UNIVERSITY OF KWAZULU-NATAL

ISOLATION AND CHARACTERIZATION OF CYTOTOXIC COMPOUNDS FROM *ANTHOSPERMUM HISPIDULUM* AND *ERIOCEPHALUS TENUIFOLIUS*

RUDZANI NTHAMBELENI

2008

ISOLATION AND CHARACTERIZATION OF CYTOTOXIC COMPOUNDS FROM *ANTHOSPERMUM HISPIDULUM* AND *ERIOCEPHALUS TENUIFOLIUS*

by

RUDZANI NTHAMBELENI

Submitted in fulfilment of the academic
requirements of the degree of
Master of Science in the
Discipline Chemistry,
School of Chemistry
University of KwaZulu-Natal

Pietermaritzburg
2008

Preface

The experimental work described in this dissertation was carried out in the School of Chemistry, University of KwaZulu-Natal, Pietermaritzburg, from January 2005 to December 2008, under the supervision of Professor Fanie van Heerden.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

Signed……

Rudzani Nthambeleni (Candidate)

I hereby certify that this statement is correct.

Signed……

Professor Fanie van Heerden (Supervisor).

Signed……

Doctor Gerda Fouche' (Co-supervisor)

Acknowledgements

This piece of work will never be accomplished without our God Almighty with His blessings, wisdom, knowledge and His power that work within me. To him I say, I am forever grateful and also the people behind my life for inspiring and guiding me through thick and thin.

I would like to express my sincere appreciation to my supervisor Professor Fanie van Heerden and my co-supervisor Dr Gerda Fouché, for their superb guidance and encouragement. Thank you for imparting your knowledge and for checking and proof reading my work for those things that I may have missed. Your comments on the research have helped tremendously.

Special thanks to Dr Vinesh Maharaj, research group leader at CSIR Bioprospecting, for granting me the opportunity to carry out my research within Bioprospecting laboratories. I really appreciate your interest, guidance and support throughout the study.

To my colleagues at CSIR, the Bioprospecting team, thank you guys for your assistance in the laboratory and support during this period. Special thanks to Natasha Kolesnikova for anticancer screening of my extracts and compounds. Many thanks to Pastor Eric Khorombi for his help especially his computer skills and prayers.
I would also like to thank Mutshinyalo Nwamadi and Dilip Jagivan for NMR analyses.

To my beloved parents, Pastor Lawrence Masia and Selinah Takalani, I am forever grateful for your love, patience, guidance, encouragement and prayers.

I wish to thank my dear husband, Bernard Ndanduleni, and our children, Ndalamo and Andani, for their patience, love, support and understanding during my studies and late-night writing. My achievements are yours, also. I love you guys.

Finally, I would like to thank the National Research Foundation and CSIR for financial support.

Abstract

Cancer is a human tragedy that strikes and kills the lives of our beloved people. With a limited number of effective anticancer drugs from natural resources currently in use, there is a real need for new, safe, cheap and effective anticancer drugs to combat this dreaded and formidable disease. Plants have a long history of use in the treatment of cancer. Several plant-derived anticancer agents including taxol, vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan and etoposide derived from epi-podophyllotoxin are in clinical use all over the world.

In this study, two endemic plant species from the Rubiaceae and Asteraceae families, namely *Anthospermum hispidulum* E.Mey. ex Sond. and *Eriocephalus tenuifolius* DC. were investigated for their anticancer properties. The organic (methanol/dichloromethane, 1:1 v/v) extracts of both plant species were found to have moderate anticancer activity against a panel of three human cancer cell lines namely, breast MCF7, renal TK10 and melanoma UACC62 at the CSIR anticancer screen. Bioassay-guided fractionation of the organic extracts of *Anthospermum hispidulum* led to the isolation of an active compound which was characterised as ursolic acid. Another compound, namely scopoletin was also isolated. The compounds isolated here are known compounds, but have not previously been reported as present in the genus *Anthospermum.*

Bioassay-guided fractionation of the organic extracts of *Eriocephalus tenuifolius* resulted in the isolation of 8-*O*-isobutanoylcumambrin B as the active constituent. This compound is reported to have been isolated from related plant species; however its biological activity is not known. The compounds pectolinagenin, hispidulin, friedelinol and tetracosanoic acid were also isolated, but did not show any significant anticancer activity. The structures of all compounds isolated in this study were elucidated using nuclear magnetic resonance spectroscopy, mass spectroscopy and also by comparison with data reported in the literature.

List of Abbreviations

$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
CH_2Cl_2	Dichloromethane
CHO	Chinese Hamster Ovarian
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance Spectroscopy
COSY	Correlated Spectroscopy
CSIR	Council for Scientific and Industrial Research
d	doublet
dd	doublet of doublets
ddd	doublet of doublets of doublets
DEPT	Distortionless Enhancement Polarization Transfer
DMSO	Dimethylsulfoxide
DMSO-d_6	deuterated dimethylsulfoxide
EI-MS	Electron Impact Mass Spectroscopy
ESI-MS	Electrospray Ionization Mass Spectroscopy
EtOAc	Ethyl acetate
FAB	Fast Atom Bombardment
GC-MS	Gas Chromatography-Mass Spectrometry
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
^1H NMR	Proton (^1H) Nuclear Magnetic Resonance spectroscopy

HR-MS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
IC_{50}	Concentration at which 50% inhibition is achieved
J	Spin-spin coupling constant in Hz
Lit	literature
m	multiplet
Me	Methyl
MeOH	Methanol
mg	milligram
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	mass-to-charge ratio
NCI	National Cancer Institute
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
pLDH	parasite lactate dehydrogenase
ppm	Parts per million
sp.	Species
s	singlet
SANBI	South African National Biodiversity Institute
SI	selectivity index

t	triplet
TGI	Total Growth Inhibition
TLC	Thin-Layer Chromatography
UCT	University of Cape Town
UV	Ultra Violet Spectroscopy
VLC	Vacuum Liquid Chromatography
^{o}C	Degrees Celsius
1D	One Dimensional
2D	Two Dimensional
[α]	Specific Rotation
δ	NMR chemical shift in ppm
μg/mL	Microgram per milliliter
et al	and others
i.e.	that is

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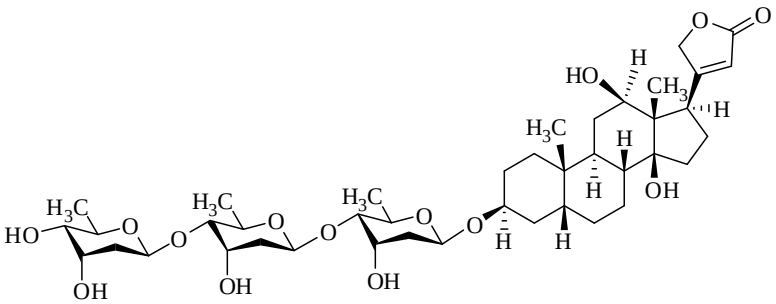
Chapter 1

Introduction

For decades, natural products have been a source of drugs and drug leads. According to a recent survey by Newman and Cragg (2007) of the United States National Cancer Institute (NCI), 63% of the 974 small molecule new chemical entities introduced as drugs worldwide during the period 1981-2006 can be traced to or were inspired by natural products. Examples of important drugs obtained from plants are digoxin (**1.1**) from *Digitalis purpurea* (Fig. 1.1), quinine (**1.2**) and quinidine (**1.3**) from *Cinchona officinalis* (Fig. 1.2), vincristine (**1.4**) and vinblastine (**1.5**) from *Catharanthus roseus* (Fig. 1.3), atropine (**1.6**) from *Atropa belladonna* (Fig. 1.4) and codeine (**1.7**) and morphine (**1.8**) from *Papaver somniferum* (Fig. 1.5). It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin (Rates 2001; Newman et al. 2003; Cragg et al. 2005; Butler 2004; McChesney et al. 2007).



1.1

Figure 1.1: Structure of digoxin (**1.1**) from *Digitalis purpurea*

Source: http://www.one-garden.org/Indig/foxglove.htm

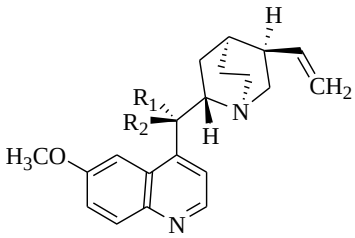


Figure 1.2: Structures of quinine (**1.2**) and quinidine (**1.3**) from *Cinchona officinalis*

Source: http:// www.lewis-clark.org/media/NewImages/PHILADELPHIA/med_ cinchona.jpg

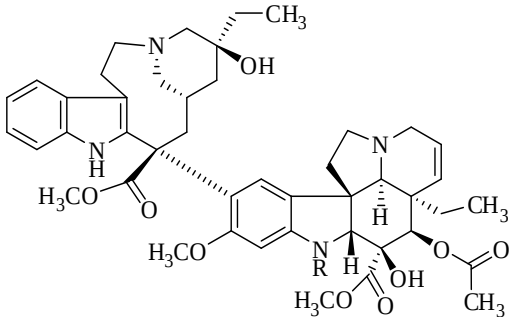


Figure 1.3: Structures of vinblastine (**1.4**) and vincristine (**1.5**) from *Catharanthus roseus*

Source: http://www.dkimages.com/discover/previews/794/20145149.JPG

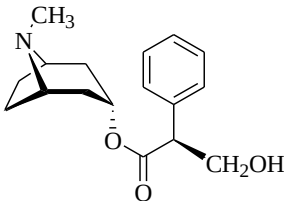


Figure 1.4: Structure of atropine (**1.6**) from *Atropa belladonna*

Source: http://upload.wikimedia.org/wikipedia/commons/thumb/3/31/Atropa_belladonna _220605.jpg/684px-Atropa_belladonna_220605.jpg

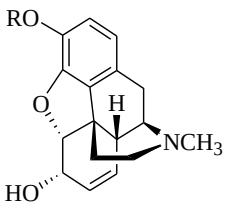


Figure 1.5: Structures of codeine (**1.7**) and morphine (**1.8**) from *Papaver somniferum*
Source: http://www.maltawildplants.com/PAPV/Papaver_somniferum_subsp_setigerum.php

The search for anticancer agents from plant sources began in the 1950's at the United States National Cancer Institute (NCI) and the NCI has since made major contributions to the discovery of new naturally occurring anticancer agents. In spite of the success of the natural products approach to anticancer drug discovery, reports on plants used for the treatment of cancer are rare in South Africa (Steenkamp and Gouws 2006). As a result, a collaborative research programme was initiated between the Council for Scientific and Industrial Research (CSIR) in South Africa and the NCI, aimed at the screening of plant extracts and identification of potentially new anticancer drug leads.

An anticancer screening technology was implemented at the CSIR in 1999 with a panel of three human cancer cell lines namely, breast MCF7, renal TK10 and melanoma UACC62. These cell lines were selected because of their high sensitivity to detect anticancer activity.

Plant extracts that exhibit anticancer activity against these three human cell lines are then screened by the NCI against sixty cell lines organized into sub-panels representing leukaemia, melanoma, cancer of the lung, colon, kidney, ovary and central nervous system.

Two endemic plants from the Rubiaceae and Asteraceae families, namely *Anthospermum hispidulum* E.Mey. ex Sond. and *Eriocephalus tenuifolius* DC., respectively, were investigated as part of this research programme and their organic extracts were found to have moderate anticancer activity against the panel of three human cancer cell lines at the CSIR.

The aims of this study were:

- To screen extracts of *Anthospermum hispidulum* and *Eriocephalus tenuifolius* for anticancer activity.
- To isolate and purify compounds from the active organic extracts of *A. hispidulum* and *E. tenuifolius.*
- To elucidate structures of the isolated compounds using nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) techniques.
- To identify active ingredients.

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Chapter 2

Literature Review

2.1 What is cancer?

Cancer is the generic term for a group of diseases characterized by uncontrolled growth and proliferation of abnormal cells. If the spread is not controlled, it can result in death. Cancer is caused by both external factors (tobacco, chemicals, radiation and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions and mutation that occur from metabolism) (Fig. 2.1). These causal factors may act together or in sequence to initiate or promote carcinogenesis. Most cancers can be treated and some cured, depending on the specific type, location and stage. The main treatments are surgery, radiation therapy, chemotherapy, hormone therapy, biological therapy and targeted therapy (American Cancer Society 2007).

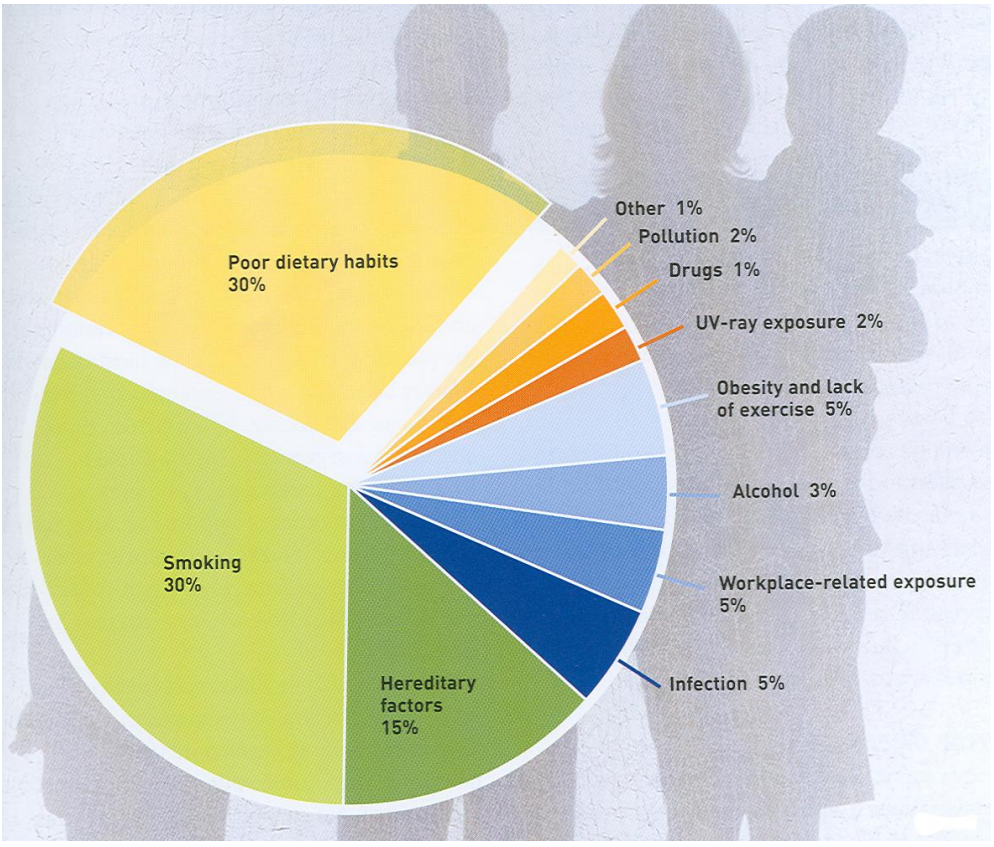


Figure 2.1: Causes of cancer (Beliveau and Gingras 2007)

There are more than 100 different types of cancer, which are grouped into five major categories:

- **Carcinoma** – cancer that originates in the skin or in tissue that line or cover internal organs. For example, breast, prostate, lung and colon cancer.
- **Sarcoma** - cancer that originates in supportive and connective tissues such as bones, cartilage, fat, muscle and blood vessels.
- **Leukemia** - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
- **Lymphoma and myeloma** - cancers that originate in the cells of the immune system.
- **Central nervous system cancers** - cancers that begin in the tissues of the brain and spinal cord.

Most cancers are named for the organ or type of cell in which they originate. For example, cancer that begins in the breast is called breast cancer (National Cancer Institute 2007).

According to the Cancer Association of South Africa (2005), South African men and women, in order of prevalence, are affected by the following cancers:

Women:

Breast cancer; cervical cancer; colo-rectal cancer; lung cancer and oesophageal cancer

Men:

Prostate cancer; lung cancer; oesophageal cancer; bladder cancer and colo-rectal cancer

2.2 Cancer and its impact

Cancer is a growing public health problem worldwide. It affects all people irrespective of age, gender, race and socio-economic status. More than 11 million people are diagnosed with cancer every year. It is estimated that there will be 16 million new cases every year by 2020. About 12.5% of all deaths worldwide are caused by cancer. That is more than the percentage of deaths caused by HIV/AIDS, tuberculosis and malaria put together (World Health Organization 2005).

Chronic diseases, including cancer, are on the increase internationally and in South Africa, due to unhealthy lifestyle habits. In South Africa approximately 1 in 4 people may become a cancer statistic in their lifetime and almost everyone knows someone who has been affected by the disease.

Cancer has long been the second leading cause of death in the United States of America after heart disease, accounting for about a quarter of all deaths. About 1,444,920 new cancer cases are expected to be diagnosed in 2007 (this figure does not include non-invasive cancers) and about 559,650 Americans are expected to die of cancer this year.

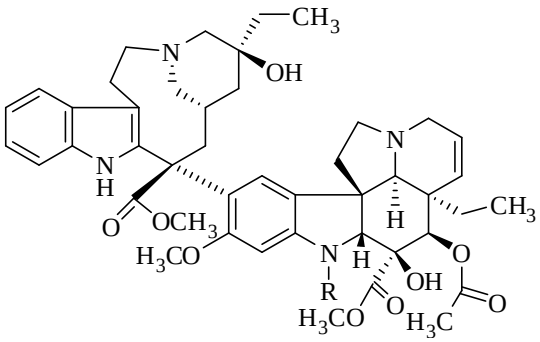
The National Institute of Health (NIH) has estimated the costs from cancer in 2006 to be 206.3 billion US dollars. It is also important to note that 77% of all cancers diagnosed are in people 55 years of age or older (American Cancer Society 2007). With cancer taking such a toll on the population, both in lives and cost, the discovery of effective anticancer drugs has become very important.

2.3 Plants as a source of anticancer agents

Over 60% of currently used anticancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms (Rates 2001; Newman et al. 2003; Cragg et al. 2005; McChesney et al. 2007). Plants have a long history of use in the treatment of cancer (Hartwell 1982). In his review, Hartwell lists more than 3000 plant species that have reportedly been used in the treatment of cancer. Graham et al. (2000) had also added another 350 plant species to Hartwell's list. These statistics clearly demonstrate the importance and potential of plants as a primary source for drug discovery and development.

Well-known examples of the plant-derived anticancer drugs in clinical use or development are the so-called vinca alkaloids, vinblastine (**2.1**) and vincristine (**2.2**), which are isolated from the Madagascar periwinkle, *Catharanthus roseus*. *C. roseus* is used by various cultures for the treatment of diabetes, and vinblastine (**2.1**) and vincristine (**2.2**) were first discovered during an investigation of the plant as a source of potential oral hypoglycaemic agents (Schwartsmann et al. 2002; Cragg and Newman 2003; Kintzios 2006).

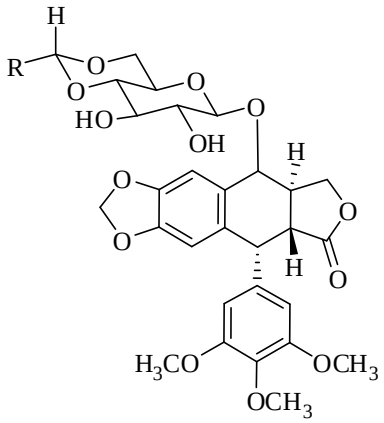
Vinblastine (**2.1**) has been shown to be a very effective treatment for Hodgkin's disease and has also been used to treat breast cancer, Kaposi's sarcoma and other diseases. It was also found to be the first drug of choice in the treatment of many forms of leukaemia and since the 1950s it has increased the survival rate of childhood leukemias by 80%. Vincristine (**2.2**) is widely used, in combination with other anticancer agents, in the treatment of acute lymphocytic leukaemia in children, and for certain lymphomas and sarcomas, small cell lung cancer, cervical and breast cancer (Hostettmann et al. 2000; Mahidol et al. 2000; Kong et al. 2003).

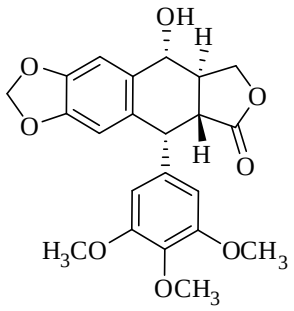


2.1 Vinblastine, R= CH_3,

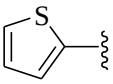
2.2 Vincristine, R= CHO

The two clinically active agents, etoposide (**2.3**) and teniposide (**2.4**), which are semi-synthetic derivatives of the natural product epipodophyllotoxin, may be considered being more closely linked to a plant originally used for the treatment of cancer. Epipodophyllotoxin is an isomer of podophyllotoxin (**2.5**), which was isolated as the active antitumor agent from the roots of various species of the genus *Podophyllum*. Etoposide (**2.3**) is used in the treatment of small cell lung cancer, testicular cancer, Kaposi's sarcoma, lymphoma and leukaemia, while teniposide (**2.4**) has proved to be active against acute lymphatic leukaemia and neuroblastoma, non-Hodgkin's lymphoma and brain tumours in children (Holthuis 1988; Cragg et al. 1994).



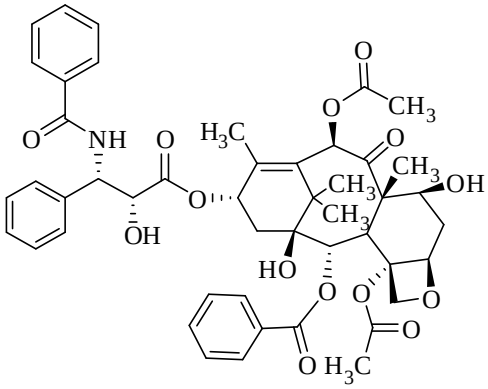


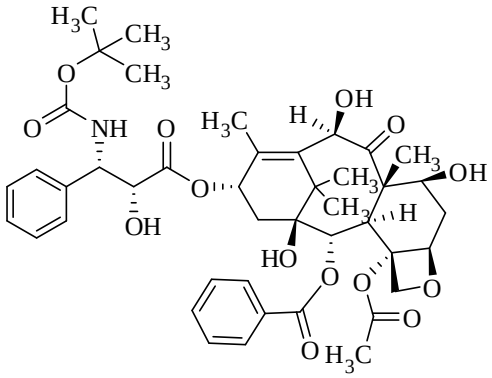
2.3 Etoposide, R = CH_3,

2.4 Teniposide, R = 

2.5 Podophyllotoxin

Another example of a plant-derived anticancer drug under clinical use is paclitaxel, more commonly known by its trademark name Taxol (**2.6**). Taxol, a complex terpene-based molecule isolated from the bark of the Pacific or American yew tree *Taxus brevifolia* is used in the treatment of refractory ovarian, breast and non-small cell lung cancer (NSCLC), and has also shown efficacy against Kaposi's sarcoma. A paclitaxel analog, docetaxel (**2.7**), is used in the treatment of breast cancer and NSCLC (Cragg and Newman 2005).

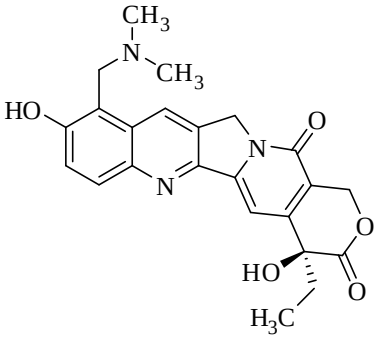




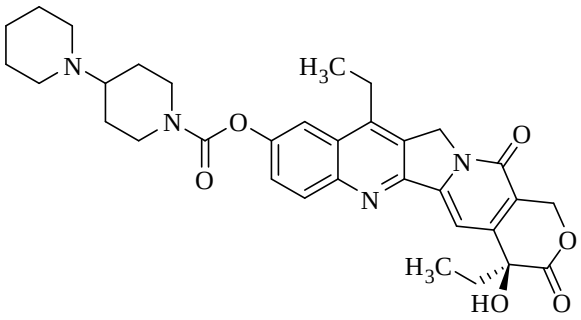
2.6 Taxol

2.7 Docetaxel

The clinically active agents, topotecan (**2.8**) and irinotecan (**2.9**) are semi-synthetically derived from camptothecin, isolated from the Chinese ornamental tree, *Camptotheca acuminata.* Topotecan (**2.8**) is used for the treatment of ovarian and small cell lung cancers, while irinotecan (**2.9**) is used in the treatment of metastatic colorectal cancer (Kong et al. 2003).

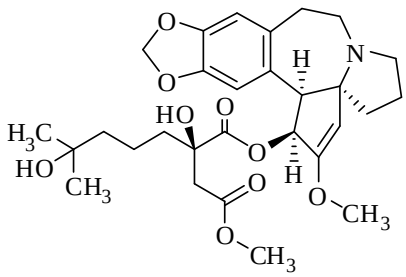


2.8 Topotecan

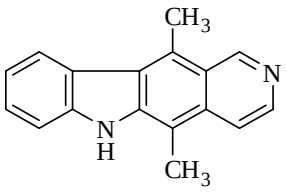


2.9 Irinotecan

Other examples of plant-derived agents in clinical use are homoharringtonine (**2.10**), an alkaloid isolated from the Chinese tree, *Cephalotaxus harringtonia* var *drupacea* and elliptinium, a derivative of ellipticine (**2.11**), isolated from species of several genera of Apocynaceae family, including *Bleekeria vitensis*, a Fijian medicinal plant with reputed anticancer properties. Homoharringtonine has shown efficacy against various leukemias, while elliptinium is marketed in France for the treatment of breast cancer (Cragg and Newman 2005; Mans et al. 2000).



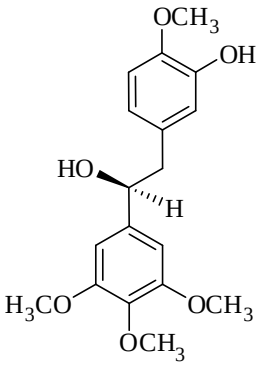
2.10 Homoharringtonine



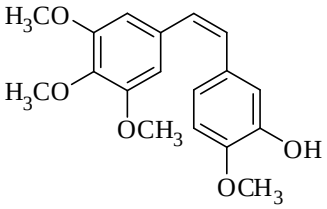
2.11 Ellipticine

Examples of plant-derived anticancer agents in clinical development are the combretastatins (**2.12**), isolated from the bark of the South African "bush willow" tree, *Combretum caffrum*. These compounds are a family of stilbenes which act as antiangiogenic agents, causing vascular shutdowns in tumours and resulting in tumour necrosis when tested against solid tumours. Combretastatin A_4 (**2.13**) has been found to

show strong cytotoxicity against a wide variety of human cancer cell lines, including multiresistant cancer cell lines. It is also stated that it is the most cytotoxic phytomolecule isolated so far. (Cragg and Newman 2005; Srivastava et al. 2005; Gurib-Fakim 2006; Simoni et al. 2006).

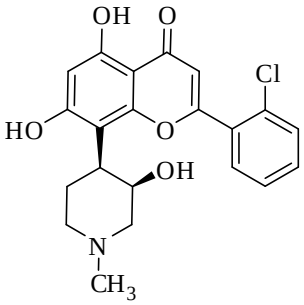


2.12 Combretastatin



2.13 Combretastatin A_4

Flavopiridol (**2.14**), a synthetic flavone derived from the plant alkaloid rohitukine, which was isolated from *Dysoxylum binectariferum*, is currently in phase II clinical trials against a broad range of tumours. It was found to be the first anticancer agent that targets cell cycle progression (Cragg et al. 2005; Cragg and Newmann 2005; Mans et al. 2000).



2.14 Flavopiridol

The potential use of higher plants as sources of new drugs is still poorly explored. Of the estimated 250,000-500,000 plant species existing worldwide, only a small percentage has been investigated phytochemically, and a fraction submitted to biological or pharmacological screening is even smaller (Calixto et al. 2000).

South Africa in particular, is blessed with a rich plant biodiversity of about 24 300 indigenous plants (Mulholland and Drewes 2004), representing about 10% of the world's

higher plants. Approximately 3000 plants are reported to be used medicinal and to have medicinal properties, but only a few have been described or studied (Coetzee et al. 1999; Taylor et al. 2001).

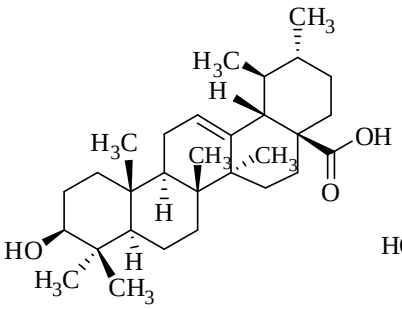
The potential of using plants as anticancer agents was recognized in the 1950s by the U.S. National Cancer Institute (NCI) under the leadership of the late Dr. Jonathan Hartwell. The NCI, in its search for anticancer agents has tested 35,000 species of higher plants. Many of these have shown reproducible anticancer effects, and the active principles have been extracted from most of these and their structures determined. However, Cragg and Newman (2005) indicated that no new plant-derived clinical anticancer agents except Taxol (Christopher 1993) have, as yet, reached the stage of general use, but a number of agents are in preclinical development.

2.4 Classes of plant-derived anticancer agents

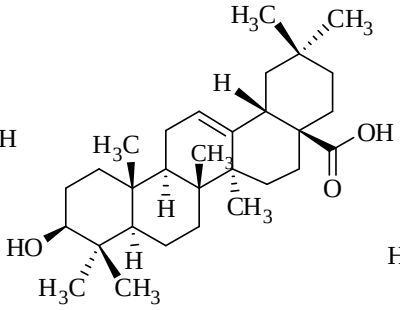
Kintzios (2006) classified plant-derived natural products with antitumour properties into thirteen distinct chemical groups. Of these, alkaloids, phenylpropanoids and terpenoids were found to be the best documented for their tumour cytotoxicity. Only the triterpenoids, sesquiterpene lactones, flavonoids and coumarins will be discussed, since these classes were isolated in this study.

2.4.1 Triterpenoids

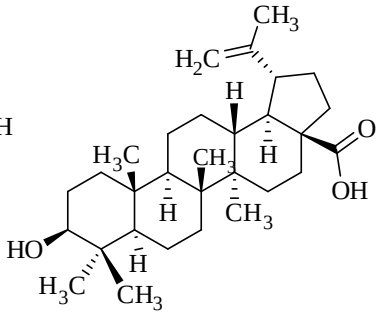
Triterpenoids are terpenoid-type compounds made from six isoprene units. These are naturally-occurring compounds with a C_{30} skeleton and are present as secondary metabolites in plants (Harborne and Baxter 1993). Examples are ursolic acid (**2.15**), oleanolic acid (**2.16**) and betulinic acid (**2.17**).



2.15 Ursolic acid



2.16 Oleanolic acid

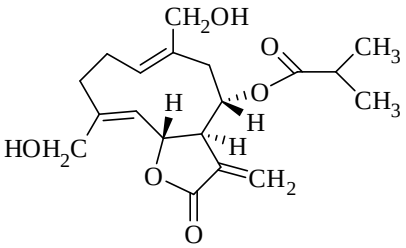


2.17 Betulinic acid

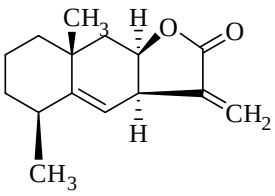
2.4.2 Sesquiterpene lactones

Sesquiterpene lactones are a class of natural sesquiterpenoids, which are chemically distinct from other members of the group through the presence of a γ-lactone system. This class of compounds are biologically very active and are classified into four major groups: germacranolides with a 10-membered ring (Harborne and Baxter 1993), e.g., alatolide (**2.18**); eudesmanolides with two fused six-membered rings, e.g., alantolactone (**2.19**); guaianolides with a five-membered ring fused to a seven and a methyl substituent at C-4, e.g., archangelolide (**2.20**); and pseudoguaianolides, as guaianolides but a methyl substituent at C-5, e.g., ambrosin (**2.21**).

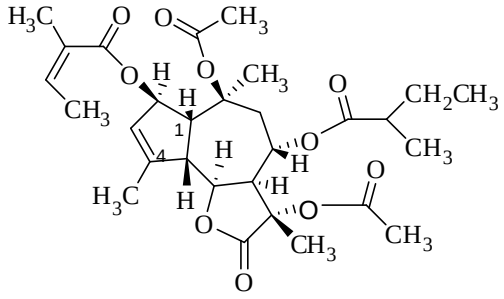
Sesquiterpene lactones form one of the largest groups of cytotoxic and antitumour compounds of plant origin. Most of these active sesquiterpene lactones are found in species of the Asteraceae, although some of them originate in Magnoliaceae, Apiaceae and even fungi. A wide variety of sesquiterpene lactones isolated from plant extracts have demonstrated other pharmacological activities which include expectorant, blood pressure lowering, cholinergic, hypoglycaemic, anti-asthmatic and anti-inflammatory activity (Picman 1986; Harborne and Baxter 1993).



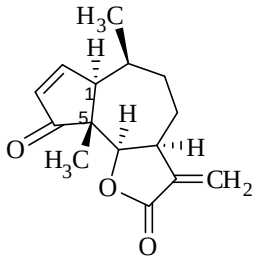
2.18 Alatolide



2.19 Alantolactone



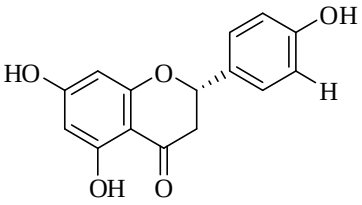
2.20 Archangelolide



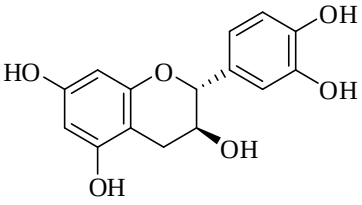
2.21 Ambrosin

2.4.3 Flavonoids

Flavonoids are compounds that are responsible for the colour of flowers, fruits and sometimes leaves. Flavonoids protect the plant from UV-damaging effects and play a role in pollination by attracting insects or birds by their colours. Their basic structure is 2-phenyl chromane or Ar-C3-Ar skeleton (Harborne and Baxter 1993). Flavonoids are categorized as flavones, flavanones, flavonols, dihydroflavonols, flavan-3-ols, flavan-3,4-diols and flavans. The best known flavonoids are the flavanones, e.g., naringenin (**2.22**); and the flavanon-3-ols or dihydroflavonols, e.g., (+)-catechin (**2.23**).



2.22 **Naringenin**

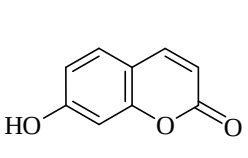


2.23 (+)-Catechin

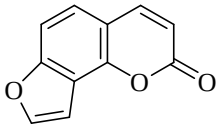
2.4.4 Coumarins

Coumarins are shikimate-derived, benzo-α-pyrone derivatives that are present in plants both in a free state and as glycosides. Most natural coumarins contain a hydroxyl group at the C-7 position. Some coumarins are phytoalexins and are manufactured by the plant in the event of an infection by bacteria and fungi. Scopoletin is among such compounds (Harborne and Baxter 1993).

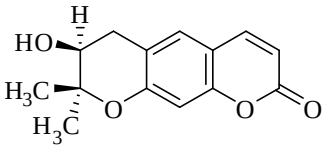
There are three major classes: the hydroxycoumarins, such as umbelliferone (**2.24**), the furanocoumarins, e.g., angelicin (**2.25**), and the pyranocoumarins, e.g., decursinol (**2.26**).



2.24 Umbelliferone



2.25 Angelicin



2.26 Decursinol

2.5 Conclusion

Cancer is a human tragedy that strikes and kills the lives of our beloved people. With limited number of effective anticancer drugs from natural resources currently in use, there is a real need for new, safe, cheap and effective anticancer drugs to combat this dreaded and formidable disease.

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Chapter 3

Materials and Methods

3.1 Plant collection

Two basic methods of plant collections were employed in this study, the random method and ethnobotanical method. In the random method, plants were randomly selected based on what was available and what could be identified. The ethnobotanical method relied on the knowledge of local, indigenous people (in this case, traditional healers). Plants were collected by two botanists, Hans Vahrmeijer and Jean Meyer. Voucher specimens were deposited and identified at the South African National Biodiversity Institute (SANBI) in Pretoria.

3.2 Processing and extraction of plant material

The collected plant materials were oven-dried at 30-60 ºC and the drying time and temperature varied depending on the nature of the plant part. The dried plant materials were milled to a coarse powder and stored at ambient temperature prior to extraction.

The dried and ground plant materials were extracted with methanol/dichloromethane (1:1) for a period of 1-2 days at room temperature. The solvent was evaporated using a rotary evaporator at 50-60 ºC and then further dried either in a dessicator for 24 h or in a fume hood for 5 days. The extracts were stored in a cold room at -20 ºC until used.

3.3 Liquid-liquid partitioning of extracts

A liquid-liquid partitioning step was employed to separate the extracts based on polarities. The organic extract was first dissolved in methanol/water (9:1) and then partitioned with hexane three times using a separatory funnel. The combined hexane layers and the methanol from the methanol/water layer were evaporated under reduced pressure.

Additional water was added to the remaining water layer and further partitioned, three times with dichloromethane. The combined dichloromethane layers were concentrated, whilst the water layer was freeze-dried.

3.4 Chromatography

3.4.1 Column Chromatography

Different sized columns, ranging from 1.5-8 cm in diameter, were used depending on the amount of sample available and the purification stage. Separation of crude extracts was generally carried out on a column using silica gel 60 (0.063-0.2 mm). Flash column chromatography was carried out using 35-75 micron flash silica gel (Merck). All separations were carried out under gravity.

3.4.2 Thin-layer chromatography

Thin-layer chromatography was carried out on pre-coated glass plates (Merck, SIL G-25 UV_{254}, 20 cm x 20 cm). The plates were first viewed under ultraviolet (UV) light (shortwave 254 nm and long wave 366 nm) and then sprayed with a vanillin- H_2SO_4 (1 g vanillin in 100 ml H_2SO_4) solution and heated at 100 ºC for few minutes to detect compounds.

3.4.3 Preparative thin-layer chromatography

Compounds which were visible under UV light were isolated using this technique. The glass plates were lined with the sample, 1.5 cm from the bottom of the plate. The samples were loaded onto the plates by using a Pasteur pipette containing the sample from one end of the plate to the other end. The plates were then developed in a chromatography tank and the compounds of interest were detected under UV light. The bands were then removed, dissolved in methanol and dichloromethane and then filtered to remove the silica gel from the filtrate containing compounds of interest.

3.4.4 Vacuum Liquid Chromatography

This technique was used to fractionate crude extracts. A sintered glass funnel was used, dry packed with a suitable adsorbent such as silica gel of TLC grade. The packing was done under vacuum to achieve maximum packing density. The solvent was poured and sucked dry under vacuum. The sample was loaded in the form of dry slurry and vacuum reapplied to settle the sample. A small amount of silica gel was added on it and filter paper (round, of sintered funnel circumference) was placed on it. Then the column was eluted with appropriate solvent mixture (or solvent of increasing polarity). In each attempt the column was sucked to dryness.

3.4.5 Gas chromatography-Mass Spectrometry (GC-MS)

GC-MS analyses were carried out on an Agilent 7890A GC instrument coupled to a 5975C inert Mass Selection Detector (MSD) system provided with an HP-5MS capillary column.
GC-MS analysis conditions: Injection temperature: 250 ^{o}C; mode: pulsed splitless; detector temperature: 300 ^{o}C; capillary column: 30 m, 0.32 mm, 0.25 μm film thickness. The temperature program was as follows: from 45 ^{o}C (1 min) to 280 ^{o}C (0 min) at 25 ^{o}C /min then to 320 ^{o}C (1 min) at 5 ^{o}C/min. The injection volume was 1 μl. Helium was used as a carrier gas at a flow rate of 1.2 ml/min. MS was in EI mode at 70 eV.
The identification of the compounds in the chromatographic profiles was achieved by comparison of their mass spectra with a NIST library data base.

3.5 Spectroscopy

3.5.1 Nuclear Magnetic Resonance (NMR Spectroscopy)

NMR spectroscopy was carried out on either a 400 MHz Varian Unity Inova spectrometer or on Bruker Avance III 400 or 500 MHz spectrometers at the CSIR and University of KwaZulu-Natal, respectively. All spectra were recorded at room temperature in either deuterated chloroform, deuterated methanol, a mixture of deuterated chloroform and methanol or deuterated dimethylsulphoxide. The chemical shifts were all reported in ppm

relative to tetramethylsilane (TMS). Structural characterizations were carried out using a combination of 1D (^{1}H, ^{13}C) and various 2D (DEPT, COSY, HSQC, HMBC and NOESY) experiments.

3.5.2 Mass Spectrometry (MS)

Mass spectra were obtained from a coupled HPLC-UV/MS instrument with a triple pole Quattro LC Micro mass spectrometer or on a Waters LCT Premier operating in the ESI positive or ESI negative modes at the CSIR and University of KwaZulu-Natal respectively. High-resolution mass spectroscopy was performed at the University of Witwatersrand, South Africa on a VG 70SEQ MS instrument, operated at 8 kV in the positive EI mode.

3.6 Physical constants

3.6.1 Optical Rotation

Optical rotations were recorded at room temperature on a Perkin Elmer 241 Polarimeter at 589 nm using a sodium lamp as a light source and a 1 dm cell.

3.6.2 Melting points

Melting points for crystalline compounds isolated were determined using a Reichert hot stage apparatus and are uncorrected.

3.7 Biological assays

3.7.1 *In vitro* anticancer assay

Anticancer assays were performed at the CSIR, South Africa and also at the National Cancer Institute (NCI) in the United States of America, in accordance with the protocol of

the Drug Evaluation Branch, National Cancer Institute (Monks et al. 1991; Kou et al. 1993; Leteurtre et al. 1994). Extracts and compounds were assayed in the 3-cell line panel consisting of TK10 (renal), MCF7 (breast), and UACC62 (melanoma). The extracts or compounds were tested at a single concentration (100 μg/ml) and the culture was incubated for 48 hrs.

End point determinations were made with a protein-binding dye, sulforhodamine B (SRB). The growth percentage was evaluated spectrophotometrically versus controls not treated with test agents. Results for each extract/compound were reported as the growth percentage of the treated cells, compared to that of the untreated control cells. Extracts and compounds which reduced the growth of two of the cell lines by 75% or more, were further tested at five serial dilution concentrations ranging from 6.25 - 100 μg/ml for extracts or 10 fold dilutions for compounds.

The results of the five-dose assays were reported as total growth inhibition (TGI). The assay results for the extracts screened were separated into four categories: inactive (TGI > 50 μg/ml), weak (15 μg/ml < TGI < 50 μg/ml), moderate (6.25 μg/ml < TGI < 15 μg/ml) and potent (TGI < 6.25 μg/ml).

Extracts from the latter two categories were selected for further *in vitro* testing for selective cytotoxicity against panels of human cancer cell lines at the NCI. The NCI panel of 60 cell lines included leukemia (L) lines, non-small cell lung cancer (NSCLC) lines, colon cancer (CL) lines, central nervous system cancer (CNSC) lines, melanoma (M) lines, ovarian cancer (OC) lines, renal cancer (RC) lines, prostate cancer (PC) lines and breast cancer (BC) lines.

The results from NCI were reported as mean $\log_{10}$ functions of the three response parameters, GI_{50} (50% growth inhibition), TGI (drug concentration that is indicative of the cytostatic effect of the test agent) and LC_{50} (50% lethal concentration indicative of the cytotoxic effect of the test agent), calculated for each cell line. The *in vitro* results were classified as follows:

log GI_{50} >1.10 to 1.5, the extract was considered weakly active

log GI_{50} > 0 to 1.0, the extract was considered moderately active

log GI_{50} < 0, the extract was considered potent

3.7.2 *In vitro* antiplasmodial assay

This assay was carried out at the Department of Pharmacology, University of Cape Town. Samples were prepared as a 2 mg/ml stock solution in 10% DMSO or 10% methanol and were sonicated to enhance solubility. Stock solutions were stored at -20 ºC. The test samples were tested in duplicate against a chloroquine sensitive (D10) strain of *Plasmodium falciparum.* Continuous *in vitro* cultures of asexual erythrocytes stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976). Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase (pLDH) assay using a modified method described by Makler (1993). Chloroquine (CQ) was used as the reference drug in all experiments. A full dose-response was performed for all samples to determine the concentration inhibiting 50% of parasite growth (IC_{50} value). Test samples were tested at a starting concentration of 100 μg/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations, with the lowest concentration being 0.2 μg/ml. CQ was tested at a starting concentration of 100 ng/ml. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability.

Dose-response curves were constructed using non-linear dose-response curve fitting analyses with GraphPad Prism v.4.0 software. The concentration of the drug that inhibits 50% of the parasites (IC_{50} values) was established from the dose-response curves using GraphPad Prism.

3.7.3 *In vitro* Cytotoxicity Assay

Cytotoxicity assays were conducted by the Department of Pharmacology, University of Cape Town (UCT). This assay was performed on Chinese Hamster Ovarian (CHO) cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) calorimetric assay. The MTT assay is used as a colorimetric assay for cellular growth and survival and compares well with other available assays (Mosmann 1983; Rubinstein et al. 1990). The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The test samples were tested in triplicate on one occasion.

Samples were dissolved in methanol/water (1:9). Stock solution (2 mg/ml) was prepared and stored at -20 ºC until use. The highest concentration of methanol to which the cells were exposed to had no measurable effect on the cell viability. Emetine was used as the positive control in all cases. The initial concentration of emetine was 100 µg/ml, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/ml. The same dilution technique was applied to all test samples with an initial concentration of 100 µg/ml to give 5 concentrations, with the lowest concentration being 0.010 µg/ml. In the initial stage of the experiments, the cells were adjusted to a concentration of 10^5/ml and 100 µl of this cell suspension were seeded in all wells except in column 1 (blank) in a 96-well culture plate (costar). The plates were incubated at 37 ºC for 24 h in a humidified 5% CO_2 – air atmosphere. After the incubation period, the medium was carefully aspirated out of the wells and 100 µl of the different test substances (drug solutions) were added in quadruplicate to columns 3 through to 9. A further 100 µl of culture medium was then added to all of the wells containing cells and drugs (columns 3 to 9), and 200 µl of medium was dispensed to the wells in column 1 (blank) and column 2 (cells and no drug). The microplate was then incubated at 37 ºC for 48 h.

After the 48 h incubation period, 25 µl of sterile MTT (5 mg/ml in PBS) was added to each well and incubation was continued for 4 h at 37 ºC. The plates were then centrifuged at 2050 rpm for 10 minutes and the supernatant was carefully aspirated from the wells, ensuring that the formazan crystals were not disturbed. The formazan crystals were dissolved in DMSO (100 µl) and the plate was gently shaken for 5 min on a microtitre plate shaker. The plate was blanked on the wells in column 1 and the absorbance of the crystals was measured at λ 540 nm on a microtitre plate reader (Cambridge Technologies). The cell viability was calculated in each well using the formula:

$$\% \text{ cell viability} = \frac{A_{\lambda 540} \text{ test well (cells + drug)}}{A_{\lambda 540} \text{ control well (cells + no drug)}} \text{ X } 100$$

The concentration of drug that inhibits 50% of the cells (IC_{50} values) for these samples were obtained from dose-response curves, using a non-linear dose-response curve fitting analyses via GraphPad Prism v.4 software.

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Chapter 4

Bioassay-guided isolation of cytotoxic components from *Anthospermum hispidulum* E.Mey. ex Sond.

4.1 Introduction

4.1.1 Review of Anthospermeae

The tribe Anthospermeae belongs to the huge, mostly woody, tropical and subtropical family Rubiaceae, one of the largest of all angiosperm families, with about 637 genera and more than 10 000 species (Bremer et al. 1995; Bremer et al. 1999; Mongrand et al. 2005; Natali et al. 1995). Anthospermeae and Theligoneae are the only tribes in the family Rubiaceae which are comprised of wind-pollinated taxa (Puff 1986a).

According to Kirkbribe (1982), Anthospermeae is one of the most advanced tribes in the family, with foliaceus stipules, herbaceous habitat and fleshy fruits. This tribe is widely distributed and occurs in tropical, subtropical and temperate regions of the southern hemisphere. It consists of approximately a dozen genera and comprises several hundred species and is believed to be closely related to the tribe Paederieae (Puff 1982; Puff 1986a).

The subtribe, Anthosperminae is restricted to Africa, Arabia in the Yemen Arab Republic, Madagascar, the Canary Islands and Madeira and is characterized by dry, dehiscent fruits with the exception of dry, indehiscent fruits in a few *Nenax* species, and unisexual and /or protandrous hermaphrodite flowers (Puff 1982; Puff 1986a). Anthosperminae comprises of five genera, *Anthospermum, Galopina*, *Nenax*, *Carpacoce* and *Phyllis.*

Anthospermum L., the largest genus with thirty-nine species, is widely distributed in Africa south of the Sahara and in Madagascar. The highest concentration of taxa is found in Southern Africa; 21 species (29 taxa) occur in this flora area, of which 18 spp. (26 taxa) are endemic; of these, 10 spp. (16 taxa) are endemic to the south-western Cape region

(map, Fig. 4.1). This genus comprises large to medium-sized shrubs, dwarf shrubs, short-lived sub-shrubs, or perennial herbs (Dyer 1975; Puff 1982; 1986a; 1986b).

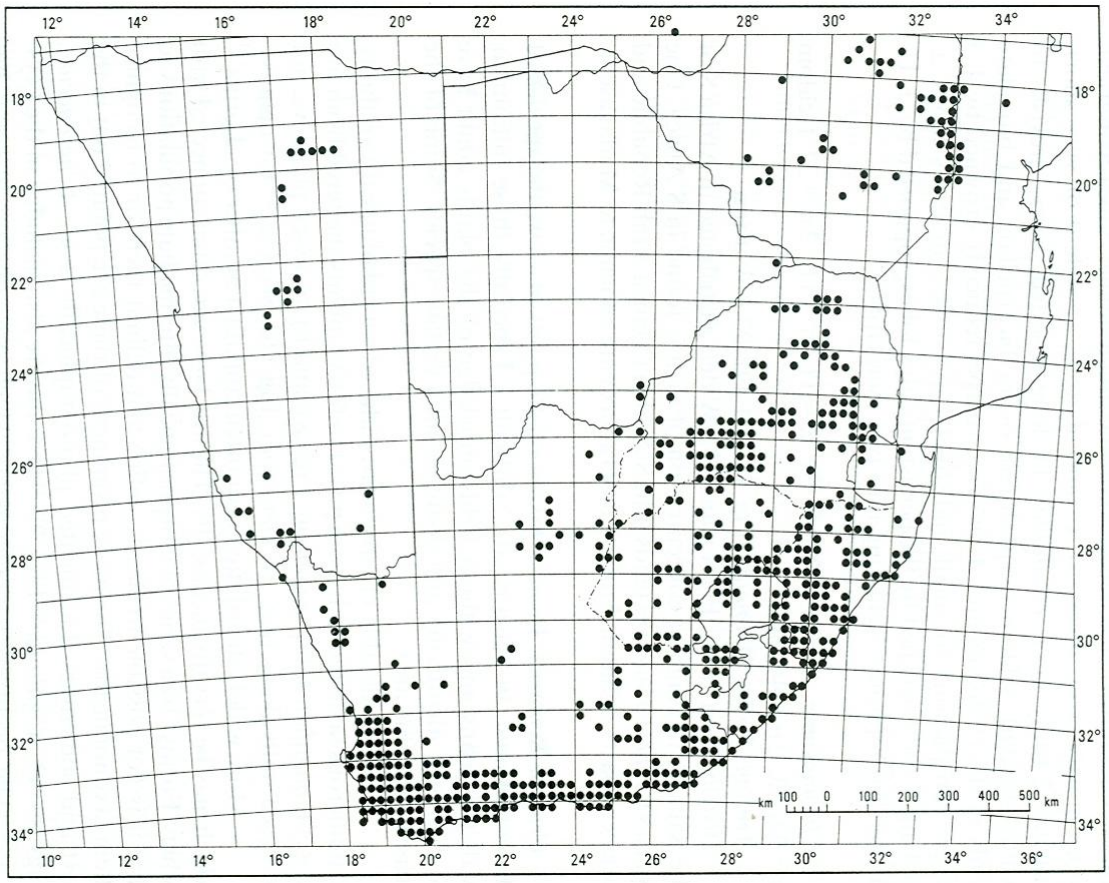


Figure 4.1: Geographical distribution of *Anthospermum* in Southern Africa (Puff 1986a)

4.1.2 Background on *Anthospermum hispidulum* E.Mey. ex Sond.

Anthospermum hispidulum (Fig. 4.2) is a dwarf shrub with often thick, woody roots, non-dioecious hairy species with large funnel shaped male and female flowers, long corolla tubes, and small, narrow fruits. The leaves are arranged decussately and the stems are about 15-40 cm long. *A. hispidulum* is believed to be closely allied to *A. whyteanum* and is found growing on rocky ridges, in cracks of rocky sheets, on rocky outcrops in grassland, on koppies, on rock ledges or krantzes, or amongst boulders, less commonly in rocky grassland, often occurring over sandstone, quartzite or old granite (Puff 1986a; Puff 1986b).

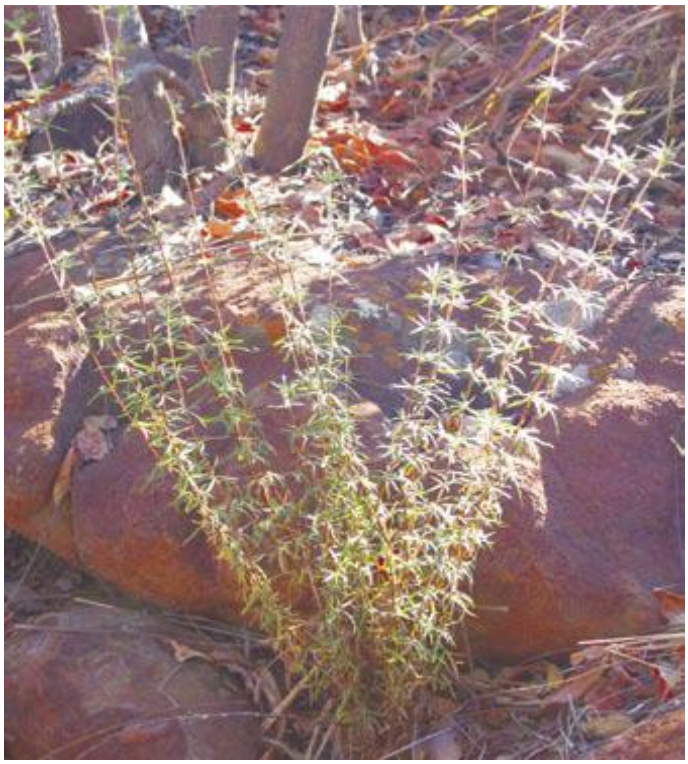


Figure 4.2: *Anthospermum hispidulum*

The distribution of *A. hispidulum* (Fig. 4.3) is limited to only four provinces in South Africa (Limpopo, KwaZulu-Natal, Eastern Cape and Free State) and Swaziland (Puff 1986a; Puff 1986b).

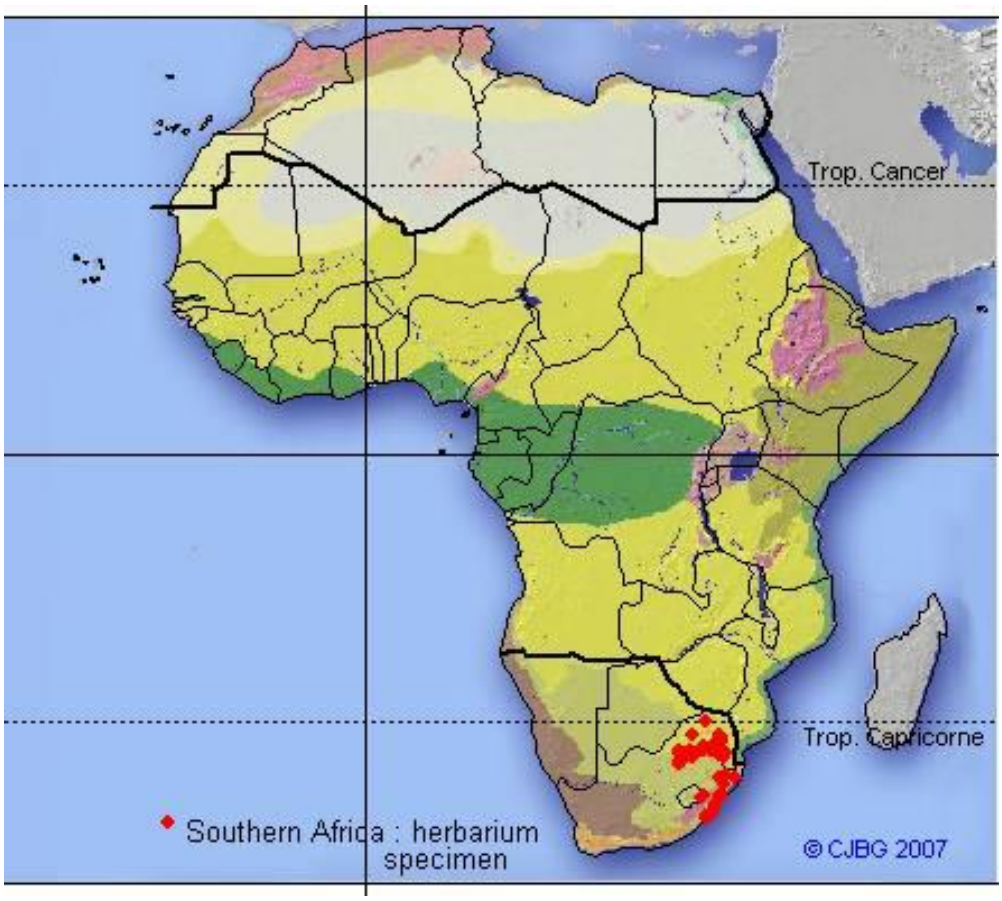


Figure 4.3: Geographical distribution of *Anthospermum hispidulum* in Southern Africa

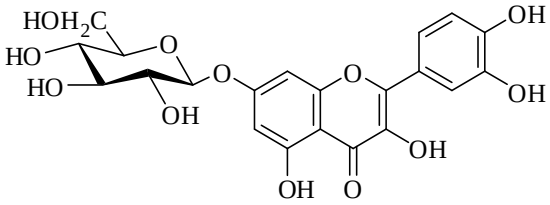
Source: http://www.ville-ge.ch/cjb/bd/africa/details.php?langue=an&id=86047

4.1.3 Ethnopharmacology

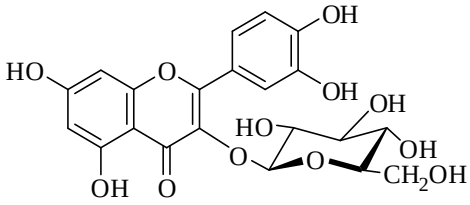
To our knowledge, there is no published ethnopharmacology information on *A. hispidulum*, but other species of the genus *Anthospermum* have long been used in folk medicine to treat various ailments. A decoction of *A. rigidum* is used by the Tswana and Kwena people to relieve toothache. The roots of *A. pumilum* are used as a remedy for painful menstruation and also during pregnancy. *A. hedyotideum* and *A. rigidum* are both used as charms to hasten convalences and also for various other protective purposes (Hutchings et al. 1996; Watt and Breyer-Brandwijk 1962).

4.2 Phytochemical studies

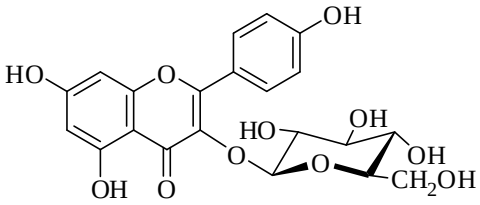
From a phytochemical point of view, no previously reported investigations were found for *A. hispidulum*. Moreover, only a few species of the genus *Anthospermum* have been investigated for their chemical constituents. The flavonoids quercetin 7-*O*-glucoside (**4.1)**, quercetin 3-*O*-glucoside (**4.2)** and kaempferol 3-*O*-glucoside (**4.3)**, were isolated from the leaves of *A. littoreum*, *A. galpinii*, *A. aethiopicum*, *A. usambarense* and *A. emirnense.* Iridoid glycosides, e.g. asperuloside (**4.4)**, were isolated from *A. aethiopicum* (Harborne and Baxter 1993; Puff 1986a).



4.1



4.2



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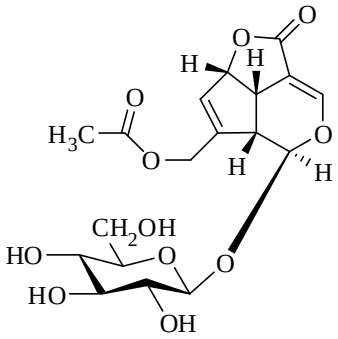


4.4

4.3 Isolation of active compounds

Leaves of *A. hispidulum* collected from Steenkampsberg near Lydenberg, Limpopo province in South Africa were dried, ground and extracted with methanol/dichloromethane (1:1) for 24 hours at room temperature (Fig. 4.4). The organic crude extract was assayed and found to have moderate anticancer activity (Chapter 3) against a panel of three human cancer cell lines at CSIR and weak activity (Chapter 3) against a panel of sixty human cancer cell lines at the NCI. This extract was subjected to liquid-liquid partitioning between hexane, methanol/water and dichloromethane. This led to three fractions i.e. a

hexane, dichloromethane and aqueous fraction. Of these, only the dichloromethane fraction retained activity in the CSIR screen.

The dried dichloromethane extract was fractionated by column chromatography using silica gel as the stationary phase and different solvent mixtures (hexane, dichloromethane, acetone and methanol) of increasing polarity as the mobile phase. Seven fractions were obtained and repeated flash chromatography of these fractions led to the isolation of three compounds; scopoletin (**4.5)**, ursolic acid (**4.6)** and oleana-11,13-diene (**4.7)**.

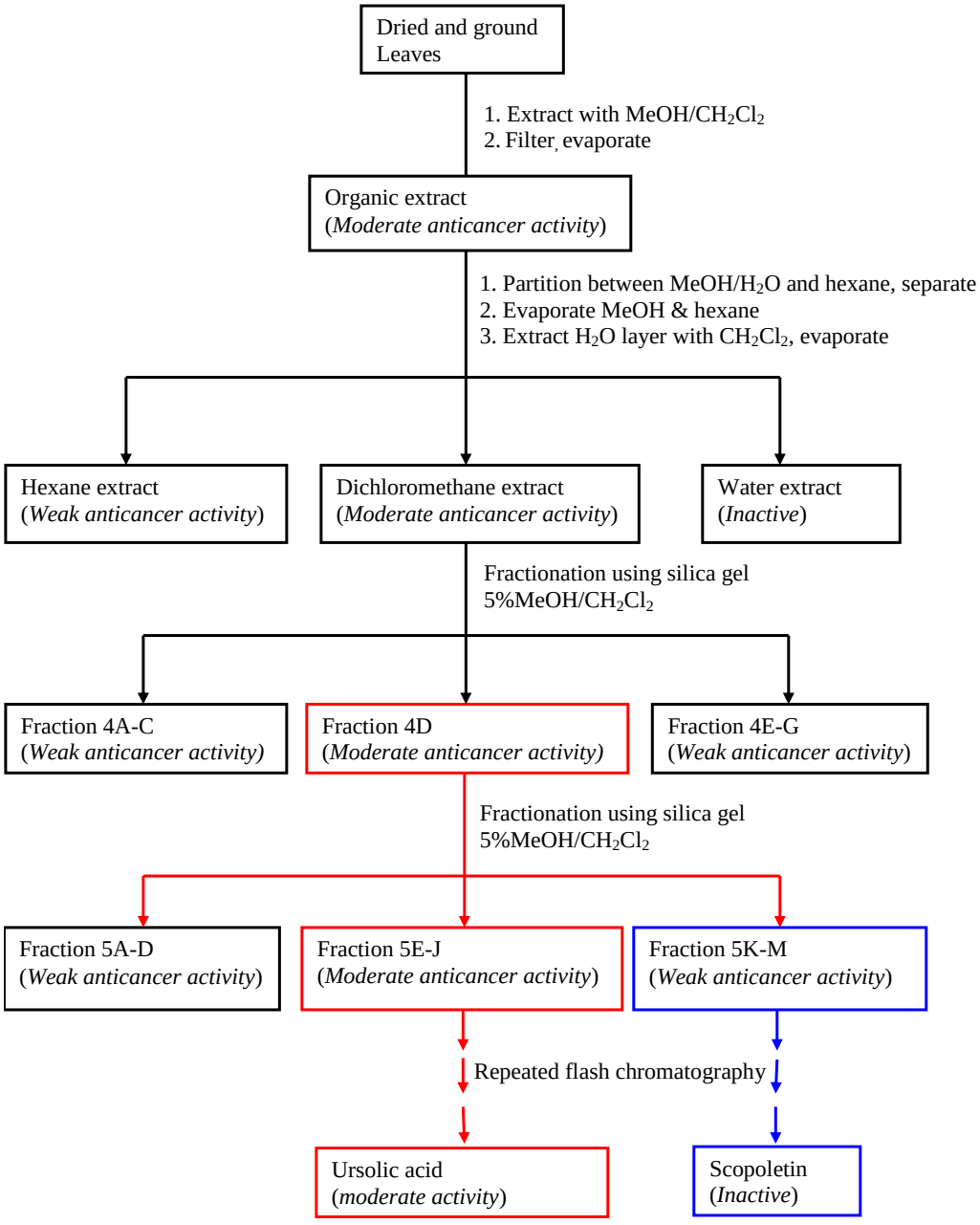


Figure 4.4: Flow diagram illustrating the purification of the organic extract

4.3.1 Structural elucidation of scopoletin (4.5)

Compound **4.5** was isolated as yellow crystals. The high resolution EI mass spectrum gave a molecular ion signal $[M]^+$ at *m/z* 192.04358, corresponding to a molecular formula of $C_{10}H_8O_4$ and a distinct fragment peak at *m/z* 121.02964 due to $[M\text{-}C_3H_3O_2]^+$ (Fig. 4.5)

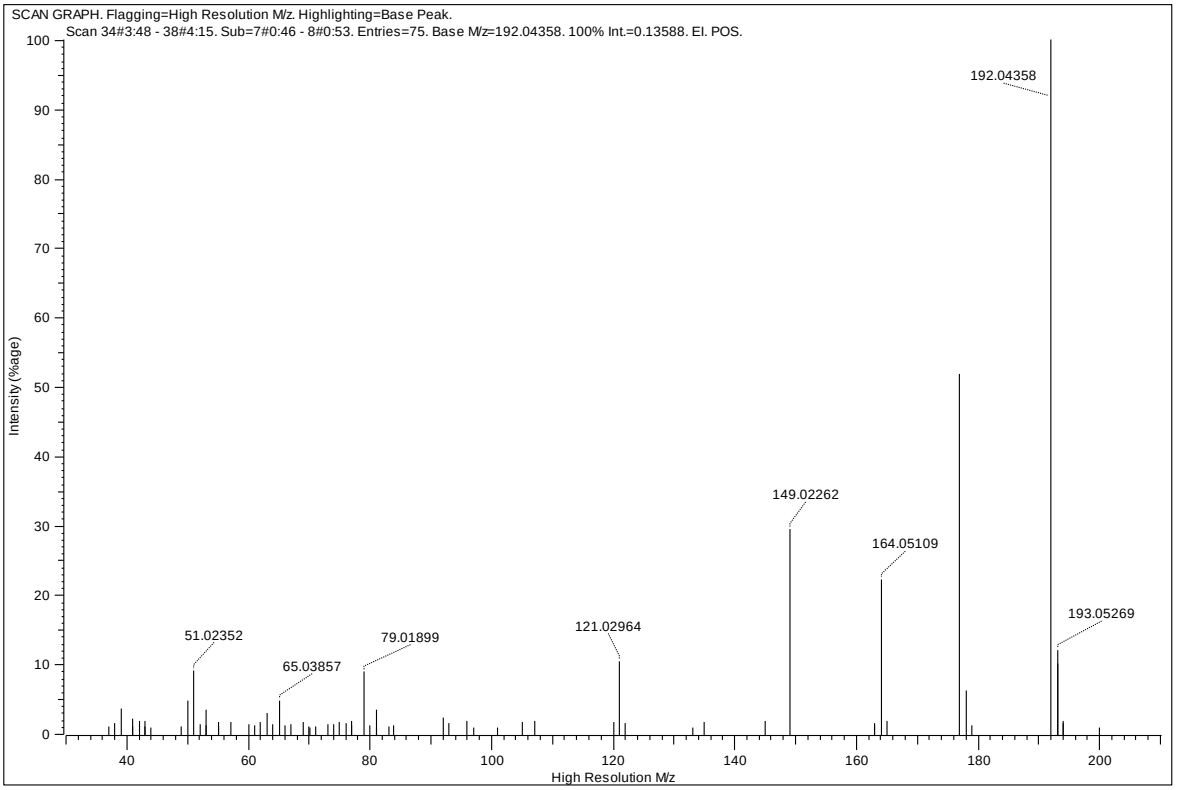


Figure 4.5: High-resolution electron ionization mass spectrum of compound **4.5**

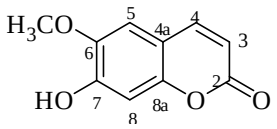
The ^1H NMR spectrum (Plate 1) showed a pair of doublets at δ_H 6.22 and δ_H 7.55 (each 1H, d, *J* 9.5 Hz), assigned to H-3 and H-4 of an α-pyrone ring system. It also showed the presence of two aromatic proton singlets in the region δ_H 6.80 – 6.88 and methoxyl protons at δ_H 3.91. The ^{13}C NMR spectrum (Plate 2) showed nine sp^2 carbon signals, which were assigned by using the DEPT spectrum (Plate 3) as four CH carbons and five quaternary carbons including an ester carbonyl at δ_C 161.5 and three oxygenated carbons at δ_C 144.2, 149.9 and 150.5.

Compound **4.5** was identified as 7-hydroxy-6-methoxycoumarin, also known as scopoletin. NMR data corresponded with the reported data (Kayser and Kolodziej 1995) (Table 4.1).

Table 4.1: ^{13}C and ^{1}H NMR spectral data for scopoletin (**4.5**) in $CDCl_3$

Carbon number	**Carbon type**	**δ_C**	**δ_H (*J* in Hz)**	**HMBC**	**Literature* δ_C**	**Literature* δ_H (*J* in Hz)**
2	-C=O	161.5		H-4	160.9	
3	-CH	113.6	6.22 *d* (9.5)		113.3	6.20 *d* (9.5)
4	-CH	143.4	7.55 *d* (9.5)	H-5	144.6	7.85 *d* (9.5)
4a	-C	107.7		H-8, H-3	109.7	
5	-CH	111.7	6.80 *s*	H-4	112.0	6.84 *s*
6	-C	149.9		H-8	151.9	
7	-C-OH	144.2		H-5	145.6	
8	-CH	103.4	6.88 *s*		103.7	6.59 *s*
8a	-C	150.5		H-5, H-4	151.8	
OCH_3	$-CH_3$	56.6	3.91 *s*		56.7	3.86 *s*

*- Kayser and Kolodziej 1995, Compound was dissolved in acetone-d_6



4.5

4.3.2 Structural elucidation of ursolic acid (4.6)

Compound **4.6** was isolated as white crystals. The FAB mass spectrum of compound **4.6** showed a molecular ion signal $[M]^+$ at *m/z* 456.0 which corresponds to the molecular formula $C_{30}H_{48}O_3$. Fragment ions at *m/z* 439 $[M\text{-}OH]^+$, 411 $[M\text{-}COOH]^+$ and 393 $[M\text{-}CH_3O_3]^+$ were also observed in the mass spectrum (Fig 4.6).

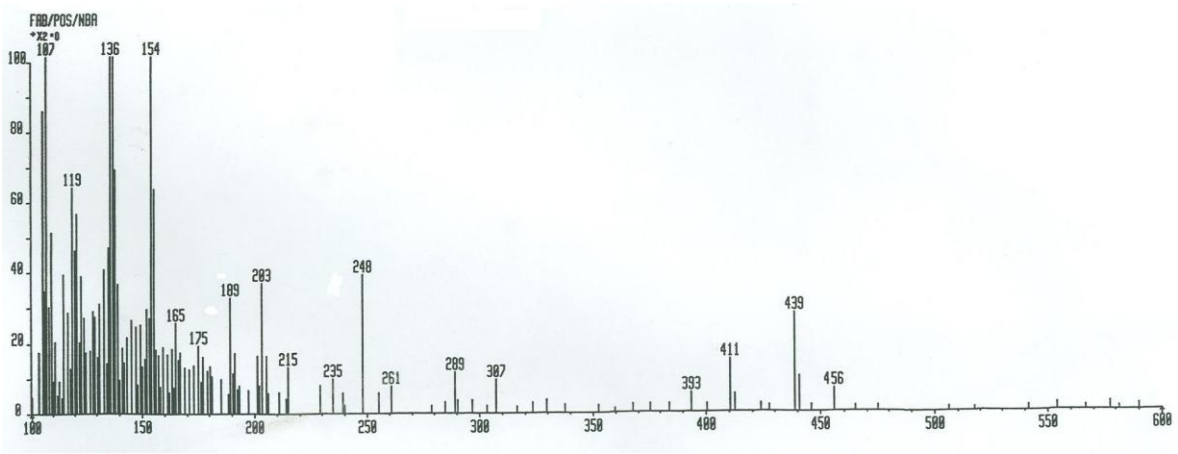


Figure 4.6: The FAB mass spectrum of compound **4.6**

The ^{1}H NMR spectrum (Plate 6) showed that compound **4.6** has five three-proton singlets indicating tertiary methyls at δ_H 0.70, 0.74, 0.84, 0.90 and 1.01 and two doublets of secondary methyls at δ_H 0.78 (3H, *d, J* 6.5 Hz) and 0.86 (3H, *d, J* 6.1 Hz). It further showed an olefinic proton at δ_H 5.16 (1H, *t, J* 3.5 Hz, H-12), a proton doublet at δ_H 2.11 (1H, *d, J* 11.8 Hz, H-18) and a doublet of doublets at δ_H 3.11(1H, *dd, J* 9.0, 7.0 Hz, H-3). These data suggested that compound **4.6** possessed an ursene skeleton.

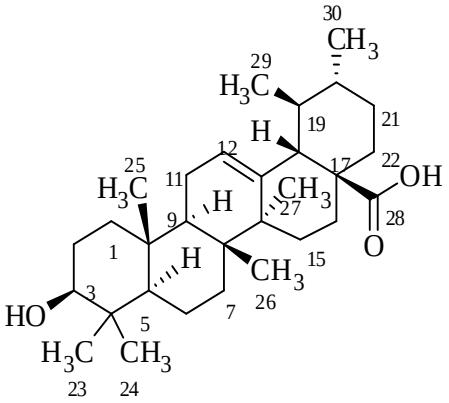
The ^{13}C NMR spectrum (Plate 7) showed the presence of 30 carbon signals: a carboxylic acid at δ_C 180.8, olefinic carbons at δ_C 125.8 and 138.5, an oxygenated carbon at δ_C 79.0, and seven methyls at δ_C 15.6, 15.8, 17.1, 17.2, 21.3, 23.7 and 28.3. Therefore, the structure of **4.6** was assigned as ursolic acid. MS and ^{13}C NMR spectral data of compound **4.6** are in agreement with those reported in the literature (Thanakijcharoenpath and Theanphong 2007) for ursolic acid.

Table 4.2 ^{13}C and ^{1}H NMR spectral data for ursolic acid (**4.6**) in $CDCl_3$ / CD_3OD

Carbon number	δ_C	δ_H (*J* in Hz)	**Literature*** δ_C	**Literature*,#** δ_H (*J* in Hz)
1	38.9		38.2	
2	27.1		27.0	
3	79.0	3.11(1H, *dd*, *J* =9.0, 7.0)	76.8	2.99 (1H, *dd*, *J* =10.5, 5.2)
4	39.0		38.5	
5	55.6		54.8	
6	18.6		18.0	
7	33.4		32.7	
8	39.8		39.1	
9	47.9		47.0	
10	37.2		36.5	
11	23.6		22.8	
12	125.8	5.16 (1H, *t*, *J* = 3.5)	124.6	5.12 (1H, *br t*, *J* = 3.4)
13	138.5		138.2	
14	42.4		41.6	
15	29.9		27.5	
16	24.5		23.8	
17	48.1		46.8	
18	53.2	2.11 (1H, *d*, *J* = 11.8)	52.4	2.10 (1H, *d*, *J* = 11.6)
19	39.4		38.5	
20	39.2		38.4	
21	30.9		30.2	
22	37.1		36.3	
23	28.3	0.90 (3H, *s*)	28.2	0.88 (3H, *s*)
24	15.6	0.74 (3H, *s*)	16.1	0.67 (3H, *s*)
25	15.8	0.70 (3H, *s*)	15.2	0.86 (3H, *s*)
26	17.2	0.84 (3H, *s*)	16.9	0.74 (3H, *s*)
27	23.7	1.01 (3H, *s*)	23.3	1.03 (3H, *s*)
28	180.8		178.3	
29	17.1	0.86 (3H, *d*, *J* = 6.1)	17.0	0.80 (3H, *d*, *J* = 6.4)
30	21.3	0.78 (3H, *d*, *J* = 6.5)	21.0	0.89 (3H, *d*, *J* = 8.9)

*- Thanakijcharoenpath and Theanphong 2007

#- Compound was dissolved in DMSO-d_6



4.6

4.4 Biological activity of the crude extract and isolated compounds

4.4.1 *In-vitro* anticancer activity

Anticancer assays were performed at both CSIR and the NCI. *A. hispidulum* was identified through random screening at the CSIR and the dichloromethane/methanol extract exhibited moderate anticancer activity when tested against a panel of three human cancer cell lines at the CSIR (Fig. 4.7; TGI value of between 12.5 and 25 µg/ml), while weak anticancer activity (Fig. 4.10; average log GI_{50} of 1.41) against 60 human cancer cell lines was observed at the NCI. Bioassay-guided fractionation of the crude extract was conducted and led to the isolation of two compounds. Compounds **4.5** and **4.6** were evaluated for anticancer activity at the CSIR. Ursolic acid (**4.6**) showed a significant improvement in activity (Fig. 4.9; TGI value between 6.25 and 12.5 µg/ml), while scopoletin (**4.5)** did not show any significant activity in all three cell lines (Fig. 4.8). Therefore, ursolic acid (**4.6**) can be considered as an active ingredient since its activity compares well to that of the crude extract.

The results from CSIR are presented as dose-response curves for each sub-panel (melanoma, breast and renal). The dose response curve is a plot of percent growth (PG) against the concentration for each cell line. The response parameters GI_{50} (50% growth inhibition), TGI (total growth inhibition) and LC_{50} (lethal concentration killing 50% of cells) are interpolated values from these graphs representing the concentrations at which PG is +50, 0 and -50 respectively

The results from NCI were reported as mean $\log_{10}$ functions of the three response parameters, GI_{50} (50% growth inhibition), TGI (drug concentration that is indicative of the cytostatic effect of the test agent) and LC_{50} (50% lethal concentration indicative of the cytotoxic effect of the test agent), calculated for each cell line.

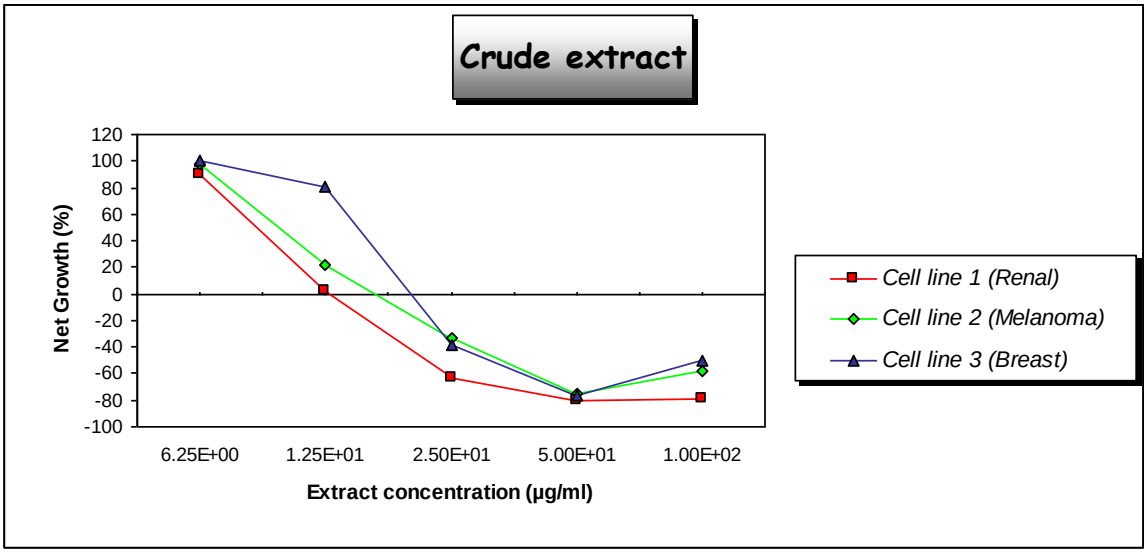


Figure 4.7: Dose-response curve of the crude extract

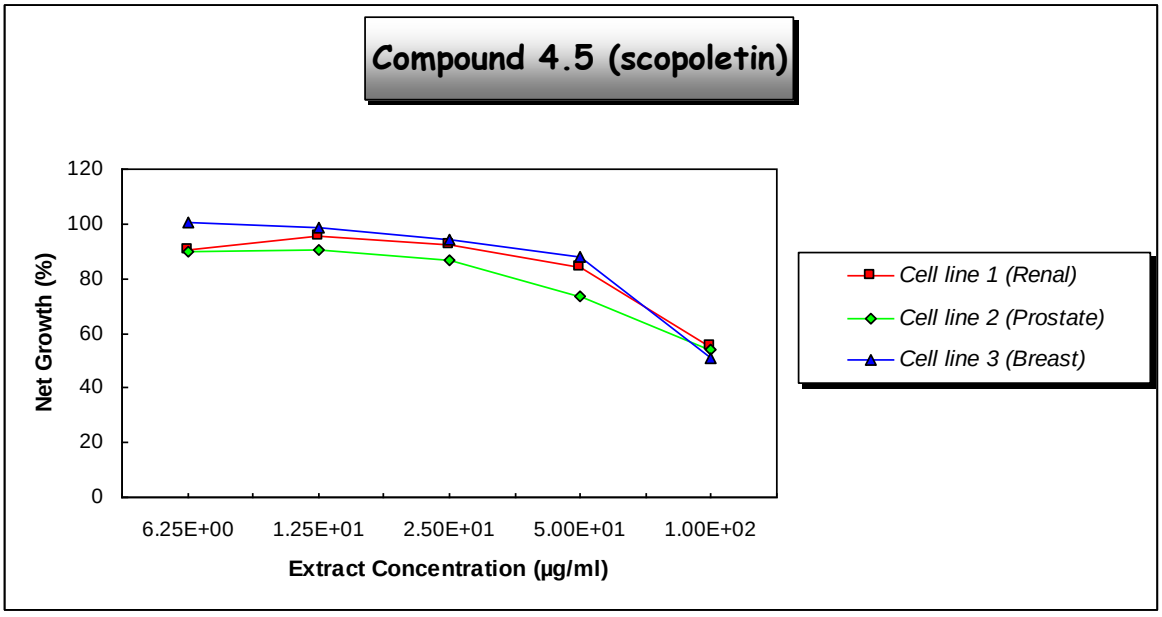


Figure 4.8: Dose-response curve of scopoletin **4.5**

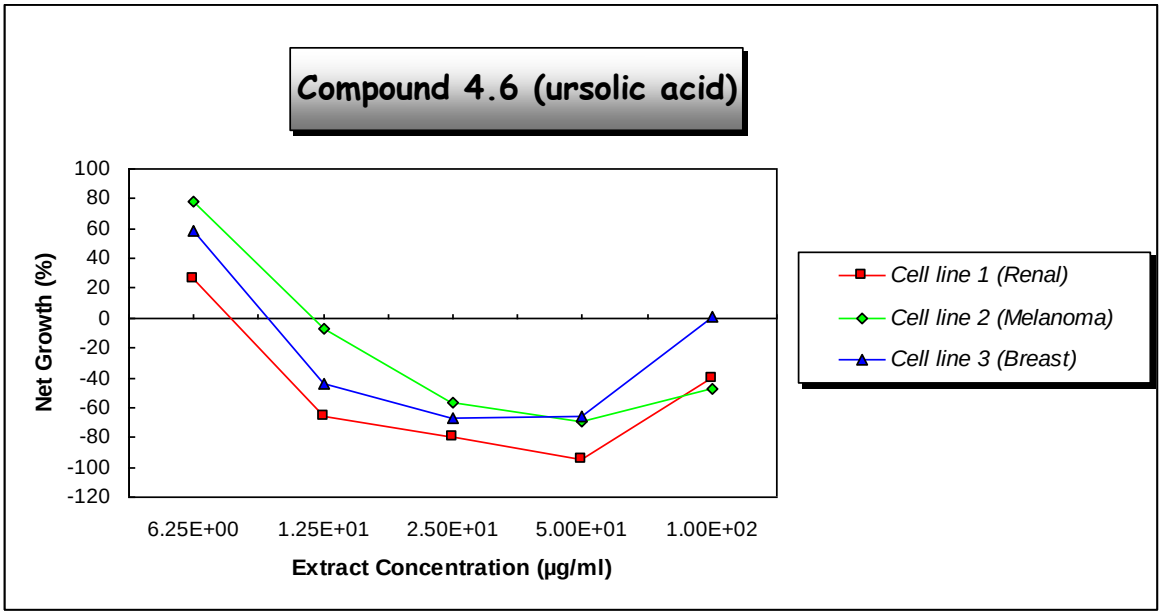


Figure 4.9: Dose-response curve of ursolic acid **4.6**

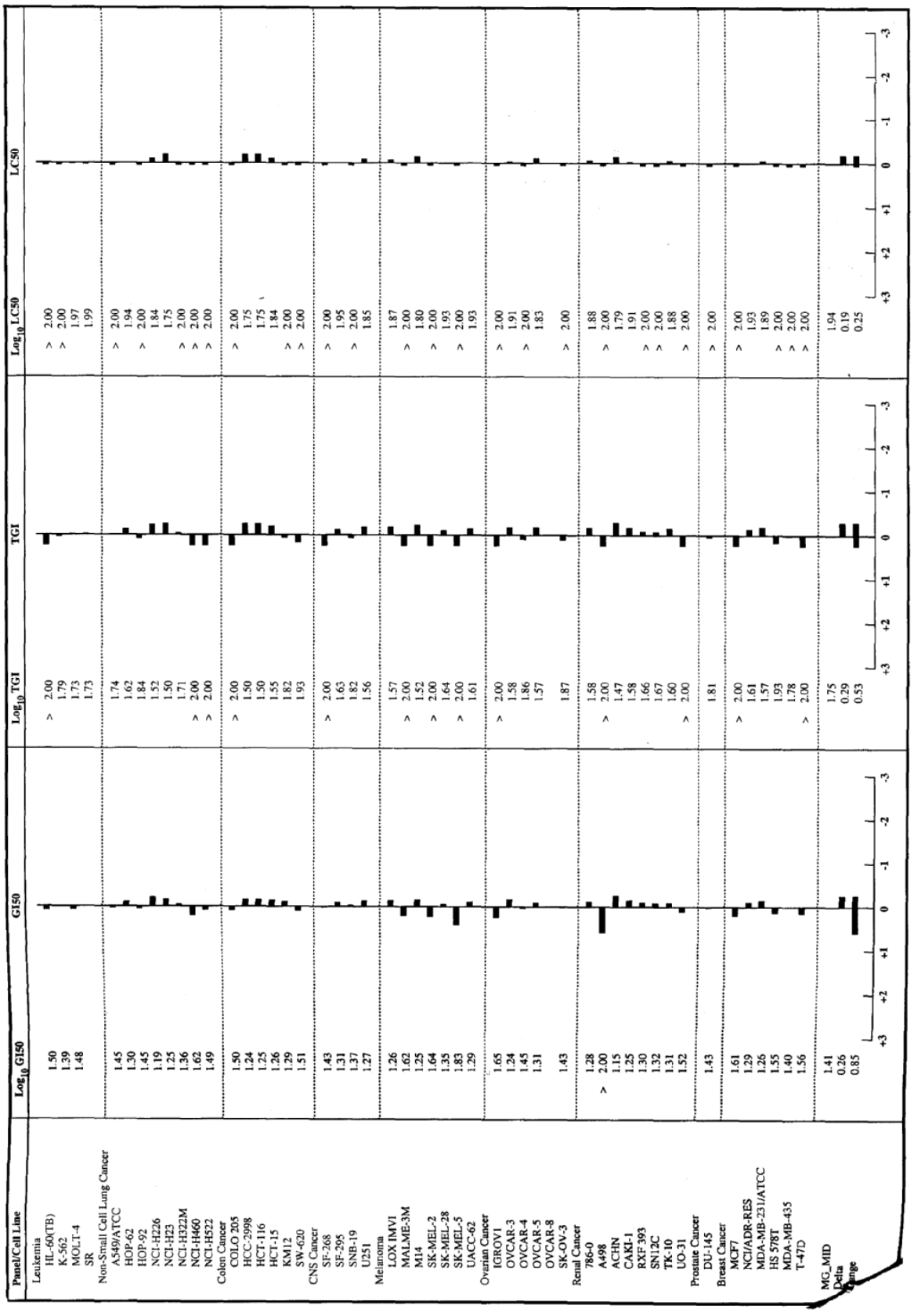


Figure 4.10: Mean $\log_{10}$ functions of the three response parameters, GI_{50}, TGI and LC_{50} of the crude extract

4.5 Conclusion

Bioassay-guided fractionation of the organic extract of *A. hispidulum* resulted in the isolation of scopoletin **(4.5**) and ursolic acid (**4.6**). Ursolic acid (**4.6**) exhibited moderate anticancer activity and it is most likely that the activity observed in the crude extract can be attributed to ursolic acid (**4.6**).

Literature searches indicated that ursolic acid (**4.6**) and its derivatives have been patented for their anticancer properties by Japanese scientists (Ishida et al. 1990). Ursolic acid (**4.6**) has also been reported to possess a wide range of pharmacological activities, including anti-HIV, anti-inflammatory, antitumor, hepatoprotective, anti-ulcer, antimicrobial and anti-hyperlipidemic (Liu 1995; Kashiwada et al. 2000; Dzubak et al. 2006).

Scopoletin (**4.5**) did not show any activity in all three cell lines. However, it has been reported to possess many biological activities, including anti-inflammatory effect (Muschietti et al. 2001), inhibition of monoamine oxidase (Yun et al. 2001) and inducible nitric oxide synthase (Kang et al. 1999; Kim et al. 1999), hepatoprotective effects (Kang et al. 1998), antibacterial (Kayser and Kolodziej 1997) and antioxidant activity (Shaw et al. 2003) and cytotoxicity (Moon et al. 2007).
Compounds isolated here are known compounds, but have not been previously reported as present in the genus *Anthospermum.*

4.6 Experimental

4.6.1 General

Materials and Methods are described in Chapter 3.

4.6.2 Plant Material

The leaves of the plant were collected in April 2004 from Steenkampsberg near Lydenberg, Limpopo province in South Africa. The plant was identified by the South African National Biodiversity Institute (SANBI) in Pretoria as *Anthospermum hispidulum* E.Mey. ex Sond. (Genspec number 8438000 32) and a voucher specimen was deposited in the herbarium of SANBI. The leaves (6.5 kg) were oven dried at 50 ^{o}C overnight and milled to a coarse powder.

4.6.3 Extraction and Isolation

The dried and ground leaves of *Anthospermum hispidulum* (4.9 kg) were extracted with methanol/dichloromethane (1:1) for 24 h at room temperature, after which the solvent was evaporated using a rotary vacuum evaporator at 50 ^{o}C and then further dried in a desiccator for 24 h. The resulting extract (106.7 g) was dissolved in methanol/water (9:1) and then partitioned between hexane, dichloromethane and water/methanol (Fig. 4.4). The dichloromethane residue (30 g) was fractionated by vacuum liquid chromatography (VLC) on silica gel in a stepwise fashion using solvent mixtures of increasing polarity from hexane to dichloromethane, ethyl acetate, acetone and methanol. Seven fractions (labelled fraction 4A-G) were obtained and then further fractionated using flash chromatography as follows: Fraction 4D (5.5 g) was fractionated by flash chromatography using 5% methanol/dichloromethane as eluant, resulting into 13 sub-fractions (fraction 5A-M). Repeated flash chromatography of sub-fractions 5E-J (1.3 g) using 5% methanol/dichloromethane as eluant led to the isolation of compound **4.6** (25 mg).

Sub-fractions 5K, L and M (2.1 g) were combined and further subjected to flash chromatography employing acetone/chloroform (1:9) as eluant to yield a fluorescing blue compound which was identified as 7-hydroxy-6-methoxycoumarin (compound **4.5**, 8.5 mg).

4.6.4 Physical data

4.6.4.1 Physical data for scopoletin (4.5)

Physical description: yellow crystals

Molecular formula: $C_{10}H_8O_4$

Yield: 8.5 mg

UV maxima: 203.0, 227.6 and 343.4 nm

EI-MS m/z: 192.04

Melting Point: 200-204 ^{o}C [lit. 202-204 ^{o}C (Kayser and Kolodziej 1995)]

^{1}H NMR: Plate 1

^{13}C NMR: Plate 2

DEPT: Plate 3

HSQC: Plate 4

HMBC: Plate 5

4.6.4.2 Physical data for ursolic acid (4.6)

Physical description: white crystals

Molecular formula: $C_{30}H_{48}O_3$

Yield: 25 mg

FABMS m/z: 456

Melting Point: 286-288 ^{o}C [lit. 282-283 ^{o}C (Thanakijcharoenpath and Theanphong 2007)]

Optical rotation: $[\alpha]_D$ +61.0, c = 0.054, MeOH/$CHCl_3$ [lit. $[\alpha]_D$ +67.5, c = 1.0, KOH (Budavari et al. 1989)

^{1}H NMR: Plate 6

^{13}C NMR: Plate 7

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Chapter 5

Bioassay-guided isolation of cytotoxic components from *Eriocephalus tenuifolius* DC.

5.1 Introduction

5.1.1 Review of the genus *Eriocephalus* L.

The genus *Eriocephalus* L., commonly known as 'wild rosemary', 'Cape snow bush', 'kapokbos' or 'asmabossie' belongs to the largest flowering plant family Asteraceae, which has about 1535 genera and 25000 species worldwide. This family is found in abundance on every continent except Antarctica. It is most concentrated in Southern Africa (Botswana, Lesotho, Namibia and South Africa). In South Africa, there are about 250 genera and 2300 species of Asteraceae. *Eriocephalus* L., is considered to be a distinct genus in the tribe Anthemideae, whose species are characterized by an aromatic scent and dissected leaves (Bremer and Humphries 1993; Muller et al. 2001; Njenga et al. 2005; Njenga 2005; Viljoen at al. 2006; Germishuizen et al. 2007).

The genus *Eriocephalus* comprises thirty-two species (total of 42 taxa), all endemic to Southern Africa. Its name is derived from the Greek words *erion cephalus* meaning wool headed. Its distribution covers the whole of the Flora of Southern Africa (FSA) region except Gauteng and KwaZulu-Natal provinces, with the highest concentration of taxa in the Western and Northern Cape provinces (Muller et al. 2001; Njenga 2005).

Eriocephalus species occur in a variety of habitats ranging from coastal (Fig. 5.1) to inland on plains, mountains and the desert. Morphologically, members of the genus are many-stemmed, sparsely to much-branched, erect to spreading, sometimes spinescent shrubs, rarely suffrutices, and 0.2-2.0 m high and in diameter and are often aromatic. With the exception of *E. pinnatus* and *E. longifolius*, members of *Eriocephalus* display anomalous secondary growth, resulting in the splitting of older plants into independent daughter plants (Muller et al. 2001).

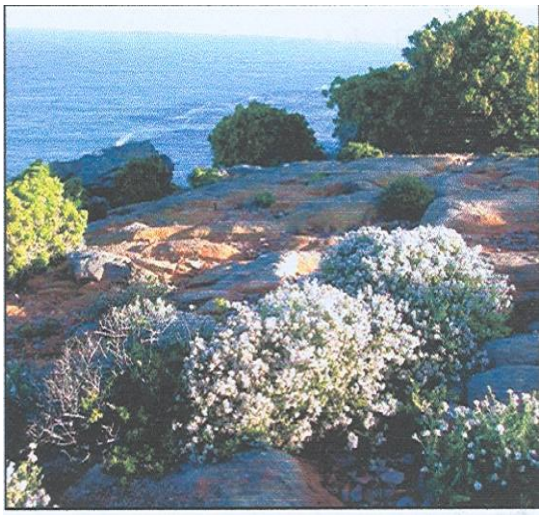


Figure 5.1: *Eriocephalus africanus* showing a spreading habit in a rocky coastal habitat

Eriocephalus L. is also characterized by the heterogeneous heads, the female ray florets with bifid style, mostly ligulate, the disc florets being tubular, five-toothed, and male with perfect stamens and simple, club shaped and truncate styles. Traditionally the genus is placed in the tribe Anthemideae, subtribe Anthemidinae. However, this genus in now grouped together with *Brachylaena, Tarchonanthus* and *Osmitopsis* in the *Lasiospermum* group with other South African genera (Zdero et al. 1987; Muller et al. 2001).

5.1.2 Background on *Eriocephalus tenuifolius* DC.

Eriocephalus tenuifolius DC. (Fig. 5.2), commonly known in Afrikaans as boegoekapok or klein-bergkapokbossie, is a rigid, erect, many-stemmed perennial shrub that reaches a height of 0.3-1.3 m. *E. tenuifolius* is closely related to *E. punctulatus,* and these species, can be separated by their leaf size, peduncle length, the length of the subtending leaves of the peduncles and their distribution. The other distinguishing character is the fact that *E. punctulatus* is hardly browsed whereas *E. tenuifolius* is heavily browsed (Muller et al. 2001).

Figure 5.2: *Eriocephalus tenuifolius*

The distribution of *E. tenuifolius* (Fig. 5.3) is restricted to five provinces in South Africa (Mpumalanga, Free State, Eastern Cape, Western Cape and Northern Cape) and Lesotho.

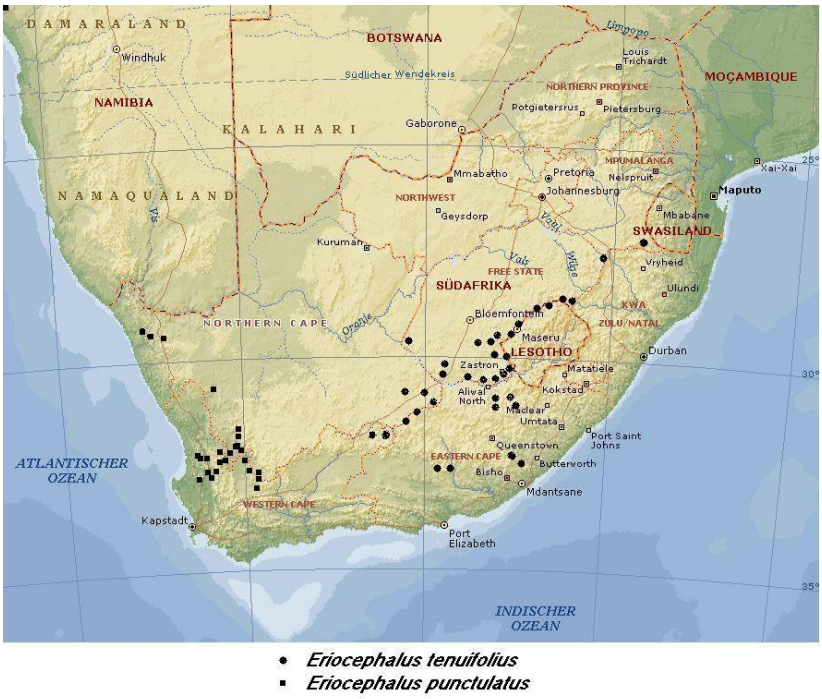


Figure 5.3: Geographical distribution of *Eriocephalus tenuifolius* in Southern Africa

5.1.3 Ethnopharmacology

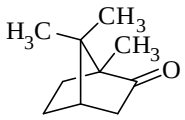
Several species of *Eriocephalus* L. are economically important as traditional herbal remedies and as perfumes in fragrance industries. *E. africanus*, a fragrant herb, has been used to treat oedema, colds, coughs, gastrointestinal disorders, dermal infections, gynaecological conditions and is also used as a rosemary substitute for culinary flavouring purposes. It is also used in the treatment of stress related ailments and depression (Van Wyk and Gericke 2000; Njenga 2005; Njenga and Viljoen 2006; Merle et al. 2007). The Griqua and the Nama used *E. ericoides, E. racemosus, E. africanus, E. punctulatus* and *E. umbellatus* as diuretics and diaphoretics (Watt and Breyer-Brandwijk 1962; Van Wyk et al. 1997). *E. punctulatus* is also used in the treatment of stomach diseases (Mierendorff et al. 2003; Njenga and Viljoen 2006). The oils from *E. punctulatus* and *E. africanus* have a wide application in perfumes, skin care preparations and as blend oils in beauty care products (Njenga and Viljoen 2006).

No special use is published concerning *E. tenuifolius.* Claims have been made by a traditional doctor, the late Solomon Mahlaba who brought the plant as a substitute for buchu (Muller et al. 2001), to CSIR. This study was prompted by this claim and because of the anticancer in-house screen at the CSIR, it was decided to test both the aqueous and organic extracts of *E. tenuifolius* for anticancer properties. Initial *in vitro* results of the organic extract against a panel of three human cancer cell lines at CSIR looked promising.

5.2 Phytochemical studies

Zdero et al. (1987) isolated camphor (**5.1**), linalyl acetate (**5.2**), nerolidol (**5.3**), squalene (**5.4**), flavonoids e.g. quercetin (**5.5**) and several sesquiterpene lactones: guaianolides e.g. estafiatin (**5.6**), eudesmanolides e.g. ivangustin (**5.7**), germacranolide e.g. parthenolide (**5.8**), derivatives of costic acid (**5.9**), a seco-eudesmane diketone (**5.10**) and a chrysanthemol derivative (**5.11)** from nine Namibian *Eriocephalus* species (*E. africanus, E. ericoides, E. scariosus, E. ambigius, E. merxmullerii, E. pauperrimus, E. kingesii, E. giessii* and *Eriocephalus sp*).

The chemistry of other species is relatively unknown and undocumented. To our knowledge, there is no published information on the phytochemistry of *E. tenuifolius*.



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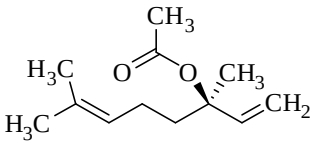

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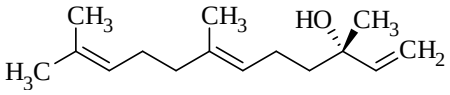

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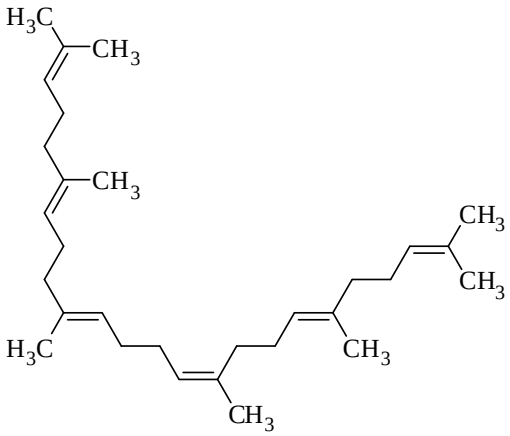

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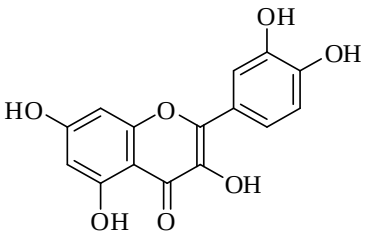

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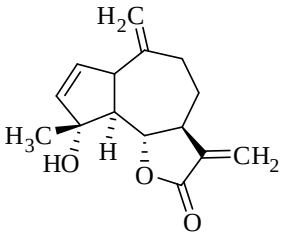

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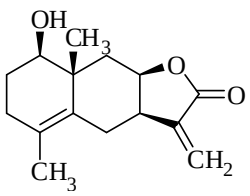

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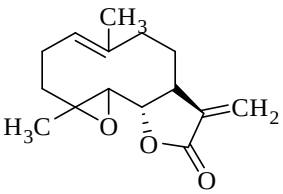

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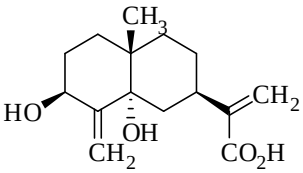

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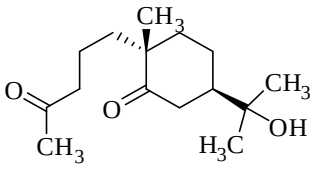

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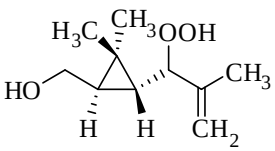


5.11

5.3 Isolation of active compounds

The aerial parts of *E. tenuifolius* collected from Free State province in South Africa was dried, ground and sequentially extracted with purified water and methanol/dichloromethane (1:1) (Fig. 5.4). Both aqueous and organic extracts were assayed and the organic extract was found to have moderate anticancer activity (Chapter 3) against a panel of three human cancer cell lines at CSIR. The organic extract was fractionated by column chromatography on silica gel using 2-5% methanol/dichloromethane. Ten fractions were obtained and repeated fractionation of these fractions led to the isolation of the active compound, 8-*O*-isobutanoylcumambrin B (**5.12)** and other four compounds; pectolinagenin (**5.13)**, hispidulin (**5.14)**, compound **5.15** and compound **5.16**.

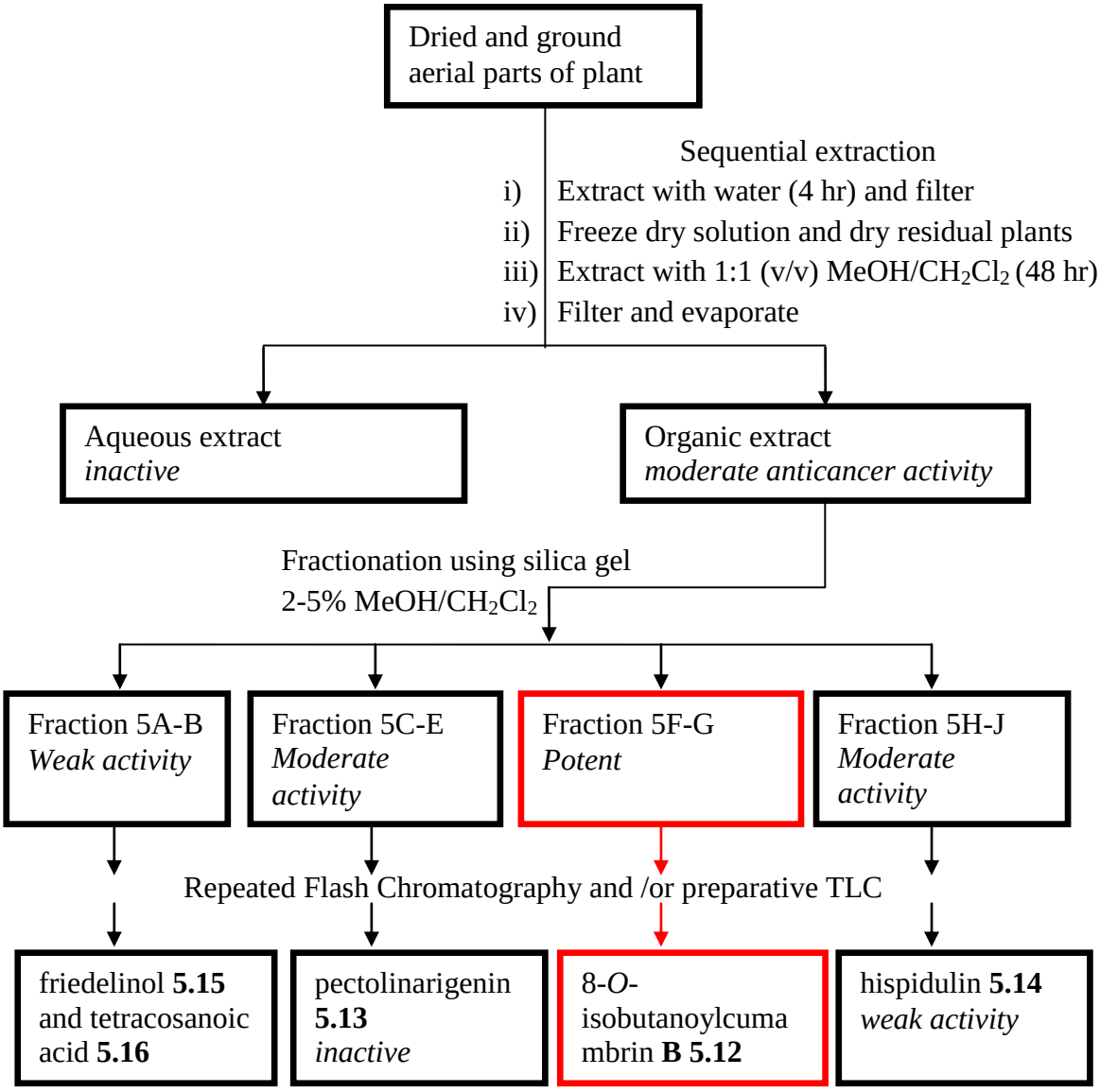


Figure 5.4: Flow diagram illustrating the purification of the organic extract

5.3.1 Structural elucidation of 8-*O*-isobutanoylcumambrin B 5.12

Compound **5.12** was isolated as white crystals. The ESI$^-$-TOF mass spectrum (Fig. 5.5) showed a molecular ion $[M-H]^-$ peak at *m/z* 333.1693, corresponding to a molecular formula $C_{19}H_{26}O_5$.

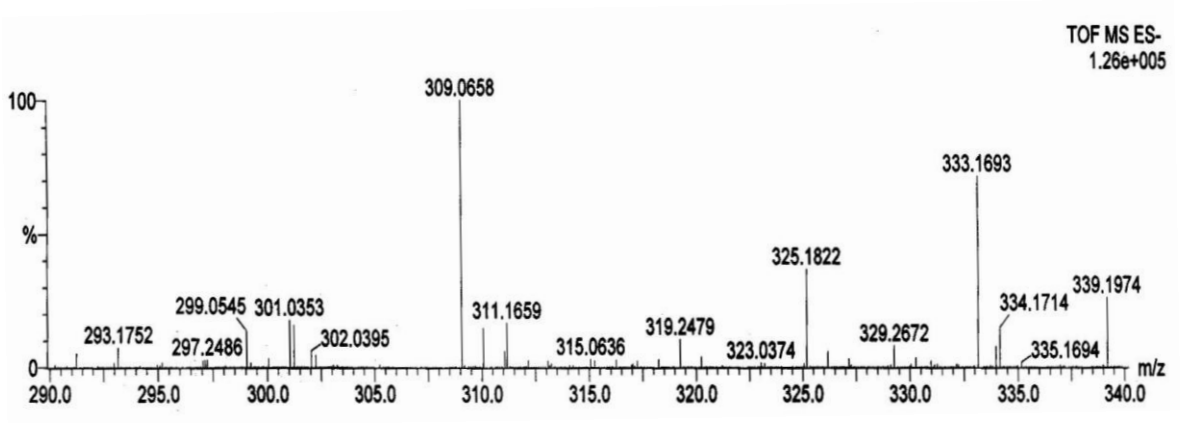


Figure 5.5: The ESI$^-$ TOF mass spectrum of compound **5.12**

The ^{1}H NMR spectrum (Plate 8, Table 5.1) of compound **5.12** showed two doublet signals at δ_H 6.19 (1H, *d, J* 3.5 Hz, H-13) and 5.50 (1H, *d, J* 3.1 Hz, H-13), a broad doublet signal at δ_H 1.81 (1H, *br d, , J* 16.7 Hz, H-9β), three doublet of a doublet signals at δ_H 2.31 (1H, *dd, J* 16.7, 5.6 Hz, H-9α), 2.80 (1H, *dd, J* 9.5, 8.6 Hz, H-5) and 4.03 (1H, *dd, J* 10.5, 9.5 Hz, H-6), a broad doublet of a doublet at δ_H 2.25 (1H, *br dd, J* 15.5, 7.8 Hz, H-2), a doublet of a doublet of a doublet at δ_H 5.22 (1H, *ddd, J* 9.2, 5.5,1.5 Hz, H-8), a triplet of a triplet at δ_H 3.89 (1H, *tt, J* 9.4, 3.2), a broad triplet at δ_H 2.11 (1H, *br t, J* ~ 12.5 Hz, H-2), and two singlets at δ_H 1.23 (3H, *s*, H-14) and 1.93 (3H, *s*, H-15).

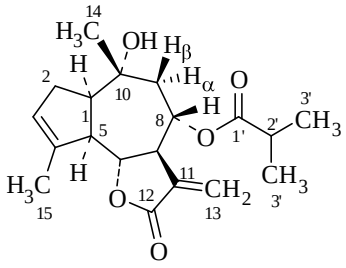
The ^{13}C NMR spectrum (Plate 9, Table 5.1) showed nineteen carbon signals, consisting of two carbonyl carbon signals at δ_C 169.4 (C-12) and 176.0 (C-1'), three oxygenated carbon signals at δ_C 73.1 (C-8), 73.7 (C-10) and 80.3 (C-6), four methyl carbon signals at δ_C 17.9, 18.8, 18.9 and 33.7 and four olefinic carbon signal at δ_C 121.1, 125.5, 138.7 and 143.8. These spectral data suggested that compound **5.12** was a quaiane-type sesquiterpene lactone (Zdero et al. 1987). The ^{1}H and ^{13}C spectral data of compound **5.12** are listed in Table 5.1.

Compound **5.12** was identified as 8-*O*-isobutanoylcumambrin B. The NMR data corresponded with the reported data (Davies-Coleman et al. 1992) (Table 5.1).

Table 5.1: ^{13}C and ^{1}H NMR spectral data for compound **5.12** in $CDCl_3$.

Carbon number	**Carbon type**	**δ_C**	**δ_H (*J* in Hz)**	**Literature* δ_C**	**Literature* δ_H (*J* in Hz)**
1	-CH	54.2	2.60 *m*	54.2	
2	-CH_2	33.5	2.25 *br dd* (15.5, 7.8) 2.11 *br t* (~12.5)	33.6	
3	-CH	125.5	5.52 *br s*	125.7	5.47 *t* (1.5)
4	-C	143.8		143.7	
5	-CH	54.5	2.80 *dd* (9.5, 8.6)	54.5	
6	-CH	80.3	4.03 *dd* (10.5, 9.5)	80.4	3.98 *t* (11)
7	-CH	46.8	3.89 *tt* (9.4, 3.2)	46.9	3.85 *m*
8	-CH	73.1	5.22 *ddd* (9.2, 5.5, 1.5)	73.2	5.16 *m*
9α 9β	-CH_2	38.8	2.31 *dd* (16.7, 5.6) 1.81 *br d* (16.7)	38.8	2.28 *dd* (16.0, 6.0)
10	-C	73.7		73.7	
11	-C	138.7		139.6	
12	-C=O	169.4		169.4	
13	-CH_2	121.1	6.19 *d* (3.5) 5.50 *d* (3.1)	121.1	6.13 *d* (3.0)
14	-CH_3	33.7	1.23 *s*	33.8	1.20 *s*
15	-CH_3	17.9	1.93 *s*	17.8	1.88 *t* (1.5)
1'	-C=O	176.0		176.0	
2'	-CH	34.2	2.65 *septet* (7.0)	34.1	2.60 *m*
3'	-CH_3	18.8, 18.9	1.25 *d* (6.9)	18.8, 18.9	1.21 *d* (7.0)

* Davies-Coleman et al. 1992. Compound was dissolved in $CDCl_3$.



5.12

In the HMBC spectrum (Plate 11, Fig 5.6), the hydrogen signal at δ_H 1.23 (H-14) showed correlations with carbon signals at δ_C 38.8 (C-9) and 54.2 (C-1). The methyl proton at δ_H 1.93 (H-15) correlated with carbon signals δ_C 125.5 (C-3) and 143.8 (C-4). Correlations were also observed between methyl proton at δ_H 1.25 (H-3') with carbonyl carbon signal at δ_C 176.0, between methylene protons at δ_H 6.19 (H-13) and carbon signals at δ_C 46.8 (C-7), 138.7 (C-11) and 169.4 (C-12).

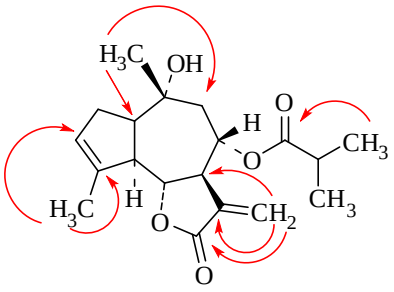


Figure 5.6: HMBC correlations of compound **5.12**

5.3.2 Structural elucidation of pectolinarigenin 5.13

Compound **5.13** was isolated as yellow crystals. The ESI^- mass spectrum (Fig.5.7) showed a molecular ion $[M-H]^-$ peak at *m/z* 313.10, corresponding to a molecular formula $C_{17}H_{14}O_6$. The peaks observed in the spectrum at *m/z* 283.04 and *m/z* 267.99 were due to the loss of a methoxy and hydroxyl group, respectively.

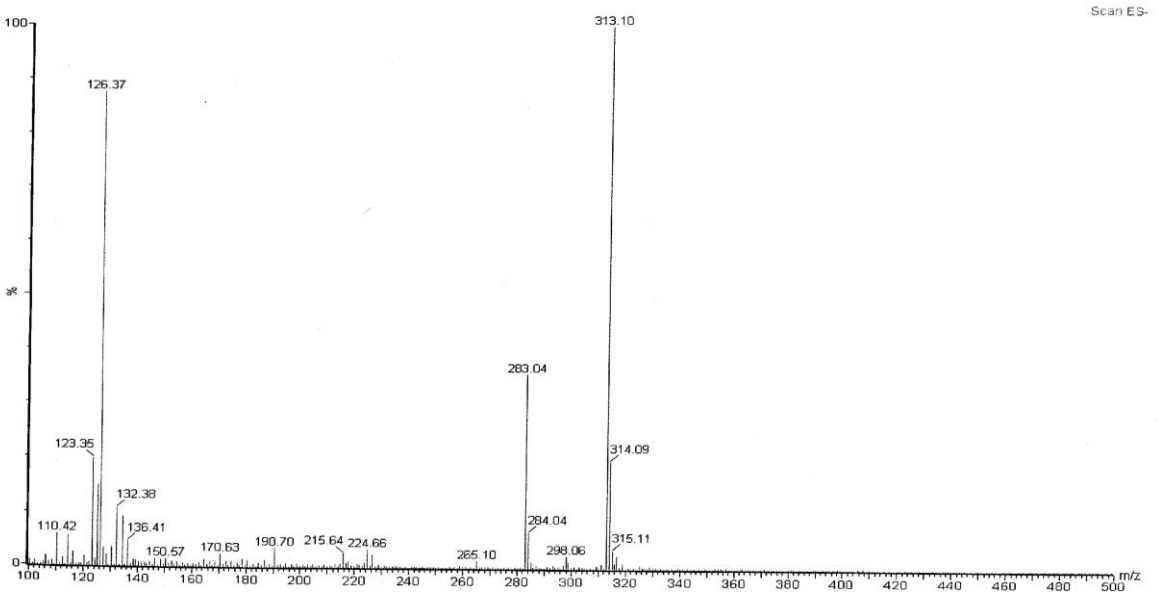


Figure 5.7: The ESI$^-$ mass spectrum of compound **5.13**

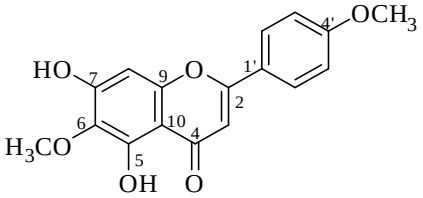
The ^{1}H NMR spectrum (Plate 12, Table 5.2) of compound **5.13** showed two doublets of similar coupling constants at δ_H 7.11 (2H, *d, J* 8.9 Hz, H-3', H-5'), and 8.04 (2H, *d, J* 8.9 Hz, H-2', H-6'). It further showed two singlets at δ_H 3.86 (3 H, *s*, 6-OMe) and 3.76 (3H, *s*, 4'-OMe). Proton signals at δ_H 13.04 (*s*, 5-OH), 6.87 (1H, s, H-3) and 6.62 (1H, s, H-8) were also observed.

The ^{13}C NMR spectrum (Plate 13, Table 5.2) showed seventeen carbon signals, consisting of a carbonyl carbon signals at δ_C 182.1, six oxygenated carbon signals at δ_C 131.4, 152.4, 152.7, 157.4, 162.3 and 163.3. Two peaks at δ_C 114.5 (C-3') and 128.3 (C-2') showing double intensities were also observed in the ^{13}C NMR spectrum. The complete assignments of ^{1}H and ^{13}C chemical shifts of compound **5.13** are listed in Table 5.2.

Table 5.2: ^{13}C and ^{1}H NMR spectral data for pectolinarigenin **5.13** in DMSO-d_6.

Carbon number	**Carbon type**	**δ_C**	**δ_H (*J* in Hz)**	**Literature* δ_C**	**Literature* δ_H (*J* in Hz)**
2	-C	163.3		163.2	
3	-CH	103.0	6.87 *s*	103.2	6.83 *s*
4	-C=O	182.1		182.3	
5	-C-OH	152.7	13.04 *s*	152.6	13.01 *s*
6	-C	131.4		131.5	
7	-C-OH	157.4		157.5	
8	-CH	94.3	6.62 *s*	94.5	6.59 *s*
9	-C	152.4		152.9	
10	-C	104.1		104.3	
1'	-C	122.8		123.0	
2'	-CH	128.3	8.04 *d* (8.9)	128.4	7.99 *d* (9.0)
3'	-CH	114.5	7.11 *d* (8.9)	114.7	7.08 *d* (9.0)
4'	-C	162.3		162.5	
5'	-CH	114.5	7.11 *d* (8.9)	114.7	7.08 *d* (9.0)
6'	-CH	128.3	8.04 *d* (8.9)	128.4	7.99 *d* (9.0)
6-OCH_3	-CH_3	59.9	3.76 *s*	60.1	3.74 *s*
4'-OCH_3	-CH_3	55.5	3.86 *s*	55.7	3.84 *s*

* Lim et al. 2008, Compound was dissolved in DMSO-*d_6*



5.13

The HMBC spectrum (Plate 16, Fig. 5.8), showed correlations between the proton at δ_H 3.86 (OCH_3) with the carbon signal at δ_C 162.3 (C-4'), between the hydrogen signal at δ_H 3.76 (OCH_3) and the carbon signal at δ_C 131.4 (C-6), between the hydrogen signal at δ_H 7.11 (H-5') with the carbon signal at δ_C 122.8 (C-1') and between the hydrogen signal at δ_H 6.62 (H-8) and the carbon signals at δ_C 104.1 (C-10) and 131.4 (C-6). It further showed correlations between the proton signal at δ_H 8.04 (H-6') with the carbon signal at δ_C 163.3 (C-2).

The correlations observed in the NOESY experiment (Plate 17, Fig. 5.8), between proton signal at δ_H 7.11 (H-5') and proton signal at δ_H 3.86 (OCH_3) indicate that the methoxy group at δ_H 3.86 is linked to C-4' position.

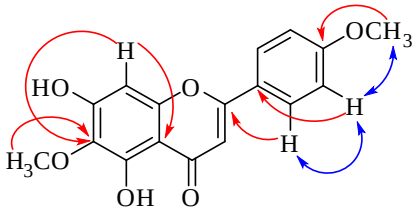


Figure 5.8: HMBC (⟶) and NOESY (⟷) correlations of compound **5.13**

From the spectral evidence, the structure of compound **5.13** was determined to be 5,7-dihydroxy-6,4-dimethoxyflavone (known as pectolinarigenin). The structure was also verified by comparison of the NMR data with those reported in the literature (Lim et al. 2008).

5.3.3 Structural elucidation of hispidulin 5.14

Compound **5.14** was isolated as yellow crystals. The ESI^- mass spectrum (Fig. 5.9) showed a molecular ion $[M-H]^-$ peak at *m/z* 299.08, corresponding to a molecular formula $C_{16}H_{12}O_6$. The mass spectrum showed a fragment at *m/z* 268.99, corresponding to the fragment which was formed after the removal of a methoxy group from the molecular ion peak.

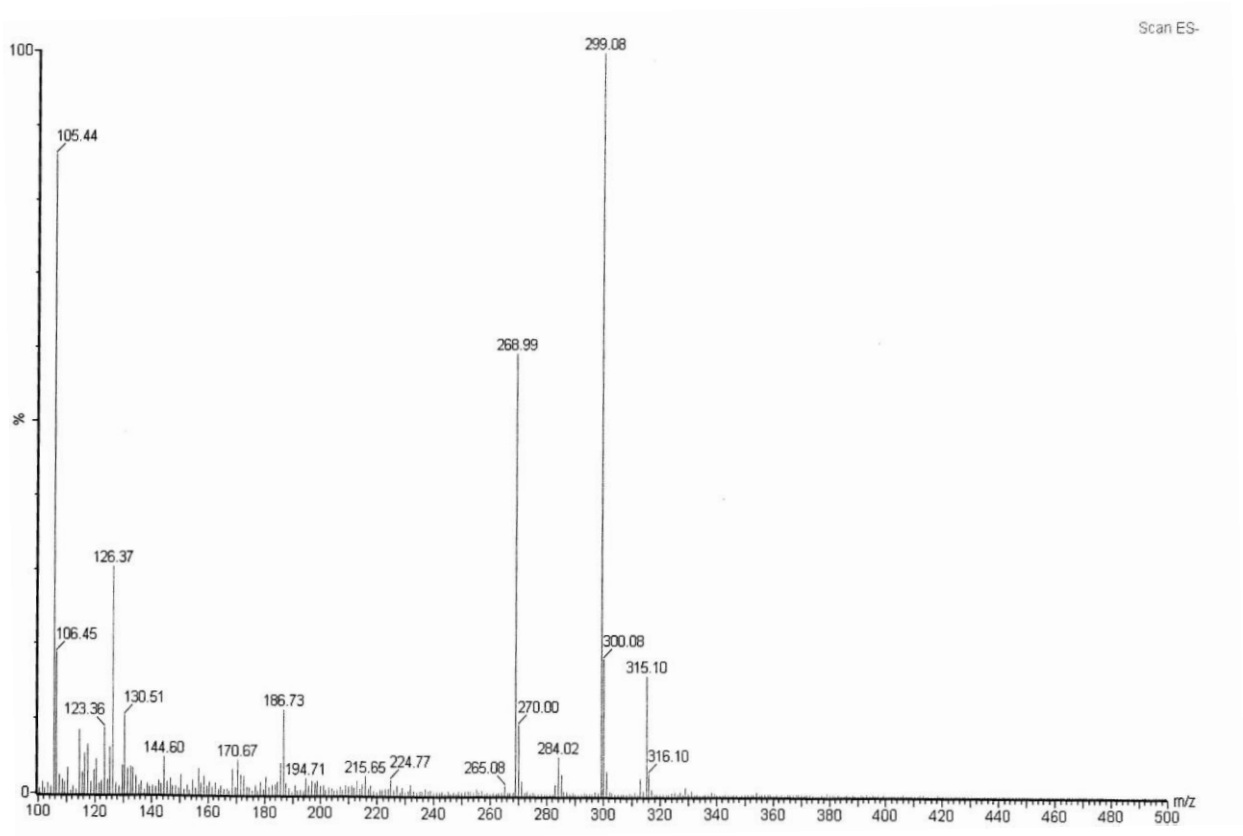


Figure 5.9: The ESI$^-$ mass spectrum of compound **5.14**

The ^{1}H NMR spectrum (Plate 18, Table 5.3) of compound **5.14** showed six signals, two doublets at δ_H 6.91 (2H, *d*, *J* 8.7 Hz, H-3', H-5') and 7.91 (2H, *d*, *J* 8.7 Hz, H-2', H-6'), four singlets at δ_H 3.74 (3 H, *s*, 6-OMe), 6.59 (1H, s, H-8), 6.77 (1H, s, H-3) and δ_H 13.06 (*s*, 5-OH).

The ^{1}H NMR spectrum of compound **5.14** was similar to that of compound **5.13**, except for the presence of only one methoxyl proton signal.

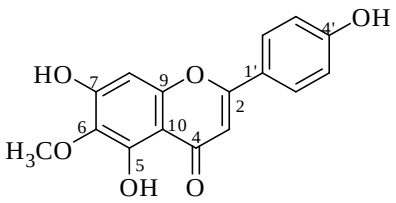
The ^{13}C NMR spectrum (Plate 19, Table 5.3) showed sixteen carbon signals, with two peaks at δ_C 115.9 (C-3') and 128.4 (C-2') showing double intensities, a carbonyl carbon signal at δ_C 182.1, a methoxyl signal at δ_C 59.9 and six oxygenated carbon signals at δ_C 131.3, 152.4, 152.7, 157.2, 161.1 and 163.8. The complete assignments of ^{1}H and ^{13}C chemical shifts of compound **5.14** are listed in Table 5.3

.

Table 5.3: ^{13}C and ^{1}H NMR spectral data for hispidulin **5.14** in DMSO-d_6.

Carbon number	**Carbon type**	**δ_C**	**δ_H (*J* in Hz)**	**Literature[#] δ_C**	**Literature[#] δ_H (*J* in Hz)**
2	-C	163.8		163.8	
3	-CH	102.3	6.77 *s*	102.4	6.75 *s*
4	-C=O	182.1		182.1	
5	-C-OH	152.7	13.06 *s*	152.8	13.05 *s*
6	-C	131.3		131.4	
7	-C-OH	157.2		157.3	
8	-CH	94.2	6.59 *s*	94.2	6.57 *s*
9	-C	152.4		152.4	
10	-C	104.0		104.0	
1'	-C	121.2		121.2	
2'	-CH	128.4	7.91 *d* (8.7)	128.4	7.90 *d* (8.8)
3'	-CH	115.9	6.91 *d* (8.7)	115.9	6.90 *d* (8.8)
4'	-C-OH	161.1		161.2	
5'	-CH	115.9	6.91 *d* (8.7)	115.9	6.90 *d* (8.8)
6'	-CH	128.4	7.91 *d* (8.7)	128.4	7.90 *d* (8.8)
6-OCH_3	-CH_3	59.9	3.74 *s*	59.9	3.73 *s*

[#] Hase et al. 1995. Compound was dissolved in DMSO-d_6.



5.14

Correlations between H-6' (δ_H 7.91) and both H-3 (δ_H 6.77) and H-5' (δ_H 6.91) were observed in the NOESY spectrum (Plate 23, Fig. 5.10).

From the HMBC spectrum (Plate 22, Fig. 5.10), the methoxyl proton at δ_H 3.74 (OCH_3) correlates with the carbon signal at δ_C 131.3 (C-6), hydrogen signal at δ_H 6.59 (H-8) with carbon signals at δ_C 131.3 (C-6) and 104.0 (C-10). These correlations suggested that the methoxyl group is positioned at C-6.

The HMBC spectrum further showed correlations between hydrogen signal at δ_H 6.91 (H-3') with the carbon signal at δ_C 121.2 (C-1').

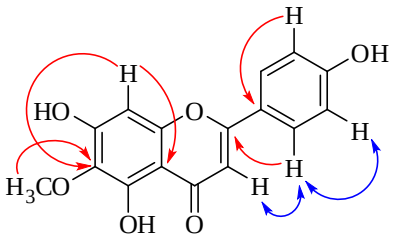


Figure 5.10: HMBC (⟶) and NOESY (⟷) correlations of compound **5.14**

The structure of compound **5.14** was assigned as hispidulin. MS, ^{1}H and ^{13}C NMR spectral data of compound **5.14** are in agreement with those reported in the literature for hispidulin (Hase et al. 1995).

5.3.4 Structural elucidation of friedelinol 5.15

Compound **5.15** was isolated as white crystals. The GC/MS spectrum (Fig. 5.11) showed a molecular ion $[M]^{+}$ peak at *m/z* 428.4, corresponding to the molecular formula $C_{30}H_{52}O$. The ions at *m/z* 413.4, 275.2, 205.2 and 69.1 were due to the loss of a methyl, $C_{11}H_{21,}$ $C_{16}H_{30}O$ and $C_{25}H_{43}O$ groups respectively. The National Institute of Standards and Technology (NIST) library search gave a 99% probability that compound **5.15** is a friedelinol.

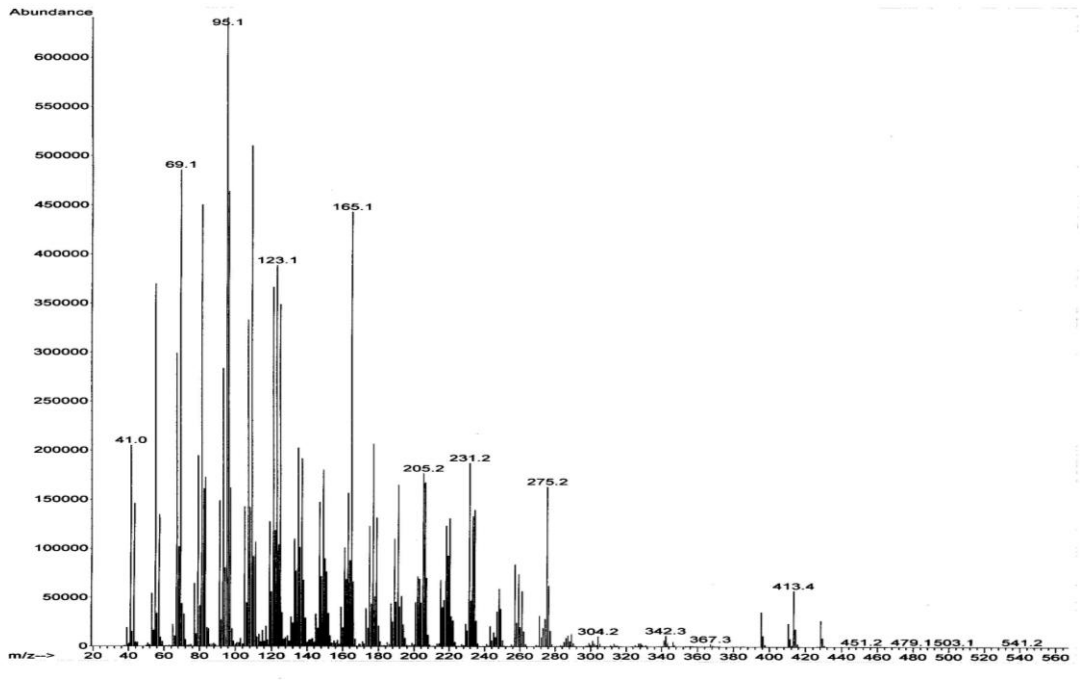


Figure 5.11: GC-MS spectrum of compound **5.15**

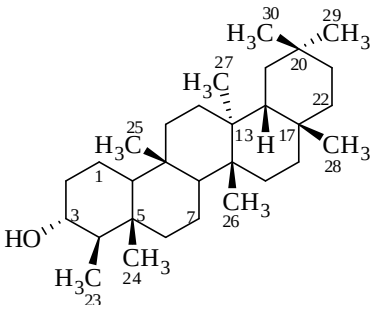
The ^{1}H NMR spectrum (Plate 24) showed the presence of one doublet methyl signal at δ_H 1.02 (3H, *d*, *J* 6.1 Hz, 23-Me) and seven methyl singlet methyl signals at δ_H 0.88 (3H, *s*, 24-Me), 0.89 (3H, *s*, 25-Me), 0.94 (3H, *s*, 29-Me), 0.95 (3H, *s*, 26-Me), 0.97 (3H, *s*, 27-Me), 1.03 (3H, *s*, 30-Me), and 1.18 (3H, *s*, 28-Me).

The ^{13}C NMR spectrum (Plate 25, Table 5.4) showed the presence of thirty carbon signals. Eight methyl carbon signals at δ_C 35.0, 32.1, 31.8, 20.1, 18.6, 18.2, 16.4, and 11.6 were assigned for C-29, C-28, C-30, C-26, C-27, C-25, C-24 and C-23 respectively. One oxygenated carbon signal at δ_C 72.7 assigned for C-3 was also observed. The ^{13}C NMR shifts were found to be identical with ^{13}C NMR reported spectrum (Table 5.4) of 3β-friedelinol (Salazar et al. 2000).

Table 5.4: ^{13}C and ^{1}H NMR spectral data for compound **5.15** in $CDCl_3$.

Carbon number	**Carbon type**	**δ_C**	**δ_H (*J* in Hz)**	**Literature[#] δ_C**	**Literature[#] δ_H (*J* in Hz)**
1	-CH_2	15.8		16.2	
2	-CH_2	36.1		36.1	
3	-CH	72.7		71.6	
4	-CH	49.2		49.6	
5	-C	37.9		38.1	
6	-CH_2	41.8		41.9	
7	-CH_2	17.6		17.7	
8	-CH	53.2		53.3	
9	-C	37.1		37.2	
10	-CH	61.4		61.7	
11	-CH_2	35.4		35.7	
12	-CH_2	30.6		30.7	
13	-C	38.4		38.4	
14	-C	39.7		39.7	
15	-CH_2	32.4		32.3	
16	-CH_2	35.6		35.9	
17	-C	30.0		30.0	
18	-CH	42.9		42.9	
19	-CH_2	35.2		35.4	
20	-C	28.2		28.2	
21	-CH_2	32.8		32.9	
22	-CH_2	39.3		39.3	
23	-CH_3	11.6	1.02 *s*	12.1	1.02 *s*
24	-CH_3	16.4	0.89 *s*	16.6	1.10 *s*
25	-CH_3	18.2	0.88 *s*	18.4	0.89 *s*
26	-CH_3	20.1	0.95 *s*	20.1	0.99 *s*
27	-CH_3	18.6	0.97 *s*	18.7	1.02 *s*
28	-CH_3	32.1	1.18 *s*	32.1	1.16 *s*
29	-CH_3	35.0	0.94 *s*	35.0	0.97 *s*
30	-CH_3	31.8	1.03 *s*	31.9	1.0 2 *s*

[#] Salazar et al. 2000. Compound was dissolved in $CDCl_3$, with few drops of pyridine-d_5.



5.15

5.3.5 Structural elucidation of tetracosanoic acid 5.16

Compound **5.16** was isolated as white crystals. The GC/MS spectrum (Fig. 5.12) showed a molecular ion $[M]^+$ peak at *m/z* 368.4, corresponding to the molecular formula $C_{24}H_{48}O_2$. The mass spectrum showed various peaks having the difference of 28 a.m.u. (i.e. loss of 2 x CH_2) revealing the hydrocarbon nature of compound **5.16**. The NIST library search confirmed a 99% match with tetracosanoic acid.

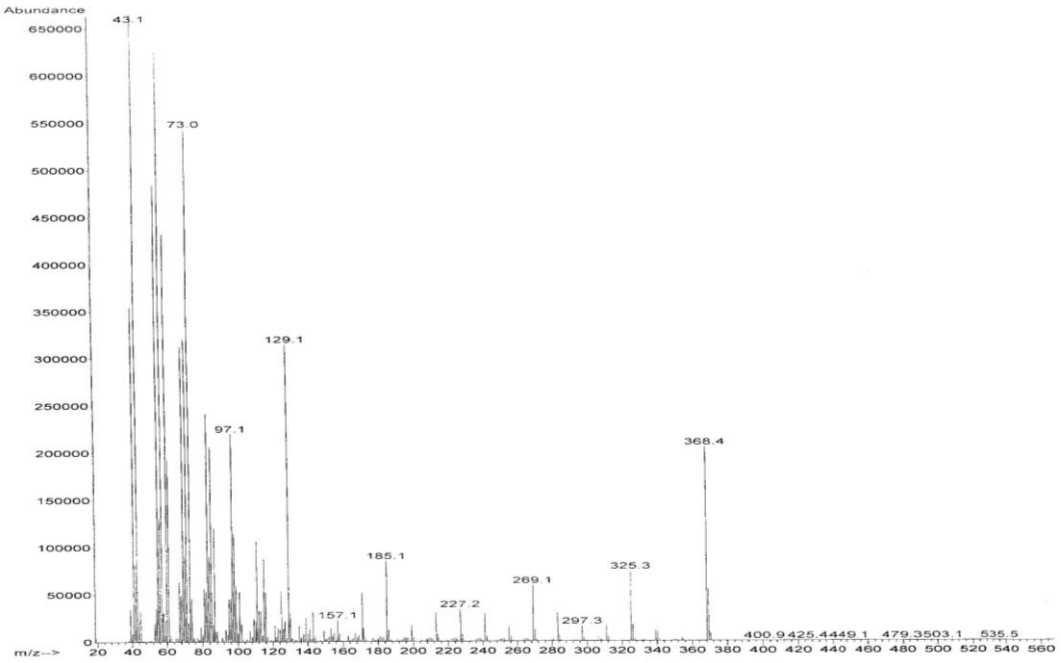
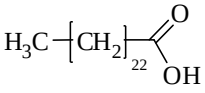


Figure 5.12: GC-MS spectrum of compound **5.16**

The ^1H NMR spectrum (Plate 26) showed only four signals, two triplets at δ_H 0.81 (*J* 6.8 Hz) and 2.28 (*J* 7.5 Hz), a broad singlet at δ_H 1.19 due to various CH_2 fragments and a multiplet at δ_H 1.57.

The ^{13}C NMR spectrum (Plate 27) showed a carboxyl carbon signal at δ_C 178.5 and a methyl carbon signal at δ_C 14.1. Methylene carbon signals were observed in the region δ_C 22.7- 33.8. From the GC-MS, ^1H and ^{13}C NMR spectral data, compound **5.15** was determined to be a tetracosanoic acid.



5.16

5.4 Biological activity of the crude extracts and isolated compounds

5.4.1 *In vitro* anticancer activity

The crude extracts and isolated compounds were assayed in the three human cell line panel consisting of renal (TK10), melanoma (UACC62), and breast (MCF7) at the CSIR.

In vitro assaying of the organic extract of *E. tenuifolius* showed that it had a moderate anticancer activity i.e. an average total growth inhibition (TGI) value of between 12.5 and 15.5 µg/ml for all three cell lines (Fig. 5.13). This organic extract was subjected to bioassay-guided fractionation and five compounds were isolated and identified. Compound **5.12** showed an increase in potency with TGI values of 2.73 µg/ml for renal cell line, 5.35 µg/ml for melanoma and 4.82 µg/ml for breast cell line (Fig. 5.14). Compound **5.14** showed selectivity for melanoma cell line with TGI value of 36.5 µg/ml (Fig. 5.16). No anticancer activity was observed for compounds **5.13**, **5.15** and **5.16** (Figs. 5.15, 5.17, 5.18 respectively).

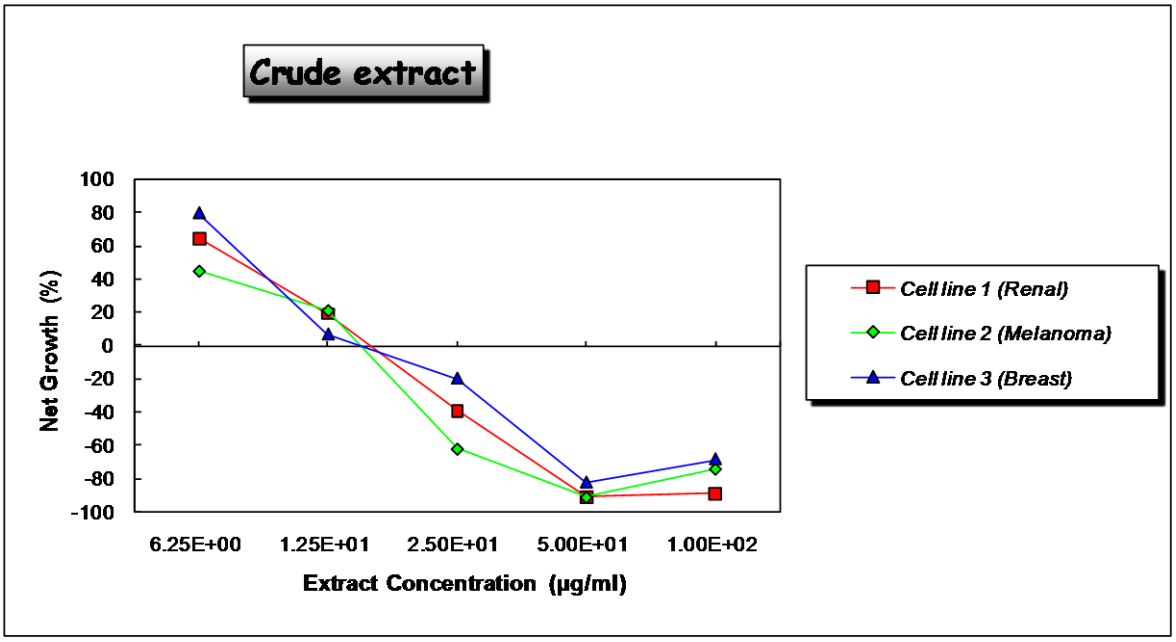


Figure 5.13: Dose-response curve of the crude extract

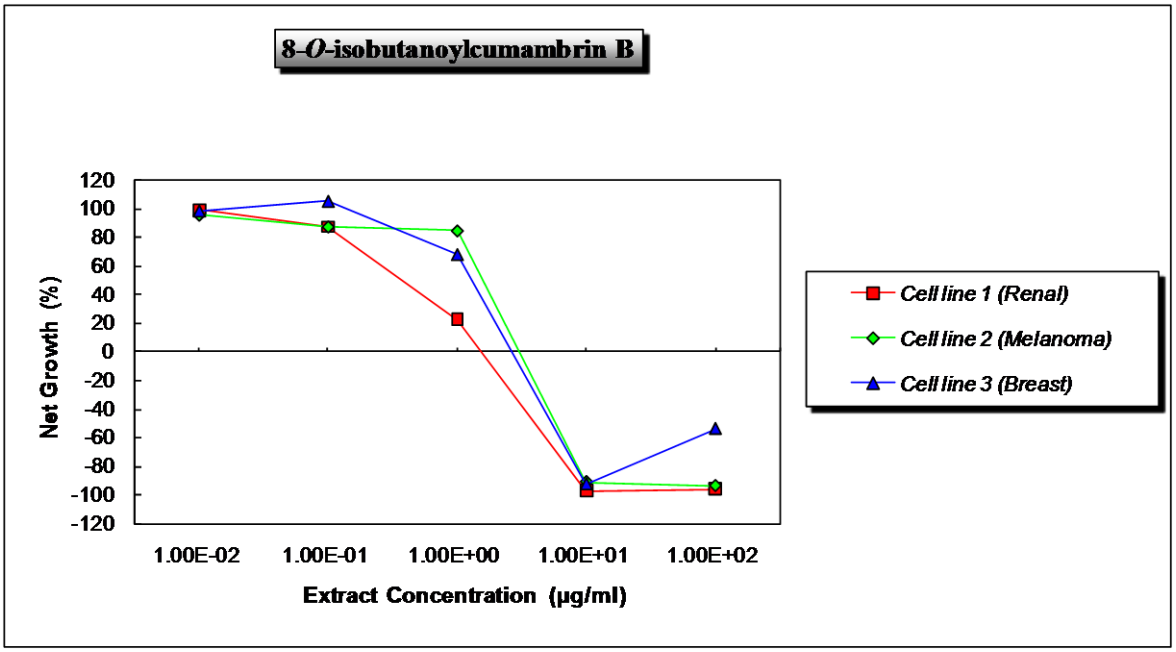


Figure 5.14: Dose-response curve of the 8-*O*-isobutanoylcumambrin B **5.12**

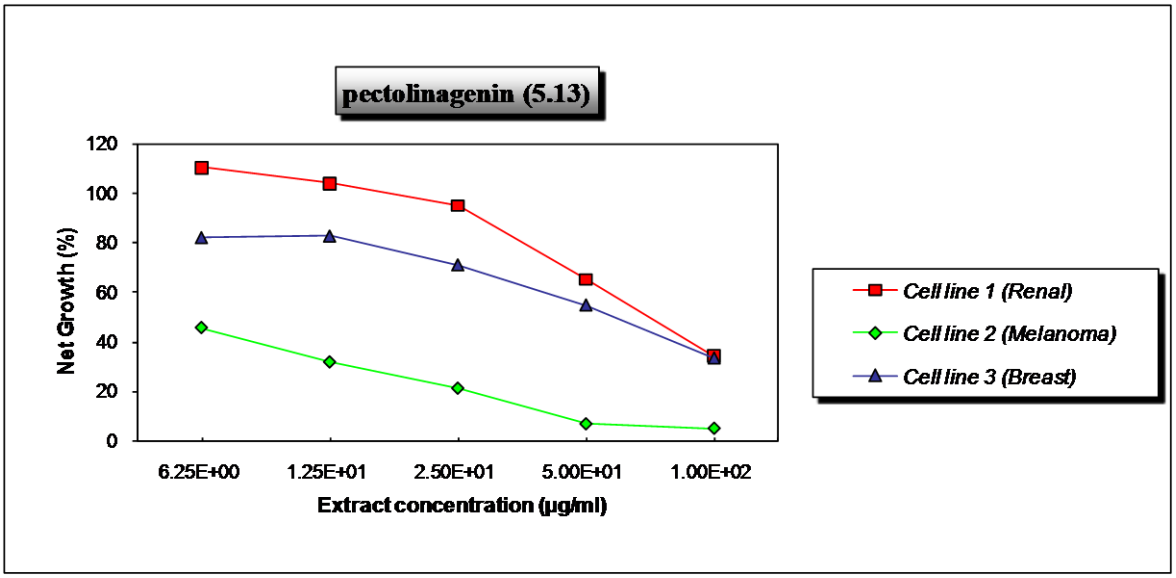


Figure 5.15: Dose-response curve of the pectolinagenin **5.13**

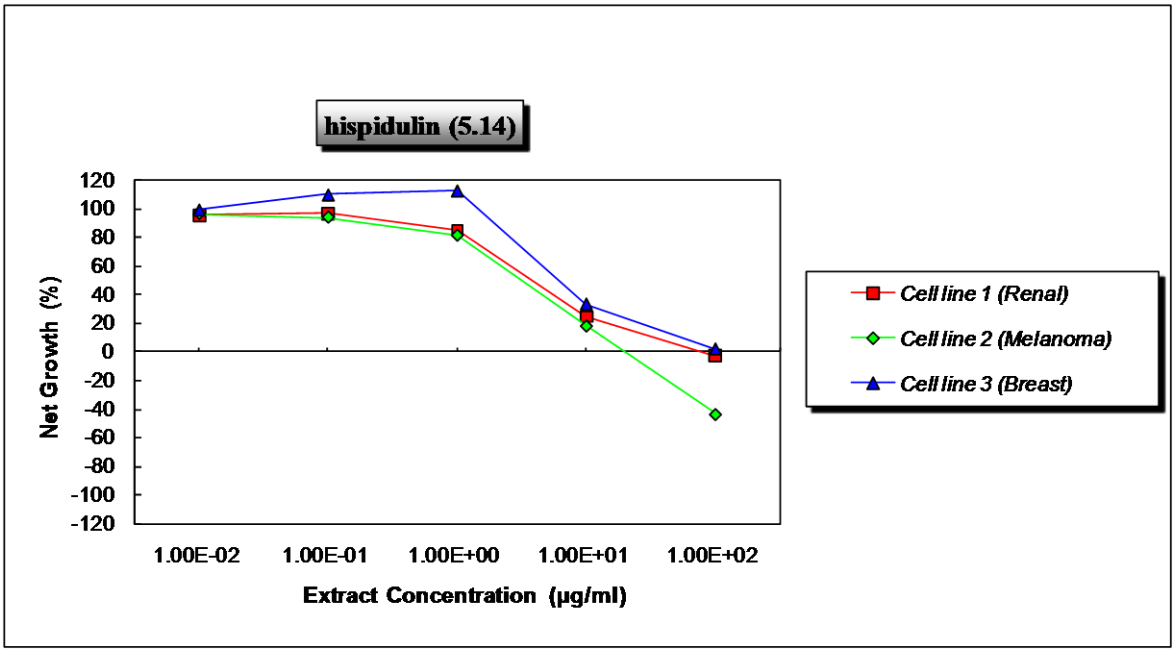


Figure 5.16: Dose-response curve of the hispidulin **5.14**

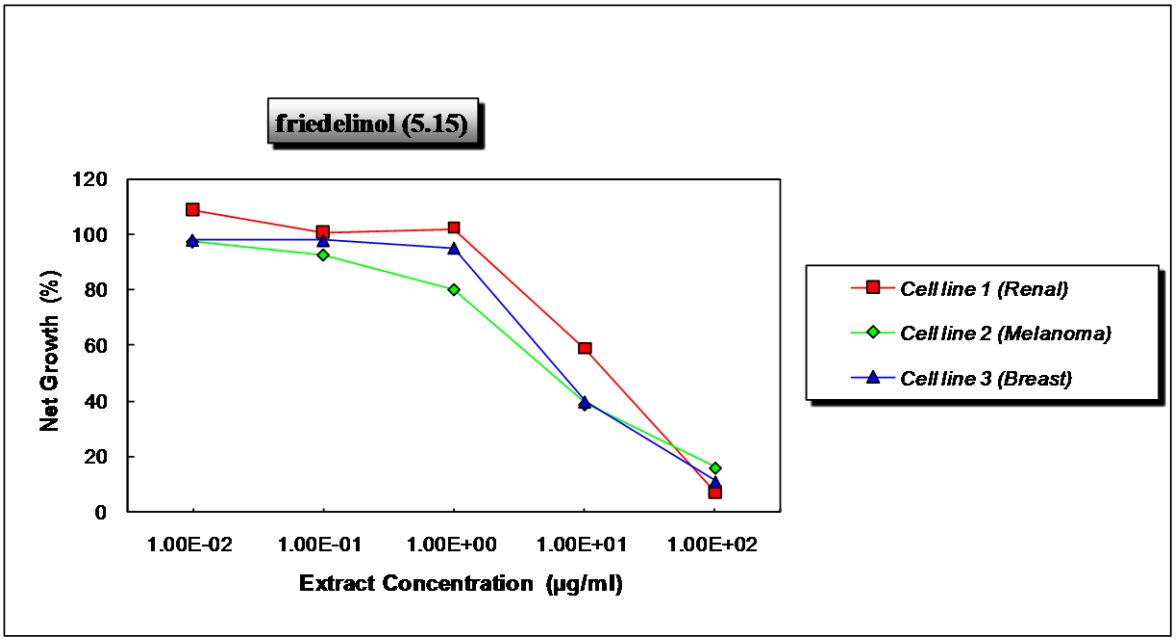


Figure 5.17: Dose-response curve of friedelinol **5.15**

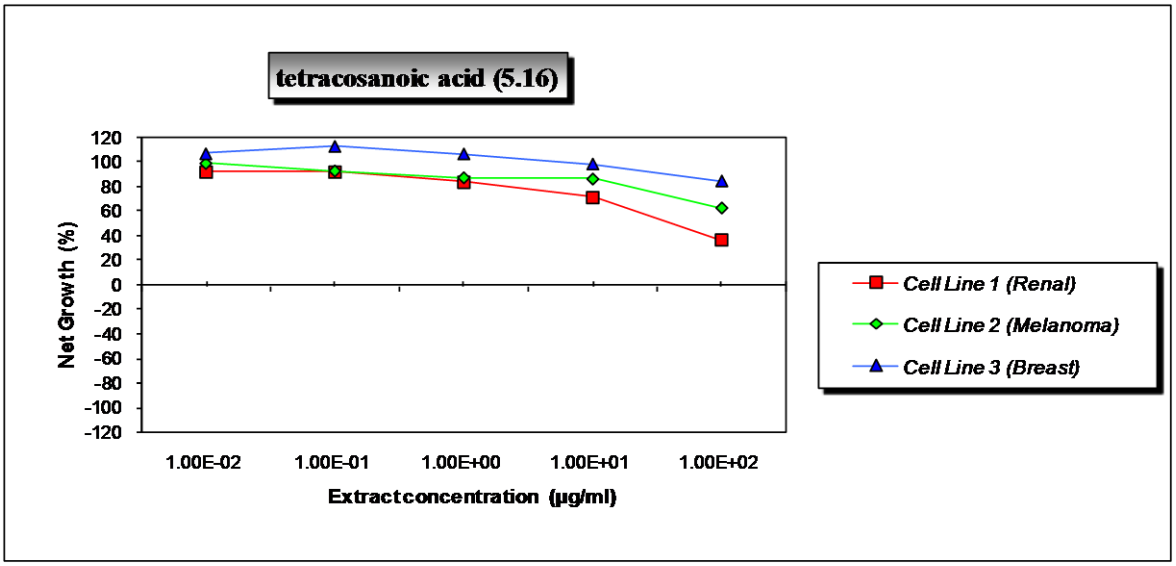


Figure 5.18: Dose-response curve of tetracosanoic acid **5.16**

5.4.2 *In vitro* antiplasmodial activity and cytotoxicity of compound 5.12

Compound **5.12** was tested for *in vitro* antiplasmodial activity against *Plasmodium falciparum* (CQS) D10 strain using the pLDH assay. The sensitivity of the D10 strain to chloroquine was also evaluated. The IC_{50} values are listed in Table 5.4.

Table 5.4: *In vitro* antiplasmodial activity of chloroquine and compound **5.12**

Tested sample	**D10 IC_{50} (µg/ml)**
Chloroquine	11.96×10^{-3}
Compound **5.12**	6.25

The *in vitro* results were classified as follows:

IC_{50} value of ≤ 5 µg/ml, the compound was considered potent.

IC_{50} value of between 5 and 10 µg/ml, the compound was considered moderately active.

IC_{50} value of ≥ 10 µg/ml, the compound was considered weakly active.

Based on these criteria, compound **5.12** can be considered moderately active against the parasite.

The *in vitro* cytotoxicity of compound **5.12** was determined against Chinese Hamster Ovarian (CHO) cells using the MTT assay. The IC_{50} and SI values of the positive control emetine and compound **5.12** are shown in Table 5.5.

Table 5.5: *In vitro* cytotoxicity of Emetine and compound **5.12**

Tested sample	**CHO IC_{50} (µg/ml)**	**SI***
Emetine	0.08	ND
Chloroquine	18.53	1549
Compound **5.12**	5.72	1

*Selectivity index (SI) = cytotoxicity IC_{50} / antiplasmodial $IC_{50;}$ ND = Not Done
For the compound not to be toxic, SI values should be greater than 100.

Compound **5.12** showed significant toxicity at the same concentration range against the CHO cell line and this is emphasized by its low SI value, so the observed antiplasmodial activity could be due to toxicity.

5.5 Conclusion

Of all compounds isolated from the aerial parts of *E. tenuifolius*, only 8-*O*-isobutanoylcumambrin B **5.12** showed potent anticancer activity against a panel of three highly sensitive human cancer cell lines (breast MCF7, renal TK10 and melanoma UACC62) at the CSIR cancer screen. Compound **5.12** also showed moderate antiplasmodial activity against *Plasmodium falciparum* (CQS) D10 strain and toxicity to CHO cells. Although compound **5.12** has been isolated previously from other *Eriocephalus* species, there are no previous reports of the compound being investigated for any biological activity.

The compounds pectolinagenin (**5.13**), hispidulin (**5.14**), friedelinol (**5.15**), and tetracosanoic acid (**5.16**), did not show any significant anticancer activity. All compounds isolated here are known compounds, but have not been previously reported as present from the species *E. tenuifolius.*

5.6 Experimental

5.6.1 General

Materials and Methods are described in Chapter 3

5.6.2 Plant Material

The aerial parts of the plant were collected in February 2005 from Free State province in South Africa. The plant was identified by the South African National Biodiversity Institute (SANBI) in Pretoria as *Eriocephalus tenuifolius* DC. (Genspec number 315732) and the voucher specimen was deposited in the herbarium of SANBI. The aerial parts of the plant (10.0 kg) were oven dried at 50 ^{o}C for two days and milled to a coarse powder.

5.6.3 Extraction and Isolation of Compounds

The dried and ground plant material of *E. tenuifolius* (4.6 kg) was sequentially extracted with 1:1 (v/v) methanol/dichloromethane and purified water. The aqueous extract was concentrated by freeze-drying. The organic solvent was evaporated using a rotary vacuum evaporator at 60 ^{o}C and then further dried in a desiccator for five days. The resulting organic extract (30.5 g) was fractionated by column chromatography on silica gel using 2-5% methanol/dichloromethane as eluant. Ten fractions (labelled 5A-J) were generated and fractionated as follows: Fraction 5A and B (1.5 g) were combined and flash chromatographed using 5-20% ethyl acetate/ hexane as eluant. Two compounds; compound **5.15** (4.5 mg) and compound **5.16** (7.8 mg**)** were isolated.

Repeated flash chromatography of fraction 5C-D (5.5 g) using 5-40% ethyl acetate/ hexane as eluant yielded a semi purified compound. Further purification by preparative TLC (9:1 chloroform/methanol) gave compound **5.13** (10.6 mg).

Repeated flash chromatography and preparative TLC (40% ethyl acetate/hexane) of fraction 5F-G (10.2 g) led to the isolation of compound **5.12** (30.5 mg).

Repeated flash chromatography and preparative TLC (9:1 chloroform/methanol) of fraction 5F-G (2.1 g) yielded compound **5.14** (15.2 mg).

5.6.4 Physical data

5.6.4.1 Physical data for 8-*O*-isobutanoylcumambrin B (5.12)

Physical description: white crystals

Molecular formula: $C_{19}H_{26}O_5$

Yield: 30.5 mg

ESI$^-$TOF-MS m/z: 333.1693

Melting Point: 264-266 ^oC

^1H NMR: Plate 8

^{13}C NMR: Plate 9

HSQC: Plate 10

HMBC: Plate 11

5.6.4.2 Physical data for pectolinarigenin (5.13)

Physical description: yellow crystals

Molecular formula: $C_{17}H_{14}O_6$

Yield: 10.6 mg

ESI$^-$-MS m/z: 313.10

Melting Point: 220-222 ^oC

^1H NMR: Plate 12

^{13}C NMR: Plate 13

COSY: Plate 14

HSQC: Plate 15

HMBC: Plate 16

NOESY: Plate 17

5.6.4.3 Physical data for hispidulin (5.14)

Physical description: yellow crystals

Molecular formula: $C_{16}H_{12}O_6$

Yield: 15.2 mg

ESI$^-$-MS m/z: 299.08

Melting Point: 298-299 ^oC

^1H NMR: Plate 18

^{13}C NMR: Plate 19

COSY: Plate 20

HSQC: Plate 21

HMBC: Plate 22

NOESY: Plate 23

5.6.4.3 Physical data for friedelinol (5.15)

Physical description: white crystals

Molecular formula: $C_{30}H_{52}O$

Yield: 4.5 mg

GC-MS m/z: 428.4

Melting Point: 287-288 ^oC

^1H NMR: Plate 24

^{13}C NMR: Plate 25

5.6.4.3 Physical data for tetracosanoic acid (5.16)

Physical description: white crystals

Molecular formula: $C_{24}H_{48}O_2$

Yield: 7.8 mg

GC-MS m/z: 368.4

Melting Point: 82-84 ^oC

^1H NMR: Plate 26

^{13}C NMR: Plate 27

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Chapter 6

Conclusion

Cancer continues to be a public health problem worldwide and a major cause of death. The number of new cases, as well as the number of individuals living with cancer is expanding continuously. Plant-derived agents are being used for the treatment of cancer. Anticancer agents including taxol, vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, and etoposide derived from epipodophyllotoxin are in clinical use all over the world. With limited number of effective anticancer drugs from natural resources currently in use, there is a real need for new, safe, cheap and effective anticancer drugs to combat this dreaded and formidable disease.

There are about 24 300 indigenous plants in South Africa and 3000 of these are reported to be used medicinal, but only a few have been described or studied. This unique resource remains untapped and could be the source of new drug leads and therefore a collaborative research programme was initiated between the CSIR and the NCI in the USA.

In this study, two endemic plant species, *Anthospermum hispidulum* and *Eriocephalus tenuifolius* were investigated for their anticancer properties. The *in vitro* assaying of the organic extracts of these plant species, showed that they had moderate anticancer activity i.e. TGI value of < 15 μg/ml against a panel of three highly sensitive human cancer cell lines (breast, melanoma and renal). Bioassay-guided fractionation of the organic extracts of *A. hispidulum* led to the isolation of an active compound which was characterised as ursolic acid, and it is most likely that the activity observed in the organic extract can be attributed to this compound. Literature searches indicated that ursolic acid and its derivatives have been patented for their anticancer properties by Japanese scientists.

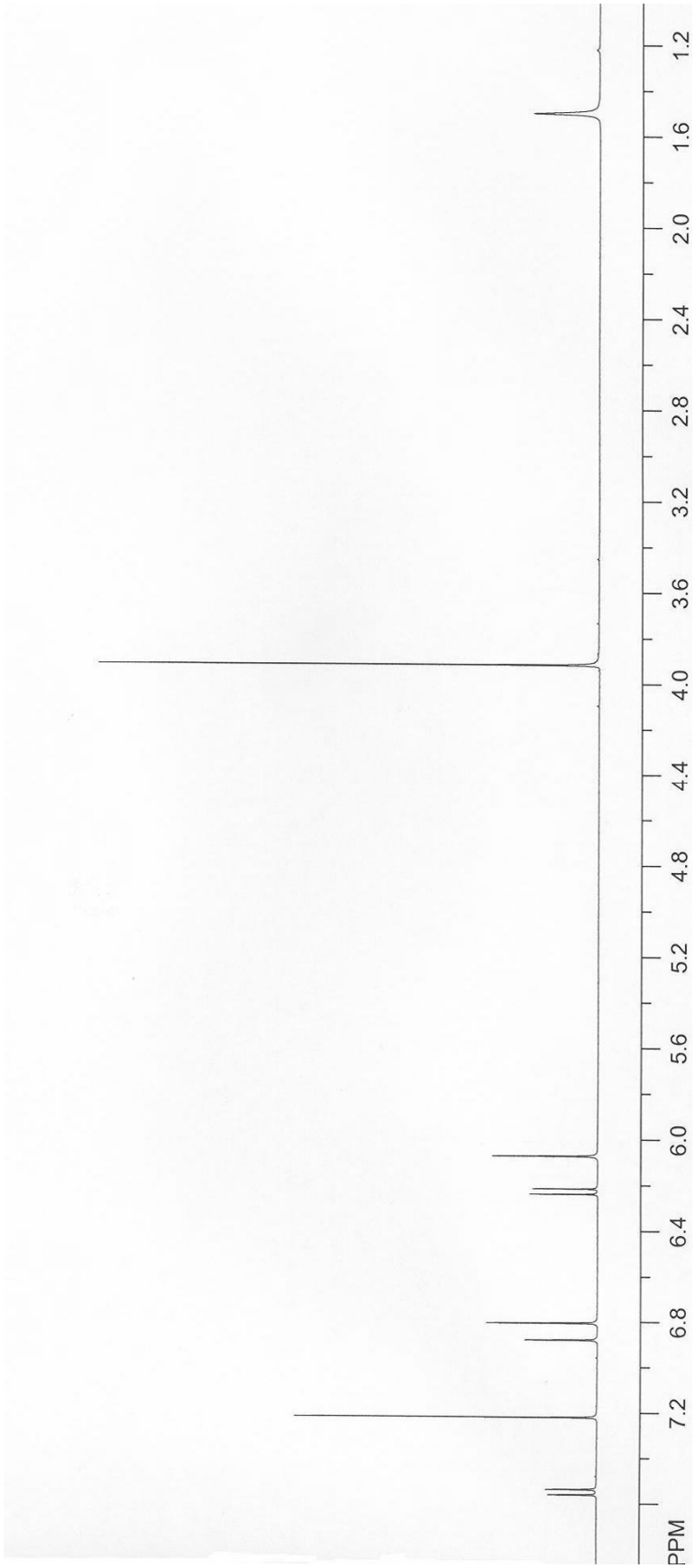
Bioassay-guided fractionation of the organic extracts of *E. tenuifolius* resulted in the isolation of 8-*O*-isobutanoylcumambrin B as the active constituent. Although 8-*O*-isobutanoylcumambrin B has been isolated previously from other *Eriocephalus* species, there are no previous reports of the compound being investigated for any biological activity. To my knowledge, this is the first report of this compound having anticancer

activity. Structural modification of this compound to improve its potency and drugability can be recommended for further studies. 8-*O*-isobutanoylcumambrin B can be further developed as new anticancer drug lead.

Bioassay-guided fractionation of these plant extracts, resulted in the isolation and identification of active ingredients, which could be used as scaffolds to develop leads with enhanced anticancer activity. The findings of the study have shown that South African plants can provide potential bioactive compounds for the development of new leads to combat cancer diseases.

Appendix

Plate 1



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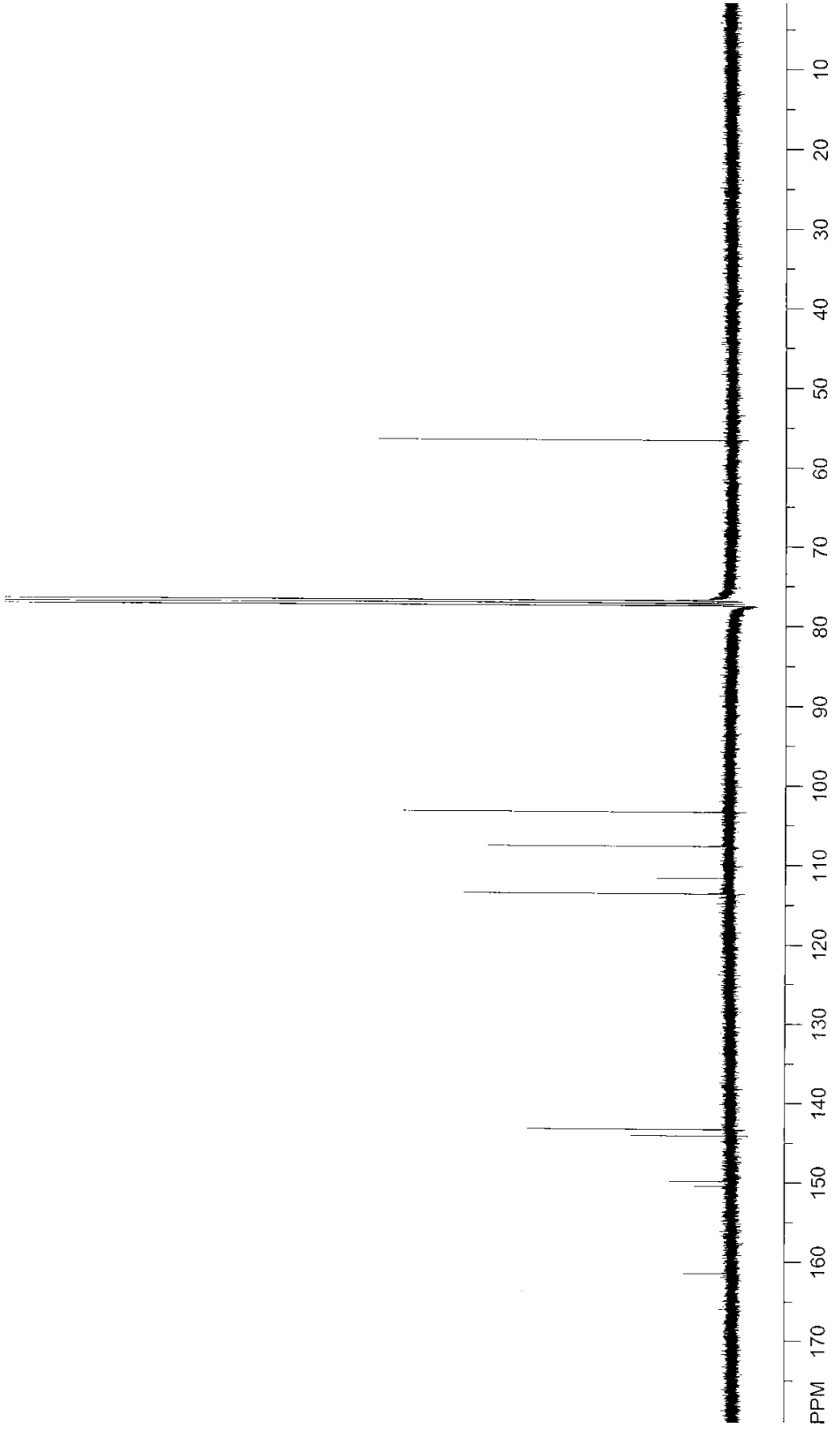

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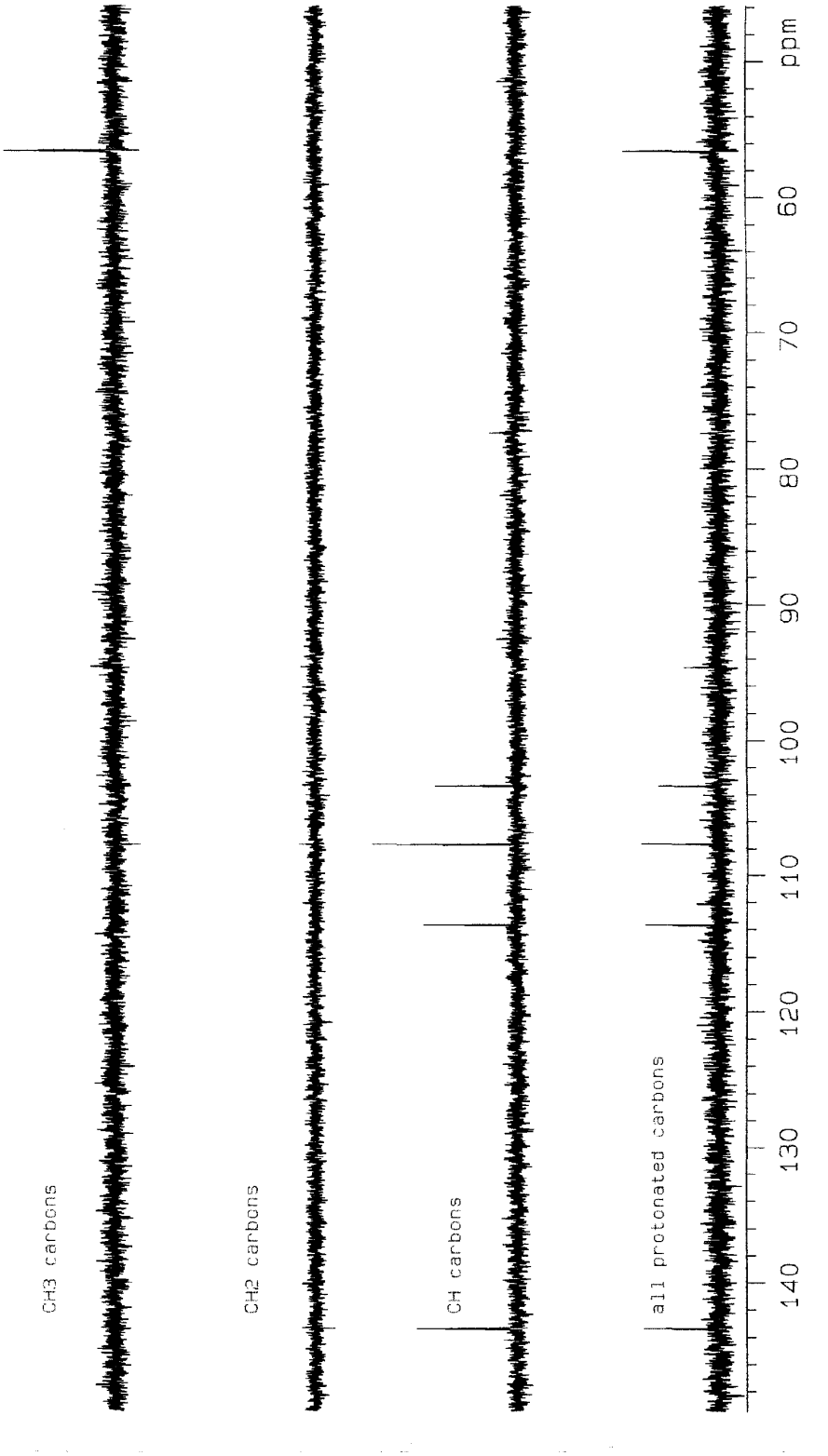
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CH2 carbons
CH carbons
all protonated carbons
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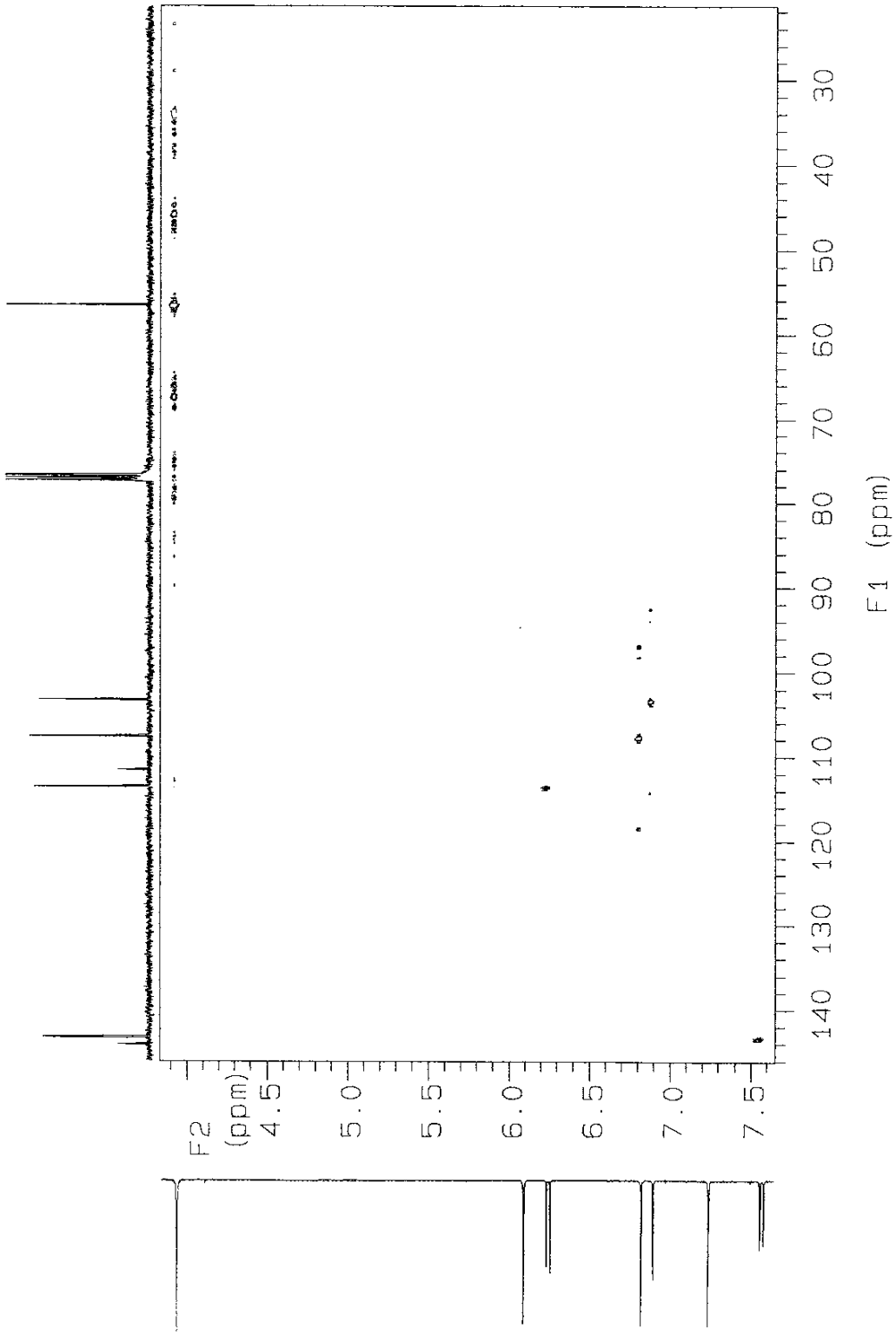
130
120
110
100
90
80
70
60
ppm

Plate 4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5.5
6.0
6.5
7.0
7.5
F1 (ppm)
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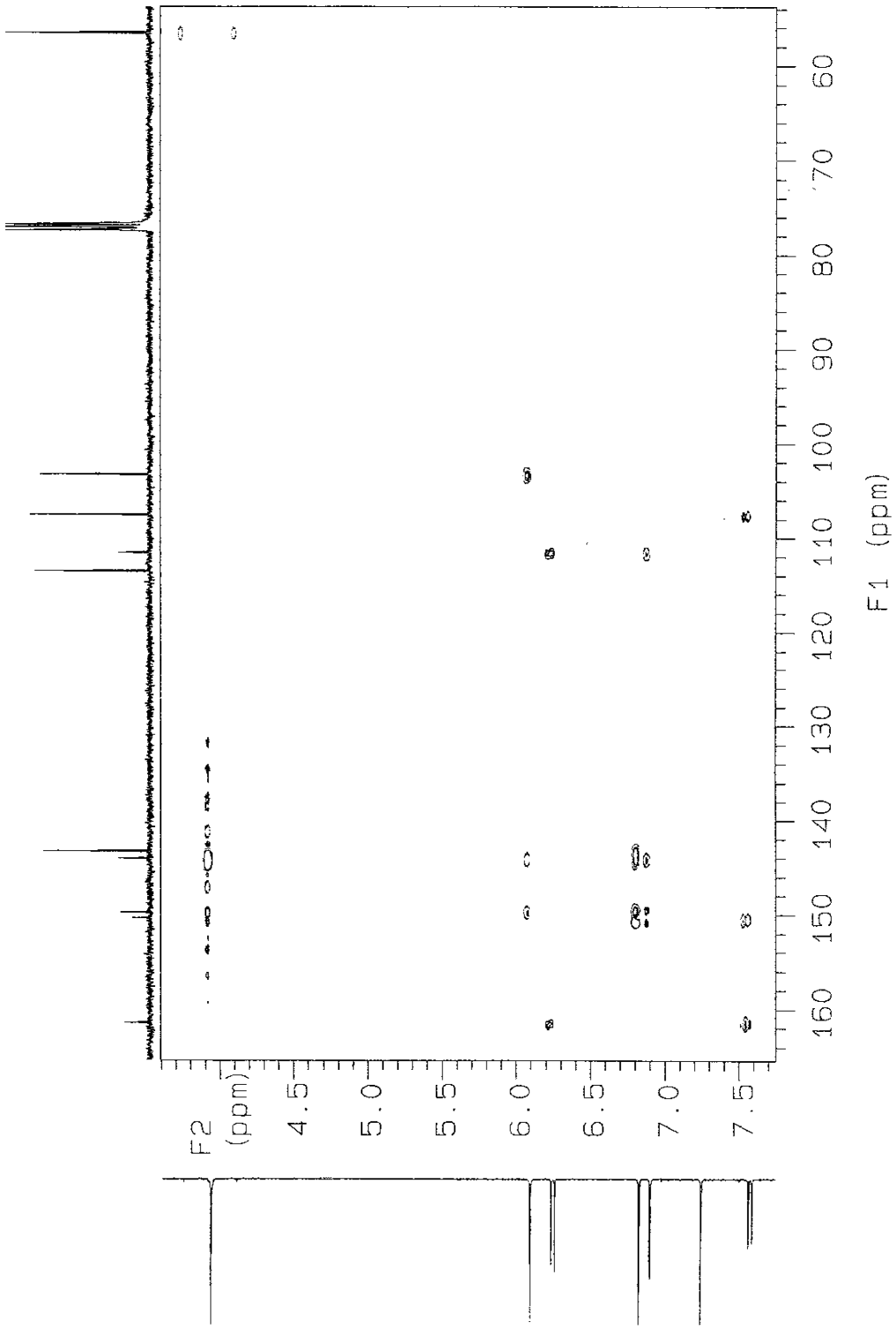
40
50
60
70
80
90
100
110
120
130
140

Plate 5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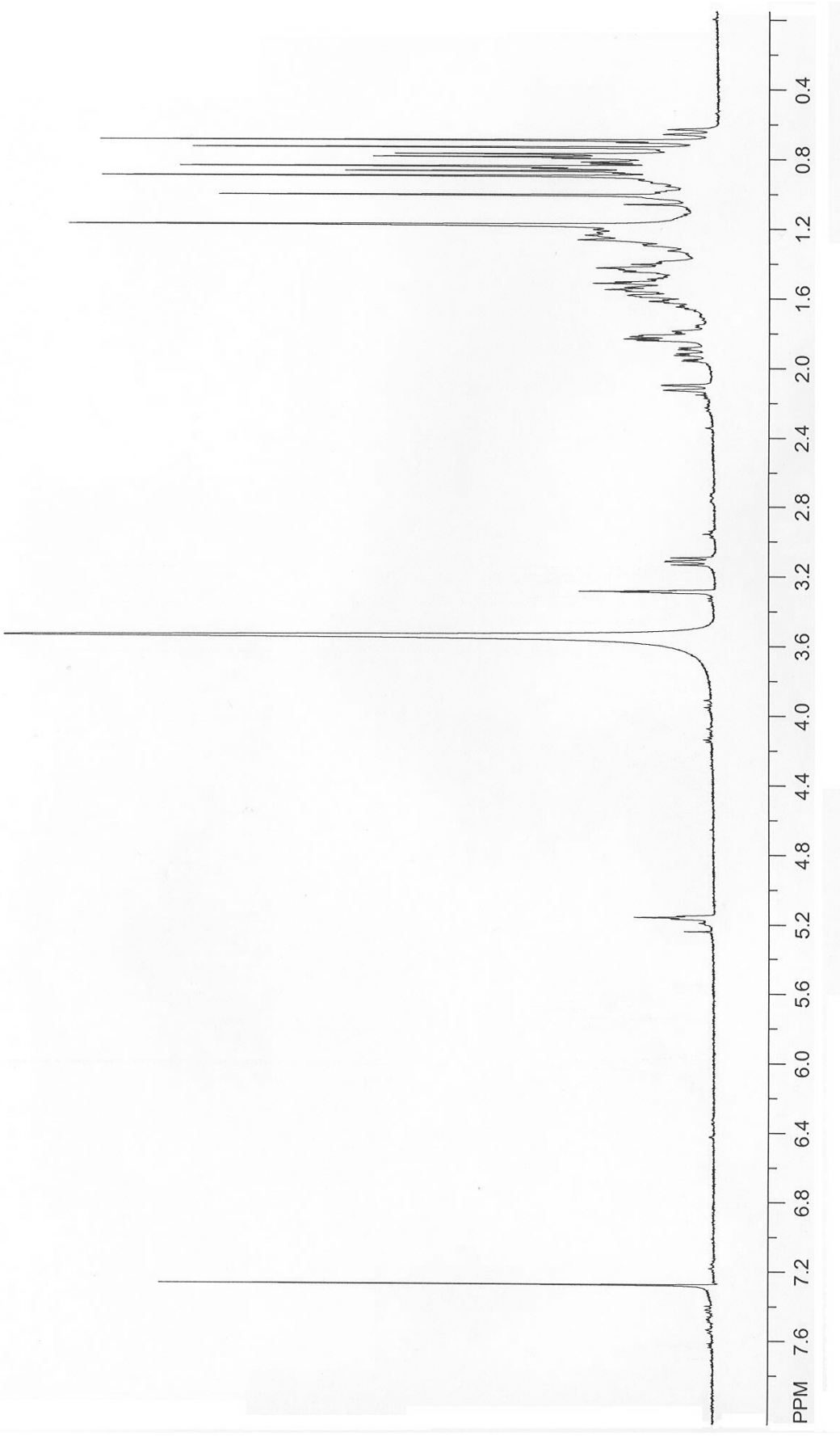
4.5
5.0
5.5
6.0
6.5
7.0
7.5
F1 (ppm)
60
70
80
90
100
110
120
130
140
150
160

Plate 6



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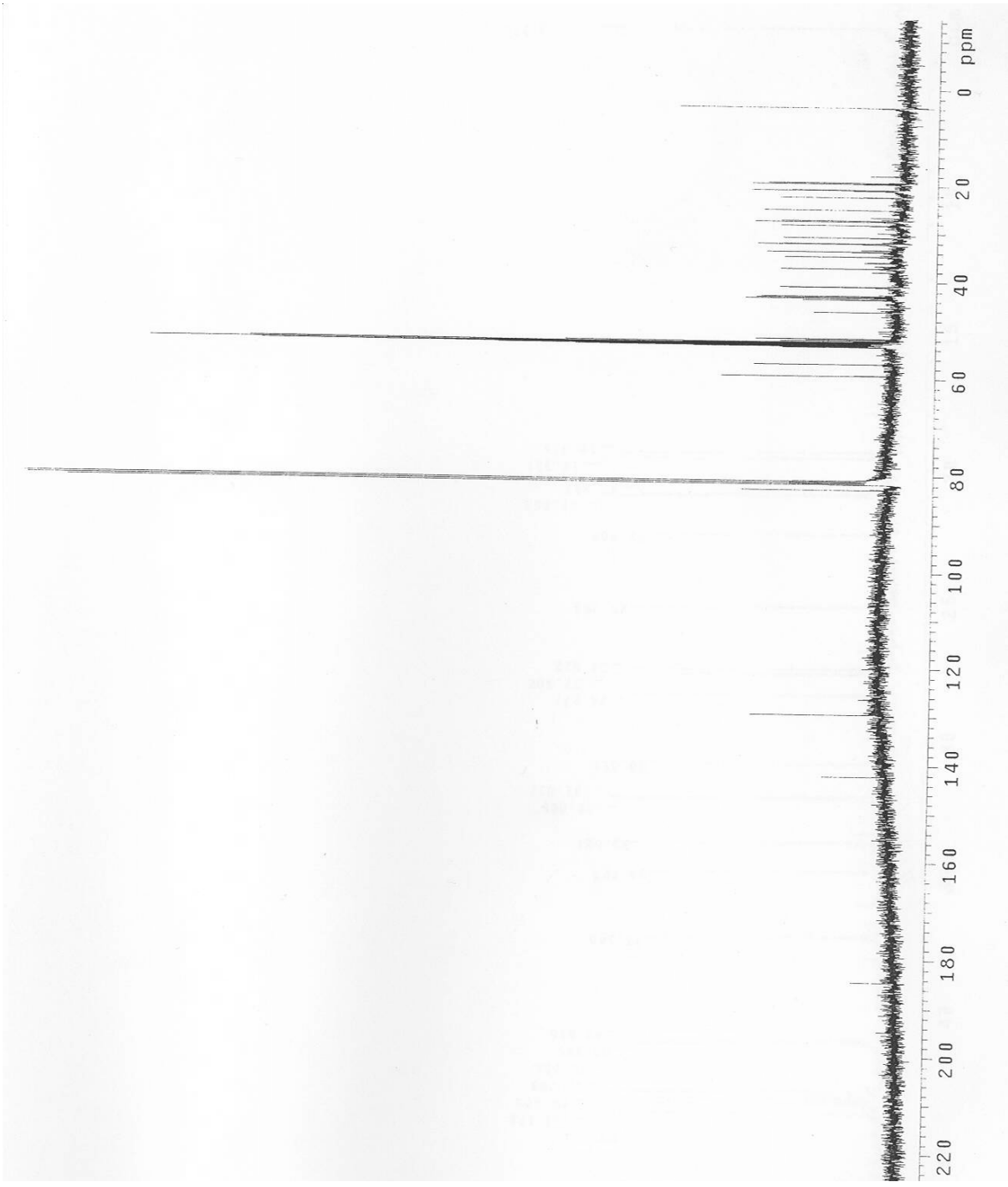

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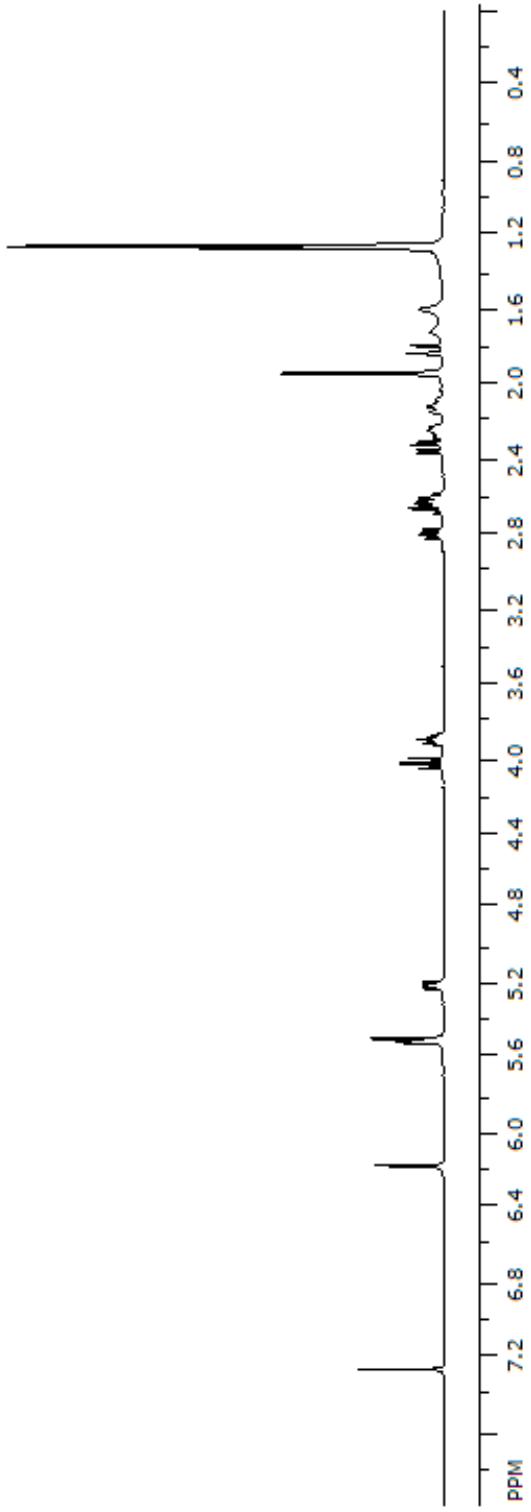

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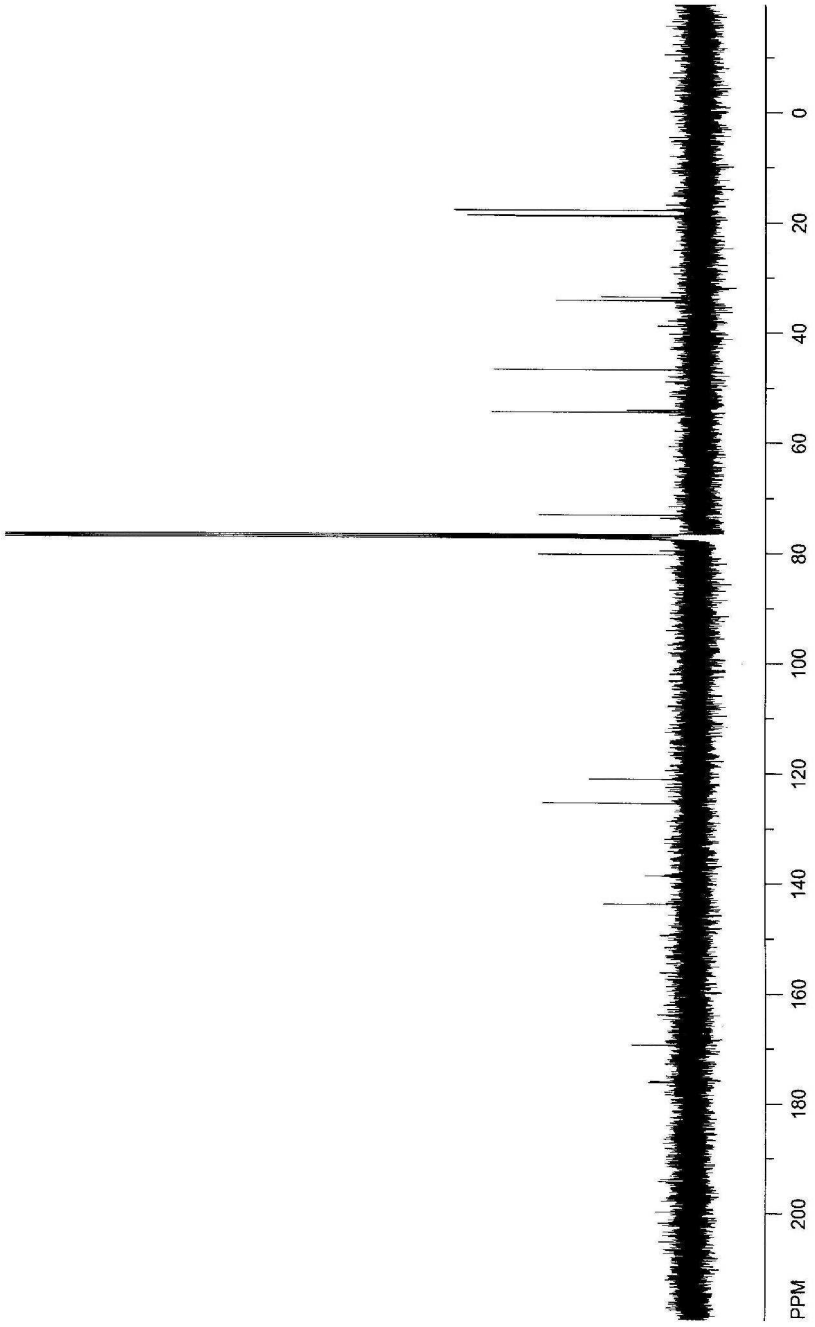

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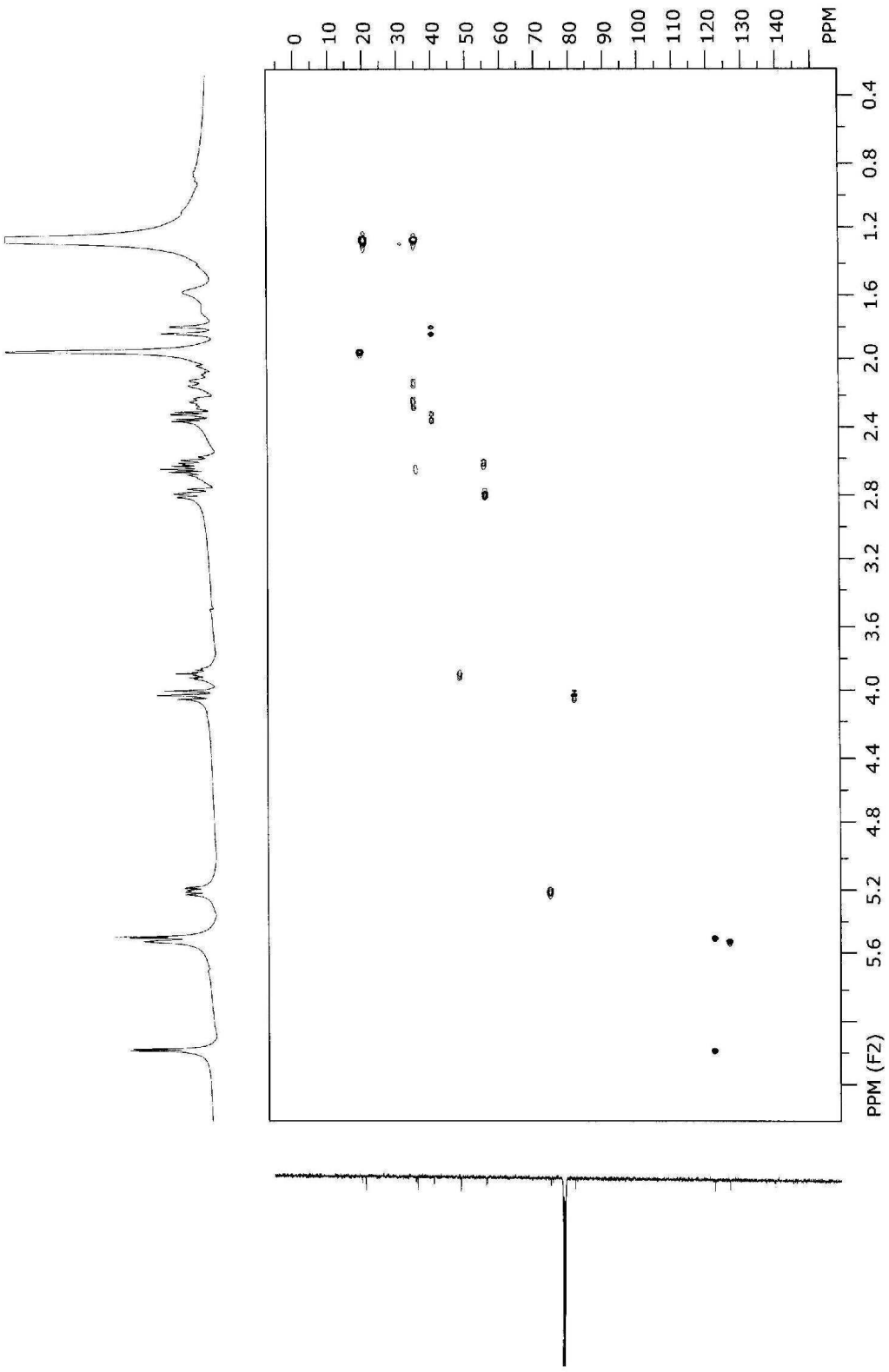
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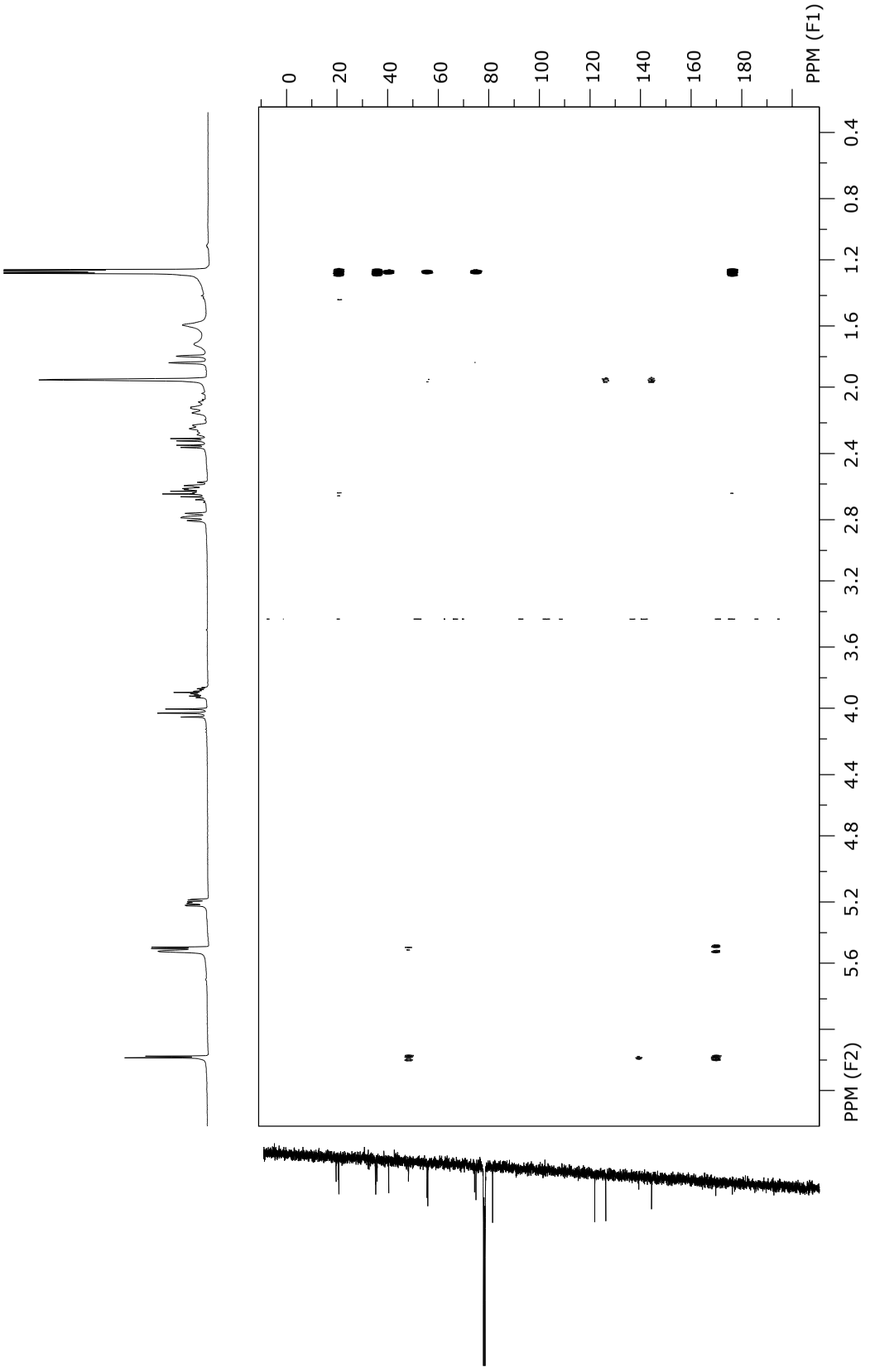
10
20
30
40
50
60
70
80
90
100
110
120
130
140
PPM
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
PPM (F2)

Plate 11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40
60
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100
120
140
160
180
PPM (F2)
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0.8
1.2
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2.4
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6

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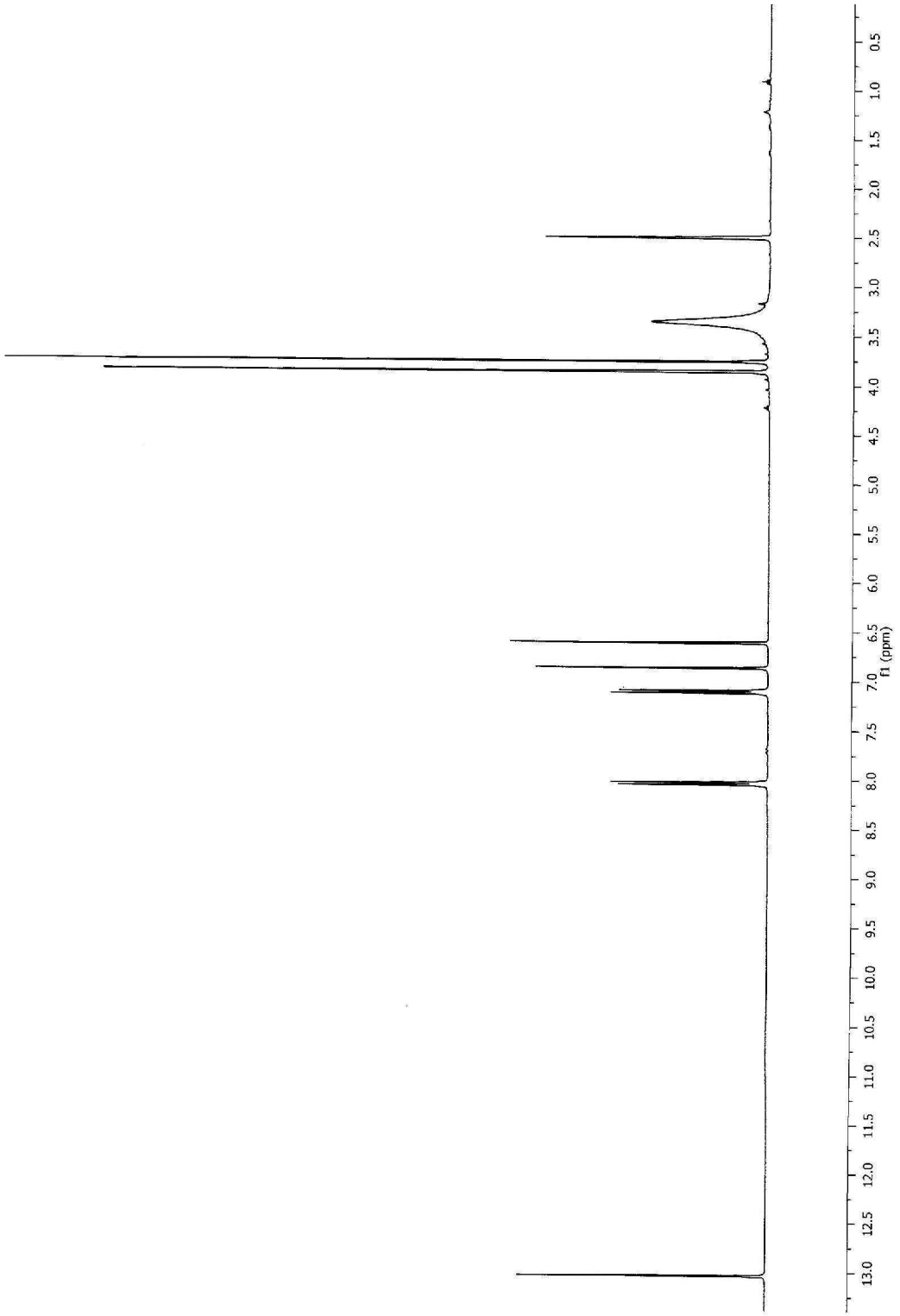
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1.0
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6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
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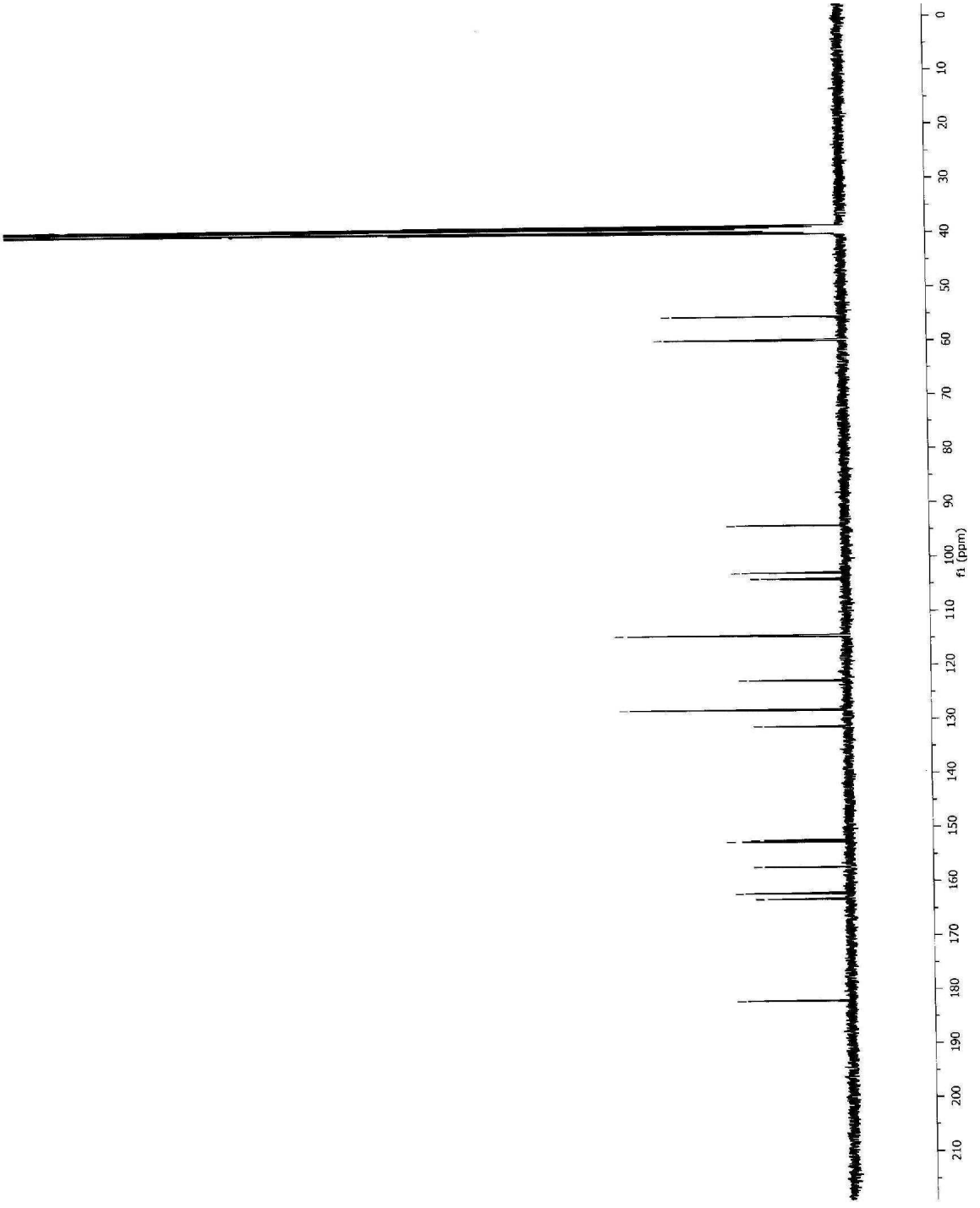
11.0
11.5
12.0
12.5
13.0
f1 (ppm)

Plate 13


0
10
20
30
40
50
60
70
80
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100
f1 (ppm)
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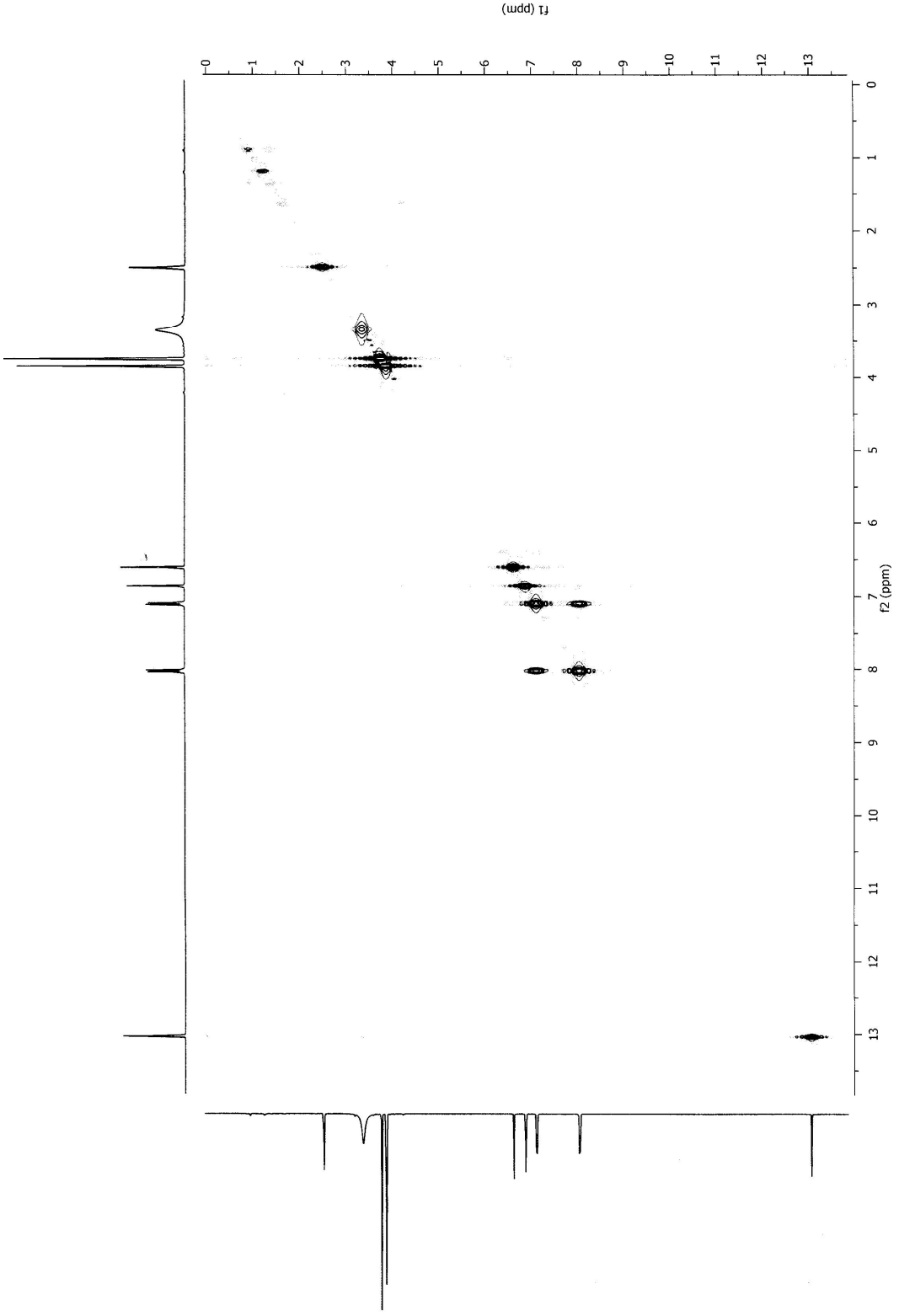
120
130
140
150
160
170
180
190
200
210

Plate 14



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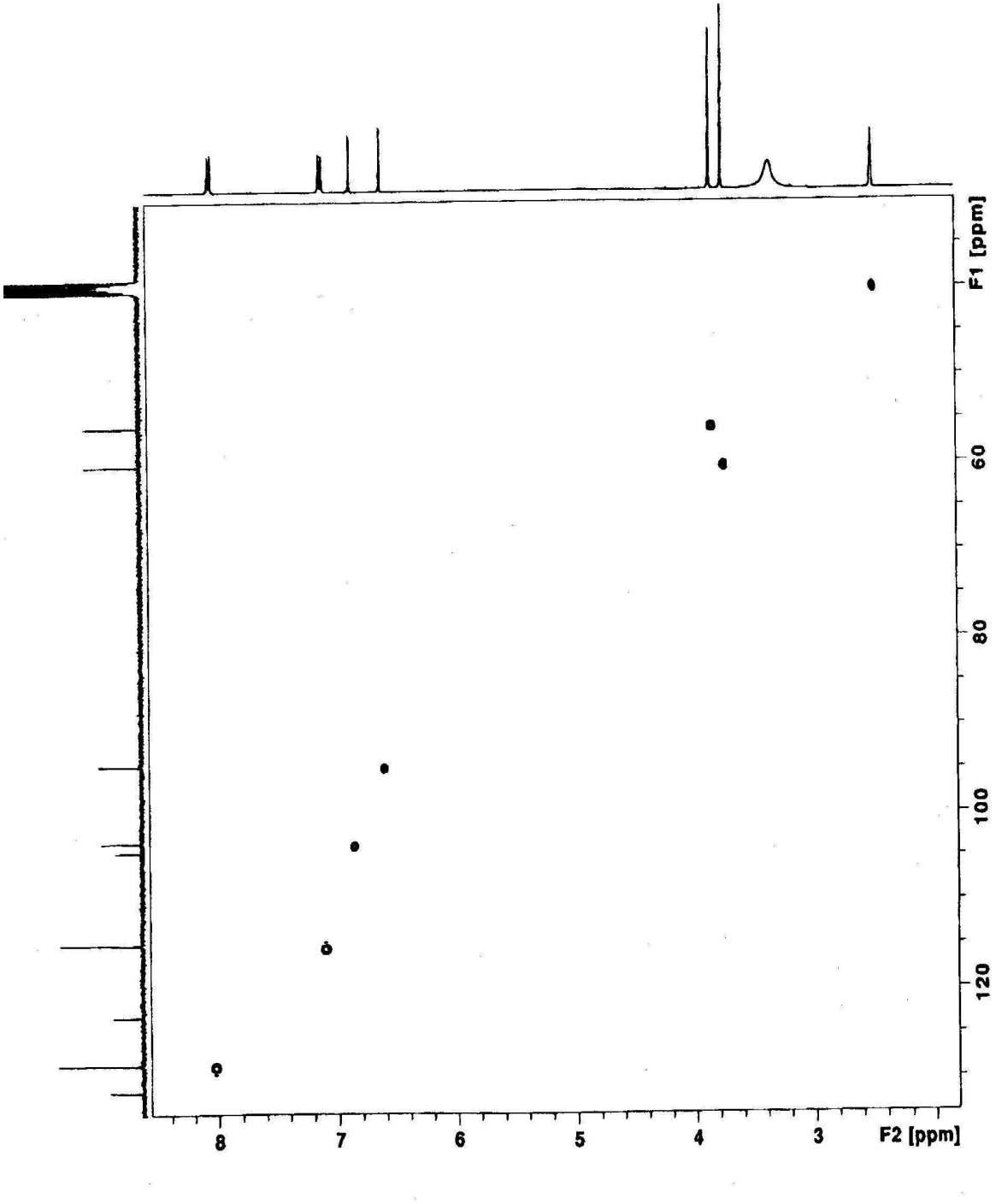

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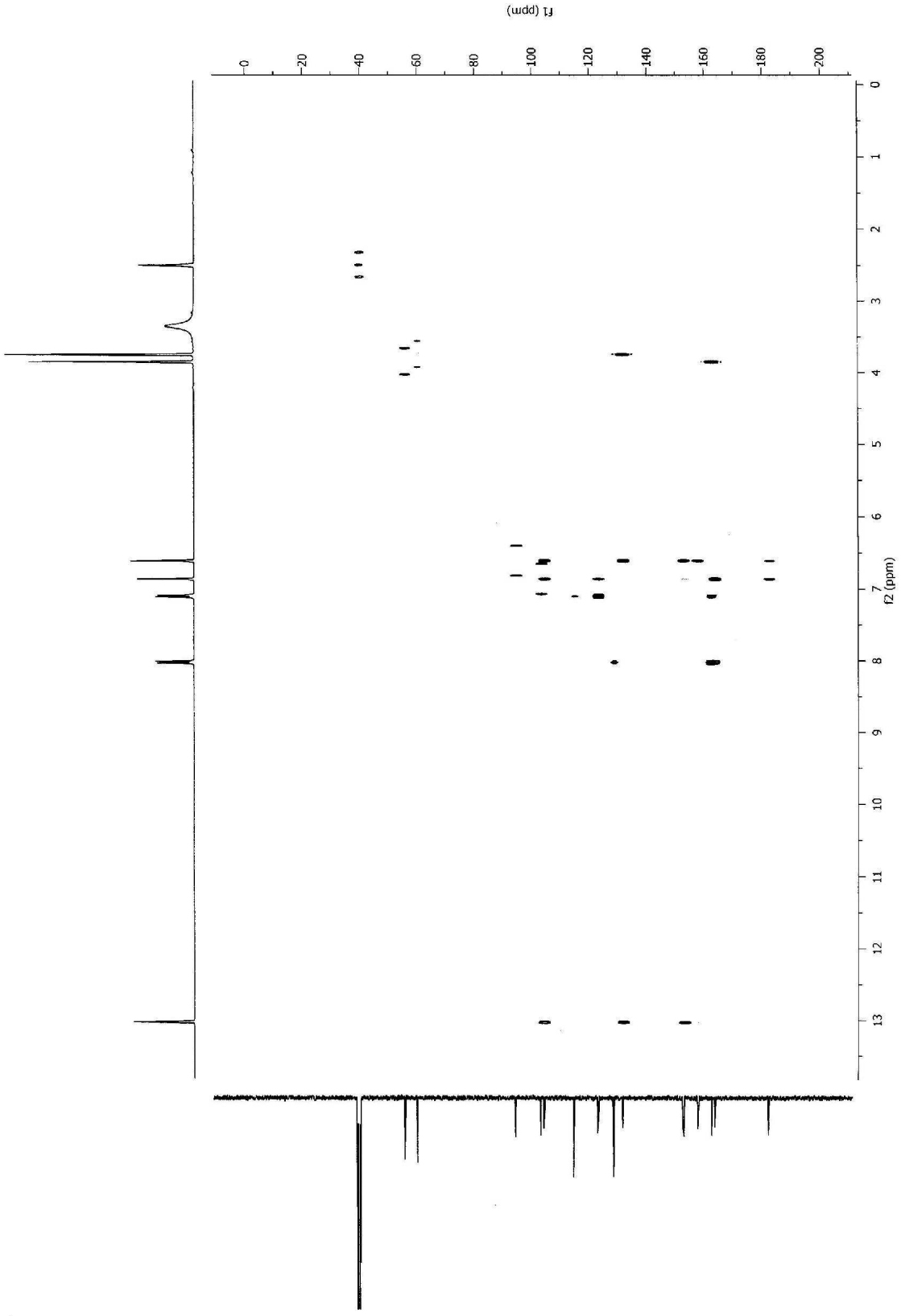
f1 (ppm)
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20
40
60
80
100
120
140
160
180
200
f2 (ppm)
0
1
2
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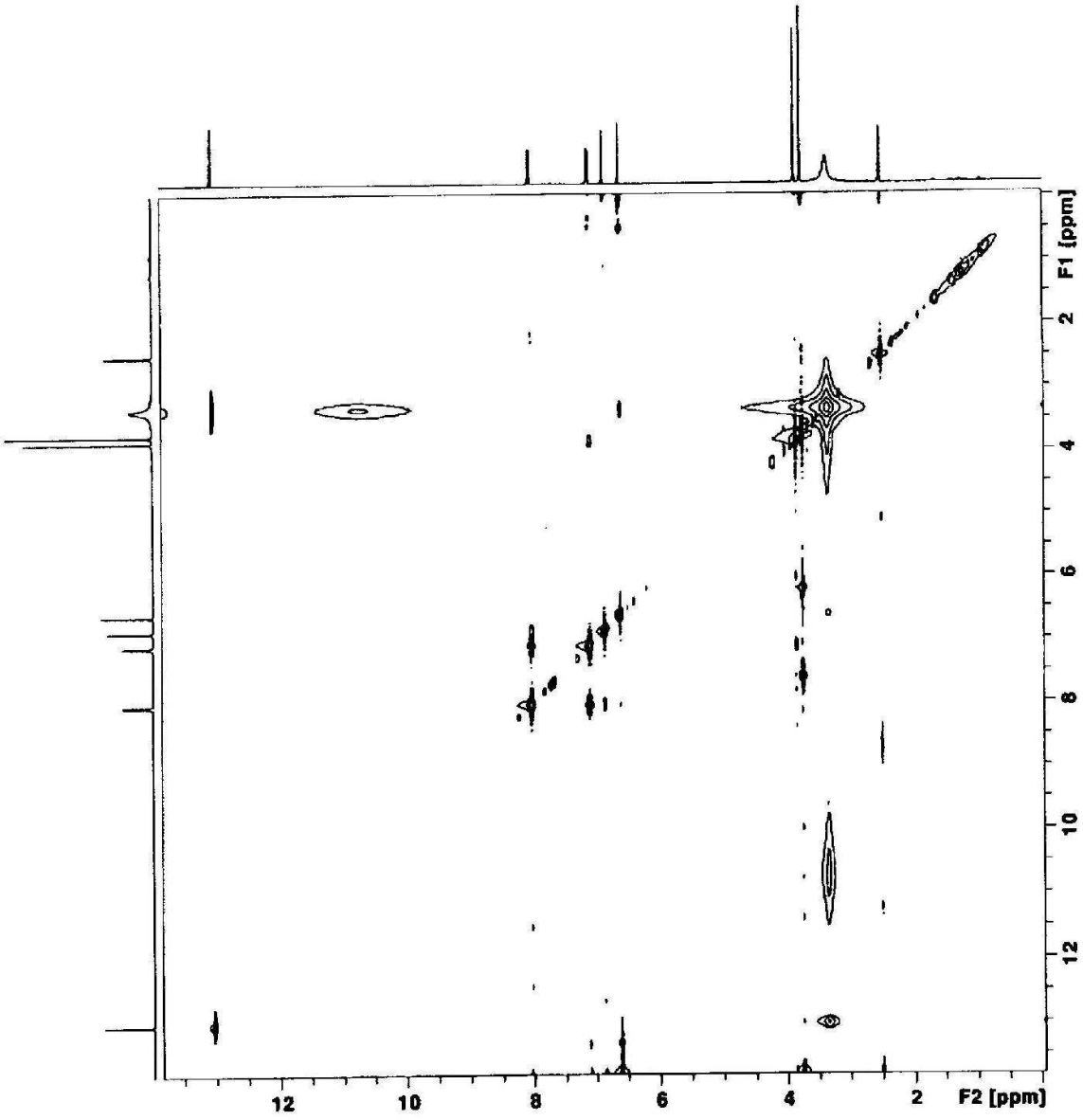
6
7
8
9
10
11
12
13

Plate 17



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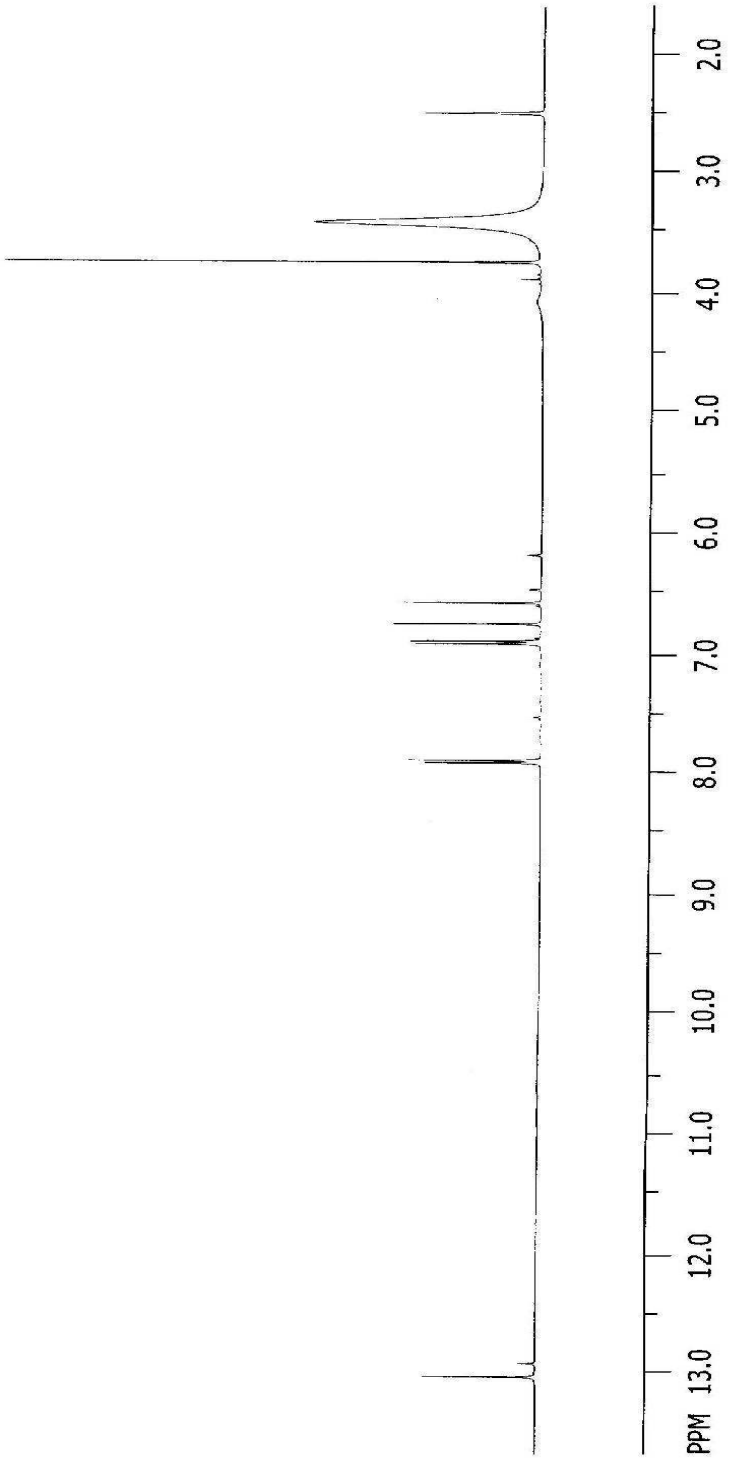

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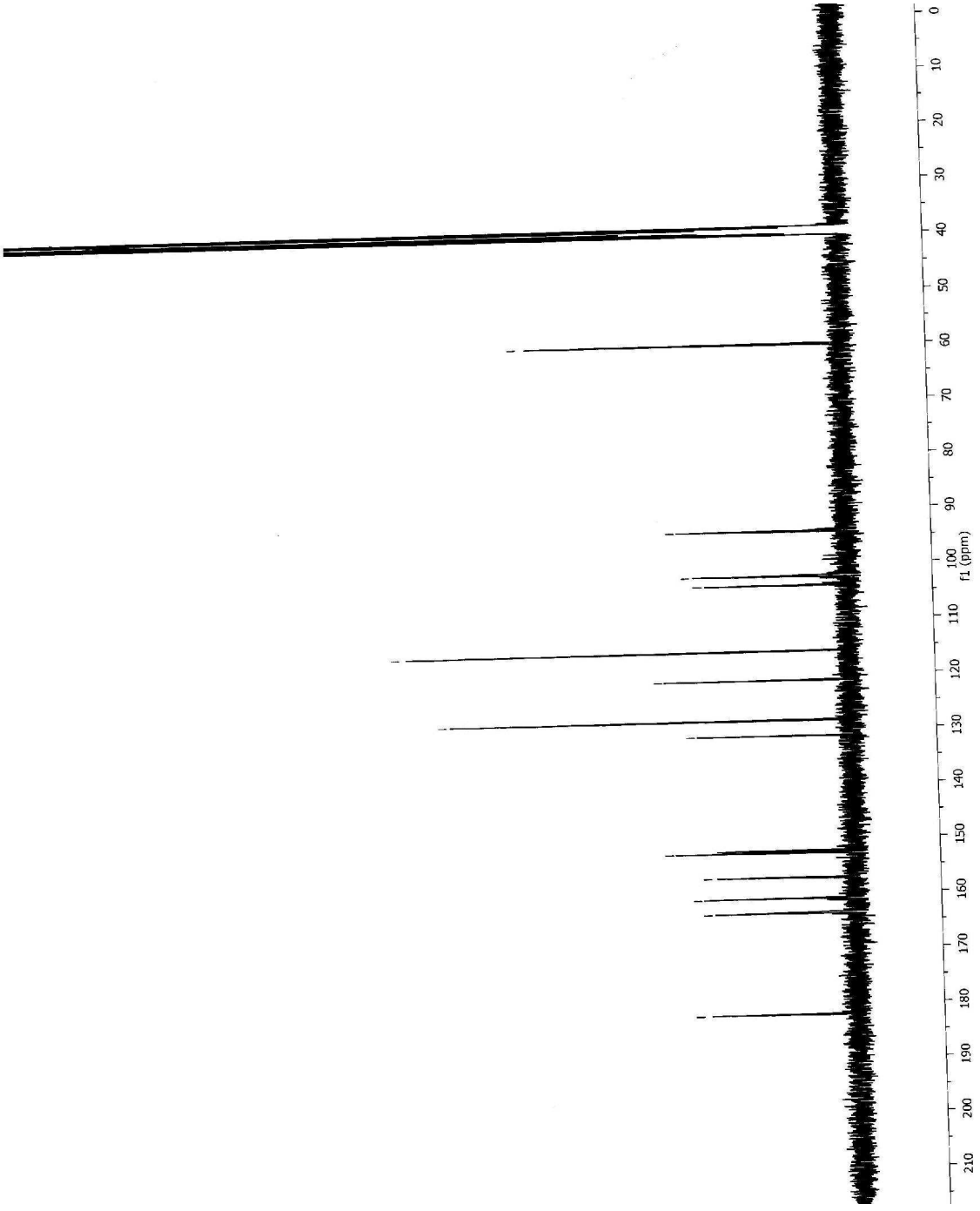
0
10
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30
40
50
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70
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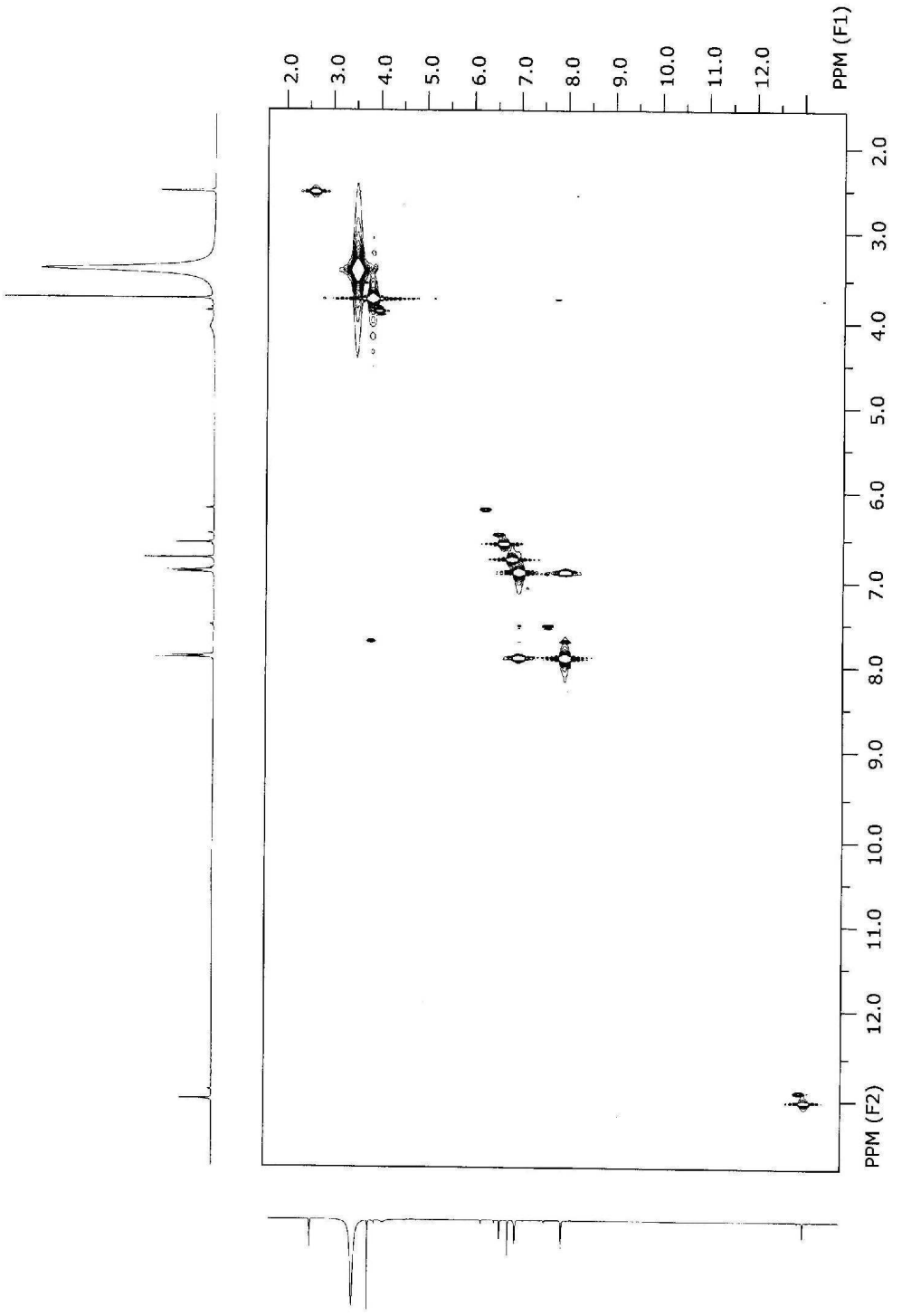
90
100
f1 (ppm)
110
120
130
140
150
160
170
180
190
200
210

Plate 20



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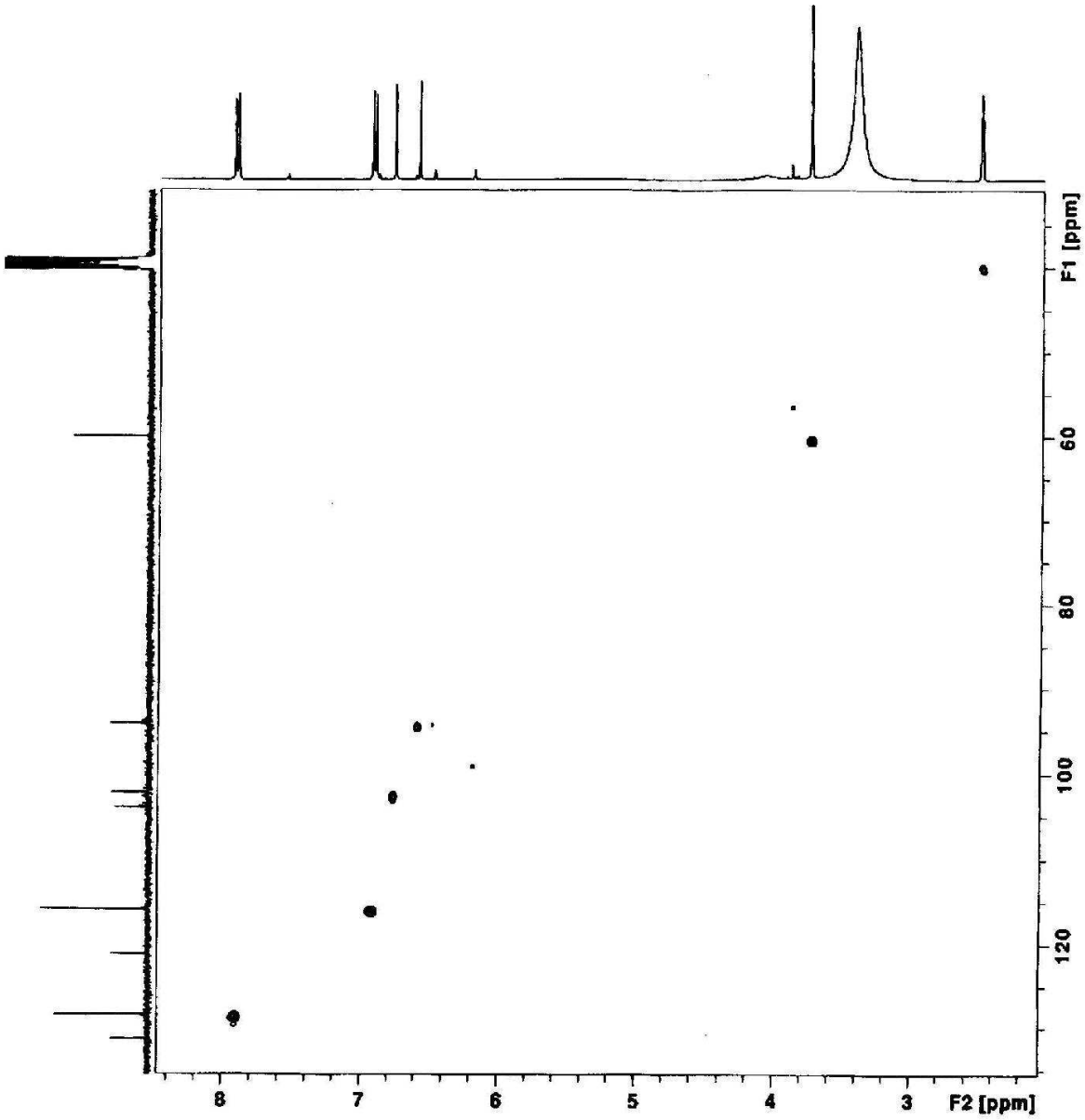

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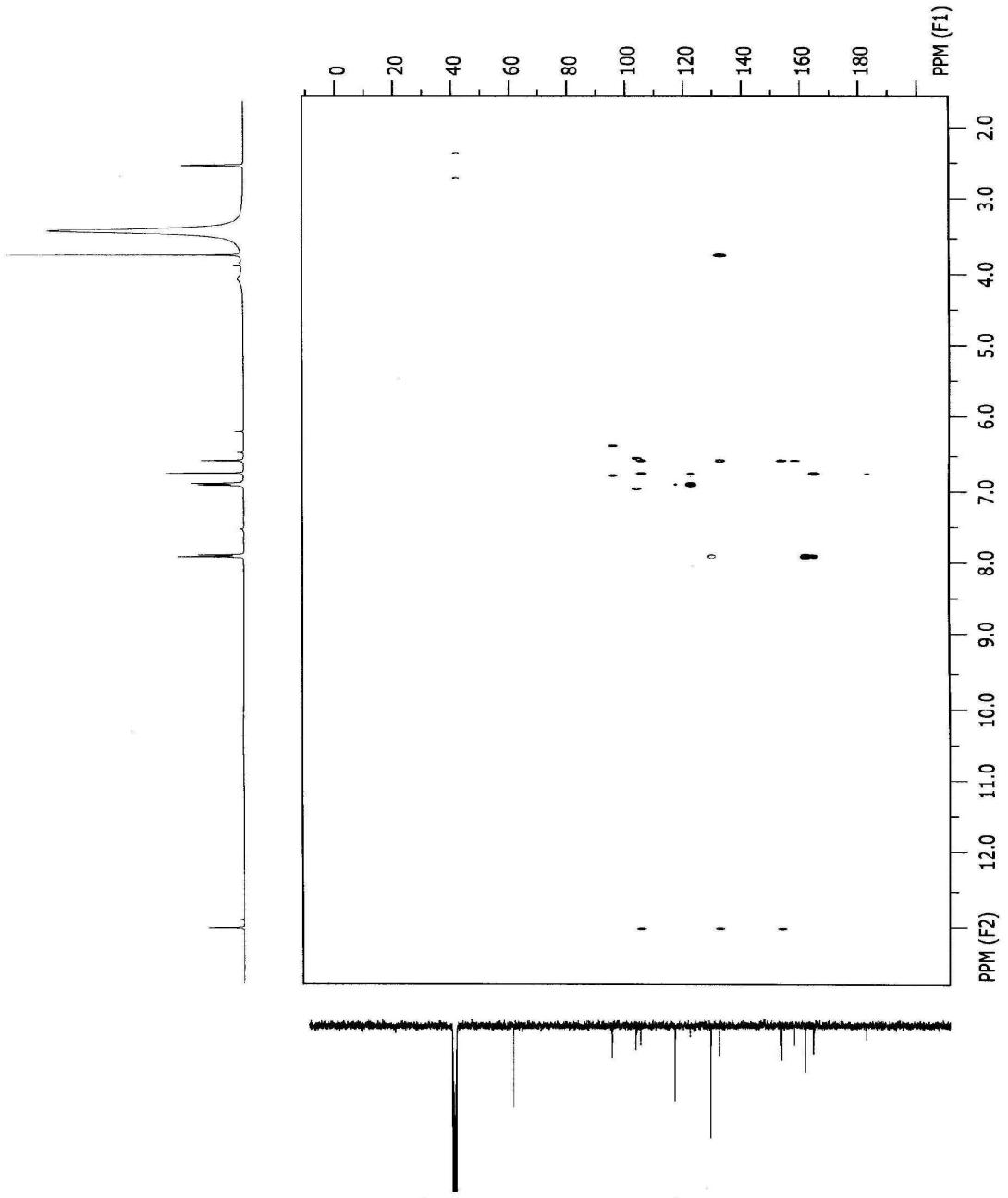
0
20
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60
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100
120
140
160
180
PPM (F1)
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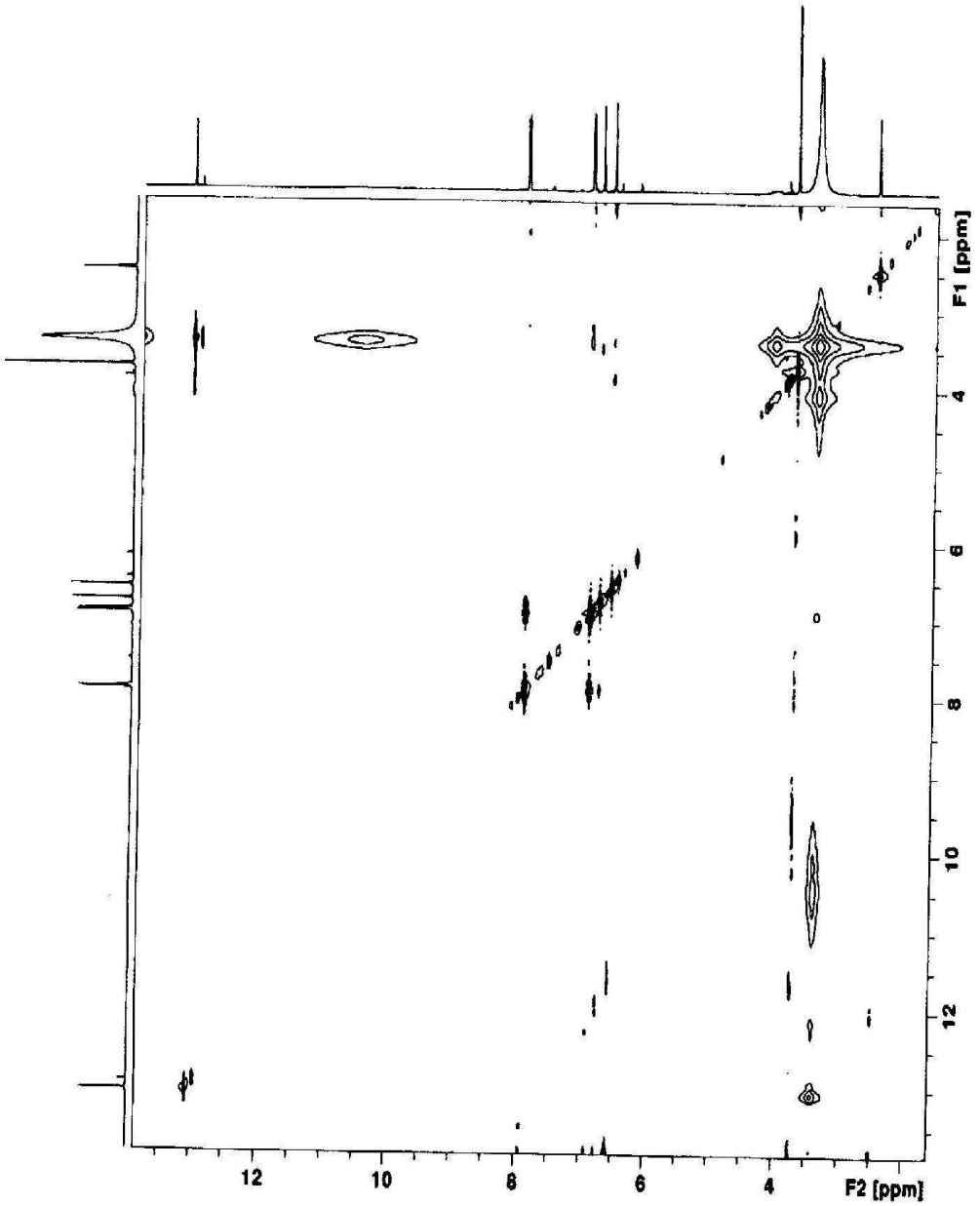
10.0
11.0
12.0
PPM (F2)

Plate 23



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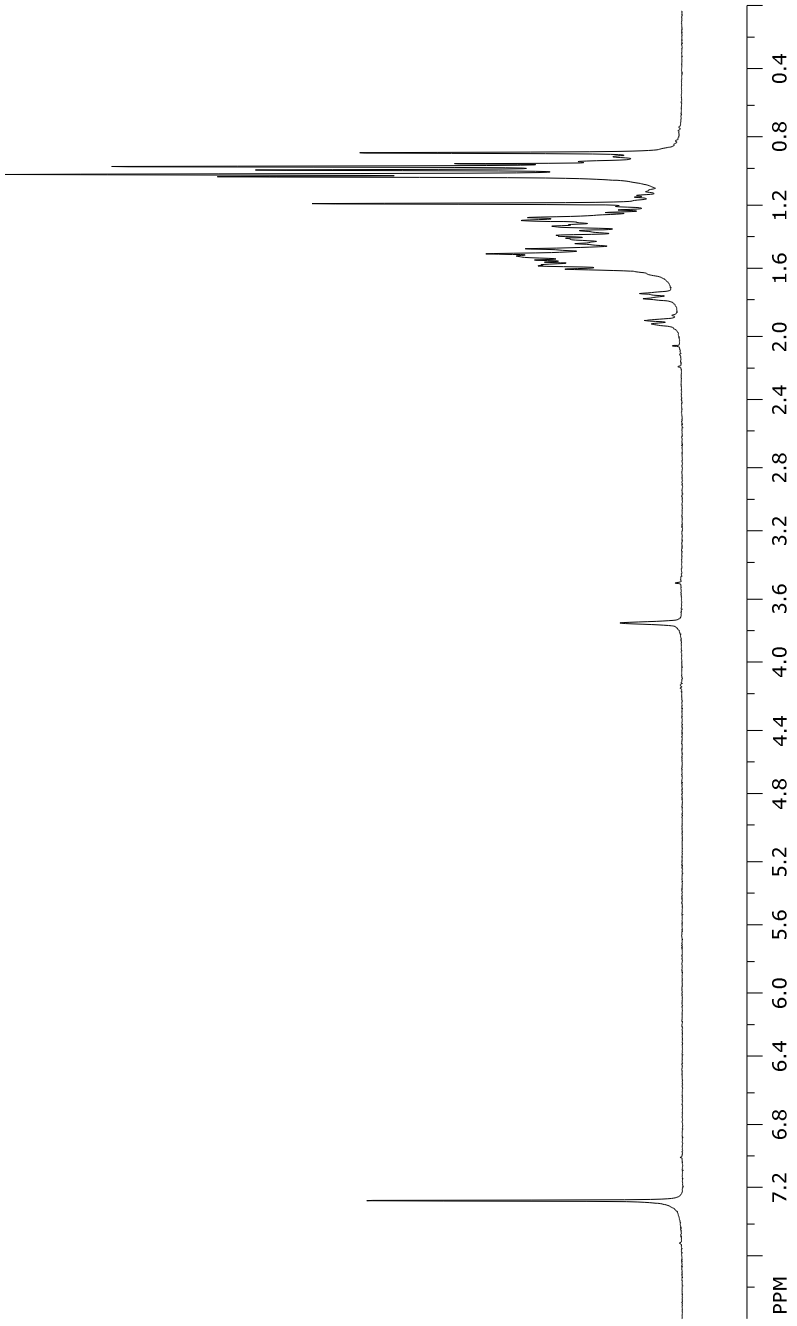

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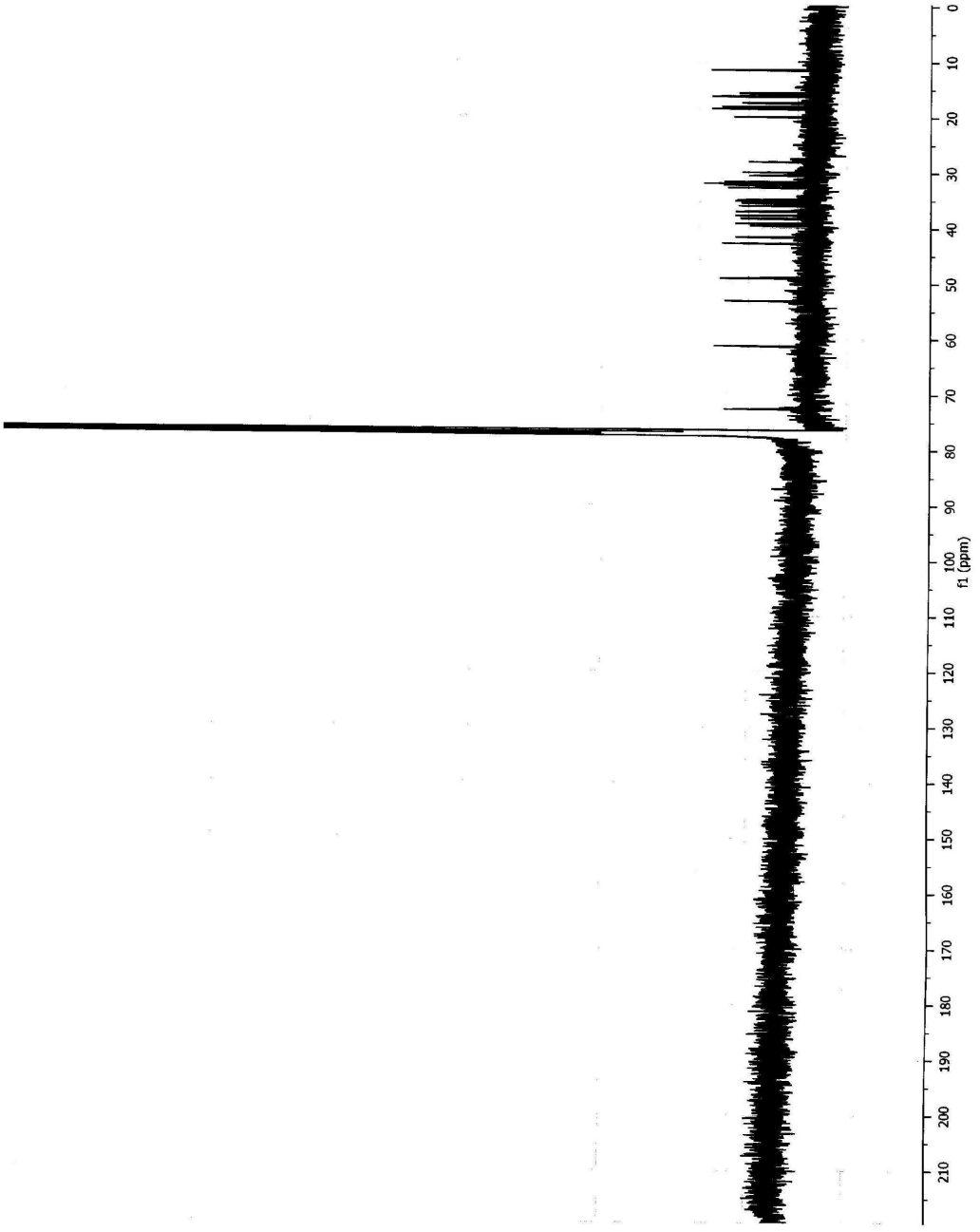
0
10
20
30
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200
210
f1 (ppm)

Plate 26

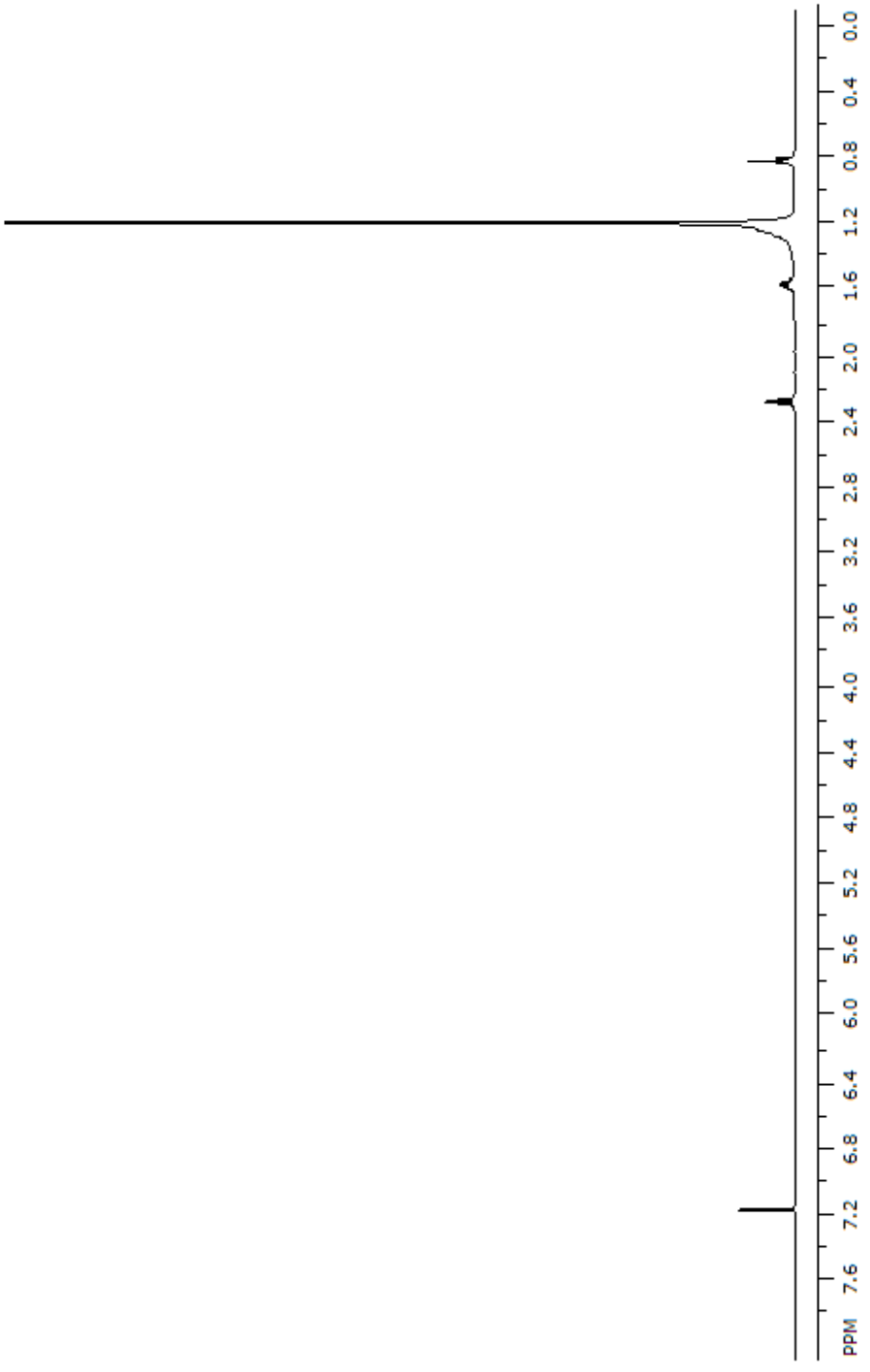

Plate 27