South African plants as a source of herbicides: Identification of a compound with phytotoxic activity from *Artemisia afra* Jacq. ex Willd.

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Master of Science

By

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DECLARATION

This is to certify that the experimental work and the results presented in this thesis are the original work of my own investigation carried-out at the School of Chemistry and Physics, University of KwaZulu-Natal, Pietermaritzburg campus and have not been submitted for any degree or diploma at any institution.

Signed……

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I hereby certify that this statement is correct

Signed…….....

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DEDICATION

This research work is dedicated to my wife for her support and encouragement and the whole of my family at large not forgetting all of those who helped in one way or the other.

ACKNOWLEDGEMENTS

I thank the almighty God for His protection and guidance and helping me to go through this fruitful long journey besides all the life dares I encountered.

I also direct my deepest appreciations to my Supervisor Professor F.R. van Heerden for her painstaking supervision. Her expert guidance, inspirations and motivation were more than helpful. This project would not have been possible without her. The human rapport established and positive instructions given are esteemed. My sincere gratitude also goes to Professor Mark D. Laing for his academic and expertise guidance in the bioassay processes.

I would also like to thank Ms. Alison Young, curator of the UKZN botanical garden, for her support and role she played during the plant collection process I am grateful to Mr F. Shaik and Dr A. Majavu, for their help in the laboratory, Mr C. Grimmer, for NMR lessons he taught me, Mrs C. Janse van Rensburg for running MS experiments of my samples, Mr. S. Ball for providing any material I requested for and all my colleagues in Organic Chemistry for their academic support most importantly the natural product group.

ABSTRACT

In this study, six medicinal plant species namely, *Artemisia afra* Jacq. ex Willd, *Eriocephalus africanus* L, *Vernonia natalensis* Sch.Bip. ex Walp belonging to the Asteraceae family and *Leonotis leonurus* (L) R.Br, *Plectranthus fruticosus* L'Hér. 'James' and *Tetradenia riparia* (Hochst.) Codd of the Lamiaceae were harvested and extracted using dichloromethane and methanol. These extracts were assayed for the germination inhibition of seeds of four different plants, namely, lettuce (*Lactuca sativa)*, radish (*Raphanus sativus*), ryegrass (*Lolium rididum*) and teff (*Eragrostic tef*). The inhibition of germination was considered a preliminary indication of phytotoxicity. Based on the results obtained, the most active plant, *A. afra* was investigated further.

The bioassays were conducted in Petri dishes with 9 cm filter papers impregnated with extract at concentrations of 25 mg/paper, 50 mg/paper and 75 mg/paper. The seeds, 10 each *Lactuca sativa* and *Raphanus sativus* and 20 and 100 each of *Lolium rididum* and *Eragrostic teff*, respectively, were used in each Petri dishes and monitored for 7 days. The DCM-MeOH (1:1) extract of *A. afra* showed the highest inhibition of germination, with 50% germination at the lowest concentration and no germination for the two higher concentrations for *L. sativa* and 40% germination at the lowest concentration and no germination at the two higher concentrations for *R. sativus*. In the bioassay with *Eragrostic teff*, the extract showed 80%, 52% and 22% germination for the three concentrations, whereas with ryegrass 95%, 80% and 0% germination at the three concentrations were observed. A bioassay-guided fractionation of the extract lead to the isolation of 7-hydroxy-6-methoxycoumarin was identified as the compound responsible for the herbicidal property of the extract. In the bioassay, the root and shoot lengths of the germinated seeds were also measured. The pure compound was assayed and it gave the highest inhibition of root and shoot lengths of *Lactuca sativa* at 75 mg/dish with a P_{cal} value of 0.000 for each compared with the control while a commercially available coumarin (5,7-dimethoxycoumarin) at the same concentration gave for shoot length P_{cal} 0.003 and root length P_{cal} 0.167.

Comparing the activity of the two compounds, the isolated coumarin from *A. afra* showed inhibition of both the root and shoot lengths whereas 5,7-dimethoxycoumarin only inhibited the shoot lengths and did not affect the root lengths in all the concentrations used.

Two other compounds, the flavone acacetin and the sesquiterpene lactone lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide were also isolated from the extract of *A. afra* and characterised

by spectroscopic techniques. The ^{13}C NMR data of the latter compound are reported for the first time.

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LIST OF ABBREVIATIONS

µL	Microliters
ADP	Adenosine diphosphate
AHAS	Acetohydroxyacid synthase
ALS	Acetolactate synthase
AMPA	Aminomethylphophonic acid
ATP	Adenosine triphosphate
BFR	German Federal Institute for Risk Assessment
cm	Centimeter
COSY	Correlated spectroscopy
D	Doublet
dd	Doublet of doublets
DAHPS	3-Deoxy-d-arabino-heptulosanate-7-phosphate synthase
DCM	Dichloromethane
DEPT	Distortionless enhancement polarization transfer
DHQS	3-Dehydroquinate synthase
DMSO	Dimethyl sulfoxide
EFSA	European food Safety Authority
ESI	Electrospray ionisation
ESPS	5-Enolpyruvyl shikimate-3-phosphate synthase
EtOAC	Ethyl acetate
Fig.	Figure
g	gram
GC-MS	Gas chromatography mass spectrometry
GP	Germination percentage
Hex	Hexane
HMBC	Heteronuclear multiple bond correlation
HRMS	High-resolution mass spectrometry
HSQC	Heteronuclear multiple quantum coherence
IAA	Indol-3-acetic acid
IARC	International Agency for Research on Cancer
IPM	Integrated pest management
IPPM	Integrated plant production management
J	Spin-spin coupling constant in Hz

LD_{50}/LC_{50}	Lethal dose or lethal concentration
m	Multiplet
MCPA	m-Chlorophenoxyacetic acid
MeOH	Methanol
mL	Milliliter
mm	Millimeter
MS	Mass spectrometry
NAA	Naphthaleneacetic acid
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
PEP	Phosphoenolpyruvic acid
PMRA	Pest Management Regulatory Agency
ppm	Parts per million
QA	quinine QA (primary acceptor)
QB	quinine QB (secondary acceptor)
R_f	Retention factor
RPM	Rotation per minute
s	Singlet
SPSS	Statistical Package for the Social Sciences
TLC	Thin-layer chromatography
UV	Ultraviolet
VLSFA	Very long chain fatty acid

CHAPTER 1

INTRODUCTION AND AIM

1.1 INTRODUCTION

The population of the world is steadily increasing and it is estimated that by 2050 the current 7 billion people will reach 9.7 billion.[1] A direct result of this, the growth in wealth and the increased use of processed food, is putting pressure on the food supply systems of the world.[2] In view of this, there is a need to produce more food to accommodate the increase in population. It is estimated that 854 million people in the world are chronically or acutely malnourished.[3] Malnourishment might not only be attributed to poverty but also to a lack of expertise and knowledge of food production and preservation and the application of pesticides during and after the production of food.

Pesticides are extensively used in agriculture to manage pests, diseases, weeds and plant pathogens in order to minimize losses and safeguard the quality of food products.[4] However, the use of synthetic pesticides are not without environmental problems. One area of major concern is that non-targeted species or organisms are affected by these chemicals. It is reported that over 98% of applied synthetic insecticides and 95% of herbicides migrate to destinations different to their targeted areas or species because they are often applied or sprayed across an entire agricultural field.[5]

Pesticides can be defined as chemicals that are used to attract and destroy pests. They come in many forms depending on the purpose for which they are used. However, they can be classified as acaricides (for the control of mites or ticks), algaecides (for the control of algae), antiseptics (for protecting materials from microorganisms), bactericides (for the control of bacteria), fungicides (for fungi control), herbicides (for weed control), insecticides (for insect control) and zoocides (for rodent control). The use of the term pesticides is so common that it is often considered to be a crop protection product.[6]

1.2 OVERVIEW OF THE HISTORY OF PESTICIDES

The use of pesticides is obviously not a new invention, its use can be dated back to at least 2500 B.C.[6a] During that time, the Sumerians used sulfur-containing compounds on their bodies. Due to the stench of these compounds, insects and mites were repelled. Egyptians documented over 800 formulations containing known substances that were used as poisons and pesticides, as

documented in 'The Ebers' Papyrus' around 1550 B.C. This is known to be the oldest medical document in the world. The Greeks on the other hand, at about 1000 B.C, fumigated their surroundings by burning sulfur to control pests. At that time, the Chinese were already using mercury- and arsenic-based preparations to control body lice and developed a biological system by using predatory ants to protect citrus groves against boring beetles and caterpillars. Considered as the father of modern botany, Theophrastus, was the first to record various pest damages to plants. He used olive oil as a herbicide by pouring it on the roots of plants. He also observed that certain weeds were associated with specific crops.[6a]

Pests have existed for centuries but attempts to control them were not intensive until the past three centuries. They were viewed by dwellers as part of nature. This attitude changed and people started to develop methods and tools for controlling pests. During the period 1750 to about 1880, Europe experienced a total revolution in agricultural crop protection that became wide spread and due to the importance placed on agriculture then, resulted in the discovery of the natural insecticides pyrethrum and derris. During the past two centuries, scientists have developed a better understanding of pests. The USA, for example, used diluted sulfuric acid, iron sulfate, copper nitrate and sodium arsenate to control broad leaf weeds in cereal crops in the 19th century.

"Paris green" the common name for cupric acetoarsenite, was used from 1865 to the 1940's to control Colorado potato beetle, a chewing pest of cotton and many other crops.[7] It was later found that this chemical also posed problems to other useful living organisms. A Swiss chemist, Paul Muller, developed a compound called DDT (dichlorodiphenyltrichloroethane), which won him the Nobel Prize in 1948 for the effectiveness of DDT in controlling insect-vectored human diseases.[7a] This chemical replaced Paris green and was used extensively in the USA. This chlorinated compound later posed environmental problems and affected birds, fishes and honey bees and its use was discontinued.[8]

Presently, they are many pesticides with about 900 structurally diverse compounds.[9] These compounds act by numerous mechanisms to control pests. A major problem associated with these synthetic chemicals, apart from not being friendly to the environment, is the resistance developed by pests to the pesticides. Resistance of pests towards pesticides has increased exponentially since the 1940's and presently there are about 500 species classified as pests that show some degree of resistance to at least one type of pesticides.[10] In view of the fact that various pests are now developing resistance to pesticides, an alternative method of developing new tools for controlling pests and at the same time causing less harm to the environment should be resorted to. Plant secondary metabolites have been identified as an alternative for synthetic pesticides with less resistance by pests and an increased eco-friendliness. These metabolites have novel target points of action, hence the probability of pests developing resistance is very low.[1]

1.3 ENVIRONMENTAL EFFECTS OF SYNTHETIC PESTICIDES

The vast array of organic compounds used as pesticides causes different effects, some of these effects can be noticed on aquatic life, on soil environment, air quality and on human health.

1.3.1 Effects of synthetic pesticides on aquatic life

Pesticides are mostly used as liquids that are sprayed on the crops and soil. Occasionally they are inculcated or injected into the soil or applied as granules or as seed treatments.[11] These pesticides are always in contact with the environment. In the USA alone, there are about 500 000 tonnes (1 billion pounds) of pesticides in use to manage weeds and other pests in many different agricultural and non-agricultural applications.[12] Notable among them are imidacloprid (**1.1**, insecticide), dimethoate (**1.2**, insecticide and acariside), cynoxanil (**1.3**, fungicide), isoproturon (**1.4**, fungicide), carbendazim (**1.5**, fungicide), phosmet (**1.6**, insecticide) carbofuran (**1.7**, insecticide), diuron (**1.8**, herbicide), methidathion (**1.9**, insecticide), linuron (**1.10**, herbicide), methiocarb (**1.11**, insecticide, acariside and molluscusides), chlorpyrifos (**1.12**, insecticide and miticide), atrazine (**1.13** herbicide), tebuconazole (**1.14**, fungicide) (Fig. 1.1).

When these pesticides are applied to agricultural lands, they remain on the surface of the crops or land for some time. When it rains during this period, the running water from the rains carries these chemicals from their point of application to streams and rivers. The residues of these synthetic chemicals remain on the surface of the water bodies affecting their quality and living organisms. The chemical explanation behind these residues on the surface of water bodies after run off, is that the majority of the pesticides are hydrophobic and have hydrolysis half-life times in excess of 1 day. An example is atrazine, a herbicide which is widely used in the USA. It has a hydrolysis half-life of 244-309 days in distilled water at 25 ºC but the half-life time is pH dependent and is 1.73 days in 2% humic acid suspensions at 25 °C (pH 4).[13]

Concerns over the prospective combined effects of co-occurring pesticides in aquatic systems resulted in several investigations on pesticide mixtures during the past 30 years. Evaluations of streams throughout Europe, USA and Africa showed that pesticides were detected and most often in mixtures.[14] Important natural assets enjoyed by millions of people all over the world usually get contaminated due to run off. Fish and other aquatic animals are greatly affected, sometimes leading to the death of thousands of them.[15] In some exceptional cases, human beings who live along streams contaminated by these synthetic chemicals are affected health wise when they unknowingly drink the water or consume fish from these streams.

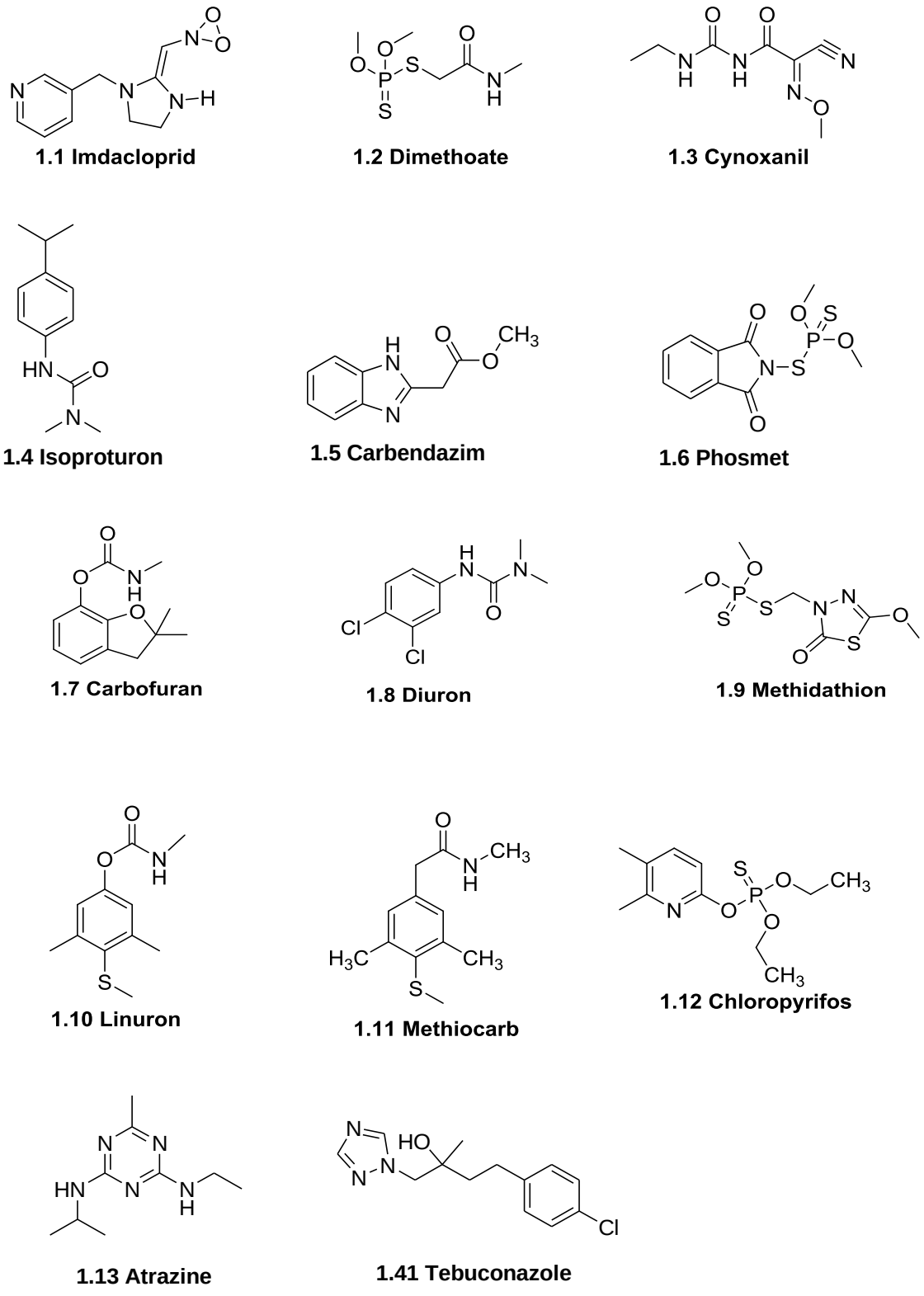


Figure 1.1. Structures of pesticides.

1.3.2 Effects of the use of synthetic pesticides on soil

The use of synthetic pesticides cause soil pollution by introducing xenobiotic chemicals into the natural soil environment. Micro-organisms are an important part of the soil ecosystem as they degrade soil organic matter and sustain biological transformation of elements.[16] Plants depend on millions of these micro-organisms for their survival as they help convey food nutrients to their rootlets. Application of synthetic pesticides break the linkage between these micro-organisms and the plants, causing or reducing the fertility of the soil to the plants. There is also bioaccumulation of these synthetic pesticides over time (especially those that contain heavy metals) on the surface of the soil leading to blockages of micro pores in the soil, since most of them are non-biodegradable. This also create problems of non-fertility of the soil. Also, the intensive use of synthetic chemicals on soil in some areas has led to a reduction in the concentration of phosphorus and the effects are normally shown by the fragile development of plants.[17]

1.3.3 Effects of the application of synthetic pesticides on air

Air quality is a measure of the amount of pollutants in the atmosphere which includes indoors and outdoors air. Synthetic pesticides used in agricultural and in urban settings have the potential to adulterate the air quality affecting human and animal health. The active ingredients of some of these pesticides stay in the atmosphere for only a short period of time, whereas others can last longer. Synthetic pesticides released for action against pests, dissipate into the surrounding, contributing to air pollution. This is normally facilitated by wind which drifts them to new places, adulterating the air quality of those areas.[18]

Most of the pesticides used are perceived to be non-toxic, which is often the most important reason why they are used widely. However, their stability allows them to rise to the stratosphere. Once there, they are decomposed by the intense ultraviolet light, releasing chlorine and bromine radicals from those that contain these elements as part of their chemical constituents, and these radicals then destroy the ozone. The rate at which this destruction is taking place is alarming. A single chlorine radical can destroy thousands of ozone molecules.[19]

1.3.4 Effects of the application of synthetic pesticides on human health

Synthetic pesticides are associated with several human health problems, ranging from short term effects like skin and eye irritation, headaches and nausea, to more serious conditions such as cancer, reproductive health problems and endocrine disruption. More serious effects such as systemic poisoning may occasionally be fatal.[20] Even minimal exposure to pesticides in the environment, food or water can lead to long-term chronic health effects. Research have revealed

that the vulnerability of a foetus to environmental pollution is related to the developmental stage of the foetus.[21] In humans, organogenesis, which normally occurs from the 3rd to the 8th week of pregnancy, is the most dangerous period for teratogenic effects by the pesticides on the foetus[22].

Cancer has also been identified as one of the several conditions caused by exposure to synthetic pesticides. The various types identified include liver cancer, prostate cancer, testicular cancer, breast cancer brain and bone cancer.[23]

Human occupational exposure is expected during production and application of pesticides, but people can also come in contact with pesticides through drift contaminations of water and food supplies. The use of synthetic pesticides to obliterate nuisance pests in the house is a vital source of exposure for many people on an indoors level.[24] The concept of pesticides being hazardous to human health is often not fully appreciated.[25]

1.4 NATURAL PRODUCTS AS HERBICIDES

From an industrial perspective, herbicides is one of the most important groups of pesticides. Weed management has been problematic since the start of agriculture. Unmanaged weeds can cause greater reduction in crop yields than the presence of any other agricultural pest. Synthetic herbicides have been relied on heavily to solve the weed problem. Due to environmental effects associated with the use of synthetic herbicides, natural products are being investigated to relegate these effects. Natural compounds with herbicidal properties are most often secondary metabolites derived from different metabolic pathways. It is known that natural products have a higher likelihood to possess some biological activity against other organisms compared to randomly selected synthetic compounds and that natural products often have novel mechanisms of action.[26] Several natural compounds discovered from plants and microorganisms have been found to inhibit the growth and reproduction of weeds, some of them have shown higher efficacy in managing weeds under field conditions than synthetic compounds.[1] Natural products-based herbicides may also have the benefit of a short environmental half-life time which makes them eco-friendly.

1.5 AIM OF THE THESIS

The concept of pesticides in a broader sense consists of all the chemicals used to control harmful organisms, including weeds. However, this is too a wide an area to investigate for a MSc project and, therefore, this project was limited to herbicides.

The aim of this investigation was to investigate the potential of a selection of South African medicinal plants as herbicides.

The specific objectives were to

- Collect a number of medicinal plants
- Dry, ground and extract these plants
- Develop a screening method to assay herbicidal activity of plant extracts and isolated compounds
- Screen extracts for herbicidal activity
- Isolate and characterize pure compounds responsible for the herbicidal activity in a selected extract (*Artemisia afra*)

This study may provide the peasant farmer with an alternative method of controlling weeds. The study may also help to eliminate the dangers of toxic synthetic chemicals that could poison human and also affect other biologically important microorganisms. However, the study does not include mode of action of isolated compounds on plants or seed germination or bioaccumulation of compound in seeds or seedlings.

The outline of the thesis is as follows: Chapter Two consists of a literature review on herbicides, Chapter Three describes the preparation of plant extracts and the evaluation of the phytotoxic effects of the crude extracts, the isolation and structural elucidation of active metabolites from the most active plant, *Artemisia afra*, is described in Chapter Four and Chapter Five is the concluding chapter.

CHAPTER 2

HERBICIDES - A REVIEW

2.1 INTRODUCTION

Losses of agricultural products due to unmanaged weed competition have been known since man gave up on hunting and embraced farming. The culture of adopting a piece of land for the cultivation of a particular food crop has been practiced since ancient times. Man has selected many species as food crops from thousands of species of plants in nature and depends on them for survival. However, monoculture as practiced by man is essentially making competition intense as evolution demands, for nutrients, water and light. In view of the above situation, inputs have to be made to eradicate weeds in order to enable crops to grow well.

During the 19th century, in agricultural systems crop rotation and human labour by way of hoeing were the predominant modes of eliminating weeds. There are several reasons why weeds have to be eliminated, apart from the competition posed to crops. Firstly, weeds may affect the speed and efficacy of harvest, secondly, weed seeds may be poisonous and can affect the health of humans and animals. Furthermore, weed seeds may also contaminate food crops such as oats affecting their market value and quality and finally, weeds act as reservoirs for plant pathogens.[27] However, with the loss of labour due to urbanization and the intense increased in farming, it became difficult to control weeds. The search for controlling weeds began with chemicals that could be used selectively.

Herbicides, also known as weed killers, are chemicals used to kill or inhibit unwanted plants. They are generally classified into two types, known as selective and non-selective herbicides. Selective herbicides are designed to kill a specific plant species without harming other desirable plants, whereas non-selective herbicides destroy all plants.[28] Although the use of herbicides have often been regarded as an agricultural practice, their use can also be extended to industrial sites, parks, gardens, lawns and railway areas to clear unwanted plants.

Herbicides accounts to more than 40% of the total global pesticide sales. In 2015 alone, the global herbicide market sales were worth US$ 25475 million.[29] The most commonly used herbicides include 2,4-D **(2.1)**, atrazine **(2.2)**, glyphosate **(2.3)**, glufosinate **(2.4)**, paraquat **(2.5)**, pendimathelin **(2.6)**, decamba **(2.7)**, fluroxypyr **(2.8)** and metolachlor **(2.9)** (Fig. 2.1). Glyphosate is the most used among the herbicides and it is the driving force in the herbicide market. Its use is projected to reach 1.35 million metric tons by 2017.[30] There are currently public outcries on

the health effects of glyphosate which may affect the market sale of this herbicide. However, natural product metabolites are offering promising alternatives to these synthetic herbicides.

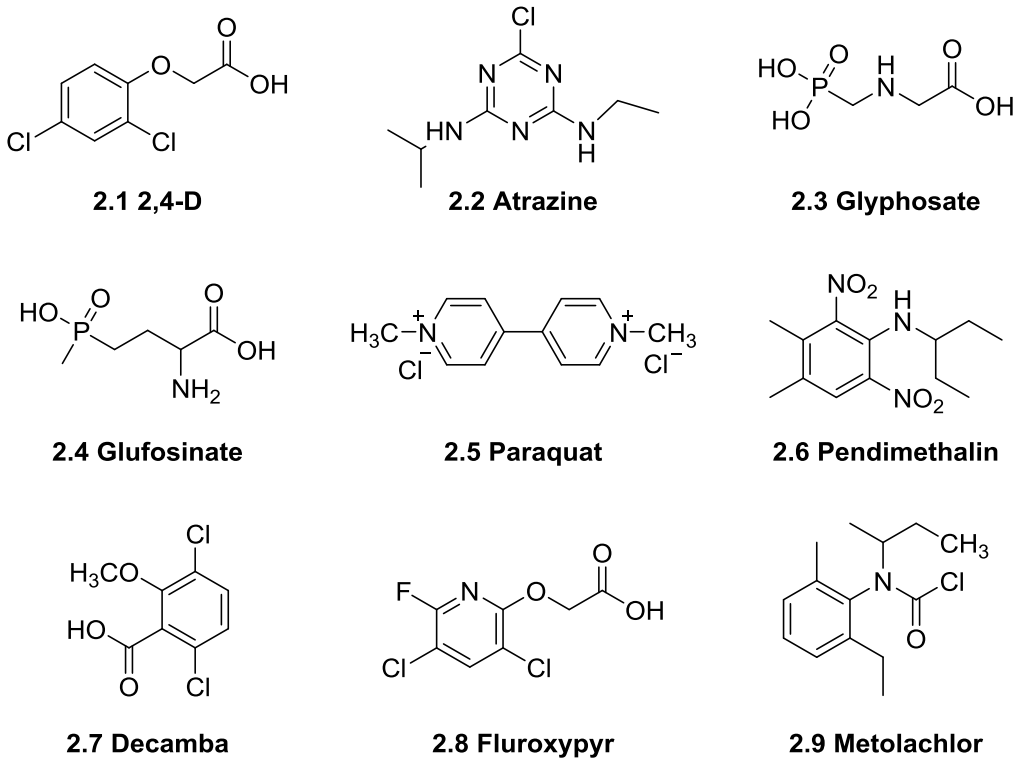


Figure 2.1. Structures of current commercial herbicides.

2.2 MODE OF ACTION OF SOME HERBICIDES

The mode of action of a herbicides is the manner in which it controls unwanted plants. It usually describes the biological processes or enzymes in the plant which are affected. There are a number of different modes of action exhibited by commercial herbicides. Based on the mode of action, commercial herbicides can be classified as follows:[31]

- Disruptors of plant cell growth (Synthetic auxins)
- Inhibitors of photosynthesis at photosystem II (PS II inhibitors)
- Inhibitors of 5-enolpyruvyl shikimate-3 phosphate (EPSP) synthase
- Inhibitors of acetyl co-enzyme A carboxylase (Inhibitors of fat synthesis/ACCase inhibitors)
- Inhibitors of acetolactate synthase (ALS inhibitors), acetohydroxyacid synthase (AHAS)
- Inhibitors of microtubule assembly
- Inhibitors of mitosis / microtubule polymerisation
- Inhibitors of carotenoid biosynthesis at the phytoene desaturase step (PDS inhibitors)

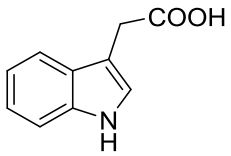
- Inhibitors of protoporphyrinogen oxidase (PPOs)
- Inhibitors of 4-hydroxyphenyl-pyruvate dioxygenase (HPPDs)
- Inhibitors of lipid synthesis (Not ACCase inhibitors)
- Inhibitors of cell division / Inhibitors of very long chain fatty acids (VLCFA inhibitors)
- Inhibitors of photosynthesis at photosystem I via electron diversion (PSI inhibitors)
- Inhibitors of glutamine synthetase
- Inhibitors of cell wall (cellulose) synthesis
- Inhibitors of auxin transport
- Inhibitors of dihydropteroate synthase (DHP inhibitors)

The most important herbicides belong to the first three classes listed above and will be discussed in more detail.

2.3 DISRUPTORS OF PLANT CELL GROWTH (SYNTHETIC AUXINS)

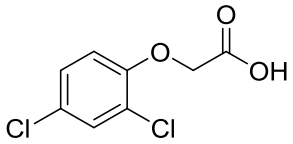
Auxins are a vital class of phytohormones. Indole-3-acetic acid (IAA) **(2.10)** is the main natural auxin in higher plants.[32] All stages of plant development are being influenced by IAA and it is generally called the master hormone and has complex interactions with other phytohormones during the plant development processes.[33] Auxins normally influence cell division and plant elongation. Some processes such as vascular tissue and floral meristem differentiation, leaf initiation, phyllotaxy, senescence, apical dominance and root formation are influenced by auxins. Auxins are also significant plant chemicals in tropical responses.[34] At lower concentrations of auxin (IAA) at cellular sites of action, plants respond by promoting growth and developmental processes but at higher concentrations and auxin activity growth is disturbed and the plant dies.

Synthetic auxins are produced not only to boost production, but also as herbicides to manage weeds. Some of the auxin-based herbicides include phenoxycarboxylic acids such as 2,4-D **(2.1)**, *m*-chlorophenoxyacetic acid (MCPA) **(2.11)** and dichlorprop-P **(2.12)**, benzoic acid, pyridinecarboxylic acids, aromatic carboxymethyl derivatives such as benazolin **(2.13)** and 1-naphthaleneacetic acid (NAA) **(2.14)**, and the quinolinecarboxylic acids quinclorac **(2.15)** and quinmerac **(2.16)** (Fig. 2.2). For weed control, apart from the concentration of these synthetic auxin herbicides, the sensitivity of the tissues which is determined by the tissue types are taken into consideration, as well as physiological stage and plant species coupled with signal transduction pathways.[34]

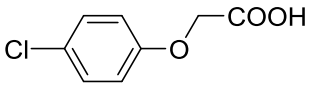


2.10 Indol-3-acetic acid (IAA)

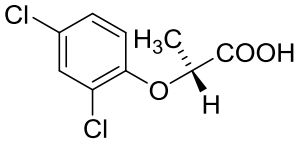
Phenoxy carboxylic acids:



2.1 2,4-D

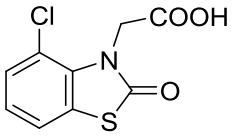


2.11 MCPA

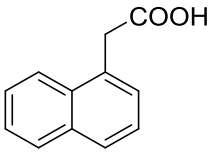


2.12 Dichlorprop-P

Aromatic carboxymethyl derivatives:

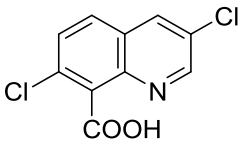


2.13 Benazolin

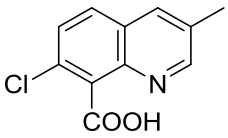


2.14 1-Naphthaleneacetic acid (1-NNA)

Quinoline carboxylic acids:



2.15 Quinclorac



2.16 Quinmerac

Figure 2.2. Structures of natural (IAA) and synthetic auxin-based herbicides.

When these auxin-based herbicides are applied, they copy the destructive and growth inhibiting pattern of IAA at a higher concentration in the active site.[35] The destructive symptoms are the same as in transgenic IAA-overproducing plants.[36] This leads to an imbalance in auxin homeostasis and interactions with other hormones in the tissue. As reported by Gilbert in 1946, "auxin herbicides cause susceptible plants to grow themselves to death".[37] His statement prevailed until research proved that there are growth abnormalities indeed and further destruction. The mechanism often considered at this point was that a continued stimulation of plant metabolism was seen as what evokes a deregulation of growth through twisted cell division and expansion, collapsing the plant growth structure.[38] Although a detailed mechanism is not well

understood, a more specific mode of action attributing to plants growth inhibition and death was considered as a result of the high level of species selectivity of some auxin herbicides coupled with their rapid action at low application rates and the fact that with some chiral compounds, only one enantiomer displays herbicidal activity (Fig. 2.3).[39]

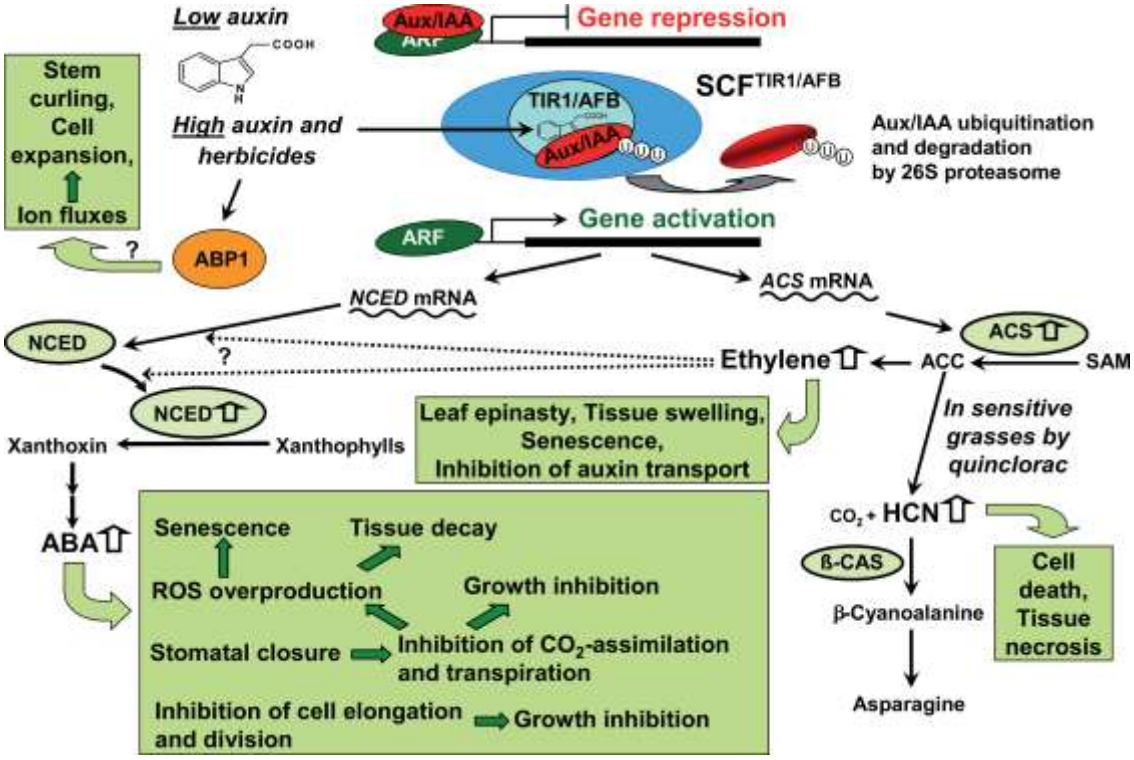


Figure 2.3. The proposed mechanism and mode of action of auxin herbicide and phytohormones indole-3-acetic acid (IAA) at supraoptimal endogenous concentrations in dicots plant species. (Source: Auxin herbicides: current status of mechanism and mode of action; Klaus Grossmann, www.interscience.wiley.com) DOI 10.1002/ps.1860).

2.4 INHIBITORS OF PHOTOSYNTHESIS AT PHOTOSYSTEM II (PS II INHIBITORS)

Photosynthesis is a process by which plants and some other living organisms convert light energy into chemical energy that can later be released in the form of ATP for other activities.[40] The general equation is given as:

$$6CO_2 + 6H_2O \longrightarrow C_6H_{12}O_6 + 6O_2$$

In the process H_2O is oxidized within the thylakoid membrane to protons, electrons and oxygen. The movement of protons and electrons through the thylakoid generates ATP and it used to reduce $NADP^+$. This energy is also used in the enzymatic reduction of CO_2 to carbohydrates within the

chloroplast stroma. Knowledge of this process has led scientist to develop herbicides which attack plants by inhibiting photosynthesis. An example of the class of herbicides that inhibits photosynthesis is the triazines, e.g. atrazine (**2.2**) (Fig. 2.4).[41]

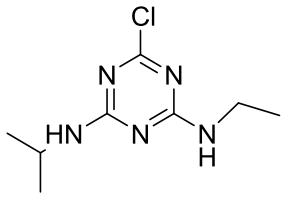


Figure 2.4. Structure of atrazine (**2.2**).

This herbicide is a photosystem II inhibitor chemical. It belongs to the triazine class of herbicides and functions by binding to the Q_B-niche on D1 protein of the photosystem II complex in chloroplast thylakoids membranes. When this happens, the flow of electrons from the primary acceptor (quinine Q_A) to Q_B is blocked and CO_2 fixation and production of ATP and $NADPH_2$ are stopped. These processes are needed for plant growth and survival, hence plant death occurs by other processes in most cases. Triple state chlorophyll which interacts with ground state O_2 to form singlet O_2 is promoted due to the inability to re-oxidize Q_A. Lipids and proteins are oxidized in the process resulting in loss of chlorophyll and carotenoids leaking in the membranes which allow cells and cell organelles to dry and disintegrate rapidly.[42]

2.5 INHIBITORS OF 5-ENOLPYRUVYLSHIKIMATE-3 PHOSPHATE (EPSP) SYNTHASE

In the early 1970's, herbicides that specifically inhibit amino acid biosynthesis were developed and made available on the market. Development of this class of herbicides became apparent due to prior knowledge of the shikimate pathway which results in the biosynthesis of aromatic amino acids and which is common to plants and some microorganisms. This class of herbicides control weed species at very low user rates and is less toxic to mammals due to the absence of the shikimate pathway in mammals.[43] An example of a compound belonging to this class of herbicides is glyphosate.

2.5.1 An overview of the shikimate pathway

In certain living systems aromatic compounds are formed from carbohydrates by a pathway known as the shikimate pathway, which consists of a seven-step metabolic process as shown in Fig. 2.5.[44] Phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate, the precursor of the aromatic amino acids and many aromatic secondary metabolites. Pathway

intermediates may also serve as substrates for other metabolic pathways. Due to the importance of this pathway, it has been a target for the design of drugs and herbicides. The pathway is important in plants, microorganisms and parasites such as the malaria parasite, but it is absent in mammals.[45] This pathway is also found in microbes that live within animals in the gut microbiome.[46]

The diagram below shows the seven steps in the shikimate pathway.

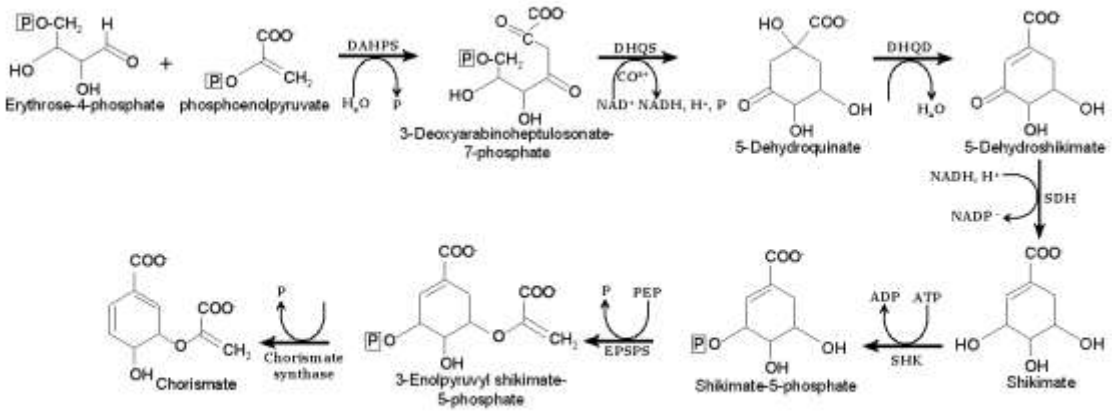


Figure 2.5. The seven steps of the shikimate pathway. DAHPS (3-deoxy-D-arabino-heptulosonate-7-phosphate synthase), DHQS (dehydroquinate synthase), ADP (adenosine diphosphate), ATP (adenosine triphosphate), PEP (phosphoenol pyruvic acid), $NADP^+$ (nicotinamide adenine dinucleotide phosphate), EPSPS (5-enolpyruvylshikimate-3-phosphate synthase).

2.5.2 Glyphosate (*N*-(phosphonomethyl) glycine) (2.3)

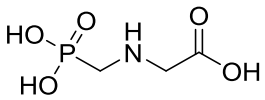


Figure 2.6. Structure of glyphosate (**2.3).**

Glyphosate (**2.3**) (Fig. 2.6) is a broad-spectrum, non-selective systemic herbicide. This chemical was first synthesized and tested as herbicide by John E Franz of the company Monsanto in 1970[47] and was soon endorsed for use as a herbicide.[48] This organophosphorus compound is used to kill weeds and grasses that compete with crops. The efficacy of this herbicide was quickly noticed after its introduction and farmers quickly adopted it as a weed control tool with the trade mark name Roundup®. In 2007 for instance, glyphosate was the most-used herbicide in farms and the

second most-used in the gardens and homes in the United States.[49] The development of genetically-modified crops such as cotton and soya which are resistant to glyphosate and enabled farmers to kill weeds without affecting the crops, has largely expanded the market for this herbicide.

During the application of glyphosate, mostly post-emergence, the molecules are normally absorbed through the leaves[50] and transferred to sites that inhibit plant growth.[51] The major role of this herbicide in plants is to inhibit the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), a key enzyme in the shikimate pathway, which is involved in the biosynthesis of amino acids such as tyrosine, tryptophan and phenylalanine.

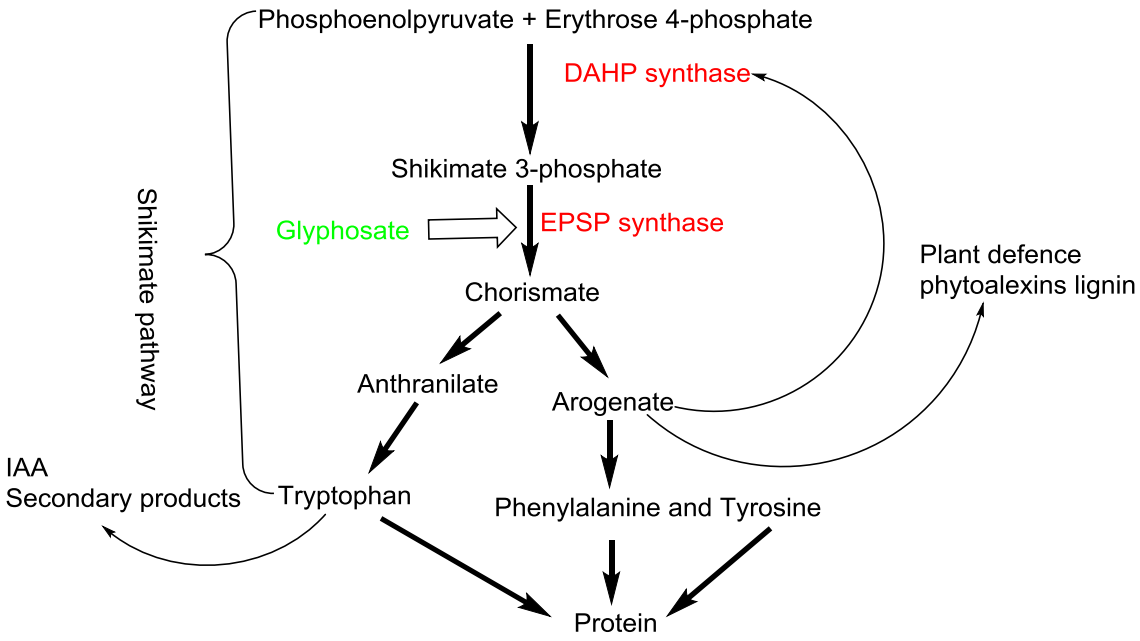
2.5.3 Mode of action of glyphosate

Glyphosate is taken up through the leave surfaces,[52] which normally varies from plant to plant. The mode of glyphosate makes it easy to translocate from leaves to the tissues that are metabolic reservoirs for sugars.[53] This then makes it easy for phytotoxic activities of glyphosate to reach meristems, young roots and leaves and other growing parts of the plant. Glyphosate can however limit its own translocation.[54]

The mode of action of glyphosate is unique[55] because it is highly effective at inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS). When glyphosate is applied, it acts as a substrate for EPSPS leading to the inhibition of this enzyme. When this happens, feedback in the pathway is also inhibited which then causes more carbon flow to shikimate-3-phosphate, converting it into high levels of shikimate.[56]

However, the lack of knowledge of the mechanism how glyphosate kills the plant steered researchers to propose several reasons and postulates for the death of the plant. One of the assumptions by researchers is that the mortality of plants is a result of the accumulation of shikimic acid in plant tissues thus inhibiting the enzymatic conversion of shikimic acid to anthranilic acid in cells.[57]

Another postulate is thus, deregulation of the pathway B inhibition of EPSPS and thus increasing carbon flow to the pathway cause shortage of carbon for other essential pathways.[53] Glyphosate is often regarded as a non-selective herbicide[58] due to the fact that it is sensitive to EPSPS which is present in all higher plants[55] and is active on a wide range of plant species. The diagram below (Fig. 2.7) shows the shikimate pathway and the site of inhibition by glyphosate.



Source: http://onlinelibrary.wiley.com/doi/10.1002/ps.1518/full [59]

Figure 2.7. Schematic representation of mode of action of glyphosate.

2.5.4 Toxicology of glyphosate

Glyphosate was produced to control many of plant species and received the approval of the European Union in 2002. It is considered by many as the least toxic pesticide.[47,56,60] It is less acutely toxic than many common chemicals with an acute oral LD_{50} for rats of greater than 4320 mg/kg.[61] Some reviews concluded that when glyphosate is used according to instructions, it should not pose a problem to human health.[62]

Another argument by researchers who explained the low toxicity and non-carcinogenicity of glyphosate is the fact that it functions by inhibiting an enzyme which is essential to plant growth but is not found in animals or humans. Therefore, it has a low risk to human health. Many regulatory agencies in the world such as Canada's pest management regulation agency (PMRA), United States EPA and European Food Safety Authority have conclude that glyphosate does not pose a cancer risk when used according to regulations and label directions. On April 11, 2015, the USA EPA declared that based on their criteria for assessing carcinogenicity, it affirmed its original stands in 1991 that glyphosate should be classify as group E (evidence of non-carcinogenicity for humans) based on a lack of convincing evidence of carcinogenicity.[63] The German Federal Institute for Risk Assessment (BFR), which carried out the health risk assessment of glyphosate on behalf of the European Community concluded and announced that in March, 2015 that glyphosate is non-carcinogenic.[64] It is considered to be biodegradable over time by soil microorganisms.

However, many people still believe that glyphosate is not without health hazards and even stressed that it is "probably carcinogenic to human". France, Sweden and The Netherland have all announced that they will not support an assessment by the European Food Safety Authority (EFSA) that glyphosate is harmless. In 2013, an independent laboratory in Germany studied the effects of glyphosate on people from 18 countries and the results showed that 44% of the participants had traces of the glyphosate in their urine.[65] The International Agency for Research on Cancer (IARC) of the WHO linked glyphosate to tumours in rats and mice and also classified as "Mechanistic" such as DNA damage to human cell from exposure to glyphosate.[66] The Swedish environment minister, said "We won't take risk with glyphosate and we don't think that the analysis done so far is good enough, we would propose that no decision is taken until further analysis has been done and the EFSA scientists have been more transparent about their considerations".[67]

Although the totality of evidence is not enough to call for the ban on glyphosate, some cities such as Chicago and Paris do not use glyphosate in their public spaces. The city of Vancouver has gone further to ban the public and private use of synthetic herbicides including glyphosate against invasive weeds such as Japanese knotweed.

2.5.5 Environmental fate of glyphosate

A number of processes occurs when glyphosate is introduced into the soil, such as the formation of complexes, uptake and metabolism by plants and biodegradation by microorganisms. The known metabolite after break down by microorganisms is aminomethylphosphonic acid (AMPA) and CO_2 is released in the process. Glyphosate is found to have a half-life ranging from 3 days to 14 days. Further investigations revealed that the major product after degradation of glyphosate by microorganisms (AMPA) even stays longer than the chemical glyphosate itself, with a half-life of 119 to 958 days.[62, 68] It has been reported by some studies that glyphosate persistence in water is also longer.[68] The manufacturer of glyphosate claims it is immobile in soil,[69] but further studies which even placed the mechanism of sorption to soil as a complex process which is not well understood, proved otherwise. However, the sorption process to metal complexes with humic acids in the soil is considered as the main binding mechanism.[62] Other research have proved that glyphosate can easily be desorbed from the soil particles in some soil types and can move in the soil environment.[70]

The persistent use of glyphosate resulted in the development of glyphosate-resistant weeds covering an estimated 120 million hectares globally in 2010. It is estimated that 23 persistent weeds species have been identified forcing the manufacturer to acknowledge the problem and

declaring that their warranty does not cover yield losses.[71] The development of resistant weeds is a threat to the glyphosate market.

2.6 ALLELOPATHIC AGENTS

Allelopathy is a biological process by which an organism produce one or more biochemicals that influence the germination, growth, survival and reproduction of other organisms.[71a] The chemicals released are called allelochemicals. The concept was first introduced by Molisch in 1937.[72] The definition is broad but appropriate because considerable research has covered microorganisms and lower plants in the production of active phytochemicals.[73] In other definitions, allelopathy can be seen as an important mechanism of plant interference mediated by the addition of plant-produced secondary products to the soil rhizosphere.[74] Allelochemicals are present in many tissues and plants and are normally released through mechanisms such as decomposition, volatilization and roots exudation.[74a] These allelochemicals have different structures and mechanisms of action and many may be considered as hit compounds for the development of future herbicides.[1,75]

There are a number of plant secondary metabolites which have been developed or have the potential to be developed as commercial herbicides.[76] Three of the classes of compounds implicated in allelopathic studies include coumarins, flavonoids and terpenoids.

2.6.1 Coumarins

Coumarins are lactones of *o*-hydroxycinnamic acid.[77] Various kinds of side chains may be present with isoprenoids being common. Several of these coumarins have been isolated from plants and have proven to possess phytotoxic activities in laboratory bioassays. Most of them have been observed to inhibit germination and seedling development.[77] Umbelliferone **(2.17)** and psoralen **(2.18)** have been studied to block mitosis in *Allium cepa* while scopoletin **(2.19)** was found to inhibit germination and development of oat roots.[78] Other coumarins with phytotoxic activities include esculetin **(2.20)**, novobiocin **(2.21)** (Fig. 2.8), which were isolated from a South American shrub, *Pilocapus goudotainus*.[79]

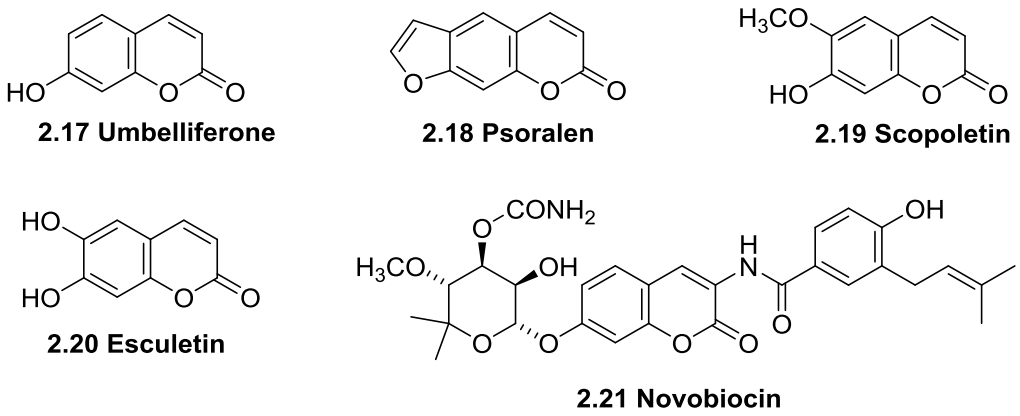


Figure 2.8. Coumarins implicated as allelochemicals.

2.6.2 Flavonoids

In some invasive plant species, flavonoids have been reported to be responsible for the allelopathic effects of those plant species, e.g. (-)-catechin **(2.22)**, found in *Centraurea maculosa*, robinetin **(2.23)** and other flavonoids isolated from *Robinia pseudoacacia*.[80] The most reported flavonoids in literature having allelopathic properties include kaempferol **(2.24)**, quercetin **(2.25)** and naringenin **(2.33)** (Fig. 2.9).[81] Flavonoids also play a vital role in plant survival.

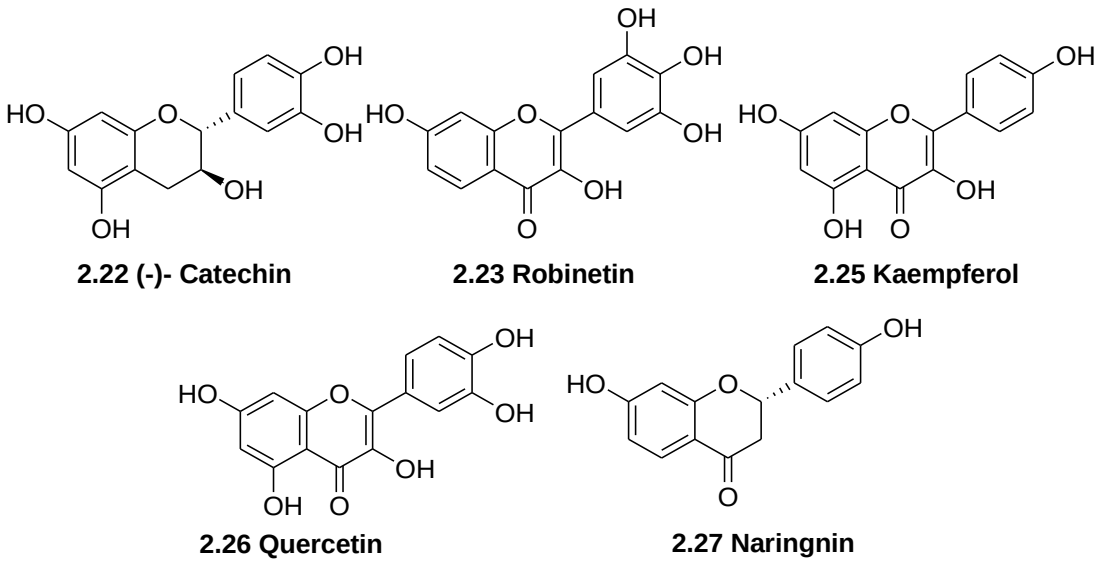
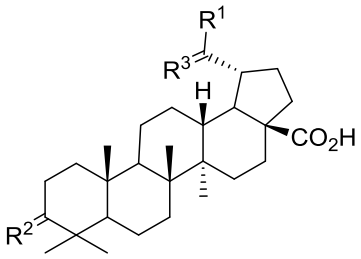


Figure 2.9. Flavonoids implicated as allelochemicals.

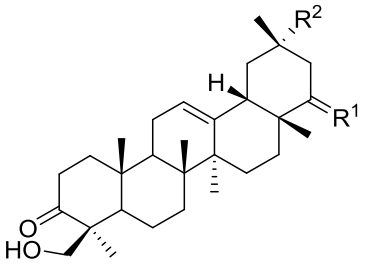
2.6.3 Terpenoids

This class is sometimes referred to as isoprenoids and consists of a diversity of natural occurring organic molecules. Terpenoids are vital for plant survival and also have some biological activities

that are beneficial to humans.[82] A group of lupane derivatives known as messagenic acid derivatives **(2.28 - 2.36)** was isolated from sweet clover in addition to oleanane triterpenes known as the mililotigenins **(2.37 – 2.39)** (Fig. 2.10).[83] These compounds were tested for allelopathic effects using both mono- and dicotyledonous seeds. Stimulation of the germination of onion and inhibition of the germination of barley were observed.



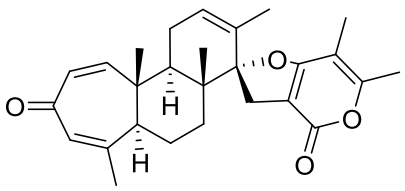
	R^1	R^2	R^3
2.28	CH_2OH	β-OH	CH_2
2.29	CHO	β-OH	CH_2
2.30	CH_2OH	O	CH_2
2.31	CH_3	β-OH	CH_2
2.32	CH_3	O	CH_2
2.33	CHO	O	CH_2
2.34	CH_3	O	O
2.35	CH_2OH	O	O
2.36	CH_2OH	β-OH, H	O



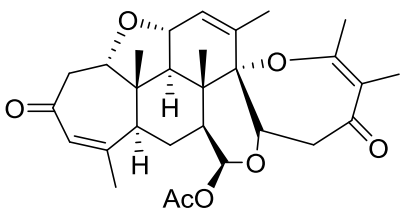
	R^1	R^2
2.37	O	CH_3
2.38	β-OH,α-H	CH_3
2.39	O	CO_2H

Figure 2.10. Triterpenes as allelochemicals.

Breviones **2.40** – **2.41** (Fig. 2.11) are diterpenoid derivatives isolated from *Penicillium brevicompactum*.[84] In the bioassay of these compounds for allelopathic effects, brevione C **(2.40)** and brevione D **(2.41)** were the most active compounds in the etiolated coleoptile elongation bioassay of wheat; they were found to inhibit the growth of the shoots by 80% and 100%, respectively.[84] Ailanthone **(2.42)** (Fig.2.12) is a quassinoid ketone isolated from *Ailanthus altissima* (tree-of-heaven). This compound was isolated as the major allelopathic phytotoxin in the plant and was shown to be a post-emergence herbicide but degrades in the field rapidly losing its effects after some days.[85]



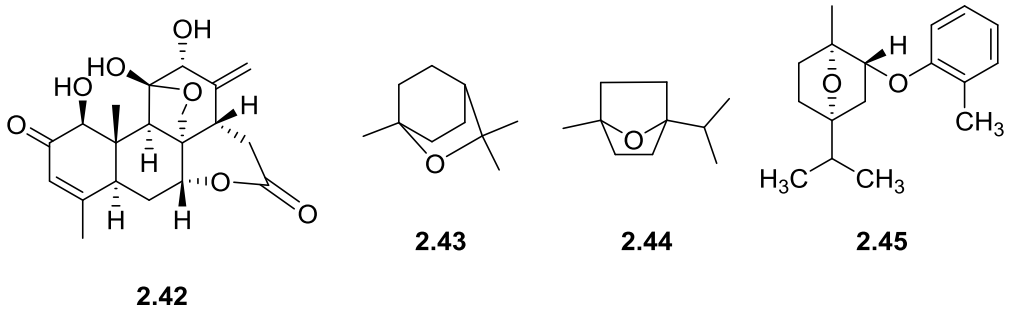
2.40



2.41

Figure 2.11. Diterpenoids implicated as allelochemicals.

The monoterpene ether 1,8-cineole **(2.43)** and its isomer 1,4-cineole **(2.44)** are the major components in many essential oil of plants and was the first compounds implicated as allelochemicals,[86] but due to the volatile nature of these compounds, their application as herbicides is limited. A benzyloxy derivative known as cinmethylin **(2.45)** was developed and was found to interrupt mitosis in plants it was applied to (Fig. 2.12).[87]

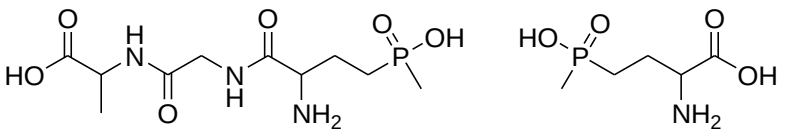


2.42 **2.43** **2.44** **2.45**

Figure 2.12. Terpenoids and monoterpenes implicated as allelochemicals.

2.7 COMMERCIAL HERBICIDES BASED ON NATURAL PRODUCTS

Although there is an interest in the application of natural products for the control of weeds in agriculture, only a few natural products have actually been developed as commercial herbicides. The most successful natural product herbicides are bialaphos **(2.46)** and glufosinate **(2.47)**,[88] which are metabolites produced by *Streptomyces* (Fig. 2.13). These compounds are available as commercial herbicides and are the only two phytotoxins produced by micro–organisms that are used in agriculture.[89] There are many reports on plants with allelopathic effects and several plant metabolites with phytotoxic activity have been identified in laboratory bioassays.[90] However, only a few of these compounds are active enough to be considered for development as herbicides. One exception is a patented corn gluten meal recently discovered as a pre-emergence herbicide.[91] The herbicidal component of this product, a dipeptide, is formed during the hydrolysis of the corn meal,[91] suggesting that micro-organisms are involved in producing the phytotoxic effects. The hydroxylate is a potent inhibitor of root system development in the germination of grass seedlings.[92]



2.46 Bialaphos **2.47 Glufosinate**

Figure 2.13. Commercial herbicides based on natural products.

Some natural product-based herbicides with their trade names are given in Table 2.1.

Table 2.1. Natural product-based herbicide formulations with their trade names.

Formulation Based	Examples
Plant essential oil-based herbicides	Weed Zap®, Bioorganic Broadleaf Killer®, EcoSmart Weed and Grass Killer®.
Orange oil (*d*-limonene)-based herbicides	Avenger Organic Weed Killer®, Worry Free Weed and Grass Killer®.
Acetic acid-based herbicides	Weed Pharm®, AllDown®, Grotek Elimaweed Weed and Grass Killer®
Fatty acid-based herbicides	Safer Moss and Algae Killer®, Safer Fast Acting Weed and Grass Killer®, Monterey Herbicidal Soap®, Natria Weed and Grass Killer®

Source: http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=6498

2.8 CONCLUSION

From this review it is clear that herbicides have become an integral part of modern agriculture. Although a large number of commercial herbicides based on synthetic chemicals are available, there are problems associated with these products such as pollution and the development of resistance in the target plant species. Herbicides developed from natural products may play a much larger role in future. However, limited research has been conducted in this area and there is a large scope for the identification of herbicidal compounds from plants and commercialization of these products.

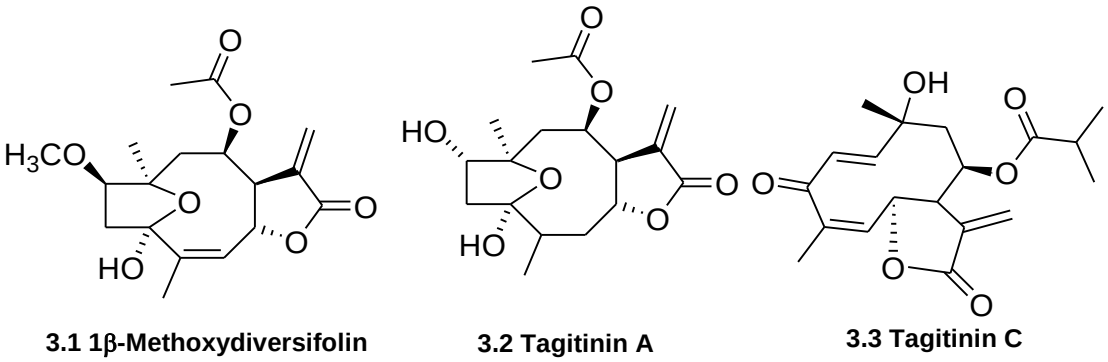
CHAPTER 3

EXTRACTION AND PHYTOTOXIC SCREENING OF SELECTED MEDICINAL PLANTS

3.1 INTRODUCTION

The introduction of synthetic herbicides have benefited farmers but also introduced new problems to the environment and crop species. Many of these herbicides are non-degradable and have long half-lives. Residues of these herbicides remain on the surface of the soil and get carried into water bodies during heavy rains polluting them in the process.[93] An over-reliance on herbicides for weed control has led to rapid evolution of herbicide-resistant weeds.[94] Research have revealed that there are also human health problems associated with the use of certain synthetic herbicides.[95]

The outcry on the effects of synthetic herbicides have triggered researchers to consider alternative methods for weed control. Some researchers are considering natural products as a source for lead compounds for the development of new herbicides, as plant secondary metabolites provide natures' biggest chemical laboratory.[1] For example, an investigation by Miranda et al. on the Mexican sunflower (*Tithonia diversifolia* (Hemsl.) A. Gray, Asteraceae) led to the isolation of phytotoxic sesquiterpene lactones.[96] The major phytotoxic components were identified as 1β-methoxydiversifolin **(3.1)**, tagitinin A **(3.2)** and tagitinin C **(3.3)** (Fig. 3.1). An important benefit of natural product-based herbicides is their relatively short environmental half-lives, which is due to the fact that few halogen substituents are present in most compounds.[97]



3.1 1β-Methoxydiversifolin **3.2 Tagitinin A** **3.3 Tagitinin C**

Figure 3.1. Sesquiterpene lactones with phytotoxic activity.

In this chapter, the investigation of six South African medicinal plants belonging to the Asteraceae and Lamiaceae families are described. The selected plants were harvested, extracted and the extracts assayed for phytotoxicity.

3.2 SELECTION AND EXTRACTION OF PLANTS

3.2.1 Selection of plants

The selection of the plants for investigation was based on ethnomedicinal usages of the plants and also on the availability of plant material. Some of these plants have been studied for various medicinal properties by researchers but little attention have been paid to them as a potential source of new herbicides. The plants selected for initial studies are collated in the Table 3.1.

Table 3.1. Selected plants and their ethnomedicinal usages.

Family and plant species	**Ethnomedicinal uses**	**Reference**
Asteraceae		
Artemisia afra Jacq. ex Willd.	• Bladder and kidney disorders • Coughs, colds, influenza, convulsions, diabetes, fever, headache, heart, inflammation • Rheumatism, stomach disorders and worms	[98]
Eriocephalus africanus L.	• Traditionally used for ailments like coughs and colds, flatulence and colic, as a diuretic and a diaphoretic • Plant decoctions are prepared and use as a hair growth stimulant and conditioner	[99]
Vernonia natalensis Sch.Bip. ex Walp.	• Leaves and roots used as charms against lightening • Used by herbalist to ensure health of mother and baby during pregnancy and child birth • Powdered bark are used to treat coughs, malaria and other feverish conditions, headache, pains in the loins and pain in the kidneys	[100]
Lamiaceae		
Leonotis leonurus (L) R.Br.	• Cold water infusions are used to relieve feverish headaches • Hot water infusions from a mixture of roots of this plant and roots of *Strychnos spinosa* Lam are taken as emetics for haemorrhoids and snake bites • Infusions of leaves and roots are taken to treat dysentery	[101]

Plectranthus fruticosus L'Hér. 'James'	• Disorders of the digestive system are treated using decoctions of this plant • Use for treating skin conditions such as skin burns • Use for treating ailments such as fever, respiratory disorders and cardiovascular disorders	[102]
Tetradenia riparia (Hochst.) Codd	• Leaf infusions are taken for coughs and sore throats • Cold water infusions of pounded leaves may be taken for chronic coughs, followed by sufficient warm water to induce vomiting • Unspecified parts are used for boils and mumps in Kenya	[103]

3.2.2 Extraction of plants

Plant parts were separated and extracted according to standard procedures as described in the experimental section (3.6.2). The masses of the extracts obtained are given in Table 3.2.

Table 3.2. Masses of dry plant material and of crude extracts.*

Plant materials	**Dry mass (g)**	**Yield of extract (DCM-MeOH) (g)**	**Yield of extract (100% methanol) (g)**
A. afra (flowers)	19.45	2.08	1.35
A. afra (leaves)	240.30	14.95	11.15
A. afra (stems)	1420	13.78	9.67
E. africanus	40.88	3.14	1.42
L. leonurus	23.82	1.06	1.01
P. fruticosus	15.45	1.16	0.28
T. riparia	250.45	11.43	3.51
V. natalensis	65.37	6.17	2.11

*For plants, apart from *A. afra*, combined aerial parts were used

3.3 SCREENING METHODOLOGY

Bioassays are defined as the evaluation of the influence of a compound on a biological system.[104] Assays for phytotoxicity must be rapid, relevant and cost effective for both natural products and synthetic chemicals. Such assays can be conducted on a whole plant or on specific plant parts in

the laboratory.[88] There is a mutual relationship between germination and seedling development and anti-germination activity on seeds by an extract or single compound which can be used as a bioassay for phytotoxic activity.[105] Parameters such as shoot and root lengths are accepted as a direct measure of phytotoxic effects.[106] Generally, phytotoxic bioassays on extracts are conducted in Petri dishes by placing a filter paper impregnated with extract in the dish and moistened the paper then with distilled water, with a control involving the same procedure but with a non-impregnated filter paper. Seeds are now introduced and the dishes sealed.[107,108] These dishes are left under controlled laboratory conditions (dark, 23 °C) for a period of 2 to 7 days after which germination, shoot and root lengths are recorded. Sometimes a number of replicates are run to obtain statistically relevant results. Bioassay of the extracts are rapid and straight forward and can provide preliminary information on plant material that contain allelochemicals.[109]

Seed of four different plants were used in this study, lettuce (*Lactuca sativa* L., Asteraceae), radish (*Raphanus sativus* L., Brassicaceae), annual ryegrass (*Lolium rididum* Gaud., Poaceae) and teff (*Eragrostic tef* (Zucc.) Trotter, Poaceae). The choice for these seeds is due to the fact that their growth properties and patterns have been studied and are well known. *L. sativa* (lettuce) was selected for most of the screening procedures due to the fact that it is very sensitive to phytotoxic compounds.[88] The target plants belong to three families, Asteraceae, Brassicaceae and Poaceae, all of which contain species classified as weeds in one or more countries. In South African some important declared weeds that are part of these families are *Campuloclinium macrocephalum* (Asteraceae, pom weed), *Rorippa nasturtium-aquaticum* (Brassicaceae, watercress) and *Cortaderia jubata* (Poaceae, pampas grass).

Another assay used in this study (Chapter 4) is the coleoptile (a sheath protecting a young shoot in monocotyledons) elongation bioassay. The procedure involves the growing of wheat in the dark for four days and then sizing the coleoptiles to 4 mm. The sized coleoptiles are exposed to the plant extract in test tubes devoid of light for 24 hours after which each is measured and compared with the control for growth inhibition. This assay was used to identify the active fraction of a plant extract. It was useful when only small amounts of material were available after serial column chromatography on the extract.[110]

3.4 RESULTS OF SCREENING EXPERIMENTS

In assessing the results of the study, the seeds that failed to germinate after 7 days were considered to be inhibited by the extract. The seeds that germinated had their root and shoot lengths measured with a ruler.

The percentage germination was calculated using the formula:

$$\text{Percentage germination} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} * 100$$

For treatments 1 (TR1), 2 (TR2) and 3 (TR3), the filter papers were impregnated with 25 mg, 50 mg and 75 mg crude extract, respectively.

The results on the effects of crude plant extracts on the germination inhibition on *L. sativa* (lettuce), *R. sativus* (radish), *E. tef* (teff) and *L. rididum* (annual ryegrass) seeds are collated in Table 3.3, 3.4, 3.5 and 3.6, respectively. Extracts of three of the plants, *Eriocephalus africanus*, *Leonotis leonurus* and *Plectranthus fruticosus*, showed almost no effect on the germination of lettuce and radish seeds and these extracts were not investigated further.

The DCM-MeOH (1:1) extract of *Vernonia natalensis* did not show much inhibition of germination in the bioassay on *L. sativa*, as treatment 1 gave 90% germination and treatment 3 showed 60%, as shown in Table 3.3. However, a greater inhibition was observed in the bioassay on *R. sativus*, with treatment 1 showing 40% germination and no germination for treatments 2 and 3, as shown in Table 3.4. With the bioassay on teff and ryegrass, some inhibition of germination was observed with treatment 3 with 20% and 0% germination, respectively, as shown in Tables 3.5 and 3.6. Pictures of the germination inhibition are shown in Fig. 3.2.

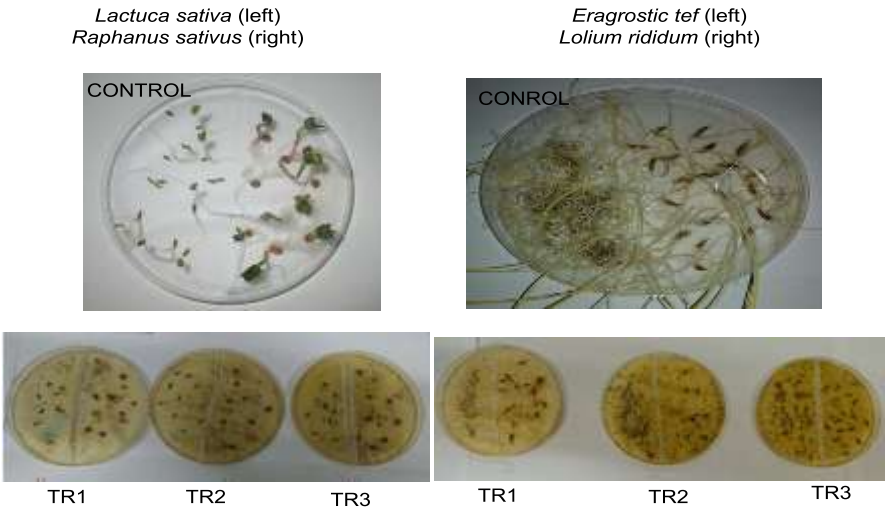


Figure 3.2. Phytotoxic bioassay of the crude DCM-MeOH extract of *V. natalensis*. Each Petri dish was divided into two parts to accommodate two different types of seed. The left hand set of dishes contain lettuce (*L. sativa*) (left half of dish) and radish (*R. sativus*) (right half of dish), whereas the right hand set of dishes contain teff (*E. tef*) (left half of dish) and ryegrass (*L. rididum*) (right half of dish).

The DCM-MeOH (1:1) extract of *Tetradenia riparia* showed minimal germination inhibition in the bioassay on *L. sativa* (Fig. 3.3). For treatment 1, a 100% germination relative to the control was recorded and for treatment 3, 60% germination was recorded, as shown in Table 3.3. However, it showed greater inhibition in *R. sativus* with treatment 1 giving 50% germination, treatment 2 10% and 0% germination for treatment 3, as shown in Table 3.4. The bioassay on teff and ryegrass seeds, on the other hand, showed some level of inhibition with treatment 1 recording 56% and 85% germination, respectively. It substantially inhibited germination in treatment 3 with teff and ryegrass seeds, recording 28% and 45% germinations, respectively, as shown in Table 3.5 and 3.6.

The MeOH extract, on the other hand, did not show significant inhibition of germination of *L. sativa* seeds with 90% germination in treatment 1 and 50% in treatment 3, as shown in Table 3.3. However, this extract inhibited germination of *R. sativus* substantially and 50%, 40% and 20% germination for treatments 1, 2 and 3, respectively, were observed as shown in Table 3.4. The assay on teff and ryegrass seeds revealed an opposite trend, though there was inhibition, the percentage germination was higher in treatment 3 with 70% and 65%, respectively. Treatment 2 on the ryegrass assay showed the highest inhibition of 20% germination, as shown in Table 3.5 and 3.6.

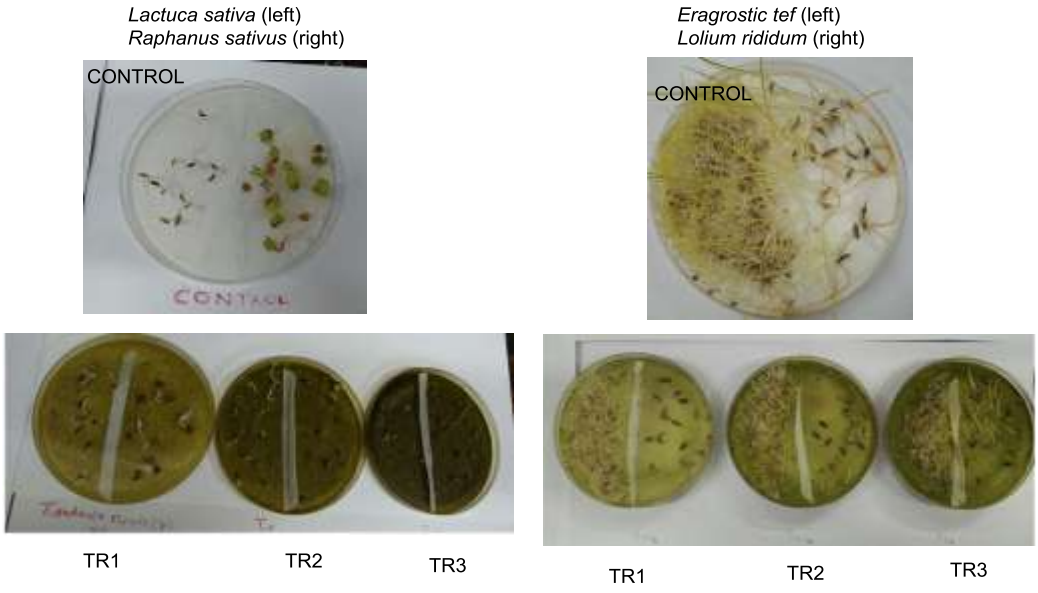


Figure 3.3. Phytotoxic bioassay of the crude MeOH extract of *T. riparia*. Each Petri dish was divided into two parts to accommodate two different types of seed. The left hand set of dishes contain *L. sativa* (left half of dish) and *R. sativus* (right half of dish), whereas the right hand set of dishes contain teff (*E. tef*) (left half of dish) and ryegrass (*L. rididum*) (right half of dish).

Among the plant extracts, the DCM-MeOH extract of *Artemisia afra* leaves showed the highest inhibition of seed germination. It inhibited both *L. sativa* and *R. sativus* seeds in treatment 1 with 50% and 40% germination, respectively, as shown in Table 3.3 and 3.4. Furthermore, no germination was observed in treatments 2 and 3, as shown in Table 3.3 and 3.4. In the screening of teff and ryegrass, the former was greatly affected with germination of 80%, 52% and 22% for treatment 1, 2 and three 3, respectively, as shown in Table 3.5, whereas 95%, 80% and 0% germination was recorded for treatments 1, 2 and 3, respectively, for ryegrass seeds as shown in Table 3.6. The crude extracts of the stems and flowers of *A. afra* also showed inhibition in the various assays. Pictures of the experiments are shown in Fig. 3.4 and 3.5.

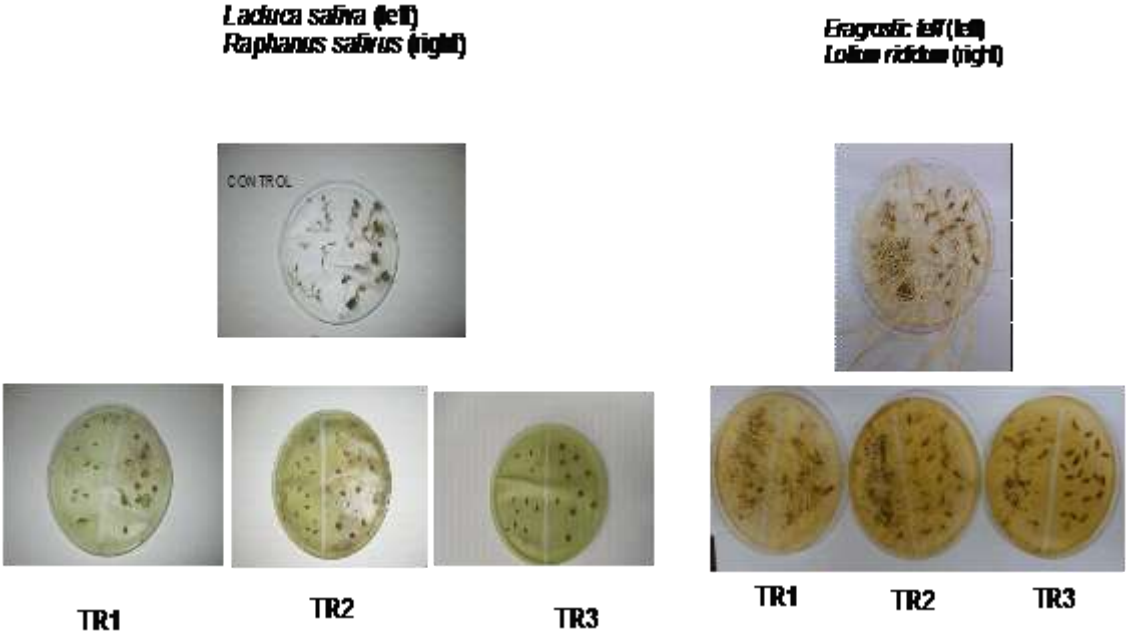


Figure 3.4. Phytotoxic bioassay of the crude DCM-MeOH extract of *A. afra* leaves. Each Petri dish was divided into two parts to accommodate two different types of seed. The left hand set of dishes contain *L. sativa* (left half of dish) and *R. sativus* (right half of dish), whereas the right hand set of dishes contain teff (*E. tef*) (left half of dish) and ryegrass (*L. rididum*) (right half of dish).

Another obvious observation made during the bioassay is that the seeds of *L. sativa* in *A. afra* treatments delay for 6 hours before germinating. A closer look at the roots of the germinated seeds after the 7 days revealed that they were stunted and had a burnt-like appearance.

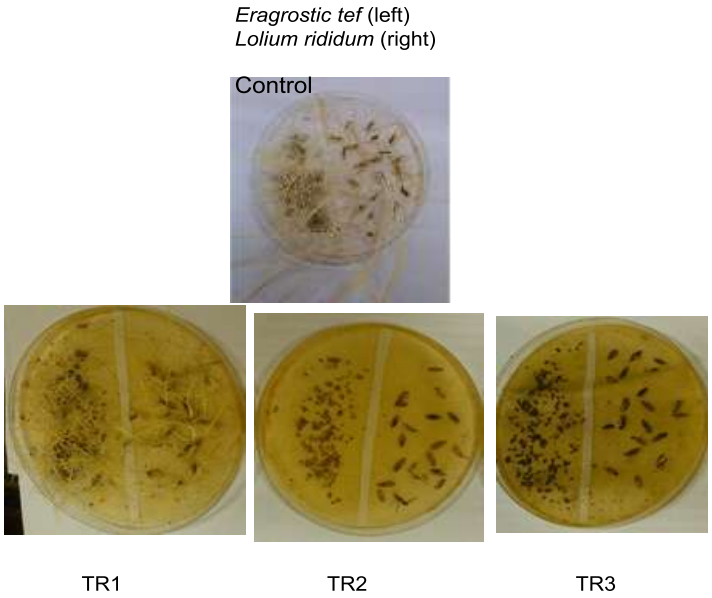


Figure 3.5. Phytotoxic bioassay of *A. afra* flowers DCM-MeOH crude extract. Each Petri dish was divided into two parts to accommodate two different types of seed, the left hand side of each dish is teff seeds while the right hand side is ryegrass seeds.

3.5 CONCLUSION

Bioassays on selected medicinal plants showed that some of the plant extracts inhibited the germination of seeds. This is an indication of the presence of phytotoxic compound(s) in the extracts of these plants and that the plants may have the potential to be developed as natural herbicides. It was also noticed that the increased in extract concentrations affected seed germination greatly which is an indicative of higher phytotoxic compound(s) present in the higher extract concentrations due to an increased in extract mass. The different seeds responded in different germination pattern which can be attributed to the fact that the seeds have different physiological make up and respond to the phytotoxic compound(s) in different ways. The most active among the plant extracts assayed was the DCM-MeOH (1:1) extract of *A. afra* leaves and this extract was selected for further investigation. The results of this investigation are described in Chapter Four of this dissertation.

Table 3.3. Effect of crude extracts of plants on the germination of *L. sativa* (lettuce) seeds.

Plant extract (mg)	**Number of seeds**	**Number of seeds germinated**	**Percentage germination**	**Root length (mm)**	**Shoot length (mm)**
Control	10	10	100	17	12
***A. afra* flowers DCM-MeOH**					
25	10	7	70	3	2
50	10	1	10	2	2
75	10	0	0	0	0
***A. afra* flowers MeOH**					
25	10	9	90	7	10
50	10	5	50	1	0
75	10	1	10	1	0
***A. afra* leaves DCM-MeOH**					
25	10	5	50	4	5
50	10	0	0	0	0
75	10	0	0	0	0
***A. afra* leaves MeOH**					
25	10	7	70	4	2
50	10	6	60	3	1.5
75	10	0	0	0	0
***A. afra* stems MeOH**					
25	10	8	80	6	10
50	10	2	20	6	11
75	10	4	40	3	9
***E. africanus* leaves DCM-MeOH**					
25	10	10	100	13	8
50	10	10	100	16	9
75	10	9	90	12	11
***L. leonorus* DCM-MeOH**					
25	10	8	80	12	14
50	10	7	70	15	10
75	10	6	60	10	9
***P. fruticosus* DCM-MeOH**					
25	10	7	70	12	13
50	10	8	80	14	11
75	10	10	100	9	12

***T. riparia* leaves MeOH**					
25	10	9	90	10	8
50	10	8	80	9	7
75	10	5	50	10	6
***V. natalensis* DCM-MeOH**					
25	10	9	90	4	10
50	10	8	80	2	7
75	10	4	40	1	7

Table 3.4. Effect of crude extracts of plants on the germination of *R. sativus* (radish) seeds.

Plant extract (mg)	Number of seeds	Number of seeds germinated	Percentage germination	Root length (mm)	Shoot length mm)
Control	10	10	100	38	19
***A. afra* flowers DCM-MeOH**					
25	10	7	70	9.6	7
50	10	1	10	3	1
75	10	0	0	1	0
***A. afra* flowers MeOH**					
25	10	8	80	13	10
50	10	3	30	6	80
75	10	2	20	5	4
***A. afra* leaves DCM-MeOH**					
25	10	4	40	4	4.2
50	10	0	0	0	0
75	10	0	0	0	0
***A. afra* leaves MeOH**					
25	10	8	80	6	2
50	10	6	60	4	5
75	10	5	50	2	2
***A. afra* stems MeOH**					
25	10	8	80	10	12
50	10	7	70	3	8
75	10	2	20	2	9
***E. africanus* leaves DCM-MeOH**					
25	10	7	70	27	16
50	10	9	90	30	18
75	10	9	90	32	16

L. leonorus **DCM-MeOH**					
25	10	8	80	30	16
50	10	7	70	28	11
75	10	6	60	19	12
P. fruticosus **DCM-MeOH**					
25	10	7	70	20	12
50	10	8	80	27	15
75	10	10	100	34	18
T. riparia **leaves DCM-MeOH**					
25	10	5	50	14	14
50	10	1	10	12	18
75	10	0	0	0	0
T. riparia **leaves MeOH**					
25	10	5	50	13	18
50	10	4	40	14	16
75	10	2	20	9	19
V. natalensis **DCM-MeOH**					
24	10	7	70	7	9
50	10	0	0	0	0
75	10	0	0	0	0

Table 3.5. Effect of crude extracts of plants on the germination of *E. tef* (teff) seeds.

Plant extract (mg)	Number of seeds	Number of seeds germinated	Percentage germination	Shoot length (mm)*
Control	**100**	**100**	**100**	**30**
***A. afra* flowers DCM-MeOH**				
25	100	60	60	15
50	100	43	43	14
75	100	22	20	14
***A. afra* leaves DCM-MeOH**				
25	100	80	80	19
50	100	52	52	11
75	100	22	22	12
***A. afra* stems MeOH**				
25	100	68	68	12
50	100	64	64	17
75	100	20	20	10
***T. riparia* leaves DCM-MeOH**				
25	100	56	56	26
50	100	45	45	20
75	100	28	28	32
***T. riparia* leaves MeOH**				
25	100	58	58	22
50	100	65	65	19
75	100	70	70	20
***V. natalensis* DCM-MeOH**				
25	100	85	85	17
50	100	42	42	14
75	100	20	20	12

*Because the teff seeds were so small, the measurement of root lengths were not possible.

Table 3.6. Effect of crude extracts of plants on the germination of *L. rididum.*

Plant extract	**Number of seeds**	**Number of seeds germinated**	**Percentage germination**	**Shoot length (mm)***
Control	20	20	100	71
***A. afra* flowers DCM-MeOH**				
25	20	15	75	23
50	20	10	50	20
75	20	4	20	10
***A. afra* leaves DCM-MeOH**				
25	20	19	95	34
50	20	16	80	11
75	20	0	0	0
***A. afra* stems MeOH**				
25	20	11	55	13
50	20	16	8	17
75	20	1	5	5
***T. riparia* DCM-MeOH**				
25	20	17	85	30
50	20	12	60	14
75	20	9	54	20
***T. riparia* MeOH**				
25	20		45	30
50	20		20	10
75	20		65	27
***V. natalensis* DCM-MeOH**				
25	20	17	85	14
50	20	4	20	7
75	20	0	0	0

*Because of the size of the seeds, the measurement of root lengths were not possible.

3.6 EXPERIMENTAL

3.6.1 Plant material

Plant material involved in this study were collected from the UKZN botanical garden under the supervision of Ms. Alison Young, the curator of the garden. The plants were identified by Ms. Young. Vouchers were deposited in the Bews Herbarium of the School of Life Sciences, UKZN, Pietermaritzburg Campus. The plants collected are given in Table 3.1.

Commercial seeds used for the phytotoxic assays were obtained from the following sources:

- *Lactuca sativa*: Starke Ayers Lettuce Commander seeds were obtained from a local nursery.
- *Raphanus sativus*: Stark Ayers Radish Cherry Belle seeds were obtained from a local nursery.
- *Triticum aestivum* (wheat): McDonalds wheat seeds were obtained from a local nursery.
- *Eragrostic tef*: obtained from a local supplier of agricultural products
- *Lolium rididum*: Agriseeds Archie annual ryegrass seeds were obtained from a supplier of agricultural products. Agriseeds is a registered New Zealand trademark.

3.6.2 Extraction of plant material

The various parts of the plants (leaves, branches and flowers) were separated from each other and cleaned from any soil particles and allowed to dry at room temperature for three weeks. The material was ground into a fine powder using a hammer mill. The masses of the dry materials are given in Table 3.2.

The ground materials were extracted sequentially with a DCM-MeOH (1:1) mixture and methanol. The plant material was first covered with the DCM-MeOH (1:1) mixture and then left on an orbital shaker for 24 h at 23 °C. The material was filtered and the solvents removed under vacuum using a rotavapor to obtain a crude extract. This process was repeated and the extract combined with the first DCM-MeOH extract. The recovered plant material was then allowed to stand at room temperature to dry, MeOH was added and the mixture was left on an orbital shaker at 23 ºC and 120 rpm for 24 h. The contents were filtered and the filtrate taken to dryness by using a rotavapor to yield the MeOH crude extract. This extraction was also repeated a second time and the two MeOH extracts were combined. The masses of the crude extracts are shown in Table 3.2.

3.6.3 Phytotoxic bioassay procedure for inhibition of germination

The viability of the seeds were evaluated before experiments on the inhibition of germination were conducted. Seeds were studied and good ones were handpicked and sterilized in a 10% sodium hypochlorite aqueous solution for 5 minutes and rinsed with distilled water. Petri dishes (plastic, 9 cm diameter) were lined with two layers of Whatman number 1 filter paper. Distilled water (5 mL) was added to the Petri dishes to moisten the filter paper. For the viability assay, 20 seeds of each species (*L. sativa*, *R. sativus*, *E. tef* and *L. rididum*) were put on the filter paper in four separate dishes. The dishes were covered with the lids and sealed with Parafilm® to prevent water loss and to maintain an internal humidity conducive for germination. The Petri dishes were left for 7 days at 23 °C in an incubator in the dark. In all four of these experiments, a 100% germination of the seeds was observed.

To assay the inhibition of germination and growth, the procedure described below was followed. To obtain different concentrations of the extracts, 25 mg, 50 mg and 75 mg of crude extract were dissolved in 5 ml solvent and was indicated as treatment 1, 2 and 3, respectively. For the DCM-MeOH extract, ethanol was used due to the fact that the plastic Petri dishes were partly dissolved by DCM, but were not affected by ethanol. The DCM-MeOH (1:1) normally extracts the medium polar compounds and therefore, ethanol, which is less polar than methanol, was used as a substitute solvent. For the methanol extract, methanol was used as the solvent. The steps below were followed to screen lettuce (*L. sativa*), radish (*R. sativus*), teff (*E. tef*) and annual ryegrass (*L. rididum*) seeds.

The extract solutions prepared were transferred into appropriate Petri dishes lined with two Whatman number 1 filter papers. The open Petri dishes were left in a fume hood for 24 hours to enable the solvent to evaporate, leaving the extracts on the filter papers. This was done to ensure that the solvent did not affect the germination which might then be wrongly attributed to an effect by the extracts.

After the solvent was removed from the Petri dishes with the filter papers, 5 ml of distilled water was added to each dish and seeds were spread evenly on top of the filter paper. Because limited amounts of extract were available, the dishes were divided into two parts and two different types of seeds were introduced into each Petri dish. In one set of dishes, 20 seeds, that is, 10 each *L. sativa* and *R. sativus*, were introduced into each dish. In another set of dishes, 100 and 20 seeds of teff and annual ryegrass, respectively, were introduced into one Petri dish. The large number of teff seeds was as a result of the extremely small size of the seeds, which made it difficult to introduce a small number of seeds. The dishes were covered with the lids and sealed with Parafilm® to prevent water loss and to maintain an internal humidity conducive for germination. For each extract concentration, the experiment was performed in triplicate.

For the control, a Petri dish lined with two Whatman number 1 filter papers was moistened with 5 ml of the solvent used to dissolve a particular extract and allowed to dry in a fume hood. This experiment was performed to confirm that solvent used does not play a role in inhibiting growth or germination.

Seeds were considered germinated when the radicle sprouted to 1 mm and above.[111,112] The experiments were monitored for 7 days.

Seed were considered inhibited when 50% of germination was recorded. The experiment for each treatment was repeated in triplicate and the average number of seeds germinated was recorded in Table 3.3. Measurement of the root and shoot lengths were by the use of a ruler.

CHAPTER 4

ISOLATION AND IDENTIFICATION OF HERBICIDAL COMPOUNDS FROM *ARTEMISIA AFRA* JACQ. EX WILLD.

4.1 INTRODUCTION

This Chapter is focused on *Artemisia afra* Jacq. ex Willd., a medicinal plant used across many cultures in South Africa for the treatment of various ailments. The selection of this plant was based on the results obtained in screening experiments for phytotoxic activity of a number of crude plant extracts, as described in Chapter 3.

4.2 LITERATURE OVERVIEW

4.2.1 The family Asteraceae

Artemisia afra is part of the Asteraceae. The Asteraceae or Compositae was investigated by Henri Cassini in the 19th century and he is often regarded as the founder of this family based on his effort to classify its members.[113] Asteraceae is the largest family in the plant kingdom and have approximately 1620 genera and more than 25000 species; thirteen subgroups have also been identified.[114]

Many species of this family have been used as ornamental plants or served as sources for medicines or essential oils.[115] Characteristic features common to all species include propagation by seeds, clustered flowers on a common base surrounded by bracts, different coloured bracts and pollination by wind and/or insects.[116]

4.2.2 The genus *Artemisia* L.

This genus was named after the Greek goddess Artemis[117] and is a large genus with over 500 species distributed worldwide. The morphological features of this genus include:

- Alternating leaves
- Usually racemose
- Capitated flowers
- Bracts in few rows

- Inflorescence.[118]

Ethnopharmacological reports on this genus indicated that many species have been used traditionally to treat various kinds of ailments. Notable among these are:

- *Artemisia absinthium* L. (use as an antispasmodic, stomach pains, cardiac stimulant and a memory enhancer)[119]
- *Artemisia afra* (treatment of cough, headache and kidney disorder)[98]
- *Artemisia annua* L. (use for treating malaria)[120]
- *Artemisia asiatica* (Pamp.) Nakai ex Kitam. (for cancer treatment and inflammation)[98]
- *Artemisia douglasiana* Besser ex Besser (treating premenstrual syndrome)[121]

4.2.3 Phytochemistry of some species in the genus *Artemisia*

Research have revealed that *Artemisia* species contain mainly terpenoids, flavonoids, coumarins, caffeoylquinic acids, sterols and acetylenes.[121a] Species like *A. absinthium*, *A. scaparia*, *A. annua*, *A. afra* and *A. maritima* are rich in terpenoids.[122] A brief phytochemical review of some of these species are presented below.

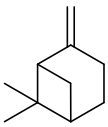
Artemisia absinthium

Source: https://za.pinterest.com/Fairyllusion/artemisia-absinthium/.[31]

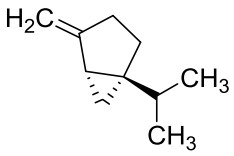
Figure 4.1. Picture of *A. absinthium*.

This species is used as a herbal medicine worldwide.[123] Nine different chemotypes have been recognized based on essential oil composition.[124] Daise et al.[125] reported in 2008 that the essential oil of *Artemisia absinthium* (Fig. 4.1) contains β-Pinene **(4.1)**, sabinene **(4.2)**, 1,8-cineole **(2.50)** and artemisia ketone **(4.3)**. Kordali et al. reported in 2005 on the composition of essential oil of this species together with *A. dracunculus* from Turkey, *A. spicigara* and *A. santonicum*. By using GC-MS analysis the authors identified 30 phytoconstituents representing 99.5% of the total oil with the predominate components being (*Z*)-anethole **(4.4)**, (*E*)-β-ocimene **(4.5)**, (*Z*)-β-ocimene **(4.6)**, methyleugenol **(4.7)** and limonene **(4.8)** (Fig. 4.2).[126] The presence of sesquiterpene

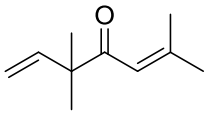
lactones[127], flavonoids, tannins, lignans, flavonols, caffeic acid and coumarins were also reported.[128, 129]



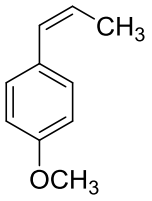
4.1 β-Pinene



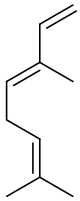
4.2 Sabinene



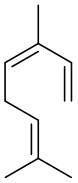
4.3 Artemisia ketone



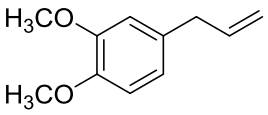
4.4 (*Z*)-Anethole



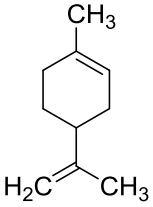
4.5 (*E*)-β-Ocimene



4.6 (Z)-β-Ocimene



4.7 Methyleugenol



4.8 Limonene

Figure 4.2. Structures of compounds identified in the essential oil of *Artemisia* species.

Artemisia scoparia

Source: http://plantdiversityofsaudiarabia.info/.[130]

Figure 4.3. Picture of *A. scoparia*.

A. scoparia (Fig. 4.3) is used as a choleretic, anti-inflammatory and diuretic agent in the treatment of hepatitis.[131] Negahban et al.[132] evaluated the essential oil of this species and realized it was active in controlling weevils.

A. scoparia contain mostly compounds common to the other species. The volatile oil was analysed by Singh et al.,[122] who identified α-pinene **(4.9)**, γ-terpinene **(4.10)** and eugenol **(4.11)**. β-Myrcene **(4.12)** was identified as the major component of the essential oil of with up to 29.27% of this compound. This monoterpene was found to be phytotoxic and inhibited the germination and growth of weed seeds of *Avena fatua*, *Phararis minor* and *Cyperus rotudus.*[122] Further investigations by Ali et al.[133] showed that in Pakistan this species contains two structurally similar compounds known as sabandin A **(4.13)** and sabandin B **(4.14)** (Fig. 4.4).

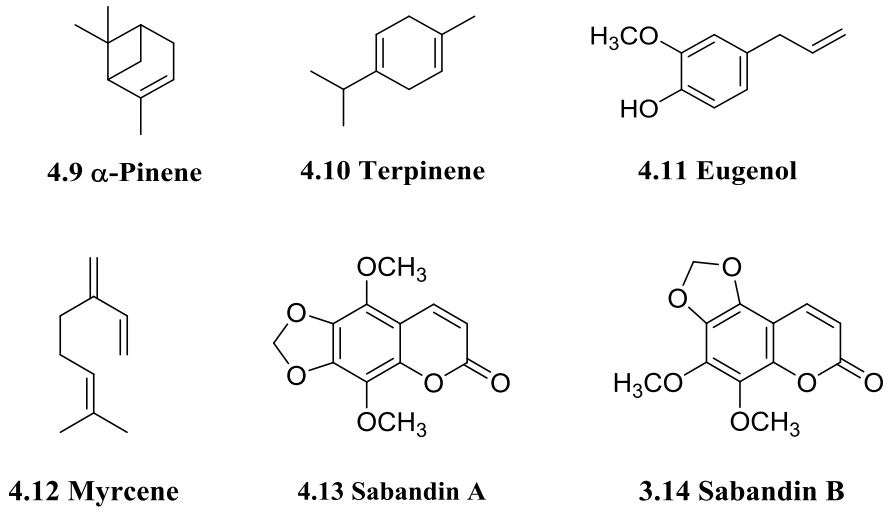


Figure 4.4. Structure of compounds identified in *A. scoparia*.

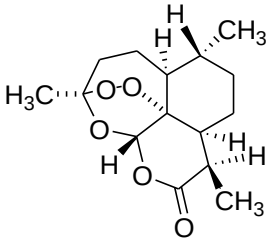
Artemisia annua

Source: http://wisflora.herbarium.wisc.edu/imagelib/imgdetails.php?imgid=1257.[134]

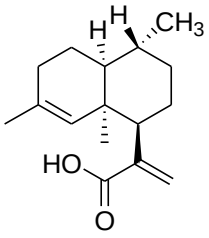
Figure 4.5. Picture of *A. annua*.

A. annua (Fig. 4.5) is an indigenous plant in Vietnam and China and has been investigated by researchers for it potent anti-malarial properties. Artemisinin **(4.15)**, which is currently used as anti-malarial drug, was found to be the major component in this species.[135] Additional sesquiterpenes found in this species include artemisinic acid **(4.16)**, arteannuin B **(4.17)** and

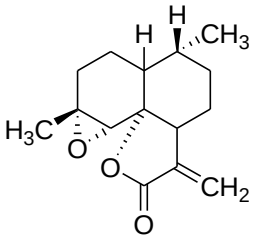
artemisitene **(4.18)**.[136] Other components identified from this species are compounds **4.19 - 4.25**. Lai-king et al.[137] isolated flavonoids **4.26 - 4.29** from the leaves of *A. annua* (Fig. 4.6).



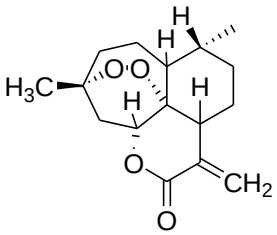
4.15 Artemisinin



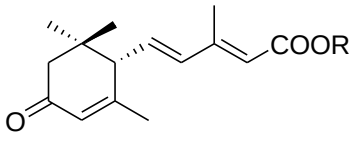
4.16 Artemisinic acid



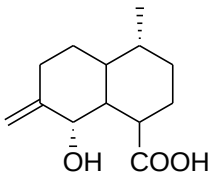
4.17 Arteannuin B



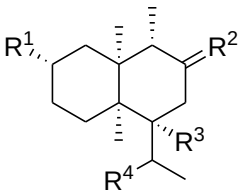
4.18 Artemisitene



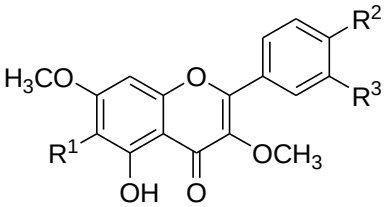
4.19 R = H Abscicic acid
4.20 R = CH_3 Abscicic acid methyl ester



4.21 Annuic acid



4.22 R^1=R^3=H, R^2=CH_2, 4(5),11(13)diene
Arteannuic alcohol
4.23 R^1=R^3=R^4=H, R^2=CH_2, 4(5)ene
Artemisinol
4.24 R^1=R^3=H, R^2=O, R^4=CH_3, 4(15),11(13)diene
Cadina-4(15)-11-diene-9-one
4.25 R^1,R^3=H, R^2=CH_2, R^4=CHO, 4(5),7(11)diene
Cadina-4-(7)-11-dien-12-al



4.26 R^1=R^2=R^3=OCH_3
Artemetin
4.27 R^1=R^3=OCH_3,R^2=OH
Chrysoplenetin
4.28 R^1=R^2=OCH_3, R^3=H
5-Hydroxy-3,4',6,7-tetramethoxyflavone
4.29 R^1=OCH_3, R^2=OH, R^3=H
Penduletin

Figure 4.6. Compounds identified in *A. annua.*

4.2.4 *Artemisia afra*

Figure 4.7. A picture of *A. afra* in the botanical garden of University of KwaZulu-Natal.

A. afra has been given different common names by different societies, such as wild wormwood, African wormwood (English), wilde-als (Afrikaans), umhlonyane (IsiXhosa), muhlonyane (IsiZulu), lengana (Setswana) and zengana (Sesotho).[138] The plant is a perennial woody shrub and grows up to 2 m tall (Fig. 4.7). The stems are normally thick at the base and softer and thinner at the top. It normally flowers in late summer between March and May. One characteristic feature of *A. afra* is that it releases a sticky sweet smell when touched or cut.[139] *A. afra* is common in South Africa with a wide distribution from the Cederberg Mountains in the Cape, northward to tropical east Africa and stretching as far north as Ethiopia. It grows at altitudes between 20-2440 m on slopes along stream sides and forest margins.[140] It also grows in the northern provinces of Gauteng and Limpopo along the Eastern parts of South Africa. It is also abundant in the Kwazulu-Natal province.[141]

A. afra has volatile and non-volatile secondary metabolites, such as monoterpenoids, sesquiterpenes, glaucolides, guaianolides and flavonoids.[140] However, the chemistry of *A. afra* revealed that there are geographical differences in the chemical composition of its secondary metabolites. It was reported that the major constituent in the oil of *A. afra* in Ethiopia was artemisyl acetate **(4.30)** (24 - 32%),[142] whereas in Kenya, 1,8-cineole (67%) was found to be the major constituent of the oil. In Zimbabwe, *A. afra* had α-thujone **(4.31)** and β-thujone **(4.32)** (combined 52%) as the major constituents,[143] while South Africa has only α-thujone as the major constituent, making up 54% of the essential oil.[144] In another study by Viljoen et al.[145], the essential oil of 17 plants from four different geographical regions were investigated. The authors observed that there was a large variation in the composition of the oil, both geographically and within a population. For the 17 plants sampled, the major components were α-thujone **(4.31)** (4 plants), β-thujone **(4.32)** (3 plants), camphor (3 plants) **(4.33)**, 1,8-cineole **(2.50)** (3 plants), artemisia ketone **(4.34)** (2 plants), artemisia alcohol **(4.35)** (1 plant) and santolinyl acetate (1 plant) (Fig. 4.8).

By GC analysis, Asfaw et al.[146] confirmed that artemisia alcohol represents 14.7% of the essential oil, while artemisia ketone (3,3,6-trimethylhepta-1,5-dien-4-one) represents 13.3%. A report on the antimicrobial activity of the essential oil of *A. afra* by Mangena et al.[147] revealed that the essential oil contains similar compounds compared to other species. Researchers found the monoterpenes 1,8-cineole **(2.50)**, α-thujone **(4.31)** and β-thujone **(4.32)** were present in the essential oil, but that camphor **(4.33)** was absent. Sesquiterpenes with glaucolide **(4.37)** and guaianolide **(4.38)** skeletons were also reported.[148] Based on the differences in chemical constituents on the essential oil of *A. afra*, it is clear that factors such as age of plant, season and geographical locations can have an influence on the chemical composition of the oil.

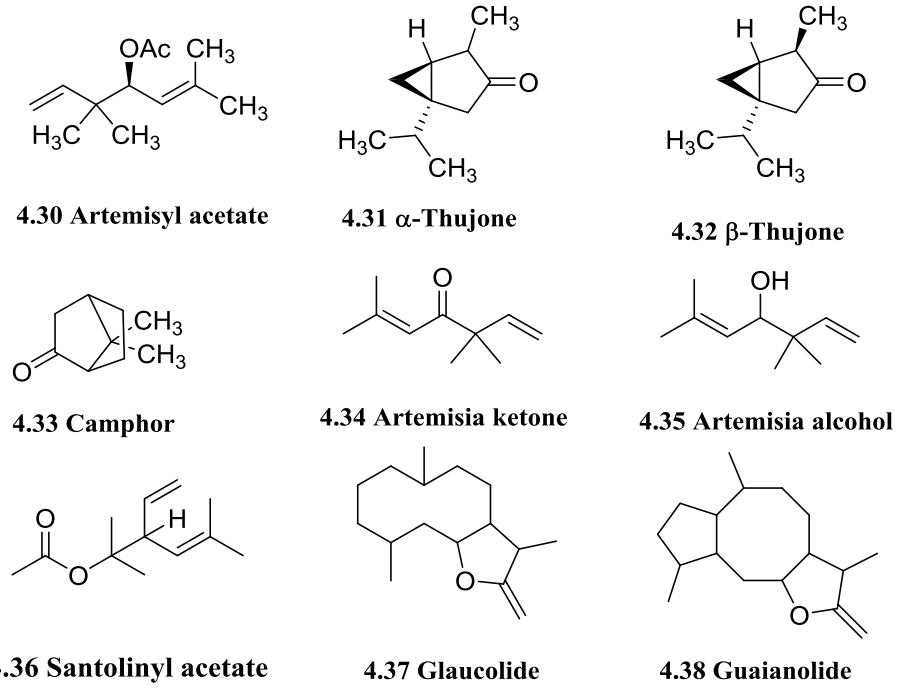


Figure 4.8. Structures of terpenoids identified in *A. afra.*

The aim of this project was to investigate South African medicinal plants as a possible source of herbicidal (phytotoxic) compounds. The crude extracts of some selected plants were screened for phytotoxicity as described in Chapter 3 and after analysis of the results, *A. afra* DCM-MeOH (1:1) extract was identified as the extract with the highest antigermination activity, hence it was selected for further investigations.

After identifying *A. afra* as a plant that produces phytotoxins, we did an in-depth literature search and found that two other research groups investigated the phytotoxic effects of this plant. Hausen et al. reported that wherever *A. afra* grows in the wild, bare soil is found around the shrub.[149] The same authors observed inhibitory effects of the aqueous extracts on the germination and seedling development of maize (*Zea mays*), bean (*Phaeseolus vulgaris*) and ryegrass (*Lolium pirenne*).[149]

Extracts of *A. afra* significantly reduced seed germination and the height and dry matter of all seedlings as compared to the control. The authors observed that the order of activity for the extracts were leaves > stems = roots. The authors also observed that the germination process was affected to a larger extent than inhibition of the growth of the seedlings. These results were confirmed by Amman and Pieterse,[150] who observed antigermination activity on a number of plant species by an *A. afra* extract. None of these authors attempted to isolate and characterize the active principle(s) from *A. afra*. In the subsequent part of our investigation, we attempted to isolate compounds in the *A. afra* extract with antigermination activity. In the next few sections, the fractionation and screening of the extract leading to the identification of a compound with antigermination activity are described.

4.3 RESULT AND DISCUSSION

4.3.1 Fractionation of crude extract

The bioassay-guided fractionation of the DCM-MeOH extract of *A. afra* is summarized in Fig. 4.9. Initially the crude extract was separate into five different fractions by eluting the column with solvents of increasing polarity. The activity was observed in fraction F_B, which was eluted with DCM-EtOAc (20:1). This indicated that the active compounds were relatively non polar. Further fractionation resulted in five subfractions of which the second (BA2) and fourth (BA4) fractions were active. This indicated that the activity is not associated with a single compound only and that more than one active compound is present. Part of fraction BA2 was further separated by column chromatography into five different fractions, of which three fractions (BA2f3 - BA2f5) were active and inhibited the growth of coleoptiles relative to the control. Analysis of the TLC profile of the active fractions showed a common compound with the same R_f which appeared as a blue spot under UV light (365 nm). The TLC profiles of the five fraction are shown in Fig. 4.10. The remaining material of fraction BA2 was subjected to column chromatography and radial chromatography as described in Section 4.4.3 to yield a pure compound identified as scopoletin (**2.19)**.

For the isolation of compounds, a bioassay-guided approach was followed. The initial approach to the assaying of antigermination activity is described in Chapter 3. When limited quantities of material were available during the fractionation processes, two alternative assays, one using smaller filter papers in the germination assay and a second assay, the coleoptile elongation bioassay, were used to assay antigermination effect and phytotoxicity. When a pure compound was isolated using the coleoptile elongation bioassay, the antigermination activity was subsequently confirmed with the bioassay on the smaller filter papers.

The flavonoid acacetin (**4.39**) was isolated from combined fractions BA2f1 and BA2f2. Not enough material was available to determine the activity of the compound. BA4 was further fractionated, but it contains a complex mixture of sesquiterpene lactones of which we could identify only one compound, la,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6a-olide (**4.40**). The structural elucidation of the three compounds **2.19**, **4.39** and **4.40** are discussed in sections 4.3.5, 4.3.6, and 4.3.7, respectively.

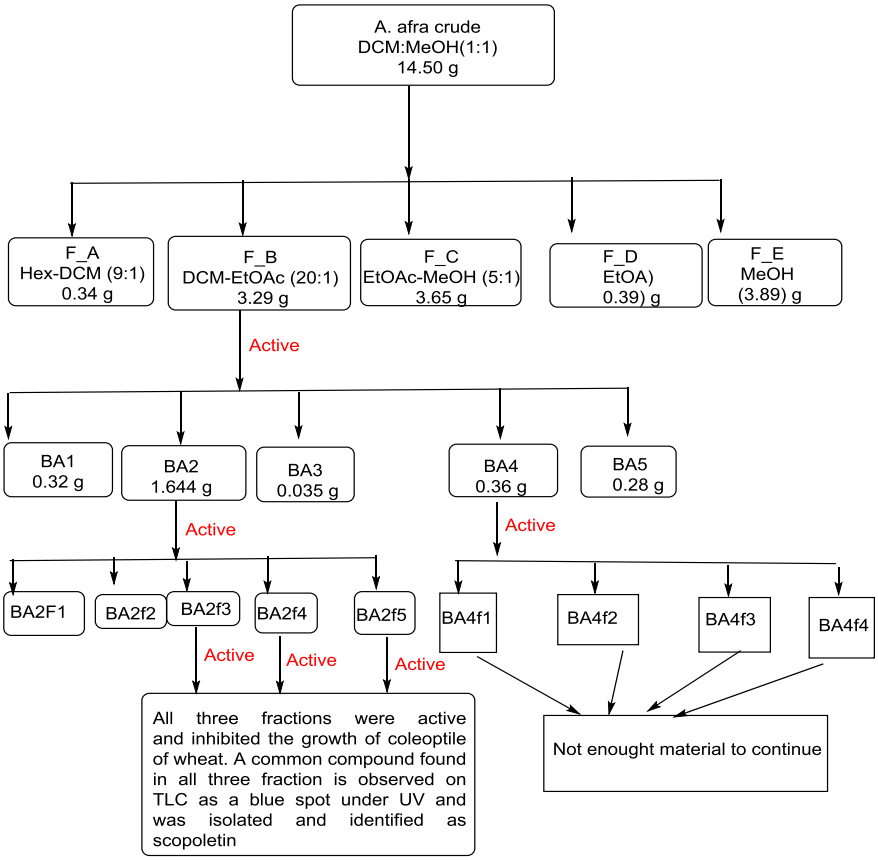


Figure 4.9. Bioassay-guided fractionation of the crude leaf extract of *A. afra*.

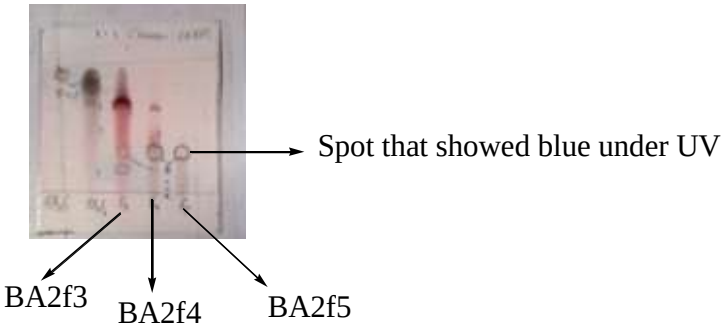


Figure 4.10. A qualitative TLC-profile of compounds of fractions BA2f3, BA2f4 and BA2f5 showing the profiles of the three fractions containing the common compound of R_f 0.53 responsible for the herbicidal activity.

4.3.2 Bioassay of fractions and compounds

The bioassay of the crude DCM-MeOH extract of the leaves of *A. afra* is illustrated in Fig. 4.11.

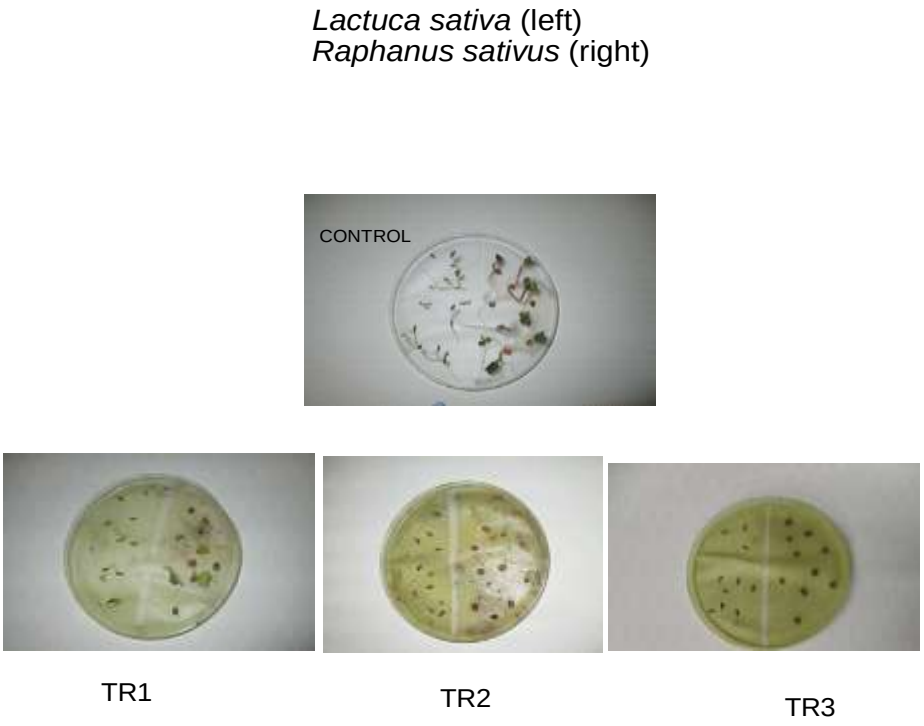


Figure 4.11. Antigermination bioassay of DCM-MeOH crude extract of *A. afra* leaves. Each Petri dish was divided into two parts to accommodate two different types of seed. The left and right hand parts of the dishes contain *L. sativa* and *R. sativus*, respectively.

The crude extract was fractionated by a column chromatography eluting with solvents of varying polarity as described in Section 4.4.2. The setup of the bioassay for the fractions obtained are shown in Fig. 4.12 (day 1).

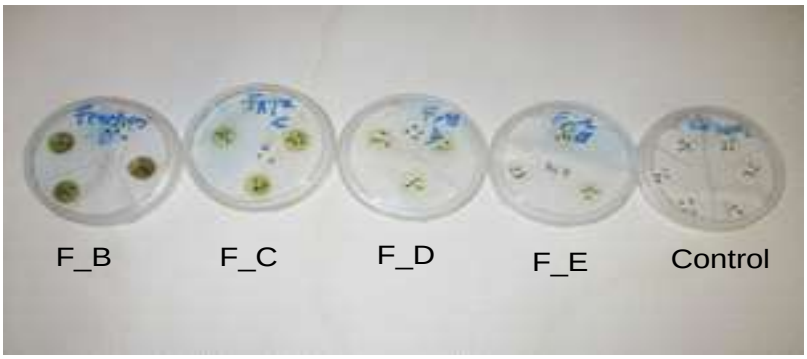


Figure 4.12. Bioassay on *L. sativa* seeds of the fractions generated from the crude fractionation by the modified method for the antigermination assay.

The assay revealed that fraction F_B was active, hence it was selected for further fractionation, whereas the other four fractions were not investigated further. Sample pictures of the result of the bioassay is shown below in Fig. 4.13.

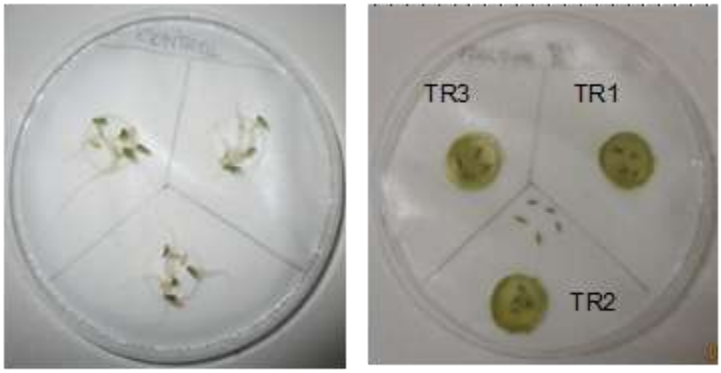


Figure 4.13. Bioassay results of F_B on *L. sativa* seeds after 7 days. No germination was observed.

This fraction was further separated as described in Section 4.4.2 to generate five new subfractions, which were all assayed. It was observed that both fractions BA2 and BA4 were active and those two were selected for further investigations whereas investigation on the other fractions were discontinued. Sample pictures of the bioassays are shown in Fig. 4.14 and 4.15, respectively.

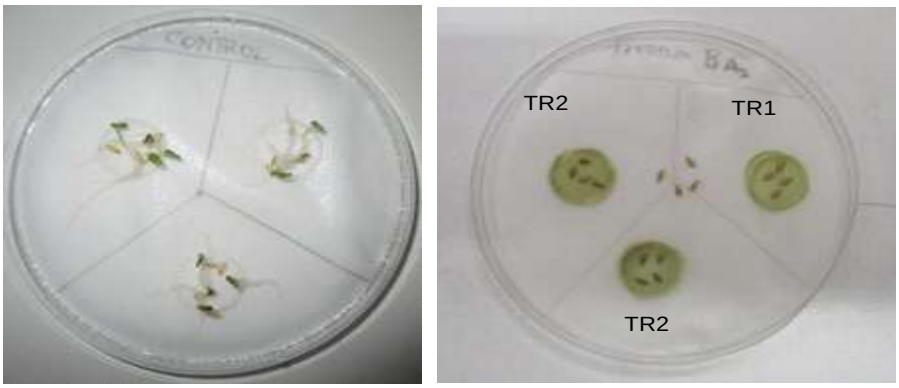


Figure 4.14. Bioassay of fraction BA2 and control on *L. sativa* seeds. No germination was observed after 7 days.

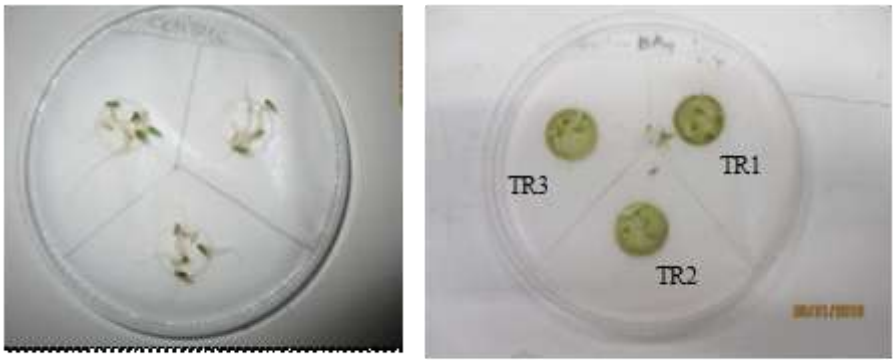


Figure 4.15. Bioassay of fraction BA4 fraction and control on *L. sativa* seeds. Germination was observed after 7 days but root length and shoot length were inhibited substantially in comparison to the control.

The BA2 fraction was further fractionated to generate five new fractions and assayed using the coleoptile elongation bioassay method described in Section 4.4.7. From the result of the bioassay, it was observed that fractions BA3f3 to BA2f5 were active and inhibited the growth of the wheat coleoptile. These three fractions had a common compound with the same R_f and appeared blue under UV. The pure compound was assayed for phytotoxicity using the modified method Section 4.4.6 and the result is shown in Fig. 4.16.

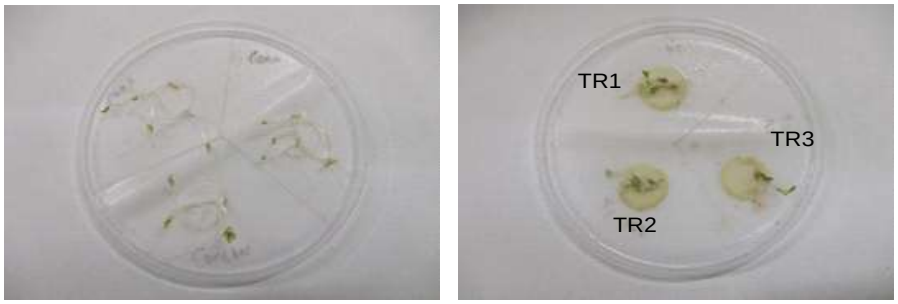


Figure 4.16. Bioassay on *L. sativa* seeds of the pure isolated 7-hydroxy-6-methoxycoumarin (**2.19**) which appeared blue under UV light.

4.3.3 T-test analysis of results of bioassay

The t-test was introduced by William Sealy Gosset who was a quality control chemist working for the Guinness Brewery Ireland.[151] The t-test is concerned with evaluating the differences

between means. It also involves comparing a test statistic to the t-distribution to determine the probability of that statistic if the study's null hypothesis is true.

The hypothesis (H_1) in this study is that the isolated compound has a phytotoxicity effect on the seed germination and subsequent development of the plant. A null hypothesis (H_0) suggests that there is no phytotoxic activity of the isolated compound (**2.19**) on the germination and subsequent development of the seeds. From the above statements, it can therefore be deduced that:

$$H_1: \delta_1 \neq \delta_2$$
$$H_0: \delta_1 = \delta_2$$

where δ_1 is the standard deviation for control values and δ_2 standard deviation of experiment values.

Analysis of the data obtained using SPSS data analysis tool of both treatments is shown in the Table 4.1.

Table 4.1. P_{cal} values of the various treatments in the experiment.

Treatments	$P_{cal.}$ (Shoot length)	$P_{cal.}$ (Root length)
TR1	0.000	0.001
TR2	0.000	0.001
TR3	0.000	0.000

The results in Table 4.1 indicate that the P_{cal} in each of the treatments for both shoot and root lengths is < $P_{crit.}$ 0.05. It can be concluded that the bioassay of compound **2.19** showed a significant difference between the shoot and root lengths compared to the control. Therefore, we reject the null hypothesis which initially suggested that there was no phytotoxic activity of the isolated compound on the germination and subsequent development of the seedlings. The complete Tables of the t-test are shown in the appendix II page 107-115

A commercial coumarin (5,7-dimethoxycoumarin, **4.41**, Fig. 4.17) which was used in comparison to the isolated compound was also assayed using the same method and the values obtained were analysed. The results showed P_{cal}. 0.936, 0.12, 0.198 for TR1, TR2 and TR3, respectively, which is greater than P_{crit}. 0.05. Therefore, we accept the null hypothesis and reject the alternative. Hence there was no significant difference between the control and the treatment in the root length of the germinated seeds, suggesting that the commercial coumarin **4.41** did not affect the roots of the seeds, in comparison with the isolated compound (**2.19**), where the root length was affected greatly. Analysis of the shoot length showed P cal. 0.016, 0.02 and 0.009 for TR1, TR2 and TR3,

respectively, which is less than $P_{crit.}$ 0.05, therefore, we reject the null hypothesis and accept the alternative. Hence there was significant difference in the shoot length of the various treatments compare to the control, suggesting that coumarin **4.41** affected the shoots length of the seedlings. The complete Table of the t-test is shown in the appendix II page 116-121

4.3.4 Phytotoxicity activity charts

The activity of the isolated compound **2.19** was compared to that of a commercially purchased coumarin (5,7-dimethoxycoumarin, **4.41**), which showed that the natural product isolated, 7-hydroxy-6-methoxycoumarin (**2.19**), was more active that the commercially purchased coumarin **4.41**. The graphic representation of the phytotoxicities of the two compounds are shown below in Fig. 4.18 and Fig. 4.19.

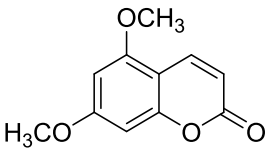


Figure 4.17. Structure of 5,7-dimethoxycoumarin (**4.41**).

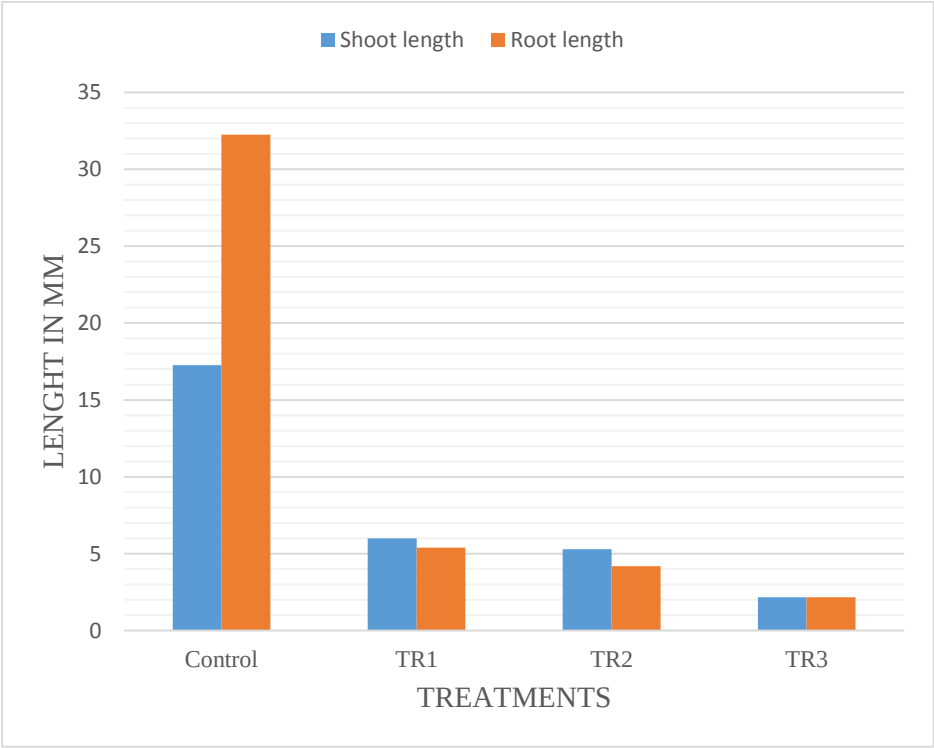


Figure 4.18. The effect of isolated 7-hydroxy-6-methoxycoumarin (**2.19**) on the shoot and root lengths of *L. sativa*.

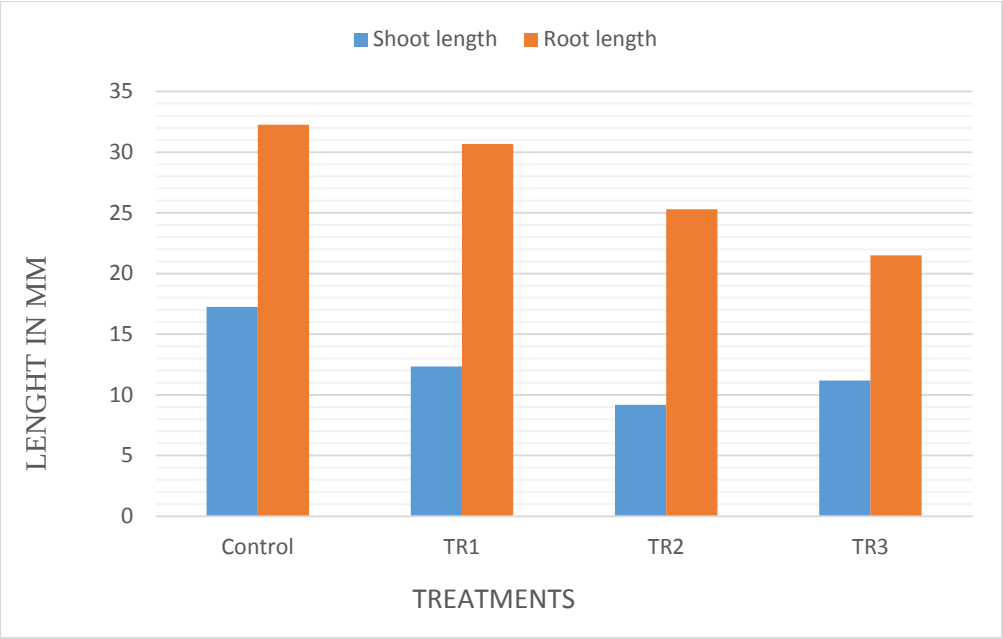


Figure 4.19. The effect of 5,7-dimethoxycoumarin (**4.41**) on the shoot and root lengths of *L. sativa*.

4.3.5 Structural elucidation of 7-hydroxy-6-methoxycoumarin (2.19)

The NMR data of **2.19** are collated in Table 4.6. In the ^{1}H NMR spectrum of **2.19** in acetone-d_6 (Plate 1), six signals were observed; a three-proton singlet at δ_H 3.90, two doublets at δ_H 7.84 (1H, d, *J* 9.5 Hz) and at δ_H 6.17 (1H, d, *J* 9.5 Hz), two aromatic singlets at δ_H 7.19 (1H, s) and at δ_H 6.79 (1H, s) and an one-proton broadened signal at δ_H 8.83. The broadened singlet is characteristic of a hydroxy proton. The COSY spectrum (Plate 3) showed a correlation between the protons resonating at δ_H 6.17 and 7.84.

In the ^{13}C NMR spectrum (Plate 2), a total of ten carbon resonances were observed. By analysing the DEPT-135 spectrum (Plate 4), the carbons were identified as a CH or CH_3 (δ_C 56.8), four alkene/aromatic CH carbons (δ_C 103.8, 110.1, 113.3 and 144.7) and five non-protonated sp^2 carbons (δ_C 112.1, 146.0, 151.1, 151.9, 161.3). In the HSQC spectrum (Plate 5), the protons resonating at δ_H 3.90 (3H, s) correlated with a carbon resonating at δ_C 56.8 (OCH_3), confirming the presence of a methoxy group. The two doublets in the ^{1}H NMR spectrum observed at δ_H 7.84 and 6.17 correlated with the carbon resonances at δ_C 144.7 and 113.3, respectively. The large chemical shift difference between these two carbons indicated that they might be part of a α,β-unsaturated carbonyl moiety with the β-carbon resonating at lower field. In the HMBC spectrum, both the proton resonances at δ_H 7.84 and 6.17 correlated with the carbon resonance at δ_C 161.3. The chemical shifts of both the protons and carbons are in agreement with shifts expected for the lactone ring of a coumarin.

The two remaining aromatic protons were observed as singlets, therefore, the aromatic ring of the coumarin is substituted at C-6 and C-7. Taking this into account this evidence, there were two possible structures for **2.19**, 6-hydroxy-7-methoxycoumarin or 7-hydroxy-6-methoxycoumarin. To differentiate between these two structures, a NOESY experiment was conducted (Plate 7). One of the aromatic protons (δ_H 7.19) showed a correlation to both H-4 of the coumarin (δ_H 7.84) and the methoxy protons, indicating that 7-hydroxy-6-methoxycoumarin (**2.19**) (scopoletin) was the correct structure. In the HMBC spectrum of **2.19** (Plate 6), a ^{1}H-^{13}C correlation was observed between the methoxy group protons (δ_H 3.90) and a carbon resonating at δ_C 146.0. The aromatic proton resonating at δ_H 6.79 correlated with carbons resonating at δ_C 110.1 and 161.3 while the δ_H 7.84 correlated with 144.7 and 151.9. The carbonyl carbon of the lactone ring of the coumarin appeared at δ_C 161.3. The two other carbons of the lactone ring resonated at δ_C 112.1 and at δ_C 144.7, respectively. The HMBC correlations observed for **2.19** are indicated in Fig. 4.20. These correlations supported the assigned structure for **2.19**. However, both 3- and 4-bond ^{1}H,^{13}C correlations were observed and without the results obtained from the NOESY spectrum, it would have been difficult to assign the substitution pattern on the benzene ring unambiguously.

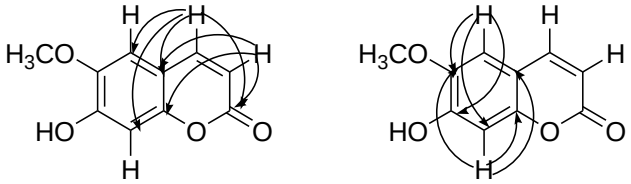
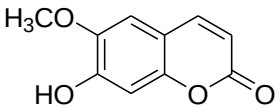


Figure 4.20 HMBC correlation observed for scopoletin



2.19 Scopoletin

The high-resolution ESI-(-)-TOF-MS spectrum showed a peak at *m/z* 191.0349 ($M-H^-$) which was in agreement with the calculated value of 191.0344 for $C_{10}H_7O_4$.

This compound has first been reported by Goodson in 1922.[152] Scopoletin has also been reported in several plants, e.g. Richard et al. isolated this compound from the roots of oats.[153] Silver et al. isolated this compound from uninfected leaves of *Hevea brasiliensis* in 2000 and also noted that scopoletin inhibits the conidial germination of *Crynespora casiicola*.[154] Several researchers have implicated this compound in biological activities such as antitumour and anti-HIV.[155]

Table 4.2. ^{1}H and ^{13}C NMR data of scopoletin (**2.19**) in acetone-d_6 (500 MHz).

Experimental						Literature[156]	
Position	δ_C	DEPT	δ_H	COSY	HMBC correlation[a]	δ_C	δ_H
2	161.3	C			H-3	160.8	
3	112.1	CH	6.17 (d, *J* 9.5)	4	H-3, 4, 5, 8	113.3	6.16 (d, *J* 9.75)
4	144.7	CH	7.84 (d, *J* 9.5)	3	H-5, OCH_3	144.7	7.82 (d, *J* 9.75)
4a	113.3	C			H-3, 4, 8	112.1	
5	110.1	CH	7.19 (1H, s)		H-3, 4, 8 OCH_3	109.9	7.17 (s)
6	146.0	C			H-5, OCH_3	146	
7	151.1	C			H-5, 8, OCH_3	151.9	
8	103.8	CH	6.79 (1H, s)		H-5	103.7	6.78 (s)
8a	151.9	C			H-5, 8	151.2	
6-OCH_3	56.8	CH_3	3.90 (3H, s)			56.7	3.80 (s)
7-OH			8.83 (br s)				

[a]HMBC correlations are from the protons stated to the indicated carbon.

4.3.6 Structural elucidation of acacetin (4.39)

An examination of the ^{1}H NMR spectrum (Plate 8) of the compound revealed the presence of five aromatic proton signals, which were observed as four doublets and a singlet, a three-proton singlet resonating at δ_H 3.90 and a broad singlet resonating at δ_H 12.91. A pair of doublets were resonating at δ_H 8.01 (2H, d, *J* 9.1 Hz) and δ_H 7.12 (2H, d, *J* 9.1 Hz). The coupling constant of 9.1 Hz indicated an *ortho* relationship between the two protons. Both doublets integrated for two protons and a *para*-disubstituted phenyl ring was identified. The remaining two doublets, each integrating for one proton, resonated at δ_H 6.53 and δ_H 6.25. The coupling constant (*J* 2.1 Hz) was an indication of a *meta* relationship. The chemical shifts of the last two protons were in agreement with a phloroglucinol-type aromatic ring. The results discussed above were in agreement with a flavonoid structure. The aromatic singlet resonance at δ_H 6.67 was characteristic of the proton alpha to the carbonyl carbon of a flavone. Therefore, this compound was a derivative of 5,7,4'-trihydroxyflavone. A broad singlet resonating at δ_H 12.91 (2H, br, s) was observed, suggesting that a hydroxy group, which was hydrogen bonded, was present. This was only possible if a free hydroxyl substituent was present at C-5 of the flavone. A singlet resonating at δ_H 3.90 (3H) could be assigned to a methoxy group. Therefore, the structure of the compound was either 5,7,-hydroxy-4'-methoxyflavone or 5,4'-dihydroxy-7-methoxyflavone. The COSY spectrum (Plate 11) confirmed coupling between the protons resonating at δ_H 8.01 and δ_H 7.12 and between the protons resonating at δ_H 6.53 and δ_H 6.25.

The ^{13}C NMR spectrum (Plate 9) showed the presence of 16 carbon atoms. Two of the carbons resonated at the same frequency at δ_C 115.6 and two other carbons also resonated at the same frequency but downfield from the initial two at δ_C 129.2. This suggested a plane of symmetry in a *p*-substituted phenyl ring. A peak at δ_C 183.2 was an indication of the presence of a ketone carbon of a flavone. Five oxygen-substituted aromatic carbons were observed at δ_C 165.7, 164.9, 163.8, 163.4 and 165.7. The rest of the spectrum consisted of resonances of a methyl carbon at δ_C 56.1, methine carbons at δ_C 104.7, 99.8 and 94.8 and non-protonated carbons at δ_C 124.5 and δ_C 105.3.

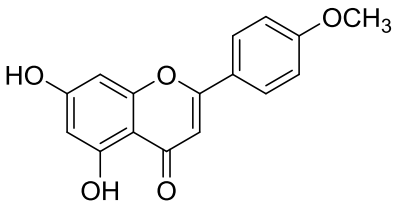
The HSQC spectrum (Plate 12) revealed the correlation of δ_H 6.53 to δ_C 94.8, δ_H 6.25 to δ_C 99.8 and δ_H 3.90 to δ_C 56.1. These data are typical of a flavonoid with a phloroglucinol-type A-ring and a *p*-substituted B-ring.

The HMBC spectrum (Plate 13) showed correlations between δ_H 8.01 (H-2',6'), δ_H 7.12 (H-3',5') and δ_H 6.67 (H-3) and the aromatic carbon at δ_C 124.5. This resonance was assigned to C-1'. The protons resonating at δ_H 8.01 (H-2',6') and) and δ_H 7.12 (H-3',5') were also correlated to a carbon resonating at δ_C 163.8, which was assigned to C-4'. This carbon also showed a correlation to the methoxy protons (δ_H 3.90). Therefore, the B-ring of **4.39** was assigned as a *para*-methoxyphenyl. The protons resonating at δ_H 6.53 and δ_H 6.25 were both correlated to the carbons resonating at δ_C 165.7 (C-7) and δ_C 105.3 (C-4a). Further correlations were observed between signals at δ_H 6.53 (H-8) and δ_C 159.0 (C-8a) and also between δ_H 6.25 (H-6) and δ_C 163.4 (C-5). These correlations enabled us to assign unambiguously the ^{13}C resonance to the A-ring carbons of the flavone.

The position of the methoxy group on the flavone was further corroborated by the NOESY spectrum (Plate 14), where a correlation was observed between the methoxy protons and the aromatic protons resonating as a two-protons double at δ_H 7.12, which were assigned to H-3' and H-5'.

Based on the NMR evidence, the structure of compound **4.39** was assigned as 5,7-dihydroxy-4'-methoxyflavone (**4.39**), also known as acacetin. The spectroscopic data of **4.39** are collated in Table 4.3. The NMR data are in agreement with published data for this compound, despite the fact that the spectra were obtained in different solvents.

The high-resolution ESI-(-)-TOF-MS spectrum showed a peak at m/z 283.0611 ($M-H^-$) which was in agreement with the calculated value of 283.0606 for $C_{16}H_{11}O_5$.



4.39 Acacetin

This compound forms part of the non-volatile component of *Artemisia afra* which have previously been reported and implicated as an antimicrobial active compound.[157] Acacetin has also been reported in several different species across the world found to possess several biological activities. Bentriz et al. studied the effects of this compound on photophosphorylation and seedling growth and development. Their findings showed that acacetin was the most active compound.[158] Yung et al. in their search for new anti-HIV active compounds, investigated *Chrysanthemum morifolium* and through a bio-guide fractionation and isolation acacetin was isolated and found to be a strong anti-HIV component.[159] Cai et al. also isolated this compound from *Chrysanthemum indicum* during their evaluation of antioxidant and phenolic compounds of 112 medicinal plants in China.[160]

Table 4.3. ^{1}H and ^{13}C NMR data of acacetin (**4.39**) in acetone-d_6 (500 MHz).

Experimental						Lit.[161] (DMSO-d_6)	
Position	δ_C	DEPT	δ_H	COSY	HMBC[a]	δ_C	δ_H
2	164.9	C			H-3, 5'	162.8	
3	104.7	CH	6.67 (1H, s)		H-1', 2, 4, 5	103.7	6.74 (1H, s)
4	183.2	C			H-3	181.7	
4a	105.3	C			5-OH, 8	105.3	
5	163.4	C			H-3, 6	162.5	
6	99.8	CH	6.25 (1H, d, *J* 2.1)	8	H-8	100.0	6.85 (1H, d, *J* 2.5)
7	165.7	C			H-6, 8	163.8	
8	94.8	CH	6.53 (1H, d, *J* 2.1)	6	H-6	94.7	6.45 (1H, d, *J* 2.5)
8a	159.0	C			H-8	156.8	
1'	124.5	C			H-3, 2', 5'	122.6	
2', 6'	129.2	CH	8.01 (1H, d, *J* 9.1)	3', 5'	H-2, 1', 3'	128.2	8.03 (d, *J* 9.0)
3', 5'	115.6	CH	7.12 (1H, d *J* 9.1)	2', 6'	H-2, 1', 4'	114.5	7.11 (d, *J* 9.0)
4'	163.8	C			2', 3', OCH_3	161.0	
4'- OCH_3	56.1	CH_3	3.90 (3H, s)			55.8	3.90 (3H, s)
5-OH			12.91 (1H, br.s)				12.83 (1H, s)

[a]HMBC correlations are from the protons stated to the indicated carbon.

4.3.7 Structural elucidation of l*α*,4*α*-dihydroxyguaia-2,10(14),11(13)-trien-12,6*α*-olide (4.40)

The ESI-(+)-HRMS spectrum indicated that the molecular formula of **4.40** was $C_{15}H_{18}O_4$ (Observed: 285.1098 ($M+Na^+$), calculated for $C_{15}H_{18}O_4Na$ 285.1103). The molecular formula was corroborated by the ^{13}C NMR spectrum (Plate 16), in which fifteen carbon resonances were observed. This indicated that the compound might be a sesquiterpenoid. The ^{13}C NMR data further indicated the presence of an ester carbon (δ_C 170.2) and six sp^2 carbons at δ_C 148.9 (C), 140.6 (CH), 138.9 (C), 134.8 (CH), 121.1 (CH_2), 114.4 (CH_2). Using the molecular formula, the number of double bond equivalents were calculated to be seven. The ^{13}C NMR data indicated the presence of a carbonyl and three double bonds and, therefore, the compound contains three rings. Other features of the ^{13}C NMR spectrum was the presence of three oxygen-containing carbons [δ_C 86.6 (C), 85.5 (C), 83.3 (CH)], two methine carbons resonating at δ_C 67.2 and 43.2, two methylene carbons resonating at δ_C 32.1 and 30.2 and a methyl carbon resonating at δ_C 24.7.

Many sesquiterpenoids contain a 5-membered methylene lactone ring. From the DEPT-135 (Plate 17) and HSQC (Plate 19) data two terminal alkene moieties were identified in **4.40**, the one methylene carbon resonated at δ_C 121.1 (CH_2) and were correlated with protons resonating at δ_H 6.27 and 5.54, whereas the other methylene carbon resonated at δ_C 114.4 and the attached protons at δ_H 4.98 and 4.78. In the ^1H NMR (Plate 15), two protons resonating at δ_H 3.30 (1H, m) (δ_C 43.2, C-7) and 4.13 (1H, dd, *J* 11.1, 9.0 Hz) (δ_C 83.3, C-6) were an indication of the presences of a lactone moiety. In the COSY spectrum (Plate 18), $^1H,^1H$ correlations between both δ_H 6.27 and δ 5.54 and the proton resonating at δ_H 3.30 (1H, m) were observed. The proton resonating at δ_H 3.30 also showed correlations to the resonances at δ_H 4.13 and the methylene protons at δ_H 2.78 and δ_H 1.44 (δ_C 32.1, C-8). A second aliphatic methylene group having its protons resonating at δ_H 2.90 and δ_H 2.32, correlating to carbon resonating at δ_C 30.2 (C-9), was identified. The COSY spectrum showed that these two methylene groups are next to each other. The proton resonating at δ_H 4.13 has coupling constants of 11.1 and 9.0 Hz, indicating that it has an axial, axial relationship with both adjacent protons. In the COSY spectrum, a correlation was observed between this proton and the proton resonating at δ_H 2.48 (d, *J* 11.2 Hz) (δ_C 67.2, C-5). These data enabled us to construct the fragment in Fig. 4.20. For clarity, atom numbers of the final compound was used here.

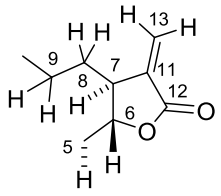
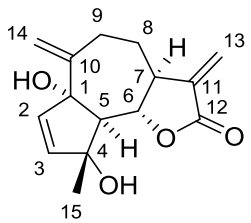


Figure 4.21. Fragment of structure **4.40.**

The HMBC spectrum (Plate 20) showed a correlation between the protons resonating at δ_H 6.27 and δ_H 5.57 to carbons resonating at δ_C 170.2 (C-12), δ_C 138.9 (C-11) and δ_C 43.2 (C-7), which proved that the methylene group (δ_H 6.27 and δ_H 5.54) was next to the carbonyl carbon of the lactone moiety. Another significant correlation was observed from the protons resonating at δ_H 4.99 and δ_H 4.78 (assigned to the second exocyclic methylene group) to carbons resonating at δ_C 148.9, δ_C 86.4 and δ_C 30.2 (C-9). The HSQC showed that these protons were on the same carbon and from the HMBC results, it was observed that the adjacent alkene carbon resonated at δ_C 114.4, with the two other neighbouring carbons an oxygen-containing quaternary carbon (δ_C 86.4, C-1) and a methylene carbon at δ_C 30.2 (C-9).

The remaining fragments of the molecule was a *cis*-alkene, as evidenced by a proton resonance at δ_H 5.99 (*J* 6.0 Hz), which had a one-bond correlation in the HSQC with the carbon resonance at δ_C 140.6, and a proton resonance at δ_H 5.62 (*J* 6.0 Hz), which had a one-bond correlation with the carbon resonance at δ_C 134.8. In the ^{1}H NMR spectrum a methyl singlet was observed at δ_H 1.34, which correlated in the HSQC with the carbon resonance at δ_C 24.7 (C-15). The only remaining signal in the ^{13}C NMR spectrum was a resonance at δ_C 82.2 (C-4), which was assigned to a quaternary oxygen-linked carbon atom. In the HMBC, correlations were observed between the methyl protons resonances (δ_H 1.34) and the carbon resonances at δ_C 67.2 (C-5), δ_C 82.2 (C-4) and one of the alkene carbons at δ_C 140.6 (C-3). Both alkene protons correlated with the two oxygen-containing carbons resonating at δ_C 86.6 (C-1) and δ_C 82.2 (C-4).

Based on the spectroscopic data, the compound was assigned lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**). The relative configuration of the compound was established by correlation observed in the NOESY between the methyl protons (H-15, δ_H 1.34) and H-6 (δ_H, 4.13) and between H-5 (δ_H 2.48) and H-7 (δ_H 3.30). The ring junction between a five and seven-membered rings can be either *cis* or *trans*. The junction with the lactone ring is *trans*, as indicated by the large coupling constant between H-6 and H-7 (*J* 9.0 Hz). Models showed that if the other ring junction was also *trans*, a correlation would have been observed in the NOESY between H-2 and one of the H-14 protons (δ_H 4.78). The absence of this correlation lead us to the conclusion that ring junction between the cyclopentene ring and the seven-membered ring is *cis*.



4.40

The NMR data of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**) are collated in Table 4.4. The only reports on the isolation of this compound is by Jakupovic et al.[162] in 1991 who isolated it from *Artemisia rutifolia* and Todorovan and Tsankova who isolated the compound from *Achillea chrysocoma*.[162] Neither of these authors have reported any ^{13}C NMR data, therefore, this is the first report on the assignment of the ^{13}C NMR spectrum of **4.40**.

Table 4.4. ^{1}H and ^{13}C NMR data of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**) in $CDCl_3$ (500 MHz).

Experimental						Literature[163]	
Position	δ_C	DEPT	δ_H	COSY	HMBC correlation[a]	δC	δH
1	86.4	C			H-2, 3, 5, 14a, 14b		
2	134.8	CH	5.99,d, *J* 6.0 Hz	3	H-3		5.79 d
3	140.6	CH	5.62, d, *J* 6.0 Hz	2	H-2, 15		5.93 d
4	82.2	C			H-2, 3, 6, 5, 15		
5	67.2	CH	2.48, d, *J* 11.2 Hz	6	H-2, 3, 6, 15		2.17 d
6	83.5	CH	4.13, dd, *J* 11.1, 9.01 Hz	5, 7	H-3, 5, 7		4.67 t
7	43.2	CH	3.9, m	8, 6	H-2, 5, 6, 13a, 13b		3.17 ddddd
8	32.1	CH_2	2.78 m 1.44 m	7, 9	H-6, 9a, 9b		
9	30.2	CH_2	2.90, td, *J* 13.0, 2.32, m	8	H-8a, 8b, 14a, 14b		
10	148.9	C			H-5, 8a, 14a, 14b		
11	138.9	C			H-7, 13a, 13b		
12	170.2	C		13, 9	H-13a, 13b		
13	121.2	CH_2	6.27, *d*, *J* 3.5 Hz 5.57, *d*, *J* 3.1 Hz		H-7		6.25 d 5.47 d
14	114.4	CH_2	14a: 4.99, br, *s* 14b: 4.78, *d*, *J* 1.2 Hz		H-8a, 8b, 9a, 9b		1.45 s
15	24.7	CH_3	1.36, *s*		H-5		1.24 s

[a]HMBC correlations are from the protons stated to the indicated carbon.

4.3.8 Phytotoxicity of coumarins and sesquiterpenes lactones

Many coumarins have been implicated as phytotoxic compounds by researchers in laboratory bioassay experiments. The work of Dastan et al. in 2014 implicated a number of sesquiterpene coumarins as phytotoxic. They also noted that these compounds are present in the roots of many species in the genus *Ferula*.[164] Phytotoxicity and inhibitory effects of simple coumarin such as

methoxycoumarins produced from certain plant roots are observed on some seeds and reported in laboratory bioassay experiments.[165]

Researchers carried out phytotoxicity studies through laboratory bioassays of some selected plants and isolated sesquiterpene lactones as compounds responsible for these activities. The work of Miranda et al.[96] on the sunflower, isolated a number of these sesquiterpene lactones. The phytotoxic potentials of these compounds have been documented by researchers through the outcome of laboratory bioassays.[166,167,168]

4.4 EXPERIMENTAL

4.4.1 General experimental procedures

Thin-layer chromatography (TLC) were performed on silica gel 60 F_{254} aluminium-based sheets (Merck). Visualization of UV-active compounds were done using a ultraviolet lamp, λ 254 nm (short wavelength) and λ 365 nm (long wavelength), while non-UV active compounds were detected by spraying with a *p*-anisaldehyde solution and heated with a heat gun. The staining solution were prepared as follows: methanol (680 mL) was cooled down in ice and sulfuric acid (32 mL) and glacial acetic acid (32 mL) added. The solution was mixed properly before *p*- anisaldehyde (4 mL) was added dropwise. The solution was stored in a fridge and used to stain TLC plates followed by heating using a heat gun and colours were observed. Isolation techniques used included column chromatography on various sizes of columns packed with silica gel 60 (0.04 – 0.063 μm, Merck). Radial chromatography was performed on a Harrison Chromatotron model 7924T On 4 mm thick rotor plates coated with silica gel 60PF_{254} (Merck). Preparative TLC plates were prepared by coating 20 x 20 cm glass plates with an aqueous suspension of silica gel 60PF_{254} (Merck).

NMR experiment were conducted on Bruker Avance III 500 operating at 500 MHz for 1H and 124 13C and Bruker Avance III 400 MHz operating at 400 MHz for 1H and 100 MHz for 13C spectrometers. 1D and 2D experiments were combine to determine the structures and include, for 1D; 1H, 13C, DEPT and for 2D; COSY, NOESY, HMBC and HSQC. Definitions such doublet (d), multiplet (m) coupling constant (*J*), double doublet (dd), triplet (t) and doublet of triplet (dt) were assigned to various peaks with such characteristics. All experiments were recorded in acetone-d_6 and chloroform ($CDCl_3$) at 30°C using 5 mm TBIZ probe, chemical shift were measured in ppm and were referenced to solvent peaks, for acetone-d_6 ^1H 2.05 and ^{13}C 29.84 and for chloroform ($CDCl_3$) ^1H 7.26 and ^{13}C 77.02.

High-resolution mass spectra were conducted on a Micromass LTC (Waters) spectrometer with electrospray ionization in either positive or negative mode with TOF detection.

GC-MS data were obtained from a Shimadzu QP2010 GCMS performed by using helium gas as a carrier transporter for the sample in the vapour state.

SPSS data analysis tool was used to analysed the data obtained, using the t-test, results with P_{cal} < P 0.05 were considered significant.

The van der Weij guillotine was manufactured by Mr. Paul Forder, a glass blower in the school chemistry and physics Pietermaritzburg.

All solvents, except hexanes (Hex), and deuterated solvents used were obtained from Merck and used without further purification. Hexanes (a mixture of hexane isomers) was distilled before use.

4.4.2 Fractionation of *A. afra* leaf extract

The crude DCM-MeOH (1:1) extract of *A. afra* (14.50 g) was fractionated by column chromatography eluting with organic solvents with an increasing order of polarity. The Table 4.5 below shows the various fractions obtained and their masses.

Table 4.5. Fractionation of crude extract and masses of fractions.

Fractions generated	**Solvent combinations volume**	**Masses (g)**
F_A	Hex-DCM (9:1)	0.337
F_B	DCM-EtOAc (20:1)	3.298
F_C	EtOAc-MeOH (5:1)	3.649
F_D	EtOAc	0.39
F_E	MeOH	3.891

The fractions above was assayed by the modified screening method described in Section 4.4.7 in order to use the minimum of fractionated material. The results showed that the phytotoxic activity was concentrated in fraction F_B and this material was selected for further fractionation.

Fraction F_B (3.29 g) was subjected to column chromatography. The crude material was dissolved in 20 mL DCM-EtOAc and loaded onto a column. The column was eluted starting with Hex-EtOAc 9:1 and then increasing the polarity of the eluent until the final eluent was 100% EtOAc. The volume of the fractions collected was 5 mL and 104 fractions were collected. The

fraction were monitored by TLC and fractions that showed the same profile were combined. Five fraction were generated and labelled BA1 to BA5. Table 4.6 below shows the fractions generated and their masses.

Table 4.6. Masses of fractions generated from the fractionation of F_B.

F_B Fraction	Mass(g)
BA1	0.324
BA2	1.644
BA3	0.035
BA4	0.364
BA5	0.283

The factions shown in Table 4.6 above were assayed using the modified method and BA2 and BA4 showed active. Only these two fractions were investigated further.

Fraction BA2 (200 mg) was subjected to column chromatography (Fig. 4.20). The starting eluent was Hex-EtOAc (9:1) and the polarity was increased by increasing the EtOAc concentration in the eluent. Eighty-six fraction (5 mL each) were collected. The fractions were evaluated by TLC and fractions with the same profile were combined to generate five new fractions labelled BA2f1 to BA2f5. Table 4.7 shows the fractions and their masses.

Figure 4.22. Column chromatography experiment on the fractionation of BA2 fraction.

Table 4.7. Masses of fractions generated after column chromatography of fraction BA2.

Fractions	**Masses (mg)**
BA2f1	10
BA2f2	81
BA2f3	55
BA2f4	22
BA2f5	5

The fractions obtained in Table.4.7 were subjected to screening by using the coleoptile elongation bioassay.

4.4.3 Isolation of 7-hydroxy-6-methoxycoumarin (scopoletin) (2.19)

The remaining 1.44 g of the active fraction BA2, which contained the compound that showed the blue colour under UV, was fractionated by column chromatography (Hex-EtOAc, 6:4). 109 fractions were collected and monitored by TLC; *p*-anisaldehyde stain solution was used to stain plates, followed by heating for colour development of compounds. An impure compound (80 mg) was obtained and this compound was further purified by using a 4 mm thick rotor plate on a Chromatotron® eluting with Hex-EtOAc (6:4) (Fig. 4.21). 75 fractions were collected at 5 mL per vial and were monitored by TLC plates. Fractions 13 – 22 gave a single spot with R_f 0.53 which has a clear blue fluorescens under UV. These fractions were combined and the solvent removed using a rotavapor under vacuum to generate a pure compound (15 mg). The physical characteristics are summarize below.

Figure 4.23. Chromatotron apparatus used to purify the impure compound obtained from the column chromatography of the BA2 fraction.

Appearances: White crystal-like substance

Empirical formula: $C_{10}H_8O_4$

^{1}H NMR (Acetone-d_6): 8.83 (s, 7-OH), 7.84 (1H, d, *J* 9.5), 7.19 (1H, s) 6.79 (1H, s) 6.17 (1H, d, *J* 9.5), 3.90 (3H, s) (Plate 1).

^{13}C NMR (Acetone-d_6): 161.3(s, C-2), 151.9 (s, C-6), 151.1 (s, C-7), 146.0 (s, C-8a), 144.7 (d, C-4), 113.3(s, C-4), 112.1 (d, C-3)' 110.1 (s, C-5)103.8 (s, C-8), 56.8 (q,-OCH_3) (Plate 2).

ESI-(-)-MS: Observed m/z 191.0349 (M-H-). Calculated for $C_{10}H_7O_4$ 191.0344.

4.4.4 Isolation of acacetin (4.39)

During the column chromatography of fraction BA2 (1.44 g) that led to the isolation of scopoletin, fraction 3 – 8 were combined based on compound profile similarities to yield 25 mg of impure acacetin. Acacetin (**4.39**) was purified by preparative TLC using the solvent system Hex-EtOAc (7:3). The compound appeared yellow on the coated silica with a R_f of 0.76.and was removed from the plate with a spatula. The scrapped silica and the compound were dissolved in EtOAc and eluted over silica gel packed in a small column with EtOAc to yield 2 mg of the pure compound. The physical characteristics are summarize below.

Appearance: White crystal-like substance

Empirical formula: $C_{16}H_{12}O_5$

^{1}H NMR (Acetone-d_6): 12.91 (1H, br s, 5-OH), 8.01 (1H, d, *J* 9.1, H-2', H-6'), 7.12 (1H, d, *J* 9.1, H-3', H-5'), 6.67 (1H, s, H-3), 6.53 (1H, d, *J* 2.08, H-8), 2.25 (1H, d, *J* 2.08 H-6), 3.90 (3H, s,OCH_3-7).

^{13}C NMR (Acetone-d_6): 183.2 (s, C-4), 165.5 (s, C-7), 164.8 (s, C-5), 163.7 (s, C-2), 163.4 (s, C-4'), 158.8 (s, 8a), 129.2 (d, C-2', C-6'), 124.4 (s, C-1') 115.4 (d, C-3', C-5'), 104 7 (s, C-4a), 104.7 (d, C-3), 99.8 (d, C-6), 94.8 (d, C-8), 56.1 (q, 7-OCH_3).

ESI-(-)-HRMS: Observed *m/z* 283.0611 (M-H^-). Calculated for $C_{16}H_{11}O_5$. 283.0606

4.4.5 Isolation of 1*α*,4*α*-dihydroxyguaia-2,10(14),11(13)-trien-12,6*α*-olide (4.40)

This compound was part of fraction BA4, which was generated from fraction F_B (Table 4.5) and was also active in the phytotoxicity assay. A bioassay-guided isolation leading to a phytotoxic

compound could not be achieved due to limited extract quantity. However, the 358 mg of the fraction was subjected to column chromatography and eluted with Hex-EtOAc (2:8) to generate 107 fractions (5 mL per vial). The fractions were monitored by TLC and those with same profile combined. Fraction 24 – 58 were combined to generate 40 mg of an impure F4C5. Preparative TLC (Hex-EtOAc, 2:8) was used to isolate compound 1α,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**) which appeared as a dark colour under UV on TLC and had a R_f of 0.58. A total of 5 mg material was obtained. The compound had a golden colour after drying under nitrogen. The physical characteristics are summarize below.

Appearances: Yellow amorphous powder

Empirical formula: $C_{15}H_{10}O_4$

^{1}H NMR (Acetone-d_6): 6.27 (1H, d, *J* 3.6 Hz, H-13), 5.99 (1H, d, *J* 5.9 Hz), 5.62 (1H, d, *J* 5.6 Hz), 5.54 (1H, d, *J* 3.1 Hz, H-13), 4.98 (1H, br s), 4.78 (1H, d, *J* 2.1 Hz), 4.13 (1H, dd, *J* 11.1, 9.0 Hz), 3.30 (1H, m), 2.48 (1H, d, *J* 11.2 Hz), 2.90 (1H, td, *J* 13.0, 5.0, H-9), 2.78 (1H, m, H-8), 2.32 (1H, m, H-9), 1.44 (1H, m, H-8), 1.34 (3H, d, *J* 11.2 Hz)

^{13}C NMR (Acetone-d_6): 170.2 (s, C-12), 148 (s, C-10), 140.6 (d, C-3), 138.9 (s, C-11), 134.8 (d, C-2), 121.2 (t, C-13), 114.4 (t, C-14,), 86.4 (s, C-1), 83.5 (d, C-6), 82.2 (s, C-4), 67.2 (d, C-5), 43.2 (d, C-7), 32.1 (t, C-8), 30.2 (t, C-9), 24.7 (q, C-15)

ESI-(+)-HRMS: Observed: 285.1098 ($M+Na^+$), calculated for $C_{15}H_{18}O_4Na$ 285.1103

4.4.6 Modification of bioassay for phytotoxicity

When limited quantities of extract or compounds were available, two alternative assays were used: 1) smaller filter papers in Petri dishes, monitoring germination of *L. sativa* seeds and 2) elongation of wheat coleoptiles. When a pure compound was isolated, the coleoptile bioassay was used but the herbicidal activity was also confirmed on smaller filter papers monitoring germination.

4.4.6.1 Modification of Petri dish bioassay

The bioassay for phytotoxicity described in Section 3.53 (9 cm filter papers) require larger amounts of material and therefore, the method was adapted to be suitable for smaller amounts of extract and/or compound. A mathematical relation was used to equate the masses and volumes

used for a 9 cm diameter filter paper to a 1.5 cm diameter filter papers. The area of the circular Petri dish was calculated using the relation below:

For a 9 cm Petri filter paper:

$$\text{Surface area} = \pi r^2 \quad (r = \text{radius of the filter paper})$$
$$= \pi(4.5)^2$$
$$= 63.643\ \text{cm}^2$$

For a 1.5 cm Petri filter paper:

$$\text{Surface area} = \pi r^2 \quad (r = \text{radius of the filter paper})$$
$$= \pi(0.75)^2$$
$$= 1.768\ \text{cm}^2$$

In the previous procedure, the three amounts of extracts used were 25 mg, 50 mg and 75 mg. Taking the surface areas into account, the corresponding masses used for the 1.5 cm filter papers were 0.6 mg (TR1), 1.3 mg (TR2) and 2 mg (TR3), respectively. These fractions were dissolved in 150 μL of solvent in order to apply them to the filter paper. The filter papers were allowed to dry in a fume hood for 24 hours. The impregnated filter papers were placed inside 9 cm Petri dish lined with Whatman No 1 filter paper and 5 mL distilled water was added. Four seeds of *L. sativa* was place in each treated 1.5 cm filter paper and the dish was sealed with Parafilm®. The control had the ethanol evaporated and distilled water used without extract. The dishes were left for a period of 7 days after which the germination, root and shoot lengths were monitored.

4.4.6.2 Coleoptile elongation bioassay

A citrate-phosphate buffer with pH 5.6 containing 2% sucrose and 400 ppm DMSO (dimethyl sulfoxide) was used for this assay. For the control, pure DMSO was used. For assaying of the samples, a specific amount of sample was dissolved in the DMSO.

For the preparation of 0.0123 M $CaHPO_4$ solution, 1.68 g of the chemical was taken and dissolved in a beaker, the content was transferred into a 1000 mL volumetric flask and the flask filled up to the mark with distilled water. For the preparation of 0.005 M citric acid solution, 1.1 g of citric acid was dissolved in distilled water in a beaker and the content transferred to a 1000 mL volumetric flask and the flask filled to the mark with distilled water. For the preparation of 2% sucrose solution, 2 g of sucrose was taken and dissolved in a beaker, the content was transferred into a 100 mL volumetric flask and made up to the mark with distilled water. For the preparation of the buffer solution, equal volumes (10 mL each) of sucrose and citric acid were combined giving a solution with a pH of 2.07. To this solution 300 mL of 0.0123 M $CaHPO_4$ solution (pH 6.47) was added to adjust the pH to 5.6.

To assay individual fractions, a single concentration of the extract in the phosphate-citrate buffer concentration was prepared for the bioassay. For these experiments, 4 mg of each fraction was dissolved in 10 µL of DMSO and added to 20 mL of the citrate-buffer solution to generate a 400 ppm solution of the extract in the phosphate-citrate buffer. The negative control solution of 400 ppm of DMSO in the citrate-phosphate buffer solution was prepared by adding 8 µL of DMSO to 20 mL of the citrate-phosphate buffer solution. The positive control contain 1,8-cineole (eucalyptol) purchased from Sigma-Aldrich. For the preparation of the solution 8 µL of eucalyptol was added to 20 mL of the citrate-phosphate buffer solution.[110] The solutions prepared were used to screen the fractions for phytotoxicity as described below.

Wheat (*Triticum aestivum)* seeds were cultured on Petri-dishes to germinate in the dark at room temperature for 4 days. The coleoptiles were separated from the roots and caryopses of the seeds and the tip of each coleoptile (2 mm) was cut off before resizing them to 4 mm using a Van der Weij guillotine (Fig. 4.22).

Figure 4.24. Van der Weij guillotine used for the resizing of the wheat coleoptile.

The assay was conducted in a series of test tubes. Four prepared coleoptile pieces were transferred into each test tubes and 2 mL of 400 ppm of the extract in phosphate-citrate buffer solution was then added to the test tube. The test tubes were wrapped with aluminium foil to prevent light from getting access to the test samples. Samples of the test tubes used in the bioassay are shown in Fig. 4.23. The tubes were transferred into an orbital shaker set at 21°C and at 150 rpm and left for 24 hours.[169] The test tubes were taken out, the solutions removed and the length of each coleoptile immediately measured with a ruler. The t-test table for the statistical analysis of the result using SPSS is given in the Appendix II page 122-124.

Figure 4.25. Prepared coleoptile samples in test tubes treated with extract phosphate-citrate buffer solutions ready to be incubated.

4.4.8 Bioassay of 7-hydroxy-6-methoxycoumarin (2.19)

The pure isolated compound was assayed using the method described in Section 4.4.6 on *L. sativa* seeds. It was active against the germination of the seeds and subsequent development of the seedlings. This bioassay was repeated tree times. Sample pictures of the bioassay are shown in Fig. 4.16.

CHAPTER 5

CONCLUSION AND FUTURE WORK

The use of synthetic herbicides has posed more problems than perceived. There are reports of weeds developing resistance towards some of these herbicides.[170] Research have also proved that the continual use of some synthetic herbicides have led to several problems ranging from pollution of water bodies and the environment to direct effects on unborn babies.[171] This situation has led researchers to consider and explore various alternatives to help eradicate these problems.

Plant secondary metabolites have been give much attention by researchers as an alternative source of herbicides and have the potential to eradicate these harmful effects. Nature provides the largest chemical laboratory and only few of its chemicals have been investigated.[172] Over the past two decades, several studies have gone into plants secondary metabolites as sources of herbicides, and several compounds such as flavonoids, coumarins, sesquiterpenes and terpenoids have been implicated as possible hits in the discovery of new herbicides.[88]

Six medicinal plants were used in this study, of which *A. afra* leaves DCM-MeOH (1:1) extract gave the best results among the extracts screened hence it was selected for further investigation. *Artemisia afra*, a medicinal plant used across several cultures in South Africa for the treatment of various ailments,[173] was investigated as a possible source of herbicidal compounds. The plant was harvested and extracted with DCM-MeOH (1:1), the crude extract was screened for phytotoxicity by preparing 3 treatments of increasing order of extract concentration, 25 mg, 50 mg and 75 mg and impregnated filter papers with each of this mass. The impregnated filter papers were placed in Petri dishes and moistened with 5 mL of distilled water and then placed the seed to be assayed inside and covered the Petri dishes with the lid and sealed with Parafilm®. Four different weed seeds *Lactuca sativa*, *Raphanus sativus*, *Lolium rididum* and *Eragrostic teff*, were used of which *L. sativa* was selected for the final phytotoxicity study. *Lactuca sativa* showed 50% germination for treatment 1 and 0% germination in treatment 2 and 3, *Raphanus sativus*, showed 40% germination in treatment 1 and 0% in treatment 2 and 3, *Eragrostic tef*, showed 80% germination for treatment 1 and 52% and 22% for treatments 2 and 3, respectively, whereas *Lolium rididum* showed 95%, 80% and 0% germinations for treatments 1, 2 and 3, respectively.

A bio-guided fractionation resulted in the identification of two major fractions being active namely BA2 and BA4. BA2 fraction was investigated further and the compound responsible for the herbicidal properties of the fraction was isolated and identified as 7-hydroxy-6-

methoxycoumarin (scopoletin). This compound was observed as a blue fluorescent spot on TLC under UV light. The phytotoxicity of this compound was compared with a commercially purchased coumarin (5,7-dimethoxycoumarin), which showed that the isolated compound from *A. afra* (7-hydrox-6-methoxycoumarin) had higher activity than 5,7-dimethoxycoumarin. Another compound isolated from this fraction was 5,7-dihydroxy-4'-methoxyflavone (acacetin). BA4 fraction which also showed herbicidal activity in the phytotoxicity assay was available in only a small amount and could not be subjected to a bio-guided assay; however, lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide was isolated from this fraction.

The herbicidal compound was duly identified, isolated and characterized as scopoletin (**2.19**). This compound has been isolated previously from *A. afra* but no journal has reported its herbicidal activity against the plant species used in this assay. Acacetin (**4.39**) has been isolated previously from this species, but this is the first time lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**) compound has been isolated from *Artemisia afra*. This investigation presents the first assignment of the ^{13}C NMR data of this compound; these data have not been reported previously.

Based on the findings of this study, we have come to the conclusion that medicinal plants may have compounds with herbicidal properties that could serve as hits in the development of herbicides. In future, more medicinal plants would be investigated as a possible source of herbicidal compounds by using laboratory bioassays. Field trials will be conducted in collaboration with researchers in relevant agriculture-related disciplines to investigate the activity and stability of the compound under real conditions.

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APPENDIX I

NMR spectra of isolated compounds

Plate 1: ^{1}H NMR spectrum of scopoletin (**2.19**)

Plate 2: ^{13}C spectrum of scopoletin (**2.19**)

Plate 3: DEPT 135 spectrum of scopoletin (**2.19**)

Plate 4: COSY spectrum of scopoletin (**2.19**)

Plate 5: HSQC spectrum of scopoletin (**2.19**)

Plate 6: HMBC spectrum of scopoletin (**2.19**)

Plate 7: NOESY spectrum of scopoletin (**2.19**)

Plate 8: ^{1}H NMR spectrum of acacetin (**4.39**)

Plate 9: ^{13}C NMR spectrum acacetin (**4.39**)

Plate 10: DEPT spectrum of acacetin (**4.39**)

Plate 11: COSY spectrum of acacetin (**4.39**)

Plate 12: HSQC spectrum of acacetin (**4.39**)

Plate 13: HMBC spectrum of acacetin (**4.39**)

Plate 14: NOESY spectrum of acacetin (**4.39**)

Plate 15: ^{1}H NMR spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**)

Plate 16: ^{13}C NMR spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**)

Plate 17: DEPT-135 spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**)

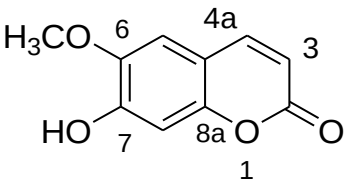
Plate 18: COSY spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**)

Plate 19: HSQC spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**)

Plate 20: HMBC spectrum of 1α,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**)

Plate 21: NOESY spectrum of 1α,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (**4.40**)

Plate 1: ^{1}H NMR spectrum of scopoletin (2.19) (500 MHz, acetone-d_6)



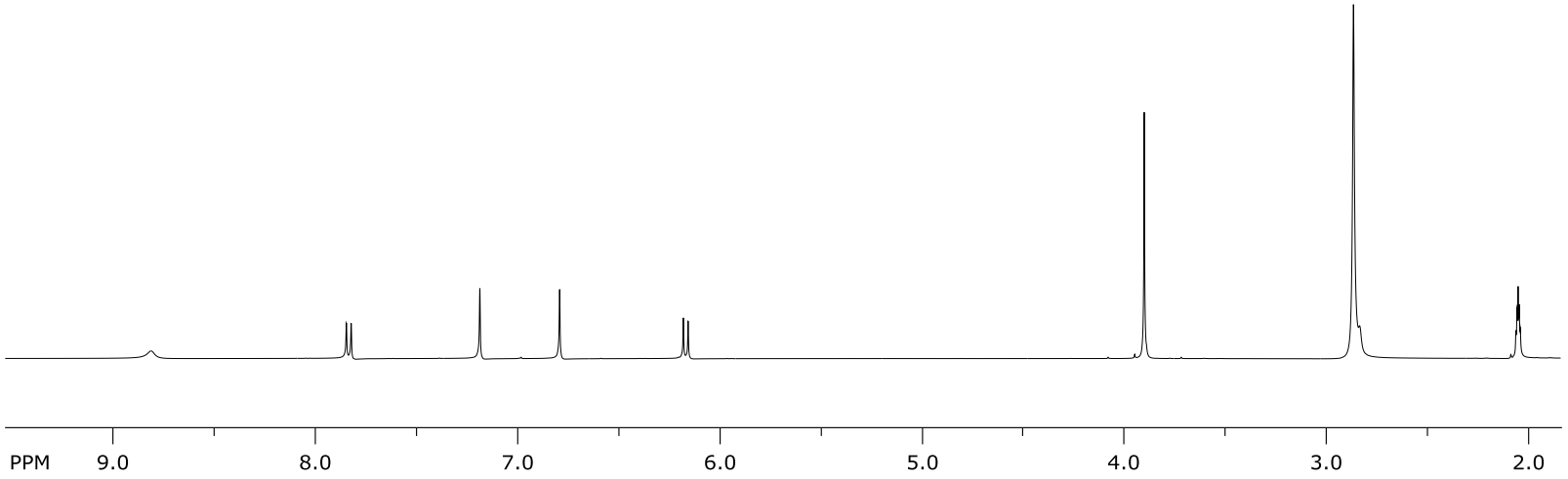


Plate 2: ^{13}C NMR spectrum of scopoletin (2.19) (125 MHz, acetone-d_6)

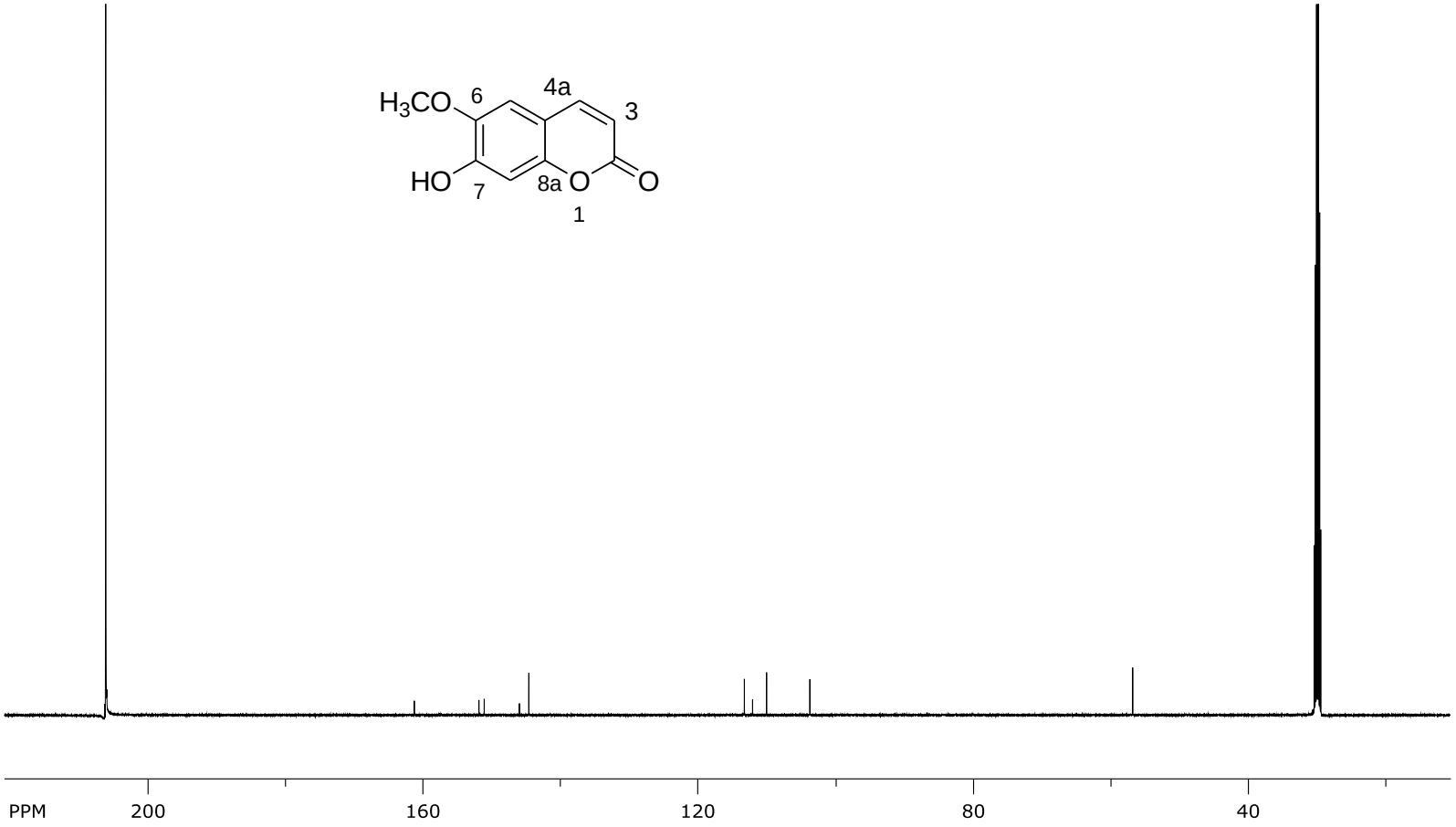


Plate 3: COSY spectrum of scopoletin (2.19) (acetone-d_6)

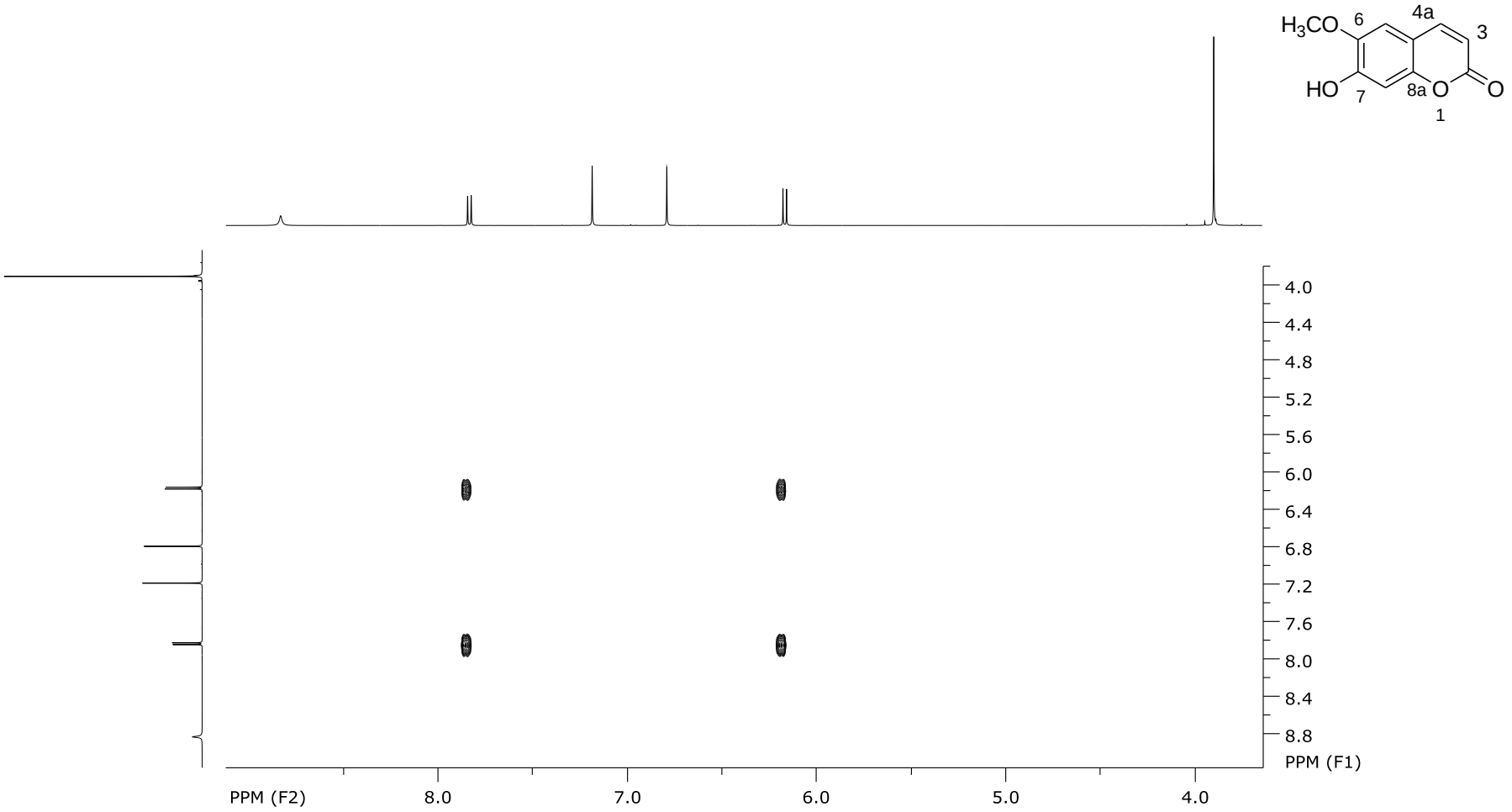
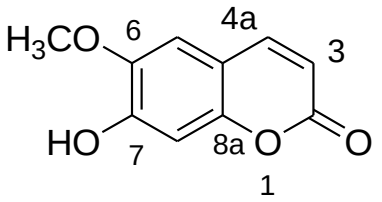


Plate 4: DEPT 135 spectrum of scopoletin (acetone-d_6)



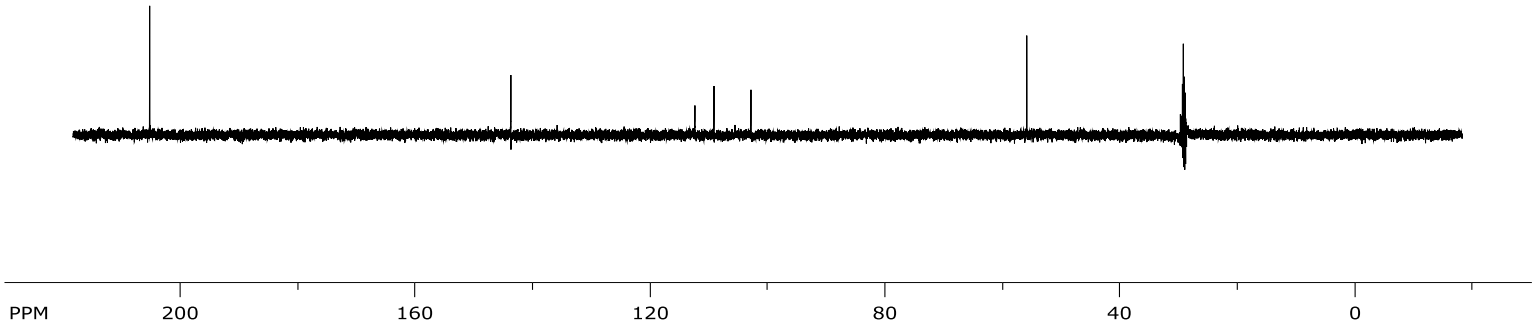


Plate 5: HSQC spectrum of scopoletin (2.19) (acetone-d_6)

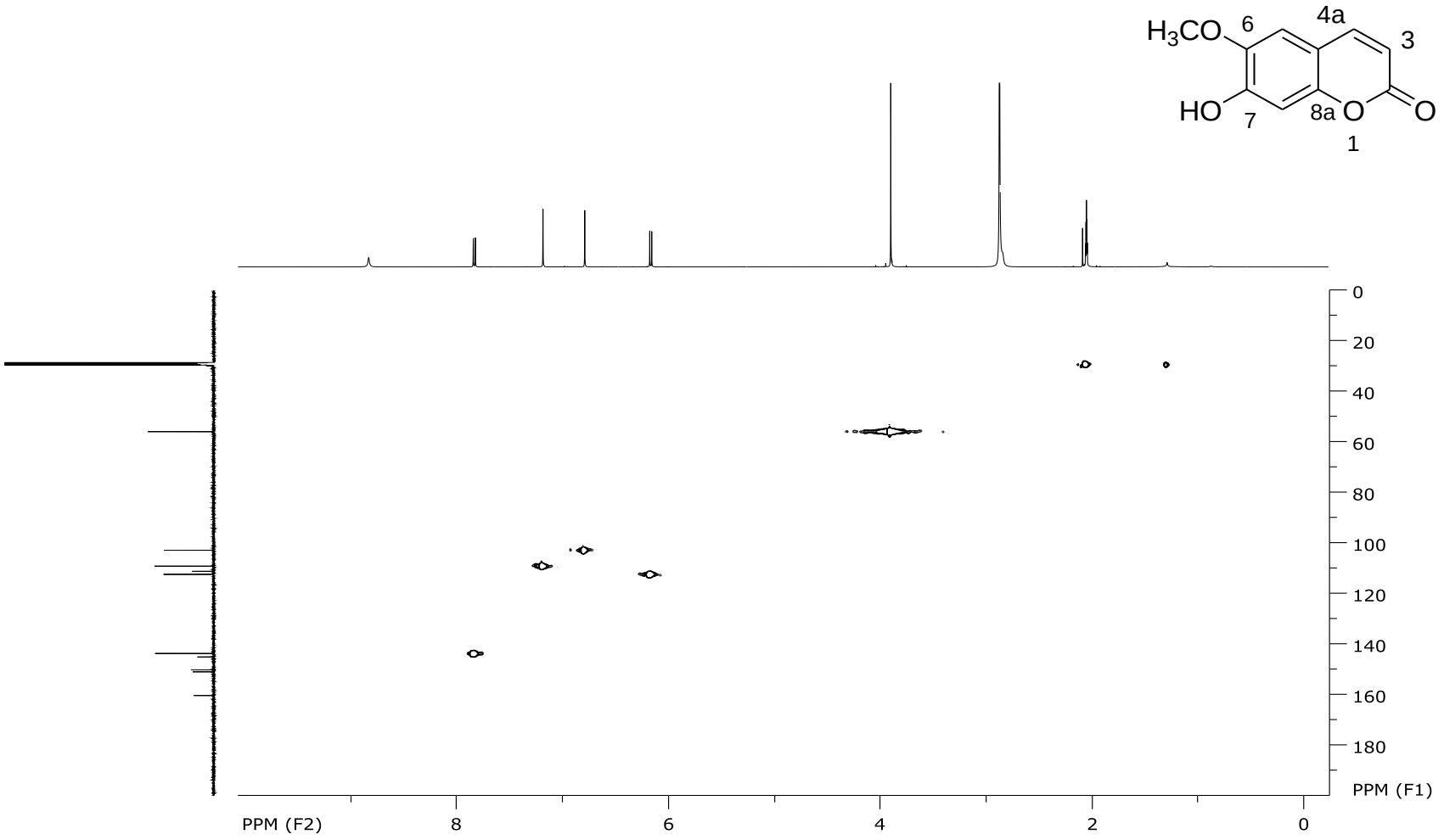


Plate 6: HMBC spectrum of scopoletin (2.19)

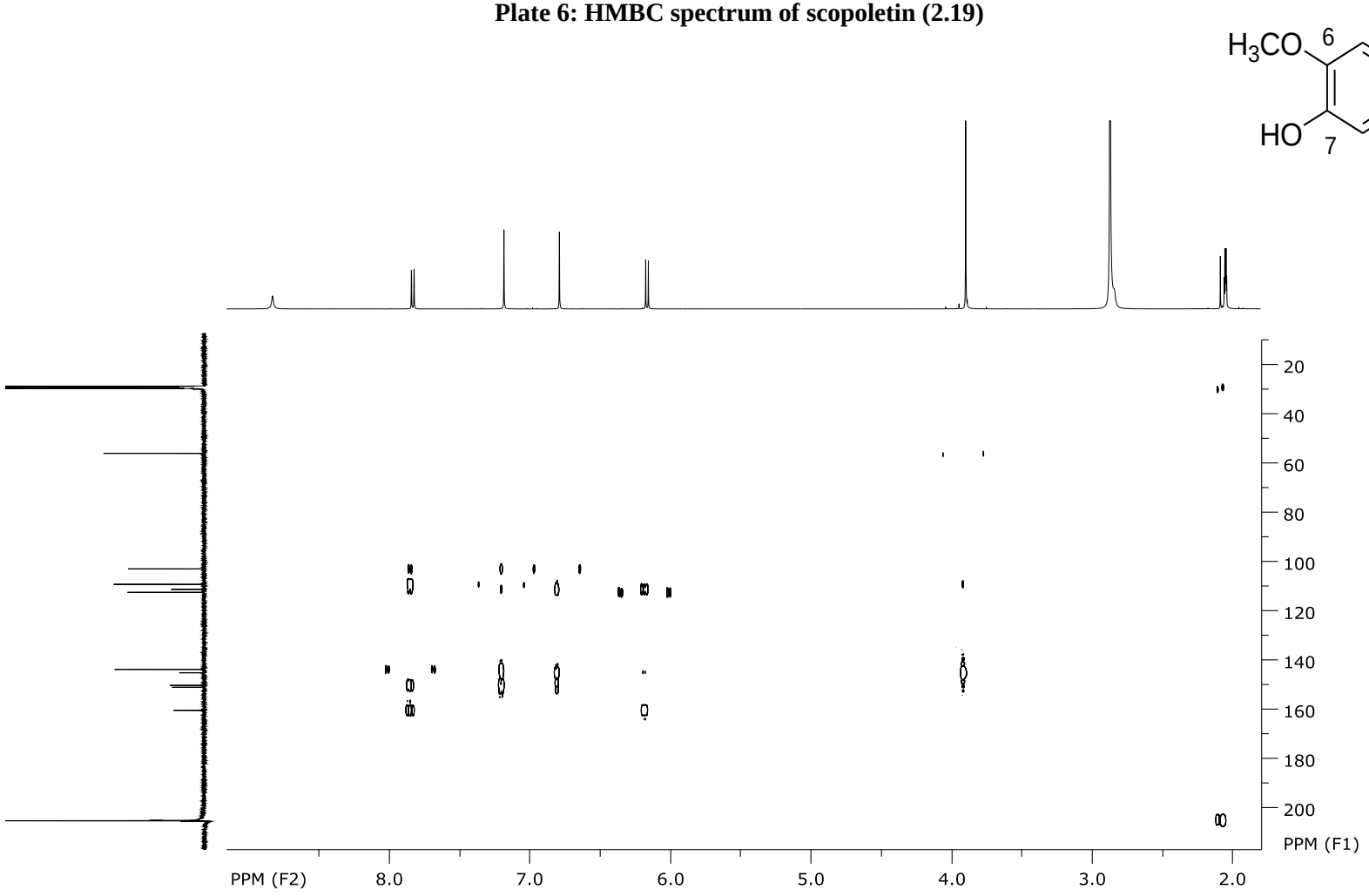


Plate 7: NOESY spectrum of scopoletin (2.19)

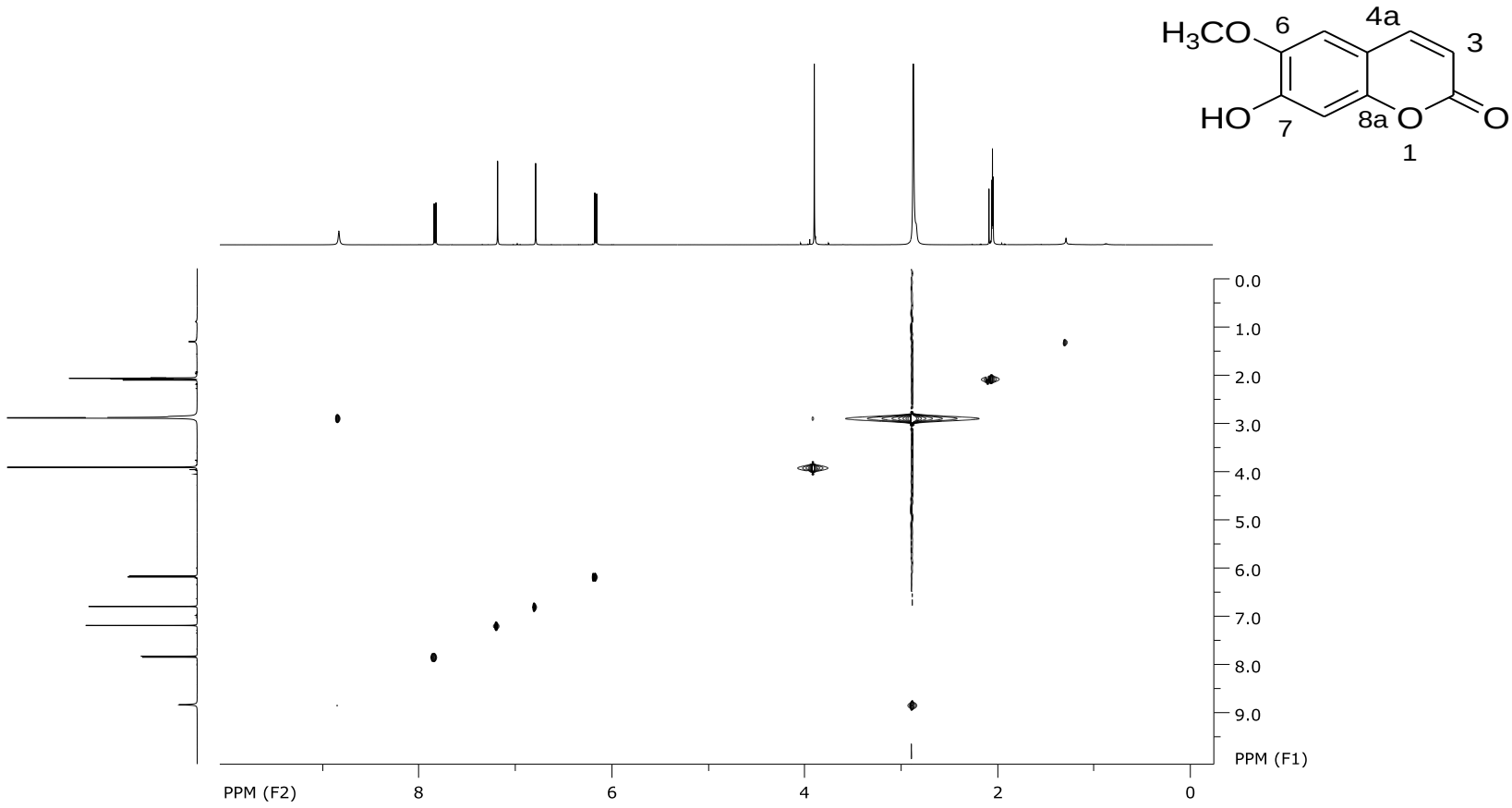


Plate 8: ^{1}H NMR spectrum of 5,7-dihydroxy-4'-methoxyflavone (acacetin) (4.39)

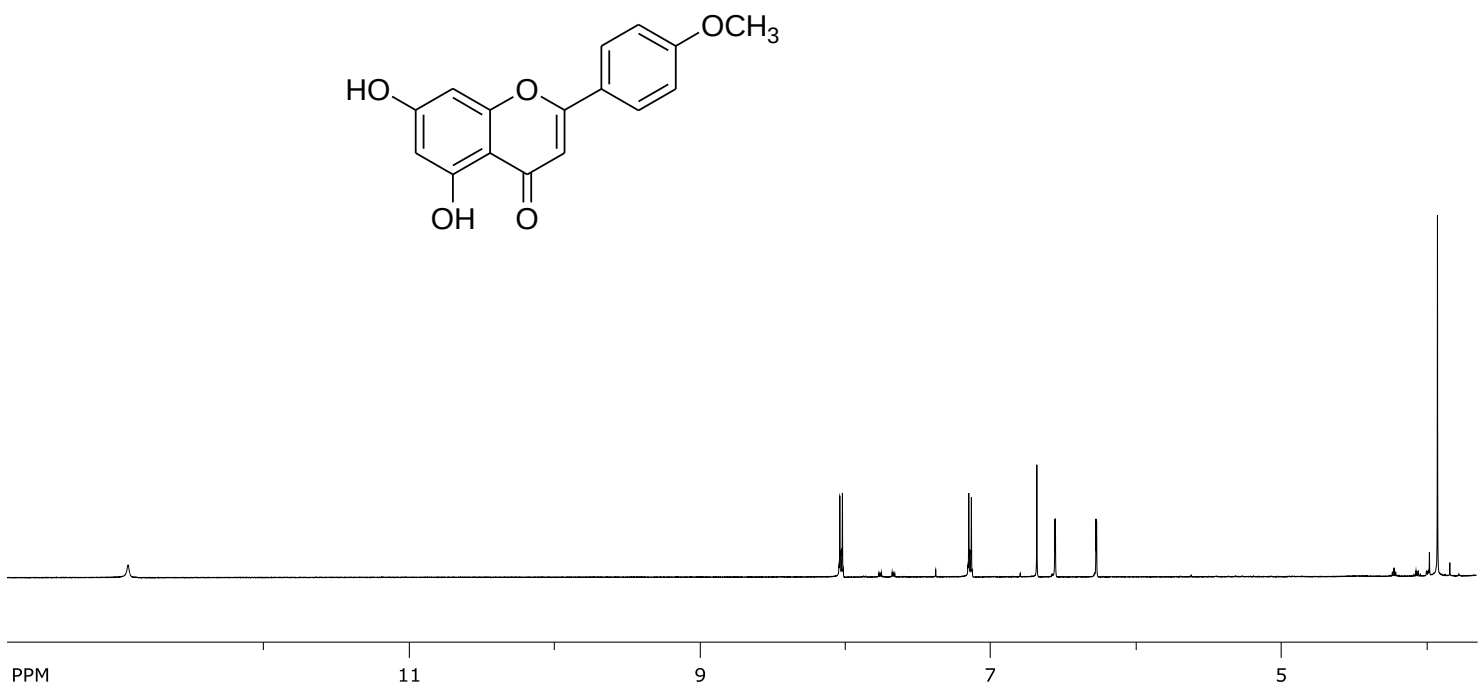


Plate 9: ^{1}C NMR spectrum of 5,7-dihydroxy-4'-methoxyflavone (acacetin) (4.39)

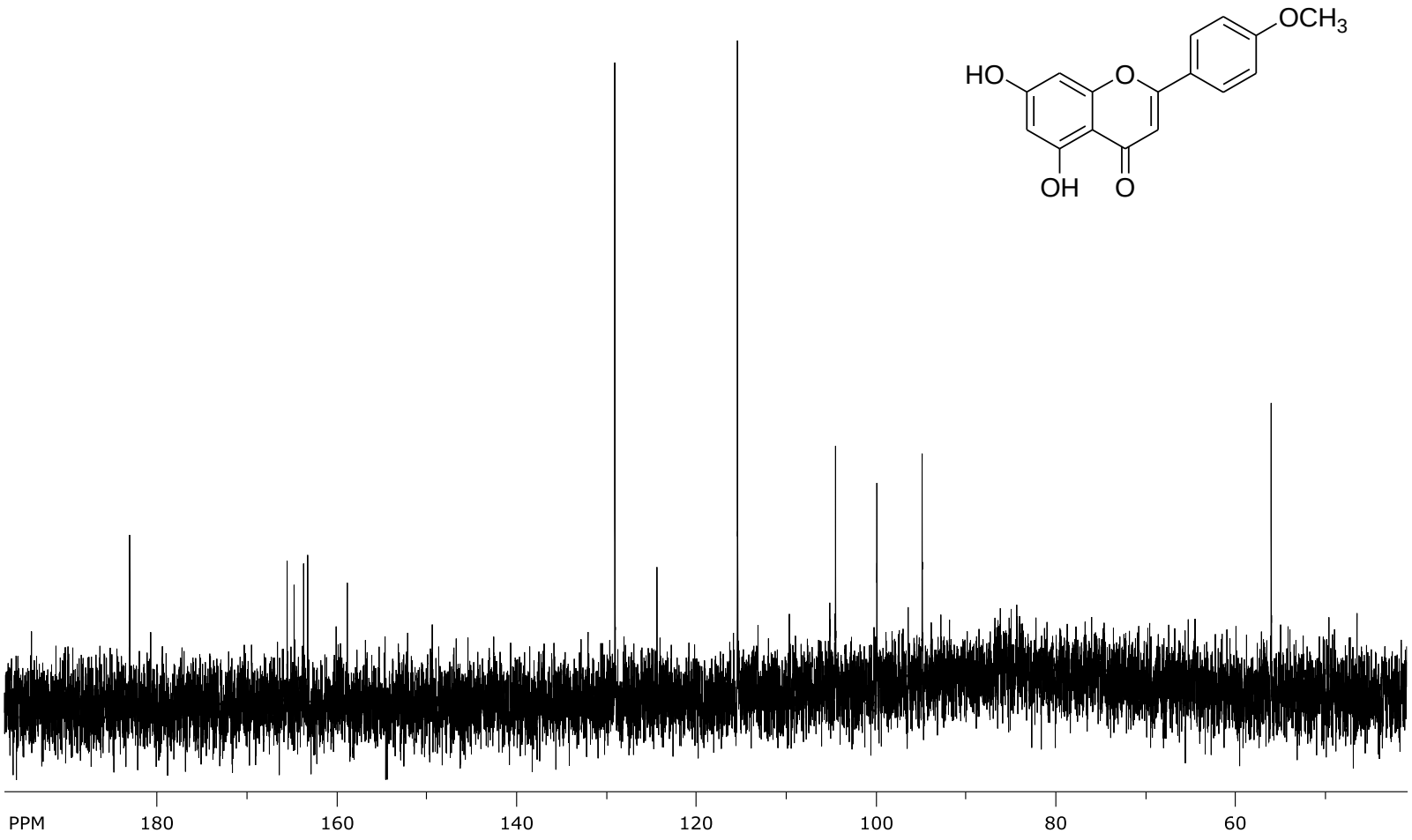


Plate 10: COSY spectrum of 5,7-dihydroxy-4'-methoxyflavone (acacetin) (4.39)

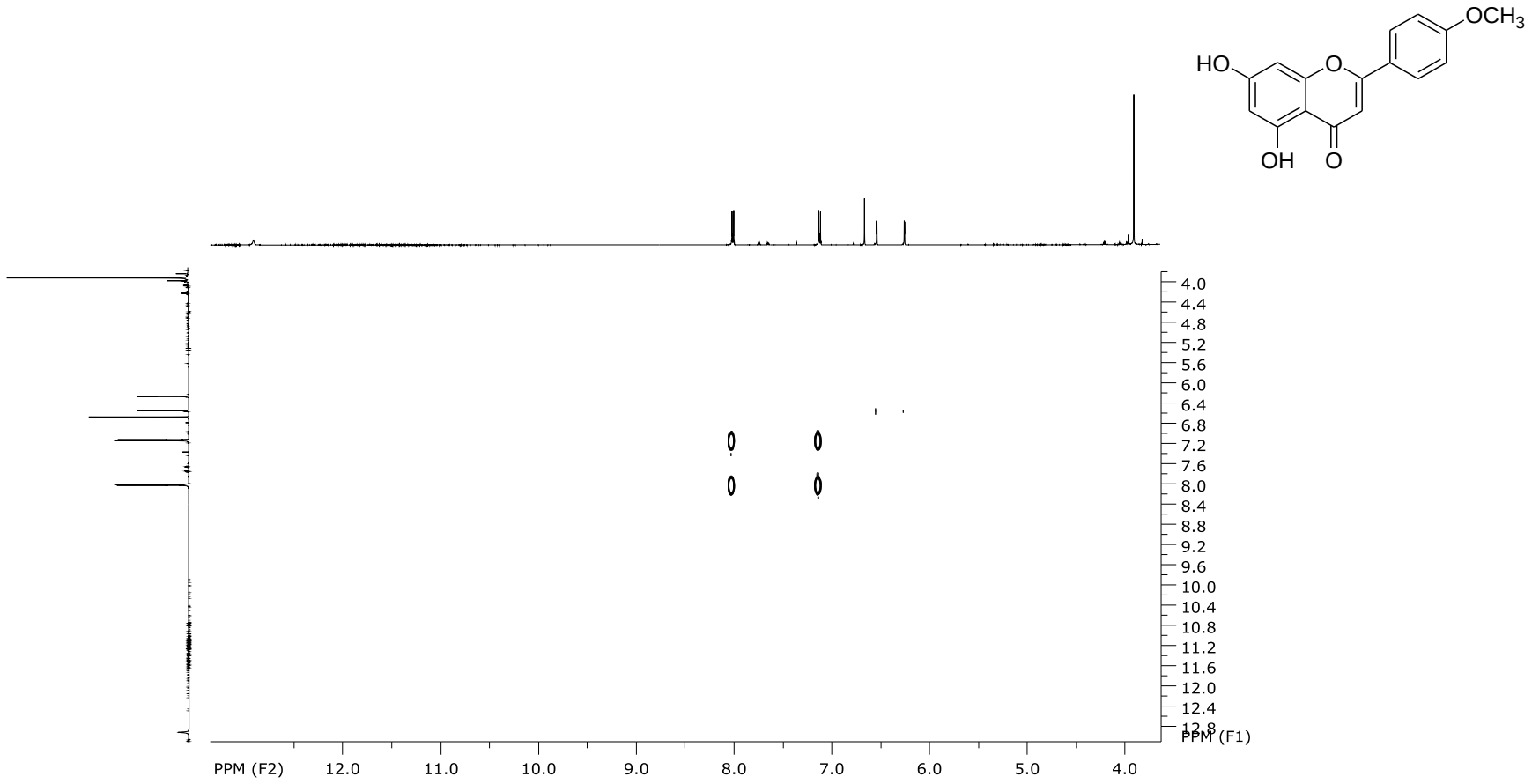
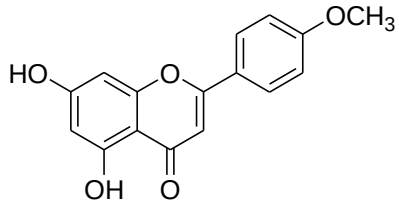


Plate 11: DEPT spectrum of 5,7-dihydroxy-4'-methoxyflavone (acacetin) (4.39)



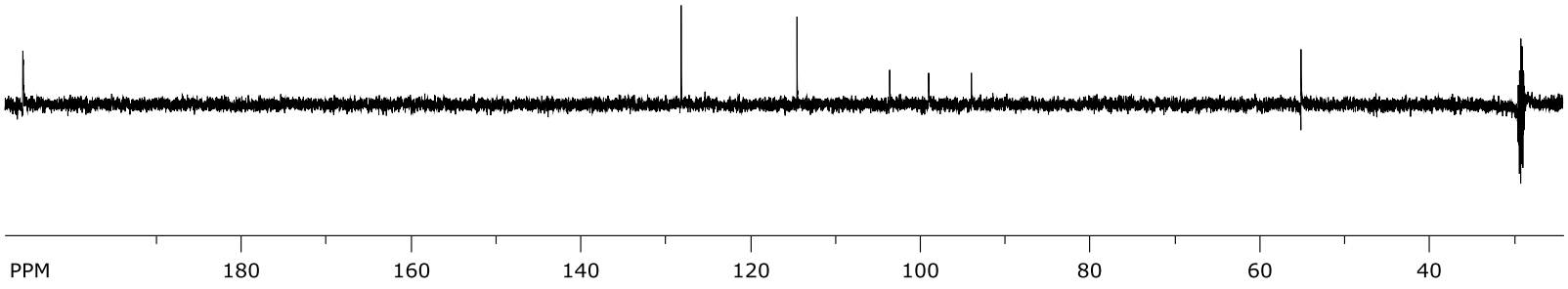


Plate 12: HSQC spectrum of 5,7-dihydroxy-4'-methoxyflavone (acacetin) (4.39)

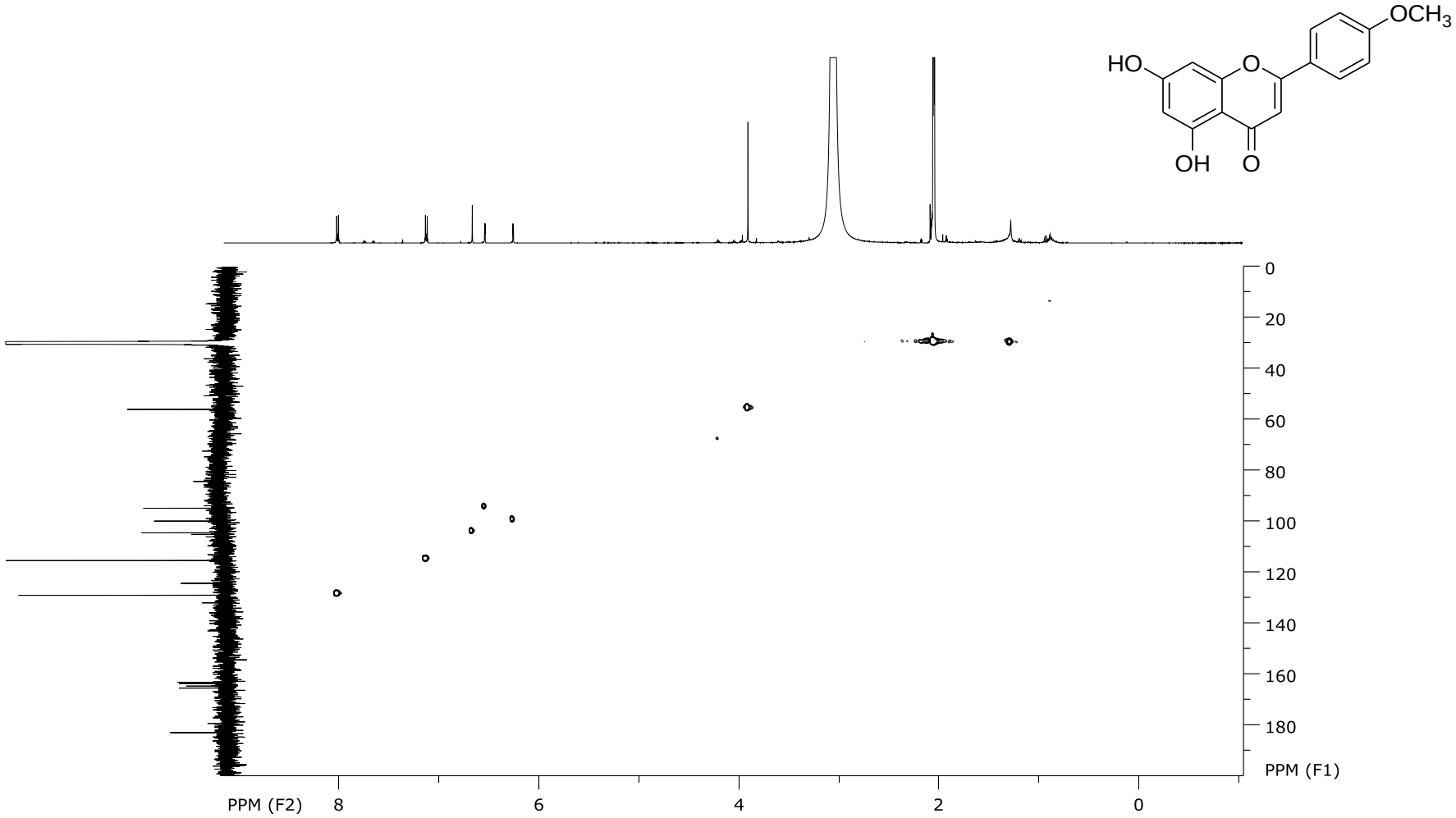


Plate 13: HMBC spectrum of 5,7-dihydroxy-4'-methoxyflavone (acacetin) (4.39)

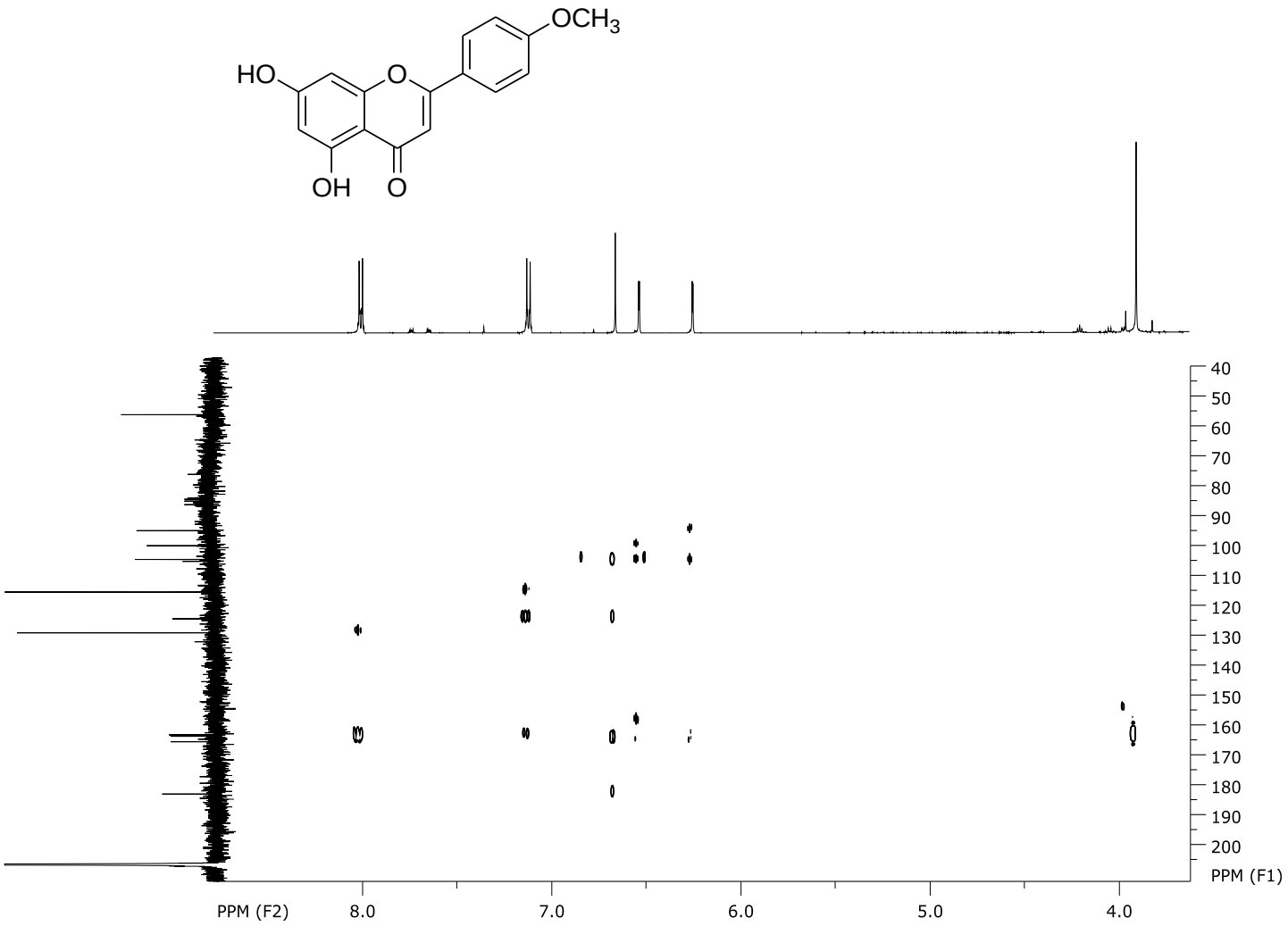


Plate 14: NOESY spectrum of 5,7-dihydroxy-4'-methoxyflavone (acacetin) (4.39)

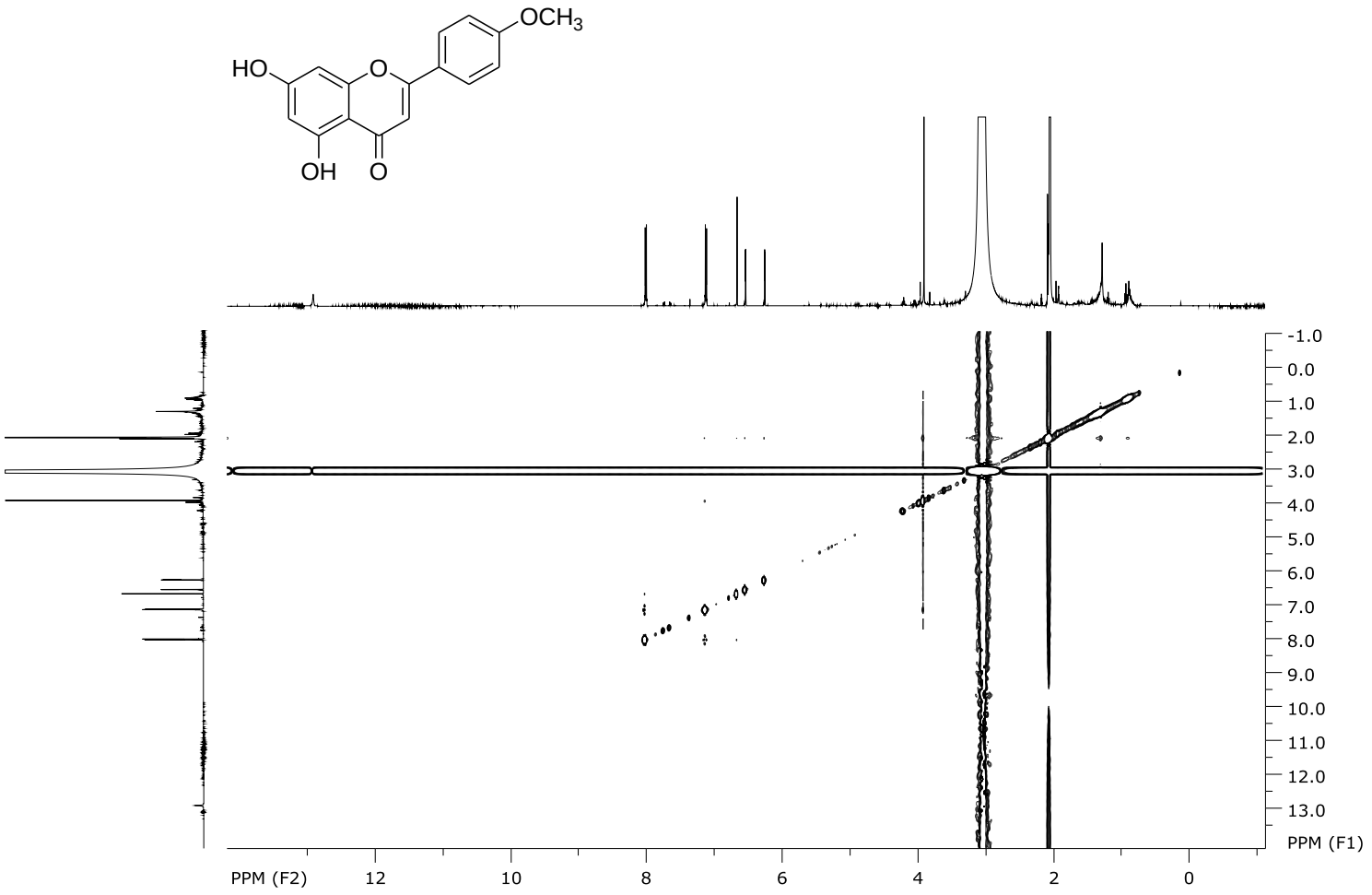


Plate 15: ^{1}H NMR spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (4.40)

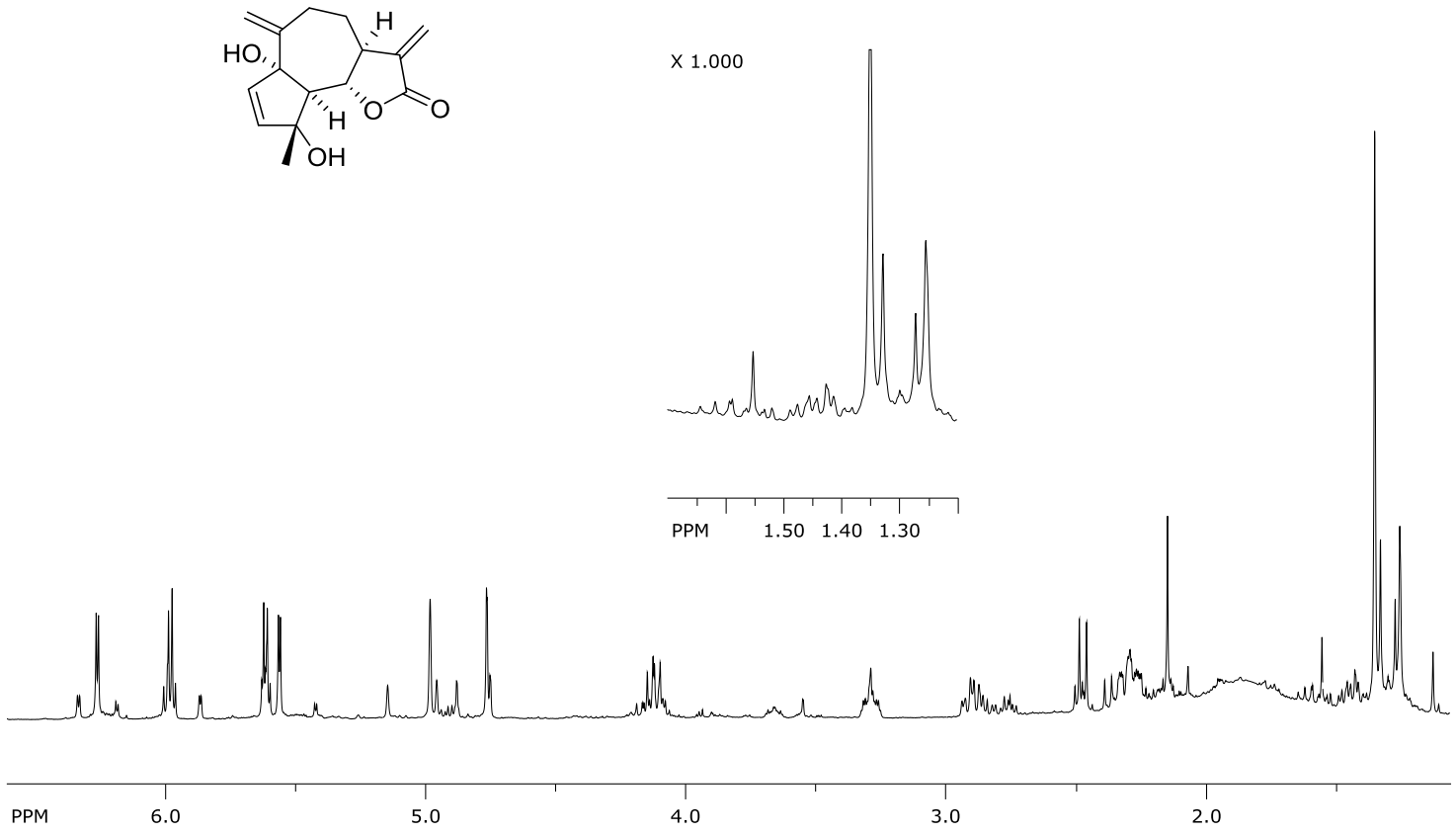


Plate 16: ^{13}C NMR spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (4.40)

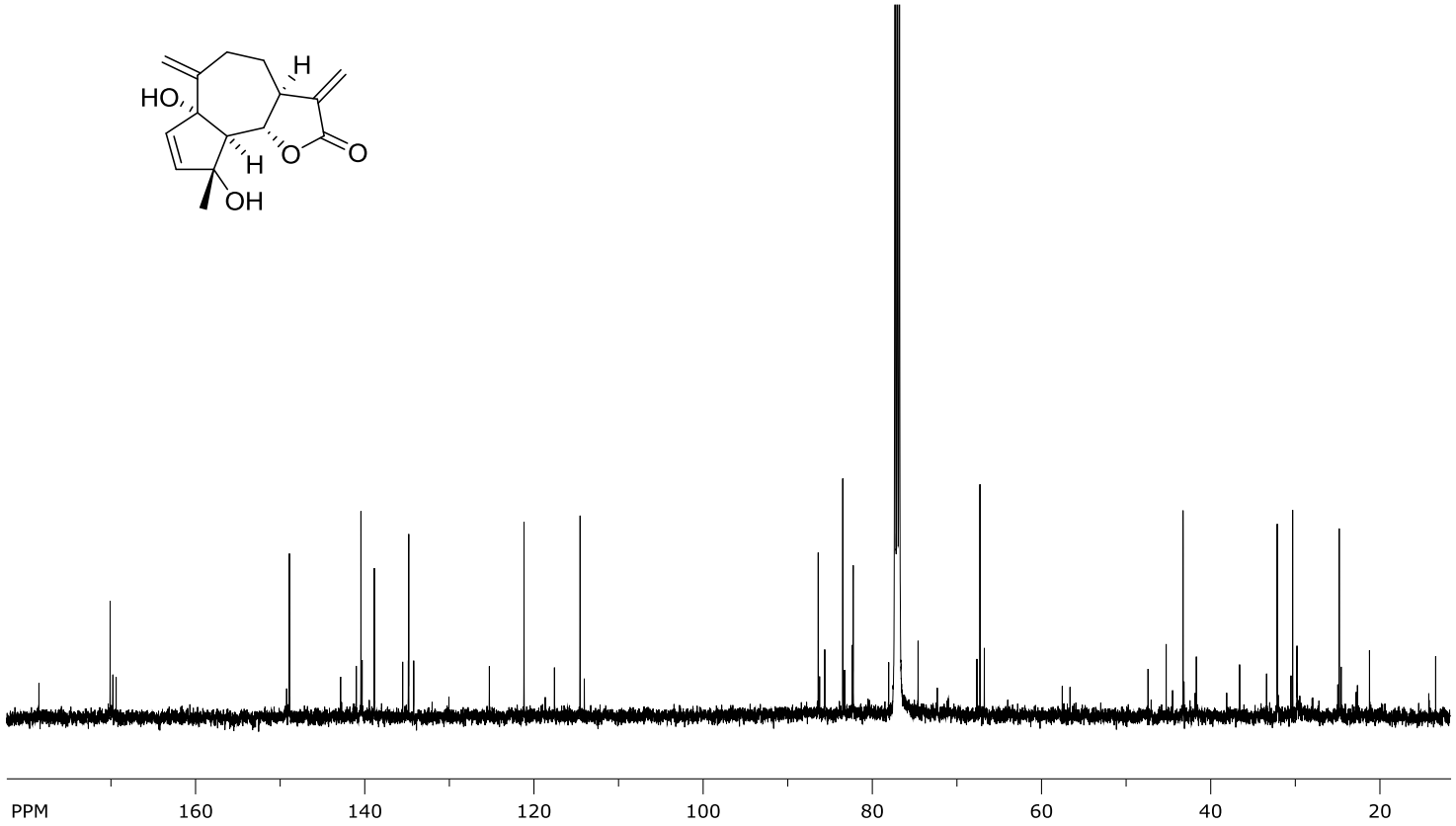
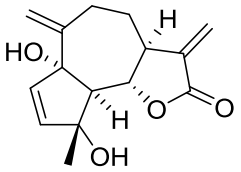


Plate 17: DEPT 135 NMR spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (4.40)



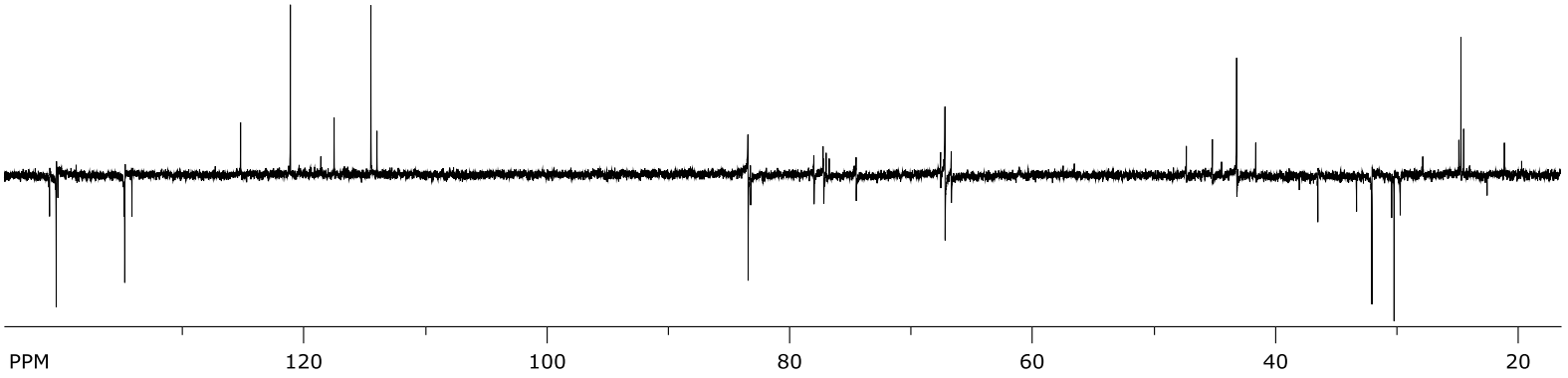


Plate 18: COSY NMR spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (4.40)

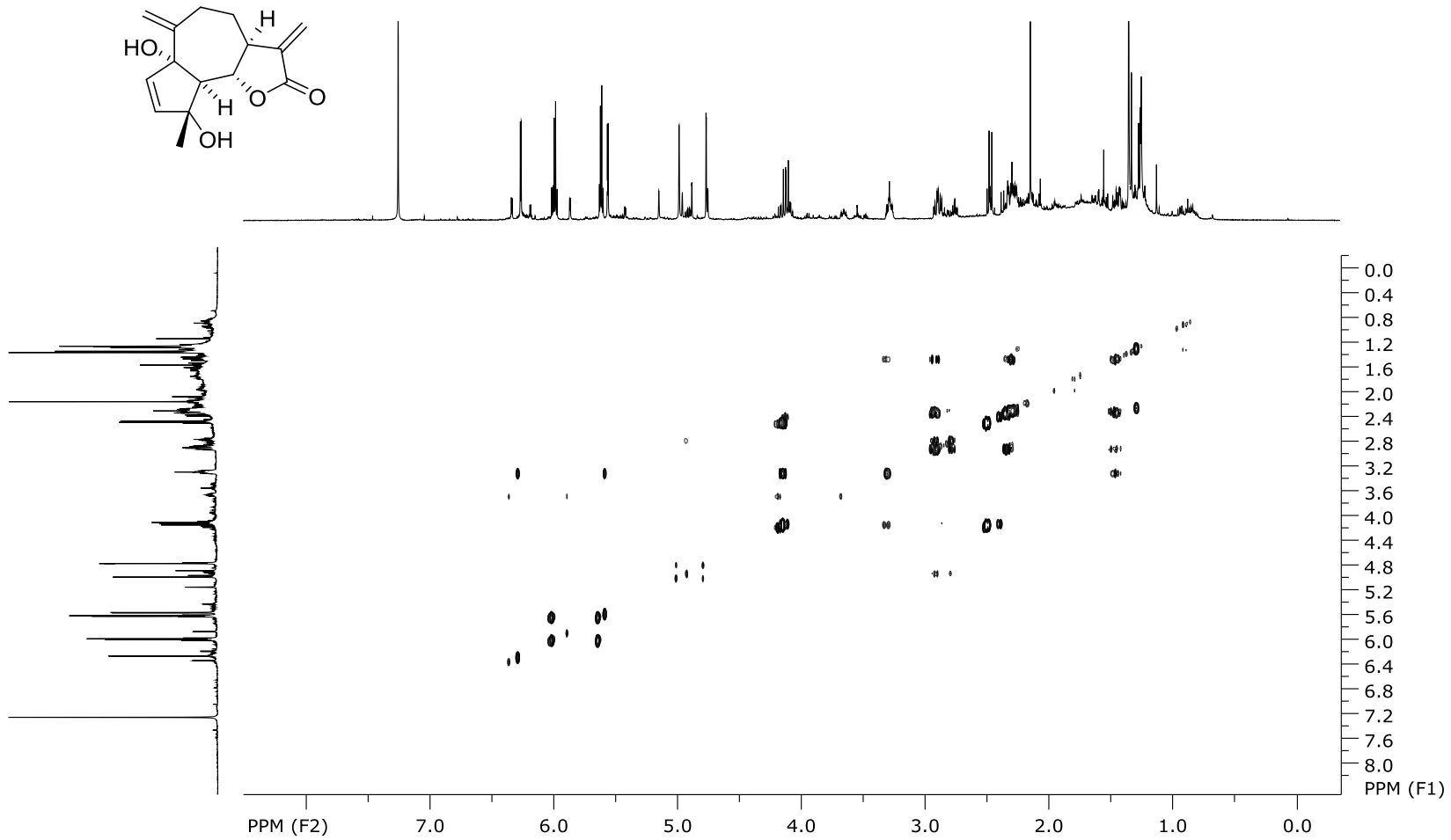


Plate 19: HSQC NMR spectrum of lα,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (4.40)

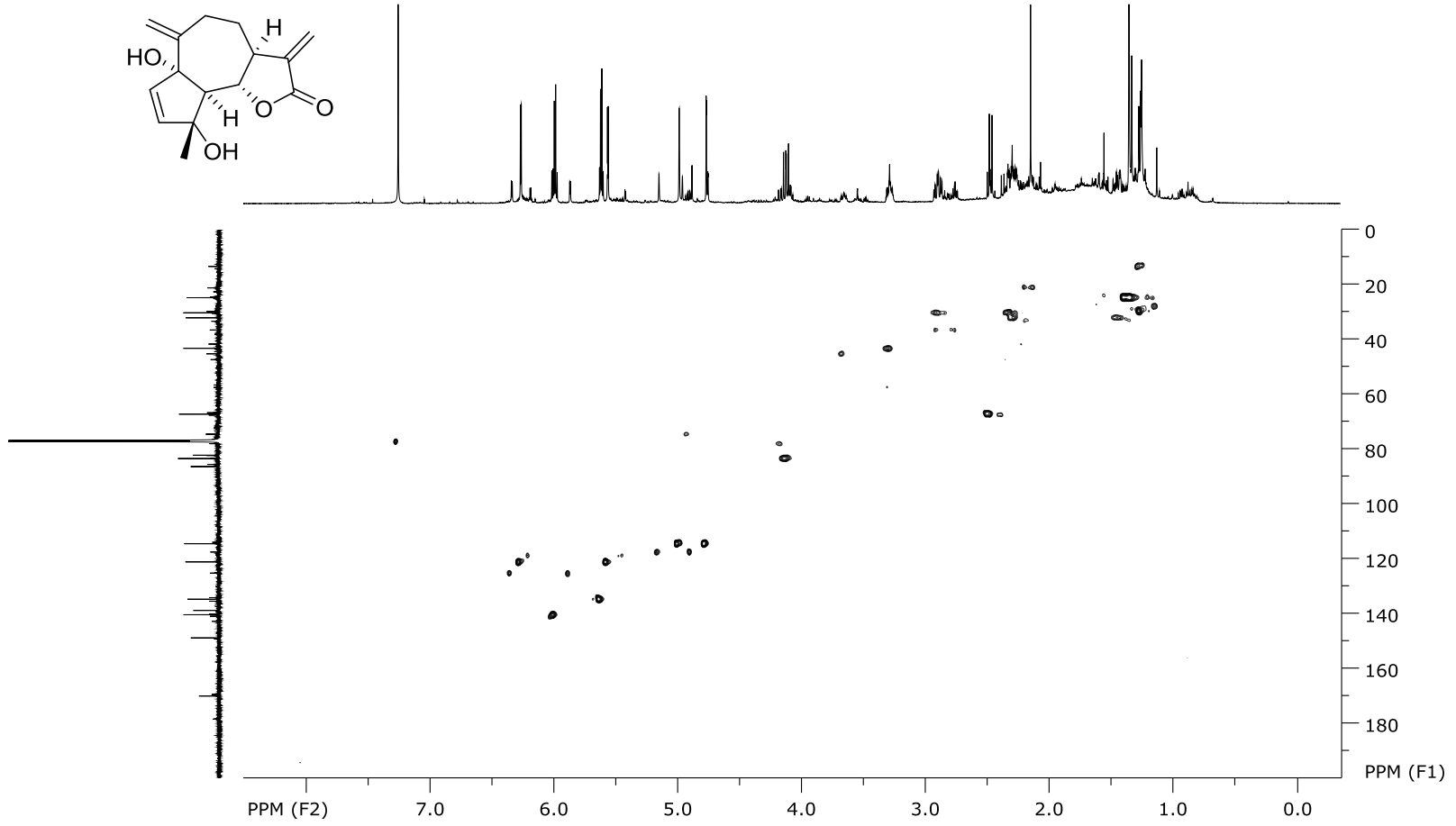


Plate 20: HMBC NMR spectrum of 1α,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (4.40)

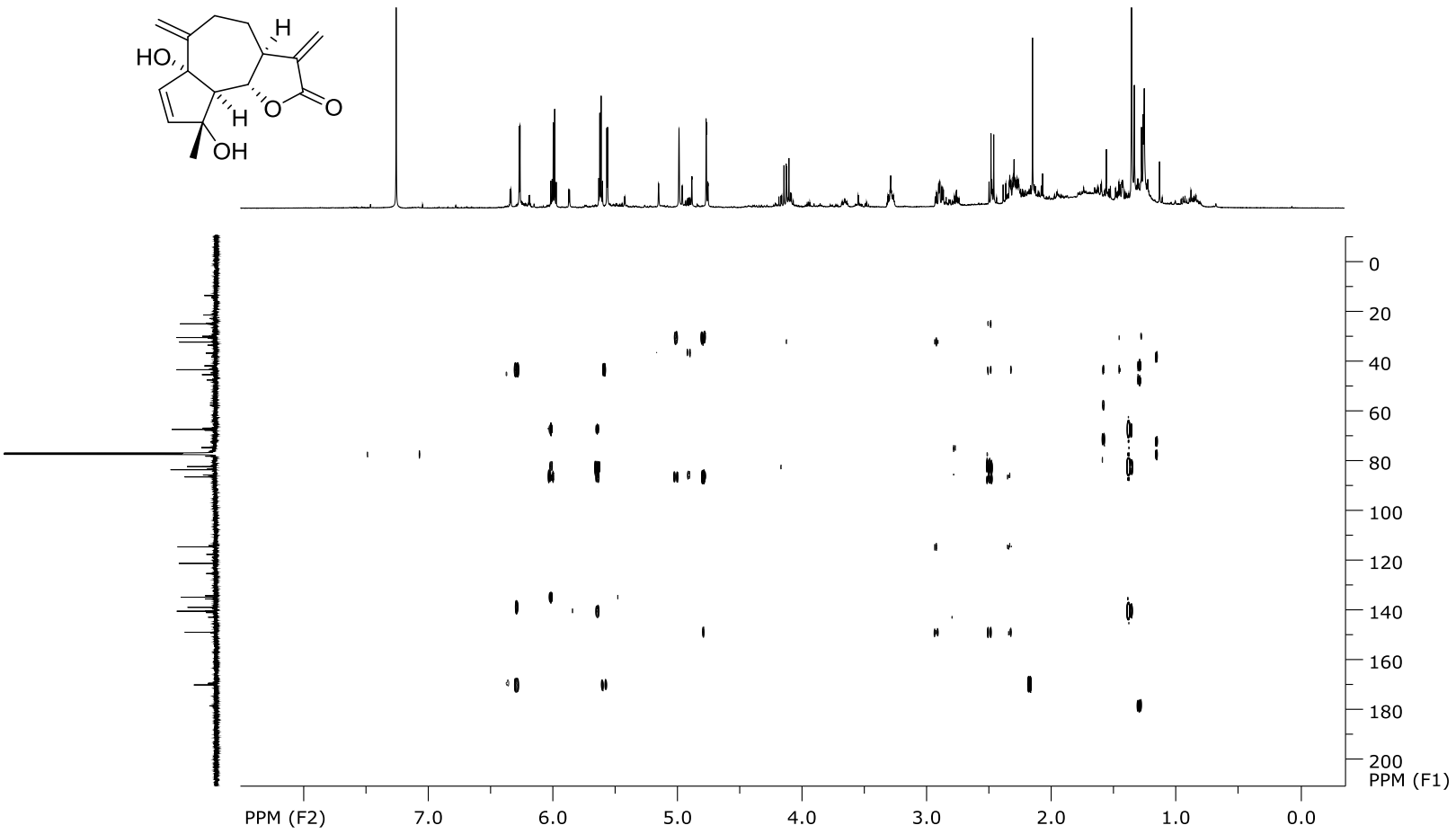
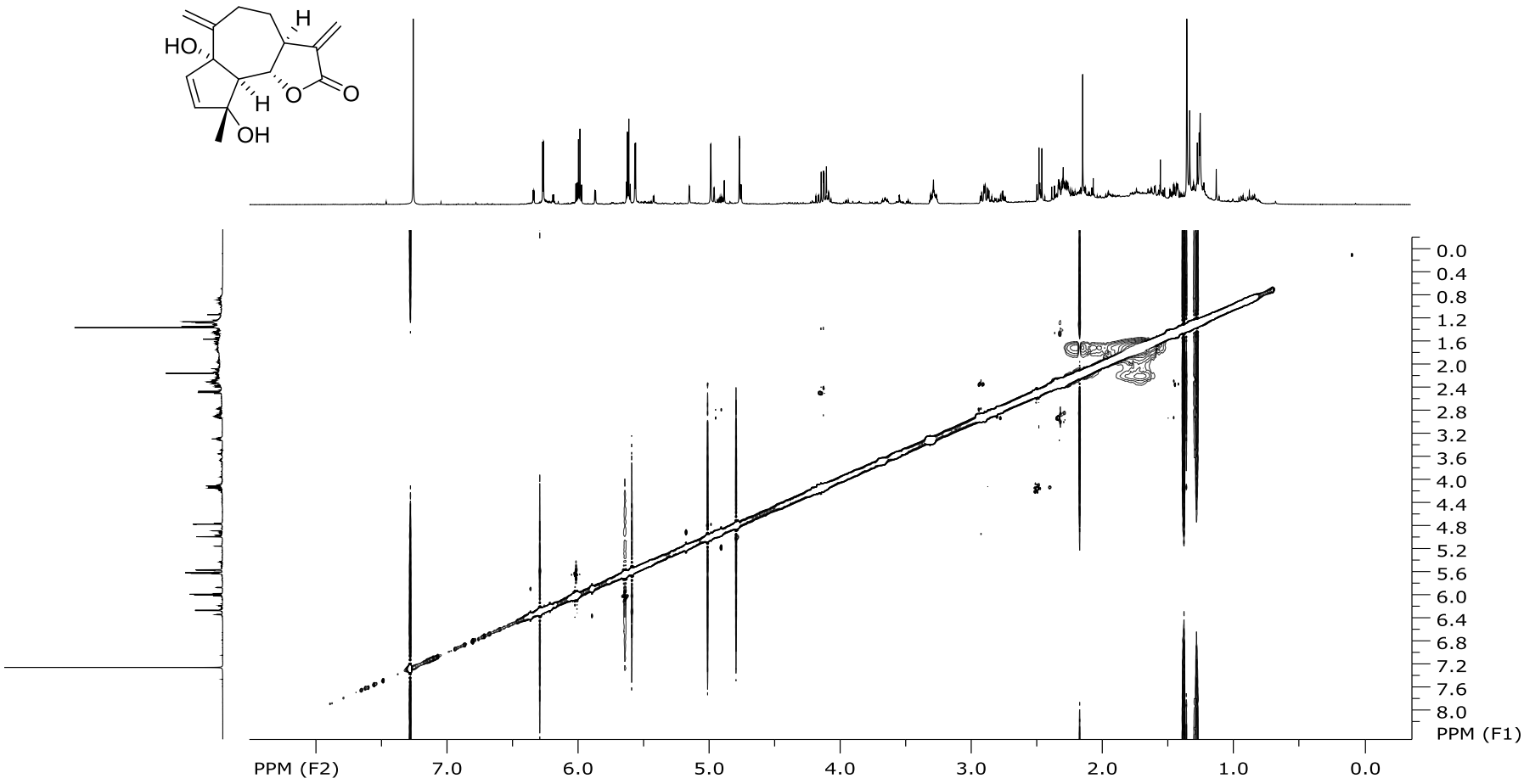


Plate 21: NOESY NMR spectrum of 1α,4α-dihydroxyguaia-2,10(14),11(13)-trien-12,6α-olide (4.40)



APPENDIX II

T-test distribution tables of treatment 1 of isolated scopoletin (2.19): Comparing shoot length to control.

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Shoot lengths (Control &TR 1)	1.00	3	6.0333	1.12398	.64893
	2.00	3	16.4333	1.25033	.72188

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Shoot lengths (Control &TR 1)	Equal variances assumed	.120	.746	-10.714	4	.000	-10.40000
	Equal variances not assumed			-10.714	3.955	.000	-10.40000

Independent Samples Test

		t-test for Equality of Means		
			95% Confidence Interval of the Difference	
		Std. Error Difference	Lower	Upper
Shoot length (Control &TR1)	Equal variances assumed	.97068	-13.09504	-7.70496
	Equal variances not assumed	.97068	-13.10706	-7.69294

T-test distribution tables of treatment 1 of isolated scopoletin (2.19): Comparing root length to control.

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Root length (Control &TR 1)	1.00	3	5.4333	3.60046	2.07873
	2.00	3	30.9333	3.93234	2.27034

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Root length (Control&TR1)	Equal variances assumed	.131	.736	-8.284	4	.001	-25.50000
	Equal variances not assumed			-8.284	3.969	.001	-25.50000

Independent Samples Test

		t-test for Equality of Means		
			95% Confidence Interval of the Difference	
		Std. Error Difference	Lower	Upper
Root length (Control&TR1)	Equal variances assumed	3.07824	-34.04656	-16.95344
	Equal variances not assumed	3.07824	-34.07269	-16.92731

T-test distribution tables of treatment 2 of isolated scopoletin (2.19): Comparing shoot length to control.

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Shoot length (Control &TR 2)	1.00	3	5.2667	1.10151	.63596
	2.00	3	16.4333	1.25033	.72188

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Shoot length (Control &TR 2)	Equal variances assumed	.110	.757	-11.607	4	.000	-11.16667
	Equal variances not assumed			-11.607	3.937	.000	-11.16667

Independent Samples Test

		t-test for Equality of Means		
			95% Confidence Interval of the Difference	
		Std. Error Difference	Lower	Upper
Shoot length (Control &TR 2)	Equal variances assumed	.96206	-13.83777	-8.49557
	Equal variances not assumed	.96206	-13.85459	-8.47874

T-test distribution tables of treatment 2 of isolated scopoletin (2.19): Comparing root length to control.

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Root length (Control &TR 2)	1.00	3	4.1667	3.05505	1.76383
	2.00	3	30.9333	3.93234	2.27034

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Root length (Control &TR 2)	Equal variances assumed	.376	.573	-9.310	4	.001	-26.76667
	Equal variances not assumed			-9.310	3.770	.001	-26.76667

Independent Samples Test

		t-test for Equality of Means		
			95% Confidence Interval of the Difference	
		Std. Error Difference	Lower	Upper
Root length (Control &TR 2)	Equal variances assumed	2.87499	-34.74891	-18.78442
	Equal variances not assumed	2.87499	-34.94503	-18.58830

T-test distribution tables of treatment 3 of isolated scopoletin (2.19): Comparing shoot length to control.

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Shoot length (Control &TR 3)	1.00	3	2.2000	.52915	.30551
	2.00	3	16.4333	1.25033	.72188

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Shoot length (Control &TR 3)	Equal variances assumed	3.962	.117	-18.158	4	.000	-14.23333
	Equal variances not assumed			-18.158	2.694	.001	-14.23333

Independent Samples Test

		t-test for Equality of Means		
			95% Confidence Interval of the Difference	
		Std. Error Difference	Lower	Upper
Shoot length (Control &TR 3)	Equal variances assumed	.78387	-16.40969	-12.05698
	Equal variances not assumed	.78387	-16.89606	-11.57060

T-test distribution tables of treatment 3 of isolated scopoletin (2.19): Comparing root length to control.

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Root length (Control &TR 3)	1.00	3	2.1667	2.46644	1.42400
	2.00	3	30.9333	3.93234	2.27034

Independent Samples Test

	Levene's Test for Equality of Variances	t-test for Equality of Means

		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Root length (Control &TR 3)	Equal variances assumed	1.104	.353	-10.734	4	.000	-28.76667
	Equal variances not assumed			-10.734	3.363	.001	-28.76667

Independent Samples Test

		t-test for Equality of Means		
			95% Confidence Interval of the Difference	
		Std. Error Difference	Lower	Upper
Root length (Control &TR 3)	Equal variances assumed	2.67997	-36.20745	-21.32589
	Equal variances not assumed	2.67997	-36.79798	-20.73535

T-test distribution tables of commercial coumarin: Comparing shoot length of TR1 and control

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Shootlength(C&Com)	1.00	3	12.4333	1.20968	.69841
	2.00	3	16.4333	1.25033	.72188

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Shootlength(C&Com)	Equal variances assumed	.014	.910	-3.982	4	.016	-4.00000	1.00443	-6.78876	-1.21124
	Equal variances not assumed			-3.982	3.996	.016	-4.00000	1.00443	-6.78996	-1.21004

T-test distribution tables of commercial coumarin: Comparing root length of TR1 and control

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Rootlength (C&Com)	1.00	3	30.7000	2.51595	1.45258
	2.00	3	30.9333	3.93234	2.27034

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Rootlength (C&Com)	Equal variances assumed	1.017	.370	-.087	4	.935	-.23333	2.69526	-7.71658	7.24992
	Equal variances not assumed			-.087	3.402	.936	-.23333	2.69526	-8.26455	7.79788

T-test distribution tables of commercial coumarin: Comparing shoot length of TR2 and control

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Shootlength_Com2	1.00	3	9.2000	1.15326	.66583
	2.00	3	16.4333	1.25033	.72188

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Shootlength_Com2	Equal variances assumed	.129	.738	-7.365	4	.002	-7.23333	.98206	-9.95997	-4.50669
	Equal variances not assumed			-7.365	3.974	.002	-7.23333	.98206	-9.96698	-4.49969

T-test distribution tables of commercial coumarin: Comparing root length of TR2 and control

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Rootlength_Com2	1.00	3	25.3333	2.62742	1.51694
	2.00	3	30.9333	3.93234	2.27034

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Rootlength_Com2	Equal variances assumed	.844	.410	-2.051	4	.110	-5.60000	2.73049	-13.18105	1.98105
	Equal variances not assumed			-2.051	3.489	.120	-5.60000	2.73049	-13.63970	2.43970

T-test distribution tables of commercial coumarin: Comparing shoot length of TR3 and control

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Shootlength_Com3	1.00	3	11.1667	.57735	.33333
	2.00	3	16.4333	1.25033	.72188

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Shootlength_Com3	Equal variances assumed	3.396	.139	-6.624	4	.003	-5.26667	.79512	-7.47428	-3.05905
	Equal variances not assumed			-6.624	2.816	.009	-5.26667	.79512	-7.89338	-2.63995

T-test distribution tables of commercial coumarin: Comparing root length of TR3 and control

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Rootlength_Com3	1.00	3	21.5333	8.80700	5.08473
	2.00	3	30.9333	3.93234	2.27034

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Rootlength_Com3	Equal variances assumed	2.980	.159	-1.688	4	.167	-9.40000	5.56856	-24.86081	6.06081
	Equal variances not assumed			-1.688	2.767	.198	-9.40000	5.56856	-27.99664	9.19664

T-test distribution tables of the negative control compare with the standard (4 mm)

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Standard (4 mm) Vs Negative	1.00	3	4.0000	.00000	.00000
	2.00	3	6.3333	.57735	.33333

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Standard (4 mm) Vs Negative	Equal variances assumed	16.000	.016	-7.000	4	.002	-2.33333	.33333
	Equal variances not assumed			-7.000	2.000	.020	-2.33333	.33333

From the table, the Leven's test for Equality of variance has $P_{cal} < 0.05$ based on this, the reading is taking from the first roll of equal variances assumed. Therefore, P_{cal} 0.002 < P_{crit} 0.05, hence there was significant difference between the standard and the negative control. This explains that the negative control did not inhibit the growth of the coleoptiles hence the DMSO in the solutions prepared had no effect on the growth of the coleoptile in the experiment.

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
Standard (4 mm) Vs Negative	Equal variances assumed	-3.25882	-1.40785
	Equal variances not assumed	-3.76755	-.89912

T-test distribution tables of positive control (eucalyptol) control compare with the standard (4 mm)

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Standard (4 mm) Vs Eucalyptol	1.00	3	4.0000	.00000[a]	.00000
	2.00	3	4.0000	.00000[a]	.00000

a. t cannot be computed because the standard deviations of both groups are 0.

Independence sample table could not be generated because there was no increase in length of coleoptiles compared with the standards (4mm). Hence t-distribution could not be computed. This means the eucalyptol inhibited the growth of the coleoptiles.

T-Test

Warnings

The Independent Samples table is not produced.

T-test distribution tables of the Treatment compare with the standard (4 mm)

Group Statistics

	Experiments	N	Mean	Std. Deviation	Std. Error Mean
Standard (4 mm) Vs Treatment	1.00	3	4.0000	.00000[a]	.00000
	2.00	3	4.0000	.00000[a]	.00000

a. t cannot be computed because the standard deviations of both groups are 0.

Independence sample table could not be generated because there was no increase in length (growth) of coleoptiles compared with the standards (4mm). Hence t-distribution could not be computed. This means the treatment inhibited the growth of the coleoptiles.