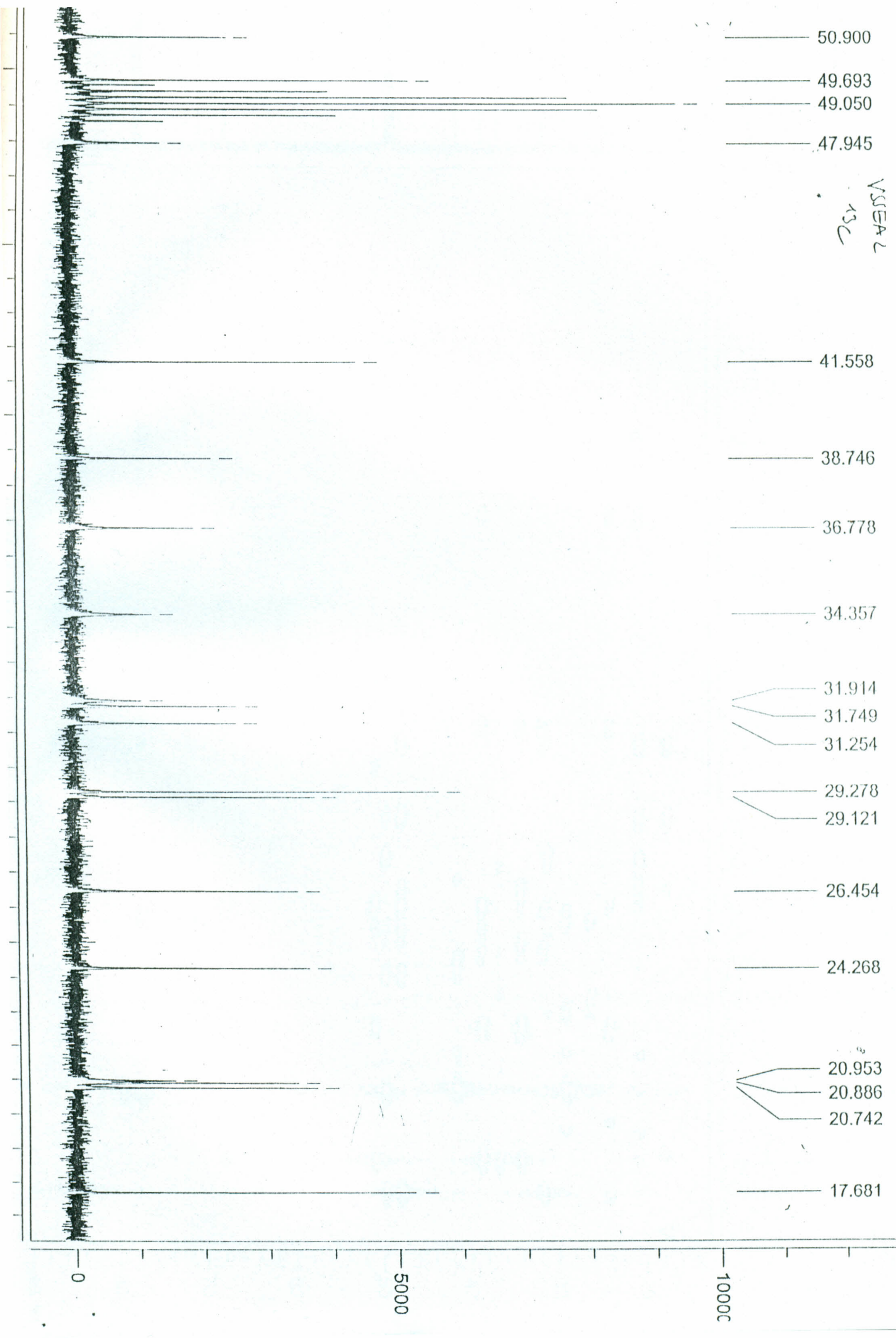
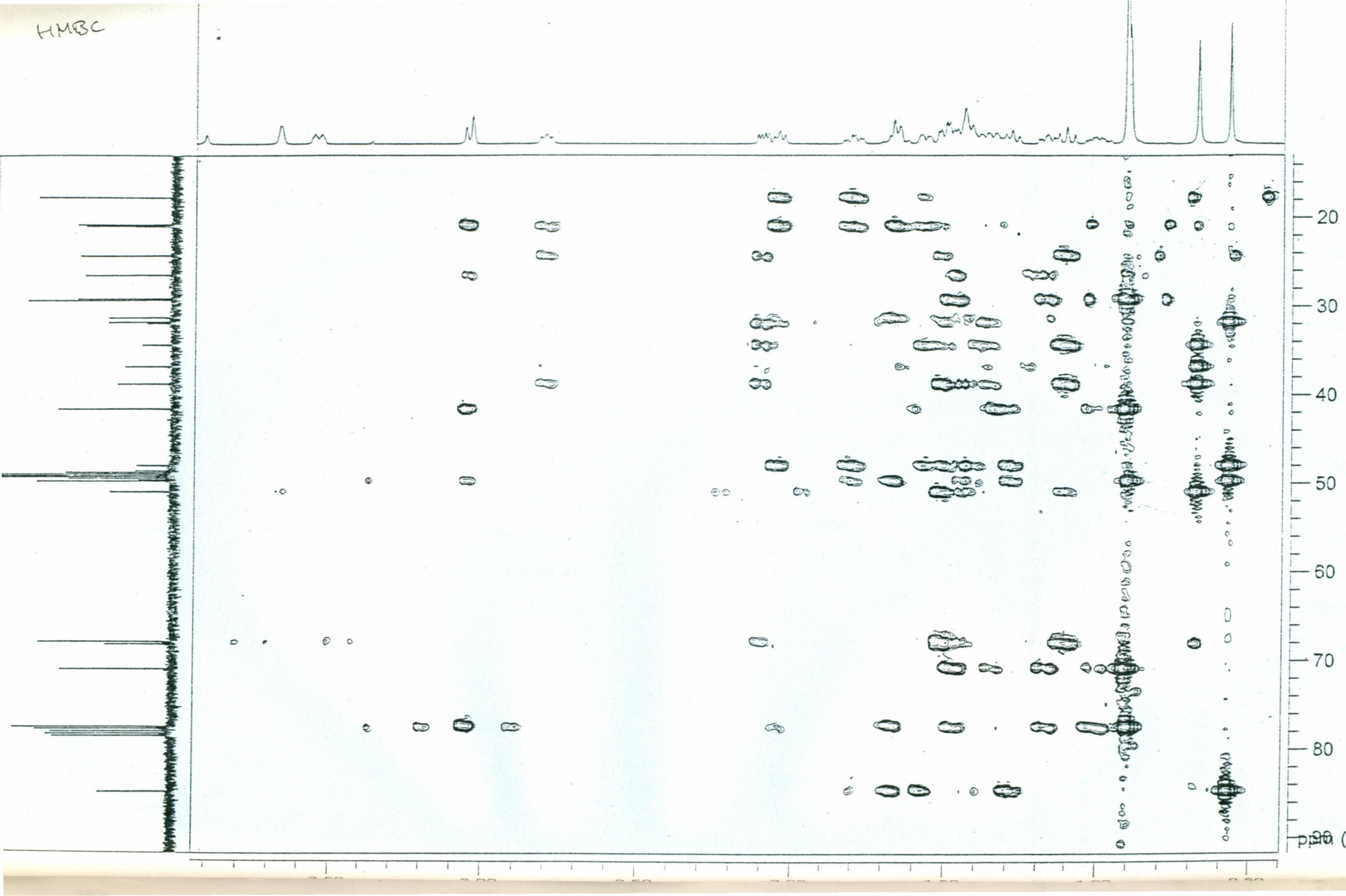
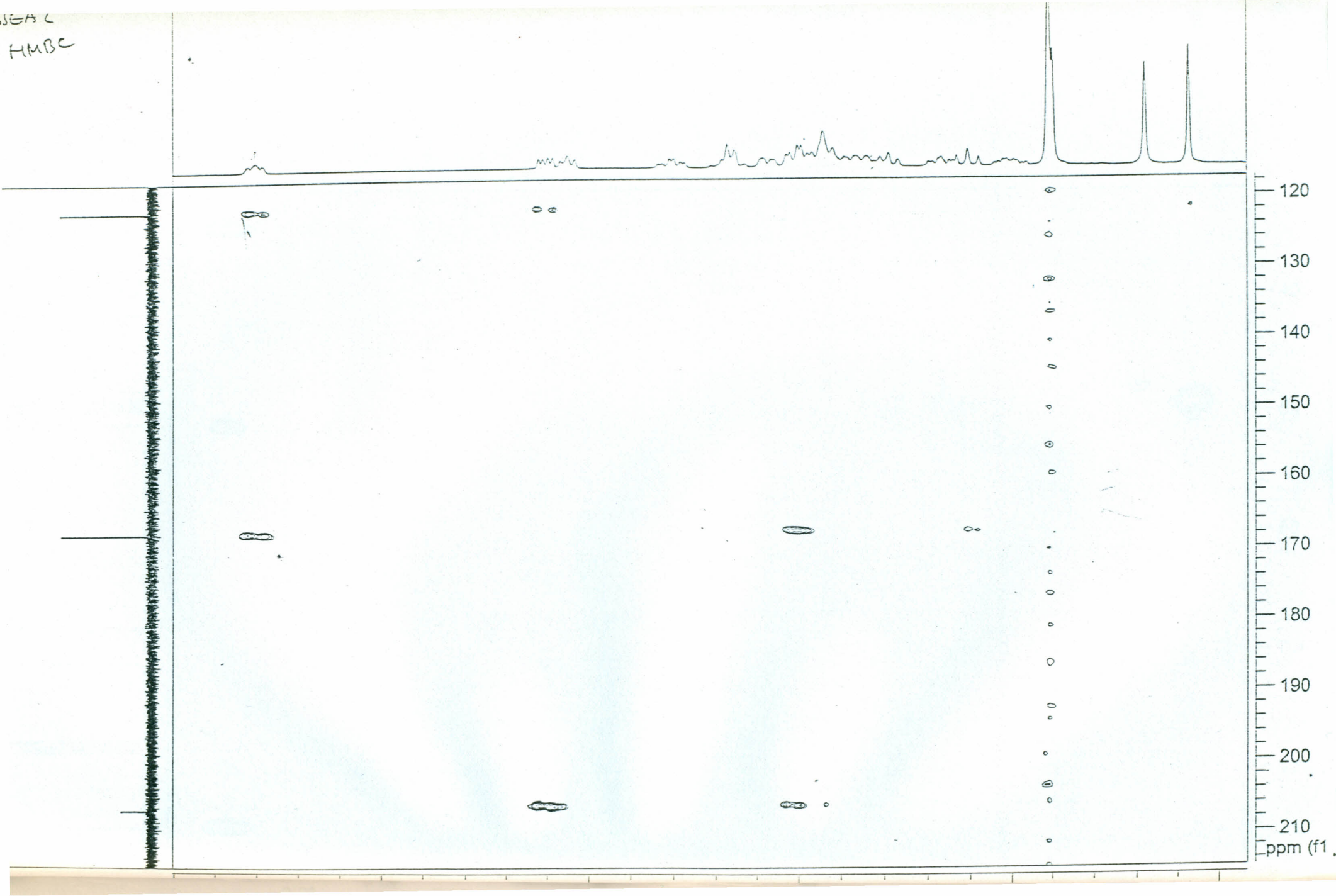
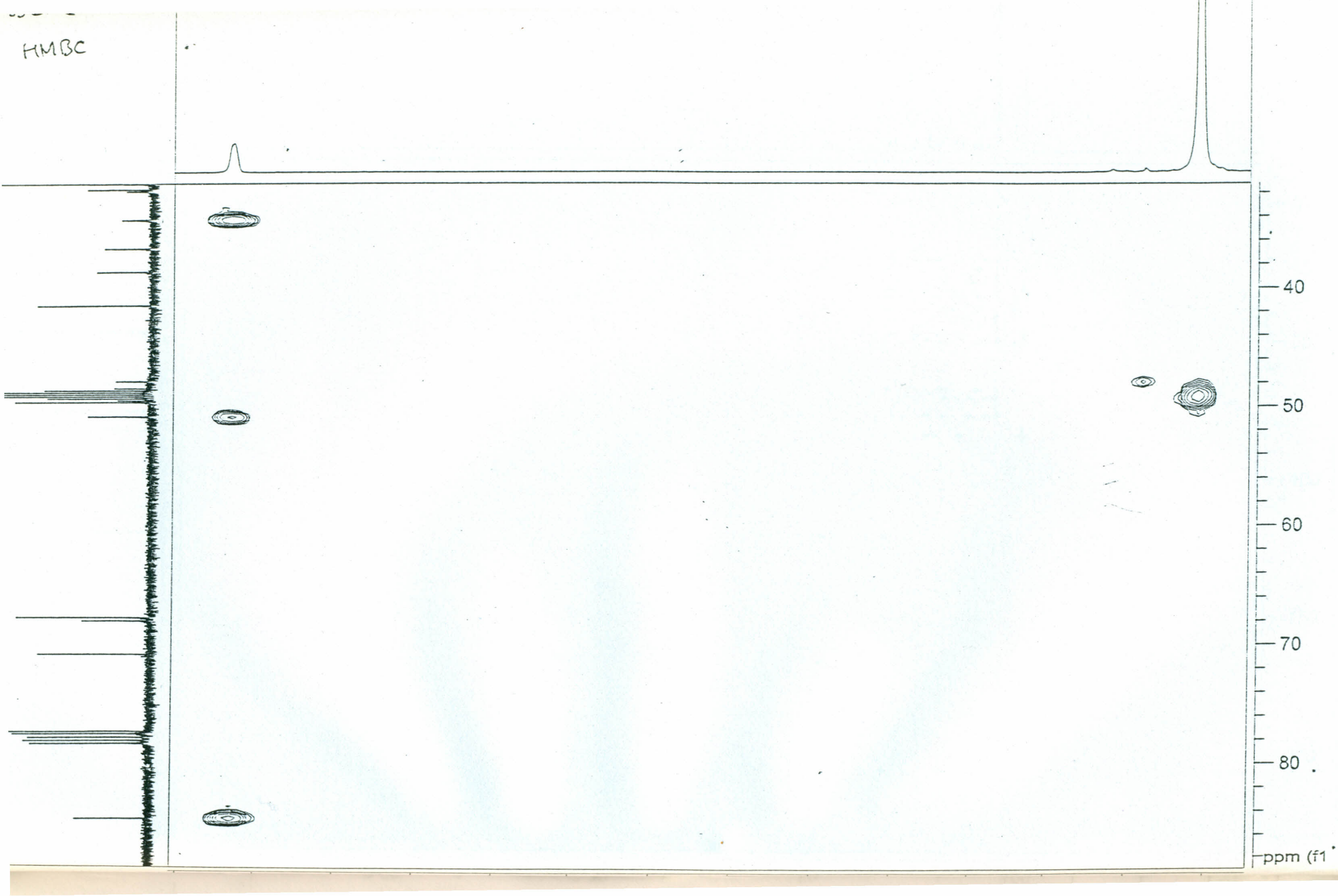
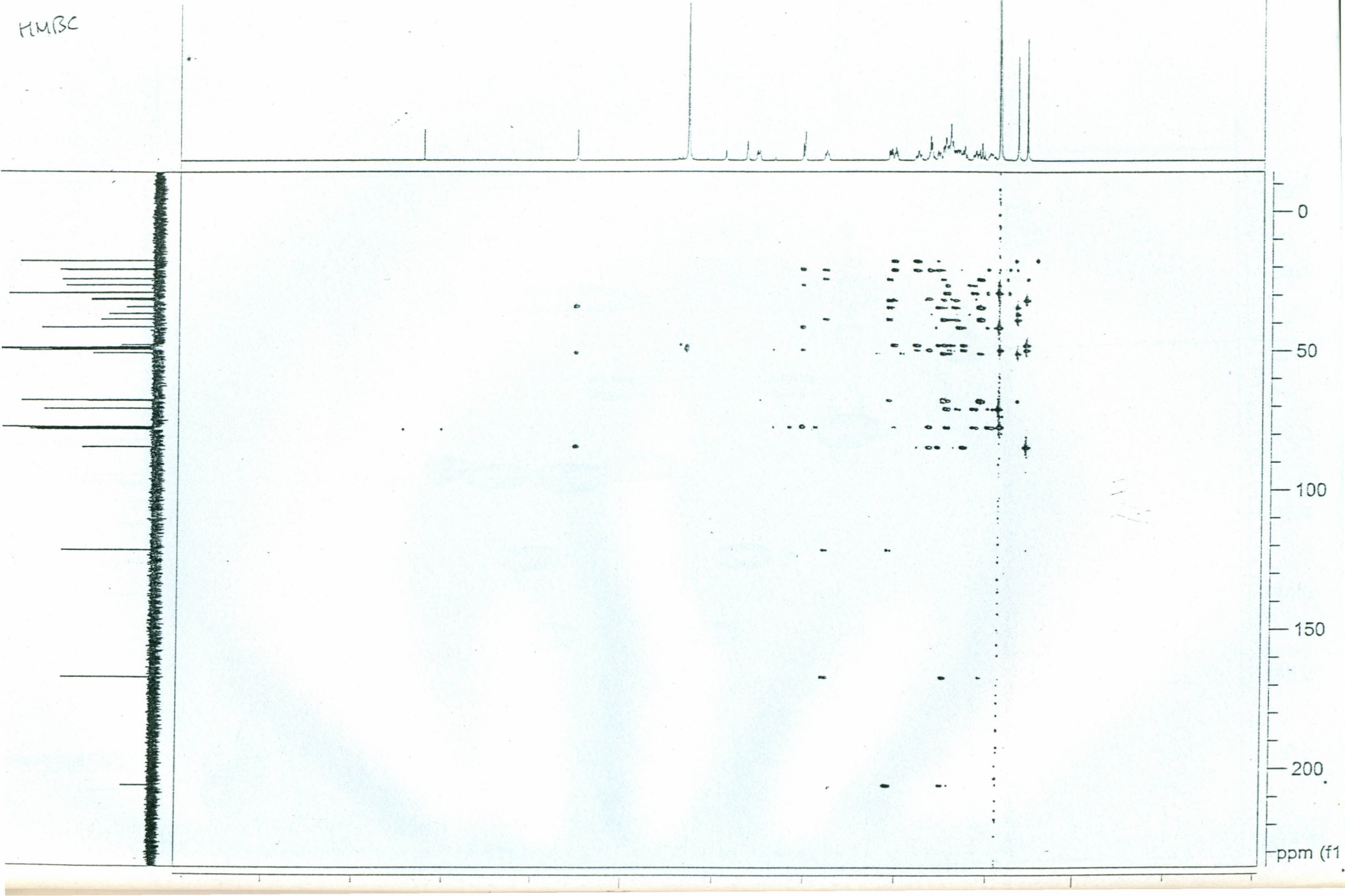
78.096
77.839
77.534
77.337
70.855
68.053
67.779
10000
5000
0

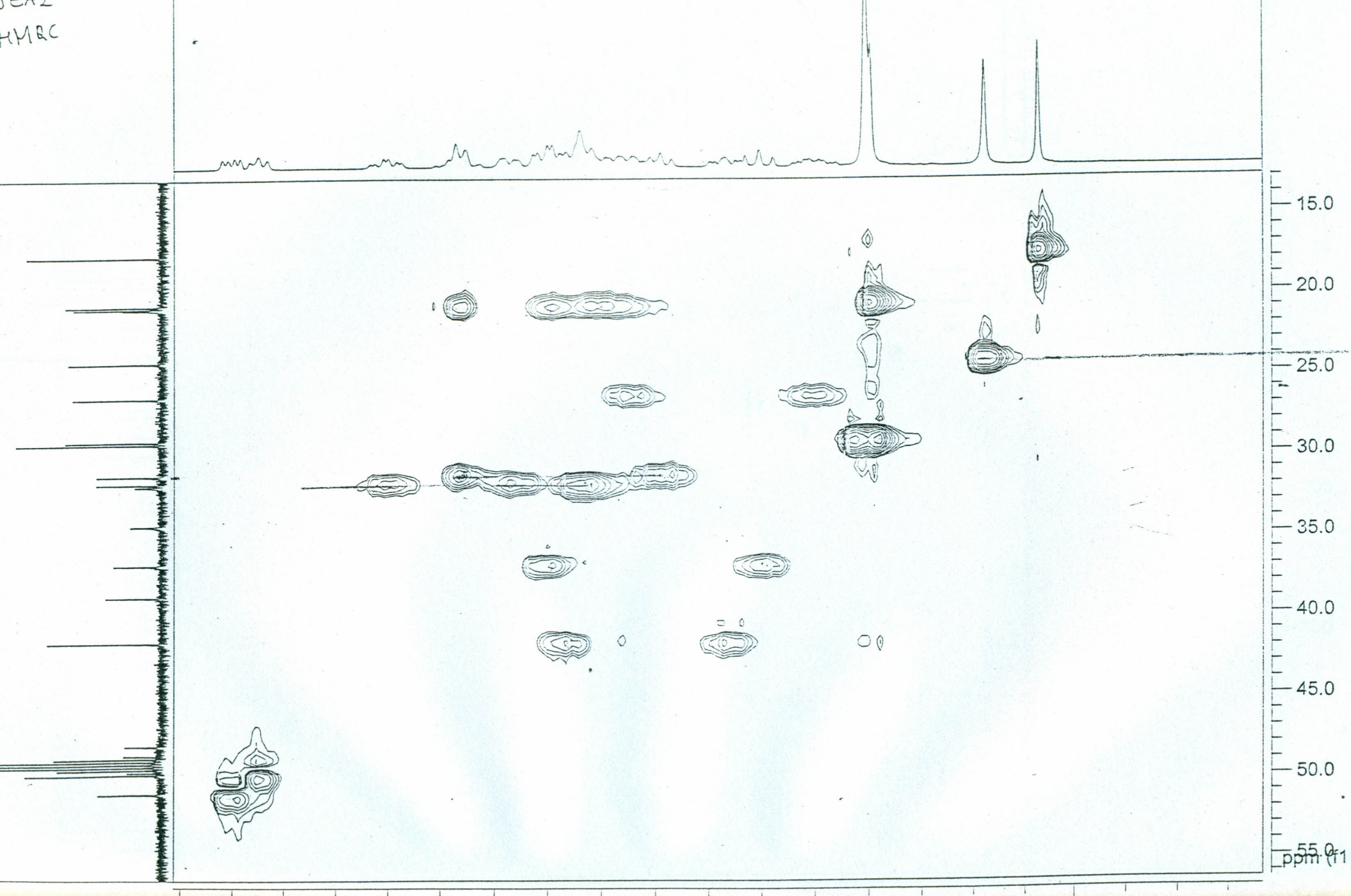

50.900
49.693
49.050
47.945
41.558
38.746
36.778
34.357
31.914
31.749
31.254
29.278
29.121
26.454
24.268
20.953
20.886
20.742
17.681
0
5000
10000

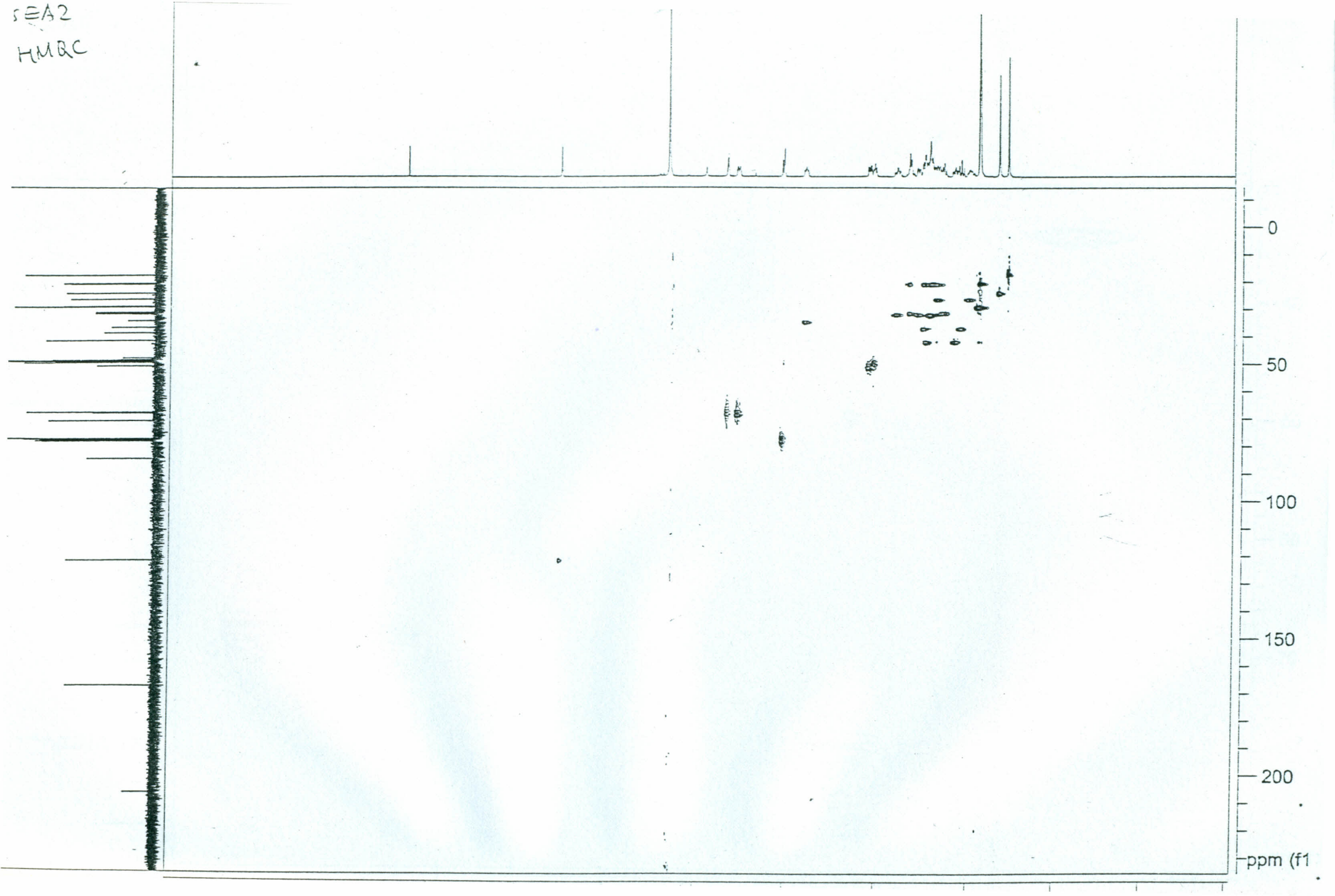

HMBC
20
30
40
50
60
70
80
90
f1 (pp


HMBC
120
130
140
150
160
170
180
190
200
210
ppm (f1

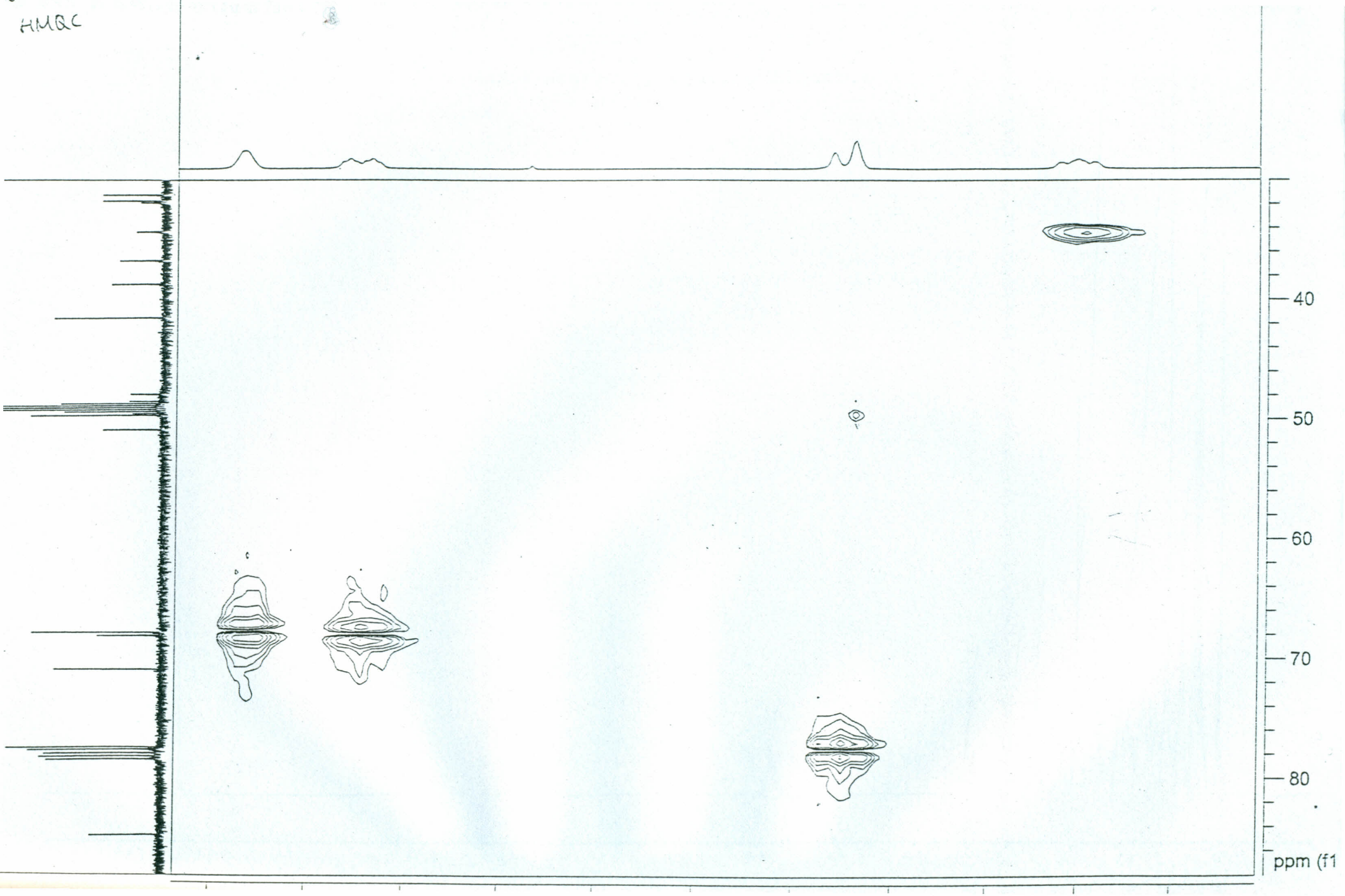

HMBC
40
50
60
70
80
ppm (f1


HMBC
0
50
100
150
200
ppm (f1

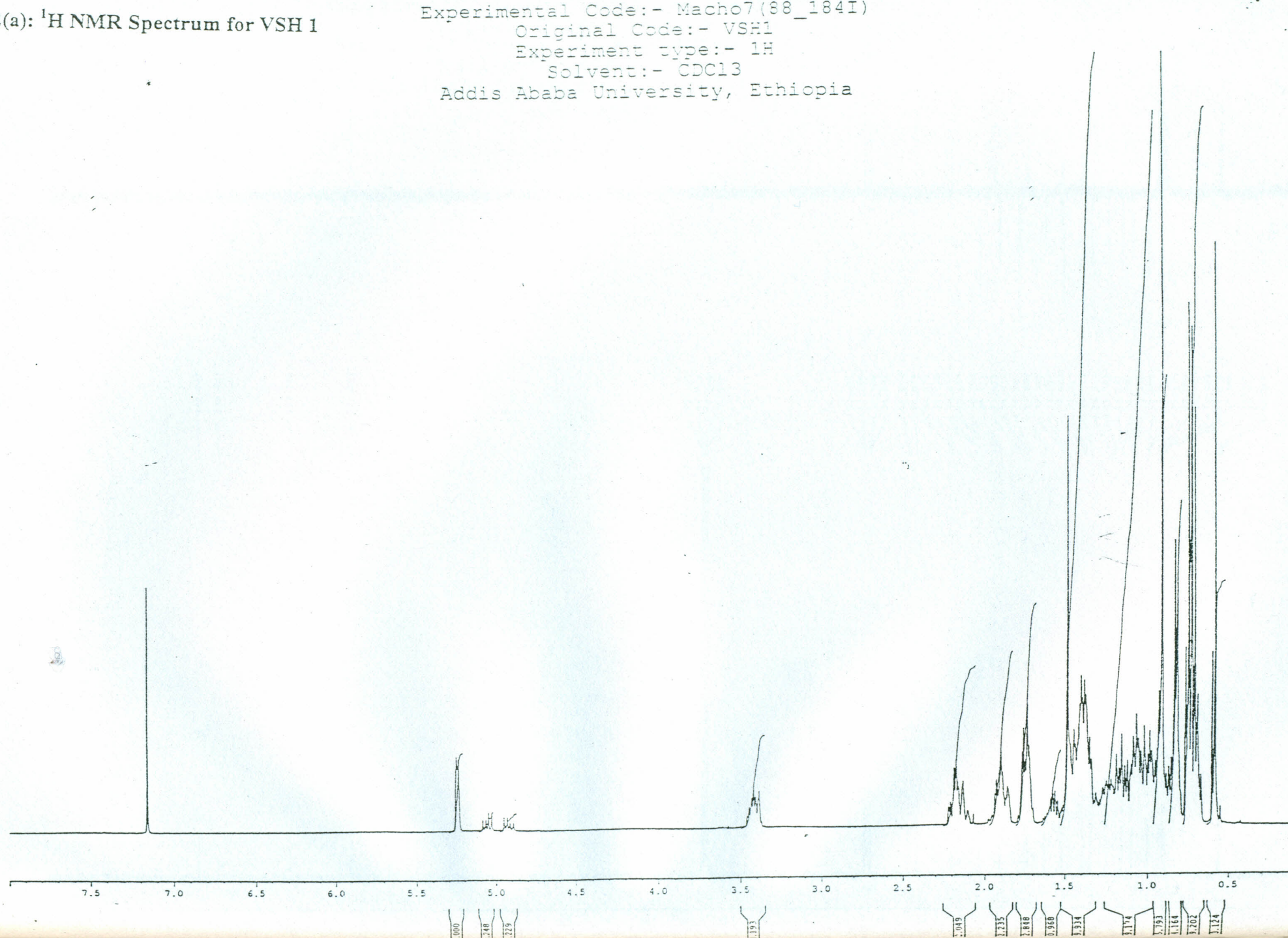

15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0
ppm (f1


5≡A2
HMBC
0
50
100
150
200
ppm (f1

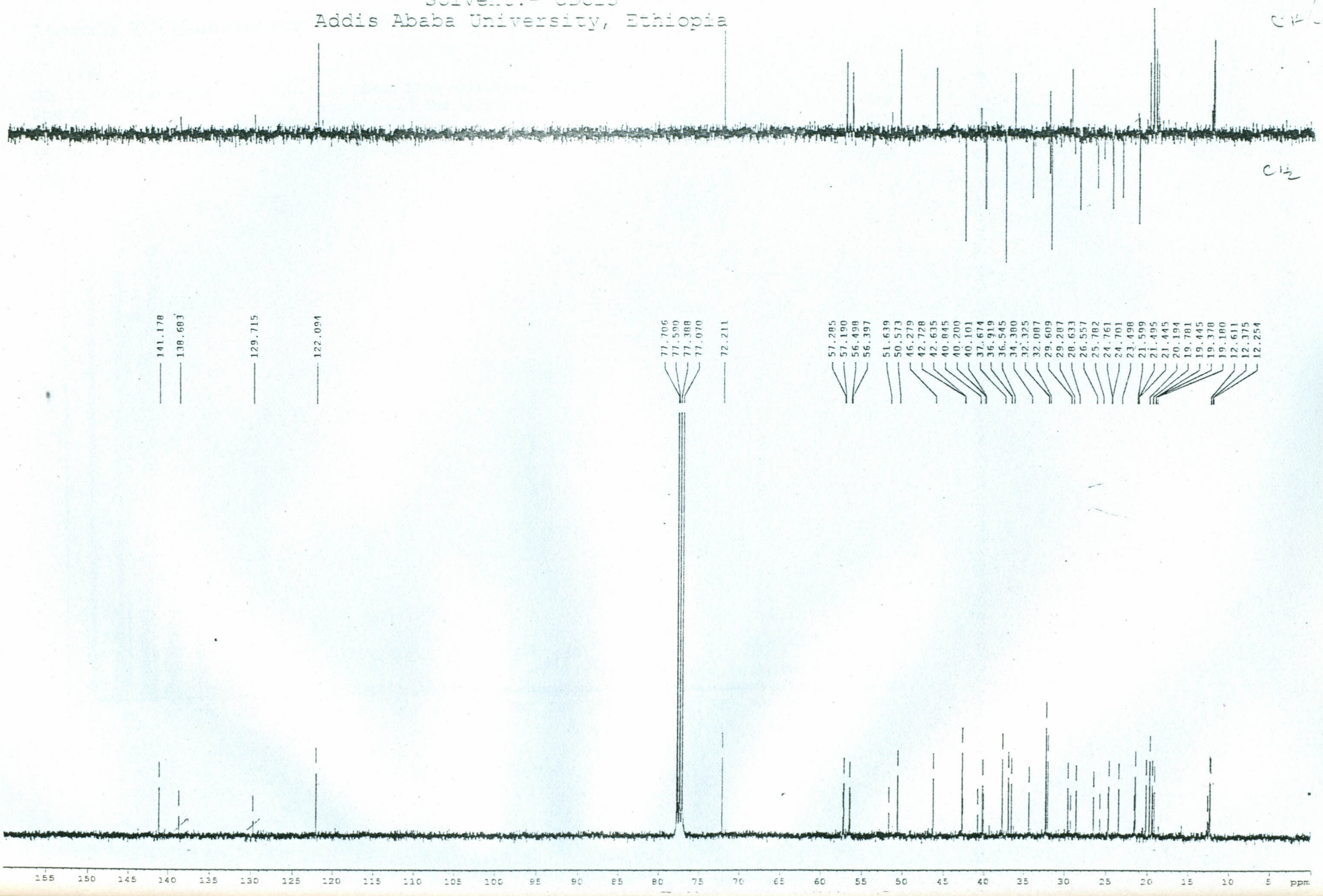
HMQC



Appendix 2(a): ^{1}H NMR Spectrum for VSH 1



Experimental Code:- Macho7(88_1841)
Original Code:- VSH1
Experiment type:- 13C and Dept-135
Solvent:- CDCl3
Addis Ababa University, Ethiopia

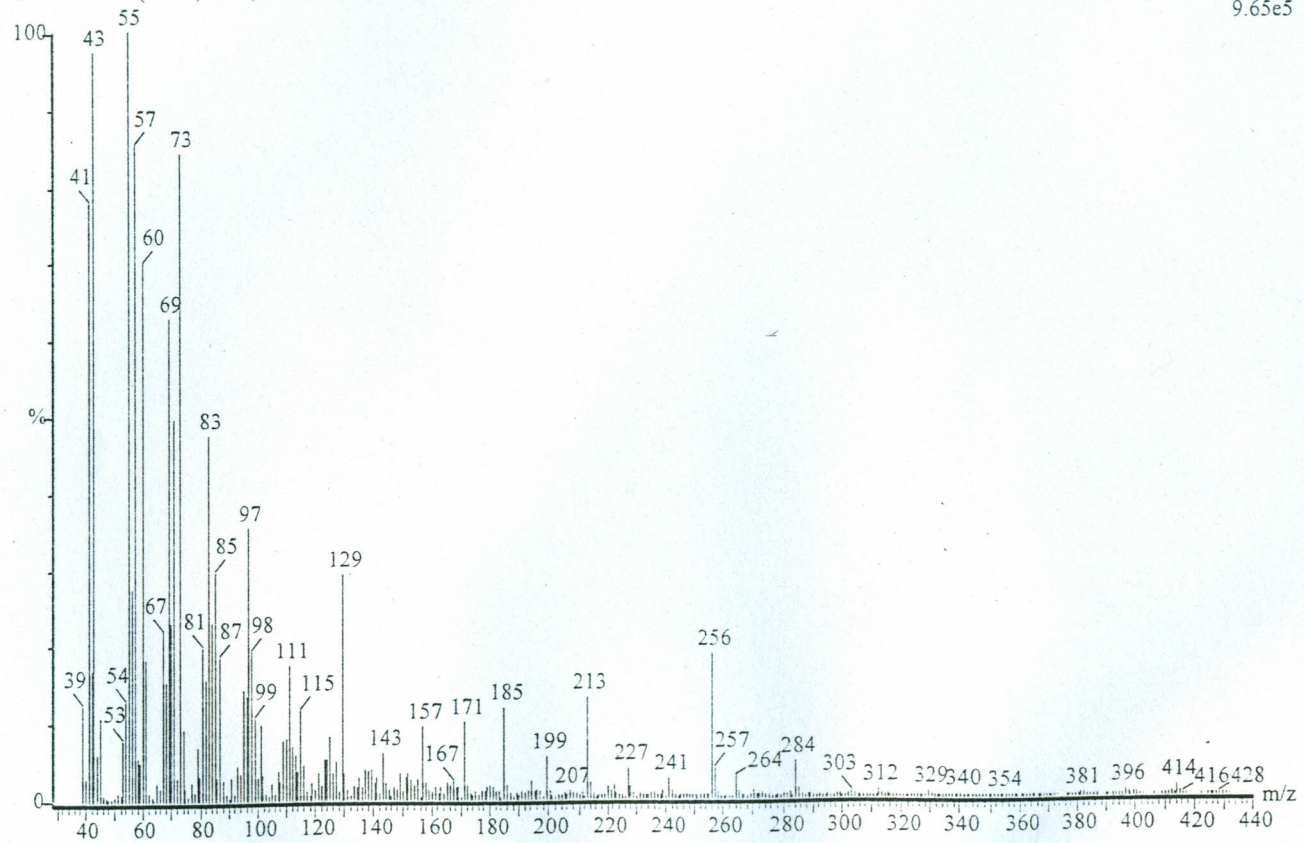


Appendix 2(c): Mass spectrum for VSH 1

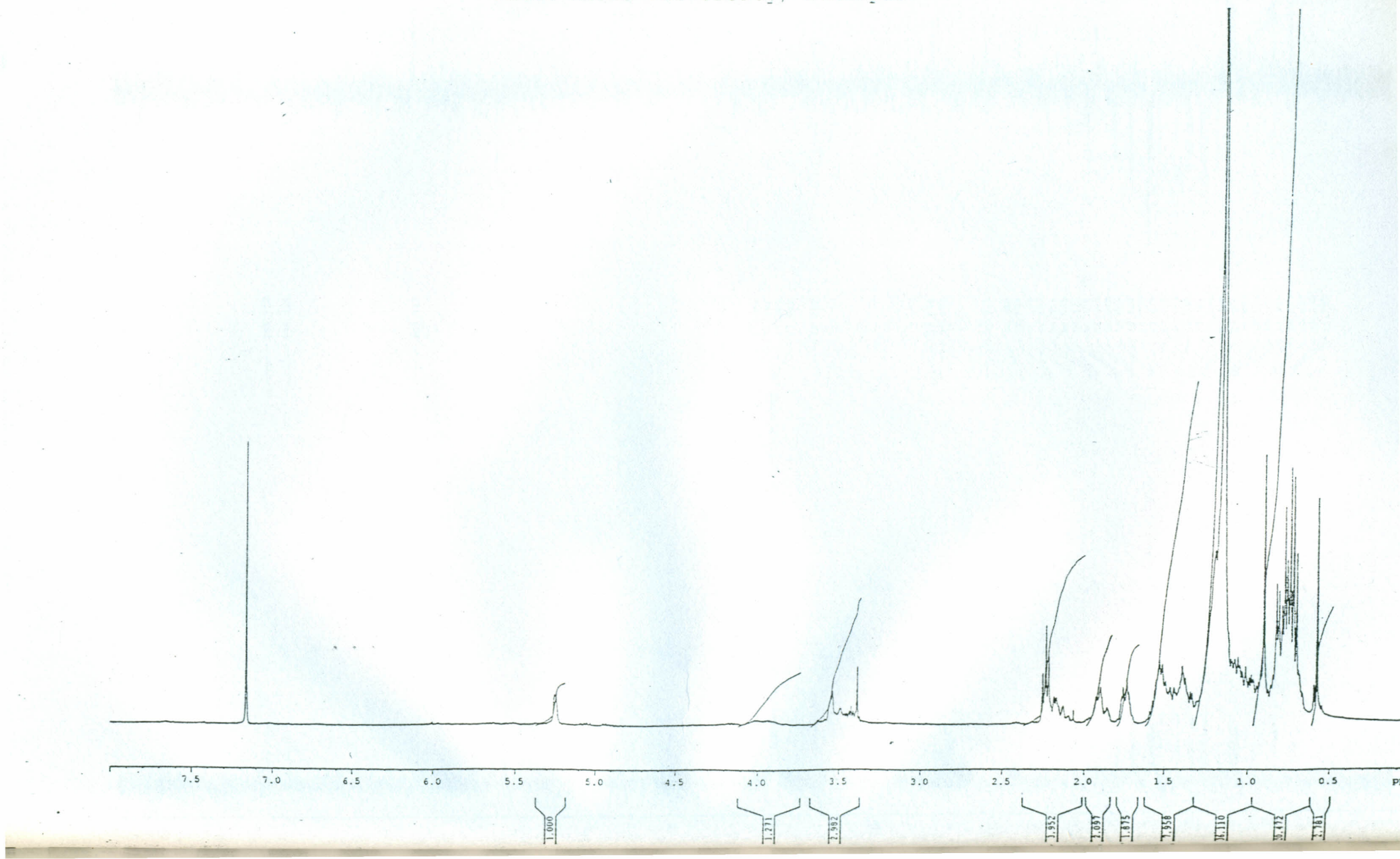
INS: VG 12-250 UPGRADE
BpM: 55
Sample VSH 1 ASSAY by SOLID PROBE
GK7115C 58 (1.959) Cm (16:61)

Date: 07-Nov-2005 Time: 13:20:12
BpI: 964591

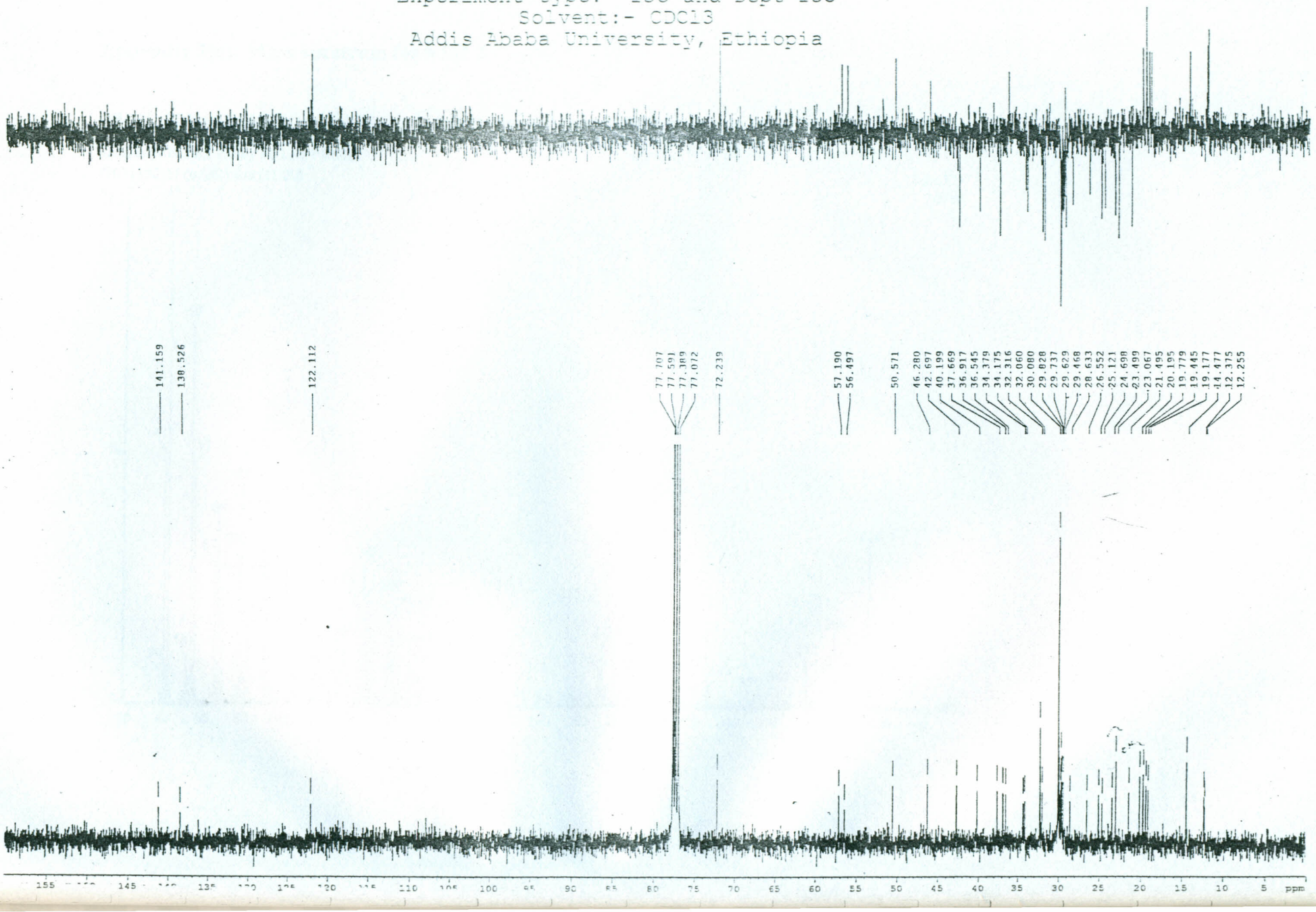
Scan EI+
9.65e5



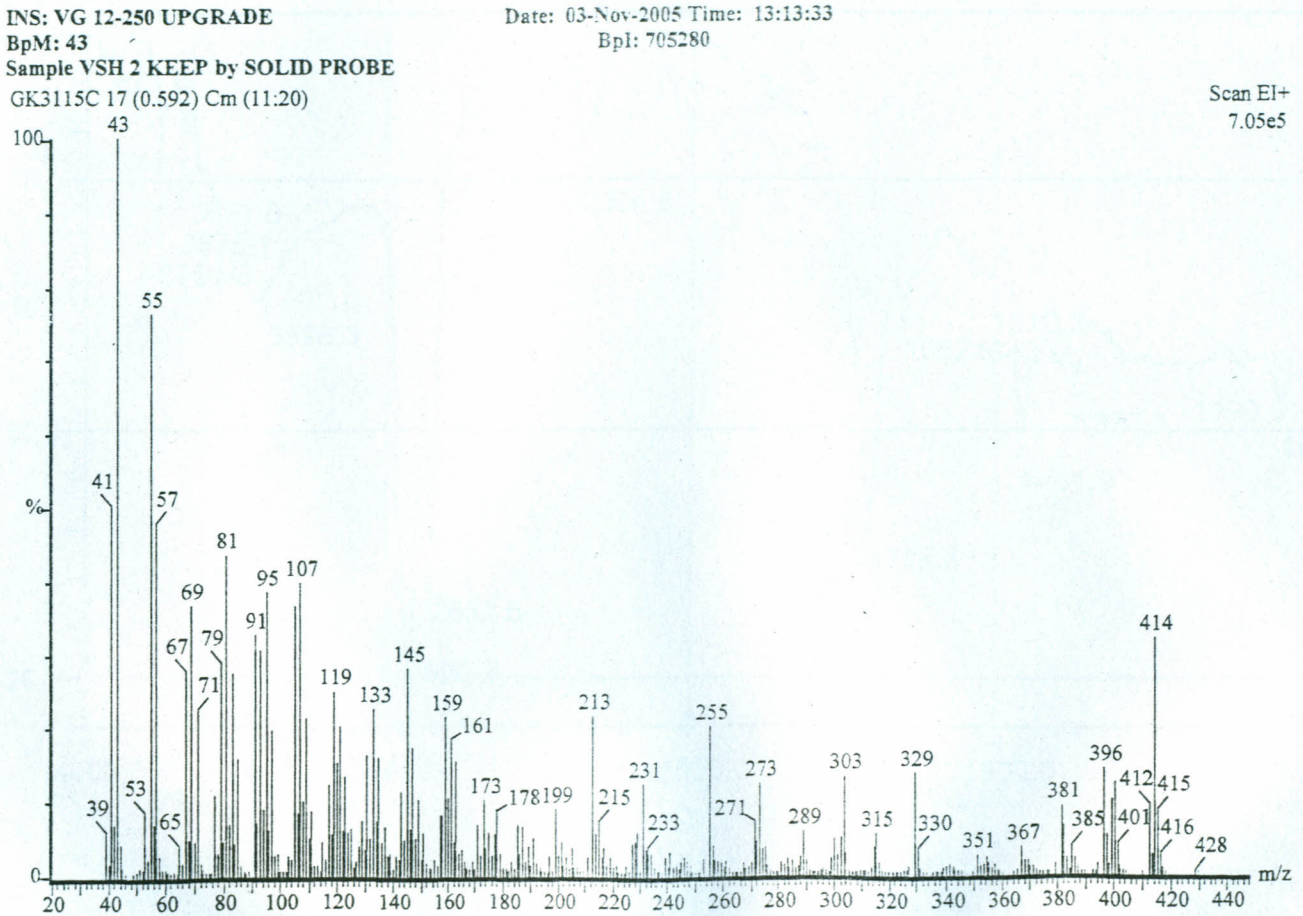
Experimental Code:- Macho8(88_184J)
Original Code:- VSH2
Experiment type:- 1H
Solvent:- CDCl3
Addis Ababa University, Ethiopia



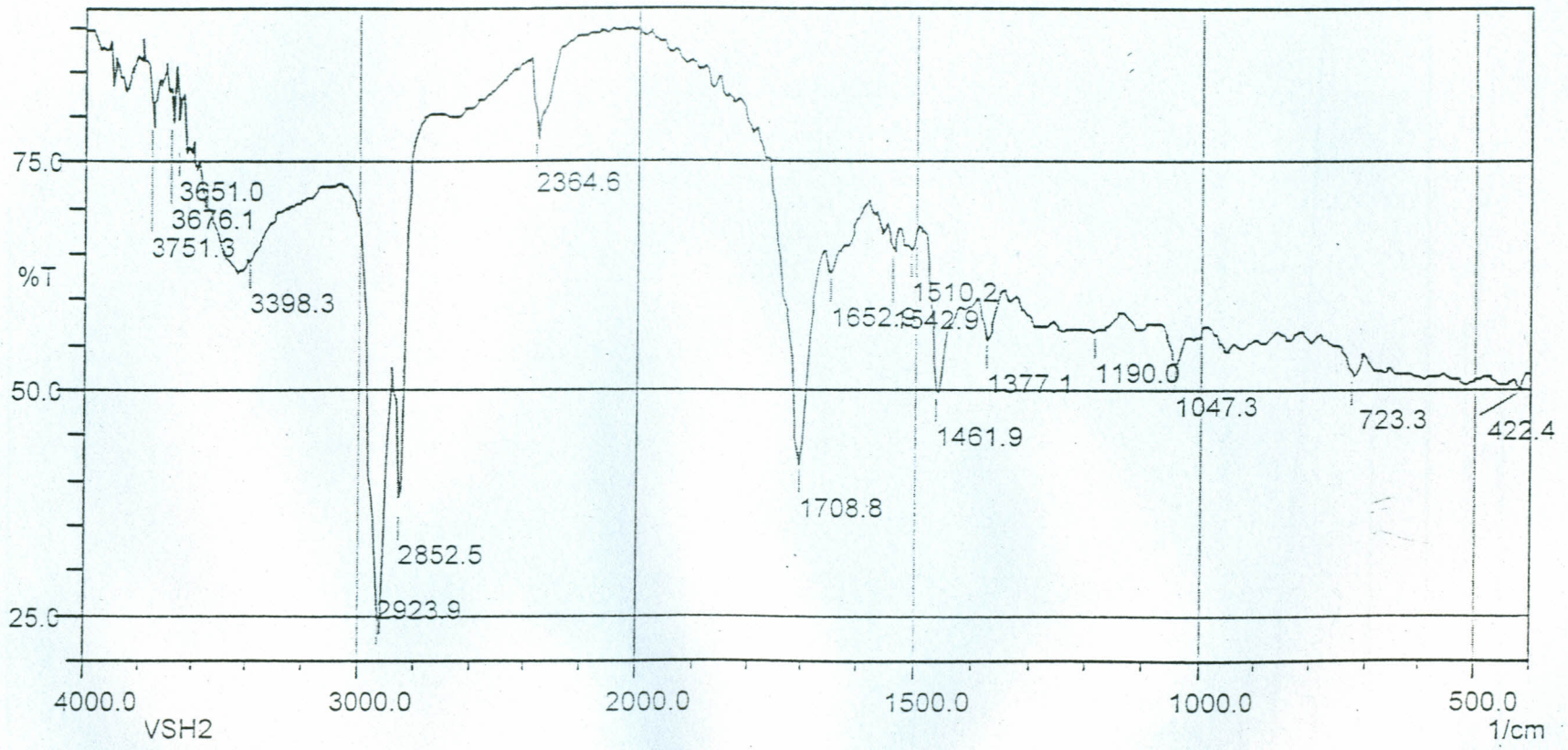
Experimental Code:- Macho8(88_184J)
Original Code:- VSH2
Experiment type:- 13C and Dept-135
Solvent:- CDCl3
Addis Ababa University, Ethiopia



Appendix 3(c): Mass spectrum for VSH 2

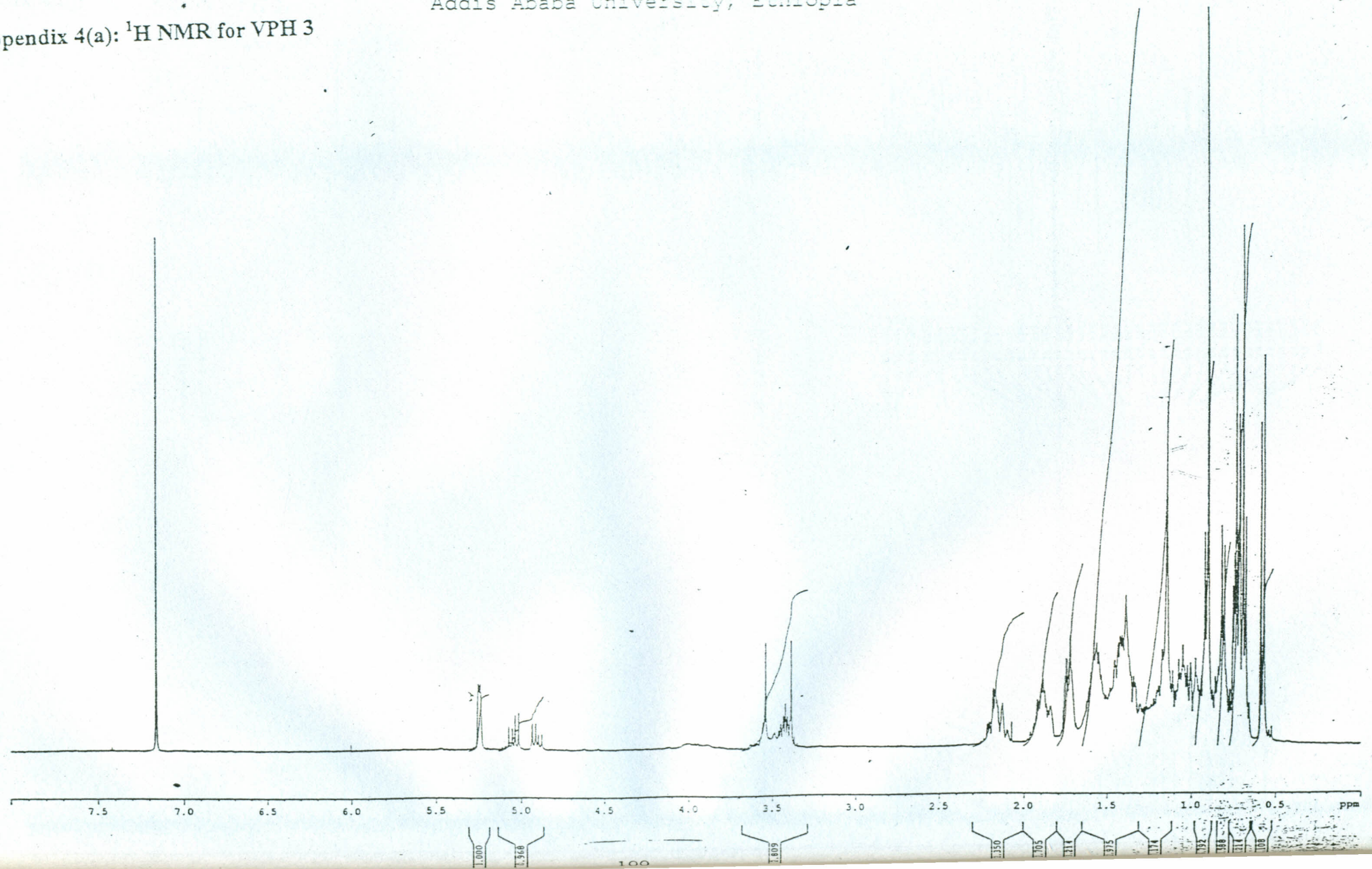


IR SPECTRUM FOR VSH 2



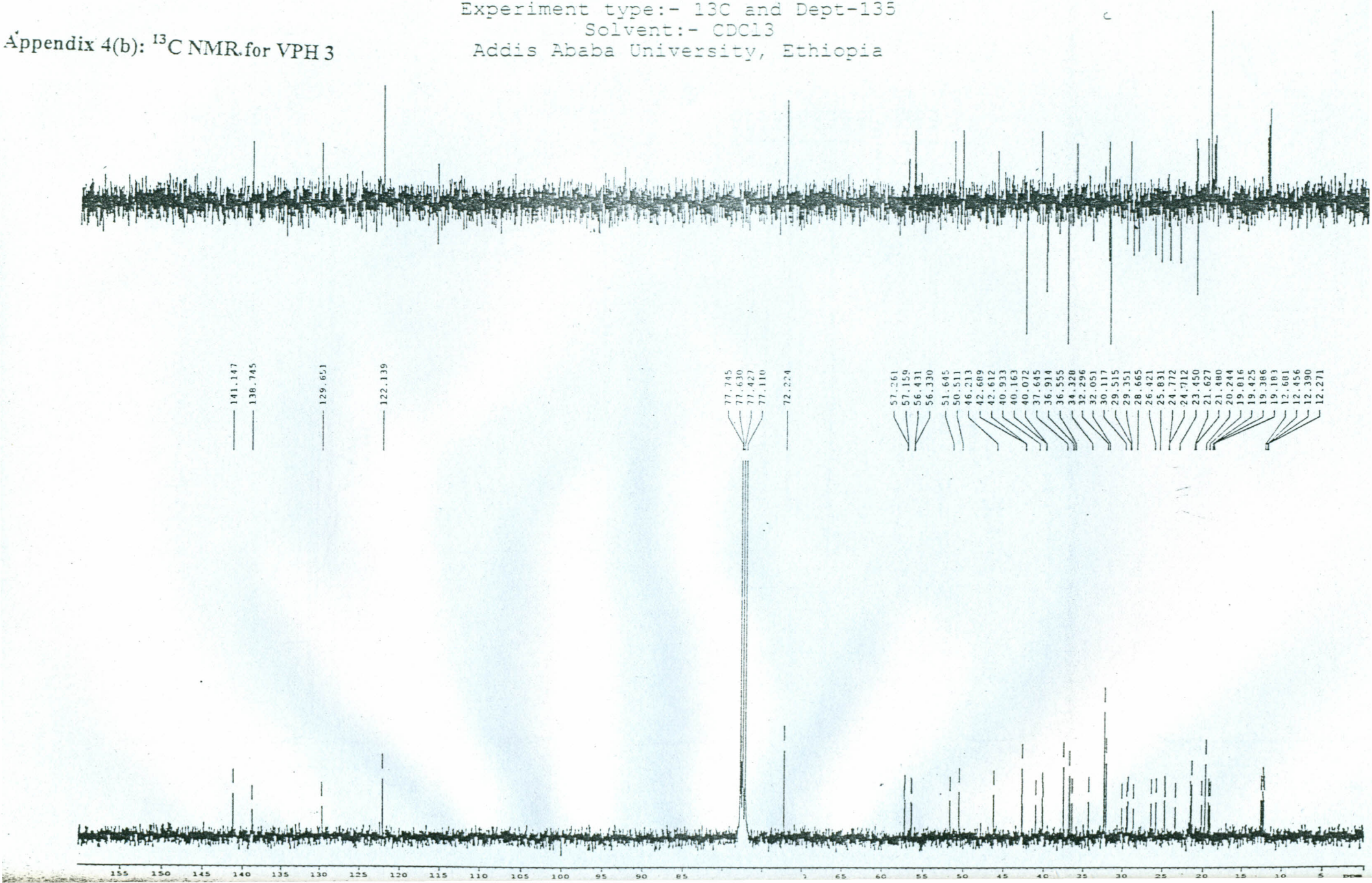
Experimental Code:- Macho3(88_184E)
Original Code:- VPH3
Experiment type:- 1H
Solvent:- CDCl3
Addis Ababa University, Ethiopia

Appendix 4(a): ^{1}H NMR for VPH 3



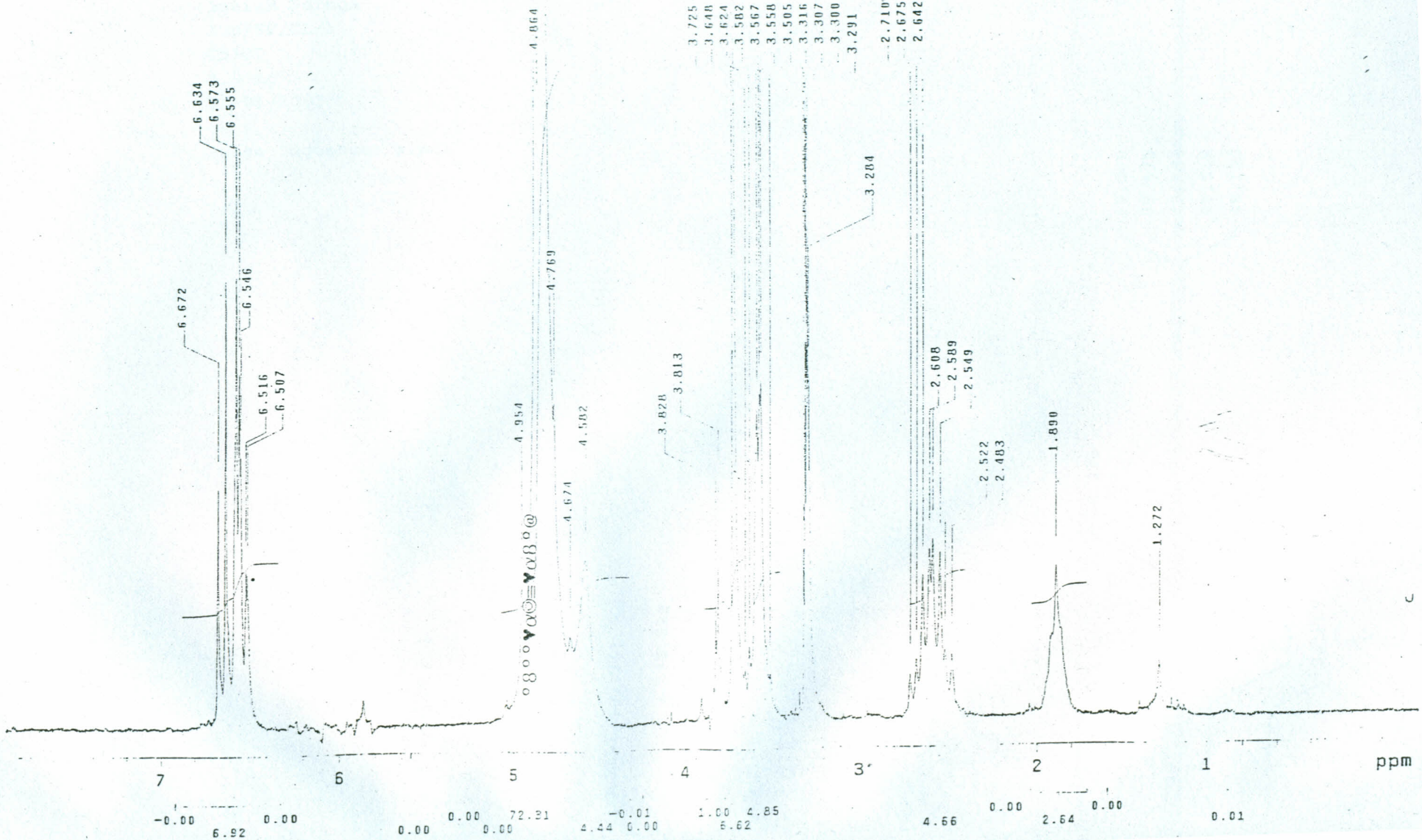
Original Code:- VPH3
Experiment type:- 13C and Dept-135
Solvent:- CDCl3
Addis Ababa University, Ethiopia

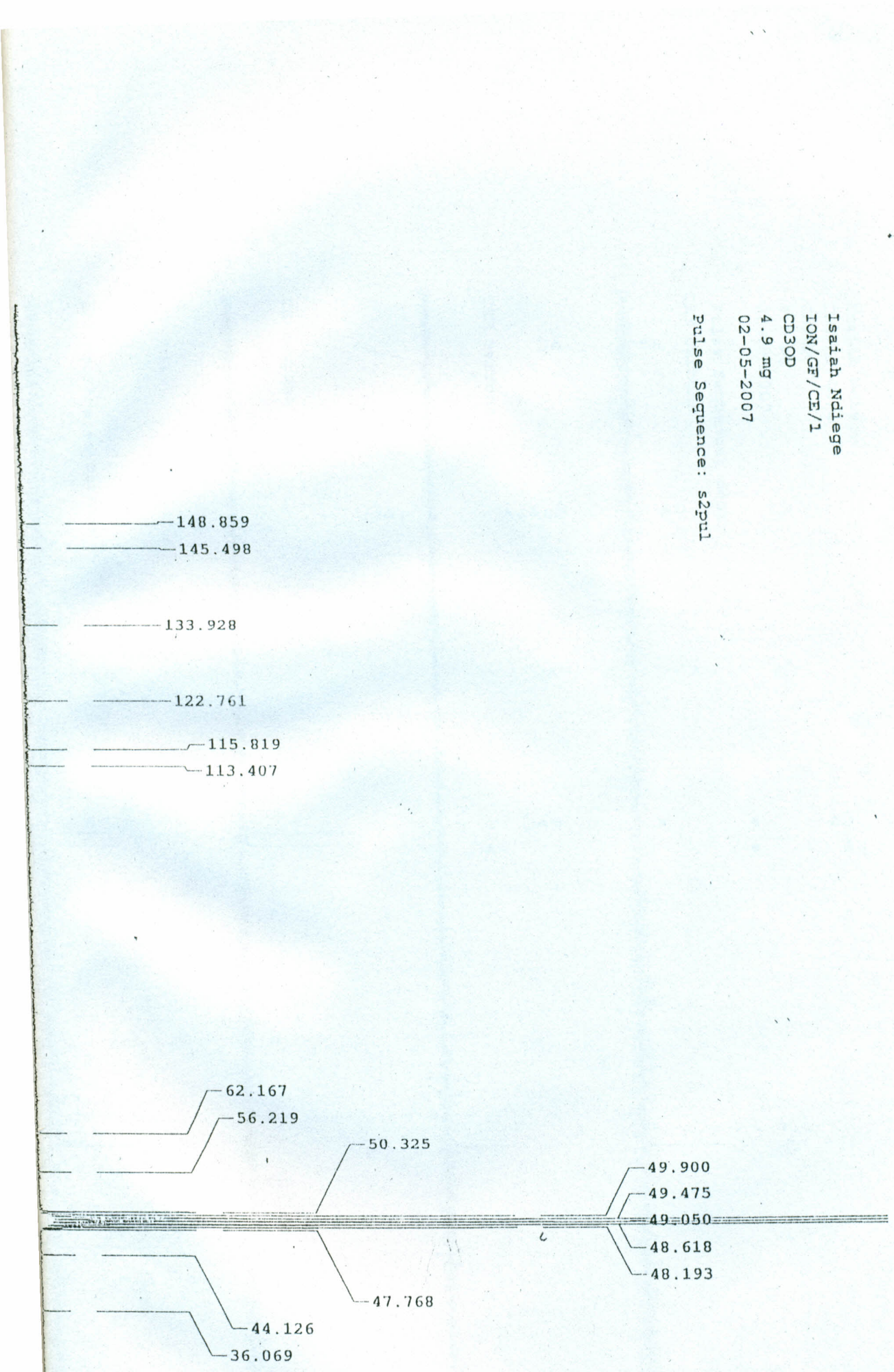
Appendix 4(b): ^{13}C NMR for VPH 3



Isaiah Ndiege
ION/OF/CE/1
CD3OD
4.9 mg
02-05-07

Pulse Sequence: s2pul




Isaiah Ndiege
ION/GF/CE/1
CD3OD
4.9 mg
02-05-2007
Pulse Sequence: s2pul
148.859
145.498
133.928
122.761
115.819
113.407
62.167
56.219
50.325
49.900
49.475
49.050
48.618
48.193
47.768
44.126
36.069

Isaiah Ndiege
ION/GF/CE/1
CD3OD
4.9 mg
02-05-2007

Pulse Sequence: dept

CH3 carbons

CH2 carbons

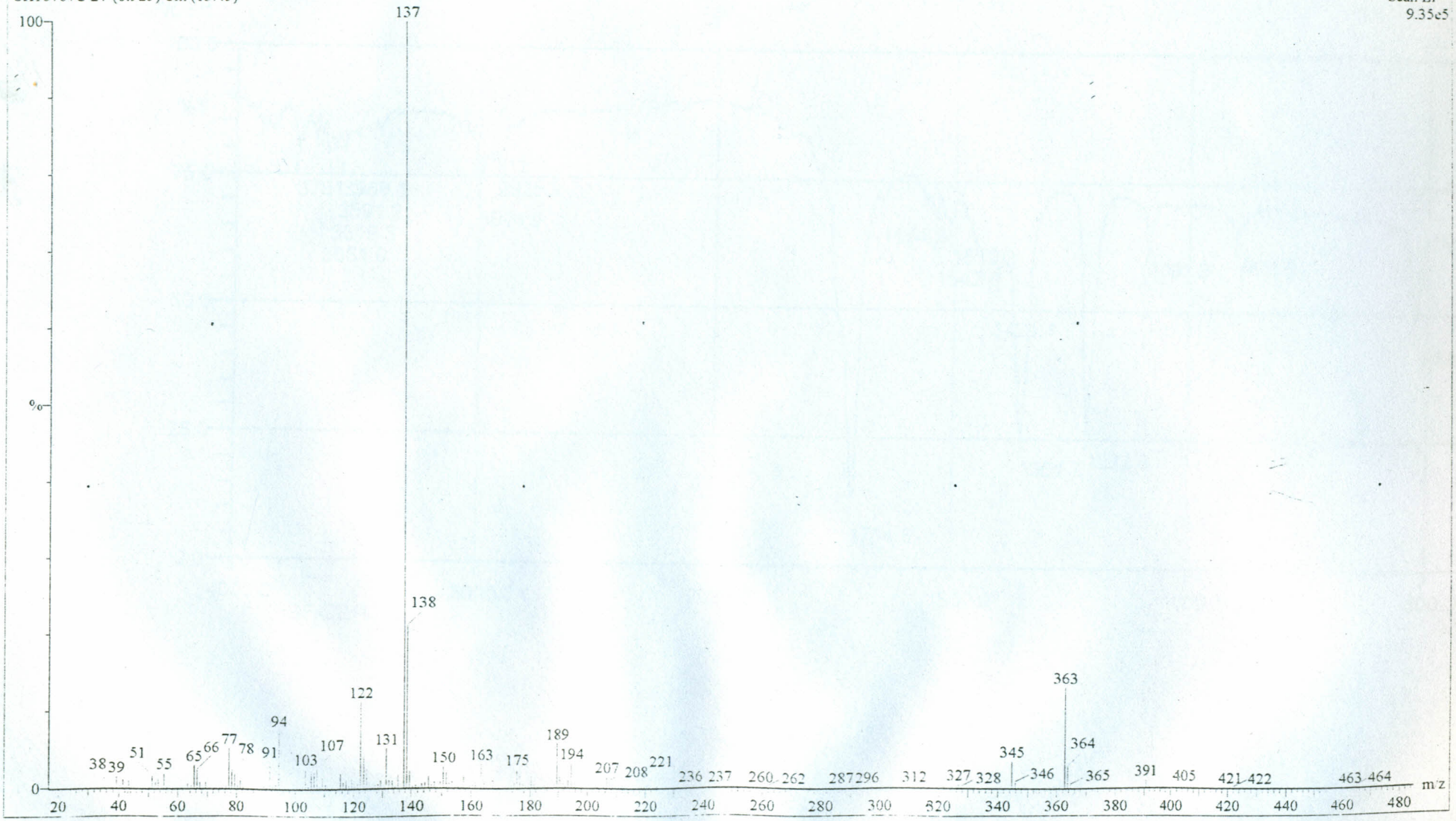
CH carbons

all protonated carbons

INS: VG 12-250 UPGRADE
BpM: 137
Sample GF/ CE/ 1 70eV by SOLID PROBE
SK10707C 27 (0.925) Cm (13:45)

Date: 10-Jul-2007 Time: 21:15:22
BpI: 934912

Scan EI+
9.35e5



IR SPECTRUM FOR
GF/CE/1