Isolation and characterization of antimicrobial compounds from berries of *Harrisonia abyssinica* (Simaroubaceae).

By

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SM11/1025/03

A Thesis submitted to the Graduate School in Partial Fulfillment of the requirements for the Award of the Degree of Master of Science in Chemistry of Egerton University

October, 2007

DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work, and has not been submitted for an award in any university for any degree as by my knowledge.

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DEDICATION

This thesis is dedicated to my husband and my kids, whose vision and energy, moral, financial and emotional support brought this work to reality.

Dr. Cheplogoi and Dr. Omolo who inspired the work and injected the impetus to strive and achieve.

ACKNOWLEDGEMENT

Utmost compliments go to my supervisors Dr. P. K. Cheplogoi and Dr. J. O. Omolo for their invaluable support. I am greatly indebted to them for their tireless guidance, valuable advice, encouragement, and their useful ideas, which culminated deep from their wide experiences. I wish to acknowledge all staff members of the Department of Chemistry, Egerton University. To mention a few, Mrs. A. Njue for her tireless guidance as it regards to the study, Mr. S. Kariuki and his technical team for availing materials and apparatus necessary for this work.

I express sincere gratitude to the Department of Dairy Egerton University, for facilitating the bioassay in the Microbiology laboratory, Mr. E. J. Ojwaka of the Department of Natural Resources, Egerton University for facilitating the collection and identifying of the berries, Mr. M. K. Langat who coordinated and did spectroscopic and MS analysis and Prof. D.A Mulholland formerly of School of Pure and Applied Chemistry University of Kwazulu –Natal South Africa and currently of the University of Surrey, UK for both NMR and MS analysis.

I thank my colleague Salome, who together we strived to achieve. I am deeply indebted to my husband Mr. S. Ochako whose energy and vision, moral, emotional and financial support motivated me all through my educational endeavors. Lastly, I thank my parents for their years of self-sacrifice. Thank you all who helped me in one way or another and did not get to be mentioned by person, may the almighty bless you abundantly.

ABSTRACT

Plants are a potential source of antimicrobial compounds. In this study, a plant from the family Simaroubaceae, *Harrisonia abyssinica,* traditionally used to treat a number of diseases was used. Research has been carried out on its stem bark, root bark and leaves and a number of chemical constituents have been isolated but no research has been carried out on its berries. Therefore the berries of this plant were chosen in order to investigate for antimicrobial compounds. The berries of the plants were collected, defatted using hexane and extracted using a mixture of methanol and dichloromethane solvents in a ratio of 1:1. The extract was screened for antimicrobial activity (agar diffusion assay) and it was found to have significant activity against tested microorganisms. Fractionation by column chromatography yielded 35 fractions that were pooled to three main fractions by the aid of TLC analysis. The three were further purified by repeated column chromatography and gel filtration method. The pure compounds were coded HRF_1, HRF_2 and HRF_3 and they were subjected to antimicrobial (agar diffusion assay) to confirm their antimicrobial activity. Compound HRF_1 showed substantial activity and therefore was chosen and screened for its antimicrobial activity using serial dilution assay. The compound was found to have a minimum inhibitory concentration (MIC) of 5 µg/ml against *Candida albicans*, 6 µg/ml against *Bacillus cereus* and >20 µg/ml against other test microorganisms. The compounds were subjected to spectroscopic techniques; 1D NMR (^{1}H NMR, ^{13}C NMR), 2D NMR (COSY, DEPT, HSQC, HMBC and NOESY). HRF_2 and HRF_3 were found to obtain similar spectral features a part from the noise signals in HRF_2 hence was found to be the same compound. The structures were confirmed using HRESIMS spectrometry. Compound HRF_1 was named harronin I and HRF_3 as harronin II, these compounds belong to a class of acetophenones and they are being isolated for the first time.

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LIST OF ACRONYMS AND ABBRIEVIATIONS

CC	Column Chromatography
TLC	Thin layer chromatography
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon -13 nuclear magnetic resonance
COSY	Correlated spectroscopy
HMBC	Heteronuclear multiple bond coherence
HSQC	Heteronuclear multiple quantum coherence
DEPT	Distortionless enhancement by polarization transfer
NOESY	Nuclear Overhauser effect spectroscopy
CH_2Cl_2	Dichloromethane
MeOH	Methanol
WHO	World Health Organization
EtOAc	Ethyl acetate
MIC	Minimum inhibitory concentration
$CDCl_3$	Deuterated chloroform
DAFTEC	Dairy and Food technology
HRESIMS	High resolution electrospray ionisation Mass Spectrometry
d	Doublet
t	Triplet
m	Multiplet
s	Singlet

CHAPTER ONE

INTRODUCTION

1.1 Background information

For many years people have developed a store for empirical information concerning therapeutic values of local plants. In the African continent long before colonization and arrival of Western values, plants were used for medicinal purposes. Extraction of medicine from herbs was indeed an integral part of the lifestyles of the people of Africa (Rukangira, 2002).

Herbal remedies have been applied for treatment of many ailments since ancient time all over the world and about 25% of current drugs are derived from plants (Wanyoike, 2004). The WHO estimates that four billion people, 80% of the world population presently use herbal medicine for some aspects of primary healthcare (Akerere, 1992). In Africa, an estimated 85% of the populations rely totally or partially on traditional medicine for their healthcare needs. WHO defines traditional medicine as, "the sum total of all the knowledge and practices whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation whether verbally or in writing" (Rukangira, 2002).

Growing resistance of pathogens to therapeutic agents has given the urgency to search for both better and safer compounds, and delivery systems. The need to treat the target more precisely has provided additional opportunities for the use of natural products (Jasper *et al.*, 1998). With the renewed interest from Western countries in herbal remedies, and the increasingly urgent need to develop new effective drugs, traditionally used medicinal plants have recently received the attention of the pharmaceutical and scientific communities (Cordell, 1981). This involves the isolation and identification of the secondary metabolites produced by the plants and used as the active principles in herbal preparations. Therefore search for appropriate medicine to counter the numerous diseases that endanger the life of man continues.

More than 50 years have passed since the modern antibiotic era opened with the clinical trial of penicillin in early 1940s. It is estimated that between 5,000 and 10,000

natural antibiotics have been isolated and characterized and at least 50,000 to 100,000 analogues have been synthesized, but clearly the vast majority fails to find medicinal use (Berdy, 1980). Most of the natural antibiotics have been isolated from soil microorganisms through intensive screening. The phytochemical screening of plant species especially ethnopharmaceutically provides a valuable baseline information in search for new pharmaceuticals (Farnsworth *et al*., 1985). The development of resistance by pathogens to many of the commonly used antibiotics provides sufficient impetus for search for more antimicrobial agents to combat infection and overcome the problem of resistance.

Plants produce diverse array of secondary metabolites, many of which have antimicrobial activity. Some of these compounds are constitutive, existing in healthy plants in their biologically active form; others such as cyanogenic glycosides occur as active precursors and are activated in response to tissue damage or pathogenic attack (Van *et al.,* 1995). Plants are the oldest source of pharmacologically active compounds, and have provided humankind with many medically useful compounds for centuries (Bennett and wellsgrove, 1994). It is estimated that more than two thirds of the population relies on plant derived drugs; some 7000 medicinal compounds used in western pharmacopoeia are derived from plants. The search for natural products to cure diseases represents an area of great interest in which plants have been the important source. Thus, phytochemical screening of plant species, especially of ethnopharmaceutical use, will provide valuable information in search for novel pharmaceuticals (Haslam *et al*., 1989).

The use of shrub *Harrisonia abyssinica* particularly in East Africa to treat numerous diseases has been used as a basis for this study in order to investigate for the medicinal compounds of its berries. In the previous research done on its leaves, roots and stem, a number of chemical constituents that have shown to exhibit biological activity have been isolated and structurally elucidated (Okorie, 1982). The shrub indeed has so far been known to treat a number of diseases like, fever, tuberculosis, malaria, bubonic plague, haemorrhoids, snakebite, dysentery, herpes simplex and other uses (Kokwaro, 1993). This research project was conducted on the berries of *H. abyssinica* to investigate for antimicrobial chemical compounds.

1.2 Statement of the problem

H. abyssinica, is traditionally widely used to treat numerous diseases. A number of chemical constituents have been isolated from the root bark, stem bark, and leaves but no research has been done on its berries despite the fact that they are suspected to harbour a lot of secondary metabolites in addition to the germplasm and storage of carbohydrates. Therefore this has prompted this study on these berries in order to investigate for antimicrobial compounds.

1.3 Objectives

The main objectives are;

1. To extract dried berries of *H. abyssinica* shrub.
2. To screen for antimicrobial activity of the crude extract.
3. To isolate and purify compounds from crude extract.
4. To carry out chemical characterization of isolated compounds.

1.4 Justification

Secondary metabolites have proved to be biologically active molecules, in higher plants secondary metabolites serve as defence agents against invading microorganisms Chemical constituents for instance limonoids, chromones, and quassinoids responsible for antimicrobial, insect antifeedant, antiplasmodial and plant growth inhibitory activities have been isolated from the root bark, stem bark and leaves of the plant *H. abyssinica*. The scientific prospects of getting a new and novel chemical compound(s) will increase the number of isolated chemical constituents and hence support the role of this herbal medicine in managing microbial pathogens therefore validate the role of traditional medicine in provision of primary health care.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal Plants

Plants have traditionally served as a main weapon against pathogens; in fact even Neanderthals knew and made use of medicinal plants (Solecki, 1975). It is estimated by WHO that between 60 and 90% of Africa's population rely on medicinal plants to meet totally or partially their health care needs. The importance of traditional medicine as a source of primary health care was first officially recognized by WHO in the primary healthcare Declaration of Alma (1978) and has been globally addressed since 1976 by the Traditional Medicine Program of WHO (Akerele, 1992). The main problem facing the use of traditional medicines is the proof requirement that the active components contained in medicinal plants are useful, safe and effective. The scientific proof then becomes the most important thing in order to eliminate the concern of using medicinal plants as drugs for alternative treatment.

2.1.1 Challenges and constraints in the use of herbal medicine

The herbal medicine trade is booming business worldwide. Herbal medicine represents an estimated $60 billion annual turnover in the global market; this represents 20% of the overall drug market (Alok, 1991; Kong, 1982). Despite this huge usage of herbal medicine, several arguments have been advanced against their use such as;

(i) The herbalist is not competent to diagnose.
(ii) No data on long-term toxicity of herbs have been documented.
(iii) Absence of standard dosage can lead to overdose or under dosage.
(iv) Herbal preparations are generally unhygienic and poorly packed.
(v) Dosage forms are usually voluminous and difficult to cope with

These are strong and valid arguments and concerns. Moreover, some of the herbal prescriptions especially those accompanied by ritualistic institutions are sometimes extremely difficult to rationalize scientifically (Ivan, 1991).

One area in which traditional medicine has not developed in the same way as Western medicine is in the construction of precision methodologies for testing its effectiveness. Often those trained in Western medicine have taken this to mean

indigenous is not effective. But the apparent absence of Western-type methods for testing the efficacy of indigenous medicine should not be seen as evidence of its ineffectiveness (Van *et al*., 1995).

2.2 The family Simaroubaceae

The family Simaroubacea*e* is most abundantly represented in tropical America, Mexico to Argentina and Africa. It is grouped in the order Rutales. The family is known to have diverse range of secondary metabolites. It has played prominent role in folk medicine. Plants of the Simaroubaceae family produce a series of degraded triterpene limonoids and related quassinoids. The medicinal properties associated with these plants have been attributed to the bitter constituents namely the quassinoids. Some of these plants are used in folk medicine for anthelmentic and antiamoebic properties (Wright, 2005).

The species *Hannoa chlorantha* and *H. klaineana* are traditionally used in traditional medicine of Central African countries against fevers, diarrhoea, worm diseases and malaria. Previous phytochemical studies of these plants have led to the isolation of several quassinoids, alkaloids, coumarins and limonoids (Grieco *et al*., 1999). Chemical investigations of *H. chlorantha* have resulted in the isolation of quassinoids; chaparrinone (**1**), 14-hydroxychapparrinone (**2**), 15-deacetylundulatone (**3**) and 6-α-trigloylaxyglaucarubol (**4**) that have showed to have good activity against *Plasmodium. faciparam* (Francois *et al*., 1998). In concert with the research program in the area of anti cancer quassinoids, chemical investigation of the species *Castela polyandria* has resulted in the isolation of more C_{20} quassinnoids; 1-*epi*-holocanthone **(5)**, 15-*O* -acetylglaucarubol **(6)**, 1-*epi*-5-iso-glaucarubolone (**7**), 15-acetyl-$\Delta^{4.5}$ glaucarubol (**8**), 1-*epi*-glaucarubolone (**9**), $\Delta^{4.5}$-glaucarubol (**10**) and 15-*O*-acetyl-5(S)-polyandrol (**11**) (Grieco *et al*.,1999).

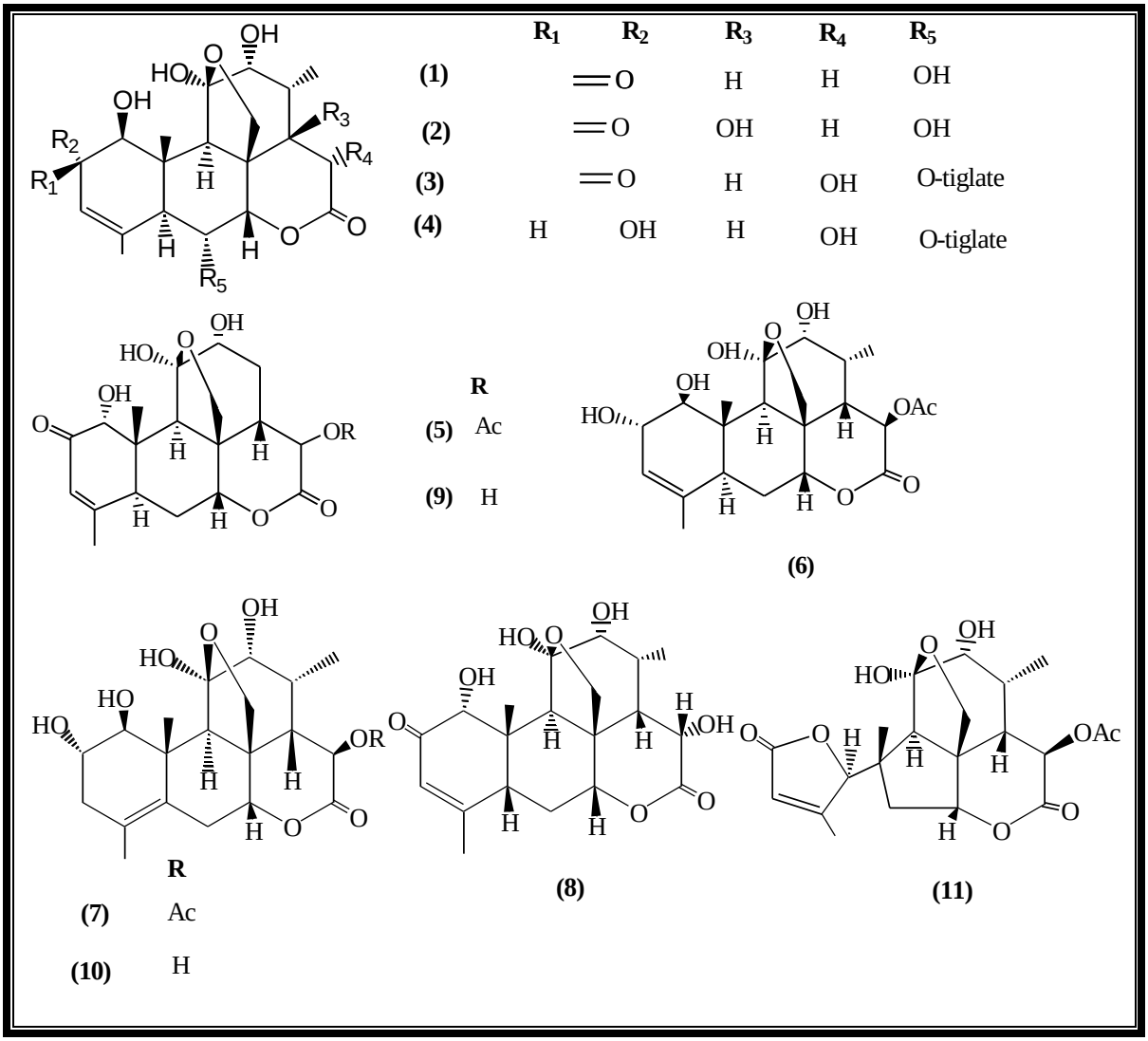


Figure 1: Compounds 1-11

Investigation carried out on the roots of *Eurycoma harmandiana* resulted in the isolation of unusual 15α-OH quassinoids; iandonoside A (**12**), iandonoside B (**13**), iandone (**14**) and one known coumarin, scopoletin (**15**) (Kanchanapoom *et al*., 2001). Attention has been focused on quassinoids as several of them have shown promising antitumour, antiviral, antimalarial, antileukemic and antifeedant properties (Kanchanapoom *et al*., 2001). The species *Brucea amarissima* is used in Asia for treatment of dysentery, malaria and cancer. As regards bioactive components, quassinoids from *B. amarissima* have been reported to have interesting biological effects, such as antimalarial, antitumour and antiamoebic activities. Further studies on the seeds

of the plant resulted in the isolation of a quassinoid glycoside, javanicosides I (**16**) (Kim *et al*., 2004).

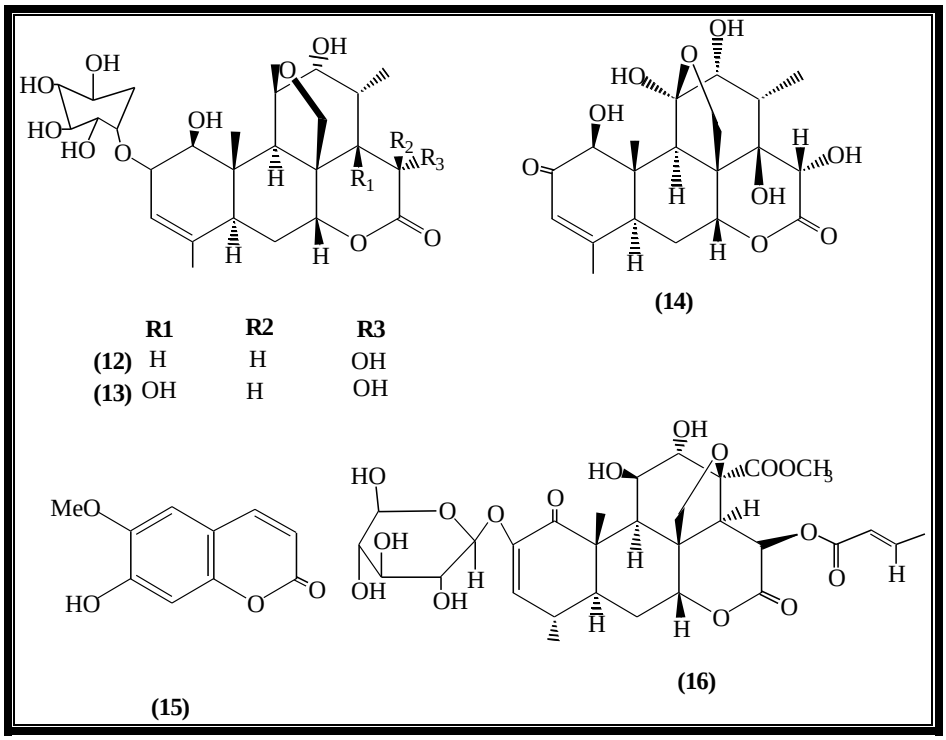


Figure 2: Compounds 12-16

2.3 The genus *Harrisonia*

In this genus three species have been phytochemically investigated; *H. perforata*, *H. brownii* and *H. abyssinica*. They are widely used in Africa and Southeast Asia as tropical medicine. Compounds in the classes' limonoids, chromones, quassinoids and coumarins have been isolated from these plants.

2.3.1 *H. perforata*

This species is used in folk medicine for treatment of itching in Middle Vietnam; the root of this plant is used in folk medicine in South China for the prevention and treatment of malaria, boils and in Indonesia the root bark is a remedy of diarrhoea (Sung *et al*., 1994). The chemical studies carried out on the species led to the isolation of a limonoid, perforatilonone (**17**) (Sung *et al*., 1994). Further studies have resulted in the isolation of chromones, perforatins C-G (**18-22**) in addition to known chromones, heteropeucenin-7-methyl ether (**23**)**,** heteropeucenin-5-methoxy-7-methyl ether (**24**); 2-hydroxymethylallopataeroxylin-5-methyl ether (**25**) (Balde *et al*., 1987); perforatin A

(**26**); perforatic acid (**27**) and perforatic acid methyl ester (**28**). A coumarin, scopoletin (**15**)**,** together with a phenyl propanoid coniferyl aldehyde have also been isolated (Tanaka *et al*., 1995)

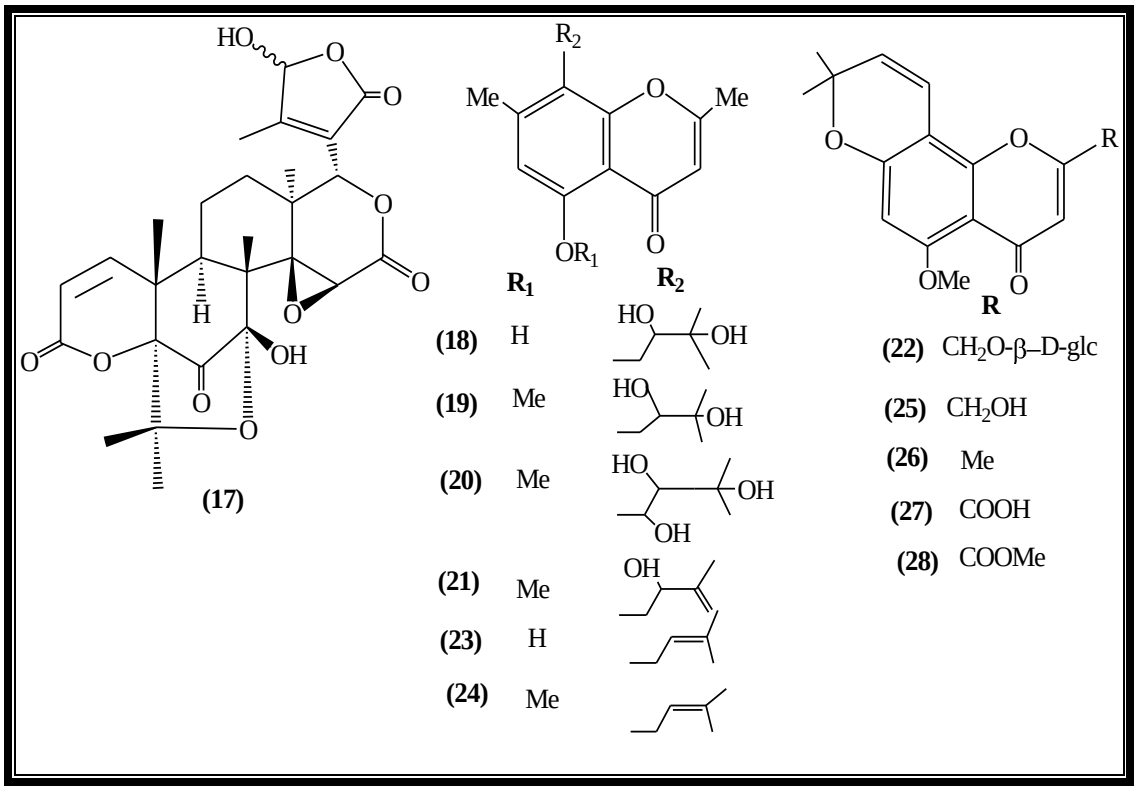


Figure 3: Compounds 17-28

2.3.2 *H. Brownii*

The species is widely distributed in Southeast Asia and its root is used in the treatment of dysentery and cholera. Chemical studies have led to the isolation of limonoids; brownin A (**29**) and brownin B (**30**) (Koike *et al*., 1993). The study of the root bark led to the isolation of two rearranged limonoids; brownin D and brownin E (Mitsunaga *et al.,* 1993a) and the a study carried out on the bark and wood of the same plant has led to isolation of more limonoids brownin C (**31**) and brownin G (**32**) (Mitsunaga *et al*., 1993b).

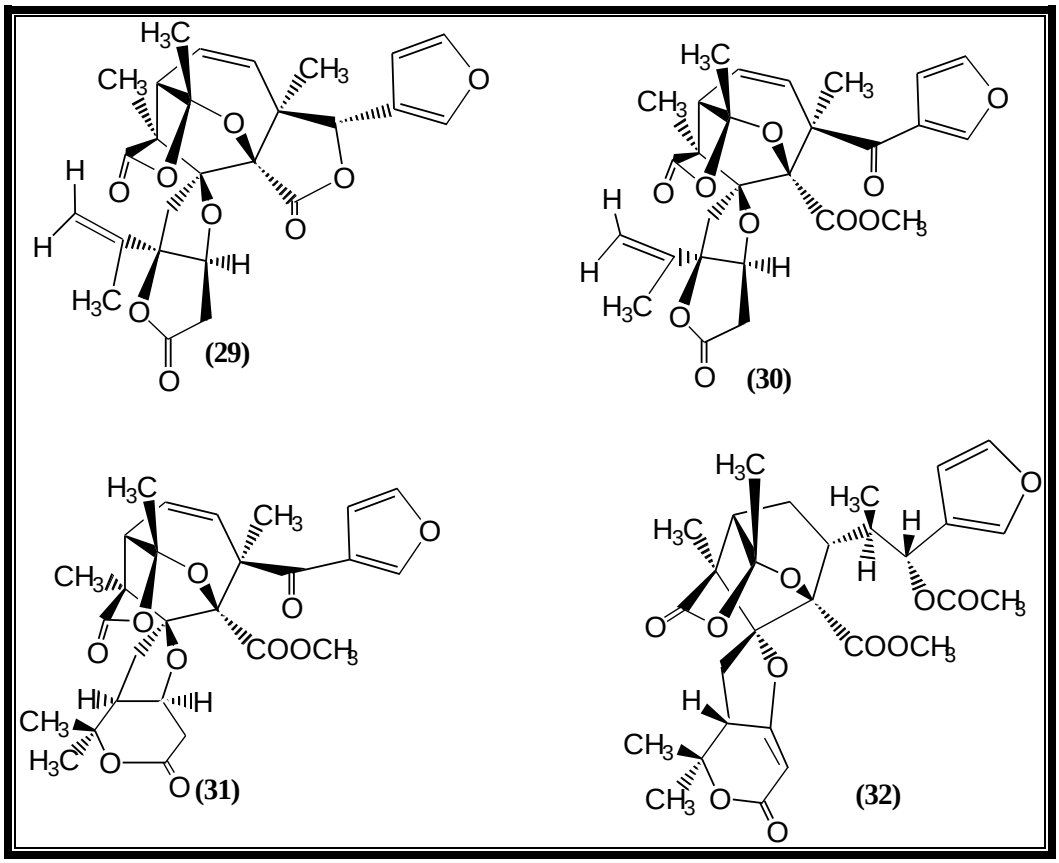


Figure 4: Compounds 29 -32

2.3.3 *H. abyssinica*

H. abyssinica, is a spreading and much branched tree up to 6 m high, trunk and stems thorny with re-curved spines at base long and whip like, leaves-pinnate leaflets 4-8 pairs with terminal leaflets; paler below rachis narrowly winged leaflets obscurely toothed, leaves often with pain prickles at base; leaflets 3.8 x 3.8 cm, oblanceoalate to elliptic, apex rounded, base narrow entire or create, rachis and midrib slightly hairy when young, flowers small and white in erect terminal panircles, fruits, small red berries depressed globule about 0.64 cm in diameter (Irvine, 1961). The East African shrub *H. abyssinica*, also known locally as Omindi or Pedo (Luo), Kidori (Digo), Orongonwe (Kuria), Mukurumando (Swahili) (Kokwaro, 1993), grows along the coast of East Africa and in Western Kenya.

Figure 5: The plant *H. abyssinica*

It is used in Kenya and Tanzania as a remedy for treatment of fever, bubonic plague, tuberculosis, haemorrhoids, malaria and snakebite (Kokwaro, 1993). The pulp and roots or leafy tips in water is drunk or used in the Ivory Coast as an anema for venereal diseases and dysentery. A decoction of the root bark is drunk in palm wine as a laxative (Irvine, 1961). The root of this plant is used in a folk medicine in south China for prevention and treatment of malaria and boils. The plant is also used to treat skin diseases. Chemical constituents that have been isolated are limonoids of these obacunone **(33),** harrisonin, 12β-acetoxy harrisonin and pedonin have been obtained from Kenyan samples (Hassanali *et al*., 1987) whereas obacunone, atalantolide (**34),** 5-dehydrooriciopsin were found in Nigeria samples (Okorie 1982; Balde *et al*., 1988). The large variance in the chemical constituents of the East and West African samples of *H. abyssinica* and absence of a quassinoid has led to more study of the plant (Okorie, 1982). This together with more interest in compounds from *H. abyssinica* with effect on germination of S*triga* (Rugutt, 1996) prompted researchers to re-examine the extracts of the plant.

Further investigations of the methanol extract of the root bark of this plant has led to the isolation of a new limonoid 11β, 12β-diacetoxyharrisonin (**35**) (Rajab *et al*., 1999). In addition perforaquassin A (**36),** a quassinoid has also been isolated. More research of the plant has led to a new limonoid, deoxyobacunone (**37**) being isolated (Rugutt *et al*., 2001).

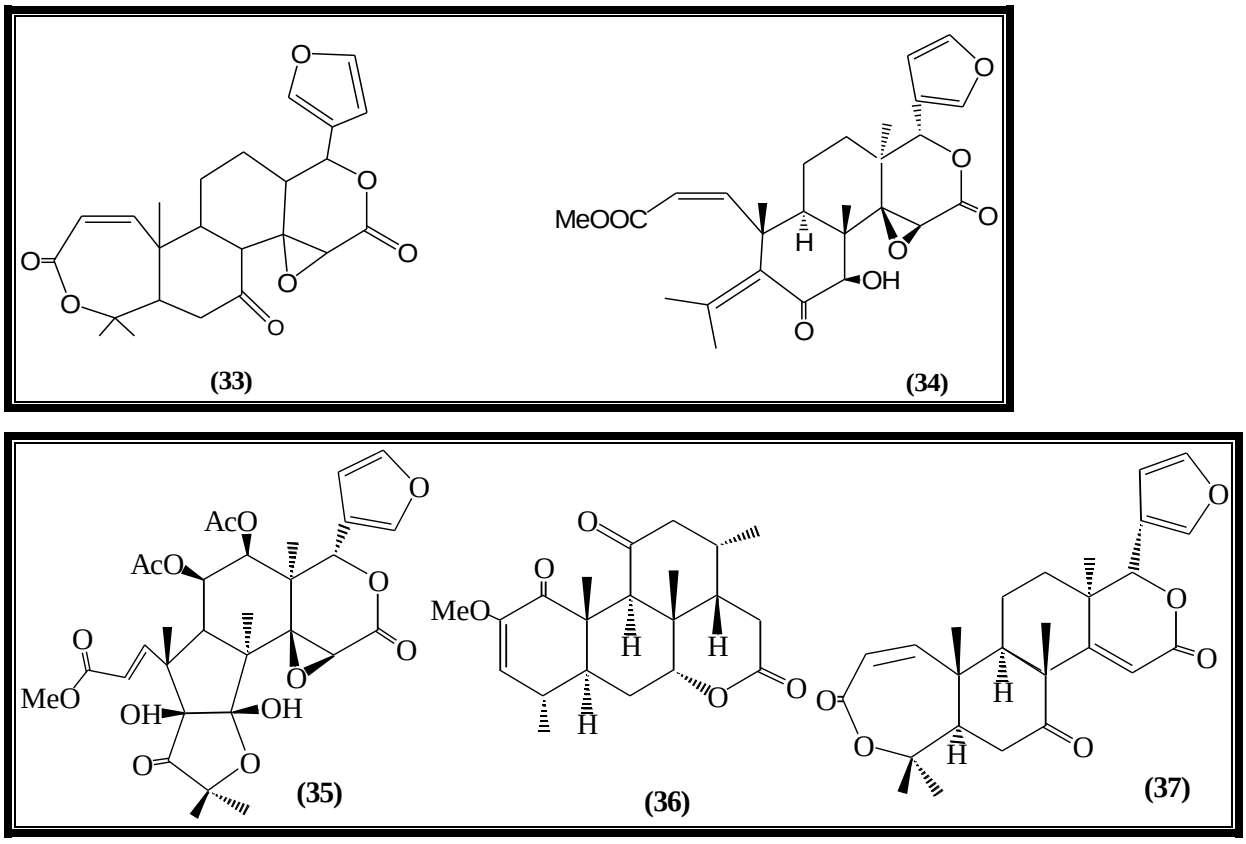


Figure 6: Compounds 33-37

A cycloterpene, 4β,14-dimethyl-9,19-cyclo-5α,9β-ergost-24(28)-en-3-one (cycloabyssinone) (**38**) has been isolated from the stem bark of *H. abyssinica* and identified by NMR, GC- MS spectroscopic methods (Balde *et al.*, 2001).

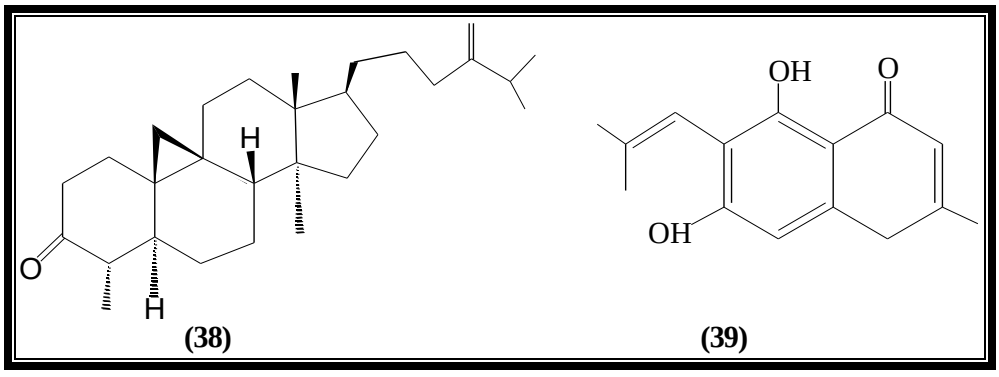


Figure 7: Compounds 38-39

Examination of the roots of the plant yielded three known chromones peucenin (**39**)**,** alloptaeroxylin and *O*-methylalloptaeroxylin (**26**) (Okorie, 1982). A chromone, 2-hydroxymethylalloptaeroxylin **(25)** has also been isolated from the plant.

2.4 Activity of the chemical constituents of *H. abyssinica*

Crude extracts and isolated chemical constituents of the root and stem bark of this plant have shown to exhibit insect antifeedant, antimicrobial, cytotoxic and plant growth inhibitory activities (Hassanali *et al*., 1987).

2.4.1 Antibacterial activity of the root extract *H. abyssinica*

In an ethnopharmacological survey carried out (Fabry *et al*., 1998), extracts of *H. abyssinica* (roots) among others were screened against 105 strains of bacteria from seven genera (S*taphylococcus, Enterococcus, Pseudomonas, Escherichia, Klebsiella, Salmonella Mycobacterium). H. abyssinica* had minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of greater or equal to 8 mg/ml and was concluded that it may serve as a source of compounds with therapeutic potency.

Fabry *et al*., (1996) evaluated the activity of the root extracts against 12 strains of *Helicobacter pylori.* It was found to have a 50% minimum inhibitory concentration (MIC_{50}) of 250 μg/ml and MIC_{90} of less than 4000 μg/ml with a range of 125-4000 μg /ml. It was concluded that these plants contain compounds with antimicrobial activity against *H. pylori*.

2.4.2 Plant growth inhibitory activity against *Striga hermonthica* seeds.

Deoxyobacunone and other limonoids; obacunone, harrisonin, 12β-acetoxyharrisonin and pedonin have shown to exhibit significant plant growth inhibitory activity against *Striga hermonthica* seed. The study provided useful information regarding the functionalities required for activity of limonoids against *Striga* seeds (Rugutt *et al*., 2001).

2.4.3 Antiplasmodial activity

The antiplasmodial activity of methanolic plant extract of *H. abyssinica* has been examined (Tahir *et al*., 1999). The extract was tested on a chloroquine sensitive strain and a chloroquine resistant strain of *P. falciparum* in Sudan. *H. abyssinica* stem bark was found to inhibit the resistant strain more efficiently than the sensitive strain with IC_{50} values of 4.7 μg/ml and 10 μg/ml, respectively. Thus it was concluded that some plants used in traditional medicine possess a potent antiplasmodial activity.

2.4.4 Insect antifeedant activities

A limonoid, pedonin, that showed insect antifeedant properties against the African crop pests *Eldana sacharina, Maruca testulalis*, army-worm *Spodoptera exempta*, has been isolated from the roots of *H. abyssinica* (Hassanali *et al*., 1987).

2.5 Acetophenones

These are products of the polyketide pathway, compounds in which the carbocyclic-skeleton is wholly polyketide-derived. The probable biosynthetic route leading to acetophenones is depicted below (Quader *et al*, 1991).

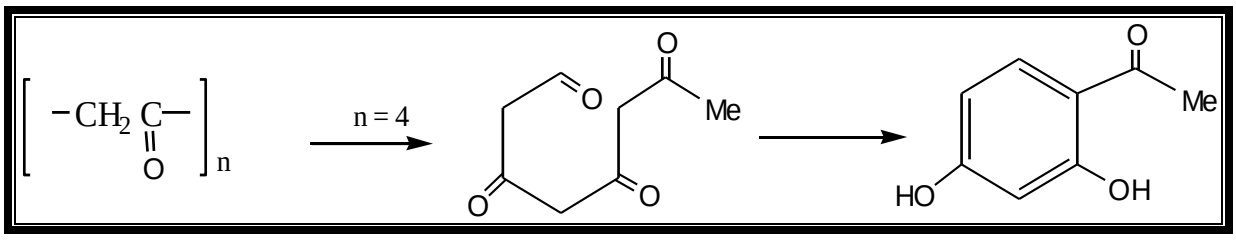


Figure 8: Biosynthetic route to acetophenones from acetate

The distribution of acetophenones appears limited and taxonomically restricted in the Rutaceae (Quader *et al*, 1991). Approximately 20 structures have been recorded and these (skeleletal types **A-E** in fig 8), and their sources, are listed in Table 1. Furthermore all the genera involved belong to Englers tribes, Zanthoxyleae and Boranieae of the subfamily Rutoideae. An examination of the aerial parts of *Acradenia frankliniae* and *A. euodiforrmis* (Rutaceae), in the study carried out to review the distribution of acetophenones confirmed that in both species the major secondary metabolites are prenylated acetophenones. The main components from both species were characterized as xantholin (**40**), franklinone (**41**)**,** franklinol (**42**), octandrenolone **(43)** and therefore are most potential as chemotaxonomic markers (Quader *et al***.,** 1991).

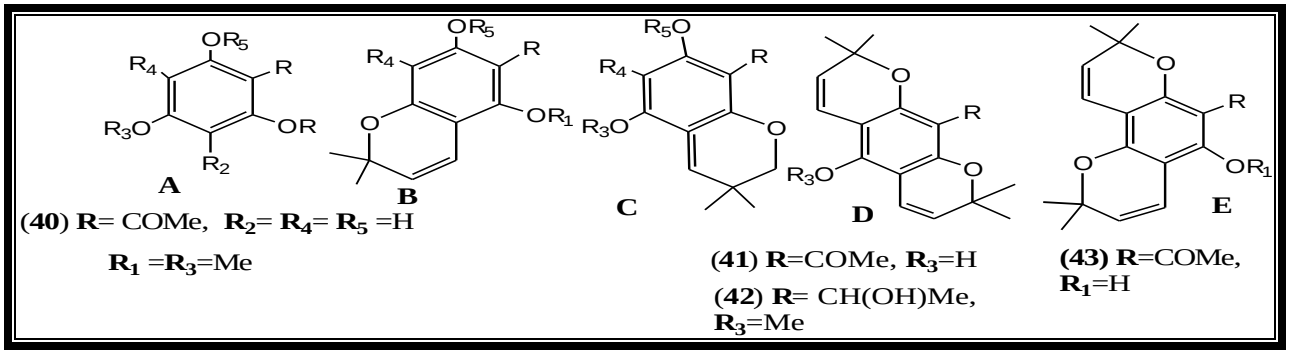


Figure 9: Five skeletal types of acetophenones found in the Rutaceae

Table 1: Acetophenones of the Rutaceae ; Structure and distribution

Trivial name	Type	R	R_1	R_2	R_3	R_4	R_5	Distribution
Xanthoxylin (40)	A	COMe	Me	H	Me	H	H	*Zanthoxylum*
								Melicope xanthoxyloides
								Acradenia frankliniae
Methoxyxathoxylin	A	COMe	Me	OMe	Me	H	H	*Zanthoxylum*
Methylxanthoxylin	A	COMe	Me	Me	Me	H	H	*Acradenia frankliniae*
Acronylin	A	COMe	Me	Pre	H	H	H	*Acronychia pedunculata*
								Melicope lnuu-ankenda
Dermethylacronylin	A	COMe	H	Pre	H	H	H	*Acronychia pedunculata*
	A	COMe	Me	Pre	H	Pre	H	*Acronychia pedunculata*
								Melicope lunu-ankenda
Acrovestone	A-dimer							*Acronychia pedunculata*
	A	$CH\text{-}CH_2$	Me	H	Me	H	Me	*Ziera chevalieri*
	A	$CH\text{-}CH_2$	Me	-O-CH2-		H	Me	*Ziera species*
Melicopol	A	CO_2Me	Ger	CH_2OH	H	H	H	*Melicope broadbentiana*
Methylmelicopol	A	COMe	Ger	CH_2OH	Me	H	H	*Melicope broadbentiana*
Evodionol	B	COMe	Me	-	-	H	H	*Melicope vitiflora*
								Melicope lunu-ankenda
								Melicope simplex
Evodionol methyl ether	B	COMe	Me	-	-	H	Me	*Acradenia frankliniae*
								Melicope lunu-ankenda
Evodione	B	COMe	Me	-	-	OMe	Me	*Melicope elleryana*
Isoevodionol	B	COMe	H	-	-	H	Me	*Melicope lunu-ankenda*
Alloevodione	C	COMe	-	-	Me	H	H	*Melicope elleryana*
Alloevodione methyl ether	C	COMe	-	-	Me	H	Me	*Acradenia frankliniae*
								Melicope lunu-ankenda
								Melicope elleryana
Franklinone (41)	D	COMe	-	-	Me	-	-	*Acradenia frankliniae*
Franklinol (42)	D	CH(OH)Me	-	-	Me	-	-	*Acradenia euodiiformis*
Franklinene	D	$CH=CH_2$	-	-	Me	-	-	*Acradenia euodiiformis*
Octandrenolone (43)	E	COMe	H	-	-	-	-	*Acronychia pedunculata*
								Acradenia euodiiformi

Pre= $CH_2 = C(Me)_2$; Ger= $CH_2CH = C\{Me\}\{CH_2\}_2$ $CH= C(Me)_2$. Types **A-E** represents the five ring systems known to occur among acetophenones in Rutaceae

In the phytochemical study of the species *Evodia merrilli* Kachira and Sasaki ex. Kachira (Rutaceae) acetophenones, 4-(1'-geranyloxy-2, 6, β-trihydroxy-3-dimethylallyacetophenone (**44**)**,** 2-(1'-geranyloxy-2,6,β-trihydroxyacetophenone (**45**), 4-(1'-geranyloxy)-2, 6-dihydroxy-3-isopentenylacetophenone (**46**) and 2-(1'-geranyloxy)-4,6-dihydroxyacetophenone (**47**) have been isolated (Lin *et al*., 1993). The acetophenones have been isolated from plants used traditionally to treat bacterial and fungal infections (Farnsworth *et al.,* 1985).

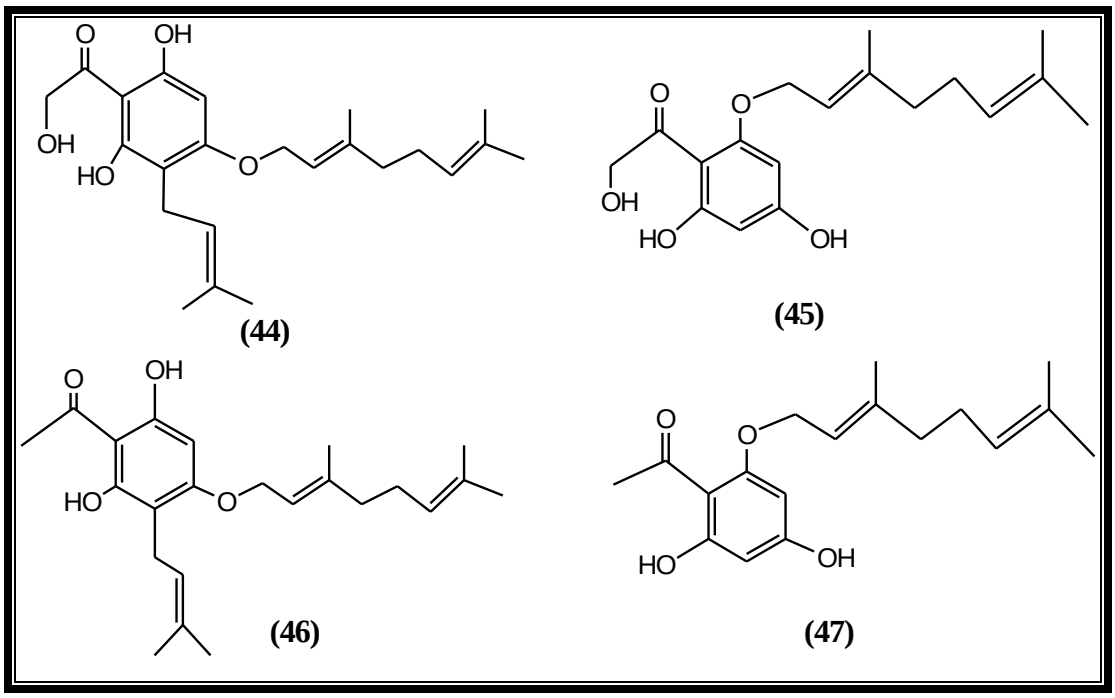


Figure 10: Compounds 44-47

Within the Rutaceae, a number of phloroglucinol oxygenation pattern substituted with prenyl, geranyl and farnesyl substituents are known, many of which exhibit similarly modified and cyclized side chains (Auzi *et al*., 1997). Leaves from four collections of *Bosistoa pentacocca,*, two of the variety *connaricarpa* and one each of the type variety and var. *dryanderensis* examined gave five phloroglucinal derivatives; pentacoccol (**48**), pentacoccol-5-methyl ether (**49**)**,** 3-hydroxy-dihydropyranopentacoccol (**50**), 2'-(1-hydroxyisopropyl) furanopentacoccol (**51**) and furanopentacoccol (**52**) which are biosynthesized from the combination of three acetate units, whereas in the acetophenones four acetate units are involved (Auzi *et al*., 1997).

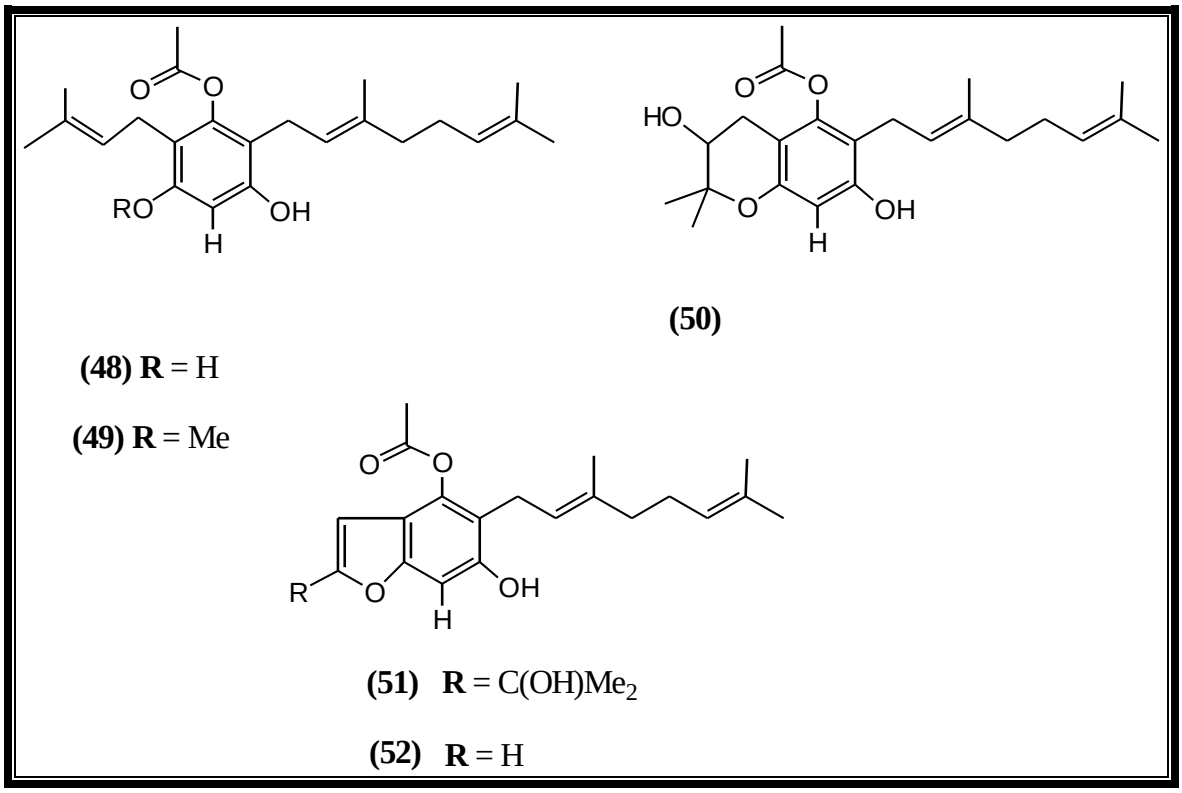


Figure 11: Compounds 48-52

Analysis of peel extracts from γ-irradiated mature oranges (C*itrus sinensis*) revealed the presence of novel metabolites/phytoalexin; 4-(3-methyl-2-butenoxy) isoacetophenone (**53**), which possess antifungal activity against C*ladosporium cucumerium* as well as antioxidant activity (Dubery *et al*., 1999).

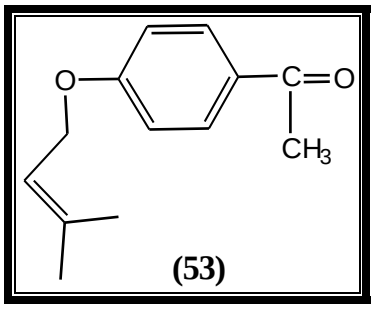
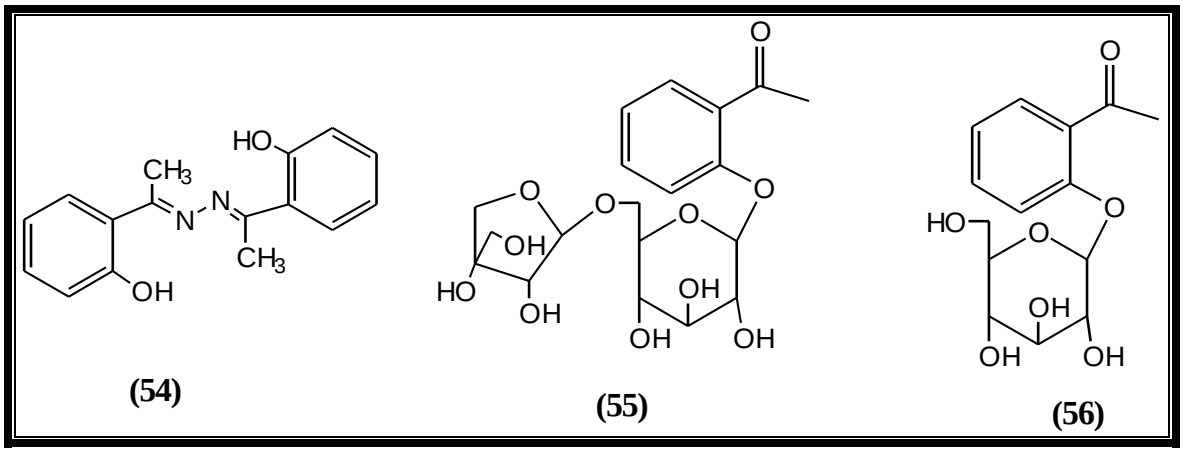


Figure 12: Compound 53

The phytochemical investigations of the root bark and the roots of *Chione venosa* (Rubiaceae), popularly known as Bois Bande in native West Indian islands, yielded

acetophenones derivatives; *O*-hydroxy-acetophenone-azine (**54**), acetophenone-2-*O*-[β-apiofuranosyl]-glucopyranoside (**55**) and acetophenone-2-*O*-D-glucopyranoside (**56**) among other compounds. The tree grows in the island rain forest and the stem bark and roots are offered for sale at the local markets as drug (Lendl *et al*., 2005)



(54) **(55)** **(56)**

Figure 13: Compounds 54-56

2.6 Test organisms

a) *Candida albicans*

It is a yeast cell which is most frequent etiological agent for candidiasis, common opportunistic fungal infection. It is known to be the cause of the following clinical implications; pulmonary infections, vaginitis, urinary tract infections, dermatitis, fungemia, mycotic keratitis, meningitis, osteomylitis, peritonitis and mycocarditis.

It is present in most humans as part of normal microbial flora. In patients who are immunocompromised e.g. HIV/AIDS, prematurely born babies, leukemic's and burn patients, the yeast can turn into a deadly pathogen causing systemic infections (Schmid, 2002).

b) *Bacillus cereus*.

These are gram positive and are the cause of serious infections in immunocompromised hosts. However its pathogenic role is doubtful. It is known to be resistant to β–lactam based drugs (they produce β-lactamases which it uses to overcome the drugs) and this makes it resistant to penicillin and all the other related antimicrobial agents (Freeman, 1979).

c) *Salmonella typhimurium*.

Are flagellated bacilli pathogenic bacteria, and are known to cause typhoid fever. The infectious nature of typhoid was apparent in 1856 when William Budd on the basis of epidemiological evidence indicated that transmission of the disease was by sewage contamination. The typhoid bacillus was discovered by Eberth in 1880 in the mesenteric glands and the spleen of persons dying from typhoid fever and was cultured in 1884 by Gaffky. *S. typhimurium* are gram negative and they stain readily with usual dyes such as methylene blue, and infection in human is almost always by ingestion of the microorganism usually as contamination of food, milk or even water (Schmid, 2002).

d) *Escherichia coli.*

E. coli is gram-negative bacteria and so as part of normal intestinal flora except for a few enteropathic strains, which are commensal in the gastrointestinal tract. However there are potentially pathogenic strains elsewhere in the body where they cause pyogenic infections. Diagnosis is by fluorescent antibody methods, which are available for rapid presumptive diagnosis of enteropathic *E. coli* in rectal swabs. Tests for the production of enterotoxin by isolated strains are also possible (Freeman, 1979).

e) *Staphylococcus aureus*

It is a gram-positive bacteria and a pathogen in humans; its mode of action is usually by production of a number of enzymes and toxins that contribute substantially to its ability to cause disease (Freeman, 1979).

2.7 Mode of action of antimicrobial agents

The biochemical mechanisms that underlie the action of compounds used in the fight against microorganisms is based on the essential metabolic process of the organism. This selective toxicity can be due to distinct differences between the metabolism of the host and that of the target organism, due to the selective binding to grossly different metabolic structures or due to the differences in permeability of cells to chemotherapeutic agents. It is essential to note that for a newly isolated compound with no previous reported mode of action it may not be easy to suggest mechanism of action from the chemical structure.

Some of the modes of antimicrobial agents are:

i) Inhibition of folic acid synthesis

ii) Inhibition of peptidoglycan synthesis.

iii) Inhibition of protein synthesis.

iv) Inhibition of nucleic acid synthesis

v) Disruption of the integrity of cell membrane. (Schmid, 2002).

2.8 Antimicrobial activity tests

These are *in vitro* tests carried out to check for the chemotherapy of drug against specified pathogenic microorganisms. Methods that were used in these *in vitro* tests include agar diffusion, and broth dilution tests (Ryle *et al*., 1981).

2.8.1 Agar diffusion assay

Agar diffusion tests measure the inhibition of test microorganism growth by a drug diffusing from a point source. Typically the inoculum of test microoganisms is seeded into sterile molten agar (45°C) medium swirled gently to ensure homogeneity before being poured into plates. After cooling samples are commonly loaded onto filter paper discs and placed on the surface of the solidified medium. During incubation, the organism grows but zones of no growth develop around the disks containing agents that inhibit growth. Zones of inhibition may as well not be observed if the agent is not active against that microorganism. The size of the zone of inhibition caused by the diffusion of the agent into the agar is directly related to the degree of susceptibility of the organism to that agent.

2.8.2 Serial dilution assay

It involves adding decreasing amounts of the sample concentrations into micro titer plate wells containing a defined liquid inoculum of the test microorganism. After incubation for 18-24 hours each well is examined for the amount of microorganism growth, compared to an untreated control. The lowest concentration of the drug that inhibits (prevents) growth is called minimum inhibitory concentration (MIC). The use of 96-well micro titer plates permits the testing of 8 samples at 12 concentrations including zero against a single microbe at a time.

2.9 Nuclear Magnetic Resonance (1D and 2D) Spectroscopy

Over the past fifty years nuclear magnetic resonance spectroscopy, commonly referred to as NMR, has become the pre-eminent technique for determining the structure of organic compounds. Of all the spectroscopic methods, it is the only one for which a

complete analysis and interpretation of the entire spectrum is normally expected and is concerned with the magnetic properties of certain atomic nuclei. NMR is non-destructive and with modern instruments good data may be obtained from samples weighing less than a milligram (Kemp, 1987).

Proton NMR (^{1}H-NMR) offers three important parameters, which can be extracted; the chemical shift (δ), ^{1}H-^{1}H coupling constants ($^nJ_{HH}$; n=number of intervening bonds) and the signal intensity. Studying a molecule by NMR enables the deduction of the position of the nuclei in the molecule and how many different kinds of chemical environments. The position of atoms in the molecule are dependent on electron density about the environment, which relates most importantly to inductive and resonance effects, transmitted through bonds and the anisotropic effects through space (Brown *et al.*, 1988).

Carbon-13 NMR (^{13}C-NMR) spectra are routinely recorded under ^{1}H broad - band (BB) decoupling. Thus a significant improvement of signal/noise ratio is achieved, because the signals of the insensitive ^{13}C nuclei appear as narrow singlets without any splitting due to ^{13}C-^{1}H, coupling. In addition, the Nuclear Overhauser Effect (NOE) may enhance the signal intensities thereby as much as three fold (Duddeck *et al.*, 1998). However this is accompanied by a complete loss of ^{13}C-^{1}H coupling information. The number of hydrogen atoms adjacent to a carbon can no longer be determined. In ^{1}H coupled spectra obtainable by the so called gated decoupling technique the carbon signal are split owing to the large one bond ^{13}C-^{1}H coupling constants ($^1J_{CH}$, between 120 and 200 Hz) (Kalinowski *et al.*, 1984). Doublets are observed for CH, triplets for CH_2 and quartets for CH_3 fragments, possibly over range of several parts per million (ppm). Often these multiplets contain further fine splittings from couplings over more than one bond and may overlap severely so that an ambiguous assignment is possible (Duddeck *et al.*, 1998).

To escape the above discrepancy, the so-called off-resonance decoupling technique was invented at the beginning of routine ^{13}CNMR spectroscopy. The effect of partial ^{1}H decoupling was achieved by irradiation of a selective proton frequency near to the ^{1}H resonance range (Kalinowski *et al.*, 1984). This led to invention of another important technique, INEPT (Insensitive Nuclei Enhanced by Polarization Transfer), in

which a J-modulation is accompanied by a polarization transfer (PT) from protons to coupled carbons. With this method however signals of quaternary carbons do not appear because the experiment is generally optimised to accomplish PT via large bond coupling. Nevertheless, such quaternary carbon signals can easily be detected by comparison of the INEPT spectrum with the normal ^{1}H broad – band decoupled ^{13}C NMR spectrum.

A further improvement has been introduced by the DEPT (Distortionless Enhancement by Polarization Transfer) technique (Derome, 1987). Its advantage compared to INEPT, is a pulsed sequence so that during the evolution time the loss of magnetization due to transversal relaxation is less severe. Moreover DEPT is clearly less sensitive to missetings of parameters such as pulse widths of delays (as function of coupling constants) (Shaka *et al*., 1987). The so-called spectral editing enables us to prepare DEPT spectra in such a way that only CH, CH_2 and CH_3 signals are displayed.

2D NMR spectroscopic analysis such as COSY, NOESY, HMBC and HMQC were necessary so as to determine the interactions of the atoms in the molecule. Two-dimensional ^{1}H-^{1}H correlation like COSY (correlation spectroscopy) provides information about the connectivity of atom within the molecule emerging from internuclear couplings. Two-dimensional ^{13}C-^{1}H one bond correlation for example HETCOR (Heteronuclear correlation) are necessary since it connects ^{1}H signals with ^{13}C coupling. HMQC (Heteronuclear multiple-Quantum correlation) is an inverse technique to HETCOR as it records ^{1}H nuclei coupled to ^{13}C spins. This information is very important for structure elucidation purposes. Other important NMR data can be derived from two-dimensional ^{13}C-^{1}H long-range correlation techniques. Heteronuclear Multiple-Bond correlation (HMBC) is generally conducted without ^{13}C decoupling so that correlation via one or more bond can be discerned.

2.10 Mass spectrometry

Mass spectrometry also is very important since is provides information on the relative molecular mass and the chemical formula of the compound. Mass Spectrometry is based on slightly different principles to the other spectroscopic methods (Ashcroft, 1997). The sample has to be introduced into the ionisation source of the instrument. Once inside the ionisation source, the sample molecules are ionised, because ions are easier to manipulate than neutral molecules. The ionization process can either produce a

molecular ion which will have the same molecular weight and elemental composition of the starting analyte, or it can produce a fragment ion which corresponds to a smaller piece of the analyte molecule. These ions are extracted into the analyser region of the mass spectrometer where they are separated according to their mass to charge ratios (m/z). The separated ions are detected and this signal sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of an m/z spectrum (Wiley, 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Equipment

Autoclave (Danofass 59407-3 NO. 375 2 ATO PROVET 1977), weighing balance (EK-1200GD max 1200g d=0.1g), Vacuum pump (Type 349\2), Mill (MILWAUKEE WINSOONSIN 53207 model NO.311), Refrigerator, Incubator (Carbolite Sekonic pocketcorder SK-50P), Analytical balance (PRECISA 310 SWISS QUALITY), rotary evaporator type 349/2, Oven – Struess Electrolux. These were used in addition to other more equipment.

3.2 Chemicals and reagents

Ethyl acetate, methanol, distilled water, nutrient agar, nutrient broth, dichloro-methane, hexane, silica gel60, sephadex LH20.

3.3 Apparatus and other materials

Erlenmeyer conical flasks, Bunsen burner, glass vials, Buchner funnel, round bottomed flasks 500 ml, measuring cylinders, glass Petri dishes, paper discs (Rundfilter 6mm Schleicher& Scheull, Dasseln Germany), forceps, filter papers (Macharey Nagel), aluminium foil, cotton wool, parafilm, scissors, column, TLC plates (20x20 cm Macharey Nagel Duren), 96-microtitre well plates, adjustable volume (analogue) micropipette

3.4 Laboratory strains of micro organisms used for testing

Fungus	Source
Candida albicans (wild type)	-KEMRI
Bacteria	
Lactobacillus casei (wild type)	-KEMRI
Salmonella typhimurium (ATCCC 14028/AMZ)	-KEMRI
Staphylococcus aureus (S140)	-KEMRI
Escherichia coli	-KEMRI
Bacillus cereus	-KEMRI

KEMRI- Kenya Medical Research Institute

3.5 Collection and taxonomic identification of the plant berries

The plant berries were collected from South Sakwa, Bondo District of Kenya. A taxonomist from Botany Department of Egerton University identified the plant (reference number MK/HR/04) and the sample specimen was deposited in East African Herbarium.

3.6 Preparation of the crude extract

The seeds of the plant were air dried under shade, away from sunlight to avoid any photo induced decomposition and transformation of compounds present. The dried plant seeds were ground using a mill (MILWAUKE WISCONSIN 53207 model NO.311) to a fine powder and stored in standard sample paper bags under dry conditions at low temperature.

The powdered material was defatted with hexane and was soaked in methanol and dichloromethane mixture in a ratio 1:1 for 72 hours. Some of the solvents used in this research project were general-purpose reagents. The solvents were always distilled before use to get rid of impurities. Filtration was carried out and the solvent evaporated under reduced pressure to give a crude extract that was preserved at 4°C. Evaluation of antimicrobial activity of the crude extract was done using strains of microorganisms (sec 3.4).

3.7 Thin layer chromatography

The crude extract was spotted on aluminium coated silica TLC plates (20x20 cm Machary Nagel Duren), and then developed in chambers saturated with various solvent systems. Different solvent systems were prepared from combination ratios of methanol, dichloromethane, ethyl acetate and hexane. The developed plates were allowed to dry and were visualised by spraying with anisaldehyde before heating at 110°C for 15 minutes. The plates with the best separation were used to determine solvent mixture for efficient separation of the compounds. The results obtained using TLC were translated to column chromatography using the same adsorbent.

3.8 Column chromatography

The column was used to fractionate the crude extract into enriched fractions. The extract (20 g) was added to 5 g of silica gel drop wise and the solvent was allowed to

evaporate. This was repeated until all the dissolved extract was adsorbed on to the silica gel.

The column was slurry packed with silica gel suspended in dichloromethane. The column was initially mounted vertically and was halfway filled with dichloromethane. To prepare the slurry, 40g of silica gel was added to 100 ml of dichloromethane in a 250 ml Erlenmeyer flask. It was degassed using a sunction pump and slurry was poured into the column, which was simultaneously drained at the bottom. The column was washed with dichloromethane and drained at the same time until all the slurry was packed into the column. The silica gel adsorbed sample was introduced and the sample was anchored in place with acid washed sand so as to avoid turbulence while adding the mobile phase. The column was eluted using discrete solvent gradient mobile phase of varying polarities. The solvent varying polarity ratios used were:

100% dichloromethane

95% dichloromethane: 5% ethyl acetate

90% dichloromethane: 10% ethyl acetate

In each case, fractions were collected differently for each solvent gradient system as much as possible. TLC analysis was used to determine the purity of the eluent fractions. Fractions were pooled into three main fractions, concentrated under reduced Pressure and chromatographed on silica gel repetitively. Further purification was carried out by size exclusion method using Sephadex LH20. Purified fractions were further tested for activity. Each of the fractions was transferred into a screw-capped vial and then kept for further analysis at 4^{o}C.

3.9 Preparation of the test plates for antimicrobial test

3.9.1 Resuscitation of test microoganisms

The test microorganisms were obtained from the DAFTEC department of Egerton University. The deep frozen microorganisms were obtained and left to thaw at room temperature.

3.9.2 Inoculation and incubation of resuscitated microorganisms

A wire loop was sterilized by passing it several times over a flame of a Bunsen burner. The inoculum was then picked using the wire loop and spread on the upper

portion of the broth for each test microbes in duplicate. These were incubated at 37°C for 24 hours.

3.9.3 Preparation of culture petri dishes

About 7 g of nutrient agar was weighed and transferred into 500 ml conical flask. Into the conical flask containing the nutrient agar, 250 ml of distilled water was added. The mixture was swirled to dissolve completely. The flask was sealed using a cotton wool and aluminium foil. And then, it was autoclaved for 30 minutes at about 120^{o}C then allowed to cool to 45°C in a warm bath. The cool agar was used to harvest each of the test microorganisms from the resuscitated colonies. The harvested microorganisms were each seeded into separate 250 ml of nutrient agar in a conical flask under sterile conditions. The flasks were swirled to distribute the microorganisms uniformly. The seeded medium was dispersed into sterile Petri dishes. When agar solidified the plates were used immediately or kept at 4°C, except one plate for each microorganism that were incubated at 37°C for 18-24 hours to ascertain whether the microorganisms were still viable.

3.9.4 Antimicrobial activity testing using agar diffusion assay

Samples (5 mg) of crude extract and isolated compounds were dissolved in 1 ml of methanol. About 10 µl of the crude extract and the isolated compounds were pipetted onto paper disk placed on a sterile glass using a self-loading pipette. The paper discs were left to dry in air then carefully placed on the surface of the Petri dishes containing cultured microorganisms in nutrient agar. The test plates were then incubated at 37°C for 24 hours. The inhibition growth of the microorganisms was determined by measuring the diameter in mm.

3.9.5 Serial dilution assay

The pure compound(s) were diluted (over the ranges of 0 – 20 µg/ml) into micro titre wells, containing liquid medium each with defined innocula of test microorganisms. Pure methanol (100%) was used as a negative control. They were incubated for 24 hrs at 37°C and the lowest concentration of the sample that showed no visible growth was recorded as the minimum inhibitory concentration (MIC).

3.10 Characterisation of the active compounds

The compounds obtained were analysed using spectroscopic techniques namely 1D, 2D NMR, and the spectra interpreted to give proposed structures. The structures were confirmed using MS spectrometry.

3.10.1 NMR spectroscopy

NMR spectroscopy was carried out on a Varian Unity-Inova 400 MHZ spectrometer. The purified compounds were dissolved in deuterated chloroform ($CDCl_3$) in a clean dry micro vial. The solution was shaken to ensure that all the solute was dissolved.

The solution was then transferred into an NMR tube using a clean pipette. The NMR tube was then placed on the probe for analysis. The same sample was used to obtain the spectral (^{1}H, ^{13}C, DEPT, NOESY, HMBC and COSY) data for the compound. Data was acquired from the NMR machine as computer print outs, all spectra were recorded at room temperature using Deuterated chloroform ($CDCl_3$).

3.10.2 Mass Spectrometry

Mass spectrometry was carried out using Quadrupole-TOF mass spectrometer that was operated in ESI (electron spray ionisation) coupled to Ultra Performance Liquid Chromatography system with a UV detector.

3.10.3 Optical Rotation

Optical rotations were measured at room temperature using model ADP 220 Polarimeter with 20 cm tube length. The pure compounds were measured in grams, dissolved in 30 ml of methanol and percentage concentrations obtained which were then loaded to the 20 cm tube. The optical rotations were recorded in radians.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Column chromatography

From the first column 35 fractions were collected. They were spotted on TLC plate and on visualization using anisaldehyde spraying reagent gave three major spots. The fractions were pooled into three main fractions and were concentrated under reduced pressure. They were chromatographed repetitively and finally purified on sephadex LH20. The three pure compounds obtained were coded HRF_1, HRF_2 and HRF_3. TLC analysis was used to determine the purity of the compounds and the plates showed that HRF_2 and HRF_3 had nearly same retardation factors. Compound HRF_I (92 mg) was obtained as a light yellow crystalline solid while compound HRF_2 (45 mg) and HRF_3 (240 mg) were obtained as light grey paste.

4.2 Agar diffusion assay

At the beginning of the study it was hypothesized that compounds present in the berries of the plant could be active against the test microorganisms. Bioassay guided fractionation led to isolation of HRF_1, HRF_2 and HRF_3. Primary screen *in vitro* agar diffusion assay of the crude extract of *H. abyssinica* displayed very high activity against *C. albicans*, *E. coli* and *B. cereus* and *L. cereus*, while it showed significant activity against *S. typhimurium* and *S. aureus* (table 2) and therefore this supports the use of decoctions from *H. abyssinica* as an alternative herbal medicine. Agar diffusion assay using chromatographic compounds displayed decreased activity in HRF_1, HRF_2 and HRF_3 and these could be attributed to the long duration of storage or possibilities of the three compounds reinforcing each other in the crude extract could not be ruled out.

The compound HRF_1 displayed significant activity on all the test microorganisms that is it had inhibition zones of 12-15 mm (table 2). Compounds HRF_2 and HRF_3 displayed significant activity against four test organisms (*C. albicans*, *E. coli* and *B. cereus* and *L. cereus*), inhibition zones of (10-11 mm) and insignificant activity against other test organisms (<10 mm). HRF_2 and HRF_3 displayed similar inhibition zones (Table 2). Due to the significant activity in HRF_1, its activity was evaluated using serial dilution assay, The compound was found to have minimum inhibitory concentrations (MIC) of 5 µg/ml against *C. albicans*, 6 µg/ml against *B. cereus* and > 20 µg/ml against

other test microorganisms and therefore concluded that the compound could exhibit potent activity. In both methods the solvent methanol was used as a control and it had no bioactivity effect hence the results was independent of the effects of the solvents.

Table 2: Table showing antimicrobial activity of the extract and the isolated compounds, diameter of inhibition zones in mm

Microorganism/ Sample	Crude extract	HRF_1	HRF_2	HRF_3	MeOH
C. albicans	30	15	10	11	-
B. cereus	35	14	11	10	-
E. coli	30	13	10	11	-
S. typhimurium	10	12	8	9	-
S. aureus	20	12	8	8	-
L. casei	17	12	10	10	-

Key:

(-) -no activity

<10 mm- non significant activity

10-19 mm -significant activity

>20 mm- high activity

MeOH- negative control

4.3 Serial dilution assay

Table 3: MIC values of HRF_1

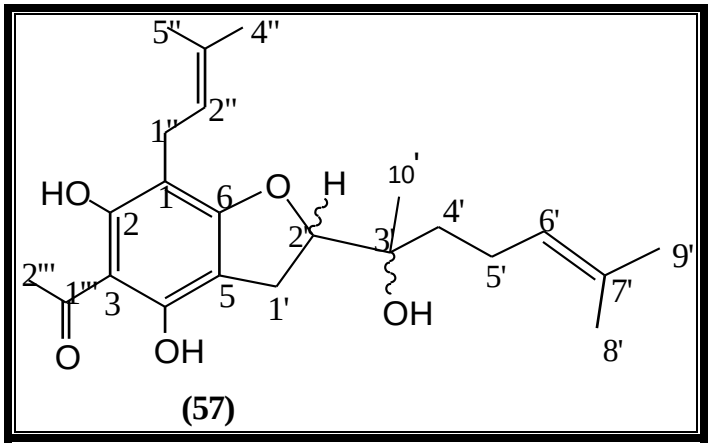
Organism	MIC (µg /ml)
C. albicans	5
B. cereus	6
E. coli	>20
S. typhimurium	>20
S. aureus	>20
L. casei	>20
MeOH	-

4.4 Structural elucidation of HRF_1 (harronin I)

Table 4: ^{1}H- NMR, ^{13}C-NMR, DEPT and HMBC data for HRF_1 (harronin I)

HRF1				
POSITION	^{1}H (*J* in Hz)	^{13}C	DEPT	HMBC (H $\rightarrow$C)
1		101.47	C	
2		161.71	C	
3		105.61	C	
4		156.59	C	
5		103.39	C	
6		164.28	C	
1’	3.03 dd (9.3) 2.99 dd (9.4)	26.82	CH_2	4, 5, 6, 2’, 3’
2’	4.68 t (9.3)	90.15	CH	1’, 5, 6, 10’
3’		73.85	C	
4’	1.51 t (10.0)	37.09	CH_2	2’, 3’, 5’, 6’
5’	2.08 dt (6.6, 10.0)	21.88	CH_2	4’, 6’, 7’
6’	5.09 t (6.6)	123.89	CH	5’, 8’, 9’
7’		132.20	C	
8’	1.66 s	25.64	CH_3	5’, 6’, 9’
9’	1.59 s	17.63	CH_3	5’, 6’, 7’, 8’
10’	1.24 s	22.30	CH_3	2’, 3’, 4’
1”	3.22 d (7.1)	22.23	CH_2	1, 2, 3, 5, 6, 2”,3”
2”	5.20 t (7.1)	121.40	CH	1, 4”, 5”
3”		134.81	C	
4”	1.76 s	17.80	CH_3	2”,3”, 5”
5”	1.72 s	25.78	CH_3	2”, 3”, 4”
1”’		203.36	C	
2”’	2.60 s	32.75	CH_3	1”’, 3

The structure of compound HRF_1 was determined by; ^1H NMR, ^{13}C NMR, COSY, DEPT, HSQC, NOESY and HMBC. The ^1H NMR of HRF_1 showed characteristic olefinic proton absorptions at δ 5.20 t (J = 7.14 Hz) and 5.09 t (6.68), an isolated methyl group δ 1.24(s), a set of four methyl protons attached to a quaternary carbon at δ 1.76 (s), 1.72 (s), 1.66 (s), 1.59 s, an oxygenated methine carbon at δ 4.68, isolated methyl protons next to a carbonyl carbon at δ 2.60 s and two non equivalent protons at δ 3.03 and δ 2.99. The ^{13}C NMR exhibited 23 carbon resonances, a characteristic sp^2 carbonyl carbon at δ 203.36 and 10 sp^2 hybridised carbons within the range (δ 100–164.28) and 12 sp^3 hybridised carbons between δ 90-17.25. The DEPT experiment showed that there are 13 protonated carbons (three methine, four methylene and six methyl carbons) and therefore 10 quaternary carbons. Examination of COSY helped in assigning the different patterns in compound, the experiment indicated the presence of two isolated structural units (-CH_2 (1')-CH (2')-COH (3')- CH_2 (4')-CH_2 (5')-CH (6')-C (7')-$(CH_3)_2$ (8'& 9') and (-CH_2 (1")-CH (2")- C(3")- $(CH_3)_2$ (4"&5"). All protons were located by HMBC results (table 4) and these correlations were verified by NOESY. The HMBC results also confirmed the correlation between the CH_3 protons (2'") δ 2.60 with the carbonyl carbon δ 203.36 (1'"). From this interpretation the structure (**57**) was therefore proposed and named harronin I (**57**. The compound belongs to the class of compounds called acetophenones. Full NMR and MS spectra are attached as appendices.



(57)

Figure 14: Structural formula of harronin I

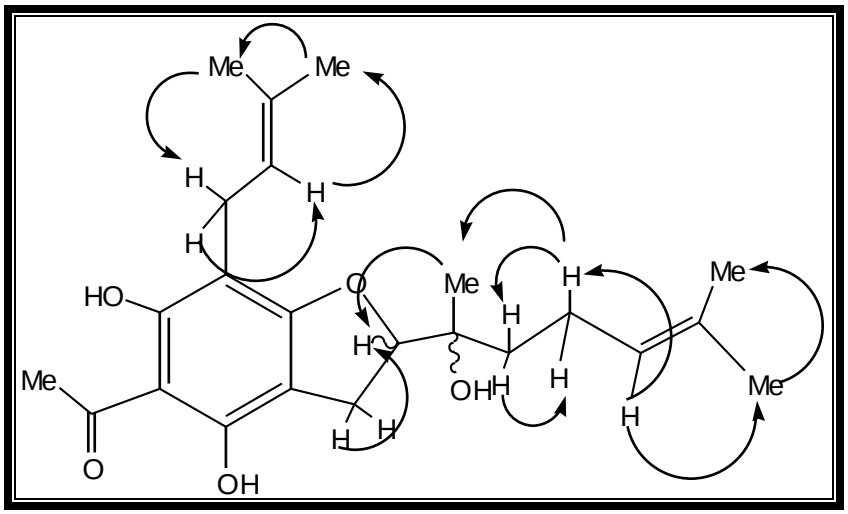


Figure 15: Selected NOESY correlations of harronin I

The molecular formula from ^{13}C and ^{1}H NMR data of harronin I was $C_{23}H_{32}O_{5,}$ which gives the unsaturation number that gives complementary information to that of the structure of the compound**.** Unsaturation number of $C_{23}H_{32}O_5$ is given by

$$\frac{2 \text{ x no.of carbons} + 2 - \text{no.of hydrogens}}{2}$$

$$= \frac{2x23 + 2 - 32}{2}$$

$$= 8$$

This was accounted for by the aromatic ring, one carbonyl, two double bonds and five member cyclic ring. The melting point of compound **(57)** was 105-108°.

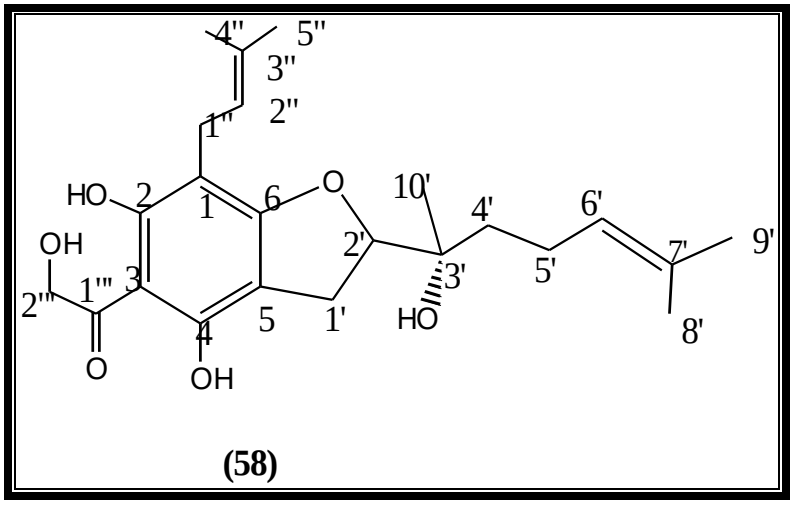
4.5 Structural elucidation of HRF_3 (harronin II)

Table 5: ^{1}H- NMR, ^{13}C-NMR, DEPT and HMBC data for HRF_3 (harronin II)

HRF_3				
POSITION	^{1}H (*J* in HZ)	^{13}C	DEPT	HMBC(H $\rightarrow$C)
1		102.98	C	
2		162.00	C	
3		102.98	C	
4		159.23	C	
5		104.05	C	
6		165.50	C	
1’	3.03 dt (9.5, 19.6)	26.69	CH_2	4, 5, 6, 2’, 3’
2’	4.65 t (9.8)	90.27	CH	1’,4’, 5, 6, 10’
3’		73.86	C	
4’	1.50 s	37.35	CH_2	2’, 3’, 5’, 6’
5’	2.08 m	22.02	CH_2	4’, 6’, 7’
6’	5.09 t (7.07)	123.83	CH	
7’		132.91	C	
8’	1.66 s	25.75	CH_3	5’, 6’, 9’
9’	1.59 s	17.62	CH_3	5’, 6’, 7’, 8’
10’	1.25 s	22.26	CH_3	2’, 3’, 4’
1”	3.20 d (7.14)	21.91	CH_2	1, 2, 3, 5, 6,
2”	5.18 t (7.2)	121.35	CH	1, 4, 5”
3”		134.12	C	
4”	1.75 s	17.79	CH_3	2”,3”, 5”
5”	1.69 s	25.65	CH_3	2”, 3”, 4”
1”’		203.36	C	
2”’	4.69 s	68.18	CH_2	1”’,3

The features of HRF_3 **(58)** in the ^{1}H NMR spectra Table 5, were virtually the same as those of HRF_1 **(57)** except for methylene protons at δ 4.69 due to oxidation of methyl protons at δ 2.60 s in **(57)** next to carbonyl group in the ^{1}H NMR spectrum. The ^{13}C looked like that of (**57**) except for an additional oxygenated methylene carbon at δ 68.18 (C-2”’) in **(58)** instead of a methyl group at δ 32.3 (C-2”’) in **(57)**. The HMBC experiments clearly showed the correlations from methylene protons to the carbonyl carbon (table 5). On this basis the compound has been characterised as an acetophenone and is been isolated for the first time hence named harronin II. The compound has a melting point of 120-122°. The ^{1}H NMR spectra of compound HRF_2 when analysed

showed similar absorption signals as those of HRF_3 apart from the noise signals due to impurities. Full NMR and MS spectra are attached at the back as appendices.



(58)

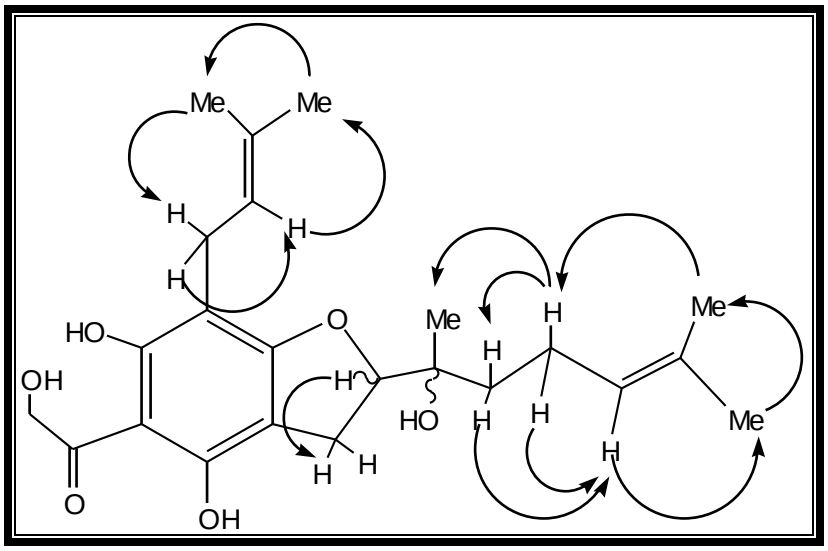


Figure 16: Structural formula of harronin II (HRF_3) and its NOESY correlations

4.6 Optical rotation results

From the proposed structures the compounds were observed to contain chiral centres and therefore optical activity was measured. A polarimeter measures the rotation of plane polarized light. The light from sodium lamp is filtered so that it contains just one wavelength (sodium D line) which then passes through the sample cell containing a solution of the optically active compound. On leaving the sample cell the polarize light encounters another polarizing filter which is movable with a scale allowing the operator to read the observed rotation, α which was measured in radians.

Specific rotation [α] (characteristic physical property) of a given compound was found by,

Observed rotation, α for (HRF_1) harronin I = - 0.9 radians x 57.3 = - 51.59°

Concentration was 0.001g/ml

Length was 20cm = 2dm

$$\text{For HRF}_1\ [\alpha]^{19} = \frac{\alpha(\text{observed})}{\text{conc(g/ml)x length(dm)}}$$

$$= \frac{-51.59^\circ}{0.001\text{x}2}$$

$$= -25795^\circ$$

$$= -25795^\circ + 71(0^\circ) = -235^\circ$$

Observed rotation, α for (HRF_3) harronin II =- 0.4 radians x 57.3 = - 22.92°

Concentration was 0.01g/ml

$$\text{For HRF}_3\ [\alpha]^{19} = \frac{-22.92^\circ}{0.01\times 2}$$

$$= -1146^\circ$$

$$= -1146^\circ + 3(0^\circ) = -66^\circ$$

$$\textit{Note}: 360^\circ \equiv 0^\circ$$

4.7 Mass spectrometry

The compound Harronin I gave an $[M + H]^+$ ion at *m/z* 389.2335 (calculated for $C_{23}H_{32}O_5$, 388.2328) in the HRESITOFMS suggesting the molecular formula $C_{23}H_{32}O_5$. Harronin II gave an $[M + H]^+$ ion peak at *m/z* 405.2277 (calculated for $C_{23}H_{32}O_6$, 404.502). This confirms the structures of the two compounds. MS spectra are attached at the back as appendices.

CHAPTER FIVE

GENERAL CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Extraction of the plant berries gave a crude extract that showed very high activity against all the tested microorganisms. The good activities obtained for the extract supports the use of this plant as an herbal medicine. The bioassay guided fractionation gave compounds that were mid-polar as they were eluted by a solvent system of moderate polarity. The activity of these compounds using agar diffusion assay were; significant for HRF_1 (harronin I) (inhibition zones of 12-15 mm) and non significant for HRF_2 and HRF_3 (harronin II) (inhibition zones of 8-11 mm). Due to significant activity of harronin I it was further subjected to serial dilution assay and it gave minimum inhibitory concentration (MIC) values ranging from 5 to >20 µg/ml. Isolation of bioactive agents has demonstrated that medicinal activity of this plant is a reality as they have demonstrated high potency. The present results show that there are plenty of unexploited natural resources of compounds which can be used to control bacteria.

The proposed structures of these compounds were obtained using the NMR spectroscopic, mass spectrometry and other physical methods. HRF_2 and HRF_3 had quite similar inhibition zones, retardation factors and NMR absorption signals apart from the noise signals observed in HRF_2 which eventually explain why the two fractions gave the same compound. Bioassay guided research has revealed new harronin I (**57**), harronin II (**58**) from the class of acetophenones and which is being reported for the first time from this plant (*H. abyssinica*).

5.2 Recommendations

The following recommendations were made in relation to this study:

1. That harronin I which showed significant activity need to be screened against other strains of microorganisms to enrich its potency.
2. That the Harronin II which showed non-significant activity need to be further screened using serial dilution assay.
3. That if possible their dosage should be determined. This will serve to enrich the chemical potency in search for new antimicrobial compounds
4. That the active compounds should be subjected to *in vivo* to ascertain their activity and eliminate the prediction that may be, observed activities were due to non-selective toxicity of the compounds.
5. Extensive research should be done on these compounds, to determine toxicity LD_{50} (the dose which can kill half the population or the test group i.e. the lethal dose) and other relevant information so that the use of *H. abyssinica* in alternative medicine can be fully exploited

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APPENDICES

A_1: ^1H NMR for HRF_1 (harronin I)

hhrf1.hrf1 in cdcl3
probe=5mmASW

Pulse Sequence: s2pul

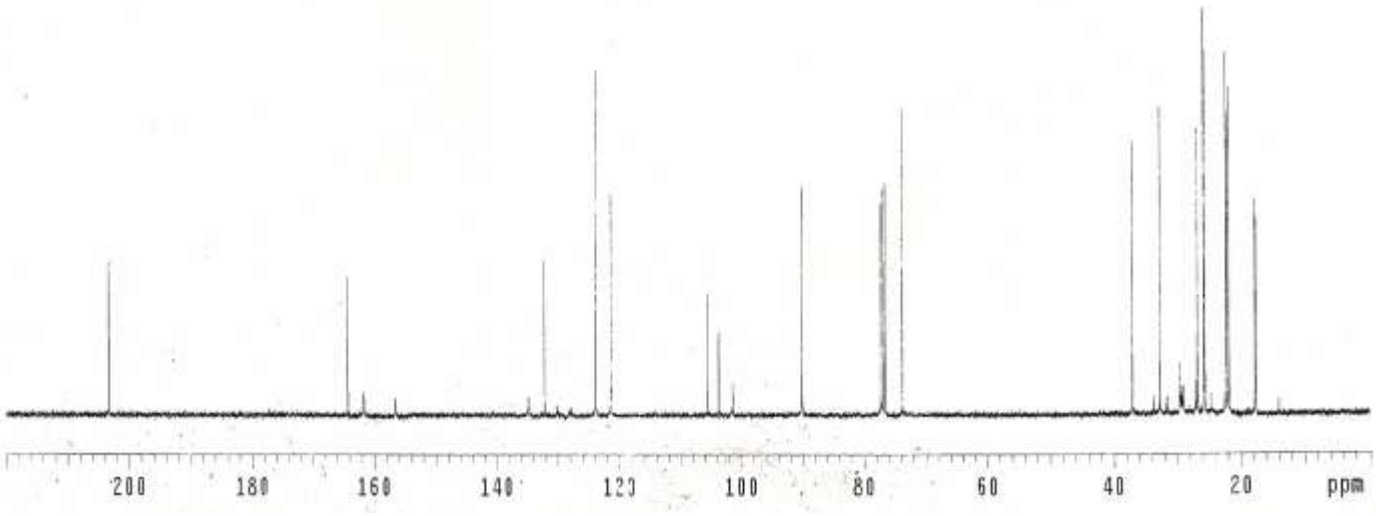
INDEX	FREQUENCY	PPM	HEIGHT	INDEX	FREQUENCY	PPM	HEIGHT
1	2895.544	7.240	5.4	40	607.525	1.519	9.2
2	2085.929	5.216	3.8	41	605.877	1.515	10.8
3	2079.885	5.201	6.6	42	603.679	1.509	10.6
4	2078.603	5.197	8.4	43	599.284	1.498	11.3
5	2077.505	5.195	6.9	44	597.636	1.494	9.8
6	2071.461	5.179	4.2	45	595.439	1.489	9.3
7	2038.132	5.096	4.0	46	591.959	1.480	4.7
8	2036.850	5.093	3.5	47	589.029	1.473	8.1
9	2032.272	5.081	6.7	48	587.198	1.468	5.7
10	2030.990	5.078	8.6	49	581.521	1.454	3.7
11	2029.891	5.076	7.2	50	515.045	1.288	9.0
12	2024.031	5.061	4.3	51	511.565	1.279	9.1
13	1882.655	4.707	9.1	52	508.086	1.270	8.4
14	1873.499	4.684	16.5	53	495.267	1.238	120.5
15	1865.258	4.664	9.8	54	489.407	1.224	20.7
16	1292.978	3.233	15.2	55	339.607	0.849	3.7
17	1285.836	3.215	14.4				
18	1214.599	3.037	17.7				
19	1212.402	3.031	18.0				
20	1206.541	3.017	16.3				
21	1203.062	3.008	16.6				
22	1040.077	2.601	150.0				
23	854.750	2.137	3.6				
24	848.157	2.121	4.5				
25	845.044	2.113	4.6				
26	837.302	2.095	6.2				
27	830.943	2.078	6.2				
28	820.322	2.051	5.3				
29	815.743	2.040	5.1				
30	813.363	2.034	4.9				
31	805.671	2.014	3.7				
32	707.331	1.769	73.5				
33	684.623	1.712	65.4				
34	661.731	1.655	71.2				
35	636.093	1.590	75.9				
36	627.853	1.570	4.7				
37	621.626	1.554	4.9				
38	617.597	1.544	3.6				
39	613.752	1.535	8.7				

A_2: ^{13}C NMR for HRF_1 (harronin I)

chrf1.hrf1 in cdcl3
probe=5mmASW

Pulse Sequence: s2pul

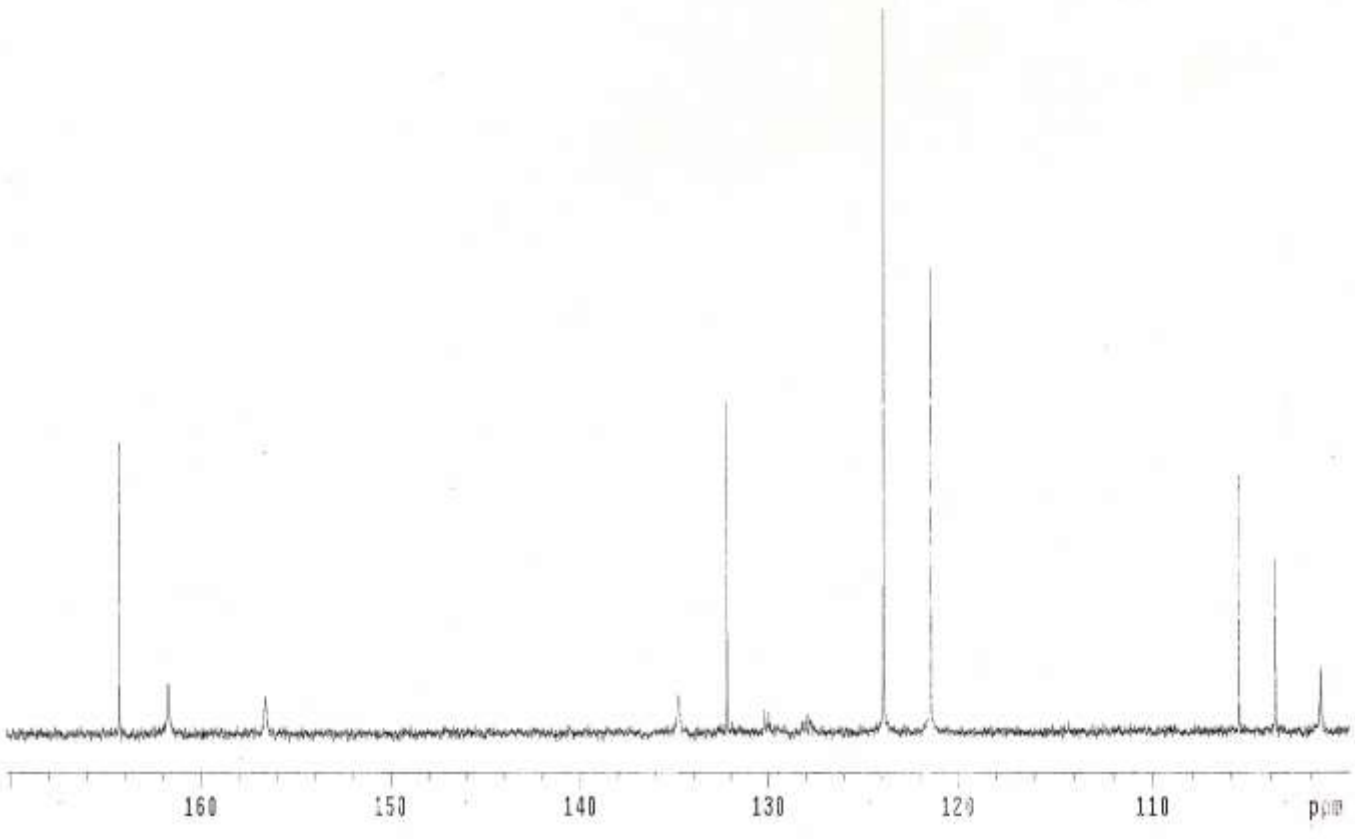
INDEX	FREQUENCY	PPM	HEIGHT
1	20451.011	203.363	18.9
2	16520.581	164.279	17.1
3	13294.663	132.201	19.3
4	12459.961	123.901	42.5
5	12210.292	121.418	27.3
6	10620.817	105.612	15.1
7	10434.595	103.761	10.2
8	9066.773	90.159	28.3
9	7774.757	77.311	26.3
10	7743.446	77.000	27.9
11	7711.310	76.680	28.6
12	7427.034	73.854	37.8
13	3729.713	37.089	33.7
14	3291.431	32.730	37.9
15	2980.787	29.641	6.4
16	2697.334	26.822	35.2
17	2591.040	25.765	50.0
18	2578.680	25.642	44.9
19	2242.492	22.299	44.5
20	2234.252	22.217	37.2
21	2200.469	21.881	40.4
22	1790.122	17.801	26.7
23	1772.818	17.629	24.1



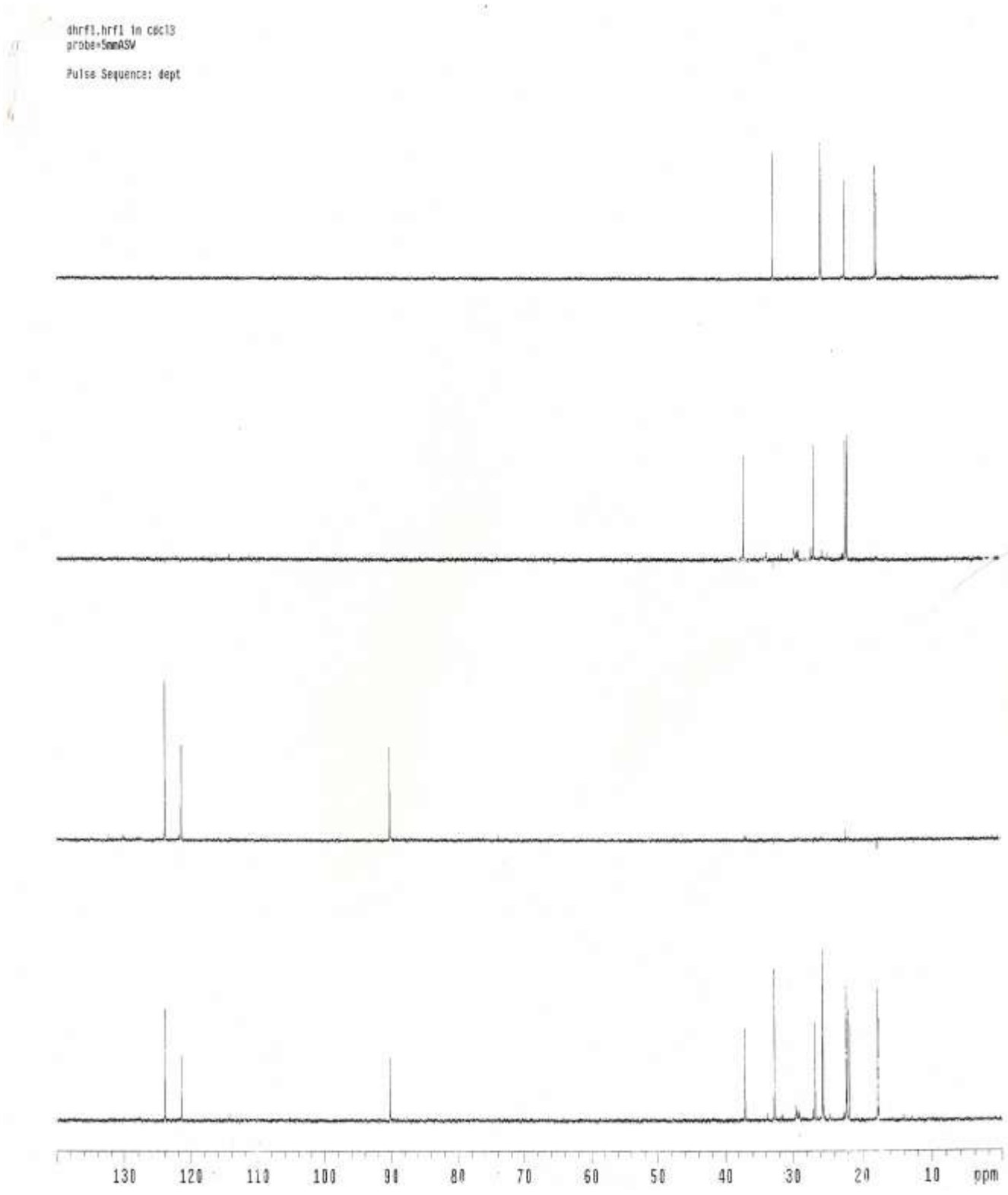
chrf1.hrf1 in cdcl3
probe=5mmASW

Pulse Sequence: s2pul

INDEX	FREQUENCY	PPM	HEIGHT
1	16520.581	164.279	34.0
2	16262.673	161.714	5.7
3	15746.855	156.585	4.1
4	13556.691	134.806	4.3
5	13294.663	132.201	38.5
6	13093.609	130.201	2.4
7	13071.361	129.980	2.3
8	12857.124	127.850	2.8
9	12459.961	123.901	64.9
10	12210.292	121.418	54.3
11	10620.817	105.612	30.1
12	10434.595	103.761	20.3
13	10204.702	101.474	7.6



A_3: DEPT Experiment for HRF_1 (harronin I)



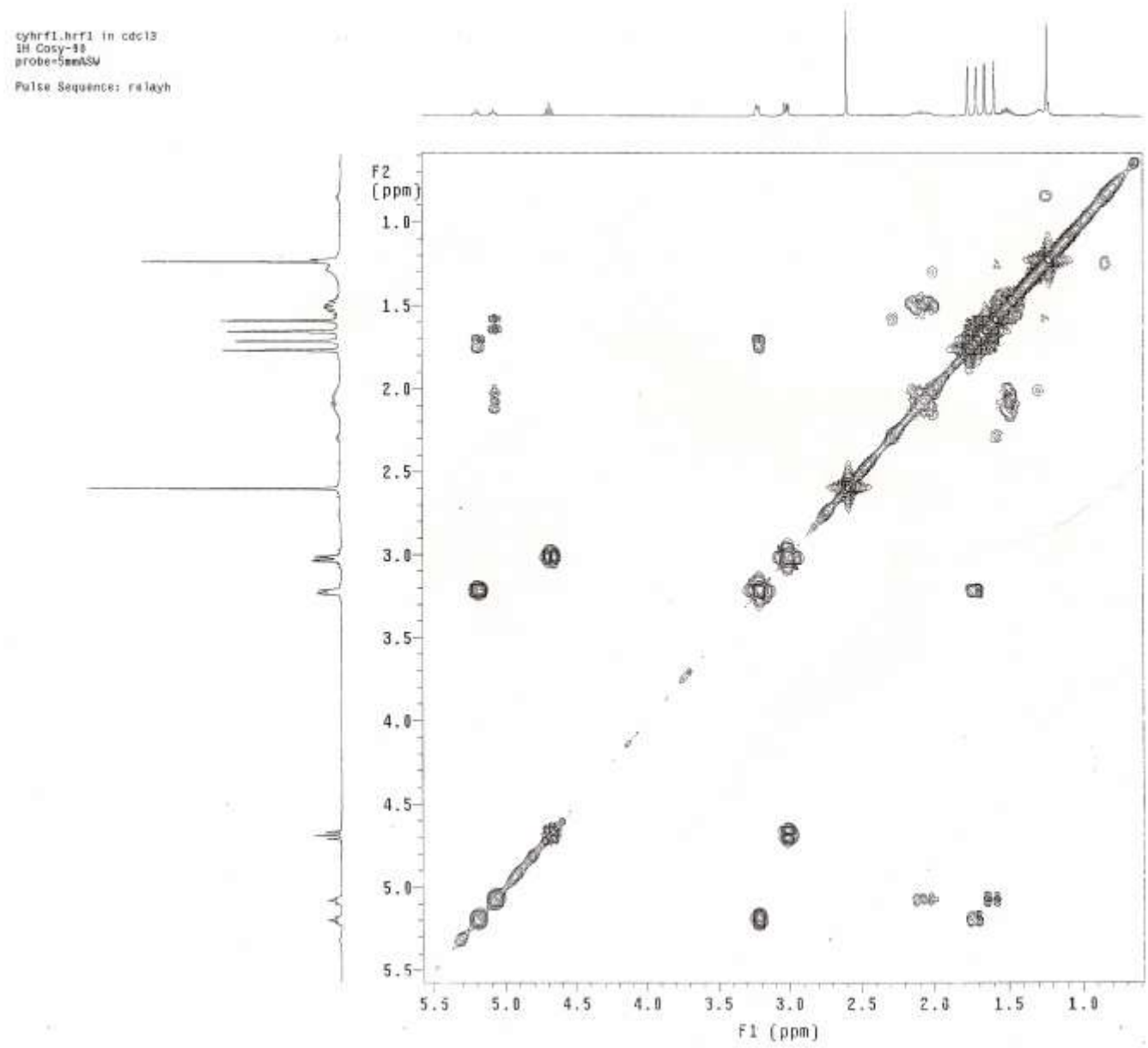
ADEPT SPECTRUM ANALYSIS

index		frequency	ppm	intensity
1	D	12460.0	123.886	51.109
2	D	12210.3	121.404	30.330
3	D	9066.8	90.149	29.794
4	T	3729.8	37.084	33.280
5	Q	3291.4	32.726	41.300
6	T	2697.3	26.819	36.615
7	Q	2591.0	25.762	43.515
8	Q	2578.7	25.639	34.929
9	Q	2242.5	22.297	31.497
10	T	2234.2	22.215	38.191
11	T	2200.5	21.879	39.996
12	Q	1790.1	17.799	35.852
13	Q	1772.8	17.627	27.212

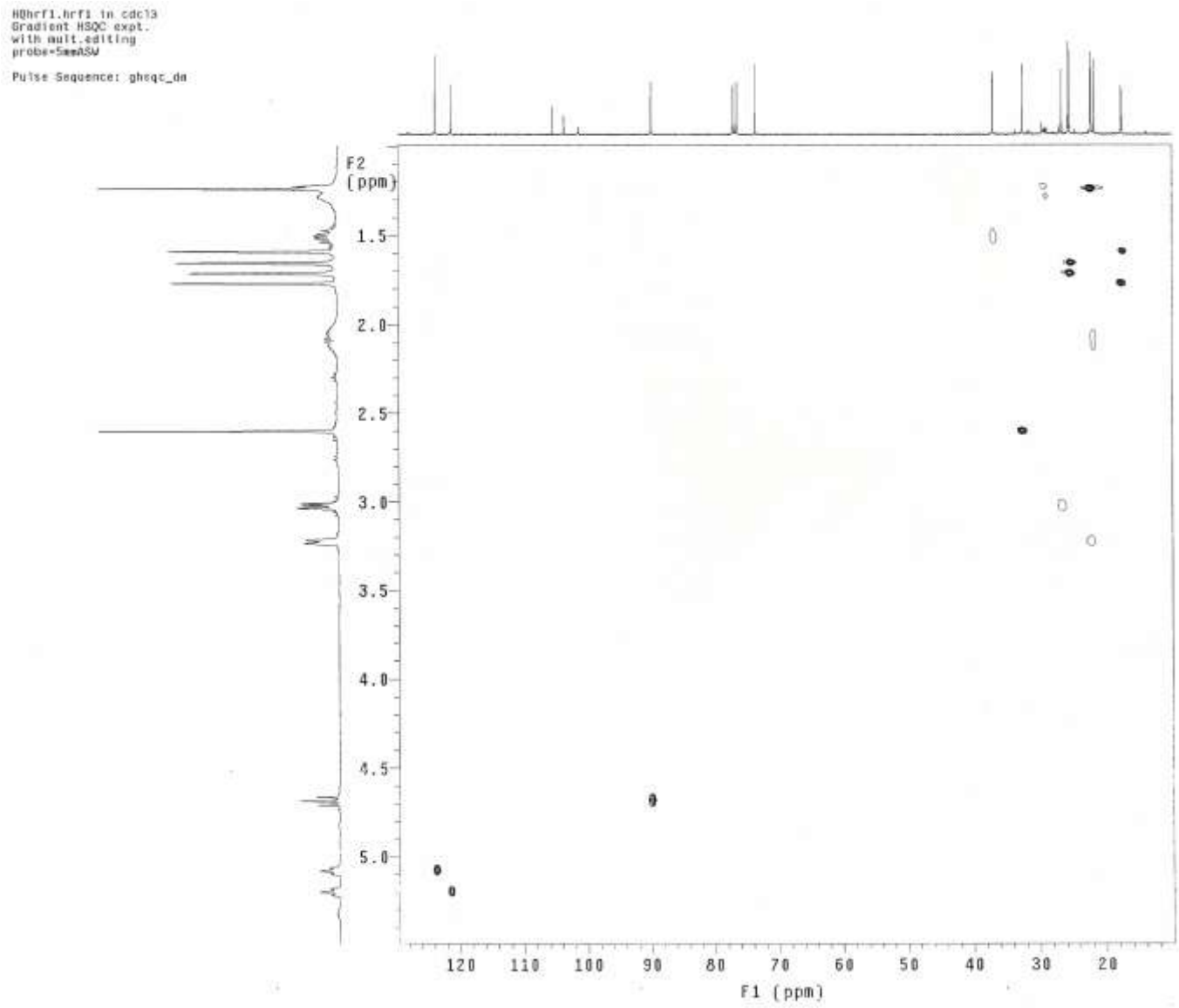
Number of protonated carbons: 13

CH : 3
CH2: 4
CH3: 6

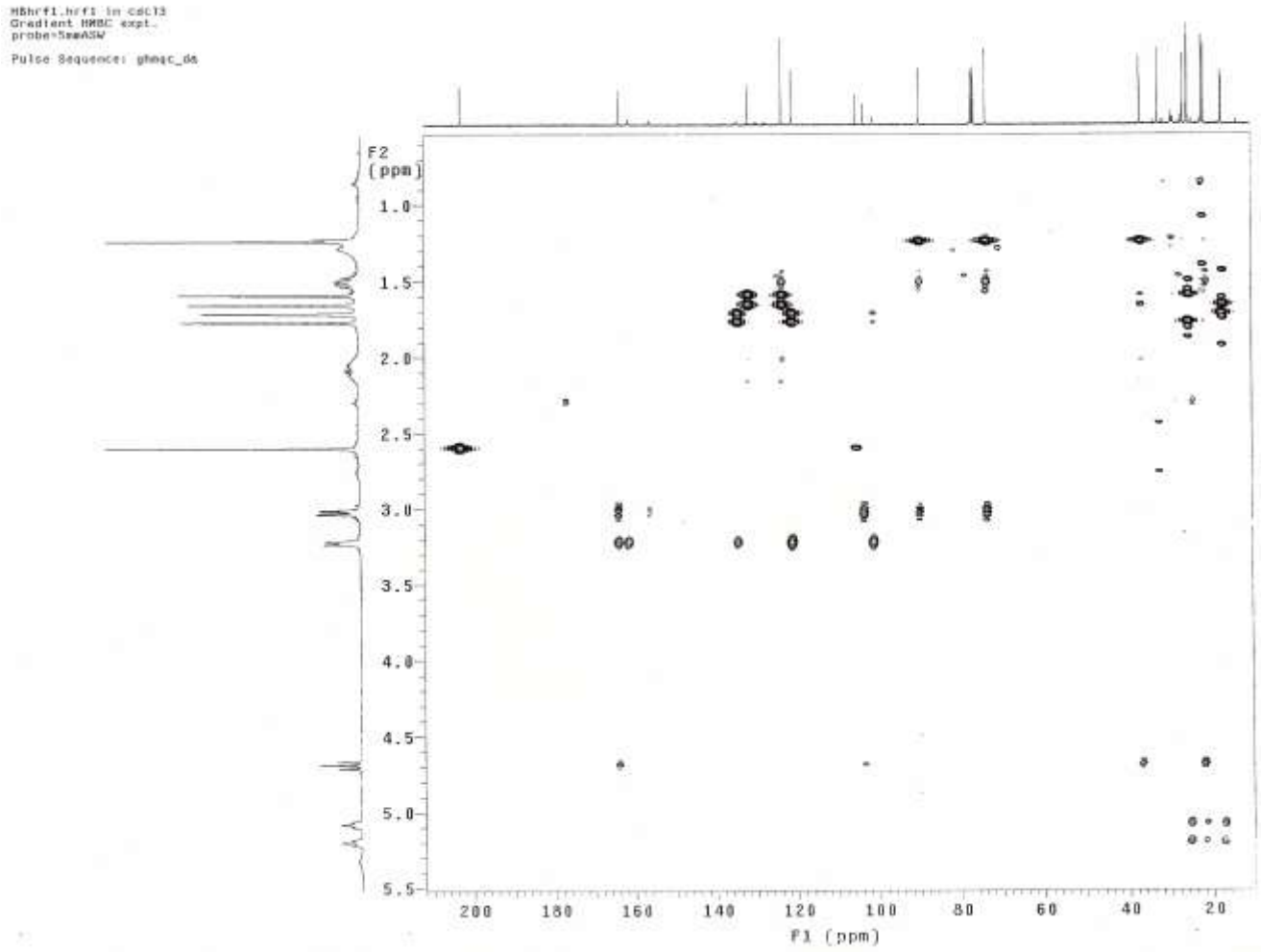
A_4: COSY Experiment for HRF_1 (harronin I)



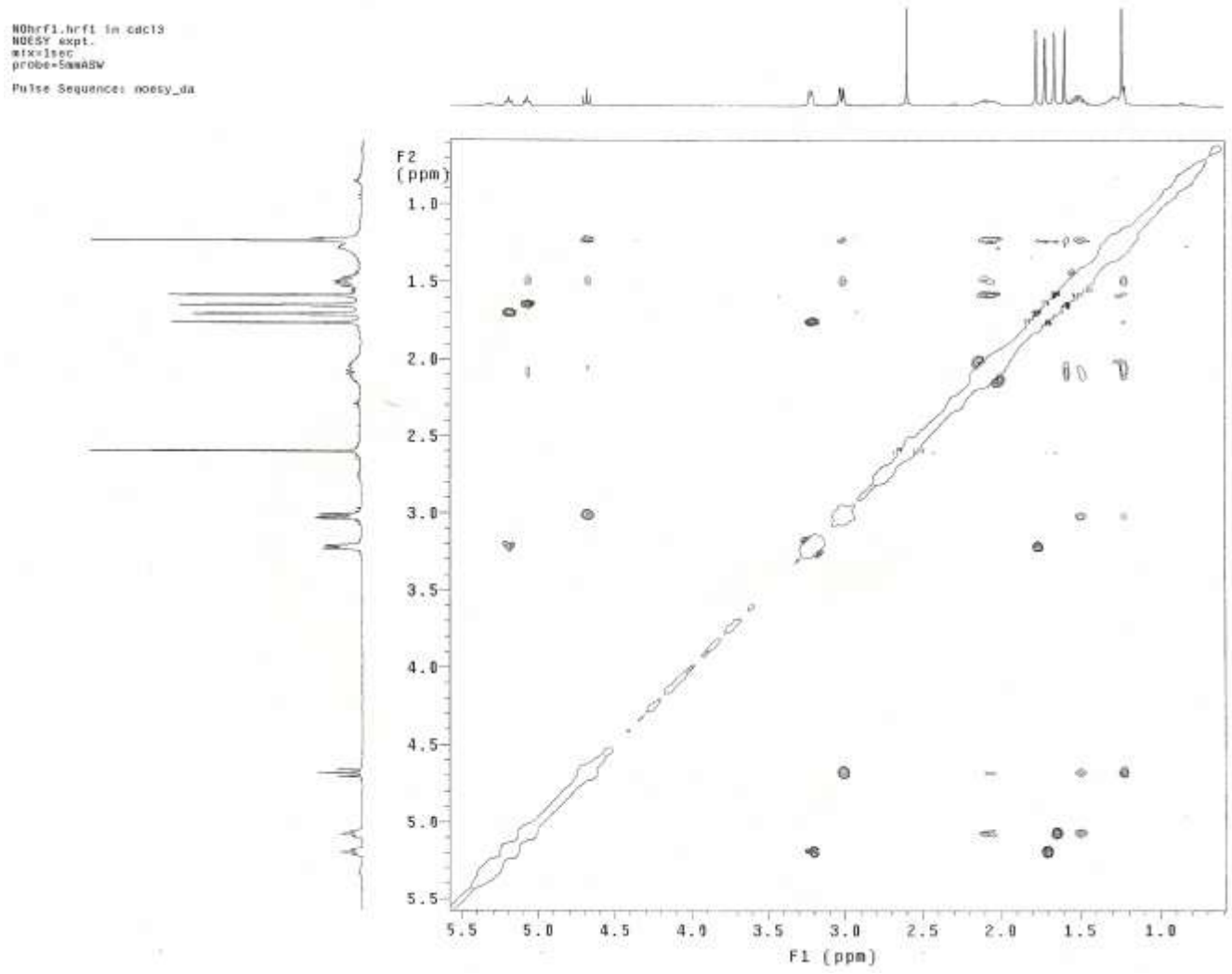
A_5: HSQC Experiment for HRF_1 (harronin I)



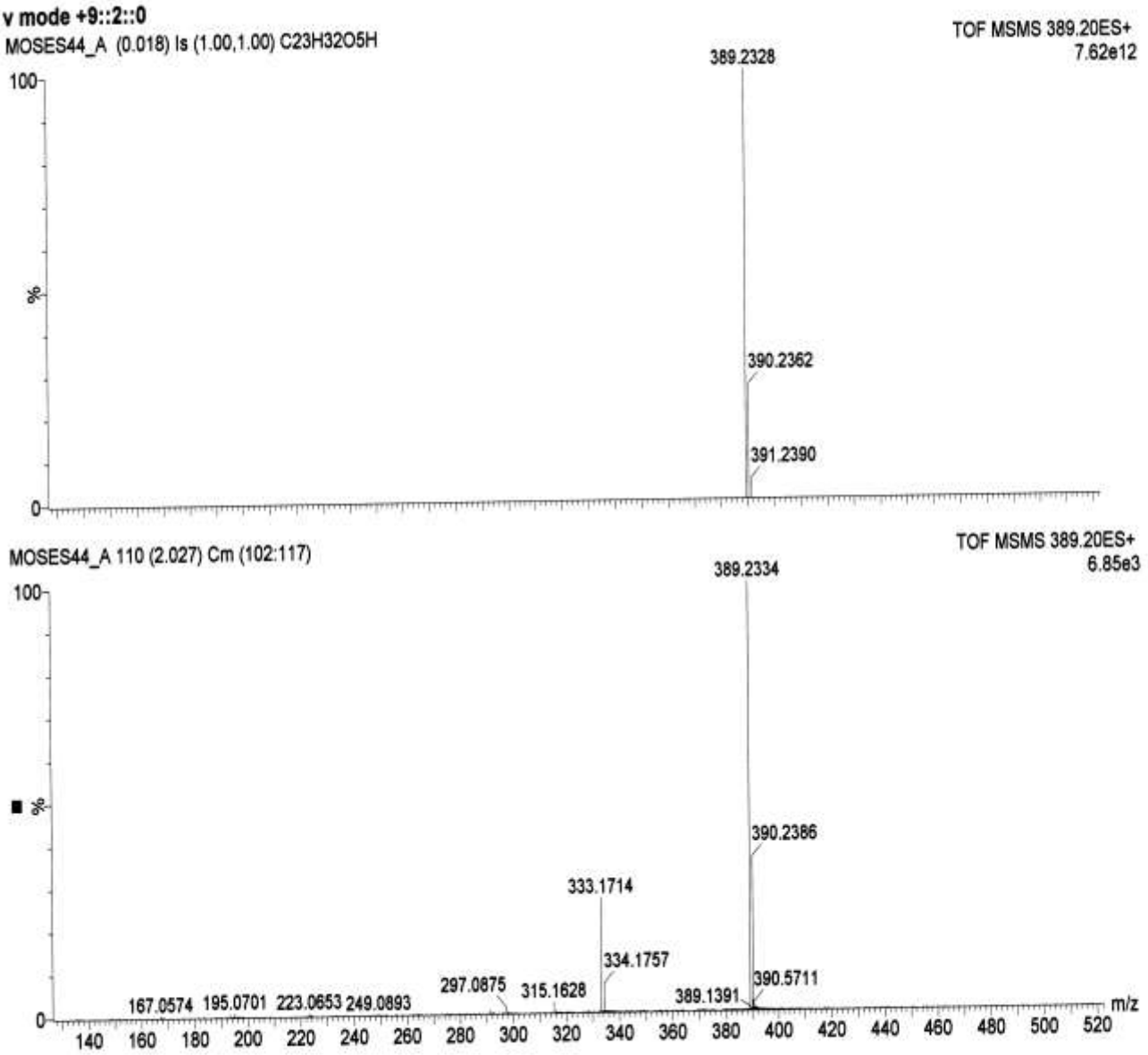
A_6: HMBC Experiment for HRF_1 (harronin I)



A_7: NOESY Experiment for HRF_1 (harronin I)



A_8: Mass Spectrum for HRF_1 (harronin I)

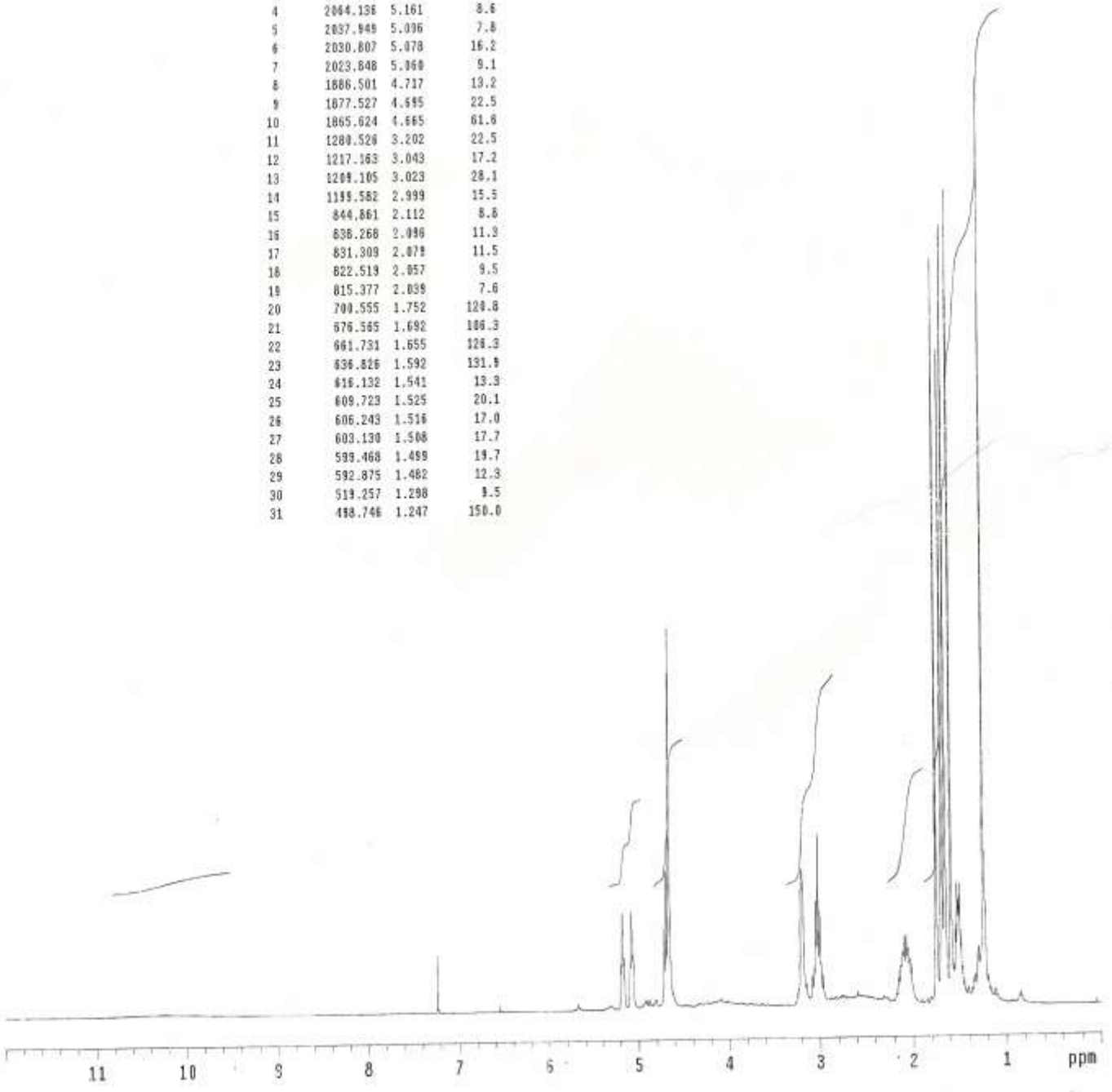


A_9: ^{1}H NMR for HRF_3 (harronin II)

hhrf3.hrf3 in cdcl3
probe=5mmASV

Pulse Sequence: s2pul

INDEX	FREQUENCY	PPM	HEIGHT
1	2895.544	7.240	9.3
2	2078.603	5.197	7.4
3	2071.278	5.179	15.7
4	2064.136	5.161	8.6
5	2037.949	5.096	7.8
6	2030.807	5.078	16.2
7	2023.848	5.060	9.1
8	1886.501	4.717	13.2
9	1877.527	4.695	22.5
10	1865.624	4.665	61.6
11	1280.526	3.202	22.5
12	1217.163	3.043	17.2
13	1208.105	3.023	28.1
14	1199.582	2.999	15.5
15	844.861	2.112	8.8
16	836.268	2.090	11.9
17	831.309	2.079	11.5
18	822.519	2.057	9.5
19	815.377	2.039	7.6
20	700.555	1.752	120.8
21	676.565	1.692	106.3
22	661.731	1.655	126.3
23	636.826	1.592	131.9
24	616.132	1.541	13.3
25	609.723	1.525	20.1
26	606.243	1.516	17.0
27	603.130	1.508	17.7
28	599.468	1.499	19.7
29	592.875	1.482	12.3
30	519.257	1.298	9.5
31	498.746	1.247	150.0

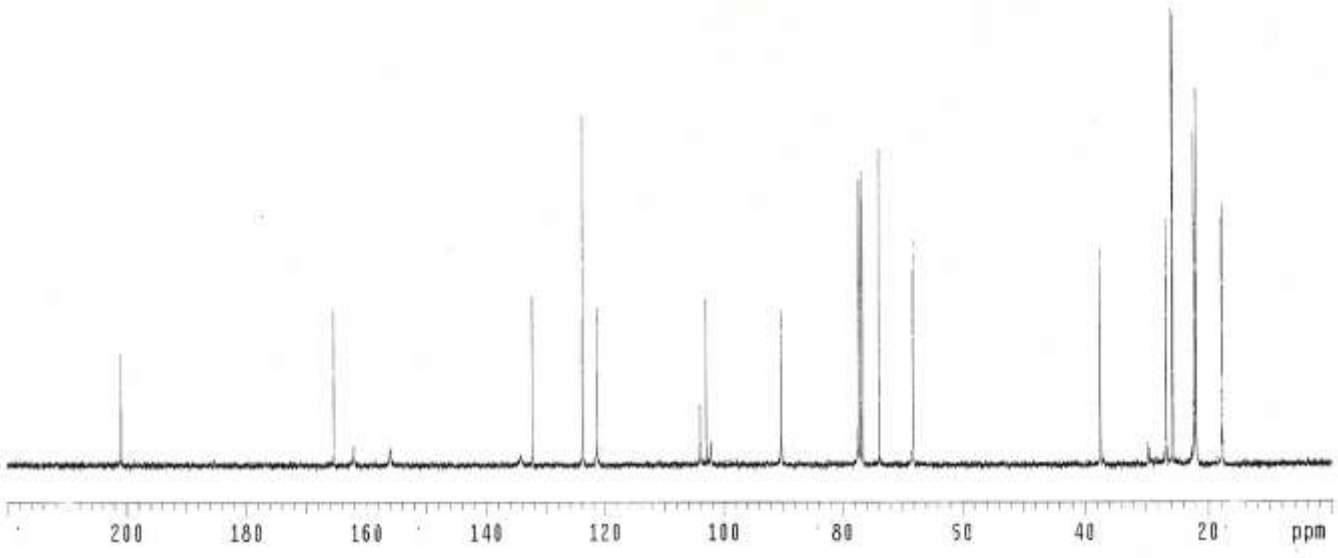


A_{10}: ^{13}C NMR for HRF_3 (harronin II)

chrf3.hrf3 in cdcl3
probe=5mmASW

Pulse Sequence: s2pul

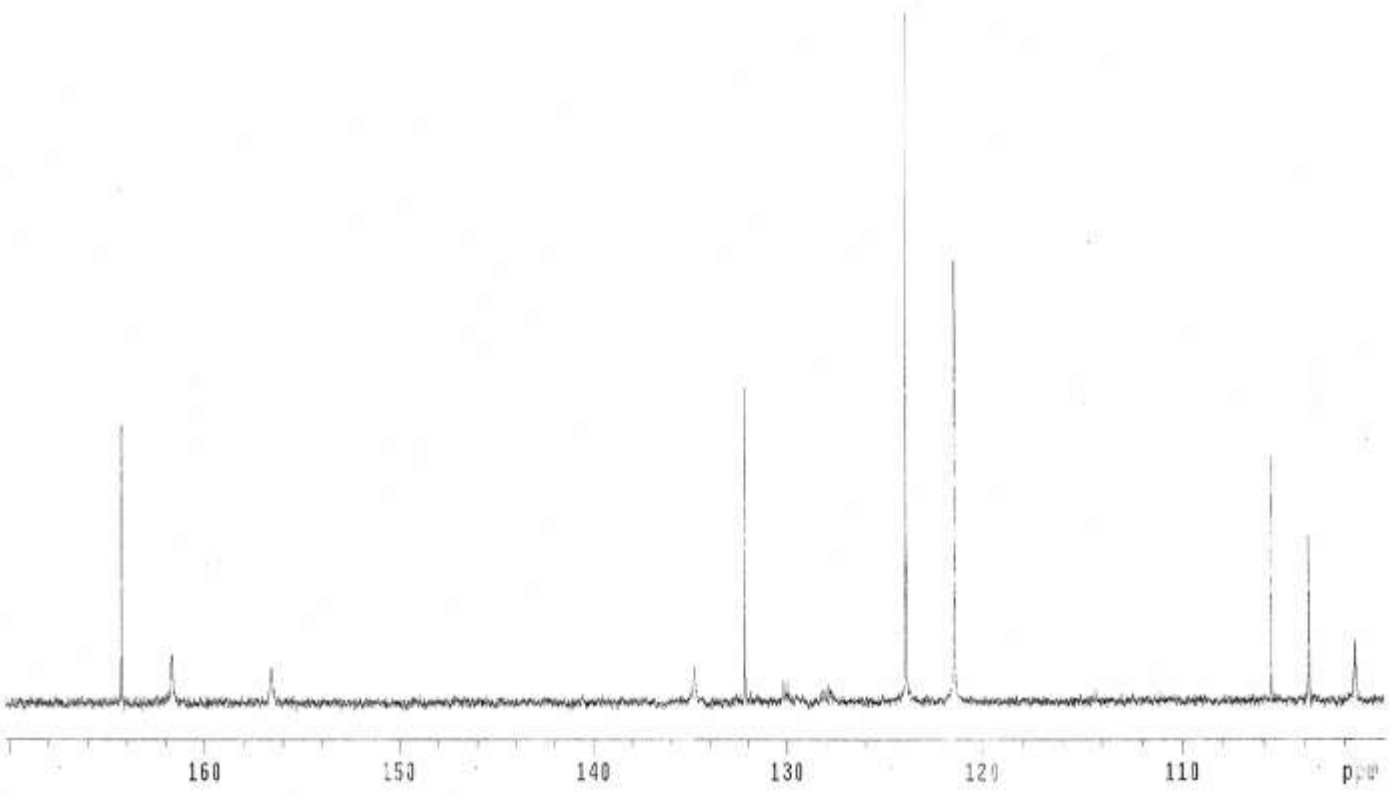
INDEX	FREQUENCY	PPM	HEIGHT
1	20212.878	200.995	14.8
2	16643.356	165.500	20.6
3	13303.726	132.291	22.3
4	12452.545	123.827	46.1
5	12203.700	121.352	20.7
6	10460.139	104.015	7.9
7	10355.492	102.974	21.9
8	9078.308	90.274	20.4
9	7775.581	77.320	37.6
10	7743.446	77.000	37.5
11	7712.134	76.689	36.7
12	7427.858	73.862	41.7
13	6856.009	68.175	29.8
14	3756.161	37.351	28.6
15	2684.150	26.691	32.4
16	2589.391	25.749	60.0
17	2579.504	25.650	59.4
18	2239.196	22.266	45.7
19	2214.476	22.021	33.0
20	2202.948	21.906	49.5
21	1789.298	17.793	32.6
22	1772.818	17.629	34.3



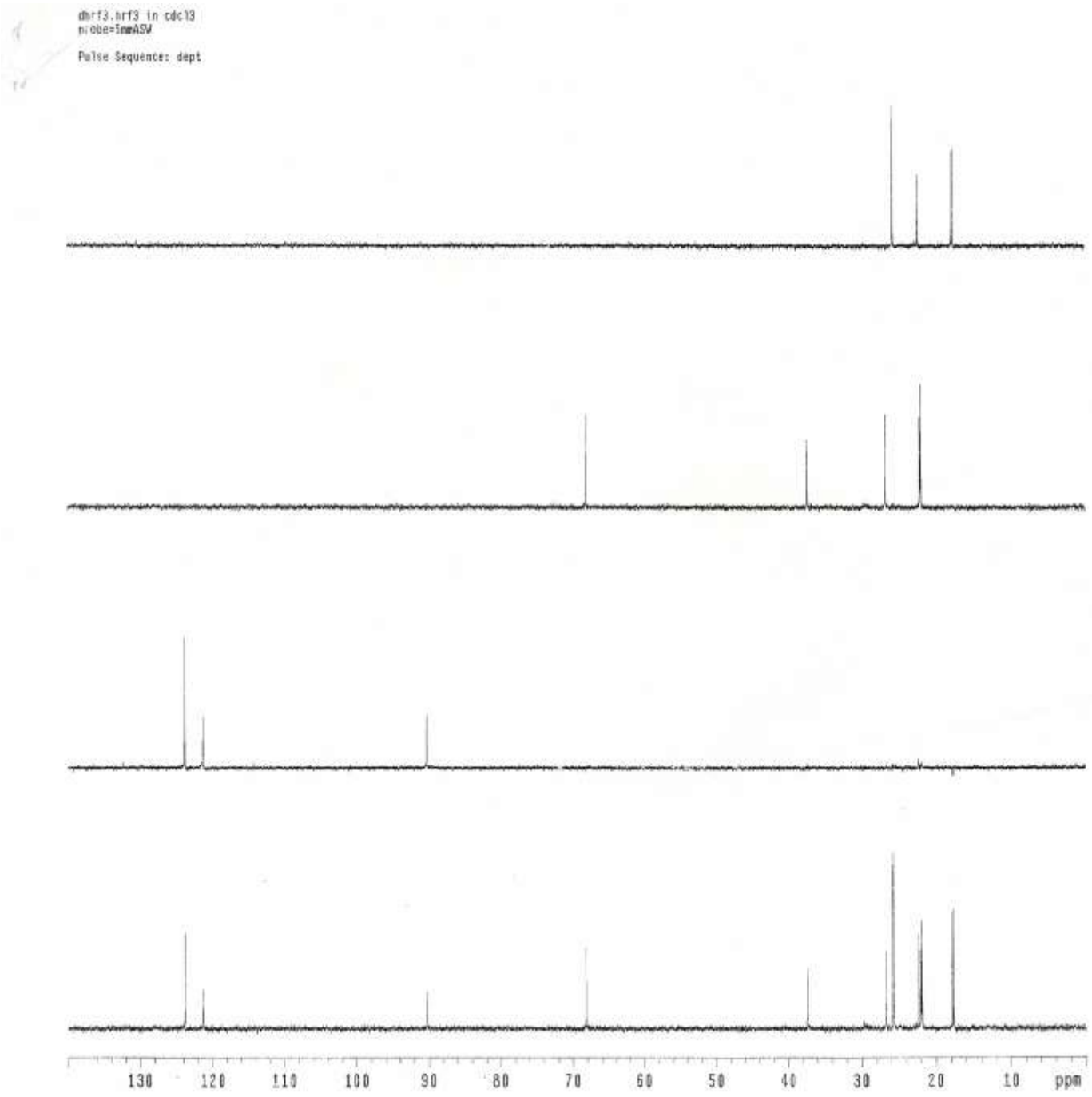
chrf1.hrf1 in cdcl3
probe=5mmASW

Pulse Sequence: s2pul

INDEX	FREQUENCY	PPM	HEIGHT
1	16520.581	164.279	34.0
2	16262.673	161.714	5.7
3	15746.855	156.585	4.1
4	13556.691	134.806	4.3
5	13294.663	132.201	38.5
6	13093.609	130.201	2.4
7	13071.361	129.980	2.3
8	12857.124	127.850	2.0
9	12459.961	123.901	84.9
10	12210.292	121.418	54.3
11	10620.817	105.612	30.1
12	10434.595	103.761	20.3
13	10204.702	101.474	7.6



A_{11}: DEPT Experiment for HRF_3 (harronin II)



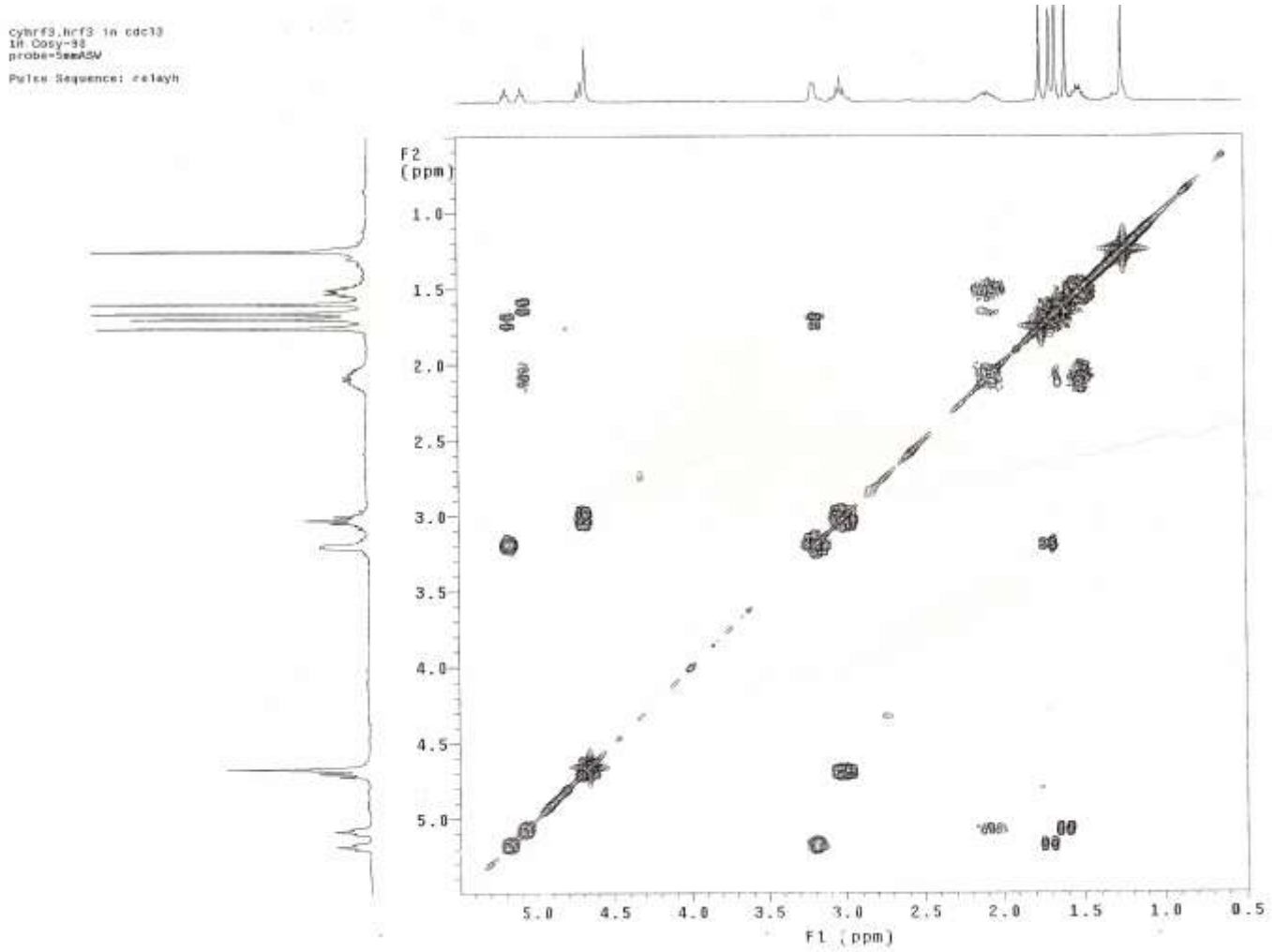
ADEPT SPECTRUM ANALYSIS

index		frequency	ppm	intensity
1	D	12452.5	123.813	45.022
2	D	12204.5	121.347	17.399
3	D	9078.3	90.263	18.393
4	T	6856.0	68.168	31.961
5	T	3756.2	37.347	23.551
6	T	2684.1	26.688	32.227
7	Q	2589.4	25.746	44.079
8	Q	2579.5	25.647	48.753
9	Q	2239.2	22.264	24.521
10	T	2214.5	22.018	31.143
11	T	2202.9	21.903	42.428
12	Q	1789.3	17.791	33.398
13	Q	1772.8	17.627	34.151

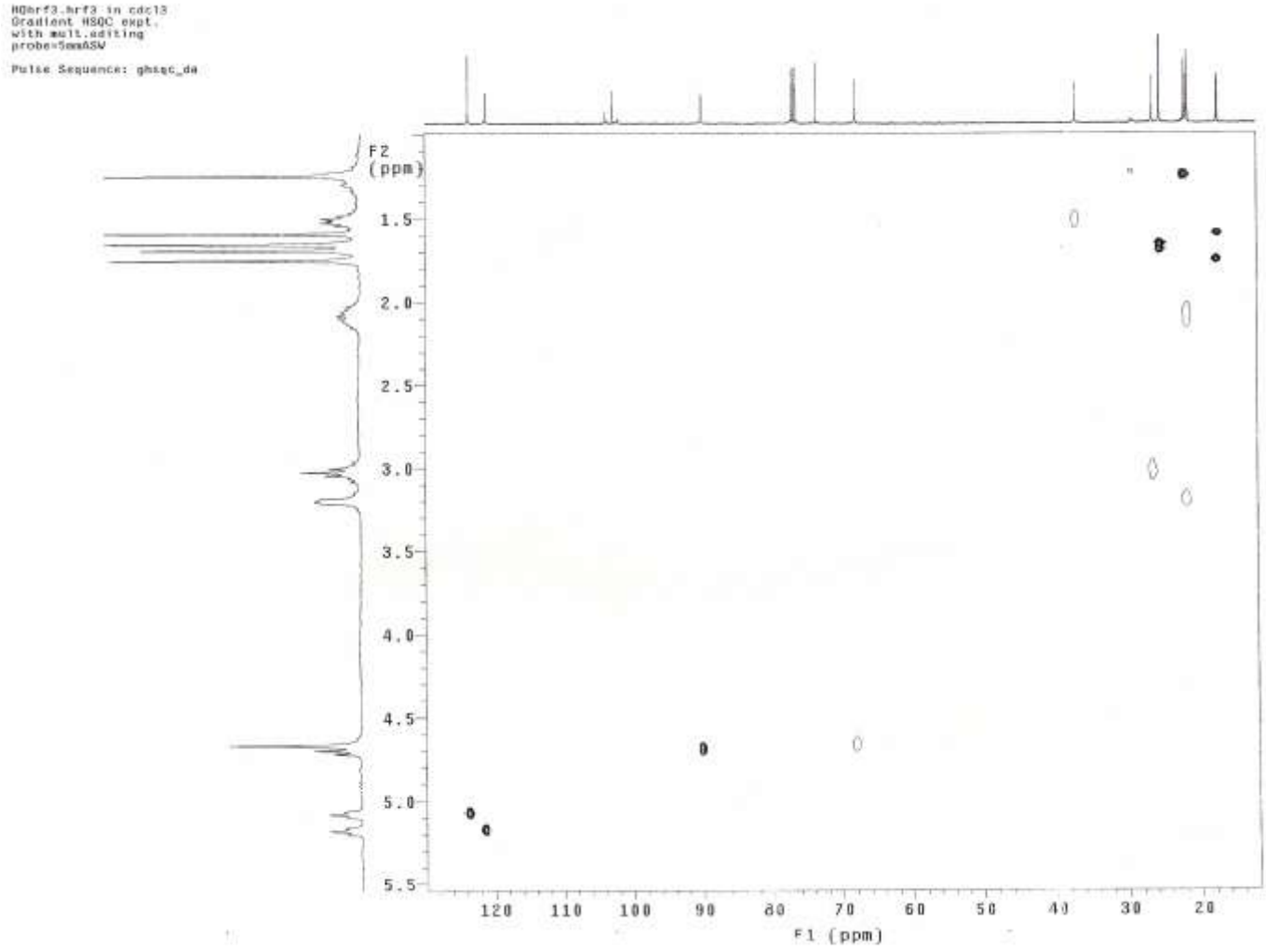
Number of protonated carbons: 13

CH : 3
CH2: 5
CH3: 5

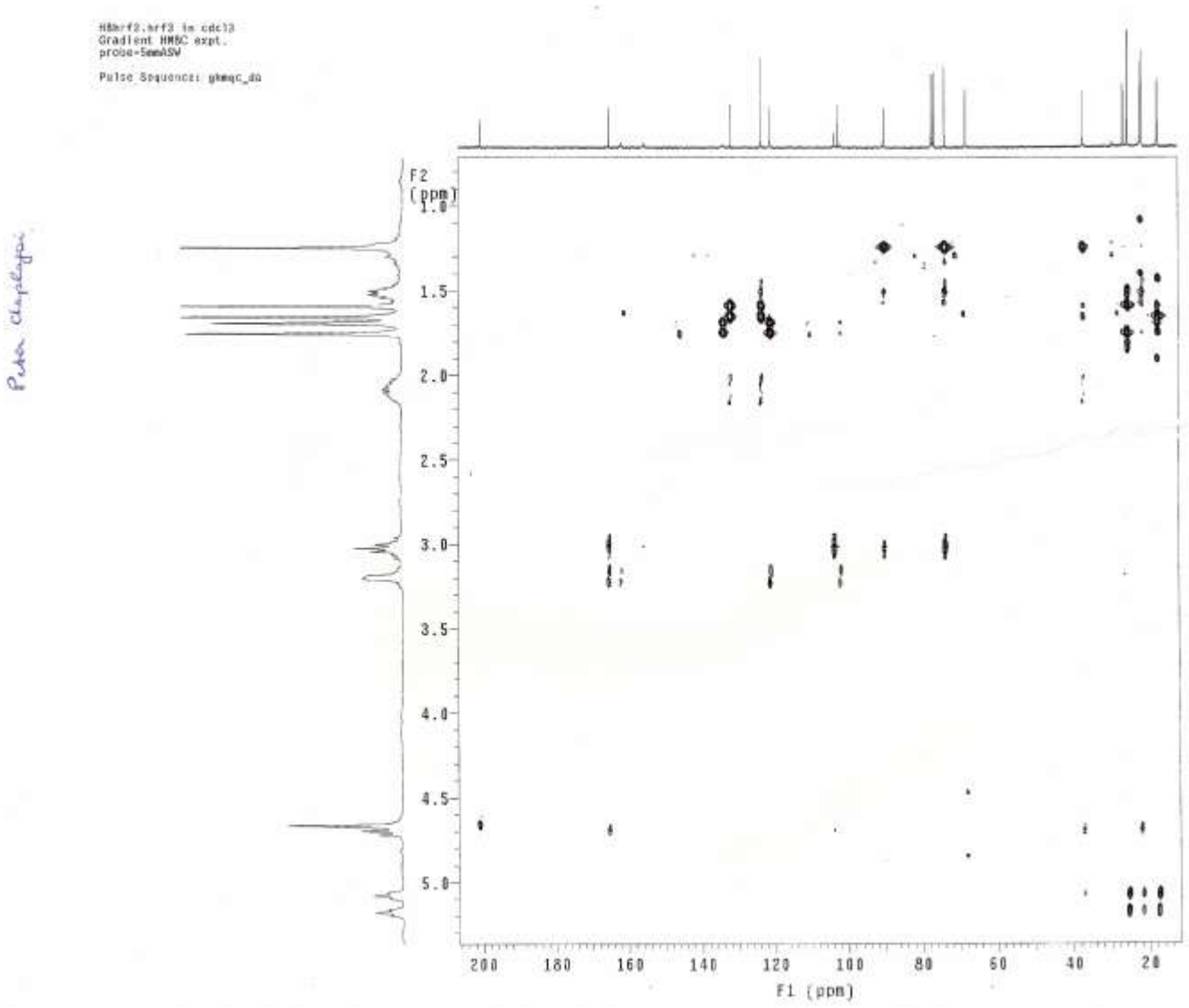
A_{12}: COSY Experiment for HRF_3 (harronin II)

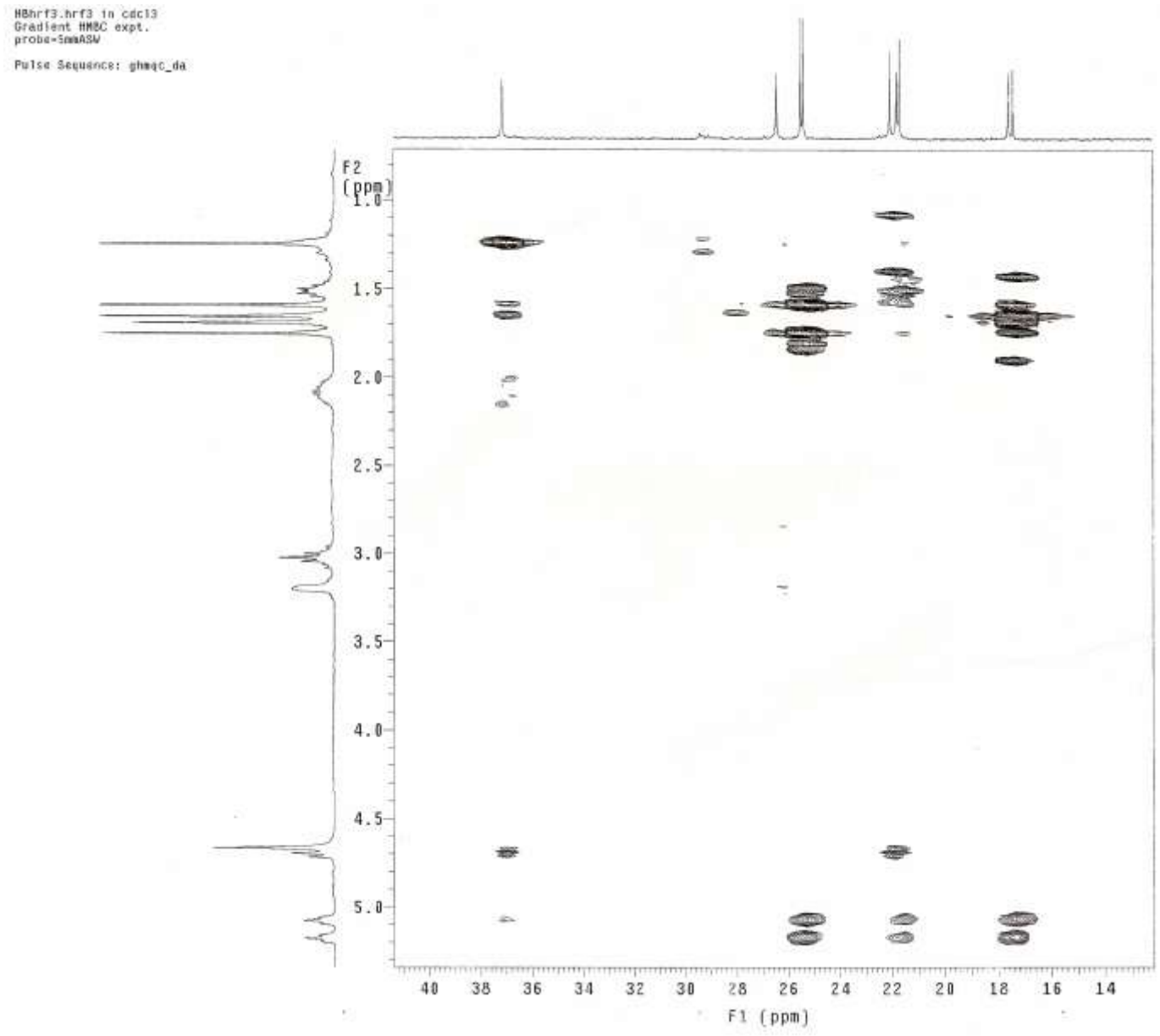


A_{12}: HSQC Experiment for HRF_3 (harronin II)

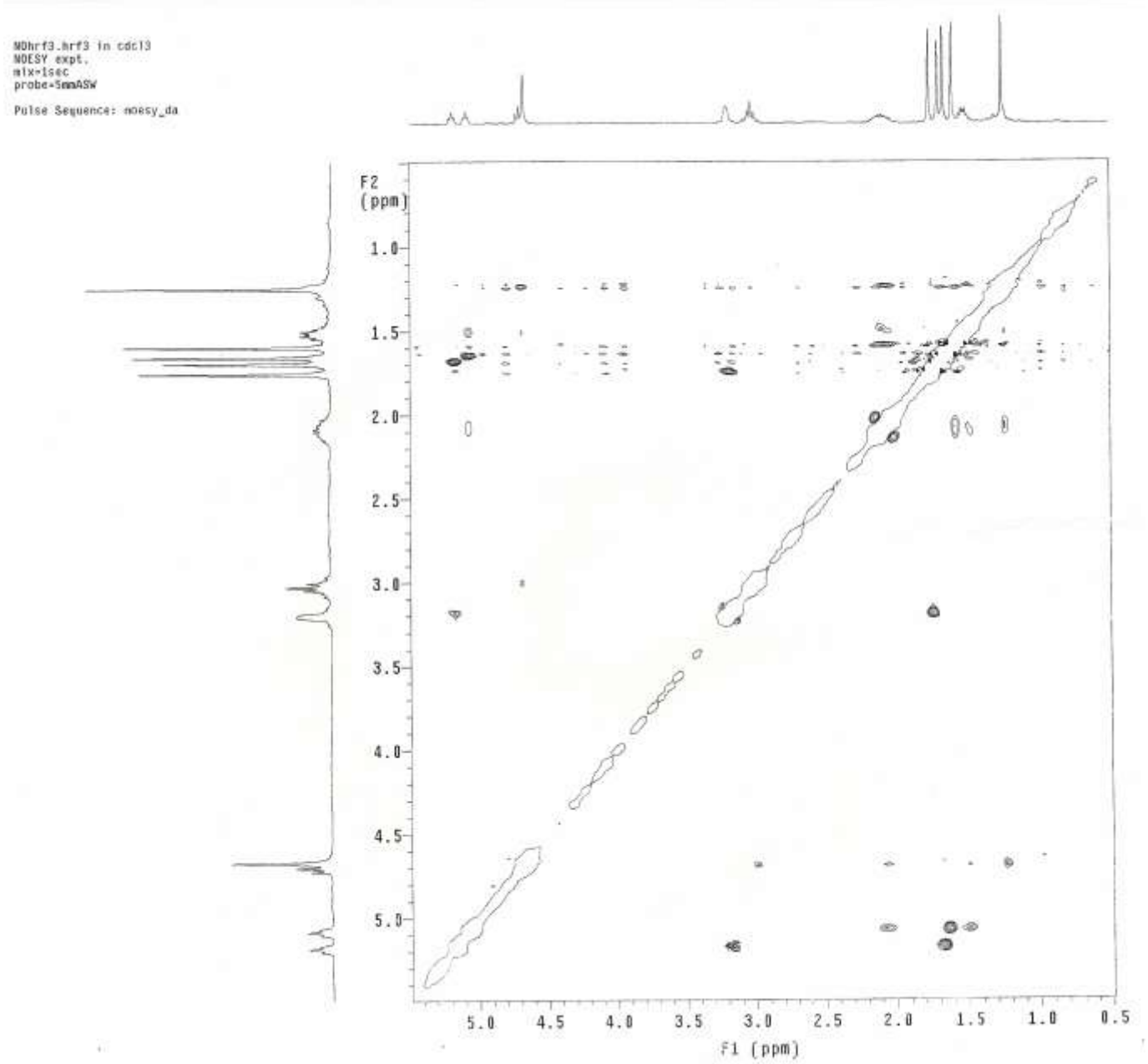


A_{14}: HMBC Experiment for HRF_3 (harronin II)




HBhrf3.hrf3 in cdcl3
Gradient HMBC expt.
probe-5mmASW
Pulse Sequence: ghmqc_da
F2 (ppm)
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
40
38
36
34
32
30
28
26
24
22
20
18
16
14
F1 (ppm)

A_{15}: NOESY Experiment for HRF_3 (harronin II)



A_{16}: Mass Spectrum for HRF_3 (harronin II)