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The biological activity and phytochemistry of selected *Hermannia* species

Ayesha Bibi Essop

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Pharmacy.

November, 2005

DECLARATION

I, Ayesha Bibi Essop, declare that this dissertation is my own work. It is being submitted in fulfillment for the degree, Master of Pharmacy, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____

_____ day of _____, 2005.

DEDICATION

I dedicate this dissertation,

with love, to my parents

Hoosen and Sheerin Essop.

For loving me always,

Supporting me always,

And teaching me to dream

ACKNOWLEDGEMENTS

1. I must express my absolute appreciation to my supervisor, Professor Alvaro M. Viljoen for all the support, encouragement and enthusiasm provided throughout the duration of my project. I am eternally grateful for all that he has given and do not have enough words to express my heartfelt gratitude.
2. I am also in debt to my co-supervisor, Professor Dulcie A. Mulholland and to Dr Chantal Koorbanally whose assistance has been pivotal to the completion of this project.
3. I also acknowledge and appreciate all assistance provided by Mrs Sandy F. van Vuuren, who tirelessly supervised all microbiological aspects of this project. In. addition, I am grateful to Dr Robin van Zyl who was exceptionally helpful with the antimalarial and toxicity aspects of this study.
4. I would also like to thank Miss Petra Snijman from the Medical Research Council for her assistance in teaching me the ABTS assay as well as helping me to implement the assay within the department. I also appreciate her wonderful hospitality during my stay in Cape Town.
5. I am further indebted to Dr Paul Steenkamp and Mr Nial M. Harding for their technical guidance and assistance with the HPLC analysis. I must also thank the Department of Chemistry and especially Mr Tommy van der Merwe for their assistance in determining the physical data of the isolated compounds.
6. I also acknowledge Mr Jan Vlok and Dr John Manning for assisting in the collection and identification of the plant material.
7. I am also indebted to the National Research Foundation (Indigenous Knowledge Systems) for the financial assistance without which this research would not have been possible.

8. I would like to thank my friends, Miss Rupal Patel, Miss Jacqui Lalli, Miss Maria Paraskeva and Miss Ellen Huang for all their support and friendship without which I would never have survived.
9. I also acknowledge and appreciate Ms Susan Kemp, Mr Muhsin Hendriks and Dr Haroon Essa. Thank you for all the love and support. Words can never express how much I cherish all that you bring to my life.
10. Finally, thank you to my parents. Thank you for supporting and loving me as well as always being on my side. I appreciate all that you have given to me and every sacrifice that you have endured to ensure that I have only the best that life has to offer.

ABSTRACT

Traditional medicines form a significant part of the lives of many people around the world and in South Africa almost 60 % of people consult traditional healers in addition to the modern medical services available. Plants form a significant part of traditional healing and hence, selected species of a traditionally used plant genus, *Hermannia*, were chosen for biological and chemical investigation to determine a scientific basis for the traditional use of these plants.

A phytochemical investigation was carried out, firstly using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and then isolation and identification of compounds from various *Hermannia* species. TLC analysis indicated significant similarities between the various species with only *H. saccifera* displaying chemical anomalies. This was further corroborated by the HPLC analysis although very conservative profiles were produced. Isolation of compounds from *H. saccifera* yielded a novel labdane compound, *E*-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene, as well as two flavones, 5,8- dihydroxy-6,7,4'- trimethoxyflavone and cirsimaritin which have previously been isolated. In addition, two commonly found compounds, lupeol and β-sitosterol were isolated from *H. cuneifolia* and *H. salviifolia* respectively. This is the first report on the isolation and identification of all five compounds from *Hermannia* species.

Antimicrobial activity was assessed using two methods i.e. minimum inhibitory concentrations as well as the death kinetics assay. Minimum inhibitory concentrations were determined using four Gram-positive and two Gram-negative bacteria as well as two yeasts. All species investigated indicated antimicrobial activity with *H. saccifera* showing good activity against *S. aureus* and *B. cereus*. *E*-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene isolated from *H. saccifera* indicated activity (MIC = 23.6 µg/ml against *S.*

aureus) although the activity was less than that of the crude extract (MIC = 19.5 µg/ml), thus, demonstrating that there are a number of compound contributing to the promising activity of the crude extract. This was further corroborated by the bioautograms developed of the *H. saccifera* extract. Time-kill studies on *H. saccifera* against *S. aureus* indicated that at concentrations of 0.1, 0.25 and 0.5 % bacteriostatic activity was observed while at 0.75% the extract achieved complete bactericidal activity after 240min.

Free radical scavenging activity was assessed using the 2,2-diphenyl-1-picrylhydrazy (DPPH) and 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assays. Ten of the twelve species indicated good activity with *H. cuneifolia* demonstrating the most promising activity (IC_{50} = 10.26 µg/ml for DPPH and 10.32 µg/ml for ABTS). Two of the isolated compound, 5,8- dihydroxy-6,7,4'- trimethoxyflavone and cirsimaritin displayed insignificant activity.

The 5-lipoxygenase assay was used to assess the anti-inflammatory activity of *Hermannia* species. All species exhibited intermediate activity with the exception of *H. cuneifolia* (IC_{50} = 15.32 µg/ml). In addition, four isolated compounds, 5,8- dihydroxy-6,7,4'-trimethoxyflavone, cirsimaritin, lupeol and β-sitosterol showed moderate inhibition of the enzyme indicating that while these compounds do contribute to the activity of the extracts they are not individually responsible for any significant activity.

Antimalarial activity was assessed using the titrated hypoxanthine incorporation assay while toxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay. Only three species indicated any good antimalarial activity i.e. *H. saccifera*, *H. muricata* and mostly *H. trifurca* (IC_{50} = 25.30, 28.17 and 18.80 µg/ml respectively). However, the activity of *H. saccifera* and *H. trifurca*

are probably due to a general cytotoxicity as these species exhibited a low safety index. All other species appear safe for use.

Several *Hermannia* species have indicated *in vitro* biological activity in a number of assays which is related to their use in traditional medicines to treat a number of disease states. Hence, a scientific basis, albeit *in vitro*, has been established for the use *Hermannia* species in traditional healing.

CONFERENCES

Ayesha B. Essop, Alvaro M. Viljoen, Dulcie A. Mulholland, Sandy F. van Vuuren, Chantal Koorbanally (2004) *Hermannia:* Antibacterial Activity and Phytoconstituents of Selected Species of a Previously Unresearched Genus Used in Traditional Medicine. Podium presentation at the 25th Annual Congress of Academy of Pharmaceutical Sciences, Rhodes University, Grahamstown, South Africa (Abstract, see Appendix I).

Ayesha B. Essop, Alvaro M. Viljoen, Dulcie A. Mulholland, Sandy F. van Vuuren (2004) *Hermannia:* The Biological Activity and Phytoconstituents of an Unexplored Genus Used in African Traditional Medicine. Podium presentation at the RAU Botany Symposium, Rand Afrikaans University, Johannesburg, South Africa (Abstract, see Appendix II). Winner of the Aspen Gold Medal for the best MSc presentation.

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CHAPTER 1: GENERAL INTRODUCTION

1.1. PLANTS AND THEIR USE AS TRADITIONAL MEDICINES:

Plants are essential for all life on earth since they convert solar energy into organic compounds and have the remarkable capacity of producing carbohydrates, proteins, fats and vitamins and, most importantly, oxygen. In addition, the "phytochemical laboratory" produces countless secondary metabolites. These compounds often are the most conspicuous of the constituents of a plant and have been in use for many purposes since prehistoric times. Constituents with special properties often occur in a high concentration and sometimes with great purity in a particular plant (Neuwinger, 1994).

A number of theories have been proposed as to why these compounds are produced but it is highly likely that many of them are produced as part of a chemical defense system to protect the plant from attack. Examples of this defense include the synthesis of antimicrobial compounds by plants, which may be infected by bacteria and fungi. Whatever the reasons for the presence of these compounds in Nature, they are an invaluable resource that can be used to find new molecules for the pharmaceutical domain.

A perceived benefit of compounds derived from nature is that they are 'ecofriendly' and that they may be produced as a renewable resource by growing the plants. This approach has both advantages and disadvantages over the synthetic production of biologically active agents. But synthetic chemistry cannot readily mimic the ability of organisms to produce such structurally complex and diverse natural product molecules. This incredible genetic resource could potentially generate millions of natural products to be assessed for biological activity.

While plants have adapted to the diverse habitats of the world through their physical and biochemical modifications, human populations have adapted largely through generation and application of knowledge. Today, traditional societies throughout the world posses a wealth of such knowledge which they have accumulated during prolonged interaction with the natural world, and which remains fundamental to their physical, spiritual and social well-being (Cotton, 1996).

The history of pharmacy was for centuries identical with the history of Pharmacognosy, or the study of *materia medica*, which were obtained from natural sources, mostly plants (van Wyk and Wink, 2004). Written records about medicinal plants date back at least 500 years and may possibly extend to as early as the Sumerians. In addition, archaeological evidence suggests an even earlier use than this. However, the strong bond between plants and human health began to unwind in 1897, when Friedrich Bayer and Co. introduced synthetic acetyl salicylic acid (aspirin) to the world. Thus the twentieth century became a triumph for the synthetic-chemistry-dominated pharmaceutical industry, which replaced natural extracts with synthetic molecules.

However, this benefit of modern drugs is felt primarily in developed countries (Raskin *et al.,* 2002). An estimated one-third of the world lacks regular access to essential drugs, with this figure rising to 50% in the poorer parts of Africa and Asia (WHO, 2000) and thus, these people are reliant on their own knowledge of traditional medicines to cater for their health care requirements.

It is likely that the profound knowledge of herbal remedies in traditional cultures developed through trial and error over many centuries, and that the most important cures were carefully passed on verbally from one generation to the next. It is hard to dismiss medical claims of safety and efficacy when a plant medicine has been used by these

traditional cultures for centuries without evidence of serious side effects (Heinrich *et al.,* 2004). It is only within the last few decades that research results generated have given us a much better understanding of the scientific rationale behind many natural remedies. Phytomedicines often contain a mixture of substances that have additive or even synergistic effects, so that the health benefits are difficult to test and verify. Plant medicine or phytomedicines may have subtle effects on several different biochemical pathways and receptors in the body-mind continuum that may all contribute directly and indirectly to restore equilibrium and balance (van Wyk and Wink, 2004).

African traditional medicine, itself, is the oldest and perhaps most diverse of all medicine systems. The biological and cultural diversity of Africa that constitutes the cradle of mankind is reflected in the marked regional differences in healing practices. Unfortunately, the various systems are poorly recorded and remain so to this day (van Wyk and Wink, 2004).

African traditional medicine in its varied forms is a holistic system involving both body and mind. The healer typically diagnoses and treats the psychological basic of an illness before prescribing medicines to treat the symptoms. The Khoi-San people of Southern Africa, nowadays considered to be the most ancient of all cultures, have an extraordinarily diverse *materia medica* which typically includes general tonics, fever remedies, sedatives, diuretics, laxatives and numerous wound healing plants (van Wyk and Wink, 2004). This is not exceptional, however, when considering that of the estimated 300 000 species of plants which exist throughout the world (Cotton, 1996), Southern Africa has the richest temperate flora and encompasses a rich floristic diversity. There are approximately 24 000 taxa of 368 families, including more than 10% of the world's vascular plant flora on less than 2.5% of the Earth's land surface (Germisthuizen and Meyer, 2003).

1.1.2. Extent of traditional use and its importance in the South African context

It is thought that 80% of the population of the world live in less developed countries and the World Health Organization estimates that about 80% of these people rely exclusively on traditional medicine, for which medicinal plants form the 'backbone' to satisfy their primary health-care needs (WHO, 2003).

There are an estimated 200 000 traditional healers in South Africa, and up to 60% of South Africans consult these healers, usually in addition to modern biomedical services. With South Africa's immense floristic and cultural diversity, it is not surprising to find that approximately 3 000 species of plants are used as medicines, and of these, some 350 species are the most commonly used and traded medicinal plants (van Wyk and Wink, 2004).

The problem is that the usefulness of these traditional remedies has not been systematically investigated, and the country is therefore not in a position to benefit from this significant genetic resource and indigenous knowledge base. Such an evaluation is urgent, since urbanization and other factors are rapidly reducing the availability of first-hand information on traditional medicines (Long and Li, 2003). Since medicinal plants and plant-derived medicine are so widely used in traditional cultures all over the world and are becoming increasingly popular in modern society as natural alternatives to synthetic chemicals (van Wyk and Wink, 2004), it is a necessity to obtain information on compounds contained within the extracts that possess biological activity, their contribution to the entire effect of the extracts which may include synergistic or antagonistic effects, as well as potential toxicity of the extracts as well as the constituents.

1.1.3. Pharmacognosy, ethnobotany and the necessity for research into traditional medicines.

Pharmacognosy is the study of medical products derived from our living environment, especially those derived from plants and fungi while ethnobotany studies the relationship between humans and plants in all its complexity. Many drugs that are commonly used today came into use through the study of indigenous remedies – that is, through the bioscientific investigation of plants used by people throughout the world. Ethnobotany and Pharmacognosy are interdisciplinary fields of research that look specifically at the empirical knowledge of indigenous peoples concerning medicinal substances, their potential health benefits and the potential toxicity risks associated with such remedies (Heinrich *et al.,* 2004).

The popularity of traditional medicines has led to increasing concerns over their safety, quality and efficacy. In many countries the herbal medicines market is poorly regulated and products may be neither registered nor controlled. National surveillance systems used to monitor and evaluate adverse effects are rare (Camejo-Rodrigues *et al.,* 2003). This is surprising when you consider that during the latter part of the 20th century herbalism has become mainstream worldwide partly due to the perception that herbal remedies are somehow safer and more efficacious than remedies that are pharmaceutically derived (Elvin-Lewis, 2001).

In addition, chemo diversity in Nature, such as in plants, still offers a valuable source for novel lead discovery (Tringali, 2001). There are a number of approaches that can be used to discover new drug leads from nature. In the ethnobotanical approach, knowledge of the use of a particular plant by indigenous people is used to direct a search for a drug lead. In

this case, observation of a particular usage of a plant, allows the collection of that plant and its subsequent testing for biological activity.

It is estimated that there are some 10-100 million species of organisms living on earth. Higher plants form a small group of some 250 000 species of which only 6% have been investigated for biological activity and 15% for their chemical constituents. To date, some 139 000 secondary metabolites have been isolated, the major groups being alkaloids and terpernes. Thus, we have only scratched the surface of this wonderful resource of natural chemicals with its vast potential for the development of new drugs for medicinal use (Heinrich *et al.,* 2004).

1.2. THE GENUS *HERMANNIA*:

1.2.1. Selection, description and distribution

Hermannia is a genus of the subfamily Byttnerioideae and tribe Hermanieae of the family Malvaceae (previously Sterculiaceae).There are about 180 species of *Hermannia* found worldwide. Eleven species occur in tropical Africa, three in America, one or more in Australia and about 162 species in Southern Africa (Leistner, 2000).

The genus was introduced by Tournefort, but as taxonomic priority is conventionally started with Linnaeus's Species Plantarum of 1753, it is usually ascribed to Linnaeus. Tournefort named the genus after Paul Hermann who was a professor of Botany at Leyden in the latter part of the seventeenth century.

The wide diversity of species in a restricted geographical region is suggestive of a recent origin and diversification of the species. The lack of reported variation in chromosome counts may be further evidence in favor of this interpretation, or may reflect a limited sampling of the species of the genus. On the other hand the genus seems less derived than

the other genera of the tribe (e.g. in the presence of 5-locular ovules with pluri-ovulate locules, which is a widespread condition in Byttneroideae, whereas the other genera show reduction in both the number of locules and ovules). Combining this observation with the disjunctive distribution suggests that it may be worth testing the hypothesis that *Hermannia* is polyphyletic (Hinsley, 2003).

These are generally herbs, shrublets or undershrubs and are frequently stellate-pubescent, sometimes glandular. Leaves are entire, toothed or pinnatifid with stipules often foliaceaous. Flowers are axillary and solitary or binate or in terminal pseudoracemes or panicles. Calyx is 5-lobed and the tube globose to campnulate. Petals number five and are obovate or oblong, sessile or clawed, spirally twisted; claw with infolded margin. Five stamens occur opposite petals, and are either longer or shorter than the ovary. The filaments are connate at the base, and are sometimes almost free and linear with broad membraneous wings or are somewhat cruciform. Anthers are mostly lanceolate, slightly or distinctly two-lobed at the apex. Ovary is sessile or shortly stalked, five–lobed, five-locular, with few to many ovules in each locule and is hairy. The five styles that occur are capitate. Fruit are five-locular, loculicidal capsule and are sometimes horned. Seeds are reniform, and usually ribbed (Leistner, 2000).

1.2.2. Traditional uses and extent of usage

The genus *Hermannia* has been utilized traditionally by a diversity of people such as the European, Tswana, Kwena, Southern Sotho, Xhosa and Zulu for a wide variety of uses such as fever, cough, respiratory diseases such as asthma, wound plaster, burns, stomachache, purgative, diaphoretic, heartburn, flatulence in pregnant women, colic, hemorrhoids as well as syphilis and eczema. In addition specific species of *Hermannia* that have been chosen to be researched have the following uses:

Hermannia incana:

The Xhosa use a decoction of the root for dysuria. The decoction is blue if boiled in an iron vessel and greenish in a "tin" one and becomes starchy when cold.

Hermannia salviifolia:

This plant is used to make a tea with aromatic properties and a decoction of the root is an old-fashioned European household remedy for fits. An ointment made from the plant together with *Losbostemon fruticosus* and *Psoralea decumbens* is an old Cape remedy for "roos" (erysipelas or eczema).

Hermannia cuneifolia:

The Europeans apply and infusion or a decoction of this plant to sores and take the preparation internally (Watt and Breyer-Brandwijk, 1962).

Hermannia althaeifolia:

This plant was cultivated in Europe during the 18th century and was used medicinally as an aromatic tea against syphilis (Shearing, 1997).

1.2.3. Previous research conducted on the genus

Most ethnobotanical literature refers to the extensive traditional use of *Hermannia* and, thus, it is remarkable that limited research has been conducted on this genus implying that very little information is available on the chemical constituents, pharmacology and toxicity of *Hermannia*. Two papers have been published regarding the antimicrobial and anti-inflammatory activity as well as the antimalarial activity of one species of *Hermannia* i.e. *H. depressa* (Reid *et al.,* 2005 and Clarkson *et al.,* 2004). Since this is the only species of *Hermannia* to be investigated, this pioneer evaluation of the possible activity is necessary to gauge the potential pharmacological use and chemical composition of this genus.

1.3. RATIONALE:

1.3.1. Choice of *Hermannia* species as a research topic

It is unlikely that the ethnic population of Southern Africa would describe the various and extensive uses for this genus if the plants did not possess some biological activity and it would be interesting to note the possible correlation between the traditional use of the genus and the actual biological activity. In addition, since this is, surprisingly, one of the first studies to be conducted on this genus, isolation of any active compounds could produce interesting and biologically active compounds that may play an important role in the treatment of diseases.

1.3.2. Choice of the studied biological activities

There may be correlation between traditional usage and pharmacological action, such as isolation of anti-pyretic principals from a 'fever' remedy, but even so, it may be different from our expectations. Therefore, extracts of plants based on traditional usage should not only be tested for the activity expected, but should also be subjected to a battery of tests, since some important modern drugs have been developed from plants which have been used for a different purpose entirely (Heinrich *et al.,* 2004). Thus, four activities were assayed, all related to the possible activity indicated by the traditional use. In addition, toxicity of the plants species was also determined.

The treatment of respiratory diseases, wounds, burns, dysuria, piles as well as suppurating wounds suggest that the plants my contain compounds with antimicrobial activity as infection plays an important role in the promotion of these disease states. Thus assessment of this activity against a diversity of pathogens is essential to obtain the scope of activity portrayed by the species.

In addition, inflammation is a significant factor in the above-mentioned diseases as it is the initial response to tissue injury. The treatment of these disease states may thus involve some form of anti-inflammatory activity and therefore to obtain an idea of the method in which these species function in the treatment of disease, an anti-inflammatory assay is necessary.

The role of free radical reactions in biology has become an area of intense interest. It is generally accepted that free radicals play an important role in the development of tissue damage and pathological events in living organisms and thus there is an increased interest in the natural antioxidants contained in medical and dietary plants, which are candidates for the prevention of oxidative damage (Velázquez *et al.,* 2003). Considering the extensive use of the genus *Hermannia* to treat a number of diseases for which free radicals may act as a primer, it is necessary to establish the free radical scavenging activity of the plant extracts.

Research previously conducted on related genera such as *Melochia* reported the presence of alkaloids within that genus (Kapadia *et al.,* 1977) and potentially may occur in the various species of *Hermannia.* Alkaloids are most often associated with antimalarial activity and thus this activity was assessed. In addition some species are used to treat 'fever' which in many areas especially in Africa, may be related to a malarial infection (Addae-Kyereme *et al.,* 2001).

During the course of their evolution, many plant species have been protected by their ability to accumulate toxic compounds (Cotton, 1996). The quantity of these toxins found in the plants and their ability to cause damage to cells and cell death is an important factor to be assessed. A plant may have potent biological activity but should it also prove to be

highly toxic, it cannot be used to any benefit in humans and thus, toxicity testing is essential.

1.4. OBJECTIVES OF THE STUDY:

The study has been completed with the following objectives:

- To phytochemically characterize the species using thin layer chromatography and HPLC/UV.
- To isolate and identify of phytochemicals from lead crude plant extracts using column chromatography, thin layer chromatography, nuclear magnetic resonance spectroscopy and mass spectroscopy.
- To screen selected species for antibacterial activity using minimum inhibitory concentrations, the death kinetics assay and bioautographic assays.
- To screen selected species for antioxidant activity using DPPH and ABTS antioxidant assays and to determine the concentration of selected species to reduce 50 % of free- radicals (IC_{50}).
- To screen of selected species for anti-inflammatory activity using the 5-lipoxygenase enzyme assay and to determine the concentration of species to inhibit 50 % of 5-lipoxygenase activity (IC_{50}).
- To screen of selected species for anti-malarial activity using titrated hypoxanthine incorporation assay.
- To establish the potential toxicity that is portrayed by these plants and to relate the toxicity to biological activity.
- To provide a scientific basis for the traditional use and indigenous knowledge which has developed around this genus.

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CHAPTER 2: STUDIED SPECIES, PLANT COLLECTION AND PREPARATION OF SAMPLES

2.1. STUDIED SPECIES:

There are approximately 180 species of *Hermannia* worldwide of which about 162 species occur in Southern Africa (Leistner, 2000). Ten species were originally chosen for investigation based on the extent of traditional use as well as availability and accessibility for collection of the plant material. Later two species were added to the investigation as they became available. A collecting permit was obtained from Cape Nature Conservation.

2.1.1. *Hermannia althaeifolia*

This plant is also known by the vernacular names 'Wolhaar Poproos', 'Bokkiesblom' and 'Pokkiesblom'. It is a shrublet with soft hairy branches. The leaves are wrinkled and roughly toothed and are softly hairy. The stipules are large and leaf-like. Flowers occur in axillar clusters and are bright orange-yellow with a swollen, hairy calyx (Fig 2.13). Flowering occurs from August to March. The plants occur on clay flats and slopes or along sandy watercourses from Namaqualand to Uniondale. They are perennial plants that may grow up to 50 cm in height. They are sometimes grazed (Shearing, 1997) Fig 2.1 indicates the distribution of this species in South Africa.

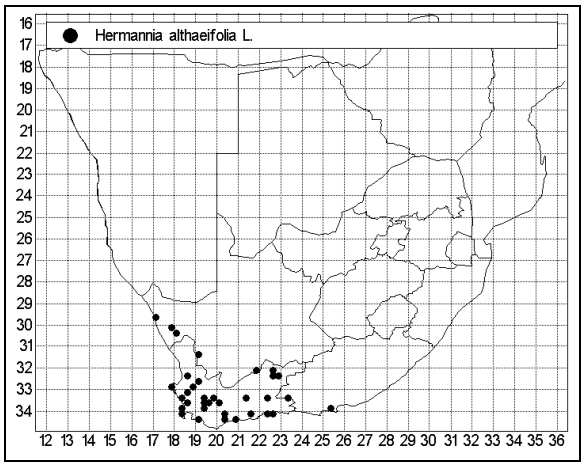

Fig. 2.1: Distribution map of *H. althaeifolia.* (All distribution maps purchased and included with permission from the South African National Biodiversity Institute)

2.1.2. *Hermannia cuneifolia*

Also known as ‘Agtdaegeneesbos’, this plant is an aromatic shrub that may grow up to 50 cm in height. The leaves are wedge-shaped and are slightly hairy. It has bright yellow to orange flowers that are pendulous (Fig 2.14). They are plants that are drought resistant and well grazed by animals. They occur throughout the Cape and one locality in Lesotho in seasonal streams and wide range of habitats (Shearing, 1997) (Fig 2.2).

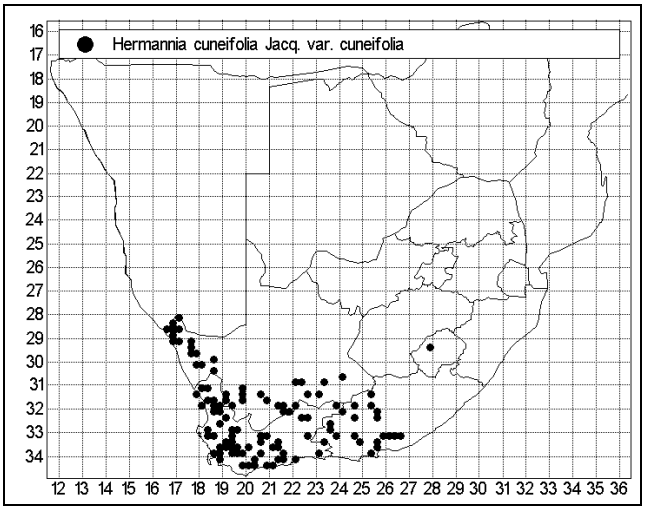

Fig. 2.2: Distribution map of *H. cuneifolia.*

2.1.3. *Hermannia flammula*

The plant is also called ‘Poprosie’ by the local population. It is a shrublet which grows up to 250-900mm in height. The flowers are red in color (Fig 2.15) and the plants occur on the lower slopes of hills. The plants occur in regions from Caledon to Uniondale as well as the Little Karoo (Fig 2.3).

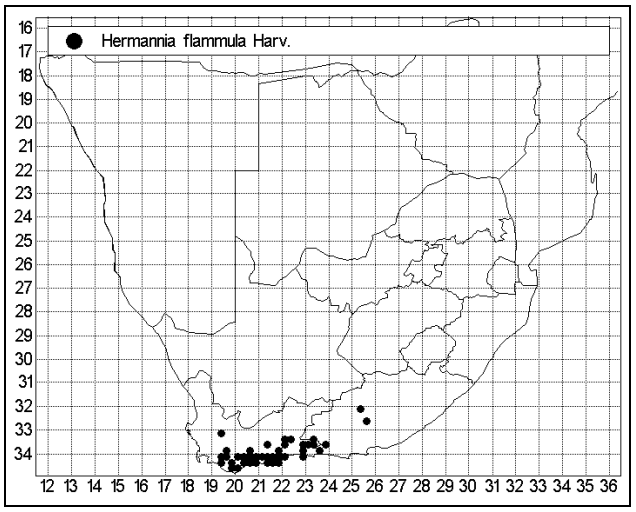

Fig. 2.3: Distribution map *of H. flammula.*

2.1.4. ***Hermannia holosericea***

This is a velvety shrub that grows to 0.3 to 1.2 m in height. The flowers are yellow. The plants grow on coastal limestone to middle inland slopes from Bredasdorp and Montagu to Oudshoorn as well as Mossel Bay to Uitenhage in the Eastern Cape (Fig 2.4).

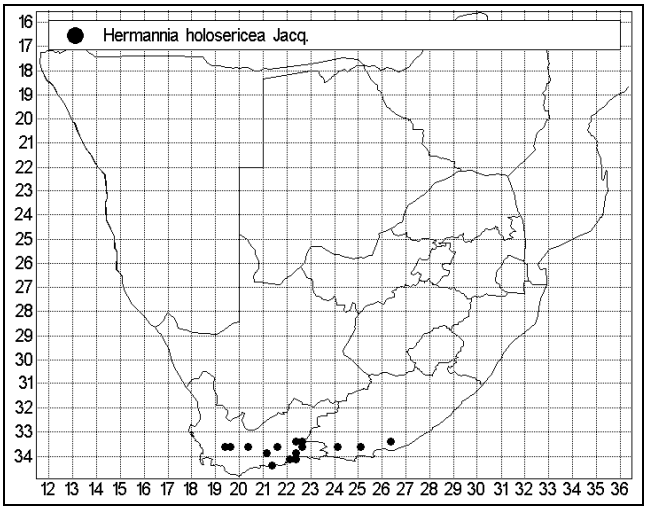

Fig. 2.4: Distribution map of *H. holosericea.*

2.1.5. *Hermannia incana*

The plant is a grey-leafed shrub which grows up to 2 m in height. The flowers are yellow in color (Fig 2.16). The plants grow on the dry lower to middle slopes in regions between Worcester to Outshoorn as well as the Peninsula to George in the Karoo (Fig 2.5).

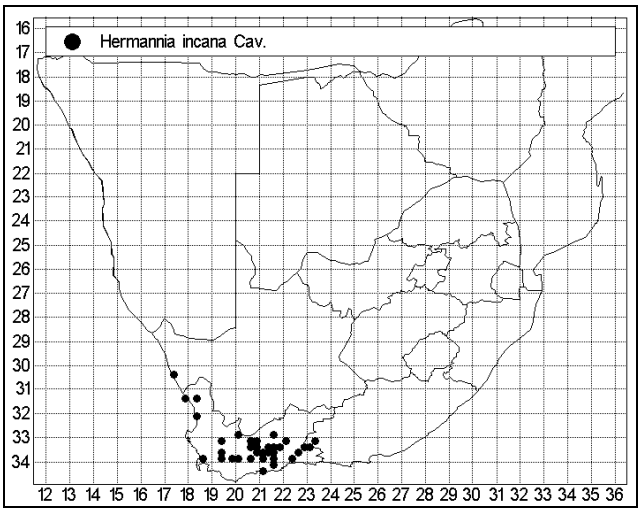

Fig. 2.5: Distribution map of *H. incana.*

2.1.6. *Hermannia involucrata*

This is a golden-yellow shrub that may grow to over 1 m. It bears flowers that are yellow to orange in color (Fig 2.17). They grow on shaded lower slopes and occur at Ladismith, Humansdorp and Uniondale (Fig 2.6).

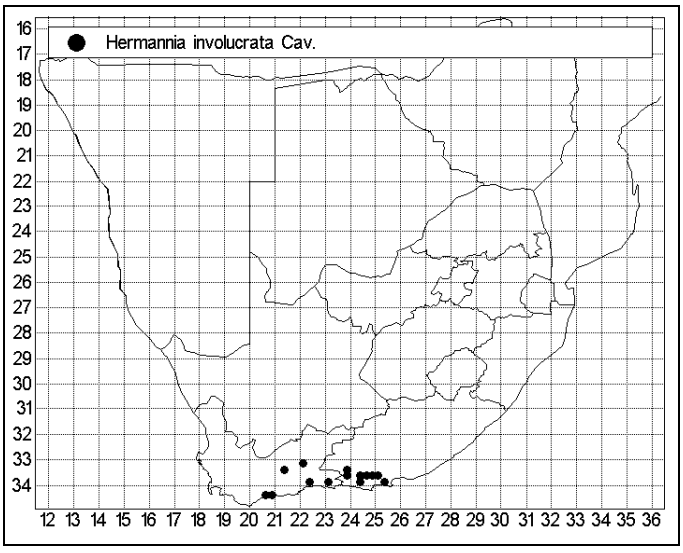

Fig. 2.5: Distribution map of *H. involucrata.*

2.1.7. *Hermannia lavandufolia*

This plant is a spreading shrublet that has velvety grey leaves. It grows to between 0.3 to 1 m and has flowers that are colored yellow to orange (Fig 2.18). They occur on the lower coastal slopes in the region of Caledon to Mossel Bay (Fig 2.7).

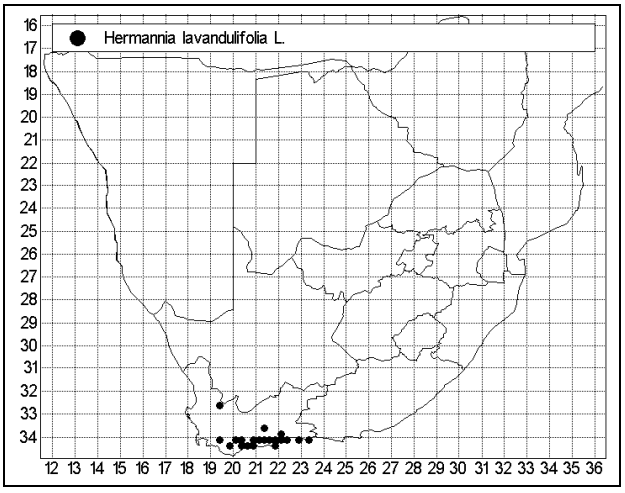

Fig. 2.7: Distribution map of *H. lavandufolia.*

2.1.8. *Hermannia muricata*

Hermannia muricata is a flexuose shrublet which grows up to 200 mm in height. The plant bears flowers that yellow to orange in color (Fig 2.19). They occur in the river valleys from Clanwilliam, Piketberg, Worcester, to Uniondale basically the Karoo and Namaqualand regions (Fig 2.8).

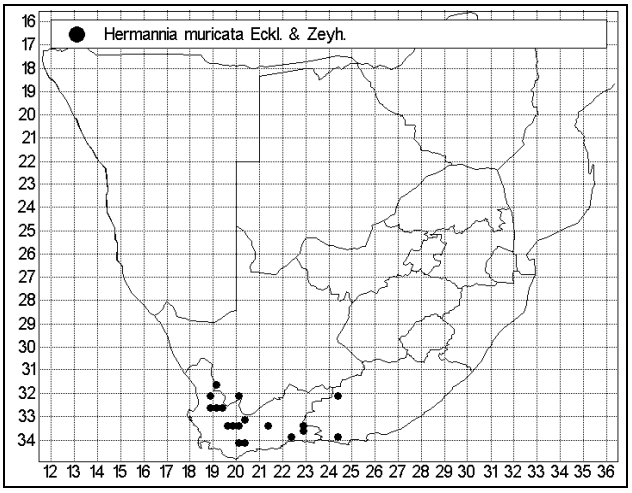

Fig. 2.8: Distribution map of *H. muricata.*

2.1.9. *Hermannia saccifera*

Also known as 'Komynbossie', this is an erect or sprawling woody shrublet that may grow up to 300 mm. The flowers are yellow (Fig 2.20) and the plants occur on the lower coastal slopes of the region Bredasdorp to Uitenhage (Fig 2.9).

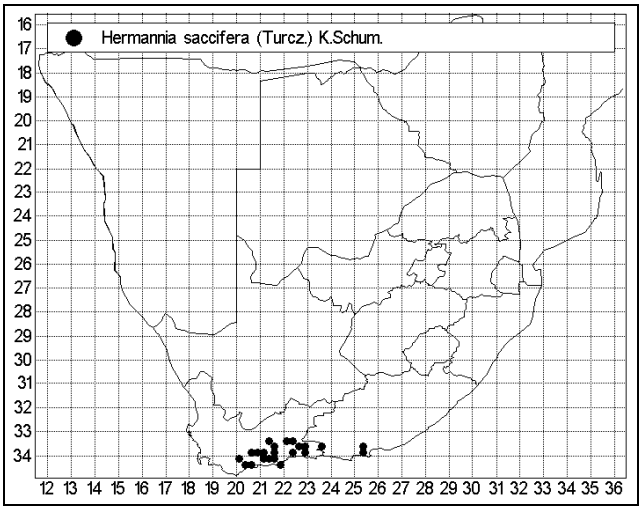

Fig. 2.9: Distribution map of *H. saccifera*

2.1.10. *Hermannia salviifolia*

Hermannia salviifolia is a variable shrub which has roughly pubescent leaves and may grow to over 1 m in height. It has flowers that are a yellow-orange color. The plants occur on the coastal flats or lower slopes of the region Peninsula to Port Elizabeth (Fig 2.10).

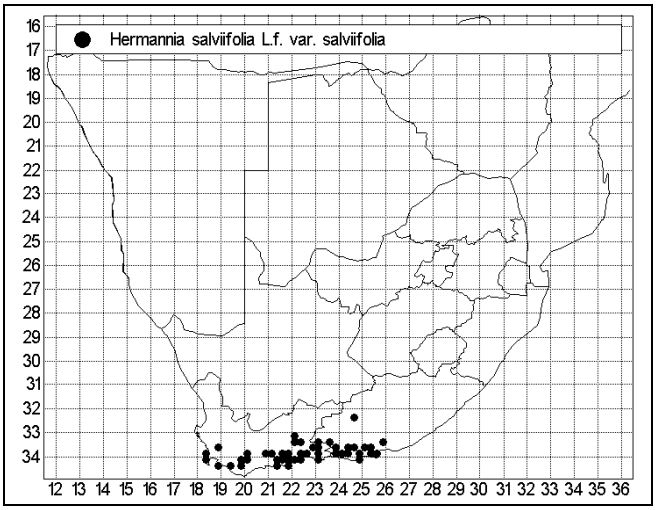

Fig. 2.10: Distribution map of *H. salviifolia*

2.1.11. *Hermannia scabra*

This is an erect or spreading shrublet that grows up 600 mm. The flowers are yellow in color or may be a fading reddish color. They occur on the middle slopes of hills from Gifberg to Clanwilliam as well as Malmesbury (Fig 2.11).

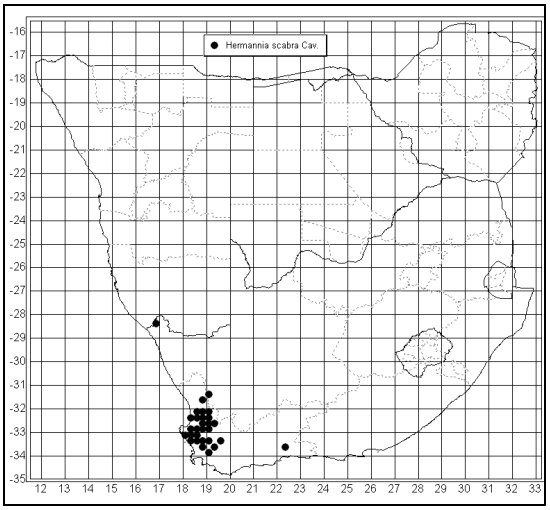

Fig. 2.11: Distribution map of *H. scabra*

2.1.12. *Hermannia trifurca*

This is a twiggy shrub that grows up to 1 m. The flowers are a pink to purple color. The plants occurs on the flats or lower slopes in areas from Clanwilliam to Malmesbury as well as Ceres, Worcester and in the Namaqualand as well as Namibia (Bond and Goldblatt, 1984) (Fig 2.12).

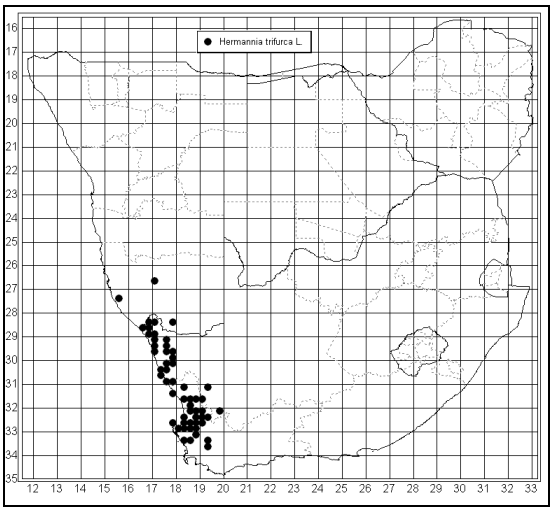

Fig. 2.12: Distribution map of *H. trifurca*



Fig. 2.13: *H. althaeifolia*



Fig. 2.14: *H. cuneifolia*



Fig. 2.15: *H. flammula*



Fig. 2.16: *H. incana*



Fig. 2.17: *H. involucrata*



Fig. 2.18: *H. lavandufolia*



Fig. 2.19: *H. muricata*



Fig. 2.20: *H. saccifera*

2.2. COLLECTION OF PLANT MATERIAL

Fresh plant material of selected *Hermannia* species was collected from natural populations in the Cape region of South Africa (Table 2.1). The taxonomy was confirmed by botanists at the National Botanical Institute (NBI) in Pretoria, South Africa and voucher specimens have been maintained at the Department of Pharmacy and Pharmacology at the University of the Witwatersrand in Johannesburg.

Table 2.1: Selected species of *Hermannia* studied

Species	Locality	Vegetation Type	Date of Collection	Voucher Number
H. althaeifolia	Uniondale, in Langkloof near turnoff to Daskop	Fynbos	06/11/2003	2834
H. cuneifolia	De Rust, on hill south of town	Renosterveld, Succulent Karoo	05/11/2003	2828
H. flammula	Uniondale, in Langkloof near turnoff to Daskop	Fynbos	06/11/2003	2832
H. holosericea	De Rust, on hill south of town	Renosterveld, Succulent Karoo	05/11/2003	2827
H. incana	De Rust, on hill south of town	Renosterveld	05/11/2003	2829
H. involucrata	Uniondale, next to road near Daskop	Renosterveld	06/11/2003	2831
H. lavandufolia	Oudsthoorn, northern end of Paerdepoort, near farm Heimersrivier.	Succulent Karoo	06/11/2003	2835
H. muricata	Uniondale, in Langkloof near turnoff to Daskop	Fynbos	06/11/2003	2833
H. saccifera	Dysselsdorp, next to road near farm, Leenblad	Renosterveld, Succulent Karoo	06/11/2003	2830
H. salviifolia	Oudtshoorn, next to national road to George near Blossoms	Succulent Karoo	06/11/2003	2836
H. scabra	Farm Kersefontein, Darling district	Fynbos	16/09/2004	AV 1165
H. trifurca	Farm Kersefontein, Darling district	Fynbos	16/09/2004	AV 1166

2.3. PREPARATION OF SAMPLES:

2.3.1. Process of extraction

A thin layer chromatography plate indicated that leaves, flowers and stems contained similar compounds and hence, these parts were combined before crushing. Fresh plant material was dried through air drying. When plant material was completely dry, it was crushed into powder which was then used in various assays and preparative chromatography.

To conduct assays on the crude extracts, the pulverized plant material was extracted (5 g) in a conical flask using the necessary solvent by adding approximately 50 ml of solvent to the flask and placing the flask in a water bath at 40° C for three hours after which the solvent was filtered off into a separate flask. This was repeated three times to maximize the extraction. The solvent was then evaporated utilizing a Büchi Rotavapor R-114 and the weight of the extract was obtained. These extracts were then diluted in suitable solvents specific for each assay to varying concentration and were thus utilized to determine the various biological activities.

Isolation of compounds required a larger quantity of powdered plant material (500 g) which was extracted in a cylinder through which the solvent could be removed easily. Solvent was added to the crushed plant material and extracted for 48 hours during which the solvent was removed and fresh solvent added three times. The solvent was then evaporated using a Büchi Rotavapor R-114 and the weights of the extracts were determined.

2.4. REFERENCES:

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CHAPTER 3: PHYTOCHEMICAL ANALYSIS

3.1. INTRODUCTION:

Chromatographic procedures are the most diverse and the most widely used techniques in fractionation of extracts. All chromatography relies on the differential distribution of compounds between two phases, one of which moves relative to the other. These phases are called the mobile and stationary phase, respectively. The mobile phase is a fluid, which can be either liquid, a gas, or a supercritical fluid. The stationary phase usually appears to be a solid consisting of fine particles (Houghton and Raman, 1998).

Chromatography is a separation process. The analysis is accomplished by first separating a mixture into its individual components and then, monitoring these with a detector for quantitative determination and/or qualitative identification. Optimizing the chromatographic process implies generating sufficient resolution between adjacent components as quickly as possible. The individual constituents of a mixture are separated as a result of their different physical and chemical interactions with the mobile phase (the solvent) and with the stationary phase (the column packing) (Henschen *et al.*, 1985).

Chromatography is based on the act that a dynamic equilibrium is established between the concentration of a solute in two phases. This dynamic equilibrium consists between molecules of the solutes continually passing between the two phases. At any one time the concentration ratio, i.e. ratio of number of molecules in each phase, is constant. However, each molecule spends time in both phases and the proportion of time spent in a phase depends on the relative attractions of the substance for the two phases. The concentration ratio at equilibrium is called the distribution coefficient and the name for the attraction for any phase is called the affinity, i.e. a compound that moves more slowly is said to have a

higher affinity for the stationary phase than the one that moves more quickly (Houghton and Raman, 1998).

Thin layer chromatography (TLC) is a solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (moving phase). Solids most commonly used in chromatography are silica gel (SiO_2 x H_2O) and alumina (Al_2O_3 x H_2O). Both of these adsorbents are polar, but alumina is more so. Silica is also acidic. Alumina is available in neutral, basic, or acidic forms. TLC is a sensitive, fast, simple, and inexpensive analytical technique. It is a micro technique; as little as 10^{-9}g of material can be detected, although the sample size is from 1 to $100x10^{-6}$ g. TLC involves spotting the sample to be analyzed near one end of a sheet of glass or plastic that is coated with a thin layer of an adsorbent. The sheet, which can be the size of a microscope slide, is placed on end in a covered jar containing a shallow layer of solvent. As the solvent rises by capillary action up through the adsorbent, differential partitioning occurs between the components of the mixture dissolved in the solvent the stationary adsorbent phase. The more strongly a given component of a mixture is adsorbed onto the stationary phase, the less time it will spend in the mobile phase and the more slowly it will migrate up the plate. The following are some common uses of TLC:

1. To determine the number of components in a mixture.
2. To determine the identity of two substances.
3. To monitor the progress of a reaction.
4. To determine the effectiveness of a purification.
5. To determine the appropriate conditions for a column chromatographic separation.
6. To monitor column chromatography (Fried and Sherma, 1996)

A large number of spray reagents have been produced which when applied to a layer forms colors from colorless substances. Some of the spray reagents are very specific, but many others will react with a broadly based type of compound. Thus these spray reagents due to the color reactions may be useful in determining the nature of the compounds forming zones on the TLC plate (Houghton and Raman, 1998).

High performance liquid chromatography (HPLC) uses high pressure to force eluent through a closed column packed with micron-size particles that provide exquisite separations of picograms to micrograms of analyte. Essential components include a solvent delivery system, a sample injection valve, a detector, and a recorder or computer to display results. If a solute can diffuse rapidly between the mobile and stationary phases, then plate height is decreased and resolution increases. In liquid chromatography, we increase the rate of mass transfer by reducing the dimensions of the stationary particles, thereby reducing the distance through which solute must diffuse in both phases (Harris, 1995).

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on a chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted and the detection and sensitivity parameters may also be controlled. There are many types of detectors that can be used with HPLC such as Refractive Index, Ultra-violet, Fluorescent, Radiochemical, Electrochemical, Near-Infra Red, Mass Spectroscopy, Nuclear Magnetic Resonance, and Light Scattering.

Ultra-violet detectors measure the ability of a sample to absorb light. UV detectors have a sensitivity to approximately 10^{-8} or 10^{-9} gm/ml. This can be accomplished at one or several wavelengths such as by utilizing Fixed Wavelength, Variable Wavelengths, or Diode

Array detectors. Photodiode Array detectors can be used to measure and detect samples over the entire UV to visible spectrum. These detectors record the entire spectrum at once in a fraction of a second. They are highly beneficial tools in identification and analysis of a sample compound (Parriott, 1993)

HPLC is unquestioningly the most widely used of all of the analytical separation techniques. The reasons for the popularity of the method are its sensitivity, its ready adaptability to accurate quantitative determination, its suitability for separating nonvolatile species or thermally fragile ones, and above all, its widespread applicability to substances that are of prime interest to industry, to many fields of science, and to the public. Examples of such materials include amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, terpenoids, pesticides, antibiotics, steroids, metal-organic species, and a variety of inorganic substances (Skoog and Leary, 1992). High performance liquid chromatography is now a firmly established separation technique which can be used in a variety of ways in a laboratory dealing with plant products, both for preparative and analytical purposes (Linskens and Jackson, 1987).

3.2. METHOD:

3.2.1. Thin layer chromatography

Plant extracts of ten species [chloroform: methanol (1:1)] as well as two isolated compounds were utilized for TLC analysis. Analysis of *H. scabra* and *H. trifurca* were not conducted as these species were added to the investigation at a later stage. All samples were made up to concentrations of 50 mg/ml in chloroform: methanol (1:1). Solutions of samples (2 µl in volume) were applied to 0.2 mm silica-gel, aluminium-backed TLC plates (Macherey-Nagel Art. 818133) after which the plates were developed in one of the following TLC systems:

- TLC 1: methanol: water: ethyl acetate (1.65: 1.35: 10)
- TLC 2: toluene: ethyl acetate (6:4)
- TLC 3: toluene: dioxan: ethyl acetate (9: 2.5: 1)

Plates were developed up to 9 cm. Thereafter the plates were dried and examined under UV light (UV_{254nm} and UV_{365nm}) after which they were sprayed with anisaldehyde/sulphuric acid spray reagent (anisaldehyde: conc. H_2SO_4: methanol [1:2:97]), using an atomizer. Plates were heated for 5-10 min at 100°C.The spray reagent enabled visible evaluation of the plates.

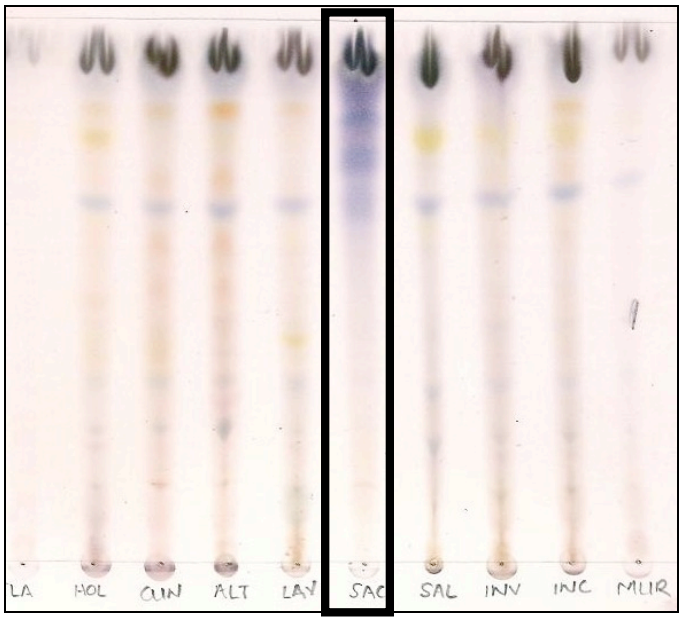
3.2.2. HPLC/UV

HPLC/UV was overseen by Dr Paul Steenkamp from the Forensic Toxicology Research Unit, Forensic Chemical Laboratory in Johannesburg. A Waters 2690 HPLC System (Phenomenex Aqua C18 column, 250 x 2.1 mm) equipped with a 996 photodiode array (PDA) detector was used. The mobile phase which was added over time at a flow rate of 0.2 ml/min, was a 10% acetonitrile in 10 µM aqueous formic acid solvent. The ratio was changed through a linear gradient to 90 % acetonitrile in 10 % 10 µM aqueous formic acid at 40 min. This ratio was maintained for 10 min after which the solvent ratio was changed back to the original starting solution. HPLC profiles were analyzed by convolution of retention times and UV profiles using Empower® software.

3.3. RESULTS:

Thin-layer chromatography of ten species when examined under UV light indicated few compounds present. However, anisaldehyde spray reagent indicated that many compounds are present in the extract that had not been visible under UV light. Plates developed in TLC 1, 2 (Fig. 3.1. and Fig. 3.2) and 3 (Fig. 3.3.) indicated many similarities in the phytochemical composition of all ten species. However, the TLC plates also indicate a

distinct difference being portrayed by *H. saccifera*, which when compared to the other species being investigated, indicates anomalous compounds which are not present in the other species. This chemical diversity is evident in all TLC systems utilized. In addition, further TLC analysis indicated that β-sitosterol and lupeol are present in the ten species investigated (Fig. 3.4. and Fig. 3.5.)



Legend: For all TLC Plates

1 - *H. flammula*
2 – *H. holosericea*
3 – *H. cuneifolia*
4 – *H. althaeifolia*
5 – *H. lavandufolia*
6 – *H. saccifera*
7 – *H. salviifolia*
8 – *H. involucrata*
9 – *H. incana*
10 – *H. muricata*

Fig. 3.1: TLC plate of ten species of *Hermannia* developed in TLC: 1. The distinct and characteristic profile of *H. saccifera* is shown in the black box.

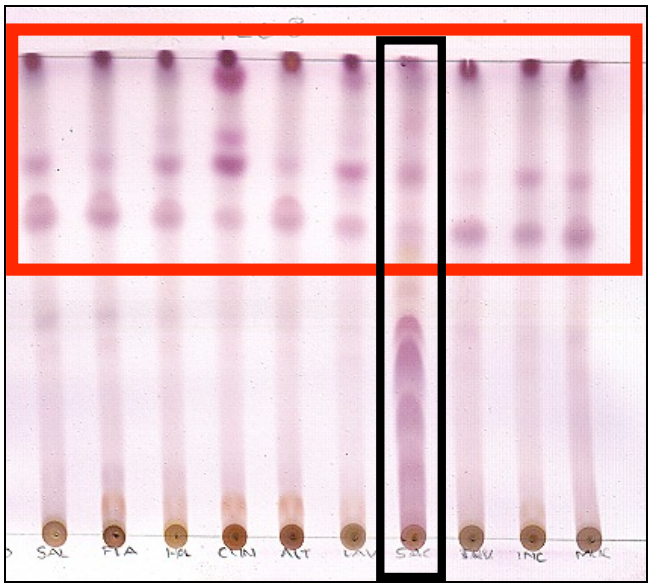


Fig. 3.2: TLC plate of ten species of *Hermannia* developed in TLC: 2. The area boxed-in in red refers to similar compounds present in all species of *Hermannia* being investigated. The black box refers to *H. saccifera*.

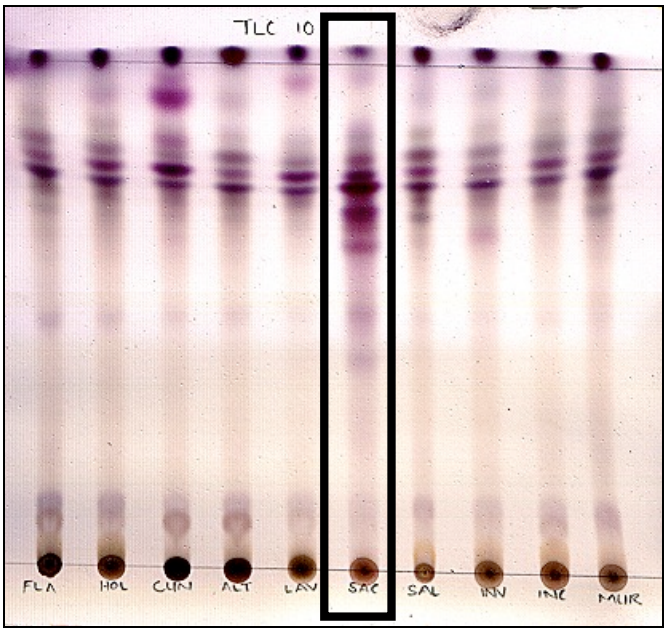


Fig. 3.3: TLC plate of ten species of *Hermannia* developed in TLC: 3. Species show distinct similarities with only *H. saccifera* appearing to contain compounds not present in the other species studied.

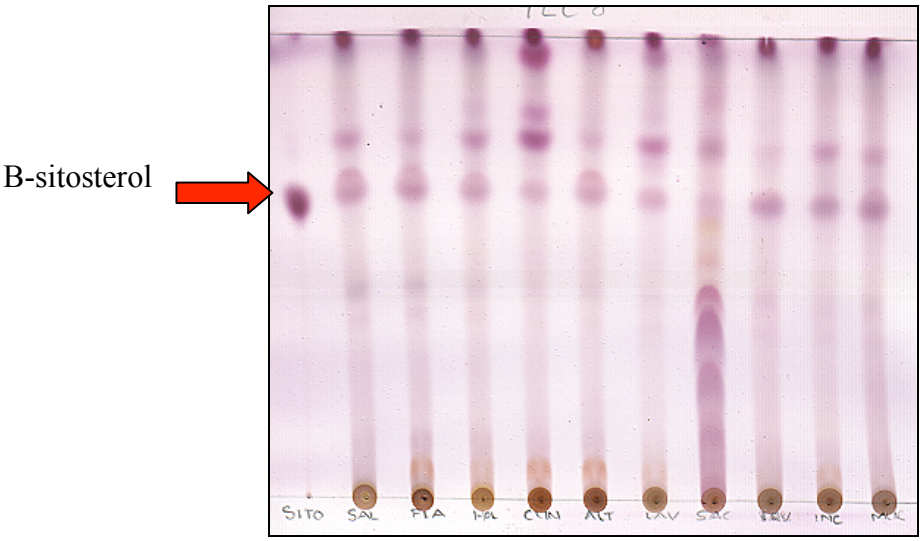


Fig: 3.4. TLC plate indicating the presence of β-sitosterol in ten species of *Hermannia* being investigated.

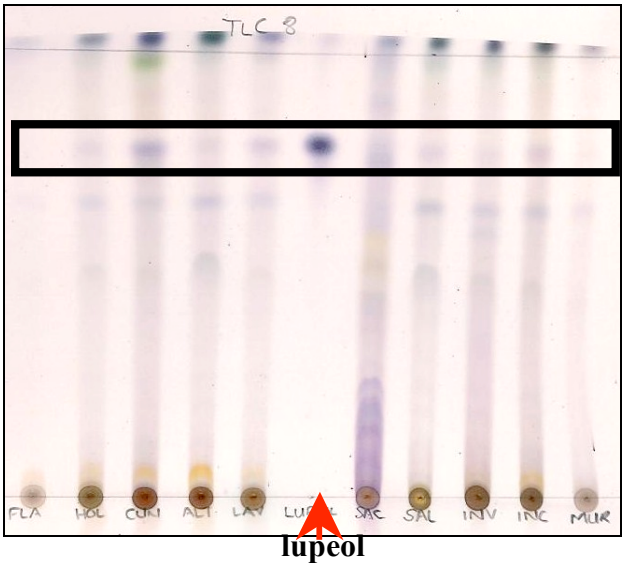


Fig. 3.5: TLC plate indicating the presence of lupeol in ten species of *Hermannia* being investigated.

Fig 3.18 shows the HPLC profiles for all twelve selected species. Some similarities are indicated from these, which were confirmed using retention times and UV profiles for specific compounds. Tables 3.1 - 3.13 indicate the retention times and percentage area obtained for peaks found in each of the species analyzed as well as the isolated compound investigated.

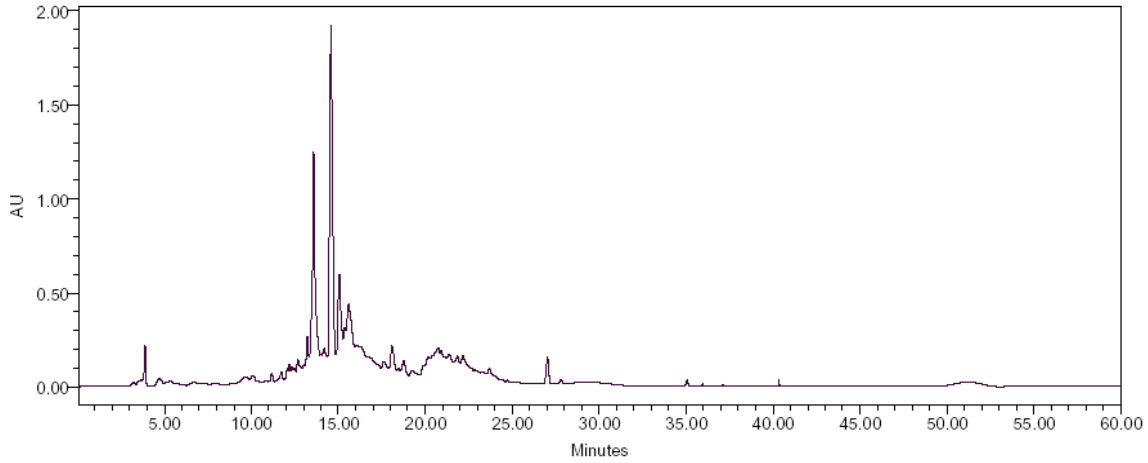


Fig. 3.6: HPLC profile for *H. althaeifolia.*

Table 3.1: HPLC retention time and percentage area for peaks from *H. althaeifolia.*

Retention Time	Absorbance maxima (nm)	% Area
3.8	201.6 and 207.4	3.0
13.2	202.8, 226.2, 285.1 and 336.1	2.9
13.5	201.6 and 278.0	28.8
14.5	207.4 and 278	37.6
15.0	201.6 and 278.0	10.8
15.3	202.8 and 281.5	3.0
15.5	201.6 and 279.2	10.8
18.0	201.6 and 278.0	3.3
27.0	229.7, 266.2 and 313.5	2.4

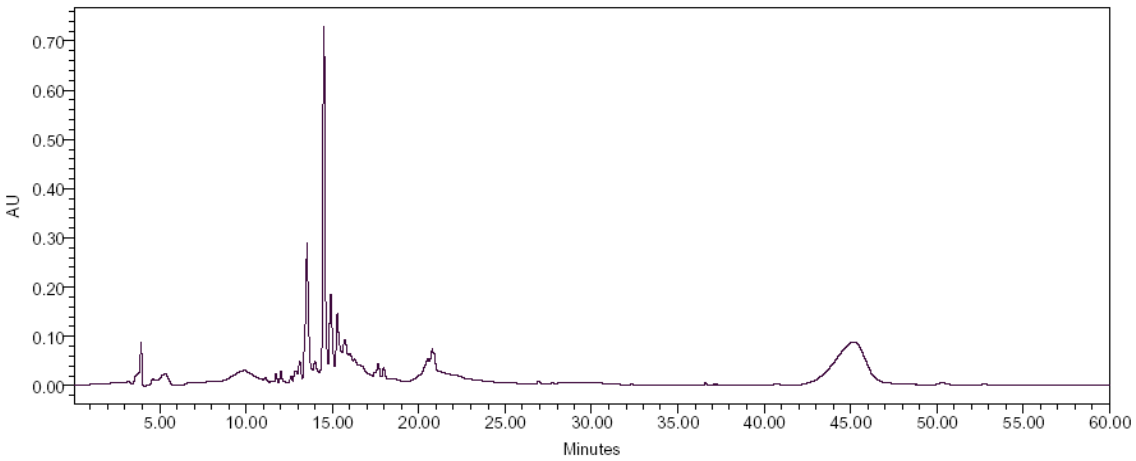


Fig. 3.7: HPLC profile for *H. cuneifolia.*

Table 3.2: HPLC retention time and percentage area for peaks from *H. cuneifolia.*

Retention Time	Absorbance maxima (nm)	% Area
3.9	201.6 and 207.4	2.6
9.92	209.8, 260.3 and 296.9	7.0
13.1	202.8 and 279.2	1.1
13.5	201.6 and 278.0	9.3
13.9	201.6 and 279.2	1.6
14.5	205.1 and 278.0	17.7
14.9	202.8 and 279.2	5.5
15.2	202.8 and 279.2	5.7
15.7	202.8 and 279.2	4.2
16.0	202.8 and 279.2	2.1
16.2	202.8 and 279.2	2.0
16.5	201.6 and 279.2	2.7
20.5	203.9 and 278.0	1.8
20.6	202.8 and 278.0	2.7
20.7	201.6 and 278.0	2.6
20.9	202.8 and 272.1	2.5
45.159	228.5, 274.4, 324.2 and 408.8	28.2

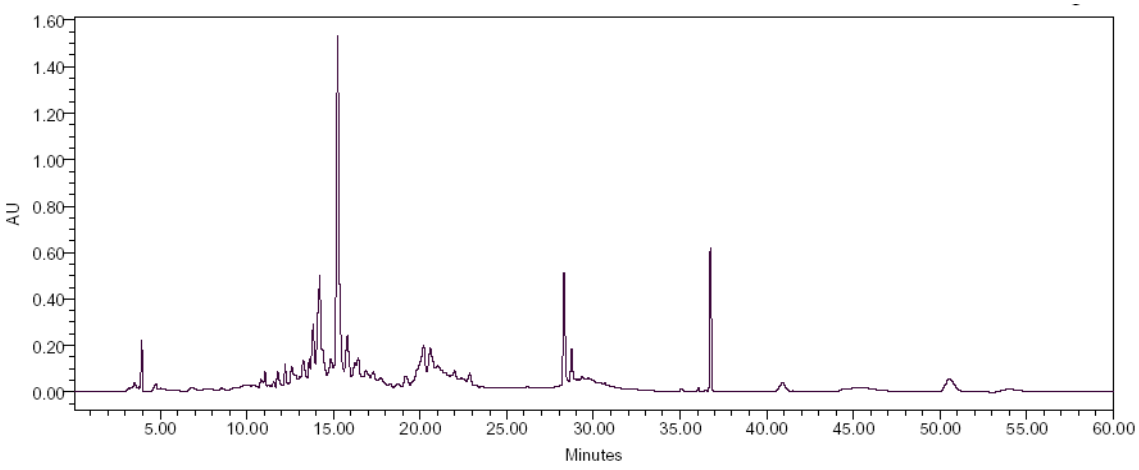


Fig. 3.8: HPLC profile for *H. flammula.*

Table 3.3: HPLC retention time and percentage area for peaks from *H. flammula.*

Retention Time	Absorbance maxima (nm)	% Area
3.9	207.4 and 307.6	2.8
13.8	202.8, 226.2, 283.9 and 336.1	5.4
14.1	201.6 and 278.0	14.2
14.8	201.6 and 274.4	2.7
15.2	206. and 278.0	37.3
15.8	202.8, 275.6 and 369.2	5.8
16.4	203.9 and 275.6	2.3
20.2	351.6	6.2
20.5	201.6, 285.1 and 327.8	3.0
21.0	203.9 and 278.0	3.46
28.3	267.4 and 313.5	8.7
36.7	229.7 and 276.8	8.6

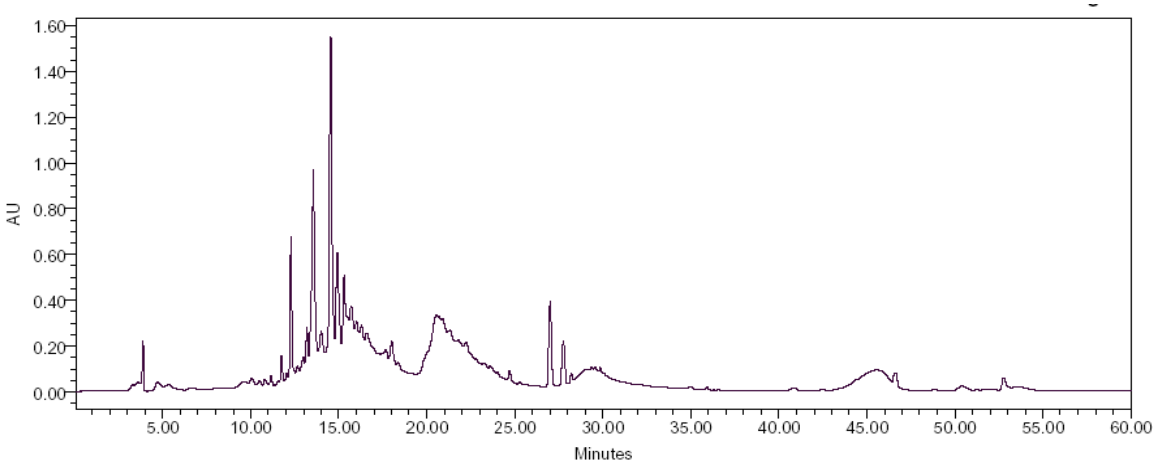


Fig. 3.9: HPLC profile for *H. incana.*

Table 3.4: HPLC retention time and percentage area for peaks from *H. incana.*

Retention Time	Absorbance maxima (nm)	% Area
3.8	201.6 and 207.4 nm	2.1
12.2	207.4 and 270.9	6.5
13.1	202.8, 283.9 and 336.1	2.9
13.5	203.9 and 278.0	16.0
14.0	202.8 and 279.2	3.9
14.5	206.3 and 278.0	22.7
14.9	202.8 and 278.0	8.9
15.3	202.8 and 279.2	8.8
15.7	202.8 and 279.2	5.4
16.0	202.8 and 279.2	3.3
20.5	202.8 and 278.0	10.4
27.0	266.2 and 313.5	5.4
27.7	228.5, 267.4 and 312.3	3.2

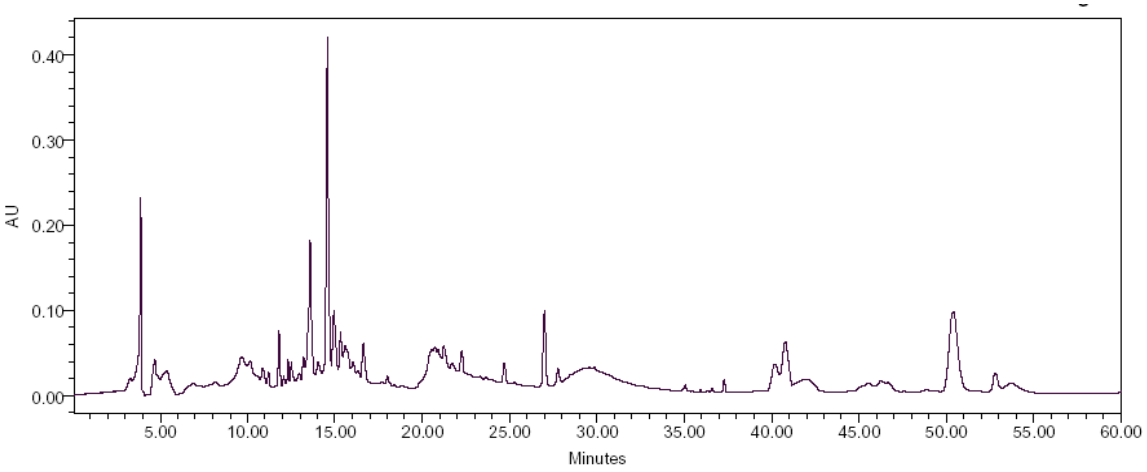


Fig. 3.10: HPLC profile for *H. involucrate.*

Table 3.5: HPLC retention time and percentage area for peaks from *H. involucrata.*

Retention Time	Absorbance maxima (nm)	% Area
3.8	201.6 and 207.4	11.1
11.8	219.1 and 378.0	3.0
13.5	202.8 and 278	12.6
14.5	202.8 and 278	24.3
14.9	282.7 and 326.6	5.3
15.3	201.6 and 278.0	2.9
16.6	201.6, 256.7 and 352.8	2.7
26.9	266.2 and 313.5	5.9
40.7	241.4 and 324.2	13.4
50.3	216.8, 241.4 and 323.0	18.4

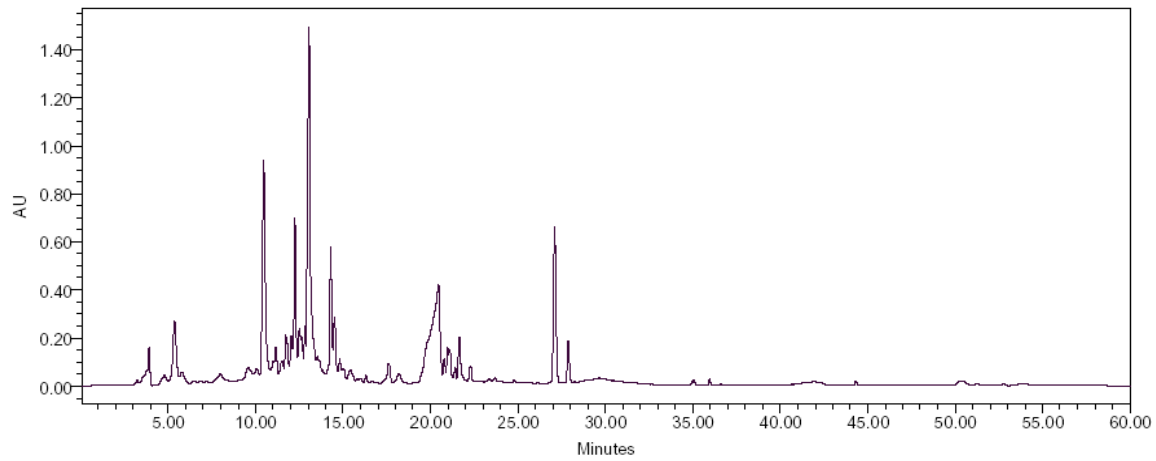


Fig. 3.11: HPLC profile for *H. holosericea.*

Table 3.6: HPLC retention time and percentage area for peaks from *H. holosericea.*

Retention Time	Absorbance maxima (nm)	% Area
3.9	206.3	2.5
5.3	220.3 and 282.7	4.7
10.4	206.3 and 269.7	14.6
11.7	219.1 and 278.0	2.5
12.2	206.3 and 269.7	6.8
12.5	202.8 and 282.7	3.9
13.0	203.9, 225.0, 256.7, 287.4, 319.4	23.3
14.2	203.9 and 278.0	5.8
14.5	202.8and 278.0	2.6
19.8	295.7 and 331.3	4.2
20.4	278.0	16.9
21.6	206.3, 255.6 and 293.4	2.2
27.0	266.2 and 313.5	9.5

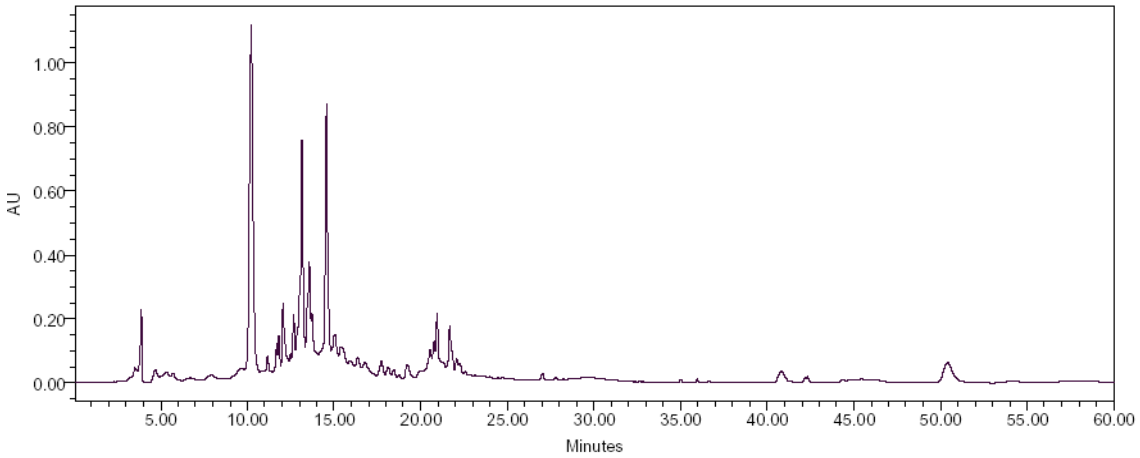


Fig. 3.12: HPLC profile for *H. lavandufolia.*

Table 3.7: HPLC retention time and percentage area for peaks from *H. lavandufolia.*

Retention Time	Absorbance maxima (nm)	% Area
3.8	201.6 and 207.4	3.8
10.2	201.6 and 289.8	34.0
11.8	203.9 and 279.2	1.4
12.0	201.6 and 278.0	4.2
12.6	202.8 and 275.6	2.4
13.1	202.8 and 278.0	16.4
13.5	202.8 and 278.0	8.2
14.5	202.8 and 278.0	15.7
15.0	203.9 and 278.0	2.3
17.7	202.8, 256.7 and 354.0	1.3
20.5	265.0 and 343.2	1.3
20.9	203.9, 254.4 and 354.0	2.6
21.6	327.8	2.9
50.4	216.8, 241.4 and 323.0	2.7

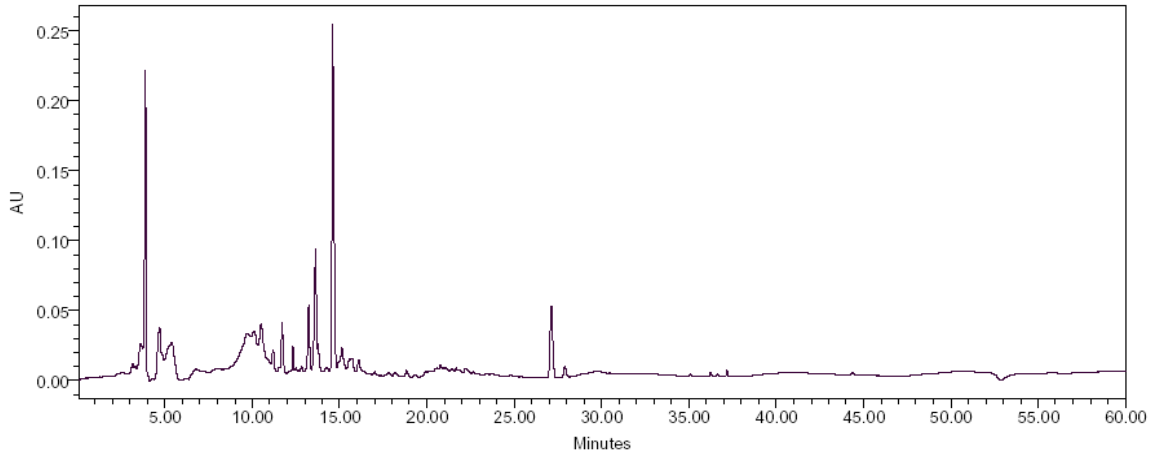


Fig. 3.13: HPLC profile for *H. muricata.*

Table 3.8: HPLC retention time and percentage area for peaks from *H. muricata.*

Retention Time	Absorbance maxima (nm)	% Area
3.6	207.4	3.8
3.8	207.4 and 278.0	23.0
4.6	207.4	4.9
10.5	207.4 and 278.0	3.5
11.7	219.1 and 278.0	3.5
13.2	203.9, 226.2, 256.7, 278.4, 325.4	5.2
13.6	201.6 and 279.2	13.2
14.6	203.9 and 278.0	31.7
15.1	201.6 and 376.4	1.8
27.1	266.2 and 313.5	8.8

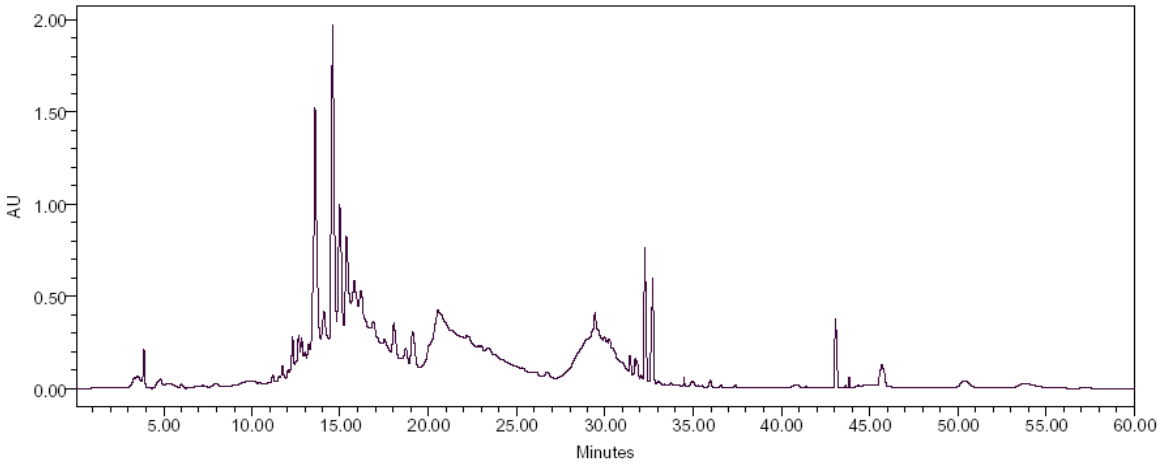


Fig. 3.14: HPLC profile for *H. saccifera.*

Table 3.9: HPLC retention time and percentage area for peaks from *H. saccifera.*

Retention Time	Absorbance maxima (nm)	% Area
3.8	201.6 and 207.4	1.0
12.3	207.4 and 267.4	1.0
12.6	203.9 and 278.0	1.0
13.5	202.8 and 278.0	13.1
14.0	201.6 and 279.2	2.1
14.5	207.4 and 278.0	15.3
14.9	201.6 and 278.0	6.6
15.3	201.6 and 278.0	5.4
15.7	201.6 and 280.3	3.6
16.1	201.6, 255.6 and 354.0	1.9
18.0	202.8 and 278.0	1.5
19.1	203.9, 255.6 and 354.0	1.9
20.5	202.8, 268.5 and 356.3	31.5
29.4	201.6, 293.4 and 336.1	2.3
32.2	278.0 and 330.1 (Flavonoid)	4.3
32.7	278 and 336.1 (Flavonoid)	3.5
43.0	292.2	2.4
45.6	260.3	1.5

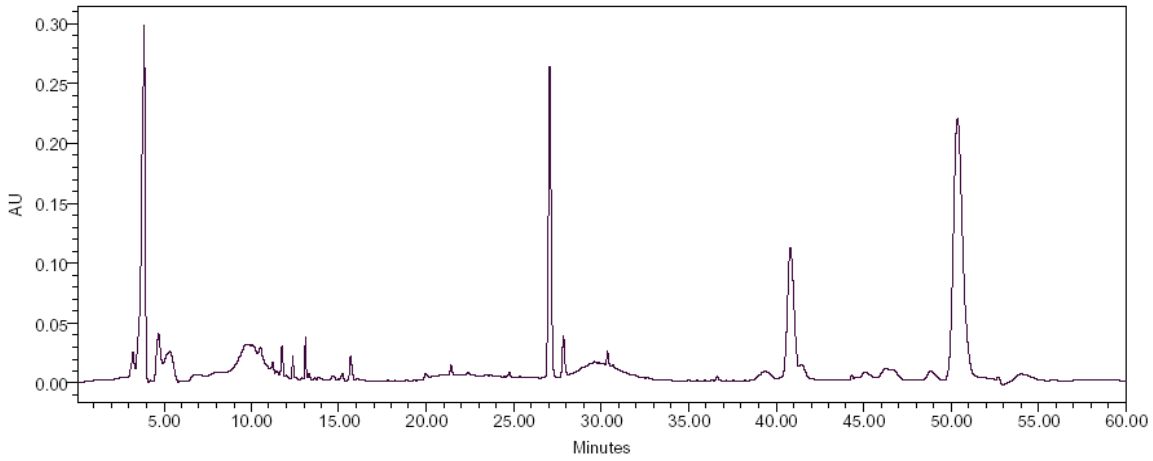


Fig. 3.15: HPLC profile for *H. salviifolia.*

Table 3.10: HPLC retention time and percentage area for peaks from *H. salviifolia.*

Retention Time	Absorbance maxima (nm)	% Area
3.2	201.6 and 283.9	1.2
3.8	206.3 and 278.0	22.7
4.6	207.4	2.4
11.7	219.1 and 278.0	0.8
12.3	206.3 and 268.5	0.4
13.0	222.7 and 275.6	0.8
15.6	206.3 and 343.2	0.9
27.0	266.2 and 313.5	14.8
27.8	266.2 and 312.3	1.6
30.3	267.4 and 312.3	0.3
40.8	216.8, 241.4 and 323.0	13.3
50.3	216.8, 241.4 and 323.0	40.2

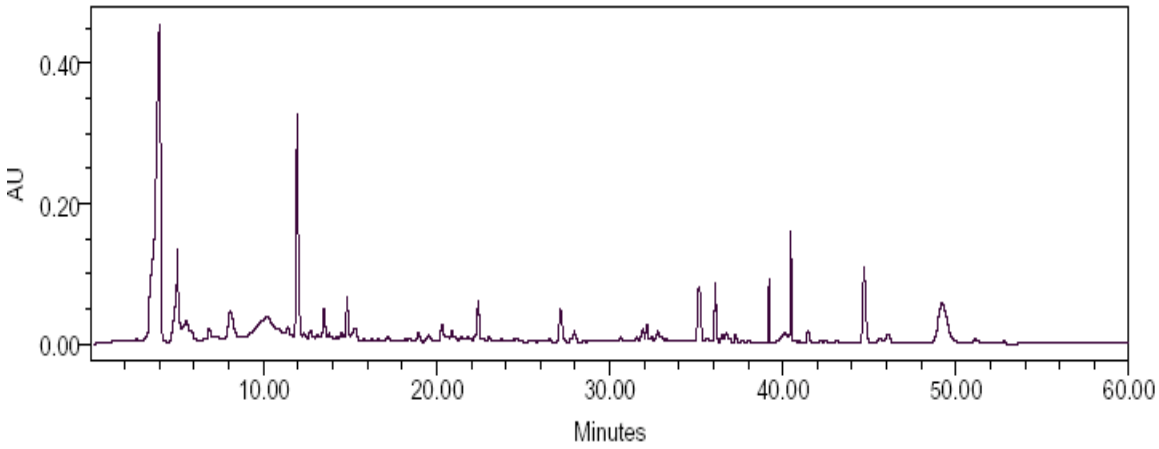


Fig. 3.16: HPLC profile for *H. scabra.*

Table 3.11: HPLC retention time and percentage area for peaks from *H. scabra.*

Retention Time	Absorbance maxima (nm)	% Area
3.9	202.8 and 259.1	40.7
5.	259.1	7.4
8.0	207.4	5.2
11.9	218.0 and 278.0	12.2
13.4	206.3, 285.1 and 327.8	2.1
14.8	206.3, 280.3 and 323.0	2.5
22.3	205.1 and 274.4	2.4
27.1	210.9, 266.2 and 313.5	2.7
35.1	235.6	4.5
36.0	234.4	2.9
40.4	237.9	3.3
44.7	267.4, 331.3, 446.1 and 475.1	5.2
49.2	219.1, 241.4 and 323.0	8.5

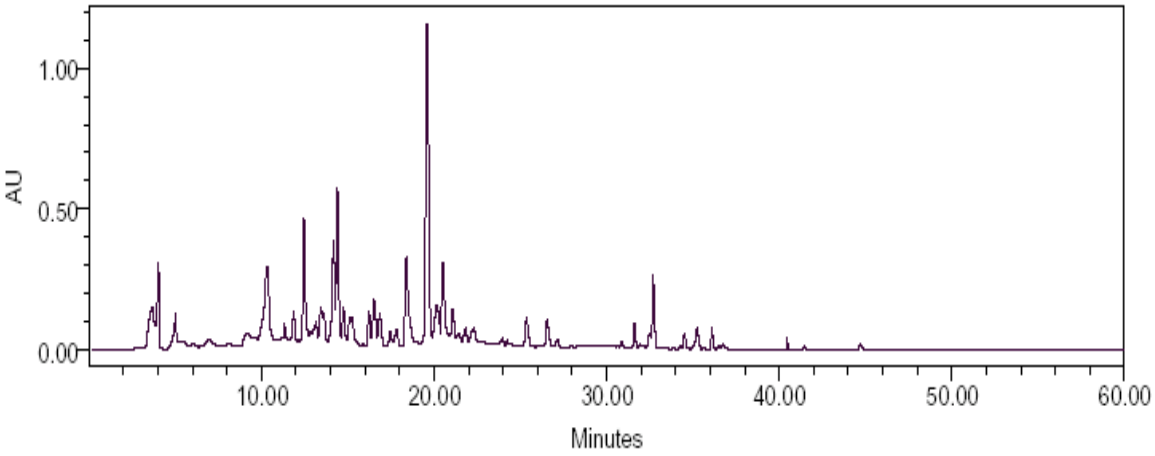


Fig. 3.17: HPLC profile for *H. trifurca.*

Table 3.12: HPLC retention time and percentage area for peaks from *H. trifurca.*

Retention Time	Absorbance maxima (nm)	% Area
3.6	265.0	5.8
4.0	205.1	4.4
10.2	202.8 and 289.8	12.7
12.4	206.3 and 269.7	8.0
14.1	201.6 and 266.2	7.0
14.3	201.6 and 265.0	9.3
16.5	206.3 and 274.4	3.6
18.3	202.8 and 276.8	8.2
19.5	247.3	24.4
20.1	206.3 and 336.1	5.3
20.4	205.1 and 243.8	6.9
32.6	234.4 and 331.3	3.8

Table 3.13: HPLC retention time and percentage area for peaks of isolated flavones.

Compounds	Retention Time	Absorbance maxima (nm)	% Area
Compound 2	31.9	278.0 and 334.9 (Flavone)	43.0
Compound 3	32.5	278.0 and 336.1 (Flavone)	

Peak analysis indicates that similar compounds are present in certain species. *H. althaeifolia*, *H. cuneifolia*, *H. incana*, *H .involucrata*, *H. lavandufolia*, *H. saccifera and H. scabra* all indicate a compound with the retention time of approximately 3.8 min which corresponds to the UV absorbance with maxima at 201.6 and 207.4 nm. In addition, *H. involucrata*, *H. lavandufolia*, *H. saccifera* and *H. scabra* contain a compound with retention time of approximately 13.5 min and the absorbance maxima of 202.8 and 278

nm. *H. involucrata* and *H. lavandufolia* indicate a compound with the retention time of 14.5 and absorbance maxima at 202.8 and 278 nm. *H. althaeifolia* and *H. saccifera* contain a compound, retention time 14.5 min and absorbance maxima at 207.4 and 278 nm.

H. saccifera alone portrays two compounds with retention times of 32.2 and 32.7 nm with maxima at 278.0 and 330.1 nm as well as 278 and 336.1 nm respectively (Fig 3.19). These compounds appear to occur in this species only. Analysis of flavone compounds isolated from *H. saccifera* occur at 31.9 and 32.5 min and indicate UV spectra that are very similar with peaks occurring at 278.0 and 334.9 nm and 278.0 and 336.1 nm respectively (Fig. 3.20).

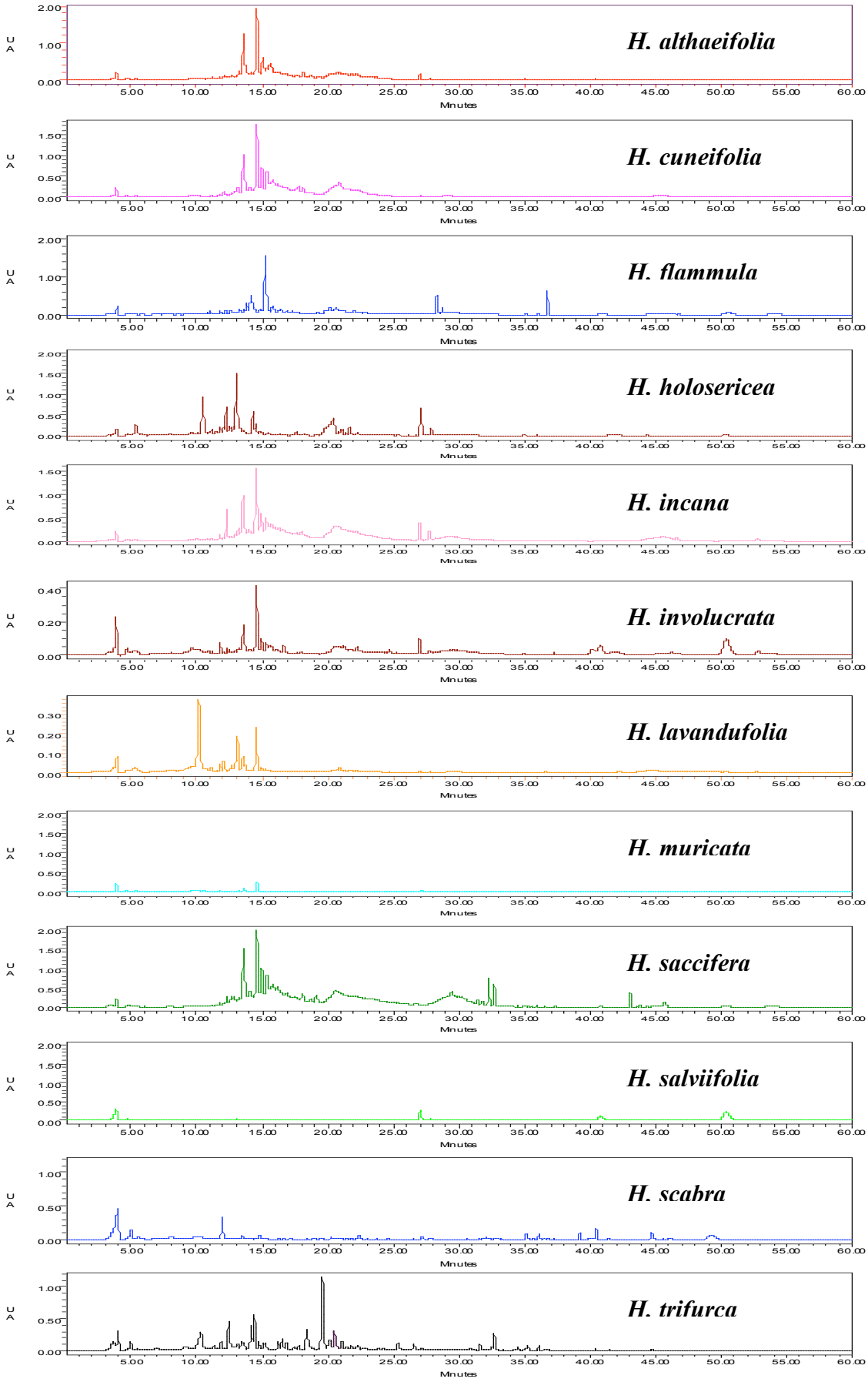


Fig. 3.18: HPLC profiles for all twelve species of *Hermannia*

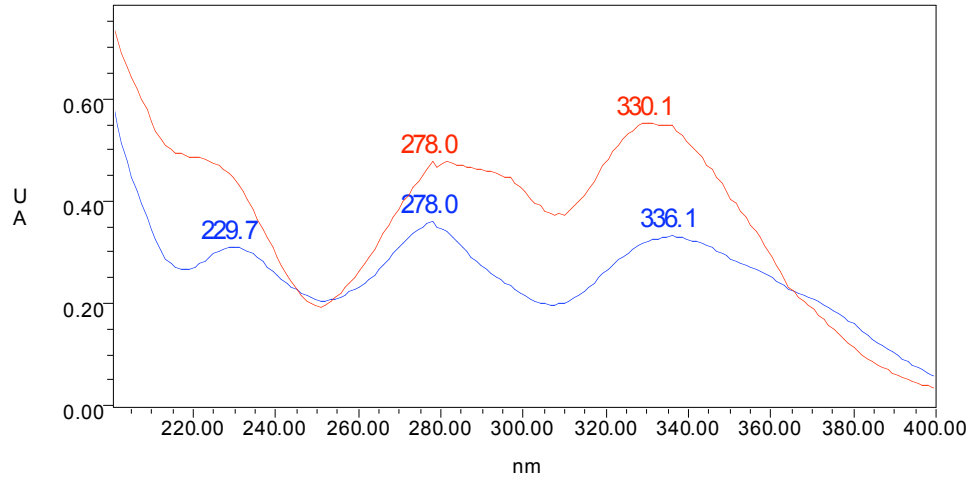


Fig. 3.19: UV spectra of compounds present in *H. saccifera* with retention times of 32.269 and 32.707 min.

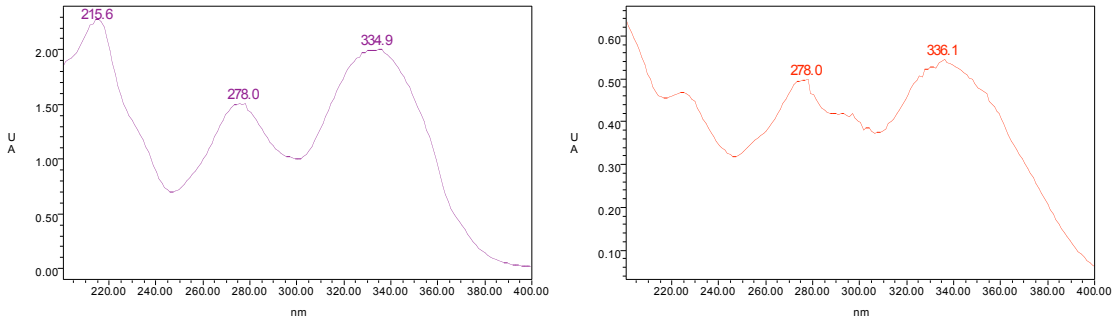


Fig. 3.20: UV spectra for flavone compounds, cirsimaritin (1) and 5,8- dihydroxy-6,7,4'-trimethoxyflavone.

3.4. DISCUSSION:

Thin layer chromatography analysis provided some insight into the chemical composition of the ten species of *Hermannia* being investigated. The profiles viewed under UV light were conservative indicating that many compounds contained in the extract are poor chromophores and are thus, unable to absorb UV light. Spraying with anisaldehyde reagent provided better insight into the phytochemical complexity of the species. In addition, both

isolated compounds (β-sitosterol and lupeol) could not be viewed under UV light while they were observed after spraying since they are also poor chromophores.

Remarkable similarity between the various species being investigated was seen (Fig. 3.1., 3.2. and 3.3.). This was confirmed in all TLC systems used. Different solvent systems may be used for different classes of compounds based on the polarity of organic solvent being used (Ahmad and Beg, 2001), and thus, three different TLC systems were used. All three systems indicated the similarity of compounds present indicating that there are many different classes of common compounds that are present in the species being investigated, indicating the possible taxonomic importance of these similar compounds.

TLC analysis, further, indicated that *H. saccifera*, while containing some of the common compounds, appears to be chemical anomalous to the other species. This distinct difference can bee seen in all TLC systems utilized. Further, antimicrobial bioautgrams indicated that these distinct compounds appear to be responsible for the good antimicrobial activity of *H. saccifera*, which is considerably greater than any of the other species (Chapter 5). Thus, isolation of these compounds was imperative to identify these interesting and possibly novel compounds that possessed promising biological activity.

β-sitosterol and lupeol were also present in all species investigated. This is not an unusual occurrence since these compounds are common in the plant kingdom. However, they have been shown to possess some biological activity (Awad *et al.,* 2004; Geetha and Varalakshmi, 2000; Ziegler *et al.,* 2004) and thus, may contribute to the pharmaceutical significance of these plants

The HPLC profiles appear to be very conservative and few compounds were detected through HPLC/UV. This suggests that many compounds contained within the plant are not

good chromophores i.e. that the compounds do not contain certain molecules that have the ability to absorb light. Thus, many interesting and possibly bioactive compounds would not have been detected and would, therefore, require further analysis. Isolation of compounds in Chapter 4 confirmed the presence of several compounds which are poor chromophores.

Common compounds elute at retention time of 3.8, 13.5 as well as two compounds at 14.5 min. The UV spectra obtained for these retention times confirm that these are similar compounds found in the different species. Although these compounds do not appear in all species, they may be present in trace amounts that would not have been detected on the HPLC/UV. These compounds may therefore be characteristic of the genus, thus indicating a chemotaxonomic potential for these compounds.

H. saccifera has two peaks at retention times of 32.2 and 32.7 nm and λ_{max} at 278.0 and 330.1 nm as well as 278 and 336.1 nm respectively. These λ_{max} correspond with that suggested for flavones since typical flavone UV spectra exhibit two major absorption peaks in the region 240-400 nm (Mabry *et al*., 1970). This corresponds with the two flavone compounds isolated from *H. saccifera* as described in Chapter 4, namely cirsimaritin and 5,8- dihydroxy-6,7,4'- trimethoxyflavone. These peaks do not appear to occur in the other species being analyzed as can be seen in the HPLC profiles displayed in Fig 3.18.

Considering the conservative nature of these HPLC profiles, it may be useful to conduct further HPLC analysis utilizing a different detector which would be able to analyze compounds that do not absorb UV light. One possible suggestion would be fluorescent detectors, which measure the ability of a compound to absorb then re-emit light at given wavelengths. Another detector that may be useful is a refractive index detector, which measures the ability of sample molecules to refract light (Parriott, 1993). Thus, although

the photodiode detector is a useful tool and is widely utilized, it is less useful in these plants as they do not contain many compounds that are good chromophores.

3.5. CONCLUSION:

TLC analysis has identified common compounds in the various species of *Hermannia* investigated which was further corroborated by the HPLC/UV profiles obtained. This aspect may be useful for taxonomic purposes for identification of species belonging to the genus, *Hermannia*. *H. saccifera* was shown to be chemically anomalous when compared to the other species, providing some insight into the extensive antimicrobial activity portrayed by the plant in comparison to the other selected species. *H. saccifera,* in addition, appears to be the only species investigated that contains the two isolated flavones, cirsimaritin and 5,8- dihydroxy-6,7,4'- trimethoxyflavone. Further, lupeol and β-sitosterol are common compounds in all species and may contribute to the biological activity of these plants. The conservative nature of the HPLC/UV profiles suggests the presence of many compounds that are poor chromophores and therefore, further analysis must be conducted using different detectors to provide a greater insight of the phytochemical composition of this genus.

3.6. REFERENCES:

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CHAPTER 4: ISOLATION OF COMPOUNDS FROM *HERMANNIA* SPECIES

4.1. INTRODUCTION:

Chemodiversity in Nature offers a valuable source for novel lead discovery. However, the discovery of drugs from Nature is complex. The biomass must be collected, dried and extracted into a suitable organic solvent to give an extract, which is then screened in a bioassay to assess biological activity. Active extracts are then fractionated using bioassay guided fractionation, in which chromatographic techniques are used to separate the extract into its individual components; the biological activity is checked at all stages until a pure active compound is obtained.

Although biological activity testing is on-going, structure elucidation is necessary to determine the three-dimensional structure of the active compounds. This is done through a variety of techniques such as nuclear magnetic resonance spectroscopy and mass spectrometry. This will enable a literature search to be done to establish whether a compound is novel, what chemical class it belongs to and whether that type of compound has previously been reported to possess biological activity in the bioassay of interest or other bioassays (Heinrich *et al*., 2004).

Nuclear magnetic resonance (NMR) is a spectroscopic technique that reveals information about the environment of magnetically active nuclei. Under proper conditions, such nuclei absorb electromagnetic radiation in the radio-frequency region governed by their chemical environment. This environment is influenced by chemical bonds, molecular conformations, and dynamic processes. By measuring the frequencies at which these absorptions occur and their strengths, it is usually possible to deduce facts about the structure of the molecule

being examined. NMR spectroscopy is commonly used in organic chemistry to elucidate molecular structures and conformations by studying ^{1}H and ^{13}C nuclei (Oehler, 2005).

The combination of gas chromatography (GC) for separation and mass spectrometry for detection and identification of the components of mixtures of compounds is an important analytical tool in the research and commercial laboratory. Gas chromatography involves the absorptive interaction between the components with a column packing which leads to differential separation of the components of the mixture, which are passed in order through a detector flow cell. The mass spectrometer ionizes injected material and focuses these ions and their fragmentation products through a magnetic mass analyzer, and then collects and measures the amounts of each of the selected ions in a detector (McMaster and McMaster, 1998). The data obtained when matched to a library and coupled with NMR forms an excellent basis for identifying compounds.

It is important to note that, by following only a bioactivity-guided fractionation procedure, isolation of known plant constituents with recognized activity may occur. Furthermore, interesting lead compounds, which do not exhibit the tested activity, will simply be missed. Thus, in order to avoid the time-consuming isolation of known constituents, many identification techniques are used at the earliest stage of isolation to detect compounds with interesting structural features and to target their isolation.

Given the complexity of the process described above, it is not surprising that many natural product leads fail to make their way onto the market. The expense, complexity and time of the natural drug lead process have influenced against natural products in the past, but the fact remains that natural products are a tried and tested source and there are many examples of compounds originally isolated from natural sources that are now produced

synthetically and are used pharmaceutically such as aspirin as well as many alkaloid compounds such as quinine, vinblastine and vincristine.

The most important strength of natural products is their complex chemistry and structural diversity (Heinrich *et al*., 2004). The phytochemistry of the genus, *Hermannia,* has not previously been investigated and thus, an important resource for compound diversity remains unexplored. In addition to producing possible novel biologically active compounds, the plants may provide interesting structural diversity which may be utilized as lead compounds for further research and enhancement of biological activity. The good biological activity of *H. saccifera* as portrayed in the antimicrobial assays (Chapter 5) suggested that this plant may be a source of interesting biologically active compounds and hence it was chosen for compound isolation. In addition *H. cuneifolia* and *H. salviifolia* were chosen due to their extensive use in traditional medicine.

4.2. METHOD:

4.2.1. General Methods

4.2.1.1. Nuclear Magnetic Resonance Spectroscopy (NMR Spectroscopy)

Nuclear magnetic resonance spectroscopy was performed on a 400MHz Varian UNITY-INOVA spectrophotometer. All spectra were recorded at room temperature in deuterated methanol (CD_3OD) and deuterated chloroform ($CDCl_3$). The chemical shifts were all recorded in parts per million (ppm) relative to TMS. For deuterated methanol, the spectra were referenced according to the central line $\delta_C = 49.0$ and $\delta_H = 4.80$ or $\delta_H = 3.30$. For deuterated chloroform, the spectra were referenced according to the central line at $\delta_C = 76.6$ and $\delta_H = 7.24$.

4.2.1.2. Mass Spectrometry (MS)

The compounds were run on a Finnigan 1020 GC-MS spectrometer using either injection or solid probe methods.

4.2.1.3. Optical Rotation

Optical rotation was determined using a Jasco DIP-370 digital polarimeter. Samples were dissolved in chloroform to a concentration of 2 mg/ml.

4.2.1.4. Infrared

Infrared spectra were recorded on a Bruker Vector 22 infrared spectrometer using the thin-film technique with chloroform as a solvent.

4.2.1.5. General Chromatography

Thin-layer and column chromatographic techniques were employed for the process of separation and isolation. Different sized columns were used in column chromatography, ranging from 1-5 cm in diameter, the size being dependant on the amount of sample available and the purification stage. Separation of the crude extract was carried out on a column using Macherey-Nagel Art. 815330 as well as Merck Art. 9385 silica gel. Final purification was found to be most successful when use was made of an open, 0.75 cm diameter Pasteur pipette column also packed with Merck Art. 9385 silica gel. All separations were carried out under gravity. Both the column and thin-layer chromatography made use of chemically pure hexane, dichloromethane, methanol, toluene and ethyl acetate (Rochelle Chemicals). Thin layer chromatography was carried out on 0.2 mm silica-gel, aluminium-backed plates (Macherey-Nagel Art. 818133 and Merck Art. 5554). The plates were analyzed under UV (254 and 366 nm) and then developed using anisaldehyde (Fluka) : conc. H_2SO_4 (Rochelle Chemicals) : methanol [1:2:97] spray reagent.

4.2.1.6. Dry Packing

This procedure is employed for extracts that do not dissolve in a relatively non-polar solvent. These, generally, refer to methanol extracts as the extract dissolved in methanol cannot be loaded onto a column as separation would not occur. Hence, dry packing is undertaken. The extract is dissolved in a minimal amount of methanol, after which the extract is mixed with silica gel until the extract is absorbed onto the silica gel. The result is a fine, dry powdery extract which is left to dry and then loaded onto the column.

4.2.2. Extraction and isolation of compounds from *H. saccifera*

The powdered plant material (500 g) was extracted using acetone (1.5 L) over a period of 48 hours at room temperature during which the solvent was removed and fresh solvent added three times. All extracts were combined and the extract was concentrated using a Büchi Rotavapor R-114 to yield 43.82 g of extract. This was applied to two TLC plates and developed in toluene: ethyl acetate (6:4). One plate was examined under UV and then developed using anisaldehyde : conc. H_2SO_4 : methanol [1:2:97] spray reagent while a bioautogram was produced for the other to identify antimicrobially active compounds. The extract (25 g) was then absorbed onto silica gel and dry packed onto a silica gel column (Φ 4 cm). Polarity based fractionation was undertaken using the following systems:

Fraction 1: hexane: dichloromethane (9:1)

Fraction 2: hexane: dichloromethane (9:1)

Fraction 3: dichloromethane: methanol (6:1)

Fraction 4: dichloromethane: methanol (6:1)

Fraction 5: methanol (100%)

Bioactivity was confirmed in fraction 3 and 15.09 g was dry packed onto a second silica gel (Φ 5 cm) column. Toluene: ethyl acetate (6:4) was utilized to subfractionate the extract

to produce 153 subfractions. Fractions 51-60 (0.638 g) indicated bioactivity and the sample was again dry packed onto a column (Φ 1 cm). The mobile phase used was a hexane: ethyl acetate step gradient. The fractions (5 ml each) eluted are according to the following solvent system:

Fractions 1-12: 5% ethyl acetate in hexane

Fractions 13-54: 10% ethyl acetate in hexane

Fractions 54-72: 15% ethyl acetate in hexane

Fractions 73-92: 20% ethyl acetate in hexane

Fractions 93-125: 30% ethyl acetate in hexane

Fractions 126-136: 50% ethyl acetate in hexane

Fraction 137: 100% ethyl acetate

Fraction 138: 100% methanol

Compound **1** was eluted in fractions 32-37, compound **2** was recrystalized from fractions 42-45, compound **3** was recrystalized from fractions 78-84 and compound **4** was eluted from fractions 108-125. Compounds **1** and **4** were purified using a dichloromethane: methanol step gradient system utilizing a Pasteur pipette column. Compounds **2** and **3** were washed with hexane to remove any remaining impurities (Fig. 4.1). Compound **4** was isolated in insufficient quantities to provide adequate data for identification.

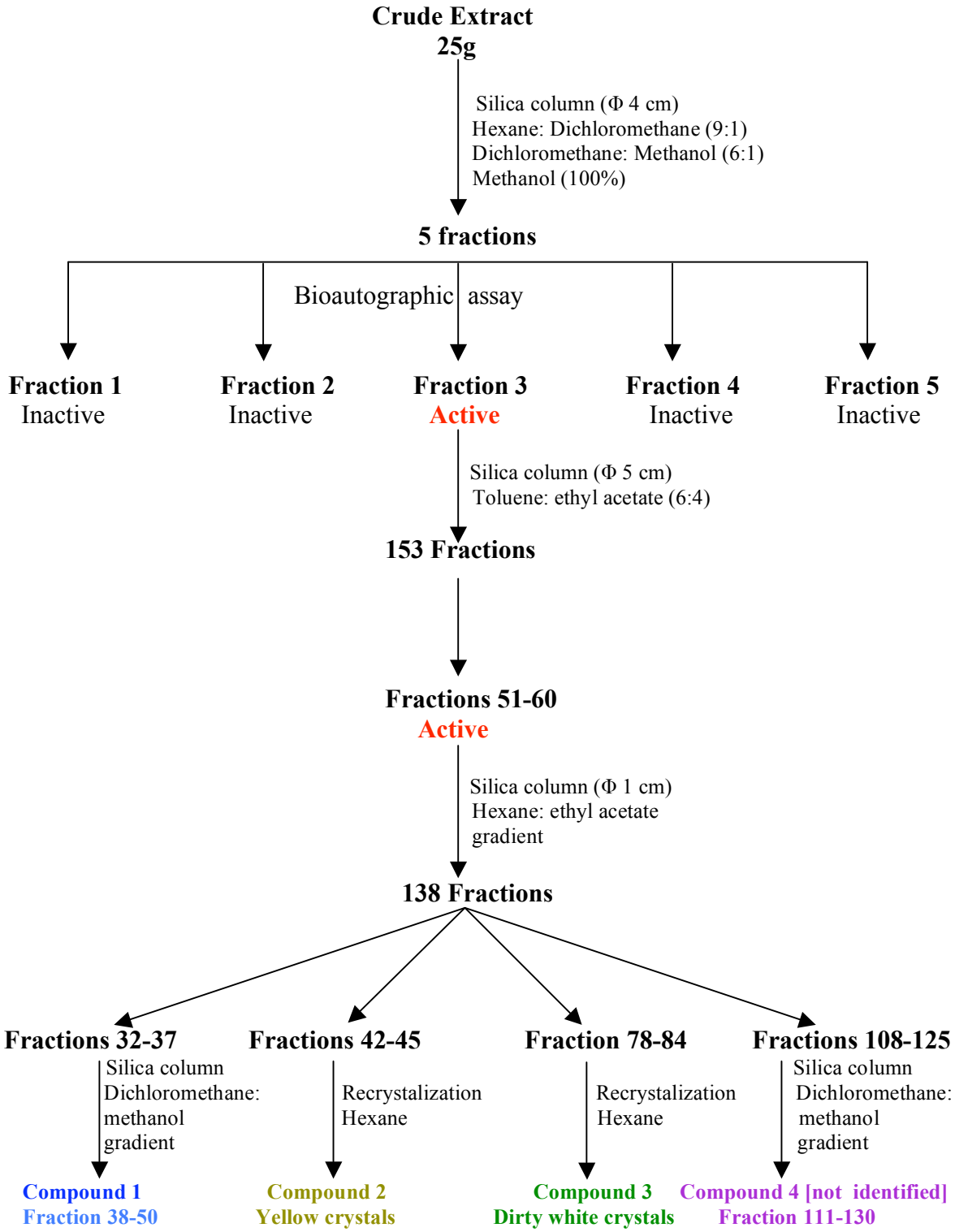


Fig. 4.1: Schematic representation of the purification steps for compounds isolated from *H. saccifera.*

4.2.3. Extraction and isolation of compounds from *H. cuneifolia* and *H. salviifolia*

4.2.3.1. *H. cuneifolia*

The powdered plant material (500 g) was extracted using acetone (1.5 L) over a period of 48 hours at room temperature during which the solvent was removed and fresh solvent added three times. All extracts were combined and the extract was concentrated using a Büchi Rotavapor R-114 to yield 20.30 g of extract. The extract was then absorbed onto silica gel and dry packed onto a silica gel column (Φ 4 cm). Polarity based fractionation was undertaken using the following systems:

Fraction 1: hexane: dichloromethane (9:1)

Fraction 2: hexane: dichloromethane (9:1)

Fraction 3: dichloromethane: methanol (6:1)

Fraction 4: Methanol (100%)

Fraction 5: Methanol (100%)

In an effort to isolate compounds found in the very polar methanol fraction (F5), the fraction (3.9 g) was rechromatographed on a silica gel column using a step gradient solvent system of dichloromethane: methanol. Fractions of 50 ml each were collected from the column to produce 469 subfractions. Each subfraction was then concentrated by evaporating off the solvent. Compound **5** was eluted from subfractions 5-6.

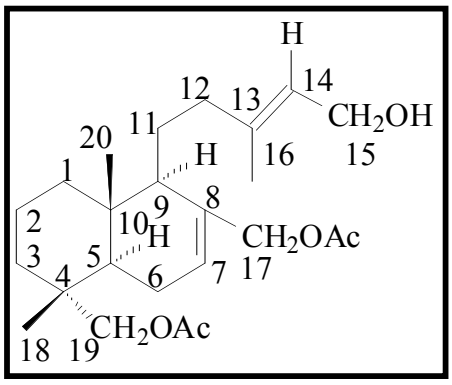
4.2.3.2. *H. salviifolia*

The powdered plant material (500 g) was extracted using 1.5 L of acetone over a period of 48 hours at room temperature during which the solvent was removed and fresh solvent added three times. All extracts were combined and the extract was concentrated using a Büchi Rotavapor. The non-polar compounds were partitioned into dichloromethane and the liquid extract was decanted to produce 7.91 g of dichloromethane extract. This was

then chromatographed on a silica gel column using the step gradient system of dichloromethane: ethyl acetate, again 50 ml fractions were collected to produce 333 fractions. Fraction 21 was rechromatographed on silica gel once again using the gradient system of dichloromethane: ethyl acetate. Subfractions of 1 ml were collected to produce 55 fractions. Compound **6** was eluted from fraction 50-55.

4.3. RESULTS AND DISCUSSION:

4.3.1. Identification of Compound 1, *E*-17, 19-diacetoxy – 15 – hydroxylabda - 7,13 - diene



[1]

The LRMS of compound **1** indicated a molar mass of 406 g mol^{-1}, which, in conjunction with the ^{1}H and ^{13}C NMR spectra (Appendix III), indicated a molecular formula of $C_{24}H_{38}O_5$. The molecule was found to be a labdane diterpenoid with three of the methyl groups being oxidized to primary alcohols, two of which had been further acetylated. The two acetate groups were indicated by two acetate methyl group proton resonances at δ 2.06 and δ 2.05 in the ^{1}H NMR spectrum. The remaining methyl groups occurred at δ 1.65, δ0.89 and δ 0.77. The ^{13}C NMR spectrum indicated the presence of two acetate carbonyl carbon resonances at δ 171.3, δ 170.9, two tri-substituted double bonds, and three oxymethylene carbons (δ 59.4, δ 67.5 and δ 72.7). The first double bond was placed in the 13, 14-position with C-13 (δ 139.9) showing correlations in the HMBC spectrum

(Appendix III) with the 2H-15 resonances which occurred as a broad doublet at δ 4.13. This resonance showed coupling in the COSY spectrum (Appendix III)with the H-14 resonance at δ 5.38 and the vinyl methyl group proton resonance at δ 1.65 which was assigned to 3H-16.

The two methine carbon resonances at δ 44.0 and δ 51.7 were assigned to C-5 and C-9 respectively. The HMBC spectrum showed correlations between the C-5 resonance and the 3H-20 (δ 0.77) methyl group proton resonance, as well as the methyl group proton resonance at C-4 (δ 0.89). Further correlations were seen between C-5 and the pair of oxymethylene protons at δ 3.67 and δ 3.76. This indicated that one of the methyl groups at C-4 had been oxidized. The C-9 resonance showed correlations in the HMBC spectrum with the 3H-20 methyl group proton resonance (δ 0.77) and the third oxymethylene proton resonance which occurred as a pair of doublets at δ 4.42 and δ 4.56. These were assigned to the 2H-17 oxymethylene protons at C-8. The C-5 resonance showed coupling in the COSY spectrum with the 2H-6 resonance at δ 1.96, which was, in turn, seen to be coupled to the H-7 resonance at δ 5.75. Thus the second double bond was placed at C-7, and the fully substituted resonance at δ 133.9 was assigned to C-8. The HMBC spectrum indicated correlations between the acetate carbonyl resonances and the 2H-17 resonance and the oxymethylene group attached at C-4. Hence acetate groups were placed at these positions and a primary hydroxyl group was present at C-15.

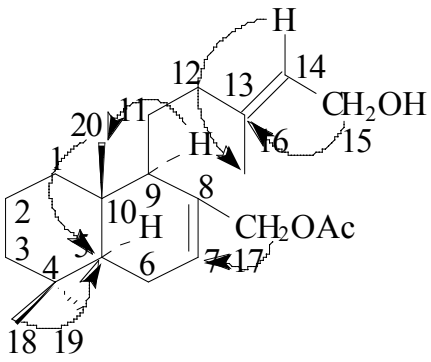


Fig. 4.2: HMBC correlations for Compound **1**

A correlation in the NOESY spectrum (Appendix III) between the 2H-15 proton resonance and the 3H-16 resonance indicated they were *cis* to each other. A correlation between the 3H-20 methyl group proton resonance and the methyl group at C-4, indicated, the methyl group at C-4 was in the β-configuration, and hence the acetylated oxymethylene group was α. The optical rotation of $[\alpha]_D$ = + 5.2632 (chloroform) indicated that the compound belonged to the normal labdane series. (Zdero *et al.,* 1991). Thus the structure was determined to be *E*-17, 19-diacetoxy – 15 – hydroxylabda - 7,13 - diene.

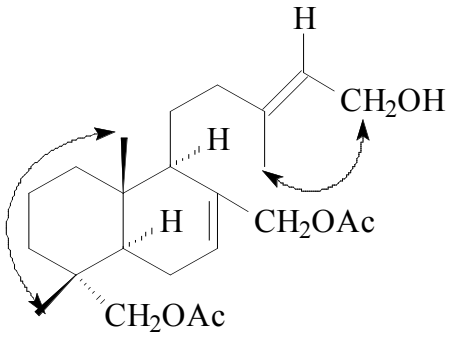


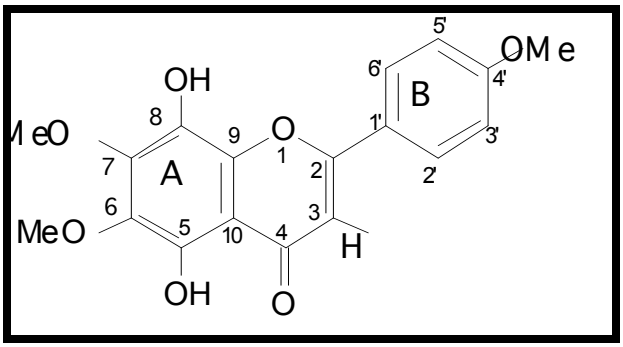
Fig. 4.3: NOESY correlations for Compound **1**

This compound is a novel compound which has not been previously isolated. The novel nature of the compound suggests that the compound should be investigated for numerous bioactivities to determine its potential use in the treatment of disease. In addition since it has been isolated from *H. saccifera,* it would be interesting to note its contribution to the healing ability of the plant when utilized in traditional medicine. Due to the limited quantity of material available for investigation only the antimicrobial activity of the compound was explored as described in Chapter 5.

Table 4.1: ^{1}H, ^{13}C, COSY, NOESY, HMBC correlations for Compound **1** ($CDCl_3$).

Carbon	**^{1}H**	**^{13}C**	**HMBC**	**COSY**	**NOESY**
1	1.82, 1.86	38.3			
2	0.86, 1.51	17.8			
3	1.38	35.9			
4	-	36.5/36.3			3H-20
5	1.46 dd	44.0	3H-20	2H-6	
6	1.96 m 2H	23.8		H-7	
7	5.75 bs	128.2			
8	-	133.9			
9	1.82 m	51.7	3H-20, 2H-17		
10	-	36.5			
11	1.57, 1.30	24.9			
12	2.10, 1.95	41.2			
13	-	139.9	2H-15	H-14, 3H-16	
14	5.38 bt	123.6			
15	4.13 bd	59.4			3H-16
16	1.65 s	16.3			2H-15
17	4.42 d 12.6 4.56 d 12.6	67.5			
18	0.89 s	17.6			
19	3.67 d 10.9 3.76 d 10.9	72.7			
20	0.77 s	14.0			
Acetatate C=O	-	171.3, 170.9			
Acetate CH_3	2.06, 2.05	21.2, 21.1			

4.3.2. Identification of Compound 2, 5,8- dihydroxy-6,7,4'- trimethoxyflavone



[2]

The mass spectrum of compound **2** showed a M^+ peak at *m/z* 344. This was consistent with the molecular formula of $C_{18}H_{16}O_7$.

The ^{1}H NMR spectrum (Appendix IV) showed a pair of doublets at δ 7.83 (J = 8.7) and δ 6.96 (J = 8.7). These were attributed to the *para*-disubstituted proton of ring-B, H-2'/6' and H-3'/5' respectively.

The ^{1}H NMR spectrum also showed a single proton resonance at δ 6.57 which was attributed to H-3. Three methoxy group singlet resonances were seen at δ 4.09, δ 3.95 and δ 3.93. These resonances were typical of those of a flavone-type structure (Fig. 4.4).

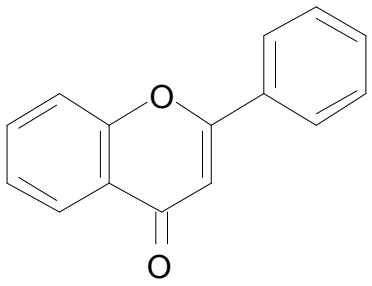


Fig. 4.4: Typical flavone-type structure.

The ^{13}C NMR spectrum (Appendix IV) showed the presence of eighteen carbon resonances which were resolved using the HSQC spectrum into three methyl and five methine carbon

resonances. The ^{13}C NMR spectrum also showed the presence of a carbonyl carbon resonance at δ 183.1.

The COSY spectrum (Appendix IV) showed the typical coupling between the *para*-disubstituted protons of H-2'/6' and H-3'/5'. The NOESY spectrum (Appendix IV) showed a spatial correlation between H-2'/6' and the resonance at δ 6.57 which indicated that this resonance was H-3. Another correlation was seen between H-2'/6' and the methoxy group at δ 3.95 and therefore the methoxy group was assigned to H-4'. In addition, a NOESY correlation was seen between the methoxy groups at δ 4.09 and δ 3.93 which implied that these were adjacent methoxy groups.

The positions of the methoxy groups were confirmed by reference to literature which indicated that for hydroxyflavones whose NMR spectra are in $CDCl_3$, the methoxy group signals at the 7-position were exhibited at a fairly low field shift in the range of 4.12 – 4.16 (Horie *et al*., 1995). Thus the methoxy group at δ 4.09 was assigned as C-7. Further, Horie *et al*. (1995) indicated that although the difference between the 6- and 7- methoxy group signals were similar to that between 7- and 8- methoxy, the 6-methoxy group is more affected that the 8-methoxy group by the solvent such that the difference between the 6- and 7- methoxy signals (Δδ 0.11 – 0.16) are larger than that between 7- and 8- methoxy (Δδ 0.02 – 0.06). In this case, the difference was Δδ 0.16, thus confirming that the second methoxy group most likely occurred at C-6 rather than C-8. Biosynthetically, these compounds always have an oxygenated substituent at C-5 and C-7 and hence a hydroxyl group was placed at C-5. This was confirmed by the ^{1}H NMR spectrum which showed a broad singlet resonance at δ 12.52 which indicated the presence of a hydroxyl group at C-5.

The HMBC spectrum (Appendix IV) showed a ^{3}J correlation between the carbonyl carbon resonance and H-3, as expected. The methoxy group resonance at δ 3.95 was assigned to C-4' because of HMBC correlations between the carbon resonance C-4' (δ 159.3) and the methoxy group protons as well as to H-2'/6' and H-3'/5'. A hydroxy group was assigned to C-8 to complete the fully-substituted ring-A. All other assignments of the quaternary carbons such as C-2, C-10 and C-1' were confirmed by HMBC correlations and are listed in Table 4.2 and are indicated in Fig 4.5. below.

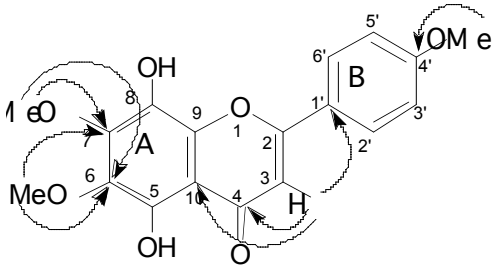


Fig. 4.5: HMBC correlations for Compound 2.

A literature search revealed that compound **2** was 5,8- dihydroxy-6,7,4'-trimethoxyflavone (Horie *et al*., 1995). The correlations are listed in Table 4.2 and serve to confirm the structure.

5,8-Dihydroxy-6,7,4'-trimethoxyflavone has previously been isolated from the genus *Ocimum* (Grayer *et al.*, 2001) as well as *Nepeta* (Lamiaceae) (Jamzad *et al.,* 2003). This compound has the unusual 5,8-dihydroxy-6,7-dimethoxy A-ring substitution pattern that may provide valuable and characteristic chemotaxonomic information. It is an aglycone 'external flavonoid' that is lipophilic in nature. They are amongst the rarely occurring flavonoids but this may be due to their disintegration which occurs during the process of isolation (Jamzad *et al.,* 2003). The external flavonoids are especially common in species which grow in the wild arid and semi-arid regions, and are presumably present to provide the plant protection against harmful UV radiation (Grayer *et al.,* 1996). These chemically

unstable surface flavones with an 8-hydroxylated group may be stored in some plants as the more stable 8-*O*-glycosides. The immediate precursor of 5,8-dihydroxy-6,7,4'-trimethoxyflavone is probably the 8-deoxy derivative salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone) (Grayer *et al.*, 2001) (Fig. 4.6)

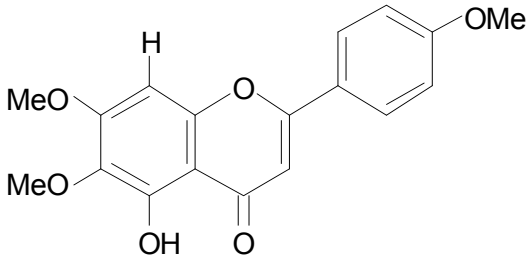
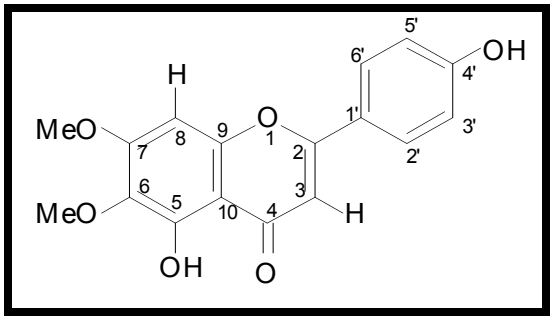


Fig. 4.6: Structure of Salvigenin, probable precursor of 5,8-dihydroxy-6,7,4'-trimethoxyflavone.

Table 4.2: ^{1}H, ^{13}C, COSY, NOESY, HMBC correlations for Compound **2** ($CDCl_3$) as well as literature values in $CDCl_3$ (Horie *et al.,* 1995)

	^{1}H	^{1}H Lit	^{13}C	HMBC	COSY	NOESY
1						
2			162.1	H-3, H-2'/6'		
3	6.57*s*	6.58*s*	103.7			H-2'/6'
4			183.1	H-3		
5			145.7			
6			137.0	6-OMe		
7			153.0	7-OMe		
8			130.0			
9			149.5			
10			106.9	H-3		
1'			123.5	H-3, H-3'/5'		
2'6'	7.83*d*	7.91*d*	128.4		H-3'/5'	H-3,H-4',H-3'/5'
3'5'	6.97*d*	7.02*d*	116.2		H-2'/6'	H-2'/6'
4'			159.3	H-3'/5', H-2'/6'		H-2'/6'
5-OH	12.4*s*	12.34*s*				
8-OH						
6-OMe	3.93*s*	3.98*s*	61.15			H-7-OMe
7-OMe	4.09*s*	4.14*s*	61.7			H-6-OMe
4'-OMe	3.95*s*	3.89*s*	62.2			

4.3.3. Identification of Compound 3, cirsimaritin



[3]

The mass spectrum of compound **3** showed a M^+ peak at *m/z* 314. This was consistent with the molecular formula of $C_{17}H_{14}O_6$.

The ^{1}H NMR spectrum (Appendix V) showed a pair of doublets at δ 7.89 (*J* = 8.9) and δ 6.93 (*J*=8.9). These were attributed to the *para*-disubstituted protons of ring-B, H-2'/6' and H-3'/5' respectively. The ^{1}H NMR spectrum, in addition showed single proton resonances at δ 6.81 and δ 6.65 which were attributed to H-8 and H-3 respectively. These were typical of the splitting pattern for a flavone-type structure. Two proton methoxy group singlet resonances were seen at δ 3.97 and δ 3.82.

The ^{13}C NMR spectrum (Appendix V) indicated the presence of seventeen carbon resonances which were resolved using the HSQC (Appendix V) into two methyl and six methine carbon resonances. The HSQC spectrum correlated the proton resonances to their corresponding carbons. The ^{13}C NMR spectrum also showed the presence of a carbonyl carbon resonance at δ 184.2.

The COSY spectrum (Appendix V) showed typical coupling between the *para*-disubstituted protons of H-2'/6' and H-3'/5'. The NOESY spectrum (Appendix V) showed a spatial correlation between H-2'/6' and the resonance at δ 6.65, therefore this resonance was attributed to H-3. Another correlation was seen between the single proton resonance

on the A-ring and one of the methoxy groups. This proton was assigned as H-8 by the HMBC (Appendix V) correlation seen between C-9, C10 and C-7 to this proton at δ 6.81.

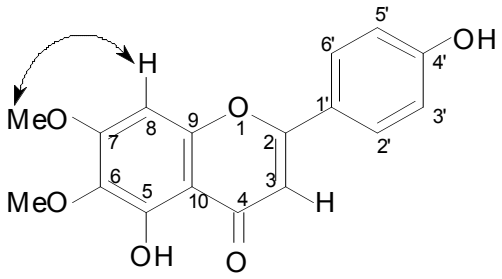


Fig. 4.7: NOESY correlations for Compound **3**.

The positions of the methoxy groups at C-6 and C-7 were also confirmed using the HMBC spectrum. The C-6 carbon resonance (δ_C 132.4) showed a HMBC correlation to the methoxy group attached at this position as well as a ^4J correlation to H-8. The C-7 carbon resonance at δ 160.6 showed a HMBC correlation to its corresponding methoxy group substituent as well as to H-8. If a methoxy group was placed at C-5, there would be no correlation to the A-ring proton. Biosynthetically, these compounds always have an oxygenated substituent at C-5 and C-7 and hence a hydroxyl group was placed at C-5. The presence of the hydroxyl at C-5 could not be confirmed on the ^1H NMR spectrum as the deuterated methanol was used as a solvent.

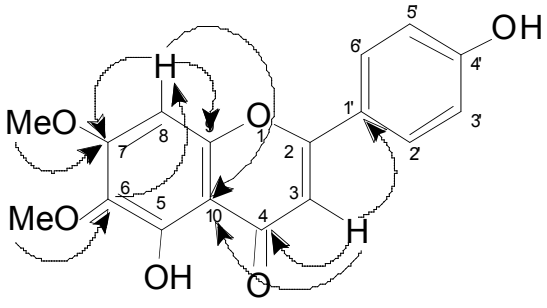


Fig. 4.8: HMBC correlations for Compound **3**.

A literature search revealed that Compound **3** was 5,4'-dihydroxy-6,7-dimethoxyflavone (Hase *et al*., 1995) also known as cirsimaritin. All correlations are listed in Table 4.3 and

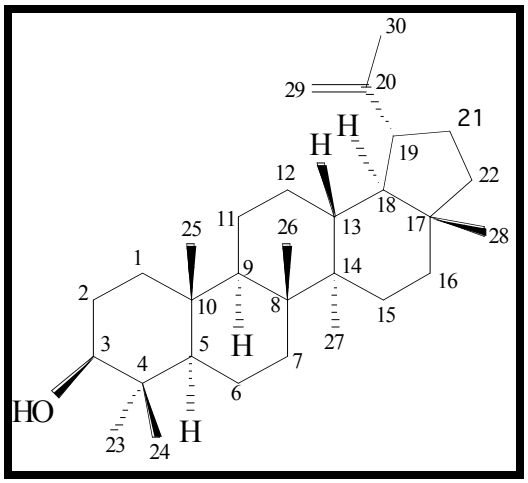
serve to confirm the structure. Differences in data from that of the literature are due to different solvents being utilized when NMR spectroscopy was conducted.

Cirsimaritin is also one of the so called ‘external flavonoids’ or ‘surface flavonoids’. These compounds are aglycones and are, therefore, lipophilic constituents. They are not as universal in occurrence in higher plants as the more polar flavonoid glycosides, which occur in the vacuoles in the plant cells. They are therefore useful as taxonomic characters at various levels of classification (Jamzad *et al.*, 2003). Further, cirsimaritin has also been indicated as being one of the main effective antioxidant phenolic compounds (Santos-Gnomes *et al.*, 2002), which will be considered in Chapter 6. Additionally, these lipophilic flavonoids may protect plants against infection by microorganisms, as several have been shown to have antibacterial or antifungal activities (Grayer *et al.,* 1996).

Table 4.3: ^{1}H, ^{13}C, COSY, NOESY, HMBC correlations for Compound **3** (CD_3OD) as well as literature values in $CDCl_3$ (Hasrat *et al*., 1997).

	^{1}H	^{1}H Lit	^{13}C	^{13}C Lit	HMBC	COSY	NOESY
1							
2			166.7	164.0	H-3, H-2'/6'		
3	6.65*s*	6.85*s*	103.8	102.6			H-2'/6'
4			184.0	182.1	H-3		
5			169.3	152.6			
6			132.4	131.9	6-OMe,H-8		
7			160.6	158.6	7-OMe, H-8		
8	6.82*s*	6.93*s*	92.3	91.5			7-OMe
9			152.4	152.0	H-8		
10			105.1	105.1	H3, H8		
1'			123.1	121.0	H-3		
2'6'	7.89*d*	7.96*d*	129.6	128.4		H-3'/5'	H-3, H-3'/5'
3'5'	6.93*d*	6.94*d*	117.1	115.9		H-2'/6'	H-2'/6'
4'			162.9	161.3	H-2'/6'		
6-OMe	3.82*s*	3.74*s*	61.1	60.0			
7-OMe	3.97*s*	3.93*s*	57.0	56.4			H-8

4.3.4. Identification of Compound 5, lupeol



[5]

The ^{1}H NMR spectrum (Appendix VI) suggested that a lupane structure was present. Two broad singlets at δ 4.55 and δ 4.67 (2H, brs, 2 x H-29) were due to the non-equivalent methylene protons at C-29. This was confirmed by the ^{13}C NMR spectrum (Appendix VI) which showed signals at δ 109.3 and δ 150.9 due to the C-29 and C-20 carbons of the double bond. A downfield shifted methyl proton resonance at δ 1.66 in the proton spectrum indicated a vinyl methyl group in the proton spectrum and this resonance was assigned to 3H-30. Six tertiary methyl group proton resonances occurred at δ 0.74, 0.77, 0.81, 0.92, 0.95 and 1.01 and were due to H-24, H-28, H-27, H-23 and H-26 respectively.

Also present in the proton spectrum was a broad doublet at δ 3.16 ascribed to H-3 and indicating the presence of a β-hydroxyl group at C-3. Deshielding caused by the oxygen atom of the hydroxyl group at C-3 shifted the methane signal downfield to δ 79.02 in the carbon spectrum.

The ^{13}C NMR spectrum showed the presence of 30 carbon atoms, which supported the proposed structure. The methine group at δ 55.3 was ascribed to C-5 and the fully

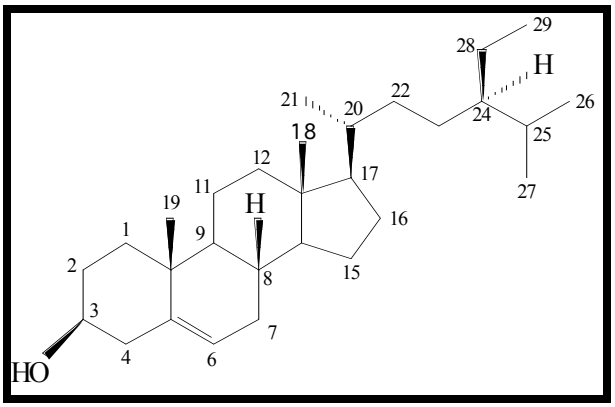
substituted carbon at δ 38.8 was ascribed to C-4 and are characteristic of a triterpenoid with the two methyl groups attached at C-4.

The compound **5** isolated was thus found to be 3β-hydroxylup-20(29)-ene, which is commonly known as lupeol. This is a triterpenoid which is common in nature and has previously been isolated from many plant species including Asteraceae (Akihisa *et al.* 1996*)*, *Euclea divinorum* (Mebe *et al.,* 1998)*, Crataeva nurvala* (Geetha *et al.* 1998) as well as *Pimenta racemosa* var. *ozua* (Fernándes *et al.,* 2001). Thus, the data for lupeol was assigned by comparison with literature data (Aratanechemuge *et al.,* 2004)**.** It has been shown to possess anti-inflammatory (Geetha and Varalakshmi*,* 2001) as well as antimicrobial and cytotoxic activity (Ajaiyeoba *et al.,* 2003). Thus, this compound was evaluated for activity against the 5-lipoxygenase enzyme (Chapter 7) as well as antioxidant activity (Chapter 6)

Table 4.4: ^{1}H, ^{13}C NMR spectral data for Compound **5** ($CDCl_3$) as well the literature values in CD_3OD (Aratanechemuge *et al.,* 2004).

Carbon Number	^{1}H	^{1}H Lit in CD_3OD	^{13}C	^{13}C Lit in CD_3OD
1		1.65	38.7	38.7
2		1.59	27.4	27.4
3	3.16 *dd*	3.20 *dd*	79.0	79.0
4			38.8	38.8
5		0.68	55.3	55.3
6		1.40	18.3	18.3
7		1.32	34.2	34.3
8			40.8	40.8
9		1.29	50.4	50.4
10			37.1	37.1
11		1.20	20.9	20.9
12		1.07	25.1	25.1
13		1.68	38.0	38.1
14			42.8	42.8
15		1.00	29.7	27.4
16		1.37	35.5	35.6
17			43.0	42.9
18		1.37	48.3	48.3
19		2.38	47.9	47.9
20			150.9	150.9
21		1.37	29.8	29.8
22		1.37	40.0	39.9
23	0.95 *s*	0.97 *s*	28.0	27.9
24	0.74 *s*	0.76 *s*	15.3	15.4
25	0.81 *s*	0.83	16.1	16.1
26	1.01 *s*	1.03	15.9	15.9
27	0.92 *s*	0.94	14.5	14.5
28	0.77 *s*	0.79	18.0	17.9
29	4.55 *brs,* 4.67 *brs*	4.54 *brs*, 4.67 *brs*	109.3	109.3
30	1.66 *s*	1.68s	19.3	19.3

4.3.5. Identification of Compound 6, β-sitosterol



[6]

Comparison of the ^{1}H NMR spectrum (Appendix VII) of Compound **6** with that of library spectra suggested that this compound was β-sitosterol. The ^{13}C NMR spectrum (Appendix VII) showed an oxymethine carbon resonance occurring at δ 71.8 and this was typical for a carbon atom attached to an oxygen. This was therefore assigned to C-3. The fully substituted carbon resonance at δ 141.0 indicated the presence of one trisubstituted double bond and these resonances were assigned to C-5 and C-6 respectively.

A multiplet at δ 5.32 in the ^{1}H NMR was assigned to the H-6 proton. Another multiplet at δ 3.50 could be ascribed to H-3α, with a hydroxyl group attached at C-3β. Chemical shifts for the methyl group protons correlated with the literature values and were assigned as follow: δ 0.66 (3H, s, H-18), δ 0.78 (3H, d, *J*=7.2, H-29), δ 0.91 (3H, d, J=6.4, H-21), δ 0.99 (3H, s, H-19).

This compound is a common triterpenoid and is quite widely distributed within the plant kingdom.

4.4. CONCLUSION:

Three compounds have been isolated from the previously undescribed species, *Hermannia saccifera*. These have been identified as two flavones, 5,8- dihydroxy-6,7,4'-trimethoxyflavone and cirsimaritin, as well as one diterpene, E-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene. The presence of leaf surface flavonoids may prove useful as chemotaxonomic indicators for the genus. In addition, two known compounds, lupeol and β-sitosterol have been isolated from *H. cuneifolia* and *H. salviifolia* respectively. Although these compounds are fairly prominent throughout the plant kingdom, this is the first isolation of them from *Hermannia* species. All isolated compounds may possess biological activity and thus, may be contributors to the healing ability of *Hermannia* species and hence, their individual activities in certain assays were determined as detailed in forthcoming chapters.

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CHAPTER 5: ANTIMICROBIAL ACTIVITY

5.1. INTRODUCTION:

A healthy person lives in harmony with the microbial flora that help protect them from invasion by pathogens (Beers and Berkow, 1999). An infection is a disease caused by a pathogen. It is the presence of a replicating organism, associated with tissue damage that defines a condition as an infection (Bannister *et al.*, 2000).

When penicillin became widely available during the Second World War, it was a medical miracle, rapidly vanquishing the biggest wartime killer - infected wounds. Discovered initially by a French medical student, Ernest Duchesne, in 1896, and then rediscovered by Scottish physician Alexander Fleming in 1928, the product of the soil mold *Penicillium* crippled many types of disease-causing bacteria. But just four years after drug companies began mass-producing penicillin in 1943, resistant microbes began to appear (Lewis, 1995).

The development of resistance in pathogenic bacteria is the greatest threat to the use of antimicrobial agents for therapy of bacterial infections. Since the introduction of penicillin, the discovery of each new antimicrobial compound has been followed by emergence of antimicrobial resistance (Aarestrup, 1999). Microorganisms can adapt to environmental pressures in a variety of effective ways, and their response to antibiotic pressure is no exception, with resistance occurring via the following mechanisms (Cloete, 2003):

- limited diffusion of antimicrobial agents through the biofilm matrix,
- interaction of antimicrobial agents with the biofilm matrix (cells and polymer),
- enzyme mediated resistance,
- level of metabolic activity within the biofilm,
- genetic adaptation,
- efflux pumps and,
- outer membrane structure.

An inevitable consequence of antimicrobial usage is the selection of resistant microorganisms. Overuse and inappropriate use of antibiotics has fueled a major increase in prevalence of multi-drug resistant pathogens, leading some to speculate that we are nearing the end of the antibiotic era. Unfortunately, as the need has grown in recent years, development of novel drugs has slowed. Thus, pending identification of new compounds, it seems that over the next decade we will have to rely on currently available families of drugs (Katzung, 2001).

The ability of plants to produce diverse chemical compounds that are frequently used for the defense of the plant suggests that new, innovative compounds that possess antimicrobial activity may be found in these plants. Many traditionally used plants are utilized for diseases associated with infection and, thus, it is not unreasonable to assume that some of these plants may contain novel compounds that are so desperately needed to ensure that medicines will be available for future generations, when resistance to the currently available antibiotics has overruled any use of those life-saving drugs.

5.2. METHOD:

Two methods were utilized in the determination of antibacterial activity for the twelve species of *Hermannia* i.e. minimum inhibitory concentration as well as the death kinetics assay. Isolation of active compounds was directed by a bioautographic assay. Acetone extracts were used for all assays.

5.2.1. Minimum inhibitory concentration assay

5.2.1.1. Principle of the method

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. They are considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials (Andrews, 2001). The method investigates the ability of the microorganisms to produce visible growth in broth containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent that, under *in vitro* conditions, prevents the appearance of visible growth of a microorganism within a defined period of time is recorded as the MIC (EUCAST, 2003). Tetrazolium salts are used to indicate biological activity because the colorless compounds acts as an electron acceptor and is reduced to a colored product (maroon) by biologically active organisms (Eloff, 1998).

5.2.1.2. Protocol

A 96 well, sterile microculture plate was used to quantitate the antimicrobial activity as described by Eloff (1998) (Fig 5.1). The test solution (100 µl) was serially diluted 50 % with water and 100 µl of a three hour culture grown at 37°C in Mueller-Hinton broth was added to each well. The covered microplates were then, incubated overnight at 37°C. Eight cultures were used to ascertain the range of activity exhibited by *Hermannia* species:

- *Staphylococcus aureus* (ATCC 12600) (Gram-positive)
- *Staphylococcus epidermidis* (ATCC 2223) (Gram-positive)
- *Enterococcus faecalis* (ATCC 29212) (Gram-positive)
- *Bacillus cereus* (ATCC 11778) (Gram-positive)
- *Klebsiella pneumoniae* (NTCC 9633) (Gram-negative)
- *Pseudomonas aeruginosa* (ATCC 9027) (Gram-negative)
- *Candida albicans* (ATCC 1023) (Yeast)
- *Cryptococcus neoformans* (ATCC 901) (Yeast)

To indicate bacterial growth, 40 µl of *p*-iodonitrotetrazolim violet (INT) dissolved in water was added to all microwells and then incubated at 37°C for 6 hours after which the lowest concentration at which inhibition of the micro-organism occurred was recorded. The assay was conducted with $N \geq 2$. The control utilized was ciprofloxacin for the bacteria while Amphotericin B was used for the yeasts. Controls were used to determine the sensitivity of micro-organisms.

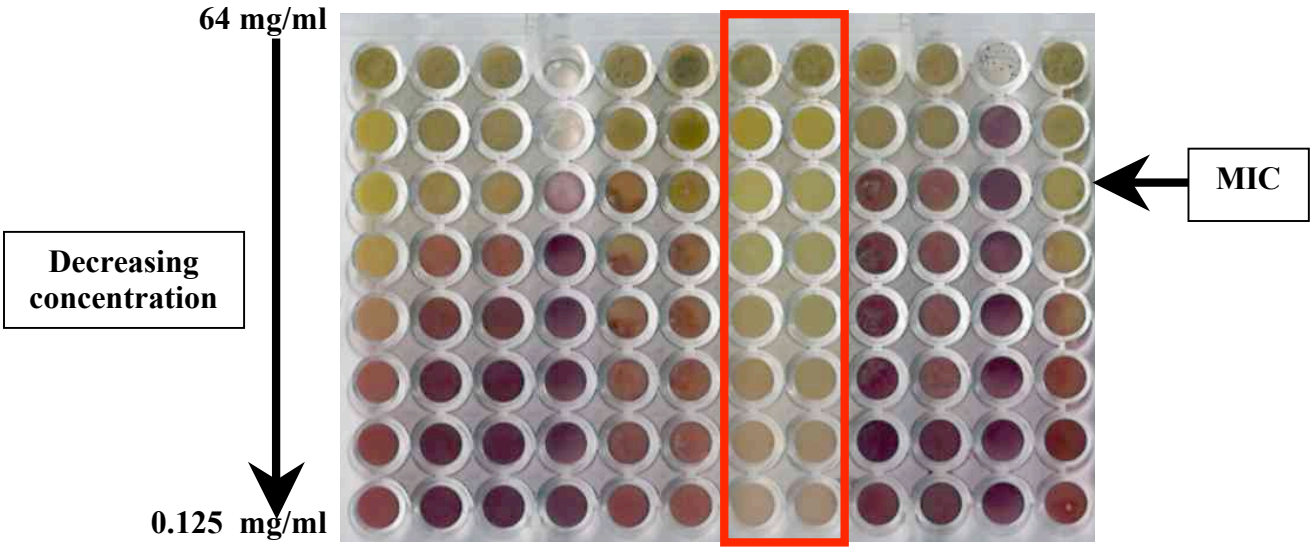

Fig. 5.1: Example of MIC plate. Maroon wells indicate growth of organism has occurred while the yellow wells indicate inhibition of microorganism growth. Concentrations decrease from 64 mg/ml to 0.125 mg/ml.

5.2.2. Bioautographic assay

5.2.2.1. Principle of the method

Thin-layer chromatography is the simplest and cheapest method of detecting plant constituents. Bioautography is a very convenient way of testing plant extracts and pure substances for their effects against pathogens. It can be employed in target-directed isolation of active constituents. One of the bioautographic methods that have been described is agar diffusion in which the antimicrobial agent is transferred from the chromatogram to an inoculated agar plate through a diffusion process. It is applicable to a broad spectrum of microorganisms. It produces well defined zones of inhibition and is not sensitive to contamination. Active compounds are transferred by diffusion from the stationary phase to the agar layer which contains the microorganism. After incubation, the plate is sprayed with a tetrazolium salt which is converted to a formazan dye by the microorganism. Inhibition zones are observed as clear spots against a purple background (Marston and Hostettmann, 1999).

5.2.2.2. Protocol

A thin-layer chromatography (TLC) plate to which the extracts had been applied, was developed in the solvent system toluene: ethyl acetate (6:4). The plate was placed in ultra violet light for one hour to eliminate any contamination that may have been present. The plates were placed on a, previously poured, fifteen milliliter agar base in a Petri dish and covered with fifteen milliliters of agar, inoculated with 150 µl of *Staphylococcus aureus* (ATCC 12600). The plate was allowed to predefuse for one hour in the fridge and incubated at 37 °C overnight.

After 24 hours, the bacteria-covered plate was sprayed, using an atomizer, with a 2 mg/ml *p*-iodonitrotetrazolium violet (INT) aqueous solution. The bacteria stained dark red by the INT such that zones of inhibition were clearly visible where bacterial growth has been inhibited. Zones on the TLC plate that remained clear indicated compounds that exhibit antibacterial activity (Marston and Hostettmann, 1999).

5.2.3. Death kinetics assay

5.2.3.1. Principle of assay

Death kinetics or time-kill studies are commonly used to investigate new antimicrobial agents (Tam *et al.,* 2005). Data collected from time-kill studies have provided critical information regarding the rate and extent of bactericidal activity. These data have significantly enhanced our understanding regarding the dynamic relationships which exist between antimicrobial agents and their effects on bacteria (Klepser *et al.,* 1998). The diversity of various bacterial responses to antibiotics cannot easily be understood without establishment of a time-kill curve. A typical time-kill curve generated by subjecting the test organism to exposure at constant antibiotic concentrations normally consists of a lag phase, a log-linear killing phase, a second lag phase as well as a regrowth phase (Li, 2000).

The determination of death kinetics enables the differentiation between microbiostatic and microbiocidal effects (Christoph and Stahl-Biskup, 2001). Thus, time-kill curves are figurative representations of bacterial concentrations (CFU/ml) in subcultures taken serially from cultures, usually in liquid media, containing antibiotics from which killing kinetics can be derived (European Society of Clinical Microbiology and Infectious Diseases, 2000)

5.2.3.2. Protocol

The inactivation broth death kinetic method described by Christoph and Stahl-Biskup, (2001) was used to determine the antimicrobial activity of *H. saccifera* which was chosen based on the promising results obtained in the MIC determination. *Staphylococcus aureus* (ATCC 12600) was cultured overnight at 37ºC on Tryptone Soya Agar (TSA). The colonies were then seeded into sterile 0.9 g/L NaCl solution. Serial dilutions (10^{-1} to 10^{-4}) were carried out in 0.9 g/L NaCl which provided a final colony count in the lowest dilution of 173 colonies per plate. In addition, the optical density of the solution (absorbance) of bacteria was determined as 0.023. Suspensions (final volume of 50 ml) were made containing 0.1, 0.25, 0.5 and 0.75 % w/v of an acetone extract of *H. saccifera* which was first dissolved in 2.5 ml of acetone and thereafter added to Tryptone Soya Broth and was placed in a shaking water bath at 37ºC. A control was run in the same way without the addition of the extract. Aliquots of 1 ml were sampled after 0, 5, 15, 30, 60, 120 and 240 min as well as at 8 and 24 hours and were transferred to 9 ml of inactivation broth containing 0.1 % peptone, 5 % lecithin and 5 % yeast extract and then vortex stirred. Four serial dilutions were performed in 0.9 g/L NaCl solution (1 in 10 dilutions) from the inactivation broth. Samples of each dilution (100 µl) as well as from the inactivation broth were plated on TSA. The plates were then incubated for 24 hours at 37°C after which the

number of colonies were counted. The assay was performed in duplicate. Activity was determined by plotting $\log_{10}$ colony counts (CFU/ml) against time.

5.3. RESULTS:

5.3.1. Minimum inhibitory concentration

Table 5.1 indicates the MIC results for all twelve species of *Hermannia* as well as the isolated compound **[1]**. All twelve species investigated indicated activity against all organisms tested. Most values ranged from 2-4 mg/ml with only *H. flammula* indicating limited activity against *C. albicans* and *S. aureus* with values of greater than 16 and 8 mg/ml respectively. In addition, *H. althaeifolia, H. holosericea* and H. *scabra* produced values of 8 mg/ml against *E. faecalis*.

The most activity, however, was obtained by *H. saccifera* which indicated 0.0195 mg/ml (19.5 µg/ml) as the MIC against both *S. aureus* and *B. cereus* and an MIC of 0.125 mg/ml against *E. faecalis*. *H. salviifolia* and *H. scabra* had MIC values of 0.5 mg/ml against *P. aeruginosa*, as did *H. muricata*, *H. cuneifolia*, *H. saccifera* and *H. scabra* against *C. neoformans*.

The novel compound, *E*-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene, isolated from *H. saccifera* produced an MIC of 0.0263 mg/ml (26.3 µg/ml) against *S. aureus*, 0.0943 mg/ml (94.3 µg/ml) against *E. facials* as well as 0.0472 mg/ml (47.2 µg/ml) against *B. cereus.*

The results indicate that the species tested have similar antimicrobial activity against both Gram-negative and Gram-positive organisms as well as against the yeasts.

5.3.2. Bioautographic assays

The bioautogram for *H. saccifera* on *S. aureus* (ATCC 12600) indicated that there are at least two to three compounds that are responsible for antimicrobial activity of the plant (Fig 5.2) and thus served as a focus for isolation.

In addition, a bioautogram conducted on *H. althaeifolia*, showed that while the crude extract did not possess visible antimicrobial activity, the fraction obtained from the column chromatography did have activity (Fig 5.3).

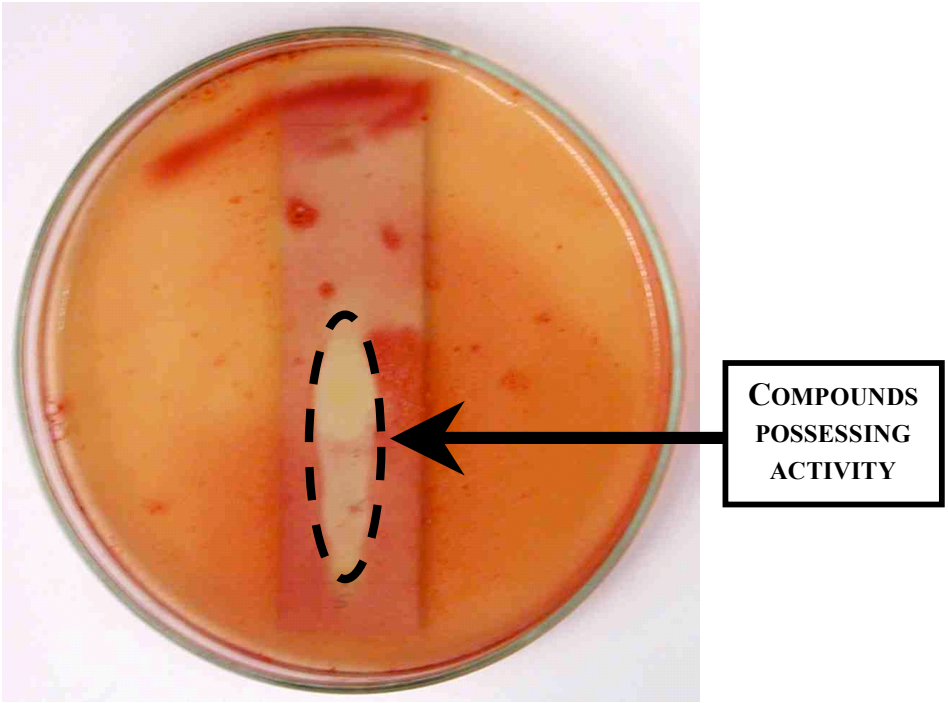

Fig. 5.2: Bioautogram of crude extract of *H. saccifera* on *S. aureus* (ATCC 12600) indicating two or three compounds that possess antimicrobial activity.

Table 5.1: MIC values for *Hermannia* species [mg/ml] (N≥ 2).

Species	*S. aureus* **ATCC 12600**	*S. epidermidis* **ATCC 2223**	*E. faecalis* **ATCC 29212**	*B. cereus* **ATCC 11778**	*K. pneumoniae* **NTCC 9633**	*P aeruginosa* **ATCC 9027**	*C. albicans* **ATCC 1023**	*C. neoformans* **ATCC 901**
H. althaeifolia	4	4	8	4	4	4	4	1
H. cuneifolia	2	2	4	2	2	2	4	0.5
H. flammula	8	4	4	2	2	4	>16	4
H. holosericea	2	2	8	2	2	2	4	2
H. incana	2	2	4	2	2	1	8	1
H. involucrata	2	2	4	2	2	1	2	0.5
H. lavandulifolia	4	2	4	2	2	1	4	4
H. muricata	2	4	4	2	2	2	4	0.5
H. saccifera	0.0195	1	0.125	0.0195	2	2	1	0.5
H. salvifolia	2	2	4	2	1	0.5	2	1
H. scabra	4	2	8	1	1	0.5	2	0.5
H. triflora	4	4	4	6	3	1	4	1
E-17, 19-diacetoxy – 15–hydroxylabda - 7,13 - diene	0.0236	nd*	0.0943	0.0472	nd*	nd*	nd*	nd*
Control [µg/ml]	2.5	0.625	1.25	0.625	1.25	0.0178	>2.5	0.313

* nd = not determined due to insufficient sample

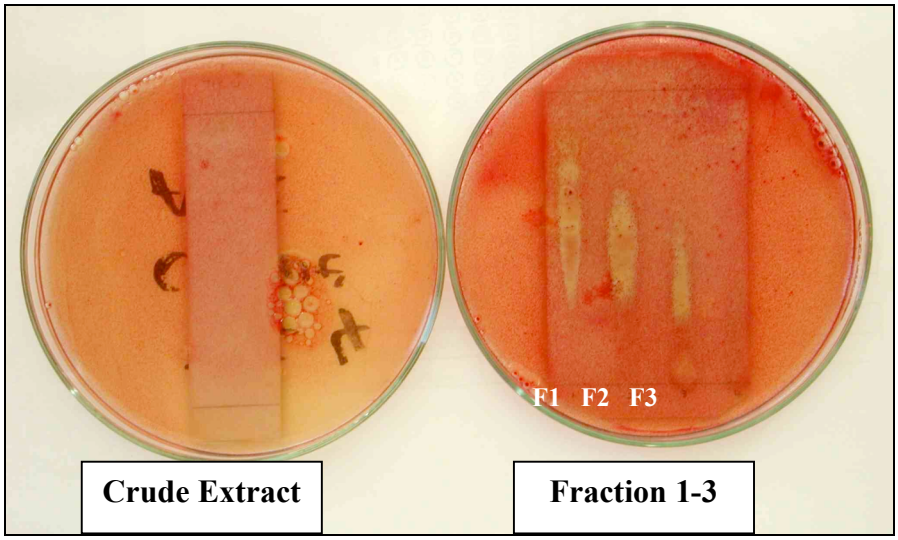

Fig. 5.3: Bioautogram of *H. althaeifolia* on *S. aureus* (ATCC 12600) indicating lack of antimicrobial activity in the crude extract with activity being present in fraction 1-3.

5.3.3. Death kinetics assay

The time-kill plot of *H. saccifera* is displayed in Fig 5.4. The antibacterial activity of all concentrations was noticeable after 60 min of exposure time. The activity of this plant is concentration dependant with exposure to the plant extracts over time resulting in a reduction of colony forming units. All concentrations exhibited antibacterial activity over time with most concentrations achieving at least a 4-5 $\log_{10}$ reduction in bacterial count after 8 hours. Concentrations of 0.1, 0.25 and 0.5 % indicated bacteriostatic activity while the 0.75 % (7.5 mg/ml) extract achieved complete bactericidal activity after 240 min. Regrowth of microorganisms did not occur at any concentration being investigated within the 24 hour test period.

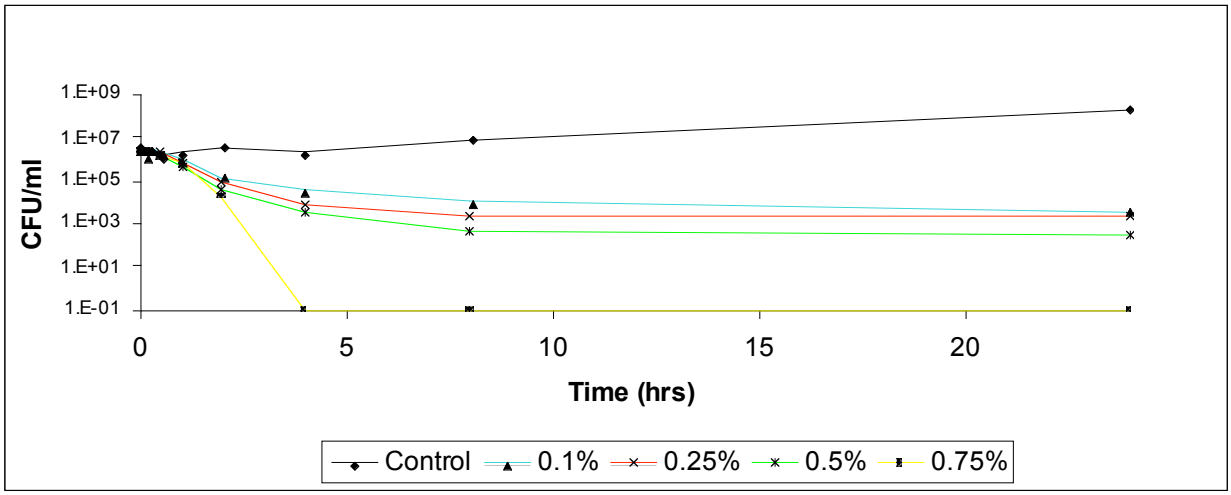

Fig. 5.4: Death kinetics of *Staphylococcus aureus* exposed to various concentrations of *H. saccifera.*

5.4. DISCUSSION:

The crude extracts of all twelve plants possessed inhibitory effects on the test organisms selected and this activity was shown to be a concentration dependant inhibition. Duarte *et al.* (2005) suggested that extracts presenting an MIC below 2.0 mg/ml should be considered as having potential antimicrobial activity. Further, Aligiannis *et al.* (2001) proposed a classification for plant materials based on MIC results as follows: strong inhibitors – MIC up to 0.5 mg/ml; moderate inhibitors – MIC between 0.6 and 1.5 mg/ml; weak inhibitors above 1.6 mg/ml. Thus, while most extracts do possess potential antimicrobial activity, many exhibit weak activity. However, a few species as listed in Table 5.1 have indicated moderate activity. In addition, *H. cuneifolia*, *H. involucrata, H. muricata, H. salviifolia* and *H. scabra* have indicated strong activity against organisms, *P. aeruginosa* and *C. neoformans*.

It is interesting to note that the species had broad spectrum activity that was inhibitory to Gram-positive and Gram-negative bacteria as well as yeasts. Generally, previous research conducted on plant extracts has indicated that they are more active against Gram-positive rather than Gram-negative bacteria (Lin *et al.,* 1999). *Hermannia* could be producing compounds with broad spectrum activity against a host of micro-organisms, making the plant very useful in the treatment of infections. In addition, antifungal agents are amongst the most expensive antibiotics. Thus, readily accessible and inexpensive alternative remedies for treatment of fungal infections, is required (Salie *et al.*, 1996). The species were found to produce good antifungal activity against the two yeasts tested, and may, thus, be useful as an antifungal agent.

The most promising results were obtained for *H. saccifera* against *S. aureus* (ATCC 12600) as well as *B. cereus* (ATCC 11778) with MICs of 19.5 µg/ml. It must be taken into consideration that these are MIC values for crude extracts and it is expected that the isolated active compound should have increased activity. This activity is promising as it is highly active against *S. aureus* which known to be resistant to many antimicrobial agents (Lechner *et al.*, 2004). In addition, the plant is highly active against *B. cereus* which is also a Gram-positive organism. *Hermannia depressa* which is the only species of *Hermannia* investigated for antimicrobial activity, to date, ranges from 0.195 to 3.125 mg/ml against *S. aureus* and *B. cereus* (Reid *et al.,* 2005) indicating that *H. saccifera* has much greater activity than any other *Hermannia* species tested. Thus, the isolation of these compounds were imperative to identify the actual compounds responsible for this activity. The bioautogram (Fig 5.2) indicated that there were two to three compounds contributing to this activity. Isolation was attempted using bioassay-guided fractionation as discussed in Chapter 4.

The activity of *H. saccifera* with the MIC against *S. aureus* (ATCC 12600) being 19.5 µg/ml, prompted the testing of the plant extract in the death kinetics assay to determine the bactericidal activity over time. There was an initial decrease in viable cell counts for all concentrations including the control. After the first 30 min the viable colony count for the control increased. The lower concentrations of 0.1, 0.25 and 0.5 % showed bacteriostatic activity, as after an initial decrease in viable cell counts further growth was prevented but *S. aureus* was not killed off by the presence of the plant extract. The time-kill curve (Fig 5.4.) indicates that the activity of the plant extract against *S. aureus* is concentration dependant as well as that exposure to the plant extract over time results in a reduction in colony forming units.

The plant as indicated previously portrayed very low MIC values (i.e. 19.5 µg/ml) against the same strain of *S. aureus*. These time-kill results do not correlate with these excellent MIC results. However, bactericidal or bacteriostatic activity cannot be predicted by MIC (Krueger *et al.,* 2001) and thus, the plant may produce a low MIC value but this activity may be bacteriostatic with only high concentrations of extract i.e. 0.75% (7.5 mg/ml) producing bactericidal activity.

The results did indicate that no regrowth of micro-organisms occurred during the time period being assessed. In addition, rapid killing of micro-organisms was not observed as a decrease in viable cell count compared to the control was only observed after 60 minutes.

One of the compounds contributing to the antimicrobial activity of *H. saccifera* was isolated and identified as *E*-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene (Chapter 4). This compound produced interesting results when minimum inhibitory concentrations were

determined. The MIC against *S. aureus* was established to be 23.6 µg/ml. This was a promising result indicating that the compound does indeed contribute to the antimicrobial activity. However, this value was less than that produced by the plant extract itself which was 19.5 µg/ml. This indicates, in addition to confirming the bioautogram results, that there are a number of compounds found within the plant that together produce the total antimicrobial activity. This is further displayed in the increased MIC of 47.2 µg/ml against *B. cereus* when the crude plant extract produced an MIC of 19.5 µg/ml. Only in the case of *E. faecalis* was the result produced for the compound (94.3 µg/ml) less than that of the extract (125 µg/ml) suggesting that this compound contributes significantly to the antimicrobial activity of the plant against this bacterial species. It is therefore evident that while this novel compound may not be used as an antibacterial agent in its present form, it does indicate promising activity against several bacteria and may, thus, prove to be an interesting lead compound in the development of new antimicrobial drugs. Hence further investigation into the chemical structural components of the compound is required to determine the structure-activity relationships and thereafter, chemical synthesis of related compounds with increased activity is necessary.

One further interesting aspect was the results of the bio-autogram conducted on *H. althaeifolia*, in which the crude extract showed no antimicrobial activity yet after column fractionation activity was exhibited (Fig 5.3). This could be due to antagonism present in the crude extract which had been eliminated in the fraction. Conversely, the results may have been due to the assay not being sensitive enough to obtain activity in the crude extract in which the concentration of active compound would have been much less than that of the enriched fraction. This aspect warrants further investigation.

Staphylococcus aureus is an opportunistic organism which only becomes pathogenic when the immune system of a patient is compromised. They contribute significantly to the initiation of infection and are commonly associated with skin and wound infections (Bannister *et al.,* 2000). The results indicate that the selected species are effective against *S. aureus* especially *H. saccifera* and therefore, they can be utilized in the treatment in the above conditions. *S. epidermidis* is also associated with skin infections and again the plants activity against this microorganism with *H. saccifera* portraying the best activity with an MIC of 1 mg/ml.

The plants all exhibited promising activity against *P. aeruginosa* (ATCC 9027) which is naturally resistant to many antimicrobial agents (Konning *et al.*, 2004). Thus, the isolation of compounds from the most active extracts may produce compounds that are active against *P. aeruginosa* making it useful for these resistant organisms. This organism is associated with middle ear suppuration in children, destructive lesions of the skin, and infections of the genito-urinary tract, respiratory tract, the joints and the eye. It is also associated with a dysentery-like enteric infection and in pneumonia (Bannister *et al.,* 2000). *E. faecalis* is the cause of endocarditis, urinary tract infections, intra-abdominal infections cellulites and wound infection as well as concurrent bacteremia. *Klebsiella pneumoniae* is associated with pneumonia, bronchitis and other respiratory diseases (Beers and Berkow, 1999). *Bacillus cereus* has been implicated in gastrointestinal, wound, and ocular infections to name a few (Kotiranta *et al.*, 2000). Thus, the plant species active against these organisms has many important implications for the treatment of diseases associated with these organisms.

Activity portrayed by the selected species against *Candida albicans* may provide interesting compounds especially if the isolated compounds have increased activity compared to the crude

extracts. *Candida albicans* is an opportunistic pathogen that commonly affects immunologically compromised patients (Duarte *et al.*, 2005) and the plants may, thus, be useful in the treatment of opportunistic infections associated with HIV/AIDS. *Cryptococcus neoformans* is associated with meningitis or as generalized infection (Bannister *et al.,* 2000). The inhibition of this organism by the plant extracts suggests that the compounds producing this activity should be further investigated with regards to its ability to cross the blood brain barrier since this is a requirement for the treatment of meningitis during *in vivo* testing.

Traditional usage of these plants includes skin and wound as well as respiratory infections, burns and dysuria, amongst a few. These conditions are caused or exacerbated through a number of micro-organisms including those used in the investigation above thus, providing a scientific basis for the traditional use of the plants.

5.5. CONCLUSION:

All plants extracts from the selected *Hermannia* species exhibit antimicrobial activity with *H. saccifera* exhibiting the most potent activity with an MIC of 19.5 µg/ml against *S. aureus* and *B. cereus*. The death kinetics of *H. saccifera* indicates that the antibacterial activity is bacteriostatic rather than bactericidal with only very high concentrations of the extract producing bactericidal activity. Two to three compounds have been implicated as producing this activity in *H. saccifera*. A novel compound identified as *E*-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene produced good MIC results and may be an important lead compound in the development of new antimicrobial drugs. In addition, the activity displayed can be correlated to the traditional usage of the plants, thus providing a scientific basis for the use of these plants in traditional healing to treat infectious diseases.

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CHAPTER 6: ANTIOXIDANT ACTIVITY

6.1 INTRODUCTION:

The role of free radical reactions in biology has become an area of intense interest. Free radicals play an important role in the development of tissue damage and pathological events in living organisms. In aerobic life, lipids containing polyunsaturated fatty acids, proteins, nucleic acids and carbohydrates can be oxidized by free radical-mediated reactions (Velázquez *et al.*, 2003).

Electrons in atoms occupy regions of space known as orbitals. Each orbital can hold a maximum of two electrons, spinning in opposite directions. A free radical can be defined as any species capable of independent existence that contains one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. Since electrons are more stable when paired, radicals generally are more reactive (Halliwell, 1991).

Aerobic life uses oxygen to oxidize carbon and hydrogen rich substrates to obtain chemical energy and heat essential for life. Unfortunately, when this occurs, the oxygen molecule itself becomes reduced and forms intermediates; two of which are free radicals (Eqns 1- 4).

$O_2 + e + H^+ \rightarrow HO_2^{\bullet}$ Hydroperoxyl radical

$HO_2^{\bullet} \rightarrow H^+ + O_2^-$ Superoxide radical (1)

$O_2^- + 2H^+ + e \rightarrow H_2O_2$ Hydrogen peroxide (2)

$H_2O_2 + e \rightarrow OH^- + {}^{\bullet}OH$ Hydroxyl radical (3)

$$^{\bullet}OH + e + H^{+} \rightarrow H_2O \qquad (4)$$

Eqn 1 indicates the addition of an electron which causes oxygen to be reduced creating the free radical $HO_2^{\bullet}$. At the physiological pH of 7.4, the hydroperoxyl radical dissociates to give the superoxide anion radical (O_2^{-}) (Gutteridge, 1994). Dismutation (Eqn 2), further, causes hydrogen peroxide to be formed. In the presence of trace metals, the superoxide anion and hydrogen peroxide undergo the so-called Haber-Weiss reaction which results in the hydroxyl radical ($^{\bullet}OH$) formation (Kappus, 1987). In addition, hydroxyl radicals may also originate from hydrogen peroxide in the presence of greater amounts of reduced metal ions by the Fenton reaction (Halliwell and Gutteridge, 1984).

$$H_2O_2 + Fe^{2+} + H^{+} \rightarrow HO^{\bullet} + Fe^{3+} + H_20$$

$^{\bullet}OH$ is the most reactive radical known to chemistry. It can attack and damage almost every molecule found in living cells. Reaction of $^{\bullet}OH$ with biological molecules set off chain reactions. The ability of $^{\bullet}OH$ to stimulate the free radical chain reaction, known as lipid peroxidation, is perhaps, the best characterized biological damage caused by this molecule. In the close vicinity of membranes, $^{\bullet}OH$ attacks the fatty acid side chains of the membrane phospholipids, resulting, ultimately, in the production of lipid hydroperoxides. These disrupt membrane function through accumulation, causing it to collapse (Gutteridge, 1994). Both O_2^{-} and H_2O_2 are far less reactive than $^{\bullet}OH$ but they are not completely harmless as both can produce direct injury to a few cellular sites if they are generated in excess but $^{\bullet}OH$ remains the most deleterious in its effects.

Aerobic organisms are protected against oxygen toxicity by a natural antioxidant defense system involving enzymatic and non-enzymatic mechanisms (Velázquez *et al*., 2003). In healthy individuals, the production of free radicals is balanced by the antioxidative defense system. However, oxidative stress is generated when the balance is in favor of the free radicals as a result of an increased production or depletion of antioxidant levels (Parejo *et al.,* 2002).

The term 'antioxidant' is frequently used in biomedical literature, but rarely is it defined, with a strong implication that it refers to chemicals with chain breaking properties such as vitamin E and vitamin C. It may also be defined as "any substance that when present at low concentrations, compared to those of the oxidizable substrate, significantly delays, or inhibits, oxidation of that substrate" (Gutteridge, 1994).

Antioxidants can act at several different stages in an oxidative sequence, such as:

(1) removing oxygen or decreasing local O_2 concentrations;

(2) removing catalytic metal ions;

(3) removing key reactive oxygen species

(4) scavenging initiating radicals such as $^{\cdot}OH$, $RO^{\cdot}$, $RO_2^{\cdot}$;

(5) breaking the chain of an initiated sequence;

(6) quenching or scavenging singlet oxygen (Gutteridge, 1994).

A wide range of methods are currently used to assess antioxidant capacity, for example for measurement of prevention of oxidative damage to biomolecules such as lipids or DNA and methods assessing radical scavenging. Both *in vivo* and *in vitro* assays are used and all methods have their own advantages and limitations (van den Berg *et al.*, 1999). Radical scavenging assays relate to the generation of a different radical, acting through a variety of

mechanisms and the measurement of a range of end points at a fixed time point or over a range. Two types of approaches have been taken, namely, the inhibition assays in that the extent of the scavenging of hydrogen – or electron- donation of a pre-formed free radical is the marker of antioxidant activity, as well as assays involving the presence of an antioxidant system during the generation of the radical (Re *et al.,* 1999)

Traditional medicine all over the world is nowadays revalued by an extensive activity of research on different plant species and their therapeutic principles. Plants, generally, produce many antioxidants to control the oxidative stress caused by sunbeams and oxygen and it is thus possible that they can represent a source of new compounds with antioxidant activity (Scartezzini and Speroni, 2000) that may be useful in providing protection against various diseases such as cancers, cardio- and cerebrovascular disease which have been associated with free radical damage (du Toit *et al.*, 2001).

6.2. METHODS:

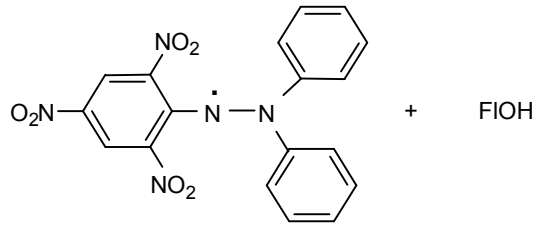
Two assays were utilized to identify the antioxidative ability of the twelve species i.e. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as well as the 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assay. Solutions of 10 mg/ml in methanol were made for each sample from which further dilutions were made. Antioxidant activity of the isolated flavone compounds, 5,8- dihydroxy-6,7,4'- trimethoxyflavone and cirsimaritin, isolated in Chapter 4, were only investigated in the DPPH assay due to the limited amounts of pure compound available.

6.2.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

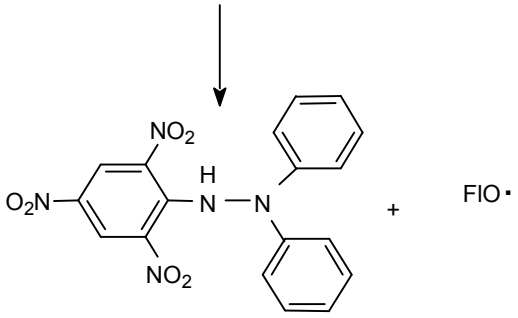
6.2.1.2. Principle of the method:

The DPPH free radical scavenging assay is a simple and widely used screen for bioactive compound discovery. The molecule, DPPH is characterized as a stable free radical by virtue of the delocalization of a spare electron over the molecule as a whole. This delocalization gives rise to a deep violet color, characterized by an absorption band at 517 nm.

When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom this gives rise to the reduced form with the loss of the violet color such that a pale yellow color forms due to the presence of the picryl group (Fig 6.1.). Antioxidant activity is evaluated by the decrease in absorption as a result of the conversion of the purple DPPH radical color to that of yellow when this reduction occurs.



DPPH radical (Purple)



Reduced DPPH (Yellow)

Fig. 6.1: Reaction of DPPH radical with antioxidative substance (FlOH).

The reaction is intended to provide the link with the reactions taking place in an oxidizing system, such as the autoxidation of a lipid or other unsaturated substance i.e. the DPPH represents free radicals formed in the system whose activity is suppressed by a substance that has a hydrogen donating ability (Molyneux, 2004).

6.2.1.3. Protocol

6.2.1.3.1. Microtitre plate method

This is a colorimetric method described by Shimada *et al.* (1992) which investigates the radical scavenging ability of a sample using the DPPH radical. Quantitative measurement of radical scavenging properties was carried out in a 96 well microtitre plate using analytical grade ascorbic acid as the reference compound (Fig 6.2). Each well contained 50 µl of sample solutions (100 µg/ml) as well as 200 µl of DPPH (0.077 $mmol.L^{-1}$) (Fluka) dissolved in methanol (Ultrafine Ltd). Negative controls contained 50 µl of sample and 200 µl methanol without any DPPH. Measurements were performed in triplicate. The microtitre plate was placed on a Labsystems Multiskan RC microtitre plate reader and shaken for two minutes after which it was allowed to react for a further 30 minutes in the dark before a reading was taken at 550 nm. Samples having activity of less than 100 µg/ml were then serially diluted and results obtained at varying concentrations. Enzfitter® version 1.05 software was used to calculate the IC_{50} values which denote the concentration of sample required to scavenge 50 % of the DPPH radical.

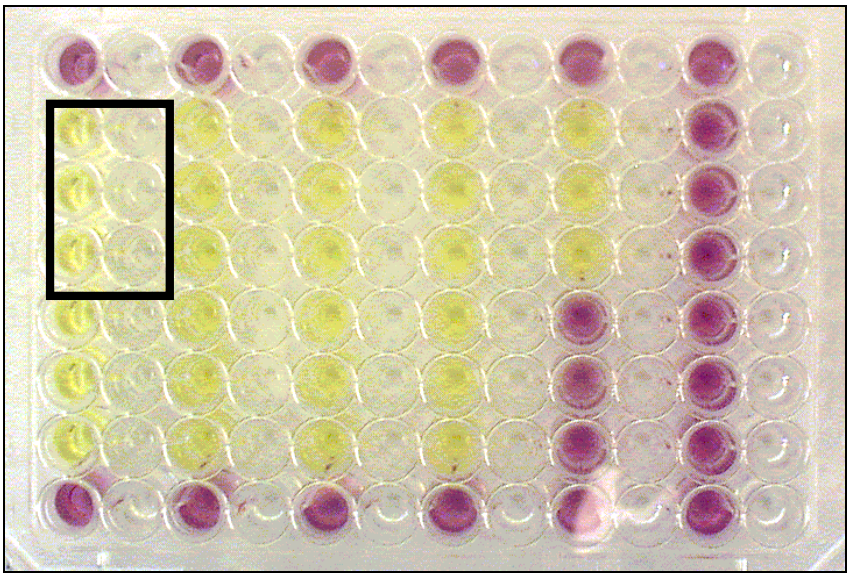


Fig. 6.2: Example of microtitre plate. The purple wells refer to the maximum reaction of DPPH while yellow wells indicate antioxidant activity. Clear wells are the control for each sample. Boxed area refers to the reaction of one sample at 100 µg/ml assayed in triplicate.

6.2.1.3.1. Thin layer chromatographic analysis

A chloroform: methanol (1:1) extract of the twelve selected species of *Hermannia* were diluted to a concentration of 50 mg/ml using methanol and 2 µl of this solution was applied to an aluminium backed silica plate (Macherey-Nagel) using a calibrated glass capillary tube (Hirschmann Laborgerate). The TLC plate was developed in a mobile phase TLC 2 as described in Chapter 3. Positive free radical scavenging / anti-oxidant activity was confirmed by spraying the plate with DPPH solution (0.04 **%** in HPLC grade methanol) using an atomizer. Color development occurred immediately. Activity was indicated by active molecules appearing as yellow zones against a purple background.

6.2.2. 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assay

6.2.2.1. Principle of the method

The ABTS antioxidant assay is based on the TEAC (Trolox Equivalent Antioxidant Capacity) assay originally described by Miller *et al.* (1993) which involves the scavenging of long-lived radical anions. The TEAC assay was based on the reduction of the accumulation of $ABTS^{\cdot}$ formed by the peroxidase activity of metmyoglobin by antioxidants. However, a major impediment to this assay is that compounds may inhibit peroxidase activity (Strube *et. al.,* 1997). Therefore, the assay has been adapted by using preformed $ABTS^{\bullet}$ (van den Berg *et al.,* 1999).

This method involves the direct production of the $ABTS^{\cdot+}$ chromophore through the reaction between ABTS and potassium persulphate (Fig 6.3). This has absorption maxima at wavelengths 645, 734, and 815 nm. The radical generated is capable of reacting with both water soluble and lipid soluble antioxidants as well as pure compounds and food extracts. Addition of antioxidants to the preformed radical cation reduces it to an extent depending on the antioxidant activity and the concentration of the antioxidant. This converts the blue/green color to colorless through scavenging of the stable $ABTS^{\cdot+}$ radical (Re *et. al.,* 1999). The degree of discoloration reflects the amount of $ABTS^{\cdot+}$ that has been scavenged and can be determined spectrophotometrically (Arts *et al.*, 2003).

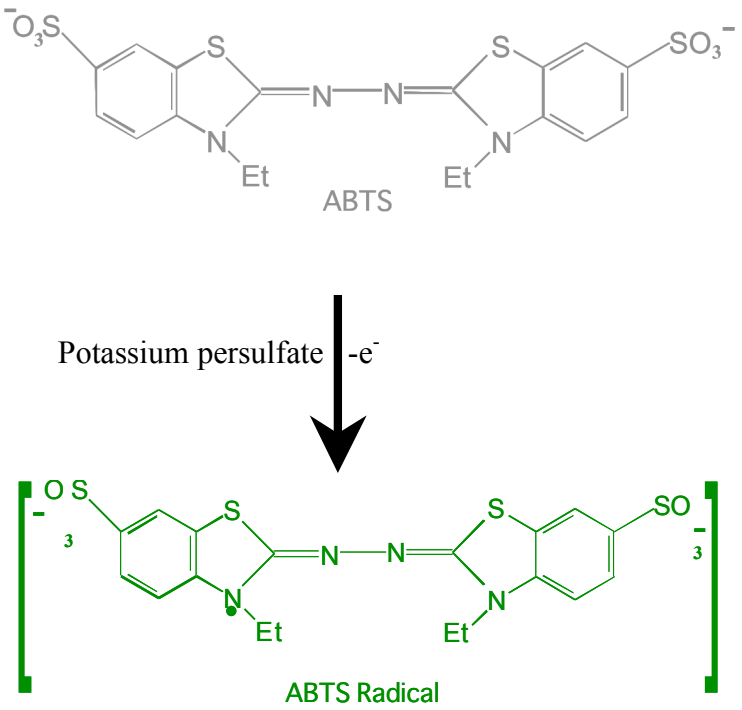


Fig. 6.3: Reaction showing the production of $ABTS^{\cdot+}$.

6.2.2.2. Protocol

The radical scavenging activity of all extracts were determined using the 2,2 - azino-bis(3-ethylbenz-thiozoline-6-sulfonic acid) (ABTS) assay described by Miller *et al*. (1993) and modified by van den Berg *et al.* (1999). A 7 mM ABTS (Sigma Aldrich) stock solution was prepared in double distilled water. The ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting 5 ml of ABTS solution with 88 µl of a 140 mM potassium persulphate ($K_2S_2O_8$) (Fluka) solution and the mixture was allowed to stand in the dark for 12-16 hours to stabilize. The radical solution is stable for two days in the dark.

Prior to the assay, the $ABTS^{\cdot+}$ solution was diluted in cold ethanol to give an absorbance of 0.70 ± 0.02 at 734 nm in a 1 cm cuvette. Ethanol and methanol were used as negative controls. The radical scavenging activity was quantitated by reacting 1 ml of $ABTS^{\cdot+}$ solution with 50 µl of sample. Trolox (6-hydroxy-2,5,7,8-tetramethylchromon-2-carboxylic acid) (Sigma Aldrich) was used as a positive control. The mixture was heated for 4 min after which the absorbance was read at 734 nm on a Specord 40 spectrophotometer. Measurements were performed in triplicate. The percentage inhibition was then plotted as a function of the concentration, from which the equation of the straight line was calculated. The concentration that produced 50 % decolorisation (IC_{50}) was determined as well as the standard deviation.

6.3. RESULTS:

Table 6.1 and Fig 6.4 summarize the radical scavenging activity of the twelve selected species of *Hermannia* in the DPPH and ABTS antioxidant assays. Most species portrayed promising antioxidant activity in both assays with *H. cuneifolia* possessing the most significant activity with a concentration to produce 50 % decolorisation (IC_{50}) of 10.26 ± 0.29 and 10.32 ± 0.34 (µg/ml) in the DPPH and ABTS assays respectively.

Ten of the twelve species possessed activity with IC_{50} ranging from 10-30 µg/ml with only one species, *H. trifurca*, indicating moderate activity and one species, *H. salviifolia,* showing limited activity in both assays. No extracts were found to exhibit a higher radical scavenging activity than the positive controls, ascorbic acid and Trolox which IC_{50} values of 2.46 ± 0.01 and 2.96 ± 0.001 µg/ml respectively.

Both isolated flavone compounds investigated, i.e. 5,8- dihydroxy-6,7,4'- trimethoxyflavone and cirsimaritin, exhibited poor activity with both compounds having an IC_{50} of greater than 100 µg/ml thus, indicating almost no activity against the DPPH radical.

In addition, the values obtained for each species in both assays indicates some correlation with the ABTS assay producing only slightly better activity in most species than the DPPH assay.

The results obtained from the TLC analysis indicate that there are a number of compounds in each species that portray antioxidant activity (Fig 6.5). Many compounds that produce activity are found in all species being investigated as can be seen from similar R_f values being obtained for these compounds. In addition, only 5,8- dihydroxy-6,7,4'- trimethoxyflavone indicates slight activity while cirsimaritin is not active against the DPPH free radical (Fig 6.6).

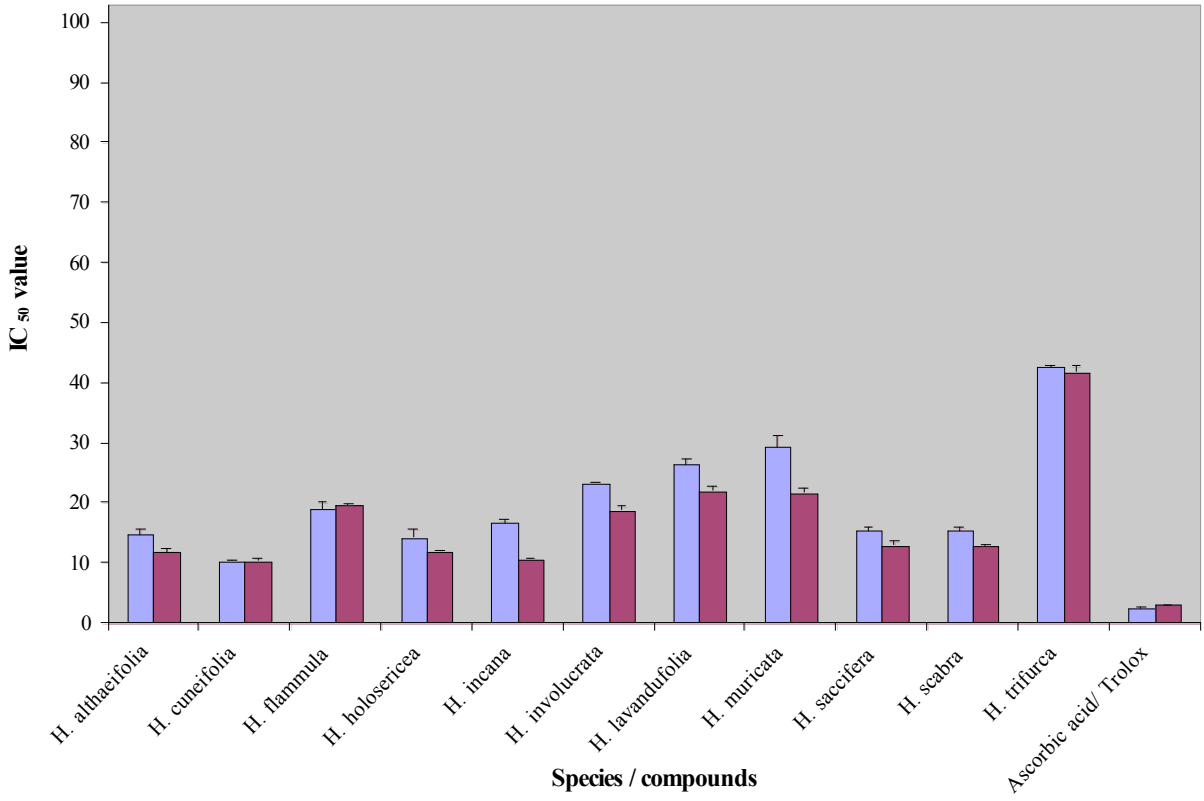
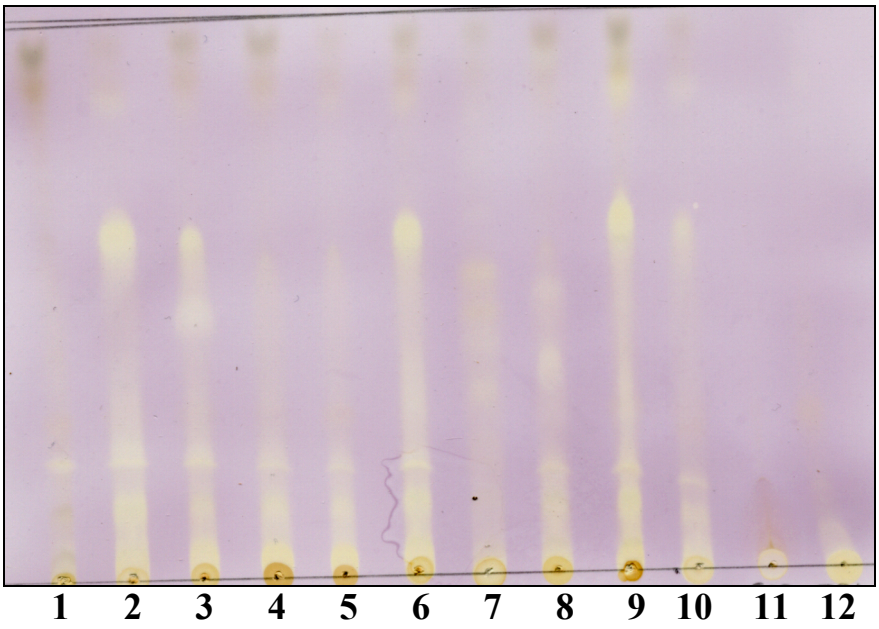


Fig. 6.4: Bar graph comparing results obtained for DPPH and ABTS assays indicating similarities between the results and the consistent lower results of the ABTS results.

Table 6.1: DPPH and ABTS antioxidant activity of selected species of *Hermannia.*

Species/Compounds	DPPH ASSAY		ABTS ASSAY	
	IC_{50} (µg/ml)	Std dev	IC_{50} (µg/ml)	Std dev
H. althaeifolia	14.73	0.79	11.86	0.64
H. cuneifolia	10.26	0.29	10.32	0.34
H. flammula	18.91	1.13	19.56	0.24
H. holosericea	14.16	1.39	11.89	0.13
H. incana	16.57	0.54	10.63	0.18
H. involucrata	23.32	0.18	18.78	0.62
H. lavandufolia	26.35	1.02	22.05	0.62
H. muricata	29.21	1.96	21.62	0.91
H. saccifera	15.41	0.65	12.94	0.56
H. salviifolia	>100		109.49	0.26
H. scabra	15.40	0.43	12.77	0.20
H. trifurca	42.66	0.35	41.74	0.99
5,8- dihydroxy-6,7,4'-trimethoxyflavone	>100			
cirsimaritin	>100			
Ascorbic acid/ Trolox	2.46	0.01	2.96	0.001



Legend: For all TLC plates

1 - *H. salviifolia*
2 - *H. flammula*
3 - *H. holosericea*
4 - *H. cuneifolia*
5 - *H. althaeifolia*
6 - *H. lavandufolia*
7 - *H. saccifera*
8 - *H. involucrata*
9 - *H. incana*
10 - *H. muricata*
11 - *H. scabra*
12 - *H. trifurca*

Fig. 6.5: TLC plate sprayed with DPPH indicating compounds contained within each of the twelve plant extracts that possess free-radical scavenging activity.

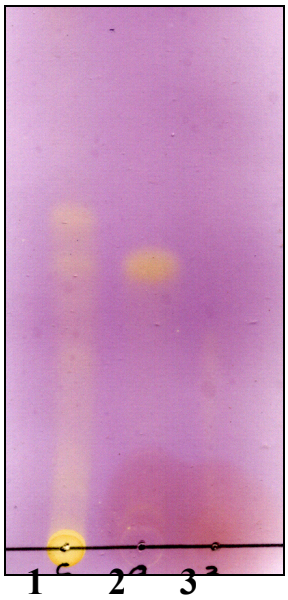


Fig. 6.6: TLC plate indicating antioxidant activity of isolated compounds, 5,8- dihydroxy-6,7,4'- trimethoxyflavone (2), cirsimaritin (3) and *H. saccifera* (1). Only 5,8- dihydroxy-6,7,4'- trimethoxyflavone of the isolated compounds produces slight activity.

6.4. DISCUSSION:

Ten of the twelve species indicated promising antioxidant activity in both the DPPH and ABTS assays with activity being dose-dependant such that increasing doses produced greater antioxidant activity. Since natural antioxidative substances usually have a phenolic moiety in their molecular structure (Dapkevicius *et al.,* 1998), this suggests that these plants may have a high polyphenolic content such as tannins. Further research to explore the nature of these antioxidative compounds may yield interesting and possibly novel bioactive compounds.

In contrast to the activity displayed by the other selected species, *H. salviifolia* indicated limited activity as a free radical scavenger. This may suggest low antioxidant content within the plant. This is interesting when it is noted that there appears to be no significant differences in the conservative HPLC profiles of *H. salviifolia* and that of *H. cuneifolia*, which displayed the greatest activity in both assays. In addition, TLC analysis of the extracts, again, does not display vital inclusions or exclusions of certain molecules between both species. It may be thus assumed that the differences in activity is not due to the content of antioxidant molecules within the plant but rather that of the quantity of molecules available to react, suggesting that *H. salviifolia* contains a lower quantity of the same free radical scavengers than *H. cuneifolia*. Further work to identify and quantitate these compounds is thus essential in explaining the antioxidative ability of these plants.

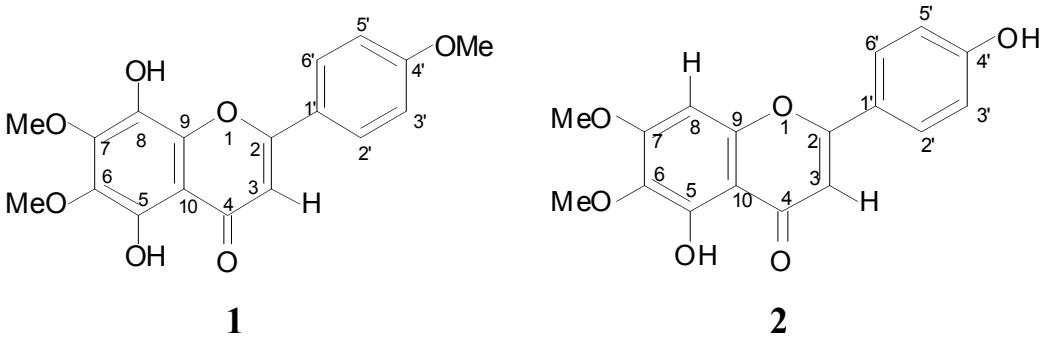
TLC analysis of the twelve species indicates that there are a number of compounds contained within each plant that possess activity. In addition, some of these compounds possessing activity are found in all species investigated, further, suggesting that the quantity of certain compounds contained within the plant is responsible for the activity.

When compared to the reference antioxidants (ascorbic acid and Trolox) the extracts exhibited lower activity. However, some extracts compared favorably to that of the reference compounds. Furthermore, these are crude extracts and not isolated compounds such that they contain many compounds that do not contribute to the total antioxidant capacity of the extracts. Isolation of pure antioxidant compounds may provide results that indicate equal or greater free radical scavenging activity than that of the reference compounds.

$DPPH^{\cdot}$ and $ABTS^{\cdot+}$ are based on their ability to scavenge a proton from surrounding molecules resulting in a loss of color by the radical which decreases the absorbance of the solution. Since these assays have the same mechanism of reaction, it is reasonable to expect that the results obtained should be relatively similar allowing for experimental error. This is indicated in the results in which similarity between the two assays are evident. However, it is important to note that in most cases, the IC_{50} values in the ABTS assay are lower than those recorded in the DPPH assay. The ABTS radical may react with a molecule that has electron- or H- donating potential (Pellegrini *et al.,* 1999). The electron donors undergo a rapid reaction with $ABTS^{\cdot+}$ while the functional hydroxyl groups are slower reacting (Pannala *et al.,* 2001). Thus, the ability of $ABTS^{\cdot+}$ to react via two mechanisms indicates that the activity displayed would be higher in this assay as compared to that of DPPH which reacts only via the acceptance of a hydrogen from a suitable donor.

The isolated flavone compounds indicated limited activity in the DPPH assay with IC_{50} values greater than 100 µg/ml. It can be assumed due to the close resemblance of the mechanism of action that the assays would thus, have exhibited limited activity in the ABTS assay as well. In addition, TLC analysis indicates that only 5,8- dihydroxy-6,7,4'- trimethoxyflavone possess slight free radical scavenging activity while cirsimaritin does not appear to possess any

activity. This result is surprising considering that these compounds have a number of hydroxyl groups that may donate a hydrogen to partake in the reaction with the radical (Fig 6.4). In addition, studies conducted by Cuvelier *et al.* (1996), as measured by accelerated autoxidation of Methyl linoleate, have indicated that cirsimaritin is an effective antioxidant compound. However, both compounds contain only two hydroxyl groups and it has been shown that an increasing number of hydroxyl groups as well as the presence of a hydroxyl group at the 5' position, which does not appear in the structure of these compounds, increases the antioxidant activity of flavonoid-type compounds such as flavones (Škerget *et al.,* 2005).



1 **2**

Fig. 6.7: Structure of isolated flavone compounds, 5,8- dihydroxy-6,7,4'- trimethoxyflavone (1) and cirsimaritin (2).

While the selected plant species indicate promising free radical scavenging activity, it must be noted, however, that there are a number of mechanisms by which antioxidants may function. The cascade leading to oxidative damage is complex and only free radical scavenging activity has been explored. Mechanism of antioxidant action can include suppressing reactive oxygen species formation, either by inhibition of enzymes or by chelating trace elements involved in

free-radical production, scavenging reactive species, and up-regulating or protecting antioxidant defenses (van Acker *et al*., 1996). While these plants do possess chain breaking potential, a selection of assays may provide a more complete indication for the extent of antioxidant activity and therefore, assays such as the hydrogen peroxide, Thiobarbituric Acid Reactive Substances (TBARS), as well as iron-chelation assays may define the antioxidant ability further. In addition, *in vitro* activity does not always correspond with activity *in vivo* and therefore, *in vivo* tests are required to verify this activity.

6.5. CONCLUSION:

Ten *Hermannia* species have indicated good *in vitro* antioxidant activity in both the DPPH and ABTS assays although the activity was less than that of the reference compound with the IC_{50} values in the ABTS assay being lower possibly due to its ability to function via two mechanisms. In addition, the two flavone compounds investigated indicated negligible free radical scavenging activity which may be attributed to the presence of only a few hydroxyl groups on the structure of both compounds. When considering the traditional uses of these plants as listed in Chapter 1, it appears that a number have been employed to treat wounds and various skin afflictions. This indicates that antioxidant activity may play a vital role in the ability of *Hermannia* species to heal. In addition, neutrophil derived reactive oxygen intermediates, such as hydrogen peroxide and superoxide anions, are responsible for the pathogenesis of various inflammatory conditions (Fernandes *et. al.,* 2004), suggesting the possible treatment of inflammation through their antioxidative abilities. Since the selected species have shown significant antioxidant activity, it may be speculated that one aspect of their healing properties may be through antioxidant activity. While this is only one aspect of the antioxidative cascade that is being investigated, the plants have indicated promising

activity which may be further explored in other antioxidant assays. A correlation is indicated between the biological activity and traditional usage of these plants which are utilized in the treatment of many diseases associated with free radical activity.

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CHAPTER 7 - ANTI-INFLAMMATORY ACTIVITY

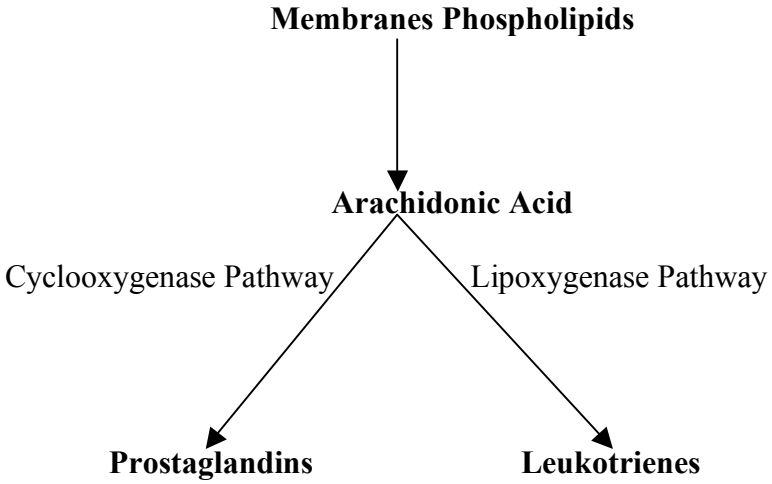
7.1. INTRODUCTION:

The word inflammation is derived from the state of being inflamed. To inflame means to "set afire". This conjures up the color red, a sense of heat and often pain. Inflammation is commonly divided into three phases: acute inflammation, the immune response and chronic inflammation (Katzung, 2001). It is controlled by the presence of a group of substances called chemical mediators, each with a specific role at some definite stage of the inflammatory reaction (Trowbridge and Emling, 1989). Histamine, serotonin, bradykinin, and the eicosanoids, prostaglandins and leukotrienes are some of the mediators, which are known as autacoids that are involved. The cell damage associated with inflammation acts on cell membranes to cause leukocytes to release lysosomal enzymes, which causes the liberation of arachidonic acid from precursor compounds.

Arachidonic acid is the most abundant and probably most important of the precursors of eicosanoids. It is a 20-carbon fatty acid that contains four double bonds. For eicosanoid synthesis to occur, arachidonic acid must be first released or mobilized from the membrane phospholipids by one or more lipases of the phospholipase A_2 type (Katzung, 2001).

Arachidonic acid is present in the cell membranes in an esterified form. Release of arachidonic acid is thought to be the rate-limiting step in a multistage biosynthetic process, which is mediated by specific enzymes that begin the process by inserting molecular oxygen on arachidonic acid. The chain of eicosanoid biosynthesis begins with trauma, infection and inflammation (Henderson, 1994). Following mobilization, arachidonic acid is oxygenated by four separate routes: the cyclooxygenase, lipoxygenase, P450 epoxygenase, and isoprostane

pathways. The metabolism of arachidonic acid by 5-, 12-, and 15-lipoxgenase results in the production of hydroperoxyeicosatetranoic acids, which are rapidly converted to hydroxy derivatives and leukotrienes (Katzung, 2001).



The metabolism of arachadonic acid by 5-lipoxygenase produces the biologically active leukotrienes which are involved in the mediation of various inflammatory disorders. They play a major role in the inflammation response to injury and have been implicated in the pathogenesis of several inflammatory diseases most noticeably asthma, psoriasis, rheumatoid arthritis and inflammatory bowel disease (Henderson, 1994). This pathway is of great interest since it is associated with asthma and anaphylactic shock. Stimulation of these inflammatory cells elevates intracellular Ca^{2+}, releases arachidonic acid and incorporates molecular oxygen by 5-lipoxygenase to yield the unstable epoxide leukotriene A_4 that may then be converted to cystennyl leukotrienes or peptidoleukotrienes (Katzung, 2001).

The action of lipoxygenases generates compounds that can regulate specific cellular responses important in inflammation and immunity (Katzung, 2001). Certain leukotrienes cause adherence of neutrophils to the endothelium of postcapillary venules and are powerful

chemotactic agents while others cause vasodilation and increased venular permeability (Trowbridge and Emling, 1989).

The role of leukotrienes as inflammatory mediators of disease has made them therapeutic targets and many inhibitors aimed at leukotrienes biosynthetic or effecter mechanisms are being developed (Henderson, 1994). Leukotrienes, thus, in addition to being an important factor in the promotion of asthma, is responsible for some of the symptoms of inflammation and, thus, inhibition of this enzyme may prove useful in the treatment of inflammation.

7.2. METHOD

7.2.1. Principle of the method

This method for the inhibition of the 5-lipoxygenase enzyme was determined by Sircar *et al.* (1983) and further modified by Evans (1987). The spectrophotometric assay involves the detection of 5-lipoxygenase inhibitory activity of test compounds which results in the inhibition of formation of the conjugated diene of linoleic acid.

The oxidation of unsaturated fatty acids containing 1-4 pentadiene structures is known to be catalyzed by 5-lipoxygenase. In the body, the biological substrate for 5-lipoxygenase is arachidonic acid which produces various eicosanoids that play a role in inflammation. However, 5-lipoxygenase enzyme is also able to interact with linoleic acid which was then chosen to be utilized in this *in vitro* assay due to its ease in handling. In addition, it has a stronger affinity for the 5-lipoxygenase enzyme resulting in an increase in the UV absorbance. 5-lipoxygenase oxidizes linoleic acid to a conjugated diene. The modification of the unsaturation site of linoleic acid (1,4-diene to 1,3-diene) causes an increase in the absorption

at 234 nm. When inhibition occurs, the absorbance is decreased and can be correlated to inhibition of the 5-liopxygenase enzyme.

7.2.2. Protocol

A starting concentration of 100 µg/ml was obtained with each sample being dissolved in DMSO (Saarchem) and Tween® 20 (Merck). 10 µl of test sample was mixed with 2.95 ml of a 0.1 M potassium phosphate buffer (pH 6.3) and 45 µl of linoleic acid (≥ 99%, Fluka), all of which was placed in a 3 ml cuvette maintained at 25°C in a thermostat bath. The initiation of the reaction was produced through the addition of 100 U of 5-lipoxygenase (Cayman) which had been diluted with an equal volume of a 0.1 M potassium buffer (pH 6.3) that had been maintained at 4°C. The increase in absorbance at 234 nm was recorded for a period of ten minutes using a single beam spectrophotometer (Specord 40) utilizing Winaspect® software. The straight-line portion of the curve was used to determine the initial reaction rate and comparison with the negative control (DMSO and Tween® 20) produced the percentage inhibition of enzyme activity. Dilutions were made of samples that displayed activity from which the concentration that produces 50 % inhibition (IC_{50}) of the enzyme was calculated using the Enzfitter® version 1.05 software. The experiment was conducted in duplicate i.e. N = 2. Nordihydroguaiaretic acid (NDGA) was used as a positive control.

7.3. RESULTS:

Table 7.1. indicates the inhibition of the 5-lipoxygenase enzyme by the various selected plant species. Eleven of the twelve plant species showed activity against 5-lipoxygenase enzyme ranging from 26.79 to 56.36 % of inhibition of the enzyme at 100 µg/ml with *H. lavandufolia* indicating the lowest activity. The only plant extract to portray good activity was *H.*

cuneifolia, which indicated 100 % inhibition at 100 µg/ml with an IC_{50} value of 15.32 ± 5.49 µg/ml.

Results for compounds isolated from various *Hermannia* species i.e. β-sitosterol, lupeol, 5,8-dihydroxy-6,7,4'- trimethoxyflavone and cirsimaritin indicated limited activity against the enzyme with an inhibition of 2.0 % and 40.0 % for β-sitosterol and lupeol respectively as well as 51.97 % and 33.86 % for 5,8- dihydroxy-6,7,4'- trimethoxyflavone and cirsimaritin respectively.

Fig 7.1 indicates the activity of all tested plant extracts and compounds in comparison indicating the extensive activity exhibited by *H. cuneifolia* and the limited activity of the other compounds in comparison.

Table 7.1: Inhibition of the 5-lipoxygenase enzyme by various species of *Hermannia* as well as compounds isolated from *Hermannia* species.

Species/Compounds	**Inhibition (100 %)**	**St. dev.**	**IC_{50} (µg/ml)**
H. althaeifolia	56.53	0.23	
H. cuneifolia	100.0		15.32 ± 5.49
H. flammula	44.77	0.96	
H. holoserisea	56.4	2.63	
H. incana	47.26	0.02	
H. involucrate	44.66	1.46	
H. lavandufolia	27.69	3.16	
H. muricata	32.11	0.87	
H. saccifera	45.8	4.65	
H. salviifolia	41.38	0.62	
H. scabra	33.07	0.00	
H. trifurca	51.97	2.23	
5,8- dihydroxy-6,7,4'-trimethoxyflavone	51.97	2.23	
Cirsimaritin	33.86	3.34	
Lupeol	42.5	3.54	
β-sitosterol	2.13	0.18	
Nordihydroguaiaretic acid (NDGA)	100		2.39 ± 0.71

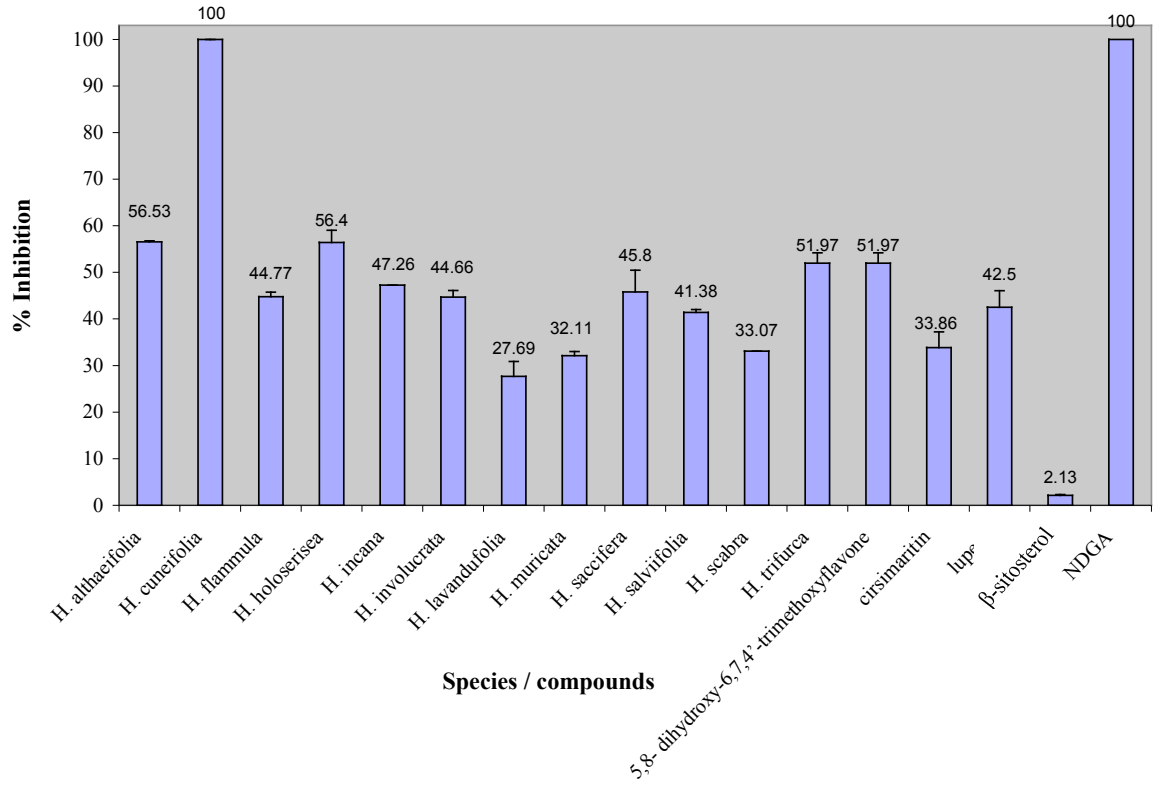


Fig. 7.1: Bar chart indicating the percentage inhibition of the 5-lipoxygenase enzyme by *Hermannia* plant extracts as well as isolated compounds, 5,8- dihydroxy-6,7,4'-trimethoxyflavone, cirsimaritin, lupeol and β-sitosterol, at 100 µg/ml.

7.4. DISCUSSION:

The extracts displayed moderate inhibition of the 5-lipoxygenase enzyme with only *H. cuneifolia* indicating activity that was promising. This, however, does not correlate with complete lack of anti-inflammatory activity. It is important to recognize that this assay relates only to the production of leukotrienes and thus, the extracts may be active in other areas of the cascade of events implicated in the inflammatory process other than the formation of leukotrienes. Conclusive evidence would be required by investigating the effects of these plants in other assay such as the cyclo-oxygenase-1 (COX-1) assay or phospholipase A_2 assay. Previous studies conducted on one species of this genus, *Hermannia depressa,* indicated good

inhibition of the COX-1 enzyme (Reid *et al.,* 2005) and hence further investigation into this aspect of the inflammatory cascade may provide added insight into the anti-inflammatory action of *Hermannia*. In addition, the inhibition of leukotrienes by the plant extracts may also be related to the inhibition of the 5-lipoxygenase activating protein (FLAP). Studies have indicated that cells, which contain 5-lipoxygenase and not FLAP, are unable to biosynthesize leukotriene products unless provided with a large excess of exogenous arachidonic acid substrate and thus, the plant may exhibit this mechanism of action. Further, the direct inhibition of leukotriene D_4 itself cannot be eliminated as this is another possible mechanism of action for the modulation of the pathway (Young, 1999).

H. cuneifolia, in contrast, displayed good activity against the 5-lipoxygenase enzyme and exhibited a concentration dependant effect such that increasing concentrations of extract produced a greater inhibition of the 5-lipoxygenase enzyme. This suggests that there may be interesting compounds present in the plant that may be contributing to this activity. As shown in Chapter 6, *H. cuneifolia* also displayed good antioxidant activity. Since antioxidant and anti-inflammatory activities are often related (Fernandes *et al.,* 2004), isolation may produce bioactive compounds that may be useful in treating inflammation as well as free radical scavenging activity. The formation of leukotrienes has been implicated in asthma and, therefore, isolation of these bioactive compounds may produce compounds that may be utilized in the treatment of these disease states.

Isolation of compounds from *H. cuneifolia* produced two compounds, lupeol and β-sitosterol which have, previously, been implicated in anti-inflammatory activity *in vivo* (Geetha and Varalakshi, 2000; Awad *et al.,* 2004). However, lupeol produced moderate activity against the 5-lipoxygenase enzyme *in vitro* while the activity of β-sitosterol was negligible. These

compounds do contribute to the total activity of *H. cuneifolia* in the inhibition of the enzyme yet they are not the major contributors to the activity of the crude plant extract. It also suggests that there are a number of compounds, occurring within the plant, that may react collectively to produce the anti-inflammatory activity observed.

Lupeol has previously been shown to exhibit good anti-inflammatory activity in the carrageenan and 12-*O*-tetradecanoylphorbol-13-acetate induced models of inflammation (Arciniegas *et al*., 2004) and the limited activity indicated by the compound is thus surprising. Studies into the mechanism of anti-inflammatory action of lupeol have indicated that its action is dependant on its ability to prevent the production of some pro-inflammatory mediators. However, it was shown that lupeol dose-dependently inhibited the production of PGE_2 but did not exhibit any significant effect on LTC_4 release, which strongly suggested that the triterpene did not affect the 5-lipoxygenase pathway (Fernádez *et al.,* 2001). Hence, the moderate activity of lupeol in the 5-lipoxygenase assay was understandable and further, confirms that lupeol may not affect leukotriene synthesis in its anti-inflammatory action.

In addition, the two flavones isolated from *H. saccifera* exhibited moderate inhibition of the 5-lipoxygenase enzyme with the flavone, 5,8- dihydroxy-6,7,4'- trimethoxyflavone, producing the best inhibition (51.97 % at 100 µg/ml). This indicates that these compounds contribute to the total inhibition of 5-liopxygenase by *H. saccifera* and are possible major contributors to this activity since the activity of 5,8- dihydroxy-6,7,4'- trimethoxyflavone is greater than that of the plant extract (45.8 %). However, further work must be undertaken as it is important to investigate either the synergistic or antagonistic activity of the compounds in order to determine their total effect on the inhibition of 5-lipoxygenase enzyme.

Yoshimoto *et al.* (1983) reported that certain flavonoids were potent and relatively selective inhibitors of the 5-lipoxygenase enzyme. However, it was determined that certain structural characteristics are necessary to produce potent activity. Flavones with no substituents would have limited activity. A cathechol structure (i.e. a vicinal diol at R_5 and R_6) in ring B appeared necessary to inhibit the 5-lipoxygenase enzyme. Further modification of the 5-OH group (R_1) decreases the inhibitory effect as well as the demethylation at position 7 (R_3) reduces the inhibitory effect of the flavones. The structural components of 5,8- dihydroxy-6,7,4'-trimethoxyflavone and cirsimaritin (Fig. 7.3) indicates most structural requirements are present thus, causing some activity. However, a lack of the cathechol structure in ring B is a possible reason for the moderate activity observed.

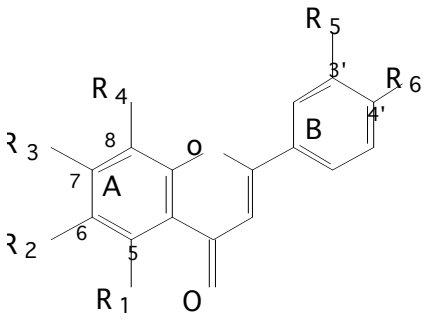


Fig. 7.2: Structure of a flavone.

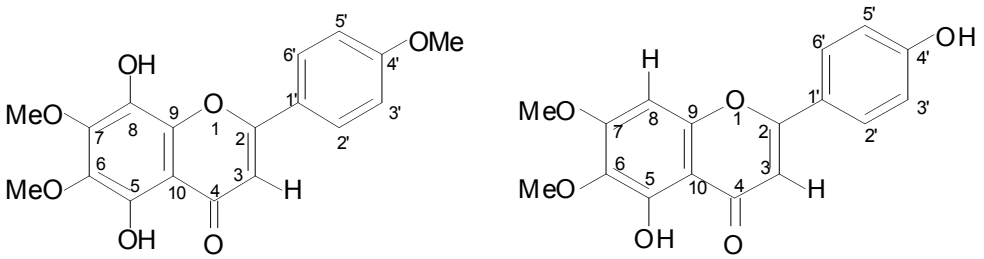


Fig. 7.3: Structure of isolated flavone compounds, 5,8- dihydroxy-6,7,4'- trimethoxyflavone (1) and cirsimaritin (2).

7.5. CONCLUSION:

The selected species of *Hermannia* investigated do not possess significant activity against the 5-lipoxygenase enzyme with the exception of *H. cuneifolia* where promising activity has been recorded. Further, compounds isolated from *Hermannia* species displayed limited inhibition against the enzyme. However, further work is necessary to determine the actual compounds possessing activity as well as to quantify them in all species to determine whether the amount of compound contained within the plant is a major contributor. In addition, while the plant extracts do not portray good activity in this assay we cannot exclude the possibility that the plants do have anti-inflammatory activity, which may be displayed at other events in the inflammatory cascade. The selected species of the genus, *Hermannia* are utilized in the treatment of a number of disease states, such as wounds, sores burns as well as hemorrhoids. Therefore, the anti-inflammatory effect portrayed by the plants may be only one aspect of the healing ability and the effect of the plants on healing may be observed at various therapeutic levels. In addition, they are also utilized in the treatment of respiratory conditions such as asthma, in which the ability to inhibit the 5-lipoxygenase is an important aspect.

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CHAPTER 8: ANTIMALARIAL ACTIVITY AND TOXICITY STUDIES

8.1. INTRODUCTION:

8.1.1. Malaria

Of all the human afflictions, the greatest toll has been exacted by malaria. Even today, malaria, which is caused by the protozoan parasites of the genus *Plasmodium,* infects and kills more people than any other infectious disease. Every year it causes clinical illness, often very severe, in 300-500 million people, 1.5-2.7 million of whom die. At present, some 90 countries or territories in the world are considered malarious, with almost half of them located in Africa south of the Sahara (Sherman, 1998).

Infection with any of the four species of *Plasmodia*, resulting in periodic paroxysms of chills, fever and sweating, anemia and splenomegaly, is the cause of the disease known as malaria. Transmission begins when a female *Anopheles* mosquito feeds on a person with malaria and ingests blood-containing gametocytes. During the following one to two weeks, gametocytes inside the mosquito reproduce sexually and develop into infective sporozoites. When the mosquito feeds on a human, it inoculates sporozoites, which quickly infect the host's hepatocytes. This does not produce clinical illness. However, schizogony occurs within infected hepatocytes; one to two weeks later they rupture and release merozoites that invade the red blood cells and there transform into rings and trophozoites. These young trophozoites grow and develop into schizonts, which then rupture the red blood cell resulting in anaemia, jaundice and eventual death (Beers and Berkow, 1999). Fig. 8.1 illustrates the simplified life cycle of *P. falciparum.*

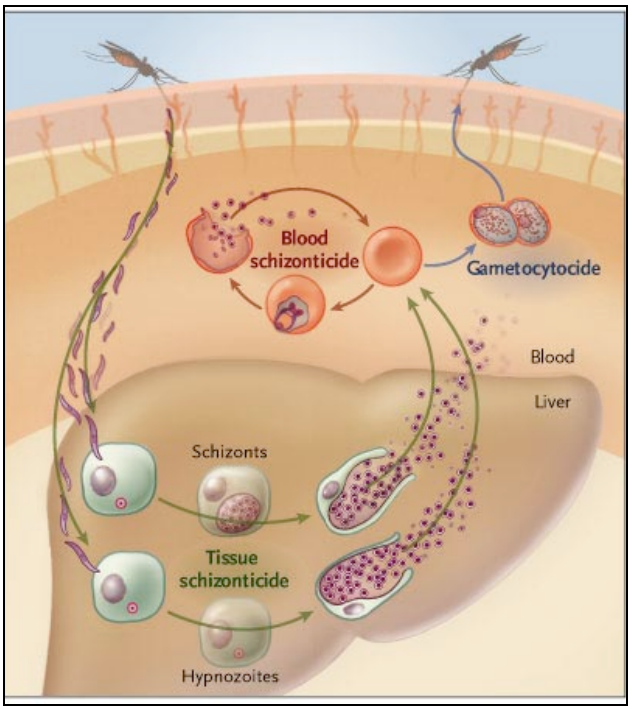

Fig. 8.1: Life cycle of *P. falciparum* in the human host (Baird, 2005).

Over the past 20 years, efforts to control malaria have been less successful (Sherman, 1998). This is largely due to the development of resistance by the malaria parasite to nearly all the antimalarial drugs used for prophylaxis and treatment, particularly in *P. falciparum* (Nwaka *et al.*, 2004). Since the treatment and control of malaria depends largely on a limited number of chemoprophylactic and chemotherapeutic agents, there is an urgent need to develop novel, affordable antimalarial treatments. Historically, the majority of antimalarial drugs have been derived from medicinal plants or from structures modeled on plant lead compounds. These include the quinoline-based antimalarials, as well as artemisinin and its derivatives.

Medicinal plants are commonly used in South African traditional healthcare to treat a range of ailments, including malaria and its associated symptoms (Clarkson *et al.*, 2004). Traditional

medicine is, therefore considered as a rich source of potential drugs for the treatment of fevers and malaria and thus it seems worthwhile to examine these plants closely using modern scientific methods (François *et al.*, 1996). To contribute towards this desperate need for further life-saving treatment, *Hermannia* species were systematically investigated for antimalarial activity.

8.1.2. Toxicity

It is difficult to believe that the 'green world' is not entirely friendly. However, a surprisingly large number of plants contain toxic substances that can kill any creature that eats enough of them (Dowden, 1994). Most common herbal remedies are fairly safe in clinical use, not because they are 'natural', but because the long history of use has uncovered some of the side effects. Traditional use, however, is not always a reliable indication of safety and, because many patients do not consider phytomedicines to be 'drugs', an association may not have been made between the remedy and the problem. Alternatively a long interval between taking the medicine and the onset of a reaction may make the connection difficult (Heinrich *et al.*, 2004).

In South Africa, poisoning by traditional remedies especially those obtained from plants is well documented (Steenkamp, 2000). Cases of acute poisoning due to traditional medicines are not uncommon, many of which have resulted in significant morbidity and mortality, with mortality estimated to be as high as 10,000 to 20,000 per annum (Popat *et al.*, 2001). Only a few plants found in traditional medicines contain pharmaco-active chemicals that are toxic. However, these seem to make a significant contribution to morbidity and mortality (Steenkamp, 2000).

The extensive use of traditional medicines in South Africa indicates an urgent need to ensure that many of these plants, that are utilized, do not contain phytochemicals that make them hazardous for use and for this reason identification of potential toxicity of medicinal herbs has been identified as a top research priority. In addition, should these plants possess extensive biological activity, this data is necessary in assessing the possibility of developing a drug from the natural source.

8.2. METHODS:

8.2.1. Antimalarial activity

Antimalarial activity of all twelve of the selected species of *Hermannia* was assessed *in vitro* using the titrated hypoxanthine incorporation assay (Desjardins *et al.,* 1979; van Zyl and Viljoen, 2002). Methanol extracts were made of all plants and were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 10 mg/ml.

8.2.1.1. Principle of assay

DNA and RNA are polymers of nucleotides. Nucleotides consist of a ribose sugar group linked to either a purine (adenine and guanine) or a pyrimidine (cytosine, uracil, and thymine) base. These bases can either be obtained via *de novo* synthesis or from the environment by the 'salvage' pathway. The malarial parasite obtains preformed purines by the salvage pathway and synthesizes pyrimidines *de novo*.

The primary purine salvaged by the parasite is hypoxanthine which can be obtained from the host plasma. Hypoxanthine is a naturally occurring purine derivative which is present in inosine monophosphate from which adenosine monophosphate and guanosine monophosphate are made. Malaria parasites (*P. falciparum*) grow and replicate in human red blood cells. This

stage of their life cycle results in the morbidity and mortality associated with the disease. Replication depends on available sources of purine and pyrimidine precursors for DNA and RNA synthesis. While parasites are able to synthesize pyrimidines *de novo*, they depend entirely on their host for pre-formed purines. Previous work showed that hypoxanthine is the preferred form of purine utilized by growing parasites (Berman *et al.,* 1991).

Desjardins *et al.* (1979) developed an *in vitro* drug-sensitivity assay based on the incorporation of tritiated-labeled hypoxanthine. This method is based on the inhibition of uptake of a radiolabeled nucleic acid precursor (hypoxanthine) during short term cultures in microtitration plates. Hypoxanthine is only incorporated into living cells thus, if the plant extract is active against the *Plasmodium* the protozoa will die and thus no hypoxanthine will be incorporated and therefore a lower value will be obtained. Inhibition of uptake of the radio labeled nucleic acid precursor by parasites, thus serves as an indicator of antimalarial activity.

8.2.1.2. Protocol

8.2.1.2.1. Culturing of parasites

The parasite strain used in these experiments was an isolate of a chloroquine-resistant *P. falciparum* - FCR-3 (van Zyl and Viljoen, 2002). The parasites were continuously maintained in a 5 % suspension of human erythrocytes with a culture medium consisting of 10.4 g/L RPMI–1640, 5.9 g/L HEPES (*N*-2-hydroethylpiperazine-*N'*-2-ethanesulfonic acid), 4.0 g/L glucose, 44 mg/L hypoxanthine and 50 mg/ml gentamicin sulphate, which was supplemented by 10 % (v/v) human plasma and 5 % (w/v) sodium bicarbonate. The culture medium was filtered through a Sterivex–GS 0.22 μm filter unit before use to ensure sterility. The plasma was heat inactivated at 56°C in a water bath for 2 hours before being centrifuged at 3000 rpm for 20 min after which it was stored at -20˚C. Citrate phosphate dextrose adenosine-1 was

added to prevent coagulation of red blood cells. Cultures containing predominantly early ring stages were used for drug-sensitivity testing. Cultures were synchronized every second day using a 5 % (w/v) D-sorbitol solution (Lambros and Vanderberg, 1979). Cells were cultured at 37°C with fresh erythrocytes added every second day. Cells were maintained at less than 5 % infected with parasites.

Blood was obtained from the South African National Blood Services. Before use blood was washed three times in phosphate buffer saline (PBS, pH 7.4) by centrifugation at 2000 rpm for 5 min after which the buffy coat and serum were discarded. PBS consisted of NaCl (8.0 g), KCl (0.3 g), $Na_2HPO_4.2H_2O$ (0.73 g) and KH_2PO_4 (0.2 g) dissolved in 1 L sterile MilliQ® water and was autoclaved. The erythrocytes were then suspended in experimental medium (hypoxanthine and gentamicin deficient culture medium) to prevent dehydration and stored at 4°C.

8.2.1.2.2. Hypoxanthine incorporation assay

A 96-well microtitre plate was used to measure antimalarial activity. The plates and parasites were prepared using strict aseptic techniques inside a laminar flow hood. Each dilution of extract (25 µl) was plated out in triplicate. Parasite suspension (200 µl) that had been adjusted to 0.5 % parasitaemia and 1 % of haematocrit, was added to the wells containing the extract. To prepare the drug-free parasite control, 200 µl of parasites were added to 8 wells containing no extract. A 1 % solution of red blood cells (RBC) that were parasite free were added to 4 wells and served as the non-parasitized erythrocyte control. Hypoxanthine-free media (25 µl) was added to these wells to ensure that the volume in each cell was correct. The plate was then incubated at 37°C in a humidified candle jar for 24 hours.

Radio labeled [^{3}H]-hypoxanthine (10 µl/plate) (5 mCi) (Amersham, UK) stabilized in ethanol:water (1:1, v/v) solution was 50 % evaporated off and 2.7 ml experimental media was added to adjust to 18 µCi. The isotope (25 µl) was added to each well and the plate was incubated for a further 24 hours. The parasite DNA was then harvested on GFB-filtermats with a Titertek® cell harvester. The filtermats were then dried and the incorporated [^{3}H]-hypoxanthine was measured in counts per minute (cpm) by a liquid beta scintillation counter determined

Percentage parasite growth of untreated parasitized and erythrocyte controls were used to express the inhibitory effects of the plant extracts on the malaria parasite.

$$\% \text{ Parasite Growth} = \frac{\text{(Plant extract) cpm} - \text{(Mean RBC control) cpm}}{\text{(Mean Parasite control) cpm} - \text{(Mean RBC Control) cpm}}$$

Logarithmic transformation of the concentration allowed sigmoid-dose response curves to be plotted from which the IC_{50} value i.e. the concentration required to inhibit 50 % of parasite growth, was obtained using the Enzfitter® software. The plant extracts were replicated in triplicate to ensure accuracy of results.

8.2.2. Toxicity testing

8.2.2.1. Principle of assay

The measurement of cell viability and growth is a valuable tool in a wide range of research areas. Viable cells can be measured by using any of several staining methods. Ideally a colorimetric assay for living cells should utilize a colorless substrate that is modified to a colored product by any living cell but not dead cells or tissue culture medium. Tetrazolium salts are attractive candidates for this purpose since they measure the activity of various dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells (Mosmann, 1983). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay is a rapid colorimetric test based on the tetrazolium salt, MTT. This is a yellow salt which is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of DMSO. The color can then be spectrophotometrically quantified. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate quantification of changes in proliferation.

8.2.2.2. Protocol

Transformed human kidney epithelium (Graham) cells were used to test the toxicity of the various plant extracts using the MTT colorimetric microtitre plate method developed by Mosmann (1983). Extracts were made up to 10 mg/ml solutions in DMSO from which further dilution were made.

8.2.2.2.1. Culturing of cells

The culture medium containing Ham F10 solution (9.38 g of Ham's F10 medium and 1.18 g of $NaHCO_3$ in 1 L of sterile water), 5 % fetal calf serum (inactivated at 56°C for 1 hour) and 50 mg/ml gentamicin sulfate was utilized to maintain the Graham cells. The culture medium was replaced every second day.

8.2.2.2.2. MTT cell proliferation assay

When cells had reached confluency, they were trypsinised using 4 ml of 0.25 % Trypsin/0.1 % Versene EDTA that was at 25°C. Experimental medium (containing Ham F10 solution (9.38 g of Ham's F10 medium and 1.18 g of $NaHCO_3$ in 1 L of sterile water), 5 % fetal calf serum (inactivated at 56°C for 2 hours) was then added and a single cell suspension of the cells was obtained. One milliliter of the suspension was used to reseed the culture, while the rest was utilized in the MTT assay. The cell density was assessed by staining them with Tryptan Blue in a 1:1 ratio and then counted with a haemocytometer. A 95 % cell viability was ensured before use of cells. The cell suspension was diluted using experimental medium to 0.25 million cells per milliliter and 180 µl of this suspension was added to the 96-well microtitre plate. The plate was incubated under a humidified environment at 37°C with 5 % CO_2 for six hours to allow the cells to adhere to the bottom of plates. The different concentrations of extracts (20 µl) were plated out in triplicate. DMSO was added to ten wells with no extract being added and this served as the cell control, while two wells served as the cell-free control with only experimental medium and DMSO added to these wells. The plates were incubated for 44 hours after which 40 µl of a 240 mM MTT solution prepared in PBS buffer (section 8.2.1.2.1) was added to each well. Thereafter the plate was incubated for a further 4 hours. The supernatant (180 µl) from each well was removed and 150 µl of DMSO added to stop the

reaction as well as solubilize the formazan crystals. The plates were shaken at 1020 rpm for 4 min and the absorbance read at 540 and 690 nm using a microplate reader (Labsystems iEMS Reader MF) and Ascent® software. The results were expressed as percentage cellular viability of the drug and cell-free controls. Sigmoid dose-response curves were obtained after logarithmic transformation of the concentration using the Enzfitter® software from which the IC_{50} values were obtained. The extracts were tested in triplicate. To determine selectivity of the extracts for the parasite and the relative toxicity profile of the extracts, the safety index was calculated by the following formula:

$$\text{Safety Index} = \frac{\text{Toxicity } (IC_{50})}{\text{Antimalarial Activity } (IC_{50})}$$

8.3. RESULTS:

The antimalarial and toxicity profiles for *Hermannia* are listed in Table 8.1. The safety indexes are included for species, *H. saccifera* and *H. trifurca*. Most species indicated moderate antimalarial activity with *H. muricata*, *H.saccifera* and *H. trifurca* showing any promising activity. *H. trifurca* exhibited the best activity with an IC_{50} value of 18.806 ± 1.113 mg/ml which is 553 fold less active than quinine as seen in Fig 8.2.

The results obtained for toxicity indicate that most species investigated have an IC_{50} value that is >200 µg/ml. Only two species indicated a value lower than 100 µg/ml with *H. saccifera* and *H. trifurca* being 61.403 ± 4.56 and 75.613 ± 6.11 µg/ml, respectively. However, the safety index for both species is considerably lower than that of the controls with *H. saccifera* and *H. trifurca* having a safety index of 2.426 and 4.021 respectively. In comparison, the controls, chloroquine and quinine have a safety index of 2092.67 and 4001.76, respectively.

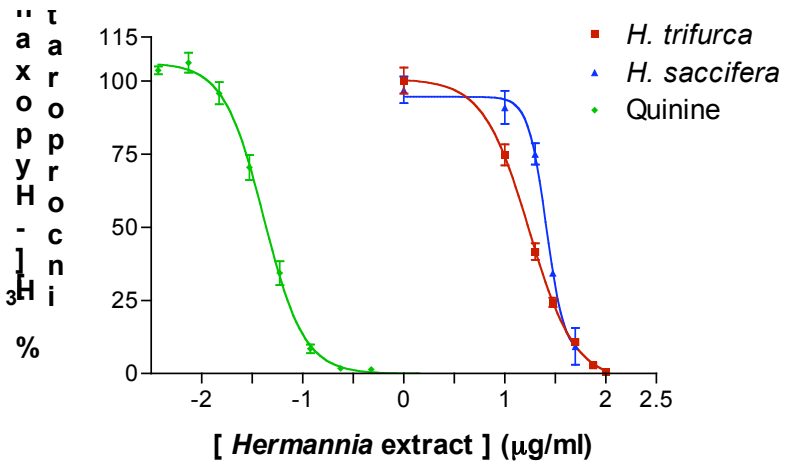

Fig. 8.2: Sigmoid-dose response curves for *H. saccifera*, *H. trifurca* and quinine.

Table 8.1: Antimalarial activity, toxicity profile and safety index for selected species of *Hermannia* in µg/ml.

Species	Antimalarial activity			Toxicity profile			Safety index
	IC_{50}	**s.d.***	**n***	**IC_{50}**	**s.d.***	**n***	
H. althaeifolia	58.49	3.96	3	>200		3	
H. cuneifolia	50.67	2.96	3	>200		3	
H. flammula	55.84	2.62	3	>200		3	
H. holosericea	52.35	2.12	3	>200		3	
H. incana	68.13	1.81	3	>200		3	
H. involucrata	46.08	3.23	3	>200		3	
H. lavandufolia	70.38	3.98	3	>200		3	
H. muricata	28.17	3.72	3	>200		3	
H. saccifera	25.30	0.96	3	61.403	4.56	3	2.43
H. salviifolia	64.38	2.16	3	>200		3	
H. scabra	88.57	2.41	3	>200		3	
H. trifurca	18.80	1.11	3	75.613	6.11	3	4.02
Chloroquine	0.06	0.003	6	125.56	5.04	3	2092.67
Quinine	0.03	0.002	6	136.06	4.06	3	4001.77

* s.d. = standard deviation
n = number of times experiment was replicated

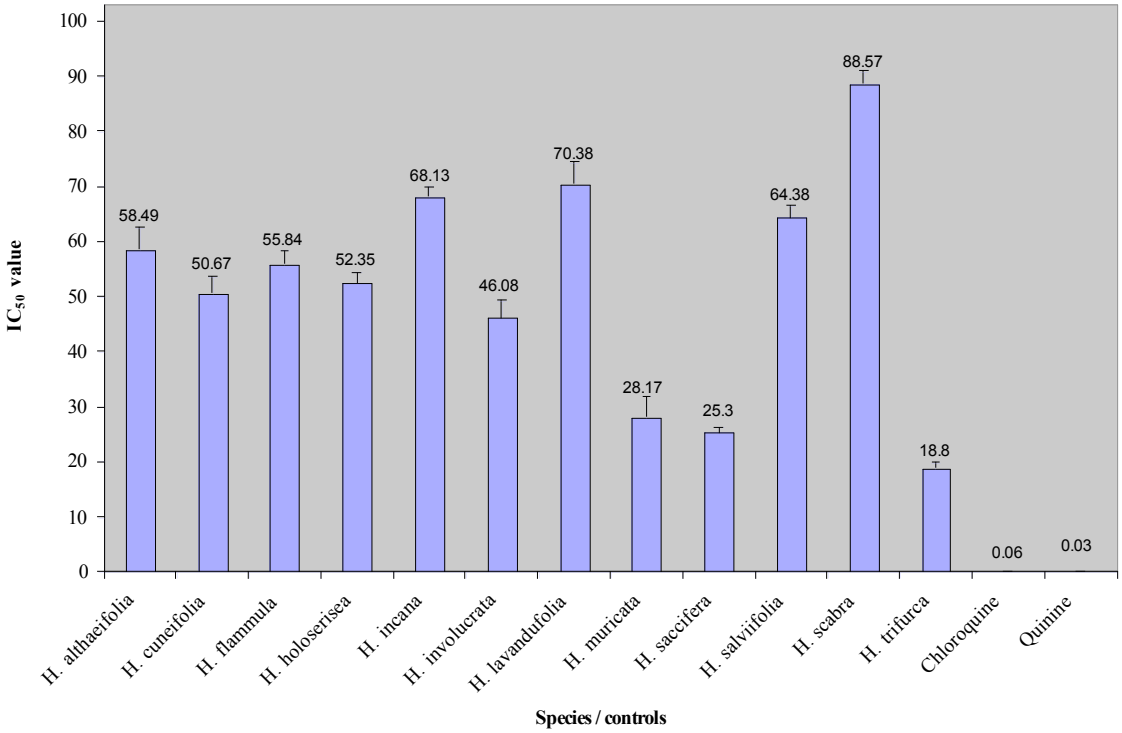

Fig. 8.3: Bar chart indicating antimalarial activity of twelve *Hermannia* plant extracts and reference compounds (IC_{50}) as well as indicating standard deviation.

8.4. DISCUSSION:

The results indicated that there was a concentration-dependant activity with an increasing concentration producing greater activity in the tritiated hypoxanthine incorporation assay (Fig 8.2). However, the activities portrayed by the various species were not promising with only *H. trifurca* indicating any promising activity (Fig 8.3.; Table 8.1). This suggests that *H. trifurca* may be a suitable species for further phytochemical investigation.

Most species of *Hermannia* appeared to be relatively non-toxic as most species displayed IC_{50} values greater than 200 µg/ml. However, the two species, *H. saccifera* and *H. trifurca*, that indicated greater toxicity are also those species that showed activity in the antimalarial assay. These plants portrayed a concentration dependant effect with increasing concentrations

causing increasing cell death. This possibly indicates that the antimalarial activity is not specific for these plants, but rather their activity is based on a general toxicity of living cells which is highlighted by the low safety index displayed in Table 8.1. The identification of compounds that are active may provide further evidence or explanations of the relationship between activity and toxicity of these species.

H. saccifera has previously indicated excellent activity against a number of micro-organisms in the antimicrobial activity assays (Table 5.2.). This activity may be related to the toxicity and non-specific cell-death that is caused by these plants. Identification of active compounds present in *H. saccifera* is essential to obtain a clearer understanding of the wide spectrum of activity that is displayed by this plant. In addition, further studies on these compounds should establish possibilities of the use of the compounds in the treatment of microbial diseases relating to safety of their use.

H. muricata has displayed antimalarial activity with an IC_{50} value of 28.176 µg/ml. In addition the toxicity profile indicates that the IC_{50} value is >200 µg/ml with a safety index of >7.098 (Table 8.1.). The safety index is higher than that of the other two active species and while still being lower than the controls, the accurate value may yield increased safety data, suggesting that this activity may be more specific to the malaria parasite than the other species tested. Further, *H. muricata* has not shown good antimicrobial activity further suggesting that this activity is more specific to the *Plasmodium* parasite. This requires further investigation to determine the active compounds and their potential toxicity as this species may yield novel drugs or drug templates with novel mechanisms of action against the parasite which will be of importance as the development of resistance by the parasite to current treatments spirals.

Only one species of *Hermannia* has previously been assayed for its antimalarial activity, i.e. *Hermannia depressa* (Clarkson *et al.,* 2004). The assay utilized in assessing the activity was the parasite lactate dehydrogenase assay in which it produced an IC_{50} value of 6.9 µg/ml. The IC_{50} values obtained for the various species investigated is considerably higher than this value. While the differences in the assay utilized could be contributing to the variation in values, it can be seen that certain *Hermannia* species have greater anti-malarial activity than others. Hence, it is necessary to determine the active compounds in these species to determine if one or more different compounds contribute to the activity in the various species.

8.5. CONCLUSION:

The various species of *Hermannia* investigated have indicated a degree of antimalarial activity with only *H. saccifera*, *H. muricata* and mostly *H. trifurca* possessing any promising activity. The toxicity testing of the various species indicates the species are fairly safe with the majority of species having an IC_{50} of >200 μg/ml. The safety index however indicates that the activity obtained by *H. saccifera* and *H. trifurca* have a non-specific action causing the death of any cell including healthy cells. On the otherhand, *H. muricata* appears to be of greater interest, as it is considerably less toxic with substantial activity and thus, further investigation is required. These species are often used to treat symptoms such as fevers, which in Africa are often related to or are used as a description of malaria. While the species do not portray extensive activity and an activity which may be non-specific, certain species have displayed good activity against *Plasmodium falciparum*. Further, the lack of *in vitro* activity should not disqualify the use of the plants as traditional antimalarials. While plant extracts may not display *in vitro* activity they may display *in vivo* activity in that the active compounds may require *in vivo* activation such as artemisinin. Therefore, it is necessary to undertake *in vivo* tests before a conclusion can be reached (Muregi *et al.,* 2003). Finally, although limited activity was portrayed, there is some scientific basis for the traditional use of this plant in the treatment of malaria as well as further indicating that traditional use of plants have over the centuries managed to determine which plants are safe as well as active, substantiating the use of the plants.

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CHAPTER 9: GENERAL CONCLUSION

- TLC analysis indicated that the species are chemically uniform with only *H. saccifera* having differences in composition. HPLC results, further, indicated conservative profiles with similar profiles being produced by all species being investigated.

- Isolation of compounds from *H. saccifera* produced a novel labdane-type compound, *E*-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene. In addition, two known flavone compounds, 5,8- dihydroxy-6,7,4'- trimethoxyflavone and cirsimaritin, were isolated. HPLC analysis indicates that the two flavone compounds occur in *H. saccifera* only. This is the first report of the presence of these compounds in *Hermannia* species.

- All twelve species exhibited antimicrobial activity. The most promising activity was displayed by *H. saccifera* from which bioguided fractionation yielded the active diterpene, *E*-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene. The compound displays promising activity (23.6 µg/ml against *S. aureus*) which, although higher MIC values were determined than the crude plant extract (19.5 µg/ml against *S. aureus*), may be a possible lead compound in the search for new potent antibacterial compounds.

- The death kinetics assay conducted on the crude extract of *H. saccifera* indicated that this activity is bacteriostatic rather than bactericidal with only high concentrations (0.75 %) indicating bactericidal activity.
- Potential free radical scavenging activity was exhibited by ten of the twelve species investigated in both the DPPH and ABTS assays. The ABTS results were found to be

consistently higher due to its ability to function via two mechanisms. *H. cuneifolia* displayed the most promising activity (IC_{50} = 10.26 µg/ml in DPPH assay). Surprisingly, the two flavone compounds investigated had negligible free radical scavenging activity which may be due to the presence of fewer hydroxyl groups present on the molecule.

- The *Hermannia* species investigated displayed limited inhibition of the 5-lipoxgenase enzyme with the exception of *H. cuneifolia* with produced good activity (IC_{50} = 15.32 µg/ml). The two flavones as well as lupeol and β-sitosterol that have been isolated indicate moderate activity in the assay, indicating that these compounds do contribute to the activity of the plant extracts. The flavone compounds, in addition, lack chemical structural features that are required to potentiate the inhibition of the 5-lipoxygenase enzyme.

- *Hermannia* species displayed moderate antimalarial activity with only *H. saccifera*, *H. muricata* and mostly *H. trifurca* possessing good activity. However, toxicity studies indicate that the activity of *H. saccifera* and *H. trifurca* may be due to a non-specific destruction of cells including healthy cells. *H. muricata*, thus, may be a potential source of antimalarial compounds due to its superior activity in the antimalarial assay as well as the favorable safety index.

- The biological activity observed in *Hermannia* species provides a scientific basis for the use of the plants in traditional medicines with each facet of activity contributing to the ultimate healing ability of the plant.

Recommendations for further work

- HPLC should be conducted using a detector which will be able to detect compounds that are poor chromophores. A refractive light or electrochemical detector may be useful.

- Further isolation is required to determine compounds producing the various biological activities portrayed by the plant and the activity of these compounds require quantification. In addition, the synergistic or antagonistic properties of these compounds should be determined.

- The chemical structure of *E*-17, 19-diacetoxy - 15 - hydroxylabda - 7,13 - diene should be reinvestigated to determine the structural-activity relationships of the compound and then compound manipulation may be utilized to improve the antimicrobial activity of the compound. In addition, the compound should be investigated for biological activities other than antimicrobial activity to determine the full spectrum of its use in the process of healing. However, more material is first required to record the physical data as well as to determine the toxicity of these compounds.

- Antioxidant activity may be displayed by various mechanisms and hence the plant extracts should be investigated in other assays to determine if the activity is limited to free radical scavenging ability. In addition, active compounds should be quantitated within each plant species.

- While plant extracts indicated limited inhibition of the 5-lipoxygenase enzyme, there are many events in the inflammation cascade that may be interrupted, hence decreasing

the symptoms of inflammation in the body. The plants should be investigated using different assays that assess other events in the cascade.

APPENDIX I

Hermannia: Antibacterial Activity and Phytoconstituents of Selected Species of a Previously Unresearched Genus Used in Traditional Medicine

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PURPOSE:

To evaluate the antibacterial activity of selected species of the genus, *Hermannia*, and to isolate and identify the bioactive compounds, as well as other phytoconstituents present within the various species.

METHODS:

Ten species of *Hermannia* were chosen due to their widespread use in traditional medicine. Plants were collected and crushed into powder. Acetone was used to obtain extracts from 5mg of powder of all selected species. Broad screen assays were carried out using the disc diffusion method on the following bacteria: *Staphylococcus aureus, Baccilus cereus, Escheria coli.* 500g of active species were then extracted using acetone and were fractionated using column chromatography firstly in groups sharing similar physicochemical characteristics. Bioguided fractionation, column chromatography and preparative thin-layer chromatography were utilized to isolate bioactive compounds. Pure compounds were characterized using NMR spectroscopy.

500g of certain species were selected, based on traditional use, were extracted using dichloromethane: methanol (1:1). Some phytoconstituents were isolated and characterized using methods described above. High performance liquid chromatography was used to

evaluate the compounds present in all species and ultra-violet spectrums were compared for similarities.

RESULTS:

Of the ten species selected, two species viz. *H. althaeifolia* and *H. cuneifolia* were found to be antibacterially active. Isolation using bioguided fractionation supplied interesting aromatic compounds. Active compounds were confirmed using bioautographic assays and disc diffusion.

Species were found to be similar in character with regard to phytoconstituents present.

CONCLUSIONS:

Hermannia is a genus that is widely used in traditional medicine for ailments such as respiratory infections. The results indicate that certain species are antibacterially active and isolated compounds have potential use in this area. Thus, potentially, this neglected genus may be, extensively pharmacologically active.

The similarity of phytoconstituents within the selected species suggests a possibility of similar compounds across the board within the species of the genus.

APPENDIX II

Hermannia: The Biological Activity and Phytoconstituents of an Unexplored Genus Used in African Traditional Medicine

Ayesha B. Essop[*1], Alvaro M. Viljoen[1], Dulcie A. Mulholland[2], Sandy F. van Vuuren[1].
[1]Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 2001.
[2] Natural Products Research Group, School of Chemistry, University of KwaZulu-Natal, Durban, 4041

PURPOSE:

To evaluate the biological activity and phytoconstituents of selected species of the previously unresearched genus, *Hermannia*, that is used in African traditional healing.

METHODS:

Ten species of *Hermannia* were chosen due to their widespread use in traditional medicine. Plants were collected and crushed into powder. Acetone extracts of all selected species were assessed for antibacterial activity, firstly using the broad screen disc diffusion assay on *S. aureus* (ATCC 12600), *B. cereus* (ATCC 11778) and *E. coli* (ATCC 1175) after which, the minimum inhibitory concentrations of the most active species were determined. Bioautograms were used to indicate compounds that possessed activity.

Methanol extracts were used to evaluate the antioxidant activity of the ten selected species utilizing the DPPH assay and the IC_{50} for each plant was calculated.

Characterization of the phytoconstituents of two species, *H. salviifolia* and *H. cuneifolia* was attempted. A methanol: dichloromethane (1:1) extract was placed on a column and separated using a gradient solvent system.

Five compounds were isolated from the above species of which two, lupeol and β-sitosterol, have been characterized. Lupeol and the crude extract of *H. cuneifolia,* from which lupeol was isolated, were assessed for *in vitro* anti-inflammatory activity using the 5-lipoxygenase assay.

Finally, HPLC-UV-MS was used to analyze the chemical diversity of the ten species.

RESULTS:

Three species, *H. cuneifolia*, *H. althaeifolia* and *H. saccifera* possessed antimicrobial activity with *H. saccifera* indicating remarkable activity with a MIC of 19.5 µg/ml against two bacterial species.

Nine species possessed promising antioxidant activity with IC_{50} of between 10-30 µg/L.

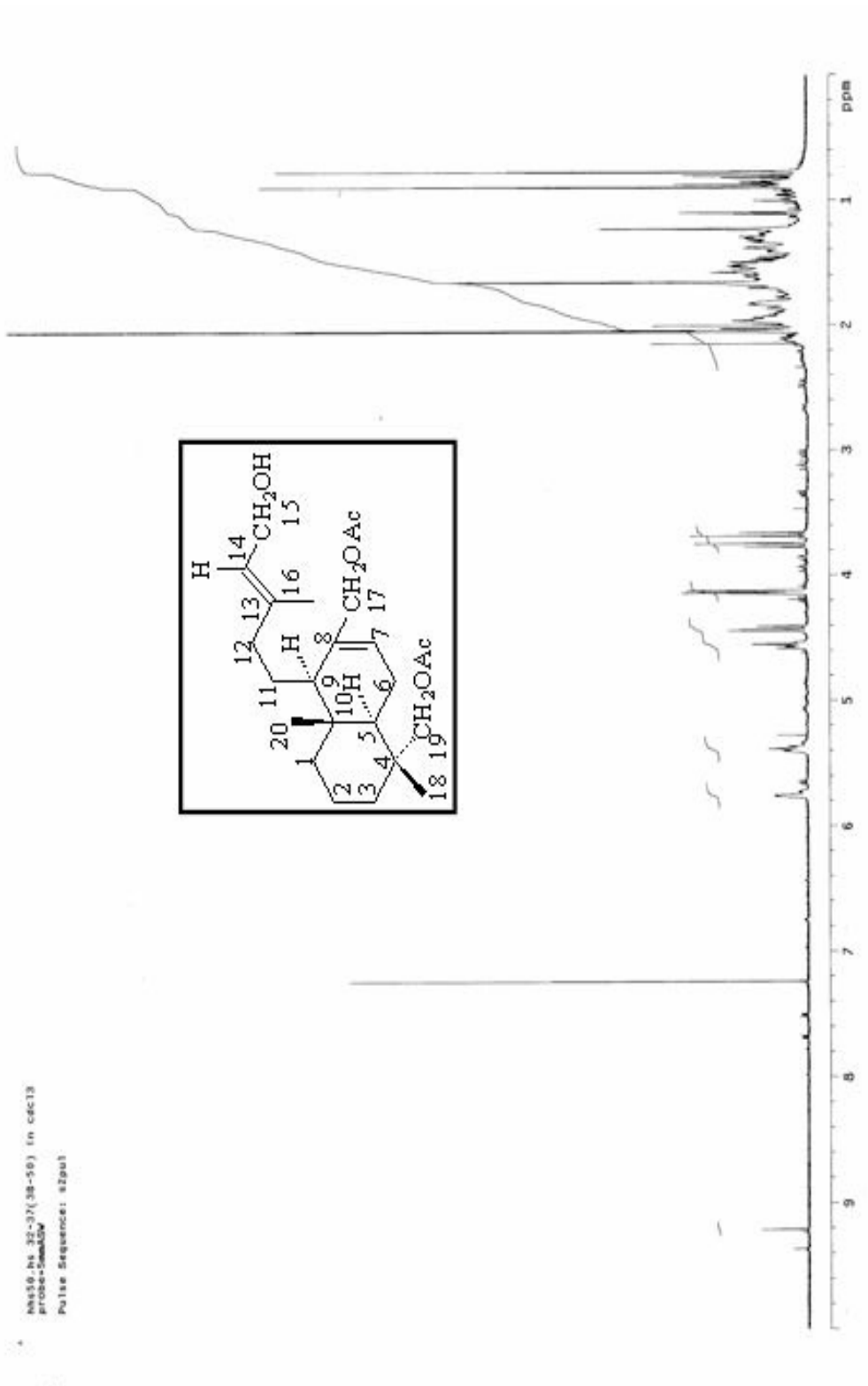
Five compounds were isolated of which two have been characterized as being lupeol and β-sitosterol. In addition lupeol inhibited the 5-lipoxygenase enzyme by 78% at 100ppm. However, the crude extract of *H. cuneifolia* totally inhibited the enzyme.

Finally, HPLC profiles and corresponding UV spectrums indicate extensive similarities between the species, which is corroborated by the TLC plates with the exception of *H. saccifera*, which appears to be chemically anomalous.

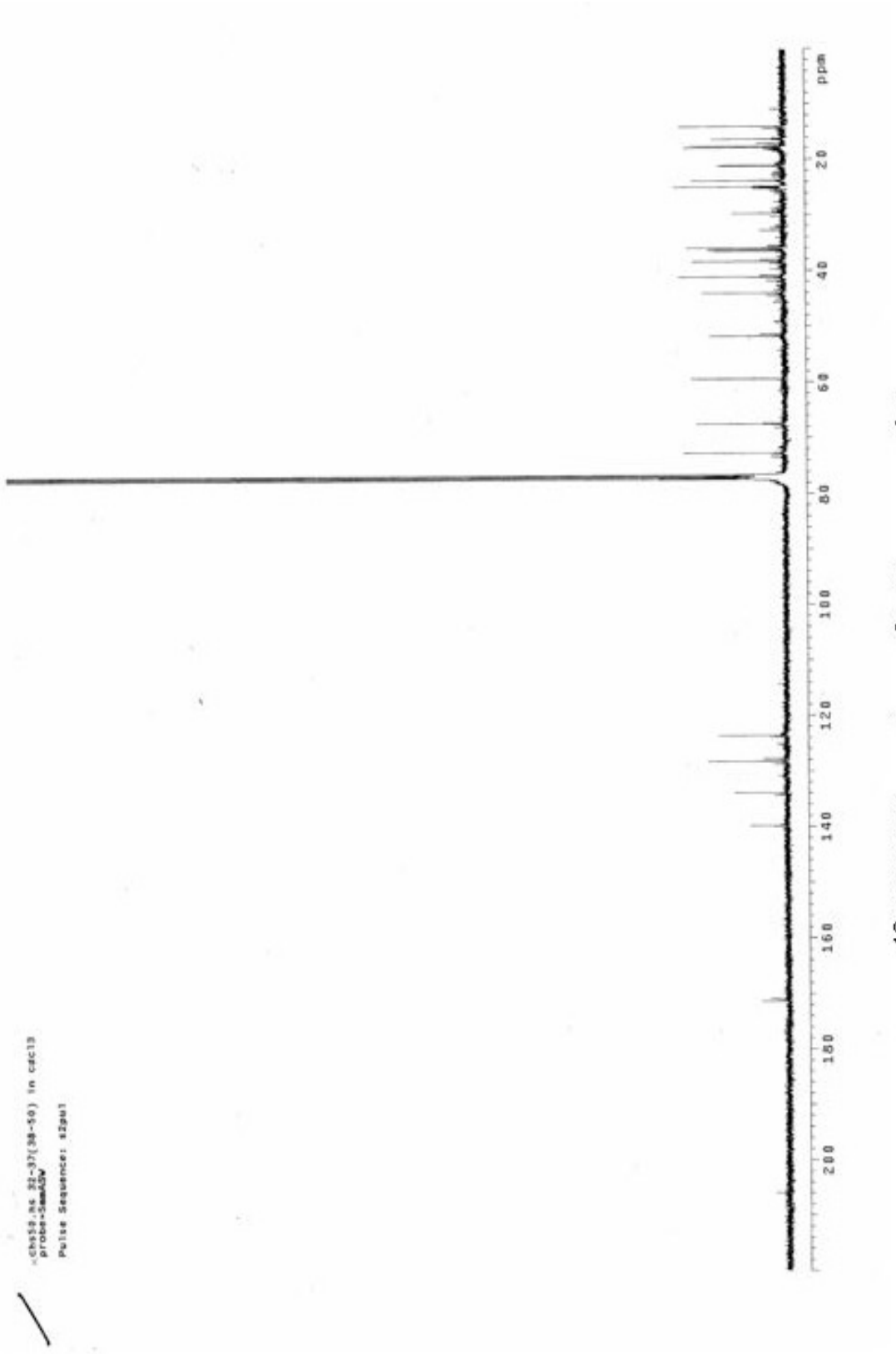
CONCLUSIONS:

The results indicate some correlation, albeit in vitro, between the biological activity and traditional use of these plants which include treating wounds and burns as well as infections Since this is, surprisingly, the first study to be conducted on this genus, isolation of these active compounds may produce interesting bioactive compounds that may play an important role in the treatment of diseases.

APPENDIX III



1H NMR spectrum for Compound 1



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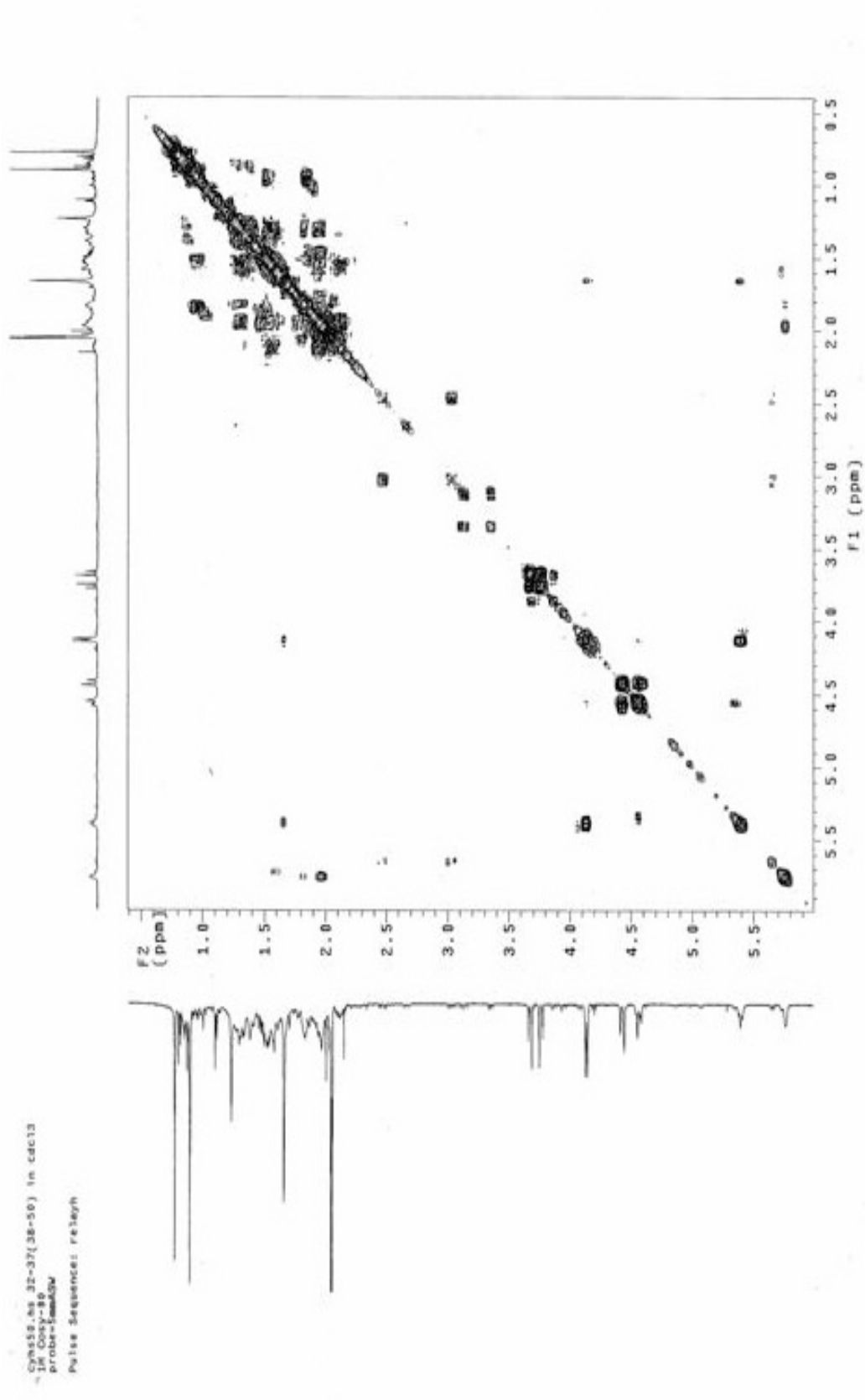
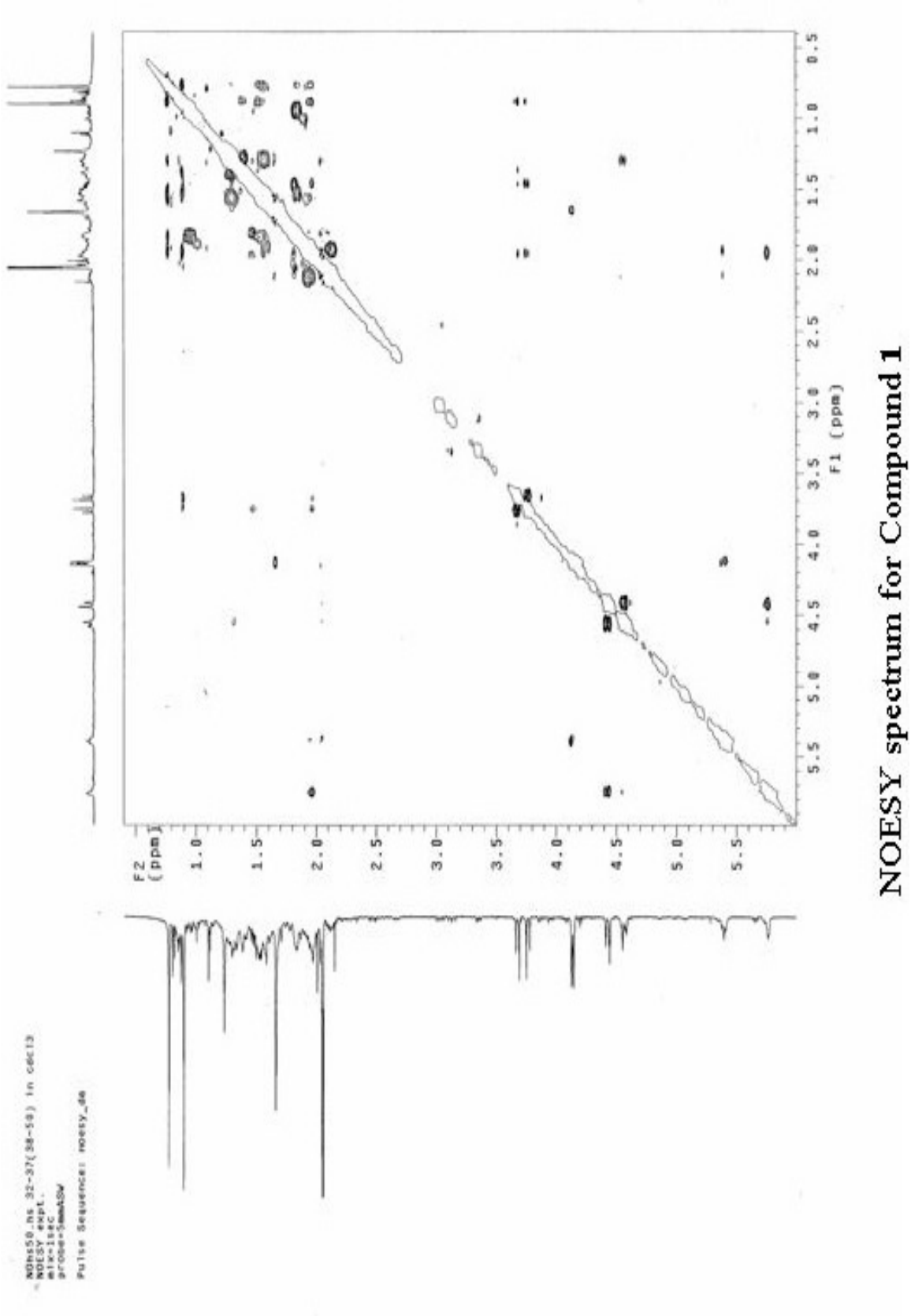
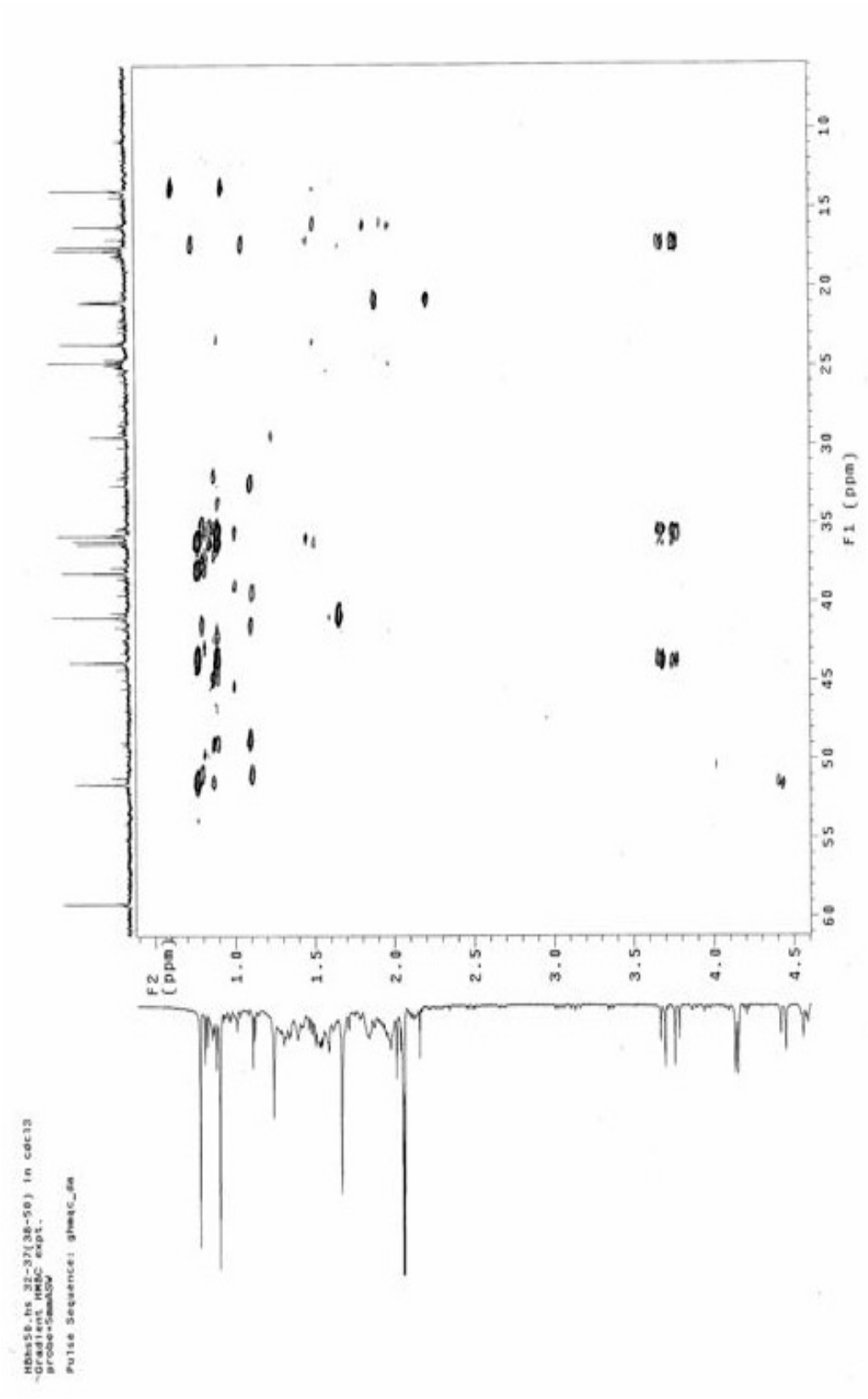


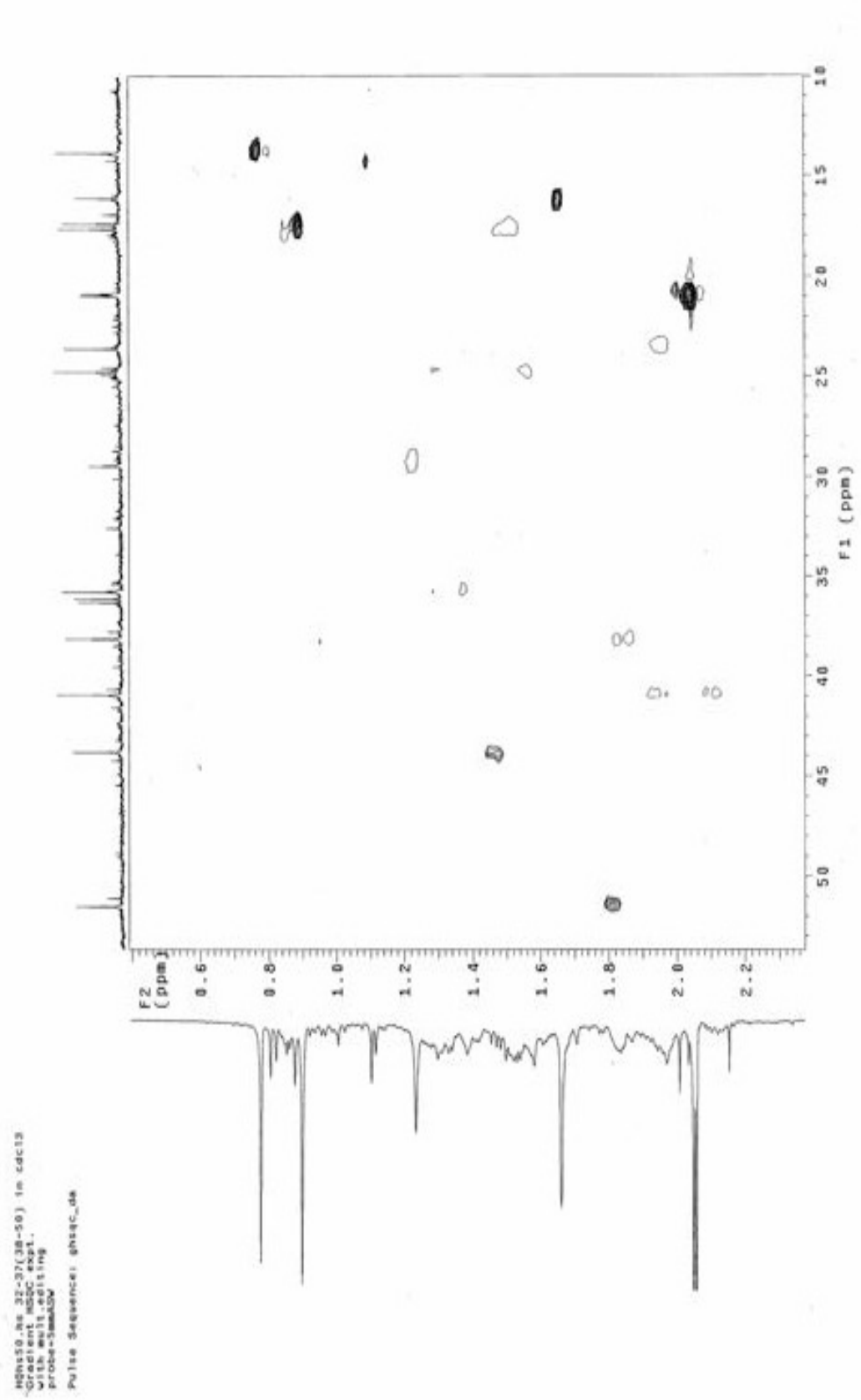
COSY spectrum for Compound **1**



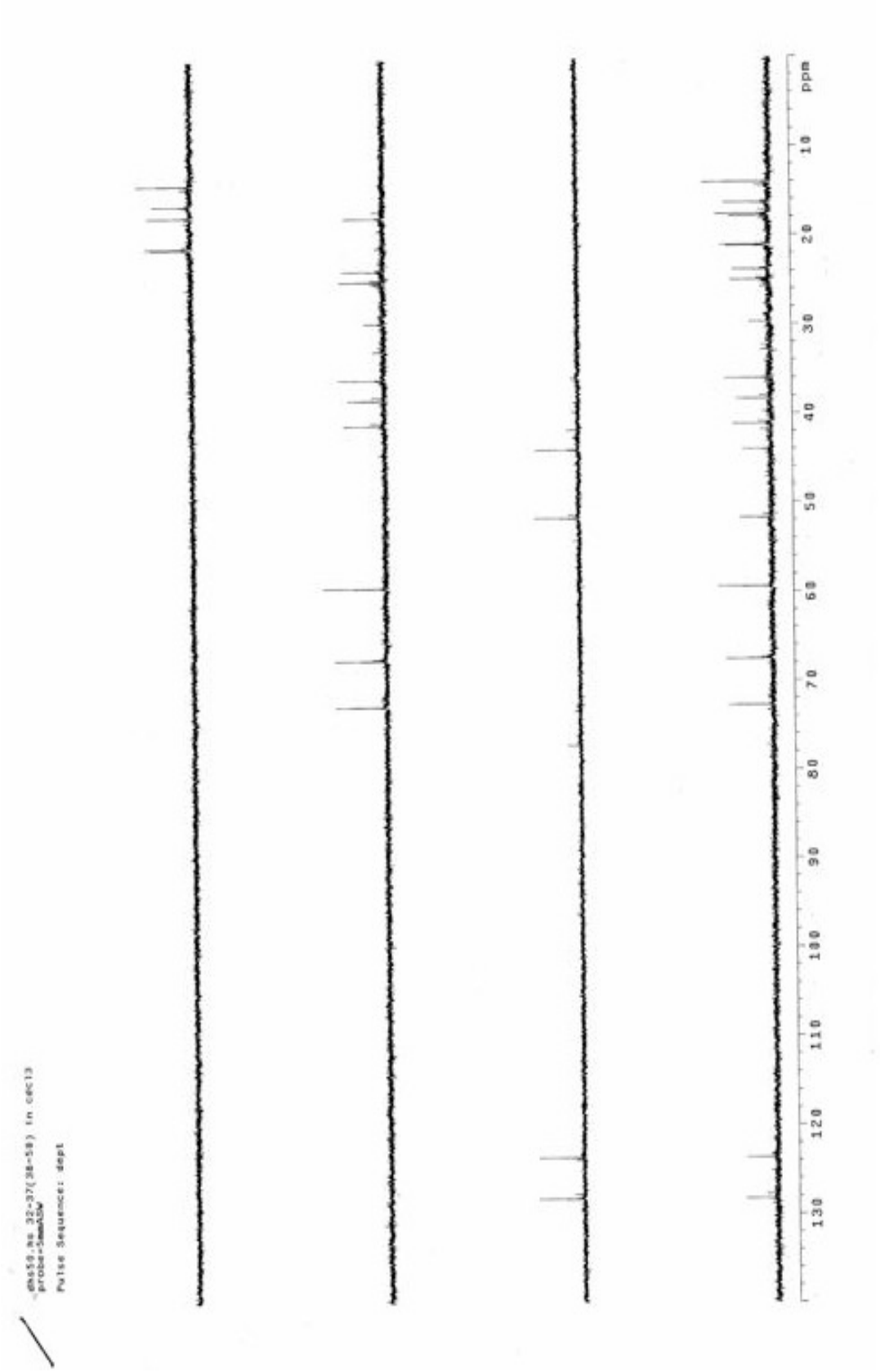
NOESY spectrum for Compound **1**



HMBC spectrum for Compound **1**

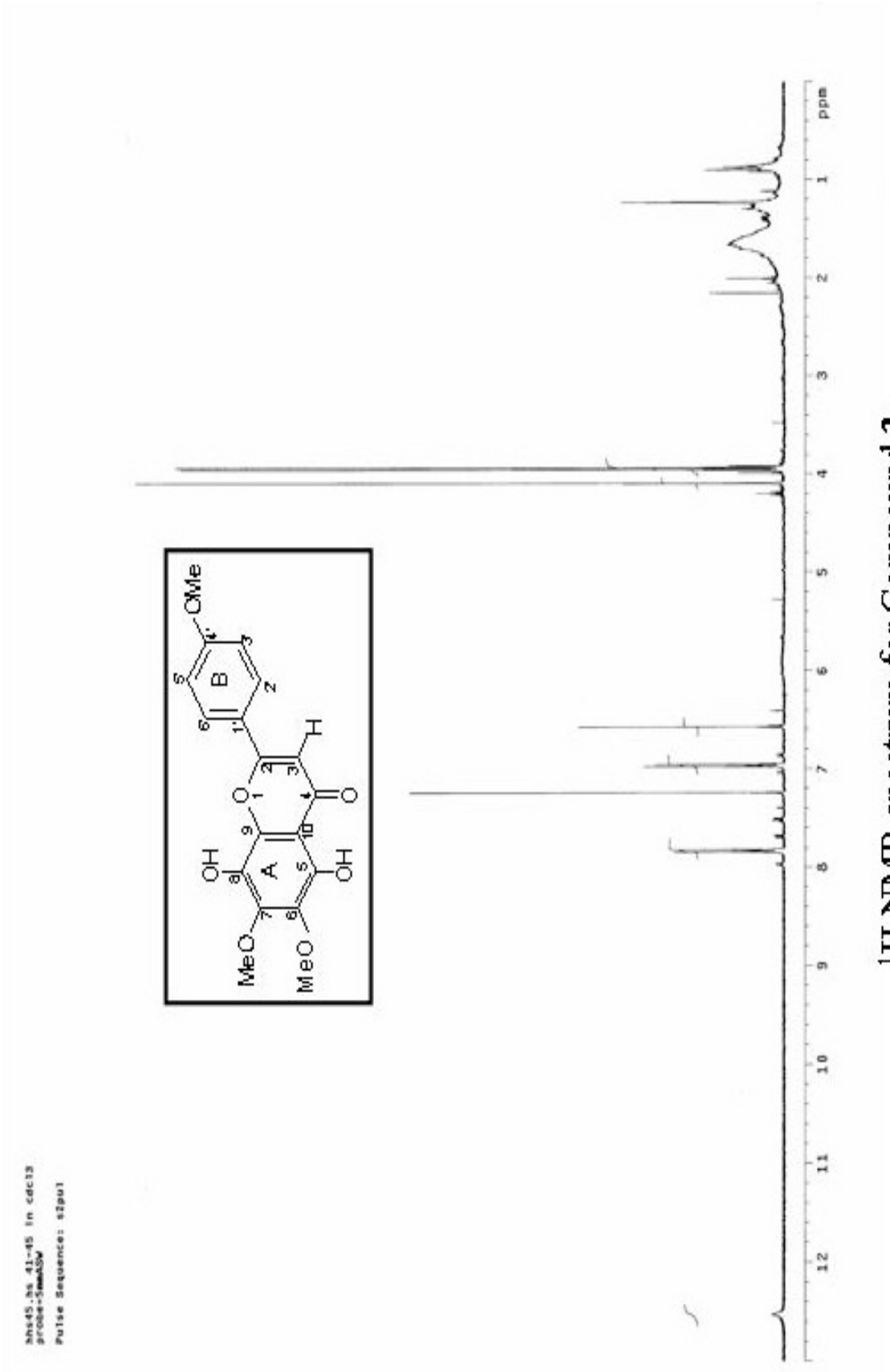


HSQC spectrum for Compound **1**

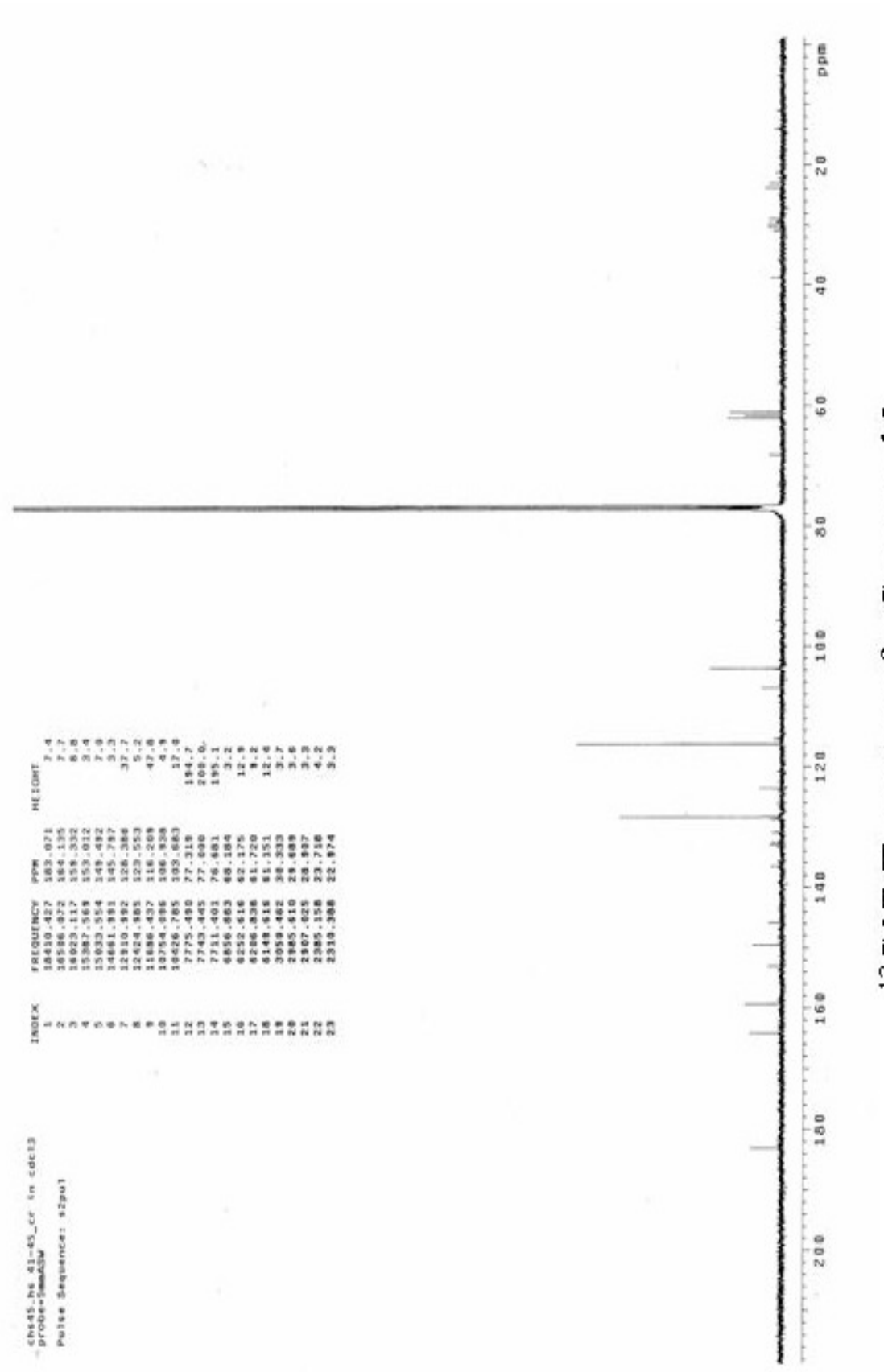


ADEPT spectrum for Compound 1

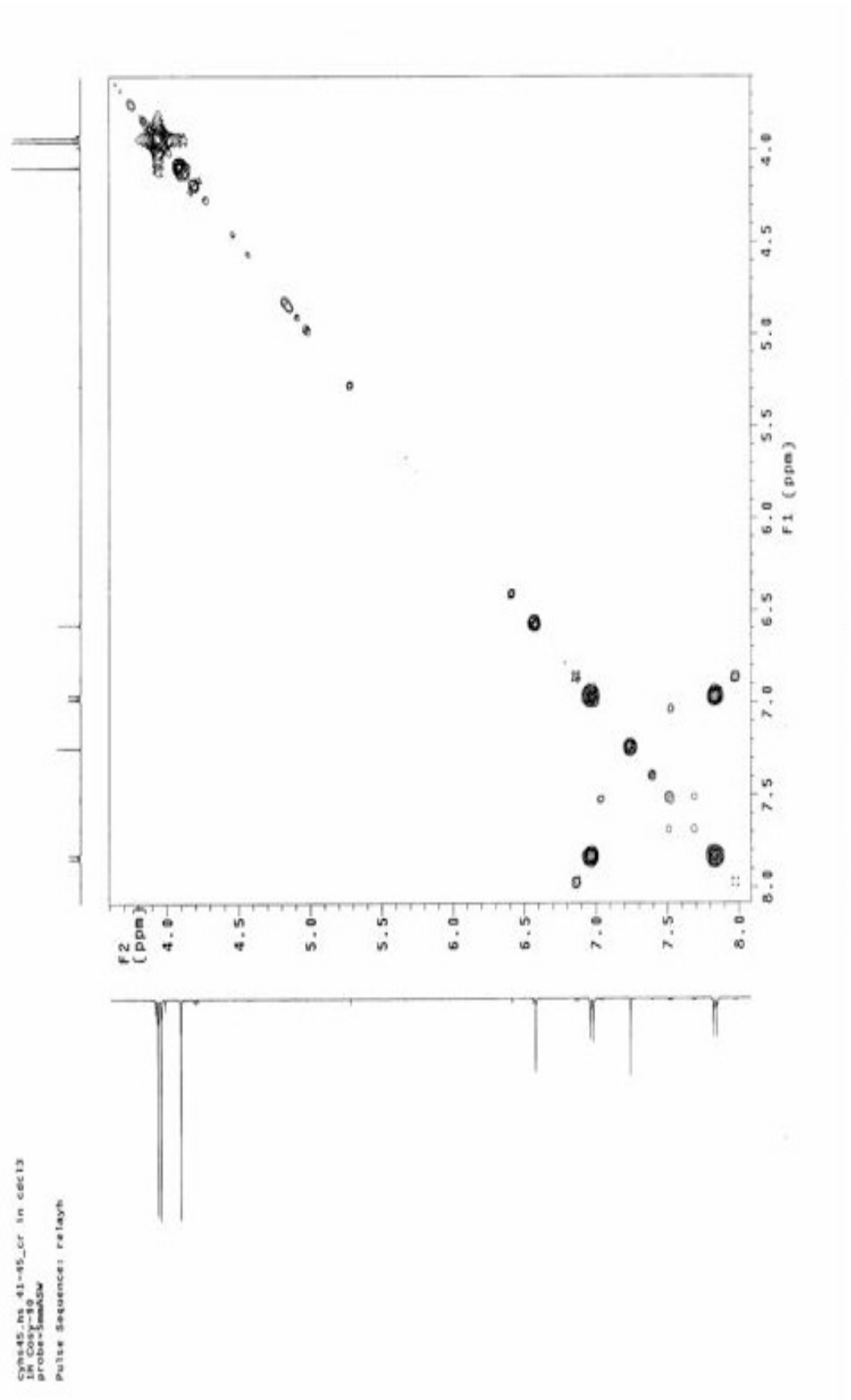
APPENDIX IV



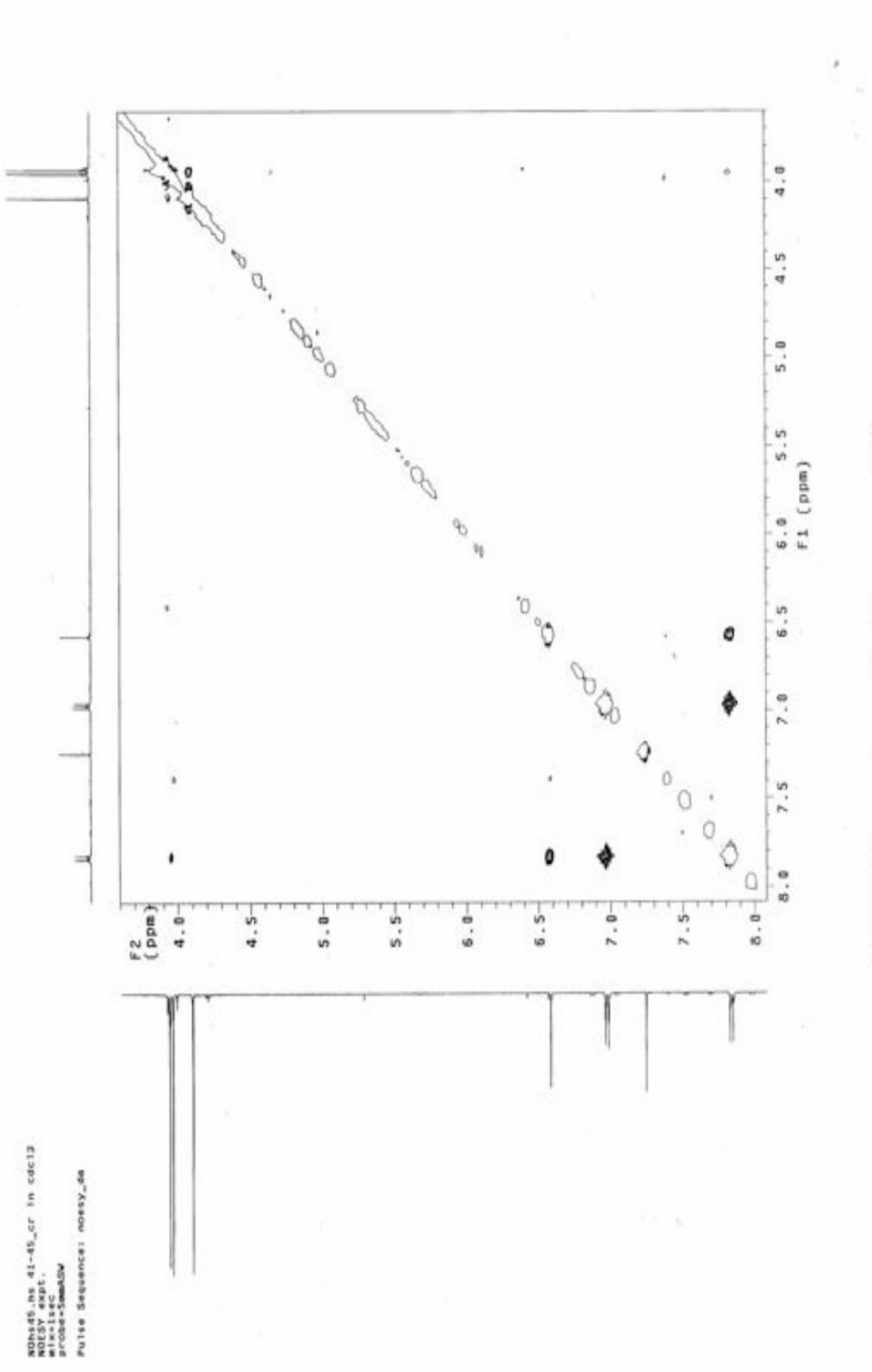
^{1}H NMR spectrum for Compound **2**



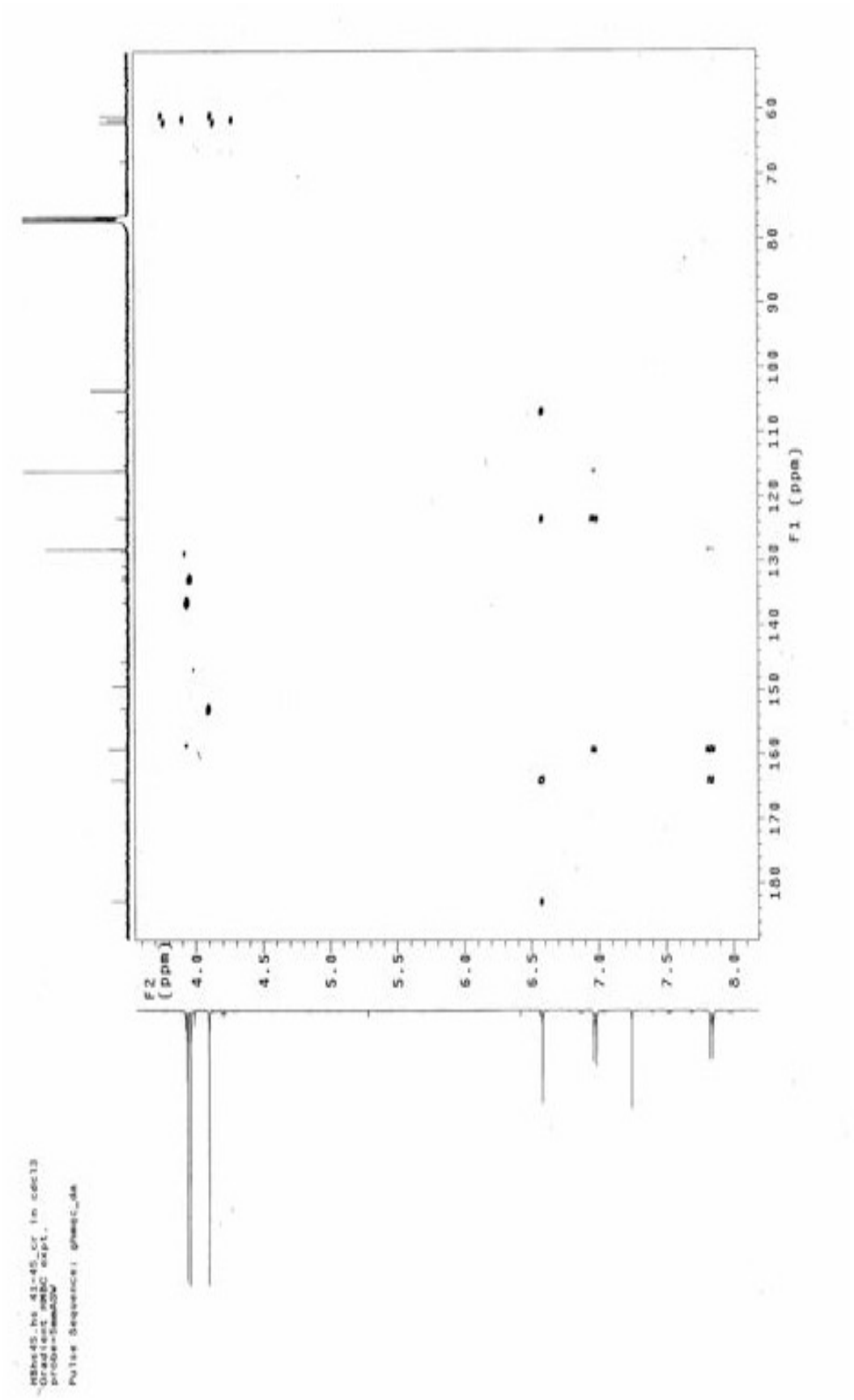
^{13}C NMR spectrum for Compound **2**



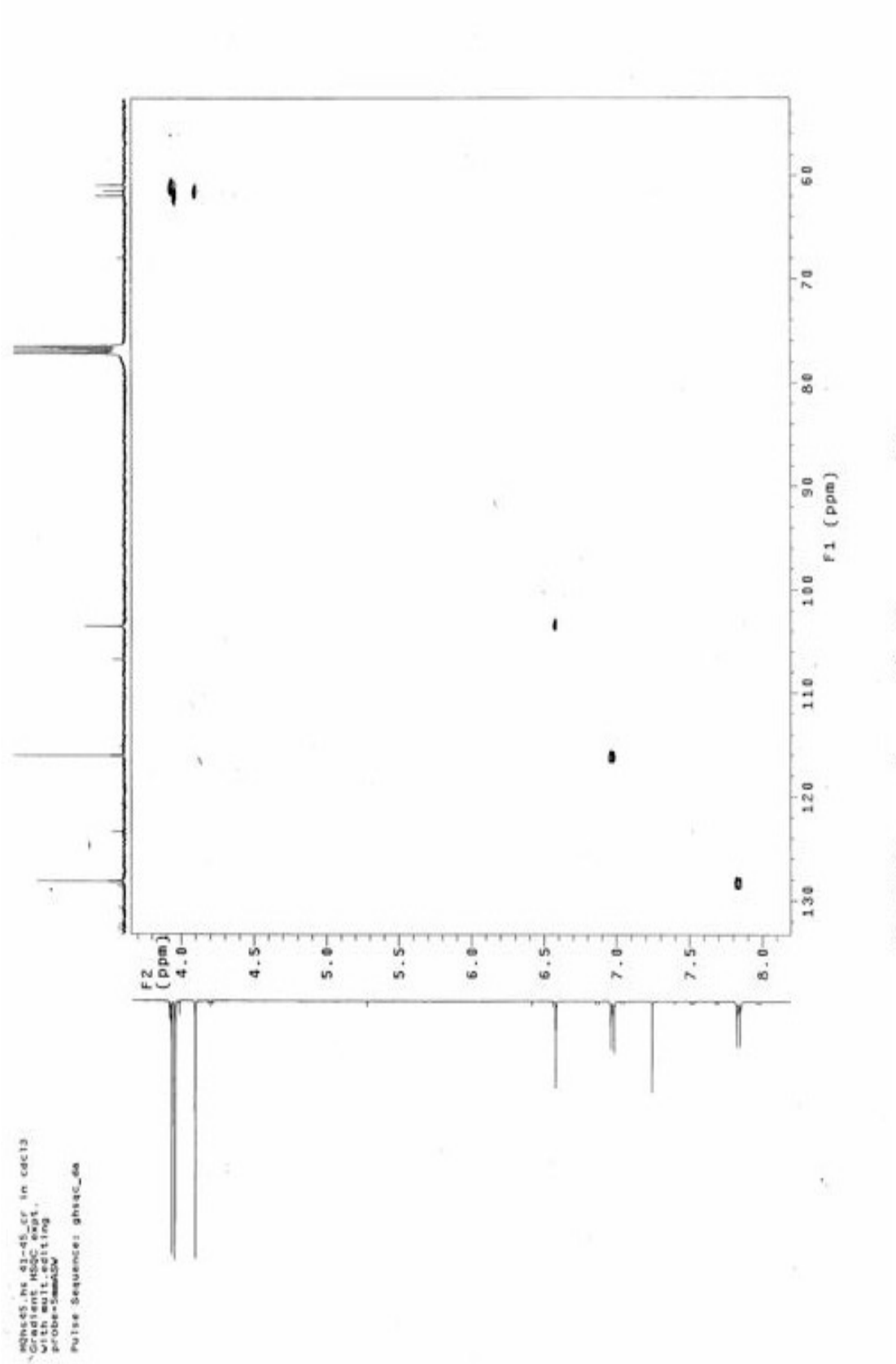
COSY spectrum for Compound 2



NOESY spectrum for Compound 2

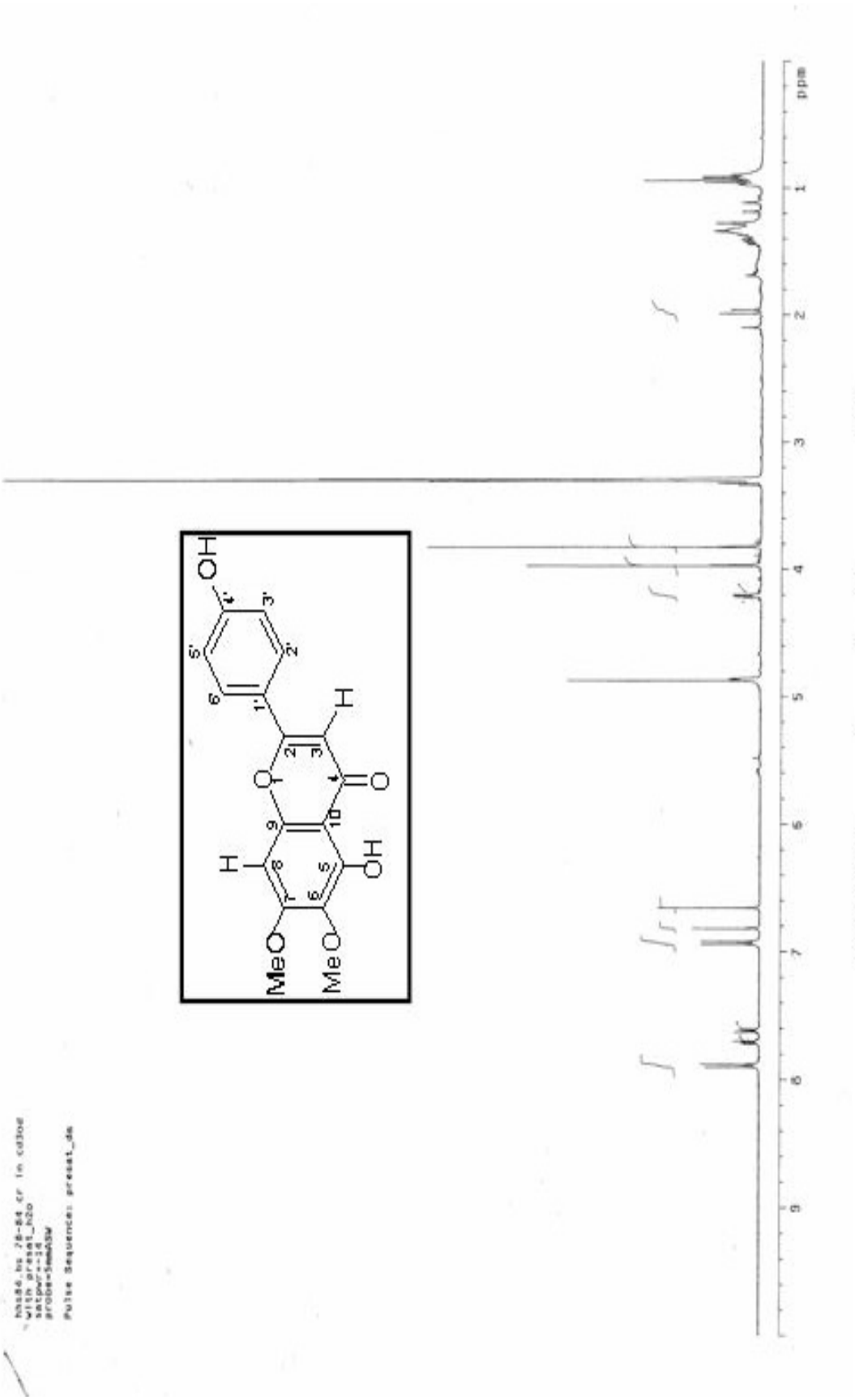


HMBC spectrum for Compound 2

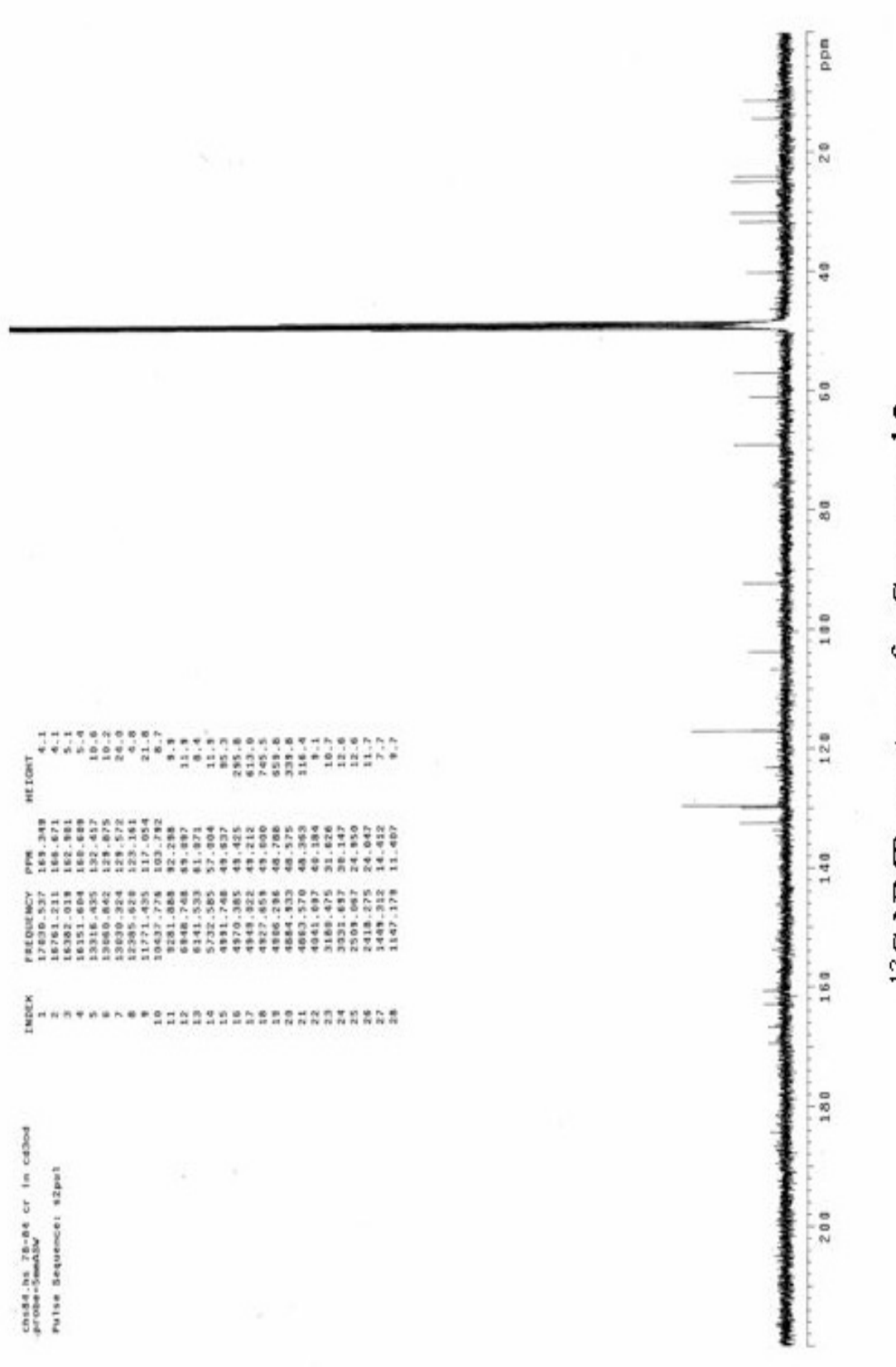


HSQC spectrum for Compound 2

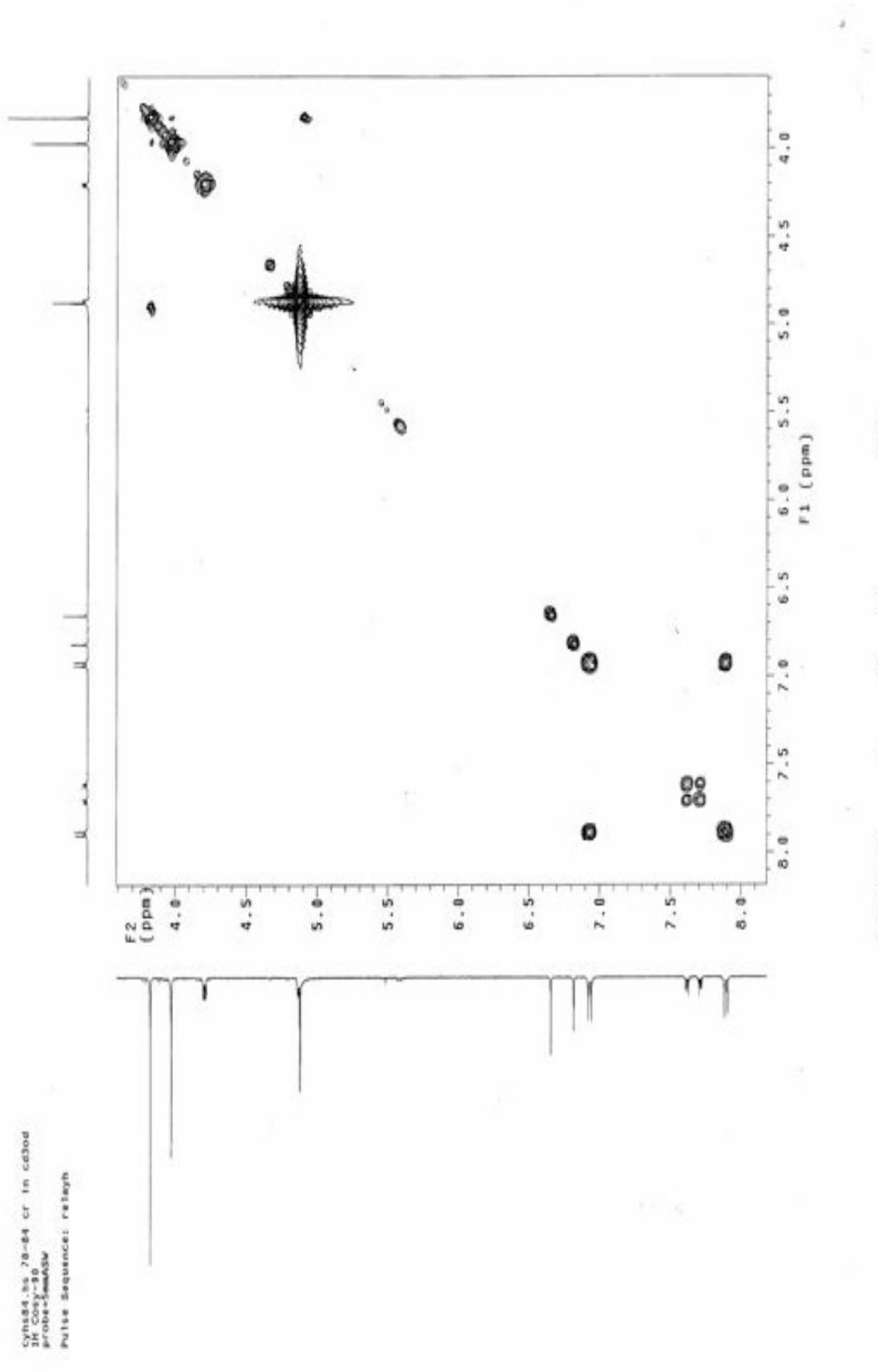
APPENDIX V



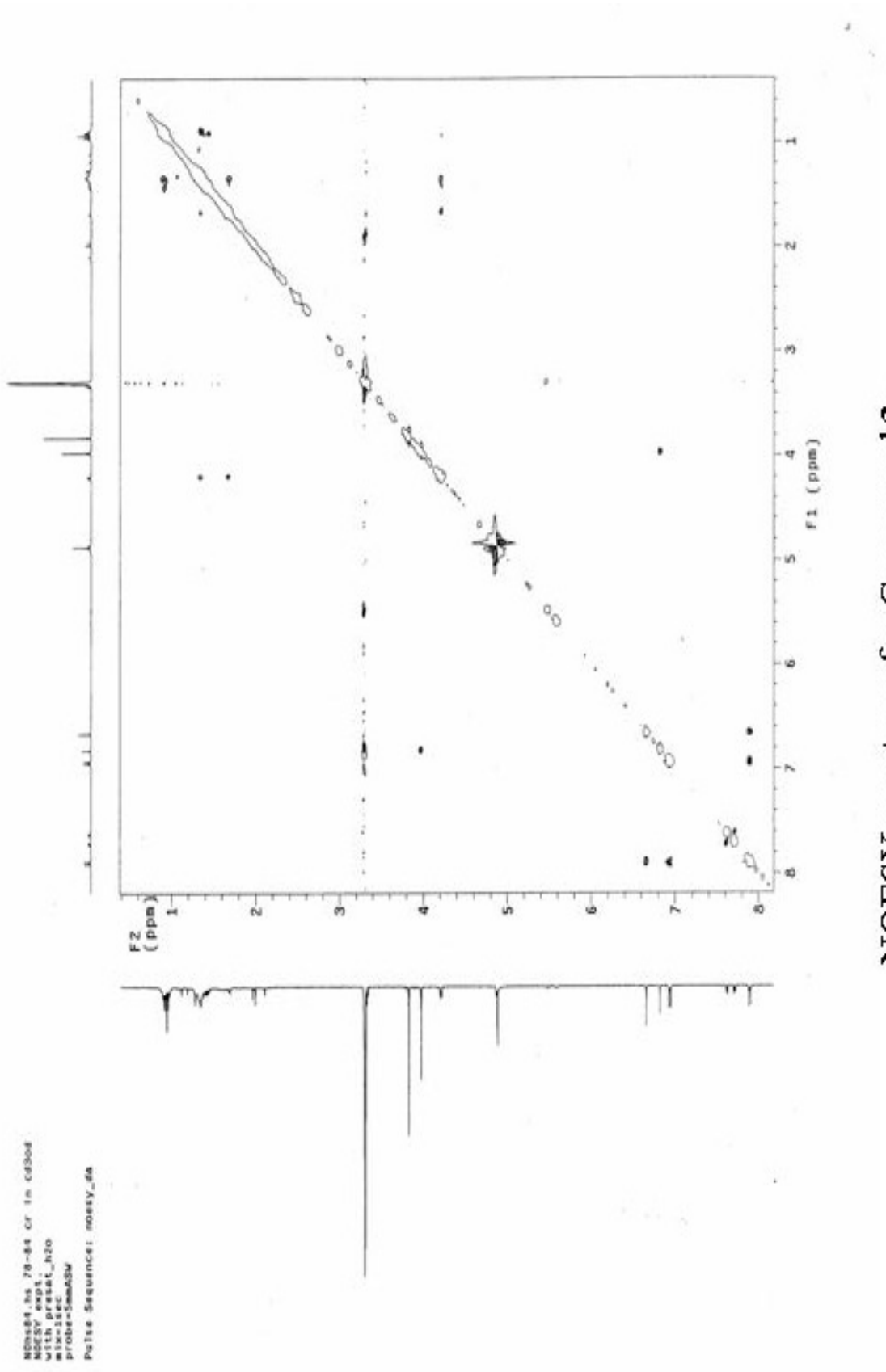
^{1}H NMR spectrum for Compound 3



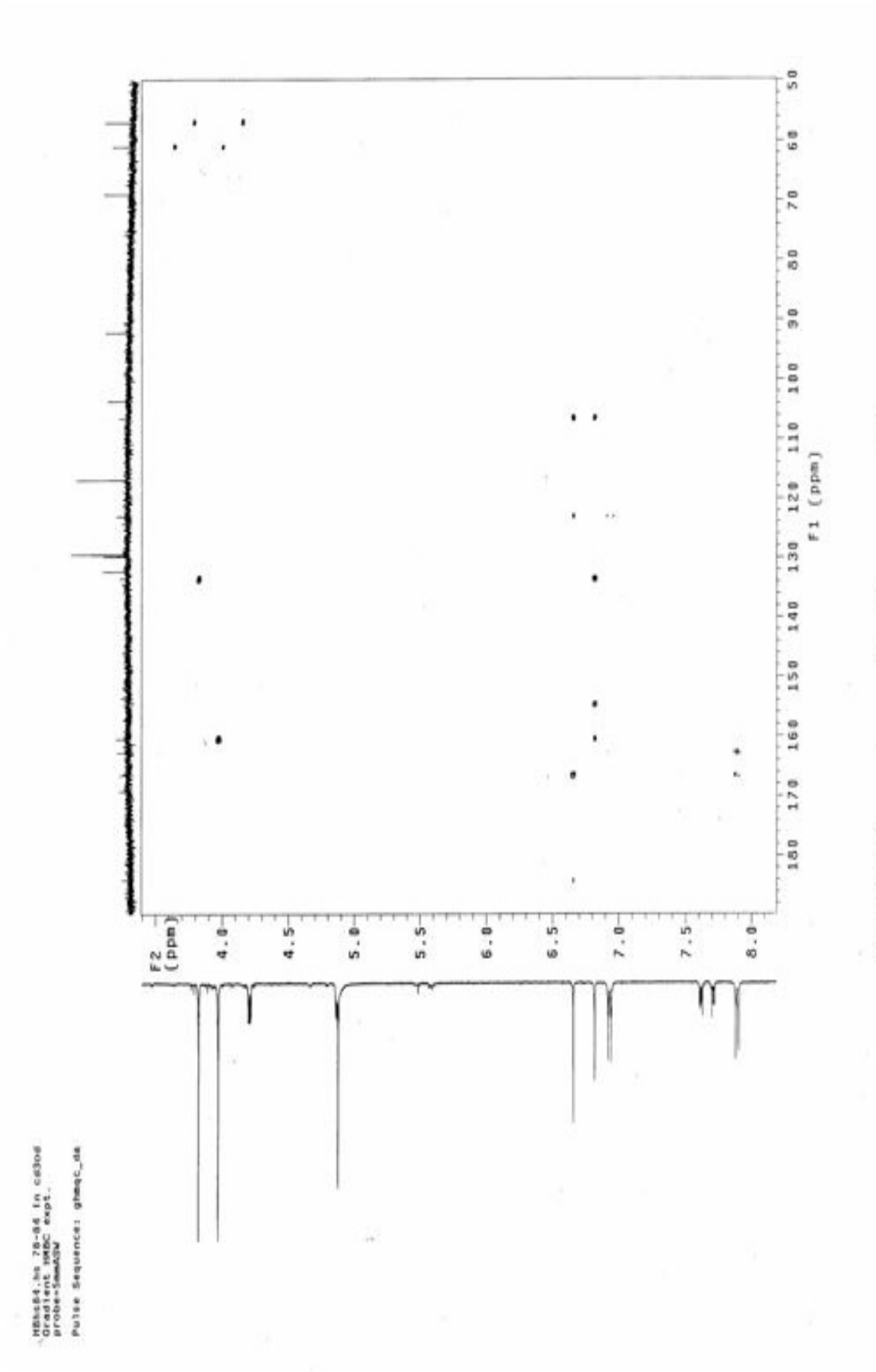
^{13}C NMR spectrum for Compound **3**



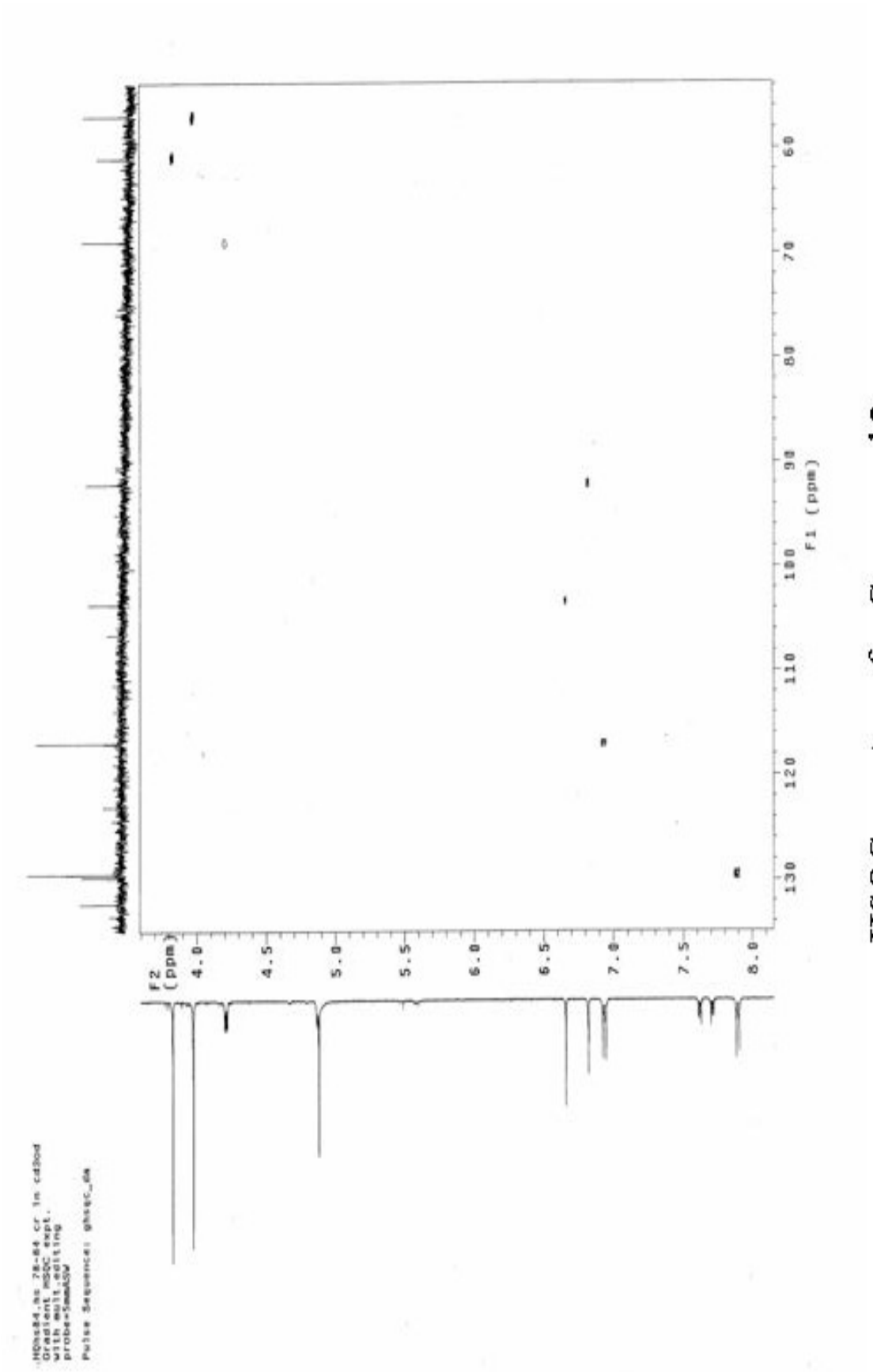
COSY spectrum for Compound **3**



NOESY spectrum for Compound **3**

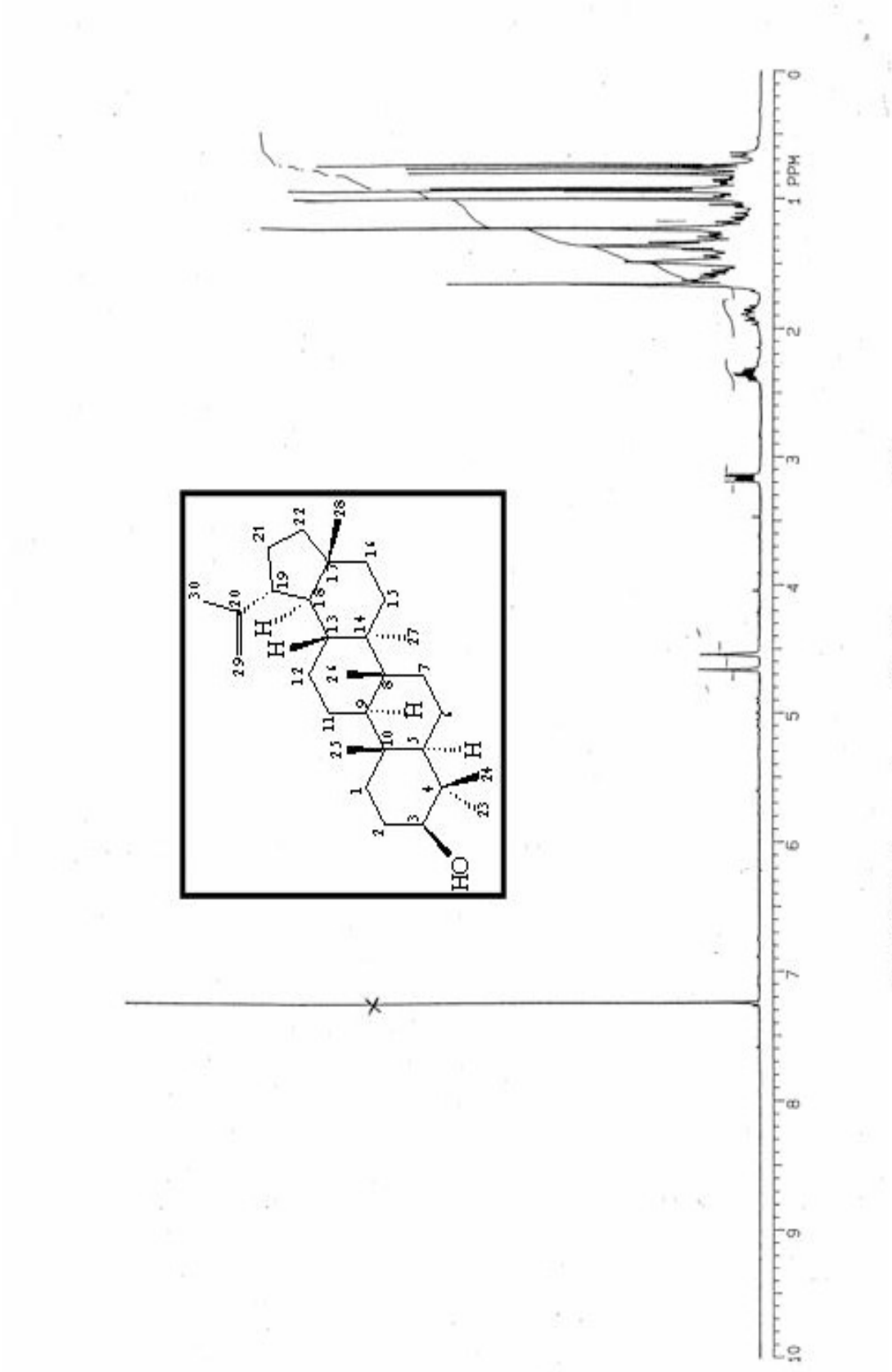


HMBC spectrum for Compound **3**

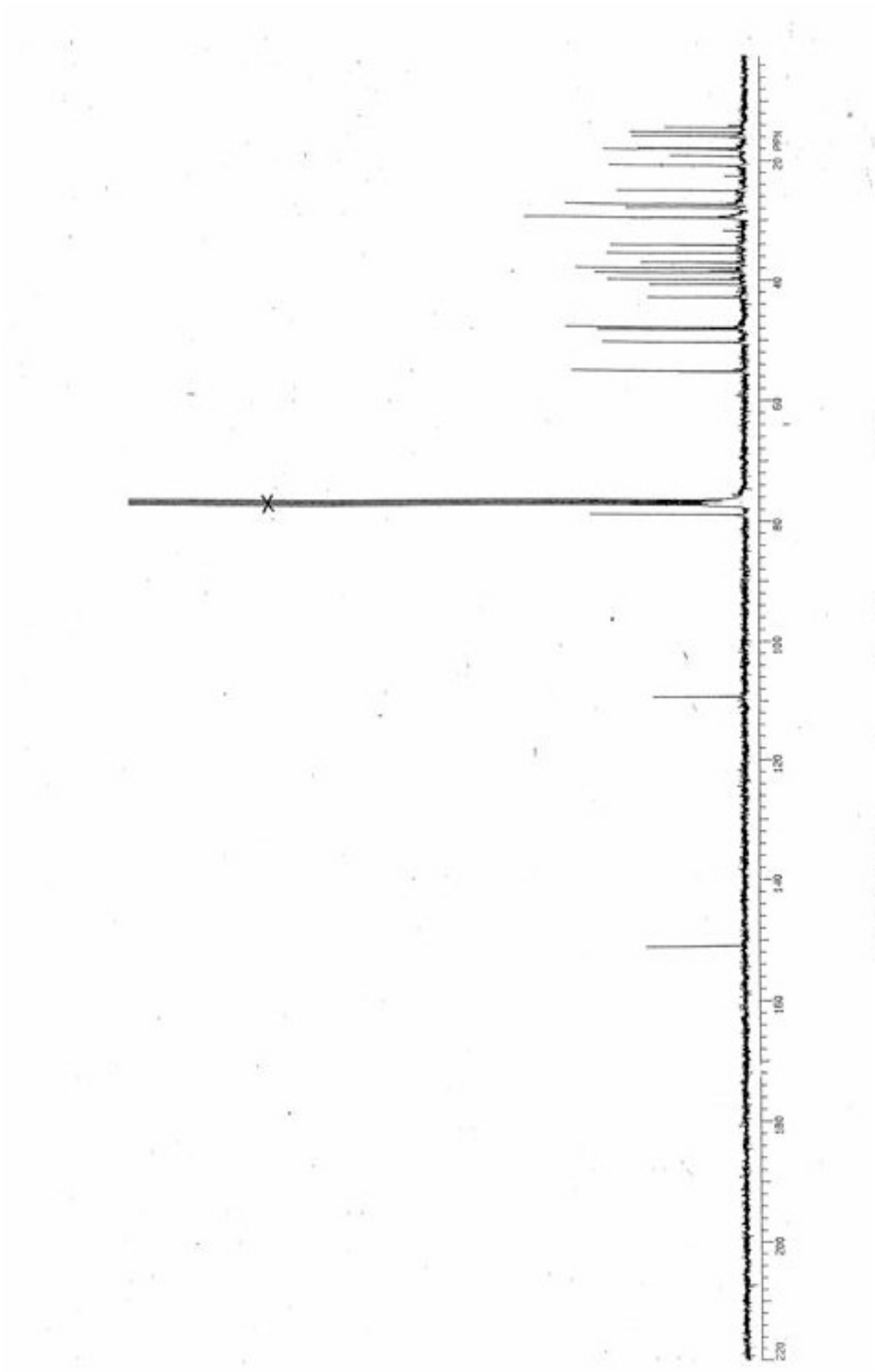


HSQC spectrum for Compound **3**

APPENDIX VI

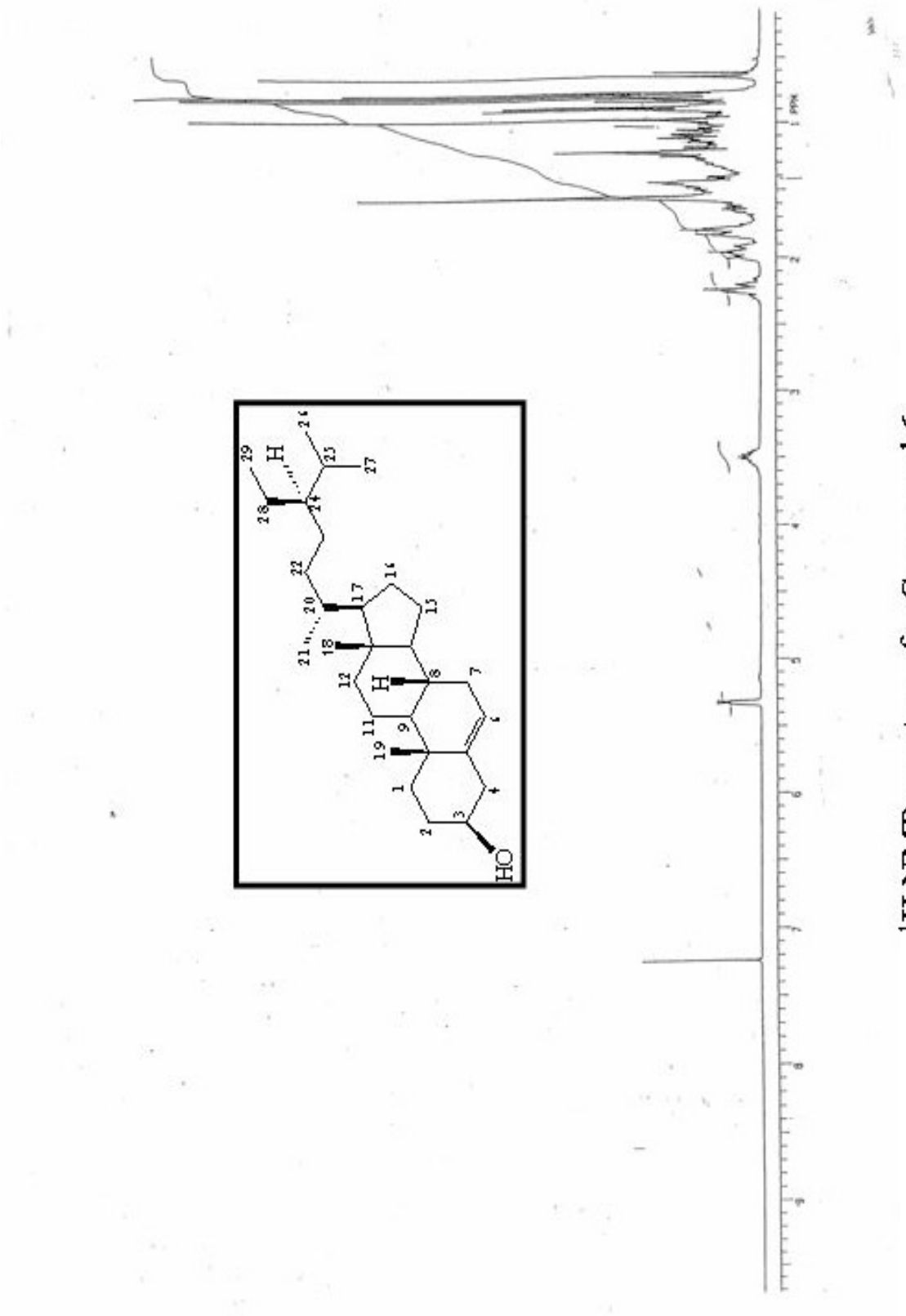


^{1}H NMR spectrum for Compound **5**

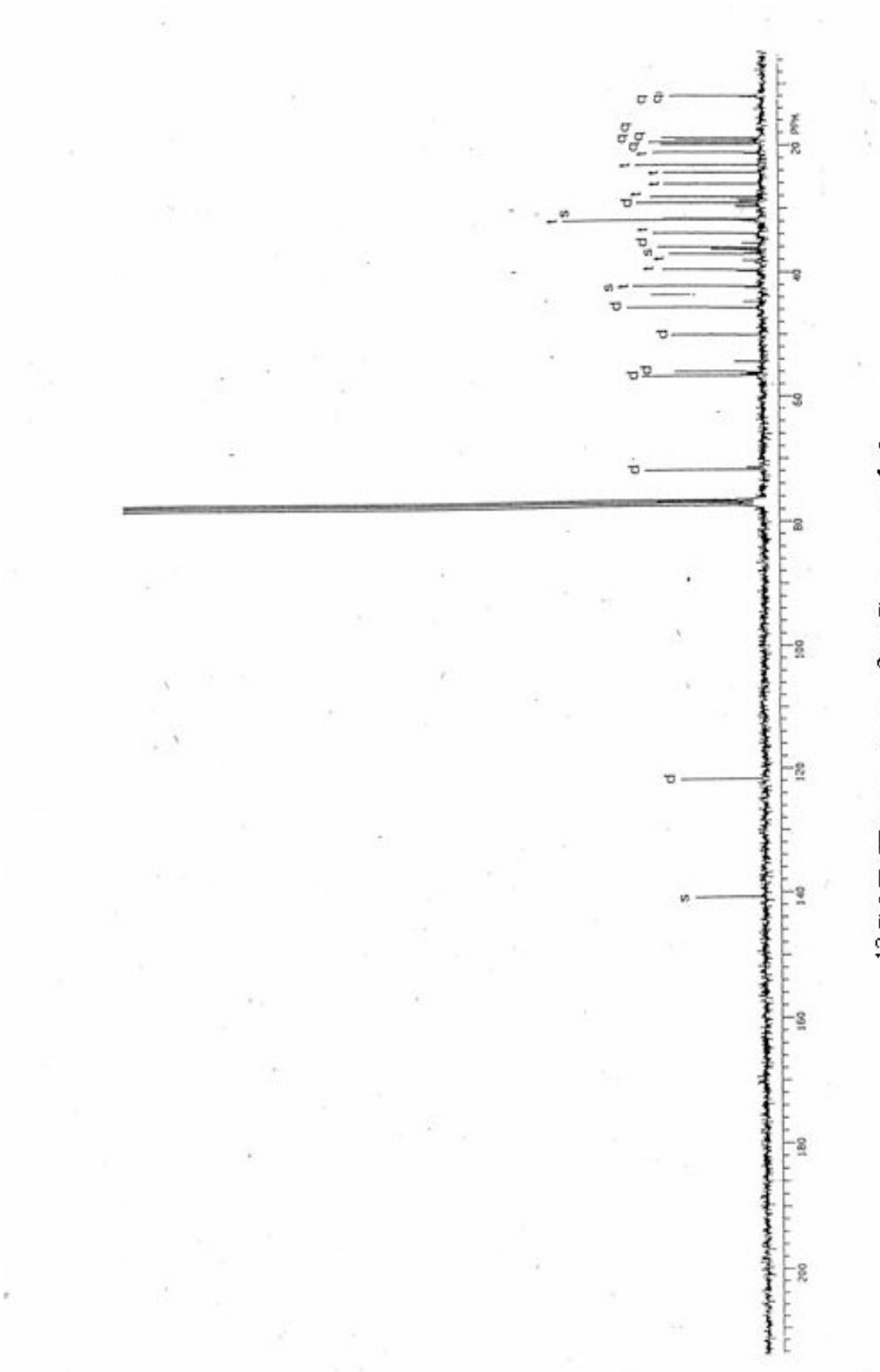


^{13}C NMR spectrum for Compound **5**

APPENDIX VII



^{1}H NMR spectrum for Compound **6**



^{13}C NMR spectrum for Compound **6**